

Chemical and Functional Properties
of Food Components Series



Food Colorants Chemical and Functional Properties

EDITED BY
Carmen Socaciu



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Food Colorants

***Chemical and
Functional
Properties***

Chemical and Functional Properties of Food Components Series

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Food Colorants

Chemical and Functional Properties

EDITED BY

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Preface

We live, more and more, in a globalized society, looking to cycles and chains that integrate knowledge and interdisciplinary areas, looking for the welfare and health of human beings.

In this context, a scientific approach related to food colorants should follow the “chain” from light to health, looking to pigments as key molecules able to transfer light energy to the biochemical and sensorial properties of cells, tissues, organisms, and finally to be used as ingredients to improve food quality, safety, and appearance. Therefore, this book may be thought of as a monograph that provides integrative images of the scientific characteristics, functionalities, and applications of color molecules (pigments) as colorants in food science and technology, and finally their impacts on health.

The seven sections in this book deal with updated information about the relationships of the chemical natures and functional properties of various natural pigments and synthetic molecules that are used to color food. Sections 1 through 3 develop fundamental aspects regarding the physics and (bio)chemistry of color and mechanistic views of the stability and availability of pigments, looking to their actions *in vitro* and *in vivo* and to indicators of their impacts on health. Sections 4 and 5 discuss technological aspects regarding the occurrence of pigments in food matrices, stability during storage and processing, the production of food colorants by conventional technologies, new environmentally friendly technologies and formulations, and, most important, advanced biotechnologies for producing natural colorants. Analysis of natural and synthetic colorants and advanced techniques developed in recent years are covered in Section 6. Finally, Section 7 details colorant quality and safety supervision, assessment of possible risks, and quality assurance related to international regulations. Lists of formerly and newly approved colorants in the food additive category are also discussed.

Each Section provides new information about the main classes of pigments: chlorophylls, carotenoids, polyphenols, especially colored flavonoids and anthocyanins, betalains, and other natural pigments (curcuminoids, monascinoïdes, cochineal lacs, carmine, caramel) and synthetic colorants. New approaches to the biosynthesis of colorants by microalgae and microorganisms and the use of genetic engineering to produce colorants are updated based on progress reported in recent years. The information available in current world literature is critically evaluated and presented in a concise and systematic form.

Many structure–function relationships of food colorants are stressed in this book, helping readers understand the effects of their biosynthesis, structures, and function modifications during food storage and processing conditions, and their influences on food quality and safety. This knowledge is necessary to control the rate of

undesirable degradation in foods and to select optimum parameters in the food processing industry.

This volume benefits from the contributions of 22 outstanding scientists from the United States, Brazil, and five European countries who are well known for their competence, sound backgrounds, and personal research experience in food science and technology and related fields such as biophysics, biochemistry, biotechnology, analytical chemistry, quality management, and food safety.

The book is addressed primarily to food science researchers, PhD students, post-graduates, and graduates, as well as food scientists and technologists working in the food industry and food quality control, and in relevant educational fields where it may serve as a condensed, systematic and valuable source of information for university lectures and practical courses. Many topics should be interesting for students of chemistry, biology, biochemistry, food technology, and biotechnology and also for nutritionists and technical staffs in food processing plants. Some sections may be of interest to individuals interested in food quality, journalists, and politicians interested in recent problems of food, nutrition, and health.

This book draws an integrative image of the scientific characteristics and applications of pigments as colorants in food science and technology. All aspects of food colorants are touched — from fundamental, to analytical, technological, quality assurance and safety aspects, to their impacts on health. Using the valuable professional expertise of an international team of scientists and experts in the fields covered by the book, it presents updated knowledge and underlines the key findings in this domain.

I would like to thank Prof. Dr. Zdzislaw E. Sikorski, the editor of the Chemical and Functional Properties of Food Components series, for his confidence and help with this challenging task. I would like also to thank Prof. Dr. Horst A. Diehl for his valuable suggestions and help in revising chapters. I am grateful, too, to my son, Michael Socaciu, MD, for his skillful technical help.

Prof. Dr. Carmen Socaciu

Editor

Carmen Socaciu was born in Cluj-Napoca, Romania and earned a BSc in chemistry in 1976, an MSc in 1977, and a PhD in 1986 from the University Babes-Bolyai in Cluj-Napoca, an important academic centre located in the Transylvania region. Dr. Socaciu worked as a researcher in medical and cellular biochemistry for more than 10 years, and became a lecturer in 1990 and full professor in 1998 in the Department of Chemistry and Biochemistry of the University of Agricultural Sciences and Veterinary Medicine (USAMV) in Cluj-Napoca. She extended her academic background in pure chemistry (synthesis and instrumental analysis) to the life sciences (agrifood chemistry and cellular biochemistry). Her fields of competence are directed especially toward natural bioactive phytochemicals (carotenoids, phenolics, flavonoids), looking to advanced methods of extraction and analysis and to their *in vitro* actions on cellular metabolism, their effects as functional food ingredients, and their impacts on health.

Dr. Socaciu held post-doctoral positions and research fellowships with outstanding European university groups including the University of Bordeaux I, France (Prof. Dr. M. Gleizes, Plant Cellular Physiology, 1991 and 1992), University of Bern, Switzerland (Prof. Dr. H. Pfander, Institute of Organic Chemistry, 1991 and 1998), University of Liverpool, United Kingdom (Dr. G. Britton, Biochemistry, 1998), and University of Bremen, Germany (Prof. Dr. Horst A. Diehl, Membrane Biophysics, 2000 through 2005). Since 2000, Dr. Socaciu has served as a PhD supervisor in food biotechnology, a scientific counsellor on the Faculty of Agriculture, and a member of the Senate of USAMV. In 2001, she was named the director of the Research Centre on Chemistry and Biochemistry of Plant Pigments, at USAMV, a centre authorized by the Romanian Council for Higher Education and Research.

Dr. Socaciu has been a director or partner in about 20 international educational or research programs (NATO, Erasmus, EU FP5 and FP6 programs, and bilateral international research collaborations with many European groups). She has also been the leading investigator named on more than 20 national grants, all focused on plant and food pigments, valorization of phytochemicals from food waste, and characterization of antioxidant phytochemicals from vegetables and fruits. She is an active member of many national and international scientific societies, serves on editorial committees, and acts as an evaluator of research projects at national and European levels.

She coordinates an international master's program in food quality, manages Romanian participation in the EU COST 926 Action program titled "Impact of New Technologies on the Health Benefits and Safety of Bioactive Plant Products" (2004 through 2010), and represents her university in the EU Socrates Thematic Network's Integrating Safety and Environmental Knowledge into Food Studies (ISEKI) program intended to achieve sustainable development in the EU. She has presented nearly 200 publications at conferences, in scientific journals, and as contributions to books.

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Section 1

1 Physics of Color

Horst A. Diehl

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1.1 INTRODUCTION

With regard to choice and consumption of food, all human sensory perceptions are involved. Among them, vision is the most important one for selecting food and appreciating its quality. Color is an intrinsic property of food. A color change of food often is caused by a quality change. Consumers are attracted by the color of a food product. This implies three main consequences for food producers:

1. Food quality should be controlled by optical inspection.
2. Food processing steps may change food color.
3. Colorants may be added to food as preservatives or simply to attract consumers.

Intrinsic food colorants can be conserved more or less during food processing. The pigments that color the original living biological material often possess essential functional properties like anti-oxidative effects, radical scavengers or are transmitters of signals or energy. In this way, intrinsic food colorants are involved in synergistic effects that they perform as components of molecular complexes. These supra-molecular structures may, at least partly, be disturbed during food processing. Visual inspections cannot evaluate those functional properties and rarely distinguish between intrinsic and added food colorants. However, spectroscopic methods allow qualitative examinations. Therefore, the evaluation of food color is an essential topic in food technology.

To approach the topic, we review in Section 1.2 the roles of light and colors in nature. Basic optical phenomena are shown to modulate the color impressions of biological objects. A more extended description is dedicated to the physical basis of light and color in Section 1.3. While the whole electromagnetic spectrum is considered, the visible part receives the most emphasis. It should be pointed out that the invisible parts of the electromagnetic spectrum also affect food colorants. Moreover, essential methods to analyze food colorants and use food colorants as labels to investigate structure and quality of food are run on instruments working in these spectral regions. The dual nature of light is considered with regard to its use for inspecting food and its destructive effects on food colorants. The relevant spectroscopic methods are presented. Section 1.4 deals with the physical instrumentation for analyzing light and color and discusses practical, well-defined methods that, if correctly applied, reveal reproducible results. However, for physiological and psychological reasons, the perception of color by humans differs among individuals; and one individual's perception may vary, depending on background and subjective personal conditions. Section 1.5 explains individual perceptions of color and brightness and the problems of standardization of individual perceptions. Standardization in terms of light absorbance, light reflection, light scattering, and light detection has been developed and will be reported in this chapter. Standardization in terms of human color perception cannot be performed yet as generally as is possible with physical spectrometry. This chapter also summarizes the basic problems that determine the colorimetry of foods and explains that colorimetry is a kind of spectrometry that combines physical and biological aspects.

1.2 ROLE OF LIGHT AND COLOR IN NATURE

Light is the primary carrier of photosynthetic energy and also the initial producer of natural food colorants. To speak about the color of an object is to speak simultaneously about the illuminating light source, light transmitting medium, object properties, eye sensitivity, and conventions about color scales. Teleologically viewed, food color has two ambivalent main functions:

1. To mediate attraction to symbiotically acting living beings (e.g., bees)
2. To serve as an indicator of appropriate food sources (mainly for animals) and to assess food quality (mainly human perceptions)

Let us for the moment restrict our considerations to the objective properties. We have to take into account two main physical phenomena:

1. According to the laws of geometrical optics, we have to deal with reflection, refraction, transmission, and absorption of light by biological matter. These effects are determined by the surface of a sample and by the properties of the bulk material that usually is inhomogeneous and thus provides a kind of internal surface. Visible light irradiating a food species will, at least partially, be reflected by the surface itself and, if the species is partially transparent, also be reflected from inner surfaces. The light

reflected from inner surfaces travels a longer distance and interference with the surface reflected wavelengths may occur and cause color impressions to the onlooker. The effect depends strongly on the conditions of irradiation and the viewer's position.

2. Many biological objects modulate the light in a way that is noticed preferentially by certain species and thus determines the behavioral ecology of that species. The spectral sensitivity of the retinal light receptors is a species-bound property. Spectral light modulation happens because of a spectral dependence of the reflected light on the angle of light reflection or by fluorescence effects. The physical phenomenon behind this dynamic is the interference of light waves during internal reflections that typically occurs in the feathers of birds and in the armored shields of insects. An impressive example is the throat of the purple-throated mountain gem (*Lampornis calolaema*), whose reflected color depends strongly on the angle under which it is observed.¹ Another spectral light modulation occurs in the case of fluorescent matter. When an object is illuminated by ultraviolet (UV) light, a bright fluorescence emission in the visible spectral region may appear. This has impressively been demonstrated with another bird, the budgie (*Melopsittacus undulatus*), under different light conditions.¹

The same properties hold for colorants in food: interference colors, illumination conditions, and fluorescence partially determine the appearance of food.

The laws of geometrical optics strongly determine the appearance of food and food colorants, depending in detail on the transparency of the matter and how homogeneous it is.

The spectral variations caused by the interference phenomena become relevant when a food contains tightly adjoining dense structures like feathers, fish scales, or the shells of crustaceans.

Natural food and food colorants show weak or no fluorescence. But food may be incorporated easily by fluorescing pigments that impart bright colors to the matter when it is irradiated by blue or UV light, where usually fluorophores are excited to emit light in the visible region. Comparing food colors under daylight and under UV light helps to identify artificial color additions.

1.3 PHYSICAL NATURE OF LIGHT AND COLOR

About 100 years ago, Albert Einstein established the modern understanding of light and color. Based on it, up to now, tremendous development of optical technologies including laser technology and color analysis methods has taken place. Also, the interaction of light with biological matter evolved since then from an empirical description to a basic understanding. The interaction of light with inorganic and organic matter follows the same laws.

Basic biologic processes like photosynthesis and vision are fairly well understood. However, the perception of light by individuals is not easy to describe in physical terms because the light receptors differ considerably among species and

individuals. The sensory impressions of light and color by individuals cannot be standardized completely. Consequently, the automation of sensory visual evaluation is limited to cases of exactly reproducible frame conditions. Objects that are substantially identical but show varying surface ripple structures or contain varying degrees of moisture will yield different results. Light shining on an object may be diffuse or more focused, or the angle between the illuminating light beam to the object and the light beam from the object to the viewer's position may change. All these factors will impact error in measurements. In this section, we deal only with the basic physical properties of light.

1.3.1 DUALISM OF LIGHT AS WAVE OR ASSEMBLY OF PHOTONS

Light and color are fascinating to mankind. Many philosophies and theories related to light and color have arisen over the years. Quantum theory gave us a complete description but the theory is beyond our ability to visualize. In both experiments and in our daily experience, light shows one of two faces: an electromagnetic wave or an assembly of particles known as “photons.” The effects of light on food colorants and on their chemical and functional properties in foods follow that dichotomy. Light transmits energy to food. This can cause a warming effect based on the wave nature of light or the quantum nature of light can quickly destroy molecular complexes or even molecules. Detecting and analyzing methods are based on these principles. The most important techniques are absorption and reflection spectrometry in the visible and ultraviolet regions, light scattering, infrared spectroscopy, Raman spectroscopy, fluorescence spectroscopy, electron spin resonance spectroscopy, nuclear magnetic spectroscopy, x-ray diffraction and neutron diffraction.

What visualization can we get from *light as an electromagnetic wave*? An electromagnetic wave is not like a surface wave on a pond if we throw a stone into it, or a material wave-like sound. It consists of rapidly oscillating electric and magnetic fields that are strictly coupled to each other and travel with light velocity. The oscillations of the electric and magnetic field take place with the light frequency, which is in the order of magnitude of 10^{15} Hz. For simple situations, Figure 1.1 shows a snapshot of how the electric and magnetic field are correlated to each other for only half a wave period. For the next half wave period, both fields change their signs. Movable charges of molecules (electrons or ions) will allow the electromagnetic wave to enter if the charged particles are able to move with the frequency of the wave. Consequently, the frequencies at which the electric molecular charge can oscillate will be absorbed from the light. This is the “classical” visualization of colorimetry. Food colorants usually are pigments that contain conjugated double bonds and conjugated double bonds contain mobile electrons and therefore are easily detectable by light absorption and related effects.

The visualization of light as an *assembly of photons* moving with light velocity dates back to Isaac Newton and was formulated quantitatively by Max Planck and Albert Einstein. Formula [1] below connects basic physical values:

$$E = h \nu \quad [1]$$

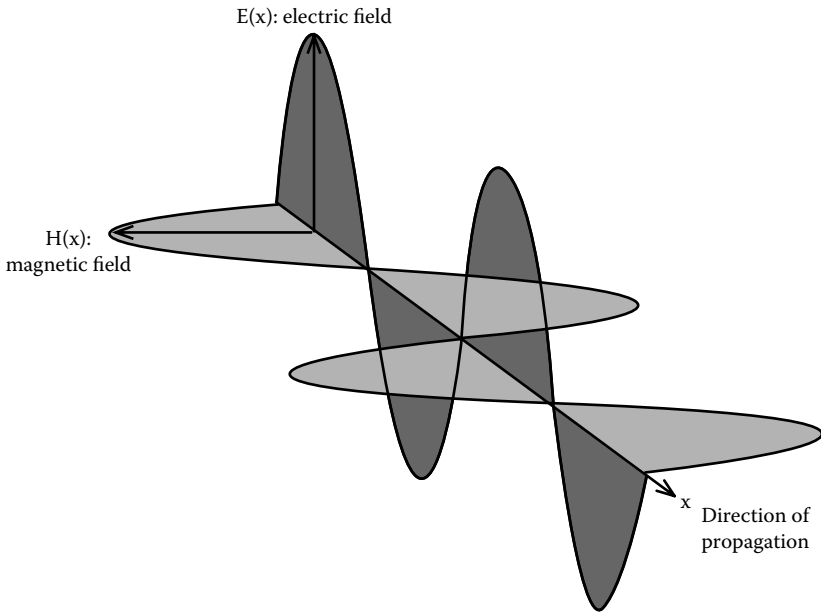


FIGURE 1.1 Electromagnetic wave. At any time the elongations of the electric wave $E(x)$ and of the magnetic wave $H(x)$ into space appear perpendicular to each other. The figure shows two full periods of the electromagnetic wave.

where E is the energy of a single photon, h is a natural constant named Planck's constant ($h = 6,626 \times 10^{-34}$ Js), and ν is a frequency strictly correlated to a monochromatic color — the frequency which in the imaging of light as a wave represents the frequency of the oscillating wave. Basic relation [2] below determines the counter-correlation of light wave frequency ν and light wavelength λ :

$$c = \lambda \nu \quad [2]$$

where c is the velocity of light ($c = 299792$ km/s).

As an example, we look to lutein, a natural colorant that is the ubiquitous main carotenoid in chloroplasts of all green plants and also is found widely in algae, fruits, and flowers. Its spectral absorption peaks at about 445 nm. From formula [2], we calculate the corresponding wave frequency to be $6.74 \times 10^{14} \text{ s}^{-1}$. With this we obtain from formula [1] the quantum energy of one single absorbed photon to be $4.47 \times 10^{-19} \text{ J} = 2.79 \text{ eV}$ (electronvolt). This energy is enough to decompose or photodissociate biological molecules. However, lutein is stable and its presence in plant cells and in mammalian retinal pigment epithelial cells, where it concentrates via food in a unique way, provides a very effective protection against violet and ultraviolet radiation. The absorbed energy does not decompose the lutein molecule. It is dissipated over the many accessible vibrational energy levels of the molecule and thus it relaxes to its unexcited state ready for a new absorption. This provides a protection of the retina against UV irradiation. Lutein, like other carotenoids, is also active in

scavenging organic radicals that have been produced by photons or by chemicals. In these situations, lutein molecules are decomposed and must be replaced from the food cycle.

The long wavelength absorbances of colorants (in the red and infrared spectral regions) lead to a warming effect of the biological matter. This provides good growing conditions in living matter and causes decay in prepared food.

1.3.2 ELECTROMAGNETIC SPECTRUM OF LIGHT WITH REGARD TO ITS IMPACT ON MATTER AND ITS BASE FOR ANALYTICAL TOOLS

We recognize food colorants only because of their interactions with visible light. Considering their chemical and functional properties, we must be aware of the fact that the human eye has a very limited ability to sense light and that the interactions of foods and food colorants with light are not limited to these boundaries. This must be taken into account, particularly because under both, natural light (sunshine) and artificial light (incandescent lamps), “visible light” and “not-visible light” commonly appear. The whole range of light frequencies is called the *electromagnetic spectrum of light*. It is presented in Figure 1.2; the small range of visible light is marked. The methods used to detect food colorants and study their impacts on foods use the full electromagnetic spectrum. The impacts of the different regions of the electromagnetic spectrum on food quality and food colorants increase with quantum energy and light intensity.

Based on the scheme of Figure 1.2, we present the most important effects and methods. We start from the long wavelength side, the *radio frequencies*, where the wavelengths are in the order of magnitude of meter to kilometer. These waves do not have an impact on food colorants and foodstuffs. Their quantum energies ($<10^{-23}$ J) are far too low to cause molecular stress. Only at very high intensities, near those of an emitting radio antenna, a weak warming of matter may take place. For

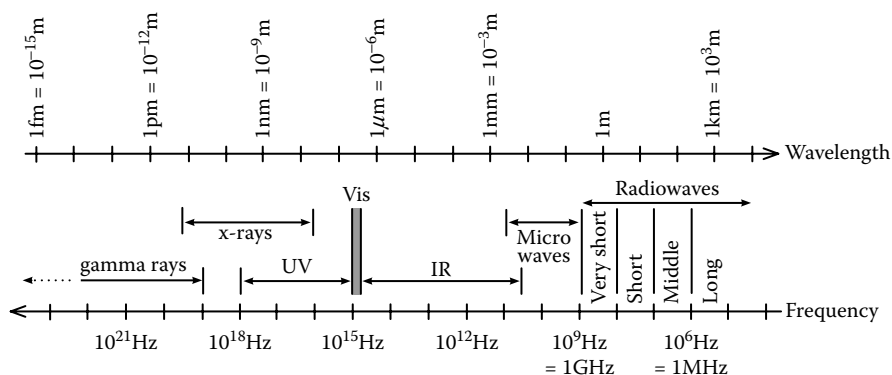


FIGURE 1.2 Electromagnetic spectrum. The upper scale shows the wavelength range and the lower scale the frequency range of the electromagnetic waves. The most characteristic denominations of the spectral regions are plotted between them. The small shadowed area between UV (ultraviolet) and IR (infrared) is the visible region to which the human retina is sensitive.

analytical purposes, radio waves are eminently important because nuclear magnetic resonance (NMR) spectroscopy is based on them. Those nuclei that have nuclear momentum (unpaired protons) are able to absorb radio waves if they are brought into a strong magnetic field. The isotopes of this kind that are of biological relevance are the proton ^1H , the carbon isotope ^{13}C , the oxygen isotope ^{17}O , and the widely naturally abundant phosphorus isotope ^{31}P .

The intensity of the absorption of microwave energy is a measure of the abundance of that isotope. The potency of the NMR spectroscopy is not only its ability to quantify the concentration of an isotope, but to check the environment into which an isotope is embedded. This is possible because the magnetic resonance and thus the absorption frequency prove to be sensitive to the spins of neighboring atoms and to structural features of the probe. Therefore, NMR spectroscopy is more a tool for scientific structural analyses than for daily food (colorant) inspection. For a detailed study of the NMR techniques used in food science we recommend books by Macomber and Pochapsky.^{2,3}

The frequency range of short radio waves overlaps with that of long wave microwaves — from about 1 m to about 100 μm . Microwaves are of high technical importance. They cover the radar frequencies and the frequencies at which cellular (mobile) telephones work. Their impacts on food colorants, foods, and biological materials are similar to those of radio waves but the warming effect is more distinct. If microwave use is limited to the topics discussed in this book, no precautions are necessary.

While nuclear magnetic resonance spectroscopy is based on radio waves, electron spin resonance (ESR) spectroscopy, also known as electron paramagnetic resonance (EPR) spectroscopy, is based on microwaves. Like NMR, ESR is of more importance as a scientific tool than as a routine instrument for analyzing food colorants. Microwaves can be absorbed by paramagnetic molecules if they are brought into a proper magnetic field. Paramagnetic molecules, like transition metals, possess unpaired electrons, in their electron shells. For example, colorants, free radicals originating from photoreactions or preservatives, and incorporated spin labels that are stable organic radicals bound to biomolecules can be used to trace metabolism or to check the efficiencies of radical scavengers.

Biological free radicals occur as more or less stable intermediate products during many enzymatic reactions. Flavin and heme radicals and porphyrin molecules are relevant examples of food colorants. Extended literature surveys are available.^{4,5}

In Figure 1.2, the shorter spectral wavelengths lead to the infrared (IR) region, that extends from about 100 μm down to about 1 μm . Because the quantum energy is still low in this region, about 10^{-18} J, no molecular destruction is caused, but this is the spectral region where thermal energy transition is most relevant. By far, most of the energy from the sun arrives via the IR region of the spectrum. Analytically, IR absorption spectroscopy has outstanding relevance for evaluating the molecular compositions of biological matter. The molecular excitation is achieved via energy absorption by intra-molecular vibrations. The only vibrations that can be stimulated are those that change the dipole moment of a molecule. Therefore homo-nuclear molecules like O_2 or N_2 are not infrared-active, but most functional

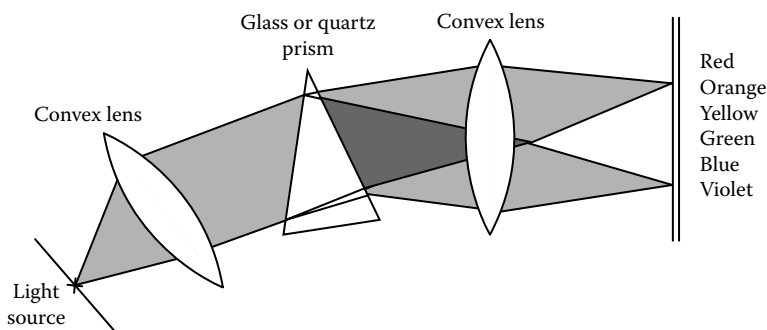


FIGURE 1.3 Light dispersion by a glass or quartz prism. Violet light is deflected most and red light is deflected least from the direction of the original polychromatic light beam. If the prism monochromator is replaced by a grating monochromator (not shown), the deflection order of the monochromatic light is the reverse.

groups of biomolecules do not have symmetrical centers and can be investigated by IR spectroscopy.

Modern IR spectrometers and also Raman spectrometers (see below) are constructed as Fourier transform spectrometers and called FT-IR or FT-Raman spectrometers. They do not contain light dispersive elements like prisms (Figure 1.3) or gratings that are used in the UV-Vis spectral region. The essential part of an FT spectrometer is a Michelson interferometer that does not produce an absorption or reflectance or scattered light spectrum of the sample but an interferogram containing the corresponding spectral characteristics of the sample. This interferogram is automatically analyzed by a Fourier transform operation, a purely mathematical procedure. Compared to conventional dispersive spectrometers, FT spectrometers allow detection of a complete spectrum in an essentially shorter time because all frequencies are measured in parallel. In an FT spectrometer, the exciting light beam is not narrowed by a monochromator slit; therefore a higher light intensity is achieved and the signal-to-noise ratio is enhanced considerably.²⁴

Another challenging analytical technique is attenuated total reflectance (ATR) spectroscopy, which has been successfully applied in the IR spectral region and also in the visible region. The physical principle of ATR is based on the fact that a light beam in a medium with a high refraction index, e.g., a diamond crystal will be totally reflected at the crystal surface when it strikes the surface. If the crystal surface is in tight contact with a substrate to be analyzed, the striking light wave will enter the substrate but only to a depth that corresponds to a small fraction of the light wavelength. This way the light beam is partially absorbed by the substrate and the corresponding spectral pattern is negatively imprinted onto the totally reflected light beam. ATR-FT-IR spectroscopy is currently the most effective method for online *in situ* analysis. Simultaneous qualitative and quantitative detections are possible.²⁵

Raman spectroscopy works in the same spectral region as infrared spectroscopy but their physical principles and applicabilities are fundamentally different. Com-

plementary types of molecules can be “seen” by Raman V and IR spectroscopy: Raman spectroscopy senses those molecules that do not change their dipole moments under vibration but change their polarizability. This means that molecules such as O_2 and N_2 that are not seen by IR spectroscopy can be seen by Raman spectroscopy. Different vibrations from the same molecule may be detected by IR or Raman spectroscopy, depending on the changing dipole moment or a changing polarizability, respectively, of that vibration in action; e.g., the asymmetric stretching vibration of CO_2 is IR active and its symmetric stretching vibration is Raman active. Therefore both methods complete each other with respect to the recognition and analysis of molecules, especially biomolecules and food colorants.

Raman spectroscopy detects the scattering of light, not its absorption. Superposed on the frequency of the scattered light are the frequencies of the molecular vibrations. The detection occurs in the IR spectral region while the excitation happens in the visible region. Since laser light sources have become well developed, Raman spectroscopy has become an important tool for the analysis of biomolecules.

Raman spectroscopy is well suited for food analysis because the high water content of food that is a disadvantage for IR spectroscopy does not disturb Raman spectroscopy.

With the more advanced technique of resonance Raman spectroscopy, the light source is tuned to the absorption frequency of the molecule and the Raman lines of the molecule are scattered with a high intensity while the other lines remain weak. This is the way chromophores like food colorants allow tracing of material all along a food chain. The Raman spectra of carrots, carrot juice, and pure β -carotene can be followed all along the food chain *in situ*, directly in the plant tissue or food matrix. Many other chromophores can be traced. Mixtures and adulterations of foods like oils, juices, creams and so on can be identified reliably. The fundamentals of IR and Raman spectroscopy,⁶ their characteristic group frequencies,⁷ and a detailed Raman/IR atlas of organic compounds may be of further help.⁸ To analyze substrates, which by visible light may be affected or excited to fluorescence and thus disturb the measurement, near-infrared (NIR) light is used to probe molecular vibrations instead of visible light. NIR-FT-Raman spectroscopy is a fast, safe and non-destructive method to analyze food and food colorants.²³

At shorter wavelengths, the infrared region (Figure 1.2) merges into the visible and further into the ultraviolet region (UV-Vis) of the spectrum. This is the region where both molecular vibrations and also molecular electronic transitions are stimulated. With respect to food colorants, the UV-Vis region is the most relevant one, useful for (1) identifying the pigment, (2) quantifying its concentration, and (3) estimating the food quality.

The pigment identification is performed by the recognition of its characteristic absorption spectra. The pigment quantification is revealed by the extent of absorption (optical density). Each of these properties may be modulated by the direct environment into which the pigment molecule is incorporated. We look at this in some detail.

The most suitable spectroscopic method for food colorants is UV-Vis absorption spectrometry. To quantify the concentration of a food colorant the light transmission, T , must be measured:

$$T = I/I_0 \quad [3]$$

where I_0 is the incident light intensity to the specimen and I is the transmitted light intensity. There is no linear correlation between T and the concentration c of the colorant. Only the logarithm of the reciprocal transmission, $1/T$, is directly correlated to the colorant concentration:

$$\log 1/T = \log I_0/I = E = \epsilon c d \quad [4]$$

The right part of equation [4], $E = \epsilon c d$, represents Lambert-Beer's law. E is called the extinction, c is the substance concentration, and d is the thickness of the sample. The E values span from 0 (this is the case when all light is transmitted and no absorption takes place, i.e., $I = I_0$) to infinity, ∞ (this is the case of maximal extinction when no incident light is transmitted, i.e., $I = 0$). Realistic E values that can be correctly measured by normal spectrometers range between 0 and 2. Instead of using the E expression for extinction, A for absorbance is often used. E and A are dimensionless values, i.e., numbers without units. Nevertheless, OD, the symbol for optical density, is often added to E and A in order to clarify their meanings.

In formula [4] ϵ is the extinction coefficient of the substance. It is a substance-specific property of the dimension [(concentration) $^{-1}$ times (length) $^{-1}$]. Usually c is measured in moles/liter and d in centimeters, corresponding to the light paths in normal cuvettes. Then ϵ is given in the unit mole $^{-1}$ liter centimeter $^{-1}$. For conformity we use this, according to the *Système International d'Unités* (SI); the unit should be given in square meter/mol (m ^{2} mol $^{-1}$). Chemists still like to use another unit system defined as the theoretical absorbance of a solution of 1% (w/v) concentration (i.e., 1 g in 100 ml) in a cuvette of 1 cm path length, often abbreviated as $E_{1\%}^{1\text{ cm}}$. Based on that unit, they give only the numerical value and the unit is kept in mind. To give an example: In the *Carotenoid Handbook* (page 3) you find for β -carotene that A_{2590} is referred to as the specific absorption coefficient.⁹ Accordingly, using the molecular mass 536 for β -carotene, we obtain the extinction coefficient $\epsilon = 158121$ mole/liter/cm. Some call the corresponding numerical value the molar extinction coefficient. Assuming we measure an extinction of $E = 0.1$ OD of a β -carotene solution in a 1 cm cuvette ($d = 1$ cm) we obtain from equation [4] the concentration

$$c = E / \epsilon d = 0.63 \text{ } \mu\text{M} \text{ (M = mole/liter)}. \quad [5]$$

To avoid misunderstandings, the recommendation is to always use complete physical values consisting of a numerical value and the corresponding unit, and in calculations to use the physical values and not only the numerical values.

In colorants, the spectral absorption pattern and the extinction coefficient are determined by the chromophore and rarely by side groups of the molecule. β -carotene and zeaxanthin contain the same chromophore but their molecular masses are 536 and 568, respectively. Therefore $\epsilon_{\text{zeaxanthin}} = 536/568 \epsilon_{\beta\text{-carotene}} = 149213$ mol $^{-1}$ liter cm $^{-1}$. This does not mean that all spectral details are changed only by that factor.

The fine structures of β -carotene and zeaxanthin are different because of different side groups and slightly different electronic configurations.

The extinction coefficients of carotenoids have been listed completely⁹ but solvent effects can shift the absorption patterns. If a colorant molecule is transferred into a more polar environment, then the absorption will be subjected to a bathochromic (red) shift. If the colorant molecule is transferred into a more apolar environment, the absorption will be subjected to a hypsochromic (blue) shift. If a carotenoid molecule is transferred from a hexane or ethanol solution into a chloroform solution, the bathochromic shift will be 10 to 20 nm.

Also bound to the UV-Vis spectral area is fluorescence spectrometry. It is most important with respect to those fluorescent food colorants that have been incorporated into food. In detail it helps to (1) identify a colorant by the spectral pattern of fluorescence excitation and emission spectra, (2) quantify its concentration by the fluorescence emission intensity, (3) qualify the environment into which the colorant molecule is embedded, and (4) perform structural research on the food matter into which the colorant is incorporated.

Molecular fluorescence is observed from molecules that contain aromatic, heterocyclic, and condensed ring systems. Betaxanthins have been identified as pigments responsible for visible fluorescence in flowers.¹⁰ These molecules, after photonic excitation, emit their absorbed excitation energy by photons of a quantum energy that is a bit smaller than that of the exciting photon (Stokes shift). The energy difference of the exciting and emitted photons is dissipated radiationless within the matter, as to a bigger extent it is dissipated from molecules that do not fluoresce. The radiationless molecular energy dissipation activates molecular internal vibrations that serve as the basis for the Raman resonance spectrometry method discussed earlier. The basic physics of fluorescence spectrometry and its biochemically oriented applications have been compiled by Lakowicz.¹¹

To identify a colorant, its excitation and emission spectra must be measured. This can be done under standard conditions if the colorant has been extracted from a foodstuff. Usually the spectral patterns taken from real conditions will not deviate too much from standard conditions. One must be aware that the main spectral patterns are determined by the chromophore of the colorant and that further molecular identification needs to recognize special fine structures of the spectra or employ additional analytical tools.

To quantify the concentration of a colorant, one must consider that linearity between the colorant concentration and the fluorescence emission intensity exists only at very low concentrations. The reason for deviation from linearity may be re-absorption of the emission light by other fluorophores or formation of dimers. If no extraction and controlled dilution of the fluorescent colorant are performed, the colorant quantification will be only qualitative.

To qualify the environment into which the colorant molecule is embedded, the actual fluorescence spectrum is compared with the one under standard conditions. If the fluorescence emission spectrum is shifted to longer wavelengths (bathochromic shift), it can be concluded that the molecular environment is of a more polar nature or is polarized by the excited fluorophore. Conversely, a fluorescence shift to shorter wavelengths (hypsochromic shift) indicates a transfer of the fluorophore from a polar

to an unpolar environment. Moreover, other molecules, whether fluorescent or not, may be present in the food and absorb the emission light of the fluorophore. This environmental property contributes also to the non-linearity between concentration and fluorescence emission intensity.

To perform structural research on a food stuff into which a colorant is incorporated, special properties of fluorescing molecules are exploited: fluorescence efficiency, fluorescence lifetime, fluorescence quenching, radiationless energy (Foerster) transfer, stationary or time-dependent fluorescence polarization and depolarization.¹¹ Generally, if food colorants fluoresce, they allow very sensitive investigations which in most cases cannot be surpassed by other methods.

The most common methods for separating, controlling, and calibrating spectroscopic methods with regard to food colorants are chromatographic methods, e.g., thin layer chromatography (TLC). The combination gas chromatography and mass spectrometry (GC/MS), and high pressure liquid chromatography (HPLC) combined with a photodiode array or fluorescence detector. These have been applied extensively to analysis of natural and synthetic colorants.¹²

In continuation of our tour of the electromagnetic spectrum (Figure 1.2) toward shorter wavelengths, at a range of around 100 nm we arrive at the region of x-rays (quantum energies around 10^{-16} J) and further to γ -rays (quantum energies below 10^{-14} J) — emissions from radioactive material. Here the quantum energies of photons are extremely high compared to the spectral regions we discussed so far. The high quantum energies are destructive to food colorants and thus they are not used to analyze them. Some countries allow food sterilization by x-rays or radioactive rays. Food colorants will be affected by this treatment. Food colorants could even be used to detect those irradiations.

1.4 PHYSICAL DETECTING DEVICES FOR LIGHT AND COLOR

In the preceding section, we presented principles of spectroscopy over the entire electromagnetic spectrum. The most important spectroscopic methods are those in the visible spectral region where food colorants can be perceived by the human eye. Human perception and the physical analysis of food colorants operate differently. The human perception with which we shall deal in Section 1.5 is difficult to normalize. However, the intention to standardize human color perception based on the abilities of most individuals led to a variety of protocols that regulate in detail how, with physical methods, human color perception can be simulated. In any case, a sophisticated instrumental set up is required. We present certain details related to optical spectroscopy here. For practical purposes, one must discriminate between measurements in the absorbance mode and those in the reflection mode. The latter mode is more important for direct measurement of colorants in food samples. To characterize pure or extracted food colorants the absorption mode should be used.

Any spectroscopy procedure starts with a light source. An ideal source would be a lamp emitting white light, i.e., a source that provides light intensity uniformly

distributed over the whole visible spectral region, but such an ideal light source is not available. Incandescent lamps like tungsten bulbs approach the requirement partially but are insufficient in the blue and UV regions where high-pressure mercury lamps are recommended. High-pressure xenon arc lamps emit well over the whole spectral region, simulating better than any other source the spectral emissions of sunlight. Special light sources applied particularly in colorimetry involve additional filter systems. They will be treated in the next section along with information about human color perception.

To identify a certain pigment, monochromatic light is required, but the assessment of food quality by a human inspector or by a device requires normal daylight. The evaluation procedure, however, to be discussed in the next section, also involves decomposition into monochromatic (red, green, and blue) regions. Monochromatic dissection of a continuous spectrum by monochromators can be achieved in three ways:

1. The light beam passes through a filter system. The filters may be colored glass or special interference filters that allow very narrow spectral cuts.
2. The light enters into a glass or quartz prism (that is also transmissible by UV light) and there it is dispersed into the colors contained in the entering light beam. From the rainbow-like dispersed spectrum, the color wanted is taken by screening off the other colors. The principle of optical dispersion is shown in Figure 1.3.
3. The light beam shines on a diffraction grating. Each line on the grating scatters the light in all directions. By means of geometrical optics (lenses or mirrors), the scattered light is brought to interference, which results in a spectral decomposition of the entered light beam.

To finally detect the light after its interaction with the sample to be evaluated for its spectral absorptivity or reflectivity, optoelectric devices are employed. They transform light signals into electric signals. Two different main types are in use.

Based on the photoelectric effect, electrons in evacuated tubes (photoelectrons) are released from a metal surface if it is irradiated with photons of sufficient quantum energy. These are simple photocells. Photomultipliers are more sophisticated and used in modern spectrophotometers where, via high voltage, the photoelectrons are accelerated to another electrode (dynode) where one electron releases several electrons more, and by repetition up to more than ten times a signal amplification on the order of 10^7 can be obtained. This means that one photon finally achieves the release of 10^7 electrons from the anode, which easily can be measured as an electric current. The sensitivity of such a photomultiplier resembles the sensitivity of the human eye adapted to darkness. The devices described are mainly used in laboratory-bound spectrophotometers.

Solid-state photodetectors are semiconductors. Because of their small size and the fact that they do not need high voltage, they are suited for portable instruments. Several subtypes are in use:

1. Photodiodes are the modern analogues to photocells. They increase their electrical resistance under light impact which, as part of an electric circuit, can be measured easily. Many current instruments display diode arrays instead of a single diode. Tens of photodiodes are arranged in a tight area. They are exposed to the sample bound spectrum where they respond to the color that corresponds to their positions in the diode array. A rapid, periodically performed electrical interrogation of all diodes (sequence periodicity in the order of milliseconds) reveals a quasi-stationary stable spectrogram. More sophisticated than photodiodes are phototransistors. They amplify internally the photoelectric effect, but the sensitivity of a photomultiplier cannot be achieved.
2. Based on the photoresistivity levels of certain crystals like Se, Ge, Si, CdS, and PbS, photoconductors are constructed. Under light impact, they reduce their electrical resistance up to about 10^4 times. They are used in portable instruments like light exposure meters.
3. Based on the photovoltaic effect, photoelements are constructed from crystals like Se, Si, Cu_2O or metallic Cu. On light impact, they create an electric potential by internally separating positive and negative charges. They are actually in use to transform sunlight photons into electric energy and also as light meters.

The devices described are mostly used in portable instruments. For a deeper understanding, extended studies are recommended.^{13,14} After a general review of the most important parts of optical spectrometers, we proceed now to colorimetric particularities.

1.5 INDIVIDUAL PERCEPTIONS OF COLOR AND BRIGHTNESS AND STANDARDIZATION PROBLEMS

About 7% of men and 0.5% of women perceive colors in significantly different ways and individuals do not perceive colors identically. Older people perceive colored surfaces as having less chromatic content than do younger ones. For this reason, human communication about colors is based on different codes.

Nevertheless, a mutual understanding about colors does work because common rules have been implemented by education, habituation, socially approved behavior, and properties that appear to individuals simultaneously, e.g., the vertical signal order of traffic lights. However, those rules are of limited value when color perception is the base for aesthetic appreciation as is the case for many industrial products and food products. In order to meet the demands of as many consumers as possible, producers look for a “standard consumer” who is most representative of the group. This requires establishment of a reliable measurement procedure that can be reproduced easily and be adapted to the various conditions under which it is applied: light conditions, more or less opaque or translucent objects, object surface structures, etc. These measurement procedures were created more than a century ago and have

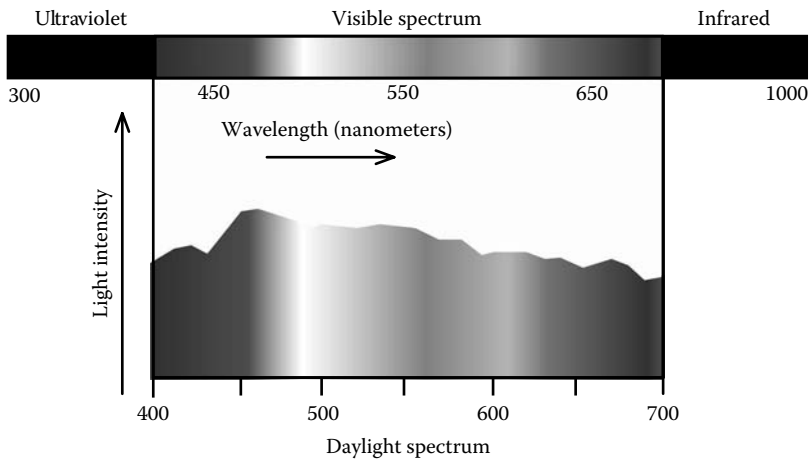


FIGURE 1.4 Daylight spectrum at noon. The maximum intensity is in the green spectral region where the human retina is most sensitive. (Adapted from HunterLab Version 1.4, 12.)¹⁸

been revised several times in order to become more generally applicable to reality. Before we deal with the state of the art, we shall deal with the nomenclature in the field. The notions used in literature are not always consistent.

The principal attributes of object colors are hue, lightness, and saturation.¹⁶ *Hue* is the quality that normally identifies a color such as red, green, and blue. *Lightness* is related to the paired connotation of light and dark when considering color as a source of reflected light. Lightness is the light reflected by a surface in comparison to a white surface under a identical illumination. A related term is *brightness*, a term that designates total light from an illuminated object or reflected from a surface. Lightness and brightness are grouped into a single term called *value*, although the lightness and value terms are commonly used interchangeably.

Saturation is the clarity of a color. It can also be understood as the intensity of hue in comparison to brightness. A saturated color looks clear and bright; an unsaturated color appears pale, muddy, or dull.

Our short review of standardization procedures starts again with illumination. Figure 1.4 shows the dispersed spectrum of the light source which is most relevant for observers: daylight. Most instruments use incandescent lamps that emit very weakly in the blue and UV regions and are therefore well suited in situations where no fluorescence should be excited.

The object under test may be examined by its absorbance or reflectance of light.

Any color signal received from the subject will be rated by the spectral sensitivity of the eye. The physiological bases for this are three types of cone-shaped receptors on the human retina that are sensitive to red, green, and blue. The color measurement techniques have been established on this tri-colorimetric system. They simulate human perception by the relative extent to which the observer estimates the relative share of which of these three components contributes to his color impression from an object.

Under the guidance of the International Commission on Illumination (Commission Internationale de l'Eclairage or CIE), several color description systems and notions have been developed and have established the present procedures:

- The CIE system (1931) introduced the standard observer looking at an object under an angle of 2° . At that time it was thought that the cones were mainly located in the foveal area of the retina. After realizing that the cones were spread beyond the fovea, the experiments were repeated and resulted in observation of an object by a standard observer under an angle of 10° . Later CIE systems were refined from there. Figure 1.5 demonstrates these geometrical conditions and the corresponding tri-stimulus values. The 10° standard observer is recommended for its better

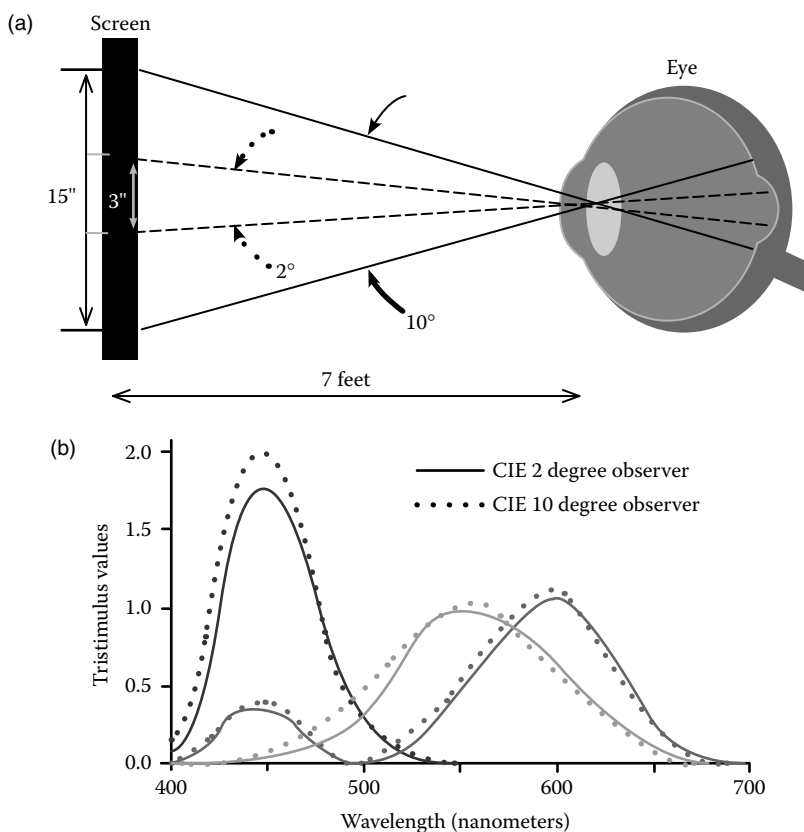


FIGURE 1.5 Two standardized geometrical conditions under which the human eye is disposed to observe light impressions from colored objects. (a) The angles of 2° and 10° under which the resting eye is supposed to register a colored object. (Adapted from HunterLab Version 1.4, 37).¹⁸ (b) The corresponding “tristimulus” eye sensitivity (red, green, blue) according to the standardizations plotted in (a). (Adapted from HunterLab Version 1.4, 36.)¹⁸

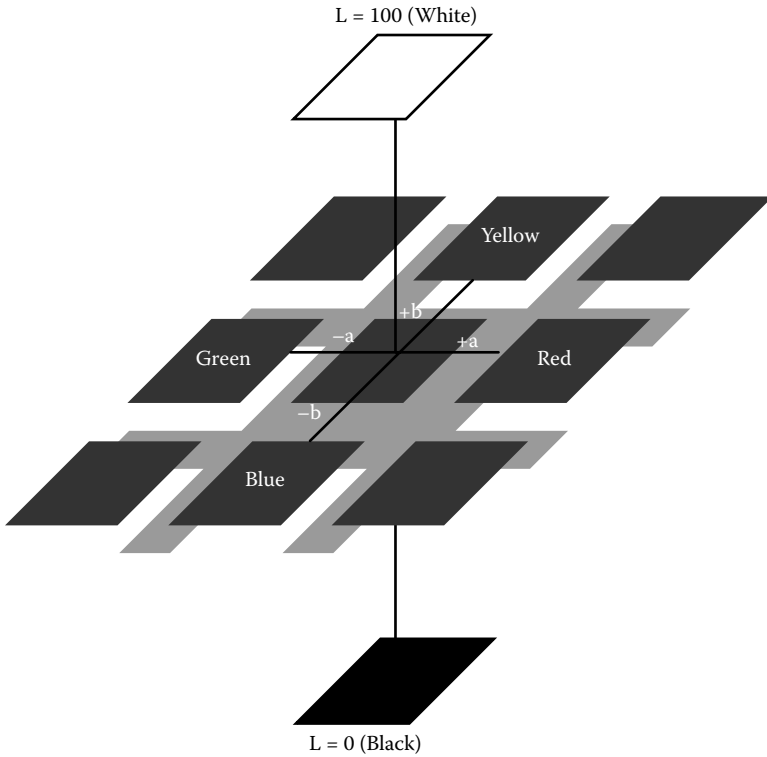


FIGURE 1.6 Human color perception according to the opponent color theory. The description establishes a three-dimensional space where any color perception must be coordinated to each of the three notion pairs: white–black, red–green, and yellow–blue. L (lightness) axis: 0 = black, 100 = white. a = (red–green) axis: red has positive and green negative values. b (blue–yellow) axis: yellow has positive and blue negative values. (Adapted from HunterLab Version 1.4, 63.)¹⁸

correlation with average visual assessments made with large fields of view, typical for most commercial applications.

- The HunterLab system (1958) was the first to use the opponent color theory stating that the red, green, and blue cone responses are re-mixed into opponent coders as they move up the optic nerve to the brain.¹⁷ Based on that theory, the HunterLab color space is three-dimensional and rectangular¹⁸ (see Figure 1.6).
- The CIELAB system (1976) strictly standardizes the light source and the observer. CIE recommends three standard sources, A is an incandescent lamp, and B and C are lamps provided with different two-cell Davis-Gibson liquid filters that simulate noon daylight and average daylight, respectively.^{16,20} Since the main object of the system is to obtain colorimetric results for normal tri-chromats (people with normal color vision), the standard observer must represent the human population with normal

color vision. To determine a standard observer, the procedure is to check 15 to 20 people who have normal color vision and are subjected to a defined experimental test. The mean value is taken as the standard observer. Further system improvements have been made with regard to the color difference formula (CIE, 2000) and with regard to chromatic adaptation formulae to describe color appearances under different viewing conditions.^{20,21}

All these systems have been put into mathematical formulae that can be transferred to each other, but possibly different measuring conditions must be taken into account. The British Standards Institute and the International Standards Organization have edited general guidance and test methods for the assessment of the colors of foods.²²

REFERENCES

1. Pohland, G. and Mullen, P., Farben aus der Vogelperspektive, *Biol. Uns. Zeit*, 2, 131, 2005.
2. Macomber, R.S., *A Complete Introduction to Modern NMR Spectroscopy*, 1st ed., John Wiley & Sons, New York, 1998, 382.
3. Pochapsky, C. and Pochapsky, S., *NMR for Physical and Biological Scientists*, Garland Science, London, 2006, 350.
4. Gerson, F. and Huber, W., *Electron Spin Resonance Spectroscopy of Organic Radicals*, 1st ed., Wiley-VCH, Weinheim, 2003, 464.
5. Rhodes, C.J. (Ed.), *Toxicology of the Human Environment: The Critical Role of Free Radicals*, Taylor & Francis, London, 2000, 512.
6. Wartewig, S., *IR and Raman Spectroscopy, Fundamental Processing, Spectroscopic Techniques: An Interactive Course*, Wiley-VCH, Weinheim, 2003, 175.
7. Socrates, G., *Infrared and Raman Characteristic Group Frequencies*, John Wiley & Sons, New York, 2004, 366.
8. Schrader, B., *Raman/Infrared Atlas of Organic Compounds*, 2nd ed., Wiley-VCH, Weinheim, 1989, 1118.
9. Britton, G., Liaaen-Jensen, S., and Pfander, H. (Eds.), *Carotenoids Handbook*, Birkhäuser Verlag, Basel, 2004.
10. Gandia-Herrero, F., Escribano, E., and Garcia-Carmona, F., Betaxanthins as pigments responsible for visible fluorescence in flowers, *Planta*, 222, 586, 2005.
11. Lakowicz, J.R., *Principles of Fluorescence Science*, 2nd ed., Kluwer Academic/Plenum, New York, 1999.
12. Gennaro, M.C., Abrigo, C., and Cipolla, J., High-performance liquid chromatography of colours and its relevance in forensic chemistry, *J. Chromatogr.*, 674, 281, 1994.
13. Banwell, C.N. and McCash E.M., *Molekülspektroskopie*, R. Oldenbourg Verlag, München, 1999, 417 (translated from *Fundamentals of Molecular Spectroscopy*, 4th ed., McGraw-Hill International, U.K., 1994).
14. Schmidt, W., *Optical Spectroscopy in Chemistry and Life Sciences*, 1st ed., Wiley-VCH, Weinheim, 2005, 370.
15. Hammes G.C., *Spectroscopy for the Biological Sciences*, 1st ed., Wiley-VCH, Weinheim, 2005, 184.
16. Delgado-Vargas, F. and Paredes-Lopez, O., *Natural Colorants for Food and Nutritional Uses*, CRC Press, Boca Raton, FL, 2003, chap. 2.

17. Derrington, A.M., Krauskopf, J., and Lennie, P., Chromatic mechanisms in lateral geniculate nucleus of macaque, *J. Physiol.*, 357, 241, 1984.
18. Hunter, Richard S. 2001. The Basics of Color Perception and Measurement. Hunter Associates Laboratory, Inc., Ed., January, 2006. <http://www.hunterlab.com/pdf/color.pdf>.
19. Billmeyer, F.W. and Saltzman, M., *Principles of Color Technology*, John Wiley & Sons, New York, 1981.
20. Luo, M. R., Cui, G., and Rigg, B., The development of the CIE 2000 colour-difference formulae: CIEDE 2000, *Color Res. Appl.*, 26, 340, 1986.
21. Li, C., Luo, M.R., Rigg, B., and Hunt, R.W.G., CMC 2000 Chromatic adaptation transform: CMCCAT2000, *Color Res. Appl.*, 27, 49, 2002.
22. British Standard Institution, *Methods for the Sensory Analysis of Foods*, BS 5929-10:1999/ISO11037:1999, London, 1999.
23. Schulz, H., Baranska, M., and Baranski, R., Potential of NIR-FT-Raman spectroscopy in natural carotenoid analysis, *Biopolymers*, 77, 212, 2005.
24. Gremlich, H.U. and Yan, B., *Infrared and Raman Spectroscopy of Biological Materials*, Marcel Dekker, Inc., Basel, 2000.
25. Mirabella, F.M., *Modern Techniques in Applied Molecular Spectroscopy*, John Wiley & Sons, New York, 1998.

Section 2

Biochemistry of Color: Pigments

2.1 Chlorophylls: Properties, Biosynthesis, Degradation and Functions

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Patrícia Sinnecker*

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2.1.1 INTRODUCTION

The chemistry and elucidation of the structures of chlorophylls have received much attention for a long time, not only because of their significance as colorants but also because of their importance in living systems. Chlorophylls are the most abundant natural pigments and are the sources of the green color in all plants, algae, ferns, mosses, and some bacteria that are able to capture light energy for photosynthesis. Chlorophylls were first documented in higher plants in early 1818 by Pelletier and Caventow and isolated by Sorby in 1873.¹ In 1913, Willstätter and Stoll published a monograph summarizing most of the research findings of Willstaetter and collaborators and ushered in the modern era in the field of chlorophyll chemistry. They established

completely conclusive investigations stating that porphyrins and chlorophylls are chemically related pigments with the same basic cyclic tetrapyrrole structure. Since these pioneer works, the number of known chlorophyll-like structures has increased steadily. Several comprehensive published reviews have cited the most up-to-date status regarding the constitutions, chemical properties, and occurrences of these compounds.¹⁻⁴

The presence of tetrapyrrole structures in the simplest living organisms all the way through higher plants and mammals evidenced by the phylogenetic reconstruction of evolution provides an insight into their importance in cellular metabolism. They are active in photosynthesis, electron transfer, transportation of oxygen and other diatomic gases, protection from reactive forms of oxygen, and this could explain their universal distribution. Naturally occurring tetrapyrroles found in all organisms can be discriminated into two major classes of pigments according to their chemical structure: (1) linear, as in phycobilins (orange/red and blue) or (2) cyclic, as in chlorophylls (green) and haem (pink or red). All cyclic and most linear tetrapyrroles are thought to be linked to proteins that help stabilize the chromophores. Consequently free haems and chlorophylls probably do not exist in living cells, or at least do not accumulate.⁵

Closed ring tetrapyrroles, also named porphyrins, contribute to the greatest range of colors among all classes of natural pigments, from purple and blue protein complexes found in avians and certain algae, to the red haem pigments in the animal and plant kingdom and the blue-green chlorophyll-derived pigments in all photosynthetic tissues. The array of different colors and shades are mostly determined by the structures of the tetrapyrrole rings and the peripheral substitutions and less by the centrally complexed metals. A small group of highly colored pigments known as the phycobilins are open tetrapyrroles covalently bonded to proteins. The detachment from these proteins renders orange-red phycoerythrins and blue phycocyanins that are abundant in algae (Rodophyta and Cryptophyta) and photosynthetic bacteria.

This chapter brings together information concerning structural features, spectral characteristics, distributions, and functions of major chlorophylls in photosynthetic organisms. Other topics discussed include biosynthesis and degradation in senescent plants and ripening fruits and potential biological properties of chlorophylls.

2.1.2 STRUCTURES AND NOMENCLATURE

Chemically, the basic skeleton of a cyclic tetrapyrrole is a large planar ring structure in a symmetric arrangement in which the four pyrrole rings are joined together by methine ($-C=$) bridges and the four nitrogen atoms are coordinated with a central metal atom. The complete carbon numbering of this macrocycle from C-1 to C-20 in a clockwise sequence was introduced in 1980 by the IUPAC-IUB Joint Commission on Biochemical Nomenclature.⁶ This nomenclature has been increasingly implemented by scientists due to its simplicity and precision, replacing the previous numbering system (from 1 to 8 and alpha to delta for the methine bridges) created by Fischer and his colleagues in the first half of the last century. The changes affected not only the carbon numbering but also the pyrrole rings that were previously designated by Roman numerals from I to IV and now are lettered A to D. The IUPAC rule and numbering system has been adopted in this text.

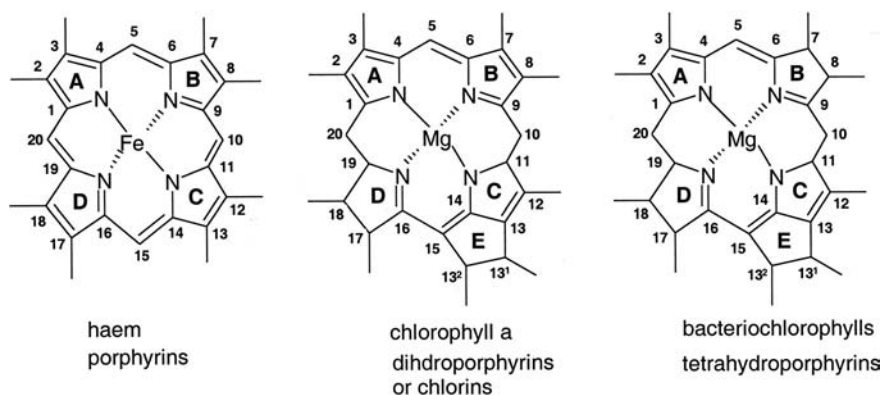


FIGURE 2.1.1 Basic structures of porphyrins, dihydroporphyrins, and tetrahydroporphyrins.

Porphyrinogens are cyclic tetrapyrrole precursors which give rise to three basic groups of molecules, discriminated by their state of oxidation: porphyrins, dihydroporphyrins and tetrahydroporphyrins; the basic structures are depicted in Figure 2.1.1.

Porphyrins have the highest degree of unsaturation among the tetrapyrrole macrocycles. Although fixed double bond positions are shown in Figure 2.1.1, the molecules are resonance hybrids of several possible conjugated double-bond arrangements. They represent the prosthetic group of a large family of proteins that is present throughout the animal and plant kingdoms. Molecules that do not contain metals seem to be precursors or degradation products. Among porphyrins, the most intensively studied molecules have been haemoglobin and myoglobin, the red pigments of blood and muscles, respectively, that are responsible for the transport of oxygen. These pigments are always red or pink due to their common prosthetic group.

Reduced porphyrins are dihydroporphyrins, collectively known as chlorophylls or chlorins, in which the saturated carbon atoms are located between C-17 and C-18 (ring D); thus chlorophylls do not belong exactly to porphyrins, but even so, they still show extended conjugated systems of carbon-carbon double-bonds. Chlorophylls, but not porphyrins like haem pigments, contain an additional fifth isocyclic ring E (cyclopentanone). The two additional carbons of this ring, previously numbered C-9 and C-10 (Fischer system), are now 13¹ and 13². Chlorophyll molecules centrally bind a Mg²⁺ instead of the very reactive Fe²⁺ that is characteristic for haem. If this magnesium ion is replaced by hydrogen, chlorophylls are converted into pheophytins, and when replaced by Cu²⁺, Zn²⁺, or other metal ions, they are transformed into the so-called metallo-complexes. A chlorophyll also differs from a haem pigment by containing a long hydrophobic side-chain at C-17, derived from a monounsaturated isoprenoid alcohol named phytol (C₂₀H₃₉OH), esterified to propionic acid, which is typical for natural chlorophylls in higher plants.

Esterification increases the lipophilic character of the pigments that has been recognized as an important factor for interactions with the peptide chains of proteins.⁷ The hydrolysis of this side chain results in chlorophyllides and the concomitant removal of the Mg²⁺ ion in pheophorbides. Only a limited number of natural chlorophylls in plants and photosynthetic organisms has been described and is well

known; these chlorophylls are known by their trivial names as chlorophyll a, b, c, d, and e, the latter being merely a negligible and less studied derivative. All higher plants, at the onset of the natural process of senescence in leaves and ripening of fruits, contain only chlorophylls a, b, and their respective breakdown derivatives: pheophytins, chlorophyllides, and pheophorbides that are further metabolized into colorless derivatives. Chlorophyll b differs from chlorophyll a by having an aldehyde group ($-\text{CHO}$) at C-7 in place of the methyl group.

The typical isocyclic ring E present in chlorophylls is susceptible to a number of different modifications such as epimerization, which produces stereoisomers by inversion of the configuration at C-13² of their parent pigments. These 13²-epichlorophylls, known as chlorophylls a' and b', are minor pigments. They are considered artifacts produced in the course of handling plant extracts and sometimes are also found in small amounts in heated and deep-frozen vegetables. In the old Fischer system of nomenclature that can still be found in some literature, these epimers were named 10-epichlorophylls.

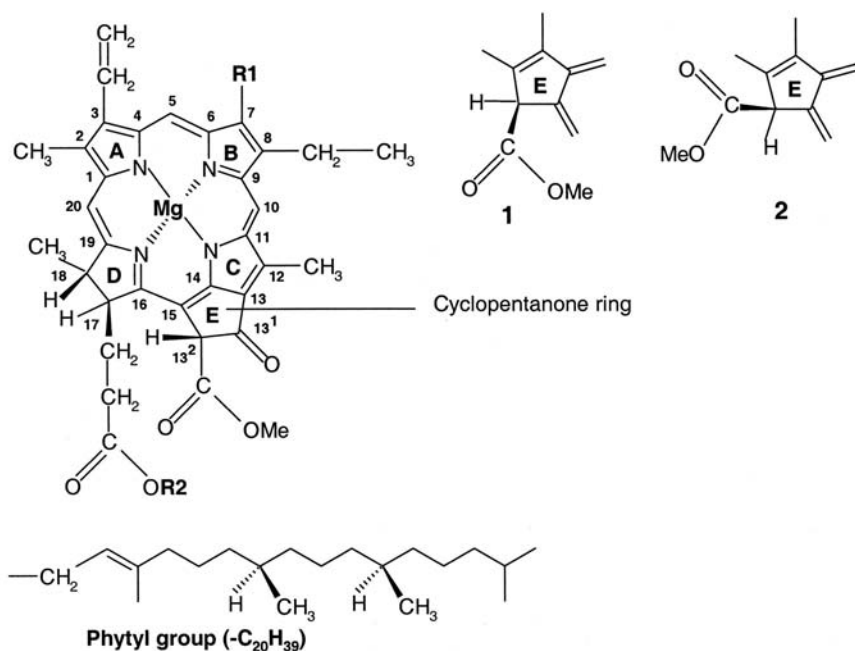
Allomerized chlorophylls are oxidized compounds at C-13², forming the C-13² OH-chlorophyll catabolites. Other common positions for modifications and/or oxidation have been found at C-3, C-7, and C-8. Pyroderivatives of chlorophylls and their degradation products, usually found in heated and processed vegetables, lack the carbomethoxy group ($-\text{COOCH}_3$) at C-13² of ring E, which is replaced by hydrogen.

From these structural features it is interesting to note that each molecule of chlorophylls a and b consists of a hydrophilic part (tetrapyrrole macrocycle) and a hydrophobic portion (long terpenoid chain of phytol esterifying the acid group at C-17). Figure 2.1.2 shows the structures and nomenclature of chlorophylls a and b and their major breakdown derivatives.

Chlorophyll c is represented by a group of pigments with slightly different structures, differentiated according to their chromatographic and spectroscopic properties.⁸⁻¹² The number of representatives in the group of chlorophylls c has increased since the early 1970s. At least five structures have already been described; but the nomenclature is still confusing. Chlorophylls c₁ and c₂ are identical with those of protochlorophyllide and (8-vinyl) protochlorophyllide, respectively, except for the acrylate side chain instead of a phytol at ring D, and some authors suggest that protochlorophyllide c would be a more appropriate name.^{2,7} Despite being named as chlorophylls, they are not chlorins but true porphyrins, because of the unreduced ring D between C-17 and C-18. Most of them show a non-esterified acrylic acid side chain at C-17, which results in a profound change in solubility since unesterified molecules are much more polar. Recently, however, a non-polar structure, esterified with monogalactosyl diacylglycerol instead of phytol has been described.^{11,12}

Chlorophyll d is considered to arise from chlorophyll a, being structurally similar except for the vinyl group at C-3, which is oxidized to a formyl group.¹⁴ This pigment is considered to be a minor constituent accompanying chlorophyll a in some Rhodophyta (red algae) and Chrysophyta (golden algae).¹⁵ However, recently it was discovered that chlorophyll d is the major pigment (> 90%) in free-living Cyanobacteria that lives in visible-light depleted and NIR enhanced environments.

Tetrahydroporphyrins are found only in some photosynthetic bacteria (bacteriochlorophylls) and, besides a reduced ring D, they have an additional reduction at



Compound	Mg ²⁺	R1	R2	Isocyclic ring (E)
Chlorophyll <i>a</i>	+	CH ₃	Phytyl	1
Chlorophyll <i>b</i>	+	CHO	Phytyl	1
Chlorophyll <i>a'</i>	+	CH ₃	Phytyl	2
Chlorophyll <i>b'</i>	+	CHO	Phytyl	2
Chlorophyllide <i>a</i>	+	CH ₃	H	1
Chlorophyllide <i>b</i>	+	CHO	H	1
Pheophytin <i>a</i> *	-	CH ₃	Phytyl	1
Pheophytin <i>b</i> *	-	CHO	Phytyl	1
Pheophorbide <i>a</i> *	-	CH ₃	H	1
Pheophorbide <i>b</i> *	-	CHO	H	1

* In pheophytins and pheophorbides Mg²⁺ is replaced by 2H⁺.

FIGURE 2.1.2 Structural formulas and nomenclature of chlorophylls *a* and *b* and some derivatives. The two positive charges on the central magnesium ion are balanced by two negative charges shared randomly among the four pyrrole-nitrogens. The arrangements of the ten double bonds within the ring may also vary.

ring B between C-7 and C-8. In some photosynthetic bacteria and algal groups, the phytol can be replaced by other alcohols such as geranylgeraniol and farnesol.²

Table 2.1.1 shows the structures and chemical and spectroscopic properties of well-known chlorophylls and bacteriochlorophylls. However, it is likely that more structures (chlorophyll-type pigments) will be discovered with further studies on certain less known algal groups since slight differences in their molecular structures and constituents outside the macrocycle have been identified.

TABLE 2.1.1
Structures and Chemical and Spectroscopic Properties of Major Chlorophylls and Bacteriochlorophylls

Pigment	Absorption Maxima (nm)	Major Occurrence	Color	Functional Group at C-3	Functional Group at C-7	Functional Group at C-8	Functional Group at C-17	C-7-C-8 Bond	C-17-C-18 Bond
Dihydroporphyrins									
Chlorophyll a	432,669 ^a	Universal	Blue-green	-CH=CH ₂	-CH ₃	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-phytyl	Double	Single
Chlorophyll b	459,647 ^a	Land plants and green algae	Green	-CH=CH ₂	-CHO	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-phytyl	Double	Single
Chlorophyll d	445,686 ^b	Some red algae and Cyanobacteria	Blue-green	-CHO	-CH ₃	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-phytyl	Double	Single
Tetrahydroporphyrins									
Bacteriochlorophyll a	365, 772 ^c	Purple bacteria	Grey-pink	-COCH ₃	-CH ₃	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-phytyl	Single	Single
Bacteriochlorophyll b	368, 795 ^d	Purple sulfur bacteria	Brown-pink	-COCH ₃	-CH ₃	=CHCH ₃	-CH ₂ CH ₂ COO-phytyl	Single	Single
Bacteriochlorophyll c	428, 660 ^d	Green sulfur bacteria	Green	-C ₂ H ₃ -OH	-CH ₃	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-farnesyl	Single	Single
Bacteriochlorophyll d	424, 654 ^d	Green sulfur bacteria	Green	-C ₂ H ₃ -OH	-CH ₃	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-farnesyl	Single	Single
Bacteriochlorophyll e	456, 646 ^d	Green sulfur bacteria	Green	-C ₂ H ₃ -OH	-CHO	-CH ₂ CH ₃	-CH ₂ CH ₂ COO-farnesyl	Single	Single
Porphyrins									
Chlorophyll c ^a	446, 578, 626 ^e	Various algae	Yellow-green	-CH=CH ₂	-CH ₃	-CH ₂ CH ₃	-CH=CHCOOH	Single	Double
Chlorophyll c ^b	450, 576, 630 ^e	Various algae	Yellow-green	-CH=CH ₂	-CH ₃	-CH=CH ₂	-CH=CHCOOH	Single	Double
Chlorophyll c ^c	450, 582, 628 ^e	Various algae	Yellow-green	-CH=CH ₂	-COOCH ₃	-CH=CH ₂	-CH=CHCOOH	Single	Double

^a , ^b , ^c , ^d and ^e correspond to solvents: 80% acetone, ether, methanol, acetone and diethyl ether/1% pyridine, respectively.

Original data derived from References 1, 5, 7, 12, 15, 16, 17, 18, and 19.

^{a, b, c, d} and ^e correspond to solvents: 80% acetone, ether, methanol, acetone and diethyl ether/1% pyridine, respectively.

Original data derived from References 1, 5, 7, 12, 15, 16, 17, 18, and 19.

2.1.3 SPECTROSCOPIC PROPERTIES

2.1.3.1 ULTRAVIOLET (UV)–VISIBLE (VIS) ABSORPTION SPECTRA

Intact chlorophyll structures absorb strongly in the red and blue regions of the visible spectrum due to the conjugated double-bond system that imparts a green color to chlorophyll-containing organisms, and molar extinction coefficients vary between 10^4 and 10^5 M/cm. Chlorophylls and their green derivatives are distinguishable from each other according to the specific structural characteristics of the macrocycle, the peripheral groups, and the nature of the central metal ion that may have different effects on each resonance form and strongly influence the profile of their UV-Vis absorption spectra.^{5,16}

The absorbance spectrum of chlorophyll a shows two dominant bands: the Q-band in the 669 nm region and what is known as the Soret band at 432 nm. Although chlorophyll b is similar to chlorophyll a, except for having an aldehyde group in place of the methyl group at C-7, this small structural difference between both molecules generates significant differences in absorption spectra. The absorption maxima of chlorophyll b is shifted toward the green region of the spectrum, showing two dominant bands: one around 644 nm and the other near 455 nm, being responsible for the different green hues of the pigments — blue-green for chlorophyll a and yellow-green in chlorophyll b. If the Soret band in the violet or near-violet region is not detected, porphyrin structures have been broken.¹

Chlorophylls c have characteristic bands between 578 and 630 nm and between 443 and 450 nm that correspond to the Q-band and the Soret band, respectively.¹²

The absorption maxima of chlorophyll d, as expected, is very close to that of chlorophyll a, due to their structural similarity: it has a formyl group instead of a vinyl group at C-3 but is otherwise identical with chlorophyll a.⁷

The spectral characteristics of bacteriochlorophylls differ from each other, depending on their peripheral side chains, and the Q band varies between 646 and 795 nm, while the Soret band ranges between 365 and 456 nm. Bacteriochlorophylls absorb in the infrared, in addition to the blue part of the spectrum.¹⁷

The water-soluble phycobilin pigments, phycoerythrin and phycocyanin, absorb strongly at 495, 540, and 565 nm, and in the 600 to 640 nm region, respectively, indicating that they absorb wavelengths of visible light that are not efficiently absorbed by chlorophylls and carotenoids. Photosynthetic rates are high at these absorption maxima, indicating the unique role of phycobilins as primary light absorbers.¹⁸

Chlorophylls and related compounds are soluble in most organic solvents like acetone, methanol, ethanol, petroleum ether, and diethyl ether due to the hydrophobic character of the phytol chain and of other alcohols, eventually present. Nevertheless, the position of the absorption maxima and the shape of the spectrum can vary by some nanometers depending on the surroundings of the pigments (solvent, temperature, bond to protein, etc.). For instance, the dielectric properties of the organic solvent alter the spectral characteristics of chlorophylls due to hydrogen bonds and dipole–dipole interactions between the solvent–water mixtures, contributing to the formation of aggregates.¹⁹ Consequently, the measurement of pigment concentration requires extraction with a solvent for which specific or molar absorbance coefficients

have been established. A more detailed discussion about quantitative measurements can be found in Chapter 6.

2.1.3.2 FLUORESCENCE SPECTRA

Since chlorophylls absorb light, the energy is communicated to them and the chromophores are lifted from their normal low-energy state to an energy-rich state, which explains their ability to emit photons to de-excite and, therefore, to emit fluorescence. In solution, both a and b chlorophylls are fluorescent, but the fluorescence spectrum of chlorophyll a shows greater sensitivity at the maximum and minimum wavelengths when compared to chlorophyll b, and spectra contain only one main band because the emission always originates from the first excited state. Pigments in diethyl ether solutions are excited at 453 nm at room temperature and the fluorescence emission is measured at 646 and 666 nm for chlorophylls a and b, respectively. Spectra of chlorophylls are affected by temperature, concentration of molecules, and aggregation in solvents.^{1,20} Pheophytins a and b fluorescence spectra are similar to their corresponding parent chlorophylls' spectra.

Other spectroscopic properties such as nuclear magnetic resonance (NMR), mass spectrometry (MS), infra-red (IR), and circular dichroism (CD) spectra of chlorophyll compounds and derivatives have been valuable tools for structural elucidation.^{12,16}

2.1.4 DISTRIBUTION OF CHLOROPHYLLS IN PHOTOSYNTHETIC ORGANISMS

The porphyrins found in fossil fuels, biochemical evidence, and modern phylogenetics all assisted in reconstructing their evolutionary history. Data revealed that plants are descended from multicellular algae and various green algae groups have been proposed to be the ancestors, given that algae dominated the oceans of Precambrian times over 700 million years ago. It is assumed that between 500 and 400 million years ago, some algae became terrestrial by developing a series of adaptations to help them survive on land. Estimates for annual chlorophyll synthesis and degradation range up to 10⁹ tons chlorophyll per year on Earth, of which about one-third is from terrestrial and two-thirds from aquatic (and mainly marine) environments.¹⁵

In the aquatic milieu, algae — a wide variety of photosynthetic organisms ranging from tiny bacteria-sized (1 to 5 μm) phytoplankton to macroalgae, the kelps (*Macrocystis* spp.) reaching up to 30 m in length, can be found in salt and freshwater ecosystems.²¹ However, the microscopic marine plants called phytoplankton (primarily diatoms, dinoflagellates, and Cyanobacteria) are the true bases of the marine food chain due to their photodynamic properties: capturing sunlight and transducing energy for the production of organic compounds.

Because of the role these algae play in the oceans' biological productivity and their impacts on climate due to the removal of carbon dioxide, satellite sensors have been employed to measure the chlorophyll a contents in oceans, lakes, and seas to indicate the distribution and abundance of biomass production in these water bodies. Detection is set at the specific reflectance and absorption wavelengths of the light from the upper layer of the ocean where photosynthesis occurs.

The reflection of light from organisms containing chlorophyll is highest at the absorption minimum around 550 nm and lowest at the absorption maximum around 440 nm. The chlorophyll content is then calculated, after calibration and correction from the reflection ratio at 440 nm, by reflection at 550 nm. The calibration requires the determination of the chlorophyll content in a given area (or volume) by conventional standard methods. This kind of estimation does not fit well when photosynthetic organisms are located in deeper water layers and there may be errors due to the fact that many marine organisms contain accessory photosynthetic pigments in addition to chlorophyll.⁷

In a similar way, microalgal biomass on the sediment surface can be estimated by measuring the chlorophyll contents in benthic microalgae, which are single-celled microscopic plants that inhabit the top 0 to 3 cm of a sediment surface and are sometimes referred to as microphytobenthos. These organisms are the primary food resources of benthic grazers such as shellfish and numerous finfish species.

The algae can be loosely defined as photosynthetic organisms that are classified into the kingdom Protista, excluding the land plants, with a perplexing array of cell morphologies, lifecycles, and habitats. They have been classified as belonging to several taxonomic lineages.²¹ The major divisions are blue-green algae (Cyanobacteria [also called blue-green algae]), Rhodophyta (red algae), Chlorophyta (green algae), Euglenophyta (euglenoid), Glaucocystophyta, Chromophyta (brown, golden, yellow-green algae and diatoms), and Pyrrophyta (dinoflagellates). All of them contain at least chlorophyll a. On average, 1.5% of algal organic matter is chlorophyll a.²² Chlorophyll b occurs only in green algae. Blue-green algae are often classified as algae by mistake because of the chloroplasts within the cells. Actually these organisms are photosynthetic bacteria classified as Cyanobacteria.

The remaining chlorophylls c, d, and e have been found in some Chromophyta (Chromista) algae such as brown algae and brown and red seaweeds and also in the single-celled marine algae making up the phytoplankton of the oceans. One of the characteristic properties of some algal species is the presence of other pigments with light absorbing capacities, e.g., phycobilins in cyanobacteria and red algae and carotenoids in Chromista that make them appear yellow or brown. These organisms make the most efficient use of light by stimulating the synthesis of pigments at the available wavelengths with minimum expenditures of metabolic energy.²³

Terrestrial plants are divided into two groups: nonvascular plants lacking lignin-impregnated conducting cells and vascular plants containing specialized transporting cells. Nonvascular plants are the simplest of all land plants. They generally grow only as tall as 1 or 2 cm because they lack the woody tissue necessary for support on land. The nonvascular plants include liverworts and mosses while the vascular plants consist of nonseed plants like ferns and seed plants including conifers and flowering plants. All the terrestrial plants, from mosses to flowering plants, contain chlorophyll a, which is usually accompanied by the variant chlorophyll b.

Due to the high quantities of chlorophylls a and b found in all land plants, these two types of pigments have been the most widely studied. They coexist in all the edible parts of vegetables, whether roots, stems, leaves, flowers, fruits or seeds, at least at a certain developmental stage of maturing. The approximate proportion of chlorophyll a and chlorophyll b is usually 3 to 1, but that varies depending on genus, species, growth conditions, and environmental factors, particularly high levels of

exposure to sunlight. Ratios varying from 2.5 to 4.0 have been reported.²⁴ In higher plants, these compounds are found in the chloroplasts of photosynthetic tissues, where they are noncovalently bound to polypeptides, phospholipids, and tocopherols, accompanied by carotenoids, and held within hydrophobic membranes named thylakoids.

2.1.5 BIOSYNTHESIS AND DEGRADATION

2.1.5.1 BIOSYNTHESIS OF CHLOROPHYLLS IN HIGHER PLANTS

The biosynthesis of the tetrapyrrole macrocycle and its branches leading to haem and chlorophylls has been covered in detail in several reviews^{3,7,13,25,26} and will be concisely described in this section. Tetrapyrrole biosynthesis occurs entirely in the plastids and is composed of several enzymatic steps starting from 5-aminolevulinic acid (ALA), which is the key precursor of porphyrins and the source of their carbon and nitrogen.

The most accepted mechanism for ALA formation in almost all photosynthetic tissues is the C-5 pathway proposed by Beale and Castelfranco (1974)²⁷ and Beale (1978),²⁸ which assumes that the 5-carbon molecule of glutamate or α -ketoglutarate is converted to ALA (Figure 2.1.3). However, a second or C-4 pathway proposed previously by Granick (1950)²⁹ cannot be excluded and may be active, parallel to the C-5 pathway, or when the C-5 pathway is blocked. Via this second mechanism, ALA is the result of the condensation of succinyl CoA with glycine in the mitochondria by ALA synthase. Since this enzyme is found only in a few plant tissues, this mechanism has not been sufficient to explain the appearance of ALA in photosynthetic tissues in general. The C-5 pathway occurs also in some bacteria, while the C-4 pathway is the only one for ALA synthesis in animals and fungi.⁷

Most of the subsequent steps of tetrapyrrole synthesis are identical in plants, animals, and bacteria. The pathway includes synthesis of the monopyrrole porphobilinogen from two molecules of ALA by the action of ALA dehydratase with the elimination of two molecules of water, followed by the assembling of a linear tetrapyrrole hydroxymethylbilane from four molecules of porphobilinogen, ring closure and two modification reactions of side chains. This produces the first tetrapyrrole macrocycle, uroporphyrinogen III. Therefore, eight molecules of ALA are necessary to form one tetrapyrrole.

All natural and most studied tetrapyrroles with known biological functions (chlorophylls, haem, and bilins) are derived from uroporphyrinogen III. Subsequently, enzymatic modification of the side chains (acetic and propionic acid) attached to each of the four pyrrole rings, catalyzed by uroporphyrinogen decarboxylase and coproporphyrinogen oxidase, yields protoporphyrinogen IX. In the next step, this macrocycle is oxidized in the presence of light and molecular oxygen and six atoms of hydrogen are removed to form the stable chromophore. This structure containing a planar system of 11 conjugated double bonds determines the spectroscopic properties of chlorophylls. All these steps from ALA to protoporphyrin IX can be visualized in Figure 2.1.4.

Up to this point, the biosynthesis steps are identical for both chlorophyll and haem, but depending on which metal is inserted in the center of the porphyrin, the pathway branches to form one or another. The insertion of iron is followed by

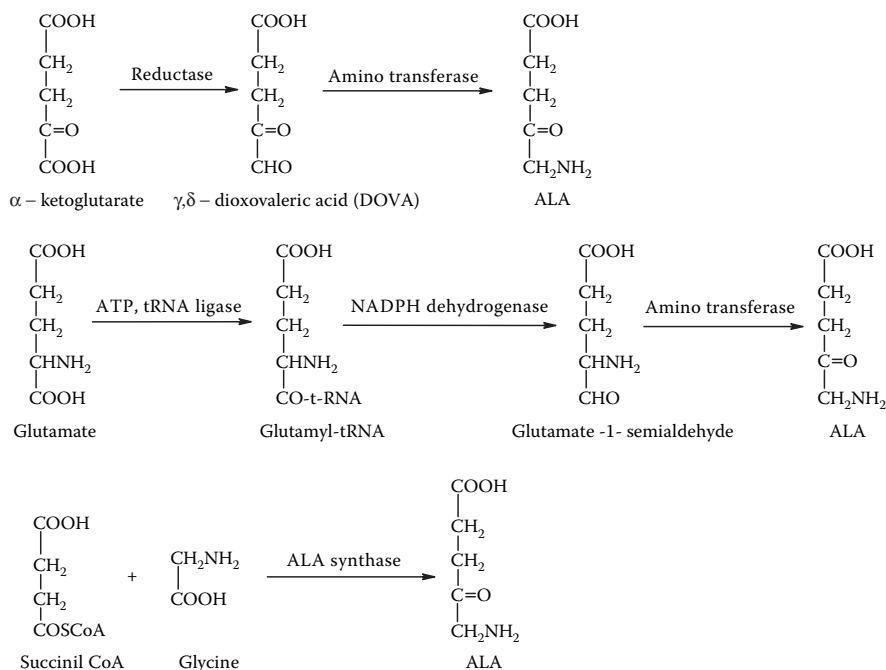


FIGURE 2.1.3 Synthesis of 5-aminolevulinic acid (ALA) by the C-5 pathway (from α-ketoglutarate or glutamate) and the C-4 pathway (condensation of succinyl CoA with glycine).

additional steps to produce haem, but if magnesium is inserted the molecule becomes chlorophyll. The ability to produce chlorophylls seems to be restricted to photosynthetic organisms (green plants, most algae and some bacteria). The magnesium protoporphyrin chelatase is highly specific for magnesium insertion and excludes other metal ions like zinc. Other metals can be introduced into porphyrins nonenzymatically, at this point or later in the process, and metallo-complexed intermediates are accepted by enzymes involved with chlorophyll synthesis.⁸

Esterification of the propionic acid side chain at C-13 (ring C) with a methyl group catalyzed by S-adenosyl-L-methionine-magnesium protoporphyrin *O*-methyltransferase yields protoporphyrin IX monomethyl ester (MPE), which originates protochlorophyllide by a β-oxidation and cyclization of the methylated propionic side chain. This molecule contains a fifth isocyclic ring (ring E), the cyclopentanone ring that characterizes all chlorophylls.

The “greening process” occurs with a photoreduction of the protochlorophyllide by the loss of the double bond between C-17 and C-18 of ring D to form dehydroprotoporphyrin (chlorin), also named chlorophyllide, imparting a green color to the molecule. This process is mostly light-dependent; it is performed by an enzyme called NADPH-protochlorophyllide oxidoreductase (POR). In the absence of light, etiolated seedlings stay pale yellow, turning green when exposed to light. This light-dependent mechanism occurs predominantly in angiosperms and is responsible for oxygenic photosynthesis. Cyanobacteria, green algae, pteridophytes, and gymno-

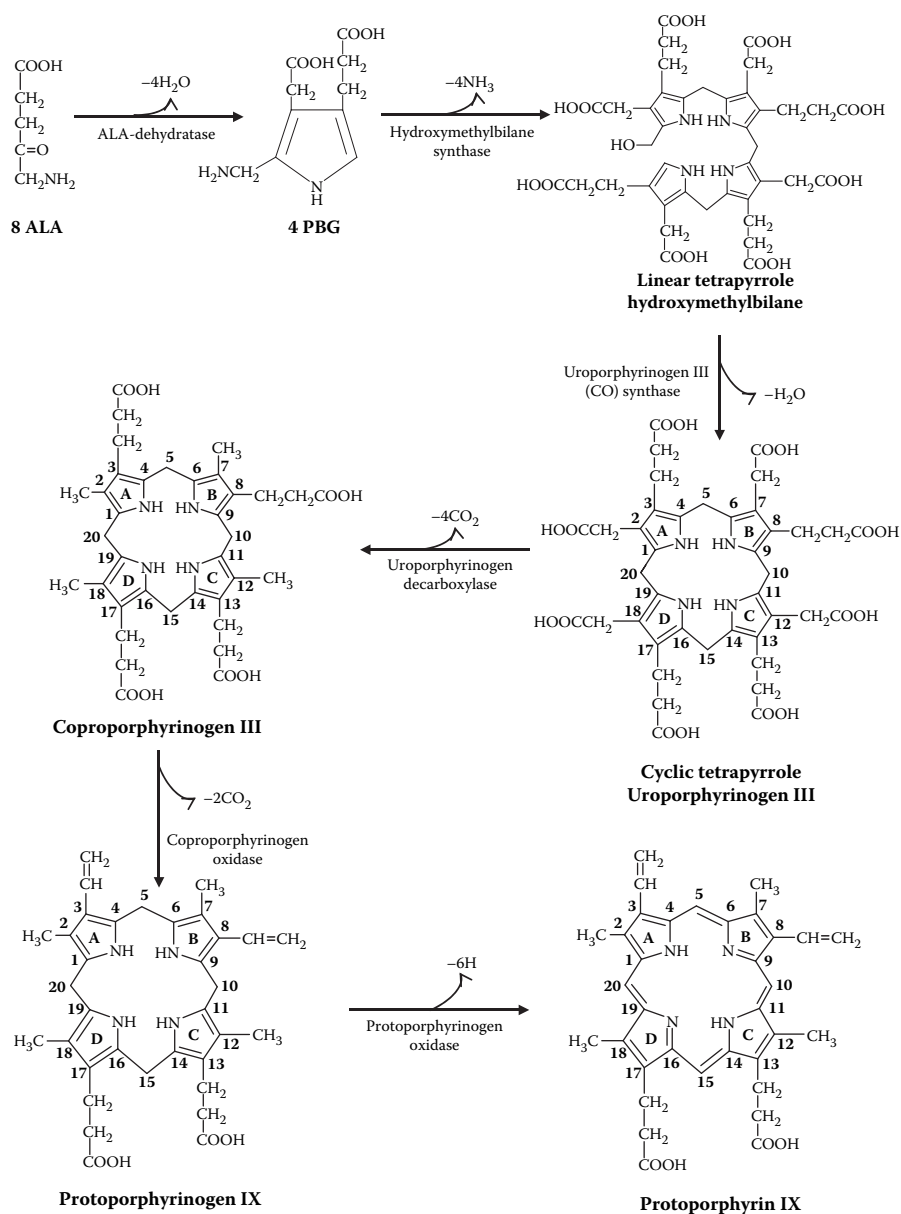


FIGURE 2.1.4 Biosynthesis steps of porphyrins from ALA to protoporphyrin IX.

sperms contain, besides a light-mediated pathway, a light-independent protochlorophyllide reduction to synthesize chlorophyll in darkness by using a completely different set of enzymes.^{25,30,31}

The last step in the synthesis of chlorophyll involves the attachment of phytol, the tetra-isoprene alcohol ($C_{20}H_{39}OH$), by esterification of the propionic acid residue at C-17 of ring D, by the enzyme chlorophyll synthase, which shows both synthesis and degradation activities. The presence of phytol gives the molecule a lipophilic character, facilitating the interaction with peptide chains from the thylakoid membrane.³⁰ Another parallel pathway admits the esterification with geranylgeraniol, followed by three gradual hydrogenation steps to form phytol. At this stage, chlorophyll is ready to be incorporated into protein complexes to form the stable light-harvesting antennae complexes of photosynthetic organisms. Figure 2.1.5 summarizes the chemical transformation from protoporphyrin IX to chlorophyll a.

It is generally assumed that the early steps of biosynthesis in chlorophylls a and b are identical. Chlorophyll b is formed by an additional step to transform the methyl group at C-7 of ring B into an aldehyde group, but it is not precisely known at which point of biosynthesis the oxygenation occurs, before or after phytylation. Therefore, oxygenation of the methyl group could occur at either the stage of chlorophyll a, chlorophyllide a, or even protochlorophyllide a.

Chlorophyll b occurs as an accessory pigment of the light-harvesting systems in land plants and green algae, and comprises one-third (or less) of total chlorophyll.³² The biosynthesis of chlorophyll b has been an area of active research particularly regarding its compartmentalization in chloroplast membranes, identification of the gene for chlorophyllide a oxidase, and characterization of the enzymes involved.³³

The reverse, formation of chlorophyll a from chlorophyll b, has been discussed as a process involved in reorganization of the photosynthetic apparatus during acclimation to different light environments due to the differences in absorption maxima between various pigments. Acclimation implies redistribution of chlorophylls between the different chlorophyll-protein complexes. Reduction of chlorophyll b to chlorophyll a must also play a role in the process of chlorophyll degradation, because during senescence of higher plants, chlorophyll b disappears together with chlorophyll a, but the degradation products are entirely derived from chlorophyll a. The key enzyme of chlorophyll degradation, pheophorbide a monooxygenase, accepts only pheophorbide a, as will be discussed later.³⁴

In accordance with the structure of chlorophyll c, it is hypothesized that its biosynthesis comes from protochlorophyllide a by dehydrogenation of the side chain at C-17.⁹ Chlorophyll d should arise from chlorophyll a by oxidation of the C-3-vinyl residue, but at which stage of chlorophyll biosynthesis this occurs is unknown. The biosynthesis of bacteriochlorophylls seems to follow the same general pathway of higher plants, according to studies performed with chlorophyllide and bacteriochlorophyll enzymes.¹³

Whereas the biosynthesis of chlorophylls a and b in higher plants has been described in detail, the synthesis and regulation of related substances found in less well-known algal groups and lower plants are largely unknown and will be areas of scientific interest in the future. Different and new types of chlorophylls and related substances have been reported and little is known about their possible biological

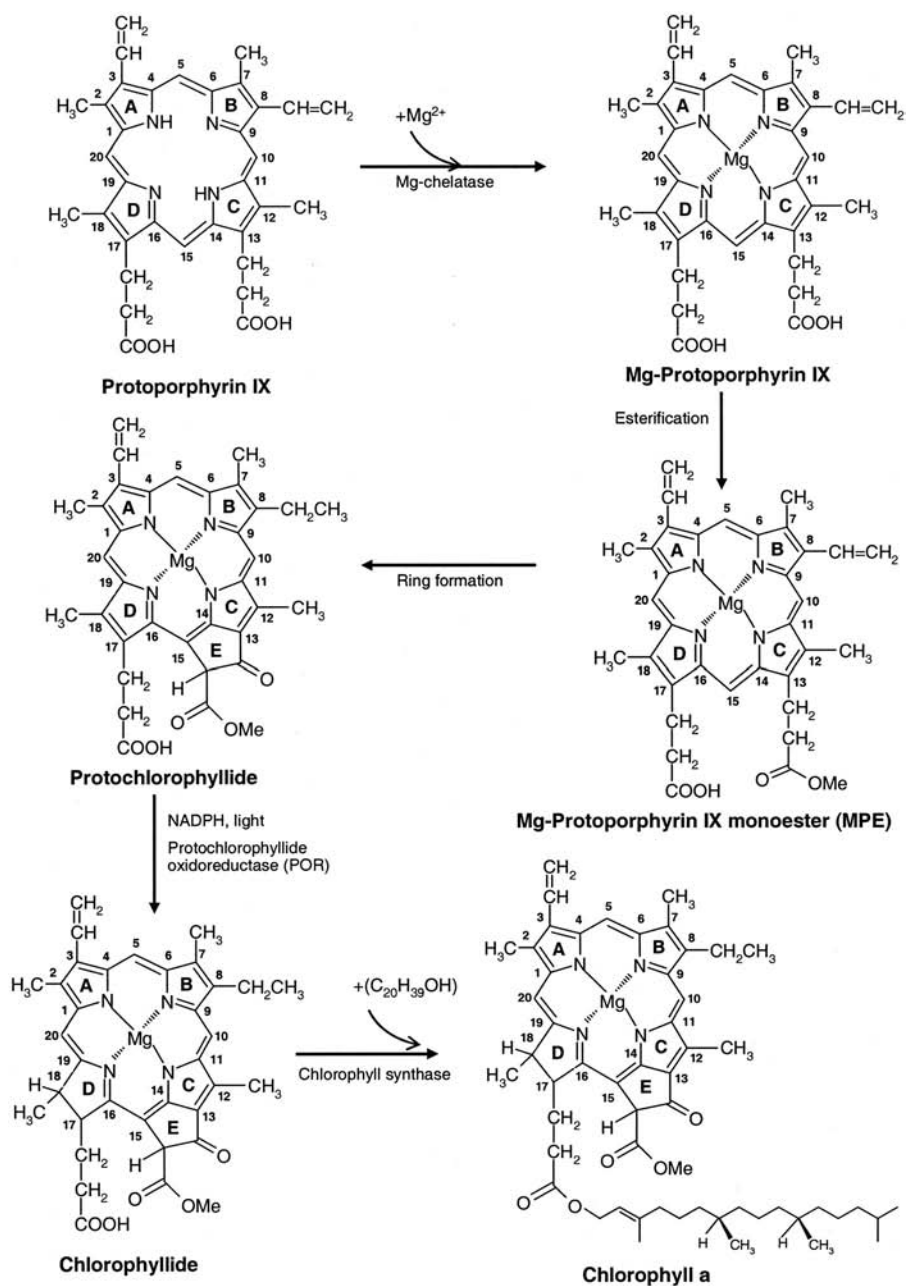


FIGURE 2.1.5 Biosynthesis steps of porphyrins from protoporphyrin IX to chlorophyll a.

adaptations to particular environmental conditions where their biological functions, sometimes closely related to the synthesis of other unrelated molecules, are fundamental for survival. However, during the past several years, much progress has been made and more is expected in the identification of synthesis and degradation intermediates, elucidation of the molecular mechanisms, and cloning of the genes for the enzymes.^{35,36}

2.1.5.2 CHLOROPHYLL DEGRADATION DURING PLANT SENESCENCE AND FRUIT RIPENING

Disappearance of chlorophyll during fruit ripening and leaf senescence or normal turnover in photosynthetic tissues indicates programmed slowing of photosynthesis. The process was largely unknown and only during the last 20 years has significant research progress been made. Several linear tetrapyrrolic chlorophyll catabolites were isolated from green algae *Chlorella protothecoides* and senescent higher plants, and the similarities of their structures corroborate close relationships between degradation pathways. Since the phylum Chlorophyta is believed to be the ancestor of higher plants, it is reasonable to hypothesize on a unique chlorophyll pathway in all green plants.^{37,38}

The whole process of chlorophyll disappearance in vascular plants is a complex multistep pathway, much as chlorophyll biosynthesis is, but for didactic reasons it can be abbreviated into two main stages. The first group of reactions produces greenish derivatives while the more advanced steps produce colorless compounds by an oxidative ring opening, analog to the oxygenolytic rupture of the porphyrinoid macrocycle of haem. It is a very rapid process and despite considerable efforts, the detection of intermediates is difficult.³⁸⁻⁴²

The early stages of catabolism correspond to the replacement of Mg by two H atoms under acidic conditions and/or by the action of Mg-dechelataase and the cleavage of the phytol chain by the enzyme chlorophyllase. The still greenish intermediates are pheophytins, chlorophyllides, and pheophorbides with intact tetrapyrrole rings.^{43,44}

The late degradation stages are responsible for effective de-greening through rapid formation of several colorless linear tetrapyrroles. Labeling experiments with oxygen isotopes and heavy water demonstrated a high region- and stereo-selective oxygenolytic opening of the macrocycle ring between C-4 and C-5, catalyzed by the action of pheophorbide a monooxygenase (PaO) yielding the red chlorophyll catabolite (RCC).^{37,39,45-47} In higher plants, the activity of this enzyme is restricted to pheophorbide a since the degradation products are entirely derived from chlorophyll a.⁴⁸ Pheophorbide b is a competitive inhibitor of this enzyme. Additionally, the absence of type b catabolites strongly supports the hypothesis that chlorophyll b must be enzymatically converted into chlorophyll a before degradation.⁴⁹

RCC is very unstable and rapidly reduced to a primary fluorescent chlorophyll catabolite (pFCC) by red chlorophyll catabolite reductase (RCCR). In subsequent steps pFCC is converted to different fluorescent chlorophyll catabolites (FCCs). These chemically rather labile compounds are further converted into colorless nonfluorescing chlorophyll catabolites (NCCs) by a nonenzymatic deconjugation of the four

pyrrolic rings. Peripheral hydroxylations and sometimes conjugations with hydrophilic groups increase their polarity, facilitating their exportation to the vacuoles.^{38,50}

These NCCs have been suggested to be the terminal products of chlorophyll breakdown accumulated in the vacuoles of senescent higher plants. However, evidence indicates that NCCs can be further oxidized to rust-colored products when air is present, and in some senescent leaves they were broken into fragments of monopyrroles.^{51,52} In addition, isolation of a urobilinogen-like catabolite from de-greened primary leaves of barley (*Hordeum vulgare*, cv. Lambic), raises the question whether the urobilinogenoids are peculiar to warm-blooded organisms or common intermediates in the chlorophyll catabolic pathway.⁵³

The joint reactions of PaO and RCCR are responsible for the loss of photodynamic activity has long been considered a necessary detoxification process during senescence. This process is the result of the deconjugation of the π -electron system. The photodynamic properties of chlorophylls are essential to convert light energy into chemical energy during photosynthesis, but during leaf senescence the photodynamism would cause premature cell death.^{54–56} Besides that, the formation of open chain NCCs that have more flexible conformations is believed to contribute to their transport and remobilization within the nitrogen pool and disruption from protein complexes, allowing for the reutilization of nutrients.^{40,57} In contrast with the available information about chlorophyll catabolism in higher plants, little is known about chlorophylls or bacteriochlorophylls from marine organisms like green algae. A monooxygenase seems to be responsible for cleaving the chlorophyll macrocycle of *Chlorella protothecoides*, but RCCR is absent and RCC-like compounds are excreted into the medium.^{58,59} Considering the variable pressure of oxygen in the deep sea environment, an oxygenolytic mechanism may not be the only way to degrade chlorophylls in marine photosynthetic organisms.³⁸

The study of the biochemical pathways, the functions of enzymes, and the structures of catabolites formed may give an insight into the regulation of the de-greening process. Research in this area will also enable understanding of the roles of genes that are assumed to encode proteins involved in regulation of chlorophyll degradation at the molecular level. Stay-green mutants have been useful models for elucidating the de-greening mechanism.^{41,60} The knowledge of the mechanisms of degradation and related processes will be potentially useful to industry, agriculture, and horticulture by controlling both the retardation and the acceleration of chlorophyll degradation before natural onset of senescence. Retardation may keep leaves green and result in higher yields of chlorophyll in plants for food colorant purposes.

2.1.6 FUNCTIONS

2.1.6.1 FUNCTIONS OF CHLOROPHYLLS IN PHOTOSYNTHETIC TISSUES

Chlorophylls play a vital and central role in photosynthesis, creating the basis for the animal food chain on which most living organisms depend. The initial step of photosynthesis involves absorption of light by the light-harvesting antennae complexes and funnelling the resulting electronic excitation to the photosynthetic reaction

center, where the energy is used for the conversion of carbon dioxide and water into carbohydrates with the liberation of oxygen. The chlorophylls are organized in these light-harvesting complexes found in subcellular organelles known as chloroplasts, more specifically located in the lipid (or thylakoid) membranes, creating a maximum area of absorption. The major function of chlorophylls is to capture sunlight and funnel light energy to the reaction center where it is subsequently used for the conversion of carbon dioxide and water into carbohydrates with the liberation of oxygen. The differences among the photosynthetic organisms are related to the structures of various light-harvesting complexes, whereas the reaction centers were conserved during evolution.

The complex process of oxygenic photosynthesis includes a light (or photo-chemical) stage and a dark stage that takes place in the thylakoid membrane and in the stroma of chloroplasts, respectively. The absorbed whole energy of the quantum produces an electronic excitation that is transferred to the reaction center where the light energy is converted into chemical energy as reduced nicotinamide dinucleotide (NADPH) and adenosine triphosphate (ATP). Simultaneously, oxygen is released. In the dark, these energy-rich molecules reduce CO_2 to synthesize simple carbohydrate-phosphorylated glucose, fructose, and sucrose.

The photosynthetic pigments are noncovalently linked to proteins or peptides of the thylakoid membrane through a coordination bond between the central Mg of the chlorophyll and the histidine residue of protein, the most electronegative ligand.⁶¹ The complexes are assembled into two functional cooperative systems, named photosystem I (PS I) and photosystem II (PS II). PS II is concerned with the removal of hydrogen from water, which is split into oxygen, protons, and electrons ($2\text{H}_2\text{O} \rightarrow \text{O}_2 + 4\text{H}^+ + 4\text{e}^-$). PS I promotes the reduction of NADP. Electrons flow from PS II to PS I through a series of intermediate electron carriers, which means they move from the outer side to the inner side of the thylakoid membrane.^{3,5,13,62} Photosynthetic membranes are formed from repeated assembled photosynthetic units, each consisting of a network called an antenna chlorophyll–protein complex that harvests light and funnels it to the reaction centers that are the starting points of the electron transport chain. The entire chlorophyll molecule is a resonance hybrid of several possible double-bond arrangements which enable the pigments to capture light in the form of photons and then pass the energy onto neighboring molecules until a concentration of energy occurs.

In green algae and vascular plants, chlorophyll a is the primary cofactor of the reaction center while the antenna complexes (light-harvesting apparatuses) contain variable amounts of chlorophyll a and b. Chlorophyll b is involved only with light harvesting, whereas chlorophyll a is also involved in energy transduction within the chloroplast membranes. In other photosynthetic algae (Cyanobacteria, Rodophyta, Chromista) the remaining chlorophylls, c and d and/or phycobilins, not only augment the light-harvesting properties, but also replace chlorophyll a in PS II.^{12,63}

In photosynthetic bacteria, (i.e., Chlorobiaceae) the bacteriochlorophylls support photosynthesis at low light intensities, and they accomplish this activity by using a unique antenna complex known as a chlorosome in which the pigments are located. Since these bacteria are strict anaerobes, photosynthesis is nonoxygenic.¹⁷

The chlorophyll–protein complexes located in the hydrophobic thylakoid membrane are accompanied by xanthophylls, certain carotenes, and tocopherols (depend-

ing on the species of the organism) playing an auxiliary role. Carotenoids play an important role in protecting the lipid membrane from oxidation damage by scavenging excess energy captured by chlorophyll and reactive forms of oxygen (singlet oxygen) inevitably generated during the photosynthesis process.⁶³ The tocopherols also act as antioxidants, quenching singlet oxygen and trapping free radicals and peroxide radicals. Therefore, these compounds are effective natural antioxidant agents protecting chlorophyll and the environmental system of photosynthesis from lipid oxidation and photo-oxidation.

2.1.6.2 BIOLOGICAL ACTIVITIES OF CHLOROPHYLLS IN HUMANS

In addition to their use as foods and pharmaceutical colorants, chlorophylls — the green pigments responsible for photosynthesis in plants — also take part in nutrition of humans. The high levels and ubiquity of chlorophylls in a variety of leafy green vegetables raise questions about their bioavailability and metabolism and whether they may exert any biological function. Currently, there is considerable interest in studying chlorophylls, not only because of their coloring properties, but also for their health-related biological activities.

Evidence from a large body of *in vitro* and *in vivo* studies has indicated that green and raw vegetables may reduce the risk of certain types of cancers, coronary heart diseases, cataracts, diabetes, and other chronic and age-related diseases. However, there is a gap in knowledge about their health promoting properties. The question is whether these may be ascribed to bioactive phytochemicals like carotenoids, vitamins C and E, and phenolic compounds or also attributed to chlorophylls. The possibility that natural chlorophylls and their semi-synthetic water-soluble derivatives may protect an organism against these diseases, especially colon cancer, created an expectancy about potential health benefits, and a substantial increase of interest in research focusing on cancer and chlorophyll has been observed in recent years.

Nevertheless, despite being the most abundant pigments in nature, chlorophylls have rarely been included in biological experiments, first due to the difficulty in purifying these pigments and the chemical instability of the molecules, and also because of the high costs involved. Natural chlorophylls are so unstable that in most research the commercially available semi-synthetic copper chlorophyllin, a saponified mixture of natural chlorophylls, has been used as a model for several experimental designs. Copper chlorophyllin is easily available, water-soluble, and significantly more stable than natural chlorophyll.

Natural chlorophylls are not known to be toxic, based on their long history of consumption and also because they are believed to be absorbed in very small amounts. However, information regarding their absorption and metabolism is almost nonexistent. Copper chlorophyllin has a long history of therapeutic use without reported side effects. Since the 1960s it has been suggested to be used topically to accelerate wound healing by slowing the growth of anaerobic bacteria in persistent open wounds in humans. Several reports also indicate that orally administered Cu chlorophyllin may decrease subjective judgment of urinary and fecal odor in incontinent patients and reduce odors in patients with colostomies and ileostomies.⁶⁴ Chlorophyllin has also been reported to be effective in decreasing the

fishy odors attributed to patients with trimethylaminuria, a genetic disorder characterized by the inability to metabolize trimethylamine.⁶⁵

Rapid accumulation of scientific data, especially during the last 15 years, has associated chlorophyll compounds to bioactivities, as long as the chlorophyll derivatives conserve their basic porphyrin ring structures. Chlorophylls and some of their degradation products found in common vegetables, including pheophytins, pheophorbides, chlorophyllides, and semi-synthetic chlorophyllin derivatives exhibit antimutagenic and antioxidant activities that have served as the foci of research in various test systems related to their prospective chemopreventive properties.^{66,67}

Endo and his colleagues (1985)⁶⁸ were the first to suggest the ability of chlorophylls to act as effective electron donors. Sato et al. (1986)⁶⁹ identified Cu isochlorin e_4 , the major component in commercial sodium-copper chlorophyllin, as being capable of minimizing lipid peroxidation. However, it was found that antioxidant activities of natural chlorophylls are variable, depending on their structures, and are always significantly lower than the activities observed for metallo-chlorophyll derivatives.^{70,71} These metallo-chlorophylls also protected mitochondria against oxidative damage induced by reactive oxygen species, *in vitro* and *ex vivo*.⁷²

The main effect of chlorophylls and chlorophyllins reported in literature is their ability to protect an organism against many mutagens and carcinogens from dietary and environmental origins that could cause DNA damage. A positive relationship has been established between the chlorophyll contents of various vegetable extracts and their abilities to inhibit mutations in the Ames Salmonella system and, in addition, cytotoxic effects were observed against tumor cells.^{73,74}

Regarding chemopreventive properties, the most studied and most accepted theory demonstrated by *in vitro* and *in vivo* study models is that the intact porphyrin ring can form complexes with planar aromatic carcinogens like aflatoxin B1, polycyclic aromatic hydrocarbons, heterocyclic amines, and smoke condensates acting as receptor molecules, thus inhibiting their uptake and bioavailability from the gut, or scavenge free radicals from carcinogens.^{73,75} Both metal-free and metallo-chlorophyll derivatives demonstrated similar dose-dependent inhibitory activities against benzo[*a*]pyrene.⁷⁰ Although the complex formation depends on an intact chemical structure of the porphyrin nucleus, the absence of the central metal is not a guarantee that pheophytins (lacking central metal ions) or different types of molecules would have similar beneficial effects. Although the possibility of complex formation between chlorophylls or their derivatives with a broad range of compounds in the intestines has been reported with increasing frequency, questions regarding the binding constants for different complexes may impact the *in vivo* relevance of chlorophylls as health-promoting phytochemicals.

In a clinical trial performed in China, the administration of 300 mg/day of copper chlorophyllin to humans who had detectable levels of serum aflatoxin due to unavoidable food contamination resulted in a 50% reduction of median urinary levels of aflatoxin-DNA adducts.⁷⁶ If health benefits from consuming natural chlorophylls were confirmed, it would be easy to add green leafy vegetables to a daily diet to obtain the benefit. Since leafy vegetables contain usually up to 200 mg chlorophylls/100 g fresh weight, the intake of approximately 1 to 2 cups of raw spinach/day

would furnish the same amount of chlorophyll as the 300 mg of chlorophyllin reported to decrease the damage of DNA by aflatoxin.

This interceptor theory does not seem to be the only protective mechanism in operation. Inhibition of cytochrome P450 enzymes related to the bioactivation of mutagens and toxic radical scavenger activities have been proposed to integrate the different modes of action. Other investigations have reported the involvement of chlorophyllin in inducing apoptosis in human colon cells, which may be important in limiting cancer cell invasion and metastasis.^{75,77}

There is also evidence that individual chlorophyll derivatives exhibit cytostatic and cytotoxic activities against tumor cells.⁶⁶ Studies have been started on electronic structures, in particular the electronic state of the porphyrin macrocycle, and progress in this area is expected regarding photodynamic therapy for tumors, since the strong absorption of light in the visible region is effective for laser excitation. Nevertheless, little is known to date about the influences of peripheral groups on the electronic state of the macrocycle π system in chlorophyll derivatives.¹⁶

Diets high in red meat and low in green vegetables have been associated with increased colon cancer risk and the opposite has been postulated for diets rich in green vegetables. A plausible explanation for an increased colon cancer risk is that dietary haem is metabolized in the gut to a factor that increases colonic cytotoxicity and hyperproliferation, which are considered important risk factors in the development of cancer. In this sense, it has been shown that spinach and isolated natural chlorophyll, but not sodium-copper chlorophyllin, prevented the proliferation of colonic cells and may therefore reduce colon cancer risk. It has been speculated that haem and chlorophylls, due to their hydrophobicity, form a complex, thus preventing the metabolism of haem.⁷⁸

Surprisingly, other studies including tumor promotion have reported conflicting results in the colon. The chemoprotective effects attributed to copper chlorophyllin contrast with its tumor inducing and genotoxic activity observed in a colon carcinogenesis model in which cancer in rats was induced by dimethylhydrazine. However, the underlying mechanism of the tumor promoting activity remains unclear.⁷⁹

Scientific evidence of these properties is still incomplete because most studies employed different experimental designs. A substantial body of research in the area of biological activity is still needed to achieve a better understanding of the absorption and metabolism of chlorophylls. The uneven responses, the use of poorly defined pigments, and the employment of different biological assays have become barriers to further studies of the possible role of chlorophylls in reducing disease risks by dietary management.

The variability in chlorophyllin composition and conditions of testing may result in ambiguous, sometimes not-reproducible biological effects.⁷⁵ In addition, thermally induced degradation of copper chlorophyllin, causing it to lose the central copper ion or affecting the porphyrin ring structure, may alter antimutagenic and anticarcinogenic properties. Therefore, the establishment of standards of identity and quality of commercial copper chlorophyllin preparations is strongly encouraged.⁸⁰

In addition to the porphyrin nucleus, the phytol tail that esterifies the propionic acid side chain at C-17 may be hydrolyzed enzymatically during storage or processing. Cleavage of the phytol chain during digestion is unlikely. Free phytol is quickly

absorbed, oxidized to phytanic acid, and then reduced to phytanic, which is further metabolized into pristanic acid. These intermediates can be found in human serum and tissues and their origin is always dietary chlorophyll. Phytanic acid was found to have a physiological role, being capable of promoting white adipose differentiation which may be relevant for the treatment of several human disorders. Phytanic acid is a lipophilic ligand and is likely to mediate cell signaling and activate nuclear receptors that regulate gene expression.^{81–83}

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REFERENCES

1. Schwartz, S.J. and Lorenzo, T.V., Chlorophylls in foods, *Crit. Rev. Food Sci. Nutr.*, 29, 1, 1990.
2. Rüdiger, W. and Schoch, S., Chlorophylls, in *Plant Pigments*, Goodwin, T.W., Ed., Academic Press, London, 1988, chap. 1.
3. Gross, J., Chlorophylls, in *Pigments in Vegetables: Chlorophylls and Carotenoids*, Gross, J., Ed., Van Nostrand Reinhold, New York, 1991, 2.
4. Schoefs, B., Plant pigments: properties, analysis, and degradation, *Adv. Food Nutr. Res.*, 49, 41, 2005.
5. Hendry, G.A.F., Chlorophylls and chlorophyll derivatives, in *Natural Food Colorants*, 2nd ed., Hendry, G.A.F. and Houghton, J.D., Eds., Chapman & Hall, London, 1996, chap. 5.
6. Merritt J.E. and Loening, K.L., IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN): Nomenclature of tetrapyrroles: recommendations, 1978, *Eur. J. Biochem.*, 108, 1, 1980.
7. Rüdiger, W., Chlorophyll metabolism: from outer space down to the molecular level, *Phytochemistry*, 46, 1151, 1997.
8. Garrido, J.L., Zapata, M., and Muñoz, S., Spectral characterization of new chlorophyll c pigments isolated from *Emiliania huxleyi* (Prymnesiophyceae) by high performance liquid chromatography, *J. Physiol.*, 31, 761, 1995.
9. Porra, R.J., Recent progress in porphyrin and chlorophyll biosynthesis, *Photochem. Photobiol.*, 65, 492, 1997.
10. Jeffrey, S.W., Structural relationships between algal chlorophylls, in *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*, Jeffrey, S.W., Mantoura, R.F.C. and Wright, S.W., Eds., UNESCO, Paris, 1997, 566.
11. Garrido, J.L. et al., The main non-polar chlorophyll c from *Emiliania huxleyi* (Prymnesiophyceae) is a chlorophyll c2-monogalactosyldiacylglyceride ester: a mass spectrometry study, *J. Physiol.*, 36, 497, 2000.
12. Helfrich, M. et al., Chlorophylls of the c family: absolute configuration and inhibition of NADPH: protochlorophyllide oxidoreductase, *Biochim. Biophys. Acta*, 1605, 97, 2003.
13. Scheer, H., Structure and occurrence of chlorophylls, in *Chlorophylls*, Scheer, H., Ed., CRC Press, Boca Raton, 1991, 3.

14. Miyashita, H. et al., Chlorophyll d as a major pigment, *Nature*, 383, 402, 1996.
15. Larkum, A. W. D. and Kühl, M., Chlorophyll d: the puzzle resolved, *Trends Plant Sci.*, 10, 355, 2005.
16. Nonomura, Y. et al., Spectroscopic properties of chlorophylls and their derivatives: influence of molecular structure on the electronic state, *Chem. Phys.*, 220, 155, 1997.
17. Blankenship, R.E., Identification of key step in the biosynthetic pathway of bacteriochlorophyll c and its implications for other known and unknown green sulfur bacteria, *J. Bacteriol.*, 186, 5187, 2004.
18. Houghton, J.D., Haem and bilins, in *Natural Food Colorants*, 2nd ed., Hendry, G.A.F. and Houghton, J.D., Eds., Chapman & Hall, London, 1996, chap. 6.
19. Tuszynski, W. et al., The observation of chlorophyll a aggregates with plasma desorption mass spectrometry, in *Proceedings of the 5th International Conference of Ion Formation from Organic Solids (IFOS V)*, Hedin, A., Sundqvist, B.U.R. and Benninghoven, A., Eds., Wiley, Chichester, England, 1989.
20. Tan, Y.A., Low, K.S., and Chong, C.L., Rapid determination of chlorophylls in vegetable oils by laser-based fluorometry, *J. Sci. Food Agric.*, 66, 479, 1994.
21. Bhattacharya, D. and Medlin, L., Algal phylogeny and the origin of land plants, *Plant Physiol.*, 116, 9, 1998.
22. Van den Hoek, C., Mann, D.G., and Jahns, H.M., *Algae: An Introduction to Phycology*, Cambridge University Press, Cambridge, UK, 1995, 623.
23. Green, B.R. and Dunford, D.G., The chlorophyll-carotenoid proteins of oxygenic photosynthesis, *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, 47, 685, 1996.
24. Gross, J., Chlorophylls, in *Pigments in Fruits*, Gross, J., Ed., Academic Press Inc., London, 1987, chap. 1.
25. Thomas, H., Chlorophyll: a symptom and a regulator of plastid development, *New Phytol.*, 136, 163, 1997.
26. Adamson, H.Y., Hiller, R.G., and Walmsley, J., Protochlorophyllide reduction and greening in angiosperms: an evolutionary perspective, *J. Photochem. Photobiol. B: Biol.*, 41, 201, 1997.
27. Beale, S.I. and Castelfranco, P.A., The biosynthesis of δ -aminolevulinic acid in plants. II. Formation of ^{14}C - δ -Aminolevulinic acid from labeled precursors in greening plant tissues, *Plant Physiol.*, 53, 297, 1974.
28. Beale, S.I., δ -Aminolevulinic acid in plants: its biosynthesis, regulation and role in plastid development, *Annu. Rev. Plant Physiol.*, 29, 95, 1978.
29. Granik, S., Magnesium vinyl pheoporphyrin a_5 , another intermediate in the biological synthesis of chlorophyll, *J. Biol. Chem.*, 183, 713, 1950.
30. Malkin, R. and Niyogi, K., Photosynthesis, in *Biochemistry and Molecular Biology of Plants*, Buchanan, B.B. et al., Eds., American Society of Plant Physiologists, Rockville, MD, 2000, 575.
31. Rüdiger, W., Biosynthesis of chlorophylls a and b: the last steps, in *Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications*, 25, Grimm, B. et al., Eds., Springer, Dordrecht, 2006, chap. 14.
32. Folly, P., *Catabolisme de la chlorophyllide b, structures, mécanismes et synthèses*, These présentée à la Faculté des Sciences de l'Université de Fribourg pour l'obtention du grade de Docteur ès sciences naturelles, Fribourg, 2000.
33. Eggink, L.L., Park, H., and Hooper, J.K., The role of chlorophyll b in photosynthesis: hypothesis, *BMC Plant Biol.*, 2001, 192 doi.10.1186/1491-2229-1-2.
34. Scheumann, V., Schoch, S., and Rüdiger, W., Chlorophyll: A formation in the chlorophyll b reductase reaction requires reduced ferredoxin, *J. Biol. Chem.*, 273, 35102, 1998.

35. Suzuki, J.Y., Bollivar, D.W., and Bauer, C.E., Genetic analysis of chlorophyll biosynthesis, *Annu. Rev. Genet.*, 31, 61, 1997.
36. Armstrong, G. and Apel, K., Molecular and genetic analysis of light-dependent chlorophyll biosynthesis, *Methods Enzymol.*, 297, 237, 1998.
37. Curtly, C., Engel, N., and Gossauer, A., Evidence for a monooxygenase-catalyzed primary process in the catabolism of chlorophyll, *FEBS Lett.*, 364, 41, 1995.
38. Kräutler, B. and Hörtensteiner, S., Shlorophyll catabolites and the biochemistry of chlorophyll breakdown in *Chlorophylls and Bacteriochlorophylls: Biochemistry, Biophysics, Functions and Applications*, Grimm, B. et al., Eds., Springer, The Netherlands, 2006, 237.
39. Hörtensteiner, S. et al., The key step in chlorophyll breakdown in higher plants: Cleavage of pheophorbide a macrocycle by a monooxygenase, *J. Biol. Chem.*, 273, 15335, 1998.
40. Hörtensteiner, S., Chlorophyll breakdown in higher plants and algae, *Cell. Mol. Life Sci.*, 56, 330, 1999.
41. Takamiya, K., Tsuchiya, T., and Ohta, H., Degradation pathway(s) of chlorophyll: What has gene cloning revealed? *Trends Plant Sci.*, 5, 426, 2000.
42. Prušinská, A. et al., Chlorophyll breakdown in senescent Arabidopsis leaves: Characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction, *Plant Physiol.*, 139, 52, 2005.
43. Heaton, J.W. and Marangoni, A.G., Chlorophyll degradation in processed foods and senescent plant tissues, *Trends Food Sci. Technol.*, 7, 8, 1996.
44. Mangos, T.J. and Berger, R.G., Determination of major chlorophyll degradation products, *Z. Lebensm. Unters. Forsh. A*, 204, 345, 1997.
45. Mühlecker, W. et al., Tracking down chlorophyll breakdown in plants: Elucidation of the constitution of a “fluorescent” chlorophyll catabolite, *Angew. Chem. Int. Ed. Engl.*, 36, 401, 1997.
46. Rodoni, S. et al., Partial purification and characterization of red chlorophyll catabolite reductase, a stroma protein involved in chlorophyll breakdown, *Plant Physiol.*, 115, 677, 1997.
47. Oberhuber, M. and Kräutler, B., Breakdown of chlorophyll: electrochemical bilin reduction provides synthetic access to fluorescent chlorophyll catabolites, *Chembiochem*, 3, 104, 2002.
48. Kräutler, B., Unravelling chlorophyll catabolism in higher plants, *Biochem. Soc. Trans.*, 30, 625, 2002.
49. Hörtensteiner, S., Vicentini, F., and Matile, P., Chlorophyll breakdown in senescent cotyledons of rape, *Brassica napus* L.: enzymatic cleavage of pheophorbide a *in vitro*, *New Phytol.*, 129, 237, 1995.
50. Oberhuber, M. et al., Breakdown of chlorophyll: A nonenzymatic reaction accounts for the formation of the colorless “nonfluorescent” chlorophyll catabolites, *PNAS*, 100, 6910, 2003.
51. Suzuki, Y. and Shioi, Y., Detection of chlorophyll breakdown products in the senescent leaves of higher plants, *Plant Cell Physiol.*, 40, 909, 1999.
52. Kräutler, B. and Matile, P., Solving the riddle of chlorophyll breakdown, *Acc Chem. Res.*, 32, 35, 1999.
53. Losey, F.G. and Engel, N., Isolation and characterization of a urobilinogenoidic chlorophyll catabolite from *Hordeum vulgare* L., *J. Biol. Chem.*, 276, 8643, 2001.
54. Matile, P., Hörtensteiner, S., and Thomas, H., Chlorophyll degradation, *Annu. Rev. Plant. Physiol. Plant Mol. Biol.*, 50, 67, 1999.

55. Hörtensteiner, S., The loss of green color during chlorophyll degradation by a pre-requisite to prevent cell death? *Planta*, 219,191, 2004.
56. Pruzinská, A. et al., In vivo participation of red chlorophyll catabolite reductase in chlorophyll breakdown, *The Plant Cell*, 19, 369, 2007.
57. Iturraspe, J., Moyano, N., and Frydman, B., A new 5-formylbilinone as the major chlorophyll a catabolite in three senescent leaves, *J. Org. Chem.*, 60, 6664, 1995.
58. Engel, N. et al., Chlorophyll catabolism in *Chlorella protothecoides*: Isolation and structure elucidation of a red bilin derivative, *FEBS Lett.*, 293, 131, 1991.
59. Hörtensteiner, P. et al., Chlorophyll breakdown in *Chlorella protothecoides*: characterization of degreening and cloning of degreening-related genes, *Plant Mol. Biol.*, 42, 439, 2000.
60. Roca, M. and Mínguez-Mosquera, M.I., Chlorophyll catabolism pathway in fruits of *Capsicum annum* (L.): stay-green versus red fruits, *J. Agric. Food Chem.*, 54, 4035, 2006.
61. Eggink, L.L. et al., The role of chlorophyll b in photosynthesis: hypothesis, *BMC Plant Biol.*, 1, 2, 2001. Available from: <http://www.biomedcentral.com/1471-2229/1/2>.
62. Davis, M.S., Forman, A., and Fajer, J., Ligated chlorophyll cation radicals: their function in photosystem II of plant photosynthesis, *PNAS*, 76, 4170, 1979.
63. Ritz, T. et al., Efficient light harvesting through carotenoids, *Photosyn. Res.*, 66, 125, 2000.
64. Young, R.W. and Beregi, J.S. Jr., Use of chlorophyllin in the care of geriatric patients, *J. Am. Geriatr. Soc.*, 28, 46, 1980.
65. Yamazaki, H. et al., Effects of the dietary supplements, activated charcoal and copper chlorophyllin, on urinary excretion of trimethylamine in Japanese trimethylaminuria patients, *Life Sci.*, 74, 2739, 2004.
66. Chernomorsky, S., Segelman, A., and Poretz, R.D., Effect of dietary chlorophyll derivatives on mutagenesis and tumor cell growth, *Teratog. Carcinog. Mutag*, 19, 313, 1999.
67. Ferruzzi, M.G. and Blakeslee, J., Digestion, absorption and cancer preventative activity of dietary chlorophyll derivatives, *Nutr. Res.*, 27, 1, 2007.
68. Endo, Y., Usuki, R., and Kaneda, T., Antioxidant effects of chlorophyll and pheophytin on the autoxidation of oils in the dark. II. The mechanism of antioxidative action of chlorophyll, *J. Am. Oil Chem. Soc.*, 62, 1387, 1985.
69. Sato, M. et al., Effect of sodium copper chlorophyllin on lipid peroxidation. IX. On the antioxidative components in commercial preparations of sodium copper chlorophyllin, *Chem. Pharm. Bull.*, 34, 2428, 1986.
70. Ferruzzi, M.G. et al., Antioxidant and antimutagenic activity of dietary chlorophyll derivatives determined by radical scavenging and bacterial reverse mutagenesis assays, *J. Food Sci.*, 67, 2589, 2002.
71. Lanfer-Marquez, U.M., Barros, R.M.C., and Sinnecker, P., Antioxidant activity of chlorophylls and their derivatives, *Food Res. Int.*, 38, 885, 2005.
72. Kamat, J.P., Boloor, K.K., and Devasagayam, P.A., Chlorophyllin as an effective antioxidant against membrane damage *in vitro* and *ex vivo*, *Biochim. Biophys. Acta*, 1487, 113, 2000.
73. Breinholt, V. et al., Mechanisms of chlorophyllin anticarcinogenesis against aflatoxina B1: Complex formation with the carcinogen, *Chem. Res. Toxicol.*, 8, 506, 1995.
74. Dashwood, R.H. et al., Chemopreventive properties of chlorophylls towards aflatoxin B₁: A review of the antimutagenicity and anticarcinogenicity data in rainbow trout, *Mutat. Res.*, 399, 245, 1998.

75. Blum, C.A. et al., Promotion versus suppression of rat colon carcinogenesis by chlorophyllin and chlorophyll: Modulation of apoptosis, cell proliferation, and β -catenin/Tcf signaling, *Mutat. Res.*, 523, 217, 2003.
76. Egner, P.A. et al., Chlorophyllin intervention reduces aflatoxin-DNA adducts in individuals at high risk for liver cancer, *Proc. Nat. Acad. Sci. USA*, 98, 14601, 2001.
77. Carter, O., Bailey, G.S., and Dashwood, R.H., The dietary phytochemical chlorophyllin alters E-cadherin and β -catenin expression in human colon cancer cells: International Research Conference on Food, Nutrition and Cancer, *J. Nutr.*, 134, 3441S, 2004.
78. De Vogel, J. et al., Green vegetables, red meat and colon cancer: Chlorophyll prevents the cytotoxic and hyperproliferative effects of haem in rat colon, *Carcinogenesis*, 26, 387, 2005.
79. Nelson R.L. Chlorophyllin, an antimutagen, acts as a tumor promoter in the rat dimethylhydrazine colon carcinogenesis model, *Anticancer Res.*, 12, 737, 1992.
80. Dashwood, R.H., The importance of using pure chemicals in (anti) mutagenicity studies: Chlorophyllin as a case point, *Mutat. Res.*, 381, 283, 1997.
81. Ma, L. and Dolphin, D., The metabolites of dietary chlorophylls, *Phytochemistry*, 50, 195, 1999.
82. Schlüter, A. et al., The chlorophyll-derived metabolite phytanic acid induces white adipocyte differentiation, *Int. J. Obes.*, 26, 1277, 2002.
83. Arnhold, T., Elmazar, M.M.A., and Nau, H., Prevention of vitamin A teratogenesis by phytol or phytanic acid results from reduced metabolism of retinol to the teratogenic metabolite, all *trans*-retinoic acid, *Toxicol. Sci.*, 66, 274, 2002.

2.2 Carotenoids as Natural Colorants

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2.2.1 CLASSIFICATION AND CHEMISTRY

The carotenoids are the most widely distributed group of pigments, occur naturally in large quantities, and are known for their structural diversity and various functions.¹ The carotenoids constitute a widespread class of natural pigments that occur in all three domains of life: in the eubacteria, the archaea, and the eucarya.² Carotenoids are ubiquitous organic molecules, but they are not produced by the human body. They have been found to be essential to human health based on the nutritional understanding of vitamin A (retinol) and β -carotene.³

New research has demonstrated that carotenoids may also lend additional health benefits that may possibly reduce the risk of certain types of chronic diseases such as cancer and heart disease.⁴ Carotenoids are also important natural sources of orange, yellow, and red food coloring for the food and beverage industries.⁵

Carotenoids are lipid-soluble pigments responsible for many of the brilliant red, orange, and yellow colors in edible fruits (lemons, peaches, apricots, oranges, strawberries, cherries, etc.), vegetables (carrots, tomatoes, etc.), fungi (chanterelles), flow-

TABLE 2.2.1
Distribution of Carotenoids in Some Foods

Carotenoid	Source
Lycopene	Tomato, watermelon, pink grapefruit, papaya, guava, rose hip
β -Carotene	Carrot, apricot, mango, red pepper, kale, spinach, broccoli
α -Carotene	Carrot, collard green, pumpkin, corn, yellow pepper, cloudberry
Lutein + zeaxanthin	Kale, spinach, broccoli, pea, Brussels sprout, collard green, lettuce, corn, egg yolk
β -Cryptoxanthin	Avocado, orange, papaya, passion fruit, pepper, persimmon

Source: Adapted from Osganian, S.K. et al., *Am. J. Clin. Nutr.*, 77, 1390, 2003.

ers, and also in birds, insects, crustaceans, and trout.⁶⁻¹¹ Table 2.2.1 shows the distribution of carotenoids in some foods.¹²

Carotenoids are also present in animal products such as eggs, lobsters, greyfish, and various types of fish.⁶ In higher plants, they occur in photosynthetic tissues and chloroplasts where their color is masked by that of the more predominant green chlorophyll. The best known are β -carotene and lycopene but others are also used as food colorants: α -carotene, γ -carotene, bixin, norbixin, capsanthin, lycopene, and β -apo-8'-carotenal, the ethyl ester of β -apo-8-carotenic acid. These are lipid-soluble compounds, but the chemical industry manufactures water-dispersible preparations by formulating colloid suspensions by emulsifying the carotenoids or by dispersing them in appropriate colloids.⁶

In 1831, Wackenroder isolated an orange pigment from a carrot (*Daucus carota*) and coined the term *carotene* from the Latin word *carota*. Later, in 1837, Berzelius assigned the name xanthophylls to the yellow pigments of autumn leaves. Today more than 650 different carotenoids have been isolated from natural sources and identified, and more than 100 have been found in fruits and vegetables.¹³ Actually, this number has been exceeded if we consider that many carotenoids have been isolated from marine organisms¹⁴ with annual production estimated at 100 million tons.¹⁵

Most of this amount is in the form of fucoxanthin in various algae and in the three main carotenoids of green leaves: lutein, violaxanthin, and neoxanthin. Others produced in much smaller amounts but found widely are β -carotene and zeaxanthin. The other pigments found in certain plants are lycopene and capsanthin (Figure 2.2.1).¹⁶ Colorant preparations have been made from all of these compounds¹⁷ and obviously the composition of a colorant extract reflects the profile of the starting material. Carotenoids are probably the best known of the food colorants derived from natural sources.¹⁸

In general, carotenoids in foods are C_{40} tetraterpenoids comprised of eight C_5 isoprenoid (ip) units (Figure 2.2.2) whose order is inverted at the molecule center, joined head to tail, except at the center where a tail-to-tail linkage reverses the order, resulting in a symmetrical molecule.^{1,7} This produces the parent C_{40} carbon skeleton from which all the individual variations are derived.¹⁹

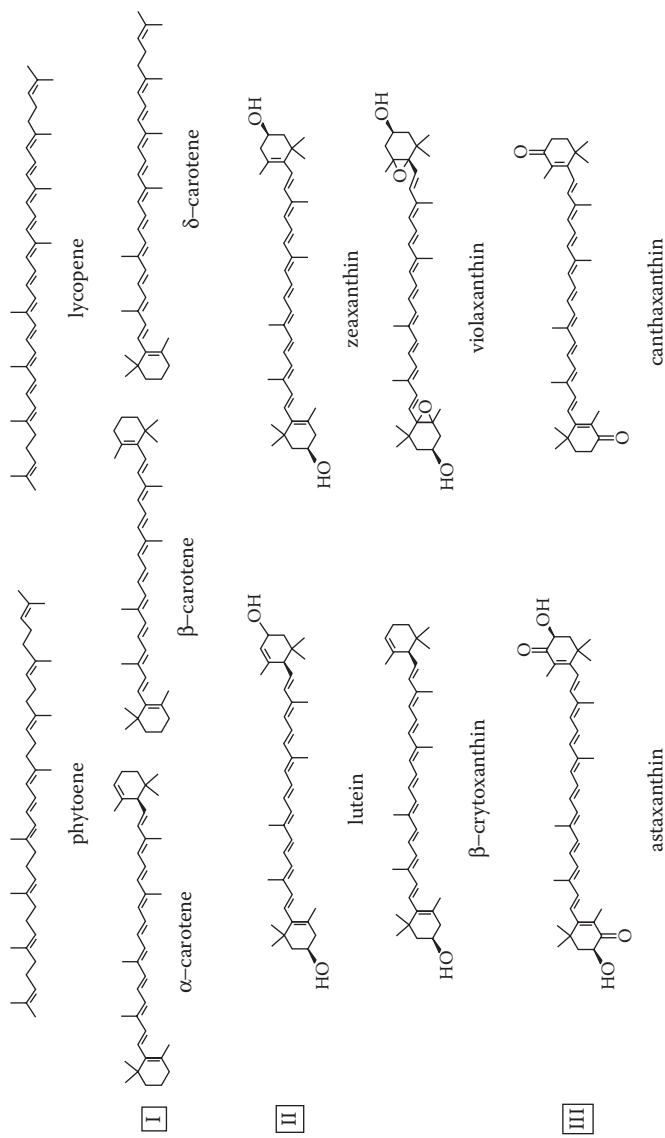


FIGURE 2.2.1 Structures of common carotenoids (I. Main carotenes, II. Xanthophylls, III. Animal carotenoids).

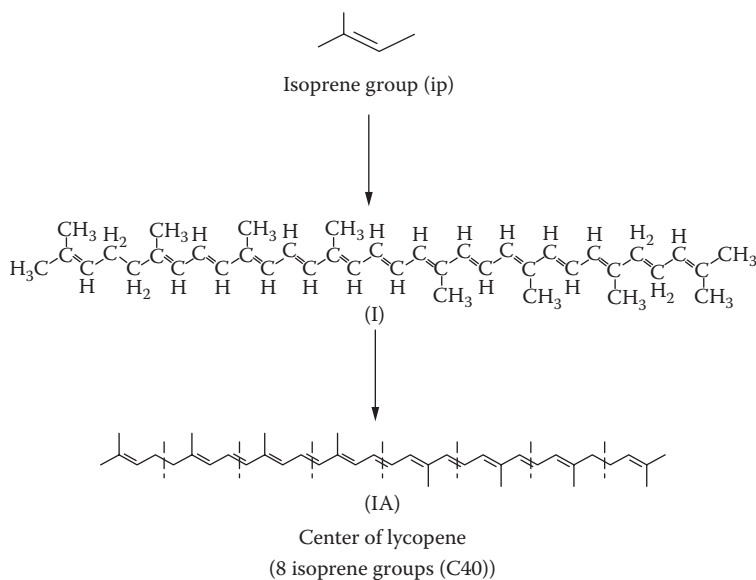


FIGURE 2.2.2 Structure of carotenoid (Source: Adapted from Goodwin, T.W., *Biochemistry of the Carotenoids*, Chapman & Hall, New York, 1980.)

All carotenoids can be considered as lycopene (C₄₀H₅₆) derivatives by reactions. This basic skeleton can be modified in many ways including cyclization at one and/or both ends of the molecule to give different end groups, changes in hydrogenation level, dehydrogenation and introduction of oxygen-containing functional groups, rearrangement, double bond migration, methyl migration, chain elongation, chain shortening, isomerization, or combinations of these processes resulting in a great diversity of structures.^{1,7,20,21}

There are basically two types of carotenoids; those that contain one or more oxygen atoms are known as xanthophylls; those that contain hydrocarbons are known as carotenes.²⁰ Common oxygen substituents are the hydroxy (as in β -cryptoxanthin), keto (as in canthaxanthin), epoxy (as in violaxanthin), and aldehyde (as in β -citraurin) groups.¹ Both types of carotenoids may be acyclic (no ring, e.g., lycopene), monocyclic (one ring, e.g., γ -carotene), or dicyclic (two rings, e.g., α - and β -carotene). In nature, carotenoids exist primarily in the more stable all-trans (or all-E) forms, but small amounts of cis (or Z) isomers do occur.^{1,22}

The most characteristic feature of the carotenoid structure is the long system of alternating double and single bonds that forms the central part of the molecule. This constitutes a conjugated system in which the electrons are effectively delocalised over the entire length of the polyene chain. This portion of the molecule (chromophore) is responsible for the molecular shape, chemical reactivity, and light-absorption in the visible region of the spectra and hence the colors of carotenoids.^{19,22} At least seven conjugated double bonds are needed for the carotenoids to impart color; phytofluene, with five such bonds, is colorless (Table 2.2.2). The color deepens as the conjugated system increases, thus lycopene (11 double bonds) is red. Cycliza-

TABLE 2.2.2
Characteristics of Common Food
Carotenes and Xanthophylls

Name	Characteristics
Phytofluene	Acyclic, colorless
Lycopene	Acyclic, red
ζ-Carotene	Acyclic, light yellow
δ-Carotene	Monocyclic (1β ring), red-orange
γ-Carotene	Monocyclic (1β ring), red-orange
β-Carotene	Bicyclic (2β rings), orange
α-Carotene	Bicyclic (1β ring, 1ε ring), yellow
β-Cryptoxanthin	Bicyclic (2β rings), orange
α-Cryptoxanthin	Bicyclic (1β ring, 1ε ring), yellow
Zeaxanthin,	Bicyclic (2β rings), yellow-orange
Lutein	Bicyclic (1β ring, 1 ring), yellow
Violaxanthin	Bicyclic, yellow
Astaxanthin	Bicyclic (2β rings), red

Sources: Adapted from Rodriguez-Amaya, D.B., *Carotenoids and Food Preparation*, USAID/OMNI, Washington, DC, 1997; Takyi, E.E.K., *Bioavailability of Carotenoids from Vegetables*, CRC Press, Boca Raton, FL, 2001.

tion causes some limitations; hence even though β- and α-carotenes have the same number of conjugated double bonds as lycopene, they are orange and orange-red, respectively. The intensity of food color depends on which carotenoids are present, their concentrations, physical states, and the presence or absence of other plant pigments such as chlorophyll.²²

Some carotenoid derivatives are associated with beneficial effects on human health. Carotenoids containing retinoid structures (β-ionone rings), such as the α- and β-carotenes, serve as precursors of provitamin A. Carotenoids can act as good singlet oxygen quenchers and free radical scavengers due to the many double bonds present in their structures.²³ Handelman²⁴ suggested that the following structural properties may contribute to antioxidant functions of carotenoids:

1. A multiplicity of closely spaced energy levels between the excited state and ground state of the carotenoid, such that the carotenoid can dissipate excited state energy via small collisional exchanges with the solvent.
2. Minimal tendency for the excited-state carotenoid to sensitize other molecules.
3. Resonance states in the excited state carotenoid allowing delocalisation and stabilisation of the excited state.
4. Multiple potential sites on the carotenoid for attack by active oxygen. Each double bond in the polyene chain of a carotenoid can exist in two

configurations, trans or cis geometrical isomers. The presence of a cis double bond creates greater steric hindrance between nearby hydrogen atoms and/or methyl groups, so that cis isomers are generally less stable thermodynamically than the trans form. Most carotenoids occur in nature predominantly or entirely in the all-trans form.

In plants, the carotenoids are located and accumulated in specialized subcellular organelles called plastids, concretely in the chloroplasts — accompanying chlorophylls and chromoplasts.²⁵ The chloroplasts are present in all photosynthetic tissues, where practically all the carotenoids are present in the form of chlorophyll–carotenoid–protein complexes at the level of the thylakoid membranes.²⁶ In green leaves, carotenoids are free and nonesterified, and their compositions depend on the plant and developmental conditions. Some leaves of gymnosperms accumulate not very common carotenoids in oily droplets, which are extraplastidial: rhodoxanthin in some members of the families Cupressaceae and Taxaceae and semi- β -carotenone in young leaves of cycads. In reproductive tissues, liliaxanthin has been found in white lilies and crocetin has been found in crocus species stigmas; in flowers more than 40 pigments exclusive of petals have been identified.

Fruits are yet more prodigious than flowers in their synthetic abilities. More than 70 characteristic carotenoids have been described and classified as those with minimal quantities, higher quantities, and specific carotenoids, for example, capsanthin and capsorubin in pepper fruits.^{8,25,27}

Interestingly, carotenoids have been identified in wood. Samples of oak (*Quercus robur* L., *Quercus petrae* Liebl., and *Quercus alba* L.), chestnut (*Castanea sativa* Mill.), and beech (*Fagus silvatica* L.) were studied at different ages and sections. Lutein and β -carotene were identified in oak wood and also in other deciduous species. These carotenoids may be the origins of β -ionone and more than 30 other norisoprenoid substances identified in oak wood. Considering that carotenoids are hydrophobic and not soluble in sap, it is suggested that the *in situ* formation of carotenoids in living cells occurs in the sapwood. It was reported that sapwood was richer in β -carotene than lutein, and the ratio was reversed in the heartwood. Also, it was found that lutein could be used as a marker to distinguish between wood samples.²⁸

2.2.2 PHYSICAL CHARACTERISTICS

The physical properties of pure carotenoids, especially their poor stability and low solubility, are particularly significant.²⁹ Carotenoids are unstable in the presence of light and oxygen.^{30–32} The central chain of conjugated double bonds is oxidatively cleaved chemically at various points, giving rise to a family of apocarotenoids. Most carotenoids, but not vitamin A, also serve as singlet oxygen quenchers. In essence, singlet oxygen, which is an electronically excited and highly reactive form of oxygen, interacts with the highly conjugated, ground state carotenoid to yield triplet states of both molecules. The triplet state of oxygen is its less active ground state, whereas the triplet carotenoid returns to the ground state by the emission of thermal energy. Carotenoids can also serve as antioxidants and free radical quenching agents. Carotenoids interact rapidly with free radicals and with oxygen, thereby inhibiting the

TABLE 2.2.3
Light Absorbances of Selected Carotenoids

Carotenoid	Solvent	Absorption Maximum (nm)	E _{1cm} ^{1%}
Canthaxanthin	Light petroleum	466	2200
α-Carotene	Light petroleum	422, 444, 474	2800
β-Carotene	Light petroleum	425, 453, 479	2592
β-Cyptoxanthin	Light petroleum	425, 452, 479	2386
Lutein	Ethanol	421, 445, 475	2550
Lycopene	Light petroleum Ethanol	444, 472, 502	3450
Neoxanthin		416, 439, 467	2243
Violaxanthin	Ethanol	420, 443, 470	2550
Zeaxanthin	Light petroleum	426, 452, 479	2348

Sources: Britton, G., in *Carotenoids 1B: Spectroscopy*, Birkhauser Verlag, Basel, 1995, 13; De Ritter, A.E., in *Carotenoids as Colorants and Vitamin A Precursors*, Academic Press, New York, 1981, 815.

propagation step of lipid peroxidation. Carotenoids serve this function best at low oxygen tensions; indeed, carotenoids can be pro-oxidants in 100% oxygen.¹⁹

The carotenoids as a group are extremely hydrophobic molecules with little or no solubility in water. They are thus expected to be restricted to hydrophobic areas in cells, such as the inner cores of membranes, except when association with protein allows them access to an aqueous environment.¹⁹ Their chemical structures make carotenoids very insoluble in water, but they are fat soluble.³³ Carotenoids in the food matrix are relatively stable during typical thermal processing.³⁴ Several precautions are necessary in handling carotenoids, e.g., carrying out experiments under dim light, evaporation by rotary evaporator under nitrogen gas flow, storage in the dark under nitrogen or argon at -20°C , and use of antioxidants such as butylated hydroxyanisol, pyrogallol, or ascorbic acid.^{33,36}

Because of their highly conjugated double-bond systems, carotenoids show characteristic ultraviolet and visible absorption spectra.³⁷ For most carotenoids, three peaks or two peaks and a shoulder absorb in the range of 400 to 500 nm. Light absorbances of selected carotenoids are shown in Table 2.2.3. Both the wavelength maximum and E_{1cm}^{1%} are significantly affected by the solvent used. Thus, for all-trans β-carotene, the wavelength maximum and E_{1cm}^{1%} are 453 nm and 2592 in petroleum ether, 453 nm and 2620 in ethanol, 465 nm and 2337 in benzene, 465 nm and 2396 in chloroform, and 484 nm and 2008 in carbon disulfide. The cis isomers not only absorb less strongly than the all-trans isomer, but also show a so-called cis peak of absorbance at 330 to 340 nm.^{37,38}

2.2.3 CHEMICAL PROPERTIES

The fundamental chemistry of carotenoid radicals and the reactions with oxidizing agents, peroxy radicals, etc., is important for evaluating the proposed actions of

carotenoids as antioxidants. The electron-rich conjugated double bond structure is primarily responsible for the excellent ability of β -carotene to physically quench singlet oxygen without degradation, the chemical reactivity of β -carotene with free radicals, and its instability toward oxidation.^{19,39} Oxidation, the major cause of carotenoid loss, depends on available oxygen and the carotenoid involved, and is stimulated by light, heat, peroxides, metals such as iron, and enzymes, while inhibited by antioxidants such as tocopherols and ascorbic acid. Oxidation therefore leads to complete loss of activity while isomerization leads to reduced activity.²²

The overall size and shape of a molecule are extremely important in relation to the properties of a carotenoid and hence to function. All colored carotenoids in the all-trans configuration have extended conjugated double bond systems and are linear, rigid molecules. The cis isomers, however, are no longer simple linear molecules. Their overall shapes differ substantially from those of the all-trans forms, so their ability to fit into subcellular structures may be greatly altered.¹⁹ During isomerization, the carotenoid molecules fold back and change from the naturally occurring trans form to the cis form. The conditions necessary for the isomerization and oxidation of carotenoids are likely to exist in home preparations, industrial processing, and during storage of foods. The polyene chain is the cause of the instability of carotenoids, including their susceptibility to oxidation and geometric isomerization. Heat, light, and acids promote isomerization of trans carotenoids, their usual configuration in nature, to the cis form.⁴⁰

Carotenoid radicals — Many of the important oxidations are free-radical reactions, so a consideration of the generation and properties of carotenoid radicals and of carbon-centered radicals derived from carotenoids by addition of other species is relevant. The carotenoid radicals are very short-lived species. Some information has been obtained about them by the application of radiation techniques, particularly pulse radiolysis. Carotenoid radicals can be generated in different ways.^{41,42}

1. *Oxidation* — Oxidizing radicals with high redox potential can remove one electron from the carotenoid molecule to yield a radical cation: $\text{CAR} - e^- \rightarrow \text{CAR}^+$ (e.g. $\text{CAR} + \text{R} \rightarrow \text{CAR}^+ + \text{R}$).
2. *Reduction* — The addition of one electron to the carotenoid molecule would give the radical anion: $\text{CAR} + e^- \rightarrow \text{CAR}^-$.
3. *Hydrogen abstraction* — The abstraction of a hydrogen atom $\text{H}^\cdot$ from a saturated carbon atom in a position allylic to the polyene chain can generate a resonance-stabilized neutral radical by homolytic cleavage of a C-H bond: $\text{CAR} = \text{X} - \text{H}$. Then $\text{X} - \text{H} + \text{R}^\cdot \rightarrow \text{X}^\cdot + \text{RH}$.
4. *Addition* — The addition of a radical species such as a peroxy radical $\text{ROO}^\cdot$ or the hydroxyl radical $\text{HO}^\cdot$ to the polyene chain could generate a carotenoid-adduct radical: $\text{CAR} + \text{ROO}^\cdot \rightarrow \text{CAR} - \text{OOR}^\cdot$.

In the carotenoid radicals, the unpaired electron is highly delocalized over the conjugated polyene chromophore. This has a stabilizing effect and also allows subsequent reactions. The cation and anion radicals can be detected by their characteristic spectral properties, with intense absorption in the near-infrared region.

2.2.3.1 MAJOR CAROTENOIDS

Beta-carotene — This compound occurs in nature, usually associated with a number of chemically closely related pigments and extracts that have been used as food colorants for many years. β -Carotene is a carotenoid with the many conjugated double bonds seen in lycopene, forming a connected double ring structure.⁴³ Most β -carotene applied today is manufactured by synthesis, resulting in a molecule equivalent to that found in nature. However, several natural sources are available and are increasingly used to replace the synthetic variant.⁴⁴ It is derived from green leaves, where it functions as a photoenergy transfer medium and as a photoprotectant in the light-harvesting complexes of the chloroplasts.⁴⁵

β -Carotene is found in the form of a crystalline powder ($C_{40}H_{56}$, mol wt 536.9, β , β -carotene). It is insoluble in water and ethanol and not very soluble in vegetable fats. In chloroform, the maximum spectrometric absorption is found between 466 and 496 nm. β -Carotene is sensitive to oxygen (air), heat, light, and humidity.⁶

β -Carotene plays a crucial role in human health since it is the major source of vitamin A for most people throughout the world.⁴⁶ It has vitamin A activity: 1 g of β -carotene corresponds to 1.67 million IU of vitamin A and the vitamin activity of 0.6 mg of β -carotene is almost equivalent to 0.3 mg of vitamin A. The antioxidant properties of β -carotene are currently the subjects of special attention because they may be involved in the mechanisms of preventing certain types of cancers.⁶

In the spirulina, photosynthetic microalgae rich in proteins, the level of β -carotene can reach 10% of the dry matter.⁶ Although it is present in hundreds of dark green vegetables, the most concentrated sources of β -carotene are carrots, squashes, pumpkins, and mangos.⁴⁷ Peaches, apricots, and papayas are the major fruit sources and yellow-orange fleshed varieties of sweet potatoes and cassavas are the other major sources in some diets. Most of the world's major cereals contain very little β -carotene but small amounts are present in maize and grain legumes. High-carotenoid rice is being developed.⁴⁵

Lutein and Zeaxanthin — Lutein is a major component of many plants. It is a component of most of the carotenoid extracts suggested as food colorants.⁴⁸ Lutein is the dominant xanthophyll in leafy green and yellow vegetables, which are the primary human sources of carotenoids.⁴⁹ Lutein has a structure similar to β -carotene with a hydroxyl group on the ionone ring at each end of the molecule.⁴⁸ As its name indicates, it is a dihydroxy carotenoid and the presence of the polar groups alters its properties so that it is easily separated from the hydrocarbon carotenoids. Lutein has one end group β and one ϵ end group. Zeaxanthin is symmetric and has two β end groups. Both lutein and zeaxanthin are dihydroxy carotenoids with the hydroxyl groups located on the 3 and 3' carbons. In lutein, the hydroxyl group is allylic to the isolated double bond in the ϵ ring.

The maximum spectrometric absorption of lutein ($C_{40}H_{56}O_2$, mol wt 568.9, xanthophyll, (3R,3',6',6'- β , ϵ -carotene-3,3'-diol) is found between 453 and 481 nm. Its solubility in ethanol is greater than that of the carotenoids.⁶ It is somewhat less sensitive to oxidation and heat degradation than β -carotene. It contributes yellow color.⁴⁸

Although present in free form in leaves, the acyl (palmitate) esters normally occur in fruits and flowers. Rich sources of lutein include spinach, kale, and broccoli. The main sources in the human diet are green leafy vegetables. Immature legumes (peas), unripe fruit (green peppers), and egg yolks are also good sources.^{46,49}

Zeaxanthin ($C_{40}H_{56}O_2$, mol wt 568.9, (3R,3R') β,β -carotene-3.3'-diol) is a constitutional isomer of lutein and it differs from lutein structurally in subtle but important ways.⁵⁰ This dihydroxy carotenoid is mainly derived from maize as its name suggests, although traces are found in many foods. It is chromatographically difficult to separate from its isomer lutein.⁴⁵

Zeaxanthin is the abundant xanthophyll in only a small number of food sources and is the dominant xanthophyll in orange peppers and Gou Zi Qi or lycium mill (*Lycium chinense*) berries, probably the richest sources.^{51,52}

Lycopene — This compound is the major pigment in tomatoes and is one of the major carotenoids in the human diet. Lycopene is a long hydrocarbon chain with 11 conjugated double bonds and it lacks the characteristic ring structures.^{53,54} It is a long chain conjugated hydrocarbon and its structure suggests that it would be easily oxidized in the presence of oxygen and isomerized to cis compounds by heat. Both of these reactions occur in purified solutions of lycopene but in the presence of other compounds normally present in tomatoes, lycopene is more stable.⁵⁵

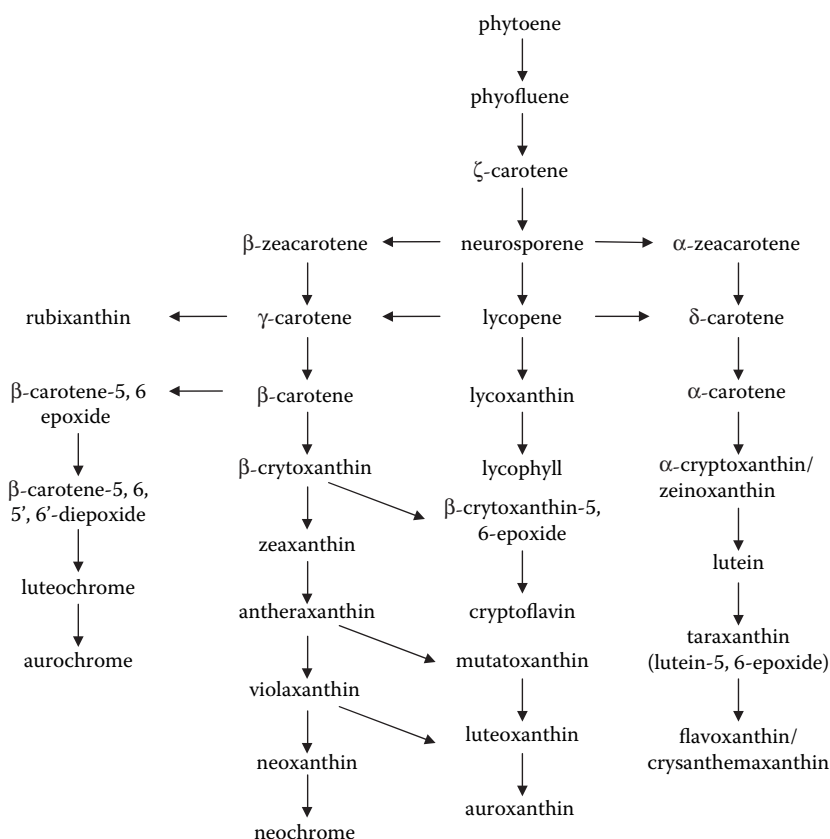
Lycopene ($C_{40}H_{56}$, mol wt 536.9, ψ,ψ -carotene) has maxima of absorption at 446, 472, and 505 nm (for the trans form). It is soluble in chloroform and benzene, and virtually insoluble in methanol and ethanol. The β -apo-8'-carotenal trans form is widespread in nature in citrus fruits, vegetables, and grasses. Often a synthetic carotenoid in the form of a fine purple crystalline powder, insoluble in water, slightly soluble in ethanol and vegetable oils, and very soluble in chloroform is used. This pigment is heat-sensitive.

Lycopene is a bright red pigment that colors several ripe fruits, vegetables, and flowers. Tomato and tomato products are the main dietary sources of this carotenoid, although it is also found in watermelons, guavas, pink grapefruits, and in small quantities in at least 40 plants.^{45,56} The absorption of lycopene in the human gut is increased by heat treatment, probably because the breakdown of the plant cells makes the pigment more accessible.⁴⁸

2.2.4 BIOSYNTHESIS

Carotenoids are predominantly synthesized in nature by photosynthetic plants, algae, bacteria, and some fungi.^{57,58} Animals can metabolize carotenoids in a characteristic manner, but they are not able to synthesize carotenoids. The total global biosynthesis of carotenoids is estimated to be in excess of 100 million tons per year.⁵⁷

Subsequent cyclizations, dehydrogenations, oxidations, etc., lead to the individual naturally occurring carotenoids, but little is known about the biochemistry of the many interesting final structural modifications that give rise to the hundreds of diverse natural carotenoids. The carotenoids are isoprenoid compounds and are biosynthesized by a branch of the great isoprenoid pathway from the basic C_5 -terpenoid precursor, isopentenyl diphosphate (IPP). The entire biosynthesis takes place in the chloroplasts (in green tissues) or chromoplasts (in yellow to red tissues),



encoded by nucleus genes. In carotenoids, the isoprenoid chain is built up from mevalonic acid (MVA) by prenyl transferases to the C₂₀ level, as geranylgeranyl diphosphate, and two molecules of this are joined tail to tail to give 15-cis phytoene as the first product with the C₄₀ carotenoid skeleton, which is catalysed by the phytoene synthase (PSY). See Figure 2.2.3.

Phytoene is colorless but undergoes a series of desaturation reactions, each of which creates a new double bond and extends the chromophore by two conjugated double bonds. The end product is lycopene, produced via the successive intermediate phytofluene, ζ -carotene, and neurosporene by the combined action of phytoene desaturase (PDS) and ζ -carotene desaturase (ZDS). The light absorption maximum shifts progressively to longer wavelengths as the chromophore is extended and lycopene, with 11 conjugated double bonds, absorbs maximally ($A_{\max}$) at 470 to 500 nm and is strongly colored orange-red. The phytoene in higher plants appears to be formed as the 15Z isomer, although lycopene and the other colored carotenoids are generally in the all-E form. Isomerization from Z to E must therefore take place during the desaturation sequence but the stage at which this occurs has not been established unequivocally. The lycopene molecule may then undergo cyclization,

the branch point that later gives the great variety of xanthophyll structures, to form six-membered rings at one end or both ends of the molecule, e.g., β rings and ϵ rings. This reaction is catalyzed by two lycopene cyclases. Lycopene β -cyclase catalyses a two-step reaction that forms one β -ionone ring at each end of the lycopene molecule to give β,β -carotene. Lycopene ϵ -cyclase creates only one ring to produce δ -carotene from lycopene or β,ϵ -carotene from γ -carotene (with only one β ring).

The introduction of oxygen functions and other structural modifications of end groups including esterification then follow as the final stages of biosynthesis. Thus zeaxanthin and lutein are formed by the introduction of two hydroxy groups at C-3 and C-3' of β,β -carotene and β,ϵ -carotene, respectively by the action of hydroxylases. Following hydroxylation, an epoxyde group can be introduced at positions 5 and 6 of the 3-hydroxy- β ring. In this way zeaxanthin is converted into violaxanthin via antheraxanthin by introducing, respectively, two and one 5,6 epoxyde groups.

Schemes have been proposed for the formation of a variety of other end groups by rearrangement of a 3-hydroxy-5,6-epoxy- β ring end group.¹³ In the case of hydroxycarotenoids, it is common in fruits (red pepper, lemon peel, etc.) for hydroxycarotenoids to occur naturally as esters with different fatty acids.⁵⁹ It is assumed that fatty acid carotenoid esters are formed conventionally by esterification of the hydroxy groups with the appropriate acyl-CoA, but the biochemistry of the process has not been studied.⁶⁰ Because of the number of combinations of reactions that are possible in the two ends of the molecule, the conventional pathways that can be constructed can look very complicated. However, the picture is greatly simplified when considered in terms of sequences of reactions that can occur in one end of the molecule or the other. Thus the formation of zeaxanthin from lycopene involves only two reactions, namely β -cyclization and hydroxylation, at each end group. For instance, the exotic-looking cyclopentanone end group of capsanthin and capsorubin requires only two additional reactions, namely an epoxydation and a rearrangement. Each reaction, whether it occurs in only one or in both end groups, is catalyzed by a particular enzyme.⁶¹

2.2.4.1 OCCURRENCE OF CAROTENOIDS

Over 600 carotenoids occur in plants, animals, and microbes. Since only higher plants and photosynthetic microorganisms can synthesize carotenoids, animals appear to be incapable of synthesizing them. Carotenoids in animals all come from dietary sources. They also occur in some nonphotosynthetic bacteria, yeasts, and molds, where they may carry out protective functions against damage by light and oxygen.⁶² Carotenoids are mainly obtained from plant sources such as carrots, green leafy vegetables, spinach, oranges, and tomatoes. Animal sources include calf liver, whole milk, butter, cheddar cheese, and eggs.

Typically several different carotenoids occur in plant tissues containing this class of pigments. Carotenoids are accumulated in chloroplasts of all green plants as mixtures of α - and β -carotene, β -cryptoxanthin, lutein, zeaxanthin, violaxanthin, and neoxanthin. These pigments are found as complexes formed by noncovalent bonding with proteins. In green leaves, carotenoids are free, nonesterified, and their compositions depend on the plant and developmental conditions. In reproductive

tissues, liliaxanthin in white lily and crocetin in *Crocus* sp. stigmas have been found; in flowers more than 40 pigments exclusive of petals have been identified. Certain flowers synthesize (1) highly oxygenated carotenoids, frequently 5,8-epoxydes, (2) β -carotenes, and (3) species-specific carotenoids. Fruits are more prodigious in their synthetic abilities than flowers.^{8,25,27} Plant carotenoids are red, orange, and yellow lipid-soluble pigments found embedded in the membranes of chloroplasts and chromoplasts.

The red algae Rhodophyta contain α - and β -carotene and their hydroxylated derivatives. The main pigments of Pyrrophyta are peridinin, dinoxanthin, and fucoxanthin. Chrysophyta accumulates epoxy, allenic, and acetylenic carotenoids, and between them fucoxanthin and diadinoxanthin. In Euglenophyta, eutreptiellanone has been found. The principal carotenoids in Chloromonadophyta are diadinoxanthin, heteroxanthin, and vaucheriaxanthin. Alloxanthin, monadoxanthin, and crocoxanthin characterized in Chryptophyta while the Phaeophyta are characterized by their main pigment, fucoxanthin.^{8,25}

Approximately 80 different carotenoids are synthesized by photosynthetic bacteria. The accumulated carotenoids have certain characteristics:

1. Most carotenoids are aliphatic, but some carotenoids in Chlorobiaceae and Chloroflexaceae have aromatic or β -rings.
2. They contain aldehydes with crossover conjugations and tertiary methoxy groups.
3. Various classes of carotenoids are present in each species.
4. All carotenoids are bound to the light harvesting complexes or reaction centers in membranal systems of bacterial cells.
5. Structural elements such as allenic or acetylenic bonds, epoxydes, furanoxides, and C_{45} or C_{50} carotenoids are not found.

In vivo, one of the main groups of carotenoids are the sulfates of eritoxanthin sulfate and of the caloxanthin sulfates. The sulfates of carotenoids are not associated with pigment-protein complexes, for example, they are neither part of the light harvesting complexes nor of the reaction centers. In nonphotosynthetic bacteria, carotenoids appear sporadically and when present, they have unique characteristics. Some Staphylococci accumulate C_{30} carotenoids, flavobacteria C_{45} and C_{50} , while some mycobacteria accumulate C_{40} carotenoid glycosides.⁸

Carotenoid distribution in fungi, nonphotosynthetic organisms, are apparently capricious, but they usually accumulate carotenes, mono- and bicyclic carotenoids, and lack carotenoids with ϵ rings. Plectanixanthin in Ascomycetes and canthaxanthin in *Cantharellus cinnabarinus* have been found.⁸

Both chlorophylls and carotenoids occur in all green leaves, but their color is masked by chlorophyll in photosynthetic tissues. When the chlorophylls break down as leaves senesce (mature), the yellow and orange carotenoids persist and the leaves turn yellow.³³ Carotenoids are responsible for the colors of familiar animals such as lobsters, flamingos, and fish. Often people are unaware of the chemical nature of food colorants.⁶³

Lycopene, lycoxanthin, and lycophyll are rarely encountered; they are found in trace amounts in tomatoes. Rubixanthin, derived from γ -carotene, is the main pigment of rose hips and also occurs in appreciable levels in *Eugenia uniflora*.⁶⁴ The α -cryptoxanthin and zeinoxanthin xanthophylls are widely distributed, although generally at low levels. β -Cryptoxanthin is the main pigment of many orange-fleshed fruits such as peaches, nectarines, papayas, persimmons, tree tomatoes, and *Spondias lutea*, but occurs rarely as a secondary pigment. Lutein is normally present in plant tissues at considerably higher levels than is zeaxanthin, which is the predominant carotenoid in leaves, green vegetables, and yellow flowers. β -Carotene is the preponderant pigment of many foods and whatever zeaxanthin is formed is easily transformed to antheraxanthin, particularly violaxanthin.

Lutein appears to undergo limited epoxidation. Because of its facile degradation, the violaxanthin epoxycarotenoid may be underestimated in foods, as shown for mangos.⁶⁵ The most prominent examples are capsanthin and capsorubin, the predominant pigments of red peppers. Other classical examples of unique carotenoids are bixin, the major pigment of annatto, a food colorant, and crocetin, the main coloring component of saffron. Astaxanthin is the principal carotenoid of certain fish such as salmon and trout, and most crustaceans (shrimp, lobsters, and crabs). The intermediates in the transformation of dietary carotenoids, such as echinenone and canthaxanthin, are often detected as accompanying minor carotenoids. Tunaxanthin is also a major carotenoid of fish.

2.2.5 FUNCTIONS

The structural, chemical, and physical properties of carotenoids produce varied biological functions and actions. The conjugated polyene chromophore determines the light absorption properties, color, and also the photochemical properties of a molecule and consequent light harvesting and photoprotective actions. The polyene chain is also the feature mainly responsible for the chemical reactivity of carotenoids toward oxidizing agents and free radicals, and hence for any antioxidant role.¹⁹

2.2.5.1 LIGHT ABSORPTION

The absorption of light energy by an organic molecule produces a higher-energy excited state of that molecule. In the case of carotenoids, the relevant transition is a $\pi \rightarrow \pi^*$ transition in which one of the bonding π electrons of the conjugated double bond system is promoted to a previously unoccupied π^* antibonding orbital. The π electrons are highly delocalized and the excited state is of comparatively low energy, so the energy required to bring about the transition is relatively small and corresponds to light in the visible region in the wavelength range of 400 to 500 nm. Carotenoids are therefore intensely colored yellow, orange, or red. The relationship between chromophores and light absorption properties, widely used in the identification of carotenoids, is developed more fully elsewhere.³⁷

Carotenoids also assist chlorophylls in harvesting light. Carotenoids absorb wavelengths of blue light which chlorophylls do not. The energy that carotenoids harvest in the blue range of the spectrum and transfer to chlorophyll contributes

significantly to photosynthesis. The growth and development of plants are often stimulated by light, and carotenoids have sometimes been implicated as the photo-receptors of light that trigger these responses.³³

In plants, carotenoids function as accessory light harvesting pigments that absorb light energy, which is then transferred to chlorophyll for use in photosynthesis.⁶⁷ Carotenoids also act as UV light scavengers, protecting plants from photooxidation and its adverse effects, preventing cell damage from singlet oxygen. Plants also use carotenoids during times of stress, injury, or severe light exposure, in order to protect themselves from further infection and oxidative damage.⁶⁷

2.2.5.2 PHOTOSYNTHESIS

Carotenoids are essential to plants for photosynthesis, acting in light harvesting and especially in protection against destructive photooxidation. Without carotenoids, photosynthesis in an oxygenic atmosphere would be impossible. Some animals use carotenoids for coloration, especially birds (yellow and red feathers), fish and a wide variety of invertebrate animals, where complexation with protein may modify their colors to blue, green or purple.⁶⁸

The main pigments involved in photosynthesis are chlorophylls and carotenoids. Carotenoids perform two well known functions in photosynthesis. Potentially harmful oxidizing compounds are generated during photosynthesis. The carotenoids occur in photosynthetic tissues along with chlorophyll to protect them from photooxidative damage. It has been proposed that carotenoids as light harvesting compounds evolved from anaerobic organisms, then generalized to all the aerobic photosynthetic organisms.⁶⁹ The carotenoids have been shown to be active in the light gathering process. In *Chlorella*, other green algae, and higher plants, the light absorbed by the carotenoids is used at low efficiency. In diatoms and brown algae, the energy transfer is comparable to chlorophyll in which the main pigment is fucoxanthin. In the photosynthetic process, two photosystems are involved. More carotenes are generally found in photosystem I and more xanthophylls in photosystem II.⁷⁰

2.2.5.3 PROVISION OF COLOR

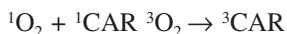
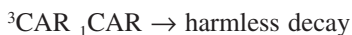
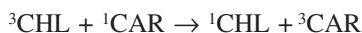
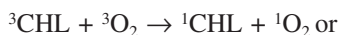
Outside of photosynthesis, plant carotenoids also serve as pigments that, along with anthocyanins and betalins, provide color to flowers, ripening fruit, and other plant parts. Common examples of carotenoids having this role are found in sunflowers, marigolds, bananas, peaches, oranges, tomatoes, peppers, melons, and yellow corn. Two root crops, carrots and sweet potatoes, also acquire their color from carotenoids. The color attracts insects, birds, and bats for pollinating flowers.³⁷ In nonphotosynthetic tissues, carotenoids determine or contribute to the colors of flowers and fruits.⁷¹

2.2.5.4 PHOTOPROTECTION

Carotenoids protect photosynthetic organisms against potentially harmful photooxidative processes and are essential structural components of the photosynthetic antenna and reaction center complexes.⁷¹ Plant carotenoids play fundamental roles as accessory pigments for photosynthesis, as protection against photooxidation, and

as structural determinants in plastid pigment–protein complexes. The role of these pigments is determined primarily by whether a tissue is photosynthetic or nonphotosynthetic. In photosynthetic tissues, photoprotection against harmful oxygen species is their most important function.⁷²

The photoprotective role of carotenoids is demonstrated in plant mutants that cannot synthesize essential leaf carotenoids. These mutants are lethal in nature since without carotenoids, chlorophylls degrade, their leaves are white in color, and photosynthesis cannot occur.³³ Generally, the carotenoids are effective for visible light but have no effects in ultraviolet, gamma, or x-radiation. The reactions are listed as follows:



As can be seen in these reactions, carotenoids may protect photosynthetic bacteria at various levels by quenching the singlet-excited state of O_2 or the triplet-excited state of chlorophyll. The ground states of oxygen would be ${}^3\text{O}_2$ and for CHL the triplet state. The carotenoids may be the preferred substrates for oxidation or may act in quenching reactive species.⁷⁰

It has been established that carotenoid structure has a great influence in its antioxidant activity; for example, canthaxanthin and astaxanthin show better antioxidant activities than β -carotene or zeaxanthin.^{73–75} β -Carotene also showed prooxidant activity in oil-in-water emulsions evaluated by the formation of lipid hydroperoxides, hexanal, or 2-heptenal; the activity was reverted with α - and γ -tocopherol. Carotenoid antioxidant activity against radicals has been established. In order of decreasing activity, the results are lycopene > β -cryptoxanthin > lutein = zeaxanthin > α -carotene > echineone > canthaxanthin = astaxanthin.⁷⁶

2.2.5.5 VITAMIN A PRECURSORS

In animals, the major function of carotenoids is as a precursor to the formation of vitamin A.⁷⁰ Carotenoids with provitamin A activity are essential components of the human diet, and there is considerable evidence that they are absorbed through the diet and often metabolized into other compounds.³² Beyond their important role as a source of vitamin A for humans, dietary carotenoids, including those that are not provitamin A carotenoids, have been implicated as protecting against certain forms of cancer and cardiovascular disease.³³

It is assumed that in order to have vitamin A activity a molecule must have essentially one-half of its structure similar to that of β -carotene with an added molecule of water at the end of the lateral polyene chain. Thus, β -carotene is a potent provitamin A to which 100% activity is assigned. An unsubstituted β ring with a C_{11} polyene chain is the minimum requirement for vitamin A activity. γ -Carotene, α -carotene, β -cryptoxanthin, α -cryptoxanthin, and β -carotene-5,6-epoxide all have single unsubstituted rings.⁷⁷ Recently it has been shown that astaxanthin can be converted to zeaxanthin in trout if the fish has sufficient vitamin A. Vitiated astaxanthin was converted to retinol in strips of duodenum or inverted sacks of trout intestines. Astaxanthin, canthaxanthin, and zeaxanthin can be converted to vitamin A and A_2 in guppies.⁷⁰

REFERENCES

1. Rodriguez-Amaya, D.B. and Kimura, M., *Harvest Plus Handbook for Carotenoid Analysis Harvest Plus Technical Monograph 2*, Washington, International Food Policy Research Institute and International Center for Tropical Agriculture, 2004.
2. Britton, George, 2006. Occurrence. *The Carotenoids Page*. November 1. <http://dcb-carot.unibe.ch/occur.htm>.
3. Otles, Semih, 2006. Carotenoids. *Carotenoids*. October 15. <http://eng.ege.edu.tr/~otles/ColorScience/carotenoids.htm>.
4. Chaudhry, Y., Carotenoids: natural food colors and health benefits, Symposium 12 Interaction of Natural Colors with Other Ingredients, July 19, 2003, GNT USA Inc., Tarrytown, NY.
5. Otles, S. and Atl, Y., Karotenoidlerin insan sagligi icin onemi, *Muhendislik Bilimleri Dergisi*, 3, 249, 1997.
6. Linden, G. and Lorient, D., *New Ingredients in Food Processing*. Woodhead Publishing, Cambridge, U.K., 1999.
7. Goodwin, T.W., *Biochemistry of the Carotenoids*, Vol. 1, 2nd ed., Chapman & Hall, New York, 1980.
8. Goodwin, T.W., Biosynthesis of carotenoids: an overview, *Meth. Enzymol.*, 330, 112, 1992.
9. Gordon, H.T. and Bauernfeind, J.C., Carotenoids as food colorants, *Crit. Rev. Food Sci. Nutr.*, 18, 59, 1982.
10. Hari, R.K., Patel, T.R., and Martin, A.M., An overview of pigment production in biological systems: functions, biosynthesis, and applications in food industry, *Food Rev. Int.*, 10, 49, 1994.

11. Wong, D.W.S., Colorants, in *Mechanism and Theory in Food Chemistry*, Avi Publishing, Westport, CT, 1989.
12. Osganian, S.K. et al., Dietary carotenoids and risk of coronary artery disease in women, *Am J Clin Nutr.*, 77, 1390, 2003.
13. Britton, G. and Hornero-Mendez, D., Carotenoids and colour in fruit and vegetables, in *Phytochemistry of Fruit and Vegetables.*, Tomas-Barberan, T.A. and Robins, R.J., Eds., Clarendon Press, Oxford, 1997, 11.
14. Tsushima, M., Fujiwara, Y., and Matsuno, T., Novel marine di-Z-carotenoids: cucumariaxanthins A, B and C from the sea cucumber *Cucumaria japonica*, *J. Nat. Prod.*, 59, 30, 1996.
15. Francis, F.J., Carotenoids, in *Colorants*, Eagan Press, St. Paul, MN, 1999.
16. Francis, F.J., Pigments and other colorants, in *Food Chemistry*, 2nd ed., Fennema, O.R., Ed., Marcel Dekker, New York, 1985, 545.
17. Bauernfeind, J.C., *Carotenoids as Colorants and Vitamin A Precursors*, Academic Press, New York, 1981.
18. Francis, F.J., *Handbook of Food Colorants*, Eagan Press, St. Paul, MN, 1999.
19. Britton, G., Structure and properties of carotenoids in relation to function. *FASEB J.*, 9, 1551, 1995.
20. Haila, K., Effects of carotenoids and carotenoid-tocopherol interaction on lipid oxidation *in vitro*, etc., University of Helsinki, Department of Applied Chemistry and Microbiology, Helsinki, 1999.
21. Rodriguez-Amaya, D.B., *Carotenoids and Food Preparation: The Retention of Provitamin A Carotenoids in Prepared, Processed, and Stored Foods*, USAID/OMNI, Washington, D.C., 1997.
22. Takyi, E.E.K., *Bioavailability of Carotenoids from Vegetables versus Supplements in Vegetables, Fruits and Herbs in Health Promotion*, CRC Press, Boca Raton, FL, 2001.
23. Foote, C., Photosensitized oxidation and singlet oxygen: consequences in biological systems, in *Free Radicals in Biology*, Pryor, W.A., Ed., Academic Press, New York, 1976.
24. Handelman, G.J., Carotenoids as scavengers of active oxygen species, in *Handbook of Antioxidants*, Cadenas, E. and Packer, L., Eds., Marcel Dekker, New York, 1996, 259.
25. Goodwin, T.W. and Britton, G., Distribution and analysis of carotenoids, in *Plant Pigments*, Goodwin, T.W., Ed., Academic Press, London, 1988, 62.
26. Sitte, P., Falk, H., and Liedvogel, B. Chromoplasts, in *Pigments in Plants*, Czygan, F.Ch., Ed., Gustav Fischer, Stuttgart, 1980, 117.
27. Lichtenhaler, H.K., Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, *Meth. Enzymol.*, 148, 350, 1987.
28. Masson, G. et al., Demonstration of the presence of carotenoids in wood: quantitative study of cooperage oak, *J. Agric. Food Chem.*, 45, 1649, 1997.
29. Klaui, H., *Carotenoids and Their Applications in Natural Colours for Food and Other*, Applied Science, London, 1981, 91.
30. Frickel, F., Chemistry and physical properties of retinoids, in *The Retinoids*, Vol. 1, Sporn, M.B. et al., Eds., Academic Press, Orlando, 1984, 7.
31. Dawson, M.I. and Hobbs, P.D., Synthetic chemistry of retinoids, in *The Retinoids*, Sporn, M.B. et al., Eds., Raven Press, New York, 1994, 5.
32. Isler, H. and Gutmann, U.S., *Carotenoids*, Birkhauser Verlag, Basel, 1971.
33. Anon., 2006. Carotenoids. *Macmillan Science Library: Plant Sciences*. October 15. www.bookrags.com/research/carotenoids-plsc-01.
34. Nguyen, M.L. and Schwartz, S.J., Lycopene stability during food processing, *Proc. Exp. Biol. Med.*, 218, 101, 1998.

35. Ferruzzi, M.G., and Schwartz, S.J., Overview of chlorophylls in foods, in *Current Protocols in Food Analytical Chemistry*, Schwartz, S.J., Ed., John Wiley & Sons, New York, 2001.
36. Oliver, J. and Palou, A., Chromatographic determination of carotenoids in foods, *J. Chromatogr. A*, 881, 543, 2000.
37. Britton, G., UV/visible spectroscopy, in *Carotenoids 1B: Spectroscopy*, Britton, G. et al., Eds., Birkhauser Verlag, Basel, 1995, 13.
38. De Ritter, A.E., Carotenoid analytical methods, in *Carotenoids as Colorants and Vitamin A Precursors*, Bauernfeind, J.C., Ed., Academic Press, New York, 1981, 815.
39. Krinsky, N.I., The biological properties of carotenoids, *Pure Appl. Chem.*, 66, 1003, 1994.
40. Falconer, M.E. et al., Carotene oxidation and off-flavor development in dehydrated carrot, *J. Sci. Food Agric.*, 15, 857, 1964.
41. Simic, M. C., Carotenoid free radicals, *Meth. Enzymol.*, 213, 444, 1992.
42. Lafferty, J., Truscott, T.C., and Land, E.J., Electron transfer reactions involving chlorophylls a and b and carotenoids, *J. Chem. Soc. Farad. Trans.*, 74, 2760, 1978.
43. Burri, B.J., Clifford, A.J., and Dixon, Z.R., Beta-carotene depletion and oxidative damage in women, in *Natural Antioxidants and Anticarcinogens in Nutrition, Health and Disease*, Kumulainen, J.T. and Salonen, J.T., Eds., Royal Society of Chemistry, Stockholm, 1999, 231.
44. Nielsen, S.R. and Holst, S., Developments in natural colourings, in *Color in Food: Improving Quality*, MacDougall, D., Ed., Woodhead Publishing, Cambridge, U.K., 2002.
45. Faulks, R.M. and Southon, S., Carotenoids, metabolism and disease, in *Handbook of Nutraceuticals and Functional Foods*, CRC Press, Boca Raton, FL, 2001.
46. Burri, B.J., Beta-carotene and human health: a review of current research, *Nutr. Rev.*, 17, 547, 1997.
47. Holden, J.M., Eldridge, A.L., Beecher, G.R., Buzzard, I.M., Bhagwat, S.A., Davis, C.S., Douglass, Larry, W., Gebhardt, S.E., Haytowitz, D.B., and Schakel, S., 1998. Data table. *USDA-NCC Carotenoid Database for U.S. Foods—1998*. October 20, 2006. <http://www.nal.usda.gov/fnic/foodcomp/Data/car98/car98.html>.
48. Francis, F.J., Food colorings, in *Colour in Food: Improving Quality*, Woodhead Publishing, Cambridge, U.K., 2002.
49. Landrum, J.T., Bone, R.A., and Herrero, C., Astaxanthin, cryptoxanthin, lutein, and zeaxanthin, in *Phytochemicals in Nutrition and Health*, CRC Press, Boca Raton, FL, 2002.
50. Landrum, J.T. and Bone, R.A., Lutein, zeaxanthin and the macular pigment, *Arch. Biochem. Biophys.*, 385, 28, 2001.
51. Scott, K.J. and Hart, D.J., The carotenoid composition of vegetables and fruits commonly consumed in the U.K., Norwich Laboratory, 1994.
52. Khachik, F., Beecher, G.R., and Smith, J.C., Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer, *J. Cell. Biochem.*, 22, 236, 1995.
53. Stahl, W. and Sies, H., Lycopene: a biologically important carotenoid for humans? *Arch. Biochem. Biophys.*, 336, 1, 1996.
54. Sies, H. and Stahl, W., Lycopene: antioxidant and biological effects and its bioavailability in the human, *Proc. Soc. Exp. Biol. Med.*, 218, 121, 1998.
55. Francis, F.J., *Colorants*. American Association of Cereal Chemists, St. Paul, MN, 1998.
56. Nguyen, M.L. and Schwartz, S.J., Lycopene, in *Natural Food Colorants*, Lauro, G.J. and Francis, F.J., Eds., Marcel Dekker, New York, 2000, 153.

57. Britton, G., Liaaen-Jensen, S., and Pfander, H., Carotenoids today and challenges for the future, in *Carotenoids*, Vol. 1, Britton, G. et al., Eds., Birkhauser Verlag, Basel, 1995, 13.
58. Weedon, B.C.L., Occurrence, in *Carotenoids*, Isler, O., Ed., Birkhauser Verlag, Basel, 1971, 29.
59. Minguez-Mosquera M.I. and Hornero-Mendez, D., Changes in carotenoid esterification during the fruit ripening of *Capsicum annuum* cv. Bola. *J. Agric. Food Chem.*, 42, 640, 1994.
60. Britton, G., Overview of carotenoid biosynthesis, in *Carotenoids*, Vol. 3, Britton, G. et al., Eds., Birkhauser Verlag, Basel, 1998.
61. Hirschberg, J., Carotenoid biosynthesis in flowering plants, *Curr. Op. Plant Biol.*, 4, 210, 2001.
62. Anon., 2002. Carotenoids: What are carotenoids? What do carotenoids do? *Astaxanthin Biochemical Properties*, Mera Pharmaceuticals, Inc. October 1, 2006. www.astaxanthin.org/carotenoids.htm.
63. Klaüi, H. and Bauernfeind, J.C., Carotenoids as food color, in *Carotenoids as Colorants and Vitamin A Precursors*, Bauernfeind, J.C., Ed., Academic Press, New York, 1981, 48.
64. Cavalcante, M.L. and Rodriguez-Amaya, D.B., Carotenoid composition of the tropical fruits *Eugenia uniflora* and *Malpighia glabra*, in *Food Science and Human Nutrition*, Charalambous, G., Ed., Elsevier, Amsterdam, 1992, 643.
65. Mercadante, A.Z. and Rodriguez-Amaya, D.B., Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango, *J. Agric. Food Chem.*, 46, 128, 1998.
66. Young, A. and Britton, G., *Carotenoids in Photosynthesis*, Chapman & Hall, London, 1993.
67. Demming-Adams, B., Gilmore, A.M., and Adams, W.W., *In vivo* functions of carotenoids in higher plants, *FASEB J.*, 10, 403, 1996.
68. Estes, A.M., 2002. Carotenoids. *The Carotenoids*. October 1, 2006. <http://members.aol.com/profchm/carot.html>.
69. Delgado-Vargas, F., Jiménez, A.R., and Paredes-López O., Natural pigments: carotenoids, anthocyanins, and betalains — characteristics, biosynthesis, processing, and stability, *Crit. Rev. Food Sci. Nutr.*, 40, 173, 2000.
70. Kenneth, L.S., Carotenoid pigments, in *Encyclopedia Of Food Science and Technology*, 2nd Ed., Vol. 1, Wiley-Interscience, New York, 2000.
71. Koornneef, M., Genetic aspects of abscisic acid, in *A Genetic Approach to Plant Biochemistry*, Blonstein, A.D. and King, P.J., Eds., Springer, New York, 1996, 35.
72. Bartley, G.E. and Scolnik, P.A., Plant carotenoids: pigments for photoprotection, visual attraction, and human health, *Plant Cell*, 7, 1027, 1995.
73. Liebler, D.C., Antioxidant reactions of carotenoids, *Carotenoids Hum. Health*, 691, 20, 1993.
74. Mathews-Roth, M.M., Carotenoids in erythropoietic protoporphyria and other photosensitivity disease, *Carotenoids Hum. Health*, 691, 127, 1993.
75. Palozza, P. and Krinsky, N.I., Antioxidant effects of carotenoids *in vivo* and *in vitro*: an overview, *Meth. Enzymol.*, 213, 403, 1992.
76. Miller, N.J. et al., Antioxidant activities of carotenes and xanthophylls, *FEBS Lett.*, 384, 240, 1996.
77. Rodriguez-Amaya, D.L., *A Guide To Carotenoid Analysis in Foods*, International Life Sciences Institute Press, Washington, D.C., 2001.

2.3 Stability and Analysis of Phenolic Pigments

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2.3.1 STABILITY OF PHENOLIC COMPOUNDS

2.3.1.1 STABILITY OF ANTHOCYANINS

As frequently mentioned in the literature, anthocyanins co-exist in equilibrium in four different forms. The pH conditions shift this equilibrium toward a variety of structural forms, with the direct consequences of color changes of these pigments.¹ As a rule, at pH above 4, yellow compounds (chalcone form), blue compounds (quinoid base), or colorless compounds (methanol form) are produced. Anthocyanins have the highest stabilities at a pH between 1 and 2 since the flavylium cation is the most stable predominant form.

Factors influencing anthocyanin stability are diverse and widely discussed in the literature. The influence of the specific structures of anthocyanins (glycosylation, acylation with aliphatic or aromatic acids,² pH, temperature, light, presence of metal ions, oxygen and sugar content, and effects of sulfur dioxide have been covered and partially clarified.³ Of all these parameters, the storage temperature seems to be primary and it has been possible to determine first order degradation kinetics⁴: di-glucosylated anthocyanins are more stable than mono-glucosylated anthocyanins, and acylation by aromatic acids increases the stability of a given anthocyanin.^{5,6} Finally, Turker et al.⁷ studied effects of this phenomenon on black carrot juice (*Daucus carota* L.) kept at 4.25 and 40°C for 90 days. Monomer anthocyanins and the corresponding colorant intensity decreased with the time-temperature combina-

tion, whereas the polymer fraction (brown pigments) exhibited the reverse. Finally, the stabilizing effect of acylation was in this case confirmed again.

The effects of pH under model conditions (0.6 to 5.5 for 24 hr) were covered by Nielsen et al.¹ for four anthocyanins (3-*O*-glucoside, glycosylated cyanidin, rutinoside, and delphinidin rutinoside). After 24 hr, over 90% of the anthocyanins were intact up to pH 3.3, while instability greatly increased at pH greater than 4.5.

This high instability of anthocyanins has a direct consequence on possible color stabilization actions. In fact, fruit juices rich in anthocyanins, especially berry-based juices, need to be stabilized to preserve the product's original color and its potential health benefits. Eiro and Heinonen⁸ clearly demonstrated the color stabilizing and amplifying effects of phenolic acids on anthocyanins, the stabilizing effects of caffeic acid and rutin on blood orange juice already having been demonstrated by Maccarone et al.⁹ In 2004, Rein and Heinonen¹⁰ refined these results, demonstrating that sinapic acid preferentially boosts the color of strawberry juice, while ferulic and sinapic acids evenly boost the colorant intensity of raspberry juice. This effect was attributed to co-pigmentation, with the ferulic and sinapic acids leading to the synthesis of new compounds.

Of the other factors that may be behind color degradation, the addition of ascorbic acid brought about a notable acceleration in degradation of anthocyanins under model conditions, as in the analyzed matrix.¹¹ The influence of sugars on anthocyanin stability remains a controversial subject. Certain authors do not mention any effect with a model solution of commercial anthocyanidin-based pigments with or without sugar (10°Bx), whereas anthocyanidin degradation in the presence of sugar is frequently mentioned in the literature. In 2006, Hubbermann et al.¹² demonstrated that in a gel-form model solution, colorant stability increased with the acid p_{Ka} (tartric, acetic, ascorbic) and decreased with salt concentration (sodium citrate and tartrate). This effect could be attributed to the decrease of water activity. The same authors were able to demonstrate that in the event of formation of hydrocolloid gels after heat treatment, fructose brought about acceleration in anthocyanin degradation.

Color change during anthocyanin degradation may be tracked using a chromametry system. A decrease in the red color parameter is a good indicator of degradation of these pigments, while an increase in that parameter is a possible indicator of browning (possibly associated with polymer formation).¹² The influence of co-pigmentation of pyruvic acid with four anthocyanins (3-*O*-cyanidin glucoside, 3-*O*-cyanidin rutinoside, 3-*O*-cyanidin sambubioside, and 3-*O*-cyanidin sophoroside) was studied by Oliveira et al.¹³ At pH 1 to 2, a loss of saturation ($\Delta C^* < 0$) and an increase in luminosity of the solution ($\Delta L^* > 0$) were observed in model solutions containing the co-pigments anthocyanin-pyruvic acid in comparison with 3-*O*-cyanidin glucoside only. These solutions were in this case much less colored than the anthocyanin-only solution under the same pH and concentration conditions. For pH 5 to 7, the reverse phenomenon was observed, with the co-pigment solutions more colored than the control anthocyanin. Thus the special behavior of these co-pigments according to the pH may directly influence their application in food industry.

Co-pigmentation of anthocyanins generally produces more intensely colored and more stable pigments than anthocyanin only. Two types of co-pigmentation reactions are mentioned in the literature.⁸ The first one involves intramolecular

interactions via covalent bonds between the chromophore moiety of the anthocyanin and organic acids, aromatic groups, or flavonoids (or a combination of all three). The second type of intramolecular interaction, more frequently encountered in fruits and berries, involves the establishment of weak hydrophobic bonds between the flavonoids and anthocyanins.

The effects of co-pigmentation on the absorption spectrum of the anthocyanin in question are two-fold: a hyperchromic effect (increase in intensity of absorbance to the molecule's $\lambda_{\max}$), and a bathochromic effect (shift from $\lambda_{\max}$ to higher wavelengths). Intermolecular co-pigmentation and its effects on the compound's absorbance spectrum were studied by Eiro and Heinonen.⁸ 3-Malvidin glucoside is the anthocyanin with the greatest spectral sensitivity to co-pigmentation, with the strongest co-pigments for all anthocyanins being ferulic and rosmarinic acid. The immediate reaction of rosmarinic acid with 3-malvidin glucoside causes the greatest bathochromic (+19 nm) and hyperchromic effects (260% increase in colorant intensity). However, we should note that the pigment newly obtained by adding rosmarinic acid is unstable. Finally, in the discussion of co-pigment solutions, the colorant intensity of 3-pelargonidin glucoside greatly increases in the presence of ferulic and caffeic acids.

Anthocyanin–flavonoid association, although a minority constituent in gooseberries (~1% of total anthocyanins),¹⁴ may strongly influence the extract color. This association also stabilizes the biological activity attributed to the corresponding anthocyanins. The stability of the complexes of seven phenolic compounds (catechin, epicatechin, procyanidin B₂, caffeic acid, *p*-coumaric acid, myricetin, and quercetin) with 3-malvidin glucoside (the principal anthocyanin in *Vitis vinifera*) was monitored for 60 days at 25°C.¹⁵ Regardless of the co-pigment-to-pigment ratio used, the complex and therefore the anthocyanin–flavonoid or anthocyanin–phenolic acid association was confirmed. Flavan-3-ols, and more particularly procyanidin B₂ were the least effective co-pigments, while flavonols were considered the most effective. They also caused the statistically greatest bathochromic effects.

The co-pigmentation effects of flavan-3-ols during storage of the solutions are manifested as increases in hue value, so these co-pigments are the weakest in terms of browning prevention. Finally, regardless of the cofactor, anthocyanin stabilization during storage via the phenomenon of co-pigmentation was not revealed, as similar degradation ratios were observed in the presence or absence of these co-factors.

2.3.1.2 STABILITY OF CURCUMINOIDS

The rhizome of the *Curcuma longa* plant is called turmeric and is extensively used as a flavoring and coloring agent.^{16,17} The three main compounds that are responsible for the yellow-orange color of turmeric belong to the curcuminoids family: curcumin, demethoxycurcumin, and bis-demethoxycurcumin (Figure 2.3.1). These compounds are also referred to as curcumin I, II and III, respectively, by some authors. They exist under equilibrium between keto and enol forms. Curcumin (1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione) is the major pigment and precursor of the curcuminoids. It contains two ferulic acid molecules linked via a methylene bridge at the C atoms of the carboxyl groups.

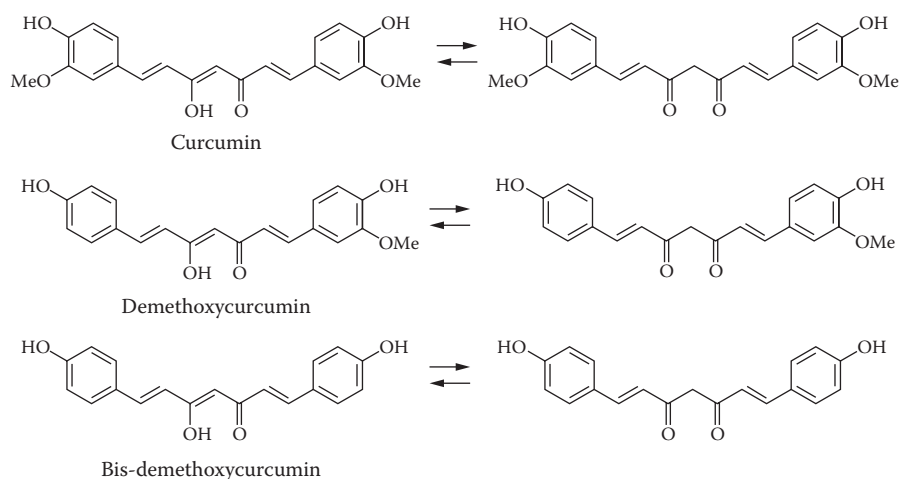


FIGURE 2.3.1 Chemical structures of curcuminoids.

Curcumin is poorly soluble in water (partition coefficient octanol/water, $\log P = 3.29$)¹⁸ and is highly unstable in neutral and alkaline solutions. Ninety percent of curcumin is degraded within 30 min when placed in a 0.1 M phosphate buffer at pH 7.2, and gives rise to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal (major degradation product), vanillin, ferulic acid, and feruloyl methane.¹⁹ Degradation of curcumin can be prevented by the addition of antioxidants such as ascorbate or N-acetyl-cysteine, suggesting the involvement of an oxidative mechanism underlying this degradation process.²⁰ Curcumin is also light-sensitive and photochemical degradation leads to the formation of vanillin, vanillic acid, ferulic aldehyde, ferulic acid, and 4-vinylguaiacol.²¹

2.3.2 ANALYSIS OF ANTHOCYANINS AND THEIR AGLYCONES (ANTHOCYANIDINS)

As we have seen above, anthocyanins comprise an aglycone fraction commonly known as anthocyanidin and a frequently acylated osidic substituent. This characteristic leads to two different approaches for the analysis of these pigments: (1) a direct anthocyanin analysis without a hydrolysis stage requiring identification of a number of molecules (several hundreds in the plant kingdom) or (2) an analysis of the anthocyanidin fraction only after hydrolysis of the anthocyanins present in the medium.

The overall anthocyanin analysis is generally conducted using the Giusti and Wrolstad method²² based on the differences in absorbance of anthocyanins at pH 1 and pH 4.5. Then the pigment content is determined using the coefficient of molar extinction of the predominant anthocyanin. It should be noted that this technique only allows dosing of anthocyanins with a color difference between the two pH values (due to transition to the flavylium cation form). A more global analysis of total anthocyanin content may be conducted by direct spectrophotometry of the

solution to be analyzed, calibrating in parallel using purely the predominant anthocyanin. For matrices especially rich in anthocyanins (such as red fruits), using a microchamber will generally be preferable to diluting the sample in an acidic medium, as this dilution could lead to structural modifications of the anthocyanins.

Anthocyanins are water-soluble pigments, as acylation of the carbon skeleton by glycosides increases the polarity of these molecules. Furthermore, according to the pH conditions, the heterocyclic oxygen atom may be found in cation form, further increasing the molecule's polarity.

Based on these properties, anthocyanins are mainly extracted with polar solvents such as methanol, acetone, or chloroform, most often with added water and hydrochloric or formic acid.²³ Acidification stabilizes nonacylated compounds and also converts the anthocyanidin fraction into its cation form.²⁴ Optimizing the extraction solvent for the plant material analyzed, and therefore the anthocyanidins present in the medium, is generally an essential precursor to characterizing the anthocyanidins. Although methanol is the most frequently cited compound in the literature,^{25,26} Giusti et al.⁶ demonstrated that using acetone (often diluted in water) is preferable for anthocyanidin extraction from red fruits. This solvent actually provides better extraction reproducibility, and decreases the risks of pectin precipitation. Finally, its greater volatility means the medium can be concentrated at a lower temperature, thus reducing the risks of hydrolysis.

The medium is most often acidified with hydrochloric acid (0.1% v/v)²⁷ but it should be noted that in light of the work of Revilla et al.,²⁸ the hydrochloric acid concentration should not exceed 0.12 mole/liter to prevent risks of anthocyanidin hydrolysis. Formic acid (2% v/v), with a greater volatility than hydrochloric acid, is preferred because it prevents risks of hydrolysis during the extract concentration stage.²⁹

After extraction, an extract purification stage is generally recommended. This is most often done by liquid–solid exchange using resins such as Sephadex, Amberlite XAD-7, or C₁₈ mini-columns.^{30,31} All the polar compounds are first trapped on the resin, and then in succession the sugars, acids, and other polar compounds (excluding polyphenolic compounds), polyphenolic compounds (excluding anthocyanidins), and anthocyanidins are respectively eluted with acidified water (HCl 0.01% v/v), ethyl acetate, and acidified methanol (HCl 0.01% v/v).

Anthocyanidins are primarily separated by high performance liquid chromatography (HPLC) using deactivated C₁₈ columns, thereby preventing interactions with the free hydroxyl groups of the anthocyanidins.²⁴ The anthocyanidins are mainly eluted according to their polarity, with an increasing order of elution under conventional elution conditions as follows: delphinidin, cyanidin, petunidin, pelargonidin, peonidin, and malvidin (the most polar compounds are the most strongly retained). Finally, monoglycosylated anthocyanidins have retention times generally greater than their corresponding aglycones, whereas diglucosides are less strongly retained than the corresponding monoglucosides.

Although chromatographic conditions are extremely variable and widely discussed in the literature, broad trends may nonetheless be perceived. A binary solvent such as acidified water/acidified methanol or acidified water/acidified acetonitrile is primarily used because acidification of the solvent converts nearly all anthocyanidins

into the flavylum cation form (96% at pH 1.5). Of the many solvent gradients mentioned in the literature, the bibliographic work of da Costa et al.²⁴ stands out because the authors propose different methods and bibliographical references according to whether wine and red grape anthocyanins, red fruits, or plants with high acylated anthocyanin content (red radish, elderberry) are involved.

Although anthocyanins were identified by acid and alkaline hydrolysis for a long time, the most recent works cite mass spectrometry detection with an ionization source [electrospray ionization (ESI)].^{25,27,32} The fragmentation of the molecules provides essential information for compound identification, such as the mass of anthocyanin and its corresponding aglycone (anthocyanidin). In parallel, a library covering the spectra of pure anthocyanins will have been prepared to compare the reference spectrum with the target molecule. The information provided by HPLC analysis in combination with a UV/visible photodiode array detector (retention time, compound absorption spectrum), will guide the selection with even greater accuracy. It should also be noted that tandem mass spectroscopy using argon as the target gas is able to produce cascade fragmentation of the molecule, thereby yielding precise information on the constituent fragments of the compound. In this case, the target molecule is fragmented initially in the first quadrupole, and then the selected m/z fragment is again subjected to fragmentation in the second quadrupole. In the case of multiple fragmentations, ionization energies of variable intensity are applied (usually 15 to 30 eV)³³ to compare the various fragmentation paths of the target molecule.

Among the new analysis techniques for anthocyanidins and their corresponding aglycones, the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) technique was compared to HPLC combined with mass spectrometry by Wang et al.²⁹ This comparison conducted on whortleberries demonstrated that while the MALDI-TOF technique cannot distinguish isomers, it can quickly (in about 4 min) distinguish anthocyanins of different masses. This technique must be considered as a complementary part of analyses not requiring isomer distinction.

2.3.3 ANALYSIS OF FLAVONOIDS

The analysis of flavonoids in the plant kingdom has been covered by hundreds of publications, with the number of works increasing exponentially since the widespread use of HPLC. Among the many bibliographical reviews in this field, the work of Robards and Antolovich³⁴ is a review of analytical techniques used for flavonoid analysis in fruits. For the different compounds predominant in the matrix, the sample treatment, extraction method (type of solvent used), purification method (absorption on resins), type of column, and chromatographic conditions are set out and synthesized. It should be noted that the extraction and purification stage is essential. At this stage, an extract with a uniform content of beneficial molecules is obtained but without interfering nonphenolic compounds. Flavonoids are for the most part stable compounds with a variety of solubility levels in medium to high polarity solvents such as ethanol, methanol, acetone, or dimethyl formamide (DMF). However, certain classes of flavonic compounds such as flavones, isoflavones, and flavonols are more hydrophobic, in which case they are extracted using lower polarity solvents such as

chloroform or an ethyl acetate/methanol mixture. The preparation of the extracts remains an essential stage in metabolite characterization and every separation step may considerably influence flavonoid recovery. The bibliographical study conducted by Widmer and Martin³⁵ reveals the influence of the purification steps and the possible adsorption of flavonoids for each filtration type and support.

Flavonoids are frequently analyzed after a preliminary hydrolysis stage to split the molecule concerned into its aglycone and glycosidic fractions. Hot acid hydrolysis is the most commonly used method for this purpose. Vinson et al.^{36,37} suggest initial extraction of “free” polyphenols with a methanol/water mixture (50/50 v/v) for 3 hr at 90°C followed by extraction of glycosylated polyphenols under the same conditions but with the solvent mixture acidified (HCl at 1.2 N). However, it should be noted that this hydrolysis stage may lead to degradation in the aglycone, and therefore cause compound identification errors.

Regarding chromatographic conditions, the bibliographical review presented by Merken and Beecher³⁸ must be noted. Their work sets out a very complete synthesis of HPLC conditions, noting column types and solvent gradients suited for identification of anthocyanins, flavanols, and flavones in many plant matrices. Among the many works produced on this subject, the most used extraction solvents are acetone and methanol (commonly diluted in water 50/50 or 70/30 v/v). The work of Merken and Beecher³⁹ should be noted because the authors present a useful analysis method for aglycone flavonoids. Extraction on lyophilized material is conducted under reflux for 2 hrs in an acidified methanol solution (methanol/acidified water, HCl 1.2 N, 50/50 v/v) with added antioxidant (TBHQ at 0.5 g/l).⁴⁰ A linear solvent gradient on a C18 column at 1 ml/min is applied (Table 2.3.1).

The chromatographic conditions as mentioned above must in most cases be adapted to the matrix under study, i.e., to the predominant flavonoid classes. Paganga et al.⁴¹ studied aglycone flavonoids in apples, onions, and tomatoes, and were able to develop another solvent gradient enabling very good separation of the different aglycones (Table 2.3.2).

Just as with anthocyanin analysis, the advent of HPLC/mass spectrometer coupling made it possible to avoid acid hydrolysis for flavonoid identification. Määttä

TABLE 2.3.1
Solvent Gradients for Separation of Aglycone
Flavonoids³⁹

Time (min)	Water ^a (%)	Methanol ^a (%)	Acetonitrile ^a (%)
0	90	6	4
5	85	9	6
30	71	17.4	11.6
60	0	85	15
61	0	6	4
66	90	6	4

^a All solvents were acidified.

TABLE 2.3.2
Solvent Gradients for Separation of
Aglycone Flavonoids⁴²

Time (min)	Methanol/Water ^a (20/80 v/v) (%)	Acetonitrile (%)
0	95	5
10	95	5
50	50	50
55	95	5
60	95	5

^a Acidified solvent (HCl 0.1%).

et al.⁴² set out a complete polyphenol identification work for berries, suggesting a single solvent gradient for identification of molecules by mass spectrometry and quantification by UV/visible spectrophotometry. A linear gradient of 5 to 30% of acetonitrile/water (v/v) containing 1% formic acid in 20 min was used for molecule identification and quantification.

2.3.4 ANALYSIS OF CURCUMIN IN PLANTS AND IN BIOLOGICAL FLUIDS

Regarding the data concerning the stability of curcumin in solution, it is advisable to perform extraction under acidic conditions and to avoid direct light exposure. However, it will also depend on the material from which curcumin has to be extracted.

Extraction of curcuminoids from turmeric usually requires drying and grinding the rhizomes with a mill. The mode of drying and temperature and the particle size after grinding can affect the extraction yield. Best results were obtained when samples were dried at 50°C for 24 hr compared with 105°C.⁴³ Some authors recommend drying the rhizomes in sunlight to avoid variations in raw material and storing the samples in dark bags at low temperature.⁴⁴ It is difficult to compare the results of extraction yields among published data, because of the variabilities of the starting materials (i.e., species and varieties of turmeric). However, curcuminoid content usually accounts for 2 to 8% (w/w) of the dry weight.

Classical extraction is achieved by mixing the samples with an organic solvent (solid–liquid extraction) such as acetonitrile, methanol, or ethanol, used either in the pure form or as a mixture or aqueous solution.^{45–47} Extraction time can be reduced by sonicating the samples.^{48,49}

Braga et al.⁵⁰ compared the efficiencies of several processes, i.e., hydrodistillation, low pressure solvent extraction, and Soxhlet and supercritical fluid extraction. For each process, the influences of several parameters (duration, temperature, nature of solvent) were also evaluated. These authors concluded that the Soxhlet method performed with ethanol/isopropanol 1/100 v/v for 2 hr and 30 min was the most effective. Sun et al.⁵¹ used solid phase extraction to concentrate (nine times) a

turmeric light petroleum extract. Stationary phase was tributyl phosphate resin and an 80% yield was obtained. Supercritical carbon dioxide extraction is better adapted to volatile turmeric compounds than curcuminoids. However, the addition of ethanol as a co-solvent gives satisfying yields.⁴³

Since many biological properties are attributed to curcumin, several studies have investigated the bioavailability of curcumin in animals and in humans and looked for plasmatic and tissue metabolites. Like other phenolic compounds, curcumin is metabolized by the body at different levels (intestine, liver, colon). Therefore, curcumin is mainly found conjugated to either sulfate or glucuronide; reduced forms such as tetrahydrocurcumin can also be conjugated.⁵² Because no commercial standards of these conjugates are available, prior enzymatic hydrolysis of the samples is required to release the native form that will be subsequently extracted. Acidification is necessary to ensure the stability of curcumin, but concentrated HCl, and phosphate, ammonium acetate, or citrate buffers can be employed as long as final pH is <6.¹⁹

Because of the high protein content in plasma and tissues, more apolar solvents than the ones used for vegetal material are needed to disrupt noncovalent curcumin-protein bonds. Extraction procedures for most tissues have been reported, with satisfying recovery yields. Ethyl acetate is the most often employed and good recovery yields are reported, whatever the starting tissue is.⁵³⁻⁵⁵ Nevertheless, for very low concentrations better recoveries are obtained from plasma with the use of chloroform.⁵⁵ Acetone can be used for extraction from freeze-dried feces, with a 100% recovery of curcumin.⁵⁶ The choice of the solvent is also guided by the method of analysis chosen. Yuan et al.¹⁶ compared petroleum ether, ether, hexane, ethyl acetate, and methylene chloride for subsequent capillary electrophoresis (CE) analysis. The first three gave rise to high emulsification. Ethyl acetate was not selective enough so methylene chloride was chosen.

The choice of the method of analysis depends on the question to address. Spectrophotometry is sufficient for total curcuminoid content determination in a turmeric extract. Separation techniques coupled to mass spectrometry detection and MALDI-TOF are highly sensitive techniques that are more adapted to the identification of metabolites in biological fluids such as urine or plasma.^{57,58}

Because curcuminoids are the major pigments in *Curcuma*, it is possible to determine their contents in an extract easily by spectrophotometry or spectrofluorimetry,⁵⁹ assuming that the different molecules have the same responses. However, no information regarding the proportions of the various curcuminoids can be obtained with this method, which may be of importance in distinguishing varieties of *Curcuma*. Still, the two main methods used to separate, characterize, and quantify curcuminoids are HPLC and CE. Most of the time, detection is achieved by coupling with UV absorption. Coupling of a mass spectrometer to liquid chromatography increases sensitivity compared to UV detection, but this is mainly of interest in *in vivo* studies to identify the natures of curcumin metabolites. CE has several advantages over HPLC, for example, separation can be achieved very quickly with small sample volumes and with lower amounts of solvents.⁶⁰ However, compared with HPLC, few effective methods have been developed to date^{16,48,49} and only one is dedicated to the study of curcuminoid content in biological (urine) samples.¹⁶

Several HPLC methods combining sensitivity and specificity have been developed for curcumin determination. Separation is most of the time achieved by reverse phase chromatography, using octadecyl (C_{18}) stationary phases. The mobile phase is generally composed of a mixture of acidified water and an organic modifier, classically acetonitrile or methanol.^{43,45,47,48} Detection is made in the 425- to 430-nm region for curcuminoids, whereas mammalian reduced metabolites of curcumin such as tetrahydrocurcumin or hexahydrocurcumin lose their yellow coloration and display maximal absorption wavelengths close to 280 nm.⁵² A short selection of different available quantifying methods of curcuminoids and their metabolites in vegetal or animal material is reported in Table 2.3.3.

TABLE 2.3.3
Sensitive Methods for Analysis of Curcuminoids and Their Metabolites

Method	Sample	Separation Conditions	LOQ	Reference
HPLC/UV 425 nm	Turmeric powder	Column: Waters Bondapak C ₁₈ 300 × 4.6 mm i.d. Mobile phase: (A) MeOH, (B) 20% CH ₃ COOH, (C) ACN	0.05 µg	61
HPLC/UV 425 nm	Ethanollic extracts	Hamilton HPR-1 ACN/Water 55/45	10.2 ng	47
HPLC/MS (thermospray)	Powder	Column: Supelcosil 250 × 4.6 mm, p.s. 5 µm Mobile phase: 1% citric acid/ACN	186 pg	46
Spectrofluorometry λ _{exc} = 397 nm λ _{em} = 508 nm	Mustard, yogurt	None	0.08 ng/ml	59
CE/DAD 470 nm	Rhizome	Uncoated fused-silica capillary 370 × 50 µm Current = 150 to 160 µA Voltage = 20 kV t ^c = 27°C Running buffer: 20 mM phosphate, 14 mM β-cyclodextrine, 50 mM NaOH (pH 12.1)	0.01 mg/ml	48
CE/Amperometric Detection	Rhizome	Uncoated fused-silica capillary 360 × 25 µm Voltage = 16 kV Running buffer: 15 mM phosphate pH 9.7	3 × 10 ⁻⁸ M	51

Continued.

TABLE 2.3.3 (Continued)
Sensitive Methods for Analysis of Curcuminoids and Their Metabolites

Method	Sample	Separation Conditions	LOQ	Reference
CE/DAD	Powder	Uncoated fused-silica capillary 40.2 cm $\times$ 50 μ m i.d. Voltage = -15 kV t° = 25° C Microemulsion buffer: 50 mM phosphate buffer pH 2.5, 1.1% (v/v) n-octane, 180 mM SDS, 890 mM n-octanol, 25% (v/v) 2-propanol Column: Waters Symmetry Shield RP18, 150s $\times$ 3.9 mm, p.s. 5 μ m Mobile phase: (A) 0.01% ammonium acetate, (B) ACN	11 ppm	49
HPLC/DAD/MS DAD: 420 nm MS: electrospray, negative ion mode HPLC/DAD 262 nm	Hepatic tissue, plasma, bile		5–10 nM	54
HPLC/DAD 280 nm	Plasma and urine THC in plasma and urine	Column: Waters Symmetry Shield 3.9 $\times$ 150 mm, p.s. 5 μ m Mobile phase: ACN/MeOH/water/acetic acid (41/23/36/1 v/v/v/v) Column: Waters Symmetry Shield 3.9 $\times$ 150 mm, p.s. 5 μ m Mobile phase: (A) ACN, (B) 0.01% ammonium sulphate, pH 7.8, (C) ACN/MeOH/water/acetic acid (41:23:36:1, v/v/v/v)	0.2 μ g/ml 0.05 μ g/ml	62 63
HPLC/UV 430 nm	Plasma	Column: Waters Symmetry Shield C18 150 $\times$ 3.9 mm, p.s. 5 μ m Mobile phase: 1% (w/v) citric acid, pH 3.0/THF 50:50 (v:v)	2.5 ng/ml	55
HPLC/UV 420 nm	Plasma, kidney, liver, spleen, brain, intestine	Column: Waters Nova-pak C ₁₈ 150 $\times$ 3.9 mm, 5 μ m p.s. Mobile phase: THF/1% citric acid, pH 3.0 40/60 (v/v)	5 ng/ml	52
CE/DAD 266 nm	Urine	Fused-silica capillary 50 cm $\times$ 50 μ m i.d. $\times$ 375 μ m o.d. Voltage: 25 kV, t° = 30° C Running buffer: 15 mM Na ₂ B ₄ O ₇ pH 10.8, 10% MeOH	n.a.	16

THF = tetrahydrofuran. ACN = acetonitrile, p.s. = particle size. i.d. = internal diameter. o.d. = outer diameter. MeOH = methanol. MS = mass spectrometry. DAD = diode array detector. n.a. = not available. THC = tetrahydrocurcumin. λ_{exc} = excitation wavelength. λ_{em} = emission wavelength.

REFERENCES

1. Nielsen, I.L.F. et al., Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography: investigation of their pH stability and antioxidative potency, *J. Agric. Food Chem.*, 51, 5861, 2003.
2. Stintzing, F. et al., Color and antioxidant properties of cyanidin-based anthocyanin pigments, *J. Agric. Food Chem.*, 50, 6172, 2002.
3. Rodriguez-Saona, L.E. et al., Color and pigment stability of red radish and red-fleshed potato anthocyanins in juice model systems, *J. Food Sci.*, 64, 451, 1999.
4. Marti, N. et al., Influence of storage temperature and ascorbic acid addition on pomegranate juice, *J. Sci. Food Agric.*, 82, 217, 2001.
5. Baublis, A. et al., Anthocyanin pigments: comparison of extract stability, *J. Food Sci.*, 59, 1219, 1994.
6. Giusti, M.M. et al., Molar absorptivity and color characteristics of acylated and nonacylated pelargonidin-based anthocyanins, *J. Agric. Food Chem.*, 47, 4631, 1999.
7. Turker, N. et al., Effect of storage temperature on the stability of anthocyanins of a fermented black carrot (*Daucus carota* var. L.) beverage: shalgam, *J. Agric. Food Chem.*, 52, 3807, 2004.
8. Eiro, M.J. and Heinonen, M., Anthocyanin color behavior and stability during storage: effect of intermolecular copigmentation, *J. Agric. Food Chem.*, 50, 7461, 2002.
9. Maccarone, E. et al., Stabilization of anthocyanins of blood orange fruit juice, *J. Food Sci.*, 50, 901, 1985.
10. Rein, M.J. and Heinonen, M., Stability and enhancement of berry juice color, *J. Agric. Food Chem.*, 52, 3106, 2004.
11. Garcia-Viguera, C. and Bridle, P., Influence of structure on colour stability of anthocyanins and flavylium salts with ascorbic acid, *Food Chem.*, 64, 21, 1999.
12. Hubbermann, E.M. et al., Influence of acids, salt, sugars and hydrocolloids on the colour stability of anthocyanin rich black currant and elderberry concentrates, *Eur. Food Res. Technol.*, 223, 83, 2006.
13. Oliveira, J. et al., Color properties of four cyanidin-pyruvic acid adducts, *J. Agric. Food Chem.*, 54, 6894, 2006.
14. McDougall, G.J. et al., Anthocyanin-flavanol condensation products from black currant (*Ribes nigrum* L.), *J. Agric. Food Chem.*, 53, 7878, 2005.
15. Gomez-Miguez, M. et al., Influence of different phenolic copigments on the color of malvidin 3-glucoside, *J. Agric. Food Chem.*, 54, 5422, 2006.
16. Yuan, K. et al., Application of capillary zone electrophoresis in the separation and determination of the curcuminoids in urine, *J. Pharm. Biomed. Anal.*, 38, 133, 2005.
17. Leu, T.H. and Maa, M.C., The molecular mechanisms for the antitumorogenic effect of curcumin, *Curr. Med. Chem. Anticancer Agents*, 2, 357, 2002.
18. Cooper, D.A. et al., Evaluation of the potential for olestra to affect the availability of dietary phytochemicals. *J. Nutr.*, 127, 1699S, 1997.
19. Wang, Y.J. et al., Stability of curcumin in buffer solutions and characterization of its degradation products, *J. Pharm. Biomed. Anal.*, 15, 1867, 1997.
20. Oetari, S. et al., Effects of curcumin on cytochrome P450 and glutathione S-transferase activities in rat liver, *Biochem. Pharmacol.*, 51, 39, 1996.
21. Tonnesen, H.H. and Karlsen, J., Studies on curcumin and curcuminoids. VI. Kinetics of curcumin degradation in aqueous solution, *Z. Lebensm. Unters. Forsch.*, 180, 402, 1985.

22. Giusti, M.M. and Wrolstad, R.E., Characterization and measurement with UV-visible spectroscopy, in *Current Protocols in Food Analytical Chemistry*, Wrolstad, R.E., Ed., John Wiley & Sons, New York, 2001.
23. Schoefs, B., Determination of pigments in vegetables. *J. Chromatogr. A*, 1054, 217, 2004.
24. Da Costa, C. et al., Analysis of anthocyanins in foods by liquid chromatography, liquid chromatography-mass spectrometry and capillary electrophoresis, *J. Chromatogr. A*, 881, 403, 2000.
25. Wu, X. et al., Characterization of anthocyanins and proanthocyanidins in some cultivars of Ribes, Aronia and Sambucus and their antioxidant capacity, *J. Agric. Food Chem.*, 52, 7846, 2004.
26. Cabrita, L. and Andersen, O.M., Anthocyanins in blue berries of *Vaccinium padifolium*, *Phytochemistry*, 52, 1693, 1999.
27. Longo, L. et al., Identification of anthocyanins in *Rhamnus alaternus* L. berries, *J. Agric. Food Chem.*, 53, 1723, 2005.
28. Revilla, E. et al., Comparison of several procedures used for the extraction of anthocyanins from red grapes, *J. Agric. Food Chem.*, 46, 4592, 1998.
29. Wang, J. et al., Comparison between HPLC and MALDI-TOF-MS analysis of anthocyanins in high bush blueberries, *J. Agric. Food Chem.*, 48, 3330, 2000.
30. Hong, V. and Wrolstad, R.E., Use of HPLC separation/photodiode array detection for characterization of anthocyanins, *J. Agric. Food Chem.*, 38, 708, 1990.
31. Osmianski, J. and Lee, C.Y., Isolation and HPLC determination of phenolic compounds in red grapes, *Am. J. Enol. Vitic.*, 41, 204, 1990.
32. Wu, X. and Prior, R.L., Identification and characterization of anthocyanins by high performance liquid chromatography-electrospray ionization-tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains, *J. Agric. Food Chem.*, 53, 3101, 2005.
33. Giusti, M.M. et al., Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization, *J. Agric. Food Chem.*, 47, 4657, 1999.
34. Robards, K. and Antolovich, M., Analytical chemistry of fruit bioflavonoids: a review, *Analyst*, 122, 11R, 1997.
35. Widmer, W.W. and Martin, S.F., Interferences with naringin and neohesperidin analysis by high performance liquid chromatography, *Proc. Flo. Hort. Soc.*, 105, 149, 1992.
36. Vinson, J.A., Flavonoids in foods as *in vitro* and *in vivo* antioxidants, *Adv. Exp. Med. Biol.*, 439, 151, 1998.
37. Vinson, J.A. et al., Determination of quantity and quality of polyphenol antioxidants in foods and beverages, *Meth. Enzymol.*, 335, 103, 2001.
38. Merken, H.M. and Beecher, G.R., Measurement of food flavonoids by high performance liquid chromatography: a review, *J. Agric. Food Chem.*, 48, 577, 2000.
39. Merken, H.M. and Beecher, G.R., Liquid chromatographic method for the separation and quantification of prominent flavonoid aglycons, *J. Chromatogr.*, 897, 177, 2000.
40. Hertog, M.G.L. et al., Optimization of a quantitative HPLC determination of potentially anticarcinogenic flavonoids in vegetables and fruit, *J. Agric. Food Chem.*, 40, 1591, 1992.
41. Paganga, G. et al., The polyphenolic content of fruit and vegetables and their antioxidant activities: what does a serving constitute? *Free Radical Res.*, 30, 153, 1999.
42. Määttä, K.R. et al., High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: Ribes species, *J. Agric. Food Chem.*, 51, 6736, 2003.

43. Chassagnez-MÈndez, A.L. et al., Supercritical C₂ extraction of curcumins and essential oil from the rhizomes of turmeric (*Curcuma longa* L.), *Ind. Eng. Chem. Res.*, 39, 4729, 2000.
44. Manzan, A.C. et al., Extraction of essential oil and pigments from *Curcuma longa* [L.] by steam distillation and extraction with volatile solvents, *J. Agric. Food Chem.*, 51, 6802, 2003.
45. He, X.G. et al., Liquid chromatography–electrospray mass spectrometric analysis of curcuminoids and sesquiterpenoids in turmeric *Curcuma longa*), *J. Chromatogr. A*, 818, 127, 1998.
46. Hiserodt, R. et al., Characterization of powdered turmeric by liquid chromatography–mass spectrometry and gas chromatography–mass spectrometry *J. Chromatogr. A*, 740, 51, 1996.,
47. Taylor, S.J. and McDowell, I.J., Determination of the curcuminoid pigments in turmeric (*Curcuma domestica* Val) by reversed-phase high performance liquid chromatography, *Chromatographia*, 34, 73, 1992.
48. Lechtenberg, M. et al., Quantitative determination of curcuminoids in *Curcuma* rhizomes and rapid differentiation of *Curcuma domestica* Val. and *Curcuma xanthorrhiza* Roxb. by capillary electrophoresis, *Phytochem. Anal.*, 15, 152, 2004.
49. Nhujak, T. et al., Microemulsion electrokinetic chromatography for separation and analysis of curcuminoids in turmeric samples, *J. Sep. Sci.*, 29, 666, 2006.
50. Braga, M.E. et al., Comparison of yield, composition, and antioxidant activity of turmeric (*Curcuma longa* L.) extracts obtained using various techniques, *J. Agric. Food Chem.*, 51, 6604, 2003.
51. Sun, X. et al., Capillary electrophoresis with amperometric detection of curcumin in Chinese herbal medicine pretreated by solid-phase extraction, *J. Chromatogr. A*, 962, 117, 2002.
52. Pan, M.H. et al., Biotransformation of curcumin through reduction and glucuronidation in mice, *Drug. Metab. Dispos.*, 27, 486, 1999.
53. Garcea, G. et al., Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration, *Br. J. Cancer*, 90, 1011, 2004.
54. Ireson, C. et al., Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat *in vivo*, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production, *Cancer Res.*, 61, 1058, 2001.
55. Pak, Y. et al., Sensitive and rapid isocratic liquid chromatography method for the quantitation of curcumin in plasma, *J Chromatogr B Analyt. Technol. Biomed. Life Sci.*, 796, 339, 2003.
56. Wahlstrom, B. and Blennow, G., A study on the fate of curcumin in the rat, *Acta Pharmacol. Toxicol. (Copenh.)*, 43, 86, 1978.
57. Liu, A. et al., Validated LC/MS/MS assay for curcumin and tetrahydrocurcumin in rat plasma and application to pharmacokinetic study of phospholipid complex of curcumin, *J. Pharm. Biomed. Anal.*, 40, 720, 2006.
58. May, L.A. et al., Detection and quantitation of curcumin in mouse lung cell cultures by matrix-assisted laser desorption ionization time of flight mass spectrometry, *Anal. Biochem.*, 337, 62, 2005.
59. Navas-Diaz, A. and Ramos-Peinado, M.C., Fluorometric determination of curcumin in yogurt and mustard, *J. Agric. Food Chem.*, 40, 56, 1992.
60. Watanabe, T. and Terabe, S., Analysis of natural food pigments by capillary electrophoresis, *J. Chromatogr. A*, 880, 311, 2000.

61. Jayaprakasha, G.K. et al., Improved HPLC method for the determination of curcumin, demethoxycurcumin, and bisdemethoxycurcumin, *J. Agric. Food Chem.*, 50, 3668, 2002.
62. Heath, D.D. et al., Curcumin in plasma and urine: quantitation by high-performance liquid chromatography, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 783, 287, 2003.
63. Heath, D.D. et al., Tetrahydrocurcumin in plasma and urine: quantitation by high performance liquid chromatography, *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, 824, 206, 2005.

2.4 N-Heterocyclic Pigments: Betalains

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2.4.1 CLASSIFICATION AND BIOSYNTHESIS

The term *betalains* (Latin: beta = beet) was coined in 1968 by Mabry and Dreiding for the yellow and red N-heterocyclic pigments from cactus pear and red beet, which until then had been erroneously called flavocyanins (betaxanthins, Greek: xanthos = yellow) and nitrogenous anthocyanins (betacyanins, Greek: kyaneos = blue), respectively.¹ In 1963 and 1964, betanin and indicaxanthin were the first betacyanin and betaxanthin structurally elucidated from red beet (*Beta vulgaris* L.) and cactus pear fruits (*Opuntia ficus indica* [L.] Mill.), respectively.^{2,3} One year later, the term *betalamic acid* was proposed for their mutual biosynthetic precursor.⁴

Betalains represent immonium derivatives of betalamic acid and are subdivided into the red-violet betacyanins and the yellow-orange betaxanthins. It is most intriguing that anthocyanins and betalains mutually exclude each other — they have never been found together in the same plant.^{5,6} Moreover, Vogt provided evidence that betalains appeared after the anthocyanins on an evolutionary scale.⁷ These facts indicate that anthocyanins and betalains replace each other with respect to their particular functions in the plant tissue such as pollinator attraction, upholding the antioxidant potential, and shielding against noxious ultraviolet (UV) light.^{8,9} While both pigment classes share the shikimate pathway (Figure 2.4.1), differentiation commences at the aroenate level, leading to either tyrosine (betalains) or phenylalanine (anthocyanins).

Whereas the anthocyanins bear hues from orange (pelargonidin) to red (cyanidin) to blue (delphinidin), the betalains may be subdivided into distinct yellow-orange (betaxanthins) and red-violet structures (betacyanins). On the other hand, betalainic

plants may contain leucoanthocyanidins and proanthocyanidins, but lack the enzymatic provision to generate 3-hydroxy-anthocyanidins.^{15–19}

The betalain pathway splits into three main routes, the first leading to *cyclo-dopa* and its glucoside. After scission of *dopa*, either 4,5-secodopa or 2,3-secodopa is generated, the latter procuring the betalamic acid isomer muscaflavin typical of fungi belonging to the *Hygrophorus*, *Hygrocybe* and *Amanita* genera.^{10,12} The pathway typical of higher plants is continued via the 4,5-secodopa structure mainly yielding betalamic acid, the key precursor for most betacyanins and betaxanthins. For deeper insights into betalain biosynthesis and its regulation, the reader may consult two excellent reviews published recently.^{13,20} However, it is not ultimately understood whether condensation of amino acids or amines with betalamic acid to yield betaxanthins proceeds spontaneously and/or is controlled by enzymatic action.^{21–24} For betacyanins, current research is directed to clarify whether *cyclo-dopa* is glycosylated before or after condensation with betalamic acid.^{25–28} Furthermore, it has not yet been unveiled how specific color patterns in betalainic plants are achieved and by which mechanisms these may be controlled.¹³ The endogenous enzymes in particular may regulate betalain turnover *in vivo* and these have received scarce attention.²⁰

2.4.2 PHYSICAL AND CHEMICAL PROPERTIES

Betalains are vacuolar plant pigments. Hence their hydrophilic nature is comprehensible. Although they are slightly soluble in ethanol and methanol, water is the best suited solvent both for stability and solubility reasons. In contrast to the anthocyanins, the betalains are even more polar as can be demonstrated by shorter retention times in RP-HPLC and lower solubilities in alcoholic solutions.²⁹ The varying polarities may also be beneficially used to separate anthocyanins from betalains on an RP-18 solid-phase extraction cartridge (Stintzing, unpublished data).

In addition, the extinction coefficients of anthocyanins are smaller than those for betalains, i.e., 11,300 to 29,000 L/mol*cm have been reported for the most common anthocyanin 3-glucosides, while for betaxanthins and betacyanins, 48,000 L/mol*cm and 60,000 L/mol*cm values, respectively, have been published, translating into higher coloring capacities of the latter.^{30–32} Some chemical tests by which anthocyanins may be differentiated from betalains have been compiled by Delgado-Vargas and co-workers.³³ The color changes upon acidification or alcalization are most characteristic.

While the betalains visibly change to a canary yellow tint at alkaline pH through release of betalamic acid, color strength and brilliance will increase for anthocyanins at a pH value below 3 and rapidly decrease at low acid and neutral pH. As reported earlier, betalains are sensitive to the presence of metals, sulfur dioxide, light exposure, high water activity, enzymatic action, pH and elevated temperatures.^{9,34,35} The betaxanthins are most stable at pH 5.5 to 7.^{36,37} Betacyanins are considered to exhibit optimum stability at pH 5 to 6, while betalamic acid remains intact at pH 9.^{38–40} Betalain degradation under adverse pH conditions has not been thoroughly investigated with respect to resulting degradation products.^{33–35,41} In contrast, detailed

investigations of the effect of thermal exposure on betalain stability have only very recently been of renewed research interest.^{42–51}

2.4.3 PRODUCTION OF BETALAIN COLORS AND COLORING FOODSTUFFS

Since biotechnological production of betalain colors is still economically unfeasible, the main focus of the food industry is directed toward the exploitation of food crops for pigment extraction.^{52–57} Economic considerations include high initial color yield, optimization of the extraction process, and high color stability during processing. Furthermore, a good hygienic status, neutrality in taste and smell, and legal requirements need to be considered.

Red Beet — Red beet juices, concentrates and powders are the typical applications for coloring purposes authorized in Europe and North America and still represent the sole betalainic source used commercially.⁵⁸ Red beet application was supported by research activities conducted by Von Elbe and co-workers starting in the early 1970s. Usually, whole unpeeled beets are processed; more than 30% color is lost by removal of the peels.^{59,60} This is noteworthy because the greatest polyphenoloxidase activity, which is deleterious to both betacyanins and betaxanthins, is located in the peel. Previous blanching presents a tool to inactivate unfavorable enzymatic action.^{61–63} In theory, the oxidizing and hydroxylating activities of polyphenol oxidase action require monophenolic or diphenolic structures rarely found in betaxanthins and betacyanins and only after previous hydrolysis by β -glucosidase activity.^{64–68} Hence, for enzymatic betalain degradation, a concerted action of glucoside-cleaving enzymes, polyphenoloxidases and peroxidases is required.^{69–73} In general, small beets are favored because they accumulate higher betalain concentrations.^{59,74,75}

Tissue comminution is usually performed by milling, followed by acidification of the resulting mash through the addition of citric acid until reaching pH 4. Lowering the pH will preclude the action of polyphenol oxidases while peroxidases may still be active until the filtered juice is heated to a temperature exceeding 75°C.^{76,77} Through acidification, a lower thermal load, i.e., pasteurization instead of sterilization, suffices to secure microbial stability. Thus, pigments will be less severely affected.

In addition to chamber filtration, ultrafiltration has been successfully applied.^{78,79} More recent approaches using pulsed electric fields for pigment extraction have not yet entered industrial practice.^{80,81} Besides technologically oriented work focused on stability aspects during processing, an extensive breeding program initiated in the 1980s resulted in a color yield improvement of 200%.^{74,82–85} High pigment–low solid beets were suggested for food coloring purposes.⁸⁶ Notably, comparatively little research has been dedicated to exploiting alternative food sources. This is even more surprising since red beet preparations are afflicted with adverse flavors of geosmin and methoxy-pyrazine derivatives, high nitrate levels, and the risk of carry-over of earth-bound germs.^{9,35} On the other hand, red beet is economically satisfactory because annual crop yields of 50 to 70 tons per hectare with 40 to 200 mg betanin/100 g have not yet been reached by any other betalainic crop.

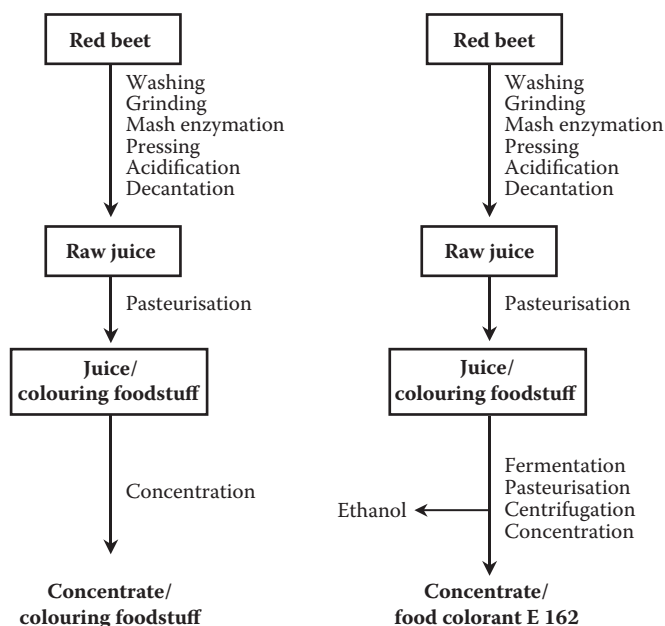


FIGURE 2.4.2 Production scheme of a coloring foodstuff (left) and a food colorant (right) from red beet.

Most importantly, a clear differentiation between color preparations derived from natural sources requiring E number declarations (i.e., E 162 for beet red) and coloring foodstuffs derived from typical food commodities should be made (Figure 2.4.2). For the latter, only physical unselective extraction based on oil or water followed by concentration through heating is allowed, following the recent trend for clean-labelled food.^{9,87}

The so-obtained fruit or vegetable extracts may be applied *quantum satis* to support the particular color characteristics of foods. They are characterized by the typical smells and tastes of the color crop sources. These colored extracts are labeled as ingredients, e.g., red beet extract. On the other hand, if the color extract is fermented with yeasts or molds to remove sugars for achieving a higher tinctorial strength after five- to seven-fold concentration until reaching 65°Bx, the resulting product is considered a natural colorant.^{88–91} The same applies to coloring preparations that have previously been denitrified.^{92–95}

During fermentation, the betacyanins turned out to be more stable than the betaxanthins, which is assumed to be due to their thermal stability rather than different tendencies of pigments toward microbial degradation.⁹⁶ Besides these biological tools, beet extracts may also be purified by column chromatographic techniques. After removal of sugars, salts, and phenolics, the nature-derived color preparation will, however, require E number labeling.⁹⁷

Amaranth — A great potential has been forecasted for grain and leaf amaranth both for nutritional and ornamental purposes.^{98–101} The dye of amaranth leaves (Ama-

ranthaceae) is used as a food color in Bolivia and northwestern Argentina for alcoholic beverages, in Mexico and the southwest United States as a colorant for maize dough, and in Ecuador for different kinds of foods.^{99,100} In China, amaranth is authorized for the production of a natural dye.¹⁰² In a breeding and selection program initiated in China in 1996, 388 genotypes of 37 species from 8 genera were studied.¹⁰³ Cultivated species exhibited higher color contents than wild species, and total betacyanin contents ranged from 46 to 199 mg/100g fresh plant material.^{104,105}

Due to the high number of acylated betacyanin structures, a satisfactory stability was achieved in spray-dried preparations.^{104,106} Because of the wider adaptability of amaranth plants compared to red beets, the former were proposed as new sources of natural food colorants. On the other hand, saponins amounting to 0.1% of dry matter and dopamine contents of about 6 mg/g fresh weight need to be carefully considered prior to their use in foods.^{99,107,108}

Cactus Pear — Since 1998, cactus pear fruits (*Opuntia* sp.) are the focus of the production of betalain-based color preparations to extend the narrow hue range of red beet preparations.^{109–111} The chromatic properties of *Opuntia ficus-indica* cv. ‘Rossa’ were found to be comparable to those of red beet.¹¹¹ Together with orange fruits, different shades should be achievable. Therefore, a process for the production of a yellow-orange cactus pear juice from *O. ficus-indica* cv. ‘Gialla’ has been established which was extended to spray-dried powders and coloring concentrates on a semi-industrial scale.^{112,113} Processing of a red-purple cactus pear at laboratory scale has been reported while a deep-red colored concentrate from *O. stricta* was found to be competitive to cochineal, red beet and commercial anthocyanic extracts.^{114,115} Although current pigment yields range from 15 to 80 mg/100 g fruit, new hybrids are promising higher yields up to 100 mg/100 g.^{111,116} It is thus expected that increased breeding efforts will strengthen the position of cactus pears as sources of purple, red, and yellow-orange hues and their use will have a bright future.

2.4.4 STABILITY OF BETALAIN PREPARATIONS IN COLORED FOODS

A small number of studies have dealt with betalain stability in colored food.^{33,117,118} In most cases, purified or nonpurified pigment solutions and juices were investigated with respect to their color stability in the presence of heat, light, varying a_w , metal ions, and oxygen. In general, betalains are considered to be most stable at near neutral conditions in foods that are devoid of sulfites, protected from oxygen and light, and stored for short times at cooling temperatures. Hence, typical food commodities colored with betalains include dairy products, fruit fillings for bakery products, relishes, various instant products, confectionary, meat substitutes, and sausages.^{33,35}

2.4.5 LEGISLATION

Synthetic colors are important from a regulatory point of view, but they lack consumer acceptance. They are increasingly rejected and considered unwholesome. In

some cases, they were shown to be toxic and were consequently prohibited as occurred in 1977 when food authorizations for six azo compounds were withdrawn in Germany.^{119,120} In addition, artificial colors may cause adverse physiological reactions that again served to restrict their use.^{119–122} Therefore, systematic searches for natural substitutes have become important concerns both for industry and academia.¹²³ The acceptance of natural and nature-derived alternatives is being promoted psychologically by portraying them as healthy and of good quality.

Food coloring is restricted by law to prevent misuse that may lead to deception of consumers related to reduced value or usability. For this purpose, the European Union implemented food colorant guidelines in 1994 based on the understanding that food coloration presents a technological need. While European Parliament and Council Directive 94/36/EC lists colors and their uses in food, the European Commission Directive 95/45/EC contains specific purity criteria for colors in foodstuffs, e.g., a maximal lead content of 20 ppm.^{58,124,125}

Since national food legislation within the European Union varies and differences also exist with regard to United States, Asian, and South American legislation, coloring of foods, drugs, and cosmetics is indeed a most crucial issue for manufacturers.¹²⁶

In Europe, a coloring foodstuff, although not legally defined, is considered a plant product used to color food, e.g., a concentrate or powder from beet, carrot or elderberry. Since the latter is exclusively obtained by means of physical processes, it is considered a food based on its characteristic ingredients and flavor. If, however, a selective extraction process is applied, the resulting product is regarded as a colorant and needs labeling with an EU number, e.g., E 162 for beet red, E 163 for anthocyanins.⁸⁷ In this respect, a coloring foodstuff classified as food may be used internationally, although specific labeling requirements will apply.

The United States Code of Federal Regulations (CFR) 21.73.250/260 covers fruit and vegetable use for coloring (<http://www.cfsan.fda.gov/~dms/col-toc.html>; <http://www.cfsan.fda.gov/~dms/opa-col2.html>). The Joint Food and Agricultural Organization (FAO)/World Health Organization (WHO) Expert Committee on Food Additives Guidelines FAO 52/1 and FAO 52/2 are analogous to 94/36/EC and may be instrumental for food manufacturers dealing with food coloring. Current updates for international trade agreements may be retrieved at <http://www.codexalimentarius.net>.

REFERENCES

1. Mabry, T.J. and Dreiding, A.S., The betalains, in *Recent Advances in Phytochemistry*, Mabry, T.J., Alston, R.E., and Runeckles, V.C., Eds., Appleton-Century-Crofts, New York, 145, 1968.
2. Wyler, H., Mabry, T.J., and Dreiding, A.S., Zur Struktur des Betanidins Über die Konstitution des Randenfarbstoffes Betanin, *Helv. Chim. Acta*, 46, 1745, 1963.
3. Piattelli, M., Minale, L., and Protta, G., Isolation, structure and absolute configuration of indicaxanthin, *Tetrahedron*, 20, 2325, 1964.
4. Wyler, H., Wilcox, M.E., and Dreiding, A.S., Umwandlung eines Betacyans in ein Betaxanthin, Synthese von Indicaxanthin aus Betanin, *Helv. Chim. Acta*, 48, 361, 1965.

5. Kimler, L. et al., On the question of the mutual exclusiveness of betalains and anthocyanins, *Taxon*, 19, 875, 1970.
6. Stafford, H.A., Anthocyanins and betalains: evolution of the mutually exclusive pathways, *Plant Sci.*, 101, 91, 1994.
7. Vogt, T., Substrate specificity and sequence analysis define a polyphyletic origin of betanidin 5- and 6-*O*-glucosyltransferase from *Dortheanthus bellidiformis*, *Planta*, 214, 492, 2002.
8. Ibdah, M. et al., Spectral dependence of flavonol and betacyanin accumulation in *Mesembryanthemum crystallinum* under enhanced ultraviolet radiation, *Plant Cell Environ.*, 25, 1145, 2002.
9. Stintzing, F.C. and Carle, R., Functional properties of anthocyanins and betalains in plants, food, and in human nutrition, *Trends Food Sci. Technol.*, 15, 19, 2004.
10. Gill, M. and Steglich, W., Pigments of fungi (Macromycetes), *Progr. Chem. Organic Nat. Prod.*, 51, 1, 1987.
11. Springob, K. et al., Recent advances in the biosynthesis and accumulation of anthocyanins, *Nat. Prod. Rep.*, 20, 288, 2003.
12. Steglich, W. and Strack, D. Betalains, in *The Alkaloids: Chemistry and Pharmacology*, Brossi, A., Ed., Academic Press, San Diego, 39, 1990.
13. Strack, D., Vogt, T., and Schliemann, W., Recent advances in betalain research. *Phytochemistry*, 62, 247, 2003.
14. Winkel-Shirley, B., Biosynthesis of flavonoids and effect of stress, *Curr. Opin. Plant Biol.*, 5, 218, 2002.
15. Bate-Smith, E.C., The phenolic constituents of plants and their taxonomic significance, *Bot. J. Linn. Soc.*, 58, 95, 1962.
16. Bittrich, V. and Amaral, M.D.C.E., Proanthocyanidins in the testa of centrospermous seeds, *Biochem. System. Ecol.*, 19, 319, 1991.
17. Kolodziej, H., Occurrence of procyanidins in *Nelia meyeri*, *Phytochemistry*, 23, 1745, 1984.
18. Shimada, S. et al., Dihydroflavanol 4-reductase cDNA from non-anthocyanin-producing species in the Caryophyllales, *Plant Cell Physiol.*, 45, 1290, 2004.
19. Shimada, S., Inoue, Y., and Sakuta, M., Anthocyanidin synthase in non-anthocyanin-producing Caryophyllales species, *Plant J.*, 44, 950, 2005.
20. Zryd, J.-P. and Christinet, L., Betalains, in *Plant Pigments and their Manipulation*, Davies, K., Ed., Ann. Plant Rev., 14, CRC Press/Blackwell, Oxford, 2004, 185.
21. Böhm, H. and Mäck, G., Betaxanthin formation and free amino acids in hairy roots of *Beta vulgaris* var. *lutea* depending on nutrient medium and glutamate or glutamine feeding, *Phytochemistry* 65, 1361, 2004.
22. Hempel, J. and Böhm, H., Betaxanthin pattern of hairy roots from *Beta vulgaris* var. *lutea* and its alteration by feeding of amino acids, *Phytochemistry*, 44, 847, 1997.
23. Schliemann, W., Kobayashi, N., and Strack, D., The decisive step in betaxanthin biosynthesis is a spontaneous reaction, *Plant Physiol.*, 119, 1217, 1999.
24. Stintzing, F.C., Schieber, A., and Carle, R., Amino acid composition and betaxanthin formation in fruits from *Opuntia ficus-indica*, *Planta Med.*, 65, 632, 1999.
25. Hans, J., Brandt, W., and Vogt, T., Site-directed mutagenesis and protein 3D-homology modelling suggest a catalytic mechanism for UDP-glucose-dependent betanidin 5-*O*-glucosyltransferase from *Dortheanthus bellidiformis*, *Plant J.*, 39, 319, 2004.
26. Sasaki, N. et al., Detection of UDP-glucose:cyclo-DOPA 5-*O*-glucosyltransferase activity in four o'clocks (*Mirabilis jalapa* L.), *FEBS Lett.*, 568, 159, 2004.

27. Sasaki, N. et al., Isolation and characterization of cDNAs encoding an enzyme with glucosyltransferase activity for *cyclo*-dopa from four o'clocks and feather cockscombs, *Plant Cell Physiol.*, 46, 666, 2005.
28. Sasaki, N. et al., Amaranthin in feather cockscombs is synthesized via glucuronylation at the *cyclo*-DOPA glucoside step in the betacyanin biosynthetic pathway, *J. Plant Res.*, 118, 439, 2005.
29. Stintzing, F.C., Trichterborn, J., and Carle, R., Characterisation of anthocyanin–betalain mixtures for food colouring by chromatic and HPLC-DAD-MS analyses, *Food Chem.*, 94, 296, 2006.
30. Giusti, M.M. and Wrolstad, R.E., Characterization and measurement of anthocyanins by UV-visible spectroscopy, in *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*, Wrolstad, R.E. et al., Eds., John Wiley & Sons, New York, 2005, 19.
31. Trezzini, G. F. and Zryd, J.P., Characterization of some natural and semi-synthetic betaxanthins, *Phytochemistry*, 30, 1901, 1991.
32. Wyler, H. and Meuer, U., Zur Biogenese der Betacyane: Versuche mit [2–14C]-Dopaxanthin. *Helv. Chim. Acta*, 62, 1330, 1979.
33. Delgado-Vargas, F., Jiménez, A.R., and Paredes-López, O., Natural pigments: carotenoids, anthocyanins and betalains: characteristics, biosynthesis, processing, and stability. *Crit. Rev. Food Sci. Nutr.*, 40, 173, 2000.
34. Herbach, K.M., Stintzing, F.C., and Carle, R., Betalain stability and degradation: structural and chromatic aspects, *J. Food Sci.*, 71, R41, 2006.
35. Stintzing, F.C., Schieber, A., and Carle, R., Rote Bete als färbendes Lebensmittel eine Bestandsaufnahme. *Obst Gem. Kartoffelver. Fruit Vegetable Potato Process.* 85, 196, 2000.
36. Cai, Y. et al., Chemical stability and colorant properties of betaxanthin pigments from *Celosia argentea*. *J. Agric. Food Chem.*, 49, 4429, 2001.
37. Stintzing, F.C., Schieber, A., and Carle, R., Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear (*Opuntia ficus-indica* (L.) Mill.) by high-performance liquid chromatography-electrospray ionization mass spectrometry, *J. Agric. Food Chem.*, 50, 2302, 2002.
38. Von Elbe, J.H., Maing, I.-Y., and Amundson, C.H. Color stability of betanin, *J. Food Sci.*, 39, 334, 1974.
39. Von Elbe, J.H., Stability of betalaines as food colors, *Food Technol.*, 29, 42, 1975.
40. Kimler, L. et al., Betalamic acid, a new naturally occurring pigment, *J. Chem. Soc./Chem. Commun.*, 21, 1329, 1971.
41. Jackman, R.L. and Smith, J.L., Anthocyanins and betalains, in *Natural Food Colorants*, 2nd ed., Hendry, G.F. and Houghton, J.D., Eds., Blackie, London, 1996, 245.
42. Herbach, K.M., Stintzing, F.C., and Carle, R., Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations, *J. Food Sci.*, 69, C491, 2004.
43. Herbach, K.M., Stintzing, F.C., and Carle, R., Thermal degradation of betacyanins in juices from purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) monitored by high-performance liquid-chromatography-tandem mass spectrometric analyses, *Eur. Food Res. Technol.*, 219, 377, 2004.
44. Herbach, K.M., Stintzing, F.C., and Carle, R., Identification of heat-induced degradation products from purified betanin, phyllocactin and hylocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry, *Rapid Commun. Mass Spectrom.*, 19, 2603, 2005; 20, 1822, 2006.

45. Herbach, K.M., Stintzing, F.C., and Carle, R., Stability and color changes of thermally treated betanin, phyllocactin, and hylocerenin solutions, *J. Agric. Food Chem.*, 54, 390, 2006.
46. Herbach, K.M. et al., Structural and chromatic stability of purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) betacyanins as affected by the juice matrix and selected additives, *Food Res. Int.*, 39, 667, 2006.
47. Herbach, K.M. et al., Effects of processing and storage on juice colour and betacyanin stability of purple pitaya (*Hylocereus polyrhizus*) juice, *Eur. Food Res. Technol.*, 224, 649, 2007.
48. Mosshammer, M.R., et al., Impact of thermal treatment and storage on color of yellow-orange cactus pear (*Opuntia ficus-indica* [L.] Mill. cv. 'Gialla') juices, *J. Food Sci.*, 71, C400, 2006.
49. Mosshammer, M.R. et al., Stability of yellow-orange cactus pear (*Opuntia ficus-indica* [L.] Mill. cv. 'Gialla') betalains as affected by the juice matrix and selected food additives. *Eur. Food Res. Technol.*, 225, 21, 2007.
50. Wybraniec, S., Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beet root (*Beta vulgaris* L.) monitored by LC-MS/MS, *J. Agric. Food Chem.*, 53, 3483, 2005.
51. Wybraniec, S. and Mizrahi, Y., Generation of decarboxylated and dehydrogenated betacyanins in thermally treated purified fruit extract from purple pitaya (*Hylocereus polyrhizus*) monitored by LC-MS/MS, *J. Agric. Food Chem.*, 53, 6704, 2005.
52. Böhm, H. and Rink, E., Betalains, *Cell Cult. Somatic Cell Gen. Plants*, 5, 449, 1988.
53. Jiménez-Aparicio, A. and Gutiérrez-López, G., Production of food related colorants by culture of plant cells, *Adv. Exp. Med. Biol.*, 464, 195, 1999.
54. Hunter, C.S. and Kilby, N.J., Betalains-their accumulation and release *in vitro*, *Meth. Mol. Biol.*, 111, 403, 1999.
55. Mukundan, U. et al., pH-mediated release of betalains from transformed root cultures of *Beta vulgaris* L., *Appl. Microbiol. Biotechnol.*, 50, 241, 1998.
56. Schroeder, W. and Böhm, H., Once more: secondary metabolite concentrations in whole plants and in cell cultures derived therefrom, *J. Plant Physiol.*, 145, 126, 1995.
57. Yang, R.Y.K. et al., Plant-cell bioreactors with simultaneous electroporabilization and electrophoresis, *J. Biotechnol.*, 100, 13, 2003.
58. Otterstätter, G., *Coloring of Food, Drugs and Cosmetics*, Marcel Dekker, New York, 1999.
59. Lee, Y.N. and Wiley, R.C., Betalaine yield from a continuous solid-liquid extraction system as influenced by raw product, post-harvest and processing variables, *J. Food Sci.*, 46, 421, 1981.
60. Wiley, R.C. and Lee, Y.-N., Recovery of betalains from red beets by a diffusion-extraction procedure, *J. Food Sci.*, 43, 1056, 1978.
61. Im, J.S., Parkin, K.L., and Von Elbe, J.H., Endogenous polyphenoloxidase activity associated with the "black ring" defect in canned beet (*Beta vulgaris* L.) root slices, *J. Food Sci.*, 55, 1042, 1059, 1990.
62. Shih, C.C. and Wiley, R.C., Betacyanine and betaxanthine decolorizing enzymes in the beet (*Beta vulgaris* L.) root, *J. Food Sci.*, 47, 164, 172, 1981.
63. Lee, C.Y. and Smith, N.L., Blanching effect on polyphenol oxidase activity in table beets. *J. Food Sci.*, 44, 82, 86, 1979.
64. Escribano, J. et al., Characterization of monophenolase activity of table beet polyphenol oxidase: determination of kinetic parameters on the tyramine/dopamine pair, *J. Agric. Food Chem.*, 45, 4209, 1997.

65. Gandía-Herrero, F., Escribano, J., and García-Carmona, F., Betaxanthins as substrates for tyrosinase: an approach to the role of tyrosinase in the biosynthetic pathway of betalains, *Plant Physiol.*, 138, 421, 2005.
66. Gandía-Herrero, F., Escribano, J., and García-Carmona, F., Characterization of the activity of tyrosinase on betaxanthins derived from (*R*)-amino acids, *J. Agric. Food Chem.*, 53, 9207, 2005.
67. Escribano, J., Cabanes, J., and García-Carmona, F., Characterisation of latent polyphenol oxidase in table beet: effect of sodium dodecyl sulphate, *J. Sci. Food Agric.*, 73, 34, 1997.
68. Steiner, U., Schliemann, W., and Strack, D., Assay for tyrosine hydroxylation activity of tyrosinase from betalain-forming plants and cell cultures, *Anal. Biochem.*, 238, 72, 1996.
69. Escribano, J. et al., Subcellular localization and isoenzyme pattern of peroxidase and polyphenol oxidase in beet root (*Beta vulgaris* L.), *J. Agric. Food Chem.*, 50, 6123, 2002.
70. Martínez-Parra, J. and Muñoz, R., An approach to the characterization of betanine oxidation catalyzed by horseradish peroxidase, *J. Agric. Food Chem.*, 45, 2984, 1997.
71. Martínez-Parra, J. and Muñoz, R., Characterization of betacyanin oxidation catalyzed by a peroxidase from *Beta vulgaris* L. roots, *J. Agric. Food Chem.*, 49, 4064, 2001.
72. Wasserman, B.P. and Guilfoy, M.P., Peroxidative properties of betanin decolorization by cell walls of red beet, *Phytochemistry*, 22, 2653, 1983.
73. Wasserman, B.P., Eiberger, L.L., and Guilfoy, M.P., Effect of hydrogen peroxide and phenolic compounds on horseradish peroxidase-catalyzed decolorization of betalain pigments, *J. Food Sci.*, 49, 536, 557, 1984.
74. Nilsson, T., The pigment content in beetroot with regard to cultivar, growth, development and growing conditions, *Swed. J. Agric. Res.*, 3, 187, 1973.
75. Watson, J.F. and Gabelman, W.H., Seasonal changes and cultivar differences in pigment concentrations and percent dissolved solids in roots of table beets, *J. Am. Soc. Hort. Sci.*, 107, 713, 1982.
76. Parkin, K.L. and Im, J.S., Chemical and physical changes in beet (*Beta vulgaris* L.) root tissue during simulated processing: relevance to the "black ring" defect in canned beets, *J. Food Sci.*, 55, 1039, 1053, 1990.
77. Wiley, R.C. et al., Efficiency studies of a continuous diffusion apparatus for the recovery of betalaines from the red table beet, *J. Food Sci.*, 44, 208, 1979.
78. Bayindirli, A., Yildiz, F., and Özilgen, M., Modeling of sequential batch ultrafiltration of red beet extract, *J. Food Sci.*, 53, 1418, 1998.
79. Lee, Y.N. et al., Purification and concentration of betalaines by ultrafiltration and reverse osmosis, *J. Food Sci.*, 47, 465, 475, 1982.
80. Chalermchat, Y., Fincan, M., and Dejmek, P., Pulsed electric field treatment for solid-liquid extraction of red beetroot pigment: mathematical modelling of mass transfer, *J. Food Eng.*, 64, 229, 2004.
81. Fincan, M., DeVito, F., and Dejmek, P., Pulsed electric field treatment for solid-liquid extraction of red beetroot pigment, *J. Food Eng.*, 64, 381, 2004.
82. Von Elbe J.H. and Goldman, I.L., The betalains, in *Natural Food Colorants*, Lauro, G.J. and Francis, F.J., Eds., Marcel Dekker, New York, 2000, 11.
83. Gaertner, V. and Goldman, I.L., Pigment distribution and total dissolved solids of selected cycles of table beet from a recurrent selection program for increased pigment, *J. Am. Soc. Hort. Sci.*, 130, 424, 2005.
84. Goldman, I.L. and Navazio, J.P., History and breeding of table beet in the United States, *Plant Breed. Rev.*, 22, 357, 2003.

85. Nilsson, T., Statistical analysis of individual variation and sampling technique when determining the pigment content in beetroot, *Swed. J. Agric. Res.*, 3, 201, 1973.
86. Wolyn, D.J. and Gabelman, W.H., Selection for betalain pigment concentrations and total dissolved solids in red table beets. *J. Am. Soc. Hort. Sci.*, 115, 165, 1990.
87. Stich, E., Kloos, K., and Hoeck, H., Natur, Technik und Recht: miteinander, nicht gegeneinander. *Zucker Süßwaren Wirtsch.*, 52, 10, 1999.
88. Adams, J.P., Von Elbe, J.H., and Amundson, C.H., Production of a betacyanine concentrate by fermentation of red beet juice with *Candida utilis*, *J. Food Sci.*, 41, 78, 1976.
89. Drdák, M. et al., Effect of fermentation on the composition of the red beet pigments. *Ztschr. Lebensm. Unters. Forsch.*, 188, 547, 1989.
90. Drdák, M. et al., Red beet pigment composition: effects of fermentation by different strains of *Saccharomyces cerevisiae*, *J. Food Sci.*, 57, 935, 1992.
91. Sobkowska, E., Czapski, J., and Kaczmarek, R., Red table beet pigment as food colorant. *Int. Food Ingrid.*, 3, 24, 1991.
92. Czapski, J., Maksymiuk, M., and Grajek, W., Analysis of biodenitrification conditions of red beet juice using the response surface method, *J. Agric. Food Chem.*, 46, 4702, 1998.
93. Grajek, W.H. and Walkowiak-Tomczak, D., Factors influencing the denitrification rate of red beet juice by the bacteria *Paracoccus denitrificans*, *J. Agric. Food Chem.*, 45, 1963, 1997.
94. Kolb, E. et al., Potential nitrosamine formation and its prevention during biological denitrification of red beet juice, *Food Chem. Toxicol.*, 35, 219, 1997.
95. Walkowiak-Tomczak, D., Microbiological denitrification of red beet juice, *Eur. Food Res. Technol.*, 215, 401, 2002.
96. Pourrat, H. et al., Purification of red beetroot dye by fermentation, *Biotechnol. Lett.*, 5, 381, 1983.
97. Vergeront, T.A., Von Elbe, J.H., and Amundson, C.H., Large-scale isolation of betalains by gel filtration, *Process Biochem.*, 15, 2, 15, 1980.
98. Brenner, D.M. et al., Genetic resources and breeding of *Amaranthus*, *Plant Breed. Rev.*, 19, 227, 2000.
99. Guzmán-Maldonado, S.H., and Paredes-López, O., Functional products of plants indigenous to Latin America: amaranth, quinoa, common beans, and botanicals, in *Functional Foods: Biochemical and Processing Aspects*, Mazza, G., Ed., Technomic Publishing, Lancaster, PA, 1998, 293.
100. Teutonico, R.A. and Knorr, D., Amaranth: composition, properties, and applications of a rediscovered food crop, *Food Technol.*, 39, 49, 1985.
101. Singh, G., Kawatra, A., and Sehgal, S., Nutritional composition of selected green leafy vegetables, herbs and carrots, *Plant Foods Human Nutr.*, 56, 359, 2001.
102. Cai, Y., Sun, M., and Corke, H., Colorant properties and stability of *Amaranthus* pigments, *J. Agric. Food Chem.*, 46, 4491, 1998.
103. Cai, Y.-Z., Sun, M., and Corke, H., Characterization and application of betalain pigments from plants of the *Amaranthaceae*, *Trends Food Sci. Technol.*, 16, 370, 2005.
104. Cai, Y., Sun, M., and Corke, H., Identification and distribution of simple and acylated betacyanins in the *Amaranthaceae*, *J. Agric. Food Chem.*, 49, 1971, 2001.
105. Cai, Y. et al., Characterization and quantification of betacyanin pigments from diverse *Amaranthus* species, *J. Agric. Food Chem.*, 46, 2063, 1998.
106. Cai, Y.Z. and Corke, H., Production and properties of spray-dried *Amaranthus* betacyanin pigments, *J. Food Sci.*, 65, 1248, 2000.

107. Oleszek, W., Junkuszew, M., and Stochmal, A., Determination and toxicity of saponins from *Amaranthus cruentus* seeds, *J. Agric. Food Chem.*, 47, 3685, 1999.
108. Schliemann, W. et al., Betalains of *Celosia argentea*, *Phytochemistry*, 58, 159, 2001.
109. Stintzing, F.C., Schieber, A., and Carle, R., Amino acid composition and betaxanthin formation in fruits from *Opuntia ficus-indica*, *Planta Med.*, 65, 632, 1999.
110. Stintzing, F.C., Schieber, A., Carle, R., Phytochemical and nutritional significance of cactus pear, *Eur. Food Res. Technol.*, 212, 396, 2001.
111. Stintzing, F.C., Schieber, A., and Carle, R., Evaluation of colour properties and chemical quality parameters of cactus juices, *Eur. Food Res. Technol.*, 216, 303, 2003.
112. Mosshammer, M.R., Stintzing, F.C., Carle, R., Development of a process for the production of a betalain-based colouring foodstuff from cactus pear, *Innov. Food Sci. Emerg. Technol.*, 6, 221, 2005.
113. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Evaluation of different methods for the production of juice concentrates and fruit powders from cactus pear, *Innov. Food Sci. Emerg. Technol.*, 7, 275, 2006.
114. Rodríguez-Hernández, G.R. et al., Spray-drying of cactus pear juice (*Opuntia streptacantha*): effect on the physicochemical properties of powder and reconstituted product, *Drying Technol.*, 23, 955, 2005.
115. Castellar, M.R., Obón, J.M., and Fernández-López, J.A., The isolation and properties of a concentrated red-purple betacyanin food colourant from *Opuntia stricta* fruits, *J. Sci. Food Agric.*, 86, 122, 2006.
116. Castellar, R. et al., Color properties and stability of betacyanins from *Opuntia* fruits, *J. Agric. Food Chem.*, 51, 2772, 2003.
117. Vareltsis, K.P. and Buck, E.M., Color stability and sensory attributes of chicken frankfurters made with betalains and potassium sorbate versus sodium nitrite, *J. Food Protect.*, 47, 41, 1984.
118. Dhillon, A.S. and Maurer, A.J., Evaluation of betalain pigments as colorants in turkey summer sausages, *Poultry Sci.*, 54, 1272, 1975.
119. Combes, R.D. and Haveland-Smith, R.B., A review of the genotoxicity of food, drug and cosmetic colours and other azo, triphenylmethane and xanthene dyes, *Mutation Res.*, 98, 101, 1982.
120. Classen, H.G., Elias, P.S., and Hammes, W.P., *Toxikologisch-hygienische Beurteilung von Lebensmittelinhalt und usatzstoffen sowie bedenklicher Verunreinigungen*, Paul Parey, Berlin, 1987.
121. Lucas, C.D., Hallagan, J.B., and Taylor, S.L., The role of natural color additives in food allergy, *Adv. Food Nutr. Res.*, 43, 195, 2001.
122. Sasaki, Y.F. et al., The comet assay with 8 mouse organs: results with 39 currently used food additives, *Mutation Res.*, 519, 103, 2002.
123. Downham, A. and Collins, P., Colouring our foods in the last and next millennium, *Int. J. Food Sci. Technol.*, 35, 5, 2000.
124. Muermann, B., Das neue Zusatzstoffrecht Teil 1: Allgemeine Grundsätze, *Ernähr. Umsch.*, 45, 162, 1998.
125. Muermann, B., Das neue Zusatzstoffrecht Teil 3: Farbstoffe in Lebensmitteln, *Ernähr. Umsch.*, 45, 237, 1998.
126. Wissgott, U. and Bortlik, K., Prospects for new natural food colorants, *Trends Food Sci. Technol.*, 7, 298, 1996.

2.5 Other Natural Pigments

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2.5.1 QUINONES

2.5.1.1 STRUCTURE AND NOMENCLATURE

Quinones constitute a large group of natural pigments, widely distributed in higher plants but also present in fungi, lichens, and some invertebrate animals.¹ The common structural feature of quinones is the presence of two ketone groups on a monocyclic or polycyclic aromatic skeleton. The two ketone groups can be located on the same ring or on different rings, but it is necessary to have a conjugation involving π (from greek electrons) electrons of carbon–carbon double bonds. Depending on their structures, quinones can be categorized as benzoquinones, naphthoquinones, anthaquinones, or extended quinones. In addition to ketone groups, some other functional groups such as methyl, hydroxyl, methoxyl, or glycosylated phenol can be found in natural quinones. Some quinones with important biological functions, for example, ubiquinones, have long prenyl side chains. The so-called extended quinones such as aphins are complex structures that represent dimerization products of naphthoquinones or anthaquinones. Figure 2.5.1 represents the general quinone structure and some important natural representatives.

2.5.1.2 BIOSYNTHESIS

Quinones represent a very large and heterogeneous class of biomolecules. Three major biosynthetic pathways contribute to the formations of various quinones. The aromatic skeletons of quinones can be synthesized by the polyketide pathway and by the shikimate pathway. The isoprenoid pathways are involved in the biosynthesis of the prenyl chain and in the formation of some benzoquinones and naphthoquinones.¹

The polyketide pathway begins with a condensation of acetyl-CoA with a malonyl-CoA, forming acetoacetyl-CoA. It is followed by a number of decarboxylative condensations with malonyl-CoA, yielding an oligopolyketide. The aromatic ring is formed by the cyclization (aromatization) of polyketides. The polyketide pathway is controlled by a multifunctional enzyme known as polyketide synthase (PKS). Fungal PKS belongs to the class I PKS enzymes and is a single polypeptide containing up to eight domains such as ketosynthetase (KS), acyltransferase (AT), and dehydratase (DH). This pathway was intensively investigated in the production of fungal quinones and is involved as well in the biosynthesis of mycotoxins and antibiotics.^{2,3,4} As an example, aurofusarin, a golden yellow naphthoquinone, is biosynthesized by this pathway in the *Gibberella zeae* fungus.⁵ Other fungal, lichen, and plant benzoquinones, anthaquinones and extended quinones are biosynthesized by the same route.

The shikimate pathway is the major route in the biosynthesis of ubiquinone, menaquinone, phyloquinone, plastoquinone, and various colored naphthoquinones. The early steps of this process are common with the steps involved in the biosynthesis of phenols, flavonoids, and aromatic amino acids. Shikimic acid is formed in several steps from precursors of carbohydrate metabolism. The key intermediate in quinone biosynthesis via the shikimate pathway is the chorismate. In the case of ubiquinones, the chorismate is converted to *para*-hydroxybenzoate and then, depending on the organism, the process continues with prenylation, decarboxylation, three hydroxylations, and three methylation steps.^{6,7}

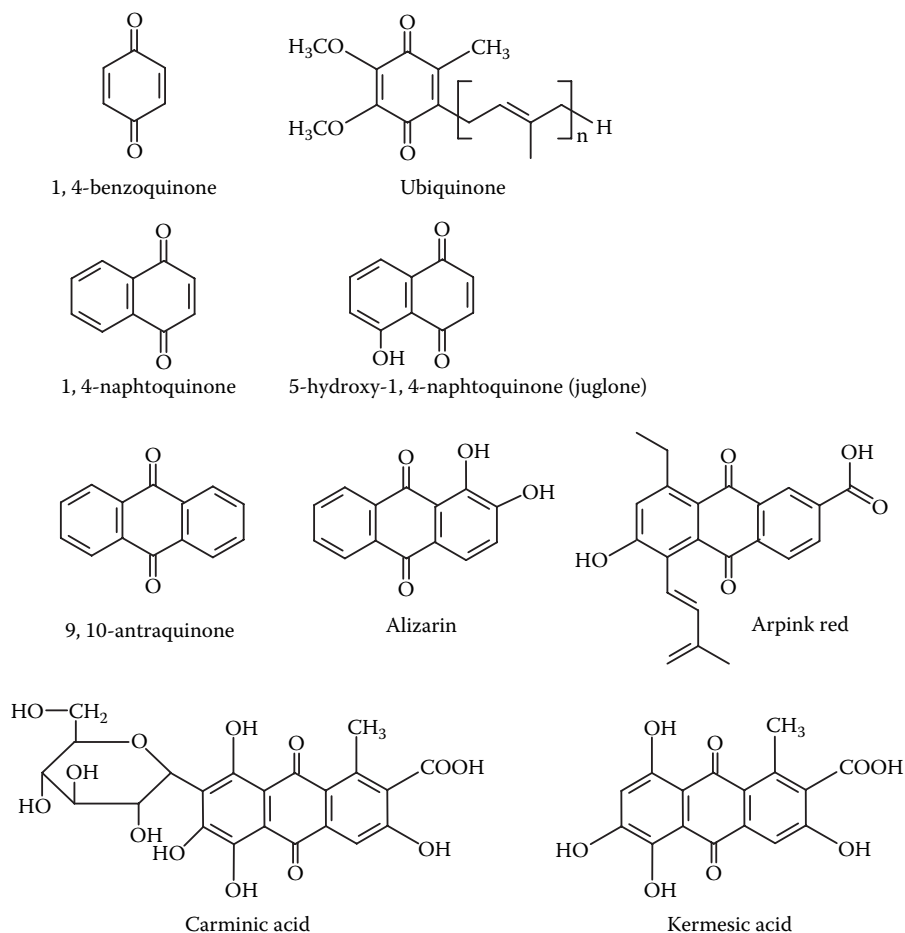


FIGURE 2.5.1 General quinone structure and some important natural representatives.

In the case of menaquinones, the chorismate is converted to isochorismate. The formation of the second ring of menaquinone and other naphthoquinones requires 2-ketoglutarate, coenzyme A and thiamine pyrophosphate, with *o*-succinylbenzoate as an intermediate. Further chemical modifications of the naphthoquinone ring occur, leading to the formation of the two ketone groups, prenyl side chain, and C-methyl group. There are important differences in ubiquinone and menaquinone formation after the key step of chorismate formation, but all the methyl groups are provided by S-adenosylmethionine and the same enzyme catalyses the C-methylation in both molecules.^{6,7} The juglone and lawsone naphthoquinones are biosynthesized by the same pathway.

The third pathway involved in the quinones biosynthesis is the isoprenoid route. This pathway is primarily important for the formation of prenyl side chains of prenylquinones (ubiquinone, menaquinone, plastoquinones, etc.). The side chains of ubiquinones and prenylated naphthoquinones derive from polyprenyl diphosphates.

The isoprenoid side chains of quinones are biosynthesized mainly by the mevalonic acid pathway from acetyl-CoA. Another pathway to biosynthesizing isoprenoids is the so-called non-mevalonate route by which isopentenyl diphosphate (IPP) is formed from glyceraldehyde 3-phosphate and pyruvate.⁸ The key molecule is the farnesyl diphosphate (FPP) that accepts other IPP molecules to form polyprenyl diphosphates.

The lengths of the polyprenyl chains (numbers of isoprene residues) vary in a species-specific way. Ubiquinone side chains in eukaryotic cells, for example, contain six to ten isoprene residues. A *para*-hydroxybenzoate-poly prenyltransferase catalyzes the introduction of an isoprenoid chain in the aromatic ring of quinones. Secondly, the isoprenoid pathway can supply carbon atoms for the formation of naphthoquinone and anthraquinone rings. Incorporation studies revealed evidence that the biosynthesis of chimaphilin and alkannin involves the shikimate pathway for the A ring and the mevalonate pathway for the B ring. In the biosynthesis of alizarin, the A and B rings are formed following the same route as in the case of juglone, and the C ring derives from mevalonate. Phenolic coupling is a process encountered in the formation of the dimers and other condensation products of naphthoquinones and anthraquinones.¹

2.5.1.3 PHYSICAL AND CHEMICAL PROPERTIES

Most quinones are solids and form crystals; their solubility is dependent on their structures. The quinones containing phenol, carboxylic, or glycosyl groups are water-soluble or soluble in alkaline solutions. Carminic acid is soluble both in water and ethanol, kermesic acid is soluble only in water, while alizarin is sparingly soluble in boiling water and soluble in alkalis and in ethanol. Almost all quinones are soluble in organic solvents. Ubiquinone, menaquinone, and plastoquinone are lipophilic compounds, soluble in lipids and organic solvents. The complex polycyclic quinones have poor solubilities both in water and organic solvents.

Light absorption of quinones depends on their skeletons and is also strongly influenced by the presence of various substituents. The absorption spectra of 1,4-benzoquinone presents an intense absorption band (band I) at 240 nm, a medium-intensity absorption band (band II) around 285 nm, and a weak absorption band (band III) at 434 nm. Due to their weak absorption levels in the visible region, unsubstituted benzoquinones are not important as pigments.

The introduction of a substituent, especially a free or methylated hydroxyl group, determines a bathochromic shift of band II in the visible region. The 1,4-naphthoquinone absorption bands are at 245, 257, and 335 nm and the bands are at 243, 263, 332, and 405 nm for anthraquinone. The introduction of a substituent (especially a hydroxyl group) in the aromatic ring of a naphthoquinone determines a strong bathochromic effect (up to 100 nm) and some UV bands are shifted into the visible (vis) region.

A similar effect can be observed in anthraquinones, mainly for the presence of an hydroxyl group. The ionization of hydroxyl groups under basic conditions also undergoes a bathochromic shift.¹ Alizarin has two absorption bands in the vis region, situated at 567 and 609 nm; carminic acid has a visible absorption maximum at around 500 nm and kermesic acid at 498 nm.

Quinones cover a wide range of colors, from pale yellow, to orange, red, purple, and brown. Anthraquinone, for example, has a molar extinction coefficient of $45,000 \text{ M}^{-1}\text{cm}^{-1}$ at 251.25 nm while benzoquinone has a molar extinction coefficient of $24,300 \text{ M}^{-1}\text{cm}^{-1}$ at 239.8 nm. The main chemical property of quinones is the reversible reduction of ketone groups to the phenol group. It is extremely important for ubiquinone and plastoquinone in exerting their biological functions as electron carriers. Natural quinones bearing phenol groups are slightly acidic and can form salts.

2.5.1.4 OCCURRENCE

Quinones are among the most widespread pigments in nature, characterized by great structural diversity. Prenylated quinones such as ubiquinones, menaquinone, and plastoquinone serve important biological functions in animal and plant tissues. Ubiquinones are ubiquitous molecules. Plastoquinone is located in chloroplasts; menaquinone (vitamin K_2) is produced by microorganisms, and phyloquinone (vitamin K_1) by plants. They do not make important contributions to the color because they have weak absorption levels in the visible region. Important quinone pigments are the derivatives of 1,4-naphthoquinone and 9,10-anthraquinone. They can be found in higher plants, microorganisms, and also in arthropods and echinoderms.

In higher plants, quinones can be found in leaves, flowers, fruits, roots, bark and heartwood. There is a long history of utilization of quinones in dyes. The best-known example is the lawsone naphthoquinone present in henna, a natural dye obtained by crushing leaves of *Lawsonia alba*. Another well known naphthoquinone is juglone, isolated from buds, nut hulls, and roots of the walnut tree (*Juglans regia*). Juglone can also occur in glycosylated form, hydrojuglone β -D-glucopyranoside, representing 6 to 8% dry weight in young organs.⁹ Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) is a yellow pigment identified in walnut roots, leaves, and bark, in the roots of *Plumbago zeylanica*, and in Droseraceae, Ancestraladaceae, and other plants.^{10,11} The yellow pigment lapachol (2-hydroxy-3(3-methylbut-2-enyl)-1,4-naphthoquinone and its α -lapachone and β -lapachone derivatives, all of which have important biological properties, have been isolated from the stems and seeds of the South American *Tabebuia avellaneda* (Bignoneaceae) tree.

The best-known anthraquinone in plants is alizarin, found in madder root of *Rubia tinctorum*, along with purpurin, rubiadin and lucidin-primeveroside. Rhubarb (genus *Rheum*) contains an orange anthraquinone emodin, also distributed in other higher plants such as *Aloe vera*, *Rumex acetosa*, and in fungi and lichens. *Hypericum perforatum* L., well known as the medicinal St. John's Wort plant, produces hypericin, a naphthodiantrone.¹ Because hypericin is considered a biologically active compound, *Hypericum* extracts and products are standardized according to their hypericin and hyperforin (an antibiotic derived from phloroglucinol) contents.

The *Mollisia fallens* fungus produces mollisin — a yellow substituted 1,4-naphthoquinone. *Aspergillus* spp. biosynthesize anthraquinones and derivatives such as norsolorinic acid, averantin, averufin, versicolorin I and nidurufin, involved or associated with the aflatoxin biosynthetic pathway in *Aspergillus* spp.¹² Other fungi such as *Polyporus rutilans* and *Helminthosporium* spp. accumulate important amounts of different quinones reaching up to 20 to 23 % of dry weight.¹³ The

Penicillium oxalicum var. *Armeniaca* CCM 8242 strain produces an anthraquinone-type pigment related to carmine and patented as Arpink Red. The pigment is produced during batch submerge cultivation, harvested, and purified. It is presently under evaluation by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) for use as a food colorant.¹⁴

From the cultures of streptomycetes were isolated anthacyclonones like aklavinone.¹ Various aromatic polyketide antibiotics produced by fungi and bacteria have quinoid structures, for example, doxorubicin, rhodomycin, and actinorhodin.⁴

The most important anthaquinones to be used as food colorants are the pigments produced by Coccidae insects. The red anthaquinone carminic acid, a C-glucoside derivative, is obtained from the bodies of female *Coccus cacti* L. (*Dactylopius coccus* Costa) insects. Another insect, *Laccifer lacca* Kerr (formerly *Coccus lacca*) produces lac (or lake) which contains laccaic acids (A, B, C, and D) that are closely related to kermesic acid, a yellow to red pigment produced by *Kermes ilices* (formerly *Coccus ilicis*).¹ In the hemolymphs of various aphids, extended quinones like protoaphins are found and converted after the deaths of the insects to a final product known as red erythroaphin. The spinochromes and echinochromes are strongly colored oxygenated naphthazarins or juglones isolated from sea urchins, brittle stars, and starfish.¹ They are found as calcium or magnesium salts and are natural iron chelators.¹⁵

2.5.1.5 FUNCTIONS, BIOLOGICAL EFFECTS, AND UTILIZATION

Although they are pigments, quinones make only small contributions to the colors of tissues and organisms that produce them. Quinones play an important role in the coloration of some fungi, lichens, insects (Coccidae), and echinoderms, but they rarely contribute to the external colors of higher plants.

Ubiquinone, known also as coenzyme Q, plays a crucial role as a respiratory chain electron carrier transport in inner mitochondrial membranes. It exerts this function through its reversible reduction to semiquinone or to fully hydrogenated ubiquinol, accepting two protons and two electrons. Because it is a small lipophilic molecule, it is freely diffusible within the inner mitochondrial membrane. Ubiquinones also act as important lipophilic endogenous antioxidants and have other functions of great importance for cellular metabolism.¹⁶

Plastoquinone in plant chloroplasts and menaquinone in bacteria play similar roles in electron transport. Phylloquinone (vitamin K₁) and menaquinone (vitamin K₂) are essential for humans. They function mainly as lipid co-factors in blood clotting. Recently, a novel family of redox enzymes — the quinoenzymes — was characterized. Quinoenzymes use o-quinone co-factors derived from amino acids. The most important o-quinone co-factors are topaquinone (TPQ), tryptophan tryptophylquinone (TTQ), lysine tyrosylquinone (LTQ), and the copper-complexed cysteinyl-tyrosyl radical, all of them formed by post-translational modifications, and pyrroloquinoline quinone (PQQ) constructed from glutamate and tyrosine. TPQ and LTQ are found on the active sites of copper-containing amine oxidases while TTQ and the copper-complexed cysteinyl-tyrosyl radical appear to be utilized in bacterial and fungal redox processes. PQQ acts as noncovalent co-factor in simple sugar and alcohol dehydrogenases from prokaryotic organisms. Interest in PQQ is increasing

because of its presence in food (including human milk), its remarkable antioxidant properties, and its role as a growth-promoting factor.^{17,18} Whether PQQ is a co-factor also acting as a vitamin in mammalian organisms is still subject to debate.

Quinones are implicated in defense and in protection because they are often toxic to other organisms. The best example is the juglone which acts as a herbicide against a large number of plants. However, some studies showed that juglone can be involved in plant developmental processes, promoting cell division, cell elongation and adventitious root formation in blackberries.⁹ Alkylated 1,4-benzoquinones were identified in the defensive secretions of neotropical harvestmen, *Goniosoma longipes* (Gonyleptidae), and they proved effective against some ants, spiders, and even frog species.^{19,20} Several other arthropods use quinones in defense mechanisms: opilionides (*Acanthopachylus aculeatus*), coleoptera (*Melolontha* sp.), isoptera (*Macrotermes* sp.), etc. Some fungi produce naphthoquinones and anthraquinones with antiprotozoan, antibacterial, and antifungal properties. *Streptomyces* sp. synthesize a large number of compounds with quinoid structures — tetracyclines, anthracyclines, and actinorhodins — used as antibiotics or in cancer chemotherapy.⁴

A number of quinones have proven toxic or irritating for humans. Hypericin is known to cause phototoxicity, but it is also used as an antidepressant and antiviral agent. Lawsone from henna can induce severe hemolytic anemia.^{21,22} Because of the cytotoxic effects of quinones, they were studied as potential anticancer agents. Emodin isolated from *Rumex acetosa* showed cytotoxic effects on five cultured human tumor cell lines and antimutagenic effects on *Salmonella typhimurium* and *Escherichia coli*.²³ Emodin also directly targeted androgen receptors to suppress prostate cancer cell growth *in vitro* and prolonged the survival of transgenic mice *in vivo*.²⁴ Lapachone (and derivatives) from *Tabebuia* showed antibacterial activities on some methicillin-resistant *Staphylococci* and were atoxic when applied as topical preparations in healthy rabbits.²⁵ In another study β -lapachone induced apoptosis and growth inhibition in human bladder carcinoma T24 cells.²⁶ Rats treated with juglone and plumbagin showed lower incidences and smaller multiplicities of tumors in the entire intestine compared with those exposed to a carcinogen alone in azoxymethane-induced intestinal carcinogenesis.²⁷

2.5.2 NON-POLYMERIC N-HETEROCYCLIC PIGMENTS (OTHER THAN TETRAPYRROLES AND BETALAINS)

2.5.2.1 STRUCTURE AND NOMENCLATURE

Several non-polymeric N-heterocyclic pigments other than betalains will be briefly presented in this chapter, even if they do not belong to a unique class of chemical compounds.

Purines — These molecules have basic skeletons of purine heterocycles. Adenine and guanine, intrinsic components of nucleic acids, are also ubiquitous molecules. Related molecules are isoguanine, xanthine, and uric acid.

Pterins — These are pigments derived from pteridine skeletons. All natural pterins are 2-amino-4-hydroxypteridines bearing various substituents at C₆ and C₇ and having different oxidation states of N₅ and N₈.

Flavins — These are isoalloxazine derivatives methylated at C₆ and C₇, with substituents at N₉. The most important flavin, riboflavin, has a ribityl group (derived from ribitol) at N₉.

Phenazines — These are dibenzopyrazine derivatives with functional groups (hydroxy-, carboxy-) at C₁ and C₆ and an oxygen or methyl group at N₅ and N₁₀. There are also more complex structures, substituted phenazines, terpenoidal, and carbohydrate-containing phenazines and phenazines derived from saphenic acid.²⁸

Phenoxazines — A phenoxazine has a ring skeleton that differs from phenazine by the fact that the nitrogen at position 5 is replaced by an oxygen atom.¹

Figure 2.5.2 illustrates the chemical structures of some N-heterocyclic non-polymeric pigments.

2.5.2.2 BIOSYNTHESIS

The biosynthesis processes of purines, pterins, and flavins are closely related. Both pterins and flavins are synthesized via the guanosine triphosphate (GTP) purine intermediate.

As a component of nucleic acids, the purine ring of guanine is biosynthesized by the same well-known pathway in all living organisms. The process starts from the phosphorybosylpyrophosphate (PRPP) that receives from glutamine an amino group, forming 5-phospho-β-D-rybosylamine. The purine ring of this structure is built up by a sequence of reactions involving glycine, N¹⁰-formyltetrahydrofolate, glutamine, CO₂, aspartate, and energy from adenosine triphosphate (ATP). The key intermediate is the inosinate (IMP). IMP is further dehydrogenated to xanthylate (XMP), which takes an amino group from glutamine, forming guanylate (GMP). The GMP is then phosphorylated to yield GTP.

From GTP the first pterin, 7,8-dihydroneopterin triphosphate (DHN-TP), is synthesized and converted to 7,8-dihydroneopterin (DHN), the key compound in the biosynthesis of folate, biopterins, and pterin pigments of animals.^{1,30} A DHN aldolase catalyzes the cleavage of DHN to 6-hydroxymethyl-7,8-dihydropterin (HMDHP). In plant mitochondria, HMDHP, p-aminobenzoate (PABA), and glutamate are combined to produce folate. PABA is synthesized in the plastids from chorismate by two reactions.^{30–32} Folates are synthesized *de novo* in bacteria, fungi, and plants. For mammals and other higher animals, folates must be supplied in the diet because they are essential co-factors for reactions involving the transfer of one-carbon units.

For the synthesis of drosopterin, tetrahydrobiopterin, sepiapterin, 7-oxopterin and isoxanthopterin, DHN-TP is first converted to the common intermediate 6-pyruvoyl-tetrahydropterin. The biosynthesis of pteridines was studied in zebrafish in relation with the differentiation of neural crest derivatives. The key intermediate in the synthesis of 7-oxobiopterin is the sepiapterin. Pteridins are produced in xanthophores and erythrophores of fish and amphibian species.³³

Riboflavin (vitamin B₂) is the most important representative of flavins. Riboflavin is biosynthesized in plants and bacteria, including those of intestinal flora. GTP is the precursor of flavins, supplying the pyrimidine ring, the nitrogen atoms of the pyrazine ring, and the ribityl side chain.³⁴ The process begins with the hydrolytic cleavage of the imidazole ring and the release of pyrophosphate. Subsequently a

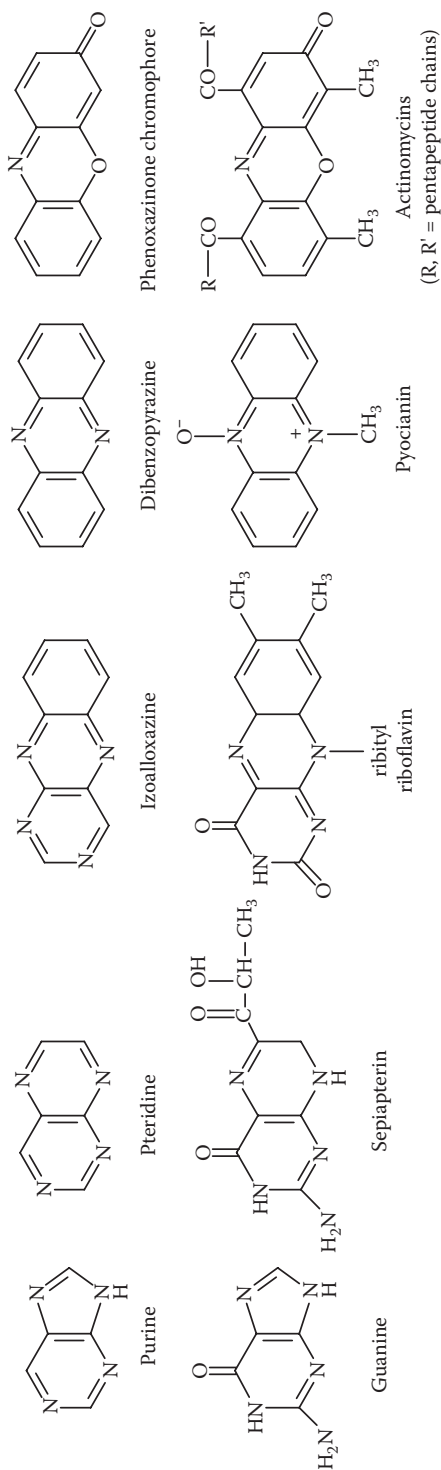


FIGURE 2.5.2 Chemical structures of some N-heterocyclic non-polymeric pigments.

deamination, a reduction of the ribityl side chain, and a diphosphorylation occur. The intermediate is condensed with 3,4-dihydroxybutanone 4-phosphate and give rise to 6,7-dimethyl-8-ribityllumazine. This compound is dismutated to form riboflavin and a secondary product that is recycled. The biosynthesis pathway of riboflavin in plants is much closer to that of eubacteria than to the pathways of fungi and archaea.^{35,36}

Phenazines — The phenazines are biosynthesized by the shikimic acid pathway, through the intermediate chorismic acid. The process was studied using different strains of *Pseudomonas* species, the major producers of phenazines. The best-known phenazine, pyocyanine, seems to be produced from the intermediate phenazine-1-carboxylic acid (PCA). Although intensive biochemical studies were done, not all the details and the intermediates of conversion of chorismic acid to PCA are known. In the first step, PCA is N-methylated by a SAM-dependent methyltransferase. The second step is a hydroxylative decarboxylation catalyzed by a flavoprotein monooxygenase dependent on NADH. PCA is also the precursor of phenazine-1-carboxamide and 1-hydroxyphenazine from *Pseudomonas* species.^{28,37,38}

Phenoxazines — The two main types of phenoxazines are the ommochromes and the microbial phenoxazines. The biosynthesis of ommochromes occurs via the kynurenine pathway. The tryptophan amino acid is converted to formylkynurenine and then to kynurenine and 3-hydroxykynurenine. Not all the steps of ommochrome synthesis are completely elucidated yet. Ommatins are dimers and ommins are oligomers of 3-hydroxykynurenine.^{1,39} The papiliochromes are derived from tyrosine as well as from the tryptophan pathway. The key intermediate in the formation of papiliochromes is N-beta-alanyldopamine (NBAD). Papiliochromes are synthesized in special wing scale cells, before melanins.^{40,41}

2.5.2.3 PHYSICAL AND CHEMICAL PROPERTIES

Purines and pterins are sparingly soluble in water, soluble in diluted acid or alkali and polar solvents, but insoluble in non-polar organic solvents. Flavins and flavin nucleotides are very soluble in water and soluble in polar organic solvents. Phenazines are generally soluble in water and, depending on their structures, soluble in some organic solvents. Riboflavin can participate in oxidation and reduction processes, an important property in exerting biological functions. Pterins can also undergo oxidoreduction reactions but they do not present this behavior *in vivo*.

Purines absorb only ultraviolet light and they contribute to structural colors (white and silver) in animals. Pterines are generally yellow, orange, or red pigments. Because they are amphoteric molecules, the absorption spectra depend on the pH and present three or two absorption maxima, usually one in the visible region. Sepiapterin has an absorption maximum at 340 nm in 0.1M NaOH and at 410 nm in 0.1M HCl.⁴² Leucopterin has three maxima: 240, 285, and 340 nm. Xanthopterin has two: 255 and 391 nm. Because they are conjugated with proteins, pterins show bathochromic shifts *in vivo*.¹ They also present fluorescence when excited with UV light.

The excitation and emission wavelengths used for fluorescence detection in HPLC analysis are 350 and 450 nm for isoxanthopterin and 340 and 450 nm for 2,4,7-trioxopteridine, respectively.⁴² The absorption spectra of riboflavin present

maxima at 223, 267, 373, 445, and 475 nm and it has a molar extinction coefficient of $33,000 \text{ M}^{-1}\text{cm}^{-1}$ at 266.5 nm.⁴³ Riboflavin appears yellow in solution and also presents strong green fluorescence in UV light. Phenazines can be yellow, purple, or blue, having several absorption bands in UV and at least one between 400 and 600 nm. Ommochromes have absorption in the UV and visible regions between 440 and 500 nm. *In vivo*, they are yellow-brown, purple, or almost black.¹

2.5.2.4 OCCURRENCE

Purines — Adenine and guanine are ubiquitous molecules serving as components of nucleic acids and nucleotides. Guanine, hypoxanthine and in some cases adenine are produced by iridophores, special types of pigment cells located in the epidermis of lower vertebrates.³³ Guanine is the most frequent purine and accumulates as microcrystals or granules, mainly in fish skin and scales. Guanine and hypoxanthine are also found in the irises of some bird species.⁴⁴ Uric acid, xanthine, and isoguanine may also contribute to the external colors of animals.¹

Pterins — These compounds are mostly known as pigments of butterflies, moths, and other insects but they are also found in fish, amphibians, reptiles, and crustaceans. Leucopterin is a white compound identified in the cabbage white butterfly, but the white color of the wings is of structural origins. Other colored pterins such as the yellow chrysopterin and the red erythropterin contribute to the bright colors of butterflies, for example, the orange sulfur *Colias eurytheme* butterfly.⁴⁵ Pterins are also found in the eyes of insects; e.g., drosiopterin which is found in *Drosophila melanogaster*,¹ and in the irises of many birds.^{44,46–48}

In vertebrates, pterins are located in xanthophores and erythrophores, in special organelles called pterinosomes. Sometimes pterins coexist with carotenoids, sharing contributions to orange and red colors. The most common pterin is the yellow sepiapterin, which is accompanied in zebrafish, for example, by 7-oxobiopterin and isoxanthopterin.⁴² The orange spots of male guppies (*Poecilia reticulata*), small teleost fishes, contain both carotenoids and drosiopterin.⁴⁹ A lot of other marine animals such as ascidians accumulate purines, pterins, and melanins.³⁹

Pterins make no contributions to the colors of plants and microorganisms. One important pterin is the folate produced by plants and microorganisms. Folate and its derivatives are present in plants in various concentrations in mitochondria, cytosols, vacuoles, and plastids. The total amount of folic acid depends on the plant species, on the developmental stage, and on external factors. Good sources of folates are beans, lentils, spinach, and wheat germ.³²

Flavins — The most important flavin is riboflavin (vitamin B₂), the precursor of the flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) flavo-coenzymes and of flavoproteins. They are widespread in all living organisms, but are biosynthesized only in plants and microorganisms.³⁶ Even so, riboflavin makes no contributions to the colors of plants, and makes small contributions to colors of some microorganisms. The best sources of riboflavin are vegetables and milk, but an important amount of vitamin B₂ is obtained by fermentation processes. So-called flavinogenic microorganisms (bacteria, yeasts, and fungi) are used to provide riboflavin for human and animal nutrition. Some microorganisms now or formerly used

for the production of riboflavin include *Clostridium acetobutylicum*, *Ashbya gossypii*, *Candida famata*, and *Bacillus subtilis*.⁵⁰

Phenazines — This large class of compounds includes more than 6,000 natural and synthetic representatives. Natural phenazines are secondary metabolites of certain soil and marine microorganisms. The main phenazine producers are *Pseudomonas* and *Streptomyces* species.¹ *Pseudomonas* strains produce the most simple phenazines: tubermycin B (phenazine-1-carboxylic acid), chlororaphine, pyocyanin, and iodinine. Pyocyanin is a blue pigment while chlororaphine is green; both are produced by *Pseudomonas aeruginosa*. They can be seen in infected wounds of animal and human skins. Iodinine is a purple phenazine produced by *Pseudomonas aureofaciens*.

Besides the compounds mentioned above, *Streptomyces* strains produce more complex structures. The hydroxyacetic acid derivatives, griseoluteins A and B, were isolated from *Streptomyces griseoluteus*. *Streptomyces anulatus* produces C-isoprenylated phenazines, endophenazines A through C, and *Streptomyces prunicolor* synthesizes the N-monoterpenoids, benthocyanins A through C. Few carbohydrate-containing phenazines derived from 6-deoxy-L-glucopyranosides were isolated from marine *Streptomyces* species. Other complex and unusual phenazines were isolated from *Methanosarcoma mazei*, *Pelagibacter variabilis* (halophile bacterium), *Vibrio* strains, and *Erwinia herbicola*.²⁸

Phenoxazines — The most important microbial phenoxazines are the actinomycins, red compounds produced in *Streptomyces* species. An actinomycin contains a substituted phenoxazinone chromophore bearing two identical pentapeptide lactone chains. Ommochromes are widespread in the eyes of insects and arthropods, in the integuments of arthropods and cephalopods, and in eggs and worm tissues (*Bombyx mori*). Xanthommatin is the brown pigment in the eyes of wild-type *Drosophila melanogaster*.¹ The tobacco hornworm hemolymph, *Manduca sexta*, contains a yellow-colored protein containing ommatin D.⁵¹ Papiliochromes are yellow complex kynurenine derivatives found in the wings of Papilionidae (e.g. *Papilio glaucus*, *Papilio xuthus*).^{40, 41}

2.5.2.5 FUNCTIONS, BIOLOGICAL EFFECTS, AND UTILIZATION

Pterins — Colored pterins are important in the pigmentation of butterflies, other insects, fish, amphibians, and reptiles, as sexual color displays. The red drosoterpine that accumulates only in the orange spots of males contributes to the sexual coloration of poeciliid fish.⁴⁹ The pterins accumulating in vertebrate eyes together with purines act as light reflectors.^{1,48} Several other pterin derivatives play important metabolic roles. Folate and folic acids serve as vitamins for humans who are not capable of producing them. Folate as the tetrahydrofolate (THF) is involved in the reactions of single-carbon unit (C1) transferases essential for the biosynthesis of dTMP nucleotides and subsequently of DNA. Rich folate diets or folate supplementations are strongly recommended during pregnancy and infancy when intensive biosynthesis of DNA occurs.

Folic acid deficiency is also related to megaloblastic anemia. Tetrahydrobiopterin is a co-factor for phenylalanine, tyrosine, and tryptophan hydroxylases — enzymes

involved in the biosynthesis of catecholamines, serotonin, and melanins. More recently, pteridine derivatives have been investigated regarding their interactions with free radicals. Several studies showed that 7,8-dihydroneopterin released from human macrophages, when exposed to the gamma-interferon immune stimulant, acts as an antioxidant. 7,8-Dihydroneopterin protects the erythrocytes, inhibits low-density lipoprotein oxidation, and inhibits oxidized low-density lipoprotein-induced cell death in cell culture.^{52–54} Still, the antioxidant activity of pterins is not clear; it seems that they can act as antioxidants or prooxidants, depending on the specific experimental conditions.⁵⁵

Flavins — Riboflavin is first of all essential as a vitamin for humans and animals. FAD and FMN are coenzymes for more than 150 enzymes. Most of them catalyze redox processes involving transfers of one or two electrons. In addition to these well known and documented functions, FAD is a co-factor of photolyases, enzymes that repair UV-induced lesions of DNA, acting as photoreactivating enzymes that use the blue light as an energy source to initiate the reaction. The active form of FAD in photolyases is their two-electron reduced form, and it is essential for binding to DNA and for catalysis. Photolyases contain a second co-factor, either 8-hydroxy-7,8-didemethyl-5-deazariboflavin or methenyltetrahydrofolate.⁵⁶

Folate and FAD are also components of cryptochromes, proteins widespread in living organisms. Cryptochromes are considered photolyase sequence homologues with no DNA repair activities but with blue light-activated factors. Cryptochromes regulate growth and development in plants and seem to be responsible for the synchronization of circadian rhythms in animals and human.^{56–58}

The FMN form of riboflavin is also co-factor for phototropin, a light-activated autophosphorylating serine and threonine kinase. Phototropins are chromoproteins serving as photoreceptors for phototropism. The two FMN molecules act as blue light-absorbing chromophores.^{59,60} Phototropins (phot1 and phot2) regulate phototropism, chloroplast movement, stomatal opening, and leaf expansion in plants.⁶¹

Phenazines — A large number of natural and synthetic phenazines display biological effects. Most of them have antimicrobial effects, but some have also shown antitumor, antimalaria, and antiparasitic activities. The physiological role of phenazines is still unclear, but it is supposed that their accumulation protects the producing organism against other microorganisms due to their antibiotic properties. Some complex phenazines such as benthocyanins showed radical scavenging properties and inhibited lipid peroxidation. Several phenazines also induced cytotoxicity against different tumor cells.²⁸ Pyocyanin is found in high concentrations in the sputum of cystic fibrosis patients and it is probably implicated in pulmonary tissue damage.⁶² Phenazines produced in the rhizospheres of plants protect against *Fusarium* and *Phythium* in chickpeas and beans.³⁷

Phenoxazines — The microbial phenoxazines like actinomycins are well-known antibiotics. Actinomycin D produced by *Streptomyces antibioticus* is an effective antineoplastic agent that inhibits nucleic acid synthesis. The main function of ommochromes is to act as screening pigments in the eyes of insects and other arthropods, as pattern pigments in the integument, and as excretion products of excess tryptophan.^{1,51}

2.5.3 MELANINS

2.5.3.1 STRUCTURE AND NOMENCLATURE

Melanins are complex polymeric structures, which are usually mixtures of macromolecules. Melanins are classified as eumelanins, phaeomelanins and allomelanins.⁶³

Eumelanins — These melanins are considered polymers derived from tyrosine derivatives, mainly 5,6-dihydroxyindole-2-carboxylic acid (DHICA) and dihydroxyindole (DHI), with high degrees of cross-linking. *In vivo* eumelanins are associated with proteins and with metals, most frequently copper, zinc, or iron.

Phaeomelanins — This group consists of sulfur-containing polymers composed of benzothiazines, derived from cysteinyl-DOPA.

Allomelanins — These are structurally different compounds containing little or no nitrogen. They are considered polymers of phenolic compounds like catechol.¹ Fungi produce melanin pigments, predominantly dihydroxyphenylalanine (DOPA)-melanin and dihydroxynaphthalene (DHN)-melanin.^{64–66}

2.5.3.2 BIOSYNTHESIS

Melanins are produced in mammals in two types of cells of different developmental origin: (1) the melanocytes of the skin, hair, choroids and iris and (2) the retinal pigment epithelium (RPE). Specialized organelles of the melanocytes, the melanosomes, synthesize and store eumelanins and phaeomelanins.

Melanin biosynthesis in animals is a complex process starting with the L-tyrosine amino acid. In the first step, L-tyrosine is converted first into DOPA and then into dopaquinone, a process catalyzed by tyrosinase. In the biosynthesis of eumelanins, dopaquinone undergoes a cyclization to form dopachrome and subsequently a tautomerization into 5,6-dihydroxyindole-2-carboxylic acid (DHICA). DHICA is further oxidized to indole-5,6-quinone-2-carboxylic acid, the precursor of DHICA eumelanins. Tyrosinase-related proteins TRP-2 and TRP-1, respectively, are responsible for the last two steps, and they are under the control of the tyrosinase promoter.

Dopachrome also undergoes a nonenzymatic reaction to form dihydroxyindole (DHI), the precursor of DHI-eumelanins. For the formation of phaeomelanins, dopaquinone is first transformed in cysteinyl-DOPA and then in cysteinyl-dopaquinone which suffers a nonenzymatic polymerization. The polymerization of monomers and the association of melanins with proteins is not yet completely elucidated and may involve other intermediates.^{67–69}

2.5.3.3 PHYSICAL AND CHEMICAL PROPERTIES

Because of their very complex chemical structures and heterogeneity, melanins are difficult to extract, separate, and characterize from tissues. Eumelanins are insoluble in water and organic solvents. They can be extracted from tissues with strong chemicals that are capable of removing lipids, proteins, and other tissue components but also lead to the formation of degradation products. Enzymatic procedures were developed for the isolation of eumelanins from mammalian hair and irises. The first step is sequential digestion with protease, proteinase K, and papaine in the presence

of dithiothreitol.⁷⁰ The identification of monomers is made after chemical reaction with specific reagents and identification of degradation products.

Eumelanins are chemically stable and inert but they are bleached after exposure to strong light and hydrogen peroxide. Melanins show strong and broad UV-visible absorption with shapes similar to those of amorphous disordered semiconductors. Structural, optical, and electrical properties of melanins were recently investigated and a model of a natural melanin film as a network of nanoaggregates of polymeric units was proposed to explain the x-ray diffraction results.⁷¹ Pheomelanins are also very stable, insoluble in water, but soluble in diluted alkali. They show strong absorption between 500 and 550 nm. Allomelanins are soluble in alkali, relatively stable at temperatures below 100°C and in alkali solutions, reducers, and salts, but bleached by strong oxidants.⁷²

2.5.3.4 OCCURRENCE

Eumelanins are produced mainly by animals, but sometimes they also occur in plants. Eumelanins provide black and brown colors of animals and can be found as granules in hair, feather, fur, skin, scales, and integuments. They are also produced in inner tissues such as choroid, iris, RPE, and inner ear.^{1,67–69} One of the best-known melanins is the sepiomelanin produced and accumulated in the ink of *Sepia officinalis* cuttlefish.⁷³ It consists of a mixture of oligomeric structures, of which 75% are derived from 5,6-dihydroxyindole-2-carboxylic acid and 20% from dihydroxyindole.⁷⁴ Pheomelanins are yellow, red, or brown pigments found in red hair, freckles, and feathers. Neuromelanin is a complex structure found in substantia nigra that seems to contain both eumelanins and pheomelanins, together with other amino acids and oxidation products derived from DOPA.^{75,76} Malignant melanocytes (melanoma cells) present up-regulated melanogenesis and defective melanosomes, accumulating large amounts of melanin⁷⁷ and 5-cysteinil-DOPA; the last is excreted in large amounts in the urine of patients with melanoma metastasis.⁷⁸

Allomelanins are black pigments produced by plants and fungi. Allomelanins form black spots on leaves and flowers and accumulate in beans and seeds (e.g., *Osmanthus fragrans*)^{1,72,79} and spores of fungi (e.g., *Tuber melanosporum*).⁸⁰ Melanin-type compounds were isolated from tea and tea polyphenols and they showed scavenging and antioxidant properties.⁸¹ Fungal melanins are produced in *Aspergillus nidulans* and *A. niger*, *Alternaria alternata*, *Cryptococcus neoformans*, *Wangiella dermatitis*, etc.⁶⁵

2.5.3.5 FUNCTIONS, BIOLOGICAL EFFECTS, AND UTILIZATION

The most evident function of melanins is providing pigmentation to mammals, invertebrates, and plants as well. In animals, melanin pigmentation is important for camouflage and display and even in defense (e.g., cuttlefish ink).^{68,73} In invertebrates, melanin is involved in immunity, wound healing, and sclerotization (cuticular hardening).⁶⁷ Accumulation of melanin in the skin was considered a protective response to the damages induced by UV radiation. Several studies covered the photoprotective role of melanins against UV light.^{68,82} Their photoprotective effect was proven also

in RPE cells by an EPR (electron paramagnetic resonance) study.⁸³ It seems that melanin protects cells by absorbing the photodamaging blue light and scavenging the reactive oxygen species.^{84–86} Melanins can bind toxic metals, and this property may have a role in antioxidant defense and also in transcutaneous metal excretion.^{68,87}

The role of allomelanins is not completely understood but it seems that their accumulation is related to the maturation of truffles.⁸⁸ Plant melanins show antioxidant,⁸⁹ liver protecting,⁹¹ and antitumor⁹¹ properties when tested in various experimental models. Recently it was shown that fungal melanins are linked to virulence in some human pathogenic and phytopathogenic fungi and they protect fungal cells.^{64–66}

2.5.4 IRIDIODS

2.5.4.1 STRUCTURE AND NOMENCLATURE

Iridoids are a group of monoterpenoids having methylcyclopentane skeletons. Iridoids have divided into true iridoids and secoiridoids. The true iridoids have a cyclopentanodihydropyran ring, while secoiridoids have the cyclopentane ring opened. Iridoids can be aldehydes, alcohols, lactones, esters, and even alkaloids. Iridoids can be found in free form but they often exist as glycosides. Secoiridoids are almost all glycosides; some are alkaloidal glycosides.^{93–95} Iridoids and secoiridoids show a large diversity of structures. Several hundreds of representatives have been isolated and characterized.

2.5.4.2 BIOSYNTHESIS

Iridoids are biosynthesized along the well-known pathway of terpenoids, from the immediate precursor, the geranyldiphosphate. The terpenoid origin of the iridoid skeleton was proven by incorporation studies with labeled mevalonic acid and geranyldiphosphate (GPP). The formation of iridoids is known to occur through two main routes. In the first route, GPP is transformed successively into 10-hydroxygeraniol, 10-oxogeraniol, iridodial, and iridotrial which forms deoxyloganic acid — the precursor of many iridoids such as loganin, secologanin, derived secoiridoids, and complex indole alkaloids. In the second route, the intermediates are 8-*epi*-iridodial, 8-*epi*-iridotrial, and 8-*epi*-deoxyloganic acid. Through this pathway are formed the decarboxylated carbocyclic iridoid (e.g., aucubin) and a few unusual secoiridoids.^{92,93,95} Several other subroutes were identified in the formation of various iridoid structures.⁹⁵ The alternative terpenoid pathway (triose phosphate/pyruvate) was demonstrated for the secologanin biosynthesis in *Catharantus roseus* cell culture.⁹⁶ Secologanin is the key precursor in the formation of the majority of terpene alkaloids.

2.5.4.3 PHYSICAL AND CHEMICAL PROPERTIES

The solubility of iridoids depends on their state (free, glycosylated, acetylated), but usually they are extracted with polar solvents: methanol, ethanol, aqueous alcohols, and rarely acetone. Iridoid glycosides are more or less stable; some of them are very sensitive to acids and alkalis. Some iridoid glycosides such as aucubin suffer color modification after chemical or enzymatic hydrolysis; they give first a blue to green

color and finally a black precipitate. In order to avoid decomposition, the plant should be soaked in the solvent and heated for the inactivation of hydrolytic enzymes.⁹² Iridoids can react with primary amines, amino acids and proteins, giving various colored products.^{97,98}

The most studied iridoid is geniposide from *Gardenia jasminoides* Ellis fruits. Genipin, the aglycone of geniposide, is liberated by enzymatic hydrolysis, and then reacts with glycine, lysine, or phenylalanine, giving rise to the gardenia blue pigment. The blue pigments are stable at temperatures between 60 and 90° C and under light irradiance of 5,000 to 20,000 lux. The pigments are more stable at alkaline pH than at neutral or acidic pH.⁹⁹ Red pigments can also be obtained from the reactions of geniposidic acid with amino acids.¹⁰⁰ Aucubin, another iridoid glucoside, gives a red pigment after condensation with glutamic acid.¹⁰²

2.5.4.4 OCCURRENCE

Iridoids and their related alkaloids are widely spread in angiosperms and are found in 13 orders and 70 families including Rutales, Buxales, Hamamelidales, Cornales, Loasales, Gentianales, etc. Important iridoids are loganin, found in high amounts in *Strychnos nux-vomica* and in *Catharanthus roseus*, and secologanin found especially in Caprifoliaceae.

The most studied for their applications as pigments are the iridoids from cape jasmin fruits (*Gardenia jasminoides* Ellis). Gardenia fruits contain geniposide and gardenoside glycosides as major compounds. Other common iridoids are aucubin from *Aucuba japonica* and Plantago sp., catalposide from Catalpa, jasminine from Jasminum, etc. An interesting compound is the volatile nepetalactone iridoid from catmint, *Nepeta cataria*.⁹²⁻⁹⁵

2.5.4.5 FUNCTIONS, BIOLOGICAL EFFECTS, AND UTILIZATION

In plants, iridoid glycosides often act as feeding attractants and stimulants for larvae of Lepidopterae. The catnip essential oil has been formulated and marketed as an alternative repellent for protection against biting arthropods but it seems less effective than synthetic compounds.¹⁰² On the other hand, insects sequester iridoids of plant origin (e.g., derivatives of aucubin) that serve as feeding deterrents to birds. Structures related to iridoids occur in the defense secretions of ants and other insects. Plants containing iridoids have been used in traditional medicine for a long time. Iridoids are bitter-tasting compounds and they have been used as tonics and cholagogues. Some iridoids show also laxative, diuretic, and sedative effects. Several other biological effects were proven including antiinflammatory, antimicrobial, analgesic, and antitumor effects.^{92,94,103-105}

The main industrial utilization of iridoids remains the production of gardenia blue and gardenia yellow pigments used for coloring liqueurs, candies, noodles, etc. Gardenia blue is largely used in Asia as the only blue pigment of plant origin. Gardenia yellow is colored because of its carotenoid and flavonoid contents; its iridoid fraction is colorless. Gardenia yellow shows hepatotoxicity in rats¹⁰⁶ and more recently was found to be genotoxic but not mutagenic when tested *in vitro*.¹⁰⁷ In a chronic study in rats, gardenia blue color showed a lack of carcinogenicity.¹⁰⁸

2.5.5 INORGANIC NATURAL PIGMENTS

Some inorganic compounds are used as food additives and food colorants. They include titanium dioxide, carbon black, iron oxides, ultramarine, and calcium carbonate. Some of them are important for properties other than the ability to impart color. Titanium is the most commonly used inorganic pigment in food and will be briefly discussed below.^{13,109,110}

2.5.5.1 TITANIUM DIOXIDE

Titanium dioxide (E171, CI white 6) is a white, opaque mineral occurring naturally in three main forms: rutile, anatase, and brookite. More than 4 million tons of titanium dioxide are produced per year and it is widely used for industrial applications (paints, inks, plastics, textiles) and in small amounts as a food colorant.^{109,110}

Production and properties — Titanium oxide is mainly produced from ilmenite, a titaniferous ore (FeTiO_3). Rutile and anatase are relatively pure titanium dioxide (TiO_2) forms. Titanium oxide pigment is produced via chloride or sulfate processes via the treatment of the titanium oxide ore with chlorine gas or sulfuric acid, followed by a series of purification steps. High-purity anatase is preferred for utilization in the food industry. It may be coated with small amounts of alumina or silica to improve technological properties.

Titanium dioxide is an amorphous white powder characterized by brightness and a very high refractive index (2.4). It is insoluble in water and organic solvents, and is a very stable material, resistant to light, pH variation, oxidation, etc. TiO_2 is available in oil-dispersible and water-dispersible forms.^{13,109,110}

Utilization and toxicology — Titanium dioxide is used to provide whiteness and opacity in paints, inks, plastics, textiles, paper, toothpaste, and food. The most important food applications are in dairy products, icings, confectionery, and toppings.^{13,109–111} Titanium dioxide is used in low-fat products to add creaminess. It also serves as a photocatalyst under UV light and can be used as antibacterial agent.¹¹²

The utilization of TiO_2 as a food colorant in the United States is limited to 1% by weight. The JECFA has not established an ADI, considering TiO_2 to be self-regulated under GMP. A sensitive method using inductively coupled plasma optical emission spectrometry was established for the quantification of TiO_2 in food.¹¹³

Animal studies showed low acute toxicity in rats ($\text{LD}_{50} > 25$ g/kg body weight/day) and mice ($\text{LD}_{50} > 10$ g/kg body weight/day) and non-carcinogenicity and genotoxicity in long-term studies.^{13,109,110} It was considered that TiO_2 is poorly absorbed in mammals, but it was found in human gut-associated lymphoid tissue¹¹⁴ and also showed potential genotoxicity in Chinese hamster ovary-K1 cells.¹¹⁵

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REFERENCES

1. Britton, G., *The Biochemistry of Natural Pigments*, Cambridge University Press, Cambridge, 1983.
2. Hopwood D.A. and Sherman, D.H., Molecular genetics of polyketides and its comparison with fatty acid biosynthesis, *Annu. Rev. Genet.*, 24, 37, 1990.
3. Hutchinson, R.C., Microbial polyketide synthases: more and more prolific, *Proc. Natl. Acad. Sci. USA*, 96, 3336, 1999.
4. Schneider, G., Enzymes in the biosynthesis of aromatic polyketide antibiotics, *Curr. Opin. Struct. Biol.*, 15, 629, 2005.
5. Kim, J.E. et al., Putative polyketide synthase and laccase for biosynthesis of aurofusarin in *Gibberella zeae*, *Appl. Environ. Microbiol.*, 71, 1701, 2005.
6. Meganathan, R., Biosynthesis of menaquinone (vitamin K₂) and ubiquinone (coenzyme Q): a perspective on enzymatic mechanism, *Vitamins Hormones*, 61, 173, 2001.
7. Meganathan, R., Ubiquinone biosynthesis in microorganisms, *FEMS Microbiol Lett.*, 203, 131, 2001.
8. Rohmer, M., The discovery of a mevalonate-independent pathway for isoprenoid biosynthesis in bacteria, algae and higher plants, *Nat. Prod. Rep.*, 16, 565, 1999.
9. Duroux, L. et al., Insight into naphthoquinone metabolism: β -glucosidase-catalysed hydrolysis of hydrojuglone β -pyranoside, *Biochem. J.*, 333, 275, 1998.
10. Binder, R.G., Benson, M.E., and Flath, R.A., Eight 1,4-naphthoquinones from *Juglans*, *Phytochemistry*, 28, 2799, 1989.
11. Sandur, S.K. et al., Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) suppresses NF-kappa B activation and NF-kappa B-regulated gene products through modulation of p65 and I-kappa-B-alpha kinase activation, leading to potentiation of apoptosis induced by cytokine and chemotherapeutic agent, *J. Biol. Chem.*, 281, 17023, 2006.
12. Shier, W.T. et al., Yellow pigments used in rapid identification of aflatoxin-producing *Aspergillus* strains are anthraquinones associated with the aflatoxin biosynthetic pathway, *Bioorg Chem.*, 33, 426, 2005.
13. Delgado-Vargas, F. and Paredes-Lopez, O., Natural pigments: global perspective, in *Natural Colorants for Food and Nutraceutical Uses*, CRC Press, Boca Raton, FL, 2003, chap. 6.
14. Sardaryan, E. et al., Arpink Red: meet a new natural red food colourant of microbial origin, in *Pigments in Food: More Than Colors*, Dufossé, L., Ed., Université de Bretagne Occidentale, Quimper, 2004, 207.
15. Lebedev, A.V., Ivanova, M.V., and Levitsky, D.O., Echinochrome, a naturally occurring iron chelator and free radical scavenger in artificial and natural membrane systems, *Life Sci.*, 76, 863, 2005.
16. Turunen, M., Olsson, J., and Dallner, G., Metabolism and function of coenzyme Q, *Biochim. Biophys. Acta*, 1660, 171, 2004.
17. Stites, T.E., Mitchell, A.E., and Rucker, R.B., Physiological importance of quinoenzymes and the O-quinone family of cofactors, *J. Nutr.*, 130, 719, 2000.
18. Magnusson, O.T. et al., Quinone biogenesis: structure and mechanism of PqqC, the final catalyst in the production of pyrroloquinoline quinone, *Proc. Natl. Acad. Sci. USA*, 101, 7913, 2004.
19. Machado, G. et al., Chemical defense in harvestmen (Arachnida, Opiliones): do benzoquinone secretions deter invertebrate and vertebrate predators? *J. Chem. Ecol.*, 31, 2519, 2005.

20. Hara, M.R. et al., A comparative analysis of the chemical nature of defensive secretions of Gonyleptidae (Arachnida, Opiliones, Laniatores), *Biochem. Syst. Ecol.*, 33, 1210, 2005.
21. Raupp, P. et al., Henna causes life threatening haemolysis in glucose-6-phosphate dehydrogenase deficiency, *Arch. Dis. Child.*, 85, 411, 2001.
22. McMillan, D.C., et al., Role of oxidant stress in lawsone-induced hemolytic anemia, *Toxicol. Sci.*, 82, 647, 2004.
23. Lee, N.J. et al., Antimutagenicity and cytotoxicity of the constituents from the aerial parts of *Rumex acetosa*, *Biol. Pharm. Bull.*, 28, 2158, 2005.
24. Cha, T.L. et al., Emodin down-regulates androgen receptor and inhibits prostate cancer cell growth, *Cancer Res.*, 65, 2287, 2005.
25. Pereira, E.M. et al., *Tabebuia avellaneda* naphthoquinones: activity against methicillin-resistant staphylococcal strains, cytotoxic activity and *in vivo* dermal irritability analysis, *Ann. Clin. Microbiol. Antimicrob.*, 22, 5, 2006.
26. Lee, J.I. et al., Beta-lapachone induces growth inhibition and apoptosis in bladder cancer cells by modulation of Bcl-2 family and activation of caspases, *Exp. Oncol.*, 28, 30, 2006.
27. Sugie, S. et al., Inhibitory effects of plumbagin and juglone on azoxymethane-induced intestinal carcinogenesis in rats, *Cancer Lett.*, 127, 177, 1998.
28. Laursen, J.B. and Nielsen, J., Phenazine natural products: biosynthesis, synthetic analogues, and biological activity, *Chem. Rev.*, 104, 1663, 2004.
29. Thöny, B., Auerbach, G., and Blau, N., Tetrahydrobiopterin biosynthesis, regeneration and functions, *Biochem. J.*, 347, 1, 2000.
30. Mouillon, J.M. et al., Folate synthesis in higher-plant mitochondria: coupling between the dihydropterin pyrophosphokinase and the dihydropterolate synthase activities, *Biochem. J.*, 363, 313, 2002.
31. Goyer, A. et al., Folate biosynthesis in higher plants. cDNA cloning heterologous expression and characterization of dihydroneopterin aldolases, *Plant. Physiol.*, 135, 103, 2004.
32. Rebeille, F. et al., Folates in plants: biosynthesis, distribution and enhancement, *Physiol. Plant*, 126, 330, 2006.
33. Ziegler, I., The pteridine pathway in zebrafish: regulation and specification during the determination of neural crest cell-fate, *Pigment. Cell. Res.*, 16, 172, 2003.
34. Bacher, A. et al., Biosynthesis of vitamin B₂ (Riboflavin), *Annu. Rev. Nutr.*, 20, 153, 2000.
35. Fischer, M. and Bacher, A., Biosynthesis of flavocoenzymes, *Nat. Prod. Rep.*, 22, 324, 2005.
36. Fischer, M. and Bacher, A., Biosynthesis of vitamin B₂ in plants, *Physiol. Plant*, 126, 304, 2006.
37. Mavrodi, D.V. et al., Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1, *J. Bacteriol.*, 183, 6454, 2001.
38. Mavrodi, D.V., Blankenfeldt, W., and Thomashow, L.S., Phenazine compounds in fluorescent *Pseudomonas* spp. biosynthesis and regulation, *Annu. Rev. Phytopathol.*, 44, 417, 2006.
39. Takeuchi, K. et al., A genome-wide survey of genes for enzymes involved in pigment synthesis in an ascidian, *Ciona intestinalis*, *Zool. Sci.*, 22, 723, 2005.
40. Umebachi, Y., Papiliochrome; a new pigment group of butterfly, *Zool. Sci.*, 2, 163, 1985.

41. Koch, P.B. et al., Insect pigmentation: activities of beta-alanyldopamine synthase in wing color patterns of wild-type and melanic mutant swallowtail butterfly, *Papilio glaucus*, *Pigment Cell Res.*, 13, Suppl 8, 54, 2000.
42. Ziegler, I. et al., Development of the pteridine pathway in the zebrafish, *Danio rerio*, *J. Biol. Chem.*, 275, 18926, 2000.
43. Du, H. et al., Photochem CAD: a computer-aided design and research tool in photochemistry, *Photochem. Photobiol.*, 68, 141, 1998.
44. Hudon, J. and Muir, A.D., Characterization of the reflective materials and organelles in the bright irides of North American blackbirds (Icterinae), *Pigment Cell Res.*, 9, 96, 1996.
45. Rutowski, R.L. et al., Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colia eurytheme*, *Proc. R. Soc. B*, 272, 2329, 2005.
46. Oliphant, L.W., Pteridines and purines as major pigments of the avian iris, *Pigment Cell Res.*, 1, 129, 1987.
47. Tillotson, H.M. and Oliphant L.M., Iris stromal pigment cells of the ringed turtle dove, *Pigment Cell Res.*, 3, 319, 1990.
48. Hudon, J. and Oliphant, L.W., Reflective organelles in the anterior pigment epithelium of the iris of the European starling, *Sturnus vulgaris*, *Cell Tiss. Res.*, 280, 383, 1995.
49. Grether, G.F., Hudon, J., and Endler, J.A., Carotenoid scarcity, synthetic pteridine pigments and the evolution of sexual coloration in guppies (*Poecilia reticulata*), *Proc. R. Soc. Lond. B*, 268, 1245, 2001.
50. Stahmann, K.-P., Revuelta, J.L., and Seulberger, H., Three biotechnical processes using *Ashbya gossypii*, *Candida famata* and *Bacillus subtilis* compete with chemical riboflavin production, *Appl. Microbiol. Biotechnol.*, 53, 509, 2000.
51. Martel, R.R. and Law, J.H., Purification and properties of an ommochrome-binding protein from the hemolymph of the tobacco hornworm, *Manduca sexta*, *J. Biol. Chem.*, 266, 21392, 1991.
52. Giese, S.P., Maghzal, G., and Glubb, D., Protection of erythrocytes by the macrophage synthesized antioxidant 7,8 dihydroneopterin, *Free Radic. Res.*, 34, 123, 2001.
53. Giese, S.P. and Cato, S., Inhibition of THP-1 cell-mediated low-density lipoprotein oxidation by the macrophage-synthesised pterin, 7,8-dihydroneopterin, *Redox Rep.*, 8, 113, 2003.
54. Baird, S.K. et al., OxLDL induced cell death is inhibited by the macrophage synthesised pterin, 7,8-dihydroneopterin, in U937 cells but not THP-1 cells, *Biochim. Biophys. Acta*, 1745, 361, 2005.
55. Oettl, K. and Reibnegger, G., Pteridine derivatives as modulators of oxidative stress, *Curr. Drug Metab.*, 3, 203, 2002.
56. Sancar, A., Structure and function of DNA photolyase and cryptochrome blue-light photoreceptors, *Chem. Rev.*, 103, 2203, 2003.
57. Sancar, A., Regulation of the mammalian circadian clock by cryptochrome, *J. Biol. Chem.*, 279, 34079, 2004.
58. Partch, C. L. et al., Posttranslational regulation of mammalian circadian clock by cryptochrome and proteinphosphatase 5, *Proc Natl Acad. Sci. USA*, 103, 10467, 2006.
59. Briggs, W.R., Christie, J.M., and Salomon, M., Phototropins: a new family of flavin-binding blue light receptors in plants, *Antioxid. Redox Signal.*, 3, 775, 2001.
60. Briggs, W.R. et al., The phototropin family of photoreceptors, *Plant Cell*, 13, 993, 2001.
61. Corchnoy, S.B. et al., Intramolecular proton transfers and structural changes during the photocycle of the LOV2 domain of phototropin, *J. Biol. Chem.*, 278, 724, 2003.

62. Wilson, R., et al. Measurements of *Pseudomonas aeruginosa* phenazine pigments in sputum and assessment of their contribution to sputum sol toxicity for respiratory epithelium, *Infect. Immun.*, 56, 2515, 1988.
63. Nicolaus, R.A., *Melanins*, Hermann, Paris, 1968.
64. Butler, M.J. and Day, A.W., Fungal melanins: a review, *Can. J. Microbiol.*, 44, 1115, 1998.
65. Jacobson, E.S., Pathogenic roles for fungal melanins, *Clin. Microbiol. Rev.*, 13, 708, 2000.
66. Langfelder, K. et al., Biosynthesis of fungal melanins and their importance for human pathogenic fungi, *Fungal Genet. Biol.*, 38, 143, 2003.
67. Sugumaran, M., Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects, *Pigment Cell Res.*, 15, 2, 2002.
68. Riley, P.A., Melanin, *Int. J. Biochem. Cell. Biol.*, 29, 1235, 1997.
69. Murisier, F. and Beerman, F., Genetic of pigment cells: lessons from the tyrosinase gene family, *Histol. Histopathol.*, 21, 567, 2006.
70. Novellino, L., Napolitano, A., and Prota, G., Isolation and characterization of mammalian eumelanins from hair and irides, *Biochim. Biophys. Acta*, 1475, 295, 2000.
71. Capozzi, V. et al., Optical and photoelectronic properties of melanin, *Thin Solid Films*, 511, 362, 2006.
72. Wang, H. et al., Isolation and characterization of melanin from *Osmanthus fragrans* seeds, *LWT Food Sci. Technol.*, 39, 496, 2006.
73. Bandaranayake, W.M., The nature and role of pigments of marine invertebrates, *Nat. Prod. Rep.*, 23, 223, 2006.
74. Pezzella, A. et al., An integrated approach to the structure of sepia melanin: evidence for a high proportion of degraded 5,6-dihydroxyindole-2-carboxylic acid units in the pigment backbone, *Tetrahedron*, 53, 8281, 1997.
75. Odh, G. et al., Neuromelanin of the human substantia nigra: a mixed-type melanin, *J. Neurochem.*, 62, 2030, 1994.
76. Double, K.L. et al., Structural characteristics of human substantia nigra neuromelanin and synthetic dopamine melanins, *J. Neurochem.*, 75, 2583, 2000.
77. Riley, P.A., Melanogenesis and melanoma, *Pigment Cell Res.*, 16, 548, 2003.
78. Rorsman, H., The pigmented life of a red hair: bibliographic review, *Pigment Cell Res.*, 17, 191, 2004.
79. De Angelis, F. et al., Partial structures of truffle melanins, *Phytochemistry*, 43, 1103, 1996.
80. Harki, E., Talou, T., and Dargent, R., Purification, characterisation and analysis of melanin extracted from *Tuber melanosporum* Vitt., *Food Chem.*, 58, 69, 1997.
81. Sava, V.M. et al., Isolation and characterization of melanic pigments derived from tea and tea polyphenols, *Food Chem.*, 73, 177, 2001.
82. Krol, E.S. and Liebler, D.C., Photoprotective action of natural and synthetic melanins, *Chem. Res. Toxicol.*, 11, 1434, 1998.
83. Seagle, B.L. et al., Melanin photoprotection in the human retinal pigment epithelium and its correlation with light-induced cell apoptosis, *Proc. Natl. Acad. Sci. USA*, 102, 8978, 2005.
84. Rozanowska, M. et al., Free radical scavenging properties of melanin: interaction of eu- and pheo-melanin models with reducing and oxidising radicals, *Free Rad. Biol. Med.*, 26, 518, 1999.
85. Sarna T. et al., Loss of melanin from human RPE with aging: possible role of melanin photooxidation, *Exp. Eye Res.*, 76, 89, 2003.

86. Wang, Z., Dillon, J., and Gaillard, E. R., Antioxidant properties of melanin in retinal pigment epithelial cells, *Photochem. Photobiol.*, 82, 474, 2006.
87. McGraw, K.J., The antioxidant function of many animal pigments: are there consistent health benefits of sexually selected colourants? *Anim. Behav.*, 69, 757, 2005.
88. Harki, E., Bouya, D., and Dargent, R., Maturation-associated alterations of the biochemical characteristics of the black truffle *Tuber melanosporum* Vitt., *Food Chem.*, 99, 394, 2006.
89. Hung, Y.C. et al., Antioxidant activity of melanins derived from tea: comparison between different oxidative states, *Food Chem.*, 78, 233, 2002.
90. Sava, V.M. et al., The liver-protecting activity of melanin-like pigment derived from black tea, *Food Res. Int.*, 36, 505, 2003.
91. Kamei, H. et al., Suppression of growth of cultured malignant cells by allomelanins, plant-produced melanins, *Cancer Biother. Radiopharm.*, 12, 47, 1997.
92. Inouye, H., Iridoids, in *Methods in Plant Biochemistry*, vol. 7, Terpenoids, Charlwood, B.V. et al., Eds., Academic Press, London, 1991, 132.
93. Jensen, S.R., Plant iridoids, their biosynthesis and distribution in angiosperms, in *Ecological Chemistry and Biochemistry of Plant Terpenoids*, Harborne, J.B. et al., Eds., Clarendon Press, Oxford, 1991, 133.
94. Harborne, J.B., *Phytochemical Dictionary: A Handbook of Bioactive Compounds from Plants*, Taylor & Francis, London, 1999.
95. Jensen, S.R., Franzyk, H., and Wallander, E., Chemotaxonomy of the Oleaceae: iridoids as taxonomic markers, *Phytochemistry*, 60, 213, 2002.
96. Contin, A. et al., The iridoid glucoside secologanin is derived from the novel triose phosphate/pyruvate pathway in a *Catharantus roseus* cell culture, *FEBS Lett.*, 434, 413, 1998.
97. Fujikawa, S. et al., Brilliant skyblue pigment formation from Gardenia fruits, *J. Ferment. Technol.*, 65, 419, 1987.
98. Park, J.-E. et al., Isolation and characterization of water-soluble intermediates of blue pigments transformed from geniposide of *Gardenia jasminoides*, *J. Agric. Food Chem.*, 50, 6511, 2002.
99. Paik, Y.S. et al., Physical stability of the blue pigments formed from geniposide of Gardenia fruits: effects of pH, temperature, and light, *J. Agric. Food Chem.*, 49, 430, 2001.
100. Moritome, N. et al., Formation of red pigment produced from geniposidic acid and amino compound, *J. Food Sci. Technol. Mysore*, 39, 345, 2002.
101. Moritome, N., Nakashima, K., and Fujii, S., The red pigment prepared from aucubin and amino acid, *J. Jap. Soc. Food Sci. Tech.*, 44, 760, 1997.
102. Chauhan, K.R. et al., Feeding deterrent effects of catnip oil components compared with two synthetic amides against *Aedes aegypti*, *J. Med. Entomol.*, 42, 643, 2005.
103. Samuelsen, A.B., The traditional uses, chemical constituents and biological activities of *Plantago major* L: a review, *J. Ethnopharm.*, 71, 1, 2000.
104. Konoshima, T. et al., Cancer chemopreventive activity of an iridoid glycoside, 8-acetylharpagide, from *Ajuga decumbens*, *Cancer Lett.*, 157, 87, 2000.
105. Koo, H.J. et al., Anti-inflammatory evaluation of gardenia extract, geniposide and genipin, *J. Ethnopharmacol.*, 103, 496, 2006.
106. Yamano, T. et al., Hepatotoxicity of gardenia yellow color in rats, *Toxicol. Lett.*, 44, 177, 1988.
107. Ozaki, A. et al., Genotoxicity of gardenia yellow and its components, *Food Chem. Toxicol.*, 40, 1603, 2002.

108. Imazawa, T. et al., Lack of carcinogenicity of gardenia blue colour given chronically in the diet to F344 rats, *Food Chem. Toxicol.*, 38, 313, 2000.
109. Francis, F.J., Food coloring, in *Colour in Food: Improving Quality*, MacDougall, D.B., Ed., CRC Press, Boca Raton, FL, 2002, chap. 12.
110. Francis, F.J., *Colorants*, Eagan Press, St. Paul, MN, 1999, chap. 11.
111. Phillips, L.G. and Barbano, D.M., The influence of fat substitutes based on protein and titanium dioxide on the properties of low fat milks, *J. Dairy Sci.*, 80, 2726, 1997.
112. Choi, J.Y. et al., Photocatalytic antibacterial effect of TiO₂ film formed on Ti and TiAg. *J. Biomed. Mater. Res. B Appl. Biomater.*, epub., 2006.
113. Lomer, M.C.E. et al., Determination of titanium dioxide in foods using inductively coupled plasma optical emission spectrometry, *Analyst*, 125, 2339, 2002.
114. Powell, J.J. et al., Characterisation of inorganic microparticles in pigment cells of human gut associated lymphoid tissue, *Gut*, 38, 390, 1996.
115. Lu, P.J., Ho, I.C., and Lee, T.C., Induction of sister chromatid exchanges and micro-nuclei by titanium dioxide in Chinese hamster ovary-K1 cells, *Mutat. Res.*, 414, 15, 1998.

Section 3

*Pigment Stability, Bioavailability,
and Impacts on Human Health*

3.1 Plant Pigments as Bioactive Substances

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3.1.1 INTRODUCTION

According to epidemiological studies, there is convincing evidence that fruits and vegetables reduce the risk of cancers,¹⁻³ cardiovascular diseases,⁴⁻⁸ and also Alzheimer's disease, cataracts, and age-related functional decline.⁹ Such protective effects are supported by numerous epidemiological studies and data obtained from cells and animals. Health benefits of fruits and vegetables are attributed to their non-energetic fractions rich in fibers, vitamins [A (by providing provitaminic A carotenoids), B, C, and K] and minerals.

Fruits and vegetables also contain other bioactive substances such as polyphenols (including well-known pigments: anthocyanins, flavonols) and non-provitamin A carotenoids (mainly lycopene, lutein, and zeaxanthin) that may have protective effects on chronic diseases. Polyphenols and carotenoids are known to display antioxidant activities, counteracting oxidative alterations in cells. Besides these antioxidant properties, these colored bioactive substances may exert other actions on cell signaling and gene expression.

The purpose of this chapter is to provide an overview of our present knowledge about the health benefits of pigments, particularly their effects on chronic diseases. We examine the effects of lipophilic (carotenoids, chlorophylls) and hydrophilic pigments (anthocyanins and flavones-flavonols), and curcumin. Descriptive and mechanistic studies are reviewed in regard to common chronic diseases.

3.1.2 LIPOPHILIC PIGMENTS

Carotenoids are the most commonly studied pigments related to common chronic degenerative diseases, while knowledge about the role of chlorophylls in human health is lacking. Carotenoids are the most widespread group of pigments in nature (more than 600 have been characterized structurally). Animals and humans cannot synthesize them and plant foods are therefore the primary sources for humans. The beneficial effects of carotenoids are attributed to a small portion of the hundreds of carotenoids found in nature; only about ten are found in plasma and tissues and only two (lutein and zeaxanthin) in the retina and lens of the eye. The most studied carotenoids are β -carotene and lycopene (belonging to the subgroup of carotenes) as well as lutein, zeaxanthin, and β -cryptoxanthin (belonging to the subgroup of xanthophylls). About 50 carotenoids such as α - and β -carotene and β -cryptoxanthin display provitamin A activities.

3.1.2.1 ESTIMATION OF DAILY CAROTENOID INTAKE

Daily consumption of various fruits, vegetables, and derived juices contributes to human intake of carotenoids. The estimation of carotenoid intakes has been made possible through publication of the qualitative and quantitative carotenoid contents of commonly consumed foods.^{10–12} Average intake estimates in the United States are around 6.5 mg/day. In seven countries in Europe, the average total carotenoid intake based on the sum of the five carotenoids was approximately 14 mg/day. When dietary source of carotenoids were analyzed, carrots appeared as the major sources of β -carotene in all countries except Spain, where spinach was the main contributor.

Carrots were also the main sources of α -carotene, whereas tomatoes and tomato products were the major sources of lycopene. Lutein was mainly provided by peas in the Republic of Ireland and United Kingdom. Spinach was found to serve as the major source in other countries. Lutein and zeaxanthin xanthophylls are found in a wide variety of fruits and vegetables, particularly green leafy vegetables, but also in some animal products such as egg yolks. In all countries, β -cryptoxanthin was obtained primarily from citrus fruits.

Health benefits of carotenoids are related to their bioavailability and thus their absorption. Plasma concentration is considered a good biomarker of fruit and vegetable consumption.¹⁰ Table 3.1.1 shows plasma carotenoid levels in EPIC study subjects from 16 European locations. EPIC was the first large cross-sectional study analyzing plasma carotenoid levels in several European populations.¹⁰

TABLE 3.1.1
Mean Plasma Carotenoid Levels
($\mu\text{mol/l}$) in EPIC Study Subjects from
16 European Areas

Carotenoid	Plasma Level		
	Minima	Maxima	Mean
α -Carotene	0.06	0.32	0.14
β -Carotene	0.21	0.68	0.44
β -Cryptoxanthin	0.11	0.53	0.29
Lycopene	0.43	1.32	0.73
Lutein	0.26	0.7	0.41
Zeaxanthin	0.05	0.13	0.09

Source: From Al-Delaimy, W.K. et al., *Public Health Nutr.*, 7, 713, 2004.

3.1.2.2 EPIDEMIOLOGICAL STUDIES

Many epidemiological studies have analyzed the correlations between different carotenoids and the various forms of cancer and a lot of conclusions converge toward protective effects of carotenoids. Many studies were carried out with β -carotene.^{13,14} The SUVIMAX study, a primary intervention trial of the health effects of antioxidant vitamins and minerals, revealed that a supplementation of β -carotene (6 mg/day) was inversely correlated with total cancer risk.¹⁵ Intervention studies investigating the association between carotenoids and different types of cancers and cardiovascular diseases are reported in Table 3.1.2 and Table 3.1.3.

Carotenoids and prostate cancer — Numerous epidemiological studies including prospective cohort and case-control studies have demonstrated the protective roles of lycopene, tomatoes, and tomato-derived products on prostate cancer risk; other carotenoids showed no effects.^{16–19} In two studies based on correlations between plasma levels or dietary intake of various carotenoids and prostate cancer risk, lycopene appeared inversely associated with prostate cancer but no association was reported for α -carotene, β -carotene, lutein, zeaxanthin, or β -cryptoxanthin.^{21,22} Nevertheless, a protective role of all these carotenoids (provided by tomatoes, pumpkin, spinach, watermelon, and citrus fruits) against prostate cancer was recently reported by Jian et al.²²

Giovannucci¹⁶ reviewed 72 epidemiological studies including ecological, case-control, dietary, and blood specimen-based investigations of tomatoes, tomato-based products, lycopene, and cancer. Thirty-five studies reported an inverse association between tomato intake or circulating lycopene levels and risk of several types of cancers. More recently, the same authors analyzed data from 17 studies based on the relation between prostate cancer and lycopene or tomatoes. Eight showed significant inverse correlations between lycopene or tomato intake and incidence of

TABLE 3.1.2
Summary of Supplementation Studies Examining Intake of Carotenoids and Incidence of Cancers

Study Design	Dose and Time	Carotenoid	Variables	Association	References
Womens' Health Study Primary prevention trial (Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study)	50 mg/alternate days, 2.1 yr	β -Carotene	Lung cancer	0	38
	20 mg/day, 5 to 8 yr	β -Carotene	Lung cancer	+	35
			Gastric cancer	0	45
			Urinary cancer	0	48
			Colorectal cancer	0	43
Placebo-controlled chemoprevention trial [Beta-Carotene and Retinal Efficacy Trial (CARET)]	30 mg/day carotene + 25,000 IU retinal palmitate, 4 yr	β -Carotene	Lung cancer and total mortality	+	36
Primary prevention trial (Physicians' Health Study)	20 mg/alternate days, 12 yr	β -Carotene	Lung cancer risk	0	37
Primary prevention trial (SU.VI.MAX)	6 mg/day beta-carotene, 7.5 years, in combination with other antioxidants	β -Carotene	Total cancer risk	–	15
Supplementation study	Oleoresin (equivalent to 30 mg/day lycopene), 3 wk	Lycopene	PSA and tumors	–	24
Supplementation study	Tomato sauce-based pasta (equivalent to 30 mg lycopene/day), 3 wk	Lycopene	Serum and prostate levels	+	25
Supplementation study	Tomato sauce-based pasta (equivalent to 30 mg lycopene/day), 3 wk	Lycopene	Serum and prostate levels	+	23
			PSA	–	
			Leukocyte oxidative DNA damage	–	

0 = No association. – = Inverse association. + = Positive association. ATBC = α -Tocopherol, β -Carotene Cancer Prevention Study. PSA = Prostate-specific antigen.

TABLE 3.1.3
Intervention Studies Relating Carotenoids and Cardiovascular Diseases

Study Design	Dose and Time	Carotenoid	Variables	Association	References
Supplementation study (European Multicenter, Placebo-Controlled Supplementation Study)	15 mg/day, 20 wk (capsules of natural extracts)	Lutein, lycopene, α - and β -carotene	Serum level	+	70
Randomized cross-over dietary intervention study	20 to 150 mg/day, 1 wk (tomato juice, spaghetti sauce)	lycopene	Biomarkers associated with CVD	0	
			Serum level	+	50, 68
			Lipid, protein and DNA oxidation	-	
			LDL oxidation/serum lipid peroxidation	-	
Supplementation study	60 mg/day, 3 mo (tomato lycopene)	Lycopene	Plasma LDL cholesterol level	-	71
Supplementation study (Multicenter Skin Cancer Prevention Study)	50 mg/day, 4.3 yr	β -Carotene	Mortality from CVD	0	66
Primary prevention trial (Beta Carotene and Retinol Efficacy Trial)	30 mg/day carotene, 25,000 IU retinol	β -Carotene + vitamin A	Risk of death from CVD	0	36
Primary prevention trial (α -Tocopherol, β -Carotene Cancer Prevention Study)	20 mg/day, 6 yr	β -Carotene	Non-fatal MI and fatal CHD	0	67
			First major coronary event	+	67
Primary prevention study (Physicians' Health study)	50 mg/day, 13 yr	β -Carotene	Non-fatal and fatal MI	0	36
Secondary prevention study (Heart Protection Study)	20 mg/day, 5 yr	β -Carotene	Fatal or non-fatal vascular events	0	76
Secondary prevention study (α -Tocopherol, β -Carotene Cancer Prevention Study)	20 mg/day, 5.3 yr	β -Carotene	Non-fatal MI and fatal CHD	+	75

- = Inverse association. + = Positive association. 0 = No association. CHD = coronary heart disease. CVD = cardiovascular disease. MI = myocardial infarction. IMT = intima media thickness. CCA-IMT = common carotid artery intima media thickness. LDL = low-density lipoprotein.

prostate cancer, three reported inverse but not significant correlations, and six reported no associations.²¹

Intervention trials confirmed this protective role of lycopene on prostate cancer risk. Three primary intervention studies evaluated the effect of lycopene supplementation on prostate cancer risk or on certain risk markers such as prostate-specific antigen (PSA) plasma concentration or oxidative alterations of leucocyte DNA.^{23–25} All showed increases of plasma and prostate lycopene levels after diet supplementation with lycopene and inverse correlations between tumor incidence and risk biomarkers.

Carotenoids and breast cancer — Among seven case-control studies investigating the correlation between different carotenoid plasma levels or dietary intakes and breast cancer risk, five showed significant inverse associations with some carotenoids.^{26–29} In most cases, this protective effect was due to β -carotene and lutein. However, one (the Canadian National Breast Screening Study³¹) showed no association for all studied carotenoids including β -carotene and lutein. More recently, another study³² even demonstrated a positive correlation between breast cancer risk and tissue and serum levels of β -carotenes and total carotenenes. Nevertheless, these observational results must be confirmed by intervention studies to prove consistent.

Carotenoids and lung cancer — Data concerning the role of carotenoids in lung cancer are not convincing. Although prospective studies converge toward an inverse association between carotenoid dietary intake or serum level and lung cancer risk, the compounds presenting protective effects differ according to the studies. Only one study³³ showed a protective effect of the main carotenoids, namely lutein, zeaxanthin, lycopene, β -cryptoxanthin, and β -carotene. By contrast, in the Tin Corporation study, Ratnasinghe et al.³⁴ reported a positive correlation between lung cancer and serum levels of lutein, zeaxanthin, β -cryptoxanthin, and β -carotene.

Thus, data concerning the role of carotenoids in lung cancer are inconclusive. Moreover, results from intervention trials investigating a supplementation of β -carotene were surprising (Table 3.1.2). Two large randomized intervention trials of β -carotene supplementation having lung cancer as the primary study endpoint were published: the Alpha-Tocopherol, Beta-Carotene (ATBC) Cancer Prevention Study³⁵ and the Beta-Carotene and Retinol Efficacy Trial (CARET).³⁶ Both studies showed that supplementation of β -carotene led to an increase of lung cancer incidence. By contrast, two other large intervention trials reporting data concerning the effects of β -carotene supplementation on lung cancer, namely the Physicians' Health Study³⁷ and The Women's Health Study,³⁸ showed no differences between the β -carotene and placebo groups. Thus, the current data concerning the association between carotenoids and lung cancer do not really demonstrate a possible protective role of carotenoids. Although observational studies show an inverse association, this trend is not confirmed by intervention trials.

Carotenoids and urino-digestive cancers — On the whole, findings from epidemiological studies did not demonstrate a protective role of carotenoids against colorectal, gastric, and bladder cancers. Indeed, most prospective and case-control studies of colorectal cancer showed no association with dietary intake or plasma level of most carotenoids.^{39–42} Only lycopene and lutein were shown to be protective against colorectal cancer.^{39,41} Otherwise, findings from the ATBC study⁴³ showed no effect of β -carotene supplementation on colorectal cancer.

Data concerning gastric cancer are scarce. The prospective Netherlands Cohort Study⁴⁴ found no correlation between lutein dietary intake and gastric cancer risk, whereas findings from the Physicians' Health Study³⁷ and the ATBC study⁴⁵ reported no effect of β -carotene on gastric cancer incidence. Two case-control studies^{46,47} and three intervention trials (ATBC,⁴⁸ CARET,³⁶ and the Physicians' Health Study³⁷) showed no association of β -carotene, lycopene, lutein, zeaxanthin, and β -cryptoxanthin.

Thus, with regard to findings from epidemiological studies, it is difficult to conclude a protective role of carotenoids in cancer. Although observational studies converge toward an inverse association between some carotenoids and certain forms of cancer, intervention studies do not follow this trend; most of them show the absence of effect and even positive correlations between carotenoids and cancers. Moreover, it is important to note that carotenoid doses used in intervention trials (20 to 50 mg/day) led to plasma concentrations higher than those found after fruit and vegetable consumption, so these results are not really representative of results from fruit and vegetable consumptions.

Carotenoids and cardiovascular diseases — Numerous epidemiological studies aimed to study the relationship of carotenoids and cardiovascular diseases (CVDs) including coronary accident risk and stroke.^{49,50} It appeared then that observational studies, namely prospective and case-control studies, pointed to a protective effect of carotenoids on myocardial infarct and stroke, but also on some atherosclerosis markers such as intima media thickness (IMT) of the common carotid artery (CCA) and atheromatous plaque formation.

Among 27 prospective and case-control studies, 16 reported inverse associations between some carotenoids and CVDs, taking plasma or serum concentration as carotenoid biomarkers (11 of 16 studies), dietary intake (5 of 16 studies), or adipose tissue level (1 of 16 studies). With regard to the findings from the studies based on CVD risk, only two^{51,52} of seven presented significant inverse associations of carotenoids, particularly lycopene and β -carotene, whereas five studies^{53,54,55,57} of nine showed inverse correlations between myocardial infarcts and lycopene and/or β -carotene; the others presented no associations.^{58–61}

Some prospective and case-control studies also investigated the relationship of carotenoids and the evolution of CCA-IMT. Although the EVA study showed no association between total carotenoids and IMT,⁶² others like the ARIC study,⁶³ the Los Angeles Atherosclerosis Study,⁶⁴ and the Kuopio Ischaemic Heart Disease Risk Factor Study⁶⁵ demonstrated the protective role of isolated carotenoids such as lycopene, lutein, zeaxanthin, and β -cryptoxanthin on IMT. Thus, findings from prospective and case-control studies have suggested that some carotenoids such as lycopene and β -carotene may present protective effects against CVD and particularly myocardial infarcts and intima media thickness, a marker of atherosclerosis.

However, intervention trials investigating the effects of β -carotene and lycopene supplementation on CVD have not reported convincing results (Table 3.1.3). Among the seven studies reviewed herein, four primary prevention trials, namely the Multicenter Skin Cancer Prevention Study,⁶⁶ the Beta Carotene and Retinol Efficacy Trial,³⁶ the ATBC cancer prevention study,⁴⁸ and the Physicians' Health Study³⁷ have shown no association between a supplementation of β -carotene and risk of death from CVD or fatal and non-fatal MI.

Recent findings from the ATBC study⁶⁷ even showed that β -carotene supplementation increased the post-trial risk of a first-ever non-fatal MI. Two secondary prevention trials, the Heart Protection Study and the ATBC presented similar results. The former showed no association between β -carotene and fatal or non-fatal vascular events and the latter reported significantly increased risks of fatal coronary events in the β -carotene-supplemented group. Results of clinical trials focused on the effects of carotenoids on CVD biomarkers are controversial. Although carotenoid supplementation increased serum levels,^{68–70} only lycopene was shown to be inversely associated with lipid, protein, DNA and LDL oxidation, and plasma cholesterol levels.^{68,69,71}

Epidemiological data on carotenoids and cerebral infarcts or strokes indicate a protective effect of β -carotene and lycopene. Indeed, the Basel prospective study,⁷² the Kuopio Ischaemic Heart Disease Risk Factor study,⁷³ and the Physicians' Health Study⁷⁴ have shown an inverse correlation between carotenoid plasma level and risk of stroke. In the same way, Hirvonen et al.⁷⁵ demonstrated, in findings from the ATBC cancer prevention study, an inverse association between β -carotene dietary intake and stroke. However, clinical data on carotenoids and stroke are nonexistent and they are needed to confirm this possible protective effect of carotenoids on stroke.

Although numerous epidemiological studies reported protective effects of β -carotene on CVD risks and biomarkers, clinical trials did not follow this trend. This divergence may depend on several factors. For example, in observational studies, β -carotene is essentially an indicator of a diet rich in fruits and vegetables that also contain other carotenoids and vitamins. Thus, the protective effects of β -carotene may be due to the consumption of β -carotene-rich foods rather than the ingestion of β -carotene alone. This compound may act in synergy and complementarity with other carotenoids, vitamins, or polyphenols. Moreover, it is also possible that carotenoids may prevent cellular damage at physiologic concentrations and that their ability to protect against cellular damage disappears at the higher doses used in the supplementation studies. Otherwise, it was shown by El-Agamey et al.⁷⁷ that high doses of carotenoids may have prooxidant effects.

Carotenoids and ocular diseases — Age-related ocular diseases such as cataracts and macular degeneration, the leading cause of irreversible blindness, are common problems in the elderly populations of western countries. These diseases are thought to result from damages caused photochemically and non-photochemically by oxidative stress to various cell types in the eye.⁷⁸ Thus, fruit and vegetable antioxidant nutrients such as carotenoids and vitamin E may influence this oxidative process through their ability to scavenge free radicals, as mentioned earlier and thereby reduce oxidative damage in lens tissues. Among carotenoid pigments found in humans, lutein and zeaxanthin are present only in macula, retina, and lens and are referred to as macular pigments or MPs.^{79–81} Their eye tissue concentration can reach 1 mmol/l, which is 500 times higher than concentrations in other tissues.⁸²

Comprehensive reviews published by Snodderly⁸³ and Beatty et al.⁸⁴ explore the evidence for a protective function by the macular pigment against age-related macular diseases and the mechanisms by which it might act. The antioxidant properties of lutein and zeaxanthin recently reviewed by Young and Lowe⁸⁵ may reduce the degree to which oxidative damage promotes these diseases. Otherwise, because these

carotenoids also absorb blue light, they may reduce photochemical damage that would otherwise occur in the retina when exposed to light at these wavelengths.⁸⁶

3.1.2.3 MECHANISMS OF ACTION

The protective effects of carotenoids against chronic diseases appear to be correlated to their antioxidant capacities.⁸⁵ Indeed, oxidative stress and reactive oxygen species (ROS) formation are at the basis of oxidative processes occurring in cardiovascular incidents, cancers, and ocular diseases. Carotenoids are then able to scavenge free radicals⁸¹ such as singlet molecular oxygen ($^1\text{O}_2$) and peroxyl radicals particularly, and protect cellular systems from oxidation.

Convincing evidence indicates that ROS generated both endogenously and also in response to diet and lifestyle factors may play a significant role in the etiology of atherosclerosis and CHD.^{87,88} Indeed, free radicals are responsible for LDL oxidation, which is involved in the initiation and promotion of atherosclerosis.⁸⁸ Thus, protection from LDL oxidation by antioxidants such as carotenoids may lead to protection against human CHD.

Lutein and zeaxanthin are mainly accumulated in the macula of the human retina and may be protective against age-related increases in lens density and cataract formation. Zeaxanthin is specifically concentrated in the macula, especially in the fovea. Lutein is distributed throughout the retina. Their protective effect in part is due to the ability to quench ROS species and filter out high-energy blue light.⁸⁹ These pigments protect underlying cell layers from potential light damage by filtering blue light. It has been shown that these xanthophylls are located in domains formed from unsaturated lipids. This suggests that they may act as antioxidants against lipid oxidation, a mechanism through which lutein and zeaxanthin protect the retina from age-related macular diseases.⁹⁰

The most potent antioxidant among various carotenoids is lycopene.^{91,92} In this regard, lycopene can trap singlet oxygen and reduce mutagenesis in the Ames test. Besides these actions, lycopene was shown to display numerous beneficial biological effects involving anti-inflammatory, anti-mutagenic, and anti-carcinogenic activities.^{93–95}

Lycopene at physiological concentrations may inhibit human cancer cell growth.⁹⁶ The mechanisms include inhibition of prostatic IGF-I signaling, IL-6 expression, and androgen signaling. Moreover, lycopene improves gap-junctional communication via the up-regulation of connexin 43 and induces phase II drug metabolizing enzymes as well as oxidative defense genes. Interestingly, a combination of low concentrations of lycopene with 1,25-dihydroxyvitamin D₃ exhibits a synergistic effect on cell proliferation and differentiation.⁹⁷ Other potential mechanisms such as stimulation of xenobiotic metabolism, inhibition of cholesterologenesis, modulation of cyclooxygenase pathways, and inhibition of inflammation will be considered.⁹⁸

3.1.3 HYDROPHILIC PIGMENTS

Betacyanins and anthocyanins are the major hydrophilic pigments in our diet, and most of the literature focusing on health essentially concerns anthocyanins — the largest group of water-soluble pigments in the plant kingdom. They belong to the

family of flavonoids (a subclass of the huge class of polyphenols), and are characterized by their ability to form flavylium cations. Anthocyanins are responsible for most of the red, blue, and purple colors of fruits, vegetables, and flowers.

Six anthocyanidins are commonly found in plants and plant-derived foods and beverages: pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin. Their structures differ in the number and position of hydroxyl and methoxyl groups on the flavan nucleus. The most commonly occurring anthocyanidin is cyanidin, and all these anthocyanidins are found in plants as glycosides with or without acylation, leading to around 400 different structures.

Besides anthocyanins, a common class of flavonoids occurring in plants and plant-derived food products is the flavonol group. The main aglycone structures are quercetin and kempferol, which occur in plants as glycosides. Flavonols and flavones (luteolin and apigenin) are light yellow water-soluble phenolic pigments, reported as excellent co-pigments conferring better stabilities to anthocyanins by stacking mechanisms.

3.1.3.1 ESTIMATION OF DAILY INTAKE OF POLYPHENOLS

The levels of intake of polyphenols vary considerably among diets, depending on the types and quantities of plant foods consumed. Dietary intake data for polyphenols are limited. The main reason is the lack of detailed composition tables. In fact, polyphenols represent a wide variety of diverse structures belonging to different subclasses (flavonoids, phenolic acids, lignans, proanthocyanidins, and others) and many phenolic compounds escape high performance liquid chromatography (HPLC) and ultraviolet (UV) quantification, because of a lack of commercially available standards. The United States Department of Agriculture (USDA) recently published a food database on flavonoids.⁹⁹ Such a database is extremely useful for epidemiological studies on the relationship of dietary flavonoids and health.

Concerning anthocyanin intake in the U.S., an estimation by Kühnau¹⁰⁰ reported 215 mg/day in summer and 180 mg/day in winter. These values were shown to be underestimated for wine consumers.¹⁰¹ However, a very recent estimation reported by Wu et al. corresponded to 12 mg/day.¹⁰² The estimation of flavonols ranged from 4 mg/day in Finland to 30 mg/day in Denmark. The mean flavonol intake was 21 mg/day in the U.S., 15 mg/day in Japan, and 17 mg/day in Germany.

3.1.3.2 EPIDEMIOLOGICAL STUDIES

Flavonoids and cardiovascular diseases — Data are not sufficient to confirm protective effects of flavonoids against degenerative diseases, because only fifteen prospective studies are reported in the literature and they took into account only three classes of flavonoids: catechins, flavones, and flavonols. Nevertheless, among the fifteen studies,¹⁰³ six displayed significant reductions of risk for cardiovascular disease, seven noted tendencies to risk reduction, and two noted insignificant increases. These results suggest a protective effect from high consumption of flavones, flavonols, and catechins against cardiovascular diseases. In addition, three recently published case-control studies^{104–106} confirmed the protective effect against vascular pathologies.

Flavonoids and cancers — Data concerning the role of flavonoids in cancer are not sufficient. A possible protective effect of flavonoids (catechins, flavones, and flavonols) was found for lung cancer. Among four prospective studies, only two converged toward an inverse association between flavonoid dietary intake and lung cancer risk. In addition, an inverse association has been found between flavonoid consumption and asthma or chronic bronchial obstruction.^{107,108} Experiments in mice fed quercetin for 11 weeks revealed that the lung appears as a target displaying the higher concentrations of quercetin.¹⁰⁹ This result may explain the possible protective effects of quercetin at lung level.

Flavonoids and neurodegenerative diseases — The literature on this subject remains extremely rare. We know that during normal aging, the brain undergoes morphological and functional modifications leading to declines of motor and cognitive performances. These declines are increased by neurodegenerative diseases including amyotrophic lateral sclerosis, Alzheimer's disease, and Parkinson's disease. It was recently reported that polyphenols provided by berries may reverse age-related declines in neuronal signal transduction as well as cognitive and motor deficits.¹¹⁰ This topic is an emerging and promising one.

3.1.3.3 MECHANISMS OF ACTION

Epidemiological studies and intervention trials with food and beverages rich in flavonoids are not conclusive although flavonoids were recognized to display numerous antioxidant, anti-inflammatory, anti-tumoral, and anti-microbial activities. The antioxidant capacity of flavonoids has been largely reported in numerous *in vitro* and *ex vivo* systems. Numerous reviews^{111–113} have been published on the antioxidant properties of flavonoids. Degenerative diseases are largely associated with oxidative mechanisms that may be counteracted by flavonoids.

Under normal physiological conditions, cells possess endogenous protection mechanisms against ROS: enzymatic mechanisms such as catalase, the superoxide dismutase/glutathione peroxidase system, and glutathione S-transferase; and non-enzymatic ones such as glutathione. When defense mechanisms decrease, ROS increase and biomolecules (lipids, proteins, nucleic acids, etc.) in cells may be oxidized. These oxidations are responsible for cell aging¹¹⁴ and are involved in carcinogenesis^{115,116} and in neurodegenerative diseases such as Alzheimer's and Parkinson's diseases.^{117,118}

Oxidative lesions of DNA constitute the initiation step of carcinogenesis. Flavonoids are strong antioxidants that prevent DNA damage. Numerous experimental studies have noted the beneficial actions of flavonoids on multiple cancer-related biological pathways: carcinogen bioactivation, cell signaling, cell cycle regulation, angiogenesis, oxidative stress, and inflammation.¹¹⁹ Recently it has been reported that apigenin may inhibit human lung cancer angiogenesis by inhibiting hypoxia-inducible factor-1- α and vascular endothelial growth factor expression.¹²⁰

LDL when oxidized is recognized to play a crucial role in the development of atherosclerosis. It was thought that flavonoids could also protect LDL against oxidation, especially by limiting the degradation of vitamin E, the main antioxidant in LDL. Other beneficial effects of flavonoids have been reported: inhibition of platelet

aggregation and adhesion, induction of endothelium-dependent vasodilation, and inhibition of enzymes involved in lipid metabolism.¹²¹ Nevertheless, it has been shown that flavonoids are poorly absorbed and extensively metabolized into conjugates of glucuronate and sulfate with or without methylation of the catechol group. Thus, the antiradical effect is improbable because of the low plasmatic concentrations of flavonoids and the structural differences of circulating metabolites compared with the parent molecule.

It is possible that dietary flavonoids participate in the regulation of cellular function independent of their antioxidant properties. Other non-antioxidant direct effects reported include inhibition of prooxidant enzymes (xanthine oxidase, NAD(P)H oxidase, lipoxygenases), induction of antioxidant enzymes (superoxide dismutase, glutathione peroxidase, glutathione S-transferase), and inhibition of redox-sensitive transcription factors.¹²²

3.1.4 CURCUMIN

Curcumin (diferuloyl methane) is the main pigment of turmeric. It is widely used as a colorant and preservative agent. No data regarding its daily intake in western countries are available; intake may reach 80 to 200 mg in adult Indians.¹²³ To date, no study has explored the effect of curcumin consumption on the incidence of diseases, but many beneficial effects on health have been reported in cell and animal models. These include anti-carcinogenic, anti-diabetic, anti-atherosclerotic, and anti-Alzheimer's disease properties.¹²⁴

The most convincing data have been obtained from rodent models of cancers that revealed protective effects of curcumin against cancers of all sites.¹²⁵ Curcumin is able to inhibit all steps of cancer processes, initiation, progression, and promotion. These observations, combined with the apparent lack of toxicity of curcumin for doses up to 8 g/day for 3 mo¹²⁶ suggest potential uses of curcumin as an anti-carcinogenic chemoprotective agent.¹²⁷

Curcumin possesses strong antioxidant capacities, which may explain its effects against degenerative diseases in which oxidative stress plays a major role. As previously described for flavonoids, it is unlikely that curcumin acts as a direct antioxidant outside the digestive tract since its concentration in peripheral blood and organs is very low (near or below 1 μ M, even after acute or long-term supplementation). Indeed, it has been shown that the intestinal epithelium limits its entry into the body, as reflected by absorption studies in various models (portal blood perfusion, everted bags).^{128,129}

A recent study¹³⁰ indicates that curcumin vectorization significantly improves its bioavailability in rats. Glucuronide and/or sulfate conjugates of curcumin have been identified in the plasma of humans and rodents. In addition, reduced forms of curcumin (tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol) in free and conjugated forms have also been characterized.^{131–134} Indeed, it is thought that most of curcumin's anti-carcinogenic properties come from its ability to modulate transcription factors involved in detoxification and antioxidant responses. Curcumin was shown to be able to activate nrf2 and to inhibit NF- κ B, leading to an increase in cellular stress defenses (heme oxygenase-1, phase 2 enzymes) and a decrease in pro-inflammatory phenotype (by diminishing COX2 expression for

example), respectively.^{135–137} Curcumin can also trigger apoptosis or block a variety of transformed cell lines in the G2/M phase of the cell cycle, and abolish invasiveness via the decrease of MMP-9.¹³⁸

The molecular mechanisms underlying these effects have been extensively reviewed.^{139,140} Moreover, it has been proposed that curcumin may be employed as a sensitizing agent against tumors by decreasing multidrug-resistant protein (Pgp, ABCG2, ABCC1) activities.^{141,142} These properties combined with the capacity of inhibiting the intestinal CYP3A,¹⁴³ which is responsible for the metabolism of most drugs, raise the possibility that concomitant intake of curcumin and drugs or xenobiotics may increase their intestinal absorption and therefore their therapeutic or toxic effects.

Some metabolites of curcumin (particularly tetrahydrocurcumin) may also participate in producing the observed effects of curcumin in different models because these metabolites display greater stabilities than the parent curcumin molecule at physiological pH.¹³¹ Recent data show similar modes of action of curcumin metabolites regarding antioxidant enzyme induction and inhibition of multidrug-resistant proteins.^{144,145} Additional data indicate that curcumin may even act against other types of diseases such as atherosclerosis^{146,147} and Alzheimer's disease.^{148,149}

3.1.5 CONCLUSION

Epidemiologic studies support the principle that a diet rich in fruits and vegetables can decrease the risks of degenerative diseases. The main plant pigments, carotenoids, flavonoids, and also curcumin, appear to be protective. They display antioxidant properties. However, numerous studies related to their bioavailability and their effects at cellular or tissue levels suggest that their beneficial effects may be brought about by other mechanisms. To clearly establish the biological activities of plant pigments, numerous difficulties arise in studies. The first one relates to the high concentrations of the plant pigments delivered to the target tissues or cells in most intervention studies. Such high concentrations do not reflect the normally circulating concentrations that are particularly low for flavonoids and curcumin (< 1 μ M), and extremely low for anthocyanins (5 to 40 nM).

The second major difficulty is that cells and tissues in the body are exposed to numerous metabolites displaying different structures compared to the parent molecules present in plant foods. For example, it has been suggested that the metabolites of lycopene may be responsible for reducing the risk of developing prostate cancer. These metabolites may interact with nuclear receptors such as PPARs, LXR, and others.¹⁵⁰ Future research is needed to produce metabolites (enzymatically or chemically) in order to elucidate their cellular mechanisms and thus clarify their effects on human health.

REFERENCES

1. CNERNA, *Alimentation et cancer. Evaluation des données scientifiques*, Riboli et al Eds., Tec Doc, Lavoisier, Paris, 1996.

2. World Cancer Research Fund and American Institute for Cancer Research, *Food, Nutrition and the Prevention of Cancer: A Global Perspective*, Banta Book Group, Menasha, WI, 1997.
3. Committee on Medical Aspects of Food and Nutrition Policy, Nutritional aspects of the development of cancer, report of the Working Group on Diet and Cancer, Her Majesty's Stationery Office, London, 1998.
4. Ness, A.R. and Powles, J.W., Fruit and vegetables, and cardiovascular disease: a review, *Int. J. Epidemiol.*, 26, 1, 1997.
5. Law, M.R. and Morris, J.K., By how much does fruit and vegetable consumption reduce the risk of ischaemic heart disease: response to commentary, *Eur. J. Clin. Nutr.*, 53, 903, 1999.
6. Panagiotakos, D.B. et al., Consumption of fruits and vegetables in relation to the risk of developing acute coronary syndromes; the CARDIO2000 case-control study, *Nutr. J.*, 2, 2, 2003.
7. Dauchet, L. et al., Fruit and vegetable consumption and risk of stroke: a meta-analysis of cohort studies, *Neurology*, 65, 1193, 2005.
8. Dauchet, L. et al., Fruit and vegetable consumption and risk of coronary heart disease: a meta-analysis of cohort studies, *J. Nutr.*, 136, 2588, 2006.
9. Youdim, K.A. and Joseph, J.A., A possible emerging role of phytochemicals in improving age-related neurological dysfunctions: a multiplicity of effects, *Free Radic. Biol. Med.*, 30, 583, 2001.
10. Al-Delaimy, W.K. et al., Plasma levels of six carotenoids in nine European countries: report from the European Prospective Investigation into Cancer and Nutrition (EPIC), *Public Health Nutr.*, 7, 713, 2004.
11. Ritenbaugh, C. et al., New carotenoid values for foods improve relationship of food frequency questionnaire intake estimates to plasma values. *Cancer Epidemiol. Biomarkers Prev.*, 5, 907, 1996.
12. O'Neill, M.E. et al., A European carotenoid database to assess carotenoid intakes and its use in a five-country comparative study, *Br. J. Nutr.*, 85, 499, 2001.
13. van Poppel, G. and Goldbohm, R.A., Epidemiologic evidence for beta-carotene and cancer prevention, *Am. J. Clin. Nutr.*, 62, 1393S, 1995.
14. Hercberg, S., The history of beta-carotene and cancers from observational to intervention studies: what lessons can be drawn for future research on polyphenols? *Am. J. Clin. Nutr.*, 81, 218S, 2005.
15. Hercberg, S. et al., The SU.VI.MAX Study: a randomized, placebo-controlled trial of the health effects of antioxidant vitamins and minerals, *Arch. Intern. Med.*, 164, 2335, 2004.
16. Giovannucci, E., Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature, *J. Natl. Cancer Inst.*, 91, 317, 1999.
17. Giovannucci, E. et al., A prospective study of tomato products, lycopene, and prostate cancer risk, *J. Natl. Cancer Inst.*, 94, 391, 2002.
18. Campbell, J.K. et al., Tomato phytochemicals and prostate cancer risk, *J. Nutr.*, 134, 3486S, 2004.
19. Etminan, M. et al., The role of tomato products and lycopene in the prevention of prostate cancer: a meta-analysis of observational studies, *Cancer Epidemiol. Biomarkers Prev.*, 13, 340, 2004.
20. Gann, P.H. et al., Lower prostate cancer risk in men with elevated plasma lycopene levels: results of a prospective analysis, *Cancer Res.*, 59, 1225, 1999.
21. Giovannucci, E. et al., A prospective study of tomato products, lycopene, and prostate cancer risk, *J. Natl. Cancer Inst.*, 94, 391, 2002.

22. Jian, L. et al., Do dietary lycopene and other carotenoids protect against prostate cancer? *Int. J. Cancer*, 113, 1010, 2005.
23. Chen, L. et al., Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention, *J. Natl. Cancer Inst.*, 93, 1872, 2001.
24. Kucuk, O. et al., Effects of lycopene supplementation in patients with localized prostate cancer, *Exp. Biol. Med. (Maywood)*, 227, 881, 2002.
25. van Breemen, R.B. et al., Liquid chromatography–mass spectrometry of cis- and all-trans-lycopene in human serum and prostate tissue after dietary supplementation with tomato sauce, *J. Agric. Food Chem.*, 50, 2214, 2002.
26. Potischman, N. et al., Breast cancer and dietary and plasma concentrations of carotenoids and vitamin A, *Am. J. Clin. Nutr.*, 52, 909, 1990.
27. Freudenheim, J.L. et al., Premenopausal breast cancer risk and intake of vegetables, fruits, and related nutrients, *J. Natl. Cancer Inst.*, 88, 340, 1996.
28. Dorgan, J.F. et al., Relationships of serum carotenoids, retinol, alpha-tocopherol, and selenium with breast cancer risk: results from a prospective study in Columbia, Missouri (United States), *Cancer Causes Control*, 9, 89, 1998.
29. Zhang, S. et al., Dietary carotenoids and vitamins A, C, and E and risk of breast cancer, *J. Natl. Cancer Inst.*, 91, 547, 1999.
30. Toniolo, P. et al., Serum carotenoids and breast cancer, *Am. J. Epidemiol.*, 153, 1142, 2001.
31. Terry, P. et al., Dietary carotenoids and risk of breast cancer, *Am. J. Clin. Nutr.*, 76, 883, 2002.
32. Zaroukian, S. et al., Correlation between nutritional biomarkers and breast cancer: a case-control study, *Breast*, 14, 209, 2005.
33. Holick, C.N. et al., Dietary carotenoids, serum beta-carotene, and retinol and risk of lung cancer in the alpha-tocopherol, beta-carotene cohort study, *Am. J. Epidemiol.*, 156, 536, 2002.
34. Ratnasinghe, D. et al., Serum carotenoids are associated with increased lung cancer risk among alcohol drinkers, but not among non-drinkers in a cohort of tin miners, *Alcohol*, 35, 355, 2000.
35. Rautalahti, M. et al., Beta-carotene did not work: aftermath of the ATBC study, *Cancer Lett.*, 114, 235, 1997.
36. Omenn, G.S. et al., Risk factors for lung cancer and for intervention effects in CARET, the Beta-Carotene and Retinol Efficacy Trial, *J. Natl. Cancer Inst.*, 88, 1550, 1996.
37. Hennekens, C.H. et al., Lack of effect of long-term supplementation with beta carotene on the incidence of malignant neoplasms and cardiovascular disease, *New Engl. J. Med.*, 334, 1145, 1996.
38. Lee, B.W. et al., Association between diet and lung cancer location, *Am. J. Respir. Crit. Care Med.*, 158, 1197, 1998.
39. Slattery, M.L. et al., Carotenoids and colon cancer, *Am. J. Clin. Nutr.*, 71, 575, 2000.
40. Terry, P. et al., Dietary carotenoid intake and colorectal cancer risk, *Nutr. Cancer*, 42, 167, 2002.
41. Erhardt, J.G. et al., Lycopene, beta-carotene, and colorectal adenomas, *Am. J. Clin. Nutr.*, 78, 1219, 2003.
42. Nkondjock, A. and Ghadirian, P., Dietary carotenoids and risk of colon cancer: case-control study, *Int. J. Cancer*, 110, 110, 2004.
43. Albanes, D. et al., Effects of supplemental alpha-tocopherol and beta-carotene on colorectal cancer: results from a controlled trial (Finland), *Cancer Causes Control*, 11, 197, 2000.

44. Botterweck, A.A. et al., Vitamins, carotenoids, dietary fiber, and the risk of gastric carcinoma: results from a prospective study after 6.3 years of follow-up, *Cancer*, 88, 737, 2000.
45. Malila, N. et al., Dietary and serum alpha-tocopherol, beta-carotene and retinol, and risk for colorectal cancer in male smokers, *Eur. J. Clin. Nutr.*, 56, 615, 2002.
46. Garcia, R. et al., High intake of specific carotenoids and flavonoids does not reduce the risk of bladder cancer, *Nutr. Cancer*, 35, 212, 1999.
47. Zeegers, M.P. et al., Are retinol, vitamin C, vitamin E, folate and carotenoids intake associated with bladder cancer risk? Results from the Netherlands Cohort Study, *Br. J. Cancer*, 85, 977, 2001.
48. Virtamo, J. et al., Effects of supplemental alpha-tocopherol and beta-carotene on urinary tract cancer: incidence and mortality in a controlled trial (Finland), *Cancer Causes Control*, 11, 933, 2000.
49. Tavani, A. et al., Risk factors for breast cancer in women under 40 years, *Eur. J. Cancer*, 35, 1361, 1999.
50. Rao, A.V., Lycopene, tomatoes, and the prevention of coronary heart disease, *Exp. Biol. Med. (Maywood)*, 227, 908, 2002.
51. Kristenson, M. et al., Antioxidant state and mortality from coronary heart disease in Lithuanian and Swedish men: concomitant cross-sectional study of men aged 50, *Br. Med. J.*, 314, 629, 1997.
52. Osganian, S.K. et al., Dietary carotenoids and risk of coronary artery disease in women, *Am. J. Clin. Nutr.*, 77, 1390, 2003.
53. Street, D.A. et al., Serum antioxidants and myocardial infarction: are low levels of carotenoids and alpha-tocopherol risk factors for myocardial infarction? *Circulation*, 90, 1154, 1994.
54. Kohlmeier, L. et al., Lycopene and myocardial infarction risk in the EURAMIC Study, *Am. J. Epidemiol.*, 146, 618, 1997.
55. Tavani, A. et al., Beta-carotene intake and risk of nonfatal acute myocardial infarction in women, *Eur. J. Epidemiol.*, 13, 631, 1997.
56. Klipstein-Grobusch, K. et al., Dietary antioxidants and risk of myocardial infarction in the elderly: the Rotterdam Study, *Am. J. Clin. Nutr.*, 69, 261, 1999.
57. Osganian, S.K. et al., Dietary carotenoids and risk of coronary artery disease in women, *Am. J. Clin. Nutr.*, 77, 1390, 2003.
58. Gaziano, J.M. et al., A prospective study of consumption of carotenoids in fruits and vegetables and decreased cardiovascular mortality in the elderly, *Ann. Epidemiol.*, 5, 255, 1995.
59. Kardinaal, A.F. et al., Association between beta-carotene and acute myocardial infarction depends on polyunsaturated fatty acid status: the EURAMIC study (European Study on Antioxidants, Myocardial Infarction, and Cancer of the Breast), *Arterioscler. Thromb. Vasc. Biol.*, 15, 726, 1995.
60. Hak, A.E. et al., Plasma carotenoids and tocopherols and risk of myocardial infarction in a low-risk population of U.S. male physicians, *Circulation*, 108, 802, 2003.
61. Sesso, H.D. et al., Dietary lycopene, tomato-based food products and cardiovascular disease in women, *J. Nutr.*, 133, 2336, 2003.
62. Bonithon-Kopp, C. et al., Combined effects of lipid peroxidation and antioxidant status on carotid atherosclerosis in a population aged 59–71 years: The EVA (Etude sur le Vieillissement Arteriel) study, *Am. J. Clin. Nutr.*, 65, 121, 1997.
63. Iribarren, C. et al., Association of serum vitamin levels, LDL susceptibility to oxidation, and autoantibodies against MDA-LDL with carotid atherosclerosis: a case-control study, *Arterioscler. Thromb. Vasc. Biol.*, 17, 1171, 1997.

64. Dwyer, J.H. et al., Oxygenated carotenoid lutein and progression of early atherosclerosis: the Los Angeles atherosclerosis study, *Circulation*, 103, 292, 2001.
65. Rissanen, T.H. et al., Serum lycopene concentrations and carotid atherosclerosis: the Kuopio Ischaemic Heart Disease Risk Factor Study, *Am. J. Clin. Nutr.*, 77, 133, 2003.
66. Greenberg, E.R. et al., Mortality associated with low plasma concentration of beta carotene and the effect of oral supplementation, *JAMA*, 275, 699, 1996.
67. Tornwall, M.E. et al., Effect of alpha-tocopherol and beta-carotene supplementation on coronary heart disease during the 6-year post-trial follow-up in the ATBC study, *Eur. Heart J.*, 25, 1171, 2004.
68. Agarwal, S. and Rao, A.V., Tomato lycopene and low density lipoprotein oxidation: a human dietary intervention study, *Lipids*, 33, 981, 1998.
69. Rao, A.V. and Agarwal, S., Bioavailability and *in vivo* antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer, *Nutr. Cancer*, 3, 199, 1998.
70. Olmedilla, B. et al., Serum status of carotenoids and tocopherols in patients with age-related cataracts: a case-control study, *J. Nutr. Health Aging*, 6, 66, 2002.
71. Fuhrman, B. et al., Hypocholesterolemic effect of lycopene and beta-carotene is related to suppression of cholesterol synthesis and augmentation of LDL receptor activity in macrophages, *Biochem. Biophys. Res. Commun.*, 233, 658, 1997.
72. Gey, K.F. et al., Poor plasma status of carotene and vitamin C is associated with higher mortality from ischemic heart disease and stroke: Basel Prospective Study, *J. Clin. Invest.*, 71, 3, 1993.
73. Rissanen, T.H. et al., Low serum lycopene concentration is associated with an excess incidence of acute coronary events and stroke: the Kuopio Ischaemic Heart Disease Risk Factor Study, *Br. J. Nutr.*, 85, 749, 2001.
74. Hak, A.E. et al., Prospective study of plasma carotenoids and tocopherols in relation to risk of ischemic stroke, *Stroke*, 35, 1584, 2004.
75. Hirvonen, T. et al., Intake of flavonoids, carotenoids, vitamins C and E, and risk of stroke in male smokers, *Stroke*, 31, 2301, 2000.
76. Heart Protection Study Collaborative Group, MRC/BHF Heart Protection Study of antioxidant vitamin supplementation in 20,536 high-risk individuals: a randomised placebo-controlled trial, *Lancet*, 360, 23, 2002.
77. El-Agamey, A., Carotenoid radical chemistry and antioxidant/pro-oxidant properties, *Arch. Biochem. Biophys.*, 430, 37, 2004.
78. Cruickshanks, K.J. et al., Sunlight and the 5-year incidence of early age-related maculopathy: the Beaver Dam eye study, *Arch. Ophthalmol.*, 119, 246, 2001.
79. Handelman, G.J. et al., Carotenoids in the human macula and whole retina, *Invest. Ophthalmol. Vis. Sci.*, 29, 850, 1988.
80. Johnson, E.J. et al., Relation among serum and tissue concentrations of lutein and zeaxanthin and macular pigment density, *Am. J. Clin. Nutr.*, 71, 1555, 2000.
81. Krinsky, N.I. et al., Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye, *Annu. Rev. Nutr.*, 23, 171, 2003.
82. Schmitz, H.H. et al., Analysis of carotenoids in human and animal tissues, *Meth. Enzymol.*, 214, 102, 1993.
83. Snodderly, D.M., Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins, *Am. J. Clin. Nutr.*, 62, 1448S, 1995.
84. Beatty, S. et al., The role of oxidative stress in the pathogenesis of age-related macular degeneration, *Surv. Ophthalmol.*, 45, 115, 2000.
85. Young, A.J. and Lowe, G.M., Antioxidant and prooxidant properties of carotenoids, *Arch. Biochem. Biophys.*, 385, 20, 2001.

86. Landrum, J.T. et al., The macular pigment: a possible role in protection from age-related macular degeneration, *Adv. Pharmacol.*, 38, 537, 1997.
87. Halliwell, B., Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? *Lancet*, 344, 721, 1994.
88. Witztum, J.L., The role of oxidized LDL in the atherogenic process, *J. Atheroscler. Thromb.*, 1, 71, 1994.
89. Whitehead, A.J. et al., Macular pigment: a review of current knowledge, *Arch. Ophthalmol.*, 124, 1038, 2006.
90. Wisniewska, A. and Subczynski, W.K., Accumulation of macular xanthophylls in unsaturated membrane domains, *Free Radic. Biol. Med.*, 40, 1820, 2006.
91. Kris-Etherton, P.M. et al., Bioactive compounds in foods: their role in the prevention of cardiovascular disease and cancer, *Am. J. Med.*, 113, 71, 2002.
92. Bhuvaneswari, V. and Nagini, S., Lycopene: a review of its potential as an anticancer agent, *Curr. Med. Chem. Anticancer Agents*, 5, 627, 2005.
93. Karas, M. et al., Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells, *Nutr. Cancer*, 36, 101, 2000.
94. Agarwal, S. and Rao, A.V., Tomato lycopene and its role in human health and chronic diseases, *CMAJ*, 163, 739, 2000.
95. Heber, D. and Lu, Q.Y., Overview of mechanisms of action of lycopene, *Exp. Biol. Med. (Maywood)*, 227, 920, 2002.
96. Wertz, K. et al., Lycopene: modes of action to promote prostate health, *Arch. Biochem. Biophys.*, 430, 127, 2004.
97. Amir, H. et al., Lycopene and 1,25-dihydroxyvitamin D3 cooperate in the inhibition of cell cycle progression and induction of differentiation in HL-60 leukemic cells, *Nutr. Cancer*, 33, 105, 1999.
98. Elliot, R., Mechanisms of genomic and non-genomic actions of carotenoids, *Biochim. Biophys. Acta Molecular Basis Dis.*, 1740, 147, 2005.
99. USDA Food Composition Data (database on the Internet), Nutrient Data Laboratory, Beltsville, MD, Release 19, <http://www.ars.usda.gov/Services/docs.htm?docid=5121>, 2006.
100. Kuhnau, J., The flavonoids: a class of semi-essential food components: their role in human nutrition, *World Rev. Nutr. Diet*, 24, 117, 1976.
101. Clifford, M.N., Anthocyanins: nature, occurrence, and dietary burden, *J. Sci. Food Agric.*, 80, 1063, 2000.
102. Wu, X. et al., Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption, *J. Agric. Food Chem.*, 54, 4069, 2006.
103. Arts, I.C. and Hollman, P.C., Polyphenols and disease risk in epidemiologic studies, *Am. J. Clin. Nutr.*, 81, 317S, 2005.
104. Lagiou, P. et al., Intake of specific flavonoid classes and coronary heart disease†: a case-control study in Greece, *Eur. J. Clin. Nutr.*, 58, 1643, 2004.
105. Lagiou, P. et al., Flavonoid classes and risk of peripheral arterial occlusive disease: a case-control study in Greece, *Eur. J. Clin. Nutr.*, 60, 214, 2006.
106. Tavani, A. et al., Intake of specific flavonoids and risk of acute myocardial infarction in Italy, *Public Health Nutr.*, 9, 369, 2006.
107. Tabak, C. et al., Chronic obstructive pulmonary disease and intake of catechins, flavonols, and flavones: the MORGEN Study, *Am. J. Respir. Crit. Care Med.*, 164, 61, 2001.
108. Garcia, V. et al., Dietary intake of flavonoids and asthma in adults, *Eur. Respir. J.*, 26, 449, 2005.

109. de Boer, V.C. et al., Tissue distribution of quercetin in rats and pigs, *J. Nutr.*, 135, 1718, 2005.
110. Andres-Lacueva, C. et al., Anthocyanins in aged blueberry-fed rats are found centrally and may enhance memory, *Nutr. Neurosci.*, 8, 111, 2005.
111. Rice-Evans, C.A. et al., Structure–antioxidant activity relationships of flavonoids and phenolic acids, *Free Radic. Biol. Med.*, 20, 933, 1996.
112. Lairon, D. and Amiot, M.J., Flavonoids in food and natural antioxidants in wine, *Curr. Opin. Lipidol.*, 10, 23, 1999.
113. Heim, K.E. et al., Flavonoid antioxidants: chemistry, metabolism and structure–activity relationships, *J. Nutr. Biochem.*, 13, 572, 2002.
114. Beckman, K.B. and Ames, B.N., The free radical theory of aging matures, *Physiol. Rev.*, 78, 547, 1998.
115. Ames, B.N. et al., Oxidants, antioxidants, and the degenerative diseases of aging, *Proc. Natl. Acad. Sci. USA*, 90, 7915, 1993.
116. Galan, P. et al., Antioxydants et prévention, *Cah. Nutr. Diét.*, 32, 359, 1997.
117. Morel, Y. and Barouki, R., Influence du stress oxydant sur la régulation des gènes, *Med. Sci.*, 14, 713, 1998.
118. Bonnet, A.M. and Houeto, J.L., Pathophysiology of Parkinson's disease, *Biomed. Pharmacother.*, 53, 117, 1999.
119. Le Marchand, L. et al., Intake of flavonoids and lung cancer, *J. Natl. Cancer Inst.*, 92, 154, 2000.
120. Liu, L.Z. et al., Apigenin inhibits expression of vascular endothelial growth factor and angiogenesis in human lung cancer cells: implication of chemoprevention of lung cancer, *Mol. Pharmacol.*, 68, 635, 2005.
121. Reed, J., Cranberry flavonoids, atherosclerosis and cardiovascular health, *Crit. Rev. Food Sci. Nutr.*, 42, 301, 2002.
122. Manach, C. et al., Polyphenols and prevention of cardiovascular diseases, *Curr. Opin. Lipidol.*, 16, 77, 2005.
123. Grant, K.L. and Schneider, C.D., Turmeric, *Am. J. Health Syst. Pharm.*, 57, 1121, 2000.
124. Bengmark, S., Curcumin, an atoxic antioxidant and natural NF-kappaB, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: a shield against acute and chronic diseases, *J. Parenteral Enteral Nutr.*, 30, 45, 2006.
125. Sharma, R.A. et al., Curcumin: the story so far, *Eur. J. Cancer*, 41, 1955, 2005.
126. Cheng, A.L. et al., Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions, *Anticancer Res.*, 21, 2895, 2001.
127. Sarkar, F.H. and Li, Y., Using chemopreventive agents to enhance the efficacy of cancer therapy, *Cancer Res.*, 66, 3347, 2006.
128. Wahlstrom, B. and Blennow, G., A study on the fate of curcumin in the rat, *Acta Pharmacol. Toxicol. (Copenh)*, 43, 86, 1978.
129. Ravindranath, V. and Chandrasekhara, N., Absorption and tissue distribution of curcumin in rats, *Toxicology*, 16, 259, 1980.
130. Marczyklo, T.H. et al., Comparison of systemic availability of curcumin with that of curcumin formulated with phosphatidylcholine, *Cancer Chemother. Pharmacol.*, 60, 171, 2007.
131. Pan, M.H. et al., Biotransformation of curcumin through reduction and glucuronidation in mice, *Drug Metab. Dispos.*, 27, 486, 1999.
132. Asai, A. and Miyazawa, T., Occurrence of administered curcuminoid as glucuronide and glucuronide/sulfate conjugates in rat plasma, *Life Sci.*, 67, 2785, 2000.

133. Ireson, C. R. et al., Metabolism of the cancer chemopreventive agent curcumin in human and rat intestine, *Cancer Epidemiol. Biomarkers Prev.*, 11, 105, 2002.
134. Garcea, G. et al., Detection of curcumin and its metabolites in hepatic tissue and portal blood of patients following oral administration, *Br. J. Cancer*, 90, 1011, 2004.
135. Jeong, G.S. et al., Comparative effects of curcuminoids on endothelial heme oxygenase-1 expression: ortho-methoxy groups are essential to enhance heme oxygenase activity and protection, *Exp. Molec. Med.*, 38, 393, 2006.
136. Ireson, C. et al., Characterization of metabolites of the chemopreventive agent curcumin in human and rat hepatocytes and in the rat *in vivo*, and evaluation of their ability to inhibit phorbol ester-induced prostaglandin E2 production, *Cancer Res.*, 61, 1058, 2001.
137. Sharma, R.A. et al., Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance, *Clin. Cancer Res.*, 10, 6847, 2004.
138. Parodi, F.E. et al., Oral administration of diferuloylmethane (curcumin) suppresses proinflammatory cytokines and destructive connective tissue remodeling in experimental abdominal aortic aneurysms, *Ann. Vasc. Surg.*, 20, 360, 2006.
139. Leu, T.H. and Maa, M.C., The molecular mechanisms for the antitumorigenic effect of curcumin, *Curr. Med. Chem. Anticancer Agents*, 2, 357, 2002.
140. Thangapazham, R.L. et al., Multiple molecular targets in cancer chemoprevention by curcumin, *AAPS J.*, 8, 443, 2006.
141. Chearwae, W. et al., Biochemical mechanism of modulation of human P-glycoprotein (ABCB1) by curcumin I, II, and III purified from turmeric powder, *Biochem. Pharmacol.*, 68, 2043, 2004.
142. Chearwae, W. et al., Modulation of the function of the multidrug resistance-linked ATP-binding transporter ABCG2 by the cancer chemopreventive agent curcumin, *Mol. Cancer Ther.*, 5, 1995, 2006.
143. Zhang, W. et al., Impact of curcumin-induced changes in P-glycoprotein and CYP3A expression on the pharmacokinetics of peroral celirolol and midazolam in rats, *Drug Metab. Dispos.*, 35, 110, 2007.
144. Murugan, P. and Pari, L., Antioxidant effect of tetrahydroxycurcumin streptozotocin-nicotinamide induced diabetic rats, *Life Sci.*, 79, 1720, 2006.
145. Limtrakul, P. et al., Modulation of the function of three ABC drug transporters, P-glycoprotein (ABCB1), mitoxantrone resistance protein (ABCG2) and multidrug resistance protein 1 (ABCC1) by tetrahydrocurcumin, a major metabolite of curcumin, *Mol. Cell Biochem.*, 296, 85, 2007.
146. Arafa, H.M.M., Curcumin attenuates diet-induced hypercholesterolemia in rats, *Med. Sci. Monit.*, 11, 228, 2005.
147. Peschel, D. et al., Curcumin induces changes in expression of genes involved in cholesterol homeostasis, *J. Nutr. Biochem.*, 18, 113, 2007.
148. Yang, F. et al., Curcumin inhibits formation on amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid *in vivo*, *J. Biol. Chem.*, 280, 5892, 2005.
149. Ng, T.P. et al., Curry consumption and cognitive function in the elderly, *Am. J. Epidemiol.*, 164, 898, 2006.
150. Lindshield, B.L. et al., Lycopeneoids: are lycopene metabolites bioactive?, *Arch. Biochem. Biophys.*, 15, 458, 2007.

3.2 Bioavailability of Natural Pigments

Alexandrine During

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3.2.1 INTRODUCTION

Bioavailability is commonly defined as the fraction of the ingested pigment that is absorbed and is available in the bloodstream for its utilization in normal physiological function or for storage.¹ The term bioavailability thus covers several *in vivo* processes: (1) release of the pigment from the food matrix and its solubilization in the gut lumen (bioaccessibility), (2) uptake of the pigment by the intestinal cell followed by its secretion into the blood circulation (absorption), and (3) circulation of the pigment in the bloodstream and its delivery to target tissues where it is stored and/or utilized for its biological activities. During these different processes, the pigment can be metabolized or broken down into metabolites or products of degradation that may have specific actions in the body and thus should be considered when studying pigment bioavailability.

A close relationship exists between physicochemical properties of pigment molecules and their ability to be absorbed and thus to exhibit biological functions. Carotenoids are hydrophobic molecules that require a lipophilic environment. *In vivo*, they are found in precise locations and orientations within biological membranes. For example, the dihydroxycarotenoids such as lutein and zeaxanthin orient themselves perpendicularly to the membrane surface as “molecular rivets” in order to expose their hydroxyl groups to a more polar environment.

In contrast, the carotenes such as β -carotene and lycopene may position themselves parallel to the membrane surfaces to remain in a more lipophilic environment in the inner cores of the bilayer membranes.² To move through an aqueous environment, carotenoids can be incorporated into lipid particles such as mixed micelles in the gut lumen or lipoproteins in the blood circulation and they can also form complexes with proteins with unspecific or specific bindings.

Specific carotenoid–protein complexes have been reported in plants and invertebrates (cyanobacteria, crustaceans, silkworms, etc.), while data on the existence of carotenoproteins in vertebrates are more limited.³ As alternatives for their water solubilization, carotenoids could use small cytosolic carrier vesicles.⁴ Carotenoids can also be present in very fine physical dispersions (or crystalline aggregates) in aqueous media of oranges, tomatoes, and carrots.⁵ Thus these physicochemical characteristics of carotenoids as well as those of other pigments are important issues for the understanding of their bioavailability.

3.2.2 APPROACHES FOR ASSESSING PIGMENT BIOAVAILABILITY

3.2.2.1 INTRODUCTION

In this module, an emphasis is placed on the different methods that have been used for assessing the bioavailability of food pigments such as carotenoids. Different *in vivo* and *in vitro* approaches can be used to estimate pigment bioavailability from foods in humans.

3.2.2.2 *IN VIVO* APPROACHES

3.2.2.2.1 Balance Methods

In the traditional balance method, the apparent absorption of a carotenoid or any other pigment is determined by the difference between its intake (input) and its excretion (output).⁶ This technique requires careful measurements of all input and output of the compound of interest. The approach has the advantage of being non-invasive and thus able to be used directly on human subjects.

Practically, this technique gives only a rough estimate of the amount or percent of the compound absorbed because (1) it does not account for the formation of degradation or oxidation products occurring in the gastrointestinal tract, possibly by action of the intestinal microflora, i.e., for carotenoids, the extent of broken-down products formed in the intestinal tract is unknown, (2) it does not differentiate in fecal samples between exogenous compounds from foods or supplements and endogenous compounds that can be resecreted into the lumen via the bile or pancreatic route or via the intestinal cells, (3) it does not account for the loss of the compound via the skin, i.e., for carotenoids, the loss from skin by exfoliation is considered negligible, even though it has never been truly quantified, and finally (4) it also requires sufficient knowledge of the urinary excretion of the compound and its metabolites, i.e., for carotenoids, no urinary excretion of free or conjugated carotenoids has been found and thus the main excretion route of those compounds is considered to be fecal. By using this approach, apparent absorption levels of β -carotene between 32 and 100% were found in humans⁷ — values that were largely overestimated when compared with values obtained via more recent and sophisticated approaches.

As an alternative approach, the traditional balance method can be combined with the gastrointestinal lavage technique consisting of washing the entire gastrointestinal tract before and after the ingestion of the compound of interest given either in a single dose or in food. These additional wash-out steps allow shortening of the residence time of the compound in the gut, thus minimizing the formation of degradation and oxidation products. This approach was applied for measuring carotenoid absorption in humans, which was estimated between 17 and 47% of the dose.⁸

3.2.2.2.2 Total Plasma Responses

In the total plasma response approach, the bioavailability of a compound is determined by measuring its plasma concentration at different times (up to weeks) after single or long-term ingestion of the compound from supplements or food sources. Generally, a plasma concentration-versus-time plot is generated, from which is determined the area-under-curve (AUC) value used as an indicator of the absorption of the compound. Here, the term “relative” bioavailability is more appropriate since AUC values of two or more treatments are usually compared. This is in contrast to “absolute” bioavailability for which the AUC value of the orally administered compound is compared to that obtained with intravenous administration taken as a reference (100% absorption).

The plasma response approach gives only an estimation of the bioavailability for several reasons: (1) the intestinal metabolism of the compound of interest is often not accounted for, i.e., provitamin A carotenoids (mainly β -carotene) are partly metabolized into retinol and retinyl esters during passage through the intestinal mucosa, (2) the presence of a circulating endogenous pool that decreases the sensitivity of this method, i.e., for carotenoids, because of their relatively high baseline levels in humans, doses higher than physiological doses ($> 5\text{ mg/day}$) had to be used to reveal changes in plasma carotenoid responses, and (3) the absolute absorption cannot be determined by this approach unless an injectable form of the compound is available to estimate its plasma clearance rate.

By using this approach, the mean AUC values for lutein and β -carotene were 59.6 ± 9.0 and $26.3 \pm 6.4 \mu\text{mol/hr/L}$, respectively, during the first 440 hr after ingestion of separate single doses by eight human subjects.⁹ These values may indicate that lutein is absorbed at twice the level of absorption of β -carotene in humans; however, intestinal metabolites of β -carotene were not accounted for in the study.⁹ To overcome the major problem of distinguishing the circulating endogenous pool from the newly absorbed pool, the plasma triglyceride-rich lipoprotein fraction response approach was developed.

3.2.2.2.3 Postprandial Chylomicron Responses

This approach can be used only for fat-soluble compounds that follow the same lymphatic route to be transported to the liver as carotenoids. The bioavailability of the compound of interest is determined by monitoring the appearance of the compound and its newly formed intestinal metabolites in the postprandial chylomicron fraction of plasma [also called the density $< 1.006 \text{ kg/L}$ fraction or triglyceride-rich lipoprotein (TRL) fraction because it is generally a mixture of chylomicrons (CMs) and very low density lipoproteins (VLDLs)] as a function of the time after ingestion.

As for whole plasma, AUC values for the CM-associated compound are used as indicators of its absorption. This method has the advantage of distinguishing the newly absorbed and endogenous pools. Indeed, the first (exogenous) pool is primarily associated with CMs which, after a meal, are produced by intestinal cells and secreted into the lymph. In contrast, the endogenous pool is mostly associated with hepatic-originated HDL fractions in the bloodstream. By using this approach, the mean value of β -carotene absorption was 11% assuming central cleavage (i.e., two molecules of retinyl esters formed per molecule of β -carotene) or 17% assuming eccentric cleavage (i.e., one molecule of retinyl ester formed per molecule of β -carotene).¹⁰

The postprandial CM response approach has its limitations too. The isolation of the CM fraction alone from plasma is a problem. First, by using a conventional ultracentrifugation method combined with a density gradient, it is difficult to obtain a density $< 1.006 \text{ kg/L}$ fraction that contains only CM freed from liver-derived VLDL. Second, CMs represent a broad population of particles varying in size and density. Immediately after entering the bloodstream, newly secreted CMs are targeted by lipoprotein lipases and have their sizes reduced to become "remnants."

The recovery of the entire CM population in the TRL fraction, which is dependent on centrifugation parameters (speed, time, and density gradient used) must be

determined in order to assess accurately the bioavailability of the CM-associated compound. Intra- and inter-individual variations in responses can be another problem. For instance, after ingesting a single dose of β -carotene in standardized conditions by 10 men, inter-individual variations in the AUC responses of β -carotene and retinyl esters in TRL fractions were very high: 42 and 36%, respectively.¹⁰

Large variations in responses make the data difficult to interpret, particularly when comparing different treatments, and thus this approach may require the use of a large number of subjects to observe possible statistical differences. Finally, the degree of polarity of the compound of interest is another important factor to keep in mind since that can affect its location in the CMs and in turn its possible exchanges with other high-density and low-density lipoproteins present in the bloodstream.

In contrast with the hydrocarbon carotenes primarily located in the cores of the CM particles, xanthophylls are present at the surfaces of the CM particles, making their exchanges with other plasma lipoproteins easier.¹¹ Therefore, if some exchanges occur between lipoproteins, AUC (or absorption) values of the newly absorbed compound in the TRL fraction will be underestimated. Based on all these considerations, the present approach is more appropriate to determine the “relative” bioavailability of a compound derived from various treatments within one subject and/or within one study.

3.2.2.2.4 Isotopic Labeling Techniques

By using a compound labeled with either radioisotopes (^3H , ^{14}C) or stable isotopes (^2H , ^{13}C), its bioavailability can be assessed by following the appearance of the isotopically labeled material in whole plasma, the plasma TRL fraction (if applicable), tissue biopsies, feces, or urine samples from minutes up to months following the ingestion of a single dose or multiple doses.¹² One of the advantages of using multiple doses is to reach a plateau of isotopic enrichment at a plasma level that is generally higher than that obtained after a single dose and thus facilitate detection with the possibility of reducing the dose size.

The compound of interest can be labeled either by extrinsic or intrinsic methods. Extrinsic labeling uses chemical reactions to incorporate an isotope into a compound. Intrinsic labeling is the result of biological incorporation of an isotope into a compound by growing plants on $^2\text{H}_2\text{O}$ or on $^{13}\text{CO}_2$ so that the labeled compound of interest is in the plant food matrix and consumed as it. By successfully labeling nutrients in kale with ^{13}C , the bioavailability of ^{13}C - β -carotene and ^{13}C -lutein from kales was thus investigated in humans.¹³ Isotopic labeling approaches have several advantages including the ability to (1) clearly distinguish between the newly absorbed and endogenous pools, (2) easily follow the appearance of newly formed metabolites, and (3) estimate the “absolute” absorption of the compound of interest.

For human studies, the choice of stable isotopes is limited because radioisotopes are associated with ionization radiation and thus with some potential harmful effects for humans. Studying the bioavailability of compounds labeled with stable isotopes requires complex techniques such as gas chromatography coupled with mass spectrometry (GC-MS), liquid chromatography coupled with MS (LC-MS), and atmo-

spheric pressure chemical ionization coupled with LC-MS (LC-APCI-MS), which can detect levels as small as the femtomolar level (10^{-5} mol).

Using high performance liquid chromatography plus GC-MS and a compartmental model, the absorption of β -carotene was estimated as 22% (17.8% as intact β -carotene and 4.2% as retinoids) after ingestion of a single high dose of β -carotene-d8 (40 mg) in oil by one adult subject.¹⁴ This value was close to the 9 to 17% values obtained in earlier human lymph cannulation studies using radioisotopes.^{15,16}

Despite health concerns, radioisotopes present some advantages compared to stable isotopes; they have relative long half-lives, they can be administered at lower doses, avoiding the perturbation of the endogenous pool size, and their use increases the sensitivity of the method of detection. In that regard, a relatively new approach using trace amounts of ^{14}C -labeled compounds coupled with the accelerator mass spectrometry (AMS) technique has been developed for studying the kinetics of phytochemicals in humans at physiological doses.¹⁷ By using this highly sensitive AMS method of detection (attomolar level or 10^{-18} mol) and minute radiation doses (safe for human health), the apparent absorption of ^{14}C - β -carotene was estimated as 43% in an adult subject after ingestion of a single dose of ^{14}C - β -carotene (306 μg) in oil.¹⁸ However, the estimation in this study was done by ^{14}C mass balance between the dose and the stool excretion. Finally, isotopic labeling techniques require that a labeled form of the compound of interest is available and in general involve expensive instrumentations that are often associated with labor-intensive sample preparation and limited numbers of human subjects.

In sum, different methods quantify the intestinal absorption of food pigments in humans, for example, the intake–excretion balance and plasma response approaches. Both methods provide only rough estimates of intestinal absorption per se. More recent approaches using isotopes coupled with mass spectral analysis of a compound and its newly synthesized metabolites isolated from whole plasma or from the TRL plasma fraction are the most promising methods in terms of accurate measurement of absolute absorption. However such studies are costly and complex, and the data generated about carotenoid bioavailability are currently limited and difficult to compare from study to study due to the use of different experimental designs. Although such methods have great promise in assessing nutrient bioavailability from different food sources in humans, they do not provide mechanistic information about the absorption process.

3.2.2.3 *IN VITRO* APPROACHES

Based on the limitations of using human subjects, simple alternative *in vitro* models were developed to investigate mechanisms involved in the intestinal absorption process of a compound of interest and to screen the relative bioavailability of a compound from various food matrices. However, the data generated from *in vitro* approaches must be taken with caution because they are obtained under relatively simplified and static conditions compared to dynamic physiological *in vivo* conditions. Indeed, the overall bioavailability of a compound is the result of several complex steps that are influenced by many factors including factors present in the gastrointestinal lumen and intestinal cells as described later. Nevertheless, these *in vitro* approaches are useful tools for guiding further studies in humans.

3.2.2.3.1 Caco-2 Cell Model

In culture, the human colon carcinoma cell line Caco-2 spontaneously differentiates at confluency into polarized cells with enterocyte-like characteristics. The principle of this approach consists of following the passage of the compound of interest from the apical or “lumen-like” sides to the basolateral or “lymph-like” sides of Caco-2 cells, thus following the absorption of the compound *per se*. One obligate step for fat-soluble nutrients such as carotenoids to cross the intestinal barrier is their incorporation into CMs assembled in the enterocytes. Under normal cell culture conditions, Caco-2 cells are unable to form CMs. When supplemented with taurocholate and oleic acid, Caco-2 cells were reported to assemble and secrete CMs.¹⁹

Thus, in our laboratory, an *in vitro* cell culture system was developed to study the intestinal absorption of carotenoids,²⁰ which can be applied to other food pigments as well. The *in vitro* model is schematized in Figure 3.2.1 and consists of a 3-week differentiated Caco-2 cell monolayer cultured on a membrane. At the zero time of the experiment, oleic acid, taurocholate, and ³H-glycerol are added to the apical sides of Caco-2 cells. Radioactive glycerol is employed to follow the pools of newly synthesized ³H-triglycerides and ³H-phospholipids. Under these conditions mimicking the *in vivo* postprandial state, the carotenoid is delivered to cells using the Tween 40 method.²¹ After incubation, an aliquot of the basolateral medium is subjected to a lipoprotein fractionation that yields large CMs, small CMs, VLDLs and non-lipoprotein fractions (Figure 3.2.1).

In this *in vitro* system, the presence of serum in cell culture medium is not necessary, but the type of transwell is important (the total amount of ³H-triglycerides secreted was two-fold higher when using 3 μ m versus 1 μ m pore size transwells), and oleic acid supplementation is required for the formation and secretion of CMs as well as the transport of β -carotene through Caco-2 cells. Finally, the presence of Tween 40 does not affect CM synthesis and secretion in this *in vitro* cell culture system. Thus, CMs secreted by Caco-2 cells were characterized as particles rich in newly synthesized ³H-triglycerides (90% of total secreted) containing apolipoprotein B (30% of total secreted) and ³H-phospholipids (20% of total secreted) and with an average diameter of ~60 nm. These characteristics are close to those of CMs secreted *in vivo* by enterocytes.²⁰

In contrast to previous *in vivo* models, this *in vitro* model provides the possibility of dissociating experimentally two important processes of intestinal absorption: cellular uptake and secretion. Under conditions mimicking the postprandial state (taurocholate/oleic acid supplementation), differentiated Caco-2 cells were able to (1) take up carotenoids at the apical sides and incorporate them into CMs and (2) secrete them at the basolateral sides associated with CM fractions.²² Using this approach, the extent of absorption of β -carotene through Caco-2 cell monolayers after 16 hr of incubation was 11.2%, a value falling within the *in vivo* range (9 to 22%).^{10,14–16} Of the total amount of β -carotene secreted, 78% was associated with the two CM fractions and 10% with the VLDL fraction.²²

This *in vitro* approach thus has a great potential for studying the intestinal absorption processes of carotenoids and other pigments. It is important to note the existence of several clones isolated from the parent Caco-2 cell line that can be used for studying

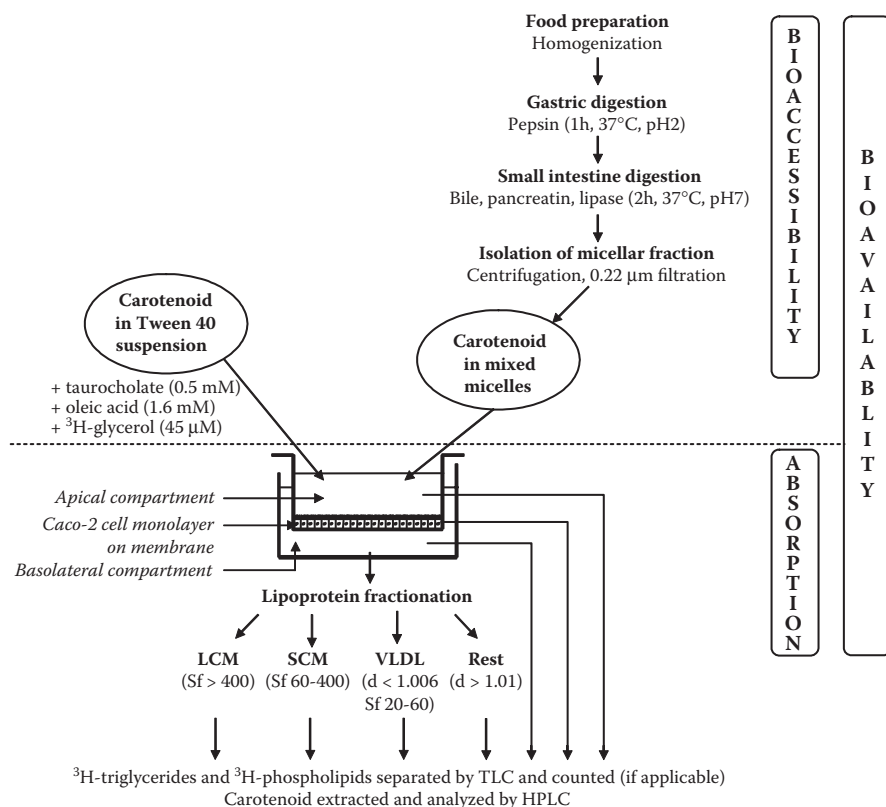


FIGURE 3.2.1 *In vitro* digestion/Caco-2 cell model combination approach to assess carotenoid bioavailability. LCM = large chylomicrons. SCM = small chylomicrons. VLDL = very low density lipoproteins.

compound bioavailability. Lately, the use of the TC7 clone for this type of study has been favoured, perhaps due to its higher viability in the presence of mixed micelles.²³

In our study, the parent Caco-2 cells were more efficient than the TC7 cells in terms of both CM formation and secretion and β -carotene transport.²² As an alternative to the Tween 40 method, mixed micelles can also be utilized as more physiological vehicles for presentation of lipophilic compounds to intestinal cells. These mixed micelles contain usually at least one fatty acid (i.e., oleic acid), one monoglyceride (i.e., monoolein), one phospholipid (i.e., phosphatidylcholine), and one bile salt (i.e., sodium taurocholate). Finally, it is important to note that a hydrophilic compound can be applied at the apical sides of Caco-2 cells, directly solubilized in the cell culture medium, and probably will be secreted at the basolateral sides mostly associated with the non-lipoprotein fraction (Figure 3.2.1).

In this model, no attempt is made to reproduce the *in vivo* physiochemical conditions occurring in the lumen of the human small intestine during digestion. This cell culture model only provides information about the intestinal absorption and metabolism processes of the compound. Using this cell culture system in con-

junction with an *in vitro* digestion procedure can be useful for screening the relative bioavailabilities of carotenoids and other pigments from different types of food matrices *in vitro*.

3.2.2.3.2 *In Vitro* Digestion Approach

In the *in vitro* digestion method, the compound of interest is transferred from the food matrix to a bile salt micelle suspension that simulates the *in vivo* digestion process. This *in vitro* digestion procedure was first developed to estimate iron availability from meals and since then has been modified and applied to studying carotenoid bioaccessibility from various food matrices.^{24–27} This approach assesses the bioaccessibility of the compound from a certain meal before it is presented to and taken up by intestinal cells.

The procedure includes four successive steps: (1) food preparation, (2) gastric digestion, (3) small intestinal digestion, and (4) isolation of the micellar fraction (Figure 3.2.1). First, the food or meal (cooked or uncooked) is mixed with a saline solution and homogenized to make a puree, a process that simulates mastication (step 1). Next, an aliquot of the homogenized food is acidified to pH 2 and incubated in the presence of porcine pepsin at 37°C for 1 hr to mimic gastric digestion (step 2). After incubation, an aliquot of the digesta is neutralized to pH 7.5 and further digested in the presence of bile extract, porcine pancreatin, and lipase at 37°C for 2 hr to simulate small intestinal digestion (step 3). An aliquot of the final digesta is then centrifuged to isolate the aqueous fraction that contains micelles from residual oil droplets and particles of food (step 4).

It is important to indicate here that a hydrophilic compound will not be necessarily associated with the micelles but still will be found in the aqueous fraction isolated in step 4. Using this *in vitro* digestion approach, extents of carotenoid transfer from various food matrices to the micellar phase were 25 to 40% for lutein, 12 to 18% for α - and β -carotene, and less than 0.5% for lycopene,²⁴ indicating that lutein was more bioaccessible than the carotenes and suggesting that transfer is a function of the polarity of the carotenoid molecule as suggested *in vivo*.²⁸ By using this *in vitro* digestion approach, three factors that emerged appeared to influence compound bioaccessibility: the chemical structure of the compound, the food matrix, and the food processing (for more details see Section 3.2.3). This *in vitro* digestion procedure was also used to assess the bioaccessibility of anthocyanins from various food matrices.^{29,30}

3.2.2.3.3 *In Vitro* Digestion/Caco-2 Cell Model Combination Approach

The combination of the *in vitro* digestion method with the Caco-2 cell culture model presents the advantage of following a compound of interest from its release from the food matrix through its secretion at the basolateral sides of cells (Figure 3.2.1). Thus, after completing the *in vitro* digestion protocol (see above), the resulting aqueous micellar fraction including the compound is filtered by passage through a 0.22 μm filter and then diluted with the cell culture medium before application at the apical sides of differentiated Caco-2 cells grown on membranes.

To date only one study has been conducted using this complete *in vitro* digestion/Caco-2 cell model approach as represented in Figure 3.2.1 to assess lutein bioavailability from meals and a supplement.³¹ As a rapid way to estimate bioavailability, Caco-2 cells have been often grown directly on plastic plates,^{25,32–34} making the system even more “static” and for which only the cellular uptake, but not the full absorption process, is studied. Nevertheless, this *in vitro* approach is a promising method for studying the bioavailability of carotenoids and other food pigments.

Indeed, data generated by using a partial or complete *in vitro* method (digestion procedure and/or (un)differentiated Caco-2 cells grown on plates or transwells) are similar to those observed *in vivo*: (1) the increased accessibility of carotenoids from cooked versus uncooked meals,^{26,33} (2) the increased accessibility of carotenoids from green leafy vegetables when cooked with oil versus without oil,²⁷ (3) the increased uptake of carotenoids from mixed micelles containing lysophosphatidylcholine versus phosphatidylcholine,³⁴ (4) the selective absorption of β -carotene isomers in favor of all-trans forms versus cis forms,²² (5) the reduction of β -carotene uptake in the presence of phytosterols,³⁵ and finally (6) the increased absorption of carotenoids from micelles obtained with an *in vitro* digestion procedure in the presence versus absence of tomato peels.³⁶ These facts support the relevance of such an *in vitro* digestion/Caco-2 cell approach for assessing the bioavailability of food pigments as a suitable and cost-effective alternative method.

3.2.3 BIOACCESSIBILITY OF PIGMENTS FROM FOODS

3.2.3.1 INTRODUCTION

The bioaccessibility of a compound can be defined as the result of complex processes occurring in the lumen of the gut to transfer the compound from a non-digested form into a potentially absorbable form. For carotenoids, these different processes include the disruption of the food matrix, the disruption of molecular linkage, the uptake in lipid droplets, and finally the formation and uptake in micelles.³⁷ Thus, the bioaccessibility of carotenoids and other lipophilic pigments from foods can be characterized by the efficiency of their incorporation into the micellar fraction in the gut. The fate of a compound from its presence in food to its absorbable form is affected by many factors that must be known in order to understand and predict the efficiency of a compound's bioaccessibility and bioavailability from a certain meal.^{37–39}

A number of factors described as influencing carotenoid bioavailability were regrouped under the SLAMENGHI mnemonic.³⁷ Species of carotenoid, Linkages at molecular level, Amount of carotenoids consumed in a meal, Matrix in which the carotenoid is incorporated, Effectors of absorption and bioconversion, Nutrient status of the host, Genetic factors, Host-related factors, and Interactions among these variables. Only the factors that affect the micellarization of the compound in the gut are discussed and summarized in Table 3.2.1.

3.2.3.2 PHYSICOCHEMICAL CHARACTERISTICS OF PIGMENTS

The *degree of lipophilicity* of a pigment molecule can play a major role in its bioaccessibility. Obviously, a compound with a lower lipophilic character will be

TABLE 3.2.1
Factors Influencing Bioaccessibility
of Pigments from Foods

Physicochemical properties of compound

Lipophilic character

Configuration

Degree of linkage

Release of compound from food matrix

Type of food matrix

Subcellular location of compound

Food processing

Intraluminal factors

Nutrients: lipids, fibers, other carotenoids

Bile salts

pH

Microflora

better released from a food matrix because of its higher solubility in an aqueous environment such as in the gastrointestinal lumen. For instance, when comparing the extents of solubilization of different carotenoids, they systematically showed a decreasing order: lutein > β -carotene > lycopene in the micellar fractions obtained by *in vitro* digestion of processed foods^{24,25} and in the micellar phase of the duodenum *in vivo* after eating a meal enriched with these three carotenoids.²⁸ It is interesting to note here that the extent of absorption of these three carotenoids through Caco-2 cells followed a different order: β -carotene > lutein > lycopene (11, 7.5, and 2%, respectively).²²

The *configuration* of the molecule can also be another factor affecting the degree of micellarization of a compound in the lumen. For instance, *cis* isomers of β -carotene present a greater solubilization in mixed micelles *in vitro*⁴⁰ and in the duodenal micellar phase *in vivo*²⁸ than all-*trans* β -carotene. Despite their higher efficiency of micellarization, *cis* isomers of β -carotene are less absorbed by Caco-2 cells²² and also *in vivo*⁴¹ than the all-*trans* forms.

The *degree of linkage* of a compound may also affect its bioaccessibility in the gut. It is generally admitted that a compound linked with other molecules (e.g., via esterification, glycosylation, etc.) is not absorbed as well as its free form and thus it must be hydrolyzed in the gut in order to be taken up by enterocytes. Due to the presence of hydroxyl or keto groups on their molecules, the xanthophylls (lutein, zeaxanthin, and β -cryptoxanthin) are found in both free and esterified (monoester or diester) forms in nature, but few studies have been conducted to date to assess the bioavailabilities of these esters.

One recent report involving the use of the *in vitro* digestion procedure noted that the micellarization of zeaxanthin from digested foods was dependent on its degree of esterification with transfer efficiency levels of 80, 44, and 11%, respectively, for the free form, monoesters, and diesters of zeaxanthin.⁴² *In vivo* studies^{43,44}

indicate, however, comparable bioavailabilities for both free and esterified forms of lutein and β -cryptoxanthin, suggesting that xanthophyll esters are efficiently hydrolyzed by an enzymatic system in the gastrointestinal lumen.

The hydrolysis of zeaxanthin esters by a carboxyl ester lipase indeed enhanced both the incorporation of zeaxanthin in the micellar phase and uptake of zeaxanthin by Caco-2 cells.⁴² As mentioned earlier, carotenoids can also be linked to proteins by specific bindings in nature and these carotenoid–protein complexes may slow the digestion process and thus make their assimilation by the human body more difficult than the assimilation of free carotenoids. Anthocyanins are usually found in a glycosylated form that can be acetylated and the linked sugars are mostly glucose, galactose, rhamnose, and arabinose.

The positions, numbers, and types of sugars on the anthocyanin molecule influence its bioaccessibility. Indeed, a recent human study reported that the acylation of anthocyanins resulted in a significant decrease of anthocyanin recoveries in plasma and urine.⁴⁵ In addition, anthocyanins form linkages with aromatic acids, aliphatic acids, and methyl ester derivatives, which can also affect their passage through the intestinal barrier.

3.2.3.3 RELEASE OF PIGMENTS FROM FOOD MATRIX

The release of a compound from the food matrix in which it is incorporated is a determining process for its bioavailability and is largely influenced by the physicochemical characteristics of the compound, the type of food matrix, the subcellular location of the compound in plant tissues, and the food processing. The *food matrix type* greatly influences the compound bioaccessibility.

For carotenoids, the type of matrix varies from relatively simple matrices in which the free carotenoid is dissolved in oil or encapsulated in supplements to more complex matrices in which the carotenoid is within plant foods. It is clear that the efficiency of the process by which the compound becomes more accessible in the gastrointestinal tract is inversely related to the degree of complexity of the food matrix. Carotenoid bioavailability is indeed far greater in oil or from supplements than from foods and usually the pure carotenoid solubilized in oil or in water-soluble beadlets is employed as a reference to calculate the relative bioavailability of the carotenoid from other foods.³⁷

In relation to its physicochemical properties, a compound can be trapped in different *subcellular locations* of the cells that constitute the food matrix, making it more or less extractable from the matrix during the digestion process in the gastrointestinal tract. For instance, in plants, carotenoids are localized (1) in the chloroplasts, entrapped with the light-harvesting complex in the thylakoid membranes (in dark green leafy vegetables), (2) in the chromoplasts, dissolved in lipid droplets (in orange and yellow fruits, pumpkins, and sweet potatoes) or associated with membranes in crystalline form (in carrots and tomatoes), or (3) as mentioned above, in protein complexes. Several *in vivo* studies reported that β -carotene bioavailability was greater from carrots, broccolis, green peas, and fruits than from dark green, leafy vegetables,^{46–48} suggesting that chloroplasts may be less efficiently disrupted in the gastrointestinal tract than chromoplasts.

The *food processing* can also affect compound release from the food matrix. Food preparation methods such as juicing, blending, chopping, and/or moderate heating usually improve the carotenoid bioavailability from many plant foods, probably as a result of the increased compound bioaccessibility by weakening the cell wall of plant tissues, dissociating the carotenoid-protein complexes, and/or dissolving the crystalline carotenoid forms. For instance, β -carotene bioavailability from carrots was increased by 70% by juicing raw carrots.⁴⁹ Lutein bioavailability from spinach was improved by chopping leaf spinach.⁴⁷ In addition, lycopene bioavailability from tomatoes was enhanced by cooking and processing them into paste.⁵⁰ Similarly, the bioavailability of non-acylated anthocyanins in purple carrots, but not that of acylated anthocyanins, was increased by cooking.⁴⁵ Note that excessive heating and processing may affect the structural stability of a compound in food (as reported for carotenoids) by causing their isomerization from the naturally occurring all-trans form to cis forms, their oxidation, or even their photo-bleaching resulting in the production of new species that impact on bioavailability.

3.2.3.4 INTRALUMINAL FACTORS

Different factors including nutrients, bile salts, pH, and microflora present in the gastrointestinal tract during the digestion process can affect the bioaccessibility of a compound (Table 3.2.1). The compound of interest is generally consumed together with other *nutrients* present in the meal and, once the compound and these nutrients are released from the food matrix during the same period, they may interact in the intestinal lumen.

Dietary fats, fibers, and other carotenoids have been reported to interfere with carotenoid bioaccessibility. It is clear that by their presence in the gut, *lipids* create an environment in favor of hydrophobic compounds such as carotenoids. When arriving in the small intestinal lumen, dietary fats stimulate bile flow from the gall-bladder and therefore enhance the micelle formation, which in turn could facilitate the emulsification of carotenoids into lipid micelles. Without micelle formation, carotenoids are poorly absorbed; a minimum of 3 g of fat in meal is necessary for an efficient absorption of carotenoids,⁵¹ except for lutein esters that require higher amounts of fat.⁵²

In addition to the amount of fat, the type of fat may play a role in the micellarization of carotenoids. In phospholipid-stabilized triglyceride emulsions, the solubility of β -carotene and zeaxanthin increased with decreasing chain-length of fatty acids in triglycerides⁵³ although *in vivo* β -carotene CM response was markedly diminished when β -carotene was ingested with medium-chain rather than long-chain triglycerides in humans.⁵⁴ Phospholipids also decrease carotenoid absorption,⁵⁵ probably via the reduction of micelle formation, resulting in a decreased carotenoid micellarization in the gut. In that regard, bile salt sequestrant agents, such as cholestyramine or non-absorbable fat replacers such as sucrose polyester (Olestra), which are known to primarily disrupt micelle formation in the lumen also decrease carotenoid bioavailability.⁵⁶

Similarly, dietary *fibers* are known to interact with bile acids in the intestinal lumen and thus increase bile salt excretion in feces, resulting in decreased numbers

and/or sizes of mixed micelles, which may in turn affect the absorption of lipophilic nutrients such as cholesterol and carotenoids. For instance, the bioavailability of β -carotene, lycopene, and lutein was reduced markedly by different dietary fibers in humans.⁵⁷ The carotenoid of interest is generally ingested together with other *carotenoids* from the meal and it is now clear that carotenoids interact during their absorption.^{22, 58} One possible mechanism to explain these interactions is that two carotenoids compete for their incorporation into mixed micelles as shown *in vitro*.⁵⁹

For a lipophilic compound, the *bile salt* composition in mixed micelles is a determining factor for its incorporation inside the micelle and thus its bioaccessibility. *In vitro* carotenoid micellarization was indeed affected by the types and concentrations of bile salts present in mixed micelles.^{60, 61} The intraluminal intestinal *pH* also plays a role in carotenoid bioaccessibility. In the gastrointestinal tract, *pH* values range from 2 to 7. Carotenoid solubility in *in vitro* mixed micelles was decreased with acidic *pH* < 5,^{59, 60} while β -carotene was absorbed to a greater extent under acidic intraluminal conditions than under alkaline conditions.⁶¹ Thus, under increased luminal hydrogen ion concentration, mixed micelles containing the carotenoid could precipitate,⁵⁹ but also see their diffusion increased by reduction of the negative surface charges of intestinal cell membranes.⁶¹

Finally, the intestinal *microflora* is another factor that can affect carotenoid bioaccessibility, since some bacteria are able to hydrolyze conjugated bile salts. Carotene bioavailability was improved when the intestinal microflora were partially eliminated in rats, probably by decreasing both the intestinal transit time and the pool of bile salts in the jejunum.⁶² In the colon, the microflora can catalyze the breakdown of anthocyanins into more simple compounds, first by hydrolyzing glycosides into aglycones and then by metabolizing the aglycones into various aromatic compounds such as some phenolic acids that may then be absorbed and conjugated with glycine, glucuronic acid, or sulfate.⁶³

3.2.4 ABSORPTION, METABOLISM, AND TISSUE DISTRIBUTION OF MAJOR FOOD PIGMENTS

Among all food pigments, we have the most knowledge about the carotenoids related to their absorption and metabolism on a molecular basis.

3.2.4.1 CAROTENOIDS

3.2.4.1.1 Introduction

Carotenoids constitute a group of liposoluble pigments that are widely spread in nature and are responsible for the yellow, orange, red, and purple colors of many fruits, flowers, birds, insects, and marine animals. They are found in plant food sources such as carrots, squash, and dark-green leafy vegetables for β -carotene, carrots for α -carotene, tomatoes and watermelon for lycopene, kale, peas, spinach, and broccoli for lutein, and sweet red peppers, oranges, and papayas for β -cryptoxanthin. β -Carotene is one of the most abundant carotenoids found in the human diet and the most potent vitamin A precursor of all the provitamin A carotenoids.

In the United States, the daily intake of β -carotene is around 2 mg/day.⁶⁴

Several epidemiological studies have reported that consumption of carotenoid-rich foods is associated with reduced risks of certain chronic diseases such as cancers, cardiovascular disease, and age-related macular degeneration.^{65,66} These preventive effects of carotenoids may be related to their major function as vitamin A precursors and/or their actions as antioxidants, modulators of the immune response, and inducers of gap-junction communications.⁶⁷ Not all carotenoids exert similar protective effects against specific diseases. By reason of the potential use of carotenoids as natural food colorants and/or for their health-promoting effects, research has focused on better understanding how they are absorbed by and metabolized in the human body.

3.2.4.1.2 Intestinal Carotenoid Absorption

More than 600 carotenoids have been isolated from natural sources, but only about 60 have been detected in the human diet — about 20 in human blood and tissues. β -Carotene, α -carotene, lycopene, lutein, and β -cryptoxanthin are the five most prominent carotenoids present in the human body.

The *in vivo* intestinal absorption of carotenoids involves several crucial steps: (1) the release of carotenoids from the food matrix, (2) the solubilization of carotenoids into mixed lipid micelles in the lumen, (3) the cellular uptake of carotenoids by intestinal mucosal cells, (4) the incorporation of carotenoids into CMs, and (5) the secretion of carotenoids and their metabolites associated with CMs into the lymph (Figure 3.2.2). Until recently, based on earlier rat studies,^{60,68} the intestinal absorption of carotenoids was thought to be a passive diffusion process. Use of the *in vitro* Caco-2 cell model mentioned above (Figure 3.2.1) revealed (1) saturation of β -carotene transport for concentrations (15 μ M) far higher than physiological concentrations, (2) discrimination within β -carotene isomers for their transport — the 9-*cis* and 13-*cis* isomers were taken up by cells to only one-fifth the extent of the all-*trans* form, (3) differential transport among individual carotenoids as follows: all-*trans* β -carotene (11%) $\approx$ α -carotene (10%) > lutein (7%) > lycopene (2.5%), and (4) carotenoid interactions during their transport, especially between non-polar carotenoids (β -carotene/ α -carotene and β -carotene/lycopene). All these observations suggest that the intestinal absorption of carotenoids is a facilitated transport process perhaps mediated by specific transporters.²²

A scavenger receptor with a high homology to mammalian scavenger receptors, i.e., scavenger receptor class B, type I (SR-BI) and cluster determinant 36 (CD36), was reported to mediate the cellular uptake of carotenoids in *Drosophila*.⁶⁹ The identification of such transporters in the human intestine constituted an exciting challenge in carotenoid research. Only very recently has the involvement of SR-BI in the intestinal transport of both β -carotene and lutein been shown in mammals, either by using brush border membrane vesicles made from wild-type or SR-BI knockout mice⁷⁰ or by applying a blocking antibody against SR-BI on the TC7/Caco-2 cells or the parent Caco-2 cells.^{71,72}

In addition to SR-BI, it is suggested that other lipid transporters such as Niemann-Pick type C1-like 1 protein (NPC1L1) and ATP-binding cassette transporter, subfamily A (ABCA1) may also participate in intestinal carotenoid transport.⁷² Indeed, by using

Caco-2 cells and ezetimibe, a potent inhibitor of cholesterol absorption in humans, it was reported that (1) carotenoid transport was inhibited by ezetimibe up to 50% and the extent of that inhibition diminished with increasing polarity of the carotenoid molecule, (2) the inhibitory effects of ezetimibe and the antibody against SR-BI on β -carotene transport were additive, and (3) ezetimibe may interact physically with cholesterol transporters as previously suggested^{73,74} and also down-regulate the gene expression of three surface receptors, SR-BI, NPC1L1, and ABCA1.

The hypothesis of the participation of those cholesterol transporters (NPC1L1 and ABCA1) in the carotenoid transport remains to be confirmed, especially at the *in vivo* human scale. If the mechanism by which carotenoids are transported through the intestinal epithelial membrane seems better understood, the mechanism of intracellular carotenoid transport is yet to be elucidated. The fatty acid binding protein (FABP) responsible for the intracellular transport of fatty acids was proposed earlier as a potential transporter for carotenoids.⁶¹ FABP would transport carotenoids from the epithelial cell membrane to the intracellular organelles such as the Golgi apparatus where CMs are formed and assembled, but no data have illustrated this hypothesis yet.

3.2.4.1.3 Carotenoid Metabolism

In intestinal cells, carotenoids can be incorporated into CMs as intact molecules or metabolized into mainly retinol (or vitamin A), but also in retinoic acid and apocarotenals (see below for carotenoid cleavage reactions). These polar metabolites are directly secreted into the blood stream via the portal vein (Figure 3.2.2). Within intestinal cells, retinol can be also esterified into retinyl esters.

Both intact carotenoids and their apolar metabolites (retinyl esters) are secreted into the lymphatic system associated with CMs. In the blood circulation, CM particles undergo lipolysis, catalyzed by a lipoprotein lipase, resulting in the formation of CM remnants that are quickly taken up by the liver. In the liver, the remnant-associated carotenoid can be either (1) metabolized into vitamin A and other metabolites, (2) stored, (3) secreted with the bile, or (4) repackaged and released with VLDL particles. In the bloodstream, VLDLs are transformed to LDLs, and then HDLs by delipidation and the carotenoids associated with the lipoprotein particles are finally distributed to extrahepatic tissues (Figure 3.2.2). Time-course studies focusing on carotenoid appearances in different lipoprotein fractions after ingestion showed that CM carotenoid levels peak early (4 to 8 hr) whereas LDL and HDL carotenoid levels reach peaks later (16 to 24 hr).

Carotene cleavage enzymes — Two pathways have been described for β -carotene conversion to vitamin A (central and eccentric cleavage pathways) and reviewed recently.⁷⁵ The major pathway is the *central cleavage* catalyzed by a cytosolic enzyme, β -carotene 15,15-oxygenase (BCO; EC 1.13.1.21 or EC 1.14.99.36), which cleaves β -carotene at its central double bond (15,15') to form retinal. Two enzymatic mechanisms have been proposed: (1) a dioxygenase reaction (EC 1.13.11.21) that requires O_2 and yields a dioxetane as an intermediate⁷⁶ and (2) a monooxygenase reaction (EC 1.14.99.36) that requires two oxygen atoms from two different sources (O_2 and H_2O) and yields an epoxide as an intermediate.⁷⁷

TABLE 3.2.2
Retinol Equivalence of Dietary
Provitamin A Carotenoids

1 retinol activity equivalent (RAE) =

1 μg of all-trans retinol

2 μg of all-trans β -carotene in oils or supplements

12 μg of all-trans β -carotene in foods

24 μg of other provitamin A carotenoids in foods

Source: Institute of Medicine, 2001.⁸⁰

Regardless of the mechanism, the final product is retinal, a direct precursor of retinol (or vitamin A) by reduction through short-chain dehydrogenase and reductase activity and of retinoic acid by irreversible oxidation through aldehyde dehydrogenase activity. The stoichiometry of the central cleavage reaction is two moles of retinal formed per mole of β -carotene cleaved.⁷⁸ Recently, BCO was characterized as a protein of ~550 amino acids (mol wt ~65 kDa) that has well-conserved sequences among the different species including humans.

In order to exhibit provitamin A activity, the carotenoid molecule must have at least one unsubstituted β -ionone ring and the correct number and position of methyl groups in the polyene chain.⁷⁹ Compared to all-trans β -carotene (100% provitamin A activity), α -carotene, β -cryptoxanthin, and γ -carotene show 30 to 50% activity and cis isomers of β -carotene less than 10%. Vitamin A equivalence values of carotenoids from foods have been recently revised to higher ratio numbers⁸⁰ (see Table 3.2.2) due to poorer bioavailability of provitamin A carotenoids from foods than previously thought when assessed with more recent and appropriate methods.

The second pathway is the *eccentric cleavage* that occurs at double bonds other than the central 15,15'-double bond of the β -carotene molecule to produce different products called β -apocarotenals with various chain lengths. Because only trace amounts of apocarotenals were detected *in vivo* from tissues of animals fed β -carotene⁸¹ and these compounds can be formed non-enzymatically from β -carotene auto-oxidation,⁸² the existence of this pathway was controversial until recently. The identification of β -carotene 9',10'-oxygenase (BCO2), which acts specifically at the 9',10' double bond of β -carotene to produce β -apo-10'-carotenal and β -ionone,⁸³ provided clear evidence of the eccentric cleavage pathway *in vivo*. Lycopene was also reported as a substrate for BCO2 activity.

The existence of BCO2 suggests the possibility of other yet unidentified eccentric cleavage enzymes that would cleave carotenoid molecules at other double bonds [i.e., (7',9'), (11',12'), or 13',14')]. Based on *in vitro* observations,⁸⁴ it was suggested that eccentric cleavage may occur preferentially — when antioxidants are insufficient under conditions such as smoking and diseases involving oxidative stress and/or when high β -carotene levels are present. In contrast, under normal physiological conditions, when antioxidants are adequate, central cleavage would be the predominant pathway.

The two major sites of β -carotene conversion are the intestine and liver in humans. The liver seems to have a greater capacity for metabolizing β -carotene to vitamin A than the intestine.^{14,85} In rats, BCO activity was also reported to be higher in the small intestine and liver, followed by brain, lung, and kidney.⁸⁶ In agreement with the tissue distribution of BCO activity, high levels of human BCO mRNA were reported in the jejunum, liver, and kidney, whereas lower levels were present in the prostate, testes, ovaries, and skeletal muscles.⁸⁷

3.2.4.1.4 Transport and Tissue Distribution

In fasting human serum, the hydrocarbon carotenes (β -carotene and lycopene) are found primarily in LDL, while the xanthophylls (lutein, zeaxanthin, and β -cryptoxanthin) are more evenly distributed between LDLs and HDLs.^{88, 89} As mentioned earlier and contrary to the carotenes, the xanthophylls are primarily located at the surfaces of lipoprotein particles, making them more likely to exchange between plasma lipoproteins. This hypothesis may explain their equal distribution (or apparent equilibrium) between LDLs and HDLs.

In humans, carotenoids were reported in liver, adrenals, testes, kidneys, lungs, skin, eyes, and adipose tissues. Adipose tissue seems to be the main storage site, together with the liver accounting for at least 80% of carotenoid storage.⁹⁰ It was suggested that the tissue distribution of carotenoids may correlate with the LDL uptake in tissues expressing LDL receptors at their surfaces,⁹¹ but this does not explain why some tissues show marked enrichment in specific carotenoids, i.e., the human macula accumulates specifically the two xanthophylls, lutein and zeaxanthin.

3.2.4.2 ANTHOCYANINS

3.2.4.2.1 Introduction

Anthocyanins represent a large group of water-soluble plant pigments that are responsible for the red, blue, and purple colors of many fruits and vegetables and also of autumn leaves. They belong to the class of flavonoids within the large polyphenol family. Anthocyanins are present in blackberry, chokeberry, cherry, eggplant, red cabbage, blue grape, and grape skin extracts. They exist normally as glycosides; the aglycone compounds alone (anthocyanidins) are extremely unstable. Most frequently found in nature are the glycosides (mono- or di-glucosides and acylated mono- or di-glucosides) of cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin.

Cyanidin is the most common anthocyanin in foods. In addition, anthocyanins are stabilized by the formation of complexes with other flavonoids (co-pigmentation). In the United States, the daily anthocyanin consumption is estimated at about 200 mg.⁹² Several promising studies have reported that consumption of anthocyanin-rich foods is associated with reductions of the risks of cancers^{93, 94} and atherosclerosis⁹⁵ and with preventive effects against age-related neuronal and behavioral declines.⁹⁶ These beneficial effects of anthocyanins might be related to their reported biological actions such as modulators of immune response and as antioxidants.⁹⁷ Knowledge of anthocyanin bioavailability and metabolism is thus essential to better understand their positive health effects.

3.2.4.2.2 Intestinal Absorption

Anthocyanins are poorly absorbed from the gastrointestinal tract and the mechanisms involved remain unclear. These compounds are usually recovered in very small amounts in human serum after oral ingestion (less than 1% of the dose)⁹⁸ or in the IN fraction after *in vitro* digestion (about 5%).³⁰ Unlike other polyphenols, anthocyanins constitute an exception because intact glycosides are recovered in the body (without deglycosylation prior to absorption).^{98–100} This may be explained by either the instability of the free aglycone form or by a specific mechanism of absorption for anthocyanins.

Several findings support the idea of specific transports for anthocyanins. A large dose size (700 μmol versus 350 μmol of total anthocyanins) significantly reduced plasma responses of both acylated and non-acylated anthocyanins, suggesting a saturation for their absorption at a dose of 350 μmol or even lower.⁴⁵ Seventeen of twenty tested anthocyanins were ligands of bilitranslocase, an organic anion membrane carrier for sulfobromophthalein, bilirubin, and nicotinic acid present in the gastric mucosa cells, with a higher affinity for mono- and di-glucosyl anthocyanins than the corresponding aglycone form. This indicates that bilitranslocase may be involved in the intestinal transport of anthocyanins.¹⁰¹ Finally, the urinary excretion of cyanidin glycosides was reduced after simultaneous ingestion of elderberry juice and sucrose, compared to juice alone, supporting the idea that intestinal sugar carriers may play a role in anthocyanin absorption.¹⁰² Sugar supplementation led to a saturation of the glucose transporter used also by the glucose moiety of the cyanidin glycoside molecule for its entrance into enterocytes, resulting in a decrease of anthocyanin intake.¹⁰²

The exact site of anthocyanin absorption is not fully known; recently it was suggested that the stomach is one of the preferential sites for the process in humans.¹⁰³ Interestingly, anthocyanin glycosides are efficiently absorbed in rats (up to 25%) from both stomach¹⁰⁴ and small intestine,¹⁰⁵ suggesting that anthocyanins have more than one site of absorption along the intestinal tract.

3.2.4.2.3 Metabolism

Anthocyanins appear in the blood circulation and urine as intact (glycosylated), methylated, glucurono- and/or sulfo-conjugated forms.^{98,106} This indicates that anthocyanin glycosides in the human body may undergo hydrolysis (to form free aglycone) and/or methylation and/or glucuronidation and/or sulfation (to form conjugated forms) as was reported for other polyphenol derivatives.¹⁰⁷ In polyphenol metabolism, these different steps are catalyzed by specific enzymes and mounting evidence indicates that these enzyme activities may be involved in anthocyanin metabolism as well.

Potential enzymes involved in anthocyanin metabolism — The lactase phlorizin hydrolase (LPH; EC 3.2.1.108) present only in the small intestine on the outside of the brush border membrane¹⁰⁸ and the cytosolic β -glucosidase (CBG; EC 3.2.1.1) found in many tissues, particularly in liver,¹⁰⁷ can catalyze the *deglycosylation* (or hydrolysis) of polyphenols. LPH may play a major role in polyphenol metabolism

because it deglycosylates a wide range of polyphenol glucosides. No clear evidence of the participation of these two enzymes in anthocyanin metabolism has been reported yet, probably related to the difficulty in detecting the unstable aglycones (products of hydrolysis of anthocyanin glycosides) in human plasma. In rats, however, aglycones (cyanidin and peonidin) were recently reported in plasma, urine, and jejunum.^{109,110}

Catechol-O-methyltransferase (COMT; EC 2.1.1.6) is located in many tissues and catalyzes the *methylation* of polyphenols. The methylation is a well-established pathway in the metabolism of flavonoids such as those that undergo 3',4'-dihydroxylation of ring B excreted as 3'-O-methyl ether metabolites in rat bile.¹¹¹ Recently, the apparent methylation of both cyanidin-3-glucoside and cyanidin-3-sambubioside (cyanidin is an anthocyanin with a 3',4'-dihydroxylation of ring B) to peonidin-3-glucoside and peonidin-3-sambubioside was reported in humans.⁹⁸ In rats, this transformation occurred mainly in the liver and was catalyzed by COMT.¹¹⁰

Among the large group of UDP-glucuronosyl transferases (UDPGT, UGT; EC 2.4.1.17) located in the endoplasmic reticula of many tissues, the UGT1A family is the one that is more specifically involved in the *glucuronidation* of polyphenols that occurs mainly in the liver, but also in the intestine and kidney. The presence of glucuronide forms of anthocyanins (peonidin monoglucuronide and cyanidin-3-glucoside monoglucuronide or pelargonidin monoglucuronides) was reported in the urine of humans after consumption of elderberry anthocyanins⁹⁸ or strawberry anthocyanins,¹⁰⁶ confirming the *in vivo* glucuronidation of anthocyanins. Two possible pathways may explain the formation of these monoglucuronides *in vivo* (Figure 3.2.3). In the first pathway, the anthocyanin glycoside would be first hydrolyzed to the aglycone in the intestine and then rapidly absorbed. In the liver, the aglycone may be methylated and then conjugated with glucuronic acid by action of a UDPGT.

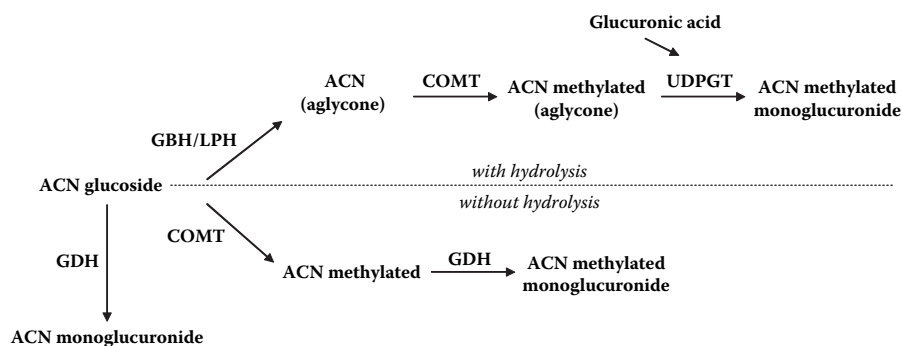


FIGURE 3.2.3 Two possible pathways for the formation of anthocyanin monoglucuronides (as proposed for cyanidin-3-glucoside and pelargonidin-3-glucoside).^{98,106} CAN = anthocyanin. GBH = cytosolic β -glucosidase. LPH = lactase phlorizin hydrolase. COMT = catechol-O-methyltransferase. UDPGT = UDP-glucuronosyl transferase. GDH = UDP-glucose dehydrogenase.

In the second pathway, the (methylated or not) anthocyanin glucoside may be absorbed intact and serve as a substrate for the UDP-glucose dehydrogenase enzyme (GDH; EC 1.1.1.22) that converts the glucose form into the corresponding glucuronide form,⁹⁸ possibly in both human liver and small intestine.

Phenol sulfotransferases (P-SST, SULT; EC 2.8.2.1) constitute a small group of cytosolic enzymes that are widely distributed and catalyze the *sulfation* of polyphenols. Recently, a sulfate conjugated form of anthocyanin (the sulfo-conjugate of pelargonidin) was detected in urine samples after subjects ingested strawberries providing 179 μmol pelargonidin-3-glucoside,¹⁰⁶ providing evidence of the existence of this pathway in anthocyanin metabolism.

Thus, anthocyanin metabolism seems to be controlled by several enzymatic pathways (Figure 3.2.3), but the sites (tissues) where these enzymatic activities take place still remain unclear. In that regard, knowledge about the tissue distribution and transport of anthocyanins and their metabolites *in vivo* may be helpful (see below). More investigations are thus necessary to fully elicit anthocyanin metabolism and its related actions. Conjugated forms of anthocyanins may dramatically alter the bioactive properties of these compounds, since glucuro- and sulfo-conjugations are considered the major detoxification pathways of many drugs and xenobiotic compounds.

3.2.4.2.4 Transport, Tissue Distribution and Excretion

Polyphenols are not found free in the blood but are bound to plasma proteins.⁶³ Albumin is the primary protein responsible for the bindings of several polyphenols and their metabolites (i.e., quercetin, kempferol, isorhamnetin), but no data are available for anthocyanins. However it is probable that anthocyanin derivatives also bind to albumin and the degree of that binding may affect the clearance rate and the delivery to tissues of these compounds as well.

When given either by the intraperitoneal or intravenous route, anthocyanins were rapidly distributed into rat tissues with primary accumulations in kidney, skin, liver, heart, and lung.¹¹² A recent study confirmed the deposition of anthocyanins in liver, kidney, and also in the stomach, jejunum and brain of rats fed blackberry anthocyanin-enriched diets.¹⁰⁹ The stomach exhibited only native blackberry anthocyanins, while in other organs (jejunum, liver, and kidney) native, methylated, and glucuronidated anthocyanins were identified. The highest proportion of methylated forms was found in liver. Circulating forms of anthocyanins in plasma included native anthocyanins, methylated and/or glucuronidated derivatives, and aglycones, providing evidence of anthocyanin metabolism in various tissues.¹¹²

Finally, the fact that anthocyanins can reach the brain represents a beginning of an explanation of the purported neuroprotection effects of anthocyanins. Anthocyanins may be eliminated via urinary and biliary excretion routes.^{109,112} The extent of elimination of anthocyanins via urine is usually very low (< 0.2% intake) in rats¹⁰⁹ and in humans,⁹⁸ indicating either a more pronounced elimination via the bile route or extensive metabolism. As mentioned earlier, in the colon, non-absorbed or biliary excreted anthocyanins can be metabolized by the intestinal microflora into simpler break-down compounds such as phenolic acids that may be (re)absorbed and conjugated with glycine, glucuronic acid, or sulfate and also exhibit some biological

activities.⁶³ Protocatechuic acid, a metabolite of cyanidin 3-O- β -glucoside, was present in rat plasma and its concentration was eight times higher than the intact form,¹¹⁰ and may result from degradation of the aglycone form of cyanidin 3-O- β -glucoside in the colon. However, in this study, the exact origin of protocatechuic acid was not determined.

3.2.4.3 BETALAINS

Betalains are water-soluble nitrogen-containing pigments responsible for the red and violet (betacyanins class) and yellow (betaxanthins class) colors found in many flowers, fruits, and occasionally in vegetative tissues of plants of most families of the Caryophyllales order (except the Caryophyllaceae and Molluginaceae families, which have anthocyanins instead).¹¹³

As for anthocyanins, betalains are found in vacuoles and cytosols of plant cells. From the various natural sources of betalains, beetroot (*Beta vulgaris*) and prickly pear cactus (*Opuntia ficus indica*) are the only edible sources of these compounds. In the food industry, betalains are less commonly used as natural colorants from plant sources than anthocyanins and carotenoids, probably related to their more restricted distribution in nature. To date, red beetroot is the only betalain source exploited for use as a natural food coloring agent. The major betalain in red beetroot is betanin (or betanidin 5-O- β -glucoside). Prickly pear fruits contain mainly (purple-red) betanin and (yellow-orange) indicaxanthin and the color of these fruits is directly related to the betanin-to-indicaxanthin ratio (99 to 1, 1 to 8, and 2 to 1, respectively in white, yellow, and red fruits).¹¹⁴

Betalains have shown strong antioxidant activities in biological environments such as membranes and LDLs,^{114,115} suggesting that the consumption of betalain-colored foods may exert protective effects against certain oxidative stress-related diseases (i.e., cancers) in humans. Beetroot has been used as a treatment for cancer in Europe for several centuries. The high content of betanin in red beetroot (300 to 600 mg/kg) may be the explanation for the purported cancer chemopreventive effects of beets.

Despite their potential health-promoting effects as dietary antioxidants, the fate of betalains in humans has been poorly studied. Betalain bioavailability was first demonstrated in humans by the appearance of betacyanins in urines after ingestion of beetroot extract¹¹⁶ and red beet juice,¹¹⁷ indicating that these compounds are indeed absorbed. Although intact betacyanins (betanin and isobetanin) appeared rapidly in human urine with a maximum excretion rate observed within 2.5 to 8 hr,¹¹⁷ betacyanin recoveries in human urine were usually low (< 1% of the dose) over 24 hr post-dose, suggesting that either the bioavailability of betacyanins from red beetroot is low or that renal clearance is a minor excretion route for these compounds.

After ingestion of cactus pear fruit pulp, both betanin and indicaxanthin were found in human plasma (with AUC_{0-12h} values of 0.46 and 29.2 nmol/hr/mL, respectively), partly associated with LDL, and in urine (3 and 76%, respectively, of the ingested compounds),¹¹⁸ indicating that indicaxanthin was better absorbed than betanin. The bioavailability of indicaxanthin from prickly pear fruit pulp was 20 times that of betanin, suggesting differences in the fates of the two classes of betalains (betacyanin and betaxanthins) in the human body. In rats, betanin appeared to be

easily degraded in the gastrointestinal tract,¹¹⁹ but no data are available about betaxanthin degradation in the intestine or even about betalain metabolite formation *in vivo*. Clearly, more investigations are necessary to better understand the intestinal transport, metabolism, and tissue distribution of betalains.

REFERENCES

1. Jackson, M.J., The assessment of bioavailability of micronutrients: introduction, *Eur. J. Clin. Nutr.*, 51, S1, 1997.
2. Britton, G., Structure and properties of carotenoids in relation to function, *FASEB J.*, 9, 1551, 1995.
3. During, A. and Harrison, E.H., Intestinal absorption and metabolism of carotenoids: insights from cell culture, *Arch. Biochem. Biophys.*, 430, 77, 2004.
4. Gugger, E.T. and Erdman, J.W., Intracellular β -carotene transport in bovine liver and intestine is not mediated by cytosolic proteins, *J. Nutr.*, 126, 1470, 1996.
5. Kläui, H. and Bauernfeind, J.C., Carotenoids as food colors, in *Carotenoids as Colorants and Vitamin A Precursors: Technological and Nutritional Applications*, Bauernfeind, J.C., Ed., Academic Press, New York, 1981, chap. 2.
6. Bowen, P.E., Mobarhan, S., and Smith, J.C., Carotenoid absorption in humans, *Methods Enzymol.*, 214, 3, 1993.
7. Rao, C.N. and Rao, B.S.N., Absorption of dietary carotenes in human subjects, *Am. J. Clin. Nutr.*, 23, 105, 1970.
8. Shiau, A. et al., Assesment of the intestinal retention of β -carotene in humans, *J. Am. Coll. Nutr.*, 13, 369, 1994.
9. Kostic, D., White, W.S., and Olson, J.A., Intestinal absorption, serum clearance, and interactions between lutein and β -carotene when administered to human adults in separate or combined oral doses, *Am. J. Clin. Nutr.*, 62, 604, 1995.
10. Van Vliet, T., Schreurs, W.H.P., and Van den Berg, H., Intestinal β -carotene absorption and cleavage in men: response of β -carotene and retinyl esters in the triglyceride-rich lipoprotein fraction after a single oral dose of β -carotene, *Am. J. Clin. Nutr.*, 62, 110, 1995.
11. Tyssandier, V. et al., Carotenoids, mostly the xanthophylls, exchange between plasma lipoproteins, *Int. J. Vitam. Nutr. Res.*, 72, 300, 2002.
12. Van Lieshout, M., West, C.E., and Van Breemen R.B., Isotopic tracer techniques for studying the bioavailability and bioefficacy of dietary carotenoids, particularly β -carotene, in humans: a review, *Am. J. Clin. Nutr.*, 77, 12, 2003.
13. Novotny, J.A. et al., Plasma appearance of labeled β -carotene, lutein, and retinol in humans after consumption of isotopically labeled kale, *J. Lipid Res.*, 46, 1896, 2005.
14. Novotny, J.A. et al., Compartmental analysis of the dynamics of β -carotene metabolism in an adult volunteer, *J. Lipid Res.*, 36, 1825, 1995.
15. Goodman, D.S. et al., The intestinal absorption and metabolism of vitamin A and β -carotene in man, *J. Clin. Invest.*, 45, 1615, 1966.
16. Blomstrand, R. and Werner, B., Studies on the intestinal absorption of radioactive β -carotene and vitamin A in man, *Scand. J. Clin. Lab. Invest.*, 19, 339, 1967.
17. Le Vuong, T. et al., Phytochemical research using accelerator mass spectrometry, *Nutr. Rev.*, 62, 375, 2004.
18. Dueker, S.R. et al., Long-term kinetic study of β -carotene, using accelerator mass spectrometry in an adult volunteer, *J. Lipid Res.*, 41, 1790, 2000.

19. Luchoomun, J. and Hussain, M.M., Assembly and secretion of CM by differentiated Caco-2 cells. Nascent triglycerides and preformed phospholipids are preferentially used for lipoprotein assembly, *J. Biol. Chem.*, 274, 19565, 1999.
20. During, A. and Harrison, E.H., An *in vitro* model to study the intestinal absorption of carotenoids, *Food Res. Int.*, 38, 1001, 2005.
21. During, A., Albaugh, G., and Smith, J.C., Characterization of β -carotene 15,15-dioxygenase activity in TC7 clone of human intestinal cell line Caco-2 cells, *Biochem. Biophys. Res. Commun.*, 249, 467, 1998.
22. During, A. et al., Carotenoid uptake and secretion by Caco-2 cells: β -carotene isomer selectivity and carotenoid interactions, *J. Lipid. Res.*, 43, 1086, 2002.
23. Salvini, S. et al., Functional characterization of three clones of the human intestinal Caco-2 cell line for dietary lipid processing, *Br. J. Nutr.*, 87, 211, 2002.
24. Garrett, D.A., Failla, M.L., and Samara, R.J., Development of an *in vitro* digestion method to assess carotenoid bioavailability from meals, *J. Agric. Food Chem.*, 47, 4301, 1999.
25. Ferruzzi, M.G., Failla, M.L., and Schwartz, S.J., Assessment of degradation and intestinal cell uptake of carotenoids and chlorophyll derivatives from spinach puree using an *in vitro* digestion and Caco-2 cell model, *J. Agric. Food Chem.*, 49, 2082, 2001.
26. Hedren, E., Diaz, V., and Svanberg, U., Estimation of carotenoid accessibility from carrots determined by an *in vitro* digestion method, *Eur. J. Clin. Nutr.*, 56, 425, 2002.
27. Hedren, E., Mulokozi, G., and Svanberg, U., *In vitro* accessibility of carotenes from green leafy vegetables cooked with sunflower oil or red palm oil, *Int. J. Food Sci. Nutr.*, 53, 445, 2002.
28. Tyssandier, V. et al., Processing of vegetable-borne carotenoids in the human stomach and duodenum, *Am. J. Physiol. Gastrointest. Liver Physiol.*, 284, G913, 2003.
29. Perez-Vicente, A., Gil-Izquierdo, A., and Garcia-Viguera, C., *In vitro* gastrointestinal digestion study of pomegranate juice: phenolic compounds, anthocyanins, and vitamin C, *J. Agric. Food Chem.*, 50, 2308, 2002.
30. McDougall, G.J. et al., Assessing potential bioavailability of raspberry anthocyanins using an *in vitro* digestion system, *J. Agric. Food Chem.*, 53, 5896, 2005.
31. Chitchumroonchokchai, C., Schwartz, S.J., and Failla, M.L., Assessment of lutein bioavailability from meals and a supplement using simulated digestion and Caco-2 human intestinal cells, *J. Nutr.*, 134, 2280, 2004.
32. Liu, C.S., Glahn, R.P., and Liu R.H., Assessment of carotenoid bioavailability of whole foods using a Caco-2 cell culture model coupled with an *in vitro* digestion, *J. Agric. Food Chem.*, 52, 4330, 2004.
33. Garrett, D.A., Failla, M.L., and Sarama, R.J., Estimation of carotenoid bioavailability from fresh stir-fried vegetables using an *in vitro* digestion/Caco-2 cell culture model, *J. Nutr. Biochem.*, 11, 574, 2000.
34. Sugawara, T. et al., Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human intestinal cells, *J. Nutr.*, 131, 2921, 2001.
35. Fahy, D.M., O'Callaghan, Y.C., and O'Brien, N.M., Phytosterols: lack of cytotoxicity but interference with β -carotene uptake in Caco-2 cells in culture, *Food Addit. Contam.*, 21, 42, 2004.
36. Reboul, E. et al., Enrichment of tomato paste with 6% tomato peel increases lycopene and β -carotene bioavailability in men, *J. Nutr.*, 135, 790, 2005.
37. Castenmiller, J.J.M. and West, C.E., Bioavailability and bioconversion of carotenoids, *Annu. Rev. Nutr.*, 18, 19, 1998.

38. Van het Hof, K. et al., Dietary factors that affect the bioavailability of carotenoids, *J. Nutr.*, 130, 503, 2000.
39. Borel, P., Factors affecting intestinal absorption of highly lipophilic food microconstituents (fat-soluble vitamins, carotenoids and phytosterols), *Clin. Chem. Lab. Med.*, 41, 979, 2003.
40. Levin, G. and Mokady, S., Incorporation of all-trans or 9-cis- β -carotene into mixed micelles *in vitro*, *Lipids*, 30, 177, 1995.
41. Gaziano, J.M. et al., Discrimination in absorption or transport of β -carotene isomers after oral supplementation with either all-trans or 9-cis β -carotene, *Am. J. Clin. Nutr.*, 61, 1248, 1995.
42. Chitchumroonchokchai, C. and Failla, M.L., Hydrolysis of zeaxanthin esters by carboxyl ester lipase during digestion facilitates micellarization and uptake of the xanthophylls by Caco-2 human intestinal cells, *J. Nutr.*, 136, 588, 2006.
43. Bowen, P.E. et al., Esterification does not impair lutein bioavailability in humans, *J. Nutr.*, 132, 3668, 2002.
44. Breithaupt, D.E. et al., Plasma response to a single dose of dietary β -cryptoxanthin esters from papaya (*Carica papaya* L.) or non-esterified β -cryptoxanthin in adult human subjects: a comparative study, *Br. J. Nutr.*, 90, 795, 2003.
45. Kurilich, A.C. et al., Plasma and urine responses are lower for acylated versus nonacylated anthocyanins from raw and cooked purple carrots, *J. Agric. Food Chem.*, 53, 6537, 2005.
46. Micozzi, M.S. et al., Plasma carotenoid response to chronic intake of selected foods and β -carotene supplements in men, *Am. J. Clin. Nutr.*, 55, 1120, 1992.
47. Van het Hof, K.H. et al., Bioavailability of carotenoids and folate from different vegetables: effect of disruption of the vegetable matrix, *Br. J. Nutr.*, 82, 203, 1999.
48. De Pee, S. et al., Orange fruit is more effective than are dark-green, leafy vegetables in increasing serum concentrations of retinol and β -carotene in school children in Indonesia, *Am. J. Clin. Nutr.*, 68, 1058, 1998.
49. Törrönen, R. et al., Serum β -carotene response to supplementation with raw carrots, carrot juice of purified β -carotene in healthy non-smoking women, *Nutr. Res.*, 16, 565, 1996.
50. Gartner, C., Stahl, W., and Sies, H., Lycopene is more bioavailable from tomato paste than from fresh tomatoes, *Am. J. Clin. Nutr.*, 66, 116, 1997.
51. Jayaranjan, P., Reddy, J.P. and Mohanram, M., Effect of dietary fat on absorption of β -carotene from green leafy vegetables in children, *Indian J. Med. Res.*, 70, 53, 1980.
52. Roodenburg, A.J. et al., Amount of fat in the diet affects bioavailability of lutein esters but not α -carotene, β -carotene and vitamin E in humans, *Am. J. Clin. Nutr.*, 71, 1187, 2000.
53. Borel, P. et al., Carotenoids in biological emulsions: solubility, surface-to-core distribution, and release from lipid droplets, *J. Lipid Res.*, 37, 250, 1996.
54. Borel, P. et al., Chylomicron β -carotene and retinyl palmitate responses are dramatically diminished when men ingest β -carotene with medium-chain rather than long-chain triglycerides, *J. Nutr.*, 128, 1861, 1998.
55. Baskaran, V., Sugawara, T., and Nagao, A., Phospholipids affect the intestinal absorption of carotenoids in mice, *Lipids*, 38, 705, 2003.
56. Weststrate, J.A. and van Het Hof, K.H., Sucrose polyester and plasma carotenoid concentrations in healthy subjects, *Am. J. Clin. Nutr.*, 62, 591, 1995.
57. Riedl, J. et al., Some dietary fibers reduce the absorption of carotenoids in women, *J. Nutr.*, 129, 2170, 1999.

58. Van den Berg, H. Carotenoid interactions, *Nutr. Rev.*, 57, 1, 1999.
59. Tyssandier, V., Lyan, B., and Borel, P., Main factors governing the transfer of carotenoids from emulsion lipid droplets to micelles, *Biochim. Biophys. Acta*, 1533, 285, 2001.
60. El-Gorab, M. and Underwood, B.A., Solubilization of β -carotene and retinol into aqueous solutions of mixed micelles, *Biochim. Biophys. Acta*, 306, 58, 1973.
61. Hollander, D. and Ruble, P.E., β -Carotene intestinal absorption: bile, fatty acid, pH, and flow rate effects on transport, *Am. J. Physiol.*, 235, E686, 1978.
62. Grolier, P. et al., The bioavailability of α - and β -carotene is affected by gut microflora in the rat, *Br. J. Nutr.*, 80, 199, 1998.
63. Manach, C. et al., Polyphenols: food sources and bioavailability, *Am. J. Clin. Nutr.*, 79, 727, 2004.
64. Lachance P., Dietary intake of carotenes and the carotene gap, *Clin. Nutr.*, 7, 118, 1988.
65. Van Poppel, G., Epidemiological evidence for β -carotene in prevention of cancer and cardiovascular disease, *Eur. J. Clin. Nutr.*, 50, 55S, 1996.
66. Seddon, J.M. et al., Dietary carotenoids, vitamins A, C, and E, and advanced age-related macular degeneration, *JAMA*, 272, 1413, 1994.
67. Krinsky, N.I. and Johnson, E.J., Carotenoid actions and their relation to health and disease, *Mol. Asp. Med.*, 26, 459, 2005.
68. El-Gorab, M.I., Underwood, B.A., and Loerch, J.D., The roles of bile salts in the uptake of β -carotene and retinol by rat everted gut sacs, *Biochim. Biophys. Acta*, 401, 265, 1975.
69. Kiefer, C. et al., A class B scavenger receptor mediates the cellular uptake of carotenoids in *Drosophila*, *Proc. Natl. Acad. Sci. USA*, 16, 10581, 2002.
70. Van Bennekum, A. et al., Class B scavenger receptor-mediated intestinal absorption of dietary β -carotene and cholesterol, *Biochem.*, 44, 4517, 2005.
71. Reboul, E. et al., Lutein transport by Caco-2 TC-7 cells occurs partly by a facilitated process involving the scavenger receptor class B type I (SR-BI), *Biochem. J.*, 387, 455, 2005.
72. During, A., Dawson, H.D., and Harrison, E.H., Carotenoid transport is decreased and expression of the lipid transporters SR-BI, NPC1L1, and ABCA1 is down-regulated in Caco-2 cells treated with ezetimibe, *J. Nutr.*, 135, 2305, 2005.
73. Altmann, S.W. et al., The identification of intestinal scavenger receptor class B, type I (SR-BI) by expression cloning and its role in cholesterol absorption, *Biochim. Biophys. Acta*, 1580, 77, 2002.
74. Altmann, S.W. et al., Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption, *Science*, 303, 1201, 2004.
75. During, A. and Harrison, E.H., Intestinal absorption and metabolism of carotenoids: insights from cell culture, *Arch. Biochem. Biophys.*, 430, 77, 2004.
76. Olson, J.A. and Hayaishi, O., The enzymatic cleavage of beta-carotene into vitamin A by soluble enzymes of rat liver and intestine, *Proc. Natl. Acad. Sci. USA*, 54, 1364, 1965.
77. Leuenberger, M.G., Engeloch-Jarret, C., and Woggon, W.-D., The reaction mechanism of the enzyme-catalyzed central cleavage of β -carotene to retinal, *Angew. Chem. Int. Ed.*, 40, 2613, 2001.
78. Nagao, A. et al., Stoichiometric conversion of all trans- β -carotene to retinal by pig intestinal extract, *Arch. Biochem. Biophys.*, 328, 57, 1996.
79. Wirtz, G.M. et al., The substrate specificity of β , β -carotene 15,15'-monooxygenase, *Helv. Chim. Acta*, 84, 3201, 2001.

80. Institute of Medicine, *Dietary Reference Intakes for Vitamin A, Vitamin K, Arsenic, Boron, Chromium, Copper, Iodine, Iron, Manganese, Molybdenum, Nickel, Silicon, Vanadium, and Zinc*, National Academies Press, Washington, 2001.
81. Barua, A.B. and Olson, J.A., β -Carotene is converted primarily to retinoids in rats *in vivo*, *J. Nutr.*, 130, 1996, 2000.
82. Handelman, G.J. et al., Characterization of products formed during the autoxidation of beta-carotene, *Free Radic. Biol. Med.*, 10, 427, 1991.
83. Kiefer, C. et al., Identification and characterization of a mammalian enzyme catalyzing the asymmetric oxidative cleavage of provitamin A, *J. Biol. Chem.*, 276, 14110, 2001.
84. Yeum, K.J. et al., The effect of α -tocopherol on the oxidative cleavage of β -carotene, *Free Radic. Biol. Med.*, 29, 105, 2000.
85. During, A. et al., β -Carotene 15,15-Dioxygenase activity in human tissues and cells: evidence of an iron dependency, *J. Nutr. Biochem.*, 12, 640, 2001.
86. During, A. et al., Assay of β -carotene 15,15-dioxygenase activity by reverse phase high-pressure liquid chromatography, *Anal. Biochem.*, 241, 199, 1996.
87. Lindqvist, A. and Andersson, S., Biochemical properties of purified recombinant human beta-carotene 15,15-monooxygenase, *J. Biol. Chem.*, 277, 23942, 2002.
88. Krinsky, N.I., Cornwell, D.G., and Oncley, J.I., The transport of vitamin A and carotenoids in human plasma, *Arch. Biochem. Biophys.*, 73, 233, 1958.
89. Clevidence, B.A. and Bieri, J.G., Association of carotenoids with human plasma lipoproteins, *Methods Enzymol.*, 214, 33, 1993.
90. Kaplan, L.A., Lau, J.M., and Stein, E.A., Carotenoid composition, concentrations, and relationship in various human organs, *Clin. Physiol. Biochem.*, 8, 1, 1990.
91. Parker, R.S., Carotenoids in human blood and tissues, *J. Nutr.*, 119, 101, 1989.
92. Clifford, M.N., Anthocyanins: nature, occurrence, and dietary burden, *J. Sci. Food Agric.*, 80, 1063, 2000.
93. Kang, S.Y. et al., Tart cherry anthocyanins inhibit tumor development in Apc(Min) mice and reduce proliferation of human colon cancer cells, *Cancer Lett.*, 194, 13, 2003.
94. Zhao, C. et al., Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth, *J. Agric. Food Chem.*, 52, 6122, 2004.
95. Kaplan, M. et al., Pomegranate juice supplementation to atherosclerotic mice reduces macrophage lipid peroxidation, cellular cholesterol accumulation and development of atherosclerosis, *J. Nutr.*, 131, 2082, 2001.
96. Youdim, K.A. et al., Short-term dietary supplementation of blueberry polyphenolics: beneficial effects on aging brain performance and peripheral tissue function, *Nutr. Neurosci.*, 3, 383, 2000.
97. Youdim, K.A. et al., Potential role of dietary flavonoids in reducing microvascular endothelium vulnerability to oxidative and inflammatory insults, *J. Nutr. Biochem.*, 13, 282, 2002.
98. Wu, X., Cao, G., and Prior, R.L., Absorption and metabolism of anthocyanins in elderly women after consumption of elderberry or blueberry, *J. Nutr.*, 132, 1865, 2002.
99. Matsumoto, H. et al., Orally administered delphinidin 3-rutinoside and cyanidin 3-rutinoside are directly absorbed in rats and humans and appear in the blood as the intact forms, *J. Agric. Food Chem.*, 49, 1546, 2001.
100. Murkovic, M., Adam, U., and Pfannhauser, W., Analysis of anthocyan glycosides in human serum, *Fresenius J. Anal. Chem.*, 366, 379, 2000.
101. Passamonti, S., Vrhovsek, U., and Mattivi F., The interaction of anthocyanins with bilitranslocase, *Biochem. Biophys. Res. Commun.*, 296, 631, 2002.

102. Müllerder, U., Murkovic, M., and Pfannhauser, W., Urinary excretion of cyanidin glycosides, *J. Biochem. Biophys. Methods*, 53, 61, 2002.
103. Passamonti, S. et al., The stomach as a site for anthocyanins absorption from food, *FEBS Lett.*, 544, 210, 2003.
104. Talavéra, S. et al., Anthocyanins are efficiently absorbed from the stomach in anesthetized rats, *J. Nutr.*, 133, 4178, 2003.
105. Talavéra, S. et al., Anthocyanins are efficiently absorbed from the small intestine in rats, *J. Nutr.*, 134, 2275, 2004.
106. Felgines, C., Strawberry anthocyanins are recovered in urine as glucuro- and sulfo-conjugates in humans, *J. Nutr.*, 133, 1296, 2003.
107. Scalbert, A. and Williamson, G., Dietary intake and bioavailability of polyphenols, *J. Nutr.*, 130, 2073S, 2000.
108. Day, A.J. et al., Dietary flavonoid and isoflavone glycosides are hydrolyzed by the lactase site of lactase phlorizin hydrolase, *FEBS Lett.*, 468, 166, 2000.
109. Talavéra, S. et al., Anthocyanin metabolism in rats and their distribution to digestive area, kidney, and brain, *J. Agric. Food Chem.*, 53, 3902, 2005.
110. Tsuda, T., Horio, F., and Osawa, T., Absorption and metabolism of cyanidin 3-O-beta-D-glucoside in rats, *FEBS Lett.*, 449, 179, 1999.
111. Shaw, I.C. and Griffiths, L.A., Identification of the major biliary metabolite of (+)-catechin in the rat, *Xenobiotica*, 10, 905, 1980.
112. Lietti, A. and Forni, G., Studies on *Vaccinium myrtillus* anthocyanosides. II. Aspects of anthocyanins pharmacokinetics in the rat, *Arzneimittelforschung*, 26, 832, 1976.
113. Strack, D., Vogt, T., and Schliemann, W., Recent advances in betalain research, *Phytochemistry*, 62, 247, 2003.
114. Butera, D. et al., Antioxidant activities of Sicilian prickly pear (*Opuntia ficus indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin, *J. Agric. Food Chem.*, 50, 6895, 2002.
115. Tesoriere, L. et al., Increased resistance to oxidation of betalain-enriched human low density lipoproteins, *Free Radic. Res.*, 37, 689, 2003.
116. Watts, A.R. et al., Beeturia and the biological fate of beetroot pigments, *Pharmacogenetics*, 3, 302, 1993.
117. Frank, T. et al., Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans, *Pharmacol. Res.*, 52, 290, 2005.
118. Tesoriere, L. et al., Absorption, excretion, and distribution of dietary antioxidant betalains in LDLs: potential health effects of betalains in humans, *Am. J. Clin. Nutr.*, 80, 941, 2004.
119. Krantz, C., Monier, M., and Wahlstrom, B., Absorption, excretion, metabolism and cardiovascular effects of beetroot extract in the rat, *Food Cosmet. Toxicol.*, 18, 363, 1980.

3.3 Antioxidant and Prooxidant Actions and Stabilities of Carotenoids *In Vitro* and *In Vivo* and Carotenoid Oxidation Products

Catherine Caris-Veyrat

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3.3.1 INTRODUCTION

Many reviews have been written about the antioxidant activities of carotenoids.¹⁻⁵ Some also describe prooxidant activities.^{6,7} In consequence, only selected points about this very broad subject will be presented in the first part of this chapter. Linked to these properties and important for food nutritional value is the stability of caro-

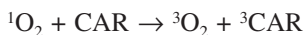
tenoids to oxygen. Some literature data on this subject will be presented later in this chapter. Finally, the recent increasing interest in carotenoid oxidation products will be reviewed in the last part of this chapter.

3.3.2 ANTIOXIDANT ACTIVITY

The ability of carotenoids to act as antioxidants is closely related to their long-chain conjugated polyene structures (see Section 2.2 in Chapter 2). Two main types of antioxidant actions can be distinguished: singlet oxygen quenching and reactions with radicals. The first mechanism occurs *in vivo* in plants and has been extensively studied *in vitro*. Reactions with radicals of different types have also been extensively studied *in vitro* under different conditions but their occurrence *in vivo* is still a matter of discussion.

3.3.2.1 *IN VITRO*

One of the natural roles of carotenoids in plants is to physically quench the highly reactive singlet oxygen produced from triplet oxygen and the triplet state of chlorophyll produced in presence of light. Through this action, carotenoids turn to an excited triplet state that returns to the ground state by losing the extra energy under the form of heat.



Carotenoid chemical structure is usually not affected by the physical quenching. Another mechanism can occur in which a carotenoid chemically quenches singlet oxygen and is thus transformed in derived products.^{8,9}

Among the carotenoids tested, lycopene has been shown *in vitro* to be the most efficient singlet oxygen quencher.¹⁰ More recently singlet oxygen quenching by carotenoids has been evaluated in model membrane systems¹¹ like liposomes.¹² Carotenoids chemically and physically quench singlet oxygen and are also able to react with free radicals¹² by electron transfer or addition reactions.² As lipophilic molecules, carotenoids are good potential antioxidants against radicals formed during lipid peroxidation *in vivo* and they have been widely studied in *in vitro* systems of lipid peroxidation.

Many different methods have been used to evaluate the antioxidant capacities of isolated molecules, carotenoids, and other natural antioxidants and of foods and food extracts containing antioxidants. It is not the purpose of this chapter to review all the methods, but some general points can be made. First, when using only one test to evaluate the antioxidant capacities of carotenoids, one should be very careful in the interpretation of obtained data.¹³ Indeed, different results can be obtained with different tests applied to the same molecules. At least two different methods should be used to evaluate the antioxidant activity of a molecule or a food extract.¹⁴ Second, lipophilicity is an important factor to consider in testing the antioxidant activities

of carotenoids. The method used to test their antioxidant capacities should take this important parameter into account and one should make sure that the carotenoids are well dissolved in the reaction media, especially when using a water-soluble radical (e.g., ABTS) or radical initiator (e.g., AAPH).

Another point is the concentration of the antioxidant which, in order to have physiological relevance, should be in the physiological range, i.e., not above 1 to 5 μM . Finally, when evaluating antioxidant capacities of foods and food extracts, one should take into account the presence of all the possible antioxidant molecules (phenols, vitamin E, etc.) to explain the results because interactions can occur between antioxidant molecules.

As mentioned earlier, physiological concentrations of carotenoids *in vivo* are in the micromolar range, mainly because of limited bioavailability. Also, the antioxidant efficiencies of carotenoids after absorption are probably limited. Concentrations before absorption are much higher and can justify possible antioxidant actions *in vivo*. To test this hypothesis, Vulcain et al.¹⁵ developed an *in vitro* system of lipid peroxidation in which the oxidative stress is of dietary origin (metmyoglobin from meat) and different types of antioxidants (carotenoids, phenols) are tested.

3.3.2.2 *In Vivo*

In plants, and more specifically in leaves, the antioxidant role of carotenoids is well demonstrated because they quench singlet oxygen as noted earlier. However, the antioxidant role of carotenoids in humans is still under debate.

Experimental evidence in humans is based upon intervention studies with diets enriched in carotenoids or carotenoid-containing foods. Oxidative stress biomarkers are measured in plasma or urine. The inhibition of low density lipoprotein (LDL) oxidation has been postulated as one mechanism by which antioxidants may prevent the development of atherosclerosis. Since carotenoids are transported mainly via LDL in blood, testing the susceptibility of carotenoid-loaded LDL to oxidation is a common method of evaluating the antioxidant activities of carotenoids *in vivo*. This type of study is more precisely of the *ex vivo* type because LDLs are extracted from plasma in order to be tested *in vitro* for oxidative sensitivity after the subjects are given a special diet.

Results obtained in *in vivo* and *ex vivo* experiments are of various types. Some studies have found positive effects^{16–18} of the consumption of *carotenoids* or foods containing *carotenoids* on the markers of *in vivo* oxidative stress, even in smokers.¹⁹ Other studies demonstrated no effects of carotenoid ingestion on oxidative stress biomarkers of lipid peroxidation.^{20,21} It should be noted that for studies using food, the activity observed may also be partly due to other antioxidant molecules in the food (phenols, antioxidant vitamins) or to the combination of actions of all the antioxidants in the food.

In atherosclerosis and other heart diseases, the role of carotenoids as antioxidants is probable,²² but for these types of diseases and also for other degenerative diseases such as cancers, non-antioxidant activities constitute other possible prevention mechanisms.²³ These activities are, for example, stimulation of gap junction communications between cells,²⁴ and the induction of detoxifying enzymes. The

effects may at least partly be due to effects of the carotenoids on the modulation of gene expression.²⁵

3.3.3 PROOXIDANT ACTIVITY

A molecule that has a prooxidant effect can be defined as a molecule that can react with reactive oxygen species (ROS) to form compounds more deleterious to biomolecules than the ROS alone. Possible prooxidant activity of carotenoids was for the first time mentioned by Burton and Ingold.²⁶ Since then, many other examples of loss of antioxidant activity or prooxidant activity have been illustrated and reviewed in the literature.^{27,28} Increasing oxygen partial pressure (pO_2) and/or carotenoid concentration can convert a carotenoid from antioxidant to prooxidant. Thus, depending on the environment, the same molecule can exert either antioxidant or prooxidant activity.^{6,7}

3.3.3.1 *IN VITRO*

Various types of cell-based *in vitro* studies have shown that carotenoids can exert prooxidant effects under certain conditions. Most of these studies show in fact decreases in antioxidant efficacy of carotenoids with increasing carotenoid concentration; examples of true prooxidant effects are rarer.²⁷ It is also important to pay attention to the experimental conditions and their biological relevance. Indeed, carotenoids have sometimes been proven to (1) exert prooxidant activity in an atmosphere of pure oxygen, (2) never occur *in vivo*, or (3) appear in concentrations that they would never reach *in vivo*.

Two main mechanisms by which a carotenoid can become a prooxidant have been proposed and reviewed:²⁷

1. Carotenoid reactions with ROS or RNS (reactive nitrogen species) would generate prooxidative products.⁹
2. High concentrations of carotenoids may increase the permeability of membranes to toxins and radicals.

An example of an experiment showing a loss of antioxidant efficacy is the work of Lowe et al.²⁹ who studied the abilities of supplementary carotenoids to protect cells against oxidatively induced DNA damage and maintain membrane integrity. Both lycopene and β -carotene afforded protection against DNA and membrane damage at physiological concentrations (1 to 3 μM). At higher concentrations (4 μM), the ability to protect the cells and membrane was lost and the authors claimed that “the presence of carotenoids may actually serve to increase the extent of DNA damage.”

Numbers of *in vitro* studies have tried to better explain how β -carotene may become a prooxidant *in vitro* in the presence of cigarette smoke. For instance, Palozza et al.³⁰ recently showed that at pO_2 ranging from 100 to 760 mmHg (pO_2 present in lung = 100 to 150 mmHg), β -carotene acted as a prooxidant in a dose-dependent manner in the presence of cigarette smoke condensate in rat lung microsomal membranes.

3.3.3.2 *IN VIVO*

No prooxidant role of carotenoids has been demonstrated *in vivo*, but carotenoids are suspected to exert a prooxidant activity role after the negative results of the CARET³¹ and ATBC³² studies, which noted increased risks of lung cancers in subjects taking supplements containing high doses of β -carotene. Subjects participating in the studies were either asbestos workers or heavy smokers, thus considered at risk. β -Carotene was given at doses closer to pharmacological than physiological levels. These two parameters certainly influenced the results of the two studies. Since then, researchers have tried to understand whether prooxidant effects of carotenoids appear in the presence of cigarette smoke,³³ and a number of *in vitro* studies have been focused on elucidating possible prooxidant mechanisms.

3.3.4 STABILITY TO OXYGEN

Carotenoids are known to be sensitive to oxygen, and are said to be unstable in presence of air. Because of their polyene structures, carotenoids are susceptible to reactions with the so-called ROS that may be radicals (O_2^- , $HO\cdot$) or non-radicals (H_2O_2 , $^1O_2$).

The dioxygen molecule exists in two forms: a triplet or ground state in which it is a stable biradical and a singlet or excited state in which it is not a radical. Reactions of carotenoids with singlet oxygen have already been presented in this chapter and we now focus on the reactions of carotenoids and oxygen in the ground or triplet state.

The reaction of a molecule with ground state oxygen is commonly called *autoxidation*, defined as “spontaneous oxidation” in air of a substance, not requiring a catalyst.”³⁴ However, because molecular oxygen is in triplet form under its ground state and most biomolecules are under singlet form, reactions between them are spin forbidden, although they can occur at very slow speeds (less than $10^{-5} \text{ M}^{-1} \text{ s}^{-1}$) over time frames of days.³⁴ Direct reactions between biomolecules such as carotenoids and dioxygen are either very slow or when quicker are probably catalyzed by metal traces or light.

The mechanism of the non-radical and non-metal-initiated autoxidation of carotenoids has been studied in experimental models using organic solvents and flows of oxygen. The first insights into the mechanism were given by El-Tinay and Chichester³⁵ who studied the reaction between β -carotene and oxygen in toluene at 60°C in the dark. They found overall zero-order reaction kinetics and an activation energy of 10.20 kcal/mol. Products of the reaction tentatively identified were 5,6- and 5,8-epoxides, 5,6;5',6'- and 5,8;5';8'-diepoxides of β -carotene, and polyene carbonyl (not further identified). Thus, the authors deduced that the site of the “initial attack” of oxygen was on the terminal carbon-carbon double bond that has the highest electron density in the polyene chain. The authors concluded that an “associated intermediate complex between β -carotene and oxygen” with a “free radical character” existed.

A similar experimental model (β -carotene, toluene, 60°C, oxygen, 120 min) was later tested by Handelman et al.³⁶ Using HPLC and mass analysis, the authors could

tentatively identify a 5,6-epoxide of β -carotene, apo-carotenals, and some compounds that were not identified. Using comparable experimental conditions but lower temperature (β -carotene, benzene or tetrachloromethane, 30°C, oxygen, darkness, 48 and 77 hours), Mordi et al.³⁷ published the identification of the mono- and di-epoxides of β -carotene previously detected together with (Z)-isomers, apo-carotenals of different lengths, volatile short compounds, and unidentified minor or oligomeric compounds. The authors suggested the result was a radical-mediated reaction in which the initiation process involved the formation of a diradical of β -carotene that evolved into the different products of the reaction.

The results of the three studies are not completely comparable because some experimental conditions were different and some (duration, presence or absence of light) were not indicated. However, very similar types of products were found. Note that in the first two examples, the influence of the experimental temperature used (60°C) on the autoxidation of β -carotene should be taken into account because thermal degradation can also yield epoxy-carotenoids and apo-carotenals.³⁸ However, the 30°C temperature was probably a factor influencing the reaction noted. Takahashi et al.³⁹ proposed a kinetic model for autoxidation of β -carotene in organic solutions on the basis of an autocatalytic free radical chain reaction mechanism. Another mechanism was also proposed according to which triplet oxygen is added to an undisturbed carotene.⁴⁰ The calculated energy needed for the reaction is 18 kcal/mol, which is in agreement with the experimental value of $E_a = 16$ kcal/mol.

The speed of autoxidation was compared for different carotenoids in an aqueous model system⁴¹ in which the carotenoids were adsorbed onto a C-18 solid phase and exposed to a continuous flow of water saturated with oxygen at 30°C. Major products of β -carotene were identified as (Z)-isomers, 13-(Z), 9-(Z), and a di-(Z) isomer; cleavage products were β -apo-13-carotenone and β -apo-14'-carotenal, and also β -carotene 5,8-epoxide and β -carotene 5,8-endoperoxide. The degradation of all the carotenoids followed zero-order reaction kinetics with the following relative rates: lycopene > β -cryptoxanthin > (E)- β -carotene > 9-(Z)- β -carotene.

Studies of the autoxidation of carotenoids in liposomal suspensions have also been performed since liposomes can mimic the environment of carotenoids *in vivo*. Kim et al. studied the autoxidation of lycopene,⁴² β -carotene,⁴³ and phytofluene⁴⁴ in liposomal suspensions and identified oxidative cleavage compounds. Stabilities to oxidation at room temperature of various carotenoids incorporated in pig liver microsomes have also been studied.⁴⁶ The model took into account membrane dynamics. After 3 hr of reactions, β -carotene and lycopene had completely degraded, whereas xanthophylls tested were shown to be more stable.

Interestingly, early examples of carotenoid autoxidation in the literature described the influence of lipids and other antioxidants on the autoxidation of carotenoids.^{46,47} In a study by Budowski et al.,⁴⁷ the influence of fat was found to be prooxidant. The oxidation of carotenoids was probably not only caused by molecular oxygen but also by lipid oxidation products. This now well-known phenomenon called co-oxidation has been studied in lipid solutions, in aqueous solutions catalyzed by enzymes,⁴⁸ and even in food systems in relation to carotenoid oxidation.⁴⁹ The influence of α -tocopherol on the autoxidation of carotenoids was also studied by Takahashi et al.⁵⁰ who showed that carotene oxidation was suppressed as

long as the tocopherol remained in the system, thus α -tocopherol protected β -carotene from autoxidation.

Studies on carotenoid autoxidation have been performed with metals. Gao and Kispert⁵¹ proposed a mechanism by which β -carotene is transformed into 5,8-peroxide- β -carotene, identified by LC-MS and ¹H NMR, when it is in presence of ferric iron (0.2 eq) and air in methylene chloride. The β -carotene disappeared after 10 min of reaction and the mechanism implies oxidation of the carotenoid with ferric iron to produce the carotenoid radical cation and ferrous iron followed by the reaction of molecular oxygen on the carotenoid radical cation. Radical-initiated autoxidations of carotenoids have also been studied using either radical generators like AIBN^{26,35,36} or NBS.³⁵

In conclusion, oxidation of carotenoids by molecular oxygen, the so-called autoxidation process, is a complex phenomenon that is probably initiated by an external factor (radical, metal, etc.) and for which different mechanisms have been proposed. The autoxidation of a carotenoid is important to take into account when studying antioxidant activity because it can lower the apparent antioxidant activity of a carotenoid.¹⁵

3.3.5 CAROTENOID OXIDATION PRODUCTS

3.3.5.1 OCCURRENCE IN NATURE

As described in the preceding paragraphs, oxidation products of carotenoids can be formed *in vitro* as a result of their antioxidant or prooxidant actions or after their autoxidation by molecular oxygen. They can also be found in nature, possibly as metabolites of carotenoids. Frequently encountered products are the monoepoxide in 5,6- or 5',6'-positions and the diepoxide in 5,6;5',6' positions or rearrangement products creating furanoid cycles in the 5,8 or 5',8' positions and 5,8;5',8' positions, respectively. Products like apo-carotenals and apo-carotenones issued from oxidative cleavages are also common oxidation products of carotenoids also found in nature. When the fission occurs on a cyclic bond, the C-40 carbon skeleton is retained and the products are called seco-carotenoids.

Seventy naturally occurring carotenoid epoxides have been referenced⁵² and 43 of them have been fully characterized. These compounds can be formally considered oxidation products as defined above, but they first have the status of carotenoids. They are indeed found *in vivo* and are possibly biosynthesized from the corresponding non-oxidized carotenoids. If carotenoids containing epoxide functions have been found in humans, the epoxidation reaction has not yet been proven to occur in humans.

Some 117 naturally occurring apo-carotenoids, 88 of which have been fully identified and another 6 naturally occurring seco-carotenoids have been referenced as carotenoids,⁵² thus representing around 15% of the carotenoids numbered to date (see Figure 3.3.1). This subfamily of carotenoids would be even larger if we consider the retinoids and norisoprenoids. However, these compounds are excluded by nomenclature rules^{53,54} that dictate that they are not deemed to be carotenoids because of the absence of two central methyl groups (at C20 and C20'). Retinoic acid, retinal,

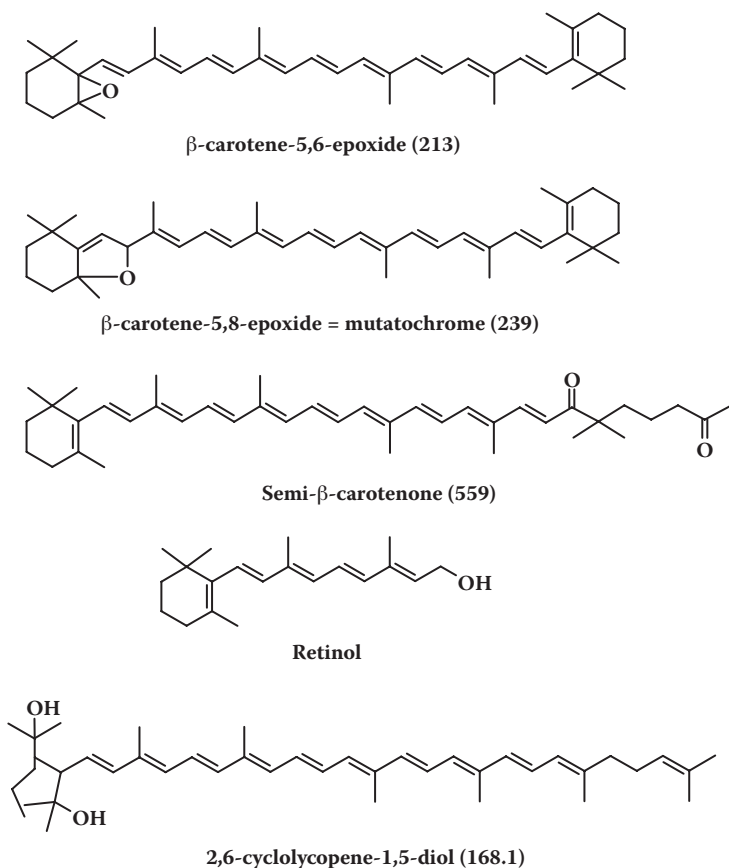


FIGURE 3.3.1 Chemical structures of carotenoid oxidation products occurring in nature. The compound numbers correspond to those cited in Britton, G. et al., *Carotenoids Handbook*.⁵²

and retinol (vitamin A) can be considered as carotenoid oxidation products of provitamin A carotenoids like β -carotene or β -cryptoxanthin; they are formed in humans by enzymatic cleavage.

Such an enzyme was partially purified via cloning of its encoding cDNAs from different organisms^{55–58} and was shown to be a monooxygenase-type enzyme.⁵⁹ Recently a 9',10'-monooxygenase from the ferret, a good model for studying carotenoid metabolism in humans, was shown to oxidize β -carotene and also 5-(Z) and 13-(Z) lycopene *in vitro* at the 9',10' carbon-carbon double bond,⁶⁰ thus producing the corresponding apo-carotenals and apo-lycopenals.

Apo-8'-lycopenal and apo-12'-lycopenal were found to occur *in vivo* in rat liver.⁶¹ The findings on the biosynthetic route to apo-carotenals in animals and the discovery of an enzyme catalyzing the asymmetric cleavage of carotenoids have generated heightened interest in carotenoid oxidation products and their possible biological role *in vivo*.

There are few naturally occurring oxidation products that do not belong to the families of epoxides or apo-carotenoids. One of those is the metabolite of lycopene known as 2,6-cyclo-lycopene-1,5 diol found in human plasma and at lower levels in tomato products.⁶²

3.3.5.2 FORMATION IN ABIOTIC SYSTEMS

Carotenoid oxidation products were first produced in abiotic systems in order to help in the structural identification of carotenoids.⁶³ Carotenoids were oxidized expressly to form small fragments that could be analyzed with the techniques then available. The chemical structures of the parent carotenoids were deduced from the structures of their oxidation products. For example, step-wise degradation by oxidation with alkaline potassium permanganate or chromic acid and ozonolysis were used to obtain large fragments of carotenoids that could be used to deduce the carotenoid structures.⁶³ More recently, oxidation by manganese dioxide was used as a chemical derivatization in microscale tests to elucidate the presence of allylic primary and secondary hydroxy groups in carotenoids, with the allylic aldehyde or ketone formed exhibiting a bathochromic shifted UV/Vis spectrum.⁶⁴

Carotenoid oxidation products were not only formed from the parent molecules in order to elucidate structure, they were also obtained by partial or total synthesis or by direct oxidation of carotenoid precursors. Thus, apo-8'-lycopenal was synthesized in 1966⁶⁵; more recently, the ozonide of canthaxanthin was obtained by chemical oxidation of canthaxanthin.⁶⁶

We developed and applied two oxidation methods to lycopene and β -carotene. The first chemical oxidation method was performed in biphasic medium using the potassium permanganate hydrophilic oxidant.⁶⁷ Cetyltrimethylammoniumbromide was the phase transfer agent used to achieve contact of the hydrophilic oxidant with the lycopene lipophilic carotenoid dissolved in methylene chloride/toluene (50/50, v/v).

Analysis of the reaction mixture with HPLC-DAD-MS revealed the presence of (1) apo-lycopenals and apo-lycopenones derived from a single oxidative cleavage, and (2) diapolyycopene-dials derived from a double oxidative cleavage of lycopene which thus lost the two ψ -end groups of lycopene. No apo-lycopenoic acids were found in the reaction mixture, indicating that under our experimental conditions, no further oxidation of apo-lycopenals by potassium permanganate occurred. This oxidation method allowed the production of the complete range of the possible apo-lycopenals formed by oxidative cleavage of conjugated carbon-carbon double bonds of lycopene and also six diapolyycopene-dials. This opens the possibility of preparing these compounds for further use by preparative HPLC.

In the second oxidation method, a metalloporphyrin was used to catalyze the carotenoid oxidation by molecular oxygen. Our focus was on the experimental modeling of the eccentric cleavage of carotenoids. We used ruthenium porphyrins as models of cytochrome P450 enzymes for the oxidation studies on lycopene⁶⁷ and β -carotene.⁶⁸ Ruthenium tetraphenylporphyrin catalyzed lycopene oxidation by molecular oxygen, producing (*Z*)-isomers, epoxides, apo-lycopenals, and apo-lycopenones.

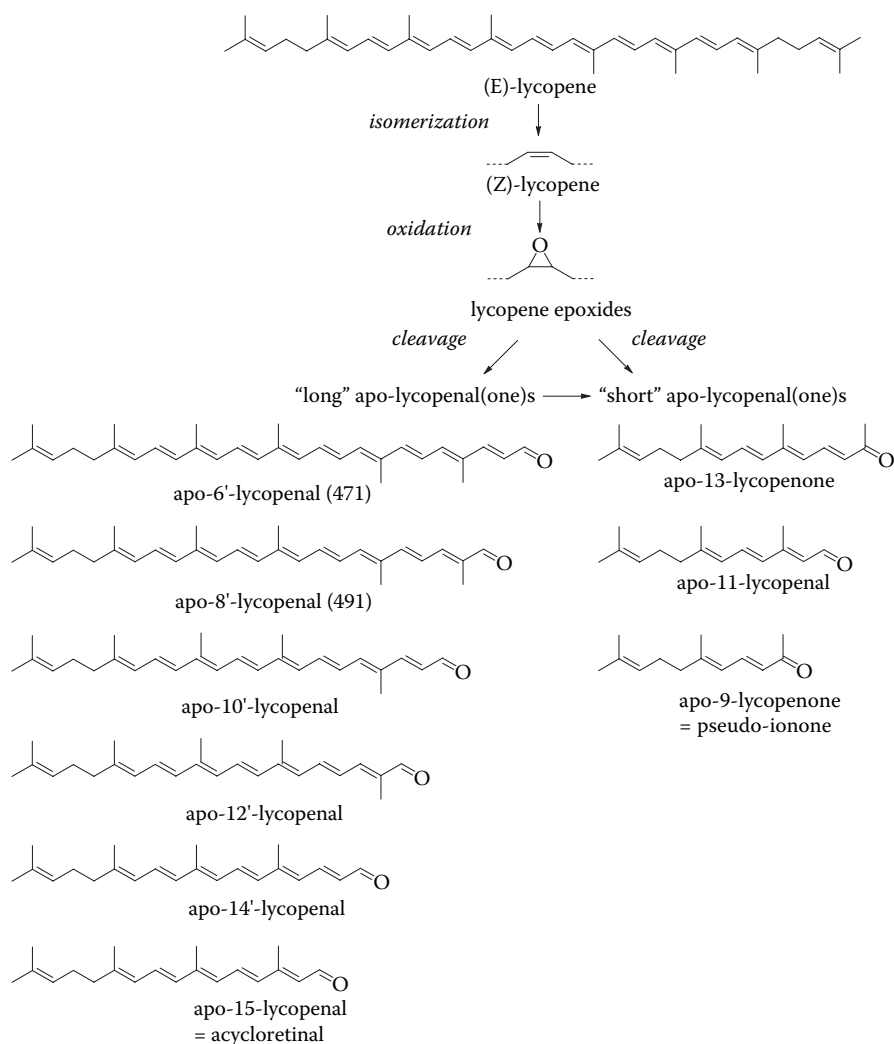


FIGURE 3.3.2 Hypothesized mechanism of formation of lycopene oxidation products in an abiotic system.⁶⁷ The compound numbers correspond to those cited in Britton, G. et al., *Carotenoids Handbook*.⁵²

The evolution in time of these different products suggests a possible mechanism (see Figure 3.3.2) for the oxidation of lycopene by molecular oxygen catalyzed by a metalloporphyrin: lycopene (Z)-isomers would be the first products formed and would be quickly oxidized into lycopene “in-chain” epoxides, which in turn would undergo oxidative cleavage to form apo-lycopenals or apo-lycopenones. The longer-chain apo-lycopenals (apo-15- to apo-6'-lycopenal) could be oxidized by the metalloporphyrin/O₂ system and thus be cleaved into shorter apo-lycopenals or apo-lycopenones (apo-9-lycopenone, apo-11-lycopenal, apo-13-lycopenone).

A similar system, but with a more hindered porphyrin (tetramesitylporphyrin = tetraphenylporphyrin bearing three methyl substituents in ortho and para positions on each phenyl group), was tested for β -carotene oxidation by molecular oxygen. This system was chosen to slow the oxidation process and thus make it possible to identify possible intermediates by HPLC-DAD-MS analysis. The system yielded the same product families as with lycopene, i.e., (Z)-isomers, epoxides, and β -apo-carotenals, together with new products tentatively attributed to diapocarotene-dials and 5,6- and/or 5,8-epoxides of β -apo-carotenals. The oxidation mechanism appeared more complex in this set-up.

The ready availability of carotenoid oxidation products through chemical methods will facilitate their use as standard identification tools in complex media such as biological fluids, and enable *in vitro* investigation of their biological activity. Moreover, these studies can help reveal the mechanisms by which they can be chemically or biochemically cleaved *in vivo*.

3.3.5.3 BIOLOGICAL EFFECTS *IN VIVO*

In the natural world, carotenoid oxidation products are important mediators presenting different properties. Volatile carotenoid-derived compounds such as norisoprenoids are well known for their aroma properties.⁶⁹ Examples include the cyclic norisoprenoid β -ionone and the non-cyclic pseudoionone or Neral. Carotenoid oxidation products are also important bioactive mediators for plant development,⁷⁰ the best-known example being abscisic acid. Apo-carotenoids act as visual and volatile signals to attract pollination and seed dispersal agents in the same way as carotenoids do, but they are also plant defense factors and signaling molecules for the regulation of plant architecture.

Vitamin A (retinol) and retinoic acid are carotenoid oxidation compounds that are very important for human health. The main functions of retinoids relate to vision and cellular differentiation. With the exception of retinoids, it was only about 10 years ago that other carotenoid oxidation products were first thought to possibly exert biological effects in humans and were implicated in the prevention^{71,72} or promotion of degenerative diseases. A review on this subject was recently published.⁷³

The underlying mechanisms involved in the activities of carotenoid oxidation products are due either to a possible role as precursors of retinoids that would be the active species for positive effects or to their own specific activities. This latter case is illustrated by the activity of non-provitamin A carotenoid oxidation products such as those derived from lycopene. However, biological effects of carotenoid oxidation products other than retinoids are only hypothesized *in vivo* in humans, which hypothesis has been used as the basic principle to justify *in vitro* studies of these compounds.

3.3.5.4 BIOLOGICAL EFFECTS *IN VITRO*

Different types of apparently beneficial activities have been demonstrated *in vitro* for carotenoid oxidation products, including induction of gap-junctional communications,⁷⁴ growth inhibition of leukemia and cancer cells,⁷⁵⁻⁷⁷ induction of apoptosis

in cultured cells,^{78,79} and gene activation.⁸⁰ However, to date, none of the compounds tested in the cited studies were detected *in vivo* in human plasma, other biological fluids, or tissues. The oxidative metabolite of lycopene, 2,6-cyclolycopene-1,5-diol found in humans also showed *in vitro* up-regulation of connexin 43 gene expression⁷² and *in vitro* growth inhibition of human prostate cancer cells.⁸¹

Carotenoid oxidation products are also supposed to have detrimental effects *in vivo*. As mentioned earlier, they are suspected to be involved in the adverse effects of high doses of β -carotene supplementation in smokers and asbestos workers (CARET³¹ and ATBC³² studies) and in smoke-exposed ferrets.⁸² The mechanisms potentially involved have been investigated *in vitro*. β -Apo-8'-carotenal, an eccentric cleavage oxidation product of β -carotene, was shown to be a strong inducer of CYP1A1 in rats, whereas β -carotene was not active.⁸³ Cytochrome P450 (CYP 450) enzymes thus induced could enhance the activation of carcinogens and the destruction of retinoic acid.⁸⁴

Another study showed that a mixture of oxidative metabolites of β -carotene, but not β -carotene, was able to increase the binding of benzo[a]pyrene to DNA.⁸⁵ Other mixtures of β -carotene cleavage products have been shown to induce oxidative stress *in vitro*,⁸⁶ exert cytotoxic⁸⁷ and genotoxic effects,⁸⁸ and inhibit gap junction inter-cellular communications.⁸⁹ It has been suggested that these detrimental effects could possibly occur *in vivo* following the intake of high doses of carotenoids.

Carotenoid oxidation products, as carotenoids, may exert protective or detrimental effects on human health. Efforts must be made to try to identify them *in vivo* where they may appear in lower quantities than carotenoids. Studies of abiotic systems can provide great support for their identification and the comprehension of their stability and reactivity.

REFERENCES

1. Palozza, P. and Krinsky, N.I., Antioxidant effects of carotenoids *in vivo* and *in vitro*: an overview, in *Methods in Enzymology*, Packer, L., Ed., Academic Press, 1992, 403.
2. Edge, R., McGarvey, D.J., and Truscott, T.G., The carotenoids as anti-oxidants: a review, *J. Photochem. Photobiol. B*, 41, 189, 1997.
3. Kiokias, S. and Gordon, M.H., Antioxidant properties of carotenoids *in vitro* and *in vivo*, *Food Rev. Int.*, 20, 99, 2004.
4. Young, A.J., Phillip, D.M., and Lowe, G.M., Carotenoid antioxidant activity, in *Carotenoids in Health and Disease*, Krinsky, N.I., Mayne, S.T., and Sies, H., Eds., Marcel Dekker, New York, 2004, 105.
5. Krinsky, N.I. and Johnson, E.J., Carotenoid actions and their relation to health and disease, *Molec. Asp. Med.*, 26, 459, 2005.
6. Martin, H.D. et al., Anti- and prooxidant properties of carotenoids, *J. Prakt. Chem.*, 341, 302, 1999.
7. Young, A.J. and Lowe, G.M., Antioxidant and prooxidant properties of carotenoids, *Arch. Biochem. Biophys.*, 385, 20, 2001.
8. Stratton, S.P., Schaefer, W.H., and Liebler, D.C., Isolation and identification of singlet oxygen oxidation products of beta-carotene, *Chem. Res. Toxicol.*, 6, 542, 1993.

9. Fiedor, J. et al., Cyclic endoperoxides of beta-carotene, potential pro-oxidants, as products of chemical quenching of singlet oxygen, *Biochim. Biophys. Acta Bioenerg.*, 1709, 1, 2005.
10. Di Mascio, P., Kaiser, S., and Sies, H., Lycopene as the most efficient biological carotenoid singlet oxygen quencher, *Arch. Biochem. Biophys.*, 274, 532, 1989.
11. Cantrell, A. et al., Singlet oxygen quenching by dietary carotenoids in a model membrane environment, *Arch. Biochem. Biophys.*, 412, 47, 2003.
12. Fukuzawa, K. et al., Rate constants for quenching singlet oxygen and activities for inhibiting lipid peroxidation of carotenoids and alpha-tocopherol in liposomes, *Lipids*, 33, 751, 1998.
13. Frankel, E.N. and Meyer, A.S., The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants, *J. Sci. Food Agric.*, 80, 1925, 2000.
14. Schlesier, K. et al., Assessment of antioxidant activity by using different *in vitro* methods, *Free Radic. Res.*, 36, 177, 2002.
15. Vulcain, E. et al., Inhibition of the metmyoglobin-induced peroxidation of linoleic acid by dietary antioxidants: action in the aqueous versus lipid phase, *Free Rad. Res.*, 39, 547, 2005.
16. Rao, A.V. and Agarwal, S., Bioavailability and *in vivo* antioxidant properties of lycopene from tomato products and their possible role in the prevention of cancer, *Nutr. Cancer Int. J.*, 31, 199, 1998.
17. Bub, A. et al., Moderate intervention with carotenoid-rich vegetable products reduces lipid peroxidation in men, *J. Nutr.*, 130, 2200, 2000.
18. Visioli, F. et al., Protective activity of tomato products on *in vivo* markers of lipid oxidation, *Eur. J. Nutr.*, 42, 201, 2003.
19. Kim, H.S. and Lee, B.M., Protective effects of antioxidant supplementation on plasma lipid peroxidation in smokers, *J. Toxicol. Environ. Health A*, 63, 583, 2001.
20. Gaziano, J.M. et al., Supplementation with beta-carotene *in vivo* and *in vitro* does not inhibit low density lipoprotein oxidation, *Atherosclerosis*, 112, 187, 1995.
21. Sutherland, W.H.F. et al., Supplementation with tomato juice increases plasma lycopene but does not alter susceptibility to oxidation of low-density lipoproteins from renal transplant recipients, *Clin. Nephrol.*, 52, 30, 1999.
22. Rao, A.V.R. and Agarwal, S., Role of antioxidant lycopene in cancer and heart disease, *J. Am. Coll. Nutr.*, 19, 563, 2000.
23. Stahl, W., Ale-Agha, N., and Polidori, M.C., Non-antioxidant properties of carotenoids, *Biol. Chem.*, 383, 553, 2002.
24. Stahl, W. and Sies, H., Effects of carotenoids and retinoids on gap junctional communication, *Biofactors*, 15, 95, 2001.
25. Bertram, J.S., Carotenoids and gene regulation, *Nutr. Rev.*, 57, 182, 1999.
26. Burton, G.W. and Ingold, K.U., β -carotene, an unusual type of lipid antioxidant, *Science*, 224, 569, 1984.
27. Lowe, G.M., Vlismas, K., and Young, A.J., Carotenoids as prooxidants, *Molec. Asp. Med.*, 24, 363, 2003.
28. Palozza, P., Evidence for pro-oxidant effects of carotenoids *in vitro* and *in vivo*: implications in health and disease, in *Carotenoids in Health and Disease*, Krinsky, S.T.M. and Sies, H., Eds., Marcel Dekker, New York, 2004, 127.
29. Lowe, G.M. et al., Lycopene and beta-carotene protect against oxidative damage in HT29 cells at low concentrations but rapidly lose this capacity at higher doses, *Free Rad. Res.*, 30, 141, 1999.

30. Palozza, P. et al., Dual role of beta-carotene in combination with cigarette smoke aqueous extract on the formation of mutagenic lipid peroxidation products in lung membranes: dependence on pO_2 , *Carcinogenesis*, 2006.
31. Omenn, G.S. et al., Effects of a combination of beta-carotene and vitamin A on lung cancer and cardiovascular disease, *New Engl. J. Med.*, 334, 1150, 1996.
32. The Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study Group, *New Engl. J. Med.*, 330, 1029, 1994.
33. Truscott, T.G., Beta-carotene and disease: a suggested prooxidant and anti-oxidant mechanism and speculations concerning its role in cigarette smoking, *J. Photochem. Photobiol. B*, 35, 233, 1996.
34. Miller, D.M., Buettner, G.R., and Aust, S.D., Transition metals as catalysts of "autoxidation" reactions, *Free Rad. Biol. Med.*, 8, 95, 1990.
35. El-Tinay, A.H. and Chichester, C.O., Oxidation of beta-carotene: site of initial attack, *J. Org. Chem.*, 56, 2290, 1970.
36. Handelman, G.J. et al., Characterization of products formed during the autoxidation of β -carotene, *Free Rad. Biol. Med.*, 10, 427, 1991.
37. Mordi, R.C. et al., Oxidative degradation of beta-carotene and β -apo-8-carotenal, *Tetrahedron*, 49, 911, 1993.
38. Onyewu, P.N., Ho, C.T., and Daun, H., Characterization of β -carotene thermal degradation products in a model food system, *J. Am. Oil Chem. Sci.*, 63, 1437, 1986.
39. Takahashi, A., Shibasaki-Kitakawa, N., and Yonemoto, T., Kinetic model for autoxidation of beta-carotene in organic solutions, *J. Am. Oil Chem. Sci.*, 76, 897, 1999.
40. Martin, H.D. et al., Chemistry of carotenoid oxidation and free radical reactions, *Pure Appl. Chem.*, 71, 2253, 1999.
41. Henry, L.K. et al., Effects of ozone and oxygen on the degradation of carotenoids in an aqueous model system, *J. Agric. Food Chem.*, 48, 5008, 2000.
42. Kim, S.J. et al., Formation of cleavage products by autoxidation of lycopene, *Lipids*, 36, 191, 2001.
43. Kim, S.J., Cleavage products formed through autoxidation of zeta-carotene in liposomal suspension, *Food Sci. Biotech.*, 13, 202, 2004.
44. Kim, S.J., Kim, H.L., and Jang, H.G., Oxidative cleavage products derived from phytofluene by pig liver homogenate, *Food Sci. Biotech.*, 14, 424, 2005.
45. Socaciu, C., Jessel, R., and Diehl, H.A., Carotenoid incorporation into microsomes: yields, stability and membrane dynamics, *Spectrochim. Acta A Mol. Biomol. Spect.*, 56, 2799, 2000.
46. Lisle, E.B., The effect of carcinogenic and other related compounds on the autoxidation of carotene and other autoxidizable systems, *Cancer Res.*, 11, 153, 1951.
47. Budowski, P. and Bondi, A., Autoxidation of carotene and vitamin A: influence of fat and antioxidants, *Arch. Biochem. Biophys.*, 89, 66, 1960.
48. Grosch, W. and Laskawy, G., Co-oxidation of carotenes requires one soybean lipoxygenase isoenzyme, *Biochim. Biophys. Acta*, 575, 439, 1979.
49. Perez-Galvez, A. and Minguez-Mosquera, M.I., Structure-reactivity relationship in the oxidation of carotenoid pigments of the pepper (*Capsicum annuum* L.), *J. Agric. Food Chem.*, 49, 4864, 2001.
50. Takahashi, A., Shibasaki-Kitakawa, N., and Yonemoto, T., A rigorous kinetic model for beta-carotene oxidation in the presence of an antioxidant, alpha-tocopherol, *J. Am. Oil Chem. Soc.*, 80, 1241, 2003.
51. Gao, Y.L. and Kispert, L.D., Reaction of carotenoids and ferric chloride: equilibria, isomerization, and products, *J. Phys. Chem. B*, 107, 5333, 2003.

52. Britton, G., Liaaen-Jensen, S., and Pfander, H., *Carotenoids Handbook*, Birkhäuser Verlag, Basel, 2004.
53. IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN), Nomenclature of carotenoids (rules approved 1974), *Pure Appl. Chem.*, 41, 107, 1975.
54. IUPAC Commission on the Nomenclature of Organic Chemistry and IUPAC-IUB Commission on Biochemical Nomenclature, in *Carotenoids*, Isler, O. Ed., Birkhäuser Verlag, Basel 1971, 851.
55. von Lintig, J. and Vogt, K., Filling the gap in vitamin A research: molecular identification of an enzyme cleaving beta-carotene to retinal, *J. Biol. Chem.*, 275, 11915, 2000.
56. Wyss, A. et al., Cloning and expression of b,b-carotene 15,15-dioxygenase, *Biochem. Biophys. Res. Comm.*, 271, 334, 2000.
57. Paik, J. et al., Expression and characterization of a murine enzyme able to cleave beta-carotene: formation of retinoids, *J. Biol. Chem.*, 276, 32160, 2001.
58. Redmond, T.M. et al., Identification, expression, and substrate specificity of a mammalian beta-carotene 15,15-dioxygenase, *J. Biol. Chem.*, 276, 6560, 2001.
59. Leuenberger, M.G., Engeloch-Jarret, C., and Woggon, W.D., The reaction mechanism of the enzyme-catalyzed central cleavage of beta-carotene to retinal, *Ang. Chem. Int. Ed.*, 40, 2614, 2001.
60. Hu, K.Q. et al., The biochemical characterization of ferret carotene-9,10 -monooxygenase catalyzing cleavage of carotenoids *in vitro* and *in vivo*, *J. Biol. Chem.*, 281, 19327, 2006.
61. Gajic, M. et al., Apo-8-lycopenal and apo-12-lycopenal are metabolic products of lycopene in rat liver, *J. Nutr. Biochem.*, 136, 1552, 2006.
62. Khachik, F. et al., Identification, quantification, and relative concentrations of carotenoids and their serum metabolites in human milk and serum, *Anal. Chem.*, 69, 1873, 1997.
63. Karrer, P. and Jucker, E., *Carotenoids*, Elsevier, Amsterdam, 1950.
64. Uebelhart, P. and Eugster, C.H., Synthesen von enantiomerenreinen Apoviola-xanthin-säuren, -olen- und -alen (Persicaxanthin, Sinensiaxanthin und b-Citraurin-epoxid) und ihrer furanoiden Umlagerungsprodukte, *Helv. Chim. Acta*, 71, 1983, 1988.
65. Surmatis, J.D. et al., Total synthesis of rhodovibrin (OH-P481), anhydorrhodovibrin (P481), and rhodopin, *J. Org. Chem.*, 31, 186, 1966.
66. Zurcher, M. and Pfander, H., Oxidation of carotenoids II. Ozonides as products of the oxidation of canthaxanthin, *Tetrahedron*, 55, 2307, 1999.
67. Caris-Veyrat, C. et al., Cleavage products of lycopene produced by *in vitro* oxidations: characterization and mechanisms of formation, *J. Agric. Food Chem.*, 51, 7318, 2003.
68. Caris-Veyrat, C. et al., Mild oxidative cleavage of beta, beta-carotene by dioxygen induced by a ruthenium porphyrin catalyst: characterization of products and of some possible intermediates, *New J. Chem.*, 25, 203, 2001.
69. Winterhalter, P. and Rouseff, R., *Carotenoid-Derived Aroma Compounds*, Series, A.S. Ed., American Chemical Society, Washington, 2001, 1.
70. Bouvier, F. et al., Oxidative tailoring of carotenoids: a prospect towards novel functions in plants, *Trends Plant Sci.*, 10, 187, 2005.
71. Khachik, F., Beecher, G., and Smith, J.C., Lutein, lycopene, and their oxidative metabolites in chemoprevention of cancer, *J. Cell. Biochem.*, 22, 236, 1995.
72. King, T.J. et al., Metabolites of dietary carotenoids as potential cancer preventive agents, *Pure Appl. Chem.*, 69, 2135, 1997.

73. Wang, X.D., Carotenoid oxidative/degradative products and their biological activities, in *Carotenoids in Health and Disease*, Krinsky, S.T.M. and Sies, H., Eds., Marcel Dekker, New York, 2004, 313.
74. Aust, O. et al., Lycopene oxidation product enhances gap junctional communication, *Food Chem. Toxicol.*, 41, 1399, 2003.
75. Hu, X.M. et al., Inhibition of growth and cholesterol synthesis in breast cancer cells by oxidation products of beta-carotene, *J. Nutr. Biochem.*, 9, 567, 1998.
76. Ben-Dor, A. et al., Effects of acyclo-retinoic acid and lycopene on activation of the retinoic acid receptor and proliferation of mammary cancer cells, *Arch. Biochem. Biophys.*, 391, 295, 2001.
77. Tibaduiza, E.C. et al., Excentric cleavage products of beta-carotene inhibit estrogen receptor positive and negative breast tumor cell growth *in vitro* and inhibit activator protein-1-mediated transcriptional activation, *J. Nutr.*, 132, 1368, 2002.
78. Sommerburg, O. et al., Oxidation derived metabolites of β -carotene are able to initiate apoptosis in S-type SHEP neuroblastoma cells, *Free Rad. Biol. Med.*, 33, S332, 2002.
79. Zhang, H. et al., A novel cleavage product formed by autooxidation of lycopene induces apoptosis in HL-60 cells., *Free Radic. Biol. Med.*, 35, 1653, 2003.
80. Ruhl, R. et al., Carotenoids and their metabolites are naturally occurring activators of gene expression via the pregnane X receptor, *Eur. J. Nutr.*, 43, 336, 2004.
81. Pastori, M. et al., Lycopene in association with alpha-tocopherol inhibits at physiological concentrations proliferation of prostate carcinoma cells, *Biochem. Biophys. Res. Com.*, 250, 582, 1998.
82. Wang, X.D. et al., Retinoid signaling and activator protein-1 expression in ferrets given beta-carotene supplements and exposed to tobacco smoke, *J. Natl. Cancer Inst.*, 91, 60, 1999.
83. Gradelet, S. et al., Beta-apo-8-carotenal, but not beta-carotene, is a strong inducer of liver cytochromes P4501A1 and 1A2 in rat, *Xenobiotica*, 26, 909, 1996.
84. Liu, C., Russell, R.M., and Wang, X.D., Exposing ferrets to cigarette smoke and a pharmacological dose of beta-carotene supplementation enhance *in vitro* retinoic acid catabolism in lungs via induction of cytochrome P450 enzymes, *J. Nutr.*, 133, 173, 2003.
85. Salgo, M.G. et al., Beta carotene and its oxidation products have different effects on microsome mediated binding of benzo[a]pyrene to DNA, *Free Rad. Biol. Med.*, 26, 162, 1999.
86. Augustin, W. et al., Beta-carotene cleavage products induce oxidative stress by impairing mitochondrial functions: brain mitochondria are more sensitive than liver mitochondria, *Free Rad. Biol. Med.*, 33, S326, 2002.
87. Hurst, J.S. et al., Toxicity of oxidized beta-carotene to cultured human cells, *Exp. Eye Res.*, 81, 239, 2005.
88. Sommerburg, O. et al., Cytotoxic and genotoxic effects due to beta-carotene cleavage products possibly formed in inflamed lung tissue, *Free Rad. Biol. Med.*, 36, S56, 2004.
89. Yeh, S.L. and Hu, M.L., Oxidized [beta]-carotene inhibits gap junction intercellular communication in the human lung adenocarcinoma cell line A549, *Food Chem. Toxicol.*, 41, 1677, 2003.

Section 4

*Food Pigments:
Major Sources and Stability
during Storage and Processing*

4.1 Chlorophylls in Foods: Sources and Stability

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4.1.1 INTRODUCTION

Green vegetables and fruits constitute food sources for carbohydrates, vitamins, minerals, and dietary fiber. They have low fat and protein contents and are gaining increasing importance in the human diet. They are considered food colorants and ingredients that produce attractive naturally colored products and can reinforce color. However, the consumption of foods rich in phytochemicals including natural pigments has also been related to certain biological functions, health benefits, and diet trends.

Natural food colors have become pleasant connotations for consumers over the years since the replacement of synthetic pigments would result in healthier foods. Due to the increasing public concern for food safety, chlorophyll must be considered in the search for food-grade natural green colorants. The addition of dried green vegetables or chlorophyll extracts would be valuable for restoring or reinforcing the natural levels of pigments because the preservation of the green color during food processing is sometimes poor and can lead to an undesirable change of product color.¹

Despite the ubiquitous distribution of chlorophylls in all photosynthetic plants, quantitative information exists only for a few vegetables. The most common edible plants lack definitive data and consequently no information is available about chlorophyll distribution in current food composition tables. Still more difficult is to find analytical data in literature about the individual amounts of chlorophyll a and b and their respective derivatives.

It is well known that chlorophyll contents in plants vary greatly, depending upon metabolic processes that are reliant primarily on physiological, genetic, and biochemical factors but also on external influences: climatic factors, agricultural practices, season, geographic growing region, post-harvest handling, storage, and plant part considered. There are pronounced differences in chlorophyll contents among stems, leaves, peels, and pulps of vegetables and fruits. Absolute values reported in the literature should be used only as general guides due to wide biological variabilities.

In general, it can be stated that higher chlorophyll contents are mainly found in leaves and, the more colored they are, the higher amounts of chlorophyll they have. Therefore, the highest amounts (as much as 1.5 to 2.0% fresh weight) can be found in fully developed leaves of spinach, parsley, kale, and green cabbage, for example. Senescence of plants and ripening of fruits causes a sharp decrease in chlorophyll content due to the programmed natural biochemical process of chlorophyll breakdown, which ensures their complete transformation into colorless catabolites. However, some fruits are exceptions and retain high chlorophyll contents even in the ripe stages: avocado, cucumber, kiwi, green-fleshed muskmelon (*Cucumis melo*), and certain tomato, apple, and pear cultivars. Stay-green mutant species have been the foci of many studies in recent years and have increased our knowledge about the genes that control chlorophyll degradation.²

The aim of this chapter is to provide a concise synopsis of the factors that promote degradation during post-harvest handling, processing, and storage, and the strategies to preserve the green color of the most commonly consumed chlorophyll-rich foods. Some considerations about the production and characteristics of natural and semi-synthetic chlorophyll derivatives for use as food colorants are also presented.

4.1.2 FOOD SOURCES RICH IN CHLOROPHYLLS

The wide distribution of chlorophylls throughout the plant kingdom facilitates the scrutiny of chlorophyll-rich vegetables for human nutrition with a view to their direct consumption. However, until now, little standardized information was available in the literature regarding the absolute total chlorophyll contents and the ratios of chlorophylls a and b in raw materials. The different analytical methods of extraction and quantification and the lack of data about moisture contents (that may vary considerably among varieties and preparations) can also influence the final contents of pigments. These factors have largely contributed to the discrepancies found in similar food samples.

Therefore, data obtained from raw material should be accompanied by data about water content or alternatively should be expressed on a dry weight basis but this demands an additional step of drying fresh food. Additionally, in some investigations related to changes in chlorophyll contents during processing and storage, the percentage of chlorophyll retention is reported but absolute values are not. Published tables on chlorophyll contents still show gaps and systematical research is strongly recommended. All these observations imply that it is extremely difficult to collect information and achieve accurate estimates of chlorophyll consumption by humans.

Not-senescent and fresh-cut plants are almost devoid of degradation products like pheophytins and pheophorbides because chlorophylls associated with caro-

tenoids and proteins within plant structures are very stable against the photodegradative environment. All higher plants contain both a and b chlorophylls; the a form generally predominates by a 3:1 margin. The generation of accurate analytical data started to be explored only with the development of sophisticated analytical methods such as HPLC and MS, which enabled the separation, identification, and correct quantification of individual chlorophyll derivatives, namely pheophytins and pheophorbides. Most analytical results are reported as total chlorophyll contents or the sum of chlorophyll a plus chlorophyll b.^{3,4}

Quantitative chlorophyll content data for some fruits and vegetables have been collected over the years and are listed in reviews or can be found in specific reports, mainly in the context of effects of processing. Among the still limited data available, for example, Gross⁵ presents in her book extended review of various edible plants citing the amounts of chlorophyll a, chlorophyll b, and total chlorophyll analyzed by several authors, expressed as dry and/or fresh weight, taking into consideration different species, cultivars, and plant parts. In addition, detailed discussions about color changes during ontogenesis and influences of environmental factors are presented for some commonly consumed vegetables. The same author earlier published a list of 13 fruits, presenting the total chlorophyll amounts (fresh weight basis) according to the cultivar, part of the fruit, and ripening stage. She also discussed the changes in chlorophyll and carotenoid contents during the ripening of certain fruits.⁶

Several authors have not only dealt with the quantification of chlorophylls in fresh foods, but focused their studies on pigment-level influencing factors such as food preparation, methods of cooking, plant species, harvest time, stage of development, and part of the plant considered. In fact, Bohn et al.⁷ reported chlorophyll contents in 22 frequently consumed vegetables and fruits, although the objective was to evaluate the relevance of chlorophylls as a source of dietary magnesium. Burns, Fraser and Bramley⁸ used HPLC to analyze different pigment contents (carotenoids, tocopherols and chlorophylls) expressed on a dry weight basis in 10 commonly consumed fruits and vegetables. Among broccoli, green pepper, and lettuce, only the lettuce contained significant amounts of chlorophylls.

Kopsell and colleagues⁹ investigated 23 leafy *Brassica oleracea* cultivars over two growing seasons to characterize the variability of their chlorophyll, lutein, and β -carotene contents, aiming to evaluate the correlation of levels of pigments and genetic differences or environmental factors. Turkmen et al.¹⁰ reported pigment and color changes caused by boiling, steaming, and microwaving treatments of six green vegetables (squash, green beans, peas, leek, broccoli, spinach). In recent years the amounts of chlorophylls in vegetables and fruits have been reported in literature but, in general, data are not easily comparable.

A selection of some edible vegetables containing the highest chlorophyll contents was gathered from these publications and is shown in Table 4.1.1.^{7-9,11-19} Original data were collected from literature and recalculated to the same unit in order to facilitate comparisons. Spinach, kale, mustard, and parsley corresponded to the richest chlorophyll-containing leafy vegetables, and reported amounts varied between 60 and 200 mg per 100 g fresh weight. Other parts of the plants like stems, seeds, fruits, pulp, and pods contained lower amounts, around 1 to 8 mg per 100 g fresh weight.

TABLE 4.1.1

Total Chlorophyll Contents of Some Vegetables and Fruits

Common Name (Species)	Chlorophyll Content (mg/100 g fresh wt)	Reference
Dasheen (<i>Colocasia esculenta</i>)	1262 ^a	Maharaj, Sankat (1996) ¹¹
Kale (<i>Brassica oleracea</i>)	187	Khachik, Beecher, Whittaker (1986) ¹²
	181	Khachik, Beecher, Whittaker (1986) ¹²
	154–367	Kopsell et al. (2004) ⁹
	104–262	Kopsell et al. (2004) ⁹
Spinach (<i>Spinacia oleracea</i> L.)	127	Khachik, Beecher, Whittaker (1986) ¹²
	79	Bohn et al. (2004) ⁷
	180	Yamauchi, Watada (1985) ¹³
	92	Piagentini, Güemes, Pirovani (2002) ¹⁴
Mustard (<i>Brassica juncea</i> L.)	125	Kaur, Manjerkar (1975) ¹⁵
Parsley (<i>Petroselinum crispum</i>)	63	Bohn et al. (2004) ⁷
Rocket salad (<i>Eruca vesicaria sativa</i> Mill)	41	Bohn et al. (2004) ⁷
Cress (<i>Lepidium sativum</i>)	31	Bohn et al. (2004) ⁷
Lettuce (<i>Lactuca sativa</i>)	288 ^a	Burns et al. (2003) ⁸
	25	Bohn et al. (2004) ⁷
	2–109	Mou (2005) ¹⁶
Broccoli (<i>Brassica oleracea</i>)	8	Khachik, Beecher, Whittaker (1986) ¹²
	2 ^a	Burns et al. (2003) ⁸
Endive (<i>Cichorium endivia</i>)	10	Bohn et al. (2004) ⁷
Leek (<i>Allium ampeloprasum porrum</i> L.)	9	Bohn et al. (2004) ⁷
Basil (<i>Ocimum basilicum</i>)	281 ^a	Rocha, Lebert, Marty-Audouim (1993) ¹⁷
Green pepper (<i>Capsicum annum</i> L.)	80 ^a	Burns et al. (2003) ⁸
Brussels sprout (<i>Brassica oleracea</i>)	6	Khachik, Beecher, Whittaker (1986) ¹²
	5	Khachik, Beecher, Whittaker (1986) ¹²
Green pea (<i>Pisum sativum</i>)	5	Bohn et al. (2004) ⁷
Sugar pea (<i>Pisum sativum</i>)	8	Bohn et al. (2004) ⁷
Green bean (<i>Phaseolus vulgaris</i> , L.)	8	Bohn et al. (2004) ⁷
Avocado (<i>Persea americana</i>)	40	Gross, Ohad (1983) ¹⁸
Kiwi (<i>Actinidia chinensis</i>)	2	Bohn et al. (2004) ⁷
Grape (<i>Vitis vinifera</i>)	1	Bohn et al. (2004) ⁷
Grape (<i>Vitis vinifera</i>) Riesling	2	Gross (1984) ¹⁹

Original data recalculated to mg/100 g.

^a Data expressed on dry weight basis.

There are some other less-known chlorophyll-rich vegetables consumed in specific regions of the world. For instance, dasheen bush (*Colocasia esculenta* Linn Schott var. *esculenta*), a popular vegetable cultivated in Trinidad and Tobago and other tropical countries, has around 1.26 g total chlorophyll per 100 g dry weight, similar to spinach.¹¹ Probably, the number of high chlorophyll-containing leaves will increase as more vegetables are investigated as potential alternative food sources.

Regardless of the final product type, chlorophyll-containing green vegetables can be either added directly to foods as ingredients or added as color extracts and in this case becoming food additives. Natural chlorophylls are considered safe due to their long history of human consumption as regular components of all green vegetables.

Considering the high amounts of chlorophylls in spinach leaves, dehydrated spinach is frequently added to foods as an easy and inexpensive way to impart a natural green color and a healthy ingredient to a specific food (e.g., dried spinach in powder form as an ingredient of green pasta). However, a more widespread use of natural chlorophyll is still impracticable due to lack of stability of the green pigments during food processing and storage.

Commercially produced metal-substituted chlorophylls such as copper chlorophylls and copper chlorophyllins that can be obtained by chemical modification of natural chlorophylls have better stability, solubility, and tinctorial strength, but they cannot be considered natural food colorants and will be discussed later.

4.1.3 CHLOROPHYLL STABILITY DURING STORAGE AND PROCESSING

Leafy vegetables and some fruits in particular are rich sources of chlorophylls. However, they are ranked among the most perishable post-harvest products and must be consumed within a few days after harvest or subjected to preservation methods to extend their freshness. Their typical green color is, if not the most important sensory attribute, an extremely important parameter of quality. Any discoloration can lead to rejection by consumers as the bright green color is intuitively linked with freshness.

All leafy horticultural commodities show high transpiration rates due to their large surface areas and this poses the additional problem of accelerating senescence. Therefore, a great challenge of most processes has been the maintenance of the original food color along with other characteristics perceived as quality issues by consumers.

Approaches for lengthening the shelf lives of fresh-packed vegetables are freezing, cooling, application of modified atmosphere packaging, and gamma irradiation. Chlorination, which has been used industrially to wash and sort fruits and vegetables, is not well tolerated by some leaves.²⁰ In recent years, consumption of fresh ready-to-eat and frozen vegetables has increased greatly, following dietary recommendations for eating healthier foods, creating a demand for fresh-cut, hygienized, and other minimally processed vegetables.

For example, the preservation of green color and the nutritional and sensory quality of unwrapped fresh-cut broccoli heads (*Brassica oleracea* L.) were prolonged from 5 to 28 days at 1°C under a modified atmosphere created by micro-

perforated and non-perforated polypropylene films.²¹ Polymeric films and CO₂-enriched atmosphere inside a package reduce gas and vapor permeability, and slow respiration and ethylene production. Furthermore, the addition of agents like KMnO₄, which absorbs ethylene, and sorbitol, which reduces water activity may avoid water loss and diffusion of volatile off-flavors and odors produced during storage under anaerobic conditions.^{14,20}

Among several types of packaging, low-density polyethylene (LDPE) films showed the best results in extending shelf life of fresh broccoli.²² Good maintenance of chlorophyll content was also found in sugar pea pods in modified atmosphere packaging.²³ Modified atmosphere was also beneficial in extending the shelf life of green asparagus spears (*Asparagus officinalis* L.) up to 4 weeks when combined with refrigeration temperature (2°C).²⁴

Low temperature storage preserves chlorophylls; however, cold stored products may develop chilling injury symptoms. For example, green beans stored at 4°C maintained brighter green color and better quality than those stored at 8 or 12°C, but developed latent chilling injuries after 8 days of storage that became evident when the pods were transferred to 20°C.²⁵

Many companies now are looking to irradiation as an effective means of reducing microbial contamination. The feasibility of gamma radiation in combination with storage at 8 to 10°C was studied to ensure microbiological safety and maintain physicochemical and sensory characteristics of fresh raw coriander leaves with a radiation dose of 1.0 kGy. This treatment was efficient for bacterial decontamination and elimination of potential pathogens without altering sensory attributes like color.²⁶ The advantage of irradiation is that it causes fewer changes to the sensory attributes of spices than blanching, application of fumigants, steam, microwaves, and other treatments.

Freezing is a very efficient method of preserving vegetables for long periods, but even at usual freezing temperatures (−18°C) vegetables undergo changes in their nutritional and organoleptic characteristics such as development of off-flavors, decreased firmness, color losses, and diminution of vitamins. Several chemical and enzymatic reactions may cause chlorophyll degradation, but often these changes are associated with enzymatic activity and therefore inactivation of enzymatic activities was considered essential. Consequently, the main preservation techniques are blanching, followed by freezing, and canning.²⁷

Blanching is a very common thermal treatment to inactivate enzymes that catalyze down-grading reactions during storage. Chlorophyllase catalyzes the hydrolysis of the phytol ester from the porphyrin ring, forming chlorophyllides. Magnesium dechelataase catalyzes the removal of the Mg²⁺ ion from the tetrapyrrolic ring, leading to the formation of pheophytins and pheophorbides. Oxidative enzymes such as lipoxygenases, chlorophyll oxidase, and peroxidases contribute to the loss of green color and accumulation of oxidized chlorophyll catabolites (13²-OH-chlorophylls).^{28,29}

Considering the increasing demands for seasoning herbs, Lisiewska, Kmiecik, and Shupski³³ performed studies to prolong the shelf life of dill by protecting the greenness. Dill could be stored up to 6 months at −20°C without blanching but the authors recommend inactivation of enzymes if longer periods of storage are needed.

Although blanching produces a reduction of the concentrations of oxygen in plant tissues and thus better retention of pigments at the first moment, the acidic medium progressively promotes the replacement of the centrally located magnesium ion from the chlorophyll molecule by two atoms of hydrogen, producing pheophytins after prolonged storage. Pyropheophytins and phyropheophorbides that lack the carbomethoxy group at C-10 are also readily encountered even after mild processing conditions. Additionally, photooxidation and other oxidative reactions seem to be involved in later stages of the degreening process by contact with oxidized lipids in the presence of oxygen.¹¹

In general, thermally processed green vegetables exhibit faded colors and poor color retention because the disruption of the plant cells and tissues and the denaturation of proteins attached to the chlorophyll molecules exposes the pigments to a harsh environment. Food ingredients and processing conditions greatly influence the rate and pathway of chlorophyll degradation. Chlorophylls are extremely sensitive to low pH, high temperatures, and length of heat treatment, in addition to the presence of salts, enzymes and surface-active ions.

Various conditions of food processing induce structural and chemical changes on cells and tissues that often result in dramatic color changes. Presumably, chlorophylls quickly undergo a combination of distinct types of enzymatic and/or chemical reactions, finally resulting in unwelcome brownish degradation products such as pheophytins and pheophorbides. In metabolically active tissues, these catabolites can be further enzymatically degraded to colorless compounds and the color changes.^{28,31} Figure 4.1.1 presents a representation of the most likely chlorophyll degradation pathways and the major types of chlorophyll catabolites found in plant tissues and in processed foods.

Chlorophylls are degraded in harvested and processed foods and the importance of this research area is reflected by the widespread research undertaken to measure the array of compounds resulting from chlorophyll breakdown simultaneous with the development of methods to preserve the original typical green colors of fresh foods. Although attempts to improve and to maintain the quality of processed green vegetables have been numerous, they cannot be considered completely successful yet.^{32,33} Possible combined strategies to control chlorophyll degradation include the maintenance of neutral pH joined to high-temperature short-time processing; heat inactivation of chlorophyllase with minimal conversion of chlorophyll to pheophytins; the addition of antioxidants to prevent the induction of chlorophyll oxidation by light, peroxidases and/or lipoxygenases and finally, control of the ionic strengths of food products.²⁸ Unfortunately all these methods are of limited value for long preservation. Freezing, drying, and canning, sometimes preceded by blanching, are the main techniques presently employed.

A widely reported method for retaining intact chlorophylls, at least for a certain period, is the addition of alkalizing agents. Leaves of Indian spinach (*Beta vulgaris* var. *bengalensis*), amaranth (*Amaranthus tricolor*) and fenugreek (*Trigonella fenum graecum*) blanched at 95°C in water followed by a potassium metabisulfite dip and subsequent drying retained their original chlorophyll contents.³⁴ Like some other highly perishable leafy vegetables, the previously mentioned dasheen leaves (*Colocasia esculenta* Linn Schott var. *esculenta*) cultivated extensively in tropical coun-

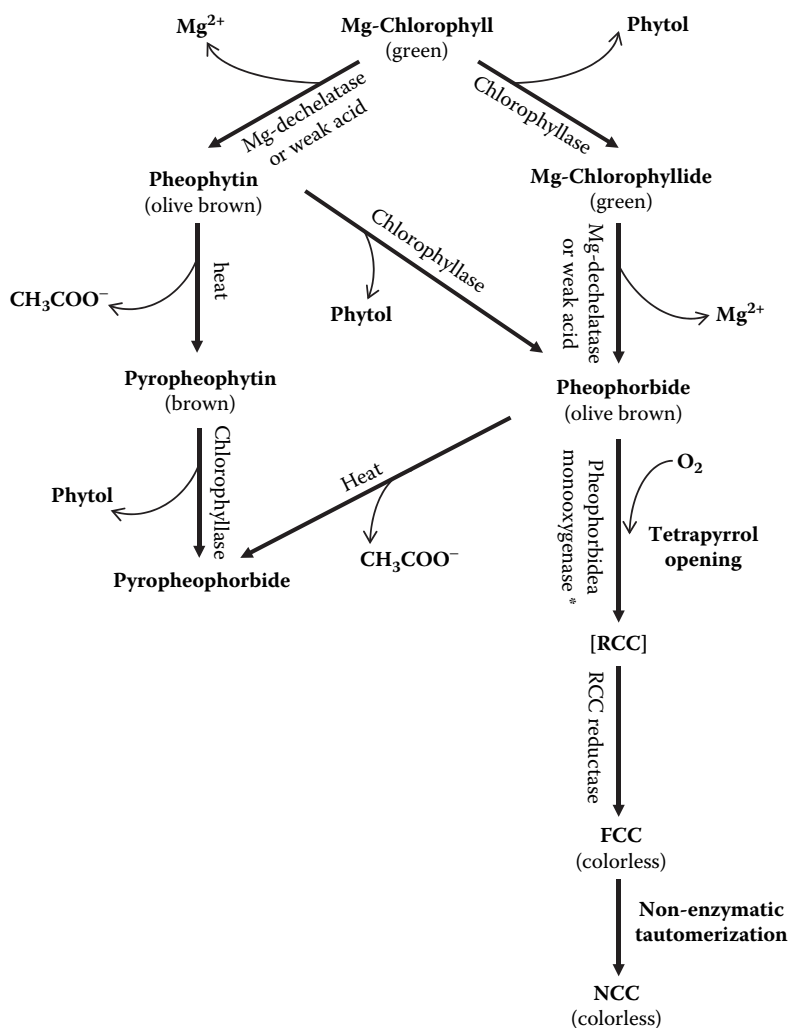


FIGURE 4.1.1 Possible chlorophyll degradation pathways in plant tissues or in processed foods. *Pheophorbide a monooxygenase is specific for pheophorbide a. RCC = red chlorophyll catabolite. FCC = fluorescent chlorophyll catabolite. NCC = non-fluorescent chlorophyll catabolite.

tries yielded a superior dehydrated product compared with unblanched and steam-blanching leaves after a water and magnesium carbonate blanching infusion pretreatment.¹¹ The thermally processed leaves showed minimal loss of green color with no signs of browning, and their organoleptic properties were comparable to those of fresh harvested products. Besides these individual approaches, in general, a good retention of chlorophyll a can be achieved by using blanching water at pH 7 or higher and salts of magnesium, calcium, sodium, or ammonium. Some surface-active agents are also known to have some stabilizing effects on chlorophyll degradation.³⁵

It is worth mentioning that the amounts of pigments immediately after blanching or cooking vegetables and fruits are usually higher than in their native state. This can be explained by a greater extractability of the pigments after processing because heating promotes cell wall rupture, thus facilitating the release of pigments from cells.

The degradation of chlorophylls in olives during fermentation is a combination of enzymatic activity and chemical changes, converting as expected, chlorophylls into pheophytins and pheophorbides, but oxidative changes cannot be excluded.³⁶ It has been reported that the content and the types of chlorophyll and carotenoid pigments present in recently extracted virgin oils stored for one year are indicators of their history prior to marketing.³⁷

The rate and pathway of chlorophyll degradation differ among highly processed canned foods and minimally processed foods like fresh-packed salads. Thermally treated green vegetables lose their metabolic activity and chlorophyll breakdown occurs mainly by external factors. During prolonged thermal treatments such as canning, most chlorophylls are chemically converted to pheophytins and then further transformed into C-13² epimers and pyropheophytins.^{38–40}

As most plant foods are not consumed raw, the effect of food handling, cooking procedures, and industrial processing on their appearance and acceptance has been widely investigated by using kinetic models for chlorophyll degradation in green tissues based on rate constants and activation energies.⁴¹ This approach is essential to understand and to predict quality changes that occur during thermal processing and eventually to open new prospects for industrial application. Chlorophyll undergoes degradation over storage time following a first-order exponential decay, represented by the equation:

$$\text{total chlorophyll (t)} = C_0 e^{-kt}$$

where (t) is the total chlorophyll concentration over time, C_0 is the initial chlorophyll concentration, k is the constant rate of chlorophyll degradation, and t is time (days).

Studies of chlorophyll degradation in heated broccoli juices over the 80 to 120°C range revealed that chlorophylls degrade first to their respective pheophytins and then to other degradation products in what can therefore be described as a two-step process. Both chlorophyll and pheophytin conversions followed a first-order kinetics, but chlorophyll a was more heat sensitive and degraded at a rate approximately twice that of chlorophyll b.^{38,40} This feature had been observed by other authors. Temperature dependence of the degradation rate could adequately be described by the Arrhenius equation.⁴¹

Teng and Chen³⁹ reported that degradation of both chlorophylls a and b in spinach leaves fitted a first-order kinetics, but the rate constant (min^{-1}) was dependent upon the length of heating and methods of cooking: higher rates were observed by microwave cooking or blanching than by steaming or baking. Wet heating methods (blanching and steaming) produced higher amounts of pheophytins, and authors suggest that moist heat facilitates the liberation of organic acids from the matrix of spinach promoting pheophytinization. Alternatively, the degradation of visual green color of vegetables measured by the parameters (a^* and a^*/b^*) of the CIELAB system (Commission International d'Eclairage) follows a first-order kinetics, where

the rate constant increases with the temperature of cooking, demonstrating the relationship between perceptible color attributes of the vegetables and chlorophyll contents.^{42,35} Kinetics of color and pigment degradation were also evaluated in fruits and vegetables such as peas,^{43,44} broccoli,⁴¹ spinach,^{35,40} coleslaw, pickles, and olives.^{36,45} In vegetables in which enzymes are still active, kinetic modeling using uniresponse methods to estimate the respective rate constants was found inadequate but could be improved by introduction of the parameter related to chlorophyllase activity by creating a multiresponse modeling.⁴⁶

4.1.4 CHARACTERISTICS OF CHLOROPHYLL-BASED FOOD COLORANTS

4.1.4.1 NATURAL CHLOROPHYLL FOOD COLORANTS

Natural chlorophylls for the food colorant market have been extracted from an assortment of green leaves, but usually land plants such as several pasture grasses, lucerne (*Medicago sativa*) and nettles (*Urtica dioica*) have been chosen.^{4,47} The choice of raw material must take into account high-yield production, availability and convenience of harvesting and drying, chlorophyll content, facility of extraction, and the desirability of low chlorophyllase activity. Within these parameters, some terrestrial fibrous plants that do not fulfill these attributes must be excluded despite their high chlorophyll contents. Sometimes the production process is not continuous and is hindered by interrupted supplies of raw material that are often restricted to short periods during the year. This is particularly true in temperate climates.

Traditionally, dried or powdered plant material is used and extracts can be obtained by mixing the material with food-grade solvents like dichloromethane or acetone followed by washing, concentration, and solvent removal. The result is an oily product that may contain variable amounts of pheophytins and other chlorophyll degradation compounds usually accompanied by lipid-soluble substances like carotenoids (mainly lutein), carotenes, fats, waxes, and phospholipids, depending on the raw material and extraction techniques employed. This product is usually marketed as pheophytin after standardization with vegetable oils.

Lipophilic chlorophyll crude extracts are also suitable for the manufacture of water-soluble chlorophyllins by hydrolyzing the ester bond of the hydrophobic phytol chain with dilute alkali and introducing sodium or potassium. The acidic groups at 13¹ and 13² of ring E of the macrocycle are also neutralized by their conversion to sodium or potassium salts. This crude product can be further purified to remove lipophilic contaminants. Therefore, the term “chlorophyllins” must be understood as a mixture of several water-soluble, usually metal-free greenish chlorophyll-derived compounds formed during the various steps of processing the raw material.⁴⁷

Although chlorophyll and chlorophyllin colorants seem to be easily obtained, in practice their production as natural food colorants is rather difficult. The sensitivity of chlorophylls to certain enzymes, heat, and low pH, and their low tinctorial strength greatly limit their manufacture and application as food additives, principally when the pigments are isolated from the protective environment of the chloroplasts. The well-known instability of chlorophylls prompted extensive research for developing

methods to prevent degradation. Procedures for their isolation, analysis, and concentration from cheaper sources have been improved. Modern methods for drying raw sources under closely controlled temperatures (obtaining cubes or pellets) are efficient to deactivate both chlorophyllase and Mg-dechelataase that would readily form discolored pigments.⁴⁷

However, there are also different economic constraints to producing natural green colorants. The first is limitation of supplies of adequate amounts of raw material mentioned earlier. Production of pigments using conventional plant cultural traits depends on climatic conditions, plant cultivars and varieties, seasons, and processing that may cause color variation. Second, the production and marketing of a new and improved natural pigment extract is underlain by regulations covering food additives to be marketed. A final and important consideration is how much a colorant will cost. Certainly, the use of natural chlorophyll colorants suffers inherently from high production costs. Their reduced chemical stability also implies an increase in costs in comparison to synthetic pigments.

In the face of the difficulties and limitations related to producing and applying natural chlorophylls as food colorants, recent progress in research and technical advances has suggested at least three areas to be explored. Future prospects are related to the genetic mapping of genes responsible for the regulation and longer chlorophyll retention during senescence, creating an expectance in developing stay-green species. Additionally, higher amounts of intact chlorophylls in final products can be expected from either cultivars with reduced chlorophyllase activity or from cultivars with higher initial chlorophyll contents.¹

A second potential area to be explored may be biochemical encapsulation of chlorophylls to protect and prolong their stability. New techniques of encapsulation produce a milieu capable of quenching active forms of O₂ (radicals and ¹O₂) that mimic the protective environment of chlorophylls in the thylacoid membranes.

A third choice may be the commercial exploration of chlorophyll c, indicated in the literature to be more stable than chlorophylls a and b and found in abundance in marine organisms such as green microalgae (*Chlorella* spp.), cyanobacterium *Spirulina platensis*, and single-celled phytoplankton.⁴ On the other hand, in spite of the abundance of algae, the chlorophylls are extracted with difficulty, and extracts are often contaminated by high levels of undesirable metallic ions. The need for a proper and expensive infrastructure also hinders productivity. On the basis of these considerations, the use of authentic natural chlorophylls as food colorants represents a challenge with a number of seriously limiting factors.

4.1.4.2 SEMI-SYNTHETIC CHLOROPHYLL FOOD COLORANTS

In recent years, metallo-chlorophylls and metallo-chlorophyllins have been considered alternatives to their natural chlorophyll counterparts due to their enhanced color potency, and greater stability against moderate heat, dilute acids, and oxidative agents in general, not to mention their alleged biological activities.

The spontaneous and occasional regreening of certain green plants during storage was first noticed more than 50 years ago. The pigmentation related to the original color was assumed to arise from a stable complex formation between the porphyrin

ring of chlorophyll and several transition metals, mainly zinc and copper. At that time, the uneven green spots were considered color defects. In the following years, efforts were dedicated to studying the formation of such complexes in order to take advantage of their color stabilizing properties by adding zinc or copper salts to green vegetables before thermal treatment.

Commercially available stable green metallo-chlorophyll colorants could be produced and in 1984 Segner et al.⁴⁸ patented the process to preserve the green color in canned vegetables under the “Veri-Green” trade name. Some years later, the formation and stability of these complexes were found to be dependent on the type of metal, pH, ionic concentration, temperature, and chlorophyll species — which could explain the unpredictable color changes observed in the beginning.⁴⁹

The replacement of Mg^{2+} or two H^+ ions by metal ions at the center of the porphyrin rings of chlorophylls, chlorophyllins, pheophytins, or pheophorbides, or a mixture of all these in green plant part extracts produced diverse semi-synthetic colorants, permitted as food additives. Metallo-chlorophylls and metallo-chlorophyllins that differ greatly in solubility have been produced commercially and introduced into the European Community market and other countries as colorants for foods, pharmaceuticals, and food supplements. These preparations exhibit outstanding stability against acids and oxidants, but in contrast, the heat stability of Cu-chlorophyllin seems to be similar to the natural chlorophylls and thermal degradation following first-order reaction kinetics. This raises the possibility of color degradation in foods submitted to mild to severe heat treatment.⁵⁰

Commercial food grade water-soluble Cu-chlorophyllin is the most notable among these preparations. Copper chlorophyllins are produced from crude natural chlorophyll extracts followed by the hydrolysis of the phytol and methyl esters, cleavage of the cyclopentanone (E) ring in dilute alkali, and the replacement of magnesium by copper.⁵¹

Several purification steps are necessary to remove interferents. This particular product, marketed as its sodium or potassium salt in liquid or powdered form, is the most widely used water-soluble green colorant of natural origin producing a mint green (blue-green) color. Yellow colorants are frequently added to achieve other tones of green. The powder dissolves easily in water giving slightly alkaline solutions but precipitates in acidic pH; special forms are required to stabilize it in acidic media. The complexes are extremely stable and, as long as copper is chelated (thus not being bioavailable), they have been considered safe for consumption.⁴⁷ Nevertheless, concerns have been raised regarding the potential adverse impacts on humans if the exposure due to higher dietary intakes of these colorants increases. Future studies should determine the physiological bases: whether the body is able to handle and how it handles intakes of supplemental copper chlorophyllin.

Chemical analysis revealed that commercial food grade copper chlorophyllin is not a single, pure compound, but is a complex mixture of structurally distinct porphyrins, chlorin, and non-chlorin compounds with variable numbers of mono-, di-, and tri- carboxylic acid that may be present as either sodium or potassium salts. Although the composition of different chlorophyllin mixtures may vary, two compounds are commonly found in commercial chlorophyllin mixtures: trisodium Cu (II) chlorin e_6 and disodium Cu (II) chlorin e_4 , which differ in the number of

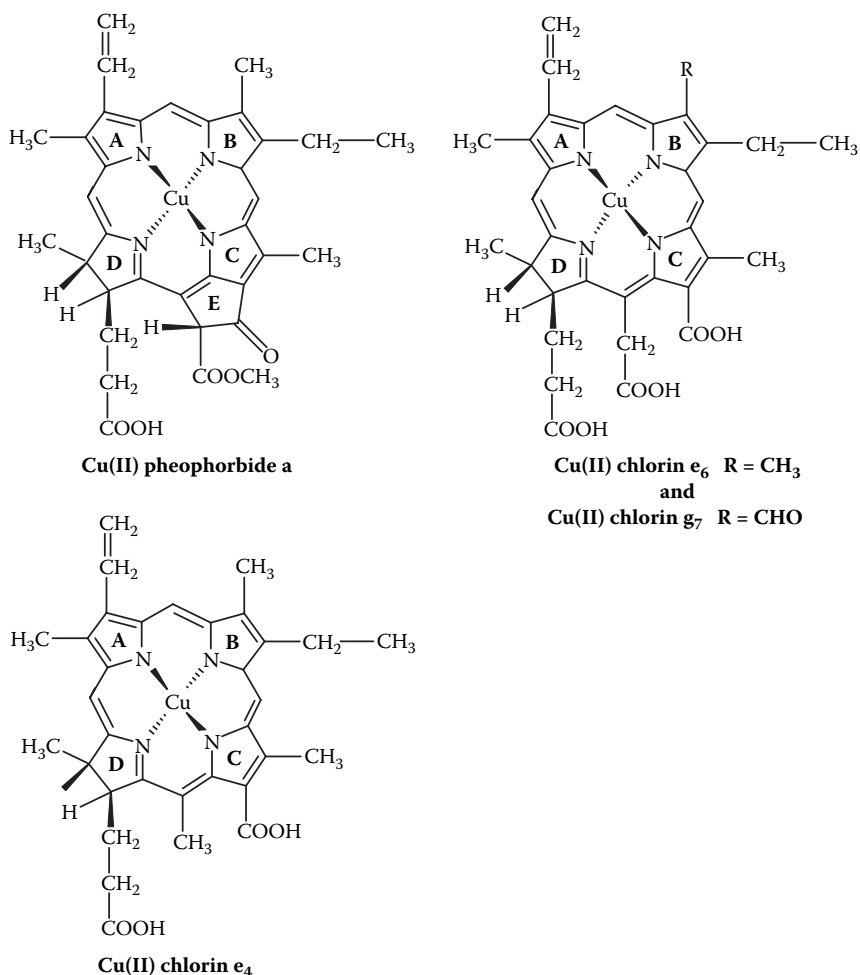


FIGURE 4.1.2 Structures of major copper chlorophyllin components. For Cu(II) chlorin e₆, **R** = CH₃. For Cu(II) chlorin g₇, **R** = CHO.

carboxylic groups. Cu (II) chlorin g₇ is similar to Cu (II) chlorin e₆, but derives from chlorophyll b. Frequently, Cu (II) pheophorbide a can also be found.⁵² Depending on the various extractions and purification processes employed, some compounds lack metal ions. Figure 4.1.2 shows the chemical structures of the major copper chlorophyllin components.

Lipid-soluble food grade copper chlorophyll is manufactured similarly by extraction of adequate plant material, followed by replacement of magnesium by copper, and purification steps to remove carotenoids, waxes, sterols, oils, and other minor components that are co-extracted.⁵³ Commercial copper chlorophylls may vary physically, ranging from viscous resins to fluid dilutions in edible oils as well as granulated forms and emulsions standardized with edible vegetable oil. Colors may vary

slightly according to the ratio of chlorophyll a to chlorophyll b contents and the presence of carotenoids and other coloring matter.

It must be remembered that copper chlorophylls and copper chlorophyllins are chemically modified natural extracts and therefore should not be called natural.

For trade purposes, the major demand is for water-soluble chlorophyll derivatives. Even fat-soluble copper chlorophyll colorants can be mixed with permitted emulsifiers to yield water-miscible forms marketed as liquid or spray dried powders. These food colorants can be used in food and beverages such as dairy products, pastas, soups, gums, confectionary products, drinks, bakery products, extruded products, and green white chocolate. Beyond usage in foods, these green pigments are used for cosmetic and toiletry items (shampoos, foams, gels, soaps) and in the pharmaceutical trade (deodorants, mouthwashes). Botanical extracts in tablets and powders have been commercialized as dietary supplements with reported beneficial biological activities.⁴

The use of copper chlorophyllin as a food colorant is permitted in the European Community, Japan, and other countries. Its use is legally limited in the United States, where it is mostly used in oral hygiene as a common dietary supplement and as an over-the-counter medicine. Only in 2002, a petition was filed with the U.S. Food and Drug Administration for its use as a colorant in dry beverage mixes. The Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) established 1500 mg/kg body weight as the “no observed effect level” (NOEL) of sodium copper-chlorophyllin and the agency calculated an acceptable daily intake (ADI) of 450 mg/person/day for a 60 kg human by applying a 200-fold safety factor to the NOEL.⁵⁴

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REFERENCES

1. Schoefs, B., Plant pigments: properties, analysis, degradation, *Adv. Food Nutr. Res.*, 49, 42, 2005.
2. Pruzinská, A. et al., In vivo participation of red chlorophyll catabolite reductase in chlorophyll breakdown, *The Plant Cell*, 19, 369, 2007.
3. Ferruzzi, M.G. and Schwartz, S., Overview of chlorophylls in foods, in *Current Protocols in Food Analytical Chemistry*, John Wiley & Sons, New York, 2001, Suppl. 1, Unit F4.1.
4. Hendry, G.A., Chlorophylls, in *Natural Food Colorants: Science and Technology*, Lauro, G.J. and Francis, F.J., Eds., Marcel Dekker, New York, 2000, 344.
5. Gross, J., Chlorophylls, in *Pigments in Vegetables: Chlorophylls and Carotenoids*, Gross, J., Ed., Van Nostrand Reinhold, New York, 1991, 3.
6. Gross, J., Chlorophylls, in *Pigments in Fruits*, Gross, J., Ed., Academic Press, London, 1987, chap. 1.

7. Bohn, T. et al., Chlorophyll-bound magnesium in commonly consumed vegetables and fruits: relevance to magnesium nutrition, *J. Food Sci.*, 69, S347, 2004.
8. Burns, J., Fraser, P.D., and Bramley, P.M., Identification and quantification of carotenoids, tocopherols and chlorophylls in commonly consumed fruits and vegetables, *Phytochemistry*, 62, 939, 2003.
9. Kopsell, D.A. et al., Variation in lutein, β -carotene and chlorophyll concentrations among *Brassica oleracea* cultigens and seasons, *Hort. Sci.*, 39, 361, 2004.
10. Turkmen, N. et al., Effects of cooking methods on chlorophylls, pheophytins and colour of selected green vegetables, *Int. J. Food Sci. Technol.*, 41, 281, 2006.
11. Maharaj, V. and Sankat, C.K., Quality changes in dehydrated dasheen leaves: effects of blanching pre-treatments and drying conditions, *Food Res. Int.*, 29, 563, 1996.
12. Khachik, F., Beecher, G.R., and Whittaker, N.F., Separation, identification and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography, *J. Agric. Food Chem.*, 34, 603, 1986.
13. Yamauchi, N. and Watada, A.E. Regulated chlorophyll degradation in spinach leaves during storage. *J. Am. Soc. Hort. Sci.*, 116, 58, 1991.
14. Piagentini, A.M., Güemes, D.R., and Pirovani, M.E., Sensory characteristics of fresh-cut spinach preserved by combined factors methodology, *J. Food Sci.*, 67, 1544, 2002.
15. Kaur, B. and Manjekar, S.P., Effect of dehydration on the stability of chlorophyll and β -carotene content of green leafy vegetables available in northern India, *J. Food Sci. Technol. (India)*, 12, 321, 1975.
16. Mou, B., Genetic variation of β -carotene and lutein contents in lettuce, *J. Amer. Soc. Hort. Sci.*, 130, 870, 2005.
17. Rocha, T., Lebert, A., and Marty-Audouin, C., Effect of pretreatments and drying conditions on drying rate and colour retention of basil (*Ocimum basilicum*), *Lebensm.-Wiss. u. Technol.*, 26, 456, 1993.
18. Gross, J. and Ohad, I., *In vitro* fluorescence spectroscopy of chlorophyll in various unripe and ripe fruit, *Photochem. Photobiol.*, 37, 195, 1983.
19. Gross, J., Chlorophyll and carotenoid pigments of pigment of grapes (*Vitis vinifera* L.), *Gartenbauwiss*, 49, 180, 1984.
20. DeEll, J.R. et al., Addition of sorbitol with KMnO_4 improves broccoli quality retention in modified atmosphere packages, *J. Food Qual.*, 29, 65, 2006.
21. Serrano, M. et al., Maintenance of broccoli quality and functional properties during cold storage as affected by modified atmosphere packaging, *Postharvest Biol. Technol.*, 39, 61, 2006.
22. Jacobsson, A., Nielsen, T., and Sjöholm, I., Effects of type of packaging material on shelf-life of fresh broccoli by means of changes in weight, colour and texture, *Eur. Food Res. Technol.*, 218, 157, 2004.
23. Pariasca, J.A.T. et al., Effect of modified atmosphere packaging (MAP) and controlled atmosphere (CA) storage on the quality of snow pea pods (*Pisum sativum* L. var. *saccharatum*), *Postharvest Biol. Technol.*, 21, 213, 2000.
24. Tenorio, M.D., Villanueva, M.J., and Sagardoy, M., Changes in carotenoids and chlorophylls in fresh green asparagus (*Asparagus officinalis* L.) stored under modified atmosphere packaging, *J. Sci. Food Agric.*, 84, 357, 2004.
25. Monreal, M., De Ancos, B., and Cano, M.P., Influence of critical storage temperatures on degradative pathways of pigments in green beans (*Phaseolus vulgaris* Cvs. Perona and Boby), *J. Agric. Food Chem.*, 47, 19, 1999.
26. Kamat, A. et al., Potential application of low dose gamma irradiation to improve the microbiological safety of fresh coriander leaves, *Food Control*, 14, 529, 2003.

27. Giannakourou, M.C. and Taoukis, P.S., Kinetic modeling of vitamin C loss in frozen green vegetables under variable storage conditions, *Food Chem.*, 83, 33, 2003.
28. Heaton, J.W. and Marangoni, A.G., Chlorophyll degradation in processed foods and senescent plant tissues, *Trends Food Sci. Technol.*, 7, 8, 1996.
29. López-Ayerra, B., Murcia, M.A., and García-Carmona, F., Lipid peroxidation and chlorophyll levels in spinach during refrigerated storage and after industrial processing, *Food Chem.*, 61, 113, 1998.
30. Simpson, K.L., Chemical changes in natural food pigments, in *Chemical Changes in Food during Processing*, Richardson, T. and Finley, J.W., Eds., AVI Publishing, Westport, CT, 1985, 409.
31. Schwartz, S.J. and Lorenzo, T.V., Chlorophylls in foods, *Crit. Rev. Food Sci. Nutr.*, 29, 1, 1990.
32. Funamoto, Y. et al., Effects of heat treatment on chlorophyll degrading enzymes in stored broccoli (*Brassica oleracea* L.), *Postharvest Biol. Technol.*, 24, 163, 2002.
33. Lisiewska, Z., Kmiecik, W., and Shupski, J., Contents of chlorophylls and carotenoids in frozen dill: effect of usable part and pre-treatment on the content of chlorophylls and carotenoids in frozen dill (*Anethum graveolens* L.), depending on the time and temperature of storage, *Food Chem.*, 84, 511, 2004.
34. Negi, P.S. and Roy, S.K., Effect of drying conditions on quality of green leaves during long term storage, *Food Res. Int.*, 34, 283, 2000.
35. Nisha, P., Singhal, R.S., and Pandit, A.B., A study on the degradation kinetics of visual green colour in spinach (*Spinacea oleracea* L.) and the effect on salt therein, *J. Food Eng.*, 64, 135, 2004.
36. Mínguez-Mosquera, M.I., Gandul-Rojas, B., and Mínguez-Mosquera, J., Mechanism and kinetics of the degradation of chlorophylls during the processing of green table olives, *J. Agric. Food Chem.*, 42, 1089, 1994.
37. Gallardo-Guerreiro, L. et al., Effect of storage on the original pigment profile of Spanish virgin olive oil, *J. Am. Oil Chem. Soc.*, 82, 33, 2005.
38. Schwartz, S.J. and Lorenzo, T.V., Chlorophyll stability during continuous aseptic processing and storage, *J. Food Sci.*, 56, 1059, 1991.
39. Teng, S.S. and Chen, B.H., Formation of pyrochlorophylls and their derivatives in spinach leaves during heating, *Food Chem.*, 65, 367, 1999.
40. Canjura, F.L., Schwartz, S.J., and Nunes, R.V., Degradation kinetics of chlorophylls and chlorophyllides, *J. Food Sci.*, 56, 1639, 1991.
41. Weemaes, C.A. et al., Kinetics of chlorophyll degradation and color loss in heated broccoli juice, *J. Agric. Food Chem.*, 47, 2404, 1999.
42. Tijssens, L.M.M., Schijvens, E.P.H.M., and Biekman, E.S.A., Modelling the change in colour of broccoli and green beans during blanching, *Innovative Food Sci. Emerging Technol.*, 2, 303, 2001.
43. Steet, J.A. and Tong, C.H., Degradation kinetics of green color and chlorophylls in peas by colorimetry and HPLC, *J. Food Sci.*, 61, 924, 1996.
44. Ryan-Stoneham, T. and Tong, C.H., Degradation kinetics of chlorophyll in peas as a function of pH, *J. Food Sci.*, 65, 126, 2000.
45. Heaton, J.W., Lencki, R.W., and Marangoni, A.G., Kinetic model for chlorophyll degradation in green tissue, *J. Agric. Food Chem.*, 44, 399, 1996.
46. Boekel, M.J.S., Kinetic modeling in food science: a case study on chlorophyll degradation in olives, *J. Sci. Food Agric.*, 80, 3, 2000.
47. Humphrey, A.M., Chlorophyll as a color and functional ingredient: interaction of natural colors: 12th World Congress of Food Science and Technology. *J. Food Sci.*, 69, c422, 2004.

48. Segner, W.P. et al., Process for the preservation of green color in canned green vegetables, U.S. Patent 4,473,591, September 25, 1984.
49. LaBorde, L.F. and Von Elbe, J.H., Chlorophyll degradation and zinc complex formation with chlorophyll derivatives in heated green vegetables, *J. Agric. Food Chem.*, 42, 1100, 1994.
50. Ferruzzi, M.G. and Schwartz, S.J., Thermal degradation of commercial grade sodium copper chlorophyllin, *J. Agric. Food Chem.*, 53, 7098, 2005.
51. Kephart, J.C., Chlorophyll derivatives: their chemistry, commercial preparation and uses, *Econ. Bot.*, 9, 3, 1955.
52. Scotter, M.J., Castle, L., and Roberts, D., Method development and HPLC analysis of retail foods and beverages for copper chlorophyll (E 141[i]) and chlorophyllin (E 141[ii]) food colouring materials, *Food Addit. Contam.*, 22, 1163, 2005.
53. Sarkar, D., Sharma, A., and Talukder, G., Chlorophyll and chlorophyllin as modifiers of genotoxic effects, *Mutat. Res.*, 318, 239, 1994.
54. U.S. Food and Drug Administration, Listing of color additives exempt from certification: sodium copper chlorophyllin, 21CFR Part 73, 67 *Fed. Reg.* 35429, May 20, 2002.

4.2 Carotenoids in Foods: Sources and Stability during Processing and Storage

Adriana Z. Mercadante

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4.2.1 INTRODUCTION

Carotenoid-rich extracts can be used for coloring purposes and serve as good sources of bioactive compounds. Breeding or genetic manipulation can substantially increase the carotenoid contents of plants, resulting in carotenoid-rich foods that can be applied either as direct sources of nutrients or as raw materials for extracting natural yellow to red colorants.

Processing has become an important part of the food chain and many types of food products can be found on the market, allowing the population to choose

according to need, taste, and purchasing power. Another advantage of processed products is that they are available all year round, whereas tropical fruits and perennial foods have short harvesting seasons and shelf lives. Moreover, processing during peak harvest decreases losses and price. In addition, processing of vegetables and fruits has been found to facilitate the release of carotenoids from their food matrixes, enhancing the bioavailability of these compounds.

Nevertheless, processing can cause degradation of labile nutrients including carotenoids. Due to several conjugated double bonds, carotenoids are susceptible to degradation under high temperature, low pH, and in the presence of light and reactive oxygen species, among other factors. Degradation leads to a reduction of the active carotenoids or to their transformation into *cis* isomers and/or degradation products with different colors and properties. Usually, *cis* isomers are thermodynamically less stable than their correspondent *trans* forms due to a decreased tendency to crystallization. Despite claims that industrial processes are often responsible for the degradation of bioactive compounds, losses of nutrients during domestic preparation are even more considerable.

Comparison of published data regarding the extension of carotenoid degradation is a difficult task for several reasons. Different foods are processed and stored under different combinations of temperature and time conditions; processing and storage conditions are often partially described; and methods used for calculating retention of carotenoids in foods are often not reported. In addition, the inherent food composition plays a crucial role because carotenoid structures, their initial concentrations, and the presence and concentrations of enzymes, antioxidant, and prooxidant compounds influence the degree of degradation. Although carotenoids are fairly stable in natural food environments, these pigments are much more labile when food cells are damaged as during pulping or cutting, and even more fragile when extracted or dissolved in organic solvents.

Insights into the mechanisms of carotenoid degradation can be followed in model systems that are more easily controlled than foods and the formation of initial, intermediate, and final products can also be more easily monitored. However, extrapolation to foods must be done with caution because simple model systems may not reflect the nature and complexity of a multicomponent food matrix and the interactions that can occur. In addition, even in model systems, one must keep in mind that carotenoid analysis and identification are not easy tasks.

4.2.2 FOOD SOURCES OF MAJOR CAROTENOIDS

Many countries have food composition databases but only a few present the compositions of some carotenoids. The U.S. Department of Agriculture's NCC Carotenoid Database covers 215 foods and cites levels of α -carotene, β -carotene, lycopene, β -cryptoxanthin, lutein plus zeaxanthin, and also zeaxanthin in a more limited number of foods.¹ An electronic version of this database is available at <http://www.ars.usda.gov/nutrientdata>.

The different levels of carotenoids in foods reported in the literature may simply arise from the use of different items and/or varieties since the same common names are sometimes applied to items with different scientific names. To overcome this

miscommunication, foods should be unambiguously named and described as pointed out in Section 6.2 in Chapter 6. Other well-known factors that influence the contents of carotenoids in vegetables and fruits are cultivar or variety (genetic variation), growing site and conditions (soil and weather), and stage of maturity at analysis.

In addition, genetic manipulation can be used to change the original carotenoid profile for accumulation of a desired specific carotenoid or produce transgenic foods with high carotenoid contents. To change the carotenoid profiles of tomatoes, transgenic lines containing a bacterial carotenoid gene (*crtI*) encoding the phytoene desaturase enzyme that converts phytoene into lycopene were produced.² As a consequence of this gene expression, the β -carotene content increased about three-fold, up to 45% of the total carotenoid content. However, the total carotenoid levels decreased from 2850 to 1372 $\mu\text{g/g}$ dry weight due to decreased lycopene levels from 2436 $\mu\text{g/g}$ (85%) to 733 $\mu\text{g/g}$ (53%).² In transgenic canola seeds in which a bacterial phytoene synthase (*crtB*) gene was overexpressed, the resultant embryos from those transgenic plants were visibly orange due to accumulation of 394 $\mu\text{g/g}$ of α - and 949 $\mu\text{g/g}$ of β -carotene in their mature seeds, compared to absence of α -carotene and 3 $\mu\text{g/g}$ of β -carotene in the control seeds.³

4.2.2.1 PROVITAMIN A CAROTENOIDS

Tables 4.2.1 and 4.2.2 show, respectively, major sources of β -carotene and other provitamin A carotenoids, especially α -carotene and β -cryptoxanthin. Since *cis* isomers have different biological and physical–chemical properties than their corresponding all-*trans* carotenoids, whenever available, their distribution was included in the tables. The structures of β -carotene *cis* isomers are shown in Figure 4.2.1, whereas the structures of the other provitamin A carotenoids are presented in Figure 6.2.1 in Chapter 6.

Green leaves and carrots represent the most important dietary sources of β -carotene because they are available all around the world throughout the year. Some native leaves found in tropical countries (Table 4.2.1), such as Brazil⁴ and India⁵ had much higher β -carotene levels than most commercial vegetables.^{6–10} Similar results were obtained for some native leafy vegetables harvested in Kenya.¹¹ On the other hand, native Chinese vegetables¹² showed lower β -carotene contents, ranging from 4 to 23 $\mu\text{g/g}$, than those found in common leafy vegetables.^{6–10} As can be seen in Table 4.2.1, independently of leafy sources, 9-*cis*- β -carotene was found in higher amounts (10 to 12%) than 13-*cis*- β -carotene (4 to 6%) in fresh green leaves.^{6,13,14} This high proportion of *cis* isomers in green vegetables prior to processing is probably due to the ability of chlorophylls present in these vegetables to act as sensitizers for β -carotene photoisomerization.

Among 19 cultivars of carrots, the contents of β -carotene varied from 46 to 103 $\mu\text{g/g}$ and of α -carotene from 22 to 49 $\mu\text{g/g}$.¹⁵ Carrots of the cultivar Nantes grown in Brazil showed the lowest level¹⁶ and an unspecified cultivar from Spain had intermediate levels⁹ of both carotenoids (Tables 4.2.1 and 4.2.2). The distribution of α - and β -carotene isomers in fresh carrots was investigated.^{6,14,16,17} Results reported included the absence of α - and β -carotene *cis* isomers in unspecified cultivars,^{14,17} 3% of 9-*cis*- β -carotene and 3% of 9-*cis*- α -carotene in cultivar Nantes,¹⁶ 9% of 9-

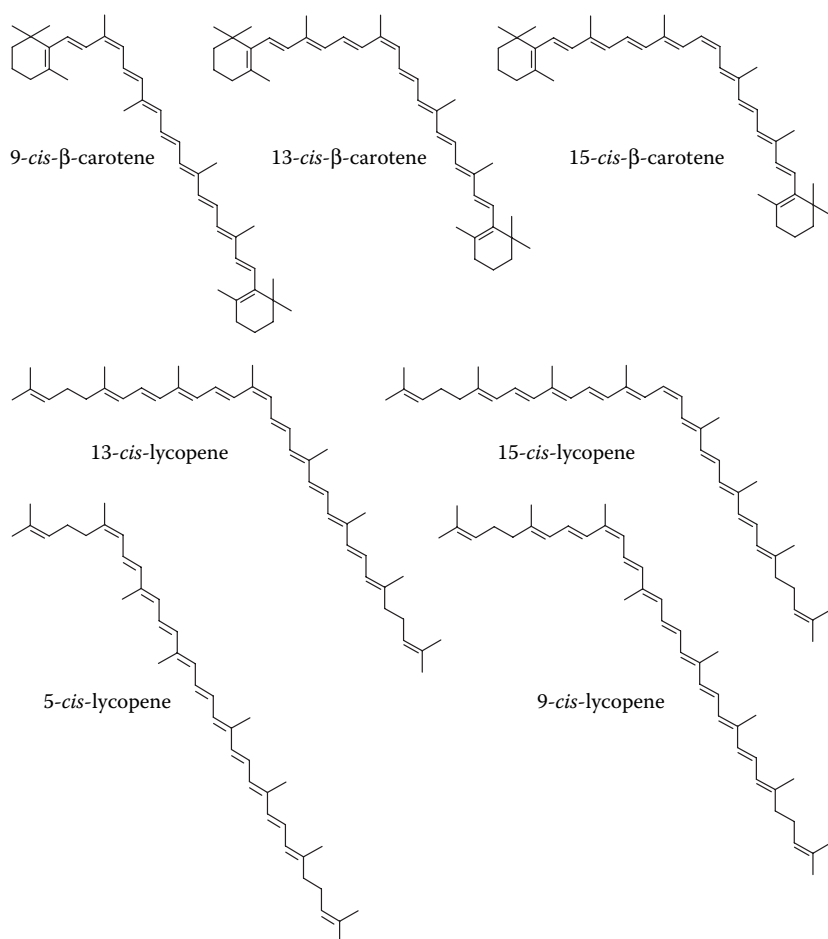


FIGURE 4.2.1 Structures of *cis*-isomers of β -carotene and lycopene found in foods.

cis- and 3% of 13-*cis*- β -carotene along with 1% of 9-*cis*- and 1% of 13-*cis*- α -carotene in cultivar Danvers.⁶ These results indicate that in fresh orange vegetables such as carrots, the proportion of β -carotene *cis* isomers is lower as compared to green vegetables, and that the predominant isomer is 9-*cis*- β -carotene.

Some cultivars of sweet potatoes for human consumption are also good sources of β -carotene since their contents can achieve 218 $\mu\text{g/g}$ as in cultivar Acadian¹⁸ (Table 4.2.1). Although separation of *cis* isomers was not carried out in the later study, small amounts of 13-*cis*- β -carotene were found in fresh sweet potatoes of an unspecified cultivar,⁶ whereas no *cis* isomers of β -carotene were found in this fresh vegetable in other studies.^{14,17}

In tropical and subtropical regions, fruits also contribute to the β -carotene supply. The mango, one of the most consumed tropical fruits, showed a wide range of carotenoids, especially β -carotene contents, depending on cultivar, plantation weather conditions, and degree of ripening^{19–21} (Table 4.2.1). Fresh fruits and pro-

TABLE 4.2.1
Rich Food Sources of β -Carotene and Its *Cis* Isomer Distribution

Source and Reference	Total β -Carotene ($\mu\text{g/g}$ f.w.)	<i>Cis</i> Isomer Distribution (%)			
		all- <i>trans</i> -	9- <i>cis</i> -	13- <i>cis</i> -	others
kale ⁶	47	86	10	4	n.d.
kale ⁷	146	n.d.	n.d.	n.d.	n.d.
cv. Manteiga ⁸	44–54 ^a	n.d.	n.d.	n.d.	n.d.
cv. Tronchuda ⁸	57–60 ^a	n.d.	n.d.	n.d.	n.d.
spinach ⁶	33	83	12	5	n.d.
spinach ⁷	67	n.d.	n.d.	n.d.	n.d.
spinach ⁹	33	n.d.	n.d.	n.d.	n.d.
Italian spinach ¹³	1220–1275 ^b	83	12	5	n.d.
spinach ¹⁴	397 ^b	79	10	6	6
leaves of caruru ⁴	110	n.d.	n.d.	n.d.	n.d.
leaves of mentruz ⁴	85	n.d.	n.d.	n.d.	n.d.
leaves of taioba ⁴	67	n.d.	n.d.	n.d.	n.d.
leaves of serralha ⁴	63	n.d.	n.d.	n.d.	n.d.
leaves of botla benda ⁵	126	n.d.	n.d.	n.d.	n.d.
leaves of Yerramolakakaura ⁵	119	n.d.	n.d.	n.d.	n.d.
leaves of mulla thotakura ⁵	109	n.d.	n.d.	n.d.	n.d.
carrot ⁹	66	n.d.	n.d.	n.d.	n.d.
carrot ¹⁴	534 ^b	100	0	0	0
cv. Nantes Duke ¹⁵	84	n.d.	n.d.	n.d.	n.d.
cv. Nantes Fancy ¹⁵	79	n.d.	n.d.	n.d.	n.d.
cv. Narbonne F ₁ BZ ¹⁵	103	n.d.	n.d.	n.d.	n.d.
cv. Nantes ¹⁶	34	97	3	0	0
cv. Campinas IAC ¹⁶	46	n.d.	n.d.	n.d.	n.d.
sweet potato ⁶	76	97	<1	3	0
cv. Centennial ¹⁸	149	n.d.	n.d.	n.d.	n.d.
cv. Acadian ¹⁸	218	n.d.	n.d.	n.d.	n.d.
mango, cv. Keitt ²⁰	7–15	n.d.	n.d.	n.d.	n.d.
cv. Haden ¹⁹	13	>99	0	<1	0
cv. Tommy Atkins ¹⁹	16	>99	0	<1	0
cv. Kent ²¹	57 ^b	80	0	20	0
cv. Tommy Atkins ²¹	46 ^b	80	0	20	0
cv. Kaew ²¹	139 ^b	84	7	9	0
acerola					
cv. Waldy Cati 2003 harvest ²⁸	3	100	0	0	0
cv. Waldy 2004 harvest ²⁸	6	100	0	0	0
cv. Olivier 2003 harvest ²⁸	9	100	0	0	0
cv. Olivier 2004 harvest ²⁸	17	100	0	0	0
salak ²⁶	30	n.d.	n.d.	n.d.	n.d.
banana, orange flesh ²⁷	59–64	n.d.	n.d.	n.d.	n.d.
palm fruit <i>E. oleifera</i> ³³	993	41	24	35	0
cv. Melanococa ³²	612	67	22	7	5

Continued.

TABLE 4.2.1 (Continued)
Rich Food Sources of β -Carotene and Its *Cis* Isomer Distribution

Source and Reference	Total β -Carotene ($\mu\text{g/g}$ f.w.)	<i>Cis</i> Isomer Distribution (%)			
		all- <i>trans</i> -	9- <i>cis</i> -	13- <i>cis</i> -	others
<i>E. guineensis</i> , cv. Psifera ³³	206	43	27	31	0
cv. Dura Dumpy ³³	634	40	28	32	0
cv. Tenera ³³	411	56	13	31	0
<i>E. guineensis</i> ³²	132	69	15	15	0
<i>E. guineensis</i> $\times$ <i>oleifera</i> ³²	224	63	20	12	7
buriti ¹⁹	365	99	<1	1	0

f.w. = fresh weight. n.d. = not determined.

^a Range for leaves harvested in winter and summer.

^b Amount in dry weight.

cessed juices from *Citrus* are consumed in great quantities all over the world, being orange juice the most globally accepted fruit juice. *Citrus* contains a complex mixture of carotenoids, with different qualitative and quantitative compositions depending on the species,²² cultivar,^{22,23} maturity stage,²⁴ and region of harvest.²⁵ Among 25 genotypes of *Citrus*, juice made from mandarins, satsumas, and clementines showed much higher β -cryptoxanthin levels²² (Table 4.2.2) compared to several orange cultivars: Valencia (1 to 8 $\mu\text{g/mL}$),^{23,24} Pera (4 $\mu\text{g/mL}$),²³ Hamlin (1 $\mu\text{g/mL}$),²³ Cara-cara (2 $\mu\text{g/mL}$),^{22,23} and Shamouti (3 $\mu\text{g/mL}$).^{22,23} On the other hand, orange cultivars, with low β -cryptoxanthin levels showed as major carotenoids either violaxanthin (1 to 7 $\mu\text{g/mL}$), lutein (1 to 4 $\mu\text{g/mL}$), or zeaxanthin (1 to 4 $\mu\text{g/mL}$).²²⁻²⁴

Less common fruits were also reported to be good sources of β -carotene (Table 4.2.1). For example, salak showed the highest β -carotene content among 18 fruits consumed in Indonesia.²⁶ Some locally grown Micronesia fruits with high cultural acceptability also showed high levels of β -carotene, although most cultivars analyzed were not documented by cultivar name.²⁷ Among 13 local banana cultivars, one locally named uht en yap, which has orange flesh, presented the highest β -carotene content compared to other cultivars of local fruits, such as giant swamp taro and seeded and unseeded breadfruit.²⁷

Cultivar and harvest year greatly influenced β -carotene contents of acerola fruits, reaching a difference of *ca.* 2.5 times in a comparison of the same acerola cultivar harvested at the same plantation during two consecutive harvest years.²⁸ In addition, differences up to 3 times were found in a comparison of the β -carotene contents of two acerola cultivars (Table 4.2.1).²⁸

In processed products of the tropical fruit caju²⁹ and in some cultivars of persimmons,³⁰ all-*trans* β -cryptoxanthin was found to be the major carotenoid, contributing to 31 to 38% of the total carotenoid contents in both fruits (Table 4.2.2). Although β -cryptoxanthin was not the major carotenoid in three cultivars of sea buckthorn berries,³¹ their contents were higher than those found in other fruits (Table 4.2.2).

TABLE 4.2.2

Rich Food Sources of Other Provitamin A Carotenoids

Source and Reference	Provitamin A Carotenoid ($\mu\text{g/g}$ f.w.)
carrot ⁹	α -carotene (29)
cv. Nantes Duke ¹⁵	α -carotene (39)
cv. Nantes Fancy ¹⁵	α -carotene (42)
cv. Nelson F ₁ BZ ¹⁵	α -carotene (49)
cv. Nantes ¹⁶	α -carotene (22)
cv. Campinas IAC ¹⁶	α -carotene (21)
palm fruit <i>E. oleifera</i> ³³	all- <i>trans</i> - α -carotene (342), 13- <i>cis</i> - α -carotene (144), β -cryptoxanthin (14)
<i>E. guineensis</i> , cv. Psifera ³³	all- <i>trans</i> - α -carotene (14), 13- <i>cis</i> - α -carotene (6)
cv. Dura Dumpy ³³	all- <i>trans</i> - α -carotene (223), 13- <i>cis</i> - α -carotene (87)
cv. Tenera ³³	all- <i>trans</i> - α -carotene (94), 13- <i>cis</i> - α -carotene (64)
<i>E. guineensis</i> ³²	all- <i>trans</i> - α -carotene (78), 9- <i>cis</i> - α -carotene (8)
<i>E. guineensis</i> $\times$ <i>oleifera</i> ³²	all- <i>trans</i> - α -carotene (59), 9- <i>cis</i> - α -carotene (13), 13- <i>cis</i> - α -carotene (10)
<i>E. melanococa</i> ³²	all- <i>trans</i> - α -carotene (194), 9- <i>cis</i> - α -carotene (31), 13- <i>cis</i> - α -carotene (11)
buriti ¹⁹	all- <i>trans</i> - α -carotene (80), 13- <i>cis</i> - α -carotene (2), γ -carotene (37)
Willowleaf mandarin ²²	β -cryptoxanthin (10 ^a)
Wase satsuma ²²	β -cryptoxanthin (18 ^a)
Hansen mandarin ²²	β -cryptoxanthin (12 ^a)
clementine ²²	β -cryptoxanthin (9 ^a)
caja ²⁹	all- <i>trans</i> - β -cryptoxanthin (6-8), <i>cis</i> - β -cryptoxanthin (<1)
sea buckthorn berry	
cv. Hergo ³¹	β -cryptoxanthin (8-12)
cv. Askola and cv. Leikora ³¹	β -cryptoxanthin (5-19)
persimmon, cv. Sharon ³⁰	β -cryptoxanthin (6)
cv. Rojo Brillante ³⁰	β -cryptoxanthin (7)

f.w. = fresh weight.

^a amount in $\mu\text{g/mL}$.

Among the best-known sources of β -carotene are fruits grown in some families of palm trees. The best known is the palm fruit whose orange to red color oil contains high levels of carotenoids. The genus *Elaeis* comprises two species, *E. guineensis* originating from West Africa and *E. oleifera*, which is native to South America. The contents of β -carotene depend on the species and variety of the palm fruit pulp used as raw material to obtain the palm oil (Table 4.2.1). Due to the high level of lipase activity, the palm fruits are immediately submitted to heat treatment such as blanching³² or sterilization³³ after harvest to avoid oil rancidity. Thus, commercial palm oils always contain high amounts of *cis* isomers that can constitute as much as 60% of total β -carotene content (Table 4.2.1). Nevertheless, the contents of β - and α -carotene, their total usually surpassing 80% of total carotenoid content, were still very high in palm oils from different species^{32,33} and varieties,³³ confirming palm oil as an excellent source of provitamin A (Table 4.2.1 and Table 4.2.2). Although not as widely used as palm oil, another example from another family of palm tree

also rich in α - and β -carotenes is buriti,¹⁹ native to the Amazonian region (Tables 4.2.1 and Table 4.2.2).

4.2.2.2 LYCOPENE

Lycopene is well known as the predominant carotene in tomatoes, accounting for 65 to 98% of the total colored carotenoid content, depending on the cultivar (Table 4.2.3). The levels of lycopene in fresh tomatoes for salad varied from 21 to 79 $\mu\text{g/g}$.^{34,35} In tomatoes for processing and deep-red tomatoes, the level of lycopene can be as high as 623 $\mu\text{g/g}$.^{9,34,36} More than 80% of the tomatoes produced are consumed in the form of processed products, and most commercial products contain much higher lycopene levels than fresh tomatoes^{37,38} (Table 4.2.3).

Freeze-dried tomato powders obtained from whole tomato fruits and from their pulp after centrifugation, containing 474 and 5399 $\mu\text{g/g}$ dry weight, respectively, were developed for use as additives for food fortification.³⁹ *Cis* isomers of lycopene were determined in only a few studies.^{37,38} The 5-*cis*-, 9-*cis*-, and 13-*cis*-+15-*cis*-lycopene were the isomers found in commercial tomato products.³⁸ The structures of lycopene *cis* isomers are shown in Figure 4.2.1 and the structure of the all-*trans* isomer is displayed in Figure 6.2.1 in Chapter 6.

Other common fruits are also good sources of lycopene that represents 87 to 95% of the total carotenoid content in several seeded and seedless watermelon cultivars,⁴⁰ 56 to 66% in different papaya cultivars,⁴¹ and 24 to 58% in pink grapefruits⁴² (Table 4.2.3).

Among tropical fruits, red guavas, containing 76 to 86% of lycopene in relation to total carotenoid, can be considered good sources of lycopene.⁴³ An indigenous Southeast Asia fruit called gac in Vietnam and used as a rice colorant shows an intense red color in the seed membrane (seed pulp or aril) of the ripe fruit; the mesocarp, characterized by its yellow color, is discarded. Although some discrepancies can be found in the literature regarding the lycopene content in gac fruits (Table 4.2.3), this fruit is an extraordinarily rich source of lycopene.⁴⁴⁻⁴⁶

4.2.2.3 LUTEIN AND ZEAXANTHIN

Among commonly consumed vegetables and fruits, dark green leafy vegetables (spinach, kale, and parsley)^{10,47-50} are the most important sources of lutein (Table 4.2.4). Regarding the distribution of *cis* isomers of lutein in fresh green vegetables, lower amounts were found in broccoli⁴⁹ as compared to green leaves in which 9-*cis*- and 13-*cis*-lutein were found in higher proportions than 9'-*cis*- and 13'-*cis*-lutein.^{49,50} The presence of 15-*cis*-lutein was reported in only one study.⁴⁸

On the other hand, widely consumed vegetables and fruits are poor sources of zeaxanthin. The most commonly consumed are corn,⁴⁸⁻⁵² green leafy vegetables,^{4,8,10,48} persimmons,^{30,51} and orange juice^{23,24} (Table 4.2.5). The *cis* isomers of zeaxanthin were determined in very few foods; 9-*cis*- and 13-*cis*-zeaxanthin were found in corn.⁴⁸⁻⁵⁰

Red peppers are good sources of zeaxanthin (Table 4.2.5) although it represents less than 15% of total carotenoid content.⁵³⁻⁵⁵ Sea buckthorn, a berry fruit native to

TABLE 4.2.3
Rich Food Sources of Lycopene and Its *Cis* Isomer Distribution

Source and Reference	Total Lycopene (µg/g f.w.)	Cis Isomer Distribution (%)				
		all- <i>trans</i> -	5- <i>cis</i> -	9- <i>cis</i> -	13- <i>cis</i> -	others ^a
tomato for salad						
cv. Monika ³⁴	73	98	n.d.	n.d.	n.d.	2
cv. Carmen ³⁵	35	n.d.	n.d.	n.d.	n.d.	n.d.
common ⁹	21	n.d.	n.d.	n.d.	n.d.	n.d.
cv. Canary Islands ⁹	16	n.d.	n.d.	n.d.	n.d.	n.d.
tomato for processing	117	>99	n.d.	n.d.	n.d.	<1
cv. Jovanna ³⁴						
cv. BOS 3155 ³⁶	173–237 ^b	n.d.	n.d.	n.d.	n.d.	n.d.
pear-shape ⁹	623	n.d.	n.d.	n.d.	n.d.	n.d.
tomato paste ³⁷	555	n.d.	n.d.	n.d.	n.d.	n.d.
tomato paste ³⁸	520	96	4	<1	<1 ^c	<1
hot ketchup ³⁸	95	88	7	2	3 ^c	1
ketchup ³⁷	172	n.d.	n.d.	n.d.	n.d.	n.d.
ketchup ³⁸	30	77	11	5	7 ^c	1
ready sauce for pasta ³⁷	160	n.d.	n.d.	n.d.	n.d.	n.d.
raw ³⁸	92	67	14	6	5 ^c	8
cooked ³⁸	93	60	14	8	7 ^c	11
canned tomatoes ³⁸	71	84	5	3	5 ^c	3
tomato juice ³⁷	108	n.d.	n.d.	n.d.	n.d.	n.d.
red palm oil ³⁸	450	16	19	6	30 ^c	29
watermelon, cv. Ole ⁴⁰	76	93	n.d.	n.d.	n.d.	7
cv. Xite ⁴⁰	112	90	n.d.	n.d.	n.d.	10
papaya, cv. Solo ⁴¹	17–31	n.d.	n.d.	n.d.	n.d.	n.d.
cv. Formosa ⁴¹	15–30	n.d.	n.d.	n.d.	n.d.	n.d.
cv. Tailandia ⁴¹	32–47	n.d.	n.d.	n.d.	n.d.	n.d.
grapefruit						
cv. Ray Ruby ⁴²	21	n.d.	n.d.	n.d.	n.d.	n.d.
cv. Star Ruby ⁴²	33	n.d.	n.d.	n.d.	n.d.	n.d.
red guava, cv. IAC-4 ⁴³	53	n.d.	n.d.	n.d.	n.d.	n.d.
aril of gac fruit ⁴⁴	348	n.d.	n.d.	n.d.	n.d.	n.d.
aril of gac fruit ⁴⁵	1547–3054	87–97	n.d.	n.d.	n.d.	3–13
aril of gac fruit ⁴⁶	268–705	n.d.	n.d.	n.d.	n.d.	n.d.

f.w. = fresh weight. n.d. = not determined.

^a Not identified.

^b Only all-*trans*-lycopene.

^c Corresponds to 13-*cis*- + 15-*cis*-lycopene.

TABLE 4.2.4
Rich Food Sources of Lutein and Its *Cis* Isomer Distribution

Source and Reference	Total Lutein ($\mu\text{g/g}$ f.w.)	Cis Isomer Distribution (%)				
		all- <i>trans</i> -	9- <i>cis</i> -	9'- <i>cis</i> -	13- <i>cis</i> -	13'- <i>cis</i> -
broccoli ¹⁰	8	n.d.	n.d.	n.d.	n.d.	n.d.
broccoli ⁴⁷	18 ^a	n.d.	n.d.	n.d.	n.d.	n.d.
fresh ⁴⁹ broccoli	84 ^b	99	0	0	1	0
microwaved ⁴⁹ broccoli	102 ^b	96	0	0	4	0
kale ¹⁰	186	n.d.	n.d.	n.d.	n.d.	n.d.
kale ⁴⁸	150	87	3	5	5 ^c	n.d.
fresh ⁴⁹ kale	515 ^b	100	0	0	0	0
canned ⁴⁹ kale	627 ^b	78	7	6	9	0
spinach ¹⁰	95	n.d.	n.d.	n.d.	n.d.	n.d.
spinach ⁴⁷	44	n.d.	n.d.	n.d.	n.d.	n.d.
spinach ⁴⁸	92	92	2	<1	5 ^c	n.d.
fresh ⁴⁹ spinach	881 ^b	97	0	0	3	0
canned ⁴⁹ spinach	923 ^b	86	5	3	6	0
fresh ⁵⁰ spinach	443 ^b	79	9	2	7	3
blanched ⁵⁰ spinach	369 ^b	85	3	2	7	3
parsley ¹⁰	138	n.d.	n.d.	n.d.	n.d.	n.d.
parsley ⁴⁷	102	n.d.	n.d.	n.d.	n.d.	n.d.
parsley ⁴⁸	108	92	3	<1	4 ^c	n.d.
leaves of caruru ⁴	237 ^d	n.d.	n.d.	n.d.	n.d.	n.d.

f.w. = fresh weight. n.d. = not determined.

^a Reported as lutein + zeaxanthin.

^b Amount in dry weight.

^c Determined as 15-*cis*- + 15'-*cis*-lutein.

^d Reported as lutein + violaxanthin.

Asia, also contains high amounts of zeaxanthin,^{31,51} representing 82 to 86% of the total of main carotenoids in some cultivars of this berry.³¹ Moreover, the best-known source of zeaxanthin is wolfberry, a small fruit native to China, with zeaxanthin dipalmitate accounting for 89% of total carotenoid in dried fruits.⁵¹ In another study using fresh wolfberries, zeaxanthin corresponded to only 17%.⁵⁶

4.2.2.4 UNUSUAL CAROTENOIDS

Spices such as paprika, saffron, and annatto, which are traditionally employed in different parts of the world, contain unique carotenoids. Figure 4.2.2 shows the structures of these carotenoids.

Thousands of paprika varieties belong to the *Capsicum* genus and they vary widely in their sizes, shapes, colors, flavors, and pungency levels.⁵⁵ The major carotenoids found in red paprika are capsanthin and capsorubin, which possess κ end group. Yellow pigments such as lutein, zeaxanthin, violaxanthin, and β -carotene

TABLE 4.2.5
Rich Food Sources of Zeaxanthin and Its *Cis* Isomer Distribution

Source and Reference	Total Zeaxanthin ($\mu\text{g/g}$ f.w.)	Cis Isomer Distribution (%)			
		all- <i>trans</i> -	9- <i>cis</i> -	13- <i>cis</i> -	9'- <i>cis</i> -
corn ⁵¹	1	n.d.	n.d.	n.d.	n.d.
canned ⁴⁸ corn	3	93	7	0	0
fresh ⁴⁹ corn	24 ^a	99	0	<1	0
microwaved ⁴⁹ corn	24 ^a	83	0	17	0
fresh ⁵⁰ corn	9 ^a	93	2	5	0
canned sterilized ⁵⁰ corn	7 ^a	75	3	22	0
cv. White Shoepeg ⁵² corn	0.3	n.d.	n.d.	n.d.	n.d.
cv. Golden Whole Kernel ⁵² corn	2	n.d.	n.d.	n.d.	n.d.
caruru leaves ⁴	8	n.d.	n.d.	n.d.	n.d.
persimmon ⁵¹	<1	n.d.	n.d.	n.d.	n.d.
cv. Sharon ³⁰	3	n.d.	n.d.	n.d.	n.d.
cv. Rojo Brillante ³⁰	4	n.d.	n.d.	n.d.	n.d.
orange juice, cv. Valencia ^{23,24}	1–4 ^b	n.d.	n.d.	n.d.	n.d.
cv. Sanguinelli ²³	4 ^b	n.d.	n.d.	n.d.	n.d.
cv. Pera ²³	2 ^b	n.d.	n.d.	n.d.	n.d.
cv. Cara-cara ²³	2 ^b	n.d.	n.d.	n.d.	n.d.
fresh pepper, orange ⁵¹	30	n.d.	n.d.	n.d.	n.d.
red ⁵¹ fresh pepper	168	n.d.	n.d.	n.d.	n.d.
orange Thai chili ⁵¹	26	n.d.	n.d.	n.d.	n.d.
red pepper, cv. Belrubi ⁵³ fresh pepper	600 ^a	n.d.	n.d.	n.d.	n.d.
variety Agridulce ⁵⁴ red pepper	106	93	7 ^c	n.d.	n.d.
variety Bola ⁵⁴ red pepper	47	88	12 ^c	n.d.	n.d.
sea buckthorn ⁵¹	23–33	n.d.	n.d.	n.d.	n.d.
cv. Askola ³¹	64–151	n.d.	n.d.	n.d.	n.d.
cv. Hergo ³¹	96–115	n.d.	n.d.	n.d.	n.d.
cv. Leikora ³¹	30–61	n.d.	n.d.	n.d.	n.d.
wolfberry, dried ⁵¹	824	n.d.	n.d.	n.d.	n.d.
fresh ⁵⁶	24	n.d.	n.d.	n.d.	n.d.

f.w. = fresh weight. n.d. = not determined.

^a Amount in dry weight.

^b $\mu\text{g/mL}$.

^c *Cis* isomer not identified.

were also detected in paprika. In red paprika, capsanthin was always found to be the major carotenoid, either in varieties from Hungary, accounting, for example, from 27% in red *C. grossum* to 45% in *C. abbreviatum pendens*,⁵⁵ as well as in varieties from Spain representing from 23% in cultivar NAN to 54% in cultivar AlbarxM.CA.^{53,54} The capsanthin contents can reach as much as 636 $\mu\text{g/g}$ of fresh weight in red cultivars.⁵⁴ Although the *Capsicum* genus is the main source of these red pigments, they were also found in fruits of *Asparagus officinalis*.⁵⁷

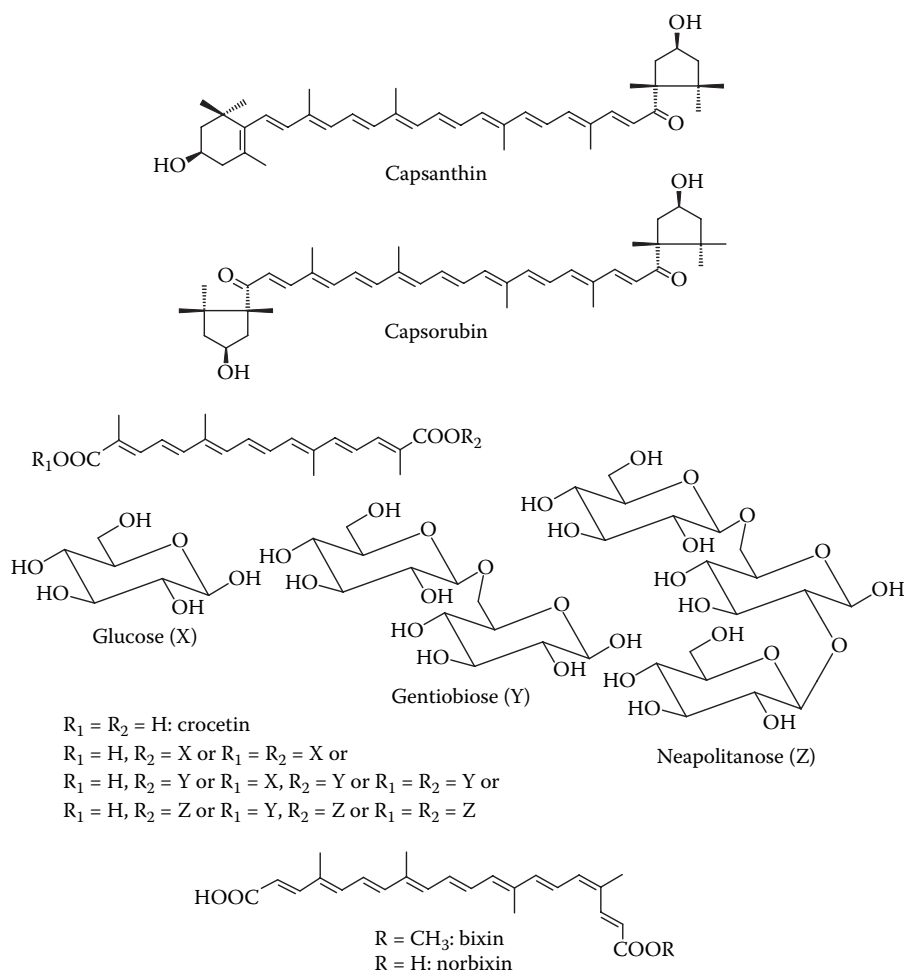


FIGURE 4.2.2 Structures of carotenoids found in paprika (capsanthin and capsorubin), saffron and gardenia (crocetin derivatives), and annatto (bixin and norbixin).

Carotenoid glycosyl esters are compounds containing an apocarotenoid acid esterified with a cyclic sugar at the anomeric carbon atom. The major carotenoid glycosyl esters found in stigmas of saffron and in gardenia fruits are derivatives of the unusual C_{20} diapocarotenoid acid called crocetin (8,8'-diapocarotene-8,8'-dicarboxylic acid) esterified with one or two glucose, gentiobiose, or neapolitanose sugar moieties.^{58,59} These compounds are known for their coloring properties and water-soluble behavior, in contrast to most families of carotenoids.

Bixin, a diapocarotenoid with a *cis* configuration at the carbon 9' and two carboxylic groups (one methylated), accounts for more than 80% of the total carotenoid content in annatto seeds, and has only been encountered to date in these seeds.⁶⁰ The amount of red pigments in annatto seeds varies from 1.5 to 4%, depending on the variety. Bixin is the main pigment in commercial annatto powder

and oil-soluble preparations, whereas for water-dispersible colors, extraction is carried out with alkali that furnishes norbixin as the main pigment.⁶¹ In Brazil, a condiment called “colorifico” used for coloring foods consisting of ground annatto seeds mixed with corn flour contained 1540 to 3540 µg/g of bixin.⁶²

4.2.3 EFFECTS OF TEMPERATURE ON CAROTENOID STABILITY

The effects of temperature on carotenoid content can be considered from three perspectives: (1) evaluation of stability or retention of carotenoids, (2) study of the chemical changes (isomerization, oxidation, epoxy–furanoid rearrangement), and (3) their effects on the nutritional value and other carotenoid actions in humans. The first two topics are discussed in the following sections. The third is presented in Section 3.2.4.1 of Section 3.2.

Since it is easier to control and change the conditions of carotenoid studies carried out in model systems, information on degradation kinetics (reaction order model, degradation rate, and activation energy) and products formed are often derived from such studies.

4.2.3.1 MODEL SYSTEMS

Because of its fundamental role as a precursor of vitamin A and the availability of β-carotene standard in crystalline form, the thermal degradation of β-carotene in model systems has been a subject of intense research.

The products formed after heating dried β-carotene at 180°C for 2 hr in a sealed ampoule (S1); with air circulation (S2); stirring with starch and water (S3); and during extrusion process (S4) were isolated.^{63–65} In all systems, 5,6-epoxy-β-carotene (*trans* and two *cis* isomers), 5,8-epoxy-β-carotene (*trans* and four *cis* isomers), and 5,6,5,6-diepoxy-β-carotene were identified, along with 5,6,5,8-diepoxy-β-carotene in systems S3 and S4.⁶³ Later on, along with the epoxides previously found, 5 β-apocarotenals with 20 to 30 carbons, β-caroten-4-one, and 6 different β-carotene *cis* isomers were isolated in systems S3 and S4, whereas lower numbers of degradation products were found in the other systems.⁶⁵

Although no kinetic data were reported, *trans*-5,8-epoxy-β-carotene was the major compound formed in systems S2, S3, and S4, followed by *trans*-5,6-epoxy-β-carotene (major in S1), *cis*-5,8-epoxy-β-carotene, *trans*-5,8,5,8-diepoxy-β-carotene, and β-apo-8-carotenal.⁶⁵ Losses of only 7.5% of the initial β-carotene contents were found in S1 and 15% in S2, which increased to 40% under stirring (S3) and to 92% under stirring and high pressure as in S4.⁶⁵ Formation of epoxides or apocarotenals, both from β-carotene, was proposed as a degradation mechanism by heating.^{64,65}

After heating β-carotene at 97°C for 3 hr under oxygen in aqueous medium, 7 degradation compounds, 4 of them identified as β-carotene epoxides, were isolated; 52 volatiles were separated (21 identified and 15 quantified).⁶⁶ The major volatiles found were 5,6-epoxy-β-ionone and dihydroactinidiolide (DHA), the latter being the only volatile compound formed during heating at 30°C for 3 hr, indicating that it was the first volatile compound produced during heating treatment of β-carotene.⁶⁶

TABLE 4.2.6
Observed Rate Constants (k_{obs}) for Thermal Degradation of Carotenoids

Carotenoid and Reference	Model System	Temperature (°C)	k_{obs} (min ⁻¹)
all- <i>trans</i> - α -carotene ⁶⁷	crystal, under air (isomerization to 13- <i>cis</i> - α -carotene)	150	0.0323
all- <i>trans</i> - α -carotene ⁶⁷	crystal, under air (isomerization to 9- <i>cis</i> - α -carotene)	150	0.0038
9- <i>cis</i> - β -carotene ⁷¹	safflower seed oil, dark, under air	75	0.0008
		85	0.0018
		95	0.0059
all- <i>trans</i> - β -carotene ⁶⁷	crystal, under air (isomerization to 13- <i>cis</i> - β -carotene)	150	0.0125
all- <i>trans</i> - β -carotene ⁶⁷	crystal, under air (isomerization to 9- <i>cis</i> - β -carotene)	150	0.0043
all- <i>trans</i> - β -carotene ⁷¹	safflower seed oil, dark, under air	75	0.0007
		85	0.0020
		95	0.0054
all- <i>trans</i> - β -carotene ⁷⁰	chlorophyll A, dark, under air,		
	methyl stearate	60	0.022
	methyl oleate	60	0.013
	methyl linoleate	60	0.006
lycopene ⁷¹	safflower seed oil, dark, under air	75	0.0018
		85	0.0036
		95	0.0086
lycopene ⁷³	dry thin layer of lycopene, dark, under air	50	0.0075
		100	0.0124
		150	0.1651
lutein ⁷¹	safflower seed oil, dark, under air	75	0.0006
		85	0.0022
		95	0.0045
bixin ⁷⁴	H ₂ O/EtOH (8:2), dark, under air	70	0.004
		98	0.020
		125	0.091

These authors proposed a reaction mechanism with β -carotene monoepoxides and diepoxides as intermediates for volatile formation.

Four *cis* isomers of β -carotene (13,15-di-*cis*-, 15-*cis*-, 13-*cis*-, and 9-*cis*-) and three of α -carotene (15-*cis*-, 13-*cis*-, and 9-*cis*-) were formed during heating of their respective all-*trans* carotene crystals at 50, 100, and 150°C.⁶⁷ Isomerization catalyzed by heat was considered as a reversible first-order degradation reaction — a *trans*-to-*cis* conversion two- to three-fold slower than the backward (*cis*-to-*trans*) reaction (Table 4.2.6). The 9-*cis*- and 13-*cis*- were the major β -carotene isomers formed and the 13-*cis*- formed at a two- to three-fold faster rate than 9-*cis*- β -carotene.⁶⁷ In this system, α -carotene showed lower stability than β -carotene (Table 4.2.6).⁶⁷ The activation energy (E_a) was not reported since practically no degradation was observed

during 30 min of heating at 50 and 100°C. The fact that the k_{obs} values found for thermal degradation of α - and β -carotene crystals⁶⁷ were lower compared to other studies (Table 4.2.6) can be attributed to the highest stability of crystals since carotenoid isomerization does not occur in crystalline forms.⁶⁸ After heating a β -carotene standard dissolved in toluene at 98°C for 60 min, 85% of the initial content was retained with concomitant formation of 13-*cis*- β -carotene (6 to 28%) and of the 9-*cis*- isomer (2 to 5%).⁶⁹

On the other hand, isomerization of all-*trans* β -carotene was found to be comparatively faster in a model containing methyl fatty acid and chlorophyll heated at 60°C (Table 4.2.6), resulting in 13-*cis*- β -carotene as the predominant isomer.⁷⁰ The first-order degradation rate of β -carotene significantly decreased with the increased number of double bonds in the methyl fatty acid, probably due to competition for molecular oxygen between β -carotene and the fatty acid.⁷⁰ Since the systems were maintained in the dark, although in the presence of air, the addition of chlorophyll should not catalyze the isomerization reaction.

The degradations of all-*trans* β -carotene, 9-*cis*- β -carotene, lycopene, and lutein were evaluated during heating safflower seed oil at 75, 85, and 95°C.⁷¹ Using adjustment to first-order reaction for all carotenoids, the observed thermal degradation constant rate (k_{obs}) for lycopene was about twice higher than those found for the other carotenoids, whereas no significant difference was found in the stabilities of the β -carotene isomers (Table 4.2.6).⁷¹ The calculated E_a was 26.2 kcal/mol for all-*trans*- β -carotene, 25.1 kcal/mol for 9-*cis*- β -carotene, 19.8 kcal/mol for lycopene, and 24.9 kcal/mol for lutein.⁷¹ Heating β -carotene at all temperatures formed 13-*cis*- in higher amounts, 9-*cis*- β -carotene, and an unidentified *cis* isomer. Although several degradation products were formed during lycopene and lutein heating, they were not identified.⁷¹

Isomerization of lycopene in tomato oleoresin increased at 75 and 100°C, reaching the formation of eight unidentified lycopene geometrical isomers in tomato oleoresin stored at 100°C.⁷² The k_{obs} of 0.2597/min was higher, whereas the E_a (11.7 kcal/mol) was lower than that observed for lycopene standard heated in safflower oil⁷¹ at 75°C.

Lycopene dispersible as dry thin layers in glass vials was heated at 50, 100, and 150°C⁷³ (Table 4.2.6). At 50°C, isomerization dominated in the first 9 hr; however, degradation was later favored. On the other hand, at 100 and 150°C, degradation proceeded faster than isomerization. Using a first-order kinetic model, the calculated E_a for lycopene was reported by the authors as 11.6 kcal/mol. Although *cis* isomer identification was not confirmed by standards, the mono-*cis* forms of lycopene, 5-*cis*-, 9-*cis*-, 13-*cis*-, and 15-*cis*-, degraded at the same rate as did all-*trans* lycopene, whereas formation of two di-*cis* lycopene isomers showed increasing trends during heating.⁷³

The kinetics of the thermal degradation of bixin in a water:ethanol (8:2) solution was studied as a function of temperature (70 to 125°C).⁷⁴ Figure 4.2.3 shows the UV-vis spectral changes, with the consumption of the visible band of bixin (400 to 500 nm) accompanied by an increase in the absorbance below 400 nm, without the presence of clear isosbestic points. In addition, as can be seen in the inset of Figure 4.2.3, the degradation rate was strongly dependent on the monitoring wavelength.

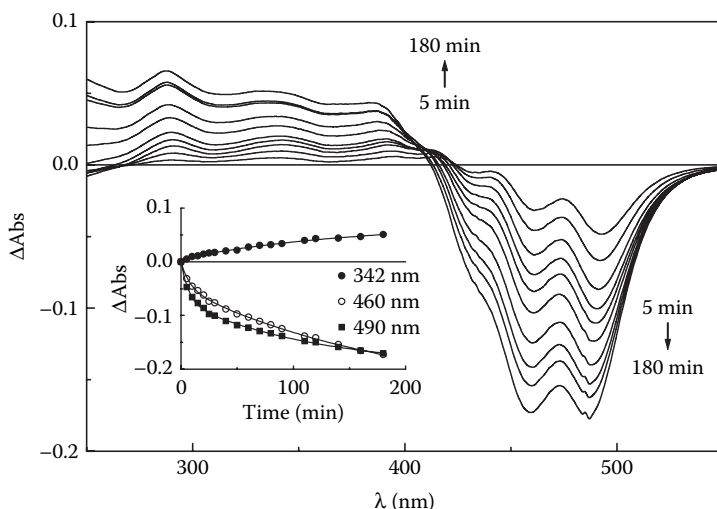


FIGURE 4.2.3 UV-vis spectral changes and ΔAbs obtained by heating bixin in water:ethanol (8:2) at 92°C. Inset shows kinetic profile at several wavelengths, with the solid lines representing the fitting of experimental data from the sum of two exponential functions. From Rios, A.O., Borsarelli, C.D., and Mercadante, A.Z., *J. Agric. Food Chem.*, 53, 2307, 2005. With permission.

Therefore no trustworthy results for kinetic analysis could be obtained from the UV-vis absorption spectra due to the formation of bixin isomers and degradation products at different rate constants.⁷⁴

The decay of bixin and the formation of several products were confirmed by HPLC, allowing mathematical modeling to a bi-exponential reaction order to give the rate constant values for the formation of the primary products from bixin (Table 4.2.6) and the energy barriers for each step.⁷⁴ The di-*cis* isomers were formed immediately ($E_a = 15$ kcal/mol) together with the decay of bixin, followed by its slow consumption, indicating their role as reaction intermediates.⁷⁴ In fact, the di-*cis* isomers could easily revert to bixin ($E_a = 3$ kcal/mol) or yield the primary C-17 degradation product, with an energy barrier of 6.5 kcal/mol. In turn, an energy level of 24 kcal/mol was necessary for the isomerization of the 9'-*cis*- double bond in bixin to all-*trans* bixin, explaining its slower formation.⁷⁴ These transformations were accompanied by the formation of *m*-xylene as the major volatile,⁷⁵ involving the di-*cis* isomer as an intermediate of the mechanism of thermal degradation of bixin.^{74,76}

In summary, some conclusions can be drawn from the carotenoid model systems submitted to heating:

1. When formation of isomers or colored degradation products occurs, separation of such compounds by HPLC must be carried out in order to obtain correct k_{obs} and E_a values.
2. Isomerization is the main reaction that occurs during heating at atmospheric pressure and at temperatures below 100°C.

3. Formation of oxidation products from β -carotene, such as epoxides and apocarotenals, occurs only at extreme conditions of combined temperature, time, or pressure.
4. 13-*cis*- β -Carotene is formed at higher rates than 9-*cis*- β -carotene because the activation energy for *trans*-to-*cis* isomerization about the central double bond is lower than that about the other double bonds.⁷⁷

Since k_{obs} values reported in different studies for the same carotenoid at the same temperature showed differences of at least one order of magnitude, it is imperative to confirm the kinetic reaction order model by conducting experiments with different carotenoid concentrations and at different temperatures.

4.2.3.2 FOOD SYSTEMS

Food processing involves changes in structural integrity of matrices, producing both negative effects such as loss of carotenoids due to isomerization and oxidation and positive effects due to increased bioavailability. As a result of losses of soluble solids into the water medium and increased carotenoid extraction efficiency due to tissue softening and disruption of the carotenoid-protein complexes during processing, increased carotenoid concentrations during processing are often noted in the literature.^{9,49,52,78}

Among thermal processes, canning caused the largest *trans*-to-*cis* isomerization of provitamin A carotenoids, increasing the total *cis* isomers by 39% for sweet potatoes, 33% for carrots, 19% for collards, 18% for tomatoes, and 10% for peaches; 13-*cis*- β -carotene was the isomer formed in highest amounts.¹⁴

Canning at 121°C for 30 min was also responsible for the highest losses of carotenoids in carrot juice, reaching 60% for β - and α -carotene, whereas the lutein level decreased 50%, all accompanied by the formation of 13-*cis*- β -carotene in the largest amount, followed by 13-*cis*-lutein and 15-*cis*- α -carotene.⁷⁹ Canning ($T_{\text{max}} = 121^\circ\text{C}$, $F = 5$) of sweet corn resulted in a decrease of lutein by 26% and zeaxanthin by 29%, accompanied by increased amounts of 13-*cis*-lutein, 13'-*cis*-lutein, and 13-*cis*-zeaxanthin.⁵⁰ The relative amounts of *cis* isomers of lutein, mainly the 13-*cis*, increased by 15% and of 13-*cis*-zeaxanthin by 20% after corn canning.⁴⁹

Canning of spinach increased 9-*cis*- β -carotene from 10 to 15%, whereas the percentage of 13-*cis*- β -carotene remained unchanged in 6%.¹⁴ The levels of all-*trans* β -carotene in blanched Italian spinach (boiling water for 1 min) decreased 33% after freeze drying and 43% after simulated solar drying; both losses were accompanied by increased percentages of 9-*cis*- and 13-*cis*- β -carotene from, respectively, 12 and 5% in fresh to 15 and 6% after freeze drying and 17 and 6% after solar drying processes.¹³

Losses of 45 to 48% in the β -carotene contents and formation of *cis* isomers were also verified by pasteurization of carrot juice at 110 and 120°C for 30 sec.⁷⁹ No significant effects on *trans*-to-*cis* isomerization of α - and β -carotene isomers were observed after acidification and heating of carrot juice at 105°C for 25 sec.⁷⁹ In addition, an increase of only 3% in the *cis* isomers of provitamin A carotenoids was observed after orange juice pasteurization.¹⁴

Extensive carrot blanching (100°C for 60 min) caused losses of 26 to 29% in total β -carotene content, along with increased 13-*cis*- β -carotene contents up to 10% after pasteurization ($T_{\max}$ 95°C, $P = 3$) and to 14% after sterilization ($T_{\max}$ 121°C, $F = 5$).⁸⁰ However, unheated juices produced from carrots blanched at 80°C for 10 min were devoid of *cis* isomers, and further pasteurization or sterilization processes formed only 13-*cis*- β -carotene, at 2 and 5%, respectively.⁸⁰

The addition of grape oil to carrot juice before heat treatment enhanced the 13-*cis*- β -carotene formation,⁸⁰ probably due to partial dissolution of crystalline carotene present in the intact carrot in lipid droplets, since solubilization of carotenes during blanching is a pre-requisite for formation of *cis* isomers. This assumption was supported by the fact that the addition of grape seed oil during juice processing resulted in significantly higher levels of *cis* isomers compared to the control sample.⁸⁰

Similar effects could be observed in oil-rich foods such as palm fruits in which losses of all-*trans* α - and all-*trans* β -carotene were found to be, respectively, *ca.* 23 and 44% after sterilization.³³ In terms of relative composition after processing, all-*trans* α -carotene decreased from about 30 to 23% and all-*trans* β -carotene from 65 to 27%, whereas the *cis* isomers increased as follows: 13-*cis*- α -carotene from less than 1 to 10%, 13-*cis*- β -carotene from 3 to 23%, and 9-*cis*- β -carotene from less than 1 to 18%.³³

When drying of mango slices was performed using an overflow tray dryer (75°C for 3 to 3.5 hr), the relative amount of 13-*cis*- β -carotene significantly increased from *ca.* 25 to 37% accompanied by losses of 7 and 30% of all-*trans* β -carotene, respectively in mango cultivars Kent and Tommy Atkins, most probably due to oil content.²¹ In addition, the formation of 9-*cis*-isomers was negligible, whereas complete degradation of violaxanthin ester occurred.²¹ Similar results were obtained by drying mango pieces under hot air at 60°C for 20 hr; violaxanthin and β -carotene decreased by *ca.* 41% compared to freeze drying.⁸¹

Higher degradation rates of lycopene compared to those of β -carotene in model systems were not observed in food systems. In fact, the amount of *trans*- and *cis*-lycopene isomers remained almost unchanged during processing of tomato juice and even of tomato paste, whereas increased levels of *cis*- β -carotene were found during these processes.³⁴ Very similar results were reported for different tomato varieties submitted to thermal treatment in water or water:oil (8:2) at 100°C for 30 min; lycopene, prolycopene, δ -carotene, and γ -carotene did not undergo isomerization and in contrast an average of 21% of β -carotene and 27% of lutein *cis* isomers were found.⁸² These differences can be explained by the structural specificities of the carotenes, such as molecular shape, ease of crystal formation, further organization in multilayers or aggregates, and their storage at different locations in cells. Once in the aggregated form, lycopene molecules may be able to resist further structural change. On the contrary, β -carotene with two bulky β -ionone rings may not be able to assemble easily into an ordered and stable structure, as can lycopene molecules.⁸²

Aside from isomerization, transformation of the 5,6-epoxy to the 5,8-furanoid group is a common alteration during heating treatments of carotenoids. Violaxanthin was found to be the major carotenoid in mangoes; however, in commercially processed mango juice, violaxanthin was not detected while auroxanthin, not present in the

fruits, appeared at an appreciable level due to the conversion of the 5,6-epoxide groups of violaxanthin to the 5,8-furanoxide groups of auroxanthin.²⁰ Losses of 35 to 66% of violaxanthin and 34 to 100% of 5,6-epoxy-lutein were observed during steaming or microwaving of green vegetables.⁷⁸ Violaxanthin decreased 46%, *cis*-violaxanthin 20%, and antheraxanthin 25%, whereas no changes of β -carotene and β -cryptoxanthin levels were observed after pasteurization of orange juice at 90°C for 30 sec.⁸³

Based on these studies, the major consequence of food thermal processing on carotenoids is the *trans*-to-*cis* isomerization and the rearrangement from 5,6-epoxy to the 5,8-furanoid ring. Moreover, independently of the food matrix or thermal process, the predominant *cis* isomers of β -carotene, lutein, zeaxanthin, and β -cryptoxanthin formed in processed red, yellow, and orange fruits and vegetables is the 13-*cis* form (and 13'-*cis* for asymmetric carotenoids) followed by smaller quantities of 9-*cis* and 15-*cis* isomers.^{14,49,79} However, for processed green vegetables, 9-*cis*- β -carotene is the predominant *cis* isomer followed by 13-*cis*- β -carotene.^{13,14,69} In addition, carotenoids within their natural matrices are stable toward isomerization reactions, as carotenoids can be arranged in crystalline form and their dissolution is a major factor leading to degradation.

4.2.4 CHANGES DURING STORAGE

4.2.4.1 POST-HARVEST RIPENING

Carotenoid levels in vegetables also vary depending on the maturity at harvest. Dark green mature leaves of leafy vegetables such as kale typically have higher carotenoid contents than the paler inner leaves or young ones.⁸⁴ In fruits whose color when ripe is due to pigments other than carotenoids (strawberries) and in fruits that remain green (kiwi and olive fruit), the carotenoid content decreases during ripening.^{85,86}

In contrast, carotenogenesis continues in most carotenoid-containing fruits and vegetables such as apricot, mango, orange, muskmelon, papaya, pepper, persimmon, and tomato. For example, in cherry tomato fruits sorted according to ripening stages into five groups, lycopene contents significantly increased during ripening as follows, 4 $\mu\text{g/g}$ in green-yellow, 22 $\mu\text{g/g}$ in green-orange, 45 $\mu\text{g/g}$ in orange-red, 69 $\mu\text{g/g}$ in light red, and 104 $\mu\text{g/g}$ in fully red skin.⁸⁷ Moreover, all carotenoids followed the same tendency during ripening.⁸⁷

Mangoes are usually picked at the mature green or partially ripe stage to allow sufficient time for transport and commercialization before turning ripe. No qualitative change was seen in the carotenoid composition of mangoes from the mature green to the ripe stage.²⁰ On the other hand, pronounced quantitative changes took place on ripening, total carotenoid content rising from 12 to 38 $\mu\text{g/g}$ in cultivar Keitt and from 17 to 51 $\mu\text{g/g}$ in cultivar Tommy Atkins from the mature green to the ripe stage, the major increases occurring in the β -carotene and violaxanthin levels.²⁰

4.2.4.2 INFLUENCE OF LIGHT

A group of competing reactions takes place when carotenoids are exposed to light. When the incidence of light energy overlaps the carotenoid absorption region,

photoisomerization competes with photodegradation reactions, whereas in the presence of a sensitizer and light, not in the same range that carotenoid absorbs, isomerization and oxidation reactions occur, often with the excited triplet carotenoid state as an intermediate.^{88,89} The predominant reaction will depend on the light intensity and irradiation wavelength, temperature, solvent, and presence of catalysts or sensitizers in the medium.

4.2.4.2.1 Model Systems

In dark conditions, the spontaneous isomerization of carotenoids occurs in solution; the rate is dependent on temperature, solvent, and carotenoid structure. In the case of β -carotene, 13-*cis*- β -carotene was formed approximately three times faster than the 9-*cis*- isomer at room temperature^{90,91} and at 150°C.⁶⁷

On the other hand, under cool white fluorescent light (250 ft-c) at 28°C, the formation of 9-*cis*- β -carotene was favored over formation of the 13-*cis*- isomer.⁹⁰ Light appeared to catalyze the formation of 9-*cis*- β -carotene and/or this isomer is more stable under light than 13-*cis*- β -carotene since 9-*cis*- β -carotene was found in higher amounts in samples stored under light conditions than in the dark.⁹⁰ The first-order photodegradation rate of all isomeric forms of β -carotene in acetonitrile/methanol was $2.78 \times 10^{-5}/\text{min}$, whereas a greater change for all-*trans* β -carotene occurred, $3.06 \times 10^{-5}/\text{min}$, indicating that although *trans*-to-*cis* isomerization took place, the photodegradation of all-*trans* β -carotene was the predominant reaction taking place under the above conditions.⁹⁰

A degradation rate of the same kinetic order and magnitude, $1.67 \times 10^{-5}/\text{min}$, was also observed for β -carotene dissolved in hexane during storage at -5°C under fluorescent light with 2000 lux intensity.⁹² Although *cis* isomer rate values were not given, 9-*cis*-, 13-*cis*-, and 15-*cis*- β -carotene isomers increased along with formation of 13,5-di-*cis*- β -carotene, followed by losses of all *cis* isomers after 12 to 14 hr of illumination.⁹²

In contrast, zero-order reaction rate kinetics was established for the degradation of β -carotene and capsanthin (free and diesterified) under white fluorescent light (1000 lux) or in the dark at different temperatures between 15 and 45°C when these carotenoids were dissolved in cyclohexane and absolute ethanol; while in an aqueous medium (Tween 20 added) the degradation followed first-order reaction.⁹³ Under all conditions tested, β -carotene was the most labile of the pigments, with k values of $1.69 \times 10^{-2}/\text{min}$ in cyclohexane, $2.72 \times 10^{-2}/\text{min}$ in ethanol, and $3.33 \times 10^{-4}/\text{min}$ in water at 25°C under light.⁹³ De-esterification of capsanthin increased its stability in aqueous medium and decreased in non-polar solvent, whereas in a medium with intermediate polarity, both forms of capsanthin had similar stability degrees.⁹³ The degradation rates observed by Minguez-Mosquera and Jaren-Galan⁹³ for β -carotene in organic solvents were three orders of magnitude lower than k values reported in other studies,^{90,92} most probably due to the zero-order kinetic assignment.

After illumination for 60 min with fluorescent lamps with total intensity of 3750 lux at 20°C, degradation rates of all-*trans* β -carotene and all-*trans* lutein, both dissolved in toluene, were very similar: 21%, accompanied by only marginally increasing in the levels of *cis* isomers of β -carotene and lutein.⁶⁹ On the other hand,

β -carotene was found to be less stable than α -carotene and lutein in a powder composed of carotenoids extracted from carrot pulp and encapsulated with sucrose/gelatin by spray-drier when stored at temperatures of 4, 25, and 45°C in the dark and at 25°C under fluorescent light (1500 lux).^{94,95} Degradation of all carotenoids followed first-order kinetics, the k_{obs} values at 25°C under dark and light being, respectively, 5.17×10^{-4} and $9.67 \times 10^{-4}/\text{min}$ for β -carotene, 4.33×10^{-4} and $8.17 \times 10^{-4}/\text{min}$ for α -carotene, and 1.33×10^{-4} and $2.50 \times 10^{-4}/\text{min}$ for lutein.⁹⁴ The 13-*cis* type isomers of carotenoids dominated under dark storage, while the formation of the 9-*cis* type was favored under light storage; in addition, 13,15-di-*cis*- β -carotene was also formed under illumination.⁹⁵

Degradation of bixin dissolved in a water medium with 0.2% Tween 80 followed first-order rate, either in the dark or under fluorescent light (700 lux), both at 21°C.⁹⁶ As expected, degradation was much higher under light ($k_{\text{obs}} = 3.79 \times 10^{-3}/\text{min}$) than in dark conditions ($k_{\text{obs}} = 7.25 \times 10^{-5}/\text{min}$).⁹⁶

Only 6% of the initial total lycopene prepared as a thin film on the surface of each vial remained after 144 hr under fluorescent light (2000 to 3000 lux) at 25°C under N_2 .⁷³ Lycopene degradation occurred as a first-order reaction at $2.93 \times 10^{-4}/\text{min}$, and the concentration of all lycopene mono-*cis* isomers already present in the sample, 5-*cis*-, 9-*cis*-, 13-*cis*- and 15-*cis*-, showed an inconsistent change in this period.⁷³ Nevertheless, formation of lycopene di-*cis* isomers was observed after 32 hr of light exposure and when considering relative percentage, loss of 13% of all-*trans*-lycopene occurred while an increase of 11% for total *cis* isomers was found after 144 hr.⁷³

In general, the above studies show that both isomerization and degradation reactions proceed simultaneously under light incidence in the same region as carotenoids absorb, apparently requiring lower energy barriers than those observed for thermal isomerization and degradation. The formation of 9-*cis*- β -carotene is favored over the 13-*cis* isomer under light. As already stated in Section 4.2.3.1, the kinetic order rate model should be confirmed by carrying out additional experiments with different carotenoid concentrations.

4.2.4.2.2 Food Systems

The degradation of carotenes followed the first-order rate law during storage of a commercial vegetable juice, in which tomatoes and carrots are the major carotenoid sources, under constant light intensity of 230 ft-c at 4°C and in the presence of air.⁹⁷ Lycopene degraded at a much slower rate ($k_{\text{obs}} = 4.2 \times 10^{-5}/\text{min}$) compared to α -carotene ($k_{\text{obs}} = 1.9 \times 10^{-4}/\text{min}$) and β -carotene ($k_{\text{obs}} = 2.1 \times 10^{-4}/\text{min}$). Since negligible degradation of these carotenes was found in samples not exposed to light, the air present in the bottle headspace had practically no influence on carotene degradation.⁹⁷

Losses from 21 to 23% in β -carotene, α -carotene, and lutein contents were observed during storage of carrot juice under light (1500 lux) at 25°C for 12 wk.⁹⁸ The losses were accompanied by increased concentrations of the 13-*cis* isomer type of β -carotene, α -carotene, and lutein during dark storage, while the formation of 9-*cis*- β -carotene, 9-*cis*- α -carotene, and 13-*cis*-lutein was favored under light storage.⁹⁸

In contrast, the amounts of *cis* isomers remained fairly constant in carrot juice after 12 days of storage in the dark at 28°C.⁹⁰ When exposed to light (250 ft-c), degradation of total carotenes in carrot juice was not a significant factor, and although the 9-*cis* isomer appeared already in carrot juice samples after 1 day, it reached only 5% maximum in 35 days.⁹⁰ Moreover, no losses of all-*trans* β -carotene, 13-*cis*- β -carotene, α -carotene, and lutein contents were found in carrot slices during 12 days of storage at 4°C in dark or light (2000 lux) conditions, while a loss of 47% of 9-*cis*- β -carotene occurred.⁹⁹

In spinach leaves exposed to white fluorescent light (2000 lux) at 4°C for 8 days, losses of carotenoids occurred: 22% for lutein, 41% for all-*trans* β -carotene, 45% for 13-*cis*- β -carotene and neoxanthin, 48% for 9-*cis*- β -carotene, and 60% for violaxanthin, along with losses of 42% of chlorophyll *b* and 30% of *a*.⁹⁹ Since chlorophyll is a sensitizer capable of generating the highly reactive singlet oxygen species, both photodegradation and photosensitized reactions took place at the same time. Under the same conditions in darkness, *cis* isomerization was not important since lower losses occurred: 0% for violaxanthin, 3% for lutein, 7% for neoxanthin, 10% for 9-*cis*- β -carotene, 12% for 13-*cis*- β -carotene, and 18% for all-*trans* β -carotene.⁹⁹ In another study, higher losses were observed during storage in the dark of minimally processed kale at 7 to 9°C for 5 days: 33% for neoxanthin, 27% for lutein, 21% for violaxanthin, and 14% for β -carotene.⁸⁴

Lycopene stability was evaluated in tomato pulp stored at -20, 5, and 25°C under oxygen atmosphere, reduced pressure, and fluorescent light or dark conditions.¹⁰⁰ Under all conditions, degradation was adjusted to the pseudo first-order rate kinetics, giving, as expected, faster rate values for increased temperature, presence of oxygen, and light. At 25°C, lycopene degradation rates were 1.74×10^{-5} /min under light and oxygen, 1.53×10^{-5} /min under dark and oxygen, and 0.50×10^{-5} /min in the dark and under vacuum.¹⁰⁰ Since similar behavior was observed at the lowest temperatures, these results indicated that oxygen was more deleterious to lycopene than light, although light intensity was not reported in this study.

The comparison of the light effect on carotenoids in foods is very difficult to carry out because different foods with different isomer compositions are employed at the beginnings of experiments. The presence of large molecules offers some photoprotection to carotenoids in food systems, either by complexation with proteins as found in carrots or acting as a filter to reduce the light incidence. Different storage conditions are often found because different light intensities are used or sometimes they are not even reported and experiments are carried out under air, N₂, or in a vacuum.

SCIENTIFIC NAMES

acerola (*Malpighia emarginata* DC.), annatto (*Bixa orellana* L.), banana (*Musa troglodytarum*), botla benda (*Abutilon indicum*), breadfruit with seeds (*Artocarpus mariannensis*), breadfruit without seeds (*Artocarpus altilis*), buriti (*Mauritia flexosa* or *M. vinifera* Mart.), carrot (*Daucus carota* L.), caruru (*Amaranthus viridis* L.), clementine (*Citrus clementine* Hort. ex Tan.), gac fruit (*Momordica cochinchinensis* Spreng), gardenia (*Gardenia jasminoides* Ellis), giant swamp taro (*Cryotosperma*

chamissonis), Hansen mandarin (*Citrus reticulada* Blanco), kale (*Brassica oleracea* var. *acephala*), kiwi (*Actinidia chinensis* Planch.), mentruz (*Lepidium pseudodidymum*), mulla thotakura (*Amaranthus spinosus*), olive (*Olea europaea* L.), orange (*Citrus sinensis* L.), palm fruit (*Elaeis guineensis* or *E. oleifera*), parsley (*Petroselinum hortense*), pepper (*Capsicum annum* L.), persimmon (*Diospyros kaki*), saffron (*Crocus sativus* L.), sea buckthorn (*Hippophae rhamnoides* L.), serralha (*Sonchus oleraceus*), spinach (*Spinacia oleracea* L.), strawberry (*Fragaria x ananassa* Duch.), taioba (*Xanthosoma* spp.), tomato (*Lycopersicon esculentum*), Wase satsuma (*Citrus unshiu* Marc.), watermelon (*Citrullus lanatus*), Willowleaf mandarin (*Citrus deliciosa* Ten.), wolfberry (*Lycium barbarum* L.), yerramolakakaura (*Amaranthus* sp).

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REFERENCES

1. Holden, J.M. et al., Carotenoid content of U.S. foods: an update of the database, *J. Food Compos. Anal.*, 12, 169, 1999.
2. Römer, S. et al., Elevation of the provitamin A content of transgenic tomato plants, *Nat. Biotechnol.*, 18, 666, 2000.
3. Shewmaker, C.K. et al., Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects, *Plant J.*, 20, 401, 1999.
4. Mercadante, A.Z. and Rodriguez-Amaya, D.B., Carotenoid composition and vitamin A value of some native Brazilian green leafy vegetables, *Int. J. Food Sci. Technol.*, 25, 213, 1990.
5. Bhaskarachary, K. et al., Carotene content of some common and less familiar foods of plant origin, *Food Chem.*, 54, 189, 1995.
6. Sweeney, J.P. and Marsh, A.C., Effect of processing on provitamin A in vegetables, *J. Am. Diet. Assoc.*, 59, 238, 1971.
7. Khachik, F., Beecher, G.R., and Whittaker, N.F., Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography, *J. Agric. Food Chem.*, 34, 603, 1986.
8. Mercadante, A.Z. and Rodriguez-Amaya, D.B., Carotenoid composition of a leafy vegetable in relation to some agricultural variables, *J. Agric. Food Chem.*, 39, 1094, 1991.
9. Granado, F. et al., Carotenoid composition in raw and cooked Spanish vegetables, *J. Agric. Food Chem.*, 40, 2135, 1992.
10. Müller, H., Determination of the carotenoid content in selected vegetables and fruit by HPLC and photodiode array detection, *Z. Lebensm. Unters. Forsch. A*, 204, 88, 1997.
11. Gomez, M.I., Carotene content of some green leafy vegetables of Kenya and effects of dehydration and storage on carotene retention, *J. Plant Foods*, 3, 231, 1981.
12. Wills, R.B.H. and Ranga, A., Determination of carotenoids in Chinese vegetables, *Food Chem.*, 56, 451, 1996.

13. Nyambaka, H. and Ryley, J., An isocratic reversed-phase HPLC separation of the stereoisomers of the provitamin A carotenoids (α - and β -carotene) in dark green vegetables, *Food Chem.*, 55, 63, 1996.
14. Lessin, W.J., Catigani, G.L., and Schwartz, S.J., Quantification of *cis-trans* isomers of provitamin A carotenoids in fresh and processed fruits and vegetables, *J. Agric. Food Chem.*, 45, 3728, 1997.
15. Heinonen, M.I., Carotenoids and provitamin A activity of carrot (*Daucus carota* L.) cultivars, *J. Agric. Food Chem.*, 38, 609, 1990.
16. Almeida, L.B. and Penteado, M.V.C., Carotenoids with provitamin A activity of carrots (*Daucus carota* L.) consumed in São Paulo, Brazil, *Rev. Farm. Bioquím. Univ. S. Paulo*, 23, 133, 1987.
17. Chandler, L.A. and Schwartz, S.J., HPLC separation of *cis-trans* carotene isomers in fresh and processed fruits and vegetables, *J. Food Sci.*, 52, 669, 1987.
18. Almeida, L.B. and Penteado, M.V.C., Carotenoids and provitamin A value of some Brazilian sweet potato cultivars (*Ipomoea batatas* Lam.), *Rev. Farm. Bioquím. Univ. S. Paulo*, 28, 145, 1992.
19. Godoy, H.T. and Rodriguez-Amaya, D.B., Occurrence of *cis*-isomers of provitamin A in Brazilian fruits, *J. Agric. Food Chem.*, 42, 1306, 1994.
20. Mercadante, A.Z. and Rodriguez-Amaya, D.B., Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango, *J. Agric. Food Chem.*, 46, 128, 1998.
21. Pott, I. et al., Quantitative determination of β -carotene stereoisomers in fresh, dried, and solar-dried mangoes (*Mangifera indica* L.), *J. Agric. Food Chem.*, 51, 4527, 2003.
22. Fanciullino, A.-L. et al., Carotenoid diversity in cultivated citrus is highly influenced by genetic factors, *J. Agric. Food Chem.*, 54, 4397, 2006.
23. Dhuique-Mayer, C. et al., Varietal and interspecific influence on micronutrient contents in citrus from the Mediterranean area, *J. Agric. Food Chem.*, 53, 2140, 2005.
24. Gama, J.J.T. and Sylos, C.M., Major carotenoid composition of Brazilian Valencia orange juice: identification and quantification by HPLC, *Food Res. Int.*, 38, 899, 2005.
25. Mouly, P.P. et al., Differentiation of several geographical origins in single-strength Valencia orange juices using quantitative comparison of carotenoid profiles, *J. Agric. Food Chem.*, 47, 4038, 1999.
26. Setiwan, B. et al., Carotenoid content of selected Indonesian fruits, *J. Food Compos. Anal.*, 14, 169, 2001.
27. Englberger, L. et al., Further analyses on Micronesian banana, taro, breadfruit and other foods for provitamin A carotenoids and minerals, *J. Food Compos. Anal.*, 16, 219, 2003.
28. De Rosso, V.V. and Mercadante, A.Z., Carotenoid composition of two Brazilian genotypes of acerola (*Malpighia puniceifolia* L.) from two harvests, *Food Res. Int.*, 38, 1073, 2005.
29. Hamano, P.S. and Mercadante, A.Z., Composition of carotenoids from commercial products of caju (*Spondias lutea*), *J. Food Compos. Anal.*, 14, 335, 2001.
30. De Ancos, B., Gonzalez, E., and Cano, M.P., Effect of high-pressure treatment on the carotenoid composition and the radical scavenging activity of persimmon fruit purees, *J. Agric. Food Chem.*, 48, 3542, 2000.
31. Raffo, A., Paoletti, F., and Antonelli, M., Changes in sugar, organic acid, flavonol and carotenoid composition during ripening of berries of three seabuckthorn (*Hippophae rhamnoides* L.) cultivars, *Eur. Food Res. Technol.*, 219, 360, 2004.

32. Trigueiro, I.N.S. and Pentead, M.V.C., Stereochemical isomers composition and vitamin A value of Brazilian palm oils, *Arch. Latinoamer. Nutr.*, 46, 334, 1996.
33. Trujillo-Quijano, J.A. et al., Carotenoid composition and vitamin A values of oils from four Brazilian palm fruits, *Fat Sci. Technol.*, 92, 222, 1990.
34. Abushita, A.A., Daoud, H.G., and Biacs, P.A., Change in carotenoids and antioxidant vitamins in tomato as a function of varietal and technological factors, *J. Agric. Food Chem.*, 48, 2075, 2000.
35. Niizu, P.Y. and Rodriguez-Amaya, D.B., New data on the carotenoid composition of raw salad vegetables, *J. Food Compos. Anal.*, 18, 739, 2005.
36. Takeoka, G.R., Processing effects on lycopene content and antioxidant activity of tomatoes, *J. Agric. Food Chem.*, 49, 3713, 2001.
37. Tonucci, L.H. et al., Carotenoid content of thermally processed tomato-based food products, *J. Agric. Food Chem.*, 43, 579, 1995.
38. Schierle, J. et al., Content and isomeric ratio of lycopene in food and human blood plasma, *Food Chem.*, 59, 459, 1997.
39. Lavelli, V. et al., Properties of tomato powders as additives for food fortification and stabilization, *J. Agric. Food Chem.*, 49, 2037, 2001.
40. Perkins-Veazie, P. et al., Carotenoid content of 50 watermelon cultivars, *J. Agric. Food Chem.*, 54, 2593, 2006.
41. Kimura, M., Rodriguez-Amaya, D.B., and Yokoyama, S.M., Cultivars differences and geographic effects on the carotenoid composition and vitamin A value of papaya, *Lebensm.-Wiss. u.-Technol.*, 24, 415, 1991.
42. Rouseff, R.L. et al., Determination of β -carotene and other hydrocarbon carotenoids in red grapefruit cultivars, *J. Agric. Food Chem.*, 40, 47, 1992.
43. Padula, M. and Rodriguez-Amaya, D.B., Characterisation of the carotenoids and assessment of the vitamin A value of Brazilian guavas (*Psidium guajava* L.), *Food Chem.*, 20, 11, 1986.
44. Aoki, H. et al., Carotenoid pigments in gac fruit (*Momordica cochinchinensis* Spreng), *Biosci. Biotechnol. Biochem.*, 66, 2479, 2002.
45. Ishida, B.K. et al., Fatty acid and carotenoid composition of gac (*Momordica cochinchinensis* Spreng) fruit, *J. Agric. Food Chem.*, 52, 274, 2004.
46. Vuong, L.T. et al., *Momordica cochinchinensis* Spreng. (gac) fruit carotenoids reevaluated, *J. Food Compos. Anal.*, 19, 664, 2006.
47. Heinonen, M.I., Carotenoids in Finnish foods: vegetables, fruits, and berries, *J. Agric. Food Chem.*, 37, 655, 1989.
48. Humphries, J.M. and Khachik, F., Distribution of lutein, zeaxanthin, and related geometrical isomers in fruit, vegetables, wheat, and pasta products, *J. Agric. Food Chem.*, 51, 1322, 2003.
49. Updike, A.A. and Schwartz, S.J., Thermal processing of vegetables increases *cis* isomers of lutein and zeaxanthin, *J. Agric. Food Chem.*, 51, 6184, 2003.
50. Aman, R. et al., Application of HPLC coupled with DAD, APcI-MS and NMR to the analysis of lutein and zeaxanthin stereoisomers in thermally processed vegetables, *Food Chem.*, 92 753, 2005.
51. Weller, P. and Breithaupt, D.E., Identification and quantification of zeaxanthin esters in plants using liquid chromatography-mass spectrometry, *J. Agric. Food Chem.*, 51, 7044, 2003.
52. Scott, C.E. and Eldridge, A.L., Comparison of carotenoid content in fresh, frozen and canned corn, *J. Food Compos. Anal.*, 18, 551, 2005.
53. Almela, L. et al., Carotenoid composition of new cultivars of red pepper for paprika, *J. Agric. Food Chem.*, 39, 1606, 1991.

54. Mínguez-Mosquera, M.I. and Hornero-Méndez, D., Formation and transformation of pigments during the fruit ripening of *Capsicum annum* cv. *Bola* and *Agridulce*, *J. Agric. Food Chem.*, 42, 38, 1994.
55. Deli, J. and Molnár, P., Paprika carotenoids: analysis, isolation, structure elucidation, *Curr. Org. Chem.*, 6, 1197, 2002.
56. Lam, K.W. and But, P., The content of zeaxanthin in Gou Qi Zi, a potential health benefit to improve visual acuity, *Food Chem.*, 67, 173, 1999.
57. Deli, J., Matus, Z. and Tóth, G., Carotenoid composition in the fruits of *Asparagus officinalis*, *J. Agric. Food Chem.*, 48, 2793, 2000.
58. Pfister, S. et al., Isolation and structure elucidation of carotenoid-glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne), *J. Agric. Food Chem.*, 44, 2612, 1996.
59. Carmona, M. et al., Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-MS, *J. Agric. Food Chem.*, 54, 973, 2006.
60. Mercadante, A.Z. and Pfander, H., Carotenoids from annatto: a review, *Recent Res. Devel. Agric. Food Chem.*, 2, 79, 1998.
61. Mercadante, A.Z., Composition of carotenoids from annatto, in *Chemistry and Physiology of Selected Food Colorants*, Ames, J.M. and Hofmann, T.F., Eds., ACS Symposium Series 775, Washington, 2001, chap. 6.
62. Tocchini, L. and Mercadante, A.Z., Extraction and determination of bixin and norbixin, by HPLC, in annatto spice ("colorifico"), *Ciênc. Tecnol. Aliment.*, 21, 310, 2001.
63. Marty, C. and Berset, C., Degradation of *trans*- β -carotene during heating in sealed glass tubes and extrusion cooking, *J. Food Sci.*, 51, 698, 1986.
64. Marty, C. and Berset, C., Degradation products of *trans*- β -carotene produced during extrusion cooking, *J. Food Sci.*, 53, 1880, 1988.
65. Marty, C. and Berset, C., Factors affecting the thermal degradation of all-*trans* β -carotene, *J. Agric. Food Chem.*, 38, 1063, 1990.
66. Kanasawud, P. and Crouzet, J.C., Mechanism of formation of volatile compounds by thermal degradation of carotenoids in aqueous medium. 1. β -Carotene degradation, *J. Agric. Food Chem.*, 38, 237, 1990.
67. Chen, B.H., Chen, T.M., and Chien, J.T., Kinetic model for studying the isomerization of α - and β -carotene during heating and illumination, *J. Agric. Food Chem.*, 42, 2391, 1994.
68. Gaier, K., Angerhofer, A., and Wolf, H.C., The lowest excited singlet states of all-*trans*- β -carotene single crystals, *Chem. Phys. Lett.*, 187, 103, 1991.
69. Aman, R., Schieber, A., and Carle, R., Effects of heating and illumination on *trans-cis* isomerization and degradation of β -carotene and lutein in isolated spinach chloroplasts, *J. Agric. Food Chem.*, 53, 9512, 2005.
70. Liu, M.H. and Chen, B.H., Relationship between chlorophyll *a* and β -carotene in a lipid-containing model system during heating, *Food Chem.*, 61, 41, 1998.
71. Henry, L.K., Catignani, G.L., and Schwartz, S.J., Oxidative degradation kinetics of lycopene, lutein and 9-*cis* and all-*trans* β -carotene, *J. Amer. Oil Chem. Soc.*, 75, 823, 1998.
72. Hackett, M.M. et al., Thermal stability and isomerization of lycopene in tomato oleoresins from different varieties, *J. Food Sci.*, 69, C536, 2004.
73. Lee, M.T. and Chen, B.H., Stability of lycopene during heating and illumination in a model system, *Food Chem.*, 78, 425, 2002.

74. Rios, A.O., Borsarelli, C.D., and Mercadante, A.Z., Thermal degradation kinetics of bixin in an aqueous model system, *J. Agric. Food Chem.*, 53, 2307, 2005.
75. Scotter, M.J., Castle, L., and Appleton, G.P., Kinetics and yields for the formation of coloured and aromatic thermal degradation products of annatto in foods, *Food Chem.*, 74, 365, 2001.
76. Scotter, M.J., Characterization of the coloured thermal degradation products of bixin from annatto and a revised mechanism for their formation, *Food Chem.*, 53, 177, 1995.
77. Zechmeister, L., *Cis-trans* isomerization and stereochemistry of carotenoids and diphenylpolyenes, *Chem. Rev.*, 34, 267, 1944.
78. Khachik, F. et al., Effect of food preparation on qualitative and quantitative distribution of major carotenoid constituents of tomatoes and several green vegetables, *J. Agric. Food Chem.*, 40, 390, 1992.
79. Chen, B.H., Peng, H.Y., and Chen, H.E., Changes of carotenoids, color, and vitamin A contents during processing of carrot juice, *J. Agric. Food Chem.*, 43, 1912, 1995.
80. Marx, M. et al., Effects of thermal processing on *trans-cis*-isomerization of β -carotene in carrot juices and carotene-containing preparations, *Food Chem.*, 83, 609, 2003.
81. Chen, J.P., Tai, C.Y., and Chen B.H., Effects of different drying treatments on the stability of carotenoids in Taiwanese mango (*Mangifera indica* L.), *Food Chem.*, 100, 1005, 2007.
82. Nguyen, M., Francis, D., and Schwartz, S., Thermal isomerisation susceptibility of carotenoids in different tomato varieties, *J. Sci. Food Agric.*, 81, 910, 2001.
83. Lee, H.S. and Coates, G.A., Effect of thermal pasteurization on Valencia orange juice color and pigments, *Lebensm.-Wiss. U.-Technol.*, 36, 153, 2003.
84. Azevedo-Meleiro, C.H. and Rodriguez-Amaya, D.B., Carotenoid composition of kale as influenced by maturity, season and minimal processing, *J. Sci. Food Agric.*, 85, 591, 2005.
85. Gross, J., *Pigments in Fruits*, Academic Press, London, 1987.
86. Roca, M. and Mínguez-Mosquera, M.I., Changes in chloroplast pigments of olive varieties during fruit ripening, *J. Agric. Food Chem.*, 49, 832, 2001.
87. Raffo et al., Nutritional value of cherry tomatoes (*Lycopersicon esculentum* Cv. Naomi F1) harvested at different ripening stages, *J. Agric. Food Chem.*, 50, 6550, 2002.
88. Fujii, R. et al., *Cis-to-trans* isomerization of spheroidene in the triplet state as detected by time-resolved absorption spectroscopy, *J. Phys. Chem. A*, 106, 2410, 2002.
89. Montenegro, M.A. et al., Model studies on the photosensitized isomerization of bixin, *J. Agric. Food Chem.*, 52, 367, 2004.
90. Pesek, C.A. and Warthesen, J.J., Kinetic model for photoisomerization and concomitant photodegradation of β -carotenes, *J. Agric. Food Chem.*, 38, 1313, 1990.
91. Pesek, C.A., Warthesen, J.J., and Taoukis, P.S., A kinetic model for equilibration of isomeric β -carotenes, *J. Agric. Food Chem.*, 38, 41, 1990.
92. Chen, B.H. and Huang, J.H., Degradation and isomerization of chlorophyll *a* and β -carotene as affected by various heating and illumination treatments, *Food Chem.*, 62, 299, 1998.
93. Mínguez-Mosquera, M.I. and Jaren-Galan, M., Kinetics of the decolouring of carotenoid pigments, *J. Sci. Food Agric.*, 67, 153, 1995.
94. Chen, B.H. and Tang, Y.C., Processing and stability of carotenoid powder from carrot pulp waste, *J. Agric. Food Chem.*, 46, 2312, 1998.
95. Tang, Y.C. and Chen, B.H., Pigment change of freeze-dried carotenoid powder during storage, *Food Chem.*, 69, 11, 2000.

96. Barbosa, M.I.M.J., Borsarelli C.D., and Mercadante, A.Z. Light stability of spray-dried bixin encapsulated with different edible polysaccharide preparations, *Food Res. Int.*, 38, 989, 2005.
97. Pesek, C.A. and Warthesen, J.J., Photodegradation of carotenoids in a vegetable juice system, *J. Food Sci.*, 52, 744, 1987.
98. Chen, B.H., Peng, H.Y., and Chen, H.E., Stability of carotenoids and vitamin A during storage of carrot juice, *Food Chem.*, 57, 497, 1996.
99. Kopas-Lane, L.M. and Warthesen, J.J., Carotenoid photostability in raw spinach and carrots during cold storage, *J. Food Sci.*, 60, 773, 1995.
100. Sharma, S.K. and Le Maguer, M., Kinetics of lycopene degradation in tomato pulp solids under different processing and storage conditions, *Food Res. Int.*, 29, 309, 1996.

4.3 Anthocyanins in Foods: Occurrence and Physicochemical Properties

Adriana Z. Mercadante and Florinda O. Bobbio

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4.3.1 INTRODUCTION

In the past decade, general interest and research activities focusing on anthocyanins have considerably increased. This increased interest is not only based on the common knowledge that these water-soluble pigments can be used as possible alternatives to artificial food colorants, but also it relates to their bioactive properties. When search-

ing the Chemical Abstract Service and Medline for the word *anthocyanin*, 910 articles were cited in 2005 compared to 305 articles in 1995.

The total number of different anthocyanins reported to be isolated from plants was 539.¹ However, the number of anthocyanins found in foods is much smaller. Although a large number of papers were published regarding anthocyanin composition in several foods, investigators in most studies used only chromatographic and chemical behaviors as bases for identification. In this chapter, we considered only papers in which identification was based at least on mass spectrometry (MS). In fact, the use of only MS and UV-visible information can easily lead to misidentification as the following example shows.

A minor peak separated from boysenberry was found to have a mass at m/z 757 and was assigned as cyanidin-3-(6''-*p*-coumaryl-glucoside)-5-glucoside² and was also considered to be cyanidin-3-(2-glucosyl)-6-(rhamnosyl)glucoside³, consistent with its molecular weight of 757 μ . Nuclear magnetic resonance (NMR) analysis confirmed that cyanidin-3-(2-(glucosyl)-6-(rhamnosyl)glucoside was the correct assignment.³

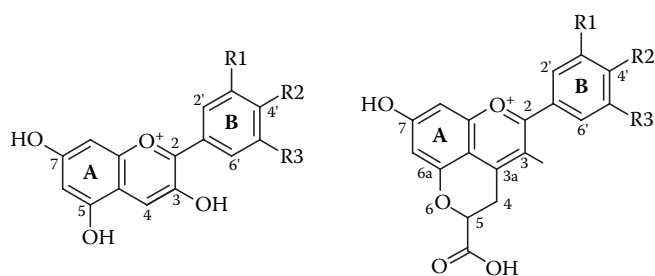
The assignment of sugar configuration was not properly investigated in most papers. In addition, few data regarding amounts of specific anthocyanins in foods are available because anthocyanin standards are expensive, are not easily available, and are very difficult to prepare in a laboratory.

Anthocyanins would be the ideal substitutes for synthetic red colorants based on their bright colors varying from orange red to blue, water solubility, and non-toxicity. Nevertheless the use of these pigments in foods has been hampered by poor stability, which is affected by physical and chemical factors such as temperature, pH, light, solvent, and pigment structure as well as factors prevailing during food processing and storage. In order to overcome this problem, intense research has been done on stabilization of anthocyanins and elucidation of the high stability of wine colors.

4.3.2 STRUCTURES AND PHYSICOCHEMICAL PROPERTIES

Anthocyanins are glycosides of anthocyanidin, aglycone possessing a fundamental skeleton of 2-phenylbenzopyrylium, known as the flavylium cation (Figure 4.3.1). More than 90% of all anthocyanins isolated in nature are based only on the following six anthocyanidins: pelargonidin (plg), cyanidin (cyd), peonidin (pnd), delphinidin (dpd), petunidin (ptd), and malvidin (mvd), which as shown in the figure, are differentiated by the substitution pattern on the B ring. Recently, the structure of a novel type found especially in wine and called pyroanthocyanin was described (Figure 4.3.1).

Most anthocyanins in foods contain one or two monosaccharide units, most commonly occurring at position 3, sometimes at position 3,5, and more rarely at position 3,7. The sugar moieties are connected to the anthocyanidins through hemiacetalic linkages. The monosaccharides found in foods are β -D-glucopyranose, β -D-galactopyranose, α -rhamnopyranose, D-arabinopyranose and D-xylopyranose (Figure 4.3.2). The following disaccharides have also been found linked to anthocyanidins in foods: 2- β -glucopyranosyl- β -glucopyranoside (sophorose), α -L-rham-



Anthocyanidins	Substitution pattern		
	R1	R2	R3
Pelargonidin (plg)	H	OH	H
Cyanidin (cyd)	OH	OH	H
Delphinidin (dpd)	OH	OH	OH
Peonidin (pnd)	OCH ₃	OH	H
Petunidin (ptd)	OCH ₃	OH	OH
Malvidin (mvd)	OCH ₃	OH	OCH ₃

FIGURE 4.3.1 Structures of anthocyanidins and pyroanthocyanidins occurring in foods.

nopyranosyl-(1,6)- β -glucopyranoside (rutinose), 2- α -L-xylopyranosyl-glucopyranoside (sambubiose), 2- β -xylopyranosyl- β -galactopyranoside (lathyrose), and 3- β -glucopyranosyl- β -glucopyranoside (laminariobiose) (Figure 4.3.2).

Four anthocyanin species exist in equilibrium under acidic conditions at 25°C,^{4,5} according to the scheme in Figure 4.3.3. The equilibrium constant values determine the major species and therefore the color of the solution. If the deprotonation equilibrium constant, K_a , is higher than the hydration constant, K_h , the equilibrium is displaced toward the colored quinonoidal base (A), and if $K_h > K_a$ the equilibrium shifts toward the hemiacetalic or pseudobase form (B) that is in equilibrium with the chalcone species (C), both colorless.^{4,5} Therefore, the structure of an anthocyanin is strongly dependent on the solution pH, and as a consequence so is its color stability, which is highly related to the deprotonation and hydration equilibrium reaction constant values (K_a and K_h).

4.3.3 OCCURRENCE IN FOODS

4.3.3.1 ANTHOCYANIDINS

The most widespread anthocyanidin in foods is cyanidin, as can be seen in Table 4.3.1 through Table 4.3.4. It was found in amounts greater than 10% of the total anthocyanin content in 39 of 44 different fruits listed in Table 4.3.1 and in 9 of 13 vegetables shown in Table 4.3.3. On the other hand, cyanidin was presented in high amounts only in non-*Vitis vinifera* species, such as cv. Concorde^{10,11} and Cynthiana²¹ (Table 4.3.2).

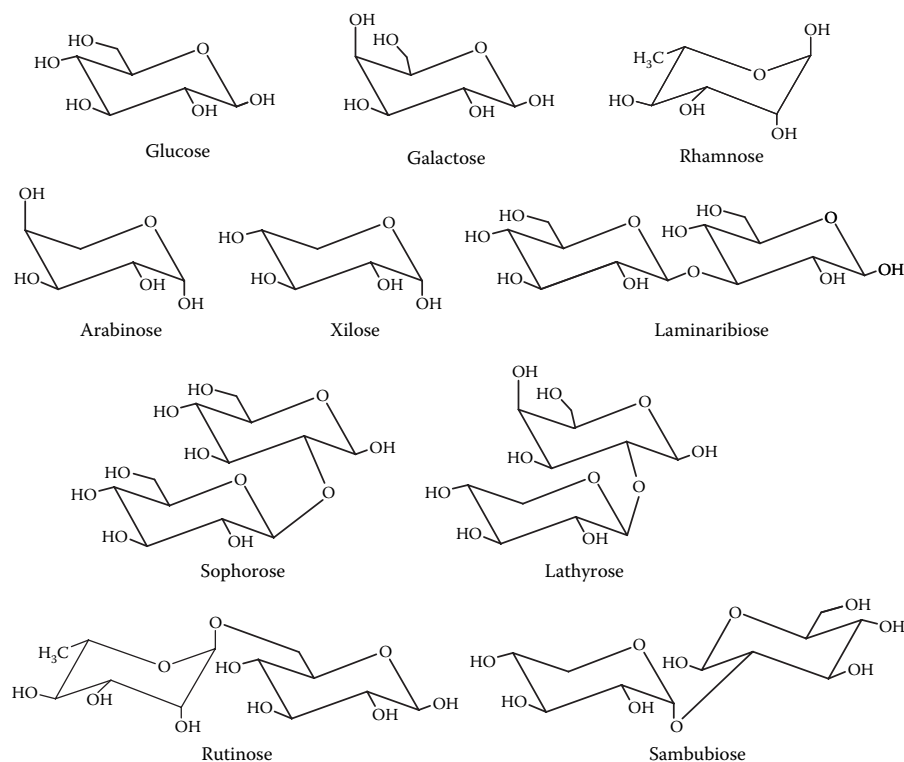


FIGURE 4.3.2 Structures of common mono- and disaccharides occurring in anthocyanins from foods.

Cyanidin was the only aglycone detected in apple cultivars Fuji and Gala,^{10,11} in some kinds of berries,^{15,16,20} black chokeberry,^{23,24} dwarf dogwood,³⁸ elderberry,^{24,39} litchi,⁴¹ and in some berry fruits of genus *Rubus* which includes blackberries^{10,11,21,22,45} in addition to hybrid berries like marionberry^{10,11} and boysenberry,^{3,32} and in stone fruits such as nectarines, plums, and peaches.^{10,11} As shown in Tables 4.3.3 and 4.3.4, cyanidin was also the only aglycone found in red leaf lettuce,^{11,60} chive,⁵⁹ *Allium victorialis*,⁵⁵ garlic,⁶¹ red cabbage,^{11,23,52,60} and pistachio nuts.^{11,60}

Delphinidin is less widespread in fruits and vegetables, being detected in amounts higher than 10% of the total anthocyanin content only in 9 of 44 fruits listed in Table 4.3.1, and in 2 of 13 vegetables shown in Table 4.3.3. It is also found in *Vitis vinifera* grape cultivars Nerello Mascalese,⁵¹ Pallagrello, and Piediroso Vesuvio,⁵⁴ in non-*Vitis vinifera* species such as cultivars Concorde^{10,11} and Cynthiana,²¹ and in hybrid red grapes such as Isabelle^{49,54} and Clinton.⁴⁹ Unlike cyanidin, which is not found as a major anthocyanidin in grains, delphinidin is a major aglycone in different types of beans,^{11,60, 73–76,83} lentils,⁷⁷ soybeans,^{76,80} blue wheat,⁷⁹ and bambara groundnuts.⁷² The anthocyanins from eggplants^{11,60} and black lentils⁷⁷ contain only this aglycone.

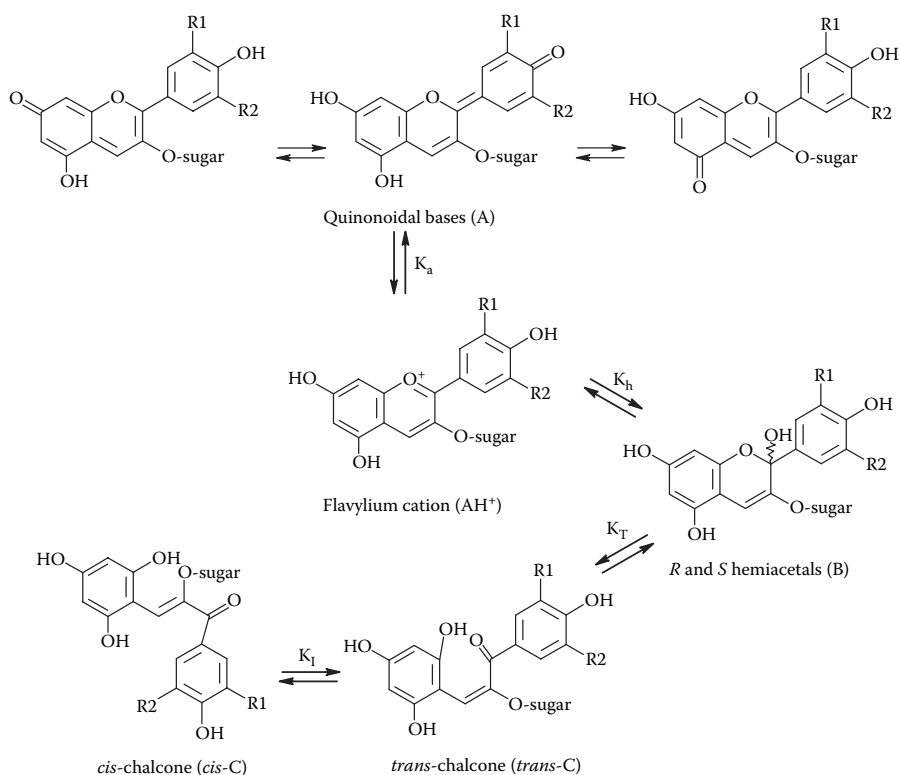


FIGURE 4.3.3 Interconversion pathways of various forms of anthocyanins in acidic aqueous medium.

Pelargonidin is even less widespread in fruits and vegetables, being detected in amounts above 10% of the total anthocyanin content only in 4 of 44 fruits listed in Table 4.3.1, in 2 of 13 vegetables shown in Table 4.3.3 and in some beans (Table 4.3.4). In addition, regarding grapes (Table 4.3.2), pelargonidin was found only in small amounts in cv. Rubired⁵³ and cv. Concorde.^{10,11,52,53} While strawberries are known as the best sources of pelargonidin, it is the only aglycone found in *Ruscus aculeatus* berries,¹⁸ red radishes,^{11,52,60} and some potato hybrids and clones.^{52,63,64}

Petunidin was detected in amounts above 10% of the total anthocyanin content in only 4 of the 44 fruits listed in Table 4.3.1, in 1 of 13 vegetables shown in Table 4.3.3, in 2 kinds of beans and in 1 nut (Table 4.3.4). Petunidin was found in different grape species such as cv. Cabernet Sauvignon,⁵⁰ variety Isabelle,⁵⁴ cv. Pallagrello, cv. Piediroso Vesuvio,⁵⁴ cv. Nerello Mascalese,⁵¹ and cv. Clinton.⁴⁹ None of the foods listed in the four tables presented petunidin as the sole aglycone.

Peonidin is even less widespread in fruits and vegetables, being detected in amounts higher than 10% of the total anthocyanin content in only 4 of 44 fruits listed in Table 4.3.1, in sweet potatoes^{69–71} and in purple corn cv. Morado.^{39,81,82} It was found as one of the principal anthocyanidins in cv. Cabernet Franc,⁴⁹ cv. Rubi-

TABLE 4.3.1
Distribution of Anthocyanins in Fruits

Source and Reference	Total Content (mg/100 g)	Major ^a	Minor ^b
açai ⁶⁻⁸	50 d.w. 300–4630 d.w. n.d.	cyd-3-glu, cyd-3-rut cyd-3- α -rha, plg-3- α -rha	cyd-3-sam, pnd-3-glu, pnd-3-rut n.d.
acerola ⁹ apple ^{10,11}	12 f.w. 1–2 f.w. 342 f.w.	cyd-3-gal cyd-3-gal dpd-3-glu, cyd-3-glu, ptd-3-glu, pnd-3-glu, mvd-3-glu	cyd-3-ara, pnd-3-gal, cyd-7-ara, cyd-3-xyl cyd-3-ara plg-3-glu
cv. Red delicious cv. Fuji and cv. Gala bagaçu ¹²	21700 f.w. n.d.	cyd-3-glu, cyd-3-rut dpd-3- β -glu-7,3,5-tri-(6''-cou)- β -glu	pnd-3-glu, pnd-3-rut dpd-3,7-di- β -glu-3,5-di-(6''-cou)- β -glu, dpd-3,7,3,5-tetra-(6''-cou)- β -glu
bayberry <i>Laurus nobilis</i> ¹³ berry <i>Dianella nigra</i> and <i>D. tasmanica</i> ¹⁴	n.d. n.d. n.d.	cyd-3-(4'''-ace)-rut cyd-3-lat	n.d. n.d.
berry <i>Eurya japonica</i> ¹⁵ berry <i>Fatsia japonica</i> ¹⁶	8500 f.w. 7480 f.w. 2370 f.w. 949 f.w.	dpd-3-rut, ptd-3-rut plg-3-glu, plg-3-rut cyd-3-rut, plg-3-rut cyd-3-glu, cyd-3-rut	cyd-3-rut, plg-3-rut, pnd-3-rut, mvd-3-rut plg-3-(cis-cou)-glu, plg-3-(trans-cou)-glu n.d. n.d.
berry <i>Rhamnus alaternus</i> ¹⁷ berry <i>Ruscus aculeatus</i> ¹⁸ berry <i>Smilax aspera</i> ¹⁹ berry Tasmanian pepper ²⁰			

TABLE 4.3.1 (Continued)
Distribution of Anthocyanins in Fruits

Source and Reference	Total Content (mg/100 g)	Major ^a	Minor ^b
cherry, tart			
cv. Balaton ^{35,36}	23–52 f.w.	cyd-3-glu-rut, cyd-3-rut	cyd-3-ara-rut, pnd-3-rut, cyd-3-glu
cv. Montmorency ³⁶	4 f.w.		
<i>Coriaria myrtifolia</i> L. ³⁷	10719 d.w.	pnd-3-gal, pnd-3-glu, mvd-3-gal, mvd-3-glu	dpd-3-gal, dpd-3-glu, cyd-3-gal, cyd-3-glu, ptd-3-gal, ptd-3-glu
cranberry ^{10,11}	140 f.w.	cyd-3-gal, cyd-3-ara, pnd-3-gal, pnd- 3-ara	pnd-3-glu, mvd-3-ara
dwarf dogwood ³⁸	n.d.	cyd-3-(2''-β-glu)-β-gal, cyd-3-(2''-β- glu)-β-glu, cyd-3-gal, cyd-3-glu	*
elderberry ^{24,39}	1374 f.w.	cyd-3-glu, cyd-3-sam	cyd-3,5-diglu, cyd-3-sam-5-glu
gooseberry ²⁴	2–10 f.w.	cyd-3-glu, cyd-3-rut, cyd-3-(6''-cou)- glu	pnd-3-glu, pnd-3-rut, cyd-3-(6''-caf)-glu
honeysuckle ⁴⁰	116–593 f.w.	cyd-3-glu	cyd-3,5-diglu, cyd-3-rut, plg-3-glu, pnd-3-glu, pnd-3-rut
lichi ⁴¹	48 f.w.	cyd-3-rut	cyd-3-glu
marionberry ^{10,11}	300 f.w.	cyd-3-glu, cyd-3-rut	cyd-3-(diox)-glu
muntries ²⁰	37 f.w.	dpd-3-glu, cyd-3-glu	n.d.
nectarine ^{10,11}	7 f.w.	cyd-3-glu	cyd-3-rut
peach ^{10,11}	5 f.w.	cyd-3-glu	n.d.

plum, black ^{10,11}	125 f.w.	cyd-3-glu, cyd-3-rut	n.d.	
plum, regular ^{10,11}	19 f.w.	cyd-3-gal, cyd-3-glu, cyd-3-rut	cyd-3-(6''-ace)-glu	
plum, Illawarra ²⁰	870 f.w.	cyd-3-glu	plg-3-glu	
plum, Burdekin ²⁰	273 f.w.	cyd-3-glu	n.d.	
plum, Davidson's ²⁰	57 f.w.	dpd-3-sam, cyd-3-sam, pnd-3-sam	pid-3-sam	
<i>Passion suberosa</i> L. ⁴²	n.d.	dpd-3-glu, cyd-3-glu, ptd-3-glu, ptd-3-(6''-mal)-glu	plg-3-glu, dpd-3-(6''-mal)-glu, cyd-3-(6''-mal)-glu, plg-3-(6''-mal)-glu	
purple passion fruit ⁴²	n.d.	cyd-3-glu	plg-3-glu, cyd-3-(6''-mal)-glu	
raspberry, Molucca ²⁰	115 f.w.	cyd-3-glu, cyd-3-rut	plg-3-rut	
red currant ²⁴	12 f.w.	cyd-3-rut, cyd-3-sam, cyd-3-xyI-rut	cyd-3-glu, dpd-3-sam, cyd-3-sop, cyd-3-glu-rut	
red raspberry ^{10,11,43,44}	92 f.w.	cyd-3-sop, cyd-3-sop-5-rha or cyd-3-(2 ^G -glu-rut)**, cyd-3-glu, cyd-3-rut	cyd-3,5-diglu, plg-3-sop, cyd-3-glu-rut**, cyd-3-xyI-rut**, plg-3-glu, plg-3-(2 ^G -glu-rut)**, plg-3-rut	
<i>Rubus pinnatus</i> and <i>R. rigidus</i> ⁴⁵	n.d.	cyd-3-β-glu, cyd-3-rut	n.d.	
strawberry ^{10,11,46-48}	n.d.	plg-3-β-glu	cyd-3-glu, cyd-3-(mal-glu)-5-glu, plg-3-rut, plg-3-(6-mal)-glu, plg-4(5-cbp)-3-glu, plg-3-(ace)-glu	
	21-42 f.w.			

ace = acetyl; ara = arabinoside; cou = coumaryl; cyd = 5-carboxypyran; diox = dioxalyl; dpd= delphinidin; d.w. = dry weight; f.w. = fresh weight; gal = galactoside; glu = glucoside; lat = lathyruside; mal = malonyl; mvd = malvidin; myl = maly; n.d. = not determined; plg = pelargonidin; pnd = peonidin; ptd = petunidin; rut = rutinose; sam = sambubioside; sop = sophorose; xyl = xyloside.

^a Based on peak size of HPLC chromatogram or percent above 10.

^b Only anthocyanins exceeding 1% listed.

^c Commercial juices, amount in mg/100 ml.

* No relative amount nor HPLC chromatogram given.

** MS data fragments suggest anthocyanins may also be cyd-3-sop-5-rha and cyd-3-sam-5-rha.

TABLE 4.3.2
Distribution of Anthocyanins in Grapes

Cultivar and Reference	Total Content (mg/100 g)	Major ^a	Minor ^b
Cabernet Franc ⁴⁹	n.d.	mvd-3-glu, mvd-3-(ace)-glu, pnd-3-(cou)-glu mvd-3-(cou)-glu	ptd-3,5-diglu, dpd-3-glu, pnd-3,5-diglu, ptd-3-glu, pnd-3-glu, pnd-(ace)-3-glu
Cabernet Sauvignon ^{1,50,51}	34 d.w. 38 f.w.	dpd-3-glu, ptd-3-glu, mvd-3-glu, mvd-3-(6''-cou)-glu 3-(6''-ace)-glu, mvd-3-(6''-cou)-glu	cyd-3-glu, ptd-glu-pyr, pnd-3-glu, dpd-3-(6''-ace)-glu, cyd-3-(6''-ace)-glu, ptd-3-(6''-ace)-glu, pnd-3-(6''-ace)-glu, dpd-3-(6''-cou)-glu, mvd-3-(6''-caf)-glu, cyd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu, pnd-3-(6''-cou)-glu
Clinton ⁴⁹	n.d.	ptd-3,5-diglu, dpd-3-glu, ptd-3-glu, pnd-3-glu, mvd-3-(ace)-glu, mvd-3-(cou)-glu	dpd-3,5-diglu, cyd-3,5-diglu, mvd-3,5-diglu, cyd-3-glu, dpd-3-(ace)-glu, dpd-3-(6-cou)-5-diglu, dpd-3-(6-cou)-glu, ptd-3-(6-cou)-glu, ptd-3-(6-cou)-glu
Concorde ^{10,11,52,53}	120 f.w.	dpd-3-glu, cyd-3-glu, mvd-3-glu, dpd-3-(6''-cou)-glu	dpd-3,5-diglu, cyd-3,5-diglu, ptd-3,5-diglu, mvd-3,5-diglu, ptd-3-glu, plg-3-glu, pnd-3-glu, mvd-3-glu, dpd-3-(6''-ace)-glu, dpd-3-(6''-cou)-5-diglu, cyd-3-(6''-ace)-glu, dpd-3-(6''-cou)-5-diglu, cyd-3-(6''-ace)-glu, ptd-3-(6''-ace)-glu, mvd-3-(6''-cou)-5-diglu, mvd-3-(cou)-5-diglu, dpd-3-(6''-cou)-glu, mvd-3-(6''-caf)-glu, cyd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu, pnd-3-(6''-cou)-glu
Cynthiana ²¹	89 f.w.	dpd-3-glu, cyd-3-glu, mvd-3-(ace)-glu	ptd-3-glu, pnd-3-glu, mvd-3-glu, dpd-3-(ace)-glu, cyd-3-(ace)-glu, mvd-3-(ace)-glu, dpd-3-(cou)-glu, ptd-3-(cou)-glu, mvd-3-(cou)-glu

Isabelle ^{49,54}	n.d.	dpd-3-glu, ptd-3-glu, mvd-3-glu, pnd-3-glu	dpd-3,5-diglu, cyd-3,5-diglu, ptd-3,5-diglu, pnd-3,5-diglu, mvd-3,5-diglu, cyd-3-glu, ptd-3-(6''-ace)-glu, pnd-3-(6''-ace)-glu, mvd-3-(6''-ace)-glu, cyd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu, pnd-3-(6''-cou)-glu, mvd-3-(6''-cou)-glu, mvd-3-(6''-caf)-glu, dpd-3-glu-pyr, dpd-3-(6''-cou)-glu-pyr, pnd-3-glu-acet, mvd-3-glu-acet, ptd-3-(6''-caf)-5-diglu, dpd-3-(6''-ace)-5-diglu, dpd-3-(6''-cou)-5-diglu, dpd-3-(6''-fer)-5-diglu, ptd-3-(6''-cou)-5-diglu, pnd-3-(6''-cou)-5-diglu, mvd-3-(6''-cou)-5-diglu
Nerello Mascalese ⁵¹	52 d.w.	dpd-3-glu, ptd-3-glu, pnd-3-glu, mvd-3-glu	cyd-3-glu, mvd-3-(6''-cou)-glu
Nero d'Ávola ⁵¹	137 d.w.	mvd-3-glu, mvd-3-(6''-cou)-glu	dpd-3-glu, ptd-3-glu, dpd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu, pnd-3-(6''-cou)-glu, mvd-3-(6''-ace)-glu
Pallagrello and Piedirosso Vesuvio ⁵⁴	n.d.	dpd-3-glu, ptd-3-glu, mvd-3-glu	cyd-3-glu, pnd-3-glu, dpd-3-(6''-ace)-glu, cyd-3-(6''-ace)-glu, ptd-3-(6''-ace)-glu, pnd-3-(6''-ace)-glu, mvd-3-(6''-ace)-glu, dpd-3-(6''-cou)-glu, cyd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu, pnd-3-(6''-cou)-glu, mvd-3-(6''-cou)-glu, mvd-3-(6''-caf)-glu, pnd-3-glu-acet
Red ^{10,11}	27 f.w.	cyd-3-glu, pnd-3-glu, mvd-3-glu, mvd-3-(6''-cou)-glu, pnd-3-(6''-cou)-glu	dpd-3-glu, ptd-3-glu, mvd-3-(6''-ace)-glu, cyd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu
Rubired ⁵³	n.d.	pnd-3,5-diglu, mvd-3,5-diglu, mvd-3-(6''-cou)-5-diglu	dpd-3,5-diglu, cyd-3,5-diglu, ptd-3,5-diglu, dpd-3-glu, ptd-3-glu, plg-3-glu, pnd-3-glu, mvd-3-glu, dpd-3-(6''-cou)-5-diglu, ptd-3-(6''-cou)-5-diglu, dpd-3-(6''-cou)-glu, ptd-3-(6''-cou)-glu, mvd-3-(6''-cou)-glu

ace = acetyl; acet = acetaldehyde derivative; cou = coumaryl; cyd = cyanidin; dpd = delphinidin; d.w. = dry weight; fer = ferulyl; f.w. = fresh weight; glu = glucoside; mvd = malvidin; n.d. = not determined; plg = pelargonidin; pnd = peonidin; ptd = petunidin; pyr = pyruvic derivative.

^a Based on peak size of HPLC chromatogram or percent above 10.
^b Only anthocyanins exceeding 1% listed.
^c Commercial juices, amount in mg/100 ml.

TABLE 4.3.3
Distribution of Anthocyanins in Vegetables

Source and Reference	Total Content (mg/100 g)	Major ^a	Minor ^a
<i>Allium victorialis</i> ⁵⁵ black carrot ⁵⁶⁻⁵⁸	n.d. 5–174 d.w.	cyd-3-(3''-mal)-glu, cyd-3-(3'',6''-dimal)-glu cyd-3-(2''-xyl-gal), cyd-3-(2''-xyl-6'''-(6''-sin-glu)-gal, cyd-3-(2''-xyl-6''-(6'''-fer-glu)-gal, cyd-3-(2''-xyl-6''- (6'''-cou-glu)-gal	cyd-3-glu, cyd-3-(6''-mal)-glu cyd-3-(2''-xyl-6''-glu-gal), cyd-3-(2''-xyl-6''-(caf)-glu-gal), cyd- 3-(2''-xyl-6''-(phb)-glu-gal), pnd-xyl-glu-gal, plg-xyl-gal, pnd- xyl-gal, plg-(fer)-xyl-glu-gal, pnd-(fer)-xyl-glu-gal, pnd-(cou)- xyl-glu-gal
chive ⁵⁹ eggplant ^{11,60} garlic ⁶¹	n.d. 86 f.w. n.d.	cyd-3-(6''-mal)-glu, cyd-3-(3'',6''-dimal)-glu dpd-3-rut-5-gal, dpd-3-rut cyd-3-glu, cyd-3-(6''-mal)-glu, cyd-3-(3'',6''-dimal)-glu ptd-3-(4''-cou)-rut-5-glu, ptd-3-(4''-cou)-rut-5-glu plg-3-rut-5-glu, plg-3-(4''-cou)-rut-5-glu, plg-3-(fer)- rut-5-glu	cyd-3-glu, cyd-3-(3''-mal)-glu dpd-3-rut-5-glu cyd-3-(3''-mal)-glu ptd-3-(4''-caf)-rut-5-glu, pnd-3-(4'''''-caf)-rut-5-glu plg-3-rut, plg-3-(4''-fer)-rut-5-glu, plg-3-(fer)-rut
potato cv. Norwegian ⁶² potato hybrids and clones ^{52,63,64} purple mashua ⁶⁵	n.d. 2–40 f.w.	dpd-3-sop-5-rha, cyd-3-sop-5-rha, dpd-3-glu-5-(ace)- rha, dpd-3-sop-5-(ace)-rha	dpd-3-glu, cyd-3-sop, dpd-3-glu-5-rha, cyd-3-glu, cyd-3-rut
genotype DP 0224, genotype AR8 5241 genotype AGM 5109 red cabbage ^{1,12,52,60}	132 f.w. 123 f.w. 46 f.w. 322 f.w.	cyd-3-diglu-5-glu, cyd-3-(sin)-diglu-5-glu, cyd-3- (sin,sin)-diglu-5-glu	cyd-3-glu-5-glu, cyd-3-(sin)-diglu-5-glu, cyd-3-(caf,cou)-diglu- 5-glu, cyd-3-(fer)-triglu-5-glu, cyd-3-(sin)-triglu-5-glu, cyd-3- (sin)-diglu-5-glu, cyd-3-(sin,cou)-diglu-5-glu, cyd-3-(cou)- diglu-5-glu, cyd-3-(fer,fer)-diglu-5-glu, cyd-3-(sin,fer)-diglu- 5-glu

red leaf lettuce ^{11,60}	2 f.w.	cyd-3-(6''-mal)-glu	cyd-3-glu, cyd-3-(3''-mal)-glu, cyd-3-(6''-ace)-glu
red onion ^{11,59,60,66-68}	49 f.w.	cyd-3-glu, cyd-3-lam, cyd-3-(6''-mal)-glu, cyd-3-(6''-mal)-lam-4'-glu, cyd-7-(6''-mal)-lam-4'-lam, cyd-3,5-digu, cyd-3-(6''-met-mal)-glu, pnd-3-(6''-mal)-glu, cyd-3-(3''-mal)-glu, pnd-3-glu, 5-cbp-cyd-3-glu, 5-cbp-cyd-3-(6''-mal)-glu	
red radish ^{11,52,60}	100 f.w.	plg-3-(cou)-digu-5-(mal)-glu, plg-3-(fer)-digu-5-(mal)-glu	plg-3-(fer)-digu-5-(mal)-glu, plg-3-(caf)-digu-5-(mal)-glu, plg-3-(cou)-digu-5-glu, plg-3-(fer)-digu-5-glu, plg-3-(fer,caf)-digu-5-(mal)-glu, plg-3-(fer,fer)-digu-5-(mal)-glu, plg-3-(cou,cou)-digu-5-(mal)-glu, plg-3-(cou,fer)-digu-5-(mal)-glu, plg-(cou)-3-sop-5-glu, plg-(fer)-3-sop-5-glu, plg-(cou)-3-sop-5-glu, plg-(fer,mal)-3-sop-5-glu
sweet potato, red or purple ⁶⁹⁻⁷¹	n.d.	cyd-3-(6''-caf)-sop-5-glu, pnd-3-(6''-caf)-sop-5-glu, cyd-3-(6''-caf-fer)-sop-5-glu, pnd-3-(caf-fer)-sop-5-glu, cyd-3-(6''-caf,phb)-sop-5-glu, cyd-3-(6''caf,caf)-sop-5-glu, pnd-3-(6''-caf,caf)-sop-5-glu, pnd-3-(6''-caf,phb)-sop-5-glu, fer,caf)-sop-5-glu	

ace = acetyl; caf = caffeyl; cbp = carboxypyran; cou = coumaryl; cyd = cyanidin; d.w. = dry weight; fer = ferulyl; f.w. = fresh weight; gal = galactoside; glu = glucoside; lam = laminaribioside; mal = malonyl; met = methyl; mvd = malvidin; n.d. = not determined; pen = pentoside; phb = *p*-hydroxybenzyl; plg = pelargonidin; pnd = peonidin; ptd = petunidin; rut = rutinose; sin = sinapyl; xyl = xyloside.

^a Based on peak size of HPLC chromatogram or percent above 10.

^b Only anthocyanins exceeding 1% listed.

TABLE 4.3.4
Distribution of Anthocyanins in Grains and Nuts

Source and Reference	Total Content (mg/100 g)	Major ^a	Minor ^a
bambara groundnut ⁷²	n.d.	dpd-3-glu, ptd-3-glu, mvd-3-glu	n.d.
bean cv. Tolosa ⁷³	24 f.w.	cyd-3-glu, plg-3-glu	cyd-3,5-diglu, plg-3,5-diglu, cyd, pnd, plg
black bean		dpd-3-glu, ptd-3-glu, mvd-3-glu	dpd-3,5-diglu, ptd-3,5-diglu, dpd-3-gal, mvd-3,5-diglu, ptd-3-gal
cv. UI911 ⁷⁴	213 f.w.		
cv. KG98010 ⁷⁵	214 f.w.		
cv. KG9728 ⁷⁵	278 f.w.		
undefined cv. ^{11,60}	45 f.w.		
black turtle ⁷⁶	n.d.		
black lentil ⁷⁷	n.d.	dpd-3-(2-β-glu-α-ara)	n.d.
black rice ^{78,79}	228 f.w.	cyd-3-glu	cyd-diglu, cyd-3-rut, pnd-3-glu
black soybean ^{76,80}	158–202 d.w. 1530–2040 d.w.	dpd-3-glu, cyd-3-glu	ptd-3-glu
blue wheat			
cv. Purendo ⁷⁹	15 f.w.	dpd-3-glu, dpd-3-rut, cyd-3-glu, cyd-3-rut	ptd-3-glu, ptd-3-rut, mvd-rut

corn, blue cv. Shaman ⁷⁹	23 f.w.	cyd-3-glu, cyd-(suc)-glu	plg-3-glu, cyd-(mal)-glu, cyd-(suc,suc)-glu
corn, purple cv. Morado ^{39,81,82}	n.d.	cyd-3-glu, pnd-3-glu, cyd-3-(6''-mal)-glu	plg-3-glu, plg-3-(6''-mal)-glu, pnd-3-(6''-mal)-glu
Guatemalan bean ⁸³ (<i>P. coccineus</i>)	n.d.	dpd-3-glu, met-cyd, cyd, met-cyd	dpd-3-glu-5-pen, dpd, plg, mvd
kidney bean ⁸³	50–390 d.w.	dpd-3-glu, ptd-3-glu, cyd-3-glu, met-cyd	ptd-3,5-diglu, dpd-3-gal, dpd-3-glu, mvd-3,5-diglu, pnd-3-glu, dpd, mvd-3-glu, met-dpd, cyd
pistachio ^{1,60}	8 f.w.	cyd-3-gal, cyd-3-glu	n.d.
red bean ⁷⁵	44–74 d.w.	cyd-3-glu, plg-3-glu	cyd-3,5-diglu, dpd-3-glu
small red bean ^{11,60,76}	7 f.w. 30 d.w.	cyd-3-glu, plg-3-sam, plg-3-glu	n.d.

ara = arabinoside; cyd = cyanidin; d.w. = dry weight; f.w. = fresh weight; gal = galactoside; glu = glucoside; mal = malonyl; met = methyl; mvd = malvidin; n.d. = not determined; pen = pentoside; plg = pelargonidin; pnd = peonidin; ptd = petunidin; rut = rutoside; sam = sambubioside; suc = succinyl.

^a Based on peak size of HPLC chromatogram or percent above 10.

^b Only anthocyanins exceeding 1% listed.

red,⁵³ cv. Red,^{10,11} cv. Isabelle,⁴⁹ cv. Nerello Mascalese,⁵¹ and cv. Clinton⁴⁹ grapes. None of the foods listed in the four tables presented peonidin as the only aglycone.

Malvidin was detected in amounts greater than 10% of the total anthocyanin content only in 4 of 44 fruits listed in Table 4.3.1, in none of the 13 vegetables shown in Table 4.3.3, and in 2 grains and nuts presented in Table 4.3.4. Malvidin is widespread in all types of grapes; being one of the major anthocyanidins found in grapes shown in Table 4.3.2. None of the foods listed in the tables presented malvidin as the sole aglycone.

Cyanidin, peonidin, and pelargonidin were detected in free forms in the Tolosa bean variety.⁷³ This was the first report regarding the presence of aglycones in natural form in vegetable samples. On the basis of the data obtained by HPLC-MS and chemical tests, 3-methyldephinidin and 3-methylcyanidin were identified in scarlet red runner beans and in kidney beans.⁸³

3-Desoxyanthocyanidins are unusual types of anthocyanins in which the position 3 of the oxygen of the flavylum cation is not substituted. Apigeninidin, derivatives of luteolinidin, and 7-methylapigeninidin were isolated in low concentrations from grains and leaf sheaths of *Sorghum caudatum*.⁸⁴ In *Sorghum bicolor*, 3-desoxyanthocyanidins were produced in response to stress caused by both pathogenic and non-pathogenic fungus infection. The major ones were luteolinidin, 5-methoxyluteolinidin, apigeninidin, and the caffeoyl ester of arabinosyl-5-apigeninidin.⁸⁵ It is interesting that concentrations of anthocyanin decreased significantly in inoculated sorghum but not in uninoculated plants.⁸⁶

Four cyanidins with 4'-glucosidation were recently isolated in minor amounts from pigmented scales of red onion. Two were the only anthocyanins with B ring sugar substitutions and no glycosyl moieties in anthocyanidin position 3, whereas the other two were also glycosylated at the usual position 3.⁶⁷

In recent years, several color-stable 4-substituted anthocyanins originating from the reaction of malvidin 3-glucoside and acylated derivatives with pyruvic acid have been discovered in small amounts in red wine. These pigments were formed by cycloaddition of pyruvic acid involving both C-4 and the hydroxyl at C-5 of malvidin, giving for example carboxypyranomalvidin (vitisidin A)^{87,88} or by direct reaction between anthocyanins and hydroxycinnamic acids forming all anthocyanin vinylphenol-type adducts in red wines.⁸⁹ These kinds of vinylcatechol, vinylphenol, and vinylguaiacol adducts are also formed during storage of juices such as black carrot⁵⁸ and blood orange.²⁹ The pyranoanthocyanins were absent in fresh black currant seed extract and their levels in the extract increased gradually over time.⁹⁰ However, four methylpyranoanthocyanins detected in black currant seeds were later shown to be the oxidative cycloaddition products of acetone and natural anthocyanins.^{91,92} Although more stable, these compounds showed hypsochromic shifts compared to the aglycon from which they originated.

In addition, four 5-carboxypyranocyanidins have been isolated from acidified methanolic extracts of the edible scales as well as from the dry outer scales of red onions.⁶⁷ Two of the structures were the 3-glucoside and 3-(6-malonyl)-glucoside of the 4-substituted cyanidin. However, the two analog pigments methylated at either the terminal carboxyl group of the acyl moiety or at the aglycone carboxyl were most probably formed by esterification with acidified methanol during the isolation

process. Another 3-glucoside with the new 4-substituted aglycone, 5-carboxypyranopelargonidin, has been isolated in small amounts from strawberries.⁴⁸ These data suggest that carboxypyrananthocyanins may have a more widespread occurrence beyond wines.

Four novel dimeric flavonoids were isolated from strawberry extracts, comprising an anthocyanin unit (pelargonidin 3- β -glucopyranoside) connected with a C-C binding to four different flavan-3-ols, catechin(4 α →8)pelargonidin 3- β -glucopyranoside, epicatechin(4 α →8)pelargonidin 3- β -glucopyranoside, afzelechin(4 α →8)pelargonidin 3- β -glucopyranoside, and epiafzelechin(4 α →8)pelargonidin 3- β -glucopyranoside.⁹³ These purple pigments may be formed by direct reactions of anthocyanins with flavanols involving nucleophilic additions, as already proposed for some of the pigments contributing to the color of red wines.^{94–96}

4.3.3.2 GLYCOSIDES

Glucose is the most widespread sugar, both as a component of major and minor anthocyanidins, in most foods presented in Table 4.3.1 through Table 4.3.4. However, this sugar was absent in the anthocyanins isolated from acerola,⁹ apples,^{10,11} some berries,^{15–17,19} cranberries,^{10,11} and sweet cherries.^{10,11,34} On the other hand, glucose was found as the only sugar linked to all anthocyanins, also at position 3, in baguaçu,¹² camu-camu,³³ Cedar Bay cherries,²⁰ mumtries,²⁰ *Passion* sp. fruits,⁴² peaches,^{10,11} Illawarra and Burdekin plums,²⁰ red leaf lettuces,^{11,60} chive,⁵⁹ *Allium victorialis*,⁵⁵ garlic,⁶¹ black soybean,^{76,80} black rice,^{78,79} blue corn,⁷⁹ purple corn,^{39,79,81,82} and bambara groundnuts.⁷² Glucose alone was also found at positions 3,5 in red cabbage,^{11,23,52,60} in red beans,⁷⁵ and in bean cv. Tolosa.⁷³ A different pattern with glucose unities linked at positions 3,7,3',5' was found in *Dianella nigra* and *D. tasmanica* berries.¹⁴

It is interesting that glucose was the only sugar found in all the red grapes listed in Table 4.3.2. In fact, the skins of *Vitis vinifera* red grape cultivars contained only the 3-monoglucoside form, while other *Vitis* species and hybrid red grapes (non-pure *Vitis vinifera*) also showed 3,5-diglucoside anthocyanins.⁵⁴

Galactose is the second most widespread monosaccharide in foods, being found in apples,^{10,11} dwarf dogwood,³⁸ cranberries,^{10,11} black chokeberries,^{23,24} highbush blueberries,^{10,11,21} *Vaccinium padifolium* blueberries,^{30,31} *Coriaria myrtifolia*,³⁷ black carrots,^{56–58} eggplants,^{11,60} pistachios,^{11,60} and black and kidney beans.^{11,60,76,83}

Anthocyanins from apples,^{10,11} cranberries,^{10,11} black chokeberries,^{23,24} blackberries of an unknown cultivar,^{10,11} highbush blueberries,^{10,11,21} *Vaccinium padifolium* blueberries,^{30,31} cv. Balaton black cherries,^{35,36} and black lentils⁷⁷ contained arabinose. This pentose was not found in any of the vegetables presented in Table 4.3.3. Even less widespread is xylose, found in Red Delicious apple cultivars,^{10,11} black chokeberries,^{23,24} different cultivars of blackberries,²¹ evergreen blackberries,²² red currants,²⁴ *Vaccinium padifolium* blueberries,^{30,31} different cultivars of red raspberry,^{10,11,43,44} and black carrots.^{56–58}

It is worth noting that whenever present, galactose, arabinose, and xylose appeared in combination in most foods. None of the foods listed in the four tables contained one of these monosaccharides as the only sugar linked to the anthocyanins.

Another interesting point is that glucosyl transferase and galactosyl transferase apparently exerted different activities depending on the blueberry cultivar.²¹

Despite the fact that α -L-rhamnosylpyranoside usually occurs in the anthocyanins as the disaccharide rutinose, α -rhamnose was found in acerola, linked at position 3 to cyanidin and pelargonidin⁹ and to delphinidin in *Vaccinium padifolium* blueberries,^{30,31} and at the 5 position in black raspberries,^{10,11} red raspberries,^{10,11} and purple mashua.⁶⁵ The D- and L-configurations were not assigned in any of these studies.

Rutinose is the most widespread disaccharide in foods, being the sugar moieties of anthocyanins present in proportions above 10% of the total anthocyanin contents in several fruits such as in açai,⁶⁻⁸ several berries,^{13,15,17-20} *Rubus* sp.,^{10,11,45} marionberries,^{10,11} black currants,²³⁻²⁶ red currants,²⁴ boysenberries,^{3,32} sweet and tart cherries,^{10,11,34-36} gooseberries,²⁴ litchis,⁴¹ plums,^{10,11} and black and red raspberries.^{10,11,43,44} Rutinose was also detected in a few vegetables and grains such as eggplants,^{11,60} potatoes,^{52,62-64} and blue wheat.⁷⁹ It is worth mentioning that rutinose was the only sugar found in *Rhamnus alaternus* berries,¹⁷ *Eurya japonica*,¹⁵ and *Smilax aspera*.¹⁹

Sophorose was found linked at position 3 of the main anthocyanins from boysenberries,^{3,32} red raspberries,^{10,11,43,44} sweet potatoes,⁶⁹⁻⁷¹ and purple mashua.⁶⁵ This disaccharide was also found in minor anthocyanins from red currants,²⁴ blood oranges,²⁷⁻²⁹ and red radishes.^{11,52,60}

Sambubiose was less widespread, as a constituent of the major anthocyanins from red currants,²⁴ elderberries,^{24,39} Davidson's plums,²⁰ black raspberries,^{10,11} and small red beans,^{11,60,76} and linked to minor anthocyanins from açai⁶⁻⁸ and *Vaccinium padifolium* blueberries.^{30,31} Considering the anthocyanins identified at least with MS, as far as we are concerned, this disaccharide was not reported in vegetables, cereals, or grains.

Among the disaccharides with more limited occurrence in anthocyanins, lathyrrose was found in dark red berries of *Fatsia japonica*¹⁶ and linked to one of the major anthocyanins in black carrots.⁵⁶⁻⁵⁸ Until now, laminariobiose was only detected in red onions.⁵⁹ A very rare disaccharide 2''- β -glucopyranosyl- β -galactopyranoside was found in anthocyanins from dwarf dogwood.³⁸

Only a few trisaccharides have been identified in anthocyanins from foods, all at the 3 position: 6''- α -rhamnopyranosyl-2''- β -xylopyranosyl- β -glucopyranoside in minor amounts from *Vaccinium padifolium* blueberries^{30,31} and 2''-xylopyranosyl-6'''-glucopyranosyl-galactopyranoside in major anthocyanins from black carrots.⁵⁶⁻⁵⁸

4.3.3.3 ACYL GROUP

Advances in analytical procedures resulted in several reports on anthocyanins acylated with hydroxycinnamic acids (*p*-coumaric, caffeic, ferulic, sinapic, and 3,5-dihydroxycinnamic acids), hydroxybenzoic acids (*p*-hydroxybenzoic and gallic acids), and aliphatic acids (malonic, acetic, malic, oxalic, succinic and tartaric acids). However, not all of them were found in anthocyanins isolated from foods. Among the 44 fruits listed in Table 4.3.1, 15 presented acylated anthocyanins as did 12 of 13 vegetables shown in Table 4.3.3 and 2 of the 9 grains cited in Table 4.3.4. On the other hand, acylated anthocyanins were found in all grapes from *Vitis* species, although at different abundance levels, as can be seen in Table 4.3.2. A higher

percentage of acylated anthocyanins in a specific grape cultivar indicates that they may confer greater color stability to the wines of the cultivar (see Section 4.3.5.2).

p-Coumaryl acid is most frequently found connected to position 6 of the glucose moiety as in black currants,^{23–26} gooseberries,²⁴ black carrots,^{56–58} and all species of *Vitis* (Table 4.3.2). Up to four 6-*p*-coumarylglucoside units were located at different aglycone positions in *Dianella* berries.¹⁴ In potatoes, the *p*-coumaryl unit was found linked at position 4 to the rhamnose moiety of 3-rutinoside.^{52,62–64} The evidence for this acyl group location was not reported in anthocyanins from some foods such as *Ruscus aculeatus* berries,¹⁸ red cabbage,^{11,23,52,60} and red radishes.^{11,52,60} None of the anthocyanins from grains and nuts (Table 4.3.4) were acylated with *p*-coumaric acid.

Most of the anthocyanins acylated with caffeic acid have this cinnamyl moiety linked to a glucosyl position 6, as in gooseberries,²⁴ some grape cultivars,^{10,11,50–54} black carrots,^{56–58} red cabbage,^{11,23,52,60} red radishes,^{11,52,60} and sweet potatoes.^{69–71} Some anthocyanins isolated from species of potatoes have a caffeoyl group located at position 4 of the rhamnosyl unit of the rutinose disaccharide.^{52,62–64}

The ferulyl unit is found in most anthocyanins linked to glucose at position 6 within a glycosidic moiety linked to the aglycone 3 position as in the Isabelle grape variety,⁵⁴ sweet potatoes,^{69–71} and black carrots.^{56–58} As caffeic acid, the ferulyl unit occurred attached to the 4-hydroxyl group of the rhamnose unit of 3-rutinoside in potatoes.^{52,62–64} However, the position of this cinnamic acid was not assigned in anthocyanins from red cabbages^{11,23,52,60} and red radishes.^{11,52,60} It is interesting that acylated anthocyanins with ferulyl acids were the predominant pigments comprising 43 to 84% of the total anthocyanin contents in 15 black carrot cultivars,⁵⁷ unlike levels found in most foods.

Among the hydroxycinnamic acids, anthocyanins acylated with sinapic acid are not widespread in foods; they have been isolated only in black carrots^{56–58} and red cabbages.^{11,23,52,60} Anthocyanins that are acylated with *p*-hydroxybenzoic acid were only found in black carrots^{56–58} and sweet potatoes.^{69–71} This acyl group was located within the 6 position of a glucoside moiety.

Since anthocyanins acylated with aliphatic acids are sensitive to acids,⁹⁷ as verified, for example, in fruits of *P. suberosa* when extracted with methanol containing 0.1% HCl,⁴² their occurrence in foods may be underestimated. Malonyl acylation in glucose at its position 6 was found in anthocyanins from blood oranges,^{27–29} *Passion* species fruits,⁴² strawberries,^{10,11,46–48} red leaf lettuce,^{11,60} and purple corn cv. Morado.^{39,81,82} This acyl group was also detected in non-specified locations in blackberries,²¹ evergreen blackberries,²² red radishes,^{11,52,60} and blue corn cv. Shaman.⁷⁹ Dimalonyl substitution at both 3'' and 6'' positions of the sugar moieties positions of anthocyanins were isolated from red onions,^{11,59,60,66–68} chives,⁵⁹ *Allium victorialis*,⁵⁵ and garlic.⁶¹ These rare 3'',6''-dimalonylglucosides constituted more than 50% of the total anthocyanin contents of red onions⁵⁹ while malonated anthocyanins constituted 87% of the total anthocyanin contents of garlic.⁶¹

One acetyl group was identified at position 6 in highbush blueberries,^{10,11} plums,^{10,11} red leaf lettuce,^{11,60} and all grapes (Table 4.3.2) and at position 4 in *Eurya japonica* berries.¹⁵ Although acetyls were found in anthocyanins from strawberries^{10,11,46–48} and purple mashua,⁶⁵ the positions of this acyl group were not assigned.

Oxalic acid is another dicarboxylic acid with restricted occurrence isolated from blackberries,^{10,11,21} evergreen blackberries,²² marionberries,^{10,11} and blood oranges.^{27–29} However, evidence for the linkage position of this acyl group was only reported for blood oranges at position 6 of the 3-glucose moiety. Anthocyanins acylated with malyl acid were detected only in blackberries of an unknown cultivar.^{10,11}

4.3.4 FACTORS AFFECTING STABILITY OF ANTHOCYANINS

Considerable studies have been done on the effects of the most important chemical and physical factors involved in the degradation of anthocyanins (temperature, light, pH, SO₂, metal, sugar, and oxygen) in model systems and food extracts. In addition, anthocyanin concentrations, its chemical structures, and media compositions are fundamental factors influencing stability.

4.3.4.1 STRUCTURE

Stabilities and colors of anthocyanins are dependent on the nature and number of sugars attached to the flavylium ion and the nature and number of acids linked to the glycosylic moiety. Tint and hue, however, are related to the numbers and positions of hydroxyl and methoxyl substituents in the flavylium ion. In addition, 3-deoxy-anthocyanins that are yellow due to dehydroxylation of the carbon at C-3 have higher stabilities than the corresponding 3-hydroxy anthocyanins that in turn are red but lose much of their stability.

The pK_a value (2.43) of the hydration constant of the cyanidin was found to be lower than the pK_a values of glycosylated and acylated cyanidins, meaning lower resistance of the anthocyanidin to hydration.⁹⁸ The stability of nonacylated 3,5-diglucosides was lower compared to the 3-glucoside because the 5 position markedly lowered the hydration constant due to decreased electron density of the pyrilium ring that favors nucleophilic attack by water, enhancing hemiacetal formation.⁹⁸

On the other hand, anthocyanins that contained aromatic acyl groups showed higher stabilities than the unacylated pigments that were quickly decolorized by hydration at position 2 of the anthocyanidin nucleus.⁹⁹ In fact, the exceptional stabilities of a few anthocyanins found in flowers throughout the whole pH range is attributed to their more complex structures.¹⁰⁰ Examples are zebrinin composed of cyanidin with three molecules of glucose, one molecule of arabinose, and four unities of caffeic acid (cyanidin 3-(6-(2,5-di-caffeyl- α -L-arabinofuranosyl)- β -D-glucopyranosyl-7,3'-di-(6-caffeyl- β -D-glucopyranosyl glucoside) from *Zebrina pendula* flowers¹⁰¹ and ternatin-A1 composed of delphinidin with seven molecules of glucose, four molecules of *trans*-*p*-coumaric acid, and one unity of malonic acid (delphinidin 3-(6'-malonyl- β -D-glucopyranosyl)-3'',5''-di-(6-*trans*-4-(6-*trans*-4- β -D-glucopyranosyl-*p*-coumaryl)- β -D-glucopyranosyl-*p*-coumaryl)- β -D-glucopyranoside).¹⁰²

The greater stabilities of very complex anthocyanins are in many cases achieved simply by intramolecular copigmentation since the long substituents linked to the aglycone can fold over the flavylium cation, hindering the addition of water at position 2 of the chromophore through hydrogen bonding between the phenolic

hydroxyl groups in the anthocyanin and the aromatic acids and hydrophobic interaction between the flavylium ring and the aromatic acyl groups by a sandwich-type stacking.^{103–106} Besides the influence on stability, when the glycosylic moiety linked to the chromophore is long enough to fold over the 2-benzopyrylium system, the addition of water is hindered, thus stabilizing the color.¹⁰⁵

The structure complexity also interferes with the equilibria in acidic medium, as shown in Figure 4.3.3. The deprotonation equilibrium constant value (K_a) of zebrinin was higher than the hydration constant (K_h), leading to the formation of a greater amount of colored quinonoidal with no formation of the colorless species, pseudobase or chalcone.¹⁰⁵

The influence of *trans*–*cis* configurations of the acids attached to the glycosylic moieties of the anthocyanins on the color stabilization of anthocyanins was reported for the first time in two sets of complex coumaryl acylated anthocyanins.¹⁰⁷ The simple change from the *trans*- to *cis* isomer of the coumaryl moieties in both malvidin 3-[6-(4-(*p*-coumaryl- α -L-rhamnopyranosyl)- β -D-glucopyranoside)]-5-(β -D-glucopyranoside) and delphinidin 3-[6-(*p*-coumaryl)- β -D-glucopyranoside]-5-[6-(malonyl)- β -D-glucopyranoside] produced increases in the tint intensity and in the stability of the pigments due to the formation of folded structures (intramolecular copigmentation). The fact that the distance of acyl residues to the chromophore is shorter in the *cis* configuration enhanced the protective effect against hydration at the C-2 position.¹⁰⁷

The β configuration of the 3-D-glucosylation of malvidin, cyanidin and delphinidin improved their stabilities over the α configuration.¹⁰⁸ Through the calculation of thermodynamic parameters, the anthocyanin stability was explained by a net of intramolecular H bondings within the sugar moiety and between the sugar and the chromophore.¹⁰⁸ Pigment complexes of anthocyanins with metals and other substances, especially polyphenols, led to stable compounds such as commelinin and protocyanin from blue corn flower — both pigments probably consisting of six anthocyanins, six flavone molecules, and two metal ions with molecular weights near 10,000.¹⁰⁹

4.3.4.2 TEMPERATURE AND pH

The thermal degradation of anthocyanins, both in extracts and model systems, was reported to follow first-order reaction kinetics in all studies. The stability of anthocyanins and all pigments found in foods decreased with increases in temperature.

Cyanidin 3-glucoside and cyanidin 3-rutinoside were degraded at 100°C in weak acidic solutions at pH range 1 to 4 under aerobic and anaerobic conditions.¹¹⁰ The degradation revealed that cyanidin 3-glucoside was more stable under both conditions.¹¹⁰

The thermal stability of the anthocyanins from sunflower hulls (genotype Neagra de Cluj) extracted with SO₂ in different concentration solutions was verified by heating for various durations at 65 to 95°C and at a pH range from 1 to 5. The degradation increased with the increasing of SO₂ in the extraction solvent.¹¹¹

The colors and stabilities of anthocyanin extracts from purple- and red-flesh potatoes were less affected by pH and temperature than commercial concentrates of

purple carrots and grapes.¹¹² The kinetics of anthocyanin degradation followed a first-order reaction; while changes in the CIELAB parameters lightness (L^*) and hue (h_{ab}) followed a zero-order kinetics, and chroma also followed between first and order changes.¹¹²

Natural and pasteurized raspberry pulps were not very sensitive to color deterioration under processing and canning conditions, but the color practically disappeared after 50 days of storage at 37°C.¹¹³ Temperature also changed the relative proportion of red raspberry anthocyanins (cyanidin and pelargonidin 3-glucosides) in juice concentrate.¹¹³ After 3 mo of storage at 20°C, significant changes in anthocyanins were observed while at -20°C, only minor changes occurred.¹¹⁴

Storage stabilities of blood orange juices and concentrates with different total soluble solids concentrations and at different temperatures were investigated under identical conditions. The half-life values of juices and concentrates decreased with increases of °Brix value and temperature.¹¹⁵ The stability of red cabbage extracts in soft drink model systems subjected to heat and light was higher than stability levels of black currant grape skin and elderberry extracts and also presented lower sensitivity to photodegradation at pH range 3 to 7.¹¹⁶

The kinetics of photochemical and thermal degradation of pelargonidin, delphinidin, malvidin, and cyanidin in aqueous acidic media were investigated.¹¹⁷ All four anthocyanidins followed similar degradation patterns but through different pathways involving excitation of the flavylum ion. In all cases, the final product was identified as 2,4,6-tri-hydroxybenzaldehyde originating from ring A, in addition to 3,4,5-hydroxybenzoic acid from delphinidin, 4-hydroxybenzyl-3,5-dimethoxybenzoic acid from malvidin, 3,4-dihydroxy benzoic acid from cyanidin, and 4-hydroxybenzoic acid from pelargonidin, all originating from ring B.¹¹⁷ Glucosylation of the hydroxyl group in the C-3 position increased the quantum yield of the photochemical reaction.¹¹⁷

Studies of the influence of pH on the color of anthocyanins were conducted with petanin (petunidin 3-[6-(4-*trans-p*-coumaroyl- α -L-rhamnopyranosyl)- β -D-glucopyranoside]-5-glucopyranoside) and cyanidin 3- β -D-glucopyranoside over 60 days at a pH ranging from 1 to 9 and at different temperatures. At strong acidic pH, a solution of simple anthocyanin showed stability similar to that of the more complex one. However, as the pH rose, cyanidin 3-glucoside lost stability, while petanin underwent a color change toward blue at pH 8.1.¹¹⁸

4.3.4.3 ASCORBIC ACID

The mechanism proposed by Jurd¹¹⁹ and later reinforced by Poei-Langston and Wrolstad¹²⁰ for the degradation of anthocyanins in the presence of ascorbic acid (AA) consisted of direct condensation of AA on carbon 4 of the anthocyanin molecule, causing the loss of both. In other mechanism, the loss of anthocyanin color caused by AA occurred due to oxidative cleavage of the pyrilium ring by a free radical mechanism in which the AA acts as a molecular oxygen activator, producing free radicals.¹²¹ The addition of flavonol exerted a protective effect of the anthocyanins in the presence of AA, probably by competition with the anthocyanins in the preference for condensation reactions.¹²² Kinetic analysis indicated that anthocyanin destruction by AA also fit first-order degradation reactions in all studies.

Ascorbic acid (330 mg/L) was added to solutions of malvidin 3-glucoside, malvidin-3,5-diglucoside, and flavylium salts with different substituents in position 4 at pH 2.¹²³ The anthocyanin flavylium salt structures played an important role in the stability of anthocyanins and the diglucosides faded more slowly than the monoglucosides. The flavylium cation showed a more marked decrease in the presence of AA in comparison to the anthocyanins.¹²³ The influence of anthocyanin structures rather than the presence of AA in the color changes was observed. In addition, the anthocyanin was protected from AA degradation; however the type of flavylium cation had no significant influence on the rate of AA degradation.¹²³

Acerola is considered one of the best natural sources of AA and for this reason, the influence of this component on the stability of anthocyanins from acerola extracts was determined and compared to results from açai, which contain no detectable AA.¹²⁴ The stability of acerola was hampered by the high concentration of AA in the fruit. Moreover, the addition of AA to the açai solution in concentrations equivalent to the concentration in the acerola caused a dramatic decrease of the stability of açai. The decrease was not sufficient to match the k_{obs} value observed in acerola, indicating the existence of stabilizing compounds in the açai.¹²⁴

The color stability of strawberry syrup with or without fortification with anthocyanins from black currants (cyanidin and delphinidin 3-glucosides and 3-rutinosides) and/or AA with levels equivalent to those existing in black currant syrup was investigated.¹²⁵ Black currant-fortified strawberry anthocyanins showed higher stability; the addition of AA diminished stability.¹²⁵

Ascorbic acid retention and loss of anthocyanin contents in blood orange juices during refrigerated storage were investigated using CIELAB parameters.¹²⁶ Concurrent losses of anthocyanins and AA were observed, but the rate of reactivity depended on the anthocyanin structure.¹²⁶ When no anthocyanin was present, AA degraded 100% after 9 days, while in the presence of malvidin 3-glucoside and malvidin 3,5-diglucoside after 9 days, 15 and 23% of AA, respectively, remained.¹²⁷ This effect may have been provided by the antioxidant properties attributed to anthocyanins.¹²⁷

4.3.4.4 SUGARS

The effect of added sugar on anthocyanin stability depends on the anthocyanin structure, concentration, and type of sugar. Reducing and non-reducing sugars had destructive effects on the stabilities of anthocyanins from black currants.¹²⁸ The anthocyanin thermostability was reduced when sucrose concentration increased from 0 to 20%, while further concentration increases to 40% had positive effects on the stabilities of pigments. On the other hand, the thermostabilities of pigments decreased linearly with increases of fructose concentration,¹²⁸ probably due to the formation of furaldehydes.¹²⁹

The anthocyanin stabilities of grape-marc, elderberry, and black currant extracts were lower in all sucrose (100 g/L)-added systems as compared to the controls at pH values of 3, 4, and 5, whereas the brown index did not change with the addition of sugar.¹³⁰ On the contrary, a protective effect of 20% sucrose added to frozen crowberries¹³¹ and strawberries¹³² was reported and the stabilities of anthocyanin juices from aronia were higher in syrups than in diluted extracts.¹³³ Roselles pre-

served with honey browned heavily after storage, while sucrose acted as a protector at the same level of water activity.¹³⁴ Although most anthocyanic extracts showed lower stabilities in sugar-added systems, no statistical analysis was carried out to verify the significance of this difference.

4.3.4.5 OTHER FACTORS

Anthocyanins were decolorized at pH 3.0 by the addition of sodium sulfite at the C-2 or C-4 of the chromophore, a reaction that was rapidly reversible on acidification.¹³⁵ Sulfur dioxide, EDTA, and a combination of sulfur dioxide and EDTA exerted very small effects on the losses of anthocyanins in strawberry purées and juices during 10 wk of storage at -20°C .¹³⁶ Conversely, the addition of sulfur dioxide and storage at 20°C slowed the anthocyanin losses and concurrently decreased the formation of polymeric compounds, especially in purées. EDTA had a slight effect on color stability.¹³⁶

The stability of cyanidin 3-glucosyl-arabioside was investigated in different solvents, water, and dimethyl sulfoxide (DMSO, an aprotic solvent) under the same conditions.¹³⁷ The k_{obs} values unexpectedly showed that the anthocyanin was more stable in water solution. DMSO was chosen as a solvent so that it would make the addition of DMSO to C-2 difficult and thus the anthocyanin would be more stable in DMSO than in a water solution. However, since the experiment took place in acidic solution ($\text{pK}_{\text{a}} > \text{pK}_{\text{h}}$), the preferential addition at C-2 or C-4 of the flavylium ion probably took place by a protonated molecule of DMSO.¹³⁷

The degradation kinetics of malvidin 3-glucoside in ethanolic solutions under conditions simulating wine accelerated with the increase of ethanol concentration, probably because the extent of anthocyanin self-association decreased with elevated ethanol concentration.¹³⁸

Strawberries and raspberries were submitted to high hydrostatic pressures from 200 to 800 mPa at 18 and 21°C , followed by storage at 4, 20, and 30°C . The greatest stability of the anthocyanins was observed in raspberries submitted to 200 mPa pressure followed by 800 mPa, both stored at 4°C .¹³⁹

Attempts to stabilize anthocyanins by complex inclusion with α - and β -cyclodextrins failed; on the contrary, a discoloration of anthocyanin solutions was observed.^{140–142} Thermodynamic and kinetic investigations demonstrated that inclusion and copigmentation had opposite effects. In the anthocyanins, the *cis*-chalcone colorless structure is the best species adapted to inclusion into the β -dextrin cavity, shifting the equilibrium toward colorless forms.^{141–142}

4.3.5 STABILIZATION

Research into the copigmentation of anthocyanins started as early as 1913 when Willstätter and Everest¹⁴³ determined the chemical structure of cyanidin 3-glucoside isolated from blue cornflowers and red roses, and attributed the color changes to different pH levels in cell saps. This theory, however, was questioned and in 1916, Willstätter and Zollinger,¹⁴⁴ revising the previous work, proposed a new theory according to which the colors of the anthocyanins varied significantly by the effects

of other substances (later named copigments) present in the sap that may have competed with the colored anthocyanins.¹⁴⁵ The extensive work done on the copigmentation of anthocyanins with flavonols and their effects on flower colors opened new avenues for the applications of copigmented anthocyanins in foods.^{98,146–149}

4.3.5.1 SELF-ASSOCIATION

Stability of anthocyanins can be attained by self-association, that is, when two or more anthocyanin molecules are associated. This effect was verified by increasing the concentration of the cyanidin 3,5-diglucoside solution from 10^{-4} M to 10^{-2} M with a consequent bathochromic shift in maximum wavelength absorption in the visible region.¹⁵⁰

The color of malvidin 3-glucoside in aqueous solution can be stabilized by self-association or copigmentation with the *cis*-chalcone form, according to the pH of the solution.⁵

4.3.5.2 INTERMOLECULAR COPIGMENTATION

The poor stability of anthocyanins with simple structures can be overcome by intermolecular copigmentation reaction, that is, an association with different compounds, especially polyphenolic ones, stabilized by π - π intermolecular and hydrogen bonds.¹⁵¹ However, this complex dissociates at high temperatures.¹⁵¹

The magnitude of the copigmentation is influenced by pH value, pigment and copigment concentrations, chemical structure of anthocyanin, temperature, and ionic strength of the medium.¹⁵² As to the effect of the solvent, the important issue is the hydrogen-bonded molecular structure of the liquid water, not the polarity of the medium.¹⁵²

The best cofactors are typically flavonoid derivatives that contain many hydroxyl groups, the most favorable at position 3 of the flavones.¹⁵³ The strongest cofactors have electron-rich systems that associate with electron-poor compounds such as the flavylium cation.

The effects of catechin, epicatechin, procyanidin B2, caffeic acid, *p*-coumaric acid, myricetrin, and quercetrin on the color intensity and stability of malvidin 3-glucoside at a molar ratio of 1:1 under conditions similar to red wine were evaluated.¹⁵⁴ Flavan 3-ols appeared to have the lowest protective effects and flavonols the highest; strong color changes were visually perceptible.¹⁵⁴ In the complexation of malvin chloride and natural polyphenols, flavonol glycosides by far exerted the best protector effect.¹⁵⁵

Polyhydroxylated flavones, isoflavones, and aurone sulfonates improved the photostabilities of anthocyanins due, according to the authors, to the formation of a complex by juxtaposition of the pigment and copigment and stabilization by π - π ring interaction, H-bonding, and ionic bonding of electron-deficient flavylium ions and negativity-charged sulfonate groups.¹⁵¹ The sulfonation of flavonoids improved their abilities to complex with anthocyanins, hindering the hydration reaction and shifting the equilibria toward the colored quinonoidal base.¹⁵¹ Based on the reactions between caffeine and three flavylium salts, it was concluded that

copigmentation is a phenomenon that changes the molar fraction distribution of the species in the equilibrium.¹⁵⁶

Stabilization of crude and purified anthocyanin extracts from açai by the addition of tannic acid resulted in a 65% half-life increase of anthocyanins from the crude extract and 610% of the half-life of the purified one.¹⁵⁷ Although tannic acid was considered an efficient copigment,^{158,159} in general all cinnamic acids give unpleasant odors and tastes to solutions.

Chlorogenic acid enhanced the colors of strawberry and chokeberry juices in concentrations higher than those of the anthocyanins present. However, the effects on purified pigments were lower, indicating the presence of other stabilizing compounds in the juices.¹⁶⁰

The colors of muscadine grape juice and wine were enhanced by copigmentation with formonetin, biochanin A, and prunetin, the major isoflavonoids extracted from red clove leaves, at ratios of 1:2, 1:4, and 1:8.¹⁶¹ The 1:8 ratio representing the maximum amount of isoflavonoids used due to its limiting solubility yielded the maximum color enhancement when compared to standard solutions of anthocyanins, at 23 and 60°C.¹⁶¹

In 1967, Timberlake and Bridle¹⁶² proposed that copigmentation complex formation reactions between cyanidin and quercetin in aqueous buffered solutions took place between the colored forms of the flavylum cation (AH^+) cyanidin at pH 3.0 and the quinoidal base (A) at pH 5.0.

Malvidin 3-glucoside from grape skins was copigmented with several procyanidins of a homogeneous series in a wine-similar solution in order to evaluate the influence of the copigment structure on the strength of complexation.¹⁶³ Copigments used were (+)-catechin, (–)-epicatechin, dimers with C₄–C₆ and C₄–C₂ linked interflavonoids, a dimer esterified with gallic acid, and a trimer. To reduce the strengths of complexed anthocyanins in order to make the reaction solutions similar to wines, ethanol was added although it was reported to impair the complexation reaction.¹⁶⁴ The results showed that association strength is related to the stereoisomeric form such as asymmetries of the side groups of pyranic rings of the copigments rather than to structures.¹⁶³ Depending on its conformation, a copigment may result in a special hydrogen-bonded network between water and procyanidins modifying the water network in favor of a more or less important interaction with malvidin 3-glucoside.¹⁶³

The influence of the pH on the copigmentation reaction was verified in the reaction of malvidin 3-glucoside and (–)-epicatechin at pH ranges from 2 to 6.¹⁶⁵ The disappearances of the pigment and copigment were observed over the whole pH range since the nucleophilic addition of epicatechin onto malvidin 3-glucoside took place. Although the formed final products were not the same, a progressive decrease in the concentrations of both reagents during the reaction occurred at rates increasing with pH value, the reactions following first-order kinetics.¹⁶⁵

4.3.5.3 EFFECTS OF ALDEHYDES

In the intermolecular reactions between anthocyanins and flavonoids mediated by acetaldehyde, new compounds linked by an ethyl bridge are formed. Three new compounds were detected by the reaction of malvidin 3-glucoside and proanthocyan-

anidin B mediated by acetaldehyde under wine-like conditions. Two were assigned to enantiomers containing malvidin 3-glucoside covalently bonded to proanthocyanidin linked through C-8 by an H-C-CH₃ ethyl bridge.¹⁶⁶

A pigment derived from the acetaldehyde-mediated condensation between (+)-catechin and malvidin 3-glucoside was prepared and its properties were compared to those of malvidin 3-glucoside in aqueous solution.¹⁶⁷ With a pH increase from 2.2 to 5.5, the color of the formed pigment solution shifted toward violet at pH 5.5, whereas similar solutions of malvidin 3-glucoside were almost colorless at pH 4.0. This behavior indicated that the anthocyanin moiety of the new pigment was more protected against water attack. The new pigment was more stable with regard to bleaching by SO₂ than malvidin glucoside, but was more sensitive to degradation in aqueous solution. The cleavage of the bridge linking the anthocyanin to the catechin is the first step in its degradation; malvidin-glucoside is the major product formed.¹⁶⁷

In the reaction of malvidin 3-glucoside and catechin mediated by acetic, isovaleric, propionic, isobutyric, formic, and 2-isobutyric aldehydes, oligomeric compounds formed by a reaction between flavanol-flavanol (colorless) and colored flavanol-anthocyanins were detected. All compounds revealed bluish and darkening color effects, measured by CIELAB parameters.¹⁶⁸ Direct reactions of small molecules such as 4 vinylphenol, pyruvic acid, flavonols, and anthocyanins were detected in wine model solutions^{96,169} and identified in wines.⁸⁹ The same pigments were also identified in strawberry and raspberry juices by the addition of ferulic or siringic acid.¹⁷⁰

The physicochemical properties of 8,8-methyl-methine catechin-malvidin 3-glucoside isomers (*R* and *S*) and catechin-ethyl-malvidin-3-glucoside were compared with malvidin 3-glucoside.¹⁷¹ The ethyl-linked catechin-malvidin 3-glucoside presented higher stability toward hydration than the malvidin 3-glucoside, most probably related to the self-association that protects the pigments from water attack. The ethyl linkage may confer flexibility to the compound, so further protection from hydration and bisulfite discoloration is provided by an intramolecular association.¹⁷¹

Cyanidin 3-glucoside, cyanidin 3-rutinoside, cyanidin 3-sophoroside, and cyanidin 3-sambubioside reacted with pyruvic acid in solutions at pH 1.0, 2.0, 5.0, and 7.0.¹⁷² The anthocyanin-pyruvic acid adducts formed showed higher resistance to decoloration toward pH variation and to decoloration by SO₂. The different sugar moieties in the adducts affected all the CIELAB color parameters.¹⁷²

4.3.6 FINAL REMARKS

Fortunately, the appearance in the last years of new, more accurate methods and sophisticated equipments permitted the isolation of anthocyanins on a preparative scale and allowed the identification of extremely complex and stable anthocyanins.

Studies of the stability and stabilization of anthocyanins are still required, based on the extreme importance of those pigments for food colors. Modern HPLC-MS equipment also allows us to easily follow the copigmentation reactions in detail, calculate their kinetic and thermodynamic parameters, identify the products formed during the reactions, and thus shed new light on the stability and stabilization of these pigments. Since anthocyanins play important roles as natural colorants for

foods and participate in red wine aging, our understanding of these reactions and the anthocyanin structures is extremely important. Unfortunately, the anthocyanins that have exceptional stability in weak acid or neutral solution are more frequently found in flowers than in foods. These anthocyanins may in the future be produced in fruits and vegetables by cell culture, breeding, or even by genetic modifications.

SCIENTIFIC NAMES

açaí (*Euterpe oleracea* Mart.), apple (*Malus pumila* L.), aronia (*Aronia melanocarpa* Elliot), baguaçu (*Eugenia umbelliflora* Berg), bambara groundnut (*Vigna subterranea*), bean (*Phaseolus vulgaris* L.), blackberry – evergreen (*Rubus laciniatus* Willd.), black carrot (*Daucus carota*), black currant (*Ribes nigrum* L.), blueberry (*Vaccinium* spp.), black blueberry (*Vaccinium corymbosum* L.), black raspberry (*Rubus occidentalis*), Burdekin plum (*Pleiogynium timorense* DC. Leenh), Cedar Bay cherry (*Eugenia carissoides* F. Muell.), chive (*Allium schoenoprasum*), chokeberry (*Aronia melanocarpa* Elliot), cranberry (*Vaccinium macrocarpon* Ait. or *V. oxycoccus* L.), cornflower (*Centaurea cyanus*), Davidson's plum (*Davidsonia pruriens* F. Muell. var. *pruriens*), elderberry (*Sambucus nigra* L.), grape (*Vitis vinifera*), grape cv. Concord (*Vitis labrusca*), honeysuckle (*Lonicera caerulea* L. or *L. nitida* L.), Illawarra plum (*Podocarpus elatus* R. Br. ex Endl.), lentil (*Lens culinaris*), marionberry (*Rubus ursinus*), mashua (*Tropaeolum tuberosum* Ruiz and Pavon), Molucca raspberry (*Rubus moluccanus* var. *austropacificus* van Royen), muntry (*Kunzea pomifera* F. Muell.), muscadine grape (*Vitis rotundifolia*), passion fruit (*Passiflora edulis* L.), peach (*Prunus persica* L.), pina boca (*Solanum stenotomum*), plum (*Prunus domestica* L.), potato (*Solanum tuberosum* L.), red clove (*Trifolium platense*), red raspberry (*Rubus idaeus* L.), red cabbage (*Brassica oleracea*), red onion (*Allium cepa*), radish (*Raphanus sativus*), strawberry (*Fragaria x ananassa* Duch.), sweet cherry (*Prunus avium* L.), sweet potato (*Ipomoea batatas*), Tasmanian pepper berry (*Tasmanian lanceolata* R. Br.)

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REFERENCES

1. Andersen, Ø.M. and Jordheim, M., The anthocyanins, in *Flavonoids: Chemistry, Biochemistry and Applications*, Andersen, Ø.M. and Markham, K.R., Eds., CRC Press, Boca Raton, 2006, 472.
2. Wada, L. and Ou, B., Antioxidant activity and phenolic content of Oregon caneberries, *J. Agric. Food Chem.*, 50, 3495, 2002.
3. McGhie, T.K., Rowan, D.R., and Edwards, P.J., Structural identification of two major anthocyanin components of boysenberry by NMR spectroscopy, *J. Agric. Food Chem.*, 54, 8756, 2006.

4. Brouillard, R. and Delaporte, B., Chemistry of anthocyanin pigments. 2. Kinetic and thermodynamic study of proton-transfer, hydration, and tautomeric reactions of malvidin-3-glucoside, *J. Am. Chem. Soc.*, 99, 8461, 1977.
5. Houbiers, C. et al., Color stabilization of malvidin 3-glucoside: self-aggregation on the of flavylum cation and copigmentation with the Z-chalcone form, *J. Phys. Chem. B*, 102, 3578, 1998.
6. Gallori, S. et al., Polyphenolic constituents of fruit pulp of *Euterpe oleracea* Mart. (açai palm), *Chromatographia*, 59, 739, 2004.
7. Lichtenthaler, R. et al., Total oxidant scavenging capacities of *Euterpe oleracea* Mart. (açai) fruits, *Int. J. Food Sci. Nutr.*, 56, 53, 2005.
8. Schauss, A.G. et al., Phytochemical and nutrient composition of the freeze-dried Amazonian palm berry, *Euterpe oleraceae* Mart. (açai), *J. Agric. Food Chem.*, 54, 8598, 2006.
9. Hanamura, T., Hagiwara, T., and Kawagishi, H., Structural and functional characterization of polyphenols isolated from acerola (*Malpighia emarginata* DC) fruit, *Biosci. Biotechnol. Biochem.*, 69, 280, 2005.
10. Wu, X. and Prior, R.L., Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries, *J. Agric. Food Chem.*, 53, 2589, 2005.
11. Wu, X. et al., Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption, *J. Agric. Food Chem.*, 54, 4069, 2006.
12. Kuskoski, E.M. et al., Characterization of anthocyanins from the fruits of baguaçu (*Eugenia umbelliflora* Berg), *J. Agric. Food Chem.*, 51, 5450, 2003.
13. Longo, L. and Vasapollo, G., Anthocyanins from bay (*Laurus nobilis* L.) berries, *J. Agric. Food Chem.*, 53, 8063, 2005.
14. Bloor, S.J., Deep blue anthocyanins from blue *Dianella* berries, *Phytochemistry*, 58, 923, 2001.
15. Terahara, N., Yamaguchi, M.-A., and Shizukuishi, K., Cyanidin 3-acetylrutinoside in *Euria japonica* berries, *Phytochemistry*, 27, 3701, 1988.
16. Terahara, N. et al., Cyanidin-3-lathyroside from berries of *Fatsia japonica*, *Phytochemistry*, 31, 1446, 1992.
17. Longo, L., Vasapollo, G., and Rescio, L., Determination of anthocyanins in *Rhamnus alaternus* L. berries, *J. Agric. Food Chem.*, 53, 1723, 2005.
18. Longo, L. and Vasapollo, G., Determination of anthocyanins in *Ruscus aculeatus* L. berries, *J. Agric. Food Chem.*, 53, 475, 2005.
19. Longo, L. and Vasapollo, G., Extraction and identification of anthocyanins from *Smilax aspera* L. berries, *Food Chem.*, 94, 226, 2006.
20. Netzel, M. et al., Sources of antioxidant activity in Australian native fruits. Identification and quantification of anthocyanins, *J. Agric. Food Chem.* 54, 9820, 2006.
21. Cho, M.J. et al., Flavonoid glycosides and antioxidant capacity of various blackberry, blueberry and red grape genotypes determined by high-performance liquid chromatography/mass spectrometry, *J. Sci. Food Agric.*, 84, 1771, 2004.
22. Stintzing, F.C. et al., A Novel zwitterionic anthocyanin from evergreen blackberry (*Rubus laciniatus* Willd.), *J. Agric. Food Chem.*, 50, 396, 2002.
23. Degenhardt, A., Knapp, H., and Winterhalter, P., Separation and purification of anthocyanins by high-speed countercurrent chromatography and screening for antioxidant activity, *J. Agric. Food Chem.*, 48, 338, 2000.
24. Wu, X. et al., Characterization of anthocyanins and proanthocyanidins in some cultivars of *Ribes*, *Aronia*, and *Sambucus* and their antioxidant capacity, *J. Agric. Food Chem.*, 52, 7846, 2004.

25. Slimestad, R. and Solheim, H., Anthocyanins from black currants (*Ribes nigrum* L.), *J. Agric. Food Chem.*, 50, 3228, 2002.
26. Nielsen, I.L. et al., Quantification of anthocyanins in commercial black currant juices by simple high-performance liquid chromatography. Investigation of their pH stability and antioxidative potency, *J. Agric. Food Chem.*, 51, 5861, 2003.
27. Lee, H.S., Characterization of major anthocyanins and the color of red-fleshed budd blood orange (*Citrus sinensis*), *J. Agric. Food Chem.*, 50, 1243, 2002.
28. Dugo, P. et al., Characterization of the anthocyanin fraction of Sicilian blood orange juice by micro-HPLC-ESI/MS, *J. Agric. Food Chem.*, 51, 1173, 2003.
29. Hillebrand, S., Schwarz, M., and Winterhalter, P., Characterization of anthocyanins and pyranoanthocyanins from blood orange [*Citrus sinensis* (L.) Osbeck] juice, *J. Agric. Food Chem.*, 52, 7331, 2004.
30. Cabrita, L. and Andersen, Ø.M., Anthocyanins in blue berries of *Vaccinium padifolium*, *Phytochemistry*, 52, 1693, 1999.
31. Cabrita, L., Froystein, N.A., and Andersen, Ø.M., Anthocyanin trisaccharides in blue berries of *Vaccinium padifolium*, *Food Chem.*, 69, 33, 2000.
32. Cooney, J.M., Jensen, D.J., and McGhie, T.K., LC-MS identification of anthocyanins in boysenberry extract and anthocyanin metabolites in human urine following dosing, *J. Sci. Food Agric.*, 84, 237, 2004.
33. Zanatta, C.F. et al., Determination of anthocyanins from camu-camu (*Myrciaria dubia*) by HPLC-PDA, HPLC-MS, and NMR, *J. Agric. Food Chem.*, 53, 9531, 2005.
34. Mozetic, B. and Trebse, P., Identification of sweet cherry anthocyanins and hydroxycinnamic acids using HPLC coupled with DAD and MS detector, *Acta Chim. Slov.*, 51, 151, 2004.
35. Wang, H. et al., Quantification and characterization of anthocyanins in Balaton tart cherries, *J. Agric. Food Chem.*, 45, 2556, 1997.
36. Chandra, A., Rana, J., and Li, Y., Separation, identification, quantification, and method validation of anthocyanins in botanical supplement raw materials by HPLC and HPLC-MS, *J. Agric. Food Chem.*, 49, 3515, 2001.
37. Escribano-Bailón, M.T. et al., Anthocyanin composition of the fruit of *Coriaria myrtifolia* L., *Phytochem. Anal.*, 13, 354, 2002.
38. Slimestad, R. and Andersen, Ø.M., Cyanidin 3-(2-glucosyl)galactoside and other anthocyanins from fruits of *Cornus suecica*, *Phytochemistry*, 49, 2163, 1998.
39. Schwarz, M. et al., Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins, *Biochem. Eng. J.*, 14, 179, 2003.
40. Chaovanalikit, A., Thompson, M.M., and Wrolstad, R.E., Characterization and quantification of anthocyanins and polyphenolics in blue honeysuckle (*Lonicera caerulea* L.), *J. Agric. Food Chem.*, 52, 848, 2004.
41. Sarni-Manchado, P. et al., Phenolic composition of litchi fruit pericarp, *J. Agric. Food Chem.*, 48, 5995, 2000.
42. Kidøy, L. et al., Anthocyanins in fruits of *Passiflora edulis* and *P. suberosa*, *J. Food Compos. Anal.*, 10, 49, 1997.
43. Mullen, W., Lean, M.E.J., and Crozier, A., Rapid characterization of anthocyanins in red raspberry fruit by high-performance liquid chromatography coupled to single quadrupole mass spectrometry, *J. Chromatogr. A*, 966, 63, 2002.
44. Beekwilder, J. et al., Antioxidants in raspberry: on-line analysis links antioxidant activity to a diversity of individual metabolites, *J. Agric. Food Chem.*, 53, 3313, 2005.
45. Byamukama, R. et al., Anthocyanins from fruits of *Rubus pinnatus* and *Rubus rigidus*, *J. Food Compos. Anal.*, 18, 599, 2005.

46. Tamura, H., Takada, M., and Yoshida, Y., Pelargonidin-3-*O*-(6-*O*-malonyl- β -D-glucoside) in *Fragaria x ananassa* Duch. cv. Nyoho, *Biosci. Biotech. Biochem.*, 59, 1157, 1995.
47. Lopes-da Silva, F., et al., Identification of anthocyanin pigments in strawberry (cv. Camarosa) by LC using DAD and ESI-MS detection, *Eur. Food Res. Technol.*, 214, 248, 2002.
48. Andersen, Ø.M. et al., Anthocyanin from strawberry (*Fragaria ananassa*) with the novel aglycone, 5-carboxypyranopelargonidin, *Phytochemistry*, 65, 405, 2004.
49. Favretto, D. and Flamini, R., Application of electrospray ionization mass spectrometry to the study of grape anthocyanins, *Am. J. Enol. Vitic.*, 51, 55, 2000.
50. Revilla, I. et al., Identification of anthocyanin derivatives in grape skin extracts and red wines by liquid chromatography with diode array and mass spectrometric detection, *J. Chromatogr. A*, 847, 83, 1999.
51. Ruberto, G. et al., Polyphenol constituents and antioxidant activity of grape pomace extracts from five Sicilian red grape cultivars, *Food Chem.*, 100, 203, 2007.
52. Giusti, M.M. et al., Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization, *J. Agric. Food Chem.*, 47, 4657, 1999.
53. Wang, H., Race, E.J., and Shrikhande, A.J., Characterization of anthocyanins in grape juices by ion trap liquid chromatography-mass spectrometry, *J. Agric. Food Chem.*, 51, 1839, 2003.
54. Mazzuca, P. et al., Mass spectrometry in the study of anthocyanins and their derivatives: differentiation of *Vitis vinifera* and hybrid grapes by liquid chromatography/electrospray ionization mass spectrometry and tandem mass spectrometry, *J. Mass Spectrom.*, 40, 83, 2005.
55. Andersen, Ø.M. and Fossen, T., Anthocyanin with an unusual acylation pattern from stem of *Allium victorialis*, *Phytochemistry*, 40, 1809, 1995.
56. Kammerer, D., Carle, R., and Schieber, A., Detection of peonidin and pelargonidin glycosides in black carrots (*Daucus carota* ssp. sativus var. atropurpureus Alef.) by high-performance liquid chromatography/electrospray ionization mass spectrometry, *Rapid Commun. Mass Spectrom.*, 17, 2407, 2003.
57. Kammerer, D., Carle, R., and Schieber, A., Quantification of anthocyanins in black carrot extracts (*Daucus carota* ssp. sativus var. atropurpureus Alef.) and evaluation of their color properties, *Eur. Food Res. Technol.*, 219, 479, 2004.
58. Schwarz, M., Wray, V., and Winterhalter, P., Isolation and identification of novel pyranoanthocyanins from black carrot (*Daucus carota* L.) juice, *J. Agric. Food Chem.*, 52, 5095, 2004.
59. Fossen, T. et al., Characteristic anthocyanin pattern from onions and other *Allium* spp, *J. Food Sci.*, 61, 703, 1996.
60. Wu, X. and Prior, R.L., Identification and characterization of anthocyanins by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains, *J. Agric. Food Chem.*, 53, 3101, 2005.
61. Fossen, T. and Andersen, Ø.M., Malonated anthocyanins of garlic *Allium sativum* L., *Food Chem.*, 58, 215, 1997.
62. Fossen, T. et al., Anthocyanins from a Norwegian potato cultivar, *Food Chem.*, 81, 433, 2003.
63. Naito, K. et al., Acylated pelargonidin glycosides from a red potato, *Phytochemistry*, 47, 109, 1998.
64. Rodríguez-Saona, L.E., Giusti, M.M., and Wrolstad, R.E., Anthocyanin pigment composition of red-fleshed potatoes, *J. Food Sci.*, 63, 458, 1998.

65. Chirinos, R. et al., High-performance liquid chromatography with photodiode array detection (HPLC-DAD)/HPLC–mass spectrometry (MS) profiling of anthocyanins from Andean mashua tubers (*Tropaeolum tuberosum* Ruiz and Pavón) and their contribution to the overall antioxidant activity, *J. Agric. Food Chem.*, 54, 7089, 2006.
66. Terahara, N., Yamaguchi, M.-A., and Honda, T., Malonylated anthocyanins from bulbs of red onion, *Allium cepa* L., *Biosci. Biotechnol. Biochem.*, 58, 1324, 1994.
67. Fossen, T. and Andersen, Ø.M., Anthocyanins from red onion, *Allium cepa*, with novel aglycone, *Phytochemistry*, 62, 1217, 2003.
68. Fossen, T., Slimestad, R., and Andersen, Ø.M., Anthocyanins with 4'-glucosidation from red onion, *Allium cepa*, *Phytochemistry*, 64, 1367, 2003.
69. Odake, K. et al., Chemical structures of two anthocyanins from purple sweet potato, *Ipomoea batatas*, *Phytochemistry*, 31, 2127, 1992.
70. Goda, Y. et al., Two acylated anthocyanins from purple sweet potato, *Phytochemistry*, 40, 1809, 1995.
71. Terahara, N. et al., Six diacylated anthocyanins from the storage roots of purple sweet potato, *Ipomoea batatas*, *Biosci. Biotechnol. Biochem.*, 63, 1420, 1999.
72. Pale, E. et al., Anthocyanins from Bambara groundnut (*Vigna subterranea*), *J. Agric. Food Chem.*, 45, 3359, 1997.
73. Macz-Pop, G.A. et al., Natural occurrence of free anthocyanin aglycones in beans (*Phaseolus vulgaris* L.), *Food Chem.*, 94, 448, 2006.
74. Takeoka, G.R. et al., Characterization of black bean (*Phaseolus vulgaris* L.) anthocyanins, *J. Agric. Food Chem.*, 45, 3395, 1997.
75. Choung, M.-G. et al., Anthocyanin profile of Korean cultivated kidney bean (*Phaseolus vulgaris* L.), *J. Agric. Food Chem.*, 51, 7040, 2003.
76. Yoshida, K. et al., Structural analysis and measurement of anthocyanins from colored seed coats of *Vigna*, *Phaseolus*, and *Glycine* legumes, *Biosci. Biotech. Biochem.*, 60, 589, 1996.
77. Takeoka, G.R. et al., Delphinidin 3-O-(2-O-β-D-glucopyranosyl-α-L-arabinopyranoside): a novel anthocyanin identified in Beluga black lentils, *J. Agric. Food Chem.*, 53, 4932, 2005.
78. Hu, C. et al., Black rice (*Oryza sativa* L. indica) pigmented fraction suppresses both reactive oxygen species and nitric oxide in chemical and biological model systems, *J. Agric. Food Chem.*, 51, 5271, 2003.
79. Abdel-Aal, E.-S.M., Young, J.C., and Rabalski, I., Anthocyanin composition in black, blue, pink, purple, and red cereal grains, *J. Agric. Food Chem.*, 54, 4696, 2006.
80. Choung, M.-G. et al., Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.), *J. Agric. Food Chem.*, 49, 5848, 2001.
81. de Pascual-Teresa, S. de, Santos-Buelga, C., and Rivas-Gonzalo, J.C., LC–MS analysis of anthocyanins from purple corn cob, *J. Sci. Food Chem.*, 82, 1003, 2002.
82. Escribano-Bailón, M.T., Santos-Buelga, C., and Rivas-Gonzalo, J.C., Anthocyanins in cereals, *J. Chromatogr. A*, 1054, 129, 2004.
83. Macz-Pop, G.A. et al., New flavanol-anthocyanin condensed pigments and anthocyanin composition in Guatemalan beans (*Phaseolus* spp.), *J. Agric. Food Chem.*, 54, 536, 2006.
84. Pale, E. et al., 7-O-methylapigeninidin, an anthocyanidin from *Sorghum caudatum*, *Phytochemistry*, 45, 1091, 1997.
85. Lo, S.-C. et al., Phytoalexin accumulation in sorghum: identification of a methyl ether of luteolinidin, *Physiol. Mol. Plant Pathol.*, 49, 21, 1996.
86. Wharton, P.S. and Nicholson, R.L., Temporal synthesis and radiolabelling of the sorghum 3-deoxyanthocyanidin phytoalexins and the anthocyanin, cyanidin 3-dimallyl glucoside, *New Phytol.*, 145, 457, 2000.

87. Bakker, J. et al., Identification of an anthocyanin occurring in some red wines, *Phytochemistry*, 44, 1375, 1997.
88. Fulcrand, H. et al., A new class of wine pigments generated by reaction between pyruvic acid and grape anthocyanins, *Phytochemistry*, 47, 1401, 1998.
89. Schwarz, M., Wabnitz, T.C., and Winterhalter, P., Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigments in red wines, *J. Agric. Food Chem.*, 51, 3682, 2003.
90. Lu, Y., Foo, L.Y., and Sun, Y., New pyranoanthocyanins from blackcurrant seeds, *Tetrahedron Lett.*, 43, 7341, 2002.
91. Lu, Y., Sun, Y., and Foo, L.Y., Novel pyranoanthocyanins from black currant seed, *Tetrahedron Lett.*, 41, 5975, 2000.
92. Lu, Y. and Foo, L.Y., Unusual anthocyanin reaction with acetone leading to pyranoanthocyanin formation, *Tetrahedron Lett.*, 42, 1371, 2001.
93. Fossen, T., Rayyan, S., and Andersen, Ø.M., Dimeric anthocyanins from strawberry (*Fragaria ananassa*) consisting of pelargonidin 3-glucoside covalently linked to four flavan-3-ols, *Phytochemistry*, 65, 1421, 2004.
94. Jurd, L., Anthocyanidins and related compounds. XI. Catechin-flavylium salt condensation reactions, *Tetrahedron*, 23, 1057, 1967.
95. Remy, S. et al., First confirmation in red wine of products resulting from direct anthocyanin-tannin reactions, *J. Sci. Food Agric.*, 80, 745, 2000.
96. Salas, E. et al., Demonstration of the occurrence of flavanol-anthocyanin adducts in wine and in model solutions, *Anal. Chim. Acta*, 513, 325, 2004.
97. Harborne, J.B., The natural distribution in angiosperms of anthocyanins acylated with aliphatic dicarboxylic acid, *Phytochemistry*, 25, 1887, 1986.
98. Stintzing, F.C. et al., Color and antioxidant properties of cyanidin-based anthocyanin pigments, *J. Agric. Food Chem.*, 50, 6172, 2002.
99. Asen, S., Stewart, R.N., and Norris, K.H., Copigmentation of anthocyanins in plant tissues and its effect on color, *Phytochemistry*, 11, 1139, 1972.
100. Saito, N. and Osawa, Y., Platycanin, a new acylated anthocyanin in Chinese bell flower, *Platycodon grandiflorum*, *Phytochemistry*, 10, 445, 1971.
101. Idaka, E. et al., Structure of zebrinin, a novel acylated anthocyanin isolated from *Zebrina pendula*, *Tetrahedron Lett.*, 28, 1901, 1987.
102. Terahara, N. et al., Structure of ternatin-A1, the largest ternatin in the major blue anthocyanins from *Clitoria ternatea* flowers, *Tetrahedron Lett.*, 31, 2921, 1990.
103. Goto, T. et al., Structure of gentiodelphin, an acylated anthocyanin isolated from *Gentiana makinoi*, that is stable in dilute aqueous solution, *Tetrahedron Lett.*, 23, 3695, 1982.
104. Goto, T. et al., Structure of platycanin, a diacylated anthocyanin isolated from the Chinese bell flower *Platycodon grandiflorum*, *Tetrahedron Lett.*, 24, 2181, 1983.
105. Brouillard, R., The chemistry of anthocyanin pigments. 7. Origin of the exceptional color stability of the zebrina anthocyanin, *Phytochemistry*, 20, 143, 1981.
106. Brouillard, R., The *in vivo* expression of anthocyanin color in plants, *Phytochemistry*, 22, 1311, 1983.
107. George, F. et al., Influence of *trans-cis* isomerisation of coumaric acid substituents on colour variance and stabilisation in anthocyanins, *Phytochemistry*, 57, 791, 2001.
108. Borkowski, T. et al., The effect of 3-*O*- β -glucosylation on structural transformations of anthocyanidins, *Food Res. Int.*, 38, 1031, 2005.
109. Goto, T. and Kondo, T., Structure and molecular stacking of anthocyanins: flower color variation, *Angew. Chem. Int. Edit.*, 30, 17, 1991.

110. Adams, J.B., Thermal degradation of anthocyanins with particular reference to the 3-glucoside of cyanidin. I. In acidified aqueous solutions at 100°C, *J. Sci. Food Agric.*, 24, 747, 1973.
111. Mok, C. and Hettiarachchy, N.S., Heat-stability of sunflower-hull anthocyanin pigment, *J. Food Sci.*, 56, 553, 1991.
112. Reyes, L.F. and Cisneros-Zevallos, L., Degradation kinetics and colour of anthocyanins in aqueous extracts of purple- and red-flesh potatoes (*Solanum tuberosum* L.), *Food Chem.*, 100, 885, 2007.
113. Ochoa, M.R. et al., Physical and chemical characteristics of raspberry pulp: storage effect on composition and color, *Lebensm.-Wiss. u.-Technol.*, 32, 149, 1999.
114. Withy, I.M. et al., Storage changes in anthocyanin content of red raspberry juice concentrate, *J. Food Sci.*, 58, 190, 1993.
115. Kirca, A. and Cemeroglu, B., Degradation kinetics of anthocyanins in blood orange juice and concentrate, *Food Chem.*, 81, 583, 2003.
116. Dyrby, M., Westergaard, N., and Stapelfeldt, H., Light and heat sensitivity of red cabbage extract in soft drink model systems, *Food Chem.*, 72, 431, 2001.
117. Furtado, P., et al., Photochemical and thermal-degradation of anthocyanidins, *J. Photochem. Photobiol. A-Chem.*, 75, 113, 1993.
118. Fossen, T., Cabrita, L., and Andersen, Ø.M., Colour and stability of pure anthocyanins influenced by pH including the alkaline region, *Food Chem.*, 63, 435, 1998.
119. Jurd, L., Some advances in the chemistry of anthocyanins-type plant pigments, in *The Chemistry of Plant Pigments*, Chichester, C.O., Ed., Academic Press, New York, 1972, 123.
120. Poei-Langston, M.S. and Wrolstad, R.E., Color degradation in an ascorbic acid-anthocyanin-flavonol model system, *J. Food Sci.*, 46, 1218, 1981.
121. Iacobucci, G.A., and Sweeny, J.G., The chemistry of anthocyanins and related flavylum salts, *Tetrahedron*, 39, 3005, 1983.
122. Shrikhande, A.J. and Francis, F.J., Effect of flavonols on ascorbic and anthocyanin stability in model systems, *J. Food Sci.*, 39, 904, 1974.
123. Garcia-Viguera, C. and Bridle, P., Influence of structure on color stability of anthocyanins and flavylum salts with ascorbic acid, *Food Chem.*, 64, 21, 1999.
124. De Rosso, V.V. and Mercadante, A.Z., The high ascorbic acid content is the main cause of the low stability of anthocyanin extracts from acerola, *Food Chem.*, 103, 935, 2007.
125. Skrede, G., et al., Color stability of strawberry and black currant syrups, *J. Food Sci.*, 57, 172, 1992.
126. Choi, M.H., Kim, G.H., and Lee, H.S., Effects of ascorbic acid retention on juice color and pigment stability in blood orange (*Citrus sinensis*) juice during refrigerated storage, *Food Res. Int.*, 35, 753, 2002.
127. Wang, H., Cao, G.H., and Prior, R.L., Oxygen radical absorbing capacity of anthocyanins, *J. Agric. Food Chem.*, 45, 304, 1997.
128. Rubinskiene, M. et al., Impact of various factors on the composition and stability of black currant anthocyanins, *Food Res. Int.*, 38, 867, 2005.
129. Debicki-Pospisil, J. et al., Anthocyanin degradation in the presence of furfural and 5-hydroxymethylfurfural, *J. Food Sci.*, 48, 411, 1983.
130. Malien-Aubert, C., Dangles, O., and Amiot, M.J., Color stability of commercial anthocyanin-based extracts in relation to phenolic composition: protective effects by intra- and intermolecular copigmentation, *J. Agric. Food Chem.*, 49, 170, 2001.
131. Kallio, H. et al., Comparison of the half-lives of the anthocyanins in the juice of chowberry, *Empetrum nigrum*, *J. Food Sci.*, 51, 408, 1986.

132. Wrolstad, R.E. et al., Influence of sugar on anthocyanin pigment stability in frozen strawberries, *J. Food Sci.*, 55, 1064, 1990.
133. Plocharski, W., Zbroszczyk, J., and Lenartowicz, W., Aronia fruit (*Aronia melanocarpa* Elliot) as a natural source of anthocyanin colorants. II. The stability of the color of aronia juices and extracts, *Fruit Sci. Rep.*, 16, 41, 1989.
134. Tsai, P.J., Hsieh, Y.Y., and Huang, T.C., Effect of sugar on anthocyanin degradation and water mobility in a roselle anthocyanin model system using O-¹⁷ NMR, *J. Agric. Food Chem.*, 52, 3097, 2004.
135. Adams, J.B. and Woodman, J.S., Thermal degradation of anthocyanins with particular reference to the 3-glucosides of cyanidin. 2 – Anaerobic degradation of cyanidin-3-rutinoside at 100°C and pH 3.0 in presence of sodium sulfite, *J. Sci. Food Agric.*, 24, 763, 1973.
136. Bakker, J. and Bridle, P., Strawberry juice color — The effect of sulfur-dioxide and EDTA on the stability of anthocyanins, *J. Sci. Food Agric.*, 60, 477, 1992.
137. Bobbio, F.O., Do Nascimento, M.T.V., and Bobbio, P.A., Effect of light and tannic acid on the stability of anthocyanin in DMSO and in water, *Food Chem.*, 51, 183, 1994.
138. Tseng, K.C. et al., Degradation kinetics of anthocyanin in ethanolic solutions, *J. Food Process Preserv.*, 30, 503, 2006.
139. Suthanthangjai, W., Kajda, P., and Zabetakis, I., The effect of high hydrostatic pressure on the anthocyanins of raspberry (*Rubus iudaeus*), *Food Chem.*, 90, 193, 2005.
140. Yamada, T., Komiya, T., and Akaki, M., Formation of an inclusion complex of anthocyanin with cyclodextrin, *Agric. Biol. Chem.*, 44, 1411, 1980.
141. Dangles, O. et al., Two very distinct types of anthocyanins complexation — copigmentation and inclusion, *Tetrahedron Lett.*, 33, 5227, 1992.
142. Dangles, O., Wigand, M.C., and Brouillard, R., Anthocyanin anti-copigment effect, *Phytochemistry*, 31, 3811, 1992.
143. Von Willstätter, R. and Everest, E., Untersuchungen über die anthocyane: I. Über den farbstoff der kornblume, *Justus Liebigs Ann. Chem.*, 401, 189, 1913.
144. Von Willstätter, R. and Zollinger, E.H., XVI. Über die farbstoffe der weintraube und der heidelbeere, II, *Justus Liebigs Ann. Chem.*, 412, 195, 1916.
145. Robinson, G.M. and Robinson, R., A survey of anthocyanins, *Biochem. J.*, 25, 1687, 1931.
146. Asen, S., Factors affecting formation of anthocyanin-flavonol co-pigment complexes and their importance on flower color, *Plant Physiol.*, 47, 20, 1971.
147. Asen, S., Norris, K.H., and Stewart, R.N., Effect of pH and concentration of anthocyanin-flavonol co-pigment complex on the color of “better timer” roses, *J. Am. Soc. Hort. Sci.*, 96, 770, 1971.
148. Van Teeling, C.G., Cansfield, P.E., and Gallop, R.A., Anthocyanin complex isolated from syrup of canned blueberries, *J. Food Sci.*, 36, 1061, 1971.
149. Scheffeldt, P. and Hrazdina, G., Co-pigmentation of anthocyanins under physiological conditions, *J. Food Sci.*, 43, 517, 1978.
150. Timberlake, C.F. and Bridle, P., The anthocyanins, in *The flavonoids*, Harborne, J.B., Mabry, T.J., and Mabry, H., Eds., Chapman & Hall, London, 1975, 214.
151. Sweeny, J.G., Wilkinson, M.M., and Iacobucci, G.A., Effect of flavonoid sulfonates on the photobleaching of anthocyanins in acid solutions, *J. Agric. Food. Chem.*, 29, 563, 1981.
152. Mazza, G. and Brouillard, R., The mechanism of copigmentation of anthocyanins in aqueous solutions, *Phytochemistry*, 29, 1097, 1990.
153. Chen, L.J. and Hrazdina, G., Structural aspects of anthocyanin-flavonoid complex formation and its role in plant color, *Phytochemistry*, 20, 297, 1981.

154. Gomez-Miguez, M. et al., Influence of different phenolic copigments on the color of malvidin 3-glucoside, *J. Agric. Food Chem.*, 54, 5422, 2006.
155. Cai, Y., Lilley, T.H., and Haslam, E., Polyphenol anthocyanin copigmentation, *J. Chem. Soc., Chem. Commun.*, 380, 1990.
156. Pina, F., Caffeine interaction with synthetic flavylium salts. A flash photolysis study for the adduct involving 4,7-dihydroxyflavylium, *J. Photochem. Photobiol. A Chem.*, 117, 51, 1998.
157. Bobbio, F.O. et al., Stability and stabilization of the anthocyanin from *Euterpe oleracea* Mart, *Acta Alimentaria*, 31, 371, 2002.
158. Maccarone, E., Maccarrone, A., and Rapisarda, P., Color stabilization of orange fruit juice by tannic acid, *Int. J. Food. Sci. Technol.*, 22, 159, 1987.
159. Bailoni, M.A., Bobbio, P.A., and Bobbio, F.O., Stability of the anthocyanins from *Acalypha hispida* and copigmentation effect, *Acta Alimentaria*, 28, 161, 1999.
160. Wilska-Jeszka, J. and Korzuchowska, A., Anthocyanins and chlorogenic acid copigmentation — Influence on the colour of strawberry and chokeberry juices, *Z. Lebensm. Unters. Forsch.*, 203, 38, 1996.
161. Talcott, S.T., Pelle, J.E., and Brenes, C.H., Red cloves isoflavonoids as anthocyanin color enhancing agents in muscadine wine and juice, *Food Res. Int.*, 38, 1205, 2005.
162. Timberlake, C.F. and Bridle, P., Flavylium salts anthocyanidins and anthocyanins. Structural transformations in acid solutions, *J. Sci. Food Agric.*, 18, 473, 1967.
163. Berké, B. and de Freitas, V.A.P., Influence of procyanidin structures on their ability to complex with oenin, *Food Chem.*, 90, 3, 453, 2005.
164. Dangles, O and Brouillard, R., Polyphenol interactions. The copigmentation case — thermodynamic data from temperature-variation and relaxation kinetics. Medium effect, *Can. J. Chem.-Rev. Can. Chim.*, 70, 2174, 1992.
165. Dueñas, M., Fulcrand, H., and Cheynier, V., Formation of anthocyanin-flavanol adducts in model solutions, *Anal. Chem. Acta*, 563, 15, 2006.
166. Francia-Aricha, E.M. et al., New anthocyanin pigments formed after condensation with flavanols, *J. Agric. Food Chem.*, 45, 2262, 1997.
167. Escribano-Bailón, T. et al., Color and stability of pigments derived from the acetaldehyde-mediated condensation between malvidin 3-O-glucoside and (+)-catechin, *J. Agric. Food Chem.*, 49, 1213, 2001.
168. Pissarra, J. et al., Reaction between malvidin 3-glucoside and (+)-catechin in model solutions containing different aldehydes, *J. Food Sci.*, 68, 476, 2003.
169. Romero, C. and Bakker, J., Interactions between grape anthocyanins and pyruvic acid, with effect of pH and acid concentration on anthocyanin composition and color in model solutions, *J. Agric. Food Chem.*, 47, 3130, 1999.
170. Rein, M.J. et al., Identification of novel pyranoanthocyanins in berry juices, *Eur. Food Res. Technol.*, 220, 239, 2005.
171. Dueñas, M., et al., UV-visible spectroscopic investigation of the 8,8-methylmethine catechin-malvidin-3-glucoside pigments in aqueous solutions: structural transformations and molecular complexations with chlorogenic acid, *J. Agric. Food. Chem.*, 54, 189, 2006.
172. Oliveira, J. et al., Color properties of four cyanidin-pyruvic acid adducts, *J. Agric. Food Chem.*, 54, 6894, 2006.

4.4 Betalains in Food: Occurrence, Stability, and Postharvest Modifications

Florian C. Stintzing and Reinhold Carle

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4.4.1 DEFINITIONS AND STRUCTURES

Betalains are N-heterocyclic water-soluble pigments deposited in vacuoles. Their common precursor is betalamic acid consisting of a 1,7-diazaheptamethine system, an extended π -electron system exhibiting a canary yellow color. Betalamic acid may condense with *cyclo*-dopa to yield betanidin, the common precursor aglycon of the red betacyanins. Betanidin in turn may be glycosylated and/or acylated, yielding 29

genuine structures known to date. Due to stereoisomerism at C₁₅ their number is doubled, except for neobetanin (14,15-dehydrobetanin) which is devoid of the chiral center at C-15.¹

The yellow analogues, the betaxanthins, are composed of betalamic acid with amino acids or amines, respectively, amounting to 26 structures known to occur naturally.²⁻⁴ Structures unambiguously assigned by NMR spectroscopy usually carry trivial names derived from the plant material from which they have been first isolated. The substitution patterns of betacyanins and betaxanthins hitherto reported together with their particular plant sources are listed in Table 4.4.1 and 4.4.2, respectively.

4.4.2 DISTRIBUTION, NATURAL FUNCTIONS, AND ACTIONS

Betalains are known to occur in 13 plant families of the Caryophyllales and have never been found to co-occur with anthocyanins in the same plant.⁴⁶⁻⁴⁸ The presence of betalains in some genera of higher fungi such as *Amanita*, *Hygrocybe*, and *Hygrophorus* represents a paradigm of chemical convergence. While their occurrence is quite limited as compared to the anthocyanins, betalain presence in edible plant material is even more restricted.⁴⁹⁻⁵² The structural diversity of beta-cyanins and betaxanthins found in food plants is given in Figure 4.4.1A and 4.4.1B.

4.4.3 CURRENT AND POTENTIAL FOOD SOURCES

4.4.3.1 AMARANTH

The Amaranthaceae include 65 genera and about 1000 species of annual and perennial plants, shrubs, and trees from tropical, subtropical, and temperate zones of Africa, South America, and south east Asia.⁵³ Traditionally used in folk medicine, some members are used for pigment exploitation.⁵⁴ Consequently, studies of the betalain pattern and the potential for processing amaranth into coloring preparations have been carried out.⁵⁵ Amaranth plants may be subdivided into grain and vegetable or leafy types. They are well known as ornamental plants and recent studies were dedicated to the colored inflorescences of *Celosia* sp.⁵⁶ However, their broader use for food coloring outside their region of origin, where no traditional usage exists, remains doubtful.

4.4.3.2 RED BEET

Starting in the early 1970s, red beets have been applied commercially since the pioneering work by Von Elbe and co-workers.^{57,58} At that time, red beet betalains consisting of betanin, isobetanin, prebetanin, and smaller quantities of vulgaxanthin I and vulgaxanthin II, respectively, were proposed as viable alternatives to synthetic colorants, especially in low-acid foods such as dairy and meat products.^{6,25,42,59-63} In the latter commodities, red beet was even considered as a replacement for nitrite to achieve reddening, but of course it lacks the antimicrobial activity of nitrite. Moreover, red beet addition contributes to higher nitrate levels of the particular food which represents a potential source of nitrite.

TABLE 4.4.1
Substitution Patterns of Genuine Betacyanins of Higher Plants

Betacyanin (Bc)	Substituent	Occurrence	References
5-O-glycosides			
Amaranthin	Glucuronyl-glucose	Amaranthus sp.; Bougainvillea sp.	5–8
Betanin (syn. phytolaccanin)	Glucose	Amaranthus sp.; Beta sp., Hylocereus sp., Opuntia sp., Phytolacca sp., Portulaca sp.	9–13
Bougainvillein r-I	Sophorose	Bougainvillea sp.; Hylocereus sp.;	14–16
		Mesembryanthemum sp.	
Celosianin I	Coumaroyl-glucuronyl-glucose	Celosia sp.	10,11
Celosianin II	Feruloyl-glucuronyl-glucose	Celosia sp.	10,11
2-Decarboxybetanin	Glucose	Beta sp.	17,18
Malonyl-2-Decarboxybetanin	Malonyl-glucose	Schlumbergera sp.	17
Hylocerein	3-Hydroxy-3-methyl-glutaryl-glucose	Hylocereus sp.	15,19
Iresinin I	Glucuronyl-3-hydroxy-3-methyl-glutaryl-glucose	Iresine sp.	10,11,20
Lampranthin I	Coumaroyl-glucose	Lampranthus sp.	21
Lampranthin II	Feruloyl-glucose	Beta sp., Lampranthus sp., Mesembryanthemum sp., Phytolacca sp.	2,12,16,21
Neobetatin (=14,15-Dehydrobetanin)	Glucose	Beta sp., Opuntia sp.	4,22–24
Phyllocactin	Malonyl-glucose	Beta sp., Hylocereus sp., Opuntia sp.	4,14,15,19
Prebetanin	6-Sulfatyl-glucose	Beta sp.; Phytolacca sp.	12,23,25
Riviniain	3-Sulfatyl-glucose	Rivina sp.	26
Phytolacca-Bc ^a	Feruloyl-apiose	Phytolacca sp. Schlumbergera sp.	12,27

Continued.

TABLE 4.4.1 (Continued)
Substitution Patterns of Genuine Betacyanins of Higher Plants

Betacyanin (Bc)	Substituent	Occurrence	References
Schlumbergera-Bc I ^a	Malonyl-apiose	Schlumbergera sp.	27
Schlumbergera-Bc II ^a	Feruloyl-malonyl-apiose	Schlumbergera sp.	27
Mesembryan themum-Bc I ^a	Feruloyl-di-glucose	Mesembryanthemum sp., Beta sp.	4,16
Mesembryan themum-Bc II ^a	Di-feruloyl-di-glucose	Mesembryanthemum sp.	16
Mesembryan themum-Bc III ^a	Tri-feruloyl-di-glucose	Mesembryanthemum sp.	16
6-O-glycosides			
Bougainvillea v	Sophorose	Bougainvillea sp.	5,28
Gomphrenin I	Glucose	Basella sp., Bougainvillea sp., Gomphrena sp., Opuntia sp., Phytolacca sp.	4,5,10-13, 29-31
Gomphrenin II	Coumaroyl-glucose	Gomphrena sp.	10,31
Gomphrenin III	Feruloyl-glucose	Gomphrena sp.	10,31
Bougainvillea-Bc I ^a	6-Caffeoyl-sophoroside	Bougainvillea sp.	5
Bougainvillea-Bc II ^a	6"-Coumaroyl-sophorose	Bougainvillea sp.	5
Bougainvillea-Bc III ^a	6-Coumaroyl-sophorose	Bougainvillea sp.	5
Bougainvillea-Bc IV ^a	2"-sophorosyl-6-caffeoyl-6"-coumaroyl-sophorose	Bougainvillea sp.	5
Bougainvillea-Bc V ^a	2"-glucosyl-6-caffeoyl-6"-coumaroyl-sophoroside	Bougainvillea sp.	5
Bougainvillea-Bc VI ^a	2"-glucosyl-6,6"-di-coumaroyl-sophoroside	Bougainvillea sp.	5
Bougainvillea-Bc VII ^a	6,6"-di-coumaroyl-sophoroside	Bougainvillea sp.	5

^a No trivial name established.

TABLE 4.4.2**Substitution Patterns of Genuine Betaxanthins of Higher Plants**

Betaxanthin (Bx)	Substituent	Occurrence	References
Dopaxanthin	Dopa	Glottiphyllum sp., Lampranthus, sp., Portulaca sp.	32–35
Humilixanthin	5-Hydroxynorvaline	Rivina sp.	36
Indicaxanthin	Proline	Opuntia sp., Beta sp.	2,4,34,37–39
Miraxanthin I	Methionine sulfoxide	Mirabilis sp.	40,41
Miraxanthin II	Aspartic acid	Mirabilis sp.	41,42
Miraxanthin III	Tyramine	Beta sp., Mirabilis sp.	2,41,42
Miraxanthin V	Dopamine	Beta sp., Mirabilis sp.	2,42
Muscaaurin VII	Histidine	Beta sp., Opuntia sp.	2,4,39
Portulacaxanthin I	Hydroxyproline	Portulaca sp.	43
Portulacaxanthin II	Tyrosine	Beta sp., Portulaca sp.	2,44
Portulacaxanthin III	Glycine	Beta sp., Portulaca sp.	2,44
Vulgaxanthin I	Glutamine	Beta sp., Opuntia sp., Portulaca sp.	2,4,23,34, 39,40,45
Vulgaxanthin II	Glutamic acid	Beta sp., Portulaca sp.	2,23,34,35, 40,45
Vulgaxanthin III	Asparagine	Beta sp.	2
Vulgaxanthin IV	Leucine	Beta sp., Opuntia sp.	2,39
Ala-Bx ^a	Alanine	Beta sp.	2
Gaba-Bx ^a	γ -Amino butyric acid	Beta sp., Opuntia sp.	2,39,45
HisA-Bx ^a	Histamine	Beta sp.	2
Ile-Bx ^a	Isoleucine	Beta sp., Opuntia sp.	2,39
Met-Bx ^a	Methionine	Opuntia sp.	4
MeTyr-Bx ^a	3-Methoxytyramine	Beta sp., Celosia sp.	2,13
Phe-Bx ^a	Phenylalanine	Beta sp., Celosia sp., Opuntia sp.	2,39
Ser-Bx ^a	Serine	Beta sp., Opuntia sp.	2,39
Trp-Bx ^a	Tryptophan	Beta sp., Celosia sp.	2,13,39
Val-Bx ^a	Valine	Beta sp., Opuntia sp.	39

^a No trivial name established.

A great number of further investigations addressed betalain stability related to pH, oxygen, light, and temperature.^{64–66} Technological studies were also carried out to establish a production scheme for industrial processing of red beets into juice concentrate. To increase pigment contents in the final product, a fermentation step was proposed to remove sugars, thus achieving a greater color intensity of the resulting 65°Bx concentrate. Moreover, breeding strategies were early considered to be successful for increasing the pigment content of the raw material.

The fortunate constellation of scientists at Wisconsin facilitated further achievements toward increasing pigment content in red beets. In a recurrent selection program, betalain contents of red beet crops were enhanced by 200%.^{67,68} This example should encourage both food scientists and plant breeders to join forces and invest in the improvement of promising color crops.

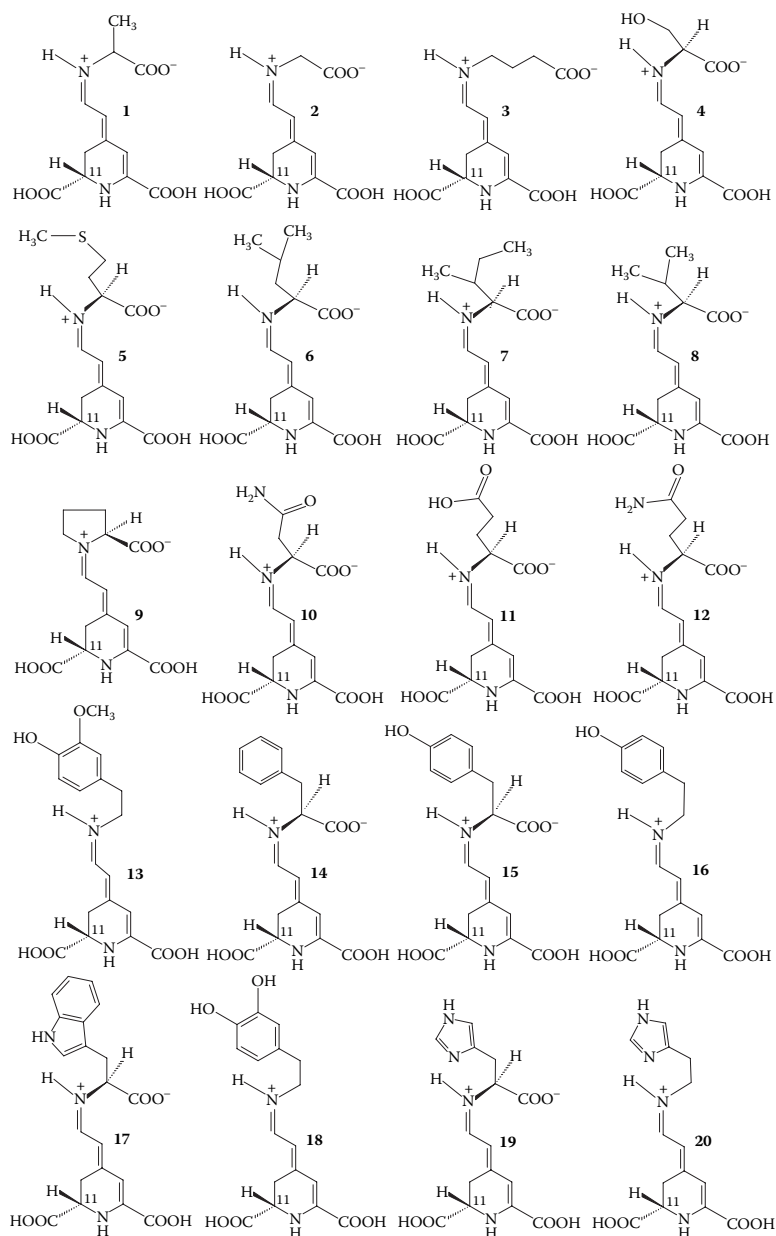


FIGURE 4.4.1A Betaxanthins (bx) from food. 1. Alanine –bx. 2. Glycine bx. 3. γ-aminobutyric acid –bx. 4. Serine –bx. 5. Methionine –bx. 6. Leucine –bx. 7. Isoleucine –bx. 8. Valine –bx. 9. Proline bx. 10. Asparagine bx. 11. Glutamic acid bx. 12. Glutamine bx. 13. Methoxytyramine bx. 14. Phenylalanine –bx. 15. Tyrosine –bx. 16. Tyramine –bx. 17. Tryptophan –bx. 18. Dopamine –bx. 19. Histidine –bx. 20. Histamine –bx.

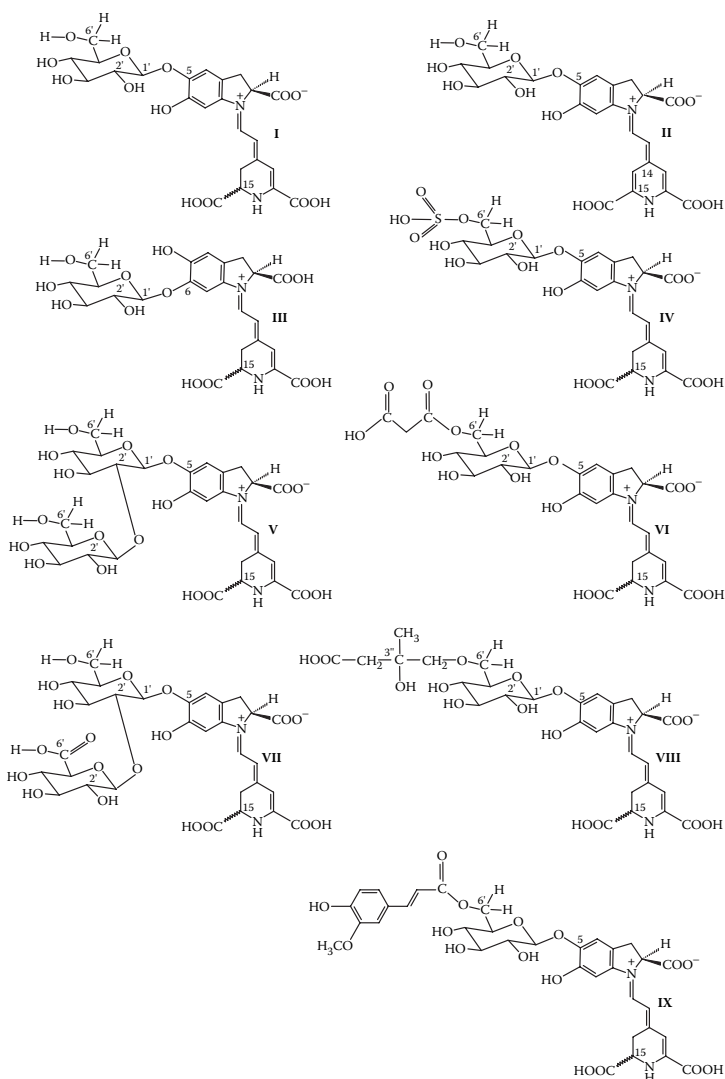


FIGURE 4.4.1B Betacyanins from foods. I. Betanin. II. Neobetanin. III. Gomphrenin I. IV. Prebetanin. V. Bougainvillein r-I. VI. Phyllocactin. VII. Amaranthin. VIII. Hylocerenin. IX. Lampranthin II.

The adverse properties of red beet preparations are without doubt, their high nitrate content, their unpleasant smell due to pyrazine derivatives and geosmin with a low odor threshold, and finally the risk of carry-over of earth-bound germs.^{66,69–74} Since nitrate has been associated with nitrite and nitrosamine formation, the latter exhibiting cancer-inducing properties, procedures have been developed to reduce the nitrate content of red beet preparations.^{75,76} This has been achieved by microbial denitrification.^{77–79}

Geosmin formation has been associated with *Streptomyces* species that generate the smell of wet earth.^{80,81} It has therefore been intriguing to investigate whether red beet alone has the potential to produce geosmin. Using cell cultures, Lu and co-workers unambiguously demonstrated that geosmin formation was an endogenous trait of beetroot.^{82,83} Geosmin removal may be desired and has been achieved by ultrafiltration patented by the Henkel Company.⁸⁴ Due to culture conditions, earth-bound yeasts and Bacillaceae spores attached to the roots may be easily carried over into the refined color preparation, thus representing a serious hazard. Therefore, special requirements apply, i.e., yeast counts must remain below 10/g for dairy products.⁸⁵

4.4.3.3 YELLOW BEET

Although the subjects of early analytical investigations in the late 1970s, yellow beets, also known as golden beets, have not yet been widely introduced on the market as vegetables or as sources of yellow pigments. The pigment profile responsible for the bright yellow tint was first characterised by Strack and Reznik³⁴ and Savolainen and Kuusi⁸⁶ and was found to consist of up to ten constituents, four of which were assigned to vulgaxanthin I, vulgaxanthin II, indicaxanthin, and betalamic acid. A more recent study by Stintzing and co-workers revealed an even more complex pattern.³⁹

Yellow beets may be valuable companions for red beets in establishing color blends, thus allowing a broader color range than obtainable with red beets alone. In addition, food-derived yellow water-soluble pigments are scarce and golden beets may enlarge the candidate list of betalainic food sources. According to our observations, yellow beets have considerable browning potential, which may have discouraged potential producers of food colorants (Stintzing and coworkers, unpublished data). While the same browning potential is also inherent to red beets, the yellow color of the golden variety is overpowered by oxidized phenolics, thus rendering its preparations visibly unattractive. In contrast, red beets exhibit the potential to compensate for this brownish tint by their red-purple pigments.

High levels of polyphenol oxidase activities are held responsible for the undesirable appearance, but till now, the browning potentials of different red and golden beet varieties appeared to be unknown.^{87,88} Because of the apparent need for yellow water-soluble color preparations, our current investigations are aimed at developing a line for processing yellow beets into juice and concentrate. To obtain a product of brilliant yellow appearance, efficient strategies for browning inhibition are elaborated. Together with CIE L*C*h° measurements, HPLC-DAD represents a powerful tool for accompanying and evaluating technological studies. Apart from being used as sources for food coloring uses, yellow beets are delicious vegetables that exhibit less earthy aromas than red beets and add new color to everyday dishes.

4.4.3.4 SWISS CHARD

Swiss chard, also known as foliage beet, silver beet, or perpetual beet, is mostly known as a green-white vegetable; the colored cultivars are less widespread.

Zakharova and co-workers studied a red variety in 1997 to gain a closer insight into its polyphenol oxidase activity.⁸⁹ More recently, a study addressing the antioxidant properties of a red colored Swiss chard was published.⁹⁰ However, the pigments were erroneously addressed as anthocyanins.

Since it was reported that the Bright Lights cultivar presented a number of colors, a thorough investigation was dedicated to the pigment profiles of yellow, orange, red, and violet petioles.^{2,91} Applying a highly powerful HPLC separation, a total of 19 betaxanthins could be characterized, some of which were new structures. The color gamut ranging from yellow to orange to red and violet was found to be due to a fine-tuned blend of betacyanins and betaxanthins. The yellow and the violet petioles exclusively contained yellow and red betalains, respectively, while increasing proportions of betacyanins and constant betaxanthin concentrations were responsible for the orange and red tonalities.²

These principles are most important to obtain tailor-made coloring foodstuffs allowing a larger variety of shades. Whether colored Swiss chard petioles may gain importance for food coloring in addition to their culinary value is doubtful. Due to the low pigment contents of 4–8 mg/100g petiole, exploitation for food coloring purposes does not appear to be economically viable. Reaching a hectare yield of 35 to 40 tons annually, only 50% of the harvested material is colored suitable for extraction. Hence, breeding activities to enhance pigment contents are considered prerequisite to rendering Swiss chard more economically feasible.

4.4.3.5 CACTUS PEAR

Cactus pear, previously known as prickly pear, has been mainly studied for its green fleshed pads known as nopalitos.⁹² It originates from Mexico but is cultivated in Chile and Peru, mainly for cochineal production, and also in the U.S. (California and Texas), South Africa, India, Israel, and the Mediterranean, amounting to a total of 100,000 hectares under cultivation.⁹³ The cactus pear fruit is a berry with many seeds and a mean weight of 160 g. Its fruit pulp covers a color range from deep purple to green.^{94,95}

The overall fruit composition was studied thoroughly starting in the early 1980s, when Askar and El-Samahy reported extraordinarily high amino acid contents.⁹⁶ Later investigations characterized cactus pears as sweet fruits containing fructose and glucose with a high sugar-to-acid ratio and a faint melon–cucumber-like taste.^{94,95} Most interestingly, the presence of high taurine levels in South African cactus pears and lower levels in Italian cultivars were described.^{38,97} Since then, taurine has been mainly believed to be of animal origin. As a result, cactus pears were considered as alternative sources for natural taurine, especially for functional drinks.^{94,98,99}

Proline, however, is the predominant amino acid and most interestingly, its betalamic acid adduct indicaxanthin is the major betalain pigment in cactus pear.¹⁰⁰ Proline functions as an osmolyte, accumulating in water- and heat-stressed plant tissues, and in comparison to other amino acids, it exhibits an extraordinarily high solubility of 1623 mg/L water at 25°C.^{101–108}

Odoux and Domínguez-López were the first to propose cactus pears for food coloring purposes.¹⁰⁹ In-depth research was carried out by Stintzing and co-workers

since 1999.^{94,110,111} Color blends with pitahaya have been systematically investigated and demonstrate the viability of cactus fruits for color preparations.¹¹² Until recently, high pectin content along with the concomitant presence of reducing sugars and high amino acid contents potentially leading to brown Maillard-type products have been the main hurdles related to cactus pear processing. Therefore, a line for the production of cactus pear juice at semi-industrial scale has been established and extended to coloring concentrates and fruit powders from the most common European cactus pear, *Opuntia ficus indica* cv. Gialla.^{113,114} In a parallel investigation, purple cactus pears were used as starting materials for spray-dried powders.¹¹⁵

These studies demonstrate that the processing of cactus pears into coloring preparations is feasible. It is now up to companies to exploit the enormous potential of these fruits. The by-products from *Opuntia* processing such as seeds and peels could be further processed into vegetable oil and pectin, respectively.

4.4.3.6 PITAHAYA

Originating from tropical regions of North and South America, pitahayas are now grown in Guatemala, Nicaragua, Vietnam, Taiwan, Thailand, Indonesia, Israel, the U.S. and Australia. Their fruits are oblongate, 8 to 10 cm long, with a mean weight of 350 to 450 g. The seeds are small and digestible, comparable to those from kiwi.¹¹⁶ In the strictest sense, pitayas originate from columnar cacti belonging to the *Cereus* and *Stenocereus* subfamilies. On the other hand, pitahayas are the fruits from the *Hylocereus* and *Selenicereus* vine cacti.

Pitahayas, usually called pitayas, comprise all fruits belonging to the *Hylocereus* and *Selenicereus* species. While the latter produce white-fleshed fruits such as *Selenicereus megalanthus*, fruits derived from *Hylocereus* sp. may exhibit a white, red, or even red-purple fruit pulp. In parallel studies, the pigments responsible for the red-purple color of *H. polyrhizus* were identified as betanin, phyllocactin, and the 3-hydroxy-3-methyl-glutaric acid adduct of betanin, later named hylocerenin.¹⁵⁻¹⁹ Various clones have been investigated with respect to their pigment profiles exhibiting varying patterns among clones.¹¹⁷

4.4.4 STABILITY AND POSTHARVEST MODIFICATIONS

4.4.4.1 PARAMETERS AFFECTING STABILITY

Since several previous reviews have treated betalain stability, the interested reader is referred to those for more detailed information.^{64-66,91,111,118} This chapter is intended to present some general aspects of handling betalainic foodstuffs.

As soon as betalains are extracted from the vacuoles through plant tissue compartmentalization, they are prone to degradation. Factors supporting stabilization or rather destabilization are summarized in Figure 4.4.2.

Two main groups can be discerned (1) endogenous factors such as plant enzymes like polyphenoloxidase, peroxidase, and β -glucosidase and (2) conditions prevailing in the extraction medium will decide the fates of betalain pigments among which temperature, oxygen, and pH are considered the most important conditions.

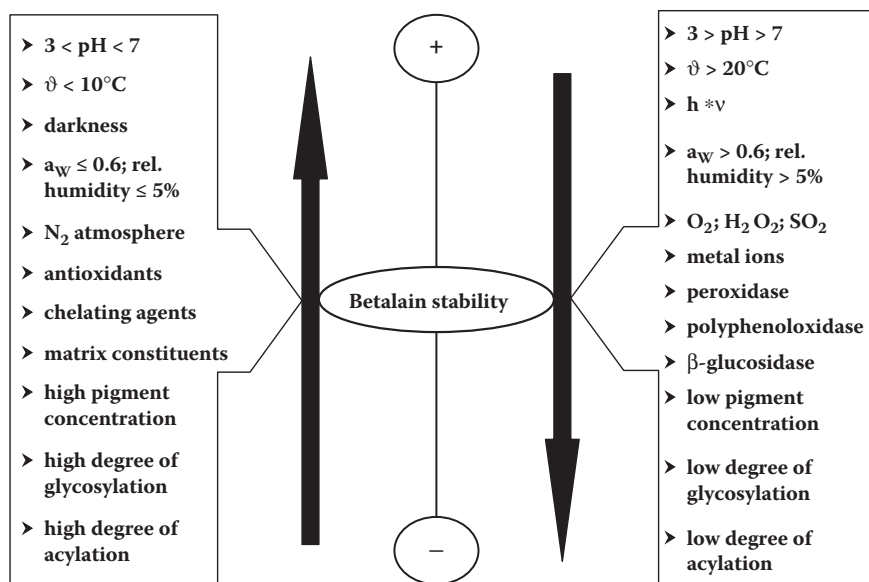


FIGURE 4.4.2 Factors influencing betalain stability.

pH value — It is generally accepted that betalains are most stable in a broad pH range from 3 to 7.¹¹⁹ In contrast to the more common anthocyanins, they exhibit unchanged tint and retain their coloring power.^{111,120} The pH optima of betalains only differ slightly. Interestingly, color retention is optimum at the pH of the respective plant source, i.e., red beet betalains are most stable at the pH of red beet around 5.5 to 6.0.^{61,121–24} Shifting the pH to values below 3 will result in color loss, although the exact mechanisms have not yet been elucidated.¹¹⁸ In alkaline media, the betalains will be cleaved into their biosynthetic precursors, betalamic acid and *cyclo*-dopa (betacyanins) or amino compounds (betaxanthins), all of which may further degrade, thus ultimately leading to color fading.^{66,91,111} As a rule, betacyanins are considered more resistant to acidic media, while betaxanthins are most stable at neutral pH. These characteristics may change according to their respective chemical structures. In general, betalains are most suited for low-acid foods such as dairy and meat products as well as ice cream.

a_w value — As with other natural colorants, a greater water activity is detrimental to color stability through cleavage as is the case in alkaline solutions. Along with greater water content, both the mobility of food components and oxygen solubility will be increased, adding up to betalain destabilization.¹²⁵ As a result, betalains may be applied successfully to fruit fillings, creams, instant products such as soups, and confectionary items.^{64,66,126} On the other hand, color preparations are usually spray-dried or concentrated to at least 65°Bx to secure maximum stability during storage and transportation.^{127–129}

Endogenous and exogenous enzymes — While the role of β -glucosidase affecting betalains is quite clear, the roles of polyphenoloxidase and peroxidase are less obvious. Betacyanin glycosides may be cleaved into the corresponding aglycones, the

latter being highly unstable and oxidized further. On the other hand, acylated betacyanins will not be affected and remain stable toward both endogenous glucosidase activity and side activities that may stem from pectinolytic preparations during mash enzymation.^{15,20,29} Microorganisms intentionally applied for denitrification and also for sugar removal prior to concentration or lactic acid fermentation may be the third source of glycoside cleaving activities.^{76–79,129–133} Polyphenoloxidases and peroxidases released from the comminuted plant tissue will be inactivated only by heat.⁸⁷ Hence, the period between mashing and heating of the filtered juice should be minimized. While peroxidases affect betalain stability by radical formation with concomitant pigment fading, polyphenoloxidases chiefly oxidize betalain aglycones and accompanying colorless phenolics, thereby unfavorably affecting color. Finally, acidification presents a feasible tool for reducing the activities of endogenous enzymes.^{134–136}

Metal ions — Several investigations have proven that Sn^{2+} , Al^{3+} , Ni^{2+} , Cr^{2+} , Fe^{2+} , Fe^{3+} , and Cu^{2+} accelerate betalain color loss, the latter two being the most deleterious.^{137–139} Metal ions may originate from the crop, from soil accompanying the crop, and from the processing machinery. The first source can hardly be influenced, but earth-borne metal contamination can be minimized by extensive washing. The potential contamination should not pose a problem when suitable processing equipment is used. To reduce oxidative damage by metal ions, the use of citric acid is recommended for acidification and as a chelating agent in the manufacture of pigment preparations.¹⁴⁰

Oxygen — Oxygen is a substrate for all of the above-mentioned oxidative enzymes; it catalyzes metal-induced oxidation and also directly affects betalain stability, especially beyond their optimum pH levels.^{121,141} Nitrogen flushing may be recommended, but will rarely be applied under normal processing conditions. Although the oxygen solubility in water is improved when water temperature is lowered, oxidative events are assumed to proceed more slowly in cool environments, thus improving overall pigment retention.

Light — In plant tissues, betalains function as UV-screens, readily absorbing light energy.^{16,142} Hence, as is true for all natural pigments, betalains will degrade when exposed to light, especially in the presence of oxygen and at pH deviating from the stability optimum.^{58,143,144} Generally, processing and storage should be carried out in the absence of deleterious light exposure. Consequently, products that are sold in transparent packaging should be subjected to extensive testing under real illumination conditions. It should also be considered that light exposure will heat the food products, thus aggravating color loss. Interestingly, the effect of light is only measurable below 25°C; above that it is overpowered by thermal damage.¹⁴³

Temperature — Temperature is the most critical factor related to betalain stability.^{64,65,91,118,144} However, thermal processing is required to inactivate enzymes and prevent microbial spoilage. To avoid the heat burden of sterilization conditions for preparations with pH values above 4.3 such as red beet or cactus pear, acidification to pH 4 by citric acid addition is advantageous. Thus, pasteurization below 100°C is sufficient to achieve microbial stability. However, since betalain degradation is enforced from at temperatures higher than 30°C, color losses during processing are inevitable. In conclusion, strategies for maximizing pigment retention are complex and need to be scrutinized to assure optimum betalain protection and color yield.

4.4.4.2 MODEL FOOD SYSTEMS

Most published data deals with model solutions to assess the major factors influencing betalain stability, among which pH and temperature are most frequently addressed. Until recently, total color loss was assessed by spectrophotometric monitoring of the decline at the wavelength of maximum absorption. To predict color fading over time, kinetic data were derived therefrom, most often obeying first-order decay principles.

Only Schwartz and Von Elbe attempted to investigate the degradation products related to thermal treatment and found 15-decarboxylated betanin in addition to betalamic acid and *cyclo*-dopa-glucoside.¹⁴⁵ These findings were scrutinized recently when red beet and purple pitahaya juice were exposed to heat in a varying range of holding times and produced a great number of different dehydrogenated and decarboxylated products.^{146,147} Subsequently, studies of both aqueous and ethanolic solutions yielding similar degradation products were conducted.^{148,149} Very recently, the structural characterizations of the novel structures and color alterations thereby induced were investigated in depth.^{146,147,150,151} While decarboxylation of betacyanins at C₁₅ and C₂ did not affect visual appearance, 17-decarboxy structures exhibited an orange hue.^{8,151}

In addition, dehydrogenated structures were found to cause a hypsochromic shift.^{146,147,151} Since, with the exception of neobetanin, these betacyanin degradation products were found not to be native to betalain crops, it is suggested that these peculiar structures may be used as heat indicators to assess the thermal loads of the respective products have undergone during production.

4.4.4.3 REAL FOOD SYSTEMS

Although a protective matrix effect has been assumed to be responsible for improved betalain stability, investigations on the tinctorial stability of foods colored with betalain preparations are scarce.^{123,153,154} Hávílková et al. performed a 10-day stability study of soft drinks at a betalain concentration of 2.5 g/100 g at pH 3.¹⁵⁴ The addition of 50 mg ascorbic acid/100 g beverage solution was shown to improve overall color stability. Temperatures below 5°C were recommended for optimum pigment retention. Cai and Corke applied amaranth pigments to jelly, ice cream, and model beverages at different concentrations over a period of 20 weeks.¹⁵⁵ While stability was reasonable for ice creams at -18°C and jellies at 4 and 14°C, respectively, the latter faded at 25°C, and especially at 37°C. In consideration of the little data available, more extended studies are warranted for assessing betacyanin and betaxanthin retention and considering different matrices such as foods rich in starch, protein, or pectin to target novel betalain applications for food coloring.

4.4.5 CURRENT AND FUTURE PERSPECTIVES

4.4.5.1 BETALAINIC CROPS

Betalains have recently regained importance due to continuing interest in natural food colorants. Currently, red beet is the only food source commercially exploited, although amaranth, Swiss chard, yellow beet, and cactus fruit represent promising

future color crops. The processing of cactus fruit into colored preparations bears good prospects because (1) schemes for processing cactus fruits into juices and concentrates are established and ready for industrial scale-up, (2) *Opuntia* plants exhibit great genetic potential to be exploited for increasing pigment yield without the necessity of GMO plants but rather by appropriate selection and breeding strategies, and (3) *Opuntia* is an ecologically viable crop showing an excellent climatic adaptability and high water-use efficiency, making it a suitable crop for cultivation in both arid and semi-arid regions.^{94,114–116,156–159}

Enlarging the current acreages and thereby reducing the costs of the fruit is considered the last hurdle for cactus exploitation on an industrial scale. Due to dropping market prices for cochineal, the red anthraquinone pigment from female lice settling on cactus pear pads, plantations that are no longer needed for cochineal harvest could be converted into fruit production. On the other hand, screening of cactus fruits for their coloring potential is still very rare and requires intensification. Joint research activities of food scientists and agriculturists to extend our knowledge about cactus therefore appears of utmost importance.

4.4.5.2 TECHNOLOGICAL ASPECTS

From a technological view, measures to stabilize betalains during the production process need to be elaborated. Some approaches have been described in the earlier literature, mainly for red beets and only recently for alternative crops such as cactus pears and red-violet pitahayas.^{86,114,115,122,138,139,144, 160,161} Despite being indispensable for practical application, systematic studies on the stability of betalain preparations in real food systems during storage are lacking.

4.4.5.3 NUTRITIONAL ASPECTS

Besides their interesting color application values, betalainic plants are also worthwhile from a nutritional standpoint. Research on this topic has recently been resumed with great scientific vigor in both *in vitro* and *in vivo* studies on red beets, amaranth, red-colored Swiss chard, red-violet pitahayas, and especially cactus pears.^{90,92,97,162–182} In the future, betalainic color crops will be benchmarked because of their pigment structure and quantity and also because of the individual and synergistic activities of their components such as colorless phenolics, amino compounds, peptides, proteins, and hydrocolloids.

REFERENCES

1. Strack, D., Vogt, T., and Schliemann, W., Recent advances in betalain research, *Phytochemistry*, 62, 247, 2003.
2. Kugler, F., Stintzing, F.C., and Carle, R., Identification of betalains from petioles of differently colored Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. cv. 'Bright Lights') by high-performance liquid chromatography–electrospray ionization mass spectrometry, *J. Agric. Food Chem.*, 52, 2975, 2004.

3. Steglich, W. and Strack, D. *Betalains*, in *The Alkaloids: Chemistry and Pharmacology*, Brossi, A., Ed., Academic Press, San Diego, 39, 1990.
4. Stintzing, F.C. et al., Color, betalain pattern and antioxidant properties of cactus pear (*Opuntia* sp.) clones, *J. Agric. Food Chem.*, 53, 442, 2005.
5. Heuer, S. et al., Betacyanins from bracts of *Bougainvillea glabra*, *Phytochemistry*, 37, 761, 1994.
6. Piattelli, M. and Minale, L., Pigments of Centrospermae. II. Distribution of betacyanins, *Phytochemistry*, 3, 547, 1994.
7. Huang, A.S. and Von Elbe, J.H., Stability comparison of two betacyanine pigments: amaranthine and betanine, *J. Food Sci.*, 51, 670, 1986.
8. Stintzing, F.C. et al., Betacyanins and phenolic compounds from *Amaranthus spinosus* L. and *Boerhavia erecta* L., *Ztschr. Naturforsch. C/J. Biosci.*, 59, 1, 2004.
9. Adachi, T. and Nakatsukasa, M., High-performance liquid chromatographic separation of betalains and their distribution in *Portulaca grandiflora* and related species, *Ztschr. Pflanzenphysiol.*, 109, 155, 1983.
10. Cai, Y., Sun, M., and Corke, H., Identification and distribution of simple and acylated betacyanins in the Amaranthaceae, *J. Agric. Food Chem.*, 49, 1971, 2001.
11. Piattelli, M. and Minale, L., Pigments of Centrospermae. I. Betacyanins from *Phyllocactus hybridus* Hort. and *Opuntia ficus-indica* Mill., *Phytochemistry*, 3, 307, 1964.
12. Schliemann, W. et al., Betacyanins from plants and cell cultures of *Phytolacca americana*, *Phytochemistry*, 42, 1039, 1996.
13. Schliemann, W. et al., Betalains of *Celosia argentea*, *Phytochemistry* 58, 159, 2001.
14. Piattelli, M. and Imperato, F., Betacyanins from *Bougainvillea*, *Phytochemistry*, 9, 455, 1970.
15. Stintzing, F.C., Schieber, A., and Carle, R., Betacyanins in fruits from red-purple pitaya, *Hylocereus polyrhizus* (Weber) Britton & Rose, *Food Chem.*, 77, 101, 517, 2002.
16. Vogt, T. et al., Light-induced betacyanin and flavonol accumulation in bladder cells of *Mesembryanthemum crystallinum*, *Phytochemistry*, 52, 583, 1999.
17. Kobayashi, N. et al., Formation and occurrence of dopamine-derived betacyanins, *Phytochemistry*, 56, 429, 2001.
18. Schliemann, W., Kobayashi, N., and Strack, D., The decisive step in betaxanthin biosynthesis is a spontaneous reaction, *Plant Physiol.*, 119, 1217, 1999.
19. Wybraniec, S. et al., Betacyanins from vine cactus *Hylocereus polyrhizus*, *Phytochemistry*, 58, 1209, 2001.
20. Minale, L. et al., Pigments of Centrospermae VI. Acylated betacyanins, *Phytochemistry*, 5, 1037, 1966.
21. Piattelli, M. and Impellizzeri, G., Betacyanins from *Lampranthus* sp. (Aizoaceae), *Phytochemistry*, 8, 1595, 1969.
22. Alard, D. et al., Neobetainin: Isolation and identification from *Beta vulgaris*, *Phytochemistry*, 24, 2383, 1985.
23. Kujala, T., Loponen, J., and Pihlaja, K., Betalains and phenolics in red beetroot (*Beta vulgaris*) peel extracts: extraction and characterisation. *Ztschr. Naturforsch. C/J. Biosci.*, 56, 343, 2001.
24. Strack, D., Engel, U., and Wray, V., Neobetainin: a new natural plant constituent, *Phytochemistry*, 26, 2399, 1987.
25. Wyler, H. et al., Präbetainin, ein Schwefelsäurehalbester des Betanins. Ein Beitrag zur Kenntnis der Betacyane, *Helv. Chim. Acta*, 50, 545, 1967.
26. Imperato, F., Betain 3-sulphate from *Rivina humilis*, *Phytochemistry*, 14, 2526, 1975.

27. Kobayashi, N. et al., Betalains from Christmas cactus, *Phytochemistry*, 54, 419, 2000.
28. Piattelli, M. and Imperato, F., Pigments of *Bougainvillea glabra*, *Phytochemistry*, 9, 2557, 1970.
29. Minale L, Piattelli M., and de Stefano S., Pigments of Centrospermae VII. Betacyanins from *Gomphrena globosa* L., *Phytochemistry*, 6, 703, 1967.
30. Heuer, S. et al., Betacyanins from flowers of *Gomphrena globosa*, *Phytochemistry*, 31, 1801, 1992.
31. Glässgen, W.E. et al., Betacyanins from fruits of *Basella rubra*, *Phytochemistry*, 33, 1525, 1993.
32. Impellizzeri, G., Piattelli, M., and Sciuto, S., A new betaxanthin from *Glottiphyllum longum*, *Phytochemistry*, 12, 2293, 1973.
33. Strack, D., Engel, U., and Reznik, H., High performance liquid chromatography of betalains and its application to pigment analysis in Aizoaceae und Cactaceae, *Ztschr. Pflanzenphysiol.*, 101, 215, 1981.
34. Strack, D. and Reznik, H., High-performance liquid chromatographic analysis of betaxanthins in Centrospermae (Caryophyllales), *Ztschr. Pflanzenphysiol.*, 94, 163, 1979.
35. Gandía-Herrero, F., Escribano, J., and García-Carmona, F., Betaxanthins as pigments responsible for visible fluorescence in flowers, *Planta*, 222, 586, 2005.
36. Strack, D. et al., Humilixanthin, a new betaxanthin from *Rivina humilis*, *Phytochemistry*, 26, 2285, 1987.
37. Piattelli, M., Minale, L., and Prota, G., Isolation, structure and absolute configuration of indicaxanthin, *Tetrahedron*, 20, 2325, 1964.
38. Stintzing, F.C., Schieber, A., and Carle, R., Amino acid composition and betaxanthin formation in fruits from *Opuntia ficus-indica*, *Planta Med.*, 65, 632, 1999.
39. Stintzing, F.C., Schieber, A., and Carle, R., Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear (*Opuntia ficus-indica* (L.) Mill.) by high-performance liquid chromatography-electrospray ionization mass spectrometry, *J. Agric. Food Chem.*, 50, 2302, 2002.
40. Piattelli, M., Minale, L., and Nicolaus, R.A., Pigments of Centrospermae. V. Betaxanthins from *Mirabilis jalapa* L., *Phytochemistry*, 4, 817, 1965.
41. Gandía-Herrero, F., Carcía-Carmona, F., and Escribano, J., Fluorescent pigments: new perspectives in betalain research and applications, *Food Res. Int.*, 38, 879, 2005.
42. Piattelli, M., Minale, L., and Prota, G., Pigments of Centrospermae. III. Betaxanthins from *Beta vulgaris* L., *Phytochemistry*, 4, 121, 1965.
43. Piattelli, M., Minale, L., and Nicolaus, R.A., Ulteriori ricerche sulla betaxantine, *Rend. Accad. Sci. Fis. Mat.*, 32, 55, 1965.
44. Trezzini, G.F. and Zryd, J.P., Two betalains from *Portulaca grandiflora*, *Phytochemistry*, 30, 1897, 1991.
45. Böhm, H. and Mäck, G., Betaxanthin formation and free amino acids in hairy roots of *Beta vulgaris* var. *lutea* depending on nutrient medium and glutamate or glutamine feeding, *Phytochemistry*, 65, 1361, 2004.
46. Clement, J.S. and Mabry, T.J., Pigment evolution in the Caryophyllales: a systematic overview, *Bot. Acta*, 109, 360, 1996.
47. Stafford, H.A., Anthocyanins and betalains: evolution of the mutually exclusive pathways, *Plant Sci.*, 101, 91, 1994.
48. Zryd, J.P. and Christinet, L., Betalains, in *Plant Pigments and Their Manipulation*, Davies, K., Ed., Annual Plant Reviews 14, CRC Press/Blackwell, Oxford, 2004, 185.
49. Gill, M. and Steglich, W., Pigments of fungi (Macromycetes), *Progr. Chem. Organic Natural Products*, 51, 1, 1987.
50. Gill, M., Pigments of fungi (Macromycetes), *Nat. Prod. Rep.*, 11, 67, 1994.

51. Musso, H., The pigments of fly agaric, *Amanita muscaria*, *Tetrahedron*, 35, 2843, 1979.
52. Michelot, D. and Melendez-Howell, L.M., *Amanita muscaria*: chemistry, biology, toxicology, and ethnomycology, *Mycol. Res.*, 107, 131, 2003.
53. Siqueira, J.C., Phytogeography of Brazilian Amaranthaceae, *Pesquisa Bot.*, 10, 5, 1994–1995.
54. Teutonico, R.A. and Knorr, D., Amaranth: composition, properties, and applications of a rediscovered food crop, *Food Technol.*, 39, 49, 1985.
55. Cai, Y.Z., Sun, M., and Corke, H., Characterization and application of betalain pigments from plants of the Amaranthaceae, *Trends Food Sci. Technol.*, 16, 370, 2005.
56. Cai, Y. et al., Chemical stability and colorant properties of betaxanthin pigments from *Celosia argentea*, *J. Agric. Food Chem.*, 49, 4429, 2001.
57. Von Elbe, J.H. et al., Quantitative analysis of betacyanins in red table beets (*Beta vulgaris*), *J. Food Sci.*, 37, 932, 1972.
58. Von Elbe, J.H. and Maing, I.Y., Betalains as possible food colorants of meat substitutes, *Cereal Sci. Today*, 18, 263, 1973.
59. Pasch, J.H. and Von Elbe, J.H., Red and yellow pigments from betalaines hold promise as substitutes for colors banned by the FDA, *Candy Snack Ind.*, 142, 32, 1977.
60. Pasch, J.H., Von Elbe, J.H., and Sell, R.J., Betalains as colorants in dairy products, *J. Milk Food Technol.*, 38, 25, 1975.
61. Von Elbe, J.H. et al., Evaluation of betalain pigments as sausage colorants, *J. Food Sci.*, 39, 128, 1974.
62. Vareltsis, K., Buck, E.M., and Labbe, R.G., Effectiveness of a betalains/potassium sorbate system versus sodium nitrite for color development and control of total aerobes, *Clostridium perfringens* and *Clostridium sporogenes* in chicken frankfurters, *J. Food Protect.*, 47, 532, 1984.
63. Vareltsis, K.P. and Buck, E.M., Color stability and sensory attributes of chicken frankfurters made with betalains and potassium sorbate versus sodium nitrite, *J. Food Protect.*, 47, 41, 1984.
64. Delgado-Vargas, F., Jiménez, A.R., and Paredes-López, O., Natural pigments: carotenoids, anthocyanins and betalains: characteristics, biosynthesis, processing, and stability, *Crit. Rev. Food Sci. Nutr.*, 40, 173, 2000.
65. Herbach, K.M., Stintzing, F.C., and Carle, R., Betalain stability and degradation: structural and chromatic aspects, *J. Food Sci.*, 71, R41, 2006.
66. Stintzing, F.C., Schieber, A., and Carle, R., Rote Bete als färbendes Lebensmittel — eine Bestandsaufnahme, *Obst Gem Kartoffelver./Fruit Vegetable Potato Process.*, 85, 196, 2000.
67. Gaertner, V. and Goldman, I.L., Pigment distribution and total dissolved solids of selected cycles of table beet from a recurrent selection program for increased pigment, *J. Am. Soc. Hort. Sci.*, 130, 424, 2005.
68. Goldman, I.L. and Navazio, J.P., History and breeding of table beet in the United States. *Plant Breed. Rev.*, 22, 357, 2003.
69. Acree, T.E. et al., Geosmin, the earthy component of table beet odor, *J. Agric. Food Chem.*, 24, 430, 1976.
70. Murray, K.E. and Whitfield, F.B., The occurrence of 3-alkyl-2-methoxypyrazines in raw vegetables, *J. Sci. Food Agric.*, 26, 973, 1975.
71. Parliment, T.H., Kolor, M.G., and Maing, I.Y., Identification of the major volatile components of cooked beets, *J. Food Sci.*, 42, 1592, 1977.
72. Pasch, J.H. and Von Elbe, J.H., Sensory evaluation of betanine and concentrated beet juice, *J. Food Sci.*, 43, 1624, 1978.

73. Tyler, L.D., Acree, T.E., and Smith, N.L., Sensory evaluation of geosmin in juice made from cooked beets, *J. Food Sci.*, 44, 79-81, 1979.
74. Tyler, L.D. et al., Effect of maturity, cultivar, field history, and the operations of peeling and coring on the geosmin content of *Beta vulgaris*, *J. Agric. Food Chem.*, 26, 1466, 1978.
75. Santamaria, P., Nitrate in vegetables: toxicity, content, intake and EC regulation, *J. Sci. Food Agric.*, 86, 10, 2006.
76. Kolb, E. et al., Potential nitrosamine formation and its prevention during biological denitrification of red beet juice, *Food Chem. Toxicol.*, 35, 219, 1997.
77. Czapski, J., Maksymiuk, M., and Grajek, W., Analysis of biodenitrification conditions of red beet juice using the response surface method, *J. Agric. Food Chem.*, 46, 4702, 1998.
78. Grajek, W.H. and Walkowiak-Tomczak, D., Factors influencing the denitrification rate of red beet juice by the bacteria *Paracoccus denitrificans*, *J. Agric. Food Chem.*, 45, 1963, 1997.
79. Walkowiak-Tomczak, D., Microbiological denitrification of red beet juice. *Eur. Food Res. Technol.*, 215, 401, 2002.
80. Bentley, R. and Meganathan, R., Geosmin and methylisoborneol biosynthesis in Streptomycetes. *FEBS Lett.*, 25, 220, 1981.
81. Dionigi, C.P. et al., Spore and geosmin production by *Streptomyces tendae* on several media, *J. Agric. Food Chem.*, 40, 122, 1992.
82. Lu, G. et al., Quantitative determination of geosmin in red beets (*Beta vulgaris* L.) using headspace solid-phase microextraction, *J. Agric. Food Chem.*, 51, 1021, 2003.
83. Lu, G. et al., Biosynthetic origin of geosmin in red beets (*Beta vulgaris* L.), *J. Agric. Food Chem.*, 51, 1026, 2003.
84. Behr, N., Göbel, G., and Pfeiffer, H., Patentanmeldung der Fa. Henkel KGaA, Düsseldorf. Herstellung eines Rote-Beete-Saftkonzentrats mit besserer Geschmacksneutralität und Haltbarkeit, *Ind. Obst Gem. Verwert.*, 59, 238, 1984.
85. Henry, B.S., Natural food colours, in *Natural Food Colorants*, 2nd ed., Hendry, G.F. and Houghton, J.D., Eds., Blackie, London, 1996, 40.
86. Savolainen, K. and Kuusi, T., The stability properties of golden beet and red beet pigments: influence of pH, temperature, and some stabilizers, *Ztschr. Lebensm. Unters. Forsch.*, 166, 19, 1978.
87. Im, J.S., Parkin, K.L., and Von Elbe, J.H., Endogenous polyphenoloxidase activity associated with the "black ring" defect in canned beet (*Beta vulgaris* L.) root slices, *J. Food Sci.*, 55, 1042, 1990.
88. Parkin, K.L. and Im, J.S., Chemical and physical changes in beet (*Beta vulgaris* L.) root tissue during simulated processing: relevance to the "black ring" defect in canned beets, *J. Food Sci.*, 55, 1039, 1053, 1990.
89. Zakharova, N.S. and Petrova, T.A., Investigation of betalains and betalain oxidase of leaf beet, *Appl. Biochem. Microbiol.*, 33, 539, 1997.
90. Pyo, Y.H. et al., Antioxidant and phenolic compounds of Swiss chard (*Beta vulgaris* subspecies *cycla*) extracts, *Food Chem.*, 85, 19, 2004.
91. Von Elbe, J.H., and Goldman, I.L., The betalains, in *Natural Food Colorants*, Lauro, G.J. and Francis, F.J., Eds., Marcel Dekker, New York, 2000, 11.
92. Stintzing, F.C. and Carle, R., Cactus stems (*Opuntia* spp.): a review on their chemistry, technology and uses, *Mol. Nutr. Food Res.*, 49, 175, 2005.
93. Inglese, P., Basile, F., and Schirra, M., Cactus for fruit production, in *Cacti: Biology and Uses*, Nobel, P.S., Ed., University of California Press, Berkeley, 2002, 163.

94. Stintzing, F.C., Schieber, A., and Carle, R., Phytochemical and nutritional significance of cactus pear, *Eur. Food Res. Technol.*, 212, 396, 2001.
95. Piga, A., Cactus pear: a fruit of nutraceutical and functional importance, *J. Profess. Assoc. Cactus Develop.*, 6, 9, 2004.
96. Askar, A. and El-Samahy, S.K., Chemical composition of prickly pear fruits, *Dtsch. Lebensm Rdsch.*, 77, 279, 1981.
97. Tesoriere, L. et al., Biothiols, taurine, and lipid-soluble antioxidants in the edible pulp of Sicilian cactus pear (*Opuntia ficus-indica*) fruits and changes of bioactive juice components upon industrial processing, *J. Agric. Food Chem.*, 53, 7851, 2005.
98. Reyner, L.A. and Horne, J.A., Efficacy of a "functional energy drink" in counteracting driver sleepiness, *Physiol. Behavior*, 75, 331, 2002.
99. Seidl, R. et al., A taurine and caffeine-containing drink stimulates cognitive performance and well-being, *Amino Acids*, 19, 635, 2000.
100. Kugler, F. et al., Determination of free amino compounds in betalainic fruits and vegetables by gas chromatography with flame ionization and mass spectrometric detection, *J. Agric. Food Chem.*, 54, 4311, 2006.
101. Claussen, W., Proline as a measure of stress in tomato plants, *Plant Sci.*, 168, 241, 2005.
102. Delauney, A.J. and Verma, D.P.S., Proline biosynthesis and osmoregulation in plants, *Plant J.*, 4, 215, 1993.
103. Hare, P.D., Cress, W.A., and Van Staden, J., Proline synthesis and degradation: a model system for elucidating stress-related signal transduction, *J. Exp. Bot.*, 50, 413, 1999.
104. Heuer, B., Osmoregulatory role of proline in plants exposed to environmental stresses, in *Handbook of Plant and Crop Stress*, Pessarakli, M., Ed., Marcel Dekker, New York, 1999, 677.
105. Miller, G. et al., Responsive modes of *Medicago sativa* proline dehydrogenase genes during salt stress and recovery dictate free proline accumulation, *Planta*, 222, 70, 2005.
106. Ruiz, J.M. et al., Proline metabolism and NAD kinase activity in green bean plants subjected to cold shock, *Phytochemistry*, 59, 473, 2002.
107. Schobert, B. and Tschesche, H., Unusual solution properties of proline and its interaction with proteins, *Biochem. Biophys. Acta*, 541, 270, 1978.
108. *The Merck Index*, 13th ed., Merck & Co., Inc., Whitehouse Station, NJ, 2001, 7876.
109. Odoux, E. and Domínguez-López, A., Le figuier de Barbarie: Une source industrielle de bétalaines? *Fruits*, 51, 61, 1996.
110. Stintzing, F.C., Schieber, A., and Carle, R., Cactus pear: a promising component to functional food, *Obst Gem. Kartoffelver./Fruit Vegetable Potato Process.*, 85, 40, 2000.
111. Stintzing, F.C. and Carle, R., Functional properties of anthocyanins and betalains in plants, food, and in human nutrition, *Trends Food Sci. Technol.*, 15, 19, 2004.
112. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Colour studies on fruit juice blends from *Opuntia* and *Hylocereus* cacti and betalain-containing model solutions derived therefrom, *Food Res. Int.*, 38, 975, 2005.
113. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Development of a process for the production of a betalain-based colouring foodstuff from cactus pear, *Innov. Food Sci. Emerg. Technol.*, 6, 221, 2005.
114. Mosshammer, M.R., Stintzing, F.C. and Carle, R., Evaluation of different methods for the production of juice concentrates and fruit powders from cactus pear, *Innov. Food Sci. Emerg. Technol.*, 7, 275, 2006.

115. Rodríguez-Hernández, G.R. et al., Spray-drying of cactus pear juice (*Opuntia streptacantha*): effect on the physicochemical properties of powder and reconstituted product, *Drying Technol.*, 23, 955, 2005.
116. Mizrahi, Y., Nerd, A., and Sitrit, Y., New fruits for arid climates, in *Trends in New Crops and New Uses*, Janick, J. and Whipkey, A., Eds., ASHS Press, Alexandria, VA, 2002, 378.
117. Wybraniec, S. and Mizrahi, Y., Fruit flesh betacyanin pigments in *Hylocereus* cacti, *J. Agric. Food Chem.*, 50, 6086, 2002.
118. Jackman, R.L. and Smith, J.L., Anthocyanins and betalains, in *Natural Food Colorants*, 2nd ed., Hendry, G.F. and Houghton, J.D., Eds., Blackie, London, 1996, 245.
119. Von Elbe, J.H., Stability of betalains as food colors, *Food Technol.*, 29, 42, 1975.
120. Wrolstad, R.E., Anthocyanins, in *Natural Food Colorants*, Lauro, G.J. and Francis, F.J., Eds., Marcel Dekker, Basel, 2000, 237.
121. Czapski, J., The effect of heating conditions on losses and regeneration of betacyanins, *Ztschr. Lebensm. Unters. Forsch.*, 180, 21, 1985.
122. Huang, A.S. and Von Elbe, J.H., Effect of pH on the degradation and regeneration of betanin. *J. Food Sci.*, 52, 1689, 1987.
123. Pátkai, G. and Barta, J., Decomposition of betacyanins and betaxanthins by heat and pH changes, *Nahrung*, 40, 267, 1996.
124. Savolainen, K. and Kuusi, T., The stability properties of golden beet and red beet pigments: influence of pH, temperature, and some stabilizers, *Ztschr. Lebensm. Unters. Forsch.*, 166, 19, 1978.
125. Mégard, D., Stability of red beet pigments for use as food colorant: a review, *Foods Food Ingrid. J.*, 158, 130, 1993.
126. Counsell, J.N., Jeffries, G.S., and Knewstubb, C.J., Some other natural colours and their applications, in *Natural Colours for Food and Other Uses*, Counsell, J.N., Ed., Applied Science, London, 1981, 123.
127. Cohen, E. and Saguy, I., A rapid method of determination of betanin and vulgaxanthin I in beet powder using a general purpose tristimulus colorimeter, *Ztschr. Lebensm. Unters. Forsch.*, 175, 31, 1982.
128. Koul, V.K. et al., Spray drying of beet root juice using different carriers, *Ind. J. Chem. Technol.*, 9, 442, 2002.
129. Sobkowska, E., Czapski, J., and Kaczmarek, R., Red table beet pigment as food colorant, *Int. Food Ingrid.*, 3, 24, 1991.
130. Adams, J.P., Von Elbe, J.H., and Amundson, C.H., Production of a betacyanine concentrate by fermentation of red beet juice with *Candida utilis*, *J. Food Sci.*, 41, 78, 1976.
131. Drdák, M. et al., Effect of fermentation on the composition of the red beet pigments, *Ztschr. Lebensm. Unters. Forsch.*, 188, 547, 1989.
132. Czyzowska, A., Klewicka, E., and Libudzisz, Z., The influence of lactic acid fermentation process of red beet juice on the stability of biologically active colorants, *Eur. Food Res. Technol.*, 223, 110, 2004.
133. Klewicka, E., Motyl, I., and Libudzisz, Z., Fermentation of beet juice by bacteria of genus *Lactobacillus* sp., *Eur. Food Res. Technol.*, 218, 178, 2004.
134. Martínez-Parra, J. and Muñoz, R., An approach to the characterization of betanin oxidation catalyzed by horseradish peroxidase, *J. Agric. Food Chem.*, 45, 2984, 1997.
135. Martínez-Parra, J. and Muñoz, R., Characterization of betacyanin oxidation catalyzed by a peroxidase from *Beta vulgaris* L. roots, *J. Agric. Food Chem.*, 49, 4064, 2001.
136. Ashie, I.N.A. Simpson, B.K., and Smith, J.P., Mechanisms for controlling enzymatic reactions in foods, *Crit. Rev. Food Sci. Nutr.*, 36, 1, 1996.

137. Pyysalo, H. and Kuusi, T., The role of iron and tin in discoloration of berry and red beet juices, *Ztschr. Lebensm. Unters. Forsch.*, 153, 224, 1973.
138. Pasch, J.H. and Von Elbe, J.H., Betanine stability in buffered solutions containing organic acids, metal cations, antioxidants, or sequestrants, *J. Food Sci.*, 44, 72, 81, 1979.
139. Kuusi, T., Pyysalo, H., and Pippuri, A., The effect of iron, tin aluminium, and chromium on fading, discoloration, and precipitation in berry and red beet juices, *Ztschr. Lebensm. Unters. Forsch.*, 163, 196, 1997.
140. Hole, M. and Chem, C., Chelation reactions of significance to the food industry, *Process Biochem.*, 15, 16, 24, 1980.
141. Attoe, E.L. and Von Elbe, J.H., Oxygen involvement in betanin degradation, *Ztschr. Lebensm. Unters. Forsch.*, 179, 232, 1984.
142. Ibdah, M. et al., Spectral dependence of flavonol and betacyanin accumulation in *Mesembryanthemum crystallinum* under enhanced ultraviolet radiation, *Plant Cell Environ.*, 25, 1145, 2002.
143. Attoe, E.L. and Von Elbe, J.H., Photochemical degradation of betanin and selected anthocyanins, *J. Food Sci.*, 46, 1934, 1981.
144. Herbach, K.M. et al., Structural and chromatic stability of purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) betacyanins as affected by the juice matrix and selected additives, *Food Res. Int.*, 39, 667, 2006.
145. Schwartz, S.J. and Von Elbe, J.H., Identification of betanin degradation products, *Ztschr. Lebensm. Unters. Forsch.*, 176, 448, 1983.
146. Herbach, K.M., Stintzing, F.C., and Carle, R., Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations, *J. Food Sci.*, 69, C491, 2004.
147. Herbach, K.M., Stintzing, F.C., and Carle, R., Thermal degradation of betacyanins in juices from purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) monitored by high performance liquid chromatography–tandem mass spectrometric analyses, *Eur. Food Res. Technol.*, 219, 377, 2004.
148. Wybraniec, S., Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beet root (*Beta vulgaris* L.) monitored by LC-MS/MS, *J. Agric. Food Chem.*, 53, 3483, 2005.
149. Wybraniec, S. and Mizrahi, Y., Generation of decarboxylated and dehydrogenated betacyanins in thermally treated purified fruit extract from purple pitaya (*Hylocereus polyrhizus*) monitored by LC-MS/MS, *J. Agric. Food Chem.*, 53, 6704, 2005.
150. Herbach, K.M., Stintzing, F.C., and Carle, R., Identification of heat-induced degradation products from purified betanin, phylloactin and hylocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry, *Rapid Commun. Mass Spectrom.*, 19, 2603, 2005; 20, 1822, 2006.
151. Herbach, K.M., Stintzing, F.C., and Carle, R., Stability and color changes of thermally treated betanin, phylloactin and hylocerenin solutions, *J. Agric. Food Chem.*, 54, 390, 2006.
152. Sapers, G.M. and Hornstein, J.S., Varietal differences in colorant properties and stability of red beet pigments, *J. Food Sci.*, 44, 1245, 1979.
153. Singer, J.W. and Von Elbe, J.H., Degradation rates of vulgaxanthin-I, *J. Food Sci.*, 45, 489, 1980.
154. Hávlíková, L., Míková, K., and Kyzlink, V., Heat stability of betacyanins, *Ztschr. Lebensm. Unters. Forsch.*, 177, 247, 1983.
155. Cai, Y.Z. and Corke, H., Amaranthus betacyanin pigments applied in model food systems, *J. Food Sci.*, 64, 869, 1999.

156. Felker, P. et al., A comparison of the fruit parameters of 12 *Opuntia* clones grown in Argentina and the United States, *J. Arid Environ.*, 52, 361, 2002.
157. Felker, P. et al., Comparison of *Opuntia ficus-indica* varieties of Mexican and Argentine origin for fruit yield and quality in Argentina, *J. Arid Environ.*, 60, 405, 2005.
158. Le Houérou, H.N., The role of cacti (*Opuntia* spp.) in erosion control, land reclamation, rehabilitation and agricultural development in the Mediterranean basin, *J. Arid Environ.*, 33, 135, 1996.
159. Nobel, P.S., Environmental biology, in *Agro-Ecology: Cultivation and Uses of Cactus Pear*, Barbera, G., Inglese, P., and Pimienta-Barrios, E., Eds., FAO Plant Production and Protection Paper, United Nations Food and Agricultural Organization, Rome, 132, 36, 1993.
160. Von Elbe, J.H., Schwartz, S.J., and Hildenbrand, B.E., Loss and regeneration of betacyanin pigments during processing of red beets, *J. Food Sci.*, 46, 1713, 1981.
161. Herbach, K.M. et al., Effects of processing and storage on juice colour and betacyanin stability of purple pitaya (*Hylocereus polyrhizus*) juice, *Eur. Food Res. Technol.*, 224, 649, 2007.
162. Frank, T. et al., Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans, *Pharmacol. Res.*, 52, 290, 2005.
163. Jiratan, T. and Liu, R.H., Antioxidant activity of processed table beets (*Beta vulgaris* var. *conditiva*) and green beans (*Phaseolus vulgaris* L.), *J. Agric. Food Chem.*, 52, 2659, 2004.
164. Kanner, J., Harel, S., and Granit, R., Betalains — a new class of dietary cationized antioxidants, *J. Agric. Food Chem.*, 49, 5178, 2001.
165. Netzel, M. et al., Renal excretion of antioxidative constituents from red beet in humans, *Food Res. Int.*, 38, 1051, 2005.
166. Pedreno, M.A. and Escribano, J., Correlation between antiradical activity and stability of betanin from *Beta vulgaris* L. roots under different pH, temperature and light conditions, *J. Sci. Food Agric.*, 81, 627, 2001.
167. Pellegrini, N. et al., Total antioxidant capacity of plant foods, beverages and oils consumed in Italy assessed by three different *in vitro* assays, *J. Nutr.* 133, 2812, 2003.
168. Wettasinghe, M. et al., Phase II enzyme-inducing and antioxidant activities of beetroot (*Beta vulgaris* L.) extracts from phenotypes of different pigmentation, *J. Agric. Food Chem.*, 50, 6704, 2002.
169. Wettasinghe, M. et al., Screening for phase II enzyme-inducing and antioxidant activities of common vegetables, *J. Food Sci.*, 67, 2583, 2002.
170. Lee, C.-H. et al., Betalains, phase II enzyme-inducing components from red beetroot (*Beta vulgaris* L.) extracts, *Nutr. Cancer*, 53, 91, 2005.
171. Cai, Y., Sun, M., and Corke, H., Antioxidant activity of betalains from plants of the Amaranthaceae, *J. Agric. Food Chem.*, 51, 2288, 2003.
172. Vaillant, F. et al., Colorant and antioxidant properties of red-purple pitahaya (*Hylocereus* sp.), *Fruits*, 60, 1, 2005.
173. Wu, L.C. et al., Antioxidant and antiproliferative activities of red pitaya, *Food Chem.*, 95, 319, 2006.
174. Allegra, M. et al., Mechanism of interaction of betanin and indicaxanthin with human myeloperoxidase and hypochlorous acid, *Biochem. Biophys. Res. Commun.*, 332, 837, 2005.
175. Butera, D. et al., Antioxidant activities of Sicilian prickly pear (*Opuntia ficus-indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin, *J. Agric. Food Chem.*, 50, 6895, 2002.

176. Feugang, J.M. et al., Nutritional and medicinal use of cactus pear (*Opuntia* spp.) cladodes and fruits, *Frontiers Biosci.*, 11, 2574, 2006.
177. Galati, E.M. et al., Chemical characterization and biological effects of Sicilian *Opuntia ficus-indica* (L.) fruit juice: antioxidant and antiulcerogenic activity, *J. Agric. Food Chem.*, 51, 4903, 2003.
178. Galati, E.M. et al., *Opuntia ficus indica* (L.) Mill fruit juice protects liver from carbon tetrachloride-induced injury, *Phytother. Res.*, 19, 796, 2005.
179. Gentile, C. et al., Antioxidant betalains from cactus pear (*Opuntia ficus-indica*) fruit inhibit endothelial ICAM-1 expression, *Ann. NY Acad. Sci.*, 1028, 481, 2004.
180. Tesoriere, L. et al., Supplementation with cactus pear (*Opuntia ficus-indica*) fruit decreases oxidative stress in healthy humans: a comparative study with vitamin C, *Am. J. Clin. Nutr.*, 80, 391, 2004.
181. Tesoriere, L. et al., Distribution of betalain pigments in red blood cells after consumption of cactus pear fruits and increased resistance of the cells to *ex vivo*-induced oxidative hemolysis in humans, *J. Agric. Food Chem.*, 53, 1266, 2005.
182. Tesoriere, L. et al., Biothiols, taurine, and lipid-soluble antioxidants in the edible pulp of Sicilian cactus pear (*Opuntia ficus-indica*) fruits and changes of bioactive juice components upon industrial processing, *J. Agric. Food Chem.*, 53, 7851, 2005.

Section 5

Food Colorant Production

5.1 Updated Technologies for Extracting and Formulating Food Colorants

Carmen Socaciu

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5.1.1 INTRODUCTION

From health and technological perspectives, the use of natural pigments for food coloring is preferred, although natural pigments are more complex and expensive to produce than synthetics. As a consequence, the technological improvements for extraction and formulation of natural colorants with applications to the food product industry compete with earlier developments of synthetic colors.¹ Food and beverage manufacturers are still reluctant to use natural coloring pigments directly due to their poor stability, dull shades, and rapid fading. To fulfill health and economic aims and achieve consumer satisfaction, innovative technological processes are continuously needed to improve the stability and the quality-to-price ratios for natural colorants.

This chapter will review the past ten years of new approaches in the areas of coloration and production technologies²⁻⁷ and patent developments.⁸ It also discusses

updated information regarding the extraction, purification, and functionalization of natural colors, with special focus on bioencapsulation procedures. The factors that specifically influence the stabilities of a whole range of natural colors, hydrophilic and lipophilic, for example, stability in the presence of low pH, light, and heat, are considered along with solutions in given applications.

Using appropriate raw materials (fruits, vegetables, medicinal plants, and wastes from food industries) as sources of pigments, it was possible to develop and manufacture innovative products with functional properties. Many efforts were directed toward the development of natural colors with improved qualities (increased brightness and shade). Different microencapsulated colors that vary with regard to coating, production method, and final formulation are currently available. The technologies that have been applied produce microencapsulated natural pigments as stable and healthy formulations, as emulsions, oil suspensions, gels, and powders, with or without the addition of stabilizers or antioxidants.

Considering that most food products are water based, microencapsulation of colorants was mainly developed for lipophilic pigments, such as carotenoids from marigold, annatto, and paprika; curcumin from turmeric; carmine from cochineal extracts; and chlorophyll from spinach and alfalfa. The hydrophilic colorants (caramel, flavones, and anthocyanins) are specifically compatible with water-based gel formulations (pectins and gums) or maltodextrins and are used as coating molecules for polar solid matrices such as starches. The advantage of using microencapsulated products is that their coating stays intact and therefore colors do not migrate as do conventional ones. Also their brightness is improved, thus increasing their chances for commercial use.

5.1.2 COLORANT EXTRACTION, PURIFICATION, AND CONCENTRATION

Colorant extraction procedures generally include two steps that serve to isolate an enriched crude (oleoresin or hydroalcoholic) extract of pigments from raw materials by a solid–liquid partition process, followed by specific purification steps that involve the elimination of unwanted interfering components. Traditionally, in a first step, the solid raw materials are grounded or milled and mixed with a favorable solvent for the pigment to extract. Techniques have become more sophisticated and miniaturized. In order to gain high extraction yields, various parameters were optimized (temperature, pressure, use of microwaves or ultrasound). The second step consists of the purification and fractionation of the crude extract, using solid–liquid partition methods or liquid–liquid partition followed by concentration under vacuum or membrane (ultra) filtration-based techniques.⁹

5.1.2.1 PREPARATION OF CRUDE EXTRACTS

General trends are focused on reduced-solvent extractions or adsorption-based methods — environmentally friendly solvents for both solid and liquid samples. In recent decades, advanced techniques like supercritical fluid extraction (SFE),¹⁰ pressurized liquid extraction (PLE),^{11–14} microwave-assisted extraction (MAE), ultrasound-assisted extraction,¹⁵ countercurrent continued extraction (www.niroinc.com), solid

phase extraction (SPE), and microextraction (SPME) replaced the older conventional ones (Soxhlet extraction and hydrodistillation). Material about distillation and other equilibrium-staged separation processes including distillation design, control, and SFE published after 1980 has been reviewed recently.¹⁶

A new generation of enhanced extraction technologies is based on the use of temperature above the atmospheric boiling point of the extracting solvent. In SFE and PLE, pressure is applied to the extraction system so that these high temperatures can be achieved. The fundamental difference between SFE and PLE is that SFE uses solvents near or above their critical points (generally carbon dioxide-based fluids), while PLE uses traditional aqueous and organic solvents. MAE is similar, except that the heating is via microwave irradiation and the pressure is a consequence of heating a closed system, as opposed to direct application.

The current state of analytical SFE was critically reviewed¹⁷ and no major changes of the technique have been observed. Overviews of the developments of the extraction technologies of secondary metabolites from plant materials refer to three types of conventional extraction techniques that involve the use of solvents, steam, or supercritical fluids. Each technique is described in detail with respect to typical processing parameters and recent developments. Following the discussion of some technical and economic aspects of conventional and novel separation processes, a few general conclusions about the applicabilities of the different types of extraction techniques are drawn.¹⁸

CO₂ extraction has been prevalent for the isolation of essential oils and other natural lipophilic pigments like carotenoids.¹⁹ Hot water and superheated water extraction methods are used for analytical preparation of polar pigments. The technique is commonly referred to as subcritical water extraction because the practitioners of this approach come from SFE backgrounds.

MAE shares several similarities with SFE and PLE, but includes significant differences. MAE can utilize an open or closed vessel and allows the use of acids and bases, making chemical digestion possible. MAE has found a diversity of applications directed more toward food and biological than environmental areas. Foremost among other partitioning methods is the application of ultrasound energy to facilitate extraction. Novel extraction approaches will not gain acceptance until they “prove” their performance in comparison to the alternatives. Since analytical extraction is generally a preliminary step toward the final method that produces the desired information, the advantages of time, automation, reproducibility, or solvent use must be shown. The new developing technologies are compared with existing techniques. In food-related areas, PLE, SFE, SPME, and ultrasound extraction are good alternatives to the conventional Soxhlet method, especially with large sample sizes, but Soxhlet extraction is highly efficient for sterols.^{20,21} Both SPME and SFE were found superior to the other methods, and SPME was preferred because it did not require specialized instrumentation. By far, most applications relate to carotenoid pigments. Table 5.1.1 lists patent data covering different carotenoid extraction and purification procedures.

The extraction of carotenoids from tomatoes to yield tomato seed oil, the valorization of tomato waste to obtain lycopene, and their uses in functional foods are already established.

TABLE 5.1.1
Patent Data Related to Extraction, Purification, and Formulation of Carotenoid Pigments for Food and Feed Applications

Natural Colorant Product	Type of Technological Procedure	Procedure Details	Application and Patent Reference
Lutein esters	Purification and formulation	Separation of lutein esters from complex plant extract mixture	Functional food ingredient with antioxidant properties; Bowen, P. et al., U.S. Patent 6,313,169, 2001
Free xanthophylls	Extraction and transesterification, purification	Conversion of xanthophyll esters to free xanthophylls in marigold using methanol	Food colorants; Sas, B. and Clifford, A., U.S. Patent 6,221,417, 2001
Trans-xanthophyll esters (lutein-rich)	Extraction and isomerization	Extraction of total xanthophyll esters from marigold in hydrocarbon solvent, removal of impurities and cis-isomers by alcohol washing and concentration of trans-esters	Food colorants and nutraceuticals; Levy, L., U.S. Patent 5,129,041, 1999
Zeaxanthin	Hydrolysis of carotenoid precursor (saponification) and isomerization	Preparation of carotenoid extract from plant oleoresin and hydrolysis with alkaline reagent in polar organic solvents (ether, polyhydroxyl alcohol, and ether alcohol)	Food colorants and nutraceuticals; Sanroma, V. et al., U.S. Patent 5,998,678, 1999
Zeaxanthin, lutein short-chain diesters and intermediates for canthaxanthin and astaxanthin synthesis	Hydrolysis and transesterification	Plant extracts rich in carotenoids, hydrolyzed with acetic and propionic aldehydes under controlled temperature and pressure	Feed ingredient (pigmentation of broilers and egg yolks); Torres-Cardona et al., U.S. Patent 5,969,948, 1998
Zeaxanthin-rich colorant	Isomerization of lutein to zeaxanthin	Treatment of marigold meal, oleoresin, or formulations containing lutein with strongly alkaline aqueous solutions under controlled conditions to isomerize lutein into zeaxanthin, a product with greater pigmenting activity	Poultry feed ingredient (pigmentation of broilers and egg yolks); Torres-Cardona et al., U.S. Patent 5,523,494, 1994

Carotenoid dyes	Direct extraction with high yield	Treatment of pre-dried natural starting materials with compressed gases (propane and/or butane) and organic solvents to facilitate complete extraction	Food and feed colorants; Heidlas, J. et al., U.S. Patent 5,789,647, 1998
Zeaxanthin	Isomerization of lutein (esters) into zeaxanthin by base-catalyzed agents	Heating pre-treated lutein-containing material in mixture of aqueous solution, alkali hydroxide, and dimethyl sulfoxide/organic solvent under catalysis at 50 to 120°C	Food and feed colorants; Bernhard, K. et al., U.S. Patent 5,780,693, 1998
Lutein, zeaxanthin, and capsanthin/capsorubin from marigold, wolfberry, and red pepper, respectively	Extraction, isolation isomerization, and purification by crystallization	Plant extracts containing xanthophyll diesters are saponified in composition of propylene glycol and aqueous alkali to form crystals; crystallization of xanthophylls is achieved without use of organic solvents; crystals are isolated and purified	Food supplement and additive; Ausich, R. et al., U.S. Patent 5,648,564, 1998
Xanthophylls from corn gluten meal	Extraction and saponification	Xanthophylls obtained from corn gluten meal at 50°C using ethanol with ethoxyquin as antioxidant	Food, feed, and pharmaceutical ingredient; Muralidhara, H.S. and Cornuelle, T.L., U.S. Patent 5847238, 1998
Water-soluble carotenoid glycosides	Extraction and purification	Preparation of astaxanthin diglycosides, adonixanthin-3-glycosides, astaxanthin monoglycosides by expression in transgenic microorganisms (<i>Erwinia uredovora</i> or <i>Agrobacterium aurantiacum</i>) and <i>Escherichia coli</i>	Beverage and feed additive; Misawa, N. and Yokoyama, A., Japan Patent 10,327,865, 1998
Oleoresin from spices	SFE fractional separation	SFE extraction pressures of 500 bar at 80 to 100°C; combined with countercurrent cascading mode of extraction using four extraction vessels, the rate of oleoresin extraction doubles again; rate of oleoresin extraction under these conditions is four times that of traditional batch extraction at 300 bar and 60°C	Nguyen, U., et al., U.S. Patent 5,017,397, 1991; U.S. Patent 5,120,558, 1992

Continued.

TABLE 5.1.1 (Continued)
Patent Data Related to Extraction, Purification, and Formulation of Carotenoid Pigments for Food and Feed Applications

Natural Colorant Product	Type of Technological Procedure	Procedure Details	Application and Patent Reference
Carotenoid/ curcumin/porphyrin compositions	Water dispersion without using surface-active substance	Stable dispersion of water-insoluble and/or hydrophobic natural pigment such as carotenoid, curcumin, porphyrin pigment, or vegetable carbon black in form of bodies of average size of 10 nm	Nutraceutical tablets and dragees; Isager, P.P. et al., U.S. Patent 6,190,686, 2001
Carotenoid stabilizers for riboflavin-colored food	Stabilizing agent	Addition of 0.5 ppm β -carotene to yogurt containing 200 ppm riboflavin; color did not change after 40 days at 6°C compared with control (decoloration at 1 day)	Color stabilizers for dairy products; De Potzoli, B., DE 19,720,802, 1997
Carotenoid-containing granular formulation	Emulsification and microencapsulation	Palm oil carotenoid, tocopherol, and ascorbic acid palmitate were dissolved into corn oil at 160°C for 30 min, emulsified with aqueous solution of gelatin, ascorbic acid, microencapsulated on saccharose at 70°C	Food, pharmaceutical, and cosmetic ingredients; Akamatsu, A. et al., Japan Patent 10,306,073, 1998
Lycopene formulation	Oily dispersion	Lycopene was dispersed in medium-chain triglyceride oil derived from esterification of fatty acids and glycerol; composition was stable for 3 mo at 25°C, compared with dispersion on soybean oil	Food ingredient and supplement; Schlupalius, L. et al., U.S. Patent 9,852,561, 1998

Powders containing fat-soluble vitamins and carotenoids	Emulsification and microencapsulation	Emulsion formed from vitamins and/or carotenoids, gelatins (pH 2 to 4.6), saccharose, starch, and water; emulsion drops are added to powder of starch to form microparticulates by heat treatment at 120 to 170°C to give powder	Food and pharmaceutical supplement; Mori, T. et al., Japan Patent 11,012,165, 1999
Food grade yellow colorant	Carotenoid immobilization on protein carriers	Colorant containing annatto and Ca caseinate as carrier mixed with water to be added directly to cheese milk yielding uniform colored cheese mass	Cheese additive; Talbott, P.I., U.S. Patent 9,907,233, 1998
Stable carotenoid formulation	Emulsification	Water-dispersible beadlet of β -carotene is mixed with oil to attain composition that remains stable even in presence of polyphosphates and with antioxidant action even in absence of ascorbic acid	Additive for beverages and nutraceuticals; Cox, D.J. et al., U.S. Patent 9,907,238, 1998
Water-dispersible carotenoid pigment preparation	Emulsification	Blending carotenoid pigment and soybean fiber (with tomato juice) as effective ingredient for dispersion stability	Food colorant; Koguchi, M. et al., U.S. Patent 9,908,549 1998
Carotenoid microencapsulated formulation	Emulsification and microencapsulation	Combination of liquid hydroethanolic base containing β -carotene, a UV benzophenone absorber, and tocopherol antioxidant, PEG-40 hydrogenated castor oil; encapsulation on porous silica as absorbent bead and dimethylpolysiloxane to obtain stable powder	Food and cosmetic additive; Roman, F., U.S. Patent 9,911,718, 1998
Carotenoid-cyclodextrin complexes	Microencapsulation	Preparation of decolorized complex of β -carotene with β -cyclodextrin to obtain watery texture formulation of light brown color; addition of vitamin E succinate, glycerin, and gelatin give final formulation	Food and cosmetic ingredient; Sikorski, C. et al., U.S. Patent 5,834,445 A, 1999

Plant carotenoids are still extracted at laboratory and industrial scales with solvent mixtures of ethanol and ethyl acetate, but solvent extraction always bears the risk of toxic residues in the extracts and this limits their use in large production applications in the food and pharmaceutical industries.

To extract and evaluate the color pigments from cochineals (*Dactylopius coccus* Costa), a simple method was developed.²² The procedure is based on the solvent extraction of insect samples using methanol and water (65:35, v/v) and a two-level factorial design to optimize the solvent extraction parameters: temperature, time, methanol concentration in mixture, and yield. For hydrophilic colorants that are more sensitive to temperature, water is the solvent of choice. For example, de-aerated water extraction at low temperature was applied to separate yellow saffrole and carthamine from saffron (*Carthamus tinctorius*) florets that contain about 7% yellow saffrole and 0.3% red carthamine.²³

Using combined high pressure adsorption SFE at 50 MPa and 100°C, fractionation of carotenes from paprika oleoresin successively extracted¹¹ in a time-dependent manner first free carotenoids, mainly β -carotene, followed by capsanthin and then esterified carotenoids. The selectivity was increased by the silica-based absorption system. Another interesting way to extract concomitantly pigments and flavors from Andes berries and tamarillo fruits was the use of osmotic dehydration (DIS technique: dewatering, impregnation, and soaking) using sucrose syrup as the solvent to be enriched in anthocyanins.²⁴

Another study shows that the addition of trehalose to sucrose improved the stability of freeze-dried or evaporated anthocyanins obtained from strawberry paste by ultrasonication-assisted methanol extraction.²⁵ Interestingly, yeast metabolites can contribute to the stability of anthocyanins in wine during fermentation, releasing vitisin pigments, molecules formed from pyruvic acid, anthocyanins, and acetaldehyde. These pigments are more resistant to pH changes and SO₂-induced bleaching and can liberate mannoproteins that bind to anthocyanins and tannins, protecting them from precipitation.^{26,27}

Supercritical fluid extraction — During the past two decades, important progress was registered in the extraction of bioactive phytochemicals from plant or food matrices. Most of the work in this area focused on non-polar compounds (terpenoid flavors, hydrocarbons, carotenes) where a supercritical (SFE) method with CO₂ offered high extraction efficiencies. Co-solvent systems combining CO₂ with one or more modifiers extended the utility of the SFE–CO₂ system to polar and even ionic compounds, e.g., supercritical water to extract polar compounds. This last technique claims the additional advantage of combining extraction and destruction of contaminants via the supercritical water oxidation process.¹⁴

Food and natural products represent the next largest body of work in SFE. Major topics include the isolation and characterization of high value-added fragrances and flavor compounds from novel natural materials and agricultural by-products. SFE is increasingly applied to extract oils, flavors, colorants, resins, etc., avoiding the use of hexane and petroleum fractions.

The “traditional” use of SFE involved fractional extraction in which a selected group of oleoresin components is targeted. This technique makes it possible to extract subsequently the volatile oils (light fraction, under milder conditions, 120 bar, 40°C)

and then re-extract the raw material under more severe conditions (heavy fraction, 300 bar, 60°C). Both fractions are important to a food processor. An alternative approach is “fractional separation” in which under strong pressure (500 bar, 80 to 100°C) and temperature conditions, all oleoresin components are removed quickly and efficiently. The resulting supercritical fluid is then passed through a series of separator vessels in which different fractions are selectively collected. This technique seems to offer advantages in processing costs and in finding utility for oleoresin fractions; extractions costs are only one-fourth of those required by fractional extraction.

Since industrial use of such sources (tomato waste, algae, citrus press cake) is limited because solvent residues from the extraction process constitute a food safety hazard,^{28–30} SFE and methanol were applied to extract good yields of carotenoids and chlorophylls from *Nanochloropsis gaditana*, a microalgae widely used in aquaculture.³¹ Another application of SFE was reported to extract both flavonoids and amino acid components from *Scutellaria lateriflora*.³² Many research groups^{33–36} and commercial companies are involved in production of special oils, pigments, and flavors to be used in nutraceuticals and cosmetics. They include Aromtex Ltd., Finland (www.aromtech.com), Flavex Naturextrakte GmbH, Germany (www.flavex.com), and Super Critical Extraction New Zealand Ltd., New Zealand (www.supercritical.co.nz).

Seed oils and waxes are more commonly extracted by SFE. It is the most suitable technique for non-polar compounds such as oils and waxes instead of other organic solvents, and it avoids the presence of solvent residues after extraction. Both extraction methods, the classical and SFE, still need the additional purification step of the extract, increasing the cost of the final product.

For example, the lycopene available on the market is supplied mainly by LycoRed (www.lycored.com), a company that uses a classical extraction system (ethyl acetate as solvent) and maintains a monopoly position for lycopene production on a large scale. The manufacturing of the Lyc-O-Mato[®] oleoresin (recognized by European Regulation 258/97/EC) product of LycoRed is almost identical to the production of the food additive and includes physical operations to separate the pulp from ripe tomatoes extracted according to GMPs and ISO-9002-certified procedures. The final product contains 6 to 15% lycopene; the total lycopene recovery from pulp reaches 85% and from paste around 50%.

Recently, SFE technology was reported to extract the lycopene antioxidant from tomatoes through use of the Balaban technology at the University of Florida³⁷ — an inexpensive way to turn a mountain of discarded tomato waste into a marketable commodity. Breweries use the same technology to extract flavor and aroma from hops. Using half-dried tomatoes, liquefied supercritical CO₂ gas is pumped into a vessel under high pressure and extracts carotenoids. Small amounts of ethanol added to the CO₂ enhance the extraction. When the process is finished, the pressure is decreased and the CO₂ returns to a gaseous state, leaving a concentrated mixture of carotenoids containing mainly lycopene. The purification of lycopene from the mixture requires an additional chromatography step. The highest yields were obtained at a pressure of 5,000 psi and 55°C, using 90% CO₂ and 10% ethanol.

Enzyme-mediated extractions — Selective enzyme-mediated extraction of cap-sacinoids and carotenoids from Chili Guajillo Puja using ethanol as a solvent was

recently reported³⁸ as an alternative to hexane-based extraction. After a pretreatment of the fruit tissue with mixtures of pectinases, cellulases, and hemicellulases, an extraction with ethanol was performed and high yields were obtained, namely a recovery of 87% of capsacinoids in terms of pungency and 84% of carotenoids (capsanthin and capsorubin). Enzymatic treatments rapidly destroy the cellulosic walls, facilitate better extraction, are less toxic, and need lower concentrations of solvents.

An enzyme-assisted, optimized extraction of flavonoids from winery by-products (grape skins) was recently established using different pectinolytic and cellulolytic enzymes, enzyme–substrate ratios, and time–temperature regimes of enzymatic treatment.³⁹ The optimal conditions were obtained at 50°C and 5000 ppm pectinolytic and 2500 ppm cellulolytic enzyme preparations, respectively, applied also in pilot-plant scale experiments. Significantly improved yields for most pigments (quercetin glycosides and malvidin coumaroylglucoside) were reported compared to experiments without enzyme addition. The recovery rates were comparable to those obtained with sulfite-assisted extraction of grape pomace. Pre-extraction of the pomace with hot water followed by treatment with cell wall–degrading enzymes increased the yields of phenolic compounds.

Marigold petals are rich sources of xanthophylls, mainly lutein esters. To increase the coloring power, chemical extraction of the colorant from flower meal is performed or a new enzymatic procedure is applied. It was shown that treatment with cellulases or mixed saprophyte microorganisms^{40,41} or solid state fermentation improved the xanthophyll extraction yield.⁴²

Recently, an enzymatic method was reported⁴³ to recover the three main components of industrial shrimp waste (protein, chitin, and astaxanthin) using treatments with alcalase and pancreatin. The first enzyme was more efficient in increasing the recovery of protein from 57.5 to 64.6% and of astaxanthin from 4.7 to 5.7 mg/100 g of dry waste.

The influence of enzyme maceration using pectinolytic enzyme preparations (Pectofruit and Pectofruit Press) on anthocyanin extraction at 43°C from two variants of black currant berries was studied.⁴⁴ Enzymes accelerated the extraction yield; the yield of anthocyanin extraction was similar for both enzymes and the duration did not influence the total content of released pigments.

Two-phase whole-cell biocatalysis is a very interesting method for extracting high-value bioactive cell metabolites from algae in bioreactors. This method was applied to *Dunaliella salina*, a unicellular microalga known as one of the richest sources of β -carotene. This alga overproduces β -carotene under stress (high light intensity, high salinity, or nutrient deficiency).⁴⁵ In the stress phase, the cells take up dodecane, an organic solvent (up to 13 pg/cell) and this solvent stimulates continued *in vitro* and *in vivo* release of β -carotene and its biosynthesis. Using this system, it was possible to extract β -carotene selectively from *D. salina* in a two-phase bioreactor.⁴⁶ The cells were first stressed, dodecane was then continuously recirculated through the aqueous phase containing the cells. The cells continuously produced β -carotene and newly synthesized molecules substituted the extracted part. Due to this substitution process called milking, it was possible to produce larger amounts of β -carotene than in the commercial production process.⁴⁷ The results were explained by two mechanisms: one based on the expulsion of β -carotene-containing globules

from the chloroplasts and then by exocytosis from cells, or the direct release of β -carotene from the globules as a result of alterations induced by dodecane uptake in all cell membranes.⁴⁸

5.1.2.2 ADSORPTIVE AND MEMBRANE-BASED PURIFICATION METHODS

Purification of pigments from liquid or gaseous samples using adsorptive phases started with conventional column chromatography cleanup and fractionation, and developed to a microextraction SPE-based technique in a disposal-tip pipet or packed syringe (MEPS). The technology is compatible with repeating pipets and automated liquid handling systems.

In order to obtain a highly concentrated and purified anthocyanin pigment from black chokeberry (*Aronia melanocarpa* var. Nero), juices and skin extracts were purified by SPE.

Sixteen solid-phase materials were tested on a laboratory scale and the anthocyanin and sugar content of collected fractions were determined. Among these, reverse-phase silica gels and macroreticular non-ionic acrylic polymer adsorbents such as Serdolit PAD IV or Amberlite XAD-7 turned out to be most suitable. SPE was used to investigate these materials on an enlarged scale, improving elution gradient and column purification. Amberlite XAD-7 was successfully applied in a middle-scale separation.⁴⁹

The same resin was used for the purification via downstream processing of carminic acid, the natural colorant extracted from cochineal.⁵⁰ By a direct adsorption method, a crude extract was applied on the polymeric bed gel and the adsorption kinetics studied using elution with hydrochloric acid and ethanol. The desorbed pure carminic acid concentrated under vacuum yielded a final product that complied with *Codex Alimentarius* requirements and FAO/OMS norms.

To purify, concentrate, and recover different pigments (flavonoids or anthocyanins), various ion-changing resins were used.⁵¹ Recent screenings of 13 commercial resins [acrylic or styrene-divinylbenzene (SDVB)] for the purification and specific absorption of anthocyanins^{52,53} used ethanol, methanol, and water mixtures as eluents at pH 3.5. DDVB resins (EXA-118 and EXA-90) were found most suitable using a mixture of methanol and water (1:1) for elution. The other routinely used resins like XAD-7 showed low efficiency.

Countercurrent chromatography is an automated version of liquid-liquid extraction, comparable to repeated partitioning of an analyte between two phases in a separation funnel. This technique was revolutionized by Ito (1981) and developed exponentially⁵⁴⁻⁵⁶ by application in high-speed (HSCCC) or low-speed variants (LSCCC) that assure separation on a multilayer coil that rotates around its own planetary axis and simultaneously around the “solar” axis. The coil mixed with immiscible liquids allows the retention of a stationary phase and partitioning of solutes via a hydrodynamic equilibrium, achieving the separation of phases.⁵⁷⁻⁶¹ The HSCCC technique was optimized and applied for efficient isolation of carotenoids, anthocyanins, procyanidins, and other plant pigments.⁶²⁻⁶⁴ The LSCCC method is under development and appears promising for scaled-up preparations.⁶⁵

HSCCC was successfully applied for the first time to the isolation and purification of zeaxanthin from cyanobacterium *Microcystis aeruginosa*.^{64,66} Adducts of flavonols and anthocyanins were also isolated using this method.⁶⁷

A whole protocol of extraction, purification, and drying of betalain pigments from *Amaranthus* inflorescences, using a spray drying system was reported. Frozen flowers were extracted with 80% aqueous MeOH, filtered by a 0.2- μ m nylon membrane under vacuum at 22°C, and centrifuged at 20,000g for 15 min at 4°C. The supernatant was concentrated to yield crude pigment extracts. After separation on a Sephadex LH-20 column at pH 5 to 6, the yellow betaxanthin fractions were collected and lyophilized in a Heto FD3 freeze dryer, yielding yellow powders of partially purified pigments to be used as food colorants. Freeze drying was replaced by spray drying; the costs were 50 times lower, but the quality of the powder was inferior.⁶⁸

Advanced techniques like molecularly imprinted polymers (MIPs), infrared/near infrared spectroscopy (FT-IR/NIR), high resolution mass spectrometry, nuclear magnetic resonance (NMR), Raman spectroscopy, and biosensors will increasingly be applied for controlling food quality and safety.^{69,70}

5.1.3 MACRO- AND MICROENCAPSULATED FORMULATIONS

Bioencapsulation is a technology aimed toward the immobilization and incorporation (entrapping) of a biologically active compound on or inside solid particles (microspheres) or liquid vesicles in order to stabilize, structure, and protect the active compound and allow control of its release.

Bioencapsulation facilitates light- and heat-labile molecules like many pigments, to maintain their stability and improve their shelf lives and effects. It is a rapidly expanding technology, highly specialized, with affordable costs.⁷¹ Applications of bioencapsulation focus mainly on the food ingredient sector (macro- and microemulsions, encapsulated pre- and probiotics, immobilized enzymes)^{72,73} and also on biomedical areas (encapsulation of cellular proteins or DNA, integral cells for transplantation, drugs for targeted delivery, etc.), nanomaterials, and encapsulated molecules for agricultural uses. Bioencapsulation is solving many problems of active molecule delivery with impacts on nutrition, bioprocessing, food production, agriculture, water treatment, personal care, cosmetics, pharmacy, and medicine, representing over 45% of new businesses in Europe, mainly knowledge-based SME enterprises.

Immobilization is the technique of choice in many food industry processes and especially in beverage production. Many immobilization technologies have already been tested and some are applied in the production of beer, wine, vinegar, and other food products using a traditional approach with culture adhesion (i.e., *Acetobacter* in vinegar production) or more modern approaches with entrapment of yeast biomass (i.e., sparkling wines, cheeses, and yogurts).

The generic procedure for biocapsule production includes either the dispersion of a liquid matrix as droplets (spraying, dropping, or mixing) and its stabilization by solidification or by membrane formation (micelle or liposome), drying by freezing (lyophilization), or spraying of a hot coating solution on a particle surface (fluid bed or pan rotating bed) followed by the stabilization of the solidified coating.

The first stage of a bioencapsulation process involves mechanical (dispersion or spray coating) techniques like prilling (jet cutting, disc rotating, electrostatic prilling, nozzle resonance techniques), emulsification (batch process, inline mixer, static mixer), coating (fluidization, spraying) followed by stabilization processes like gelification,⁷⁴ polymerization, drying and solvent evaporation, solidification, coalescence, and coacervation.^{75–78}

5.1.3.1 EMULSIONS AND MICROEMULSIONS

Pigments are generally labile as free molecules and the natural and simplest way to stabilize their function is to form micelles (oil-in-water or water-in-oil emulsions, depending on the major dispersion media).

The conventional food industry approach to preparing emulsions has involved high-speed mixing or use of ultrasound to form stable micelles from an oily phase, a water phase, and an emulsifier (surfactant).⁷⁹ The emulsions or macroemulsions are turbid, have droplet sizes of 0.2 to 10 μm and are kinetically stable, albeit thermodynamically unstable. Macroemulsions are the major natural food formulations (mayonnaise, yogurt, etc.) and are extensively used to prepare different commercial food products. Microemulsions are transparent isotropic solutions with particle sizes ranging from 0.005 to 0.1 μm , are thermodynamically stable, and arise from spontaneous self-assembly of the hydrophobic or hydrophilic parts of surfactant molecules. Microemulsions have found numerous applications over a wide range of areas, including pharmaceuticals, cosmetics, oil recovery, models for biological membranes, etc.^{80,81}

The application of microemulsions in foods is limited by the types of surfactants used to facilitate microemulsion formation. Many surfactants are not permitted in foods or only at low levels. The solubilization of long-chain triglycerides (LCTs) such as edible oils is more difficult to achieve than the solubilization of short- or medium-chain triglycerides, a reason why few publications on microemulsions are available, especially because food-grade additives are not allowed to contain short-chain alcohols (C3–C5).

Different methods are used in microemulsion formation: a low-energy emulsification method by dilution of an oil surfactant mixture with water and dilution of a water-surfactant mixture with oil and mixing all the components together in the final composition. These methods involve the spontaneous formation of microemulsions and the order of ingredient addition may determine the formation of the microemulsion. Such applications have been performed with lutein and lutein esters.^{82,83}

The phase inversion temperature (PIT) method is helpful when ethoxylated non-ionic surfactants are used to obtain an oil-and-water emulsion. Heating the emulsion inverts it to a water-and-oil emulsion at a critical temperature. When the droplet size and interfacial tension reach a minimum, and upon cooling while stirring, it turns to a stable oil-and-water microemulsion form.^{84,85}

High pressure homogenization may also be used to form microemulsions but the process of emulsification is generally inefficient (due to the dissipation of heat) and extremely limited as the water-oil-surfactant mixture may be highly viscous prior to microemulsion formation.^{86–91}

Oil-in water emulsions using lutein and phycocyanin mixed with vegetable oil and pea protein as emulsifiers were obtained^{92,93} in order to determine the emulsion properties (droplet size, firmness) and stability. Lutein concentration in the oil droplets and the firmness were poor and droplet size distribution was small, but the addition of phycobilin increased the firmness of the emulsion. Similar studies⁹⁴ in which microalgal biomass from *Chlorella vulgaris* and *Haematococcus pluvialis* were used to make stable emulsions with good hue and color intensities were reported. Emulsions containing carotenoid pigments and lecithins as surfactants compared to ethanol extracts were tested for hepatic cell availability and showed better stability and incorporation rates.⁹⁵

Table 5.1.1 lists patents covering emulsification as a technique to obtain carotenoid formulas. Table 5.1.2 summarizes commercial formulations containing natural colorants and food applications.

5.1.3.2 VESICULAR PIGMENT CARRIERS: LIPOSOMES, TRANSFEROSOMES, AND NIOSOMES

Liposomes — These are synthetic lipid vesicles consisting of one or more phospholipid bilayers; they resemble cell membranes and can incorporate various active molecules. Liposomes are spherical, range in size from 0.1 to 500 μm , and are thermodynamically unstable.⁹⁶ They are built from hydrated thin lipid films that become fluid and form spontaneously multilamellar vesicles (MLVs). Using sonication, freeze–thaw cycles, or mechanical energy (extrusion), MLVs are converted to small unilamellar vesicles (SUVs) with diameters in the range of 15 to 50 nm.^{96–99}

More recently, liposomes and proteoliposomes (liposomes incorporating or encapsulating one or more proteins) have been shown to be extremely suitable systems to deliver a wide variety of substances to targets of biological, biochemical, pharmacological, and agricultural interest.^{100–102} Little use of liposomes in food systems has been made until now and most applications are utilized in the medical and pharmaceutical sciences. Due to a better understanding of their functional properties and stability, improved manufacturing technologies, and lower raw material costs, it has become feasible to use liposomes to incorporate functional food components such as colorants, flavors, nutraceuticals, and antimicrobials.¹⁰³ One particular advantage of liposomes is that they consist solely of naturally occurring constituents, reducing the regulatory requirements that may prevent their applications in food systems.¹⁰⁴

Data about curcumin encapsulated in liposomes have been reported recently.¹⁰⁵ The authors encapsulated curcumin into a liposomal delivery system in order to study the *in vitro* and *in vivo* effects of this compound on proliferation, apoptosis, signaling, and angiogenesis using human pancreatic carcinoma cells. Carotenoids of different polarities and in competition with cholesterol were specifically incorporated into liposomes in order to mimic the physiological uptake by cells¹⁰⁶ and monitor their antioxidant capacities.¹⁰⁷

Oleosomes — Also called oil bodies, oleosomes are the natural equivalents of liposomes. They are found in plant seeds or fruits, filled with oils, pigments, and vitamins, and serve as specific organelles to store lipid molecules. A protocol to

TABLE 5.1.2
Commercial Formulations of Natural Colors Approved for Addition to Foods

Formulation	Code	Active Colorant	Ingredients	Color	Food Application
Anthocyanin-based colors	Anthocyanin, black carrot, E 163	A = 0.300 at 525 nm	Carrot juice, propylene glycol	Pink to Red	Acidic beverages, fruit fillings, candies and confections
	Beet powder, E 162	0.35% betanin	Red beet juice, maltodextrin	Bluish red	Condiments, gelatin products, fruit preparations, sauces, candies, power beverage products
Marigold extract (lutein-xanthophylls)	E 161b	10% lutein	Lutein, vegetable oil	Egg yellow	Chicken feeds, pet foods
	E 160d	5% lycopene	Tomato extract, vegetable oil	Orange red	Snacks, butter, margarine, vegetable oils and fats, pastas, soups, gravies, sauces.
Caramel colors, liquid forms	S.S Type IV, E 150d	CU = 115 to 120	Ammonia sulfite	Brown	Carbonated drinks, candies, baked goods, syrups, pet foods
	Type IV, soft drink grade, E 150d	CU = 240 to 250	Ammonia sulfite	Brown	Carbonated drinks, candies
	Type III, E 150c	CU = 110 to 120; 0.1% w/v solution at 610 nm using 10 mm cell	Ammonia	Brown	Soy sauce, brewery products, baked goods

Continued.

TABLE 5.1.2 (Continued)
Commercial Formulations of Natural Colors Approved for Addition to Foods

Formulation	Code	Active Colorant	Ingredients	Color	Food Application
Annatto colors (C.I. 75120); water-soluble annatto extract	E 100	2% norbixin	Annatto extract, water, potassium hydroxide	Yellow-orange	Cheeses, ice cream, bakery products
or powder; acid-proof annatto colors; oil-soluble (natural orange 4) or suspension	E 100	4% norbixin	Annatto extract, water, potassium hydroxide	Yellow-orange	Cheeses, ice cream, bakery products
	E 100	2.5 to 20% norbixin	Annatto extract, sodium carbonate	Yellow-orange	Cheeses, ice cream, bakery products
	E 100	2.5% norbixin	Annatto extract, Polysorbate 80, propylene glycol, potassium hydroxide, mono- and di- glycerides	Yellow-orange	Desserts, fruit fillings, yogurt, beverages
	E 100	1% bixin	Annatto extract in vegetable oil, mono- and di-glycerides, propylene glycol, potassium hydroxide	Yellow-orange	Butter, oils, margarines, processed cheese, fat-based products
	E 100	2% bixin	Annatto extract in vegetable oil	Yellow-orange	Butter, oils, margarines, processed cheese, fat-based products

Paprika oleoresin colors, oil- or water-soluble	E 160c	CU = 40000 to 100000	Extracts of paprika and vegetable oil	Orange-red	Meats, snack food coatings, popcorn oil, cheeses
	E 160c	CU = 40000 to 80000	Extracts of paprika and Polysorbate 80	Orange-red	Salad dressings, sauces
	E 100	1% curcumin	Turmeric oleoresin, Polysorbate 80, propylene glycol	Bright yellow at and below neutral pH	Dairy products, confectionery, salad dressings
Turmeric colors (C.I. 75300); liquid and powdered (pure) turmeric colors	E 100	6% curcumin	Turmeric oleoresin, Tween 80	Bright yellow	Dairy products, confectionery, salad dressings
	E 100	90% curcumin	Turmeric oleoresin	Bright yellow	Bakery products, sauces, confectionery, pickles, fish, vitamin tablets, snack foods
Spinach extract (C.I. 75810, natural green 3.)	Copper chlorophyll from spinach, E 141i	A = 0.15 to 0.20 at 405 nm	Spinach oleoresin, vegetable oil	Green	Bakery products, soups, vegetable oil, fat-based products.
	E 141ii	A = 0.15 to 0.20 at 405 nm	Spinach oleoresin, sodium hydroxide	Green	Beverages, frozen desserts, confectionery, sauces

CU = color unit.

isolate oleosomes has been developed, based upon the stringent washing of the oil body fraction in 9 M urea. This effectively removes most of the contaminating protein as judged by SDS-PAGE. The urea-washed oil bodies were enriched in two major oleosin proteins of M_r 19,000 and 20,000.¹⁰⁸

Oleosomes of seabuckthorn fruit flesh were isolated by physical separation techniques and their higher stabilities and antioxidant activities compared to solvent-extracted oil were demonstrated.¹⁰⁹

For skin protection, an oleosome technology was developed by Lonza Company in 2004. Natrulon, the initial product in the line, is comprised of safflower oleosomes formulated as DermaSphere. For details see <http://www.lonza.com/>.

MiCap, a U.K. technology licensing company, specializes in the microencapsulation of active ingredients within yeast cells. MiCap's unique proprietary technology uses dead yeast cells as natural capsules to protect the active ingredient. Yeast encapsulation can protect active ingredients against high temperatures and also the effects of the sun, pressure, and degradation through exposure to the air. The natural affinity of yeast cells for mucous membranes can also be exploited for targeted drug delivery. For further details visit <http://www.micap.co.uk/>.

Transferosomes represent another system of encapsulation using ultradeformable vesicle carriers for bioactive molecules, applied until now for direct transdermal drug delivery. They are built from polar lipids and have high flexibility, and are rich in unsaturated fatty acids and carotenoid pigments.¹¹⁰

Niosomes are vesicular delivery systems that result from the self-assembly of hydrated nonionic surfactant monomers.^{111, 112} Consisting of single or multiple surfactant bilayers, they resemble liposomes in structure and shape.¹¹³ However, they may have some advantages over liposomes with respect to chemical stability, lower costs, and the large number of surfactant classes available for the design of this vesicular system. These vesicles have been applied very successfully for cosmetic purposes using nonionic surfactants and cholesterol, which both solubilized and stabilized β -carotene and allowed their delivery to cultured cells at physiological concentrations. β -carotene contained in niosomes was highly resistant to sunlight, high temperatures, and oxidative stress induced by different sources of free radicals. The carotenoid was extremely stable in culture medium up to 96 hr. Moreover, it was easily taken up by both immortalized and transformed cells at carotenoid concentrations that ranged from 0.1 to 2 μ M. Niosomes also provided a convenient, non-toxic and inexpensive vehicle for β -carotene in cell culture.¹¹⁴

5.1.3.3 WATER-SOLUBLE POWDERS OBTAINED BY DRYING (FREEZE AND SPRAY DRYING) PROCESSES

Because most food matrices are water soluble, many efforts were directed to the formulation of lipophilic pigments (mainly carotenoids) into water-soluble formulations (powders or gels). For hydrophilic pigments like flavonoids, polar dried microcapsules are the most popular ways to stabilize their functionality. Extracts rich in β -carotene were encapsulated using three different encapsulation techniques (spray drying, drum drying, and freeze drying).¹¹⁵

Solid microcapsules using maltodextrins or starch as matrices were incorporated into cosmetic, pharmaceutical, dietetic, and food compositions using the interfacial crosslinking of flavonoids. Such microcapsules prevented discoloration while maintaining both the anti-free radical and antioxidant activities of the flavonoids.¹¹⁶

Kinetic studies on degradation of saffron water-soluble carotenoids (mainly crocins) encapsulated into three different amorphous matrices [pullulan and two polyvinylpyrrolidone (PVP) samples differing in their molecular weights] were carried out under different water activity (a_w) conditions (0.43, 0.53, 0.64, and 0.75) in darkness at 35°C. Degradation of the polar pigments was monitored by periodic measurements of the coloring strength. PVP 40 largely decreased the oxidation rates of crocetin glycosides, being the most effective carrier under all storage conditions.¹¹⁷

The chemical stability and colorant properties of three lyophilized betaxanthin powders from *Celosia argentea* varieties were evaluated. The aqueous solutions containing betaxanthins were bright yellow in the pH range of 2.2 to 7.0, and most stable at pH 5.5. The betaxanthins in a model system (buffer) were susceptible to heat and found to be as unstable as red betacyanins (betanin and amaranthine) at high temperatures (> 40°C), but more stable at 40°C under the exclusion of light and air. Freeze-dried (lyophilized) betaxanthins showed better storage stability (mean 95.0% pigment retention) than corresponding aqueous solutions (14.8%) at 22°C after 20 weeks. Refrigeration (4°C) significantly increased pigment retention of aqueous betaxanthins to 75.5%.⁶⁸ Using concentrated sucrose syrup as a dewatering agent, encapsulation by spray drying of an ethanolic extract using maltodextrin as a coating material or co-crystallization of sucrose syrup with ethanolic extract was successfully applied, in both cases revealing powders with longer shelf lives.¹¹⁸

5.1.3.4 GELIFICATION, COACERVATION, AND MOLECULAR INCLUSION

The polymeric carbohydrates like agar, alginates, carrageenans, and gums are excellent matrices for hydrophilic pigments. They mimic the natural pectin polymeric network that stabilizes those pigments.⁷⁷ Alginates and pectins protect and stabilize anthocyanins, increasing their color stability by intermolecular associations with polyuronic acids.¹¹⁹ Sea buckthorn juice, rich in flavonoid and carotenoid pigments as are many other bioactive antioxidant phytochemicals, was encapsulated by coacervation in furcellaran, a copolymer of β - and κ -carrageenan gels.¹²⁰

Not only hydrophilic pigments are encapsulated in such matrices. The lipophilic pigments in an emulsified formulation can be subsequently included into gel-forming hydrocolloids. A novel solvent-based method using alginates or other hydrocolloids was applied to colored edible oils including pigments.¹²¹ Bixin, the major dye found in annatto extracted from urucum (*Bixa orellana*) seeds, was encapsulated in maltodextrin-rich compositions, with different percentages of Arabic gum or sucrose.¹²²

The longest lifetime, highest incorporation yield (74%), and stabilization were observed for a fraction of “bound bixin” in 95% Arabic gum plus 5% sucrose as the matrix. The minor fraction of bixin, non-encapsulated, showed a short lifetime. When encapsulated in Arabic gum or in β -cyclodextrin compared with DE-20 and

DE-10 maltodextrin encapsulation, the best stability was obtained in the first case,¹²³ the optimal bixin-to-matrix ratio being 1:3.

Effective capsulation by molecular inclusion of natural colorants in cyclodextrins was developed by a Hungarian research group and company. Experimental results indicating the stability of cyclodextrin-complexed curcumin, curcuma oleoresin, β -carotene, and carotenoid oleoresins against light, heat, and oxygen proved the benefits of molecular encapsulation of colorants. The parent β -cyclodextrin was most effective for the curcumins, while the stability of carotenoids was greater with β -cyclodextrin complexation. Methylated β -cyclodextrin was found to be the most potent solubilizing agent for both carotenoids and curcuminoids.¹²⁴ Some carotenoid molecules, depending on their polarity, were specifically incorporated into β -cyclodextrin rings.¹²⁵

REFERENCES

1. MacDougall, D.B., Ed., *Color in Food: Improving Quality*, CRC Press, Boca Raton 2002.
2. Burdock, G.A., Ed., *Encyclopedia of Food and Color Additives*, 3-volume set, 1996.
3. Von Elbe, J.H. and Schwartz, S.J., Colorants, in *Food Chemistry*, 3rd ed., Fennema, O.R., Ed., Marcel Dekker, New York, 1996, 545.
4. Hendry, G.A.F. and Houghton, J.D., eds., *Natural Food Colorants*, 2nd edition, Blackie, London, 1996.
5. Delgado-Vargas, F. and Paredes-López, O., *Natural Colorants for Food and Nutritional Uses*, CRC Press, Boca Raton, 2003.
6. Delgado-Vargas, F., Jimeney, A.R., and Paredes-Lopez, O., Natural pigments: carotenoids, anthocyanins and betalains — characteristics, biosynthesis, processing and stability, *Crit.Rev. Food Sci. Nutr.*, 40, 173, 2000.
7. Lauro, G.J. and Francis, F.J., *Natural Food Colorants*, Marcel Dekker, New York, 2000.
8. Francis, F.J., *Handbook of Food Colorant Patents*, Food and Nutrition Press, Westport, CT, 1986.
9. Hostettmann, K. et al., in *New Developments in the Separation of Natural Products*, Fischer, N.H. et al., Eds., Modern Phytochemical Methods, vol. 25, Plenum Press, New York, 1992, 1.
10. Eggers, R., Supercritical fluid extraction (SFE) of oilseeds/lipids in natural products, in *Supercritical Fluid Technology in Oil and Lipid Chemistry*, King, J.W. and List, G.R., Eds., AOCS Press, Champaign, IL. 1996, 35.
11. Ambrogi, A., Cardarelli, D.A., and Eggers, R., Separation of natural colorants using combined high pressure extraction-adsorption process, *Latin Am. Appl. Sci.*, 33, 323, 2003.
12. Yong Ju, Z. and Howar, L.R., Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin, *J. Agric. Food Chem.*, 51, 5207, 2003.
13. Johnston, D. E., High pressure: a new dimension to food processing, *Chem. Ind.*, 499, 1994.
14. Henry, M.C. and Yonker, C.R., Supercritical fluid chromatography, pressurized liquid extraction, and supercritical fluid extraction, *Anal. Chem.*, 78, 390, 2006.

15. Salvador, P., The use of ultrasonication in the extraction of natural dyes, presented at 2nd International Congress on Pigments in Food, Portugal.
16. Ray, M.S., Equilibrium staged separations: a bibliography update (2004), *Sep. Sci. Technol.*, 40, 1145, 2005.
17. Zougagh, M., Valcarcel, M., and Rios, A., Automatic selective determination of caffeine in coffee and tea samples by using a supported liquid membrane-modified piezoelectric flow sensor with molecularly imprinted polymer, *Trends Anal. Chem.*, 23, 399, 2004.
18. Starmans, D.A.J. and Nijhuis, H.H., Extraction of secondary metabolites from plant material: a review, *Trends Food Sci. Technol.*, 7, 191, 1996.
19. Jarvenpaa, E.P., Rostiala, N., and Huopalahti, R., Utilization of supercritical fluid techniques in the fractionation of natural pigments, in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 29.
20. Shen, J. and Shao, X., Comparison of accelerated solvent extraction, Soxhlet extraction, and ultrasonic-assisted extraction for analysis of terpenoids and sterols in tobacco, *Anal. Bioanal. Chem.*, 383, 1003, 2005.
21. Shen, S. et al., Comparison of solid-phase microextraction, supercritical fluid extraction, steam distillation, and solvent extraction techniques for analysis of volatile constituents in *Fructus amomi*, *J. AOAC Int.*, 88, 418, 2005.
22. González, M. et al., Optimizing conditions for the extraction of pigments in cochineals (*Dactylopius coccus* Costa) using response surface methodology, *J. Agric. Food Chem.*, 50, 6968, 2002.
23. Ogawa, I., Yamano, H., and Miyagawa, K., Application of deaerated water in extraction of colorants from dyer's saffron florets, *Int. J. Appl. Polym. Sci.*, 74, 1701, 1999.
24. Osorio, C. et al., Application of tristimulus colorimetry to obtain natural additives from fruits. I. Color evaluation during osmotic dehydration, in *Proceedings of 4th International Congress on Pigments in Food*, Hohenheim, Germany, Carle, R. et al., Eds., Shaker Verlag, Aachen, 2006, 177.
25. Kopjar, M., Influence of trehalose addition on anthocyanin retention of evaporated and freeze-dried strawberry pastes, in *Proceedings of 4th International Congress on Pigments in Food*, Hohenheim, Germany, Carle, R. et al., Eds., Shaker Verlag, Aachen, 2006, 223.
26. Escot, S. et al., Release of polysaccharides by yeasts and the influence of released polysaccharides on color stability and wine astringency, *Austr. J. Grape Wine Res.*, 7, 153, 2001.
27. Bautista-Ortin, A.B., Improving color extraction and stability in red wines: the use of maceration enzymes and enological tannins, *Int. J. Food Sci. Technol.*, 40, 1, 2005.
28. Mendes, R.L. et al., Supercritical carbon dioxide extraction of compounds with pharmaceutical importance from microalgae, *Inorg. Chim. Acta*, 356, 328, 2003.
29. Lopez, M. et al., Selective extraction of astaxanthin from crustaceans by use of supercritical carbon dioxide, *Talanta*, 64, 726, 2004.
30. Lim, S., Jung, S.K., and Jwa, M.K., Extraction of carotenoids from *Citrus unshiu* press cake by supercritical carbon dioxide, *Food Sci. Biotechnol.*, 12, 513, 2003.
31. Macias-Sanchez, M.D. et al., Supercritical fluid extraction of carotenoids and chlorophyll *a* from *Nannochloropsis gaditana*, *J. Food Eng.*, 66, 245, 2005.
32. Bergeron, C. et al., Comparison of the chemical composition of extracts from *Scutellaria lateriflora* using accelerated solvent extraction and supercritical fluid extraction versus standard hot water or 70% ethanol extraction, *J. Agric. Food Chem.*, 53, 3076, 2005.

33. Del Valle, J.M. and De La Fuente, J.C., Supercritical CO₂ extraction of oilseeds: review of kinetic and equilibrium models, *Crit. Rev. Food Sci. Nutr.*, 46, 131, 2006.
34. McHugh, M.A. and Krukonis, V.J., *Supercritical Fluid Extraction: Principles and Practice*, 2nd ed., Butterworth-Heinemann, Boston, 1994.
35. Raventós, M., Duarte, S. and Alarcón, R., Application and possibilities of supercritical CO₂ extraction in food processing industry: an overview, *Food. Sci. Technol. Int.*, 8, 269, 2002.
36. Stastova, J. et al., Rate of vegetable oil extraction with supercritical CO₂-III: extraction from sea buckthorn, *Chem. Eng. Sci.*, 51, 4347, 1996.
37. Washam, C., An ounce of prevention from a ton of tomatoes, *Env. Health Persp.*, 113, 178, 2005.
38. Santamaria, R.I. et al., Selective enzyme-mediated extraction of capsinoids and carotenoids from Chili Guajillo Puja (*Capsicum annum L.*) using ethanol as solvent, *J. Agric. Food Chem.*, 48, 3063, 2000.
39. Kammerer, D. et al., A novel process for the recovery of polyphenols from grape (*Vitis vinifera L.*) pomace, *J. Food Sci.*, 70, 157, 2005.
40. Delgado-Vargas, F. and Paredes-López, Effects of enzymatic treatments of marigold flowers on lutein isomeric profiles, *J. Agr. Food Chem.*, 45, 1097, 1997.
41. Barzana, E. et al., Enzyme-mediated solvent extraction of carotenoids from marigold flower (*Tagetes erecta*), *J. Agr. Food Chem.*, 50, 4491, 2002.
42. Navarette-Bolanos, J.L. et al., Viable alternative processing to increase xanthophyll extraction from marigold flower (*Tagetes erecta*), in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 41.
43. Duarte de Holanda, H. and Netto, F.M., Recovery of components from shrimp (*Litopenaeus setiferus*) processing waste by enzymatic hydrolysis, *J. Food Sci.*, 71, C298, 2006.
44. Lapornik, B. et al., Influence of enzyme maceration on anthocyanin extraction from blackcurrant *Ribes nigrum* (L.) berries, in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 32.
45. Ben-Amotz, A., Katz, A., and Avron, M., Accumulation of β -carotene in halotolerant algae: purification and characterization of β -carotene-rich globules from *Dunaliella bardawil* (chlorophyceae), *J. Physiol.*, 18, 529, 1982.
46. Hejazi, M.A. et al., Selective extraction of carotenoids from the microalga *Dunaliella salina* with retention of viability, *Biotechnol. Bioeng.*, 79, 29, 2002.
47. Hejazi, M.A., Kleinegris, D., and Wijffels, R.H., Mechanism of extraction of β -carotene from microalga *Dunaliella salina* in two-phase bioreactors, *Biotechnol. Bioeng.*, 88, 593, 2004.
48. Hejazi, M.A., Holwerda, E., and Wijffels, R.H., Milking microalga *Dunaliella salina* for β -carotene production in two-phase bioreactors, *Biotechnol. Bioeng.*, 85, 475, 2004.
49. Kraemer-Schafhalter, A., Fuchs, H., and Pfannhauser, W., Solid-phase extraction (SPE): a comparison of 16 materials for the purification of anthocyanins from *Aronia melanocarpa* var Nero, *J. Sci. Food Agr.*, 78, 435, 1999.
50. Cabrera, R. and Fernandez Lahore, H.M., Downstream processing of carminic acid from raw cochineal, in *Proceedings of 4th International Congress on Pigments in Food*, Hohenheim, Germany, Carle, R. et al., Eds., Shaker Verlag, Aachen, 2006, 203.
51. Di Mauro, A. et al., Recovery of hesperidin from orange peel by concentration of extract on styrene-divinylbenzene resin, *J. Agric. Food Chem.*, 47, 4391, 1999.
52. DiMauro, A. et al., Recovery of anthocyanins from pulp wash of pigmented oranges by concentration on resins, *J. Agric. Food Chem.*, 50, 5968, 2002.

53. Scordino, M. et al., Adsorption and recovery of anthocyanins on resins, in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 78.
54. Conway, W.D. and Petroski, R.J., *Modern Countercurrent Chromatography*, ACS Symposium Series 593, American Chemical Society, Washington, 1995.
55. Ito, Y., Efficient preparative counter-current chromatography with a coil planet centrifuge, *J. Chromatogr.*, 214, 122, 1981.
56. Ito, Y. and Conway, W.D., *High Speed Countercurrent Chromatography*, Wiley Interscience, New York, 1996.
57. Degenhardt, P. and Winterhalter, E., Isolation of natural pigments by high speed CCC, *J. Liq. Chromatogr. Rel. Technol.*, 24, 1745, 2001.
58. Degenhardt, P., Winterhalter, E., Separation of natural food colorants, *Chem. Innov.*, 30, 25, 2000.
59. Degenhardt, U.H. et al., Centrifugal precipitation chromatography: a novel chromatographic system for fractionation of polymeric pigments from black tea and red wine, *J. Agric. Food Chem.*, 49, 1730, 2001.
60. Degenhardt, P. and Winterhalter, E., HSCCC: a powerful tool for the preparative isolation of bioactive compounds, in *Biologically-Active Phytochemicals in Food: Analysis, Metabolism, Bioavailability and Function*, Pfannhauser, W. et al., Eds., Royal Society of Chemistry, Cambridge, 2001, 143.
61. Köhler, N. and Winterhalter, P., Large-scale isolation of flavan-3-ol fluoroglucinol adducts by high-speed counter-current chromatography, *J. Chromatogr. A*, 1072, 217, 2005.
62. Schwarz, M. et al., Application of high-speed countercurrent chromatography to the large-scale isolation of anthocyanins, *Biochem. Eng. J.*, 14, 179, 2003.
63. Schwarz, M., Wray, V., and Winterhalter, P., Isolation and identification of novel pyranoanthocyanins from black carrot (*Daucus carota* L.) juice, *J. Agric. Food Chem.*, 52, 5095, 2004.
64. Aman, R., Isolation of carotenoids from plant materials and dietary supplements by high-speed counter-current chromatography, *J. Chromat.*, 1074, 107, 2005.
65. Köhler, N. et al., Development of a new preparative spiral-coil low speed rotary countercurrent chromatographic (spiral-coil LSRCCC) method, *J. Liq. Chromatogr. Rel. Technol.*, 27, 2547, 2004.
66. Chen, F. et al., Isolation and purification of the bioactive carotenoid zeaxanthin from the microalga *Microcystis aeruginosa* by high-speed counter-current chromatography, *J. Chromatogr.*, 1064, 183, 2005.
67. Salas, E. et al., Isolation of flavanol-anthocyanin adducts by countercurrent chromatography, *J. Chrom. Sci.*, 43, 488, 2005.
68. Cai, Y.Z. and Corke, H., Production and properties of spray-dried *Amaranthus* betacyanin pigments, *J. Food Sci.*, 65, 1248, 2000.
69. Raynie, D.E., Modern extraction techniques, *Anal. Chem.*, 78, 3997, 2006.
70. Socaciu, C., Modern strategies of chemical control for food safety assessment: Proceedings of International Symposium, USAMV, Cluj-Napoca, *Bull. USAMV*, 60, 34, 2004.
71. Jackson, L.S. and Lee, K., Microencapsulation in the food industry, *Lebensmit. Wissen. Technol.*, 24, 289, 1991.
72. Heinzen, C., Microencapsulation solve time-dependent problems of foodmakers, *Eur. Food Drink Rev.*, 3, 27, 2002.
73. Balassa, L. and Fanger, G.O., Microencapsulation in food industry, *Crit. Rev. Food Technol.*, 2, 245, 1971.

74. King, A.K., Encapsulation of food ingredients: a review of available technology, focusing on hydrocolloids, in *Encapsulation and Controlled Release of Food Ingredients*, Risch, S.J. and Reineccius, G.A., Eds., American Chemical Society, Washington, 1995, 26.
75. Dziezak, J.D., Microencapsulation and encapsulated ingredients, *J. Food Technol.*, 42, 136, 1988.
76. Shahidi, F. and Han, X.Q., Encapsulation of food ingredients, *Crit. Rev. Food Sci. Nutr.*, 6, 13, 1993.
77. Kirby, C.J., Microencapsulation and controlled delivery of food ingredients, *Food Sci. Technol. Today*, 5, 74, 1991.
78. Poncelet, D. et al., Formation of microgel beads by electrostatic dispersion of polymer solutions, *AIChE J.*, 45, 2018, 2003.
79. Israelachvili, J., The science and applications of emulsions: an overview, *Colloid Surface A*, 91, 1, 1994.
80. Tadros, T. et al., Formation and stability of nano-emulsions, *Adv. Colloid Interface Sci.*, 108, 303, 2004.
81. Tenjarla, S., Microemulsions: an overview and pharmaceutical applications, *Crit. Rev. Ther. Drug*, 16, 461, 1999.
82. Amar, I., Aserin, A., and Garti, N., Solubilization patterns of lutein and lutein esters in food grade nonionic microemulsions, *J. Agric. Food Chem.*, 51, 4775, 2003.
83. Amar, I., Aserin, A., and Garti, N., Microstructure transitions derived from solubilization of lutein and lutein esters in food microemulsions, *Colloid Surface B*, 33, 143, 2004.
84. Chmiel, O., Traitler, H., and Voepel, K., Food microemulsion formulations, U.S. Patent 5,674,549, 1997.
85. Flanagan, J. and Singh, H., Microemulsions: a potential delivery system for bioactives in food, *Crit. Rev. Food Sci. Nutr.*, 46, 221, 2006.
86. Gaonkar, A.G. and Bagwe, R.P., Microemulsions in foods: challenges and applications, *Surfactant Sci. Ser.*, 109, 407, 2003.
87. Garti, N., Aserin, A., and Fanun, M., Non-ionic sucrose esters microemulsions for food applications. Part 1. Water solubilization, *Colloid Surface A*, 164, 27, 2000.
88. Garti, N., Microemulsions as microreactors for food applications, *Curr. Opin. Colloid Int.*, 8, 197, 2003.
89. Paul, B.K. and Moulik, S.P., Microemulsions: an overview, *J. Disper. Sci. Technol.*, 18, 301, 1997.
90. Spornath, A. et al., Food-grade microemulsions based on nonionic emulsifiers: media to enhance lycopene solubilization, *J. Agric. Food Chem.*, 50, 6917, 2002.
91. Glatter, O. et al., Sugar-ester nonionic microemulsion: Structural characterization, *J. Colloid Interface Sci.*, 241, 215, 2001.
92. Batista, A.P. et al., Phycocyanin and Lutein colored food emulsions: relation between pigment concentration and structural properties, in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 118.
93. Raymundo, A. et al., Novel emulsions containing functional colorings in both phases, in *Proceedings of 2nd International Congress on Pigments in Food*, Quimica Publications, Lisbon, 2002, 189.
94. Gouveia, L., Coloring emulsions using microalgal biomass: stability studies, in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 121.
95. Grolier, P. et al., Incorporation of carotenoids in aqueous systems: uptake by cultured rat hepatocytes, *Biochim. Biophys. Acta*, 1111, 135, 1992.

96. New, R.R.C., Preparation of liposomes, in *Liposomes: A Practical Approach*, New, R.R.C., Ed., Oxford University Press, New York, 1990, 33.
97. Hope, M.J. et al., Generation of multilamellar and unilamellar phospholipid vesicles, *Chem. Phys. Lipids*, 40, 89, 1986.
98. Lasch, J., Weissig, V., and Brandl, M., Preparation of liposomes, in *Liposomes: A Practical Approach*, Torchilin J. and Weissig, V., Eds., Oxford University Press, New York, 2003, 3.
99. Mui, B., Chow, L., and Hope M.J., Extrusion technique to generate liposomes of defined size, *Meth. Enzymol.*, 367, 3, 2003.
100. Gibbs, B.F. et al., Encapsulation in the food industry: a review, *Int. J. Food Sci. Nutr.*, 50, 213, 1999.
101. Lasic, D.D., Novel applications of liposomes, *Trends Biotechnol.*, 16, 307, 1998.
102. Law, B.A. and King, J.S., Use of liposomes for proteinase addition to cheddar cheese, *J. Dairy Res.*, 52, 183, 1985.
103. Reineccius, G.A., Liposomes for controlled release in the food industry, in *Encapsulation and Controlled Release of Food Ingredients*, Risch, S.J., Ed., American Chemical Society, Washington, 1995, 113.
104. Taylor, T.M. et al., Liposomal nanocapsules in food science and agriculture, *Crit. Rev. Food Sci. Nutr.*, 45, 587, 2005.
105. Li, L., Braiteh, F.S., and Kurzrock, R., Liposome-encapsulated curcumin: *in vitro* and *in vivo* effects on proliferation, *Apopt. Signal. Angiogen. Cancer*, 104, 1322, 2005.
106. Socaciu, C., Jessel, R., and Diehl, H.A., Competitive carotenoid and cholesterol incorporation into liposomes: effects on membrane phase transition, fluidity, polarity and anisotropy, *Chem. Phys. Lipids*, 106, 79, 2000.
107. Liebler, D.C. et al., Antioxidant actions of beta-carotene in liposomal and microsomal membranes: role of carotenoid-membrane incorporation and alfa-tocopherol, *Arch. Biochem. Biophys.*, 338, 244, 1997.
108. Millichip, M. et al., Purification and characterization of oil-bodies (oleosomes) and oil-body boundary proteins (oleosins) from the developing cotyledons of sunflower (*Helianthus annuus* L.), *Biochem. J.*, 314, 333, 1996.
109. Socaciu, C. et al., Carotenoid-rich fractions in sea buckthorn berry oleosomes: separation, characterisation and stability in colloid supramolecular structures, in *Proceedings of 4th International Congress on Pigments in Food*, Hohenheim, Germany, Carle, R. et al., Eds., Shaker Verlag, Aachen, 2006, 203.
110. Nanda, A. et al., Transferosomes: a novel ultradeformable vesicular carrier for transdermal drug delivery, *Drug Del. Technol.*, 5, 1, 2005.
111. Arunothayanun, P. et al., *In vitro/in vivo* characterization of polyhedral niosomes, *Int. J. Pharm.*, 183, 57, 1999.
112. Vanlerberghe, G. and Morancais, J.L., Niosomes in perspective, *STP Pharm. Sci.*, 1, 5, 1996.
113. Baillie, A.J. et al., The preparation and properties of niosomes-non-ionic surfactant vesicles, *J. Pharm. Pharmacol.*, 37, 863, 1985.
114. Palozza, P. et al., Solubilization and stabilization of β -carotene in niosomes: delivery to cultured cells, *Chem. Phys. Lipids*, 139, 322, 2006.
115. Desobry, S.A., Netto, F.M., and Labuza, T.P., Comparison of spray-drying, drum-drying and freeze drying for β -carotene encapsulation and preservation, *J. Food Sci.*, 62, 1158, 1997.
116. Levy, M.C. and Andry, M.C., Crosslinked flavonoid microencapsulation, *Trends Food Sci. Technol.*, 7, 205, 1996.

117. Selim, K., Tsimidou, M., and Biliaderis, C.G., Kinetic studies of degradation of saffron carotenoids encapsulated in amorphous polymer matrices, *Food Chem.*, 71, 199, 2000.
118. Sinuco, D.C. et al., Application of tristimulus colorimetry to obtain natural additives from fruits. II. Color characteristics of solids, in *Proceedings of 4th International Congress on Pigments in Food*, Hohenheim, Germany, Carle, R. et al., Eds., Shaker Verlag, Aachen, 2006, 180.
119. Hubbermann, E.M. et al., Influence of acids, salt, sugars and hydrocolloids on the color stability of anthocyanin rich black currant and elderberry concentrates, *Eur. Food Res. Technol.*, 223, 83, 2006.
120. Laos, K., Lougas, T., and Vokk, R., Encapsulation of β -carotene of sea buckthorn (*Hippophae rhamnoides*) juice in furcellaran beads, in *Proceedings of 4th International Congress on Pigments in Food*, Hohenheim, Germany, Carle, R. et al., Eds., Shaker Verlag, Aachen, 2006, 256.
121. Buthe, A., Hartmeier, W., and Ansorge-Schumacher, M.B., Novel solvent-based method for preparation of alginate beads with improved roundness and predictable size, *J. Microencapsulation*, 21, 865, 2004.
122. Barbosa, M.I.J., Borsarelli, C.D., and Mercadante, A.Z., Light stability of spray-dried encapsulated bixin with different edible polysaccharide preparations, in *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 53.
123. Constant, P.B.L. et al., Spray drying microencapsulation of the bixin natural colorant, *Proceedings of 3rd International Congress on Pigments in Food*, Le Berre, Quimper, France, 2004, 154.
124. Szente, L. et al., Stabilization and solubilization of lipophilic natural colorants with cyclodextrins, *J. Inclusion Phenom. Mol. Recognit. Chem.*, 32, 81, 1998.
125. Lancrajan, I. et al., Carotenoid incorporation into natural membranes from artificial carriers: liposomes and β -cyclodextrins, *Chem. Phys. Lipids*, 112, 1, 2001.

5.2 Food Colorants Derived from Natural Sources by Processing

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5.2.1 COLORANTS OBTAINED BY EXTRACTION

5.2.1.1 TURMERIC AND CURCUMINE

5.2.1.1.1 Sources

Turmeric is an aromatic spice obtained from the dried ground rhizomes of *Curcuma longa* L., a perennial shrub that belongs to genus *Curcuma* of the Zingiberaceae family. More than 100 species of curcuma were described but *Curcuma longa* L. is

the commercially most important one. *Curcuma longa* L. is cultivated and propagated by its rootstalks. It has bright green leaves, conical yellow flowers, and reaches maturity after 7 to 10 months, when rhizomes are harvested. The dried ground rhizomes yield a bright yellow powder also known as yellow ginger or Indian saffron. The plant grows in warm and rainy regions in Asia and South America and is cultivated in India, Indonesia, China, Jamaica, and Peru. The main producer is India (more than 600,000 tons or 75% of world production); most is produced for domestic consumption.

Turmeric is used mainly as a spice, to give specific flavor and color, but also as an additive for maintaining freshness and improving the palatability and shelf lives of perishable foods. Turmeric is also well documented for its biological effects and it was widely used in traditional Indian and Chinese medicine. In Ayurveda, turmeric is used as a stomachic, tonic, and blood purifier, and also in the treatment of skin diseases.¹⁻⁵

5.2.1.1.2 Chemistry, Properties, and Extraction

Turmeric contains two main classes of compounds: the curcuminoids responsible for the yellow color and the aroma compounds. The coloring principle of turmeric consists of three major phenolic derivatives: curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Commercially available products called curcumins contain curcumin (1,7-bis(4-hydroxy-methoxyphenyl)-1,6-heptadiene-3,5-dione) as the major component (about 77% of total curcuminoids). Some other minor phenolic compounds such as cyclocurcumin and calebin were also isolated from turmeric.^{1,2,4,6} See Figure 5.2.1.

Recently, 19 diarylheptanoids, of which 6 are new, were separated and identified in turmeric by LC-ESI-MS/MS coupled to a DAD detector.⁷ More than 20 compounds were identified in the volatile oil extracted from turmeric by different meth-

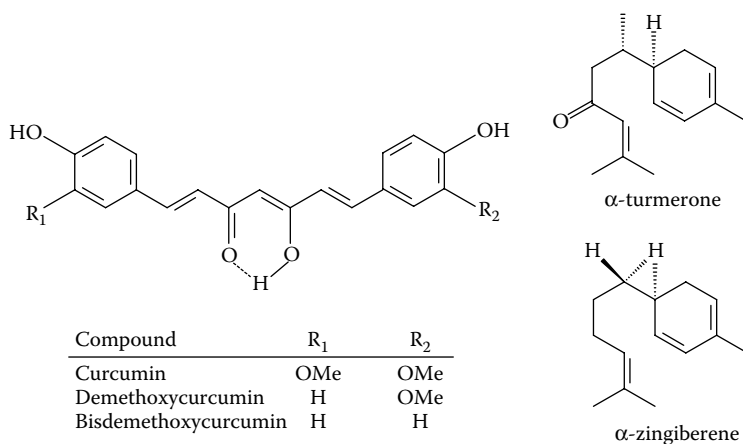


FIGURE 5.2.1 Chemical structures of curcuminoid pigments and some aroma compounds of turmeric.

ods, among them α - and β -turmerone, *ar*-turmerone, *ar*-curcumene, turmeronol, zingiberene, and phellandrene. These compounds are responsible for the specific flavor of turmeric.²

Curcuminoids are not soluble in water but are soluble in various organic solvents. Their solubility is higher in polar organic solvents and lower in aliphatic solvents. Curcumin is almost insoluble in acidic water solution but is soluble in alkali. At a pH above neutral, curcuminoids undergo rapid hydrolytic degradation, giving rise to feruloyl methane, ferulic acid, vanillin, and colored condensation products. The alkaline degradation of curcuminoids corresponds to pseudo-first-order kinetics.^{8,9} The degradation rate increases from pH 7.45 to a maximum at pH 10.2 and decreases at higher pH levels. The same behavior was observed for pure compounds, combined pigments, and oleoresins containing curcuminoids. Bisdemethoxycurcumin is most resistant in alkaline conditions, with a half life of 2200 hr at pH 7.45 and 5 hr at pH 10.2, compared to 900 hr and 0.4 hr, respectively, for curcumin.⁹ The complexation of curcumin with cyclodextrins strongly improved water solubility and stability under alkaline conditions, but the photodecomposition rate increased.^{10,11} Several patented methods have increased the stability of turmeric under light and alkaline conditions by the addition of acids (gallic, citric, gentisic) as stabilizers.⁴

In organic solvents under light exposure, curcumin decomposes and forms photolysis products that have been identified.¹² Curcuminoids were tested in a microcrystalline cellulose model system for storage stability at 21°C under various conditions (air, nitrogen, light, darkness). They were degraded following a first-order reaction under all conditions of testing. The most deleterious condition was the combination of air and light.¹³ The addition of aluminium increased the light and heat stability of curcumin in pickles and reduced curcumin decomposition caused by peroxidases.¹⁴

Curcuminoid solutions show strong absorption between 300 and 500 nm. Absorption spectra of curcumin recorded in different solvents show large red shifts in polar solvents compared with nonpolar solvents. The absorption maximum of curcumin is at 408 nm in cyclohexane, 420 nm in methanol, and 430 nm in N,N-dimethyl formamide. The absorption spectra are broad and have shoulders that indicate the presence of more than one isomeric form.¹⁵ For the estimation of curcuminoid contents in commercial products, the absorbance of ethanol solution is determined at 426 nm and an extinction coefficient of 1607 is used.¹⁶ Curcuminoids also exhibit fluorescence, depending on the solvent used. In the case of curcumin solution in methanol, when excited with a 355 nm laser pulse, the fluorescence maxima appear at 560 nm, probably because of formation of hydrogen bonds with the enolic form of the pigment. The fluorescence maxima are at lower wavelengths in less polar solvents.¹⁵ Both UV-VIS absorption and fluorescence can be used for identification and quantitative determination of curcuminoids. The physico-chemical and color characteristics of pure curcuminoid pigments were determined after chromatographic separation (TLC, HPLC).¹⁷ See Table 5.2.1.

The three commercial forms based on *Curcuma longa* L. are turmeric powder, turmeric oleoresin, and curcumin powder. Turmeric powder is obtained from dried rhizomes. The fresh material is dried under open sun ($\approx$ 11 days) or in conventional (fuel, electric) or unconventional driers.¹⁸ Dried rhizomes can be polished to improve

TABLE 5.2.1
Physicochemical and Color Characteristics of Curcuminoid Pigments

Parameter	Curcumin	Demethoxycurcumin	Bisdemethoxycurcumin
Melting point (°C)	184	172	222
$\lambda_{\max}$ in ethanol (nm)	429	424	419
Molar absorptivity in ethanol, at 425 nm ($\times 10^4$ L cm ⁻¹ mol ⁻¹)	6.73	5.78	4.95
<i>L</i> *	72.84	72.15	81.54
<i>a</i> *	16.84	1.96	-4.72
<i>b</i> *	110.06	82.73	49.44
<i>Chroma</i>	11.34	82.75	49.64
<i>Hue</i>	81.30	88.64	84.55

Source: Adapted from Péret-Almeida, L. et al., *Food Res. Int.*, 38, 1039, 2005.

their appearance and then are ground to yield a fine powder that should be stored in dark places. The turmeric powder standardized with maltodextrin contains 8 to 9% curcumin.⁴

The oleoresin is obtained from turmeric powder by solvent extraction. Solvents approved for use by European Commission are ethylacetate, acetone, carbon dioxide, dichloromethane, n-butanol, methanol, ethanol, and hexane.¹⁶ The U.S. Food and Drug Administration (FDA) also authorized the use of mixtures of solvents that include those mentioned earlier plus isopropanol and trichloroethylene.¹⁹ After filtration the solvents must be completely removed from the oleoresin.

Turmeric oleoresin is a deep orange viscous oil that can be suspended in ethanol, propylene glycol, or edible vegetable oils. Turmeric powder and turmeric oleoresin contain pigments, flavor compounds (volatile oils), resins, and fats. Both can be used as spices and as food colorants. Curcumin powder is obtained from the turmeric oleoresin by crystallization. It appears as an orange-yellow crystalline powder with a melting point at 179 to 182°C. It is soluble in ethanol, propylene glycol, and acetone and insoluble in water. Curcumin powder, known as food colorant E 100, must contain not less than 90% pigment.^{1,4,14}

5.2.1.1.3 Uses as Food Colorants

Turmeric is a common spice and coloring agent for many food items. It has the special property of imparting both color and flavor. The best-known application is as an ingredient of curry powder and other spices. It is widely used as spice in India, the Middle East, and the Far East. Other applications are coloring mustard, pickles, mayonnaise, salad dressings, oils, and cauliflower. In the European Union (EU), curcumin (E 100, CI 75300) is permitted in alcoholic beverages (*quantum satis*), jam, jellies, marmalades (100 mg/kg), sausages, pâtés (20 mg/kg), dried potato granules and flakes, non-alcoholic drinks, and confectionery.²⁰ The FDA approved turmeric powder (CI 73600) and turmeric oleoresin (CI 73615) but not curcumin

powder for general use in foods. Turmeric powder is used in the 0.2 to 60 ppm range and the oleoresin in the 2 to 640 ppm range.^{4,9} According to JECFA, the acceptable daily intake (ADI) was established at 0 to 3 mg kg/body weight based on no observed effects in studies on rats.²¹

In addition to their coloring and flavoring properties, curcuminoids have functional properties in foods. Their antioxidant effects were evaluated using standardized aqueous and ethanolic extracts of turmeric prepared as under cooking conditions. The extracts were tested for antioxidant availability during actual cooking conditions and in therapeutic applications by ORAC, DPPH, ABTS, FRAP, and inhibition of lipid peroxidation tests. The study showed that ethanol extracts were more effective as antioxidants at various levels, by reducing complexes in radical formation, radical scavenging, and membrane protection.²²

Antioxidant capacities of common individual curcuminoids were determined *in vitro* by phosphomolybdenum and linoleic acid peroxidation methods. Antioxidant capacities expressed as ascorbic acid equivalents ($\mu\text{mol/g}$) were 3099 for curcumin, 2833 for demethoxycurcumin, and 2677 for bisdemethoxycurcumin at concentrations of 50 ppm. The same order of antioxidant activity (curcumin > demethoxycurcumin > bisdemethoxycurcumin) was observed when compared with BHT (butylated hydroxyl toluene) in linoleic peroxidation tests.²³ The antioxidant activity of curcumin in the presence of ethyl linoleate was demonstrated and six reaction products were identified and structurally characterized. The mechanism proposed for this activity consisted of an oxidative coupling reaction at the 3' position of the curcumin with the lipid and a subsequent intramolecular Diels-Alder reaction.²⁴

The addition of turmeric to dill pickles with oxygen injected into the jars determined a decrease of hexanal, pentanal, (E)-2-hexenal, and heptanal concentrations. These aldehydes are responsible for the formation of oxidative off-flavors of pasteurized pickles in the presence of oxygen. At commercial coloring levels, turmeric maintained aldehyde levels in containers with controlled oxygen added — close to levels of pickles packed in glass vessels. This proved that turmeric acts as an antioxidant in this type of product.²⁵ Turmeric also showed antioxidant and antimycotic activities in butter cakes by preventing oxidation and retarding mold growth.²⁶

A water-soluble form of curcumin (prepared from mother liquor and Tween 60) was sprayed onto expanded extruded balls made from corn and defatted soybean flours and tested for stability. After 10 wk of storage (27°C, 65% relative humidity), the retention of curcumin was 77% compared to 92% of tartrazine. The effective shelf life of the product is 6 wk and retention levels of curcumin and tartrazine were 83 and 93%, respectively.²⁷ Curcumin incorporated in gels (gelatine or mixture of other gelling agents) showed good stability during storage, demonstrated by small variations of the instrumental parameters of lightness L^* and hue H^* .²⁸ Turmeric products are susceptible to microbial attack and some precautions must be taken to avoid infestation. The rhizomes and products must be treated with synthetic products or with gamma irradiation. Gamma irradiation has the advantage of being non-toxic and does not influence the color stability or antioxidant activity of turmeric.^{29,30} All these properties recommend curcumin as a potent candidate to replace the synthetic tartrazine pigment.

5.2.1.2 COCHINEAL, CARMINE, KERMES, AND LAC

5.2.1.2.1 Sources

Cochineal has been known as a pigment since ancient times, in Egypt and in pre-colonial America. Cochineal was used in cosmetics, textiles, paints, foods, and medicines. The best-known variety is the American cochineal extracted from female *Dactylopius coccus* Costa cochineal insects. The insect is a parasite on the aerial parts of cacti that belong to the *Opuntia* and *Nopalea* spp. Female insects have oval shapes, are wingless, and weigh approximately 45 mg, of which 70% is lost after drying. They reach maximum pigment content — about 22% of dried weight — just before laying eggs. About 80,000 to 100,000 insects provide 1 kg of raw cochineal dye.^{4,31}

Cochineal was introduced in Europe in the 15th century, after the colonization of the American continent, and was one of the most valuable products originating from the new world. Peru (90%), Mexico, and Spain's Canary Islands are the main producers of American cochineal today.⁴ Similar products extracted from other species were used in Europe from the earliest times. Polish cochineal is extracted from *Margarodes polonicus* (*Porphyrophora polonica*), an insect found on the roots of the *Scleranthus perennis* grass that grows in sandy areas of Europe. Armenian red is prepared from *Porphyrophora hameli* insects that grow on the roots and stems of grass species from areas of alkaline soils in Armenia.³¹

Oaks and especially *Quercus coccifera* are host plants for *Kermes ilicis*, producing the red kermes pigment. In Asia, the *Laccifer lacca* insects that grow on trees like *Schleichera oleosa*, *Ziziphus mauritiana*, and *Butea monspersma* are the sources of the red lac dye.^{4,31}

5.2.1.2.2 Chemistry, Properties, and Extraction

Cochineals contain several compounds with anthraquinonic structures; the most important is carminic acid. An uncommon chemical feature of carminic acid and its derivatives is the presence of a C-glucosidic bond (Figure 5.2.2).

Carminic acid is a water-soluble compound, stable under conditions of light and heat. It shows a maximum absorption at 518 nm in aqueous ammonia solutions and at 494 nm in diluted hydrochloric acid.²⁰ The cochineal color is pH sensitive. In

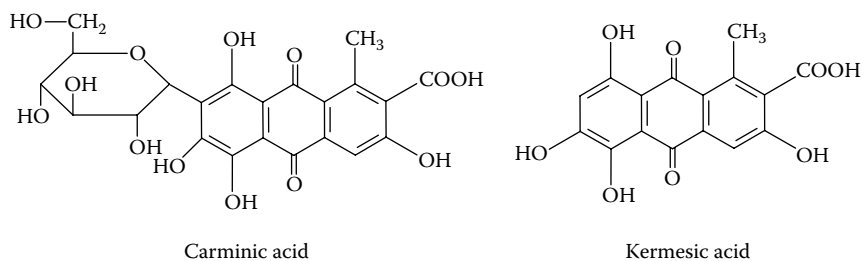


FIGURE 5.2.2 Structures of carminic and kermesic acids.

solution at pH 4, carminic acid is yellow to orange, depending on concentration. At alkaline pH and in the presence of metals (mainly aluminium), it becomes bluish red.^{4,32} Kermesic acid, flavokermesic acid, and four other pigments in addition to carminic acid were separated from *Dactylopius coccus* Costa. Although they are minor compounds, they can influence the final quality of the cochineal extract.

Better quality and higher commercial value of cochineal correlate with a higher ratio of red to yellow pigments. Combining the chromatic attributes of the extracts with the chromatographic determination of pigments, it is possible to achieve greater accuracy in the quality of the final product and in the characterization of geographical origin.^{33,34,35}

Carmines are defined as the lakes of carminic acid with various metals. The most used is carmine, the aluminium lake of carminic acid. Carmine is not soluble in water or oils and is also very stable under condition of light, heat, oxidation, and sulfur dioxide.^{4,32}

Cochineal pigments are extracted from dried bodies of female insects with water or with ethanol; the result is a red solution that is concentrated in order to obtain the 2 to 5% carminic acid concentration customary for commercial cochineal. For carmine lakes, the minimum content of carminic acid is 50%.^{4,16,36} An industrial procedure applied in Spain uses ammonium hydroxide as the extracting agent and phosphoric acid as the acidifying agent.³⁷ For analytical purposes the extraction is carried out with 2 N HCl at 100°C.³⁵ The chemical synthesis of carminic acid has also been reported and is the subject of European and United States patents.³⁸

5.2.1.2.3 Uses as Food Colorants

Cochineal, carminic acid, and carmines are approved as food colorants in the EU under code E 120, and their purity criteria are regulated.^{16,20} The amount of E 120 permitted in food ranges from 50 to 500 mg/kg. Carminic acid and carmine are considered very good food colorants due to their high stability and tinctorial properties. Solutions of carminic acid are yellow to orange, while carmines show various stable brilliant red hues.^{4,39}

In the EU, the use of cochineal derivatives is authorized for coloring alcoholic and non-alcoholic drinks, candied fruits and vegetables, red fruit preserves, confectionery, ices, bakery products, cheeses, jam, jellies, marmalades, fruit-flavored cereals, and other products.²⁰

Special applications are allowed in certain meat products: sausages, salamis, poultry products, surimi, and marinades.^{4,20} Cochineal added to processed pork meat provides a color similar to meat colored with erythrosine, but the color stability is higher.⁴⁰ Cochineal in gelatine gels did not change hue after 36 days of storage in a chamber at 22°C. It can replace synthetic pigments (tartrazine, azorubine) for coloring jellies.²⁸ Cochineal is usually provided as an alkaline solution but it can also be spray dried or delivered in other formulations containing propylene glycol, glycerine, or citric acid.⁴

In the US, cochineal derivatives are permitted in amounts ranging from 0.05 to 1.0%.³⁶ The JECFA considered acceptable a total ADI of 0 to 5 mg/kg of body weight for carmines as ammonium, calcium, potassium, or sodium salts.⁴¹ Some

concerns regarding the use of cochineal derivatives are related to adverse reactions after occupational exposure, contact, or consumption of colored food.^{41,42} Kermes and lac cannot be used as food colorants in the EU or the US.

5.2.2 COLORANTS OBTAINED BY HEAT TREATMENT: CARAMEL

5.2.2.1 INTRODUCTION AND DEFINITIONS

Caramel colors are the most widely used food coloring agents found in a wide range of foods and beverages. Caramel represents a significant segment of the color market — about 11% of total food color production. It is obtained by heating sugars in various conditions. *Caramel* is used also to define a confectionery product manufactured by heating a mixture of glucose syrup, milk, and fats. This section discusses only caramel colors — food additives used mainly to improve both the colors and aromas of various foods.

Caramel color is a dark brown or even black product used for centuries in home cooking to provide color and specific aromas to foods.^{43,44,45} The first commercial caramel was produced in Europe about 1850.⁴

According to the FDA, “The color additive caramel is the dark-brown liquid or solid material resulting from the carefully controlled heat treatment of the following food-grade carbohydrates: dextrose, invert sugar, lactose, malt syrup, molasses, starch hydrolysates and fractions thereof, sucrose.”³⁶ According to JECFA, “Caramel is a complex mixture of compounds, some of which are in the form of colloidal aggregates, manufactured by heating carbohydrates either alone or in the presence of food-grade acids, alkalis or salts; classified according to the reactant used.”⁴¹ Caramels are classified into four classes according to the method and reactant used (Table 5.2.2).

5.2.2.2 PREPARATION

The oldest way to produce caramel is by heating sucrose in an open pan, a process named caramelization. Food applications require improvement in caramel properties such as tinctorial power, stability, and compatibility with food. Caramels are produced in industry by controlled heating of a rich carbohydrate source in the presence of certain reactants. Carbohydrate sources must be rich in glucose because caramelization occurs only through the monosaccharide. Several carbohydrate sources can be used: glucose, sucrose, corn, wheat, and tapioca hydrolysates.⁴ The carbohydrate is added to a reaction vessel at 50°C and then heated to temperatures higher than 100°C. Different reactants such as acids, alkalis, salts, ammonium salts, and sulfites can be added, depending on the type of caramel to be obtained (Table 5.2.2).

The caramelization process can be conducted in open or closed vessels. The mixture obtained is cooled and filtered, and then the pH and specific gravity are adjusted by the addition of acids, alkalis, or water. The chemical composition and properties of caramel colors depend on reactants used and technical conditions such as time, temperature, moisture content, and pressure.^{4,46} During the caramelization

TABLE 5.2.2
Classes of Caramel

JECFA Classification	EU Classification	Reactants Used for Preparation	Charge
Class I	Plain caramel E 150a	With or without acids, alkalis, salts, with exception of ammonium and sulfite	–
Class II	Caustic sulfite caramel E 150b	With or without acids, alkalis, salts, in presence of sulfites (sulfurous acid, Na or K sulfite and bisulfite); no ammonium compounds used	–
Class III	Ammonia caramel E 150c	With or without acids, alkalis, salts, in presence of ammonium compounds (hydroxide, carbonate, ammonium hydrogen carbonate, phosphate); no sulfite used	+
Class IV	Sulfite ammonia caramel E 150d	With or without acids, alkalis, salts, in presence of ammonium compounds and sulfite	+

Source: Adapted from Francis, F.J., in *Colorants*, Eagan Press, St. Paul, MN, 1999; Commission Directive 95/45/EC, *Off. J. European Commun.*, L226, 22.09, 1995; JECFA, <http://www.fao.org>; Sikora, M. and Tomasik, P., *Stärke*, 46, 150, 1994.

process aroma compounds are also formed. Caramelization takes place also during heating of food products rich in sugars together with Maillard product formation.

5.2.2.3 CHEMISTRY AND PROPERTIES

A wide range of compounds are generated during the caramelization process. Sugar thermal degradation occurs through reaction sequences that include enolization (Bruijn van Eckenstein rearrangement), dehydration, cleavage, retroaldolization, aldolization, and radical reaction.⁴⁴ Volatile and non-volatile fractions are formed. The volatile fraction has been characterized but the composition of the non-volatile fraction that accounts for 95% (w/w), had not yet been completely elucidated. After a complex study based on different separation methods, the caramel components were classified according to their weights in polymers — high molecular weight condensation products and low molecular weight degradation products.

The chemical composition of caramel color is not yet fully understood but some compounds identified in the low weight fraction are considered “caramel markers.” All caramel classes contain 5-hydroxymethyl-2-furaldehyde (5-HMF). In caramel classes III and IV, 4-methylimidazole (4-MeI) has been detected, while 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) was found only in class III caramel colors.^{47–50} The analysis of five caramel III samples by SPE/HPLC-MS revealed concentrations between 28.3 and 46.8 µg/g THI and 73.3 to 187.8 for 4-MeI⁵¹ (see Figure 5.2.3).

Other compounds identified in caramels are di-D-fructose and poly(glycosyl) dianhydrides (DFAs). DFAs were found in caramels prepared from D-fructose, D-glucose, and sucrose. The analysis was done after derivatization as TMS (per-*O*-trimethylsilyl) derivatives or as TMS-oxime (per-*O*-trimethylsilyl oxime) by

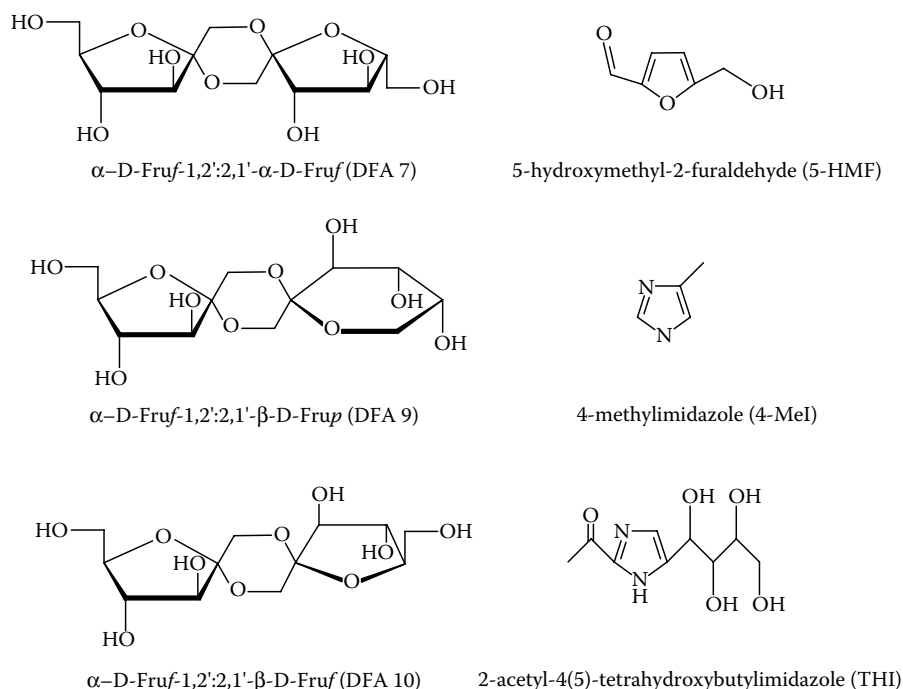


FIGURE 5.2.3 Structures of some marker molecules of caramel color.

GC/MS. A large number of DFAs were identified but their compositions and amounts depend on the nature of sugar used for caramelization. Fructose caramel contains the highest amount of DFAs (more than 39% of dry matter), while glucose caramel contains mainly glucobioses. In sucrose caramel, both types of compounds were found in similar proportions. Based on these observations, DAFs are considered suitable tracers for the determination of caramel authenticity.^{52,53}

Fourteen DFAs and some oligomers were identified in caramel obtained by thermal treatment of inulin.^{54,55} Monosaccharides (glucose, fructose), dehydration products (1,6-anhydro- β -D-glucopyranose, 1,6-anhydro- β -D-glucofuranose), disaccharides (gentiobiose and isomaltose), and oligosaccharides were also found in glucose and sucrose caramel.^{52,53}

Caramel color is a result of a complex chemical composition. The color properties are characterized by *hue index* and *tinctorial power*, calculated via the following formulas:⁴

$$\text{Hue index} = 10 \log (A_{510}/A_{610})$$

$$\text{Tinctorial power} = K = K_{560} = A_{560}/cb$$

where A = absorbance at specified wavelengths (510, 610, and 560 nm), c = concentration (g/l), and b = cell thickness.

According to EU purity criteria, color intensity is defined as the absorbance of a 0.1% (w/v) solution of caramel color solids in water in a 1 cm cell at 610 nm. The color intensity must be 0.01 to 0.12 for class I (E 150a), 0.05 to 0.13 for class II (E 150b), 0.08 to 0.36 for class III (E 150c), and 0.10 to 0.60 for class IV (E 150d).¹⁶ Ammonia caramels show the highest tinctorial power and are most commonly used as food colorants. Class I has the weakest coloring properties and is mostly used as flavor.

It was shown that the addition of ammonia and ammonium salts improves tinctorial strength. The main disadvantage of this method is the formation of 4(5)-methylimidazole (4-MeI), known to be a neurotoxic agent and THI, which has immunosuppressive effects.^{56–58} Additional methods for obtaining caramel with good tinctorial properties and devoid of 4-MeI and THI were described. The addition of magnesium oxide in catalytic amounts to caustic caramel produced an increase of 150% of tinctorial power.⁵⁹ The additions of various amino acids in free form or as salts were tested. Sodium glycinate produced the highest tinctorial strength.⁶⁰ Caramels obtained with arginine and serine at concentration of 5% (w/w) also showed good tinctorial strength; caramels obtained with serine were not completely soluble in alcohol, beer, or acidic solutions.^{60, 61} Adding alanine as a catalyst in the preparation of caramels from maltose solution decreased the acid attributes and increased the sweetness and caramel attributes (sensory analysis). It also induced a significant increase of the browning rate from 2 for maltose without alanine after 30 min to 92 for maltose with alanine after 90 min.⁶²

5.2.2.4 USE AS FOOD ADDITIVE

Caramel colors have a wide range of applications in food and beverages. They are used generally for food, and their concentration is regulated according to good manufacturing practice. The JECFA established ADIs for classes II and III (Table 5.2.3). However, in the case of ammonia caramel (class III) the European Commission specified a limit of 10 mg/kg for THI and 250 mg/kg for 4-MeI content.¹⁶ Caramel is soluble in water but insoluble in organic solvents. The total solid content of commercial preparations varies from 50 to 70% and covers a range of pH values. Caramel has other important characteristics such as emulsifying properties, stabilization of colloidal systems, improvement of shelf lives of beverages exposed to light, prevention of haze formation in beers, and even foaming properties.

An important issue in caramel applications is their compatibility with food. The compatibility is defined as the absence of flocculation, precipitation, and haze formation. These effects are the results of colloidal interactions between charged high molecular weight components of caramel and the food components; thus the charge of caramel is essential for specific food applications.⁴

In the US, more than 80% of caramel is used to color drinks such as colas and beers. Another important application is the coloring of blended whiskeys produced by mixing straight whiskeys with neutral spirits. The addition of neutral spirits to the straight whiskey produces a loss of color that can be compensated by the addition of caramel. Straight whiskeys are colored during aging for 2 yr in freshly charred oak barrels and contain furfural and 5-HMF in a ratio of 2 to 2.6:1, while the ratio

TABLE 5.2.3**Status and Application Areas of Caramel Colors**

Caramel Class	JEFCA ADI mg/kg body weight	FDA	EU	Application Area
I	Not specified	Foods generally (GMP)	Generally for food (<i>quantum satis</i>)	High-proof spirits, desserts, spice blends
II	0 to 160	Foods generally (GMP)	Generally for food (<i>quantum satis</i>)	Liqueurs, martinis; stable in presence of ethanol and tannins; least used caramel color
III	0 to 200	Foods generally (GMP)	Generally for food (<i>quantum satis</i>)	Beer, baked goods, confectionery, extruded breakfast cereals; most widely used caramel color
IV	Not specified	Foods generally (GMP)	Generally for food (<i>quantum satis</i>)	Cola-type soft drinks, soups, pet foods

GMP = Good Manufacturing Practice.

Source: Adapted from Francis, F.J., in *Colorants*, Eagan Press, St. Paul, MN, 1999; Commission Directive 95/45/EC, *Off. J. European Commun.*, L226, 22.09, 1995; U.S. Food & Drug Administration, Code of Federal Regulations, 21 CFR 73, 615; U.S. Food & Drug Administration, *Additives Listed for Use, etc.*, 1999; Downham, A. and Collins, P., *J. Food Sci. Technol.*, 35, 5, 2000; JECFA, Geneva, 2001.

for blended whiskeys is 0.2 to 1.3:1. The HPLC determination of the concentrations of furfural and 5-HMF and their ratios can be used to effectively authenticate the standards of identity of straight whiskeys.⁶⁴

Caramel color interacts with other food components. As an example, a concentration higher than 700 ppm caramel in cola increased the rate of hydrolysis of the aspartame, forming alpha-L-aspartyl-L-phenylalanine.⁶⁵ Caramelization products inhibited enzymic browning by 85.8 and 72.2% when heated at pH 4 and 6, respectively, for 90 min. The highest inhibitory activity was found for the fraction with molecular weight of 1000 to 3000.⁶⁶ Caramel is often used for adulteration of juices and other foods like honey or coffee. It can be determined by quantification of marker molecules such as 5-HMF, 4-MeI, and DFAs.^{67,68}

5.2.3 COLORANTS OBTAINED FROM FUNGI BY BIOTECHNOLOGY: MONASCUS

5.2.3.1 SOURCES OF MONASCUS PIGMENTS

Monascus pigments have been used in Asian countries for centuries as food colorants and spices and in traditional medicine. These pigments are produced by the fungi of *Monascus* genus cultivated on carbohydrate-rich substrates such as rice, wheat, corn, potatoes, and soybeans. Three species of *Monascus* identified are *pilosus*,

purpureus, and ruber. Several strains were isolated and characterized from various food sources.^{4,69–71} The main source of *Monascus* pigment is a fermented red rice called koji or angkak. *Monascus* pigments are often called anka pigments. In the traditional Chinese method, the steamed rice is inoculated with *Monascus anka* strain and incubated under controlled conditions for 20 days. A large number of fermentation processes have been elaborated and optimized, as described in Section 5.4. Japan, China, and Taiwan are the biggest producers of *Monascus* colorants.⁴

5.2.3.2 CHEMICAL STRUCTURES AND PROPERTIES

Monascus pigments belong to the azaphylone class of pigments — polyketide-derived metabolites of fungi produced mainly in the cell-bound state.⁷² The genus *Monascus* biosynthesizes six main pigments: the ankaflavin and monascin yellows; the rubropunctatin and monascorubrin oranges; and the rubropunctamine and monascorubramine red-purples.^{4,14,39,69–72} The two forms of each group differ by the aliphatic chain (C_5 or C_7) and the red-purple pigments are nitrogen analogues of the orange pigments (Figure 5.2.4).

Two yellow pigments, monacusones A ($C_{13}H_{18}O_5$) and B ($C_{17}H_{18}O_5$), were isolated and characterized from a yellow mutant of *Monascus kaoliang* grown on rice. Five new red pigments were purified, and two of them characterized by NMR, from a hyperpigmenting mutant of *Monascus purpureus* IB1 in a hexanoic acid-supplemented culture. These pigments were less hydrophobic than the usual red azaphilones.⁷³ Several other metabolites were isolated from *Monascus*, such as: monacolin K (lovastatin), known as an inhibitor of HMG-CoA reductase and used as anticholesterolemic agent; γ -aminobutyric acid, an inhibitor of nerve transmitters

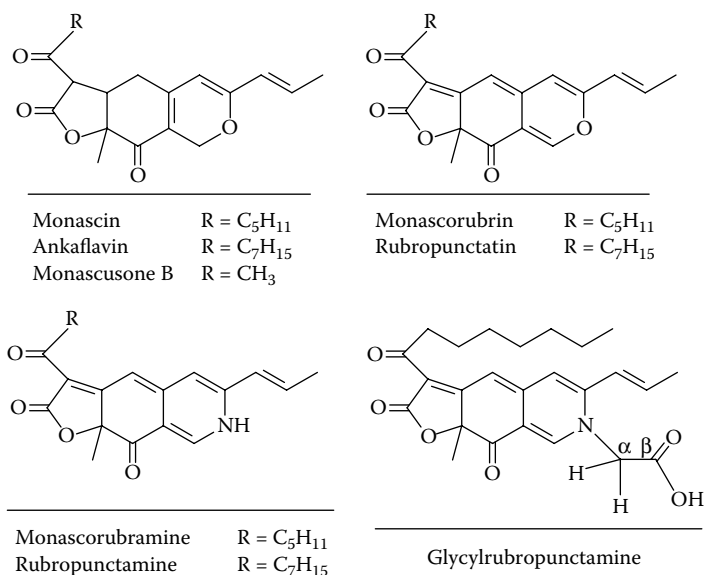


FIGURE 5.2.4 Chemical structures of some *Monascus* pigments.

and an antihypertensive agent; citrinin, a mycotoxin with antibacterial properties that is also nephrotoxic and hepatotoxic; monascodilone; and monascopyridine.^{69,74,75}

Monascus pigments are lipophilic compounds with low water solubility, but they are soluble in ethanol and other organic solvents. Monascus pigments are sensitive and fade under UV and visible light. They are stable at a pH range of 2 to 10 and at temperatures below 100°C. The colors of ethanolic solutions vary from orange at pH 3 to 4 to red at pH 5 to 6, to purple-red at pH 7 to 9.⁴ An important property of these pigments is their high affinity for compounds containing amino groups: amino acids, peptides, proteins, nucleic acids, amino sugars, amino alcohols, and chitosan. If these compounds are present in a culture medium, they react rapidly with the pigments via a ring opening and a Schiff rearrangement. This produces much more water-soluble and more light- and heat-stable compounds, for example, N-glucosyl-rubro-punctamine and N-glucosylmonascorubramine.^{4,76}

A complex experiment investigated the effects of separate additions of 20 amino acids (as precursors from culture medium) on the hue and chroma values of Monascus pigments. The yellow and orange pigments were identical regardless of amino acid addition, while the red compound varied depending on the amino acid. A range of colors from orange-red to violet-red were obtained.⁷⁷ The amino acid derivatives of Monascus pigments showed half-life improvements of 6- to 25-fold under sunlight and UV light compared to the red control pigments. The stability of complexes increased with increasing concentration. Pigment stability was decreased in both acidic and alkaline conditions, compared to neutral pH, and was dependent on the solvent used.⁷⁸ The improvement of Monascus pigment stability under various conditions is important for their use as natural colorants for foods.

5.2.3.3 USES AS FOOD COLORANTS

In Asian countries, Monascus pigments are widely used for pigmenting koji, soy sauce, tofu, bean curd, red wines, and saki. They can also be considered for coloring minced and processed meats (sausages, hams), marine products (surimi, fish paste), ketchup, ice cream, toppings, and jams. Because the bright red color associated with freshness of meat is an important factor in consumer purchasing decisions, Monascus could be one of the natural pigments used. Monascus pigments added to sausages and canned pâtés showed a stability of 92 to 98%.⁷⁹ When tested with red beet root and commercial betanin, red yeast rice protected sausages from discoloration and extended acceptability and customer willingness to purchase by about 4 days.⁸⁰ The main advantages of Monascus as a food colorant are the wide range of hues from orange to red, good stability, and availability.

The main concern regarding the utilization of *Monascus* pigments relates to the production of the citrinin mycotoxin in *Monascus* cultures. Several methods for controlling the mycotoxin production were proposed, including selection of non-toxinogenic strains, control of citrinin biosynthesis, and modifications of culture conditions.⁷¹ Despite their wide and traditional food applications in Asian countries, Monascus pigments have not been approved for use in the United States or European Union.

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REFERENCES

1. Govindarajan, V.S., Turmeric: chemistry, technology and quality, *Crit. Rev. Food Sci. Nutr.*, 12, 191, 1980.
2. Jayaprakasha, G.K., Jaganmohan Rao, L., and Sakariah, K.K., Chemistry and biological activities of *C. longa*, *Trends Food Sci. Technol.*, 16, 533, 2005.
3. Joe, B., Vijaykumar, M., and Lokesh, B.R., Biological properties of curcumin: cellular and molecular mechanism of action, *Crit. Rev. Food Sci. Nutr.*, 44, 97, 2004.
4. Francis, F.J., FD&C colorants, in *Colorants*, Eagan Press, St. Paul, MN, 1999.
5. Plotto, A., Turmeric: post-production management for improved market access for herbs and spices. Compendium on post-harvest operations. <http://www.fao.org>.
6. Park, S.Y. and Kim, D.S., Discovery of natural products from *Curcuma longa* that protect cells from beta-amyloid insult: a drug discovery effort against Alzheimer's disease, *J. Nat. Prod.*, 65, 1227, 2002.
7. Iang, H., Timmermann, B.N., and Ganf, D.R., Use of liquid chromatography–electrospray ionization–tandem mass spectrometry to identify diarylheptanoids in turmeric (*Curcuma longa* L.), *J. Chromatogr. A*, 1111, 21, 2006.
8. Tønnesen, H.H. and Karlsen J., Studies on curcumin and curcuminoids. V. Alkaline degradation of curcumine, *Z. Lebensm. Unters. Forsch.*, 180, 132, 1985.
9. Price, L.C. and Buescher R.W., Kinetics of alkaline degradation of the food pigments curcumin and curcuminoids, *J. Food Sci.*, 62, 267, 1997.
10. Szenté, L. et al., Stabilization and solubilization of lipophilic natural colorants with cyclodextrins, *J. Inclusion Phen. Mol. Recognition Chem.*, 32, 91, 1998.
11. Tønnesen, H.H., Másson, M., and Loftsson, T., Studies on curcumin and curcuminoids. XXVII. Cyclodextrin complexation: solubility, chemical and photochemical stability, *Int. J. Pharm.*, 244, 127, 2002.
12. Tønnesen, H.H., Karlsen, J., and Beijersbergen van Henegouwen, G., Studies on curcumin and curcuminoids. VIII. Photochemical stability of curcumin, *Z. Lebensm. Unters. Forsch.*, 183, 116, 1986.
13. Souza, C.R.A., Osme, S.F., and Gloria, M.B.A., Stability of curcuminoid pigments in model systems, *J. Food Proc. Preservation*, 21, 353, 1997.
14. Delgado-Vargas, F. and Paredes-Lopez, O., Other natural pigments, in *Natural Colorants for Food and Nutraceutical Uses*, CRC Press, Boca Raton, FL, 2003, chap. 9.
15. Khopde, S.M. et al., Effect of solvent on the excited-state photophysical properties of curcumin, *Photochem. Photobiol.*, 72, 625, 2000.
16. Commission directive 95/45/EC of 26 July 1995, laying down specific purity criteria concerning colours for use in foodstuffs, *Off. J. Eur. Communities*, L226, 22.09.1995.
17. Péret-Almeida L. et al., Separation and determination of the physico-chemical characteristics of curcumin, demethoxycurcumin and bisdemethoxycurcumin, *Food Res. Int.*, 38, 1039, 2005.
18. Prasad, J. et al., Study on performance evaluation of hybrid drier for turmeric (*Curcuma longa* L.) drying at village scale, *J. Food Eng.*, 75, 497, 2006.

19. U.S. Food & Drug Administration, *Code of Federal Regulations*, Vol. 1, Turmeric oleoresin, 21 CFR 73.615, 2002.
20. European Parliament and Council Directive 94/36/EC on colours for use in foodstuffs, *Off. J. Eur. Communities*, L237, 10.09, 1994.
21. World Health Organization, Series, 52, Safety evaluation of certain food additive and contaminants, International Programme on Chemical Safety, Geneva, 55, 2004.
22. Tylak, K.C. et al., Antioxidant availability of turmeric in relation to its medicinal and culinary uses, *Phytoter. Res.*, 18, 798, 2004.
23. Jayaprakasha, G.K., Jaganmohan Rao, L., and Sakariah, K.K., Antioxidant activities of curcumin, demethoxycurcumin and bisdemethoxycurcumin, *Food Chem.*, 98, 720, 2006.
24. Masuda, T. et al., Chemical studies on antioxidant mechanism of curcumin: analysis of oxidative coupling products from curcumine and linoleate, *J. Agric. Food Chem.*, 49, 2539, 2001.
25. Cleary, K. and McFeeters R.F., Effects of oxygen and turmeric on the formation of oxidative aldehydes in fresh-pack dill pickles, *J. Agric. Food Chem.*, 54, 3421, 2006.
26. Lean, L.P. and Mohamed S., Antioxidative and antimycotic effects of turmeric, lemon-grass, betel leaves, clove, black pepper leaves and *Garcinia atriviridis* on butter cakes, *J. Sci. Food Agric.*, 79, 1817, 1999.
27. Sowbhagya, H.B. et al., Stability of water-soluble turmeric colourant in an extruded food product during storage, *J. Food Eng.*, 67, 367, 2005.
28. Calvo C. and Salvador A., Use of natural colorants in food gels: influence of composition of gels on their colour and study of their stability during storage, *Food Hydrocolloids*, 14, 439, 2000.
29. Chatterjee, S., Padwal-Desai, S.R., and Thomas, P., Effect of γ -irradiation on the colour power of turmeric (*Curcuma longa* L.) and red chillies (*Capsicum annuum*) during storage, *Food Res. Int.*, 31, 625, 1998.
30. Chatterjee, S., Padwal-Desai, S.R., and Thomas, P., Effect of γ -irradiation on the antioxidant activity of turmeric (*Curcuma longa* L.) extracts, *Food Res. Int.*, 32, 487, 1999.
31. Lloyd, A.G., Extraction and chemistry of cochineal, *Food Chem.*, 5, 91, 1980.
32. Schul, I.J., An ancient but still young colorant, in *Proceedings of First International Symposium on Natural Colorants*. Francis, F.J., Ed., Hereld Organization, Hamden, CT, 1993.
33. Wouters, K. and Verhecken, A., Species recognition by HPLC and diode-array analysis of the dyestuffs, *Ann. Soc. Ent. Fr.*, 25, 393, 1989.
34. Gonzalez, M. et al., Optimizing conditions for the extraction of pigments in cochineals (*Dactylopius coccus* Costa) using response surface methodology, *J. Agric. Food Chem.*, 50, 6968, 2002.
35. Mendez, J. et al., Color quality of pigments in cochineals (*Dactylopius coccus* Costa). Geographical characterization using multivariate statistical analysis, *J. Agric. Food Chem.*, 52, 1331, 2004.
36. U.S. Food & Drug Administration, *Summary of Color Additives Listed for Use in the United States in Food, Drugs, Cosmetics and Medical Devices*, Washington, D.C., 1999.
37. Chimenos, J.M. et al., Removal of ammonium and phosphates from wastewater resulting from process of cochineal extraction using MgO-containing by-product, *Water Res.*, 37, 1601, 2003.
38. Allevi, P. et al., Synthesis of carminic acid, the colourant principle of cochineal, *J. Chem. Soc. Perkin Trans.*, 1, 575, 1998.

39. Francis, F.J., Food coloring, in *Colour in Food: Improving Quality*, MacDougall, D.B., Eds., CRC Press, Boca Raton, FL, 2002, chap. 12.
40. Madsen, H.L. et al., Cochineal as a colorant in processed pork meat: colour matching and oxidative stability, *Food Chem.*, 46, 265, 1993.
41. JECFA, Combined compendium of food additive specifications, <http://www.fao.org>.
42. Tabar-Purroy, A.I. et al., Carmine (E 120): induced occupational asthma revisited, *J. Allergy Clin. Immunol.*, 111, 415, 2003.
43. Chapell, C.I. and Howell, J.C., Characterization and specifications of caramel colours: an overview, *Food Chem. Toxicol.*, 30, 351, 1992.
44. Kroh, L.W., Caramelisation in food and beverages, *Food Chem.*, 54, 373, 1994.
45. Downham, A. and Collins, P., Colouring our foods in the last and next millennium, *J. Food Sci. Technol.*, 35, 5, 2000.
46. Myers, D.V. and Howell, J.C., Characterization and specifications of caramel colours: an overview, *Food Chem. Toxicol.*, 30, 359, 1992.
47. Licht, B.H. et al., Characterization of caramel colours I, II and III, *Food Chem. Toxicol.*, 30, 375, 1992.
48. Licht, B.H. et al., Development of specifications for caramel colours, *Food Chem. Toxicol.*, 30, 383, 1992.
49. Licht, B.H. et al., Characterization of caramel colour IV, *Food Chem. Toxicol.*, 30, 365, 1992.
50. Coffey, J.S. et al., A liquid chromatographic method for the estimation of Class III caramel added to foods, *Food Chem.*, 58, 259, 1997.
51. Klejdus, B. et al., Solid-phase extraction of 4(5)-methylimidazole (4-MeI) and 2-acetyl-4(5)-(1,2,3,4-tetrahydroxybutyl)-imidazole (THI) from foods and beverages with subsequent liquid chromatographic-electrospray mass spectrometric quantification, *J. Sep. Sci.*, 29, 378, 2006.
52. Defaye, K. and García Fernández, J.M., Protonic and thermal activation of sucrose an the oligosaccharide composition of caramel, *Carbohydrate Res.*, 256, C1, 1994.
53. Ratsimba, V. et al., Qualitative and quantitative evaluation of mono- and disaccharides in D-fructose, D-glucose and sucrose caramels by gas-liquid chromatography-mass spectrometry: di-D-fructose dianhydrides as tracers of caramel authenticity, *J. Chromatogr. A*, 844, 283, 1999.
54. Manley-Harris, M. and Richards, G.N., Di-D-difructose dianhydrides and related oligomers from thermal treatments of inulin and sucrose, *Carbohydrate Res.*, 287, 183, 1997.
55. Christian, T.J. et al., Kinetics of formation of di-D-difructose dianhydrides during thermal treatments of inulin, *J. Agric. Food Chem.*, 48, 1823, 2000.
56. Gaunt, I.F, Butterworth, K.R., and Grasso, P., Long-term toxicity study of emulsifier YN in the mouse, *Food Cosm. Toxicol.*, 15, 1, 1977.
57. Reeve, V.E. et al., Suppressive effect of 2-acetyl-4-tetrahydroxybutylimidazole on contact hypersensitivity in the Skh: HR hairless mouse, *Int. Arch. Allergy Immunol.*, 102, 101, 1993.
58. Patey, A.L. et al., Ammonia caramels: specifications and analysis, *Food Add. Contaminants*, 2, 102, 1985.
59. Sikora, M., Tomasik, P., and Palasilski, M., Enhancement of tinctorial strength of non-ammonia caramels, *Stärke*, 41, 275, 1989.
60. Sikora, M. and Tomasik, P., Alternative route to non-ammonia caramels of high tinctorial strength, *Stärke*, 41, 318, 1989.
61. Sikora, M. and Tomasik, P., Biogenic amino acids and their metal salts as catalysts of caramelization, *Stärke*, 46, 150, 1994.

62. Fadel, H.H. and Farouk A., Caramelization of maltose solution in presence of alanine, *Amino Acids*, 22, 199, 2002.
63. Joint FAO/WHO Expert Committee on Food Additives, World Health Organization, Geneva, 2001.
64. Jaganathan, J. and Dugar, S.M., Authentication of straight whiskey by determination of the ratio of furfural to 5-hydroxymethyl-2-furaldehyde, *JAOC Int.*, 82, 997, 1999.
65. Wang, R. and Schroeder, S.R., The effect of caramel coloring on the multiple degradation pathways of aspartame, *J. Food Sci.* 65, 1100, 2000.
66. Lee, G.C. and Lee C.Y., Inhibitory effect of caramelisation products on enzymic browning, *Food Chem.*, 60, 231, 1997.
67. Ciolino, L.A., Determination and classification of added caramel color in adulterated acerola juice formulations, *J. Agric. Food Chem.*, 48, 1746, 1998.
68. Montilla, A. et al., Difructose anhydrides as quality markers of honey and coffee, *Food Res. Int.*, 39, 801, 2006.
69. Blanc, P.J. et al., Pigments of *Monascus*, *J. Food Sci.*, 59, 862, 1994.
70. Mapari, S.A.A. et al., Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants, *Curr. Opin. Biotechnol.*, 16, 231, 2005.
71. Dufossé, L. et al., Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends Food Sci. Technol.*, 16, 389, 2005.
72. Jongrungruangchok, S. et al., Azaphilone pigments from a yellow mutant of the fungus *Monascus kaoliang*, *Phytochemistry*, 65, 2569, 2004.
73. Campoy, S. et al., Characterization of an hyperpigmenting mutant of *Monascus purpureus* IB1: identification of two novel pigment chemical structures, *Appl. Microbiol. Biotechnol.*, 70, 488, 2006.
74. Wild, D., Tóth, G., and Humpf, H.-U., New *Monascus* metabolite isolated from red yeast rice (Angkak, Red Koji), *J. Agric. Food Chem.*, 50, 3999, 2002.
75. Wild, D., Tóth, G., and Humpf, H.-U., New *Monascus* metabolites with pyridine structure in red fermented rice, *J. Agric. Food Chem.*, 51, 5493, 2003.
76. Hajjaj, H. et al., Production and identification of N-glucosylrubropunctamine and N-glucosylmonascorubramine from *Monascus ruber* and occurrence of electron donor-acceptor complexes in these pigments, *Appl. Environ. Microbiol.*, 63, 2671, 1997.
77. Jung, H. et al., Color characteristics of *Monascus* pigments derived by fermentation with various amino acids, *J. Agric. Food Chem.*, 51, 1302, 2003.
78. Jung, H., Kim, C., and Shin, C.S., Enhanced photostability of *Monascus* pigments derived with various amino acids via fermentation, *J. Agric. Food Chem.*, 53, 7108, 2005.
79. Fabre, C.E. et al., Production and food applications of the red pigments of *Monascus ruber*, *J. Food Sci.*, 58, 1099, 1993.
80. Martinez, L. et al., Comparative effect of red yeast rice (*Monascus purpureus*), red beet root (*Beta vulgaris*) and betanin (E-162) on colour and consumer acceptability of fresh pork sausages packaged in a modified atmosphere, *J. Sci. Food Agric.*, 86, 500, 2006.

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5.3.1 INTRODUCTION

5.3.1.1 CAROTENOID BIOSYNTHESIS: EXAMPLES OF BIOTECHNOLOGY FOR FOOD COLORANTS

Only in the past two decades has the manipulation of biologically produced pigments been informed by a substantial understanding of biochemistry and gene action. While the biotechnological engineers have some complete sets of genes involved in biosynthetic networks, they still have only rudimentary knowledge of the complex behaviors of the many other components of these systems. From an applied perspective, biotechnologists require control of the metabolic cell factory, but also require control of the supplies and demands of its resource environment. The metabolic infrastructure and the information flow through the process (cybernetics¹ or homeostasis) are being probed with pan-cellular tools such as transcriptomics, proteomics, and metabolomics, and also by new computational approaches such as metabolic flux analysis and flux balance analysis.

Truly integrative approaches, those looking across subsets of 'omics data and across metabolic networks, are still on the research horizon. In the meantime, non-integrative approaches now seek the essential components of metabolism. These include: (1) qualitative catalytic activities of enzymes; (2) quantitative flux through metabolic networks; (3) assembly and dynamics of enzyme complexes onto cellular scaffolding (metabolons); (4) supplies of small molecule cofactors and transport chains; (5) feedback mechanisms at the levels of transcription, translation, and catalysis; and (6) mechanisms of transport, sequestration, and storage (or turnover) of accumulating metabolites.² Without complete understanding of the components of metabolism, bio-engineers have been often surprised to find that integrated, complex metabolisms are resistant to manipulation.

This section will present a well-studied pigment biosynthetic system that produces carotenoids and apocarotenoids. We present examples of biochemical genetics and successes in proof-of-concept genetic engineering of pigment accumulation as well as lessons learned from unexpected results. While we focus on carotenogenesis and its manipulation in plants, we also look at a few key experiments in fungal and bacterial systems, where most of the groundwork and applications in metabolic engineering occur. This section is divided into three parts that may have more or

less relevance to particular readers. The first part defines biotechnology and the specific technologies applied to modifying pigment accumulation in plants, the second part relates the essential biochemistry and molecular genetics of carotenoid biosynthesis, and the third part discusses recent applications of the biotechnologies to pigment accumulation in edible plants.

5.3.1.2 OTHER PIGMENTS: FLAVONOIDS AND BETALAINS

We have chosen carotenoid biosynthesis as the example system for demonstrating the prospects of biotechnology of food colorants for several reasons. Carotenoid biosynthesis is the second most understood system. Multiple examples of valuable food colorant engineering in fungi, bacteria, and plants have been reported. Finally, carotenogenesis in cereal crops such as maize and rice is the primary focus of our research efforts. Hopefully, we provide the food technologist with a template with which to examine other industrially important pigment systems.

Another well-studied system is flavonoid metabolism, which often focuses on flower coloration, but includes important food colorants such as the anthocyanins. Flavonoid metabolism and biotechnology have been most astutely reviewed.^{2–10} Research on the biotechnology of betalains is a remarkably expanding field.^{3,11} Betalain researchers demonstrated how progress in genomics and molecular genetics gleaned from more studied systems in conjunction with a phyletic perspective are accelerating the pace of discovery and understanding in new fields.¹¹ We hope that our examples from carotenoid metabolism and engineering will be informative also to those with interest in these other pigment systems.

For carotenoid biosynthesis, genes for the recognized structural enzymes, catalytic activities and essential co-factors have been partially characterized and defined for a number of model crop plants as well as some niche plants such as citrus, marigold, daffodil, gentian, and others. We focus here on a few crop plants and several food colorants — β -carotene, lycopene, lutein, zeaxanthin, astaxanthin, saffron, and bixin (annatto) — for our examples. A number of excellent recent reviews of plant carotenoid biotechnology have been published from several perspectives including biochemical genetics,^{12–16} metabolic engineering,^{17–23} socio-politics,^{24–26} and health and nutrition.^{12,25,27–32} Microbial carotenogenesis is not reviewed here, but the reference section includes several reviews.^{33–39}

5.3.1.3 GENERAL CONCEPTS AND SPECIFIC TECHNIQUES IN PIGMENT BIOTECHNOLOGY

As a preface to examples of biotechnology directed at carotenoid food colorants, we briefly outline concepts in metabolic engineering and definitions of biotechnology, genetic engineering, and metabolic engineering terms. Specific technologies that will be discussed in the context of carotenoid research are gene isolation and functional testing, color complementation in heterologous systems, gene silencing (sense suppression, antisense, and RNAi), gene over-expression, metabolic engineering of pathway flux, metabolic control analysis (MCA), molecular breeding, gene shuffling, recombinant inbred linkage mapping, marker-assisted selection (MAS),

microsynteny comparison, linkage disequilibrium (LD) mapping (association genetics), and quantitative trait loci (QTL) analysis.

5.3.1.3.1 Colors of Biotechnology

Biotechnology is the application of organisms or organism-derived tools to production and process. Biotechnologies have been classified by the target area of application, for example, green biotechnology is applied science involving manipulation of organisms in agricultural endeavors, red in medical endeavors, white in industrial processes, and blue in aquatic processes.³⁸

Natural pigment production for food coloration includes the entire spectrum of biotechnologies. For example, biological production of carotenoid pigments has medical implications because carotenoids are nutritive (pro-vitamin A), antioxidant, and photoprotective. Carotenoids are produced alternately in agricultural systems (plants), industrial bioreactors (bacterial and fungi), and marine systems (cyanobacteria and algae).

Historically, biotechnology was the art and science of production organisms grown in tissue culture. Most biotechnology involves microorganisms. Often the production of secondary metabolites such as most pigments occurs after the growth phase of a bacterial, algal, or fungal culture and the amount of pigment produced is directly dependent on biomass accumulation.

Enhancement of biomass production by controlling the environment and nutrition of the organisms favors the accumulation of metabolites. For example, many environmental factors stimulate the accumulation of carotenoids in microorganisms.³⁵ Bacteria, filamentous fungi, and algae are limited by low biomass production and complicated growth requirements, while fermentation of yeasts (and other ascomycetes), combined with genetic modification may appear more promising.⁴⁰ This section examines methods of increasing carotenoid accumulation that are informed by molecular genetics rather than by culture or environmental conditions. Regulatory and public acceptance of new pigments from non-traditional sources seems to be a major impediment to the use of biotechnologically derived pigments. Nevertheless, biotechnological efforts continue and often include strategies that involve genetic manipulation of microorganisms and crop plants.

General reviews of plant genetic and metabolic engineering,^{17,19,20,33,41,42} and specific areas, such as tissue-specific gene expression,⁴³ membrane transport of substrates,^{2,21,44} engineering of transcription factors,^{3,20,42} and mathematical modeling of pathway flux^{45,46} have been published. A general resource on metabolic engineering⁴⁷ has also been recently published.

5.3.1.3.2 Genetic Engineering

Biotechnology may involve the use of genetically altered organisms and has become associated with the field of molecular biology. Genetic engineering is the addition of one or a few genes that have first been modified *in vitro* to the stably inherited chromosomes of an organism. The genes may be from the same species or another species. Genetic engineering implies *in vitro* manipulation of specific DNA sequences

and does not include changes to DNA induced by agricultural selection, applied evolution, molecular-assisted breeding, somaclonal variation, or mutation breeding.

The introduced, modified genes are often called transgenes and the plants are called transgenics. *Genetically modified organism* (GMO) is a term most commonly used for transgenics, but is a misnomer because all partners in human co-evolutionary relationships such as domesticated and bred cultivars are genetically modified by human activity. The distinction made between *in vitro* manipulation of specific DNA sequences (GM) *versus* applied evolution and conventional breeding for allelic variation is not always rational or even clear with respect to environmental and health risks.

Currently all GMOs have novel synthetic DNA sequences or sequences added from other organisms. Introduced genes are intended to add or remove a single function and thus present a value-added trait to an otherwise substantially equivalent product. Crop improvement efforts are now refocusing on targeted modification and then selection of endogenous sequences either directly *in planta* (for example, by a mutation breeding technique called TILLING⁴⁸) or via modification of native sequences *in vitro* followed by re-introduction (for example, by a technique called RNA interference or RNAi^{49,50}). Genetic engineering of whole pathways by genetic modification of pan-pathway gene regulators such as transcription factors or likewise inducing rational pleiotropic changes is just beginning.

Requirements for genetic engineering of plants — To enhance pigment accumulation, biotechnologists must understand all the factors affecting biosynthesis and accumulation. Success has been achieved despite a lack of understanding, but only by a “poke-and-hope” semi-rational approach. Completely rational, predictive engineering requires a fully characterized initial state of the system, regardless of whether one adheres to a deterministic, hierarchical control-based, or stochastic, complexity-based approach to manipulation. Some required, *a priori* knowledge for engineering is covered in Section 5.3.1.1.

Additional “hardware” requirements for genetic engineering are: (1) a genetic transformation method, (2) gene promoters and other *cis*-acting regulatory elements, and (3) a source of genes with proven function. While great progress has been made toward isolation and characterization of gene functions since the early 1980s, some deficits still exist. For example, researchers still face few choices among tissue-specific promoters, very few tested gene regulatory sequences, and many plants that are not easy to transform. Genomics technologies have produced a surplus of genetic elements and supporting information on their expression and relation to metabolism, which will give metabolic engineers the knowledge and tools they need. Current progress may be limited as the burgeoning science of bioinformatics strives to develop the implements needed to mine the full depths of complex information. Functional characterization (biology) and utilization (technology) of the genetic wealth represent the next wave of development in the post-genomic era.^{51,52}

Sources of genes — New terminology often accompanies changes of scientific paradigms,⁵³ and this was so when gene sequences suddenly became more available (and numerous) than phenotypes. Reverse genetics is a phrase coined in the 1980s to indicate genetic studies that started with a gene or protein and pursued the associated phenotype. This was opposite the previous experiences of geneticists who

collected phenotypic behaviors and pursued the underlying genetic bases (forward genetics). While the genomic revolution changed the direction of prevailing scientific endeavors, studying phenotypes with simple genetic underpinnings still has an essential impact on the understanding of gene function and metabolism. For many plants, a wealth of classical genetic information and mutant phenotype characterization became available over the past few hundred years. The pre-existing wealth of mutants in pigment phenotypes (for obvious reasons) is large^{16,54–56} and has yet to be fully exploited. The importance of classical genetics and such mutants will be illustrated below for several plants.

Genes often come from gene libraries that are complementary to messenger RNAs (cDNA libraries) from particular tissues such as maize endosperms. Interest is often focused on genes involved in the specialized metabolism of that tissue and developmental time rather than “housekeeping” genes found in all cell types. The polymerase chain reaction (PCR) and specific sequence information about genes from other organisms can often be used to produce PCR primers that allow evolutionarily related fragments of new genes to be isolated from the cDNA of the favored target organism.

Homology does not ensure analogy, so function must be demonstrated for the product of the novel gene. This is most easily done in a heterologous complementation system, for example, in transgenic *E. coli*, where the active, recombinant gene product is produced. Particularly elegant and rapid application of these techniques to isolation and understanding of pigment biosynthesis will be given here for β -carotene, lycopene, zeaxanthin, bixin, and saffron, among others. Nevertheless, expression of active, recombinant enzymes for proof of function and characterization of catalytic activity can be difficult and rate-limiting in gene discovery. Newer, facile systems for proof of function such as VIGS and RNAi that are based on removal of normal gene function from plants offer better but currently species-limited approaches. We will describe applications of such gene knockout technologies in Sections 5.3.2.2 and 5.3.3.

The sequencing of entire genomes of *Arabidopsis* and rice and the gene spaces of other plants such as maize⁵⁷ has supplied an enormous wealth of genes to metabolic engineers. Huge efforts are needed to associate these genes with phenotypes (reverse genetics) in a high throughput fashion. Another source of gene functions is map-based cloning. Genes with specific suspected functions are isolated by association of phenotypic traits with a genetic recombination-based statistical map of molecular markers on chromosomes. In a second step, the trait-associated molecular markers are then associated with a physical map of a genome and with genomic DNA clones within a library. Finally, among a few candidate genes on the position-identified genomic clone, homology and testing of function may associate a specific gene with a component of a trait.

For production of transgenics, the organism source of a gene seems to matter. One might think that modification of endogenous sequences is best, but gene silencing mechanisms often turn off highly homologous transgenes. While this phenomenon is a boon to functional analysis and genetic engineering of loss of function,⁴⁹ it has been a source of unwanted trait variation and unexpected results in some genetic engineering efforts.

Studies have shown that genes from related species are better performing transgenes.⁵⁸ Therefore, for transgenesis as well as for a comparative phyletic understanding, homologous genes from pairs of related organisms such as tomato and potato, or maize and rice, or hops and hemp are most useful. Since gene silencing is a transcript-level phenomenon, similar logic applies to the many duplicate gene functions in plants that may be directed by transgenesis with tissue-specific promoters to developmental stages that are different from their normal range (for example a transgenic shoot-specific isoform expressed in a ripening fruit as an adjunct to the endogenous fruit-specific isoform). We can offer several examples from the carotenoid literature in which a phylogenetically — framed, comparative model system is necessary and most instructive.

Control of gene expression — Promoters are sequences outside the protein coding region of a gene that influence the rate of gene product accumulation. They may be constitutive in developmental time or in space or specific to a particular ontogenic or circadian developmental time and place. For example, the plant virus promoter 35S is strong and always on in vegetative tissues of most plants, while the wheat glutelin promoter is strongly expressed during mid-endosperm development in a variety of plants.⁵⁹ Specific promoters for more tissues are needed. Synthetic promoters may be engineered. Promoter specificity is an issue for both metabolic control and also for biosafety and containment of transgenic products.

The expression of transgenes is affected by the locus of integration, a phenomenon called *position effect*. For plant transformation techniques described below, genes integrate in various and multiple positions, each having a different level of transcriptional activity. This wide variability among events (an event is a single, independent integration) can be used to select the best metabolically balanced phenotype from a range of expression-level variants. Usually at least one hundred events are needed to find production candidates with near-optimal expression, so efficient transformation becomes paramount. Position effects in pigmentation are easy to score, and we will discuss an example below. Homologous recombination that targets a gene to a specific locus is not yet available in higher plants, but understanding and advancement may come from study of the phenomenon in bryophytes.⁶⁰

Regulatory genes code products, often acting as transcription factors that simultaneously activate or suppress panoplies of functionally related genes. Such regulatory genes have great potential for metabolic control of multiple pathway points in a coordinated fashion. Progress toward understanding and use of regulatory genes for plant metabolic engineering of pigment accumulation has been reported for flavonoid biosynthetic systems and recently reviewed.^{3,20} Since little progress has been reported for regulatory genes of carotenoid biosynthesis and no carotenoid-specific transcription factors have yet been characterized, our chosen example of carotenoid biotechnology is deficient in this area.

Transformation — Most limiting for genetic manipulation of many plant species is the process of introduction of modified genes into the genome. The three major transformation techniques are: (1) bacterial-mediated,^{61,62} (2) biolistic,⁶² and (3) biolistic plastidial.⁶³ Bacterial-mediated transformation requires dipping, vacuum infiltration, or co-cultivation of explanted tissue cultures with an *Agrobacterium* or *Rhizobium* transfectant. Biolistic transformation involves the introduction of

exogenous DNA by coating the DNA onto microprojectiles (tungsten or gold dust) and shooting the particles into the cells with a gun. Both methods are followed by embryogenesis and regeneration of plants if appropriate target tissue was used or by analysis of transient expression where regeneration is not possible. Each method has problems and limitations as well as advantages.

Some methods such as floral dip with *Agrobacterium* are very species-limited (to *Arabidopsis*). Many crops (canola, maize, rice, soybean) have been more recalcitrant to transformation by *Agrobacterium* than others (tomato, tobacco). In biolistic transformation, the chromosomes are damaged by the projectiles, and endogenous DNA repair enzymes use the foreign DNA to repair the damage, resulting in transgene integration events.

Major problems with biolistic transformation are rearrangement and integration of multiple copies of transgenes that are stably inherited as linked units. Rearrangements can lead to the production of spurious coding regions and perhaps unintended proteins. Multiple transgenes often lead to co-suppression by silencing of the gene function in later generations and loss of the added trait. Since *Agrobacterium*-mediated transformation is relatively free of these problems, successful efforts have been made to bring this type of transformation to major crops such as, maize,⁶⁴ rice,⁶⁵ and soybean.^{66,67}

Plastid transformation has many advantages including compartmentalization of the gene products in the plastid; prokaryotic-like genetics allowing multiple genes to be coordinately expressed as one polycistronic messenger; and, for the many plants with uniparental inheritance of plastids, elimination of the transgene and transgene products from pollen.⁶³ Difficulties with transformation and drawbacks of some methods such as gene silencing have impacted the deployment of crops with altered pigment contents. We will describe examples in Section 5.3.3.3.

5.3.1.3.3 Molecular Breeding: Linkage Mapping and Association Genetics

Molecular breeding involves a variety of tools to associate a variation in DNA with a variation in a phenotypic trait. The associations may then be applied to crop improvement. An underlying assumption of the method is that the trait of interest is conditioned by a small number of genes with major qualitative or quantitative effects on the trait. The molecular variant (marker) then becomes predictive of the phenotypic trait and can be applied to the selection of progeny at an early stage in a breeding cycle. Marker-assisted selection (MAS) is the use of the DNA markers for applied evolution of a desired trait in a crop or horticultural variety. Linkage mapping is a prospectus study design that associates molecular markers with traits by detecting patterns in carefully prepared progenies of a bi-parental mating. The associations are based on the frequency of recombination and assortment of markers and traits among the progeny.

Linkage mapping is also called *recombinant inbred mapping*.⁶⁸ Linkage maps can often be extended from one related organism to another by maps of microsynteny (the linear conservation of gene order and linkage on chromosomes across species). Related species such as maize, sorghum, and rice^{69,70} or tomato and bell pepper⁷¹

can have extensive regions of conserved gene order. Quantitative trait loci (QTL) mapping associates variations in quantitative traits, e.g., pigment content, with molecular markers on a linkage map.⁷² QTL can also be associated with candidate genes if a second round of high-resolution mapping is possible, leading to isolation of the gene associated to the phenotype.

Using high-resolution linkage maps to isolate new genes is called *map-based cloning*. High-resolution mapping depends on having a large number of markers and appropriately inbred parents (near-isogenic lines, NILs) and a large number of progeny. Linkage disequilibrium (LD) mapping can also associate candidate loci with a DNA polymorphism, but takes advantage of retrospection of pre-existing allelic diversity within a germplasm collection. LD is the association of alleles across loci.^{73,74} LD mapping is not dependent on known pedigree or a pre-prepared recombinant inbred family. The extent (ranging from about 1 to 100 Kb) of LD varies by population and evolutionary (domestication) history of the germplasm collection and varies locally by chromosomal region.

Determining the local extent of LD sets the number of markers needed to associate a QTL with a polymorphism in a gene. Association genetics requires cost-effective detection of sequence polymorphism over extensive loci, and thus is most suited to crops for which large-scale sequence data are already available.⁷⁵ Several examples of quantitative genetics applied to basic research of carotenogenesis are described in Section 5.3.2 and specific applications of linkage analysis, QTL mapping, and LD mapping in maize are discussed in the context of biotechnology and breeding in Section 5.3.3.3.

5.3.1.3.4 Generation of Variation: Mutation Breeding, TILLING, and Directed Evolution

Mutation breeding relies on the generation of mutant loci by the application of DNA-altering treatments to plant propagules such as pollen, ovules, meristems, etc.⁵⁴ The treatment is often a mutagenic chemical or radiation. Large numbers of treated plants or asexual propagules are scored for fortuitous trait improvement and selected for use in crop improvement. With heavily mutagenized plants, further sexual mating is often needed to separate the value-added trait from spurious, deleterious mutations.

TILLING (targeted induced local lesions in genes) is a gene-targeted mutation breeding strategy.⁴⁸ Starting with information (sequence) about the gene, TILLING associates induced sequence polymorphism with traits. TILLING therefore constitutes reverse genetics (from gene to phenotype). Mutations in a gene are detected in a chemical- or radiation-mutated population. Detection of mismatched DNA duplexes (wild-type with mutant) of gene-specific PCR products identifies targeted genetic variants in pools of genomic DNA. The carefully designed genomic DNA pools from mutated plants are systematically deconstructed to identify the unique mutant plant of origin for each gene variant. Phenotypes of known mutants are then analyzed. Many independent mutations in a particular gene (alleles) can be generated and the plants analyzed. Often the mutations are truncations or mis-sense mutations, so knockout of gene function is common. Therefore, null (loss-of-function) mutants of a particular gene and an allelic series of gene variants can

be isolated and used to investigate gene function or used as phenotypic variants for crop improvement.

Directed evolution of enzymes is an *in vitro* method of generating new genes from old ones.^{33,76} Pieces of genes from different species may be shuffled together with a PCR technique to create new combinations of exons with altered or new functions. Error-prone polymerases may be used to generate sequence variation at random. Directed evolution has been successful for evolving enzyme variants that are stable in new environments or have altered substrate selection. Pigments have also been synthesized by using novel biosynthetic enzymes that have been evolved from new combinations of natural genes.^{33,77}

Transposon tagging and activation tagging have become important technologies in the cloning and analysis of plant genes.^{78,79} The cloning is a two-step process.⁸⁰ First, tagging relies on the insertion of a genetic element, an endogenous or transgenic, exogenous transposable element or a promoter or enhancer element that changes gene expression upon fortuitous integration within or near a gene function. Second, the perturbation of a gene function with a known (thus, clonable) genetic element allows rescue of surrounding sequences by inverted PCR; hence the gene is tagged for cloning. Each tagging system involves correlation of a mutant phenotype with the insertion, and so pigment phenotypes are especially amenable to tagging.

5.3.1.3.5 Metabolic Engineering

Metabolic engineering may involve the use of transgenes, but goes further by rational manipulation of substrate flow through metabolic networks and the consideration of the cell as a unit of production or cell factory. Metabolic engineering often targets the alteration of gene products that influence substrate flow, for example, over-expression of a gene coding a rate-controlling enzyme to enhance substrate flow into a pathway.

While metabolic engineers traditionally sought the rate-limiting enzyme to unlock flow through a pathway, now they understand that there may be many points of control and feedback with the metabolic network, and seek to empirically determine the dynamics of the interactions between rate controllers and other factors. For example, the sizes of metabolic precursor pools and the catabolism or sequestration of products affect accumulation as well as flux through the pathway.

Metabolic control analysis (MCA) assigns a flux control coefficient (FCC) to each step in the pathway and considers the sum of the coefficients.⁸¹ Competing pathway components may have negative FCCs. To measure FCCs, a variety of experimental techniques including radio isotopomers and pulse chase experiments are necessary in a tissue culture system. Perturbation of the system, for example, with over-expression of various genes can be applied iteratively to understand and optimize product accumulation.

Adjunctive to flux control analysis, other components of metabolism that contribute to product accumulation are needed including: (1) substrate/precursor pool sizes (metabolomics), (2) co-factor capacities (metabolomics), (3) gene expression profiles (transcriptomics and quantitative real-time PCR), (4) protein profiles (pro-

teomics), (5) rates of product catabolism (metabolomics), and (6) regulatory mechanisms (enzymology and transcript profiling). Structural components of the metabolic network must also be considered, for example, subcellular localization, metabolic channeling, metabolon assembly dynamics, and intercellular and membrane substrate transport mechanisms, all of which may be interdependent and require small molecule co-factors. Examples of some of these concepts are available for bacterial and plant carotenogenesis and are presented in Section 5.3.3.6.

5.3.2 BIOCHEMISTRY AND GENETICS

5.3.2.1 OVERVIEW

For the sake of study, the biosynthesis of carotenoid plant pigments can be divided into parts involving enzymes and their associated activities as listed in Table 5.3.1 and further detailed in Figure 5.3.1 through Figure 5.3.4. Some of the parts have common enzymatic mechanisms and may also be in distinct subcellular compartments such as cytoplasm, endoplasmic reticulum, or plastid thylakoid.

Carotenoids are C40 polyenes containing extended conjugated double bonds that absorb light and give them color. Condensation of the five-carbon phosphate-activated isoprene (C5) into longer carbon chain di-phosphate esters, e.g., C10, C15, C30, and C40, gives us the C40 backbone of the carotenoids. We also discuss the biosynthesis of the metabolic precursors for carotenoid formation, the activated isoprene units (IPPs) because genetic engineering within the precursor pathway (DOXP or MEP) and also within the carotenoid pathway has effects on carotenoid accumulation.

The polyene backbone is subject to further paired hydrogen eliminations (desaturations) and isomeric rearrangement to yield the colored polyene. Cyclization of the ends of the chains produces the carotenes. The carotenes can then be remodeled by catalyses that involve oxygen (hydroxylases, epoxidases, de-epoxidases, and oxygenase cleavage enzymes) and yield xanthophylls, ketocarotenoids, and apocarotenoids. The latter parts of the pathway lead to well-known carotenoids found in foods: lycopene, β -carotene, lutein and zeaxanthin, capsanthin and capsorubin, astaxanthin, and the apocarotenoids, bixin and saffron; and for each of these sections of the metabolic pathways, we will discuss the genes and enzymes and substrates in brief detail. This will give us a foundation to provide examples of genetic and metabolic engineering for food colorants. Figure 5.3.1 shows how all parts of the pathways to and from colored carotenoids are interrelated.

5.3.2.2 POOLS OF PRECURSORS: MEP PATHWAY TO IPP AND DMAPP

IPP and its DMAPP structural isomer are produced from glycolytic products by the methyl erythritol phosphate (MEP) pathway (Figure 5.3.1, Pathway 1). These isoprene units are condensed in a stepwise fashion to form the precursor to all carotenoids, geranylgeranyl di-phosphate (GGPP). GGPP is not solely metabolized to make carotenoids, but is a precursor for many other primary and secondary metab-

TABLE 5.3.1
Enzyme Abbreviations and Substrates

		Enzyme	Name	Substrate
1	MEP Pathway	DXS	1-deoxy-D-xylulose 5-phosphate synthase	D-glyceraldehyde-3-phosphate, pyruvate (G3P)
		DXR	1-deoxy-D-xylulose 5-phosphate reductoisomerase	1-deoxy-D-xylulose-5-phosphate (DXP)
		MCT	2-C-methyl-D-erythritol 4-phosphate cytidyltransferase	2C-methyl-D-erythritol-4-phosphate (MEP)
		MCK	4-diphosphocytidyl-2-C-methyl-D-erythritol kinase	4-diphosph-2C-methyl-D-erythritol (CDP-ME)
		MCS	2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase	4-diphosphocytidyl-2C-methyl-D-erythritol-2-phosphate (CDP-MEP)
		HDS	4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase	2C-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP)
		HDR	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate (HMBPP)
		IPPI	Isopentenyl pyrophosphate isomerase	Dimethylallyl pyrophosphate (DMAPP), isopentenyl pyrophosphate (IPP)
2	GGPPS	GGPPS	Geranylgeranyl pyrophosphate synthase	
3	Carotenoid Biosynthesis	PSY	Phytoene synthase	Geranylgeranyl pyrophosphate (GGPP)
		PDS	Phytoene desaturase	15- <i>cis</i> -phytoene; 9,15- <i>cis</i> -phytofluene
		Z-ISO	15- <i>cis</i> ζ-carotene isomerase	15- <i>cis</i> ζ-carotene
		ZDS	ζ-carotene desaturase	9,9'- <i>cis</i> -ζ-carotene; 9'- <i>cis</i> -neurosporene
		CRTISO	Carotene isomerase	7,9,9'- <i>cis</i> -neurosporene; 7',9'- <i>cis</i> -lycopene
		LCYB	Lycopene β-cyclase	All- <i>trans</i> lycopene; λ-carotene; γ-carotene
		LCYE	Lycopene ε-cyclase	All- <i>trans</i> lycopene
		HYDB	β-ring carotene hydroxylase (diiron type)	β-carotene; β-cryptoxanthin; α-carotene
		CYP97A	β-ring carotene hydroxylase (P450-type)	β-carotene; β-cryptoxanthin; α-carotene
		CYP97C	ε-ring carotene hydroxylase (P450-type)	α-carotene, zeinoxanthin
		ZEP	Zeaxanthin epoxidase	Zeaxanthin; antheraxanthin

TABLE 5.3.1 (Continued)
Enzyme Abbreviations and Substrates

		Enzyme	Name	Substrate
		VDE	Violaxanthin deepoxidase	Violaxanthin; antheraxanthin
		NXS	Neoxanthin synthase	Violaxanthin
4	Carotenoid Degradation	NCED	9- <i>cis</i> -epoxy carotenoid (cleavage) dioxygenase	Various carotenoid substrates (11,12 double bonds)
		CCD	Carotenoid cleavage dioxygenase	Various carotenoid substrates (9,10 double bonds)

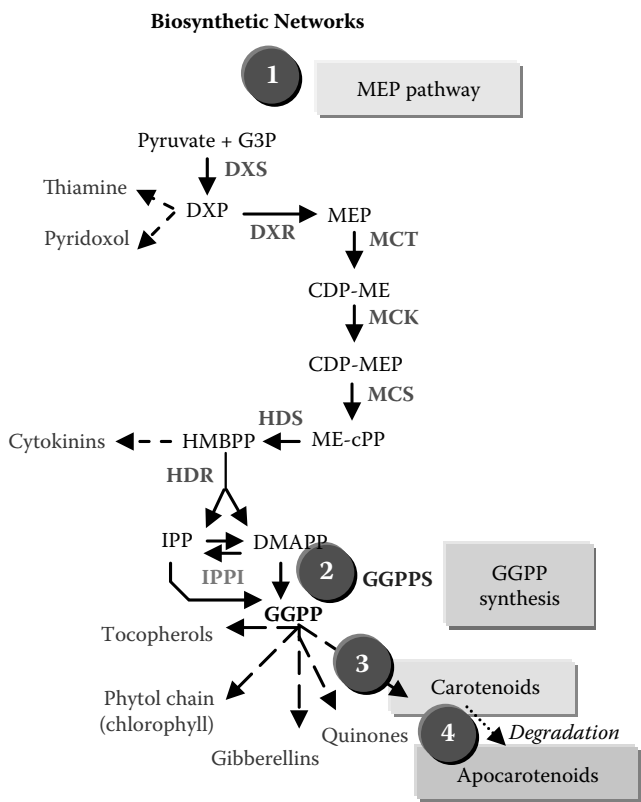


FIGURE 5.3.1 Parts of the isoprenoid pathways to carotenoids. 1 = MEP pathway. 2 = GGPP synthesis. 3 = Carotenoid biosynthetic pathway. 4 = Carotenoid degradative pathways. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

olites (Figure 5.3.1, Pathway 2). Two GGPPs are condensed to produce the extended C40 polyene carbon chain that serves as the “backbone” of all carotenoids.

In plant plastids, GGPP is formed from products of glycolysis and is eight enzymatic steps away from “central” glucose metabolism. The MEP pathway (reviewed in recent literature^{15,38,82–84}) operates in plastids in plants and is a preferred source (non-mevalonate) of phosphate-activated prenyl units (IPPs) for plastid isoprenoid accumulation, such as the phytol tail of chlorophyll, the backbones of carotenoids, and the cores of monoterpenes such as menthol, linalool, and iridoids, diterpenes such as taxadiene, and the side chains of bioactive prenylated terpenophenolics such as humulone, lupulone, and xanthohumol.⁸⁵ The mevalonic pathway to IPP that operates in the cytoplasm is the source of the carbon chains in isoprenes such as the polyisoprene, rubber, and the sesquiterpenes such as caryophyllene.

At the beginning of the MEP pathway, the glycolytic products, pyruvate and D-glyceraldehyde (GAP), are condensed in a transketolase reaction to deoxy-xylulose phosphate (DXP) by the deoxy-xylulose phosphate synthase (DXS) enzyme.^{86–88} DXP is the precursor for other pathways leading to pyridoxal and thiamine.^{82,89,90} DXP undergoes rearrangement and then is reduced by a reductionisomerase (DXR) to methyl erythritol phosphate (MEP), the first substrate committed to IPP and DMAPP.^{91,92}

The genes for *Dxs* and *Dxr* have been cloned from bacteria and plants, including *Arabidopsis*, mint, peppers, marigold (reviewed by Fraser and Bramley¹²), and recently ginkgo.⁹³ Among three *Dxs* homologues in *Arabidopsis*,⁸² only one (*Dxps-2*) has been shown to be functional to date. Other gene families in the carotenoid pathway have photosynthetic (housekeeping) tissue-specific versus storage (secondary metabolite) tissue-specific expression patterns. Both DXS and DXR have been manipulated in bacteria and plants to increase accumulation of pigments. Only DXS seems to be rate-controlling for flux into isoprenoids, although, like DXS, DXR is up-regulated in some plants during isoprenoid accumulation⁹³ (and other studies have been reviewed⁸³).

Recently, a potential cytosolic component of the MEP precursor pathway, xylulose kinase, has been cloned and tested for function in an *Escherichia coli* complementation system.⁹⁴ The kinase activates exogenous xylulose in the cytoplasm. DXP is the precursor for DXS, which resides in the plastid, suggesting the activated substrate must be transported into the plastid. Another xylulose kinase homologue in *Arabidopsis* that contains a plastid targeting sequence was not active in the *E. coli* system, suggesting that it may have some other function in the plastid. Perhaps plant and bacterial tissue cultures may be fed xylulose to condition accumulation of isoprenoid metabolites.

MEP is converted to DMAPP and IPP by five more enzymatic steps (Figure 5.3.1), only some of which have been manipulated in plants. Page et al.⁹⁵ demonstrated the functions of the latter genes coding enzymes in the metabolic pathway by gene knockout technology. Using viral-induced gene silencing (VIGS), *Hds*, *Hdr*, and *Ippi* functions were knocked out *in planta*. Since interruption of the MEP pathway affects chlorophyll and carotenoid accumulation, the virus-infected leaf tissues were non-pigmented or mottled.

Although exchange of IPP (cross-talk) between cytosols and plastids has been evidenced (cited by Page⁹⁵), the phenotypes of the VIGS knockouts of the MEP pathway show little traffic of MEP metabolites from the cytoplasm into the plastid in *Nicotiana*, and this has been confirmed by others in carrots,⁹⁶ *Arabidopsis*,⁹⁷ and strawberries,⁹⁸ except under certain interesting circumstances reviewed by Rodriguez-Concepcion.⁸³ *Hdr*, but not *Hds*, was strongly up-regulated at the transcriptional level in de-etiolating *Arabidopsis* leaves and in ripening tomato fruit,^{23,99,100} suggesting that HDR is rate-controlling. HDR over-expression studies in plants¹⁰⁰ confirmed earlier studies demonstrating rate-controlling bacterial and plant HDR enzymes expressed in *E. coli*.¹⁰¹

Even though HDR produces both DMAPP and IPP and allows some chlorophyll and carotenoids to accumulate in VIGS IPPI knockouts, IPPI seems essential to normal leaf development, and the isomerase may function to adjust the ratio of DMAPP to IPP.⁹⁵ Many studies have associated IPPI with degrees of carotenoid pigment accumulation in algae and in heterologous bacterial systems.^{101–106} IPPI may be related to cross-talk between the MVA and MEP pathways under certain developmental regimes.⁸³

5.3.2.3 POLYMERS FROM PRENYLS

GGPP is the head-to-tail condensation product of dimethylallyl di-phosphate (DMAPP) with isopentenyl di-phosphate (IPP). DMAPP is needed as the starting substrate for chain elongation of the polymer. Once produced by HDR (or IPPI), DMAPP is condensed head to tail (1 → 4') with IPP by geranyl di-phosphate synthase (GPPS) to produce GPP (C10). Further condensations of IPP onto the growing polyene by geranylgeranyl di-phosphate synthase (GGPPS) produce in sequence farnesyl di-phosphate (FPP, C15) and geranylgeranyl di-phosphate (GGPP, C20). See Figure 5.3.1, Pathway 2.

These enzymes are substrate-selective and make polymers of discrete chain lengths depending on size and features of their catalytically active pockets.¹⁰⁷ The substrate chain length specificity of GGPPS among various species has been reviewed.¹⁰⁸ Plastidial isoprenoid synthase enzymes have FPS, FPPS, and GGPPS activities. For example, in maize endosperm cytosol, a gene isolated by functional complementation for a GGPPS is a *bone fide* FPPS.¹⁰⁹ Enzymes active in different subcellular compartments are encoded by different genes active in different tissues and may have different substrate preferences. For example, *Arabidopsis* contains five GGPPS genes. Two enzymes are directed to the plastids, two to the cytosol, and one to the mitochondria.¹¹⁰ Since FPS and GGPPS are responsible for the biosynthesis of quinones, chlorophylls, and carotenoids, they have served as the foci of biotechnology techniques aimed at the accumulation of pigments. Understanding and manipulation are complicated by the many isoforms.

GGPPS functions as part of a complex metabolon. In the plastid, as shown in *Capsicum* chromoplasts,¹¹¹ GGPPS is a homodimer and associated but not integral to the plastid envelope. GGPPS is also associated with the next enzyme in the pathway as part of a holoenzyme complex.^{112,113}

The first C40 carotenoid, phytoene, is produced by head-to-head condensation of two GGPPs by an enzyme that shares homology to GGPPS and squalene synthases

(sterol biosynthesis). Formation of the C₄₀ isoprene, phytoene, is mediated by phytoene synthase or PSY (reviewed by Cunningham and by Sandmann.^{114,115} See Figure 5.3.1, Pathway 3. Phytoene is a colorless carotenoid. PSY mediates a two-step catalysis. First is the head-to-head condensation of two molecules of GGPP to produce pre-phytoene di-phosphate (PPPP). This intermediate is then rearranged to form phytoene as a precursor to all carotenoids.¹¹⁶ The route of stereo-elimination of hydrogen gives phytoene a central 15-15' Z (*Zusammen*, otherwise named *cis*) double bond (see figure by Pfander^{117,118}). The geometric isomer state of the phytoene central Z double bond is an important factor in the accumulation of carotenoids further down the pathway.

Genes coding phytoene synthase have been isolated from many plants; some of the well studied genes are listed by Fraser.¹² PSY is associated with chaperonin,^{119,120} membranes, galactolipids,¹²¹ and GGPPS dimers.^{112,113} PSY is rate-controlling for flux into carotenoids,^{58,122,123} and thus both the plant and bacterial genes have been used for genetic manipulation of carotenoid accumulation (discussed in Section 5.3.3.3).

Some plants appear to have only one *Psy* gene.¹²⁴ *Psy* gene duplications have been found in tomato,^{125,126} tobacco,¹²⁷ rice and maize,¹¹⁸ and other cereal grasses.¹²⁸ Maize is an allotetraploid among the grasses (Poaceae),¹²⁹ so more than two *Psy* genes might be expected. Interestingly, the duplications of tobacco and tomato are not the same evolutionary event as the duplication of genes in maize and rice.¹¹⁸ Thus, duplication of *Psy* is an evolutionary parallelism (convergence), having occurred multiple times in plant phylogeny.

PSY is essential for photosynthesis but over-expression in green tissues is detrimental.¹³⁰ Evolution of additional *Psy* genes may have allowed high level expression in other tissues such as roots, pollens, fruits, flowers, and seeds. Thus the co-opting of carotenoids as secondary metabolites involved in animal attraction as pigments or in response to biotic and abiotic stress occurred multiple times during plant evolution. Indeed, in both dicots and monocots, one of the *Psy* duplications has temporal and tissue-specific expression conditioning carotenoids accumulating as secondary metabolites, for example, lycopene in red tomato fruits¹¹³ and yellow xanthophylls in the endosperms of maize.¹²⁸ Pigment engineers might take the evolutionary example, and modify the expression of genes involved in secondary metabolite production, without perturbation of primary physiology. The existence of duplicate genes coding stress-inducible or storage tissue-specific isoforms of PSY allows new biotechnological approaches to pigment accumulation.

5.3.2.4 DESATURATION AND ISOMERIZATION TO COLORED

CAROTENOIDS: BIOSYNTHESIS OF LYCOPENE

Production of the linear backbone of the carotenes requires four desaturations and several isomerizations of double bonds in the polyene chain (Figure 5.3.2). The substrate of the desaturations, 15-*cis* phytoene, does not accumulate in plants, with the exception of mutants, such as maize *viviparous5*, which has a white kernel and accumulates 15-*cis* isomers of phytoene (unpublished data). Such mutants have and continue to be useful for delineation of the pathway. The desaturations introduce conjugated double bonds extending the double bond system to generate a chro-

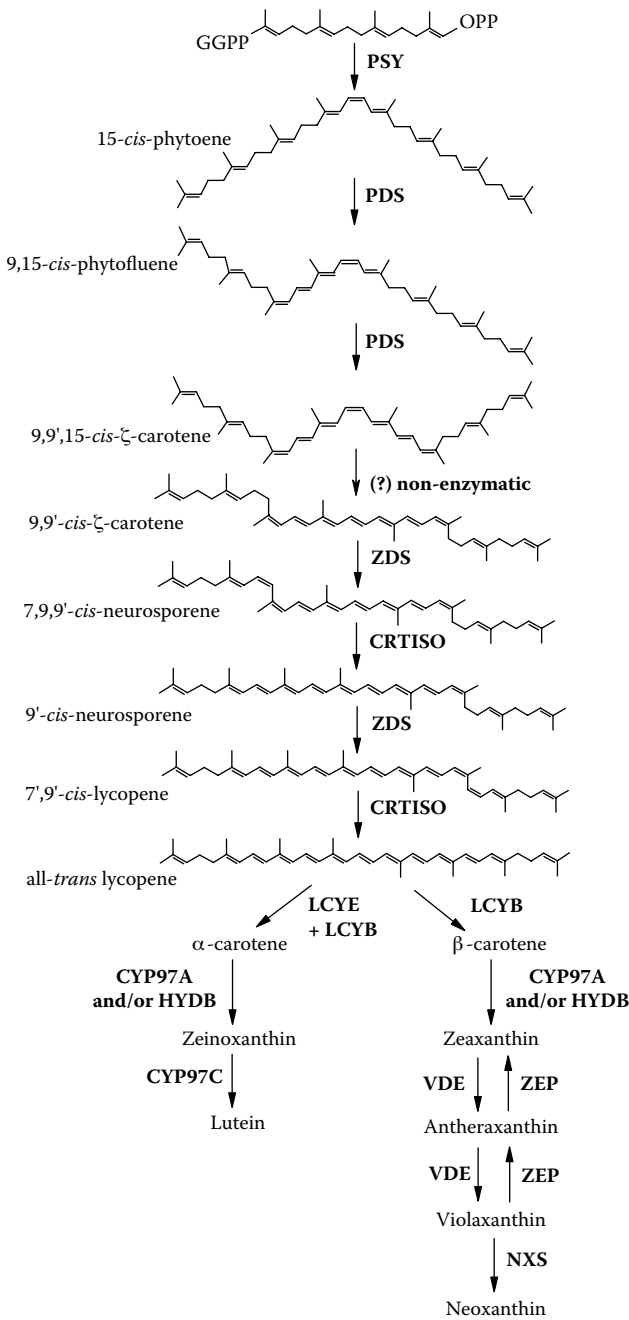


FIGURE 5.3.2 Carotenoid biosynthetic pathways in plants. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

mophore, which changes from yellow to red upon the serial desaturations. Some of the introduced double bonds are *cis*-configured.¹³¹ When lycopene accumulates (e.g., tomato), it is found as the all-*trans* isomer.¹³² The change in geometric isomer state is catalyzed by isomerase(s). The isomerization of the *cis*-double bonds to the *trans* configuration is necessary for formation of cyclic carotenes because the downstream lycopene cyclase enzymes are stereospecific for the all-*trans* configuration of lycopene.

In plants, the double bonds are introduced at paired, symmetric positions into 15-*cis* phytoene as shown in Figure 2. It was thought that only three plant enzymes, phytoene desaturase (PDS),¹³³ -carotene desaturase (ZDS),¹³⁴ and carotene isomerase (CRTISO)^{135,136} were involved in formation of all-*trans* lycopene. However, recent characterization of the maize *y9* locus has brought to light a new isomerase required in plant carotenoid biosynthesis.²⁶⁶ Maize *Y9* encodes a factor required for isomerase activity upstream of CRTISO, which has been termed Z-ISO, an activity that catalyzes the *cis* to *trans* conversion of the 15-*cis* bond in 9,15,9-tri-*cis*- ζ -carotene, the product of PDS, to form 9,9'-di-*cis*- ζ -carotene, the substrate of ZDS. Implication of *Y9* as the locus of the novel isomerase was made possible by a step-wise dissection of the carotenoid desaturation pathway in maize by a combination of molecular genetic techniques, including: (1) recombinant inbred gene mapping, (2) RT-PCR analysis of gene expression, (3) chemical complementation, and (4) HPLC analysis of geometric isomer states of pathway intermediates.^{145, 201, 266} Careful predictions and observation of accumulation of metabolites and morphological variation in pigment patterns amongst abiotically stressed or light vs. dark grown plants was crucial.²⁶⁶ Thus, identification of this "new" biosynthetic step using a maize mutant particularly well illustrates the value of classical mutant collections.¹⁶

The two desaturases are membrane-bound enzymes,^{111,112,119,137-140} and by inference, the isomerases may be also membrane-bound. Two di-hydrogen eliminations occur at the 11 and 11 positions mediated by PDS and then two more occur at the 7 and 7 positions mediated by ZDS.^{141,142} PDS introduces *trans*-configured double bonds at 11 and 11, and is thought to isomerize double bonds at 9 and 9 from *trans* to *cis*. ZDS introduces *cis*-configured double-bonds at the 7 and 7 positions.¹⁴³ Only the intermediates, phytofluene and -carotene are found among intermediate-accumulating mutants,^{144,145} so other sequences of reactions do not occur, for example phytoene is not a substrate for ZDS (no 7, 7 -polyene occurs that is not already 11, 11 desaturated) and other polyenes, such as 3,4, 3,4 lycopene do not occur naturally in plants, but have been engineered by gene shuffling.⁷⁷ Contrary to the activities of PDS and ZDS, in bacteria^{146,147} and in some fungi¹⁴⁸⁻¹⁵⁰ three to five serial desaturations of phytoene are carried-out by a single enzyme, the CRTI-type enzyme. Also the microbial CRTIs do not act with step-wise symmetry.¹³¹ Differences in the activities of the bacteria and plant enzymes are important to pigment biotechnology, because each option has been implemented in plant metabolic engineering.

Besides the capacity of CRTI to introduce all four double bonds in the conversion of phytoene to lycopene, the enzyme produces different geometric isomers than does PDS/ZDS (see graphic, side-by-side comparison in Fraser and Bramley¹²). CRTI produces all-*trans* isomers. Studies that have examined the function of the paired plant desaturases acting *together*, from *Arabidopsis*,¹⁵¹ and from maize¹⁴⁵ and from

mixed *Synnechocytis*-PDS/tomato-ZDS¹⁴³ in heterologous complementation systems, showed that among both the dicot (e.g., tomato and *Arabidopsis*) and monocot (e.g., maize) groups of higher plants, poly *cis*-pathway intermediates and products accumulate. The enzymes produce poly-*cis* lycopene (7,9,7,9-tetra *cis* lycopene). Mutants that accumulate intermediates of carotenoid biosynthesis in plants also accumulate poly-*cis* isomers^{152,153} and methods for detection of isomers of low level intermediates¹⁵⁴ will extend the demonstration of a poly-*cis* pathway to other plants. Since lycopene cyclases (LCY), which produces the cyclic carotenes, are stereoselective for all-*trans* isomers in most systems,¹⁵⁵⁻¹⁵⁹ isomerases are necessary for accumulation of cyclic carotenoids in plants (Figure 2).

The carotenoid isomerase (*CRTISO*) was the first isomerase associated with the desaturation steps and named at a time when Z-ISO was unknown to exist^{136,143,160,161} (and reviewed in references^{12,162,163}). *In vitro* analysis of substrate conversion¹⁴³ and transcript profiling *in planta*¹⁶⁴ associated CRTISO with the desaturation steps. Isaacson demonstrated that CRTISO is specific for the 7,9 or 7,9- *cis* bond configuration and is not involved in the isomerization of the 15-15-*cis* double bond to the *trans* conformation. As recently shown, Z-ISO is required for isomerization of the 15-15 *cis* double bond of phytoene produced in dark-grown tissues as well as in stressed photosynthetic tissues.²⁶⁶ Therefore, desaturation of phytoene to lycopene involves a two-step desaturation by PDS, followed 15-*cis* isomerization by Z-ISO, and then each pair of double bonds introduced by ZDS is followed by CRT-ISO-mediated isomerization of the resulting conjugated double bond pair.

Light may photoisomerize *cis*-carotenoids to the *trans*-states, especially the central 15-15 *cis*-double bond of phytoene, phytofluene, or zetacarotene.¹⁵¹ In the absence of light, *Arabidopsis* null mutants in *Crtiso* accumulate poly-*cis* lycopene¹³⁶ and maize y9 mutants accumulate the Z-ISO *cis* isomer substrate, 9,15,9-tri-*cis*- ζ -carotene.²⁶⁶ However, on the basis of mutant phenotypes (as discussed²⁶⁶), light appears to only partially compensate for lesions in the Z-ISO mutant. Interestingly in *Citrus*, there was no apparent induction of *Crtiso* during fruit ripening, while other genes coding enzymes in the pathway were up-regulated.¹⁶⁴ The genetic identification of Z-ISO will similarly lead to gene isolation, at which point analysis of Z-ISO gene expression during carotenogenesis will be feasible.

5.3.2.5 RINGING ENDS: BIOSYNTHESIS OF β -CAROTENE

Lycopene is the typical substrate for cyclization. One or both ends of the acyclic precursor can be cyclized. Cyclization can occur in one of two ways to create two different ring structures, differing only by the position of the double bond in the cyclohexane ring. Different enzymes form each of the rings, the lycopene- β -cyclase (LCYB) and the lycopene- ϵ -cyclase (LCYE), as shown in Figure 5.3.3.

While LCYB can use a linear (lycopene) or monocyclic substrate (δ -carotene or γ -carotene) to make a symmetric carotene with identical β rings on each end, LCYE introduces only one ϵ -ring into lycopene. A notable exception is the lettuce LCYE producing lactucaxanthin, which has two ϵ -rings.¹⁶⁵ The pathway bifurcates to form either β -carotene (having two β -rings) or α -carotene (having one β -ring and one ϵ -ring). The relative level of ϵ -cyclase activity influences the proportions of α -

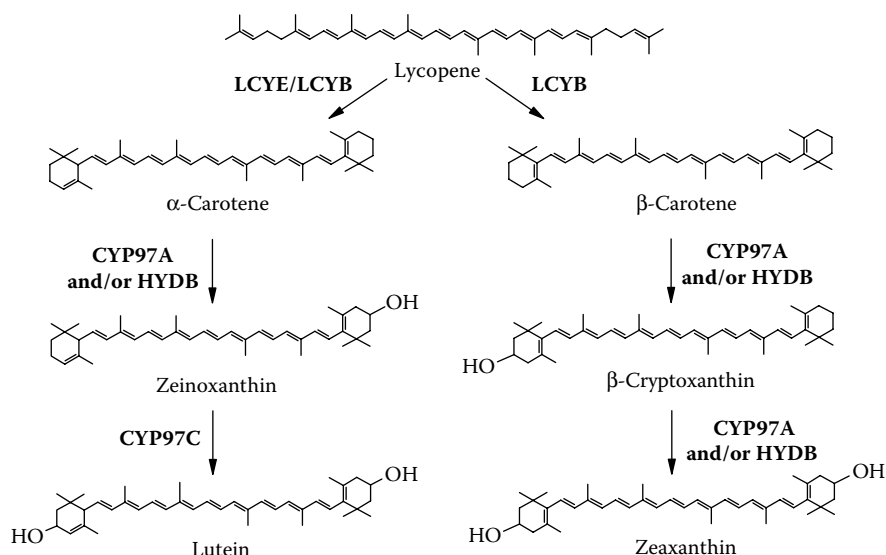


FIGURE 5.3.3A Carotenoid cyclization. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

Continued.

and β-carotene and their downstream oxygenated derivatives, the xanthophylls, lutein, and zeaxanthin.

Cunningham proposed a regulatory scheme by which the stoichiometry of the enzymes dictates the prevalence of alternate metabolons and thus the distribution of the pathway end products.¹⁵⁶ Because of the importance of lutein and zeaxanthin in the photosynthetic apparatus and xanthophyll cycle¹⁶⁶ that mitigates photo-oxidative stress, manipulation of flux through this branch point may allow engineering of photo-oxidative stress tolerance.^{167,168} Additionally, since β-carotene is the preferred food colorant and has twice the pro-vitamin A activity of α-carotene, this branch point is also a target for genetic engineering of crops. Similarly, the accumulation of xanthophylls, ketocarotenoids, and apocarotenoids used as food pigments is also effected by this pathway branch point.

5.3.2.6 HYDROXYLATION OF CAROTENES: BIOSYNTHESIS OF LUTEIN AND ZEAXANTHIN

Replacement of the hydrogen at the 3 or 3' position of the carotene ring with a hydroxyl is the next step in both branches of the pathway. Hydroxylation of the rings of the carotenes leads to biosynthesis of the xanthophylls, including the well-known lutein and zeaxanthin food pigments. Lutein is formed by hydroxylation of α-carotene; zeaxanthin is formed by hydroxylation of β-carotene.

Different monooxygenase enzymes hydroxylate the 3 position of the β- and ε-rings of α-carotene. Hydroxylation of one ring of β-carotene produces β-cryptoxanthin and hydroxylation of both β-rings produces zeaxanthin. Hydroxylation

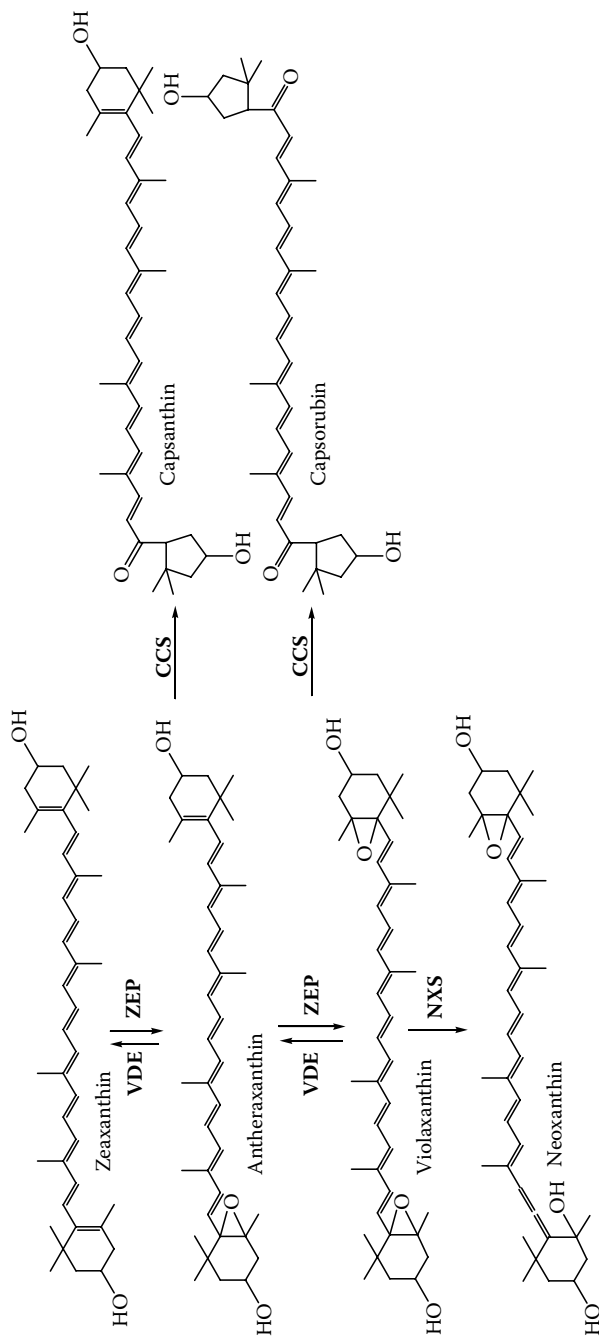


FIGURE 5.3.3B Oxidation to ketocarotenoids, capsorubin, and capsanthin. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

of the β -ring, ϵ -ring, or both rings of α -carotene produces zeinoxanthin, α -cryptoxanthin, and lutein, respectively (Figure 5.3.3A). Based on genetic studies of *Arabidopsis*, the β -ring hydroxylase was proposed to be a P450 enzyme in the CYP97 family, CYP97C, whereas the β -ring hydroxylases include the related P450 enzyme, CYP97A, and a nonheme di-iron monooxygenase, HYDB, homologous to the enzyme found in bacteria.^{169,170}

Enzyme activities for the CYP97 enzymes from rice were directly demonstrated in *E. coli*; CYP97C was shown to be specific for ϵ -rings and CYP97A was found to prefer primarily a β -ring substrate but also showed weak activity towards the ϵ -ring substrate¹⁷¹ as had been reported for the nonheme di-iron β -ring hydroxylases.¹⁷² However, it is not entirely clear whether the two structurally distinct β -ring hydroxylases function on specific branches of the pathway in plants. For example, while the P450 CYP97A could hydroxylate β -carotene in *E. coli*, it may be that the enzyme is not in a biochemical context to do so in the plant.

Furthermore, it is unclear whether the structurally distinct β -ring hydroxylases can compensate for each other if activity of one is diminished. These are important issues to address in engineering strategies for β -carotene accumulation which include blocking of β -ring hydroxylase activity. Moreover, the presence of gene families further complicates strategic engineering of provitamin A accumulation. For example, *Arabidopsis* has two genes encoding the di-iron enzyme and one gene each for the P450 enzymes. In maize, numerous genes (and pseudogenes) code for the di-iron enzyme.¹⁶

A phylogenetic analysis of HYD proteins among monocots, daffodil and crocus, as well as dicots, suggests several independent duplications of *Hyd* genes during land plant evolution.¹⁷³ Importantly, accumulation of the crocetin apocarotenoid was shown to be primarily correlated with *Hyd* transcript levels.¹⁷³ Understanding of the complex interactions and overlapping actions of hydroxylase activities and multiple hydroxylase loci is essential for breeding strategies for high-level accumulation of β -carotene requiring blocking or selection of tissue-specific null mutants of hydroxylase activity in non-photosynthetic plant tissues.

5.3.2.7 EPOXIDATION OF XANTHOPHYLLS: BIOSYNTHESIS OF ANTERAXANTHIN AND VIOLAXANTHIN

The 3-hydroxyl β -rings of zeaxanthin are further oxygenated by the introduction of 5,6-epoxy moieties by zeaxanthin epoxidase (ZEP).¹⁷⁴ A mono-epoxidated intermediate, antheraxanthin is produced, followed by the di-epoxy xanthophyll, violaxanthin, as shown in Figure 5.3.3B.

Physiologically, violaxanthin is an important component of the xanthophyll cycle;¹⁷⁵ a high light stress-induced de-epoxidation of the violaxanthin pool to the more photoprotective zeaxanthin is mediated by violaxanthin de-epoxidase (VDE). Violaxanthin and neoxanthin, an enzymatically (NXS)-produced structural isomer, are the precursors for the abscisic acid (ABA) biosynthetic pathway (Figure 5.3.1, Pathway 4 and Figure 5.3.2). In non-photosynthetic tissues, namely: ripe bell peppers, antheraxanthin and violaxanthin are precursors to the red pigments, capsanthin and capsorubin, respectively (Figure 5.3.3B).

5.3.2.8 KETOCAROTENOIDS

5.3.2.8.1 Ketolation to Capsanthin and Capsorubin

A lycopene cyclase-related enzyme, capsanthin/capsorubin synthase (CCS), converts the cyclohexane end rings of antheraxanthin or violaxanthin to cyclopentane rings to produce capsanthin or capsorubin, respectively.¹⁷⁶ The enzyme acts on the 3-hydroxy-5,6 epoxy group to produce the unusual κ -ring. Capsanthin is a mono-ketolated product; capsorubin is di-ketolated (Figure 5.3.3B). Among plants, these ketocarotenoids are limited to species of *Capsicum*. All plants make antheraxanthin and violaxanthin (they have ABA), so that transplantation of only one gene functions may allow accumulation of these red pigments in other plants. Engineering of tissue-specific expression is needed to avoid perturbations of normal physiology in photo-synthetic tissues (zeaxanthin) and seeds (ABA).

5.3.2.8.2 Astaxanthin

Astaxanthin provides wild salmon with their characteristic deep orange hue; this is a commercially valuable ketocarotenoid that has been chemically synthesized for feeding to farmed fish in order to produce optimal coloration desired for marketing. In algae and fungi, astaxanthin accumulates in response to stress. Astaxanthin is not naturally occurring in most higher plants. Whereas *Capsicum* ketocarotenoids are derived from zeaxanthin, the biosynthesis of the ketocarotenoid astaxanthin usually occurs by a different biosynthetic route that does not involve zeaxanthin.¹⁷⁷

A single β -C-4 oxygenase introduces keto groups at the 4 and 4' positions of β -carotene rings, producing the intermediate echinenone followed by canthaxanthin. The enzyme modifies both end rings, analogous to HYDB and CYP97A. A carotene hydroxylase then introduces 3 and 3' hydroxyls to each ring. Astaxanthin biosynthesis in *Adonis* utilizes a different mechanism having evolved an enzyme with similarity to plant di-iron β -ring hydroxylases.¹⁷⁸ Introduction of astaxanthin biosynthesis has been accomplished in tobacco with a combination of a unicellular algal ketolase and endogenous hydroxylase.¹⁷⁷

5.3.2.9 CAROTENOID CLEAVAGE AND APOCAROTENOIDS

Apocarotenoids are the derivatives of cleaved carotenoid chains (Figure 5.3.1, Pathway 4). Cleavage involving oxygen occurs at double bonds. These carotenoid cleavage products (CCPs) are often substrates for further enzymatic modification. While apocarotenoids with new functions such as intercellular signaling¹⁷⁹ have only recently been discovered, the origin of CCPs from carotenoids has long been suspected.

Well-known CCPs and their functions include (1) β -ionone, geraniol, and β -damascenone (fruit and flower flavor and fragrance), (2) ABA (dormancy, stress, and senescence phytohormone), (3) strigolactone (mycorrhizal fungal and parasitic weed growth stimulant),^{180–182} (4) mycorradicin and blumenin (phytoalexin antifungals), and (5) trisporic acid (suspected pheromone).¹⁸³ Their genesis is now better defined by newly isolated gene functions that were recently reviewed.^{179,183,184} They are produced by carotenoid cleavage dioxygenases (CCDs), otherwise referred to as

carotenases (Table 5.3.1).¹⁷⁹ Apocarotenoids are also produced in animals and include the retinoids: retinal (vitamin A), retinol (vision), and retinoic acid (morphogen).

The mammalian enzymes involved in vitamin A and apocarotenoid production cleave a variety of substrates (50 to 60 dietary carotenoids!) at central or eccentric double bonds and have been recently reviewed.¹⁸⁵ A mammalian CCD-homologous protein acts as a retinal isomerase,¹⁸⁵ but no plant CCD homologues reported have shown carotenoid isomerase activity. Here we will briefly describe the general properties of CCD enzymes and their role in plant physiology as it may relate to bioengineering of plant carotenoid and apocarotenoid accumulation. After the pertinent review, we will relate recent progress in molecular genetics of specific food pigments, bixin and saffron.

Plant apocarotenoids have a wide variety of structures and functions. As expected, there is a small gene family of CCDs with different cleavage sites and somewhat promiscuous substrate selection. Some CCDs are stereo-specific, for example, 9-*cis* epoxycarotenoids are the substrates for NCEDs (9-*cis* epoxy dioxygenases) that produce the precursor of ABA biosynthesis, xanthoxin. Both linear carotenoids (lycopene) and cyclic carotenoids are substrates for cleavage at various double bonds including the central 15-15' and eccentric 5-6, 7-8, 9-10, 9'-10', and 11-12 bonds. Some CCDs cleave both linear and cyclic carotenoids and may cleave the same molecule twice, e.g., both 9-10 and 9'-10' positions.

The first CCD gene cloned was *viparous* 14 from maize, for which the recessive allele conditioned ABA deficiency.¹⁸⁶ Other CCDs among several plants have been cloned by homology and functionally demonstrated. Arabidopsis was found to have nine CCD-homologous genes, of which five are involved in ABA physiology, two in morphogenic signal molecule production, one that produces two β -ionones from β -carotene, and one, CCD1, is highly promiscuous, producing a large variety of products.¹⁸³ Importantly, CCDs not only condition accumulation of dialdehydes and ketones, but also affect carotenoid turnover, and thus, may negatively impact pigment accumulation in some tissues, as seen in white chrysanthemum petals: transgenic blockage of CCD expression revealed the underlying carotenoid biosynthesis for which accumulation was ordinarily prevented through turnover mediated by CCD activity.¹⁸⁷

5.3.2.9.1 Bixin (Annatto)

Bixin, or annatto, is a widely used food colorant, an apocarotenoid produced only by the neotropical plant, *Bixa orellana*. In an elegant study using standard molecular techniques and a bacterial complementation system, the genes involved in the biosynthesis of bixin were cloned and functionally characterized.¹⁸⁸ The pathway determined by the gene isolation is shown in Figure 5.3.4A and reviewed in the recent literature.¹⁸³

The three genes that mediate the oxidative modification of lycopene to the carboxylic monomethyl ester apocarotenoid, bixin, were transferred to a variety of *E. coli* strains producing lycopene or other carotenoids to prove function and substrate range of the encoded gene products. The *B. orellana* lycopene cleavage dioxygenase (BoLCD) was specific for lycopene and did not cleave β -carotene or zeaxanthin. Thus, BoLCD, did not seem to be substrate-promiscuous, as is, for example, CCD1 of Arabidopsis. Bixin methyl ester accumulated to moderate levels

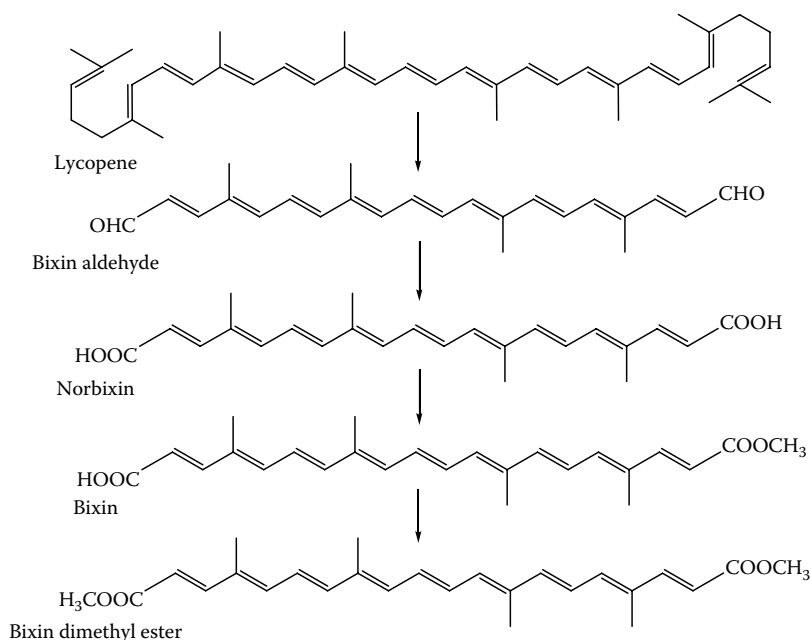


FIGURE 5.3.4A Proposed biosynthetic pathway to bixin. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

Continued.

in the heterologous host, demonstrating the function of the genes in the production of the pigment.¹⁸⁸

Genes for the predicted bixin aldehyde dehydrogenase and carboxy methyltransferase, norbixin methyltransferase, which mediates the accumulation of the apocarotenoid dimethyl ester, were cloned from *B. orellana* by using an RT-PCR primer based on other plant sequences. PCR products were used to screen a developing seed cDNA library by hybridization. Isolation of genes from cDNA of *B. orellana* was based on homology to previously cloned carotenoid cleavage dioxygenases from *Arabidopsis* and maize.^{186,189,190} Figure 5.3.4A shows the structures of intermediates and the accumulated bixin dimethyl ester. Bouvier et al. suggest that these three genes may be sufficient to genetically engineer the accumulation of bixin in tomato, which ordinarily accumulates high levels of lycopene.¹⁸⁸ Production of bixin in transgenic tomatoes or another crop may offer a cost-effective alternative to production of this food colorant in temperate zones.

5.3.2.9.2 Crocetin and Safranal

The aroma and red color of the spice saffron are partly due to the style-specific accumulation of carotenoid cleavage products produced by both enzymatic^{173,191,192} and thermal degradation.¹⁹³ M. Giaccio reviewed the renewed interest in saffron as a colorant, spice, and nutraceutical.¹⁹⁴ Crocetin is a C20 apocarotenoid derived from zeaxanthin (Figure 5.3.4B).¹⁹¹

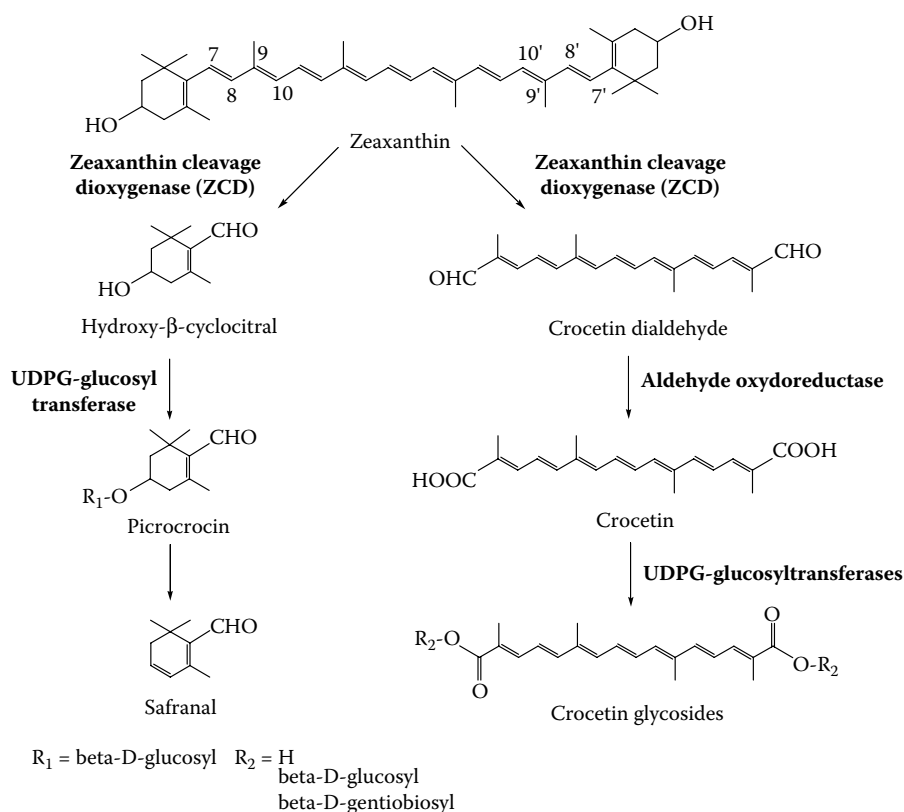


FIGURE 5.3.4B Proposed biosynthetic pathway to crocetin and safranal. Enzyme abbreviations and enzyme activities are defined in Table 5.3.1.

Two CCDs were isolated from domesticated crocus based on homology to maize *VP14*. Color complementation experiments and immunolocalization of the two CsCCDs demonstrated that one enzyme was substrate-promiscuous and localized to the cytosol, while the other was plastid-localized and specific for zeaxanthin cleavage at 7,8, 7',8'-double bonds.¹⁹² The enzyme produces crocetin dialdehyde and hydroxyl — cyclocitral. Interestingly, the crocus plastidial zeaxanthin 7,8 (7',8')-cleavage dioxygenase (CsZCD) has no N-terminal plastid targeting sequence, implying an internal plastid localization signal. The crocetin dialdehyde is a substrate for a putative oxidoreductase that produces crocetin. Both crocetin and hydroxyl-β-citral are glycosylated.

Picrocrocin is the stored precursor of the volatile safranal. Crocetin is glycosylated to form di-glycosyl, di-gentiobiosyl, mixed or mono esters, as shown in Figure 5.3.4B. Moraga et al. isolated and showed function in a complementation system of a style-specific UDP-glucosyltransferase (UDP-GTase) from crocus that is specific for products of the cleavage pathway.¹⁹⁵ While the cleavage of zeaxanthin occurs in the plastids, the glycosides of the cleavage products are stored in the vacuoles.¹⁹²

UDP-GTase may be associated with membrane transporters and is a key component of pigment accumulation for the metabolic engineer to consider.

5.3.3 CAROTENOID BIOTECHNOLOGY

Conventional breeding often focuses on introgression of a value-added trait such as disease resistance, yield increase, or accumulation of valued metabolites. Often a new trait is brought into elite commercial germplasm by mating to an exotic germplasm. Crossing brings to the selected progeny the desired trait and many other undesirable traits or inadvertently co-selected traits. Further breeding cycles are then required to reconstitute a commercially viable variety with the introgressed trait. Biotechnology aimed at crop improvement is often focused on introgression of stably inherited traits, with minimum disruption of the pre-selected adaptive state.

Biotechnologies such as mutation breeding, marker-assisted selection (MAS), and genetic engineering are thought to be superior methods of crop improvement because they increase the efficiency and accuracy of pinpoint introgression of value-added traits. Furthermore, the addition of traits from other species, for example, from bacteria to plants, can transcend the possibilities of conventional breeding. Nevertheless, biotechnology is not a panacea and presents its own problems and challenges.

5.3.3.1 HETEROLOGOUS COMPLEMENTATION IN *E. COLI*

Unicellular algal and bacterial genes were the first to be isolated and characterized and led to the isolation of most of the higher plant genes involved in carotenoid biosynthesis. Carotenogenic gene clusters from bacteria and algae^{115,147,196–198} contributed immensely to the elucidation of the carotenoid pathway.

Homology to algal genes and complementation in bacterial systems led to isolation and characterization of higher plant genes involved in carotenoid biosynthesis from a variety of model and crop species including *Arabidopsis*,^{151,156,172,199} bell pepper,²⁰⁰ maize,^{16,109,128,145,201,202} rice,^{128,199,203} soybean,¹³³ tomato,^{125,157,204} and other plants.^{93,125,134,157,201,204–208} Algal genes were essential to the isolation of higher plant genes, because the homology between bacterial genes and plant genes was generally too low to allow cloning of plant genes using hybridization-based approaches.

Transplantation of the genes to an *E. coli* expression platform allowed facile, stepwise addition of gene functions coupled with metabolite profiling and complementation of genes from other sources such as higher plants. For example, the function of a putative plant gene could be tested in the presence of a bacterial carotenogenic gene cluster which was missing the suspected gene function followed by HPLC analysis of metabolite accumulation. Whole cDNA libraries were screened visually for gene functions directly or indirectly affecting carotenoid accumulation, using a procedure called color complementation screening. Examples of using the heterologous system for isolation coupled with functional complementation are included by reference.^{106,109,124,128,172,199,202,203,209,210} The jump from algal genes to plant genes and color complementation systems exerted a dramatic impact on the pace of progress in higher plant gene cloning and functional characterization.

Complementation systems were also used to examine the consecutive actions of plant genes. Determination of the function of more than one plant gene acting in concert was especially instructive when examining the effects of geometric isomer states of intermediates on the progression through the desaturation series using PDS and ZDS from maize¹⁴⁵ and Arabidopsis.¹⁵¹ The paired desaturases were shown to mediate a poly-*cis* isomer pathway in *E. coli*, as discussed in Section 5.3.2.4.

The stereo-chemical behavior of PDS and ZDS from the phylogenetically disparate monocots and dicots as demonstrated with recombinant enzymes in *E. coli*, indicated the wide natural distribution of the poly-Z pathway among plants. The intermediates accumulating in the heterologous system and in particular plant loss-of-function mutants such as *tangerine* of tomato,¹⁵² *carotenoid and chloroplast regulation 2 (ccr2)* of Arabidopsis,¹³⁶ and of algal mutants^{211–213} allowed the possibility of isomerases that could regulate progression through the stereo-specific enzymes in the pathway. Subsequently, an isomerase was cloned by linkage analysis (map-based cloning) of the *tangerine* locus from tomato²¹⁴ and from the chemically induced mutant *ccr2* from Arabidopsis.¹³⁶ The algal isomerase was also quickly identified.^{160,161}

Following the lead from bacterial and algal systems, the combination of classical genetics and biochemical analyses, map-based cloning, heterologous expression, and morphological analyses in plants demonstrated that an isomerase was involved in the desaturation series (reviewed by Giuliano¹⁶³ and Eckardt¹⁶²), and has biochemical and morphological roles in development of carotenoid accumulation and chloroplasts,¹³⁶ thus ending a 60-year-old enigma of *cis*-carotenoid metabolic intermediates in plants. Recently, *in vitro* substrate feeding experiments with the recombinant tomato isomerase extract from *E. coli*¹⁴³ demonstrated specific substrate specificity of CRTISO,¹⁴⁵ but left open the possibility for an additional isomerase acting on 15'-*cis* phytoene, the recently discovered Z-ISO.²⁶⁶

5.3.3.2 GENETIC ENGINEERING

Proof-of-concept genetic engineering studies of plants with single or multiple transgenes aimed at the biotechnological improvement of food carotenoids have recently been cited in reviews.^{12,18,215} Transgenesis into rice to create Golden Rice has been comprehensively reviewed.⁵⁹ Despite concerns about the effectiveness of these transgenic crops as nutraceuticals,^{59,216} much progress has been made and several products may soon be released.

5.3.3.2.1 Bacterial Genes

The bacterial genes *CrtB* and *CrtI*, coding functions equivalent to plant phytoene synthase (PSY) and phytoene desaturase (PDS/ZDS/CRTISO), respectively, have been engineered into several plants with mixed and sometimes unexpected but informative results. The native bacterial genes lack a chloroplast transit peptide and a plant promoter, so these eukaryotic regulatory and targeting elements were engineered into the transgene at the DNA level. For example, the small subunit (SSU) of RuBisCo (Calvin cycle enzyme) and CaMV 35S promoter (virus) have frequently

been used for targeting and strong constitutive expression. Tissue-specific promoters for seeds and fruits have also been used and are discussed specifically in this section.

PSY was shown to be rate-controlling for flux (discussed in Section 5.3.2.3) into carotenenes in a number of systems. Hence, *CrtB* was introduced into plants under the control of several different regulatory elements. Since tomatoes are the major dietary sources of lycopene and β -carotene in some countries, tomatoes have received the most attention as targets of engineering.^{132,174,217–220} In 1995, Fray et al. reported the first use of over-expression under the control of the CaMV 35S promoter of tomato *PsyI* in tomatoes.¹³⁰ The results were not good and led to the later use of *CrtB* instead.¹²² Specifically, plants over-expressing the fruit-specific tomato PSY1 in a constitutive manner were pleiotropically affected in a number of ways: (1) dwarfism due to depletion of the GGPP precursor pool shared with the gibberellic acid growth hormone, (2) hyperaccumulation of pigment in shoots and roots leading to intense coloration and photosensitivity, (3) increase in ABA levels due to increased carotenoids, and (4) premature accumulation and little to no accumulation of carotenoids in fruits that might be attributed to gene silencing due to the use of the endogenous tomato gene.¹²

CrtB, which has low-level nucleotide sequence homology to *PsyI*, was inserted under the control of the ripening, fruit-specific polygalacturonase promoter (PG). The results were good. *CrtB* was expressed in a fruit-specific manner and targeted to the plastid and resulted in a two- to three-fold increase in carotenoids including lycopene and β -carotene.¹²² *CrtB* over-expression has also been effective in canola, carrots, and potatoes.

The case of canola is extraordinary because of the very high level accumulations (50-fold) of leaf-type carotenoids in seeds when the gene was introduced under the seed-specific promoter, napin.¹²³ The exalbuminous seeds of canola differ from those of genetically engineered rice cereal grains in that they have chloroplasts, which may explain the capacity for hyperaccumulation of carotenoids.

The food technologist may be especially interested in the fate of the carotenoids in the seed oil. Like red palm oil, the resulting carotenoid-pigmented canola oil may be more stable due to the antioxidant properties of carotenoids and may be more attractive to consumers. Alternatively, for food security concerns, transgenic soybean or canola oils and seed meals that are genetically modified for more efficient bio-diesel production may be bio-safety marked with lipid-soluble carotenoids and water-soluble anthocyanins, respectively. Potatoes are excellent potential sources of dietary carotenoids, and over-expression of *CrtB* in tubers led to the accumulation of β -carotene.²²¹ Potatoes normally have low levels of leaf-type carotenoids,²²² like canola cotyledons.

Over-expression of bacterial phytoene synthase led to only modest increases in pigment accumulation (except in the case of chloroplast-containing tissues). Attention turned to *CrtI*, one gene that might control flux through the entire four desaturation steps from phytoene to lycopene (discussed in Section 5.3.2.4). Only a modest increase in carotenoid content in tomatoes and a variety of changes in carotenoid composition including more β -carotene, accompanied by an overall decrease in total carotenoid content (no lycopene increase), resulted when *CrtI* was over-expressed under control of CaMV 35S.²²³ Apparently, the bacterial desaturase

has pleiotropic effects on the pathway and a putative induction effect on LCYB without affecting compositions of geometric isomers.^{12,220}

Manipulation of carotenogenesis with bacterial genes demonstrated the suspected ability of bacterial gene products to function in the plastid environment, the need for specific promoters, the advantage of heterologous genes in avoiding transcriptional silencing, and uncertainties surrounding communication and homeostasis within the pathway. While the bacterial functions worked, it is unclear whether they cause problems because they do not interact normally with the plastidial carotenogenic metabolon.

5.3.3.2.2 Transplanting Plant Genes

Plant phytoene synthase (*Psy*) has been used in a variety of transgenics. As noted above, *Psy1* over-expression under a strong constitutive promoter caused a decrease in carotenoid accumulation, probably due to transcription silencing.¹³⁰ Similarly, over-expression of the gene sequence backward (antisense) also silenced activity.²²⁴ In another approach to over-expression of tomato *Psy1* in fruits, a synthetic alternative in which the third position of each codon was changed in order to avoid transcriptional silencing was successful in conditioning an increase in carotenoid accumulation.

In Golden Rice, over-expression of daffodil *Psy*²²⁵ and a variety of other plant *Psy* genes including the endogenous rice gene resulted in a wide range of increased accumulation of xanthophylls.²²⁶ Because there are duplicate factors that vary in their evolutionary relationships and tissue-specific expression patterns (discussed in Section 5.3.2.3), it is notable that the maize and rice *Psy* genes in Golden Rice 2 conditioned accumulation of higher carotene accumulation compared to *Psy* genes from other plant families.

In rice endosperm that accumulated phytoene by virtue of daffodil *Psy* transgenesis, Burkhart et al. reported the failure of daffodil *Pds* over-expression in rice endosperm to condition accumulation of ζ -carotene, even though an increase in PDS antigen was detected.²²⁵

Success with transgenic expression of *Pds* or *Zds* or both has not been reported. Also not known is whether CRTISO and Z-ISO, the companion isomerases to the desaturations, will influence accumulation of intermediates or products.

The availability of precursor IPP may ultimately be most influential over accumulation of carotenoid metabolites.²²⁷ While over-expression of DXS and DXR in color complementation systems leads to hyperaccumulation of carotenoids (discussed in Section 5.3.3.3), over-expression of plant *Dxs* genes has not always been effective. Over-expression of DXS resulted in increased carotenoid accumulation in transgenic tomato⁹⁹ and Arabidopsis,²²⁸ but over-expression of daffodil DXS in rice endosperm did not increase pigment accumulation.⁵⁹

Over-expression and anti-sense constructs of LCYB have been tested in rice and tomato. In Golden Rice, daffodil LCYB was over-expressed but found to be unnecessary for accumulation of carotenes. Only PSY and CRTI are needed to accumulate carotenes and xanthophylls in the endosperm.

Apparently endogenous LCYB (and HYDB) is constitutively expressed and was not induced by the presence of CRTI,²²⁹ a not surprising fact since endogenous

expression of the pathway genes in rice endosperm had been known for some time.^{230,231} In tomatoes that accumulate high levels of lycopene, Arabidopsis LCYB over-expression with a fruit-specific *Pds* promoter resulted in increased accumulation (five-fold) of β -carotene, while antisense over-expression of an antisense tomato LCYB under a *Pds* promoter caused a small increase in lycopene only. In both cases, leaf carotenoid content was not affected. Others had similar results with the second member of the LCYB gene family in tomato.²³²

Xanthophylls do not normally occur in tomato fruits but have particular health benefits. Tomato fruits are exceptional storage organs for carotenoids and thus are natural targets for nutritional enhancement. Transgenic expression of the plant or bacterial HYDB alone did not result in accumulation of zeaxanthin, but co-transformation of Arabidopsis LYCB together with Capsicum HYDB did.^{217,218} Also in potatoes, Arabidopsis ZEP was over-expressed in both sense and antisense orientations in tubers under control of the granule-bound starch synthase (GBSS) promoter, resulting in silencing of the conversion of zeaxanthin to antheraxanthin and violaxanthin.²³³ In various situations, zeaxanthin or antheraxanthin accumulated. Also, total carotenoid content increased, reminiscent of pathway up-regulation in tomato fruits perturbed by the addition of *CrtI*.

5.3.3.2.3 Mixed Plant and Bacterial Genes

Some multigene transgenic plants have a mixture of added plant and bacterial genes. Golden Rice is a famous example. Golden Rice 1 contains the transgenes daffodil *Psy*, bacterial *CrtI*, and daffodil *Lcy*. The addition of daffodil PDS failed to confer colored carotenoids in rice endosperm.²²⁵ Unbeknownst at the time, the use of the plant two-step desaturases, PDS and ZDS, might also require the addition of companion isomerases. Based on the substrate specificity of CRTISO for the products of ZDS,¹⁴³ it is unlikely that a lack of CRTISO affects accumulation of the products of PDS, phytofluene and ζ -carotene. In any event, the lack of accumulation of products of daffodil PDS in rice endosperm precipitated the use of *CrtI*.²³⁴

Fortuitously, the bacterial gene product, CRTI, produces all-*trans* carotenoids and satisfies the stereo-chemical specificity of LYC B for all-*trans* substrates while also catalyzing the four desaturation steps from phytoene to lycopene. Nevertheless, over-expression of *CrtI* has been shown to have only a modest effect (two- to four-fold increases in tomatoes and carrots) in increasing flux through the pathway and some unexpected pleiotropic influences on activities upstream and downstream of the desaturations (reviewed by Fraser and Bramley¹² and Giuliano²²⁰).

Recently reported experiments indicate that modulation of CRTI activity with various promoters does not appreciably affect carotenoid accumulation in rice endosperm.⁵⁹ Transgenic manipulation of PDS/ZDS/CRTISO, and Z-ISO when available, for crop improvement has yet to be reported, but nevertheless may present advantages over the transgenic bacterial desaturase. For example, use of bacterial genes may have a disadvantage in deregulatory issues. The transgenesis of bacterial genes to edible plants requires higher level regulatory approval (not required for genes from one edible plant to another edible plant in the United States). Transgene products from non-crop sources, such as bacteria or daffodils, are less appealing to

the public than the use of plant genes, whose products have a history of use in food and perhaps require fewer deregulatory costs and less effort.⁵⁹

There is already some indication in genetic manipulations of carotenogenesis that transgenes from closely related species perform better than do genes encoding evolutionarily distant isozymes. In Syngenta's Golden Rice 2 which is thought to be a practical nutraceutical,²⁵ the use of maize PSY over-expression in rice endosperm led to the highest levels of carotenoid accumulation among the other PSYs tested, including Arabidopsis, carrot, daffodil, bell pepper, rice, and tomato.⁵⁸ This is in contrast to over-expression of DXS and CRTI, which had little effect on accumulation.

Now that the flux-limiting step (PSY) has been determined, partially optimized, and opened, it is compelling to give new attention to flux through the desaturations, and for reasons cited above, even more compelling to consider PDS/ZDS/isomerases coded by rice or maize genes as an alternative to *CrtI*. Maize genes may be preferable in rice because they are public,^{118,128,145,201} likely to have enough nucleotide dissimilarity to evade transcriptional silencing (discussed in Section 5.3.2.2), and may interact more appropriately with a carotenogenic metabolon.

5.3.3.2.4 Antisense Approaches and RNAi

Disruption of gene function by transcript-level attenuation is often used to assess function and can also be used to effect desired changes in accumulation of pigments. Examples of experimental gene suppression were already discussed in relation to applying VIGS technology to investigate MEP pathway functions (Section 5.3.2.2) and in manipulation of carotenogenic enzymes in tomatoes.

In a very recent study in potatoes, inhibition of LCYE accumulation was accomplished by an antisense *LcyE* driven by the patatin promoter and allowed rechanneling of lycopene toward the β -carotene branch of the pathway to produce up to 14-fold increased levels of β -carotene as well as up to 2.5-fold increased total carotenoids.²³⁵ RNAi and TILLING for manipulation of carotenogenesis have yet to be reported, but these new techniques for suppression of function and generation and selection of allelic diversity are likely to impact future research and production of varieties with enhanced pigment accumulation.

5.3.3.3 QTL AND ASSOCIATION GENETICS

Association of pigment accumulation with DNA features can inform systematics and applied breeding in crop plants. For carotenoid content among crop plants, maize and tomato are best studied by this technique. Because there is interest in accumulation of various intermediate products in the pathway as well as end products, QTLs were developed for genetic determinates that are not independent of each other.²³⁶ Some QTLs affect multiple pigment contents.

In maize, many phenotypic mutants have been associated with cloned genes by a combination of HPLC analysis of specific intermediate metabolite accumulation, RT-PCR and immunolocalization of candidate genes, and recombinant inbred mapping of candidate cDNAs.¹⁶ *PsyI* was cloned by transposon tagging²³⁷ and later shown to be functional in the color complementation system and to be the specific

isoform (not *Psy2*) expressed in maize endosperm.^{118,128,137} *Psy* was associated with the *y1* mutant by a transposable element-induced instability of pigment accumulation (somaclonal sectors)^{238,239} and genetic and recombinant inbred linkage mapping.²³⁷ *Pds* and *Zds*, which are single copy genes, were also associated with the mutants, *vp5* and *vp9*, by a series of similar studies.^{118,145,201,202,238,240–242}

QTL analysis of the intermediates in the pathway (such as β -cryptoxanthin, see Figure 5.3.2) and of total accumulated carotenoids associated the variation in content with *Y1* and *Vp9* but not with *Vp5*. Candidate genes *Psy* and *Zds* but not *Pds* were thus associated with the variation in specific and total pigment contents. These influential genes and associated markers can now be used to further select allelic variants by association genetics or TILLING and produce selection tools (MAS) to use in a conventional breeding strategy that may lead to targeted crop improvement. One such target is an increase in β -carotene content for sub-Saharan maize staples.²³⁶

Most of the DNA variation that affects pigment accumulation is thought to be associated with mutations in the *cis*-acting regulatory elements, most often upstream of the structural coding regions.²³⁶ LD mapping studies conducted on a wide collection of germplasm associated changes in the 5' *cis*-acting regulatory sequences of *Psy1* with the co-evolution of yellow endosperm during American maize domestication.^{75,243} Specifically, a transposable element in the promoter of *Psy1* seems to have led to tissue-specific gain of function, associated with novel accumulation of carotenoids in kernels, compared to the white endosperm progenitor. Again, *Psy2* was not associated with pigment accumulation in the endosperm,²⁴³ as was predicted and confirmed by classical molecular genetic studies.^{106,118,128}

Thorup et al. exploited the microsynteny of bell pepper and tomato and cloned candidate genes from each to determine correspondence of QTL.⁷¹ A comparative approach across species places consensus QTLs, candidate genes, and biochemistry in a phyletic perspective avoiding uncertainties associated with environmental and ontogenic variation in traits.

QTLs were also detected in tomato introgression lines (domestic tomatoes with single, defined chromosome regions selected from the progeny of a cross with a wild, non-pigmented variety) that correspond to candidate loci²⁴⁴ for which genes and mutants have been previously characterized. Detected QTLs included the *r* locus (*Psy*), the *Del* locus (*LcyE*), and the *B* locus. Similar to the tissue-specific expression of maize *Y1*, the *B* locus codes a fruit-specific lycopene cyclase associated with higher levels of β -carotene.¹²

The stability of QTL during applied evolution of tomatoes has also been assessed for organoleptic qualities²⁴⁵ and transcriptomics, and select metabolomics have now been applied to fruit ripening.²⁴⁶ Such phyletically broad and pan-cellular studies are at the forefront of development of an integrative approach to understanding pigment accumulation in a broader sense that transcends poke-and-hope genetic engineering.

5.3.3.4 GENERATION OF VARIATION

Experiments in directed breeding have been carried out in bacteria and are proving grounds for metabolic engineering of pigment accumulations in plants. Experi-

ments with gene shuffling have shown that novel carotenoids can be produced by mixing genes from gene libraries. C. Schmidt-Dannert et al. demonstrated gene shuffling for carotenoid functions and reviewed^{33,77,247,248} similar efforts such as those of Wang et al.²⁴⁹

Because of the ease of screening for color variants and the ability to recursively add new functions to a color complementation system, the carotenogenic model served as a testing ground for shuffling concepts. By first shuffling two *Erwinia* desaturase genes and then adding a library of shuffled lycopene cyclase genes, a directed evolution scheme produced tetrahydrolycopene and then torulene in *E. coli*.⁷⁷ Accumulation of torulene was a newly evolved function among the progenitor gene set. The addition of further downstream functions such as hydroxylase, ketolase, or glycosylase resulted also in remodeled torulenes.²⁵⁰ Accumulation of the novel products was then improved by more typical microbial production optimization.^{248,251} Further progress in directed evolution of carotenogenic genes has also been reported.^{107,252} Thus, the color complementation system has also been used effectively for testing concepts in metabolic engineering.

5.3.3.5 METABOLIC ENGINEERING

Transgenic *E. coli* accumulate comparatively low levels of carotenoids^{253,254} compared to microbial algae, yeasts, and bacteria. Many efforts^{36,37,250} have focused on increasing accumulation by manipulation of factors affecting metabolic flux and metabolite accumulation (listed and discussed in Sections 5.3.1.1 and 5.3.1.3 A) and have been reviewed.^{118,254} In bacterial systems, approaches to control can be categorized as either infrastructural (plasmids, enzymes, strains) or ultrastructural (media and feeding, environment, precursor pools, substrate flux).

Consultation of the cited literature will supply many examples of metabolic controls in microbial carotenogenesis. Influential infrastructural factors include: genetic background and strain selection, substrate feeding and optimization of growth and/or media conditions, stabilization of mRNA by alteration of gene structure, decrease of general metabolic burden by use of low copy number plasmids, use of inducible gene expression and optimization of the degree and timing of gene induction. Ultrastructural controls include: manipulation of metabolic flux by over-expression of rate-controlling enzymes, precursor pool enhancement, precursor pool balancing, removal of feedback inhibition by modification of gene and enzyme structure and sequestration of products. Some key examples are discussed in this section as a preface to prospects for higher plant metabolic engineering.

Careful empirical selection of the expression platform for carotenogenesis has included selection of the best strains for stability and degree of accumulation^{254,255} and the selection of compatible drug-resistance combinations and low copy number polycistronic plasmids to enhance product accumulation by decrease of metabolic burden.^{118,145} Matthews and Wurtzel discussed culture and induction conditions^{145,261} that have been explored in most studies. Most efforts to engineer carotenoid biosynthesis in *E. coli* focused on the genes and enzymes of the pathway and had a modest effect on improved accumulation. For example, substitution and over-expression of a GGPPS that uses IPP directly (discussed in

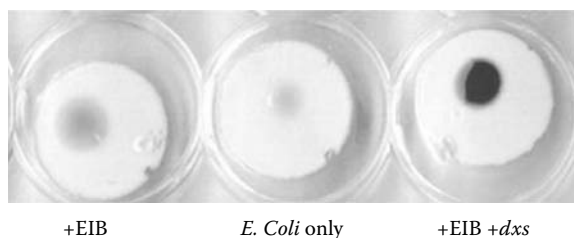


FIGURE 5.3.5 Enhancement of lycopene accumulation in *E. coli* by over-expression of DXS. Lycopene accumulation (left) is enhanced (right) when *E. coli* cells carrying a carotenoid pathway gene cassette (+EIB) are further transformed with a *dxs* gene on a multicopy plasmid (+EIB +*dxs*). Lycopene hyperaccumulation was demonstrated by Matthews and Wurtzel.²⁶¹

Section 5.3.2.3) combined with over-expression of IPPI resulted in enhanced astaxanthin accumulation to 1.4 mg/g dry cell weight (DCW).²⁴⁹ Further increases to 45 mg/g DCW were obtained by random mutagenesis of GGPPS,²⁵⁸ perhaps by altering enzyme response to substrate-level feedback inhibition.

Tunable promoters that control the expression of key carotenogenic enzymes by sensing excessive flux through glycolysis were used to test concepts of dynamic flux control using bacterial carotenogenesis.²⁵⁸ The balance of glycolytic precursors (pyruvate and G3P, see Figure 5.3.1) was modulated by over-expression of phosphoenolpyruvate synthase (PPS) or inactivation of pyruvate kinase. In conjunction with tunable promoters for PPS and IPPI expression, carotenoids accumulated five-fold over controls.²⁵⁹

The stability of phytoene desaturase and lycopene cyclase transcripts also influenced accumulation of carotenoids.²⁵⁹ Efforts in directed evolution of carotenogenic enzymes have also continued. Alternate approaches using systematic and combinatorial gene knockout targets have allowed for enhancement of carotenoid production in the absence of a *priori* assumptions of regulatory mechanisms.^{106,260}

Another approach to metabolic control of carotenoid accumulation was the modulation of the rate-controlling activities, DXS and DXR, of the MEP pathway (discussed in Section 5.3.2.2). Early experiments with the addition of DXS²⁶¹ and then DXR²⁶⁴ showed that the precursor pool (DMAPP and IPP) was limiting for accumulation of lycopene and zeaxanthin in various color complementation systems. For example, over-expression of only DXS coded on a high copy number plasmid in the presence of a carotenogenic gene cluster on a low copy number plasmid resulted in enhanced lycopene accumulation to 1.3 mg/g DCW.⁶³ The addition of a DXS transgene resulted in a striking change in pigment accumulation of colonies as shown in Figure 5.3.5.

Over-expression of multiple enzymes in plants is also possible, especially as transformation technology improves. Multiple carotenogenic genes such as *Dxs*, *Dxr*, *Hdr*, *Ippi*, *Psy*, etc. may now be coordinately expressed in a plastidic polycistron.^{16,173} Alternatively, genetic hybridization may be used to empirically effect a “balance” of gene expression and a metabolic optimum by employing either naturally diverse germplasm or primary transgenics that exhibit a wide range of position effect variation in expression. Since major control of accumulation is effected at the level

of gene transcription for plant carotenogenesis,^{16,257} such transcriptional balancing may be a good semi-rational approach.

Several basic concepts in metabolic engineering of microbial carotenogenesis have also been explored in plants. For example, over-expression of enzymes shown to be rate-controlling in bacterial dissections²⁶¹ have proven to release flux in plants. As a case in point, over-expression of CrtI or PSY led to varied and sometimes dramatically positive results (discussed in Sections 5.3.3.2 and 5.3.2). Also, modulation of precursor pools, as demonstrated in bacteria,^{99,265} occurred during over-expression of DXS in transgenic tomato²²⁸ and *Arabidopsis*,⁵⁹ while over-expression of daffodil DXS in rice endosperm did not increase pigment accumulation.¹²² Metabolic flux analysis in tomatoes³³ demonstrated that PSY is rate-controlling and confirmed empirical results of poke-and-hope genetic engineering.

One must remember that quantitative evaluations of pigment accumulation in transgenic plants are always complicated by variable position effects for individual events combined with genetic, developmental, and environmental noise; therefore more studies and statistical analysis of many events are needed for true consensus development. Also, because of difficulties of plant transformation, especially the genotype-limited applicability of optimized transformation protocols, wide-ranging empirical exploration of pigment accumulation platforms that vary in infrastructure such as genetic background, expression vector, and promoter strength is not currently tractable in plants.

The accumulation of lycopene seen in Figure 5.3.5 probably represents the maximum holding capacity of lipophilic compartments of bacterial cells. Further accumulation may require selection of alternative hosts such as yeasts or photosynthetic bacteria, or the understanding and engineering of novel sequestory structures.¹⁸⁷ In plants, product transport and storage may well limit accumulation at some point, as might the activities of degradation enzymes.¹⁹⁵ Consideration of product accumulation systems like the vacuolar deposition of crocetin glycosides²⁰ described in Section 5.3.2.8 and other membrane transporters becomes crucial.^{72,74} True application of combined transgenesis, metabolomics, and MCA has yet to be reported for plant carotenogenesis.

5.3.4 CURRENT STRATEGIES AND FUTURE PROSPECTS

Genomic and molecular tools have made great impacts on plant biotechnology and offer potential for manipulation of carotenoids as natural colorants and also for applications in human and animal health. While microbial and other non-plant systems have been successfully used, plant modification eliminates need for expensive bioreactors and offers economically feasible opportunities for less developed nations for production of nutraceuticals and other chemical products.

Plant use is less biotechnologically advanced and fundamentally more complex. The first generation of plant metabolic engineering met with mixed success and produced unanticipated results — problems that are not necessarily restricted to manipulation of carotenogenesis. The reason is that predictive metabolic engineering relies on the establishment of both needed tools and an information infrastructure

that are only just developing. The following are some of the essential tools and information required for predictive engineering of plant carotenogenesis.

1. *Characterization of gene families and identification of transcription factors to drive coordinate pathway expression:* Unlike microorganisms, agronomically important plants have gene families coding biosynthetic enzymes for which specific gene family member roles are poorly understood. These roles impact carotenoid involvement in both primary and secondary metabolism, including roles as precursors to apocarotenoids whose functions and regulation are even less understood. Although it is likely that individual members have specific functional modularity in different tissues or in response to different signals, they may also have overlapping functions. Manipulation of a gene family member to alter carotenoids in one tissue could potentially have a negative impact on carotenogenesis in other tissues. Therefore, an essential goal is a better understanding of the roles of gene family members in tissues targeted for manipulation as well as throughout the plant. Characterization of gene family members will potentially lead to identification of transcription factors that may or may not be involved in coordinate pathway induction, a valuable tool for global pathway enzyme manipulation.

2. *Understanding timing of gene expression:* Little is known about the critical timing for the expression of enzymes produced by either endogenous genes or foreign transgenes. Since carotenoid biosynthesis is nuclear encoded but plastid localized, carotenogenesis is likely intertwined with plastid development. Therefore, transgene expression to control carotenogenesis in endosperm, for example, is dependent on developmental timing of gene expression; use of a generic, albeit tissue-specific, promoter in the absence of such temporal consideration may be reflected by less than optimal results.

3. *Understanding mechanisms controlling metabolon localization in plastids of different membrane architectures:* Little is known about metabolon structure, assembly, and membrane targeting. The carotenoid biosynthetic pathway exists on plastid membranes. However, plastids have different membrane architectures and therefore tissue- and plastid-specific differences in membrane targeting of the biosynthetic metabolon can be expected. Localization in chloroplasts that harbor both thylakoid and envelope membranes differs from the envelope membranes in endosperm amyloplasts. In fact, localization on both thylakoid and envelope membranes implies that the carotenoid pathway is really not a single pathway, but a duplicated pathway that may very well have membrane-specific roles with regard to functions in primary and secondary metabolism.

Little is known of how the biosynthetic metabolon is assembled, what mechanisms control the membrane-specific targeting, and how the conversions to apocarotenoids occur. Yet the current approach to drive import of bacterial or plant genes is to use transit sequences of a stromal protein that may limit the effectiveness of the transgene. In addition, for specific applications of controlling carotenoid composition, we need to better understand the interactions of the various enzymes,

especially the structurally distinct hydroxylases that may have overlapping functions. Lastly, little is understood of substrate import into plastids and how this impacts carotenoid accumulation in time and space.

4. *Test systems to establish rate-controlling steps:* One cannot predict engineering results in genetically diverse populations or accessions based on testing a transgenic in a single model variety. Generally, a few amenable lines are routinely used for plant transformation in a given species. While such lines are important for the introduction of a new trait, they have limited value in predicting metabolic performances in diverse populations that are genetically and thereby chemically dissimilar.

How can a metabolic engineer predict when to maximize gene expression when carotenoid accumulation is linked with temporal control of gene expression during tissue development? What is the range in genotypic diversity with regard to plant chemistry? An alternative is to investigate gene expression in genetically diverse populations and carefully correlate gene expression data with plant chemistry. For example, the Wurtzel laboratory has been conducting gene transcript profiling in diverse maize germplasm and identified not only which gene family members are critical for impacting endosperm carotenogenesis, but also when during endosperm development expression is critical (Wurtzel et al., unpublished data). From these data, identification has been made of gene targets as well as developmental time frames needed for identifying better promoter and/or gene/allele choices leading to precise expression of rate-controlling enzymes.

Analysis in diverse lines can facilitate identification of useful alleles that control expression of enzymes upstream of the carotenoid pathway, a feature that would not be evident from conventional end-product screening of breeding lines. Moreover, this characterization sets the stage for marker-assisted selection of superior endogenous alleles and facilitates selection of introduced transgenes that may be necessary to supplement the genotypic contribution required for a particular plant chemical outcome.

5. *Development of multidimension pathway databases:* Current database tools do not encompass the true complexities of plants. In this regard, multidimensional databases need to be developed to integrate allelic variation with temporal, developmental, tissue-specific, and biotic and abiotic influences on pathway flux and pigment accumulation.

6. *Reliable phenotyping:* In the post-genomic era, it has become easier to measure genetic variation in a high throughput manner than to measure chemical trait data and morphological characteristics. Association studies such as QTL analysis rely on extensive genotyping of populations but also require several-fold higher levels of phenotyping. Many individuals in environmentally replicated mapping families must be phenotyped for many chemical and morphological traits in a reproducible fashion over several seasons of plant growth. Similarly, MCA is based on comparison of metabolite profiles for hundreds of metabolites.

Data production, assurance and validation are significant challenges requiring not only advanced analytical instruments, for example, for molecule separation and

identification or for high throughput videometrics, but also data handling and information flow, audit tracking, and statistical and computational advancements.

7. Predictable transformation technologies: Such needed technologies include higher-efficiency methods of gene transformation and standardized as well as more predictable results that might be obtained by targeted homologous recombination methods.

Predictable manipulation of carotenogenesis requires answers to many open questions and development of new biological, analytical, and computational tools. These are shared goals for rational, predictive metabolic engineering of secondary metabolite accumulation in plants. Advances in understanding control of carotenogenesis can be further applied to manipulation of interfacing native pathways and integration of non-native pathways. By application of comparative genomics, information gained by study and manipulation of one plant species can be further used to build resources and knowledge needed to modify and enhance related species. Multidisciplinary collaborations among fields including genomics and molecular biology, chemistry, biochemistry, ecology, biophysics, informatics, and computational science will lead to the advances necessary for making predictive metabolic engineering a reality.

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REFERENCES

1. Wiener, N., *Cybernetics or Control and Communication in the Animal and the Machine*, MIT Press, Cambridge, MA, 1948.
2. Grotewold, E., The challenges of moving chemicals within and out of cells: insights into the transport of plant natural products, *Planta* 219, 906, 2004.
3. Grotewold, E., The genetics and biochemistry of floral pigments, *Annu. Rev. Plant Biol.* 57, 761, 2006.
4. Lepiniec, L. et al., Genetics and biochemistry of seed flavonoids, *Annu. Rev. Plant Biol.* 57, 405, 2006.
5. Winkler, B.S.J., Metabolic channeling in plants, *Annu. Rev. Plant Biol.* 55, 85, 2004.

6. Winkel, B.S.J., Flavonoid biosynthesis: a colorful model for genetics, biochemistry, cell biology, and biotechnology, *Plant Physiol.* 126, 485, 2001.
7. Springob, K. et al., Recent advances in the biosynthesis and accumulation of anthocyanins, *Nat. Prod. Rep.* 20, 288, 2003.
8. Ogata, J. et al., Plant biochemistry: anthocyanin biosynthesis in roses, *Nature* 435, 757, 2005.
9. Koes, R., Verweij, W., and Quattrocchio, F., Flavonoids: a colorful model for the regulation and evolution of biochemical pathways, *Trends Plant Sci.* 10, 236, 2005.
10. Chandler, S., Commercialization of genetically modified ornamental plants, *J. Plant Biotechnol.* 5, 69, 2003.
11. Strack, D., Vogt, T., and Schliemann, W., Recent advances in betalain research, *Phytochemistry* 62, 247, 2003.
12. Fraser, P.D. and Bramley, P.M., The biosynthesis and nutritional uses of carotenoids, *Progr. Lipid Res.* 43, 228, 2004.
13. Cunningham, F.X., Regulation of carotenoid synthesis and accumulation in plants, *Pure Appl. Chem.* 74, 1409, 2002.
14. DellaPenna, D. and Pogson, B.J., Vitamin synthesis in plants: tocopherols and carotenoids, *Annu. Rev. Plant Biol.* 57, 711, 2006.
15. Bouvier, F., Rahier, A., and Camara, B., Biogenesis, molecular regulation and function of plant isoprenoids, *Progr. Lipid Res.* 44, 357, 2005.
16. Wurtzel, E.T., Genomics, genetics, and biochemistry of maize carotenoid biosynthesis, in *Recent Advances in Phytochemistry*, Romeo, J., Ed., Elsevier, Amsterdam, 2004, 85.
17. Broun, P. and Somerville, C., Progress in plant metabolic engineering, *Proc. Natl. Acad. Sci. USA* 98, 8925, 2001.
18. Sandmann, G., Römer, S., and Fraser, P.D., Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants, *Metabol. Eng.* 8, 291, 2006.
19. DellaPenna, D., Plant metabolic engineering, *Plant Physiol.* 125, 160, 2001.
20. Wurtzel, E.T. and Grotewold, E., Plant metabolic engineering, in *Encyclopedia of Chemical Processing*, 2191, 2006.
21. Yazaki, K., Transporters of secondary metabolites, *Curr. Opin. Plant Biol.* 8, 301, 2005.
22. Ralley, L. et al., Metabolic engineering of ketocarotenoid formation in higher plants, *Plant J.* 39, 477, 2004.
23. Botella-Pavia, P. and Rodriguez-Concepcion, M., Carotenoid biotechnology in plants for nutritionally improved foods, *Physiol. Plantarum* 126, 369, 2006.
24. Hoa, T.T.C. et al., Golden Indica and Japonica rice lines amenable to deregulation, *Plant Physiol.* 133, 161, 2003.
25. Al-Babili, S. and Beyer, P., Golden rice: five years on the road, five years to go? *Trends Plant Sci.* 10, 565, 2005.
26. Stein, A.J., Sachdev, H.P.S., and Qaim, M., Potential impact and cost-effectiveness of Golden Rice, *Nat. Biotech.* 24, 1200, 2006.
27. Kopsell, D.A. and Kopsell, D.E., Accumulation and bioavailability of dietary carotenoids in vegetable crops, *Trends Plant Sci.* 11, 499, 2006.
28. Higuera-Ciapara, I., Félix-Valenzuela, L., and Goycoolea, F., Astaxanthin: a review of its chemistry and applications, *Crit. Rev. Food Sci. Nutr.* 46, 185, 2006.
29. Voutilainen, S. et al., Carotenoids and cardiovascular health, *Am. J. Clin. Nutr.* 83, 1265, 2006.
30. Hussein, G. et al., Astaxanthin, a carotenoid with potential in human health and nutrition, *J. Nat. Prod.* 69, 443, 2006.

31. Tapiero, H., Townsend, D.M., and Tew, K.D., The role of carotenoids in the prevention of human pathologies, *Biomed. Pharmacother.* 58, 100, 2004.
32. Bertram, J.S. and Vine, A.L., Cancer prevention by retinoids and carotenoids: independent action on a common target, *Biochim. Biophys. Acta* 1740, 170, 2005.
33. Lee, P. and Schmidt-Dannert, C., Metabolic engineering towards biotechnological production of carotenoids in microorganisms, *Appl. Microbiol. Biotechnol.* V60, 1, 2002.
34. Prakash, B. and Paul, S.B., Microbial xanthophylls, *Appl. Microbiol. Biotechnol.* V68, 445, 2005.
35. Bhosale, P., Environmental and cultural stimulants in the production of carotenoids from microorganisms, *Appl. Microbiol. Biotechnol.* V63, 351, 2004.
36. Mijts, B.N. and Schmidt-Dannert, C., Engineering of secondary metabolite pathways, *Curr. Opin. Biotechnol.* 14, 597, 2003.
37. Rohlin, L., Oh, M.K., and Liao, J.C., Microbial pathway engineering for industrial processes: evolution, combinatorial biosynthesis and rational design, *Curr. Opin. Microbiol.* 4, 330, 2001.
38. Maury, J. et al., Microbial isoprenoid production: example of green chemistry through metabolic engineering, in *Biotechnology for the Future*, Springer-Verlag, Berlin, 2005, 19.
39. Spolaore, P. et al., Commercial applications of microalgae, *J. Biosci. Bioeng.* 101, 87, 2006.
40. Mapari, S.A.S. et al., Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants, *Curr. Opin. Biotechnol.* 16, 231, 2005.
41. Nessler, C.L., Metabolic engineering of plant secondary products, *Transgenic Res.* 3, 109, 1994.
42. Ohlrogge, J., Plant metabolic engineering: are we ready for phase two? *Curr. Opin. Plant Biol.* 2, 121, 1999.
43. Lessard, P.A. et al., Manipulating gene expression for the metabolic engineering of plants, *Metabol. Eng.* 4, 67, 2002.
44. Kunze, R., Frommer, W.B., and Flugge, U.I., Metabolic engineering of plants: role of membrane transport, *Metabol. Eng.* 4, 57, 2002.
45. Morgan, J.A. and Shanks, J.V., Quantification of metabolic flux in plant secondary metabolism by a biogenetic organizational approach, *Metabol. Eng.* 4, 257, 2002.
46. Morgan, J.A. and Rhodes, D., Mathematical modeling of plant metabolic pathways, *Metabol. Eng.* 4, 80, 2002.
47. Kholodenko, B.N. and Westerhoff, H.V., *Metabolic Engineering in the Post Genomic Era*, Horizon Bioscience, Norfolk, England, 2004.
48. Henikoff, S., Till, B.J., and Comai, L., TILLING: traditional mutagenesis meets functional genomics, *Plant Physiol.* 135, 630, 2004.
49. Mansoor, S. et al., Engineering novel traits in plants through RNA interference, *Trends Plant Sci.* 11, 559, 2006.
50. Davuluri, G.R. et al., Fruit-specific RNAi-mediated suppression of DET1 enhances carotenoid and flavonoid content in tomatoes, *Nat. Biotechnol.* 23, 890, 2005.
51. Foyer, C.H., DellaPenna, D., and Van Der Straeten, D., A new era in plant metabolism research reveals a bright future for bio-fortification and human nutrition, *Physiol. Plantarum* 126, 289, 2006.
52. Borevitz, J.O. and Ecker, J.R., Plant genomics: the third wave, *Ann. Rev. Genomics Hum. Genet.* 5, 443, 2004.
53. Kuhn, T., *The Structure of Scientific Revolutions*, University of Chicago Press, Chicago, 1962.

54. Emmanuel, E. and Levy, A.A., Tomato mutants as tools for functional genomics, *Curr. Opin. Plant Biol.* 5, 112, 2002.
55. Wurtzel, E.T., Use of a Ds chromosome breaking element to examine maize Vp5 expression, *J. Hered.* 83, 109, 1992.
56. Wurtzel, E.T., Luo, R.B., and Yatou, O., A simple approach to identify the first rice mutants blocked in carotenoid biosynthesis, *J. Exp. Bot.* 52, 161, 2001.
57. Messing, J. and Dooner, H.K., Organization and variability of the maize genome, *Curr. Opin. Plant Biol.* 9, 157, 2006.
58. Paine, J.A. et al., Improving the nutritional value of Golden Rice through increased pro-vitamin A content, *Nat. Biotechnol.* 23, 482, 2005.
59. Al-Babili, S., Hoa, T.T.C., and Schaub, P., Exploring the potential of the bacterial carotene desaturase *CrtI* to increase the beta-carotene content in Golden Rice, *J. Exp. Bot.* 57, 1007, 2006.
60. Kamisugi, Y., Cuming, A.C., and Cove, D.J., Parameters determining the efficiency of gene targeting in the moss *Physcomitrella patens*, *Nucl. Acids Res.* 33, e173, 2005.
61. Tzfira, T. and Citovsky, V., Agrobacterium-mediated genetic transformation of plants: biology and biotechnology, *Curr. Opin. Biotechnol.* 17, 147, 2006.
62. Lorrence, A. and Verpoorte, R., Gene transfer and expression in plants, *Meth. Mol. Biol.* 267, 329, 2004.
63. Maliga, P., Plastid transformation in higher plants, *Annu. Rev. Plant Biol.* 55, 289, 2004.
64. Frame, B.R., Paque, T., and Wang, K., Maize (*Zea mays* L.), *Meth. Mol. Biol.* 343, 185, 2006.
65. Datta, K. and Datta, S.K., Indica rice (*Oryza sativa*, BR29 and IR64), *Meth. Mol. Biol.* 343, 201, 2006.
66. Ko, T.S., Korban, S.S., and Somers, D.A., Soybean (*Glycine max*) transformation using immature cotyledon explants, *Meth. Mol. Biol.* 343, 397, 2006.
67. Olhoft, P.M., Donavan, C.M., and Somers, D.A., Soybean (*Glycine max*) transformation using mature cotyledonary node explants, *Meth. Mol. Biol.* 343, 385, 2006.
68. Burr, B. et al., Gene mapping with recombinant inbreds in maize, *Genetics* 118, 519, 1988.
69. Ahn, S. et al., Homoeologous relationships of rice, wheat and maize chromosomes, *Mol. Gen. Genet.* 241 (5-6), 483, 1993.
70. Bowers, J.E. et al. A high-density genetic recombination map of sequence-tagged sites for sorghum, as a framework for comparative structural and evolutionary genomics of tropical grains and grasses, *Genetics* 165, 367, 2003.
71. Thorup, T.A. et al., Candidate gene analysis of organ pigmentation loci in the Solanaceae, *Proc. Natl. Acad. Sci. USA* 97, 11192, 2000.
72. Morandini, P. and Salamini, F., Plant biotechnology and breeding: allied for years to come, *Trends Plant Sci.* 8, 70, 2003.
73. Flint-Garcia, S.A., Thornsberry, J.M., and Iv, B., Structure of linkage disequilibrium in plants, *Annu. Rev. Plant Biol.* 54, 357, 2003.
74. Morgante, M. and Salamini, F., From plant genomics to breeding practice, *Curr. Opin. Biotechnol.* 14, 214, 2003.
75. Yu, J. and Buckler, E.S., Genetic association mapping and genome organization of maize, *Curr. Opin. Biotechnol.* 17, 155, 2006.
76. Kaur, J. and Sharma, S., Directed evolution: an approach to engineer enzymes, *Crit. Rev. Biotech.* 26, 165, 2006.
77. Schmidt-Dannert, C., Umeno, D., and Arnold, F., Molecular breeding of carotenoid biosynthetic pathways, *Nat. Biotechnol.* 18, 750, 2000.

78. Brutnell, T.P., Transposon tagging in maize, *Funct. Integr. Genomics* 2, 4, 2002.
79. Brutnell, T.P. and Conrad, L.J., Transposon tagging using Activator (Ac) in maize, *Meth. Mol. Biol.* 236, 157, 2003.
80. Wurtzel, E.T. et al., Two-step cloning of the *Escherichia coli* regulatory gene *ompB*, employing phage Mu, *J. Mol. Appl. Genet.* 1, 61, 1981.
81. Raab, R.M., Tyo, K., and Stephanopoulos, G., Metabolic engineering, *Adv. Biochem. Biotechnol.* 100, 1, 2005.
82. Rodriguez-Concepcion, M. and Boronat, A., Elucidation of the methylerythritol phosphate pathway for isoprenoid biosynthesis in bacteria and plastids: a metabolic milestone achieved through genomics, *Plant Physiol.* 130, 1079, 2002.
83. Rodriguez-Concepcion, M., Early steps in isoprenoid biosynthesis: multilevel regulation of the supply of common precursors in plant cells, *Phytochem. Rev.* 5, 1, 2006.
84. Eisenreich, W., Rohdich, F., and Bacher, A., Deoxyxylulose phosphate pathway to terpenoids, *Trends Plant Sci.* 6, 78, 2001.
85. Page, J.E. and Nagel, J., *Biosynthesis of Terpenophenolics in Hop and Cannabis*, Elsevier, Oxford, 2006.
86. Sprenger, G.A. et al., Identification of a thiamin-dependent synthase in *Escherichia coli* required for the formation of the 1-deoxy-D-xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol, *Proc. Natl. Acad. Sci. USA* 94, 12857, 1997.
87. Lange, B.M. et al., A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway, *Proc. Natl. Acad. Sci. USA* 95, 2100, 1998.
88. Lois, L.M. et al., Cloning and characterization of a gene from *Escherichia coli* encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis, *Proc. Natl. Acad. Sci. USA* 95, 2105, 1998.
89. Lichtenthaler, H.K. et al., The non-mevalonate isoprenoid biosynthesis of plants as a test system for new herbicides and drugs against pathogenic bacteria and the malaria parasite, *Z. Naturforsch. C* 55, 305, 2000.
90. Lichtenthaler, H.K., The 1-deoxy-d-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 50, 47, 1999.
91. Lange, B.M. and Croteau, R., Isoprenoid biosynthesis via a mevalonate-independent pathway in plants: cloning and heterologous expression of 1-deoxy-D-xylulose-5-phosphate reductoisomerase from peppermint, *Arch. Biochem. Biophys.* 365, 170, 1999.
92. Takahashi, S. et al., A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis, *Proc. Natl. Acad. Sci. USA* 95, 9879, 1998.
93. Kim, S.M. et al., Identification of class 2 1-deoxy-D-xylulose 5-phosphate synthase and 1-deoxy-D-xylulose 5-phosphate reductoisomerase genes from *Ginkgo biloba* and their transcription in embryo culture with respect to ginkgolide biosynthesis, *Planta Med.* 72, 234, 2006.
94. Hemmerlin, A. et al., A cytosolic *Arabidopsis thaliana* D-xylulose kinase catalyzes the phosphorylation of 1-deoxy-D-xylulose into a precursor of the plastidial isoprenoid pathway, *Plant Physiol.* 106, 86652, 2006.
95. Page, J.E. et al., Functional analysis of the final steps of the 1-deoxy-D-xylulose 5-phosphate (DXP) pathway to isoprenoids in plants using virus-induced gene silencing, *Plant Physiol.* 134, 1401, 2004.
96. Hampel, D., Mosandl, A., and Wust, M., Biosynthesis of mono- and sesquiterpenes in carrot roots and leaves (*Daucus carota* L.): metabolic cross talk of cytosolic mevalonate and plastidial methylerythritol phosphate pathways, *Phytochemistry* 66, 305, 2005.

97. Laule, O. et al., Crosstalk between cytosolic and plastidial pathways of isoprenoid biosynthesis in *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci. USA* 100, 6866, 2003.
98. Hampel, D., Mosandl, A., and Wust, M., Biosynthesis of mono- and sesquiterpenes in strawberry fruits and foliage: 2H labeling studies, *J. Agric. Food Chem.* 54, 1473, 2006.
99. Lois, L.M. et al., Carotenoid biosynthesis during tomato fruit development: regulatory role of 1-deoxy-D-xylulose 5-phosphate synthase, *Plant J.* 22, 503, 2000.
100. Botella-Pavia, P. et al., Regulation of carotenoid biosynthesis in plants: evidence for a key role of hydroxymethylbutenyl diphosphate reductase in controlling the supply of plastidial isoprenoid precursors, *Plant J.* 40, 188, 2004.
101. Cunningham, Jr., F.X., Lafond, T.P., and Gantt, E., Evidence of a role for LytB in the nonmevalonate pathway of isoprenoid biosynthesis, *J. Bacteriol.* 182, 5841, 2000.
102. Kajiwar, S. et al., Expression of an exogenous isopentenyl diphosphate isomerase gene enhances isoprenoid biosynthesis in *Escherichia coli*, *Biochem. J.* 324 (Pt 2), 421, 1997.
103. Tao, L. et al., A carotenoid synthesis gene cluster from *Algoriphagus* sp. KK10202C with a novel fusion-type lycopene beta-cyclase gene, *Mol. Genet. Genomics* 379, 101, 2006.
104. Hsieh, M.H. and Goodman, H.M., The *Arabidopsis* IspH homolog is involved in the plastid nonmevalonate pathway of isoprenoid biosynthesis, *Plant Physiol.* 138, 641, 2005.
105. Shelton, D.A., Leach, D.N., and Henry, R.J., Isopentenyl pyrophosphate isomerases from *Melaleuca alternifolia* (Cheel) and their role in isoprenoid biosynthesis, *J. Hort. Sci. Biotechnol.* 79, 289, 2004.
106. Gallagher, C.E., Cervantes-Cervantes, M., and Wurtzel, E.T., Surrogate biochemistry: use of *Escherichia coli* to identify plant cDNAs that impact metabolic engineering of carotenoid accumulation, *Appl. Microbiol. Biotechnol.* 60, 713, 2003.
107. Lee, P.C. et al., Directed evolution of *Escherichia coli* farnesyl diphosphate synthase (IspA) reveals novel structural determinants of chain length specificity, *Metab. Eng.* 7, 18, 2005.
108. Sandmann, G., Carotenoid biosynthesis in microorganisms and plants, *Eur. J. Biochem.* 223, 7, 1994.
109. Cervantes-Cervantes, M. et al., Maize cDNAs expressed in endosperm encode functional farnesyl diphosphate synthase with geranylgeranyl diphosphate synthase activity, *Plant Physiol.* 220, 2006.
110. Okada, K. et al., Five geranylgeranyl diphosphate synthases expressed in different organs are localized into three subcellular compartments in *Arabidopsis*, *Plant Physiol.* 122, 1045, 2000.
111. Camara, B., Bardat, F., and Moneger, R., Sites of biosynthesis of carotenoids in *Capsicum* chromoplasts, *Eur. J. Biochem.* 127, 255, 1982.
112. Beyer, P., Weiss, G., and Kleinig, H., Solubilization and reconstitution of the membrane-bound carotenogenic enzymes from daffodil chromoplasts, *Eur. J. Biochem.* 153, 341, 1985.
113. Fraser, P.D. et al., Phytoene synthase-2 enzyme activity in tomato does not contribute to carotenoid synthesis in ripening fruit, *Plant Mol. Biol.* 40, 687, 1999.
114. Cunningham, F.X., Jr. and Gantt, E., Genes and enzymes of carotenoid biosynthesis in plants, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49, 557, 1998.
115. Sandmann, G., Carotenoid biosynthesis and biotechnological application, *Arch. Biochem. Biophys.* 385, 4, 2001.

116. Dogbo, O. et al., Carotenoid biosynthesis: isolation and characterization of a bifunctional enzyme catalyzing the synthesis of phytoene, *Proc. Natl. Acad. Sci. USA* 85, 7054, 1988.
117. Pfander, H., Carotenoids: An overview, in *Methods in Enzymology: Carotenoids, Part A: Chemistry, Separation, Quantitation, and Antioxidation*, Packer, L., Ed., Academic Press, San Diego, 1992, 9.
118. Matthews, P.D., *Carotenogenesis in Maize and Rice*, Graduate School and University Center, City University of New York, 2001.
119. Bonk, M. et al., Chloroplast import of four carotenoid biosynthetic enzymes *in vitro* reveals differential fates prior to membrane binding and oligomeric assembly, *Eur. J. Biochem.* 247, 942, 1997.
120. Bonk, M. et al., Purification and characterization of chaperonin 60 and heat-shock protein 70 from chromoplast of *Narcissus pseudonarcissus*: involvement of heat-shock protein 70 in a soluble protein complex containing phytoene desaturase, *Plant Physiol.* 111, 931, 1996.
121. Schledz, M. et al., Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering, *Plant J.* 10, 781, 1996.
122. Fraser, P. et al., Evaluation of transgenic tomato plants expressing an additional phytoene synthase in a fruit-specific manner, *Proc. Natl. Acad. Sci. USA* 99, 1092, 2002.
123. Shewmaker, C.K. et al., Seed-specific overexpression of phytoene synthase: increase in carotenoids and other metabolic effects, *Plant J.* 20, 401, 1999.
124. Scolnik, P.A. and Bartley, G.E., Nucleotide sequence of an Arabidopsis cDNA for phytoene synthase, *Plant Physiol.* 104, 1471, 1993.
125. Bartley, G.E. and Scolnik, P.A., cDNA cloning, expression during development, and genome mapping of Psy2, a second tomato gene encoding phytoene synthase, *J. Biol. Chem.* 268, 25718, 1993.
126. Fray, R.G. and Grierson, D., Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression, *Plant Mol. Biol.* 22, 589, 1993.
127. Busch, M., Seuter, A., and Hain, R., Functional analysis of the early steps of carotenoid biosynthesis in tobacco, *Plant Physiol.* 128, 439, 2002.
128. Gallagher, C.E. et al., Gene duplication in the carotenoid biosynthetic pathway preceded evolution of the grasses (Poaceae). *Plant Physiol.* 135, 1776, 2004.
129. Gaut, B.S. and Doebley, J.F., DNA sequence evidence for the segmental allotetraploid origin of maize, *Proc. Natl. Acad. Sci. USA* 94, 6809, 1997.
130. Fray, R. et al., Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from gibberellin pathway, *Plant J.* 8, 693, 1995.
131. Isaacson, T. et al., Analysis *in vitro* of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants, *Plant Physiol.* 136, 4246, 2004.
132. Bramley, P. et al., Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5, *Plant J.* 2 (3), 343, 1992.
133. Bartley, G.E. et al., Molecular cloning and expression in photosynthetic bacteria of a soybean cDNA coding for phytoene desaturase, an enzyme of the carotenoid biosynthesis pathway, *Proc. Natl. Acad. Sci. USA* 88, 6532, 1991.
134. Albrecht, M. et al., Molecular cloning and functional expression in *E. coli* of a novel plant enzyme mediating *z*-carotene desaturation, *FEBS Lett.* 372, 199, 1995.

135. Isaacson, T. et al., Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of beta-carotene and xanthophylls in plants, *Plant Cell* 14, 333, 2002.
136. Park, H. et al., Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis, *Plant Cell* 14, 321, 2002.
137. Yu, J., Localization and expression of carotenoid biosynthetic enzymes in endosperms of *Zea mays* and *Oryza sativa*, PhD thesis, City University of New York, New York, 1999.
138. Fraser, P.D., Schuch, W., and Bramley, P.M., Phytoene synthase from tomato (*Lycopersicon esculentum*) chloroplasts: partial purification and biochemical properties, *Planta* 211, 361, 2000.
139. Fraser, P.D. and Sandmann, G., *In vitro* assays of three carotenogenic membrane-bound enzymes from *Escherichia coli* transformed with different *crt* genes, *Biochem. Biophys. Res. Commun.* 185, 9, 1992.
140. Suire, C. et al., Cellular localization of isoprenoid biosynthetic enzymes in *Marchantia polymorpha*: uncovering a new role of oil bodies, *Plant Physiol.* 124, 971, 2000.
141. Goodwin, T.W., *The Biochemistry of the Carotenoids*, 2nd ed., Chapman & Hall, London, 1980.
142. Porter, J.W., Enzymatic synthesis of carotenes and related compounds, *Pure Appl. Chem.* 20, 449, 1969.
143. Isaacson, T. et al., Analysis *in vitro* of the enzyme CRTISO establishes a poly-cis-carotenoid biosynthesis pathway in plants, *Plant Physiol.* 136, 4246, 2004.
144. Robertson, D., Anderson, I., and Bachmann, M., Pigment-deficient mutants: genetic, biochemical and developmental studies, in *Maize Breeding and Genetics*, Walden, D., Ed., John Wiley & Sons, New York, 1978, 461.
145. Matthews, P.D., Luo, R., and Wurtzel, E.T., Maize phytoene desaturase and zetacarotene desaturase catalyze a poly-Z desaturation pathway: implications for genetic engineering of carotenoid content among cereal crops, *J. Exp. Botany* 54, 2215, 2003.
146. Linden, H. et al., Functional complementation in *Escherichia coli* of different phytoene desaturase genes and analysis of accumulated carotenoids, *Z. Naturforsch.* 46c, 1045, 1991.
147. Misawa, N. et al., Elucidation of the *Erwinia uredovora* carotenoid biosynthetic pathway by functional analysis of gene products expressed in *Escherichia coli*, *J. Bacteriol.* 172, 6704, 1990.
148. Bartley, G.E. et al., Carotenoid desaturases from *Rhodobacter capsulatus* and *Neurospora crassa* are structurally and functionally conserved and contain domains homologous to flavoprotein disulfide oxidoreductases, *J. Biol. Chem.* 265, 16020, 1990.
149. Goldie, A.H. and Subden, R.E., The neutral carotenoids of wild-type and mutant strains of *Neurospora crassa*, *Biochem. Genet.* 10, 275, 1973.
150. Hausmann, A. and Sandmann, G., A single five-step desaturase is involved in the carotenoid biosynthesis pathway to beta-carotene and torulene in *Neurospora crassa*, *Fungal Genet. Biol.* 30, 147, 2000.
151. Bartley, G.E., Scolnik, P.A., and Beyer, P., Two *Arabidopsis thaliana* carotene desaturases, phytoene desaturase and zeta-carotene desaturase, expressed in *Escherichia coli*, catalyze a poly-cis pathway to yield pro-lycopene, *Eur. J. Biochem.* 259, 396, 1999.
152. Clough, J.M. and Pattenden, G., Stereochemical assignment of polycopene and other poly-z-isomeric carotenoids in the fruits of the tangerine tomato *Lycopersicon esculentum* var. 'Tangella,' *J. Chem. Soc. Perkin Trans. I*, 3011, 1983.

153. Beyer, P., Mayer, M., and Kleinig, K., Molecular oxygen and the state of geometric isomerism of intermediates are essential in the carotene desaturation and cyclization reactions in daffodil chromoplasts, *Eur. J. Biochem.* 184, 141, 1989.
154. Ferruzzi, M.G. et al., Analysis of lycopene geometrical isomers in biological micro-samples by liquid chromatography with coulometric array detection, *J. Chromatogr. B Biomed. Sci. Appl.* 760, 289, 2001.
155. Cunningham, F.X. Jr. et al., Cloning and functional expression in *Escherichia coli* of a cyanobacterial gene for lycopene cyclase, the enzyme that catalyzes the biosynthesis of beta-carotene, *FEBS Lett.* 328, 130, 1993.
156. Cunningham, F.X. Jr. et al., Functional analysis of the beta and epsilon lycopene cyclase enzymes of *Arabidopsis* reveals a mechanism for control of cyclic carotenoid formation, *Plant Cell* 8, 1613, 1996.
157. Pecker, I. et al., Cloning and characterization of the cDNA for lycopene b-cyclase from tomato reveals decrease in its expression during fruit ripening, *Plant Mol. Biol.* 30, 807, 1996.
158. Ronen, G. et al., Regulation of carotenoid biosynthesis during tomato fruit development: expression of the gene for lycopene epsilon-cyclase is down-regulated during ripening and is elevated in the mutant, *Delta Plant J.* 17, 341, 1999.
159. Beyer, P., Croncke, U., and Nievelstein, V., Biochemical aspects of carotene desaturation and cyclization in chromoplasts membranes from *Narcissus pseudonarcissus*, *Pure Appl. Chem.* 66, 1047, 1994.
160. Masamoto, K. et al., Identification of a gene required for cis-to-trans carotene isomerization in carotenogenesis of the cyanobacterium *Synechocystis* sp. PCC 6803, *Plant Cell Physiol.* 42, 1398, 2001.
161. Breitenbach, J., Vioque, A., and Sandmann, G., Gene sl10033 from *Synechocystis* 6803 encodes a carotene isomerase involved in the biosynthesis of all-E lycopene, *Z. Naturforsch.* 56c, 915, 2001.
162. Eckardt, N., Tangerine dreams: cloning of carotenoid isomerase from *Arabidopsis* and tomato, *Plant Cell* 14, 289, 2002.
163. Giuliano, G., Giliberto, L. and Rosati, C., Carotenoid isomerase: a tale of light and isomers, *Trends Plant Sci.* 7, 427, 2002.
164. Kato, M. et al., Accumulation of carotenoids and expression of carotenoid biosynthetic genes during maturation in citrus fruit, *Plant Physiol.* 134, 82, 2004.
165. Cunningham, F.X. Jr. and Gantt, E., One ring or two? Determination of ring number in carotenoids by lycopene epsilon-cyclases, *Proc. Natl. Acad. Sci. USA* 98, 2905, 2001.
166. Niyogi, K.K., Safety valves for photosynthesis, *Curr. Opin. Plant Biol.* 3, 455, 2000.
167. Pogson, B.J. and Rissler, H.M., Genetic manipulation of carotenoid biosynthesis and photoprotection, *Philos. Trans. R. Soc. Lond B* 355, 1395, 2000.
168. Davison, P.A., Hunter, C.N., and Horton, P., Overexpression of [beta]-carotene hydroxylase enhances stress tolerance in *Arabidopsis*, *Nature* 418, 203, 2002.
169. Tian, L. and DellaPenna, D., Progress in understanding the origin and functions of carotenoid hydroxylases in plants, *Arch. Biochem. Biophys.* 430, 22, 2004.
170. Fiore, A. et al., Elucidation of the b-carotene hydroxylation pathway in *Arabidopsis thaliana*, *FEBS Lett.* 580, 4718, 2006.
171. Quinlan, R., Jaradat, T.T., and Wurtzel, E.T., *Escherichia coli* as a platform for functional expression of plant P450 carotene hydroxylases, *Arch. Biochem. Biophys.* In press, 2006.
172. Sun, Z., Gantt, E., and Cunningham, J., Cloning and functional analysis of the b-carotene hydroxylase of *Arabidopsis thaliana*, *J. Biol. Chem.* 271, 24349, 1996.

173. Castillo, R., Fernandez, J.A., and Gomez-Gomez, L., Implications of carotenoid biosynthetic genes in apocarotenoid formation during the stigma development of *Crocus sativus* and its closer relatives, *Plant Physiol.* 139, 674, 2005.
174. Hirschberg, J., Carotenoid biosynthesis in flowering plants, *Curr. Opin. Plant Biol.* 4, 210, 2001.
175. Demmig-Adams, B. and Adams, W.W., *The Xanthophyll Cycle*, CRC Press, Boca Raton, FL, 1993.
176. Bouvier, F. et al., Xanthophyll biosynthesis in chromoplasts: isolation and molecular cloning of an enzyme catalyzing the conversion of 5,6-epoxycarotenoid into ketocarotenoid, *Plant J.* 6, 45, 1994.
177. Mann, V. et al., Metabolic engineering of astaxanthin production in tobacco flowers, *Nat. Biotechnol.* 18, 888, 2000.
178. Cunningham, F.X. Jr. and Gantt, E., A study in scarlet: enzymes of ketocarotenoid biosynthesis in the flowers of *Adonis aestivalis*, *Plant J.* 41, 478, 2005.
179. Moise, A.R., von Lintig, J., and Palczewski, K., Related enzymes solve evolutionarily recurrent problems in the metabolism of carotenoids, *Trends Plant Sci.* 10, 178, 2005.
180. Walter, M.H., Fester, T., and Strack, D., Arbuscular mycorrhizal fungi induce the non-mevalonate methylerythritol phosphate pathway of isoprenoid biosynthesis correlated with accumulation of the "yellow pigment" and other apocarotenoids, *Plant J.* 21, 571, 2000.
181. Hans, J. et al., Cloning, characterization, and immunolocalization of a mycorrhiza-inducible 1-deoxy-d-xylulose 5-phosphate reductoisomerase in arbuscule-containing cells of maize, *Plant Physiol.* 134, 614, 2004.
182. Matusova, R. et al., The strigolactone germination stimulants of the plant-parasitic *Striga* and *Orobancha* spp. are derived from the carotenoid pathway, *Plant Physiol.* 139, 920, 2005.
183. Aldridge, M.E., McCarty, D.R., and Klee, H.J., Plant carotenoid cleavage oxygenases and their apocarotenoid products, *Curr. Opin. Plant Biol.* 9, 315, 2006.
184. Bouvier, F. et al., Oxidative tailoring of carotenoids: a prospect towards novel functions in plants, *Trends Plant Sci.* 10, 187, 2005.
185. Wyss, A., Carotene oxygenases: a new family of double bond cleavage enzymes, *J. Nutr.* 134, 246S, 2004.
186. Schwartz, S.H. et al., Specific oxidative cleavage of carotenoids by VP14 of maize, *Science* 276, 1872, 1997.
187. Ohmiya, A. et al., Carotenoid cleavage dioxygenase (CmCCD4a) contributes to white color formation in *Chrysanthemum* petals, *Plant Physiol.* 142, 1193, 2006.
188. Bouvier, F., Dogbo, O., and Camara, B., Biosynthesis of the food and cosmetic plant pigment bixin (annatto), *Science* 300, 2089, 2003.
189. Schwartz, S.H. et al., Substrate specificity and kinetics for VP14, a carotenoid cleavage dioxygenase in the ABA biosynthetic pathway, *Biochim. Biophys. Acta* 1619, 9, 2003.
190. Tan, B.C. et al., Molecular characterization of the *Arabidopsis* 9-cis epoxycarotenoid dioxygenase gene family, *Plant J.* 35, 44, 2003.
191. Pfander, H. and Schurtenberger, H., Biosynthesis of C20-carotenoids in *Crocus sativus*, *Phytochemistry* 21, 1039, 1982.
192. Bouvier, F. et al., Oxidative remodeling of chromoplast carotenoids: Identification of the carotenoid dioxygenase CsCCD and CsZCD genes involved in *Crocus* secondary metabolite biogenesis, *Plant Cell* 15, 47, 2003.
193. Carmona, M. et al., Generation of saffron volatiles by thermal carotenoid degradation, *J. Agric. Food Chem.* 54, 6825, 2006.

194. Giaccio, M., Crocetin from saffron: an active component of an ancient spice, *Crit. Rev. Food Sci. Nutr.* 44, 155, 2004.
195. Moraga, A.R. et al., Glucosylation of the saffron apocarotenoid crocetin by a glucosyltransferase isolated from *Crocus sativus* stigmas, *Planta* 219, 955, 2004.
196. Armstrong, G.A. and Hearst, J.E., Carotenoids 2: genetics and molecular biology of carotenoid pigment biosynthesis, *FASEB J.* 10, 228, 1996.
197. Sandmann, G., Carotenoid analysis in mutants from *Escherichia coli* transformed with carotenogenic gene cluster and *Scenedesmus obliquus* mutant C-6D, *Meth. Enzymol.* 214, 341, 1993.
198. Armstrong, G.A., Eubacteria show their true colors: genetics of carotenoid pigment biosynthesis from microbes to plants, *J. Bacteriol.* 176, 4795, 1994.
199. Cunningham, F.X. Jr. and Gantt, E., Identification of multi-gene families encoding isopentenyl diphosphate isomerase in plants by heterologous complementation in *Escherichia coli*, *Plant Cell Physiol.* 41, 119, 2000.
200. Hugueney, P. et al., Metabolism of cyclic carotenoids: a model for the alteration of this biosynthetic pathway in *Capsicum annuum* chromoplasts, *Plant J.* 8, 417, 1995.
201. Li, Z.H. et al., Cloning and characterization of a maize cDNA encoding phytoene desaturase, an enzyme of the carotenoid biosynthetic pathway, *Plant Mol. Biol.* 30, 269, 1996.
202. Luo, R. and Wurtzel, E.T., A maize cDNA encoding zeta carotene desaturase (accession no. AF047490, PGR99-118), *Plant Physiol.* 120, 1206, 1999.
203. Vigneswaran, A. and Wurtzel, E.T., A rice cDNA encoding phytoene desaturase (accession no. AF049356, PGR99-131), *Plant Physiol.* 121, 312, 1999.
204. Bartley, G.E. et al., A tomato gene expressed during fruit ripening encodes an enzyme of the carotenoid biosynthesis pathway, *J. Biol. Chem.* 267, 5036, 1992.
205. Armstrong, G.A., Alberti, M., and Hearst, J.E., Conserved enzymes mediate the early reactions of carotenoid biosynthesis in nonphotosynthetic and photosynthetic prokaryotes, *Proc. Natl. Acad. Sci. USA* 87, 9975, 1990.
206. Mann, V., Pecker, I., and Hirschberg, J., Cloning and characterization of the gene for phytoene desaturase (Pds) from tomato (*Lycopersicon esculentum*), *Plant Mol. Biol.* 24, 429, 1994.
207. Römer, S. et al., Expression of the genes encoding the early carotenoid biosynthetic enzymes in *Capsicum annuum*, *Biochem. Biophys. Res. Commun.* 196, 1414, 1993.
208. Misawa, N. et al., Expression of a tomato cDNA coding for phytoene synthase in *Escherichia coli*, phytoene formation *in vivo* and *in vitro*, and functional analysis of the various truncated gene products, *J. Biochem. (Tokyo)* 116, 980, 1994.
209. Linden, H., Vioque, A., and Sandmann, G., Isolation of a carotenoid biosynthesis gene coding for z-carotene desaturase from *Anabaena* PCC 7120 by heterologous complementation, *FEMS Microbiol. Lett.* 106, 99, 1993.
210. Scolnik, P.A. and Bartley, G.E., Phytoene desaturase from *Arabidopsis*, *Plant Physiol.* 103, 1475, 1993.
211. Cunningham, F. and Schiff, J., Photoisomerization of delta-carotene stereoisomers in cells of *Euglena gracilis* mutant W3BUL and in solution, *Photochem. Photobiol. Sci.* 42, 295, 1985.
212. Pows, R. and Britton, G., The roles of isomers of phytoene, phytofluene and zeta-carotene in carotenoid biosynthesis by a mutant strain of *Scenedesmus obliquus*, *Arch. Microbiol.* 115, 175, 1977.
213. Ernst, S. and Sandmann, G., Poly-cis carotene pathway in *Scenedesmus* mutant C-6D, *Arch. Microbiol.* 150, 590, 1988.

214. Isaacson, T. et al., Cloning of tangerine from tomato reveals a carotenoid isomerase essential for the production of β -carotene and xanthophylls in plants, *Plant Cell* 14, 333, 2002.
215. Taylor, M. and Ramsay, G., Carotenoid biosynthesis in plant storage organs: recent advances and prospects for improving plant food quality, *Physiol. Plantarum* 124, 143, 2005.
216. Van den Berg, H. et al., The potential for the improvement of carotenoid levels in foods and the likely systemic effects, *J. Sci. Food Agric.* 80, 880, 2000.
217. Rosati, C. et al., Metabolic engineering of beta-carotene and lycopene content in tomato fruit, *Plant J.* 24, 413, 2000.
218. Dharmapuri, S. et al., Metabolic engineering of xanthophyll content in tomato fruits, *FEBS Lett.* 519, 30, 2002.
219. Long, M. et al., Metabolite profiling of carotenoid and phenolic pathways in mutant and transgenic lines of tomato: identification of a high antioxidant fruit line, *Phytochemistry*, 67, 1750, 2006.
220. Giuliano, G., Aquilani, R., and Dharmapuri, S., Metabolic engineering of plant carotenoids, *Trends Plant Sci.* 5, 406, 2000.
221. Ducreux, L.J. et al., Metabolic engineering of high carotenoid potato tubers containing enhanced levels of beta-carotene and lutein, *J. Exp. Bot.* 56, 81, 2005.
222. Morris, W.L. et al., Carotenogenesis during tuber development and storage in potato, *J. Exp. Bot.* 55, 975, 2004.
223. Römer, S. et al., Elevation of the provitamin A content of transgenic tomato plants, *Nat. Biotechnol.* 18, 666, 2000.
224. Bird, C.R. et al., Using antisense RNA to study gene function: inhibition of carotenoid biosynthesis in transgenic tomatoes, *Biotechnology* 9, 635, 1991.
225. Burkhardt, P.K. et al., Transgenic rice (*Oryza sativa*) endosperm expressing daffodil (*Narcissus pseudonarcissus*) phytoene synthase accumulates phytoene, a key intermediate of provitamin A biosynthesis, *Plant J.* 11, 1071, 1997.
226. Paine, J.A. et al., Improving the nutritional value of Golden Rice through increased pro-vitamin A content, *Nat. Biotechnol.* 23, 482, 2005.
227. Theis, N. and Lerda, M., The evolution of function in plant secondary metabolites, *Int. J. Plant Sci.* 164 (3 Suppl.), 93, 2003.
228. Estevez, J.M. et al., 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants, *J. Biol. Chem.* 276, 22901, 2001.
229. Schaub, P. et al., Why is golden rice golden (yellow) instead of red? *Plant Physiol.* 138, 441, 2005.
230. Wurtzel, E.T. et al., Research towards improvement of the pro-vitamin A (carotenoid) content of rice endosperm, *Int. Rice Res. Notes* 21, 43, 1996.
231. Yoganathan, A., Isolation, expression and functional analysis of a cDNA encoding phytoene desaturase, a carotenoid biosynthetic enzyme from rice, *Oryza sativa* L., PhD dissertation, Graduate School and University Center, City University of New York, 1998.
232. Ronen, G. et al., An alternative pathway to beta-carotene formation in plant chloroplasts discovered by map-based cloning of beta and old-gold color mutations in tomato, *Proc. Natl. Acad. Sci. USA* 97, 11102, 2000.
233. Römer, S. et al., Genetic engineering of a zeaxanthin-rich potato by antisense inactivation and co-suppression of carotenoid epoxidation, *Metabol. Eng.* 4, 263, 2002.
234. Ye, X. et al., Engineering the provitamin A (beta-carotene) biosynthetic pathway into (carotenoid-free) rice endosperm, *Science* 287, 303, 2000.

235. Diretto, G. et al., Metabolic engineering of potato tuber carotenoids through tuber-specific silencing of lycopene epsilon cyclase, *BMC Plant Biol.* 6, 13, 2006.
236. Wong, J.C. et al., QTL and candidate genes phytoene synthase and zeta-carotene desaturase associated with the accumulation of carotenoids in maize, *Theor. Appl. Genetics* 108, 349, 2004.
237. Buckner, B. et al., Cloning of the y1 locus of maize, a gene involved in the biosynthesis of carotenoids, *Plant Cell* 2, 867, 1990.
238. Robertson, D.S., Survey of the albino and white-endosperm mutants of maize, *J. Hered.* 66, 67, 1975.
239. Buckner, B., San Miguel, P., and Bennetzen, J.L., The y1 gene of maize codes for phytoene synthase, *Genetics* 143, 479, 1996.
240. Li, Z.H., Molecular cloning and characterization of phytoene desaturase cDNA and leucine-rich repeat protein kinase cDNA from maize, PhD dissertation, Graduate School and University Center, City University of New York, 1998.
241. Hable, W.E. and Oishi, K.K., Maize phytoene desaturase maps near the viviparous5 locus, *Plant Physiol.* 108, 1329, 1995.
242. Hable, W.E., Oishi, K.K., and Schumaker, K.S., Viviparous-5 encodes phytoene desaturase, an enzyme essential for abscisic acid (ABA) accumulation and seed development in maize, *Mol. Gen. Genet.* 257, 167, 1998.
243. Palaisa, K. A. et al., Contrasting effects of selection on sequence diversity and linkage disequilibrium at two phytoene synthase loci, *Plant Cell* 15, 1795, 2003.
244. Rousseaux, M.C. et al., QTL analysis of fruit antioxidants in tomato using *Lycopersicon pennellii* introgression lines, *Theoret. Appl. Genet.* 111, 1396, 2005.
245. Chaib, J. et al., Stability over genetic backgrounds, generations and years of quantitative trait locus (QTLs) for organoleptic quality in tomato, *Theoret. Appl. Genet.* 112, 934, 2006.
246. Alba, R. et al., Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development, *Plant Cell* 17, 2954, 2005.
247. Schmidt-Dannert, C., Engineering novel carotenoids in microorganisms, *Curr. Opin. Biotechnol.* 11, 255, 2000.
248. Lee, P.C. et al., Biosynthesis of structurally novel carotenoids in *Escherichia coli*, *Chem. Biol.* 10, 453, 2003.
249. Wang, C., Oh, M.K., and Liao, J.C., Directed evolution of metabolically engineered *Escherichia coli* for carotenoid production, *Biotechnol. Progr.* 16, 922, 2000.
250. Lee, P.C. and Schmidt-Dannert, C., Metabolic engineering towards biotechnological production of carotenoids in microorganisms, *Appl. Microbiol. Biotechnol.* 60, 1, 2002.
251. Lee, P.C., Mijts, B.N., and Schmidt-Dannert, C., Investigation of factors influencing production of the monocyclic carotenoid torulene in metabolically engineered *Escherichia coli*, *Appl. Microbiol. Biotechnol.* 65, 538, 2004.
252. Lee, P.C. et al., Alteration of product specificity of *Aeropyrum pernix* farnesylgeranyl diphosphate synthase (fgs) by directed evolution, *Protein Eng. Design Sel.* 17, 771, 2004.
253. Ruther, A. et al., Production of zeaxanthin in *Escherichia coli* transformed with different carotenogenic plasmids, *Appl. Microbiol. Biotechnol.* 48, 162, 1997.
254. Wurtzel, E.T., Valdez, G., and Matthews, P. D., Variation in expression of carotenoid genes in transformed *E. coli* strains, *Biores.* J. 1, 1, 1997.
255. Jones, K.L., Kim, S.W., and Keasling, J.D., Low-copy plasmids can perform as well as or better than high-copy plasmids for metabolic engineering of bacteria, *Metab. Eng.* 2, 328, 2000.

256. Kim, S.W. et al., Over-production of beta-carotene from metabolically engineered *Escherichia coli*, *Biotechnol. Lett.* 28, 897, 2006.
257. Wang, C.W., Oh, M.K., and Liao, J.C., Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*, *Biotechnol. Bioeng.* 62, 235, 1999.
258. Farmer, W.R. and Liao, J.C., Improving lycopene production in *Escherichia coli* by engineering metabolic control, *Nat. Biotechnol.* 18, 533, 2000.
259. Smolke, C.D., Martin, V.J.J., and Keasling, J.D., Controlling the metabolic flux through the carotenoid pathway using directed mRNA processing and stabilization, *Metabol. Eng.* 3, 313, 2001.
260. Alper, H., Miyaoku, K., and Stephanopoulos, G., Construction of lycopene-overproducing *E. coli* strains by combining systematic and combinatorial gene knockout targets, *Nat. Biotechnol.* 23, 612, 2005.
261. Matthews, P.D. and Wurtzel, E.T., Metabolic engineering of carotenoid accumulation in *Escherichia coli* by modulation of the isoprenoid precursor pool with expression of deoxyxylulose phosphate synthase, *Appl. Microbiol. Biotechnol.* 53, 396, 2000.
262. Harker, M. and Bramley, P.M., Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in *Escherichia coli* increases carotenoid and ubiquinone biosynthesis, *FEBS Lett.* 448, 115, 1999.
263. Verdoes, J.C., Misawa, N., and van Ooyen, A.J., Cloning and characterization of the astaxanthin biosynthetic gene encoding phytoene desaturase of *Xanthophyllomyces dendrorhous* [letter], *Biotechnol. Bioeng.* 63, 750, 1999.
264. Kim, S.W. and Keasling, J.D., Metabolic engineering of the nonmevalonate isopentenyl diphosphate synthesis pathway in *Escherichia coli* enhances lycopene production, *Biotechnol. Bioeng.* 72, 408, 2001.
265. Enfissi, E.M.A. et al., Metabolic engineering of the mevalonate and non-mevalonate isopentenyl diphosphate-forming pathways for the production of health-promoting isoprenoids in tomato, *Plant Biotechnol. J.* 3, 17, 2005.
266. Li, F., Murillo, C., and Wurtzel, E.T., Maize Y9 encodes a product essential for 15-cis zetacarotene isomerization, *Plant Physiol.* 144, 1181, 2007.

5.4 Pigments from Microalgae and Microorganisms: Sources of Food Colorants

Laurent Dufossé

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5.4.1 INTRODUCTION

Color plays a special role in the foods we eat. For example, when confronted with a food of an unattractive color, the consumer assumes the food is of poor quality or is spoiled. Similarly, a product with an atypical color, e.g., a green cheese or a blue drink, in most cases would be rejected by the consumer.¹ Typically, one associates certain colors with certain food items such as cherry with red, lemon with yellow, and orange with carrot. Therefore, color can serve as a primary identification of food and also as a protective measure to prevent the consumption of spoiled food. Food colors create physiological and psychological expectations and attitudes that are developed by experience, tradition, education, and environment: we inevitably eat with our eyes.²

The controversial topic of synthetic dyes in food has been discussed for many years. The scrutiny and negative assessment of synthetic food dyes by the modern consumer have given rise to a strong interest in natural coloring alternatives. Some companies decided to color food with food, using mainly plant extracts and pigments from plants, e.g., red from paprika, beetroots, berries, or tomatoes; yellow from saffron or marigold; orange from annatto; green from leafy vegetables, etc.³

Penetration of fermentation-derived ingredients into the food industry continues to increase every year. Examples are thickening or gelling agents (xanthan, curdlan, gellan), flavor enhancers (yeast hydrolysate, monosodium glutamate), flavor compounds (γ -decalactone, diacetyl, methyl ketones), acidulants (lactic acid, citric acid), and others. Pigments producing microorganisms and microalgae are common in Nature. The molecules produced include carotenoids, melanins, flavins, quinones and more specifically monascins, violacein, phycocyanin, and indigo. Some fermentation-derived pigments such as β -carotene from the *Blakeslea trispora* fungus in Europe and Monascus pigments in Asia were developed later,⁴⁻⁶ and are now in use in the food industry (Figure 5.4.1).

Efforts have been made to reduce the production costs of fermentation pigments compared to costs of synthetic pigments and pigments extracted from natural

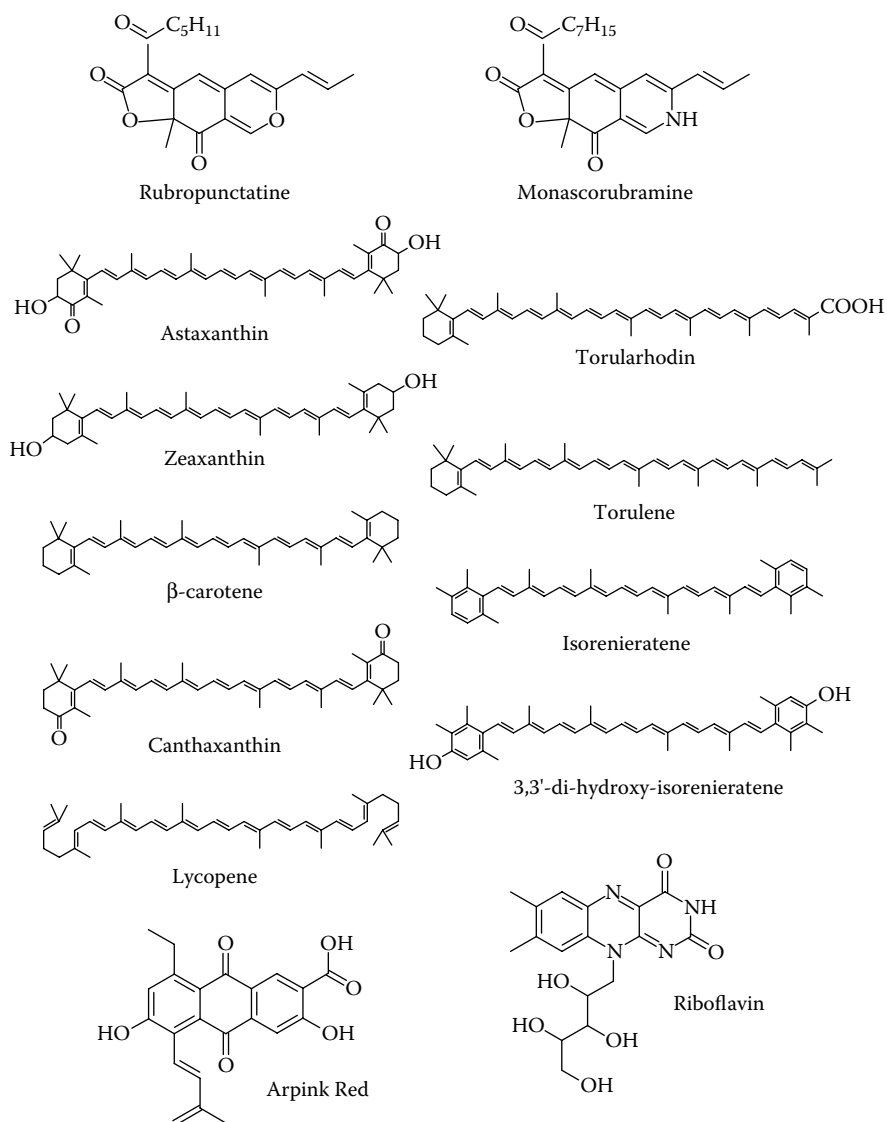


FIGURE 5.4.1 Chemical formulae of some microbial food-grade pigments.

sources. Innovations will improve the economy of pigment production by isolating new or creating better microorganisms by improving the processes. Among microalgae, two success stories arise from efficient production of carotenoids: β -carotene from *Dunaliella* and astaxanthin from *Haematococcus*. Regarding phycobiliproteins or phycocyanin, researchers should first carry out toxicological studies required before these compounds can be used as food colors.

This section focuses on research works in this field published over the past 10 years by private companies and academic laboratories. As our group recently noted,^{7,8} it is a long way from the Petri dish to the market place and thus to the placement of a food product on store shelves.

5.4.2 β -CAROTENE FROM *DUNALIELLA* MICROALGA: SALTY BUT EFFECTIVE

Algae comprise a group of non-vascular plants that are autotrophic and able to harness solar energy. This characteristic accounts for the large quantities of biomass accumulation through the photosynthesis mechanism. Algae produce a range of products including carbohydrates, proteins, essential amino acids, vitamins, pharmaceuticals, and bioactive molecules. Some algal forms have been exploited for centuries for food and health care.

Algae are classified according to their colors (1) Chlorophyceae (green), (2) Rhodophyceae (red), (3) Cyanophyceae (blue-green), and (4) Pheophyceae (brown). The major pigments include chlorophylls a, b, and c, β -carotene, phycocyanin, xanthophylls, and phycoerythrin. All these pigments have great potential for applications in foods, pharmaceuticals, and cosmetics.

Demand is increasing for natural colors that are useful in foods, pharmaceuticals, cosmetics, textiles, and as printing dyes. However, their utility is limited to few compounds because natural dyes have low tinctorial values and persistence. Due to the toxic effects of several synthetic dyes, end users are showing increasing preference for natural colors. Another major problem for natural colors is sustainability. As alga cultures are ecofriendly and renewable, there is increasing interest in using them as a source of natural colors, e.g., pigments such as phycocyanin (blue pigment from *Spirulina*), β -carotene (yellow-orange pigment from *Dunaliella*), and astaxanthin (red pigment from *Haematococcus*). See Figure 5.4.2.

5.4.2.1 β -CAROTENE FROM MICROALGAE

Algal carotenoids are present in chloroplasts as complex mixtures characteristic of each class of algae. The red Rhodophyta algae contain α and β -carotene and their hydroxylated derivatives.⁹ Chloromonadophyta contain diadinoxanthin, heteroxanthin, and vaucherixanthin. Chlorophyta are characterized by acetylenic carotenoids, namely alloxanthin, monadoxanthin, and crocoxanthin.¹⁰ *Spirulina* is known for accumulation of up to 0.8 to 1.0% w/w β -carotene. *Dunaliella* is the best carotenoid-providing organism among the algae and other organisms. Commonly cultivated species are *Dunaliella salina* and *D. bardawil*.

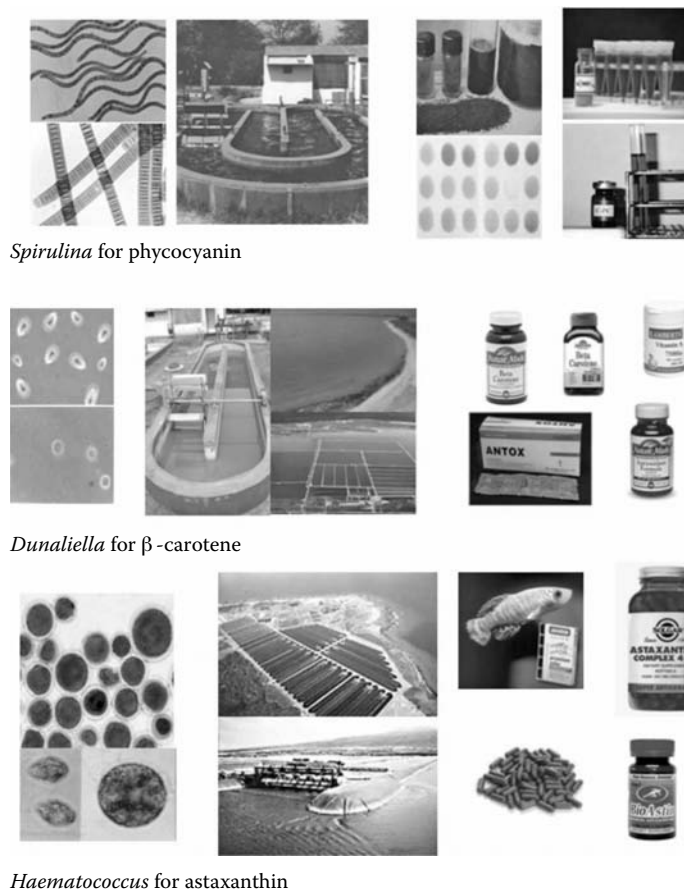


FIGURE 5.4.2 Cultivation of microalgae and utilization as natural pigments.

5.4.2.2 DUNALIELLA SPECIES FOR CAROTENOIDS

The genus *Dunaliella* includes halotolerant, unicellular, motile green algae with exceptional morphological and physiological properties belonging to family Chlorophyceae.¹¹ *Dunaliella* is devoid of rigid cell walls and contains a single, large cup-shaped chloroplast. It accumulates massive amounts of β -carotene, primarily in response to high light intensity.¹² The algae can yield three major valuable products: glycerol, β -carotene, and protein. Apart from β -carotene, this alga is a rich source of protein that has good utilization value, is rich in essential fatty acids, and can safely be used directly as a food due to its GRAS (generally recognized as safe) status. *Dunaliella* species have been shown to exhibit various biological functions such as antihypertensive, bronchodilator, analgesic and muscle relaxant, and anti-edema activities.¹³ In recent years, the species is cultivated mainly for carotenoids. On average, *Dunaliella salina* under ideal conditions can yield 400 mg of β -carotene per square meter of cultivation area.¹⁴

5.4.2.2.1 Carotenoids from *Dunaliella*: Natural versus Synthetic β -Carotene

Natural ' β -carotene' contains numerous carotenoids and essential nutrients that are not present in synthetic β -carotene. Natural β -carotene can be consumed in larger quantities because body tissues regulate its use.¹⁵ Natural sources generally contain one or two carotenoids in lower concentrations and thus may not be suitable for all applications. However *Dunaliella* contains a range of carotenoids with wider applications.

5.4.2.2.2 Applications of β -Carotene

Health benefits — Research reports indicate that natural β -carotene possesses numerous benefits for the human body and consistently supports the use of β -carotene as part of the human diet. The human body converts β -carotene to vitamin A via body tissues as opposed to the liver, hence avoiding a build-up of toxins in the liver. Vitamin A is essential for the human body in that it assists the immune system and helps battle eye diseases such as cataracts and night blindness, various skin ailments such as acne, signs of aging, and various forms of cancer.

β -carotene has antioxidant qualities. Antioxidants help mediate the harmful effects of free radicals, which are implied in over 60 life-threatening diseases including various forms of cancer, coronary heart disease, premature aging, and arthritis.^{16,17} Additionally, the antioxidant qualities of β -carotene assist the body in suppressing the effects of premature aging caused by ultraviolet (UV) rays. β -carotene is also added to numerous cosmetic and body-care products as a non-harmful colorant to improve attractiveness.

Use as food coloring — β -carotene is one of the world's leading food colorants. It has been applied to a range of food and beverage products, for example, margarines, cheeses, fruit juices, baked goods, dairy products, canned goods, confectionery, health condiments, and other items with the intent of improving appearance for customers.

Other applications — β -carotene is used in various pet foods as both a colorant and a precursor to vitamin A. It can be applied to an array of animal foods designed for dogs, cats, fish, and birds. The antioxidant and precursory vitamin A properties increase the appeal and application of β -carotene in pet foods.¹⁸ Additionally, β -carotene is an important carotenoid that may assist in improving the color of birds, fish, and crustaceans. *Dunaliella salina* can serve as a source of algal feed for fish and crustaceans. The microalgae provide carotenoids that are essential for flesh coloring, particularly of salmon and crustaceans.

5.4.2.2.3 Advantages of Production from *Dunaliella*

Algae can be cultivated easily and quickly when compared to plants. They produce very high quantities of carotenoids compared to other sources (3.0 to 5.0% w/w on a dry weight basis).⁹ They contain both *cis* and *trans* isomers of carotenoids for high bioavailability and bioefficacy,²⁰ and also contain oxygenated carotenoids (xanthophylls), which have greater bioactivity and better anticancer properties.²¹ The proteins from *Dunaliella* biomass can be utilized for bread and other products²² and whole cells can be utilized for animal, poultry, and fish foods because they are safe.²³

5.4.2.2.4 β -Carotene Production from *Dunaliella*

Dunaliella is a halotolerant organism that grows in high salt concentration (1.5 ± 0.1 M NaCl). Essentially this alga requires bicarbonate as a source of carbon and other nutrients such as nitrate, sulfate and phosphate. The initial growth phase requires 12 to 14 days in nitrate-rich medium; magnesium salt is essential as it is required for chlorophyll production. For the carotenogenesis phase, nitrate depletion along with salinity maintenance will give good results. This is the best-suited technology for coastal areas where sea water is rich in salt and other nutrients.

For carotenogenesis, nutrient, salt, or light stress is essential. The vegetative phase generally requires 5 to 10 klux and the light should be around 25 to 30 klux for carotenoid accumulation. Harvesting is done by flocculation followed by filtration and the product can be utilized directly as feed or extracted for pigments. The biomass can be utilized directly for food formulations because it has been shown to be safe. For various formulations and other applications, it can be extracted in either edible oils or food-grade organic solvents. Most pharmaceutical formulations are made with olive or soybean oil.

5.4.2.2.5 *Dunaliella* Producers

Parry's Agro Ltd. is the major industry in India producing *Dunaliella* as well as β -carotene for pharmaceutical purposes. Another Indian company producing *Dunaliella* is ABC Biotech Ltd. located in Tamil Nadu. Small commercial plants are located in Chile, Mexico, Cuba, Iran, Taiwan, and Japan. The following companies actively engage in cultivating *Dunaliella* for commercial purposes:

Betatene Ltd., Cheltenham, Australia, a division of Cognis Ltd.

Cyanotech Corp., Kailua-Kona, Hawaii, U.S.

Inner Mongolia Biological Engineering Co., Inner Mongolia, People's Republic of China

Nature Beta Technologies (NBT) Ltd., Eilat, Israel, a subsidiary of Nikken Sohonsha Co., Gifu, Japan

Tianjin Lantai Biotechnology, Nankai, Tianjin, in collaboration with the Salt Scientific Research Institute of Light Industry Ministry, People's Republic of China

Western Biotechnology Ltd., Bayswater, Australia, a subsidiary of Cognis Ltd.

Aqua Carotene Ltd., Subiaco, Australia

5.4.2.2.6 Marketed β -Carotene Products

Dunaliella natural β -carotene is distributed widely in many different markets under three categories: β -carotene extracts, *Dunaliella* powder for human use, dried *Dunaliella* for feed use. Extracted purified β -carotene is sold mostly in vegetable oil in bulk concentrations from 1 to 20% to color various food products and for personal use in soft gels usually containing 5 mg β -carotene per gel. Purified natural β -carotene is generally accompanied by the other *Dunaliella* carotenoids, primarily lutein, neoxanthin, zeaxanthin, violaxanthin, cryptoxanthin, and α -carotene for a total of approximately 15% of carotene concentration. This compound is marketed as "carotenoids mix."

5.4.3 HAEMATOCOCCUS FOR ASTAXANTHIN: RED “GOLD RUSH”

Haematococcus pluvialis is a green alga known for its ability to accumulate astaxanthin, a ketocarotenoid, up to 0.2 to 2.0% (dry weight basis). It is a high value product with applications in pharmaceuticals, nutraceuticals, and animal nutrition.²⁴ It can grow under both autotrophic and heterotrophic conditions. Astaxanthin from *Haematococcus* algae is under consideration for U.S. Food and Drug Administration clearance and several European countries approved its marketing as dietary supplement ingredient for human consumption. Its applications in many degenerative diseases and cancer prevention show its potential for technology development.

The production of the astaxanthin ketocarotenoid using the *Haematococcus pluvialis* freshwater alga is very attractive, but has fewer advantages than the *Dunaliella* β -carotene process. First *H. pluvialis* is a freshwater alga and thus open-air culture is extremely difficult due to contamination by many other undesirable algal species. *H. pluvialis* culture may require closed systems such as tubular photobioreactors and although some pilot-scale units have been tested,^{25,26} they require further refinements. However, completely closed photobioreactors with artificial light and combinations of closed photobioreactors and open culture ponds are now being used for *Haematococcus* cultivation.²⁷

Unlike *Dunaliella*, *H. pluvialis* changes from a motile, flagellated cell to a non-motile, thick-walled aplanospore during the growth cycle.^{28,29} The astaxanthin is contained in the aplanospore. This means that the physical properties (density, settling rate, cell fragility) and nutrient requirements of the cells change during the culture process, and this alters the optimum conditions for growth and carotenoid accumulation during the growth cycle.³⁰

The content of astaxanthin in the aplanospores is about 1 to 2% of dry weight and their thick walls require physical breakage before the astaxanthin can be extracted or become available to organisms consuming the alga.³¹

On the other hand, the high value of astaxanthin and the rapid growth of its main market, the aquaculture industry, means that the potential markets are very promising. The main competing product is synthetic astaxanthin, and again, the algal process has the advantage of producing a natural product. The only natural alternatives are crustacean meal, which is in limited supply and has low astaxanthin content, and the *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) yeast, which also has much lower astaxanthin content than the algae.³²

The development of a commercially viable algal astaxanthin process requires both an effective closed culture system and a selection (from Nature or via mutagenesis) of strains of *Haematococcus* with higher astaxanthin contents and abilities to tolerate higher temperatures than the wild strains.

5.4.3.1 ADVANTAGES OF ASTAXANTHIN OVER OTHER CAROTENOIDS

Some of the advantages of astaxanthin over other carotenoids include (1) better stability compared to other carotenoids, (2) high antioxidant potential (10 times

more than β -carotene and 500 times more than α -tocopherol), (3) ability to cross the blood–brain barrier easily, (4) high tinctorial properties.

5.4.3.2 ASTAXANTHIN AS NUTRACEUTICAL

Astaxanthin is a powerful bioactive antioxidant and has demonstrated efficacy in animal and human models of macular degeneration,³³ a cause of blindness in a large population. It is also helpful in treating Alzheimer's and Parkinson's diseases and is known to offer protection against cancer.

5.4.3.3 ASTAXANTHIN AS ANTIOXIDANT

Astaxanthin has been shown to be a powerful quencher of singlet oxygen as evident from *in vitro* studies.³⁴ It has stronger antioxidant activity, 10 times higher than β -carotene and more than 500 times more effective than α -tocopherol. Astaxanthin has been proposed as the super vitamin E.³⁵ Its antioxidant property has been demonstrated in a number of biological membranes³⁶ and it has shown preventive effects against aflatoxin B1 carcinogenicity.³⁷ Kobayashi and Sakamoto³⁸ showed antioxidative activity of astaxanthin under both hydrophobic and hydrophilic conditions, while Kobayashi et al.²⁹ reported *in vivo* activity against superoxide anion radicals using a whole cell assay system. Astaxanthin also exerted strong activity as an inhibitor of lipid peroxidation mediated by active forms of oxygen. Its strong antioxidative activities suggest its potential as a photoprotectant against UV irradiation.³⁹ Astaxanthin-containing preparations for prevention of light-induced aging of skin have been developed by Suzuki, Masaki, and Takei.⁴⁰

5.4.3.4 ASTAXANTHIN FOR HEALTH

Mammals lack the ability to synthesize astaxanthin or convert dietary astaxanthin into vitamin A. Unlike β -carotene, astaxanthin has no pro-vitamin activity in these animals.⁴¹

Astaxanthin has been shown in both *in vitro* and in a study with human subjects to be effective for the prevention of the oxidation of low-density protein,⁴² suggesting that it can be used to prevent arteriosclerosis, coronary artery disease, and ischemic brain development. A number of astaxanthin health products are under study.

Studies on rats had shown no toxicity of astaxanthin preparations.⁴³ Dietary administration of astaxanthin has proved to significantly inhibit carcinogenesis in the mouse urinary bladder, rat oral cavity, and rat colon.⁴⁴ In addition, it is reported to induce xenobiotic-metabolizing enzymes in rat liver.

Astaxanthin has been shown to enhance *in vitro* antibody production by mouse spleen cells stimulated with sheep red blood cells and in human blood cells *in vitro*. Furthermore, it has not exhibited any mutagenicity in an *in vitro* study at doses up to 14.4 mg/day for 2 weeks.

Yamashita⁴⁵ reported anti-inflammatory effect of astaxanthin when administered with aspirin. An oral preparation has been developed by Alejung and Wadstroem⁴⁶ for the treatment of *Helicobacter* infections of the mammalian gastrointestinal tract. Strong evidence suggested that astaxanthin modulated the humoral and non-humoral immune systems. It enhanced the release of interleukin-1 and tumor necrosis factor-

α in mice to a greater extent than canthaxanthin or β -carotene and has the greatest cytokinine-inducing activity.⁴⁷

Astaxanthin has a significant enhancing action on the production of immunoglobulins A, M, and G and on T helper cell antibody production, even when suboptimal amounts of antigen are present.

5.4.3.5 ASTAXANTHIN FOR SALMON AND TROUT FEEDS

The predominant source of carotenoids for salmonids has been synthetic astaxanthin, which has been used for pigmentation for the last 20 years with FDA approval in 1996. Natural sources of astaxanthin for commercially raised salmonids include processed crustacean waste from krill, shrimp, crab, and crawfish. However, crustacean waste products contain high amounts of moisture, ash, and chitin. Another natural source, *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) requires large amounts of feed for sufficient pigmentation, leading to higher ash contents. The efficiency of utilization of dietary astaxanthin using microalgae for flesh pigmentation of Atlantic salmon and rainbow trout was demonstrated by Torrissen, Hardy, and Shearer⁴⁸ and Storebakken.⁴⁹

For salmon, astaxanthin is considered as a vitamin essential for the proper development and survival of juveniles.⁵⁰ Choubert and Heinrich⁵¹ showed that feeding rainbow trout with algae up to 6% of the diet had no major effect on growth or mortalities and concluded the algae constituted a safe and effective source of pigment. Astaxanthin has been used to enhance the immune responses of fish and shrimp for maximum survival and growth. Also, natural microalgal astaxanthin has shown superior bioefficacy over synthetic forms. Japan granted full approval for use as a pigment in feeds and foods; registration for approval is in progress for the United States, the European Union, and Canada. A concentration of 25 to 100 ppm of carotenoids in the final feed has been considered to give desired pigmentation in various salmonid species.

However, the livestock feed market for astaxanthin, although presently small, will grow to a size comparable to the market for the synthetic compound, estimated at \$185 million. The largest market for astaxanthin is aquaculture, which accounts for 24% of total global fisheries. Production is currently valued at \$35 billion per annum and is expected to grow to \$49 billion by 2010.

5.4.3.6 ASTAXANTHIN FOR HUMANS

Limited studies have focused on dietary intake of astaxanthin by humans. In a study reported by Miki,⁵² an astaxanthin-containing drink was used to protect low-density lipoprotein from oxidation (astaxanthin was administered at doses of 3.6 to 14.4 mg/day over a 2-week period). Progressive slowing of LDL oxidation with increasing doses of astaxanthin was observed and no ill effects were reported.

When 100 mg of synthetic astaxanthin in olive-oil-containing meal was given to male volunteers,⁵³ a maximum plasma concentration of 1.24 mg/L astaxanthin was observed in the first 6 hours postprandially. The relative concentration of total astaxanthin in HDL decreased compared to the other lipoprotein fractions in the 72-hour study.

Based on a study conducted with 40 healthy volunteers, Lignell⁵⁴ reported the effect of astaxanthin on mammalian muscle function. Volunteers received one capsule of 4 mg astaxanthin each morning in association with food. No significant difference was observed between the treatment and placebo groups in any physical parameters measured.

The effect of dietary astaxanthin on the health of humans as carried out by Aquasearch Inc.⁵⁵ on 33 volunteers consuming 3.85 mg (low dose) and 19.25 mg (high dose) daily for 29 days indicated no ill effects or toxicity due to the consumption of astaxanthin as analyzed by medical and clinical parameters.

Tso and Lam⁵⁶ suggested that astaxanthin could be useful for prevention and treatment of neuronal damage associated with age-related macular degeneration and may also be effective in treating ischemic reperfusion injury, Alzheimer's disease, Parkinson's disease, spinal cord injuries, and other types of central nervous system injuries. Astaxanthin was found to easily cross the blood-brain barrier and did not form crystals in the eye.

5.4.3.7 PRODUCTION SYSTEM FOR HAEMATOCOCCUS

Haematococcus pluvialis is one of the organisms that has potential for the commercial production of astaxanthin. The challenging task with this organism is its outdoor cultivation which involves curtailment of contamination and control of environmental conditions such as light and temperature. The organism grows at 20 to 28°C, below 15 klux light intensity, and at a pH 6.8 to 7.4. Since it grows at neutral pH, contamination by bacteria, fungi, and protozoa, is the main problem. Under high light intensity, the cell growth is significantly affected. Therefore, its cultivation is reported in closed tubular photobioreactors and there are few successful reports of open pond cultivation due to its slow growth and contamination. Details of production steps are shown in Figure 5.4.3.

5.4.3.8 COMPANIES PRODUCING ASTAXANTHIN FROM HAEMATOCOCCUS

The major producers of *Haematococcus* in commercial scale worldwide are:

Mera Pharmaceuticals, Kailua-Kona, Hawaii, U.S.
 Cyanotech Corporation, Kailua-Kona, Hawaii, U.S.
 BioReal Inc., Kihei, Hawaii, U.S., a subsidiary of Fuji Chemical Industry,
 Toyama, Japan
 Parry's Pharmaceuticals, Chennai, India

5.4.3.9 ASTAXANTHIN-CONTAINING FORMULATIONS

This pigment is recognized by the U.S. Food and Drug Administration (21 Code of Federal Regulations, Part 73) as a color additive exempted from certification (Subpart A, Foods, Section 73.35, Astaxanthin). Formulations containing astaxanthin include soft gelatin capsules containing 100 mg equivalents of total carotenoids, a skin care

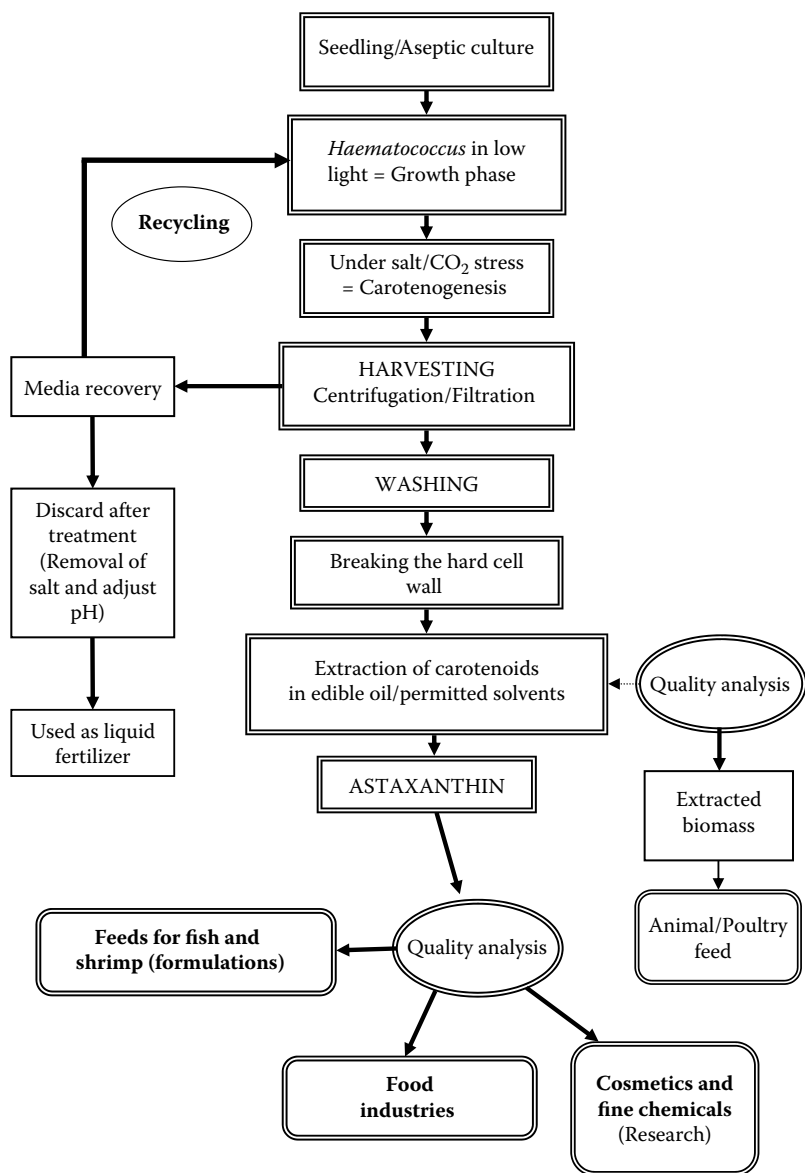


FIGURE 5.4.3 Production and utilization steps for astaxanthin from *Haematococcus*.

cream containing astaxanthin as one of the ingredients, and food and feed formulations for shrimp and fish.

Microalgae are recognized as excellent sources of pigments. Algal biomasses can be produced on a large scale in outdoor cultivation areas in various parts of the world near the equator. Algal forms are the richest sources of pigments and can be produced in a renewable manner because they sustainably produce some unique pigments and are thus highly dependable sources of pigments that can be produced in large quantities. The astaxanthin from *Haematococcus* is a potent pigment with antioxidant properties and tinctorial values. The microalgal forms discussed have great potential for the future and are already recognized as sources of colorants and nutraceuticals. Hence they will surpass other natural sources and synthetics due to their sustainability of production and renewable nature.

5.4.4 FLUORESCENT PINK FROM RED PORPHYRIDIMUM MICROALGA AND PHYCOBILIPROTEINS

The red microalga genus *Porphyridium* is a source of biochemicals possessing nutritional and therapeutic values. The biochemicals include polysaccharides that have anti-inflammatory and antiviral properties, long-chain polyunsaturated fatty acids, carotenoids such as zeaxanthin, and fluorescent phycobiliproteins.

The phycobiliproteins are accessory photosynthetic pigments aggregated in cells as phycobilisomes that are attached to the thylakoid membrane of the chloroplast.⁵⁷ The red phycobiliproteins (phycoerythrin) and the blue phycobiliprotein (phycocyanin) are soluble in water and can serve as natural colorants in foods, cosmetics, and pharmaceuticals.⁵⁸ Chemically, the phycobiliproteins are built from chromophores — bilins — that are open-chain tetrapyrroles covalently linked via thioether bonds to an apoprotein.^{59,60}

The microalgae are cultured in bioreactors under solar or artificial light in the presence of carbon dioxide and salts. The bioreactors may be closed systems made of polyethylene sleeves rather than open pools. Optimal conditions for pigment production are low to medium light intensity and medium temperatures (20 to 30°C). Pigment extraction is achieved by cell breakage, extraction into water or buffered solution, and centrifugation to separate out the filtrate. The filtrate may then be partly purified and sterilized by microfiltration and spray dried or lyophilized.

Porphyridium species are the sources of fluorescent pink color. The main *Porphyridium* phycobiliproteins are B-phycoerythrin and b-phycoerythrin. Maximum absorbance of a 1% solution of B-phycoerythrin in a 1-cm cuvette is at 545 nm, and the fluorescence emission peak is at 575 nm; molecular weight is 240 kda.⁶¹ Batch culture of *Porphyridium* species outdoors yields approximately 200 mg of colorant per liter of culture after 3 days; the phycoerythrin level in the colorant is about 15%. A higher concentration of phycoerythrin, up to 30%, can be achieved under optimal algal culture conditions.

The pinkish-red color can be used to color confections, gelatine desserts, and dairy products. The quantity of color required for 1 kg of food varies from 50 to 100 mg/kg.

The color is stable at 60°C for 30 min and has a long shelf life at pH 6 to 7. As an ingredient in dry food preparations stored under low humidity conditions, it is very stable. A number of patents have been granted for use of the red color from Porphyridium in foods.

In addition to its coloring properties, red phycoerythrin possesses a yellow fluorescence. Opportunities for exploiting this property for special effects in food are under study. A range of foods that fluoresce under natural and UV light were prepared and tested. These include transparent lollipops made from sugar solutions, dry sugar-drop candies for cake decoration (that fluoresce under UV light), and soft drinks and alcoholic beverages that fluoresce at pH 5 to 6. Fluorescent color has also been added to alcoholic beverages containing up to 30% alcohol, but the shelf life for such products is short.

This red color has not yet been approved for uses in foods or cosmetics. However, studies on rats fed with the dried biomass have not revealed any adverse growth or histological effects. Future efforts should thus be devoted toward obtaining official approval of use of the color in foodstuffs for human consumption.

5.4.5 MARINE BLUE FROM PORPHYRIDIUM AND PHYCOCYANIN: MORE BLUE FROM NATURE

The red microalga *Porphyridium aerugineum* is a source of blue color. This species is different from other red microalgae in that it lacks red phycoerythrin and its phycocyanin is C-phycocyanin rather than the R-phycocyanin that accompanies phycoerythrin found in many red algae and in other *Porphyridium* species. However, the biochemicals produced by *P. aerugineum* are similar to those of other red microalgae, e.g., sulfated polysaccharides, carotenoids, and lipids. An alternative source of C-phycocyanin is *Spirulina platensis*.⁶²

The algal extract of *P. aerugineum* is blue, with maximum absorbance at a wavelength of 620 nm and a red fluorescence with maximum emission at 642 nm. The main phycobiliprotein, C-phycocyanin, is the same type of phycocyanin found in most Cyanobacteria. The chromophores are composed of phycocyanobilins, conjugated to an apoprotein via thioether bonds.

P. aerugineum is a unicellular alga cultured under artificial or solar light in a freshwater medium supplied with CO₂, in an outdoor bioreactor developed by Arad and Yaron.⁵⁷ Algal growth was optimized for yield and for the properties of the blue color produced. The parameters that require close monitoring are light intensity and temperature, in order to avoid stress conditions that result in decreased color yield and solubility and increased biosynthesis of the polysaccharides that encapsulate the cells and are excreted into the medium.

Production of the color involves centrifugal separation of the biomass, cell breakage, and extraction. Use of a salt solution rather than water as an extraction medium increases stability of the color during extraction. Methods for partial exclusion of the polysaccharide from the color extract in order to enhance resolubilization of the dried color were developed. These processes include either microfiltration or co-precipitation of the polysaccharide with an added positively charged polysaccha-

ride. Microfiltration was also used to sterilize the solution containing the produced color prior to drying. Drying was performed either by lyophilization or by spray. After 4 days, the yield of color reached 100 mg of product per liter of batch cultured and contained 60% phycocyanin.

The blue color reached phycocyanin levels of up to 60% of the dry matter without further separation steps. The quantity required for coloring food was 140 to 180 mg of color per kilogram of blue food or drink. The polysaccharides accompanying the product stabilize the color and contribute added value by virtue of their functional nutritional properties. If the polysaccharides are separated out, antioxidants can be added to stabilize the color.

The shade of the blue color produced from *P. aeruginosa* does not change with pH. The color is stable under light, but sensitive to heat. Within a pH range from 4 to 5, the blue color produced from *P. aeruginosa* is stable at 60°C for 40 min (this is not typical of blue colors from Cyanobacteria). This property is important for food uses because many food items, particularly drinks and confections, are acidic. The blue color was added to clear Pepsi Cola® (without heat application) and to Bacardi Breezer® and these beverages retained their color for at least 1 month at room temperature.

The color was very stable in dry preparations. Sugar flowers for cake decoration maintained their color during years of storage. Foods prepared with the color include gelatin and ice cream. The color was mixed with other colorants to obtain a range of shades and hues.

The blue color from *P. aeruginosa* has not been cleared for food use by the authorities and it is not yet produced commercially. Toxicological studies carried out with other species of red microalgae have not revealed any adverse effects. Efforts should now be devoted to carrying out the required studies and procedures that will allow the use of the blue color as a substitute for synthetic colors.

5.4.6 MONASCUS PIGMENT: AN OLD STORY FOR ASIANS

5.4.6.1 SOURCES

Monascus is cultivated on solid media in Asian countries to produce a red colorant named Anka and used as a food ingredient. A Chinese medical book on herbs published in the first century first mentioned the terms “ang-kak” and “red mold rice.” Red mold rice has been used as a food colorant or spice in cooking. In 1884, the French botanist Philippe van Thieghem⁶³ isolated a purple mold on potato and linseed cakes and named it *Monascus ruber*. This ascomycete was so named because it has only one polyspored ascus. In 1895, Went⁶⁴ isolated a mold from the red mold rice obtained from a market in Java, Indonesia. This fungus was named *Monascus purpureus*, after which several other species were isolated around the world.

Monascus is often encountered in Oriental foods, especially in southern China, Japan, and southeast Asia. Currently, more than 50 patents have been issued in Japan, the United States, France, and Germany concerning the use of *Monascus* pigments

for food. Annual consumption of *Monascus* pigments in Japan increased from 100 tons in 1981 to 600 tons at the end of the 1990s and was valued at \$1.5 million. New food applications like the coloration of processed meats (sausages, hams), marine products like fish paste and surimi, and tomato ketchup were described.

5.4.6.2 *MONASCUS FUNGI*

The genus *Monascus* is divided into three species: *pilosus*, *purpureus* and *ruber* that comprise the majority of strains isolated mainly in Oriental foods.

5.4.6.3 FUNGAL METABOLITES

The main metabolites produced by *Monascus* are polyketides formed by the condensation of one acetylcoA with one or more malonylcoAs with a simultaneous decarboxylation as in the case of lipidic synthesis. They consist of the pigments, monacolins, and under certain conditions a mycotoxin.

Monascus pigments comprise a group of fungal metabolites called azaphilones that have similar molecular structures and chemical properties. Two molecular structures of *Monascus* pigments are shown in Figure 5.4.1. Ankaflavine and monascine are yellow, rubropunctatine and monascorubrine are orange, and rubropunctamine and monascorubramine are purple. The same colors exist in two molecular structures differing in the lengths of their aliphatic chains. These pigments are produced mainly in the cell-bound state.

They have low water solubility, are sensitive to heat, unstable in the pH range of 2 to 10 and fade with light. A number of methods for making water-soluble pigments have been patented. The principle is the substitution of the replaceable oxygen in monascorubrine or rubropunctatine by a nitrogen of the amino groups of various compounds such as amino acids, peptides, and proteins, changing the color from orange to purple. *Monascus* pigments can be reduced, oxidized, and react with other products especially amino acids to form various derivative products sometimes called complexed pigments. Glutamyl-monascorubrine and glutamyl-rubropunctatine were isolated from the broth of a submerged culture.⁶⁵

Stability of the pigments is affected by acidity, temperature, light, oxygen, water activity, and time. These pigments added to sausages and canned pâtés remained stable for 3 months of storage at 4°C, while their stability ranged from 92 to 98%.⁶⁶ Thus, the main patents have focused on the solubilization, stability, and the extraction in solution of pigments. The pigments can easily react in a medium with amino group-containing compounds such as proteins, amino acids, and nucleic acids to form water-soluble pigments.

A series of hypocholesteremic agents were isolated from *Monascus* and named monacolin J, K, and L. These polyketides were first isolated from cultures of *Penicillium citrinum* and they can inhibit specifically the enzyme controlling the rate of cholesterol biosynthesis. They are currently used in China in traditional and modern medicine.

Antibacterial properties of *Monascus* were first mentioned by Wong and Bau⁶⁷ in 1977. The so-called monascidin A was effective against *Bacillus*, *Streptococcus* and

Pseudomonas. It was shown that this molecule was citrinin⁶⁸ and its production by various *Monascus* species was studied using different culture media and conditions.

5.4.6.4 PRODUCTION OF CULTURES IN VARIOUS MODES

5.4.6.4.1 Submerged Cultures

Considerable contradiction exists in the published works as to the best carbon source for red pigment production in liquid cultures. Traditionally cultured on breads and rice, *Monascus* grows on every amylaceous substrate. It grows very well on starch, dextrans, glucose, maltose, and fructose. Good production of pigments was achieved using glucose and maltose. The nitrogen source seems to have more importance than the carbon source and ammonium, and peptones as nitrogen sources yielded superior growth and pigment concentrations compared to nitrate.⁶⁹ The best results were obtained using glucose and histidine. The carbon-to-nitrogen ratio was also shown to be important: at a value close to 50 g/g, growth would be favored, while in the region of 7 to 9 g/g, pigmentation would be favored.⁷⁰

5.4.6.4.2 Solid-State Cultures

The classical Chinese method consists of inoculating steamed rice grains spread on big trays with a strain of *Monascus anka* and incubating in an aerated and temperature-controlled room for 20 days. In these types of cultures, moisture content, oxygen, and carbon dioxide levels in the gas environment, as well as cereal medium composition, are the most important parameters to control.

Moisture content is a very important parameter. Red pigments were produced in plastic bags containing rice grains. It was observed that pigmentation occurred only at a relatively low initial moisture level (26 to 32%). Initial substrate moisture content regulated pigmentation as it was found that glucoamylase activity increased along with a rise in initial substrate moisture content. Therefore, at high moisture content, as high enzyme activity was produced, glucose was rapidly liberated in amounts (120 g/l) that inhibited pigmentation. The sugar was then transformed into ethanol.

As far as cultures on rice are concerned, optimal pigmentation was found at an initial moisture content of 56%, while lower moisture content led to a large decrease in pigment formation. Thus it was confirmed that solid culture was superior to liquid culture for red pigment production by *Monascus purpureus*. This result has been attributed to the de-repression of pigment synthesis in solid systems due to the diffusion of intracellular pigments into the surrounding solid matrix. In submerged culture, the pigments normally remain in the mycelium due to their low solubility in the usually acidic medium.

Levels of oxygen and carbon dioxide in the gas environment influence pigment production significantly while affecting growth to a lesser extent in solid-state culture. With *Monascus purpureus* on rice, maximum pigment yields were observed at 0.5 10^5 Pa of oxygen partial pressure in closed pressure vessels. However, high carbon dioxide partial pressures progressively inhibited pigment production, with complete inhibition at 10^5 Pa. In a closed aeration system with a packed-bed fer-

mentor, oxygen partial pressures ranging from 0.05 to 0.5 10^5 Pa at constant carbon dioxide partial pressures of 0.02 10^5 Pa gave high pigment yields with a maximum at 0.5 10^5 Pa of oxygen, whereas lower carbon dioxide partial pressures at constant oxygen partial pressures of 0.21 10^5 Pa gave higher pigment yields. Maximum oxygen uptake and carbon dioxide production rates were observed at 70 to 90 and 60 to 80 hours, respectively, depending on the gas environment. Respiratory quotients were close to 1.0 except at 0.05 10^5 Pa of oxygen and 0.02 10^5 Pa of carbon dioxide partial pressures. When studying various cereal media, it was shown that the best results were obtained using “mantou” (yeast-fermented wheat) meal.

5.4.6.5 METHODS FOR AVOIDING MYCOTOXIN PRODUCTION

In order to identify chemically the so-called monascidin A discussed by some Chinese scientists in their papers as a component suitable for the preservation of food, it was isolated and chemical investigations using mass spectrometry and NMR were undertaken. Monascidin A was characterized as citrinin which is known to be a mycotoxin responsible for nephropathies. Thus, in order to avoid the production of this toxin, various strains were screened in order to see whether all were toxigenic and it was shown that among the species of *Monascus* available in public collections, non-toxinogenic strains were obtainable.

Another way to avoid the production of citrinin may be controlling the biosynthesis of the metabolite. To accomplish that, the metabolic pathway must be investigated. The metabolic pathway is the same for citrinin and the pigment: the polyketide pathway in which condensation of acetates and malonates occurs. In the case of the pigment, there occurs at the end of the pathway an esterification of a fatty acid on the chromophore to obtain the colored molecules. Modifications of the culture conditions are possible to increase the pigment production or reduce the citrinin production: (1) the addition of fatty acids and (2) a change of the nitrogen source. Adding fatty acids to the medium was effective in favoring the synthesis of pigment, but the citrinin production remained unchanged.⁷¹

The final modification of the culture conditions was the replacement of glutamic acid by other amino acids. *Monascus ruber* was cultivated in a liquid medium containing glucose and various amino acids. Histidine was found to be the most effective nitrogen source related to citrinin production inhibition. When the pathway of histidine assimilation was investigated, it was shown that during its catabolism, one molecule of hydrogen peroxide was produced per molecule of consumed histidine and it is known that peroxidases can destroy citrinin in the presence of hydrogen peroxide. Thus, the production of citrinin can be avoided by control of the medium especially by the selection of a suitable amino acid, usually histidine.⁷²

The enormous economic potential of *Monascus* pigment has not led to commercial exploitation in the Western world, mainly because of ignorance and also reluctance to change on the part of public agencies that regulate foods. Indeed these agencies have not approved *Monascus* pigments for use in the food industry, although the pigments appear to be non-toxic if correctly used. Thus, the consumption of species of *Monascus* in the Far East for many years has not helped the pigment to gain approval in the European Union or the United States.

5.4.7 ARPINK RED FROM *PENICILLIUM OXALICUM*: A NEWCOMER

Many patents granted to Ascolor Biotech, Pardubice, Czech Republic, relate to a new fungus strain having the properties to produce a red colorant that can be applied in the food and cosmetic industries (WO9950434, CZ285721, EP1070136, US6340586). The strain *Penicillium oxalicum* var. *Armeniaca* CCM 8242 obtained from soil produces a chromophore of the anthraquinone type (Figure 5.4.1). Some strains of the same species are effective as biological control agents, e.g., reduction of the incidence of Fusarium wilt of tomato plants under glass house and field conditions.^{73,74} Other strains have been cited for production of milk-clotting enzyme.⁷⁵

The cultivation of the fungus in liquid broth requires carbohydrates (sucrose, molasses), nitrogen (corn extract, yeast autolysate or extract), zinc sulfate, and magnesium sulfate. The optimum conditions for performing the microbiological synthesis are a pH value of 5.6 to 6.2 and a temperature of 27 to 29°C. On the second day of incubation, a red colorant is released into the broth, increasing to 1.5 to 2.0 g/L of broth after 3 to 4 days. After biosynthesis of the red colorant is completed, the liquid from the broth is filtered or centrifuged for separation from the biomass. The liquid is then acidified to pH 2.5 to 3.0 to precipitate the colorant. The precipitate is dissolved in ethyl alcohol and filtered. Following removal of alcohol, the dark red powder colorant in crystalline form is obtained. The colorant produces a raspberry-red color in aqueous solution and is stable at pH over 3.5. Neutral solutions are stable even after 30 min of boiling and color shade does not change in relation to pH.⁷⁶

Much toxicological data are available on this red pigment: acute oral toxicity in mice, 90-day subchronic toxicological study, acute dermal irritation and corrosion, acute eye irritation and corrosion, anti-tumor effectiveness, micronucleus test in mice, AMES test (*Salmonella typhimurium* reverse mutation assay), estimation of antibiotic activity, and results of estimation of five mycotoxins. A new patent on Arpink Red was filed in 2001⁷⁷ with claims of anti-cancer effects of the anthraquinone derivatives and applications in the food and pharmaceutical fields.

After evaluating all the materials provided by Ascolor Biotech, the Codex Alimentarius Commission (Rotterdam meeting, March 2002) stated, "There will not be any objections to use the red coloring matter Arpink Red" in:

- Meat products in amounts up to 100 mg/kg
- Meat and meat product analogues in amounts up to 100 mg/kg
- Non-alcoholic drinks in amounts up to 100 mg/kg
- Alcoholic drinks in amounts up to 200 mg/kg
- Milk products in amounts up to 150 mg/kg
- Ice creams in amounts up to 150 mg/kg
- Confectionery in amounts up to 300 mg/kg

The JECFA (Joint FAO/WHO Expert Committee on Food Additives) evaluation process is in progress and Arpink Red was discussed at the 63rd meeting of the joint committee in Geneva in June 2004.

5.4.8 β -CAROTENE FROM BLAKESLEA: FIRST MICROBIAL PIGMENT OF A TOP FOOD INGREDIENT COMPANY

The source organism, the *Blakeslea trispora* mold, is a plant commensal of tropical plants, some strains of which produce high levels of β -carotene. The fungus exists in (+) and (–) mating types, of which the (+) type synthesizes trisporic acid, a precursor of β -carotene. After mating the two types in a specific ratio, the (–) type then produces large amounts of β -carotene. The mold has been shown to be non-pathogenic and non-toxigenic. Standard pathogenicity experiments in mice and analyses of extracts of several fermentation mashings and of the final products, β -carotene crystals, were done according to literature, while enzyme immunoassays were performed for four mycotoxins.

The production process proceeds essentially in two stages. Glucose and corn steep liquor may be used as carbon and nitrogen sources. Whey, a by-product of cheese manufacture, with strains acclimatized to lactose, has also received consideration.⁷⁸ For fermentation, seed cultures are produced from the original strain cultures and subsequently used in an aerobic submerged batch fermentation to produce a biomass rich in β -carotene. In the second stage, the recovery process, the biomass is isolated and transformed into a form suitable for isolating the β -carotene, which is extracted from the biomass with ethyl acetate, suitably purified and concentrated, and the β -carotene crystallized from the mother liquor. The final product is either crystalline β -carotene (purity > 96.0%) or is formulated as a 30% micronized suspension in vegetable oil. The production process is controlled by GMP standards, adequate hygiene control, and adequate control of raw materials. The biomass and the final crystalline product comply with an adequate chemical and microbiological specification and the final crystalline product also complies with the JECFA and European Union specifications as set out in Directive 95/45/EC for coloring matters in food.

A company formerly known as Gist-Brocades, now DSM, was the first to produce β -carotene from this fermentative source. The product was launched in 1995 at the Food Ingredients Europe meeting in London. As a first entry on the European market, its experience could be useful for other companies intending to sell fermentative food-grade pigments. Following the optimization of the fermentation process, the company addressed many issues before selling the product:

Presentation of microorganism — Fungus isolated from Nature, not genetically modified; yield improvement using classical genetics.

Guidelines for labeling — natural β -carotene, natural β -carotene from *Blakeslea trispora*, fermentative natural β -carotene, natural β -carotene from a fermentative source.

Lobbying by other β -carotene producers — In addition to approvals of mixed carotenes from palm oil, β -carotene from *Dunaliella* microalgae, and other natural products, the EU Health and Consumer Protection Directorate General was asked for an opinion on the safety of β -carotene from a dried biomass source, obtained from a fermentation process with *Blakeslea trispora* for use as a coloring matter for foodstuffs.

Safety of the fermentation-produced β -carotene — HPLC analysis, stability tests, and microbiological tests showed that the β -carotene obtained by fermentation of *Blakeslea trispora* complied with the EC specification for E 160 *aii*, including the proportions of *cis* and *trans* isomers, and was free of mycotoxins and other toxic metabolites. *In vitro* tests for gene mutations and chromosomal aberrations with the β -carotene produced by the manufacturer in the EU showed it to be free of genotoxic activity. In a 28-day feeding study in rats with the β -carotene manufactured in the EU, no adverse findings were noted at a dose of 5% in the diet, the highest level used. In conclusion, evaluation of the source organism and the production process yielded no grounds to suppose that the final crystalline product, β -carotene, differed from the chemically synthesized β -carotene used as a food colorant. The final crystalline fermentation product was shown to comply with the specification for β -carotene E 160 *aii* listed in Directive 95/45/EC. The committee considered that β -carotene produced by fermentation of *Blakeslea trispora* was equivalent to the chemically synthesized material used as food colorant and was therefore acceptable for use as a coloring agent for foodstuffs.⁷⁹

The other industrial production facilities for *B. trispora* fungal β -carotene are in Russia and the Ukraine and also in León, Spain where the start-up Vitatene operation began its activities in 2004.⁸⁰ The process was developed to achieve a yield of 30 mg of β -carotene/g dry mass or about 3 g/L.

5.4.9 ASTAXANTHIN FROM XANTHOPHYLLOMYCES *DENDRORHOUS*: LARGE-SCALE PRODUCTION SOON?

Astaxanthin (3,3-dihydroxy- β , β -carotene-4,4-dione) is widely distributed in Nature and is the principal pigment in crustaceans and salmonids. The carotenoid imparts distinctive orange-red coloration to the animals and contributes to consumer appeal in the market place.

Since animals cannot synthesize carotenoids, the pigments must be supplemented in the feeds of farmed species. Salmon and trout farming is now a huge business and feeding studies have shown that astaxanthin is very effective as a flesh pigmenter.⁸¹

Among the few astaxanthin-producing microorganisms, *Xanthophyllomyces dendrorhous* is one of the best candidates for commercial production. Therefore, many academic laboratories and several companies have developed processes that could reach industrial levels.

Several reports in the literature have shown that medium constituents among other environmental factors affect astaxanthin production in this yeast. The effects of different nutrients on *Xanthophyllomyces dendrorhous* have generally been studied in media containing complex sources of nutrients such as peptone, malt, and yeast extracts. By-products from agriculture such as molasses, enzymatic wood hydrolysates, corn wet milling co-products, bagasse or raw sugarcane juice, and grape juice were also tested. Although such media are often convenient because they contain all nutrients, they suffer from the disadvantage of being undefined and

sometimes variable in composition, which may mask important nutritional effects. For this reason, several studies are difficult to interpret in detail because of inadequate characterization of growth-limiting factors in the media. Thus, in order to elucidate the nature of nutritional effects as far as possible, chemically defined or synthetic media were used by some authors.⁸²

For example, Palágyi et al.⁸³ assayed eleven strains for their ability to utilize 99 compounds as single carbon sources. In a second study, carotenoid biosynthesis was increased at low ammonium or phosphate levels and stimulated by citrate. Factorial design and response surface methodology could be used to optimize astaxanthin production.⁸⁴ The optimal conditions stimulating the highest astaxanthin production were temperature, 19.7°C; carbon concentration, 11.25 g/L; pH, 6.0; inoculum, 5%; and nitrogen concentration, 0.5 g/L. Under these conditions, the astaxanthin content was 8.1 mg/L. Fermentation strategy also has an impact on growth and carotenoid production of *Xanthophyllomyces dendrorhous*, as shown with fed-batch or pH-stat cultures. The highest biomass obtained was 17.4 g/L.

A major drawback in the *Xanthophyllomyces dendrorhous* process is that disruption of the cell wall of yeast biomass is required prior to the addition to the animal diet. This is essential for intestinal absorption of the pigment. Investigations have described several chemical, physical, autolytic, and enzymic methods for cell wall disruption. Fang and Wang⁸⁵ used a two-stage batch fermentation technique. The first stage was for red yeast cultivation. The second stage was the mixed fermentation of the yeast and *Bacillus circulans*, a bacterium with high cell wall lytic activity.

Generation of mutants is also a starting point in optimization experiments,⁸⁶ and now is the time for metabolic engineering of the astaxanthin biosynthetic pathway.⁸⁷ Researchers should be able to manage carbon fluxes within the cells and resolve competitions between enzymes such as phytoene desaturase and lycopene cyclase.

In conclusion, the case of *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) is very peculiar as hundreds of scientific papers and patents deal with astaxanthin production using this yeast⁸⁸ and the process has not been economically efficient to date. New patents are filed almost each year, with improvement in astaxanthin yield, e.g., 3 g/g dry matter cited in U.S. Patent 20030049241.⁸⁹

5.4.10 CONCLUSION

Synthetic pigments traditionally used by food processors continue to be utilized with success; however, with the increasing consumer preference for natural food additives, natural colorants from plants now present big business and most of the research efforts within the scientific field of colorants are conducted on natural materials. Among microalgal production methods, marine background is a very positive aspect in the success of β -carotene produced by *Dunaliella salina*.

Regarding bacteria, yeasts, or fungi (Table 5.4.1), despite common belief about the high production cost of fermentation pigments, two initiatives started in Europe in the past few years: β -carotene from the filamentous fungi, *Blakeslea trispora* (produced by Gist-Brocades now DSM; approved in 2000 by the EU Scientific Committee on Food Safety) and Arpink Red from *Penicillium oxalicum* (manufactured by Ascolor Biotech). These companies invested a lot of money as any combi-

TABLE 5.4.1
Microbial Production of Pigments^a

Molecule	Color	Microorganism	Status
Ankaflavin	Yellow	<i>Monascus</i> sp. (fungus)	IP
Anthraquinone	Red	<i>Penicillium oxalicum</i> (fungus)	IP
Astaxanthin	Pink-red	<i>Xanthophyllomyces dendrorhous</i> (yeast), formerly <i>Phaffia rhodozyma</i>	DS
Astaxanthin	Pink-red	<i>Agrobacterium aurantiacum</i> (bacteria)	RP
Astaxanthin	Pink-red	<i>Paracoccus carotinifaciens</i> (bacteria)	RP
Canthaxanthin	Dark red	<i>Bradyrhizobium</i> sp. (bacteria)	RP
Lycopene	Red	<i>Blakeslea trispora</i> (fungus)	DS
Lycopene	Red	<i>Fusarium sporotrichioides</i> (fungus)	RP
Melanin	Black	<i>Saccharomyces neoformans</i> var. <i>nigricans</i> (yeast)	RP
Monascorubramin	Red	<i>Monascus</i> sp. (fungus)	IP
Naphtoquinone	Deep blood-red	<i>Cordyceps unilateralis</i> (fungus)	RP
Riboflavin	Yellow	<i>Ashbya gossypi</i> (fungus)	IP
Rubrolone	Red	<i>Streptomyces echinoruber</i> (bacteria)	DS
Rubropunctatin	Orange	<i>Monascus</i> sp. (fungus)	IP
Torularhodin	Orange-red	<i>Rhodotorula</i> sp. (yeast)	DS
Zeaxanthin	Yellow	<i>Flavobacterium</i> sp. (bacteria)	DS
Zeaxanthin	Yellow	<i>Paracoccus zeaxanthinifaciens</i> (bacteria)	RP
β -Carotene	Yellow-orange	<i>Blakeslea trispora</i> (fungus)	IP
β -Carotene	Yellow-orange	<i>Fusarium sporotrichioides</i> (fungus)	RP
β -Carotene	Yellow-orange	<i>Mucor circinelloides</i> (fungus)	DS
β -Carotene	Yellow-orange	<i>Neurospora crassa</i> (fungus)	RP
β -Carotene	Yellow-orange	<i>Phycomyces blakesleeanus</i> (fungus)	RP
Unknown	Red	<i>Penicillium purpurogenum</i> (fungus)	DS
Unknown	Red	<i>Paecilomyces sinclairii</i> (fungus)	RP

IP = industrial production. DS = development stage. RP = research project.

^a Compounds listed are already in use as natural food colorants or have high potential for such use.

nation of new source and/or new pigment drives a lot of experimental work, process optimization, toxicological studies, regulatory issues, and documentation. Time will tell whether investment was cost-effective.

Another development under process is the production of lycopene using *Blakeslea trispora* by Vitatene, a subsidiary of the Spanish Antibioticos penicillin firm. Exploration of fungal biodiversity continues, with special interest in water-soluble pigments.⁹⁰ The case of *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*) is very peculiar in that hundreds of scientific papers and patents deal with astaxanthin production using this yeast and the process has not been economically efficient up to now. Microorganisms may be used for the biosynthesis of “niche” pigments not found in plants, e.g., aryl carotenoids.^{91–94}

Research projects mixing molecular biology and pigments were investigated worldwide and it seems that current production techniques are not effective in terms

of final yield. Combinatorial genetic engineering based on increasing numbers of known carotenogenic gene sequences⁹⁵ is currently being addressed. By combining genes, some authors were able to obtain more efficient biosynthesis or new carotenoids such as multi-hydroxylated compounds. While not described in Nature, they may be very efficient as antioxidants.

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REFERENCES

1. Clydesdale, F.M., Color as a factor in food choice, *Crit. Rev. Food Sci.*, 33, 83, 1993.
2. Stich, E., Chaundry, Y., and Schnitter, C., Color: you eat with your eyes, *Int. Food Inged.*, 1, 6, 2002.
3. Dufossé, L., *Pigments in Food: More than Colors*, Université de Bretagne Occidentale Publications, Quimper, France, 2004.
4. Wissgott, U. and Bortlik, K., Prospects for new natural food colorants, *Trends Food Sci., Technol.*, 7, 298, 1996.
5. O’Carroll, P., Naturally exciting colors, *World Inged.*, 3/4, 39, 1999.
6. Downham, A. and Collins, P., Coloring our foods in the last and next millennium, *Int. J. Food Sci. Technol.*, 35, 5, 2000.
7. Dufossé, L. et al., Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends Food Sci. Technol.*, 16, 389, 2005.
8. Dufossé, L., Microbial production of food grade pigments, *Food Technol. Biotechnol.*, 44, 313, 2006.
9. South, Y. and Whittick, H., *Introduction to Phycology*. Blackwell, New York, 1987.
10. Cheng, J.Y., Don Paul, M., and Antia, N.J., Isolation of an unusually stable cis isomer of alloxanthin from a bleached autolysed culture of *Chroomonas salina* grown photoheterotrophically on glycerol: observation on cis trans isomerization of alloxanthin, *J. Protozool.*, 21, 761, 1974.
11. Avron, M. and Ben-Amotz, A., *Dunaliella: Physiology, Biochemistry and Biotechnology*, CRC Press, Boca Raton, FL, 1992.
12. Ben-Amotz, A., Kartz, A., and Avron, M., Accumulation of β -carotene in halotolerant algae: purification and characterization of β -carotene globules from *Dunaliella bardawil*, *J. Phycol.*, 18, 529, 1983.
13. Villar, R. et al., Effects of *Phaeodactylum tricornutum* and *Dunaliella tertiolecta* extracts on the central nervous system, *Planta Med.*, 58, 405, 1992.
14. Lers, A., Biener, Y., and Zamir, A., Photoinduction of massive β -carotene accumulation by the alga *Dunaliella bardawil*: kinetics and dependence on gene activation, *Plant Physiol.*, 93, 389, 1990.
15. Olson, J.A. and Krinsky, N.I., Introduction: the colorful, fascinating world of the carotenoids: important physiologic modulators, *FASEB J.*, 9, 1547, 1995.

16. Albanes, D. et al., Effect of supplemental β -carotene, cigarette smoking and alcohol consumption on serum carotenoids in alpha-tocopherol, β -carotene cancer prevention study, *Am. J. Clin. Nutr.*, 66, 366, 1976.
17. Törnwall, M.E. et al., Effect of α -tocopherol and β -carotene supplementation on coronary heart disease during the 6-year post-trial follow-up in the ATBC study, *Eur. Heart J.*, 25, 1171, 2004.
18. Cantrell, A. et al., Singlet oxygen quenching by dietary carotenoids in a model membrane environment, *Arch. Biochem. Biophys.*, 412, 47, 2003.
19. Jahnke, L.S., Massive carotenoid accumulation on *Dunaliella bardawil* induced by ultraviolet-A radiation, *J. Photochem. Photobiol. B Biol.*, 48, 68, 1999.
20. Yeum, K.J. and Russel, R.M., Carotenoids: bioavailability and bioconversion, *Annu. Rev. Nutr.*, 22, 483, 2002.
21. Roodenburg, A.J. et al., Amount of fat in the diet affects bioavailability of lutein esters but not of α -carotene, β -carotene, and vitamin E in humans, *Am. J. Clin. Nutr.*, 71, 1187, 2000.
22. Finney, K.F., Pomeranz, Y., and Bruinsma, B., Use of algae *Dunaliella* as a protein supplement in bread, *Cereal Chem.*, 61, 402, 1994.
23. Mokady, S., Abramovici, A., and Cogan, U., The safety evaluation of *Dunaliella bardawil* as a potential food supplement, *Food Chem. Toxicol.*, 27, 221, 1989.
24. Lorenz, R.T. and Cysewski, G.R., Commercial potential for *Haematococcus* microalgae as a natural source of astaxanthin, *Trends Biotechnol.*, 18, 160, 2000.
25. Chaumont, C. et al., Scaling up a tubular photoreactor for continuous culture of *Porphyridium cruentum* from laboratory to pilot plant, in *Algal Biotechnology*, Stadler, T. et al., Eds., Elsevier, London, 1988, 199.
26. Borowitzka, L.J. and Borowitzka M.A., Industrial production: methods and economics, in *Algal and Cyanobacterial Biotechnology*, Cresswell, R.C. et al., Eds., Longman, London, 1989, 294.
27. Fabregas, J. et al., Two-stage cultures for the production of astaxanthin from *Haematococcus pluvialis*, *J. Biotechnol.*, 89, 65, 2001.
28. Borowitzka, M.A., Huisman, J.M., and Osborn, A., Culture of the astaxanthin-producing green alga *Haematococcus pluvialis*. I. Effects of nutrients on growth and cell type, *J. Appl. Phycol.*, 3, 295, 1991.
29. Kobayashi, M., Kakizono, T., and Nagai, S., Astaxanthin production by a green alga. *Haematococcus pluvialis* accompanied with morphological changes in acetate media, *J. Ferm. Bioeng.*, 71, 335, 1991.
30. Sarada, R., Usha, T., and Ravishankar, G.A., Influence of stress on astaxanthin production in *Haematococcus pluvialis* grown under different culture conditions, *Process Biochem.*, 37, 623, 2002.
31. Sommer, T.R., Pott, W., and Morrissey, N.M., Utilization of microalgal astaxanthin by rainbow trout (*Oncorhynchus mykiss*), *Aquaculture*, 94, 79, 1991.
32. An, G.H., Schuman, D.B., and Johnson, E.A., Isolation of *Phaffia rhodozyma* mutants with increased astaxanthin content. *Appl. Env. Microbiol.*, 55, 116, 1989.
33. Snodderly, D.M., Evidence for protection against age-related macular degeneration by carotenoids and antioxidant vitamins, *Am. J. Clin. Nutr.*, 62, 1448S, 1995.
34. DiMascio, P. et al., Carotenoids, tocopherols and thiols as biological singlet molecular oxygen quenchers, *Biochem. Soc. Trans.*, 18, 1054, 1990.
35. Jyonouchi, H., Sun, S., and Gross, M., Effect of carotenoids on *in vitro* immunoglobulin production by human peripheral blood mononuclear cells: astaxanthin, a carotenoid without vitamin A activity, enhances *in vitro* immunoglobulin production in response to a T-dependent stimulant and antigen, *Nutr. Cancer*, 23, 171, 1994.

36. Kurashige, M. et al., Inhibition of oxidative injury of biological membranes by astaxanthin, *Physiol. Chem. Phys. Med. NMR*, 22, 27, 1990.
37. Gradelet, S. et al., Effects of canthaxanthin, astaxanthin, lycopene and lutein on liver xenobiotic-metabolizing enzymes in the rat, *Xenobiotica*, 26, 49, 1996.
38. Kobayashi, M. and Sakamoto, Y., Singlet oxygen quenching ability of astaxanthin esters from the green alga *Haematococcus pluvialis*, *Biotechnol. Lett.*, 21, 265, 1999.
39. Savouré, N. et al., Vitamin A status and metabolism of cutaneous polyamines in the hairless mouse after UV irradiation: action of β -carotene and astaxanthin, *Int. J. Vitamin Nutr. Res.*, 65, 79, 1995.
40. Suzuki, K., Masaki, H., and Takei, M., External preparation for skin, Japanese Patent 08073312 [in Japanese], 1996.
41. Jyonouchi, H. et al., Studies of immunomodulating actions of carotenoids. I. Effects of β -carotene and astaxanthin on murine lymphocyte functions and cell surface marker expression in *in vitro* culture system, *Nutr. Cancer*, 19, 93, 1991.
42. Miki, W. et al., Astaxanthin-containing drink, Japanese patent 10155459, [in Japanese], 1998.
43. Nishikawa, Y., Minenaka, Y., and Ichimura, M., Physiological and biochemical effects of carotenoid (β -carotene and astaxanthin) on rat, *Koshien Daigaku Kiyo*, 25, 19, 1997 [in Japanese].
44. Tanaka, T. et al., Suppression of azoxymethane-induced rat colon carcinogenesis by dietary administration of naturally occurring xanthophylls astaxanthin and canthaxanthin during the post-initiation phase, *Carcinogenesis*, 16, 2957, 1995.
45. Yamashita, E., Anti-inflammatory agent, Japanese Patent 07300421 [in Japanese], 1995.
46. Alejung, P. and Wadstroem, T., Oral preparation for treatment of *Helicobacter* sp. infections comprises xanthophylls, especially astaxanthin esterified with a fatty acid and derived from the alga *Haematococcus* species, World Patent 9837874, 1998.
47. Okai, Y. and Higashi-Okai, K., Possible immunomodulating activities of carotenoids in *in vitro* cell culture experiments, *Int. J. Immunopharm.*, 18, 753, 1996.
48. Torrissen, O.J., Hardy, W.H., and Shearer, K.D., Pigmentation of salmonids: carotenoid deposition and metabolism, *Rev. Aqu. Sci.*, 1, 209, 1989.
49. Storebakken, T., Krill as a potential feed source for salmonids, *Aquaculture*, 70, 193, 1988.
50. Christiansen, R., Lie, O., and Torrissen, O.J., Growth and survival of Atlantic salmon, *Salmo salar* L, fed different dietary levels of astaxanthin: first-feeding fry, *Aquacult. Nutr.*, 1, 189, 1995.
51. Choubert, G. and Heinrich, O., Carotenoid pigments of green alga *Haematococcus pluvialis*: assay on rainbow trout *Oncorhynchus mykiss*, pigmentation in comparison with synthetic astaxanthin and canthaxanthin, *Aquaculture*, 112, 217, 1993.
52. Miki, W., Biological functions and activities of animal carotenoids, *Pure Appl. Chem.*, 63, 141, 1991.
53. Sterlie, M., Bjerkeng, B., and Liaaen-Jensen, S., Blood appearance and distribution of astaxanthin E/Z isomers among plasma lipoproteins in humans administered a single meal with astaxanthin, in *Abstracts of 12th International Carotenoid Symposium*, Cairns, Australia, Abstract 2A-13, 1999, 72.
54. Lignell, S., Medicament for improvement of duration of muscle function or treatment of muscle disorders or diseases, AstaCarotene AB, Sweden, US Patent 6245218, 2001.
55. Aquasearch Inc., *Haematococcus pluvialis* and astaxanthin safety for human consumption, Technical Report TR. 3005-001, 1999.

56. Tso, M.O. and Lam, T.T., Method of retarding and ameliorating central nervous system and eye damage, United States Patent 5527533, 1996.
57. Arad (Malis), S. and Yaron, A., Natural pigments from red microalgae for use in foods and cosmetics, *Trends Food Sci. Technol.*, 3, 92, 1992.
58. Yaron, A. and Arad (Malis), S., Phycobiliproteins — blue and red natural pigments — for use in food and cosmetics, in *Food Flavors, Ingredients and Composition: Developments in Food Science*, Vol. 32, Charalambous, G., Ed., Elsevier, London, 1993, 835.
59. Roman, R.B. et al., Recovery of pure B-phycoerythrin from the microalga *Porphyridium cruentum*, *J. Biotechnol.*, 93, 73, 2002.
60. Bermejo, R. et al., Preparative purification of B-phycoerythrin from the microalga *Porphyridium cruentum* by expanded-bed adsorption chromatography, *J. Chromatogr. B*, 790, 317, 2003.
61. Glazer, A.N. and Hixson, C.S., Subunit structure and chromophore composition of rhodophytan phycoerythrins: *Porphyridium cruentum* B-phycoerythrin and b-phycoerythrin, *J. Biol. Chem.*, 252, 32, 1977.
62. Bermejo, R. et al., Expanded bed adsorption chromatography for recovery of phycocyanins from the microalga *Spirulina platensis*, *Chromatographia*, 63, 59, 2006.
63. Thieghem, (van) P., *Monascus*, genre nouveau de l'ordre des ascomycètes, *Bull. Soc. Bot. France*, 31, 226, 1884.
64. Went, F.A.F.C., *Monascus purpureus*, le champignon de l'Ang-quac. Une nouvelle thélébolée, *Ann. Sci. Nat. Bot.*, 8, 111, 1895.
65. Blanc, P.J. et al., Characterization of monascidin A from *Monascus* as citrinin, *Int. J. Food Microbiol.*, 27, 201, 1995.
66. Fabre, C.E. et al., Production and food applications of the red pigments of *Monascus ruber*, *J. Food Sci.*, 58, 1099, 1993.
67. Wong, H.C. and Bau, Y.S., Pigmentation and antibacterial activity of fast neutron- and x-ray-induced strains of *Monascus purpureus* Went, *Plant Physiol.*, 60, 578, 1977.
68. Blanc, P.J. et al., Pigments of *Monascus*, *J. Food Sci.*, 59, 862, 1995.
69. Hamdi, M., Blanc, P.J., and Goma, G., A new process for red pigment production by *Monascus purpureus*: culture on prickly pear juice and the effect of partial oxygen pressure, *Bioprocess Eng.*, 17, 75, 1995.
70. Hajjaj, H. et al., The biosynthetic pathway of citrinin in the filamentous fungi *Monascus ruber* as revealed by ¹³C-NMR, *Appl. Env. Microbiol.*, 65, 311, 1999.
71. Hajjaj, H. et al., Medium-chain fatty acids affect citrinin production in the filamentous fungus *Monascus ruber*, *Appl. Env. Microbiol.*, 66, 1120, 2000.
72. Hajjaj, H. et al., Kinetic analysis of red pigment and citrinin production by *Monascus ruber* as a function of organic acid accumulation, *Enz. Microb. Technol.*, 27, 619, 2000.
73. Larena, I., Melgarejo, P., and De Cal, A., Drying of conidia of *Penicillium oxalicum*, a biological control agent against Fusarium wilt of tomato, *J. Phytopath.*, 151, 600, 2003.
74. Larena, I. et al., Biocontrol of Fusarium and Verticillium wilt of tomato by *Penicillium oxalicum* under greenhouse and field conditions, *J. Phytopath.*, 151, 507, 2003.
75. Hashem, A.M., Purification and properties of a milk-clotting enzyme produced by *Penicillium oxalicum*, *Biores. Technol.*, 75, 219, 2000.
76. Sardaryan, E. et al., Arpink Red: meet a new natural red food colorant of microbial origin, in *Pigments in Food: More than Colors*, Dufossé, Ed., Université de Bretagne Occidentale, Quimper, France, 2004, 207.
77. Sardaryan, E., Food supplement, WO 02/1153, 2002.

78. Lampila, L.E. et al., The effect of *Blakeslea trispora* strain and type of whey on the production of β -carotene and other parameters, *Lebensm. Wiss. Technol.*, 18, 366, 1985.
79. European Commission, Opinion of the Scientific Committee on Food on β -carotene from *Blakeslea trispora*, SCF/CS/ADD/COL 158, 2000.
80. Iturriaga, E.A. et al., Strain and culture condition improvement for β -carotene production with *Mucor*, in *Methods in Biotechnology: Microbial Processes and Products*, Vol. 18, Barredo, J.L., Ed., Humana Press, Totowa, NJ, 2005, 239.
81. Johnson, E.A. and An, G.H., Astaxanthin from microbial sources, *Crit. Rev. Biotechnol.* 11, 297, 1991.
82. Flores-Cotera, L.B., Martin, R., and Sanchez S., Citrate, a possible precursor of astaxanthin in *Phaffia rhodozyma*: influence of varying levels of ammonium, phosphate and citrate in a chemically defined medium, *Appl. Microbiol. Biotechnol.*, 55, 341, 2001.
83. Palágyi, Z., Ferenczy, L., and Vagvölgyi, C., Carbon-source assimilation pattern of the astaxanthin-producing yeast *Phaffia rhodozyma*, *World J. Microbiol. Biotechnol.*, 17, 95, 2001.
84. Ramirez, J., Guttierrez, H., and Gschaedler, A., Optimization of astaxanthin production by *Phaffia rhodozyma* through factorial design and response surface methodology, *J. Biotechnol.*, 88, 259, 2001.
85. Fang, T.J. and Wang, J.M., Extractibility of astaxanthin in a mixed culture of a carotenoid over-producing mutant of *Xanthophyllomyces dendrorhous* and *Bacillus circulans* in two-stage batch fermentation, *Process Biochem.*, 37, 1235, 2002.
86. Rubinstein, L. et al., Isolation and characterization of *Phaffia rhodozyma* mutants, *Folia Microbiol.*, 43, 626, 1998.
87. Verdoes, J.C. et al., Metabolic engineering of the carotenoid biosynthetic pathway in the yeast *Xanthophyllomyces dendrorhous* (*Phaffia rhodozyma*), *Appl. Environ. Microbiol.*, 69, 3728, 2003.
88. Johnson, E.A., *Phaffia rhodozyma*: colorful odyssey, *Int. Microbiol.*, 6, 169, 2003.
89. Jacobson, G.K. et al., Astaxanthin over-producing strains of *Phaffia rhodozyma*, methods for their cultivation and their use in animal feeds, United States Patent 20030049241, 2003.
90. Mapari, S.A.S. et al., Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants, *Curr. Opin. Biotechnol.*, 16, 231, 2005.
91. Dufossé, L., Mabon, P., and Binet, A., Assessment of the coloring strength of *Brevibacterium linens* strains: spectrophotometry versus total carotenoid extraction/quantification, *J. Dairy Sci.*, 84, 354, 2001.
92. Guyomarç'h, F., Binet, A., and Dufossé, L., Production of carotenoids by *Brevibacterium linens*: variation among strains, kinetic aspects and HPLC profiles, *J. Ind. Microbiol. Biotechnol.*, 24, 64, 2000.
93. Dufossé, L. and de Echanove, C., The last step in the biosynthesis of aryl carotenoids in the cheese ripening bacteria *Brevibacterium linens* ATCC 9175 (*Brevibacterium aurantiacum* sp. nov.) involves a cytochrome P450-dependent monooxygenase, *Food Res. Int.*, 38, 967, 2005.
94. Guyomarç'h, F., Binet, A., and Dufossé, L., Characterization of *Brevibacterium linens* pigmentation using spectrophotometry, *Int. J. Food Microbiol.*, 57, 201, 2000.
95. Mijts, B.N., Lee, P.C., and Schmidt-Dannert, C., Identification of a carotenoid oxygenase synthesizing acyclic xanthophylls: combinatorial biosynthesis and directed evolution, *Chem. Biol.*, 12, 453, 2005.

Section 6

Analysis of Pigments and Colorants

6.1 Analysis of Chlorophylls

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6.1.1 INTRODUCTION

One of the major challenges in pigment analysis has always been the quantification of chlorophylls in either fresh, processed, or pigment-supplemented foods. Analyzing the amounts of chlorophylls present in green plants and water organisms has become indispensable due to the urgent search for new sources of chlorophylls to be used in the food industry. Phytoplankton have been found as yet unexploited sources of chlorophylls from fresh and marine water resources. Another point is the evaluation of plant injuries induced by various air pollutants that cause losses of chlorophylls and subsequent declines in photosynthesis. Finally, the analysis of chlorophylls has improved our knowledge about the natural processes of biosynthesis, metabolism, and degradation in senescent plants and fruit ripening.

The research into the biological activities of chlorophylls developed over the past 20 years is also important although very few *in vivo* assays concerning their potential health benefits have been performed. Far fewer studies have focused on chlorophylls in comparison to carotenoids. Efforts to stabilize chlorophylls in pro-

cessed foods have been extensive, but have not always succeeded, thus hindering more widespread applications. However, this problem has been partially solved with the development of more stable metal-complexed chlorophyll derivatives. Moreover, to replace synthetic dyes, chlorophylls have been added to processed foods to restore the natural levels of these molecules or prepare fortified products. The green color is an important criterion and marker of food quality, treatment, storage, and commercial value and it represents freshness to consumers. However, food composition tables do not show data about chlorophyll despite increasing interest of consumers in its health benefits.

Once plants are harvested, chlorophylls are invariably degraded, chemically or enzymatically, to pheophytins and pheophorbides that can be further metabolized to colorless compounds in metabolically active tissues due to the instability of chlorophyll molecules against acidic pH, light, heat, oxygen, and enzymes. In addition, not only natural degradation but also food storage and processing can cause chemical changes. The derivatives produced can be either lipophilic or hydrophilic and the different physicochemical properties should be considered when they are analyzed.

In the past, no suitable analytical methodologies were capable of investigating these multiple reactions and even today, the complete extraction and analysis of all the compounds is still a difficult task. The methods for extraction must be optimized for each sample according to the solubility of either phytylated (chlorophylls and pheophytins) or dephytylated (chlorophyllides and pheophorbides) derivatives, often requiring several repeated steps and the use of a single or a mixture of organic solvents.

Considering the low stability of the pigments, all manipulations should be carried out in a light-protected environment. Equipment and glassware should be covered with black cloth or aluminum foil; low temperatures for evaporation in the rotary evaporator or evaporation of small volumes directly under N_2 or Ar should be used. Acidic pH should be avoided. Solutions and extracts should be stored at low temperatures under inert atmosphere and all procedures should be carried out as quickly as possible.

The methods for extraction and separation of chlorophylls and their derivatives and the most common procedures of identification and quantification are described in this chapter.

6.1.2 EXTRACTION OF CHLOROPHYLLS

The accuracy of the analysis depends on procedures and precautions and its purpose is to bring all pigments into solution without alterations. Freshly harvested and undamaged plants should be preferably used and the activities of endogenous chlorophyll-degrading enzymes (chlorophyllase and Mg-dechelataase) should be inhibited by blanching, drying, or keeping plants at low temperatures. Although air drying and artificial rapid heating at closely controlled temperatures are common processes, freeze drying is the best procedure. Blanching and other thermal processes denature proteins and increase the extraction of chlorophylls. On the other hand, extended heating causes allomerization, isomerization, epimerization, and pheophytinization,

particularly during extraction from acidic tissues. The yield of pigment extraction varies according to the stage of plant development, the duration and temperature of drying, and the duration of exposure to storage conditions.

Extraction consists of removing the chlorophylls from the complex matrix of the raw material by homogenization with cold organic solvents in a blender or vortex, which causes the detachment of the chlorophyll-binding protein from the thylacoid membrane. The residue is usually extracted exhaustively and filtrates are combined and protected from light to prevent photodegradation and allomerization. In certain plants as well as algae, cell walls must be disrupted by more drastic conditions like grinding by using the mortar-and-pestle technique followed by solvent extraction.¹

Acetone alone or combined with a small quantity of water is the most common extractant of higher plants. A good choice for chlorophyll extraction is aqueous acetone with water reaching up to 20% (v/v), which improves the extractability of the solvent. As water concentration increases, fewer quantities of non-polar chlorophyll derivatives and other lipid-soluble compounds are extracted, and the endogenous enzyme chlorophyllase may be activated, causing the conversion of chlorophylls to chlorophyllides. The fresh weight water contents and gains or losses of moisture should be considered in calculating the amount of water needed to reach the desired final concentration.

Phytylated and dephytylated derivatives can be effectively separated from each other through different solubilities. It is a usual procedure to add petroleum ether to an acetone extract in a separatory funnel, allowing the less polar phytylated esters to migrate to the ether phase while the polar dephytylated derivatives remain in acetone and can be recovered with diethyl ether. Granular anhydrous Na_2SO_4 is added to remove traces of remaining water. The extracts are generally concentrated under vacuum and taken to a known volume of a specific solvent (acetone, ether, methanol, or others) before analysis. The addition of NaHCO_3 , Na_2CO_3 , dimethylaniline, or ammonium hydroxide during grinding is recommended to prevent pheophytinization when acidic tissues are extracted.² Extracts of purified chlorophylls kept in the dark in a freezer under inert atmosphere may remain stable for several months.

Other organic single or mixed polar solvents have also been used to liberate chlorophylls and carotenoids from plant tissues: N,N-dimethylformamide (DMF), dimethyl sulfoxide, ethanol, pyridine, ethyl acetate-hexane, ethanol-petroleum ether, methanol-petroleum ether, acetone-methanol, and acetone-ethyl acetate.² According to Moran and Porath,³ the use of DMF is indicated to extract chlorophylls from plants containing only minute quantities of these pigments and has also been successfully employed to extract chlorophylls from raw materials like olives containing high levels of water lipids (15 to 30%), which interfere with the usual techniques due to the formation of an emulsion.⁴ Extraction with DMF seems to be a good alternative to acetone extraction due to its lower volatility and longer storage time without significant degradation.

Schoefs⁵ reports the particularities of extraction and isolation of pigments from different vegetables and fruits, giving practical advice on a case-by-case basis.

6.1.3 CHROMATOGRAPHIC SEPARATION

High performance liquid chromatography (HPLC) has been by far the most important method for separating chlorophylls. Open column chromatography and thin layer chromatography are still used for clean-up procedures to isolate and separate carotenoids and other lipids from chlorophylls and for preparative applications, but both are losing importance for analytical purposes due to their low resolution and have been replaced by more effective techniques like solid phase, supercritical fluid extraction and counter current chromatography.⁵ The whole analysis should be as brief as possible, since each additional step is a potential source of epimers and allomers.

6.1.3.1 OPEN COLUMN AND THIN LAYER CHROMATOGRAPHY

Because plants present chlorophylls and carotenoids simultaneously, it may be useful to separate both groups from each other in a laboratory or preparative scale in order to avoid contamination in further purification steps, mainly when they are prepared in large amounts. Clean-up procedures using an open column packed with absorbents such as alumina, magnesia, polyethylene powder, powdered sucrose, DEAE-Sepharese, starch, cellulose, or MgO: HyfloSupercel are good approaches.⁶ MgO: HyfloSupercel in a proportion of 1:1 or 1:2 is the usual adsorbent. Sucrose and cellulose are interesting as they do not alter the chlorophylls, but they are tedious to work with.

A very small amount of anhydrous Na_2SO_4 is commonly added on the top of the column to absorb residual water from the sample and then the elution of the compounds is performed by a gradient of solvents with different polarities.

The most common mobile phase is a gradient of petroleum ether or hexane with increasing concentrations of acetone or diethyl ether. Development of the column should be optimized for each sample to afford a quick and effective separation to avoid band broadening. The separation can be followed visually. The most non-polar α - and β -carotenes are eluted first as a yellow band followed by the chlorophylls and other more polar carotenoids like cryptoxanthin, lutein, and zeaxanthin that frequently fuse together and appear as a single band.⁶

Fractions are concentrated under vacuum below 35°C. Alternatively, they can be purified and concentrated by solid phase extraction (SPE) before identification and quantification, which is a valuable procedure for samples containing low amounts of pigments.⁷ After concentration, fractions can be stored in small flasks, protected from light, sealed under inert atmosphere, and kept below -20°C until analysis.

Despite being a fast and relatively low-cost method, thin layer chromatography shows low resolution as it involves a large surface in contact with air, promoting photooxidation. Acidity of silica gels should be neutralized because it may trigger chlorophyll degradation by pheophytinization.⁸

6.1.3.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Standard open column and thin-layer chromatographic methods have been gradually replaced by HPLC, which is faster, more reliable, more accurate and highly reproducible for analyzing plant pigments and for opening new perspectives in chlorophyll

research. Many HPLC systems for analytical or preparative purposes have been described and tested to deal with the multiple separation problems of pigment mixtures containing compounds with wide ranges of polarities.

The first HPLC method for chlorophyll analysis was developed by Evans and coworkers, in 1975⁹ and since then a number of both normal and reversed phase HPLC methods have been employed.

A normal-phase HPLC separation seems to be useful to separate major chlorophyll derivatives, but it is not compatible with samples in water-containing solvents; an additional extraction step is required to eliminate water from the extract since its presence reduces chromatographic resolution and interferes with retention times. Besides that, the analysis cannot be considered quantitative due to the difficulty in transferring compounds from the acetone solution into the ether phase. On the other hand, an advantage of the normal-phase method is its efficacy to separate magnesium-chlorophyll chelates from other metal-chelated chlorophyll derivatives.¹⁰

Due to the hydrophobic character of chlorophylls and most derivatives, C18 (octadecyl silica or ODS)-reversed phase (RP) columns provide superior resolution. In 1981, Schwartz and collaborators,¹¹ successfully separated twelve non-polar (phytylated) chlorophyll derivatives by RP-HPLC and monitored pigment changes during thermal processing of spinach after a simple extraction step (acetone: water) and to the first time pyropheophytins were detected. The pyropheophytins are less polar and, as expected, showed a slight increase of retention time due to the removal of the C-10 carbomethoxy group ($-\text{CO}_2\text{CH}_3$). These derivatives may not have been detected due to the lack of resolution of previous HPLC methods and to the identical spectra of pheophytins and pyropheophytins.

The mobile phase for RP-HPLC is made up by selected organic solvents such as hexane, acetonitrile, heptane, propanol, diethyl ether, ethanol, methanol or acetone. A key-step for improving pigment separation is gradient elution instead of isocratic runs, which provides a good separation of non-polar carotenoids and chlorophylls. Although heating the column is sometimes suggested, it is not recommended because it can promote carotenoid isomerization and chlorophyll epimerization and allomerization which are hardly detected by HPLC methods.¹²

Many other authors, as reviewed extensively by Schwartz and Lorenzo,² and by Eder¹³ improved the C18 RP-HPLC methods that have been largely applied using similar but not exactly identical systems to separate and to quantify complex mixtures of chlorophylls and carotenoids.

Despite the good separation of phytylated chlorophyll derivatives (chlorophylls and pheophytins) and carotenoids by C18 RP-HPLC, the resolution of the dephytylated compounds (chlorophyllides and pheophorbides) has been poor due to adsorption phenomena at the silanol stationary phase. Ion-suppressing or ion-pairing techniques were proposed to suppress the dissociation of both, free hydroxyl groups of the stationary phase and the propanoic acid group at C-17 of the pigments.^{14,15} Subsequently, due to the different hydrophobic interactions with the mobile and the stationary phases, the dephytylated pigments can be separated.

Buffered tetrabutylammonium acetate (final pH 7.1) showed the best results, superior to the more popular phosphate salt, because of its high solubility in aqueous methanol and acetone and its fast reaction with chlorophylls to form hydrophobic

ion-pairs.¹⁵ For analytical purposes, it is important to incorporate the ammonium acetate since it is capable of separating not only chlorophyllides a and b from pheophorbides a and b, but pyrochlorophyllides a and b, pyropheophorbides a and b, 13-OH-chlorophylls, and some other chlorophyll-derived compounds and monopyrroles.¹⁶ Ion-suppressing species such as acetic acid are undesirable due to the acid-labile nature of the chlorophylls¹⁴ but can be used with the more stable metal-chelated chlorophylls and chlorophyllins.¹⁷ These proposed methods have been routinely applied to study post harvest and food processing-based chlorophyll degradation. Pigments are eluted according to their decreasing polarities, starting with the more polar, phytol-lacking derivatives, in this order: chlorophyllide b, chlorophyllide a, pheophorbide b, and pheophorbide a, followed by the apolar derivatives: chlorophyll b, chlorophyll a, pheophytin b, ending with pheophytin a. A typical run is completed in about 30 minutes. All pigments are well separated except the epimers that elute shortly after their corresponding parents.^{15,18}

The disadvantage of using ion pairing reagents, especially quaternary bases, is that they attack no-end, capped surfaces of silica gel and shorten column life. Column stability is an important factor for reproducibility in routine separations. The HPLC system should be frequently cleaned between runs to remove the buffer and avoid crystallization of salts. The column should also be washed with absolute methanol after each analysis to prevent column deterioration by accumulation of lipids from crude extracts. The column can be regenerated by successive washings with water; methanol; chloroform; methanol; water; 0.1M sulphuric acid and ending with water.¹⁴ In recent allomerization studies, HPLC has been the most common method for the separation of the allomers while structural analysis has been based mostly on concomitant UV-Vis, IR, MS, NMR, and surface-enhanced resonance Raman spectroscopy.¹⁹

6.1.4 IDENTIFICATION AND QUANTIFICATION

Several analytical methods are available to quantify chlorophylls and choice depends on the information needed. For quality control in industries and legislation attendance, simple and cost-effective methods represent widely used problem-solving approaches. For research purposes, more sensitive and precise methods are necessary to identify chlorophylls and derivatives simultaneously and individually.

6.1.4.1 QUANTIFICATION OF TOTAL CHLOROPHYLLS BY SPECTROSCOPIC METHODS

Because a chlorophyll molecule contains a closed circuit of ten conjugated double bonds to absorb light, spectrophotometric (UV-Vis) and fluorometric measurements are satisfactory to identify and estimate amounts of chlorophyll a and chlorophyll b, usually the only ones present in fresh plant extracts. The basis of numerous spectrophotometric determinations reported in literature is that chlorophylls strongly absorb at 500 to 700 nm in the visible region and show a large typical band around 400 nm.

Chlorophyll a and chlorophyll b are distinguishable by their typical spectral properties (Figure 6.1.1A). Each one shows a specific absorbance coefficient and E

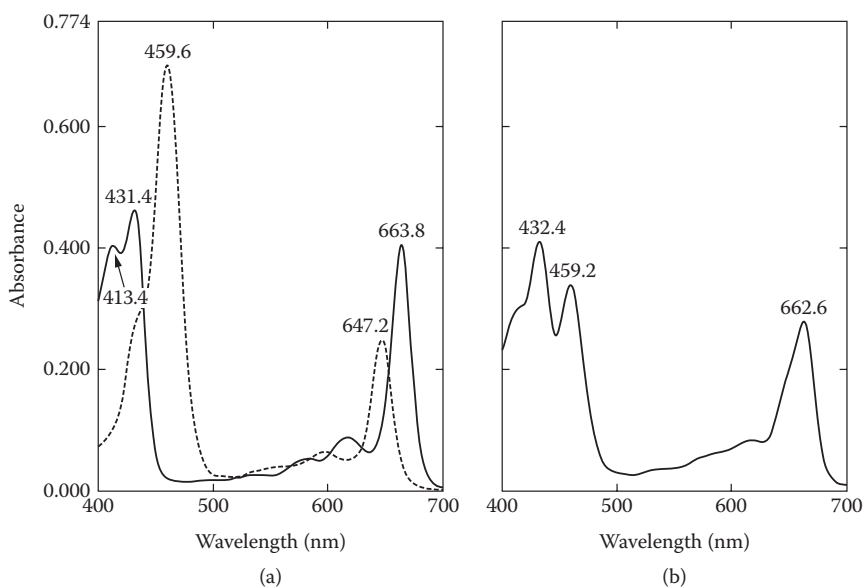


FIGURE 6.1.1 Absorption spectra. (A) Chlorophylls a and b in 80% acetone: chlorophyll a (continuous line) and chlorophyll b (dashed line). (B) Mixture of chlorophylls a and b (1:1) in 80% acetone.

(1 cm) (1%) value at its respective wavelength maximum. However, when they are together in a mixture, as occurs in nature, the typical spectrum is a result of an additive effect (Figure 6.1.1B).

Therefore, the concentration of each pigment can be estimated from the absorption coefficients at the absorption maximum wavelength of the first pigment, corrected by the absorption contribution of the second pigment by creating equations that solve for two unknown pigments.

In 1941, Mackinney²⁰ published the first specific absorption coefficients for chlorophyll a and chlorophyll b in 80% acetone, quickly followed by other reports citing different solvents. Chlorophylls form aggregates in various organic solvent–water mixtures that may interfere strongly with the absorbance maximum wavelength and the shapes of spectra.

Anon²¹ was the first to develop a set of equations for acetone to simultaneously calculate chlorophyll a and chlorophyll b in 1949. Several authors later proposed different new equations based on more adjusted and accurate extinction coefficients due to the development of higher resolution spectrophotometers adapted to each special condition. Moreover, besides 80% acetone, coefficients for diethyl ether and ethanol were also established and their respective equations developed, as reviewed by Schwartz and Lorenzo² and Eder.¹³ Solvents chosen should be those for which specific absorbance coefficients have been published to derive equations and updates should be carefully tracked for new values.

The equations (1 through 3 below) of the official method for total chlorophyll, chlorophyll a, and chlorophyll b analysis of plants published as Method 942.04 by

the Association of Official Analytical Chemists (AOAC)²² are still based on coefficients reported in 1942:

$$\text{Total chlorophyll (mg/l)} = 7.12 \times A_{660.0} + 16.8 \times A_{642.5} \quad [\text{Eq. 1}]$$

$$\text{Chlorophyll a (mg/l)} = 9.93 \times A_{660.0} - 0.777 \times A_{642.5} \quad [\text{Eq. 2}]$$

$$\text{Chlorophyll b (mg/l)} = 17.6 \times A_{642.2} - 2.81 \times A_{660.0} \quad [\text{Eq. 3}]$$

Although these equations are still frequently used, Porra and co-workers in 1989²³ recognized that the absorbance coefficients published by Mackinney²⁰ were low and proposed new values for chlorophyll a ($e_{664 \text{ nm}} = 85.95$; $e_{647 \text{ nm}} = 20.79$) and for chlorophyll b ($e_{664 \text{ nm}} = 10.78$ and $e_{647 \text{ nm}} = 51.84$) in 80% acetone, developing a new set of equations (4 through 6):

$$\text{Total chlorophyll (mg/l)} = 20.2 \times A_{645} + 8.02 \times A_{663} \quad [\text{Eq. 4}]$$

$$\text{Chlorophyll a (mg/l)} = 12.7 \times A_{663} - 2.69 \times A_{645} \quad [\text{Eq. 5}]$$

$$\text{Chlorophyll b (mg/l)} = 22.9 \times A_{645} - 4.68 \times A_{663} \quad [\text{Eq. 6}]$$

As pheophytin a and pheophytin b are the major degradation derivatives formed during extraction, food processing, and storage, some authors recommend converting chlorophylls into the more stable pheophytins by treatment with HCl, ion exchange resin, or oxalic acid to estimate the chlorophyll contents.¹³

Spectroscopic methods are easy, but the results obtained by using different sets of equations are not always comparable. Also, when the ratio of chlorophyll a to chlorophyll b is above 8, the absorption coefficients are affected due to the significant overlap of Q absorption bands of chlorophyll a and chlorophyll b. This fact is especially limiting for determinations in algae in which chlorophyll a is the main component²⁴ and in mutant organisms deficient in chlorophyll b.²⁵ However, the major limitation of spectrophotometric methods is the overlapping of the absorbance bands of individual pigments because of their similarity, especially when a high number of compounds are present in the mixture, giving rise to poor precision and on occasions even “negative” concentrations. Significant differences arise among results obtained by applying different sets of equations since chlorophyll degradation products that sometimes constitute major fractions of green pigments interfere with the results.

Spectrofluorometry presents sensitivity and selectivity greater than the absorbance spectroscopy, being more suitable for chlorophyll estimates in the nmol range and for residual amounts of derivatives in food products.²⁶ Absorbance spectroscopy is satisfactory for concentrations $\geq 1 \mu\text{M}$.²⁷ Spectrofluorometry is also more accurate for a wide range of chlorophyll a-to-chlorophyll b ratios, but it is less accurate when applied to complex sample matrices because of unpredictable quenching effects.

Moreover chlorophyll extract purity can be assessed by examining fluorescence since it is more sensitive than absorbance.

Chlorophylls and their derivatives fluoresce independently from one another and their concentrations can be calculated by equations based on the total fluorescence — the sum of individual fluorescences when together or in mixtures. Equations for 80% acetone, diethyl ether, DMF, methanol and ethanol can be found in literature.^{13,27}

Fluorometry is widely applied, particularly to marine and freshwater samples in which chlorophyll *a* and chlorophyll *c* are predominant and found at very low concentrations.²⁸ Kinetic measurements of chlorophyll fluorescence are also employed to indicate the photosynthetic activity reflecting vegetable freshness. This strategy controls chlorophyll fluorescence decrease during fruit ripening, which helps predict damage at the fruit surface before visible signs appear. Fluorescence imaging has also been used to track pollution and visualize the stress caused on leaf surfaces.⁸

However, since fluorometric methods require sophisticated instrumentation, their applicability is limited because of cost. In conclusion, spectroscopic methods usually enable crude estimates of chlorophylls in an extract, but in most cases accurate and detailed analysis of a specific composition requires separation of the mixture into individual compounds using methods such as HPLC.

6.1.4.2 IDENTIFICATION AND QUANTIFICATION OF INDIVIDUAL COMPONENTS

High performance liquid chromatography enables separation of individual pigments present in an extract. On-line single wavelength or photodiode array (PDA) UV-Vis detectors displaying the complete light absorption spectrum of an eluting peak have become the standard means for analysis. Their high sensitivity enables analysis of very small sample sizes. The crude identification of a known chlorophyll derivative is generally established by comparing the retention time and absorbance spectrum with a pure standard. Nevertheless, the correct identification of a compound based only on the absorbance spectrum of each peak is difficult, mainly when the sample is a complex mixture of many pigments (chlorophylls, carotenoids and their derivatives). Sometimes they may co-elute, causing overlapping spectra in the visible region and not reflecting the identity of each pigment individually. The nature of the chelating metal in the center and the peripheral groups specifically influence the absorption maxima wavelengths and the molar absorptivities of the Q bands of chlorophylls.²⁹

Both chlorophyll *a* and chlorophyllide *a* essentially show the same absorbance spectra — their λ_{max} at the Soret band, but the band is about 430 nm, while chlorophyll *b* and chlorophyllide *b* are shifted to longer wavelengths close to 460 nm. Pheophytinization causes an absorbance maximum shift to shorter wavelengths (λ_{max} ~410 nm) and a strong decrease in sensitivity, but pheophorbide *a* and pheophytin *a* show slightly different spectra.¹⁸ Pyropheophytins and pyropheophorbides present the same spectral properties but differ slightly from their precursors.³⁰ The epimers show identical spectra to those of their parents. Detection becomes even more complicated when degradation reactions (pheophytinization, dephyty-

lation, and pyrolysis) of both chlorophyll a and chlorophyll b occur, increasing the number of compounds significantly.

The red (Q) band around 660 nm can be an alternative wavelength for detecting all derivatives from chlorophyll a, whereas 650 nm is the wavelength for good absorption of derivatives from chlorophyll b (chlorophyllide and pheophorbide). Therefore, multi-wavelength detection is helpful to identify and quantify mixtures of pigments. The combination of photodiode array and fluorescence detection is also useful because fluorescence (excitation at 440 nm and emission at 660 nm) is particularly sensitive and more specific. This makes it the best detection method for pigments present only in small amounts.^{1,8,13}

The quantification of already identified pigments requires external or internal standards to calibrate the signal of the detector. Unfortunately, only a few chlorophyll derivatives are commercially available as standards. Therefore the preparation of the main chlorophyll a and chlorophyll b derivatives from their purified precursors is indispensable for constructing calibration curves, which is costly and time-consuming. Green leaves are extracted with acetone; and chlorophyll a and b are separated and collected in a preparative HPLC system. Pheophytins (a and b), the Mg-free derivatives) can be prepared by controlled acidification.¹¹ Chlorophyllides are prepared from the respective chlorophylls by enzymatic de-esterification with endogenous or purified chlorophyllase. The C-13 epimers of chlorophyll a or chlorophyll b can either be obtained by dissolving the precursors chlorophyll a and chlorophyll b in pyridine and leaving them to react in the dark at room temperature,¹⁵ or can be prepared by treatment with chloroform.³¹

It can be concluded that the chromatographic behavior and the absorption spectra can be enough to identify a chromophore, but not enough to confirm the identities of the individual pigments. For this reason, minimum criteria for identification have been suggested. Absorbance UV-Vis spectra should be obtained in different solvents and their profiles and molar absorbance coefficients should be compared with those of standards. Retention times of putative pigments and respective standards must be identical in thin layer chromatography and HPLC and both compounds should co-elute. Additionally, other sophisticated instrumental methods like mass spectrometry (MS), coupled or not to HPLC, infrared spectroscopy (IR) and capillary electrophoresis can provide information to aid accurate identification. MS detection is ineffective for distinguishing epimeric forms of chlorophylls. Particular advances are expected with the coupled HPLC-MS systems.³²

Due to the high mass, low volatility, and thermal instability of chlorophylls and derivatives, molecular weight determination by electron impact (EI) MS is not recommended. Desorption-ionization MS techniques such as chemical ionization, secondary ion MS, fast-atom bombardment (FAB), field, plasma- and matrix-assisted laser desorption have been very effective for molecular ion detection in the characterization of tetrapyrroles. These techniques do not require sample vaporization prior to ionization and they are effective tools for allomerization studies.^{19,33}

The most recent progress in MS analysis of chlorophylls has been obtained with the development of atmospheric ionization methods such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). These techniques have demonstrated much more sensitivity than thermospray ionization, detecting chloro-

phylls and derivatives at low nanogram amounts.³³ Other spectroscopic properties of chlorophylls, derivatives, specific functional groups, and molecular associations obtained by NMR, IR, and circular dichroism (CD) have also been helpful in elucidating the structures of the compounds.²

6.1.5 ANALYSIS OF COLORLESS CHLOROPHYLL CATABOLITES

Chlorophyll breakdown during plant senescence, a largely unknown process, has been studied over the last 20 years, revealing the complexity of the whole process. The multi-step catabolism pathway that occurs continuously, mainly in senescent plants and during fruit ripening, can be divided into at least two main stages. While the greenish catabolites resulting from the early stage of degradation are characterized by intact porphyrin rings, their intermediates are further degraded by the oxidative cleavage of the porphyrin macrocycle to colorless derivatives in the second stage.³⁴

Thus, the second stage has been recognized as essential in the degreening process and studies on the biochemical pathways and the structures of formed catabolites have given a small insight into the degreening mechanism regulation that is important for abolishing the photodynamic properties of chlorophylls due to the oxidative opening of the pheophorbide macrocycle. Research in this area also enables us to understand the role of the genes that seem to encode proteins involved in regulation of chlorophyll degradation at the molecular level.

Stay-green mutants have been useful models for elucidating the degreening mechanism and the structure–function relationship.^{34,35} The knowledge of the mechanisms of degradation and related processes may be potentially useful in industry, agriculture, and horticulture, creating a scientific basis for the development of procedures to control both retardation and acceleration of chlorophyll degradation before the natural onset of senescence. The intent is to overcome the limited and inconstant supplies of green pigments from natural plant sources.

Based on the observation that all catabolites separated on RP-HPLC fall into fluorescent and non-fluorescent categories, Ginsburg and Matile³⁶ proposed the following terminology: NCC to indicate non-fluorescent chlorophyll catabolytes and FCC for fluorescent chlorophyll catabolites. A prefix identifies the origin of the plant species, e.g., *Hv* (*Hordeum vulgare*) or *Bn* (*Brassica napus*). The catabolites are numbered starting from 1 for the first substance eluted, indicating the highest polarity. Therefore, a higher number indicates a lower polarity and vice versa. The five NCCs found in spinach (*Spinacia oleracea*) are designated *So*-NCC 1, *So*-NCC 2, *So*-NCC 3, *So*-NCC 4, and *So*-NCC 5.

Chlorophyll catabolism has been intensively studied in some plants, e.g., rapeseed, barley, spinach, tobacco, *Cercidiphyllum japonicum*, *Lolium temulentum*, *Liquidambar styraciflua* and *Arabidopsis thaliana*, which present all NCC catabolites with similar basic structures.³⁴ This suggests a uniform breakdown of chlorophyll in which the oxidative opening of pheophorbide seems to be a key step. Structural differences among the compounds have been related to at least six basic types of peripheral transformations. Some of them seem to operate either in sequence or in parallel, depending on the plant species, which caused the appearances of different

structures of NCCs. Experimental evidence suggests an accumulation of the NCCs in cell vacuoles of different senescent higher plants since the amount of NCCs found corresponded to the initial amount of chlorophyll and did not decrease strongly over several days.³⁷ Among the colorless compounds, the NCCs are the most documented to date because they are considered the final products of chlorophyll catabolism. Both RCCs and FCCs are detectable only in minute quantities and are rapidly catabolyzed further to colorless NCCs.³⁸

However, this accumulation has not been unequivocally proven. The recent identification of urobilinogenoidic linear tetrapyrroles in extracts from primary leaves of barley indicated that further degradation of the *Hv*-NCC 1 can take place.³⁹ While the monooxygenation of pheophorbide a in the earlier phases of chlorophyll breakdown in higher plants appears to be a remarkably stringent entry point, the rather diverse structures of NCCs may indicate that the later phases of the detoxification process follow less strictly regulated pathways.⁴⁰

The development and reports of methods for colorless chlorophyll derivative (RCCs, FCCs, and NCCs) analysis are relatively recent and the structures of the compounds are being elucidated by deduction from their chromatographic behaviors, spectral characteristics (UV-Vis absorbance spectra), mass spectrometry, and nuclear magnetic resonance analysis. The main obstacle is that these compounds do not accumulate in appreciable quantities *in situ* and, moreover, there are no standards for them. The determination of the enzymatic activities of red chlorophyll catabolite reductase (RCCR) and pheophorbide a monooxygenase (PAO) also helps to monitor the appearance of colorless derivatives since they are the key enzymes responsible for the loss of green color.^{35,41}

The extraction and detection methods for analyzing colorless chlorophyll catabolites are different from those used to analyze intact chlorophylls and greenish derivatives. The colorless compounds are derived from the dephytylated pheophorbide and therefore present a polar and hydrophilic character. In senescent leaves, all degreened chlorophyll catabolites have a methyl group at C-7 like that in the chlorophyll a molecule, which demonstrates that these catabolytes derive from chlorophyll a.³⁴ In the green alga *Chlorella protothecoides*,⁴² unlike in plants, NCCs that carry an aldehyde group more related to chlorophyll b have been found.

Despite the difficulties in extracting and identifying colorless catabolic products that are extremely labile and detectable only in trace amounts, several of the mysteries of chlorophyll catabolism have been revealed and about 14 non-fluorescent chlorophyll catabolytes (NCCs) from higher plants, mainly in senescent leaves, have been detected and analyzed structurally. Among them, NCCs from rapeseed (*Brassica napus*),⁴³ from *Liquidambar styraciflua*, from *Cercidiphyllum japonicum*, five NCCs from degreened leaves of spinach (*Spinacia oleracea*) and, more recently, two NCCs from tobacco (*Nicotiana rustica*)⁴⁰ and five NCCs from *Arabidopsis thaliana*⁴⁴ have been identified.

The extraction of colorless catabolites from samples is usually performed by homogenizing the ground tissue in 20 to 100 mM K₃PO₄ buffer (pH 7.0) and methanol (1:1, v/v)^{36,38} or 0.1 M Tris-HCl, pH 8.0 and methanol (1:4 v/v)⁴⁴ followed by centrifugation and analysis by RP-HPLC, either directly or after concentration on a C18 SepPak cartridge. A reversed phase system with a C18 Hypersil ODS

column and elution with methanol, K-phosphate buffer at pH 7.0, and water was successfully employed.^{38,40} Detection may be performed by photodiode array detector (200 to 600 nm) or fluorescence detector (excitation at 320 nm and emission at 450 nm).³⁴

Peaks are analyzed separately by their retention times, absorption, and fluorescence properties. RCCs show absorbance maxima near λ 500 and 316 nm. For FCCs, UV-Vis spectra show two prominent bands near 361 and 320 nm and a luminescence maximum at 436 nm and NCCs show UV-Vis spectra with absorbance maxima near 320 and 210 nm. Nevertheless, as none of these approaches is suitable for elucidating structures, it is necessary to apply additional MS and NMR analyses to fully characterize structural features. Electron spray ionization (ESI) and high-resolution FAB mass spectroscopy have been applied to elucidate the molecular formulae of colorless compounds.^{38,44}

6.1.6 COLOR ANALYSIS

All the analytical methods mentioned to separate, identify, and quantify chlorophylls and derivatives consume time, money, and samples. As alternatives, industries have been employing non-destructive methods for surface color measurements that are not only indirectly related to chlorophyll content, but may also estimate the pigments directly in tissues, leaving the sample intact and enabling serial analyses in a relatively short time. Food color affects consumer acceptance and is an important criterion for quality control. Color vision is a complex phenomenon that depends on both the total content and number of pigments and also on absorption, reflectance and emission spectra of each compound present.

The methods measuring surface color to estimate chlorophyll contents are particularly useful for classifying the maturity of fruits and vegetables and monitoring pigment modifications during food processing. A combination of visible and near-infrared spectroscopy (400 to 1100 nm) has been used to estimate chlorophyll contents in ripening fruits, vegetables, and seeds.^{45–47}

Three attributes characterize color: hue, lightness (or value), and saturation (or chroma) and they are graphically represented in color solids (e.g., Munsell solid, Hunter solid). The Munsell Color Notation is a rapid, portable, widespread, and economical system of color determination. However, as it depends on sensory evaluation by panels, many laboratories prefer when possible to replace human judgment by instrumental techniques that are easier to handle. The CIELAB established by the Commission International d'Eclairage (CIE) has become widely used with the availability of reflectance spectrophotometric instrumentation.

Correlation studies between the total chlorophyll content obtained by analytical methods and quantitative color evaluations (instrumental and visual) have presented significant correlation coefficients, showing that colorimetric methods are suitable for evaluating color characteristics and pigment contents of fresh and processed foods and also for monitoring chlorophyll changes during development of fruits, vegetables, and seeds.^{48,49} Several mathematical models have been proposed to predict total chlorophyll content and pigment modifications by color evaluation, but as modifications of the chlorophyll molecule do not always present changes in color,

the results vary from one sample to another and this approach must be considered case by case.

6.1.7 ANALYSIS OF SYNTHETIC CHLOROPHYLL-BASED FOOD COLORANTS

6.1.7.1 QUANTITATIVE PROCEDURES FOR FOOD CONTROL

The replacement of magnesium at the center of the natural chlorophyll or chlorophyllin molecule with a copper ion produces bright green complexes, commercially available as food colorants and dietary food supplements. Sodium copper chlorophyllin salts, the most notable among these preparations, are mixtures of water-soluble compounds. Trisodium copper chlorin e6 ($C_{31}H_{31}N_4Cu(COONa)_3$) and disodium copper chlorin e4 ($C_{31}H_{32}N_4Cu(COONa)_2$) are the main components reported. Select commercial preparations show compositions that vary depending on the manufacturing conditions and this complicates the determinations of identity, purity, and concentration. Despite their importance, the U.S. Food and Drug Administration has not approved an analytical method for monitoring the quality of these preparations.

In contrast to the well-established methods for identifying and quantifying naturally occurring chlorophylls, very few reports concern quantitative analysis of chlorophyllin copper complexes in color additives and in foodstuffs.⁵⁰ Analytical methods proposed are based on spectral properties, elemental analysis, chromatographic separation, and molecular structure elucidation or a combination of these procedures.

Comparative studies of the widely employed spectrophotometric readings at the Soret and Q bands (405 and 630 nm, respectively) and the elemental analysis of copper and nitrogen showed that the spectrophotometric assay based only on the Soret band can overestimate the purity of a preparation.⁵¹ Erroneous data were attributed to an increase in absorptivity at the Soret band when other colored compounds like metal-free analogs and carotenoids are present. Indeed, copper-free chlorin e6 exhibits a specific absorbance 3.6 times greater than that of its coppered counterpart. Therefore, measurements at the Q band (630 nm) and the establishment of the S:Q ratio are preferred.

Sodium copper chlorophyllin, approved by the FDA as a color additive in citrus-based dry beverage mixes, should have a ratio of absorbance (Soret:Q band) not less than 3.4 and not more than 3.9.⁵² In Europe, purity criteria of the food additives E141[i] and E141[ii], which are copper complexes of chlorophyll and chlorophyllin, respectively, are set out in the EC color specifications that include identification and spectrophotometric assay tests.⁵³

Purity levels of commercial preparations have also been estimated based on their Cu contents and compared with the theoretical values expected for fully coppered chlorophyllin based on the two major compounds: Cu(II) chlorin e4 (disodium salt) and Cu(II) chlorin e6 (trisodium salt). The expected theoretical content of copper in a pure Cu chlorophyllin complex is 9.2%, which has never been found in commercial preparations. The sodium copper chlorophyllin from Sigma-Aldrich (St. Louis, MO) has a 4.5% copper content, specified by the manufacturer with respect

to the main component Cu(II) chlorin e4 (MW = 664.3) and a calculated purity of 47.8%, which has been adopted by some researchers to calculate effective concentrations of chlorophyllins for biological studies.⁵⁴ Other commercial preparations have shown lower amounts, 2.1 to 3.3 % copper, and large discrepancies were observed among batches of the same preparation, which may indicate that some components lack the metal.⁵¹

The Cosmetic, Toiletry, and Fragrance Association⁵⁵ reported a usual range of 4 to 6% for the copper content; this value was later accepted and adopted by FDA in 1991⁵² The value corresponds to a purity of only 43.6 to 65.2%, implying significant presences of other unknown compounds such as sodium salts and contaminants from different raw materials used. As the variance in composition can alter the biological properties of these pigments, eventual side effects in biological studies should be considered.⁵⁶

Analysis of nitrogen contents could be an aid for estimating the chlorophyllin concentration, complementing the copper analysis. The Cu:N ratio of 1.1 calculated based on a Cu content of 9.2% and N content of 8.1% must be re-evaluated because both Cu and N levels found in commercial preparations are significantly lower than theoretical values.

6.1.7.2 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Similarly to the methods used to characterize natural chlorophylls, RP-HPLC has been chosen by several authors to identify the individual components in Cu chlorophyllin preparations and in foods. The same ODS columns, mobile phase and ion pairing or ion suppressing techniques coupled to online photodiode UV-Vis and/or fluorescence detectors have been used.^{50,56,57}

Usually, HPLC analysis resolves four peaks identified by co-chromatography with authentic standards as copper pheophorbide a, Cu(II) chlorin e6, Cu(II) chlorin e4, Cu rhodin g7, and their degradation products, but a sum of other colored components can also be found, for example, native chlorophylls, pheophytins, pheophorbides, and rodochlorins (free carboxyl forms of pheophorbides) besides epimers, allomers, and degradation products that have been only tentatively identified.

HPLC analysis of several batches of the same commercial sample revealed significant differences in composition and some unidentified chlorins and non-chlorin types, some lacking copper, were found.⁵⁶ Cupro analogues may be discriminated from the native substances by their different retention times and spectral characteristics. The tandem UV-Vis and fluorescence detection was found to be an important diagnostic feature since the fluorescence stands for non-coppered molecules.⁵⁰ The analysis of E141-containing foods and color formulations revealed a complex chromatogram, hardly interpreted, probably due to the various extraction and purification steps that caused interferences when applied to different food matrices. A continual background absorbance was also evidenced near the Soret band area as well as a tendency to co-elute with chemically similar compounds. In conclusion, the exceptional difficulty in extracting chlorophyllins from food matrices rich in fats, emulsifiers, and gelatin requires further development of extraction methods to assess the levels of these additives in foods.⁵⁰

REFERENCES

1. Ergun, E. et al., Simultaneous determination of chlorophyll a and chlorophyll b by derivative spectrophotometry, *Anal. Bioanal. Chem.*, 379, 803, 2004.
2. Schwartz, S.J. and Lorenzo, T.V., Chlorophylls in foods, *Crit. Rev. Food Sci. Nutr.*, 29, 1, 1990.
3. Moran, R. and Porath, D., Chlorophyll determination in intact tissues using *N,N*-dimethylformamide, *Plant Physiol.*, 65, 478, 1980.
4. Roca, M. and Mínguez-Mosquera, M.I., Involvement of chlorophyllase in chlorophyll metabolism in olive varieties with high and low chlorophyll content, *Physiol. Plant.*, 117, 459, 2003.
5. Schoefs, B., Chlorophyll and carotenoid analysis in food products. A practical case-by-case view, *Trends Anal. Chem.*, 22, 335, 2003.
6. Gross, J., Chlorophylls, in *Pigments in Vegetables: Chlorophylls and Carotenoids*, Gross, J., Ed., Van Nostrand Reinhold, New York, 1991, 3.
7. Hornero-Mendez, D., Gandul-Rojas, B., and Mínguez-Mosquera, M.I., Routine and sensitive SPE-HPLC method for quantitative determination of pheophytin a and pyropheophytin a in olive oil, *Food Int.*, 38, 1067, 2005.
8. Schoefs, B., Plant pigments: properties, analysis, degradation, *Adv. Food Nutr. Res.*, 49, 42, 2005.
9. Evans, N. et al., Applications of high-pressure liquid chromatography and field desorption mass spectrometry in studies of natural porphyrins and chlorophyll derivatives, *J. Chromatogr.*, 115, 325, 1975.
10. Canjura, F.L. and Schwartz, S.J., Separation of chlorophyll compounds and their polar derivatives by high-performance liquid chromatography, *J. Agric. Food Chem.*, 39, 1102, 1991.
11. Schwartz, S.J., Woo, S.L., and Von Elbe, J.H., High-performance liquid chromatography of chlorophylls and their derivatives in fresh and processed spinach, *J. Agric. Food Chem.*, 29, 533, 1981.
12. Vanheukelem, L. et al., Improved separations of phytoplankton pigment using temperature-controlled high-performance liquid chromatography, *Mar. Ecol. Prog. Ser.*, 14, 303, 1994.
13. Eder, R., Pigments, in *Handbook of Food Analysis*, 2nd ed., vol. 1, Nollet, L.M.L., Ed., Marcel Dekker, Inc., New York, 2005, chap. 23.
14. Shioi, Y., Doi, M., and Sasa, T., Separation of non-esterified chlorophylls by ion-suppression high-performance liquid chromatography, *J. Chromatogr.*, 298, 141, 1984.
15. Mangos, T.J. and Berger, R.G., Determination of major chlorophyll degradation products, *Z. Lebensm. Unters. Forsch. A*, 204, 345, 1997.
16. Gandul-Rojas, B., Roca, M., and Mínguez-Mosquera, M.I., Chlorophyll and carotenoid degradation mediated by thylacoid-associated peroxidative activity in olives (*Olea europaea*) cv. Hojiblanca, *J. Plant. Physiol.*, 161, 499, 2004.
17. Inoue, H. et al., Determination of copper(II) chlorophyllin by reversed-phase high-performance liquid chromatography, *J. Chromatogr. A*, 679, 99, 1994.
18. Sinnecker, P. et al., Mechanisms of soybean (*Glycine max* L. Merrill) degreening related to maturity stage and postharvest drying temperature, *Postharvest Biol. Technol.*, 38, 369, 2005.
19. Hyvärinen, K. and Hynninen, P.H., Liquid chromatographic separation and mass spectrometric identification of chlorophyll b allomers, *J. Chromatogr. A*, 837, 107, 1999.

20. Mackinney, G., Absorption of light by chlorophyll solutions, *J. Biol. Chem.*, 140, 315, 1941.
21. Arnon, D.E., Copper enzymes in isolated chloroplast. Polyphenoloxidase in *Beta vulgaris*, *Plant Physiol.*, 24, 1, 1949.
22. *Official Methods of Analysis of AOAC International*, 16th ed., Vol. 1, Cunniff, P., Ed., Association of Official Analytical Chemists, Arlington, VA, 1995, chap. 3.
23. Porra, R.J., Thompson, W.A., and Kriedemann, P.E., Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls a and b extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectroscopy, *Biochim. Biophys. Acta*, 975, 384, 1989.
24. Kouril, R. et al., On the limits of applicability of spectrophotometric and spectrofluorimetric methods for the determination of chlorophyll a/b ratio, *Photosynth. Res.*, 62, 107, 1999.
25. Chunaev, A.S. et al., Chlorophyll b and luteoxanthin-deficient mutants of *Chlamydomonas reinhardtii*, *Photosynthetica*, 25, 291, 1991.
26. Usuki, R. et al., Residual amounts of chlorophylls and pheophytins in refined edible oils, *J. Am. Oil Chem. Soc.*, 61, 785, 1984.
27. Porra, R.J., The chequered history of the development and use of simultaneous equations for the accurate determination of chlorophylls a and b, *Photosynth. Res.*, 73, 149, 2002.
28. Mantoura, R.F.C. and Llewellyn, C.A., The rapid determination of algal chlorophyll and carotenoid pigments and their breakdown products in natural waters by reversed-phase high-performance liquid chromatography, *Anal. Chim. Acta*, 151, 297, 1983.
29. Nonomura, Y. et al., Spectroscopic properties of chlorophylls and their derivatives. Influence of molecular structure on the electronic state, *Chem. Phys.*, 220, 155, 1997.
30. Buskov, S., Sørensen, H., and Sørensen, S., Separation of chlorophylls and their degradation products using packed column supercritical fluid chromatography (SFC), *J. High Resol. Chromatogr.*, 22, 339, 1999.
31. Watanabe, T. et al., Preparation of chlorophylls and pheophytins by isocratic liquid chromatography, *Anal. Chem.*, 56, 251, 1984.
32. Gauthier-Jaques, A. et al., Improved method to track chlorophyll degradation, *J. Agric. Food Chem.*, 49, 1117, 2001.
33. Schoefs, B., Chlorophyll and carotenoid analysis in food products. Properties of the pigments and methods of analysis, *Trends Food Sci. Technol.*, 13, 361, 2002.
34. Kräutler, B., Chlorophyll breakdown and chlorophyll catabolites, in *The porphyrin Handbook*, vol. 13, Kadish, K.M., Smith, K.M., Guillard, R., Eds., Elsevier Science, Amsterdam, 2003, 183.
35. Roca, M. and Mínguez-Mosquera, M.I., Chlorophyll catabolism pathway in fruits of *Capsicum annuum* (L.): stay-green versus red fruits, *J. Agric. Food Chem.*, 54, 4035, 2006.
36. Ginsburg, S. and Matile, P., Identification of catabolites of chlorophyll-porphyrin in senescent rape cotyledons, *Plant. Physiol.*, 102, 521, 1993.
37. Mühlecker, W. and Kräutler, B., Breakdown of chlorophyll: constitution of nonfluorescing chlorophyll-catabolites from senescent cotyledons of the dicot rape, *Plant Physiol. Biochem.*, 34, 61, 1996.
38. Oberhuber, M. et al., Chlorophyll breakdown — on a nonfluorescent chlorophyll catabolite from spinach, *Helv. Chim. Acta*, 84, 2615, 2001.
39. Losey, F.G. and Engel, N., Isolation and characterization of a urobilinogenoidic chlorophyll catabolite from *Hordeum vulgare* L., *J. Biol. Chem.*, 276, 8643, 2001.

40. Berghold, J. et al., Chlorophyll breakdown in tobacco: on the structure of two non-fluorescent chlorophyll catabolites, *Chemistry & Biodiversity*, 1, 657, 2004.
41. Hörtensteiner, S. et al., Evolution of chlorophyll degradation: the significance of RCC reductase, *Plant Biol.*, 2, 63, 2000.
42. Engel, N., Curty, C., and Gassauer, A., Chlorophyll catabolism in *Chlorella protothecoides*. 8. Facts and artefacts, *Plant Physiol. Biochem.*, 34, 77, 1996.
43. Hörtensteiner, S. and Kräutler, B., Chlorophyll breakdown in oilseed rape, *Photosynth. Res.*, 64, 137, 2000.
44. Pruzinská, A. et al., Chlorophyll breakdown in senescent Arabidopsis leaves. Characterization of chlorophyll catabolites and of chlorophyll catabolic enzymes involved in the degreening reaction, *Plant Physiol.*, 139, 52, 2005.
45. Mínguez-Mosquera, M.I. et al., Color-pigment correlation in virgin olive oil, *J. Am. Oil Chem. Soc.*, 68, 332, 1991.
46. Thompson, T.E., Grauke, L.J., and Young, E., Pecan kernel color: standards using the Munsell color notation system, *J. Am. Soc. Hort. Sci.*, 121, 548, 1996.
47. Tkachuk, R. et al., Determination of chlorophyll in ground rapeseed using a modified near infrared reflectance spectrophotometer, *J. Am. Oil Chem. Soc.*, 65, 381, 1988.
48. Berset, C. and Caniaux, P., Relationship between color evaluation and chlorophyllian pigment content in dried parsley leaves, *J. Food Sci.*, 48, 1854, 1983.
49. Sinnecker, P. et al., Relationship between color (instrumental and visual) and chlorophyll contents in soybean seeds during ripening, *J. Agric. Food Chem.*, 50, 3961, 2002.
50. Scotter, M.J., Castle, C., Roberts, D., Method development and HPLC analysis of retail foods and beverages for copper chlorophyll (E141[i]) and chlorophyllin (E141[ii]) food colouring materials, *Food Additives and Contaminants*, 22, 1163, 2005.
51. Chernomorsky, S., Quantitative procedure for chlorophyllin copper complex. Technical Communications, *J. AOAC Internat.*, 77, 765, 1994.
52. Code of Federal Regulations, Title 21, part 73.1125, U.S. Government Printing Office, Washington, D.C., 1991, 287.
53. EC 1995, Commission Directive 95/15/EC, of July, 1995 laying down specific criteria of purity concerning colours for use in foodstuffs. Official Journal of the European Communities, L226 of September 22, 1995, pp. 1–44.
54. Ferruzzi, M.G. and Schwartz, S.J., Thermal degradation of commercial grade sodium copper chlorophyllin, *J. Agric. Food Chem.*, 53, 7098, 2005.
55. CTFA Compendium of Cosmetic Ingredient Composition, Descriptions 1, Nikitakis, J.M. and McEwen, G.N., Eds., The Cosmetic Toiletry and Fragrance Association, Washington, D.C., 1990.
56. Chernomorsky, S. et al., Antimutagenicity, cytotoxicity and composition of chlorophyllin copper complex, *Cancer Lett.*, 120, 141, 1997.
57. Ferruzzi, M.G., Failla, M.L., and Schwartz, S.J., Sodium copper chlorophyllin: in vitro digestive stability and accumulation by Caco-2 human intestinal cells, *J. Agric. Food Chem.*, 50, 2173, 2002.

6.2 Analysis of Carotenoids

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6.2.1 INTRODUCTION

A large number of carotenoids, approximately 120, with different polarities can be found in foods¹ (Figure 6.2.1). Moreover, carotenoid concentrations in foods show a large range, generally with up to four main carotenoids at higher concentrations, along with several other carotenoids in minor or even trace amounts. These facts led to a need for chromatographic separation optimization for specific samples but required laborious quantification and generated calibration curves with large ranges of concentrations. The identification of these several minor carotenoids is also a challenging and difficult task.

In order to obtain reliable results, three steps are involved in the analysis: sampling and sample preparation; carotenoid extraction, separation, identification,

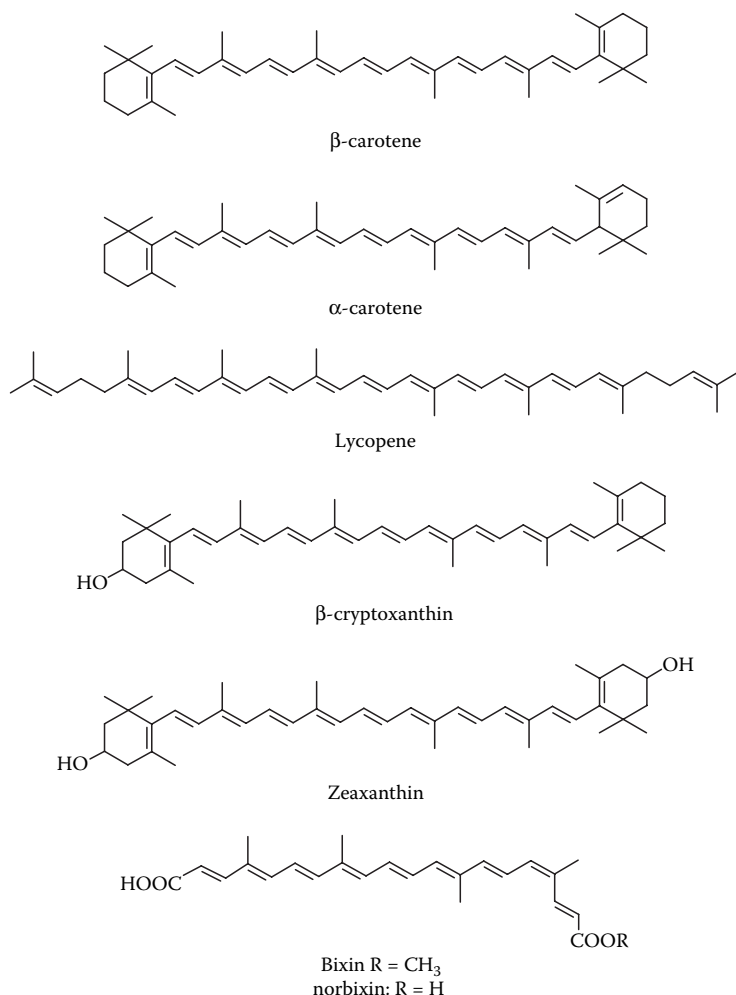


FIGURE 6.2.1 Structures of carotenoids found in foods.

and quantification; and validation of the chosen method. Another point is analytic quality control related to the day-to-day accuracy and precision of the analysis. While precision is usually evaluated by calculating coefficients of variation, known also as relative standards deviation, of several replicates of the same sample, accuracy is monitored by routine analysis of a certified standard reference material or a reference material developed in-house.

The National Institute of Standards and Technology (NIST) released certified standard materials of a baby food composite (SRM 2383) and an infant formula (SRM 1846) containing carotenoids; however, the relative uncertainties of certified values are considerably high, ranging from 20% for β -carotene (*cis* + *trans*) to 28% for lutein (including esters) and to a 47% for free lutein reference value in SRM 2383.²

An alternative for evaluating accuracy is spiking known amounts of standards to a food, as reported in several papers,³⁻⁶ although percent recoveries of spikes do not truly address the influence of the food matrix complexity on the extraction efficiency. Data evaluation procedures were developed as a manual system to assess the quality of analytical data for carotenoids in foods.⁷

A more difficult problem to overcome is the overestimation of carotenoid concentrations in processed foods due to the usually more efficient extraction of carotenoids in such foods as a result of the denaturation of the carotenoid-protein complexes and cell damage. In addition, weight changes due to loss or gain of water or fat, enzymatic oxidation of carotenoids in raw samples, and leaching of soluble solids during processing should be considered.

Independently of the objective of the study, full descriptions of the food sample units such as noting geographic region and period of collection, whether the samples were analyzed in a fresh state or preserved (frozen, dried, canned, cooked), and stating cultivars or breeds, sizes and weights, number of batches (including weight, number of vegetables or processed units), number of brands, and information on the ripe stage should be included.

6.2.2 PRECAUTIONS DURING ANALYSIS

The characteristic system of conjugated double bonds in the carotenoid molecule in which the π electrons are delocalized over the whole polyene chain is responsible for both absorption of visible light and chemical reactivity, resulting in unstable and easily destroyed compounds. Based on this information, the following precautions must be taken during analysis:

1. All operations should be carried out under diffuse light; equipment and glassware should be covered by a black cloth or aluminium foil.
2. Atmosphere should be inert; air should be replaced by a vacuum or inert gas such as nitrogen (N_2) or argon (Ar).
3. High temperature must be avoided; temperatures below 35°C should be used for evaporation of a large amount of solvent in a rotary evaporator or evaporation of small volumes directly under an N_2 or Ar stream.
4. Avoid acid in ambient air where carotenoids are handled.
5. Samples should be stored at very low temperature under inert atmosphere.
6. The analysis should be carried out in the shortest possible time.

When the aim is isolation for identification by direct probe insertion mass spectrometry (MS), plastic materials, filter papers, and blenders should be avoided to prevent contamination during extraction and chromatography. It is also very important to avoid the *cis-trans* isomerization of carotenoids in solution, which is accelerated by heat, light, acids, and active surfaces.⁸ Therefore, a pure carotenoid or even a crude extract should never be stored in solution; it should be kept completely dry in an inert atmosphere at low temperature.

6.2.3 PRE-CHROMATOGRAPHIC STEPS

A representative sampling that reflects a food sample should be planned. In the case of analysis for the Food Composition Database, nationwide composite samples should be prepared. Consideration should be given, among other factors, to the major regions of the country, the different brands of processed foods available, and consumption in industrial and rural areas.⁹

The sampling, subsampling, and sample preparation followed in our laboratory depend on the purpose of the analysis and on the food under investigation. Several units are taken at random from a market or plantation, generally about 2 kg of fresh food and three to five packs of a single lot of processed food. At the laboratory, inedible parts are removed and samples are prepared as follows. Small fruits and vegetables are homogenized in a blender and small portions are rapidly weighed. Big fruits and vegetables are quartered longitudinally and opposite sections are combined and homogenized. Leafy vegetables are cut into pieces before homogenization. In all cases, the extraction is carried out immediately after homogenization.

Detailed information about sampling should be reported. Among details to be noted are size, number, and locale of sample collection; procedure and equipment used for reduction of sample size; and whether homogenized subsamples will be joined to form one composed sample or analyzed separately. It is important to maintain the integrity of the food matrix because unwanted degradation and reactions catalyzed by enzymes and acids may occur in damaged tissues after harvesting or during sample homogenization. In case it is not possible to carry out the fresh food analysis immediately, freezing or freeze drying processes may be used to preserve the food until analysis can be performed.

6.2.3.1 EXTRACTION

The choice of the best solvent for extraction depends on the type of sample and carotenoid composition. Since most carotenoids found in foods are hydrophobic compounds, water-miscible organic solvents are generally employed for extraction of fresh or processed foods. The most used is acetone,^{3,10–13} although other solvents are also employed, e.g., tetrahydrofuran (THF) for green vegetables¹⁴ and persimmons,¹⁵ THF and methanol (MeOH)¹⁶ or MeOH for frozen orange juice,¹⁷ and MeOH with ethyl acetate (EtOAc) and petroleum ether (1:1:1) for orange juice.¹⁸ Conversely, carotenoid glycosides and glycosyl esters found in saffron and gardenia are soluble in water; thus the extraction requires a more polar solvent such as H₂O,¹⁹ ethanol (EtOH),²⁰ EtOH with H₂O (1:1),²¹ and MeOH alone or in combination with acetone.^{21,22}

Dried or freeze dried samples can be extracted with water-immiscible solvents such as EtOAc or diethyl ether.²³ For quantitative extraction, dried samples are preferably rehydrated at different times; for example, 5 to 10 min for dried mangoes,²⁴ 30 min for lyophilized red peppers²⁵ and pasta.²⁶ Rehydration is followed by extraction with acetone^{25,26} or MeOH.²⁴ Bixin and norbixin from a mix dry powder of annatto and corn can quantitatively be extracted with MeOH followed by acetone.²⁷ In order to improve pigment recovery, extruded foods require pre-digestion with enzymes to liberate the pigment from the matrix.²⁸

The addition of celite or HyfloSupercel to increase the contact surface with the extraction solvent and help filtration is often utilized during extraction.^{3,10-12} In case of interaction of such filter aids with the food, treated sand can be employed instead. Weak bases such as NaHCO_3 , MgCO_3 , or CaCO_3 (1 g/10 g sample) to neutralize acids liberated from tissues¹⁶ and antioxidants (0.1% BHT, 5% pyrogallol, ascorbic acid, and sodium ascorbate) to avoid oxidation can be added during extraction.²⁹

For qualitative extraction, a large amount of food is often used and there is no concern about loss of material. If the final objective is, for example, the production of a carotene standard, it is very useful to first extract the sample with a very polar solvent (e.g., MeOH or EtOH) that will remove water and partially remove the xanthophylls. The alcohol is discarded and the carotenes are then extracted with a suitable less polar solvent.³⁰

On the other hand, quantitative extraction requires complete and exhaustive extraction and no material can be lost. To assure complete extraction when a food is analyzed for the first time in a laboratory, it is useful to carry out two or three extractions, pool the solvents, and keep separate the next extracts to verify the presence of carotenoids. Usually four to six extractions are enough to remove the carotenoids completely from a sample. The extraction can be carried out in a blender, vortex, or with a mortar and pestle. Accelerated solvent extraction (ASE), an important extraction technique in residue analysis, currently attracts interest due to its short duration, low level of solvent use, and high extraction yield. The average recoveries for all carotenoids with the exception of norbixin ranged from 88.7 to 103.3% using manual extraction and from 91.0 to 99.6% by ASE (70 bar and temperature of 40°C); both extractions were carried out with a mixture of MeOH, EtOAc, and petroleum ether (1:1:1).⁴

The carotenoid extract obtained by extraction of fresh food with a water-soluble solvent contains large amounts of water from the sample. In order to remove the water and solvent, in the case of acetone, carotenoids are transferred to petroleum ether, diethyl ether, or a mixture by adding small portions of the solvent extract and a large amount of water in a separatory funnel. The remaining traces of water can be removed either by addition of anhydrous Na_2SO_4 or ethanol to form the azeotropic mixture.

Another option is supercritical extraction (SPE), which is frequently carried out with carbon dioxide (CO_2) because of its practical advantages: it is non-toxic and non-flammable, environmentally safe, and readily available. However, SFE is a very expensive technology. Operation conditions using CO_2 vary at temperatures between 25 and 60°C over a pressure range of 100 to 400 bar.³¹⁻³³ Under these condition ranges, CO_2 behaves as a non-polar and lipophilic solvent. The addition of small amounts of a co-solvent such as ethanol, which shows ability to induce dipole-dipole interactions and hydrogen bonding with polar functional groups, is used to improve pigment extractions from leaves.³³ Results show that the carotenoid extraction curves have the three distinct regions usually found in the extraction of natural products using supercritical fluids: constant extraction, falling (transition), and diffusion-controlled rate periods.³¹

6.2.3.2 SAPONIFICATION

Alkaline hydrolysis (saponification) has been used to remove contaminating lipids from fat-rich samples (e.g., palm oil) and hydrolyze chlorophyll (e.g., green vegetables) and carotenoid esters (e.g., fruits). Xanthophylls, both free and with different degrees of esterification with a mixture of different fatty acids, are typically found in fruits, and saponification allows easier chromatographic separation, identification, and quantification. For this reason, most methods for quantitative carotenoid analysis include a saponification step.

Although saponification was found to be unnecessary for the separation and quantification of carotenoids from leafy vegetables by high performance liquid chromatography (HPLC)¹⁴ or open column chromatography (OCC),³⁴ saponification is usually employed to clean the extract when subsequent purification steps are required such as for nuclear magnetic resonance (NMR) spectroscopy and production of standards from natural sources.

A general procedure that our laboratory generally employs is the addition of an equal amount of methanolic 10% potassium hydroxide (KOH) to an ethereal carotenoid extract. This solution is bubbled with N₂ and allowed to stand overnight at room temperature.^{3,10–12,35,36} Other conditions that shorten time at room temperature have also been used, such as saponification of the dichloromethane (CH₂Cl₂) extract with the same amount of 10% KOH in MeOH for 1 hr (peppers and fruits¹⁶) and ethereal extract treated with 30% methanolic KOH under N₂ for 3 hr (green leaves,¹⁴ vegetables and fruits³⁷).

However, complete hydrolysis of carotenoid esters sometimes is not achieved in 1 to 3 hr. The saponification degree can be verified easily by the presence of carotenol ester peaks eluting later than the peaks of β -carotene on reversed phase columns. Retinol palmitate, added as an internal standard to orange juice, also serves to indicate whether saponification is complete, since it is converted to retinol which elutes at lower retention time.³⁸ The mixture is subsequently washed with water until free of alkali in a separatory funnel. Other more polar solvents such as CH₂Cl₂ or EtOAc, and diethyl ether alone or mixture with petroleum ether can be used to increase the recovery of polar xanthophylls from the water phase.

More severe conditions, 35 ml of 35% methanolic KOH added to 10 mL extract in EtOAc and shaken for 20 min at 50°C, are necessary for the total conversion of bixin, an ester of a carotenoid acid, to norbixin in snacks.²⁸ Since saponification yields the norbixin salt (K or Na, depending on the alkali) that is soluble in the aqueous phase, the pH should be decreased to 3.5 or even lower to allow extraction of the protonated norbixin by EtOAc and diethyl ether.²⁸

Carotenoids with allylic hydroxy and keto groups such as the 3-hydroxy-4-keto group in astaxanthin which is widespread in marine animals, microorganisms, and algae undergo oxidation in the presence of alkali and air. For such samples, saponification is not recommended or must be carried out under anaerobic conditions. For this purpose, a special apparatus and procedure were developed by Schiedt et al.³⁹

Aldol condensation is another undesirable reaction that can occur during saponification. Carotenals undergo aldol condensation, with the extension of the polyene chain in the presence of alkali and acetone remaining from the extraction

step. In fact, citranaxanthin and reticulaxanthin, reported as natural carotenoids from citrus, are aldol condensation products formed from β -apo-8-carotenal and β -citraurin, respectively.⁴⁰ In such samples, the extraction can be performed with MeOH and EtOAc.

We always recommend that structural changes occurring after the saponification procedure be verified. Since the hydroxyl groups have no influence on the chromophore, the wavelength of the maximum absorption, shape, and intensity of the ultraviolet-visible (UV-Vis) spectrum would be identical for unsaponified and saponified samples.

In recent years, chromatographic methods for the simultaneous determination of free and esterified carotenoids have been developed. Since no alkaline saponification can be employed, it is necessary to clean up the extract in order to avoid detection of fatty acids that produce high background noise in MS detectors. The crude extract can be partially purified by OCC on silica gel.⁴¹ However, further purification using enzyme suspensions is often necessary. In the presence of residual triacylglycerides as in potatoes, an additional enzymatic clean-up procedure using microbial lipases for hydrolysis of triacylglycerides should be applied.⁵ The enzymes employed by Breithaupt and Bamedi⁵ do not cleave carotenoid esters, ensuring carotenoid ester stability during the clean-up procedure.

6.2.4 CHROMATOGRAPHIC SEPARATION

Chromatography is a powerful analytical method suitable for the separation and quantitative determination of a considerable number of compounds, even from complicated matrices. The separation of carotenoids can be carried out by OCC, thin layer chromatography (TLC), high performance liquid chromatography (HPLC), or a combination of them. Examples that employ capillary electrophoresis (CE) and high-speed counter-current chromatography (HSCCC) have recently been published. Since carotenoids are labile to high temperature, gas chromatography is never employed for their separation.

The choice of the most suitable chromatographic method depends on the objective of analysis. For quantitative analysis, the most popular and reliable system among all chromatographic separation techniques is HPLC, since separation resolution and speed are much higher compared to OCC and TLC. On the other hand, when amounts higher than 1 mg of pigment are required, a combination of TLC and/or OCC with semi-preparative HPLC gives very good results.^{23,42–44}

Chromatography is essentially a method of separation based on two phases, one stationary and one mobile. If the composition of the mobile phase is not changed during the separation, the term isocratic elution is used. For separation of complex mixtures with wide ranges of polarities, the composition of the mobile phase can be changed during separation, a process known as gradient elution.

6.2.4.1 STATIONARY PHASES

A large range of stationary phases is available, and according to their polarity they can be divided into normal phase and reversed phase types. Silica gel, aluminium oxide, and a nitrile-bonded-phase are normal adsorbents used to separate carotenoids

according to their polarities. Compounds with more polar substituents are more strongly adsorbed due to the formation of hydrogen bonds and dipole–dipole interactions between the functional groups and the stationary phase. Thus the retention degree depends on the numbers and types of functional groups in the carotenoid molecule, the most polar compounds eluting later than those with lower polarity; in other words, in normal phase, carotenes elute first, followed by mono-, di-, and polyhydroxy carotenoids. The mobile phase is usually relatively apolar (hexane, toluene) with increasing amounts of a more polar solvent (acetone, EtOAc, diethyl ether) to elute the most polar xanthophylls.

Basic materials, such as magnesium oxide (MgO) and calcium carbonate, demonstrate affinity for conjugated double bonds, polarity being less important. Therefore, a greater number of conjugated double bonds implies stronger retention. The mobile phase used is also non-polar (hexane, petroleum ether) with increasing amounts of a polar solvent (acetone, CH_2Cl_2 , diethyl ether) to elute the most polar xanthophylls and highly conjugated double bond carotenoids such as lycopene.

Many different reversed phase materials, especially C_{18} , are available from different manufacturers. The main differences lie in the carbon loading and nature of synthesis, monomeric or polymeric. Usually relatively high carbon loading is required to achieve retention of polar carotenoids.⁴⁵ Polymeric synthesis improves the column selectivity toward forms and groups with similar structures.⁴⁶ In the past decade, a polymeric reversed phase C_{30} column was developed specially for carotenoid separation.^{45,46} This stationary phase shows adequate retention for polar carotenoids and superior selectivity toward polar and non-polar carotenoids, especially geometrical isomers. Among reversed phase columns, the C_{30} is the only one capable of resolving peaks of geometrical isomers of asymmetrical carotenoids in which *cis* double bonds are present at the same position but at opposite ends of the molecule.⁴⁷

A variety of mobile phases have been employed for carotenoid separation by reversed phase HPLC. Most are based on MeOH or acetonitrile, with the addition of CH_2Cl_2 , THF, methyl-*tert*-butyl ether (MTBE), acetone, or EtOAc. In general, recoveries of carotenoids are higher with methanol-based systems compared to acetonitrile-based ones.⁴⁸

Considering that each stationary phase has a different mechanism of separation, the analyst should apply the most suitable phase for a particular carotenoid separation, not forgetting that it is very useful to look for previous experiences among the several examples available in the literature and that it is also possible to find different solutions for the same problem.

6.2.4.2 OPEN COLUMN CHROMATOGRAPHY

No expensive equipment is required for OCC; however, the separation efficiency depends on the analyst's experience since a new column has to be packed for each analysis. In addition, depending on the packing type (powder or slurry), stationary phase, and purpose of the separation, the separation can take from 30 min to 4 hr. The AOAC official method for the determination of carotenoids still uses OCC.⁴⁹

Separation of carotenoids from many foods was developed on a column packed with a mixture of MgO and HyfloSupercel (or celite or diatomaceous earth) at 1:1

or 1:2 proportions under vacuum, using petroleum ether with increasing amounts of diethyl ether or acetone as the mobile phase. The major carotenoids from leafy vegetables,³⁴ carrots,⁵⁰ papaya,⁵¹ roots of *Arracacia xanthorrhiza* Bancr.,⁵² and sweet potatoes⁵³ were separated by OCC on MgO and quantified. However, since carotenoid polarity alone does not account for the whole separation mechanism in this material, the elution order should be carefully considered for identification purposes. For example, the carotene lycopene elutes very close to dihydroxy carotenoids such as lutein. On the other hand, OCC on MgO is a very useful alternative for removing large amounts of colorless impurities such as lipids and waxes from a sample or carotenoid fraction by eluting large amounts of petroleum ether through the column.⁵⁴ In addition, standards of α -carotene and β -carotene can be easily prepared from carrots by OCC separation on MgO and HyfloSupercel (see Section 6.2.6.1).

An open column packed with neutral aluminium oxide (grade III) slurry is generally used for semi-preparative separation of large amounts of carotenoid extract, revealing three broad bands: (1) carotenes and epoxy-carotenes constitute the first fraction to elute with petroleum ether, (2) monohydroxy and keto-carotenoids with 50 to 80% diethyl ether in petroleum ether are next, and (3) finally, the polyhydroxy carotenoids elute with 2 to 5% diethyl ether in ethanol or MeOH.^{23,55,56}

6.2.4.3 THIN LAYER CHROMATOGRAPHY

TLC has been traditionally regarded as a simple, rapid, and inexpensive separation method, currently used mainly for preliminary examinations to give an indication of the number and variety of pigments present and help in the selection of suitable separation and purification procedures for further analysis. To avoid epoxy-furanoid rearrangements caused by inherent silica gel acidity, one pellet of a strong alkali such as KOH or NaOH should be added to the water used to make the thin layer, or in case of ready commercial plates, 0.1% triethylamine (TEA) should be added to the mobile phase.

In order to verify the minimum number of carotenoids in an extract or fraction, a very easy and fast test can be carried out with only one small piece of silica gel thin layer plate, consecutively developed with 10% diethyl ether or 4% acetone in petroleum ether for carotene separation, followed by 70% diethyl ether or 15% acetone in petroleum ether for visualization of monohydroxy xanthophyll separation, and finally with 30% acetone in petroleum ether for separation of di- and trihydroxy-carotenoids.⁵⁷

The TLC technique on a semi-preparative scale has been very useful for purification of fractions previously separated by OCC. A protocol combining OCC separation on aluminium oxide and TLC on silica gel and on MgO with kieselguhr was used in the 1970s to achieve sufficient purification of carotenoids from tomato for analysis by direct insertion electron impact MS.⁵⁸⁻⁶⁰ Using the same successful approach, many recent examples can be found in the literature such as for carotenoids from leaves,⁵⁴ orange,⁶¹ passion fruit,⁵⁶ and mango.⁶² A combination of OCC on silica gel followed by TLC on silica gel, aluminium oxide, and MgO with kieselguhr was used to isolate minor carotenoids from annatto seeds, although further HPLC was necessary to achieve enough purity for NMR spectroscopy.⁴²⁻⁴⁴

6.2.4.4 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

Most researchers would agree that among all chromatographic separation techniques, HPLC is currently the most popular and reliable system for the separation of carotenoids. It offers significant advantages in terms of separation resolution, speed, sensitivity, precision, cost, and specificity (depending on detection method). Ultra-violet (UV) and visible (Vis) single wavelength absorbance detectors have been used for many years as components of HPLC systems for carotenoid quantification, whereas coupling a photodiode array (PDA) detector to HPLC allows the determination of peak purity and UV-Vis spectrum features. The use of MS detectors has a number of advantages, for example, the sample quantity required for analysis is very small, MS provides information on carotenoid molecular mass, and fragmentation patterns indicate the presence of some groups in the carotenoid structure.

A calcium hydroxide column slurry packed in the laboratory was used to evaluate the distribution of all-*trans*-, 13-*cis*-, and 9-*cis*- isomers of β -carotene in fresh and processed vegetables and fruits.⁶³ Elution order was reported to be 15-*cis*-, 13-*cis*-, all-*trans*-, and 9-*cis*- β -carotene, using 2% *p*-methylanisole or 2% acetone in hexane as mobile phase, in a 35-min run.⁶⁴ However, a column packed with calcium hydroxide as the stationary phase is not commercially available.

Using a silica column, 16 carotenoids were separated by HPLC from carotenoid saponified extracts obtained from several cultivars of pepper.⁶⁵ A silica-based nitrile-bonded column was used for the separation of lutein, zeaxanthin, and their geometrical isomers as well as their metabolic by-products in fruits, vegetables, and wheat products. The other carotenoids were not separated since all had been eluted together at the beginning of the run.³⁷ In a 70-min run, the elution order was: 13,13'-di-*cis*-lutein, all-*trans* lutein, all-*trans* zeaxanthin, 9-*cis* lutein, 9'-*cis*-lutein, lutein epoxide, 13-*cis*- + 13'-*cis* lutein, 9-*cis*-zeaxanthin, 13-*cis*-zeaxanthin, and violaxanthin using as mobile phase hexane with CH_2Cl_2 and MeOH (75:25:0.3) containing 0.1% *N,N*-diisopropylethylamine (DIPEA) at 0.7 mL/min³⁷. The addition of DIPEA, a nucleophilic base, to the mobile phase used for the silica column was recommended in order to improve carotenoid recoveries.⁶⁶

Although some normal phase methods have been used, the majority of carotenoid separations reported in the literature were carried out by reversed phase HPLC. Among the C_{18} columns employed for determination of complete carotenoid compositions in foods, the polymeric Vydac brand is preferably used for separation of *cis* isomers.^{67,68} Several examples of different C_{18} columns and mobile phases are cited in the literature, but not all carotenoids are baseline separated in most systems. Table 6.2.1 shows some examples employing different brands of C_{18} columns.^{11,12,14,15,67-71} Acetonitrile did not improve selectivity toward separation of carotene isomers in a Vydac 201TP column⁶⁷ and resolution was strongly dependent on the Vydac column lot.⁶⁸

Most laboratories now employ C_{30} columns for separation of carotenoids from complex matrices. There are several examples for separation of carotenoids from foods such as orange,^{17,36,72} watermelon,⁷³ mango,⁶ camu-camu,³⁵ carrot,⁷⁴ spinach,⁷⁵⁻⁷⁷ tomato,³⁰ sweet corn,⁷⁷ and potato.⁵ The C_{30} column systems shown in Table

TABLE 6.2.1
HPLC Systems Employing Reversed Phase C₁₈ Columns for Separation of Carotenoids

Column	Mobile Phase	Elution Order of Carotenoids	Run Time (min)	Source and Reference
Vydac 201TP54	MeOH/DCM/H ₂ O (80:15.2:4.8) at 1 mL/min and at RT	All- <i>trans</i> α -carotene, all- <i>trans</i> β -carotene, 9- <i>cis</i> β -carotene, 13- <i>cis</i> β -carotene	45	Green leafy vegetables ⁶⁷
Vydac 218TP54	MeOH/THF (99:1) at 0.6 mL/min and at 30°C	All- <i>trans</i> -, 9- <i>cis</i> -, 13- <i>cis</i> - and 15- <i>cis</i> - isomers of β -carotene	25	Iodine-catalyzed isomerized β -carotene ⁶⁸
Vydac 218TP54	100% MeOH at 1 mL/min and at 29°C	Lutein, zeinoxanthin, β -cryptoxanthin, <i>cis</i> - β -cryptoxanthin, α -carotene, β -carotene, phytoene + phytofluene, 9- <i>cis</i> β -carotene, 13- <i>cis</i> β -carotene	16	Caja pulp ¹²
Nova-Pak	ACN/EtOAc/H ₂ O from 88:2:10 to 85:15:0 in 15 min, maintaining this proportion for 30 min at 1 mL/min and at 29°C	Neochrome + neoxanthin, violaxanthin, luteoxanthin, lutein, zeaxanthin, 5,6,5,6-diepoxy- β -cryptoxanthin, 5,6-epoxy- β -cryptoxanthin, 5,8-epoxy- β -cryptoxanthin, zeinoxanthin, β -cryptoxanthin, 5,6,5,6-diepoxy- β -carotene, 5,8-epoxy- β -carotene, α -carotene, β -carotene	45	Acerola ¹¹
Shim-pack CLC M	ACN/MeOH/EtOAc + 0.05% TEA (99:1:0) for 25 min to 60:10:30 for 5 min, maintaining this proportion for 25 min at 0.7 mL/min	Auroxanthin, antheraxanthin, violaxanthin, mutatoxanthin, β -lutein, zeaxanthin, α -cryptoxanthin or zeinoxanthin, β -cryptoxanthin, ζ -carotene, α -carotene, β -carotene	57	Valencia orange juice ⁶⁹
Kromasil	MeOH/ACN/ CH ₂ Cl ₂ /H ₂ O (50:30:15:5) + 0.1% BHT + 0.1% TEA at 2.5 mL/min and at 23°C	Neoxanthin, violaxanthin, antheraxanthin, lutein, + zeaxanthin, 5,6-epoxy-lutein, α -cryptoxanthin or zeinoxanthin, β -cryptoxanthin, α -carotene, β -carotene	35	Valencia ultrafrozen orange juices ⁷⁰

Continued.

TABLE 6.2.1 (Continued)
HPLC Systems Employing Reversed Phase C₁₈ Columns for Separation of Carotenoids

Column	Mobile Phase	Elution Order of Carotenoids	Run Time (min)	Source and Reference
Spherisorb ODS-2	Acetone/H ₂ O 75:25 maintaining for 5 min, to 95:5 in 5 min, maintaining for 7 min, to 100:0 in 5 min at 1.5 mL/min	Neoxanthin, neoxanthin isomer, neochrome, neochrome isomer, violaxanthin, violaxanthin isomer, luteoxanthin, auroxanthin, antheraxanthin, mutatoxanthin, lutein, <i>cis</i> -lutein, 5,8-epoxy- β -cryptoxanthin, 5,6-epoxy- β -cryptoxanthin, β -cryptoxanthin, β -carotene, <i>cis</i> - β -carotene	18	Acerola ⁷¹
Microsorb (Rainin Instrument Co.)	ACN/MeOH/CH ₂ Cl ₂ /HEX 75:15:5:5 maintained for 12 min, to 40:15:22.5:22.5 in 15 min at 0.5 mL/min	All- <i>trans</i> neoxanthin, 9- <i>cis</i> neoxanthin, violaxanthin, neochrome, epoxy all- <i>trans</i> lutein, 13- or 13'- <i>cis</i> -epoxy-lutein, all- <i>trans</i> lutein, 9- or 9'- <i>cis</i> -lutein, 9- or 9- <i>cis</i> -lutein, 13- or 13'- <i>cis</i> -lutein, β -apo-8-carotenal (IS), chlorophyll b, chlorophyll b', chlorophyll a, chlorophyll a', pheophytin b, pheophytin a, all- <i>trans</i> - β -carotene, 15,15'- <i>cis</i> - β -carotene, decapreno- β -carotene (IS)	35	Green leaves ¹⁴
Hypersil	MeOH/H ₂ O 75:25 (A) and EtOAc (B) from 100% to 70% A in 10 min, to 0% A in 20 min at 1 mL/min	Neoxanthin, violaxanthin, zeaxanthin, lutein, antheraxanthin, β -cryptoxanthin, lutein monoester, antheraxanthin monoester, β -cryptoxanthin monoester, lycopene, β -carotene, violaxanthin ester, lutein diester, β -cryptoxanthin ester, β -cryptoxanthin ester, zeaxanthin diester, zeaxanthin diester, zeaxanthin diester	28	Persimmon ¹⁵

AA = ammonium acetate. ACN = acetonitrile. BHT = butylated hydroxytoluene. CH₂Cl₂ = dichloromethane. HEX = hexane. IS = internal standard. MeOH = methanol. RT = room temperature. TEA = triethylamine. TBME = *tert*-butyl methyl ether. THF = tetrahydrofuran.

6.2.2 were selected after considering studies in which carotenoid identification was confirmed at least by comparison with authentic standards.^{5,30,46,76-80}

It is interesting that the retention behavior of lycopene varies dramatically depending on the stationary phase synthesis; lycopene usually elutes before α - and β -carotene in monomeric columns,¹⁵ whereas with polymeric C_{30} columns, lycopene elutes after these carotenenes.³⁰

Figure 6.2.2 shows the separations of mixtures of standards on a monomeric C_{18} column and also on a polymeric C_{30} column. The elution order on the monomeric C_{18} column is, as expected, first the dihydroxy xanthophylls (lutein and zeaxanthin), followed by the monohydroxy compounds (rubixanthin and β -cryptoxanthin), and finally by the carotenes (γ -, α -, and β -carotene). However, on the C_{30} column, rubixanthin and γ -carotene, both with 1 acyclic ψ -end group, eluted after α - and β -carotene, with two cyclic end groups.

In recent years, the methods for carotenoid determination without saponification have increased. Independently of the mobile phase and food composition, there are similar patterns of chromatographic separation on reversed phase columns. A chromatograph can be divided roughly into four zones: the first zone corresponds to free xanthophylls, the second zone to monoesterified pigments, the third zone contains carotenes, and finally the fourth zone corresponds to diesterified carotenoids.^{81,82}

Independently of the reversed phase column, the addition of TEA to the mobile phase increases carotenoid recovery from the column.^{16,74,83} Increased recoveries of 18% lutein, 33% zeaxanthin, 33% β -cryptoxanthin, 53% lycopene, 30% α -carotene, and 42% β -carotene in a Vydac column were observed after the addition of 0.1% TEA to the mobile phase.¹⁶ Recovery on a C_{30} column was also enhanced by the addition of 0.1% TEA to the mobile phase, with the peak area of lutein increasing by 26%, that of zeaxanthin by 42%, that of β -cryptoxanthin by 55%, that of lycopene by 21%, and those of α -carotene and β -carotene by 47 and 64%, respectively.⁷⁴

A solution of 0.05 M of ammonium acetate (AA) added to the MeOH used in the mobile phase also improved column recovery, and the addition of 0.05% TEA to the mobile phase containing AA further increased the recovery of all columns to up 100%.⁸³ However, the addition of 0.05 M AA to a mobile phase containing 0.05% TEA did not significantly improve carotenoid recovery in the Vydac column.¹⁶ The exact action by which TEA and AA improve carotenoid recovery is not clear, but it is suggested that these modifiers either buffer the acidity of free silanol groups or participate in hydrogen-bonding interactions with silanols from the stationary phase as well as by preventing reactions with free metal ions from the silica support.

Temperature has an influence on the retention and consequently on the capacity factors of carotenoids in HPLC columns. Usually, as the column temperature increases, the retention decreases; however, in a polymeric C_{30} column, after an initial decrease of the t_R values of *cis* isomers of carotenoids, the retention of *cis* isomers actually increases at temperatures above 35°C. This different behavior can be explained by the increased order and rigidity of the C_{30} stationary phase at lower temperatures that in turn induce preferential retention of long, narrow solutes as the *trans* isomer; and partial exclusion of bent and bulky *cis* isomers. The greater chain mobility and less rigid conformation of the C_{30} at higher temperatures may increase the contact area available for interaction with the *cis* isomers and also may lower

TABLE 6.2.2
HPLC Systems Employing Reversed Phase C₃₀ Column for Separation of Carotenoids

Column Brand and Size	Mobile Phase	Elution Order of Carotenoids	Identification Method	Run Time (min)	Source and Reference
NIST (3 μ m, 250 $\times$ 4.6 mm)	MeOH/MTBE (89:11) at 1 mL/min	13- <i>cis</i> , 13'- <i>cis</i> , all- <i>trans</i> , 9- <i>cis</i> , 9- <i>cis</i> isomers of α -carotene	PDA, NMR	60	Iodine-catalyzed photo-isomerized standard ⁷⁸
YMC (3 μ m, 250 $\times$ 4.6 mm)	MeOH/MTBE (80:20) at 1 mL/min	15- <i>cis</i> , 13- <i>cis</i> , all- <i>trans</i> , 9- <i>cis</i> isomers of β -carotene	PDA, NMR	35	Thermally isomerized standard ⁷⁹
YMC (3 μ m, 250 $\times$ 4.6 mm)	MeOH/MTBE (95:5) at 1 mL/min	13- <i>cis</i> + 15- <i>cis</i> , 13' <i>cis</i> , all- <i>trans</i> , 9- <i>cis</i> , 9' <i>cis</i> isomers of lutein	PDA, NMR	26	Thermally isomerized standard ⁷⁹
YMC (5 μ m, 250 $\times$ 4.6 mm)	MeOH/MTBE/H ₂ O from 81:15:4 to 6:90:4 in 90 min at 1 mL/min and at 20°C	Astaxanthin, capsanthin, lutein, zeaxanthin, canthaxanthin, β -cryptoxanthin, echinenone, 15- <i>cis</i> - β -carotene, 13- <i>cis</i> - β -carotene, α -carotene, all- <i>trans</i> β -carotene, 9- <i>cis</i> - β -carotene, δ -carotene, lycopene	Standards	80	Authentic standards ⁴⁶
YMC (5 μ m, 250 $\times$ 4.6 mm)	MeOH/MTBE/H ₂ O 92:4:4 (A) and 90:6:4 (B) from 100% A to 6% B in 80 min at 1 mL/min and at 20°C	13- <i>cis</i> -lutein, 13' <i>cis</i> -lutein, 13- <i>cis</i> -zeaxanthin, all- <i>trans</i> lutein, all- <i>trans</i> zeaxanthin, 9- <i>cis</i> -lutein, 9'- <i>cis</i> -lutein, 9- <i>cis</i> -zeaxanthin	PDA, HPLC-MS, HPLC-NMR, standards	80	Standards, spinach, sweet corn ⁷⁷

YMC (3 µm, 250 × 4.6 mm)	Acetone/D ₂ O (93:7) at 1 mL/min	13,15-di- <i>cis</i> -, 9,13,13'-tri- <i>cis</i> -, 13- <i>cis</i> -, 9,13,9'-tri- <i>cis</i> , all- <i>trans</i> and 9- <i>cis</i> isomers of β-carotene	HPLC -NMR	18	Source not reported ⁸⁰
ProntoSIL (3 m, 250 × 4.6 mm)	Acetone/D ₂ O (86:14) for 21 min to 97:3 in 4 min at 1 mL/min and at 22°C	13- <i>cis</i> -lutein, 13'- <i>cis</i> -lutein, all- <i>trans</i> lutein, all- <i>trans</i> zeaxanthin, 9- <i>cis</i> -lutein, 9'- <i>cis</i> -lutein, (β-apo-8-carotenal (IS), chlorophylls, 13,15-di- <i>cis</i> -β-carotene, 13- <i>cis</i> -β-carotene, 9,13-di- <i>cis</i> -β-carotene, β-carotene, 9- <i>cis</i> -β-carotene	HPLC-NMR and HPLC -MS	35	Spinach ⁷⁶
YMC (5 µm, 250 × 4.6 mm)	MeOH/MTBE/H ₂ O + 0.1% TEA 81:15:4 (A) and 6:90:4 (B), from 99% to 44% A in 39 min to 0% A in 6 min at 1 mL/min and at 35°C	C _{14,0'} -violaxanthin-C _{14,0'} , C _{14,0'} -violaxanthin-C _{16,0'} , C _{14,0'} -lutein-C _{14,0'} , C _{14,0'} -zeaxanthin-C _{14,0'} , C _{14,0'} -lutein-C _{16,0'}	PDA, HPLC-MS	45	Potato ⁵
YMC (3 µm, 250 × 4.6 mm)	MeOH + 0.1% TEA/MTBE (1:1) at 1 mL/min and at 33°C	All- <i>trans</i> β-carotene, 15- <i>cis</i> -lycopene, all- <i>trans</i> γ-carotene, 13- <i>cis</i> -lycopene, 9- <i>cis</i> -lycopene, all- <i>trans</i> -lycopene + 5- <i>cis</i> -lycopene	Co-elution with standards and PDA	33	Tomato ³⁰

D₂O = deuterated water. HPLC = high performance liquid chromatography. IS = internal standard. MeOH = methanol. MS = mass spectrometry. NMR = nuclear magnetic resonance. PDA = photodiode array detector. TEA = triethylamine. MTBE = methyl tert-butyl ether.

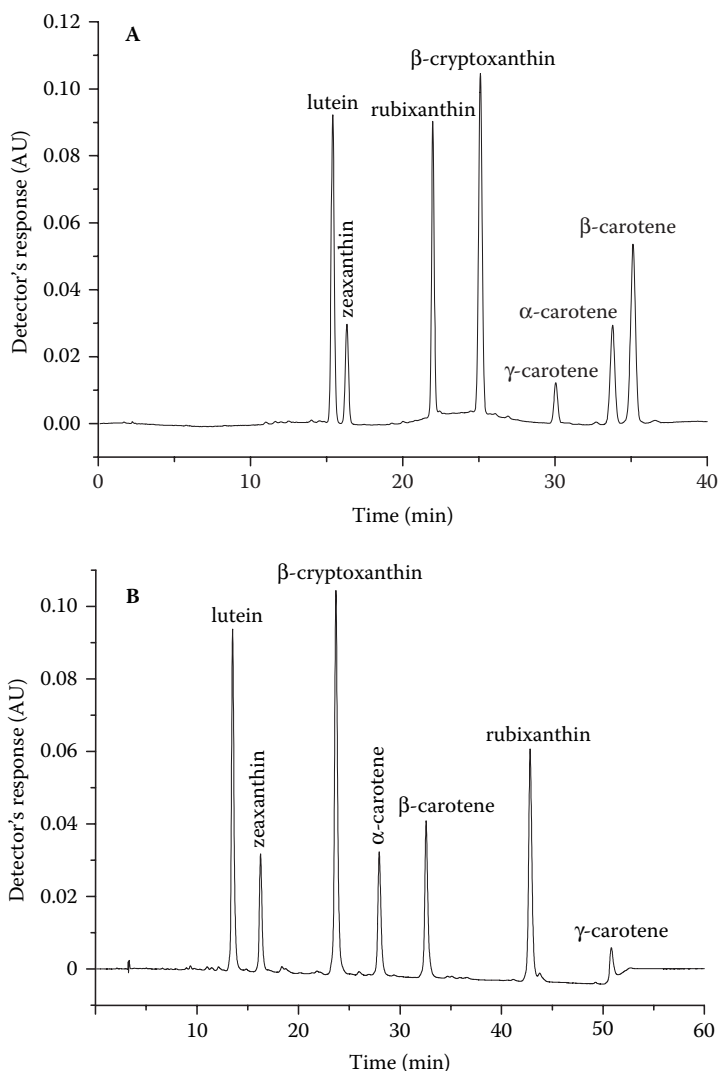


FIGURE 6.2.2 Chromatograms of carotenoid standards obtained by HPLC. Chromatographic conditions: A: C_{18} Novapak column (4 μ m, 3.9 $\times$ 300 mm), acetonitrile (0.1% TEA), H_2O , and EtOAc from 88:10:2 to 85:0:15 in 15 min at 1 mL/min. B: C_{30} YMC column (3 μ m, 4.6 $\times$ 250 mm), MeOH (0.1% TEA)/MTBE from 95:5 to 70:30 in 30 min to 50:50 in 20 min at 0.9 mL/min.

the barrier for bent solutes to penetrate the stationary phase.⁸⁴ For example, on a C_{30} column with the temperature column set at 13°C, the elution order was found to be 15-*cis*- β -carotene, 13-*cis*- β -carotene, followed by echinenone. At 38°C, 15-*cis*- eluted together with 13-*cis*- β -carotene, and both after echinenone.⁸⁴

Another important factor is the composition of the injection solvent that involves a compromise between good solubilities of sample carotenoids, compatibility with

the mobile phase, and no deformation of peaks. However, this can be a problem for reversed phase HPLC separations of carotenoids. In case of using a solvent much stronger than the mobile phase, as for example an extract dissolved in hexane and usage of MeOH as the mobile phase, carotenoids can precipitate in the head of the column, resulting in band broadening and even double or tailing peaks and inconsistent peak areas. Conversely, the use of a weak solvent injection such as MeOH will not completely dissolve the carotenoids. To overcome this problem, a successful procedure employed in our laboratory is first to dissolve the carotenoids in a known small volume of a strong solvent such as EtOAc or MTBE, followed by addition of at least the same volume of the main mobile phase constituent, MeOH or acetonitrile.

6.2.4.5 OTHER SEPARATION TECHNIQUES

Capillary electrophoresis (CE) has several unique advantages compared to HPLC, such as higher efficiency due to non-parabolic fronting, shorter analytical time, production of no or much smaller amounts of organic solvents, and lower cost for capillary zone electrophoresis (CZE) and fused-silica capillary techniques. However, in CZE, the most popular separation mode for CE, the analytes are separated on the basis of differences in charge and molecular sizes, and therefore neutral compounds such as carotenoids do not migrate and all co-elute with the electro-osmotic flow.

To overcome this problem a hybrid technique called capillary electrochromatography (CEC) combining a reversed phase column such as C₁₈ or C₃₀ with high voltage electrophoresis was first used for separation of carotenoid isomers.⁸⁴ The separation resolution for the isomers of β - and α -carotene is improved using CEC when compared to HPLC, both using C₃₀ columns.⁸⁵ Another alternative for the separation of different neutral compounds is micellar electrokinetic chromatography (MEKC) based on micellar solubilization with a charged surfactant and separation employing the CZE instrumental technique. However, no examples using MEKC to separate carotenoids were found in the literature.

Counter-current chromatography (CCC) is a very versatile separation technique that does not require a solid stationary phase. It relies simply on the partition of the sample compounds between the two phases of an immiscible solvent system. The new generation of high-speed CCC (HSCCC) instruments show improved efficiency and separation time aided by pressure and centrifugal force partition, using sample loads ranging from milligrams to grams. Purification of lycopene was achieved by HSCCC using *n*-hexane, CH₂Cl₂, and acetonitrile at a volume ratio of 10:3.5:6.5, starting from 100 mg of crude extract of tomato paste to yield 8.3 mg of lycopene at over 98% purity measured by HPLC.⁸⁶ The purification of lutein from a saponified extract of marigold flowers was carried out in a preparative HSCCC system with hexane, EtOH, and H₂O (6:4.5:1.5), yielding lutein with 96.7% purity as measured by HPLC.⁸⁷

6.2.5 IDENTIFICATION

The identification of a known carotenoid should be based, at least, on the information obtained from the following criteria: chromatographic behavior in two

different systems, UV-Vis absorption spectrum, and mass spectrum.⁸⁸ The determination of chirality and *cis-trans* configuration requires the additional applications of CD and NMR spectroscopy, respectively.⁸⁸ Some simple chemical derivatizations can also provide very useful information.⁸⁹ The information from the different spectroscopic and chromatographic methods used should be combined to determine the final structure.

6.2.5.1 ULTRAVIOLET-VISIBLE (UV-Vis) SPECTROSCOPY

The UV-Vis spectrum of a carotenoid is always examined first because it provides useful information about the chromophore and some information on the functional groups and rings conjugated to the polyene chain. Most carotenoids absorb light in the region between 400 and 500 nm; however, huge differences occur among the spectra of different carotenoids regarding both the location of the maximum absorption wavelength ($\lambda_{\max}$) and degree of spectral vibrational fine structure. A UV-Vis spectrum of good quality can easily be obtained in a spectrophotometer from 1 to 2 μg of an isolated carotenoid and from a few nanograms when obtained online with HPLC combined with a photodiode array detector (PDA).

Figure 6.2.3 shows the typical three-peak carotenoid UV-Vis spectrum and indications to calculate the spectral fine structure (% III/II) and the intensity of the *cis* peak (% $A_{\text{B}}/A_{\text{II}}$).⁹⁰ Spectral fine structure is defined as the ratio of the height of the longest wavelength absorption peak (designated III) and that of the middle absorption peak (designated II), taking the minimum between the two peaks multiplied by 100 as a baseline. The height of the *cis* peak⁹⁰ located *ca.* 142 nm below the longest wavelength absorption maximum can be indicated by the notation % $A_{\text{B}}/A_{\text{II}}$ representing the ratio of the height of the *cis* peak (A_{B}) and that of the middle main absorption peak (A_{II}) multiplied by 100.

The maximum absorption wavelengths in different solvents of many carotenoids can be found in the literature,^{1,89,90} and the % III/II values are also available for some carotenoids.^{1,90} It is common to find variations of 1 to 3 nm in $\lambda_{\max}$ for the same carotenoid in the same solvent cited in different publications. No identification based simply on the matching of recorded UV-Vis spectra with tabulated data can be done without considering the relationships of structures and the factors influencing light absorption. The principal factors that influence carotenoid UV-Vis absorption spectra are discussed below.

Number of conjugated double bonds — As the length of a chromophore increases, it becomes easier to promote the electrons to the excited state, with consequent reduced energy necessary for the transitions, and thus the absorption occurs at longer wavelengths.

Geometrical *cis-trans* isomers — The UV-Vis spectra of most *cis* isomers are similar to those of the corresponding all-*trans* isomer. However, a few consistent differences can be found in the spectra of *cis* isomers as compared to those from the corresponding all-*trans* compound: a hypsochromic shift (2 to 6 nm for mono-*cis* through 34 nm for tetra-*cis*), a decrease in absorbance, a reduction of the spectral fine structure, and the appearance of a new absorption band known as a *cis* peak. For example, in a study in which the structures were confirmed by NMR, the isomers

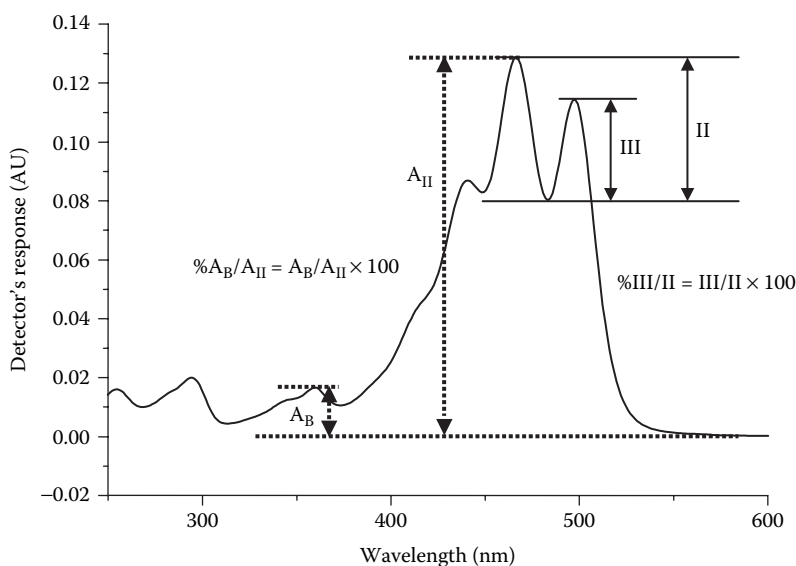


FIGURE 6.2.3 Calculations of spectral fine structure ($\% \text{ III/II}$) and intensity of *cis* peak ($\% A_B/A_{II}$).

of β -carotene showed $\% \text{ III/II} = 22$ and $\% A_B/A_{II} = 0$ for the all-*trans*-, $\% \text{ III/II} = 20$ and $\% A_B/A_{II} = 10$ for the 9-*cis*-, $\% \text{ III/II} = 4$ and $\% A_B/A_{II} = 45$ for the 13-*cis*-, and $\% \text{ III/II} = 0$ and $\% A_B/A_{II} = 56$ for the 15-*cis*- β -carotene.²³ In the same study, the values found for all-*trans*-lycopene were $\% \text{ III/II} = 65$ and $\% A_B/A_{II} = 8$, for 9-*cis*- $\% \text{ III/II} = 55$ and $\% A_B/A_{II} = 11$, for the 13-*cis*- $\% \text{ III/II} = 54$ and $\% A_B/A_{II} = 58$, and for the 15-*cis*-lycopene $\% \text{ III/II} = 43$ and $\% A_B/A_{II} = 74$. These values indicate that the fine structure ($\% \text{ III/II}$) decreases and intensity of the *cis* peak ($\% A_B/A_{II}$) increases as the *cis* double bond gets closer to the center of the molecule (Figure 6.2.4). The figure also shows that the UV-Vis spectra of 5-*cis* and all-*trans* lycopene are identical since the 5-*cis* double bond is not conjugated with the polyene chain.

Cyclic end groups — In the γ and ϵ ring end groups, the double bond is not conjugated with the polyene chain, and thus these types of rings do not interfere with the chromophore. In other words, the λ_{max} and $\% \text{ III/II}$ features of the UV-Vis spectra of neurosporene and ϵ,ϵ -carotene are virtually the same. On the other hand, in the β ring, the double bond is conjugated with the polyene chain but it is not coplanar. As a consequence of the steric hindrance of the β ring double bond, the degree of spectral fine structure ($\% \text{ III/II}$) is reduced and the λ_{max} appears at a shorter wavelength as compared to the ψ end group.

Allenic groups — Neoxanthin, a xanthophyll found in many foods, has an allenic group at the C-6,7,8 position where the two double bonds are perpendicular to each other, and the C-7,8 double bond coplanar with the polyene chain contributing effectively to the chromophore; since the C-6,7 bond is in a different plane, it makes no contribution. Therefore, neoxanthin, despite its 10 conjugated double bonds, has a UV-Vis spectrum similar to that of a conjugated nonaene such as violaxanthin.

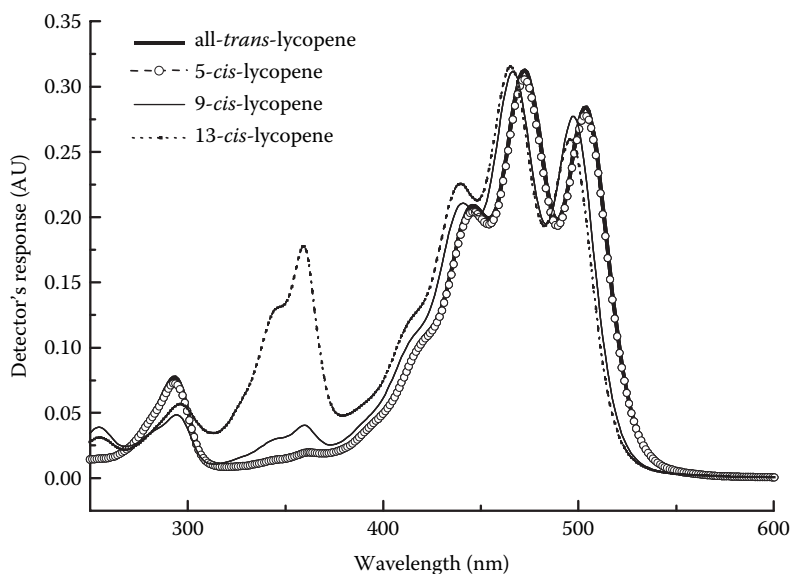


FIGURE 6.2.4 UV-Vis spectra obtained by HPLC-PDA in MeOH (0.1% TEA) and MTBE (50:50) of 5-*cis*, 9-*cis*, 13-*cis*, and all-*trans*-lycopene standards.

Carbonyl groups — A carbonyl group that is not conjugate to the polyene chain generally has no contribution to the chromophore, and therefore no influence on the UV-Vis spectrum. When a carbonyl group is in conjugation with the main polyene chain, the chromophore is extended, resulting in a bathochromic shift, and it is usually accompanied by loss of spectral fine structure. A terminal-conjugated aldehyde group leads to an increase of 25 to 40 nm in the $\lambda_{\max}$, with the increase greater for short chain compounds. Regarding fine structure, the short-chain monoaldehydes usually show some spectral fine structures that decrease as the chain lengths increase. Extension of an acyclic chromophore by a conjugated keto group increases the $\lambda_{\max}$ by *ca.* 30 nm, whereas with a cyclic end group that contains a conjugated keto group, the increase in $\lambda_{\max}$ is about 10 nm. The bathochromic effect of a carboxylic acid or ester is smaller than that of an aldehyde.

Epoxide groups — The addition of an epoxide group to a conjugated double bond removes that double bond from the chromophore and thus a hypsochromic shift occurs. The presence of a 5,6-epoxy group in a β ring causes a shift of *ca.* 6 nm to shorter wavelength and an increase in the fine structure, while a hypsochromic shift of 15 nm occurs if the epoxide at the same position is an acyclic end group. The 5,8-epoxide in a β ring has an even shorter chromophore, with $\lambda_{\max}$ at about 20 nm lower than that of β -carotene. On the other hand, 1,2-epoxides and cyclic 3,6-epoxides generally not conjugated to the chromophore do not have any influence on the UV-Vis spectra.

Hydroxyl groups — Since conjugated hydroxyl groups do not have any influence on the chromophore of the molecule, they do not have any effect on the UV-Vis spectrum. Therefore, β -carotene, β -cryptoxanthin, and zeaxanthin all

have the same UV-Vis spectra, thus making it impossible to distinguish them by UV-Vis spectroscopy.

Solvent — The transition energy responsible for the main absorption band is dependent on the refractive index of the solvent, the transition energy being lower as the refractive index of the solvent increases. In other words, the $\lambda_{\max}$ values are similar in petroleum ether, hexane, and diethyl ether and much higher in benzene, toluene, and chlorinated solvents. Therefore, for comparison of the UV-Vis spectrum features, the same solvent should be used to obtain all carotenoid data. In addition, because of this solvent effect, special care should be taken when information about a chromophore is taken from a UV-Vis spectrum measured online by a PDA detector during HPLC analysis.

6.2.5.2 MASS SPECTROMETRY

The three major parts of a mass spectrometer are the devices for the sample introduction into the ionization chamber where the ionization takes place, the mass analyzer in which the ions are separated according to their mass-to-charge (m/z) ratios, and a detector to measure the abundance of negative or positive ions. The basis of MS is the production of ions that are subsequently separated or filtered according to their m/z ratios and then detected. The most important information obtained from a mass spectrum is the molecular mass of the compound and the fragment patterns characteristic of structure features. Because carotenoids contain extended polyene chains that can stabilize a negative or positive charge, molecular ions of carotenoids have been formed using a variety of ionization techniques.

Most carotenoids provide molecular ions upon electron ionization (EI) in the gas phase with an electron beam of 70 eV, although the mass spectra of carotenoids obtained by EI show high numbers of high-intensity peaks in the region of low mass and rare peaks toward the molecular ion peak. As a consequence, characteristic peaks, even those of low intensity, can be clearly recognized in the unpopulated region. On the other hand, most of the peaks in the low mass region give little structural information since they occur in similar relative intensities in most carotenoid spectra. Additional information comes from the characteristic fragmentations such as elimination of toluene [92 mass units (u)] and *m*-xylene (106 u) from the polyene chain that help confirm the identification of the molecular ion and loss of functional groups as water from alcohols, acetic acid from acetates, and MeOH from methyl ethers. MS-EI spectra were obtained from purified carotenoids from tomato,^{58–60} mango,⁶² passion fruit,⁵⁶ and orange.^{61,91} Many data and fragments of different carotenoids measured by EI ionization have been tabulated.^{1,92–94} However, since different ionization techniques can give different fragmentation patterns for the same carotenoid, interpretation of mass spectra based on these tables must be carried out with caution.

In recent years, much effort has been devoted to developing HPLC-MS methods for natural compounds. The main difficulty lies in the different operating conditions of HPLC and MS. While HPLC operates using high flow rates, high pressures, and liquid phases near room temperature, MS uses low flow rates, high vacuum, and a gas phase ion analyzer. Various interfaces allow for online coupling of these two

instruments. The two main types of ionization techniques currently coupled to HPLC are ion spray techniques such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), and the ion desorption techniques that include fast atom bombardment (FAB) and matrix-assisted laser desorption ionization (MALDI). The most frequently used mass analyzers can also be categorized. The most common analyzers are based on ion beam transport such as magnetic field, time-of-flight (TOF) and quadrupole mass filter types and those based on ion trapping technology. These analyzers vary in their capabilities with respect to resolution, accuracy and mass range. Although all these techniques have been employed for carotenoid analysis, most methods for carotenoids now use the APCI interface that allows the detection of a molecular ion as a protonated molecule ($[M + H]^+$) and adduct ($[M + Na]^+$) both in positive mode or as a deprotonated molecule ($[M - H]^-$) in negative mode.

Positive ion FAB mass spectra obtained with a double focusing mass spectrometer produced abundant molecular ions ($[M]^{*+}$) of carotenes and xanthophylls with minimal fragmentation and no detectable thermal decomposition.⁹⁵ Fragmentation of the precursor ion was enhanced by collision-induced dissociation (CID) using helium gas.⁹⁵

Because carotenes lack heteroatoms such as oxygen to which protons or sodium cations might attach, no ions are usually detected for these hydrocarbon compounds during ESI in positive mode, although protonated molecules and sodium adducts were observed for xanthophylls under normal conditions with MeOH, MTBE, and H₂O as a mobile phase from HPLC.⁹⁶ Addition of a heptafluorobutanol oxidant at 0.1 or 0.5% produced abundant molecular ions of β -carotene with high reproducibility. Substitution of MeOH for acetonitrile produced similar limits of detection.⁹⁶

The technique of adding silver ions previously used for MALDI matrices for analysis of non-polar polymers was adapted to ESI by the addition of AgClO₄ to the analyte solution, causing carotenoid and tocopherol ionization as their Ag⁺ adducts.⁹⁷ The addition of AA favored the formation of an adduct between the glycoside esters of carotenoids and the NH₄⁺ ion in ESI-positive mode; therefore, all masses obtained corresponded to the $[M + H + NH_4]^+$ molecules.²¹ Recently, identification and quantification of carotenoids in fish eggs were carried out by a positive ESI triple quadrupole using a C₁₈ column and a mixture of MeOH, MTBE, and H₂O containing 0.1% formic acid in gradient elution.¹³ All carotenoids including β -carotene yielded abundant molecular radical cations $[M]^{*+}$ with the exceptions of astaxanthin and canthaxanthin for which the $[M + Na]^+$ adducts were dominant, with lower intensity of the $[M]^{*+}$ species. Quantification using single ion monitoring (SIM) was about 20 times more sensitive than using full scan.¹³

Using a solvent mixture of MeOH, MTBE, and H₂O, protonated molecules $[M + H]^+$ were detected for both xanthophylls and carotenes during positive ion APCI, whereas $[M]^{*+}$ and deprotonated molecules $[M - H]^-$ were detected during negative ion APCI.⁹⁸ Positive mode APCI mass spectra showed more fragmentation than negative ion analyses, and when combined with CID in the ion source, positive ion APCI produced structurally significant fragment ions that were similar to those observed using positive ion FAB ionization followed by CID and MS/MS.⁹⁵ Varying the nitrogen drier gas temperature from 300 to 400°C had no effect on the abundance

of the molecular ion species. However, changing the carrier gas temperature from 300 to 500°C increased the abundance of protonated molecular species up to a maximum value at 375°C. The background noise increased with increases in temperature throughout the range investigated.⁹⁸

APCI in positive mode ionization and triple quadrupole detection was used for determination of free and bound carotenoids in paprika, obtaining the $[M + H]^+$ and losses of fatty acids as neutral molecules from the $[M + H]^+$ with MeOH, MTBE, and H₂O as eluent from the C₃₀ column.⁸² The positions of the fatty acids on unsymmetrical xanthophylls could not be established by the MS data.

Lutein esters were separated on a C₃₀ column using a gradient of acetonitrile and MTBE containing 1 mM AA and analyzed by negative ion APCI and a triple quadrupole detector that provided abundant molecular radical cations $[M]^{*+}$ and fragments corresponding to loss of fatty acids.⁹⁹ Varying the desolvation gas temperature from 250 to 400°C had no effect on the abundance of the protonated molecules and fragments. However, the abundance of the protonated molecules and fragments changed significantly with the adjustment of the cone potentials.⁹⁹ In positive ion in-source CID experiments, along with protonated molecules and fragments, an adduct with potassium $[M + 39]^+$ was observed in the spectrum.⁹⁹

It must be underlined that independently of the MS equipment characteristics, no information about stereo-chemistry can be obtained. In fact, *cis* and *trans* isomers of the corresponding carotenoid showed identical mass spectra, as did carotenoids with epoxide groups at 5,6 and 5,8 positions. In addition, special care should be taken in assigning carotenoid molecular masses to avoid confusion due to the various ions that may be formed depending on measurement conditions.

6.2.5.3 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY AND CIRCULAR DICHROISM

NMR is the most powerful technique for structure elucidation. Through the ¹H NMR spectrum, the structural surroundings of each hydrogen atom in the carotenoid can be identified. The ¹³C NMR spectra serve to identify the type of each carbon atom and its surroundings. Thus NMR spectra are essential in the structure elucidation of a new carotenoid. Detailed ¹H and ¹³C NMR may prove knowledge of the structure beyond doubt, including the geometry of carbon-carbon double bonds and relative stereo-chemistry. However, NMR does not give information on the absolute configuration of a compound.

NMR spectrum identification is very demanding and requires training because carotenoids have great numbers of protons (most have 56 protons), and many of them are chemically similar but not exactly equivalent.

This means that most protons located in the polyene chain absorb at almost the same frequency, giving overlapped signals in the region from 6.1 to 6.7 ppm. Moreover, interaction of protons implies that the signal of a distinct proton is split and may appear as doublet, triplet, quartet, or even multiplet signals. This multiplicity turns the spectra even more complex, requiring the performance of two-dimensional (2D) NMR experiments such as homonuclear ¹H, ¹H-correlated spectroscopy (COSY) — the most useful technique for assignment of the NMR signals of carotenoids.

However, several hours of measurement are typically needed to achieve good signal-to-noise and high resolution, especially for 2D techniques. Other 2D techniques were used for structure elucidation of carotenoids from guava²³ and annatto seeds.^{42–44}

Measurement of a routine NMR spectrum requires very pure compound in greater amounts than those needed for UV-Vis and MS — about 100 to 200 µg for ¹H and 0.5 to 1 mg for ¹³C spectra. Even more material is needed for 2D techniques.

Both MS and NMR coupling to HPLC have been employed for the analysis of β-carotene isomers⁸⁰ and determination of lutein and zeaxanthin isomers in spinach, sweet corn,⁷⁷ and in retina.⁷⁶ Capillary high performance liquid chromatography with stop flow connected to NMR (600 MHz) was used for structure elucidation of all-*trans* deoxylutein II and its isomers.¹⁰⁰ Efforts are in progress to eliminate the remaining major drawbacks such as obligatory use of deuterated solvents in the mobile phase, poor sensitivity, and low throughput of HPLC-NMR analyses.

Englert¹⁰¹ published the best and greatest compilation of chemical shifts of carotenoids, including some isomers, which are essential for interpretation of NMR spectra.

The determination of the absolute configuration of a carotenoid is only possible by circular dichroism (CD) measurement. The spectrum interpretation can only be done by comparison with reference or model compounds with known chiralities. The sample requirement is as low as 5 to 50 µg, but CD facilities are not so commonly available. Buchecker and Noack¹⁰² reported experimental aspects and discussion of the relationships of carotenoid structures and CD spectra.

6.2.6 QUANTIFICATION

Carotenoid concentration in solution can be calculated taking in consideration the absorption coefficient ($A_{1\text{ cm}}^{1\%}$) or molar absorptivity (ϵ) values of the specific carotenoid according to the Beer-Lambert law. The absorption coefficient and ϵ values were determined for many carotenoids and values are available in the literature.^{89,90} These two values are related as shown in Equation 1. The concentration is calculated according to Equation 2.

$$\epsilon = \frac{A_{1\text{ cm}}^{1\%} \times \text{molecular mass}}{10} \quad [\text{Eq. 1}]$$

$$m = \frac{a \times \text{vol} \times 10^4}{A_{1\text{ cm}}^{1\%}} \quad [\text{Eq. 2}]$$

where m is weight in micrograms of carotenoid, a is absorbance, and vol is the volume used for dilution in mL.

For estimation of the total carotenoid content of a sample, generally the absorbance is measured in petroleum ether at 450 nm and a mean value of 2500 for $A_{1\text{ cm}}^{1\%}$ is used. In case a known carotenoid is present in the sample, the absorbance measured at the λ_{max} of that carotenoid and its corresponding $A_{1\text{ cm}}^{1\%}$ can be used for quantifi-

cation. The provitamin A values are calculated according to the conversion factor given by NAS-NRC,¹⁰³ 6 μg of β -carotene correspond to 1 μg of retinol equivalent (RE), and the activities are related as follows: 100% for all-*trans* β -carotene and 50% for all-*trans* β -cryptoxanthin and for all-*trans* α -carotene.¹⁰⁴

For HPLC, it is necessary to establish the relationship between the detector signal, of which the most used is peak area, and the concentrations of the pigments. Calibration curves for external quantification should be constructed for each carotenoid. Internal calibration is also used for quantification of carotenoids, using as internal standards all-*trans*- β -apo-8-carotenal,^{14,71,105} Sudan I,¹⁵ and decapreno- β -carotene.¹⁴

One point of discussion is the number of necessary concentration points to construct calibration curves. Statistics demonstrated that it is not necessary to use more than six points.¹⁰⁶ Because of the difficulty of handling carotenoid standards, a minimum coefficient of correlation of 0.9 was suggested by Khachik et al.¹⁰⁷ while Mantoura and Repeta¹⁰⁸ recommended a coefficient above 0.95. The curves should intercept close to the zero value.

The accuracy and precision of carotenoid quantification by HPLC depend on the standard purity and measurement of the peak areas; thus quantification of overlapping peaks can cause high variation of peak areas. In addition, preparation and dilution of standard and sample solutions are among the main causes of error in quantitative analysis. For example, the absorbance levels at λ_{max} of lutein in concentrations up to 10 mM have a linear relationship between concentration and absorbance in hexane and MeOH; on the other hand, the absorbance of β -carotene in hexane increased linearly with increasing concentration, whereas in MeOH, its absorbance increased linearly up to 5 mM but non-linearly at increasingly higher concentrations.¹⁰⁹ In other words, when a stock solution of carotenoids is prepared, care should be taken to ensure that the compounds are fully soluble at the desired concentrations in a particular solvent.

An interlaboratory study using mixed vegetable reference material showed average relative standard deviations (RSDs) of 23% ranging from 11% for lutein and α -carotene to 40% for lycopene.¹¹⁰ Triplicate HPLC injections of the same extract showed RSD values of 0% for β -carotene and 6.8% for lutein.¹²

6.2.6.1 STANDARDS

Traditionally, carotenoid standards are prepared in each laboratory using the best sources of each individual carotenoid, for example, violaxanthin from spinach, antheraxanthin from potatoes, capsanthin and capsorubin from paprika, α - and β -carotene from carrots, and lycopene from tomatoes.

The simplest and cheapest procedure to obtain standards is based on selective extraction followed by crystallization. A method developed to obtain lycopene from tomato residue using factorial experimental design consisted of a preliminary water removal with ethanol, followed by extraction with EtOAc and two successive crystallization processes using dichloromethane and ethanol (1:4), producing lycopene crystals with 98% purity, measured by HPLC-PDA.³⁰ Using this approach, bixin was extracted with EtOAc from annatto seeds that were previously washed with

hexane and MeOH; crystallization was achieved with the same pair of solvents used for lycopene.¹¹¹

Chromatography may be necessary to separate some carotenoids. A saponified carotenoid extract from carrots was applied to OCC on MgO and two fractions were eluted. Successive crystallization with petroleum ether and MeOH was carried out to obtain α - and β -carotene crystals, with 99 and 98% purity levels, respectively.³⁵

6.2.7 CONCLUDING REMARKS

It is recognized that carotenoid analysis is inherently difficult and errors can be introduced in all steps. Thus continued efforts focusing on analytical refinements are justified so that analytical variability is not mistaken for natural variation of samples.

HPLC continues to become more available in many countries as the price of the required equipment decreases. However, although the results obtained with HPLC show high precision, unfortunately the same is not true regarding their accuracy.

Modern instruments such as HPLC-PDA-MS take advantage of chromatography as a separation method and both PDA and MS as identification methods, providing at the same time information on chromophore and molecular weight for each individual peak in a chromatogram. The identification of a carotenoid based only on its UV-Vis spectrum obtained by PDA and elution order in a HPLC column should be avoided because it is easy to misidentify some carotenoids, especially those corresponding to the minor peaks. If MS is not available, co-chromatography with commercial or laboratory-prepared standards is imperative. A carotenoid structure is unequivocally established only by NMR spectroscopy including also CD for carotenoids with chiral centers or atoms. In addition, the assignment of the *cis* double bond position is only possible by NMR.

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REFERENCES

1. Mercadante, A.Z. and Egeland, E.S., *Handbook of Carotenoids*. Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhauser, Basel, 2004.
2. Sharpless, K.E. et al., Value assignment of retinol, retinyl palmitate, tocopherol, and carotenoid concentrations in standard reference material 2383 (baby food composite), *J. AOAC Int.*, 82, 288, 1999.
3. Assunção, R.B. and Mercadante, A.Z., Carotenoids and ascorbic acid composition from commercial products of cashew apple (*Anacardium occidentale* L.), *J. Food Compos. Anal.*, 16, 647, 2003.
4. Breithaupt, D.E., Simultaneous HPLC determination of carotenoids used as food coloring additives: applicability of accelerated solvent extraction, *Food Chem.*, 86, 449, 2004.

5. Breithaupt, D.E. and Bamedi, A., Carotenoids and carotenoid esters in potatoes (*Solanum tuberosum* L.): new insights into an ancient vegetable, *J. Agric. Food Chem.*, 50, 1715, 2002.
6. Chen, J.P.; Tai, C.Y., and Chen, B.H., Improved liquid chromatographic method for determination of carotenoids in Taiwanese mango (*Mangifera indica* L.), *J. Chromatogr. A*, 1054, 261, 2004.
7. Mangels, A.R. et al., Carotenoid content of fruits and vegetables: an evaluation of analytic data, *J. Am. Diet. Assoc.*, 93, 284, 1993.
8. Schiedt, K. and Liaaen-Jensen, S., Isolation and analysis, in *Carotenoids: Isolation and Analysis*, 1A, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 81.
9. Holden, J.M., Bhagwat, S.A., and Patterson, K.Y., Development of a multi-nutrient data quality evaluation system, *J. Food Compos. Anal.*, 15, 339, 2002.
10. Assunção, R.B. and Mercadante, A.Z., Carotenoids and ascorbic acid from cashew apple (*Anacardium occidentale* L.): variety and geographic effects, *Food Chem.*, 81, 495, 2003.
11. De Rosso, V.V. and Mercadante, A.Z., Carotenoid composition of two Brazilian genotypes of acerola (*Malpighia punicifolia* L.) from two harvests, *Food Res. Int.*, 38, 1073, 2005.
12. Hamano, P.S. and Mercadante, A.Z., Composition of carotenoids from commercial products of caju (*Spondias lutea*), *J. Food Compos. Anal.*, 14, 335, 2001.
13. Li, H. et al., Determination of carotenoids and all-*trans*-retinol in fish eggs by liquid chromatography–electrospray ionization–tandem mass spectrometry, *J. Chromatogr. B*, 816, 49, 2005.
14. Khachik, F., Beecher, G.R., and Whittaker, N.F., Separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by liquid chromatography, *J. Agric. Food Chem.*, 34, 603, 1986.
15. De Ancos, B., Gonzalez, E., and Cano, M.P., Effect of high-pressure treatment on the carotenoid composition and the radical scavenging activity of persimmon fruit purees, *J. Agric. Food Chem.* 48, 3542, 2000.
16. Hart, D.J. and Scott, K.J., Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK, *Food Chem.*, 54, 101, 1995.
17. Meléndez-Martínez, A.J. et al., Color and carotenoid profile of Spanish Valencia late ultrafrozen orange juices, *Food Res. Int.*, 38, 931, 2005.
18. Schlatterer, J. and Breithaupt, D.E., Cryptoxanthin structural isomers in oranges, orange juice, and other fruits, *J. Agric. Food Chem.*, 53, 6355, 2005.
19. Carmona, M. et al., Crocetin esters, picrocrocetin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits. Tentative identification of seven new compounds by LC-ESI-MS, *J. Agric. Food Chem.*, 54, 973, 2006.
20. Pfister, S. et al., Isolation and structure elucidation of carotenoid-glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne), *J. Agric. Food Chem.*, 44, 2612, 1996.
21. Van Calsteren et al., Spectroscopy characterization of crocetin derivatives from *Crocus sativus* and *Gardenia jasminoides*, *J. Agric. Food Chem.*, 45, 1055, 1997.
22. Meyer, P., Riesen, R., and Pfander, H., Example 10: carotenoid glycosides and glycosyl esters, in *Carotenoids: Isolation and Analysis*, 1A, Britton, G., Liaaen-Jensen, S., Pfander, H., Eds., Birkhäuser, Basel, 1995, 277.
23. Mercadante, A.Z., Steck, A., and Pfander, H., Carotenoids from guava: isolation and structure elucidation, *J. Agric. Food Chem.*, 47, 145, 1999.

24. Pott, I. et al., Quantitative determination of β -carotene stereoisomers in fresh, dried, and solar-dried mangoes (*Mangifera indica* L.), *J. Agric. Food Chem.*, 51, 4527, 2003.
25. Pérez-Gálvez, A., Hornero-Méndez, D., and Mínguez-Mosquera, M.I., Dependence of carotenoid content and temperature-time regimes during the traditional slow drying of red pepper for paprika production at La Vera County, *Eur. Food Res. Technol.*, 221, 645, 2005.
26. Pereira, M.R., Amaya-Farfan, J., and Rodriguez-Amaya, D.B., Avaliação da metodologia analítica para determinação de β -caroteno em macarrão fortificado, *Ciênc. Tecnol. Aliment.*, 18, 35, 1998.
27. Tocchini, L. and Mercadante, A.Z., Extraction and determination of bixin and norbixin, by HPLC, in annatto spice ("colorífico"), *Ciênc. Tecnol. Aliment.*, 21, 310, 2001.
28. Rios, A.O. and Mercadante, A.Z., Novel method for the determination of added annatto colour in extruded corn snack products. *Food Addit. Contamin.*, 21, 125, 2004.
29. Müller, H., Determination of the carotenoid content in selected vegetables and fruit by HPLC and photodiode array detection, *Z. Lebensm. Unters Forsch. A*, 204, 88, 1997.
30. Nunes, I.L. and Mercadante, A.Z., Production of lycopene crystals from tomato waste, *Ciênc. Tecnol. Alim.*, 24, 440, 2004.
31. De França, L.F. et al., Supercritical extraction of carotenoids and lipids from buriti (*Mauritia flexuosa*), a fruit from the Amazon region, *J. Supercrit. Fluids*, 14, 247, 1999.
32. Gnaayfed, M.H. et al., Supercritical CO₂ and subcritical propane extraction of pungent paprika and quantification of carotenoids, tocopherols, and capsaicinoids, *J. Agric. Food Chem.*, 49, 2761, 2001.
33. Sovová, H., Near-critical extraction of pigments and oleoresin from stinging nettle leaves, *J. Supercrit. Fluids*, 30, 213, 2004.
34. Mercadante, A.Z. and Rodriguez-Amaya, D.B., Carotenoid composition of a leafy vegetable in relation to some agricultural variables, *J. Agric. Food Chem.*, 39, 1094, 1991.
35. Zanatta, C.F. and Mercadante, A.Z., Carotenoid composition from the Brazilian tropical fruit camu-camu (*Myrciaria dubia*), *Food Chem.*, 101, 1543, 2007.
36. Lee, H.S. and Castle, W.S., Seasonal changes of carotenoid pigments and color in Hamlin, Earlygold, and Budd blood orange juices, *J. Agric. Food Chem.*, 49, 877, 2001.
37. Humphries, J.M. and Khachik, F., Distribution of lutein, zeaxanthin, and related geometrical isomers in fruit, vegetables, wheat, and pasta products, *J. Agric. Food Chem.*, 51, 1322, 2003.
38. Cortés et al., Identification and quantification of carotenoids including geometrical isomers in fruit and vegetable juices by liquid chromatography with ultraviolet-diode array detection, *J. Agric. Food Chem.*, 52, 2203, 2004.
39. Schiedt, K., Bischof, S., and Glinz, E., Metabolism of carotenoids and *in vivo* racemization of (3S,3S)-astaxanthin in the crustacean *Penaeus*, *Meth. Enzymol.*, 214, 148, 1993.
40. Stewart, I., and Wheaton, T.A., Conversion of β -citraurin to reticulaxanthin and β -apo-8'-carotenal to citranaxanthin during isolation of carotenoids from citrus, *Phytochemistry*, 12, 2947, 1973.
41. Breithaupt, D.E., Wirt, U., and Bamedi, A., Differentiation between lutein monoester regioisomers and detection of lutein diesters from marigold flowers (*Tagetes erecta* L.) and several fruits by liquid chromatography-mass spectrometry, *J. Agric. Food Chem.* 50, 66, 2002.

42. Mercadante, A.Z., Steck, A., and Pfander, H., Isolation and identification of new apocarotenoids from annatto (*Bixa orellana* L.) seeds, *J. Agric. Food Chem.*, 45, 1050, 1997.
43. Mercadante, A.Z., Steck, A., and Pfander, H., Isolation and structure elucidation of minor carotenoids from annatto (*Bixa orellana* L.) seeds, *Phytochemistry*, 46, 1379, 1997.
44. Mercadante, A.Z., Steck, A., and Pfander, H., Three minor carotenoids from annatto (*Bixa orellana* L.) seeds, *Phytochemistry*, 52, 135, 1999.
45. Sander, L.C., Sharpless, K.E., and Pursch, M., C₃₀ Stationary phases for the analysis of food by liquid chromatography, *J. Chromatogr. A*, 880, 189, 2000.
46. Sander, L.C. et al., Development of engineered stationary phases for the separation of carotenoid isomers, *Anal. Chem.*, 66, 1667, 1994.
47. Emenhisier C., Sander L.C., and Schwartz, S.J., Capability of a polymeric C₃₀ stationary phase to resolve *cis-trans* carotenoid isomers in reversed-phase liquid chromatography, *J. Chromatogr. A*, 707, 105, 1995.
48. Epler, K.S. et al., Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids, *J. Chromatogr.*, 595, 89, 1992.
49. *Official Methods of Analysis*, Association of Official Analytical Chemists, Arlington, VA, 1998, Method 970.64.
50. Almeida, L.B. and Penteado, M.V.C., Carotenoids with pro-vitamin A activity of carrots (*Daucus carota* L.) consumed in São Paulo, Brazil, *Rev. Farm. Bioquim. Univ. S. Paulo*, 23, 133, 1987.
51. Kimura, M., Rodriguez-Amaya, D.B. and Yokoyama, S.M., Cultivar differences and geographic effects on the carotenoid composition and vitamin A value of papaya, *Lebensm. Wiss.u. Technol.*, 24, 415, 1991.
52. Almeida, L.B. and Penteado, M.V.C., Carotenoids and pro-vitamin A value of "mandioquinha" (*Arracacia xanthorrhiza* Bancr.) consumed in São Paulo, *Rev. Farm. Bioquim. Univ. S. Paulo*, 23, 52, 1987.
53. Almeida, L.B. and Penteado, M.V.C., Carotenoids and pro-vitamin A value of white fleshed Brazilian sweet potatoes (*Ipomoea batatas* Lam.), *J. Food Compos. Anal.*, 1, 341, 1988.
54. Mercadante, A.Z. and Rodriguez-Amaya, D.B., Confirmation of the identity of α -cryptoxanthin and incidence of minor provitamin A carotenoids in green leafy vegetables, *Ciênc. Tecnol. Alim.*, 21, 216, 2001.
55. Britton, G., Example 1: higher plants, in *Carotenoids: Isolation and Analysis*, 1A, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 201.
56. Mercadante A.Z., Britton, G., and Rodriguez-Amaya, D.B., Carotenoids from yellow passion fruit (*Passiflora edulis*), *J. Agric. Food Chem.*, 46, 4102, 1998.
57. Mercadante A.Z. and Rodriguez-Amaya, D.B., Screening of carotenoids: comparison of thin-layer chromatography with high-efficiency thin-layer chromatography, with multiple development, *Ciênc. Tecnol. Alim.*, 11, 200, 1991.
58. Ben-Aziz, A., Britton, G., and Goodwin, T.W., Carotene epoxides of *Lycopersicon esculentum*, *Phytochemistry*, 12, 2759, 1973.
59. Britton, G. and Goodwin, T.W., Carotene epoxides from the Delta tomato mutant, *Phytochemistry*, 14, 2530, 1975.
60. Britton, G. and Goodwin, T.W., The occurrence of phytoene-1,2-oxide and related carotenoids in tomatoes, *Phytochemistry*, 8, 2257, 1969.
61. Meléndez-Martínez, A.J. et al., Identification of zeinoxanthin in orange juices, *J. Agric. Food Chem.*, 53, 6362, 2005.

62. Mercadante A.Z., Rodriguez-Amaya, D.B., and Britton, G., HPLC and mass spectrometric analysis of carotenoids from mango, *J. Agric. Food Chem.*, 45, 120, 1997.
63. Chandler, L.A. and Schwartz, S.J., HPLC separation of *cis-trans* carotene isomers in fresh and processed fruits and vegetables, *J. Food Chem.*, 52, 669, 1987.
64. Schmitz, H.H., Emenhiser, C., and Schwartz, S.J., HPLC separation of *cis-trans* carotene isomers using a calcium hydroxide stationary phase, *J. Agric. Food Chem.*, 43, 1212, 1995.
65. Almela et al., Carotenoid composition of new cultivars of red pepper for paprika, *J. Agric. Food Chem.*, 39, 1606-1609, 1991.
66. Kamber, M. and Pfander, H., Separation of carotenoids by high performance liquid chromatography. III. 1,2-epoxycarotenoids, *J. Chromatogr.*, 295, 295, 1984.
67. Nyambaka, H. and Ryley, J., An isocratic reversed-phase HPLC separation of the stereoisomers of the provitamin A carotenoids (α - and β -carotene) in dark green vegetables, *Food Chem.*, 55, 63, 1996.
68. Schierle, J. et al., Example 8: geometrical isomers of β , β -carotene, in *Carotenoids: Isolation and Analysis*, 1A, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 265.
69. Gama, J.J.T. and Sylos, C.M., Major carotenoid composition of Brazilian Valencia orange juice: identification and quantification by HPLC, *Food Res. Int.*, 38, 899, 2005.
70. Meléndez-Martínez, A.J., Vicario, I.M., and Heredia, F.J., A routine high-performance liquid chromatography method for carotenoid determination in ultrafrozen orange juices, *J. Agric. Food Chem.*, 51, 4219, 2003.
71. Mezadri, T., Pérez-Gálvez, A., and Hornero-Méndez, D., Carotenoid pigments in acerola fruits (*Malpighia emarginata* DC.) and derived products, *Eur. Food Res. Technol.*, 220, 63, 2005.
72. Mouly, P.P., Gaydou, E.M., and Corsetti, J., Determination of the geographical origin of Valencia orange juice using carotenoid liquid chromatographic profiles, *J. Chromatogr. A*, 844, 149, 1999.
73. Perkins-Veazie, P. et al., Carotenoid content of 50 watermelon cultivars, *J. Agric. Food Chem.*, 54, 2593, 2006.
74. Emenhiser, C. et al., Separation of geometrical carotenoid isomers in biological extracts using a polymeric C_{30} column in reversed-phase liquid chromatography, *J. Agric. Food Chem.*, 44, 3887, 1996.
75. Aman, R., Schieber, A., and Carle, R., Effects of heating and illumination on *trans-cis* isomerization and degradation of β -carotene and lutein in isolated spinach chloroplasts, *J. Agric. Food Chem.*, 53, 9512, 2005.
76. Dachtler, M. et al., Combined HPLC-MS and HPLC-NMR on-line coupling for the separation and determination of lutein and zeaxanthin stereoisomers in spinach and in retina, *Anal. Chem.*, 73, 667, 2001.
77. Aman, R. et al., Application of HPLC coupled with DAD, APcI-MS and NMR to the analysis of lutein and zeaxanthin stereoisomers in thermally processed vegetables, *Food Chem.*, 92, 753, 2005.
78. Emenhiser, C. et al., Isolation and structural elucidation of the predominant geometrical isomers of α -carotene, *J. Chromatogr. A*, 719, 333, 1996.
79. Brunner, M.R., Thermische (*E/Z*)-isomerisierung von Carotinoiden, PhD Thesis, University of Berne, Berne, 1997.
80. Strohschein, S., Pursch, M., and Albert, K., Hyphenation of high performance liquid chromatography with nuclear magnetic resonance spectroscopy for the characterization of β -carotene isomers employing a C_{30} stationary phase, *J. Pharm. Biom. Anal.*, 21, 669, 1999.

81. Khachik, F. and Beecher, G.R., Separation and identification of carotenoids and carotenol fatty acid esters in some squash products by liquid chromatography. 1. Quantification of carotenoids and related esters by HPLC, *J. Agric. Food Chem.*, 36, 929, 1988.
82. Breithaupt, D.E. and Schwack, W., Determination of free and bound carotenoids in paprika (*Capsicum annuum* L.) by LC/MS, *Eur. Food Res. Technol.*, 211, 52, 2000.
83. Epler, K.S., Ziegler, R.G., and Craft, N.E., Liquid chromatographic method for the determination of carotenoids, retinoids and tocopherols in human serum and in food, *J. Chromatogr.*, 619, 37, 1993.
84. Bell, C.M., Sander, L.C., and Wise, S.A., Temperature dependence of carotenoids on C₁₈, C₃₀ and C₃₄ bonded stationary phases, *J. Chromatogr. A*, 757, 29, 1997.
85. Sander, L.C. et al., Separation of carotenoid isomers by capillary electrochromatography with C₃₀ stationary phases, *Anal. Chem.*, 71, 3477, 1999.
86. Wei, Y. et al., Application of analytical and preparative high-speed counter-current chromatography for separation of lycopene from crude extract of tomato paste, *J. Chromatogr. A*, 929, 169, 2001.
87. Tsao, R. and Yang, R., Lutein in selected Canadian crops and agri-food processing by-products and purification by high-speed counter-current chromatography, *J. Chromatogr. A*, 1112, 202, 2006.
88. Liaaen-Jensen, S., Combined approach: identification and structure elucidation of carotenoids, in *Carotenoids: Spectroscopy*, 1B, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 343.
89. Davies, B.H., Carotenoids, in *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Goodwin, T.W., Ed., Academic Press, London, 1976, 38.
90. Britton, G., UV/visible spectroscopy, in *Carotenoids: Spectroscopy*, 1B, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 13.
91. Meléndez-Martínez, A.J. et al., Identification of isolutein (lutein epoxide) as *cis*-antheraxanthin in orange juice, *J. Agric. Food Chem.*, 53, 9369, 2005.
92. Enzell, C.R. and Back, S., Mass spectrometry, in *Carotenoids: Spectroscopy*, 1B, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 261.
93. Enzell, C.R., Francis, G.W., and Liaaen-Jensen, S., Mass spectrometric studies of carotenoids. 1. Occurrence and intensity ratios of M-92 and M-106 peaks, *Acta Chem. Scand.*, 22, 1054, 1968.
94. Enzell, C.R., Francis, G.W., and Liaaen-Jensen, S., Mass spectrometric studies of carotenoids. 2. Survey of fragmentation reactions, *Acta Chem. Scand.*, 3, 727, 1969.
95. Van Breemen, R.B., Schmitz, H.H., and Schwartz, S.J., Fast atom bombardment tandem mass spectrometry of carotenoids, *J. Agric. Food Chem.*, 43, 384, 1995.
96. Van Breemen, R.B., Electrospray liquid chromatography-mass spectrometry of carotenoids, *Anal. Chem.*, 67, 2004, 1995.
97. Rentel, C. et al., Silver-plated vitamins: a method of detecting tocopherols and carotenoids in LC/ESI-MS coupling, *Anal. Chem.*, 70, 4394, 1998.
98. Van Breemen, R.B. et al., Liquid chromatography/mass spectrometry of carotenoids using atmospheric pressure chemical ionization, *J. Mass Spectrom.*, 31, 975, 1996.
99. Tian, Q., Duncan C.J.G., and Schwartz S. J., Atmospheric pressure chemical ionization mass spectrometry and in-source fragmentation of lutein esters, *J. Mass Spectrom.*, 38, 990, 2003.
100. Hentschel, P. et al., Structure elucidation of deoxylutein II isomers by on-line capillary high performance liquid chromatography-¹H nuclear magnetic resonance spectroscopy, *J. Chromatogr. A*, 1112, 285, 2006.

101. Englert, G., NMR spectroscopy, in *Carotenoids: Spectroscopy*, 1B, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 147.
102. Buchecker, R. and Noack, K., Circular dichroism, in *Carotenoids: Spectroscopy*, 1B, Britton, G., Liaaen-Jensen, S., and Pfander, H., Eds., Birkhäuser, Basel, 1995, 63.
103. NAS-NRC, *Recommended Dietary Allowances*, National Academy of Science, Washington, 1989, 78.
104. Bauerfeind, J.C., Carotenoid vitamin A precursors and analogs in foods and feeds, *J. Agric. Food Chem.*, 20, 456, 1972.
105. Tonucci, L.H. et al., Carotenoid content of thermally processed tomato-based food products, *J. Agric. Food Chem.*, 43, 579, 1995.
106. Caulcutt, R. and Boddy, R., *Statistics for Analytical Chemists*, Chapman & Hall, London, 1983.
107. Khachik, F. et al., Separation and quantification of carotenoids in foods, *Meth. Enzymol.*, 213, 347, 1992.
108. Mantoura, R.F.C. and Repeta, D.J., Calibration methods for HPLC, in *Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods*, Jeffrey, S.W., Mantoura, R.F.C., and Wright, S.W., Eds., Unesco Publishing, Paris, 1997, 407.
109. Zang, L.Y., Sommerburg, O., and Van Kuuk, F.J.G.M., Absorbance changes of carotenoids in different solvents, *Free Rad. Biol. Med.*, 23, 1086, 1997.
110. Scott, K. et al., Interlaboratory studies of HPLC procedures for the analysis of carotenoids in foods, *Food Chem.*, 57, 85, 1996.
111. Rios, A.O. and Mercadante, A.Z., Optimization of the conditions to obtain bixin crystals and for extraction and saponification to quantify bixin in extruded snacks by HPLC, *Alim. Nutr.*, 15, 203, 2004.

6.3 Analysis of Anthocyanins

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6.3.1 INTRODUCTION

Anthocyanins are water-soluble plant pigments, responsible for the red, purple, and blue colors of many fruits, vegetables, cereal grains, and flowers. They have long been the subjects of investigation by botanists and plant physiologists because of their roles as pollination attractants and phytoprotective agents.^{1,2} In addition, anthocyanins have found considerable potential in the food industry as safe and effective food colorants.^{2,3} Interest in anthocyanins as potential natural food colors has increased in recent decades. Anthocyanin pigments are receiving extra attention due to their possible health benefits as dietary antioxidant,⁴⁻⁸ antimutagenic,^{9,10} and chemopreventive¹¹⁻²⁰ nutraceuticals that contribute to reduced incidences of chronic diseases and the prevention of obesity and diabetes.^{21,22}

Anthocyanins belong to the class of flavonoid compounds that are widely distributed as plant polyphenols. The basic structures of aglycons or anthocyanidins contain C6-C3-C6 carbon skeletons of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavylium salts.²³ The anthocyanin pigments consist of two or three parts: an aglycon base (anthocyanidin), sugars, and possibly acylating groups. Nineteen different aglycon groups are known to occur naturally in nature,²⁴⁻²⁷ although only six of those occur frequently.^{2,28} Since each aglycon may be glycosylated and/or acylated by different sugars and cinnamic and aliphatic acids, about 539 different anthocyanins were reported from plants.²⁹

An anthocyanin occurs in solution as a mixture of different secondary structures, a quinonoidal base, a carbinol pseudobase, and a chalcone pseudobase.^{2,30} In addition, different mechanisms for the stabilization of anthocyanins lead to the formation of tertiary structures such as self-association, inter-, and intra-molecular co-pigmentation.²

Quantitative and qualitative anthocyanin composition must be known in order to determine the feasibility of application of new plant materials as anthocyanin-based colorant sources and to understand the relationships of structures and functions of anthocyanins. In addition, anthocyanin compositions of fruits and vegetables have also been used to detect adulteration of anthocyanin-based products^{31,32} and as indicators of product quality.^{33,34}

Many techniques for the analysis of anthocyanins have been used for almost a century and are still of importance, along with considerable advances in technologies such as mass spectroscopy (MS) and nuclear magnetic resonance (NMR). This section summarizes the analytical procedures for quantitative and qualitative analyses of anthocyanins, including classical and modern techniques.

6.3.2 PIGMENT EXTRACTION

6.3.2.1 TRADITIONAL EXTRACTION METHODS

The extraction of anthocyanins is the first step in the determination of both total and individual anthocyanins in any type of plant tissue.³⁵ The choice of an extraction method is of great importance in the analysis of anthocyanins and largely depends on the purpose of the extraction, the nature of the anthocyanins, and the source material. A good extraction procedure should maximize anthocyanin recovery with

a minimum number of adjuncts and minimal degradation or alteration of the natural (*in vivo*) state, although one can never be sure that the extracted pigment is exactly the one occurring *in vivo*.³⁶ A knowledge of the factors that influence anthocyanin structure and stability is vital. Those factors were reviewed by Markakis (1982),³⁷ Francis (1989),³⁸ and Jackman and Smith (1996).^{28,29} It is also desirable that an extraction procedure not be too complex, hazardous, time consuming, or costly.

Anthocyanin extraction depends on a sample matrix in which anthocyanins are present and on how anthocyanins interact with the matrix. Extraction procedures usually are simpler for liquid samples than for solid or semi-solid samples. For most solid materials, crushing or pulverization requires reducing sample size and increasing surface areas of material particles with extracting solvent by mortar and pestle, blender,³⁹ or mill. Liquid nitrogen powder has been used to minimize anthocyanin degradation during extraction and maximize recovery.^{40,41} Advantages of the use of liquid nitrogen to pulverize the sample are the inhibition of enzymatic reaction by lowering temperature, the exclusion of oxygen by a nitrogen environment, and maximization of the surface area by obtaining a very fine powder.⁴²

Many solvents such as methanol, acetone, ethanol, and water are used for anthocyanin extraction due to their polar character since most anthocyanins occur naturally as glycosides. Anthocyanin glycosides have higher solubility in water than the corresponding aglycons. In addition, in most fruits and vegetables, anthocyanin pigments are located in cells near the surface.³⁴

To obtain anthocyanins closer to their natural state, a number of researchers have used neutral solvents for initial extraction such as 60% methanol, *n*-butanol, cold acetone, mixtures of acetone, methanol, and water, or simply water.³⁴ Methanol is the most common solvent used for anthocyanin extraction. Metivier et al. (1980)⁴⁴ compared the efficiency of extraction with three different solvents (methanol, ethanol, and water) and different acids, and found that methanol extraction was 20% more effective than ethanol and 73% more effective than water when used for anthocyanin recovery from grape pomace.

Ethanol and water are preferred for food use to avoid the toxicity of methanolic solutions. However, recoveries are not as great as those obtained with methanol. An alternative procedure uses acetone as an extracting solvent followed by partition with chloroform to yield an aqueous phase (containing anthocyanins, phenolics, sugars, organic acids) and an organic phase (containing the bulk of solvents and lipid materials).^{39,45-47} An advantage of this procedure is that the chloroform-acetone mixture will partition lipids, chlorophyll, and other water-insoluble materials from anthocyanins, yielding high recoveries of anthocyanins and requiring few concentration and purification steps.

Timberlake and Bridle (1971)⁴⁶ and later Wrolstad and Durst (1998)⁴⁸ compared the acetone-chloroform method of extraction to acidified methanol and, depending on the material used, the anthocyanin recoveries were comparable or up to 30% higher with the use of acetone. This procedure proved efficient for a wide range of anthocyanin-rich plants to achieve high recoveries with minimal degradation.^{39,49,50}

Acids are very important both to stabilize anthocyanins in the aqueous environment necessary for obtaining the flavylium cation that is the most stable form and also to improve extraction efficiency. However, acids may also change the native

form of the pigment in the tissue by breaking associations with metals and/or co-pigments. Concentration procedures after extraction with acidified solvent may also favor acid hydrolysis of labile acyl and sugar residues. Extraction with solvents containing HCl may result in pigment degradation during concentration⁵¹ and is one reason acylation with aliphatic acids had been overlooked in the past.⁵²

To minimize the decomposition of pigments, the use of milder pigment extraction procedures has been proposed. This involves using weaker and volatile organic acids such as formic, acetic, citric, or tartaric acids or small amounts (0.01 to 3%) of stronger more volatile acids such as trifluoroacetic acid, which could be then removed during pigment concentration.^{2,28} Low HCl concentrations on the order of 0.01 to 0.05% and procedures in the absence of acid have also been proposed.^{2,34} These procedures must be performed with care to avoid acid-dependent pigment degradation.

Maceration of crushed or ground material in methanol containing small amounts of HCl (<1%) is commonly used at refrigerated temperatures for times ranging from a few hours to overnight. The extracted material is usually too dilute for further analyses and the extraction procedure is usually followed by evaporation of the methanol using vacuum and mild temperatures (30 to 40°C). Alternatively, the plant materials and solvents can be mixed well with a laboratory blender for a few minutes or a chemical-resistant stir bar for a longer time.³⁹ Concentration of anthocyanin extracts can be done by rotary evaporation under vacuum conditions for volatile solvents or lyophilization for water.

Depending on the means of extraction, decreasing the ratio of extraction solvent to plant material may avoid the need for a concentration step.²⁸ However, quantitative extractions usually require an adequate rate of solvent to material and two or more re-extractions until a clear or faintly colored solution is obtained.³⁹

Extraction procedures must be adjusted when separated anthocyanins will be tested in biological studies. We have found that the types of acids used for anthocyanin extraction as well as their residual concentrations in the final extract may affect the results obtained from biological tests. The growth inhibitory effect of anthocyanins on HT29 (human colonic cancer) cells may be overestimated if the residual acid in the extract exerts a toxic effect on the cells. Acetic acid residues in anthocyanin extracts showed less toxicity to HT29 cells than hydrochloric acid when samples were prepared under the same extraction procedure and subjected to the same tests on HT29 cells. In addition, the procedure to remove acids affected the acid residual concentration as well in final anthocyanin extracts, with lyophilization being more successful than rotary evaporation.

6.3.2.2 MODERN SEPARATION TECHNOLOGIES

In the past decade, new sample extraction techniques have been introduced to meet stricter criteria in the areas of food and agriculture, for example, environmentally friendly, non-toxic, fast, automated, robust, and cost-efficient techniques.⁵³ Accelerated solvent extraction (ASE) and pressurized liquid extraction (PLE) are two methods developed for the extraction of chemicals of interest^{54–57} and provide high yields and efficiency from a wide range of botanical,^{54,55,57,58} animal,⁵⁹ and biological⁵⁶ samples.⁶⁰ ASE and PLE combine solvents at elevated temperatures (40 to 200°C)

and pressures (500 to 3000 psi) with an inert gas for short periods (5 to 10 min). Since anthocyanins are heat-sensitive, the rate of anthocyanin degradation is accelerated at temperatures above 70°C.⁶¹

Ju and Howard (2003)⁵⁸ studied pressurized liquid extraction of anthocyanins from dried red grape skins with six solvents at temperatures ranging from 20 to 140°C using 10.1 MPa. They found that the type of solvent and the temperature used affected the types and levels of anthocyanins in the PLE extracts.

Supercritical fluid extraction (SFE) is another modern separation technology usually employed to extract lipophilic compounds such as cranberry seed oil,⁶² lycopene,⁶³ coumarins,⁶⁴ and other seed oils.⁶⁵ Anthocyanins generally and glycosylated anthocyanins in particular were considered unsuitable for SFE due to their hydrophilic properties, since SFE is applicable for non-polar analytes. However, a small amount of methanol was applied as co-solvent to increase CO₂ polarity in anthocyanin extraction from grape pomace.⁶⁶ New applications of SFE for anthocyanin purification have been reported for cosmetic applications from red fruits.⁶⁷

6.3.3 QUANTITATIVE ANALYSIS OF ANTHOCYANINS

Frequently, the quantitative determination of anthocyanins is complicated by the presence of other compounds that may interfere with the measurements. It is desirable to express anthocyanin determinations in terms that may be compared with results from different workers. The best way to express the results is in terms of absolute quantities of anthocyanins present.³⁵ This is necessary to establish the identities of the pigments and their molar absorptivity coefficients.

The methods of quantitation of anthocyanins can be divided into three major groups: methods for samples with few or no interfering compounds, methods for samples with interfering compounds that absorb in the 480 to 550 nm range, and methods for quantitation of individual anthocyanin components.

6.3.3.1 SAMPLES WITHOUT INTERFERING MATERIALS: SINGLE pH METHOD

The total anthocyanin content can often be determined in crude extracts containing other phenolic materials by measuring absorptivity of the solution at a single wavelength (Table 6.3.1). This is possible because anthocyanins have typical absorption bands in the 490 to 550 nm region of the visible spectra — far from the absorption bands of other phenolics with spectral maxima in the UV range.³⁵

The wavelength of maximum absorption and the molar absorptivity are very dependent on pH, buffer, temperature, solvent, and the presence of other materials that may interact with anthocyanins.⁶⁸ In addition, anthocyanin absorption follows a linear relationship with concentration only when present at low levels; therefore considerable dilution is usually necessary.³⁵ Absorbance normally should vary from 0.2 to 1.0 unit in order to obey Lambert-Beer's law. However, absorbance values as high as 1.5 to 2.0 absorbance units may be valid for sophisticated new instruments.

TABLE 6.3.1
Useful Equations for Monomeric Anthocyanin and Polymeric
Color Calculation

Method	Absorbance
Single pH	$(A_{\lambda_{vis-max}} - A_{700nm})_{pH 1}$
pH differential	$(A_{\lambda_{vis-max}} - A_{700nm})_{pH 1} - (A_{\lambda_{vis-max}} - A_{700nm})_{pH 4.5}$
Total monomeric anthocyanins (mg/L) = $(A \times MW \times DF \times 1000)/$	
Degradation index = $[(A_{\lambda_{vis-max}} - A_{700nm})_{pH 1}] / [(A_{\lambda_{vis-max}} - A_{700nm})_{pH 1} - (A_{\lambda_{vis-max}} - A_{700nm})_{pH 4.5}]$	
Color density (control)	$(A_{420nm} - A_{700nm}) + (A_{\lambda_{vis-max}} - A_{700nm})$
Polymeric density (bisulfite-treated sample)	$(A_{420nm} - A_{700nm}) + (A_{\lambda_{vis-max}} - A_{700nm})$
Browning index (bisulfite-treated sample)	$A_{420nm} - A_{700nm}$
Percent of polymeric color = polymeric color density $\times 100\%$ /color density	
The above equations assume a pathlength of 1 cm. $A_{\lambda_{vis-max}}$, A_{700nm} , and A_{420nm} represent absorbance of sample at maximum wavelength, 700 nm, and 420 nm at certain pH. DF = dilution factor. MW = molecular weight. = molar absorptivity.	

6.3.3.2 SAMPLES WITH INTERFERING MATERIALS: DIFFERENTIAL AND SUBTRACTIVE METHODS

6.3.3.2.1 Determination of Total Monomeric Anthocyanins by pH Differential Method

For many situations, a simple total anthocyanin determination is inappropriate because of interference from polymeric anthocyanins, anthocyanin degradation products, or melanoidins from browning reactions.⁶⁹ In those cases, the approach has been to measure the absorbance at two different pH values. The differential method measures the absorbance at two pH values and relies on structural transformations of the anthocyanin chromophore as a function of pH.⁶⁸ Anthocyanins switch from a saturated bright red-bluish color at pH 1 to colorless at pH 4.5. Conversely, polymeric anthocyanins and others retain their color at pH 4.5. Thus, measurement of anthocyanin samples at pH 1 and 4.5 can remove the interference of other materials that may show absorbance at the $\lambda_{vis-max}$.

This concept was introduced in 1948 by Sondheimer and Kertesz who used pH values of 2 and 3.4 for analyses of strawberry jams.³⁸ Since then, the use of other pH values has been proposed. Fuleki and Francis (1968)⁶⁹ used pH 1 and 4.5 buffers to measure anthocyanin content in cranberries, and modifications of this technique have been applied to a wide range of commodities.^{70,71} The absorbance of anthocyanins at two different pH levels (1 and 4.5) is measured at $\lambda_{vis-max}$ and at 700 nm, which corrects for haze in the sample (Table 6.3.1). The total monomeric anthocy-

anins are calculated by the molecular weights and molar absorptivities of the most abundant anthocyanins or cyanidin 3-glucoside for unknown anthocyanin samples (Table 6.3.1).

The pH differential method was described as a fast and convenient assay for the quantitation of monomeric anthocyanins by Giusti (2001).⁶⁸ It was approved by the Association of Official Analytical Chemists (AOAC) in 2005 as a standard method to evaluate total monomeric anthocyanin pigment content in fruit juices, beverages, natural colorants, and wines.⁷² The degradation index is the ratio between total and monomeric anthocyanins (Table 6.3.1). The content of total anthocyanins can be obtained by the single pH method and the monomeric anthocyanin by the pH differential method.⁶⁹

6.3.3.2.2 Determining Indices of Polymeric Color and Browning by Subtractive Methods

Subtractive methods are based on the use of bleaching methods that would decolor monomeric anthocyanins but not affect interfering polymeric color. A measurement of absorbance at the visible maximum is obtained, followed by bleaching and re-measuring to give a blank reading.³⁴ The most used bleaching agents are sodium or potassium metabisulfite^{68,70,73} and hydrogen peroxide.⁷⁴

Taking advantage of the differential and subtractive methods, a few absorbance readings can be used to obtain an estimation of the amount of monomeric anthocyanin, polymeric color, color density, browning index,⁷⁰ and degradation index.⁶⁹ The absorbance at 700 nm is used to correct for haze in the sample. The absorbance at 420 nm of the bisulfite-treated sample is an index for browning (Table 6.3.1), as accumulation of brownish degradation products increases the absorption in the range of 400 and 440 nm for polyphenols such as proanthocyanidins.

Absorbance is measured at $\lambda_{\text{vis-max}}$ and 420 nm of the bisulfite-treated sample and control sample. Consequently, color density is the sum of the absorbance at $\lambda_{\text{vis-max}}$ and 420 nm of a control sample while polymeric color density is the total absorbance at $\lambda_{\text{vis-max}}$ and 420 nm of the bisulfite-treated sample (Table 6.3.1). The methodologies used to measure color density and polymeric color were developed for fruit juices that naturally have acid pH levels. However, if the material to be measured has a pH in the neutral or alkaline range or the extraction methodology affects the stability of the final pH in anthocyanin extracts, the use of a 0.1 mol/L pH 3.5 citric acid buffer instead of distilled water is recommended.⁶⁸ The ratio of polymerized color and color density is used to determine the percentages of color contributed by polymerized compounds (Table 6.3.1).

6.3.3.3 CONTENTS OF INDIVIDUAL PIGMENTS

In many cases, determination of total anthocyanin content may not be enough and information about specific individual pigments will be required. Typically this situation arises in research situations.³⁸ One analytical method for the quantitative determination of individual anthocyanins involves their separation from a mixture and measurement of each individual pigment.^{34,75} Chromatographic separation of

individual pigments is usually the first step. Formerly, the individual anthocyanins were separated by paper chromatography followed by spectrophotometric estimation of the amount of individual pigment bound to the paper. The availability of an efficient method for separation of anthocyanins such as HPLC combined with a list of molar absorptivity coefficients should simplify the quantitative estimations of individual anthocyanins.^{38,76} The use of HPLC coupled to a photodiode array detector allows for separation of anthocyanins from even complex mixtures and for the determination of the $\lambda_{\text{vis-max}}$ of each individual anthocyanin.^{77,78}

Quantitation of anthocyanins has become simple and fast since many anthocyanin standards became commercially available as external standards in the past decade. When the standards are not available, individual anthocyanins or total monomeric anthocyanins can be determined by the use of a generic external standard such as commercial cyanidin-3-glucoside or other compound structurally similar to the analytes of interest. Individual and total peak areas are measured at 520 nm or their $\lambda_{\text{vis-max}}$ and quantified using external standards by which values are typically slightly different from those via the pH differential method.⁷⁹

6.3.3.4 MOLAR ABSORPTIVITY

The single pH and pH differential methods are generally considered UV-Vis spectrophotometric methods. Regardless of the spectrophotometric method used for anthocyanin quantitation, the determination of the amount present requires an absorptivity coefficient. Absorptivity coefficient value has been reported as the absorption of a 1% solution measured through a 1-cm path at the $\lambda_{\text{vis-max}}$ or as a molar absorption coefficient. Absorptivity coefficients of some known anthocyanins have been reported by different researchers.^{68,75,76,80–83} Through the years, the values of absorptivity reported have lacked uniformity, mainly due to the difficulties of preparing crystalline anthocyanin, free from impurities, in sufficient quantities to allow reliable weighing under optimal conditions.^{35,75,76}

Another problem is that the anthocyanin mixtures may be very complicated and not all absorptivity coefficients may be known. Even when they are known, it is necessary to first evaluate whether the objective is the estimation of total anthocyanin content or the determination of individual pigments, and then to decide which absorption coefficient(s) to use. The absorptivity is dependent on both the chemical structure of the pigment and also on the solvent used, and preferably the coefficient used should be one obtained in the same solvent system as the one used in the experiment.⁶⁸ If the identity of the pigment is unknown, it has been suggested that it could be expressed as cyanidin-3-glucoside³⁸ since that is the most abundant anthocyanin in nature.

6.3.4 QUALITATIVE ANALYSIS OF ANTHOCYANINS

Many methods are currently available for the qualitative analysis of anthocyanins including hydrolysis procedures,⁴⁹ evaluation of spectral characteristics,⁷⁶ mass spectroscopy (MS),^{84–87} nuclear magnetic resonance (NMR),^{2,52,76,88} and Fourier transform infrared (FTIR) spectroscopy.^{89–91} Frequently a multi-step procedure will be used for

anthocyanin characterization with a combination of these methods. However, a purification or isolation step is frequently necessary and more than one analytic method is recommended to identify and confirm anthocyanin structures.

6.3.4.1 PURIFICATION OF CRUDE EXTRACTS

Purification of anthocyanin-containing extracts is often necessary for further structural identification. Since none of the solvents used for extraction is specific for anthocyanins, considerable amounts of other compounds may be also extracted and concentrated. The variety and concentration of other compounds will depend on the solvent and methodologies used. The presence of extraneous materials could affect the stability and/or analysis of anthocyanins. Therefore, the next step toward anthocyanin characterization is the purification of those extracts.

6.3.4.1.1 Precipitation

When appreciable amounts of pectin, proteins, lipids, unwanted polyphenols, or other compounds are suspected to be present in anthocyanin-containing extracts, some of them can be precipitated or the anthocyanins may be crystallized and separated from the others.⁹² Pectin and proteins can be removed by organic solvents such as methanol and acetone in order to reduce their solubility, then precipitated and separated by centrifugation. Gelatin was used to remove proanthocyanidin due to its high molecular weight.⁹³ Anthocyanins were reported to be precipitated early by lead acetate to achieve isolation from other materials.⁹²

6.3.4.1.2 Solid Phase Purification

Different resins have been used to clean up or pre-fractionate anthocyanins prior to isolation or characterization: ion exchange resins, polyamide powders, and gel materials. Chromatography on Dowex or Amberlite ion exchange resins and on polyamide powders [e.g., polyvinyl pyrrolidone (PVP)] has been used to isolate polar non-phenolic compounds (sugar et al.) from crude anthocyanin extracts and concentrate anthocyanins.^{94,95} Amberlite GC-50 is a weakly acid ion exchange resin that has been used to remove sugar and concentrate anthocyanins. Amberlite XAD-7 polymeric adsorbent has intermediate polarity and adsorbs either hydrophobic materials from water or hydrophilic materials from non-aqueous systems. Its application in the recovery of phenolics was useful for anthocyanin purification from other compounds that are more hydrophilic or more hydrophobic than anthocyanins.^{96,97}

Column gel chromatography can be used for further isolation of anthocyanins using Sephadex LH-20.^{96,97} Sephadex LH-20 is useful for purification of individual anthocyanins⁵² and the separation of non-polymeric and polymeric phenols.⁹⁸

C-18 Sep Pak cartridges have become popular because of their ease of use and high efficiency for fractionating anthocyanins. In an aqueous phase, anthocyanins and other hydrophobic compounds are bound while more hydrophilic compounds such as acids and sugars can be washed away with water. The water can be slightly acidified with 0.01% HCl to stabilize the anthocyanins on the C18 resin.³⁹

The use of ethyl acetate was suggested by Oszmianski and Lee (1990)^{39,99} to wash out phenolics other than anthocyanins. Finally, a relatively pure anthocyanin extract can be removed from the column with acidified methanol (0.1% HCl). Anthocyanin extracts can be enriched in this way by use of solid phase purification, which is especially helpful for diluted samples such as biological samples. Two factors in the use of these purification techniques are the stability of anthocyanins to the conditions used and the ease of anthocyanin recovery from the column.⁵²

6.3.4.1.3 High Speed Countercurrent Chromatography (HSCCC)

Typically lipids, chlorophyll, and phenolic acids can be separated by liquid–liquid partition. Lipids and chlorophyll can be removed from acetone–water extracts by chloroform³⁹ while phenolic acids have higher affinities for ethyl acetate at a pH close to neutral and water.¹⁰⁰

HSCCC is attracting attention based on its high separation scale, 100% recovery of sample, and mild operating conditions.¹⁰¹ It is a chromatographic separation process based on the partition coefficients of different analytes in two immiscible solvent systems (mobile phase and stationary phase) subjected to a centrifugal acceleration field.¹⁰²

A biphasic solvent system composed of *tert*-butyl methyl ether, *n*-butanol, acetonitrile, and water (2:2:1:5) acidified with trifluoroacetic acid has been applied to fractionate anthocyanins.^{103–105} The upper (organic) phase acts as the stationary phase and the lower (aqueous) as the mobile phase. HSCCC has been applied to obtain several anthocyanin fractions from wine,¹⁰³ red cabbage, black currants, chokeberries,¹⁰⁴ bilberries (*Vaccinium myrtillus*),¹⁰⁶ acylated anthocyanins,¹⁰⁵ and also isolate individual anthocyanins from wine.¹⁰⁷

6.3.4.2 SEPARATION OF INDIVIDUAL ANTHOCYANINS

Chromatographic separation of anthocyanins plays a major role in analyses of the patterns of phenolic compounds in crude extracts.⁵²

6.3.4.2.1 Paper and Thin Layer Chromatography

Paper chromatography (PC) and thin layer chromatography (TLC) have been used since the 1940s. Preparative PC on Whatman #3 paper, analytical PC on Whatman #1 paper, and analytical TLC on microcrystalline cellulose, silica gel, or polyamide have been applied with a variety of solvents and the behaviors of anthocyanins have been similar in all media. Two-dimensional TLC allows the separation of several compounds and has been used to clarify the anthocyanin compositions of different commodities.⁷⁵

Some of the solvent systems frequently used include *n*-butanol, acetic acid, and water (BAW, 4:1:5), *n*-pentanol, acetic acid, and water (PAW, 2:1:1, acetic acid, concentrated HCl, and water (AHW, 25:3:72), and 1% aqueous HCl (water and concentrated HCl, 97:3). In general, anthocyanins with more glycosidic substitutions

will have lower R_f values in solvents like BAW and PAW, but higher R_f in AHW and 1% HCl, while the addition of acyl groups usually has the opposite effect.⁵²

6.3.4.2.2 High Performance Liquid Chromatography

HPLC has proved to be fast and sensitive for the analyses of phenolic plant constituents, and is especially useful for the analysis of anthocyanins.⁵² The first application of HPLC to anthocyanin analyses was in 1975 by Manley and Shubiak¹⁰⁸ and it has now become the method of choice for the separation of mixtures of anthocyanins and anthocyanidins.² HPLC is now used for anthocyanin qualitative, quantitative, and preparative work, offering improved resolution compared to chromatographic procedures previously employed. It also allows for simultaneous rapid monitoring of the eluting anthocyanins.^{78,109}

The most popular system is a reversed phase column (C18), on a silica base column. However, the use of C18 on a polymer-based column has been reported to provide better resolution, especially for the separation of complex anthocyanin mixtures containing acylated pigments.^{32,71} Polymer-based columns also show better stability at low pH operating conditions.

The overall polarity and stereochemistry of anthocyanins are the key factors for their separation. The order of elution will be dependent on the hydroxyl or methoxyl substitutions of the pyrylium ring, the number and nature of sugar substituents, and the presence, number, and nature of acylating groups. In general, more glycosylations or hydroxylations increase anthocyanin mobility, while O-methylations and acylations increase the elution time. Nevertheless, this is not a strict rule; deviations of this behavior may be dependent on the nature and position of the substitutions.^{2,71} For instance, 3-rutinoides have longer retention times than the corresponding 3-glucosides because of the non-polarity imparted by the methyl group of rhamnose at position C6.

Typical mobile phases are composed of gradients of acetic, phosphoric, or formic acid in water–methanol or water–acetonitrile solvents⁵² that separate anthocyanins as their flavylium cations and can be easily detected by their absorbance at their visible $\lambda_{\max}$. Different detectors are available for monitoring eluting anthocyanins, including monochromatic or multiple wavelength spectrophotometers, photodiode array detectors, and more recently, mass spectrometers. A photodiode array detector scans UV-Vis spectral data of the eluting sample every few seconds and allows spectroscopic interpretation of the data and detection of impurities co-eluting with the compounds of interest.^{2,77,78} Anthocyanins are usually inspected at 480 to 550 nm by comparing retention times and UV-Vis spectra with respective standards.

6.3.4.2.3 Capillary Electromigration (CE)

CE was recently used for anthocyanin analysis because of its excellent resolution. This technique has different modes: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary isotachopheresis (CITP).¹¹⁰ CZE is the most popular method for anthocyanin

analysis. It is an advantageous method consisting of separation principles of both chromatography and electrophoresis; it separates analytes based on their different electrophoretic mobilities, which depend on their charged-to-size ratios.¹¹¹

About 15 anthocyanins in bilberries (*Vaccinium myrtillus* L.) were reported by Ichianagi et al. using CZE separation and MS-NMR identification.¹¹² Anthocyanin analysis of strawberry and elderberry extracts was performed by reverse HPLC at pH 1.8 and CZE using a standard silica capillary and pH 8.0 running buffer. Under these conditions, HPLC had more advantages than CZE in terms of anthocyanin separation in these extracts.¹¹³

CZE is particularly useful for separating anthocyanin dimers or polymeric anthocyanins. Calvo et al. (2004)¹¹⁴ separated 13 anthocyanins by CZE including acylated and non-acylated anthocyanins, pyranoanthocyanins, and flavonol derivatives in wine. Saenz-Lopez et al. (2004)¹¹⁵ applied CZE to analyze wine aging (1 to 14 yr) as related to monomeric anthocyanins, anthocyanin derivatives, tannins, and flavonols. Bicard et al. (1999)¹¹⁶ reported the improved detection sensitivity of anthocyanin chemical degradation analysis by CZE.

6.3.4.3 CHARACTERIZATION AND IDENTIFICATION

6.3.4.3.1 Hydrolysis of Anthocyanins

Identification of the anthocyanins of interest usually requires the breakdown of a pigment into its components for structural elucidation using acid, alkaline and/or hydrogen peroxide hydrolyses.⁵² Hydrolysis of anthocyanins by boiling them with strong acid (2 N HCl) causes release of sugars and with them any acylating group present. However, it does not affect the structure of the aglycon, which can be then separated by HPLC.^{49,117} Of the 19 known naturally occurring anthocyanidins or aglycons based on the skeleton,²⁴⁻²⁷ only 6 are widely distributed, as compared to hundreds of anthocyanins known.

Acid hydrolysis prior to HPLC simplifies the chromatogram and provides definite conclusions on the identity of the aglycons, facilitating interpretation of the data. Anthocyanidins are unstable and precautions must be taken to achieve satisfactory results, avoiding oxygen and light exposure, and cooling down rapidly after hydrolysis. As a result, anthocyanidin analyses preferably should be performed immediately following hydrolysis. Controlled acid hydrolysis has been found helpful for the rapid structural elucidation of an unknown anthocyanin. Samples are hydrolyzed for different periods and the appearance and disappearance of intermediate glycosides formed from the original pigment are monitored until finally the aglycon is left.¹¹⁸ The pattern of intermediate glycosides will reflect the numbers and sites of attachments of the sugars, e.g., a 3-diglycoside will produce only one 3-glycoside as an intermediate step, while a 3,5-diglycoside will produce two different intermediates, a 3-glycoside and a 5-glycoside.⁵²

Saponification or hydrolysis with a base (10% KOH for 8 min) will not affect the glycosidic bond, but it will break down the ester linkages of acylating groups. This procedure can be used to determine with certainty which anthocyanins are

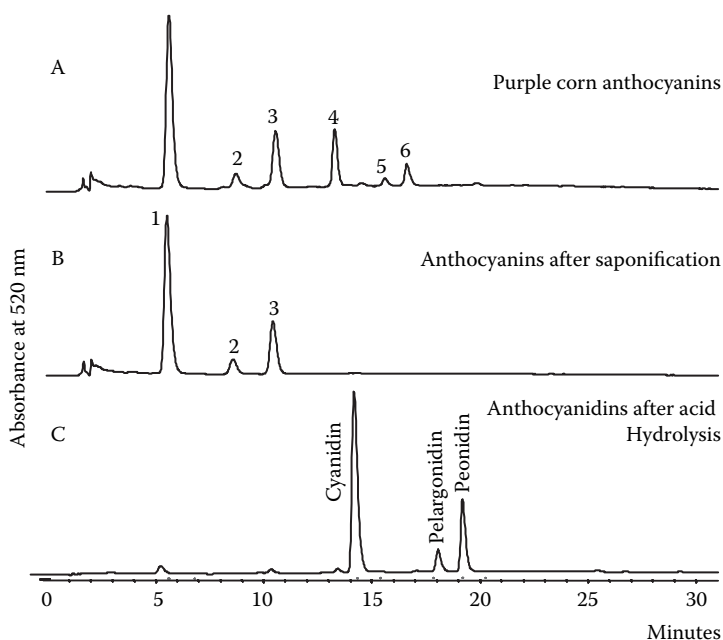


FIGURE 6.3.1 HPLC profiles of anthocyanins in purple corn cob anthocyanin-rich extract before and after saponification and acid hydrolysis: 1, cyanidin-3-glucoside; 2, pelargonidin-3-glucoside; 3, peonidin-3-glucoside; 4, cyanidin-3-(6''-malonylglucoside); 5, pelargonidin-3-(6''-malonylglucoside); and 6, peonidin-3-(6''-malonylglucoside). (Source: Jing, P. and Giusti, M.M., *J. Agric. Food Chem.*, 53, 8775, 2005.)

acylated and to separate cinnamic or aliphatic acids from the anthocyanin for later separation and determination of their identities.^{49,71,117}

For example, six peaks are present in the purple corn anthocyanin chromatograph (Figure 6.3.1A). After acid hydrolysis, only three peaks were left (Figure 6.3.1C), which were identified as cyanidin, pelargonidin, and peonidin with the aid of reference standards prepared from radish and grape anthocyanins.¹¹⁹ After alkaline hydrolysis, only the first three peaks in Figure 6.3.1A were retained in Figure 6.3.1B, which means these peaks should correspond to glycosylated anthocyanins in purple corn and the remaining peaks in Figure 6.3.1A to acylated anthocyanins.

Anthocyanin oxidation with hydrogen peroxide has been used to separate intact sugar substituents from anthocyanins. Hydrogen peroxide under alkaline conditions reacts with the anthocyanin, breaking down the basic structure of the aglycon (C2 and C3 positions of the anthocyanin) and liberating intact sugars. Anthocyanins are dissolved in methanol or water and treated with 30% H_2O_2 and allowed to stand until the pigment is bleached. The solution is then treated with NH_4OH , and finally evaporated to dryness.⁵² The intact sugars liberated can later be analyzed after derivatization using gas chromatographic procedures.

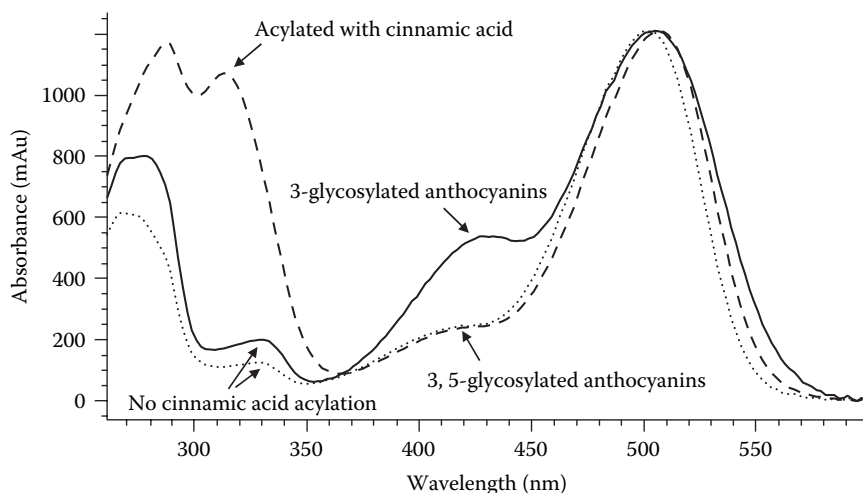


FIGURE 6.3.2 Spectral characteristics of different pelargonidin derivatives in pH 1 buffer.

6.3.4.3.2 Spectral Characteristics

Substantial information can be obtained from the spectral characteristics of anthocyanins. Two distinctive bands of absorption, one in the UV region (260 to 280 nm) and another in the visible region (480 to 550 nm) are shown by all anthocyanins. The different aglycons have different visible λ_{max} values ranging from 520 nm for pelargonidin to 546 nm for delphinidin, and their monoglycosides exhibit their visible λ_{max} levels about 10 to 15 nm lower.⁵² The shapes of the spectra may give information regarding the numbers and positions of glycosidic substitutions and numbers of cinnamic acid acylations. The ratio between the absorbance at 440 and the absorbance at the visible λ_{max} is almost twice as much for anthocyanins with glycosidic substitutions in position 3 as compared to those with substitutions in positions 3 and 5 or in position 5 only (Figure 6.3.2).¹¹⁷

However, note that anthocyanins of pelargonidin also had noticeable absorbance around 440 nm except the visible λ_{max} . The presence of glycosidic substitutions at other positions, e.g., 3,7-diglycosides, can be recognized because they exhibit different spectra from those of anthocyanins with common substitution patterns. The presence of cinnamic acid acylation is revealed by the presence of a third absorption band in the 310 to 360 nm range. The ratio of absorbance at 310 to 360 nm to the absorbance at the visible λ_{max} will give an estimation of the number of acylating groups (Figure 6.3.2).^{78,120} If the ratio is low, there is no acylation; if it exceed 0.5, one or more acylations occurred.¹¹⁷ The solvent media used for spectral determination will affect the position of the absorption bands and therefore must be taken into consideration when comparing data available.

The availability of HPLC systems coupled to photodiode array detectors allows for online spectral characterization of anthocyanins.

6.3.4.3.3 Mass Spectroscopy (MS)

The use of mass spectroscopic analyses for characterization of anthocyanins has increased dramatically over the past decade. Most reports cite the use of HPLC coupled to MS detectors or isolating individual pigments prior to the mass spectroscopic analysis.^{84,87,121–127}

Accurate molecular weight determination is very important for structure elucidation of complex anthocyanins since small components with little UV-Vis absorption, which show weak or no NMR signals, may be overlooked if the molecular weight is not known.¹²⁸ MS can be used to qualitative and quantitative analyses of anthocyanins by differentiating charged fragments in electronic and magnetic fields according to their mass-to-charge ratios. Generally, mass spectrometry is composed of three components: ion sources, mass analyzer, and detector. The ionization techniques most reported for anthocyanin analyses are fast atom bombardment (FAB) and electrospray ionization (ESI).

The introduction of FAB-MS constituted an advance in anthocyanin structural identification.⁵² FAB is an ionization technique that generates ions by bombarding the sample and liquid matrix with an atom beam. FAB-MS provides molecular ions and various fragmentation ions that give direct information on the structure of a molecule. Only a small amount of sample is required (1 to 2 μ L may be enough). After solvent evaporation, a liquid matrix (glycerol, thioglycerol, or 3-nitrobenzyl alcohol) is introduced. A neutral atom beam usually of an inert gas such as xenon or argon is then used to bombard the droplet surface from which material is sputtered. The mass-to-charge ratio of the intact molecular ion is obtained and the fragmentation of the molecule allows the side chains to be determined although their positions are ambiguous.^{2,52} FAB-MS has been used to identify or characterize anthocyanin structures such as cyanidin 3-glucoside acylated with *p*-coumaric acid from camellias,¹²⁹ nasunin from eggplants,¹³⁰ cyanidin 3-oxalylglucoside from orchids,¹³¹ and delphinidin-3-glucoside, cyanidin-3-glucoside, and petunidin-3-glucoside from seed coats of black soybeans.¹³²

ESI-MS has emerged as a powerful technique for the characterization of biomolecules, and is the most versatile ionization technique in existence today.^{85,86,133,134} This highly sensitive and soft ionization technique allows mass spectrometric analysis of thermolabile, non-volatile, and polar compounds and produces intact ions from large and complex species in solution.^{135–137} In addition, it has the ability to introduce liquid samples to a mass detector with minimum manipulation. Volatile acids (such as formic acid and acetic acid) are often added to the mobile phase as well to protonate anthocyanins. A chromatogram with only the base peak for every mass spectrum provides more readily interpretable data because of fewer interference peaks.¹³⁸ Cleaner mass spectra are achieved if anthocyanins are isolated from other phenolics by the use of C18 solid phase purification.^{85,139}

Tandem mass spectrometry (MS-MS) uses more than one mass analyzer for structural and sequencing studies that have been found very useful for anthocyanin characterization. These mass analyzers may be of the same type (triple or quadrupole)^{85,86} or hybrid such as ion trap quadrupole,^{123,140} and quadrupole-time-of-flight (TOF)¹⁴¹ for anthocyanin structural analysis.

The triple quadrupole instrument is most used in MS-MS, allowing for the formation of low-energy collisionally induced dissociation fragments.^{85,137} Individual molecules are selected by the first quadrupole mass analyzer and fragmented in the collision cell using a suitable gas, usually argon, and their fragments are detected by the second quadrupole mass analyzer.^{85,137,142–144} Consequently the signal-to-noise ratio was improved greatly by selection of a particular fragmented ion. This process provides structural information on the components of a mixture and that leads to the unambiguous identification of fragmentation pathways.¹⁴³ ESI and MS-MS were used to structurally characterize anthocyanins from radishes (*Raphanus sativus*), red-fleshed potatoes (*Solanum tuberosum*), red cabbage (*Brassica oleracea*), chokeberries (*Aronia melanocarpa*), concord grapes (*Vitis labrusca*), and roselle (*Hibiscus sabdariffa* L.) anthocyanin extracts.⁸⁵ MS-MS provided clear and characteristic fragmentation patterns for complicated anthocyanins.

The numbers and types of fragments depend on the anthocyanin structure pattern. The aglycone (anthocyanidin) ordinarily is very stable and cannot be broken easily. In most cases, cleavage of the glycosidic groups will occur to generate small amounts of anthocyanidins in addition to the intact anthocyanin molecular ions.

Fragmentation patterns can provide some insight regarding the positions of the glycosidic substitutions on an anthocyanin. For example, a 3-disaccharide usually generates one ion fragment of the aglycone in addition to the intact molecular ion (m/z). See Figure 6.3.3A. However, in the case of a 3,5-disaccharide substitution, different fragments will be produced, corresponding to the breakage of the glycosidic bonds at the 3 and 5 positions in addition to the intact molecular ion (m/z) and the aglycone ion (Figure 6.3.3B).

An exception to this rule is rutinose, a disaccharide of rhamnose and glucose by a 1-6 linkage that allows free rotation and more accessibility of the fragmentation gas.⁸⁵ This allows the formation of different fragments, such as in the case of pelargonidin 3-rutinoside present in red-fleshed potatoes and was confirmed in MS analysis of cyanidin 3-rutinoside by Tian and coworkers.⁸⁶ In the case of acylated anthocyanins, the sugar and acylating group are normally lost together since the ester bond is stronger than the glycosidic bond (Figure 6.3.3B). The type and location of the acylating group may be estimated roughly based on the fragmentation pattern.⁸⁵

The coupling of reversed phase liquid chromatography to MS allows molecules to be characterized by retention time, UV-Vis response, and mass spectral information for the individual components and fragments^{86,137} and is gaining in popularity. The development of ESI as a sensitive technique and also as a specific and versatile detector for liquid sample introduction has contributed enormously to the establishment of LC-MS as an analytical technique for mixture analysis.¹⁴⁵ LC-ESI-MS combinations have been applied to characterize anthocyanins from many plant sources such as blueberry, black currant, chokeberry, elderberry, strawberry, and red orange juices.^{146–148} LC-ESI-MS-MS illustrates molecular structures better and is very popular in the field of anthocyanin structure analysis, determining anthocyanins from fruits and berries,^{121–123} vegetables,¹⁴⁹ nuts,¹⁴⁹ grains,¹⁴⁹ medicinal plants,⁸⁴ wine,¹²⁷ and biological samples.^{87,124–126}

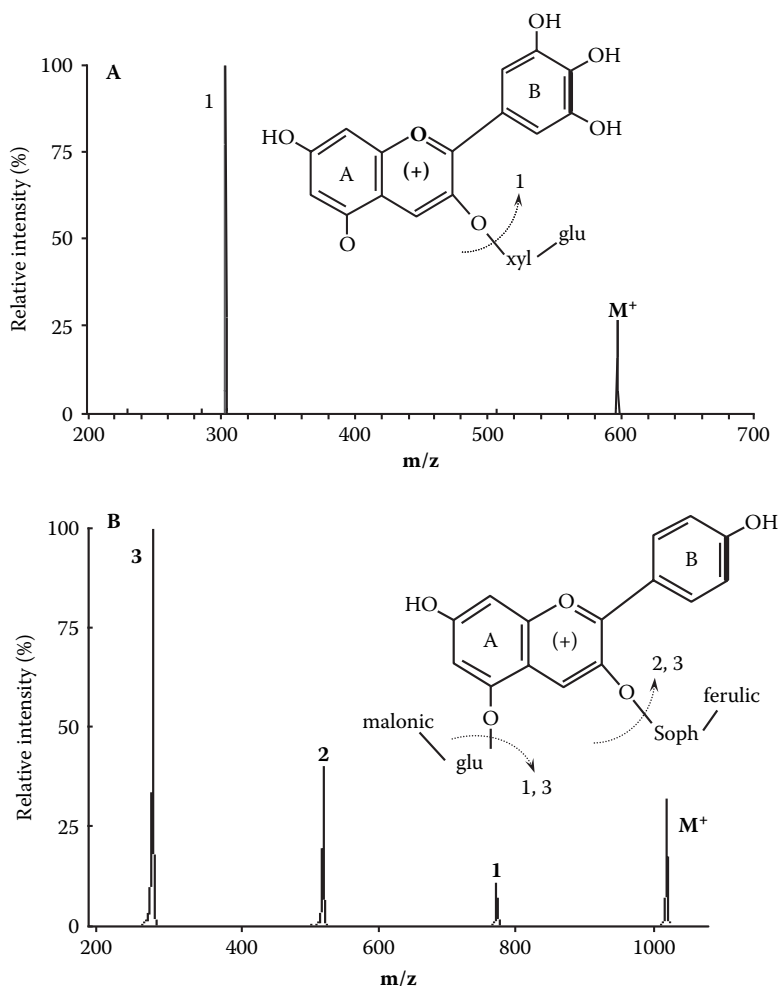


FIGURE 6.3.3 Fragment patterns of anthocyanins: A, dephinidin 3-xyl-glc from Roselle (*Hibiscus sabdariffa* L.); B, pelargonidin-3-(feruloyl-sophoroside)-5-(malonyl-glucose) from radish (*Raphanus sativus*). (Source: Giusti, M.M. et al., *J. Agric. Food Chem.*, 47, 4657, 1999.)

Different types of mass analyzers have been used for anthocyanin analysis: single or triple quadrupole mass analyzers,^{85,86} TOF mass analyzer,^{150,151} ion trap mass analyzers,^{104,152} and the combination of analyzers cited above.^{123,140}

6.3.4.3.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectroscopy is the most powerful method for structural elucidation in solution and advances in NMR techniques have made significant impacts on anthocyanin studies.^{2,52,88} Complete structural characterization of anthocyanins is possible with one- and two-dimensional NMR techniques. However, relatively large quantities of

purified materials are required for resolution of proton signals associated with sugars and positions C6 and C8 of the flavylum nucleus.²⁸

In the late 1970s, the first successful ¹H-NMR recorded on natural anthocyanins by Goto et al.¹⁵³ made it possible to determine the complete structures and stereochemistries of complex anthocyanins.¹²⁸ ¹³C-NMR has also been reported. One- and two-dimensional NMR information allowed determination of the *trans* configuration of cinnamic acid acylation, the β -glucopyranoside conformation of the sugars present, and the exact position of attachment of the acylating group to the sugar moiety.¹²⁸

The nature of the aglycon and the number of glycosylations and acylating groups present in a molecule can normally be assessed from one-dimensional ¹H-NMR. When the one-dimensional spectrum is too complex, it is useful to perform a two-dimensional J-resolved experiment to simplify the spectra and clarify the magnitude of the coupling constants. Coupling constants provides information regarding *trans* versus *cis* configurations and equatorial or axial protonations in the glycosidic moiety, as well as information regarding the configurations of the anomeric carbons in the sugars. Two-dimensional shift correlation and total correlation analyses (2D NMR COSY and TOCSY) assist in assignment of all individual proton signals from the individual sugar moieties. Acylation causes a low field shift of 0.5 to 1.0 ppm for the geminal proton relative to the non-acylated proton.⁵²

HMQC and HMBC experiments enabled us to trace connectivities between ¹H and ¹³C atoms through indirect detection of the low natural abundance nuclei ¹³C, via ¹H nuclei.¹⁵⁴ The HMQC spectra provide correlation between directly bonded ¹H and corresponding ¹³C. HMBC is a long-range heteronuclear chemical shift correlation technique that provides intra-residue multiple bond correlation; this information is valuable for confirming ¹³C and/or ¹H assignments. It also provides inter-residue multiple bond correlation between the anomeric carbon and the aglycon proton and thus serves to identify the inter-glycosidic linkages.¹⁵⁴

Two-dimensional NOESY experiments provide valuable information in mapping specific through-space internuclear distances¹⁵⁵ that could be sufficient to determine the molecular three-dimensional structure. Cross-peaks are observed in NOESY spectra between proton pairs that are close in space, typically less than 5 Å, close enough to allow through-space interactions.¹⁵⁴ The greater the signal, the closer together those hydrogens are in space.¹⁵⁶ It has been proposed that the stacking between the aromatic nuclei of the anthocyanin and the planar ring of the aromatic acid occurs via the formation of π - π hydrophobic interactions.^{81,128,157}

Many recent studies of NMR spectroscopy have been reported for structure elucidation of anthocyanins from many plant materials such as carrot,¹⁵⁸ tart berries,¹⁵⁹ boysenberries,¹⁶⁰ flowers,¹⁶¹ black soybeans,¹³² and anthocyanin and flavonol derivatives in red wine.¹⁶² Giusti et al. (1998)¹⁶³ structurally elucidated two novel diacylated anthocyanins and two monoacylated anthocyanins from radish (*Raphanus sativus*) by one- and two-dimensional NMR. Anderson et al. (2006)¹⁶⁴ applied two-dimensional NMR to characterize carboxypyrananthocyanins. Two 3-deoxyanthocyanins, luteolinidin-5-glucoside, and apigeninidin-5-glucoside were identified by Swinny et al.¹⁶⁵ using ¹H and ¹³C NMR.

6.3.4.3.5 Infrared Spectroscopy

Photon energies associated with the infrared range (2,500 to 16,000 nm) are not large enough to excite electrons, but may induce vibrational excitation of covalently bonded atoms and groups. Organic compounds will absorb infrared radiation that corresponds in energy to a wide variety of vibrational motions. This permits chemists to obtain absorption infrared spectra of compounds that are unique reflections of their molecular structures. The introduction of a sampling technique known as attenuated total reflection (ATR) has enabled qualitative and quantitative analysis of samples in a liquid or solid state without further preparation.

In the wine industry, FTIR has become a useful technique for rapid analysis of industrial-grade glycerol adulteration, polymeric mannose, organic acids, and varietal authenticity.^{166–169} Urbano Cuadrado et al. (2005)¹⁷⁰ studied the applicability of spectroscopic techniques in the near- and mid-infrared frequencies to determine multiple wine parameters: alcoholic degree, volumic mass, total acidity, total polyphenol index, glycerol, and total sulfur dioxide in a much more efficient approach than standard and reference methods in terms of time, reagent, and operation errors.

To detect adulteration of wine, Burns et al. (2002)⁸⁹ found that the ratios of acetylated to *p*-coumaroylated conjugates of nine characteristic anthocyanins served as useful parameters to determine grape cultivars for a type of wine. Our laboratory utilized mid-infrared spectroscopy combined with multivariate analysis to provide spectral signature profiles that allowed the chemically based classification of anthocyanin-containing fruits juices and produced distinctive and reproducible chemical fingerprints, making it possible to discriminate different juices.⁹⁰ This new application of ATR-FTIR to detect adulteration in anthocyanin-containing juices and foods may be an effective and efficient method^{171,172} for manufacturers to assure product quality and authenticity.

REFERENCES

1. Harborne, J.B., Distribution of anthocyanins in higher plants, in *Chemical Plant Taxonomy*, Swain, T., Ed., Academic Press, New York, 1963.
2. Strack, D. and Wray, V., The anthocyanins, in *The Flavonoids: Advances in Research since 1986*, Harborne, J.B., Ed., Chapman & Hall, London, 1994.
3. Giusti, M.M. and Wrolstad, R.E., Acylated anthocyanins from edible sources and their applications in food systems, *Biochem. Eng. J.*, 14, 217, 2003.
4. Ghiselli, A. et al., Antioxidant activity of different phenolic fractions separated from an Italian red wine, *J. Agric. Food Chem.*, 46, 361, 1998.
5. Wang, S.Y. and Lin, H.S., Antioxidant activity in fruits and leaves of blackberry, raspberry, and strawberry varies with cultivar and developmental stage, *J. Agric. Food Chem.*, 48, 140, 2000.
6. Prior, R.L., Fruits and vegetables in the prevention of cellular oxidative damage, *Am. J. Clin. Nutr.*, 78, 570S, 2003.
7. Wang, H., Cao, G., and Prior, R.L., Oxygen radical absorbing capacity of anthocyanins, *J. Agric. Food Chem.*, 45, 304, 1997.

8. Tamura, H. and Yagami, A., Antioxidative activity of mono-acylated anthocyanins isolated from Muscat Bailey A grape, *J. Agric. Food Chem.*, 42, 1612, 1994.
9. Aoki, H. et al., Inhibitory effect of anthocyanin colors on mutagenicity induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP), *Foods Food Ingrid. J. Jpn.*, 209, 240, 2004.
10. Gasiorowski, K. et al., Antimutagenic activity of anthocyanins isolated from *Aronia melanocarpa* fruits, *Cancer Lett.*, 119, 37, 1997.
11. Aziz, R.M. et al., The effect of freeze-dried blueberries on N-nitrosomethylbenzylamine tumorigenesis in the rat esophagus, *Pharmaceut. Biol.* 40 (Suppl.), 43, 2002.
12. Casto, B.C. et al., Chemoprevention of oral cancer by black raspberries, *Anticancer Res.*, 22, 4005, 2002.
13. Kresty, L.A. et al., Chemoprevention of esophageal tumorigenesis by dietary administration of lyophilized black raspberries, *Cancer Res.*, 61, 6112, 2001.
14. Bomser, J. et al., *In vitro* anticancer activity of fruit extracts from *Vaccinium* species, *Planta Med.*, 62, 212, 1996.
15. Ramirez-Tortosa, C. et al., Anthocyanin-rich extract decreases indices of lipid peroxidation and DNA damage in vitamin E-depleted rats, *Free Radic. Biol. Med.*, 31, 1033, 2001.
16. Lala, G. et al., Anthocyanin-rich extracts inhibit multiple biomarkers of colon cancer in rats, *Nutr. Cancer*, 54, 84, 2006.
17. Zhao, C. et al., Effects of commercial anthocyanin-rich extracts on colonic cancer and nontumorigenic colonic cell growth, *J. Agric. Food Chem.*, 52, 6122, 2004.
18. Malik, M. et al., Anthocyanin-rich extract from *Aronia melanocarpa* E induces a cell cycle block in colon cancer but not normal colonic cells, *Nutr. Cancer*, 46, 186, 2003.
19. Jing, P. et al., Structure-function relationship between anthocyanins and their chemoprotective properties, *4th Int. Congr. Pigment in Foods*, Sec. 2: Biological and Nutritional Properties of Pigments, 134, 2006.
20. Hagiwara, A. et al., Pronounced inhibition by a natural anthocyanin, purple corn color, of 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP)-associated colorectal carcinogenesis in male F344 rats pretreated with 1,2-dimethylhydrazine, *Cancer Lett.*, 171, 17, 2001.
21. Tsuda, T. et al., Dietary cyanidin 3-O-beta-D-glucoside-rich purple corn color prevents obesity and ameliorates hyperglycemia in mice, *J. Nutr.*, 133, 2125, 2003.
22. McDougall, G.J. and Stewart, D., The inhibitory effects of berry polyphenols on digestive enzymes, *Biofactors*, 23, 189, 2005.
23. Mazza, G. and Miniati, E., *Anthocyanins in Fruits, Vegetables, and Grains*, CRC Press, Boca Raton, FL, 1993.
24. Strack, D. and Wray, V., *The Flavonoids: Advances in Research since 1986*, Chapman & Hall, London, 1993.
25. Nygaard, A. et al., Structure determination of 6-hydroxycyanidin- and 6-hydroxydelphinidin-3-(6''-o-a -L-rhamnopyranosyl-b -D-glucopyranosides) and other anthocyanins from *Alstroemeria* cultivars, *Acta Chem. Scand.*, 51, 108, 1997.
26. Pale, E. et al., 7-O-methylapigeninidin, an anthocyanidin from *Sorghum caudatum*, *Phytochemistry*, 45, 1091, 1997.
27. Kong, J.M. et al., Analysis and biological activities of anthocyanins, *Phytochemistry*, 64, 923, 2003.
28. Jackman, R.L. and Smith, J.L., Anthocyanins and betalains, in *Natural Food Colorants*, Hendry, G.A.F. and Houghton, J.D. Eds., Blackie, London, 1996, chap. 8.
29. Andersen, Ø.M. and Markham, K.R., *Flavonoids: Chemistry, Biochemistry and Applications*, CRC Press, Boca Raton, FL, 2006.

30. Brouillard, R., Chemical structure of anthocyanins, in *Anthocyanins as Food Colors*, Markakis, P., Ed., Academic Press, New York, 1982.
31. Wrolstad, R.E. et al., Establishing criteria for determining the authenticity of fruit juice concentrates, *ACS Symp. Ser.*, 170, 77, 1981.
32. Hale, M.L., Francis, F.J., and Fagerson, I.S., Detection of enocyanin in cranberry juice cocktail by HPLC anthocyanin profile, *J. Food Sci.*, 51, 1511, 1986.
33. Spayd, S.E. and Morris, J.R., Influence of immature fruits on strawberry jam quality and storage stability, *J. Food Sci.*, 46, 414, 1981.
34. Jackman, R.L., Yada, R.Y., and Tung, M.A., Review: separation and chemical properties of anthocyanins used for their qualitative and quantitative analysis, *J. Food Biochem.*, 11, 279, 1987.
35. Fuleki, T. and Francis, F.J., Quantitative methods for anthocyanins. 1. Extraction and determination of total anthocyanin in cranberries, *J. Food Sci.*, 33, 72, 1968.
36. Brouillard, R. and Dangles, O., Flavonoids and flower colour, in *The Flavonoids: Advances in Research since 1986*, Harborne, H.B., Ed., Chapman & Hall, London, 1994, chap. 13.
37. Markakis, P., Stability of anthocyanins in food, in *Anthocyanins as Food Colors*, Markakis, P., Ed., Academic Press, New York, 1982, chap. 6.
38. Francis, F.J., Food colorants: anthocyanins, *Crit. Rev. Food Sci. Nutr.*, 28, 273, 1989.
39. Rodríguez-Saona, L.E. and Wrolstad, R.E., Extraction, isolation, and purification of anthocyanins, in *Current Protocols in Food Analytical Chemistry*, Wrolstad, R.E., et al., Eds., John Wiley & Sons, New York, 2001, 1.
40. Price, C. and Wrolstad, R.E., Anthocyanin pigments of Royal Okanagan Huckleberry juice, *J. Food Sci.*, 60, 369, 1995.
41. Wrolstad, R.E. and Heatherbell, D.A., Identification and distribution of flavonoids in Tamarillo fruit (*Cyphomandra betaceae* (Cav.) Sendt.), *J. Sci. Food Agric.*, 25, 1221, 1974.
42. Giusti, M.M. and Wrolstad, R.E., Radish anthocyanin extract as a natural red colorant for maraschino cherries, *J. Food Sci.*, 61, 688, 1996.
43. Markakis, P., Anthocyanins and their stability in foods, *CRC Crit. Rev. Food Technol.*, 4, 437, 1974.
44. Metivier, R.P., Francis, F.J., and Clydesdale, F.M., Solvent extraction of anthocyanins from wine pomace, *J. Food Sci.*, 45, 1099, 1980.
45. Abers, J.E. and Wrolstad, R.E., Causative factors of color deterioration in strawberry preserves during processing and storage, *J. Food Sci.*, 44, 75, 1979.
46. Timberlake, C.F. and Bridle, P., Anthocyanins in oetals of *Chaenomeles speciosa*, *Phytochemistry*, 10, 2265, 1971.
47. Wrolstad, R.E. et al., Influence of anthocyanin pigment stability in frozen strawberries, *J. Food Sci.*, 55, 1064, 1990.
48. Wrolstad, R.E. and Durst, R.W., Use of anthocyanin and polyphenolic analyses in authenticating fruit juices, in *Proceedings of Food Authenticity Workshop*, ENI Laboratories, Ed., Eurofins Scientific International Group, Montreal, 1998.
49. Giusti, M.M. and Wrolstad, R.E., Characterization of red radish anthocyanins, *J. Food Sci.*, 61, 322, 1996.
50. Rodríguez-Saona, L.E., Giusti, M.M., and Wrolstad, R.E., Anthocyanin pigment composition of red-fleshed potatoes, *J. Food Sci.*, 63, 458, 1998.
51. Revilla, E., Ryan, J.-M., and Martin-Ortega, G., Comparison of several procedures used for the extraction of anthocyanins from red grapes, *J. Agric. Food Chem.*, 46, 4592, 1998.

52. Strack, D. and Wray, V., Anthocyanins, in *Methods in Plant Biochemistry Vol. I. Plant Phenolics.*, Dey, P.M. and Harborne, J.B., Eds., Academic Press, San Diego, 1989, chap. 9.
53. Turner, C., Overview of modern extraction techniques for food and agricultural samples, in *Modern Extraction Techniques: Food and Agricultural Samples*, ACS Symposium Series, No. 926, Turner, C., Ed., Washington, D.C., 2006, chap. 1.
54. Peres, V.F. et al., Comparison of soxhlet, ultrasound-assisted and pressurized liquid extraction of terpenes, fatty acids and vitamin E from *Piper gaudichaudianum* Kunth, *J. Chromatogr. A*, 1105, 115, 2006.
55. Anand, R. et al., Comparison of extraction techniques for extraction of bioactive molecules from *Hypericum perforatum* L. plant, *J. Chromatogr. Sci.*, 43, 530, 2005.
56. Kuriki, K., Tajima, K., and Tokudome, S., Accelerated solvent extraction for quantitative measurement of fatty acids in plasma and erythrocytes, *Lipids*, 41, 605, 2006.
57. Tao, S. et al., Use of sequential ASE extraction to evaluate the bioavailability of DDT and its metabolites to wheat roots in soils with various organic carbon contents, *Sci. Total Environ.*, 320, 1, 2004.
58. Ju, Z.Y. and Howard, L.R., Effects of solvent and temperature on pressurized liquid extraction of anthocyanins and total phenolics from dried red grape skin, *J. Agric. Food Chem.*, 51, 5207, 2003.
59. Obana, H., Furuta, M., and Tanaka, Y., Analysis of 2-alkylcyclobutanones with accelerated solvent extraction to detect irradiated meat and fish, *J. Agric. Food Chem.*, 53, 6603, 2005.
60. Richter, B.E. et al., Accelerated solvent extraction: a technique for sample preparation, *Anal. Chem.*, 68, 1033, 1996.
61. Markakis, P.G., Livingstone, G.E., and Fellers, R.C., Quantitative aspects of strawberry-pigment degradation, *Food Res.*, 22, 117, 1957.
62. Bhagdeo, M., Ashraf-Khorassani, M., and Taylor, L.T., Analysis of supercritical CO₂ extractables from cranberry seeds, in *Modern Extraction Techniques: Food and Agricultural Samples*, ACS Symposium Series, No. 926, Turner, C. Ed., Washington, D.C., 2006, chap. 3.
63. Topal, U. et al., Extraction of lycopene from tomato skin with supercritical carbon dioxide: effect of operating conditions and solubility analysis, *J. Agric. Food Chem.*, 54, 5604, 2006.
64. Teng, W.Y., Chen, C.C., and Chung, R.S., HPLC comparison of supercritical fluid extraction and solvent extraction of coumarins from the peel of *Citrus maxima* fruit, *Phytochem. Anal.*, 16, 459, 2005.
65. del Valle, J.M. and de la Fuente, J.C., Supercritical CO₂ extraction of oilseeds: review of kinetic and equilibrium models, *Crit. Rev. Food Sci. Nutr.*, 46, 131, 2006.
66. Mantell, C., Martinez de la Ossa, E., and Rodriguez, M., Supercritical fluid extraction of anthocyanins from grape pomace, in *Solvent Extraction for the 21st Century*, Cox, M., Hidalgo, M., and Valiente, M., Eds., Barcelona, Spain, 1999.
67. Bleve, M., Ciurlia, L., and Rescio, L., Supercritical carbon dioxide extracts from red fruits: a "natural" cosmeceutical application, *Nutra. Cos.*, 4, 23, 2005.
68. Giusti, M.M. and Wrolstad, R.E., Characterization and measurement of anthocyanins by UV-visible spectroscopy, in *Current Protocols in Food Analytical Chemistry*, Wrolstad, R.E., et al., Eds., John Wiley & Sons, New York, 2001, 1.
69. Fuleki, T. and Francis, F.J., Quantitative methods for anthocyanins. 2. Determination of total anthocyanin and degradation index for cranberry juice, *J. Food Sci.*, 33, 78, 1968.

70. Wrolstad, R.E. et al., Detection of adulteration in blackberry juice concentrates and wines, *J. AOAC*, 65, 1417, 1982.
71. Wrolstad, R.E. et al., Use of anthocyanin pigment analysis for detecting adulteration in fruit juices, in *Methods to Detect Adulteration in Fruit Juice and Beverages*, Vol. I, Nagy, S. and Wade, R.L., Eds., AgScience Inc., Auburndale, FL, 1995.
72. Lee, J., Durst, R., and Wrolstad, R., AOAC official method 2005.02: total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method, in *Official Methods of Analysis of AOAC International*, Horowitz, H., Ed., AOAC, Washington, D.C., 2005.
73. Somers, T.C. and Evans, M.E., Wine quality: correlations with colour density and anthocyanin equilibria in a group of young red wines, *J. Sci. Food Agric.*, 25, 1369, 1974.
74. Swain, T. and Hillis, W.E., The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents, *J. Sci. Food Agric.*, 10, 63, 1959.
75. Francis, F.J., Analysis of anthocyanins, in *Anthocyanins as Food Colors*, Markakis, P., Ed., Academic Press, New York, 1982, chap. 7.
76. Giusti, M.M., Rodriguez-Saona, L.E., and Wrolstad, R.E., Molar absorptivity and color characteristics of acylated and non-acylated pelargonidin-based anthocyanins, *J. Agric. Food Chem.*, 47, 4631, 1999.
77. Andersen, O.M., Chromatographic separation of anthocyanin in cowberry (*Lingonberry*) *Vaccinium vitis-idaea* L., *J. Food Sci.*, 50, 1230, 1985.
78. Hong, V. and Wrolstad, R.E., Use of HPLC separation/photodiode array detection for characterization of anthocyanins, *J. Agric. Food Chem.*, 38, 708, 1990.
79. Wrolstad, R.E. et al., Analysis of anthocyanins in nutraceuticals, in *Quality Management of Nutraceuticals*, Ho, C.T. and Zheng, Q.Y., Eds., American Chemical Society, Washington, D.C., 2002, chap. 4.
80. Dangles, O., Saito, N., and Brouillard, R., Anthocyanin intramolecular copigment effect, *Phytochemistry*, 34, 119, 1993.
81. Figueiredo, P. et al., Anthocyanin intramolecular interactions: a new mathematical approach to account for the remarkable colorant properties of the pigments extracted from *Matthiola incana*, *J. Am. Chem. Soc.*, 118, 4788, 1996.
82. Hrazdina, G., Iredale, H., and Mattick, L.R., Anthocyanin composition of *Brassica oleracea* cv. Red Danish, *Phytochemistry*, 16, 297, 1977.
83. Swain, T., Analytical methods for flavonoids, in *Chemistry and Biochemistry of Plant Pigments*, Goodwin, T.W., Ed., Academic Press, London, 1965, chap. 19.
84. Chirinos, R. et al., High-performance liquid chromatography with photodiode array detection (HPLC-DAD)/HPLC-mass spectrometry (MS) profiling of anthocyanins from Andean mashua tubers (*Tropaeolum tuberosum* Ruiz and Pavon) and their contribution to the overall antioxidant activity, *J. Agric. Food Chem.*, 54, 7089, 2006.
85. Giusti, M.M. et al., Electrospray and tandem mass spectroscopy as tools for anthocyanin characterization, *J. Agric. Food Chem.*, 47, 4657, 1999.
86. Tian, Q. et al., Screening for anthocyanins using high-performance liquid chromatography coupled to electrospray ionization tandem mass spectrometry with precursor-ion analysis, product-ion analysis, common-neutral-loss analysis, and selected reaction monitoring, *J. Chromatogr. A*, 1091, 72, 2005.
87. Tian, Q. et al., Urinary excretion of black raspberry (*Rubus occidentalis*) anthocyanins and their metabolites, *J. Agric. Food Chem.*, 54, 1467, 2006.
88. Andersen, O.M. and Fossen, T., Characterization of anthocyanins by NMR, in *Handbook of Food Analytical Chemistry: Pigments, Colorants, Flavors, Texture, and Bioactive Food Components*, Wrolstad, R.E., Ed., John Wiley & Sons, New York, 2005.

89. Burns, J. et al., Variations in the profile and content of anthocyanins in wines made from cabernet sauvignon and hybrid grapes, *J. Agric. Food Chem.*, 50, 4096, 2002.
90. He, J., Rodriguez-Saona, L.E., and Giusti, M.M., Rapid differentiation of commercial anthocyanin-rich juices based on FTIR analysis, in *The 4th International Congress on Pigments in Food*, Section 1: Analysis and Chemistry of Pigments, Carle, R., Schieber, A., and Stintzing, F.C., Eds., Shaker Verlag Bmbtt, Inc., Germany, 47, 2006.
91. Downey, G. and Kelly, J.D., Detection and quantification of apple adulteration in diluted and sulfited strawberry and raspberry purees using visible and near-infrared spectroscopy, *J. Agric. Food Chem.*, 52, 204, 2004.
92. Mattick, L.R., Weirs, L.D., and Robinson, W.B., Effect of pH on the precipitation of the anthocyanins of wine as their lead salts, *Am. J. Enol. Viticult.*, 20, 206, 1969.
93. Sarni-Manchado, P. et al., Analysis and characterization of wine condensed tannins precipitated by proteins used as fining agent in enology, *Am. J. Enol. Viticult.*, 50, 81, 1999.
94. Fuleki, T. and Francis, F.J., Quantitative methods for anthocyanins. 3. Purification of cranberry anthocyanins, *J. Food Sci.*, 33, 266, 1968.
95. Chiriboga, C. and Francis, F.J., Anthocyanin recovery system from cranberry pomace, *Proc. Am. Soc. Hortic. Sci.*, 95, 233, 1970.
96. Andersen, O.M. et al., Anthocyanin from strawberry (*Fragaria ananassa*) with the novel aglycone, 5-carboxypyranopelargonidin, *Phytochemistry*, 65, 405, 2004.
97. Fossen, T., Rayyan, S., and Andersen, O.M., Dimeric anthocyanins from strawberry (*Fragaria ananassa*) consisting of pelargonidin 3-glucoside covalently linked to four flavan-3-ols, *Phytochemistry*, 65, 1421, 2004.
98. Kantz, K. and Singleton, V.L., Isolation and determination of polymeric polyphenols in wines using Sephadex LH-20, *Am. J. Enol. Viticult.*, 42, 309, 1991.
99. Oszmianski, J. and Lee, C.Y., Isolation and HPLC determination of phenolic compounds in red grapes, *Am. J. Enol. Viticult.*, 41, 204, 1990.
100. Ghiselli, A. et al., Antioxidant activity of different phenolic fractions separated from an Italian red wine, *J. Agric. Food Chem.*, 46, 361, 1998.
101. Winterhalter, P., Application of countercurrent chromatography (CCC) to the analysis of natural pigments, in *The 4th International Congress on Pigment in Food*, Section 1: Analysis and Chemistry of Pigments, Carle, R., Schieber, A., and Stintzing, F.C., Eds., Shaker Verlag Bmbtt, Inc., Germany, 47, 2006.
102. Foucault, A.P. and Chevolot, L., Counter-current chromatography: instrumentation, solvent selection and some recent applications to natural product purification, *J. Chromatogr. A*, 808, 3, 1998.
103. Salas, E. et al., Characterization of pigments from different high speed countercurrent chromatography wine fractions, *J. Agric. Food Chem.*, 53, 4536, 2005.
104. Degenhardt, A., Knapp, H., and Winterhalter, P., Separation and purification of anthocyanins by high speed countercurrent chromatography and screening for antioxidant activity, *J. Agric. Food Chem.*, 48, 338, 2000.
105. Torskangerpoll, K., Chou, E., and Andersen, O.M., Separation of acylated anthocyanin pigments by high speed CCC, *J. Liq. Chromatogr. Rel. Technol.*, 24, 1791, 2001.
106. Du, Q., Jerz, G., and Winterhalter, P., Isolation of two anthocyanin sambubiosides from bilberry (*Vaccinium myrtillus*) by high-speed counter-current chromatography, *J. Chromatogr. A*, 1045, 59, 2004.
107. Degenhardt, A., Knapp, H., and Winterhalter, P., Rapid isolation of malvidin 3-glucoside from red wine by high speed counter-current chromatography (HSCCC), *Vitis*, 39, 43, 2000.

108. Manley, C.H. and Shubiak, P., High pressure liquid chromatography of anthocyanins [food pigments], *Can. Inst. Food Technol.*, 8, 35, 1975.
109. Hong, V. and Wrolstad, R.E., Characterization of anthocyanin-containing colorants and fruit juices by HPLC/photodiode array detection, *J. Agric. Food Chem.*, 38, 698, 1990.
110. Herrero, M., Ibaniez, E., and Cifuentes, A., Analysis of natural antioxidants by capillary electromigration methods, *J. Sep. Sci.*, 28, 883, 2005.
111. Mazza, G., Cacace, J.E., and Kay, C.D., Methods of analysis for anthocyanins in plants and biological fluids, *J. AOAC Int.*, 87, 129, 2004.
112. Ichiyana, T. et al., Complete assignment of bilberry (*Vaccinium myrtillus* L.) anthocyanins separated by capillary zone electrophoresis, *Chem. Pharm. Bull.*, 52, 226, 2004.
113. Bridle, P. and Garcia-Viguera, C., Analysis of anthocyanins in strawberries and elderberries: a comparison of capillary-1 electrophoresis and HPLC, *Food Chem.*, 59, 299, 1997.
114. Calvo, D. et al., Migration order of wine anthocyanins in capillary zone electrophoresis, *Anal. Chim. Acta*, 524, 207, 2004.
115. Saez-Lopez, R., Fernandez-Zurbano, P., and Tena, M.T., Analysis of aged red wine pigments by capillary zone electrophoresis, *J. Chromatogr. A*, 1052, 191, 2004.
116. Bicard, V., Fougere, A., and Brouillard, R., Analysis of natural anthocyanins by capillary zone electrophoresis in acidic media, *J. Liq. Chromatogr. Rel. Technol.*, 22, 541, 1999.
117. Durst, R.W. and Wrolstad, R.E., Separation and characterization of anthocyanins by HPLC, in *Current Protocols in Food Analytical Chemistry*, Wrolstad, R.E., et al., Eds., John Wiley & Sons, New York, 2001, 1.
118. Francis, F.J. and Harborne, J.B., Anthocyanins in the garden huckleberry, *Solanum guineense*, *J. Food Sci.*, 31, 524, 1966.
119. Jing, P. and Giusti, M.M., Characterization of anthocyanin-rich waste from purple corn cobs (*Zea mays* L.) and its application to color milk, *J. Agric. Food Chem.*, 53, 8775, 2005.
120. Harborne, J.B., *Comparative Biochemistry of the Flavonoids*, Academic Press, London, 1967.
121. Wu, X. and Prior, R.L., Systematic identification and characterization of anthocyanins by HPLC-ESI-MS/MS in common foods in the United States: fruits and berries, *J. Agric. Food Chem.*, 53, 2589, 2005.
122. Hvattum, E., Determination of phenolic compounds in rose hip (*Rosa canina*) using liquid chromatography coupled to electrospray ionisation tandem mass spectrometry and diode-array detection, *Rapid Commun. Mass Spectrom.*, 16, 655, 2002.
123. Montoro, P. et al., Characterisation by liquid chromatography-electrospray tandem mass spectrometry of anthocyanins in extracts of *Myrtus communis* L. berries used for the preparation of myrtle liqueur, *J. Chromatogr. A*, 1112, 232, 2006.
124. Ohnishi, R. et al., Urinary excretion of anthocyanins in humans after cranberry juice ingestion, *Biosci. Biotechnol. Biochem.*, 70, 1681, 2006.
125. Felgines, C. et al., Strawberry anthocyanins are recovered in urine as glucuro- and sulfoconjugates in humans, *J. Nutr.*, 133, 1296, 2003.
126. Tomoyuki, O. et al., Determination of acylated anthocyanin in human urine after ingesting a purple-fleshed sweet potato beverage with various contents of anthocyanin by LC-ESI-MS/MS, *Biosci. Biotechnol. Biochem.*, 70, 2540, 2006.
127. Hayasaka, Y. and Asenstorfer, R.E., Screening for potential pigments derived from anthocyanins in red wine using nanoelectrospray tandem mass spectrometry, *J. Agric. Food Chem.*, 50, 756, 2002.

128. Goto, T., Structure, stability and color variation of natural anthocyanins, *Fortsch. Chem. Org. Natur.*, 52, 113, 1987.
129. Saito, N. et al., Cyanidin 3-p-coumaroylglucoside in Camellia species and cultivars, *Phytochemistry*, 26, 2761, 1987.
130. Hayashi, K. et al., Photoisomerization of the nasunin, the major eggplant anthocyanin, *Food Sci. Technol. Int. Tok.*, 4, 25, 1998.
131. Strack, D. et al., Cyanidin 3-oxalylglucoside in orchids, *J. Biosci.*, 41, 707, 1986.
132. Choung, M.-G. et al., Isolation and determination of anthocyanins in seed coats of black soybean (*Glycine max* (L.) Merr.), *J. Agric. Food Chem.*, 49, 5848, 2001.
133. Covey, T., Analytical characteristics of the electrospray ionization process, in *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry*, ACS Symposium Series, Snyder, A.P. and Anaheim, C.A., Eds., Washington, D.C., 1995, chap. 2.
134. Snyder, A.P., Electrospray: a popular ionization technique for mass spectrometry, in *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry*, ACS Symposium Series, Snyder, A.P. and Anaheim, C.A., Eds., Washington, D.C., 1995, chap. 1.
135. Black, G.E. and Fox, A., Liquid chromatography with electrospray ionization tandem mass spectrometry: profiling carbohydrates in whole bacterial cell hydrolysates, in *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry*, ACS Symposium Series, Snyder, A.P. and Anaheim, C.A., Eds., Washington, D.C., 1995, chap. 4.
136. Fenn, J.B. et al., Electrospray ion formation: desorption versus desertion, in *Biochemical and Biotechnological Applications of Electrospray Ionization Mass Spectrometry*, ACS Symposium Series, Snyder, A.P. and Anaheim, C.A., Eds., Washington, D.C., 1995, chap. 3.
137. Hutton, T. and Major, H.J., Characterizing biomolecules by electrospray ionization-mass spectrometry coupled to liquid chromatography and capillary electrophoresis, *Biochem. Soc. Trans.*, 23, 924, 1995.
138. Sagesser, M. and Deinzer, M., HPLC-ion spray-tandem mass spectrometry of flavonol glycosides in hops, *J. Am. Soc. Brew. Chem.*, 54, 129, 1996.
139. Skrede, G., Wrolstad, R.E., and Durst, R.W., Changes in anthocyanins and polyphenolics during juice processing of highbush blueberries (*Vaccinium corymbosum* L.), *J. Food Sci.*, 65, 357, 2000.
140. Pati, S. et al., Simultaneous separation and identification of oligomeric procyanidins and anthocyanin-derived pigments in raw red wine by HPLC-UV-ESI-MS, *J. Mass Spectrom.*, 41, 861, 2006.
141. Mazzuca, P. et al., Mass spectrometry in the study of anthocyanins and their derivatives: differentiation of *Vitis vinifera* and hybrid grapes by liquid chromatography, electrospray ionization mass spectrometry and tandem mass spectrometry, *J. Mass Spectrom.*, 40, 83, 2005.
142. Jennings, K.R., MS/MS instrumentation, in *Applications of Modern Mass Spectrometry in Plant Science Research*, Newton, R.P. and Walton, T.J., Eds., Clarendon Press, New York, 1996, chap. 3.
143. Lawson, G., Ostah, N., and Woolley, J.G., MS/MS studies of tropane alkaloids: Detection and determination of structure, in *Applications of Modern Mass Spectrometry in Plant Science Research*, Newton, R.P. and Walton, T.J., Eds., Clarendon Press, New York, 1996, chap. 16.
144. McLafferty, F.W. and Turecek, F., *Interpretation of Mass Spectra*, University Science Books, Sausalito, CA, 1993.

145. Gaskell, S.J., Recent and projected developments in mass spectrometric techniques, in *Applications of Modern Mass Spectrometry in Plant Science Research*, Newton, R.P. and Walton, T.J., Eds., Clarendon Press, New York, 1996, chap. 5.
146. Paola, D. et al., Characterization of the anthocyanin fraction of Sicilian blood orange juice by micro-HPLC-ESI/MS, *J. Agric. Food Chem.*, 51, 1173, 2003.
147. Nakajima, J.I. et al., LC/PDA/ESI-MS profiling and radical scavenging activity of anthocyanins in various berries, *J. Biomed. Biotechnol.*, 241, 2004.
148. Lopes-Da-Silva, F. et al., Identification of anthocyanin pigments in strawberry (cv Camarosa) by LC using DAD and ESI-MS detection, *Eur. Food Res. Technol.*, 214, 248, 2002.
149. Wu, X. and Prior, R.L., Identification and characterization of anthocyanins by high-performance liquid chromatography–electrospray ionization–tandem mass spectrometry in common foods in the United States: vegetables, nuts, and grains, *J. Agric. Food Chem.*, 53, 3101, 2005.
150. Wang, J., Kalt, W., and Sporns, P., Comparison between HPLC and MALDI-TOF MS analysis of anthocyanins in highbush blueberries, *J. Agric. Food Chem.*, 48, 3330, 2000.
151. Sugui, J.A. et al., MALDI-TOF analysis of mixtures of 3-deoxyanthocyanidins and anthocyanins, *Phytochemistry*, 48, 1063, 1998.
152. Wang, H., Race, E.J., and Shrikhande, A.J., Characterization of anthocyanins in grape juices by ion trap liquid chromatography–mass spectrometry, *J. Agric. Food Chem.*, 51, 1839, 2003.
153. Goto, T., Takase, S., and Kondo, T., PMR spectra of natural acylated anthocyanins determination of stereostructure of awobanin, shisonin and violanin (*Commelina communis*, *Viola tricolor*, *Perilla orimoides*), *Tetrahedron Lett.*, 27, 2413, 1978.
154. Agrawal, P.K., NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides, *Phytochemistry*, 31, 3307, 1992.
155. Keepers, J.W. and James, T.L., A theoretical study of distance determinations from NMR: two-dimensional nuclear Overhauser effect spectra, *J. Mag. Reson.*, 57, 404, 1984.
156. Kemp, W., *Organic Spectroscopy*, 3rd ed., W.H. Freeman, New York, 1991.
157. Figueiredo, P. et al., New aspects of anthocyanin complexation: intramolecular copigmentation as a means for colour loss? *Phytochemistry*, 41, 301, 1996.
158. Gakh, E.G., Dougall, D.K., and Baker, D.C., Proton nuclear magnetic resonance studies of monoacylated anthocyanins from the wild carrot: part 1. Inter- and intramolecular interactions in solution, *Phytochem. Anal.*, 9, 28, 1998.
159. Wang, H. et al., Quantification and characterization of anthocyanins in Balaton tart cherries, *J. Agric. Food Chem.*, 45, 2556, 1997.
160. McGhie, T.K., Rowan, D.R., and Edwards, P.J., Structural identification of two major anthocyanin components of boysenberry by NMR spectroscopy, *J. Agric. Food Chem.*, 54, 8756, 2006.
161. Tanaka, M. et al., A malonylated anthocyanin and flavonols in blue Meconopsis flowers, *Phytochemistry*, 56, 373, 2001.
162. Nuno, M. et al., Identification of anthocyanin-flavanol pigments in red wines by NMR and mass spectrometry, *J. Agric. Food Chem.*, 50, 2110, 2002.
163. Giusti, M.M., Ghanadan, H., and Wrolstad, R.E., Elucidation of the structure and conformation of red radish (*Raphanus sativus*) anthocyanins using one- and two-dimensional nuclear magnetic resonance techniques, *J. Agric. Food Chem.*, 46, 4858, 1998.
164. Jordheim, M., Fossen, T., and Andersen, O.M., Preparative isolation and NMR characterization of carboxypyrananthocyanins, *J. Agric. Food Chem.*, 54, 3572, 2006.

165. Swinny, E.E., Bloor, S.J., and Wong, H., ^1H and ^{13}C NMR assignments for the 3-deoxyanthocyanins, luteolinidin-5-glucoside and apigeninidin-5-glucoside, *Mag. Reson. Chem.*, 38, 1031, 2000.
166. Dixit, V. et al., Identification and quantification of industrial grade glycerol adulteration in red wine with Fourier transform infrared spectroscopy using chemometrics and artificial neural networks, *Appl. Spectros.*, 59, 1553, 2005.
167. Coimbra, M.A. et al., Quantification of polymeric mannose in wine extracts by FT-IR spectroscopy and OSC-PLS1 regression, *Carbohydrate Poly.*, 61, 434, 2005.
168. Moreira, J.L. and Santos, L., Analysis of organic acids in wines by Fourier-transform infrared spectroscopy, *Anal. Bioanal. Chem.*, 382, 421, 2005.
169. Pennington, N. et al., Red and white wine varietal authenticity using FTIR spectroscopy and chemometrics, in *Abstracts of Papers, 229th ACS National Meeting* American Chemical Society, San Diego, CA, 2005.
170. Urbano Cuadrado, M. et al., Comparison and joint use of near infrared spectroscopy and Fourier transform mid-infrared spectroscopy for the determination of wine parameters, *Talanta*, 66, 218, 2005.
171. Penman, K.G. et al., Bilberry adulteration using the food dye amaranth, *J. Agric. Food Chem.*, 54, 7378, 2006.
172. Wrolstad, R.E. et al., Detection of adulteration in blackberry juice concentrates and wines, *J. AOAC*, 65, 1417, 1982.

6.4 Analysis of Betalains

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6.4.1 EXTRACTION

In contrast to colorless phenolics, betalains are most soluble in water and are even more hydrophilic than the anthocyanins that readily dissolve in methanol. However, to preclude activities of endogenous enzymes during extraction, aqueous methanol at a ratio of 60:40 or 80:20 (v/v) is recommended.

While water will readily leach the pigments from the matrix, methanol will denature the protein structures of the enzymes present, thus inhibiting degradation reactions by polyphenoloxidases, peroxidases, and β -glucosidases.¹⁻⁶ As a result, it is ensured that the extract will exhibit the pattern actually present in the particular plant tissue under study. No acidification is needed; it is even considered counter-productive due to the lower stabilities of betalain pigments at acidic pH.^{7,8} A ratio of 10 parts extraction solvent to 1 part plant material is usually appropriate.⁹⁻¹¹ In some instances, sodium ascorbate is added to prevent oxidation, i.e., browning.^{9,11,12} These precautionary measures have proven especially helpful for working with betaxanthin-rich plant material exhibiting highly oxidable structures such as miraxanthin V (dopamine-betaxanthin), portulacaxanthin II (tyrosine-betaxanthin) and 3-methoxy-tyramine-betaxanthin.^{9,11-13}

Special care must be taken in the case of betacyanins acylated with dicarboxylic acids such as malonic acid, i.e., phyllocactin. Upon acidification in methanol, methyl-esterification may easily proceed, thus generating a new pigment that is not genuine

to the plant material under investigation.^{14,15} Notably, a sample work-up analogous to the one used for anthocyanins cannot be recommended and the specific stability characteristics must be taken into account. To avoid pigment decay, the resulting extracts should be carefully concentrated under vacuum at bath temperatures lower than 30°C and kept frozen until further analyses.

6.4.2 ISOLATION AND PURIFICATION

Prior to semi-preparative HPLC, matrix compounds may be present that require a purification step. In some cases, high sugar or salt contents and also pectic-like substances may preclude concentration and isolation; precipitation using 2-propanol or ethanol is then required.¹⁶ Subsequent filtration will yield a pigment solution and a colorless filtrate that may be rinsed with a mixture of one part water and two parts alcohol for complete discoloration. The filtrate volume will be reduced under vacuum before further purification. Usually, precipitation precedes desalting.

While earlier studies have reported the use of cation or anion exchange methods for betalain purification and fractionation, highly acidic conditions or high salt concentrations were required.^{17–24} Whereas the first method promotes pigment degradation, especially during the evaporation process by gradual acidity increase, the latter method simply substitutes sugars versus buffer or sodium chloride in the resulting eluates, requiring an additional desalting step.²⁵ Another approach was to mimic conditions that govern HPLC separation processes. Using a polymeric resin, Czapski elaborated a technique for separation of betalains from accompanying matrix components.²⁶ This idea was developed further when removal of sugars and salts and a separate elution of betaxanthins from betacyanins were achieved on solid phase extraction (SPE) cartridges, thereby allowing purification of betalainic solutions on a small scale.^{16,27}

While these purification procedures will be afflicted with loss of total pigment content, the genuine pigment pattern was proven to remain unchanged.^{16,27} Most recent attempts to separate anthocyanins from betalains were successful using reversed phase SPE cartridges. The samples were applied to cartridges pre-equilibrated with methanol and excessive washing with purified water of neutral pH. After application of the samples containing both betacyanins and anthocyanins, the first were eluted with neutral water, while the latter were obtained by successive elution with methanol (Stintzing, unpublished data).

It is noteworthy that trifluoroacetic acid was introduced as the most appropriate acidifier for column chromatography and solid phase extraction techniques, i.e., low boiling point due to its high acidity, requiring low amounts to reach the respective pH. Also, its high volatility allows easy evaporation thus minimizing the thermal load and acidification during concentration.

To ensure maximum pigment retention, the acidic eluates should be checked for their pH values and if required, aqueous ammonia has been proven viable for pH adjustment to reach 5 to 7 pH values. While Sephadex G types were used in earlier studies for purification and desalting, Sephadex LH-20 is the material of choice today. Since the betalains will elute with water, it is doubtful whether effective salt removal is possible. However, Sephadex LH-20 has proven excellent for removing

colorless phenolics from betalain-containing solutions and also for fractionation of betaxanthins, betacyanins, and betacyanin aglycones.^{28–33} The eluting aqueous extracts are suited for isolation of single compounds by semi-preparative HPLC for which volatile solvents such as formic or acetic acids are highly recommended, because they may be easily removed by evaporation afterward.^{9,10,25,30,34–39}

If NMR measurements are intended, the eluting betalain fractions should be very carefully concentrated under vacuum by repetitive addition of purified water or directly lyophilized before frozen storage.^{37,38} For other purposes, such as for model studies, betalain stabilization may be achieved by continuous pH control and addition of aqueous ammonia prior to concentration.^{30,40}

6.4.3 SPECTROPHOTOMETRIC CHARACTERIZATION

6.4.3.1 PIGMENT QUANTITY

After a series of well-designed experiments, Nilsson proposed a method for simultaneous spectrophotometric quantification of betaxanthins and betacyanins in red beets.⁴¹ Through this method, brownish co-absorbing substances by abstraction of the absorption at 600 nm and also the cross-absorption of betaxanthins and betacyanins was considered. Some follow-up investigations created computer models to implement this quantification method and were found appropriate for unheated samples.^{42–45}

Although spectrophotometry was reported to yield higher pigment contents than the true values, Nilsson's method is still recommended for red beet samples today.^{46,47} However, in a recent study on cactus fruit juices, Nilsson's method was found inappropriate for properly determining betalain content⁴⁸ due to different ratios of betaxanthins and betacyanins when compared to beets. The resulting suggestion was to assess the absorption at two fixed wavelengths for the yellow and the red betalains, respectively, and subsequent correction of the values by the relative ratio of the same pigment classes obtained from HPLC chromatograms at 470 nm and 538 nm, respectively. This approach has also proven valuable for colored Swiss chard petioles.⁹

Most often, quantification has been carried out by exclusively assessing the absorption at the maximum wavelength and selection of the appropriate molar extinction coefficient.^{12,49,50} Reasonable values will be obtained if the absorption at 600 to 650 nm is subtracted.

It should also be kept in mind that the pigment solution under investigation should exhibit a constant pH. McIlvaine buffer was found to be the most suitable because it contains both citric acid and phosphate ions previously reported to enhance betalain stability.^{48,51} According to our observations, a pH of 6.0 or 6.5 should be chosen for spectrophotometric quantification, and not more than 20% of the final dilution should stem from the sample.

Until 2004, no betalain standards were commercially available and current products lack the required purity. Preparative isolation from plant material is laborious and costly and the resulting standard substances vary in relative humidity, crystal water, and salts,⁵² resulting in over- or under-estimation of pigment contents

(Stintzing, unpublished observations). Analogous aspects were recently addressed in an extended study of anthocyanin quantification.⁵³ Spectrophotometric determination is still the most preferred method that has been shown to compare well with HPLC measurements.⁴⁶ Hence, only in rare cases have purified standards from a particular food commodity been used for calibration by HPLC-DAD.^{54,55}

It is expected, however, that the results obtained by HPLC or the spectrophotometric assay will differ. The latter method is more prone to over- or under-estimation by cross-absorption of the analyte and the accompanying compounds, especially in heated samples.⁴⁶ On the other hand, using HPLC quantification, a number of minor compounds may be neglected although contributing to overall pigment content because some of them do not reach the respective quantitation limit. In most cases, the sample to be quantified contains more than one major betalain. Therefore, a couple of standards are required. Alternatively, quantification may be performed on an equivalent basis assuming that an individual pigment exhibits a comparative extinction coefficient to the remaining ones, i.e., betanin for red beets and cactus pears, vulgaxanthin I for red and yellow beets, indicaxanthin for cactus pears, and amaranthin for amaranth samples.

Due to varying solvent systems, varying purities and water contents of purified pigments, different molar extinction coefficients have been reported.^{41,50,52,56–60} The most reliable ones commonly applied are 60,000 L/mol*cm for betanin, 56,600 L/mol*cm for amaranthin, and 48,000 L/mol*cm for betaxanthins.^{50,52,61} Pigment contents may be calculated with the following formulae.^{48,62}

$$\text{Betalain content [mg/g fresh weight]} = A * MW * V_f * DF / \epsilon * L * W_f$$

$$\text{Betalain content [mg/g dry weight]} = A * MW * V_d * DF / \epsilon * L * W_d$$

$$\text{Betalain content [mg/L]} = [(A * DF * MW * 1000 / \epsilon * l)]$$

where A is absorption at λ_{max} corrected by the absorption at 600 nm or even 650 nm; MW represents the molecular weights of betanin (550 g/mol), amaranthine (726 g/mol), vulgaxanthin I (339 g/mol), and indicaxanthin (308 g/mol), respectively; V is the total extract volume, DF is the dilution factor, ϵ is molar absorptivity, L equals path length, and W is the fresh (W_f) or dry weight (W_d) of the plant material.⁶³

6.4.3.2 PIGMENT QUALITY

In addition to the pigment concentration in the respective food source, the color quality is of major importance for plant material quality assessment and selection during production and storage. Color quality also strongly affects consumer purchase decisions. Since red beet is still the sole betalain source exploited commercially, quality parameters have been developed for beet preparations. The most important one is the so-called color shade representing a ratio of two absorbance values, namely for betaxanthins and for betacyanins, respectively, A (at 535 nm)/A (at 480 nm).

Color shades of 1.5 to 1.8 are optimum, while values below 1.2 are considered inadequate for food coloring purposes. This ratio may also be inversed, i.e., values from 0.65 and 0.85 considered standard.⁶⁴ Color shade values may also be derived from HPLC-DAD chromatography as recently established by Herbach and co-workers.⁶⁵ In addition, CIEL*a*b* values offer viable tools for color quality assessment. While the lightness value L^* is an indicator of color strength, a^* and b^* values are commonly translated into chroma $C^* = [(a^{*2} + b^{*2})^{0.5}]$ and hue angle $h^\circ = [\arctan^{-1}(b^*/a^*)]$ values to express color brilliance or purity and the tonality of a sample, respectively.

Whereas color monitoring in the course of food production should be performed based on the same dilutions of samples to track changes of L^* , C^* , and h° , it may be advantageous to adjust absorbance values to constant values in other cases. By setting a definite absorptivity at the maximum wavelength, i.e., 1.00 ± 0.05 , the L^* value is normalised. Thus, data interpretation is less complex and reduced to the C^* and h° coordinates, respectively.^{9,63,66,70} It may also be worthwhile to establish a total color change including L^* , C^* , and h° values. In this case, the following formula will apply: $[\Delta E^* = (\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2})^{0.5}]$ with $[\Delta L^* = (L^*_2 - L^*_1)]$, $[\Delta a^* = (a^*_2 - a^*_1)]$, $[\Delta b^* = (b^*_2 - b^*_1)]$ equivalent to $[\Delta E^* = (\Delta L^{*2} + \Delta C^{*2} + \Delta H^{*2})^{0.5}]$ with $[\Delta L^* = (L^*_2 - L^*_1)]$, $[\Delta C^* = (C^*_2 - C^*_1)]$, and $[\Delta H^* = (2 \times (\sin(h^\circ_2 - h^\circ_1)/2) \times (C^*_2 \times C^*_1)^{0.5})]$.^{71–74} CIE data may only be compared if the same illuminant and observer angle are employed, with D_{65} and 10° being the most common settings, respectively.

6.4.4 CHARACTERIZATION AND IDENTIFICATION

6.4.4.1 STANDARD PREPARATION

Achievements in betalain analyses prior to the early 1990s have been extensively reviewed elsewhere.^{17,18,75} Since no commercial standards with the required purity are yet available, structural dereplication is usually performed by comparison with extracts of known composition or by partial synthesis. The most common betacyanins, betanin and isobetanin, may be assigned by co-injection of the juice or a carefully prepared extract from red beet. These major betacyanins in beet are usually accompanied by the less polar corresponding aglycones, betanidin and isobetanidin, generated by residual β -glucosidase activity.

Since red beet color constitutes a blend of betacyanins and betaxanthins, respectively, the red beet preparation usually exhibits one major betaxanthin, vulgaxanthin I, the glutamine adduct of betalamic acid.^{76–78} The second most common betaxanthin is indicaxanthin, representing the major betalain from cactus pears.^{27,56,63} Typical sources for betacyanins may be *Amaranthus* species for amaranthin and isoamaranthin and *Gomphrena* species for gomphrenin I and isogomphrenin I. Although neobetainin has been identified in red beet and cactus pears, its genuine occurrence is not guaranteed, but may be intentionally generated upon heating at acidic pH (Stintzing and Herbach, unpublished results).^{33,63,79,80}

2-Decarboxybetanidin, the glucoside of which was reported recently in hairy root cultures of yellow beet, may be synthesized from dopamine.¹² Two further alternatives were proposed earlier. While Minale and co-workers subjected betanin

to thermal decarboxylation, Piattelli and Impellizzeri achieved 5,6-dihydroxyindol-2,3-dihydroindole by oxidative cyclization of 3-hydroxytyramine followed by condensation with betalamic acid.^{81,82} According to recent reports, 2-decarboxybetanin will be formed after thermal exposure of betanin in aqueous solutions. This was corroborated by Mosshammer and co-workers when purified pigment solutions from cactus pear juice were heated at pH 4.^{83–85}

For betaxanthins, partial synthesis is quite common and presents a viable tool for identification by co-injection experiments.^{12,27,86} Starting from a red beet extract or semi-purified betanin-isobetanin blend, alkaline hydrolysis by addition of 32% ammonia is initiated. Spectrophotometric monitoring at 424 nm allows the release of betalamic acid to be followed. Betaxanthins are obtained through the addition of the respective amino acid or amine in at least 20-fold molar excess followed by careful evaporation.⁵⁹ Since the starting material most often consists of a racemic betacyanin mixture, the resulting betaxanthin will also consist of two stereoisomers that may not easily be separated by HPLC.¹²

The feasibility of partial synthesis has been demonstrated in the course of structural confirmation of a great number of betaxanthins.^{9,12,27,86} Due to incomplete hydrolyses, residual betanin and isobetanin may also accompany the respective target betaxanthin. Most importantly, the purification of betalamic acid does not appear to be possible because it exhibits both amino and aldehyde functions (Stintzing, unpublished observations), resulting in self-condensation.⁸⁷ Therefore, data by Barth and co-workers reporting NMR data on purified betalamic acid should be carefully interpreted.⁸⁸

Forthright isolation of standard substances from known sources may be achieved by analytical and/or semi-preparative HPLC.^{30,37,38,89} Although it appears promising with respect to obtainable pigment yields, countercurrent chromatography has been applied only once for red beets but lacked sufficient separation efficiency.⁹⁰

6.4.4.2 HPLC-UV AND HPLC-DAD

Investigations relying on HPLC coupled to a UV-Vis detector should be set at 470 to 475 nm for betaxanthins and 535 to 540 nm for betacyanins. Gradient elution is commonly preferred to achieve complete separation. If a diode array detector is available, common monitoring wavelengths are 280 nm for colorless phenolics, 406 nm for betalamic acid, 470 nm for betaxanthins, and 536 nm for betacyanins.

While earlier papers cited buffer systems or aqueous *o*-phosphoric acid to achieve satisfactory peak resolution, most recent investigations involved acetic acid or formic acid systems.^{8–10,12,16,25,61,63,66,67,78,89,91,92} Representative examples are 0.2% and 1% HCOOH for betacyanins and betaxanthins, respectively, the latter requiring a lower pH for chromatographic resolution. Methanol or acetonitrile are most commonly used as modifiers, either undiluted or diluted with purified water at ratios of 60:40 or 80:20 (v/v), respectively.^{9,6,67,89} Typical HPLC fingerprints for yellow and red beet juice are shown in Figure 6.4.1.

Optimized HPLC separation allows most betaxanthins to be separated on a C18 reversed phase stationary phase according to their respective polarities.^{12,27} Considerable progress was achieved by the introduction of a highly polar silica-based column, which allowed major improvement of peak resolution, especially at early

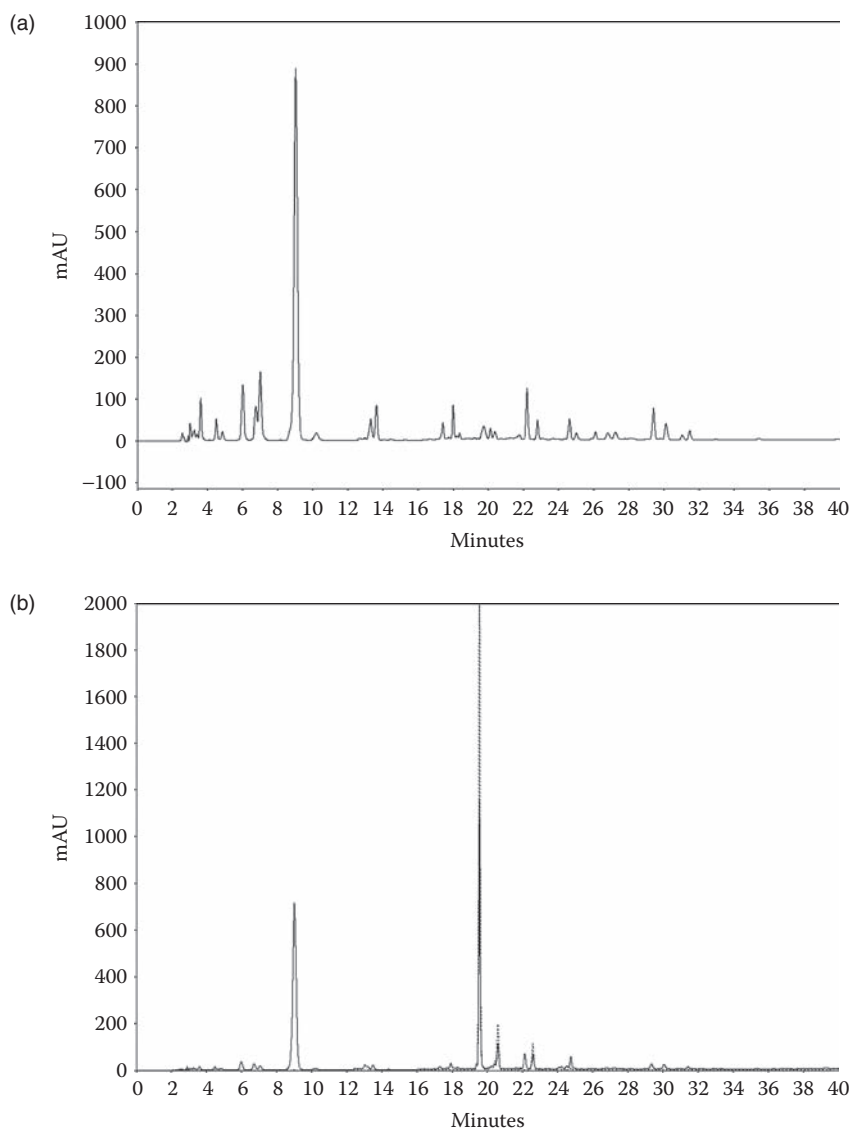


FIGURE 6.4.1 (a) HPLC fingerprint of a yellow beet juice monitored at 470 nm. The main peak is vulgaxanthin I ($t_R = 9.0$ min). No betacyanins are present. (b) HPLC fingerprint of a red beet juice monitored at 470 nm (solid line) and 535 nm (dotted line), respectively. The main betaxanthin (470 nm) is vulgaxanthin I ($t_R = 9.0$ min), while the major betacyanins (535 nm) are betanin, isobetanin, and betanidin ($t_R = 19.5, 20.6$ and 22.6 min, respectively).

retention times.⁹ While the use of a diode array detector will provide the spectral characteristics of individual compounds, it does not substantially contribute to betaxanthin identification because most betaxanthins only slightly differ in their spectral characteristics.

As a rule, indicaxanthin and portulacaxanthin I exhibit the highest absorption maxima. The lowest are usually represented by amine adducts of betalamic acid such as miraxanthin V.^{9,12,27,86} The absolute values will also be affected by the respective HPLC solvent and pH applied, so small deviations are reported for the same compounds in the literature.^{12,27} Therefore co-injection experiments with semi-synthetic standards are mandatory while access to mass spectrometric characterization usually simplifies structure identification.

6.4.4.3 HPLC-FD AND CZE

Very recently, HPLC with fluorescence detection was recommended for improving detection sensitivities of betalains.^{93,94} While this technique may be worthwhile for betaxanthin analyses, its use for betacyanins cannot be recommended. Although this technique represents a worthwhile approach requiring low amounts of solvent and sample and generally characterized by a high separation efficiency, only one study dealt with the use of capillary zone electrophoresis for betalain analyses.⁹⁵

6.4.4.4 LC-MS

In a recently published example of betaxanthin analyses in a complex food matrix, 19 betaxanthins were assigned in yellow Swiss chard petioles.⁹ Mass spectrometric measurements are even more helpful if unknown betacyanin structures are to be elucidated. While betacyanin plant materials such as red beet and amaranth may still be commercially available for coinjection experiments and comparison with samples under investigation, it may be an easier task to first optimize pigment separation followed by mass spectrometric measurements.

On the other hand, for the characterization of complex pigment profiles as recently reported for heated red beet and pitaya preparations, mass spectrometric detection is a prerequisite.^{66,67,83,84,89} Electrospray ionization techniques operating in the negative ionization mode were shown to be less sensitive and are rarely applied.^{9,89} For mass detection, highly volatile eluent compositions are required for mass detection; acetic and formic acids together with methanol or acetonitrile are the most frequently used.^{9,10,25,61,63,89,96} Although trifluoroacetic acid (TFA) was applied earlier to achieve optimum betaxanthin separation, this volatile acid cannot be recommended due to its ion-suppressing effect, which reduced detection sensitivity.²⁷ Furthermore, TFA exhibits a memory effect in the negative ionization mode, producing predominant monomers, dimers, and trimers and thus precluding proper mass signal acquisition of the analyte.

6.4.4.5 LC-NMR AND NMR

While ¹H-NMR data have been published both for betacyanins and for betaxanthins, ¹³C data of C₁₄-C₁₅ saturated betacyanins and betaxanthins have only recently become

accessible.^{10,18,25,75,37,38} Previous reports noted that highly acidic conditions were detrimental for betacyanins, thus precluding long-term data acquisition.^{10,18,25,75} The first ¹³C NMR data were obtained for neobetanin (14,15-dehydrobetanin), exhibiting a reasonable stability toward low pH values.⁸⁰ By introducing a water-based set up working at neutral and slightly acidic conditions, ¹³C data for betanin, isobetanin, phyllocactin (malonyl-betanin) and hylocerenin (3-hydroxy-3-methyl-glutaryl-beta-nin) and for indicaxanthin and miraxanthin V were reported for the first time.^{37,38}

In addition, an LC-NMR technique was presented.³⁷ By comparing NMR data obtained under continuous LC and batch conditions as well as considering literature values, the impact of solvent acidity and solvent type was clearly demonstrated.^{37,38} The proposed solvent systems have been successfully applied to mono- and bi-decarboxylated betacyanins very recently.⁹⁷ Hence, it is expected that through using this new experimental approach, further structural elucidation of betalains may be achieved in the future.

REFERENCES

1. Escribano, J., Cabanes, J., and García-Carmona, F., Characterisation of latent polyphenol oxidase in table beet: effect of sodium dodecyl sulphate, *J. Sci. Food Agric.*, 73, 34, 1997.
2. Gandía-Herrero, F., García-Carmona, F., and Escribano, J., Purification and characterization of a latent polyphenol oxidase from beet root (*Beta vulgaris* L.), *J. Agric. Food Chem.*, 52, 609, 2004.
3. Gandía-Herrero, F., Escribano, J., and García-Carmona, F., Characterization of the activity of tyrosinase on betaxanthins derived from (*R*)-amino acids, *J. Agric. Food Chem.*, 53, 9207, 2005.
4. Strack, D., Vogt, T., and Schliemann, W., Recent advances in betalain research, *Phytochemistry*, 62, 247, 2003.
5. Stintzing, F.C. and Carle, R., Functional properties of anthocyanins and betalains in plants, food, and in human nutrition, *Trends Food Sci. Technol.*, 15, 19, 2004.
6. Stintzing, F.C., Schieber, A., and Carle, R., Rote Bete als färbendes Lebensmittel — eine Bestandsaufnahme. *Obst Gem. Kartoffelver. Fruit Veg. Potato Process.*, 85, 196, 2000.
7. Von Elbe, J.H., Maing, I.-Y., and Amundson, C.H., Color stability of betanin, *J. Food Sci.*, 39, 334, 1974.
8. Stintzing, F.C. et al., Betacyanins and phenolic compounds from *Amaranthus spinosus* L. and *Boerhavia erecta* L., *Ztschr. Naturforsch. C/J. Biosci.*, 59, 1, 2004.
9. Kugler, F., Stintzing, F.C., and Carle, R., Identification of betalains from petioles of differently colored Swiss chard (*Beta vulgaris* L. ssp. *cicla* [L.] Alef. cv. Bright Lights) by high-performance liquid chromatography–electrospray ionization mass spectrometry, *J. Agric. Food Chem.*, 52, 2975, 2004.
10. Kobayashi, N. et al., Formation and occurrence of dopamine-derived betacyanins. *Phytochemistry*, 56, 429, 2001.
11. Schliemann, W. et al., Betalains of *Celosia argentea*, *Phytochemistry*, 58, 159, 2001.
12. Schliemann, W., Kobayashi, N., and Strack, D., The decisive step in betaxanthin biosynthesis is a spontaneous reaction, *Plant Physiol.*, 119, 1217, 1999.

13. Kugler, F. et al., Determination of free amino compounds in betalainic fruits and vegetables by gas chromatography with flame ionization and mass spectrometric detection, *J. Agric. Food Chem.*, 54, 4311, 2006.
14. Fossen, T., Slimestad, R., and Andersen, O.M., Anthocyanins from maize (*Zea mays*) and reed canarygrass (*Phalaris arundinaceae*), *J. Agric. Food Chem.*, 49, 2318, 2001.
15. Stintzing, F.C. et al., A novel zwitterionic anthocyanin from evergreen blackberry (*Rubus laciniatus* Willd.), *J. Agric. Food Chem.*, 50, 396, 2002.
16. Stintzing, F.C., Schieber, A., and Carle, R., Betacyanins in fruits from red-purple pitaya, *Hylocereus polyrhizus* (Weber) Britton & Rose, *Food Chem.*, 77, 101, 517, 2002.
17. Steglich, W. and Strack, D., Betalains, in *The Alkaloids: Chemistry and Pharmacology*, Brossi, A., Ed., Academic Press, San Diego, 39, 1990.
18. Strack, D., Steglich, W., and Wray, V., Betalains, in *Methods in Plant Biochemistry*, Dey, P.M. and Harborne, J.B., Eds., Academic Press, London, 421, 1993.
19. Colomas, J., Séparation des pigments bétalainiques par une méthode fondée sur la chromatographie sur gel de Séphadex appliquée à des plantules d' *Amaranthus caudatus* L. var. *pendula*, *Ztschr. Pflanzenphysiol.*, 85, 227, 1977.
20. Colomas, J., Barthe, P., and Bulard, C., Séparation et identification des bétalaines synthétisées par les tissus de tige de *Myrtillocactus geometrizans* cultivés *in vitro*, *Ztschr. Pflanzenphysiol.*, 87, 341, 1978.
21. Wilkins, C.K., Technical note: Improved separations of beet pigments, *Int. J. Food Sci. Technol.*, 22, 571, 1987.
22. Bokern, M. et al., Ferulic acid conjugates and betacyanins from cell cultures of *Beta vulgaris*. *Phytochemistry*, 30, 3261, 1991.
23. Strack, D. et al., Feruloylbetanin from petals of *Lampranthus* and feruloylamaranthin from cell suspension cultures of *Chenopodium rubrum*, *Phytochemistry* 27, 3529, 1988.
24. Döpp, H. and Musso, H., Isolierung und Chromophore der Farbstoffe aus *Amanita muscaria*, *Chem. Ber.*, 106, 3473, 1973.
25. Schliemann, W. et al., Betacyanins from plants and cell cultures of *Phytolacca americana*, *Phytochemistry*, 42, 1039, 1996.
26. Czapski, J., The effect of heating conditions on losses and regeneration of betacyanins, *Ztschr. Lebensm. Unters. Forsch.*, 180, 21, 1985.
27. Stintzing, F.C., Schieber, A., and Carle, R., Identification of betalains from yellow beet (*Beta vulgaris* L.) and cactus pear (*Opuntia ficus-indica* (L.) Mill.) by high-performance liquid chromatography-electrospray ionization mass spectrometry, *J. Agric. Food Chem.*, 50, 2302, 2002.
28. Cai, Y. et al., Chemical stability and colorant properties of betaxanthin pigments from *Celosia argentea*, *J. Agric. Food Chem.*, 49, 4429, 2001.
29. Frank, T. et al., Urinary pharmacokinetics of betalains following consumption of red beet juice in healthy humans, *Pharmacol. Res.*, 52, 290, 2005.
30. Herbach, K.M. et al., Isotope ratio mass spectrometrical analysis of betanin and isobetanin isolates for authenticity evaluation of purple pitaya-based products, *Food Chem.*, 99, 204, 2006.
31. Netzel, M. et al., Renal excretion of antioxidative constituents from red beet in humans, *Food Res. Int.*, 38, 1051, 2005.
32. Kujala, T.S. et al., Phenolics and betacyanins in red beetroot (*Beta vulgaris*) root: distribution and effect of cold storage on the content of total phenolics and three individual compounds, *J. Agric. Food Chem.*, 48, 5338, 2000.
33. Kujala, T., Loponen, J., and Pihlaja, K., Betalains and phenolics in red beetroot (*Beta vulgaris*) peel extracts: extraction and characterisation, *Z. Naturforsch C/J. Biosci.*, 56, 343, 2001.

34. Heuer, S. et al., Betacyanins from flowers of *Gomphrena globosa*, *Phytochemistry*, 31, 1801, 1992.
35. Heuer, S. et al., Betacyanins from bracts of *Bougainvillea glabra*, *Phytochemistry*, 37, 761, 1994.
36. Kobayashi, N. et al., Betalains from *Christmas cactus*. *Phytochemistry*, 54, 419, 2000.
37. Stintzing, F.C. et al., Structural investigations on betacyanin pigments by LC NMR and 2D NMR spectroscopy, *Phytochemistry*, 65, 415, 2004.
38. Stintzing, F.C. et al., First ¹³C-NMR assignments of betaxanthins. *Helv. Chim. Acta*, 89, 1008, 2006.
39. Strack, D. et al., Humilixanthin, a new betaxanthin from *Rivina humilis*, *Phytochemistry*, 26, 2285, 1987.
40. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Colour studies on fruit juice blends from *Opuntia* and *Hylocereus* cacti and betalain-containing model solutions derived therefrom, *Food Res. Int.*, 38, 975, 2005.
41. Nilsson, T., Studies into the pigments in beetroot (*Beta vulgaris* L. *vulgaris* var. *rubra* L.), *Lantbrukshög. Ann.*, 36, 179, 1970.
42. Kopelman, I.J., and Saguy, I., Color stability of beet powders, *J. Food Process. Preserv.*, 1, 217, 1977.
43. Saguy, I., Mizrahi, S., and Kopelman, I.J., Mathematical approach for the determination of dyes concentration in mixtures, *J. Food Sci.*, 43, 121, 1978.
44. Saguy, I., Kopelman, I.J., and Mizrahi, S., Computer-aided determination of beet powders, *J. Food Sci.*, 43, 124, 1978.
45. Saguy, I., Thermostability of red beet pigments (betanine and vulgaxanthine-I): influence of pH and temperature, *J. Food Sci.*, 44, 1554, 1979.
46. Schwartz, S.J., Hildenbrand, B.E., and Von Elbe J.H., Comparison of spectrophotometric and HPLC methods to quantify betacyanins, *J. Food Sci.*, 46, 296, 1981.
47. Von Elbe, J.H., Betalains, in *Handbook of Food Analytical Chemistry*. Wrolstad, R.E. et al., Eds., John Wiley & Sons, New York, 2005, 123.
48. Stintzing, F.C., Schieber, A., and Carle, R., Evaluation of colour properties and chemical quality parameters of cactus juices, *Eur. Food Res. Technol.*, 216, 303, 2003.
49. Cai, Y.Z. and Corke, H., *Amaranthus* betacyanin pigments applied in model food systems, *J. Food Sci.*, 64, 869, 1999.
50. Girod, P.-A. and Zryd, J.-P., Secondary metabolism in cultured red beet (*Beta vulgaris* L.) cells: Differential regulation of betaxanthin and betacyanin biosynthesis, *Plant Cell Tiss. Org. Cult.*, 25, 1, 1991.
51. Pasch, J.H. and Von Elbe, J.H., Betanine stability in buffered solutions containing organic acids, metal cations, antioxidants, or sequestrants, *J. Food Sci.*, 44, 72, 1979.
52. Wyler, H. and Meuer, U., Zur Biogenese der Betacyane: Versuche mit [2-¹⁴C]-Dopaxanthin, *Helv. Chim. Acta*, 62, 1330, 1979.
53. Lee, J., Durst, R.W., and Wrolstad, R.E., Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study, *J. AOAC Int.*, 88, 1269, 2005.
54. Kujala, T.S. et al., Betalain and phenolic compositions of four beetroot (*Beta vulgaris*) cultivars, *Eur. Food Res. Technol.*, 214, 505, 2002.
55. Butera, D. et al., Antioxidant activities of Sicilian prickly pear (*Opuntia ficus-indica*) fruit extracts and reducing properties of its betalains: betanin and indicaxanthin, *J. Agric. Food Chem.*, 50, 6895, 2002.
56. Piattelli, M., Minale, L., and Protà, G., Isolation, structure and absolute configuration of indicaxanthin, *Tetrahedron*, 20, 2325, 1964.

57. Piattelli, M. and Imperato, F., Betacyanins of the family Cactaceae, *Phytochemistry*, 8, 1503, 1969.
58. Schwartz, S.J. and Von Elbe, J.H., Quantitative determination of individual betacyanin pigments by high-performance liquid chromatography, *J. Agric. Food Chem.*, 28, 540, 1980.
59. Trezzini, G.F. and Zryd, J.-P., Characterization of some natural and semi-synthetic betaxanthins, *Phytochemistry*, 30, 1901, 1991.
60. Wyler, H., Wilcox, M.E., and Dreiding, A.S., Umwandlung eines Betacyans in ein Betaxanthin, Synthese von Indicaxanthin aus Betanin, *Helv. Chim. Acta*, 48, 361, 1965.
61. Cai, Y., Sun, M., and Corke, H., Identification and distribution of simple and acylated betacyanins in the Amaranthaceae, *J. Agric. Food Chem.* 49, 1971, 2001.
62. Cai, Y. et al., Characterization and quantification of betacyanin pigments from diverse *Amaranthus* species, *J. Agric. Food Chem.*, 46, 2063, 1998.
63. Stintzing, F.C. et al., Color, betalain pattern and antioxidant properties of cactus pear (*Opuntia* sp.) clones, *J. Agric. Food Chem.*, 53, 442, 2005.
64. Mégard, D., Stability of red beet pigments for use as food colorant: a review, *Foods Food Ingred. J.*, 158, 130, 1993.
65. Herbach, K.M., Stintzing, F.C., and Carle, R., Stability and color changes of thermally treated betanin, phyllocactin, and hylocerenin solutions, *J. Agric. Food Chem.* 54, 390, 2006.
66. Herbach, K.M., Stintzing, F.C., and Carle, R., Impact of thermal treatment on color and pigment pattern of red beet (*Beta vulgaris* L.) preparations, *J. Food Sci.*, 69, C491, 2004.
67. Herbach, K.M., Stintzing, F.C., and Carle, R., Thermal degradation of betacyanins in juices from purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) monitored by high-performance liquid chromatography–tandem mass spectrometric analyses, *Eur. Food Res. Technol.*, 219, 377, 2004.
68. Herbach, K.M. et al., Structural and chromatic stability of purple pitaya (*Hylocereus polyrhizus* [Weber] Britton & Rose) betacyanins as affected by the juice matrix and selected additives, *Food Res. Int.*, 39, 667, 2006.
69. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Development of a process for the production of a betalain-based colouring foodstuff from cactus pear, *Innov. Food Sci. Emerg. Technol.*, 6, 221, 2005.
70. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Colour studies on fruit juice blends from *Opuntia* and *Hylocereus* cacti and betalain-containing model solutions derived therefrom, *Food Res. Int.*, 38, 975, 2005.
71. Gonnet, J.F., Colour effects of co-pigmentation of anthocyanins revisited. 1. A colorimetric definition using the CIELAB scale, *Food Chem.*, 63, 409, 1998.
72. Herbach, K.M. et al., Effects of processing and storage on juice colour and betacyanin stability of purple pitaya (*Hylocereus polyrhizus*) juice, *Eur. Food Res. Technol.*, 224, 649, 2007.
73. Mosshammer, M.R., Stintzing, F.C., and Carle, R., Evaluation of different methods for the production of juice concentrates and fruit powders from cactus pear, *Innov. Food Sci. Technol.*, 7, 275, 2006.
74. Mosshammer, M.R. et al., Impact of thermal treatment and storage on color of yellow-orange cactus pear (*Opuntia ficus-indica* [L.] Mill. cv. 'Gialla') juices, *J. Food Sci.*, 71, C400, 2006.
75. Strack, D. and Wray, V., Anthocyanins, in *Methods in Plant Biochemistry 1: Plant Phenolics*, Dey, P.M. and Harborne, J.B., Eds., Academic Press, London, 1994, 325.

76. Piattelli, M., Minale, L., and Protà, G., Pigments of Centrospermae. III. Betaxanthins from *Beta vulgaris* L., *Phytochemistry* 4, 121, 1965.
77. Strack, D. and Reznik, H., High-performance liquid chromatographic analysis of betaxanthins in Centrospermae (Caryophyllales), *Ztschr. Pflanzenphysiol.*, 94, 163, 1979.
78. Vincent, K.R. and Scholz, R.G., Separation and quantification of red beet betacyanins and betaxanthins by high-performance liquid chromatography, *J. Agric. Food Chem.*, 26, 812, 1978.
79. Alard, D. et al., Neobetanin: isolation and identification from *Beta vulgaris*, *Phytochemistry*, 24, 2383, 1985.
80. Strack, D., Engel, U., and Wray, V., Neobetanin: a new natural plant constituent, *Phytochemistry*, 26, 2399, 1987.
81. Minale, L., Piattelli, M., and Nicolaus, R.A., Decarbossilazione termica dei betaciani e delle betaxantine, *Rend. Accad. Sci. Fis. Mat.*, 32, 165, 1965.
82. Piattelli, M. and Impellizzeri, G., 2-Decarboxybetanidin, a minor betacyanin from *Carpobrotus acinaciformis*, *Phytochemistry*, 9, 2553, 1970.
83. Wybraniec, S., Formation of decarboxylated betacyanins in heated purified betacyanin fractions from red beet root (*Beta vulgaris* L.) monitored by LC-MS/MS, *J. Agric. Food Chem.*, 53, 3483, 2005.
84. Wybraniec, S. and Mizrahi, Y., Generation of decarboxylated and dehydrogenated betacyanins in thermally treated purified fruit extract from purple pitaya (*Hylocereus polyrhizus*) monitored by LC-MS/MS, *J. Agric. Food Chem.*, 53, 6704, 2005.
85. Mosshammer, M.R. et al., Stability of yellow-orange cactus pear (*Opuntia ficus-indica* [L.] Mill. cv. 'Gialla') betalains as affected by the juice matrix and selected food additives, *Eur. Food Res. Technol.*, 225, 21, 2007.
86. Trezzini, G.F. and Zryd, J.-P., Two betalains from *Portulaca grandiflora*, *Phytochemistry*, 30, 1897, 1991.
87. Bilyk, A. and Howard, M., Reversibility of thermal degradation of betacyanines under the influence of isoascorbic acid, *J. Agric. Food Chem.*, 30, 906, 1982.
88. Barth, H. et al., Konstitution und Synthese des Muscaflavins, *Liebigs Ann. Chem.*, 12, 2164, 1981.
89. Herbach, K.M., Stintzing, F.C., and Carle, R., Identification of heat-induced degradation products from purified betanin, phyllocactin and hylocerenin by high-performance liquid chromatography/electrospray ionization mass spectrometry, *Rapid Commun. Mass Spectrom.*, 19, 2603; 20, 1822, 2006.
90. Degenhardt, A. and Winterhalter, P., Isolation of natural pigments by high speed CCC, *J. Liq. Chromatogr. Rel. Technol.*, 24, 1745, 2003.
91. Schwartz, S.J. and Von Elbe, J.H., Identification of betanin degradation products, *Ztschr. Lebensm. Unters. Forsch.*, 176, 448, 1983.
92. Stintzing, F.C., Schieber, A., and Carle, R., Amino acid composition and betaxanthin formation in fruits from *Opuntia ficus-indica*, *Planta Med.*, 65, 632, 1999.
93. Gandía-Herrero, F., Carcía-Carmona, F., and Escribano, J., Fluorescent pigments: new perspectives in betalain research and applications, *Food Res. Int.*, 38, 879, 2005.
94. Gandía-Herrero, F., García-Carmona, F., and Escribano, J., A novel method using high-performance liquid chromatography with fluorescence detection for the determination of betaxanthins, *J. Chromatogr. A*, 1078, 83, 2005.
95. Stuppner, H. and Egger, R., Application of capillary zone electrophoresis to the analysis of betalains from *Beta vulgaris*, *J. Chromatogr. A*, 735, 409, 1996.

96. Vogt, T. et al., Light-induced betacyanin and flavonol accumulation in bladder cells of *Mesembryanthemum crystallinum*, *Phytochemistry*, 52, 583, 1999.
98. Wybraniec, S., Nowak-Wydra, B., and Mizrahi, Y., ¹H and ¹³C NMR spectroscopic structural elucidation of new decarboxylated betacyanins, *Tetrahedron Lett.*, 47, 1725, 2006.

6.5 Analysis of Other Natural Food Colorants

Carmen Socaciu

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6.5.1 INTRODUCTION

The previous chapters of this book focused on the most important pigments found in plants, fungi, algae, and other organisms, looking to their structures, biosynthesis, and the major food sources of these pigments. The structural and functional properties of chlorophylls (Sections 2.1 and 4.1), carotenoids (Sections 2.2 and 4.2), anthocyanins (Sections 2.3 and 4.3), and betalains (Sections 2.4 and 4.4) were presented. To fully discuss their stabilities, availabilities, actions, and impacts on human health, Sections 3.1 to 3.3 included updated information about these important pigment properties. Looking to technological aspects, the isolation, purification, and formulation of food colorants from plants, microalgae, microorganisms, food matrices, and wastes were described (Sections 5.1, 5.3, and 5.4), along with advanced biotechnological methods of producing natural colorants (Section 5.3).

The previous chapters dedicated to analysis describe well-known and newly introduced techniques for identifying, separating, and quantifying active colored molecules. Such data are reported specifically for chlorophylls, carotenoids, anthocyanins, and betalains (Sections 6.1 through 6.4). Regarding the most relevant books and review articles that describe methods and protocols, we can point out two categories of publications. First are the scientific reviews discussing theoretical and practical ways to isolate, extract, and analyze food colored constituents and food colorants.⁸ The second group consists of technical handbooks and reports that focus

on the quality and safety of food colorants as a category of food additives.¹² These references offer backgrounds for the analytical chemistry applied to food colorants, updated by new data presented at relevant congresses like those dedicated to pigments in food held for the past 8 years.^{13–16}

This section aims to present a brief overview of the most significant literature covering general aspects of common techniques used currently for natural food colorant analysis, and specifically current protocols applied to analyze minor natural food colorants whose chemical and technological properties are presented in Sections 2.3 and 7.2. We consider here only pure colorants or extracts that seek or have received approval for use as food additives in the United States and European Union. Legislative aspects are detailed in Sections 7.1 and 7.2.

6.5.2 GENERAL AND SPECIFIC INSTRUMENTAL METHODS FOR ANALYZING NATURAL FOOD COLORANTS

Food colorants are analyzed either by direct inspection (sensorial analyses) or by physical or physicochemical instrumental methods. Direct inspections determine the sensorial attribute of color, frequently combined with assessments of smells and flavors. Visual color assessment is subjective and may be used with reliable visual evaluations controlling multiple variables.

Instrumental color measurements eliminate subjectivity, are more precise, take less time, and are simpler to perform. However, to evaluate instrumental results properly, the physics of the measurement processes must be considered. Three types of color measurement instruments are used for food: the monochromatic colorimeter, the tristimulus colorimeter, and the colorimetric spectrophotometer.

A monochromatic colorimeter measures the amount of light (in arbitrary units) reflected in a narrow spectral area of visible light. The device is basically colorblind and sees only one color: red, yellow, or green. A tristimulus colorimeter measures true color and correlates to what the eye sees, using specialized glass color filters and light detectors (up to 10 million different shades of color can be quantified). A colorimetric spectrophotometer measures the specific spectral absorption of a sample from the entire visible spectrum of light; the residual light is quantified. Details about these types of color evaluations appear in Chapter 1 of this book and elsewhere.²²

Physicochemical analysis includes three successive steps: sample preparation, identification, and quantitative evaluation. Sample preparation for color analysis is a critical step since it is needed to minimize modifications (heat- and light-induced, enzymatic, and oxidative degradations). Taking into account the labilities of pigments to light and oxygen, actual protocols for food colorants avoid the use of organic solvents and involve supercritical fluids (CO₂), liquid extraction under pressure, solid phase extractions, and countercurrent chromatography.^{23–29}

The identification of the target colorant is done directly by UV-Vis spectrometry (if the colorant is unique) or by a previous separation from a mixture. The most available techniques are planar chromatography (paper chromatography and thin layer chromatography)³⁰ and, especially, high performance liquid chromatography

(HPLC), an advanced, accurate, and well-developed technique for both qualitative and quantitative measurements based on reverse phase stationary phases,^{31–35} or ion pairs³⁶ applied for analytical or preparative purposes.

Capillary electrophoresis is increasingly used in food analysis due to its separation performance combined with the short time of analysis.^{37,38} Capillary electrophoresis recently applied to colorant measurements includes technical variants such as capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography.^{39,40}

High performance spectroscopic methods, like FT-IR and NIR spectrometry and Raman spectroscopy are widely applied to identify non-destructively the specific fingerprint of an extract or check the stability of pure molecules or mixtures by the recognition of different functional groups. Generally, the infrared techniques are more frequently applied in food colorant analysis, as recently reviewed.^{41–43} Mass spectrometry is used as well, either coupled to HPLC for the detection of separated molecules or for the identification of a fingerprint based on fragmentation patterns.⁴⁴

The current trend in analytical chemistry applied to evaluate food quality and safety leans toward user-friendly miniaturized instruments and laboratory-on-a-chip applications. The techniques applied to direct screening of colorants in a food matrix include chemical microscopy, a spatial representation of chemical information from complex aggregates inside tissue matrices,⁴⁵ biosensor-based screening,⁴⁶ and molecularly imprinted polymer-based methods⁴⁷ that serve as chemical alternatives to the use of immunosensors.

Detailed information about carotenoids found in food or extracted from food and evaluated for their potential as food colorants appeared in Sections 4.2 and 6.2. We would like to mention some new data about the utilization of pure carotenoid molecules or extracts as allowed food additives. Looking to the list of E-coded natural colorants (Table 7.2.1), we can identify standardized colorants E160a through f, E161a, and E161b as natural or semi-synthetic derivatives of carotenoids provided from carrots, annatto, tomatoes, paprika, and marigold. In addition, the extracts (powders or oleoresins) of saffron,^{56–65} paprika,^{71–73} and marigold^{74–80} are considered more economical variants in the United States and European Union.

Saffron is appreciated as much for its aroma and flavor as for its coloring properties and moreover it is the world's most expensive colorant and spice. The major yellow pigment present in both saffron stigmas and cape jasmine fruits is crocin, the gentiobiose form of the crocetin carotenoid. It is usually sold as "crocin extract," a trade term for the yellow, water-soluble food colorant obtained from cape jasmine (*Gardenia jasminoides* L.) and from saffron (*Crocus sativus* L.). In addition to the crocins, cape jasmine fruits contain iridoid and flavonoid pigments. The aroma of saffron arises from a volatile aldehyde, safranal, which is produced during processing from picrocrocin, a compound responsible for the bitter taste of saffron. In the European Community, the extract of cape jasmine fruit is described as a natural color but it has no E number. Reviews for the application of saffron extracts were recently published.^{62,66}

The quality of saffron, according to the International Organization for Standardization (ISO), is ranked through coloring strength numbers: the higher its coloring strength, the higher its value. The coloring strength is determined by crocin and is expressed as E1%/1 cm at 440 nm. Category I saffron needs a minimum of 190,

category II needs a minimum of 150, and category III a minimum of 110, determined by spectrometry (www.saffron.com). Saffron's coloring strength determines also its flavor and aroma. The aroma is determined by safranal (expressed as E1%/1 cm at 330 nm) and the bitterness is determined by picocrocin (expressed as E1%/1 cm at 257 nm). Carthamin, the active component of dyer's saffron flowers (*Carthamus tinctorius*) and geniposide, was also purified as a food and textile colorant.⁶⁷⁻⁷⁰

6.5.3 ANALYSIS OF QUINONES FROM COCHINEAL INSECTS: CARMINE AND CARMINIC ACID

Carmine extracted from cochineal insects is one of the most used natural colorings for beverages and other foods. Some representative articles refer to isolation and spectrometric analysis⁸¹⁻⁸⁵ or the use of HPLC⁸⁶⁻⁸⁸ or capillary electrophoresis (CE) to separate and characterize all cochineal pigments. Its active ingredient, carminic acid, was quantified by rapid HPLC-DAD or fluorescence spectrometry. Carminic acid, used as an additive in milk beverages, was separated within 9 min using a high-efficiency CE separation at pH 10.0 after a previous polyamide column solid phase extraction (SPE).⁸⁹

Lac and cochineal pigments are used as food color additives in many countries. These natural pigments were analyzed by CE. This technique has several advantages over thin layer chromatography, gas chromatography, and HPLC, for example, low capillary costs, reduced operating costs, small sample amounts, small production of waste materials, and short analysis time. CE is a useful technique for the analysis of natural food pigments and pigments extracted from commercial food samples by solid phase extraction.³⁸

Samples with higher protein levels (yogurts), are initially treated with hydrochloric acid and after protein precipitation the supernatant is filtered and injected into the HPLC column. The separations performed with a LiChroCART RP18 column used a mixture of acetonitrile and formic acid as the mobile phase. A baseline quantification of the carminic acid was possible in the presence of other coloring agents, with excellent recuperation, selectivity, accuracy, and precision.⁹⁰

6.5.4 TURMERIC COLORANTS: CURCUMINOIDES

The three main compounds responsible for the bright yellow-orange color of turmeric (*Curcuma longa*) belong to the diaryl heptanoid (curcuminoid) family, including curcumin, demethoxycurcumin, and bis-demethoxycurcumin as active molecules. Some authors refer to these compounds as curcumin I, II, and III, respectively. The extraction of turmeric oleoresin was optimized using ethanol, acetone, and ethylene dichloride⁹¹ and analyzed by HPLC using different protocols.⁹²⁻⁹⁴

Four different commercially available varieties of turmeric containing curcumin were isolated, separated by column chromatography, and identified by spectroscopy. The purity of the curcuminoids was analyzed by an improved HPLC method, performed on a C₁₈ column using methanol, acetic acid, and acetonitrile and detection at 425 nm. The total percentages of curcuminoids in turmeric were between 2.34

and 9.18%, the major fraction represented by curcumin (1 to 5.6%).⁹⁵ FT-Raman and Raman spectroscopy were also used for the investigation of curcumin, which is a valuable dyeing component of curcuma root. Although curcumin is present in the root at only 3 to 5%, it dominates in the Raman spectrum.⁹⁶

A rapid and simple high performance thin layer chromatographic method has been developed for the simultaneous quantitation of pharmacologically important curcumin, demethoxy curcumin, and bis-demethoxy curcumin in *Curcuma longa* and *C. amada*. The assay combines the isolation and separation of curcuminoids on silica gel 60F²⁵⁴ high performance thin layer chromatographic plates, followed by scanning of the spots at 366 nm using a UV detection mode.⁹⁷ Recently, a sensitive fluorimetric method using fluorescence enhancement by a mixed micelle formulation was applied for curcumin determination.⁹⁸

6.5.5 COLORED POLYPHENOLS: FLAVONOIDS AND PROANTHOCYANINS

Thousands of polyphenols from fruits (grapes, apples, etc.), vegetables (horse beans), and teas have been identified, many having good coloring properties, especially anthocyanins and some flavonoids. Well-documented reviews discuss the coloring capacities of some polyphenols including procyanidins.^{99,100} Detailed presentations of anthocyanin and flavonoid properties and analysis are included in Sections 2.3, 4.3, and 6.3. The soluble proanthocyanidins of the colored horse bean *Vicia faba* L. seed coats were isolated and separated by solvent partition.

The chemical characteristics of the proanthocyanidins were elucidated by total oxidation and partial degradation in the presence of phloroglucinol followed by HPLC analysis. The native extract of proanthocyanidins contained (+) gallo catechin, (–) epigallocatechin, (+) catechin, and (–) epicatechin units.¹⁰¹

Oligomeric procyanidins and major condensed tannins from the same horse beans were purified by chromatography on Sephadex LH-20 and then the active compounds were isolated by RP-HPLC using a Licrospher Li 100 Column. The structures of the purified oligomeric procyanidins were elucidated by spectroscopy, ESI-MS, and HPLC analysis. Six compounds were identified as two A-type procyanidin dimers, the procyanidin dimers B1, B2 and B3, and a procyanidin trimer.¹⁰¹ Extracts of apple fruits (*Malus domestica* cv.) were analyzed by HPLC and photodiode array detection, and the identified peaks corresponded to isorhamnetin 3-O-glucoside based on Vis spectrometry. Using atmospheric pressure chemical ionization mass spectrometry in the negative ion mode, the presence of an isorhamnetin glycoside was also supported.¹⁰²

Similarly, black tea polyphenols using counter-current chromatography were separated and characterized.¹⁰³ Grape seed and grape skin extracts are rich sources of procyanins and tannins, beside flavonoids and some anthocyanins. Their brown-reddish color is not usually used for food coloring but is used in food supplements due to its antioxidant capacity.¹⁰⁴ Various methods were used to quantify the oligomeric proanthocyanidins from grape seeds.¹⁰⁵ Their detection and quantification using various chromatographic and electrophoretic techniques with UV-Vis, diode array, and mass spectrometry were reported.⁵¹

6.5.6 CARAMEL COLORANTS

Caramel color is produced by the controlled heating of carbohydrates. It includes a heterogeneous complex mixture of low molecular weight compounds up to colloidal aggregates (details in Section 2.5). Significant progress has recently been made in the detection and chemical characterization of caramel colors^{106–109} in finished products. These tests typically involved color formation as a result of a reaction between a component of caramel color and a reagent. The generally qualitative and non-specific screening tests focused on types of caramel colors.^{110–112} More recently, advanced methods such as size exclusion chromatography,¹¹³ gel permeation,¹¹⁴ and capillary electrophoresis^{115,116} have been used for the identification and quantification of caramel color in beverages, soft drinks, and juices.

REFERENCES

1. Macrae, R., Robinson, R.K., and Sadler, M.J., Eds., *Encyclopaedia of Food Science, Food Technology and Nutrition*, Vol. II, Academic Press, San Diego, 1993.
2. Bente, D. and Charalampous, G., Eds., *Instrumental Methods in Food and Beverage Analysis*, Elsevier, Amsterdam, 1998.
3. Pare, J.R.J. and Belanger, J.M.R., *Instrumental Methods in Food Analysis*, Elsevier, Amsterdam, 1998.
4. Tunick, M.H., Palumbo, S.A., and Fratamico, P.M., *New Techniques in the Analysis of Foods*, Academic/ Plenum, New York, 1998.
5. Multon, J., Ed., *Analysis of Food Constituents*, John Wiley & Sons, New York, 1996.
6. CIE, *Technical Report: Improvement in Industrial Colour-Difference Evaluation*, Publication 142-2001, Commission Internationale de l'Eclairage, Vienna, 2001.
7. Watson, D.H., *Food Chemical Safety*, vols. 1 and 2, CRC Press, Boca Raton, FL, 2002.
8. King, S., Gates, M., and Scalettar, L., Eds., *Current Protocols in Food Analytical Chemistry*, John Wiley & Sons, New York, 2001.
9. Mabon, T.J., Color measurement of food, *Cereal Foods World*, 38, 21, 1993.
10. Pomeranz, Y. and Meloan, C.E., *Food Analysis: Theory and Practice*, 3rd ed., Chapman & Hall, New York, 1994.
11. Nollet, L.M.L., *Handbook of Food Analysis*, Vols. 1 and 2, Marcel Dekker, New York, 1996.
12. Otles, S., Ed., *Methods of Analysis of Food Components and Additives*, CRC Press, Boca Raton, FL, 2005.
13. Mínguez Mosquera, M.I., Galán, M.J., and Hornero Méndez, D., Eds., *Proc. 1st Int. Congr. on Pigments in Food Technology*, Instituto della Grassa, Sevilla, 1999.
14. Empis, J.A., Ed., *Proc. Functionalities of Pigments in Food*, Society of Portuguese Chemists, Lisbon, 2002.
15. Dufosse, L., Ed., *Proc. 3rd Int. Congr. on Pigments in Food*, Quimper, France, Pigments Publ. Impr. Le Berre, Quimper, 2004.
16. Carle, R., Schieber, A., and Stintzing, F.C., Eds., *Proc. 4th Int. Congr. on Pigments in Food, A Challenge for Life Sciences*, Hohenheim, Germany, Shaker Verlag GmbH, Germany, 2006.
17. Francis, F.J. and Clydesdale, F.M., *Food Colorimetry: Theory and Applications*, AVI, Westport, CT., 1975.

18. Hunt, R.W.G., *Measuring Colour*, 3rd ed., Ellis Horwood, Chichester, 2001.
19. Fratianni, A. et al., Estimation of color of durum wheat: comparison of WSB, HPLC and reflectance colorimeter measurements, *J. Agric. Food Chem.*, 53, 2373, 2005.
20. Papadakis, S. E. et al., A versatile and inexpensive technique for measuring color of foods, *Food Technol.*, 54, 48, 2000.
21. Mossoba, M.M., Ed., *Spectral Methods in Food Analysis*, Marcel Dekker, New York, 1999.
22. Wilson, R., Ed., *Spectroscopic Methods for Food Analysis*, John Wiley & Sons, New York, 1994.
23. Westwood, S.A., *Supercritical Fluid Extraction and Its Use in Chromatographic Sample Preparation*, Blackie, London, 1993.
24. Pimbley, D. and Patel, P.D., Agrifood applications of solid-phase extraction: a review, *Leatherhead Food RA Sci. Tech. Notes*, 197, 2000.
25. Henry, M.C. and Yonker, C.R., Supercritical fluid chromatography, pressurized liquid extraction, and supercritical fluid extraction, *Anal. Chem.*, 78, 3909, 2006.
26. Wenclawiak, B., Ed., *Analysis with Supercritical Fluids: Extraction and Chromatography*, Springer Verlag, Berlin, 1992.
27. Smith, R.M., Supercritical fluids in separation science: the dreams, the reality and the future, *J. Chromatogr. A*, 856, 83, 1999.
28. McHugh, M.A. and Krukonis, V.J., *Supercritical Fluid Extraction Principles and Practice*, 2nd ed., Butterworth-Heinemann, Boston, 1994.
29. Degenhardt, A., Knapp, H., and Winterhalter, P., Separation of natural food colorants by high-speed countercurrent chromatography, in *Chemistry and Physiology of Selected Food Colorants*, Ames, J.M. and Hofmann, T., Eds., ACS Symposium Series 775, American Chemical Society, Washington, 2001, 22.
30. Sherma, J., Planar chromatography, *Anal. Chem.*, 78, 3841, 2006.
31. Nollet, L.M.L., *Food Analysis by HPLC*, 2nd ed., Marcel Dekker, New York, 2000.
32. Gennaro, M.C., Abrigo, C., and Cipolla, G., HPLC analysis of food colors and its relevance in forensic chemistry, *J. Chromatogr. A*, 674, 281, 1994.
33. Gratzfeld-Huesgen, A. and Schuster, R., *HPLC for Food Analysis: A Primer*, Hewlett-Packard Company, Palo Alto, CA, 1996.
34. Oliver, J. and Palou, A., Chromatographic determination of carotenoids in foods, *J. Chromatogr. A*, 881, 543, 2000.
35. Sander, L.C., Sharpless, K.E., and Pursch, M.C., Stationary phases for the analysis of food by liquid chromatography, *J. Chromatogr. A*, 880, 189, 2000.
36. Henshall, A., Use of ion chromatography in food and beverage analysis, *Cereal Foods World*, 42, 414, 1997.
37. Sadecka, J. and Polonsky, J., Electrophoretic methods in the analysis of beverages, *J. Chromatogr. A*, 880, 243, 2000.
38. Watanabe, T. and Terabe, S., Analysis of natural food pigments by capillary electrophoresis, *J. Chromatogr. A*, 880, 311, 2000.
39. Watanabe, T. et al., Analysis of elderberry pigments in commercial food samples by micellar electrokinetic chromatography, *Anal. Sci.*, 14, 839, 1998.
40. Watanabe, T. et al., Separation and determination of monascus yellow pigments for food by micellar electrokinetic chromatography, *Anal. Sci.*, 13, 571, 1997.
41. Wetzel, D.L.B., Analytical near infrared spectroscopy, in *Instrumental Methods In Food and Beverage Analysis*, Wetzel, D.L.B. and Charalambous, G., Eds., Elsevier, Amsterdam, 1998.
42. Osborne, B.G., Near-infrared spectroscopy in food analysis, *Encyclopedia of Analytical Chemistry*, Meyers, R.A., Ed., John Wiley & Sons, Chichester, 2000.

43. Schulz, H., Baranska, M. and Baranski, R., Potential of NIR-FT-Raman spectroscopy in natural carotenoid analysis, *Biopolymers*, 77, 212, 2005.
44. Smedsgaard, J. and Frisvad, J.C., Using direct electrospray mass spectrometry profiling of crude extracts, *J. Microbiol. Methods*, 25, 5, 1996.
45. Navratil, M., Mabbott, G.A., and Arriaga, E.A., Chemical microscopy applied to biological systems, *Anal. Chem.*, 78, 4005, 2006.
46. Scott, A.O., *Biosensors for Food Analysis*, Royal Society of Chemistry, Cambridge, 1998.
47. Yano, K. and Karube, I., Molecularly imprinted polymers for biosensor applications, *Trends Anal. Chem.*, 18, 199, 1999.
48. Hedry, G.A.F. and Houghton, J.D., Eds., *Natural Food Colorants*, Blackie, Glasgow, 1992.
49. Hedry, G.A.F. and Houghton, J.D., Eds., *Natural Food Colorants*, 2nd Ed., Blackie, Glasgow, 1996.
50. Francis, F.J., Miscellaneous colorants, in *Colorants*, Eagan Press, St. Paul, MN, 89, 1999.
51. Tsao, R. and Deng, Z.Y. Separation procedures for naturally occurring antioxidant phytochemicals, *J. Chromatogr. B*, 812, 85, 2004.
52. Gonzales, M., Gallego, M., and Valcarcel, M., Liquid chromatographic determination of natural and synthetic colorants in lyophilized foods using an automatic solid-phase extraction system, *J. Agric. Food Chem.*, 51, 2121, 2003.
53. González, M., Gallego, M., and Valcárcel, M., Automatic screening method for the rapid and simple discrimination between synthetic and natural colorants in foods, *Anal. Chim. Acta*, 464, 237, 2002.
54. Müller, H., Determination of the carotenoid content in selected vegetables and fruit by HPLC and photodiode array detection, *Z. Lebensm. Unters. Forsch. A*, 204, 88, 1997.
55. Mercadante, A.Z. and Egeland, E.S., *Handbook of Carotenoids*, Britton, G. et al., Eds., Birkhauser, Basel, 2004.
56. Sampathu, S.R. et al., Saffron cultivation, processing, chemistry and standardization, *CRC Crit. Rev. Food Sci. Nutr.*, 20, 123, 1984.
57. Winterholter, P. and Straubinger, M., Saffron: renewed interest in an ancient spice, *Food Rev. Int.*, 16, 39, 2000.
58. International Standardization Organisation, Saffron (*Crocus sativus* L.), ISO 3632-1, Part 1, Specification, Geneva, 1993.
59. Pfister, S. et al., Isolation and structure elucidation of carotenoid-glycosyl esters in gardenia fruits (*Gardenia jasminoides* Ellis) and saffron (*Crocus sativus* Linne), *J. Agric. Food Chem.*, 44, 2612, 1996.
60. Iborra, J.L. et al., TLC preparative purification of picrocrocin, HTCC and crocin from saffron, *J. Food Sci.*, 57, 714, 1992.
61. Van Calsteren, J. et al., Spectroscopy characterization of crocetin derivatives from *Crocus sativus* and *Gardenia jasminoides*, *J. Agric. Food Chem.*, 45, 1055, 1997.
62. Carmona, M. et al., Crocetin esters, picrocrocin and its related compounds present in *Crocus sativus* stigmas and *Gardenia jasminoides* fruits: tentative identification of seven new compounds by LC-ESI-MS, *J. Agric. Food Chem.*, 54, 973, 2006.
63. Kamikura, M. and Nakazato, K., Natural yellow colours from gardenia fruit and colours found in commercial gardenia extract: analysis of natural yellow colours by high performance liquid chromatography, *J. Food Hygiene Soc. Japan*, 26, 150, 1984.
64. Solinas, M. and Cichelli, A., HPLC analysis of the colour and aroma constituents of saffron, *Ind. Alimentari*, 27, 634, 1988.

65. Watanabe, T. et al., Separation and determination of yellow and red Safflower pigments in food by capillary electrophoresis, *Biosci. Biotech. Biochem.*, 61, 1179, 1997.
66. Fekrat, H., The application of crocin and saffron ethanol-extractable components in formulation of health care and beauty care products, *Proceedings of International Symposium on Saffron Biology and Biotechnology*, Fernández, J.A. and Abdullaev, F., Eds., Albacete, Spain, 2004, p. 650.
67. Noda, N. et al., Determination of natural yellow dye from the fruits of gardenia by detecting geniposide, *J. Hygenic Chem. (Eisei Kagaku)*, 29, 7, 1983.
68. Saito, K., A new method for reddening dyer's saffron (*Carthamus tinctorius*) florets: evaluation of carthamin productivity, *Z. Lebensmitt. Untersuch. Forsch.*, 192, 343, 1991.
69. Saito, K. and Miyakawa, K., A new procedure for the production of carthamin dye from dyer's saffron flowers, *Lebensm. Wiss. Technol.*, 27, 384, 1994.
70. Saito, K., A new enzymatic method for extraction of precarthamin from dyer's saffron (*Carthamus tinctorius*) florets, *Z. Lebensmitt. Untersuch. Forsch.*, 197, 34, 1993.
71. Cserhati, T. et al., Separation and quantitation of colour pigments of chili powder (*Capsicum frutescens*) by high-performance liquid chromatography-diode array detection, *J. Chromatogr. A*, 896, 69, 2000.
72. Minguez-Mosquera, M.I. and Hornero-Mendez, D., Separation and quantification of the carotenoid pigments in red peppers, paprika and oleoresin by reversed phase HPLC, *J. Agric. Food Chem.*, 41, 1616, 1993.
73. Breithaupt, D.E. and Schwack, W., Determination of free and bound carotenoids in paprika (*Capsicum annuum* L.) by LC/MS, *Eur. Food Res. Technol.*, 211, 52, 2000.
74. Philip, T. and Berry, J.W., A process for the purification of lutein-fatty acid esters from marigold petals, *J. Food Sci.*, 41, 163, 1976.
75. Tyczkowski, J.K. and Hamilton, P.B., Preparation of purified lutein and its diesters from extracts of marigold (*Tagetes erecta*), *Poultry Sci.*, 70, 651, 1991.
76. Gregory, G.K. et al., Quantitative analysis of lutein esters in marigold flowers (*Tagetes erecta*) by high performance liquid chromatography, *J. Food Sci.*, 51, 1093, 1986.
77. Livingston, A.L., Rapid analysis of xanthophyll and carotene in dried plant materials, *J. AOAC*, 69, 1017, 1986.
78. Gau, W. et al., Mass spectrometric identification of xanthophyll fatty acid esters from marigold flowers (*Tagetes erecta*) obtained by high performance liquid chromatography and Craig countercurrent distribution, *J. Chromatogr.*, 262, 277, 1983.
79. Breithaupt, D.E., Wirt, U., and Bamedi, A., Differentiation between lutein monoester regioisomers and detection of lutein diesters from marigold flowers (*Tagetes erecta* L.) and several fruits by liquid chromatography-mass spectrometry, *J. Agric. Food Chem.*, 50, 66, 2002.
80. Fletcher, D.L. and Halloran, H.R., An evaluation of commercially available marigold concentrate and paprika oleoresin on egg yolk pigmentation, *Poultry Sci.*, 60, 1846, 1981.
81. Francis, F. J., Turmeric, carthamin and monascus, in *Colorants*, Eagan Press, St. Paul, MN, 77, 1999.
82. Lloyd, A.G., Extraction and chemistry of cochineal, *Food Chem.*, 5, 91, 1980.
83. Yamada, S. et al., Analysis of natural colouring matters in food (IV). Methylation of cochineal colour with diazomethane for analysis of food products, *J. Agric. Food Chem.*, 41, 1071, 1993.
84. Nishizawa, M. et al., Analysis of natural dyes (III). Analysis of cochineal dye and lac dye in foods and dyes, *Hokk. Eisei Kenyush.*, 35, 7, 1985.

85. Schwing-Weill, M.J. and Wechsler, S., Spectrophotometric study of carminic acid in solution: application to its determination, *Analysis*, 14, 290, 1986.
86. Wouters, J., High performance liquid chromatography of anthraquinones: analysis of plant and insect extracts and dyed textiles, *Stud. Conserv.*, 30, 119, 1985.
87. Wouters, J. and Verhecken, A., The scale insect dyes: species recognition by HPLC and diode-array analysis of the dyestuffs, *Annales Soc. Entom. France*, 25, 393, 1989.
88. Lancaster, F.E. and Lawrence, J.F., High-performance liquid chromatographic separation of carminic acid, and P-bixin, and a- and 13-norbixin, and the determination of carminic acid in foods, *J. Chromatogr. A*, 732, 394, 1996.
89. Carvalho, P.R.N. and Collins, C.H., HPLC determination of carminic acid in foodstuffs and beverages using diode array and fluorescence detection, *Chromatographia*, 45, 63, 1997.
90. Huang, H.Y. et al., Analysis of food colorants by capillary electrophoresis with large-volume sample stacking, *J. Chromatogr. A*, 995, 29, 2003.
91. George, K.M., On the extraction of oleoresin from turmeric: comparative performance of ethanol, acetone and ethylene dichloride, *Indian Spices*, 18, 7, 1981.
92. Tønnesen, H.H. and Karlsen, J., Studies on curcumin and curcuminoids VII. Chromatographic separation and quantitative analysis of curcumin and related compounds, *Z. für Lebensm. und Forsch. A*, 182, 215, 1986.
93. Taylor, S.J. and McDowell, I.J., Determination of the curcuminoid pigments in turmeric (*Curcuma domestica* Val.) by reversed-phase high performance liquid chromatography, *Chromatographia*, 34, 73, 1992.
94. Gupta, A.P., Gupta, M.M., Kumar, S., Simultaneous determination of curcuminoids in curcuma samples using high performance thin layer chromatography, *J. Liq. Chromatogr. Rel. Technol.*, 22, 1561, 1999.
95. Jayaprakasha, G.K. et al., Improved HPLC method for the determination of curcumin, demethoxycurcumin and bisdemethoxycurcumin, *J. Agric. Food Chem.*, 50, 3668, 2002.
96. Baranska, M. et al., Identification of secondary metabolites in medicinal and spice plants by NIR FT Raman microspectroscopic mapping, *Analyst*, 129, 926, 2004.
97. Pathania, V., Improved HPTLC method for determination of curcuminoids from *Curcuma longa*, *J. Liq. Chromatogr. Rel. Technol.*, 29, 877, 2006.
98. Wang, F., The sensitive fluorimetric method for the determination of curcumin using the enhancement of mixed micelle, *J. Fluoresc.*, 16, 53, 2006.
99. Francis, F.J., Miscellaneous colorants, in *Colorants*, Eagan Press, St. Paul, MN, 89, 1999.
100. Francis, F.J., Polyphenols as natural food colorants, in *Polyphenolic Phenomena*, Scalbert A., Ed., INRA, Paris, 1993, 209.
101. Merghem, R., Qualitative analysis and HPLC isolation and identification of procyanidins from *Vicia faba*, *Phytochem. Anal.*, 15, 95, 2004.
102. Schieber, A., Detection of isorhamnetin glycosides in extracts of apples (*Malus domestica* cv. "Brettacher") by HPLC-PDA and HPLC-APCIMS/MS, *Phytochem. Anal.*, 13, 87, 2002.
103. Degenhardt, A. et al., Isolation of black tea pigments using high-speed countercurrent chromatography and studies on properties of black tea polymers, *J. Agric. Food Chem.*, 48, 5200, 2000.
104. Nakamura, Y., Tsuji, S., and Tonogai, Y., Analysis of proanthocyanidins in grape seed extracts, health foods and grape seed oils, *J. Health Sci.*, 49, 45, 2003.
105. Waterhouse et al., A comparison of methods for quantifying oligomeric proanthocyanidins from grape, *Am. J. Enol. Vitic.*, 51, 383, 2000.

106. Ciolino, L.A., Determination and classification of added caramel color in adulterated acerola juice formulations, *J. Agric. Food Chem.*, 46, 1746, 1998.
107. Coffey, J.S. and Castle, L., Analysis for caramel colour (Class III), *Food Chem.*, 51, 413, 1994.
108. Coffey, J.S. et al., Analysis of caramel classes, *Food Chem.*, 58, 259, 1997.
109. Francis, F.J., Caramel, brown polyphenols and iridoids, in *Colorants*, Eagan Press, St. Paul, MN, 83, 1999.
110. Licht, B. et al., Characterization of caramel colour IV, *Food Chem. Toxicol.*, 30, 365, 1992.
111. Licht, B. et al., Characterization of Caramel Colours I, II, and III, *Food Chem. Toxicol.*, 30, 375, 1992.
112. Myers, D.V. and Howell, J.C., Characterization and specifications of caramel colours: an overview, *Food Chem. Toxicol.*, 30, 359, 1992.
113. Frischenschlager, S., Hellwig, E., and Peteuly, F., Detection and identification of caramel colors in some liquid foodstuffs, *Dtsch. Lebensm. Rundsch.*, 78, 385, 1982.
114. Hellwig, E. et al., Detection and identification of caramel by gel-permeation chromatography, *Dtsch. Lebensm. Rundsch.*, 77, 165, 1981.
115. Royle, L. et al., A new method for the identification and quantification of class IV caramels using capillary electrophoresis and its application to soft drinks, *J. Sci. Food Agric.*, 76, 579, 1998.
116. Royle, L. and Radcliffe, C.M., Analysis of caramels by capillary electrophoresis and ultrafiltration, *J. Sci. Food Agric.*, 79, 1709, 1999.

6.6 Analysis of Synthetic Food Colorants

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6.6.1 INTRODUCTION

In the past 20 years, consumers have increasingly considered synthetic colorants undesirable or harmful but they are still used extensively in many food products.¹ Official organizations in the United States and European Union have restricted the use of some synthetic colorants as additives in foods (see Table 7.3.1 in Section 7.3). The list of allowed colorants has been reduced to 21. Section 7.3 also discusses details about their structures.

The official permission to use a synthetic colorant in food is determined by its quality and safety. Detailed and accurate analysis became compulsory in order to verify purity and quantify the labeled concentrations of colorants in food. For the analysis of synthetic colorants added to food products, (1) simple and rapid methods are used to determine their presence, (2) accurate and precise methods evaluate their concentrations, or (3) certain methods evaluate their degradations to unstable and unsafe forms. This chapter is dedicated to these three methods used to identify and quantify synthetic colorants as pure or mixed pigments in foodstuffs.

The simple and rapid methods include UV-Vis spectrometry, which reveals the presence of a specific colorant according to its maxima in the absorption spectra in an extract by comparison with a reference (pure colorant extract). The identification

of individual colorants in a mixture (blended colorants contained in a food product) is performed by qualitative TLC, HPTLC, or HPLC fingerprinting.

The most economical and convenient quantitative method is direct or derived UV-Vis spectrometry, with preliminary calibration curves for each colorant to be quantified. The quantitative HPLC methodology includes separation of different colorants found in a food extract and their quantification based on previous calibration curves made by HPLC with each individual colorant, comparing retention times and peak areas. This method also offers the possibility of identifying degraded forms of genuine colorants and determining their stability during storage or processing.

In addition to chromatography based on adsorption, ion pair chromatography (IP-HPLC) and capillary electrophoresis (CE) or capillary zone electrophoresis (CZE) are new methods that became popular and are sufficiently accurate for these types of investigations. Other methods involving electrochemical responses include differential pulse polarography, adsorptive and derived voltammetry, and more recently, electrochemical sensors.

Many methods of determining individual colorants and blended compositions have been selected, critically reviewed, and published.^{2,3} Combined methods such as paper chromatography, TLC, HPLC, spectrophotometry, voltammetry, and more recently, CZE have been developed. Table 6.6.1 presents some of these methods.

6.6.2 EXTRACTION AND PURIFICATION PROTOCOLS

A sequential analysis protocol includes three steps: (1) extraction in water or other appropriate solvent for the colorant, (2) purification or concentration of the colorant, and (3) separation coupled with detection of the target molecule. Different methods of extracting synthetic colorants from foods have been developed using organic solvents followed by SPE protocols using as adsorption support RP-C18,⁴ amino materials,⁵ or Amberlite XAD-2.⁶ For qualitative evaluations, the easiest option for separating colorant molecules from unwanted ingredients found in an extract is SPE on polyamide or wool.

As an example, five different synthetic colorants (Tartrazine, Sunset Yellow, Ponceau 4R, Amaranth, and Brilliant Blue FCF) from drinks and candies were separated on a polyamide adsorbent at pH 4, eluted with an alkaline-ammonia solution.⁷ By another method, 13 synthetic food colorants were isolated from various foods using specific adsorption on wool. After elution with 10% ammonia solution and gentle warming, an absorption spectrum of the resulting colorant solution was recorded, compared to the reference spectra of pure colorants, and identified by linear regression analysis.⁸

6.6.3 QUALITATIVE EVALUATION AND SCREENING BY SPECTROMETRY AND CHROMATOGRAPHY

Government bodies, inspection services, and laboratories are increasingly interested in knowing and selecting analytical systems that provide rapid and reliable yes-or-no responses rather than detailed chemical information. Screening systems are inter-

TABLE 6.6.1
Relevant Methods for Extracting, Separating, and Identifying Synthetic Colorants

Colorant	Measurement Type	Method	Details of Analysis	Detection	Application and References
Sunset Yellow	Spectrophotometry	Direct	Ion pair formation with octadecyltrimethylammonium bromide at pH 5.6 and extraction of ion pair into n-butanol	485 nm	Soft drinks ⁶⁵
		Simultaneous spectrophotometry	Addition of acetate buffer at pH 4.5 and water isolated from food matrices by SPE using polyamide sorbent packed into 1 mm cells for spectrophotometric determination	300 to 700 nm and second derivatives analyzed by partial least squares	Beverages, candies ⁷
		Derivative spectrophotometric ratio spectrum-zero crossing	No separation step required	Multivariate calibration	Samples containing 3 dyes ^{12,31}
		Solid phase spectrophotometry		Direct detection of dyes in mixtures	
Chromatography	Reverse phase HPLC		Colorants dissolved in water or acetate buffer at pH 5, separation on Sephadex DEAE A-25 gel at pH 2.0	400 to 800 nm, calibration by partial least squares	Soft drinks ^{18,22}
			Diluted in neutralized water, separation on C18 Novapak or Bondapak using gradient elution, methanol and phosphate or ammonium buffers at pH 7	520 nm (Sunset Yellow)	Beverages ³¹

Continued.

TABLE 6.6.1 (Continued)
Relevant Methods for Extracting, Separating, and Identifying Synthetic Colorants

Colorant	Measurement Type	Method	Details of Analysis	Detection	Application and References
	Electrophoresis	Ion pair HPLC	Formation of ion pairs with cetylpyridinium chloride (CeCl) in water and extraction in butanol, separation on C8 Spherisorb using gradient of acetonitrile, methanol, CeCl/phosphate buffer	Diode array, 430 nm	Drinks, milks, cakes ⁵⁴
		Ion HPLC	Sample dissolved in water, separation on Dionex Ion Pac AS11 using gradient elution with HCl:water:acetonitrile	480 nm	Drinks, instant powders ^{15,16}
		Capillary zone electrophoresis	Buffered water solutions separated on quartz columns at voltage 28 kV in electrolyte 10 mM $\text{KH}_2\text{PO}_4/\text{Na}_2\text{B}_4\text{O}_7/3\%$ ethanol at pH 11	216 to 254 nm	Beverages ⁵¹
		High performance CZE	Direct injection of liquids, pH 9.5, borax-NaOH buffer containing 5 mM β -cyclodextrin	Diode array	Drinks, ice cream ⁴⁹
		Micellar electrokinetic capillary chromatography	Extraction of sample in water/methanol with buffer addition, tetrabutylammonium phosphate, purification on C18 Sep-Pak cartridge and elution with methanol, separation on fused-silica capillary column at 30 kV with elution of sodium deoxycholate buffer at pH 8.6/acetonitrile (17:3)	214 nm	Confectionery ⁵³
Voltammetry		Ratio derivative voltammetry	Direct measurement using hanging mercury dropping electrode (HDME)		Soft drinks ²³

Azorubine	Spectrophotometry	Square wave adsorptive voltammetry	Direct measurement of adsorptive stripping voltammetric peaks using HMDE 0.60 V and accumulation potential of -0.40V		Soft drinks ⁵⁹
		Direct	Dilution in phosphate buffer and water, analyzed in Vis region	427 nm	Beverages ⁹
		Direct	Ion pair formation with octadecyltrimethylammonium bromide at pH 5.6, extraction of ion pair into n-butanol	550 nm	Confectionery ⁶⁵
	Chromatography	Solid phase spectrometry	Sample solution mixed with 1 M HCl, ethanol and purification on Sephadex DEAE 25 gel, gel beads are filtered off, packed into 1 mm cell and absorbance measured	525 and 800 nm	Confectionery ¹⁸
		Reverse phase HPLC	Diluted in neutralized water, separation on C18 Novapak using gradient elution using methanol and phosphate buffers at pH 7	520 nm	Beverages ^{1,2,31}
		Ion pair HPLC	Formation of ion pairs with cetylpyridinium chloride (CeCl) in water and extraction in butanol, separation on C8 Spherisorb using gradient of acetonitrile, methanol, CeCl/phosphate buffer	Diode array 520 nm	Drinks, skim milk ^{34,35}
34 Synthetic colorants	Chromatography	RP-HPLC	Samples dissolved in methanol, diluted in water and injected in Spherisorb ODS-2 column, elution with water/acetonitrile (7:3) containing 5 mM octylamine/-phosphoric acid at pH 6.4	520 nm	Confectionery ³³
		RP-HPLC	Separation on STR ODS-II columns and gradient elution with 20 mM ammonium phosphate buffer (pH 6.8)/isopropanol (25:1 v:v) and acetonitrile) at 40°C	Diode array	Pure dyes ⁶⁰

Continued.

TABLE 6.6.1 (Continued)
Relevant Methods for Extracting, Separating, and Identifying Synthetic Colorants

Colorant	Measurement Type	Method	Details of Analysis	Detection	Application and References
Sunset Yellow and Quinoline Yellow Synthetic colorants	Chromatography	RP-HPLC	Separation on Sephadex DEAE A-25 gel (pH 5.0) and then on C18 silica gel (pH 5.0), absorbances of both systems measured directly in solid phase		Soft drinks, fruit liqueurs, ice cream ¹⁷
	Chromatography	RP-HPLC	Use of 10 μ m LiChrosorb RP18 column and binary eluent of methanol and aqueous 0.1 M phosphate buffer (pH 4.0) according to suitable gradient elution program in less than 20-min run time with satisfactory precision; sensitivity of spectrophotometric detection optimized, achieving for all additives considered detection limits ranging from 0.1 to 3.0 mg/l, below maximum permitted levels		Soft drinks ³⁰
14 Synthetic dyes	Electrophoresis	MEKC	Simultaneous separation (20 min) of 14 synthetic colors using uncoated fused silica capillary column operated at 25 kV and elution with 18% acetonitrile and 82% 0.05 M sodium deoxycholate in borate-phosphate buffer (pH 7.8), recovery of all colors better than 82%	214 nm	Soft drinks, confectionery ³⁷

esting alternatives because they are designed to filter samples and select only those that contain target dyes above or below a pre-set concentration threshold. The advantages of sample screening systems include cost reduction and enhancement of rapidity and simplicity.

The identification of synthetic colorants (pure or mixtures) in foods is usually carried out using spectrophotometry^{6,8-10} but the resolution of complex mixtures in food requires a previous separation of extract components by SPE and chromatographic techniques. Dual wavelength, solid phase, and derivative spectrophotometric methods^{7,10-12} combined with chemometric approaches have been used.^{8,13}

The combined use of a continuous flow system and a spectrophotometer for sample screening to discriminate between synthetic and natural colorants is also available.¹⁴ With a very simple flow system on a column packed with natural materials, one can discriminate natural and synthetic colorants. The natural (not retained) ones can be determined in the first step and the synthetic (retained) ones in the second step after their elution. For yellow, red, green, blue, and brown, natural or synthetic colorants were chosen as models. The specific maximum wavelength for each color (400, 530, and 610 nm, respectively) was selected by a diode array system. A complete discrimination of natural and synthetic colorants was obtained for concentrations of natural colorants (in the absence of synthetic ones) up to 2000 (yellow), 2000 (red), and 10,000 (brown) times that of the detection limits (DLs) of synthetic additives. This method was applied to screen fruit drinks and candies.¹⁴

To test the quality of some synthetic dyes according to standardized procedures, a screening is recommended based on TLC analysis on silica plates 60 F 254 using elutions with an ethyl acetate:pyridine:water 25:25:20 (v:v:v) mixture. To determine purity and secondary dyes (components or by-products of a dye that are not allowed to be present), successive TLC separations are recommended or, for more accurate answers, HPLC-DAD using RP-18 columns and eluents like acetonitrile and phosphate buffer.¹⁴

Spectrophotometric resolution for the discrimination of individual colorant molecules found in mixtures is lower than that of chromatographic techniques such as TLC or HPTLC and even low-cost paper chromatography.^{5,6} More expensive but more accurate determinations may be made by RP-HPLC, IP-HPLC with UV-Vis, and diode array detection.^{4,15,16}

6.6.4 QUANTITATIVE ANALYSIS

6.6.4.1 SPECTROMETRIC QUANTIFICATION

Spectrophotometric determinations aim at evaluation of actual versus permitted concentrations of synthetic colorants. Quantitative analysis of colorants resulting from these procedures can be performed by various techniques. Spectrophotometry allows individual or simultaneous quantitative analyses of colorant mixtures having similar absorption spectra.^{8,17-20}

To suggest a threshold-limited concentration of individual synthetic colorants, it was necessary to set up an imaginary concentration of such colorants to be considered as a cut-off concentration² set to twice the DL of each synthetic colorant

at each wavelength, e.g., DL = 22 ng/ml for yellow tartrazine ($\lambda_{\max}$ at 400 nm) and brown (brilliant black BN, $\lambda_{\max}$ at 610 nm) and DL = 16 ng/ml for red erythrosine ($\lambda_{\max}$ at 530 nm). A false positive response arises when the signal determines a *yes* response for a standard solution containing only natural colorants at a concentration above the cut-off value (of synthetic colorants).

A systematic study was carried out using in parallel 50 standard solutions for each concentration of three natural colorants (curcumin, carminic acid, and caramel as yellow, red, and brown, respectively). No false positive results for synthetics were obtained up to concentrations of 15 and 20 ng/ml for natural red and yellow colorants, respectively, or 110 ng/ml for natural brown colorant. The concentrations have to be high enough to prove that the screening method is able to accurately discriminate natural and synthetic colorants. To make a clear interpretation of the quantitative UV-Vis spectrum, linear regression analysis was used.⁸ Quantitative UV-Vis analysis of a dye²⁰ can be calculated according to the following formula:

$$\text{Percent of pure dye concentration} = A_{\lambda_{\max}} \times \text{dilution} / A^{1\%}_{1 \text{ cm}}$$

where $A_{\lambda_{\max}}$ is the absorption value at the absorption maximum of the dye, taken from its UV-Vis absorption spectra, dilution is the dilution factor involved, and $A^{1\%}_{1 \text{ cm}}$ is the dye-specific molar extinction coefficient (for definition see Section 1.2.3; for values see Table 6.6.2).

TABLE 6.6.2
Maxima of Light Absorptions of Synthetic Food Colorants
Determined by UV-Vis Spectrophotometry

Colorant	European Union Code	$\lambda_{\max}$ (nm)	Absorption (Extinction) $A^{1\%}_{1 \text{ cm}}$
Allura Red AC	E 129	520	
Amaranth	E 123	520	440
Azorubine	E 122	516	510
Brilliant Black BN	E 151	570	530
Brilliant Blue FCF	E 133	630	1630
Brown FK	E 154	—	—
Brown HT	E 155	—	—
Erythrosine BN	E 127	526	1100
Green S	E 142	608	—
Indigotine	E 132	610	480
Patent Blue V	E 131	638	2050
Ponceau 4R (Cochineal red)	E 124	506	430
Quinoline yellow	E 104	413	865
Red 2G	E 128	520	—
Sunset Yellow FCF	E 110	481	555
Tartrazine	E 102	426	530

Solid phase spectrophotometry proved to be an appropriate technique for the determination of colorants in foods due to its simplicity, selectivity, reasonable cost, low detection limits, and use of conventional instrumentation. This simple, sensitive, and inexpensive method allowed simultaneous determinations of Sunset Yellow FCF (SY), Quinoline Yellow, and their unsulfonated derivatives [Sudan I (SUD) and Quinoline Yellow Spirit Soluble (QYSS)] in mixtures.²² Mixtures of food colorants containing Tartrazine, Sunset Yellow, Ponceau 4R, Amaranth, and Brilliant Blue were simultaneously analyzed with Vis spectrophotometry without previous chemical separation.²³

Because of peak overlappings in the first- and second-derivative spectra, conventional spectrophotometry cannot be applied satisfactorily for quantitative analysis, and the interpretation cannot be resolved by the zero-crossing technique. A chemometric approach improves precision and predictability, e.g., by the application of classical least squares (CLS), principal component regression (PCR), partial least squares (PLS), and iterative target transformation factor analysis (ITTFA), appropriate interpretations were found from the direct and first- and second-derivative absorption spectra.^{8,24,25} When five colorant combinations of sixteen mixtures of colorants from commercial food products were evaluated, the results were compared by the application of different chemometric approaches. The ITTFA analysis offered better precision than CLS, PCR, and PLS, and calibrations based on first-derivative data provided some advantages for all four methods.⁷

Recently, a very simple spectrophotometric method was described to resolve binary mixtures of Sunset Yellow (INS 110) and Tartrazine Yellow (INS 102) food colorants by using first-derivative spectra with measurements at zero-crossing wavelengths. Before the spectrophotometric measurements were made, the dyes were adsorbed onto polyurethane foam and recovered in *N,N*-dimethylformamide. Commercial food products (gelatin and juice powder) were analyzed using the proposed method in parallel with HPLC separation. The results were in very good agreement. Therefore, the first-order derivative spectrophotometric method is accurate, precise, and reliable enough to be applied for routine analysis of food samples.²⁶

6.6.4.2 IDENTIFICATION AND QUANTIFICATION BY LIQUID CHROMATOGRAPHY

HPLC is often reported to be the technique of best choice for the quantification of food colorants. According to European Directive 94/36/EC, the quantities of synthetic colorants to be added to foods are restricted and thus reliable methods for their quantification must be established. Approved colorants, defined by E-coded numbers (Table 6.6.2), are permitted for non-alcoholic beverages, confectionery products, and even for caviar (dying fish roe). For example, a specific HPLC chromatographic method for the quantization of 14 synthetic food colorants belonging to azo dye, triphenylmethane, or quinophthalone classes (E 102, 104, 110, 122, 123, 124, 127, 128, 129, 131, 132, 133, 142, 151) was reported to check their contents in caviar.²⁷

Polyamide powder showed to be highest recovery adsorbent for quantitative determination, compared to other resins. The isolated colorants were analyzed by

RP-HPLC-diode-array, and it was found that the maximum concentrations of individual colorants regulated by the European Union were exceeded.²⁷

Recently a new method was developed for the complete liquid chromatographic separation and diode array detection of standard mixtures of the 14 most frequently used synthetic colorants.²⁸ Protocols for RP-HPLC^{12,15,28–32} and IP-HPLC^{33–35} techniques have been extensively described and the techniques were compared with micellar electrokinetic capillary chromatography,^{36,37} which has been shown to be suitable for the analysis of synthetic colorants.

Five synthetic and five natural colorants were identified and quantified in lyophilized dairy products and fatty foods using an automatic method based on solid phase extraction using a stationary phase followed by RP-HPLC C₁₈ columns for the sequential retention of colorants and diode array detection. Lyophilization of the samples coupled with the separation procedure provided clean extracts despite the complexity of the food matrices and preserved the sample for at least 2 months without changes in colorant concentrations. The detection limits achieved for the colorants were found in a wide range from 0.03 to 75 µg/g of the lyophilized sample, according to the limits established by the European Union.³⁸

Another reverse phase HPLC method for the simultaneous determination of the main artificial dyes, sweeteners, and preservatives found in soft drinks was proposed (see details in Table 6.6.1). It involves the use of a LiChrosorb RP C₁₈ column and a binary eluent consisting of a phosphate buffer (pH 4.0) containing methanol, according to a suitable gradient elution program. The sensitivity of the spectrophotometric detection was optimized by adopting a wavelength switching technique, thus achieving detection limits below the maximum permitted levels for all the considered additives. The method was applied to some commercial soft drinks and the analysis required minimum pre-treatment before direct injection.³⁰

Similarly, other thin layer separations³⁹ and reverse phase, ion pair, and anion exchange HPLC methods have been developed.^{37,15,40–44} A further method is based on direct injection of a beverage solution (homogenized and degassed) into a C₁₈ HPLC column and separation by RP-HPLC with visible detection at a single wavelength.⁴² Determination of synthetic colors in soft drinks containing fruit juice and flavor extracts was not affected by the presence of other natural colors. The results obtained from the samples confirm that the proposed method works well and is useful for serious control or screening of additions of synthetic colors to beverages. The method is therefore recommended for use by the quality control departments of soft drink producers using synthetic food colors.

6.6.4.3 ELECTROKINETIC CHROMATOGRAPHY, CAPILLARY ELECTROPHORESIS, AND VOLTAMMETRY

At present, the most promising methods for synthetic colorant analysis seem to be those based on separation approaches such as HPLC and capillary electrophoresis (CE). CE is the method of choice for the determination of synthetic dyes in biological materials while HPLC is generally a more suitable method for the identification and determination of hydrophobic natural pigments, having a better sensitivity and efficiency than CE.

The separation of some food additives including colorants by CZE^{45–52} and micellar electrokinetic chromatography (MEKC)⁵³ has been reviewed. These techniques are more affected by sensitivity problems due to smaller injection volumes.

Another method based on electrokinetic chromatography was recently developed and tested for the separation of synthetic dyes. It is known as microemulsion electrokinetic chromatography (MEEKC) and was developed to analyze eight food colorants commonly used in commercial food products. Most papers that compared separations obtained by MEEKC and MEKC concluded that MEEKC has greater separation capability than MEKC for highly hydrophobic compounds because highly hydrophobic compounds tend to be strongly retained by micelles.^{54–58} The optimal conditions developed for MEEKC were used to successfully separate colorants in several real samples without SPE sample pretreatment and a higher separation efficiency in detecting food colorants in some food samples was obtained. Although CE provided faster separations (9 min) of the eight colorants, SPE pretreatment was needed to reduce interference.⁵⁴

This separation technique produces very good results for acidic or anionic dye molecules containing carboxylic, sulfonic, and hydroxy groups that can be separated within short run times in an alkaline medium in a single analysis step.^{52,53} Natural colorants usually do not contain these functional groups; they are usually more voluminous and strongly hydrophobic, properties that complicate their determination by CE. The sample pretreatment is more difficult when CE (compared to HPLC) is used.

Direct and derivative spectrophotometric and IP-RP-HPLC methods were applied to identify and determine synthetic dyes and follow their degradation processes.³⁴ The dyes considered were Tartrazine (E 102), Quinoline Yellow (E 104), Sunset Yellow (E 110), Carmosine (E 122), Amaranth (E 123), New Coccine (E 124), Patent Blue Violet (E 131), and Brilliant Blue FCF (E 133). All are considered representative additives for soft drinks.

In conclusion, synthetic dyes can be determined in solid foods and in non-alcoholic beverages and from their concentrated formulas by spectrometric methods or by several separation techniques such as TLC, HPLC, HPLC coupled with diode array or UV-Vis spectrometry, MECK, MEECK, voltammetry, and CE.^{60–62} Many analytical approaches have been used for simultaneous determinations of synthetic food additives: thin layer chromatography,^{39,44} derivative spectrophotometry,^{10,16,18,51} adsorptive voltammetry,^{13,59} differential pulse polarography,⁶³ and flow-through sensors for the specific determination of Sunset Yellow and its Sudan 1 subsidiary in food,⁶⁴ but they are generally suitable only for analyzing few-component mixtures.

All these methods give similar results but their sensitivities and resolutions are different. For example, UV-Vis spectrophotometry gives good results if a single colorant or mixture of colorants (with different absorption spectra) were previously separated by SPE, ion pair formation, and a good previous extraction.⁶⁵ Due to their added-value capability, HPLC and CE became the ideal techniques for the analysis of multicomponent mixtures of natural and synthetic colorants found in drinks. To make correct evaluations in complex dye mixtures, a chemometric multicomponent analysis (PLS, nonlinear regression) is necessary to discriminate colorant contributions from other food constituents (sugars, phenolics, etc.).

REFERENCES

1. Downham, A. and Collins, P., Colouring our foods in the last and next millennium, *Int. J. Food Sci. Tech.*, 35, 5, 2000.
2. Analytical Methods Committee, Handling false negatives, false positives and reporting limits in analytical proficiency tests, *Analyst*, 122, 495, 1997.
3. Ashkenazi, P., Yarnitzky, C., and Casi, M., Determination of synthetic food colours by means of a novel sample preparations system, *Anal. Chim. Acta*, 248, 289, 1991.
4. AOAC Official Method 988.13, FD&C Color Additives in Foods, Rapid Cleanup for Spectrophotometric and Thin-Layer Chromatographic Identification, *AOAC Official Method of Analysis*, 46.1.05, 3, 2000.
5. Anderton, S.M., Incarvito, C.D., and Sherma, J., Determination of natural and synthetic colors in alcoholic and non-alcoholic beverages by quantitative HPTLC, *J. Liq. Chromatogr.*, 20, 101, 1997.
6. Rizova, V. and Stafilov, T., XAD-2 HPLC method of identification and determination of some synthetic colourings, *Anal. Lett.*, 28, 1305, 1995.
7. Ni, Y. and Gong, X., Simultaneous spectrophotometric determination of mixtures of food colorants, *Anal. Chim. Acta*, 354, 163, 1997.
8. Hofer, K. and Jenewein, D., Quick spectrophotometric identification of synthetic food colorants by linear regression analysis, *Z. Lebensm. Unters. Forsch.*, A204, 32, 1997.
9. Berzas Nevado, J.J. et al., Simultaneous determination of tartrazine, riboflavine, curcumin and erythrosine by derivative spectrophotometry, *Fresenius J. Anal. Chem.*, 350, 610, 1994.
10. Cruces-Blanco, C., García-Campaña, A.M., and Alés-Barrero, F., Derivative spectrophotometric resolution of mixtures of the food colourants Tartrazine, Amaranth and Curcumin in a micellar medium, *Talanta*, 43, 1019, 1996.
11. Berzas-Nevado, J.J., Cabanillas, C.G., and Contento-Salcedo, A.M., Simultaneous spectrophotometric determination of three food dyes by using the first derivative of ratio spectra, *Talanta*, 42, 2043, 1995.
12. Berzas Nevado, J.J., Resolution of ternary mixtures of Tartrazine, Sunset Yellow and Ponceau 4R by derivative spectrophotometric ratio spectrum-zero crossing methods in commercial foods, *Talanta*, 46, 933, 1998.
13. Ni, Y., Bai, J., and Jin, L., Multicomponent chemometric determination of colorant mixtures by voltammetry, *Anal. Chim. Acta*, 329, 65, 1996.
14. González, M., Gallego, M., and Valcárcel, M., Automatic screening method for the rapid and simple discrimination between synthetic and natural colorants in foods, *Anal. Chim. Acta*, 464, 237, 2002.
15. Chen, Q. et al., Determination of eight synthetic food colorants in drinks by high-performance ion chromatography, *J. Chromatogr. A*, 827, 73, 1998.
16. Berzas-Nevado, J.J. et al., Separation and determination of dyes by ion-pair chromatography, *J. Liq. Chromatogr. Rel. Technol.*, 20, 3073, 1997.
17. Capitan, F. et al., Determination of colorant matters mixtures in foods by solid-phase spectrophotometry, *Anal. Chim. Acta*, 331, 141, 1996.
18. Capitan-Vallvey, L.F. et al., Simultaneous determination of the colorants tartrazine, ponceau 4R and sunset yellow FCF in foodstuffs by solid phase spectrophotometry using partial least square multivariate calibration, *Talanta*, 47, 861, 1998.
19. Bozdogan, A., Ozgur, U.M., and Koyuncu, I., Simultaneous determination of sunset yellow and Ponceau 4R in gelatin powder by derivative spectrophotometry and partial least-squares multivariate spectrophotometric calibration, *Anal. Lett.*, 33, 2975, 2000.

20. Sayar, S. and Ozdemir, Y., First-derivative spectrophotometric determination of Ponceau 4R, Sunset Yellow and tartrazine in confectionery products, *Food Chem.* 61, 367, 1998.
21. BASF, *Analytical Tests for the Sicovit and Sicomed Colorants, Technical Information*, 8, 1996.
22. Capitan-Vallvey, L.F. et al., Simultaneous determination of sunset yellow FCF and Sudan I by HPLC, *Analyst*, 120, 2421, 1995.
23. Ni, Y. and Bai, J., Simultaneous determination of Amaranth and Sunset Yellow by ratio derivative voltammetry, *Talanta*, 44, 105, 1997.
24. El-Sayed, A.A.Y. and El-Salem, N.A., Recent developments of derivative spectrophotometry and their analytical applications, *Anal. Sci.*, 21, 595, 2005.
25. Thomas, E.V. and Haaland, D.M., Comparison of multivariate calibration methods for quantitative spectral analysis, *Anal. Chem.*, 62, 1091, 1990.
26. Vidotti, E.C., Rollemberg, M., and do Carmo E., Derivative spectrophotometry: a simple strategy for simultaneous determination of food dyes, *Quim. Nova*, 29, 230, 2006.
27. Kirschbaum, J., Krause, C., and Bruckner, H., Liquid chromatographic quantification of synthetic colorants in fish roe and caviar, *Eur. Food Res. Technol.*, 222, 572, 2006.
28. Kirschbaum, J. et al., Development and evaluation of an HPLC-DAD method for determination of synthetic food colorants, *Chromatogr. Suppl.*, 57, 115, 2003.
29. Prado, M.A. and Godoy, H.T., Determination of synthetic dyes by high performance liquid chromatography (HPLC) in jelly powder, *J. Liq. Chromatogr. Rel. Technol.*, 25, 2455, 2002.
30. Dossi, N., Simultaneous RP-LC determination of additives in soft drinks, *Chromatographia*, 63, 557, 2006.
31. Berzas Nevado, J.J. et al., A reverse phase HPLC method to determine six food dyes using buffered mobile phase, *Anal. Lett.*, 31, 2513, 1998.
32. Kiseleva, M.G., Pimenova, V.V., and Eller, K.I., Optimization of conditions for the HPLC determination of synthetic dyes in food, *J. Anal. Chem.*, 58, 685, 2003.
33. Gennaro, M.C. et al., Identification and determination of red dyes in confectionery and determination by ion-interaction HPLC, *J. Chromatogr. A*, 767, 87, 1997.
34. Gianotti, V. et al., Chemometrically assisted development of IP/RP/HPLC and spectrophotometric methods for the identification and determination of synthetic dyes in commercial soft drinks, *J. Liq. Chromatogr. Rel. Technol.*, 28, 923, 2005.
35. DeVilliers, A. et al., Evaluation of liquid chromatography and capillary electrophoresis for the elucidation of the artificial colorants brilliant blue and azorubine in red wines, *Chromatographia*, 58, 393, 2003.
36. Desiderio, C., Marra, C., and Fanali, S., Quantitative analysis of synthetic dyes in lipstick by MEKC, *Electrophoresis*, 19, 1478, 1998.
37. Chou, S.S. et al., Determination of synthetic colors in soft drinks and confectioneries by micellar electrokinetic capillary chromatography, *J Food Sci.*, 67, 1314, 2002.
38. Gonzales, M., Gallego, M., and Valcarcel, M., Liquid chromatographic determination of natural and synthetic colorants in lyophilized foods using an automatic solid-phase extraction system, *J. Agric. Food Chem.*, 51, 2121, 2003.
39. Oka, H. et al., Identification of unlawful food dyes by thin-layer chromatography-fast atom bombardment mass spectrometry, *J. Chromatogr. A*, 674, 301, 1994.
40. Tateo, F. and Bonomi, M., Fast determination of Sudan I by HPLC/APCI-MS in hot chili, spices, and oven-baked foods, *J. Agric. Food Chem.*, 52, 655, 2004.

41. Perez-Urquiza, M., Prat, M.D., and Beltran, J.L., Determination of sulphonate dyes in water by ion-interaction high-performance liquid chromatography, *J. Chromatogr. A*, 871, 227, 2000.
42. Garcia-Falcon, M.S. and Simal-Gandara, J., Determination of food dyes in soft drinks containing natural pigments by liquid chromatography with minimal clean-up, *Food Control*, 16, 293, 2005.
43. Gosetti, F. et al., Oxidative degradation of food dye E133 Brilliant Blue FCF. Liquid chromatography-electrospray ionization mass spectrometry identification of the degradation pathway, *J. Chromatogr. A*, 1054, 379, 2004.
44. Dugar, S.M., Leibowitz, J.N., and Dyer, R.H., Synthetic colorant chromatography for beverages and wine, *J. AOAC*, 77, 1335, 1994.
45. Boyce, M.C., Determination of additives in food by capillary electrophoresis, *Electrophoresis*, 22, 1447, 2001.
46. Frazier, R.A. et al., Development of a capillary electrophoresis method for the simultaneous analysis of colours, preservatives and sweeteners in soft drinks, *J. Chromatogr.*, A876, 213, 2000.
47. Frazier, R.A., Ames, J.M., and Nursten, H.E., *Method Development: Capillary Electrophoresis for Food Analysis*, Royal Society of Chemistry, Cambridge, 2000.
48. Huang, H.Y. et al., Analysis of food colorants by capillary electrophoresis with large-volume sample stacking, *J. Chromatogr. A*, 995, 29, 2003.
49. Kuo, K.L., Huang, H.Y., and Hsieh, Y.Z., High-performance capillary electrophoretic analysis of synthetic food colorants, *Chromatographia*, 47, 249, 1998.
50. Masar, M., Kaniansky, D., and Madajova, V., Separation of synthetic food colourants by capillary zone electrophoresis in a hydrodynamically closed separation compartment, *J. Chromatogr. A*, 724, 327, 1996.
51. Berzas Nevado, J.J. et al., Method development and validation for the simultaneous determination of dyes in food stuffs by capillary zone electrophoresis, *Anal. Chim. Acta*, 378, 63, 1999.
52. Pérez Urquiza, M. and Beltrán, J.L., Determination of dyes in foodstuffs by capillary zone electrophoresis, *J. Chromatogr. A*, 898, 271, 2000.
53. Thompson, C.O. and Trenerry, V.C., Determination of synthetic colours in confectionery by micellar electrokinetic capillary chromatography, *J. Chromatogr. A*, 704, 195, 1995.
54. Huang, H.Y., Determination of food colorants by microemulsion electrokinetic chromatography, *Electrophoresis*, 26, 867, 2005.
55. Hilder, E.F. et al., Separation of hydrophobic polymer additives by microemulsion electrokinetic chromatography, *J. Chromatogr. A*, 922, 293, 2001.
56. Klampfl, C.W., Solvent effects in microemulsion electrokinetic chromatography, *Electrophoresis*, 24, 1537, 2003.
57. Altria, K.D., Mahuzier, P.E., and Clark, B.J., Background and operating parameters in microemulsion electrokinetic chromatography, *Electrophoresis*, 24, 315, 2003.
58. Cahours, X. et al., MEEKC-UV versus CEC-UV-MS for the analysis of flunitrazepam and its major metabolites, *Electrophoresis*, 23, 2320, 2002.
59. Nevado, J.J.B., Square wave adsorptive voltammetric determination of sunset yellow, *Talanta*, 44, 467, 1997.
60. Ni, Y.N., Bai, J.L., and Jin, L., Simultaneous adsorptive voltammetric analysis of mixed colorants by multivariate calibration approach, *Anal. Chim. Acta*, 329, 65, 1996.
61. Wood, R. et al., *Analytical Methods for Food Additives*, Woodhead, Cambridge, 2004.

62. Suzuki, S. et al., Determination of synthetic food dyes by CE, *J. Chromatogr. A*, 680, 541, 1994.
63. Combeau, S., Chatelut, M., and Vittori, O., Identification and simultaneous determination of Azorubin, Allura red and Ponceau 4R by differential pulse polarography: application to soft drinks, *Talanta*, 56, 115, 2002.
64. Valencia, M.C. et al., A flow-through sensor for the determination of the dye Sunset Yellow and its subsidiary Sudan 1 in foods, *Quim. Anal.*, 19, 129, 2000.
65. Lau, O.W. et al., Spectrophotometric determination of single synthetic food colour in soft drinks using ion-pair formation and extraction, *Int. J. Food Sci. Technol.*, 30, 6, 793, 1995.

Section 7

*Quality and Safety of
Food Colorants*

7.1 Colorants and Food Quality Management

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Food quality management aims to realize food quality that complies with or even exceeds customer or consumer requirements. Food quality is not only affected by intrinsic properties and applied technological conditions, but is also influenced by the people who design, control, improve, and assure food quality. While the emphasis of previous chapters is on providing insights into the physics, chemistry, biochemistry, and perception of food colorants, this chapter illustrates the importance of this fundamental knowledge and information for food quality management.

Food quality management requires fundamental knowledge of the dynamic behaviors of physical properties that contribute to quality attributes like color, safety, and taste along with insight into how food handler behavior interferes with food production systems by executing quality control activities, applying quality systems, designing new products, and other activities. This chapter explains how characteristics of food colorants are taken into account in food quality management.

The first section briefly describes the role of colorants in food quality perception. Section 7.1.2 shows why food quality management is a complex discipline that requires a techno-managerial research approach to analyze the relevant complex issues. Section 7.1.3 explains the principle of control and illustrates how varying characteristics of food colorants may impact control activities in food quality management. Sections 7.1.4 and 7.1.5 cover two typical concepts that can be used to design and assure food quality and safety: the critical quality control point (CQP) approach and risk assessment. Finally, the impacts of legislation on the application and use of food colorants are explained as aspects of managing food quality (Section 7.1.6).

**7.1.1 IMPORTANCE OF COLOR FOR QUALITY
PERCEPTION OF FOOD**

Food quality has become increasingly important for consumers, as reflected by the number of legislative changes intended to regain consumer trust in food quality, the large number of quality systems developed in the last decade, and the efforts invested in new product development by the food industry.¹⁻⁷ No unequivocal concept surrounds food quality. Views on food quality differ, depending on focus (narrow or broad definition), position in the chain (primary production facility or retailer), and position in the production company (operating personnel or marketing).²

This chapter defines quality as meeting consumer or customer requirements: a product as such has no quality, but it has physical properties reflected in attributes that can be perceived as quality after consumption or use. Quality attributes can be defined as characteristics of a product or production process that are observable (by

sensory observation or via communication) and contribute to quality expectations and experiences of consumers or customers.^{8,9}

Typical attributes for evaluating food quality are safety, shelf life, color, taste, flavor, texture, health, and convenience.^{2,10} The level of a quality attribute is determined by levels of physical, microbial, physiological, chemical, and biochemical food processes, product composition, and applied technological conditions. For example, a combination of enzyme-degrading colorants, compositions and concentrations of pigments, and food structure properties contribute to a certain color level perceived by consumers. Depending on the match of expectation and experience, certain attributes will or will not be perceived as quality.⁸

Food colorants play an important role in quality perception. Color is often the first notable characteristic of a food and it influences the expectations of consumers buying the product and also influences food handlers who make quality-related decisions, for example, during visual inspections.¹¹ More specifically, color pre-determines our expectations and perceptions of flavor and taste.^{8,11} Color is interrelated with flavor intensity (detection threshold), with sweetness and salinity sensations, and also with our susceptibilities to and preferences for products.^{10,12} For example, consumers perceived a strongly red-colored strawberry-flavored drink to be sweeter than a less colored version, and yellow was associated with lemon and pink with grapefruit, but by reversing the colors, flavor perception changed.¹¹ If food color is not appealing, consumers will not enjoy the flavor and texture of the food.¹⁰

Color is often used as an indicator of food quality due to short evaluation times and cost savings.^{13,14} People consider the colors of raw materials, half fabricates, and final products in order to make decisions to accept or reject food products.¹⁵ For example, levels of anthocyanins have been used to evaluate the adulteration of various pigmented food products,¹⁶ and fruit color is a strong determinant of ripeness. The role of the food handler in controlling the colors of food products is very important because such judgments are subjective.¹⁷

Additionally, color may also serve as a key to cataloging a food as safe. Undesirable colors of meats, fruits, and vegetables warn us about potential dangers or at least of the presence of undesirable flavors.¹⁰ Color and other sensory attributes are even misused as indicators for safety. Walker and co-authors¹⁸ demonstrated that in small and medium enterprises, more than 50% of food handlers thought that they could tell whether food was contaminated with food poisoning bacteria by sight, smell, and taste. Color is thus used as a way to identify a food and judge its quality.¹¹

7.1.2 COMPLEXITY OF FOOD QUALITY MANAGEMENT

In agribusiness and the food industry, management of quality is often assumed to be a rather controllable process.¹⁹ This may be illustrated by the great attention paid to the development and implementation of quality systems in the past decade.^{1,3,20} These quality systems are commonly based on procedures and control circles as mechanisms to control and assure quality. After implementation, however, it often appears in practice that the intended results are not obtained and exact reasons are not clear.²

Luning and Marcelis¹⁹ argued that food quality management concerns the dynamic and complex character of living, perishable food systems in combination with the dynamic and often unpredictable behaviours of people involved in food production. Dealing with such complex systems means that one must cope with ambiguity (due to lack of understanding of underlying factors and mechanisms) and uncertainty (due to lack of information). In situations with high levels of ambiguity and uncertainty, outcomes are predictable. Luning and Marcelis concluded that the cybernetic control approach (like the control circles that serve as the bases of common quality systems) for managing food quality is too simple because it assumes predictable systems. Therefore they proposed another approach to analyze food quality management issues.

First, a food quality relationship model has been developed. It considers food quality (FQ) to be dependent on food behavior (FB) and human behavior (HB). FB is a function of food dynamics (FD) (such as variable pigment concentrations and differing color degradation profiles) and applied technological conditions (TCs) (such as oxygen control to maintain color concentrations). Likewise, HB is a function of human dynamics (HD) (for example, varying color perceptions due to age differences), and administrative conditions (ACs) (such as use of color cards to support visual color inspection). These relations are reflected in the food quality relationship model as:

$$FQ = f(FB, HB), \text{ whereby } FB = f(FD, TC) \text{ and } HB = f(HD, AC)^{19}$$

Second, a techno-managerial approach has been described to analyze how food and human systems interact and contribute to food quality. It involves a systematic and integrated use of theories from food technology sciences and management sciences, explicitly acknowledging dynamics and conditioning aspects of both the food and human systems.^{19,21}

Since the major objective of food quality management (FQM) is realizing food quality that complies with customer and consumer requirements, it was necessary to also identify the activities required to achieve this goal. Therefore, an FQM functions model has been developed describing which essential technological and managerial functions are necessary to realize food quality.

Technological functions concern receipt, processing, and storage of processed products. The functions deal with the actual production activities (heating, storing, transporting, etc.) and measuring activities (taking samples, analyzing, measuring, etc.) to obtain information about the status of products and processes. Besides common quality (Q) management functions known as Q control and Q assurance, the additional functions of Q design, Q improvement, and Q policy and strategy have been described. Each function concerns a set of decisions to achieve a certain goal. Each function has a different objective but all functions contribute to final food quality. For example, Q control aims at continuously evaluating performance of both technological and human processes and taking corrective actions when necessary. Q assurance activities deal with setting requirements for the quality system, evaluating its performance, and organizing necessary changes.²⁹ Each function explicitly acknowledges dynamics (in time) and conditioning (technological and administra-

tive) of both the food and human systems. In this way, the FQM functions model supports the systematic analysis of technological and managerial bottlenecks.

What does this information mean for managing the quality of colorants in food matrices? From the perspective of the techno-managerial approach, the food quality relationship model, and the food quality functions model one should gain a profound insight into the dynamic behavior of food colorants (like, profiles of color decay during food production) and into the often unpredictable decision-making behavior of food handlers (like, their variable judgment of color quality) in order to design, control, improve, and/or assure quality of food colorants at different stages of food production. In the beginning of this chapter, we stated that food quality management is complex (Section 7.2.1), which is underpinned by the complex mechanisms underlying behavior of food colorants and unpredictable behavior of food handlers in food production.

7.1.3 QUALITY CONTROL AND FOOD COLORANT CHARACTERISTICS

Although food colorants similarly contribute to perceived quality or safety, they originate from different (bio)chemical mechanisms. This section focuses on explaining the principles of quality control and illustrates how different mechanisms beyond food colorants may have consequences for quality control activities. Q control is a management function that plays a role in the distinct technological functions of receiving materials, processing food materials, and storage and distributing of processed materials.

7.1.3.1 PRINCIPLE OF QUALITY CONTROL

Quality control is intended to monitor and evaluate the performances of both food and human processes that contribute to food quality. The basic principle of this function is the control circle that involves (1) the taking of a process sample by the analysis or measuring unit, (2) determining whether process results meet set tolerances or limits, (3) judging the character and level of any discrepancy, and (4) application of corrective action to adjust the system to an acceptable level (Figure 7.1.1). A distinction is made between measuring and analysis, whereby the first involves direct measurements (e.g., pH, temperature) and the second involves taking samples, sample preparation, and actual analysis.²

The focus of Q control differs, depending on the technological function. Upon receipt of raw materials, Q control deals with measuring the product properties and deciding whether to accept or reject delivered batches. At processing, it deals with controlling the transformation processes, whereas during storage and distribution, control refers to measuring product properties to be maintained within desired tolerances. The performance of quality control activities is dependent on several technological and managerial aspects (Figure 7.1.1) including where in the process the situation is measured (or samples taken), how often, and how many, and sampling and measuring design. Profound insight is necessary in how intrinsic product param-

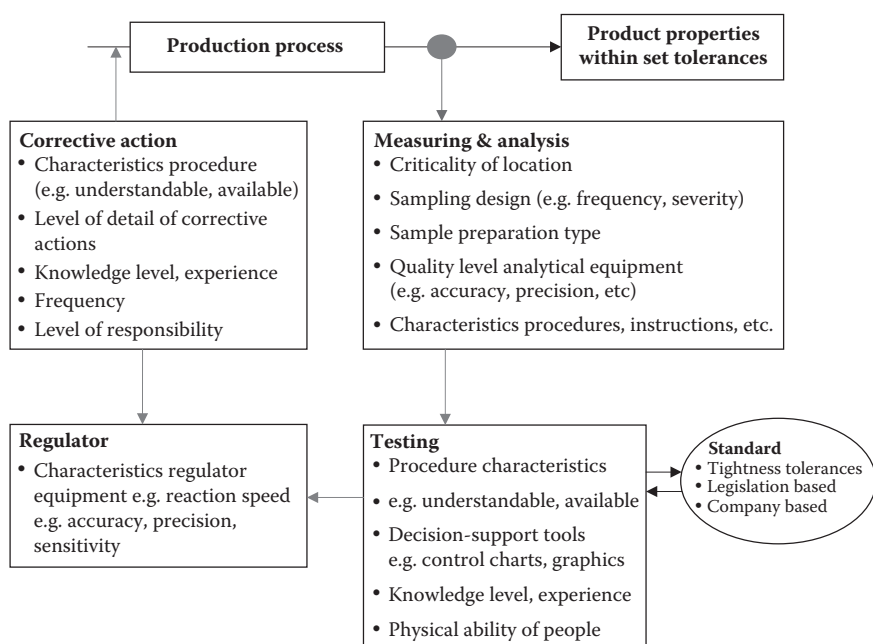


FIGURE 7.1.1 Typical technological and managerial factors influencing performance of quality control activities

eters and technological conditions affect behaviors of colorants in order to determine critical locations of control; and insight in distribution profiles of colorants is required to judge sampling frequency and number of samples.

- Sample preparation: in case of sampling this is a critical step because undesired reactions easily occur at this step (e.g., color decay due to light or oxygen) resulting in unreliable data.
- Quality of analytical equipment: specificity, accuracy, precision, and sensitivity of measuring and analyzing equipment determine the reliability of test results.
- The availability and clarity of procedures and instructions that affect the abilities of people to execute control activities is important. Unclear instructions about what to do in case of out-of-control situations will result in unpredictable decisions on quality attributes.
- Circumstances of control: specifically for visual inspections, several conditions (e.g., amount of light, time for judgment, disturbing noise, visual product complexity) contribute to how well people are able to judge quality based on visual characteristics like color.
- Availability of decision support tools like control charts and color charts is also important. In situations that are out of control, these tools support people in making homogeneous decisions dealing with quality failure (e.g., color deviation).

- Ability to control: age, experience, specific deficiencies, and other factors can impact the judgment performance of people who evaluate visual quality.^{2,17,22}

To appropriately control quality in terms of food color, one is required to have insight regarding the (bio)chemical mechanisms of colorants, how they are affected by intrinsic properties and technological conditions, where and how to measure and analyze the colorants, and how to instruct, support, and train people who judge quality based on color properties. Although a wide range of food colorants exists, from a quality control perspective, we can roughly divide them into three categories: natural colorants, formed colorants, and color additives.

7.1.3.2 CONTROLLING NATURAL COLORANTS

Natural colorants are pigments produced by living organisms such as plants, animals, fungi, and micro-organisms.¹⁰ Typical food colorants consumed daily in considerable quantities in human diets are anthocyanins, carotenoids, and chlorophylls.²³ Natural colorants are widely spread throughout nature in fruits, vegetables, seeds, and roots. They are commonly found in raw materials and unprocessed food products.²³ The biosynthesis and regulation of pigments are complex processes and the exact pathways are still unknown.

For example, carotenoids consist of a large group of compounds that are formed via complex biosynthesis routes, and the regulation of carotenoid biosynthesis seems to occur at several levels.¹⁰ Moreover, these natural pigments vary widely in physical and chemical properties and stability. Concentrations can be affected by conditions such as oxygen, sugar level, and pH in food matrices and their inherent solubilities vary widely.²³ Typical technological conditions like refrigeration, canning, dehydration, smoking, bottling, and exposure to light, air, moisture, and temperature extremes all tend to alter levels and/or compositions of natural color pigments.¹¹ For example, anthocyanin pigments can be easily degraded during processing of fruits and vegetables; high temperature, increased sugar level, pH, and ascorbic acid can affect the rate of degradation.^{10,24}

The betalains serve as another example. They are very sensitive to different technological factors. They can only be maintained in foods with short shelf lives, produced with minimum heat treatment, and packaged in a dry state under reduced levels of light, oxygen, and humidity. Betalains have several applications in gelatin desserts, confectionery, dry mixes, poultry, dairy, and meat products.¹⁰

What do these properties mean for the quality control of natural colorants? The lack of a complete understanding of the underlying biochemical mechanisms hinders specific control of colorants during cultivation and harvesting. From the FQM perspective, one deals with a high level of ambiguity reflected in the large variations in concentrations of natural colorants in raw materials that are input for subsequent food processing. During processing of materials containing natural colorants, one should understand which technological conditions and product properties are critical for colorant decay, then control these conditions to maintain color concentrations

within acceptable tolerances. Quality control of natural colorants focuses on *maintaining initial concentrations within acceptable tolerances*.

7.1.3.3 CONTROLLING FORMED COLORANTS

The second group consists of colorants formed during processing or storage. The Maillard reaction is a major process contributing to color formation. It is a non-enzymatic browning process that encompasses a whole network of reactions between amines and reducing sugars. The Maillard reaction takes place in heated, dried, and stored foods and is responsible for changes in color, flavor, and nutritive value but is also responsible for the formation of stabilizing and mutagenic compounds.^{25,26} Little information is available on the chemical structures of the hundreds of brown products formed by a series of consecutive and parallel reactions including oxidations, reductions, aldol condensations, and others.^{27,28} The simultaneous development of a number of reactions, their interactions, and a prevailing Maillard reaction can affect in different ways the overall antioxidant properties and colors of products.²⁷ Various factors involved in food processing such as precursors, thermal processing parameters, pH, and quantitative ratio of amino nitrogen to reducing sugar influence the Maillard reaction.²⁶

The reaction is notoriously difficult to control.²⁶ On the one hand, desired reactions such as color and flavor formation must be stimulated, while undesired reactions such as decay of amino acids and formation of toxic compounds should be kept below certain limits. With these types of colorants, one deals with a high level of ambiguity due to not fully understanding underlying chemical mechanisms. From a quality control perspective, the focus is on *regulating the formation process*, whereby time and temperature, for example, are important conditioning factors for achieving desired concentrations of colorants on the one hand, and keeping undesired compounds below certain limits on the other.

7.1.3.4 CONTROLLING COLOR ADDITIVES

The third group of colorants consists of the color additives used to improve appearances of food products. They reinforce the colors already present in foods and ensure uniformity of appearance from season to season and batch to batch. They also add color to virtually colorless foods such as sherbets and provide dramatic color to “fun” foods such as candies and holiday treats.^{10,11} Color additives are also used to compensate for color loss during processing and transportation and ensure desirable appearance.¹⁰

Color additives are naturally and synthetically derived. In the past 20 years, consumers have become increasingly aware of the ingredients in their foods and they desire foods be as natural as possible. This fact combined with technological developments have fueled the increases in uses of naturally derived colors.²⁹ Moreover, additives must be safe and stable in food matrices. Legislation has established which colorants may be used as food additives, taking into account toxicity of compounds and methods of assessment.^{29–32} The European Union currently allows the use of 13 naturally derived colors; but 26 colors are exempt from certification

in the United States. These colors differ in key characteristics like stability, solubility, and applicability.²³

Since additives are added to foods and have their own specific stability properties, quality focuses mainly on *controlling the addition activities* and *maintaining* added concentrations during processing and storage within desired tolerances. Quality control also focuses on keeping concentrations of additives within legislative requirements in order to guarantee food safety.

7.1.3.5 CONCLUDING REMARKS

The origin of a food colorant affects the focus of quality control (maintaining or regulating formation). In terms of ambiguity and uncertainty, quality control reduces a situation of potential chaos by collecting information intended to reduce uncertainty. Depending on the level of lack of knowledge of underlying mechanisms and understanding relationships with technological conditions, one has to accept more ambiguity.

7.1.4 CONCEPT OF CRITICAL QUALITY POINTS (CQPS)

Quality control is an important managerial function for achieving food quality and safety; the concentration is on keeping technological and managerial processes within acceptable tolerances. From consumer and legislative perspectives, more demands are put on food quality. Consumers and regulators want guarantees that food has certain quality attributes and is safe for consumption. Food companies should provide evidence that their food production processes and management systems are organized to assure that quality and safety comply within accepted tolerances. This section and Section 7.1.5 will discuss two methods: one for assessing critical process steps affecting quality attributes (like food color), also known as the critical quality point (CQP) concept, and one that can be used to assess safety, also known as risk assessment.

The CQP concept requires insight in underlying mechanisms (reducing ambiguity) to assess where undesired changes in quality attributes should be monitored and corrected in order to prevent quality defects in the end product. The risk assessment concept helps to systematically and scientifically judge if certain hazardous compounds have a high risk level. Both concepts can be used for quality design for quality assurance purposes. In this section, the concepts will be discussed from the perspective of quality assurance.

7.1.4.1 PRINCIPLE OF QUALITY ASSURANCE

The quality assurance function aims at guaranteeing and providing confidence that requirements such as product quality, safety, reliability, and service, are realized by the quality system. A quality system can be defined as the organizational structure, responsibilities, processes, procedures, and resources that facilitate the achievement of quality management.³³ A quality system is reflected in a set of descriptions, guidelines, and instructions prescribing which technological (hygienic equipment,

monitoring systems, analytical tools) and administrative conditions (procedures, training levels, number of control tasks, times) are necessary to guarantee that a certain safety or quality level is achieved.

While quality was formerly achieved by inspection of final products, it is accomplished now by prevention through controlling critical steps in the production processes along the agri-food chain.² Hazard analysis critical control points (HACCP) represent a typical example of such a preventive approach. Although this concept was developed primarily to assure food safety, the basic principle is also applicable to assuring non-safety quality attributes such as color, flavor, and nutritional value.^{2,34} This section translates the HACCP principles into a critical quality control point (CQP) concept that can be part of a system to assure food quality.

7.1.4.2 CRITICAL QUALITY POINT (CQP) CONCEPT

We have described the CQP concept from a techno-managerial perspective which means that we explicitly pay attention to the roles of both the food and human systems that constitute a food quality management system. The principle beyond the CQP concept is that one need only to control those steps in the process that are critical toward decay of the quality attributes. CQPs must be monitored, documented, and validated in such a way that the system is able to assure a certain quality level.

Within the CQP concept we distinguish three parts: (1) basic preparations, (2) CQP monitoring system development, and (3) documentation and verification development, all of which are explained in more detail below.

7.1.4.2.1 Basic Preparations

This part of the CQP concept consists of all preparations necessary before the actual CQP monitoring systems can be developed. It includes:

- Determination and organization of *appropriate resources* to design a CQP-based quality system. Typical issues to be considered include involvement of people who have practical experience, theoretical expertise, and knowledge in a CQP team, time to design the system, determination of the type of project management, funding, and management commitment.
- *Understanding the final product* to know the relevant quality attributes is important. Issues of concern are quality attributes desired by consumers (Q attribute profiling), acceptable variations in Q attributes, how the product is treated by customers and consumers, and Q attributes that easily decay and limit acceptability.
- *Understanding the production process* involves knowing the function of each step to be covered by the Q system. Typical issues that must be discussed include the different process steps, the functions of each step, the measurable technological parameters such as time, temperature, and pressure, the measuring units present, the available analysis techniques and tools, and the way the process is organized (continuous, batch, convergent, divergent, etc.).

In addition, it is necessary to determine the time span of the design project and also assess how the Q system will be continuously reviewed and updated due to changes in the process, product recipe, legislative requirements, and other factors.

7.1.4.2.2 CQP Monitoring System Development

This part concerns the actual quality control system by which the critical product and process parameters that impact final quality attributes are monitored and corrected in the production process. CQP monitoring systems can be based on the principle of a control circle as explained in Section 7.1.3. The following principles must be followed:

- *Identification and evaluation* of product properties and technological conditions that influence desired quality attributes of the end product. A typical question to be answered is: which physical, chemical, biochemical, and physiological processes (food dynamics) and technological conditions may affect a certain Q attribute? For example, compositions and concentrations of carotenoids, lipoxygenase activity, time-and-temperature conditions, light exposure, and oxygen concentration may be critical parameters affecting the orange or yellow color of a product. This step should result in a list of product properties and technological conditions that have measurable and observable influences on final quality.
- *Allocating critical steps* (CQPs). A CQP is a step (point, procedure, operation, or stage in food production) at which measures can be applied, and where control is essential to prevent or reduce quality loss in an end product. Each process step should be analyzed and evaluated to determine whether deviations of the listed product properties and technological conditions may result in unacceptable quality loss of the end product if the step is not controlled (kept within certain tolerances). Typical concerns are how product properties influence observable quality attributes in the end product, how are these properties affected by recipe or formulation (ingredient compositions, precursor levels, enzyme activities) and technological conditions (time, temperature, pressure, packaging conditions, lighting); what is the strength of the relationship, which technological conditions have the most impact (sensitivity analysis), and quality recovery steps.
- *Determination of appropriate measuring and analysis methods*. Decisions must be made on the selection of appropriate and available measuring and or analytical equipment and tools. The characteristics of the methods must be discussed in terms of specificity, accuracy, precision, sensitivity of the methods, and locations of measuring and/or sampling (off-line, at-line, on-line, in-line, non-invasive).
- *Assessment of standards and acceptable tolerances*. Decisions must be made about what is accepted as variation inherent to the overall system (due to common causes), as opposed to deviations that exceed the accepted variation of the system (due to specific causes). Typical concerns are the

desired mean value, the tolerances inherent to the system variables based on statistical analysis, the capability of the process to approach the desired standard within acceptable tolerances, the legislative norms to be met, and customer-specified requirements to be met.

- *Interpretation of test results.* The outcomes of tests are compared with norms and tolerances to determine sizes and directions of deviations. It is important to discuss how operators and QA managers who judge test results should be informed about the interpretation of results and which decision-support systems such as control charts are relevant.
- *Determining content and detail of corrective actions* to clarify which actions should be undertaken in which process situations. Issues of concern are level of differentiating corrective actions, assigning responsibilities for specific corrective actions, and explanations of actions.
- *Development procedures, instructions, guidelines, and registration forms.* These items are mentioned separately because specific procedures, instructions, handbooks, and guidelines must be developed for different CQP principles or steps. However, it is important that all people who work with these tools comply with stated procedures. Therefore it is important to consider whether the procedures and other tools are easily available, understandable, sufficiently detailed, and feasible; whether employees are committed to the procedures; and whether the procedures should be prepared by the parties involved and/or by an external organization.

The willingness to follow the procedures properly has a considerable impact on the actual performance of a monitoring system, in addition to the availability of appropriate analytical tools, methods, and equipment to monitor critical conditions.

7.1.4.2.3 Documentation and Verification System Development

Documentation and verification are two principles that support quality by assuring that food production and management systems are capable of achieving certain quality or safety levels.

- *Designing a documentation system* concerns storage of all established procedures, instructions, guidelines, flow charts, decisions about system design, and all process records so that records can be easily accessed to check process situations, perform certain activities properly, and check system design conditions. *Record keeping* is maintenance of process data needed to control production system performance, for example, information about ingredients used, completed process parameter registration forms, recorded corrective actions, packaging material specifications, etc. Typical considerations for developing such a system are how documents will be made available (online, intranet, physically present at location, personal communication, etc.), how the system can be kept up to date and who is responsible for it, how to assure reliability of information and documents, and who is permitted access to these records.

- *Determination of verification activities* is aimed at identifying and evaluating activities and processes that must be checked to determine whether the CQP-based quality system is working according to design. It is a way to assure that quality requirements will be met by the system. It includes activities such as inspections and audits and the use of classical microbiological and chemical contaminant tests to confirm that the control measures operate as designed.

Typical questions that need to be answered are whether the CQP-based system is well designed (validity), whether activities (procedures, instructions, completion of registration forms) related to the monitoring system are executed properly (verification), and whether conditions (checking temperatures, concentrations, and other quality attributes) of processes are really achieved (production process verification).

7.1.4.3 DECISION SUPPORT IN ASSESSING CQPs

Table 7.1.1 summarizes CQP concept principles and also cites typical tools, methods, and concepts that can be used to support decision making for each principle. We do not explain the techniques in detail, but we have included dedicated references explaining the principles and mechanisms. Some are highlighted below.

- *Understanding quality perception.* The starting point of a CQP-based quality assurance system should be the clear understanding of which product attributes are considered important and contribute to the quality perceptions of target consumers. This will help focus the system to control and assure only those attributes that are critical for quality perception. Decision-supporting tools can be found in the areas of consumer research, sensory studies, and new product design.^{35,36}
- *Understanding the food production system.* After the assessment of attributes considered important by customers or consumers, the next step is to understand how the dynamic product properties and applied technological conditions contribute to these quality attributes. Decisions relate to identifying which product properties and technological conditions are relevant for the specific food production system. What are the mechanistic relationships (kinetics of reaction mechanisms, precursors and agents involved) and to what extent do different food processes contribute to certain quality attributes? For example, color is a quality attribute affected by different product properties and technological conditions that are the dominating processes. To understand these processes, specific knowledge of the food chemistry, biochemistry, and physics is necessary to identify the relevant processes and evaluate them. Typical decision support tools (besides the fundamental knowledge about the mechanisms) are quality function deployment (more specifically house of quality) to systematically evaluate how consumer demands should be translated into product and process specifications,^{5,37} predictive modeling, and Monte Carlo simulations. Predictive modeling can be helpful for

TABLE 7.1.1**CQP Principles and Techniques, and Concepts to Support Decision Making**

Description (What)	Tools, Techniques and Concepts to Support Design Process (How)
1. Basic Preparations	
Determination and organization of appropriate resources	Multidisciplinary team (including people with practical and theory based information) [2]
Understanding final product and its use	Consumer research [35,36], focus group discussions, user data, complaint analysis
Understanding production process	FMEA, Pareto analysis, flow charts [2]
2. CQP Monitoring System Developments	
Identification and evaluation of critical product and process parameters affecting Q attribute	Sensory research [46], house of quality ^a [5,37]
Allocating critical process steps (where)	Predictive modeling [38], Tachugi design principles [2], Monte Carlo simulations to simulate impacts of different product and process conditions on Q attribute level [40]
Determination of appropriate measuring and analysis methods	Accuracy, specificity, precision, sensitivity, velocity, ease of use; off-line, at-line, on-line, in-line, non-invasive [2,48]
Assessment of standards and acceptable tolerances	Process capability indices [49], histograms, statistical analysis of system variation, legislation [4]
Interpretation of test results	Decision-support systems, control charts [50], color charts [17], graphic presentations [2]
Determining content and detail of corrective actions	Risk analysis [2,51]
Development procedures, instructions, registration forms	Compliance analysis [52], understandability and availability [22], organizational analysis
3. Documentation and Verification System Development	
Designing documentation system	Compliance analysis [52], understandability, availability, user friendliness, systems analysis [42–44]
Determination of verification processes	Calibration and maintenance programs [45], challenge tests [2], accelerated shelf life tests [53], auditing [2], sensory tests [46], microbial tests [47]

^a Part of quality function deployment to systematically assess relations between Q attributes and technological specifications.

understanding relationships of process conditions, product properties, and quality attributes without performing laborious and time-consuming experiments.³⁸ Monte Carlo simulations can be used to identify the impacts of specific product and process conditions on final quality attributes — a kind of sensitivity analysis supporting the assessment of critical steps.^{39–41} However, both techniques require basic understanding of mechanistic and/or empiric relationships and require experimental data to validate the modeling and simulations.

- *Understanding the monitoring system.* This requires concepts and techniques to determine the appropriateness of technical parts of the monitoring system: accuracy, specificity, precision and sensitivity of analytical tools, techniques, and measuring units, and locations of measuring and/or sampling (off-line, at-line, on-line, in-line).² Typical managerial decisions are related to the designs of the administrative conditions to support control and assurance activities in practice. This requires concepts or techniques to assess factors influencing compliance with these administrative conditions and understanding the impacts of organizational conditions on behavior.¹⁹
- *Understanding the documentation and verification system.* Typical decisions concern the design features of a documentation and verification system. Tools and techniques are necessary to systematically analyze factors influencing effective use of documentation and information systems.^{42–44} Validation involves deciding how to check system validity (is it doing what it should do?). As shown in Table 7.1.1, typical techniques and concepts with different validation or verification purposes such as auditing, calibration and maintenance programs,⁴⁵ challenge tests, sensory techniques,⁴⁶ and microbial tests⁴⁷ are available.

7.1.4.4 CONCLUDING REMARKS

The CQP concept requires a systematic analysis of underlying mechanisms contributing to final quality attributes like color. This concept can be used to design a quality assurance system designed to assure that a quality system can produce products that comply with quality requirements of customers and/or consumers. In terms of ambiguity and uncertainty, the CQP concept as part of designing a quality assurance system reduces ambiguity by providing insight into underlying mechanisms contributing to specific quality attributes. The concept also reduces uncertainty by collecting information about the performance of the quality assurance system. The effectiveness of CQP-based quality assurance systems is influenced by both managerial and technological factors.

7.1.5 RISK ASSESSMENT CONCEPT

Quantitative risk assessment is now used extensively for determination of chemical and microbial risks in food. This concept helps to systematically and scientifically judge whether certain hazardous compounds may reach unacceptable risk levels when ingested. Quantitative risk assessment can support both quality design and quality assurance; but, we discuss it from the assurance perspective. In the past decade, much attention has been paid to assessment of microbial risks due to their typical differences as compared to chemical risks:

- Microbial risks are mostly due to single exposures (except for microbial toxins); chemical risks are affected by chronic duration of exposure.
- *Responses* to infective pathogens are probably more variable as compared to chemical agents due to different subpopulations and depending on immune status.

- In contrast to chemical agents, micro-organisms are dynamic and can adapt to situations like antibiotic or heat treatment; and risks from specific micro-organisms may change over time.

Chemical contaminants are usually not reduced or removed by processing steps. Chemical risks must preferably be controlled as early as possible in the agri-food chain. Food color additives (Section 7.1.3) are chemical compounds and are considered potential risks. Therefore a safety evaluation is part of the approval of a food colorant before its use is acknowledged by legislation (see also Section 7.1.6). This section explains the principles of risk assessment and includes an example of such an assessment of a specific food colorant.

7.1.5.1 PRINCIPLES OF RISK ASSESSMENT

In order to achieve a uniform approach, the Codex Alimentarius Commission developed guidelines for risk analysis — a process consisting of risk assessment, risk management, and risk communication. Risk assessment is the scientific evaluation of known or potential adverse health effects resulting from human exposure to hazards. It consists of four elements: (1) hazard identification, (2) exposure assessment, (3) hazard characterization, and (4) risk characterization (Figure 7.1.2) and will be discussed in more detail below. Risk management is a process whereby policy alternatives to accept or minimize assessed risk are balanced and appropriate options are implemented. The aim of risk management is to identify acceptable risk levels and implement control options to comply with public health policy. Risk communication is an interactive process of information and opinion exchanges among risk assessors, risk managers, and other interested parties. Food colorants are classified as chemical compounds and we therefore focus on typical chemical-related aspects of risk assessment.

7.1.5.1.1 Hazard Identification

Hazard identification is the first step of risk assessment and includes the identification of known or potential health effects associated with a particular biological, physical, or chemical agent. Hazards can be identified by a systematic evaluation of each step in food manufacturing (up to consumption), whereby information and data must be collected from relevant sources such as scientific literature, food industry databases, and government agencies. Typical data can originate from surveillance, clinical studies, laboratory animal, epidemiological, and microbiological studies.^{2,55–59} Figure 7.1.3 shows a systematic approach to identifying hazards developed by the Scientific Steering Committee advising the European Commission.⁵⁸

7.1.5.1.2 Exposure Assessment

Exposure assessment is the qualitative and/or quantitative estimation of the probable intake of a biological, physical, or chemical agent through food, and exposure from other relevant sources. Only intakes of toxicologically significant amounts can lead

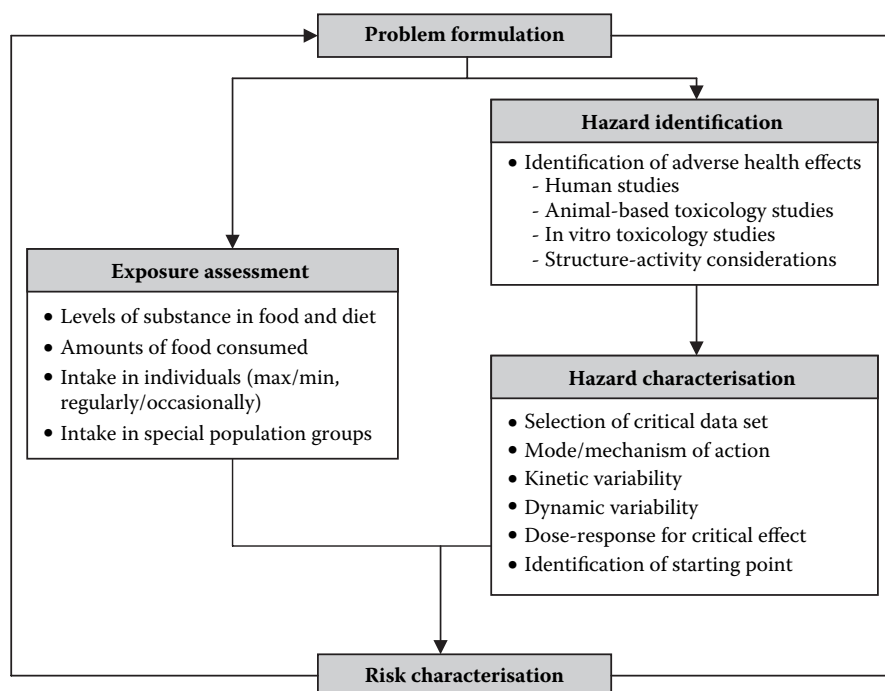


FIGURE 7.1.2 Four parts of risk assessment and required information. (Source: Renwick, A.G. et al., *Food Chem. Toxicol.*, 41, 1211, 2003. With permission.)

to adverse health effects. In the case of chemicals in foods, this exposure assessment should be based on three major issues:⁶⁰

- Quantitative determination of the *possible presence* of a chemical in individual foods and diets. This determination should consider all factors that may influence the level and characteristics of the potentially risky chemical compound throughout the food production chain.
- Determination of *potential intake* by analyzing consumption patterns of individual foods containing the relevant chemical. This aspect should evaluate how much of a food containing the relevant substance is consumed, with particular attention paid to risk groups.
- Integration of the likelihood that consumers will occasionally or regularly eat certain amounts of contaminated foods and the chance that the hazardous chemical is present in such foods at certain levels.

In order to determine the *likely contaminations* of chemicals in foods for consumption, all factors that may impact the final level should be considered systematically: initial concentrations, effect of each individual step in food production on final chemical levels, and potential reactions with other agents.

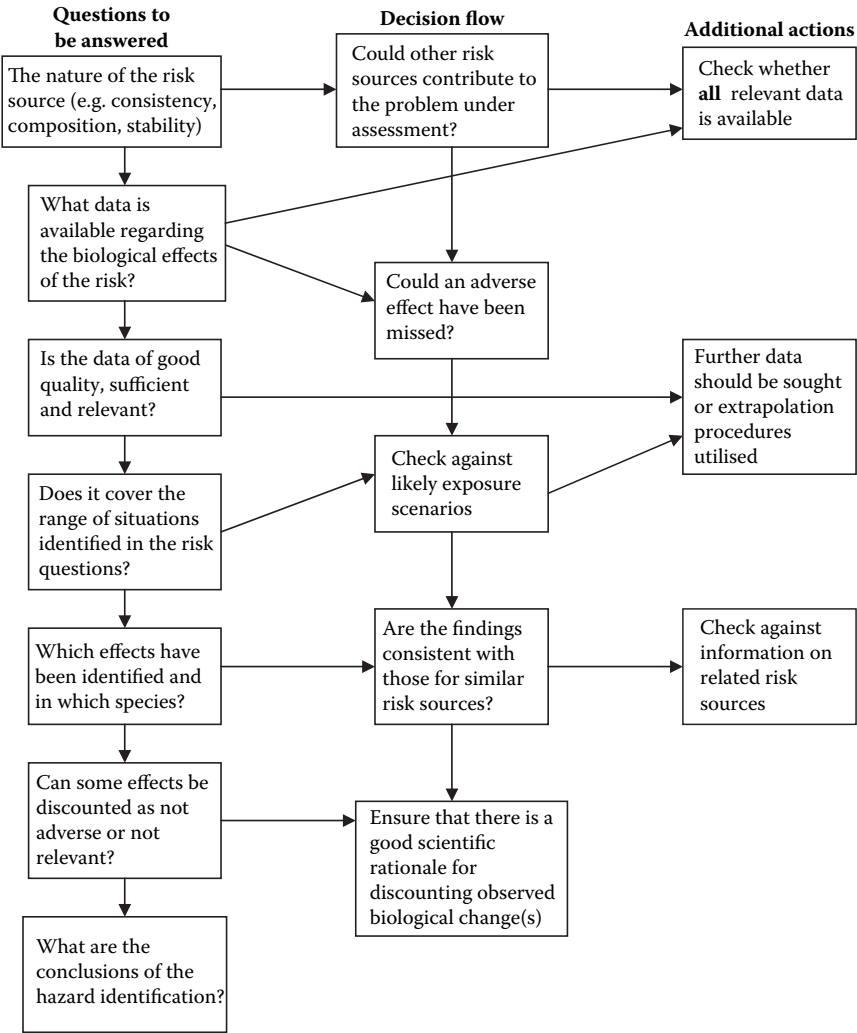


FIGURE 7.1.3 Process of hazard identification, (*Source:* SSC, First report on the harmonisation of risk assessment procedures, Part 1, October 2000.⁵⁸)

Food additives (like color additives) can be added at various processing stages, but the additions are commonly made at the latest production stage before final marketing to ensure optimal functionality of the additive in the product as sold. Residues of additives used at earlier production stages may be present still in the final formulation. However, if they do not have further functions at that stage, they are considered process aids (to help preparation but without technological effects in the finished product) and are not indicated on labels.⁶⁰ Some additives also decompose over time so that the levels present at the end of shelf life may be quite low. Several techniques and information sources can be used to assess contamination levels of chemicals:

1. Collection of data on chemical prevalence and reaction mechanisms in foods
2. Challenge testing to estimate stabilities of chemicals in specific foods
3. Historical data from food process studies
4. Examination of foods to assess typical concentrations of chemicals
5. Mathematical modeling to predict stability of chemicals due to environmental conditions
6. Market basket surveys
7. Body burden and excretion measurements^{61,62}

The choice of method will depend on what information is available and how accurate and detailed the estimate must be.⁶³

In order to determine the *potential intake*, information about portion size and consumption patterns is needed.^{61,64} Typical information that can be useful for this analysis includes:

- Consumer preferences and behavior studies that may provide information on choices and amounts of food intake
- Average portion size consumed
- Demographic data such as age distribution, susceptible groups, etc.
- Socio-economic and cultural data that may provide information about consumption patterns
- Cohort study data on population dietary exposure, dietary data from case-control studies and food consumption surveys
- Food and/or component disappearance figures
- Weights and analyses of foods^{2,62}

Data concerning use patterns of food additives and color additives are difficult to obtain. Although additives must be included on product labels in descending order of inclusion, major effort is required to evaluate even a simple presence on this basis, which would provide at best only limited information on the amounts used. In most cases, quantitative analytical controls are limited to efforts by control authorities to determine compliance with legal limits. Levels below these limits are of limited interest and are usually not published.⁶⁰

In this phase of the risk assessment, the validity and reliability of conclusions and advice to risk managers depend on the quality, reliability, and relevance of available exposure data.⁵¹ Therefore it is necessary to (1) critically review the facts from food composition tables and the reasons for differences reported by and within countries, (2) consider the way foods are categorized and thus made comparable (or not) in food consumption surveys, and (3) explore how to refine assessments as more information becomes available.⁶⁰

Risk assessors tend to build in additional uncertainty factors to avoid health-relevant underestimates. This is partly done by using screening methods designed to look for “worst case” situations. Such worst case assumptions lead to intake estimates that exceed reality. For chemicals that present potential risks, more information is needed to allow more refined screening or even the most accurate estima-

tion. Kroes and co-authors⁶⁰ suggested options for improvements, for example, the development of a pan-European food composition database, activities to better explain effects of processing on individual food chemicals, harmonization of food consumption survey methods with the option of a regular pan-European survey, evaluation of probabilistic models, and the development of models to assess exposure to food allergens.

7.1.5.1.3 Hazard Characterization

Hazard characterization is a quantitative or semi-quantitative evaluation of the nature, severity, and duration of adverse health effects associated with biological, physical, or chemical agents that may be present in food. The characterization depends on the nature of the toxic effect or hazard. For some hazards such as genotoxic chemicals, there may be no threshold for the effect and therefore estimates are made of the possible magnitude of the risk at human exposure level (dose–response extrapolation).

In contrast, other hazards such as non-genotoxic effects present a threshold of exposure below which no biologically significant effect will be revealed. Both methods usually involve the uncertainties of extrapolating from high-dose animal studies to low-dose human exposure, and from small groups of genetically homogeneous animals to the larger and more diverse human population.⁵⁹ Typical characteristics of chemical compounds that must be considered are mutagenicity, carcinogenicity, toxicity, allergy, intolerance, and decomposition rate.⁶¹

Stages in hazard characterization according to the European Commission's Scientific Steering Committee⁵⁸ are (1) establishment of the dose–response relationship for each critical effect; (2) identification of the most sensitive species and strain; (3) characterization of the mode of action and mechanisms of critical effects (including the possible roles of active metabolites); (4) high to low dose (exposure) extrapolation and interspecies extrapolation; and (5) evaluation of factors that can influence severity and duration of adverse health effects.

Dose–response assessment is the process of obtaining quantitative information about the probability of human illness following exposure to a hazard; it is the translation of exposure into harm.⁶¹ Dose–response curves have been determined for some hazards. The curves show the relationship of dose exposure and the probability of a response. Since validated dose–response relationships are scarce, various other inputs are used to underpin the hazard characterization phase of risk assessment.

NOAEL (no-observed-adverse-effect level) is defined as the highest dose at which no adverse effects are observed in the most susceptible animal species. The NOAEL is used as a basis for setting human safety standards for acceptable daily intakes (ADIs), taking into account uncertainty factors for extrapolation from animals to humans and inter-individual variabilities of humans.⁶⁵ The adequacy of any margin of safety or margin of exposure must consider the nature and quality of the available hazard identification and dose–response data and the reliability and relevance of the exposure estimations.⁵⁹ In some cases, no adverse endpoint can be identified such as for many naturally occurring compounds that are widespread in foods. In that case, an “ADI Not Specified” is assigned.⁶⁵

Benchmark dose (BMD) is an alternative method of defining an intake. The value is derived by modeling experimental data in the observed range and selecting the 95th percentile lower confidence limit of the dose causing a particular incidence of the effect.⁶⁶ The BMD is by definition greater than the threshold. Advantages of BMD are that it rewards good dose–response data because it is associated with narrower confidence intervals.⁵⁸

For chemicals, the severity of any adverse effect associated with a chemical used as a food additive is usually directly related to the dose. Severity can be measured as either the degree of damage to an individual or the probability that an individual will be affected, or a combination of these effects.^{51,65} For most chemicals, hazard characterization is based on estimations of human intake that would be below the dose necessary to produce adverse effects. For substances that can cause tissue damage, increasing the dose will tend to increase the degree of damage. For carcinogens, where only one molecule has the theoretical ability to induce a tumor, increasing the dose increases the probability that an individual will contract a tumor.^{51,65}

Hazard characterization of low molecular weight chemicals in food and diet generally uses a NOAEL or BMD as a starting point. For hazards considered to have no thresholds for their modes of action, low dose extrapolation and other modeling approaches may be applied.^{51,67,68} Since food can be considered as an extremely complex and variable chemical mixture, interactions among low molecular weight chemicals are expected to be rare because exposure levels generally are far below their NOAELs.⁶⁷

7.1.5.1.4 Risk Characterization

Risk characterization is the last step in the risk assessment procedure. It is the quantitative or semi-quantitative estimation, including uncertainties, of frequency and severity of known or potential adverse health effects in a given population based on the previous steps.⁵⁸ Risk characterization is the step that integrates information on hazard and exposure to estimate the magnitude of a risk.⁵¹ Comparison of the numerical output of hazard characterization with the estimated intake will give an indication of whether the estimated intake is a health concern.^{51,65}

The degree of confidence in the final estimation of risk depends on variability, uncertainty, and assumptions identified in all previous steps. The nature of the information available for risk characterization and the associated uncertainties can vary widely, and no single approach is suitable for all hazard and exposure scenarios.⁵¹ In cases in which risk characterization is concluded before human exposure occurs, for example, with food additives that require prior approval, both hazard identification and hazard characterization are largely dependent on animal experiments. And exposure is a theoretical estimate based on predicted uses or residue levels. In contrast, in cases of prior human exposure, hazard identification and hazard characterization may be based on studies in humans and exposure assessment can be based on real-life, actual intake measurements.⁵¹ The influence of estimates and assumptions can be evaluated by using sensitivity and uncertainty analyses.^{61,64} Risk assessment procedures differ in a range of possible options from relatively unso-

phisticated default approaches for sparse data sets to sophisticated dose–response modeling for data-rich compounds.⁵⁹

7.1.5.2 RISK ASSESSMENT OF LUTEIN FROM *TAGETES ERECTA* L.

Lutein is a naturally occurring xanthophyll pigment and an oxygenated carotenoid. It occurs along with the zeaxanthin isomeric xanthophyll in many foods, particularly vegetables and fruits. It is used as a food color additive and nutrient supplement in a wide range of applications at concentrations ranging from 2 to 330 mg/kg. Lutein is a purified extract from marigold (*Tagetes erecta* L.) oleoresin. The final product contains lutein as the major component and a smaller proportion of zeaxanthin. The Food and Agriculture Organization of the World Health Organization's Expert Committee on Food Additives prepared new specifications for lutein and evaluated its safety.⁶⁹

7.1.5.2.1 Hazard Identification

In rats, peak concentrations of radioactivity in plasma and tissues occurred about 4 hr after single oral doses of ¹⁴C lutein. Most of the radiolabel was eliminated via the feces in about 2 days; very low urinary and biliary excretions indicated poor absorption from the intestinal tract. Based on fecal excretion data, the absorption of lutein was about 30 to 40% when administered to rats in the diet as beadlets containing vitamin E (the beadlet formulation was used to enhance the stability of lutein). Ten-fold increases in dose in the range of 2 to 200 mg/kg body weight resulted in two- to three-fold increases in plasma concentrations, indicating reduced absorption at higher doses. Steady-state plasma concentrations of lutein were reached in about 3 days after the start of dietary administration, indicating a half-life of about 1 day.

In humans, peak plasma or serum concentrations of lutein occurred 11 to 16 hr after administration of a single oral dose. During daily supplementation with 20 mg of lutein, steady-state plasma concentrations were reached in about 30 days. This is consistent with an elimination half-life of 5 to 7 days.

The food matrix including its fiber and lipid content and concentrations of other carotenoids in the diet may influence the extent of absorption of carotenoid compounds. The relative absorption of lutein from a mixed vegetable diet was lower than from a diet containing pure lutein. A mixed preparation of lutein and zeaxanthin did not influence the absorption of β -carotene.

Lutein has an oral median lethal dose (LD₅₀) of >2000 mg/kg body weight in rats. In a 13-week study, oral doses of lutein of up to 200 mg/kg body weight, the highest dose tested, caused no treatment-related effects. In a 52-week study designed primarily to investigate possible adverse effects on the eyes of monkeys, lutein was administered by gavage at doses of 0.2 or 20 mg/kg body weight per day. This study was performed because adverse ocular effects were seen with canthaxanthin. No treatment-related effects were observed on a wide range of toxicological end points. Furthermore, comprehensive ophthalmic examinations including electroretinography showed no evidence of treatment-related adverse changes.

No long-term studies of toxicity or carcinogenicity were undertaken.

Lutein produced negative results in several studies of genotoxicity *in vitro* and *in vivo*. Although the committee noted that the doses used in these tests were low, it recognized that maximum feasible doses were used. No evidence of tumor-promoting activity was noted in animal models.

In a study of developmental toxicity of lutein in rats, no evidence indicated toxicity at doses up to 1000 mg/kg body weight per day, the highest dose tested.

In a 20-week multicenter intervention trial with lutein in healthy human subjects, no changes were noted in hematological or biochemical parameters after continuous daily lutein doses of 15 mg (0.25 mg/kg body weight, assuming a body weight of 60 kg). A relatively large number of human studies have examined correlations between macular degeneration and dietary intake of lutein or zeaxanthin, intakes via dietary supplements, and serum concentrations.

Although these studies were designed to look for ocular effects, no adverse effects of these xanthophylls were reported where clinical or biochemical parameters were also examined.

7.1.5.2.2 Exposure Assessment

Dietary intake data from a number of studies in North America and the United Kingdom indicate that intake of lutein from natural sources is in the range of 1 to 2 mg/day (approximately 0.01 to 0.03 mg/kg body weight per day). Simulations considering proposed levels of use as a food ingredient resulted in an estimated mean and 90th percentile of intake of lutein plus zeaxanthin of approximately 7 and 13 mg/day, respectively. Formulations containing lutein and zeaxanthin are also available as dietary supplements, but no reliable estimates of intakes from these sources were available.

7.1.5.2.3 Hazard Characterization

In several studies of toxicity, no adverse effects were documented in monkeys or humans. Taking into account data showing that lutein was not genotoxic, had no structural alert, did not exhibit tumor-promoting activity, and is a natural component of the body (the eye), the Scientific Steering Committee concluded there was no need for a study of carcinogenicity.

Lutein has some structural similarities to β -carotene, reported to enhance the development of lung cancer when given in supplement form to heavy smokers. The available data indicate that lutein in food would not be expected to have this effect. The committee was unable to assess whether lutein in the form of supplements would produce the reported effect in heavy smokers.

A 52-week study in monkeys was designed to evaluate ocular effects. Despite the absence of adverse toxicological effects at the highest dose tested (20 mg/kg body weight per day), the study was considered inappropriate for the establishment of an ADI in view of the much higher doses used in several other studies and found to be without effects. The available comparative toxicokinetic data for humans and rats indicated that studies of toxicity in rats could be used to derive an ADI.

The committee concluded that the best evaluation for this purpose was a 90-day study in rats. An ADI of 0 to 2 mg/kg body weight was allocated based on a NOEL of 200 mg/kg body weight per day (the highest dose tested in the study) and a safety factor of 100. Although the ADI was based on the results of a short-term study, the supporting data and lack of effects at much higher doses in some studies (e.g., a study of developmental toxicity) indicated that the safety factor of 100 was appropriate.

7.1.5.2.4 Risk Characterization

In view of the toxicological data and structural and physiological similarities of the lutein and zeaxanthin xanthophylls, the committee decided to include zeaxanthin in the ADI (0 to 2 mg/kg body weight) for lutein, which had a stronger toxicological database, and specify a group ADI for these two substances. The group ADI does not apply to other xanthophyll-containing extracts with lutein or zeaxanthin contents lower than the level cited in the specifications.

7.1.5.3 CONCLUDING REMARKS

The concept of risk assessment requires a profound understanding of food dynamics and technological conditions that may impact the risk levels of certain hazardous compounds. It requires that scientific information and data are collected to underpin conclusions about risk levels. Risk assessment can be used to scientifically underpin the selection of hazards that must be covered by a quality or safety assurance system (e.g., HACCP) that will improve the reliability of the system.

In terms of ambiguity and uncertainty, the risk assessment concept as part of designing a quality assurance system reduces ambiguity by providing information about underlying mechanisms contributing to food safety. Food colorants should be analyzed according to the risk assessment requirements of chemical compounds. The reliability, relevance, and validity of the outcome of a risk assessment depend greatly on the available information used to execute the assessment.

7.1.6 INTERNATIONAL LEGISLATION REGARDING FOOD COLORANTS

Food legislation is an important issue that must be concerned with food quality management. The government influences decision-making behavior in food quality management along the lines of rules and procedures that can be seen as conditioning and inspection that serve as forms of intervention in decision-making processes.

Since food colorants are used as food additives, they must also comply with legislative requirements. Food legislation in the European Union (EU) and the United States (US) differs with regard to additives and labeling. Therefore, this section provides an overview of legislation in the EU (Section 7.1.6.1) and the US (Section 7.1.6.2) and discusses colorants permitted for use in food products according to the different requirements.

7.1.6.1 EUROPEAN UNION LEGISLATION

Food additives are authorized at the EU level for all 15 member states, as well as for Norway and Iceland.³⁰ The use of food additives is controlled by legislation that is harmonized across the EU. Domestic legislation in each member state is based on the various additive directives incorporated into specific national legislation.²⁹ The community legislation on food additives consists of the following directives:

Council Directive 89/107/EEC, as amended by Directive 94/34/EC³⁰ — This framework directive provides “umbrella” legislation under which the individual additives directives are developed. It includes a definition of a food additive, exclusions from the scope of the definition, and a list of food additive categories, one of which is colors.^{29,70} Additionally, general criteria for use of food additives are described.⁷⁰

European Parliament and Council Directive 94/36/EC — This directive provides detailed rules on colors. Schedules attached to these EU regulations set out the following lists^{29,71,72}:

- Permitted colors (e.g., E 100, curcumin; E 160a, carotenes; E 163, lutein)
- Basic foodstuffs to which colors must not be added (milk, flour, bread, sugar, tea, coffee)
- Foodstuffs in which only a limited list of colors may be used (margarine to which E 160a and E 100 may be added *quantum satis*; E 160b, maximum 10 mg/kg)
- Colors that have restricted applications (e.g., E 173 may be used for external coating of sugar confectionery and for decoration of cakes and pastries *quantum satis*)
- Colors permitted generally *quantum satis* (E 101, E 140, E 160a, E 163) and colors with maximum inclusion levels for particular food categories (e.g., E 160d may not exceed 100 mg/l in non-alcoholic flavored drinks)

Commission Directive 95/45/EC, as amended by Directive 99/75/EC for colors — This specifies in detail that authorized food colors must fulfill purity criteria.³⁰

The community legislation on food additives is based on the principle that only additives that are explicitly authorized may be used. Food additives may only be authorized if their use fills a technological need, they do not mislead consumers, and they present no health hazards to consumers. Most food additives can only be used in limited quantities in certain foodstuffs. If no quantitative limits are foreseen for the use of a food additive, it must be used according to good manufacturing practices, i.e., only as much as necessary to achieve the desired technological effect.³⁰

Prior to authorization, food additives are evaluated for their safety by the Scientific Committee on Food (SCF), an expert panel that advises the European Commission on questions relating to food.³⁰ In evaluating a food color additive, the SCF allocates an acceptable daily intake (ADI), the amount of a specific color that the committee considers may be consumed safely every day throughout a lifetime. Only food colors evaluated in this way are given E numbers. The number is an indication of European safety approval and a simple and convenient way to label permitted food colors across the range of languages in the EU.⁷³

Color additives are defined by regulations. By exclusion, foodstuffs that have coloring properties (spices, spinach, malt extract) are not classified as colors. This area has posed some difficulties of definition where extracts of coloring foodstuffs are concerned, but the key factor is whether selective extraction has occurred. If such extracts retain other major components such as flavoring, they remain foodstuffs and are not considered additives. If, however, the color is concentrated at the expense of removing the other components, the resultant material becomes a color.²⁹ Detailed rules on labeling of additives in foodstuffs and on additives sold as such to food producers and consumers are covered by community legislation (Directive 2000/13/EC, Regulation 50/2000/EC, and Directive 89/107/EEC).³⁰

Colors used in food products, like other additives, must be declared on ingredients lists by category name (“color”) along with the name of the color or the E number. Colors present in mixtures with other additives (e.g., flavors) are not excluded from this requirement and must be declared because they will have a coloring function in the final product.²⁹

7.1.6.2 UNITED STATES LEGISLATION

US legislation on food additives consists of the Federal Food, Drug, and Cosmetic Act (FD&C Act), the Fair Packaging and Labeling Act, and other applicable laws including the Public Health Security and Bioterrorism Preparedness and Response Act.³¹ The FD&C Act states that foods are adulterated if they contain color additives that have not been approved as safe to the satisfaction of the Food & Drug Administration (FDA) for a particular use.⁷⁴

The use of food colors is governed by the *Code of Federal Regulations* (CFR), a compilation divided into 50 titles.²⁹ The FDA established regulations for color additives in Title 21 of the CFR, in parts 70 through 82. The parts cover different issues concerning color additives.^{31,32}

Part 70: Color additives — This part includes a definition of a color additive, restrictions on use of color additives, packaging and labeling requirements, and safety evaluation of color additives.

Part 71: Color additive petitions — These regulations describe the pre-market approval process for new color additives or new uses for listed color additives.³¹

Part 73: Listing of color additives exempt from certification — This part identifies listed color additives exempt from certification, provides chemical specifications for these color additives, and identifies uses, restrictions, labeling requirements, and requirements for certification.

Part 74: Listing of color additives subject to certification — This part identifies listed color additives subject to certification, provides chemical specifications for them, and identifies uses and restrictions, labeling requirements, and requirements for certification.

Part 80: Color additive certification — This part covers fees for certification services and certification procedures.

Part 81: General specifications and general restrictions for provisional color additives for use in foods, drugs, and cosmetics — This part contains provisional

lists of color additives, the termination dates of these lists, and information about cancellation and limitation of certificates.

Part 82: Listing of certified provisionally listed colors and specifications —

This part identifies listed color additives, provides chemical specifications for the color additives, and identifies uses and restrictions.

The FDA provides regulatory oversight of color additives used in foods, drugs, cosmetics, and medical devices. It is also concerned with certain issues related to food colorants and publishes lists of new color additives and new uses for listed color additives shown to be safe for their intended uses in the CFR. The agency also conducts certification programs for batches of color additives that must be certified before sale and monitors the use of color additives and labeling of products in the United States. The use of an unlisted color additive, the improper use of a listed color additive, or the use of a color additive that does not conform to the purity and identity specifications of the listing regulation may cause product adulteration under the provisions of the FD&C Act.³¹ Color additives fall into two categories:²⁹

Certified color additives (FD&C colors) are synthetically produced organic molecules whose purities have been checked by the FDA.

Colorants exempt from certification are derived from animal, vegetable, or mineral origin or are synthetic duplicates of naturally existing colors. They contain complex mixtures of numerous components and are listed in 21 CFR, part 73.

From a regulatory view, there is no such thing as a natural color, although it is generally accepted that colorants exempt from certification are usually naturally derived.²⁹

Additional regulations that provide specific requirements for color additives in foods are found in other parts of the CFR. Labeling of food products is found at 21 CFR 101.22(k). Color additives are sometimes called artificial colors or artificial colorings [21 CFR 101.22(a) (4)]. From a regulatory standpoint, a *colorant* is a dye or pigment used in a food contact material such as a polymer that does not migrate to food. Such materials are regulated as food additives [21 CFR 178.3297(a)], not as color additives.³¹

Use of the *natural color* and *food color* terms is not permitted because they may indicate that a color occurs naturally. The acceptable descriptions include *artificial color*, *artificial color added*, and *color added* but they do not provide any real benefit. A preferred description is to note that a product is *colored with*, then name the color source, e.g., annatto. If the name of the specific color is not included, the label declaration must also state *artificially colored* or *artificial color added*.²⁹

If a product is to be exported to different countries, an understanding of the different structures and requirements set by the European Union and United States regulatory authorities is critical.

7.1.6.3 CONCLUDING REMARKS

Color is the most obvious characteristic of a food and often predetermines the quality expectations and perceptions of consumers and food handlers who judge food. Color may provide the key to cataloging a food as safe based on good aesthetic and sensory characteristics (Section 7.1.1). A profound understanding of the physical and chem-

ical properties of colorants and the behaviors of people working with food production systems is necessary to effectively manage color quality in food production systems (Section 7.1.2).

In order to control food color, underlying mechanisms causing variation in color must be understood. Three types of colorants can be distinguished from the perspective of quality and safety control: natural colorants, formed colorants, and color additives. Depending on the type of colorant, specific strategies are required to control dynamics of colorants and achieve constant quality in terms of safety, desired color, appearance, and health (Section 7.1.3). The extent to which underlying food color-affecting mechanisms are understood determines how well the quality of food color can be predicted.

The previous chapters contribute to this understanding. A major function in food quality management deals with assuring that certain quality and safety levels (as demanded by consumers, customers, and government) are achieved by designing appropriate quality systems. Within this perspective, the concepts of critical quality points (Section 7.1.4) and risk assessment (Section 7.1.5) have been explained in detail. Both concepts require fundamental insight into the technological and managerial factors that may influence the quality and safety of colors in food systems.

Along with the principles of CQP and risk assessment, typical managerial issues determining the performance of quality and safety assurance systems have been explained (Section 7.1.4). Finally, the structures and principles of legislation related to food colorants are described (Section 7.1.6). Legislation sets clear requirements on acceptable food color levels from a safety perspective and it controls levels to be used in practice. In the beginning of this chapter, we stated that food quality management is complex (Section 7.1.2), which is underpinned by the complex mechanisms underlying behavior of food colorants and unpredictable behavior of food handlers in food production. Although a certain level of unpredictability must be accepted, thorough knowledge of the underlying mechanisms cited in this book, and increasing information obtained by appropriate monitoring and assuring systems will positively contribute to reducing levels of unpredictability (chaos).

REFERENCES

1. Ropkins, K. and Beck, A.J., Evaluation of worldwide approaches to the use of HACCP to control food safety, *Trends Food Sci. Technol.*, 11, 10, 2000.
2. Luning, P.A., Marcelis, W.J., and Jongen, W.M.F., *Food Quality Management: A Techno-Managerial Approach*, Wageningen Pers, Wageningen, 2002.
3. Van der Spiegel, M., Measuring effectiveness of food quality management, PhD thesis, Wageningen University, Wageningen, 2004.
4. Van der Meulen, B. and Van der Velde, M., *Food Safety Law in the European Union: An Introduction*, Wageningen Academic Publishers, Wageningen, 2004.
5. Costa, A.I.A., Dekker, M., and Jongen, W.M.F., Quality function deployment in the food industry: a review, *Trends Food Sci. Technol.*, 11, 306, 2000.
6. Jongen, W.M.F. and Meulenbergh, M.T.G., *Innovation of Food Production Systems: Product Quality and Consumer Acceptance*, Wageningen Academic Publishers, Wageningen, 2005.

7. Van Kleef, E., Van Trijp, H.C.M., and Luning, P., Consumer research in the early stages of new product development: a critical review of methods and techniques, *Food Qual. Pref.*, 16, 181, 2005.
8. Van Trijp, J.C.M. and Steenkamp, J.E.B.M., Consumer-oriented new product development: principles and practice, in *Innovation of Food Production Systems*, Jongen, W.M.F. and Meulenberg, M.T.G., Eds., Wageningen Pers, Wageningen, 1998, 37.
9. Luning, P.A. and Marcelis, W.J., A food quality management functions model, *Trends Food Sci. Technol.*, 18, 159, 2007.
10. Delgado-Vargas, F., Jiménez, A.R., and Paredes-López, O., Natural pigments: carotenoids, anthocyanins, and betalains: characteristics, biosynthesis, processing, and stability, *Crit. Rev. Food Sci. Nutr.*, 40, 173, 2000.
11. Griffiths, J.C., Coloring foods and beverages, *Food Technol.*, 59, 38, 2005.
12. Clydesdale, F.M., Color as a factor in food choice, *Crit. Rev. Food Sci. Nutr.*, 331, 83, 1993.
13. Miettinen, S.M. et al., Effect of emulsion characteristics on the release of aroma as detected by sensory evaluation, static headspace gas chromatography, and electronic nose, *J. Agric. Food Chem.*, 50, 4232, 2002.
14. Kvenberg, J.E. and Schwalm, D.J., Use of microbial data for hazard analysis and critical control point verification: food and drug administration perspective, *J. Food Prot.*, 63, 810, 2000.
15. Luzuriaga, D.A., Balaban, M.O., and Yeralan, S., Analysis of visual quality attributes of white shrimp by machine vision, *J. Food Sci.*, 62, 113, 1997.
16. Boyles, M.J. and Wrolstad, R.E., Anthocyanin composition of red raspberry juice: influences of cultivar, processing, and environmental factors, *J. Food Sci.*, 58, 1135, 1993.
17. Van Rossum, A., Decision-making during visual measurement: a techno-managerial approach of the decision-making process of individuals during visual measurement at critical quality control points, MSc thesis, Wageningen University, Wageningen, 2005.
18. Walker, E., Pritchard, C., and Forsythe, S., Food handlers' hygiene knowledge in small food businesses, *Food Control*, 14, 339, 2003.
19. Luning, P.A. and Marcelis, W.J., A techno-managerial approach in food quality management research, *Trends Food Sci. Technol.*, 17, 378, 2006.
20. Efstratiadis, M.M., Karirti, A.C., and Arvanitoyannis, I.S., Implementation of ISO 9000 to the food industry: an overview, *Int. J. Food Sci. Nutr.*, 51, 459, 2000.
21. Luning, P.A. and Marcelis, W.J., A stepwise food quality management research methodology, *NYAS*, 2007.
22. Bango, L., Quality behaviour along the international fruit supply chain, MSc thesis, Wageningen University, Wageningen, 2005.
23. Downham, A. and Collins, P., Coloring our foods in the last and next millennium, *Int. J. Food Sci. Technol.*, 35, 5, 2000.
24. Francis, F.J., Food colorants: anthocyanins, *Crit. Rev. Food Sci. Nutr.*, 28, 273, 1989.
25. Fay, L.B. and Brevard, H., Contribution of mass spectrometry to the study of the Maillard reaction in food, *Mass Spectr. Rev.*, 24, 487, 2005.
26. Martins, S.I.F.S., Jongen, W.M.F., and van Boekel, M.A.J.S., A review of Maillard reaction in food and implications to kinetic modelling, *Trends Food Sci. Technol.*, 11, 364, 2000.
27. Manzocco, L. et al., Review of non-enzymatic browning and antioxidant capacity in processed foods, *Trends Food Sci. Technol.*, 11, 340, 2000.
28. Yaylayan, V.A., Classification of the Maillard reaction: a conceptual approach, *Trends Food Sci. Technol.*, 8, 13, 1997.

29. Downham, A. and Collins, P., Coloring our foods in the last and next millennium, *Int. J. Food Sci. Technol.*, 35, 5, 2000.
30. European-Commission, Food additives and flavorings, retrieved January 9, 2006, http://europa.eu.int/comm/food/fs/sfp/flav_index_en.html, 2006.
31. Barrows, J.N., Lipman, A., and Bailey, C., Color additives: FDA's regulatory process and historical perspectives, *Food Safety*, Oct–Nov 2003.
32. *Code of Federal Regulations* — Title 21: Color additive regulations, retrieved January 10th, 2006, <http://www.cfsan.fda.gov/~dms/col-cfr.html>, 2001.
33. International Standards Organization, *ISO 9000:2005: Quality Management Systems: Fundamentals and Vocabulary*, 3rd ed., Geneva, 2005.
34. Peters, R.E., The broader application of HACCP concepts to food quality in Australia, *Food Control*, 9, 83, 1998.
35. Wansink, B., Response to “Measuring consumer response to food products”: sensory tests that predict consumer acceptance, *Food Qual. Pref.*, 14, 23, 2003.
36. Van Kleef, E., Consumer research in the early stages of new product development, PhD thesis, Wageningen University, Wageningen, 2006.
37. Benner, M. et al., Quality function deployment (QFD): can it be used to develop food products? *Food Qual. Pref.*, 14, 327, 2003.
38. DeVlieghere, F. et al., Modelling food safety, in *Safety in Agri-food Chains*, Luning, P.A., DeVlieghere, F., and Verhé, R., Eds., Wageningen Academic Publishers, Wageningen, 2006, 397.
39. Dekker, M. and Verkerk, R., Dealing with variability in food production chains: a tool to enhance the sensitivity of epidemiological studies on phytochemicals, *Eur. J. Nutr.*, 42, 67, 2003.
40. Albert-Gonzalez, P., Identification of sources of variation affecting food behaviour: pilot study assessing variations in lycopene content during tomato ketchup production, MSc thesis, Wageningen University, Wageningen, 2004.
41. Gao, Y., Safety assurance of vegetable oil products with respect to cost-benefits balance, MSc thesis, Wageningen University, Wageningen, 2005.
42. Peterson, M., Intranet-based service delivery: making it work, *Electr. Libr.*, 19, 19, 2001.
43. Wind, M. and Schläger, U., *E-Government Manual: Quality Criteria for a Public User-Friendly and Secure Website*, University of Bremen, Bremen, 2002.
44. Dugas, M. et al., Design and implementation of a common drug information database for a university hospital, *Pharm. World Sci.*, 25, 156, 2003.
45. Ilyukhin, S.V., Haley, T.A., and Singh, R.K., A survey of control system validation practices in the food industry, *Food Control*, 12, 297, 2001.
46. Pal, D., Sachdeva, S., and Singh, S., Methods for determination of sensory quality of foods: a critical appraisal, *J. Food Sci. Technol.*, 32, 356, 1995.
47. Maraz, A. et al., Microbial analysis of food, in *Safety in Agri-food Chains*, Luning, P.A., DeVlieghere, F., and Verhé, R., Eds., Wageningen Academic Publishers, Wageningen, 2006, 471.
48. Koutsoumanis, K., Taoukis, P.S., and Nychas, G.J.E., Development of a safety monitoring and assurance system for chilled food products, *Int. J. Food Microbiol.*, 100, 253, 2005.
49. Lin, H.C. and Sheen, G.J., An approximation approach for making decisions in assessing the capability index C-pk from the subsamples, *Comm. Stat. Sim. Comp.*, 34, 191, 2005.

50. Banens, P.J.A. et al., Control charts and stability, in *Industriële statistiek en kwaliteit*, Banens, P.J.A., Does, R.J.M.M., and Van Dongen, G.B.W., Eds., Kluwer, Deventer, 1994, 432.
51. Renwick, A.G., Risk characterization of chemicals in food, *Toxicol. Lett.*, 149, 163, 2004.
52. Henson, S. and Heasman, M., Food safety regulation and the firm: understanding the compliance process, *Food Policy*, 23, 9, 1998.
53. Ellis, D.I. and Goodacre, R., Rapid and quantitative detection of the microbial spoilage of muscle foods: current status and future trends, *Trends Food Sci. Technol.*, 12, 414, 2001.
54. Renwick, A.G. et al., Risk characterization of chemicals in food and diet, *Food Chem. Toxicol.*, 41, 1211, 2003.
55. Barlow, S.M. et al., Hazard identification by methods of animal-based toxicology, *Food Chem. Toxicol.*, 40, 145, 2002.
56. Eisenbrand, G. et al., Methods of *in vitro* toxicology, *Food Chem. Toxicol.*, 40, 193, 2002.
57. Van den Brandt, P. et al., The contribution of epidemiology, *Food Chem. Toxicol.*, 40, 387, 2002.
58. SSC, First report on the harmonisation of risk assessment procedures. Part 1. The report of the Scientific Steering Committee's working group on harmonisation of risk assessment procedures, October 26–27, 2000.
59. SSC, First report on the harmonisation of risk assessment procedures. Part 2. Appendices. The report of the Scientific Steering Committee's working group on harmonisation of risk assessment procedures, October 26–27, 2000.
60. Kroes, R. et al., Assessment of intake from the diet, *Food Chem. Toxicol.*, 40, 327, 2002.
61. SCF (Scientific Committee for Food), Principles for the development of risk assessment of microbiological hazards for the development of hygiene rules for foodstuffs as covered by the hygiene of foodstuffs directive 93/43/EEG. CS/FMH/CRIT/2 Rev 1, 1996.
62. DiNovi, M.J. and Kuznesof, P.M. 2001. Estimating exposure to direct food additives and chemical contaminants in the diet, Retrieved November 29, 2005, <http://vm.cfsan.fda.gov/~dms/opa-cg8.html>.
63. Parmar, B., Miller, P.F., and Burt, R., Stepwise approaches for estimating the intakes of chemicals in food, *Regul. Toxicol. Pharmacol.*, 26, 44, 1997.
64. Codex Alimentarius Commission, A joint FAO/WHO food standards programme. Report of 31st session of the Codex Committee on Food Hygiene, Alinorm 99/13A, 1998.
65. Tennant, D.R., Risk analysis of food additives, in *Food Chemical Safety, Volume 2: Additives*, Watson, D.H., Ed., Woodhead Publishing, Cambridge, U.K., 2002, 61.
66. Barnes, D.G. et al., Benchmark dose workshop: criteria for use of a benchmark dose to estimate a reference dose, *Regul. Toxicol. Pharmacol.*, 21, 296, 1995.
67. Dybing, E. et al., Hazard characterization of chemicals in food and diet: dose response, mechanisms and extrapolation issues, *Food Chem. Toxicol.*, 40, 237, 2002.
68. Edler, L. et al., Mathematical modelling and quantitative methods, *Food Chem. Toxicol.*, 40, 283, 2002.
69. FAO/WHO, Evaluation of certain food additives, 63rd report of Joint FAO/WHO Expert Committee on Food Additives, WHO Technical Report Series 928, 2005.

70. Council of European Communities, Directive 89/107/EEC, December 21, 1988, on the approximation of the laws of the Member States concerning food additives authorised for use in foodstuffs intended for human consumption, 1988.
71. European Parliament and Council of the European Union, Directive 94/36/EC, June 30, 1994, on colors for use in foodstuffs, *Off. J. Eur. Comm.*, 1994.
72. Flowerdew, D.W., The regulation of additives in the EU, in *Food Chemical Safety, Volume 2: Additives*, Watson, D.H., Ed., Woodhead Publishing, Cambridge, U.K., 2002, 12.
73. Food-Info, 2006. How are colors regulated in the EU? Retrieved January 10, 2006, <http://www.food-info.net/uk/qa/qa-fi11.htm>.
74. Curtis, P., The regulation of additives in the USA, in *Food Chemical Safety, Volume 2: Additives*, Watson, D.H., Ed., Woodhead Publishing, Cambridge, U.K., 2002, 12.

7.2 Natural Pigments as Food Colorants

Carmen Socaciu

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7.2.1 INTRODUCTION

Coloration of food, either with natural or synthetic color additives, should indicate good quality, assist marketing, and satisfy consumers.¹⁻³ Colorants are added to food matrices in specific technological steps in order to obtain and maintain the appropriate desired colors of food products. Colorants are also used to restore natural food colors lost by exposure to air, light, temperature, moisture, or improper storage conditions.^{4,5}

Food colorants can also provide appropriate color to colorless foods, protect flavors and vitamins (due to their antioxidant potential) during storage, or enhance the general appeal and nutritional value of foods. Color additives are classified as dyes (pigments) or lakes. Both types can be used as primary colors (pure colors used without dilution) or secondary colors (blended primary colorants diluted with solvents or other additives). Dyes are commercial water-soluble pigments, used as powders, granules, or liquids. They are generally used in beverages, dry mixes, baked goods, confections, dairy products, and pet foods.

Lakes are prepared by adsorption or precipitation of a soluble dye on an insoluble substrate (e.g., alumina). They are useful in fatty products that have insufficient moisture to dissolve dyes (coated tablets, cake mixes, hard candies, chewing gum). Lakes are insoluble in most solvents including water, have high opacity, are easily incorporated in dry media, and show higher stability to light and heat. They are effective colorants for candies, pills, fats, and oils. The main characteristics and differences between lakes and dyes are well documented.⁶

Natural pigments are formulated either as dyes (hydrophilic powders or lipophilic oleoresins) or lakes.

The permission to use food colorants is bound to their safety and is strictly regulated by specific laws controlled at national and international levels. Individual country laws differ according to specific protocols, doses, and interpretations. Currently, the European Union (EU) has authorized 43 colorants as food additives⁷ and the United States (US) has authorized about 30.⁸

7.2.2 GENERAL REGULATIONS IN THE UNITED STATES AND EUROPEAN UNION

The approved color additives appear on positive lists issued by the Food and Drug Administration in the US, the EU, and Japan, but the colorants permitted in each market vary considerably.^{9–12} US and EU regulatory organizations provide provisional and permanent lists of approved color additives. The permanently listed additives are considered safe for use in cosmetic and toiletry products by the regulatory bodies. Provisionally listed color additives are those on which some safety studies are still to be undertaken or their test results are under review. The Japanese regulations include only a permanent list of color additives.

Food legislation in the US was implemented in 1938 and since then has been improved dynamically.¹³ The Food, Drug and Cosmetic Act classified specific colorants to be used in food and non-food products (FD&C colorants). Each colorant has a color index (CI) number and CI name. Colorants are also identified by their Chemical Abstract Service (CAS) registry code numbers. The two groups are *Certifiable Colorants* and *Exempt Colorants*.

Certifiable Colorants — These are synthetic, organic colorants, dyes, or lakes that can be used only after previous certification of the FDA obtained by the manufacturer of the colorant. These colorants are admitted for usage only after the acknowledgment of analyses indicating the purity specifications and assignment of an FDA certification number. The number must be mentioned on the package in which the colorant is sold and on all associated documents. The food company that purchases the colorant is obliged to register the specific use of the colorant in its production protocols. If a colorant is certifiable for foods, drugs, or cosmetics, its name is preceded by *FD&C*. If it is approved for use only in drugs and cosmetics, *D&C* must precede the name. If approved for use in externally applied drugs and cosmetics, the name of the colorant is preceded by *Ext. D&C*. Examples of colorant names are FD&C Yellow No. 5, D&C Red No. 6, Ext. D&C Violet No. 2, and FD&C

Yellow No. 5 Al Lake. Detailed information about certifiable colorants is presented in Section 7.3.

Exempt Colors — Defined as natural organic or inorganic colors, their certification is not required, but purity specifications must be followed by both the manufacturing and consuming companies. They are permitted for use under the Code of Federal Regulation (CFR).⁸ This category in the US includes 26 colorants derived from natural sources (vegetables, fruits, minerals, animals) and man-made colorants originating from natural sources by processing or via biotechnology (e.g., caramel color produced by sugar heating). The names of these colorants are based on a common name (caramel, carmine), chemical structure (titanium dioxide and iron oxide), or origin (grape skin extract, paprika oleoresin). The natural colorants included in the US category are annatto extract, β -apo-8'-carotenal, β -carotene, beet powder, canthaxanthin, caramel color, carrot oil, cochineal extract (carmine), cottonseed flour (toasted, partially defatted, and cooked), ferrous gluconate, fruit juice, grape color extract, grape skin extract (enocyanin), paprika, paprika oleoresin, riboflavin, saffron, titanium dioxide, turmeric, turmeric oleoresin, vegetable juice (see Table 7.2.1).

Although the 26 exempt colors are permitted for food use by title 21 of the CFR, part 73, the permission is restricted to certain food matrices, e.g., grape skin extract is restricted to use only in beverages; marigold (*Tagetes*) meal may be used only for chicken food. Regarding claims of natural versus synthetic food colorant classifications, various countries have developed different interpretations and some are more restrictive.^{10,13–16} The FDA does not discriminate between natural and synthetic colorants; both categories are subjected to similar verifications. The labeling of exempt colors must state *color added*, *colored with ...*, or *... color* to indicate the type of colorant.

The EU laws follow three principles related to consumer health: (1) protection, (2) fraud prevention, and (3) trade barriers. The union tried to harmonize the laws of different countries, particularly in recent years when the enlargement of the European Community became dynamic. Color Directive 94/36/EC contains “horizontal” provisions that refer to common laws in different countries and “vertical” directives that apply to specific foods. The EU directives take into account the recommendations of the Scientific Committee for Food (SCF), the Codex Alimentarius Commission, and the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA).

The EU directive includes a list of colorants (coded as E numbers, from E 100 to E 180) suitable for use as food additives and specifies limits of impurities. For details about their codes, sources, and properties see Table 7.2.1. An update of EU legislation on food-related issues was published recently.¹⁷ In addition to the numbered E colorants, other new natural colors for use in the future as natural colorants are under examination (see positions 25 through 34 in Table 7.2.1).^{17–19}

We have observed a decreasing interest in the development of new synthetic colorants, while increased efforts are directed toward the discovery of new natural pigments and the development of extractions and formulations of natural colorants.²⁰

TABLE 7.2.1
Natural Food Colorants Authorized as Exempt by EU and US Certifying Organizations

#	Natural Food Colorants	EU Code	US-CI certification/ADI	Source; Color; Specific Properties
1	Curcumin, turmeric, turmeric oleoresin	E 100	Yes, 75300	Plant rhizome (<i>Curcuma longa</i>); orange-yellow; strong odor and bitter, sharp taste
2	Riboflavin (ii) riboflavin-5-phosphate	E 101	Yes	Semisynthetic, obtained from bacterial fermentation (<i>Blakeslea trispora</i> and <i>Ashbya gossypii</i>); yellow-green; fluorescent light-sensitive, bitter taste
3	Carminic acid and carmine, cochineal extract	E 120	Yes	Female cochineal insect; orange to red, pink to red
4	Chlorophyll grass	E 140	–	Lucerne and nettle; green to olive; labile to photo-oxidation
5	Chlorophyllins (copper complex of chlorophyll)	E 141	–	Grass, lucerne, and nettle; bluish green; not claimed as natural on food labels
6	Caramel colors (I, II-sulfite, III-ammonia, IV-ammonia sulfite)	E 150a–d	Yes	Food-grade carbohydrates; brown
7	Carbo medicinalis (vegetable carbon)	E 153		Burned plant material; black
8	Carotenes (mixtures including β -carotene), β -carotene	E 160a	Yes, 75130 Yes, 40800	Palm oil or fermentation of <i>Blakeslea trispora</i> and <i>Ashbya gossypii</i> ; yellow to orange; good pH stability, oil-soluble, easily oxidized
9	Bixin, norbixin annatto extracts	E 160b	Yes, 75120	Seeds of annatto (<i>Bixa orellana</i>); orange-red
10	Capsanthin, capsorubin, paprika, paprika oleoresin	E 160c		Paprika (<i>Capsicum annum L.</i>); reddish (carmine) orange
11	Lycopene	E 160d		Tomato (<i>Lycopersicum esculentum</i>); orange red

12	β -Apo-8-carotenal	E 160e	Yes, 40820	Semi-synthetic, from β -carotene; orangish red
13	Ethyl ester of β -apo-8-carotenoic acid	E 160f	Yes, 40825	Semi-synthetic from β -carotene; orangish red
14	Canthaxanthin	E 161a		Salmon, shrimp; semi-synthetic from β -carotene; orange and pink
15	Lutein	E 161b		Aztec marigold (<i>Tagetes sp.</i>) petals; orange and yellow
16	Betain, dehydrated beets	E 162	Yes	Red beet root; pink to red; heat-, light-, and oxygen-sensitive
17	Anthocyanins, grape color extract, grape skin extract	E 163	Yes	Elderberries, black grape skins, black carrots, red cabbages; pink-red to mauve-blue; pH-dependent, heat- and oxidation-sensitive
18	Calcium carbonate	E 170	Yes, 77220	Mineral rocks; white; surface dye
19	Titanium dioxide	E 171	ADI-NE, 77891	Mineral rocks; white; surface dye
20	Iron oxides and hydroxides, synthetic	E 172	Yes, 77490	Mineral rocks; yellow, red, black; surface dyes
21	Aluminium	E 173		Mineral rocks; white; surface dye
22	Silver	E 174		White; surface dye
23	Gold	E 175		Yellow; surface dye
24	Rubin BK	E 180		Red; surface dye
25	Algal meal, dried	No	ADI-NE	Green
26	Carrot oil	No	ADI-NE	Orange-red
27	Corn endosperm oil	No	ADI-NE	Yellow
28	Cotton seed flour, toasted, defatted	No	ADI-NE	Green
29	Ferrous gluconate	No	ADI-NE	Red
30	Fruit juice	No	ADI-NE	Red
31	Paprika	No	ADI-NE	Red
32	Saffron	No	ADI-NE	Yellow-orange
33	Tagetes (marigold) meal and extract	No	ADI-NE	Orange
34	Vegetable juice	No	ADI-NE	Red

Note: Existing colorants, positions 1–24; new colorants under certification, positions 25–34. CI = Certification index. ADI = Acceptable daily intake. NE = Non-estimated.

7.2.3 SAFETY ASPECTS: TOXICITY AND HEALTH PROTECTION

According to the 21 CFR 73, the FDA coordinates the color additive regulations and imposes the safe use of each food colorant. In 1993, the FDA published the *Redbook of Colorants*, which sets forth the toxicological principles for safety assessments of direct food and color additives used in foods, and a review of the status of food additives in the US was published.¹³

7.2.3.1 HEALTH PROTECTION

Today's consumers look for ingredients made from colored natural sources. The preference for using food-based coloring agents instead of non-food sources is a natural response to the rapidly expanding health-conscious market.¹⁹

Naturally pigmented extracts with coloring properties are made exclusively from healthy fruits and vegetables and manufactured by means of physical processes using only water. Based on the high contents of dietary phytochemicals found in fruits and vegetables, these extracts are not only safe for daily consumption; but have health benefits that are related to prevention of cancer, cardiovascular diseases, age-related eye degeneration, and other conditions.^{19,21,22}

Gesellschaft für Nahrungsmitteltechnologie (GNT), a well-known German-Dutch company developing new colored ingredients for functional foods or nutraceuticals, is a pioneer in manufacturing foods with coloring properties, and is a worldwide distributor of natural food colorants for the food and beverage industry (www.gnt-group.com). In 1992, after extensive research, GNT began to cultivate black carrot as a new source of red anthocyanin colorings, one of today's most significant raw materials used in the development of natural colors, applying exclusively physical processes. Unlike other anthocyanins, its color is less susceptible to degradation from heat and light exposure, extending the shelf life of the end product. Black carrot exhibits a wide array of red hues ranging from strawberry to burgundy wine. Its extract is FDA classified as vegetable juice color (position 34, Table 7.2.1), has kosher approval, and meets many of the necessary requirements of foods having coloring properties. GNT recently introduced pumpkin as a new source of a yellow coloring agent.

7.2.3.2 TOXICOLOGY

According to the European 94/36/EC directive, restrictions on utilization of colorants are claimed for different food categories based on their safety characteristics. In general, the toxicological studies are performed by animal testing although alternative *in vitro* methods are increasingly used.^{14,15,23} Many colorants considered natural under US regulations (exempt colorants) are mentioned in Table 7.2.1. SCF bases safety approval of colorants on specified acceptable daily intakes (ADIs). If the concentration of a colorant in food surpasses the ADI, the colorant is excluded from the positive list and may not be added. This requirement excepts coloring pigments that are normally found in foods. *Natural* is a term to be avoided because it may

suggest the idea to consumers that a colorant is a food natural pigment and not a food color additive. Section 7.1 describes all aspects linked to quality management of food colorants including risk analysis and characterization, a pre-requisite for using any food colorant.²³

The quantitative measurement of toxicity level is expressed by parameters like NOEL (no observed effect level), NOAEL (no observed adverse effect level), and ADI (acceptable daily intake). The NOEL values are divided by 100 to obtain ADI values. The 100 safety factor derives from 10×10 , where the 10s represent the animal-to-human conversion rate and the human variability factor. Currently, the most useful index of safety is the ADI, expressed as milligrams of test substance per kilogram of body weight (ppm), with the recommendation not to eat more than the ADI per day. The FDA, EU, and WHO agree on the ADI principle.

ADI values are determined by chronic toxicity studies, not acute toxicity measurements. Acute toxicity is expressed as LD₅₀ (the dosage that causes the deaths of 50% of tested animals) and can be extended for different periods (e.g., 1 week or 100 days). Generally for food colorants, the LD₅₀ values for short-term experiments are rarely used, having been replaced by long-term ADI values. For example, β -carotene has an ADI of 5 ppm per day, that is, an average of 350 mg for a person who weighs 70 kg. Table 7.2.2 shows ADI values determined for different E-coded colorants. Examples of food products that may contain specific colorants and their maximum allowed concentrations (ADIs) are presented in Table 7.2.3.

7.2.4 NATURAL COLORANTS IN FOOD PRODUCTS

The food industry, following scientific and technological developments and market demands,^{6,24} takes into account consumer wishes to have more naturally colored foods and adapts its methodologies to safer ways of producing food. In recent decades, we experienced a shift from exclusive use of certifiable synthetic colorants to exempt colorants and to natural complex extracts.

Food colorants can be classified based on their solubilities and applications. The limiting factor for their efficacy is stability in the watery environments found in most food matrices. Generally the factors that deteriorate colorant stability and efficacy are physical (temperature, light), chemical (oxidizing or reducing agents, acids, alkalis), and biological (enzymes, microorganisms). Each category of colorants to be used by food enterprises has individual specifications elaborated by the colorant manufacturer and certified by the FDA, EU, or joint FAO/WHO agencies.

To prepare homogeneous solutions of water-soluble colorants, the powders or granules are dissolved in water or hydrophilic solvents (propylene glycol or glycerin), considering the solubility limits (1.5 to 4%) recommended by the manufacturer and the need to be free of minerals that may induce sediment formation. To improve the stability of food colorants, specific permitted diluents and support matrices are used: lecithin (E 322), emulsifiers like polysorbates (E 432 through E 436), monoglycerides and diglycerides (E 471), esters of organic acids with glycerin (E 472a–e), esters of sucrose with fatty acids (E 475), sorbitan esters (E 494 and E

TABLE 7.2.2
Plant Extracts Included on Lists of Natural Colorants

#	Natural Colorant and Source	Properties and Formulation	Application	Relevant Literature for Quality Specifications
1	Carminic acid = extract of cochineal insect (<i>Dactylopius</i> spp.), protein-bound glycoside of anthraquinone Carmine = aluminium lake of carminic acid	Very soluble in water, color changes according to pH; orange color obtained in acidic media, turns to violet to red by increasing pH from 5 to 7; carminic acid has good stability to heat, light and oxygen; extracted with water or aqueous alcohol at 90 to 100°C by batch or continuous process; carminic acid is permitted and widely used in food industries in US and EU; has good solubility in water; cochineal is traditional source for carminic acid; extracts concentrated to provide solutions of 2 to 5% (maximum) pigment content; use of protolytic enzymes reported to improve extraction yields considerably; carmine commonly traded as powder with carminic acid content of 40 to 60%; liquid aqueous alkaline forms (and spray-dried derivatives) available with carminic acid content of 2 to 7%	Competes with beet root red (betanin) and anthocyanins in food coloring; main limitation is insolubility at low pH; typical applications: soft and alcoholic drinks, bakery and dairy products, confectionery at dosage levels ranging from 0.1 to 0.5%	Extraction, ⁵² spectrometry, ⁵³⁻⁵⁵ HPLC ^{56,57}
2	Crocin , from stigmas of crocus (saffron) or fruits of cape jasmine <i>Gardenia jasminoides</i>	Saffron extract widely used at typical levels of 0.1 to 0.2% (weight for weight) to impart characteristic flavor and heat-stable yellow color; does not have E number in EU; falls into natural extract category	Spice for bakery products and confectionery, European regional dishes, such as paella	Specifications, ⁵⁸⁻⁶⁰ HPLC, ^{61,62} spectrometry ⁶³

3	Bastard (false) saffron , from safflower (dyer's saffron)	Content of carthamin in Indian safflower florets ranges from 0.3 to 0.6%; improved methods for extraction from safflower and alternative production by tissue culture reported recently, mainly by Japanese researchers	Dyestuff use of safflower limited to traditional applications in countries such as India; offered as food colorant in developed countries as a natural vegetable extract; textile colorant with mordant gives nuances like pink, red, rose, crimson to scarlet Beverages, dairy products, confectionery	Extraction ⁶⁴⁻⁶⁷
4	Curcuminoids , yellow colorants, from turmeric (Curcuma)	Three curcuminoid pigments present in rhizomes (curcumin, desmethoxycurcumin, bis-desmethoxycurcumin); turmeric oleoresin = oily concentrated extract; pure curcumin obtained from refined oleoresin, free of volatile oil, is less oil-soluble; turmeric, its oleoresin, and curcumin are universally permitted as food additives; formulation by incorporation in food-grade solvent and emulsifier, usually polysorbate, to become water-soluble product; in US, curcumin oleoresin, and turmeric are approved as natural colors. In EU, curcumin is on additive list as E 100 Principal pigment is lutein, present as esters of palmitic and myristic acids; E number is 161; marigold extract has not been assigned E number and is traded as vegetable extract; competes with lutein extracted from alfalfa grass; pigment supplied as liquid extract on edible vegetable oil carrier at concentration of 5 to 12% lutein; recommended dosage levels range from 0.05 to 0.8% (weight for weight) according to application; marigold extract not approved for use as food additive in US		Specifications, ⁶⁸ extraction, ^{69,70} HPLC ⁷¹
5	Marigold meal, marigold oleoresin , from flowers of <i>T. erecta</i> L. or Aztec marigold		Additive (dried flower meal or solvent extract) to poultry feed, to enhance the yellow color of flesh and egg yolks; minor use of extract as food colorant; typical applications: salad dressings, ice cream, dairy products, other foods with high fat contents, soft drinks, bakery products, jams and confectionery	Spectrometry, ^{72,73} HPLC ⁷⁴⁻⁷⁷

Continued.

TABLE 7.2.2 (Continued)
Plant Extracts Included on Lists of Natural Colorants

#	Natural Colorant and Source	Properties and Formulation	Application	Relevant Literature for Quality Specifications
6	Paprika , powdered spice, orange-red, paprika oleoresin as food colorant	Pigments in paprika are mixtures of carotenoids in which capsanthin and capsorubin dominate; they are oil-soluble, stable to heat and pH variation but deteriorate in light; oleoresin obtained by extraction with organic solvent (hexane); sold as solution in edible vegetable oil; water-miscible forms of oleoresin are also available, incorporate polysorbates or are emulsions with gum arabic; paprika products sold by grade quality, standardized by American Spice Trade Association (ASTA) color scale; commercial oleoresins available in strengths ranging from 40,000 to 100,000 ASTA color units; strongest has pigment content of approximately 10%; extract/oleoresin listed by EU as natural color and assigned E 160c. In US paprika oleoresin is included on FDA list of approved natural colors for incorporation in foods and beverages	Meat products, soups, pickles, snacks, bread crumbs; presence of flavors of specific oleoresins restricts use as food coloring in confectionery and desserts	Extraction and quantification ^{68,78}

TABLE 7.2.3**Food Products That May Contain Specific Colorants and Maximum Allowed Concentrations**

Food Product	Colorant Allowed	Maximum Level (ppm)
Malt bread	E 150a-d	NMM
Breakfast cereals	E 120, E 150c, E 160a-c, E 162, E 163	25–200
Chips (potato)	E 100	NMM
Butter	E 160a	NMM
Margarine, emulsions of fats in water	E 100	10 for E 160b
Cheese	E 160a,b,	
	E 120, E 140, E 141, E 160a–c, E 163	1.5–50 for E 160b
Vinegars and aromatic wines	E 150a–d, E 163	NMM
Bitter beverages	E 100, E 101, E 102, E 104, E 120, E 122, E 123, E 124, E 129	100 mg/l
Fruit and vegetable juices	E 160a, E 160d, E 160e	NMM
Beer and cider	E 150a–d	NMM
Vegetables conserved in vinegar, salt or oil	E 101, E 140, E 150a–d, E 141, E 160, E 163	NMM
Jams and marmalades	E 100, E 140, E 141, E 150a–d, E 160c, E 161b, E 162, E 163, E 104, E 110, E 120, E 124, E 142	100 only for synthetic colorants
Sausages, meat pastes, fish products	E 100, E 101, E 120, E 124, E 150a–d, E 160a, E 160c, E 162	20–100
Hamburger	E 120, E 150a–d	100 only for E 120

NMM = No maximum level mentioned.

495), and viscosity regulating powders (E numbers above 500) such as silicates (E 552, E 554, E 559).

7.2.4.1 NATURAL COLORANTS IN BEVERAGES

Beverage markets are huge and production requires both synthetic colorants and naturally colored ingredients.^{25,26} In the past, dry-mix and carbonated beverages were attractive, brightly colored, and stable. No natural colorants were chosen because of higher costs and lower stabilities, except the caramel colorants used in Coca-Cola and Pepsi Cola. At present, natural colorants are becoming increasingly popular, especially in juice-containing beverages. The globalization of extraction and concentration techniques led to wider use of concentrated natural ingredients. They are easy to transport and to store and contribute to the popularity of naturally colored beverages in accordance with consumers' growing interest in quality and health protection.

More natural colorants are wanted for beverage applications, but certain issues must be considered: technical properties such as stability, sensitivity to light, pH, and temperature, and interactions with other ingredients and packaging. Some natural

exempt colorants are used at concentrations of approximately 0.03 to 0.25% (annatto, cochineal, carmine, elderberry, grape skin extracts, caramel, β -carotene), others only as concentrates (turmeric and paprika oleoresins). The FDA recently approved the use of a natural food colorant extracted from non-GMO, lycopene-rich tomatoes and known as Tomat-O-Red. This colorant is rich in natural lycopene and recommended for use in foods and beverages. In Europe, use of pure lycopene (food additive E 160d) above certain levels is forbidden, but Tomat-O-Red is available in three formulations. It is ready to use and highly stable under a wide range of temperatures and pH values. It can be used to color a variety of food products including beverages, dairy products, confectionery products, and baked goods. Tomat-O-Red claims also to provide health advantages along with its function as a colorant, based on studies showing the efficacy of lycopene as a cancer-preventing agent due to its powerful antioxidant potential. Three commercially available formulations, a 2% ready-to-use liquid formulation, an 8% oleoresin, and a 10% free-flowing powder are available (www.lycored.com).

French researchers provided an alternative to the tartrazine synthetic colorant (E 102), valorizing a phloridzine oxidation product (POP) generated as a by-product of the cider industry.²⁷ Phloridzine is a polyphenol specific to apples and shows good antioxidant capacity. When apples are pressed to yield juice, phloridzine, oxygen, and polyphenoloxidase enzyme combine to form POP. This brilliant yellow natural colorant with nuances dependent on pH level can be incorporated easily into water-based foods such as beverages (juices, syrups) and confectionery creams because it is stable during production processes. Details about the specific formulations of these colorants are presented in Section 5.1.

7.2.4.2 NATURAL COLORANTS IN DAIRY PRODUCTS

Dairy products are generally colloidal emulsions and suspensions of oil in water (milk, yogurt) or water in oil (butter, margarine, ice-cream). Accordingly, the polarity and solubility of colorants are essential factors to be considered when a stable composition is needed. Milk-based and whey-based beverages have become more popular in recent years, especially as low-calorie mixtures with fruit juices in which some synthetic colorants are used (FD&C Red No. 3; 0.08 to 0.16 g/l for lakes). Dairy product companies now produce such beverages using flavonoids and anthocyan-rich natural juices to color milk, whey-based, and fermented products.²⁸

Yogurt is generally colored with hydrophilic cochineal extracts, carmine, beet juice, or FD&C Red No. 3, all of which are resistant to microbial attack. Generally, the colorant is added to the fruit preparation, but its stability must be good at low pH levels. For example, beet juices are directly added to yogurt without the heat processing that may degrade the beet pigments. The most commonly used red colorants in yogurt are acid-proof cochineal extract and water-soluble carmine. Both are heat-stable at pH 2.8 to 7.0, resistant to microbial attack, and produce bright and appealing shades. Studies of the discoloration of annatto-colored cheese under varying conditions (source of colorant, cooking temperature, pH, and emulsifying agent) were reported.^{30,31} Annatto emulsions showed less stability than annatto solutions and suspensions during continuous heating.³² GNT's Exberry® product, is a new

fruit and vegetable extract replacing annatto; it is used as additive in Cheddar and Gouda cheese production.

7.2.4.3 NATURAL COLORANTS IN CONFECTIONS

Many sweets (confections) must be colored, a strong point in their attractiveness for consumers. The commonly colored products are candies (starch jellies, candy cream centers, pan-coated candies, and hard candies), tablets, wafers, oil-based coatings, and chewing gums.

Candy starch jellies include sugar and (modified) starch boiled to a certain viscosity and poured into a starch mold to form semi-solid jelly. Water-soluble synthetic colorants are generally added at concentrations of approximately 6% before the mixture is placed in gel-forming blocks. The shape and thickness of the final semi-transparent gel and subsequent coating with sugar sand may cause the color to become shaded. Natural colorants are rarely used for such applications due to their low stability to temperature and pH.

Liquid colorants must be homogeneously incorporated into warm candy cream centers. Light discoloration is unacceptable and natural colorants are preferred. Red cochineal extracts (containing 3 to 4% carminic acid) are used at pH > 4 and turn orange at pH < 4 when added at the end of heat processing. Carmine lakes in alkali solutions are water soluble and may be incorporated in creams to yield bright magenta-red shades. The color can be changed by blending with yellow components (β -carotene or annatto). Beet juice may also provide red color but with lower heat stability and thus lower coloring power. Anthocyanins found in red cabbage, elderberry, black currant, grape juice, and grape seed concentrates are stable at pH < 3.8 and used for interior fillings but not for jellies due to their lower stability.

Orange shades are realized with lipophilic natural colorants like paprika oleoresin, β -carotene, and canthaxanthin after previous emulsification to yield water-dispersible forms. Yellow shades can be achieved using turmeric as a water-soluble solution, but the solution is light sensitive. To maintain constant color, 3 to 6 ppm of β -carotene may be added. Stable brown coloration is obtained from caramel; a concentrated syrup is easily incorporated, well flavored and stable in creams.³³

Colorants must be introduced into the coating syrups during production of pan-coated candies. Water-soluble colorants may be used but lake pigments as dispersions are preferred. Pan-coated candies require higher concentrations of colorants than jellies or creams; they require 30 to 60 coatings of colored syrup.⁶

Opaque coatings are obtained by combining colorants and titanium oxide, and also using FD&C lake pigments. These dispersions may also contain stabilizers, preservatives, and viscosity regulators. Efficiency is determined by the coating equipment (rotational pan) and procedure used. The critical points are temperature (< 40°C), dry matter (68 to 72%) and the ratio of tablet to coating syrup. Synthetic colorants are still preferred for coating because of better stability and lower cost, but the interest in natural colorants (turmeric, carmine, beet juice, β -carotene, red cabbage) in panned candies continues to increase rapidly.

Hard-boiled candies do not tolerate water after cooking, limiting the use of water-soluble colorants in vacuum cookers. The preferred method is to disperse these

colorants into melted, hot candy slabs or disperse in glycerine or propylene glycol. To polish candies, mixtures of colored syrup together with a glossy mixture of carnauba wax and bee wax can be used as dry powders or slurries in alcohols or oils.

Other confectionery products like dextrose tablets use different FD&C lakes 0.1%, dry blended with sucrose, mannitol, or sorbitol. Fat candies mainly need oil-based dispersion coatings but may also accept water-soluble colorants. For gum products (sticks, balls), colored lakes blended with turmeric or carmine suspended in glycerine give bright colors. Coloring failures in candies and beverages arise from similar causes. The homogeneity of colorant preparation is critical for an efficient coating.

7.2.4.4 NATURAL COLORANTS IN BAKED FOODS

The use of natural versus synthetic colorants and water-soluble versus oil-soluble colorants depends on the cereal or flour matrix, baking temperature regime, pH, and ability of the colorant to dissolve in the appropriate solvent. Because baked foods contain fats, the colorants must be oil-dispersible, e.g., FD&C lakes or oil-soluble natural colorants. For a good dispersion in dough, water-soluble colorants are added at 1.5 to 3% concentration. Lakes are preferred for water-based coatings due to their better light stability and non-migration from the matrix. Liquid solvents such as propylene glycol or glycerine mixed with lecithin are recommended for cookie fillings because they incorporate well into vegetable oils.

The natural colorants used often for bakery products are annatto extract or annatto plus turmeric blends (0.02 to 0.06 %) to obtain yellow-orange shades. Crackers are colored with annatto extract, turmeric and paprika oleoresins, or caramel. Turmeric may be used also in combination with FD&C colorants.³³

7.2.5 COLORED PHYTOCHEMICALS IN FUNCTIONAL FOODS AND NUTRACEUTICALS

Considering the concerns of consumers for synthetic colorants and interest in natural formulas, many food manufacturers seek alternative healthy solutions to replace colorants, even the regulated ones from positive lists (like β -carotene), with colored fruit and vegetable extracts to be used as functional food ingredients or nutraceuticals (food supplements).^{34,35,36}

In the past two decades new high-tech products were developed by using nature's raw materials rich in dietary phytochemicals and applying new scientific knowledge. Extracts with complex compositions (characterized by advanced analytical techniques) are presented as concentrated formulations with beneficial actions (nutraceuticals) or as functional food ingredients with health-promoting capacities.^{21,22,37} *Functional foods* is a collective term for foods and food ingredients that offer preventive health benefits in addition to good taste and nutritional value. This market has been exploding in recent years and continues to grow.

Many people do not eat sufficient quantities of fruits and vegetables. Professional food and cancer associations are alarmed because of the increasing rates of nutrition-related illnesses and recommend diets rich in fruits and vegetables. Epi-

demiological studies showed that diets of increased fruit and vegetables have positive effects on health.

Fruit and vegetable extracts that contain natural, mixed dietary phytochemicals instead of isolated substances are healthy alternatives, offer high standardized contents of dietary phytochemicals, and are often very color intensive. Dietary phytochemicals such as flavonoids, anthocyanins, betalains, chlorophylls, and carotenoids have complementary effects due to bioactive antioxidant substances found in fruits and vegetables proven to be very protective, for example, by reducing risks of acquiring cancer and cardiovascular diseases by up to 50%. International health organizations therefore recommend a minimum of five daily servings of fruits and vegetables.^{37–44}

For example, GNT's Nutrifood® is marketed as an active functional food ingredient for various applications such as dairy products, beverages, cereals, and food supplements. It is made from extracts of tomatoes, carrots, and pumpkins containing lycopene, β -carotene, lutein, and flavonoids. Nutrifood concentrates are standardized according to their total contents of anthocyanins, carotenoids, and polyphenols. Despite the natural compositions of these fruit and vegetable extracts, standardization is necessary with regard to major bioactive substances such as carotenoids and anthocyanins. Since the individual compounds are present in their natural forms, the risk of over-dosage is very low.

Seabuckthorn (*Hippophae rhamnoides*) fruits, very rich in phytochemicals and demonstrated to be excellent sources of natural food colorants (carotenoids and flavonoids) are increasingly used as food ingredients and nutraceuticals (www.proplanta.ro).^{45,46}

Arpink Red™, a new natural food colorant of fungal origin, was recently launched on the market (<http://www.ascolor-biotec.cz>). It is a red colorant produced biotechnologically from a *Penicillium oxalicum* strain. It belongs to an anthraquinone class of pigments and shows anticancer effects when used in food supplements.⁴⁷ The biodiversities of fungi and algae open new possibilities for producing natural colorants as alternatives to existing additives.^{48,49}

7.2.6 CONCLUSIONS

The present market for food colorants is estimated at 1 billion USD, while the natural food colorant market is only one-third of it. Synthetic colorants have achieved better results than natural or nature-identical colorants until now because of greater stability and higher ratios of coloring yield.

In recent decades, the synthetic colorant market has declined, to the benefit of the natural-oriented market and consumers. Excluding FD&C Red 40 and Red 28, the synthetic colorants are now as well accepted as they were. In addition to the decreasing enthusiasm for chemicals in food, the high costs of toxicological studies also inhibit the development and approval of new synthetic colorants. The existing technologies used for the extraction, concentration, and purification of natural plant pigments to be used as food colorants still produce lower yields and the final products are still expensive.

To overcome this situation, considerable research and technological development must be invested for the extraction and formulation of natural colorants (see Section 5.1) extracted from plants or produced by biotechnology *de novo* or via bioconversions of colorant precursors *in vitro* (extensively presented in Sections 5.3 and 5.4). Biotechnological products are still seen with some reserve by consumers but in the future they will certainly compete with natural or environmentally friendly products obtained by applying smart technologies to ecological agricultural products. The positive lists of colorants will be enlarged and some will be replaced by standardized natural extracts. The isolations, purifications, and properties of new pigments as potential natural food colorants are reported every year. Their acceptance requires extensive investigation and certification based on more harmonized regulations of the United States, Europe, Asia, and elsewhere.

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REFERENCES

1. Clydesdale, F.M., Color as a factor in food choice, *Crit. Rev. Food Sci. Nutr.*, 33, 83, 1993.
2. Goyle, A. and Gupta, R.G., Use of synthetic colors in food, *Sci. Cult.*, 64, 241, 1998.
3. Francis, F.J., Pigments and other colorants, in *Food Chemistry*, 2nd ed., Fennema, O.R., Ed., Marcel Dekker, New York, 1985.
4. Zollinger, H., *Color: A Multidisciplinary Approach*, Wiley-VCH, New York, 1999.
5. Hurstel, O. and Meldie, J., Les colorants alimentaires, *Ind. Alim. Agr.*, 9, 234, 1994.
6. Francis, F.J., *Colorants: Practical Guide for the Food Industry*, Eagan Press, St. Paul, MN, 2000.
7. European Parliament and Council Directive 94/36/EC of June 30, 1994, on colours for use in foodstuffs, *Off. J. European Comm.*, 237, 17, 1994.
8. *Code of Federal Regulations*, 21 Color Additive Regulations, US Food and Drug Administration, <http://www.cfsan.fda.gov>, 2001, retrieved 2006.
9. Evaluation of certain food additives and contaminants, World Health Organization Technical Report Ser. 922, 1, 2004. Erratum in World Health Organization Technical Report Ser. 928, 157, 2005.
10. Parker, L.E., Regulatory approaches to food coloration, in *Developments in Food Colors 2*, Applied Science Publishers, London, 1984.
11. Otterstatter, G., *Coloring of Food, Drugs and Cosmetics*, Marcel Dekker, New York, 1999.
12. Marmion, D.M., *Handbook of US Colorants For Foods, Drugs and Cosmetics*, 3rd ed. John Wiley & Sons, New York, 1991.
13. FDA, *Everything Added to Food in the United States*, CRC Press, Boca Raton, 1993.
14. Clydesdale, F.M., *Food Additives: Toxicology, Regulation, and Properties*, CRC Press, Boca Raton, 1997.
15. Francis, F.J., Safety of natural food colorants, in *Natural Food Colorants*, Hendry, G.A.F. and Houghton, J.D., Eds, Blackie, Glasgow, 1996.

16. Hallagan, J.B., Allen, D.C., and Borzelleca, J.F., The safety and regulatory status of food, drug and cosmetic color additives exempt from certification, *Food Chem. Toxicol.*, 33, 515, 1995.
17. Arvanitoyannis, I. S., Choreftaki, S., and Tserkezou, P., An update of EU legislation (directives and regulations) on food-related issues (safety, hygiene, etc.), *Int. J. Food Sci. Technol.*, 40, 1021, 2005.
18. Joint FAO/WHO Expert Committee on Food Additives, *Toxicological Evaluation of Some Food Colours, Enzymes, Flavour Enhancers, Thickening Agents, and Certain Other Food Additives*, WHO Food Series, Geneva, 6, 74, 1975.
19. Anonymous, Natural colorants set for growth in Europe, *Confection*. Jan. 28, 1998.
20. Francis, F.J., *Handbook of Food Colorant Patents*, Food and Nutrition Press, Westport, CT, 1986.
21. Kumar, J.K., and Sinha, A.K., Resurgence of natural colourants: a holistic view. *Nat. Prod. Res.* 18, 59, 2004.
22. Socaciu, C., Antioxidant phytochemicals: chemical characterization, function and actions, *Bull. USAMV A*, 57, 22, 2002.
23. Tennant, D.R., Risk analysis of food additives, in *Food Chemical Safety, Vol 2*, Watson, D.W., Ed., Woodhead, Cambridge, 2002.
24. Hutchings, J., *Colour, Appearance, Expectations and the Food Industry*, Kluwer/Plenum, New York, 2002.
25. Timberlake, C.F., Colours in beverages, *Food Flav. Ingred. Process. Pack.*, 4, 14, 1982.
26. Holst, S., Natural colors for food and beverages, *Food Mktg. Technol.*, 14, 14, 2000.
27. Sanoner, P. et al., Colouring hydrosoluble yellow preparation derived from dehydrochalcones, International Patent WO 2005/049598, 2005.
28. Jing, P. and Giusti, M.M., Characterization of anthocyanin-rich waste from purple corn cobs (*Zea mays* L.) and its application to color milk, *J Agric Food Chem.*, 53, 8775, 2005.
29. Francis, F. J., Food colorants: anthocyanins, *Crit. Rev. Food Sci. Nutr.*, 28, 273, 1987.
30. Evans, W.C., Annatto: a natural choice, *Biologist*, 47, 181, 2000.
31. Hong, C.M., Wendorff, W.L., and Bradley, R.L., Jr., Factors affecting light-induced pink discoloration of annatto-colored cheese, *J. Food Sci.*, 60, 94, 1995.
32. Shumaker, E.K. and Wendorff, W.L., Factors affecting pink discoloration in annatto-colored pasteurized process cheese, *J. Food Sci.*, 63, 828, 1998.
33. Francis, F.J., Turmeric, carthamin and monascus, in *Colorants*, Eagan Press, St. Paul, MN, 1999, 77.
34. EU Scientific Committee on Food, Opinion on Request for Use of Algal Beta-Carotene as a Food Colour, June 13, 1997.
35. European Commission, Health & Consumer Protection Directorate General, Opinion of the Scientific Committee on Food on β -Carotene from *Blakeslea trispora*, June 22, 2000.
36. Dalzell, J.M., *LFRA Ingredients Handbook, Food Colours*, Leatherhead, 1997.
37. Francis, F.J., Polyphenols as natural food colorants, in *Polyphenolic Phenomena*, Scalbert A., Ed., INRA, Paris, 1993, 209.
38. Jimenez Aparicio, A. and Gutierrez Lopez, G., Production of food related colorants by culture of plant cells: the case of betalains., *Adv. Exp. Med. Biol.*, 464, 195, 1999.
39. Berset, C., Propriétés fonctionnelles des colorants alimentaires naturels, *Ind. Alim. Agr.*, 1, 5, 1994.
40. Bidlack, W.R. et al., *Phytochemicals as Bioactive Agents*, Technomic Lancaster, PA, 2000.

41. Ames, J.M. and Hofmann, T., Selected natural colorants in foods and beverages, *J. Am. Chem. Soc.*, 775, 1, 2001.
42. Pszczola, D.E., Antioxidants: from preserving food quality to quality of life, *Food Technol.*, 55, 51, 2001.
43. Francis, F.J., Less common natural colorants, in *Natural Food Colorants*, 2nd Ed., Hendry, G.A.F. and Houghton, J.D., Eds., Blackie, Glasgow, 1996.
44. Hendry, G.A.F., Chlorophylls and chlorophyll derivatives, in *Natural Food Colorants*, 2nd Ed., Hendry, G.A.F. and J.D. Houghton, Eds., Blackie, Glasgow, 1996, 131.
45. Singh, V., ed., *Seabuckthorn, A Multipurpose Wonder Plant*, Vol. I, Indus International, India, 2005.
46. Socaciu, C. et al., Carotenoid-rich fractions in sea buckthorn berry oleosomes: separation, characterization and stability in colloid supramolecular structures, *Proc. 4th Int. Congr. Pigments in Food*, October 9–12, 2006, Shaker Verlag GmbH, Stuttgart, Germany.
47. Sardayan, E., Food supplement, US Patent 0105864, 2004.
48. Lorenz, R.T. and Cysewski, G.R., Commercial potential for Haematococcus microalgae as a natural source of astaxanthin, *Trends Biotechnol.*, 18, 160, 2000.
49. Mapari, S.A.S. et al., Exploring fungal biodiversity for the production of water-soluble pigments as potential natural food colorants, *Curr. Opin. Biotech.*, 16, 231, 2005.
50. O'Callaghan, M.C., Biotechnology in natural food colours, in *Natural Food Colorants*, 2nd ed., Hendry, G.A.F. and Houghton, J.D., Eds., Blackie, Glasgow, 1996.
51. Francis, F.J., Miscellaneous colorants, in *Colorants*, Eagan Press, St. Paul, MN, 1999, 89.
52. Lloyd, A.G., Extraction and chemistry of cochineal, *Food Chem.*, 5, 91, 1980.
53. Nishizawa, M. et al., Analysis of natural dyes (III). Analysis of cochineal dye and lac dye in foods and dyes, *Hok. Eisei Kenyush.*, 35, 7, 1985.
54. Schwing-Weill, M.J. and Wechsler, S., Spectrophotometric study of carminic acid in solution: application to its determination, *Analysis*, 14, 290, 1986.
55. Yamada, S. et al., Analysis of natural colouring matters in food (IV). Methylation of cochineal colour with diazomethane for analysis of food products, *J. Agric. Food Chem.*, 41, 1071, 1993.
56. Wouters, J., High performance liquid chromatography of anthraquinones: analysis of plant and insect extracts and dyed textiles, *Stud. Conserv.*, 30, 119, 1985.
57. Wouters, J. and Verhecken, A., The scale insect dyes: species recognition by HPLC and diode-array analysis of the dyestuffs, *Ann. Soc. Entom. France*, 25, 393, 1989.
58. International Standardization Organisation. ISO 3632-1 Specification: Saffron (*Crocus sativus* L.), Part 1, Geneva, 1993.
59. Sampathu, S.R. et al., Saffron cultivation, processing, chemistry and standardization, *CRC Crit. Rev. Food Sci. Nutr.*, 20, 123, 1984.
60. Kamikura, M. and Nakazato, K., Comparison of natural yellow colours from saffron and gardenia fruit, *Bull. Nat. Inst. Hygenic Sci. (Eisei Shikenjo Hokoku)*, 103, 157, 1984.
61. Solinas, M. and Cichelli, A., HPLC analysis of the colour and aroma constituents of saffron, *Ind. Aliment.*, 27, 634, 1988.
62. Kamikura, M. and Nakazato, K., Natural yellow colours from gardenia fruit and colours found in commercial gardenia extract. Analysis of natural yellow colours by high performance liquid chromatography, *J. Food Hygiene Soc. Japan*, 26, 150, 1984.
63. Noda, N. et al., Determination of natural yellow dye from the fruits of gardenia by detecting geniposide, *J. Hygenic Chem. (Eisei Kagaku)*, 29, 7, 1983.

64. Saito, K., A new method for reddening dyer's saffron (*Carthamus tinctorius*) florets: evaluation of carthamin productivity, *Zeits. Lebens, Unter. Forsch.*, 192, 343, 1991.
65. Saito, K. and Kawasaki, H. Comparative studies on the distribution of quinoidal chalcone pigments in extracts from insect wastes and intact tissues of dyer's saffron florets, *Zeits. Lebens, Unter. Forsch.*, 194, 131, 1992.
66. Saito, K., A new enzymatic method for extraction of precarthamin from dyer's saffron (*Carthamus tinctorius*) florets, *Zeits. Lebens, Unter. Forsch.*, 197, 34, 1993.
67. Saito, K. and Miyakawa, K., A new procedure for the production of carthamin dye from dyer's saffron flowers, *Lebensm. Wiss. Technol.*, 27, 384, 1994.
68. ASTA, *Cleanliness Specifications for Unprocessed Spices, Seeds and Herbs*, American Spice Trade Association, Englewood Cliffs, NJ, 1992.
69. George, K.M., On the extraction of oleoresin from turmeric: comparative performance of ethanol, acetone and ethylene dichloride, *Ind. Spices*, 18, 7, 1981.
70. Hendry, G.A.F. and Houghton, J.D., Eds., *Natural Food Colourants*, Blackie, Glasgow, 1992.
71. Taylor, S.J. and McDowell, I.J., Determination of the curcuminoid pigments in turmeric (*Curcuma domestica* Val.) by reversed-phase high performance liquid chromatography, *Chromatographia*, 34, 73, 1992.
72. Fletcher, D.L. and Halloran, H.R, An evaluation of commercially available marigold concentrate and paprika oleoresin on egg yolk pigmentation, *Poultry Sci.*, 60, 1846, 1981.
73. Gau, W. et al., Mass spectrometric identification of xanthophyll fatty acid esters from marigold flowers (*Tagetes erecta*) obtained by high performance liquid chromatography and Craig countercurrent distribution, *J. Chromatogr.*, 262, 277, 1983.
74. Gregory, G.K. et al., Quantitative analysis of lutein esters in marigold flowers (*Tagetes erecta*) by high performance liquid chromatography, *J. Food Sci.*, 51, 1093, 1986.
75. Livingston, A.L., Rapid analysis of xanthophyll and carotene in dried plant materials, *J. AOAC*, 69, 1017, 1986.
76. Philip, T. and Berry, J.W., A process for the purification of lutein-fatty acid esters from marigold petals, *J. Food Sci.*, 41, 163, 1976.
77. Tyczkowski, J.K. and Hamilton, P.B., Preparation of purified lutein and its diesters from extracts of marigold (*Tagetes erecta*), *Poultry Sci.*, 70, 651, 1991.
78. Minguez-Mosquera, M.I. and Hornero-Mendez, D., Separation and quantification of the carotenoid pigments in red peppers, paprika and oleoresin by reversed phase HPLC, *J. Agric. Food Chem.*, 41, 1616, 1993.

WEB SOURCES ON NATURAL FOOD COLORANTS

<http://www.gsu.edu/~mstnrhx/edsc84/dye.htm>
<http://www.foodcolour.net/>
<http://www.neelikon.com/foodcol.htm>
<http://www.standardcon.com/food%20colo>
<http://www.rohadyechem.com/index1.shtml>
<http://www.ukfoodguide.net/enumeric.htm>
<http://www.agsci.ubc.ca/courses/fnh/410/modules.htm#Colour>
<http://www.raise.org/natural/pubs/dyes/annex.stm>
<http://www.dyeman.com/NATURAL-DYES.html>

<http://vm.cfsan.fda.gov/~lrd/colorfac.html>

<http://www.fda.gov/opacom/backgrounders/coloradd.html>

<http://crucial.ied.edu.hk/Foodchem/addcolor.html>

<http://www.ascolor-biotec.cz>

<http://www.ourfood.com>

www.thomasnet.com/metro-new-york/food-colors

7.3 Synthetic Colorants

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7.3.1 INTRODUCTION

Color is an important feature of food, and consumers often associate it with quality, taste, and flavor. Colorants were used for centuries to improve the appearances of foods, cosmetics, and clothing. Until the 19th century, the colorants used were of natural origin like henna for hair dying or saffron for providing color and flavor to food. During the 19th century, inorganic color compounds such as copper sulfate and red lead were used to color foods, from tea leaves to cheese. At the same time, the rapid development of chemical synthesis led to the industrial production of a large number of organic synthetic colorants. More than 80 synthetic colorants were available in 1907, mostly derived from coal tar and petroleum, and some were used as food additives without proper safety evaluations. Several reported health problems, intoxications, and even deaths were related to the consumption of foods containing synthetic colorants.

Colorants were the first food additives subjected to governmental regulation in the United States (US). After successive toxicological evaluations, the Food and Drug Administration established a list of permitted colorants and lakes. Only 7 synthetic pigments (and 2 others with restrictions) and 6 of their lakes are now permitted as food colorants in the US while 17 are permitted in the European Union (EU); see Table 7.3.1.¹⁻⁸

Despite the new orientation toward utilization of natural compounds, synthetic colorants are still used as food additives. Synthetic colorants are easy to produce, stable, less expensive, and have better coloring properties than natural colorants. Still, synthetic colorants are considered to belong to concern level III, a category

TABLE 7.3.1
Synthetic Food Colorants Used as Certifiable Dyes or Lakes and Current Status

#	Food Colorant	Color	CAS #	Color Index	EU Code	EU Status	FDA Code	FDA Status	JECFA Status
1	Allura Red AC	Yellowish red	25956-17-6	16035	E 129	Yes	Red No. 40	Yes	Yes
2	Amaranth	Red	915-67-3	16185	E 123	Yes	Red No. 9	No	Yes
3	Azorubine (Carmoisine)	Red	3567-69-9	14720	E 122	Yes	—	No	Yes
4	Brilliant Blue FCF	Greenish blue	3844-45-9	42090	E 133	Yes	Blue No. 1	Yes	Yes
5	Brilliant Black BN	Black	2519-30-4	28440	E 151	Yes	Black No. 1	No	Yes
6	Brown FK	Brown	8062-14-4	—	E 154	Yes	—	No	No ADI
7	Brown HT	Brown	4553-89-3	20285	E 155	Yes	Brown No. 3	No	Yes
8	Citrus Red No. 2	Red	6358-53-8	12156	—	—	Citrus Red No.2	Yes (limited)	Not to be used
9	Erythrosine	Bluish pink	16423-68-0	45430	E 127	Yes	Red No. 3	Yes	Yes
10	Fast Green FCF	Bluish green	2353-43-9	42053	—	No	Green	Yes	Yes
11	Fast Red E	Red	2302-96-7	16045	—	No	Red No. 4	No	No ADI
12	Green S	Green	860-22-0	44090	E 142	Yes	—	No	No ADI
13	Indigotine	Deep blue	860-22-0	73015	E 132	Yes	Blue No. 2	Yes	Yes
14	Lithol Rubine BK	Deep red	5281-04-9	15850	E 180	Yes	—	No	No ADI
15	Orange B	Orange	15139-76-1	—	—	No	Orange B	Yes (limited)	Not listed
16	Patent Blue V	Blue	3536-49-0	42051	E 131	Yes	—	No	No ADI
17	Ponceau 4R (Cochineal Red A)	Red	2611-82-7	16255	E 124	Yes	—	No	Yes
18	Red 2G	Red	3734-67-6	18050	E 128	Yes	—	—	Yes
19	Quinoline Yellow	Yellow	8004-72-0	47005	E 104	Yes	—	—	Yes
20	Sunset Yellow	Reddish yellow dye and lake	2783-94-0	15985	E 110	Yes	Yellow No. 6	Yes	Yes
21	Tartrazine	Lemon yellow dye and lake	1934-21-0	19140	E 102	Yes	Yellow No. 5	Yes	Yes

Source: European Union (EU),⁶ US Food & Drug Administration (FDA),⁸ and JECFA World Health Organization (WHO) regulations.^{17,18}

that requires the strictest safety evaluations.² The use of synthetic colorants is subjected to strict rules.⁶⁻⁸

7.3.2 LIST OF SYNTHETIC COLORANTS USED (EU AND FDA REGULATIONS) AS FOOD ADDITIVES

Synthetic food colorants are chemically synthesized compounds that have a large variety of structures. The structures (Figure 7.3.1) and main properties of some of these pigments are presented below. Depending on their structural characteristics, synthetic pigments used as food colorants can be classified as follows:

Azo dyes: Allura Red AC, Amaranth, Azorubine, Brilliant Black BN, Brown FK, Brown HT, Lithol Rubine BK, Ponceau 4R, Red 2G, Sunset Yellow, Tartrazine

Triarylmethane (triphenylmethane) dyes: Brilliant Blue FCF, Fast green FCF, Green S, Patent Blue V

Quinophthalon dyes: Quinoline yellow

Xanthene dyes: Erythrosine (see below)

Indigo dyes: Indigotine

Allura red AC (E 129, FD&C Red No. 40, CI Food Red 17) is a mono azo dye that consists essentially of 6-hydroxy-5-(2-methoxy-5-methyl-4-sulfophenyl) azo-2-naphthalenesulfonic acid sodium salt (or disodium 2-hydroxy-1-(2-methoxy-5-methyl-4-sulfonato-phenylazo)-naphthalene-6-sulfonate). The calcium and potassium salts are also permitted.

Allura red is a dark red powder or granules soluble in water, insoluble in ethanol. The maximum absorption in water is at 504 nm, at pH 7 ($E_{1\text{cm}}^{1\%} = 540$). Allura red is synthesized via the classical process of diazotization. It was introduced in the US in the early 1980s to replace amaranth (E 123).⁷⁻¹¹

Amaranth (E 123, CI Food Red 9) is a mono azo dye, with the chemical name trisodium 3-hydroxy-4(4-sulfonato-1-naphthylazo)-2,7-naphthalenedisulfonate) (or trisodium 2-hydroxy-1-(4-sulfonato-1-naphthylazo) naphthalene-3,6-disulfonate). The calcium and potassium salts are also permitted. Amaranth is a reddish-brown powder or granules, soluble in water, sparingly soluble in ethanol, with a maximum absorption in water at 520 nm ($E_{1\text{cm}}^{1\%} = 440$). It has been banned in the US since 1976.⁷⁻¹¹ Amaranth can be used also as a dye for cosmetics, synthetic fibers, leather, papers, and some plastics.

Azorubine (E 122, CI Food Red 3, carmoisine) is a mono azo dye. The chemical name is disodium salt of 4-hydroxy-3-(4-sulfonato-1-naphthylazo) naphthalene-1-sulfonate. The calcium and potassium salts are also permitted. Azorubine is a red to maroon powder or granules, soluble in water, sparingly soluble in ethanol, with a maximum absorption in water at 516 nm ($E_{1\text{cm}}^{1\%} = 510$). It is not permitted by the FDA as food colorant.⁷⁻¹¹

Brilliant Blue FCF (E 133, FD&C Blue No. 1, CI Food Blue 2) is a triaryl-methane dye: disodium 3-[N-ethyl-N-[4-[[4-[N-ethyl-N-(3-sulfonatobenzyl)-amino] phenyl] (2-sulfonatophenyl)methylene]-2,5-cyclohexadiene-1-ylidene] ammoniomethyl] benzenesulfonate (or disodium (4-(N-ethyl-3-sulfonato-benzylamino) phenyl)- α -(4-N-ethyl-3-sulfonatobenzylamino)cyclohexa-2,5-dienylidene)-toluene-2-sul-

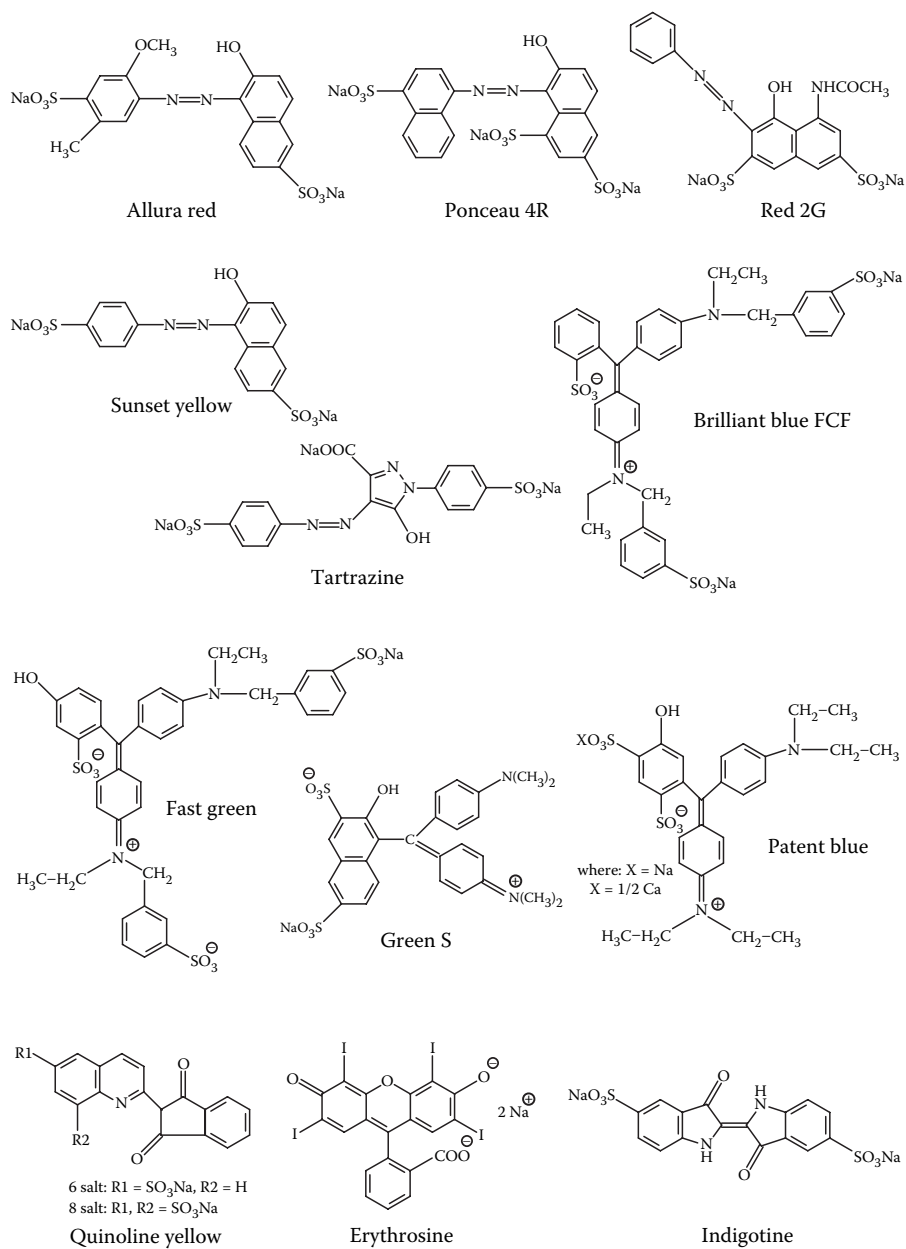


FIGURE 7.3.1 Chemical structures of some synthetic food pigments.

fonate) and its isomers. The calcium and potassium salts are also permitted. Brilliant blue is a synthetic dye obtained from coal tar. It is a reddish-blue powder or granules, soluble in water, slightly soluble in ethanol, with a maximum absorption in water at 630 nm ($E_{1\text{cm}}^{1\%} = 1630$). It is also used as an additive in cosmetics and for protein coloration.⁷⁻¹¹

Brilliant Black BN (E 151, Black PN, CI Food Black 1) is a bis azo dye: tetrasodium 4-acetamido-5-hydroxy-6-[7-sulfonato-4-(4-sulfonato-phenylazo)-1-naphthylazo]-1,7-naphtha-lene-disulfonate. The calcium and potassium salts are also permitted. It is a black powder or granules, soluble in water, sparingly soluble in ethanol, with a maximum absorption in water at 570 nm ($E_{1\text{cm}}^{1\%} = 530$). It is not permitted in the US.⁷⁻¹¹

Brown FK (E 154, CI Food Brown) is a mixture of six mono, bis, and tris azo compounds.

- I Sodium 4-(2,4-diaminophenylazo) benzenesulfonate
- II Sodium 4-(4,6-diamino-m-tolylazo) benzenesulfonate
- III Disodium 4,4'-(4,6-diamino-1,3-phenylenebisazo)-di(benzenesulfonate)
- IV Disodium 4,4'-(2,4-diamino-1,3-phenylenebisazo)-di(benzenesulfonate)
- V Disodium 4,4'-(2,4-diamino-5-methyl-1,3-phenylene-bisazo)di(benzene-sulfonate)
- VI Trisodium 4,4',4''-(2,4-diaminobenzene-1,3,5-trisazo)tri-(benzene-sulfonate)

The proportion of components in the mixture should not exceed 26% (I), 17% (II), 17% (III), 16% (IV), 20% (V), and 16% (VI) according to EU regulations. Brown FK is often diluted with sodium chloride. It is a red-brown powder or granules, soluble in water (orange solution) and sparingly soluble in ethanol. It is not permitted in the US and is restricted to certain foods in the EU.⁷⁻¹¹

Brown HT (E155, CI Food Brown 3, Chocolate brown) is a bis azo dye, with the chemical name disodium 4,4'-(2,4-dihydroxy-5-hydroxymethyl-1,3-phenylenebisazo) di(naphthalene-1-sulfonate). The calcium and potassium salts are also permitted. Brown HT is a reddish-brown powder or granules, soluble in water, insoluble in ethanol, with a maximum absorption in water at 460 nm, pH 7, ($E_{1\text{cm}}^{1\%} = 403$). It is not permitted in the US.⁷⁻¹¹

Citrus Red No. 2 is a mono azo dye, principally 1-(2,5-dimethoxyphenylazo)-2-naphthol. It has limited application, only in the US (see Table 7.3.1 and Table 7.3.2).

Erythrosine (E 127, FD&C Red No. 3, CI Food Red 14) is a xanthene dye named disodium salt of 2-(2,4,5,7-tetraiodo-3-oxido-6-oxoxanthen-9-yl)benzoate monohydrate (or disodium salt of 9-(o-carboxyphenyl)-6-hydroxy-2,4,5,7-tetraiodo-3-isoxanthone monohydrate); it is also called tetraiodofluorescein. The calcium and potassium salts are also permitted. Erythrosine is a red powder or granules, soluble in water and in ethanol, with a maximum absorption in water at 526 nm, pH 7, ($E_{1\text{cm}}^{1\%} = 1100$),^{7-9,11} shows fluorescence. It is obtained from fluorescein after precipitation and treatment with iodine. Fluorescein is synthesized from resorcinol and phthalic anhydride.¹⁰ Erythrosine can be also used in inks, as a dental plaque disclosing agent,¹³ and as biological stain.

TABLE 7.3.2
Synthetic Food Colorants and Their Uses as Food Additives

Color	ADI According to JECFA ^{17,18}	Utilization and Limits in Foods According to EU and US Rregulation
Allura Red AC	0 to 7 mg/kg bw	Specific uses: bitter soda, bitter wine, other non-alcoholic flavored drinks alone or combined with other colorants (100 mg/l); luncheon meat (25 mg/kg), breakfast sausages with minimum cereal content of 6% (25 mg/kg); general uses: nonalcoholic flavored drinks (100 mg/l), candied fruits and vegetables (100 mg/l), red fruit preserves (200 mg/kg), confectionery (300 mg/kg), decorations and coatings (500 mg/kg), fine bakery wares (200 mg/kg), edible ices (150 mg/kg), flavored processed cheese (100 mg/kg), desserts including flavored milk products (150 mg/kg), sauces, seasonings, pickles, relishes, chutneys, and piccalillis (500 mg/kg), mustard (300 mg/kg), fish and crustacean pastes (100 mg/kg), precooked crustaceans (250 mg/kg), salmon substitutes (500 mg/kg), surimi (500 mg/kg), fish roe (300 mg/kg), smoked fish (100 mg/kg), extruded or expended snacks (200 mg/kg), other snacks (100 mg/kg), edible cheese rind (<i>quantum satis</i>), complete formula for weight control and nutritional supplements (50 mg/kg), liquid food supplement integrators (100 mg/kg), solid food supplement integrators (300 mg/kg), soups (50 mg/kg), meat and fish analogues based on vegetable proteins (100 mg/kg), other spirit beverages (200 mg/l), fruit wine, cider, perry, aromatized fruit wines (200 mg/l); ¹⁶ FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts consistent with good manufacturing practice; ⁸ JECFA: 50 mg/kg limit in milk and 300 mg/kg in other foodstuffs ^{18,19}
Amaranth ^a	0 to 0.5 mg/kg bw	Aperitif wines and spirit drinks including products with less than 15% alcohol (30 mg/l); can be used in combination with other colorants, but not to exceed 100 mg/l; fish roe (30 mg/kg)

Azorubine (Carmoisine)	0 to 4 mg/kg bw	Specific uses: americano (50 mg/l), bitter and wine, (50 mg/l); general uses: non-alcoholic flavored drinks (50 mg/kg), candied fruits and vegetables, red fruit preserves, confectionery, decorations and coatings, fine bakery wares (50 mg/kg), edible ices (50 mg/kg), flavored processed cheese, desserts including flavored milk products (50 mg/kg), sauces, seasonings, pickles, relishes, chutneys, and piccalillis, mustard, fish and crustacean pastes, pre-cooked crustaceans, salmon substitutes, surimi, fish roe, smoked fish extruded or expended snacks, other snacks, edible cheese rind (<i>quantum satis</i>), complete formula for weight control and nutritional supplement, liquid food supplement integrators, solid food supplement integrators, soups, meat and fish analogues based on vegetable proteins, other spirit beverages, fruit wines, cider, perry, aromatized fruit wines; where not mentioned, maximum level may not exceed amounts mentioned for Allura Red AC ^{b,6} Processed mushy and canned garden peas (20 mg/kg) and all foodstuffs and amounts mentioned for Allura Red general use; FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts consistent with GMP; ⁸ JECFA: amount limited to 150 mg/kg in fermented milk and 100 mg/kg in baked goods ^{18,19} All foodstuffs and amounts mentioned for Allura Red general use Kippers (20 mg/kg) ⁶ All foodstuffs and amounts mentioned for Azorubine general use Permitted only for coloring skins of oranges, not intended for processing; maximum concentration is up to 2 ppm of whole fruit. ⁸
Brilliant Blue FCF	0 to 10 mg/kg bw	Cocktail and candied cherries (200 mg/kg), Bigarreaux cherries in syrup and in cocktails (150 mg/kg) ⁶ ; FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts consistent with GMP; ⁸ JECFA: can be used up to 300 mg/kg in various foods. ^{18,19} FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts consistent with GMP; ⁸ JECFA: can be used up to 100 mg/kg in various foods. ^{18,19}
Brilliant Black BN	0 to 1 mg/kg bw	—
Brown FK ^a	No ADI allocated	Specific uses: jam, jellies, marmalades, other similar fruit preparations including low-caloric products (100 mg/kg), processed mushy and canned garden peas (10 mg/kg); ^{b,6} can be used in all other foodstuffs in amounts mentioned for Allura Red general use. ^{b,6}
Brown HT	0 to 1.5 mg/kg bw	All foodstuffs and amounts mentioned for Allura Red general use. ^{b,6} FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts mentioned for Allura Red (including dietary supplements) in amounts consistent with GMP; ⁸
Citrus Red No. 2	Not to be used	JECFA: can be used up to 300 mg/kg in various foods. ^{18,19}
Erythrosine ^a	0 to 0.1 mg/kg bw	—
Fast Green FCF	0 to 25 mg/kg bw	—
Fast Red E	No ADI allocated	—
Green S	No ADI allocated	—
Indigotine	0 to 5 mg/kg bw	—

Continued.

TABLE 7.3.2 (Continued)
Synthetic Food Colorants and Their Uses as Food Additives

Color	ADI According to JECFA ^{17,18}	Utilization and Limits in Foods According to EU and US Rregulation
Lithol Rubine ^a	No ADI allocated	Edible cheese rind, <i>quantum satis</i>
Orange B	Not listed	Approved only in US; may be safely used only for coloring casings or surfaces of frankfurters and sausages, not more than 150 ppm by weight of finished food. ⁸
Patent Blue	No ADI allocated	All foodstuffs and amounts mentioned for Allura Red general use ^{b,6}
Ponceau 4R (Cochineal Red A)	0 to 4 mg/kg bw	Specific use: americano (100 mg/l), bitter and wine (100 mg/l), jam, jellies, marmalades, similar fruit preparations including low-caloric products(100 mg/kg), chorizo sausage, salchichon (250 mg/kg), sobrasada (200 mg/kg); ^{b,6} all foodstuffs and amounts mentioned for azorubine general use ^{b,6}
Red 2G ^a	0 to 0.1 mg/kg bw	Breakfast sausages with a minimum cereal content of 6% (20 mg/kg), burger meat with minimum vegetable or cereal content of 4% (20 mg/kg) ⁶
Quinoline Yellow	0 to 10 mg/kg bw	Specific use: americano (100 mg/l), bitter soda and wine (100 mg/l), jams, jellies, marmalades, similar fruit preparations including low-caloric products(100 mg/kg); all foodstuffs and amounts mentioned for Allura Red general use ^{b,6}
Sunset Yellow FCF	0 to 2.5 mg/kg bw	Specific uses: bitter soda and wine (100 mg/l), jam, jellies, marmalades, similar fruit preparations including low-caloric products (100 mg/kg), sobrasada (135 mg/kg); all foodstuffs and amounts mentioned for Allura red general use; ^{b,6} FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts consistent with GMP; ⁸ JECFA: can be used up to 300 mg/kg in various foods. ^{18,19}
Tartrazine	0 to 7.5 mg/kg bw	Specific use: americano (100 mg/l), bitter soda and wine (100 mg/l), processed mushy and canned garden peas (100 mg/kg); ⁶ all foodstuffs and amounts mentioned for Allura Red general use; ^{b,6} FDA: can be safely used generally for coloring foods (including dietary supplements) in amounts consistent with GMP; ⁸ JECFA: can be used up to 300 mg/kg in various foods. ^{18,19}

ADI = acceptable daily intake, estimate of amount of a substance in food or drinking water, expressed as mg/kg body weight, that can be ingested daily over a lifetime without appreciable risk (weight of standard human = 60 kg); bw = body weight.

^a Colorants permitted for certain uses only.

^b If colorants are used in combination, the sum of individual amounts should not exceed quantity cited in parentheses.

Fast Green FCF (FD&C Green No. 3, CI Food Green 3) is a triarylmethane dye related to Brilliant Blue, the disodium 3-[N-ethyl-N-[4-[[4-[N-ethyl-N-(3-sulfonatobenzyl)amino]-phenyl](4-hydroxy-2-sulfonatophenyl)methylene]-2,5-cyclohexadien-1-ylidene]ammonio-methyl]-benzenesulfonate. Fast green is a red to brown-violet powder or crystals, soluble in water, sparingly soluble in ethanol, with a maximum absorption in water at 625 nm. It is not permitted as food colorant in the EU.⁷⁻¹¹

Fast Red E (Red No. 4, CI Food Red 4) is a mono azo dye consisting mainly of disodium 2-hydroxy-1-(4-sulfonato-1-naphthylazo) naphthalene-6-sulfonate. It is a red-brown powder or granules, soluble in water, sparingly soluble in ethanol. It is not permitted for use in the US and EU.⁷⁻¹¹

Green S (E 142, CI Food Green 4, Brilliant Green BS) is a triarylmethane dye, with the chemical name sodium N-[4-[[4-(dimethylamino)phenyl](2-hydroxy-3,6-disulfo-1-naphthalenyl)-methylene]-2,5-cyclohexadien-1-ylidene]-N-methylmethanaminium. The calcium and potassium salts are also permitted. Green S is a dark blue or green powder or granules, soluble in water, slightly soluble in ethanol, with a maximum absorption in water at 632 nm ($E_{1\text{cm}}^{1\%} = 1720$). It is not permitted as a food colorant in the US.⁷⁻¹¹ It can also be used as biological stain.

Indigotine (E 132, FD&C Blue 2, CI Food Blue 1, Indigo Carmine) belongs to the class of indigoid dyes. Indigotine is a mixture of 3,3'-dioxo-2,2'-bi-indolyldiene-5,5'-disulfonate and disodium 3,3'-dioxo-2,2'-bi-indolyldiene-5,7'-disulfonate. The calcium and potassium salts are also permitted. Indigotine is a dark blue powder or granules, soluble in water, sparingly soluble in ethanol, with a maximum absorption in water at 610 nm ($E_{1\text{cm}}^{1\%} = 480$).^{7-9,11} It is obtained from the sulfonation of indigo, by heating with sulfuric acid. The indigo can be obtained by several chemical procedures, most commonly the fusion of N-phenylglycine (prepared from aniline and formaldehyde) in a molten mixture of sodamide and sodium and potassium hydroxides under ammonia pressure. In former times, indigo was purified from the plants of the genus *Indigofera*, but now it is almost entirely produced by chemical synthesis. Indigo is widely used as fabric and wool dye¹⁰ and has application in diagnostic procedures — evaluation of renal function and chromoscopic colonoscopy.¹⁴

Lithol Rubine BK (E 180, CI Pigment Red, Rubin Pigment, Carmine 6B) is a mono azo dye, chemical name calcium 3-hydroxy-4-(4-methyl-2-sulfonatophenylazo)-2-naphthalenecarboxylate. It is a red powder, slightly soluble in hot water (90°C), insoluble in cold water, insoluble in ethanol. The absorption maximum is 442 nm in dimethylformamide with $E_{1\text{cm}}^{1\%} = 200$. It is not permitted in the US and is restricted to cheese coloring in the EU.⁷⁻¹¹

Orange Red is a mono azo dye; its chemical is disodium salt of 1-(4-sulfophenyl)-3-ethylcarboxy-4-(4-sulfonaphthylazo)-5-hydro-xypyrazole. It has limited application, only in the US (see Table 7.3.1 and Table 7.3.2).

Patent Blue V (E 131, CI Food Blue 5, Patent Blue 5) is a triarylmethane dye, the calcium or sodium salt of 2-[(4-diethylaminophenyl)(4-diethylimino-2,5-cyclohexadien-1-ylidene)methyl]-4-hydroxy-1,5-benzenedisulfonate. It is a dark-blue powder, soluble in water, slightly soluble in ethanol. The absorption maximum is 638 nm in water, pH 5, with $E_{1\text{cm}}^{1\%} = 2000$. Patent blue is not permitted for use as

a food colorant in the US.⁷⁻¹¹ It has several applications in medicine: vital dye in chromovitrectomy¹⁵ and in labelling sentinel lymph nodes.¹⁶

Ponceau 4R (E 124, CI Food Red 7, Cochineal Red A, New Coccine) is a mono azo dye consisting essentially of trisodium d-2-hydroxy-1-(4-sulfonato-1-naphthylazo)-6,8-naphthalenedisulfonate. It is a reddish powder or granules, soluble in water, sparingly soluble in ethanol. The absorption maximum is 505 nm in water, $E_{1\text{cm}}^{1\%} = 430$. It is a suspected carcinogen and cannot be used as a food colorant in the US and other countries.⁷⁻¹¹

Red 2G (E 128, CI Food Red 10, Azogermanine) is a mono azo dye, 8-acetamido-1-hydroxy-2-phenylazo-3,6-naphthalenedisulfonate. The calcium and potassium salts are also permitted. Red 2G is a red powder or granules, soluble in water, sparingly soluble in ethanol. The absorption maximum is 532 nm in water, $E_{1\text{cm}}^{1\%} = 620$. It is not permitted as food colorant in the US. It can be used as a dye for inks, paper, fabrics, and histology stains.⁷⁻¹¹

Quinoline yellow (E 104, CI Food Yellow 13) is a quinophthalone dye consisting of a mixture of disulfonates (minimum 80%), monosulfonates (maximum 15%), and trisulfonates (maximum 7%) as sodium salts, obtained by the sulfonation of 2-(2-quinoly1)-1,3-indandione. The calcium and potassium salts are also permitted. Quinoline yellow is a yellow powder or granules, soluble in water, sparingly soluble in ethanol. The absorption maximum is at 411 nm in aqueous acetic acid solution, pH 5, $E_{1\text{cm}}^{1\%} = 865$. It is not permitted as food colorant in the US.⁷⁻¹¹

Sunset Yellow (E 110, FD&C Yellow No. 6, CI Food Yellow 3, Orange Yellow S) is a mono azo dye, essentially disodium 6-hydroxy-5-(4-sulfonatophenylazo)-2-naphthalene-6-sulfonate. The calcium and potassium salts are also permitted. Sunset yellow is an orange red powder or granules, soluble in water, sparingly soluble in ethanol. The absorption maximum is at 485 nm in water, pH 7, $E_{1\text{cm}}^{1\%} = 555$ under in the same conditions.^{7-9,11} Sunset yellow is synthesized by the coupling of 2-naphthol-6-sulfonic acid with the diazonium salt of sulfanilic acid.¹⁰

Tartrazine (E 102, FD&C Yellow No. 5, CI Food Yellow 4) is a mono azo dye containing a pyrazolone ring. The chemical name is trisodium 5-hydroxy-1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-H-pyrazole-3-carboxylate. Tartrazine is a light orange powder or granules, soluble in water, sparingly soluble in ethanol. It has an absorption maxima at 426 nm in water, $E_{1\text{cm}}^{1\%} = 530$.^{7,9} Tartrazine is obtained in a two-stage process: condensation of phenylhydrazine-*p*-sulfonic acid with sodium ethyl oxaloacetate and coupling of the product with diazotized sulfanilic acid.

7.3.3 LIMITS OF COLORANT CONCENTRATIONS IN FOODS

In the EU, the use of color additives in food was settled by two directives: 94/36/EC, which establishes the list of permitted colors, and 95/45/EC, which deals with purity criteria for colors.^{6,7} Directive 94/36/EC also contains five annexes:

1. List of permitted food colors
2. List of foodstuffs that may not contain added colors

3. List of foodstuffs to which only certain permitted colors may be added
4. List of colors permitted only for certain uses
5. List of colors permitted in foodstuffs other than those mentioned in Lists 2 and 3

The regulation is still in use, with amendments covering the purity of mixed carotene from algae, Sunset Yellow FCF, and titanium oxide.⁸

Synthetic colorants are classified by the FDA as certified color additives and are defined as synthetically produced organic molecules that have their purities checked by the FDA.^{2,4,5} A second category, colorants exempt from certification, includes naturally derived (animal, vegetal, mineral) compounds or their synthetic duplicates. Table 7.3.2 presents a summary of synthetic colorants and their utilization as food additives.

7.3.4 SYNTHETIC FOOD COLORANT FORMULATIONS

Synthetic food dyes are produced as fine powders or granules. Powders present the advantage of easy dissolution or incorporation in dry mixes but raise problems of dusting or clumping during manipulation. The granular form is easier and safer to handle, but it dissolves more slowly and cannot be incorporated into dry mixes. For certain applications, liquids, dispersions, gels, pastes, and even pre-weighted sachets containing colorants are used.^{3,4,5,19} In the forms of powders and granules, synthetic dyes show good solubility in water, propylene glycol, and glycerol. Results depend on the water percentage and temperature. In powder forms, synthetic colorants impart their color by dissolving in the product to be colored.⁴

In the food industry, synthetic dyes can be used also in the form of lakes obtained by precipitation of a soluble colorant onto an insoluble base. There are several insoluble bases, but only alumina is permitted for food application by FDA and EU regulation. All the synthetic food dyes can be obtained and used in food in the form of aluminium lakes, except erythrosine due to concerns about inorganic iodine content.

For preparing lakes, a solution of aluminium sulfate (or chloride) is mixed with sodium carbonate, forming fresh alumina $\text{Al}(\text{OH})_3$. The colorant is then added and adsorbed on the surface of alumina. Usually the content of colorant in the lake ranges from 10 to 40%.⁴ The product is filtered, washed with water, dried, and milled. The product is allowed to contain unreacted alumina but must not contain more than 0.5% HCl-insoluble matter and not more than 0.2 % ether-extractable matter.^{4,6,10} Lakes are insoluble in most solvents used for pure dyes, and they have high opacity and better stability to light and heat. Lakes impart their color by dispersion of solid particles in the food.⁴ The coloring properties of lakes depend on particles, crystal structures, concentrations of dye, etc.

Lakes have several applications in the food and pharmaceutical industries including coloration of candies and confectionery products, fats and oils, bakery products, dry mixes for powdered desserts and soups, pet foods, pill coatings, etc. The diluents used for lakes (oils, propylene glycol, glycerol, sugar syrup) must respect the regulations regarding purity, for example, conformity with FDA's GRAS (generally recognized as safe) requirement.⁴

Synthetic food colorants offer the primary colors (red, green, blue) and others offer yellow and orange. The food, textile, and cosmetic industries continue to need wider ranges of shades and hues. These can be obtained by the process of colorant blending.

Several blends of colorants were established in order to produce desired hues. To obtain orange color, one must mix the following colorants (parts per weight shown in parentheses): Allura Red (25), Tartrazine (20), and Sunset Yellow (55). Food applications must take into account the fact that various colorants have different properties or can suffer chemical modifications in the specific conditions inherent in a food product. In such cases, the blend composition and color measurements must be made in the product intended to be colored.⁴

7.3.5 SYNTHETIC COLORANT STABILITY

Synthetic colorants generally show good stability in foods. Under certain manufacturing conditions, they can lose colors, precipitate, or react with other food components (e.g., proteins) to cause color fade. The stability of synthetic colorants may also be influenced by pH, temperature, light, redox systems, and the presence of other additives or trace metals.^{4,20} Table 7.3.3 presents an overview of FD&C synthetic dyes and their stabilities under various conditions.⁴ Among other synthetic colorants permitted in the EU, amaranth shows good light and heat stability, but fades with ascorbic acid; carmoisine is stable in the presence of light, heat, and acids; Ponceau 4R is stable in the presence of light, heat, and acids, but fades moderately with ascorbic acid and SO₂; Patent Blue shows excellent light and heat stability but fades with ascorbic acid and SO₂.⁵

TABLE 7.3.3
Stability of FD&C pigments³

Colorant	pH ¹ Stability after 1 Week				Acids ²	Alkalies ²	Sulfur Dioxide ²	Ascorbic Acid ²	Heat ²	Light ²
	3	5	7	8						
FD&C Blue No. 1	sf	vsf	vsf	vsf	5	4	4	4	5	4
FD&C Blue No. 2	af	af	cf	fc	2	2	2	3	4	1
FD&C Green No. 3	sf	vsf	vsf	sf	5	2	4	4	4	4
FD&C Yellow No. 5	naf	naf	naf	naf	5	5	4	2	4	5
FD&C Yellow No. 6	naf	naf	naf	naf	5	5	4	2	3	4
FD&C Red No. 3	ins	ins	naf	naf	2	5	5	2	5	2
FD&C Red No. 40	naf	naf	naf	naf	5	4	2	2	3	5

¹ sf = slight fade, vsf = very light fade, af = appreciable fade, cf = considerable fade, fc = complete fade, naf = no appreciable fading, ins = insoluble.

² 1 = very poor, 5 = good.

³ Adapted from Francis, F.J., in *Colorants*, Eagan Press, St. Paul, MN, 1999, chap. 5.

Because synthetic pigments are unsaturated compounds, they are unstable in the presence of oxidizing or reducing agents. They fade in the presence of metal ions (Zn, Cu, Fe) at both acid and alkaline pH levels, especially at high temperatures.²⁰ Synthetic food colorants interact with food components or additives such as citric acid, acetic acid, malic acid, and tartaric acid. Among FD&C colorants, only FD&C Blue No. 2 (indigotine) shows considerable or complete fading after 1 week in 10% solutions of the above mentioned acids. Indigotine is also sensitive to alkalis; it fades with sodium bicarbonate, carbonate, and ammonium hydroxide (10% solution).

Common additives in food are sulfur S(IV) oxospecies. Quinoline Yellow and Tartrazine show excellent stability toward S(IV) oxospecies while erythrosine, Red 2G, and Green S show good stability.^{20,21} All other colorants show fair stability, except indigotine, which fades.²² In the presence of metabisulfite, Sunset Yellow FCF is degraded to a lemon yellow compound identified as 1-(4'-sulfo-1-phenylhydrazo)-keto-3,3,4-trihydronaphtalene-4,6-disulfonic acid by NMR and FAB-MS techniques.²³

The most significant interactions of synthetic dyes are those with ascorbic acid, sorbic acid, sulfur (IV) oxospecies, nitrates, and nitrites.²⁰ Synthetic colorants have good stability in the presence of sugars (cerelease, dextrose, sucrose), except for indigotine which suffers considerable fade.³ The oxidative degradation of brilliant blue FCF in the presence of potassium persulfate and under natural sunlight gives rise to dark blue compounds and finally to uncolored species identified by HPLC-MS and tandem mass spectrometry.²⁴

One of the main concerns regarding azo dyes is related to the possibility of their reduction by azoreductases, with the formation of unsulfonated aromatic amines with potential carcinogenicity.

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REFERENCES

1. Francis, F.J., Food coloring, in *Color in Food: Improving Quality*, MacDougall, D.B., Ed., Boca Raton, 2002, chap. 12.
2. Delgado-Vargas, F. and Paredes-Lopez, O., Pigments as food colorants, in *Natural Colorants for Food and Nutraceutical Uses*, CRC Press, Boca Raton, 2003, chap. 4.
3. Delgado-Vargas, F. and Paredes-Lopez, O., Inorganic and synthetic pigments, in *Natural Colorants for Food and Nutraceutical Uses*, CRC Press, Boca Raton, 2003, chap. 5.
4. Francis, F.J., FD&C colorants, in *Colorants*, Handbook Series, Eagan Press, St. Paul, MN, 1999, chap. 5.
5. Downham, A. and Collins, P., Coloring our foods in the last and next millennium, *J. Food Sci. Technol.*, 35, 5, 2000.

6. European Parliament and Council Directive 94/36/EC on colors for use in foodstuffs, *Off. J. Eur. Commun.*, L237, 10.09.1994.
7. Commission Directive 95/45/EC, July 26, 1995, laying down specific purity criteria concerning colors for use in foodstuffs, *Off. J. Eur. Commun.*, L226, 22.09.1995.
8. U.S. Food & Drug Administration, Summary of color additives listed for use in the United States in food, drugs, cosmetics and medical devices, Washington, D.C., 1999.
9. JECFA, online edition, Combined compendium of food additive specifications, <http://www.fao.org>.
10. Stern, P.W., Food, drug and cosmetic colors, in *Pigment Handbook*, Vol. 1., Lewis, P.A., Ed., John Wiley & Sons, New York, 1988, 925.
11. Food and Agriculture Organization of United Nations and World Health Organization, *Summary of Evaluations Performed by the Joint FAO/WHO Expert Committee on Food Additives* (JECFA 1956–2005), International Life Sciences Institute, Washington, 2006.
12. Commission directive 2006/33/EC, March 20, 2006, amending Directive 95/45/EC as regards Sunset Yellow FCF (E 110) and titanium dioxide (E 171), *Off. J. Eur. Commun.*, L82/10, 2006.
13. Wood, S., et al., Erythrosine is a potential photosensitizer for the photodynamic therapy of oral plaque biofilms, *J. Antimicrob. Chemother.*, 57, 680, 2006.
14. Hurlstone, D.P., et al., Indigo carmine-assisted high-magnification chromoscopic colonoscopy for the detection and characterisation of intraepithelial neoplasia in ulcerative colitis: a prospective evaluation, *Endoscopy*, 37, 1186, 2005.
15. Mennel, S. et al., Patent blue: a novel vital dye in vitreoretinal surgery, *Ophthalmologica*, 220, 190, 2006.
16. Pfutzner, W. et al., Intraoperative labeling of sentinel lymph nodes with a combination of vital dye and radionuclide tracer results in sentinel lymph node-positive patients, *J. Deutsch. Dermatol. Ges.*, 4, 229, 2006.
17. JECFA, World Health Organization, Rome, 1992.
18. JECFA, World Health Organization, Beijing, China, 2000.
19. Madkins, B. and Scafer, B., Colors: new ideas about old petfood colors, *Petfood Ind.*, 98, 24, 1998.
20. Scooter, M.J. and Castle, L., Chemical interactions between additives in foodstuffs: a review, *Food Addit. Contam.*, 21, 93, 2004.
21. Adams, J.B., Food-additive interactions involving sulphur dioxide and ascorbic acids: a review, *Food Chem.*, 59, 401, 1997.
22. Wedzicha, B.L., *Chemistry of Sulphur Dioxide in Foods*, Elsevier, Amsterdam, 1984.
23. Damant, A., Reynolds, S., and Macrae, R., The structural identification of a secondary dye produced from the reaction between sunset yellow and sodium metabisulphite, *Food Addit. Contam.*, 6, 273, 1989.
24. Gosetti, F. et al., Oxidative degradation of food dye E 133, Brilliant Blue FCF: liquid chromatography–electrospray mass spectrometry identification of the degradation pathway, *J. Chromatogr. A*, 1054, 379, 2004.

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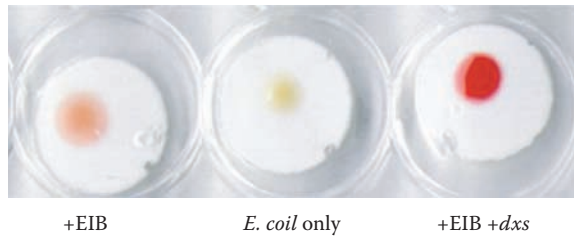


FIGURE 5.3.5 Enhancement of lycopene accumulation in *E. coli* by over-expression of DXS. Lycopene accumulation (left) is enhanced (right) when *E. coli* cells carrying a carotenoid pathway gene cassette (+EIB) are further transformed with a *dxs* gene on a multicopy plasmid (+EIB +*dxs*). Lycopene hyperaccumulation was demonstrated by Matthews and Wurtzel.²⁶¹



Spirulina for phycocyanin



Dunaliella for β -carotene



Haemafococcus for astaxanthin

FIGURE 5.4.2 Cultivation of microalgae and utilization as natural pigments.

Drawing on the expertise of internationally known, interdisciplinary scientists and researchers, **Food Colorants: Chemical and Functional Properties** provides an integrative image of the scientific characteristics, functionality, and applications of color molecules as pigments in food science and technology, as well as their impact on health. The book emphasizes the structure-function relationships of pigment molecules to examine advances in biosynthesis and biotechnology, degradation during storage and processing, and the effect of these changes on food quality and safety. Understanding the mechanisms of color formation, perception, stability, and degradation assists in selecting optimum processing parameters to ensure improved food quality and a positive impact on health.

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