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# Natural Pigments: Carotenoids, Anthocyanins, and Betalains — Characteristics, Biosynthesis, Processing, and Stability

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# Natural Pigments: Carotenoids, Anthocyanins, and Betalains — Characteristics, Biosynthesis, Processing, and Stability

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**ABSTRACT:** Pigments are present in all living matter and provide attractive colors and play basic roles in the development of organisms. Human beings, like most animals, come in contact with their surroundings through color, and things can or cannot be acceptable based on their color characteristics. This review presents the basic information about pigments focusing attention on the natural ones; it emphasizes the principal plant pigments: carotenoids, anthocyanins, and betalains. Special considerations are given to their salient characteristics; to their biosynthesis, taking into account the biochemical and molecular biology information generated in their elucidation; and to the processing and stability properties of these compounds as food colorants.

**KEY WORDS:** color, carotene, xanthophyll, betacyanin, betaxanthin, food.

## I. INTRODUCTION

Pigments produce the colors that we observe at each step of our lives, because pigments are present in each one of the organisms in the world, and plants are the principal producers. They are in leaves, fruits, vegetables, and flowers; also, they are present in skin, eyes, and other animal structures; and in bacteria and fungi. Natural and synthetic pigments are used in medicines, foods, clothes, furniture, cosmetics, and in other products. However, natural pigments have important functions other than the imparted beauty, such as the following: we could not have photosynthesis or probably life all over the world without chlorophylls and carotenoids. In animals how could oxygen and carbon dioxide be transported without hemoglobin or myoglobin? Under stress conditions plants show the synthesis of flavonoids; the quinones are very important in the conversion of light into chemical energy. The melanins act as a protective screen in humans and other vertebrates, and in some fungi melanins are essential for their vital cycle; last but not least, a lot of pigments have a well-known pharmacological activity in sickness such as cancer and cardiovascular diseases, and this has stressed pigment importance for human beings.[162,196,259,327]

Additionally, since time immemorial human beings have associated product qualities with their colors, this is especially true for meals. Historically, at the beginning of the food industry consumers did not take care about the kind of pigments used in food coloring (natural or synthetic), but recently people have shown their phobia to synthetic pigments when the concepts "synthetic pigments" and "illness" were associated, and when the attributed pharmacological benefits of natural pigments came into consideration. However, the natural pigments that are permitted for human foods are very limited, and the approval of new sources is difficult because the U.S. Food and Drug Administration (FDA) considers the pigments as additives, and consequently pigments are under strict regulations.[80,144,507,508]

Thus, an adequate understanding of the actual sources of pigments will contribute to their better use. In this review we present the basic information about pigments focusing our attention on the natural pigments. At this time, it must be emphasized the ubiquity of pigments in living organisms (plants, fungi, bacteria, among others), the variety of chemical structures, and the large quantity of information generated for each pigment group. Consequently, it is not our major goal to present a thorough review of all pigment groups. This would be impossible in a single document, and although we present here a global overview about natural pigments our work focuses on the more representative pigments in the main natural producers, namely, plants, which in addition are the more highly consumed as food products. In the following pages, we emphasize their salient characteristics; their biosynthesis, taking into account the biochemical and molecular biology information generated for their elucidation, and the processing and stability properties of these pigments as food colorants.

## II. PIGMENTS IN GENERAL

### A. Definition

Pigments are chemical compounds that absorb light in the wavelength range of the visible region. Produced color is due to a molecule-specific structure (chromophore); this structure captures the energy and the excitation of an electron from an external orbital to a higher orbital is produced; the nonabsorbed energy is reflected and/or refracted to be captured by the eye, and generated neural impulses are transmitted to the brain where they could be interpreted as a color.[196]

### B. Classification

#### *1. By Their Origin*

Pigments can be classified by their origin as natural, synthetic, or inorganic. Natural pigments are produced by living organisms such as plants, animals, fungi, and microorganisms. Synthetic pigments are obtained from laboratories. Natural and synthetic pigments are organic compounds. Inorganic pigments can be found in nature or reproduced by synthesis.[29]

#### *2. By the Chemical Structure of the Chromophore*

Also, pigments can be classified by taking into account the chromophore chemical structure as:[510]

Chromophores with conjugated systems: carotenoids, anthocyanins, betalains, caramel, synthetic pigments, and lakes.

Metal-coordinated porphyrins: myoglobin, chlorophyll, and their derivatives.

### 3. By the Structural Characteristics of the Natural Pigments

Moreover, natural pigments can be classified by their structural characteristics as:[29,196]

Tetrapyrrole derivatives: chlorophylls and heme colors.
Isoprenoid derivatives: carotenoids and iridoids.
*N*-heterocyclic compounds different from tetrapyrroles: purines, pterins, flavins, phenazines, phenoxazines, and betalains.
Benzopyran derivatives (oxygenated heterocyclic compounds): anthocyanins and other flavonoid pigments.
Quinones: benzoquinone, naphthoquinone, anthraquinone.
Melanins.

### 4. As Food Additives

By considering the pigments as food additives, their classification by the FDA is[150,162,510]

**Certifiable.** These are manmade and subdivided as synthetic pigments and lakes.

**Exempt from certification.** This group includes pigments derived from natural sources such as vegetables, minerals, or animals, and manmade counterparts of natural derivatives.

## III. NATURAL PIGMENTS

## A. Distribution

### 1. Tetrapyrrole Derivatives

These compounds have a structure with pyrrole rings in linear or cyclic arrays. Figure 1 shows some common structures of tetrapyrrole derivatives. Phytochrome is very common in algae *(Rhodophyta*, *Cryptophyta*), and bilin is this basic structure (lineal array). In the cyclic compounds, we can mention the heme group (the porphyrin ring is bonded to an iron atom); this group is present in hemoglobin and myoglobin, present in animals, and also in cytochromes, peroxidases, catalases, and vitamin $B_{12}$ as a prosthetic group, all of them with a wide distribution. However, chlorophylls constitute the most important subgroup of pigments within the tetrapyrrole derivatives. Chlorophyll is mainly present in the chloroplasts of higher plants and most algae. The number of known chlorophyll structures have shown increments through the years: up until 1960 only three structures had been described, 20 up until 1970, and more than 100 up until 1980. Higher plants, ferns, mosses, green algae, and the prokaryotic organism prochloron present only two chlorophylls ("a" and "b"), and the rest of them have been found in other groups such as algae and bacteria.[60,87,196,279,404]

### 2. Isoprenoid Derivatives

Isoprenoids, also called terpenoids, represent a big family of natural compounds; they are found in all kingdoms where they carry out multiple functions (hormones, pigments, phytoalexins). Over 23,000 individual isoprenoid compounds have been identified and many new structures are reported each year. By their abundance and structure, two subgroups of compounds are considered pigments: quinones and carotenoids. However, in addition, and only recently, iridoids is a third group of plant isoprenoid compounds that have acquired some relevance. Quinones are considered another group because not all of them are produced by this biosynthetic pathway. On the other hand, carotenoids as a major point of our review are described below. In relation to iridoids, these are found in about 70 families (Capriofilaceae, Rubiaceae, Cornaceae, among others) grouped in some 13 orders. Saffron *(Crocus sativus* L.) and cape jasmine fruit (Gardenia jasminoids Ellis) are the best-known iridoid-containing plants, but their colors are more importantly influenced by carotenoids.[406]

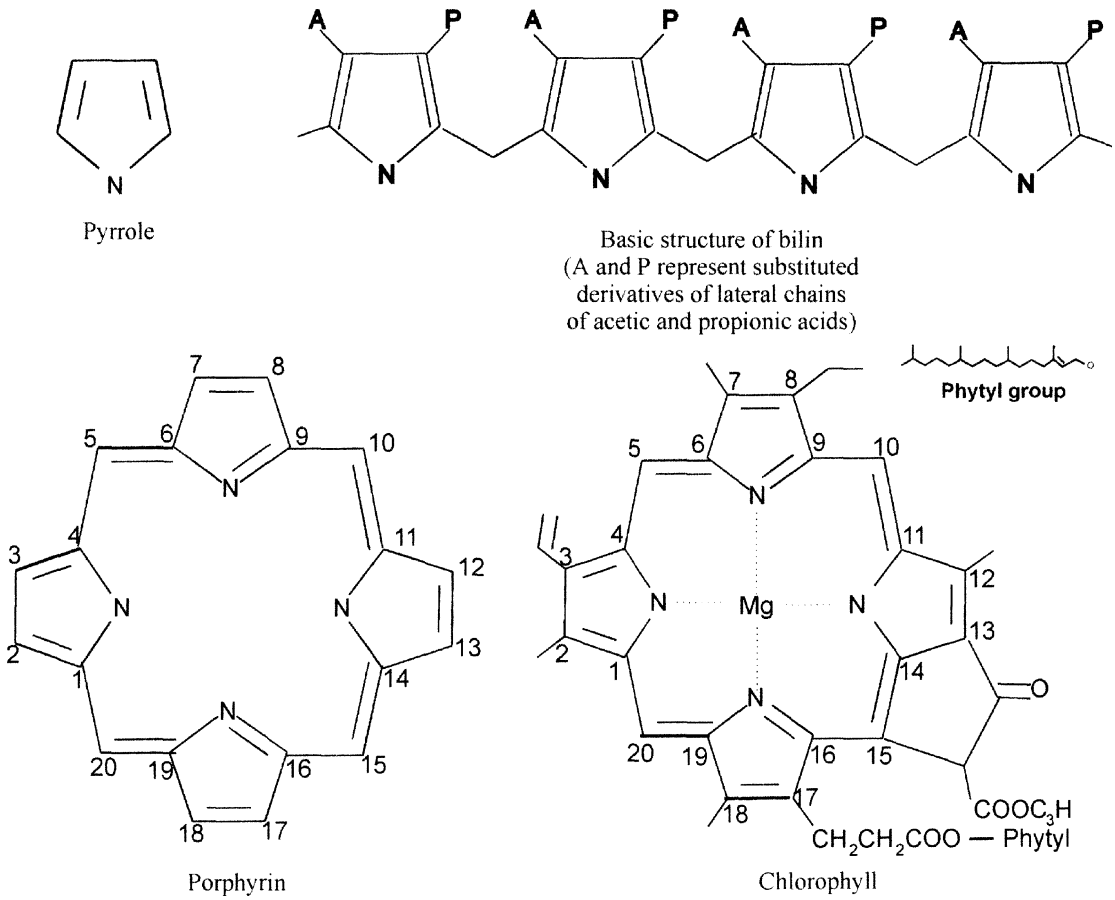


**FIGURE 1**. Basic structure of porphyrinic pigments (pyrrol) and of some porphyrinic pigments with biological importance.



### *3. N-Heterocyclic Compounds Different from Tetrapyrroles*

Figure 2 shows some structures for compounds of this group.

#### *a. Purines*

As most of the nucleotides, purines are found in two macromolecules: deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). These molecules are an essential component of life, thus they are present in each living organism. Free purines have been found in animals (golden and silvery fish).[196]

#### *b. Pterins*

The pteridin ring system is probably present in every form of life. Most of the natural pteridins have an amino group at C-2 and an hydroxyl group at C-4. Also, 2,4-dihydroxipteridins have been described as important components in the flavin biosynthesis. Pterins are responsible for color in some insects, in vertebrate eyes, human urine, and bacteria *(Lactobacillus casei* and *Streptomyces faecalis* R.).[152,196]

#### *c. Flavins*

In these compounds a pteridin and a benzene ring are condensed. Riboflavin is the main

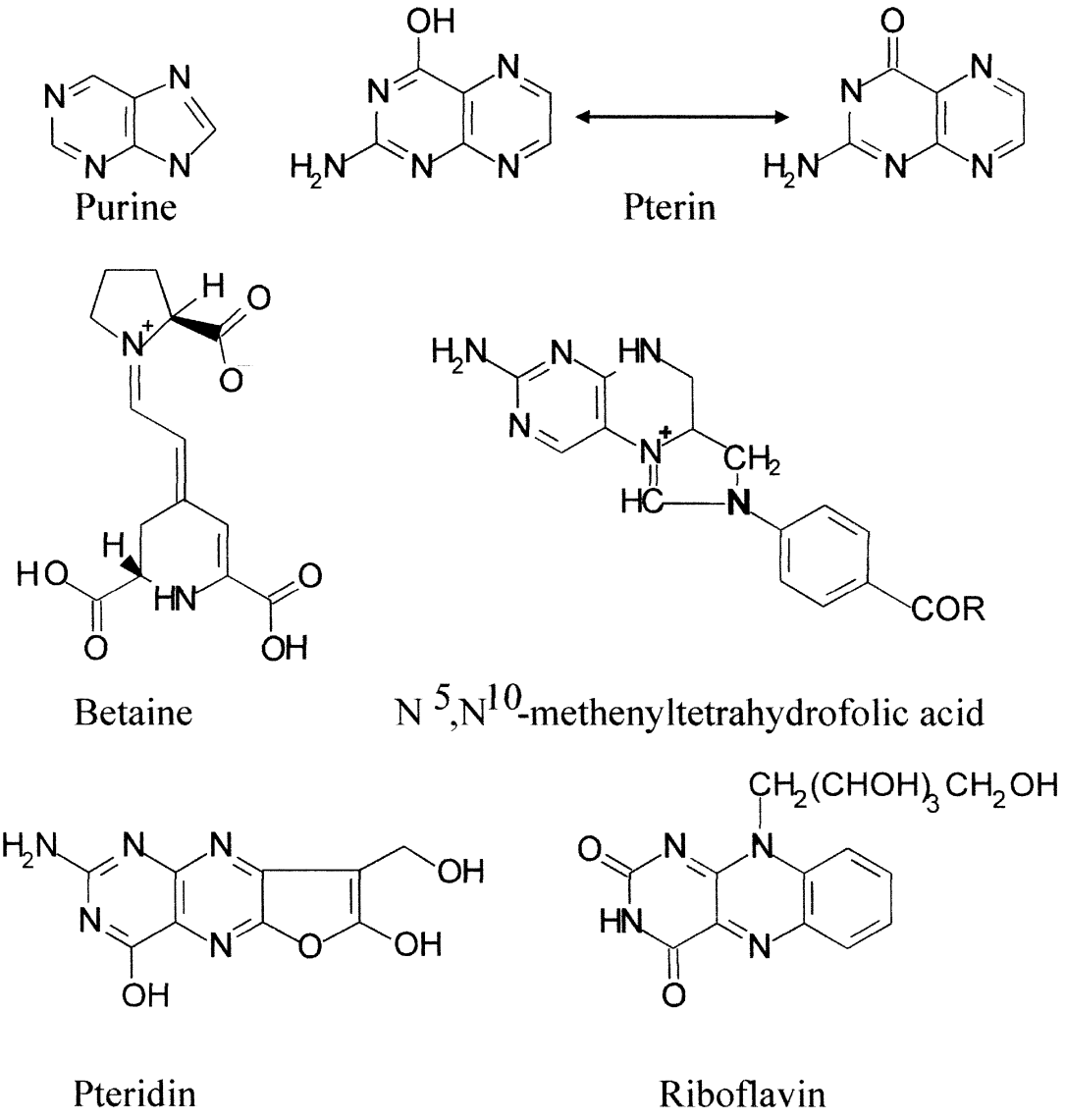


**FIGURE 2.** *N*-heterocyclic compounds.

compound of this group, and it is synthesized in all live cells of microorganisms and plants. Riboflavin is found in milk. Other sources are a wide range of leafy vegetables, meat, and fish.[87,152,196]

### *d. Phenazines*

They are found in bacteria.[196]

### *e. Phenoxazines*

They are found in fungi and insects.[196]

### *f. Betalains*

As a main component of our review, this subgroup of compounds are discussed below.

## *4. Benzopyran Derivatives*

The most studied secondary metabolites are the flavonoids. These are phenolic compounds with two aromatic rings bonded by a C3 unit (central pyran ring) and divided in 13 classes based on the oxidation state of the pyran ring and on the characteristic color: anthocyanins, aurons, chalcones, yellow flavonols, flavones, uncolored flavonols, flavanones, dihydroflavonols, dihydrochalcones, leucoanthocyanidins, catechins, flavans, and isoflavonoids. Figure 3 shows some flavonoid structures. Each type of flavonoid can be modified by hydroxylation, methylation, acylation, and glycosylation to obtain a great natural diversity of compounds. Flavonoids are water soluble and show a wide distribution in vascular plants. They are present in each part of the plant. More than 5000 flavonoids have been chemically characterized, and new structures are described continuously.[194,259]

In the flavonoids, the anthocyanins are the most important pigments; they produce colors from orange to blue in petals, fruits, leaves, and roots and are discussed below. Flavonoids also contribute to the yellow color of flowers, where they are present with carotenoids or alone in 15% of the plant species.[259]

The aurones are present in flowers *(Bidens* sp., *Cosmos* sp., *Dahlia* sp.), wood *(Rhus* sp., *Schinopsis* sp.), bryophytes *(Funaria hygrometrica*), and in Cypreceae plants. They are more common in inflorescences, seeds, and leaves. The chalcones are common in mixtures with aurones to generate the anthclor pigment in the Compositae). In nature, it is uncommon to find them without substitutions, but hydroxylated have been described in wood and peels of trees in the genus *Acacia, Rhus, Macherium,* and *Adenanthera*. Flavonols are ubiquitous in woody angiosperms, common in shrubby angiosperms, and exceptionally in inferior plants. Flavones and flavanones are found free or glycosylated in leaves of angiosperms. Flavanones are especially common in Rosaceae, Rutaceae, legumes and Compositae. Dihydrochalcones are found mainly hydroxylated in apple and in some species of Rosaceae, Ericaceae, Fagaceae, legumes, and Salicaceae. Leucoanthocyanidins (flavan 3,4-diols) are widely distributed in plants, and they have been isolated from wood and peel of trees (particularly *Acacia*) and methylated, and C-alkylated leucoanthocyanidins have been identified in a variety of sources (e.g., *Neuroautanenia amboensis*, *Marshallia* sp.). Catechins flavan 3-ols) and flavans are found mainly in leaves. Catechins and epicatechins are among the commonest flavonoids known, sharing a distribution almost as widespread as quercetin in the Dicotyledoneae. The 3,4,5-trihydroxy B-ring flavan 3-ols gallocatechin and epigallocatechin have also a wide distribution (paralleling myricetin), especially in more primitive plants (the Coniferae being outstanding). Many flavans are lipid soluble and appear to be leaf-surface constituents. Isoflavonoids have the B ring in C-3 instead of C-2, and many natural products are in this group: isoflavones, rotenoids, pterocarps, and coumestans. Isoflavones are the most common in legumes, especially in Lotoideae, although they have also been reported in Amaranthaceae, Iridicaceae, Miristicaceae, and Rosaceae.[29,157,193,259]

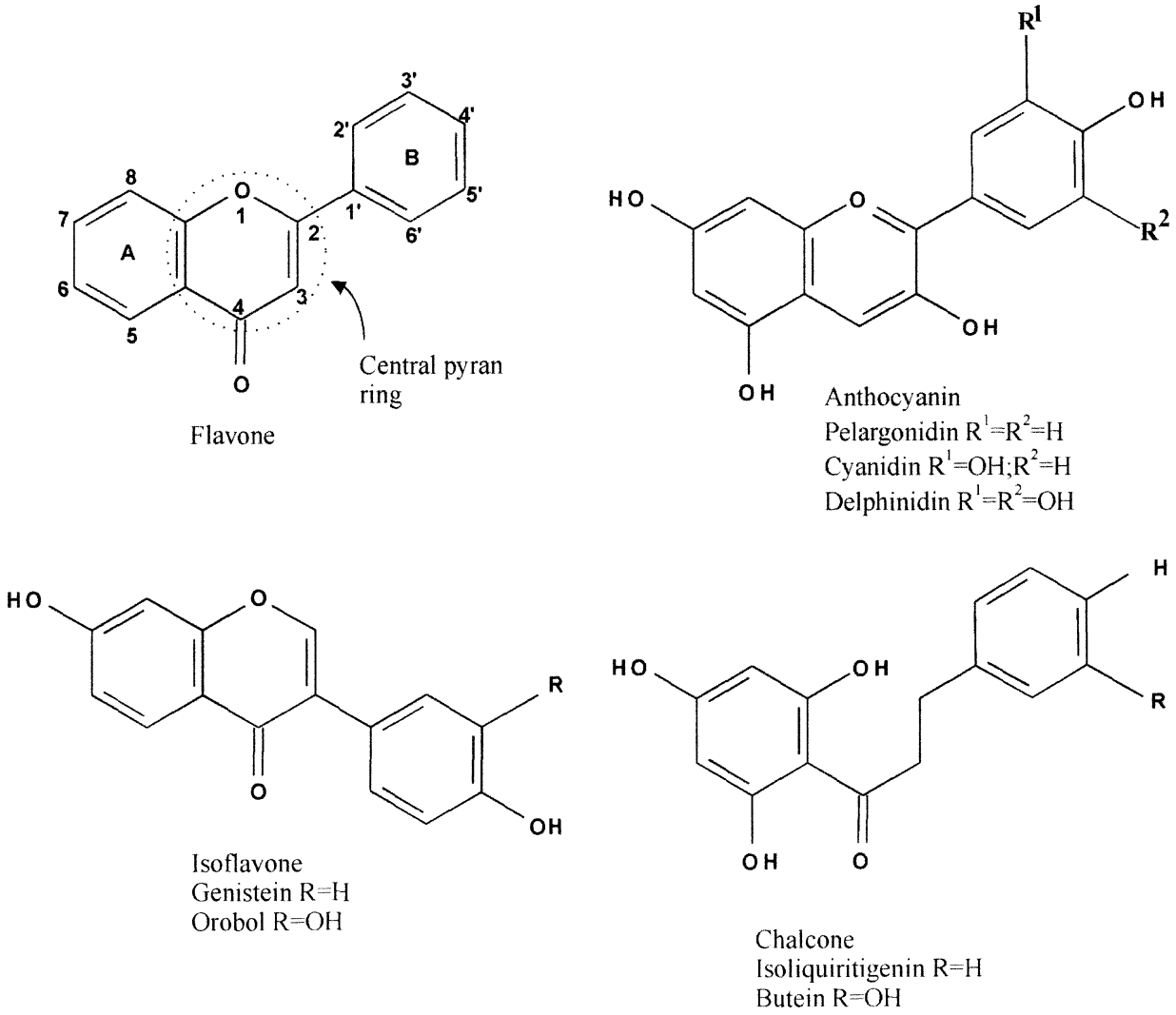


**FIGURE 3.** Benzopyran derivatives.

### 5. Quinones

Quinones have a great number of coloring compounds (Figure 4). This group is the biggest one in number and structural variation. Also, they are more widely distributed than other natural pigments (with the exception of carotenoids and melanins). The basic structure consists of a desaturated cyclic ketone that is derived from an aromatic monocyclic or polycyclic compound. Quinones can be divided by their structure as benzoquinones, naphthoquinones, anthraquinones, and miscellaneous quinones; moreover, dibenzoquinones, dianthraquinones, and dinaphthoquinones have been reported. The variability in the kind and structure of substituents conduce to large number of quinones. Quinones are found in plants: plastoquinones are found 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. Many quinones are byproducts of the metabolic pathways and a few organisms (fungi) produce large quantities. In general, quinones produce yellow, red, or brown colorations, but quinone salts show purple, blue, or green colors.[196,464]

### 6. Melanins

Melanins are nitrogenous polymeric compounds whose monomer is the indole ring (Figure 5). In general, melanins are not homopolymers but present a mixture of macromolecules. Melanins are responsible of many of the black, gray, and brown colorations of animals, plants, and microorganisms. Eumelanins are widely distributed in vertebrate and invertebrate animals. Phaemelanins are macromolecules of mammals and birds. Allomelanins have been described in seeds, spores, and fungi, and esclorotins in arthropods.[64,196,465]

## B. Functions

Up to 1898 the increased interest on color in organisms was due to three main reasons: (1) the color phenomena is conspicuous for the survival of animals and plants; (2) the relation between color and evolution theories; and (3) their importance in comparative physiology. Thus, the studies on pigments were greatly impulsed by their multiple functions.[396]

In agreement with the distribution and abundance, the most important pigments in higher plants were chlorophylls, carotenoids, and anthocyanins when considering their capacity to impart colors. By the same criteria, algae phycobilins are considered important pigments. Other pigments have less importance as colorant substances because they are present in low quantities.[465] In the following paragraphs we describe other functions of this colorant substance.

### 1. Tetrapyrrole Derivatives

The heme groups show chemical combination with metals (Fe, V, Cu, Mg) to form metal porphyrins. The metal atom is bonded to the nitrogen atoms of the heme group by a coordination bond. The most important metal porphyrins are formed with Fe. In these compounds, nature has exploited the change of the valence states of iron from ferric ($Fe^{+3}$) to ferrous ($Fe^{+2}$) and vice versa to establish a system for electron transport. This system gives the connection between the intracellular dehydrogenases with atmospheric oxygen and in consequence the support for the transport and storage of oxygen, an essential component for life. Additionally, the bonding of metal to cytochrome permitted joining the air oxygen with the metabolic substrates in the cell, contributing with electron transportation, and consequently providing the energy for vital processes.[396] Catalase and peroxidase are enzymes with an heme cofactor and have a participation in reduction-oxidation (redox) reactions of living organisms. Vitamin $B_{12}$ with its heme group participates in chemical-biological reactions that involve rearrangements.[196]

The most important function of chlorophylls is in the photosynthetic process. Chlorophylls are porphyrins and have a phytyl group conferring on them hydrophobic characteristics. The metal bonded in chlorophylls is magnesium in-

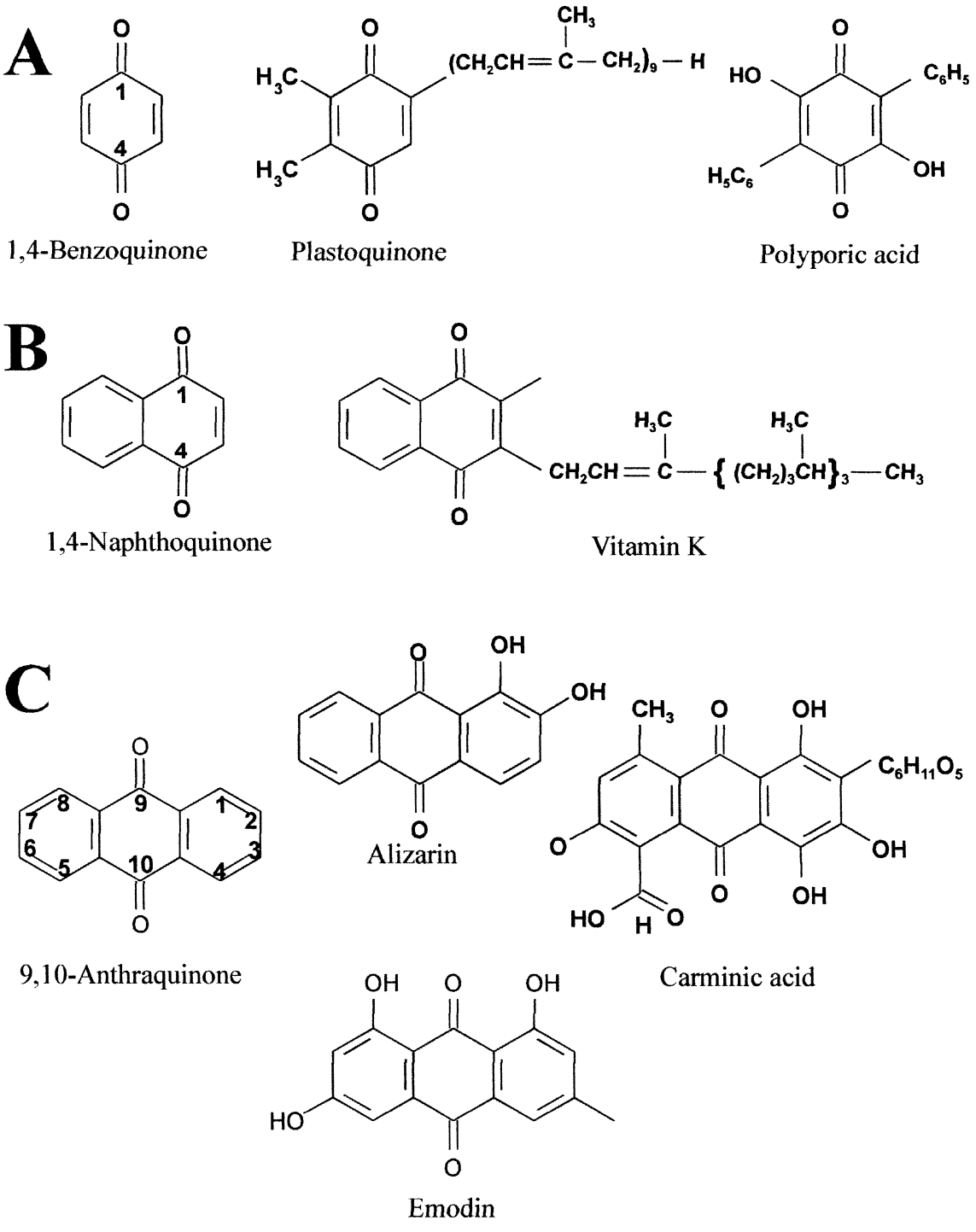


**FIGURE 4**. Basic structures of Quinones (A, B, and C) and some of their most known compounds.

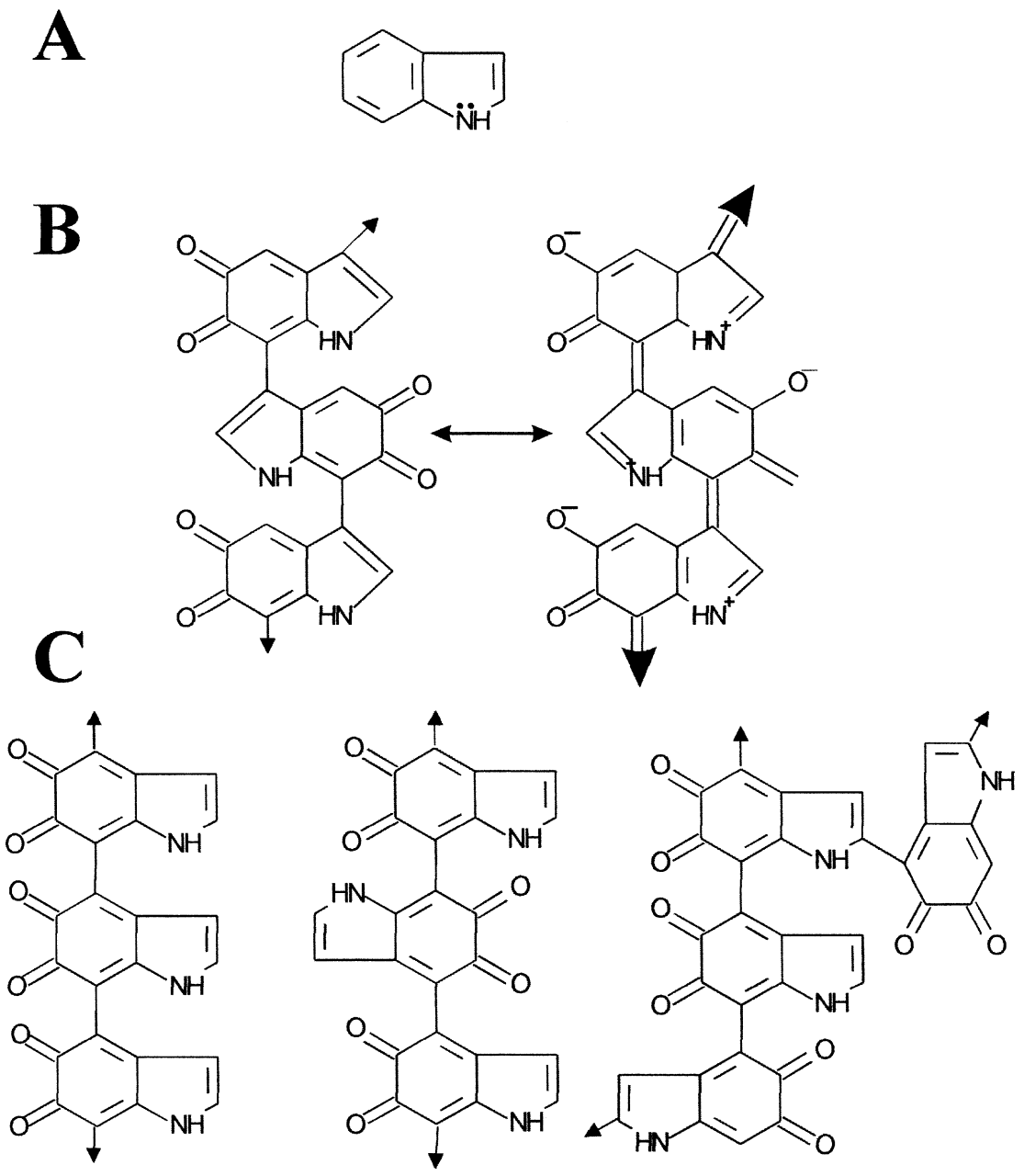


**FIGURE 5.** Melanin-related structures and some properites. (A) Basic structure (indolic ring). (B) Resonance structures that are probaly involved in the process of color. (C) Some of the suggested forms of indolic polymerization to form melanins. The arrows show the points and sense of polymerization.

stead of the iron in the heme. These compounds absorb light in the visible region, red (peak at 670 to 680 nm) and blue (peak at 435 to 455 nm). The reflection and/or transmittance of the nonabsorbed green light (intermediate wavelength) gives the characteristic green color of plants and chlorophyll solutions. Light absorption is carried out by the porphyrin ring and in the absorption process a π-electron is excited to the higher energy π*-orbital. Part of the luminosity energy is absorbed by the chlorophyll molecules, where it is transformed into biochemical energy. Normally, in this process an electron is lost and transferred to an acceptor molecule. This process needs highly specialized chlorophyll molecules that are associated with membranes (reaction centers $P_{680}$ and $P_{700}$). A small portion of chlorophyll molecules shows this function, but the reaction centers are the starting point of the chain of electron transportation that will finally produce the energy in the form of ATP and $NADPH_2$, and these molecules support all the plant metabolism. Photosynthesis as known nowadays cannot be possible without chlorophyll. Thus, chlorophyll is a very important molecule for life all over the world.[404]

Among the linear tetrapyrroles, bilins pigment the integuments of many invertebrates (worms and insects). In algae, the function of phycobilin is similar to chlorophyll as accessory pigments in light harvesting, transmitting this energy to chlorophyll molecules in the photosynthetic membrane. It has been described that pigment content in cyanobacteria and some algae is regulated by light.[196]

Phytochrome is a blue-green photochromic pigment and controls a wide variety of metabolic and developmental processes in green plants. The chromophore of phytochrome is a lineal tetrapyrrole and has been shown that the activity of many enzymes is regulated by phytochrome at biochemical and transcriptional level (mRNA species). Phytochrome is involved in the germination, flowering, ripeness, anthocyanin, and protein synthesis.[196]

## 2. N-Heterocyclic Compounds Different from Tetrapyrroles

The main function of purines is to serve as structural components in DNA and RNA, where all the information for the survival of organisms is described. Some pterins are growth factors: rhizopterin *(Streptomyces faecalis*), pteroylglutamic acid *(Lactobacillus casei* and chickens), other growth factors are teropterin, vitamin $B_c$, folinic acid, and probably the most important pterin is folic acid, which is an essential vitamin involved in the transference of methyl groups and in the insertion of two carbon atoms in the pyrimidine ring.[152,196] Flavins are in the molecule of flavinadenin mono- (FMN) and di-nucleotide (FAD), important molecules in the redox reactions of organisms, where they are found. FMN and FAD are coenzymes of many enzymes (e.g., nitrate reductase, pyruvate decarboxylase). Riboflavin is an essential vitamin in animals.[152] Phenazines are in bacteria and have bacteriostatic properties. Phenoxazines are in fungi and insects; for example, *Streptomyces* sp. produces actinomycin (antibiotic).[196]

## 3. Benzopyran Derivatives

### a. Antioxidant

Several reports have shown the antioxidant activity of flavonoids, which is important in the development of their functions such as scavenging of radicals and disease treatment and prevention. Rutin inhibited malonaldehyde formation from ethyl arachidonate by 70% at the level of 0.125 μmol. With ethyl linoleate, naringin, galangin, and rutin exhibited dose-related activities and showed inhibitions of 30% at 0.5 μmol. It has been pointed out that the major mode of flavonoids as antioxidants is in their ability to scavenge free radicals, and that hydroxylation of the B-ring is an important contributor to such activity; in particular, hydroxyl groups at the 3′- and 4′- positions of the B-ring exhibited the highest antioxidant activity, but it was lower than the observed with BHT.[484] It was shown that luteolin has good antioxidant activity evaluated by the β-carotene bleaching method, and it was suggested that C2-C3 double bond and C4 keto group seem to be essential for high antioxidant activity (quercetin > (+)-catechin). Also, it was shown that aglycone flavonoids are more potent antioxidants. However, luteolin did not exhibit quality antioxidant activity

with the α,α-diphenyl-β-picrylhydrazyl (DPPH) method, and quercetin and other flavonoids were better, and a similar trend was obtained with the Rancimat method.[484] Another study used 18 different intestinal bacteria to metabolize eriocitrin (eriodictyol 7-rutinoside), which is an antioxidant in lemon fruit. It was shown that in a first step eriocitrin is transformed to eriodictyol by different bacteria except *Clostridium* spp., but from eriodictyol *Clostridium* spp. produced 3,4-dihydroxyhydrocinnamic acid and phloroglucinol. Interestingly, eriodictyol showed higher activity than eriocitrin or α-tocopherol.[324] The antioxidant activity of wines has also been evaluated, and it has been reported that almost 96% of the activity can be explained by the content of catechin, *m*-coumaric acid, epicatechin, *cis*-polydatin, and vanillic acid, showing higher correlations with several flavonoids (catechin, myricetin, quercetin, rutin, epicatechin, cyanidin, and malvidin 3-glucoside). Wine flavonoids also show a good peroxynitrite scavenging activity; this was demonstrated by removing the activity with the elimination of these compounds, an interesting aspect of the antioxidant profile of wine.[356]

### b. Color and Sexual Processes in Plants

Most of the pigments in flowers are flavonoids, and they impart colors in the range red or purple associated with anthocyanins to yellow associated with aurones and chalcones. Also, flowers contain flavonols and flavanones that by themselves do not have colors, but modify the coloration by complexation with anthocyanins and/or metallic ions (copigmentation). The coloration patterns of flowers attract insects to anthers (masculine organ) and pistils (feminine organs) and contribute to the pollinating process, and as a matter of fact it is well known that many species accumulate flavonoids in these organs. The most common flavonoids in anthers are anthocyanins, flavonols, and chalcones. Also, flavonoids have been involved in the sexual process: in the cross- pollination of two varieties of forsythia, rutin, and quercetin are required. Another important aspect: animals are attracted by fruit color, and after the consumption the seeds are dispersed through the excrement.[193,259,455] It has been established that flavonols are required to induce pollen germination *in vivo* and *in vitro*. Some of the structural requirements of compounds to develop this function are a double bond between carbons two and three, a keto group at carbon four, and a hydroxyl group at carbon three of the heterocycle, and only the flavonol class has all these features. It has been established that phenylalanine ammonia-lyase (PAL) might play an essential role in the development of microspores to mature grains. The introduction of the sweet potato PAL into tobacco generated transgenic plants with reduced pollen fertility (10 to 45%). It was suggested that pollen reduction in PAL activity could reduce the levels of flavonols and a reduction of levels of sporollenin of the pollen cell walls, and consequently affect the development, germination, and growth of pollen tubes in tobacco. Thus, PAL activity in the anther tapetum is a significant factor in the development of pollen.[301] p100 cDNA of *Solanum tuberosum* is up-regulated in pistils after pollen tube growth and, much more transiently, by touching the stigma. This cDNA clone shows homology with various isoflavone reductase-like sequences that have been involved in the response to pathogen attack and stress conditions. In potato pistils was shown that not only pollination but also the physical presence of the pollen tubes in the pistil is a constant stimulus for expression. An antioxidant protective activity has been proposed for p100 because oxygen radicals are natural byproducts of metabolism, and a high metabolism is required for the fast-growing pollen tubes.[483]

### c. Photoprotection

Severe illumination produces and oxidative stress in plant tissues and flavonoids protect them from damage. One of the responses of UV-illuminated seedlings is the transcriptional activation of flavonoid biosynthetic genes. Also, it must be considered that UV-B radiation itself modify the pigment composition and consequently induces a reduction of the radiation levels over plant tissues. Moreover, it has been established that better UV-B photoprotectors are the noncolored flavonoids (flavones, flavonols, and isoflavonoids). Additionally, it is known that the production of pyrimidine dimers and 6,4-photoproducts

is higher in plants without flavonoids.[196,259,327] Middleton and Teramura[314] showed that flavonoids protect soybean but without an effect on the photosystems, and they suggested that carotenoids develop this function. In 1992, it was shown that quercetin and its glycoside (rutin), acting as antioxidants, reduced the rate of formation of malondialdehyde and lipid peroxidation in chloroplast thylakoids of wheat under severe illumination; it was suggested that flavonoids acted by trapping the singlet oxygen produced in the biological reactions.[96]

### *d. Defense Mechanism in Plants*

When attacked by pathogens, some plants shoot their flavonoid biosynthesis (phytoalexins, as those from *Broussonetia papyrifera* and *Narcissus pseudonarcissus*); interestingly, these flavonoids are different to those induced under severe illumination. Sorghum *(Sorghum bicolor*) synthesizes the phytoalexins apigeninidin and luteolinidin (deoxyanthocyanidin flavonoids) as a response to attempted infection by the fungus *Colletotrichum graminicola* that causes the anthracnose disease. It was reported that susceptible plants lose the ability to respond rapidly to fungal infection. It has been observed that after synthesis of phytoalexins in inclusions within the cell under attack, the pigments released were observed to accumulate in the fungus.[443]

The major rice pathogens are *Xanthomonas oryzae* pv. *Oryzae* (the bacterial blight pathogen), *Pyricularia oryzae* (the fungal blast pathogen), and *Rhizoctonia solani* (the fungal sheath blight pathogen); several flavonoids with variable activity have been reported. Naringenin inhibited the growth of *X. Oryzae,* naringenin, and kaempferol, the spore germination of *P. oryzae*. It was established that nonpolar flavonoids showed higher inhibition than their polar counterpart, but none of the tested compounds showed inhibition to *Rhizoctonia.* Also, cyanidin and peonidin glycosides (anthocyanins) showed growth inhibition of *Xanthomonas*.[354] Downy mildew caused by *Plasmopara viticola* (Berk et Curt.) is an important disease threatening the world's viticultural areas. Three *Vitis* sp.*(V. vinifera* cv. Grenache, susceptible; *V. rupestiris* cv. du Lot, intermediate resistant; and *V. rotundifolia* cv. Carlos, resistant) were studied. It was determined that the flavonoid compounds were induced following infection by *P. viticola* in both the resistant and in the intermediate resistant species. However, the kind of flavonoids was different. In the resistant species, necrosis of the stomatal tissues occurred after infection, resulting in the cessation of fungal growth of the tissue. This response was also observed in the species with intermediate resistance, but response time was longer in the last one. Thus, it is clear that flavonoids play a key role in the high resistance of *V. rotundifolia* and that precocity in the resistance of *Vitis* sp. to *P. viticola* is very important.[111] Flavonoids of sour cherries were extracted and assayed against the ascomycete *Leucostoma persoonii* that causes the perennial canker on the bark of peach, plum, and sweet cherry. In general, all tested flavonoids slowed down the mycelial growth. It was shown to have significant differences in the toxicity of aglycones and glucosides, showing higher toxicity the aglycones; therefore, suggesting glucoside hydrolysis as a defense reaction. Naringenin and chrysin showed the highest fungistatic effects.[167] *Erwinia carotovora* causes blackleg and soft-rot of potatoes by macerating activity in the host tissue. The main enzymes of this bacterium are pectate lyases (PL), and the transformation of potato with PL was used as a protection mechanism. It was determined that transgene was expressed in different tissues and tuber disks of transformants inhibited the *Erwinia* growth. Interestingly, a strong induction of PAL was observed in the transformant with the high inhibitory effect that was a marker for the activation of defense-related genes. Thus, the resistance might be based on the liberation of phenolic compounds and phenol oxidases.[501] Isolated in *Arabidopsis thaliana* was a sulfotransferase cDNA (RaR047) that regulation is developmentally regulated and associated with stages of active plant growth. RaR047 was induced under stress or pathogen attack. RaR047 showed a maximal expression in the incompatible interaction between 3 and 7 days after inoculation for *Xanthomonas campestris* pv. *campestris* and between 7 and 24 h after inoculation with *Pseudomonas syringae* pv. *maculicola*. It was suggested that sulfotransferase could be involved in the molecular communication between the inter-

acting organisms; in particular, it was proposed that the RaR047 product participates in the synthesis of toxic compounds directed against the pathogen during the resistance reaction.[268]

Several studies have been carried out to induce virus resistance, and flavonoids have shown antiviral activity against tomato ringspot virus (TomRSV). The most effective compounds were quercetin, quercetin 7,4′-dimethyl ether, quercetin 3,7,4′-trimethyl ether, and fisetin 4′-methyl ether, causing a 67 to 76% inhibition of TomRSV infectivity when applied at 10 μg $ml^{-1}$. Quercetin did not affect viral replication. When quercetin was used in combination with RNA, an increment in the infectivity was observed. Thus, it was concluded that inhibitory effect of quercetin is dependent on the presence of the coat protein for antiviral activity, possibly by the inhibition of uncoating. Flavonoids are proposed as a class of compounds with potent antiviral activity that can be used to reduce or eliminate viruses from plant material.[294] On the other hand, leucoanthocyanins are accumulated in some seeds and make them less attractive for the consumption by animals or the attack of other pests. Additionally, these leucoanthocyanins provide a seed protection against digestive enzymes, permitting that after the excretion the seed can germinate. The flavan from *Lycoris radiata* bulbs was found to be antifeedant for the larvae of the yellow butterfly *Euroma hecabe mandarina*.[193,259,455] Studying the root bark and stem wood of *Erythrina sigmoidea*, the methylene chloride extract was found to be active against the Gram-positive bacteria *Staphylococcus aureus*. After successive purifications, it was isolated with the bioactive flavonoid neobavaisoflavone, which showed a good inhibitory action compared with streptomycin sulfate.[346] Also, it has been established that proanthocyanidins are the main components of tannin. In particular, bark procyanidins are dominant (Monterrey pine 50%, Scots pine 75%, and birch 65%), being prodelphinidins in a higher proportion. It has been suggested that proanthocyanidins contribute to protecting internal plant tissues (phloem, cambium, and xylem) against invasion by pathogens because they are particularly abundant in the more exposed outer bark.[302]

It was proposed that flavonoids isolated from *Helianthus annuus* possibly play roles in the allelopathic activity of sunflower. They principally influence, the shoot growth of seedling, but germination and radical length can be affected by chalcones. Tambulin did not show any effect against germination and radical length of tomato and barley but inhibited shoot growth of tomato (approximately 25%) and barley (22%). Kukulkanin B and heliannone A differ in the presence of an additional methyl group in heliannone A; however, their activities are quite different. Heliannone A inhibited germination of tomato (20%) and barley (35%) and slightly affected barley shoot growth length. On the other hand, kukulkanin B only showed inhibitory activity on the shoot growth of tomato.[290]

### *e. Other Ecological Functions*

Studying the loblolly pine *(Pinus taeda)* needles, it was shown that increments in catechin and proanthocyanidin concentration are generated by the effect of $O_3$ exposure. It was suggested that these increments are an adaptive response by acting as an antioxidant, and it was proposed that elevated catechin levels might also be a suitable biochemical indicator of forest damage.[51]

Flavonoids are important compounds used as ecological indicators.[169] It has been reported considerable quantitative and qualitative differences of flavonoids in propolis and has been concluded that such differences were dependent mainly on plant ecology and the variety of the bee.[261] Moreover, it has been established that bee pollen can be used to identify their plant origin, for example, the major compound in almond bee pollen is 8-methoxykaempferol 3-glycoside, while jara bee pollen contained mainly quercetin and isorhamnetin 3-glycosides. Thus, flavonoids can be used as a markers of pollens.[470] The flavonoid profile has been used to evaluate the evolution in the Phaseolinae: it was determined that kaempferol and quercetin glycosides are the main flavonoids, whereas the glycosidic pattern was 3,7-*O*-diglycoside and 3-*O*-glycoside. These features represent intermediate to advanced evolutionary characters among the Phaseolinae flavonoids. The flavone frequency was higher than in other subtribes, thus indicating a highly advanced position in the subtribe Phaseolinae within the subfamily Papilionoideae, which was sup-

**TABLE 1**
**Pharmacological Activities of Some Flavonoids**

| Flavonoid | Activity |
|---|---|
| Rutin, silibin | Against disorders of the respiratory system |
| Naringenin | Antimicrobial and antifungic on skin |
| Quercetin, morin, procyanidin, pelargonidin | Antiviral |
| (+)-Catechin, 3-*O*-Metil-(+)-Catechin, naringenin | Against ulcers |
| Butrin, isobutrin | Hepatic disorders and viral hepatitis |
| Chisin, floretin, apigenin, quercetin, kaempferol, baicaelin | Antiinflammatory |
| Epicatechin | Against diabetes |
| Genistein, kaempferol, sophoricoside | Antifertility |
| Galangin | Activity against *Staphylococcus epidermis* |
| Proanthocyanidins | Astringent for digestive system, diuretic, cardiac tonic, in the treatment of high blood pressure |

Adapted from Refs. 10, 198, 509.

ported by the C-glycosylation and C-acylation profiles, and it was concluded that flavonoids of these Phaseolinae species correspond to an intermediate evolutionary level.[522]

On the other hand, leaf waxes of 32 *Aeonium* species were obtained, and 32 flavonoids were identified as methyl ethers of kaempferol, 6-hydroxykempferol, quercetin, myricetin, and scutellarein (6-hydroxyapigenin). It was suggested that waxes of different plants of the same species contained the same principal flavonols, with intraspecific variation limited to the minor flavonoids. It was determined that in the *Aeonium* genus the occurrence of myricetin methyl ethers and 6-hydroxykaempferol methyl ethers is taxonomically most significant.[450] In addition, flavonoids have been used to characterize wines, for example, in Pinot Noir wine, the highest concentrations of catechin and epicatechin were found, while Cabernet Sauvignon shows quercetin (typical of red wines).[444] Citrus has been studied by considering their flavonoid composition, which are quantitative fingerprints of the juices: the predominant flavonoids in lemon were hesperidin and eriocitrin, which were also present in addition to neoponcirin in lime, while pummelo contains almost exclusively naringin. In grapefruit, the profile was more complex with neohesperidoside as the main component. Based on this information, naringin is used as a chemotaxonomic marker to distinguish sweet orange from other citrus varieties, and is especially used as indicator of grapefruit addition to orange juice.[398]

### *f. Pharmacological Effects*

It has been clearly established that flavonoids in natural products (e.g., grape, soybean, peanut, wine, tea) have a good antioxidant activity. In some instances a better activity than in the commercial antioxidants has been determined.[159] Additionally, flavonoids contribute to the protection and/or regeneration of antioxidants. It has been suggested that the flavonoid action is by trapping free radicals and that flavonoids are antimutagenic that can reduce the atherogenesis and the risk for strokes, and that are modulators of arachidonic acid metabolism. This last point involves flavonoids in many metabolic processes.[159,198,338] Interestingly, flavonoids are ubiquitous of plants and a person can consume up to 1 g of them. This quantity implies that flavonoids can reach pharmacological concentrations in the organism. Thus, it is interesting to point out that many flavonoids have assigned pharmacological activities (Table 1), and that many medicinal plants are known and used by their flavonoid content. Moreover, it has been determined that flavonoid structure has the responsibility of a large part of the activity. Also, it has been shown that different substitutes give a molecule with an specific polarity that permits

the presence of privileged bonds in the active sites.[10] This characteristic could have a relation with the antioxidant activity, because it is known that variations in glycosylation and/or acylation induce differences in the activity and stability of flavonoids.[233] From an acetone extract of the root bark of *Ormosia monosperma* several flavonoids were isolated, and it was observed that 2,3-dihydroauriculatin showed a moderate activity against oral microbial organisms such as *Streptococcus mutants, Porphyromonas gingivalis,* and *Actinobacillus actinomycetemcomitans*, each at 6.3 μg $ml^{-1}$.[234] From *Ononis spinosa* subsp. *Leeiosperma*, two flavonoids with biological activity were isolated: spinonin showed a moderate activity against *P. aeruginosa* and higher activity was obtained with ononin against β-hemolytic *Streptococcus* (minimal inhibitory concentration or MIC = 25 μg/ mL).[256] It was shown that some flavonoids (isorhamnetin, rhamnetin, and quercetin) diminished the total serum cholesterol levels when rats were fed with these compounds; atherogenic index, bile acids, serum triacylglycerols, and thiobarbituric acid-reactive substances (TBARS) tended to low as well. Flavonoids inhibited the generation of superoxide anion (higher with rhamnetin) and the oxidation of linoleic acid (up to 84% with quercetin). The activities of superoxide dismutase and xanthine oxidase did not vary, thus the flavonoid abilities could be ascribable in part to the scavenging of free radicals and in particular to their antioxidative activities.[198]

Studies on oregano extracts showed that galangin and quercetin are active desmutagens against the mutagenicity of Trp-P-2 (3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole), a liver-specific carcinogen, and other mutagens and it was suggested that flavonoid desmutagenicity plays an important role in cancer prevention; however, it is convenient to mention that under certain conditions these flavonoids did not inhibit mutagenicity, that is, in the presence of a buffered mixture of polychlorinated biphenyls, essential cofactors, NADP, and glucose-6-phosphate to form the S9 mix.[249] It has been reported that quercetin or its analogs could be interesting substitutes of camptothecin, which is used in the treatment of cancer and produces undesirable secondary reactions. It was proposed that all of these substances act at the level of the union of topoisomerase enzymes with DNA. Flavones can either stabilize the catalytic topoisomerase I-DNA intermediate or inhibit the DNA binding of the free enzyme. Thus, in view of the relatively low toxicity of quercetin and related antitumor activity, its use as an anti-cancer drug seems feasible.[43]

It is well known that iron could induce the generation of harmful reactive species and that special proteins are required to capture iron (transferrin, ferritin, heme proteins) and to prevent its reaction with oxygen species. Released iron induces the peroxidation of membrane lipids and hemolysis, and it has been established that quercetin inhibits the harmful effect of free iron probably by forming a complex with it (in assays with erythrocytes). Interestingly, it has been suggested that the release of iron in a redox-active form correlated with the generation of senescent antigen (the antigen appearing on aged erythrocytes), and quercetin induces the chelation of iron at intracellular level and delays the formation of senescent antigen, prolonging the storage time of blood in blood stores.[145] Also, it was shown that an hydroxyl group at C-3 and carbonyl at C-4 of the C-ring are necessary to bind iron. From ethyl acetate extracts of peppermint, sage, and thyme, luteolin was identified as the main active compound, showing desmutagenicity against Trp-P-2. It was suggested that mutagenicity generated from consumption of 1 g of broiled meat could be mitigated with very small amounts of these herbs: 2.8 mg of peppermint, 13 mg of sage, or 0.9 mg of thyme. A correlation was identified between a desmutagenic mechanism of flavonoids and their antioxidant potency.[413] Also, it was suggested that the choleretic, antirheumatic, and diuretic activities of dandelions could be related with the content of luteolin glycosides (7-glucoside and 7-diglucosides).[506] Also, luteolin glycosides have been isolated from *Vitex agnus-castus* (e.g., luteolin 6-*C*-[4″-methyl-6″-*O*-*trans*-caffeoylglucoside]; luteolin 6-*C*-[6″-*O*-*trans*-caffeoylglucoside]) and these compounds showed cytotoxic activity against P388 lymphocytic leukemia cells $IC_{50}$ (values were 0.1 for 4′ to 5-dihydroxy-3,3′,6,7-tetramethoxy-flavone and 0.31 for luteolin).[214] Trypsin and leucine aminopeptidase have been implicated in a variety of

pathological process such as acute pancretitis, inflammatory process, various cancers, etc. Interestingly, most flavonoids showed inhibitory properties against trypsin aminopeptidase (strongest inhibition with quercetin and myricetin), but only 3′,4′-dihydroxyflavone and quercetin inhibited leucine aminopeptidase. From the studies with the trypsin aminopeptidase inhibition, it was established that hydroxyl groups at C-5 and C-7 in ring A are essential, while a double bond at position C-2, C-3 in ring C and hydroxylation at both the C-3′ and C-4′ enhance the inhibitory activity. On the other hand, inhibition of leucine aminopeptidase by flavonoids requires of a double bond at C-2, C-3.[358] L868276 is a synthetic analog of flavopiridol that is obtained from *Dysoxylum binectariferum*. These substances are used to inhibit the growth of breast and lung carcinoma cell lines by its effect on the cyclin-dependent kinase activities. Interestingly, it has been shown that flavopiridol do not inhibit other protein kinases.[113]

*Brysonima crassifolia* (known in México as nanche) extracts have been used in traditional medicine by Mixe Indians in the treatment of gastrointestinal disorders and skin infections, and *in vitro* assays have shown that ethanolic extracts inhibited the nematode multiplication ($IC_{50}$ = 175 ppm). In this extract the main components were proanthocyanidins.[168] In Brazil, decoctions or infusions of *Stryphnodendron adstringens* are used traditionally in the treatment of leukorrhoea, diarrhoea, and as antiinflammatory. An acetone-$H_2O$ extract was analyzed and prodelphinidins with bioactive pyrogallol units (epigallocatechin derivatives) were identified and proposed as the pharmacological active compounds.[122] In *Guazuma ulmifolia*, which is used in the treatment of diarrhoea by the Mixe Indians were identified procyanidins (epicatechins). These compounds inactivate the cholera toxin, and toxin-binding activity increased with their molecular weight.[219] Also, flavonoids have been involved in the inhibition of several individual steps related to the thrombosis process, and one of the most active compounds was the biflavonoid hinokiflavone. The activity of this flavonoid has been suggested that is mediated by the inhibition of induction of tissue factor expression by interleukin-1.[270] Other biflavonoids (e.g., amentoflavone, agathisflavone, robusta- flavone) showed activity against the human immunodeficiency virus (HIV) reverse transcriptase (HIV-1 RT). It was indicated that biflavones with two apigenin units linked either with C-C or C-O-C bonds (robustaflavone, hinokiflavone) exhibited significant inhibitory activity. However, in a whole cell assay (human PBM cells infected with HIV-1 strain (LAV-1) morelloflavone exhibited potent inhibitory activity (promising anti-HIV activity), while it possessed moderate activity in the HIV-1 RT, emphasizing the importance of cellular mechanisms in flavonoid action. It was concluded that the presence of an unsaturated double bond at C-2, C-3 and three hydroxyl groups at C-5, C-6, and C-7 positions were prerequisites for inhibition of RT.[284]

From the ethanolic extract of the stem bark of *Mitrella hentii*, it was isolated linderatin. This compound was tested *in vitro* on a non-small-cell bronchopulmonary lung carcinoma type of cancer that represents 80% of all human bronchopulmonary cancers that is highly chemoresistant to medical treatment. It was reported that only (-)-linderatin exhibited a significant activity toward this type of carcinoma ($IC_{50}$ = 3.8 μg $mL^{-1}$).[34] It is interesting to mention that quercetin or its structural analog fisetin) enhanced the estradiol-induced tumorigenesis in hamsters. The effect of quercetin was mediated by increased levels of *S*-adenosyl-L-homocysteine (SAH) which inhibited the methylation of 2- and 4-hydroxyestradiol by catechol *O*-methyltransferase. Also, it was shown that low renal pools of *S*-adenosyl-L-methionine contributed to this phenotype.[527] The estrogenic activity of isoflavonoids has been reported, and 7-isopropoxyisoflavone is now sold on the market ('Osten' from Takeda Chemical Industries, Ltd.) as a therapeutic drug for osteoporosis. Also, it has been reported that daidzein increased the cell number of mouse osteoclasts at a very low concentrations.[466] One of the main flavonoids in teas is scutellarein, which has been used as a diuretic, antiinflammatory, and antiasthmatic drug.[218] The wide spectrum of resistance of cancer cells to natural agents has been assigned in part to the overexpression of proteins that belongs to the ATP binding cassette transporter proteins (ABC). Multidrug resistance protein (MRP), pertains to ABC proteins, has been cloned from a drug-resistant small cell lung cancer line. Several flavonoids

(genistein, kaempferol and flavopiridol) have shown inhibition of MRP-mediated transport of anti-cancer drugs by a direct interaction of flavonoids on the active site of MRP. Additionally, it has been suggested that a glucose moiety in the flavonoid structure plays an important role for its activity.[218]

Flavonoids have also been proposed in the treatment of obesity. Extracts of *Cassia nomame* have lipase-inhibitory activity, and it was determined that (2S)-3′,4′,7-trihydroxyflavan-(4α→ 8)-catechin has a significant inhibition of the porcine pancreatic lipase ($IC_{50}$ = 5.5 μ*M*). However, interestingly, an oligomeric fraction showed the most potent inhibitory effect ($IC_{50}$ = 0.20 μ*M*). This oligomer was mainly composed of 3′,4′,7-trihydroxyflavan and catechin.[199]

### 4. Quinones

As colorant substances, quinones are not very important. They only provide coloration in some higher organisms and microorganisms: in members of the Echinodermata family, quinones contribute to the pigmentation of spines, shell, ovaries, and eggs. In microorganisms, *Polyporus rutilans* is a fungus and accumulates up to 23% dry weight (d.w.) of polyporic acid, a terpenyl quinone of bronze color. *Helminthosporium gramineum* reaches up to 20% d.w. of quinones (islandicin, crisophanol, emodin). Some bacteria produce good quantities of quinones; *Streptomyces coelicor* accumulates up to 15% d.w.[464]

On the other hand, some quinones have been used as food colorants at the industrial level. *Rubia tinctoreum* Linn. is obtained from an extract composed of quinones (alizarin, xanthopurpurin, rubiadin, purpurin, etc.). The peel of *Coprosoma acerosa* A. contained 3-hydroxi-2-methyl-anthraquinone, methyl-ether of rubiadin, and lucidin, etc. From the root of *Rumex chinensis* is obtained a denticulate, which in China is used as a substitute of rhubarb. However, the most important quinones at the industrial level are the anthraquinones: carminic acid is obtained from the Mexican species of *Dactylopuis coccus*; carmesic acid is obtained from *Laccifer lacca* Kerr.[464]

Quinones are very reactive compounds due their structural characteristics. Between all the quinone reactions only one is common to all of them, a reversible reduction. By this reaction, quinones participate in the redox reactions of the organisms where they are present. Ubiquinone and plastoquinone are essential components of the electron transport in mitochondrial membranes (ubiquinone) and chloroplasts (plastoquinone). Ubiquinone is an hydrophobic compound of low molecular weight, and it can be found in its reduced or oxidized state: ubiquinone is lipid soluble and can diffuse through the membranes to act as an electron carrier between the components of the respiratory chain. Plastoquinone has similar characteristics to ubiquinone, and it is involved in the electron transport between PSI and PSII in the photosynthetic process.[196,464]

Moreover, it has been reported that several enzymes have a quinone as cofactor. Methanol dehydrogenase has a pyrroloquinoline quinone (PQQ) as cofactor, and also other bacterial dehydrogenases are quinone dependent (quinoproteins). These enzymes are involved in the oxidation of substrates such as alcohols, amines, and sugars. Additionally, it has been found that some copper-dependent amino oxidases (AO) have an alternate cofactor called topaquinone (TPQ). Several methyl-amine dehydrogenases have the tryptophan tryptoquinone (TTQ) as a cofactor. TPQ is a cofactor ubiquitous in bacteria, yeasts, plants, and mammals. Bacterial and yeast TPQ enzymes permit them to grow in different amines as a nitrogen source, and in some instances as source of carbon. In plants, the main function of the AO is in the production of hydrogen peroxide to heal wounds by the induction of new cell wall. Moreover, it has been suggested that plant AO participate in the growth process by the regulation of polyamine levels. The AO functions in animals are more elusive and diverse. The well-defined enzyme is the mammal lysyl-oxidase that catalyzes the connective tissue maturation by the crossover of elastin and collagen. On the other hand, some evidence indicates that free PQQ has other functions in eukaryotes: in the production of superoxides in neutrophiles (neuroprotector); prevents cataracts by exposure to cortisone; edemas induced by carrageenans; liver injury by hepatotoxins (carbon tetrachloride). In plants, it has been shown that PQQ stimulates 5 to 10 times the conversion of

acetyl-CoA to hydroxymethylglutaryl-CoA (HMG-CoA).[258] However, new methodologies for the assay of PQQ (bioassay and GC-MS) have shown their presence in Gram-negative bacterium, and it has been reported in only one Gram-positive bacteria *(Amycolatopsis methanolica*). However, as such, it was concluded that PQQ is not present in materials obtained from plants and animals, consequently, previous reports about their presence in these organisms must come from contamination.[323]

**Defense mechanisms.** *Streptomyces* sp. produces the antibiotic tetracycline. Many insects (Dyctiopters, Diplopodes, and Opiplions) produce alkylbenzenequinones as a defense mechanism. The African tree *Mansonia altissima* excretes mansonons that protect it from fungi and insect attack.[465] In addition to the pharmacological contributions mentioned above, it has been reported that several natural laxatives rhubarb, senna) have mixtures of anthraquinones and anthrones of the emodine type, and vitamin K functions as a cofactor of the prothrombines (coagulation factor). At the industrial level, skikonin is the most important quinone with pharmacological activity, and it is produced by tissue cultures of *Lithospermum erythrorhizon.*[196,464]

### 5. Iridoids

These compounds are not particularly important as colorants and their relevance, until a few years ago, had been restricted to be taxonomic markers. Iridoid glucosides are only found in more advanced dicotyledonous plants. Remarkably, secoiridoids are biosynthetic precursors of alkaloids.[406]

### 6. Melanins

In general, melanins are not essential for the growth and development but induce an increment in the possibilities of organism survival by acting as a defense mechanism. Melanins have been also associated with the immune response. It has been observed that rice resistance to *Piricularia oryze* is associated with the induction of phenol oxidase/polyphenol oxidase and the synthesis of melanin. In fungi, melanins provide resistance to stress conditions.[32,64,456,465]

By considering the pharmacological effect, it has been found that allomelanins (free of proteins) from plants, black bean, soybean, and sesame suppress the growth of tumorigenic cells of animals and mammals. Also, it has been mentioned that melanin inhibits the *in vitro* infection of the lymphocyte cell line by the HIV-1 virus. However, the free radicals in melanin molecules could produce a toxic effect in cells or the growth inhibition by melanin association with minor elements such as iron and copper. It has been reported that soluble melanins and their precursors (catecholamines, 3-hydroxyquinurenine, 3-hydroxyanthranilic acid, catechol) can induce degenerative processes in connective tissues, blood vessels, heart valves, liver, and other tissues, similarly to that observed in the illness called alcaptonuria. Parkinson's disease and the normal aging process are characterized by the loss of pigmented neurons in nigra substance, which correlates with the accumulation of neuromelanin and with the oxidative conditions in that brain region, and it has been hypothesized that neuromelanin acts as a protector under oxidative stress in normal individuals, but in those with Parkinson's this molecule has the potential to exacerbate the oxidative conditions through the generation of hydrogen peroxide or through the liberation of active metals for the redox process. This information emphasizes the importance of protection by antioxidants in the normal human metabolism, in diseases, or in the aging process.[32,248]

## C. Importance as Food Colorants

### 1. Reasons to Use Color Additives

Color is associated with many aspects of our life. For example, the main factors to evaluate food quality are color, flavor, and texture, but color can be considered the most important of them, because if it is not appealing consumers will not enjoy the flavor and texture of any given food. Color may as well give the key to cataloge a food as safe, with good aesthetic and sensorial characteristics: the undesirable colors in meat, fruits, and vegetables warn us about a potential danger or at least of the presence of undesirable

flavors, among other reactions. Many studies have emphasized the relation of color with the flavor detection threshold, with the sweetness or salinity sensations, with the susceptibility and preference for products.[80,162,339] Nowadays, people know that foods have their own natural colors, but most people live in big cities, far from the places of food production, and processing and transportation of foods are required. During such events, foods may lose some of their properties and some additives must be added to permit such products having desirable appearance and are safe for consumption. Thus, additives are used to emulate colors that are found in natural products such as pepper, red beet, grapes, saffron, meat, and shrimp.[58,144] Moreover, it is possible to enumerate the reasons to use food colorants:[150]

1. To restore the original food appearance because of the occurrence of changes during processing and storage.
2. To assure the color uniformity to avoid variations in tone color by seasonal varia tions.
3. To intensify colors that are normally found in food and the consumer will associate this improved color with food quality.
4. To protect the flavor and light susceptible vitamins.
5. To give to food an attractive appearance, whereas without the additive food will not be an appetizing item.
6. To preserve the identity or character by which food is recognized.
7. To help in the visual assignation of the food quality.

### 2. Importance of Natural Colorants

Since the early civilizations, natural products were used to give an attractive presentation to man-made products. Saffron and other species were used frequently to provide yellow color in a variety of foods and evidence exists that butter was pigmented with these products. The first reports regarding the use of derived colors of minerals dated from the ninteenth century; however, some of them caused serious health problems. Lead chromate and copper sulfate were used to pigment candies and sauerkraut, but in the pigmentation process arsenic and other venomous impurities were added frequently. Also, in that epoch began the use of tar colorants and other petroleum derivatives in the processing of foods, medicines, and cosmetics.[144] Synthetic colorants have been used for many years, in 1938 it was recognized the use of approximately 200, in 1970 to 1965, and nowadays only seven can be used in food pigmentation.[144,150] However, in the last 30 years synthetic additives have been severely criticized, and consumers show a phobia toward these products and consequently people prefer the natural colorants.[157,161] In the period 1960 to 1970 in the U.S., the environmental activist movements attacked the food additives, and this attitude was converted in a world-wide phenomenon. In general, this movement was against the technologies that have a harmful effect on the environment, and they used a very particular association between food and nutrition. The criticism of these groups was focused on the fast food and was difficult for them to attack these products as a group. However, colorants were an easy target; they indicated that pigments only have a cosmetic value, and, on the other hand, they could cause damage. The activist consumers reviewed the concepts health and found it harmful to many foods and diets, and companies began with the aim of producing healthier foods. They started using the nutritional characteristics as a sale tool, a strategy that has previously failed, but after the social movement was converted into a total success; thus, a world-wide tendency to use natural colorants was generated. At the present time, most people interpret the content of chemical products as a contaminant and the tendency has been reinforced, and all seems to indicate that it will continue to in the future.[158] To emphasize this situation, it should be pointed out that up to 1986 356 patents on natural colorants have been registered and for the synthetics only 71.[156] Nowadays, the number of advantages of natural over synthetic colorants have been increased because of the pharmacological properties of the natural pigments. In 1994, the market of natural colorants had an estimated value of $250 million U.S.

dollars, and 65% of this corresponded to color additives for food, with an annual growth rate of 5 to 10% in relation to 3 to 5% for the synthetic pigments. However, it is necessary to note that synthetic colorants have well-known advantages over the natural ones based on the higher pigmenting power, stability, storage, facility in the processing, and they are cheaper and available in unlimited quantities.[507] On the other hand, some products have a good market value only if they are colored with natural products: in the manufacture of Cheddar cheese, only the annato pigment is used;[161] in the pigmentation of poultry products, synthetic colorants are not adequate.[297] With the information mentioned above and by considering the large number of papers about natural colorants, one tends to believe that the area of these food color additives is very active, but this is a mistake because most of them are not applicable at the industrial level because they have not covered the economical, legal, and safety requirements that most governments have established for food additives. Nowadays, the number of approved colorants for food industry is very limited, and this phenomenon is observed practically all over the world (Table 2), probably with the exception of Japan. Colorants produced by the Monascus fungi are well-known pigments. Traditionally, Monascus pigments are obtained from fermented products of rice and bread and have been used as food colorants and medicinal agents. Interestingly, iridoids and Monascus pigments have remarkable properties, such as a good range of colors and stability, that can be used as food colorants; however, none of these groups are approved in neither the U.S. nor in the European Union.[58]

## D. Some Regulatory Aspects About Color Additives

Color additive regulation has an older history than any other additive regulation, with the exception of that of preservatives. These two kinds of additives were the subject of a special legislation by the U.S. Congress in the act of 1900 on colors and preservatives. In this act it was pointed out that the government had the responsibility to investigate the safety of these products before issuing any regulation. With the 1906 act, the control on the color additives was greater than on the other additives. In 1938, the Food, Drug & Cosmetic Act (FD&C) was established and published a list of tar colorants that could be used as food additives; also, it was decreed that the FDA had the authority to certify the color lots. With this, color additives were the first substances that must be revised before their commercialization. In the first years, synthetic colors were prohibited as health-harmful products. The next important change came in 1958; additives were redefined and a new classification with three categories appeared: (1) substances approved by the FDA or the USDA (United States Department of Agriculture) during 1938 to 1958 (prior-sanctioned substances); (2) substances that are Generally Recognized as Safe (GRAS substances), which did not require of a previous FDA evaluation to be commercialized; and (3) all the remaining added substances in the food supply food additives), which must be evaluated by the FDA before commercialization.

All new and color additives were included in the last category. Moreover, in 1958 included was 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 appropriated, for the evaluation of the safety of food additives, to induce cancer in man or animal". In 1960 and with the FD&C amendment, Congress established that all color additives required the prior FDA approbation for their commercialization. It may appear that it did not difference existed between this and the regulation before 1958. However, the new regulation also included prior-sanctioned colors (before 1958). When the 1900 act was published in 1912, 80 tar-derived colors had been used in food industry, the number was reduced to 12 after the FD&C act, and nowadays only 7 are approved. Additionally, color additives have another disadvantage, that is, all must be considered as additives and not as GRAS substances, thus the regulations for color additives are stronger than for other additives, and certainly up to date there exists no scientific basis to establish differences between color and other additives.[507,508]

On November 9 1990, an act was approved in which the producers are obliged to label all products

**TABLE 2**
**Approved Colors for Food Industry in the European Union and in the Food and Drug Administration (FDA) of USA**

| Color | CEE | FDA |
|---|---|---|
| **Certifiable** | | |
| Red allure AC | No | Yes (red #40) |
| Brilliant blue | No | Yes (blue #1) |
| Carmosine | Yes (E122) | No |
| Eritrosin | Yes (E127) | Yes (red # 3) |
| Fast green FCF | No | Yes (green # 3) |
| Indigotine | Yes (E132) | Yes (blue # 2) |
| Ponceau 4R | Yes (E124) | No |
| Sunset yellow FCF | Yes (E110) | Yes (yellow # 6) |
| Tartrazine | Yes (E110) | Yes (yellow # 5) |
| Polymeric colors | No | No |
| Citric red # 2 | No | No |
| **Exempt of certification** | | |
| Annato extract | Yes | Yes |
| Dehydrated red beet | Yes | Yes |
| Ultramarine blue | | Yes |
| Canthaxanthin | No | Yes |
| Caramel | Yes | Yes |
| β-apo-8'-carotenal | No | Yes |
| β-carotene | Yes | Yes |
| *Dactylopuis coccus* extract | Yes | Yes |
| Meal of cotton seeds | Yes | Yes |
| Iron glutamate | No | Yes |
| Skin grape extract | Yes | Yes |
| Iron oxide | No | Yes |
| Fruit juices | Yes | Yes |
| Vegetable juices | Yes | Yes |
| Algae meals | No | No |
| *Tagetes* and extracts | Yes? | No |
| Carrot oil | Yes | Yes |
| Oil of corn endosperm | Yes | Yes |
| Paprika | Yes | Yes |
| Paprika oleoresin | Yes | Yes |
| Riboflavin | Yes | Yes |
| Saffron | Yes | Yes |
| Titanium dioxide | No | Yes |
| Turmeric | Yes | Yes |
| Turmeric oleoresin | Yes | Yes |
| Chlorophyll | Yes | No |
| Xanthophylls, flavoxanthins, rubiaxanthin, zeaxanthin and other natural products with some of these carotenoids | Not all | Not all |

under certain regulation. With respect to color, the act established that all certified colors used in foods must be indicated in the label, but the exempt of certification (this includes the accepted natural pigments) must be generically grouped as colorants. This act gave a clear advantage to the use of natural over synthetic colorants, because consumers are suspicious about synthetics. On the other hand, the exemption of certification additives are from common natural products: red beet, carrot, fruits, pepper, among others, and regulation could be expected to be less severe for these products; however, it is clear that FDA policies do not share this idea, and the research for natural colorant sources is uncertain

because of the difficulty in getting FDA approval. In this respect, the history of color additive amendment is disastrous; red #40 is a colorant that was developed in the 1960s and approved by FDA in 1974, the only synthetic colorant approved in the last 75 years that it is still used). In general, the approbation process is expensive, a synthetic colorant requires $2.5 millions dollars only for the FDA evaluations, and in the European Union about $1 million dollars. Nevertheless, the regulations for the GRAS substances are very successful: olestra lipid substitute) was invented in 1968, this product was cataloged as an additive and it was approved by the end of 1996, 28 years after its invention; on the other hand, the inventors of the high fructose corn syrup (HFCS) decided that their product was a GRAS substance, thus HFCS was immediately commercialized.[144,229,310,507,508] Additionally, FDA uses the term indirect additives to group those additives used in the pigmentation of animal meals, and finally animals will be used as human food. Something that it is not easy to understand.[161]

Undoubtedly, it is technologically feasible to obtain new colorants from plants and microorganisms, but the greater obstacles for the introduction of new colorants are (1) the current legislation; (2) cost of manufacture, which includes that of the biotechnological culture or production; and (3) the acceptance of unknown materials by consumers.[507]

## IV. SOME IMPORTANT PLANT PIGMENTS: CAROTENOIDS, ANTHOCYANINS, AND BETALAINS

### A. Carotenoids

#### 1. Definition

In general, carotenoids are compounds comprised of eight isoprenoid units (ip) whose order is inverted at the molecule center (Figure 6). All carotenoids can be considered as lycopene ($C_{40}H_{56}$) derivatives by reactions involving: (1) hydrogenation, (2) dehydrogenation, (3) cyclization, (4) oxygen insertion, (5) double bond migration, (6) methyl migration, (7) chain elongation, (8) chain shortening.[179]

#### 2. Classification

Carotenoids are classified by their chemical structure as: (1) carotenes that are constituted by carbon and hydrogen; (2) oxycarotenoids or xanthophylls that have carbon, hydrogen, and, additionally, oxygen.

Also, carotenoids have been classified as primary or secondary. Primary carotenoids group those compounds required by plants in photosynthesis (β-carotene, violaxanthin, and neoxanthin), whereas secondary carotenoids are localized in fruits and flowers (α-carotene, β-cryptoxanthin, zeaxanthin, antheraxanthin, capsanthin, capsorubin).[279] Some carotenoid structures are presented in Figure 7.

#### 3. Distribution

Carotenoids are the widest distributed group of pigments. They have been identified in photosynthetic and nonphotosynthetic organisms: in higher plants, algae, fungi, bacteria, and at least in one species of each form of animal life. Carotenoids are responsible for many of the brilliant red, orange, and yellow colors of fruits, vegetables, fungi, flowers, and also of birds, insects, crustaceans, and trout.[179,180,182,196,510] Only microorganisms and plants can synthesize carotenoids *de novo*; carotenoids in animals come from these two sources, although they can be modified during their metabolism to be accumulated in tissues.[179] More than 300 carotenoids have been identified up to 1972, and around 600 up to 1992. Actually, this number has been exceeded by considering that many carotenoids have been isolated from marine organisms.[477] Total production of carotenoids in nature has been estimated at $10^8$ ton/year, most of which is concentrated in four carotenoids: fucoxanthin, in marine algae; and lutein, violaxanthin, and neoxanthin, in green leaves.[200]

##### a. Higher Plants

Carotenoids are accumulated in chloroplasts of all green plants as a mixture of α- and β-carotene, β-cryptoxanthin, lutein, zeaxanthin,

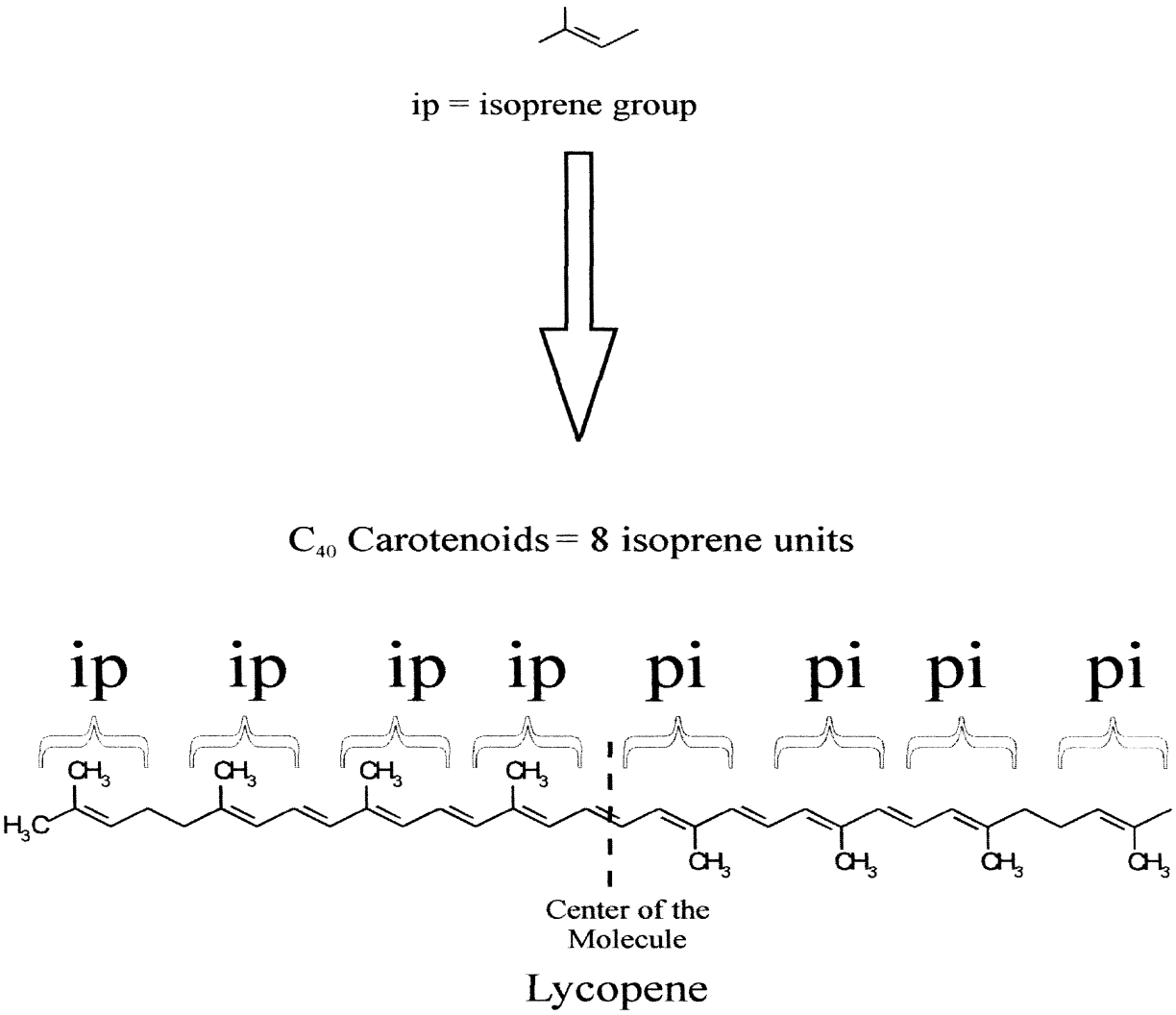


**FIGURE 6**. Carotenoid structure. (Adapted from Ref. 179.)

violaxanthin, and neoxanthin. These pigments are found as complexes formed by a noncovalent bonding with proteins. In green leaves, carotenoids are free, nonesterified, and the composition depends on the plant and developmental conditions. Some leaves of gymnosperms accumulate not very common carotenoids in oily droplets, which are extraplastidial: rhodoxanthin in some members of the families Cupressaceae and Taxaceae, and semi-β-carotenone in young leaves of cycads. In reproductive tissues the following have been found: liliaxanthin in white lily and crocetin in *Crocus* sp. stigmas; in flowers more than 40 pigments exclusive of petals have been identified. Flowers have been identified that synthesize: (1) highly oxygenated carotenoids, frequently 5,8-epoxydes; (2) principally β-carotenes; and (3) carotenoids that are species specific (e.g., eschscholzxanthin in poppies). Fruits are yet more prodigious in their synthetic ability than flowers. More than 70 characteristic carotenoids have been described and have been classified as those with minimal quantities, higher quantities, and specific carotenoids, for example, capsanthin and capsorubin in pepper fruits.[180,181,279] Interestingly, carotenoids have been identified in wood: samples of oak *(Quercus robur* L., *Quercus petrae* Liebl., and *Quercus alba* L.), chestnut (*Castanea sativa* Mill.), and beech (*Fagus silvatica* L.) were studied at different ages and sections. Lutein and β-carotene were identified in

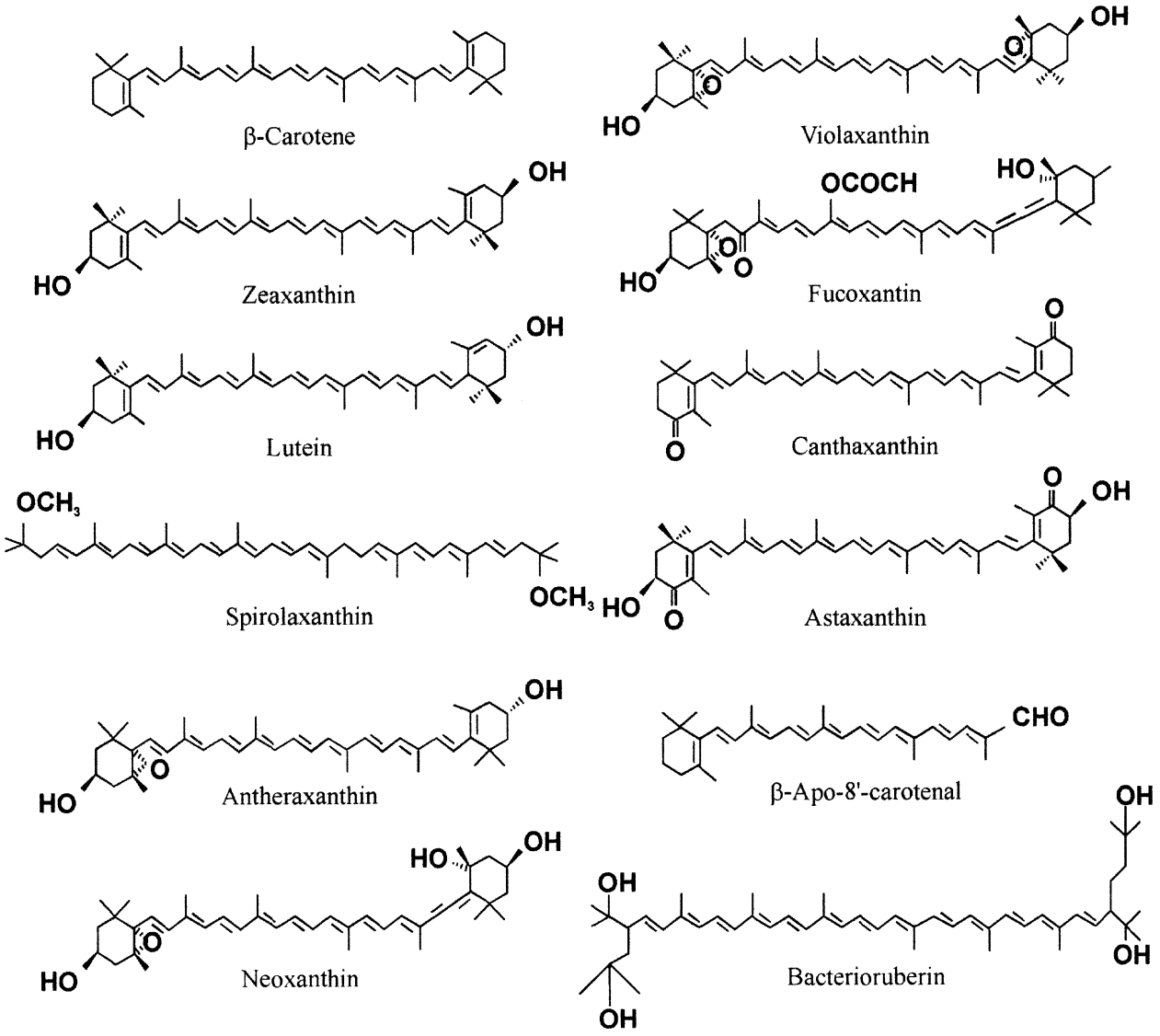


**FIGURE 7**. Structures of common carotenoids.

oak wood and also in others deciduous species. These carotenoids could be the origin of β-ionone and more than 30 other norisoprenoid substances identified in oak wood. Considering that carotenoids are hydrophobic and not soluble in sap, it is suggested the *in situ* formation of carotenoids in the living cells is in the sapwood. It was reported that sapwood was richer in β-carotene than lutein, and the ratio was reversed in the heartwood. Also, it was found that lutein could be used as a marker to distinguish between the wood samples.[299]

### *b. Algae*

Carotenoids are in chloroplasts as complex mixtures that are characteristic of each class; the exceptions are Chlorophyta carotenoids, which have a tendency to accumulate the pigments characteristics of higher plants. The red algae Rhodophyta have α- and β-carotene and their hydroxylated derivatives. In the Pyrrophyta, the main pigments are peridinin, dinoxanthin and fucoxanthin. Chrysophyta accumulates epoxy-, allenic-, and acetylenic-carotenoids, and between them fucoxanthin and diadinoxanthin. Eutreptielanone has been found in Euglenophyta. The principal carotenoids in Chloromonadophyta are diadinoxanthin, heteroxanthin, and vaucheriaxanthin. Chryptophyta is characterized by their acetylenic carotenoids, for example, alloxanthin, monadoxanthin, and crocoxanthin. While the Phaeophyta is characterized by its main pigment, fucoxanthin.[180,181]

### *c. Bacteria*

Approximately 80 different carotenoids are synthesized by photosynthetic bacteria. Usually, the characteristics of the accumulated carotenoids are (1) most of carotenoids are aliphatic, but in Chlorobiaceae and Chloroflexaceae some carotenoids have aromatic or β-rings; (2) aldehydes with crossover conjugations and tertiary methoxy groups; (3) various classes of carotenoids in each species; (4) all carotenoids are bound to the light-harvesting complexes or reaction centers in membranal systems of bacterial cells; and (5) usually structural elements are not found, that is, allenic or acetylenic bonds, epoxydes, furanoxides $C_{45}$ or $C_{50}$ carotenoids. *In vivo*, one of the main groups of carotenoids are the sulfates of eritoxanthin sulfate and of caloxanthin sulfates. The sulfates of carotenoids are not associated with pigment-protein complexes, for example, they are neither part of the light harvesting complexes nor of the reaction centers. In non-photosynthetic bacteria, carotenoids appear sporadically and when present, they have unique characteristics, for example, some *Staphylococcus* accumulate $C_{30}$ carotenoids, flavobacteria $C_{45}$ and $C_{50}$, while some mycobacteria accumulate $C_{40}$ carotenoid glycosides.[180]

### *d. Fungi*

Carotenoid distribution in fungi, non- photosynthetic organisms, are apparently capricious, but they usually accumulate carotenes, mono- and bi-cyclic carotenoids, and without carotenoids with ε-rings. For example, plectaniaxanthin in Ascomycetes and canth-axanthin in *Cantharellus cinnabarinus* has been found.[180]

## *4. Biosynthesis: Biochemistry and Molecular Biology*

### *a. Biochemistry*

Carotenoids as terpenoids are synthesized by the isoprenoid pathway (Figure 8).[185] Most of the pathways have been elucidated, but more information is required about the involved enzymes.[179,180,181] Isopentenyl pyrophosphate (IPP) is the common precursor of many of the isoprenoid compounds (Figure 8), thus sophisticated mechanisms of control must exist to assure the production of the appropriate levels of these compounds in the context of the metabolic pathway, developmental stage, and environmental conditions.[305] Initial steps of the pathway involve the fusion of three molecules of acetyl-CoA to pro-

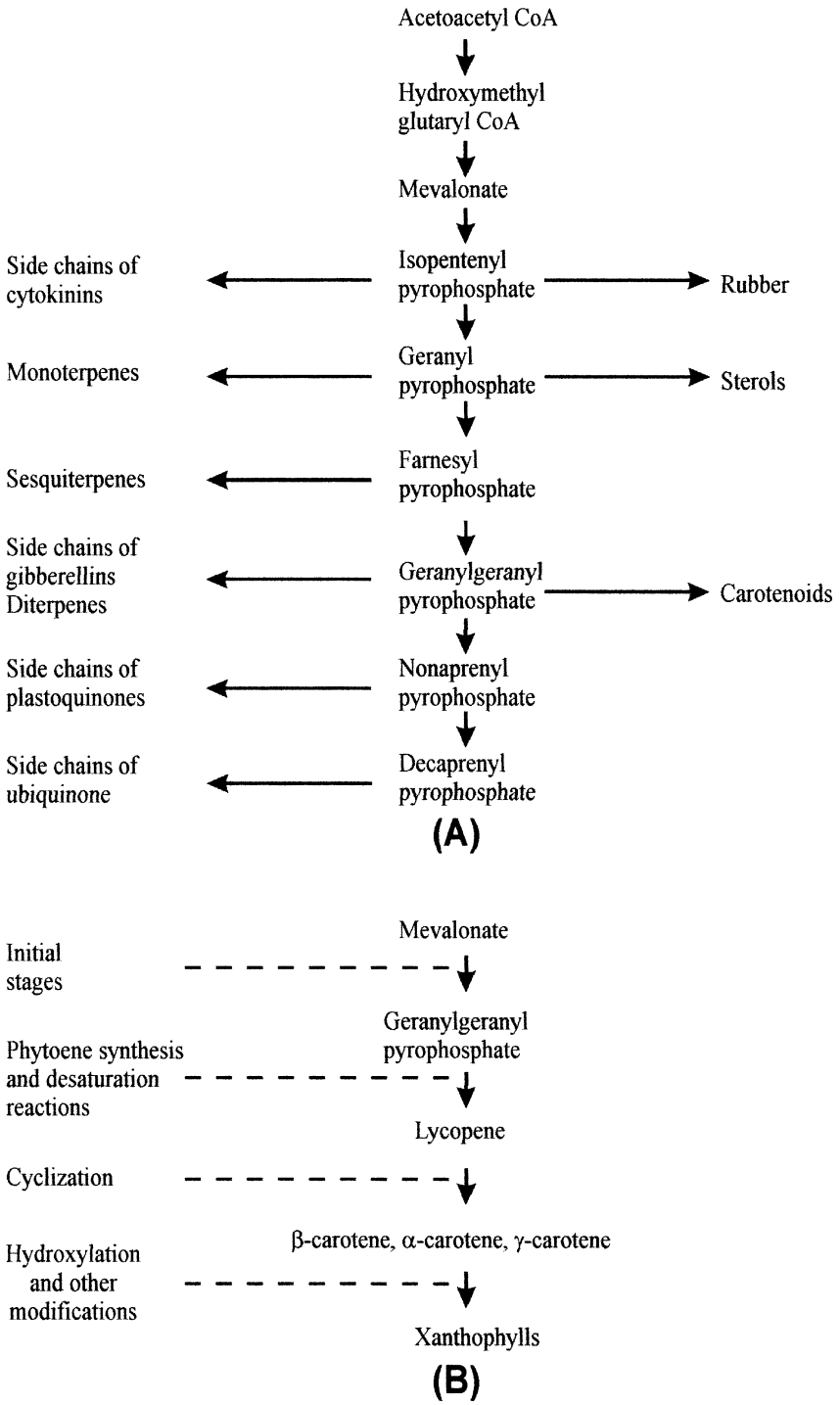


**FIGURE 8**. (A) Pathway of biosynthesis of isoprenoid compounds. (B) Proposed stages for the carotenogenesis pathway. (Adapted from Ref. 185.)

duce 3-hydroxi-3-methylglutaryl-coenzyme A (HMG-CoA). In animals and yeasts these reactions are catalyzed by two enzymes, the acetyl-CoA transferase and the HMG-CoA synthase. However, these reactions have not been studied extensively in plants, but apparently the reaction must be similar, a cabocationic mechanism. Evidence exists that only one enzyme catalyzes both reactions with $Fe^{+2}$ and quinone as cofactors.[305]

The conversion of HMG-CoA to mevalonate is catalyzed by the HMG-CoA reductase. This enzyme is very important in animals, limiting the stage of the reaction, thus this enzyme is highly regulated. The HMG-CoA reductase activity has been detected in endoplasmic reticulum, mitochondria, and plastids, and catalyzes two steps of reduction and uses NADPH as a cofactor.[305]

IPP synthesis from mevalonate is well characterized in plants (Figure 9A). Mevalonate is phosphorylated sequentially by the enzymes mevalonate kinase and after by mevalonate-5-pyrophosphate kinase to form 5-pyrophosphomevalonate. This compound suffers a decarboxylating elimination catalyzed by pyrophosphomevalonate decarboxylase that requires ATP and a divalent cation.[185,305] These enzymatic activities have been characterized in plant extracts or *in vitro.* It seems that mevalonate kinase is feedback regulated by phosphomevalonate and other allylic-diphosphate intermediates. It has been shown in potato that mevalonate diphosphate decarboxylase is induced by pathogen attack or by treatment with chemical products. It could be possible that the main controversy is in the subcellular localization of these enzymes.[185]

In 1997 Lichtenhaler et al.[280] proposed an alternative pathway for the IPP synthesis in the chloroplasts of higher plants. These authors used labeled glucose (1-$^{13}$C-glucose) to grow *L. gibba, D. carota,* and *H. vulgare.* They then analyzed the labeling in sterols produced in cytoplasm and found that they were synthesized from acetate through the mevalonate pathway as it had been described previously. However, the labeling pattern of carotenoids, phytyl, and plastoquinone were different (produced in plastids). The pattern suggested an alternative pathway that uses pyruvate and 3-phosphoglycerate (from the glycolysis pathway) to obtain D-1-deoxy-xylulose-5-phosphate, which is rearranged to IPP. Thus, they suggested the existence of an alternative pathway for isoprenoid synthesis in higher plants.

Isopentenyl pyrophosphate is the basic unit for constructing terpenoids of longer chains. IPP itself is not reactive enough to start the condensation reactions. Thus, the first step is its isomerization to dimethyl-allyl pyrophosphate (DMAPP), the reaction is catalyzed by the IPP isomerase with a divalent metallic ion as cofactor. The next step is the condensation of IPP and DMAPP to form geranyl pyrophosphate (GPP). After this, two molecules of IPP are condensed to GPP to obtain the geranylgeranyl pyrophosphate (GGPP) by catalysis with GGPP synthase. The GGPP synthase has been purified to homogeneity, and the activity has been corroborated by *in vitro* reactions.[123] The enzyme has requirements for two ions, $Mg^{+2}$ or $Mn^{+2}$, per catalytic site. The ion is bonded to the pyrophosphate group of GPP for a better salient group. The ionization of the allylic pyrophosphate leads to the formation of an allylic carbocation with a stabilized charge. The electrophilic addition of the IPP molecule generates the intermediate carbocation of 15 carbons that is attacked by another IPP molecule to obtain the GGPP (Figure 9B). Because GGPP is a precursor of many other compounds (phytyls, phylloquinones, plastoquinones, taxol, tocopherol, gibberellic acid, diterpenoid phytoalexins, and diterpenes), it is possible that the transformation of GGPP, in this branching of the pathway, is highly regulated by complex mechanisms and is possible to be compartmentalized. These assumptions are supported by the existence of multiple genes for the GGPP synthase of *Arabidopsis*.[24,93,185,266,305]

The first specific step for the synthesis of carotenoids is the condensation of two molecules of GGPP to *cis*-phytoene ($C_{40}$). The reaction has the prephytoene pyrophosphate PPPP) as an intermediate (Figure 10A).[185] In tomato and pepper it has been shown that one enzyme phytoene synthase) catalyzes both steps.[172] Complexes with IPP isomerase, GGPP synthase, and phytoene synthase activities have been isolated.[70] In the mechanism, two GGPP molecules are condensed, initially hydrogen and one of the pyrophosphate groups are

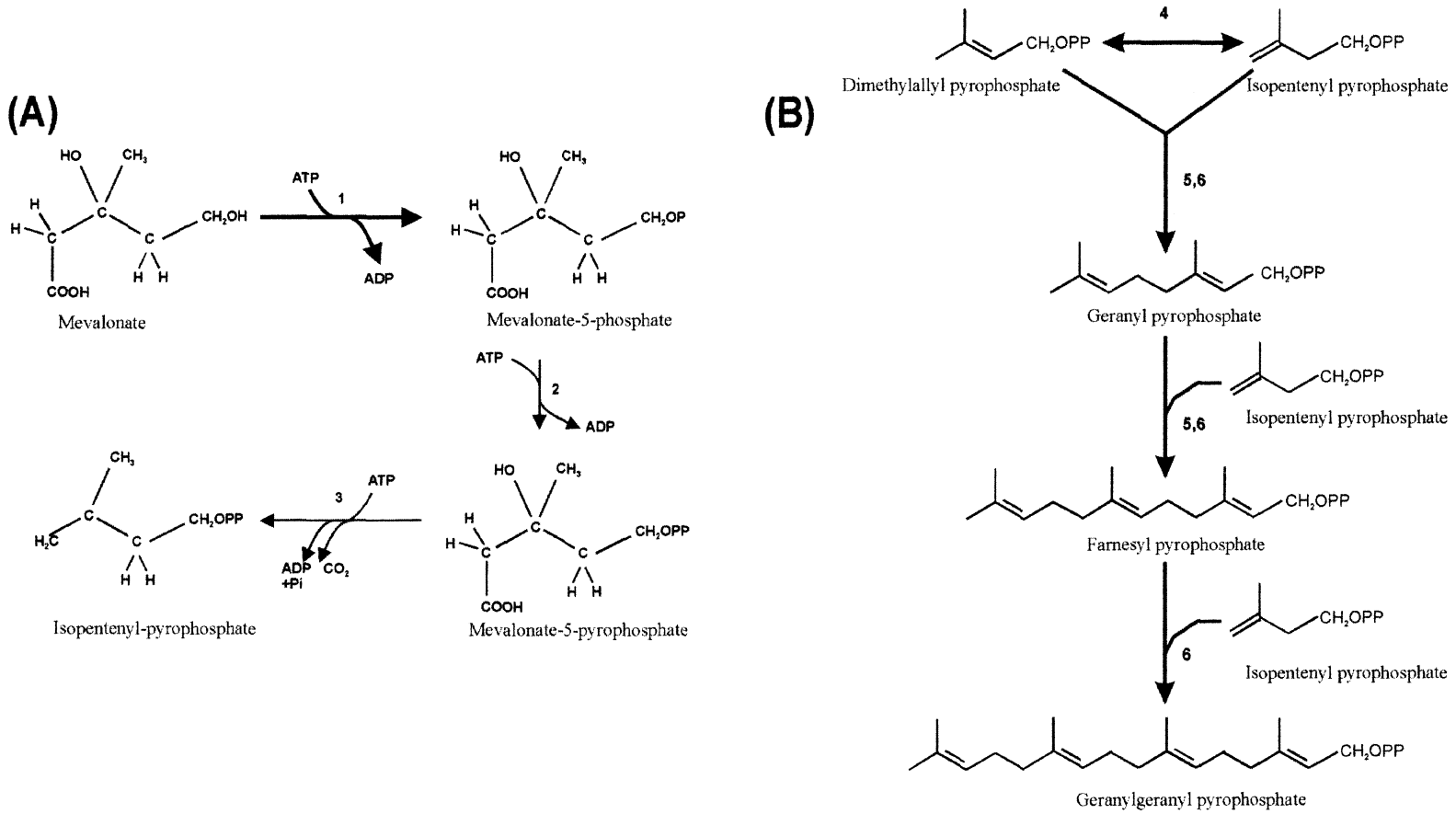


**FIGURE 9.** Initial stages in the carotenogenesis pathway. (A) Isopentenyl pyrophosphate biosynthesis. (B) Isopentenyl pyrophosphate polymerization to geranylgeranyl pyrophosphate. The involved enzymes are (1) Mevalonate kinase; (2) Mevalontate-5-pyrophosphate isomerase; (3) Mevalonate-5-pyrophosphate decarboxylase; (4) Isopentenyl pyrophosphate isomerase; (5) Farnesyl pyrophosphate synthase; (5) Geranylgeranyl pyrophosphate synthase. (Adapted from Refs. 185, 422.)

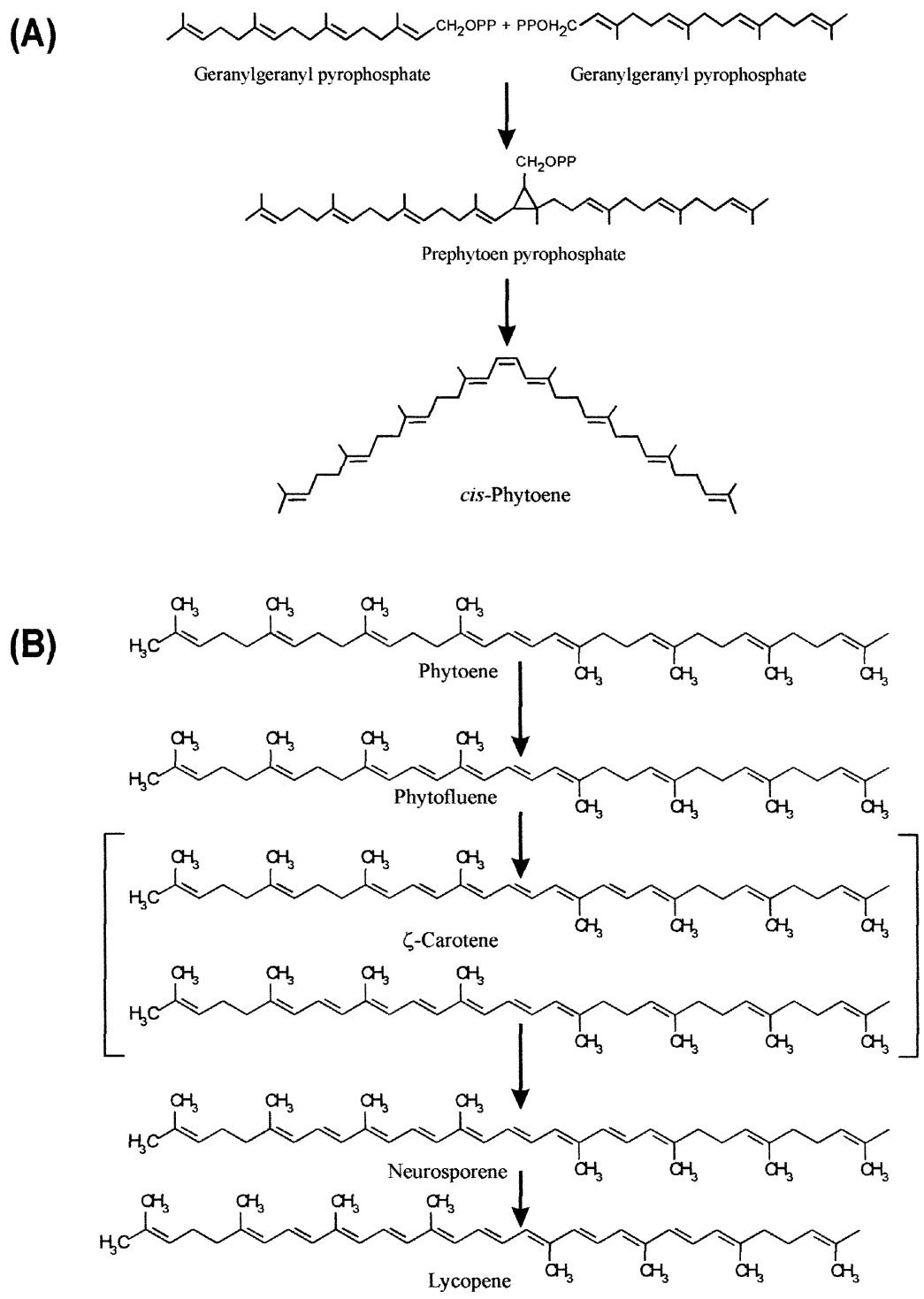


**FIGURE 10.** (A) Phytoene biosynthesis and (B) desaturation reactions to form lycopene. (Adapted from Refs. 179,185.)

produced to obtain PPPP. Afterward, the PPPP molecule is rearranged to form phytoene. Most of the organisms (especially higher plants, algae, and fungi) synthesize 15,15′-*cis*-phytoene. The 15,15′-*cis*-phytoene has the basic structure of the carotenoids, and subsequent reactions involve chemical transformations of this structure. However, most of natural carotenoids have a *trans* configuration, and it seems that the *cis-trans* transformation does not require any specific isomerase.[14,24,93]

Later, phytoene suffers four desaturation reactions to give phytofluene, ζ-carotene, neurosporene, and finally lycopene (Figure 10B).[24,179] It has been established that iron could play an important role in the desaturation electron-transport chain involved in phytoene desaturation. This conclusion was reached by growing sycamore *(Acer pseudoplatanus* L.) cells in an iron-deprived medium, producing a large accumulation of phytoene and corresponding diminution of carotenes and xanthophylls.[362] Additionally, studies on chromoplasts of *Narcissus pseudonarcissus* have been established that quinone compounds (plastoquinone/plastohydroquinone) are involved in the redox reactions in membranes. Membrane oxidation leads to a loss of desaturase activity and the main redox active components in chromoplast membranes, quinones and tocopherols, become oxidized. Then, NADPH is able to reactivate desaturase reactions by the reduction of these components. By considering that chromoplasts showed a pronounced NADPH-dependent respiratory activity, it is thus considered that phytoene desaturation and chromorespiration are overlapping biochemical phenomena by using in the redox process the same quinone pool.[2,341]

Lycopene is converted to β- or ε-carotene by lycopene cyclases (Figures 11A and 11B); these enzymes accept other acyclic substrates, such as neurosporene. In *Capsicum annuum* the direct conversion of lycopene to β-carotene catalyzed by lycopene cyclase has been detected.[71] This reaction requires FAD as a cofactor, and the proposed mechanism is shown in Figure 11A. However, this reaction is not yet perfectly defined.[14,24,179]

The hydroxylation reactions occur in an early stage, but different structural and functional modifications (epoxy, furanoxy, and oxy derivatives) are believed to happen in the last stages of the biosynthetic pathway (Figure 11C).[68,72] The first xanthophylls are formed from cyclic carotenoids (e.g., β-carotene) by hydroxylation in the 3 and 3′ positions of the ionone ring, followed by the epoxydation in the 5,6 and 5′,6′ positions. By considering the high diversity of xanthophylls in nature, nowadays a lot of information is required to completely define this route (enzymes, reaction mechanisms). In pepper, the capsanthin capsorubin synthase that catalyzed the conversion of antheraxanthin and violaxanthin to the ketocarotenoids capsanthin and capsorubin, respectively, has been purified to homogeneity.[14,24,69] In 1996, a protein of 43 kDa was purified from lettuce thylakoids that corresponded to a violaxanthin deepoxydase. This was the first reported enzyme that catalyzes the conversion of violaxanthin and antheraxanthin into zeaxanthin. Also, it was established that acidic conditions and ascorbate in lumen are required for the reaction.[399] Afterward, the violaxanthin deepoxydase from spinach was isolated and shown that it is a monomer of 46 to 50 kDa molecular weight.[201]

By using *Arabidopsis thaliana* mutants, it was shown that plants with deficiencies in carotenoid synthesis also had low levels of abscisic acid (ABA), and it was suggested that ABA was formed by the oxidative breakdown of epoxyxanthophylls, specifically it was related with violaxanthin and antheraxanthin.[24,293] This was supported by the very recent isolation of genes of the biosynthetic pathway of ABA from *Nicotiana plumbaginifolia*, *Arabidopsis*, and maize.[383] The fast conversion of *cis*-xanthoxin (obtained from epoxyxanthophylls) to *cis*-ABA was reported in tomato plants, and it was observed that *cis*-xanthoxin levels were significantly induced by water stress in older plants.[515]

### *b. Biosynthesis Regulation*

Regulation of the carotenoid biosynthesis is complex, as been observed for most of the terpenoids because it seems that regulation must occur at several levels. Evidence shows that carotenoid biosynthesis is restricted to specific tissues, where they are used. Carotenoids are produced in fruit chromoplasts during the maturation process (or-

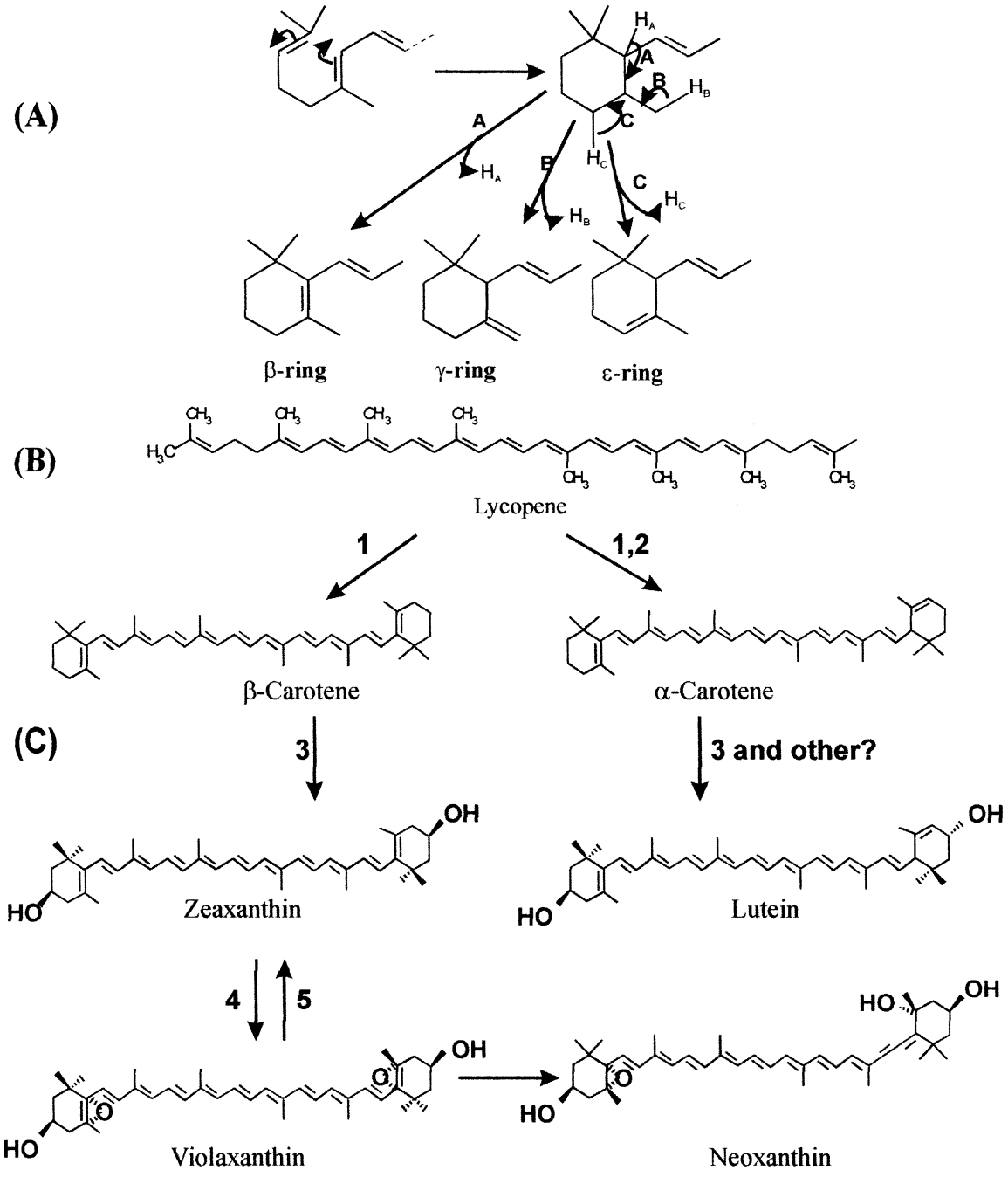


**FIGURE 11**. (A) Proposed mechanism for ring formation of carotenoids. Examples of (B) cyclization reactions of lycopene, and (C) hydroxylation and other modifications. Some of the involved enzymes are (1) β-lycopene cyclase; (2) ε-lycopene cyclase; (3) β-carotene hydroxylase; (4) zeaxanthhin epoxidase; (5) violaxanthin de-epoxidase. (Adapted from Ref. 90, 179.)

gan-specific regulation). Also, it has been determined that GGPP synthase activity is in chromoplast stroma (tissue-specific regulation).[24] Enzymes of carotenoid biosynthesis, and other related proteins, are functional in chloroplast/chromoplast but are codified by nuclear genes; then, the correspondent precursors are posttranscriptionally imported toward plastids. This could suggest the existence of transcriptional or posttranscriptional pathway control. However, although some genetic mutants in fruit ripening and carotenoid accumulation have been identified (from maize, tomato, and *Arabidopsis*), none of the regulatory genes have been isolated.[24,93]

Studies on carotenogenesis provided significant advances thanks to use of mutants of oxygenic photosynthetic organisms. However, the identification of mutants block in the initial steps of the biosynthesis pathway has proven to be difficult because of cells with chlorophyll that are simultaneously exposed to illumination and oxygen, in the absence of carotenoids, suffer photooxidative damage and then die. Additionally, mutations in the final stages are masked by endogenous chlorophylls; thus, few mutants in carotenoid synthesis have been isolated. In plants, the situation is not so discouraging: potential lethal mutants affecting green photosynthetic tissues can survive in heterozygosis, and carotenogenic mutants in flower and fruits do not necessarily affect the viability. In particular, a collection of mutants affected in carotenoid biosynthesis in leaves and endosperm of maize have been used.[14]

Interestingly, exogenous regulatory agents have been used to direct the carotenoid synthesis or block catabolism; they are attractive approaches for producing fruits or vegetables with improved characteristics. 2-(4-chlorophenylthio)-triethylamine hydrochloride (CPTA) and *N,N*-diethyl nonylamine cause an accumulation of the red carotenoid lycopene. 2-(4-ethylphenoxy)-triethylamine hydrochloride has been used to improve orange color by postharvest application induce the accumulation of xanthophylls in endocarp). Esters of 2-diethylaminoethanol induce the accumulation of β-carotene, while *p*-bromobenzylfurfurylamine applied on grapefruit produces poly-cis lycopene prolycopene), which has a characteristic orange color contrasting with the red color of lycopene.[293]

### *c. Molecular Biology of Carotenogenesis*

**Starting models.** Biochemical studies have outlined the carotenoid biosynthetic pathway; however, the biochemical approach for studying the involved enzymes have shown to be inefficient, because most of them are in membranes and are difficult to isolate. On the other hand, carotenoids are very important for human beings, and scientists work to control the carotenogenesis pathway (overproduce or change). It is clear that pathway control requires a well-defined pathway and that molecular biology has permitted advances in this area.

The current knowledge on the molecular biology of the carotenogenesis pathway is derived from studies of specific organisms. Complete sequences of clusters of genes for carotenogenesis have been reached in the photosynthetic prokaryotes *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. Much data on the carotenogenesis pathway have been reported for *Synechococcus*, and all the genes of some nonphotosynthetic bacteria, for example, *Myxococcus xanthus, Mycobacterium aurum, Thermus thermophilus,* and some bacteria *Erwinia* sp. Also, the pathway is well defined in the fungi *Neurospora* and *Phycomyces*.[13,25,160,221,321] It was isolated and characterized the *Neurospora* wc-2 gene that codes for a second central regulator of blue light responses. The coded proteins represent the first two putative GATA transcription factors that have been characterized in any organism being involved in light-activated gene regulation rather than in a general transcriptional regulation.[285] Gene clusters have been observed in some microorganisms but not in eukaryotes or cyanobacteria; however, generated information from microorganisms and from the carotenogenesis genetics of higher plants helped to identify carotenogenic genes of cyanobacteria and of higher plants *(Zea mays, Lycopersicon esculentum, Narcissus pseudornarcissus,* and *Capsicum annuum*).[14,23,420] Altogether, these models provided the essential tools to obtain the plant carotenogenesis genes reported up to date (Table 3).[422] In general, carotenogenesis eukaryotic genes seem to be in only one copy with the exemption of phytoene

**TABLE 3**
**Some Genes (genomic or cDNA Clones) Isolated from Plants That Are Directly Related to the Carotenogenesis Pathway[a]**

| | Enzyme that is coded by the gene[b] | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Plant | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
| *Arabidopsis thaliana* | ● | ● | ● | | ● | ● | ● | ● | ● | ● | ● | | ● | | ● | ● |
| *Capsicum annuum* | | | | | | ● | ● | ● | ● | ● | | ● | | | | ● |
| *Lycopersicon esculentum* | ● | | | | | | ● | ● | | ● | | | | | | ● |
| *Zea mays* | | | | | | | ● | ● | | | | | | | | |
| *Narcissus pseudonarcissus* | | | | | | | ● | ● | | ● | | | | | | |
| *Nicotiana tabacum* | ● | | | | | | | | | ● | | | | | | ● |
| *Glycine max* | | | | | | | ● | ● | | | | | | | | |
| *Cucumis melo* | | | | | | | ● | | | | | | | | | |
| *Oryza sativa* | | | | | ● | | | ● | ● | | | | | | | |
| *Brassica campestris* | | | | | | ● | | | | | | | | | | ● |
| *Catharanthus roseus* | | | | | | ● | | | | | | | | | | |
| *Lupinus albus* | | | | | | ● | | | | | | | | | | |
| *Nicotiana benthamiana* | | | | | | | | ● | | | | | | | | |
| *Sinapsis alba* | | | | | | ● | ● | ● | | | | | | | | |
| *Haematococcus pluvialis* | | | | | | | | | | | | | | ● | | |
| *Clarkia breweri* | | | | | ● | | | | | | | | | | | |
| *Clarkia xantiana* | | | | | ● | | | | | | | | | | | |
| *Lactuca sativa* | | | | | | | | | | | | | | | ● | |

[a]Adapted from Refs. 422, 498.

[b](1) 3-hydroxy-3-methyl glutaryl coenzyme A reductase; (2) mevalonate kinase; (3) mevalonate 5-pyrophosphate decarboxylase; (4) isopentenil pyrophosphate synthase; (5) isopentenyl pyrophosphate isomerase; (6) geranylgeranyl pyrophosphate synthase; (7) phytoene synthase; (8) phytoene desaturase; (9) ζ-carotene desaturase; (10) β-lycopene cyclase; (11) ε-lycopene cyclase; (12) capsanthin capsorubin synthase; (13) β-carotene hydroxylase; (14) β-carotene ketolase; (15) violaxanthin de-epoxidase; (16) zeaxanthin epoxidase.



synthase PSY) and GGPP synthase of pepper.[90,278,423]

**Initial stages (Figure 9).** Mevalonate synthesis is catalyzed by HMG-CoA reductase (HMGR). In animal systems isoprenoid biosynthesis is strongly regulated at HMGR level, and it was suggested that in plants mevalonate-synthesis catalyzed by HMGR is also the first major rate-limiting step. However, plants obtain more products from the mevalonate pathway than animals, thus some tissues and organelles have multiple HMGR genes (*Arabidopsis*, *Hevea*, and *Solanum*). These genes are differentially regulated: different genes are expressed in different parts and developmental stages.[272] On the other hand, one cDNA clone of mevalonate kinase has

been isolated from *Arabidopsis* and IPP isomerase from some plants and yeasts. All the information indicates that reactions catalyzed by HMGR, mevalonate kinase, and IPP isomerase are not limiting steps of carotenoid biosynthesis.[93,185,305]

The number of plant prenyltransferase genes isolated is very limited, for example, GGPP synthase cDNAs from *C. annuum*[266] and from *Arabidopsis.*[421] The pepper cDNA was obtained by using antibodies against the purified GGPP synthase in a cDNA expression library;[266] this cDNA was used as a probe to obtain the *Arabidopsis* gene.[421] However, most of the information about prenyltransferases come from animal and microbial systems. Prenyltransferases have high homology between different kingdoms. They are structurally and functionally conserved, suggesting the presence of a common ancestor for prokaryotes and eukaryotes.[13] However, GGPP synthase of pepper chromoplasts segregates with eubacterial prenyltransferases, thus this gene could be transferred from a bacterial symbiont. Also, a consensus region has been observed, DDXX(XX)D, which must have an important role in substrate bonding.[305] Recently, a new *GGPP synthase* (*GGPS6*) gene from *Arabidopsis thaliana* was isolated. GGPP synthase showed a strong amino acid homology with other GGPP synthases. This is the first GGPP synthase localized in mitochondria; thus, it was suggested that this isozyme produces the precursors of the side chain of isoprenoid quinones, and, importantly, that GGPP synthase could be produced in different organelles rather than organelle-specific production and posterior distribution.[528]

Phytoene synthesis and desaturation reactions (Figure 10). Tomato phytoene synthase was the first cDNA clone obtained for a carotenogenic enzyme that was isolated by using the correspondent cyanobacterial cDNA.[389] Later on, the existence of two phytoene synthase genes was discovered: one leaf-specific (PSY1) and another fruit specific(PSY2). In contrast to tomato, pepper PSY expression is not induced by fruit ripening.[400] It was suggested that differences between these materials could be assigned to differences between climacteric and non-climacteric fruits.[24] Schedz et al.[423] isolated the PSY cDNA clone from daffodil by using an heterologous tomato PSY1 probe; the gene was in one copy (Southern blot). They reported two enzyme forms: one soluble and inactive in stroma and another active, which are membrane bound. The carotenoid content was increased during flower development; however, the mRNA level was constant during the process. Also, there was an increment of the PSY protein level that was not related with activity, because the soluble PSY protein was only increased. It was reported that membrane galactolipids are necessary for PSY activity, in particular an aldopyranose is necessary to activate and lipids are necessary to membrane enzyme bounding. Additionally, sequence analysis showed that PSY is membrane integral protein, and it was suggested the existence of postranscriptional regulation mechanisms that involve the membrane redox state.[423] In 1996,[65] phytoene synthase *y1* gene was identified from maize and evidence of multiple copies was presented. Interestingly, *y1* is responsible of leave and endosperm carotenogenesis.

When tobacco plants were transformed with PSY1 cDNA, an increment was observed in carotenoid content but with a reduction in gibberellic acid and chlorophyll levels. This information suggested the existence of a common GGPP pool for these compounds. On the other hand, the cDNAs clones for GGPS, PSY, and PDS were isolated from white mustard (*Sinapis alba*). It was established that PSY transcript is up-regulated by light during etioplast/chloroplast conversion, but neither GGPS nor PDS. Also, it was shown that this response is mediated by phytochrome in mutants the PSY mRNA level was not induced). Thus, enhanced carotenoid biosynthesis should occur only after the light-dependent development of structured chloroplast membranes.[498]

Phytoene desaturases (PDS) of anoxygenic photosynthetic microbes catalyzes the conversion of phytoene to lycopene or neurosporene; in contrast, PDS cDNAs of *Arabidopsis*, tomato, and soybean catalyzes the transformation phytoene to ζ-carotene.[420] In both models, a dinucleotide binding site are identified in their coding sequence.[26] Norris et al.[343] used *Arabidopsis* mutants that accumulate phytoene to map three loci (Figure 12). Two of these loci do not correspond to the PDS gene locus. Thus, at least three genes are required in the phytoene desaturation; it was showen that mutants showed deficiencies in plastoquinone and α-tocopherol accumulation. Then a complementation assay with quinone biosynthesis

intermediates (homogenistic acid or plastoquinone) was used and carotenoid production was recovered. It was concluded that plastoquinones and/or tocopherols are essential components for phytoene the desaturation reaction in higher plants. It was proposed that plastoquinone is an important component in electron transportation and ubiquinone was proposed as the transporter of anoxygenic photosynthetic organisms, PDS expressed in *E. coli* is active, where plastoquinone is absent. In general, it was concluded that quinones are universal adapter molecules in the carotenoid desaturation reactions.[343]

Corona et al.[86] studied carotenogenesis regulation in tomato fruits. During tomato development, carotenoid content was increased (approximately 10 times), which corresponded to PDS mRNA increment. A 2-kb region was identified upstream of the PDS gene that shows a *cis*-activation of the chromoplast-specific PDS expression. Also, it was reported that chromoplast differentiation and PDS expression are coordinately regulated in petals and tomato fruits, and it suggested the presence of a "chromoplast factor" that activates the PDS promoter. This relation was not found in tobacco, which does not have chromoplasts in its leaf structure but accumulates good carotenoid levels. It was proposed that PDS could be regulated by the end product (probably β-carotene, xanthophylls, or abscisic acid) that the effect of specific inhibitors activates the PDS promoter, and that etioplasts also accumulate carotenoids.[86] Recently, maize PDS cDNA clone was isolated. This enzyme catalyzes the transformation of phytoene to ζ-carotene, an heterologous complementation assay was used in the demonstration. With these results the requirement of two enzymes for the desaturation of phytoene to lycopene in higher plants were confirmed and observed both in mono- and dicotyledonous plants. Southern blot analysis showed only one gene copy. Three possible regulatory locus *vp2,* and especially *vp9*, were reported because recessive alleles accumulate ζ-carotene. Northern analysis showed that mRNA level was constant despite the carotenoid content increments. It was pointed out that maize PDS regulation contrasts with that observed in the temporal control of PSY and PDS of tomato fruits.

Cyclization (Figures 11A and 11B). The initial step to clone a plant β-lycopene cyclase was the isolation by a complementation analysis of the gene of the cyanobacteria *Synechococcus*.[91] By using this sequence, β-lycopene cyclase cDNAs were obtained from tobacco and tomato.[364] Protein sequences are conserved between plants and cyanobacteria but different in bacteria. The enzyme showed activity in the cyclization of neurosporene (neu) to β-zeacarotene and of lycopene to β-carotene, suggesting that the enzyme could perform two cyclizations. However, β-lycopene cyclase was not capable of cyclizing ζ-carotene, and it was proposed that the presence of a double bond in the 7 to 8 or 7′ to 8′ is required for the cyclization process. Sequence analysis showed FAD or NAD dependence. Also, the enzyme was expressed in an active form in *E. coli*, showing that membrane special elements are not required. β-lycopene cyclase mRNA decreased during fruit ripening, contrary to the observation with PSY and PDS, and a transcriptional regulation mechanism was proposed. Up to date, information of regulatory mechanisms of fruit carotenogenesis does not exist, but it is clear that regulation must be different in relation to leaves. Also, a locus B that seems to be a β-lycopene cyclase regulatory element was identified, although its mode of action is unknown.[364]

As could be expected from the evidence mentioned above, β- and ε-lycopene cyclases cDNAs were obtained from *Arabidopsis thaliana* (Figure 13).[90] The importance of 7 to 8 or 7′ to 8′ insaturation for the cyclization process was reiterated. It was found that ε-lycopene cyclase is a monocyclase that cannot introduce a second ε-ring where previously another exists. It was concluded that lycopene cyclization is a key process in plant and algae carotenoid biosynthesis, because the relation between these two enzymes gives the balance between cyclic carotenoids with or without an ε-ring. Also, it was reported that these genes are in one copy.[90]

**Hydroxylation and other modifications (Figure 11C).** *A. thaliana* β-carotene hydroxylase cDNA was isolated. Hydroxylation activity of the ε-ring was low, and by considering the differences in chirality of β- and ε-rings, the presence of an ε-carotene hydroxylase was suggested.[376] In addition, the Zeaxanthin epoxydase gene was identified, and it was shown that epoxyxanthophylls are the precursors of abscisic

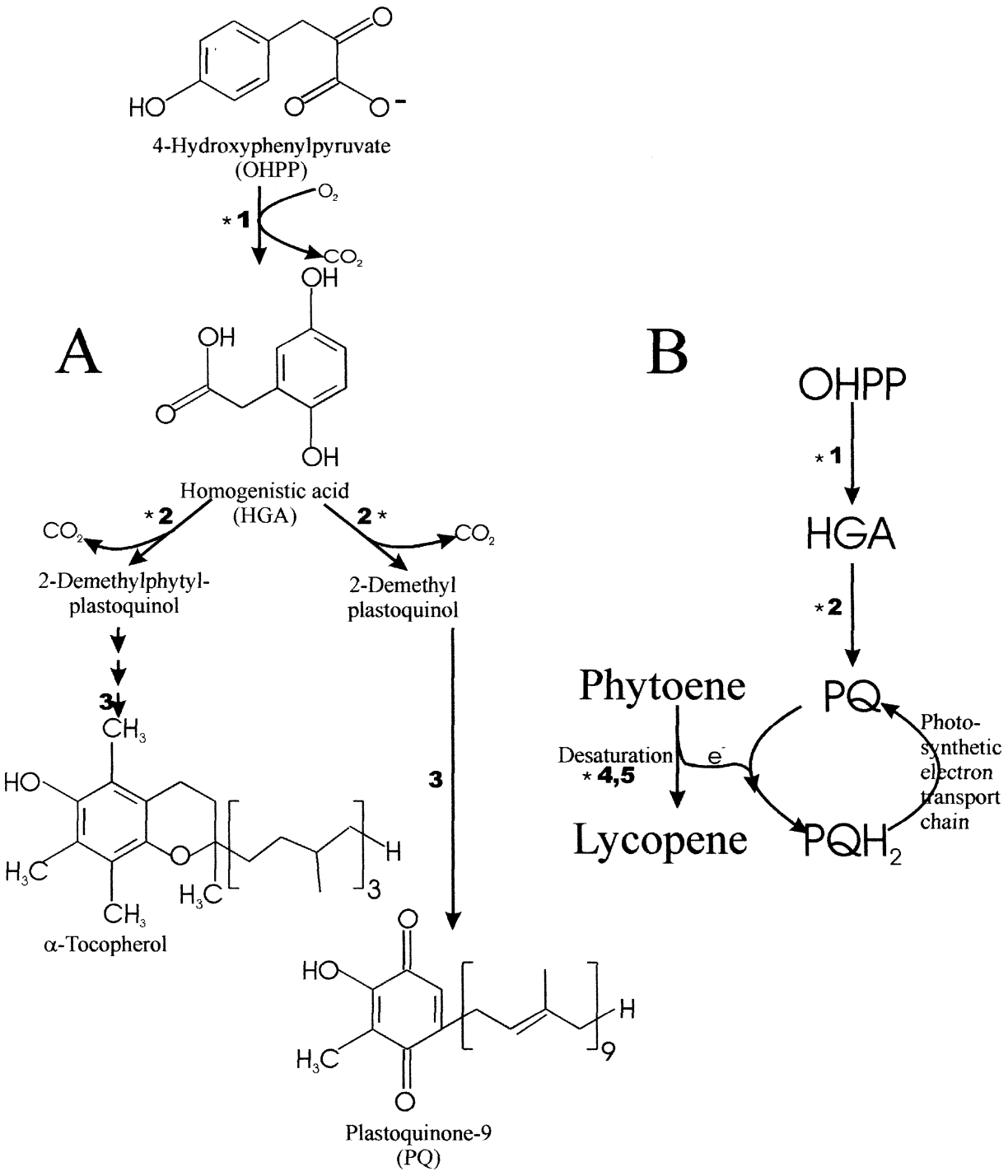


**FIGURE 12.** (A) Pathway for α-tocopherol and (B) model for the participation of plastoquinone in carotenoid desaturations. Involved enzymes are (1) OHPP dioxygenase; (2) Phytyl/Prenyl transferase; (3) methyl transferase; (4) phytoene desaturase; (5) ζ-carotene desaturase. Arabidopis plants with mutations on the steps signaled with stars were analyzed. (Adaped from Ref. 343.)

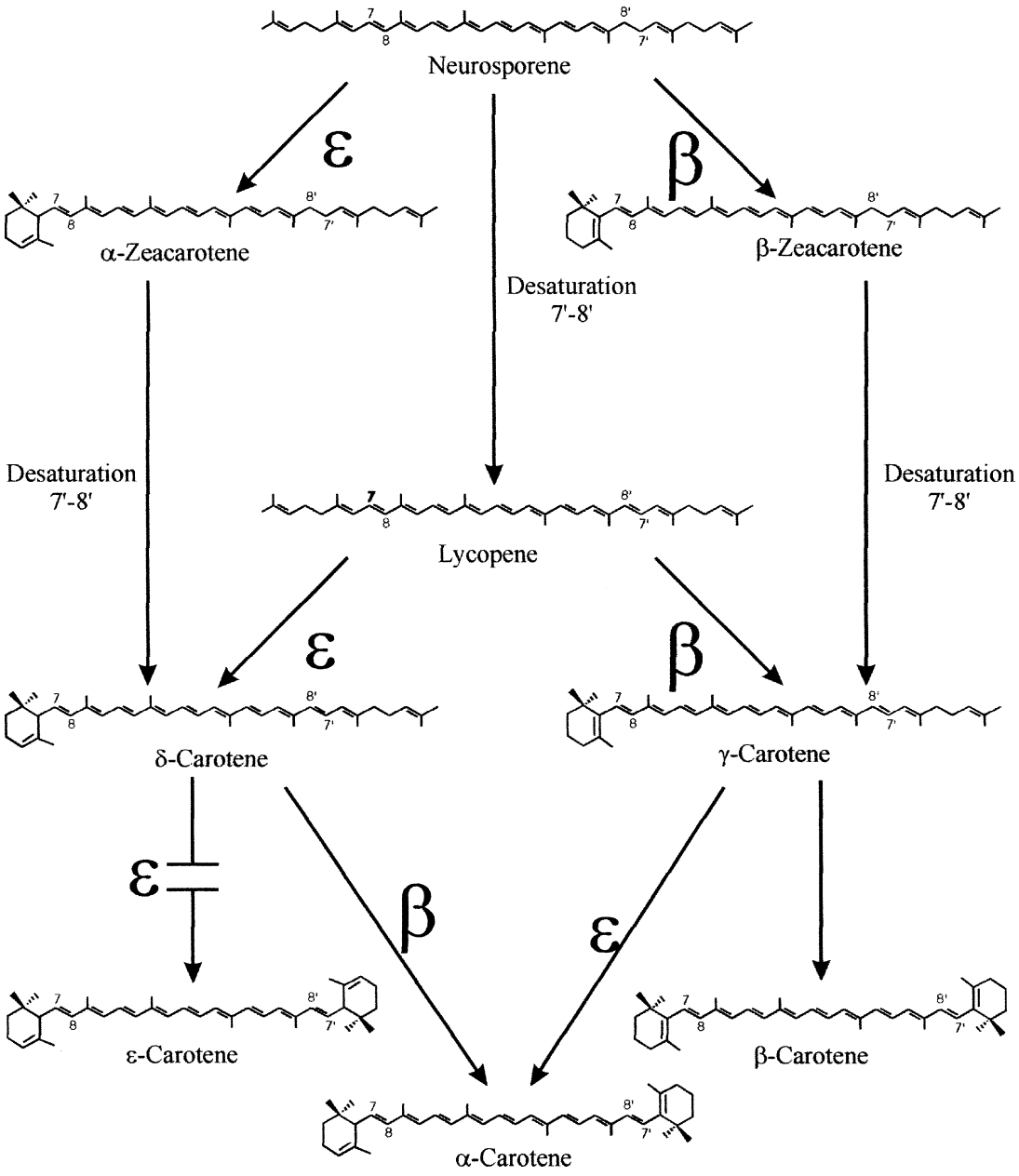


**FIGURE 13.** Carotenoid cyclization reactions in Arabidopsis. the involved enzymes are (e) ε-lycopene cyclase; (b) β-lycopene cyclase. Numbered double bonds are required for the cyclase activities. Observed activities are represented by solid arrows. Broken arrows are proposed activities. Parallel lines over the arrow indicate that the enzymatic activity was observed but no product was detected. (Adapted from Refs. 90, 364.)

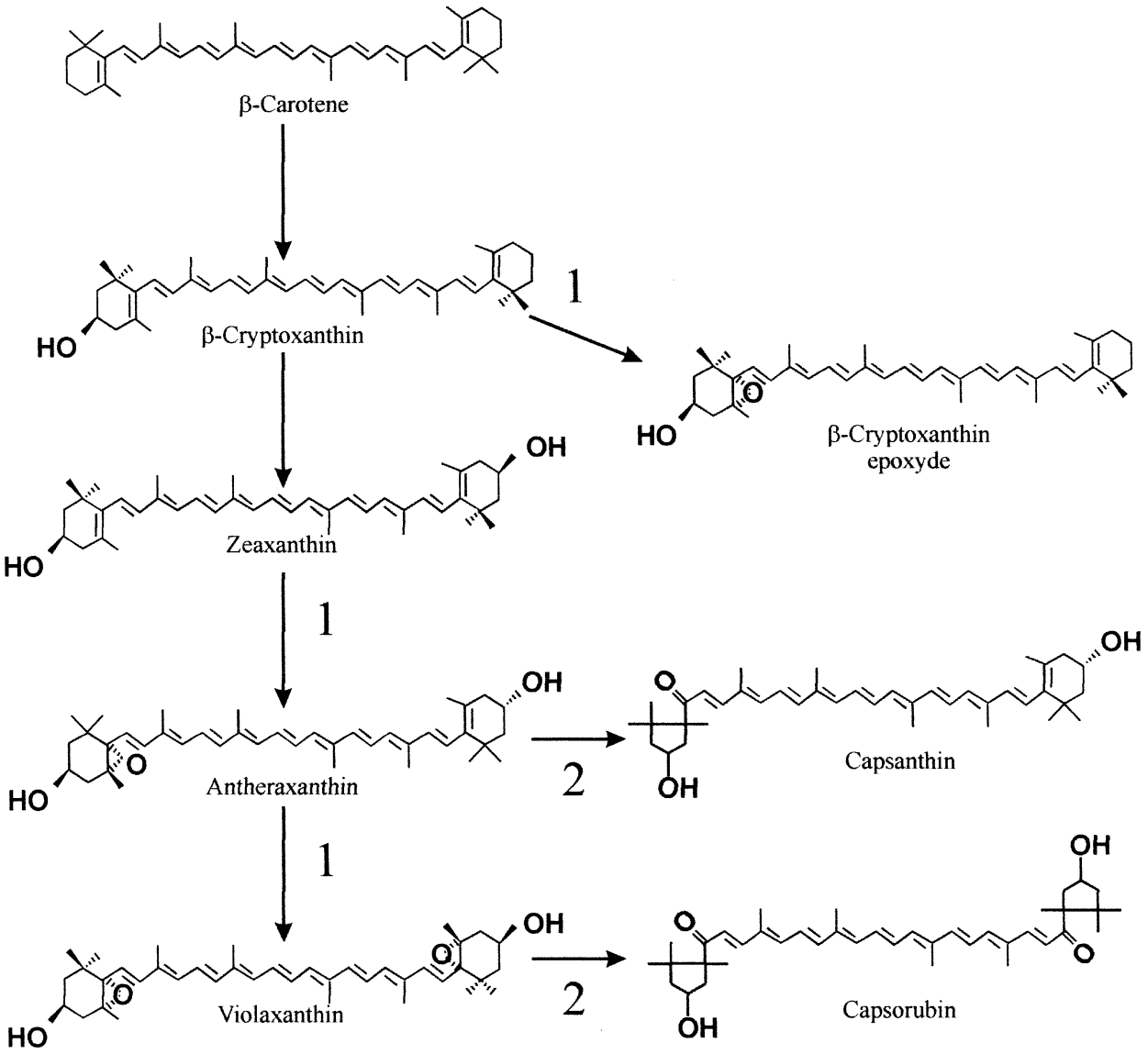


**FIGURE 14.** Hydroxylation and other modifications in the carotenoid production by pepper fruits. Enzymes involved in the latter steps are (1) zeaxanthin epoxidase and (2) capsanthin capsorubin synthase. (Adapted from Ref. 54.)

acid (ABA). The enzyme catalyzes the conversion of zeaxanthin to antheraxanthin and violaxanthin.[295]

Carotenogenesis of pepper *(Capsicum annuum*) fruit is one of the better studied models, and molecular biology has permitted the elucidation at the enzymatic level the last biosynthetic steps for its major carotenoids, capsanthin, and capsorubin (Figure 14).[24]

In the elucidation of carotenogenesis regulatory mechanisms, some interesting genes have been isolated. A cDNA that codes for a protein similar to pepper fibrillin was isolated.[490] This protein does not contain cysteines and has several aspartic and/or glutamic residues arranged in tandem; it is peripheral in thylakoid membranes; the mRNA level have a temporal and tissue specific regulation and shows increments during flower development up to anthesis, then decreases; and mRNA level is higher in corolla. Similar results were observed in melon and watermelon (fibrilar chloroplasts), tomato (crystalline chromoplasts), and orange (globulous chromoplasts). It was concluded that some apoproteins act as chromoplast

sequestrate agents of carotenoids, and altogether gene expression of these proteins and those of PSY1 and PDS genes are strongly regulated at the transcriptional level. Something similar has been observed with chloroplastidial proteins. Interestingly, corolla-specific carotenoid-associated protein (CHRC) is highly unstable and shows a fast disappearance when it is not bounded to carotenoids.[490] It was established that the CHRC expression is up-regulated in cucumber (*Cucumis sativus*) corollas by $GA_3$. Moreover, carotenoid-associated genes were not affected, and it was suggested that modulation of carotenogenesis by $GA_3$ is at the level of carotenoid sequestration. Thus, CHRC up-regulation is proposed to be crucial for enhanced carotenoid accumulation while preserving the structural organization of the organization of chromoplast.[491]

Nowadays, molecular biology has prospected new horizons to produce materials with a high content of biological active carotenoids (e.g., with vitamin A activity) or to introduce new carotenoids.[13] The *crtI* gene of *Erwinia uredovora* codes for phytoene desaturase. *Nicotiana tabacum* was transformed with *crtI* via *Agrobacterium tumefaciens*. Transformed plants were herbicide resistant: norfluorazon, fluridone, diflufenican, flurtamone, and fluorochloridone were inhibitors of phytoene desaturase, and KM143-958, J852, and LS80707 interfere with ζ-carotene desaturase. Interestingly, higher levels of β-carotene and its xanthophyll derivatives (e.g., violaxanthin) and lower levels of lutein were observed in transformed tobacco, while total carotenoid content was conserved between transformed and control plants. The meaning of the change in the xanthophyll pattern have not yet been explained.[13] Another important goal of biotechnology is the production of important crops of high human consumption with enhanced carotenoid content. Rice endosperm does not contain carotenoids, and to obtain plants that produce carotenoids it was necessary to introduce carotenogenesis genes. Rice plants were transformed via biolistic with the phytoene desaturase gene of *Narcissus pseudonarcissus*; this rice formed phytoene, the precursor of colored carotenoids. The high phytoene production with transformants was 0.74 μg phytoene/g dry seed weight. It was mentioned that these results were attractive because the goal was 2 μg β-carotene/g dry seed weight to cover the minimal requirements of young children (100 μg retinol equivalents). Thus, the introduction of the next carotenogenesis enzymes (ζ-carotene desaturase and lycopene cyclase) could generate rice transformants with good levels of β-carotene.[13,66]

## 5. Functions

### a. Color

Carotenoids provide colors to flowers, seeds, fruit, and to some fungi, and color has an important role in reproduction: coloration attracts animals that disperse pollen, seeds, or spores. In *Phycomyces blakesleanus* it was observed that intracellular accumulation of excess carotenoids disturb the mating recognition system, which appears to be involved in the later stages of mating by inhibiting the cell-to-cell recognition systems.[351]

### b. Photosynthesis

Main pigments involved in photosynthesis are chlorophylls and carotenoids. Carotenoids have two well-known functions in photosynthesis: (1) accessory pigments in light harvesting, and (2) as photoprotectors against oxidative damages. It has been proposed that carotenoids as light harvesting compounds evolved from anaerobic organisms, then generalized to all of the aerobic photosynthetic organisms. One of the carotenoid structural characteristics is their ability to absorb visible light: p delocalized electrons suffer a photoinduced transformation in which a singlet state ($s_2$) is produced, then energy is efficiently transferred to chlorophyll (chl) to form singlet chl with a slightly higher energy. The physical structure of chloroplasts facilitate the transference of energy absorbed by carotenoids to chl. In thylakoidal membranes, carotenoids are bound to chls and proteins to form specific complexes called photosystem I (PSI) and photosystem II (PSII). It is known that PSI is a pigment protein complex functioning as a

plastocyanin: ferredoxin oxidoreductase. This process is highly endergonic ($\Delta G° = 77$ kJ mole$^{-1}$) utilizing light energy to drive the reaction. PSI complex is composed of 11 to 17 different polypeptide subunits, about 90 chl "a" molecules, 10 to 15 β-carotene molecules, two phylloquinone molecules, and three [4Fe-4S] clusters.[424] PSII functions as a water-plastoquinone oxidoreductase. It is a membranal complex that comprises more than 25 different proteins, and the heart of the complex is the reaction center (RC) consisting of the D1 and D2 proteins. In all the redox process, quinones have an important participation. It has been suggested that all pigments involved in photosynthesis are bound to proteins of the individual thylakoid membranes, and certainly in bacteria it has been established that bacteriochlorophyll molecules are bound to His residues of α and β apoproteins. Even less is known about how carotenoids are bound to the photosynthetic apparatus, but it has been proposed that carotenoids span the membrane, with the groups interacting with polar amino acids near the membrane surface, and the middle parts making van der Walls contacts with hydrophobic side groups of the protein.[21] Main carotenoid in PSI is β-carotene and lutein in PSII. In PSII, all β-carotene is located in the complex nucleus, very near of the reaction center, while xanthophylls are bound to the remaining chl "α" and "β" molecules in the energy-harvesting antenna.[14,61,81,179,314,349] Wild-type *Arabidopsis thaliana* and ABA-3 mutant, which lacks of epoxydase activity (contains only lutein and zeaxanthin), were used to study the function of carotenoids in the light harvesting complex II (LHCII). It was found the same chl a/b ratio in both wild-type and ABA-3 mutant, but differences were detected in the carotenoid levels. The wild-type ratio was 7/5/2/1 chl a/chl b/lutein/neoxanthin, whereas in ABA-3 mutant it was 7/5/1.4/0.6 chl a/chl b/lutein/zeaxanthin. This study showed that the predominant sequential energy transfer is from carotenoids (predominantly lutein) to chl "b" and then to chl "a" in wild-type *Arabidopsis*. In contrast, mutants showed substantial carotenoid to chl "a" energy transfer. It was suggested that zeaxanthin is the component in ABA mutant that transfers energy to chl a.[84] The photosynthetic efficiency, $O_2$ evolution, and nonphotochemical quenching was similar between mutants and wild type. However, under strong light stress and long exposition time, the ABA mutants showed an accelerated photoinhibition compared with wild-type leaves.[139]

Carotenoid functions are greatly determined by their associated proteins. These proteins are mainly membranal, usually hydrophobic, which bound carotenoids by noncovalent bonds.[183] Also, it has been established that protein correct folding in the photosynthetic complexes requires chl and carotenoids.[186] A cDNA of protein that bound fucoxanthin-chl was isolated from *Heterosigma carterae*. This protein showed a high similarity with proteins that bind chl a/b. With this evidence, divergent evolution was suggested, while the energy-harvesting complexes of algae evolved their ability to bind accessory pigments (chl's and carotenoids) independently to increase their absorption spectra and consequently to have a more efficient energy utilization.[131]

On the other hand, *Phaffia rhodozyma* exposed to singlet oxygen increased the astaxanthin content as a protection mechanism.[428] Phytoene desaturase mutants of *Chlamydomonas reinhardtii* showed severe defects during PSII assembly, PSII repair during photoinhibition, degradation of D1 protein, and in the binding of D1 with PSII. Cells without β-carotene did not show PSII activity, and they did not have D1 protein. It was concluded that, in addition to its role in photosynthesis and photoprotection β-carotene stabilizes PSII or permits the correct assemble of D1 and PSII, protecting D1 of a fast degradation. Also, it was proposed that phytoene desaturase is plastoquinone dependent; plastoquinone redox state could limit β-carotene biosynthesis, which limits the PSII assemble, and this regulates the plastoquinone redox state. Consequently, PSII could be adjusted to illumination conditions by a fast turnover of β-carotene, D1 protein, and its corresponding assemble into PSII.[471] In addition, it has been shown that synthesis of D1 protein increases during the transference of *Chlamydomonas* cells from low light to high light conditions, and it was suggested that it tends to favor the mitigation of photoinhibition and to ensure the function of photosystem II.[436]

### c. Xanthophyll Cycle

When leaves are exposed to high illumination, epoxy xanthophyll groups are removed of violaxanthin to initially form antheraxanthin and then zeaxanthin. This is one of the plant protection mechanisms against light damage. The number of carotenoid molecules is higher in sun-exposed leaves than darkness maintained leaves. Also, xanthophyll cycle carotenoids (violaxanthin, antheraxanthin, and zeaxanthin) are increased in sun-exposed leaves. This phenomenon is very important, sun exposed leaves in a fast-growing stage use not more than 50% of absorbed energy during the stage of maximum radiation (midday), and in some species only 10% is used. Thus, 50 to 90% of absorbed light is in excess and must be eliminated in order to avoid cellular damage. Xanthophyll cycle is a process that makes the energy dissipation easy and protects the photosynthetic apparatus. It has been established that energy transference from chl to zeaxanthin is theoretically possible, and this gives support to the observed zeaxanthin increments under high illumination. Moreover, xanthophyll cycle carotenoids are associated with the energy harvesting complexes PSI and PSII.[14,349]

Xanthophyll cycle involving violaxanthin, antheraxanthin, and zeaxanthin is ubiquitous of higher plants and green and brown algae. In *Dunaliella salina* Teod. and *Dunaliella bardawil* the accumulation of β-carotene has been observed in response to a combination of high light, hypersalinity, and nutrient stress. Also, substantial amounts of zeaxanthin and a continued operation of the xanthophyll cycle have been observed. Interestingly, during diurnal illumination variations, stressed (nutrient and high salt concentration) cells did not show high xanthophyll levels at midday in contrast with nonstressed cells. It was explained that in stressed cells high levels of xanthophylls are maintained, which work as an adaptative function to protect the photosynthetic apparatus.[369]

### d. Antioxidant

*In vivo* and *in vitro* studies have shown that carotenoid photoprotective role is related to its antioxidant activity or with modulation of other cellular antioxidants. Also, it has been established that carotenoid structure has a great influence in its antioxidant activity; for example, canthaxanthin and astaxanthin show better antioxidant activity than β-carotene or zeaxanthin.[281,300,355] Chlorophyll-sensitized photooxidation (4000 lx) of soybean oil was studied in the presence of carotenoids (lutein, zeaxanthin, lycopene, isozeaxanthin, or astaxanthin). It was found that higher carotenoid concentrations decreased the peroxide value of soybean oil by quenching singlet oxygen, and it was shown that longer chromophores favor this reaction. Thus, astaxanthin with 13 conjugated double bonds was the best (in methylene chloride).[274] Packer[352] evaluated the antioxidant activity of different carotenoids by *in vitro* assays. It was reported that antioxidant activity depends on the used system.[352] Other studies indicated an isomer-specific activity of carotenoids: using *in vitro* assays, it was determined that *cis*-lycopene isomers showed better absorption than β-carotene and were more efficient in singlet oxygen quenching.[447]

The antioxidant activity of lutein, lycopene, annato, β-carotene, and γ-tocopherol was evaluated on triglycerides by the effect of air and light. It was reported that lutein, lycopene, and β-carotene act as prooxidants, favoring the formation of hydroperoxides; however, if a small quantity of γ-tocopherol is added to these pigments, the phenomenon is reverted and they act as antioxidants with a higher activity than γ-tocopherol. Annato showed antioxidant activity, however, this could be assigned to unknown fluorescent compounds.[189] β-Carotene also showed prooxidant activity in oil-in-water emulsions evaluated by the formation of lipid hydroperoxides, hexanal or 2-heptenal; the activity was reverted with α- and γ-tocopherol. With this evidence, it was suggested that tocopherols effectively protect β-carotene against radical autoxidation.[204] Antioxidant activity of capsanthin and lutein was evaluated using chlorophyll as photosensitizer. Capsanthin was a better antioxidant, and it was concluded that the antioxidant activity depended on the number of double bonds, keto groups, and that cyclopentane rings in the carotenoid structure enhanced their activity. It was suggested that carotenoids can be used

in foods to prevent degradation of other components.[100] Reactivity of singlet and triplet states was evaluated in carotenoid photodegradation; it was found that canthaxanthin is more stable than β-carotene. β-Carotene could be degraded in oxygen absence (in toluene), but canthaxanthin showed good stability. It was established that carbonyl groups facilitate energy transference by giving greater stability because they have less diradical character, thus transformation between excited states is easier. Also, it was suggested the use of ketocarotenoids as food antioxidant. Interestingly, research has shown than astaxanthin is a better agent to destroy free radicals than other carotenoids.[340] Miller et al.[315] evaluated carotenoid antioxidant activity against radicals and established the following order of activities decreasing): lycopene > β-cryptoxanthin > lutein = zeaxanthin > α-carotene > echineone > canthaxanthin = astaxanthin. Lycopene showed three times more activity than γ-tocopherol, and it was concluded that antioxidant activities are influenced by polarities that are increased with the presence of functional groups in terminal rings.[315]

### *e. Pharmacological Effects*

Many diseases, such as cancer and strokes, involve oxidative processes mediated by free radicals. Carotenoids, by their antioxidant effect, can show benefits in such diseases; however, this function is not completely demonstrated *in vivo*. Carotenoids are an integral part of membranes. Carotenes are immersed in membranes, but xanthophylls showed a variable membranal position, polar groups in xanthophylls affect their position and mobility. Consequently, carotenes are able to react efficiently only with radicals generated inside the membrane. While zeaxanthin, with their polar groups aqueous exposed, is able to react with radicals produced in that zone. Additionally, it has been suggested that carotenoids influence the strength and fluidity of membranes, thus affecting its permeability to oxygen and other molecules. Also, it has been determined that carotenoids have a remarkable effect in the immune response and in intercellular communication.[61,79,94,217] β-carotene, canthaxanthin, 4-hydroxy-β-carotene, and the synthetic retro-dehydro-β-carotene show an efficient induction of the gap junctional communication (GJC) in murine fibroblasts. The GJC stimulation was more than five times, depending on the carotenoid. β-Carotene and retrohydro-β-carotene were the most efficient inducers of GJC, indicating that the presence of a six-member ring is important in the GJC induction; carotenoids with five-member rings showed little activity. Interestingly, the induction of GJC did not show correlation with the quenching of singlet oxygen. Thus, it was suggested that GJC and antioxidant activities in cancer prevention operate independently of each other.[448] The importance of carotenoids with rings of six members was emphasized by the isolation of a carotenoid-binding protein (CCBP) of 67 kDa from ferret liver. Carotenoids with substituted β-ionone rings, without β-rings, or with an intact β-ring with a shorter lineal chain were not bonded by CCBP. Consequently, it was showed that CCBP binds β-carotene mole per mole with high efficiency and specificity. Thus, CCBP may play a major role in the storage, transport, and targeting of β-carotene in mammalian systems. Additionally, it was suggested that the high affinity between CCBP and β-carotene may protect the carotenoid from degradation, and its antioxidant activity must be better.[388] There exists evidence of the effectiveness of β-carotene in the treatment of certain kinds of cancer, for example, smoking-related cervical intraepithelial neoplasia and cervical and stomach cancer.[94] Siefer et al.[438] showed that β-carotene affects the immune response in rats, and by this means tumor growth is inhibited.[438] In contrast, it was reported that retinoic acid and citral act as suppressors of this system. However, it was demonstrated that retinoic acid regulates the γ-interferon (IFN-γ) gene, which has an important role in practically all the stages of the immune and inflammatory responses.[78,217]

More than 600 carotenoids are known, and 50 of them are consumed in meals to be transformed into the essential nutrient vitamin A. After their absorption, these carotenoids are metabolized by an oxidative rupture to retinal, retinoic acid, and small quantities of breakdown products. Carotenoids are transported by plasma lipoproteins. Carotenes are mainly associated with low-density

lipoproteins, while xanthophylls show a uniform distribution between the low- and high-density lipoproteins.[360] Vitamin A is required in the vision process, epithelial maintenance, mucose secretion, and reproduction.[348,385,460] In the process of senile macular degeneration, retinol is related to the induction of a gene cascade permitting the phagocytosis of damaged cell in retina; this process is critical for the photoreceptor survival.[138] Also, it has been established that retinoids affect many biological process, such as cellular proliferation, differentiation, and morphogenesis. Moreover, retinoids have been used in treatments of certain kinds of cancers and some dermatological activities. Additionally, it is mentioned that a diet with deficiencies in vitamin A or supplemented with an excess of retinoic acid could induce teratogenesis.[306]

Differentiation is a complex process, during development of the monoblastic cell line U-937, the activation of both the retinoic acid receptor (RAR) and the 9-*cis*-RA receptor (RXR) are required. Thus, it was suggested that different combinations of retinoids produced different pharmacological responses in the specificity and potency in cancer therapy.[53,133,187] Sun et al.[453] found that retinoic acid induces the response of proteins associated with damage by ultraviolet light in F9 and NIH3T3 cells. However, this induction does not show a correlation with the repairer of DNA damage.[453] Another research showed that retinoic acid conduces to differentiation of F9 cells by an increment of cellular communication. It was suggested that this process is mediated by the protein connexin 43 that induces the expression of important molecules for cellular adhesion.[79,94,133] Currently, the effect of retinoic acid in arthritis treatment is controversial.[267] In these studies, synthetic retinoic acid (Am-80) was used, and a suppression in the incidence of arthritis, back-feet swelling, and bone destruction in collagen-treated rats was observed. However, 13-*cis*-retinoic acid did not show the above-mentioned effects, and this suggest that Am-80 could pertain to a family of antiinflammatory compounds but shows some secondary toxic effects similar to those observed with other retinoids.[267] Ang et al.[9] studied mice embryogenesis and found that retinoic acid has an important role during the gastrulation process. In that stage, alcohol dehydrogenase class IV was identified, and it was proposed that negative effects of alcohol consumption during fetus development could be caused by the inhibition in retinoic acid synthesis, catalyzed by alcohol dehydrogenase, which conduces to a failure in function of retinoic acid receptor (necessary for normal development).[9]

Retinoic acid (RA) has also been related to the aging process. RA affects the gene expression levels through its interaction with triiodothyronine receptor (TR) and RAR. Interestingly, when the TR and RAR mRNA levels were analyzed in the brain of young, adult, or aged rats, lower values were found. Additionally, it was showed that RA supplementation increased the TR and RAR mRNA levels. Also, the transglutaminase activity was reduced in aged rats and recovered after RA treatment. Transglutaminase has involved the memory process.[385]

Prostaglandins are substances that have been involved in several physiological process, and in particular prostaglandin $D_2$ has been involved in the endogenous sleep promotion, modulation of several central actions (regulation of body temperature, release of the luteinizing hormone), etc. Recently, it was shown that prostaglandin D (PGD) synthase binds retinoic acid and retinol. In addition, it was demonstrated that *all-trans*-retinoic acid inhibits its enzymatic action but not retinol. With the generated information, it was suggested that retinoids may regulate the synthesis of $PGD_2$, and that PGD synthase may be a transporter of retinoids to the place where they are required. Thus, it was suggested that PGD synthase plays a critical role in regulating the development of neurons by the regulation of the transfer of all-*trans*- or 9-*cis*-retinoic acid to RAR or RXR in the immature nerve cell.[457] Additionally, it has been mentioned that the overexpression of the cyclooxygenase gene (a key enzyme in the formation of prostaglandins) is an early and central event in colon carcinogenesis.[217]

In contrast with that discussed above, it was shown in transgenic mice that inhibition of the transcription activator protein-1 (AP1) has an important antitumor promotion activity, but the activation of the retinoic acid-responsive element has not. AP1 regulates the expression of genes

with the recognition sequence designed as 12-*O*-tertadecanoylphorbol-13-acetate (TPA)-responsive element (TRE) in their promoter region, and many TPA-responsive genes include several protooncogenes (e.g., *c-fos*, *c-jun*). Thus, the design of specific inhibitors of the transcription of AP1may be an interesting strategy in the treatment of cancer.[227]

Carpenter et al.[75] observed that mixtures of canthaxanthin with low-density lipoproteins (LDL) inhibited macrophage formation from human monocytes. However, if canthaxanthin and LDL were added simultaneously (but without previous mixture) to cellular medium, it was not observed to any effect. The same was noted with β-carotene, but with zeaxanthin the opposite was found. It was explained that all evaluated carotenoids show good antioxidant activity, and observed differences are caused because they act at different levels: zeaxanthin can quench radicals in the aqueous phase, while β-carotene inhibits lipid peroxidation; on the other hand canthaxanthin was the most potent agent in the inhibition of methyl linoleate, and it was concluded that antioxidant activity depends on the particular system: radical, carotenoid, microenvironment, etc. Thus, diets with carotenoid mixtures are recommended instead of having just one particular carotenoid, because *in vivo* a great variability of radicals and microenvironments take place.

Carotenoids have been considered that provide benefits in age-related diseases, against some forms of cancer (in especial lung cancer), strokes, macular degeneration, and cataracts. However, most studies relate dietary components with sickness incidence or symptoms; thus, these studies cannot establish a direct cause-effect relationship. On the other hand, it is clear that carotenoids in association with other components of fruits and vegetables seem to have a protective effect against some chronic diseases and precancerous conditions. Additionally, in some studies β-carotene was supplied to smokers, and it was found that cancer mortality indexes were higher in smokers than in their respective controls.[14,349,460,529] It has been signaled that a combined supply of β-carotene, α-tocopherol, and selenium reduces stomach cancer mortality, and it was pointed out that the consumption of marine algae (especially *Phaeophyta*) diminished the risks of being affected by certain types of cancer. Also, it was established that the main antitumor agent is not β-carotene, but other components (carotenoids) that are present in algae. In this sense, mixtures of carotenoids (α-carotene, fucoxanthin, and halocintiaxanthin) have shown a higher inhibitory activity than β-carotene in proliferation of human neuroblastoma cells. In addition, α-carotene showed higher antitumorigenic activity than β-carotene in rat cancer induced by glycerol, and it was mentioned that carotenoids with an ε-ring (absent in β-carotene) have higher inhibitory activity.[94,337]

The antimutagenicity of carotenoids in Mexican green peppers *Capsicum annuum*) were studied. The experiment was carried out by determining the number of revertants in the plate assay with *Salmonella typhimurium*. The antimutagenicity inhibition by nitroarenes was higher than 90%. Pepper carotenoids were more efficient antimutagens than pure β-carotene, suggesting that other carotenoids (e.g., lutein, zeaxanthin) in the pepper extracts showed a synergistic effect with β-carotene. Also, it was mentioned that the antimutagen activity might be from blocking the entrance of toxic compounds into the cell or by their antioxidant activity.[384] Also, the antimutagenicity activity of carotenoids of Aztec marigold (*Tagetes erecta*) was evaluated. It was concluded that lutein was the compound with a higher activity on marigold extracts, but similar to the observation mentioned for the pepper extracts, the mixture of carotenoids in the marigold extract had higher antimutagenicity activity. In addition, it was suggested that lutein and 1-nitropyrene (mutagen) formed an extracellular complex that limits the bioavailability of 1-nitropyrene and consequently its mutagenicity.[177]

Böhm et al.[44] indicated that carotenoids, α-tocopherol radicals, and ascorbic acid develop their function by diminishing the content of harmful nitrogenous compounds. Also, a synergistic effect between β-carotene and vitamins E and C was observed in cellular protection. It was explained that β-carotene not only destroys oxyradicals but repairs tocopherol radicals produced when α-tocopherol destroys oxyradicals. Additionally, it was suggested that low antioxi-

dant levels (e.g., ascorbic acid) in smokers, in contrast with non-smokers, could be related with an apparent failure in the recycling of α-tocopherol by β-carotene.[44]

Carotenoids protect lab animals of UV-induced inflammation and certain type of cancers. Historically, carotenoid supplementation has been used in the treatment of diseases produced by light sensitivity, which are usually hereditary: 84% of patients with erythropoietic protoporphyria, consuming diets supplemented with β-carotene, increased by a factor of 3 their ability to resist sunlight exposition without presenting symptoms. Also, carotenoids have been used in other photosensitivity diseases: congenital porphyria, sideroblastic anemia, and have shown only a limited success in treatment of polymorphic light eruption, solar urticaria *Hydroa vacciforme*, *Porphyria variegata*, *Porphyria cutanea tarda*, or actinic reticuloid.[300,529]

Lutein and zeaxanthin have been considered as protective agents against aging macular degeneration and senile cataracts.[460] Also, it has been suggested that β-carotene suppress the increment of hormones related to stress syndrome.[197]

Certainly, up to date studies on anti-carcinogenic activity have produced unexpected results. Massive studies the α-tocopherol, β-carotene [ATBC] cancer prevention study, the β-carotene and retinol efficacy trial [CARET], and the physicians health study) gave no results or do not inclusively give a higher incidence of cancer. Nowadays it is thus premature to enunciate final conclusions regarding the potential role of carotenoids in the therapeutics of degenerative diseases. However, the consumption of fruits, vegetables, and fortified foods with antioxidants is encouraged.[94]

## 6. Methodological Aspects

It is evident that new methodologies usually give better results and confidence when identifying and quantitating the carotenoid components, an important aspect if we consider that biological activity of different carotenoids is different, for example, not only β-carotene is source of vitamin A, and not all carotenoids show this activity.

Many methodologies used in the analyses of organic compounds are inadequate for carotenoid analyses. Their study is complicated by the great variability in nature, the existence of many isomers, great structural similarity, instability, presence of substances that interfere the determination, and the absence of an exact quantitation method.[252,480]

The stages to be considered for carotenoid studies are extraction, saponification, separation, identification, and quantitation, and the development of a good work depends on our knowledge of carotenoid properties:[61,112]

1. Carotenoids are lipids and thus soluble in other lipids or in nonpolar solvents.
2. Carotenoid color is imparted by conjugated double bonds that are mainly in *trans* configuration (in their natural form), in extended form; hence, they are lineal and rigid molecules. The properties of *cis*-carotenoids show substantial differences with the *trans* ones; they are more easily solubilized, absorbed, and transported. Factors generating isomerization are free halogens, acids, excessive light, and high temperatures.
3. Conjugated double bonds in carotenoids are highly delocalized, and consequently carotenoids have a low-energy excited state. Hence, the energy of transition is in the visible region (400 to 500 nm); thus,they are intensely colored yellow, orange, or red). Also, because of this property carotenoids are involved in photosynthesis and photoprotection.
4. The polyene structure of carotenoids makes themselves highly reactive molecules. The structure is rich in electrons and susceptible to be attacked by electrophilic reagents responsible for carotenoid instability against oxidation and give them their radical characteristics. Carotenoids in the crystalline state are susceptible to oxidation after their isolation; their degradation is very quick if stored in the presence of oxygen traces. Electrons are delocalized but not with a uniform distribution, electronic density is higher at molecule ends, and these are their more reactive part: deepoxydation reaction

preferentially occurs in carbons 7 and 8, diminishing the reactivity by the presence of keto groups (astaxanthin and canthaxanthin).

### *a. Extraction*

**With solvents.** Carotenoids are soluble in lipids or in nonpolar solvents, except when they form complexes with proteins and sugars. Hence, they are extracted with nonpolar solvents. If the tissue is previously dried, then water-immiscible solvents are used such as petroleum or ethyl ether; with the fresh materials acetone or ethanol are used, which have two functions, extracting and dehydrating solvents. Solvents used in extraction must be pure (without oxygen, acids, halogens) to avoid degradation. Up to now, no solvent is optimal for the extraction of all carotenoids: carbon disulfide is the best solvent, but volatility, flammability, toxicity, and degradation limit its use. Chloride solvents are good, but they show high toxicity; free peroxide ether, despite its efficiency, is not used because of its flammability and volatility; other solvents such as hexane, heptane, and isooctane are not so good for extraction, but their other characteristics are favorable. On the other hand, it must be considered which compounds will be extracted: polar solvents (such as acetone, methanol, ethanol) are good with xanthophylls but not with carotenes. As a general rule, the extraction process consists of the removal of hydrophobic carotenoids from an hydrophilic medium. The use of nonpolar solvents is not recommended because of penetration through the hydrophilic mass that surrounds pigments is limited, while slightly polar solvents dissolve poorly carotene in dried samples and solubility diminish in fresh samples. Thus, it was postulated that complete extraction can be reached by using samples with low moisture, and slightly polar plus nonpolar solvents.[112] Literature describes different solvent systems used in carotenoid extraction. In general, extraction must be carried out very quickly, avoiding contact with light, oxygen exposure, and high temperatures in order to minimize degradation.[60]

Currently, industrial extraction consists of pressing and solvent extraction of materials: material is milled and pelleted, mixed with hexane, and heated in a special recipient covered with a vapor jacket; hexane is eliminated in a film evaporator and afterward by vacuum distillation, and the main problems to be solved are to diminish pigment degradation, increase extraction performance, and solve safety and environmental problems. More than 50% of the pigments is lost during this extraction process; oil extraction industries emitted into the environment 210 to 430 million liters of hexane that together with other organic compounds can produce nitrogen oxides and other pollutants. These products in the last stage generate ozone and other highly dangerous photochemical oxidants.[403] With these perspectives, several researchers have proposed alternative extraction solvents. Heptane has been used, and the following characteristics have been mentioned: lower volatility than hexane, similar extraction efficiency, and good quality product (oil).[83] When mixtures of heptane-isopropanol or only isopropanol are used, more antioxidants are extracted and oils with enhanced stability are obtained, and considering that isopropanol has lower flammability than hexane, this solvent could be a good alternative in oil extraction.[247,381]

Enzymatic and/or aqueous extraction. Food industries have used enzymatic methods to obtain a diversity of products: maize starch, gluten and starch of wheat, gelatin, deboned meat, among others. The main advantages of these procedures are specificity, moderated temperature and pH, treatments are mild, secondary products are scarce, and the final product is almost not affected. In enzymatic processing, enzymes with mixed activities are used because of cell wall complexity.[106,119,132,298,353] Successful application of enzymes have been reported in oil extractions, and extraction performance increments in the range 50 to 90% have been reported, and byproducts have shown higher digestibility than control.[124,403]

Aqueous extraction has been proposed since 1950 as an alternative to organic solvent. This technology was implemented because of safety

and the cheapness of the process, which is based on oil-water insolubility and phases are separated by differences in density. Up to now, many reports have been published on extraction by simultaneous enzymatic and aqueous processes: in oil extraction of avocado, olive, coconut, rapeseed, maize, cocoa, among others.[52,97,106] However, reports on the application of this technology in pigment extraction are scarce. Pommer[377] proposed an extraction method of vegetable oleoresins, and between them pigments where plant material is mixed with water and an water-immiscible organic acid. Organic acid was selected with a linear chain of 6 to 12 carbons. Optionally, enzymes are used in the extraction process, and pigment-extraction efficiency was enhanced for pigments of marigold (*Tagetes erecta*) flowers. On the other hand, Delgado-Vargas and Paredes-López[120] reported a procedure in which enzymatic treatment and aqueous extraction were carried out in order to obtain marigold flower meals with a higher carotenoid content.

**Supercritical fluid extraction (SFE).** The above-described method appears to have the requirements to be considered a good extraction process: fast, simple, and cheap. However, it generally implies that considerable time is needed for it to be carried out, and a concentration stage may be necessary as well. Consequently, SFE has been considered to perform a better extraction process.

Up to 1986, only two works using SFE had been reported, but in the period 1986-mid-1989 26 works were published; this large increment could be explained because this technique shows the following advantages: rapidity, solvent strength can be controlled, and the solvents used are gases friendly to the environment and with low toxicity. Interestingly, with this method the concentration stage is avoided because solvents are eliminated immediately under environmental conditions.[104,203,387] With these characteristics, SFE has been used commercially to obtain caffeine from coffee and hop oil.[387] Lutein and carotene were extracted from protein leaf concentrate by using $CO_2$ as a solvent in SFE, and it was shown that conditions can be manipulated to make a selective extraction.[143] β-Carotene SFE of sweet potato was three to five times more efficient than traditional methods, but isomerization was observed with this extraction process.[445] SFE and traditional extraction processes were compared in the extraction of carrot carotenoids. The highest efficiency was obtained by using ethanol (10%) as a co-solvent, and it was reported that the traditional method extracts more α-carotene than SFE. Interestingly, vitamin A equivalents obtained by SFE were higher than by traditional methods and extraction time was diminished from 6 to 1 h.[22] Despite the above mentioned, no commercial process of pigment SFE is in use nowadays.

### *b. Saponification*

As mentioned above, xanthophylls are usually esterified,[279] which produces additional analyses complications, for example, a pigment with two hydroxyl groups can be without one or two positions esterified, which requires both separation and identification. Thus, saponification obtains less complex mixtures when only nonesterified pigments appear. Another advantage of saponification is chlorophyll destruction in the saponified samples.[112]

Most carotenoids are stable under alkaline treatments; thus, the use of methanolic solutions of potassium hydroxide is a common method of saponification, sometimes at environmental temperature or by heating.[11,59,179,252] When carotenoids are sensitive (e.g., astaxanthin and fucoxanthin), alternatively lipases have been used.[59] *Candida cylindracea* lipase has been used in the saponification (darkness, under nitrogen atmosphere, and 4 h) of red palm oil and neither loss nor isomerization of β-carotene was observed.[282] In carotenoid extraction from paprika, it was reported that carotene is sensitive to alkaline saponification, thus mild conditions were evaluated and samples were saponified with potassium methoxide.[237]

### *c. Separation*

Separation methods can be classified as nonchromatographic and chromatographic. Nonchromatographic method uses mainly phase

partition, for example, by using petroleum ether and aqueous methanol (90%). Carotenoids are dissolved and nonpolar compounds recovered in epiphase, petroleum ether.[59] Also, this method has been used in lutein purification by using hexane:acetone:toluene:absolute ethanol (10:7:7:6 v/v) and then hexane.[478] Another example: xanthophyll esters are separated by Craig countercurrent distribution using dimethylformamide:dichloromethane:hexane (8:2:10 v/v) as solvent.[166]

In chromatographic methods adsorbents have been used such as sucrose, cellulose, starch, $CaCO_3$, $Ca_3(PO_4)_2$, $Ca(OH)_2$, CaO, $MgCO_3$, MgO, $ZnCO_3$, $Al_2O_3$, silicic acid, silica gel, kieselguhr, Microcel C, and mixtures. A general strategy for obtaining a pure carotenoid uses open column chromatography in alumina followed by thin layer chromatography (TLC) in silica, MgO-kieselguhr G TLC, and silica TLC again, with different solvent systems. The criteria for choosing a solid support depends on the carotenoid to be purified; for example, alumina must not be used to separate astaxanthin because of oxidation problems; additionally, alumina can produce isomerization of other carotenoids. Magnesium oxide is not recommendable when acetone is used as solvent; a possibility of solvent polymerization reaction exists. Alumina and silica separations are based on polarity; on the other hand, magnesium oxide or calcium hydroxide permits separation by number and type of double bonds in the structure; calcium hydroxide or zinc (II) carbonate separates *cis-trans* isomers. It is usually recommended to use each one of these three supports (alumina, magnesium oxide, and calcium hydroxide) to reach the separation, and, generally, preceded by silica or alumina open-column chromatography.[59,60] Nowadays, open column chromatography is used as a prepurification stage to separate groups of carotenoids with similar characteristics in special flash open column chromatography.[264,308]

In chromatography, TLC is still used because new advantages have been incorporated into this technique in addition to its main characteristics: low cost and simplicity. Also, it must be pointed out that this is the starting technique used in whatever purification, and that is the wider used technique.[59,60,378] In addition, it is possible to use reverse phase TLC chromatography to reach the separation.[235]

High-performance liquid chromatography (HPLC) is the preferred column chromatography to carry out the qualitative and quantitative analyses of carotenoids.[60] This technique is ideal because carotenoids can be monitored easily with the UV-visible detector, and methodology has converted it into a powerful technique with the introduction of the diode array detector (DAD), which permits detection at several wavelengths and simultaneous tentative identification by UV-spectral analyses. In addition, information about purity of compounds is obtained with a DAD detector.[57,427,480] In general, HPLC shows the following advantages over the traditional methods: greater sensitivity, resolution, reproducibility, and speed of analysis; additionally, it is possible to use inert conditions.[36,181,415,480]

It has been reported that HPLC has reproducibility advantages inclusive in relation to supercritical fluid chromatography.[36] Additionally, separation of carotenoid geometric isomers has been tried with reverse phase $C_{18}$ column but with limited success.[427] However, a successful separation was reached by using a column with polymeric surface $C_{30}$. The resolution of isomers was higher for polar and nonpolar carotenoids than $C_{18}$ resolution.[415] This column has been evaluated in the separation of isomers of lutein, zeaxanthin, β-cryptoxanthin, α- and β-carotene and lycopene, which were produced by iodine isomerization. The main isomers identified were 9-, 9′-, 13-, 13′-, 15-*cis* and *all-trans* carotenoids. It has been commented that a good separation is a prerequisite to make more exact determinations of provitamin A content, isomeric profiles in biological tissues, comparative physiological roles between the *cis* and *trans* configurations, purity determination, and purification of carotenoids.[136] This column has been used in the evaluation of biological samples with excellent results.[117,118] When a $C_{34}$ column was evaluated, better resolution was obtained for only some isomers, and, in general, the results were comparable to those obtained with the $C_{30}$ column.[33] On the other hand, with $Ca(OH)_2$ columns, a good resolution has been obtained in the separation of carotene geometric isomers with 5, 7, and 11 double bonds.[425]

All the information indicates that HPLC analyses are required when analyzed carotenoids are minority compounds in a given sample.[1] Also, it is necessary to establish the content of bioactive compounds, such as provitamin A precursors. The analysis of leafy vegetables used in the Indonesian food table (spinach, cassava, papaya, mango, sweet shoot, and jointfir spinach) showed that with HPLC were obtained lower values for provitamin A than previously reported values.[228]

### *d. Characterization*

**Spectroscopy.** The most important technique in carotenoid analyses is UV-visible spectroscopy, which gives information about the presence of rings, carbonyl groups, and isomeric effects.[59,92,112,179,181] In this analysis, absorption maxima, form, and fine structure of spectra are characteristic of the molecule's chromophore. Nowadays, the introduction of DAD permits to make *in situ* identifications immediately after HPLC separation.[60]

Infrared spectroscopy gives information on the kind of bonds and atoms in the analyzed compound, while nuclear magnetic resonance (NMR) of proton and carbon permits the assigning of these atoms to a certain structure. However, the technique used most in carotenoid analysis is mass spectroscopy (MS), mainly because of the sample quantity required for analysis is very small. Mass spectroscopy provides information on carotenoid MW, and fragmentation pattern helps us to determine the carotenoid structure.[179] However, it is very important to choose an adequate MS equipment because of carotenoid instability, and one of the initial and most successful MS ionization techniques is fast atom bombardment (FAB).[200,426] The main advantage of this methodology is that MW is determined unambiguously; the molecular ion usually appears, and one of its disadvantages is a poor fragmentation pattern, thus structural elucidation limited and serial detectors must be used to differentiate between geometric isomers. Nowadays, new techniques have been introduced: HPLC coupled with an MS detector (LC/MS), a very convenient technique by considering intrinsic carotenoid instability, and MS with an electrospray detector that is 100 times more sensitive than conventional techniques (picomolar).[480] Another ionization technique is Atmospheric Pressure Chemical Ionization (APCI), which produces a better fragmentation pattern than FAB or electrospray and shows a good compatibility with HPLC equipment, and, recently, matrix-assisted laser desorption ionization was introduced and its detection limit is in a femtomolar-attomolar range.[251,480] These detectors have been used in several studies to identify carotenoids from biological samples, and it has been established that carotenoid identification must cover at a minimum the following criteria: co-chromatography with authentic samples, UV visible, and mass spectra.

RAMAN spectroscopy and circular dichroism have permitted the study of carotenoids in biological systems.[200,435] Kull and Pfander[264] studied canola carotenoids and used UV visible, MS, NMR, and circular dichroism to identify several isomers of luteoxanthin and violaxanthin. Also, it was mentioned that in nature both *trans* and *cis* isomers exist.

Photoacoustic spectroscopy (PA) was used to evaluate pigment composition of paprika. It was possible to identify peaks or shoulders in the visible spectrum region that corresponds to carotenoids or chlorophylls. With the generated information, it was possible to distinguish between different samples of paprika, and, when PA was used in the near infrared region (800 to 1000 nm), semiquantitative information on total pigment composition was obtained.[489]

**Chemical tests.** By considering carotenoid chemical structure, several simple tests are used to corroborate the presence of chemical groups: 5,6-epoxydes react with HCl to form 5,8-epoxyde isomers; this chemical modification is accompanied by a hypsochromic shift of 7 to 22 nm for monoepoxydes and 40 nm for diepoxydes. Allyl alcohols treated with HCl are dehydrated, and another double bond is formed with a consequent change in their UV visible spectra. Another test to evaluate allyl alcohols is the reaction with HCl in methanol to obtain methyl esters. To determine the presence of aldehyde groups, aldol condensation was carried out by treatment with acetone in a basic system producing an extension in the con-

jugated system, then spectra showed a change toward higher wavelengths. In carotenoids with carbonyl groups, these can be identified by reaction with hydrides in ethanol or tetrahydrofuran; thus, a hypsochromic change of 20 to 30 nm and finest spectra are observed. Another simple test is iodine isomerization, which produces a mixture of equilibrium isomers (*cis-trans*); if the starting pigment is *all-trans*, a hypsochromic shift is observed (3 to 4 nm), while if it is *cis*, hyperchromic (1 to 3 nm).[112,179]

Silver nitrate is used to discriminate between β and ε carotenoid rings. Carotenoids are separated by TLC (preferable in silica gel) and then developed with a methanolic solution of silver nitrate. Carotenoids with β-rings produce a bathochromic shift; the shift depends on the number of β-rings, for example, after development zeaxanthin spot presents red tones, while lutein spot has yellow tones.[236]

**Evaluation of pigmentation efficiency.** Color is a complex process and color evaluation also, because we need to evaluate eye perception and brain interpretation, and pigment deposition and color perception do not show a direct relationship. All attempts are only approaches to reality. The most common used methods are (1) sample carotenoid extraction followed by UV-visible spectroscopy analysis,[11,313] and (2) the use of photoelectric instruments that measure reflectance. Hunter-Lab is the most common equipment; it is based on tristimulus effect.[31,297] Several studies have shown that reflectance colorimetry is a good descriptor of the pigment content from different samples (pepper, red beet, marigold). This research has given high correlation coefficients (> 0.9), and it has been concluded that the assessment of pigment content by color determination is highly feasible at industrial level.[121,230,394]

## 7. Importance As Food Colors — Stability, Processing, and Production

Carotenoids have been used as food colors for centuries: saffron, pepper, leaves, and red palm oil have carotenoids as their main color components. Color of carotenoids, together with beneficial properties such as vitamin A precursors and antioxidants, have led to their wide application in the food industry; preparations to apply them in oily or aqueous media have been produced, including emulsions, colloidal suspensions, and complexes with proteins. These preparations have found applications to pigment margarine, butter, fruit juices and beverages, canned soups, dairy and related products, deserts and mixes, preserves and syrups, sugar and flour confectionery, salad dressings, meat, pasta and egg products, among others,[257] and, interestingly, other important areas of application of carotenoids have been emerged. It has been reported that corn carotenoids inhibit the synthesis of aflatoxin by *Aspergillus flavus* (90%) and by most of the *A. parasiticus* (30%) strains. Interestingly, carotenoids with an α-ionone ring showed higher inhibition (25%) than the β-ionone ring. Thus, lutein and α-carotene showed higher activities than β-carotene or zeaxanthin, and it was determined that several corn inbred lines have high enough levels of carotenoids to affect aflatoxin formation in the endosperm (0.09 to 72 μg/g). It was shown that the antioxidant activity of carotenoids are not responsible of inhibition of aflatoxin synthesis, and it was suggested that they may affect cell membranes enough to indirectly modify polyketide synthase activity (cytosolic enzyme) or by direct interaction with the synthase or other enzymes of aflatoxin synthesis.[344]

### a. Stability in Model Systems

In practice, it is very important to take into account the carotenoid instability, and the establishment of processing conditions must consider this aspect.[297] However, most studies have been carried out with β-carotene. It has been reported that some pepper components facilitate β-carotene oxidation. β-Carotene oxidation was accelerated by increased levels of linolenic acid. The ascorbic acid effect depends on its concentration and if copper ions are present. It was mentioned that high aqueous activity favored prooxidant activity. Additionally, high ascorbic concentrations (100 μmol/g of cellulose) in the presence of copper ions induce a greater antioxidant effect, and under these conditions prooxidant activity of

peroxidase was inhibited. Moreover, it was concluded that ascorbic acid concentration needed for food protection depends on the content and identity of lipids present.[250]

At incubation conditions (37°C, 5% $CO_2$, darkness), β-carotene was slightly stable during 48 h and showed fast degradation with the effect of UV and visible light in the presence of oxygen; the degradation rate was increased with increments in $O_2$ turnover. β-Carotene was stabilized with antioxidants (e.g., BHT); it was thus concluded that degradation is by the effect of free radicals.[419]

Another study evaluated the effect of temperature (100°C by different times) on aqueous suspensions of β-carotene and lycopene. It was considered that this treatment is representative of those commonly applied on vegetables. Volatile compounds were trapped in column or in organic solvents then analyzed by gas chromatography (flame ionization detector) and mass spectrometry. The main degradation products of β-carotene were identified as 2,6,6-trimethyl-cyclohexanone; β-cyclo-citral; 5,6-epoxy-β-ionone and dihydroactinidiolide. The products of lycopene degradation were 2-methyl-2-hepten-6-one; pseudo-ionone; 6-methyl-3,5-hepadien-2-one; geranial, and neral, and the following nonvolatile compounds of β-carotene degradation were identified: aurochrome; mutatochrome; 5,6,5′,6′-diepoxy-β-carotene and 5,6-epoxy-β-carotene.[89]

An oxidation study of β-carotene by peroxide radicals was carried out at 37°C in benzene, and alkylperoxyl radicals were generated by 2,2′-azobis (2,4-dimethylvaleronitril). The analysis of oxidation products suggest that β-carotene is destroyed by a free-radical-addition mechanism. Products were analyzed by HPLC, UV-visible spectroscopy, MS, and NMR (proton and carbon-13).[519]

On the other hand, many studies have been carried out to evaluate the kinetics of carotenoid degradation and/or biosynthesis. Degradation mechanisms based on free radicals have been proposed by using solid supports (carboxymethylcellulose, $Al_2O_3$ or MgO).[174] Also, it has been established that low $a_w$ (around 0.3) diminish the carotenoid degradation.[174,192] Photodegradation 2690 lx) of aqueous β-carotene has been studied. The models were in matrixes of gelatin/sugar and acacia gum/sugar. In first-order kinetics in the degradation and isomerization reaction was observed. Also, it was shown that in illuminated aqueous β-carotene, the main isomers formed were 9-*cis*-, 13-*cis*-, and *all-trans*-β-carotene. In darkness, 13-*cis*-isomer was favored, while 9-*cis* was under light conditions, showing a lower stability of 13-*cis*-β-carotene.

Another study showed that *all-trans*-β-carotene isomerization depends on which solvents were dissolved. Faster reactions were observed in nonpolar solvents, identifying the higher rate of formation for the 13-*cis*-β-carotene.[368] However, other studies showed contrasting results: β-carotene, diesterified capsanthin, and capsanthin dissolved in anhydrous (cyclohexane) or aqueous ethanol, water) solvents were illuminated (1000 l×). Degradation in anhydrous medium followed a zero-order kinetics and of first-order in aqueous medium. In addition, it was shown that deesterified pigments were more stable in aqueous than in nonpolar medium, while at intermediate polarity both forms esterified and deesterified) showed similar stability. β-Carotene was more labile than capsanthin, and as in other studies degradation increased with temperature.[319]

Crystals of α- and β-carotene were isomerized by temperature, light, and iodine. The main components in isomerized samples were *all-trans*-, 15-*cis*-, 9-*cis*-, and 13-*cis*-β-carotene and *all-trans*-, 15-*cis*-, 13-*cis*-, and 9-*cis*-α-carotene (in decreasing order). Minimal isomerization was observed in the range 50 to 100°C/30 min, but it was considerable at 150°C/30 min. The photoisomerization reactions were of first-order: reaction constant was higher for the conversion toward *all-trans*-β-carotene, and with α-carotene the 13-*cis*-isomer was favored. With iodine catalyzed reaction, β-carotene degradation followed a first-order kinetics, while isomerization produced first- and second-order kinetics.[98]

Photostability (sunlight 8 h/day) of marigold pigments was studied in aqueous emulsions with Arabic gum and/or mesquite gum. Stability in emulsions with mesquite gum was better 3.83 times) than with Arabic gum. Also the synergistic effect between the two gums was observed. Highest stability was reached at pH 5.0. Kinetic deg-

radation was of zero-order. It was concluded that polyelectrolytic nature and MW of gums are important in color protection, and dark emulsion hue could act as a sun filter.[486]

### *b. Processing and Stability in Foods*

Processing effects were analyzed in tomato and green vegetables (broccoli, spinach, and green beans). Moderate processing did not produce carotenoid modifications, but a prolonged heating (1 h boiling) conduces to total destruction of epoxycarotenoids; it was shown that under this condition only the most sensitive carotenoids are highly destroyed. Boiled tomato showed an identical carotenoid profile with fresh tomato, and differences in content were only detected.[253] A similar result was obtained with vegetables used in Spain, and it was mentioned that carotenoid saponification results in losses that are more critical in xanthophyll than in carotenes.[184]

The effect of freezing and canning of kiwi was analyzed by TLC, HPLC, UV-visible spectroscopy and chemical tests. The principal components of fresh and frozen kiwi were 9′-*cis*-neoxanthin, *trans*- and *cis*-violaxanthin, auroxanthin and lutein. In frozen kiwi stored for 6 months (-18°C), the carotenoid profile was similar to fresh fruit, but in addition antheraxanthin was detected. On the hand, canned kiwi showed a complicated carotenoid profile because of thermal treatment. Neoxanthin and violaxanthin were some of the degraded xanthophylls.[74]

Processing of fresh pepper to obtain paprika was studied. Carotenes and xanthophylls were extracted with acetone and identified by UV-visible, infrared spectroscopy, and chemical tests; quantitation was carried out by UV-visible spectrometry. It was reported that fast drying induces the carotenoid destruction and increments were observed by slow drying of pepper. It was proposed that an adequate drying process could conduce carotenoid synthesis: in bola pepper, increments of red carotenoids were observed (capsanthin, capsorubin, and capsolutein); however, this effect was not observed in agridulce pepper. Also, it was mentioned that during milling process a high reduction in carotenoid content was observed, and the most affected carotenoid was β-carotene followed by β-cryptoxanthin and zeaxanthin, while the most stable were capsanthin and capsorubin. Also, it was observed that drying process produces a net carotenoid biosynthesis that is enhanced by illumination: drying under darkness conditions increased the carotenoid content in 15%, while under illumination it was 47%. Higher increments were observed in β-carotene, cryptoxanthin, zeaxanthin, and capsanthin, while violaxanthin and capsorubin were destroyed. The biosynthesis occurred in a first stage (35 to 40% of moisture), then degradation was observed.[318,320]

The effect of mango processing on carotenoid content was evaluated. Mango was sliced and stored at vacuum or frozen (-40°C) conditions during 6 months; other samples were commercially canned. Fresh and frozen fruits showed similar characteristics. Canned mango showed great changes in color and carotenoid profile. The most stable carotenoid was β-carotene.[73]

*All-trans*-β-carotene isomerization sensitized by chlorophyll was studied. Main formed isomers were 9-, 13-, and 15-*cis*-β-carotene, with a higher proportion of the 9-*cis*-isomer. In control without chlorophyll, isomerization was imperceptible during the first 2, then a quick increment was observed during the first day and then remained constant. The main isomers under these conditions were 9- and 13-*cis*-β-carotene. The chlorophyll sensitizer effect supports the idea that *cis*-isomers in photosynthetic tissues are not artifactual.[350]

Chen et al.[99] studied the effect of juice carrot processing on its carotenoid content. Pasteurization 105°C/25 s) neither produced a considerable variation in the isomeric profile nor in the carotenoid content. Treatment at 110°C/30 s produced the degradation of 45% of β-carotene while the isomers formed were in decrement order): 13-*cis*- > 15-*cis*- > 9-*cis*- > 13,15-di-*cis*-β-carotene, α-Carotene pattern was similar, but the main isomer was 15-*cis*-α-carotene. Lutein degradation was around 30%, and the main isomers produced were 13-*cis* > 9-*cis*. At 120°C/30 s; 48% of β-carotene was lost. In the canning process (121°C/30 min), an increment in β-carotene content was observed, and the main isomers were 9-*cis*- and 13,15-*di*-*cis*-β-carotene. α-Carotene was reduced

in 60% and isomers were degraded after the canning. Destroyed lutein was around 50%. Color diminution was higher in more drastic treatments. Also, vitamin A destruction showed a similar pattern: 55.7% of β-carotene was lost during canning.[99] In contrast, it has been reported that canning of vegetables (e.g., broccoli, cantaloupe, carrots, etc.) produces an isomerization all-*trans*- to *cis*-isomers of carotenoids. In sweet potatoes the highest isomerization was observed and was the lowest in broccoli. In processed red, yellow, and orange vegetables, the 13- and/or 13′-*cis*-isomer are favored, while in green vegetables it is the 9-*cis*-isomer. The preponderance of the 9-*cis*-carotenoids in green vegetables was assigned to the effect of chlorophyll as a sensitizer. Interestingly, the total carotenoid content was maintained after canning and the provitamin A concentration was higher. Later observation may be due to an increased extraction efficiency due to the disruption of carotenoprotein complexes, inactivation of carotene-oxidizing enzymes, and/or loss of soluble solids into the liquid canning medium.[277]

Blanching (98°C /5 min), cooking (98°C for 15, 30, and 60 min) and sun drying (25°C ± 6°C) are traditional processing practices. These methods were used in the processing of Tanzanian vegetables used for human consumption (amaranth, cowpea, peanut, pumpkin, and sweet potato leaves). In general, blanching resulted in a reduction of β-carotene and in an increment of α-carotene concentration. Cooking enhanced the quantity of carotenoids extracted, and sun drying reduced the concentration of carotenoids in all the evaluated vegetables. Also, it was shown that thermal processing increased the vitamin A activity of all vegetables, but in amaranth leaves a reduction was observed. Thus, blanching and cooking could be considered as advantageous processes for increasing the amount of provitamin A available when vegetables are consumed. It was suggested that consumption of 100 g of dry weight of these vegetables cover the recommended daily intake for vitamin A for both children and adults.[333] In the processing of bathua (*Chenopodium album*) and fenugreek (*Trigonella foenum graecum*) leaves that are consumed in India, blanching, cooking (using hydrogenated fat), and sun drying resulted in the reduction of carotenoids. It was shown that cooking in the presence of fat, carotenoid, and vitamin A reductions are lower because fat may dissolve and protect carotenoids. It was recommended that blanching for a short time (5 min) and cooking in a pressure cooker are better processing conditions for the retention of β-carotene.[512]

During egg dehydration and storage, the effect of α-tocopherol supplementation was studied. It was shown that gas heater drying produces higher loses than electric resistance drying. It is explained that during gas drying higher quantities of nitrogen oxides and other free radicals are formed, pepper oleoresin acts as a prooxidant, and α-tocopherol stabilizes carotenoids and probably permits the antioxidant action of carotenoids.[269]

In the processing of saffron, drying temperatures lower than 30°C or higher than 60°C produced a greater carotenoid degradation. The optimum temperature of drying was 40 ± 5°C, independently of type of drying (sun or furnace drying). Losses of 56.9 to 70.2% after 1 year of storage were observed (15 to 35°C, 10 to 12% moisture).[386]

To preserve color in carotenoid pigmented-foods, the antidiscoloring effect of green tea polyphenols [epigallocatechin 3-gallate (EGCg), gallocatechin (GC), gallocatechin 3-gallate (GCg), epigallocatechin (EGC), -)-epicatechin EC) or (+)-catechin (C)] in beverages and in margarine was assayed. In general, it was observed that tea polyphenols and several tea catechins have stronger effects against the discoloration of β-carotene than L-ascorbate (widely used to prevent discoloration) in the aqueous (beverages) and oily (margarine) systems. The strength of antidiscoloring activity was GCg, EGC, EGCg, GC >> gallic acid (GA) > L-ascorbate, EC, C. According to the observed order, it was established that activity depends on the presence of an hydroxyl group at the 5′ position, and it was suggested that the hydrogen radical from the phenolic hydroxyl group of catechin stabilizes the peroxide radical formed during the oxidation process. Thus, tea polyphenols might be used as excellent antidiscoloring compounds of β-carotene.[479]

Poultry feed pigmented with marigold oleoresin (lutein is the main carotenoid) was exposed

to sunlight. Poultry fed with sun-exposed feed showed better pigmentation than control feed (maintained in darkness). It was proposed that sunlight induces conversion of lutein to redder pigments (e.g., astaxanthin).[149] However, in our lab it was shown that sunlight illumination produces a favorable equilibrium toward *all-trans*-lutein isomer *(trans*-isomer showed redder hues and high pigmenting efficiencies), but redder carotenoids were not observed. Additionally, we showed a better egg yolk-pigmenting efficiencies of marigold meals exposed to sunlight than controls; and also we demonstrated that other components different to carotenoids are participating in pigmentation efficiency.[268]

Marigold pigments have also been used in the pigmentation of shrimp *(Penaeus vannamei*). It was found that saponified extracts were better pigmenting agents than esterified marigold extracts; its study suggested that lutein and zeaxanthin (main carotenoids of marigold) can be metabolized into astaxanthin and deposited in shrimp and better colorations could be obtained than with astaxanthin supplemented feed.[487] On the other hand, in the pigmentation of white-fleshed fish, carotenoids must be eliminated from the corn gluten meal (CGM) used in feeds. In the corn gluten bleaching, it was established that carotenoid extraction with ethanol is a good process, but economical benefits could be enhanced by using soybean flour (5% substitution level) as a bleaching agent by its content of lipoxygenase and because the high content of protein in soybean.[359]

The use of high pressure (9000 times over atmospheric pressure) to kill contaminating organisms in food has been proposed.[210] It was reported that sensorial characteristics, such as flavor and color, are unscathed. In Japan, this methodology has been used in the preparation of fruit jams, yoghurts, and yomogimochi (steamed rice paste mixed with wild herbs). However, some vegetables showed undesirable characteristics after pressure treatment: grapes were hardened, cabbage were softened, and mushroom and potato were oxidized. It was suggested that the killing of organisms by pressure could be assigned to modifications of protein structure by affecting ionic bonds and/or ionic interactions, consequently, protein activity is also modified. Another suggestion indicates that membranes are modified and the cell content is lost.

Kinetic studies of carotenoid degradation have been also carried out in food systems and in general, as in model systems, they are characterized by first-order kinetics. When carrot juice was used as model, photodegradation and photoisomerization reaction were slower than in synthetic models, thus some protective factors must be present in juice. In carrot juice, the main isomer was 13-*cis*-β-carotene.[367] In other studies, carrot juice was acidified, pasteurized, and then subjected to light or dark storage at different temperatures (4, 25, and 35°C) for 3 months. Temperature had a negative effect on lutein content (higher degradation at 35°C), and it was observed that the formation of 13-*cis*-lutein is favored. Under light conditions the behavior was similar, although lutein degradation was higher than in darkness storage. α-Carotene showed a similar behavior than lutein; however, 13-cis-α-carotene is formed preferentially in dark conditions (although the 9-*cis*-isomer increases also), and 9-cis-α-carotene is the preferred isomer in light conditions. The concentration change of β-carotene and its isomers was similar to the other carotenoids, but β-carotene was more susceptible to degradation. Vitamin A degradation showed a correlation with carotenoid degradation.[101] In saffron, pigment degradation showed a first-order reaction with the effect of light, heat, and pH.[475] During olive processing, lutein and β-carotene did not show variations, while violaxanthin and neoxanthin content decreased in parallel with auroxanthin and neochrome increments. By considering that total carotenoids remained constant, thus processing produced a conversion of 5,6-epoxyde carotenoids (violaxanthin, neoxanthin) to 5,8-epoxydes (auroxanthin, neochrome) with first-order kinetics.[317]

Koplas-Lane and Warthesen[262] studied spinach and carrot photostability (2000 l ×/8 days). Carotenoids were degraded with first-order kinetics. Lutein was the most and violaxanthin the least stable carotenoids. Carrot carotenoids were not affected by light and showed higher stability

than spinach carotenoids. Interestingly, during carrot storage in darkness/6°C, chromogenesis was observed and samples showed 11% more total carotenoids after 60 days of storage.[262]

Stability of carotenoid extracts from saffron (*Crocus sativus* L.) was studied at different water activities (0.11, 0.23, 0.33, 0.43, 0.53, 0.64, and 0.75) and temperatures (25, 40 and 60°C). Carotenoid degradation under all storage conditions and with a first-order kinetics was observed. Also, a higher rate of degradation associated with increments in water activities was shown. This result contrasts with those obtained with oil-soluble carotenoids. Crocin, the main carotenoid in saffron, is water soluble, and it was suggested that more available water could enhance the mobility of solution components (carotenoids and free radicals), inducing a higher degradation. Also, it was mentioned that higher stability of carotenoids might be reached at temperatures below the glass transition temperature, where solution components are at fixed positions and degradation reactions are more difficult.[474]

### c. Production of Carotenoids in Bioreactors

An important approach for carotenoid production consists of the culture of algae and bacterial strains. The fungus *Blakeslea trispora* is a well-known β-carotene producer, and it has been shown that cell growth and β-carotene production are enhanced by using surfactant agents such as Span and Triton, except with Triton X-100.[254] Another interesting model for β-carotene production is *Phycomyces blakesleeanus*, and mutants *carS* have been reported that accumulate up to 100 times the wild-type level (2 to 5 mg β-carotene/g dry mycelium). *CarS* mutants were exposed to *N*-methyl-*N*′-nitrosoguanidine and the mutant S276 was obtained. S276 contained 9.2 ± 1.6 mg of β-carotene/g dry mycelium, and it was suggested that mutated gene product probably activates carotenogenesis.[307] The alga *Haematococcus lacustris* contains large amounts of astaxanthin esters and has been considered as a potential source of astaxanthin. Recently, it was established that higher light intensities resulted in the accumulation of more astaxanthin esters in the cells: culture at 90 μmol $m^{-2}$ $s^{-1}$ of light intensity and quickly rising to 190 μmol $m^{-2}$ $s^{-1}$ permitted obtaining the maximum carotenoid content in *H. lacustris*. Also, echineone and canthaxanthin were identified in the cultures, supporting the idea that astaxanthin is synthesized from β-carotene via echineone and canthaxanthin.[520] Other studies *in vivo*[518] and *in vitro*[110] have shown that high astaxanthin production required high levels of oxygen (aerobic conditions). Additionally, it has been shown that cell growth requires low C/N ratios and astaxanthin production of high C/N ratios. The results showed that pigment production by *X. dendrorhous* is affected by fermentation, respiration, and anabolism. It was suggested that high $O_2$ levels are required for NADH oxidation, inhibiting the ethanol production (fermentation) and enhancing respiration. On the other hand, astaxanthin production required large amounts of NADPH and high C/N ratios, and high oxygen levels favor this condition. Thus, to optimize the astaxanthin production, it is necessary to optimize the synthetic pathway of astaxanthin and optimize the metabolic pathway anabolic and respiratory), and, consequently, it was proposed that optimal astaxanthin production is reached by a two-stage cultivation: the growth stage (first) in medium with low C/N ratio and the astaxanthin production stage (second) in high C/N ratio.[518] Also, it was suggested that the addition of ethanol during the second stage enhanced the production of astaxanthin 2.2 times more than control without ethanol.[517] Pine (*Pinus pinaster*) wood meals were used in the production of carotenoids. Pine meals were treated with acid and enzymatic hydrolysis. The enzymes used were cellulases from *Trichoderma reesei* (Celluclast) and cellobiase from *Aspergillus niger* (Novozym). This material was incubated with preproliferated broths of *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*), and it was determined that enzymatic hydrolysates behave as suitable culture media for proliferation of *X. dendrorhous*, reaching good growth rates (up to 0.07 $h^{-1}$) with high cell yield (0.47 g biomass/g consumed glucose) and carotenoid concentrations up to 1.8 mg carotenoids/l. Thus, it was suggested that wood hydrolyzates are potential substrates for carotenoid production with *X. dendrorhous*.[357] Astaxanthin production was also assayed in thin stillage as substrate and a nitrosoguanidine mutantof

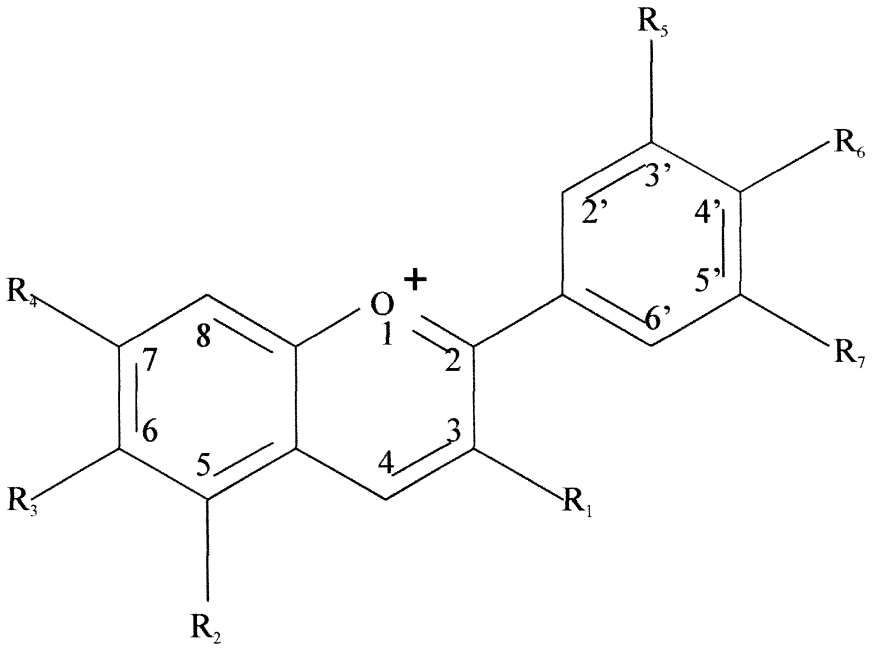


**FIGURE 15.** Basic structure of anthocyanidin pigments in which $R_x$ could be H, OH, or $OCH_3$ depending of the considered pigment. The most common accepted nomenclature for numbering carbons is indicated inside the structure.

*X. dendrorhous* called JB2. The carotenoid production was increased 2.3 times in relation to the wild-type cells (1.54 μg carotenoid/mg dry weight). Thus, it was suggested that a strain like JB2 has potential for commercial production of astaxanthin from corn byproducts.[50] Also, *Haematococcus pluvialis* has been suggested for the production of astaxanthin, and recently compactin-resistant mutants were isolated (compactin is an inhibitor of the HMGR that strongly blocks cholesterol formation). Selected mutants showed growth comparable with wild-type cells, while the astaxanthin content was increased in 1.4 to 2.0 times. The specific HMGR activity was reduced in 14 to 27%, and it suggested the presence of specific HMGR for astaxanthin production.[109]

## B. Anthocyanins

### 1. Definition

Anthocyanins are the most important group of pigments, after chlorophyll, that are visible to the human eye.[195] Chemically, anthocyanins from the Greek *anthos*, a flower, and *kyanos*, dark blue) are flavonoids (flavan like), and consequently based on a C15 skeleton with a chromane ring bearing a second aromatic ring B in position 2 (C6-C3-C6) and with one or more sugar molecules bonded at different hydroxylated positions of the basic structure (Figure 15). Anthocyanins are substituted glycosides of salts of phenyl-2-benzopyrilium (anthocyanidins).[87,455]

### 2. Classification

The basic C6-C3-C6 anthocyanin structure is the source of an infinity of colors produced by its chemical combination with glycosides and/or acyl groups (Figure 15) and by its interaction with other molecules and/or media conditions.[62] Harborne and Gryer[195] mentioned the existence of 17 anthocyanidins, with differences in the number and position of hydroxyl groups and/or methyl ether groups, but six of them are the most common anthocyanidin constituents of this kind of pigments (Table 4). From these 17 structures combinations have arisen with at least one sugar

**TABLE 4**
**Anthocyanidins Found in Nature**

| Name[a] | Position of substitution | Some of the produced colors |
|---|---|---|
| **Substituted with a characteristic hydroxyl group** | | |
| Apigeninidin | 5,7,4' | Orange |
| Aurantinidin | 3,5,6,7,4' | Orange |
| Cyanidin | 3,5,7,3',4' | Magenta and Crimson |
| Delphinidin | 3,5,7,3',4',5' | Purple, mauve and blue |
| 6-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 |
| **Substituted with a characteristic methyl ether group** | | |
| 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 | 7,3' | Red |

[a] Underlined names represent the most common anthocyanidins.
Adapted from Refs. 157, 193.



molecule to obtain anthocyanin compounds. Thus, anthocyanins have also been classified in agreement with the number of sugar molecules that constitute their molecules (i.e., monosides, biosides, triosides) and, interestingly, the number of probable compounds is greatly increased by taking into account the sugar diversity and all the possible structural points of glycosylation, although the order of sugar occurrence in natural anthocyanins is glucose, rhamnose, xylose, galactose, arabinose, and fructose. Additionally, many anthocyanins have shown in their structures ester bonds between sugars and organic acids i.e., acylated anthocyanins), and in nature the most common acyl groups are coumaric, caffeic, ferulic, *p*-hydroxy benzoic, synapic, malonic, acetic, succinic, oxalic, and malic.[157] Moreover, substitution of hydroxyl and methoxyl groups influences the color of anthocyanins. Increments in the number of hydroxyl groups tend to deepen the color to a more bluish shade. On the other hand, increments in the number of methoxyl groups increase redness (Table 4). With all of these facts in mind, it must be not difficult to understand the gamut of colors observed in nature that is produced from a single structure.

### *3. Distribution*

Anthocyanins are responsible for many of the attractive colors, from scarlet to blue, of flowers, fruits, leaves, and storage organs.[193,195] They are almost universal in higher plants, but in general anthocyanins seem absent in the liverworts, algae, and other lower plants, although some of them have been identified in mosses and ferns.[455] The type of anthocyanins in plants

is so variable that some ornamental plants present only one main type of anthocyanin *(Dianthus, Petunia*), whereas others have mixtures *(Rosa, Tulipa,* and *Verbena*). On the other hand, some fruits are a source of one anthocyanin: cyanidin in apple, cherry, fig, and peach; delphinidin in eggplant and pomegranate; some fruits have two main anthocyanins such as cherry sweet and cranberry (cyanidin and peonidin), while others have several anthocyanins (grape). In general, the anthocyanin concentration in most of the fruits and vegetables goes from 0.1 up to 1% d.w.[455]

Anthocyanins are vacuolar pigments and in this organelle the presence of membrane bound bodies called anthocyanoplasts has been proposed; these structures are formed while pigment synthesis is in operation, and eventually they are dispersed to produce a totally pigmented vacuole. In flowers, anthocyanins are almost exclusively located in epidermal cells, and only occasionally in the mesophyll. On the other hand, in the leaves of rye *(Secale cereale*) they are restricted to the mesophyll cells.[195] Interestingly, flavones co-occur with anthocyanins and participate in the color of plants as copigments (substances that contribute to anthocyanin coloration by protecting the anthocyanin molecules). The copigmentation mechanism is unique to the anthocyanin family. Anthocyanins also react with alkaloids, amino acids, benzoic acids, coumarin, cinnamic acids, and a wide variety of other flavylium compounds. This weak association is termed intermolecular copigmentation. Intramolecular copigmentation is due to the acylation in the molecule, and it is more effective than intermolecular; in acylated anthocyanins, it is suggested that acyl groups interact with the basic anthocyanin structure, avoiding the formation of the hydrated species. The basic role of copigments is to protect the colored flavylium cation from the nucleophilic attack of the water molecule.[28,410] Other important phenomena that show a great contribution to color is the self-association of anthocyanins, and in some models the contribution of metal complexation have been suggested (Figure 16).[63,146,157,193, 417]

## 4. Biosynthesis: Biochemistry and Molecular Biology

### a. Biochemistry

The phosphorylated compounds phosphoenolpyruvate (glycolytic pathway) and erythrose-4-phosphote (pentose phosphate respiratory pathway and Calvin cycle) are the precursors of shikimic acid. Shikimic acid and acetate are the precursors of the primary aromatic building stones of many phenolic compounds, including anthocyanins. Shikimate pathway gives several organic acids, such as cinnamic, *p*-coumaric, caffeic, ferulic, chlorogenic, and phenylalanine. By using chemogenetic studies and labeled precursors, it was shown that the shikimic acid product, phenylalanine, is incorporated in the C6-C3 (B aromatic rings and carbons corresponding to the central pyran ring) portion of the basic flavonoid structure (Figure 3). On the other hand, ring A and the oxygen of the central pyran ring are provided by acetyl-CoA. In addition, it is remarkable that elucidation of anthocyanin biosynthesis and genetics have been carried out mainly by using undifferentiated cells of parsley, maize, snapdragon, and petunia.[193,195,455]

Anthocyanin biosynthesis pathway could be divided into two main parts: (A) precursors of the general phenylpropanoid metabolism, and (B) specific steps toward flavonoid biosynthesis (Figure 17). In the first part, phenylalanine is converted to *p*-coumaryl-CoA by effect of three enzymes: phenylalanine ammonia-lyase (PAL), cinnamate-4-hydroxylase (C4H), and 4-coumaryl-CoA ligase (4CL). *p*-Coumaryl-CoA is the main precursor of flavonoids, lignin, and other phenylpropanoids (Figure 17A). In the second part (Figure 17B), 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. Chalcone synthase has been isolated and characterized from several sources *(Dianthus, Verbena, Anthirrhinum*). In the next step, chalcone shows a stereospecific isomerization to naringenin by the enzyme chalcone isomerase (CHI). Different CHI and tissue-specific isoenzymes have been charac-

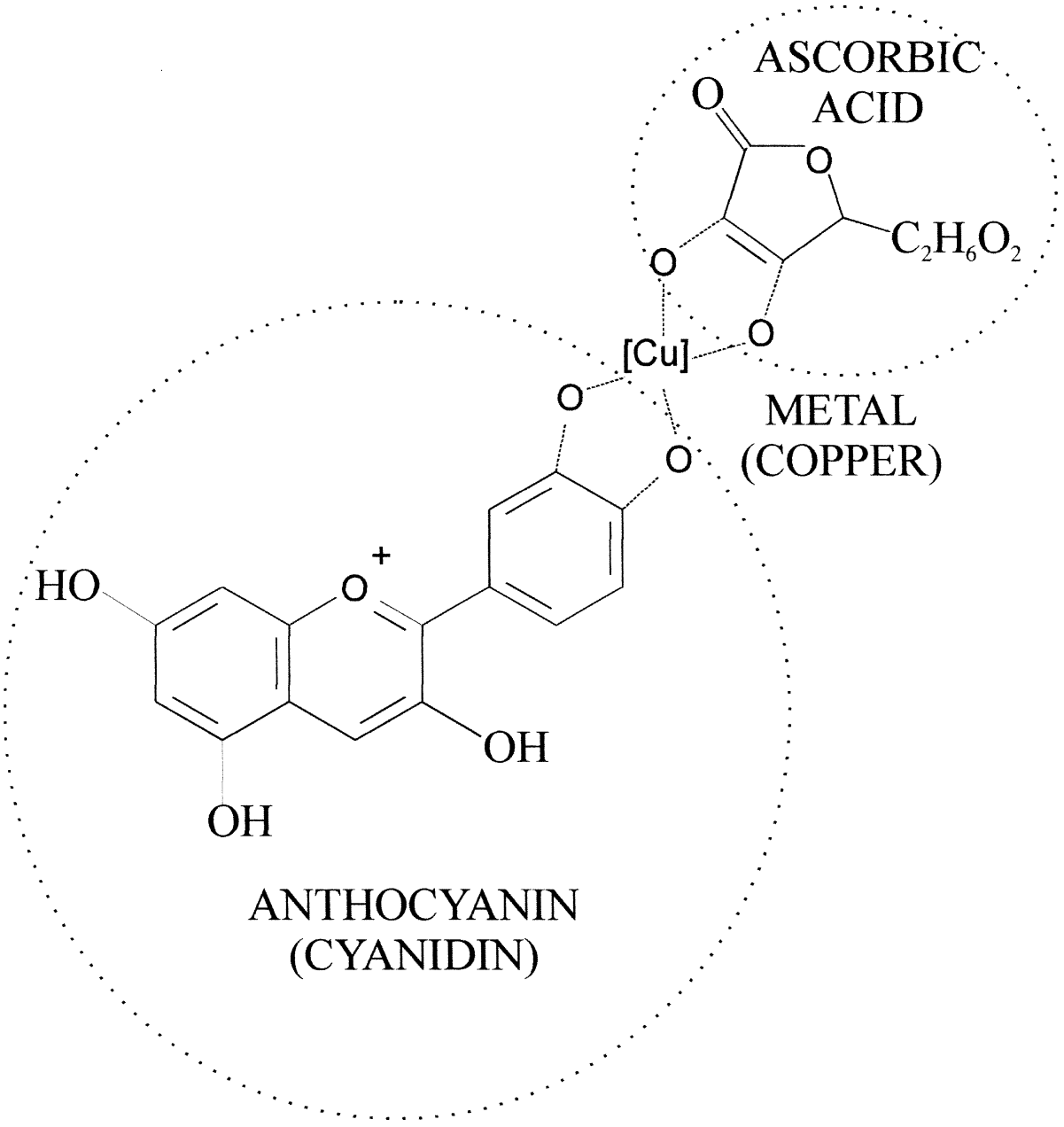


**FIGURE 16.** Suggested mechanism of formation for the complex anthocyanin-metal-ascorbic acid. (Adapted from Ref. 417.)

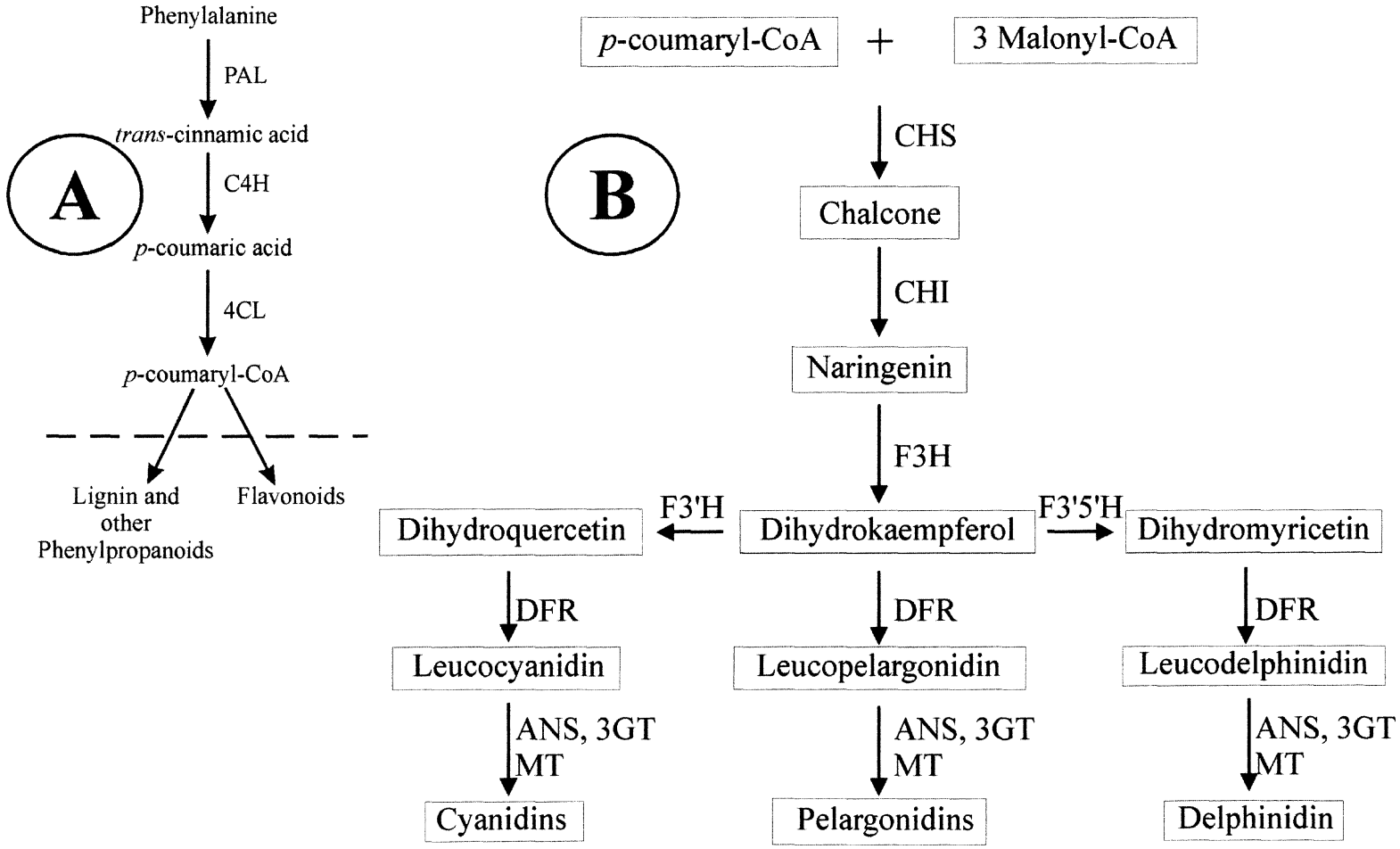


**FIGURE 17**. Anthocyanin biosynthesis pathway. (A) General phenylpropanoid metabolism. Enzymes involved: PAL, phenylalanine amonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaryl:CoA ligase. (B) Specific steps of anthocyanin biosynthesis. Enzymjes involved: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, F3′H, F3′5′H, flavonol hydroxylases; DFR, dihydroflavonol-4-reductase; ANS, anthocyanin synthase; 3GT, glucosyl transferase; MT, methyl transferase. (Adapted from Refs. 193, 216, 328.)

terized. Narigenin is the common precursor to flavonoids and isoflavonoids; naringenin flavanone) is converted to dihydrokaempferol flavone), by a dioxygenase or a monooxygenase, depending on the tissue. In petunia, the enzyme flavanone-3-hydroxylase (F3H) (dioxygenase) is involved. In the next stage, dihydrokaempferol is converted to leucoanthocyanidin by dihydroflavonol-4-reductase (DFR), which is NADPH or NAD dependent according to the plant source. This intermediate has been demonstrated by using acyanic flowers produced by different gene blocks and by the characterization of the flavan-3,4-*cis*-diol-4-reductase (catalyzes the catechin synthesis). Further ahead in the pathway, leucoanthocyanidins are transformed to the colored anthocyanidins. This conversion is not well characterized, but involves an oxidation and a dehydration step; gene mutations corresponding to these activities have been identified in several plant species *(A2* in maize, *Candi* in snapdragon, *ant17* in petunia), and this enzymatic activity has been called as anthocyanidin synthase ANS). The route is followed by the anthocyanidin-anthocyanin conversion that involves a glycosylation reaction. In this process the best described enzyme is the UDP-glucose:flavonoid 3-*O*-glucosyl-transferase (GT). Glycosyl transferases have shown a pronounced specificity with regard to substrate, position, and sugar to be transferred. Recently, the first glycosyltransferase that catalyzes the transfer of a sugar galactose) other than glucose to anthocyanidins was reported. The galactose:cyanidin galactosyltransferase (CGT) was characterized from a cell suspension culture of *Daucus carota* L. In this system a xylosyltransferase activity that is separated by anion-exchange chromatography of the galactosyltransferase activitywas also observed, suggesting the presence of two separate enzymes, each catalyzing a specific glycosyl transfer. Also, it was proposed that glycosyltransferases act in sequential reactions, initiating with the galactosyltransferase and followed by the reaction catalyzed by xylosyltransferase.[401] Hydroxylation and methylation occur at different stages of flavonoid biosynthesis. In particular, anthocyanin methylation is a late step, and the isolated flavonoid *O*-methyltransferases (MT) have shown a great specificity, thus multiple methylation requires multiple enzymes. In *Petunia hybrida* flowers at least four methyltransferases have been involved *(Mt1, Mf1, Mt2,* and *Mf2*).

On the other hand, it has been proposed that acyl transferases show high specificity, and in some instances acylation must precede glycosylationin the acylation of anthocyanins. Acyl transferases have been described in *Silene, Matthiola, Callistephus, Dendranthema,* and *Zinnia*; they catalyze the transfer of 4-coumaryl or caffeoyl groups to the corresponding acylated anthocyanins, and succinyl transfer is a noncommon event. An acyltransferase from blue flowers of *Centaurea cyanus* was isolated. This enzyme catalyzes the transfer of the succinyl moiety from succinyl-CoA to 3-glucosides of cyanidin and pelargonidin, but not to 3,5-diglucosides. Also, it was reported that the same enzyme catalyzes the malonylation at similar rates of succinylation.[513] Another important modification is sulfation, and sulfate transferase cDNA was isolated from *Flaveria bidentis*.[6] The enzymes of anthocyanin synthesis are probably cytoplasmic and attached to the vacuolar membranes, and it is likely that after the glycosylation, anthocyanins are transported into the vacuole.[151,193,195,216]

In anthocyanin-producing cells of sweet potato were identified vacuoles containing large amounts of pigment and protein. This protein of 24 kDa (VP24), which accounted for more than 50% of the total protoplast protein showed a similar pattern of accumulation to that of anthocyanin. It was observed that VP24 began to appear in vacuoles at nearly the same time as the accumulation of anthocyanin became detectable, and it was suggested that VP24 is involved in the intravacuolar accumulation of anthocyanin.[345]

### *b. Biosynthesis Regulation*

Little is known about the regulation of the biosynthetic enzymes of flavonoid synthesis, and much of the knowledge has emerged from molecular biology studies.[62,328] The pronounced substrate specificity of the 4-coumarate:CoA ligase of different plants has been shown, indicating that this enzyme is an important regulation point to

establish the distribution of CoA esters in plants. Additionally, external and developmental factors showed an interdependent response of flavonoid biosynthetic enzymes. At least three photoreceptors have been suggested that control flavonoid biosynthesis (red/far red, blue UV-A, or cryptochrome and UV-B), and anthocyanin biosynthesis showed different responses depending on the light quality. In parsley plants, enzyme activities of flavonoid synthesis are high only in young cotyledons and leaves, and a decrement was observed in the later stages of development.[195,216] In *Arabidopsis*, mutants in several of these photoreceptors have been identified and some of them involve products of the *Cop, Det,* and *Fusca* genes.[326] In Petunia, mutations at four loci *(An1, An2, An4,* and *An11*) have regulatory effects on transcription of at least six structural anthocyanin biosynthetic genes.[216]

### *c. Molecular Biology of Anthocyanin Biosynthesis*

Flavonoid biosynthesis has been selected as a favorite pathway to carry out studies at the molecular biology level; thus, this is the best known pathway of secondary metabolism. Many cDNA and genomic clones involved in flavonoid synthesis have been characterized, and some of them have been cloned in bacteria, providing an excellent model to produce the corresponding protein and to induce the *in vitro* production of valuable flavonoid metabolites.[151] In 1983, chalcone synthase gene was the first gene cloned of flavonoid biosynthesis by using differential screening, which was also employed to isolate *PAL, 4CL,* and *F3H* from parsley. Moreover, antibodies were used in an expression library to clone the genes *CHI* and *F3H*. Interestingly, flavonoid biosynthesis pathway was a model to design a new technique for cloning genes transposon tagging), and between others several regulatory genes were isolated.[125,481] In addition, new methodologies have been used to clone anthocyanin genes. Rapid amplification of cDNA Ends (RACE) by PCR was used to isolate the chalcone synthase gene 2 *(chs2*) from potato, and by Southern analysis it was shown that *chs* genes of potato comprise a family of at least six individual members.[238] On the other hand, differential display was used to identify four new *chs* genes from morning glories.[163] In addition, two clones with high homology with flavonol synthase and anthocyanidin synthase were identified by using *A. thaliana*-expressed sequence tag (EST). This EST permitted the isolation of the first flavonol synthase gene.[366] CHS is encoded by a multigene family in most of the species examined to date. Only *Arabidopsis* and *Antirrhinum* appear to contain single gene copies. It was suggested that the evolution of CHS genes is a highly fluid gene family where copy number varies greatly among plant lineages and where functional divergence may occur repeatedly.[130] Interestingly, another *chs*-related cDNA was isolated from *Gerbera hybrida (Gchs2*). *Gchs2* did not show correlation with the flavonol and anthocyanin accumulation; its pattern of expression and accepted substrates were different. Thus, it was suggested that *Gchs2* represents a new family of genes that have a common evolutionary origin with *chs* genes.[205] In addition, it was proposed that *Gchs2* emerged as the result of the duplication of a single gene, followed by subsequent differentiation during the evolution of *Gerbera*.[206]

Other studies have evaluated the temporal and spatial control of flavonoid biosynthetic genes. Some genes showed organ-specific and others an stage-specific regulation, and several researchers have been characterizing the gene promoter regions.[141,409] In maize it has been suggested that changes in kernel colors resulted from changes in *cis* regulatory elements and not changes in either regulatory protein function or in the enzymatic loci, suggesting that differences in 5′ or 3′ regulatory sequences or methylation patterns could be involved.[191] The influence of DNA methylation was also demonstrated in transformed petunia plants. Petunia plants that show dihydrokaempferol accumulation (defective in *dfr* gene) were transformed with the corresponding enzymes of maize *(A1*) and gerbera *(gdfr*). *gdfr* transformants showed higher pigmentation than *A1* transformants, and, particularly, it was observed that *A1* transgene inactivation was associated with a higher GC content and an increased methylation state.[134] Interestingly, studies on chalcone synthase (CHS) promoter of French bean showed that exists a

differential utilization of *cis* regulatory elements for stress-induced (elicitors, wounding, and infection) and tissue-specific expression, and it was indicated that this multiple combination of distinct *cis* elements contributes to the complex expression pattern of *CHS* genes.[140] It has been estimated that at least 30 genes are required for flavonoid biosynthesis, and within these different mutations have defined regulatory proteins whose absence prevents the synthesis of multiple enzymes (probable transcription factors). It has been shown that chalcone synthase activity is under the control of other genes *(f* in *Matthiola*, *niv* in *Antirrhinum*, and *c2* in *Zea mays*). In maize two different families of regulatory genes have been established, C1 and R. The expression of one of these genes induced a pleiotropic effect that in some instances is observed as an increment in the anthocyanin biosynthesis, and it has been observed that maize anthocyanin synthesis requires the expression of at least one gene of each family.[125,328] Additionally, it has been observed that regulatory systems of anthocyanin synthesis are conserved between plant families: transformation of petunia plants with genes of the C1 and R families induces the accumulation of anthocyanins. In petunia three regulatory mutants *(an1, an2, an11*) showed accumulation of dihydroflavonols, and it has been suggested that *An2* gene is an *R*-homologue, while *An1* is *C1*-homologue.[125] Also, it was shown that the *Antirrhinum del* gene, tomato *Lc* gene, and the maize *R* gene family are structurally and functionally homologous.[175,178] When plants of two *Solanaceae* species (tomato and tobacco) were transformed with the *del* gene, an increment in anthocyanins was observed in the pigmented regions, but this effect was not found in *del*-transformed *Arabidopsis*, indicating that some genes may not be able to function effectively in all plant hosts.[329]

To complicate more the understanding of the anthocyanin biosynthesis regulation, negative regulators have been identified in *Arabidopsis* seeds *(fus* and *banylus*). The presence of *fus* and *banylus* products prevents the accumulation of pigments. Additionally, other negative regulators have been isolated *(eluta* from *Antirrhinum* flower, *C1-1* from maize aleurone layer, *fusca* from *Arabidopsis* embryo). It has been proposed that *banylus* can direct the biosynthetic pathway toward different metabolites.[3] It is clear that regulation is a very complex process, and, as mentioned above developmental (maturation, germination) and external signals (light, temperature) are involved: VP-1 is a pleiotropic protein that activates *C1* specifically, during seed maturation of maize, by interacting synergistically with one or more ABA-regulated transcription factors. However, there is no evidence that VP-1 can bind directly to DNA, and it has been proposed that protein-protein interactions have a principal role in the activation of promoters of downstream genes.[304] In maize and petunia, it was shown that duplicated sequences of regulatory genes exert a critical role in gene regulation. Therefore, the copy number must be strictly controlled by as yet unidentified mechanisms, but indicating that DNA methylation is involved.[467] Moreover, it has been established that maize-anthocyanin biosynthetic pathway can be considered as cold-regulation genes that in addition are related with water stress but not with heat stress. It was observed that a moderate cold (10ºC) enhances the transcription and/or stabilities for the anthocyanin biosynthesis genes; however, at 5ºC the biosynthetic ability is damaged. At 10ºC stress, it was observed a dramatic increase in transcript abundance for anthocyanin regulatory and structural genes, but only a slight increase in anthocyanin pigmentation that is increased severalfold after plants are shifted back to higher temperatures, showing that 10ºC stress impairs postranscriptional processes important for anthocyanin biosynthesis.[108] By contrast, low temperature enhances the pigmentation and chalcone synthase gene expression in petunia flowers, similarly to the observed in apple skin and in flower buds.[326,442]

In eggplant hypocotyl-tissue was identified a P450 cDNA clone that is inducible by white light. At the same time, white light induces the accumulation of CHS and DFR mRNA in a coordinated fashion, and it is proposed that this P450 enzyme catalyzes the hydroxylation of the B-ring in flavonoids, that is, the P450 isolated clone encodes for a 3′,5′-hydroxylase that acts during anthocyanin biosynthesis.[468] Interestingly, it has been shown that phytochrome stimulates anthocyanin biosynthesis through the induction of 3′,5′ cyclic mono-

phosphate (cGMP) acting alone or together with calcium.[55] In *Perilla frutescens*, all the anthocyanin biosynthetic genes were coordinately induced by strong light (*chs, f3h, dfr, ldox, 3gt, aat*).[176] In *Arabidopsis*, it was shown that light-induced signals converge on the *fusca* genes that relay the information to different plant responses (cell expansion, chloroplast differentiation, tissue-specific changes).[322] In addition, it the *hy4* gene that codes for the CRY1 protein and have the characteristics of a blue-light photoreceptor was identified. Thus, CRY1 must be responsible for anthocyanin biosynthesis under UV-light irradiation.[283] It was found that UV-A and UV-B induce different responses. UV-A induction of CHS expression does not appear to involve calmodulin, whereas UV-B response does. Consequently, the light signaling pathway showed differences between them and with phytochrome-mediated response.[27,107] On the other hand, the gibberellin GA3 stimulates the anthocyanin synthesis on petunia corollas, while it is completely inhibited in cultured carrot cells. In petunia corollas, GA3 is essential for the induction of *chs*, *chi*, and *dfr* expression, probably by effect of a regulatory protein receptor), because after GA3 addition exists a lagging stage until the response can be observed. Interestingly, it was observed that the presence of sucrose is required to observe the effect of GA3.[325,326,502] Similarly, it has been shown that the cytokinin benzyladenine (BA) treatment induces a red pigmentation in *Arabidopsis* seedling, showing that CHS and DFR appear to be transcriptionally activated, while PAL and CHI are controlled postransciptionally.[116]

The last step in anthocyanin biosynthesis involves the transfer and deposition of the red and purple pigments in vacuoles. In maize, this step is carried out by the protein encoded by the *bronze-2* gene (*bz2*), and it was shown that *bz2* codes for a glutathione-*S*-transferase (GST) with activity in maize, transformed *A. thaliana* plants, and *E. coli*. In *bz2* mutants, cyanidin-3-glucoside is accumulated in cytoplasm, whereas in *bz2* plants anthocyanins are transferred to vacuole. It was suggested that anthocyanin import into vacuoles could occur as anthocyanin-glutathione conjugates, paralleling the mechanism by which plants eliminate herbicides and xenobiotics.[296]

The world market of flowers is very important, and the knowledge of the biosynthetic route of anthocyanins and molecular biology techniques allows to modifying flower colors.[215,220,243] Petunia DFR can not reduce dihydrokaempferol, thus wild type petunias do not produce pelargonidin pigments. Petunia plants were transformed with maize DFR and mutant petunias that accumulate pelargonidin were obtained.[312] On the other hand, petunia flowers with reduced pigmentation were obtained by transforming with the *chs* gene of petunia in anti-sense.[482] White-flowering transgenic chrysanthemums have been produced by trans-inactivation of the main *chs* gene, and a similar strategy was used to obtain a range of color for carnation and rose flowers.[88,188] Interestingly, the effect of anthocyanins on sexual process has been proposed to be manipulated by molecular biology as an alternative to produce male sterile hybrid crops.[326] In alfalfa *f3h* correlates with flavonoid accumulation, and particularly *f3h* expression in pollen grains and ovary probably could be associated with plant fertility.[95] It was also demonstrated in tobacco that flavonoids do not only determine flower color, but also play an essential role in the development of male gametophyte. The stilbene synthase that competes for primary precursors with chalcone synthase was overexpressed, and, consequently, flavonoid production was impaired (reduced color) and male-sterile plants were obtained.[147] On the other hand, the rapid quality improvement of some cultivars requires of selection at the seedling stage, and molecular biology provides excellent tools to develop this selection. Random-amplified polymorphic DNA (RAPD) was used to identify redder apple cultivars, and one marker was identified for this characteristic. It was suggested that this marker is associated with a regulatory gene of the anthocyanin biosynthesis.[102]

Another interesting function was suggested by transforming a wine yeast strain with the anthocyanin-β-glucosidase gene of *Candida molischiana*. Transformed *S. cerevisiae* produces wines with quality characteristics comparable to those obtained with the wild-type strain. This new strain is of industrial relevance mainly in the production of white wines with lower red color, although they were obtained from red grape variet-

ies. Additionally, transformed *S. cerevisiae* was proposed for the production of wines with higher levels of resveratol and terpenols from the hydrolysis of their glycosidic precursors.[414] As previously mentioned, food industry is interested in pigments with higher stability and acylation of anthocyanins produces more stable pigments. Anthocyanidin-3 glucoside rhamnosyltransferase (UFGT) of *Antirrhinum majus* was used to transform lisianthus (*Eustoma grandiflorum* Grise.). Transformed lisianthus plants were able to produce a novel 3-*O*-glucosylated flavonols. Interestingly, C-3 glucosylated anthocyanins have never been observed in this plant.[433] On the other hand, sometimes it is recommendable to diminish the quantity of anthocyanins and to increase the content of other metabolites. The presence of condensed tannins (CT) in alfalfa has supported a higher sheep productivity higher weight and wool production) than CT-free alfalfa. Recently, it was shown that cultivars with higher CT content could be obtained by molecular biology. It has been established that *dfr* is involved in CT biosynthesis, and some *Lotus corniculatus* plants transformed with the *Antirrhinum majus dfr* cDNA showed increased CT levels.

## 5. Functions

### a. Color and Ecological Functions

As flavonoids, anthocyanins are benzopyran derivatives. Thus, anthocyanins also showed similar functions in plants to those described in the corresponding section for flavonoids: antioxidant, photoprotection, defense mechanism, as well as other ecological functions (symbiosis phenomena). In particular, anthocyanins are the most important pigmenting compounds between the flavonoids, and consequently they show an interesting role in several reproductive mechanisms of plants such as pollination, seed dispersal, and antifeedant. Interestingly, it has been observed that cyanidin-3-glucoside is inhibitory to the larval growth in tobacco budworm *Heliothis viriscens*, and consequently anthocyanins could be considered agents of biological control. Additionally, anthocyanins have been proposed as taxonomic markers, although this goal has not been achieved yet because of nowadays only a limited number of species levels within families have been investigated. However, interestingly, the preferred presence of cyanidin has been suggested as a marker of ancestral plants.[193,195,455] In this aspect, anthocyanins with substitution (sugar or acid) in the sugar 3-position have only been reported in the genus *Allium*;[153] in the genus *Begonia* a pattern of six anthocyanins as marker (from a survey of 200 begonia cultivars)was suggested,[105] and acylated anthocyanins with 5-*O*-(6-*O*-hydroxycinnamoylglucoside), where hydroxycinnamic acids are coumaric or caffeic acids, have only been reported in the *Gentiana* genus.[223]

### b. Marker for Good Manufacturing Practices in Food Processing

Anthocyanins have been used to evaluate the adulteration of some pigmented food products.[56] Prune juice is a product in which brown color is developed by the reaction of phenolic compounds and/or anthocyanins, and it is possible the adulteration of prune juice with other fruit juices improve its color. To control this possible source of adulteration**,** it is believed that prune juice can have only traces of anthocyanins, while the adulterated juice will show increased levels.[485] Also, anthocyanin profiles have been used to determine the authenticity of fruit jams. With this kind of analyses, it was determined that labeled black cherry jams in reality were prepared with common red cherries (less expensive fruit). In addition, it was suggested that adulteration of blackberry jams with strawberries can be detected with analysis of the relation between pelargonidin and cyanidin 3-glucoside. Also, it was pointed out that this methodology is very efficient because anthocyanins are pretty stable during jam manufacture.[165]

### c. Pharmacological Effects

Reports on biological activities of anthocyanins are scarce, but considering the wide distribu-

tion of anthocyanins, it is reasonable to assume that humans are well conditioned to large consumptions of these compounds. In a survey with Italian subjects, anthocyanin daily intake was in the range 25 to 215 mg/person, depending on gender and age, and this intake is largely enough to induce pharmacological effects. The consumption of wine flavonoids has been correlated with low incidences of coronary hearth diseases (French paradox), and, similarly, chokeberry (*Anonia melanocarpa*) extracts have shown very strong nutraceutical properties. Moreover, anthocyanins possesses bactericidal, antiviral, and fungistatic activities. They exhibit a strong antioxidant activity that prevents the oxidation of ascorbic acid, provides protection against free radicals, shows inhibitory activity against oxidative enzymes, and has been considered as important agents in reducing the risk of cancer and heart disease.[58] Also, there is information indicating that 3′- and 4′-OH in the B ring structure are determinants for the radical scavenging potential in flavonoids with a saturated 2,3-double bond, and that different patterns of hydroxylation and glycosylation may modulate their antioxidant properties.[499] It has been mentioned that an increased number of hydroxyl substituents on the B-ring produce higher antioxidant activities when are glucosides, but the activity is weaker with aglycones.[476] These two hydroxyl groups are important in protecting ascorbic acid against oxidation by chelating metal ions. When the anthocyanin-metal chelate is formed, a 20- to 25-nm shift in the visible spectra is observed, which in the presence of ascorbic acid induces a further shift of 10 to 15 nm. It was proposed the formation of the stable complex anthocyanin-metal-ascorbic acid, where ascorbic acid acts as a copigment. Thus, improved colors are obtained with the intrinsic protection of ascorbic acid from oxidation (Figure 16).[417]

The effect of pure anthocyanins against lipid peroxidation has been studied. Liposomes were used to evaluate the inhibition in the production of malondialdehyde. All evaluated anthocyanins were better agents against lipid peroxidation than α-tocopherol (up to seven times). Also, it was demonstrated that anthocyanins have scavenging properties against $^{\cdot}OH$ and $O_2^{-}$. In addition, it was mentioned that $^{\cdot}OH$ scavenging is better with aglycones of high number of OH groups in the B-ring, opposite to that observed with other flavonoids, while $O_2^{-}$ scavenging is independent of the glycosylation state but also increases with the number of hydroxyl groups, similar to the observed with other flavonoids.[476] In the red colorant extracted from *Anonia* a mixture of anthocyanins (cyanidin, cyanidin-3-glucoside and cyanidin-3,5-diglucoside) and polyphenol substances called bioflavonoids (leucoanthocyanidins, catechins, and flavonols) has been identified. In particular, bioflavonoids have shown activities to improve the permeability and strength of capillaries, to accelerate the ethanol metabolism, and to reduce inflammatory and edematic reactions.[286] Similar effects have been observed with crude extracts of *Rubus occidentalis*, *Sambucus nigra*, and *Vaccinium myrtillus*.[157,195]

## 6. Methodological Aspects

### a. Extraction

Anthocyanin, like flavonoids in general, have aromatic rings containing polar substituent groups (hydroxyl, carboxyl, and methoxyl) and glycosyl residues that altogether produce a polar molecule. Consequently, they are more soluble in water than in nonpolar solvents, but depending on the media conditions anthocyanins could be soluble in ether at a pH value where the molecule was unionized. These characteristics aid in the extraction and separation of anthocyanin compounds.[193] Conventional methods of pigment extraction usually employ dilute hydrochloric acid in methanol. Methanol containing 0.001% HCl was the most effective, but HCl is corrosive, and methanol produces toxic effects after human exposition; consequently, food scientists frequently prefer the use of other extraction systems. Among other solvents, one finds ethanol and water, 80 and 27% as effective as methanol, respectively. Additionally, it must be taken into account that aromatic acyl acid linkages are relatively stable in dilute HCl/MeOH mixtures, but aliphatic dicarboxyl acyl groups (malonic, malic, oxalic) are susceptible to diluted acids, and different methodologies must be considered. It is recommended to use weaker

acids (acetic, formic, perchloric) during extractions and to monitor the acidity during the process. With methanol, citric acid is the most effective organic acid followed by tartaric, formic, acetic, and propionic; with water, the best acids are acetic acid, citric, tartaric, and hydrochloric.[58,157,195,455] Recently, an aqueous extraction process for anthocyanins from sunflower hulls was evaluated. It was shown that extraction with sulfurous water (1000 ppm $SO_2$) was better than the traditional extraction with ethanol:acetic-acid:water system. Also, it was mentioned that 1 h of extraction was enough to reach a complete extraction of pigments. It was suggested that possible reasons for the improved extraction with $SO_2$ are the interaction of anthocyanins with $HSO_3^-$ ions, which improves the anthocyanin solubility and the diffusion through cell walls.[164]

### *b. Separation*

The initial attempts for the separation of anthocyanins were based on the adsorption in paper chromatography or in other suitable adsorbents.[455] Nowadays, thin layer chromatography (TLC) is widely used, because this technique has shown continuous innovations and still keeps its advantages (practical and very cheap). For preparative work, droplet counter current chromatography has been applied to separate the anthocyanins of black currant. On the other hand, a general patent for the purification of anthocyanins involves selective absorption on a finely divided oxide such as silicic acid, titanium oxide, or alumina, which is coated with a styrene polymer.[157] However, undoubtedly, the main developments of recent years in the research of anthocyanins is the introduction of HPLC for their separation and quantitation.[193,195] Interestingly, it is possible to distinguish zwitterionic anthocyanins by their HPLC chromatographic separation.[195]

### *c. Characterization*

**Spectroscopy.** In general, color is evaluated by spectrophotometry.[157] Isolated pigments have been studied by UV-visible spectroscopy. All flavonoids show high absorbance in the range of 250 to 270 nm (UV region), and, particularly, anthocyanins have an intense absorption in the range of 520 to 560 nm (visible region). It has been suggested that UV absorption could be assigned mainly to ring A, while the visible to the pyran and ring B. With this methodology, researchers suppose that identity between the isolated pigments and those found in living tissue is the same; however, the main problems are the generation of artifacts during the extraction process. To solve this problem, model systems such as anthocyanins stored in synthetic vacuoles have been developed. With UV-visible spectroscopy, it is also possible to detect glycosylation on B-ring, because of the spectra show an hypsochromic shift in relation to the unglycosylated B-ring. Anthocyanin acylation is also observed by this methodology: in the presence of $AlCl_3$ a bathochromic shift is observed, only if the 3′- and 5′-OH groups are free (nonacylated).[462] Moreover, acylated anthocyanins show a maximum absorbance in the 310-nm region, and it has been found that the ratio of absorbance at the acyl maximum wavelength to the absorbance at the visible maximum wavelength ($A_{max}$acyl/ $A_{max}$visible ratio) is a measure of the molar ratio of acyl group to anthocyanin.[173] Additionally, visible absorption is the best tool to observe the copigment effect: visible spectra of anthocyanins show a hyperchromic effect, increment of the intensity of this maximum resulting in a more colored species, and a bathochromic shift by a solvation effect.[62,63,195] Heating causes a hypsochromic and hypochromic effect that is restored by copigment regeneration after cooling in the malvin-rutin systems; however, the reversibility was not observed in the malvin-quercetin system. However, in both systems the copigment reaction was a spontaneous thermodynamic process (negative $\Delta G$).[20] On the other hand, visible absorption and RAMAN spectroscopy have been used in the study of pigments in the living tissue. RAMAN spectroscopy has been used to show the anthocyanin substitution pattern. The presence of phenyl ring substitutions on benzopyrylium produces clear spectral modifications. On the other hand, the anthocyanin spectral features are modified by glycosylation, showing that perturbations are dependent of the sugar

**TABLE 5**
**Some Chemical Tests for Anthocyanin Characterization**

| Reaction conditions (anthocyanin in) | Detection: Structural characteristic | Detection: 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 band do not decrease thus 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 is reverted after HCl addition |



nature. Interestingly, 5-glycosides present higher modifications than 3-glycosides.[63,309] The coupling of the diode array detector (DAD) into the HPLC methodology has permitted the tentative identification of the separated anthocyanins, as was described with carotenoids. In addition, with the introduction of innovated methodologies such as NMR and mass spectrophotometry, anthocyanin compounds have been identified conclusively. Proton NMR has been used to study the self-association of anthocyanin molecules, while carbon-13 NMR spectroscopy has been used to define the sequence, position, and configuration of sugar residues in flavonoid glycosides. NMR methodology has been used with heteronuclear shift correlation through multiple quantum coherence (HMQC) to show the configuration of anthocyanin glycosides. β configuration was observed with glucosyl, galactosyl, and xylanopyranosyl groups and α configuration with rhamnosyl and arabinopyranosyl groups. Moreover, 1H-1H COSY and DIFNOE have been used in structure characterization to assign the NMR proton signals.[365] In addition, mass spectrometry increased its potential with the introduction of the FAB-mass detector, which permitted observing an intense peak for the molecular ion, something difficult with the previously designed detectors; anthocyanins are unstable and with low volatility. Nowadays, methodological advances have been conducted on other mass detectors that inclusively are coupled with the HPLC equipment (see above discussion about carotenoids).[19] Interestingly, new structures have been elucidated, and anthocyanins with caffeoylglucose and malonyl substitutions are perhaps the most notable.[18,291] Another methodology used to study the copigmentation phenomena is circular dichroism.[62,193,195]

**Chemical tests.** A large number of chemical tests have been developed to determine the anthocyanin structure (some of them are shown in Table 5), and a general procedure could be envisioned, but this must be modified depending on the analyzed material.[193] After separation, isolated anthocyanins were hydrolyzed with mineral acid and glycoside bonds were disrupted, then anthocyanidins were methylated, and after other acidic hydrolysis the nature of the anthocyanidin and the positions of glycosylation were determined.[455] When anthocyanin molecules show aliphatic acids in their structures, a zwitterion is observed that can be detected by electrophore-

sis.[157] Additionally, to determine the occurrence of phenolic acids as esters, the extract is concentrated and resolubilized with 6N HCl and hydrolyzed (100°C/60 min), the solution is extracted with ethyl acetate and then evaporated under vacuum and the residue prepared to be analyzed by HPLC.[19] The presence of B-ring-substituted sugars is easily detected by an hypsochromic spectral shift.[157]

**Evaluation of pigmenting efficiency.** In the evaluation of visual color, reflectance colorimetry has been the better approach because it shows a good correlation with the chemical composition, and as above mentioned, lightness has been used to determine the pigment concentration; saturation (purity) reflects the spectral properties and the chromaticity coordinates give an appreciation of the various mixed pigments.[62,157] In this sense, a method has been developed to assess the anthocyanin concentration by reflectance measurements in red raspberry fruit, and it is reported that the best predictor is the hue parameter ($r = 0.73$).[330]

## 7. Importance as Food Colors — Stability, Processing, and Production

### a. Stability in Model Systems

Anthocyanins are of great economic importance as fruit pigments and thus also as pigments of fruit juices and wines.[195] Interestingly, one of the main claims of the food industry is for natural colorants to replace synthetic red dyes, and anthocyanins are the principal candidates, but, interestingly, enocyanin and lees (sediment of the grape juice tanks) preparations are the only anthocyanin sources approved by FDA (in the U.S.) to be used for human food, while the main use is in the production of beverages and soft drinks.[58,157] However, anthocyanin instability limits their use, and different preparations have been evaluated to avoid anthocyanin degradation.[87] The color of anthocyanins is provided by its resonating structure, but resonance phenomena also confers its intrinsic instability. Moreover, it has been established that instability has a direct relation with the number of hydroxyl groups and indirect with the number of methoxyl groups. In addition, the glycosylation level showed a clear effect on stability, diglucosides are more stable than monoglucosides, but browning is more favorable in diglucosides by the presence of the additional sugar molecule. Also, it has been showed that hydroxylation at position 4 changes the colors toward red tones.[157]

The equilibrium colored-uncolored anthocyanin structures affects the stability of products on storage, because the $R^+$ form is the most stable.

$$R^+ + H_2O \longrightarrow ROH + H^+$$

Colored catonic form — Uncolored pseudobase form

The reactive species was found to be the anthocyanin carbonium ion ($R^+$), which reacts with a bisulfite ion to form a colorless 2-bisulfite adduct product.[127]

In solution, anthocyanin molecules are present in an equilibrium between the colored cationic form and the colorless pseudobase. This equilibrium is directly influenced by pH. Acidic pH is favorable for the colored form that diminished with pH increments. Some anthocyanins are red in acid solutions, violet or purple in neutral solutions, and blue in alkaline pH. This is the reason that most colorants containing anthocyanins can only be used at pH values below four.[58] Acylation hinders hydrolysis of the red flavylium cationic form, allowing preferential formation of the blue quinonoidal bases. Interestingly, acylated pigments retain more color at the higher pH values than unmodified anthocyanins. In addition, acylated anthocyanins show an improved resistance to other factors such as heat, light, and $SO_2$. In particular, in *Vitis vinifera* wines were identified the anthocyanins vitisins in which C-4 positions of their structures are substituted, and consequently these pigments give greater colors, higher resistance to color loss with sulfur dioxide, and higher pH values.[18] Thus, this group of anthocyanins has been proposed as potential food colorants. Moreover, it is also very important that acylation produces a major absorption band at pH values above four, and, consequently, acylated anthocyanins will be highly colored at pH > 4,

contrasting with the conventional compounds that are uncolored at these conditions.[58,127,157,161,193] As a matter of fact, a great number of studies have been carried out to find new acylated pigments, and several ones with novel characteristics not previously reported) have been characterized (Table 6). Interestingly, malonylated anthocyanins are produced in purple sunflower seeds, which is one of the most important oilseed crops, and, consequently, the purple-hulled residue obtained after oil extraction from seeds provides an excellent source of red colorant (cyanidin derivatives) that has been suggested as a colorant of food, pharmaceutical, and cosmetic products.[303] In addition, several of the acylated anthocyanins have shown highly desirable stability, and the structures of some of them are presented in Figure 18. In particular, the improved stability of the *Ajuga reptans* anthocyanins was assigned to the presence of two cinnamic acid groups in the anthocyanin structure,[461] and it has been suggested that long acylation groups permitted anthocyanins to adopt a folded conformation, which protects the basic anthocyanin structure of degradative factors.[146,458] To corroborate the effect of acylation on anthocyanin stability, anthocyanin extracts of *Sambucus nigra* and *S. canadiensis* were compared. Anthocyanins of *S. nigra* have a free hydroxyl group on C-5, while in *S. canadiensis* a glucosyl residue acylated with *p*-coumaroyl is present. It was observed that *S. canadiensis* extracts were more stable than those of *S. nigra*. It was shown that C-5 glucosylation does not represent a crucial factor on heat stability, but acylation does. However, it was also shown that C-5 glycosidation, as well as acylation, has an important contribution to color stability for light. In addition, the following decreasing order of stability tolight was reported: acylated diglucosides > nonacylated diglucosides > monoglucosides.[232] A comparable high stability of C-5 acylated anthocyanins was also observed with the pigments from red radish.[173]

**TABLE 6**
**Reports on Acylated Anthocyanins**

| Plant model | Remarks | Ref. |
|---|---|---|
| Verbena flowers (*Verbena hybrida*) | First report of a diacylated anthocyanin: Pelargonidin (3-*O*-(6-*O*-(malonyl)-β-*D*-glucopyranoside)-5-*O*-(6-*O*-(acetyl)-β-*D*-glucopyranoside) | 469 |
| Blue flowers of *Hyacinthus orientalis* | Identification of anthocyanins with a *cis-p*-coumaric acid as acylglucosyl moiety | 222 |
| Geranium flowers (*Geranium sylvaticum*) | First structure elucidation of a monoacetylated anthocyanin 3,5-diglycoside with identical sugars | *7* |
| Gentiana flowers (*Gentiana makinoi*) | Identification of anthocyanins in which the 5-*O*-glucose attached to anthocyanidin is acylated with hydroxycinnamic acid | 224 |
| Stem of *Allium victorialis* | First report on acylation of the 3”-position in the sugar moiety of any anthocyanin. Malonylation at this position provides better stability than 6”-acylation | 8 |
| Water lily (*Nymphaéa* X *morliacea*) | First report involving a diacylation, which involves gallic acid as one of the acyl moieties and produces a bathochromic shift (5 nm) on the maxima of the visible spectra | 154 |

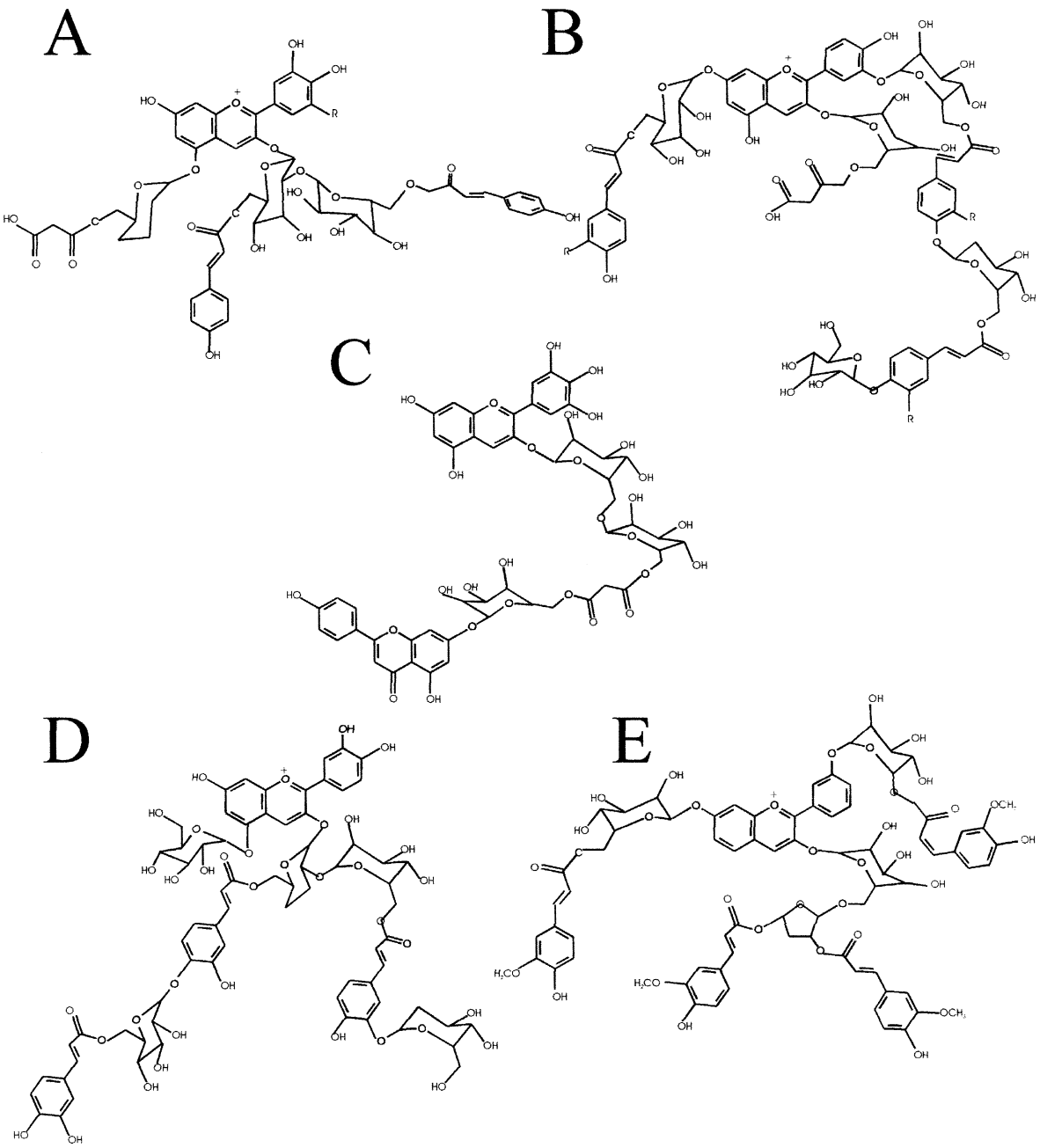


**FIGURE 18.** Acylated anthocyanins with improved stability under different process conditions. These pigments were obtained from (A) *Ajuga reptans*, (B) *Bletilla striata*, (C) *Eichhornia crassiped*, (D) *Ipomoea purpurea*, and (E) *Tradescantia pallida*.

**TABLE 7**
**Processing and Stability Studies on Anthocyanin Pigments in Different Food Systems**

| Model | Conditions | Effects | Ref. |
|---|---|---|---|
| Blackberry juice | Different temperatures and addition of aldehydes | Disappearance rate followed a first-order kinetics and was higher in the presence of aldehyde and dependent of temperature | 114 |
| Quick frozen strawberries | Addition of different levels of sucrose | Sugar addition stabilizes the pigment content ad produce a reduction in browning reactions. The protective effect was assigned to inhibition of degradative enzymes and to steric interference with condensation reactions | 511 |
| Grape musts | Addition of glutathione | Quinones are involved in anthocyanin degradation because of this is inhibited by glutathione | 103 |
| Barley | Heating (40–100ºC) | Hordeumin anthocyanin showed an improved stability because of it is constituted by a molecular complex between anthocyanins and polyphenols | 347 |
| Marashino cherries | Brined cherries pigmented with red radish anthocyanin extracts (RAE) | The coloration imparted by RAE and FD&C Red No. 40 was similar; the kinetics of degradation followed first-order kinetics | 173 |



On the other hand, anthocyanin production in leaves of *Ajuga pyramidalis* was compared with that produced in cell cultures. The main anthocyanin was 3-*O*-(6-*O*-(E)-ferulyl)-2-*O*-[(6-*O*(E)-ferulyl)-β-D-glucopyranosyl-β-D-glucopyranosyl]-5-*O*-(6-*O*-malonyl)-β-D-glucopyranosylcyanidin; and it was determined that anthocyanins from cell cultures showed higher stability than those obtained from *in vivo* extracts. It was explained that in cell cultures copigmenting agents such as flavonols, phenolic acids, and tannins could accumulate, which may be absent in plant.[292]

Condensation reactions have been reported by the reaction of acetaldehyde, anthocyanins, and flavan-3-ols, producing the increment of color (up to seven times). It is believed that acetaldehyde forms a bridge between the two flavonoids, and consequently condensation reactions could proceed and contribute to the polymeric color.[157,193,242] Moreover, the formation of new anthocyanins by the reaction of malvidin 3-monoglucoside and procyanidin B2 in the presence of acetaldehyde 15ºC/4 months) was observed. Three new pigments were observed and their visible spectra showed a bathochromic displacement, in relation to the anthocyanin by the condensation of these compounds. Interestingly, these compounds showed an improved stability.[155] Compounds with similar characteristics to the synthesized ones have been detected in red wines.

### *b. Processing and Stability in Foods*

Anthocyanin pigments can be destroyed easily during the processing of fruits and vegetables, and considering food color as an appealing characteristic, many studies have been carried out to understand the anthocyanin properties and to obtain better products with minimal degradation (Table 7). High temperature, increased sugar level, pH, and ascorbic acid can affect the rate of destruction.[157,510] Temperature has been reported to induce a logarithmic destruction of pigment with time of heating at a constant temperature. Bleaching by effect of

heat occurs because of the above-described equilibrium is changed toward the uncolored forms. It has been suggested that flavonoid structure is opened to form chalcone, which is degraded further to brown products.[157] However, interestingly, it has been observed that optimal conditions permit the regaining of color on cooling if there is sufficient time (several hours) for the reconversion.

In slightly alkaline solutions (pH 8 to 10) highly colored ionized anhydro bases are formed. At pH 12, these hydrolyze rapidly to fully ionized chalcones.[58]

Knowledge of anthocyanin characteristics have allowed the development of a large number of products and processing conditions that obtain high-quality colored products. Pomace extracts were freeze dried on DE 20 maltodextrin, and this preparation showed good shelf life (2 months at 50°C/0.5 aw and more than 5 years with $a_w < 0.3$). Also, improved stability was obtained in concentrated extracts of fermented elderberries. The described process was as follows: berries were homogenized, fermented with *C. cereviceae* var. *malaga* (room temperature, pH = 4.5), and a concentrated juice is obtained (4% d.w. of anthocyanins). Additionally, the use of this product to enhance the color of spiced wines is mentioned. Four patents have been developed to extract the colorant from blue corn by using a simple acidified aqueous extraction. A patent to obtain the sorghum colorant involves alcoholic extraction and purification on a silica gel column. While other patents have been developed for anthocyanin extraction from black mulberries, elderberries, *Viburnum* berries, rowanberries, red sweet potatoes (*Ipomoea batatas*), and for a variety of plant sources. In addition, a successful extraction procedure to isolate pigments using 350 ppm $SO_2$ in water and ion exchange chromatography was reported. Also, a purification process was implemented that involves selective absorption on inorganic oxides such as silica gel, titanium oxide, or alumina, all of them coated with a styrene polymer.[157]

During processing and commercialization of fresh products, color is the main factor of choice, and several attempts have been carried out to preserve the "fresh" appearance of fruits when stored. In fresh strawberry fruits, $CO_2$ treatments have been assayed to preserve their attractive appearance. It was found that skin color was significantly affected by different $CO_2$ treatments, evaluated by reflectance colorimetry; however, internal color decreased markedly. Color disappearance was correlated with low anthocyanin levels. Additionally, it was suggested that this opposite behavior (skin and internal) could be produced by different anthocyanin profiles in the analyzed structures; higher concentrations of cyanidin 3-glucoside are observed in external tissue (higher stability), while pelargonidin glycosides are in the internal tissue. Moreover, the accumulation of phenolic compounds is greater in external tissue, in agreement with the protective role assigned previously.[170] In addition, other methodologies have been proposed to change metabolic pathways to reach a higher color preservation. In frozen strawberries, it was shown that sugar addition has an stabilizing effect on the total monomeric anthocyanins, suggesting that sugar can be employed to enhance the shelf life of colored products (Table 7).[511] Litchi is a tropical fruit and within 2 to 3 days after harvest its pericarp becomes desiccated and turns brown. To diminish the losses, litchis were coated with chitosan (1.0 to 2.0%) and stored (4ºC/90% relative humidity). The use of chitosan delayed changes of contents of anthocyanin, flavonoid, and total phenolics. Interestingly, the activities of polyphenol oxidase and peroxidase, which have been involved in anthocyanin degradation, are inhibited. It is suggested that a plastic coating forms a protective barrier on the surface of the fruit and reduces the supply of oxygen for enzymatic oxidation of phenolics.[523] Also, anthocyanin profiles have been proposed as an indication of inadequate processing conditions. In red raspberry juices, it was found that bad processed samples showed higher levels of polymeric color instead of the monomeric anthocyanin pigments, and also this was observed as a product of poor quality.[56] Another important factor to preserve color during fresh-fruit storage is the elimination of mold contamination, which has a deleterious effect on anthocyanin polymerization.[405] In general, it is considered that light has deleterious effects on anthocyanin stability and light exposure of natural colored beverages must be avoided. It has been reported that other flavonoids flavone, isoflavone, and aurone sulfonates) increase the

photostability.[157] On the other hand, light intensity has a profound effect on an apple's color, because the light-exposed peel contains twice as much anthocyanin as a shaded peel. Interestingly, a correlation was shown between UFGT (UDPGal:flavonoid-3-*O*-glycosyltransferase) activity and anthocyanin accumulation with an UFGT activation by ethephon; it suggests a regulatory importance of UFGT in anthocyanin synthesis in apple.[245] Also, in apple it has been observed that methyl jasmonate is associated with the 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.[142] To modify the apple fruit appearance, the use of magnesium and urea as a source of nitrogen was attempted, and a better final coloration was observed, but this was mainly affected by the increase in chlorophyll and carotenoid content, while the anthocyanin level remained the same.[393] Oxygen has a negative effect on anthocyanin stability, while ascorbic acid could have a negative or positive effect, depending on the media conditions. Additionally, it has been suggested that flavonols act as free radical scavengers to protect anthocyanin molecules. Anthocyanins are very reactive toward metals, and they form stable complexes with tin, copper, and iron; it has been proposed that metal complexes could be used as colorants.[417]

The overall color changes may be due to a number of factors such as pigment degradation, pigment polymerization, reactions with other components of the formulation, nonenzymatic browning, oxidation of tannins, and other reactions completely unrelated to the added colorant.[157] Anthocyanins can be degraded by a number of enzymes found in plant tissue: glycosidases, polyphenoloxidases, and peroxidases. Glycosidases produce anthocyanidins and sugars, and anthocyanidins are very unstable and rapidly degraded. Polyphenoloxidase catalyzes the oxidation of *o*-dihydrophenols to *o*-quinones that further react to brown polymers; however, this reaction is more favorable for other phenols. Thus, pigment loss can be reduced by blanching to inactivate these enzymes. Addition of 30 ppm $SO_2$ will inhibit phenolase activity in sour cherry juice. Gallotannin will inactivate phenolase through a direct condensation system. The destruction of anthocyanins by enzymatic activity could be important in the design of an extraction procedure and perhaps in the final formulation in a food.[157]

### *c. Production of Anthocyanins by Plant Tissue Culture*

Plant tissue culture is a potential source of production of anthocyanins, in view of the quoted price ($1250 to $2000/kg) and the expanding market of natural anthocyanins. Moreover, plant cell culture could ensure a continuous supply of uniform-quality anthocyanin pigments that cannot be produced by other biotechnological approaches; however, to date no food colorant obtained by this system has been commercialized, and the main bottleneck is the low yield of secondary metabolites.[58,157,202]

It has been considered that bioreactor production is prohibitive because present technological limitations make the process very expensive; a commercial pigment production by plant cell tissue culture could be achieved only when a fully automated predictable process with a more advanced technology could be established.[442] One of the main problems with plant cell suspensions is the heterogeneity of media due to the existence of cells as large aggregates that cause problems of oxygen diffusion, mass transfer, cell ejection of the liquid media, and reduced growth rates. Additionally, the conditions favoring high growth do not favor production, and frequently high production conditions are detrimental for growth. In particular, maximal growth of strawberry cells in suspension cultures was found at 30ºC, but lower temperature induced an increased level of anthocyanin pigment (the maximum at 20ºC).[524] Thus, processes by stages have been developed (a first stage for cell growth and a second one for anthocyanin production), and models have been designed for batch and semicontinuous anthocyanin production to introduce the concept of primary and secondary metabolism because of the continuous competence for precursors.[441]

Anthocyanin production by cell cultures has been evaluated from grapes (2 to 3 mg/g fresh weight),[514] *Euphorbia milli*,[516] and carrot,[492] among

others. In general, stable production by plant cell cultures has not been reached. However, some cultures of *Ajuga reptans* have produced anthocyanins for more than 10 years, but the analyses of five lines showed quantitative differences between the lines, and it was observed that 5′-substituted anthocyanins seem to decrease more easily during repeated subcultures.[67] In wild carrot (*Daucus carota*), clonal variability has been observed to obtain low- and high-pigmented cells. Interestingly, it was shown that high-pigmented cells have an intermediate limitation reflected by a considerable increment in pigment production after supplementation with dihydroquercetin, naringenin, and 4-coumarate.[492]

Remarkably, anthocyanin production is light dependent. Darkness cell cultures showed an intense pigmentation after illumination,[418] although darkness cultured cells, *Aralia cordata* among others, have shown anthocyanin accumulation. Cultured cells of *Perilla frutescens* need light irradiation for 7 days from the initial cultivation period for the formation of anthocyanin pigments.[526] Interestingly, it was observed that light cultured cells are positively influenced by the treatment with auxins and cytokinins, while cultures in darkness are not affected.[411] In callus cultures of *Oxalis linearis*, anthocyanin production was promoted by cytokinins but repressed by auxins such as NAA and 2,4-D.[311] Another factor analyzed in plant cell pigment production is the pH, and considering the stability characteristics of anthocyanins the optimum pH is mainly acidic (pH < 7), but it was found that suspension cultures of strawberry cells showed the highest anthocyanin production at pH 8.7. It was observed that the time profile ratio of pigmented cells did not change with the variation in initial medium pH, suggesting that initial pH affected anthocyanin synthesis by changing the anthocyanin content inside the pigmented cells only.[525]

Several substrates have been assayed to induce increments in anthocyanin production. In cell suspension cultures of *Vitis*, phenylalanine addition induces the cessation of cell division and the induction of anthocyanin biosynthesis. Additionally, it was shown that *Vitis* cell division is limited by nutrients in medium, such as sucrose, phosphate, and nitrate or ammonium, and that nutrient limitation induces the activities of PAL and CHS enzymes, suggesting an association of phenylalanine addition with enzyme induction, and a similar behavior was observed with strawberry cells.[115,246,524] With *Fragaria anansa* cell cultures, riboflavin addition had a 3.2 times greater production than in control medium, but only under light conditions.[331] In carrot cell, suspension cultures were found that favor fructose growth, while anthocyanin production is better with glucose, suggesting quantitative differences in the metabolism of these two sugars.[530] In cultured cells (e.g., *Vitis vinifera, Aralia cordata, Fragaria anansa*), high sucrose and low nitrate are desireable for anthocyanin production. It has been considered that although sucrose is an essential nutrient, it also acts as an osmotic agent when used at high concentrations. Interestingly, it was observed that high ammonium concentration (24 m*M*) promotes the anthocyanin acylation, which is important for the food industry based on the improved stability of acylated pigments.[213,331,332,411,418] It has been proposed that reduced nitrate favors anthocyanin synthesis by favoring the uptake into vacuoles by a tonoplast-associated transport mechanism.[213]

Recently, the use of extracts of cultures or conditioned media to improve the anthocyanin production by tissue cultures has proposed. Culture filtrates and cell extracts of *B. cereus*, *S. aureus* and *E. coli* (among others) were used as elicitors of anthocyanin production in carrot cell cultures, obtaining increments up to 77%.[454] It has been found that conditioned media (CM) from strawberry cell cultures promote anthocyanin accumulation in cell suspensions of strawberry. The anthocyanin accumulation was sixfold higher in CM than in control media. On the other hand, rose cells were transferred to CM from strawberry cells, and it was observed that anthocyanin accumulation was induced. Thus, it the presence of some unknown material(s) released (heterologous conditioning factor) during culture that contributed to anthocyanin accumulation was proposed.[332,412]

## C. Betalains

### *1. Definition*

The term "betalains" was introduced by Mabry and Dreiding;[288] this was supported by structural and biogenetic considerations. In a strict sense, betalains do not belong to alkaloids because they are acidic in nature due to the presence of several carboxyl groups. Originally, betalains were called "caryophyllinenroth" and successively renamed "rübenroth" and "chromoalkaloids".[371,395]

Chemically, betalain definition embraces all compounds with structures based on the general formula shown in Figure 19; therefore, they are immonium derivatives of betalamic acid.[375,451] The chromophore of betalains can be described as a protonated 1,2,4,7,7-pentasubstitued 1,7-diazaheptamethin system.[371]

Betacyanin structures (Figure 20A**)** show some variations in the acyl groups and sugar moieties, while betaxanthin (Figure 20B) exhibits the same dihydropyridine moiety but show conjugation with several amines and amino acids.

Betanidin is the basic structural unit of most of the betacyanins, followed by its $C_{15}$ epimer, the isobetanidin.[374] A considerable number of different betacyanins can be obtained with glycosidation of one of the hydroxyl groups located at positions 5 and 6 (Figure 20A).

Betaxanthins are constituted of different proteinogenic and nonproteinogenic amino acids, as well as biogenic amine-conjugated moieties of betalamic acids. More than 200 amino acids found in plants may potentially give rise to betaxanthin structures.[451] The archetypal compound representing betaxanthins is the indicaxanthin, isolated from prickly pear (cactus fruits of *Opuntia ficus-indica*).[371]

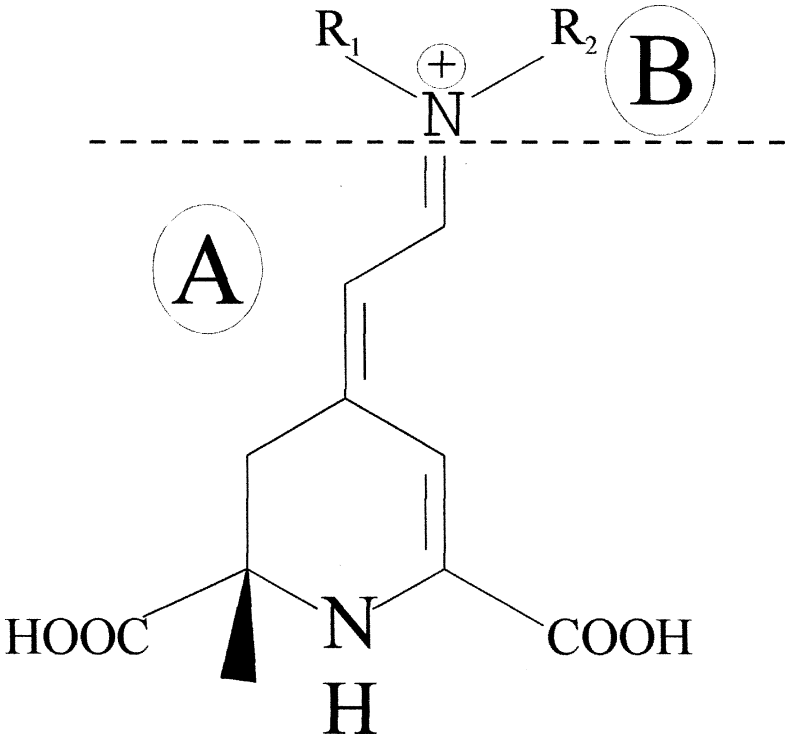


**FIGURE 19.** Betalain general formula. (A) Betalamic acid moiety is present in all betalain molecules. (B) The structure will represent a betacyanin or a betaxanthin, depending on the identity of the $R_1$ and $R_2$ residues. (Adpated from Ref. 46.)

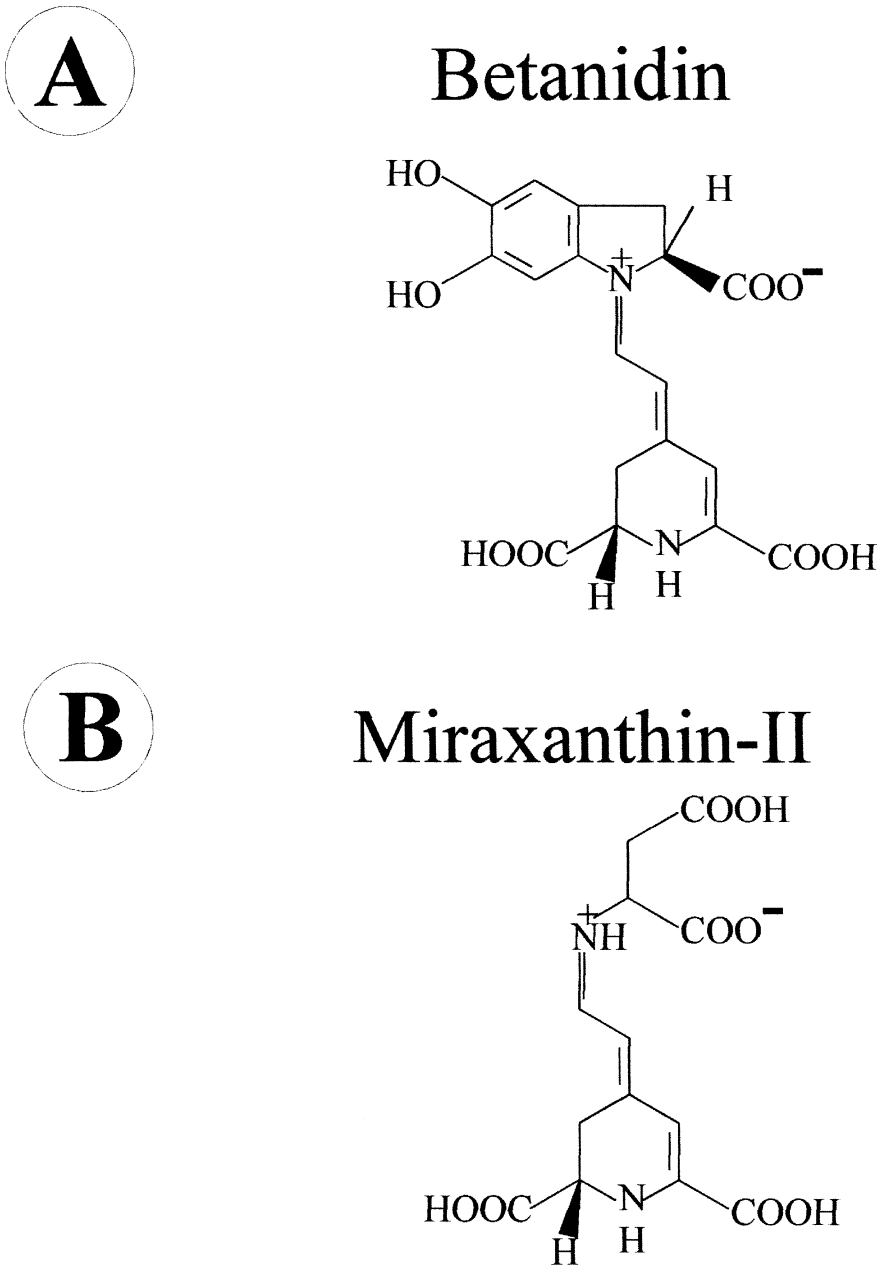


**FIGURE 20.** Betanidin is an example of betacyanins (A), while miraxanthin II is of betaxanthins (B). (Adapted from Refs. 375, 451.)

## 2. Classification

They can be divided into two structural groups, the yellow betaxanthins (from Latin *beta*, red beet and Greek *xanthos*, yellow) and red-purple betacyanins (*kyanos*, blue color), depending on $R_1$-*N*-$R_2$ moieties. More than 50 betalains are well known, and all of them have the same basic structure, in which $R_1$ and $R_2$ may be hydrogen or an aromatic substituent. Their color is attributable to the resonating double bonds.[46] Conjugation of a substituted aromatic nucleus to the 1,7-diazaheptamethinium chromophore shifts the absorption maximum from 480 nm in yellow betaxanthins to 540 nm in red-purple betacyanins.[451]

In some earlier papers, the terms "betalaines", "betacyanines", and "betaxanthines" were used; the terminal letter "e" was added by Fisher and Dreiding[148] so the names would conform to the I.U.P.A.C. nomenclature; at present, these terms can be used without the terminal "e".

Betacyanins and betaxanthins can be classified using their chemical structures. Betacyanin structures show variations in their sugar (e.g., 5-*O*–D-Glucose) and acyl groups (e.g., feruloyl), whereas betaxanthins show conjugation with a wide range of amines (e.g., glutamine) and amino acids (e.g., tyrosine) in their structures. Table 8 shows some well-studied betalains.

## 3. Distribution

Among higher plants the occurrence of betalains is restricted to the *Caryophyllales*[289] and those found in certain higher fungi such as *Amanita*, *Hygrocybe*, and *Hygrosporus*.[451] Betalains of higher plants are in different organs.[402] They produce red, yellow, pink, and orange colors in *Aizoaceae* and Portulacaceae flowers,[473] and purple pigmentation in Cactaceae fruits and in red-beet root (Chenopodiaceae).[370] Betalains are in bracts, for example, *Bougainvillea* (Nyctagynaceae) possesses a wide range of colors;[372] they are also in seeds of *Amaranthus*,[37] in leaves of *Teloxis* and in stems.[12]

Common names and classification of different betacyanins and betaxanthins are standardized, and they are usually assigned in agreement with their botanical genus.[371] In the betacyanin group, amaranthin-I was obtained from *Amaranthus tricolor*, betanin from *Beta vulgaris*, and gomphrenin-I from *Gomphrena globosa*. While in the betaxanthin group, miraxanthin occurs in flowers of *Mirabilis jalapa*, vulgaxanthin-I and II have been found in root of *Beta vulgaris*, and portulaxanthin has been isolated from the petals of *Portulaca grandiflora*.[375]

Up to date more than 50 structures of naturally occurring betalains have been identified. A considerable number of different betacyanins may be derived from two basic compounds, betanidin (*2S*, 15S) and isobetanidin (*2S*, 15*R*) by glycosidation of one of the hydroxyl groups located at position 5,[374] for example, betanin, which occurs as the 5-*O*-glucoside, and the less-occurring position 6, for example, gomphrenin-II, which is a 6-*O*-glucoside. No betacyanin is known to have both positions substituted with sugar residues.[375] A few biosides are known: amaranthin the betanidin 5-*O*-[2–*O*-(β-D-glucopyranosyluronic acid) β-D-glucopyranoside], and its epimer isoamaranthin is in the leaves of *A. caudatus*.[38] Moreover, two epimeric betacyanins, bougainvillein-r-I and isobougainvillein-r-I, have been isolated from *B. glabra*.[372]

The isomeric 5-*O*-β-cellobiosides have been produced by deacylation of the pigments aleracin-I and II from *P. oleracea*.[45] The only known 6-($2^G$-glucosylrutinosides) of betanidin and isobetanidin have been obtained by deacylation of bougainvillein-V. Betalain glycosides can be esterified with hydroxycinnamic acids (ferulic and *p*-coumaric acids), for example, celosianin-I 4-coumaroylamaranthin) in *Chenopodium rubrum* and *Lampranthus sociorum*;[48] in addition, sulfuryl and malonyl betacyanins are known, for example, rivianin (betanin-3′-sulfate) from *Riviana humilis* and phyllocactin (malonic acid 6′-half-ester of betanin) from *Phyllocactus hybridus*.[370] The decarboxybetanidin, isolated from *Carpobrotus acinaciformis*, is unique among betacyanins in containing a modified aglycone moiety.[371]

There are about 15 naturally occurring betaxanthins; the indicaxanthin from *Opuntia ficus-indica* was the first crystallized.[375] In total,

**TABLE 8**
**Some Fully Identified Naturally Occurring Betalains**

| Betalain[a] | Residue[b] | Ref. |
|---|---|---|
| ***Aglycones*** | | |
| Betanidin | — | 375 |
| ***Betanin group*** | | |
| Betanin | 5-*O*-Glc | 46 |
| Phyllocactin | 5-*O*-Glc | 373 |
| Lampranthin-I | 5-*O*-Glc | 48 |
| **Amaranthin group** | | |
| Amaranthin | 5-*O*-Glc-2-*O*-GlcU | 451 |
| Celosianin II | 5-*O*-Glc-2-*O*-GlcU | 449 |
| **Bougainvillein** | | |
| Bougainvillein | 5-*O*-Glc-2-*O*-Glc | 372 |
| ***Gomphrenin group*** | | |
| Gomphrenin-I | 6-*O*-Glc | 316 |
| ***Betaxanthins*** | | |
| DOPAxanthin | DOPA | 371 |
| Indicaxanthin | Proline | 371 |
| Portulaxanthin-II | Glycine | 473 |
| Vulgaxanthin-I | Glutamic acid | 375 |

[a] Names were standardized by Strack et al.[451]
[b] Abbreviations: **Glc** β-*D*-Glucose; **GlcU** β-*D*-Glucoronic acid; **DOPA** 3,4-dihidroxyphenylalanine.



eight of the naturally occurring betaxanthins contain non-protein amino acids.[472]

## 4. Biosynthesis: Biochemistry and Molecular Biology

### a. Biochemistry

**Initial stages (Figure 21A).** The determination of the chemical structure of betalains and of their biosynthetic intermediates contributed to the establishment the corresponding biosynthetic pathway; in addition, feeding experiments with isotopically labeled precursors and *in vitro* cell cultures were important tools in such discovering.[46,375] However, very few enzymes involved in betalain synthesis have been purified and characterized.[211,273,434,451] Figure 21 summarizes the propounded biosynthetic pathway. In detail, betalains are considered secondary metabolites; they derive from shikimic acid and from the tyrosine aminoacid.[371] In their basic structure, the phenyl group is bonded to a lateral *n*-propyl chain giving place to a $C_6$-$C_3$ unit.[35] Biogenesis of betalains from tyrosine has not been thoroughly understood, and only a few enzymes involved in the biosynthetic route have been identified.[47,273]

Two molecules of tyrosine are required in the biosynthesis of one molecule of betacyanin or

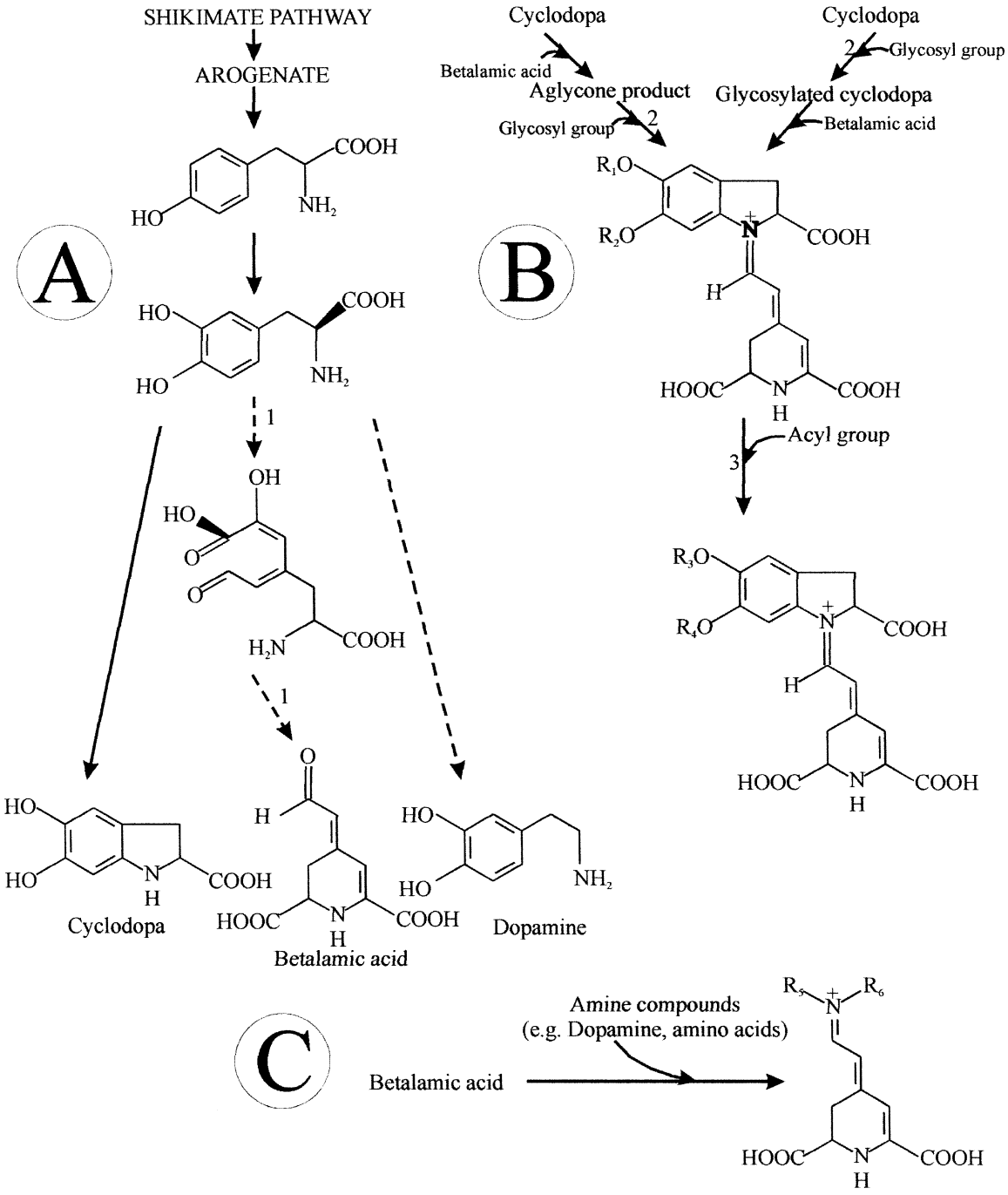


**FIGURE 21**. Pathway proposed for betalain biosynthesis. (A) Intial stage, (B) betacyanin biosynthesis. Betacyanins may be glycosylated at position $R_1$ and $R_6$ and or acyglycosylated at postion $R_3$ or $R_4$. (B) Betaxanthin biosynthesis. $R_5$ and $R_6$ represent lateral chains of amine compounds. Some of the enzymatic activities involved in betalain biosynthesis are (1) DOPA 4,5-dioxygenase, (2) glycosyltransferase, and (3) acyltransferase. (Adapted from Refs. 46, 451.)

betaxanthin. Initially, two molecules of 3-hydroxy-L-phenylalanine (L-DOPA) are formed.[335,375] The hydroxylation of tyrosine to L-DOPA is recognized as the first step in the biogenesis of betalains because experiments with radioactive precursors have shown good incorporation of labeled [$C^{14}$]-tyrosine into amaranthin and betanin, the major betacyanins in *Amaranthus tricolor* seedlings and red-beet (*Beta vulgaris*), respectively.[135] The assumption has been that the first enzyme is a phenol-oxidase complex catalyzing both the conversion of tyrosine to L-DOPA by a monophenol oxidase and the dehydrogenation of the latter to *O*-quinone by a diphenol oxidase.

Recent work on the enzymology of betalain biosynthesis has focused on the toadstool "fly agaric" (*Amanita muscaria*) mushroom of the *Agaricales.* In this basidomycete, the accumulation of betalain pigments is restricted to the cuticle of the cap and its subject to developmental regulation.[451] There is a rapid increase in betalain levels during the process of development from young fungi, which are still entirely covered by the veil, to mature specimens. Recently, Müeller et al.[335] characterized a tyrosinase from pileus of *Amanita muscaria*. This enzyme was located only in the colored parts of the fungi, and it was demonstrated that tyrosinase catalyzes the reaction of tyrosine hydroxylation to L-DOPA, confirming its involvement in betalain biosynthesis. Tyrosinase also shows diphenolase activity, and it seems to be an heterodimer of two subunits with molecular weights of 27 and 30 kDa, something unusual for tyrosinases.

On the other hand, one L-DOPA molecule is transformed into DOPA-quinone, which is spontaneously converted to cyclo-DOPA, while betalamic acid is formed from the second molecule of L-DOPA by means of a reaction catalyzed by DOPA-dioxygenase.[336] In the proposed metabolic pathway betalamic acid arises from L-DOPA. L-DOPA is cleaved at the C4-C5 bond of the aromatic ring to form an intermediate (4,5-seco-DOPA) that is cyclized, forming an heterocyclic system. It has been suggested that 4,5-seco-DOPA is produced by an "extradiol-cleavage" of L-DOPA.[463]

The oxidative disruption of the L-DOPA ring appears to be analogous to the aromatic rings fission of catechols, and the last reactions are catalyzed by specific dioxygenases. These enzymes contain Fe ion (both ferric and ferrous states) as an essential cofactor. Girod and Zrÿd[171] isolated the DOPA 4,5-dioxygenase from *A. muscaria*. This enzyme as other extradiol-cleaving dioxygenases is an oligomer that catalyzes the 4,5-extradiol disruption of L-DOPA leading, via 4,5-secoDOPA to betalamic acid.[463] It was shown by affinity chromatography that DOPA 4,5-dioxygenase is composed of a varying number of identical 22-kDa subunits. Another DOPA dioxygenase (DOPA 3,4-dioxygenase) was also extracted from *A. muscaria*; this enzyme catalyzes the 2,3-ring-opening reaction that yields the muscaflavin pigment, a compound that has never been found in plants. The betalamic acid may condense with the imino-group of cyclo-DOPA to produce the red-purple betacyanins (Figure 21B) or with the imino or amino group of amino acids to give the yellow betaxanthins (Figure 21C).[451]

**Betacyanin biosynthesis (Figure 21B).** Betacyanins are formed through the reaction of cyclo-DOPA with betalamic acid followed by glycosylation, or by condensation of the cyclo-DOPA glycosides with betalamic acid.[209] In general, it could be mentioned that condensation of betalamic acid with cyclo-DOPA to betanin formation and the subsequent glucosylation reaction to betanidin 5-*O*-β-glucoside, main compound of red-beet, remain unknown. Complete glycosylation takes place with cyclo-DOPA followed by condensation with betalamic acid. On the other hand, free betanidin can be stored as the main component in betalain-producing cells, and it has been shown that betanidin can be the main receptor of UDP-glucose from glucosyl-transferase during betanin biosynthesis.[208,209] It may be possible that the sequence of reactions depends on the plant genus.

Betanin glucosylation catalyzed by uridine 5′-diphospho-glucose: betanidin 5-*O*-β-glucosyltransferase (5-GT) was demonstrated by Heuer and Strack,[208] who described the occurrence of one of the two proposed pathways of betacyanin formation, the transfer of glucose to 5-hydroxy group of betanidin in the formation of betanin.

On the other hand, Heuer et. al.[209] described a new glucosyltransferase, the 6-*O*-glucosytransferase (6-GT) that catalyzes the regiospecific transfer of glucose to the 6-hydroxy group of betanidin in the formation of gomphrenin I, analogs to 5-GT. Both GT were extracted from cell cultures of *Dorontheanthus bellidiformis*. The 5-GT showed three isoforms and the 6-GT only one; both enzymes are monomers with a molecular weight near 55 kDa. Nowadays, it has been shown that both enzymes catalyze the indiscriminate glucose transfer from UDP-glucose to hydroxyl groups of betanidin, flavonols, anthocyanins, and flavones. Interestingly, it was observed that GT catalyzes the formation of 7-*O*-glucosides, but in a minor extent.[493] Based on these results, it could possibly be that 5-GT and 6-GT have a phylogenetic relation with flavonoid glucosyltransferases. Subsequently, acylation of glycosylated betanidins is through acyl group donation from 1-*O*-acylglucosides. It is important to point out that this reaction catalyzed by an acyltransferase seems to be an exclusive biosynthetic mechanism of betalain-producing plants, contrasted with the analogous reaction in the biosynthesis of flavonoids where acylated flavonoids are produced by the hydroxycinnamoyl-coenzyme-A pathway.[47]

Another enzyme involved in betacyanin formation is an acyltransferase (HCA), which 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 (amaranthin) in *Chenopodium rubrum*;[48,49] the products formed are celosianin I (4-coumaroylamaranthin) and celosianin II (feruloylamaranthin), as shown in Figure 22. This enzyme exhibits a molecular weight near 70 kDa.

HCA could also catalyze the formation of 4-coumaroyl and feruloyl-derivatives in *Beta vulgaris* (lampranthin II), *Gomphrena globosa* (gomphrenin III), *Lampranthus sociorum* (celosianin I and II), and *Iresine lindenii* (lampranthin II).[47]

**Betaxanthin biosynthesis (Figure 21C).** Little is known about the betaxanthin biosynthesis.[472] However, it has been suggested the interchange of basic compounds as one of the main routes.[451] A spontaneous condensation between betalamic acid and an amine group inside the vacuole has been based on genetic and biochemical studies on clones of *P. grandiflora*. In a recent work, Hempel and Böhm[207] found two new betaxanthins in hairy root cultures of *Beta vulgaris* var. Lutea, vulgaxanthin III, and IV, when the culture media was supplemented with the corresponding L-amino acids. This feeding experiment provides arguments for a spontaneous condensation of betalamic acid with amino acids or amines in the course of betaxanthin biosynthesis.

In other experiments, the administration of L-DOPA to violet petals of flowers in *Portulaca grandiflora* led to the biosynthesis of betaxanthins not present in natural plants. These results show that L-DOPA administration elicits the formation of betaxanthins, which are absent in untreated flowers.[397] In addition, nine L- and D-isomers of amino acids were supplied to seedlings and individual hairy roots strains of *Beta vulgaris*, var. Lutea; the increment of betaxanthin concentration and also the appearance of new betaxanthins was observed.[207] Therefore, the theory of a spontaneous condensation of betalamic acid with amino acids or amines in the course of betaxanthin biosynthesis is supported.

On the other hand, the activity of a betacyanin and betaxanthin-decoloring enzymes has been postulated in *Beta vulgaris*,[271,437] *Amaranthus tricolor*,[135] and *Phytolacca americana*;[265] the results suggest there is an enzyme complex with two acidic and two basic enzymes that contain a metal ion in the active site. Earlier works described this complex like a "peroxidase" enzyme.[231] Parra and Muñoz[361] confirmed that horseradish peroxidase catalyzes the oxidation of betanin. HPLC analysis showed a red intermediate and several yellow products (presumably of a polymeric nature) being betalamic acid, one of the final products. Zakharova et al.[521] described the occurrence of a betalain oxidase in three species of *Amaranthus*. This enzyme was found mainly in cell walls of *A. caudatus*, and it was suggested that pigment yield can be increased by regulating the activity of this enzyme during plant tissue extraction.

Watson and Goldman[500] reported dominant alleles at two tightly linked loci (R and Y) of red

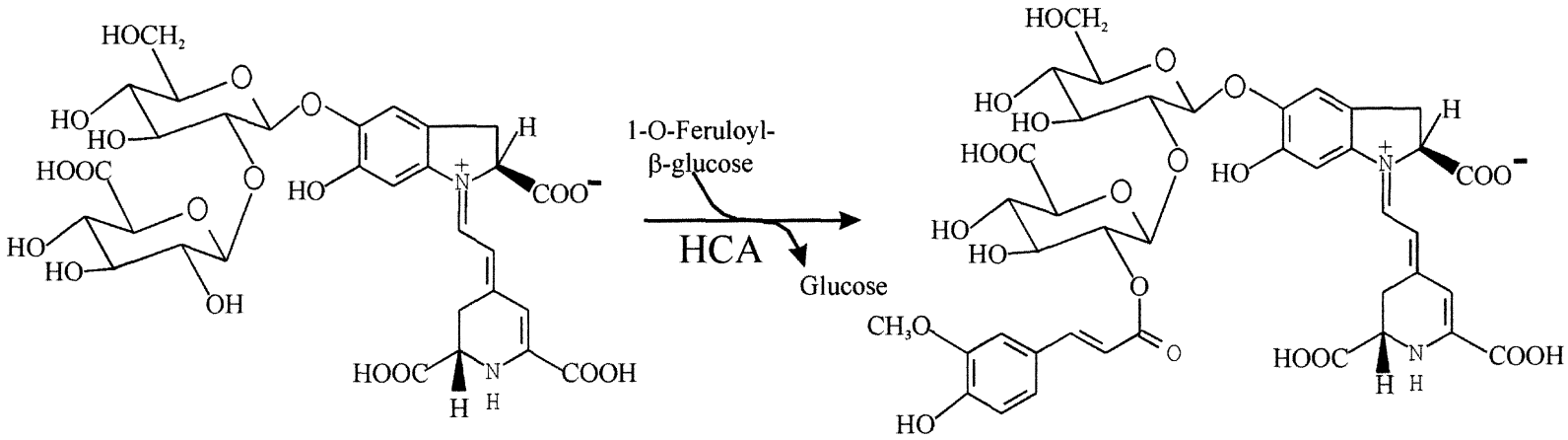


**FIGURE 22** Amaranthin acylation catalyzed by the enzyme 1-*O*-hydroxycinnamoyl-transferase (HCA) in the biosynthesis of celosianin-II, an acylated betacyanin. (Adapted from Ref. 49.)

beet. These alleles are involved in the production of betalain pigment. In addition, it has been shown that several alleles in these loci influence the production and distribution of betalains. The authors also suggested the existence of genes that play an important role in betalain synthesis.

### *b. Biosynthesis Regulation*

In plants, betalain biosynthesis is subject to complex regulation. Pigments are accumulated only in certain tissues and at specific stages of development. Their synthesis has been shown to be regulated by light and cytokinins.[135] It has been shown that effects of DOPA, light, and kinetin exposure are different between models producing betacyanins or betaxanthins. The betanin synthesis was only induced under the influence of DOPA or with the combination of kinetin and light. On the other hand, betaxanthins were synthesized only when the seedlings were fed with DOPA, and a significant increment of this production was obtained by light and kinetin supplementation.[37]

The presence of free betalamic acid in plants, which produce betaxanthins or betacyanins and betaxanthins, and its absence in plants, which produce only betacyanins, suggest a regulatory mechanism during its biosynthesis.[451] A coordinated condensation mechanism of betalamic acid with cyclo-DOPA or glycolized-cyclo-DOPA has been suggested in plants that produce betacyanins; then the accumulation of betalamic acid is arrested. On the other hand, control mechanisms in betaxanthin-producing plants must be different to allow the accumulation of betalamic acid (Mueller et al., 1997b).[46] Regulatory mechanisms on betalain formation are largely unknown, but the involvement of photoregulation and hormone control has been suggested.[395]

Cell lines produced from red beet showed a range of cell colors via specific induction methods. Phenotypic color ranged from white/green through yellow, orange and red to deep violet, representing all types of pigments found in redbeet plant. Differences in callus phenotypes were associated with cells of markedly different morphologies; cells were classified into two groups: white, orange, and violet phenotypes, and green yellow and red phenotypes. The selective expression of betaxanthin and betacyanin appeared to occur through a limited number of discrete, stable, and differentiated states, because only four colored phenotypes were isolated.[171]

In absence of specific information, it is only possible to speculate about the regulatory mechanism affecting the gene expression during phenotypic transitions of plant cell cultures. It has been proposed that DNA transposition or specific rearrangements such as translocations, inversions, breakage, and fusion are involved. It has been also suggested that such rearrangements could result in the relocation of particular genes, either within the same chromosome or to another chromosome. Moreover, it is possible that regulation of pigment synthesis is so tightly coupled to cellular morphology that an enhancement of gene expression never occurs in the yellow and red cells.[171]

Some inhibitors of cell division were used in *Phytolacca americana* suspension cultures, and a reduced betacyanin accumulation was observed, even though their modes of action are known to be different. It was suggested that inhibitory effects on betacyanin accumulation are due to the inhibition of cell division as a result of lack of DNA synthesis and that betacyanin biosynthesis and accumulation are correlated not only with DNA synthesis, but also with the progression of the cell cycle.[212]

### *c. Molecular Biology of Betalain Biosynthesis*

Up to now, very few of the enzymes involved in betalain synthesis have been purified and characterized, despite the importance of betalains as natural food colorants.[211] The only enzyme activities that have been described from higher plants are enzyme preparations that catalyze the glycosylation of betanidin and enzymes involved in betalain degradation, but work regarding genes is scarce.

Two clones encoding polyphenol oxidase were isolated from a cDNA library constructed from a log-phase suspension culture of *Phytolacca americana*, producing betalains. Spatial and tem-

poral expressions were investigated by Northern blot analysis of total RNA from various organs of *Phytolacca* plants. Transcripts of the two clones were found to be 2.1 and 2.3 kb. Both transcripts were present only at substantial levels in the ripening of betalain-containing fruits.[244]

Hinz et al.[211] described the cloning and regulation of the gene *dodA* from basidiomycete *A. muscaria*. This constitutes the first effort to clone genes of the betalain biosynthetic pathway. *dodA* codes for a DOPA dioxygenase. The cDNA library was constructed from the cap tissue of young specimens where betalain synthesis had not been yet begun. Southern blot analysis showed that DOPA dioxygenase is encoded by a single-copy gene in *A. muscaria*.[211] Müeller et al.[334,336] transformed white petals of *Portulaca grandiflora* with the *dodA* gene of *A. muscaria*, and the formation of yellow and violet spots that contained betalain and muscaflavin pigments was observed, indicating that the fungal enzyme DOPA dioxygenase was expressed in an active form in plants.

Notwithstanding these promissory results, it is obvious that much more research work is needed in order to unravel the processes involved in biosynthesis and regulation of betalains.

## 5. Functions

### a. Taxonomic Markers

Even before the structure of betalains was evident, the importance of betalain pigments in plant taxonomy and systematic distribution was clear. Betalains are in eight families: Amaranthaceae, Aizoaceae, Basellaceae, Chenopodiaceae, Cactaceae, Nyctaginaceae, Phytolaccaceae, and Portulacaceae. Nowadays, it is known that 9 of 11 families of the *Caryophyllales* order contain betalains.[402] The recent addition to the list of betalain families is Didieraceae, a small family from Madagascar.[451]

Only two families of Caryophyllales, viz., Caryophyllaceae and Molluginaceae, lack betalains and possess anthocyanins. This suggests an early differentiation of the Caryophyllales into groups with different kinds of pigments. Rosendal-Jensen et al.[402] constructed a phenogram that shows the relationship between betalains, glucosinolates, and polyacetylenes in flowering plants.

This remarkable correlation between chemical and morphological characters has led to propose that the order Centrospermae, including Cactaceae, must be reserved for betalain-containing families, while the anthocyanin-containing ones (Caryophyllaceae and Molluginaceae) must be separated into the related but distinct order Caryophyllales.[375]

The totally different chemical structure of betalains and anthocyanins,[158] the fact that they are mutually exclusive, and the restricted distribution of betalains are good arguments in favor of the paramount taxonomic significance of these pigments.

Such arguments are not lessened by the finding of their occurrence in the Basidiomycetes of higher fungi, which do not have any phylogenetic relation to flowering plants. This coincidental occurrence could be a case of chemical convergence under evolutionary phase.[371] Considering the crucial dependence of a living system on the economy of energy and our growing knowledge about the chemoecology of secondary products in nature, this assumption can be feasible.[451]

### b. Ecological and Physiological Aspects

As in the case of other secondary metabolites, it is impossible to assign a definite function to betalains in the economy of the organisms that produce them.[375] When pigments are in flowers or fruits they may have a role as attractants for vectors (insects or birds) in the pollination process and in seed dispersal by animals, such as anthocyanins.[371,503]

The occurrence in other plant parts (e.g., leaves, stem, root) may be devoid of immediate function. However, it has been suggested that betalain accumulation in red beet root is related to the storage of carbohydrates as a physiological response under stress conditions.[260] In addition, the transient coloration of many seedlings and the reddening of senescent leaves of several plants of Caryophyllales order (e.g., *Kochia scoparia*) have no obvious physiological or ecological reasons. Whatever its significance, the process resembles

the analogous phenomenon observed in anthocyanin-producing species. Betalains are also produced in injured tissues, normally not pigmented, possibly as a defense mechanism against infection. This physiological response was only observed in plants possessing specific factors that have been associated with two novel antifugal proteins.[263]

Interestingly, it must be mentioned that betalains from *Beta vulgaris* (betanin and vulgaxanthin) are effective inhibitors of indoleacetic acid (IAA) oxidase and that betanin counteracts the inhibitory effect of IAA on wheat root elongation.[375] It means that betalains could be considered as potential modifiers of auxin metabolism; notwithstanding, there is no evidence that supports the betalains role as *in vivo* regulators of IAA activity.

### c. Pharmacological Effects

Although structurally related to alkaloids, betalains have no toxic effects in the human body, as can be deduced from the fact than they are present in considerably high amounts in certain foodstuffs, such as red-beet, prickly pear fruits, and *Amaranthus* seeds.[46] Therefore, betalains represent a safe natural alternative to some synthetic color additives that are currently in use. Interestingly, there is no upper limit to the recommended daily intake.[158]

On the other hand, in betanin tested for mutagenic and carcinogenic activity an absence of mutagenicity in five *Salmonella typhimurium* strains was observed, and it did not initiate or promote hepatocarcinogenesis in levels of 50 mg/kg of weight of pure betanin or diets containing 2000 mg/kg of betacyanin.[429,431] Notwithstanding, after ingestion of these products (particularly red beet), betanin occasionally appears in the urine, an effect known as beeturia or betaninuria. The etiology and mechanism of this disorder are still controversial.[371] There are a very few pharmacological applications of betalains. Recently, they have received attention because betanin has shown antiviral and antimicrobial activities (e.g., *Pythium debaryum*, a pathogenic fungi in red-beet). In some places in Mexico, an infusion of *Bougainvillea* bracts mixed with honey is used widely for a cough. However, in both cases the action mechanisms are still unknown.

Finally, in a recent work, the importance of some natural pigments as nutraceutical ingredients was reviewed.[382] It was suggested that betalains like anthocyanins, β-carotene, and various vegetable and fruit extracts must be used for their potential health benefits. For example, yellow betaxanthins, in addition to their potential role as natural food colorant, may be used as a means of introducing essential dietary amino acids into foodstuffs, giving rise to an "essential dietary colorant".[273] Another new interesting area includes foods that can generate their own light ("biolume products") in which betalains could play an important role, suggesting how exciting the area of natural colorants has become. As other topics, it is necessary to emphasize the need of more research in these new areas.

## 6. Methodological Aspects

### a. Extraction

Betalains-containing material (raw plant or cell culture) are generally macerated or ground. Pigments can be extracted with pure water, cold, or at room temperature, although in most cases the use of methanol or ethanol solutions (20 to 50% v/v) is necessary to achieve complete extraction.[375] Sometimes, the necessity of an aerobic juice fermentation (e.g., *Saccharomyces cerevisiae, Aspergillus niger*) in order to reduce free sugars and then to increase the betacyanin content has been reported.[379] In both procedures, the inactivation of degradative enzymes by a short heat treatment of the extract (70ºC, 2 min) could be desirable, although this may destroy some of the pigments. Betacyanins can be precipitated by a slight acidification with hydrochloric acid or with acidified ethanol (0.4 to 1% HCl); subsequently, by the addition of 95% aqueous ethanol yields betaxanthins.[39,373]

Degradation of betanin may occur very fast and destruction of complex pigments should also be avoided, because acid-acylated betacyanins are rapidly deacylated and such pigments can be over-

looked.[451] In such a situation, extraction should be carried out with cold water for long-term and darkness conditions.

### *b. Separation*

**Ion-exchange and column chromatography.** Ionic-exchangers are the most widely used adsorbents in fractionation, as much as in separation; then gel filtration is used.[374] In a simple and rapid procedure, plant extract must be stirred with the ion-exchanger resin (e.g., Dowex 50W-X2, Merck I, DEAE-Sephadex A25, etc.), which adsorbs the betalains (nonionic interaction). Subsequently, resin is washed with aqueous HCl (0.1% v/v) and pigments are eluted with water followed by final separation on a chromatographic column (e.g. Polyamide, Polyclarc-AT, or polyvinylpyrrolidone, Sephadex G-15 and G-25).

The chromatographic and electrophoretic properties from unknown plant materials can be compared with those reported in the literature for known pigments (e.g., Piatelli and Minale,[374] for betacyanins; Minale et al.[316] for acylated betalains; Piatelli and Imperato;[370] von Elbe et al.;[496] Piatelli;[371] and Steglich and Strack[449] for betalains in general).

**Electrophoresis and thin layer chromatography (TLC).** Paper electrophoresis using pyridine and formic or acetic acid as solvents or in cellulose are common and reliable methods for betacyanin detection,[380] because they migrate first as immobile zwitterions (pH 2), followed as monoanions (pH 2 to 3.5), and finally as *bis*-anions (pH 3.5, 7.0). In the case of betaxanthins, the mobility may be related to indicaxanthin, and betacyanins are related with the mobility of betanin.[374] Electrophoresis can be carried out using pyridine-citric acid solvent, voltage gradient of 5.6 volts/cm, and a temperature of 4ºC.[496] Recently, capillary zone electrophoresis (CZE) has been used for the analysis of betalains, particularly from *Beta vulgaris*.[452] This technique was carried out with a fused-silica capillary at 15ºC and at a constant voltage of -22 kV, and it has permitted the separation of betanin, isobetanin, and their corresponding aglycones. CZE has been used successfully for the separation and characterization of betalains; however, time analysis is longer, but it could be shorten if separation is focused on the two major red pigments, leaving the aglycones unseparated. Betaxanthins can also be quantitated by CZE with an acceptable resolution.

By contrast, thin layer chromatography (TLC) is not widely used for betalains because its low Rf values; however, Bilyk[41] developed a preparative TLC system in a 0.5-mm cellulose-coated plate using two different mobile phases: isopropanol-ethanol-water-acetic acid in a ratio of 6:7:6:1 (v/v) in the first solvent mixture and a 11:4:4:1 (v/v) ratio in the second one. When acid is incorporated in the developing solvent, betalain mobility on the TLC plate is facilitated due to protonation of the betacyanin carboxyl group. The acid anion provides an electrically neutral system by its interaction with the quaternary nitrogen. The same effect occurs with betaxanthins.[41] Good betaxanthin separations was also obtained on diethylaminoethyl cellulose plates using isopropanol-water-acetic acid (13:4:1 v/v).[451] No indicator is needed for visualization of the separated pigments because they are clearly observed on the TLC plate with their natural colors.

**High-performance liquid chromatography (HPLC).** The HPLC technique has become the method of choice for chromatographic separation, rapid quantification, and tentative identification of betalains. The first application was done by Vicent and Scholz[488] using a $C_{18}$ column with a gradient run using tetrabutylammonium in paired ion system as the mobile phase. The most useful column supports are $C_8$ and $C_{18}$ reversed phase (e.g., Nucleosil, LiChrosorb, μBondpack, etc.), with particle sizes between 3 to 10 μm, while the most used solvents are water-methanol or water-acetonitrile mixtures, acidified with acetic, formic, or phosphoric acid.[451] HPLC elution order of pure crystalline pigments was as follows: betanin, betanidin, isobetanin and isobetanidin.[432] This evidence was based on an acid hydrolysis of the glycosides to yield aglycones and isomerization of betanin to isobetanin occurring.[488]

More recently, Pourrat et al.[379] analyzed a fermented red-beet root extract using a reversed-phase $C_{18}$ column and ion-pairing and methanol-water as mobile phase, and the elution order was

**TABLE 9**
**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 | Moderate mobility | None |
| · *n*-butanol-acetic acid-water (BAW) | Low/intermediate mobility | High mobility |
| · Aqueous solvents | | |
| Column chromatography | Elution with water | Elution with methanol/HCl mixtures |
| · cationic resins | | |

Adapted from Refs. 375, 451.



betanin, isobetanin, betanidin, isobetanidin, and prebetanin for the betacyanins and vulgaxanthin I followed by vulgaxanthin II for the betaxanthins. Another good example of betaxanthin characterization was carried out by Trezzini and Zrÿd.[472] Betalamic acid was conjugated with both protein and non-protein amino acids to yield a series of betaxanthins. They described retention characteristics for 15 naturally occurring pigments such as portulaxanthin-I, miraxanthin-II, vulgaxanthin-I, among others. Such products could be used as HPLC standards for unknown pigments.

### *c. Characterization*

**General procedures.** In most cases it is impossible to distinguish between anthocyanins and betalains visually. However, the extract source is an indication for the presence of betalains or anthocyanins because in plants the presence of one is mutually exclusive to the other. It is important to remember that betalains are characteristic pigments in plants members of Caryophyllales. Preliminary tests have been developed to easily distinguish between betacyanins and anthocyanins (Table 9) using the color exhibited at different pHs and their temperature.[451]

**Spectroscopy.** Betalain analysis as that of other colored compounds has been based basically on UV-visible spectroscopy. As a matter of fact, red violet betacyanins absorbs around λmax = 540 nm, while yellow betaxanthins at λmax = 480 nm, and the starting studies of betalain identification were supported in this methodology. In addition, structural modifications of betalains have been followed by UV-visible spectroscopy.[287,288,375] However, in the 1980s spectroscopy showed enormous progress and nowadays chemical characterization must be carried out considering at least HPLC separation and UV-visible, MS, and NMR spectroscopies: rigorous characterization of betanin, lampranthins, cellosianins, neobetanin, among others, was established thanks to these methodologies.[449,451,452]

**Chemical tests.** Chemical methods for the synthesis and degradation of pigments are very important in betalain research. Some of these established methods are well described in more detail by Strack et al.,[451] and they are briefly summarized in this section. A number of color reactions based on changes in pH have been proposed to distinguish between betalains and anthocyanins.

Acid hydrolysis (dilute aqueous HCl) of betanin gave a mixture of both aglycones, betanidin, and its 15*R* epimer isobetanidin; this

mixture is easily separated by chromatographic methods. On the other hand, enzyme-catalyzed hydrolysis produces only betanidin.[374] Moreover, heating by prolonged time produces the cleavage of betanin into betalamic acid and cycloDOPA 5-*O*-glycoside.[371] After alkali fusion, betanidin was split into 4-methylpyridine-2,6-dicarboxylic acid, 5,6-dihydroxy-2,3-dihydroxyindol, and formic acid; together, these fragments revealed the carbon structure of betanidin. Epimerization at $C_{15}$ is also observed by betanidin treatment with diluted alkali or citric acid solution (5% aqueous); in both reactions a betanin-isobetanin ratio of 3:2 was observed, while the isobetanin produced a 2:3 ratio under the same treatment conditions. In addition, alkaline treatment, for example, alkaline hydrolysis during deacylation of gomphrenin-II to gomphrenin-I, a 1:1 betacyanin-isobetacyanin ratio can be obtained.

On the other hand, betaxanthin analyses involve methodologies for amino acid analysis. They are hydrolyzed with 1 N aqueous HCl or 0.6 N ammonia to obtain betalamic acid and free amino acids.[375] The reaction of betanin in ammonia alkaline solution with an excess of amino acids are used in betaxanthin synthesis. This reaction is followed by monitoring the increments of the betaxanthin maximum (absorption at 475 nm) or decrements of betanin maximum at 540 nm. Thus, vulgaxanthin II can be obtained mixing betanin in 0.6 ammonia solution with an excess (10 *M*) of glutamic acid; this base exchange method has also been applied for the synthesis of indicaxanthin, miraxanthin, and other betaxanthins. However, nonnaturally occurring betaxanthins can be obtained with serine, phenylalanine, threonine, and lysine.[472]

Indicaxanthin oxidation with peroxyacetic acid yields L-aspartic acid, a reaction used to demonstrate the 11*S* configuration of this betaxanthin. Betanin has the 15*S* configuration at the dihydropyridine ring, as indicaxanthin was obtained via amino acid exchange from betanin. This is in accord with the isolation of *S*-betalamic acid after the degradation of many betaxanthins with alkali treatment.[451] The formation of neo-derivatives demonstrated the tendency of the dihydropyridine ring to amortize. Thus, treatment of betanidin with diazomethane gives di-*O*-methylneobetaninidin dimethyl ester in good yield (Figure 23), exhibiting an absorption at 403 nm, which can be shifted, with the addition of acid, to 513 nm.[375] Also, neo-derivatives can be obtained from betaxanthins on treatment with diazomethane, whereas esterification with $CH_3OH$-HCl or $CH_3OH$-$BF_3$ affords the normal esters.

**Quantification and pigmenting efficiency.** Nilsson[342] developed a method to measure both red and yellow betalains of *Beta vulgaris* without prior separation of betacyanins and betaxanthins. Their quantitative determination mainly involved spectrophotometry, where the absorbance at the maximum wavelength ($\lambda$) is translated into concentration by means of the appropriate absorptivities. Another method is based on electrophoretic separation of individual pigments followed by the measurement of the color intensity of the separated bands in a densitometer. The result was expressed as peak area in $cm^2$, which was determined with the aid of an integrator after correcting the baseline; thus, the results are translated to concentration comparing them with a betanin standard curve.[496]

A computer-aided determination, based on previously reported absorptivity values, has been performed by Saguy et al.[408] This method uses a nonlinear curve fitting of the spectrum with a predicted function of the individual pigments (e.g., betanin, betalamic acid, vulgaxanthin-I). The proposed procedure is rapid and accurate, avoiding the laborious and time-consuming separation steps. Schwartz and von Elbe[432] developed a method to quantify individual betalains by HPLC using the molar absorptivity of each pigment instead of absorptivity values. This method provides a more accurate determination of the total betalain content.

It must be pointed out that discrepancies between spectrophotometric and HPLC methods have been observed. Differences up to 15% have been reported after an extended heat treatment of betalains. Such differences have been attributed to degradation products or interfering substances formed during processing.[430] Previous fermentation of red beet juice is recommended for betalain quantification because free sugars are degraded and betalain content of the

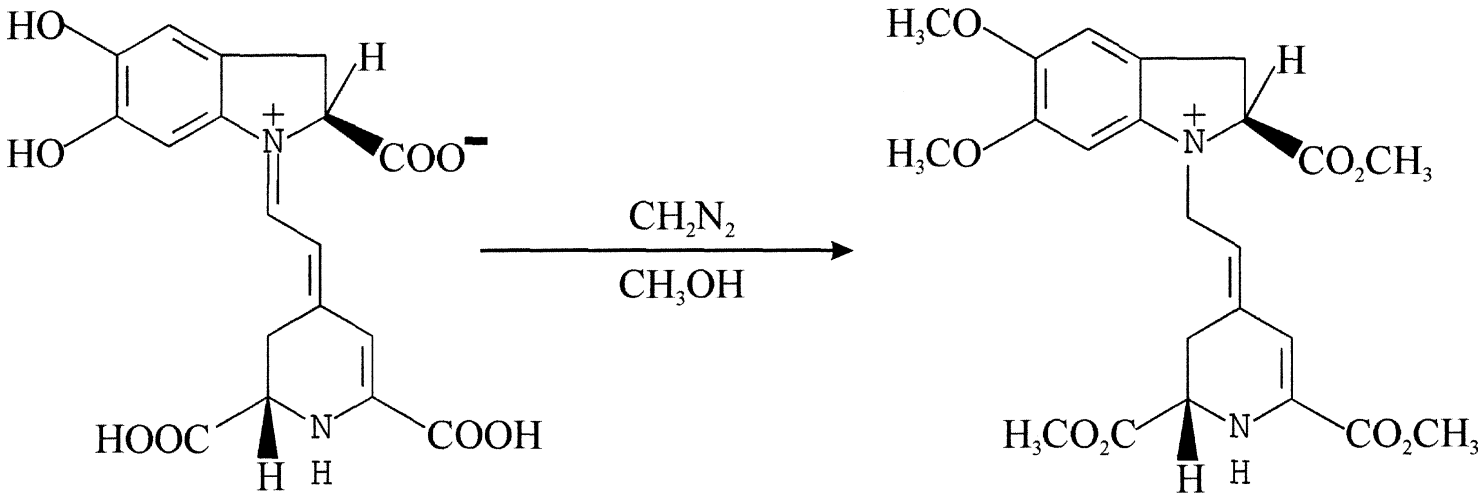


**FIGURE 23.** Aromatization of dihydropyridine ring to form a neoderivated. (Adapted from Ref. 451.)

extract is enriched; thus, HPLC is more easily performed.[379]

Interestingly, it has been shown that betalain quantitation by capillary zone electrophoresis is in close agreement with the HPLC determination.[452]

On the other hand, pigment efficiency is usually measured in terms of CIELAB parameters. It means that triestimulus colorimetry is the best methodology to carry out such measurements.[158,230] Values provided by universal colorimeters are described in previous sections ("L", "a", and "b").

Sapers and Hornstein[416] reported the Hunter color values for standardized dilutions of juice from 48 red beet cultivars. "a" values varied over a relatively narrow range being slightly lower in samples with higher "b" values. The analyses of the 20 most highly pigmented cultivars produced values in the range 8.5 to 17.2. Interestingly, it was reported that "b" values could be used in the estimation of the betaxanthin-betacyanin.

## 7. Importance as Food Colors — Stability, Processing, and Production

### a. Stability in Model Systems

When betalains are used as food colorants, color stability is a major concern. There are several factors that have been recognized to affect the stability of these pigments:

*pH*. The hue of betalains is unaffected at the pH between 3.5 to 7; the values of most foods are in this range. Betalain solutions in this pH range showed a similar visible for betacyanins and betaxanthins. Betacyanin maximum is in the wavelength (λ) range 537 to 538 nm, while betaxanthin maximum is between 475 to 477.497 Below pH 3.5, λ shifts toward a lower wavelength, and above 7 the change is toward a longer wavelength; out of the pH range 3.5 to 7 the intensity of the visible spectra decreases.[225]

Stability of betanin solutions is pH dependent. Huang and von Elbe[225,226] have shown that optimal pH for maximum betanin stability in the presence of oxygen is between 5.5 to 5.8. Red beet solutions showed their maximum stability at pH 5.5, the normal pH for beets. In addition, vulgaxanthin I was most stable between pH 5.0 to 6.0, and it was more stable in juice than in purified extracts, while optimal pigment stability in reconstituted powders was noted at pH 5.7.[440]

**Temperature.** Temperature also shows a clear effect on betalain stability.[129] Thermal kinetic degradation of betanin has been evaluated by several authors.[4,129,225,226,408,495] It has been reported that thermostability of betanin solutions is pH dependent and partially reversible. Heating of betanin solutions produces a gradual reduction of red color, and eventually the appearance of a light brown color. von Elbe et al.[495] observed a first-order reaction kinetics for betanin degradation by heating.

Saguy et al.[408] developed a thermal kinetic degradation model using a nonlinear least-square technique; this model enables one to predict the retention of betalains under variable conditions of temperature and time. Thermal degradation of betalains produced activation energies $E_A$) in the range 17 to 21 Kcal·mol$^{-1}$ for the forward reaction, whereas the reverse reaction showed values between 0.6 to 3.5.[225,408] It was also reported that $E_A$ values showed a pH dependence. Betalamic acid and cycloDOPA-5-*O*-glycoside have been reported as the probable intermediates for betanin degradation.[226,408] Interestingly, the regeneration (reverse reaction) involves a Schiff's base condensation of the amine group of cycloDOPA-5-*O*-glycoside with the aldehyde group of betalamic acid; betanin is rapidly formed when both compounds are mixed in solution.[226] Altamirano et al.[4] reported the lowest stability of betanin and the lowest $E_A$ in a water-ethanol model system, supporting the idea that the first step of the thermal betanin degradation is the nucleophilic attack on the >N+ = CH- structure of betanin. Ethanol has a high electron density on the oxygen atom; therefore, it is a strong nucleophilic agent that diminishes the betanin stability.

**Light.** Von Elbe et al.[495] found that rate of betanin degradation increased 15.6% after pigment daylight exposure at 15ºC. Degradation of light-exposed betalains followed a first-order kinetic. In addition, it was observed that degradation was higher at pH 3.0 ($k$ = 0.35 days$^{-1}$) than at pH 5.0 ($k$ = 0.11 days$^{-1}$), when betacyanins were exposed to fluorescent light. On the other hand, at dark-

ness conditions betacyanins were most stable ($k$ = 0.07 days$^{-1}$).[416] Attoe and von Elbe[16] showed an inverse relationship between betalain stability and light intensity in the range 2200 to 4400 lux). It is explained that visible light absorption excites π electrons of the pigment chromophore to a more energetic state (π*). This would cause a higher reactivity or a lowered activation energy for the molecule ($E_A$ = 25 Kcal·mol$^{-1}$ in darkness and 19.2 in illumination). The effect of UV and gamma irradiation on betanin stability was reported by Aurstad and Dahle;[17] total pigment destruction was reported by the treatments with 120 h of UV radiation or with 100 krad of gamma radiation. Nevertheless, these results, the photodegradation mechanisms for betalains remain to be determined.

**Water activity.** Recognizing the importance of water in many degradation reactions, it is not surprising that water activity ($a_w$) is included among the primary factors affecting the betalain stability and/or color of a food product containing these pigments.[494] Because the degradation reaction does involve water, the greatest stability of betalains has been reported in foods or model systems of low moisture and $a_w$.[82] Pigment degradation follows first-order kinetics, and stability increases with decreasing $a_w$.[407] It has been established that $a_w$ has a pronounced exponential effect on pigment stability. Pigment stability decreases in one order of magnitude when $a_w$ was increased from 0.32 to 0.75.[82]

On the other hand, Simon et al.[439] studied the influence of $a_w$ on the stability of betanin in various water-alcohol model systems. In all cases, it was observed a rate-constant dependence with $a_w$.[439,494] The increase in stability of betanin with decreasing $a_w$ may be attributable to reduced mobility of reactants or limited oxygen solubility.

Consequently, high moisture content produces a high degradation rate. Furthermore, specification of $a_w$ alone without the moisture content is not enough to predict pigment stability.

**Oxygen.** Oxygen causes a product darkening and loss of color. Von Elbe et al.[495] stored buffered betanin solutions at pH 7 under atmosphere of air and nitrogen for 6 days at 15ºC; it was observed that color degradation increases up to 15% due to air conditions. Betanin reacts with molecular oxygen, producing pigment degradation in air-saturated solutions.[15] Degradation kinetic under air atmosphere follows a first-order model, but deviates from first-order in the absence of oxygen. As mentioned above betanin degradation is a partially reversible reaction,[15] and it has been reported that in order to increase the recovering of pigment it is necessary to have the samples under low levels of oxygen. Thus, heated betanin solutions (pH 4.75, 130 min, 15ºC) under low oxygen levels showed an increased betanin retention from 54 to 92%.[225] Reaction reversibility was responsible for the deviation from the first-order degradation kinetics of betanin in the absence of oxygen.

Several methods have been reported to prevent the destruction or to improve the stability of pigments, including degassing, addition of antioxidants and stabilizers, control of pH, minimal heath treatment, among others,[5,15,40,190,363] and these efforts have been directed to their application in food products.

### *b. Processing and Stability in Foods*

The sensitivity of betalains to different factors suggests that their application as food colorants is limited. Based on these properties, betalains can be used in foods with a short shelf-life, produced by a minimum heat treatment, and packaged and marketed in a dry state under reduced levels of light, oxygen, and humidity.[390,497]

Betalains have several applications in foods, such as gelatins desserts, confectioneries, dry mixes, poultry, dairy, and meat products.[87,497] Table 10 summarizes some applications of betalain pigments in food products. The amount of pure pigment required in these foods groups to obtain the desired hue is relatively small and for most applications does not exceed 50 ppm of betalains, calculated as betanin. Problems associated with betalain degradation and pigment recovery during the processing operations are of economic importance and must be solved to betalains displace the application of synthetic dyes in some food products. The effectiveness of commercial betalains depends largely on a continuous availability of highly pigmented sources, the use of cold and modified storage atmospheres prior to

**TABLE 10**
**Applications of Beet Root Powder as Natural Color in Food Products**

| Food products | Shade | Level |
|---|---|---|
| *Dairy products*: | | |
| · Strawberry yogurt | Rose–pink | 0.09% |
| · Ice creams | Pink | 0.25% |
| | Rose–pink | 0.20% |
| *Meat products:* | | |
| · Sausages | Pink | 600 mg/100 g |
| · Cooked ham | Pink–brown | 0.17% |
| Dry powder beverages | Strawberry | 1.2% |
| | Raspberry | 1.5% |
| | Blackcurrant | 1.0% |
| Water ices | Strawberry–red | 0.5 to 1.0% |
| | Raspberry | 0.5 to 1.0% |
| Marzipan | Pastel–red | 0.4% |
| | Bluish–red | 2 mg/cm$^2$ |
| Baked goods | Pink–brown | 2.5% |
| Biscuit creams | Pink | 0.28% |
| | Brown | 1.6% |
| Hard candies | Pink | 0.1% |
| Jellies | Raspberry–red | 0.2% |
| *Fruit cocktails* | Raspberry–red | 2.0% |

Adapted from Ref. 87.



processing, efficient enzymatic control, handling practices, extraction procedures, purification, concentration, and finishing operations e.g., freeze, spray, and vacuum drying).

Nowadays, beet roots represent the main commercial source of betalains (concentrates or powders).[382] Many factors during the pre- and postharvest period and during processing influence the recovery of these natural beet colorants. In addition, recent efforts are centered around the betalain content in red beets through selective breeding. Initially, high pigment content is very important. The average pigment content of beets is approximately 130 mg/100 g fresh weight,[416,496,497] but new red beet varieties produce around 450 to 500 mg/100 g fresh weight. Furthermore, this value is increasing as advanced selection is developed.[382]

Commercial preparations of beet pigment for use as food colorants are available as either juice concentrates (produced by concentrating juice under vacuum to 60 to 65% total solids) or powders (produced by freeze or spray drying). These preparations contain from 0.3 to 1% of pigment.[42,76,77,497] They show a variety of colors, depending on their content of yellow pigments, and may have a beet-like odor and flavor. The remainder of the solids is mainly sugars (75 to 80%), ash (8 to 10%), and protein (10%). On a laboratory scale, betalains can be obtained by employing reverse osmosis,[276] ultrafiltration,[30,391,392] solid -liquid extraction,[275,505] and dif-

fusion.[504] These processes have been shown to be efficient on the recovery of betalains from raw beet tissue when compared with conventional hydraulic techniques.[391] As approximately 80% of beet juice solids consist of fermentable carbohydrates and nitrogenous compounds, a fermentation process to remove these materials has been widely employed.[128] The yeast *Candida utilis* and *Saccharomyces cerevisiae* have been used in the fermentative process, whereas a strain of *Aspergillus niger* not only destroyed free sugars but also enriched the colorant in betanin.[379] The powder obtained from fermented juice contained five to seven times as much as the betacyanin obtained in the powder from raw juice (on a dry weight basis).

### c. Production of Betalains by Plant Tissue Culture

Cell tissue culture has been a very useful tool in the study of various aspects of biochemistry, enzymology, genetics, and biosynthesis of betalains,[273] and, interestingly, betalain production by plant cell culture will represent an excellent option in the future; it has a number of advantages over conventional procedures. Mainly with this methodology, it is possible to control quality and availability of pigments independently of environmental changes.[126] Nevertheless, the productivity of the bioreactor systems must be increased over 0.168 mg/g dry weight/day[240,446] and the cost reduced below $0.15 U.S. dollars/l in order to be considered economically feasible.[241] Thus, a successful betalain production will depend on process optimization to maximize yields, and, consequently, suitable downstream recovery techniques must be available.[382]

Betalain production has been detected in cell cultures of plant species belonging to five families of Caryophyllales.[46] Betalain accumulation of betalains in beet callus culture was reported by Constabel and Nassif-Makki[85] and also has been demonstrated in cell cultures of *P. grandiflora*,[137] *A. tricolor*,[37] *O. microdasys*,[239] *Ch. rubrum*,[35] and *P. americana*.[212] Plant cell cultures are generally deep-red or purple colored; it means that betacyanins are dominant over betaxanthins. Some models do not produce betaxanthins. It has been also established that main betacyanin in most of the studied models is betanin. Schwitzguébel et al.[434] observed that individual *Beta vulgaris* callus cultures contained cells exhibiting a variety of colors either nonpigmented, yellow, orange, red, or purple. The range of observed pigmentation was due to the presence of the deep red-purple betacyanins and to the yellow betaxanthins contained within the cell vacuole.[171]

Recently, plant hairy roots have become of interest as an alternative for cell culture because their infinite and active proliferation in a phytohormone-free medium and their ability to synthesize and accumulate valuable betalains at comparable levels to those found in plants.[459] Extracellular production of betalains accompanied by pigment release has been obtained in hairy root cultures under oxygen starvation.[255] However, both plant cell and hairy root cultures are influenced by a variety of physical and chemical factors implicated in the production of betalain pigments, and some of them, such as growth regulators, light, nitrogen, carbon, and microelements, are widely discussed by Böhm and Rink[46] and Leathers, et al.[273]

In addition, it is important to consider that betalain production by cell or hairy root cultures are mainly empirical, and it is not supported by a strong knowledge of the underlying mechanisms of biosynthesis and regulation. Nevertheless, in some instances, such as in the production of *B. vulgaris* betalains, it is possible to obtain cultures producing specific pigments in comparable or even larger quantities than in the tissues of the original plant.[446]

Recently, Hempel and Böhm[207] administrated nine L-amino acids to hairy root cultures of *Beta vulgaris* var. Lutea. Two betaxanthins, portulaxanthin II and vulgaxanthin I, were produced predominantly, while minor quantities of muscaauri-VII, dopaxanthin, and indicaxanthin were synthesized *de novo*. These results are very important because the possibility of betaxanthin production at commercial level is opened, and, interestingly, red beet is one of the best known betalain production models, being easiest the methodology standardiza-

tion. Nowadays, the selection of a bioreactor and cultivation techniques for optimal culture growth and betalain production is one of the most important issues to be solved.[241] Much data are now available about the growth and production kinetics in suspension cultures;[273] however, most of them are not suitable for the design of large-scale processes, and the growth and production kinetics must be studied under well-defined conditions and at different steady states using the type of bioreactor selected for the large-scale process.[241]

## V. FUTURE TRENDS

Since the 1960s people have shown a clear preference for natural products, including pigments, because more nutritious and healthy characteristics have been associated with them. Remarkably, food scientists consider that this consumer trend will be maintained in the future. In this sense, food technologists will continue affronting the problems of availability and stability of natural pigments in order to replace the synthetic ones. Moreover, one of the main problems to be solved, that natural colors approved by FDA and European Union do not cover all ranges of colors (e.g., blue). Thus, many research groups are looking for new sources of natural pigments; however, these efforts have vanished, because under the current legislation the FDA or the European Union approval of new natural sources of pigments is very difficult, contrasting with politics followed in Japan. Consequently, it is expected that the world global market must contribute to the implementation of more realistic laws. On the other hand, it has been clearly established that technology development has introduced new methodologies or processes to avoid the intrinsic instability and solubility problems of natural pigments, and today most of these problems can be solved through technological processes. In addition, the pigment research area is very wide, and natural pigments with improved characteristics (new colors and improved stability) have been discovered, being that their application differed by the above-mentioned reasons. Thus, a more adequate and updated legislation will provide a broad range of natural colors, and importantly with better stability characteristics.

It is convenient now to point out that plant pigment production is limited, being that pigments are secondary metabolites. Furthermore, agriculture must continue focusing on food production for a growing population, and, undoubtedly, food color does not matter for millions of people all over the world as much as basic food production. However, the demand for better natural-colored foods by an important sector of the society will be increased because it is highly likely that future studies will increase people's conscience about the positive health benefits of natural pigment consumption. Consequently, better production systems for natural pigments will be required higher productivities in smaller areas). Then, research interests will evolve, and two areas will be the future of natural pigment production: the generation of crops with improved characteristics, and pigment production at the industrial level and under controlled conditions. An interesting goal of food research will continue being the production of highly consumed crops (e.g., rice, corn, wheat, oat, bean) with improved chemical composition, plus better functional performance, and at the same time favoring their nutraceutical properties, and genetic-engineered plants with higher productivity and with modified biosynthetic pathways as well. In order to produce such crops, the understanding of the involved metabolic pathways must be wider and deeper. In this sense, more studies must be carried out to have a complete vision of the biosynthesis and regulation of carotenoid and betalain plant production, while nowadays those performed on the anthocyanin biosynthetic pathway will continue focusing on regulatory aspects. Remarkably, the most impressive advances in these aspects have been reached using molecular biology techniques, and this will be so in the near future.

Interestingly, model systems for pigment production under controlled conditions are now available, but production at the industrial level has not been feasible yet. Thus, carotenoid production by yeasts, bacteria and fungi, and anthocyanin and betalain production by plant tissue cultures require the development of better biotechnological approaches.

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