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# Pigments from natural sources: An overview

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**Abstract**
Colorants are mainly used to impart a distinctive appearance to each step of our lives. They are present everywhere in the nature. In mainly edible things like vegetables, fruits and animals also. Pigments can also be prepared synthetically in the laboratory. Both natural and synthetic pigments are used in food, cosmetics and pharmaceutical products to impart their color as well as pharmacological effect exerted by them. Synthetic pigments getting less popular due to their health concerns. Here to use natural pigments it become very important to know its every aspect. Here we have selected four major pigments obtained from natural edible sources and tried to summarize them like chlorophyll, carotenoids, anthocyanins and betalains etc. They can also be obtained from sources other than plants like bacteria and algae. They are having multiple uses along with their coloring properties. They can be extracted and separated. So the present review reflects distribution, functions, extraction, separation and other special concerns of above said pigments.

**Keywords:** Pigments; Chlorophyll; Betalains; Carotenoids and Anthocyanin

## 1. 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 [1].

Additionally, since time immemorial human beings have associated product qualities with their colors, this is especially true for meals[2]. 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[3].

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. It would be impossible to summarize all aspects of natural pigments in a single document. Here the work is focused on the more representative pigments in the main natural producers, namely, plants, which in addition are the more highly consumed as food products.

### 1.1 Pigments

#### 1.1.1 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.

#### 1.1.2 Classification

##### 1.1.2.1 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 [1].

##### 1.1.2.2 By the Chemical Structure

Chromophore Chlorophyll, carotenoids, flavonoids, betalains, and miscellaneous pigments.

##### 1.1.2.3 According to FDA

###### 1.1.2.3.1 Certified

These are manmade and subdivided as synthetic pigments and lakes.

#### 1.1.2.3.2 Exempt from certification

This group includes pigments derived from natural sources such as vegetables, minerals, or animals, and manmade counterparts of natural derivatives [4].

## 2. Natural Pigments

### 2.1 Chlorophylls

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. 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 [5].

Two of them, chlorophyll a and chlorophyll b, are of particular interest in food coloration because they are common in green plant tissues, in which they are present in the approximate ratio 3:1, respectively. As food pigments, chlorophylls impart their green color to many leafy (spinach, lettuce, etc.) and nonleafy (green beans and peas, asparagus, etc.) vegetables and to unripe fruits.

### 2.2 Carotenoids

Carotenoids are compounds comprised of eight isoprenoid units (ip) whose order is inverted at the molecule center (Figure 1).

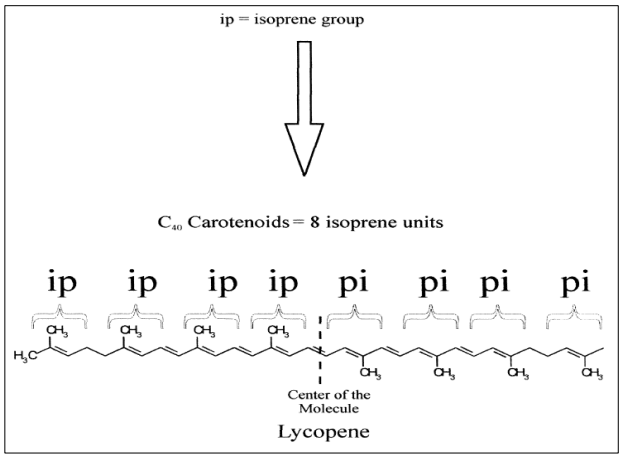


**Fig 1:** Carotenoid structure [6]

All carotenoids canbe considered as lycopene ($C_{40}H_{56}$) derivatives by reactions involving[6]:

(1) Hydrogenation
(2) Dehydrogenation
(3) Cyclization
(4) Oxygen insertion
(5) Double bond migration
(6) Methyl migration,
(7) Chain elongation
(8) Chain shortening

#### 2.2.1 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 [7].

#### 2.2.2 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 [1, 6, 8, 9].

#### 2.2.2.1 Higher Plants

Carotenoids are accumulated in chloroplasts of all green plants as a mixture of α- and β-carotene, β-cryptoxanthin, lutein, zeaxanthin, violaxanthin, and neoxanthin. These pigments are found as complexes formed by a non-covalent bonding with proteins. 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 [8, 10, 11].

#### 2.2.2.2 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. Eutreptielanonehas been found in Euglenophyta. The principal carotenoids in Chloromonadophytaarediadinoxanthin, heteroxanthin, and vaucheriaxanthin [8, 10].

#### 2.2.2.3 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 methoxygroups; (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, allenicoracetylenic bonds, epoxydes, furanoxides C45 or C50 carotenoids [8].

#### 2.2.2.4 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,

plectaniaxanthinin Ascomycetes and canth-axanthin in Cantharellus cinnabarinus has been found [8].

### 2.2.3 Functions

#### 2.2.3.1 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 [12].

#### 2.2.3.2 Photosynthesis

Carotenoids have two well-known functions in photosynthesis: (1) accessory pigments in light harvesting, and (2) as photo protectors against oxidative damages. One of the carotenoid structural characteristics is their ability to absorb visible light: p delocalized electrons suffer a photo induced transformation in which a singlet state (s2) is produced, then energy is efficiently transferred to chlorophyll (chl) to form singlet chl with a slightly higher energy. 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: ferrodoxin oxidoreductase. 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 [13]. 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[14]. 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 xanthophyllsare bound to the remaining chl "α" and "β" molecules in the energy-harvesting antenna [6, 15-19]. Carotenoid functions are greatly determined by their associated proteins. These proteins are mainly membranal, usually hydrophobic, which bound carotenoids by noncovalent bonds [20].

A cDNA of protein that bound fucoxanthin-chl was isolated from Heterosigmacarterae. 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 [21].

#### 2.2.3.3 Xanthophyll Cycle

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 [15, 19].

Xanthophyll cycle involving violaxanthin, antheraxanthin, and zeaxanthin is ubiquitous of higher plants and green and brown algae. In Dunaliellasalina Teod. and Dunaliellabardawil 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. It was explained that in stressed cells high levels of xanthophylls are maintained, which work as an adaptative function to protect the photosynthetic apparatus [22].

#### 2.2.3.4. Antioxidant

In vivo and in vitro studies have shown that carotenoid photo protective 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 [23-25]. 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 [26]. 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 [27]. Miller etal. evaluated carotenoid antioxidant activity against radicals and established the following order of activities decreasing): lycopene > β-cryptoxanthin> lutein = zeaxanthin> β-carotene >echineone>canthaxanthin = astaxanthin. Lycopene showed three times more activity than β-tocopherol, and it was concluded that antioxidant activities are influenced by polarities that are increased with the presence of functional groups in terminal rings [28].

#### 2.2.3.5. 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. it has been determined that carotenoids have a remarkable effect in the immune response and in intercellular communication [16, 29-31]. 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 [30]. Siefer *et al.* showed that β-carotene affects the immune response in rats, and by this means tumor growth is inhibited [32]. 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[33]. 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 urticarial Hydroavacciforme, Porphyriavariegata, Porphyria cutaneatarda, or actinic reticuloid[24]. Lutein and zeaxanthin have been considered as protective agents against aging macular degeneration and senile cataracts [34]. Also, it has been suggested that β-carotene suppress the increment of hormones related to stress syndrome [35].

Certainly, up to date studies on anti-carcinogenic activity have produced unexpected results. Massive studies the β-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 [30].

### 2.2.4. Methodological Aspects

#### 2.2.4.1. Extraction

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. The extraction process consists of the removal of hydrophobic carotenoids from a 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[36]. 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[37].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) [38]. 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 [39, 40].

Supercritical fluid extraction (SFE) is the other alternative method for extraction. 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 [41-43]. SFE has been used commercially to obtain caffeine from coffee and hop oil[43]. 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 [44].

#### 2.2.4.2. Saponification

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 [45, 46]. When carotenoids are sensitive (e.g., astaxanthin and fucoxanthin), alternatively lipases have been used [47]. 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 [48].

#### 2.2.4.3 Separation

Separation methods can be classified as non-chromatographic and chromatographic. Non chromatographic 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. 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[47]. Nowadays, open column chromatography is used as a prepurification stage to separate groups of carotenoids with similar characteristics in special flash open column chromatography [49, 50].

High-performance liquid chromatography (HPLC) is the preferred column chromatography to carry out the qualitative and quantitative analyses of carotenoids. 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 [51-53].

#### 2.2.4.4. Characterization

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[6]. 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) [54, 55]. 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) [53].

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 [56]. RAMAN spectroscopy and circular dichroism have permitted the study of carotenoids in biological systems [54, 57]. 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), semi quantitative information on total pigment composition was obtained [58].

#### 2.2.4.5 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 formonoepoxydes and 40 nm for diepoxydes. Allylalcohols treated with HCl are dehydrated, and another double bond is formed with a consequent change in their UV visible spectra. 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) [6, 36].

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 [59]. 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, and (2) the use of photoelectric instruments that measure reflectance. Hunter-Lab is the most common equipment; it is based on tristimulus effect [60, 61].

### 2.3. Anthocyanins

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 2). Anthocyanins are substituted glycosides of salts of phenyl-2-benzopyrilium (anthocyanidins).

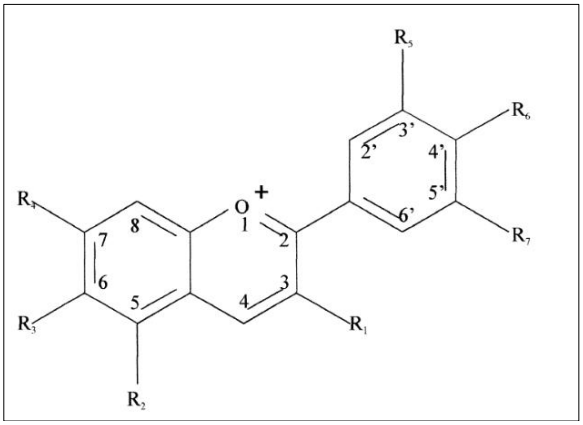


**Fig 2:** Basic structure of anthocyanidin pigments in which Rx could be H, OH, or $OCH_3$ depending of the considered pigment. The most common accepted nomenclature for numbering carbons is indicated inside the structure [62, 63].

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 and by its interaction withother molecules and/or media conditions [64]. Harborne and Gryer 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 1:** Anthocyanidins Found in Nature

| Substituted with a characteristic hydroxyl group | | |
|---|---|---|
| **Name** | **Position of substitution** | **Some of the produced colors** |
| 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 |

From these 17 structures combinations have arisen with at least one sugar 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., acylatedanthocyanins), and in nature the most common acyl groups are coumaric, caffeic, ferulic, p-hydroxy benzoic, synapic, malonic, acetic, succinic, oxalic, and malic [65].

**2.3.1. Distribution**

Anthocyanins are responsible for many of the attractive colors, from scarlet to blue, of flowers, fruits, leaves, and storage organs [66, 67]. 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. The type of anthocyanins in plantsis 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) [63].

**2.3.2. Functions**

**2.3.2.1. Color and Ecological Functions**

Anthocyanins also showed similar functions in plants to those described 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 Heliothisviriscens, 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 [66, 67].

2.3.2.2. Marker for Good Manufacturing

Practices in Food Processing Anthocyanins have been used to evaluate the adulteration of some pigmented food products [68]. 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[69]. Also, anthocyanin profiles have been used to determine the authenticity of fruit jams. With this kind of analyses, it was determined that labeled blackcherry 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 [70].

**2.3.2.3 Pharmacological Effects**

Anthocyanins possess 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[71]. 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 [72]. 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 ·OH and $O^{2-}$. In addition, it was mentioned that ·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 [73]. 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 [74].

### 2.3.3. Methodological Aspects

#### 2.3.3.1. 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[66]. 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 [63, 65, 67, 71].

#### 2.3.3.2. Separation

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[65]. However, undoubtedly, the main developments of recent years in the research of anthocyanins is the introduction of HPLC for their separation and quantitation. Interestingly, it is possible to distinguish zwitterionic anthocyanins by their HPLC chromatographic separation [67].

#### 2.3.3.3 Characterization

In general, color is evaluated by spectrophotometry [65]. 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 UV-visible spectroscopy, it is also possible to detect glycosylation on B-ring, because of the spectra show a 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) [75]. 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 [64, 67, 76]. Raman spectroscopy has been used to show the anthocyanin substitution pattern. The presence of phenyl ring substitutions on benzopyrylium produces clear spectral modifications [76, 77].

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. B-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[78]. 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 [79].

#### 2.3.3.4. Chemical tests

A large number of chemical tests have been developed to determine the anthocyanin structure, and a general procedure could be envisioned, but this must be modified depending on the analyzed material [66]. 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 [63]. When anthocyanin molecules show aliphatic acids in their structures, a zwitterion is observed that can be detected by electrophoresis. The presence of B-ring-substituted sugars is easily detected by a hypsochromic spectral shift [65]. 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 [64, 65]. 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 ($r = 0.73$) [80].

## 2.4. Betalains

The term “betalains” was introduced by Mabry and Dreiding; this was supported by structural and biogenetic considerations. Originally, betalains were called “caryophyllinenroth” and successively renamed “rübenroth” and “chromoalkaloids”. Chemically, betalain definition embraces all compounds with structures based on the general formula shown in Figure; therefore, they are immonium derivatives of betalamic acid. The chromophore of betalains

can be described as a protonated 1, 2, 4, 7, 7-pentasubstitued 1,7-diazaheptamethin system [81-83].

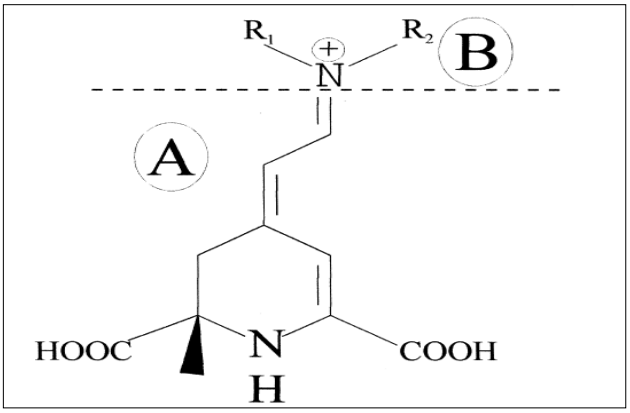


**Fig 3:** Betalain general formula. (A) Betalamic acid moiety is present in all betalain molecules. (B) The structure will represent a betacyanin or a betaxanthin, depending on the identity of the $R_1$ and $R_2$ residues.

**2.4.1. 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 [84]. 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.

**2.4.2. Distribution**

Among higher plants the occurrence of betalains is restricted to the Caryophyllales and those found in certain higher fungi such as Amanita, Hygrocybe, and Hygrosporus [85]. Betalains of higher plants are in different organs. They produce red, yellow, pink, and orange colors in Aizoaceae and Portulacaceae flowers, and purple pigmentation in Cactaceae fruits and in red-beet root (Chenopodiaceae) [82, 86]. Betalains are in bracts, for example, Bougainvillea (Nyctagynaceae) possesses a wide range of colors; they are also in seeds of Amaranthus in leaves of Teloxis and in stems[87]. 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. 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, 15R) by glycosidation of one of the hydroxyl groups located at position 5 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. There are about 15 naturally occurring betaxanthins; the indicaxanthin from Opuntia ficus-indica was the first crystallized [82].

**2.4.3. Functions**

**2.4.3.1. 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. The recent addition to the list of betalain families is Didieraceae, a small family from Madagascar [85, 86]. 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. The totally different chemical structure of betalains and anthocyanins, 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 [82].

**2.4.3.2. 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. 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. 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 [82, 88]. 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. 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 [89].

**2.4.3.3. 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. 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 [65]. 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 [82]. 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. 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" [90].

### 2.4.4. Methodological Aspects

#### 2.4.4.1 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 [82]. 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[91]. In both procedures, the inactivation of degradative enzymes by a short heat treatment of the extract (70 ºC, 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 [82].

#### 2.4.4.2. Separation

##### 2.4.4.2.1. Ion-exchange and column chromatography

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 (non-ionic 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 [82].

##### 2.4.4.2.2. Electrophoresis and thin layer chromatography

Paper electrophoresis using pyridine and formic or acetic acid as solvents or in cellulose are common and reliable methods for betacyanin detection, because they migrate first as immobile zwitterions (pH 2), followed as monoanions (pH 2 to 3.5), and finally as bisanions (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 [82]. Electrophoresis can be carried out using pyridine-citric acid solvent, voltage gradient of 5.6 volts/cm, and a temperature of 4 ºC. Recently, capillary zone electrophoresis (CZE) has been used for the analysis of betalains, particularly from Beta vulgaris [92]. This technique was carried out with a fused-silica capillary at 15 ºC 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. Bilyk developed a preparative TLC system in a 0.5-mm cellulose-coated plate using two different mobile phases: isopropanolethanol-water-acetic acid in a ratio of 6:7:6:1 (v/v) in the first solvent mixture and an 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 [93].

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 using a C18 column with a gradient run using tetrabutylammonium in paired ion system as the mobile phase. The most useful column supports are C8 and C18 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. HPLC elution order of pure crystalline pigments was as follows: betanin, betanidin, isobetanin and isobetanidin. This evidence was based on an acid hydrolysis of the glycosides to yield aglycones and isomerisation of betanin to isobetanin occurring [55, 85].

#### 2.4.4.3. Characterization

Preliminary tests have been developed to easily distinguish between betacyanins and anthocyanins using the color exhibited at different pHs and their temperature [85]. 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 $\lambda max$ = 540 nm, while yellow betaxanthins at $\lambda max$ = 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 [82].

#### 2.4.4.4. Chemical tests

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 15R epimer isobetanidin; this mixture is easily separated by chromatographic methods. On the other hand, enzyme-catalyzed hydrolysis produces only betanidin. Moreover, heating by prolonged time produces the cleavage of betanin into betalamic acid and cycloDOPA 5-O-glycoside. 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. 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 [82]. 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 [94].

Their quantitative determination mainly involved spectrophotometry, where the absorbance at the maximum wavelength (λ) 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 [95]. A computer-aided determination, based on previously reported absorptivity values, has been performed by Saguy *et al.* [96]. 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 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. Interestingly, it has been shown that betalain quantitation by capillary zone electrophoresis is in close agreement with the HPLC determination. 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 [92].

## 3. Future trends

Exploration of new sources for dyes can certainly help in increasing the shade range of natural dyes with good coloring properties. However, extensive research on the safety of these materials to humans and the environment would be needed before propagating their usage as everything of natural origin may not be safe. Nature is known to produce poisonous substances also; therefore thorough toxicological evaluations for the new sources are necessary.

## References


1. Hari RK, Patel TR, Martin AM. An overview of pigment production in biological systems: functions, biosynthesis, and applications in food industry, Food Rev. Int., 1994; 10(1):49-70.
2. Clydesdale FM. Colour as a factor in food choice, Crit. Rev. Food Sci. Nutr., 1993; (331):83-101.
3. FDA/IFIC, Regulation of color additives, Food and Drug Administration, FDA/IFIC Brochure, 1993. Wodicka VO., Regulation of food: where have webeen? Food Technol., 1996; 50(3):106-109.
4. FDA. Colour additives: FDA"s regulatory process and historical perspectives. Food and Drug Admin, 2003. www.cfsan.fda.gov/~dms/col-regu.html (accessed April 2016).
5. Britton G. Carotenoids, Method. Plant Biochem., 1991; 7:473-518.
6. Goodwin TW. Biochemistry of the Carotenoids. Plants. 2nd. ed., Chapman and Hall, New York, 1980; 1:1-95.
7. Lichtenhaler HK. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Method.Enzymol., 1987; 148:350-382.
8. Goodwin TW. Biosynthesis of carotenoids: an overview, Method. Enzymol., 1992; (214):330-340.
9. Gordon HT, Bauernfeind JC. Carotenoids as food colorants, Crit. Rev. Food Sci. Nutr., 1982; 18:59-97.
10. Goodwin TW, Britton G. Distribution and analysis of carotenoids. In: Goodwin TW, Ed., Plant Pigments. Academic Press, London, 1988, 62-132.
11. Lichtenhaler HK. Chlorophylls and carotenoids: pigments of photosynthetic biomembranes, Method. Enzymol., 1987; 148:350-382.
12. Ootaki T, Yamazaki Y, Noshita T, Takahashi S. Excess carotenoids disturb prospective cellto-cell recognition system in mating responses of Phycomycesblakesleanus. Mycoscience, 1996; 37:427-435.
13. Scheller HV, Naver H, Møller BL. Molecular aspects of photosystem I, Physiol. Plant., 1997; 100:842-851.
14. Barber J. Nield EP, Zheleva D, Hankamer B. The structure, function and dynamics of photosystem two.Physiol.Plant., 1997; 100:817-827.
15. Armstrong GA, Hearst JE. Genetics and molecular biology of carotenoid pigment biosynthesis, FASEB J., 1996; 10:228-237.
16. Britton G. Structure and properties of carotenoids in relation to function, FASEB J., 1995; 9:1551-1558.
17. Cogdell RJ, Gardiner AT. Functions of carotenoids in photosynthesis, Method. Enzymol., 1992; (214):185-193.
18. Middleton EM, Teramura AH. The role of flavonol glycosides and carotenoids in protecting soybean from ultraviolet-B damage, Plant Physiol., 1993; 103:741-752.
19. Olson JA, Krinsky NI. Introduction. The colourful, fascinating world of the carotenoids: important physiologic modulators, FASEB J., 1995; 9:1547-1550.
20. Gottfried DS, Steffen MA, Boxer SG. Large protein-induced dipoles for a symmetric carotenoid in a photosynthetic antenna complex, Science, 1991; (251):662-665.
21. Durnford DG, Aebersold R, Green BR. The fucoxanthin-chlorophyll proteins from a chromophyte alga are part of a large multigene family: structural and evolutionary relationships to other light harvesting antennae, Mol. Gen. Genet., 1996; 253:377-386.
22. Phillips LG, Cowan AK, Rose PD, Logie MRR. Operation of the xanthophyll cycle in non-Downloaded, 2015; 15:20-14. By: [Cinvestav del Ipn] At.
23. Liebler DC. Antioxidant reactions of carotenoids. In: Canfield LM, Krinsky NI, Dunastable JA. Eds., Carotenoids in Human Health, Annals of the New York Academy of Sciences, New York, 1993; 691:20-31.
24. Mathews-Roth MM. Carotenoids in erythropoietic protoporphyria and other photosensitivity disease. In: Canfield LM, Krinsky NI, Dunastable JA. Eds., Carotenoids in Human Health. Annals of the New York Academy of Sciences, New York, 1993; 691:127-138.
25. Palozza P, Krinsky NI. Antioxidant effects ofcarotenoidsin vivo and in vitro: an overview, Method. Enzymol., 1992; (213):403-420.
26. Haila KM, Lievonen SM, Heinonen MI. Effects of lutein, lycopene, annatto and γ-tocopherolon autoxidation of triglycerides, J. Agric. Food Chem.,1996; 44(8):2096-2100.
27. Chen CW, Shu CK, Ho CT. Photosensitized oxidative reaction of (2,5-dimethyl-4-hydroxy-32H)-furanone, J. Agric. Food Chem., 1996; 448:2361-2365.

28. Miller NJ, Sampson J, Candeias LP, Bramley PM, Rice-Evans CA. Antioxidant activitiesof carotenes and xanthophylls, FEBS Lett., 1996; 384:240-242.
29. Clairmont A, Tessmann D, Sies H. Analysis for connexin 43 gene expression induced by retinoic acid in F9 teratocarcinoma cells, FEBS Lett., 1996; 397:22-24.
30. Charleux JL. Beta-carotene, vitamin C, vitamin E: the protective micronutrients, Nutr. Rev., 1996; 54(11):S109-S114.
31. Hong WK, Sporn MB. Recent advances in chemoprevention of cancer. Science, 1997; 278:1073-1077.
32. Siefer E, Rettura G, Levenson SH. Carotenoids and cell-mediated immune responses. In Charalambous G, Inglett G, Eds., The Quality of Foods and Beverages, Chemistry and Technology,Academic Press, New York, 1981; 2:335-347.
33. Parker RS. Absorption, metabolism, and transport of carotenoids, FASEB J., 1996; 10:542-551.
34. Taylor-Mayne S. Beta-carotene, carotenoids, and disease prevention in humans, FASEB J., 1996; 10:609-701.
35. Hasegawa T. Anti-stress effect of □-carotene. In: Canfield LM, Krinsky NI, Dunastable JA. Eds., Carotenoids in Human Health. Annals of the New York Academy of Sciences, New York, 1993; 691:281-283.
36. De Ritter E, Purcell AE. Carotenoid analytical methods. In: Bauernfeind JC. Ed. Carotenoids as Colorants and Vitamin A Precursors. Academic Press, New York, 1981; 815-882.
37. Rosenthal A, Pyle DL, Niranjan K. Aqueous and enzymatic processes for edible oil extraction, Enz.Microb. Technol., 1996; 19:400-420.
38. Conkerton EJ, Wan PJ, Richard OA. Hexane and heptane as extraction solvents for cotton seed: a laboratory-scale study, J. Am. Oil. Chem. Soc., 1995; 72(8):963-965.
39. Kamal-Eldin A, Appelqvist LA. The effects of extraction methods on sesame oil stability, J Am. Oil. Chem. Soc., 1995; 72(8):967-969.
40. Proctor A, Bowen DJ. Ambient-temperature extraction of rice bran oil with hexane and isopropanol, J Am. Oil. Chem. Soc., 1996; 73(6):811-813.
41. Chester TL, Pinkston JD, Raynie DE. Supercritical fluid chromatography and extraction, Anal. Chem., 1992; 64(12):153-170.
42. Hawthorne SB. Analytical-scale supercritical fluid extraction, Anal. Chem., 1990; 62(11):633A-642A.
43. Randolph TW. Supercritical fluid extractions in biotechnology, Trends Biotechnol., 1990; 8:78-82.
44. Favati F, King JW, Friedrick JP, Eskins K. Supercritical $CO_2$ extraction of carotene and lutein from leaf protein concentrates, J Food Sci., 1988; 53(5):1532-1536.
45. AOAC. Official Methods of Analysis, Association of Official Analytical Chemists. Washington DC, 1980. Britton G, General carotenoid methods, Method.Enzymol., 1985; 111(B):113-149.
46. Khachik F, Beecher GR, Goli MB, Lusby WR. Separation and quantitation of carotenoids in foods, Method.Enzymol., 1992; 213(A):142-167.
47. Britton G. General carotenoid methods, Method.Enzymol., 1985; 111(B):113-149.
48. Ittah Y, Kanner J, Granit R. Hydrolysis study of carotenoid pigments of paprika Capsicum annuum L. variety Lehava by HPLC/photodiode array detection, J. Agric. Food Chem., 1993; 41:899-901.
49. Kull D, Pfander H. Isolation and identification of carotenoids from the petals of rape (Brassica napus), J Agric. Food Chem., 1995; 43(11):2854-2857.
50. Mercadente AZ, Rodriguez-Amaya DB, Britton G. HPLC and mass spectrometric analysis of carotenoids from mango. J Agric. Food Chem., 1997; 45:120-123.
51. Bramley PM. Analysis of carotenoids by high performance liquid chromatography and diode array detection, Phytochemical Anal., 1992; 3:97-104.
52. Schoefs B, Bertrand M, Lemoine Y. Separation of photosynthetic pigments and their precursors by reversed-phase high-performance liquid chromatography using a photodiode-array detector, J.Chromatogr.A, 1995; 692: 239–245.
53. Van Breemen RB. Innovations in carotenoid analysis using LC/MS, Anal. Chem., 1996; 68(9):299A-304A.
54. Haugan JA, Akermann T, Liaaen-Jensen S. Isolation of fucoxanthin and peridinin, Method. Enzymol.1992; 213(A):231-245.
55. Schmitz HH, Van Breemen B, Schwartz SJ. Fast-atom bombardment and continuous-flow fastatom bombardment mass spectrometry in carotenoid analysis, Method. Enzymol., 1992; 213(A):322-336.
56. Kaufmann R, Wingerath T, Kirsch D, Stahl W, Sies H. Analysis of carotenoids and carotenol fatty acid esters by matrix-assisted laser desorption ionization (MALDI) and MALDI-post source decay mass spectrometry, Anal. Chem., 1996; 238:117-128.
57. Seibert M, Picorel R, Kim JH, Cotton TM. Surface enhanced Raman scattering of photosynthetic membranes and complexes, Method. Enzymol.,1992; 213(A):31-42.
58. Vinha CA, Hass U. Qualitative and semi quantitative analysis of dried fruits and seasoning products of paprika using photo acoustic spectroscopy, J. Agric. Food Chem., 1997; 45(4):1273-1277.
59. Isaksen M, Francis GW. Silver ion spray reagent for the discrimination of the β and ε end groups in carotenoids on thin-layer chromatograms, Chromatographia, 1990; 29(7-8):363-365.
60. Belbin AA. Colours in oils. INFORM, 1993; 4(6):648-654.
61. Marusich WL, Bauernfeind JC. Oxicarotenoids in poultry feeds. In: Bauernfeind JC. Ed., Carotenoids as Colorants and Vitamin A Precursors, Academic Press, New York, 1981, 319-462.
62. Counsell JN, Jeffries GS, Knewstubb CJ. Some other natural colours and their applications. In: Counsell JN. and Dunastable JA., Eds., Natural Colors for Foods and Other Uses. Applied Science, London, 1979, 122-151.
63. Swain T, Bate-Smith EC. Flavonoid Compounds. In: Florkin M, Mason HS. Eds., Comparative Biochemistry Constituents of Life Part A, Academic Press, New York, 1962; III:755-809.
64. Brouillard R, Dangles O, Jay M, Biolley JP, Chirol N. Polyphenols and pigmentation in plants. In: Scalbert A. Ed., Polyphenolic Phenomena. Paris: INRA, 1993, 41-47.
65. Francis FJ. Food colorants: anthocyanins, Crit. Rev. Food Sci. Nutr., 1989; 28(4):273-314.

66. Harborne JB. The flavonoids: recent advances. In: Goodwin TW. Ed., Plant Pigments. Academic Press, London, 1988, 298-343.
67. Harborne JB, Grayer RJ. The anthocyanins. In Harborne JB. Ed. The Flavonoids. London: Chapman and Hall Ltd, 1988, 1-20.
68. Boyles MJ, Wrolstad RE. Anthocyanin composition of red raspberry juice: influences of cultivar, processing, and environmental factors, J. Food Sci. 1993; 58:1135-1141.
69. Van Gorsel H, Li C, Kerbel EL, Smits M, Kader AA. Compositional characterization of prune juice. J Agric. Food Chem., 1992; 40:784-789.
70. García-Viguera C, Zafrilla P, Tomás-Barberán FA. Determination of authenticity of fruit jams by HPLC analysis of anthocyanins, J Sci. Food Agric. 1997; 73:207-213.
71. Bridle P, Timberlake CF. Anthocyanins as natural food colour-selected aspects, Food Chem., 1997; 58(1-2):103-109.
72. Wang H, Cao G, Prior RL. Oxygen radical absorbing capacity of anthocyanins, J Agric. Food Chem., 1997; 45: 304-309.
73. Tsuda T, Shiga K, Ohshima K, Kawakishi S, Osawa T. Inhibition of lipid peroxidatin and the active oxygen radical scavenging effect of anthocyanin pigments isolated from Phaseolus vulgaris L, Biochem. Pharmacol., 1996; 52:1033-1039.
74. Lozovik G. Nutraceutical properties of Anonia colorant. In: International Congress and Symposium on Natural Colorants. Puerto de Acapulco, México, 1996.
75. Terahara N, Oda M, Matsui T, Osajima Y, Saito N, Toki K. *et al*. Five new anthocyanins, ternatins, A3, B4, B3, B2, and D2, from Clitoria ternata flowers, J Nat. Prod., 1996; 59:139-144.
76. Brouillard R. The in vivo expression of anthocyanin colour in plants, Phytochemistry, 1983; 22:1311-1323.
77. Merlin JC, Statoua A, Cornard JP, Saidi-Idrissi M, Brouillard R. Resonance RAMAN spectroscopic studies of anthocyanins and anthocyanidins in aqueous solutions, Phytochemistry, 1994; 35:227-232.
78. Pedersen AT, Andersen OM, Aksnes DW, Nerdal W. Anomeric sugar configuration of anthocyanin O-pyranosides determined from heteronuclear one-bond coupling constants, Phytochemical Anal., 1995; 6:313-316.
79. Baldi A, Romani A, Mulinacci N, Vincieri FF, Casetta B. HPLC/MS application to anthocyanins of Vitis vinifera L., J. Agric. Food Chem., 1995; 43:2104-2109.
80. Moore PP. Estimation of anthocyanin concentration from color meter measurements of red raspberry fruit, Hortscience, 1997, 32:135.
81. Mabry TJ, Dreiding AS. The betalaines In: Mabry R, Alston E, Runeckles VC. Eds., Recent Advances in Phytochemistry, Appleton Century Crofts, New York, 1968; 1:145-160.
82. Piatelli M. Betalains. In: Goodwin TW. Ed. Chemistry and Biochemistry of Plant Pigments. Academic Press, New York, 1976; 1:560-596.
83. Reznik H, Betalains. In: Pigments in plants. Czygan F. E., Ed., Akademie Verlag 1981, 370-392.
84. Böhm H, Rink E. Betalains. In: Cell culture and somatic cell genetics of plants. Academic Press, New York, 1988, 449-463.
85. Strack D, Steglich W, Wray V. Betalains. In: Methods in Plant Biochemistry Academic Press, Orlando, 1993; (8):421-450.
86. Rosendal-Jensen S, Juhl-Nielsen B, Dahlgren R. Use of chemistry in plant classification, Rev. Latinoamer. Quím, 1989; 1:66-89.
87. Bianco-Colomas J, Hugues M. Establishment and characterization of a betacyanin producing cell line of Amaranthus tricolor inductive effects of light and cytokinin, J Plant Physiol., 1990; 136:734-739.
88. Weiss MR. Floral color change: a widespread functional convergence, Am. J. Bot., 1995; 82(2): 167–185.
89. Kragh KM, Nielsen JE, Nielsen KK, Dreboldt S, Mikkelsen JD. Characterization and localization of new antifungal cysteine-rich proteins from Beta vulgaris, Mol. Plant-Microbe Inter., 1995; 8(3):424-434.
90. Leathers R, Davin C, Zrÿd J. Betalain producing cell cultures of Beta vulgaris L. red beet), In Vitro Cell Dev. Biol., 1992; 28:39-45
91. Pourrat A, Lejeune B, Grand A, Pourrat H. Betalains assay of fermented red beet root extract by high performance liquid chromatography, J Food Sci., 1988; 53(1):294-295.
92. Stuppner H, Egger R. Application of capillary zone electrophoresis to the analysis of betalains from Beta vulgaris. J Chromatogr A., 1996; 735:409-413.
93. Bilyk A. Thin-layer chromatographic separation of beet pigments. J Food Sci., 1981; 46:298-299.
94. Trezzini GF, Zrÿd JP. Characterization of some natural and semi-synthetic betaxanthins, Phytochemistry, 1991; 30(6):1901-1903.
95. von Elbe JH, Sy SH, Maing IY, Gabelman WH. Quantitative analysis of betacyanins in red table beets (Beta vulgaris), J Food Sci., 1972; 37:932-934.
96. Saguy I, Kopelman IJ, Mizrahi S. Computer aided determination of beet pigments, J. Food Sci., 1978; 43:124-127.