PLANT PIGMENTS: PROPERTIES, ANALYSIS, DEGRADATION

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I. INTRODUCTION

What do children choosing sweets in a shop and their adults selecting fruits in a grocery have in common? Both use the appearance and decide to buy the most colorful items. This very daily example shows that the colors of foods have a strong influence on our perception of acceptability and palatability (Blendford, 1995; Hendry and Houghton, 1996; Newsome, 1990). "Plant pigment" is a generic expression used to designate a large number of colored molecules synthesized by photosynthetic organisms. On the basis of their chemical structure, they can be classified into four families: tetrapyrroles (e.g., chlorophyll), carotenoids (e.g., β-carotene), polyphenolic compounds (e.g., anthocyanins), and alkaloids (e.g., betalains). An example of the chemical structure of a member of each family of compounds is presented in Figure 1. The number of molecules belonging to each family is quite large, so several volumes would be necessary to describe their particular properties. The description of the physicochemical properties of pigments has been the topic of comprehensive reviews and books (Britton, 1988; Britton *et al*., 1995; Gross, 1991; Schoefs, 2002).

Plant pigments have positive roles in human health (Franceschi *et al*., 1994; Gerster, 1993; Groten *et al*., 2000; Kumpulainen and Salonen, 1996; Mayne *et al*., 1994; Shim *et al*., 2003). Although animal tissues contain plant pigments, these tissues are not able to synthesize them. Therefore, the pigments must be obtained from food (Baker, 1992; Castenmiller *et al*., 1999; Depee *et al*., 1998). Once assimilated, pigments enter the biochemical pathways along which they may be eventually modified (Parkinson and Brown, 1981). For instance, β-carotene is split into two parts, one of these serving as a precursor of vitamin A (Goswani and Barua, 2003), an important molecule for vision, skin protection, and cell growth. Several studies have established the minimum daily intake of these valuable pigment molecules. Because consumption of fresh foods has decreased and that of processed foods has increased, food colors have become an important aspect of the pigment food formulation process. Unfortunately, during food

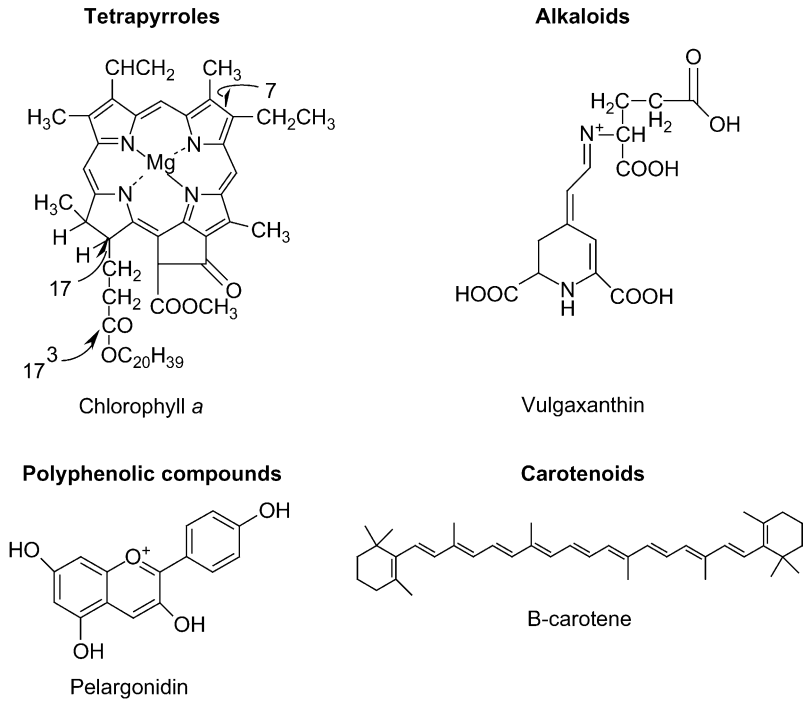


FIG. 1 Example of the chemical structure of a member of each family of pigments synthesized by plants.

processing, the natural pigment content may be altered or destroyed. A very demonstrative example is the color change that occurs during the processing of mangoes or pineapple flesh. In the fresh fruit, the main carotenoid is the diepoxy-carotenoid violaxanthin (yellow), which during processing is transformed to the faint-yellow auroxanthin, a furanoid carotenoid (Mercadante and Rodriguez-Amaya, 1998) (see Figure 4). To restore the natural level in pigments in the processed products, extracted pigments are incorporated into the final food products (Britton, 1995; Burton and Ingold, 1984; Garcia-Viguera *et al*., 2000; Palozza and Krinsky, 1991). Similarly the preparation of fortified products requires the addition of pigments to the products (Smith, 1991). Regardless of the type of final products, the pigments are incorporated either as they naturally occur or as a chemically modified form (e.g., chlorophyllin or glycosylation of crocetin) (Dufresne *et al*., 1999). As a consequence of these additional needs, the demand in natural colorants has increased compared to synthetic dyes (Joppen, 2003; Pszczola, 1988). However, this increase cannot always be satisfied due to

the limited supply of raw materials because the production of pigments using conventional plant cultivation methods is influenced by climatic conditions, plant cultivars, and varieties (Rodriguez-Amaya, 2000). Consequently part of plant pigment research is oriented to finding new sources of pigments. This quest is not only directed toward finding natural alternatives to synthetic dyes but also with the aim to discover new taxons and new procedures to produce pigments, for instance, from cell cultures (Table I). Pigment production by cell or microalgal cultures is especially interesting because these methods can be established independently of the climatic influences. In addition, yields can be predicted (Rodriguez-Amaya, 2000) and eventually improved by applications of elicitors or through genetic engineering (Ye *et al*., 2000).

For all the reasons cited previously, pigment analysis constitutes a real analytical challenge. To take this challenge, one must have a diversified number of analytical procedures able to rapidly and precisely quantify the different pigment levels in samples. Addressing these issues is never straightforward. With some samples, like bottled waters, analysis is very simple.

TABLE I
EXAMPLES OF NEW SOURCES FOR PIGMENTS SHOWING POTENTIAL FOR INDUSTRIAL PRODUCTION

Pigment produced	New sources	References
Anthocyanins	Cacti	Carle and Stintzing (2000); Stintzing *et al.* (2000)
	Sunflower (seed hulls)	Gürenç and Karaali (2000)
	Cell cultures of *Perilla*	Aoki *et al.* (2000); Ravishankar and Venkataraman (1990); Zhong *et al.* (1991)
	Cell culture of *Daucus carota* (carrot)	Ozeki and Komamine (1985)
	Cell culture of *Vitis vinifera* (vine)	Do and Cormier (1990); Hirasuna *et al.* (1991)
Carotenoids	Pumpkin	Chaudry and Stich (2000)
	Green microalgae	Cordevo *et al.* (1996); Kopecky *et al.* (2000); Ravishankar *et al.* (2000); Usha *et al.* (1999)
Betalains	Cell cultures of *Chenopodium rubrum*	Berlin *et al.* (1986)
	Cell cultures of *Phytolacca americana*	Sakuta *et al.* (1987)
	Cell cultures of *Beta vulgaris* (fodder beet)	Ravishankar *et al.* (2000)

Other samples are highly complex mixtures, requiring careful processing and storage conditions to ensure an unchanged level of pigments and color. This is especially important in the case of fortified foods, which create nutritional expectations from both the health authorities and the consumers. The development of powerful analytical methods is also of prime importance in the control of quality. Obviously, this requires a precise knowledge of the pigment composition of original products (Mercadante *et al.*, 1998; Minguez-Mosquera *et al.*, 1995a).

In this contribution, the molecular structures, the general physicochemical properties of plant pigments, and the mechanisms involved in pigment degradation are first described. Then follows a review on invasive and noninvasive methods used for pigment analysis, including the mechanisms involved in pigment degradation. Finally, several examples of analytical procedures are presented.

II. SPECTROSCOPIC, MOLECULAR STRUCTURES, AND CHEMICAL PROPERTIES

A. COMMON PROPERTIES

The absorption of light by pigment molecules results from the presence of conjugated carbon–carbon double bonds, which form the chromophore. Inside the chromophore, the electrons can be considered as belonging to this system as a whole rather than to an individual atom. When light is absorbed by the chromophore, the whole energy of the quantum is communicated to it and the chromophore is lifted from its normal state of lowest energy (ground state) to an energy-rich state (excited state). According to the Bohr theory of molecular structure, a molecule can exist only in a series of discrete states of electronic energies, which correspond to the absorbance bands [for a full description of the absorbance phenomenon, see Rabinovich (1956)]. From the comparison of the absorbance spectrum obtained with molecules from the same family, it was concluded that only the modifications in the circuit of the electrons along the chromophore are reflected in the absorbance spectrum of the pigment and, therefore, can be detected by using spectroscopic methods. As an example, one can compare the tremendous modifications in the absorbance spectrum of chlorophyll *a* caused by the replacement of the methyl side-group of C7 by an aldehyde side-group (Figure 2).

A molecule in an excited state can deexcite through several ways. If the molecule is well protected from the interactions with other molecules, the simplest pathway to rapidly lose its excitation energy is to emit a photon

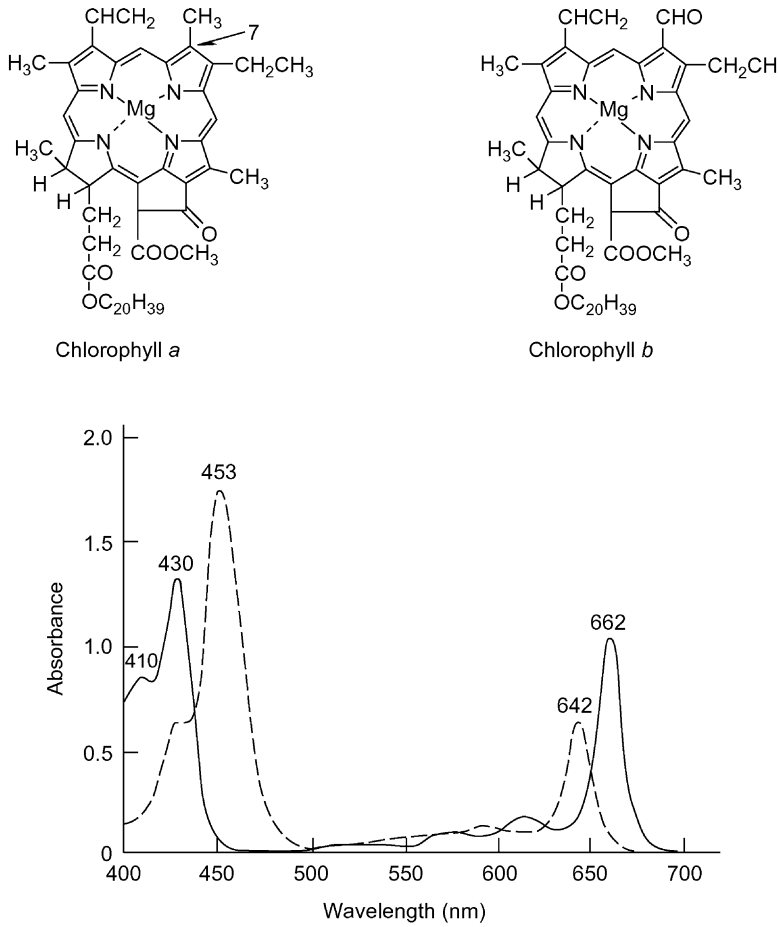


FIG. 2 Spectral modifications in the absorbance spectrum of chlorophyll *a* (*full line*) triggered by the replacement of the methyl side-group at the C7 position by an aldehyde side-group, yielding chlorophyll *b* (*dashed line*). The structures of chlorophyll *a* and chlorophyll *b* molecules are also presented.

back into the space. This is called *fluorescence*. Tetrapyrrole molecules can deexcite by emitting fluorescence. The chlorophyll fluorescence spectrum contains only one main band because the emission always originates from the first excited state. Fluorescence is in competition with other pathways that are internal conversion and transition to the triplet state. In the case of carotenoids, the fluorescence intensity is very weak (Koyama and Hashimoto, 1993) because the main deexcitation pathway occurs through the transition to the triplet state, which is nonfluorescent.

B. CAROTENOIDS

Carotenoids are responsible for most of the yellow to red-orange colors of fruits and vegetables (Table II). The basic structure of a carotenoid molecule is a symmetrical tetraterpene skeleton formed by tail-to-tail linkages of two geranylgeranyl diphosphate molecules (C20 unit). The end-groups of the basic structure are modified into six-membered rings yielding to monocyclic and dicyclic carotenoid such as β-carotene (Figure 1), the most ubiquitous carotenoid. The carotenoid group of molecules can be divided into two families of compounds: the carotenes, which are devoid of oxygen function (like β-carotene), and the xanthophylls, which contain at least one oxygen function (like violaxanthin; see Figure 5 for the structure). Carotenoids are lipophilic compounds and are usually water insoluble, although some xanthophylls may be water soluble because they harbor very strongly polar groups, such as polysaccharides. A good example is crocetin glycosyl

TABLE II
COLOR OF THE MAIN TYPES OF PIGMENTS FOUND IN PLANTS[a]

Family	Subfamily	Color	Found in
Tetrapyrroles	*Chlorophylls* (E140)	Green	Vegetables, fruits, juices, oils, sweets
	Phycocyanobilins	Blue or red	Cyanobacteria, red algae, chocolates, food complements
Carotenoids	*Carotenes* (E160)	Yellow to red	Vegetables, fruits, seeds, roots, sweets, juices, oils
	Xanthophylls (E161)	Yellow to red	Vegetables, flowers, fruits, sweets, cheese, juices, oils
Polyphenolic compounds	*Annatto* (E160b)	Blue to red	Red fruits, vegetables, seeds, flowers, roots, cheese, wines, syrups
	Anthocyanins (E163)		
	Flavonols	Yellow to cream	Fruits, teas
	Tannins	Brown	Wines
	Phytomelanins	Black to brown	Fruits, seed coats
	Curcumin (E100)	Yellow	*Curcuma*
Alkaloids	*Betalains*	Yellow to red-violet	Tissues originating from Caryophyllales and fodder beet
	Indigo	Blue to pink	Drinks

[a]The E*xyz* number refers to the European Community nomenclature for food colorants (Anonymous, 1995).

pigments that give the color to saffron stigmata. For a comprehensive description of the chemical properties of carotenoids, see the review by Britton *et al.* (1995). Solubilization of non–water-soluble carotenoids can be strongly improved by complexation of the pigment with cyclodextrin.

Carotenoids can exist in two configurations depending on the relative disposition of the four substituents around carbon–carbon double bonds. The configuration may be important for color intensity (Coultate, 1996) and for carotenoid assimilation (Storebakken and No, 1992; Torrissen *et al.*, 1989).

C. TETRAPYRROLES

The family of tetrapyrroles can be arranged in two classes depending on whether they are closed like chlorophyll (typically green) or open like phycocyanobilins (red or blue) (Table II). Since the pioneer works by Willstäter and Stöll (1913) on the chlorophyll structure, more than 50 different chlorophyll-related molecules have been reported (Scheer, 1996).

Basically, chlorophyll molecules are conjugated tetrapyrroles, to which a cyclopentanone ring has been added. The macrocycle is planar and binds into the center of an atom of Mg^{2+}. Release of this ion converts chlorophyll to pheophytin. When pheophytin binds one Cu^{2+} or Zn^{2+} ion, it is converted to Cu- or Zn-chlorophyll. All naturally occurring chlorophyll molecules have a propionic acid residue at position 17. The position 17(3) is generally esterified with a long-chain alcohol, usually phytol. Chlorophyll *b* differs from chlorophyll *a* by the presence of an aldehyde residue instead of a methyl residue at position 7 (Figure 2). Chlorophyll *a* and chlorophyll *b* are the most abundant pigments in land plants, in the skin of unripened fruits, and in green algae. Chlorophyll *b* can be considered typical for these organisms. The inner seed coat from Cucurbitaceae, though green, does not contain chlorophyll molecules but several protochlorophyllide esters (Schoefs, 2000a,b, 2001b). Besides chlorophyll *a*, brown algae and diatoms contain pigments similar to protochlorophyllide and protochlorophyll: the chlorophyll *c* and chlorophyll *c* esters (Garrido *et al.*, 2000). Because the ring IV of chlorophyll *c* and chlorophyll *c* ester molecules is not reduced (between the C17 and C18 carbons), these molecules are not true chlorophyll molecules (Figure 3). In addition, chlorophyll *c* and chlorophyll *c* ester molecules differ from true chlorophyll molecules by the nature of the esterifying residue at position 17, which is an acrylic residue in chlorophyll *c* and chlorophyll *c* ester molecules and a propionic residue in true chlorophyll molecules. From the description of the structure, it is clear that photosynthetic tetrapyrroles—chlorophyll *a*, chlorophyll *b*, and chlorophyll *c*

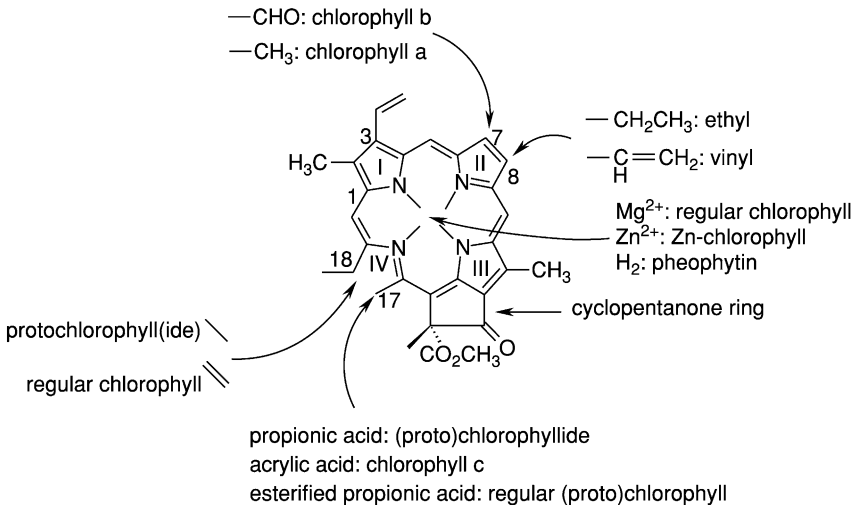


FIG. 3 Diversity in the structure of closed tetrapyrroles.

esters—are made up of a hydrophilic part, the macrocycle, and by a hydrophobic part, the phytol chain (Figures 2 and 3). The most hydrophilic segments of the macrocycle are the cyclopentanone ring (Figure 3) and the propionic ester group (position 17). Therefore, nonesterified macrocycles are much more polar than the esterified ones. The nature of the other side-groups and their configuration modify the polarity of the molecules.

Besides the family of closed tetrapyrrole molecules, there is a smaller group of pigments: the phycocyanobilin group, which is only abundant in cyanobacteria, red algae, and cryptomonad algae. The main forms of phycocyanobilins are the blue phycocyanobilin and the red phycoerythrobilin (Figure 4). *In situ* phycocyanobilins are covalently bound to particular proteins, forming different pigment–protein complexes, the so-called *phycobiliproteins* (Figure 4). These phycobiliproteins assemble to form phycobilisomes, which serve as light harvesters in the photosynthetic process. The regulation circuits of the biosynthetic and degradation pathways of phycobilisomes have been reviewed by Geiselmann *et al.* (2004). The pigment–protein complexes are stable at pH levels of 5 to 9 but may precipitate at a pH level lower than 5 (Sarada *et al.*, 1999). The protein–phycoerythrin complexes found in red algae and some cyanobacteria are potentially very interesting for several food and nonfood industries (Le Jeune *et al.*, 2003).

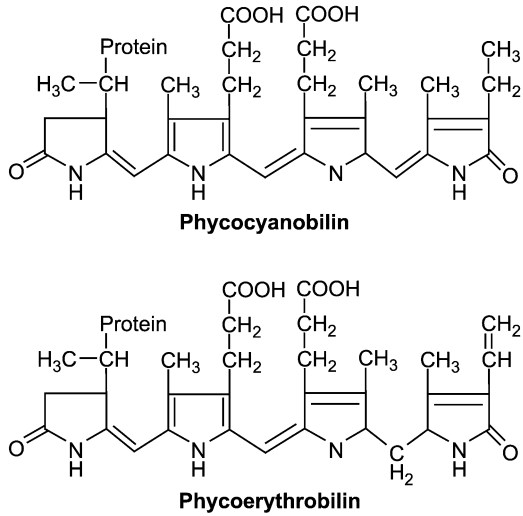


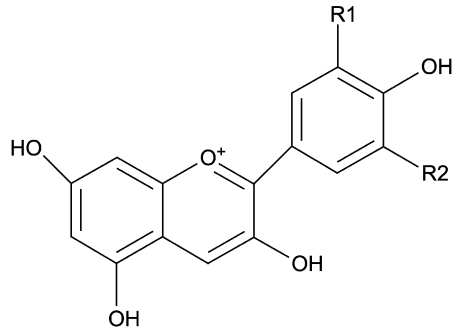
FIG. 4 Diversity in the structure of open tetrapyrroles.

D. PLANT POLYPHENOLIC COMPOUNDS

Anthocyanins constitute the largest family of colored phenolic compounds. They are responsible for colors ranging from salmon and pink, through scarlet, violet to purple, and blue of a large variety of fruits, flowers, petals, leaves, and vegetables. Therefore, these tissues constitute commercially valuable sources of anthocyanins (Table II) (Counsell *et al.*, 1981). Anthocyanin compounds have antioxidant and antimutagenesis properties (Furatu *et al.*, 1998; Yoshimoto *et al.*, 1999) and are responsible for the so-called *French paradox* (Renaud and Lorgeril, 1992), which says that despite a much higher consumption of saturated fats in France, the risk of arteriosclerosis and coronary heart diseases has a lower incidence of death in the French population than in other industrial countries.

Anthocyanins are glycosides of polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium or flavilyum salts. The aglycones are known as *anthocyanidins,* which from the chemical point of view are flavonoids. There are six natural anthocyanidins (Coultate, 1996) (Table III). The various possibilities of glycosylation or acylation regarding both the position and the nature of the side-groups increase the number of anthocyanins by a factor 15 to 20 times (Harborne and Mabuy, 1982). As the data reported in Table IV show, the differences in glycosylation and acylation around ring B have a strong impact on color and tinctorial strength (Giusti *et al.*, 1999b). An increase in the hydroxyl substitution around ring B results in a shift of

TABLE III
STRUCTURE OF THE SIX NATURAL ANTHOCYANIDINS



Anthocyanin name	R1	R2
Pelargonin	H	H
Cyanidin	OH	H
Peonidin	OCH_3	H
Delphinidium	OH	OH
Petunidium	OCH_3	OH
Malvidin	OCH_3	OCH_3

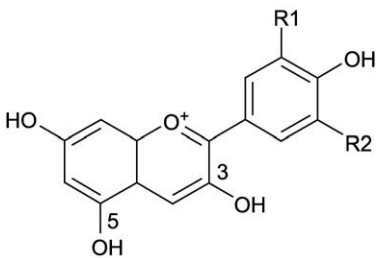
the absorption maximum to longer wavelengths, to yield a blue hue. The presence of additional acylation with cinnamic acids produces similar effects (Dangles *et al.*, 1993).

One crucial aspect of the chemistry of anthocyanins is the instability of color to pH changes (Hong and Wrolstad, 1990). Each cook knows that red cabbage turns blue-violet during cooking. This change can be reversed by the addition of some drops of vinegar or pieces of apple during cooking. At acidic pH levels, anthocyanins are red, whereas under basic conditions, they are blue-violet. An intensive search led to the discovery that anthocyanin molecules with complex patterns of glycosylation and acylation present remarkable stability to acidity, heat treatment, and light exposure (Dangles *et al.*, 1993; Francis, 1992). The improved stability has been attributed to the intramolecular and intermolecular co-pigment, self-association, metal complexing, and presence of inorganic salts (Brouillard, 1983; Goto, 1987). The phenomenon of co-pigmentation and self-association plays an important role in color diversity that each single anthocyanin chromophore may generate (Gonnet, 1998).

As it can be deduced from the description of the chemical structures of anthocyans and anthocyanidins, polyphenolic pigments are usually water soluble (Figure 1). Noticeable exceptions are the curcumin oils (yellow),

TABLE IV

INFLUENCE OF THE AGLYCONE SUBSTITUTION ON THE PIGMENT COLOR PROPERTIES (pH < 7)[a]

	Type of substitution				
	Aglycone	5-glucosidic	3-glucosidic	3,5-glucosidic	Acylation with cinnamic acid but not with malonic acid
L*		>	=	>	<
Hue		<	<	<	<
Chroma		=	<	=	<
Position of the absorbance maximum (nm)	513	509	503	500	Bathochromic shift

[a]For the position of carbon 3 and carbon 5, see Table III.

which correspond to a second class of plant polyphenolic compounds. Curcumin consists of two vinyl guaiacol groups joined by a β-diketone unit.

E. ALKALOIDS

The alkaloids are the last group of pigment molecules reviewed in this chapter. Alkaloids are secondary plant products, which very often exhibit pharmacological properties. Among the alkaloid family of compounds, only the betalain subgroup of molecules presents the unique property of being highly colored. In fact, betalain pigments are anthocyanin-like pigments (Figure 1) but represent an evolutionary divergence from the anthocyanidin-producing plants because betalain pigments are derived from different biosynthetic pathways. In the plant phylum, betalain molecules are synthesized in some families of the Caryophyllales order (Cactacae, Chenopodiacae, Amaranthaceae, Phytolaccaceae, and Basellaceae). The betalain family of compounds is divided into red to red-violet betacyanins and the yellow betaxanthins, which are acylated and nonacylated glycosides of aglycones, respectively. Betaxanthins differ from betacyanins by the conjugation of a substituted aromatic nucleus to the 1,7-diazaheptamethinium chromophore. These pigments maintain their appearance over a wide range of pH levels, from 4 to 7.

III. CHEMICAL MODIFICATIONS OCCURRING DURING FOOD TREATMENTS AND STORAGE

Everyone has observed that fruits like banana, citrus, orange, tomato, paprika, and so on are first green and then turn colored, to yellow or red, during ripening. This change in color usually operates through the combination of chlorophyll degradation pathway and synthesis of carotenoids, which are induced by ethylene (Fraser *et al*., 1994; Gross, 1991). Consequently, the reduction of ethylene emission by stored fruits and vegetables is a common process used to keep fruits and vegetables green for a longer period. Conversely, the treatment of fruits with ethylene is used successfully to promote ripening of tomatoes (Iwahori and Lyons, 1970) and to improve the peel color of citrus.

Although the chlorophyll degradation in fruits is not completely elucidated, it appears that the initial steps are similar to those observed during plant senescence (Adachi *et al*., 1992; Bertrand and Schoefs, 1999; Ma and Shimokawa, 1998; Matile *et al*., 1999). Similar color modifications, which reflect pigment degradation, have also been observed with anthocyanins (Markakis, 1982). These pigment degradations occur *in situ*. Extracted

pigments are even more sensitive to degradation, and stability of natural pigments is one of the major features of food industries. Several basic studies have been conducted to understand the ways of degradation and to increase the stability of pigments during food processing and storage. In the following sections, the principal factors responsible for pigment degradations are shortly reviewed.

A. DEGRADATION DUE TO ENZYMATIC ACTIVITIES

Pigment degradations during processing may arise from the disruption of cellular structure. This is accompanied by the liberation of enzymes, such as lipoxygenase (Irvine and Anderson, 1953; Irvine and Winkler, 1950), peroxidase (Martinez Parra and Munoz, 1997), polyphenol oxidase (Kobrehel *et al*., 1972, 1974), and/or betalain oxidase. These enzymes are involved in the decrease of the xanthophyll content during durum wheat milling (Borrelli *et al*., 1999) or the pigment content of red beet (Martinez Parra and Munoz, 1997; Shih and Whiley, 1981), respectively. The enzyme activity can be stopped during blanching (see later discussion). For instance, red-palm oil fruits are sterilized immediately after harvest to inactivate lipase enzyme, which would otherwise promote rancidity. This process provokes a considerable increase in the proportion of *cis*-α- and *cis*-β-carotene (Trujillo-Quijano *et al*., 1990).

B. DEGRADATION BY HEAT

Food processing often requires one or more heating steps, which may allomerize chlorophyll molecules (Minguez-Mosquera and Gandul-Rojas, 1995) and even transform the green chlorophyll in dull olive-green pheophytin and pheophorbide (pheophytin without phytol; see Figure 3 for the structure) [bean: Muftugil (1986); parsley: Berset and Caniaux (1983); pea: Buckle and Edwards (1970); Aczél (1971); spinach: Schwartz and Lorenzo (1991); tea: Kohata *et al*. (1998)]. *In situ*, these reactions are catalyzed by enzymes (chlorophyllase, Mg-dechelatase, lipoxygenase) (Bertrand and Schoefs, 1999), which can be inactivated by heating or during extraction with organic solvents. In some solvents, chlorophyllase can remain active, and heat inactivation requires several minutes at 90 °C. Short steaming (20–60 seconds), like that used during processing of tea leaves, may not inactivate the enzyme. Kohata *et al*. (1998) report that inactivation is only reached during tea-leaf raffination, a process that heats the leaves at 120 °C for 30 minutes. In corn, lipoxygenases and peroxidases are inactivated after 6 and 8 minutes of blanching, respectively, whereas broccoli requires only 90 seconds (Barrett *et al*., 2000). The difference in the inactivation time may be

explained by the different heat-transmission capacities. Regardless of these considerations, the severity of the heating steps should be limited to maintain the color, texture, flavor, nutritional quality (Lim *et al.*, 1989; Maccarone *et al.*, 1996; Theerakulkait *et al.*, 1995), and the pheophorbide content. In Japan, the amount of pheophorbide in the food products is regulated by the Food and Health Administrations (e.g., pheophorbide: 160 mg/100 g). One common way to avoid accumulation of pheophorbide is to let it rebind a metal. Under certain conditions, pheophytin molecules can rebind divalent metals, principally copper and zinc ions, regenerating the green color, which is usually brighter (Coultate, 1996; Jones *et al.*, 1977; LaBorde and von Elbe, 1990). This phenomenon is called *regreening*. The replacement capacity of chlorophyll *b* derivatives is much less than that of chlorophyll *a* (LaBorde and von Elbe, 1990; von Elbe *et al.*, 1986). The chlorophyll derivatives were found more stable than the original chlorophyll, especially to acids (Humphrey, 1980). Formation of metallo-chlorophylls has a potential use as a green colorant for beverages and as a means to avoid accumulation of chlorophyll degradation product during processing. Canjura *et al.* (1999) designed a method based on continuous aseptic processing to favor the replacement of the chlorophyll-Mg^{2+} ion by Zn^{2+}. Again, care should be taken not to exceed the permitted value (U.S. Food and Drug Administration [FDA]: 75 mg/kg). Alternatively, one may use a sodium-copper-chlorophyllin compound, which is more water soluble than regular chlorophyll, as chlorophyllin does not have a phytol chain. The Cu content of chlorophyllin makes it furthermore more resistant to heat (Coultate, 1996).

Thermal treatments also promote carotenoid degradations (including isomerization and oxidation) and carotenoid–protein complex disruptions (Boskovic, 1979; Pereira *et al.*, 1999; Rodriguez-Amaya, 1989). Both modifications decrease the product's nutritive value. In fact, *cis*-carotenoid isomers are less assimilated than the corresponding *trans*-isomers (Storebakken and No, 1992; Torrissen *et al.*, 1989). In addition, *cis*-β-carotene and *cis*-γ-carotene have a reduced vitamin A activity (Zeichmeister, 1962). This is especially true when severe heat treatments, such as those required to achieve commercial sterility, are applied (Robertson, 1985; Schwartz *et al.*, 1981). Chlorophyll degradation also occurs during storage at 4 °C, with chlorophyll *a* being more sensitive than chlorophyll *b* (Schwartz and Lorenzo, 1991).

C. DEGRADATION DUE TO ACIDIFICATION

In this chapter, the instability of many plant pigments to acidity has already been mentioned. For example, many polyphenolic compounds change their color depending on the pH level (Ikan, 1991). Under acidic

conditions, the red flavylium ions prevail. This could represent a disadvantage for the use of polyphenols as a colorant because they are unstable and degraded under weak acidic or basic pH levels. This is exemplified by a study of the stability of anthocyanin-3-glucoside extracted from grape (i.e., delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside). The effect of pH during accelerated storage studies, performed at 55 °C, showed that no pigment molecule remains after 4 days at a pH level of 3. The degradation is accelerated when the pH level is increased to 5 (Baublis *et al.*, 1994; Sarni-Manchado *et al.*, 1996). Such pronounced degradation has a strong influence on the color, which turns from red-orange to yellowish. This change becomes visually noticeable only when the degradation of anthocyanins has reached 60%. A similar result has been found with other anthocyanin preparations. The degradation of anthocyanins extracted from grapes can be largely prevented by the addition of a 4-vinylphenol side-group (Sweeny and Iacobucci, 1983), but this modification causes a somewhat larger precipitation of the pigment. In contrast to the natural anthocyanins, the 4-vinylphenyl derivatives are protected against discoloration by SO_2 (Sarni-Manchado *et al.*, 1996). Acylation of anthocyanins improves their stability during processing and storage (Rommel *et al.*, 1992). In monoacylated anthocyanins, only one side of the pyrylium ring can be protected against the nucleophilic attack of water, and therefore, only a weak intermolecular effect might occur (Brouillard, 1983). Differences in glycosidic substituents at the C3 position of the aglycon and the position of the acyl group in the sugar moiety may explain the different stabilities (Table V). The mechanism

TABLE V
INFLUENCE OF THE ACYL GROUP POSITION AT THE AGLYCONE ON THE ANTHOCYANIN STABILITY TO HEAT

Aglycon	Source	Acylation groups (position)		Stability (half-life week)	Reference
		Malic acid	p-coumaric acid		
Sophorose	Red radish	+ (5)	+ (3)	22 (25 °C) 120 (4 °C)	Rodriguez-Saona *et al.* (2000)
Rutinose	Red-fleshed potato	− (5)	+ (3)	11 (25 °C) 110 (4 °C)	Rodriguez-Saona *et al.* (2000)

of protection is still not determined, but specialists suggest that the acylating group is protecting the oxonium ion from hydration, thereby preventing the formation of the hemiketal or chalcone forms (Francis, 1989). Diacetylated anthocyanins can also be stabilized by a sandwich type of stacking caused by hydrophobic interactions between planar residues of the acyl groups and the positively charged pyrylium nucleus. This configuration would diminish the formation of the pseudobase (Brouillard, 1981; Goto and Kondo, 1991).

Acids also induce chemical modifications of carotenoids. For instance, juice preparation requires acidic conditions, which trigger the spontaneous conversion of 5,6- and 5,6′-epoxide groups of violaxanthin, lutein epoxide, and antheraxanthin to 5,8- and 5′,8′-furanoid epoxides (Coultate, 1996; Gross, 1991). This conversion has a dramatic effect on the color because furanoids are essentially colorless. To be convinced about this fact, it is enough to compare the flesh color of fresh (solid line) and canned (dashed line) pineapples (Coultate, 1996) (Figure 5).

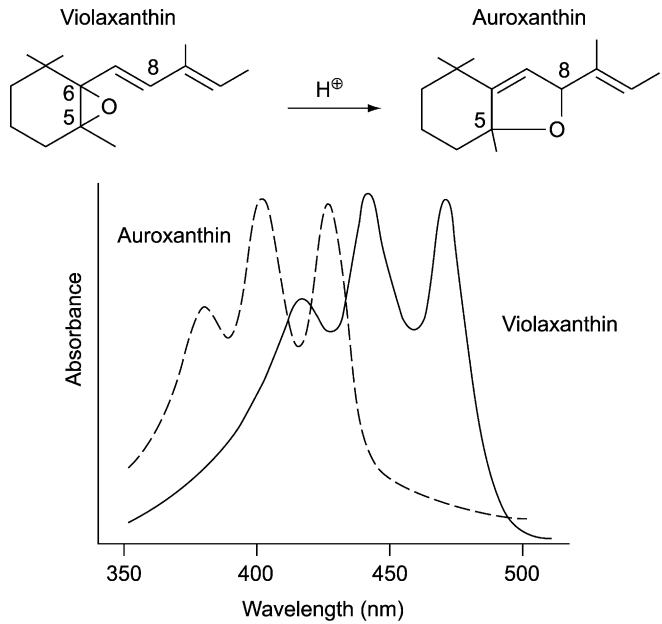


FIG. 5 Effect of processing on the color of pineapple flesh. The structure and spectral modifications occurring during the processing are displayed.

D. DEGRADATION DUE TO OXYGEN EXPOSURE

Exposure to oxygen is deleterious, particularly in dried food such as dehydrated carrot slices in which carotenoid oxidation and bleaching occur rapidly because of the formation of activated oxygen species (Coultate, 1996).

E. DEGRADATION DUE TO LIGHT

Modifications of food color reflecting changes in pigment composition may also happen during storage. For instance, the appearance of pink in cheddar cheese, colored with annatto, when stored under fluorescent light used in grocery stores dairy cases, is well known (Boyd, 2000; Hong *et al.*, 1995). Betalains are also degraded during storage. For instance, vulgaxanthin, which represents 5% of the colored molecules of red beet extract, is less stable than the major colorant betacyanin. Therefore, when stored, red beet extracts develop a bluish shade. The original color may be restored by adding carotenoids.

F. DETECTION OF ADULTERANTS AND CONTROL OF QUALITY

The use of different colorants in food is regulated by the legislation of each country. These legislations are becoming progressively more restrictive, and the number of colorants allowed is limited and strictly controlled (Anonymous, 1995; Francis, 1987). In addition, many degradation products of natural colorants can impart flavor and odor (Tonnesen and Karlsen, 1985). For instance, the appearance of unwanted odor may result from the formation of β-ionone due to the breakdown of β-carotene molecules (Coultate, 1996). Therefore, an important aspect of food research is the search for adulterants and/or the presence of toxic compounds in preparations. High-performance liquid chromatography (HPLC)–electrospray ionization (ESI)–mass spectrometry (MS) was used to analyze anthocyanin extracts from red-fleshed potato. The HPLC profile presents five major peaks corresponding to nonacylated anthocyanins and anthocyanins acylated with either ferulic acid or p-coumaric acid (Giusti *et al.*, 1999a). In addition, the ESI-MS profiles may reveal presence of several toxic alkaloids as in red-fleshed potato (Rodriguez-Saona *et al.*, 1998).

Nuclear magnetic resonance spectroscopy (NMRS) has also been used to detect adulteration in olive oil (Mannina *et al.*, 2003). To improve the color of virgin olive oil, chlorophyll molecules are sometimes added. This addition may be detected by NMRS because chlorophyll and pheophytin, the regular tetrapyrrole pigments of olive oil, give a different spectrum.

IV. METHODS OF ANALYSIS: AN OVERVIEW

A. GENERAL CASES

Because plant pigments have a chromophore made of conjugated double bonds, they react easily with acids, bases, oxygen, heat, and light (anthocyanins: Aoki *et al*., 2000; betalains: Carle and Stintzing, 2000; carotenoids: Anguelova and Warthesen, 2000; Martin *et al*., 1999; Pfander *et al*., 2000; tetrapyrroles: Bertrand *et al*., 2004; Salin *et al*., 1999). Special care should be taken when manipulating extracted pigments (see earlier discussion). This is important when a sample containing chlorophyll molecules is exposed to light because excited chlorophyll molecules efficiently transfer the excitation energy to the triplet state of oxygen, resulting in the formation of activated oxygen species, which are very reactive and can oxidize other organic molecules, including other pigments, lipids, or proteins. For this reason, care should be taken during extraction and analysis. The production of singlet oxygen by chlorophyll is not restricted to aqueous pigment extracts but also occurs in less polar solvents like in oils (Anguelova and Warthesen, 2000; Haila *et al*., 1997). For this reason, oils containing a nonnegligible amount of chlorophyll or chlorophyll-like molecules should be stored in the dark and at reduced temperature (Chen *et al*., 1997; Jung and Min, 1991), especially if the content in carotenoids is low, as it is the case in pumpkin seed oil (Schoefs, 2000a,b, 2001b). The presence of carotenoids reduces the formation and the action of activated oxygen species (Goulson and Warthesen, 1999; Palozza and Krinsky, 1991).

B. SPECTROSCOPIC METHODS

The absorbance spectrum can be considered the fingerprint of pigments (for limitations, see later discussion). Therefore, absorbance spectroscopy constitutes the simplest way to identify and quantify the major pigments in a mixture (Schoefs, 2000b, 2002). Once identified, it is possible to use a set of equations to estimate their respective concentrations (Bertrand and Schoefs, 1997; Bertrand *et al*., 2004; Jeffrey and Humphrey, 1975). Because the environment of the pigment (solvent, temperature, ligation to protein, etc.) strongly influences the position and the shape of the spectrum, the crude absorbance spectrum of intact tissues is usually useless for direct measurements of the pigment concentration (Borsari *et al*., 2002). Accurate measurements of pigment concentration require pigment extraction with a solvent for which equations have been established or at least in which specific (or molar) absorbance coefficients have been determined. With these data, one can establish a new equation set, adapted to the particular

situation. The precision of the measurements depends on the type of the device used, the precision in the determination of the position of the absorbance maxima, and of course the accuracy of the absorption coefficient used for the calculation. For instance, Porra *et al.* (1989) reevaluated the extinction coefficients for chlorophyll *a* and chlorophyll *b* by comparing the concentration of chlorophyll standards as determined by the measure of magnesium amount by atomic absorption spectroscopy and by spectrophotometric determination. These data were used to derive new equation sets, which were found significantly different from those used previously. Therefore, it is advisable to regularly check the literature for new values and equation sets. The major limitations of pigment identification on the sole basis of the absorbance spectrum is the overlapping of the absorbance bands of the pigments in the mixture. This difficulty makes the method less efficient when the number of pigments is higher than three. For completeness, it is necessary to add that the *cis*-carotenoid isomers can be recognized, because the absorbance spectrum presents an additional band in the UV region (Figure 6). The position of the *cis*-double bound is reflected in the ratio of A_{cis}/A_{MAX}.

The total phenols in an anthocyanin preparation can be routinely determined using the Folin-Ciocalteau procedure (Singleton and Rossi, 1965).

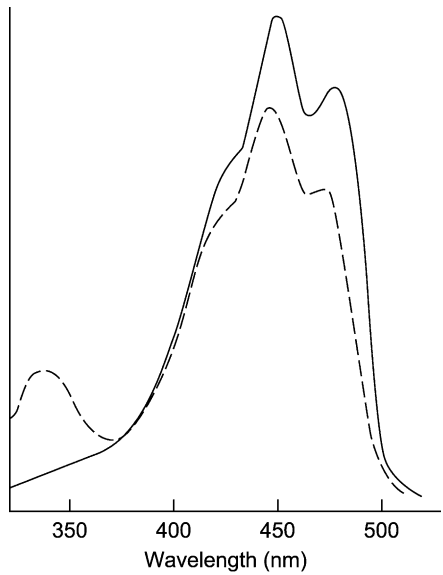


FIG. 6 Comparison of the ultraviolet-Vis absorbance spectra of all–*trans*-β-carotene (*full line*) and *cis*-β-carotene (*dashed line*).

Fluorescence spectroscopy can be more diagnostically helpful because of the selective excitation of pigments but useless in the case of carotenoids, because their fluorescence is very weak (Koyama and Hashimoto, 1993).

In some cases, other spectroscopic methods can be used to identify pigments on the basis of particular structural features. For instance infrared (IR) spectroscopy was used to reveal the presence of an allelic group in the carotenoids fucoxanthin, alloxanthin, and bastaxanthin (Britton *et al.*, 1995). IR spectroscopy was also used to establish the details of the light-induced oxygen-dependent bleaching of the food colorant chlorophyllin (Bertrand *et al.*, 2004; Chenery and Bowring, 2003; Salin *et al.*, 1999).

In conclusion, spectroscopic methods usually permit crude identification of pigments in an extract, but in most cases, the specific composition remains obscure. Therefore, obtaining details on the composition of a mixture of pigments requires additional analyses, which often involve separation of the mixture into its components using methods such as chromatography.

C. CHROMATOGRAPHIC SEPARATION

1. Open column

Various phases such as powdered sucrose, DEAE-Sepharose, cellulose, or MgO/Hyflosupercel have been used to achieve chlorophyll and carotenoid separation (Omata and Murata, 1983; Strain *et al.*, 1971; Wasley *et al.*, 1970). The all–*trans*-β-carotene can be separated from 9-*cis* and 13-*cis* isomers using an open column filled with calcium hydroxide. For phycobilins, an hydroxylapatite column can be used (Siegelman and Kycia, 1978). Methods to separate anthocyanins using gel filtration have been proposed (Somers, 1966). Although today open-column chromatography is mostly used to clean extracts or for preparative purposes, it may still be used for analytical purposes. For example, Somers and Evans (1977) reported that the formation of aggregates of glucoside anthocyanins is typical for wine aging and is related to the color stability. This natural process can be strongly accelerated by adding acetaldehyde. Therefore, upon addition of this compound to a young wine, it can "appear" old. In an attempt to find an analytical method, which would establish the "real" age of a wine, Johnson and Morris (1996) used a silica gel column, together with a gradient of formic acid to separate the different glucoside anthocyanin aggregates from red wines. Using this technique, the anthocyanins were eluted into four fractions. The two first fractions contained free anthocyanins, whereas the two last ones contained polymerized and condensed anthocyanins (Datzberger *et al.*, 1991). When the amounts of anthocyanins in each fraction from the nontreated and the treated wine were compared, a clear

difference appeared because the treated wines had more aggregates of monoglucoside anthocyanins than the nontreated ones. This may be explained by the fact that acetaldehyde accelerates preferentially the condensation of monoglucoside pigments, but only slightly diglucoside ones (Johnson and Morris, 1996).

2. *Thin-layer chromatography*

Thin-layer chromatography (TLC) is often used because it is a fast, effective, and relatively inexpensive analytical method. Special care should be taken when the separation is done on silica gel, because its slight acidity may trigger pigment degradations such as epoxide-furanoxide rearrangement in carotenoids (Schiedt and Liaaen-Jensen, 1995) and chlorophyll pheophytinization. Therefore, it is necessary to neutralize the acidity of silica before pigment separation. Unfortunately, separation of compounds with similar structures is usually rather difficult using TLC. This is well illustrated by comparing the chromatograms obtained from the pigments extracted from pumpkin seed oil and separated by TLC (Figure 7A) or by HPLC (Figure 7B). Using TLC, three bands are found: The two green bands correspond to protochlorophyll (band 2) and protopheophytin (protochlorophyll molecules, which have lost their Mg^{2+} atom) (band 3) pigments, whereas the yellow band reflects the presence of carotenoids (band 1). When the same sample was analyzed by HPLC, 14 peaks were obtained. As a consequence, TLC methodologies have been progressively supplemented by more efficient separation techniques, such as HPLC (Braun and Zsindely, 1992). Nevertheless, both open-column chromatography and TLC methods are still useful for cleaning extracts or preparing large amounts of pigments (He *et al.*, 1998).

3. *High-performance liquid chromatography*

Photosynthetic pigments, chlorophylls, and carotenoids have a clear hydrophobic character and are usually analyzed by C18–reversed-phase (RP) columns. A C30-RP appeared on the market. The C30-RP is particularly efficient in the separation of carotenoids because the interactions of the pigments and the stationary phase are maximized by the similar size. With this phase, many *cis* isomers of the same carotenoid are separated from each other (Emenhiser *et al.*, 1995; Lacker *et al.*, 1999; Sharpless *et al.*, 1996). This C30-RP has been successfully applied to the determination of saponified carotenoids in orange juice (Rousseff *et al.*, 1996). However, when a mixture is complex, coelutions may become rapidly limiting and less selective stationary phases, such as the C18-RP, are therefore preferably

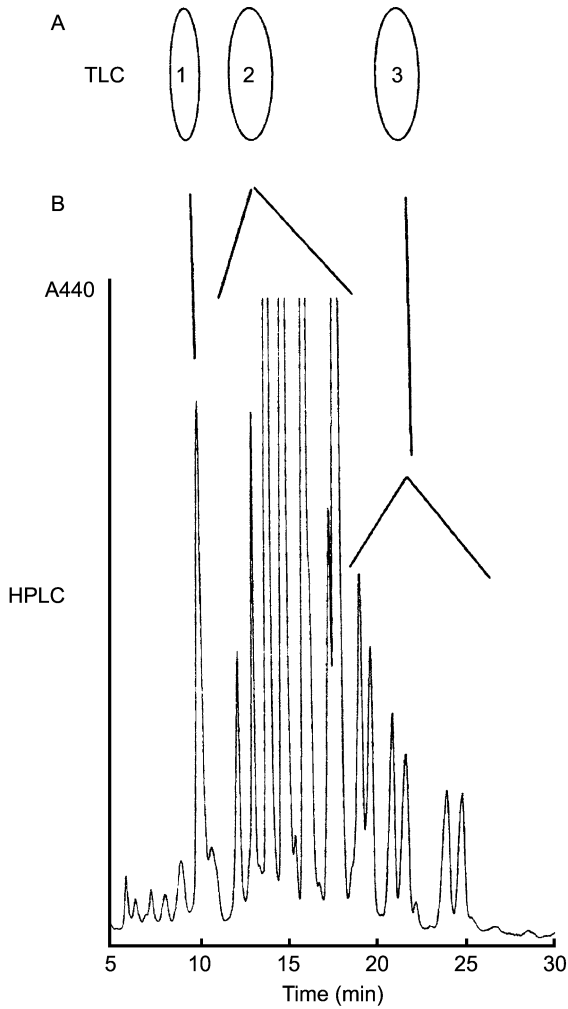


FIG. 7 Chromatogram of the pigments from pumpkin seed oil. (A) Thin-layer chromatography: petroleum ether/acetone 80/20 V:V). (B) High-performance liquid chromatography: methanol, acetonitrile, methylene chloride (see Schoefs, 2000a).

used (Schoefs *et al.*, 1995, 1996). The mobile phase used for the separation of hydrophobic molecules is usually made up of organic solvents, except when very polar molecules like glycosyl esters of carotenoid or chlorophyll *c* are present in the mixture. In this latter case, a polar organic solvent mixed with a small amount of water is recommended (Dufresne *et al.*, 1999; Latasa *et al.*, 2001).

To improve pigment separation, heating the column is sometimes proposed. Vanheukelem *et al.* (1994) found that carotenoids were optimally separated at 60 °C, whereas chlorophylls and chlorophyll-related molecules were best separated at 30 °C. This procedure is, however, not recommendable because heating can trigger carotenoid isomerization and chlorophyll epimerization and allomerization. These modifications are not detected using usual HPLC methods (Hyvärinen and Hynninen, 1999). Therefore, care should be taken to employ proper chromatographic conditions to ensure that pigments do not escape the analysis. To illustrate this possibility, the HPLC elution profiles of pigments from pumpkin seed oil using two RP HPLC methods recommended for the analysis of plant pigments (Ilik *et al.*, 2000; Schoefs *et al.*, 1995) are compared (Figures 7B and 8).

FIG. 8 High-performance liquid chromatogram of the pigments extracted from pumpkin seed oil. Elution program: solvent A: 100% methanol; solvent B: methanol-hexane (4/1 v/v). Linear gradient solvent B from 0% to 100% in 28 minutes and then isocratic elution for 6 minutes.

HPLC has also been used to evaluate the purity of commercially available chlorophyllin (Chernomorsky *et al.*, 1997, Schoefs, 2001a, 2002).

Although most of the methods used for the separation of photosynthetic pigments only require organic solvents, the elution mixtures necessary to separate more hydrophilic compounds, such as anthocyanins, are usually a mixture of organic and aqueous solvents (Sarni-Manchado *et al.*, 1996). Before analysis of anthocyanins by HPLC, it is recommended to purify the extract on a C18-RP cartridge previously activated with acidified methanol (Hong and Wrolstad, 1990). Anthocyanins and other phenolics are adsorbed onto the phase, whereas sugars, acids, and other water-soluble molecules are washed away with 0.01% aqueous HCl. Addition of ethyl acetate elutes phenolic compounds other than anthocyanins, which are eluted using acidified methanol (0.01% HCl v/v) (Oszmianski and Lee, 1990). Using a similar protocol Takeoka *et al.* (1997) analyzed the anthocyanin content of seed coat from black bean by HPLC. Three anthocyanins were found: delphinidium 3-glucose, petunidin 3-glucose, and malvidin 3-glucoside. Because the A_{ACYL}/A_{VIS} ratio reflects the molar ratio of the cinnamic acid and anthocyanin (Harborne, 1958), the use of a photodiode array detector allows determination of additional information about the acylation or the glycosylation patterns of anthocyanins (Hong and Wrolstad, 1990). For instance, absence of an absorbance peak between 300 and 350 nm in the absorbance spectra of the eluted pigments suggests that none is acylated with aromatic amino acids (Andersen, 1988). HPLC analyses of anthocyanins from radish established that the eight anthocyanins separated were acylated (Giusti and Wrolstad, 1996a). Comparison of the absorption spectra of acylated and nonacylated compounds revealed that acylation shifts the anthocyanin color to the shorter wavelengths (Giusti and Wrolstad, 1996a,b). HPLC elution programs for anthocyanins are constantly being improved, and today one can separate up to 15 anthocyanins within a single 30-minute run (Giusti *et al.*, 2000). One difficulty, but not the least, with the determination and quantification of anthocyanins by HPLC is that there is a general lack of availability of pure anthocyanin standards.

Supplementing HPLC with a diode-array detector and fast computers has greatly increased the analytical power of HPLC. Using such detectors, one can follow simultaneously elution on the full UV–Vis range (190–800 nm). This possibility guarantees that each pigment can be followed at its absorbance maximum, that is, with the maximum sensitivity.

4. Supercritical and subcritical HPLC

Supercritical fluid extraction has been suggested as an alternative method for selective one-step isolation of carotenoids without degradations (but see Britton *et al.*, 1995). For instance, Favati *et al.* (1988) isolated β-carotene

and lutein from leaf concentrates at 40 °C and pressure ranging between 29 and 70 MPa. From the analytical point of view, supercritical extraction is compatible with supercritical fluid chromatography because the two techniques can share the mobile phase and some devices, favoring the development of extraction and separation methodologies. Because the elution strength of pure CO_2 in respect to the carotenoids is rather weak, it is necessary to add co-solvents to increase the carotenoid solubility in CO_2. The solubility parameter theory predicts that the maximum solubility of a compound is reached when the solubility parameter of the solvents equals that of the solute. For instance, β-carotene can be extracted at 43 °C and approximately 70 MPa (Favati *et al.*, 1988). Unfortunately, some equipments do not allow elution at such an elevated pressure. Therefore, new conditions of pressure and temperature should be found using a diagram connecting the variations of pressure to gas densities for different temperatures (Smith, 1988). At lower temperature, densities similar to those reached at higher temperatures can be obtained for lower pressures. These conditions are referred to as *subcritical conditions.* Using this technique, Ibanez *et al.* (1998) successfully separated β-carotene from lycopene in less than 10 minutes. The best separation was obtained at 10 °C under a pressure of approximately 35 MPa, with a home-packed capillary column containing deactivated silonal groups with an octamethyl-cyclotetrasiloxane reagent. This column cannot be used at higher temperatures because the structure of the phase is broken at a high temperature, forming O-Si-CH_3 bonds with the silica phase.

5. *Capillary HPLC*

Pigments with very similar structures might be difficult to separate using classic RP-C18 HPLC. This is the case of zeaxanthin and lutein. To overcome this difficulty, a particular HPLC method should be applied (Darko *et al.*, 2000). Alternatively a capillary electrophoresis column can be used. This last method was successfully applied to the separation of zeaxanthin and lutein from eye humor (Karlsen *et al.*, 2003). The good separation and the fast elution of the pigments obtained by these authors suggest that capillary electrophoresis is suitable for routine analysis of pigments contained in tiny samples.

None of the methods described is entirely suitable for the elucidation of pigment structure. Although the spectral fingerprint is often sufficient to identify the chromophore, it does not contain enough information to determine the complete structure of the pigment (Schoefs, 2000b, 2001b). The missing information can be partly deduced from the chromatographic behavior and from the comparison of the obtained retention data with

literature. Other methods such as MS have to be applied to fully characterize the structure of the molecule (Bertrand *et al.*, 2004; Schoefs, 2001a).

D. MASS SPECTROMETRY

Because of their high mass, low volatility, and thermal instability, chlorophylls and carotenoids have long presented special analytical challenges to MS. The use of newer methods, such as chemical ionization, secondary ion MS, fast-atom bombardment, and field-, plasma-, and matrix-assisted laser desorption—for which the necessity of sample vaporization before the ionization is suppressed—opened ways to the molecular ion detection and, thus, to direct molecular weight determination. Unfortunately, these methods produced additional signals in the region of lower masses, reflecting the presence of chlorophyll degradation products, such as 10-OH chlorophyll *a* (m/z 908) or pheophytin *a* (m/z 870) and additional signals at m/z 482, m/z 556, and m/z 615. From these results, whether the additional signals reflect molecules present in the sample before the analysis or whether their formation results from pigment degradation during sample preparation or analysis is difficult to determine (Bertrand *et al.*, 2004; Schoefs, 2002). Most recent progress in MS analysis of tetrapyrroles has been obtained with the development of atmospheric ionization methods, that is, atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI). The APCI technique, in combination with reversed HPLC, has been shown to be efficient for detecting chlorophyll *a* and its nine degradation products on low nanogram levels. The procedure is approximately 1000 times more sensitive than the thermospray ionization (Eckhardt *et al.*, 1991). To demonstrate the potential of electrospray MS in chlorophyll research, ESI. interface was employed with an ion trap mass spectrometer as mass analyzer (Fenn *et al.*, 1989; Hutton and Major, 1995; Schoefs, 2001a). ESI is a mild ionization technology feature that can obtain a high proportion of the chlorophyll *a*–protonated molecular ion $(M + H)^+$, m/z 893.5. It is remarkable that the spectrum was obtained with approximately 2 pmol of the compound. Further structural information has been obtained with the unique MS^N capability of the ion trap analyzer, allowing consecutive dissociation of side-chain functional groups from the selected precursor ion (up to eight steps) (Schoefs, 2001a). The MS^N procedure is highly selective and enables consecutive cleavage of at least seven functional groups around the porphyrin, providing valuable structural information about the tetrapyrrole. Using MS methods, chlorophyll allomers and their derivatives produced during fruit and vegetable processing (Minguez-Mosquera *et al.*, 1995b) have been isolated and their structure elucidated (Hyvärinen and Hynninen, 1999).

A C4-RP HPLC method coupled to ESI-MS has been described for the analysis of the different components of phycobilisome from the cyanobacterium *Synechocystis* PCC 6803 (Zolla and Bianchetti, 2001).

Although anthocyanins have positive charges at low pH levels and are very soluble in water and alcohol because of their high mass, low volatility, and thermal instability, their high MW, which ranges from a few hundred to a few thousands (Terahara *et al.*, 1990), made their analysis by MS methodologies difficult. As in the case of chlorophyll, the ESI method appears especially suitable for the ionization of this family of labile, nonvolatile polar compounds. Using this method, Giusti *et al.* (1999a,b, 2000) obtained structural information on a minor anthocyanin component of grape juice and red cabbage.

A limitation of MS in the analysis of anthocyanins and alkaloids arises from its inability to differentiate between diastereoisomeric forms of sugars. Therefore, this method cannot provide information on the exact glycosidic substitutions other than the number of carbon atoms and the presence of methyl side-groups in the sugars. To get more information on the structure of these substitutions, it is necessary to use MS-MS technology. When applied to the anthocyanins from grape and red cabbage, typical patterns were obtained (Guisti *et al.*, 2000). The MS-MS resulted in the cleavage of glycosidic bonds only between the flavilium ring and the sugar directly attached to it. In the case of acylated anthocyanins, the fragmentation pattern allows a rough determination of the localization of the acylating group (Giusti *et al.*, 2000).

ESI-MS has also been successfully applied to determination of the molecular structure of other non–water-soluble pigments such as the polyphenolic yellow molecules contained in turmeric extracted from the rhizome of *Curcuma longa* (He *et al.*, 1998).

All the methods described are invasive techniques that are time consuming and often expensive. To reduce sampling and analytical costs, and to speed up the analyses, noninvasive analytical procedures have been developed with the aims to characterize the pigment content of samples and, in the case of food products, to estimate the impact of a color change on the visual perception of the product.

V. PIGMENT IDENTIFICATION AND QUANTIFICATION: THE PROBLEM OF STANDARDS

In the preceding sections, the different analytical methods that can be used to identify pigments were presented. One crucial point is the calibration of the signal detector. This question requires standards, ideally identical to each

pigment under consideration. Those standards are not always commercially available and, therefore, should be prepared. Relative to this problem, Schiedt and Liaaen-Jensen (1995) have defined the minimum criteria for the identification of carotenoids: (1) The absorbance spectra in the UV-Vis region, obtained in at least two different solvents, should be in agreement with the chromophore suggested; (2) the chromatographic properties of putative pigment and standard must be identical in TLC (R_f) and HPLC (t_R): both compounds should co-elute; and (3) a mass spectrum should be obtained, which allows at least confirmation of the molecular mass. Although such ''rules'' were not specified for the identification of other natural pigments, similar criteria are suggested. Even when the identification and detector calibration have been performed according to these rules, pigment quantification may not be straightforward. For instance, the amount of pigments in a sample may be apparently higher after a treatment, such as heating, than before! Such a paradox, which was frequently reported in blanched and cooked vegetables or fruits (Stahl and Sies, 1992), has been attributed to the greater extractability of the pigments after cooking. It is believed that heating triggers cell wall rupture, facilitating release of the pigments.

A. NONINVASIVE METHODS

The success of noninvasive methodologies will lie in the development of remote and noncontact ''sampling'' methodologies. Several possibilities have been described (Andrews and Dallin, 2003). In this chapter, only some methodologies using changes in the light properties are developed.

B. EVALUATION OF THE COLOR AND ITS PERCEPTION

The visual impression of a colored object mainly depends on the pigments in a sample. This feeling is affected by morphological factors such as the presence of epidermal hair or cuticular waxes and the shape and the orientation of the cells in the epidermis and the subepidermis. In fact, pigments and surface topography act together to selectively absorb, reflect, and refract the incident visible light, which will eventually be sensed by eyes. When light strikes the eye, it is detected by one of the three color sensors of the retina: a red, a green, or a blue one. It is known that the information is not sent as an individual color but as a red/green signal, a yellow/blue signal, or a black/white signal. The signals, generated at the retina level, are transduced through the optic nerve to the brain and interpreted as color. When all the wavelengths are reflected by an object, the eyes see it as white, whereas when all are absorbed, the object appears black.

Although it is out of the scope of this review to explain in detail the basis of color measurement (Wyszecki and Stiles, 1987), it is, however, necessary to briefly describe the principle of the color measurement to facilitate the understanding of the results. Each color can be described by a set of three parameters: (1) the hue, which is the dominant shade; (2) the saturation or chroma, which measures how much color is present; and (3) the lightness, which corresponds to the degree of darkness of a particular color. Therefore, for each unique set of illuminants or observed conditions, it is necessary to describe a different hue, saturation, and lightness. By using a standard illuminant and a standard observer, the amount of light reflected by an object can be converted into the hue, saturation, and lightness values. Then a sample can be compared to any standard with these three attributes. In 1976 the Commission Internationale de l'Eclairage (CIE) adopted a standard method of calculating color attributes, known as the CIE Lab Color Space. The lightness coefficient L* ranges from black (0) to white (100), whereas the coordinates a^* and b^* designate the color on a rectangular-coordinate grid perpendicular to the L* axis. The color at the grid origin is achromatic (i.e., gray; $a^* = 0$, $b^* = 0$). On the horizontal axis, positive and negative a values indicate the hue of redness (positive values) and greenness (negative values), whereas on the vertical axis, b^* indicates yellowness (positive values) and blueness (negative values). The hue H* is defined as the arctang (b*/a*). The CIE Lab Color Space method has been used frequently to characterize the *in situ* color modifications occurring in fish flesh fed with various diets (Akhtar *et al.*, 1999; Bjerkeng *et al.*, 1997), during semolina milling (Borrelli *et al.*, 1999), and anthocyanin production by *Perilla* cell culture (Aoki *et al.*, 2000). In a study comparing the stability of natural colorants and dyes in gels with different levels of sugar, a good correlation between the sensory and instrumental ranking was found. This indicates that the values of L* and H* are good representatives of the lightness and hue of the gels, respectively. This study also established that the value of L* is solely dependent on the pigment concentration and that the sugar concentration has no influence on the L* value (Calvo and Salvador, 2000).

It is necessary to emphasize that no change in color does not necessarily mean that there is no modification in the pigment composition. In fact, there are conflicting reports in the literature on the correlation between color measurement and pigment composition. This is especially true when several pigments have to be monitored (Lancaster *et al.*, 1997). Several mathematical combinations of the parameters have been proposed to predict the pigment modifications occurring during food processing (Gomez *et al.*, 1998; Ma and Shimokawa, 1998; Steet and Tong, 1996). Some authors also use the hue angle (for the definition, see Wyszecki and Stiles, 1987) to characterize color modifications (Akhtar *et al.*, 1999; De Ell

and Toivonen, 1999). Other methods, based on reflected and scattered light, were used to derive estimated pigment content (Kawashima and Nakatani, 1998) and to predict color (McClements *et al.*, 1998), but they are less popular.

C. USE OF CHLOROPHYLL FLUORESCENCE TO ESTIMATE THE FRESHNESS OR THE MATURITY OF PLANTS

The freshness of vegetables can be assessed using chlorophyll fluorescence kinetic measurements, which reflect the photosynthetic activity. It is out of the scope of this chapter to explain in detail the background of the development of this method, but the interested reader is referred to a review by Rohacek and Bartak (1999). In brief, the chlorophyll fluorescence yield depends on the reduction state of the photosystem II primary acceptor, Q_A. When all the Q_A acceptors are oxidized, for instance, after a period of complete darkness, the fluorescence yield, denoted F_0, is minimal. In contrast, when all the Q_A acceptors are reduced, for example, during a saturating light pulse, the level of fluorescence, denoted Fm, is maximal. The state of the photosynthetic apparatus can be estimated using the Fv/Fm ratio with $Fv = Fm - F_0$. Healthy plants exhibit an Fv/Fm ratio of approximately 0.8. When the photosynthetic process is inactivated, such as in senescent plants or in cold-stored plants, the ratio decreases (De Ell and Toivonen, 1999). Tian *et al.* (1996) showed that the ratio could be a sensitive indicator of responses of broccoli to hot water treatment, even before visual changes were noted. Other parameters can be obtained from chlorophyll fluorescence kinetic to characterize the physiological state of the plants (Rohacek and Bartak, 1999).

With the development of the CCD camera, we now can visualize the global fluorescence emission of an organ such as a leaf or a fruit. This new dimension of measurement differs from the classic one in that it is not focused on one cell or a small group of cells, allowing fluorescence imaging (Buschmann *et al.*, 2000). This technology has many potential applications. For instance, Nebdal *et al.* (2000) developed a strategy to measure the decrease of chlorophyll fluorescence from the lemon peel during ripening. On the basis of fluorescence data, they defined robust parameters allowing the prediction of damage at the lemon surface before the appearance of visible signs. This methodology can also be applied to trace chlorophyll in highly colored tissues such as redishing tomatoes. The development of chlorophyll fluorescence imaging in fields other than agriculture is also very promising, and it has already been used to follow pollution and even to visualize the stress caused at the leaf surface by an insect walking on the leaf (Bown *et al.*, 2002).

Other spectroscopic methodologies such as infrared and Raman spectroscopies should be developed to allow the determination of the presence of molecules in intact samples (Chenery and Bowring, 2003).

VI. EXTRACTION AND ANALYSIS: CASE BY CASE

It may be useful to have a set of analytical protocols specially formatted for particular samples. However, the complete extraction of pigments often requires several steps and may use a mixture of several solvents. This is especially true when the sample contains pigments of different polarities or pigments that are present in a complex matrix (Britton *et al.*, 1995; Taungbodhitham *et al.*, 1998). In rare cases, pigments can be extracted in a one-step process (Kopecky *et al.*, 2000). The aim of the following discussion is not to make an exhaustive list of the extraction and analytical methods described in literature, but more to provide representative selected examples, which can then be used as reference (Table VI).

A. PIGMENTS FROM INTACT FRUITS AND VEGETABLES

1. Chlorophyll and carotenoid molecules

Chlorophyll and carotenoid molecules can be extracted with acetone using a homogenizer. To extract lycopene from tomatoes, Thompson *et al.* (2000) used a mixture of organic solvents composed of hexane, acetone, and ethanol (50:25:25 v/v/v). The extraction should be repeated until no color is observed. All fractions are pooled in a separatory funnel and partitioned with diethyl ether. NaCl solution (e.g., 10% w/v) can be added to help the transfer of the pigments in the nonpolar phase. The water phase is discarded and the pigmented layer is dried with anhydrous Na_2SO_4 (e.g., 2% w/v).

When necessary, the pigments can be saponified with KOH-methanol (10% w/v) and left for some time with periodic shaking. The pigments are then transferred to diethyl ether by adding distilled water, and the organic phase is washed with water until neutrality is reached. Finally, the aqueous phase is removed. To dry the pigments, the organic phase is filtered on a bed of anhydrous Na_2SO_4.

a. Determination of the pigments.

TLC: Separation can be carried out on silica gel GF60 plates in a presaturated chamber. The elution mixture is composed of petroleum ether (bp: 65–95 °C), acetone and diethylamine (10:4:1 v/v/v) (Hornero-Méndez and Minguez-Mosquera, 1998).

TABLE VI

SUMMARY OF THE METHODS USED TO EXTRACT AND ANALYZE PIGMENTS FROM VARIOUS SOURCES

Products	Pigment analyzed	Extraction	Pretreatment before analysis	Separation methods
Fruits and vegetables	Carotenoids, tetrapyrroles	Various organic solvents	−	TLC, HPLC
	Anthocyanins	Acetone, chloroform	+	HPLC
Seeds	Anthocyanins	1% HCl in methanol	+	HPLC
Flower	Saffron	Cold water	−	HPLC
Juices and drinks	Carotenoids	Tetrahydrofuran	−	TLC, HPLC
Oil	Carotenoids	Dilution in an organic solvent	(+)	Open column, TLC, HPLC
	Tetrapyrroles			
Pasta	Carotenoids	Various organic solvents	+	Open column, TLC, HPLC
Vegetable puree	Carotenoids	Acetone	−	TLC, HPLC
	Tetrapyrroles			
Cheese	Carotenoids	Water/tetrahydrofuran	+	HPLC
Textile	Saffron	Water/pyridine	−	HPLC
Pharmaceutical products	Pigment-cyclodextrin complexes	Polar solvents for dilution	−	HPLC
Fish flesh	Astaxanthin	Various organic solvents	−	HPLC
Urine and plasma	Carotenoids	Ethyl acetate/methanol	+	HPLC
Humor eyes	Carotenoids	—	+	HPLC

Note: HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

HPLC: Many HPLC methods have been described in the literature (Hornero-Méndez and Minguez-Mosquera, 1998; Schoefs, 2002, 2003, 2004; Thompson *et al.*, 2000). When the carotenoid composition is complex, as in passiflora fruit, which contains more than 10 carotenoids, it might be necessary to first separate the different groups of carotenoids. This can be done using an open column packed with alumina. Fraction 1, which contains carotenes and epoxi-carotenoids, is eluted with petroleum ether; fraction 2, which is composed of monohydroxy- and keto-carotenoids, is eluted with 70–90% diethyl ether in petroleum ether; and fraction 3, made up of polyhydroxy-carotenoids, is eluted with 0–30% ethanol in ether. The pigments contained in individual fractions can be further separated using particular TLC or HPLC methods (Mercadante *et al.*, 1998).

2. *Anthocyanins*

During extraction with acetone and chloroform (Giusti and Wrolstad, 1996a,b), it is advisable to prepurify the extract on acidic methanol-activated C18 minicolumns (Hong and Wrolstad, 1990) before analysis by HPLC.

B. ANTHOCYANINS IN SEEDS

There are different methods to separate anthocyanins from seeds. Comparing the extraction efficiency of the different solvents, it appears that the best is 1% HCl in methanol (Gao and Mazza, 1996; Gürenç and Karaali, 2000).

C. WATER-SOLUBLE CAROTENOIDS FROM FLOWER TISSUES

An illustration is given by the stigma of saffron that are shaken during 30 minutes in cold water (4 °C). The solution is heated to 60 °C for 30 minutes and allowed to stand in the dark for 24 hours. The clear supernatant contains the pigments (Tsatsaroni *et al.*, 1998).

D. PIGMENTS FROM JUICES AND DRINKS

1. *Carotenoids*

The juice is first mixed with tetrahydrofuran. The nonpolar pigments are transferred in petroleum ether. The water phase is discarded and the organic phase is washed with water. This is repeated until the water phase becomes colorless. The ether extracts are pooled and dried on anhydrous sodium sulfate. When the juice contains pulp, it is advisable to remove it

by centrifugation. The isolated pulp is dispersed in distilled water and extracted as explained earlier (Arena *et al.*, 2000).

2. *Determination of the pigments*

Carotenoids can be analyzed using the methods previously described. When the extract is enriched in chlorophyll derivatives, the HPLC method described by Canjura *et al.* (1999) may be used. It consists of an isocratic elution (9 minutes) of a hexane/isopropanol (98.3:1.7 v/v) mixture, followed by a linear gradient (2 minutes) to yield a mobile phase with equal parts of hexane/isopropanol (98.3:1.7 v/v) and hexane/isopropanol (98.3/3.0 v/v). This mixture is held during 7 minutes to increase the hexane/isopropanol (98.3/3.0) fraction to 100%.

E. PIGMENTS FROM OIL

Pigments can be separated from the uncolored molecules of oils using a silica gel column, TLC (Ellsworth, 1971), or HPLC (Goulson and Warthesen, 1999) before further analysis. Alternatively, the oil can be directly injected in an HPLC (Schoefs, 2000a). For a separate analysis of the fatty acid moiety, the pigments should be saponified with KOH-methanol (10% w/v) and left 10 minutes with periodic shaking at room temperature. Then the pigments are transferred by addition of water to diethyl ether. The aqueous phase is reextracted twice. The pigmented diethyl ether phase is dried and stored.

F. CAROTENOIDS FROM RAW AND COOKED PASTA

The raw pasta is comminuted in a blender, followed by cylinder milling to achieve a powder passing a 40-mesh screen. The pigments are then extracted using a ternary mixture of hexane/acetone/ether (10:7:6 v/v/v), followed by a cold saponification for 1 hour (Pereira *et al.*, 1999). The pigments are then transferred to petroleum ether. A similar procedure can be used to extract pigments from cooked pasta, but acetone is the preferred solvent and the pigments should not be saponified before analysis.

A fast analytical method consists of the separation of β-carotene from the other carotenoids using an open column packed with MgO/hyflosupercel (1/2). β-Carotene is eluted with 4% diethyl ether in petroleum ether. The remaining adsorbed pigments are eluted together with acetone, transferred in petroleum ether and separated on TLC (3% methanol in benzene). The presence of carotenoid epoxide is seen by exposing TLC to HCl vapors, which triggers a change in the colored spots from yellow or orange to blue or green (Gross *et al.*, 1972). For the determination of *cis*-isomers, a C30-RP-HPLC

column is recommended (Schmitz *et al.*, 1995). Alternatively, a calcium hydroxide column can be used.

G. PIGMENTS FROM VEGETABLE PUREES

The pigments from vegetable purees are extracted with acetone and the solution is filtered through a filter paper. Then the pigments are dried over a bed of anhydrous Na_2SO_4. For tetrapyrroles, a method separating chlorophyll *a* and chlorophyll *b* from their degradation products is recommended.

H. CAROTENOIDS FROM CHEESE

Cheese mostly contains the carotenoids bixin and norbixin. To extract these pigments, cheese is crushed in a solution of water/tetrahydrofuran (1:1 v/v) and centrifuged. The supernatant is biphasic: The aqueous phase contains norbixin, and the organic phase contains mainly bixin. Norbixin and bixin can be separated by HPLC using a linear gradient starting with 100% of water/acetone (3:2 v/v) to 100% of water/acetonitrile (1:4 v/v) in 15 minutes (Tricard *et al.*, 1998).

I. PIGMENTS FROM TEXTILE

Textiles may be colored with natural colorants such as saffron or curcumin. Saffron pigments can be extracted from cotton and wool fibers using a pyridine-water mixture (25/75 v:v). This method is, however, not able to extract curcumin from the same fibers (Tsatsaroni *et al.*, 1998).

J. PIGMENTS IN PHARMACEUTICAL PRODUCTS: THE PIGMENT–CYCLODEXTRIN COMPLEXES

A general way used to increase the stability of natural colorants is to complex them with cyclodextrins. Cyclodextrins are oligosaccharides composed of six, seven, or eight glucopyranose units (referred to as α-, β-, and γ-cyclodextrin, respectively). These molecules have a doughnut shape with an interior cavity. The cavity is hydrophobic, whereas the external surface is hydrophilic. The unique property of cyclodextrin is to form an inclusion complex by incorporation of guest molecules in the cavity. As the external surface is hydrophilic, the guest molecule–cyclodextrin complex can be easily solubilized. For instance, the polyphenolic yellow pigments curcumin and

curcuminoids are nonsoluble in water unless they are complexed to cyclodextrin (Tonnesen *et al.*, 2002). Stability studies have shown that the pigment–cyclodextrin complexes may be more stable than the noncomplexed pigments. For example, the stability of the red color of ketchup versus temperature and decomposition of rutin in water is improved by complexation with cyclodextrins. Because the diameter of a chlorophyll molecule exceeds the size of cyclodextrins, chlorophyll cannot be completely included into the cavity of cyclodextrins. Nevertheless, chlorophyll–cyclodextrin complexes show improved water solubility in cold and warm waters and color stability to light (Sato *et al.*, 2000). A detailed study on the benefit for the solubility and stability of curcumin brought by cyclodextrin has indicated that substituents on the cyclodextrin molecule have little influence on photodegradation, whereas the interior of the cavity may be of some importance. Besides the positive effects of cyclodextrins, these compounds may trigger pigment fading, as observed with β-cyclodextrin-callistephin (Dangles *et al.*, 1992a,b). This effect is, however, not observed with α-cyclodextrins (Lewis *et al.*, 1995). Therefore, the type of cyclodextrin should be carefully chosen.

Because the cyclodextrin–pigment complexes are much more soluble than the noncomplexed pigments, particular chromatographic methods should be used for their analysis (Cserhati and Forgacs, 2003). For instance, to analyze curcumin–cyclodextrin complexes, Tonnesen *et al.* (2002) used a C18-RP-HPLC (Nova Pack column). The mobile phase was citric acid and acetonitrile (60/40 v:v).

K. PIGMENTS FROM ANIMAL TISSUES

A section on pigments in animal tissues may appear at first glance out of the scope of a chapter on plant pigments. However, when one looks carefully at the biochemistry of animal cells, it becomes clear that almost no plant pigment can be synthesized by these cells. On the other hand, some of them cross the intestine and are found in cells (Parkinson and Brown, 1981). This is especially the case for carotenoid molecules, which are eventually transformed.

1. Carotenoids from fish flesh

Extraction: Akhtar *et al.* (1999) recommend to finely grind the sample with magnesium sulfate. The pigments are then extracted by stirring the sample/salt mixture with acetone for 1 hour, followed by paper filtration. The water is removed by passing the extract through anhydrous Na_2SO_4.

Analysis: It is known that salmonids accumulate red carotenoids in their flesh. Several HPLC methods can be used to separate the red

carotenoids (Schoefs *et al.*, 2001). For instance, the method of Kopecky *et al.* (2000) separates astaxanthin from its esters and from canthaxanthin. The esterification state of the consumed astaxanthin, either free or esterified, is not important because only free astaxanthin is deposited in the flesh (Storebakken and No, 1992; Torrissen *et al.*, 1989). On the other hand, the ingested astaxanthin stereoisomer molecules cannot be epimerized by the fish and are deposited unchanged in the same extent in the flesh (Foss, 1984). Thus, the composition of astaxanthin in flesh reflects the diastereoisomer composition of the meal feed. Consequently, the diastereoisomer composition of the fish flesh can be used to determine the origin of the fish. In fact, in the natural environment, the astaxanthin stereoisomer composition varies largely among taxa. For instance, *Haematococcus pluvialis* (green alga) accumulated the (3*S*,3′*S*)-isomer of astaxanthin, whereas crustaceans contain a highly racemized mixture of astaxanthin isomers (Matsuno *et al.*, 1984; Rendström *et al.*, 1981a,b). In fish farms, farmers can supplement the feed with either shrimp meal, with the red yeast *Phaffia rhodozyma*, or with *Haematococcus pluvialis* (Johnson and An, 1991; Nelis and De Leenheer, 1991). Because *Phaffia* accumulates exclusively (3*R*,3′*R*)-astaxanthin, the separation of the stereoisomers can be used to determine whether a fish is grown on a farm.

The HPLC methods proposed by Aas *et al.* (1987) can be used to distinguish between astaxanthin stereoisomers in a fish flesh. These methods can separate the stereoisomers after transformation to their respective dicamphenate esters. However, to avoid coelution between 7,8-didehydro- and 7,8,7′,8′-tetradihydro-astaxanthin and (3S,3′S)-*cis* isomers of astaxanthin, the astaxanthin fraction should be purified by TLC on alkaline plates (Bjömland *et al.*, 1989). A Pirckle covalent L-leucine column is preferable, especially for routine analyses, since the separation is achieved in 18 minutes (Turujman, 1993). In addition, this method is able to separate the *cis* and *trans* isomers.

Noninvasive methods were also used to establish whether the absorption and deposition of astaxanthin are affected by feeding the fish with or without astaxanthin supplementation in all or alternated meals (Wathne *et al.*, 1998). The redness a* of the salmon fed with astaxanthin in alternating meals was higher than that for fish fed with a mixture of colored and uncolored meals. A variation in the lightness L* was also observed. Wathne *et al.* (1998) found that the total carotenoid concentration in the flesh could be estimated using the value a*. However, the equation cannot be applied for the determination of the carotenoid content in other studies because the value of the coefficients depended on the fish physiological state and on the fish taxon.

2. *Pigments from urine and plasma*

One can find several methods in the literature for the detection of polyphenolic compounds and carotenoids in urine and plasma. The early methods used a aqueous-based matrix only (Heath *et al.*, 2003). The major disadvantage of these methods was the absence of protein extraction before HPLC analysis. Heath *et al.* (2003) overcomes this difficulty by treating the samples with an extracting reagent composed of ethyl acetate and methanol (190/10 v:v) to transfer the carotenoid molecules from the aqueous medium to the organic phase, which can then be analyzed by a C18-RP-HPLC (Water Symmetry Shield column protected by a guard column) using a mobile phase composed mostly with organic solvents (actonitrile/methanol/water/acetic acid 41/23/36/1 v:v:v:v). The six major carotenoids found in human plasma are β-carotene, lycopene, β-cryptoxanthin, lutein, α-carotene, and zeaxanthin (Ford *et al.*, 2003; Karlsen *et al.*, 2003). A C18-RP-HPLC method was also applied to the detection of curcumin in plasma and urine from humans after absorption of pure curcumin (Heath *et al.*, 2003). As food, curcumin is mostly taken from curry powders and is usually in a mixture with other yellow pigments such as demethoxycurcumin, bisdemethoxycurcumin, and dihydrocurcumin (Tang and Eisenbrand, 1992). To separate these compounds, He *et al.* (1998) used a C18-RP-HPLC similar to that described by Heath *et al.* (2003). In the absence of standards, the identification of the eluted molecules cannot be performed on the sole basis of the UV-Vis spectrum, and ESI-MS was used (He *et al.*, 1998).

Sharpless *et al.* (1996) used a C30-RP-HPLC column to differentiate the various carotenoid isomers contained in human plasma.

3. *Pigments from eyes*

Carotenoids lutein and zeaxanthin have a protective role in the eyes (Krinsky *et al.*, 2003). Sampling and handling of minute biological samples containing degradable pigments is an extremely challenging task. Emphasis should be placed on the sampling technique to prevent and avoid degradations during sampling and sample processing. This is especially the case with the detection of carotenoid molecules in eye humor as the starting volume is as small as 4 μl. To remove the proteins from the sample before analysis by HPLC, the sample is treated with a mixture composed of isopropanol and BHT (2,6-di-*tert*-butyl-4-methylphenol), which precipitates the proteins. The supernatant, which contains the carotenoid molecules, can be analyzed by HPLC or by capillary liquid chromatography equipped with a C30 column (mobile phase: water/methanol 2/98 v:v). Using this method, Yeum

et al. (1995) found that human aqueous humor is much simpler than that of plasma because it contains only lutein and zeaxanthin.

VII. FUTURE TRENDS

Research on plant pigments is fascinating because it involves many methods and a deep knowledge in several fields. Plant pigment studies should be continued because they may procure several immediate interesting impressions, as well as commercial and health advantages. For these advantages, one should have the most powerful methodologies to analyze pigment composition of samples. Most of the available methods are destructive, time consuming, and cost consuming. Therefore, the development of rapid and nondestructive methods should progress further. In establishing these tools, it is necessary to correlate physical parameters such as firmness, soluble solids, dry matter, and color with physiological data (e.g., respiration and photosynthesis). These methods could involve those techniques such as delayed luminescence (Triglia *et al.*, 1998), infrared spectroscopies (Bakeev, 2003; Chenery and Bowring, 2003; Slaughter *et al.*, 1996), NMRS (Borsari *et al.*, 2002), noncontact sampling (Andrews and Dallin, 2003), and others, which in the past were more restricted to basic research.

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REFERENCES

Aas, G.H., Bjerkeng, B., Halten, B., and Storebakken, T. 1987. Idoxanthin, a major carotenoid in to flesh of artic char (*Salvenius alpinus*) fed diets containing astaxanthin. *Aquaculture* **150**, 135–142.

Aczél, A. 1971. Data on the color change of green peas. *Lebensm. Wiss. Technol.* **4**, 167.

Adachi, M., Tsuzuki, E., and Shimokawa, K. 1992. Effect of ethylene on degreening of intact radish (*Raphanus sativus* L.) cotyledons. *Sci. Hort.* **65**, 1–9.

Akhtar, P., Gray, J.I., Cooper, T.H., Garling, D.L., and Booren, A.M. 1999. Dietary pigmentation and deposition of α-tocopherol and carotenoids in rainbow trout muscle and liver tissue. *J. Food Sci.* **64**, 234–239.

Andersen, O.M. 1988. Semipreparative isolation and structure determination of pelargonidin 3-*O*-β-L-rhamnopyranosyl-(1 β 2)-β-D-glucopyranoside and other anthocyanins from the tree *Dacrycarpus dacrydioides*. *Acta Chem. Scand. B.* **42**, 462–468.

Andrews, J. and Dallin, P. 2003. Getting to grips with the process: Extractive and remote "sampling". *Spectrosc. Eur.* **15**, 23–26.

Anguelova, T. and Warthesen, J. 2000. Degrading lycopene, α-carotene, and β-carotene during peroxidation. *J. Food Sci.* **65**, 71–75.

Anonymous. 1995. Commission Directive 95/45, L226.

Aoki, H., Koda, T., Ichi, T., and Nakamura, M. 2000. Production of perilla color pigment by *Perilla* of cell culture. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 1–9. SIC Publishing, Hamden.

Arena, E., Fallico, B., and Maccarone, E. 2000. Influence of carotenoids and pulps in the color modification of blood orange juice. *J. Food Sci.* **65**, 458–460.

Bakeev, K.A. 2003. Near-infrared spectroscopy as a process analytical tool. *Pharm. Technol. Eur.* September, 27–32.

Baker, D.H. 1992. Applications of chemically defined diets to the solution of nutrition problems. *Amino Acids* **2**, 1–12.

Barrett, D.M., Garcia, E.L., Russell, G.F., Ramirez, E., and Shirazi, A. 2000. Blanch time and cultivar effects on quality of frozen and stored corn and broccoli. *J. Food Sci.* **65**, 534–540.

Baublis, A., Spomer, A., and Berger-Jimenez, M.D. 1994. Anthocyanin pigments: Comparison of extract stability. *J. Food Sci.* **59**, 1219–1223.

Berlin, J., Sieg, S., Strack, D., Bokern, M., and Harms, H. 1986. Production of betalaines by suspension cultures of *Chenopodium rubrum* L. *Plant Cell Tissue Organ. Cult.* **5**, 163–174.

Berset, C. and Caniaux, P. 1983. Relationship between color evaluation and chlorophyllin pigment content in dried parsley leaves. *J. Food Sci.* **48**, 1854–1857.

Bertrand, M. and Schoefs, B. 1997. Working with photosynthetic pigments: Problems and precautions. *In* "Handbook of Photosynthesis" (M. Pessarakli, ed.), pp. 49–69. Marcel Dekker, New York.

Bertrand, M. and Schoefs, B. 1999. Photosynthetic pigment metabolism in plants under stress. *In* "Handbook of Plant and Crop Stress" (M. Pessarakli, ed.), pp. 527–543. Marcel Dekker, New York.

Bertrand, M., Garrido, J., and Schoefs, B. 2004. Analysis of photosynthetic pigments: An update. *In* "Handbook of Photosynthesis" (M. Pessarakli, ed.), 2nd Ed. Marcel Dekker, New York.

Bjerkeng, B, Refstie, S., Fjalestad, K.T., Storebakken, T., Rodbotten, M., and Roem, A.J. 1997. Bioavailability of all-*E*-astaxanthin and *Z*-isomers of astaxanthin in rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* **157**, 63–82.

Bjömland, T.S., Liaaen-Jensen, S., and Throndsen, J. 1989. Algal carotenoids. Part 41. Carotenoids of the marine chrysophyte *Pelagococcus subviridis*. *Phytochemistry* **28**, 3347–3353.

Blendford, D. 1995. Colouring consumers' perceptions. *Food Ingredients Analysis Int.* **17**, 10–11.

Borrelli, G.M., Troccoli, A., Di Fonzo, N., and Fares, C. 1999. Durum wheat lipoxygenase activity and other parameters that affect pasta color. *Cereal Chem.* **76**, 335–340.

Borsari, M., Ferrari, E., Grandi, R., and Saladini, M. 2002. Curcuminoids as potential new iron-chelating agents: Spectroscopic, polarographic, and potentiometric study on their Fe(III) complexing ability. *Inorg. Chim. Acta* **328**, 61–68.

Boskovic, M. 1979. Fate of lycopene in dehydrated tomato products: Carotenoid isomerization in a food system. *J. Food Sci.* **44**, 84–86.

Bown, A.W., Hall, D.E., and McGregor, K.B. 2002. Insect footsteps on leaves stimulates the accumulation of 4-amino-butyrate and can be visualized through increased chlorophyll fluorescence and superoxide production. *Plant Physiol.* **129**, 1430–1433.

Boyd, W.A. 2000. Annatto and other carotenoid food colorants in dairy application. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 24–31. SIC Publishing, Hamden.

Braun, T. and Zsindely, S. 1992. The comparative standing of individual instrumental analytical techniques. *Trends Anal. Chem.* **11**, 267–269.

Britton, G. 1988. "The Biochemistry of Natural Pigments". Cambridge University Press, Cambridge.

Britton, G. 1995. Carotenoids today and challenges for the future. *In* "Carotenoids" (G. Britton, S. Liaaen-Jensen, and H. Pfander, eds), Spectroscopy, Vol. 1B, pp. 13–62. Birkhäuser, Basel.

Britton, G., Liaaen-Jensen, S., and Pfander, H. 1995. "Carotenoids", Vol. 1–3. Birkhaüser, Basel.

Brouillard, R. 1981. Origin of the exceptional colour stability of *Zebrina* anthocyanin. *Phytochemistry* **20**, 143–145.

Brouillard, R. 1983. The *in vivo* expression of anthocyanin colour in plants. *Phytochemistry* **22**, 1311–1323.

Buckle, K.A. and Edwards, R.A. 1970. Chlorophyll, color, and pH changes in H.T.S.T. (high temperature-short time) processed green pea puree. *J. Food Technol.* **5**, 173–186.

Burton, G.W. and Ingold, K.U. 1984. β-carotene: an unusual type of lipid antioxidant. *Science* **244**, 569–573.

Buschmann, C., Langsdorf, G., and Lichtenthaler, H.K. 2000. Imaging of the blue, green, and red fluorescence emission of plants: An overview. *Photosynthetica* **38**, 483–491.

Calvo, C. and Salvador, A. 2000. Use of natural colorants in food gels. Influence of composition of gels on their colour and study of their stability during storage. *Food Hydrocolloids* **14**, 439–443.

Canjura, F.L., Watkins, R.H., and Schwartz, S.J. 1999. Color improvement and metallo–chlorophyll complexes in continuous flow aseptically processed peas. *J. Food Sci.* **64**, 987–990.

Carle, R. and Stintzing, F.C. 2000. Cacti: A source for natural food colourants. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 43–56. SIC Publishing, Hamden.

Castenmiller, J.J.M., West, C.E., Linssen, J.P.H., van het Hof, K.H., and Voragen, A.G.J. 1999. The food matrix of spinach is a limiting factor in determining the bioavailability of beta-carotene and to a lesser extent of lutein in humans. *J. Nutr.* **129**, 349–355.

Chaudry, Y. and Stich, E. 2000. Pumpkin: A source of natural food color. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 56–70. SIC Publishing, Hamden.

Chen, C.W., Lee, T.C., and Ho, C.T. 1997. Antioxidative effect and kinetics study of capsanthin on the chlorophyll-sensitized photoxidation of soybean oil and selected flavor compounds. *In* "Spicies" (S.J. Rich and C.T. Ho, eds), pp. 188–198. ACS Symposium Series 660, American Chemical Society, Washington, DC.

Chenery, D. and Bowring, H. 2003. Infrared and Raman spectroscopies imaging in biosciences. *Spectrosc. Eur.* **15**, 8–14.

Chernomorsky, S., Rancourt, R., Sahai, D., and Poretz, R. 1997. Evaluation of commercial chlorophyllin copper complex preparations by liquid chromatography with photodiode array detection. *J. AOAC Int.* **80**, 433–435.

Cordevo, B., Otero, A., Patino, M., Arredondo, B.O., and Fabregas, J. 1996. Astaxanthin production from the green alga *Haematococcus pluvialis* with different stress conditions. *Biotechnol. Lett.* **18**, 213–218.

Coultate, T.P. 1996. "Food. The Chemistry of Its Compounds" 3rd Ed. Royal Society of Chemistry, Cambridge.

Counsell, J.N., Jeffries, G.S., and Knewstubb, C.J. 1981. Some other natural colours and their applications. *In* "Natural Colours for Food and Other Uses" (J.N. Counsell, ed.), pp. 123–151. Applied Science Publishers, London.

Cserhati, T. and Forgacs, E. 2003. "Cyclodextrins in Chromatography". Royal Society of Chemistry, Cambridge.

Dangles, O., Stoeckel, C., Wigand, M.C., and Brouillard, R. 1992a. Two very distinct types of anthocyanin complexation: Copigmentation and inclusion. *Tetrahedron Lett.* **33**, 5227–5230.

Dangles, O., Wigand, M.C., and Brouillard, R. 1992b. Anthocyanin anti-copigment effect. *Phytochemistry* **31**, 3811–3812.

Dangles, O., Saito, N., and Brouillard, R. 1993. Anthocyanin intramolecular copigment effect. *Phytochemistry* **34**, 119–124.

Darko, E., Schoefs, B., and Schoefs, B. 2000. An improved LC method for the analysis of photosynthetic pigments of higher plants. *J. Chromatogr.* **876A**, 111–116.

Datzberger, K., Steiner, I. Washuttl, J., and Kroyer, G. 1991. Methods for the fast analysis of anthocyanins and anthocyanidins in red wine. *Z. Lebensm. Unters. Forsch.* **193**, 462–464.

De Ell, J.R. and Toivonen, P.M.A. 1999. Chlorophyll fluorescence as an indication of physiological changes in cold-stored broccoli after transfer to room temperature. *J. Food Sci.* **64**, 501–503.

Depee, S., West, C.E., Permaesih, D., Martuti, S., Muhilal, A., and Hautvast, J.G.A.J. 1998. Orange fruit is more effective than dark-green vegetables in increasing serum concentrations of retinol and β-carotene in school children in Indonesia. *Am. J. Clin. Nutr.* **68**, 1058–1067.

Do, C.B. and Cormier, F. 1990. Accumulation of anthocyanins enhanced by a high osmotic potential in grape (*Vitis vinifera* L.) cell suspension. *Plant Cell Rep.* **9**, 143–146.

Dufresne, C., Cormier, F., Dorion, S., Niggli, U.A., Pfister, S., and Pfander, H. 1999. Glycosylation of encapsulated crocetin by a *Crocus sativus* L. cell culture. *Enzyme Microbial. Technol.* **24**, 453–462.

Eckhardt, C.B., Keely, B.J., and Maxwell, J.R. 1991. Identification of chlorophyll transformation products in a lake sediment by combined liquid chromatography and mass spectrometry. *J. Chromatogr.* **557**, 271–278.

Ellsworth, R.K. 1971. Simple method for separation of milligram quantities of photochlorophyll *a* from seed oils in extracts of whole pumpkin seeds. *Anal. Biochem.* **39**, 540–542.

Emenhiser, C., Sander, L.C., and Schwartz, S.J. 1995. Capability of polymeric C30 stationary phase to resolve *cis-trans*-carotenoid isomers in reversed phase column for the reparation and identification of saponified orange juice carotenoids. *J. Chromatogr. A.* **707**, 205–216.

Favati, F., King, J.W., Friedrich, J.P., and Eskins, K. 1988. Supercritical carbon dioxide extraction of carotene and lutein from leaf protein concentrates. *J. Food Sci.* **53**, 1532–1535.

Fenn, J.B., Mann, M., Meng, C.K., Wong, S.F., and Whitehouse, C.M. 1989. Electrospray ionization for mass spectrometry of large biomolecules. *Science* **246**, 64–71.

Ford, E.S., Liu, S., Mannino, D.M., Giles, W.H., and Smith, S.J. 2003. C-reactive protein concentration and concentrations of blood vitamins, carotenoids, and selenium among United States adults. *Eur. J. Clin. Nutr.* **57**, 1157–1163.

Foss, P. 1984. Carotenoids in diets for salmons. I. Pigmentation of rainbow trout with the individual optical isomers of astaxanthin in comparison with canthaxanthin. *Aquaculture* **41**, 213–226.

Franceschi, S., Bidoli, E., Vecchia, C., Talamini, R., D'Avanzo, B., and Wegni, E. 1994. Tomatoes and risk of digestive–tract cancers. *Int. J. Cancer* **59**, 181–184.

Francis, F.J. 1987. Lesser-known food colorants. *Food Technol.* **41**, 62–68.

Francis, F.J. 1989. Food colorants: Anthocyanins. *Crit. Rev. Food Sci. Nutr.* **28**, 273–315.

Francis, F.J. 1992. A new group of food colorants. *Trends Food Sci.* **3**, 27–31.

Fraser, P.D., Truesdale, M.R., Brid, C.R., Schuch, W., and Bramley, P.M. 1994. Carotenoid biosynthesis during tomato fruit development (evidence for tissue-specific gene expression). *Plant Physiol.* **105**, 405–413.

Furatu, S., Suda, I., Nishiba, Y., and Yamakawa, O. 1998. High tert-butylperoxyl radical scavenging activities of sweet potato cultivars with purple flesh. *Food Sci. Technol. Int. Tokyo* **4**, 33–35.

Gao, L. and Mazza, G. 1996. Extraction of anthocyanin from purple sunflower hulls. *J. Food Sci.* **61**, 600–603.

Garcia-Viguera, C., Espin, J.C., Soler, C., and Wichers, H.J. 2000. Antioxidant activity of anthocyanin colorants. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 88–94. SIC Publishing, Hamden.

Garrido, J.L., Otero, J., Maestro, M.A., and Zapata, M. 2000. The main nonpolar chlorophyll *c* from *Emiliana huxleyi* (Prymnesiophyceae) is a chlorophyll c2-monogalactosyldiacylglyceride ester: A mass spectrometry study. *J. Phycol.* **36**, 497–505.

Geiselmann, H., Houmard, J., and Schoefs, B. 2004. Regulation of phycobilisome biosynthesis and degradation in Cyanobacteria. *In* "Handbook of Photosynthesis" (M. Pessarakli, ed.), 2nd Ed. Marcel Dekker, New York.

Gerster, H. 1993. Anticarcinogenic effect of common carotenoids. *Int. J. Vitam. Nutr. Res.* **63**, 93–121.

Giusti, M.M. and Wrolstad, D.E. 1996a. Characterization of red radish anthocyanins. *J. Food Sci.* **61**, 322–326.

Giusti, M.M. and Wrolstad, R.E. 1996b. Radish anthocyanin extract as a natural red colorant for maraschino cherries. *J. Food Sci.* **61**, 688–694.

Giusti, M.M., Rodriguez-Saona, L.E., Griffin, D., and Wrolstad, D.E. 1999a. Electrospray and tandem mass spectrometry as tools for anthocyanin characterization. *J. Agr. Food Chem.* **47**, 4657–4664.

Giusti, M.M., Rodriguez-Saona, L.E., Griffin, D., and Wrolstad, D.E. 1999b. Molar abortivity and color characteristics of acylated and non-acylated pelargonin-based anthocyanins. *J. Agr. Food Chem.* **47**, 4631–4637.

Giusti, M.M., Wrolstad, D.E., and Rodriguez-Saona, L.E. 2000. The use of electrospray and tandem mass spectrometry as a tool for the characterization of anthocyanin pigments. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 95–109. SIC Publishing, Hamden.

Gomez, R., Pardo, J.E., Navarro, F., and Varon, R. 1998. Colour differences in paprika paper varieties (*Capsicum annuum*) cultivated in a greenhouse and in the open air. *J. Sci. Food Agr.* **77**, 268–272.

Gonnet, J.F. 1998. Color effects of co-pigmentation of anthocyanins revisited—1. A colorimetric definition using the CIELAB scale. *Food Chem.* **63**, 409–415.

Goswani, B.C. and Barua, A.B. 2003. Synthesis of tracer labelled [11,12-H^3]-β-carotene. *J. Labelled Compounds Radiopharm.* **46**, 843–849.

Goto, T. 1987. Structure, stability and color variation of natural anthocyanins. *Prog. Chem. Org. Nat. Prod.* **52**, 113–158.

Goto, T. and Kondo, T. 1991. Structure and molecular stacking of anthocyanin-flower color variation. *Angew. Chem. Int. Ed. Engl.* **30**, 17–33.

Goulson, M.J. and Warthesen, J.J. 1999. Stability and antioxidant activity of β–carotene in conventional and high oleic canola oil. *J. Food Sci.* **64**, 996–999.

Gross, J. 1991. "Pigments in Vegetables. Chlorophyll and Carotenoids". Van Nostrand Reinhold, New York.

Gross, J., Gabai, M., and Lifshitz, A. 1972. Carotinoids in juice of Shamouti orange. *J. Food Sci.* **36**, 466–473.

Groten, J.P., Butler, W., Feron, V.J., Kazianowski, G., Renwicks, A., and Walker, R. 2000. An analysis of the possibility for health implications of joint action and interactions between food additives. *Regul. Toxicol. Pharmacol.* **31**, 77–91.

Gürenç, A. and Karaali, A. 2000. Extraction and characterization of anthocyanins in sunflower seed hulls. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 135–145. SIC Publishing, Hamden.

Haila, K.M., Nielsen, B.R., Heinonen, M.I., and Skibsted, L.H. 1997. Carotenoid reaction with free radicals in acetone and toluene at different oxygen partial pressure. *Food Res. Technol.* **204**, 81–87.

Harborne, J.B. 1958. The chromatographic identification of anthocyanin pigments. *J. Chromatogr.* **1**, 473–488.

Harborne, J.B. and Mabuy, T.J. 1982. The flavonoids: Advances in research. *In* ''Anthocyanins'', pp. 158–188. Chapman and Hall, London.

He, X-G., Lin, L-Z., Lian, L-Z., and Lindenmaier, M. 1998. Liquid chromatography-electrospray mass spectrometric analysis of curcuminoids and sesquiterpenoids in curmeric (*Curcuma longa*). *J. Chromatogr. A.* **818**, 127–132.

Heath, D.D., Pruitt, M.A., Brenner, D.E., and Rock, C.L. 2003. Curcumin in plasma and urine: Quantitation by high-performance liquid chromatography. *J. Chromatogr. A.* **783**, 287–295.

Hendry, G.A. and Houghton, J.D. 1996. ''Natural Food Colorants''. Chapman & Hall, London.

Hirasuna, T.J., Shuler, M.L., Lackney, V.K., and Spanswick, R.M. 1991. Enhanced anthocyanin production in grape cell cultures. *Plant Sci.* **78**, 107–120.

Hong, V. and Wrolstad, R.E. 1990. Use of HPLC separation/photodiode array detection for characterization of anthocyanins. *J. Agr. Food Chem.* **38**, 708–715.

Hong, C.M., Wendorf, W.L., and Bradley, R.L. 1995. Effects of packaging and lighting on pink discoloration and lipid oxidation of annatto-colored cheeses. *J. Dairy Sci.* **78**, 1896–1902.

Hornero-Méndez, D. and Minguez-Mosquera, M.I. 1998. Isolation and identification of the carotenoid capsolutein for *Capsicum annuum* as cucurbitaxanthin A. *J. Agric. Food Chem.* **46**, 4087–4090.

Humphrey, A.M. 1980. Chlorophyll. *Food Chem.* **5**, 57–67.

Hutton, T. and Major, H.J. 1995. Characterizing biomolecules by electrospray ionization-mass spectrometry coupled to liquid chromatography and capillary electrophoresis. *Biochem. Soc. Trans.* **23**, 924–927.

Hyvärinen, K. and Hynninen, P.H. 1999. Liquid chromatographic separation and mass spectrometric identification of chlorophyll *b* allomers. *J. Chromatogr. A.* **837**, 107–116.

Ibanez, E., Lopez-Sebastian, S., Tabera, J., and Reglero, G. 1998. Separation of carotenoids by subcritical fluid chromatography with coated, packed capillary columns and neat carbon dioxide. *J. Chromatogr. A.* **823**, 313–319.

Ikan, R. 1991. ''Natural Products. A Laboratory Guide''. Academic Press, San Diego.

Ilik, P., Kouril, R., Fiala, J., Naus, J., and Vacha, F. 2000. Spectral characterization of chlorophyll fluorescence in barley leaves during linear heating. Analysis of high-temperature fluorescence rise around 60 °C. *J. Photochem. Photobiol. B Biol.* **59**, 103–114.

Irvine, G.N. and Winkler, C.A. 1950. Factor affecting the color of macaroni. II. Kinetic studies of pigment destruction during mixing. *Cereal Chem.* **27**, 205–218.

Irvine, G.N. and Anderson, J.A. 1953. Variation in principal quality factors of durum wheats with a quality prediction test for wheat or semolina. *Cereal Chem.* **30**, 334–342.

Iwahori, S. and Lyons, J.M. 1970. Maturation and quality of tomatoes with preharvest treatments of 2-chloroethyl-phosphonic acid. *J. Am. Hort. Sci.* **95**, 88–91.

Jeffrey, S.W. and Humphrey, G.F. 1975. New spectrophotometric equations for determining chlorophylls *a, b,* c_1, and c_2 in higher plants, algae, and natural phytoplankton. *Biochem. Physiol. Pflanzen* **167**, 191–194.

Johnson, E.A. and An, G.-H. 1991. Astaxanthin from microbial sources. *Crit. Rev. Biotech.* **11**, 297–326.

Johnson, T.V. and Morris, J.R. 1996. Separation of anthocyanin pigments in wine by low pressure column chromatography. *J. Food Sci.* **61**, 109–111.

Jones, I.D., White, R.C., Gibbs, E., Butler, L.S., and Nelson, L.A. 1977. Experimental formation of zinc and copper complexes of chlorophyll derivatives in vegetable tissue by thermal processing. *J. Agric. Food Chem.* **25**, 149–153.

Joppen, J. 2003. Back to nature. *Food Eng. Ingredients* **28**, 12–13.

Jung, M.Y. and Min, D.B. 1991. Effects of quenching mechanisms of carotenoids on the photosensitized oxidation of soybean oil. *J. Am. Oil Chem. Soc.* **68**, 653–658.

Karlsen, A., Alexander, G., Blomhoff, R., and Gundersen, T.E. 2003. Capillary high-performance liquid chromatography determination of lutein and zeaxanthin in aqueous humor of a single mouse eye. *J. Chromatogr. B.* **795**, 17–23.

Kawashima, S. and Nakatani, M. 1998. An algorithm for estimating chlorophyll content in leaves using a video camera. *Ann. Botul.* **81**, 49–54.

Kobrehel, K., Laignelet, B., and Feillet, P. 1972. Relation between the peroxidase and polyphenoloxidase activities of hard wheats and the browning of dough. *C. R. Acad. Agric. Fr.* **58**, 1099–1106.

Kobrehel, K., Laignelet, B., and Feillet, P. 1974. Factors of macaroni brownness. *Cereal Chem.* **51**, 675–684.

Kohata, K., Hanada, K., Yamauchi, Y., and Horie, H. 1998. Pheophorbide a content and chlorophyllase activity in green tea. *Biosci. Biotechnol. Biochem.* **62**, 1660–1663.

Kopecky, J., Schoefs, B., Loest, K., Stys, D., and Pulz, A. 2000. Microalgae as a source for secondary carotenoid production: a screening study. O. *Arch. Hydrobiol. Algol. Stud.* **98**, 153–168.

Koyama, Y. and Hashimoto, H. 1993. Spectroscopic studies of carotenoids in photosynthetic systems. *In* "Carotenoids in Photosynthesis" (G. Britton and A.J. Young, eds), pp. 327–408. Chapman & Hall, London.

Krinsky, N.I., Landrum, J.T., and Bone, R.A. 2003. Biologic mechanisms of the protection role of lutein and zeaxanthin in the eye. *Annu. Rev. Nutr.* **23**, 171–201.

Kumpulainen, J.T. and Salonen, J.T. 1996. "Natural Antioxidants and Food Quality in Atherosclerosis and Cancer Prevention". The Royal Society of Chemistry, Cambridge.

LaBorde, L.F. and von Elbe, J.H. 1990. Zinc complex formation in heated vegetable purees. *J. Agric. Food Chem.* **38**, 484–487.

Lacker, T., Stroschein, S., and Albert, K. 1999. Separation and identification of various carotenoids by C30 reversed-phase high-performance liquid chromatography coupled to UV and atmospheric pressure chemical ionization mass spectrometric detection. *J. Chromatogr. A.* **854**, 37–44.

Lancaster, J.E., Lister, C.E., Reay, P.F., and Triggs, C.M. 1997. Influence of pigment composition on skin color in a wide range of fruit and vegetables. *J. Am. Soc. Hortic. Sci.* **122**, 594–598.

Latasa, M., van Lenning, K., Garrido, J.L., Scharek, R., Estrada, M., Rodriguez, F.F., and Zapata, M. 2001. Losses of chlorophylls and carotenoids in aqueous acetone and methanol extracts prepared by RPHPLC analysis of pigments. *Chromatographia* **53**, 385–391.

Le Jeune, H., Joubert, Y., Vilaume, C., and Fleurence, J. 2003. Propriétés et intérêt biotechnologique d'un pigment algal: la R-phycoérythrine. *Biofutur.* **236**, 34–38.

Lewis, C.E., Walker, J.R.L., and Lancaster, J.E. 1995. Effect of polysaccharides on the colour of anthocyanins. *Food Chem.* **54**, 315–319.

Lim, M.H., Velasco, P.J., Pangborn, R.M., and Whitaker, J.R. 1989. Enzymes involved in off-aroma formation in broccoli. *In* "Quality Factors in Fruits and Vegetables" (M.J. Comstock, ed.), pp. 73–82. ACS Symposium Series 405. American Chemical Society, Washington, DC.

Ma, X.-W. and Shimokawa, K. 1998. *In vitro* studies of ethylene-enhanced chlorophyll degrading peroxidase and its reaction products in banana (*Musa sapientum* L.). *J. Jpn. Soc. Hortic. Sci.* **67**, 3–15.

Maccarone, E., Campisi, C., Cataldi Lupo, M.C., Fallico, B., and Nicolosi Asmundo, C. 1996. Thermal treatment effects on the red orange juice constituents. *Ind. Bevande* **25**, 335–341.

Mannina, L., Sobolev, A.P., and Segre, A. 2003. Olive oil as seen by NMR and chemometrics. *Spectrosc. Eur.* **15**, 6–14.

Markakis, P. 1982. Anthocyanins as food additives. *In* "Anthocyanins as Food Colors" (P. Markakis, ed.), pp. 245–253. Academic Press, New York.

Martin, H-D., Ruck, C., Schmidt, M., Sell, S., Beutner, S., Mayer, B., and Walsh, R. 1999. Chemistry of carotenoid oxidation and free radical reactions. *Pure Appl. Chem.* **71**, 2253–2262.

Martinez Parra, J. and Munoz, R. 1997. An approach to the characterization of betanine oxidation catalyzed by horseradish peroxidase. *J. Agric. Food Chem.* **45**, 2984–2988.

Matsuno, T., Maoka, T., Katsuyama, M., Ookubo, M., Katagiri, K., and Timura, H. 1984. Carotenoids in shellfish. VII. Carotenoids of the spindle shell *Fusinis perplexus*. *Bull. Jpn. Soc. Sci. Fish* **50**, 1589–1592.

Matile, P., Hörstensteiner, S., and Thomas, H. 1999. Chlorophyll degradation. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50**, 67–95.

Mayne, S.T., Janerich, D.T., Greenwald, P., Chorost, S., Tucci, C., Zaman, M.B., Melamed, R., Kiely, M., and McKneally, M.F. 1994. Dietary β-carotene and lung cancer risk in U.S. nonsmokers. *J. Natl. Cancer Inst.* **86**, 33–38.

McClements, D.J., Chantopromchal, W., and Clydesdale, F. 1998. Prediction of food emulsion color using light scattering theory. *J. Food Sci.* **63**, 935–939.

Mercadante, A.Z., Britton, G., and Rodriguez-Amaya, D.B. 1998. Carotenoids from yellow passion fruit (*Passiflora edulis*). *J. Agric. Food Chem.* **46**, 4102–4106.

Mercadante, A.Z. and Rodriguez-Amaya, D.B. 1998. Effects of ripening, cultivar differences, and processing on the carotenoid composition of mango. *J. Agric. Food. Chem.* **46**, 128–130.

Minguez-Mosquera, M.I. and, Gandul-Rojas, B. 1995. High performance liquid chromatographic study of alkaline treatment of chlorophyll. *J. Chromatogr. A.* **690**, 161–171.

Minguez-Mosquera, M.I., Homero-Mendez, D., and Gandul-Rojas, B. 1995a. Detection of bixin, lycopene, canthaxanthin, and β-apo-8′-carotenal in products derived from red pepper. *JAOAC Int.* **78**, 491–496.

Minguez-Mosquera, M.I., Gallardo-Guerrero, L., Homero-Mendez, D., and Garrido-Fernandez, J. 1995b. Anomalous transformation of chloroplastic pigments in gordal variety olives during processing for table olives. *J. Food Prot.* **58**, 564–569.

Muftugil, N. 1986. Effect of different types of blanching on the color and the ascorbic acid and chlorophyll contents of green beans. *J. Food Proc. Preserv.* **10**, 69–76.

Nebdal, L., Soukupova, J., Whitmarsh, J., and Trtilek, M. 2000. Postharvest imaging of chlorophyll fluorescence from lemons can be used to predict fruit quality. *Photosynthetica* **38**, 571–579.

Nelis, H.J. and De Leenheer, A.P. 1991. Microbial sources of carotenoid pigments used in foods and feeds. *J. Appl. Bacteriol.* **70**, 181–191.

Newsome, R.L. 1990. Natural and synthetic coloring agents. *In* "Food Additives" (A.L. Branen, P.M. Davidson, and S. Salminen, eds), pp. 327–345. Marcel Dekker, New York.

Omata, T. and Murata, N. 1983. Preparation of chlorophyll *a*, chlorophyll *b*, and bacteriochlorophyll *a* by column chromatography with DEAE-sepharose CL-6B and sepharose CL-6B. *Plant Cell Physiol.* **22**, 1093–1100.

Oszmianski, J. and Lee, C.Y. 1990. Isolation and HPLC determination of phenolic compounds in red grapes. *Am. J. Enol. Vitic.* **41**, 204–206.

Ozeki, Y. and Komamine, A. 1985. Effect of inoculum density, zeatin and sucrose on anthocyanin accumulation in carrot suspension cultures. *Plant Cell Tissue Organ Culture* **5**, 45–53.

Palozza, P. and Krinsky, N.I. 1991. The inhibition of radical-initiated peroxidation of microsomal lipids by both α-tocopherol and β-carotene. *Free Radical Biol. Med.* **11**, 407–414.

Parkinson, T.M. and Brown, J.P. 1981. Metabolic fate of food colorants *Ann. Rev. Nutr.* **1**, 175–205.

Pereira, M.R., Amaya-Farfan, J., and Rodriguez-Amaya, D. 1999. β-Carotene content in Brazilian fortified pasta. *Food Control* **10**, 81–85.

Pfander, H., Traber, B., and Häberli, A. 2000. Oxidation products of carotenoids in food. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 213–220. SIC Publishing, Hamden.

Porra, R.J., Thompson, W.A., and Kriedemann, P. 1989. Determination of accurate extinction coefficients and simultaneous equations for assaying chlorophylls *a* and *b* with for different solvents: Verification of the concentration of chlorophyll standards by atomic absorption spectroscopy. *Biochim. Biophys. Acta* **975**, 384–394.

Pszczola, D.E. 1988. Natural colors: Pigments of imagination. *Food Technol.* **52**, 70–82.

Rabinovich, E.I. 1956. "Photosynthesis and Related Processes", Vol. 2, Part 2. Interscience Publishers, New York.

Ravishankar, G.A. and Venkataraman, L.V. 1990. Food applications of plant cultures. *Curr. Sci.* **59**, 914–920.

Ravishankar, G.A., Sarada, R., Tripathi, U., and Bhagyalakshmi, N. 2000. Production of food colors by plant cell and microalgal cultures. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 221–252. SIC Publishing, Hamden.

Rendström, B., Borch, G., Skulberg, O.M., and Liaaen-Jensen, S. 1981a. Natural occurrence of enantiomeric and meso-astaxanthin. Part 3. Optical purity of (3*S*,3'*S*)-astaxanthin from *Haematococcus pluvialis*. *Phytochemistry* **20**, 2561–2564.

Rendström, B., Borch, G., and Liaaen-Jensen, S. 1981b. Natural occurrence of enantiomeric and meso-astaxanthin. 4. Ex-shrimp (*Pandalus borealis*). *Comp. Biochem. Physiol.* **69 B**, 621–624.

Renaud, S. and Lorgeril, M. 1992. Wine, alcohol, platelets and the French paradox for coronary heart disease. *Lancet* **339**, 1523–1526.

Robertson, G.L. 1985. Changes in the chlorophyll and pheophytin concentrations of kiwifruit during processing and storage. *Food Chem.* **17**, 25–32.

Rodriguez, D.B., Raymundo, L.C., Lee, T.C., Simpson, K.L., and Chichester, C.O. 1976. Carotenoid pigment changes in ripening *Mormordica charantia* fruits. *Ann. Bot.* **40**, 615–624.

Rodriguez-Amaya, D.B. 1989. Critical review of provitamin A determination in plant foods. *J. Micronutr. Anal.* **5**, 191–225.

Rodriguez-Amaya, D.B. 2000. Factor influencing carotenoid composition in foods. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 252–263. SIC Publishing, Hamden.

Rodriguez-Saona, L.E., Giusti, M., and Wrolstad, R.E. 1998. Anthocyanin pigment composition of red-fleshed potatoes. *J. Food Sci.* **63**, 458–463.

Rodriguez-Saona, L.E., Giusti, M., and Wrolstad, R.E. 2000. Properties of anthocyanin extracts from radish and red-fleshed potatoes. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods, Neutraceuticals, Beverages, Confectionery & Cosmetics" (J.F. Francis, ed.), pp. 264–282. SIC Publishing, Hamden.

Rohacek, K. and Bartak, M. 1999. Technique of the modulated chlorophyll fluorescence: Basic concepts, useful parameters, and some applications. *Photosynthetica* **37**, 339–363.

Rommel, A., Wrolstad, R. E., and Heatherbell, D.A. 1992. Blackberry juice and wine: Processing and storage effects on anthocyanin composition, color and appearance. *J. Food Sci.* **57**, 385–391.

Rousseff, R., Raley, L., and Hofsommer, H.-J. 1996. Application of diode array detection with a C-30 reversed phase column for the separation and identification of saponified orange juice. *J. Agric. Food Chem.* **44**, 2176–2181.

Sakuta, M., Takagi, T., and Komamine, A. 1987. Growth related accumulation of betacyanin in suspension cultures of *Phytolacca americana* L. *J. Plant Physiol.* **125**, 337–345.

Salin, M.L., Avarez, M.L., Lynn, B.C., Habulihaz, B., and Fountain, A.W. 1999. Photooxidative bleaching of chlorophyllin. *Free Radical Res.* **31**, 97–105.

Sarada, R., Manoj, G.P., and Ravishankar, 1999. Cyanin from *Spirulina* sp.: Influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. *Process Biochem.* **34**, 795–801.

Sarni-Manchado, P., Fulcrand, H., Souquet, J-M., Cheynier, V., and Moutounet, M. 1996. Stability of the color of unregistered wine anthocyanin-derived pigments. *J. Food Sci.* **61**, 938–941.

Sato, M., Mikuni, K., and Hashimoto, H. 2000. Cyclodextrins as stabilizers for natural colorants. *In* "Proceedings of the 4th International Symposium on Natural Colorants for Foods,

Neutraceuticals, Beverages, Confectionery & Cosmetics'' (J.F. Francis, ed.), pp. 288–297. SIC Publishing, Hamden.

Scheer, H. 1996. Tetrapyrroles in photosynthesis. *In* ''Biochemistry, Biophysic and Molecular Biology of Photosynthesis'' (B. Schoefs, F. Franck, and J. Aghion, eds). *Bull. Soc. R. Sci. Liège* **65**, 310–321.

Schiedt, K. and Liaaen-Jensen, S. 1995. Isolation and analysis. *In* ''Carotenoids'' (G. Britton, S. Liaaen-Jensen, and H. Pfander, eds), ''Isolation and Analysis'', Vol. 1A, pp. 81–108. Birkhäuser, Basel.

Schmitz, H.H., Emenhiser, C., and Schwartz, S.J. 1995. HPLC separation of geometric carotene isomers using a calcium hydroxide stationary phase. *J. Agric. Food Chem.* **43**, 1212–1218.

Schoefs, B. 2000a. Pigment analysis of pumpkin seed oil. *In* ''Der Ölkürbis *(Cucurbita pepo)*'' (M. Murkovic, ed.), pp. 50–59. Technische Universität Graz, Graz.

Schoefs, B. 2000b. The pigment compositing pumpkin seed oil. *In* ''Proceedings of the 4th International Symposium on Natural Colorants'' (J. Francis, ed.), pp. 85–92. SCI Publishers, Hamden.

Schoefs, B. 2001a. Analysis of tetrapyrrole pigments in food stuffs. *Int. Lab.* **33**, 34–39.

Schoefs, B. 2001b. Analyse des Farbstoffe in Kürbiskernöl. *Lebendmittel und Biotechnologie* **1**, 8–10.

Schoefs, B. 2002. Chlorophyll and carotenoid analysis in food products. Properties of the pigments and methods of analysis. *Trends Food Sci. Technol.* **13**, 361–371.

Schoefs, B. 2003. Chlorophyll and carotenoid analysis in food products. A practical case-by-case view. *Trends Anal. Chem.* **22**, 335–339.

Schoefs, B. 2004. Determination of pigments in vegetables. *J. Chromatogr. A* **1054**, 217–226.

Schoefs, B., Bertrand, M., and Lemoine, Y. 1995. Improved liquid chromatographic method for the analysis of photosynthetic pigments of higher plants. *J. Chromatogr. A* **692**, 239–245.

Schoefs, B., Lemoine, Y., and Bertrand, M. 1996. Reversed-phase HPLC separation of photosynthetic pigments and their precursors. *Am. Biotechnol. Lab.* **14**, 18–22.

Schoefs, B., Rmiki, N., Rachidi, J., and Lemoine, Y. 2001. The accumulation of astaxanthin in *Haematococcus pluvialis* cultivated in light-light stress condition requires a cytochrome P450-dependent enzyme and an active synthesis of fatty acids. *FEBS Lett.* **500**, 125–128.

Schwartz, S.J. and Lorenzo, T.V. 1991. Chlorophyll stability during continuous aseptic processing and storage. *J. Food Sci.* **56**, 1059–1062.

Schwartz, S.J., Woo, S.L., and von Elbe, J.H. 1981. High-performance liquid chromatography and their derivatives in fresh and processed spinach. *J. Agric. Food Chem.* **29**, 533–535.

Sharpless, K.E., Thomas, J.B., Sanders, L.C., and Wise, S.A. 1996. Liquid chromatographic determination of carotenoids in human serum using an engineered C30 and a C18 stationary phase. *J. Chromatogr. B* **678**, 187–196.

Shih, C.C. and Whiley, R.C. 1981. Betacyanin and betaxanthin decolorizing enzymes in the beet (*Beta vulgaris* L) root. *J. Food Sci.* **47**, 164–166, 172.

Shim, J.S., Kim, J.H., Cho, H-Y., Yum, Y.N., Kim, S.H., Park, H-J., Shim, B.S., Choi, S.H., and Kwon, H.J. 2003. Irreversible inhibition of CD13/aminopeptidase N by the angiogenic agent curcumin. *Chem. Biol.* **10**, 695–704.

Siegelman, H.W. and Kycia, J.H. 1978. Algal biliproteins. *In* ''Handbook of Physiological Methods'' (J.A. Hellebust and J.S. Craigie, eds), Vol. 2, pp. 71–81. Cambridge University Press, Cambridge.

Singleton, V.L. and Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic phosphotungstic acid reagents. *Am. J. Enol. Vitic* **16**, 144–158.

Slaughter, D.C., Barrett, D., and Boersig, M. 1996. Nondestructive determination of soluble solids in tomatoes using near infrared spectroscopy. *J. Food Sci.* **61**, 695–697.

Smith, R.M. 1988. Supercritical Fluid Chromatography. RSC Chromatography Monographs, London.

Smith, J. 1991. ''Food Additive User's Handbook''. Van Nostran Reynold, New York.

Somers, T.C. 1966. Wine tannins. Isolation of condensed flavonoid pigments by gel filtration. *Nature* **209**, 368–370.

Somers, T.C. and Evans, M.E. 1977. Spectral evaluation of young red wines: Anthocyanin equilibria, total phenolics, free and molecular sulfur dioxide, "Chemical Age". *J. Sci. Food Agric.* **28**, 279–287.

Stahl, W. and Sies, H. 1992. Uptake of lycopene and its geometrical isomers is greater from heat-processed than unprocessed tomato juice in humans. *J. Nutr.* **122**, 2161–2166.

Steet, J.A. and Tong, C.H. 1996. Degradation kinetics of green color and chlorophylls in peas by colorimetry and HPLC. *J. Food Sci.* **61**, 924–931.

Stintzing, F.C., Schierber, H., and Carle, R. 2000. Cactus pear—a promising component to functional food. *Obst. Gemüse. Kartoffelverarbeitung*.

Storebakken, T. and No, H.K. 1992. Pigmentation of rainbow trout. *Aquaculture* **100**, 209–229.

Strain, H.H., Cope, B.T., and Svec, W.A. 1971. Analytical procedures for the isolation, identification, estimation, and identification of the chlorophylls. *Meth. Enzymol.* **23A**, 452–476.

Sweeny, J.C. and Iacobucci, G.A. 1983. Effect of substitution on the stability of 3-deoxyanthocyanidins in aqueous solutions. *J. Agric. Food Chem.* **31**, 531–533.

Takeoka, G.R., Dao, L.T., Full, L.T., Wong, R.Y., Harden, L.A., Edwards, R.H., and Berrios, J.D.J. 1997. Characterization of black bean (*Phaseolus vulgaris* L.) anthocyanins. *J. Agric. Food Chem.* **45**, 3395–3400.

Tang, W. and Eisenbrand, G.F. 1992. "Chinese Drug of Plant Origin". Springer Verlag, Berlin.

Taungbodhitham, A.K., Jones, G.P., Wahqvist, M.L., and Briggs, D.R. 1998. Evaluation of extraction method for the analysis of carotenoids in fruits and vegetables. *Food Chem.* **63**, 577–584.

Terahara, N., Saito, N., Honda, T., Toki, K., and Osajima, Y. 1990. Structure of ternatin A1, the largest ternation in the major blue anthocyanins from *Clitoria ternatea* flowers. *Tetrahedron Lett.* **31**, 2921–2924.

Theerakulkait, C., Barret, D.M., and McDaniel, M.R. 1995. Lipoxygenase in sweet corn germ: Isolation and physicochemical properties. *J. Food Sci.* **60**, 1034–1040.

Thompson, K.A., Marshall, M.R., Sims, C.A., Wei, C.I., Sargent, S.A., and Scott, J.W. 2000. Cultivar, maturity, and heat treatment on lycopen content in tomatoes. *J. Food Sci* **65**, 791–795.

Tian, M.S., Woolf, A.B., Bowen, J.H., and Fergusson, I.B. 1996. Changes in color and chlorophyll fluorescence of broccoli florets following hot water treatment. *J. Am. Soc. Hort. Sci.* **121**, 310–313.

Tonnesen, H.H. and Karlsen, J. 1985. Studies of curcumin and curcuminoids. 5. Alkaline degradation of curcumin. *Z. Lebensm. Unters. Forsch* **180**, 132–134.

Tonnesen, H.H., Masson, M., and Loftsson, T. 2002. Studies of curcumin and curcuminoids. 27. Cyclodextrin complexation, solubility, chemical and photochemical stability. *Int. J. Pharm.* **244**, 127–135.

Torrissen, O.J., Hardy, R.W., and Shearer, K.D. 1989. Pigmentation of salmonoids-carotenoid deposition and metabolism. *Rev. Aquat. Sci.* **1**, 209–215.

Tricard, C., Cazabeil, J.M., and Medina, B. 1998. Identification of natural dyes added to food products. *Sci. Aliments* **18**, 25–40.

Triglia, A., La Malfa, G, Musumeci, F., Leonardi, C., and Scordino, A. 1998. Delayed luminescence as an indicator of tomato fruit quality. *J. Food Sci.* **63**, 512–515.

Trujillo-Quijano, J.A., Rodriguez-Amaya, D.B., Esteves, W., and Plonis, G.G. 1990. Carotenoid composition and vitamin A value of oils from four Brazilian plant fruits. *Fat. Sci. Technol.* **6**, 222–226.

Tsatsaroni, E., Liakopoulou-Kyriakides, M., and Eleftheradis, I. 1998. Comparative study of dyeing properties of two yellow natural pigments—effects of enzymes and proteins. *Dyes Pigments* **37**, 307–315.

Turujman, S.A. 1993. Rapid direct resolution of the stereoisomers of all-*trans* astaxanthin on a pirkle covalent L-leucine column. *J. Chromatogr.* **631**, 197–199.

Usha, T., Sarada, R., Ramachandra, Rao, S., and Ravishankar, G.A. 1999. Production of astaxanthin in *Haematococcus pluvialis* cultured in various media. *Bioresource Technol.* **68**, 197–199.

Vanheukelem, L., Lewitsu, J., Kata, T.M., and Craft, N.E. 1994. Improved separations of phytoplankton pigments using temperature controlled high performance liquid chromatography. *Mar. Ecol. Prog. Ser.* **114**, 303–313.

von Elbe, J.H., Huang, A.S., Attoe, E.L., and Nank, W.K. 1986. Pigment composition and color of conventional and Veri-Green canned beans. *J. Agric. Food Chem.* **34**, 52–54.

Wasley, J.W.F., Scott, W.T., and Holt, S. 1970. Chlorophyllides *c*. *Can. J. Botul.* **48**, 376–383.

Wathne, E., Bjerkeng, B., Storebakken, T., Vassvik, V., and Odland, A.B. 1998. Pigmentation of Atlantic salmon (*Salmo Salar*) fed astaxanthin in all meals or alternating meals. *Aquaculture* **159**, 231–317.

Willstäter, R. and Stöll, A. 1913. ''Untersuchungen über Chlorophyll''. Springer Verlag, Berlin.

Wyszecki, G. and Stiles, W.S. 1987. ''Color Science''. Wiley & Sons, New York.

Ye, X., Al-Babili, S., Kloti, A., Zhang, J., Lucca, P., Beyer, P., and Potrykus, I. 2000. Engineering the provitamin A (β-carotene) biosynthetic pathway into (carotenoid free) rice endosperm. *Science* **287**, 303–305.

Yeum, K.J., Taylor, A., Tang, G., and Russel, R.M. 1995. Measurement of carotenoids, retinoids, and tocopherols in human lenses. *Invest. Ophthalmol. Vis. Sci.* **36**, 2756–2761.

Yoshimoto, M., Okumo, S., Yoshinaga, M., Yamakawa, O., Yamaguchi, M., and Yamada, J. 1999. Antimutagenicity of sweetpotato (*Ipomoea batatas*) roots. *Biosci. Biotechnol. Biochem.* **63**, 537–541.

Zeichmeister, L. 1962. ''*Cis-trans* Isomeric Carotenoids, Vitamin A and Arylpolyenes''. Academic Press, New York.

Zhong, J.J., Seki, M., Furusaki, S., and Furuya, T. 1991. Effect of light irradiation on anthocyanin production by suspended cultures of *Perilla frutescens*. *Biotech. Bioeng.* **36**, 653–658.

Zolla, L. and Bianchetti, M. 2001. High-performance liquid chromatography coupled on-line with electrospray ionization mass spectrometry for the simultaneous separation and identification the *Synechocystis* PCC 6803 phycobilisome proteins. *J. Chromatogr.* **912A**, 269–279.