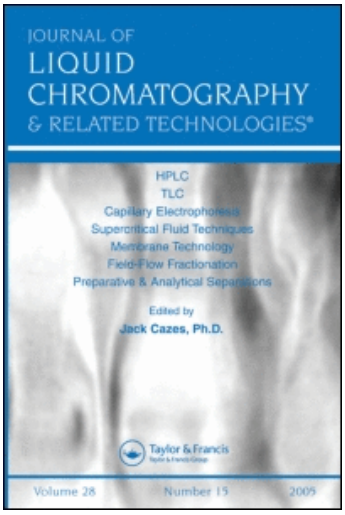


Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

High Performance Liquid Chromatography of Plant Pigments - A Review

S. J. Schwartz[a]; J. H. Von Elbe[a]
[a] Department of Food Science, University of Wisconsin-Madison Madison, WI

To cite this Article Schwartz, S. J. and Von Elbe, J. H.(1982) 'High Performance Liquid Chromatography of Plant Pigments - A Review', Journal of Liquid Chromatography & Related Technologies, 5: 12, 43 — 73
To link to this Article: DOI: 10.1080/01483918208082215
URL: http://dx.doi.org/10.1080/01483918208082215

JOURNAL OF LIQUID CHROMATOGRAPHY, 5(Suppl. 1), 43-73 (1982)

High Performance Liquid Chromatography of Plant Pigments - A Review

S. J. Schwartz

and

J. H. von Elbe

Department of Food Science

University of Wisconsin-Madison

Madison, WI 53706

ABSTRACT

High performance liquid chromatography (HPLC) has found wide application for qualitative and quantitative analysis of plant pigments. HPLC methods developed for the analysis of carotenoids, chlorophylls, flavonoids and betalaines are summarized. Compared to other chromatographic techniques the methods are precise, sensitive and less time consuming.

Sample preparation prior to chromatography is generally minimal and often a simple extraction step is sufficient. The high resolution capability of HPLC has made the separation of closely related pigments possible and has provided a means for major advances into the study of plant pigments.

Introduction

Separation and identification of plant pigments has been largely restricted to paper, thin-layer and column chromatography. Gas chroma-

tography (GC) has found only limited application in the analysis of pigments. High performance liquid chromatography (HPLC) was developed as a complementary tool to GC, particularly for the separation of non-volatile ionic compounds. It eliminated the need for derivatization and possible heat degradation of labile pigments. This technique offers many advantages. The high resolution capabilities of HPLC columns allows for the separation of complex mixtures of plant pigments. Analysis times, including sample preparation, are generally less than in other chromatographic methods, and in many instances, separation is complete within 30 min. The interaction of light energy with pigments makes them particularly suitable for HPLC methods which utilize spectrometry as a means of detection. The high sensitivity of ultraviolet-visible and fluorescence detectors make analysis of small sample sizes possible. Larger quantities can also be chromatographed by preparative HPLC which is particularly useful for the collection of individual components. Qualitative and quantitative methods have been developed for screening of plant pigments. These methods have provided for a large number of advances into the study of plant pigments and will continue to enhance the research efforts in this field.

Carotenoids

The carotenoids are the most widely distributed natural pigments found in higher plants. They range in color from red to yellow and are located in the chloroplast. In plants, their presence is often masked by chlorophyll. In his classical chromatography experiments for the separation of leaf pigments, Tswett (1906) observed both chlorophylls and carotenoids. The major carotenoid, carotene, was isolated and crystallized and later found to contain closely related isomers. Classical structural studies began in the 1920's when large enough quantities of carotenoids could be separated. The discovery that β-carotene has potential vitamin A activity (Steenbock, 1919) stimulated further research on carotenoid

pigments. Today over 400 naturally occurring carotenoids are known. The major carotenoids are ß-carotene, lutein, violaxanthin and neoxanthin. Those occurring in smaller amounts are α-carotene, ß-cryptoxanthin, zeaxanthin and antheraxanthin (Fig. 1). These compounds have been in the food supply throughout evolution and some are used today as colorants in foods and feeds. Some common carotenoids and their sources are shown in Table 1 (Isler, 1979). The distribution of carotenoids in plants and algae has been reviewed in detail (Goodwin, 1976).

The carotenoids are a class of compounds composed of isoprene units. Differences in carotenoid structures are derived by hydrogenation, dehydrogenation, cyclization and oxidation of the isoprenoid unit. The complex nature of these compounds has resulted in many trival names for various carotenoids. Definitive rules for the nomenclature of carotenoids have been published by IUPAC (1960).

The carotenoids are fat soluble pigments and are extracted by benzene, ether, carbon disulfide, ethanol or chloroform. In some instances, preliminary purification is required and usually involves basic hydrolysis of lipids or esters of hydroxylated carotenoids followed by chromatography. Care should be exercised to prevent long term exposure of the carotenoids to oxygen and light. Prolonged adsorption to certain stationary phases during chromatography should be minimal to avoid alteration of the pigments. Techniques for the isolation and purification of carotenoids have been reviewed by Liaaen-Jensen (1971) and Moss and Weedon (1976).

The advent of HPLC has provided a means for major advances into the study of carotenoid pigments. The highly conjugated structure of the carotenoids provides for their detection by ultraviolet and visible spectrophotometers. Table 2 lists some carotenoids and the wavelengths at which maximum light absorption occurs. The high resolution capability of HPLC has made the separation of closely related carotenoids possible, which previously was tedious or impossible.

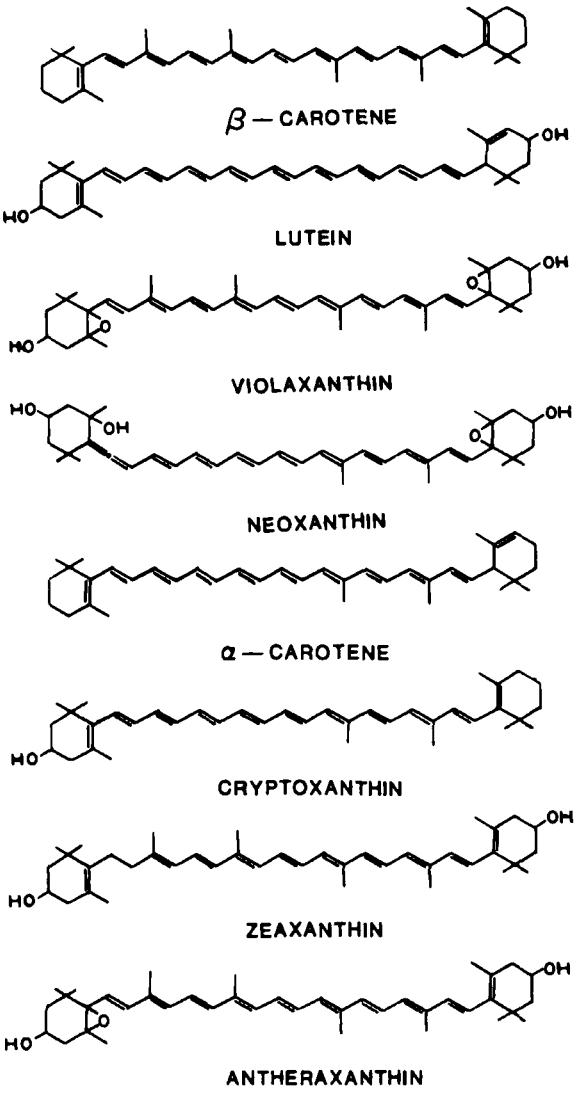


Figure 1. Structures of some carotenoids.

Table 1. Some carotenoids and their sources.

Class	Major Carotenoids	Other Carotenoids	Source
Carotenes	β-Carotene	α-Carotene	Carrots
	β-Carotene	α-Carotine	Red palm oil
		γ-Carotene	
	β-Carotene	Xanthophylls	Alfalfa
	Lycopene	Carotene	Tomato
Xanthophylls	Cryptoxanthin	Zeaxanthin	Corn
	Lutein	Carotenes	Alfalfa
	Capsanthin	-	Paprica
	Astaxanthin		Salmon
Carotenoic Acid	Crocin Crocetin	β-Carotene	Saffron
	Bixin	-	Annato

Many carotenoid pigments co-exist with chlorophylls and are often simultaneously analyzed in plant extracts (Abaychi and Riley, 1979; Baumann and Grimme, 1979; Davies and Holdsworth, 1980; De Jong and Woodlief, 1978; Eskins and Dutton, 1979; Eskins et al., 1977; Iriyama et al., 1978; Stransky, 1978).

The first HPLC technique utilized to separate carotenes was developed by Sweeney and Marsh (1970). A column packed with calcium and magnesium hydroxides was used to study carotene stereoisomers in vegetables. The method was applied to investigate the effects of food processing on isomerization. p-Methylanisole in petroleum ether (1.5%) was employed as the mobile phase to resolve seven isomers in less than one hour. Stewart and Wheaton (1971) employed HPLC to separate carotenoids and xanthophylls from citrus peels. The carotenoids were separated on a magnesium oxide column while a zinc carbonate column was used to separate xanthophylls.

Table 2. Some carotenoids and their wavelengths at maximum light absorption.

Carotenoid	Maximum Light Absorption			Solvent
Lycopene (trans)	505	474	446	
(cis)	502	470	444	
ß-Carotene	497	466		Chloroform
Zeaxanthin	483	451		Ethanol
α-Carotene	485	454		Chloroform
γ-Carotene	494	462	437	Petroleum Ether
	508	475	446	Chloroform
Astaxanthin	513	493	476	Pyridene
Bixin	509	475	443	Chloroform
Cryptoxanthin	480	452		
Crocetin	464	436	411	Pyridin
Crocin	464	434		Methanol

1. Compiled from: Merck Index 9th Edition Merck Co., Inc., Rahway, NJ (1976).

The mobile phase was a mixture of tert-pentyl alcohol in n-hexane containing 1% BHT as an antioxidant. Elution was accomplished by step-gradient system increasing the alcohol concentration. Twenty-three pigments were resolved of which thirteen were identified. Carotenoids, α- and ß-carotene and cryptoxanthin were analyzed by Reeder and Park (1975) to measure the provitamin A activity of orange juice. The method employs alumina as a stationary phase and 0.01% BHT in benzene:n-hexane (3:5 v/v) as mobile phase for the separation of carotenes. A silica stationary phase with a 0.01% BHT in tetrahydrofuran:n-hexane (1:5 v/v) as mobile phase was used for the separation of cryptoxanthin. The effect of carotenoids on the color of citrus products and problems associated with their analyses

by HPLC have been reviewed by Stewart and Leuenberger (1976). Stewart (1977a) expanded the method by Reeder and Park to separate five carotenoids in orange juice (α, β and ζ carotene and α and β cryptoxanthin). The method employs a single magnesia column and gradient elution system composed of a mixture of n-hexane and acetone or a mixture of hexane, acetone and water as the mobile phase. The method was later applied to measure carotenoid content during citrus fruit maturation (Stewart, 1977b). In these methods, detection of the pigment was monitored at 440 nm. Thompson and Maxwell (1977) applied a reverse-phase column and methanol:water mixture (99:1 v/v) as eluant to separate β-carotene from margarine, infant formula and fortified milk. The β-carotene was detected by monitoring the eluant at 453 nm. Hajibrahim et al. (1978) analyzed carotenoid and porphyrin pigments from geochemical sediments using silica gel as the stationary phase. A concave gradient system composed of 1-75% acetone in hexane or 2-50% acetone in hexane was employed. Detection of carotenoids in the eluant was monitored at 451 nm. This study was undertaken to assess the potential of HPLC in "fingerprinting" petroporphyrin distributions. The scope and limitations of HPLC on silica for the separation of carotenes, diols, cis-trans isomers and diastereoisomers in comparison with common TLC and paper chromatographic systems have been investigated by Fiksdahl et al. (1978). β-Carotene and lycopene were quantified in tomato samples by Zakaria et al. (1979). A reverse-phase column and a mixture of chloroform in acetonitrile was used as the eluant. Pigments were monitored in the eluant at 470 nm. Separation was complete within 15 min. The authors suggested that their method gives a more reliable evaluation of the vitamin A content in fruits and is more accurate than the current method used by the AOAC to establish vitamin A composition of foods. Landen and Eitenmiller (1979) combined high performance gel permeation chromatography with non-aqueous reverse-phase HPLC to simultaneously quantitate β-carotene and retinyl palmitate in oil and

margarine. The advantage of this approach is that it allows analysis without prior saponification of the sample. Saffron pigments were monitored at 440 nm using a reverse-phase column and acetonitrile as the mobile phase (Pfander et al., 1980). Four pigments, α, ß and γ carotene and lycopene were well resolved within fifteen min.

Chlorophylls

The green pigments of photosynthetic organisms are known collectively as the chlorophylls. They were first documented by Pelletier and Caventow (1818) in a study of the color of leaves and were later isolated by Sorby in 1873. Tswett (1906) separated the chlorophylls by sugar column chromatography into two components and was able to resolve them from several other yellow carotenoid pigments. The two green components were referred to as chlorophyll α and ß but are now named a and b. Chlorophylls a and b are found in higher plants and green algae in approximately a 2:1 (a:b) ratio. Chlorophyll c is found together with chlorophyll a in marine algae, dinoflagellates and marine diatoms (Dougherty et al., 1966, 1970). Chlorophyll d was discovered to be a minor constituent of red algae (Rhodophyta) by Manning and Strain (1943).

Numerous investigations comprising almost 100 years lead to the complete structure elucidation of chlorophyll. Reviews related to these discoveries have been previously published (Aronoff, 1960, 1966; Holt, 1966; Seely, 1966; Strain and Svec, 1966). The structures of chlorophylls a and b are shown in Figure 2. Chlorophyll c is closely related to chlorophyll a and b (Dougherty et al., 1966) and chlorophyll d is similar to chlorophyll a except the vinyl group is replaced by a formyl group (Holt, 1961). Historically, a number of generic names for the chlorophylls and their derivatives have been accepted and are outlined in Table 3 (Jackson, 1976). Figure 3 indicates the relationship of chlorophyll to their major derivatives. Chlorophylls are Mg complexes. The central Mg

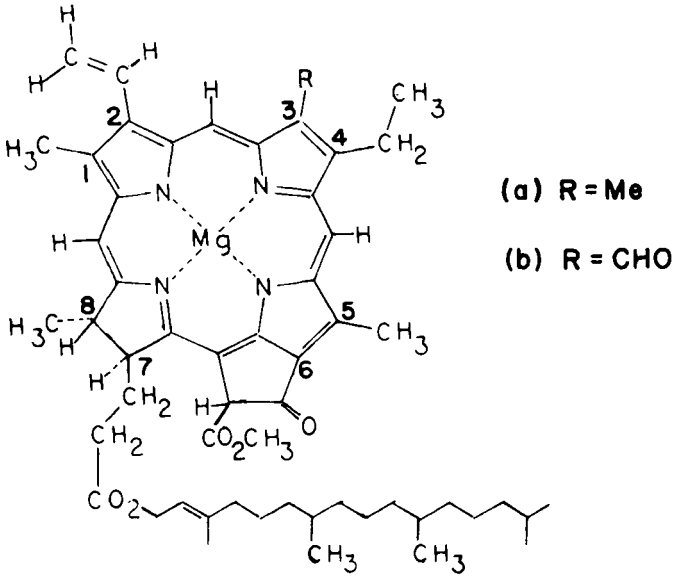


Figure 2. Structures of chlorophylls a and b.

atom is easily removed particularly under acidic conditions replacing it with hydrogen and thus forming the pheophytins. Hydrolysis of the phytyl group of pheophytin forms the pheophorbides. Loss of the phytyl group without removal of the Mg atom, usually by enzymatic treatment with chlorophyllase, produces the chlorophyllides. The wavelengths of maximum light absorption and absorptivity values of the chlorophylls and their derivatives are shown in Table 4.

Extraction of the chlorophylls can be achieved by using a mixture of petroleum ether-methanol or acetone. The pigments are then transferred to ether and purified by chromatography. Several methods of isolation and purification are reviewed by Jackson (1976) and covered in detail by Strain and Svec (1966).

The sensitivity of the chlorophylls to absorb light radiation and fluorescence is ideal for the development of analytical methodology

Table 3. Nomenclature of chlorophyll derivatives.

Phyllins: chlorophyll derivatives containing magnesium.

Pheophytins: the magnesium-free derivatives of the chlorophylls.

Chlorophyllide: the acid derivative resulting from enzymic or chemical hydrolysis of the C_7 propionate ester.

Chlorophyllase: the enzyme present in leaves which catalyses hydrolysis of the C_7 propionate ester.

Pheophorbides: the products containing a C_7 propionic acid resulting from removal of magnesium and hydrolysis of the phytyl ester. The corresponding 7-propionate methyl (or ethyl) ester is, however, somewhat unsystematically named as methyl (or ethyl) pheophorbide.

"Meso" compounds: derivatives in which the C-2 vinyl group has been reduced to ethyl.

"Pyro" compounds: derivatives in which the C-10 carbomethoxy group has been replaced by hydrogen.

Chlorins e: derivatives of pheophorbide a resulting from cleavage of the isocyclic ring; these are usually given a subscript number, e.g. chlorine e_6 specifies a product with six oxygen atoms (in three ester groups).

Rhodins g: the corresponding derivatives from pheophorbide b.

Purpurins: derivatives of chlorins in which C-10 has been oxidized.

Porphyrinogens: hexahydro derivatives of porphyrins in which four pyrrole rings are joined through saturated methylene bridges.

Adapted from: Jackson (1976).

involving spectrophotometry. The speed, efficiency and high resolution capabilities of HPLC coupled with spectrophotometric detectors provides an excellent technique to monitor, isolate, identify and quantitate these compounds.

The first application of HPLC to the analysis of chlorophyll pigments was reported by Evans et al. (1975). These authors utilized a reverse-phase column and a mixture of ethyl acetate in petroleum ether as the mobile phase. Resolution of porphyrins and chlorophyll derivatives was achieved. Detection of the pigments in the eluant was monitored at about

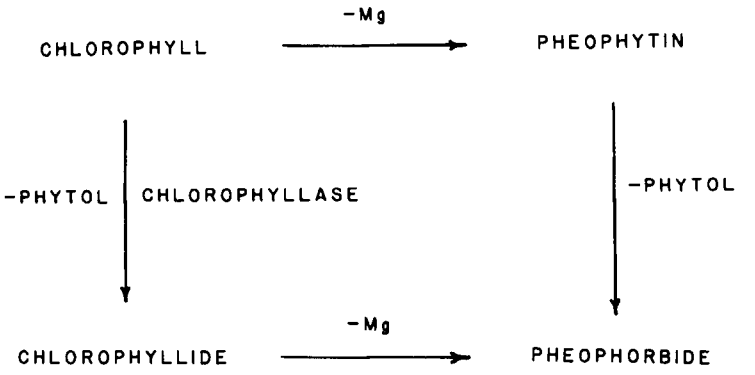


Figure 3. Relationship of chlorophyll to some of its derivatives.

400 nm for maximum sensitivity. Pigment fractions were collected and the field desorption mass spectra was obtained. Chromatographic retention data and mass spectral analyses provided complete characterization of pigment constituents. Eskins et al. (1977) developed a preparative HPLC method for plant pigments separation of the diatom Nitzschia closterium using a reverse-phase column and a stepwise gradient system starting with 80% methanol and ending with an ether-methanol mixture. The eluant was monitored for pigment concentration at 440 nm. Separation was complete in approximately 270 min. Good resolutions of chlorophylls a and c, pheophytin, carotenoids, neofucoxanthin, diadinoxanthin, diatoxanthin and carotene was obtained. The method was also applicable to separate spinach chlorophylls and carotenoids. Jacobsen (1978) described a quantitative fluorometric method for the separation of chlorophylls and pheophytins a and b from phytoplankton pigments by normal phase (silica gel) HPLC. The method involves an isocratic system employing a mixture of acetone:ligroine (20:80 v/v) as the mobile phase. Comparison of the HPLC method with classical spectrophotometric and fluorometric analyses indicated inaccuracy in the latter methods. Chlorophylls a and b were separated from interfering compounds in algae by Shoaf (1978). The pigments were

Table 4. Maximum wavelengths of light absorption and their respective absorptivity values for chlorophylls a and b and some of their derivatives in diethyl ether.[1]

Compound	Maxima λ (nm)	Molar Absorptivity (x 10^3)
Chlorophyll a	428.9	135.8
	662	100.7
Chlorophyllide a	428	80.6
	662	54.2
Pheophytin a	408	135.6
	668	66.8
Pheophorbide a	408.5	83.9
	667	41.6
Chlorophyll b	453	200.9
	642.5	72.8
Chlorophyllide b	452	99.0
	641	32.9
Pheophytin b[2]	NA	NA
	655	37.0
Pheophorbide b	432	151.9
	654	31.6

[1]Compiled from: Brown (1968).

[2]White, et al. (1963).

NA = Not available.

separated on a reverse-phase column using a mixture of methanol and water (95:5 v/v) as the eluant and detection was achieved at 654 nm. Rebeiz et al. (1978) separated cucumber chlorophylls a and b and pheophytins a and b using a reverse-phase column with a ternary solvent system consisting of

methanol, acetone and water. Picomole concentrations of pigments were detected by a spectrofluorometric detector. A micro-method for the qualitative and quantitative analysis of photosynthetic pigments in spinach was developed by Iriyama et al. (1978). Chromatograms were developed in a silica gel column using mixtures of 1-10% isopropyl alcohol in hexane as the mobile phase. Elution patterns were monitored at 380 nm. Yoshiura et al. (1978) resolved a mixture of chlorophylls a, a', b, b' and pheophytin using a silica gel column and a stepwise gradient consisting of 8, 10 and 12% acetone in hexane. Eluants were monitored for pigments at 380 nm. This method was applied to determine the purity of pigment isolates by Iriyama et al. (1979).

Polyethylene powder as a stationary phase for HPLC has been utilized for the separation and isolation of bacteriochlorophylls (Chow et al., 1978) and may be applicable to plant chlorophylls. Tobacco pigments, chlorophyll a, b, pheophytin, lutein, violaxanthin, neoxanthin and carotene were monitored and quantified at 425 nm by De Jong and Woodlief (1978) using a silica gel column and a stepwise gradient mixture containing heptane, diethyl ether and acetone. The method was applied to study the chlorophyll concentration in tobacco leaves as a function of tobacco genotype and leaf position on the stalk. Complex mixtures of pheophytins esterified with different diterpene alcohols have been separated on a reverse-phase column to further research in the identification of chlorophyll biosynthetic intermediates. Elution patterns were monitored at either 667 or 410 nm for maximum sensitivity using an eluant of methanol:acetone (90:10 v/v) (Schoch et al., 1978). Stransky (1978) developed a method for the quantitative estimation of chloroplast pigments. Spinach chlorophylls a, b and carotenoids, neoxanthin, violaxanthin, antheraxanthin, lutein, zeaxanthin and β-carotene were monitored at 445 nm using a silica gel column with isooctane:98% ethanol (9:1 v/v) as eluant. Abaychi and Riley (1979) separated phytoplankton pigments on a Partisil-10 stationary

support employing a mobile phase consisting of light petroleum, acetone, dimethylsulphoxide and diethylamine (75:23.25:1.5:0.25 v/v). Resolution of seven chlorophylls and derivatives and nine different carotenoids were detected and quantified at 440 nm. Baumann and Grimme (1979) resolved more than 10 different pigments from the green algae Chlorella fusca by using a reverse-phase column and a stepwise methanol-water gradient. Both chlorophylls and carotenoids were monitored at 440 nm. Eskins and Dutton (1979) separated chlorophylls a and b as well as carotenoid pigments neoxanthin, violaxanthin, lutein and carotene using a reverse-phase column. The method utilizes a linear gradient solvent program. The initial solvent conditions were methanol:water (90:10 v/v). Increasing the ethyl acetate content to a final concentration of methanol:water: ethyl acetate (45:5:50 v/v) after twenty min. The method was applied to measure etioplast pigments in red kidney beans (Eskins and Harris, 1981) and was found suitable for the separation of protochlorophyllides, chlorophyllides, carotenoids and several forms of chlorophyll a. Davies and Holdsworth (1980) found good separation of chlorophylls (a, c and C_2 and chlorophyllide a) and carotenoids (β-carotene, diadinoxanthin, fucoxanthin, lutein, dinoxanthin, peridinic and neoperidinin) by comparing three different reverse-phase columns. Addition of the ion-pairing agent tetrabutylammonium phosphate to the methanol-water eluant resulted in improved resolution of the complex mixtures. A variable wavelength detector at 440 and 650 nm was utilized to examine the eluant for presence of pigments. Schwartz et al. (1981) monitored changes in chlorophyll during processing of spinach. Excellent resolution of chlorophylls a, a', b, b' and pheophytins a and b was achieved using a reverse phase column. In addition, pyropheophytins were found and identified in canned spinach samples (Fig. 4). The authors utilized a gradient elution solvent system consisting of initial conditions of methanol:water (75:25 v/v) following a 10 min linear gradient to reach a final solvent composition of methanol:

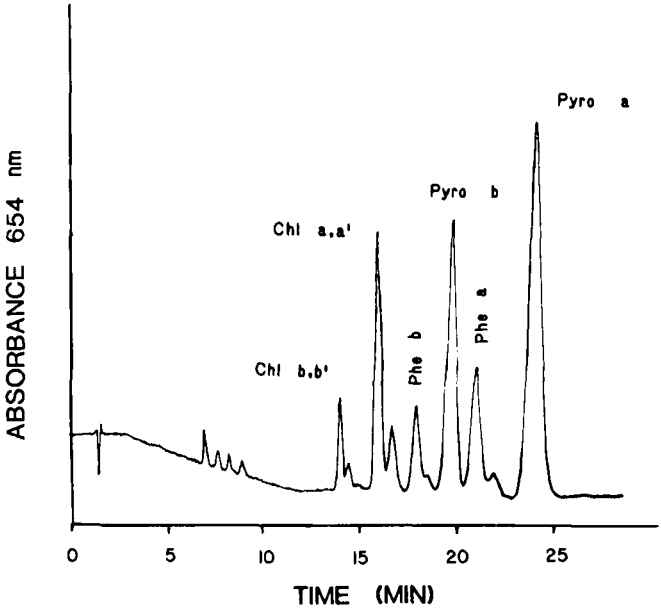
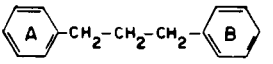


Figure 4. HPLC chromatogram of chlorophylls and their derivatives found in processed spinach. Chl = chlorophyll, Phe = pheophytin, Pyro = pyropheophytin. Adapted from Schwartz et al. (1981).

water:ethyl acetate (37.5:12.5:50 v/v). Chlorophyll pigments and their derivatives were monitored selectively at 654 nm. Brown et al. (1981) developed an HPLC method for the determination of chlorophylls a and b and their pheophytins in sediments, detritus and other epipelic samples. The pigments were resolved on a reverse-phase column and concentrations were estimated by fluorometry. Comparison of their method with standard spectrophotometric and fluorometric analysis indicated that the results obtained by the standard methods may be in error.

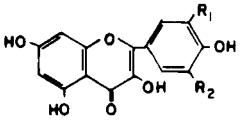
Flavonoids

The flavonoids are perhaps the most universally present phenolic compounds in the plant kingdom. The many red and blue hues of fruits and flowers can be attributed to the presence of one or more anthocyanins



BASIC STUCTURE

A

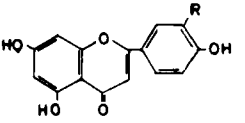


FLAVONOLS

R_1R_2 = H KAEMPFEROL

R_1 = OH, R_2 = H QUERCETIN

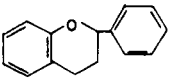
R_1R_2 = OH MYRICETIN



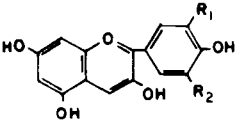
FLAVONES

R = H APIGENIN

R=OH LUTEOLIN



FLAVAN

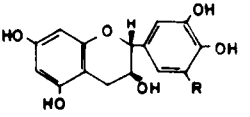


ANTHOCYANIDINS

R_1R_2=H PELARGONIDIN

R_1=OH, R_2=H CYANIDIN

R_1R_2=OH DELPHINIDIN



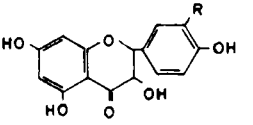
CATECHINS

R = H (+)-CATECHIN

R = OH (+)-GALLOCATECHIN

R = H (-)-EPICATECHIN

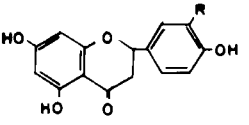
R=OH (-)-EPIGALLOCATECHIN



DIHYDROFLAVONOLS

R = H DIHYDROKAEMPFEROL

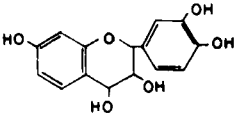
R = OH DIHYDROQUERCETIN



FLAVANONES

R=H NARINGENIN

R = OH ERIODICTYOL

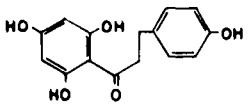


PROANTHOCYANIDINS

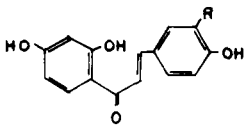
Figure 5A. Structures of the flavonoid group. True flavonoids.

belonging to the group of flavonoids. Carotenoids and flavonoids are responsible for the yellow color in many flowers. Flavonoids also account for the white color in flowers, and oxidative products of flavonoids, related phenolic compounds or flavonoid chelates produce natures' brown and black coloration.

B



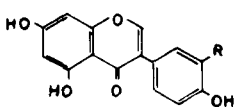
DIHYDROCHALKONES



CHALKONES

R = H ISOLIQUIRITIGENIN

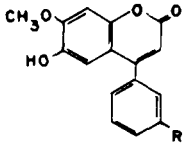
R = OH BUTEIN



ISOFLAVONES

R = H GENISTEIN

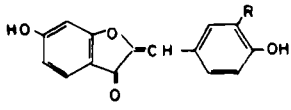
R = OH OROBOL



NEOFLAVONES

R = H DALBERGIN

R = OH STEVENIN



AURONES

R = H HISPIDOL

Figure 5B. Structures of the flavonoid group. Other compounds included in the flavonoid group.

The term flavonoid has been used for the last thirty years to embrace all compounds with the basic structure illustrated in Figure 5. It consists of two benzene rings (A and B) joined by three carbon atoms forming a γ-pyrone ring. The state of oxidation of the three carbon atoms determines the class of compound within the flavonoid group. The structures of the flavonoid group are illustrated in Figure 5A. In addition to the eight classes of flavonoids, there are five classes which are closely related in structure. These are therefore included in the flavonoid group, and their basic structures are illustrated in Figure 5B. Flavonoid compounds within each class differ in number and orientation of hydroxyl, methoxyl and other groups substituted on the benzene rings A and B. Flavonoids usually exist in plants as glycosides, that is, one or more of the hydroxyl groups are bound to a sugar moiety. The sugar-free portion of flavonoids is referred to as the aglycones. Their presence has been reported, but because of their water insolubility, are likely degradation

products formed during extraction procedures either by enzyme activity or acid solvents (von Elbe and Schaller, 1968). Detailed discussion of the chemistry, distribution and occurrence of flavonoids can be found in Geissman (1962), Harborne (1964) and Swain (1976).

The importance of flavonoids on beer stability has been well recognized. As early as 1973, Charalambous et al. applied HPLC to the separation of a number of polyphenols including catechin and quercitrin a 3-glycoside of the flavonol quercetin.

HPLC chromatography has mainly been applied to those flavonoids that are directly responsible for the color of plants. HPLC has made the separation of anthocyanins possible that previously could not be separated by other chromatographic techniques. Manley and Shubiak (1975) applied HPLC chromatography to separate enocianin, a commercially available grapeskin extract. In their work, several packing materials were evaluated. Best results were obtained with a polyamide packing and a solvent system composed of chloroform:methanol (87:13 v/v). Three monoglucosides of malvidin, petunidin and peonidin were separated within 20 min. Detection was monitored at 530 nm with a Beckman Acta III Spectrophotometer or with a fixed wavelength monitor. A reverse-phase system was used by Adamovics and Stermitz (1976) to separate anthocyanidins and flavonols. Three anthocyanidins, delphinidin, cyanidin, and pelargonidin were separated with methanol:acetic acid:water (20:5:75 v/v) as eluant. The three flavonol glycosides separated were quercetin-3-O-rutinoside, kaempferol-3-O-rutinoside, and isorhamnetin-3-O-rutinoside using two columns in series. Methanol:acetic acid:water (30:5:70 v/v) was used as the eluant. Detection was monitored at 280 nm. Separation was complete within 33 min. A similar reverse-phase system was used by Wilkinson et al. (1977) to separate six anthocyanidins (delphinidin, cyanidin, petunidin, pelargonidin, peonidin and malvidin). The column was eluted with water:acetic acid:methanol (71:10:19 v/v). The pigments were detected by monitoring at 530 nm. Akavia and Strack (1980) added acetonitrile

to the mobile phase to obtain a better solvent for the separation of anthocyanidins. A complex mixture of anthocyanins from fruits containing acylated as well as non-acylated anthocyanins were separated using a gradient elution system and by Williams et al. (1978). Non-acylated glucosides were separated using acetic acid:water (15:85 v/v) as eluant while water:acetic acid containing methanol (65:15:20 v/v) was required to separate acylated anthocyanins on a reverse-phase column. These authors point out, as one might expect, the need for temperature and pH control to obtain reproducible chromatographic data. Slight variation in either or both parameters will result in different retention times. Therefore, an internal standard is required should the data be applied for identification purposes. The effect of the polarity of the compounds on retention time is also demonstrated. The most polar anthocyanins, non-acylated diglucosides, are least retained while acylated monoglucosides show the longest retention time. Within individual groups of anthocyanins, the hydroxylation or methylation in the B ring (Fig. 5) determines their retention time. With increasing hydroxylation, retention time decreases while methylation of the hydroxyl groups increases the retention time. A reverse-phase column packing and a gradient elution system was used by Wulf and Nagel (1978), Nagel and Wulf (1979) not only to separate anthocyanins from grape varieties but also to quantify the pigments. Quantification was based on a standard curve prepared from pure malvidin-3-monoglucosides. In this work, a gradient elution solvent system composed of formic acid, methanol and water was employed. An isocratic system (formic acid: methanol:water, 10:17:73 v/v) based on the work of these authors has been applied to separate the anthocyanins in cranberries (Attoe and von Elbe, 1981). Cranberry anthocyanins had previously been separated on a reverse-phase column utilizing a mixture of methanol:acetic acid:water (37:10:53 v/v) as the mobile phase (Camire and Clydesdale, 1979).

A number of authentic flavonoids including flavones, flavonols and flavanones were separated by HPLC on a reverse-phase column using water:

acetic acid:methanol (65:5:30 v/v) as the mobile phase. The eluant was simultaneously monitored at 254 and 280 nm (Wulf and Nagel, 1976). These authors, in their study, discussed the effect of structural characteristics of these compounds on retention time. As an example, the flavonol myricetin containing a trihydroxyl substitution in the B ring elutes, because of its greater polarity, before the closely related quercetin containing two hydroxyl substitution in the B ring. Similarly, the effect of unsaturation between position 2 and 3 (Fig. 5) upon elution time is demonstrated. The flavone luteolin (unsaturated) is retained much longer on the column compared to its saturated counterpart the flavanone, eriodictyol. A number of papers have been published applying HPLC to the composition of flavonoids and other phenolic compounds in plant and food materials. In 1976, Hoefler and Coggon applied HPLC to a study of tea flavonols and tea pigments (theaflavin). Theaflavin is formed by phenolase catalyzed oxidation of epicatechin and epigallocatechin (Takino et al., 1967). The separation was accomplished on a reverse-phase column with aqueous acetic acid at various concentrations as the mobile phase. A gradient elution system on a reverse-phase column was used by Court (1977) to separate flavonoids, specifically the flavonols occurring in tobacco. HPLC because of its speed has given valuable information of the flavoniod content of plant and food materials both for screening large numbers of samples as well as quantitative data (Niemann and Koerselman-Kooy, 1977; Niemann, 1977; Galensa and Herrmann, 1978; Hardin and Stutte, 1979; Labosky and Sellers, 1980; De Loose, 1980; Niemann, 1980; Stewart et al., 1980; Wulf and Nagel, 1980; Galensa and Herrmann, 1980).

Qualitative and quantitative HPLC procedures have been applied to the study of flavones and flavanones in citrus products. In 1976, Fisher and Wheaton separated, on a reverse-phase column using water-acetonitrile as the mobile phase, naringenin and naringenin rutinoside from grape fruit juice. Fisher (1978) separated and quantified the flavanone hesperidin the

7-glycoside of hesperetin. Detection of the compounds was obtained by monitoring the eluant at 280 nm. Its content was correlated with the extraction pressure used during the processing of orange juice. The method used was a reverse-phase column and water-acetonitrile as the mobile phase. A quantitative HPLC procedure to separate five major polymethoxylated flavones in orange juice was developed by Rouseff and Ting (1979). Several mobile phases were tested. A reverse-phase column and a mobile phase of water:tetrahydrofuran:acetonitrile (72:22:6 v/v) gave optimum separation of the five compounds. In this system, a dual UV-fluorescence detector was employed to determine the presence of interfering substances. Relative concentrations of these compounds can be used to detect qualitatively the presence of one juice in the juice of another. This method was applied to mixtures of orange and tangerine juice by Ting et al. (1979). Retention time of five polymethoxylated flavones from orange peel oil have been reported by Bianchini and Gaydou (1980). Flavones found in buckwheat and oats and separated by HPLC have been studied by Strack and Krause (1978), Strack et al. (1979). The influence of isomerization and glycosylation of the monoglucoside of the flavone apigenin on retention time has been reported by Niemann and Van Brederode (1978).

Soybeans and soybean food products contain isoflavones. They are of interest because of their antioxidant properties. West et al. (1978) separated both non-glycosylated 4',6,7-trihydroxyisoflavone and genistein, 4',5,7-trihydroxyisoflavone from soy and fermented soy products. Preparation of the sample before HPLC involves defatting soy samples with petroleum ether. Separation is accomplished on a reverse-phase column with a mobile phase of water:acetonitrile (4:1 v/v). Detection of the compounds was achieved by monitoring the eluant at 260 nm. Six isoflavones were separated by HPLC in soybeans by Ohta et al. (1979), Ohta et al. (1980). The separations were accomplished with reverse-phase column and a linear gradient system of methanol ranging from 32-90% (detection at 254 nm)

HPLC investigations by Lea (1979) and Lea (1980) have given valuable information of the procyanidin, dimers of catechin and epicatechin found in foods and beverages particularly cider and wine. The author investigated several packing materials and concluded that reverse-phase system offered the greatest potential for the separation of cyanidins.

Betalains

The class of pigments known as betalains (Mabry and Dreiding, 1968) contains red and yellow pigments called betacyanins and betaxanthins, respectively. These pigments are restricted to seven families within the order Centrospermae. Betalains are found in the cell vacuoles of fruits, flowers, leaves and roots. Chemically, they are amphoteric compounds, very soluble in water and practically insoluble in organic solvents. The structure of the chromophore of betalain pigments is shown in Figure 6, and can be described as a protonated 1,2,4,7,7, tetrapentasubstituted 1,7-diazaheptamethin system. When R' does not extend the conjugation of the 1,7-diazaheptamethin system, the pigments are yellow (absorption maximum near 480 nm); if the conjugation is continued through the substitution of an aromatic group at R', the chromophore undergoes a shift toward a longer wavelength (absorption maximum near

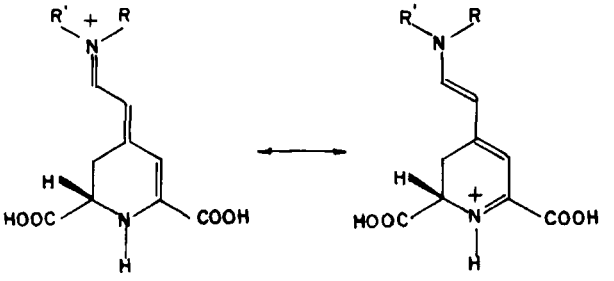
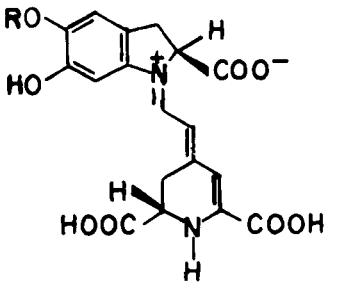


Figure 6. Chromophore of the betalain pigments.

540 nm) which accounts for the red color of the betacyanins. The most intensively studied plant containing betalains are the pigments of the red beet (Beta vulgarus). The general structures of these betacyanins and betaxanthins are given in Figure 7. The most abundant betacyanin and betaxanthin in beets are betanin and vulgaxanthin I, respectively. Each accounts for approximately 95% of either the betacyanins or betaxanthins in beets (Nilsson, 1970). The absorption spectra of these pigments are affected only slightly by pH values of the solvent. (Nilsson, 1970, von Elbe et al., 1974). The molar absorptivity values of betanin (λ_{max} = 538 nm) and vulgaxanthin (λ_{max} = 475 nm) are 65,000 and 25,000, respectively (Piattelli and Minale, 1964; Piattelli et al., 1965).

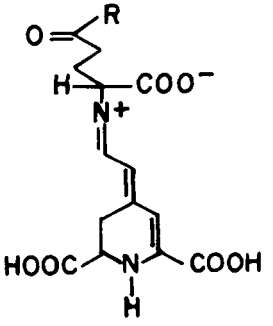


BETANIN R = GLUCOSE

BETANIDINE R = H

PREBETANINE

R = GLUCOSE-6-SULFATE



VULGAXANTHIN I R = NH_2

VULGAXANTHIN II R = OH

Figure 7. Structures of betalains in red beets.

HPLC was first applied to separate and quantify betacyanins and betaxanthins by Vincent and Scholz (1978). These authors separated the pigments of the red beet employing a reverse-phase microparticular column, ion pairing (0.005 M tetrabutyl ammonium phosphate, pH 7.5) and a methanol:water mobile phase (90:10 v/v). Quantification by HPLC of betacyanins was based on the linear response of betanin versus concentration. The assumption was made that, because of similarity in structure and the relative proportion of betanin present in beets, the same absorptivity value can be assumed for all betacyanins. Detection of the pigments employed a variable wavelength detector set at 476 nm for betaxanthins or 538 nm for betacyanins. Pigment classes were quantified as they co-eluted from the column. Quantification of individual betacyanins by HPLC found in beets was reported by Schwartz and von Elbe (1980). The authors described an isocratic and gradient separation of betacyanins. A reverse-phase column was utilized. The chromatograms were monitored at 535 nm. The mobile phase for the isocratic condition was CH_3OH/0.05 KH_2PO_4 (18:82 v/v) adjusted to pH 2.75 with H_3PO_4 (solvent A). The solvent for gradient elution separation was initial 100% solvent A and final 80% solvent A, 20% solvent B (CH_3OH). Separation time was approximately 20 min under isocratic conditions and less than 10 min under gradient elution. A gradient elution HPLC chromatogram is illustrated in Figure 8. Quantification was achieved by employing individual molar absorptivity values (betanidin hydrochloride - 54,000 and betanin hydrochloride - 65,000). Pure pigments were obtained in crystalline form by employing preparative HPLC. The major advantage over the previously described method is the quantitative estimation of each component rather than measuring all pigments co-eluting from an HPLC column in a single peak. Schwartz et al. (1981) compared spectrophometric and HPLC methods to quantify betacyanins. The authors conclude that of the two methods, HPLC is preferred particularly for the labile betacyanin pigments which may form interfering substances.

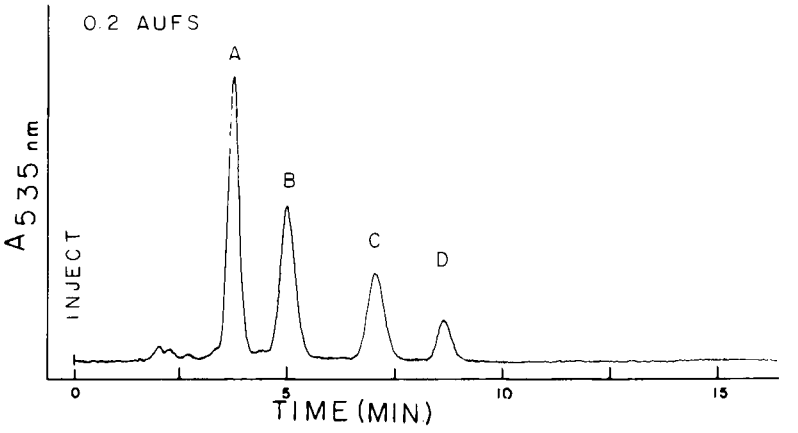


Figure 8. HPLC chromatogram of betacyanin pigments. A = betanin, B = isobetanin, C = betanidin, D = isobetanidin.

Application of the HPLC method to beet pigment losses and changes during processing have been published by von Elbe et al. (1981). In a study by Singer and von Elbe (1980) relative amounts of vulgaxanthins in beet samples were estimated by HPLC employing a column containing a strong ion exchange resin. The major advantage of this column was the longer retention factor obtained for vulgaxathin compared to the retention time on a reverse-phase column.

Summary

Qualitative and quantitative HPLC methods have found wide application for the separation and identification of a variety of plant pigments. Compared to other chromatographic techniques the methods are precise, sensitive and less time consuming. The high resolution capability of HPLC has made the separation of closely related pigments possible. Visible-ultraviolet and fluorometric detectors employed are particularly useful because of the sensitive interaction of these pigments to light energy. Sample preparation prior to chromatography is generally minimal and often

a simple extraction step is sufficient. Retention times and spectral data of individual pigments obtained during HPLC determinations have become valuable aids for identification purposes. Early methods developed for the separation of carotenoids by HPLC utilized adsorption chromatography with non-polar mobile phases. Reverse-phase columns have recently found application into the study of these pigments. Separation of chlorophylls and their derivatives have utilized largely reverse-phase stationary supports. Many of the techniques developed for the separation of carotenoids and chlorophylls are applicable for the simultaneous analysis of these pigments. The flavonoid and betalain pigments, because of their water solubility and ionic character, have been successfully separated with reverse-phase columns and polar mobile phases. The many advantages of HPLC has made it a valuable tool for the study of plant pigments.

References

Abaychi, J. K. and Riley, J. P. Anal. Chim. Acta 107,1. (1979).

Adamovics, J. and Stermitz, F. R. J. Chromatog. 129,464. (1976).

Akavia, N. and Strack, D. Z. Naturforsch. 35c,16. (1980).

Aronoff, S. In "Handbuch der Pflanzenphysiologie"(W. Ruhland, ed.), pp. 234-251. Springer-Verlag, Berlin. (1960).

Aronoff, S. In "The Chlorophylls" (L. P. Vernon and G. R. Seely, eds.), pp. 3-19. Academic Press, NY. (1966).

Attoe, E. L. and von Elbe, J. H. J. Food Sci. 46 in press. (1981).

Baumann, T. and Grimme, L. H. J. Chromatog. 170,264. (1979).

Bianchini, J. P. and Gaydou, E. M. J. Chromatog. 190,233. (1980).

Brown, L. M., Hargrave, B. T. and MacKinnon, M. D. Can. J. Fish. Aquat. Sci. 38,205. (1981).

Brown, S. R. J. Fish Res. Bd. Canada 25,523. (1968).

Camire, A. L. and Clydesdale, F. M. J. Food Sci. 44,926. (1979).

Charalambous, G., Bruckner, K. J., Hardwick, W. A. and Linnebach, A. Tech. Quart. Master Brew. Assoc. Amer. 10(2),74. (1973).

Chow, H-C., Caple, M. B. and Strouse, C. E. J. Chromatog. 151,357. (1978).

Court, W. A. J. Chromatog. 130,287. (1977).

Davies, D. and Holdsworth, E. S. J. Liq. Chromatog. 3,123. (1980).

De Jong, D. W. and Woodlief, W. G. J. Agric. Food Chem. 26,1231. (1978).

De Loose, R. Med. Fac. Landbouw. Rijksuniv. Gent. 45,81. (1980).

Dougherty, R. C., Strain, H. H., Svec, W. A., Uphams, R. A. and Katz, J. J. J. Amer. Chem. Soc. 88,5037. (1966).

Dougherty, R. C., Strain, H. H., Svec, W. A., Uphams, R. A. and Katz, J. J. J. Amer. Chem. Soc. 92,2826. (1970).

Eskins, K. and Dutton, H. J. Anal. Chem. 51,1885. (1979).

Eskins, K. and Harris, L. Photochem. Photobiol. 33,131. (1981).

Eskins, K., Scholfield, C. R. and Dutton, H. J. J. Chromatog. 135, 217. (1977).

Evans, N., Games, D. E., Jackson, A. H. and Matlin, S. A. J. Chromatog. 115,325. (1975).

Fiksdahl, A., Mortensen, J. T. and Liaaen-Jensen, S. J. Chromatog. 157,111. (1978).

Fisher, J. F. J. Agric. Food Chem. 26,1459. (1978).

Fisher, J. F. and Wheaton, T. A. J. Agric. Food Chem. 24,898. (1976).

Galensa, R. and Herrmann, K. Z. Lebensm. Unters. Forsch. 166,355. (1978).

Galensa, R. and Herrmann, K. J. Chromatog. 189,217. (1980).

Geissman, T. A. (ed.), The Chemistry of Flavonoid Compounds. The MacMillen Co. NY. (1962).

Goodwin, T. W. In "Chemistry and Biochemistry of Plant Pigments." (T. W. Goodwin, ed.), pp. 225-257. Academic Press, NY. (1976).

Hajibrahim, S. K., Tibbetts, P. J. C., Watts, C. D., Maxwell, J. R., Eglinton, G., Colin, H. and Guiochon, G. Anal. Chem. 50,549. (1978).

Harborne, J. B. (ed.). Biochemistry of Phenolic Compounds. Academic Press, NY. (1964).

Hardin, J. M. and Stutte, C. A. Anal. Biochem. 102,171. (1980).

Hoefler, A. C. and Coggon, P. J. Chromatog. 129,460. (1976).

Holt, A. S. Can. J. Bot. 39,327. (1961).

Holt, A. S. In "The Chlorophylls." (L. P. Vernon and G. R. Seely, eds.), pp. 111-117. Academic Press, NY. (1966).

Iriyama, K., Shiraki, M. and Yoshiura, M. J. Liq. Chromatog. 2,255. (1979).

Iriyama, K., Yoshiura, M. and Shiraki, M. J. Chromatog. 154,302. (1978).

Isler, O. Pure & Applied Chem. 51,447. (1979).

IUPAC. Commission on the Nomenclature of Biological Chemistry. J. Amer. Chem. Soc. 82,5583. (1960).

Jackson, A. H. In "Chemistry and Biochemistry of Plant Pigments." (T. W. Goodwin, ed.), pp. 1-57. Academic Press, NY. (1976).

Jacobsen, T. R. Mar. Sci. Comm. 4,33. (1978).

Labosky, Jr., P. and Sellers, J. A. Wood Science 13,33. (1980).

Landen, W. O. and Eitenmiller, R. R. J. Assoc. Off. Anal. Chem. 62,283. (1979).

Lea, A. G. H. J. Sci. Food Agric. 30,833. (1979).

Lea, A. G. H. J. Chromatog. 189,62. (1980).

Liaaen-Jensen, S. In "Carotenoids." (O. Isler, ed.), pp. 63-77. Birkhäuser Verlag, Basel. (1971).

Mabry, T. J. and Dreiding, A. S. In "Recent Advances in Phytochemistry." (T. J. Mabry, ed.), pp. 145-160. Appleton-Century-Croft, NY. (1968).

Manley, C. H. and Shubiak, P. Can. Inst. Food Sci. Technol. J. 8,35. (1975).

Manning, W. M. and Strain, H. H. J. Biol. Chem. 151,1. (1943).

Moss, G. P. and Weedon, B. C. L. In "Chemistry and Biochemistry of Plant Pigments." (T. W. Goodwin, ed.), pp. 149-219. Academic Press, NY. (1976).

Nagel, C. W. and Wulf, L. W. Amer. J. Enol. Vitic. 30(2),111. (1979).

Niemann, G. J. Z. Naturforsch. 32c,1015. (1977).

Niemann, G. J. Z. Naturforsch. 35c,514. (1980).

Niemann, G. J. and Koerselman-Kooy, J. W. Planta medica 31,297. (1977).

Niemann, G. J. and Van Brederode, J. J. Chromatog. 152,523. (1978).

Nilsson, T. Lantbrukshögskolans Annaler 36,179. (1970).

Ohta, N., Kuwata, G., Akahori, H. and Watanabe, T. Agric. Biol. Chem. 43,1415. (1979).

Ohta, N., Kuwata, G., Akahori, H. and Watanabe, T. Nippon Shokuhin Kogyo Gakkaishi 27,348. (1980).

Pelletier, P. J. and Caventow, J. B. Ann. Chim. et Phys. 9,194. (1818).

Pfander, H., Schurtenberger, H. and Meyer, V. R. Chimia 34,179. (1980).

Piattelli, M. and Minale, L. Phytochem. 3,307. (1977).

Piattelli, M., Minale, L. and Prota, G. Phytochem. 4,121. (1965).

Rebeiz, C. A., Bazzaz, M. B. and Belanger, F. Chromatog. Rev. 4(2). (1978).

Reeder, S. K. and Park, G. L. J. Assoc. Off. Anal. Chem. 58,595. (1975).

Rouseff, R. L. and Ting, S. V. J. Chromatog. 176,75. (1979).

Schoch, S., Lempert, U., Wieschhoff, H. and Scheer, H. J. Chromatog. 157, 357. (1978).

Schwartz, S. J., Hildenbrand, B. E. and von Elbe, J. H. J. Food Sci. 46,296. (1981).

Schwartz, S. J. and von Elbe, J. H. J. Agric. Food Chem. 28,540. (1980).

Schwartz, S. J., Woo, S. L. and von Elbe, J. H. J. Agric. Food Chem. 29, 533. (1981).

Seely, G. R. In "The Chlorophylls." (L. P. Vernon and G. R. Seely, eds.), pp. 67-103, 523-561. Academic Press, NY. (1966).

Shoaf, W. T. J. Chromatog. 152,247. (1978).

Singer, J. W. and von Elbe, J. H. J. Food Sci. 45,489. (1980).

Sorby, H. C. Proc. R. Soc. 21,442. (1873).

Steenbock, H. Science 50,352. (1919).

Stewart, I. J. Assoc. Off. Anal. Chem. 60,132. (1977a).

Stewart, I. J. Agric. Food Chem. 25,1132. (1977b).

Stewart, I. and Leuenberger, U. B. Alimenta 15,33. (1976).

Stewart, I. and Wheaton, T. A. J. Chromatog. 55,325. (1971).

Stewart, R. N., Asen, S., Massie, D. R., Norris, K. H. Biochemical Systematics and Ecology. 8,119. (1980).

Strack, D., Fuisting, K., Popovici, G. J. Chromatog. 176,270. (1979).

Strack, D. and Krause, J. J. Chromatog. 156,359. (1978).

Strain, H. H. and Svec, W. A. In "The Chlorophylls." (L. P. Vernon and G. R. Seely, eds.), pp. 22-61. Academic Press, NY. (1966).

Stransky, H. Z. Naturforsch 33,836. (1978).

Swain, T. In "Chemistry and Biochemistry of Plant Pigments." (T. W. Goodwin, ed.), pp. 425-463. Academic Press, NY. (1976).

Sweeney, J. P. and Marsh, A. C. J. Assoc. Off. Anal. Chem. 53,937. (1970).

Takino, Y., Ferritti, A., Flanagan, V., Gianturco, M. A. and Vogel, M. Can. J. Chem. 45,1949. (1967).

Thompson, J. N. and Maxwell, W. B. J. Assoc. Off. Anal. Chem. 60,766. (1977).

Ting, S. V., Rouseff, R. L., Dougherty, M. H., and Attaway, J. A. J. Food Sci. 44,69. (1979).

Tswett, M. Ber. Deut. Botan. Ges. 24,316,384. (1906).

Vincent, K. R. and Scholz, R. G. J. Agric. Food Chem. 26,812. (1978).

von Elbe, J. H., Maing, I-Y., and Amundson, C. H. J. Food Sci. 39,334. (1974).

von Elbe, J. H. and Schaller, D. R. J. Food Sci. 33,439. (1968).

von Elbe, J. H., Schwartz, S. J. and Hildenbrand, B. E. J. Food Sci. 46, *In press.* (1981).

West, L. G., Birac, P. M. and Pratt, D. E. J. Chromatog. 150,266. (1978).

White, R. C., Jones, I. D. and Gibbs, E. J. Food Sci. 28,431. (1963).

Wilkinson, M., Sweeny, J. G. and Iacobucci, G. A. J. Chromatog. 132, 349. (1977).

Williams, H., Hrazdina, G., Wilkinson, M. M., Sweeny, J. G., Iacobucci, G. A. J. Chromatog. 155,389. (1978).

Wulf, L. W. and Nagel, C. W. J. Chromatog. 116,271. (1976).

Wulf, L. W. and Nagel, C. W. Amer. J. Enol. Vitic. 29(1),42. (1978).

Wulf, L. W. and Nagel, C. W. J. Food Sci. 45,479. (1980).

Yoshiura, M., Iriyama, K. and Shiraki, M. Chem. Letts. (Tokyo) 281. (1978).

Zakaria, M., Simpson, K., Brown, P. R. and Krstulovic, A. J. Chromatog. 176,109. (1979).