Separation, Identification, and Quantification of the Major Carotenoid and Chlorophyll Constituents in Extracts of Several Green Vegetables by Liquid Chromatography[1]

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Major constituents of the extracts from five green vegetables (broccoli, cabbage, spinach, brussels sprouts, kale), several of which are members of the genus *Brassica* (Cruciferous), have been separated by high-performance liquid chromatography (HPLC) on a C-18 reversed-phase column. Three classes of compounds were shown to be present. In the order of chromatographic elution, these were xanthophylls, chlorophylls and their derivatives, and the hydrocarbon carotenoids (carotenes). The xanthophylls were identified as neoxanthin, violaxanthin, lutein epoxide, and lutein. Several mono cis isomers of xanthophylls were also shown to be present in the extracts from these vegetables. The chlorophylls were identified as chlorophylls *b* and *a* and their decomposition products pheophytins *b* and *a*. The only hydrocarbon carotenoids present in these vegetables were *all-trans*-β-carotene and its 15,15′-cis isomer. β-Apo-8′-carotenal and decapreno-β-carotene have been employed respectively as internal standards for quantification of xanthophylls and carotenes. The effect of cooking on the qualitative and quantitative distribution of carotenoids in some of the vegetables has been discussed.

INTRODUCTION

In recent years, a number of foods and constituents of foods have been studied for their inhibitory effects on carcinogenesis (National Research Council, 1982). A substantial number of these studies have demonstrated an inverse relationship between the consumption of certain fruits and vegetables and the risk of cancer (Peto et al., 1981; Shekelle et al., 1981). These studies assumed that the active factor in a diet rich in fruits and vegetables was β-carotene and related to its potential for vitamin A activity. However, many of these fruits and vegetables appear to be poor sources of β-carotene but relatively good sources of other carotenoids (oxygenated carotenoids, xanthophylls) that have little or no vitamin A activity (Beecher and Khachik, 1984). Recently, although Colditz et al. (1985) found that increased total dietary carotenoid intake was protective against cancer in an elderly population, foods high in β-carotene specifically had no association. Certain foods containing other carotenoids had significant protective effects. The epidemiological studies could be better interpreted if generation of dietary carotenoid data was accompanied by characterization and accurate quantitative measurement of all the major carotenoids found in fruits and vegetables. Current food composition tables (Souci et al., 1981; Haytowitz and Matthews, 1984) lack detailed analytical information in that they only provide data on "carotene" or vitamin A activity. To date there have been only limited literature reports that have thoroughly investigated the presence of all the major carotenoids in foods.

Sweeney and Marsh (1971) reported the effect of processing on provitamin A in vegetables and have isolated and quantified a number of cis isomers of α- and β-carotene. In their procedures, the carotenes were separated from xanthophylls and the chloroplast pigments by extraction. In the vegetables that were examined only the total xanthophylls as a percentage of the total carotenoids were reported. Several methods of isolation and detection of chlorophylls, carotenes, and xanthophylls in fruits and other organs by high-performance liquid chromatography (HPLC) have been described in the literature. Wright and Shearer (1984) have separated the carotenes, xanthophylls, and the chlorophylls of photosynthetic pigments from phytoplankton. They have investigated the rapid extraction and HPLC separation for 44 pigments from representatives of the Bacilliarophyceae, Dinophyceae, Cryptophyceae, and Cyanophyceae. Davies and Holdsworth (1980) have employed HPLC for the separation and identification of carotenoids and chlorophylls concerned in photosynthesis. A comprehensive review on the HPLC of plant pigments has been published by Schwartz and Elbe (1982). Schwartz et al. (1981) have elegantly separated and identified the chlorophylls and their derivatives in fresh and processed spinach by HPLC; however, the major carotenoid constituents were not investigated. A method for extraction and quantitation of chloroplast pigments in fruits and other organs by HPLC has recently been reported by McMahon and Gladon (1984). Although, the separation of six major chloroplast pigments was accomplished on a reversed-phase μBondapak C-18 column, employing isocratic and gradient chromatography, only chlorophylls *b* and *a* were determined quantitatively. More recently, the determination of green leaf carotenoids by HPLC for a number of plants has been reported by Takagi (1985). Perhaps the closest example to the work presented in this text has been reported by Stransky (1978), who developed a method for the separation and quantitative estimation of chlorophylls and carotenoid pigments of spinach by HPLC using a nucleosil 50 column with isooctane/98% ethanol (9:1, v/v) as eluent. The major carotenoids separated by this method were identified as β-carotene, lutein, zeaxanthin, antheraxanthin, violaxanthin, and neoxanthin. From these literature reports it appears that although the major constituents of the chloroplast have been investigated and numerous HPLC methods have been developed for their separation and identification, the generation of accurate analytical data on the individual carotenoids is yet to be explored. The majority of these

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[1]Partial support by the National Cancer Institute through reimbursable Agreement Y01-CN-30609.

methods employ HPLC conditions that result in close separation of chlorophylls and carotenoids. Since green plants have a high proportion of chlorophylls to carotenoids, the sensitivity of the detection of these pigments must be greatly reduced to attain base-line HPLC separation and accurate quantification. Under such conditions, a number of the carotenoids that may be present as minor components remain undetected. In some cases the identification of the chloroplast pigments, based on the HPLC retention time and absorption spectra alone, has resulted in ambiguous structural assignment. Finally, since the preparation of carotenoid samples for HPLC analysis requires extensive extraction and workup procedures that can be accompanied by various analytical errors, the use of an internal standard is essential (Khachik and Beecher, 1985); only a few of these reports have employed an internal standard in quantitative evaluation of carotenoids.

In this report we have addressed some of these problems and have investigated a limited number of green vegetables (broccoli, cabbage, brussels sprouts, kale) for the presence of the major carotenoids by HPLC. We have developed HPLC conditions that separated 18 components in extracts from these vegetables that were assigned to three classes of compounds: xanthophylls, chlorophylls and their derivatives, and the hydrocarbon carotenoids. The major constituents of these green vegetables have also been separated by semipreparative TLC and HPLC and have been identified by such tools as mass spectroscopy, NMR, and UV/visible spectroscopy. Pure reference compounds and internal standards (β-apo-8′-carotenal, decapreno-β-carotene) were used to quantify each component in several vegetables. In an attempt to observe possible stereochemical changes of the heat-sensitive carotenoids, some of the vegetables have been examined in both raw and cooked forms as they are commonly consumed.

EXPERIMENTAL SECTION

Apparatus. A Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Hewlett-Packard 1040A rapid-scanning UV/visible photodiode array detector. The data were stored and processed by means of a Hewlett-Packard 85-B computing system that was operated with a Hewlett-Packard Model-9121 disk drive and 7470A plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at the rate of 12 spectra/min. The HP-85B computer with a built-in integration program was used to evaluate the peak area and peak height. Absorption spectra of isolated components in various solvents were recorded on a Beckman DU-7 UV/visible spectrophotometer. Mass spectra were obtained on a V.G. 7070F mass spectrometer (V. G. Instrument Co., Stamford, CT) at 70 eV, employing a solid probe and an ion source of 225 °C. The ^{1}H NMR (400-MHz) spectra were obtained on a Bruker instrument.

Column. Separations were performed on a stainless-steel (25 cm × 4.6 mm i.d.) Microsorb C_{18} (5-μm spherical particles) column (Rainin Instrument Co.), which was protected with a Brownlee guard cartridge (3-cm length × 4.6-mm i.d.) packed with spheri-5-C_{18} (5-μm particle size). Semipreparative separations were carried out on a Rainin stainless-steel (25 cm × 10 mm i.d.) Microsorb C_{18} column (5-μm spherical particles). Analysis of the vegetable extracts often required reverse flushing of the precolumn with methanol and methylene chloride/hexane (1:1).

Reagents and Materials. The reference samples of *all-trans*-β-carotene, chlorophyll *a*, and chlorophyll *b* (Sigma, St. Louis, MO) were used without further purification. Lutein, zeaxanthin, and 15,15′-*cis*-β-carotene were provided by Hoffmann-La Roche, Basel, Switzerland. β-Apo-8′-carotenal (Fluka Chemical Corp.) and *all-trans*-decapreno-β-carotene [synthesized in our laboratory according to the method of Surmatis and Ofner (1961)] were used as internal standards. HPLC-grade solvents, methanol, acetonitrile, methylene chloride, and hexane (Fisher Scientific, Pittsburgh, PA) were used without further purification. Tetrahydrofuran was stabilized with butylated hydroxytoluene (BHT, 0.01%). Vegetables were purchased fresh from local supermarkets on the day of analysis and were cooked in a microwave oven operating at high power with a nominal output of 750 W.

Table I. Peak Identification of the Varoius Components of the Green Vegetable Extracts Separated by HPLC

chem class	peaks	components	λ monitored, nm
xanthophylls	1	*all-trans*-neoxanthin	445
	2	9′-*cis*-neoxanthin	445
	3	violaxanthin	445
	4	neochrome	445
	5	*all-trans*-lutein epoxide	445
	6	neolutein epoxidde A (13- or 13′-*cis*-lutein epoxide)	445
	7	*all-trans*-lutein	445
	8	neolutein B (9- or 9′-*cis*-lutein)	445
	9	neolutein B′ (9′- or 9-*cis*-lutein)	445
	10	neolutein A (13- or 13′-*cis*-lutein)	445
apocarotenoid	11	β-apo-8′-carotenal (int std)	455
chlorophylls	12	chlorophyll *b*	455
	13	chlorophyll *b*′	455
	14	chlorophyll *a*	430
	15	chlorophyll *a*′	430
	16	pheophytin *b*	430
	17	pheophytin *a*	410
hydrocarbon carotenoids	18	*all-trans*-β-carotene	450
	19	15,15′-*cis*-β-carotene	450
	20	decapreno-β-carotene (int std)	500

Chromatographic Procedure. The analytical separations were carried out under two sets of HPLC conditions employing eluents A and B, while the semipreparative separations were carried out employing eluent C.

1. Eluent A. An isocratic system of methanol (22%), acetonitrile (55%), methylene chloride (11.5%), and hexane (11.5%) effected the separation of *all-trans*-β-carotene and its 15,15′-cis isomer from decapreno-β-carotene (internal standard for the quantification of the hydrocarbon carotenoids) at a column flow rate of 0.70 mL/min. The chromatographic runs were monitored at 450 nm for *all-trans*-β-carotene and its 15,15′-cis isomer and at 500 nm for the internal standard.

2. Eluent B. A combination of isocratic and gradient chromatography separated the oxygenated carotenoids (xanthophylls) and β-apo-8′-carotenal (internal standard for the quantification of xanthophylls) from chlorophylls and the hydrocarbon carotenoids. An isocratic mixture of methanol (15%), acetonitrile (75%), methylene chloride (5%), and hexane (5%) at time 0 was followed by a gradient beginning at time 12 and completed at time 27 (minutes). The final composition of the gradient mixture was methanol (15%), acetonitrile (40%), methylene chloride (22.5%), and hexane (22.5%). The column flow rate was 0.50 mL/min. The chromatographic runs of the major components of the vegetable extracts were monitored at various wavelengths. These components and the wave-

Table II. Weight of the Internal Standards Added to the Vegetables at the Beginning of the Extraction and the Final Volume of the Extracts

entry	vegetable, g	β-apo-8′-carotenal, mg	decapreno-β-carotene, mg	final vol of extr, mL
1	broccoli raw, 60 g	0.25	1.08	25.0
2	cabbage raw, 150 g	0.10	0.54	10.0
3	spinach raw, 20 g	0.25	2.70	50.0
4	brussels spouts			
	raw, 70 g	0.25	1.35	25.0
	cooked, 70 g	0.25	1.35	25.0
5	kale			
	raw, 10 g	2.30	7.50	100.0
	cooked, 10 g	2.30	7.50	100.0

lengths at which they were monitored have been listed in the order of chromatographic elution in Table I. At the end of the gradient the column was reequilibrated under the initial isocratic conditions [methanol (15%), acetonitrile (75%), methylene chloride (5%), hexane (5%)] for 20 min at a flow rate of 2 mL/min and finally for 5 min at 0.5 mL/min.

3. Eluent C. An isocratic mixture of methanol (15%), acetonitrile (80%), methylene chloride (2.5%), and hexane (2.5%) and a Column flow rate of 1.5 mL/min effected the separation of xanthophylls on a semipreparative scale. This eluent was also employed at a flow rate of 0.5 mL/min with the analytical column for the purity evaluation of the various xanthophyll bands isolated from vegetable extracts by thin-layer chromatography. The purity of the xanthophylls collected by semipreparative HPLC was also checked with the analytical column utilizing this eluent. The monitoring wavelength with eluent C was 445 nm.

Preparation of the Vegetable Samples for Extraction. Vegetables were prepared for analysis in the same way they are prepared for consumption, i.e. inedible parts removed. Vegetables were washed with deionized water and drained. The cooked vegetables were heated in a microwave oven with a small amount of water for 6 min. Large pieces were reduced in size prior to homogenization (without added water) in a Cuisinart food processor (10–25 s).

Extraction. The extraction procedure was similar to that employed by Bushway and Wilson (1982). Two stock solutions of the internal standards were prepared by dissolving 5.0 mg of β-apo-8′-carotenal and 11.0 mg of decapreno-β-carotene in 100 mL of hexane. Aliquots of these two internal standards were added to each sample of vegetable, with anhydrous sodium sulfate (200% of the weight of the vegetable), and magnesium carbonate (10% of the weight of the vegetable) contained in a Waring blender. The resulting mixture was extracted with tetrahydrofuran at a moderate speed for 5 min. The extract was filtered under suction, and the solid materials were reextracted with tetrahydrofuran until the resulting filtrate was colorless. The solvent was removed on a rotary evaporator at 30 °C, and the concentrated vegetable extract was partitioned into petroleum ether and water. The water layer was washed with petroleum ether several times, and the resulting organic layers were combined, dried over sodium sulfate, and evaporated to dryness. The residue was dissolved in an appropriate volume of hexane, and samples were injected (20 μL) in duplicate for the HPLC analysis. A quantitative description of the weight of the internal standards added to each of the vegetables and the final volume of the extracts is presented in Table II. Similar extraction procedures were employed when petroleum ether (bp 30–60 °C)/acetone and diethyl ether/methanol were the extracting solvents.

Saponification. Ethereal solutions of the vegetable extracts were treated with methanolic potassium hydroxide (30%) under an atmosphere of nitrogen at room temperature for 3 h. The solution was partitioned into a saturated solution of sodium chloride and petroleum ether, and the organic layer was removed. The aqueous layer was washed with ether, and the organic layers were combined, washed several times with water, dried over sodium sulfate, and evaporated to dryness. The chlorophyll-free carotenoids (xanthophylls and the hydrocarbon carotenoids) were dissolved in an appropriate volume of hexane, and the HPLC analyses was conducted.

Separation of Xanthophylls, Chlorophylls, and the Hydrocarbon Carotenoids by Semipreparative TLC and HPLC. The general procedure described below for the separation and identification of the various components found in raw broccoli has similarly been employed for the evaluation of the major constituents of the other green vegetables. A concentrated solution of raw broccoli extract (from 500 g of raw broccoli) in hexane was chromatographed on semipreparative C-18 reversed-phase thin-layer plates (20 × 20 cm, layer thickness 200 μm; Whatman Chemical Separation Inc.) using methanol (15%), acetonitrile (80%), and methylene chloride (2.5%) and hexane (2.5%) as eluent. The following bands were separated in the order of chromatographic elution:

band	color	R_f	chemical class
1	yellow	0.56	xanthophylls
2	yellow	0.50	xanthophylls
3	yellow	0.44	xanthophylls
4	yellow	0.34	xanthophylls
5	green	0.27	chlorophylls
6	green	0.19	chlorophylls
7	yellow/green	0.15	chlorophylls
8	yellow	0.10	carotenes

Isolation of Xanthophylls. Band 1 was shown by HPLC (eluent C) to consist of two components, which were separated by semipreparative HPLC (eluent C) and were identified from their absorption and mass spectra as *all-trans*-neoxanthin [mp 142 °C, acetone/light petroleum ether; lit. mp 142–144 °C [Cholnoky et al. (1969)] and 9′-*cis*-neoxanthin (mp 134 °C, acetone/light petroleum ether; lit. mp 134 °C [Cholnoky et al. (1969)]]. The mass spectra of *all-trans*-neoxanthin and its 9′ cis isomer were identical and showed a parent ion peak at m/z 600 (86%; $C_{40}H_{56}O_4$ requires 600.418), as well as peaks at m/z 582 (100%; M − 18), 567 (53%; M − 18 − 15), 564 (84%; M − 18 − 18), 549 (78%; M − 18 − 18 − 15), 520 (44%; M − 80), 502 (95%; M − 80 − 18), and 352 (80%). The visible absorption maxima (nm) of *all-trans*-neoxanthin (hexane, λ_{max} = 412, 440, 469; benzene, λ_{max} = 427, 451, 481; carbon disulfide, λ_{max} = 440, 467, 497) and 9′-*cis*-neoxanthin (hexane, λ_{max} = 409, 436, 463; benzene, λ_{max} = 427, 447, 478; carbon disulfide, λ_{max} = 440, 463, 493) in various solvents were consistent with the chromophores involved. A solution of 9′-*cis*-neoxanthin in benzene treated with iodine according to the method described by Cholnoky et al. (1969) was shown by HPLC (eluent C) to yield *all-trans*-neoxanthin and a mixture of cis isomers. Neoxanthin and its 9′-cis isomer were converted to neochrome by the addition of a few drops of ethanolic hydrogen chloride (0.10 M). The visible absorption maxima of neochrome in various solvents were consistent with the reported absorption maxima of this compound (Cholnoky et al., 1969; Karrer and Jucker, 1950).

Band 2 was shown by HPLC (eluent C) to contain one major component as well as two minor components. These

were separated by semipreparative HPLC (eluent C) and identified from their absorption and mass spectra, in the order of chromatographic elution, as violaxanthin [major component, mp 200 °C, methanol; lit. mp 200 °C [Karrer and Jucker (1945)]], luteoxanthin, and auroxanthin. The mass spectra of these three fractions were identical and showed the molecular parent ion peaks at m/z 600 (42%; $C_{40}H_{56}O_4$ requires 600.418), as well as peaks at m/z 582 (12%; M − 18), 566 (13%; M − 18 − 16), 549 (33%; M − 18 − 18 − 15), 520 (22%; M − 80), 508 (11%; M − 92), 494 (11%; M − 106), 352 (19%), 221 (100%), and 181 (66%).

Violaxanthin. The absorption maxima of the major component of band 2 in various solvents were identical with those of violaxanthin reported in the tables of the absorption maxima for carotenoids (Ritter and Purcell, 1981). The HPLC (same conditions as above) retention time and the absorption maxima of this component were also identical with those of semisynthetic violaxanthin A prepared from epoxoidation of zeaxanthin. Treatment of violaxanthin with a few drops of ethanolic hydrogen chloride (0.1 M) afforded a mixture of luteoxanthin and auroxanthin that was completely converted to auroxanthin within minutes.

Luteoxanthin. The second minor component of band 2 was luteoxanthin, which had absorption maxima at 407, 432, and 461 nm (benzene) and 396, 422, and 448 nm (ethanol). Upon addition of ethanolic hydrogen chloride, this component was converted to auroxanthin.

Auroxanthin. The third minor component of band 2 was auroxanthin, which had absorption maxima at 379, 400, and 425 nm (hexane) and 382, 402, and 427 nm (petroleum ether).

Semisynthetic Violaxanthin. Epoxidation of zeaxanthin diacetate, prepared from zeaxanthin and acetyl chloride in pyridine, with monoperphthalic acid according to the method of Bartlett et al. (1969) gave the acetates of semisynthetic violaxanthin A, violaxanthin B, antheraxanthin A, and antheraxanthin B. These epoxides were separated by semipreparative TLC [methanol (15%), acetonitrile (75%), methylene chloride (5%), hexane (5%)] on C-18 reversed-phase plates. Isolation of the products and hydrolysis of each with methanolic potassium hydroxide (30%) at room temperature after 30 min gave semisynthetic violaxanthin A and semisynthetic violaxanthin B, which had identical visible light absorption spectra: λ_{max} = 421, 453, 483 nm (benzene). Semisynthetic antheraxanthin A and antheraxanthin B were obtained as minor products and had identical visible light absorption spectra: λ_{max} = 420, 444, 470 nm (hexane); λ_{max} = 431, 457, 487 nm (benzene). The HPLC retention time of natural violaxanthin was identical with that of semisynthetic violaxanthin A.

Band 3 was shown by HPLC (eluent C) to consist of one major and two minor components. These components were separated by semipreparative HPLC (same conditions as above) and were identified from their visible light absorption maxima and their mass spectra, in the order of chromatographic elution, as lutein epoxide [major component, mp 190 °C, methanol; lit. mp 190 °C [Cadosch and Eugster (1974)]], flavoxanthin, and a cis isomer of lutein epoxide (neolutein epoxide A). All of these compounds had identical mass spectra and showed molecular parent ion peaks at m/z 584 (22%; $C_{40}H_{56}O_3$ requires 584.881), as well as peaks at m/z 566 (43%; M − 18), 548 (49%; M − 18 − 18), 537 (38%; M − 16 − 16 − 15), 522 (46%; M − 16 − 16 − 15 − 15), 504 (14%; M − 80), 492 (12%; M − 92), 352 (17%), 236 (71%), 221 (25%), 181 (17%), 91 (100%), and 83 (64%). Addition of a few drops of ethanolic hydrogen chloride to lutein epoxide resulted in the formation of flavoxanthin.

Band 4 was shown by HPLC (eluent C) to consist of one major and three minor components, which were separated by semipreparative HPLC (eluent C) and identified from their absorption and mass spectra as *all-trans*-lutein [mp 192 °C, methanol; lit. mp 193 °C [Karrer and Jucker (1950)]], neolutein B, neolutein B′, and neolutein A. The mass spectra of these components were identical and showed molecular parent ion peaks at m/z 568 (8%; $C_{40}H_{56}O_2$ requires 568.881), as well as peaks at m/z 550 (100%; M − 18), 532 (15%; M − 18 − 18), 476 (2%; M − 92), 458 (18%; M − 92 − 18), 430 (3%; M − 138), 392 (3%; M − 158 − 18), 324 (2%; M − 138 − 106), 133 (40%), 109 (37%), 91 (41%), and 43 (24%). The HPLC (eluent C) retention time, ^{1}H NMR (Englert, 1982), and the visible light absorption spectra (Ritter and Purcell, 1981) of *all-trans*-lutein were identical with those of an authentic sample of this compound and the literature values. The visible absorption maxima (nm) of neolutein B (benzene, λ_{max} = 423, 450, 483; carbon disulfide, λ_{max} = 446, 467, 497) and neolutein B′ (benzene, λ_{max} = 427, 452, 481; carbon disulfide, λ_{max} = 448, 468, 498) in various solvents were consistent with the values expected for mono cis isomers of lutein. The visible absorption spectrum of neolutein A in the HPLC solvents (eluent B) contained an intense cis peak in the near-UV region. Owing to the long accumulation time, neolutein A was partially converted to *all-trans*-lutein during preparative HPLC.

Stereoisomerization of *all-trans*-Lutein. A solution of *all-trans*-lutein was allowed to reflux in hexane under an atmosphere of nitrogen for 6 h. The mixture was examined by HPLC (eluent C) and was found to consist of *all-trans*-lutein, neolutein B, neolutein B′, and neolutein A. The HPLC retention times and the absorption spectra of these isomers were identical with that of lutein isomers isolated from the vegetable extracts.

Isolation of Chlorophylls and Their Derivatives. The green band 5 was shown by HPLC (eluent B) to consist of a major and a minor component, which were separated by semipreparative HPLC (eluent A) and identified from their ^{1}H NMR and absorption spectra as chlorophyll *b* (major component) and chlorophyll *b′* (the C-10 epimeric isomer of chlorophyll *b*). The HPLC retention time and the absorption spectrum of the isolated chlorophyll *b* were identical with those of an authentic sample of this compound.

Examination of band 6 by HPLC (eluent B) revealed the presence of one major and two minor components that were separated by semipreparative HPLC (eluent A) and identified from their ^{1}H NMR and absorption spectra as chlorophyll *a*, chlorophyll *a′* (the C-10 epimeric isomer of chlorophyll *a*), and pheophytin *b*. The chromatographic retention time and the absorption spectrum of chlorophyll *a* were identical with those of an authentic sample of this compound.

Band 7 was shown by HPLC (eluent B) to consist of a major fraction that was identified from its absorption spectrum as pheophytin *a*. Treatment of chlorophylls *b* and *a* with a few drops of ethanolic hydrogen chloride (0.1 M) was shown by HPLC (eluent B) to afford pheophytins *b* and *a*, respectively, in nearly quantitative yield.

Isolation of the Hydrocarbon Carotenoids. Band 8 was shown by HPLC (eluent A) to consist of *all-trans*-β-carotene and its 15,15′-cis isomer, which were identified by comparison of their absorption spectra and chromatographic retention times with authentic samples of these compounds. The absorption spectrum of 15,15′-*cis*-β-

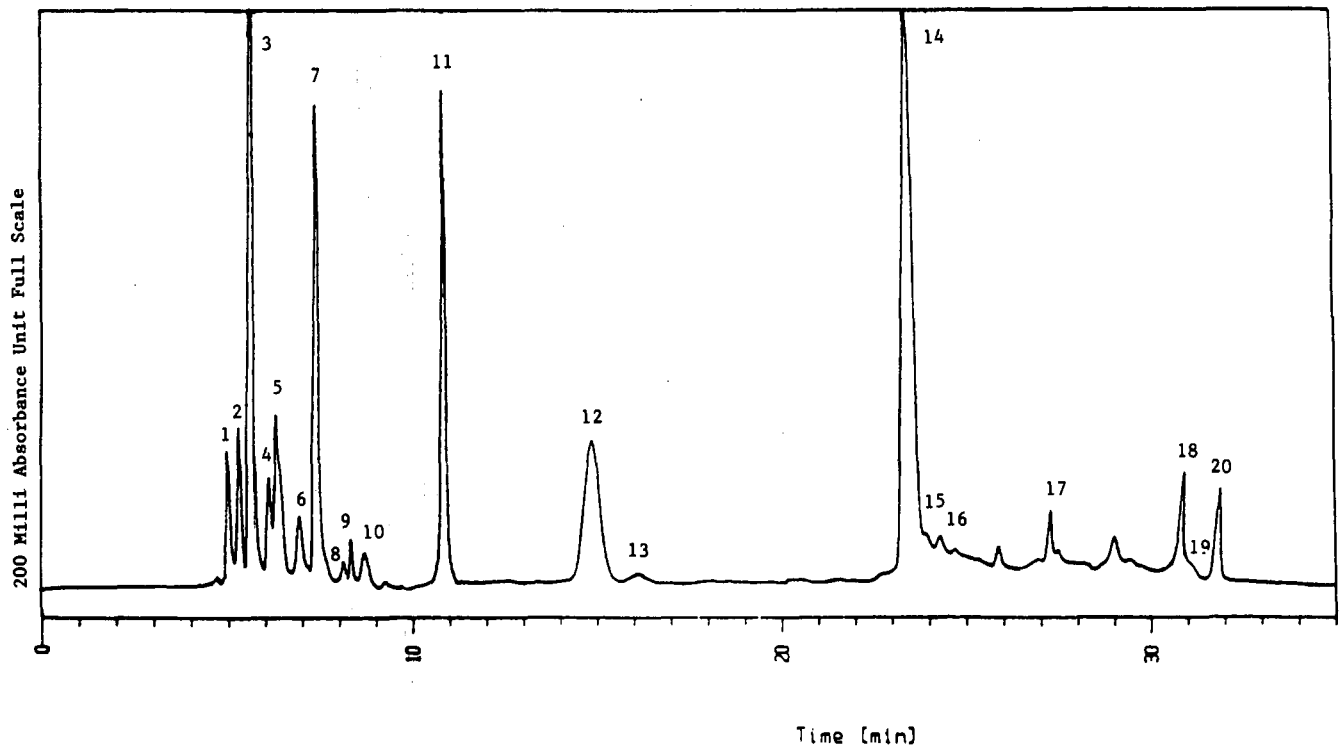

Figure 1. HPLC profile of raw brussels sprouts extract. Chromatographic conditions (eluent B) and peak identification described in text.

carotene contained a strong cis peak in the near-UV region at 334 nm, which is characteristic of the central cis isomer of carotenoids (Vetter et al., 1971).

Purification of Reference Samples and Preparation of Calibration Curves. The reference samples of neoxanthin, violaxanthin, and lutein epoxide accumulated from semipreparative TLC and HPLC were further purified by rechromatography on silica gel plates (20 × 20 cm, layer thickness 250 μm; Whatman Chemical Separation Inc.) using light petroleum ether/acetone (2:1) as eluent. These analytically pure samples were employed for the preparation of the calibration curves. The calibration curves for xanthophylls and carotenes were obtained by area measurement of pure reference compounds at various concentrations. These carotenoids were also quantified from calibration curves obtained by plotting the area ratios of pure reference compounds to that of a constant amount of their respective internal standards (β-apo-8′-carotenal for xanthophylls and decapreno-β-carotene for carotenes) at various concentrations. Since the carotenoids quantified by both methods gave similar results (less than 4% difference), the average values of the two methods were adapted. The preparation of the calibration curves for *all-trans*- and 9′-*cis*-neoxanthin was accompanied by partial stereoisomerization; therefore, these components were quantified from a calibration curve that represented an equilibrium between these isomers. A pure solution of 9′-*cis*-neoxanthin reached an equilibrium in which 20% of the all-trans isomer was present, and this equilibrium remained unchanged throughout the preparation of the calibration curve for these components. Therefore, the quantitative measurements of *all-trans*- and 9′-cis-neoxanthin were based on the total area of these isomers at various concentrations. The calibration curve for neochrome was prepared by acid treatment of the mixture of neoxanthins at various concentrations, which afforded this compound quantitatively. The cis isomers of lutein and lutein epoxide were quantified from the calibration curves prepared for their all-trans compounds. Since 15,15′-*cis*-β-carotene was not resolved and it appeared as a tailing shoulder on the *all-trans*-β-carotene peak, the area corresponding to this cis isomer was included in the integration of the *all-trans*-β-carotene peak. The chlorophylls and pheophytins were quantified from calibration curves obtained by area measurement of pure reference compounds at various concentrations. Calibration curves for pheophytins *b* and *a* were obtained by acid treatment of the solutions of chlorophylls *b* and *a* at various concentrations, respectively. The area corresponding to chlorophylls *b′* and *a′* (the C-10 epimeric isomers of chlorophylls *b* and *a*) were included in the area of chlorophylls *b* and *a*, respectively. Similarly the area corresponding to pheophytins *b′* and *a′* (the C-10 epimeric isomers of pheophytins *b* and *a*), which appeared in the chromatograms of the extracts from cooked vegetables, were combined with that of their respective pheophytins. The calibration curves prepared for xanthophylls, chlorophylls, and carotenes gave good linearity over a wide range of concentration and had relative standard deviations of less than 5%.

RESULTS AND DISCUSSION

Most of the green vegetables studied in this report are "cruciferous" vegetables of the genus *Brassica*. These were chosen for investigation owing to the epidemiological and experimental studies, which have indicated that the consumption of the green vegetables of the genus *brassica* may reduce the risk of development of certain cancers (Graham et al., 1978; Haenszel et al., 1980). A non-*Brassica* vegetable, i.e, spinach (*Spinacia oleracea*), high in carotenoids, was examined to provide a representative sample of various anatomical parts of the plant, i.e leaves, flowers, etc. The major constituents of these green vegetables consist of three classes of compounds. In the order of chromatographic elution on a C-18 reversed-phase column these are (a) xanthophylls (oxygenated carotenoids), (b) chlorophylls and their derivatives, and (c) hydrocarbon carotenoids. The chromatogram of raw brussels sprouts (Figure 1)

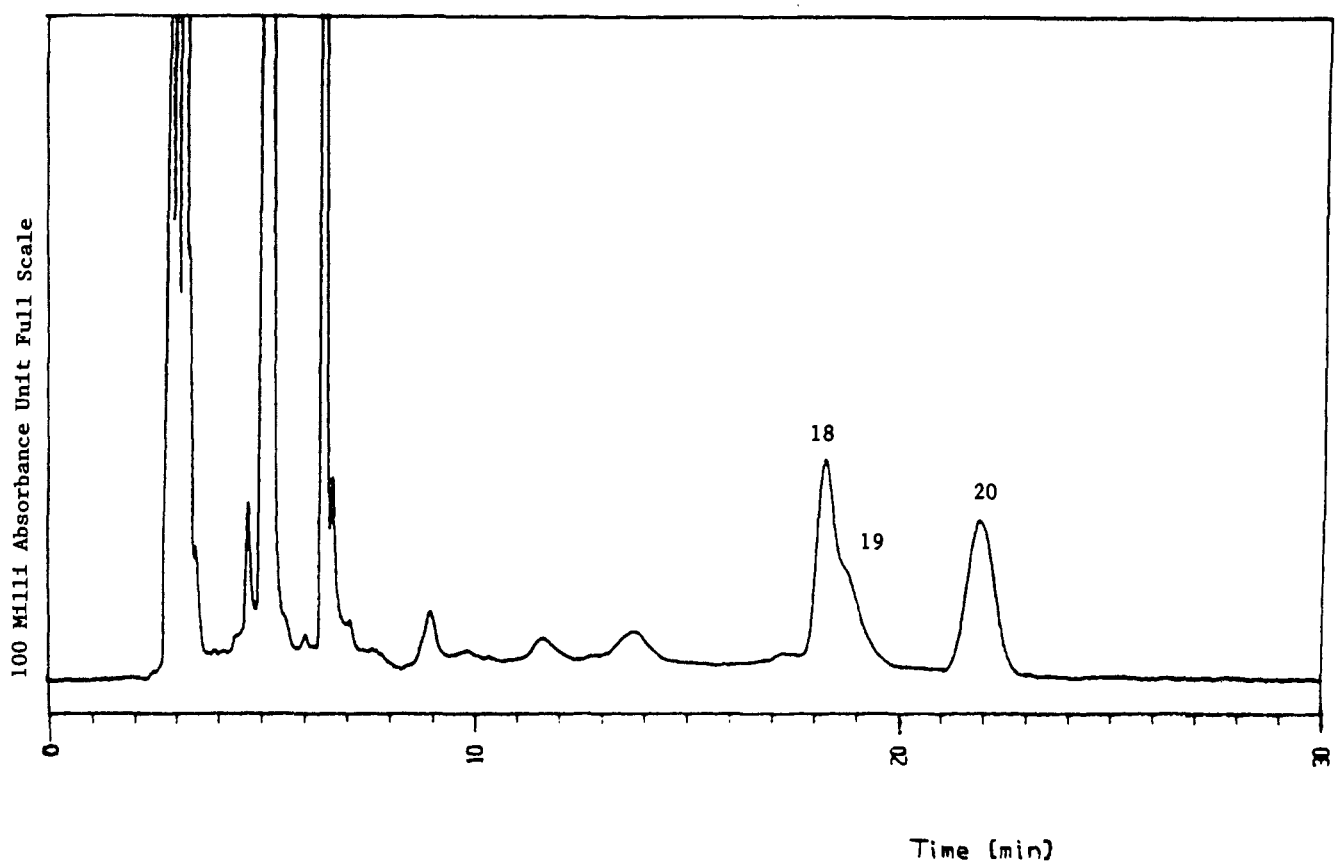

Figure 2. HPLC profile of raw brussels sprouts extract. Chromatographic conditions (eluent A) and peak identification described in text.

shows the presence of the typical components of these various classes of compounds. A combination of isocratic and gradient elution chromatography (eluent B) separated the various components within 33 min. The xanthophylls (peaks 1–10) and the internal standard, β-apo-8′-carotenal (peak 11), are eluted under isocratic conditions, which is followed by a gradient that elutes the chlorophylls and their derivatives (peaks 12–17) as well as the hydrocarbon carotenoids (peaks 18 and 19) and decapreno-β-carotene (peak 20, internal standard). Although under these conditions (eluent B), the above components are well separated, owing to the greater abundance of xanthophylls and chlorophylls with respect to the hydrocarbon carotenoids, the latter have been quantified employing isocratic HPLC conditions (eluent A). This isocratic HPLC condition has been particularly developed for the separation of α- and β-carotene and its 15,15′-cis isomer from decapreno-β-carotene (Khachik and Beecher, 1985). Under these conditions, *all-trans*-β-carotene (peak 18) and its 15,15′-cis isomer (peak 19), which were shown to be the only hydrocarbon carotenoids present in these series of the green vegetables, separated from decapreno-β-carotene (internal standard) within 23 min. The separation of these carotenes employing isocratic HPLC conditions allows the quantification of these components at higher concentrations and therefore minimizes the analytical errors arising from possible lack of reproducibility of the gradient as well as the peak area measurements at higher dilutions. The isocratic chromatographic separation for the hydrocarbon carotenoids in raw brussels sprouts has been shown in Figure 2.

Peak Identification. The abundant xanthophylls, chlorophylls, and the hydrocarbon carotenoids found in these green vegetables and their corresponding HPLC peaks as identified by spectroscopy and chromatography as well as chemical methods are shown in Table I. The details of the identification and the chemistry of these compounds will be described later in this text.

Qualitative Distribution of Carotenoids and Chlorophylls. The general chromatographic profiles of the other green vegetables are very similar to that of raw brussels sprouts described above. The major differences among these vegetables appear to be the level of concentration at which the various components are present. For example, the chromatograms from the extracts of raw broccoli and cabbage (data not shown) show the presence of the same components; however, the relative concentration of lutein epoxide (peak 5) with respect to the other xanthophylls present in this vegetable is found to be much higher than that observed in raw spinach and kale. These similarities are consistent with the extensive surveys carried out by Strain (1966), who has shown that the leaves of higher plants usually contain the same carotenoids. These carotenoids have been characterized as β-carotene, lutein, violaxanthin, and neoxanthin. Detailed qualitative and quantitative distribution of carotenoids in photosynthetic tissues has been reported by Goodwin (1980).

Examination of the extracts from the cooked vegetables revealed some interesting structural transformations. A comparison between the chromatogram of cooked brussels sprouts (Figure 3) with the chromatogram of its raw extract (Figure 1) indicated that a number of xanthophylls were destroyed as the result of the cooking process. The only surviving xanthophylls in the cooked vegetables were lutein (peak 7) and its stereoisomers (peaks 8–10) as well as minor quantities of neochrome (peak 4). Since small amounts of neochrome were shown to be present in some of the raw vegetable extracts, it is not clear at this point whether the surviving neochrome is in part a product of thermal isomerization of neoxanthin. Similar results were obtained with the extract from cooked kale. The destruction of xanthophylls in the cooked vegetables is not surprising as these

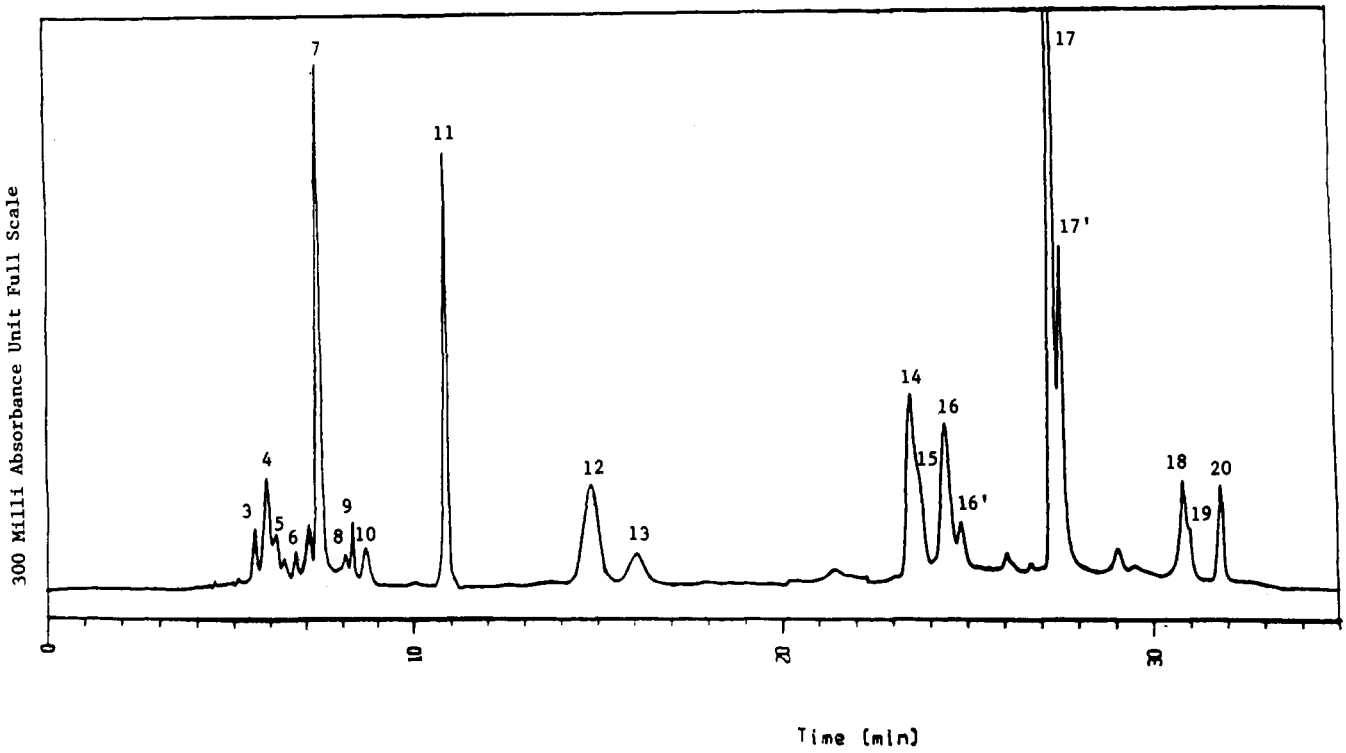

Figure 3. HPLC profile of cooked brussels sprouts extract. Chromatographic conditions (eluent B) and peak identification described in text.

compounds are found to be very sensitive to treatment with heat, light, and trace amounts of acids, and they are readily subjected to rearrangement, stereoisomerization, and degradation. No significant isomerization of lutein (peak 7) to its stereoisomers (peaks 8–10) is observed as a result of cooking. The cooking process also effects the conversion of chlorophyll *b* (peak 12) and chlorophyll *a* (peak 14) into pheophytin *b* (peak 16) and pheophytin *a* (peak 17), respectively. The hydrocarbon carotenoids, *all-trans*-β-carotene (peak 18) and its 15,15′-cis isomer (peak 19), are not greatly affected, and no significant stereoisomerization is observed with the application of heat. It must be pointed out that the degradation, rearrangement, and stereoisomerization of carotenoids and chlorophylls are expected to be greatly influenced by the severity and the length of cooking as well as the means by which the vegetables were cooked (i.e., frying, steaming, boiling, microwave cooking, etc.). Therefore, these factors would be expected to effect the proportion of the various components in the cooked vegetables. In the present study, the vegetables were investigated in the cooked form in an attempt to provide an overall picture of the possible structural changes of the carotenoids in comparison to the raw forms. However, more carefully designed experiments will be necessary in order to establish the detailed trend of these structural transformations.

Structural Elucidation. The structural elucidation of the various components of xanthophylls, chlorophylls, and the hydrocarbon carotenoids was based on the absorption and mass spectral data as well as comparison of the HPLC retention times of unknowns with those of reference compounds. In some cases the structures were established by means of chemical reactions and/or by partial synthesis of the compounds in question. Since detailed procedures for the isolation and identification of various components have been given in the Experimental Section, attention will be focused only on the structural identification of the compounds that involve chemical reactions and partial synthesis.

(*a*) *Xanthophylls.* The major xanthophylls found in green vegetables are listed in the order of chromatographic elution, in Table I. The identification of these compounds by mass spectroscopy was often accompanied by confusing results, since the 5,6-epoxides and their rearrangement products (furanoxides) gave identical fragmentation patterns. The rearrangement of the 5,6- to the 5,8-epoxides, either thermally or under the influence of electron bombardment on the probe, has been suggested (Cholnoky et al., 1969) to be responsible for identical breakdown patterns observed for these compounds in mass spectroscopy. The *cis*-carotenoids also exhibited an identical fragmentation pattern to that of their all-trans compounds. The presence of the characteristic fragments in the mass spectra of xanthophylls at m/z M − 18 − 18 indicated the presence of the hydroxy groups. The mass spectra of the xanthophylls also contained fragments at m/z M − 92 (loss of toluene), M − 106 (loss of xylene), M − 158 (loss of a precursor of 2,6-dimethylnaphthalene), which are typical of the fragmentation reactions of carotenoids. The xanthophylls with epoxy end groups were particularly identified from the fragmentation patterns of their mass spectra, which showed the presence of fragments at m/z M − 80 (loss of C_6H_8) and M − 106, as well as fragments at m/z 221 and 181. The fragments at m/z M − 80 and M − 106 are typically formed from 5,6- and 5,8-epoxides involving nonconventional in-chain elimination reactions. The detailed mechanism of the fragmentation reactions of carotenoids has been thoroughly investigated by Enzell and Wahlberg (1980) and Vetter et al. (1971). The mass spectral data coupled with UV/visible light absorption properties of individual xanthophylls provided valuable information on the nature of the end group and the chromophores involved.

all-trans-Neoxanthin (known as neoxanthin X) and 9′-*cis*-neoxanthin (natural neoxanthin) were identified from their UV/vis light absorption and mass spectra. The absorption maximum of 9′-*cis*-neoxanthin in various solvents exhibited a hypsochromic shift of 4 nm in comparison to

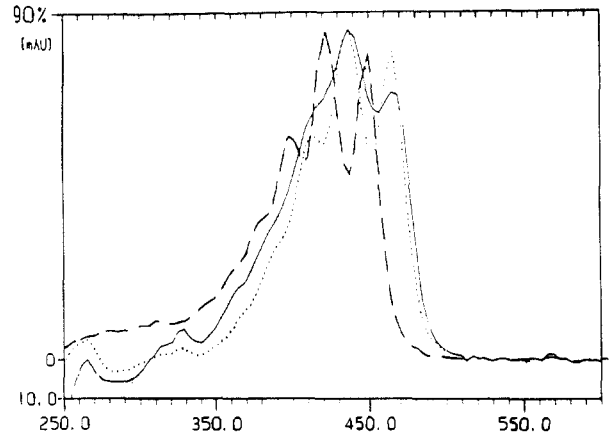

Figure 4. UV/vis light absorption spectra of *all-trans*-neoxanthin [(—) λ_{max} = 438 nm], 9′-*cis*-neoxanthin [(···) λ_{max} = 438 nm], and neochrome [(- - -) λ_{max} = 422 nm] in the HPLC solvent system (eluent C) described in text.

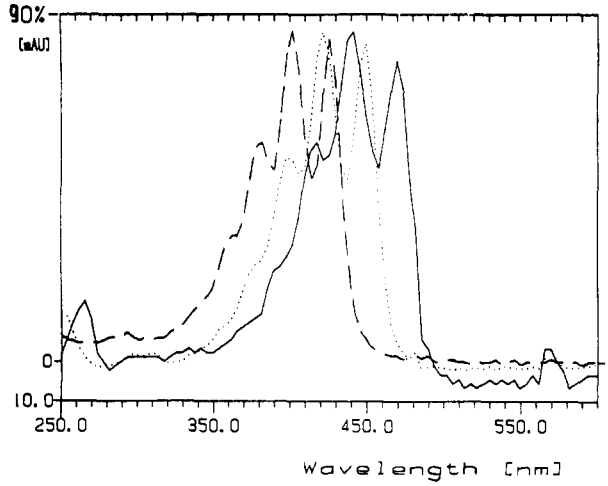

Figure 5. UV/vis light absorption spectra of violaxanthin [(—) λ_{max} = 442 nm], luteoxanthin [(···) λ_{max} = 422 nm], and auroxanthin [(- - -) λ_{max} = 402 nm] in the HPLC solvent system (eluent C) described in text.

its all-trans compound. Upon addition of a few drops of ethanolic hydrogen chloride, both stereoisomers underwent the well-known epoxide–furanoxide rearrangement to afford neochrome with the expected hypsochromic shift of 16–20 nm in their absorption maxima (Karrer and Jucker, 1946; Strain, 1954; Curl and Bailey, 1961). The visible absorption spectrum of 9′-*cis*-neoxanthin in the HPLC solvents (eluent C) exhibited well-defined fine structure in comparison to its all-trans isomer. Both neoxanthins had identical absorption maxima at 438 nm [HPLC solvents (eluent C)], while the absorption maximum of neochrome appeared at 422 nm (Figure 4). The location of the cis double bond in 9′-*cis*-neoxanthin was assumed by excluding the sterically hindered cis isomers (7′-cis, 11-cis, 11′-cis) as well as the exclusion of 13-cis, 13′-cis, and 15,15′-cis isomer due to the absence of a strong cis peak in the UV/visible light absorption spectrum of 9′-*cis*-neoxanthin. Further support for a 9′-cis geometry in neoxanthin has been obtained by means of oxidative degradation under controlled conditions (Szabolcs, 1976). Neoxanthin has also been shown by Curl and Bailey (1957) to exhibit geometrical isomerism. It was found by Cholnoky et al. (1969) that *all-trans*-neoxanthin constitutes the principal isomer from maple leaves, provided that the customary saponification step is omitted. In our studies of the green vegetable extracts, in which the carotenoids were evaluated by HPLC without saponification, the 9′-*cis*-neoxanthin has consistently been the predominant isomer. The presence of *all-trans*-neoxanthin and neochrome in the green vegetables may be an artifact due to extraction and/or chromatography; however, the occurrence of natural neoxanthin (9′-cis) in chloroplast has been well established (Goodwin, 1980). Some interesting reactions of carotenoids involving the allenic end group of neoxanthin-type have been reported (Johansen and Liaaen-Jensen, 1974; Buchecker and Liaaen-Jensen, 1975). Neochrome was shown to be more heat resistant than neoxanthins and the other epoxy carotenoid as evidenced from the HPLC profiles of the cooked vegetables. The identity of this compound in the extracts from cooked brussels sprouts and kale was confirmed by addition of a few drops of ethanolic hydrogen chloride to these solutions and examination of the resulting HPLC profile. The HPLC retention time as well as the absorption spectrum of this compound was identical with that of an authentic sample of neochrome obtained from neoxanthin. The absorption maxima of neochrome (λ_{max} = 422 nm) in the HPLC solvents (eluent C) remained unchanged on acid treatment.

Violaxanthin was identified from its visible light absorption and mass spectra. Isolation of this compound by semipreparative TLC was shown by HPLC to have resulted in the formation of luteoxanthin and auroxanthin as minor products. The conversion of violaxanthin into its mono- and di-5,8-epoxides was also confirmed in the presence of trace amounts of acid. The visible light absorption spectra of violaxanthin (λ_{max} = 442 nm), luteoxanthin (λ_{max} = 422 nm), and auroxanthin (λ_{max} = 402 nm) in the HPLC solvents (eluent C; Figure 5) exhibited maxima that were consistent with the commonly observed 18–20-nm hypsochromic shift as a result of such rearrangement (Eugster and Karrer, 1957; Tsukida and Zechmeister, 1958). In an attempt to establish the presence of violaxanthin and the absence of antheraxanthin (a structurally related isomer of lutein epoxide) in the vegetables studied in this report, partial syntheses of these compounds were investigated. The epoxidation of zeaxanthin diacetate by monoperphthalic acid according to the procedure described by Bartlett et al. (1969) was shown by HPLC (eluent C) to afford two major and two minor products. The separation of these products by semipreparative TLC (eluent C) followed by hydrolysis of each component gave four products. The first two major products were tentively identified from their absorption spectra [both showed absorption maxima at 442 nm in the HPLC solvents (eluent C)] and the hydrochloric acid test as semisynthetic violaxanthin A and semisynthetic violaxanthin B. Although a mixture of the acetates of these two components were well separated by HPLC (eluent C), semisynthetic violaxanthin A and violaxanthin B appeared as partially resolved peaks. The retention time of the natural violaxanthin was identical with that of semisynthetic violaxanthin A. The absolute configurations of these epoxides (prepared in this report) are not known. On the basis of ^{1}H NMR and ORD studies, Bartlett et al. (1969) have shown that the epoxide rings in semisynthetic violaxanthin A have a different stereochemistry than that of natural violaxanthin. An absolute configuration in which the epoxide rings are trans with respect to C-3 hydroxy groups was assigned to natural violaxanthin by these workers. More recent structural elucidation of the xanthophyll epoxides has confirmed that all of these compounds exhibit a trans relationship between C-3 hydroxy and the epoxide group (Eugster, 1982). This stereochemistry is preserved on acid-catalyzed rearrangement of 5,6- to 5,8-epoxides (Weedon, 1971). Semisynthetic viola-

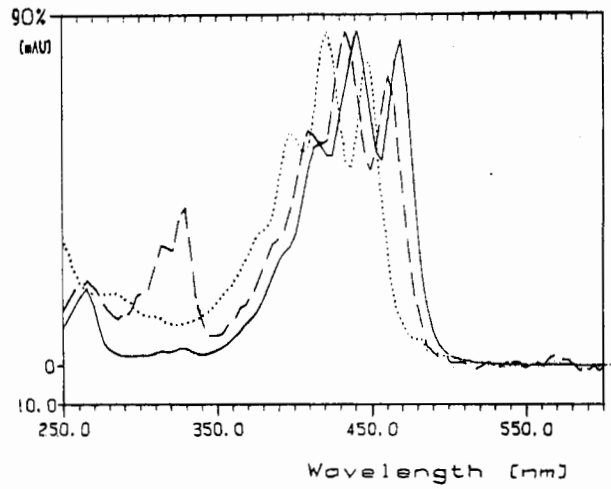

Figure 6. UV/vis light absorption spectra of *all-trans*-lutein epoxide [(—) λ_{max} = 442 nm], neolutein epoxide A [(- - -) λ_{max} = 434 nm], and flavoxanthin [(···) λ_{max} = 422 nm] in the HPLC solvent system (eluent C) described in text.

xanthin B has been reported not to exhibit a detectable ORD curve, and as a result a configuration in which a *trans*- and a *cis*-epoxide end group (with respect to the C-3 hydroxy group) are both present has been assumed for this compound (Bartlett et al., 1969). The two minor products, which were not separable by HPLC (eluent C), were tentatively identified from their absorption spectra (both showed maxima at 446 nm in the HPLC solvents) and hydrochloric acid test as semisynthetic antheraxanthin A and antheraxanthin B. These epoxides are probably a mixture of stereoisomers of antheraxanthin in which the epoxide ring may adapt a cis or trans configuration with respect to the C-3 hydroxy group.

Lutein epoxide isolated by semipreparative TLC was shown by HPLC to have partially converted to flavoxanthin. A cis isomer of lutein epoxide (neolutein epoxide A) was also shown to be present in most of the vegetable extracts that were examined by HPLC. The absorption spectra of *all-trans*-lutein epoxide (λ_{max} = 442 nm), its cis isomer (λ_{max} = 434 nm), and flavoxanthin (λ_{max} = 422 nm) in the HPLC solvents (eluent C) are shown in Figure 6. The presence of a strong cis peak in the UV/visible light absorption spectrum of the *cis*-lutein epoxide suggests that the cis double bond in this compound is probably located at a more central position (13-cis, 13′-cis, 15,15′-cis). The absorption spectrum of this compound resembles that of *syn*-neolutein epoxide A (identified as 13-cis isomer of lutein epoxide) and *syn*-neolutein epoxide A* (identified as 13′-cis isomer of lutein epoxide), which have been prepared by Szabolcs (1976) from iodine-catalyzed stereoisomerization of native lutein epoxide. The conversion of a solution of *all-trans*-lutein epoxide, isolated from the vegetable extracts, to its mono cis isomer was effected in boiling benzene. However, since the central mono cis forms (15,15′-cis) of carotenoids are known not to exist in the equilibrium mixture of isomers (Inhoffen et al., 1951), this mono cis isomer was tentively identified as 13- or 13′-*cis*-lutein epoxide. On treatment with trace amounts of acid the absorption maximum of lutein epoxide showed a 20-nm hypsochromic shift (Figure 6). Some literature reports have indicated that antheraxanthin, a structurally related isomer of lutein epoxide, is one of the constituents of raw spinach and cabbage (Stransky, 1978; Takagi, 1985). We have not observed the presence of antheraxanthin in any of the green vegetables that were investigated in the present work. The light absorption maxima of the compound that we isolated by semipreparative TLC and HPLC in various solvents are more consistent with the literature values of lutein epoxide than that of antheraxanthin (Ritter and Purcell, 1981). The absorption maxima of the product obtained from acid treatment of lutein epoxide in various solvents were also consistent with those of flavoxanthin. In addition the HPLC retention time and the light absorption maxima of semisynthetic antheraxanthin A and antheraxanthin B (λ_{max} = 446 nm), prepared by partial synthesis according to the procedure described above, were different from those of lutein epoxide (λ_{max} = 442 nm) in the HPLC solvents (eluent C). In analogy to the biosynthesis of antheraxanthin from zeaxanthin (Siefermann and Yamamoto, 1975), the biosynthetic pathway to lutein epoxide would be expected to involve lutein. Therefore, the occurrence of lutein epoxide is not surprising, since its precursor, lutein, is the most abundant xanthophyll among these vegetables. On the other hand, zeaxanthin, the precursor of antheraxanthin, was not detected in the vegetable extracts. The stereoisomers of lutein and lutein epoxide have been isolated from flowers of Helianthus Annuus, Impatiens Noli Tangere, Ranunculus Acer, and Taraxacum Officinale and in ripe hips of Rosa Canina and Rosa Rubiginosa by Toth and Szabolcs (1970).

Lutein, the most abundant xanthophyll in the green vegetables, was shown to be accompanied by three minor cis isomers, which were identified from their absorption and mass spectral data. The absorption maxima of neolutein B, in various solvents, showed a hypsochromic shift of 7–8 nm with respect to lutein, while such a hypsochromic shift for neolutein B′ was 5–6 nm. The relatively small hypsochromic shift (5–8 nm) in the absorption maxima of neolutein B and neolutein B′ with respect to that of *all-trans*-lutein suggests that these are probably mono cis isomers of lutein. The location of the cis double bonds in these isomers is not known; however, the absence of a strong cis peak in the near-UV region in the absorption spectra of these compounds and the exclusion of the sterically hindered cis isomers may suggest the possibility of 9- and 9′-cis isomers. The third minor cis isomer contained a strong cis peak in the near-UV region at 332 nm, which indicates that the cis double bond in this isomer occupies a more central position, i.e. 13-, 13′-, or 15,15′-cis. However, since a boiling solution of *all-trans*-lutein in hexane was shown to result in the formation of this cis isomer, the possibility of 15,15′-cis isomer may be ruled out. Therefore, neolutein A was tentively identified as 13- or 13′-*cis*-lutein. Neolutein A was partially converted to *all-trans*-lutein during the long accumulation time of preparative HPLC. The absorption spectra of *all-trans*-lutein (λ_{max} = 446 nm), neolutein B (λ_{max} = 442 nm), neolutein B′ (λ_{max} = 442 nm), and neolutein A (λ_{max} = 438 nm) in the HPLC solvents (eluent C) are shown in Figure 7.

(*b*) *Chlorophylls.* The major chlorophylls found in the green vegetables examined were identified as chlorophylls *b* and *a* from their ^{1}H NMR and absorption spectra as well as comparison of their HPLC retention times with those of authentic samples. Chlorophylls *b* and *a* were both accompanied by minor quantities of their C-10 epimeric isomers, which are known as chlorophylls *b′* and *a′*, respectively. These epimeric isomers have also been reported by Schwartz et al. (1981) in the HPLC separation of chlorophylls and their derivatives in fresh and processed spinach. The visible light absorption spectra of chlorophylls *b′* and *a′* were identical with those of chlorophylls *b* and *a*, respectively. Pheophytins *b* and *a*, the most common derivatives of chlorophylls *b* and *a*, were also present in most of the vegetable extracts. The conversion

Table III. Quantitative Distribution of Xanthophylls, Carotenes, and Chlorophylls in Green Vegetables

	green, vegetables, mg/100 g edible food						
	broccoli raw	cabbage raw	spinach raw	brussels sprouts raw	brussels sprouts cooked	kale raw	kale cooked
Xanthophylls							
all-trans-neoxanthin	0.21	0.07	1.39	0.36		3.01	2.76
9′-*cis*-neoxanthin	0.25	0.10	4.98	0.41		9.21	8.42
violaxanthin	0.31	0.10	4.16	0.70	0.07	6.73	0.77
neochrome		0.05	0.88	0.31	0.53	2.99	3.21
lutein epoxide	0.82	0.14	1.27	0.66	0.15	1.50	
cis-lutein epoxide (13- or 13′-cis)	0.11	0.04	1.10	0.30	0.42		
all-trans-lutein	1.77	0.28	14.4	1.34	1.18	34.2	23.5
neolutein B (9- or 9′-cis)	0.07	0.01	0.13	0.05	0.03	0.82	0.35
neolutein B′ (9′- or 9-cis)	0.11	0.01	0.51	0.07	0.02	2.19	0.69
neeolutein A (13- or 13′-cis)	0.11	0.01	0.90	0.13	0.06	2.34	1.05
total	3.76	0.81	29.7	4.33	2.46	63.0	40.8
Carotenes							
all-trans- + 15,15′-*cis*-β-carotene	0.48	0.08	6.71	0.53	0.45	14.6	12.6
total carotenoids	4.24	0.89	36.4	4.86	2.91	77.6	53.4
total (av) content from food tables (Souci et al., 1981)	1.90	0.04	4.20	0.40		4.10	
Chlorophylls							
chlorophyll *b*	1.49	0.40	20.2	1.14	0.69	46.4	29.2
chlorophyll *a*	5.77	1.13	94.6	4.61	2.35	137	85.0
pheophytin *b*	0.52	0.14	9.66	0.25	0.60	1.7	16.0
pheophytin *a*	0.12	0.05	2.12	0.01	1.50	1.69	51.2
total	7.9	1.72	127	6.01	5.14	187	181

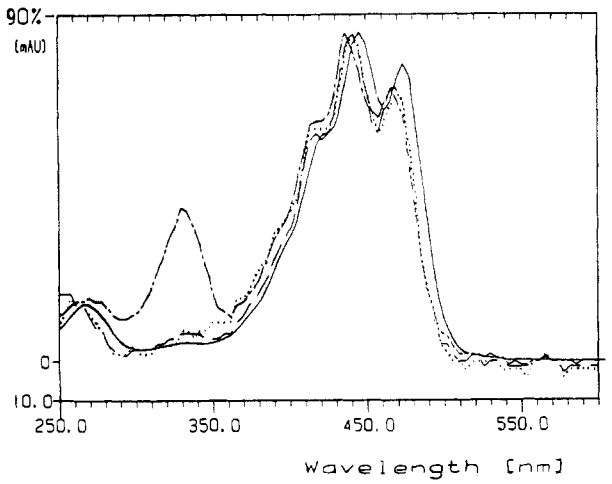

Figure 7. UV/vis light absorption spectra of *all-trans*-lutein [(—) λ_{max} = 446 nm], neolutein B [(···) λ_{max} = 442 nm], neolutein B′ [(- - -) λ_{max} = 442 nm], and neolutein A [-·-) λ_{max} = 438 nm] in the HPLC solvent system (eluent C) described in text.

of chlorophylls to pheophytins, which is readily effected as a result of heat or acid treatment, has been well documented in the literature (Kephart, 1955). The formation of pheophytin *b* (λ_{max} = 430 nm) and pheophytin *a* (λ_{max} = 410 nm) resulted in a 32- and 20-nm hypsochromic shift in the visible light absorption maxima of chlorophyll *b* (λ_{max} = 462 nm) and chlorophyll *a* (λ_{max} = 430 nm) in the HPLC solvents (eluent B), respectively. Although the chlorophylls and pheophytins also exhibit absorption maxima above 600 nm, owing to the wavelength limitation of the HPLC detector, the absorption spectra were only monitored between 200 and 600 nm. The visible light absorption spectra of the chlorophylls and pheophytins measured with a spectrophotometer between 200 and 800 nm were identical with those of their authentic samples.

(*c*) *Hydrocarbon Carotenoids.* *all-trans*-β-Carotene and its 15,15′-cis isomer were found to be the only hydrocarbon carotenoids present in the green vegetables. These were identified by comparison of their HPLC retention times and absorption spectra with those of the authentic samples. The visible light absorption spectra of *all-trans*-β-carotene (λ_{max} = 454 nm), 15,15′-*cis*-β-carotene (λ_{max} = 446 nm), and decapreno-β-carotene [internal standard (λ_{max} = 502 nm)] are shown in Figure 8. The presence of an intense cis peak (λ = 334 nm) in the absorption spectrum of 15,15′-*cis*-β-carotene is characteristic of the central cis isomer of carotenoids (Vetter et al., 1971). This central cis isomer of β-carotene, which has also been detected in the extracts from raw carrots (Khachik and Beecher, 1985), may be an artifact of extraction and/or chromatography.

Quantitative Distribution of Xanthophylls, Chlorophylls, and Carotenes. The quantitative distribution of xanthophylls, chlorophylls, and carotenes in selected vegetables is shown in Table III. The quantitative data shown in Table III for each vegetable were obtained from two consecutive extractions from one homogeneous batch of sample; therefore, these data are not necessarily representative of the levels of these compounds in the products consumed nationwide. Although the green vegetables studied in this report contain the same constituents, the concentration of the components of each class (xanthophylls, carotenes, chlorophylls) varies over a fairly wide range depending upon variation attributable to cultivar, season, and growing location. It is interesting to note that, in the case of xanthophylls, there are some similarities in the distribution percentage of the various components between different vegetables. It appears that the lutein percentages of the total xanthophylls in broccoli, spinach, and kale are higher than those of cabbage and brussels sprouts. These differences may be related to a more ef-

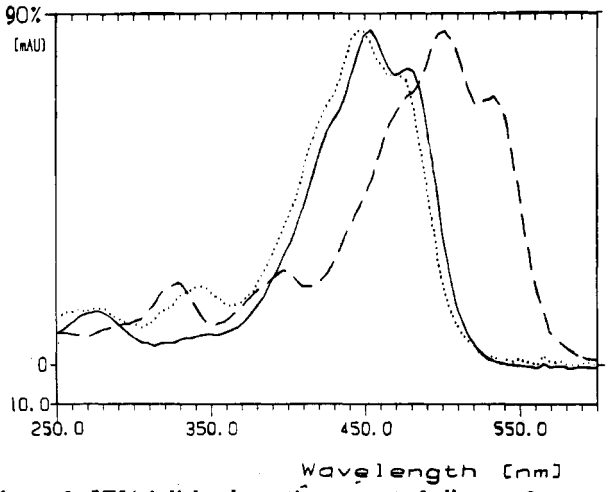

Figure 8. UV/vis light absorption spectra of *all-trans*-β-carotene [(—) λ_{max} = 454 nm], 15,15′-*cis*-β-carotene [(···) λ_{max} = 446 nm], and decapreno-β-carotene [(- - -), λ_{max} = 502 nm] in the HPLC solvent system (eluent A) described in text.

ficient conversion of lutein to lutein epoxide in cabbage and brussels sprouts. Since the hydroxylation of α- and β-carotene is known to be responsible for the formation of 3-hydroxy cyclic carotenoids and epoxy carotenoids, the absence of α-carotene in the green vegetables studied in this report may therefore be related to the conversion of this compound to lutein. We have recently shown that the extracts derived from a number of other green vegetables (lima beans, peas, green beans) contained α-carotene in addition to the chloroplast pigments described in this report (Khachik and Beecher, 1986). Therefore, under the HPLC conditions (eluents A and B) developed for the separation of carotenoids and chlorophylls in the green vegetables, α-carotene would have been well separated from β-carotene and decapreno-β-carotene (internal standard) had it been present.

The occurrence of neoxanthin, which is the most common allene in the green leaves, has also been associated with violaxanthin and lutein. There have been reports that transformation in the end group of one of these carotenoids or of zeaxanthin may perhaps be responsible for the elaboration of the allenic end group (Bonnett et al., 1969; Isoe et al., 1968; Foote and Brenner, 1968). Although, most of the vegetables examined are members of the genus *Brassica*, the differences between the percentages of neoxanthin, violaxanthin, and lutein from one vegetable to another may be attributed to these biosynthetic transformations. The photosynthetic mechanism by which these xanthophylls are formed would be expected to be greatly affected by the sources of sample variance (cultivar, growing season, location). The effect of environment on the synthesis of carotenoids in leaves has been reported by Goodwin (1980). The vegetables examined also represent various anatomical parts of the plants, which would also be expected to reflect in the distribution of the xanthophylls.

The presence of the cis isomers of xanthophylls in the vegetable extracts was initially expected to be due to an artifact of extraction and/or chromatography. Such artifacts usually result in the formation of an equilibrium mixture in which the final ratio of cis to trans isomers at equilibrium is constant. However, the quantitative evaluation of the ratios of the cis to trans isomers in various vegetables, obtained under identical extraction and chromatographic procedures, indicates that the cis isomers may not be an artifact. These changes in the ratio of cis to trans isomers are particularly noticeable in the case of 9′-*cis*- and *all-trans*-neoxanthin, which varies from 1.13 (brussels sprouts) to 3.6 (spinach) and lutein epoxide in which the ratio of trans to cis isomers varies from 1.15 (spinach) to 7.8 (broccoli). The presence of neochrome in the vegetable extracts is probably an artifact, as both the *all-trans*- and 9′-*cis*-neoxanthin readily undergo rearrangement to form this compound.

The total xanthophyll content in these vegetables is about 4–10 times greater than that of the total hydrocarbon carotenoids. Our xanthophyll values for spinach are about 2–6 times greater than those reported by Stransky (1978). This may be due to considerable differences between the chromatographic conditions, as well as the usually observed large variability of vegetable samples. There are some discrepancies between the total carotene content of the vegetables presented in this report and those of the same vegetables reported in the food tables (Souci et al., 1981). The data in the food tables have been generated on various food extracts in which the xanthophylls and the chlorophylls were removed by extraction and saponification, and the remaining carotenes were determined by colorimetric methods. The discrepancy between our data and the total carotene content from the food tables clearly demonstrates the advantage of the HPLC analysis over colorimetric methods.

In an attempt to compare the data obtained for the raw and cooked vegetables, the same batches of samples were employed for both analyses. A comparison between the total xanthophyll content of raw and cooked brussels sprouts shows that about 60% of the total xanthophylls is destroyed as a result of cooking. The loss of the total xanthophylls for raw and cooked kale is about 68%. The application of heat affects violaxanthin most, and in comparison to the raw forms only 10% and 12% of this component survive in cooked brussels sprouts and kale, respectively. There are no significant stereochemical changes in the ratio of the cis to trans isomers of neoxanthin, lutein epoxide, and lutein. However some conversion of the *all-trans*- and 9′-*cis*-neoxanthin to neochrome is noticeable. Since the degradation of the xanthophylls is a competing process, which would also be expected to contribute to the changes in the ratio of cis to trans isomers, more carefully designed experiments are required to investigate the thermally induced stereomutation of the various xanthophylls in the cooked vegetables. The much lower loss of *all-trans*-neoxanthin (8%) and its 9′-*cis*-isomer (9%) in cooked kale in comparison to brussels sprouts (100%) may be due to the much higher concentration of neoxanthins in the former; the ratio of the total neoxanthin in raw kale to that of raw brussels sprouts is 8:1. The losses of *all-trans*-β-carotene and its 15,15′-cis isomer in cooked brussels sprouts and kale were 15% and 14%, respectively.

The quantitative data on chlorophylls and pheophytins (Table III) indicate that the loss of magnesium in chlorophyll *b*, which converts this compound to pheophytin *b* for most of the raw vegetables (with the exception of kale), is taking place much more efficiently than that in chlorophyll *a*. In contrast to our finding, chlorophyll *a* has been reported to be 5 times more susceptible to pheophytin formation than chlorophyll *b* (Schanderl et al., 1962). However in cooked kale and brussels sprouts pheophytin *a* is formed 3.5 and 4 times more than that of their corresponding pheophytin *b*. The total chlorophyll loss in cooked brussels sprouts is about 14%, which predominantly results from degradation of chlorophyll *a* and pheophytin *a*, while no significant loss of chlorophylls for cooked kale is observed (3%). From these data it appears that the rate of formation of pheophytins from chlorophylls

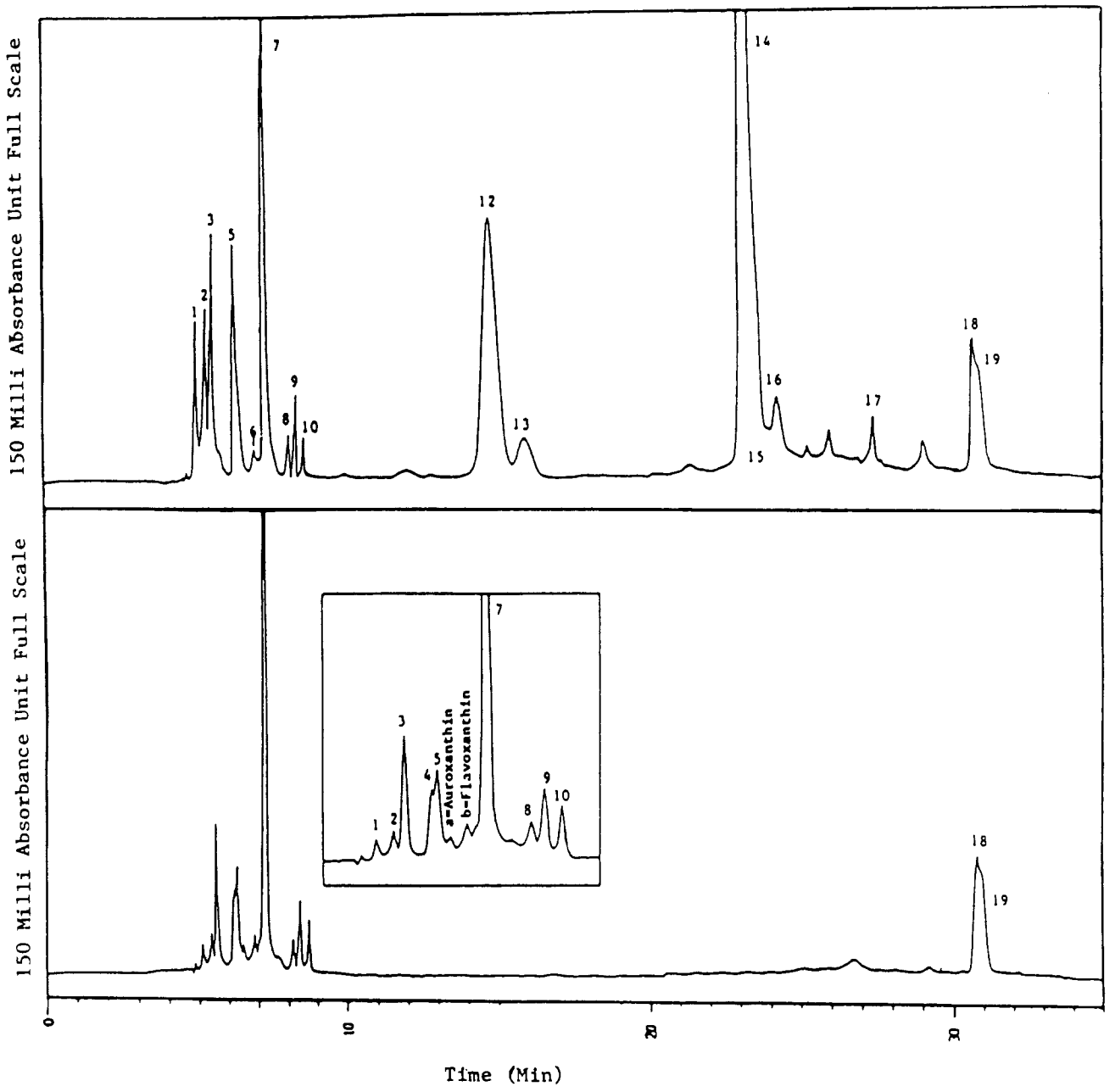

Figure 9. HPLC profiles of raw broccoli extract. Chromatographic conditions (eluent B) and peak identification described in text. Upper trace: extract before saponification. Lower trace: extract after saponification.

may be a controlled process of thermodynamics vs. kinetics. The extent to which the chlorophylls and pheophytins undergo degradation may also be a contributing factor that may, to some extent, prevent their accurate quantitative measurement.

Effect of Extraction and Saponification on Distribution of Carotenoids. The choice of the extracting solvent for naturally occurring carotenoids is very important as each class of these compounds exhibits a different solubility behavior. Tetrahydrofuran (THF), which was stabilized with butylated hydroxytoluene (BHT), was employed for the extraction of the vegetables. This solvent not only readily solubilizes the carotenoids and the chlorophylls but also by denaturing the protein-complexed carotenoids prevents the formation of emulsions. However, since this solvent is known to promote peroxide formation that may contribute to production of artifacts, several of the vegetables were extracted with a combination of light petroleum ether/acetone and diethyl ether/methanol. No significant change in both qualitative and quantitative distribution of the carotenoids and chlorophylls was indicated. The absence of the carotenoids insoluble in petroleum ether was also confirmed by employing a mixture of this solvent with 85% methanol at the partitioning stage.

The saponification of the plant extracts prior to the analysis of carotenoids has often been employed as an effective purification step for the removal of chlorophylls and lipids (Davies, 1965; Liaaen-Jensen, 1971). Although, the chlorophylls and their derivatives are readily removed from xanthophylls and carotenes by saponification, a number of carotenoids are known to be sensitive to alkaline solutions. We have investigated the effect of saponification on both qualitative and quantitative distribution of carotenoids in some of the vegetables by evaluating their HPLC profiles before and after alkali treatment. The chromatograms of raw broccoli extract before and after saponification clearly demonstrate significant differences (Figure 9). Separation of the various components in the saponified extract by semipreparative TLC and exami-

Table IV. Quantitative Evaluation of Carotenoids in Raw Broccoli Extract before and after Saponification

carotenoids	raw broccoli extract, mg/100 g: before alkali treatment	raw broccoli extract, mg/100 g: after alkali treatment	% loss due to saponification
all-trans-neoxanthin	0.25	0.04	84
9′-*cis*-neoxanthin	0.28	0.06	79
violaxanthin	0.34	0.15	56
lutein epoxide	0.90	0.27	70
neolutein epoxide A	0.10	0.08	80
all-trans-lutein	1.78	1.12	37
neolutein B	0.08	0.05	38
neolutein B′	0.11	0.08	27
neolutein A	0.12	0.10	17
all-trans- + 15,15′-*cis*-β-carotene	0.50	0.47	6
total	4.46	2.42	46

nation of the retention time and light absorption spectrum of each band revealed that no new carotenoid was formed. Trace amounts of neochrome, auroxanthin, and flavoxanthin were shown to be present, which were presumably formed by rearrangement of neoxanthin, violaxanthin, and lutein epoxide, respectively. The quantitative evaluation of these components (Table IV) showed that the saponification was accompanied by significant loss of xanthophylls, particularly the epoxycarotenoids, while the loss of carotenes was not significant. These results were obtained from treatment of raw broccoli extract with methanolic potassium hydroxide (30%) under an atmosphere of nitrogen at room temperature after 3 h. If the isolation of pure carotenoids is desirable, the saponification may be employed under milder conditions, which may minimize the loss of these pigments. Therefore, for accurate generation of quantitative data on carotenoids, which may be sensitive to alkaline treatment, this purification step should be omitted. Saponification, however, must be employed to evaluate the presence of carotenol esters that may otherwise remain undetected.

Nomenclature. For convenience the trivial names of several naturally occurring carotenoids have been used throughout this text. The trivial and the systematic names as well as chemical structures of these carotenoids in relation to the parent carotenoids with α- and β-type end groups have been tabulated by Straub (1971). In cases where definite geometrical configurations of the *cis*-carotenoids are not known, the prefixes such as neo A and neo B have been used to distinguish these compounds. Decapreno-β-carotene, which was used as an internal standard, is a synthetic carotenoid that has two isoprene units (in chain) more than that of β-carotene. This compound is known as C-50-β-carotene.

ACKNOWLEDGMENT

We thank Professor H. J. Mayer and the F. Hoffmann-La Roche & Co. (Basel, Switzerland) for their generous support throughout this research in providing us with a number of carotenoid reference samples including 15,15′-*cis*-β-carotene, lutein, zeaxanthin, and some of the epoxy carotenoids. Thanks are also due to Professor Paul H. Mazzocchi (Department of Chemistry and Biochemistry, University of Maryland) for assistance with the paper and Dr. Yiu-Fai Lam (University of Maryland) for running the NMR samples of the chlorophylls. We thank J. M. Holden and S. G. Korth (USDA, Nutrient Composition Laboratory) for their advice in selection and preparation of the vegetables.

LITERATURE CITED

Bartlett, L.; Klyne, W.; Mose, W. P.; Scopes, P. M.; Galasko, G.; Mallams, A. K.; Weedon, B. C. L.; Szabolcs, J.; Toth, G. *J. Chem. Soc. C* **1969**, 2527.

Beecher, G. R.; Khachik, F. *JNCI, J. Natl. Cancer Inst.* **1984**, *73*, 1397.

Bonnett, R.; Mallams, A. K.; McCormick, A.; Spark, A. A.; Tee, T. L.; Weedon, B. C. L. *J. Chem. Soc. C* **1969**, 429.

Buchecker, R.; Liaaen-Jensen, S. *Helv. Chim. Acta* **1975**, *58*, 89.

Bushway, R. J.; Wilson, A. M. *Can. Inst. Food Sci. Technol. J.* **1982**, *15*, 165.

Cadosch, H.; Eugster, C. H. *Helv. Chim. Acta* **1974**, *57*, 1466.

Cholnoky, L.; Gyorgyfy, K.; Ronai, A.; Szabolcs, J.; Toth, Gy.; Galasko, G.; Mallams, A. K.; Waight, E. S.; Weedon, B. C. L. *J. Chem. Soc. C* **1969**, 1256.

Colditz, G. A.; Branch, L. G.; Lipnick, R. J.; Willett, W. C.; Rosner, B.; Posner, B. M.; Hennekens, C. H. *Am. J. Clin. Nutr.* **1985**, *41*, 32.

Curl, A. L.; Bailey, G. F. *Food Res.* **1957**, *22*, 323.

Curl, A. L.; Bailey, G. F. *J. Agric. Food Chem.* **1961**, *9*, 403.

Davies, B. H. In *Chemistry and Biochemistry of Plant Pigments*; Goodwin, T. W., Ed.; Academic: London, 1965; p 489.

Davies, D.; Holdsworth, E. S. *J. Liq. Chromatogr.* **1980**, *3*(1), 123.

Englert, G. In *Carotenoids Chemistry and Biochemistry*; Britton, G., Goodwin, T. W., Eds.; Pergamon: Oxford, 1982; p 107.

Enzell, C. R.; Wahlberg, I. In *Biochemical Application of Mass Spectrometry*; Waller, G. R., Dermer, O. C., Eds.; Wiley-Interscience: New York, 1980; Chapter 13B, p 407.

Eugster, C. H. In *Carotenoid Chemistry and Biochemistry*; Britton, G., Goodwin, T. W., Eds.; Pergamon: Oxford, 1982; p 14.

Eugster, C. H.; Karrer, P. *Helv. Chim. Acta* **1957**, *40*, 69.

Foote, C. S.; Brenner, M. *Tetrahedron Lett.* **1968**, 6041.

Goodwin, T. W. In *The Biochemistry of the Carotenoids*; Chapman and Hall: New York, 1980; Vol. 1, Chapter 4, p 96.

Graham, S.; Dayal, H.; Swanson, M.; Mittelman, A.; Wilkinson, G. *JNCI, J. Natl. Cancer Inst.* **1978**, *51*, 709.

Haenszel, W.; Locke, F. B.; Segi, M. *JNCI, J. Natl. Cancer Inst.* **1980**, *64*, 17.

Haytowitz, D. B.; Matthews, R. H. In *Composition of Foods: Vegetables and Vegetables Product-Raw, Processed, Prepared*, USDA Agriculture Handbook No. 8–11; USDA: Washington, DC, Rev. 1984.

Inhoffen, H. H.; Bohlmann, F.; Rummert, G. *Liebigs Ann. Chem.* **1951**, *571*, 75.

Isoe, S.; Hyeon, S. B.; Ichikawa, H.; Katsumura, S.; Sahan, T. *Tetrahedron Lett.* **1968**, 5561.

Johansen, E.; Liaaen-Jensen, S. *Acta Chem. Scand., Ser. B* **1974**, *B28*, 949.

Karrer, P.; Jucker, E. *Helv. Chim. Acta* **1945**, *28*, 300.

Karrer, P.; Jucker, E. *Helv. Chim. Acta* **1946**, *29*, 229.

Karrer, P.; Jucker, E. In *Carotenoids*; Elsevier: Amsterdam, 1950.

Kephart, J. C. *Econ. Bot.* **1955**, *9*, 3.

Khachik, F.; Beecher, G. R. *J. Chromatogr.* **1985**, *346*, 237.

Khachik, F.; Beecher, G. R. *Separation and Identification of Carotenoids and Carotenoid Precursors Found in Green Vegetables by Liquid Chromatography*, 37th Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Atlantic City, NJ, March 10–14, 1986.

Liaaen-Jensen, S. In *Carotenoids*; Isler, O., Ed.; Birkhauser Verlag: Basel, 1971; Chapter 3, p 65.

McMahon, R. W.; Gladon, R. *J. Hort. Sci.* **1984**, *19*(2), 220.

National Research Council In *Diet, Nutrition, and Cancer*; National Academy: Washington, DC, 1982; Chapter 15, p 1.

Peto, R.; Doll, R.; Buckley, J. D.; Sporn, M. B. *Nature (London)* **1981**, *290*, 201.

Ritter, E. D.; Purcell, A. E. In *Carotenoids as Colorants and Vitamin A Precursors*; Bauernfeind, J. C., Ed.; Academic: New York, 1981; Chapter 10, p 883.

Schanderl, S. H.; Chichester, C. O.; Marsh, G. L. *J. Org. Chem.* **1962**, *27*, 2865.

Schwartz, S. J.; Woo, S. L.; Elbe, J. H. *J. Agric. Food Chem.* **1981**, *29*, 533.

Schwartz, S. J.; Elbe, J. H. *J. Liq. Chromatogr.* **1982**, *5* (Suppl. 1), 43.

Shekelle, R. B.; Lepper, M.; Liu, S.; Oglesby, P.; Shryock, A. M.; Stamler, J. *Lancet* **1981**, *2*, 1185.

Siefermann, D.; Yamamoto, H. Y. *Arch. Biochem. Biophys.* **1975**, *171*, 7a.
Souci, S. W.; Fachmann, W.; Kraut, H. In *Food Composition and Nutrition Tables 1981/1982*; Wissenschaftliche Verlagsgesellschaft: Stuttgart, 1981.
Straub, O. In *Carotenoids*; Isler, O., Ed.; Birkhauser Verlag: Basel, 1971; Chapter 12, p 771.
Strain, H. H. *Arch. Biochem. Biophys.* **1954**, *48*, 458.
Strain, H. H. In *Biochemistry of Chloroplast*; Goodwin, T. W., Ed.; Academic: London, 1966; Vol. 1, p 387.
Stransky, H. *Z. Naturforsch* **1978**, *33*, 836.
Surmatis, J. D.; Ofner, A. *J. Org. Chem.* **1961**, *26*, 1171.
Sweeney, J. P.; Marsh, A. C. *J. Am. Dietet. Assoc.* **1971**, *59*, 238.
Szabolcs, J. *Pure Appl. Chem.* **1976**, *47*, 147.
Takagi, S. *Agric. Biol. Chem.* **1985**, *49*(4), 1211.
Toth, Gy.; Szabolcs, J. *Acta Chim. Acad. Sci. Hung.* **1970**, *64*, 393.
Tsukida, K.; Zechmeister, L. *Arch. Biochem. Biophys.* **1958**, *74*, 408.
Vetter, W.; Englert, G.; Rigassi, N.; Schwieter, U. In *Carotenoids*; Isler, O., Ed.; Birkhauser Verlag: Basel, 1971; Chapter 4, p 189.
Weedon, B. C. L. In *Carotenoids*; Isler, O., Ed.; Birkhauser Verlag: Basel, 1971; Chapter 5, p 305.
Wright, S. W.; Shearer, J. D. *J. Chromatogr.* **1984**, *294*, 281.

Received for review December 2, 1985. Revised manuscript received March 25, 1986. Accepted April 22, 1986. Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

Esters and Glucosides of Hydroxycinnamic Acids in Vegetables

Michael Winter and Karl Herrmann*

Quinic, tartaric, and malic acid esters as well as glucose esters and the glucosides of hydroxycinnamic acids have been determined qualitatively and quantitatively by HPLC in tomatoes (two states of ripeness (green and red) and different types), bell pepper, eggplant, spinach, mangold, beetroot, pea, bush bean, broad bean, lettuce (outer and inner leaves separated), endive, and chicory.

INTRODUCTION

Generally naturally occurring derivatives of hydroxycinnamic acids are represented by their quinic acid or glucose esters, which often have been detected in fruit (Herrmann, 1978; Möller and Herrmann, 1983; Reschke and Herrmann, 1981; Schuster and Herrmann, 1985) and vegetables (Herrmann, 1978; Reschke and Herrmann, 1982; Brandl and Herrmann, 1983a, 1983b).

Additionally the malic acid esters of these phenolic acids could be detected in vegetables, although these compounds were found rarely in nature. Phaseolic acid (caffeoylmalic acid) represents the most detected compound of these derivatives. It has been found in beans (Scarpati and Oriente, 1960) and *Trifolium pratense* (Yoshihara et al., 1974). All of the four hydroxycinnamoyl malic acids have been isolated from small radish (Brandl et al., 1984).

Furthermore, there is another kind of hydroxycinnamic acid ester derived from tartaric acid. These compounds were found frequently in vegetables and fruits. The analysis of these derivatives will be more difficult because of the different naturally occurring stereoisomers like L- and *meso*-tartaric acid. L-Tartaric acid esters were found in grapes (Ong and Nagel, 1978), wine (Okamura and Watanabe, 1981), cider (Whiting and Coggins, 1975), and chicory (Scarpati and d'Amico, 1960).

The *meso*-tartaric acid ester of *p*-coumaric acid was found in spinach (Suzuki et al., 1970; Tadera et al., 1970; Oettmeier and Heupel, 1972a, 1972b).

Additionally, dihydroxycinnamoyltartaric acids like dicaffeoyltartaric acid were detected in lettuce (Feucht et al., 1971), endive (Wöldecke and Herrmann, 1974), and chicory (Scarpati and Oriente, 1958).

The glucosides of hydroxycinnamic acids are less distributed. In vegetables they only were found in tomatoes (Fleuriet and Macheix, 1980, 1981; Winter and Herrmann, 1984).

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To get more informed about the occurrence of these hydroxycinnamic acid derivatives, especially the glucose derivatives, we have analyzed several important species of vegetables quantitatively and qualitatively by means of HPLC.

Besides the flavonoids, which meanwhile are used as proofs for adulteration in food analysis (Siewek et al., 1984, 1985), the derivatives of hydroxycinnamic acids represent another group of interesting phenolic compounds in plants. Within the biochemical anabolism in plants these substances are precursors of the flavonoids.

Therefore, it will be imaginable that in the future they as well will be used as coindicators for proof of adulteration (Herrmann, 1979). Most of these compounds are of a sufficient stability so that they can be detected even in products like wine and must (Okamura and Watanabe, 1981; Singleton et al., 1978).

The use of hydroxycinnamic acid derivatives as proofs for adulteration will assume their sufficient determination in fruits and vegetables and there in several varieties.

MATERIAL AND METHODS

Standards. The IUPAC nomenclature is used for the quinates (e.g., chlorogenic acid (former 3-*O*-caffeoylquinic acid) is the 5′ ester and neochlorogenic acid (the former 5-*O*-caffeoylquinic acid) now will be the 3′ ester).

Chlorogenic acid is the only commercially available substance of the hydroxycinnamic acid derivatives (Roth, Karlsruhe, G.F.R.). 5′-Feruloylquinic acid was isolated from green coffee beans and 3′-*p*-coumaroylquinic acid was received from unripe Morello cherries by preparative HPLC. The isomerization of these compounds was carried out by the method of Scarpati and Esposito (1964).

Sinapoylglucose was received by Brandl and Herrmann (1983a) by preparative HPLC from garden cress. Feruloylglucose was isolated by Reschke and Herrmann (1982) from chive, and *p*-coumaroylglucose was placed at our disposal by Birkofer. Caffeoylglucose was isolated by Koeppen and Herrmann (1977). The glucosides of caffeic acid, *p*-coumaric acid, and ferulic acid were synthesized

0021-8561/86/1434-0616$01.50/0