Separation and Identification of Carotenoids and Carotenol Fatty Acid Esters in Some Squash Products by Liquid Chromatography. 1. Quantification of Carotenoids and Related Esters by HPLC

Frederick Khachik* and Gary R. Beecher

The predominant carotenoids and carotenol fatty acid esters in extracts from three varieties of squash have been separated on a C-18 reversed-phase column, employing a combination of isocratic and gradient elution high-performance liquid chromatography (HPLC). The general chromatographic profiles of the extracts from these squash products showed marked differences in both qualitative and quantitative distribution of carotenoids and carotenol fatty acid esters. Four classes of compounds have been shown to be present. In the order of chromatographic elution, these were oxygenated carotenoids (xanthophylls), carotenol mono(fatty acid esters), hydrocarbon carotenoids, and carotenol bis(fatty acid esters). The xanthophylls were identified as violaxanthin and lutein as well as several of their cis stereoisomers. The mono(fatty acid esters) were identified as lutein monomyristate, lutein monopalmitate, and violaxanthin monomyristate. The hydrocarbon carotenoids were identified as *all-trans*-α-carotene, *all-trans*-β-carotene, and its 15,15'-cis isomer. The saturated straight-chain bis(fatty acid esters) of violaxanthin, violeoxanthin (9-*cis*-violaxanthin), and lutein were shown to be present in the extracts from these squash products.

In recent epidemiological studies that have demonstrated an inverse relationship between cancer risk and the consumption of fruits and vegetables (Haenszel et al., 1972, 1976; Phillips, 1975), carotenoids have been considered as one of the possible "active" cancer inhibitory agent(s) in these foods. Indices of dietary vitamin A activity have been used to evaluate diet and cancer relationships with only modest success. A refinement of these indices has distinguished between preformed vitamin A (retinol) from animal food sources and provitamin A (primarily β-carotene) from plant food sources. In the case of foods derived from plant origin, β-carotene constitutes a major source of vitamin A activity. The current food composition tables report only total vitamin A activity that has been calculated from data obtained by direct spectrophotometric or open-column chromatography techniques (Souci et al., 1981; Haytowitz and Matthews, 1984). The improvement in analytical techniques, particularly the application of high-performance liquid chromatography (HPLC) in separation and quantification of all the major carotenoids isolated from natural products, can provide more specific detailed information of qualitative and quantitative distribution of these compounds in our food supply. We recently reported the separation, identification, and quantification of the major carotenoid and chlorophyll constituents in extracts of several green vegetables by HPLC (Khachik et al., 1986). As many as 18 components were separated, which were assigned to three classes of compounds: xanthophylls, chlorophylls and their derivatives, and the hydrocarbon carotenoids. In a similar effort, the major carotenoid constituents of several yellow/orange vegetables have been separated and quantified by HPLC (Khachik and Beecher, 1987). Accumulation of data of this nature, which attempts to evaluate the qualitative and quantitative distribution of all the carotenoids (including carotenoids with no vitamin A activity) present in food, will provide a better understanding of the role, if any, of the carotenoids in cancer prevention.

There are numerous reports on separation of various classes of carotenoids either isolated from natural sources or employed in a mixture of reference samples by HPLC. However, in these reports, the development of the HPLC techniques for carotenol fatty acid esters, one of the classes of carotenoids often isolated from natural sources, has not received much attention. One of the very rare examples of the separation of carotenol fatty acid esters by HPLC has been reported by Gregory et al. (1986), who employed this technique to separate and quantify the fatty acid esters of lutein, isolated from marigold flowers. Recently, the separation of carotenol fatty acid esters in red bell peppers by HPLC has been reported by Gregory et al. (1987). However, in addition to a number of carotenoids that were reported as unidentified, the identification of carotenol fatty acid esters was only based on absorption spectra, HPLC retention time, and thin-layer chromatography (TLC) R_f values for these compounds. Similarly the separation of carotenol fatty acid esters of paprika pigments has been reported by Fisher and Kocis (1987) that demonstrated the presence of these esters; however, individual carotenol fatty acid ester constituents of this pigment were not identified.

The carotenoid extracts isolated from natural sources suspected to contain carotenol fatty acid esters are customarily saponified to remove the fatty acids and regenerate the hydroxy carotenoids (Noga and Lenz, 1983). Depending on the nature of the carotenoids present, the saponification and extraction of carotenoids in these extracts may result in destruction or structural transformation of these compounds. Therefore, the development of a rapid HPLC method that can separate carotenol fatty acid esters within a reasonable time in the absence of saponification is required in order to assess the various carotenoid species predominant in foods as they are consumed by human beings. In addition, such HPLC methods can be subsequently applied to the quantification of various, naturally occurring carotenoids in foods.

In this report we have investigated the separation and quantification of the predominant carotenoids and carotenol fatty acid esters in extracts from several varieties of squash by HPLC. We have developed HPLC conditions

Beltsville Human Nutrition Research Center, Nutrient Composition Laboratory, U.S. Department of Agriculture—Agricultural Research Service, Building 161 E, BARC-East, Beltsville, Maryland 20705.

that separated as many as 25 carotenoids as well as several of their stereoisomers, which were assigned to four classes of compounds: xanthophylls, carotenol mono(fatty acid esters), hydrocarbon carotenoids, and carotenol bis(fatty acid esters). Pure reference compounds and synthetic internal standards (β-apo-8′-carotenol, isozeaxanthin dipelargonate) were employed in quantification of these carotenoids. Thermal stability of some of the carotenoids and the effect of cooking on their distribution in one variety of fresh cooked squash have been studied.

EXPERIMENTAL SECTION

Apparatus. The HPLC system employed for the separation of carotenoids has been described previously (Khachik et al., 1986). Absorption spectra of the carotenoids in various solvents were recorded on a Beckman DU-7 UV/visible spectrophotometer. Mass spectra were obtained on a Finnigan 4510 instrument (San Jose, CA) equipped with an Incos data system. Electron ionization (EI) spectra were obtained at 70 eV and a source block temperature of 150 °C. Desorption chemical ionization (DCI) spectra were obtained, employing ammonia as the reagent gas at a source block temperature of 60 °C.

Column. Separations were performed on a Microsorb (25-cm length × 4.6-mm i.d.) 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).

Reagents and Materials. The reference samples of *all-trans*-α- and *all-trans*-β-carotene (Sigma, St. Louis, MO) were used without further purification. Samples of zeaxanthin, lutein, and 15,15′-*cis*-β-carotene were provided by Hoffmann-La Roche, Basel, Switzerland. The purity of the reference samples was checked by HPLC as well as comparison of their extinction coefficient in various solvents with those of the published values (Khachik et al., 1988). When necessary, the reference samples were further purified by semipreparative thin-layer chromatography. Reference samples of lutein, violaxanthin, and related cis isomers were isolated from squash [procedure described in Khachik et al. (1988)]. Carotenol mono(fatty acid esters) were isolated from squash, while the bis(fatty acid esters) were synthesized from lutein and violaxanthin and the appropriate fatty acid chlorides [procedure described in Khachik et al. (1988)]. Flavoxanthin was obtained from acid treatment of lutein epoxide (Khachik et al., 1986). β-Apo-8′-carotenol was prepared from β-apo-8′-carotenal (Fluka Chemical Co.) upon reduction with lithium aluminum hydride (procedure described later in the text). Isozeaxanthin dipelargonate (internal standard) was synthesized from either canthaxanthin or β-carotene according to the procedure described in the text. Synthetic β-carotene and canthaxanthin (Fluka) were used without further purification. 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%).

Source of the Squash Samples. The scientific name for the variety of acorn squash studied in this report is *Cucurbita pepo*. Fresh samples of this squash (grown in New Jersey) were purchased from local supermarkets on the day of analysis. The scientific name for the strained squash samples, referred to as brands G and H, is *Cucurbita maxima*, which were grown and prepared by two leading baby food manufacturing companies in the United States (Gerber Products Co., Fremont, MI; H. J. Heinz Co., Pittsburgh, PA) under controlled conditions. Samples of baby food squash (brand G) of Northrup King variety, currently grown in two different locations in the United States (Fremont, MI; Asheville, NC), were provided by the food-processing company. Samples of strained butternut squash (brand H, grown in New Jersey, harvested in Sept and Oct 1985) were purchased from local supermarkets.

Chromatographic Procedure. The analytical separations were carried out under two sets of HPLC conditions employing eluents A and B.

1. Eluent A. A combination of isocratic and gradient chromatography separated the carotenoids from carotenol fatty acid esters in varieties of baby food squash (brands G and H). An isocratic mixture of methanol (15%), acetonitrile (65%), methylene chloride (10%), and hexane (10%) at time 0 was followed by a gradient beginning at time 23 min and completed at time 33 min. 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 0.7 mL/min. The chromatographic analyses with this eluent were monitored at 450 nm. At the end of the gradient the column was reequilibrated under the initial isocratic conditions [methanol (15%), acetonitrile (65%), methylene chloride (10%), hexane (10%)] for 20 min at a flow rate of 1.5 mL/min and finally for 5 min at 0.7 mL/min.

2. Eluent B. A combination of isocratic and gradient chromatography separated the carotenoids and the related fatty acid esters from raw and cooked acorn squash. An isocratic mixture of methanol (10%), acetonitrile (85%), methylene chloride (2.5%), and hexane (2.5%) at time 0 was followed by a gradient beginning at time 10 min and completed at time 40 min. The final composition of the gradient mixture was methanol (10%), acetonitrile (45%), methylene chloride (22.5%), and hexane (22.5%). The column flow rate was 0.7 mL/min. Xanthophylls, mono- and bis(fatty acid esters) of violaxanthin, and their corresponding cis isomers were monitored at 442 nm, while β-apo-8′-carotenol (internal standard) and β-carotene were monitored at 450 nm. At the end of the gradient the column was reequilibrated under the initial isocratic conditions for 20 min at a flow rate of 1.5 mL/min and finally for 5 min at 0.7 mL/min.

Preparation of the Vegetables for Extraction. Fresh acorn squash was prepared for analysis in the same way it is prepared for consumption. The squash was sliced in half. Each half was placed, cut side down, in a pyroceram dish; 36 g of water was added and dish was covered with polyethylene wrap. The raw fresh acorn squash was heated in a microwave oven for 8 min. The seed and the inedible parts were then removed before the sample was homogenized. The varieties of brands G and H of the commercially prepared baby food squash were sampled directly from their containers.

Extraction. Stock solutions of the internal standards were prepared by dissolving 75 mg of isozeaxanthin dipelargonate in 200 mL of hexane and 12.4 mg of β-apo-8′-carotenol in 100 mL of methanol. Aliquots of the appropriate internal standards were directly added to each sample of vegetable, with the extracting solvent (THF), 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 THF 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. Most of the solvent was removed on a rotary evaporator at 30 °C, and the concentrated vegetable extract was partitioned between petroleum ether and water. Occasionally salt was

Table I. Weight of the Internal Standards Added to the Squash Samples at the Beginning of the Extraction and the Final Volume of the Extracts

entry	squash variety, g	isozeaxanthin dipelargonate, mg	β-apo-8′-carotenol, mg	final vol of extr, mL
1	baby food squash brand G			
	Michigan variety, 55 g	1.12	0.00	25
	North Carolina variety, 55 g	1.12	0.00	25
2	baby food squash brand H			
	New Jersey variety, 100.3 g	3.04	0.00	50
3	fresh acorn squash (local)			
	raw, 200 g	0.00	0.298	10
	cooked, 137 g	0.00	0.496	10

added to the mixture to break the emulsion. The aqueous layer was washed with petroleum ether containing 15% methanol several times until this layer was colorless. The resulting organic layers were combined and dried over sodium sulfate, and the solvent was evaporated. The residue was dissolved in small volume of hexane, filtered through 0.45-μm disposable filter assembly (American Scientific Products, McGraw Park, IL), and transferred to an appropriate volumetric flask. 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 I.

Saponification. Ethereal solutions of the vegetable extracts were treated with methanolic potassium hydroxide (30%) under an atmosphere of nitrogen at room temperature for 6 h. The solution was partitioned into a saturated aqueous solution of sodium chloride and 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 carotenoids free from fatty acids were dissolved in a small volume of methanol and filtered through 0.45-μm disposable filter assembly into appropriate volumetric flask with methanol, and the HPLC analyses were conducted.

Synthesis of the Internal Standards. (*a*) *Isozeaxanthin Dipelargonate.* Isozeaxanthin (2.62 g, 0.0046 mol) prepared from the reduction of canthaxanthin with lithium aluminum hydride (Petracek and Zechmeister, 1956) was dissolved in benzene (200 mL), and triethylamine (23.27 g, 0.23 mol; 32 mL) was added to the solution. Pelargonoyl chloride (8.13 g, 0.046 mol) in benzene (20 mL) was stirred into the mixture in 1 h under an atmosphere of nitrogen at room temperature. After 3 h the product was washed with 5% aqueous sodium bicarbonate (2 × 150 mL) and water (2 × 150 mL) and dried over sodium sulfate. The solvent was removed under reduced pressure, and the red oily residue was recrystallized from methanol/benzene to give isozeaxanthin dipelargonate (3.12 g, 0.0037 mol, 80%), mp 60 °C dec. The mass spectrum (electron impact) of isozeaxanthin dipelargonate contained a molecular ion at m/z 848 (6%) ($C_{58}H_{88}O_4$, M_r 848) as well as fragmentations at m/z 756 (4%, M − 92), 690 [14%, M − 158 (loss of pelargonic acid)], 598 (7%, M − 158 − 92), 532 (17%, M − 158 − 158), 440 (8%, M − 158 − 158 − 92), and 333 (100%). Analysis of isozeaxanthin dipelargonate by ammonia desorption chemical ionization mass spectrometry produced ions at m/z 866 [41%, M + 18 $(M + NH_4)^+$], 883 [7%, M + 35 $[M + (NH_3)_2H]^+$], 690 [44%, M − 158 (loss of pelargonic acid)], 708 [29%, M − 158 + 18 $(M - 158 + NH_4)^+$], 725 [8%, M − 158 + 35 $[M - 158 + (NH_3)_2H]^+$], 532 (100%, M − 158 − 158), 550 [12%, $(M - 158 - 158 + NH_4)^+$], and 567 [3%, $[M - 158 - 158 + (NH_3)_2H]^+$]. The ^1H NMR (400-MHz) spectrum of the internal standard contained signals at δ 1.02 (s, 6 H) and 1.07 (s, 6 H) [Me-16, Me-17, Me-16′, Me-17′], 1.70 (s, 6 H, Me-18, Me-18′), 1.97 (s, 12 H, Me-19, Me-20, Me-19′, Me-20′), and 5.25 (t, 2 H, C-4 and C-4′). The fatty acid side chain signals appeared at δ 0.881 (s, 3 H + 3 H, methyl protons), 1.27 (m, 24 H, methylene protons), and 2.32 (t, J = 7.5 Hz) [2 H + 2 H, α- and α′-methylene protons]. The absorption maxima (nm) and absorbtivity data (extinction coefficient) of isozeaxanthin dipelargonate in various solvents [benzene, λ_{max} = 462 ($E^{1\%}$ = 1124), 490 ($E^{1\%}$ = 964); methanol, λ_{max} = 274, 426, 449 ($E^{1\%}$ = 1316), 476 ($E^{1\%}$ = 1159); petroleum ether, λ_{max} = 274, 424, 448 ($E^{1\%}$ = 1342), 475 ($E^{1\%}$ = 1175)] were consistent with the chromophore involved. The absorption spectrum of the internal standard had a maximum at 454 nm in the HPLC solvents [methanol (15%), acetonitrile (65%), methylene chloride (10%), hexane (10%)]. The purity of the internal standard was further confirmed by evaluation of at least 10 absorption spectra of this compound monitored by a rapid-scanning detector. In all cases, the absorption maximum remained at 454 nm and all spectra were superimposable.

On several occasions isozeaxanthin was prepared from β-carotene and *N*-bromosuccinimide according to the modified procedures (Petracek and Zechmeister, 1956; Entschel and Karrer, 1958). In the presence of acetic acid this reaction has been shown to result in the introduction of allylic acetoxy group in carotenoids with a β-ionone end group (Entschel and Karrer, 1958; Yasuhara et al., 1980). However, similar attempts to synthesize isozeaxanthin dipelargonate directly from β-carotene and *N*-bromosuccinimide in the presence of pelargonic acid in chloroform at −60 °C and in refluxing solution in carbon tetrachloride were unsuccessful.

(*b*) *β-Apo-8′-carotenol.* Lithium aluminum hydride (100 mg) in ether (50 mL) was added to a solution of β-apo-8′-carotenal (0.100 g, 0.24 mmol) in 5/1 benzene/ether (100 mL), and the mixture was stirred at room temperature for 30 min under an atmosphere of nitrogen. The reaction mixture was cooled to 0 °C, and cold methanol (30 mL) was added very slowly. The product was washed with water, dried over sodium sulfate, and evaporated to dryness. On addition of hexane, β-apo-8′-carotenol crystallized. The crystals were washed with cold hexane and recrystallized from hexane and petroleum ether. Analysis of β-apo-8′-carotenol ($C_{30}H_{42}O$, M_r 418) by ammonia desorption chemical ionization mass spectrometry contained molecular ions at m/z 453 [40%, $[M + (NH_3)_2H]^+$], 436 [30%, $(M + NH_4)^+$], 418 [10%, $(M + NH_4 - H_2O)^+$], and 401 [100%, $(M + H - H_2O)^+$]. The UV/visible absorption spectra in various solvents were consistent with those of the literature values (Vetter et al., 1971). The UV/visible absorption spectrum of β-apo-8′-carotenol in the HPLC solvents [methanol (10%), acetonitrile (85%), methylene chloride (2.5%), hexane (2.5%)] had maxima at 426 and 455 nm.

Preparation of the Calibration Curves. The predominant carotenoids and related fatty acid esters in baby food squash brands G and H were quantified from calibration curves obtained under HPLC conditions employing

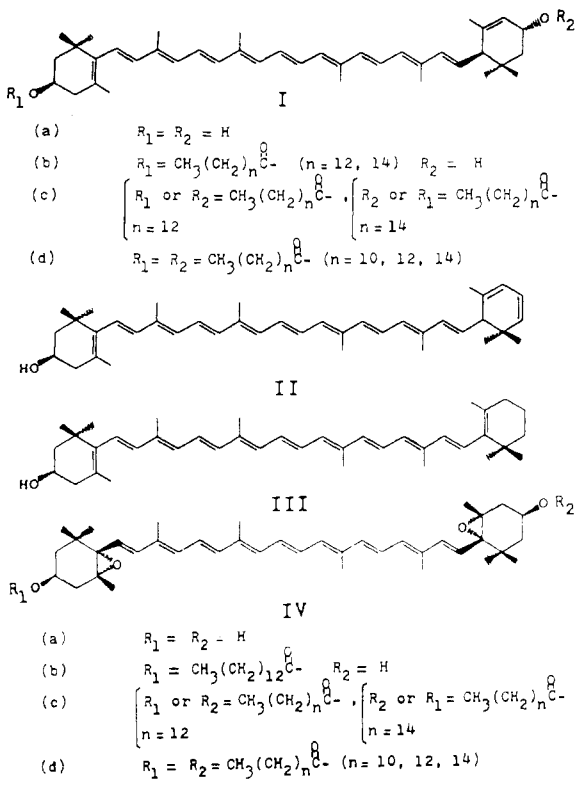

Figure 1. Chemical structures of some of the predominant carotenoids and carotenol fatty acid esters in squash products. Only the all-trans isomers are shown. Key: Ia, lutein; Ib, natural lutein mono(fatty acid esters); Ic, mixed lutein bis(fatty acid esters); Id, lutein bis(fatty acid esters); II, 3-hydroxy-2′,3′-dehydro-β,ε-carotene; III, β-cryptoxanthin, IVa, violaxanthin; IVb, violaxanthin monomyristate; IVc, mixed violaxanthin bis(fatty acid esters); IVd, violaxanthin bis(fatty acid esters).

eluent A, while the predominant carotenoids in acorn squash were quantified from separate calibration curves employing eluent B.

(*a*) *Standard Curves for Carotenoids in Baby Food Squash Brands G and H.* The calibration curves for natural lutein monomyristate, lutein monopalmitate, and α- and β-carotene were obtained by relating the ratio of the HPLC peak area of the reference samples of these carotenoids and that of a constant amount of the internal standard (isozeaxanthin dipelargonate) to the HPLC peak area of the reference samples at various concentrations. Since the HPLC peaks of neolutein monomyristate A or A′ and neolutein monopalmitate A or A′ were not resolved from their corresponding all-trans compounds, the HPLC peak areas of these cis isomers were included in the HPLC peak area of their corresponding all-trans compounds. Other carotenoids, flavoxanthin, lutein, 3-hydroxy-2′,3′-dehydro-β,ε-carotene, *cis*-β-cryptoxanthin, and lutein bis(fatty acid esters) were quantified from the HPLC response factor of their reference samples at various concentrations.

(*b*) *Standard Curves for Carotenoids in Acorn Squash.* Owing to the low abundance of the carotenoids in acorn squash and lack of availability of reference samples in large amounts, the quantification of the carotenoids in acorn squash was not based on the area ratio of these carotenoids to that of the internal standard (β-apo-8′-carotenol), but rather on the HPLC response factors of the individually isolated compounds at two or three different concentrations. The HPLC peak area of the internal standard, however, was monitored after extraction and saponification to determine the losses of carotenoids related to such workup procedures. 9-*cis*-Violaxanthin was quantified by

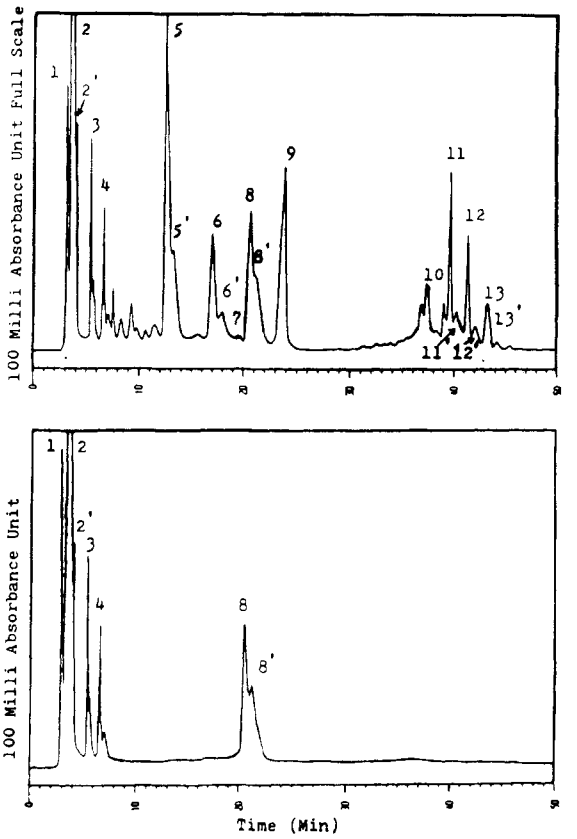

Figure 2. HPLC profiles of baby food squash brand G extract. Chromatographic conditions (eluent A) and peak identification (Table II) described in text. Upper trace: extract before saponification. Lower trace: extract after saponification.

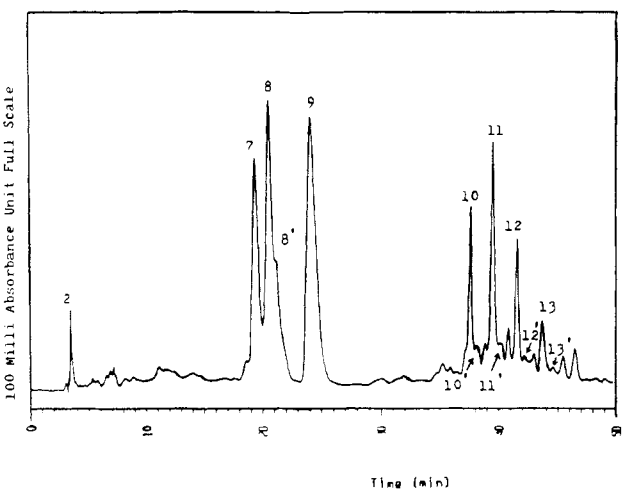

Figure 3. HPLC profile of baby food squash brand H extract. Chromatographic conditions (eluent A) and peak identification (Table II) described in text.

relating its HPLC response factor to that of *all-trans*-violaxanthin at various concentrations. Luteoxanthin, auroxanthin, *cis*-violaxanthins, and lutein were quantified from their HPLC response factors. The HPLC peak areas of *all-trans*-β-carotene and its 15,15′-cis isomer were combined, and the total β-carotene concentration was determined from the HPLC response factor of the all-trans compound.

RESULTS AND DISCUSSION

The major components of the various classes of carotenoids and carotenol fatty acid esters separated from the squash extracts and their corresponding HPLC peaks in

Table II. HPLC Peak Identification of the Predominant Carotenoids and Carotenol Fatty Acid Esters in Several Varieties of Baby Food Squash (Brands G and H)

chemical class	peak	component	RT,[a] min
xanthophyll	1	flavoxanthin	3.12
	2	*all-trans*-lutein	3.52
	2′	neolutein A or A′ (13- or 13′-*cis*-lutein)	4.00
	3	3-hydroxy-2′,3′-dehydro-β,ε-carotene	5.52
	4	9- or 9′-*cis*-β-cryptoxanthin	6.72
carotenol mono(fatty acid ester)	5	*all-trans*-lutein monomyristate (β,ε-carotene-3-monol monomyristate-3′-monol)	12.64
	5′	neolutein monomyristate A or A′	13.20
	6	*all-trans*-lutein monopalmitate (β,ε-carotene-3-monol monopalmitate-3′-monol)	17.04
	6′	neolutein monopalmitate A or A′	17.99
hydrocarbon carotenoid	7	*all-trans*-α-carotene	19.64
	8	*all-trans*-β-carotene	20.64
	8′	15,15′-*cis*-β-carotene	21.30
carotenol bis(fatty acid esters)	9	isozeaxanthin dipelargonate (int std)	23.72
	10	*all-trans*-lutein dilaurate	37.72
	10′	neolutein dilaurate A or A′	38.50
	11	*all-trans*-lutein dimyristate	39.62
	11′	neolutein dimyristate A or A′	40.40
	12	*all-trans*-lutein myristate/palmitate diester	41.60
	12′	neolutein myristate/palmitate diester A or A′	42.40
	13	*all-trans*-lutein dipalmitate	43.60
	13′	neolutein dipalmitate A or A′	44.40

[a] RT = retention times of the various components under chromatographic conditions (eluent A) described in text.

the order of elution on a C-18 reversed-phase column are shown in Tables II and III. The structures of some of the predominant squash carotenoids and their corresponding esters are shown in Figure 1. The detailed identification of these compounds by spectroscopy, partial synthesis, and chromatography will be described in Khachik et al. (1988). The chromatographic profiles of the major carotenoids and related fatty acid esters of brand G [before and after saponification] and brand H [before saponification] of baby food squash are shown in Figures 2 and 3, respectively. Chromatograms of the extract from raw and freshly cooked acorn squash purchased from a local supermarket are shown in Figure 4. Under two sets of HPLC conditions (eluents A and B) employing a combination of isocratic and gradient elution chromatography, the various classes of carotenoids and carotenol fatty acid esters in these varieties of squash were separated within 50 min. Under the HPLC conditions (eluent A) employed for separation of the carotenoids in baby food squash (brands G and H), the xanthophylls (peaks 1–4), mono(fatty acid esters) (peaks 5 and 6), the hydrocarbon carotenoids (peaks 7 and 8), and the internal standard (peak 9) are eluted under isocratic conditions, which is followed by a gradient that elutes the carotenol bis(fatty acid esters) (peaks 10–13). The predominant carotenoids in the extracts from saponified cooked acorn squash were also monitored by HPLC employing eluent B (Figure 5). Chromatographic separation of the major carotenoids in acorn squash (Figures 4 and 5) employing eluent B consists of an isocratic mixture separating the xanthophylls (peaks 1–8) and the internal standard (β-apo-8′-carotenol, peak 9) under isocratic conditions. This isocratic HPLC condition is then followed by a gradient that results in the elution of mono(fatty acid esters) (peaks 10–12), *all-trans*-β-carotene and its 15,15′-cis isomer (peaks 13 and 13′), and bis(fatty acid esters) of violaxanthin and lutein (peaks 14–25). In these chroma-

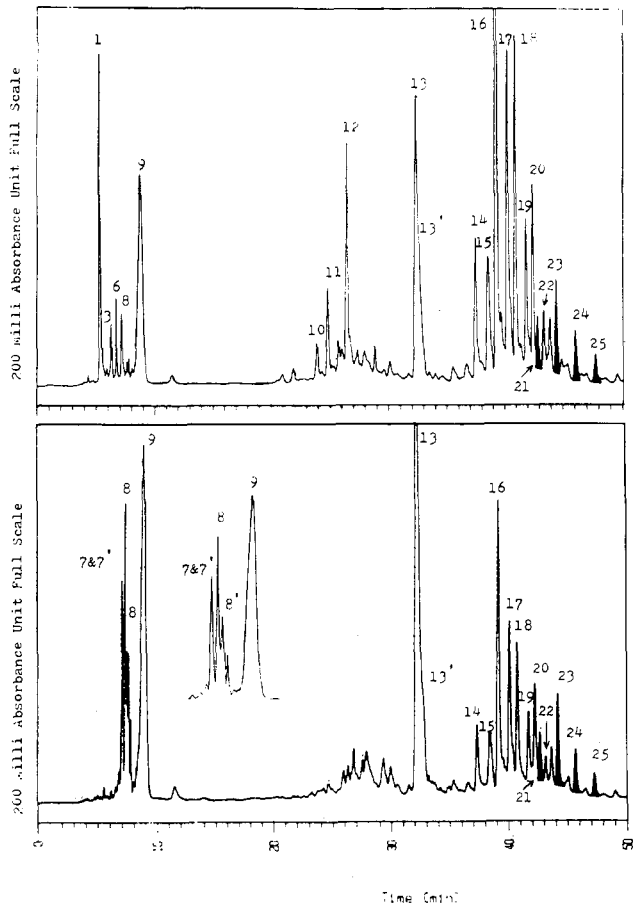

Figure 4. HPLC profiles of raw (upper trace) and fresh cooked (lower trace) acorn squash (purchased locally) extracts. Chromatographic conditions (eluent B) and peak identification (Table III) described in text. Shaded peaks are the bis(fatty acid esters) of lutein.

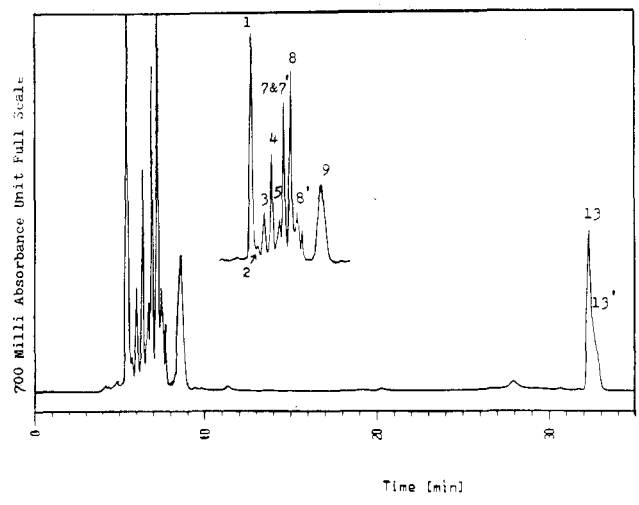

Figure 5. HPLC profile of fresh cooked acorn squash extract after saponification. Chromatographic conditions (eluent B) and peak identification (Table III) described in text.

tograms the unresolved cis isomers (appearing as a tailing shoulder on their corresponding all-trans compounds) have been designated the same number as the trans isomers but distinguished from their all-trans compouns by a prime symbol.

Qualitative Distribution of Carotenol Fatty Acid Esters. The chromatographic profiles of the varieties of squash demonstrate significant differences in qualitative distribution of carotenoids and the related fatty acid esters

Table III. HPLC Peak Identification of the Predominant Carotenoids and Carotenol Fatty Acid Esters of Acorn Squash in the Order of Chromatographic Elution on a C-18 Reversed-Phase Column

chemical class	peak	component	RT,[a] min
xanthophyll	1	*all-trans*-violaxanthin	5.43
	2	15,15′- or 13-*cis*-violaxanthin	5.73
	3	*all-trans*-luteoxanthin	6.00
	4	9-*cis*-violaxanthin (violeoxanthin)	6.34
	5	*all-trans*-auroxanthin	6.69
	6	13- or 15,15′-*cis*-violaxanthin	6.70
	7	di-*cis*-violaxanthin	6.87
	7′	di-*cis*-violaxanthin	7.07
	8	*all-trans*-lutein	7.19
	8′	neolutein A or A′ (13- or 13′-*cis*-lutein)	7.45
apocarotenoid	9	β-apo-8′-carotenol (int std)	8.48
carotenol mono(fatty acid ester)	10	9- or 9′-*cis*-violaxanthin monomyristate	23.90
	11	*all-trans*-violaxanthin monomyristate	24.78
	12	9′- or 9-*cis*-violaxanthin monomyristate	26.46
hydrocarbon carotenoid	13	*all-trans*-β-carotene	32.38
	13′	15,15′-*cis*-β-carotene	32.60
carotenol bis(fatty acid ester)	14	*all-trans*-violaxanthin dilaurate	37.50
	15	9-*cis*-violaxanthin dilaurate	38.54
	16	*all-trans*-violaxanthin dimyristate	39.26
	17	9-*cis*-violaxanthin dimyristate	40.30
	18	*all-trans*-violaxanthin myristate/palmitate	40.94
	19	9-*cis*-violaxanthin myristate/palmitate	41.90
	20	*all-trans*-violaxanthin dipalmitate	42.54
	21	*all-trans*-lutein dilaurate	42.86
	22	9-*cis*-violaxanthin dipalmitate	43.50
	23	*all-trans*-lutein dimyristate	44.46
	24	*all-trans*-lutein myristate/palmitate diester	46.06
	25	*all-trans*-lutein dipalmitate	47.70

[a] RT = retention times of the various component under chromatographic conditions (eluent B) described in text.

Table IV. Quantitative Distribution of the Predominant Carotenoids and Carotenol Fatty Acid Esters in Two Varieties of Baby Food Squash

	μg/100 g, μmol/100 g (edible food, wet wt)		
	baby food brand G		baby food brand H
	North Carolina	Michigan	
Xanthophyll			
flavoxanthin	430, 0.74	210, 0.36	–[a]
all-trans-lutein + neolutein A or A′	5340, 9.40	2640, 4.60	142, 0.25
3-hydroxy-2′,3′-dehydro-β,ε-carotene	29, 0.05	12, 0.02	–
9- or 9′-*cis*-β-cryptoxanthin	13, 0.02	8, 0.015	–
Carotene			
all-trans-α-carotene	–	–	923, 1.72
all-trans- + 15,15′-*cis*-β-carotene	990, 1.85	670, 1.25	1670, 3.11
Carotenol Mono(fatty acid ester)			
all-trans- + neolutein monomyristate A or A′	1870, 2.40	383, 0.49	–
all-trans- + neolutein monopalmitate A or A′	94, 0.12	16, 0.02	–
Carotenol Bis(fatty acid ester)			
all-trans- + neolutein dilaurate A or A′	74, 0.079	57, 0.06	297, 0.32
all-trans- + neolutein dimyristate A or A′	149, 0.15	82, 0.08	209, 0.21
all-trans- + neolutein myristate/palmitate A or A′	44, 0.04	44, 0.04	126, 0.12
all-trans- + neolutein dipalmitate A or A′	34, 0.03	26, 0.02	133, 0.12

[a] Insufficient level in aliquot of extract applied to HPLC to permit quantification.

in different cultivars of this vegetable. In cultivars of baby food squash (brands G and H), the major carotenoids are lutein, lutein mono- and bis(fatty acid esters), and β-carotene, whereas in cooked acorn squash in addition to these carotenoids violaxanthin and violeoxanthin (9-*cis*-violaxanthin) as well as their mono- and bis(fatty acid esters) are also present. Particularly noticeable is the presence of lutein and lutein mono(fatty acid esters) (lutein monomyristate, lutein monopalmitate) and the absence of α-carotene in baby food squash brand G and the absence of lutein mono(fatty acid esters) and the presence of α-carotene in baby food squash brand H. Since the hydroxylation of α-carotene is known to be responsible for the formation of lutein in plant photosynthesis (Goodwin, 1980a), it seems more likely that the mono- and bis(fatty acid esters) of lutein in squash are formed from lutein. Therefore, the absence of α-carotene and the presence of lutein and lutein mono- and bis(fatty acid esters) in baby food squash brand G may be related to a more efficient conversion of the former to the latter. However, the presence of α-carotene and the absence of lutein in baby food squash brand H may suggest a much less efficient conversion of this compound to lutein but a more efficient conversion of lutein mono(fatty acid esters) to bis(fatty acid esters). From extensive NMR studies [data presented in Khachik et al. (1988)] of the isolated native lutein monomyristate and monopalmitate and several synthetic model compounds, we have shown that the site of the ester moiety in lutein monomyristate and lutein monopalmitate is on the β- rather than the α-ionone ring. The preferential esterification of the β-ionone ring, which results in the formation of the lutein mono(fatty acid esters) is not clearly understood. The differences between the two brands of baby food squash (G and H) may also to some extent be related to degree of plant maturity. The chromatographic profiles of the extracts from baby food squash brand G grown in two different locations (Fremont, MI; Asheville, NC) show no significant differences. However, the concentration of the carotenoids and related esters in baby food squash brand G grown in Fremont (MI) is much lower than that of the same brand grown in Asheville (NC). This may be related to environmental factors affecting the biosynthesis of carotenoids (i.e., light, temperature, nitrogen metabolism, and soil nutrients). The effect of environment on the synthesis of carotenoids in seed-bearing plants has been reviewed by Goodwin (1980b). The predominant carotenoids in the extracts from fresh and cooked acorn squash are violaxanthin, violeoxanthin (9-*cis*-violaxanthin), lutein, and their corresponding fatty acid esters. The presence of violeoxanthin in fresh cooked acorn squash may be an artifact of chromatography and/or extraction. However, this compound has been isolated from yellow pansy flowers (grown in Stanford) by Strain (1954). Extensive studies on the chemistry and spectroscopic properties of violeoxanthin have concluded a 9-cis geometry for this compound with respect to *all-trans*-violaxanthin (Szabolcs and Toth, 1970; Moss et al., 1975; Szabolcs, 1976). The presence of luteoxanthin and auroxanthin in the saponified extracts from raw and fresh cooked acorn squash is probably an artifact of extraction, saponification, and/or chromatography since these compounds are the rearrangement products of violaxanthin and have been shown to gradually form from this com-

Table V. Quantitative Distribution of the Predominant Carotenoids from Saponified Extracts of Raw and Fresh Cooked Acorn Squash Purchased Locally

	μg/100 g, edible food (wet weight/dry weight)	
	raw	cooked
Xanthophyll		
all-trans-violaxanthin 13- or 15,15′-*cis*-violaxanthin	44/733	34/243
all-trans-luteoxanthin	4/67	10/71
9-*cis*-violaxanthin	7.8/130	14/100
auroxanthin	54.5/908	122/871
di-*cis*-violaxanthin	–[a]	17.4/124
all-trans-lutein + neolutein A or A′	38/633	66/471
Carotene		
all-trans- + 15,15′-*cis*-β-carotene	220/3667	490/3500
total	368.3/6138	753.4/5380

[a] Insufficient level in aliquot of extract applied to HPLC to permit quantification.

pound during prolonged chromatographic and solvent extraction procedures (Khachik et al., 1986). The effects of cooking and processing on the distribution of carotenoids in varieties of squash are discussed later in this text.

Quantitative Distribution of Carotenoids and Carotenol Fatty Acid Esters in Squash Varieties. The distribution of carotenoids and related esters in several varieties of squash is shown in Tables IV and V. The carotenoid values for baby food squash brand G grown in two different locations in the United States (North Carolina, Michigan) are also tabulated in Table IV. These data are an average of two consecutive extractions and HPLC analyses of three randomly chosen samples of each squash variety; threfore, the values presented in Tables IV and V should not be taken as representative of the carotenoid levels of these squash varieties consumed nationwide.

The total concentration of lutein and its mono- and bis(fatty acid esters), expressed in micromoles (Table IV), indicates that the levels of these compounds in the North Carolina variety of baby food squash brand G is about 2.3 times the level of the Michigan variety of the same brand. The total concentration of lutein (free lutein and lutein as mono- and bis(fatty acid esters)) of baby food squash brand G is considerably greater than those of baby food squash brand H. On the other hand, the total hydrocarbon carotenoid level in baby food squash brand H is about 3 times greater than the carotene concentration of baby food squash brand G. From Table IV it is also apparent that although the baby food squash brand H contains a very low concentration of lutein and no detectable concentration of lutein mono(fatty acid esters), the total concentration of lutein bis(fatty acid esters) in this vegetable is about 3 times greater than that of baby food squash brand G. It is important to point out that the level of volatiles in all varieties was about 94% (determined by a microwave moisture analyzer), thus suggesting that the differences in carotenoid levels were due to a combination of genetics, environment, and nutrition.

In view of the evidence that saponification and prolonged extraction may result in losses of carotenoid epoxides such as violaxanthin (Khachik et al., 1986), we attempted to quantify the individual carotenoids and carotenol fatty acid esters in raw and cooked extracts of acorn squash without saponification (Figure 4). However, the low abundance of these carotenoids and the difficulties in synthesizing reasonable quantities of the fatty acid esters of violaxanthin and violeoxanthin for the preparation of the standard curves forced us to quantify carotenoids in acorn squash after saponification of the extracts. The concentrations of the predominant carotenoids in raw and fresh cooked acorn squash based on wet and the dry weight of this vegetable are shown in Table V. The percentage volatiles decreased by only 6% when acorn squash was cooked. A comparison between the total concentration of violaxanthin, its cis isomers, and its rearrangement products (luteoxanthin, auroxanthin) in raw and fresh cooked acorn squash indicates that about 23% of these xanthophylls are destroyed as a result of cooking this vegetable. The loss of lutein and its cis isomer in the cooked acorn squash is about 26%, whereas only 5% of β-carotene was lost as result of cooking acorn squash. When the total carotenoid levels in acorn squash and the varieties of baby food squash (brands G and H) are compared, acorn squash has much lower levels of carotenoids.

Selection of the Internal Standards. In previous reports, we demonstrated the application of internal standards such as β-apo-8′-carotenal and decapreno-β-carotene (Khachik and Beecher, 1985; Khachik et al., 1986) and recently nonapreno-β-carotene (Khachik and Beecher, 1986, 1987) for quantitative determination of the various classes of the naturally occurring carotenoids (xanthophylls, hydrocarbon carotenoids) in fruits and vegetables by HPLC. However, since the major squash carotenoids were found to consist of mono- and bis(fatty acid esters) of lutein and of violaxanthin, the development of an internal standard that is structurally similar to these carotenol esters seemed appropriate. Since among dihydroxy carotenes isozeaxanthin (4,4′-dihydroxy-β-carotene) has been shown to be the most readily accessible (Petracek and Zechmeister, 1956; Entschel and Karrer, 1958), several bis(fatty acid esters) of this compound were synthesized as potential internal standards. On the basis of the HPLC retention time, isozeaxanthin dipelargonate was shown to be the most suitable internal standard for quantitative determination of carotenoids and related esters in baby food squash (brands G and H). Depending on the occurrence of carotenol esters and the chromatographic profile of the extracts from natural sources, a similar approach can be used to modify the length of the fatty acid side chain in isozeaxanthin dipelargonate to arrive at the appropriate internal standard for other HPLC separations.

In addition to simple synthesis from commercially available starting materials, isozeaxanthin dipelargonate exhibits very similar chromatographic and solubility behavior to that of α- and β-carotene. The absorption maximum (λ_{max} = 454 nm) of this internal standard in the HPLC solvents is identical with or reasonably close to that of carotenes (λ_{max} = 446, 454 nm) and lutein fatty acid esters (λ_{max} = 446 nm), which enables the chromatographic analyses of the mixture of these carotenoids to be monitored at a single wavelength (450 nm). The recovery of isozeaxanthin dipelargonate from numerous extractions of baby food squash was more than 98% as determined by the HPLC peak area of this internal standard before and after extraction. Isozeaxanthin dipelargonate was not expected to be present in the extract of squash varieties owing to the absence and/or low abundance of fatty acids with odd numbers of carbons in natural products. However, this was further confirmed by extraction and HPLC analysis of squash varieties without added internal standard. The limitation of this internal standard is in the extraction and workup procedures that involve saponification. The basic hydrolysis converts isozeaxanthin dipelargonate to isozeaxanthin; therefore, it results in its close elution and HPLC peak interference with xantho-

phylls such as lutein and zeaxanthin on a C-18 reversed-phase column.

In the case of acorn squash, where the carotenoids were quantified after saponification, β-apo-8′-carotenol was employed as an internal standard to monitor the loss of carotenoids as a result of extraction and saponification. Although this compound is not structurally similar to the oxygenated carotenoids found in acorn squash, its suitable HPLC retention time as well as its convenient synthesis from commercially available starting materials were the contributing factors in its selection as an internal standard. Under extraction, saponification, and the HPLC conditions employed, the solubility and chromatographic properties of β-apo-8′-carotenol were also shown to be similar to that of the predominant carotenoids of acorn squash. The recovery of β-apo-8′-carotenol from several extraction and saponification steps of raw and cooked acorn squash was more than 98%. This internal standard was particularly employed to monitor carotenoid losses due to saponification.

Correlation between HPLC Retention Times and the Equivalent Chain Lengths of Bis(fatty acid esters) of Carotenoids. In gas–liquid chromatography (GLC), the correlation between the number of carbons in saturated fatty acids and related esters has been extensively used to predict the elution sequence of these compounds from a gas chromatographic column (Jamieson, 1970). Under identical chromatographic conditions, a parameter known as equivalent chain length (ECL) for a number of known saturated carboxylic methyl monoesters is obtained by plotting the logarithms of the retention times of these compounds against the number of carbon atoms in their corresponding acids. ECL values of unknown esters, chromatographed under identical conditions, are then determined from the reference curve with the observed retention times.

In the present study similar correlations at first appeared to exist between the HPLC retention times of the predominant carotenol esters (Tables II and III) in squash products and the number of carbon atoms in the fatty acid side chains. For example, under the chromatographic conditions (eluent A) employed for the separation of the carotenol esters in baby food squash (brands G and H), there is a steady increase of about 3.90–4.00 min in the HPLC retention time of lutein dimyristate (Table II), as the total number of carbon atoms in the fatty acid side chains in this compound is increased by 4 to lutein dipalmitate. However, this correlation does not hold for lutein dilaurate. Furthermore, repeated HPLC injections of squash extracts and reference samples of carotenol bis(fatty acid esters) revealed that this exact retention time difference under gradient chromatographic conditions is not reproducible. This is probably related to the parameters affecting intermolecular interactions between sample and solvent molecules in liquid chromatography (i.e., dispersion, dipole, and hydrogen bonding). As the number of carbons in straight-chain fatty acids is increased by 2, some noticeable changes in the physical properties (melting points, solubility behavior) of these compounds are observed. It therefore seems more likely that the chromatographic and the solubility behavior of the carotenol bis(fatty acid esters) is influenced by the chromatographic and solubility behavior of the fatty acid side chains, which in turn may reflect in a nonsteady increase in retention times for some of these carotenol esters. In addition to data presented in this text we have recently synthesized a number of bis(fatty acid esters) (C_{10}, C_{12}, C_{12}/C_{14}, C_{14}, C_{14}/C_{16}, C_{16}) of carotenoids such as zeaxanthin, violaxanthin, auroxanthin, and β-cryptoxanthin and have extensively studied their elution sequence on a C-18 reversed-phase column under various chromatographic conditions. From these studies that will be published elsewhere, it seems quite clear that within a series of carotenol bis(fatty acid esters) of a given carotenoid as the number of carbon atoms on the fatty acid side chains is increased, the HPLC retention times of carotenol fatty acid esters are also increased.

Effect of Cooking and Processing on Distribution of Carotenoids and Carotenol Fatty Acid Esters. Since the raw samples of the varieties of the processed baby food squash were not available in the present study, it is not possible to determine the effect of cooking and processing on qualitative and quantitative distribution of carotenoids and carotenol fatty acid esters in these vegetables. The presence of the cis isomers of lutein, β-cryptoxanthin, and mono- and bis(fatty acid esters) of lutein in squash varieties may be related to the thermal isomerization of their corresponding all-trans isomers at the processing stage. However, since the cis isomers of carotenoids are often produced in organic extracts as artifacts of extraction and/or chromatography, their presence in natural products is very difficult to establish. The presence of 3-hydroxy-2′,3′-dehydro-β,ε-carotene (peak 3, Figure 2) in the extract from baby food squash brand G may well be related to lutein, which probably undergoes an acid-catalyzed dehydration to form this compound in the acidic squash (pH $\simeq$4) at the processing stage.

A comparison between the chromatograms (Figure 4) of the extracts from raw and cooked acorn squash reveals that violaxanthin (peak 1) is either destroyed or converted to its cis isomers (peaks 7 and 7′) in the cooking process. It is also interesting to note that while the mono(fatty acid esters) of violaxanthin (peaks 10–12) are completely destroyed in the cooking process, the bis(fatty acid esters) of violaxanthin and violeoxanthin survive to some extent. There seems to be no significant change in the ratio of cis/trans isomers of violaxanthin bis(fatty acid esters) as a result of cooking. The conversion of violaxanthin and violeoxanthin bis(fatty acid esters) to their corresponding luteoxanthin and auroxanthin bis(fatty acid esters) were not shown to have occurred as a result of heat treatment, and no detectable amounts of these compounds were shown to be present in the cooked vegetable extracts. Lutein bis(fatty acid esters) seem to survive the heat treatment, and no significant change in the concentration of these carotenoids is experienced. From the data obtained in the present study and our previous experience with carotenoid epoxides (Khachik et al., 1986), it seems clear that the epoxy carotenoids that contain unsubstituted hydroxyl groups are extremely sensitive to heat treatment, whereas substitution of the hydroxyl groups with long-chain fatty acids increases the thermal stability of these compounds.

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 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, prefixes such as neo A and neo A′ have been used to distinguish these compounds.

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Registry No. *all-trans*-Ia, 127-40-2; 13-*cis*-Ia, 32449-88-0; 13'-*cis*-Ia, 79464-33-8; *all-trans*-Ib (n = 12), 115095-04-0; 13-*cis*-Ib (n = 12), 115223-26-2; 13'-*cis*-Ib (n = 12), 115223-27-3; *all-trans*-Ib (n = 14), 14683-67-1; 13-*cis*-Ib (n = 14), 115223-28-4; 13'-*cis*-Ib (n = 14), 115223-29-5; *all-trans*-Ic, 104784-49-8; 13-*cis*-Ic, 115266-14-3; 13'-*cis*-Ic, 115266-15-4; *all-trans*-Id (n = 10), 23852-66-6; *all-trans*-Id (n = 12), 86853-02-3; *all-trans*-Id (n = 14), 547-17-1; 13-*cis*-Id (n = 10), 115223-30-8; 13'-*cis*-Id (n = 10), 115223-31-9; 13-*cis*-Id (n = 12), 115223-32-0; 13'-*cis*-Id (n = 12), 115223-56-8; 13-*cis*-Id (n = 14), 115223-33-1; 13'-*cis*-Id (n = 14), 115223-34-2; II, 35007-50-2; 9'-*cis*-III, 115223-25-1; 9-*cis*-III, 115223-35-3; *all-trans*-IVa, 126-29-4; 15,15'-*cis*-IVa, 24620-97-1; 9-*cis*-IVa, 26927-07-1; 13-*cis*-IVa, 75715-58-1; di-*cis*-IVa, 115182-31-5; 9-*cis*-IVb, 115182-24-6; 9'-*cis*-IVb, 115182-27-9; *all-trans*-IVb, 113464-19-0; *all-trans*-IVc, 113464-21-4; 9-*cis*-IVc, 115182-28-0; *all-trans*-IVd (n = 10), 49795-35-9; 9-*cis*-IVd (n = 10), 115182-25-7; *all-trans*-IVd (n = 12), 113531-83-2; 9-*cis*-IVd (n = 12), 115182-26-8; *all-trans*-IVd (n = 14), 113531-84-3; 9-*cis*-IVd (n = 14), 115223-24-0; *all-trans*-luteoxanthin, 1912-50-1; *all-trans*-auroxanthin, 27785-15-5; *all-trans*-β-carotene, 7235-40-7; 15,15'-*cis*-β-carotene, 19361-58-1; flavoxanthin, 512-29-8; *all-trans*-α-carotene, 432-70-2.

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