Separation and Identification of Carotenoids and Carotenol Fatty Acid Esters in Some Squash Products by Liquid Chromatography. 2. Isolation and Characterization of Carotenoids and Related Esters

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The predominant carotenoids and carotenol fatty acid esters in extracts from several varieties of squash have been isolated by preparative thin-layer chromatography (TLC) employing C-18 reversed-phase and normal-phase silica gel plates. As many as 25 components were separated and characterized in most cases on the basis of their absorption and mass spectral properties. Four classes of compounds were shown to be present: xanthophylls, hydrocarbon carotenoids, and carotenol mono- and bis(fatty acid esters). Structural elucidation of several carotenol mono(fatty acid esters) was based on the data obtained from nuclear magnetic resonance (NMR) spectroscopy of these compounds as well as NMR data obtained from several synthetic model compounds. Several stereoisomers of carotenoids and carotenol fatty acid esters were shown to be present in the extract from a variety of raw and cooked acorn squash. In some cases semipreparative high-performance liquid chromatography was employed to separate the cis isomer of carotenoids. A number of straight-chain bis(fatty acid esters) of lutein and violaxanthin, which were shown to be the major carotenoid constituents of several squash products, were prepared by partial synthesis.

In the preceding paper (Khachik and Beecher, 1988) we reported the separation and quantification of carotenoids and carotenol fatty acid esters in extract from several variety of squash by liquid chromatography. As many as 25 components were shown by HPLC to be present, which were assigned to four classes of compounds: xanthophylls, hydrocarbon carotenoids, carotenol mono- and bis(fatty acid esters). In this report we present various chromatographic techniques employed for the isolation and separation of these carotenoids, as well as detailed characterization and structural elucidation of these compounds in several varieties of this vegetable by NMR, absorption spectroscopy, and mass spectrometry. Several of the carotenol mono- and bis(fatty acid esters) have been prepared by partial synthesis from their parent hydroxy carotenoids and the appropriate fatty acid chloride.

EXPERIMENTAL SECTION

Apparatus. The high-performance liquid chromatographic system employed for analytical and preparative scale separation of the carotenoids and carotenoid fatty acid esters of squash extracts has been described elsewhere (Khachik et al., 1986). Ammonia and isobutane desorption chemical ionization (DCI) mass spectra were obtained on a Finnigan 4510 instrument equipped with an Incos data system. Samples of approximately 100 ng were deposited on the desorption probe tip and heated at a current rate of 50 mA/s from 0 to 1000 mA. Ammonia at an indicated source pressure of 0.6 Torr and a source block temperature of 60 °C provided a reagent gas ionic distribution of 28/30/100/29 (18/35/52/69 Da). Isobutane at 0.4 Torr and 60 °C produced an ionic distribution of 5/2/4/100 (18/39/43/57 Da; 18 amu from residual ammonia). DCI spectra were collected from m/z 380 to 990 with a scan cycle time of 1.0 s. The ^{1}H NMR (400-MHz) spectra were obtained on a Bruker instrument. Absorption spectra of isolated components were recorded on a Beckman DU-7 UV/visible spectrophotometer.

Chromatographic Procedures. The analytical separations were carried out employing eluents A and B, while eluents C and D were employed for the semipreparative separations. Chromatographic conditions for analytical separations employing eluents A and B have been described in Khachik and Beecher (1988). Eluent C consisted of an isocratic mixture of methanol (15%), acetonitrile (65%), methylene chloride (10%), and hexane (10%), which effected the semipreparative separations of carotenoids and related fatty acid esters at a column flow rate of 2.5 mL/min. Eluent D was employed for the semipreparative separation of the stereoisomers of violaxanthin and consisted of methanol (10%), acetonitrile (85%), methylene chloride (2.5%), and hexane (2.5%) at a column flow rate of 1.5 mL/min. The semipreparative separations were carried out on a Rainin stainless-steel (25-cm length × 10-mm i.d.) Microsorb C-18 column (5-μm spherical particles), which was protected with a Brownlee guard cartridge (3-cm length × 4.6-mm i.d.) packed with spheri-5 C-18 (5-μm particles). The purities of the isolated and synthetic carotenoids and related fatty acid esters were further evaluated by HPLC under analytical conditions employing eluents A and B.

Reagents and Materials. The reference samples of lutein, lutein monomyristate, and lutein monopalmitate were isolated from baby food squash (brand G) by semipreparative TLC (details presented later in the text). *all-trans*-Violaxanthin and its stereoisomers were isolated from the saponified extracts of raw and cooked acorn squash by preparative TLC and HPLC (details presented later in the text). Reference samples of *all-trans*-β-cryptoxanthin, zeaxanthin, and 15,15′-*cis*-β-carotene were provided by Hoffmann-La Roche, Basel, Switzerland. The appropriate bis(fatty acid esters) of lutein, violaxanthin, and zeaxanthin were obtained from lutein, violaxanthin, and zeaxanthin by partial synthesis according to the pro-

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cedure described in text. The sources of the varieties of squash samples, referred to as brands G and H and acorn, have been described in Khachik and Beecher (1988).

Isolation of Lutein. A concentrated extract from baby food squash (brand G, North Carolina Variety, 500 g) was dissolved in ether (200 mL), and it was treated with methanolic potassium hydroxide (30%) under an atmosphere of nitrogen for 6 h at room temperature. The solution was partitioned between water (250 mL) and ether (100 mL). The water layer was washed with ether several times, and the resulting organic layers were combined, dried over sodium sulfate, and evaporated to dryness. The residue was chromatographed on C-18 reversed-phase plates (1000-μm layer thickness) employing methanol (15%), acetonitrile (70%), methylene chloride (7.5%), and hexane (7.5%) as eluent. Five bands were separated.

The first and second band were identified from their UV/visible absorption maxima in various solvents as well as comparison of their HPLC retention times with authentic samples as flavoxanthin (R_f 0.60) and lutein (main yellow zone, R_f 0.50), respectively.

Band 3 was tentatively identified from its UV/visible absorption [λ_{max} = 446 nm (HPLC solvents, eluent A)] and mass spectra as 3-hydroxy-2',3'-dehydro-β,ϵ-carotene (R_f 0.41). The UV/visible absorption maxima (nm) of this compound in various solvents [hexane, λ_{max} = 331, 421 (inflection), 442.5, 470.5; ethanol, λ_{max} = 332, 422 (inflection), 444, 471.5; benzene, λ_{max} = 338.5, 433 (inflection), 455, 484] were consistent with the chromophore involved. The mass spectrum (DCI, ammonia as reagent gas) of band 3 contained an ammonium adduct ion at m/z 568 [79%, $(M + NH_4)^+$] and an ion at m/z 550 [23%, $(M + NH_4 - H_2O)^+$].

Band 4 was identified from its UV/visible absorption and mass spectra as 9-*cis*- or 9'-*cis*-β-cryptoxanthin (R_f 0.34). The UV/visible absorption maxima (nm) of this compound in various solvents [hexane, λ_{max} = 336.5, 425 (inflection), 446, 472; ethanol, λ_{max} = 337, 426 (inflection), 446.5, 472; benzene, λ_{max} = 345, 435, 457.5, 485.5] were consistent with the chromophore involved. The mass spectrum (DCI, ammonia as reagent gas) of band 4 contained an ammonium adduct ion at m/z 570 [78%, $(M + NH_4)^+$] and an ion at m/z 552 [20%, $(M + NH_4 - H_2O)^+$], indicating a subsequent loss of water. The HPLC retention time and the UV/visible absorption spectrum [λ_{max} = 450 nm, monitored by the photodiode array detector in the HPLC solvent (eluent A)] of band 4 was identical with that of the 9-*cis*- or 9'-*cis*-β-cryptoxanthin obtained from stereoisomerization of an authentic sample of *all-trans*-β-cryptoxanthin in refluxing methanol.

Band 5 was identified by comparison of its HPLC retention time and UV/visible absorption spectrum with those of authentic samples as a mixture of *all-trans*- and 15,15'-*cis*-β-carotene (R_f 0.19).

The isolated lutein (19 mg, 0.033 mmol) was shown by HPLC (eluent C) to contain 10% of neolutein A or A' (13- or 13'-*cis*-lutein). The mass spectrum (DCI/ammonia) of lutein contained peaks at m/z 586 [100%, $(M + NH_4)^+$], 569 [9%, $(M + H)^+$], 568 [8%, $(M + NH_4 - H_2O)^+$], and 551 [21%, $(M + H - H_2O)^+$]. The desorption chemical ionization mass spectrum of lutein employing isobutane as reagent gas presented a protonated molecular ion at m/z 569 [80%, $(M + H)^+$] as well as ions at m/z 551 [100%, $(M - H_2O + H)^+$] and 533 [42%, $(M - 2H_2O + H)^+$]. The UV/visible absorption maxima (nm) of the mixture of *all-trans*-lutein and its cis isomer in various solvents [ethanol, λ_{max} = 336.5 ($E^{1\%}$ = 339), 429 inflection ($E^{1\%}$ = 1990), 446.5 ($E^{1\%}$ = 2550), 473 ($E^{1\%}$ = 2122)] were consistent with the UV/visible absorption maxima of lutein reported in the literature [ethanol, λ_{max} = 445 ($E^{1\%}$ = 2550) (Ritter and Purcell, 1981)].

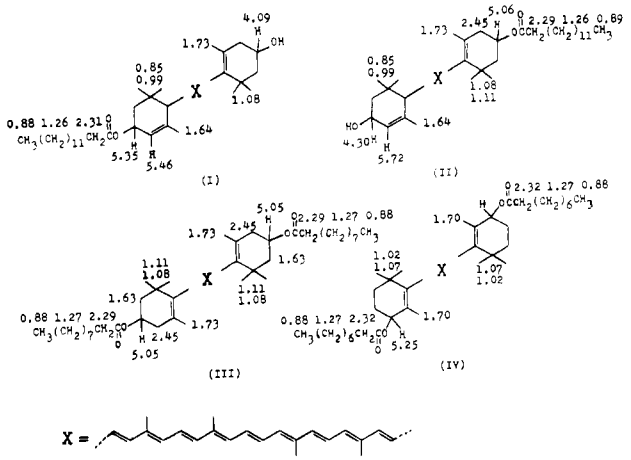

Figure 1. Proton chemical shifts (ppm) and signal assignments of some of the synthetic mono- and bis(fatty acid esters) of carotenoids employed as model compounds for structural elucidation of squash carotenoids: I, β,ϵ-carotene-3-monol-3'-monol monomyristate (semisynthetic lutein monomyristate B); II, β,ϵ-carotene-3-monol monomyristate-3'-monol (natural lutein monomyristate); III, zeaxanthin didecanoate; IV, isozeaxanthin dipelargonate.

Partial Synthesis of Lutein Monomyristate. A solution of myristoyl chloride (0.01 mmol) in benzene (2 mL) was added to a solution of lutein (0.01 mmol) and triethylamine (0.05 mmol) in benzene (10 mL), and the mixture was stirred under an atmosphere of nitrogen at 40 °C. The course of the reaction was followed by HPLC (eluent A), and at the completion of the reaction (after 30 min) four major components were shown to be present. These were tentatively identified from their HPLC retention times and their UV/visible absorption spectra as unreacted lutein (20%), semisynthetic lutein monomyristate A (37%), semisynthetic lutein monomyristate B (13%), and lutein dimyristate (20%). The product was washed several times with 10% sodium bicarbonate (3 × 15 mL) and water (2 × 20 mL) and dried over sodium sulfate. The excess of the solvent was removed on a rotary evaporator at 30 °C, and the concentrated residue was chromatographed on C-18 reversed-phase plates (1000 μm) employing acetonitrile (60%), methanol (15%), methylene chloride (12.5%), and hexane (12.5%) as eluent. Three bands were separated. Band 1 and 3 were tentatively identified from their HPLC retention times and UV/visible absorption spectra as unreacted lutein (band 1, R_f 0.70) and lutein dimyristate (band 3, R_f 0.06), respectively. Band 2 was further separated by semipreparative HPLC (eluent C) into two components that were identified by mass spectrometry and in part by NMR in the order of elution on a C-18 reversed-phase column as β,ϵ-carotene-3-monol monomyristate-3'-monol (shown to be identical with natural lutein monomyristate) and β,ϵ-carotene-3-monol-3'-monol monomyristate. These semisynthetic lutein monomyristates were shown by HPLC (eluent A) to contain minor quantities of their corresponding cis stereoisomers (tetatively identified as 13-cis or 13'-cis). The proton NMR chemical shifts of the two regioisomers of lutein monomyristates (structures I and II) including signal assignments are presented in Figure 1.

Partial Synthesis of Bis(fatty acid esters) of Lutein. A solution of an appropriate fatty acid chloride (0.018 mmol) in dry benzene (5 mL) was added to a solution of lutein (0.0018 mmol) and triethylamine (0.054

mmol) in benzene (15 mL), and the mixture was stirred under an atmosphere of nitrogen at 40 °C for 1 h. The product was sequentially washed with 10% sodium bicarbonate (3 × 30 mL), 10% methanol in water (2 × 30 mL), and water (2 × 30 mL), and it was dried over sodium sulfate. The excess of solvent was evaporated under reduced pressure, and the residue was chromatographed on *n*-silica plates (250-μm thickness) with petroleum ether/acetone (24/1) as eluent. The main yellow zone of lutein bis(fatty acid ester) was separated and further purified by recrystallization from ethanol/benzene. Lutein bis(fatty acid esters) prepared from lutein by partial synthesis were shown by HPLC (eluent A) to contain minor quantities of their corresponding 13-cis or 13′-cis stereoisomers.

Partial Synthesis of Lutein Myristate/Palmitate Mixed Diesters. A solution of lutein in benzene was added to a mixture of myristoyl chloride and palmitoyl chloride (1/1) in benzene in the presence of triethylamine, and the reaction mixture was heated at 40 °C for 1 h. The product was isolated and chromatographed as above. Examination of the isolated fraction by HPLC revealed the presence of lutein dimyristate, lutein dipalmitate, and lutein myristate/palmitate mixed diesters. The mixed diesters of lutein were tentatively identified as β,ε-carotene-3-monol monomyristate-3′-monol monopalmitate and β,ε-carotene-3-monol monopalmitate-3′-monol monomyristate. Under the chromatographic conditions employed, the two regioisomers of lutein myristate/palmitate mixed diesters could not be resolved.

Lutein Didecanoate. UV/visible absorption maxima (nm) of lutein didecanoate in various solvents [benzene, λ_{max} = 454 ($E^{1\%}$ = 1493), 483.5 ($E^{1\%}$ = 1176); light petroleum ether, λ_{max} = 440 ($E^{1\%}$ = 1795), 469 ($E^{1\%}$ = 1429), hexane, λ_{max} = 442 ($E^{1\%}$ = 1676), 470.5 ($E^{1\%}$ = 1337); ethanol, λ_{max} = 443 ($E^{1\%}$ = 1607), 471.5 ($E^{1\%}$ = 1281)] were consistent with the chromophore involved. The mass spectrum (DCI, ammonia) of this compound revealed an ammonium adduct ion at *m/z* 894 [84%, $(M + NH_4)^+$], as well as ions at *m/z* 876 [4%, $(M + NH_4 - H_2O)^+$], 802 [9%, $(M + NH_4 - 92)^+$], 788 [5%, $(M + NH_4 - 106)^+$], 722 [15%, $[M - 155\ (C_{10}H_{19}O) + H]^+$], 704 [8%, $[M - 172\ (C_{10}H_{20}O_2$, decanoic acid)$]^+$], and 532 [100%, $(M - 172 - 172)^+$]. The mass spectrum (DCI) employing isobutene as reagent gas contained a protonated molecular ion at *m/z* 877 $(M + H)^+$, as well as ions at *m/z* 705 $(M + H - 172)^+$ and 533 $(M + H - 172 - 172)^+$.

Lutein Dilaurate. UV/visible absorption maxima (nm): benzene, λ_{max} = 454 ($E^{1\%}$ = 1405), 482 ($E^{1\%}$ = 1125); light petroleum ether, λ_{max} = 440 ($E^{1\%}$ = 1630), 467.5 ($E^{1\%}$ = 1317); hexane, λ_{max} = 442 ($E^{1\%}$ = 1566), 469 ($E^{1\%}$ = 1265); ethanol, λ_{max} = 443 ($E^{1\%}$ = 1507), 469.5 ($E^{1\%}$ = 1226).

Lutein Dimyristate. UV/visible absorption maximum (nm): benzene, λ_{max} = 454.5 ($E^{1\%}$ = 1325), 483.5 ($E^{1\%}$ = 1078); light petroleum ether, λ_{max} = 441 ($E^{1\%}$ = 1534), 469.5 ($E^{1\%}$ = 1260); hexane, λ_{max} = 442.5 ($E^{1\%}$ = 1429), 471 ($E^{1\%}$ = 1174); ethanol, λ_{max} = 444 ($E^{1\%}$ = 1438), 472 ($E^{1\%}$ = 1189).

Lutein Dipalmitate. UV/visible absorption maximum (nm): benzene, λ_{max} = 456.5 ($E^{1\%}$ = 1250), 484.5 ($E^{1\%}$ = 1047) [lit. λ_{max} = 457 ($E^{1\%}$ = 1254) (Cholnoky et al., 1958)]; light petroleum ether, λ_{max} = 442.5 ($E^{1\%}$ = 1507), 470.5 ($E^{1\%}$ = 1282); hexane, λ_{max} = 443.5 ($E^{1\%}$ = 1412), 471.5 ($E^{1\%}$ = 1201) [lit. λ_{max} = 445 ($E^{1\%}$ = 1394) (Cholnoky et al., 1958)]; ethanol, λ_{max} = 445 ($E^{1\%}$ = 1201), 472.5 ($E^{1\%}$ = 1022).

Zeaxanthin Didecanoate. UV/visible absorption maxima (nm): benzene, λ_{max} = 463 ($E^{1\%}$ = 1379), 492 ($E^{1\%}$ = 1130); light petroleum ether, λ_{max} = 448 ($E^{1\%}$ = 1598), 476 ($E^{1\%}$ = 1400); hexane, λ_{max} = 450 ($E^{1\%}$ = 1551), 478 ($E^{1\%}$ = 1364); ethanol, λ_{max} = 450.5 ($E^{1\%}$ = 1139), 479 ($E^{1\%}$ = 1000). The mass spectrum (electron impact) of zeaxanthin didecanoate showed a molecular parent ion peak at *m/z* 876 (85%), as well as peaks at *m/z* 784 (10%, M − 92), 705 [34%, M − 171 ($C_{10}H_{19}O_2$)], 612 (22%, M − 172 − 92), 533 (25%, M − 172 − 171), and 440 (10%, M − 172 − 172 − 92). The proton NMR chemical shifts (Figure 1) were in agreement with the structure of zeaxanthin didecanoate.

Isolation and Characterization of Carotenoids and Related Fatty Acid Esters in Baby Food Squash (Brand G, North Carolina Variety). A concentrated solution of an extract from baby food squash (300 g, brand G) was chromatographed on semipreparative C-18 reversed-phase thin-layer plates (20 × 20 cm, layer thickness 200 μm; Whatman Chemical Separation Inc.) employing methanol (15%), acetonitrile (60%), methylene chloride (12.5%), and hexane (12.5%) as eluents. The following bands were separated in the order of chromatographic elution:

band	R_f	chemical class
1	0.69	xanthophyll
2	0.61	xanthophyll
3	0.46	xanthophyll
4	0.38	xanthophyll
5	0.30	carotenol mono(fatty acid ester)
6	0.19	carotenol mono(fatty acid ester)
7	0.14	carotene
8	0.03	carotenol bis(fatty acid ester)

Band 1 was shown by HPLC (eluent B) to consist of one component identified from its HPLC retention time and UV/visible absorption spectra in various solvents as flavoxanthin [λ_{max} = 408, 431, 459 nm (benzene); 424, 447, 475 nm (carbon disulfide); 401, 423, 449 nm (ethanol); 398, 420, 446 nm (petroleum ether)]. The UV/visible spectrum of flavoxanthin had a maximum at 422 nm in the HPLC solvents (eluents A–C). The mass spectrum of flavoxanthin with isobutane as reagent gas showed a weak protonated molecular ion peak at *m/z* 585 $(M + H)^+$, as well as peaks at *m/z* 567 $(M + H - H_2O)^+$, 543 $(M + C_4H_9 - H_2O - 80)^+$, and 487 $(M + H - H_2O - 80)^+$. The HPLC retention time of this compound was identical with that of flavoxanthin isolated from the extracts of several green vegetables (Khachik et al., 1986).

Band 2 was shown by HPLC (eluent B) to consist of one major component identified from its UV/visible absorption and mass spectra as *all-trans*-lutein. This compound was shown by HPLC to contain about 10% neolutein A or A′ (13- or 13′-*cis*-lutein), which appeared as a tailing shoulder on the *all-trans*-lutein peak. *all-trans*-Lutein was separated from neolutein A or A′ by semipreparative HPLC (eluent D). The HPLC retention times and UV/visible absorption spectra of *all-trans*-lutein [λ_{max} = 456, 486 nm (benzene); 453.5, 482 (methylene chloride); 443.5, 472 nm (methanol)], and neolutein A or A′ [λ_{max} = 338 (cis peak), 453.5, 481 nm (benzene); 338, 451, 480 nm (methylene chloride); 330, 439, 465 nm (methanol)] were consistent with those of the reference samples of these compounds isolated from the extracts of several green vegetables (Khachik et al., 1986).

Band 3 was shown by HPLC (eluent A) to consist of one component identified from its HPLC retention time and UV/visible absorption spectra as 3-hydroxy-2′,3′-dihydro-β,ε-carotene. This compound was also isolated earlier from a saponified extract of baby food squash (brand G).

Band 4 was shown by HPLC (eluent A) to consist of one component tentatively identified from its HPLC retention

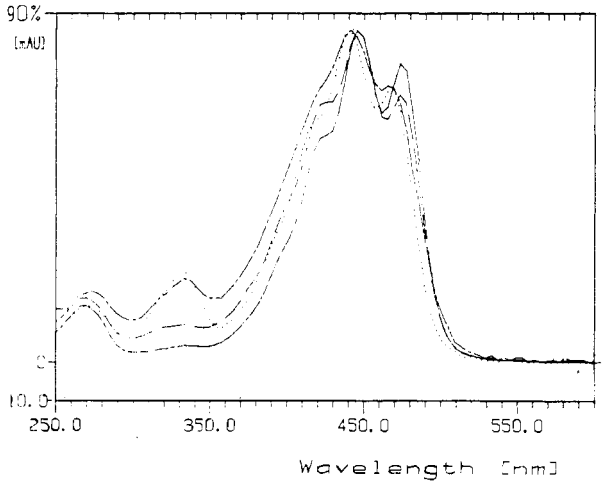

Figure 2. UV/visible absorption spectra of native *all-trans*-lutein monomyristate [(—) λ_{max} = 446 nm], neolutein monomyristate A or A′ [(···) λ_{max} = 442 nm, cis peak at 330 nm], native *all-trans*-lutein dimyristate [(- - -) λ_{max} = 446 nm], and neolutein dimyristate A or A′ [(-·-) λ_{max} = 442 nm, cis peak at 334 nm] monitored in the HPLC system (eluent A) described in the text.

time and UV/visible absorption spectra as 9-*cis*- or 9′-*cis*-β-cryptoxanthin. This compound was also isolated from a saponified extract of baby food squash (brand G).

Band 5 was shown by HPLC (eluent A) to consist of one major component (90%) and a minor component (10%) identified by NMR spectroscopy as *all-trans*-β,ε-carotene-3-monol monomyristate-3′-monol (natural lutein monomyristate) and its mono-cis isomer. The absorption spectra of *all-trans*-lutein monomyristate and its cis isomer in the HPLC solvents (eluent A) monitored by the rapid-scanning detector had maxima at 446 and 442 nm, respectively (Figure 2). The UV/visible absorption spectrum of *cis*-lutein monomyristate also contained a strong cis peak in the near-UV region at 330 nm. The UV/visible absorption spectra of the mixture of native *all-trans*-lutein monomyristate and its cis isomer in various solvents had maxima at 444, 472.5 nm (hexane); 444, 472 nm (ethanol); 440.5, 469 nm (light petroleum ether); 454.5, 484 nm (benzene); 445.5, 473 nm (acetone); 471, 501 nm (carbon disulfide); and 445.5, 473.5 nm (ethyl acetate). The mass spectrum (electron impact) of natural lutein monomyristate showed a parent ion peak at m/z 778 as well as peaks at m/z 686 (M − 92) and 672 (M − 106). The proton NMR spectrum of the mixture of native *all-trans*-lutein monomyristate and its cis isomer (Figure 3) was identical with that of synthetic β,ε-carotene-3-monol monomyristate-3′-monol (semisynthetic lutein monomyristate A).

Band 6 was shown by HPLC (eluent A) to consist of one major component (90%) and a minor (10%) component identified by NMR spectroscopy as *all-trans*-β,ε-carotene-3-monol monopalmitate-3′-monol (natural lutein monopalmitate) and its mono-cis isomer. The absorption spectra of *all-trans*-lutein monopalmitate and its cis isomer in the HPLC solvents (eluent A) monitored by the rapid-scanning detector had maxima at 446 and 442 nm, respectively. The UV/visible absorption spectrum of *cis*-lutein monopalmitate also contained a strong cis peak in the near-UV region at 330 nm. The UV/visible absorption spectra of the mixture of native lutein monopalmitate and its cis isomer in various solvents had maxima at 443.5, 472 nm (hexane); 445, 473 nm (ethanol); 441, 469.5 (light petroleum ether); 455, 484.5 nm (benzene); 446, 474 nm (acetone); 471.5, 502 nm (carbon disulfide); and 445.5, 474 (ethyl acetate). The mass spectrum (DCI, ammonia) of

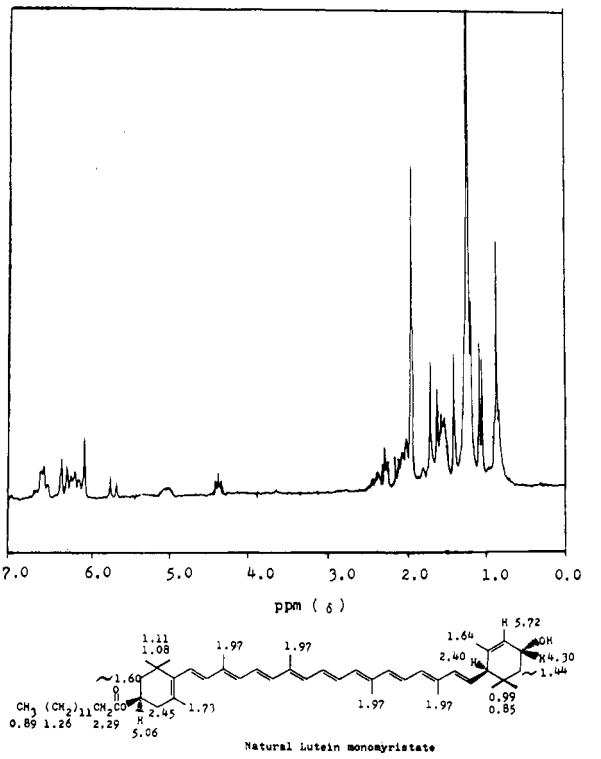

Figure 3. Proton NMR spectrum of natural lutein monomyristate including chemical shift (ppm) assignments in $CDCl_3$.

native lutein monopalmitate contained an ammonium adduct ion at m/z 824 [12%, $(M + NH_4)^+$], as well as peaks at m/z 806 [3%, $(M + NH_4 - H_2O)^+$], 732 [3%, $(M + NH_4 - 92)^+$], 718 [1%, $(M + NH_4 - 106)^+$], and 532 [100%, [M − 256 (palmitic acid) − H_2O]$^+$]. The proton NMR spectrum of the mixture of native *all-trans*-lutein monopalmitate and its mono-cis isomer was consistent with the structure of this compound and contained signals at chemical shifts identical with that of native lutein monomyristate.

Band 7 was shown by HPLC (eluent A) to consist of one major component and a minor component identified by comparison of their HPLC retention times and absorption spectra with those of the reference samples as *all-trans*-β-carotene (major component) and 15,15′-*cis*-β-carotene (minor component appearing as a tailing shoulder on the *all-trans*-β-carotene peak).

Band 8 was shown by HPLC (eluent A) to consist of seven components identified by comparison of their HPLC retention times and absorption spectra monitored by the rapid-scanning detector in the HPLC solvents (eluent A) with those of the reference samples as *all-trans*-lutein dilaurate (λ_{max} = 446 nm), *all-trans*-lutein dimyristate (λ_{max} = 446 nm, Figure 2), neolutein dimyristate A or A′ (λ_{max} = 334, 442 nm; Figure 2), *all-trans*-lutein myristate/palmitate (mixed diester, λ_{max} = 446 nm), neolutein myristate/palmitate A or A′ (mixed diester, λ_{max} = 334, 442 nm), *all-trans*-lutein dipalmitate (λ_{max} = 446 nm), and neolutein dipalmitate A or A′ (λ_{max} = 334, 442 nm).

Identification of Major Carotenoids in Brand H of Baby Food Squash. An extract from brand H baby food squash was similarly chromatographed on reversed-phase TLC plates (same conditions as above), and it was shown to consist of four major bands that were separated and identified from their HPLC retention times and absorption spectra [monitored by the rapid-scanning detector in the HPLC solvents (eluent A)] as lutein (band 1, minor component), *all-trans*-α-carotene [band 2, (λ_{max} = 446 nm)], mixture of *all-trans*-β-carotene (λ_{max} = 454 nm) and

15,15′-*cis*-β-carotene (λ_{max} = 450 nm) appearing as band 3, and a mixture of lutein bis(fatty acid esters) (band 4). Band 4 was further separated by HPLC (eluent A), and it was shown to consist of lutein dilaurate, lutein dimyristate, lutein myristate/palmitate (mixed diesters), and lutein dipalmitate.

Isolation and Characterization of Carotenoids in Acorn Squash. (*a*) *Carotenoids in a Raw/Fresh Saponified Extract.* A concentrated solution of a saponified extract of raw acorn squash (from 700 g of squash) was chromatographed on silica gel GF (Analtech, inc., Newark, DE) plates (20 × 20 cm, 1000-μm layer thickness) with 2.33/1 light petroleum ether/acetone as eluent. Four major bands were separated.

Band 1 (R_f 0.95) was identified from its HPLC retention times and UV/visible absorption spectrum as *all-trans*-β-carotene and 15,15′-*cis*-β-carotene.

Band 2 (R_f 0.45) was identified from its UV/visible and mass spectra as *all-trans*-lutein, which was shown by HPLC (eluent B) to contain minor quantities of its 13-cis or 13′-cis stereoisomers (neolutein A or A′).

Band 3 (R_f 0.35) was identified from its UV/visible absorption and mass spectra as 9-*cis*-violaxanthin. The mass spectrum (DCI, NH_3) of this compound contained a protonated molecular ion at *m/z* 601 [26%, $(M + H)^+$; $C_{40}H_{56}O_4$ requires 600.88] and 618 [23%, $(M + NH_4)^+$], as well as ions at *m/z* 585 [21%, $(M + H - CH_4)^+$] and 296 (100%). The UV/visible absorption spectrum of 9-*cis*-violaxanthin in various solvents had maxima at λ_{max} = 313.5, 328, 414, 436.5, 465.5 nm (ethanol); 313.5, 328, 412.5, 434.5, 464.5 nm (hexane); and 321, 334.5, 423.5, 447, 447 nm (benzene). Addition of a few drops of ethanolic hydrogen chloride (0.01 M) to an ethanolic solution of 9-*cis*-violaxanthin converted this compound to auroxanthin [λ_{max} = 379.5, 401, 426 nm (ethanol)].

Band 4 (R_f 0.30) was identified from its UV/visible absorption and mass spectra as *all-trans*-violaxanthin. The mass spectrum (DCI, NH_3) of this compound showed an ion at *m/z* 601 [100%, $(M + H)^+$; $C_{40}H_{56}O_4$ requires 600.88] and 618 [48%, $(M + NH_4^+)$]. The UV/visible absorption spectrum of this compound in various solvents had maxima at λ_{max} = 417, 441, 471 nm (ethanol); 416, 440, 470 nm (hexane); and 426, 451.5, 482 nm (benzene). Addition of a few drops of ethanolic hydrogen chloride (0.01 M) to an ethanolic solution of *all-trans*-violaxanthin converted this compound to auroxanthin [λ_{max} = 379.5, 401, 426 nm (ethanol)].

(*b*) *Carotenoids in a Cooked Saponified Extract.* A concentrated solution of a saponified extract of cooked acorn squash (from 700 g of cooked acorn squash) was chromatographed on silica gel GF (Analtech Inc., Newark DE) plates (20 × 20 cm, 100-μm layer thickness) employing 2.33/1 petroleum ether/acetone as eluent. In addition to bands identified as β-carotene (R_f 0.95), 9-*cis*-violaxanthin (R_f 0.35), and *all-trans*-violaxanthin (R_f 0.30) a band was isolated at R_f 0.45 that was shown by HPLC (eluent B) to consist of three components. The first two components, which were separated from the third component by semipreparative HPLC (eluent D), were tentatively identified from their UV/visible absorption spectra [monitored by photodiode array detector in the HPLC solvents (eluent D)] as a mixture of two di-*cis*-violaxanthin (λ_{max} = 430, 426 nm). Under chromatographic conditions employed, the HPLC peaks of di-*cis*-violaxanthins appeared as partially resolved peaks. The UV/visible absorption maxima of the mixture of isomeric violaxanthins in various solvents [hexane, λ_{max} = 405.5, 426, 451 nm; ethanol, λ_{max} = 405, 426.5, 452 nm] were consistent with a di-cis geometry for these compounds. Upon addition of a few drops of ethanolic hydrogen chloride to a solution of di-*cis*-violaxanthins in ethanol, both isomers were converted to auroxanthin. The third component was identified from its HPLC retention time and absorption spectrum as *all-trans*-lutein, which was accompanied by minor quantities of its 13-cis or 13′-cis stereoisomer.

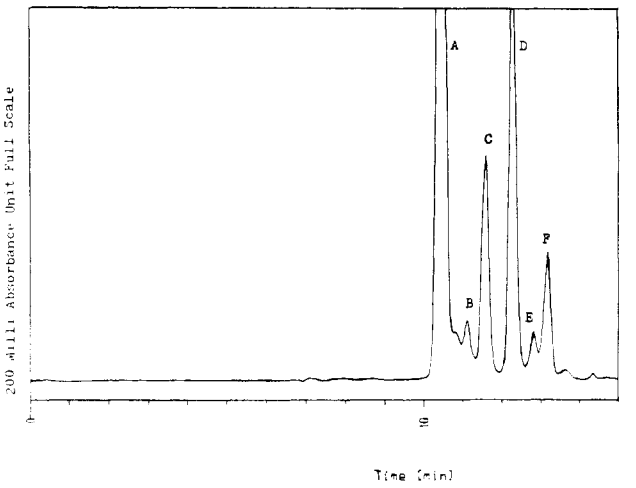

Figure 4. HPLC profile of a mixture of rearrangement and stereoisomeric products formed from thermally induced isomerization of *all-trans*-violaxanthin in refluxing methanol after 1 h. HPLC (eluent D) conditions described in the text. Key: A, *all-trans*-violaxanthin; B, 13-*cis*-violaxanthin; C, *all-trans*-luteoxanthin; D, 9-*cis*-violaxanthin (violeoxanthin); E, 9-*cis*-luteoxanthin; F, 15,15′-*cis*-violaxanthin.

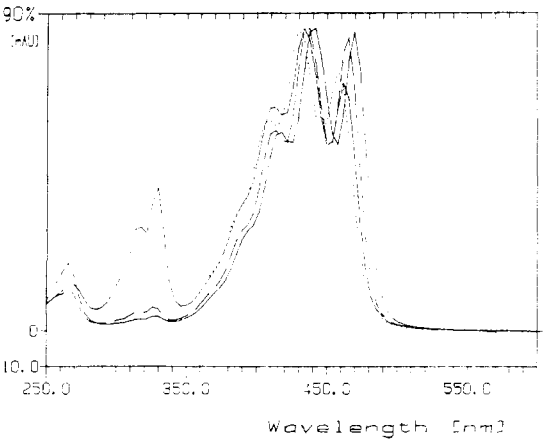

Figure 5. UV/visible absorption spectra of *all-trans*-violaxanthin [(—) λ_{max} = 442 nm], 13-*cis*-violaxanthin [(···) λ_{max} = 430 nm, cis peaks at 314 and 330 nm], 9-*cis*-violaxanthin [(- - -) λ_{max} = 438 nm, cis peaks at 314 and 326 nm], and 15,15′-*cis*-violaxanthin [(-·-) λ_{max} = 434 nm, cis peaks at 318 and 330 nm] in the HPLC solvents (eluent D) under conditions described in text.

Stereoisomerization of *all-trans*-Violaxanthin. A solution of *all-trans*-violaxanthin was refluxed in methanol under an atmosphere of nitrogen for 1 h. The resulting mixture was shown by HPLC (eluent D, semipreparative column) to consist of four major and two minor components (Figure 4). These components were separated by preparative HPLC (eluent D) and were tentatively identified from their UV/visible absorption spectra in the order of elution on a C-18 reversed-phase column as *all-trans*-violaxanthin (peak A), 13-*cis*-violaxanthin (peak B), *all-trans*-luteoxanthin (peak C), 9-*cis*-violaxanthin (peak D), 9-*cis*-luteoxanthin (peak E), and 15,15′-*cis*-violaxanthin (peak F). The UV/visible absorption spectra of the stereoisomerization products of *all-trans*-violaxanthin in the HPLC solvents (eluent D) are shown in Figures 5 and 6.

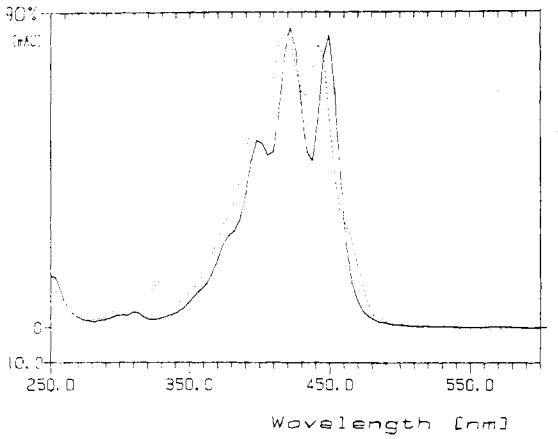

Figure 6. UV/visible absorption spectra of *all-trans*-luteoxanthin [(—) λ_{max} = 422 nm] and 9-*cis*-luteoxanthin [(···) λ_{max} = 418 nm, cis peaks at 314 and 330 nm] in the HPLC solvents (eluent D) under conditions described in the text.

Addition of a few drops of ethanolic hydrogen chloride to an ethanolic solution of each of these components resulted in the formation of auroxanthin. The HPLC retention times and the UV/visible absorption spectra of *all-trans*-violaxanthin, luteoxanthin, and 9-*cis*-violaxanthin were identical with those of the reference samples of these compounds isolated from the extract of raw and cooked acorn squash.

Stereoisomerization of *all-trans*-violaxanthin was similarly effected when a hexane solution of this compound was treated with a small crystal of iodine and the resulting mixture was kept at room temperature for 30 min.

Partial Synthesis of Fatty Acid Esters of Violaxanthin. A solution of *all-trans*-violaxanthin and 9-*cis*-violaxanthin (about 1/1) in benzene was treated with the appropriate fatty acid chloride in benzene in the presence of triethylamine, similar to the procedure described earlier for the preparation of the bis(fatty acid esters) of lutein. Violaxanthin dilaurate, dimyristate, and dipalmitate were prepared. In each case two products were formed that were identified from their UV/visible absorption spectra in the HPLC solvents (eluent B) as bis(fatty acid esters) of *all-trans*-violaxanthin (λ_{max} = 442 nm) and 9-*cis*-violaxanthin (λ_{max} = 438). Alternatively, violaxanthin bis(fatty acid esters) were also prepared by epoxidation of each of the bis(fatty acid esters) of zeaxanthin with *m*-chloroperbenzoic acid (MCPBA). Violaxanthin myristate/palmitate mixed diester was similarly prepared from epoxidation of zeaxanthin myristate/palmitate mixed diester.

RESULTS AND DISCUSSION

The HPLC separation and quantification of the predominant carotenoids and carotenol fatty acid esters in the extracts from varieties of squash have been described in Khachik and Beecher (1988). Owing to the low abundance of carotenoids the structural elucidation was in most cases based on the absorption and mass spectral data. In case of lutein monomyristate and lutein monopalmitate where sufficient levels of these compounds were accumulated by chromatography the structural elucidation was complimented by NMR spectroscopy. In the absence of NMR data the geometry of the cis carotenoids reported in this text cannot be established with certainty. However, an attempt has been made to tentatively identify these geometrical isomers. This has been assumed by exclusion of the sterically hindered cis isomers of carotenoids and evaluation of the intensity, presence, or absence of the cis peak in the UV/visible absorption spectra of the isolated fractions. Characterization of the major carotenoid components, isolated from the extracts of each squash variety, is discussed separately.

Structural Elucidation of Carotenoids and Carotenol Fatty Acid Esters in the Extracts from Baby Food Squash. (*a*) *Brand G (Grown in Asheville, NC).* The major carotenoids in this squash variety were divided into four classes of compounds. These, in the order of elution on C-18 reversed-phase TLC plates, were xanthophylls, carotenol mono(fatty acid esters), hydrocarbon carotenoids, and carotenol bis(fatty acid esters). The presence of carotenol esters in squash varieties was first established by chromatographic evaluation (HPLC) of the extracts from this vegetable before and after extraction, which demonstrated the conversion of the esters to the parent hydroxy carotenoids. Characterization of the components of each class of these compounds is described below.

Xanthophylls. Four major xanthophylls in this variety of baby food squash were identified as flavoxanthin (lutein 5,8-epoxide), lutein, 3-hydroxy-2′,3′-dehydro-β,ε-carotene, and 9-*cis*- or 9′-*cis*-β-cryptoxanthin.

Flavoxanthin was identified from its mass and absorption spectra. The mass spectrum (DCI) of this compound employing isobutane as reagent gas contained ions indicative of the expected molecular mass as well as ions due to loss of water and the loss of 80 mass units (C_6H_8) from the molecular parent ion. The loss of C_6H_8 from molecular parent ion involving nonconventional in-chain elimination is a typical fragmentation reaction of 5,8-epoxide of carotenoids (Vetter et al., 1971; Enzell and Wahlberg, 1980).

Lutein was shown to be the predominant xanthophyll in this variety of squash, and it was isolated from the extracts of this vegetable before and after saponification. Mass spectral analysis (DCI) of lutein employing ammonia and isobutane as reagent gas in both cases produced abundant adduct ions as well as ions resulting from the loss of water from the adduct ions.

3-Hydroxy-2′,3′-dehydro-β,ε-carotene was tentatively identified from its mass spectrum (DCI, ammonia as reagent gas), which produced both abundant ammonium adduct ions as well as ions due to subsequent dehydration from molecular adduct ions. The absorption spectrum of this compound in various solvents and the mixture of HPLC solvents (eluent A) resembled that of lutein. Since the mass spectrum of this compound showed the presence of only one hydroxyl group (loss of only one water molecule from the adduct ion) and the molecular mass corresponded to the loss of water from lutein, it appeared that the compound in question was one of the dehydration products of lutein. Three lutein dehydration products are possible: 3-hydroxy-2′,3′-dehydro-β,ε-carotene, 3′-hydroxy-2,3-dehydro-β,ε-carotene, 3′-hydroxy-3,4-dehydro-β,ε-carotene. The last compound contains a double bond in the β-ionone ring, which is in conjugation with the chromophoric system, and therefore its UV/visible absorption maxima in various solvents is expected to exhibit a bathochromic shift of about 10 nm from that of lutein. Since, the acid chloroform test (Petracek and Zechmeister, 1956) on the isolated compound from squash indicated that no allylic hydroxyl was present, this compound was tentatively identified as 3-hydroxy-2′,3′-dehydro-β,ε-carotene. Since the allylic dehydration of lutein catalyzed by trace of acids at the processing stage may be responsible for the formation of 3-hydroxy-2′,3′-dehydro-β,ε-carotene, the natural occurrence of this compound in this variety of squash remains uncertain.

9-*cis*- or 9′-*cis*-β-cryptoxanthin was identified from its mass spectrum (DCI, ammonia), which contained an

abundant ammonium adduct ion and an ion that indicated the loss of a single molecule of water from the adduct ion. The UV/visible absorption maxima of 9-*cis*- or 9′-*cis*-β-cryptoxanthin in various solvents showed a hypsochromic shift of 4 nm with respect to that of *all-trans*-β-cryptoxanthin, which is consistent with the small hypsochromic shift commonly observed for mono-cis carotenoids. The absence of a strong cis peak in the near-UV region in the absorption spectra of the mono-*cis*-β-cryptoxanthin in various solvents excluded the possibility of 13-cis, 13′-cis, and 15,15′-cis geometry in this compound. Therefore, with the exclusion of sterically hindered cis isomers (7-cis, 7′-cis, 11-cis, 11′-cis) a 9- or 9′-cis geometry for the isolated mono-*cis*-β-cryptoxanthin was assumed.

Carotenol Mono(fatty acid esters). The isolation of natural lutein monomyristate and lutein monopalmitate from the extract of baby food squash is the only available example of the occurrence of mono(fatty acid esters) of this compound in natural products. The identification of these compounds was based on mass spectral data, which in addition to ions indicative of the molecular mass contained ions due to loss of 92 (toluene) and 106 (xylene) mass units from the parent ion. These are typical of fragmentation reaction of carotenoids (Vetter et al., 1971). Saponification of the isolated lutein monomyristate and monopalmitate resulted in the formation of lutein. HPLC (eluent A) profile of the isolated lutein monoesters showed the presence of minor quantities of their corresponding mono-cis isomers, which appeared as tailing shoulder on their all-trans compound.

In an attempt to determine the site of the ester moiety in lutein monomyristate and monopalmitate, extensive NMR studies on several synthetic and model compounds were carried out. Incomplete esterification of lutein with myristoyl chloride gave, in addition to lutein dimyristate (20%) and unreacted lutein (20%), the two regioisomers of lutein monomyristates, which were separated by semipreparative HPLC and identified from their proton NMR spectra: β,ε-carotene-3-monol-3′-monol monomyristate [structure I, Figure 1; 13%] and β,ε-carotene-3-monol monomyristate-3′-monol [structure II, Figure 1; 37%]. The predominant esterification of the nonallylic hydroxyl group relative to the allylic hydroxyl group in lutein is probably due to the electron-withdrawing effect of the double bond in the α-ionone ring, which makes the allylic hydroxyl group in this ring slightly less reactive toward nucleophilic substitution than the nonallylic hydroxyl group in the β-ionone ring. The observed chemical shifts for two synthetic carotenoids, namely zeaxanthin didecanoate (structure II, Figure 1) and isozeaxanthin dipelargonate (structure IV, Figure 1), as well as the reported characteristic proton chemical shifts of some of the natural and synthetic carotenoids with end groups similar to that of the two regioisomers of lutein monomyristate enabled the structural elucidation of these compounds. From the chemical shifts of the two synthetic compounds (III and IV) shown in Figure 1, it appears that the methine protons adjacent to the fatty acid ester substituent in the allylic position (C-4 and C-4′ protons in compound IV, δ 5.25) are deshielded by about 0.2 ppm with respect to the nonallylic methine protons (C-3 and C-3′ protons in compound III, δ 5.05). This chemical shift difference is also in agreement with the reported chemical shifts for the allylic methine protons (C-4, C-4′) in isozeaxanthin diacetate (δ 5.26) and the nonallylic methine protons (C-3, C-3′) in zeaxanthin diacetate (δ 5.06) (Englert, 1982). The chemical shift of 5.35 ppm for the C-3′ methine proton in lutein monomyristate I versus the chemical shift of 5.06 ppm for the C-3 methine proton in lutein monomyristate II suggests that the ester moiety in the former is in the allylic position. In addition the methine proton adjacent to the hydroxy substituent in the allylic position (C-3′ proton in compound II, δ 4.30) is deshielded by about 0.21 ppm with respect to the methine proton in the nonallylic position (C-3 proton in compound I, δ 4.09), which is also in agreement with the chemical shift difference of these protons in carotenoids with similar end groups (Englert, 1982). The proton NMR spectrum of natural lutein monomyristate including signal assignment is shown in Figure 3. The NMR spectrum of natural lutein monopalmitate (data not shown) exhibits chemical shifts exactly identical with that of natural lutein monomyristate. From the chemical shift assignments it is clear that in both of the naturally occurring monoesters of lutein the site of the ester moiety is not in the allylic position. Further evidence for the regiochemistry of lutein monoesters was obtained from hydrochloric acid–chloroform test (Petracek and Zechmeister, 1956), which indicated that the allylic hydroxy group was present in the natural esters but absent in synthetic lutein monomyristate I.

The absolute configuration of the naturally occurring lutein mono- and bis(fatty acid esters) in the squash varieties reported in this text is not known with certainty. On the basis of NMR and circular dichroism (CD) data obtained on the lutein isolated from these squash products, the three centers of chirality in this compound were determined as 3*R*, 3′*R*, 6′*R*. Since as pointed out earlier [see Khachik and Beecher (1988)] carotenol fatty acid esters are most likely formed from free lutein in squash varieties, the absolute configuration of carotenol fatty acid esters would therefore be expected to be the same as that of the isolated lutein, that is 3*R*,3′*R*,6′*R*. This naturally occurring lutein (3*R*,3′*R*,6′*R*) is widely distributed not only in plants (Goodwin, 1980), but also in animals (Liaaen-Jensen, 1978). Among the eight possible stereoisomers of lutein (β,ε-carotene-3,3′-diol) with three centers of chirality, five of them have been synthesized by Mayer (1982) and two of them have recently been isolated from the integuments of marine fishes by Matsuno et al. (1986).

Hydrocarbon Carotenoids. all-trans-β-Carotene and its 15,15′-cis isomer were shown to be the only predominant hydrocarbon carotenoids in this variety of baby food squash (brand G).

Carotenol Bis(fatty acid esters). Carotenol bis(fatty acid esters) were identified by comparison of their HPLC retention times and absorption spectra with those of the reference samples of these compounds prepared by partial synthesis from lutein and the appropriate fatty acid chlorides. Lutein dilaurate, dimyristate, myristate/palmitate (mixed diesters), and dipalmitate were found to be the major carotenol diesters in this variety of baby food squash (brand G). These esters were shown by HPLC (eluent A) to be accompanied by their corresponding 13′-cis or 13-cis isomer (neo A′ or neo A). The tentative assignment of a 13-cis or 13′-cis geometry in these isomers was based on the presence of a strong cis peak at 334 nm in the UV/visible absorption spectrum of these compounds and the exclusion of the sterically hindered cis isomers. The UV/visible absorption spectra of *all-trans*-lutein dimyristate (λ_{max} = 446 nm) and neolutein dimyristate A or A′ (13- or 13′-*cis*-lutein dimyristate, λ_{max} = 442 nm, cis peak at 334 nm) in the HPLC solvents (eluent A) are shown in Figure 2.

(*b*) *Brand H (Grown in New Jersey)*. The predominant carotenoids in this variety of baby food squash were identified in an approach similar to that employed for

identification of the major squash carotenoids in brand G variety.

Structural Elucidation of Carotenoids and Carotenol Fatty Acid Esters in the Extracts from Raw and Cooked Acorn Squash. (*a*) *Raw Saponified Extract.* The major carotenoids in a saponified extract of raw acorn squash were identified from their mass and absorption spectra as *all-trans*-β-carotene and its 15,15′-cis isomer, *all-trans*-lutein and neolutein A or A′, violeoxanthin (9-*cis*-violaxanthin), and *all-trans*-violaxanthin. The absorption maxima of 9-*cis*-violaxanthin in various solvents showed a hypsochromic shift of about 4.5–5.5 nm from the absorption maxima of *all-trans*-violaxanthin. Both violaxanthin and violeoxanthin were converted to luteoxanthin and auroxanthin upon addition of a few drops of ethanolic hydrogen chloride (Strain, 1954; Eugster and Karrer, 1957; Tsukida and Zechmeister, 1958). The mass spectra (DCI, ammonia as reagent gas) of *all-trans*-violaxanthin and its 9-cis isomer were identical, and both showed a protonated molecular ion at m/z 601 $(M + H)^+$. The formation of an odd mass to charge value (m/z 601, M + H ion) in the DCI spectra of carotenoids employing ammonia as reagent gas has been related to an equilibrium reaction between carotenoids and ammonium ion (NH_4^+), which results in a proton transfer from NH_4^+ to carotenoids with regeneration of ammonia (Marty and Berset, 1986). This equilibrium is shifted toward the formation of the M + H ion as the proton affinity of the carotenoids is higher than that of ammonia. The mass spectra of violaxanthin and violeoxanthin employing $^{15}NH_3$ as reagent gas resulted in a shift in the peak at m/z 618 $(M + NH_4)^+$ to m/z 619 $(M + {}^{15}NH_4)^+$, while the ion at m/z 601 remained unchanged. Reexamination of these samples at reduced reagent gas pressure (0.3 vs 0.6 Torr) provided a spectrum in which the abundance of the ion at m/z 619 was attenuated. These data support the formation of the ion at m/z 601 by protonation or chemical reaction with the reagent gas.

(*b*) *Cooked Saponified Extract.* The major carotenoids isolated from a saponified extract of cooked acorn squash were similar to those isolated from the raw saponified extract described above; however two di-cis isomers of violaxanthin were shown to be present in the saponified extract from cooked acorn squash that were absent in the raw extract. These two di-cis isomers were not resolved by HPLC and appeared as overlapping peaks [see Khachik and Beecher (1988)]. The UV/visible absorption spectra of these di-cis isomers in the HPLC solvents (eluents B and D) had maxima at 430 and 426 nm, respectively. The hypsochromic shift of about 12–16 nm in the UV/visible absorption maxima of the compounds in question with respect to the absorption maxima of *all-trans*-violaxanthin [λ_{max} = 442 nm in the HPLC solvents (eluents B and D)] is consistent with a di-cis geometry for these compounds. The locations of the cis double bonds in di-*cis*-violaxanthins are not known. The conversion of these di-cis isomers of violaxanthin to auroxanthin was effected upon addition of a few drops of ethanolic hydrogen chloride to a solution of these compounds in ethanol. Minor quantities of *all-trans*-luteoxanthin (λ_{max} = 422 nm in the HPLC solvents) and auroxanthin (λ_{max} = 402 nm in the HPLC solvents) were also shown by HPLC (eluent B) to be present in the saponified extract from cooked acorn squash. Further evidence for the presence of 9-*cis*-violaxanthin in the extracts from raw and cooked acorn squash was obtained from thermal stereoisomerization of *all-trans*-violaxanthin.

Stereoisomerization of *all-trans*-Violaxanthin. A chromatographic profile of a solution of *all-trans*-violaxanthin after refluxing in methanol for 1 h is shown in Figure 4. The thermal and rearrangement products of this reaction were tentatively identified from their UV/visible absorption spectra monitored by the rapid-scanning detector in the HPLC solvents (eluent D): *all-trans*-violaxanthin (peak A, λ_{max} = 442 nm), 13-*cis*-violaxanthin (peak B, λ_{max} = 430 nm, cis peaks at 314 and 330 nm), *all-trans*-luteoxanthin (peak C, λ_{max} = 422 nm), 9-*cis*-violaxanthin (peak D, λ_{max} = 438 nm, cis peaks at 314 and 326 nm), 9-*cis*-luteoxanthin (peak E, λ_{max} = 418 nm, cis peaks at 314 and 330 nm), and 15,15′-*cis*-violaxanthin (peak F, λ_{max} = 434 nm, cis peaks at 318 and 330 nm). The UV/visible absorption spectra of these compounds are shown in Figures 5 and 6. The intensities of the cis peaks in stereoisomers of violaxanthin and luteoxanthin are particularly noticeable.

Carotenoids in the Unsaponified Extracts from Raw and Cooked Acorn Squash. The identification of these compounds was based on comparison of their HPLC retention times (eluent B) and absorption spectra with those of authentic samples of these compounds. Violaxanthin and violeoxanthin bis(fatty acid esters) were prepared by partial synthesis from the reaction of a 1/1 mixture of violaxanthin and violeoxanthin with the appropriate fatty acid chlorides. The extracts from raw acorn squash showed the presence of a mono(fatty acid ester) and its two possible 9- and 9′-cis isomers. The incomplete esterification of violaxanthin and violeoxanthin with myristoyl chloride in addition to violaxanthin and violeoxanthin dimyristate resulted in the formation of 9-*cis*- and 9′-*cis*-violaxanthin monomyristate and *all-trans*-violaxanthin monomyristate. The HPLC retention times and absorption spectra of these authentic samples were identical with those of the mono(fatty acid esters), which were present in the extracts from raw acorn squash. The absorption spectra of 9-*cis*-violaxanthin monomyristate (λ_{max} = 438 nm) and 9′-*cis*-violaxanthin monomyristate (λ_{max} = 438 nm) in the HPLC solvents (eluent B) showed a 4-nm hypsochromic shift from the absorption maxima of *all-trans*-violaxanthin monomyristate (λ_{max} = 442 nm), which is consistent with a small hypsochromic shift in the absorption maxima of mono-*cis*-carotenoids from those of their all-trans compounds. *all-trans*-Violaxanthin monomyristate and its 9′- and 9-cis isomers were shown to be absent in the extract from cooked acorn squash and therefore were assumed to have been destroyed as a result of the cooking process. The presence of lutein bis(fatty acid esters) in the raw and cooked extracts from acorn squash was similarly established.

ABBREVIATIONS

HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; MS, mass spectrometry.

ACKNOWLEDGMENT

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Registry No. I, 115095-05-1; II, 115095-04-0; III, 115095-07-3; *all-trans*-lutein, 127-40-2; *all-trans*-lutein monopalmitate, 14683-67-1; *all-trans*-violaxanthin, 126-29-4; 15,15′-*cis*-β-carotene, 19361-58-1; flavoxanthin, 512-29-8; 3-hydroxy-2′,3′-dehydro-β-ε-carotene, 115117-27-6; 9-*cis*-β-cryptoxanthin, 115223-35-3; 9′-*cis*-β-cryptoxanthin, 115223-25-1; *all-trans*-β-carotene, 7235-40-7; neolutein A, 32449-88-0; neolutein A′, 79464-33-8; myristoyl chloride, 112-64-1; *all-trans*-lutein dimyristate, 86853-02-3; pal-

mitoyl chloride, 112-67-4; *all-trans*-lutein dipalmitate, 547-17-1; β-ε-carotene-3-monol monomyristate-3′-monol monopalmitate, 115116-89-7; β-ε-carotene-3-monol monopalmitate-3′-monol monomyristate, 86826-05-3; *all-trans*-lutein didecanoate, 115095-06-2; *all-trans*-lutein dilaurate, 23852-66-6; *cis*-lutein monomyristate, 115182-30-4; *cis*-lutein monopalmitate, 115182-29-1; neolutein dimyristate A, 115223-32-0; neolutein dimyristate A′, 115223-56-8; neolutein myristate/palmitate A, 115266-14-3; neolutein myristate/palmitate A′, 115266-15-4; neolutein dipalmitate A, 115223-33-1; neolutein dipalmitate A′, 115223-34-2; *all-trans*-α-carotene, 432-70-2; 9-*cis*-violaxanthin, 26927-07-1; di-*cis*-violaxanthin, 115182-31-5; *all-trans*-luteoxanthin, 1912-50-1; 9-*cis*-luteoxanthin, 115182-32-6; *all-trans*-violaxanthin dilaurate, 49795-35-9; *all-trans*-violaxanthin dimyristate, 113531-83-2; *all-trans*-violaxanthin dipalmitate, 113531-84-3; *all-trans*-violaxanthin myristate/palmitate, 115095-03-9; *all-trans*-zeaxanthin myristate/palmitate, 115095-08-4; 13-*cis*-violaxanthin, 75715-58-1; 15,15′-*cis*-violaxanthin, 24620-97-1; 9-*cis*-violaxanthin monomyristate, 115182-24-6; 9′-*cis*-violaxanthin monomyristate, 115182-27-9.

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HPLC Identification of Phenols in Vidal Blanc Wine Using Electrochemical Detection

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High-performance liquid chromatography with electrochemical detection was used to identify eight nonflavanoid phenols in Vidal blanc wines. Identifications were made by comparing both the capacity factor (k') and electrochemical behavior of wine phenols to those exhibited by pure phenols in standard solutions. More components were identified by electrochemical detection than by photodiode array techniques.

The chemistry of wines made from grapes indigeneous to the northeastern United States has yet to be studied to the extent of those made from *Vitis vinifera* cultivars. Recently, several studies of the cultivar Vidal blanc have been initiated as part of an effort to improve the understanding of these wines and, ultimately, the ability of small, regional vintners to produce high-quality products. Vidal blanc is a French-American hybrid derived from the *V. vinifera* variety Ugni blanc (also known as St. Emilion in France and Trebbiano in Italy) and the *Vitis riparia* variety Seibel 4986 (Rayon d'Or). One early goal of this research was to find a method (or methods) of determining the phenolic composition of regional, French–American hybrid white wines. Because high-performance liquid chromatography (HPLC) has been used increasingly in recent years to both qualitatively and quantitatively study

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