Application of a C-45-β-Carotene as an Internal Standard for the Quantification of Carotenoids in Yellow/Orange Vegetables by Liquid Chromatography[1]

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The application of nonapreno-β-carotene as an internal standard in quantification of the hydrocarbon carotenoids extracted from carrots, sweet potato, pumpkin, and red palm oil has been thoroughly examined. Nonapreno-β-carotene is a C-45-β-carotene that has most of the requirements of an internal standard, and it can be commercially synthesized in high purity. An isocratic HPLC condition has been developed that separated lycopene, ζ-carotene, α-carotene, β-carotene, and the internal standard. The major carotenoid constituents found in the extracts from carrots, pumpkin, and red palm oil were all-trans-α-carotene, all-trans-β-carotene, and 15,15'-cis-β-carotene. Extracts from pumpkin and carrots also contained all-trans-ζ-carotene and a mono cis isomer of this compound. Lycopene was shown to be present in the extract from red palm oil, while the only hydrocarbon carotenoids detected in the extract from sweet potato were all-trans-β-carotene and its 15,15'-cis isomer. The quantitative evaluation of carotenoids in these vegetables by HPLC using the internal standard technique gave values for α- and β-carotene and lycopene similar to those obtained when these carotenes were employed as external standards.

We recently reported the application of decapreno-β-carotene as an internal standard for the quantification of the hydrocarbon carotenoids in carrots by high-performance liquid chromatography (HPLC) (Khachik and Beecher, 1985). The preparation of carotenoid samples isolated from food and natural products usually requires extensive extraction and workup procedures prior to the HPLC analysis. Since these procedures can be accompanied by various analytical errors, the use of internal standards for accurate quantification of carotenoids is essential. Although decapreno-β-carotene was shown to serve as a good internal standard, it has several disadvantages that may limit its application in the quantitative determination of hydrocarbon carotenoids. Owing to the length of the polyene chain in decapreno-β-carotene, the low solubility and unique chromatographic properties of this compound require specific HPLC conditions to elute this compound relatively close to α- and β-carotene. The much higher absorption maxima of decapreno-β-carotene (λ_{max} 502 nm) relative to those of α- and β-carotene (λ_{max} 446 and 454 nm, respectively) in the HPLC solvents [methanol (22%), acetonitrile (55%), methylene chloride (11.5%), hexane (11.5%)] require the detection of these compounds at two different wavelengths. In addition, on standing, solutions of decapreno-β-carotene in common HPLC solvents (methanol, hexane, methylene chloride, acetonitrile) undergo a much more rapid degradation than those of α- and β-carotene.

Although the synthesis of this compound is fairly straightforward, it involves a number of synthetic steps that may also limit its commercial availability. In an attempt to eliminate such problems, we recently reported the synthesis of yet another potentially useful internal standard, namely nonapreno-β-carotene. Preliminary evaluation of this compound as an internal standard for

the quantification of the hydrocarbon carotenoids found in carrots was shown to be very promising (Khachik and Beecher, 1986). This internal standard, which has one isoprene unit more than α- and β-carotene and one isoprene unit less than decapreno-β-carotene, can be readily synthesized in high purity from commercially available starting materials. In comparison with decapreno-β-carotene, the solubility and chromatographic behavior (i.e., adsorption affinity) of C-45-β-carotene on a C-18 reversed-phase column was found to be much closer to that of α- and β-carotene. This report presents a discussion of a thorough investigation of the application of this internal standard for the quantification of the hydrocarbon carotenoids from the extracts of several raw and cooked yellow/orange vegetables by HPLC. We have shown that this compound has most of the requirements of an internal standard for the quantitative determination of the hydrocarbon carotenoids. Finally, an attempt was made to investigate the effect of cooking and processing on the quantitative distribution of the hydrocarbon carotenoids in several yellow/orange vegetables.

EXPERIMENTAL SECTION

Apparatus. HPLC runs were monitored on a Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller and a Hewlett-Packard 1040A rapid-scanning UV/visible photodiode array detector. The absorption spectra of the carotenoids were monitored at 475 and 402 nm simultaneously. The data were stored and processed by means of a Hewlett-Packard 85-B computing system operated with a Hewlett-Packard Model-9121 disk drive and 7470 A plotter. The HP-85-B computing system with a built-in integration program was used to evaluate the peak area and peak height. The absorption spectra of the carotenoids were recorded between 200 and 600 nm as frequent as 1 scan/5 s (maximum scanning capability 1 scan/100 ms). Absorption spectra of isolated carotenoids in various solvents were recorded on a Beckman DU-7 UV/visible spectrophotometer. The mass spectrum of ζ-carotene was obtained on a Finnigan 4510 instrument (San Jose, CA) employing an ion source of 70 eV and a source block temperature of 150 °C.

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Column. Separations were performed on a stainless-steel (22 cm × 4.6 mm (i.d.)) Brownlee RP-18 (5-μm spherical particles) cartridge (Rainin Instrument Co.) protected in series with two Brownlee guard cartridges (each 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 lycopene and α- and β-carotene (Sigma, St. Louis, MO) were shown to be more than 96% pure as was determined by HPLC and molar absorptivity measurements in hexane and ethanol; these samples were used without further purification. 15,15'-cis-β-Carotene was donated by Hoffmann-La Roche, Basel, Switzerland. ζ-Carotene was isolated from the extract of canned pumpkin by preparative TLC and HPLC (details described in text). C-45-β-Carotene was synthesized in large quantities in our laboratory according to the procedure described in an earlier publication (Khachik and Beecher, 1986). HPLC-grade solvents, methanol, acetonitrile, and methylene chloride (Fisher Scientific, Pittsubrgh, PA), were used without further purification. Tetrahydrofuran was stabilized with butylated hydroxytoluene. Samples of a new hybrid of carrots, namely A PLUS, were donated by Dr. C. Peterson (USDA—ARS, Department of Horticulture, University of Wisconsin). Fresh sweet potato was purchased from local supermarkets on the day of the analysis. In addition, popular brands of canned carrots and sweet potato were selected. The same batches of fresh and canned pumpkin grown and processed by a food processing company (Libby, McNeill & Libby, Chicago, IL) were donated for the present study. A sample of red palm oil was obtained by scientific personnel of ORSTOM (Overseas Research Organization, National Center for Scientific Research, Republic of France) from the central market, Dakkar, Senegal.

Chromatographic Procedure. An isocratic system of methanol (22%), acetonitrile (55%), and methylene chloride (23%) and a column flow rate of 1 mL/min effected the HPLC separation of lycopene, ζ-carotene, α-carotene, β-carotene, and C-45-β-carotene (internal standard) in 22 min. Chromatographic runs were monitored at 475 nm for the detection of lycopene, α- and β-carotene, and C-45-β-carotene and at 402 nm for the detection of ζ-carotene. Scans of the absorption spectra of the carotenoids employing the photodiode array detector provided about 8–10 data points on each of the peaks as they were eluted from the HPLC column.

Preparation of the Vegetables for Extraction. The inedible parts of the vegetables were removed, large pieces were reduced in size, and the vegetables were washed with deionized water and drained. Vegetables to be analyzed cooked were heated covered, in a microwave oven (Amana Model RR-10A) for 5–6 min without added water. All the vegetable samples were homogenized in a food processor prior to extraction. Red palm oil and canned pumpkin were each mixed well and sampled directly from their containers for extraction. The percentage of voltatiles was determined by a microwave moisture analyzer (CEM Corp., Model MP), which was operated at 100% power for 3–4 min to determine the weight loss due to evaporation. An average of the percent volatiles from triplicate measurements for each vegetable was adopted.

Extraction. A known amount of a solution of the internal standard prepared from 62 mg of nonapreno-β-carotene in 250 mL of the HPLC solvents [methanol (22%), acetonitrile (55%), methylene chloride (23%)] was added to a sample of each vegetable, with anhydrous Na_2SO_4 (200% of the weight of the vegetable) and mag-

Table I. Weight of Vegetable Extracted, Weight of Internal Standard Added, and the Final Volume of the Extracts

entry	vegetable (wt, g)	nonapreno-β-carotene (int std), mg	final vol extr, mL
1	carrots		
	A+ hybrid raw (10)	3.72	50
	A+ hybrid cooked (10)	1.98	50
	canned (20)	2.48	50
2	sweet potato		
	raw (25)	2.48	50
	cooked (25)	2.48	50
	canned (25)	2.48	50
3	pumpkin		
	raw (62)	3.72	100
	canned (21)	2.48	50
4	red palm oil (16)	9.92	200

nesium carbonate (10% of the weight of the vegetable) contained in a Waring Blendor (Table I). The resulting mixture was extracted with about 150 mL of tetrahydrofuran at a moderate speed for 5 min. The extract was filtered in vacuo, 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 filtrate was partitioned into petroleum ether (150 mL) and water (150 mL). The water layer was washed with petroleum ether several times, and the resulting organic layers were combined, dried over Na_2SO_4, and evaporated to dryness. The residue was filtered through a 0.45-μm disposable filter assembly (American Scientific Products, McGaw Park, IL), and it was transferred to an appropriate volumetric flask with the HPLC solvents (same as above). A quantitative description of the weight of the internal standard added to each of the vegetables and the final volume of the extracts is presented in Table I. Samples were injected (20 μL) in triplicate for the HPLC analysis. Similar results were obtained when the addition of sodium sulfate and magnesium carbonate at the beginning of the extraction was eliminated. The vegetables were also extracted without the added internal standard, and the HPLC profile of the extracts were evaluated to determine the presence of new carotenoid peaks that may interfere with that of the internal standard.

Saponification. In cases where saponification of the vegetables was employed to evaluate the presence of carotenol esters, ethereal solutions of the extracts were treated with methanolic potassium hydroxide (30%) under an atmosphere of nitrogen at room temperature for 3 h. The solution was partitioned into a saturated solution of sodium chloride and petroleum ether, and the organic layer was removed. The aqueous layer was washed with ether, and the organic layers were combined, washed several times with water, and dried over sodium sulfate. The solvent was evaporated, and the residue was dissolved in the HPLC solvents for chromatographic analyses.

Synthesis of Nonapreno-β-carotene. Nonapreno-β-carotene (V) was synthesized in good yield (70%) from condensation of the Wittig salt of vinyl-β-ionol (III) with β-apo-8'-carotenal (IV) according to our reported procedure (Khachik and Beecher, 1986) as shown in Figure 1. Vinyl-β-ionol (II) was prepared from the Grignard reaction between vinylmagnesium bromide and β-ionone (I) in 90% yield. Recrystallization of the crude product from methanol and methylene chloride was shown by HPLC [methanol (25%), acetonitrile (55%), methylene chloride (20%)] to give nonapreno-β-carotene in high purity. The absorption spectrum of the C-45-β-carotene in the HPLC solvents had a maximum at 478 nm. The purity of the

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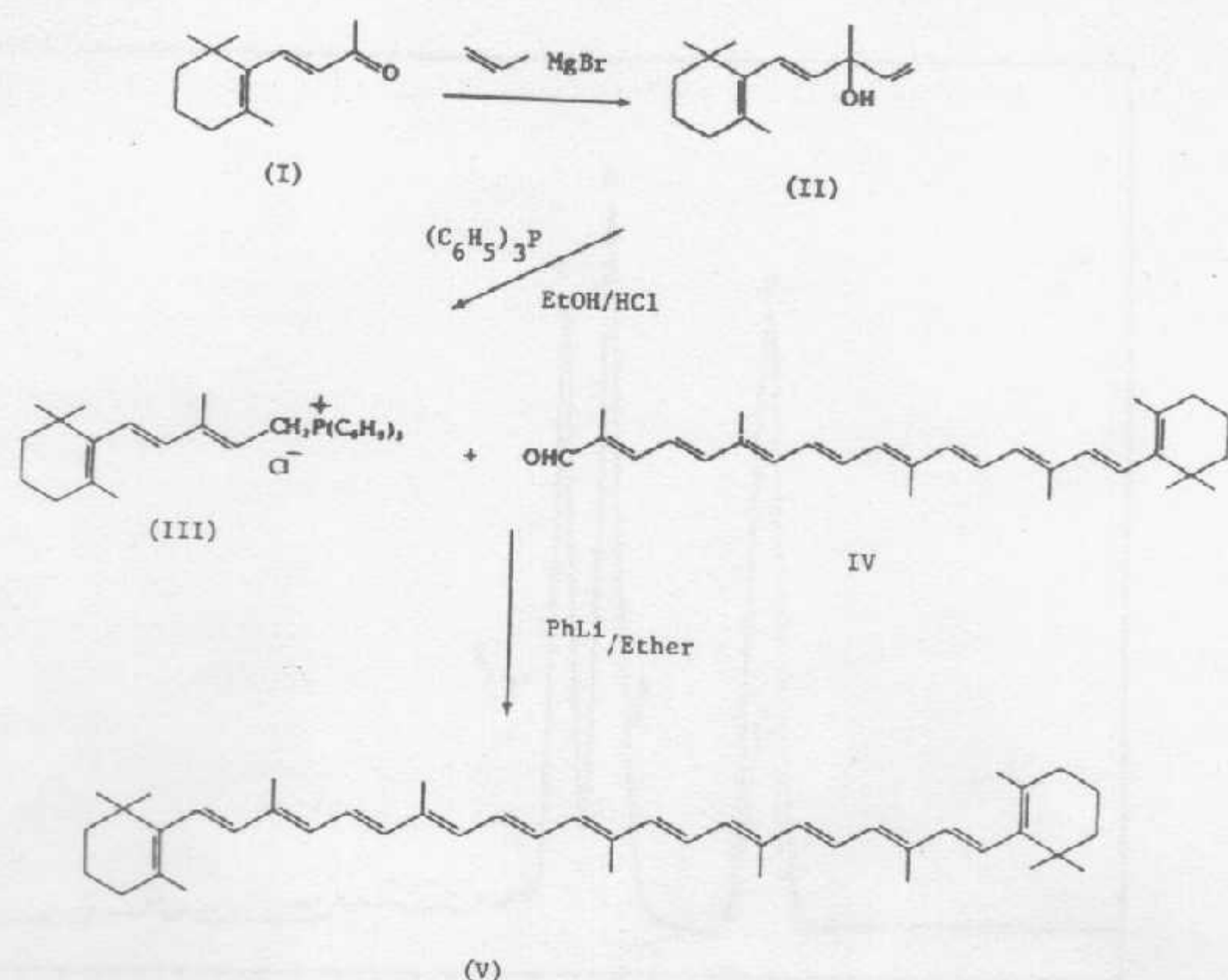

Figure 1. Chemical synthesis of nonapreno-β-carotene (internal standard).

Figure 2. UV/vis light absorption spectra in the HPLC solvent system described in the text: all-trans-α-carotene (—) λ_{max} 446 nm; all-trans-β-carotene (···) λ_{max} 454 nm; 15,15'-cis-β-carotene (---) λ_{max} 450 nm; all-trans-nonapreno-β-carotene (-·-) λ_{max} 478 nm.

internal standard was further ascertained by the evaluation of at least 10 absorption spectra of this compound monitored by the rapid-scanning detector. In all cases the absorption maximum remained at 478 nm, and all spectra were superimposable. The internal standard was also shown to be pure by plotting the peak ratios at various wavelengths.

Qualitative Identification of the Carotenoids. Lycopene and all-trans-α- and β-carotene were identified by comparison of their chromatographic retention times as well as their absorption spectra with those of the reference samples. Although the HPLC peak of 15,15'-cis-β-carotene was not resolved and appeared as a tailing shoulder on the HPLC peak of all-trans-β-carotene, as isomeric carotenes (bandwidth of about 2 min were coeluted from the HPLC column as many as 20 absorption spectra were collected between 200 and 600 nm by the rapid-scanning photodiode array detector clearly indicating the presence of the central cis isomer of β-carotene. This was particularly noticeable due to the presence of a strong cis peak in the near-UV region at 342 nm, in several of the absorption spectra of 15,15'-cis-β-carotene, which were collected and monitored by the photodiode array detector in the HPLC solvents. This band is characteristic of the central cis isomers of carotenoids (Vetter et al., 1971). The chromatographic retention time and the absorption spectrum of 15,15'-cis-β-carotene were also identical with that of an authentic sample of this compound donated by Hoffman La Roche. The UV/visible absorption spectra of all-trans-α-carotene (λ_{max} 446 nm), all-trans-β-carotene (λ_{max} 454 nm), 15,15'-cis-β-carotene (λ_{max} 450 nm), and nonapreno-β-carotene (λ_{max} 478 nm) in the HPLC solvents are shown in Figure 2. The UV/visible absorption spectrum of lycopene in the HPLC solvents had a maximum at 470 nm. Recently a method for the detection of cis–trans carotene isomers by two-dimensional thin-layer and high-performance liquid chromatography has been reported by Schwartz and Patroni-Killam (1985).

Isolation and Identification of ζ-Carotene. A partially pure sample of ζ-carotene was isolated from canned pumpkin extract by semipreparative TLC [methanol (22%), acetonitrile (55%), methylene chloride (23%)] on C-18 reversed-phase plates (20 × 20 cm, layer thickness 200 μm; Whatman Chemical Separation Inc.). This sample, which was contaminated with minor quantities of α- and β-carotene, was further purified by semipreparative HPLC [methanol (20%), acetonitrile (50%), methylene chloride (30%); flow rate 9.0 mL/min] on a stainless-steel
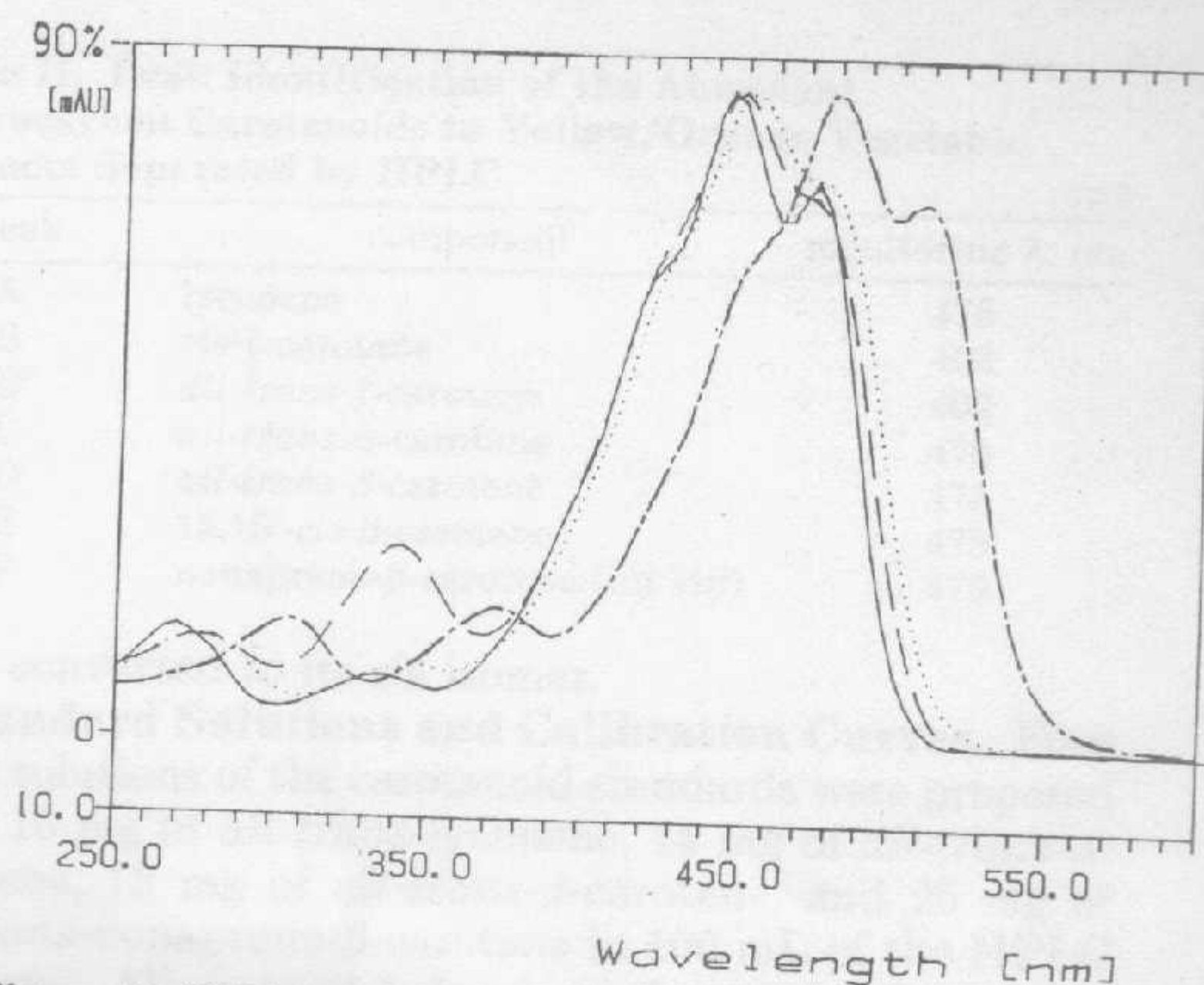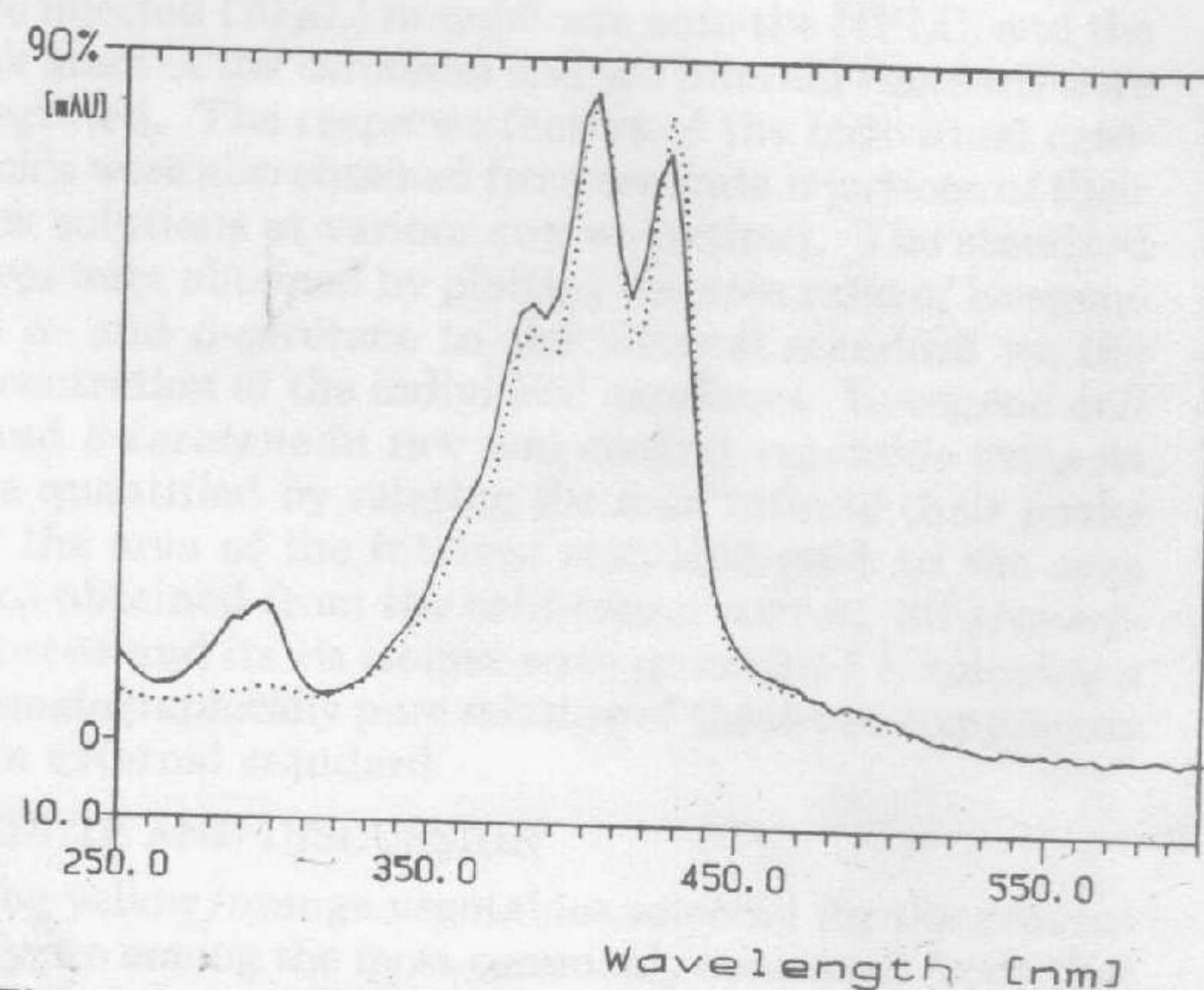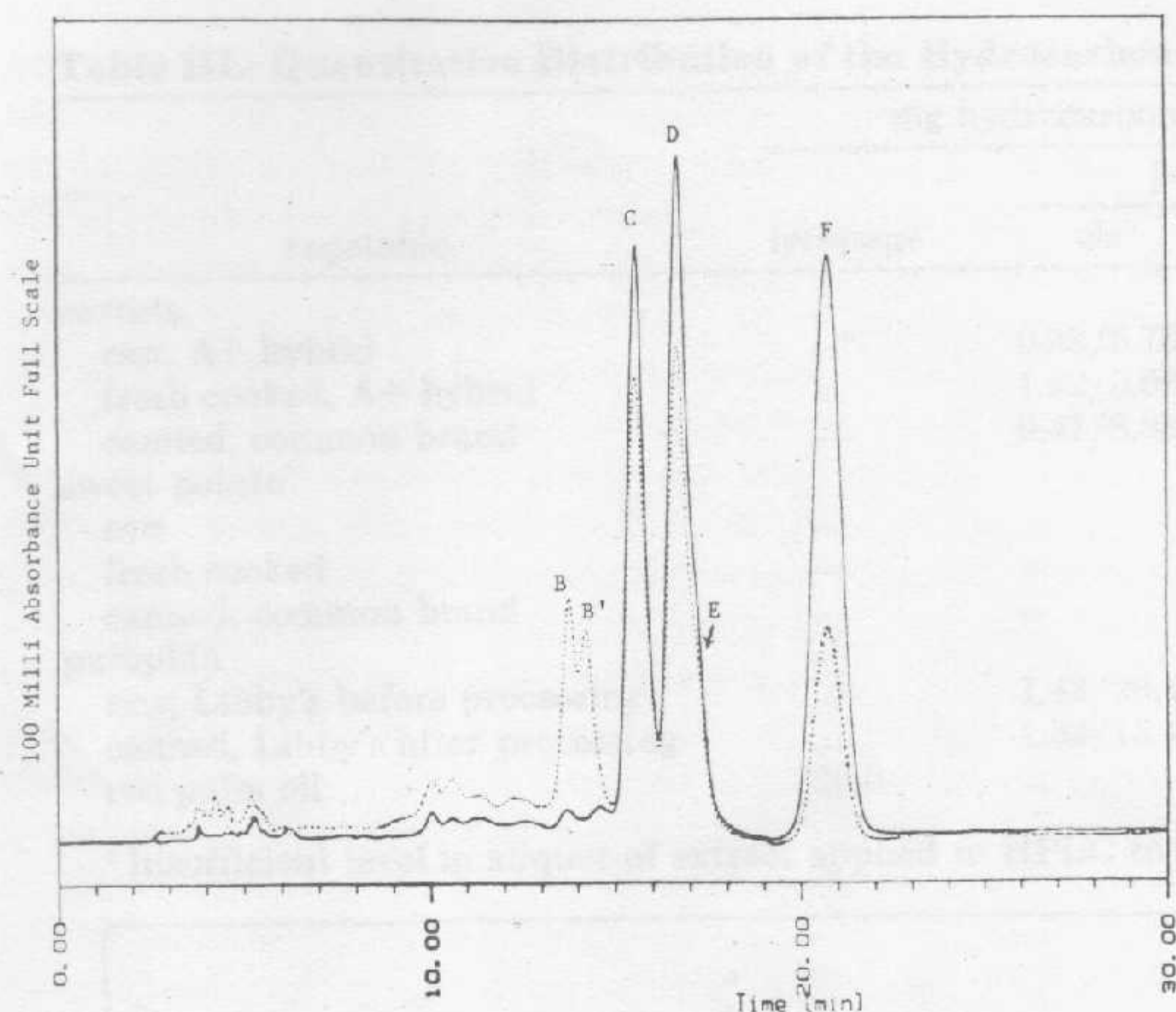

Figure 3. UV/vis light absorption spectra in the HPLC solvent system described in the text: cis-ζ-carotene (—) λ_{max} 402 nm; all-trans-ζ-carotene (···) λ_{max} 402 nm.

(35 cm × 2.5 cm (i.d.)) C-18 reversed-phase (25-μm spherical particles) column (Separation Technology, Wakefield, RI). The preparative HPLC column was protected with a guard column (2.5 cm × 2.5 cm (i.d.)) packed with C-18 reversed-phase spherical particles (25 μm). Evaluation of the chromatographically pure sample of ζ-carotene by HPLC (analytical column, conditions described in text) showed the presence of two components identified from their UV/visible absorption spectra in the HPLC solvents (Figure 3) as a mono cis isomer of ζ-carotene (λ_{max} 402 nm) and all-trans-ζ-carotene (λ_{max} 402 nm). Attempts to achieve base-line HPLC separation between cis-ζ-carotene (peak B, Figure 4) and its all-trans isomer (peak B', Figure 4), which were present in the extract from canned pumpkin (Figure 4), under various chromatographic conditions were unsuccessful. The location of the cis double bond in cis-ζ-carotene is not known; however, with the exclusion of the sterically hindered 11-cis isomer and the presence of fairly intense bands in the UV/visible absorption spectrum of this compound at 287 (inflection) and 298 nm (Figure 3), a 13-cis or 15,15'-cis geometry for this isomer is more likely. all-trans-ζ-Carotene also showed a well-defined UV/visible absorption spectrum in comparison with its cis isomer (Figure 3). The UV/visible

Figure 4. HPLC profiles of canned pumpkin extract containing nonapreno-β-carotene monitored at 402 nm (···) and 475 nm (—). Chromatographic conditions and peak identification are described in the text.

Table II. Peak Identification of the Abundant Hydrocarbon Carotenoids in Yellow/Orange Vegetable Extracts Separated by HPLC

peak	component	monitoring λ, nm
A	lycopene	475
B	cis-ζ-carotene	402
B'	all-trans-ζ-carotene	402
C	all-trans-α-carotene	475
D	all-trans-β-carotene	475
E	15,15'-cis-β-carotene	475
F	nonapreno-β-carotene (int std)	475

have converted to its cis isomer.

Standard Solutions and Calibration Curves. Four stock solutions of the carotenoid standards were prepared from 10 mg of *all-trans*-lycopene, 14 mg of *all-trans*-α-carotene, 12 mg of *all-trans*-β-carotene, and 25 mg of *all-trans*-nonapreno-β-carotene in 100 mL of the HPLC solvents. Aliquots of 1–5 mL of the stock solutions of lycopene and α- and β-carotene were mixed with a constant volume of the stock solution of the internal standard (1.5 mL) into separate 10-mL volumetric flasks, and the flasks were brought to volume with the HPLC solvents. Samples were injected (20 μL) in duplicate onto the HPLC, and the peak areas of the carotenes and the internal standard were integrated. The response factors of the individual carotenoids were also obtained from separate injections of their stock solutions at various concentrations. The standard curves were obtained by plotting the area ratio of lycopene and α- and β-carotene to the internal standard vs. the concentration of the individual carotenes. Lycopene and α- and β-carotene in raw and cooked vegetable extracts were quantified by relating the area ratio of their peaks and the area of the internal standard peak to the area ratios obtained from the calibration curves. *all-trans*-ζ-Carotene and its cis isomer were quantified employing a chromatographically pure mixture of these two compounds as an external standard.

RESULTS AND DISCUSSION

The yellow/orange vegetables selected for the present study are among the most commonly consumed foods that are known to contain high levels of hydrocarbon carotenoids. Red palm oil was also of particular interest owing to its large consumption in the third-work countries as a source of vitamin A.

The abundant hydrocarbon carotenoids found in the yellow/orange vegetables studied in this report and their corresponding HPLC peaks, in the order of chromatographic elution on a C-18 reversed-phase column, are shown in Table II. A typical chromatographic profiles of an extract from canned pumpkin containing nonapreno-β-carotene (internal standard) is shown in Figure 4. An isocratic mixture of methanol, acetonitrile, and methylene chloride has been employed to separate *cis*-ζ-carotene (peak B), and its all-trans compound (peak B'), *all-trans*-α-carotene (peak C), *all-trans*-β-carotene (peak D), and 15,15'-*cis*-β-carotene (peak E, appearing as a shoulder on the *all-trans*-β-carotene peak) from the internal standard (peak F). The chromatographic profiles of the extract from carrots (raw, cooked, canned) are similar to that of pumpkin; however, *all-trans*-ζ-carotene and its mono cis isomer are present at a much lower concentration. The chromatogram of the extract from red palm oil (Figure 5) in addition to α- and β-carotene shows the presence of lycopene (peak A), while no significant amount of ζ-carotene was detected. The only hydrocarbon carotenoids found in the extracts of sweet potato were shown by HPLC (Figure 6) to consist of *all-trans*-β-carotene (peak D) and its 15,15'-cis isomer (peak E). The presence of lycopene

absorption maxima of a mixture of *all-trans*-ζ-carotene and its cis isomer in ethanol (λ_{max} 380, 400.5, 425 nm), hexane (λ_{max} 379, 399.5, 424.5 nm), petroleum ether (λ_{max} 377.5, 398.5, 423 nm), acetone (λ_{max} 383, 404.5, 429.5 nm), and benzene (λ_{max} 387, 409, 434.5 nm) were consistent with the chromophore involved and the literature values of ζ-carotene (Ritter and Purcell, 1981). The mass spectrum of a mixture of *all-trans*-ζ-carotene and its cis isomer (low-melting oil) showed a weak parent ion peak at m/z 540 ($C_{40}H_{60}$ requires 540.914) as well as peaks at m/z 459 (M − 81), 403 (M − 137), and 69. Apart from the absence of a peak at m/z 471 (M − 69) and the presence of a peak at m/z 459 (M − 81), this fragmentation pattern is consistent with the fragmentation reactions of ζ-carotene reported in the literature (Davies, 1970; Enzell and Wahlberg, 1980). In the initial structural identification of *all-trans*-ζ-carotene and its cis isomer, it was considered that the compound identified as *cis*-ζ-carotene may be the asymmetric ζ-carotene (7,8,11,12-tetrahydrolycopene). This unsymmetrical ζ-carotene has been isolated by Davies (1970) from *Rhodospirillum rubrum* and has been suggested to be involved in biosynthetic pathway of carotene cyclization in fungi (Davies, 1970; Davies and Rees, 1973). UV/visible absorption maxima of a mixture of *all-trans*-ζ-carotene and the compound identified as *cis*-ζ-carotene is consistent with the absorption maxima of the stereoisomeric mixture of symmetrical ζ-carotenes [petroleum ether, λ_{max} 285, 296 (inflection), 377, 398, 423 nm], which has been reported (Davies, 1970) to result from iodine-induced photoisomerization of symmetrical *all-trans*-ζ-carotene isolated from carrot root. On the other hand, the UV/visible absorption maxima of an stereoisomeric mixture of the asymmetric ζ-carotenes [petroleum ether, λ_{max} 285, 296, 372, 391, 415 nm (Davies, 1970)], is not consistent with the absorption maxima of the isomeric mixture of ζ-carotenes isolated from pumpkin in the present study. Further support for the presence of an isomeric mixture of ζ-carotene in the extract from pumpkin was obtained from iodine-induced photoisomerization of the mixture, which altered the cis to trans ratio of the ζ-carotenes. When a stirring solution of *all-trans*-ζ-carotene (49%) and *cis*-ζ-carotene (51%) in hexane was exposed to indirect sunlight in the presence of a small crystal of iodine at room temperature for 1 h, 10% of the *all-trans*-ζ-carotene was shown by HPLC to

Table III. Quantitative Distribution of the Hydrocarbon Carotenoids in Yellow/Orange Vegetables

| | | mg hydrocarbon carotenoid/100 g edible food (wet wt/dry wt) | | | | mg total carotene/100 | |
| | | ζ-carotene | | | β-carotene + | | |
vegetable	lycopene	cis	all-trans	α-carotene	15,15'-cis isomer	wet wt	dry wt
carrots							
raw, A+ hybrid	...a	0.98/5.76	0.69/4.06	10.65/62.65	18.25/107.35	30.57	179.82
fresh cooked, A+ hybrid	...	1.22/5.08	0.82/3.42	15.00/62.50	25.65/106.87	42.69	177.87
canned, common brand	...	0.41/6.83	0.29/4.83	2.80/46.67	4.76/79.33	8.26	137.66
sweet potato							
raw		...	...		16.00/51.61	16.00	51.61
fresh cooked		...	...		15.16/47.38	15.16	47.38
canned, common brand		...	...		16.00/53.33	16.00	53.33
pumpkin							
raw, Libby's before processing		1.43/20.4	1.31/18.71	5.50/78.6	8.00/114.3	16.24	232.01
canned, Libby's after processing		1.34/13.4	1.22/12.2	7.42/74.2	10.82/108.2	20.8	208.0
red palm oil	20.0		...	66.88	120.5	187.4	

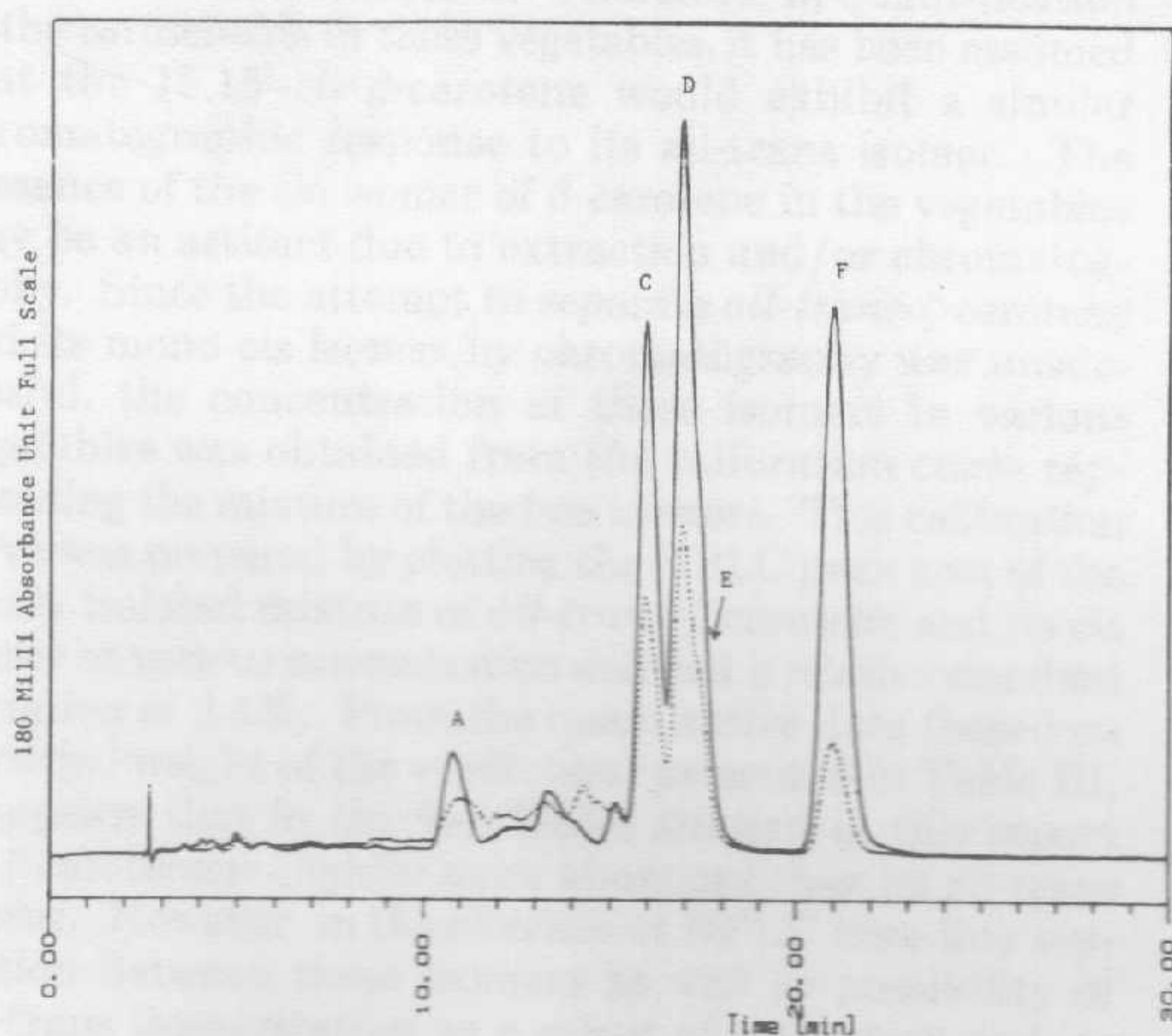a Insufficient level in aliquot of extract applied to HPLC to permit quantification.

Figure 5. HPLC profile of red palm oil extract containing nonapreno-β-carotene monitored at 402 nm (···) and 475 nm (—). Chromatographic conditions and peak identification are described in the text.
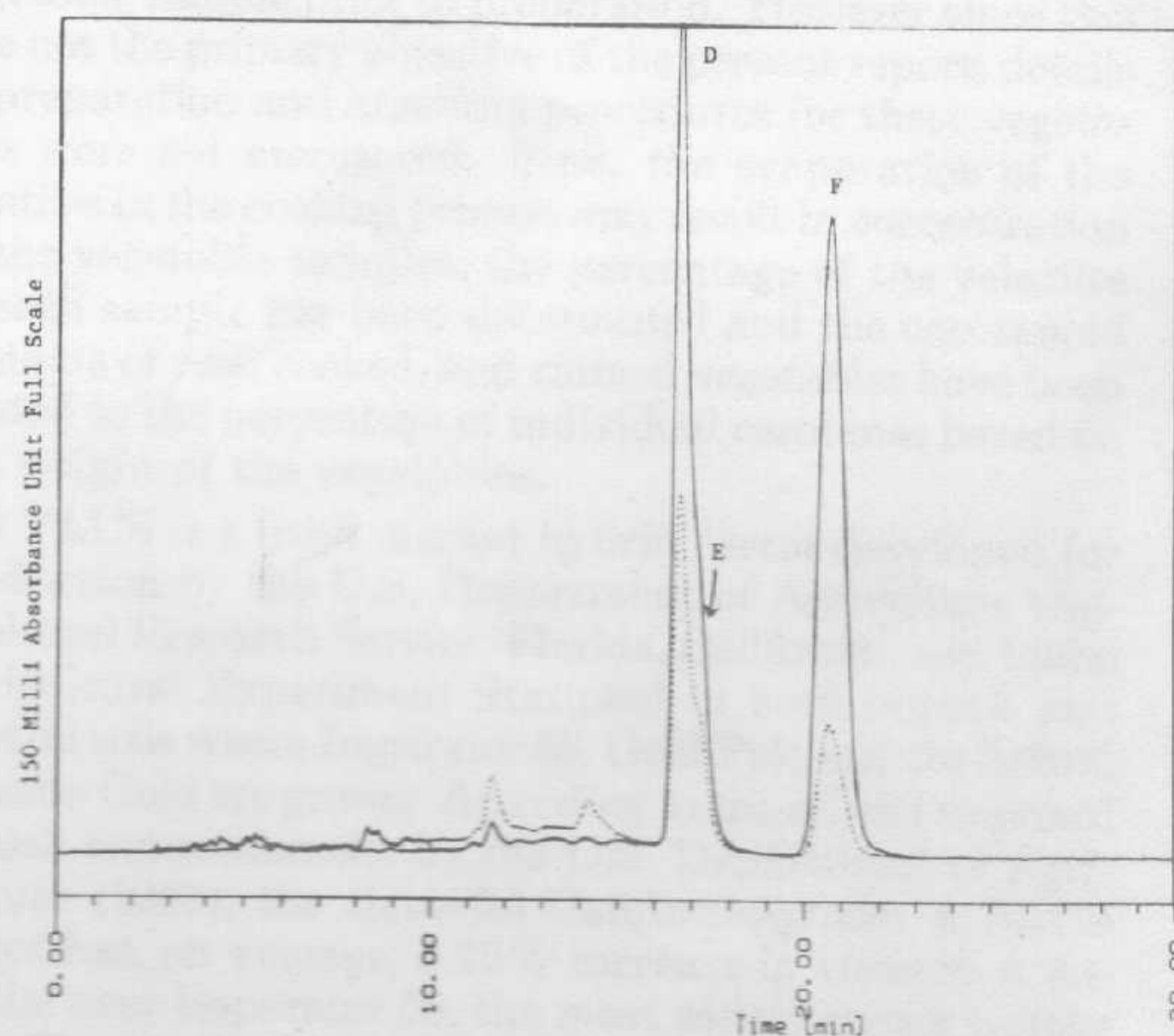

Figure 6. HPLC profile of cooked sweet potato extract containing nonapreno-β-carotene monitored at 402 nm (···) and 475 nm (—). Chromatographic conditions and peak identification are described in the text.

and ζ-carotene (a precursor of neurosporene) in these vegetables is not surprising since the photosynthetic production of β-carotene has been associated with the cyclization of these carotenes (Davies, 1970; Davies and Rees, 1973). Evaluation of the chromatographic profiles of the vegetable extracts without added internal standard indicates that no other carotenoids peak appears that may interfere with the nonapreno-β-carotene peak. Saponification of the extracts from the yellow/orange vegetables studied in this report did not alter the general chromatographic profiles of these vegetables, and no carotenol esters were found to be present. For most of the other fruits and vegetables studied, particularly the green vegetables, the carotenoids that elute last on a C-18 reversed-phase column are all-trans-β-carotene and its 15,15'-cis isomer (Khachik et al., 1986). Therefore, the application of nonapreno-β-carotene as an internal standard for the quantification of the hydrocarbon carotenoids is not limited to the vegetables studied in this report, and it can be extended to other fruits and vegetables. Preliminary evaluation of nonapreno-β-carotene as an internal standard for the determination of α- and β-carotene in human serum has also shown to be promising (Driskell, 1986). The most recent separation and quantification of carotenoids in fruits and vegetables by HPLC employing both normal- and reversed-phase columns have been reported by Bushway (1985) and by Quackenbush and Smallidge (1986).

The recovery studies on nonapreno-β-carotene obtained from two consecutive extractions on several of the raw, cooked, and canned vegetables indicated that the solubility and chromatographic behavior of this compound is very similar to that of α- and β-carotene, and more than 98% of this internal standard was recovered after extraction. These results were based on the weight of the internal standard before and after extraction as determined by the HPLC peak area of the internal standard. In the extraction and chromatographic analysis of red palm oil, where much higher concentrations of carotenoids and the internal standard were involved, the recovery of nonapreno-β-carotene was 98%. Evaluation of the HPLC response factors of solutions of nonapreno-β-carotene over long periods of time indicated that this compound is quite stable in the mixture of the HPLC solvents (acetonitrile, methanol, methylene chloride) and can be stored in solutions at low temperatures (−20 °C) for as long as 1 month. In crystalline form in an evacuated container in dark and at −20 °C, nonapreno-β-carotene has been shown to be stable for as long as 4 months.

Quantitative Determination of the Hydrocarbon Carotenoids. The quantitative distribution of the hydrocarbon carotenoids in the extract of several raw, cooked, and canned vegetables is shown in Table III. These data were generated from two consecutive extractions from one homogeneous batch of sample; therefore, these data are

Quantification of Carotenoids

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not necessarily representative of the levels of these carotenes in the products consumed nationwide. The concentrations were determined by relating the ratio of the HPLC peak area of the individual hydrocarbon carotenoids and the internal standard to the calibration curves of the carotenes at various concentrations. Only an average value of the two extractions has been presented. The calibration curves obtained from a plot of the area ratios of lycopene and α- and β-carotene peaks to that of the internal standard against the concentrations of these carotenes gave good linearity over a wide range of concentration and had a relative standard deviation of 2.0% for lycopene, 3.2% for α-carotene, and 3.1% for β-carotene. Since 15,15′-cis-β-carotene was not resolved and it appeared as a tailing shoulder on all-trans-β-carotene peak, the area corresponding to this cis isomer was included in the integration of the all-trans-β-carotene. Therefore, in quantification of the carotenoids in these vegetables, it has been assumed that the 15,15′-cis-β-carotene would exhibit a similar chromatographic response to its all-trans isomer. The presence of the cis isomer of β-carotene in the vegetables may be an artifact due to extraction and/or chromatography. Since the attempt to separate all-trans-ζ-carotene and its mono cis isomer by chromatography was unsuccessful, the concentration of these isomers in various vegetables was obtained from the calibration curve representing the mixture of the two isomers. This calibration curve was prepared by plotting the HPLC peak area of the purely isolated mixture of all-trans-ζ-carotene and its cis isomer at various concentration and had a relative standard deviation of 3.4%. From the quantitative data (based on the dried weight of the vegetables) presented in Table III, it appears that in the vegetables studied in this report cis-ζ-carotene is slightly more abundant than its all-trans isomer. However, in the absence of HPLC base-line separation between these isomers as well as possibility of cis–trans isomerization as a result of extraction and/or chromatography, the relative natural abundance of these isomers remains uncertain. The quantitative data for the hydrocarbon carotenoids employing nonapreno-β-carotene as an internal standard are in good agreement with the values obtained for these carotenes based only on the response factor of lycopene and α- and β-carotene standard solutions (data not shown).

Our total carotene content of the yellow/orange vegetables presented in this report is higher than the levels of the total carotene content of the same vegetables reported in the food tables (Souci et al., 1981; Haytowitz and Matthews, 1984). The discrepancy between these data not only may be related to the varietal differences among these vegetables but also reflects in the methods of analyses by which these numbers have been generated. In one food table (Souci et al., 1981) the total carotene contents of the vegetables have been determined by colorimetric methods, which in comparison to HPLC is not as accurate, especially if the food extracts contain hydrocarbon carotenoids other than α- and β-carotene. In another current food composition table (Haytowitz and Matthews, 1984) the total carotene contents of these vegetables have only been expressed in terms of the vitamin A active carotenoids such as α- and β-carotene. Therefore, the levels of carotenoids such as ζ-carotene and lycopene, which have no vitamin A activity, are not expected to have been included in the total carotenoid contents of foods expressed in these tables.

The carotenoid content of red palm oil has been shown to be dependent on the source of the sample as judged by the total α- and β-carotene content of the samples from seven countries (Loncin, 1976; Mueller-Mulot, 1976).

Effect of Cooking and Processing on the Quantitative Distribution of the Hydrocarbon Carotenoids. In an attempt to determine the effect of cooking and processing on the level of the hydrocarbon carotenoids in yellow/orange vegetables, samples from the same batches of raw and cooked vegetables were employed for analysis. In the case of canned carrots and sweet potato, there was no access to the original raw samples prior to processing; therefore, the levels of the carotenes in these vegetables may to some extent be accompanied by variation in the sources of sample (cultivar, growing season, location). Another variable that may contribute to changes in carotenoids results from processing these vegetables, which may be accompanied by loss and/or removal of soluble solids. In the evaluation of the effect of canning and processing, it is essential to know the history of each vegetable sample prior to preparation. However since this was not the primary objective of the present report, details of preparation and handling procedures for these vegetables were not monitored. Since the evaporation of the volatiles in the cooking process may result in concentration of the vegetable samples, the percentage of the volatiles in each sample has been determined and the carotenoid contents of raw, cooked, and canned vegetables have been related to the percentage of individual carotenes based on dry weight of the vegetables.

A PLUS is a fresh market hybrid carrot developed for production by the U.S. Department of Agriculture (Agricultural Research Service, Florida, California, and Idaho Agricultural Experiment Stations) in both organic and mineral soils where Imperator 58, Gold Pak, and the hybrid Orlando Gold are grown. According to the official standard release announcement by the U.S. Department of Agriculture (1983), the flavorful California-grown A PLUS carrot has, on average, a 76% increase in vitamin A potential over Imperator 58, the most widely grown variety in California (Peterson, 1986). A comparison between the carotene content of raw and cooked A PLUS hybrid carrots based on dry weight (Table III) indicates that the loss of the hydrocarbon carotenoids as a result of cooking is not significant. The loss of β-carotene and its 15,15′-cis isomer in cooked sweet potato is about 8%. Since a sample of canned sweet potato before processing was not available for analysis, it was not possible to determine the effect of processing on the carotenoid content of this vegetable. However, such effect has been thoroughly investigated in the case of canned pumpkin where samples of this vegetable before and after processing were donated by the Libby, McNeil Libby Co. From our findings it appears that the total loss of the carotenes as a result of processing of pumpkin is about 10%. It is interesting to note that owing to the removal of the volatiles at the processing stage, the carotenoid content of canned pumpkin for a constant weight of this vegetable is 1.3 times higher than that of the raw fresh sample. Extracts from samples of fresh pumpkin purchased locally were shown by HPLC to contain much lower levels of β-carotene than that of Libby, McNeil Libby pumpkin (grown in Illinois). In addition to β-carotene, the HPLC profile of an extract from fresh pumpkin (purchased locally) was shown to contain lutein, ζ-carotene, and a number of unidentified carotenoids, while α-carotene was only present in trace amounts (data not presented). The differences between the carotenoid profile of these pumpkin samples are probably related to the sources of sample variance (cultivar, growing season, location). The effect of cooking and processing on the content of carotene isomers in several green and yellow/orange vegetables has been reported (Panalaks and Mur-

ray, 1970; Sweeney and Marsh, 1971).

The small loss of the hydrocarbon carotenoids as a result of cooking several yellow/orange vegetables is consistent with our earlier study of the carotenoid content of several raw and cooked green vegetables (Khachik et al., 1986). In these studies it was demonstrated that while substantial levels of the oxygenated carotenoids were destroyed as a result of cooking, the loss of the hydrocarbon carotenoids in these vegetables were minimal. These findings are also consistent with the conclusions reached by Klaui and Bauernfeind (1981) in their review of the influence of cooking on carotene content of foods.

Nomenclature. The systematic names and the chemical structures of lycopene, ζ-carotene, and asymmetric ζ-carotene have been tabulated by Straub (1971). In analogy to β-carotene and decapreno-β-carotene, which consist of 8 and 10 isoprene units, respectively, C-45-β-carotene with 9 isoprene units (in chain) has been referred to as nonapreno-β-carotene (Khachik and Beecher, 1986).

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