Quantification of *cis-trans* Isomers of Provitamin A Carotenoids in Fresh and Processed Fruits and Vegetables

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A polymeric 5 μm C_{30} stationary phase for reversed phase HPLC was used to separate and quantitate geometric isomers of provitamin A carotenoids in fresh and processed fruits and vegetables. β-Carotene isomers (*all-trans*, 9-*cis*, 13-*cis*, and 15-*cis*), α-carotene isomers (*all-trans*, 9-*cis*, 13-*cis*, and 13′-*cis*), and β-cryptoxanthin isomers (*all-trans*, 13 and 13′-*cis*, and 15-*cis*) were resolved isocratically using the C_{30} stationary phase with 89:11 methanol/methyl *tert*-butyl ether as mobile phase. The percent of *cis* isomers increased 10−39% with canning. The total provitamin A carotenoid content (in micrograms per gram of dry weight of tissue) ranged from 3.5 to 907 in fresh samples and from 1.8 to 1055 in canned samples. In several fruits and vegetables, processing produced an increase of 16−50% of total measured provitamin A carotenoids relative to the fresh samples. These increases were most likely a result of increased extraction efficiency, inactivation of enzymes capable of degrading carotenoids, and/or loss of soluble solids into the liquid canning medium.

Keywords: *β-Carotene; α-carotene; β-cryptoxanthin; C_{30} reversed phase HPLC; isomers*

INTRODUCTION

In nature, carotenoids are predominantly present in the *all-trans* configuration (Zechmeister, 1962). Under certain conditions, especially during thermal processing of foods (Sweeney and Marsh, 1971; Chandler and Schwartz, 1987), *trans* double bonds are susceptible to geometric isomerization, whereby some bonds take on a *cis* configuration. *cis*-Isomers of provitamin A carotenoids, such as α-carotene, β-carotene, and β-cryptoxanthin, have provitamin A activities that are approximately 50% or less of that of corresponding *all-trans* carotenoids (Zechmeister, 1962; Sweeney and Marsh, 1973). Therefore, to accurately estimate the provitamin A content of foods and the effects of processing on their nutritional value with respect to vitamin A, the various isomeric forms of provitamin A carotenoids present in both the fresh and processed states must be accurately measured.

Both normal phase and reversed phase HPLC methods have been used to separate and quantitate provitamin A carotenoids in fruits and vegetables (Chandler and Schwartz, 1987, 1988; Quackenbush, 1987; Saleh and Tan, 1991; Godoy and Rodriguez-Amaya, 1994). Reversed phase HPLC methods utilizing C_{18} stationary phases allow for detection and partial separation of *cis/trans* isomers of provitamin A carotenoids; however, few of these methods provide baseline separation of isomers (O'Neil and Schwartz, 1992). Separation of carotene isomers has also been achieved with normal phase HPLC employing $Ca(OH)_2$ stationary phases (Chandler and Schwartz, 1987; Schmitz et al., 1995), yet these stationary phases are often variable and are not commercially available (O'Neil and Schwartz, 1992). Recently, a polymeric C_{30} stationary phase has been developed (Sander et al., 1994) that not only achieves baseline separation of common provitamin A carotenoids but also efficiently separates their geometric isomers, including α-carotene isomers (9-*cis*, 9′-*cis*, 13-*cis*, 13′-*cis*, and *all-trans*) and β-cryptoxanthin isomers (15-*cis*, 13 and 13′-*cis*, and *all-trans*) that were not previously separated (Emenhiser et al., 1996a).

Since the development of the C_{30} stationary phase, no quantitative data have been published on the distribution of geometric isomers of provitamin A carotenoids in foods. The purpose of this research is (1) to determine the effects of thermal processing on the isomerization of provitamin A carotenoids in fruits and vegetables that are the greatest contributors of provitamin A in the American diet (Block, 1994) and (2) to utilize new C_{30} HPLC methods to accurately quantitate provitamin A carotenoid isomers in fresh and processed fruits and vegetables. Taken together, these results can be used to provide more accurate information regarding vitamin A values in nutritional tables and databases and may also impact studies that correlate carotenoid isomers with specific biological activities (Levin and Mokady, 1994a,b; Gaziano et al., 1995; Stahl et al., 1995; Wang et al., 1994).

MATERIALS AND METHODS

Materials. Standards of *all-trans*-β-carotene and *all-trans*-α-carotene were purchased from Sigma Chemical Co. (St. Louis, MO), and *all-trans*-β-cryptoxanthin was a gift from Hoffmann-La Roche (Nutley, NJ). All extraction and HPLC solvents (Fisher Scientific Co., Fair Lawn, NJ) were of certified HPLC or ACS grade. The fruits and vegetables evaluated were those that contribute the most provitamin A to the American diet (Block, 1994). These include broccoli, cantaloupe, carrots, collards, orange juice, peaches, spinach, sweet potatoes, tomatoes, and tomato-based vegetarian vegetable soup. Twenty-five to 50 pounds of each food was purchased from two local stores in Raleigh, NC, thus representing two lots. Orange juice and vegetable soup, however, were pur-

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chased from a single location. Lots were kept separate throughout processing, extraction, and chromotagraphic analysis. The quantitative data presented for each food are based on the average of the two lots evaluated.

Processing. Within 24 h of purchase, samples were prepared as they are commonly consumed (e.g., skins, stems, and blemishes removed) and then cut into approximately 1 in. pieces. Half of each lot was used for processing. Canning was carried out at the North Carolina State University pilot plant according to National Canner's Association (1976) time and temperature requirements. Water was the liquid canning medium in all cases except for peaches and tomatoes, which used 40% sucrose solution and tomato juice, respectively. Fresh squeezed orange juice was pasteurized at 80 °C for 2 min in a continuous flow system. Broccoli was boiled at 100 °C for 7 min.

Extraction and Saponification. The following extraction procedures were carried out under subdued yellow light to prevent isomerization and photodegradation. The entire lot of each product (fresh and processed) was pureed in a food processor (Cuisinart, DLC-X plus) for 30−120 s. All of the canned samples were drained prior to pureeing except for canned tomatoes. Moisture analysis of the pureed tissue was performed in a vacuum oven at 68 °C and −92 kPA for 24 h. A slurry of the pureed tissue (30 g), deionized (DI) water (30 mL), calcium carbonate (1 g), and Celite (1 g) was homogenized using a Tissumizer (Tekmar SDT 1810) for 1 min and then extracted in 25 mL of methanol and filtered through No. 1 and 42 Whatman filter papers under vacuum. The filter cake was resuspended with 50 mL of 50:50 acetone/hexane, homogenized for 1 min, and filtered through the same filter papers. This acetone/hexane extraction was repeated until the filter cake was colorless. The extraction filtrates were added to a separatory funnel, and the hexane/carotenoid epilayer was saved and then washed with DI water (three times).

Due to the high pulp content, a separate extraction procedure was carried out for orange juice. Three centrifuge tubes were prepared, each with 20 g of orange juice and 10 mL of methanol. The tubes were vortexed for 30 s and centrifuged for 5 min at 5000 rpm in a Sorvall (RC-2) centrifuge. Supernatants were filtered as described above. The pellets remaining in the centrifuge tubes were each homogenized with a Tissumizer for 1 min with 25 mL of 50:50 acetone/hexane, centrifuged for 5 min at 5000 rpm, and filtered. This procedure was repeated until the pellet was colorless. The extraction filtrates were added to a separatory funnel, and the hexane/carotenoid epilayer was saved and then washed with DI water (three times).

Due to the presence of chlorophylls in broccoli, collards, orange juice, peaches, spinach, and vegetable soup, these extracts were saponified with twice the volume of saturated methanolic potassium hydroxide at room temperature under an atmosphere of nitrogen with constant stirring for 30−60 min. The hexane/carotenoid epilayer of the saponification mixture was saved and then washed with DI water until the pH was neutral.

All purified extracts were passed through anhydrous sodium sulfate to remove any contaminating water and then made up to a volume of 50 mL of hexane. Samples were stored with a nitrogen atmosphere in the dark at −20 °C. Within 48 h of the extraction, all samples were filtered through nylon 0.45 μm pore syringe filters and analyzed using HPLC.

Chromatography. To separate oxidized carotenoids and xanthophylls that coeluted with α-carotene and β-carotene under the HPLC conditions used, some extracts (carrot, orange juice, peach, tomato, and soup) were fractionated using fully activated, neutral alumina N Sep Pak cartridges (Waters, Milford, MA). (Samples that did not contain interfering carotenoids and xanthophylls were not fractionated.) Ten milliliters of hexane was passed through each cartridge to condition the cartridges for use. Concentrated extracts in approximately 1 mL of hexane were loaded onto the cartridge, followed by 5 mL of hexane. α-Carotene and β-carotene were eluted with 8 mL of 96.5:3.5 hexane/acetone, and β-cryptoxanthin was eluted with 8 mL of acetone. Collected fractions were dried with nitrogen and added back to their original volumes in hexane.

All extracts were analyzed in duplicate using reversed phase HPLC employing a polymeric 5 μm C_{30} stationary phase (4.6 mm i.d. × 250 mm) prepared at the National Institute of Standards and Technology (NIST) (Gaithersburg, MD) as described by Sander et al. (1994) and a self-packed 3 μm C_{30} guard column. An isocratic solvent system consisting of 89:11 methanol/methyl *tert*-butyl ether (MTBE) was delivered at 1 mL/min with a Waters 510 solvent delivery system. Samples were often concentrated prior to injection so that the *all-trans*-β-carotene peak was in the range of the prepared standard curves. An automatic injector (Waters, WISP Model 712) was used to inject 30−60 μL of sample dissolved in 50:50 methanol/MTBE. A linear UVIS 203 detector (Scientific Instruments, Inc., Reno, NV) with a setting of 0.02 absorbance unit full scale (AUFS) was used to record absorbances at 410 nm, the isosbestic point for β-carotene isomers and close to the isosbestic point for α-carotene and β-cryptoxanthin isomers (O'Neil and Schwartz 1992). Peaks were integrated using Dynamax chromatography software (version 1.2; Rainin Instrument Co., Woburn, MA) on a Macintosh computer (system 6.0.2, Model SE, Apple Computer, Inc., Cupertino, CA). Individual isomer concentrations were calculated using *all-trans*-β-carotene, α-carotene, and β-cryptoxanthin standard curves recorded at 410 nm.

In several samples (carrot, orange juice, peach, tomato, and vegetable soup) a ζ-carotene isomer coeluted with 13-*cis* β-carotene during the C_{30} HPLC analysis. Therefore, an additional purification step was necessary to separate the coeluting carotenoids. Extracts (50−100 μL) were solubilized in 80:20 methanol/tetrahydrofuran and injected using a Waters U6K injector and separated on a Zorbax ODS 5 μm C_{18} column (4.6 mm i.d. × 250 mm) (MacMod Analytical, Chadds Ford, PA) with 70:30:0.05 methanol/acetonitrile/triethylamine mobile phase delivered isocratically at 1 mL/min. Under these conditions, all *cis* and *trans* isomers of β-carotene eluted as a single peak. The β-carotene peak was isolated and then analyzed on the C_{30} column under the same C_{30} HPLC conditions described above. Peak areas of the C_{30} chromatograms were compared before and after elimination of the ζ-carotene to determine the percentage of the peak that was 13-*cis*-β-carotene.

Recovery Determinations. To determine recovery of the extraction, saponification, and chromatography procedures, a known amount of purified *all-trans*-β-carotene standard was added to homogenized cantaloupe tissue. Extraction, saponification, and all chromatography procedures of cantaloupe with and without added β-carotene were carried out in duplicate. Recoveries were determined to be 100%. The quantity of *cis* isomers of the sample with added *all-trans*-β-carotene was the same as in the control extract, indicating that *cis* isomers did not form during the extraction, saponification, and chromatography procedures.

Peak Identification. Individual provitamin A carotenoids were identified by (1) UV−visible absorbance spectra recorded using a Waters 996 photodiode array detector with Millennium 2010 chromatography software (revision 2.1; Milford, MA) on a Gateway 2000 personal computer (Model 4DX2-66V; North Sioux City, SD); (2) comparison of retention times and absorbance spectra to those of isomerized standards; and (3) comparison to previous carotenoid separations on C_{30} columns (Sander et al., 1994; Emenhiser et al., 1995; 1996a,b).

RESULTS AND DISCUSSION

C_{30} Reversed Phase HPLC Separation of *cis/trans* Isomers. In this research a C_{30} stationary phase for reversed phase HPLC was used to separate geometric isomers of provitamin carotenoids in fruit and vegetable extracts. This stationary phase was able to separate *cis/trans* isomers of β-carotene, α-carotene, and β-cryptoxanthin under isocratic conditions. Figure 1 illustrates the resolution of four β-carotene isomers present in representative samples of fresh and processed

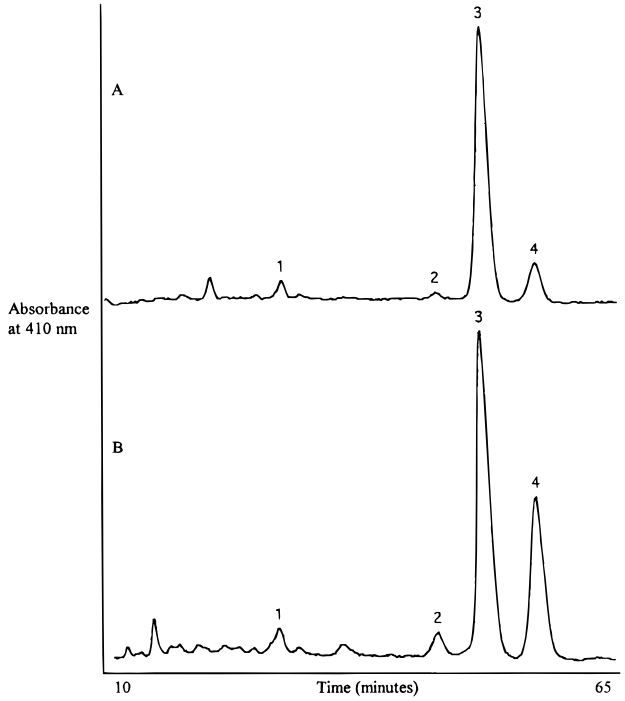

Figure 1. Reversed phase HPLC separation of β-carotene isomers in fresh (A) and canned (B) collards using a 5 μm C_{30} stationary phase and 89:11 methanol/MTBE mobile phase. Peaks: 1, 13-*cis*; 2, unidentified *cis*; 3, *all-trans*; 4, 9-*cis*.

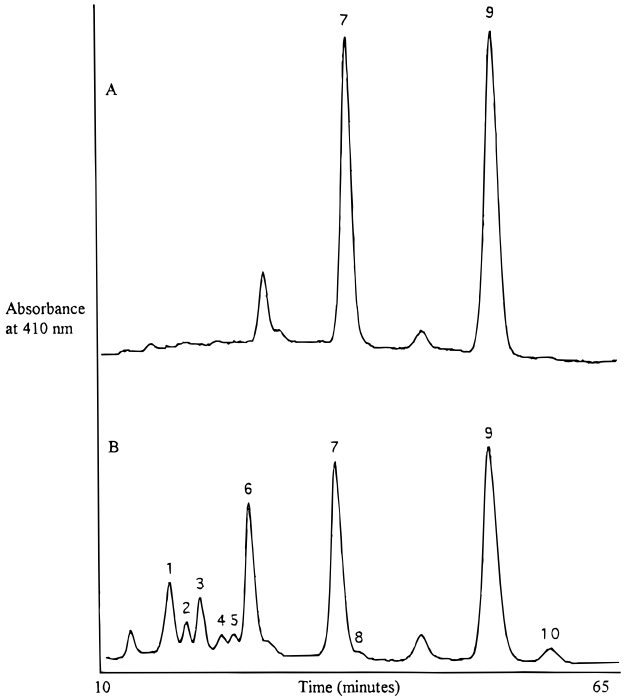

Figure 2. Reversed phase HPLC separation of α-carotene (AC) and β-carotene (BC) isomers in fresh (A) and canned (B) carrots using a 5 μm C_{30} stationary phase and 89:11 methanol/MTBE mobile phase. Peaks: 1, 13-*cis* AC; 2, unidentified *cis* AC; 3, 13′-*cis* AC; 4, 15-*cis* BC; 5, unidentified *cis* AC; 6, 13-*cis* BC; 7, *all-trans* AC; 8, 9-*cis* AC; 9, *all-trans* BC; 10, 9-*cis* BC.

collards. Figure 2 illustrates the resolution of four β-carotene and six α-carotene isomers in canned carrots. In the processed carrot chromatogram (Figure 2), the 9′-*cis*-α-carotene isomer coeluted with *all-trans*-β-carotene and therefore could not be resolved isocratically with 89:11 methanol/MTBE. However, it is likely that the amount of 9′-*cis*-α-carotene is comparable to the amount of 9-*cis*-α-carotene. This quantity is usually small as compared to the *all-trans*-β-carotene peak area and should therefore not significantly affect the calculation of *all-trans*-β-carotene concentration in carrots or other samples containing 9- and 9′-*cis*-α-carotene. Three β-cryptoxanthin isomers were resolved in peaches and orange juice. The 13-*cis*- and 13′-*cis*-β-cryptoxanthin isomers coeluted as one peak. Due to the low concentration of β-cryptoxanthin in the peaches and orange juice, 9-*cis*- and 9′-*cis*-β-cryptoxanthin were not detected; however, these isomers have been separated on the C_{30} stationary phase under similar HPLC conditions (Emenhiser et al., 1995).

Qualitative Distribution of *cis/trans* Isomers. For the majority of the samples analyzed, the *all-trans* isomer was lower in the processed samples as compared to the fresh samples on a percent basis (Table 1). This change in isomeric composition is due to *trans* to *cis* isomerization, which occurred as a direct result of the thermal processing. Canning of sweet potatoes caused the largest increase in total *cis* isomers on a percent basis (39%), followed by processing of carrots (33%), tomato juice (20%), collards (19%), tomatoes (18%), spinach (13%), peaches (10%), and orange juice (3%). Broccoli was the only vegetable that did not exhibit a change in isomer distribution; however, the processing treatment (boiling) was significantly less thermally rigorous than the canning process.

The predominant *cis* isomer of β-carotene, α-carotene, and β-cryptoxanthin in processed red, yellow, and orange fruits and vegetables was 13-*cis* (and 13′-*cis* for asymmetric carotenoids) followed by smaller quantities of 9-*cis* and 15-*cis* isomers. In green vegetables, β-carotene was the only provitamin A carotenoid detected. When processed, 9-*cis*-β-carotene was the predominant *cis* isomer followed by 13-*cis*-β-carotene, an unidentified *cis* isomer, and 15-*cis*-β-carotene. The unidentified *cis* isomer of β-carotene was detected only in the green vegetables. In the collards and spinach, the percent of 13-*cis*- and 15-*cis*-β-carotene did not change more than 2% with processing; however, the 9-*cis* isomer increased 19 and 11% in collards and spinach, respectively. In the vegetable soup the predominant *cis* isomer of β-carotene was 9-*cis* and that of α-carotene was the 13- and 13′-*cis* isomer pair. In surveys of isomeric composition of several fruits and vegetables, Kemmerer et al. (1945), Panalaks and Murray (1970), Sweeney and Marsh (1971), and Chandler and Schwartz (1987) also found the predominant *cis* isomer to be 13-*cis* except for green vegetables, for which 9-*cis* was the predominant isomer.

A significant quantity of *cis* isomers was present in all of the fresh green vegetables analyzed, with broccoli, collards, and spinach having a total of 29, 25, and 22%, respectively, of *cis* isomers of β-carotene. This high proportion of *cis* isomers in green vegetables prior to processing may be due to the ability of chlorophylls to act as sensitizers of β-carotene photoisomerization. This reaction would convert *trans*-β-carotene to *cis*-β-carotene, with 9-*cis* being the predominant isomer produced (O'Neil and Schwartz, 1995). The presence of *cis* isomers in fresh tomatoes, peaches, and oranges may also be due to chlorophylls initiating photoisomerization in these fruits in their immature state.

Quantitative Distribution of *cis/trans* Isomers. On a quantitative basis (micrograms per gram of dry weight of tissue), processing produced 50, 26, 22, 19, and 16% increases in total provitamin A carotenoids in

Table 1. Quantitative Distribution of β-Carotene, α-Carotene, and β-Cryptoxanthin Isomers in Fresh and Processed Fruits and Vegetables[a,b] and Percent Moisture of Tissue Purees

	β-carotene						α-carotene						β-cryptoxanthin					
extract	*all-trans*	9-*cis*	13-*cis*	15-*cis*	other *cis*[c]	total	*all-trans*	9-*cis*	13-*cis*	13′-*cis*	other *cis*[d]	total	*all-trans*	13/13′-*cis*	15-*cis*	total	total[e]	moisture[f]
broccoli																		
fresh	29.2	5.0	3.3	1.9	2.0	41.4											41.4	90.5
boiled	36.5	6.9	4.2	2.2	2.2	52.0											52.0	91.4
cantaloupe																		
fresh	162.8					162.8	0.9					0.9					163.7	85.4
carrot																		
fresh	534.4					534.4	372.7					372.7					907.1	89.5
canned	420.4	32.7	90.5	30.4		574.0	290.9	6.1	91.0	55.9	37.1	481.0					1055.0	91.5
collard																		
fresh	205.5	33.4	15.6	8.5	10.9	273.9											273.9	90.0
canned	229.5	128.5	18.7	9.2	23.8	409.7											409.7	91.1
orange juice																		
fresh	2.2	tr[g]	0.4	tr		2.6	1.9	tr	0.2	0.1		2.2	2.5	0.2	tr	2.7	7.5	88.6
pasteurized	1.5	tr	0.3	tr		1.8	1.3	tr	0.1	0.1		1.5	1.3	0.2	tr	1.5	4.8	88.6
peach																		
fresh	2.2	0.3	0.5	tr		3.0							0.3	0.1	0.1	0.5	3.5	90.7
canned	0.9	0.2	0.4	tr		1.5							0.2	0.1	tr	0.3	1.8	84.0
spinach																		
fresh	311.9	38.6	24.5	tr	22.5	397.5											397.5	90.8
canned	309.8	96.9	28.6	14.9	22.9	473.1											473.1	91.6
sweet potato																		
fresh	256.5					256.5											256.5	84.0
canned	191.0	25.3	76.6	19.4		312.3											312.3	88.2
tomato																		
fresh	71.0	4.8	5.8			81.6											81.6	95.2
canned	49.1	5.5	12.0	4.8		71.4											71.4	95.4
juice	40.0	4.5	10.1	4.8		59.4											59.4	95.5
vegetable soup																		
canned	93.9	7.1	5.9			106.9	70.1	0.8	4.4	4.8	2.0	82.1					189.0	86.7

[a] Data are based on an average of two lots except for orange juice and vegetable soup, which only had one lot each. Each lot was analyzed by HPLC in duplicate. [b] Concentrations are in micrograms per gram of dry weight tissue. [c] Totals the concentration of one unidentified *cis* isomer of β-carotene. [d] Totals the concentration of two unidentified *cis* isomers of α-carotene. [e] Total provitamin A carotenoid concentration includes all isomers of β-carotene, α-carotene, and β-cryptoxanthin. [f] Moistures are based on duplicate moisture determinations of each lot. [g] tr, trace.

collards, broccoli, sweet potatoes, spinach, and carrots, respectively, relative to the provitamin A content in the fresh samples (Table 1). Since it is generally observed that little or no degradation of β-carotene occurs during thermal processing (Chandler and Schwartz, 1988; Khachik et al., 1992), the increase in total provitamin A carotenoid concentration was most likely a result of increased extraction efficiency due to disruption of carotenoprotein complexes, inactivation of carotene oxidizing enzymes, and/or loss of soluble solids into the liquid canning medium (Weckel et al., 1962; Baloch et al., 1977; Ogunlesi and Lee, 1979). All of the fruits and vegetables that demonstrated an increase in total carotenoid concentration relative to the fresh samples had the canning liquid drained prior to analysis. Samples that were not drained (orange juice, tomatoes, tomato juice) showed a relative decrease in total provitamin A carotenoid concentration with processing. Canned peaches, however, were drained prior to extraction and a decrease in carotenoid concentration was observed, indicating that there may have been quantitative losses of provitamin A carotenoids with processing. Due to the relatively low concentrations of provitamin A carotenoids in orange juice, tomatoes, and peaches, small quantitative losses of provitamin A carotenoids resulted in large percentage losses. For example, in processed orange juice the quantitative loss of total provitamin A carotenoids was 2.7 μg/g of dry tissue, but on a percentage basis the loss was 36%. Losses of small quantities of provitamin A carotenoids (i.e. <5 μg/g) in processed vegetables with a high concentration of provitamin A carotenoids would not cause such a substantial percentage decrease after processing, even if the liquid canning medium was included in calculations.

Carotenoid concentrations of fruits and vegetables vary with plant variety, degree of ripeness, time of harvest, and growing and storage conditions (Gross, 1991). It is therefore difficult to compare quantitative isomer distributions of the fresh and processed fruits and vegetables analyzed in this research with previous data. In addition, few studies have analyzed fresh and processed foods that were from the same starting material, and consequently the true effects of thermal processing were not determined. In many studies data are reported on a fresh weight basis and other studies report data on a dry weight basis with no moisture information included, thus making comparisons of individual studies difficult. In a recent paper, Mangels et al. (1993) generated a carotenoid database that compiled acceptable values for carotenoid content of fruits and vegetables including median, minimum, and maximum values for five carotenoids on a fresh weight basis. This database included data from comprehensive surveys of carotenoid quantitation in fruits and vegetables by Sweeney and Marsh (1971), Bushway (1986), Bureau and Bushway (1986), and Khachik et al. (1989, 1991). Total concentrations of β-carotene, α-carotene, and β-cryptoxanthin determined in this study for fruits and vegetables on a fresh weight basis are within the range of values as reported by Mangels et al. (1993).

Prior to the development of the C_{30} stationary phase, separation of geometric isomers of asymmetrical provitamin A carotenoids in biological extracts has been difficult to achieve with reversed phase HPLC. Fur-

thermore, few HPLC methods have demonstrated simultaneous separations of the predominant *cis/trans* isomers of β-carotene and α-carotene in plant tissue under isocratic conditions or the separation of *cis/trans* isomers of β-cryptoxanthin in plant tissue. In recent work by Emenhiser et al. (1996b), separations of biological tissue extracts, including fresh and processed carrots, were achieved using the C_{30} stationary phase. To date, no other separations of fruit and vegetable extracts utilizing the C_{30} stationary phase have been published. Therefore, the separations presented in this research provide new methodology for accurate quantitation of provitamin A carotenoid isomer distribution in fresh and processed fruits and vegetables.

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