Effect of Food Preparation on Qualitative and Quantitative Distribution of Major Carotenoid Constituents of Tomatoes and Several Green Vegetables†

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The major carotenoid constituents of extracts from several raw and cooked green vegetables (broccoli, green beans, spinach), red ripe tomatoes, and tomato paste have been identified and quantified by high-performance liquid chromatography on a C_{18} reversed-phase column. The predominant carotenoids in raw green vegetables were neoxanthin, violaxanthin, lutein epoxide, lutein, α-carotene, and β-carotene. The carotenoids in tomatoes and tomato paste were lutein, 5,6-dihydroxy-5,6-dihydrolycopene, lycopene 1,2-epoxide, lycopene 5,6-epoxide, lycopene, neurosporene, γ-carotene, ζ-carotene, β-carotene, phytofluene, and phytoene. The effect of various means of cooking on the levels of carotenoids in raw and cooked (microwaved, boiled, steamed, stewed) green vegetables and tomatoes has been extensively studied. It was shown that while the epoxycarotenoids were somewhat sensitive to heat treatment, lutein and hydrocarbon carotenoids such as neurosporene, α- and β-carotene, lycopene, ζ-carotene, phytofluene, and phytoene survived the heat treatments.

INTRODUCTION

During the past decade a number of epidemiologic studies have associated the consumption of a number of cruciferous vegetables with reduced incidence of several types of cancers (Peto et al., 1981; Shekelle et al., 1981; National Research Council, 1982). The cancer-lowering effect of these foods was originally attributed to vitamin A active carotenoids (i.e., α- and β-carotene). However, many of these fruits and vegetables, in addition to vitamin A active carotenoids such as α- and β-carotene, contain substantial levels of non vitamin A active carotenoids (oxygenated carotenoids or xanthophylls). Colditz et al. (1985) found that while increased total dietary carotenoids intake was protective against cancer in an elderly population, foods high in β-carotene specifically had no association. Certain foods containing other carotenoids had significant protective effect. The epidemiologic studies could be better interpreted if collection of dietary carotenoid data is accompanied by a detailed qualitative and quantitative measurement of all of the major carotenoids in fruits and vegetables. Furthermore, since many of these foods are processed before consumption, the effects of cooking and various food preparation techniques on qualitative and quantitative distribution of carotenoids are highly significant and must be thoroughly investigated.

Several researchers have studied the effect of different methods of cooking and processing on the levels of carotenoids in foods. Panalaks and Murray (1970) (boiling, canning), Sweeny and Marsh (1971) (pressure cooking, canning), Ogunlesi and Lee (1979) (canning at high temperature), Chandler and Schwartz (1988) (canning, microwaving, baking), Almeida and Penteado (1988) (boiling), Speek et al. (1988) (boiling, frying, sun drying), Dikshit et al. (1988) (different methods of Indian cooking), and Rahman et al. (1990) have reported substantial losses of β-carotene levels in several vegetables. While Gody and Rodriguez-Amaya (1987) have reported little or no change in β-carotene levels, Gomez (1981), Bushway and Wilson (1982), and Panalaks and Murray (1970) have reported an increase in carotenoid levels as a result of cooking and processing. Most of these studies have focused on the changes in the levels of *all-trans*-α- and β-carotene and subsequent losses in vitamin A activity due to thermal degradation and isomerization of these compounds. There are very few studies that have examined the effect of processing on qualitative and quantitative distribution of all of the major carotenoids in fruits and vegetables. Gody and Rodriguez-Amaya (1987), Padula and Rodriguez-Amaya (1987), and Almeida and Panteado (1988) have shown that carotenoid epoxides are very sensitive to most food preparation conditions.

In 1986, we reported that while carotenoid epoxides in several green vegetables (broccoli, cabbage, spinach, Brussels sprouts, kale) were quite sensitive to heat treatment, the losses due to degradation of β-carotene as a result of microwave cooking were about 15% (Khachik et al., 1986). In the same study, we reported losses of 19% and 35% in the levels of lutein in microwaved cooked Brussels sprouts and kale, respectively. Although in our earlier study we thoroughly investigated the distribution

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† Presented in part at the Ninth International Symposium on Carotenoids, Kyoto, Japan, May 1990. Supported in part by the National Cancer Institute through Reimbursable Agreement YO1-CN-30609.

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of carotenoids in several green vegetables, the effect of various types of cooking on the carotenoid profiles of the vegetables was not examined. Most of the reported studies have produced conflicting results on the effect of various means of cooking on the levels of carotenoids, particularly that of β-carotene, in foods.

In this paper we have extensively examined the effect of various methods of cooking (microwaving, boiling, steaming, stewing) on the carotenoid concentration of several green vegetables (broccoli, green beans, spinach) and red ripe tomatoes. As many as 19 carotenoids were separated from the extracts of these raw and cooked vegetables on a C_{18} reversed-phase column by high-performance liquid chromatography (HPLC). With each method of cooking, the carotenoid concentrations were determined for several sources of each of the raw and cooked vegetables. Statistical analyses were employed to evaluate and interpret the effect of various means of cooking on quantitative distribution of several carotenoids in those vegetables examined. Purified reference compounds and internal standards (β-apo-8′-carotenal, ethyl β-apo-8′-carotenoate, nonapreno-β-carotene) were used to quantify each component in several vegetables.

EXPERIMENTAL PROCEDURES

Apparatus. A Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into Hewlett-Packard 1040A rapid-scanning UV–visible photodiode array detector. The data were stored and processed by a Hewlett-Packard 9000/Series 300 (ChemStation) computing system in conjunction with a Hewlett-Packard Model 9153B disk drive, color display monitor Model 35741, and a Model 7470A plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at the rate of 12 spectra/min. Absorption spectra of the carotenoids in different solvents were recorded on a Beckman DU-7 UV–visible spectrophotometer. Mass spectra were obtained from a Finnigan MAT Model 4510 mass spectrometer (San Jose, CA) equipped with an INCOS data system and a direct-exposure probe which was heated by the application of current from 0 to 1000 mA at a rate of 50 mA/s. Desorption chemical ionization (DCI) spectra were obtained, employing ammonia as the reagent gas at a source block temperature of 60 °C. Negative-ion electron capture mass spectra were produced by using methane as a buffer gas at an indicated pressure of 0.3 Torr. For negative-ion spectra, the ionizing chamber was maintained at 60 °C and spectra were collected from m/z 45 to 650.

Chromatographic Procedure. The analytical and semipreparative separations employed a combination of isocratic and gradient chromatography. An isocratic mixture of acetonitrile (85%), methanol (10%), dichloromethane (2.5%), and hexane (2.5%) at time 0 was followed by a linear gradient beginning at time 10 min and completed at time 40 min. The final composition of the gradient mixture was acetonitrile (45%), methanol (10%), dichloromethane (22.5%), and hexane (22.5%). The flow rates with the analytical and semipreparative columns were 0.7 and 2.5 mL/min, respectively. At the end of the gradient, the columns were equilibrated under the initial isocratic conditions for 20 min. The chromatographic analyses were simultaneously monitored at 470, 455, 445, 400, 350, and 286 nm.

Columns. Analytical separations used a stainless steel (25-cm length × 4.6-mm i.d.) Microsorb C_{18} (5-μm spherical particles) column (Rainin Instrument Co.) protected by a Brownlee guard cartridge (3-cm length × 4.6-mm i.d.) packed with Spheri-5 C_{18} (5-μm particle size). Semipreparative separations were carried out on a Rainin stainless steel (25-cm length × 10-mm i.d.) Microsorb C_{18} column (5-μm spherical particles), preceded by a Brownlee guard cartridge similar to that described above.

Reagents and Materials. The reference samples of *all-trans*-β-carotene (Aldrich Chemical Co., Milwaukee, WI) and *all-trans*-α-carotene (Sigma, St. Louis, MO) were purified by recrystallization (dichloromethane and methanol) and thin-layer chromatography, respectively. Reference samples of neoxanthin, violaxanthin, lutein 5,6-epoxide, and lutein were isolated from Kale (*Brassica oleracea*, variety Acephala) and purified according to published procedures (Khachik et al., 1986). Lycopene, lycopene 5,6-epoxide, and minute quantitites of lycopene 1,2-epoxide and 5,6-dihydroxy-5,6-dihydrolycopene were isolated from an extract of tomato paste (details described later in the text). Large quantities of lycopene 5,6-epoxide and 5,6-dihydroxy-5,6-dihydrolycopene were also prepared from the reaction of lycopene with Micro-Cel C (synthetic calcium silicate, Manville Products Corp., Denver, CO) according to the method of Ritacco et al. (1984a). Neurosporene was synthesized according to the method of Davis et al. (1966). γ-Carotene, ζ-carotene, phytofluene, and phytoene were isolated from the extracts of dried apricots and peaches by semipreparative TLC and HPLC (Khachik et al., 1989). Ethyl β-apo-8′-carotenoate (Fluka Chemical Corp.), β-apo-8′-carotenal (Fluka Chemical Corp.), and *all-trans*-nonapreno-β-carotene [synthesized according to the method developed in our laboratory (Khachik and Beecher, 1986)] were used as internal standards. The purity of the internal standards and the carotenoid reference samples was checked by HPLC as well as comparison of their absorptivity data in various solvents with those of published values (De Ritter and Purcell, 1981). When necessary, the samples were further purified by semipreparative TLC and HPLC. HPLC grade solvents, methanol, acetonitrile, dichloromethane, and hexane (Fisher Scientific, Pittsburgh, PA), were used without further purification. Tetrahydrofuran used for extraction was stabilized with butylated hydroxytoluene (BHT, 0.01%).

Preparation of the Raw and Cooked Vegetables for Extraction. The vegetables were prepared for analysis in the same way they are prepared for consumption. In the case of broccoli (*Brassica oleracea*, variety Botrytis), the flowerets were separated from the main stem and cut into small pieces, and three equal portions of approximately 60 g were weighed. The first portion (60 g) was extracted and analyzed raw. The second portion (60 g) was steamed over boiling water for 5 min, rinsed for 15 s under cold tap water, and dried on absorbent paper before extraction. The third portion of broccoli (60 g) was microwaved with a small amount of added water (25 mL) in a glass bowl covered with a plastic wrap for 5 min at full power (750-W maximum output). At the end of this time, the broccoli flowerets were rinsed under cold tap water for 15 s and dried on absorbent paper before extraction.

In the case of spinach (*Spinacia oleracea*), the stems and ribs were removed, and three equal portions of approximately 60 g of the leaves were weighed. The first portion (60 g) was extracted and analyzed raw, and the second portion was steamed for 3 min similar to the procedure described above for broccoli. The third portion (60 g) was microwaved (full power, 750 W) in the presence of a small amount of added water (25 mL) for 1.5 min at high power.

In the case of green beans (*Phaseolus vulgaris*), the beans were tipped and cut into 2–3-in. pieces and divided into four equal portions of approximately 60 g. The first portion (60 g) was extracted and analyzed raw. The second portion (60 g) was microwaved in the presence of a small amount of added water (20 mL) for 4 min at high power. At the end of this time, the beans were rinsed with cold water and dried on absorbent paper prior to extraction. The third portion (60 g) was boiled for 9 min in a covered pan containing boiling water (1 L), rinsed under cold tap water, and dried on absorbent paper before extraction. The fourth portion (60 g) was boiled for 1 h and processed as above prior to extraction and analysis.

In the case of tomatoes (*Lycopersicon esculentum*), the cooking method used was stewing. Tomatoes were cut into four quarters and then divided into two equal portions of approximately 120 g. The first portion was extracted and analyzed raw. The second portion was stewed as follows: The tomato pieces were further cut into smaller pieces and stewed for 8 min in a covered pan at medium heat without addition of water. At the end of this time, cold water was added to prevent further cooking. The whole mass was transferred into a blender for extraction.

Common brands of commercially prepared tomato paste (100 g) obtained from a local supermarket were sampled directly from their containers.

Table I. Weight of the Internal Standards Added to Broccoli, Spinach, Green Beans, Tomatoes, and Tomato Paste Samples at the Beginning of the Extraction and the Final Volume of the Extracts

entry	vegetable	int std, mg: β-apo-8′-carotenal	int std, mg: nonaprenoid-β-carotene	int std, mg: ethyl β-apo-8′-carotenoate	final vol extr, mL
1	broccoli (60 g)	5.20	2.16	0.00	50.0
2	spinach (60 g)	4.53	3.30	0.00	250.0
3	green beans (60 g)	0.77	0.62	0.00	25.0
4	tomatoes (120 g)	0.00	0.00	5.06	100.0
5	tomato paste (100 g)	0.00	0.00	5.06	100.0

Extraction. With the exception of minor modifications, the extraction procedure was similar to a published procedure for the extraction of green vegetables (Khachik et al., 1986). Two stock solutions of the internal standards were prepared by dissolving 65 mg of β-apo-8′-carotenal and 36 mg of nonaprenoid-β-carotene in 250 mL of a mixture of the HPLC solvents [acetonitrile (40%), methanol (20%), dichloromethane (20%), hexane (20%)], respectively. An appropriate aliquot of these internal standards, magnesium carbonate (10% of the weight of the vegetable), and tetrahydrofuran (THF, 500 mL) were added to each sample of vegetables prior to homogenization in a Waring blender. The extractions were carried out at 0 °C by immersing the Waring blender in an ice bath to prevent the degradation and isomerization of carotenoids. The extract was filtered under suction, and the solid materials were extracted repeatedly with THF until the resulting filtrate was colorless. Complete extraction of chlorophylls and carotenoids could be readily accomplished after three or four consecutive homogenizations of the vegetables. The combined THF extract was concentrated on a rotary evaporator at 35 °C and then partitioned into petroleum ether and saltwater. The organic layer was washed with water (3 × 250 mL) to remove the water-soluble materials. The water layers were combined and washed with petroleum ether (3 × 100) until the petroleum ether extract was colorless. The organic layers were combined, dried over anhydrous sodium sulfate, and evaporated to dryness on a rotary evaporator at 35 °C. The residue was dissolved in an appropriate amount of HPLC injection solvent [acetonitrile (40%), methanol (10%), dichloromethane (20%), hexane (20%)] and filtered through a 0.45-μm disposable filter assembly (American Scientific Products, McGraw Park, IL) for HPLC analyses. This HPLC injection solvent is a powerful solvent for solubilizing carotenoids and chlorophylls and does not produce chromatographic artifacts. As reported earlier (Khachik et al., 1988), this is mainly due to the polarity and solubility properties of this injection solvent, which are quite compatible with those of the HPLC mobile phase. The extraction procedure for tomatoes and tomato paste was similar to the procedure described above, with the exception that petroleum ether was replaced with dichloromethane at the partitioning stage for an efficient removal of high concentrations of lycopene in this fruit. Furthermore, a different internal standard, ethyl β-apo-8′-carotenoate, was employed in HPLC analyses of the extracts from tomatoes and tomato paste. A quantitative description of the weight of the internal standards added to each of the vegetables and the final volume of the extracts is presented in Table I.

Determination of the Volatile Content of the Vegetables. The volatile content of each of the raw and cooked green vegetables was determined by heating a known weight of the vegetable (about 5 g) in a 20-mL culture tube in a vacuum oven (Model 5831, National Appliance Co., Portland, OR) for 3.5 h at 60 °C and ≤2 mmHg. At the end of this time, the weight loss due to evaporation of the volatiles was used to determine the percentage of the volatiles in each sample.

The volatile content of raw and stewed tomatoes and tomato paste was determined by a low-temperature freeze dryer (Model 10-100, Virtis Co., Division of CENCO Medical/Health Supply Corp., Gardiner, NY) at −60 °C and ≤0.1 mmHg for 24–48 h. The volatile content of each of the raw, cooked, and processed green vegetables and tomatoes studied in this text are presented in Table II. An average of the percent volatiles from triplicate measurements for each green vegetable and tomatoes is presented.

Table II. Volatile Content of Broccoli, Spinach, Green Beans, Tomatoes, and Tomato Paste[a]

vegetable	% volatile content of raw, cooked, and processed vegetables[b]: raw	microwaved	steamed	boiled	stewed	canned
broccoli	88.7	85.2	90.4	c	c	c
spinach	88.4	86.3	88.7	c	c	c
green beans	88.9	86.2	c	89.8	c	c
tomatoes	93.8	c	c	c	94.0	c
tomato paste	c	c	c	c	c	28.3

[a] Details of volatile content determination are described in text. [b] Mean of three consecutive measurements for each batch of vegetables. [c] Data were not determined for these samples.

Isolation and Characterization of Carotenoids in Extracts from Tomato Paste. The abundant carotenoids in tomato paste were isolated by flash column chromatography (Still et al., 1978) according to the following procedure. Since flash column chromatography resulted in partial separation of various carotenoids, certain fractions were combined and then subjected to semipreparative HPLC for final purification. A concentrated extract from 300 g of red tomato paste in dichloromethane (15 mL) was chromatographed under a stream of nitrogen on a flash column (30-cm length × 4-cm i.d.) employing silica gel (88 g, 60–200 mesh, J. T. Baker Inc., Phillipsburg, NJ) as adsorbent and petroleum ether/acetone 85/15 as eluent. The flow rate was 1 in./min, and 20 fractions were collected. The volume of each fraction was about 25–35 mL.

Fractions 1–7. These fractions were combined and analyzed by HPLC on the analytical C_{18} column. Seven major components were shown to be present and were separated by HPLC on a semipreparative scale and identified by comparison of their HPLC retention times and UV–visible absorption spectra with those of authentic reference samples. In the order of chromatographic elution on a C_{18} reversed-phase column, these carotenoids were lycopene, neurosporene, γ-carotene, ζ-carotene, β-carotene, phytofluene, and phytoene.

Fractions 8–13. These fractions were combined and analyzed by HPLC on the analytical C_{18} column. Four major and several minor components were shown to be present and were separated by HPLC on the semipreparative C_{18} column and identified by comparison of their HPLC retention times, UV–visible absorption, and mass spectra with those of reference compounds. The minor components were neurosporene, γ-carotene, ζ-carotene, β-carotene, phytofluene, and phytoene. The major carotenoids in the order of chromatographic elution were as follows.

a. Lycopene 1,2-Epoxide. UV–visible absorption maxima (nm): petroleum ether, λ_{max} = 444, 470, 502 nm [lit. λ_{max} = 443, 469, 500 nm (Ben-Aziz et al., 1973)]. UV–visible absorption spectrum monitored by photodiode array detector in the HPLC solvents contained maxima at 446, 472, and 502 nm. Mass spectrum (ECNI, methane): molecular anion at m/z 552 (100%).

b. 3,4-Didehydro-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-ψ,ψ-carotene. UV–visible absorption maxima (nm): petroleum ether, λ_{max} = 414, 436–438, 466–468. UV–visible absorption spectrum monitored by photodiode array detector contained maxima at 414, 438, and 466 nm. Mass spectrum (ECNI) with methane and ammonia: molecular anion at m/z 566 (100%). Mass spectrum [ECNI, deuteroammonia (ND_3)]: molecular anion at m/z 566 (100%) indicated no exchangeable hydrogen.

c. Lycopene 5,6-Epoxide. UV–visible absorption maxima (nm): petroleum ether, λ_{max} = 430, 455, 486 [lit. λ_{max} = 428, 453, 484 nm (Ritacco et al., 1984a)]. UV–visible absorption spectrum monitored by photodiode array detector in the HPLC solvents contained maxima at 432, 456, and 486 nm. Mass spectrum (ECNI, methane): molecular anion at m/z 552 (100%).

d. Lycopene. UV–visible absorption spectrum monitored by photodiode array detector in the HPLC solvents contained maxima at 446, 474, and 502 nm. Mass spectrum (ECNI, methane): molecular anion at m/z 536 (100%).

Fractions 14–20. These fractions were combined and analyzed by HPLC on the analytical C_{18} column. In addition to lycopene 1,2-epoxide, the fractions consisted of two major components, which were separated on the semipreparative C_{18} column and identified from their UV–visible absorption and mass spectra as follows.

a. Lutein. UV–visible absorption maxima (nm): petroleum ether, λ_{max} = 422, 444, 473 nm; ethanol, λ_{max} = (423, inflection), 445, 474 nm, in agreement with literature values tabulated by De Ritter and Purcell (1981). Mass spectrum (ECNI, methane): molecular anion at m/z 568 (100%) and an anion at m/z 550 [20%, $(M - H_2O)^-$].

b. 5,6-Dihydroxy-5,6-dihydrolycopene. UV–visible absorption maxima (nm): petroleum ether, λ_{max} = 430, 455, 483 nm [lit. λ_{max} = 430, 454, 483 (Ritacco et al., 1984a)]; hexane, λ_{max} = 430, 455, 483 nm [lit. λ_{max} = 431, 456 ($E^{1\%}$ = 2820), 488 nm (Bush and Zechmeister, 1958)]. Mass spectrum (ECNI, methane): molecular anion at m/z 570 (100%). Mass spectrum (DCI, ammonia): ammonium adduct ion at m/z 588 [100%, $(M + NH_4)^+$] and 570 (100%, $(M + NH_4 - H_2O)^+$.

Isolation and Characterization of Carotenoids in Extracts of Red Ripe Tomatoes. The carotenoids from a concentrated extract of raw tomatoes (from 2000 g of red ripe tomatoes) were isolated by flash column chromatography under the same conditions described earlier for the isolation of carotenoids from tomato paste. Twenty fractions were collected and were shown to contain the same carotenoids as did tomato paste. However, the last fractions combined (fractions 14–20) in addition to lycopene 1,2-epoxide, 5,6-dihydroxy-5,6-dihydrolycopene, and lutein contained low concentrations of a number of epoxycarotenoids. These carotenoid epoxides were separated by semipreparative HPLC and identified from their absorption and mass spectra as neoxanthin, violaxanthin, and lutein epoxide. The HPLC retention times and UV–visible absorption spectra of these carotenoids monitored by a photodiode array detector in the HPLC solvents were identical with those of the authentic samples isolated from several green vegetables (Khachik et al., 1986).

Neoxanthin. UV–visible absorption maxima (nm): hexane, λ_{max} = 412, 440, 469 nm; benzene, λ_{max} = 427, 451, 481 nm, in agreement with the tabulated values in the literature (De Ritter and Purcell, 1981). Mass spectrum (ECNI, methane): molecular anion at m/z 600 (100%).

Violaxanthin. UV–visible absorption maxima (nm): petroleum ether, λ_{max} = 418, 442, 466 nm; benzene, λ_{max} = 424, 450, 482 nm, in agreement with the tabulated values of De Ritter and Purcell (1981). Mass spectrum (ECNI, methane): molecular anion at m/z 600 (100%).

Lutein Epoxide. UV–visible absorption maxima (nm): hexane, λ_{max} = 420, 444, 472 nm; ethanol, λ_{max} = 420, 444, 472 nm, in agreement with the values tabulated by De Ritter and Purcell (1981). Mass spectrum (ECNI, methane): molecular anion at m/z 584 (85%) and ions at m/z 566 due to the loss of water from the molecular anion.

RESULTS AND DISCUSSION

Qualitative Distribution of Carotenoids. *a. Raw Green Vegetables.* The major pigments of green vegetables can be classified into three groups of compounds. In the order of chromatographic elution on a C_{18} reversed-phase column these are (a) xanthophylls (oxygenated carotenoids), (b) chlorophylls and their derivatives, and (c) hydrocarbon carotenoids. The chromatogram of an extract from raw green beans (Figure 1, upper trace) shows the presence of typical components found in most of the green vegetables. A combination of isocratic and gradient elution chromatography separated the various components within 36 min. The chromatographic conditions are similar to those we reported in an earlier publication (Khachik et al., 1986). However, these conditions have been slightly modified to improve the separation of the early eluting xanthophylls in the chromatograms of the extracts from various green vegetables reported in the present study. The application of these modified HPLC conditions in separation of the various classes of carotenoids in fruits and vegetables has been demonstrated in detail (Khachik and Beecher, 1988a,b; Khachik et al., 1989, 1991a). The xanthophylls (peaks 1–4) and the internal standard, β-apo-8′-carotenal (peak 6), are eluted under isocratic conditions, which is then followed by a gradient that elutes

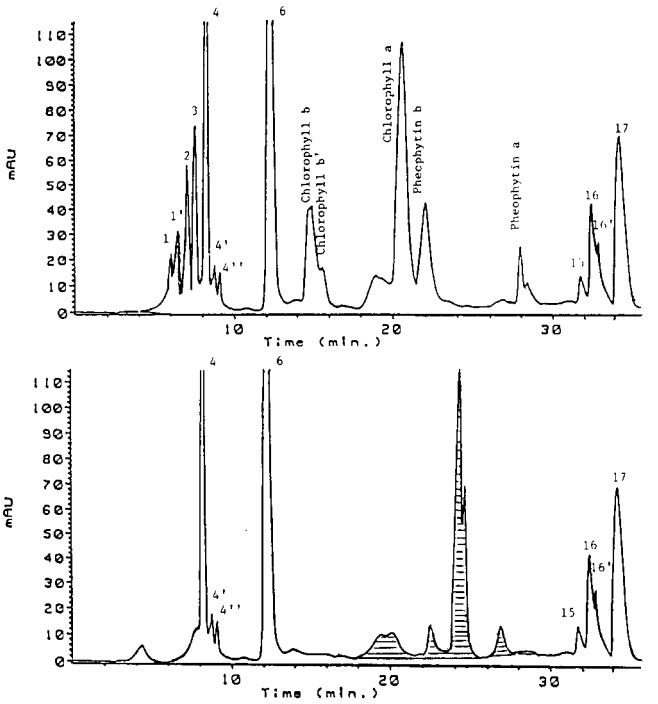

Figure 1. HPLC profiles of green bean extracts. Chromatographic conditions (eluent A) and peak identification (Table III) are described in the text. (Upper trace) Extract of raw green beans; (lower trace) extract of green beans boiled for 1 h. Shaded peaks are degradation products of chlorophylls.

the chlorophylls and their derivatives as well as the hydrocarbon carotenoids (peaks 15 and 16). A synthetic C_{45} β-carotene, namely, nonapreno-β-carotene (peak 17), which was employed as a second internal standard, elutes after β-carotene and its cis isomer (peaks 16 and 16′). This C_{45} β-carotene (Khachik and Beecher, 1986, 1987) has been found to be a more suitable internal standard than decapreno-β-carotene (Khachik and Beecher, 1985; Khachik et al., 1986), which had been employed as an internal standard for the quantification of the hydrocarbon carotenoids in our earlier publications.

A mono-cis isomer of neoxanthin (peak 1′, Figure 1), which has been tentatively identified as 9′-*cis*-neoxanthin (Khachik et al., 1986), was shown to be present in the extracts from raw and cooked green vegetables. Other geometrical isomers of carotenoids which were partially separated by HPLC were those of lutein (peaks 4′ and 4″, Figure 1) and β-carotene (peak 16′, Figure 1). The geometrical isomers of lutein in green vegetables have been tentatively identified from their UV–visible absorption spectra as a mixture of 9- and 9′-cis, 13- and 13′-cis, and 15 and 15′-cis isomers. However, the definite geometry of the cis isomers of lutein in the absence of nuclear magnetic resonance spectroscopic (NMR) data has not been established. In a recent study on separation of carotenoids from human plasma, we have isolated and identified the various geometrical isomers of lutein and have obtained NMR evidence in support of their structures (Khachik et al., 1991b,c). The geometrical isomers of lutein in these studies were effectively separated on a nitrile bonded column. An isolated fraction of lutein and its various geometrical forms from raw and cooked broccoli, spinach, and green beans were also chromatographed on a nitrile bonded column [conditions described in Khachik et al. (1991a,b)]. It was confirmed that *all-trans*-lutein was accompanied by substantial levels of 9-*cis*, 9′-*cis*, 13-*cis*, and 13′-*cis*-lutein as well as minute levels of 15- and 15′-*cis*-lutein.

Table III. Main Absorption Maxima and HPLC Peak Identification of the Various Carotenoids in Green Vegetables, Tomatoes, and Tomato Paste Extracts in the Order of Elution on a C_{18} Reversed-Phase Column

HPLC peak	carotenoid[a]	wavelength,[b] nm
1 + 1′	neoxanthin + *cis*-neoxanthin	438–440
2	violaxanthin	440–442
3	lutein 5,6-epoxide	440–442
4	*all-trans*-lutein	446
4′ + 4″	*cis*-luteins	442–444
5	5,6-dihydroxy-5,6-dihydrolycopene	456–458
6	β-apo-8′-carotenal (int std)	455
7	ethyl β-apo-8′-carotenoate (int std)	455
8	lycopene 1,2-epoxide	472–474
9	3,4-didehydro-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-ψ,ψ-carotene	438
10	lycopene 5,6-epoxide	456
11	lycopene	472–474
12	neurosporene	440
13	γ-carotene	462–464
14	ζ-carotene	400–402
15	α-carotene	448–450
16	*all-trans*-β-carotene	452–454
16′	*cis*-β-carotene	448–450
17	nonapreno-β-carotene (int std)	478
18	*all-trans* or *cis*-phytofluene	350
18′	*cis*- or *all-trans*-phytofluene	350
19	*all-trans*- or *cis*-phytoene	286
19′	*cis*- or *all-trans*-phytoene	286

[a] Cis-carotenoids have been designated with the same number as their all-trans isomers but distinguished from their all-trans compounds by prime symbols. [b] Determined in HPLC eluent employing a photodiode array detector; see the text for values in single solvents.

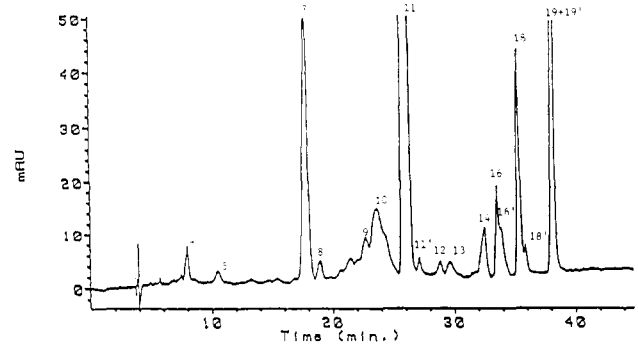

Figure 2. HPLC profile of raw tomato extract. Chromatographic conditions and peak identification (Table III) are described in the text.

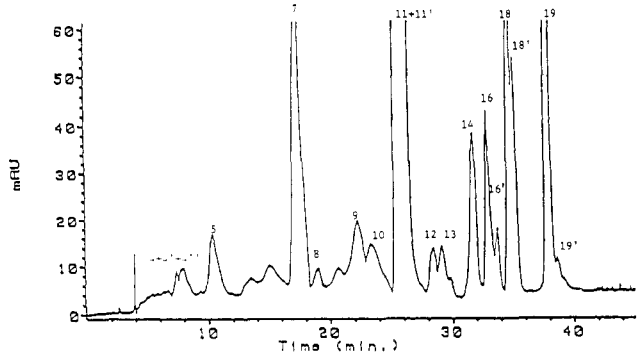

Figure 3. HPLC profile of tomato paste extract. Chromatographic conditions and peak identification (Table III) are described in the text.

The chromatograms of the green vegetables studied also show the presence of a cis isomer of β-carotene (peak 16′, Figure 1) whose HPLC peak appears as an unresolved shoulder on that of *all-trans*-β-carotene (peak 16, Figure 1). This cis isomer of β-carotene has been tentatively identified in our previous publications (Khachik et al., 1986, 1989; Khachik and Beecher, 1987) as 15,15′-*cis*-β-carotene. However, it is now believed that this cis isomer of β-carotene is probably a mixture of various geometrical isomers (i.e., 9-*cis*, 13-*cis*, and 15,15′-*cis*) of β-carotene. The geometrical isomers of hydrocarbon carotenoids such as α- and β-carotene are not generally well separated on C_{18} reversed-phase columns. However, Tsukida et al. (1982) have shown that the stereoisomers of β-carotene (four mono-*cis*- and five di-*cis*-β-carotenes) can be best separated by HPLC employing a lime (calcium hydroxide) column. This HPLC procedure has been successfully employed by Chandler and Schwartz (1987) and O'Neil et al. (1991) in separation of the cis isomers of β-carotene in raw and processed vegetables. In the present study no attempt was made to establish the geometry of the cis isomers of β-carotene in the green vegetables.

The abundant xanthophylls, chlorophylls, and hydrocarbon carotenoids found in the green vegetables reported in the present study and their corresponding HPLC peaks (Table III) were identified by spectroscopy, chemical reactions, and cochromatography with pure reference compounds similar to our earlier work with these green vegetables (Khachik et al., 1986). The major carotenoids found in the extracts of broccoli and spinach were identical with those found in green beans. However, α-carotene was absent in the extracts from broccoli and spinach.

b. Cooked Green Vegetables. A comparison between the chromatographic profiles of raw and cooked green vegetables revealed that with various means of cooking (microwaving, steaming, boiling), under moderate conditions described earlier, the integrity of carotenoids remains unchanged. The epoxycarotenoids such as neoxanthin, violaxanthin, and lutein epoxide do not undergo rearrangement to their corresponding 5,8-epoxides. However, the long-term boiling (1 h) of a sample of green beans results in complete destruction of these epoxycarotenoids as shown in the chromatogram of an extract from cooked green beans in Figure 1 (lower trace). Under various cooking conditions, the chlorophylls are readily converted to their derivatives. The separation and identification of the various derivatives of chlorophylls have recently been described by Canjura and Schwartz (1991) and van Breeman et al. (1991).

c. Raw, Cooked, and Processed Tomatoes. The major carotenoids of raw and cooked tomatoes as well as red tomato paste can be divided into three classes of carotenoids. In the order of chromatographic elution on a C_{18} reversed-phase column these are (a) hydroxycarotenoids, (b) epoxycarotenoids, and (c) hydrocarbon carotenoids. The chromatogram of an extract from raw tomatoes is shown in Figure 2. The chromatographic profiles of extracts from stewed tomatoes (chromatogram not shown) and tomato paste (Figure 3) are identical to that of raw tomatoes. The major differences among tomato paste and raw and stewed tomatoes appear to be the concentration of the various components. The individually isolated carotenoids in these tomato extracts were identified by comparison of their HPLC retention times and UV–visible absorption and mass spectra with those of the reference carotenoids.

The hydroxycarotenoids (peaks 4 and 5, Figures 2 and 3) were identified as lutein and 5,6-dihydroxy-5,6-dihydrolycopene. While lutein is the most common hydroxycarotenoid found in fruits and vegetables, the natural occurrence of 5,6-dihydroxy-5,6-dihydrolycopene has not yet been reported. This compound was first reported by Bush and Zechmeister (1958) as one of the cleavage products of the reaction between lycopene and boron tri-

Table IV. Quantitative Distribution of Xanthophylls and Carotenes in Green Vegetables[a]

vegetable	process	neoxanthin	violaxanthin	lutein 5,6-epoxide	*trans*-lutein	*cis*-lutein	total lutein	α-carotene	β-carotene
broccoli[b]	raw	0.63 ± 0.10	1.37 ± 0.09	0.64 ± 0.11	2.41 ± 0.21	0.44 ± 0.04	2.83 ± 0.20	*e*	2.33 ± 0.10
	steaming	0.58 ± 0.17	0.45 ± 0.15	0.35 ± 0.10	2.82 ± 0.26	0.45 ± 0.05	3.25 ± 0.28	*e*	2.76 ± 0.20
	microwaving	0.76 ± 0.03	0.58 ± 0.11	0.42 ± 0.06	2.79 ± 0.05	0.50 ± 0.03	3.28 ± 0.30	*e*	2.45 ± 0.07
spinach[b]	raw	2.36 ± 0.55	7.40 ± 4.00	0.50 ± 0.36	8.06 ± 2.60	1.40 ± 0.30	9.50 ± 2.90	*e*	8.90 ± 0.70
	steaming	2.47 ± 0.47	4.90 ± 1.10	*e*	9.26 ± 1.67	1.36 ± 0.25	10.60 ± 1.90	*e*	9.86 ± 0.47
	microwaving	2.26 ± 0.11	4.80 ± 1.64	*e*	7.46 ± 1.91	1.33 ± 0.23	8.73 ± 2.10	*e*	9.10 ± 0.71
green beans[c]	raw	0.13 ± 0.02	0.23 ± 0.07	0.19 ± 0.04	0.49 ± 0.06	0.12 ± 0.01	0.59 ± 0.07	0.08 ± 0.01	0.47 ± 0.05
	boiling	0.12 ± 0.02	0.08 ± 0.03	0.06 ± 0.02	0.48 ± 0.08	0.13 ± 0.02	0.61 ± 0.07	0.08 ± 0.01	0.54 ± 0.11
	microwaving	0.13 ± 0.02	0.09 ± 0.03	0.09 ± 0.03	0.60 ± 0.06	0.13 ± 0.01	0.71 ± 0.07	0.09 ± 0.02	0.53 ± 0.09
green beans[d]	raw	0.19 ± 0.03	0.23 ± 0.04	0.23 ± 0.04	0.58 ± 0.03	0.14 ± 0.09	0.69 ± 0.03	0.09 ± 0.01	0.51 ± 0.04
	boiling (1 h)	*e*	*e*	*e*	0.65 ± 0.07	0.13 ± 0.03	0.74 ± 0.01	0.10 ± 0.02	0.57 ± 0.03

[a] Amounts are expressed in milligrams per 100 g of edible food (raw). The levels are mean ± SD. [b] Mean values are for three batches. [c] Mean values are for four batches. [d] Mean values are for two batches. [e] Insufficient level in aliquot of extract applied to HPLC to permit quantification. HPLC limits of detection for carotenoids are about 1 μg/100 g of food extracted.

fluoride complexes (i.e., BF_3–etherate). 5,6-Dihydroxy-5,6-dihydrolycopene may therefore be an artifact of acid-catalyzed ring opening of lycopene 5,6-epoxide, which was shown to be present in the tomato extracts at fairly large concentrations. Although at the beginning of the extraction sufficient levels of a weak base such as magnesium carbonate are routinely added to neutralize the acids in tomatoes and prevent the formation of such artifacts, the origin of this dihydroxylycopene remains uncertain. Recently we have isolated and characterized 5,6-dihydroxy-5,6-dihydrolycopene from an extract of human plasma (Khachik et al., 1991c).

The epoxycarotenoids isolated from extracts of tomatoes were characterized as lycopene 1,2-epoxide (peak 8, Figures 2 and 3), lycopene 5,6-epoxide (peak 10, Figures 2 and 3), and a carotenoid that was tentatively identified as 3,4-didehydro-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-ψ,ψ-carotene (peak 9, Figures 2 and 3). The presence of a number of carotenoid epoxides including lycopene 1,2-epoxide and lycopene 5,6-epoxide in the extracts from tomatoes has been well established by Britton and Goodwin (1969, 1975), Ben-Aziz et al. (1973), and Kamber and Pfander (1984). The most convenient route to lycopene 5,6-epoxide and 5,6-dihydroxy-5,6-dihydrolycopene was first discovered by Ritacco et al. (1984a), who demonstrated that these compounds can be readily obtained by partial synthesis from the reaction of lycopene with Micro-Cel C (synthetic calcium silicate). These authors also proposed a mechanism for the hydroxylation of several carotenoids including lycopene on Micro-Cel C (Ritacco et al., 1984b). Following the procedures of Ritacco et al. (1984a), we readily prepared these compounds in good yield for their structural identification in tomato.

3,4-Didehydro-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-ψ,ψ-carotene was only tentatively identified from its mass and UV–visible absorption spectra, since sufficient levels of this compound were not available for measurement by NMR spectroscopy. The UV–visible absorption spectrum of this diepoxylycopene in petroleum ether had maxima at 414, 436–438, 466–468 nm, indicating the presence of nine conjugated double bonds in the polyene chain of this compound. The mass spectrum of 3,4-didehydro-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-ψ,ψ-carotene is consistent with a molecular formula of $C_{40}H_{54}O_2$ with no exchangeable hydrogens. One possible precursor to this compound is 3,4-didehydrolycopene, which may undergo selective epoxidation at the 5,6- and 5′,6′ double bonds to form this compound. Although 3,4-didehydrolycopene was not detected in the extracts from tomatoes, its occurrence in various natural products has been reported (Liaaen-Jensen, 1965; Britton et al., 1977).

Since the chromatographic profiles of raw, stewed, and processed tomatoes show the presence of the same carotenoids, the epoxylycopenes found in these samples are not likely to have been formed as a result of heat treatment during processing and sample preparation. When a large batch of raw tomatoes (from 2 kg of tomatoes) was extracted, the HPLC analysis revealed the presence of low levels of the normal chloroplast xanthophylls such as neoxanthin, violaxanthin, lutein epoxide, and lutein. These xanthophylls were only detectable in highly concentrated extracts of tomatoes. These findings are in agreement with the earlier work of Zechmeister and von Cholnoky (1936), which were later confirmed by Curl (1961).

Quantitative Distribution of Carotenoids. *a. Raw and Cooked Green Vegetables.* The quantitative distribution of xanthophylls and carotenes in broccoli, spinach, and green beans is shown in Table IV. These data were obtained from the HPLC analyses of the extracts from three or four batches of each vegetable. For a given vegetable and each method of cooking, a raw sample from the same batch was also analyzed to determine the possible losses and the recovery of carotenoids as a result of heat treatment. In all of the cooking experiments, several portions of the raw vegetables with equal weight were prepared and each portion was then cooked, extracted, and analyzed. Therefore, in all cases, the quantitative distribution of carotenoids in the cooked green vegetables obtained by HPLC was compared to those of the raw samples. This approach eliminates the errors in weight measurements due to the evaporation of volatiles in raw vs cooked samples.

Two internal standards, namely, β-apo-8′-carotenal and nonapreno-β-carotene, were employed to monitor the extraction losses of xanthophylls and carotenes from green vegetables, respectively. The recovery of these internal standards was shown by HPLC to be greater than 90%. As shown in Table II, the volatile content of the various batches of raw broccoli, spinach, and green beans is about the same.

With the exception of violaxanthin and lutein epoxide, the levels of xathophylls (neoxanthin and lutein) and carotenes (α- and β-carotene) in broccoli, spinach, and green beans under mild cooking conditions remained unchanged. This was determined by statistical evaluation which indicated that the concentration of neoxanthin (cis and trans), lutein (cis and trans), α-carotene, and β-carotene in each of the vegetables examined is not significantly changed with various methods of cooking. On the other hand, violaxanthin and lutein epoxide are quite heat labile, and even under moderate cooking conditions substantial levels of these compounds are destroyed. The percent losses of violaxanthin and lutein epoxide as a result of various moderate cooking procedures in broccoli, spinach, and green beans are shown in Table V. These losses were

Table V. Losses of Violaxanthin and Lutein 5,6-Epoxide Resulting from Various Moderate Cooking Procedures[a,b]

vegetable	process	violaxanthin	lutein 5,6-epoxide
broccoli	steaming	67	45
	microwaving	58	34
spinach	steaming	34	100
	microwaving	35	100
grean beans	boiling	65	68
	microwaving	61	53

[a] Calculated by comparison of the levels of these epoxides in raw and cooked samples as presented in Table IV. [b] The cooking conditions are described in text.

calculated from Table IV by comparing the recovery of these epoxycarotenoids in cooked vegetables with that of their raw samples.

As indicated earlier, several geometrical isomers of lutein (Figure 1, peaks 4′ and 4″) were detected in the extracts from broccoli, spinach, and green beans. A comparison between the levels of the total cis isomers of lutein in the cooked vegetables and those of the raw (Table IV) indicates that cis/trans isomerization of this compound is not influenced by the cooking conditions employed. In the present study the cooking procedures for the preparation of the green vegetables include steaming for 3–5 min, microwaving for 1.5–3 min, and boiling for 9 min. These methods of food preparation and the length of the heat treatment may be considered moderate since the qualitative and quantitative profiles of the majority of the carotenoids are not significantly changed. Under severe cooking conditions, when a sample of green beans was boiled for 1 h, the HPLC chromatogram (Figure 1, lower trace) of the extract clearly showed the complete destruction of the epoxycarotenoids such as neoxanthin, violaxanthin, and lutein epoxide. To our surprise the levels of lutein, α-carotene, and β-carotene (Table IV) in boiled (1 h) green beans were shown not to be statistically different from those of the raw beans. These results contradict one of our earlier publications (Khachik et al., 1986) in which we reported approximately 14–15% and 19–35% losses in the levels of β-carotene and lutein, respectively, in microwaved (6 min) green vegetables. The carefully designed cooking experiments in the present study clearly demonstrate that, with the exception of violaxanthin and lutein epoxide, the common chloroplast carotenoids in green vegetables are quite resistant to heat treatment. One of the factors that may lead to considerable variation in analytical data on raw and cooked vegetables is the incomplete extraction of carotenoids from the raw samples. There is no disagreement among most researchers that carotenoids are extracted much more readily from cooked vegetables than raw ones. This is presumably due to a more efficient denaturing of the carotenoid–protein complexes, which allows a more thorough and complete extraction of chlorophylls and carotenoids in green vegetables. In the present study to ensure the complete extraction of the carotenoids, the raw vegetables were repeatedly homogenized with tetrahydrofuran until the green color due to the mixture of chlorophylls and carotenoids could no longer be visually detected in the extracts.

b. Raw, Cooked, and Processed Tomatoes. The quantitative distribution of carotenoids in raw and cooked tomatoes as well as tomato paste is shown in Table VI. These data were obtained from the HPLC analyses of the extracts from three batches of tomato paste and four batches of raw and cooked tomatoes. Ethyl β-apo-8′-carotenal was employed as an internal standard for the HPLC

Table VI. Quantitative Distribution of Carotenoids in Raw and Cooked Tomatoes and Tomato Paste

carotenoids	tomatoes,[a] mg/100 g of fresh food		tomato paste,[b] mg/100 g of processed food
	raw[c]	stewed[c]	
lutein (trans + cis)	0.13 ± 0.02	0.15 ± 0.03	0.43–0.82
5,6-dihydroxy-5,6-dihydrolycopene	*d*	*d*	0.23–1.02
lycopene 1,2-epoxide	*e*	*e*	*e*
3,4-didehydro-5,6,5′,6′-diepoxy-5,6,5′,6′-tetrahydro-ψ,ψ-carotene	*e*	*e*	*e*
lycopene 5,6-epoxide	0.53 ± 0.06	0.52 ± 0.09	1.24–2.10
lycopene	3.92 ± 0.05	4.40 ± 0.30	32.7–68.2
neurosporene	0.30 ± 0.01	0.28 ± 0.05	0.90–1.19
γ-carotene	*e*	*e*	*e*
ζ-carotene	0.84 ± 0.02	0.87 ± 0.02	0.42–1.36
β-carotene (trans + cis)	0.28 ± 0.02	0.30 ± 0.01	0.26–1.66
phytofluene (trans + cis)	0.51 ± 0.08	0.54 ± 0.02	2.08–4.13
phytoene (trans + cis)	0.60 ± 0.10	0.66 ± 0.08	1.52–3.31

[a] The levels are mean ± SD. [b] Range of values for three batches. [c] Values for four batches. [d] Insufficient level in aliquot of extract applied to HPLC to permit quantification. HPLC limits of detection for carotenoids are about 1 μg/100 g of food extracted. [e] Values were not determined.

quantification of the carotenoids in tomatoes. The recovery of this internal standard from consecutive extractions was shown by HPLC to be greater than 90%. Some of the carotenoids of tomatoes and tomato paste listed in Table VI were not quantified because of their low concentration. Statistical evaluation of the levels of the individual carotenoids in raw and stewed tomatoes (8 min) revealed that concentration of carotenoids as a result of cooking remains unchanged. Furthermore, it is unclear as to whether the ring opening of carotenoids such as lycopene 5,6-epoxide to 5,6-dihydroxy-5,6-dihydrolycopene in acidic tomatoes can be thermally assisted during the dehydration of this fruit to paste. The dehydration of tomatoes to tomato paste is typically carried out at high temperature over an extended period under slight vacuum. Even under such conditions the integrity of the carotenoids is preserved and the qualitative distribution of carotenoids in tomato paste remains identical to that of raw and stewed tomatoes.

Nutritional Implication of the Distribution of Carotenoids in Raw and Cooked Green Vegetables and Tomato Paste. Although the dietary role of the vitamin A active carotenoids such as α-carotene and β-carotene in humans is well established, the nutritional implication of the oxygenated carotenoids is not known. A number of epidemiological studies have suggested that consumption of certain foods, particularly green vegetables, that are high in carotenoids lowers the incidence of several types of cancers [see review by Moon and Micozzi (1989)]. Recent interest in carotenoid-containing foods has led to numerous studies of carotenoid metabolism in humans. In addition to α- and β-carotene, as many as 16 carotenoids have recently been separated and identified in human plasma (Khachik et al., 1991a–c). A comparison between the profile of the carotenoids in human plasma and those of the green vegetables and tomatoes described earlier reveals some interesting points. For example, HPLC analyses of plasma extracts from more than several hundred human subjects in our laboratory have revealed that epoxycarotenoids such as neoxanthin, violaxanthin, lutein 5,6-epoxide, lycopene 1,2-epoxide, and lycopene 5,6-epoxide are not present in the extracts from human plasma (unpublished results). On the other hand, the hydroxycarotenoids such as lutein and 5,6-dihydroxy-5′,6′-dihydrolycopene have been isolated from plasma. In addition to α- and β-

carotene, a number of hydrocarbon carotenoids such as lycopene, neurosporene, γ-carotene, ζ-carotene, phytofluene, and phytoene also are present in human plasma. These observations suggest that epoxycarotenoids which may readily undergo degradation as a result of various methods of cooking are probably not absorbed. Furthermore, from the present study it appears that the integrity of the food carotenoids which may be significant in the diets of humans is well preserved under various cooking conditions. However, the impact of these methods of cooking on the other noncarotenoid nutrients in foods is not well established at present.

ABBREVIATIONS USED

HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet; DCI, desorption chemical ionization; ECNI, electron capture negative ionization.

ACKNOWLEDGMENT

We thank F. Hoffmann-La Roche and Co. (Basel, Switzerland) for providing us with some of the reference samples of carotenoids. We thank Dawn Harrison (USDA, Insect Neurobiology and Hormone Laboratory) for her technical support throughout this project. We also thank Dr. George Britton (Department of Biochemistry, University of Liverpool) for his helpful comments and suggestions.

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