ARTICLES

Determination of α- and β-Carotene in Some Raw Fruits and Vegetables by High-Performance Liquid Chromatography

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A high-performance liquid chromatographic method has been developed to quantify α- and β-carotene in some raw fruits and vegetables. Fresh products were extracted with stabilized tetrahydrofuran and concentrated by rotary evaporation. Samples were injected onto a Vydac 218TP54 reversed-phase column with a mobile phase of 40:56:4 acetonitrile/methanol/tetrahydrofuran. Detection was at 470 nm with a sensitivity of 0.04 AUFS. There were no interferences from the cis isomers of β-carotene, but one of the several isomers of α-carotene will interfere with β-carotene. Other carotenoids such as β-cryptoxanthin, canthaxanthin, γ-carotene, 5,6-epoxy-5,6-dihydroxy-β,β-carotene, and the 9 and 15 cis isomers of β-carotene can be separated with this system. The method has been shown to be very reproducible with coefficients of variation ranging from 2.79 to 7.26%. Results from the carotene analyses of nine fruits and vegetables from three different supermarkets are given.

INTRODUCTION

As researchers learn more about the relationship of dietary intake and human health, an accurate and specific assessment of the nutrient content of foods is becoming more important. One such group of nutrients is the provitamin A carotenoids, which are comprised of approximately 18 compounds (Underwood, 1984; Sweeney and Marsh, 1970) varying in vitamin A activity with the most prevalent and active ones found in the common raw fruits and vegetables being α-carotene, β-carotene, and the 9 and 15 cis isomers of β-carotene. β-Cryptoxanthin and γ-carotene possess vitamin A activity but are found in most foods at a low concentration (except for β-cryptoxanthin in peaches, oranges, and sweet corn). Besides their normal vitamin A function, recent research has shown that some provitamin A compounds have anticancer, antiaging, and antiulcer properties (Ong and Chytil, 1983; Moon and Itri, 1984; Wattenberg, 1983; Colditz et al.,1983; Shekelle et al., 1981; Burton and Igold, 1984; Mozsik et al., 1983; Cutler, 1984).

Antineoplastic evidence has been of two types. First, epidemiological studies have shown the existence of an inverse relationship between the risk of cancer and the consumption of foods containing β-carotene (Ong and Chytil, 1983; Moon and Itri, 1984; Colditz et al., 1983; Shekelle et al., 1981; Burton and Ingold, 1984). Second, several laboratory experiments have demonstrated the inhibition of cancer cell lines and actual tumor regression in animals given β-carotene (Ong and Chytil, 1983; Moon and Itri, 1984; Wattenburg, 1983).

Antiulcer properties were observed by Mozsik et al. (1983). They were able to show that β-carotene and β-cryptoxanthin were involved in the cytoprotective injury of the gastric mucosa.

Antiaging effects of carotenoids were demonstrated by Cutler (1984). He was able to show that a positive correlation exists between the concentration of carotenoids in serum and brain tissue with the maximal life span potential of mammalian species.

Because of their important role in nutrition and in some diseases and because of their different degrees of vitamin A activity, there is a need for a simple, rapid, and specific method for individual provitamin A carotenoids in foods. Such a procedure would be beneficial to food scientists, horticulturists, nutritionists, cancer researchers, and epidemiologists. Of the three types of chromatographic methods used for carotenoid determinations—open-column, thin-layer, and high-performance liquid chromatography—HPLC is the best. The other two procedures are very time consuming, can transform carotenoids to their stereoisomers, and cannot distinguish between stereoisomers.

High-performance liquid chromatographic techniques are considered to be the quickest, simplest, and most reproducible methods of analyzing complex mixtures of carotenoids in foods and other substances. The most extensive system has been developed by Sweeney and Marsh (1970) for fruits and vegetables in which most of the prevalent provitamin A carotenoids can be separated. The disadvantages are that this method is lengthy due to an initial open-column step and it employs two HPLC columns that are unavailable commercially. Furthermore, some of the carotenoids could be changed to isomers with this technique. A similar method was developed for citrus fruit in that a column not commercially available was used (Stewart, 1977).

Since 1977 HPLC methods for carotenoids have employed commercially available columns. Both normal and reversed-phase procedures have been developed (Giuseppe et al., 1978; Fiksdahl et al., 1978; Zakaria et al., 1979; Lander and Etenmiller, 1979; Moss, 1979; Braumann and Grimme, 1981; Stancher and Zonta, 1982; Bushway and Wilson, 1982; Hsieh and Karel, 1983; Neils and DeLeanheir, 1983; Gillan and Johns, 1983; Will and Ruddat, 1984; Quackenbush and Smallidge, 1984) with and without gradients, but the most popular packing has been C_{18}. Of these numerous methods, only one, that of Quackenbush and Smallidge (1984), has begun to deal with the complexity of food carotenoids. They were able to rapidly separate several carotenoids including β-carotene and some of its stereoisomers on using a Vydac 201 column with a nonaqueous solvent system. The best results were obtained from a gradient procedure although in the isocratic mode one of the two major β-carotene isomers was separated. Lycopene also interfered with α-carotene under certain conditions.

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This paper describes an isocratic HPLC procedure that is quick, simple, and reproducible and can separate some carotenoids and their isomers in fruits and vegetables. Furthermore, it was used to quantify α-carotene and β-carotene in nine fruits and vegetables from three area supermarkets.

MATERIALS AND METHODS

Materials. *trans*-α-Carotene and lycopene were obtained from Sigma Chemical Co. St. Louis, MO, while trans β-carotene was purchased from Fluka Chemical Corp., Hauppauge, NY. γ-Carotene, canthaxanthin, 5,6-epoxy-5,6-dihydroxy-β,β-carotene, and β-cryptoxanthin were gifts from Hoffmann-LaRoche, Nutley, NJ, and Basel, Switzerland. The stereoisomers of α- and β-carotene were obtained by irradiating 25 mg/100 mL of each solution using the procedure of Zechmeister (1960). Stabilized tetrahydrofuran was bought from VWR Scientific, Bridgeport, NJ, while the acetonitrile and methanol, all HPLC grade, were obtained from Fisher Scientific Co., Fair Lawn, NJ. All fruits and vegetables were brought from three stores (with different suppliers) in the Bangor, ME, area.

Preparation of Standards. Stock solutions of α-carotene and β-carotene were prepared by weighing 25 mg of each into 100-mL actinic volumetric flasks and bringing each to volume with THF. A working standard was made by putting 0.5 mL of each stock into a 25-mL volumetric and bringing to volume with THF. Stock solutions were stable for 4–6 months if kept at −20 °C under nitrogen while the working standard was stable for 1 week under refrigeration. Standard purities were checked by using Beer's law. Irradiated β-carotene, γ-carotene, β-cryptoxanthin, and canthaxanthin, and 5,6-epoxy-5,6-dihydro-β,β-carotene were not of a sufficient amount to prepare working standards. Small amounts were dissolved in stabilized THF and used to identify unknown carotenoids.

Liquid Chromatographic System. The chromatograph consisted of a Waters Model 510 pump (Waters Associates, Milford, MA), a Waters U6K injector, a Waters M490 variable-wavelength UV–vis detector, and an Omniscribe dual-pen recorder (Houston Instrumens, Austin, TX).

Liquid Chromatographic Conditions. The isocratic separation was performed on a 5-μm Vydac 218TP54 column with a solvent system of acetonitrile/methanol/stabilized tetrahydrofuran 40:56:4. Flow rate of 1 mL/min and detection at 470 nm.

Extraction and Analysis. A 5-g sample of fruit or vegetable was weighed into a 125-mL Erlenmeyer flask followed by the addition of 70 mL of THF. The sample was extracted for 1 min with a Polytron (Brinkman Instruments, Westbury, NY) at a speed of 6 and filtered through a Buchner funnel fitted with 7-cm Whatman #42 filter paper. The filter cake and paper were reextracted with 70 mL of THF at a speed of 10 for 2 min, making sure the filter paper was shredded. Combined filtrates were brought to a final volume of 200 mL and an 80-mL aliquot was evaporated to dryness by rotary evaporation at 40 °C. Once dried, the sample was dissolved in 10 mL of THF. If foods contained low levels of carotenes like asparagus, lettuce, white grapefruit, nectarines, and green peppers, the entire filtrate was evaporated to dryness and redissolved in 5 mL of THF.

Five microliters of each product and working standard was injected into the HPLC. Standard was injected first followed by three injections of an extracted food and then standard. Peak height was used for quantitation since it was shown to be linear vs. concentration over the working range.

Reproducibility Studies. Eleven products (1–3 lb each) were ground in a Kitchen Aid to obtain a homogeneous sample. Six 5-g subsamples were removed and extracted and quantified by using the procedure described above. Coefficients of variation were used to determine the reproducibility of this method.

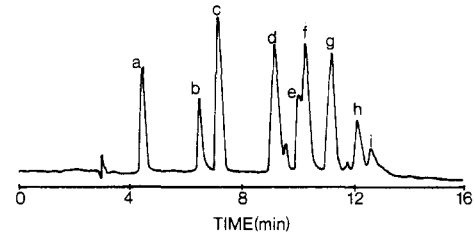

Figure 1. High-performance liquid chromatogram of carotenoids in a mix standard. Peaks: a, canthaxanthin, b, β-cryptoxanthin; c, 5,6-epoxydihydroxy-β,β-carotene; d, lycopene; e, γ-carotene; f, α-carotene; g, β-carotene; h, 9-*cis*-β-carotene; i, 15-*cis*-β-carotene.

Table I. Reproducibility of α- and β-Carotene Determination in 12 Raw Fruits and Vegetables

product	mean μg of carotene/100 g[a]		coeff of variation, %	
	α-carotene	β-carotene	α-carotene	β-carotene
spinach	ND[b]	3842		4.21
carrots	2045	7293	3.20	4.59
beet greens	ND	2181		3.13
sweet potatoes	ND	8288		2.83
asparagus	ND	362		4.02
white grapefruit	ND	84		5.91
lettuce	ND	83		5.04
broccoli	ND	545		7.26
pink grapefruit	ND	144		5.71
nectarines	ND	103		3.52
green peppers	ND	129		4.42
squash	584	1275	3.14	3.61

[a] Each mean is from six determinations. [b] ND = none detected at a limit of 1 μg/100 g.

Supermarket Studies. Nine products were analyzed for this study from three area supermarkets. Three different samples of each item (each sample approximately 1 lb) were purchased from the three stores. The samples were ground in a Kitchen Aid, and a 5-g subsample was removed for analysis. Extraction and quantification were the same as described above.

Absorbance Ratios and Spectra. Each quantifiable carotene from every fruit and vegetable was checked for identity and purity by using absorbance ratios and visible spectra. The absorbance ratios used were 462 nm/440 nm, 455 nm/480 nm and 462 nm/470 nm while the visible scans were made from 340 to 500 nm.

RESULTS AND DISCUSSION

Typical chromatograms depicting the separation of a mix standard and sample are shown in Figures 1 and 2, respectively. With this method nine carotenoids were separated in 13 min, and only two were not 100% resolved (γ-carotene and α-carotene). This was not a problem since most fruits and vegetables do not usually contain γ-carotene and if they do the concentration is low. However, the major food carotenoids were resolved including the difficult to separate α-carotene and the cis isomers of β-carotene. Separation of β-carotene and its cis isomers was extremely important since these isomers were found in most fruits and vegetables (Figure 2). Also spinach like the other produce (when this method is used) contain many other identified carotenoids. One of the several isomers of α-carotene will interfere with β-carotene analysis. This was shown by injecting an irradiated α-carotene standard. The products analyzed here containing α-carotene did not appear to have this α-carotene isomer since

Table II. α- and β-Carotene Content of Nine Raw Fruits and Vegetables from Three Local Supermarkets

product	SM[b]	mean μg of carotene/100 g[a] α-carotene		mean μg of carotene/100 g[a] β-carotene		range μg of carotene/100 g α-carotene	range μg of carotene/100 g β-carotene
spinach	1	ND		5069	271		4784–5323
	2	ND		4040	265		3771–4300
	3	ND		4505	82		4424–4588
broccoli	1	ND		692	12		679–702
	2	ND		686	85		619–782
	3	ND		559	113		429–637
asparagus	1	ND		392	9		382–399
	2	ND		371	53		323–428
	3	ND		189	37		163–232
squash	1	ND		2399	411		2154–2873
	2	ND		7457	749		6613–8044
	3	ND		3410	448		2961–3857
carrots	1	3774	504	8056	945	3361–4313	7088–8976
	2	4020	701	6832	888	3374–4765	5841–7555
	3	3978	1046	11117	442	3160–5157	10700–11500
pink grapefruit	1	ND		515	84		458–611
	2	ND		184	43		146–231
	3	ND		138	54		88–196
white grapefruit	1	7	2	14	5	4–8	9–18
	2	8	3	13	5	4–9	9–18
	3	9	4	15	5	5–13	10–19
green pepper	1	ND		71	3		68–72
	2	ND		79	6		73–83
	3	ND		92	14		83–109
sweet potato	1	ND		9161	3302		5395–11562
	2	ND		8572	359		8194–8908
	3	ND		10743	2359		8298–13005

[a] Each mean is from three determinations. [b] SM = supermarket.

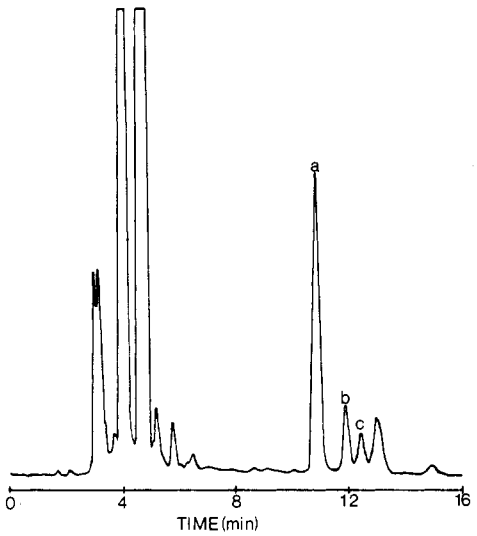

Figure 2. High-performance liquid chromatogram of carotenoids in a spinach extract. Peaks: a, β-carotene; b, 9-*cis*-β-carotene; c, 15-*cis*-β-carotene.

the β-carotene peak was shown to be pure by using absorbance ratios. However, this is something that must be taken into account especially with cooked foods.

Recovery studies were not performed since the extraction procedure was a modification of one developed previously in our laboratory (Bushway and Wilson, 1982). The modifications were such that they would not affect the recovery, and the recoveries on the old method were 100%.

For determining the repeatability of this method and also the homogenicity of the grinding procedure, 12 products were analyzed six times each for their α- and β-carotene content. The results are given in Table I. Coefficients of variation ranged from 2.83 to 7.26% with most below 5%. The results indicate that the method is very reproducible and that sample homogenicity is not a problem with the grinding technique.

Conformation of the α- and β-carotene peaks for each produce was done by taking visible spectra from 340 to 500 nm and absorbance ratios at three sets of wavelengths All the data collected on each product indicated chromatographically pure peaks and correct identity.

Nine fruits and vegetables from three different area supermarkets were analyzed for their α-carotene and β-carotene content (Table II). As one would expect the carrots, sweet potatoes, squash, and spinach had the highest β-carotene levels with white grapefruit the least. What was unexpected was the almost nonexistence of α-carotene. Also from Table II, one can see much carotene variation in the same product from different stores. The produce with the greatest amount of store variation were asparagus, squash, pink grapefruit, and white grapefruit while spinach, broccoli and green peppers had the least variation. This can be extremely important when trying to do epidemiological and nutritional studies. Furthermore, there was not one store that consistently had produce with the highest or lowest carotene content.

This new HPLC method for the determination of α- and β-carotene in fruits and vegetables is rapid and precise and would be useful in nutritional labeling and for studying the effects of differnt cultural and processing treatments on these two carotenes. Furthermore, it would also be useful in quantifying and studying β-carotene isomers if analytical standards were available.

Registry No. 9-*cis*-β-Carotene, 13312-52-2; β-carotene, 7235-40-7; β-cryptoxanthin, 472-70-8; canthaxanthin, 514-78-3; δ-carotene, 472-93-5; 5,6-epoxy-5,6-dihydroxy-β,β-carotene, 100840-35-5; α-carotene, 432-70-2; lycopene, 502-65-8; 15-*cis*-β-carotene, 19361-58-1.

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Oxidation of β-Carotene by Bovine Milk Lactoperoxidase–Halide–Hydrogen Peroxide Systems

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The halide-mediated peroxidase-catalyzed oxidation of β-carotene was studied as a model system for lipid peroxidation. Of the halide ions tested, Br^-, I^-, and SCN^- all caused some degree of β-carotene oxidation at pH 7.0 and 4.4. No oxidation was observed with Cl^-, however, which is consistent with Cl^- not being a substrate for lactoperoxidase. The β-carotene oxidation caused by the lactoperoxidase–I^-–H_2O_2 system was pH dependent, the rate of oxidation being 100-fold higher at pH 4.4 than at pH 7.0. When both I^- and SCN^- were present, the oxidation rate was reduced. At least partially, this interference between the two substrates was due to competition for the substrate-binding sites on the enzyme.

The enzyme lactoperoxidase (LP) (EC 1.11.1.7), which occurs in milk from several species, forms an antibacterial system together with the substrates thiocyanate (SCN^-) and hydrogen peroxide (H_2O_2) (Reiter et al., 1963; Björck et al., 1975; Reiter, 1978a, 1979). The general product of the peroxidase-catalyzed reaction is presumed to be hypothiocyanite ($OSCN^-$) (Hoogendoorn et al., 1977), but other compounds might also be formed (Hogg and Jago, 1970; Björck and Claesson, 1980; Pruitt et al., 1982). Hypothiocyanite reacts with protein sulfhydryl groups, and the antibacterial effect is inhibited by sulfhydryl-containing reducing compounds, like cysteine and dithiothreitol (Aune and Thomas, 1978; Thomas and Aune, 1978). LP can also catalyze the peroxidation of the halides bromide and iodide (Morrison and Schonbaum, 1976).

Lipid peroxidation might be mediated by peroxidase-catalyzed oxidation of halides (Benenson et al., 1980). A direct bleaching of β-carotene was observed with horseradish peroxidase (Ben Aziz et al., 1971). Kanner and Kinsella (1983a,b) have developed a model system for the study of the peroxidation of β-carotene and linoleate, mediated by the LP-catalyzed peroxidation of various halides. By dissolving β-carotene in the presence of a detergent it is possible to get a stabilized water solution in which it is possible to directly follow its oxidation.

This paper deals with the halide-mediated oxidation of β-carotene by lactoperoxidase and SCN^-, I^-, and Br^- at pH 4.4 and 7.0 and the interaction between SCN^- and I^- at different concentrations.

MATERIALS AND METHODS

Chemicals. LP was purchased from Boehringer Mannheim (Mannheim, West Germany). H_2O_2 (30%, analytical grade) and β-carotene (synthetic, 97%) were from Merck (Darmstadt, West Germany). All other chemicals used were of analytical grade, and double-distilled water was used in all solutions. According to the specifications given by the manufacturer the sodium chloride contained less than 0.005% Br^- and 0.001% I^-.

Aqueous β-Carotene Solution. A 25-mg portion of β-carotene and 0.9 mL of Tween 80 were dissolved in 25 mL of chloroform. A 1-mL portion of this solution was evaporated to dryness under vacuum and the residue dissolved immediately in 10 mL of 0.25% EDTA solution and filtered through filter paper. Thereafter, 40 mL of 0.01 M sodium acetate buffer (pH 4.6) was added. The β-carotene solution was prepared on the day of the experiment (Ben Aziz et al., 1971). The final concentration of β-carotene was adjusted to ~14 μM, as determined spectrophotometrically.

β-Carotene Oxidation Assay. The amount of unreacted β-carotene was measured as the absorption at 460 nm. The absorbance measurements were carried out on

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