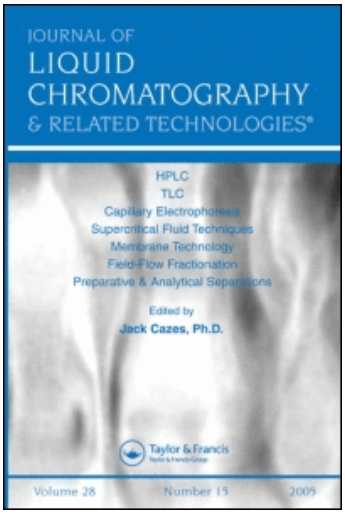

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REVERSE PHASE HPLC SEPARATION OF CIS- AND TRANS-CAROTENOIDS AND ITS APPLICATION TO β-CAROTENES IN FOOD MATERIALS

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ABSTRACT

Four trans-carotenoids, β-carotene, α-carotene, lutein and lycopene, were isomerized with light energy under iodine catalysis and the isomeric mixtures were subjected to reverse phase chromatography on a 5μ VYDAC TP-201 column with mobile phases of methanol-chloroform to separate the major isomers. The amounts and properties of the cis-compounds conformed with separations obtained by others from lime and alumina columns. In each case, the predominant isomers behaved as 13-cis- and 9-cis-isomers, except for the lycopene set which included isomers not previously separated. Use of the system for rapid analysis of 13-cis, 9-cis- and trans-β-carotene in some common food materials was demonstrated. The percentage of cis-isomers in the β-carotene fraction ranged from 1.6 in raw carrot to 29.6 in canned peach.

INTRODUCTION

cis-Isomerism is a widely recognized property of carotenoids. Extensive studies by Zechmeister et al (1,2) established a pattern of isomerism effected by light or heat exposure upon many pure

trans-carotenoids, which, for steric reasons, favored 9-cis- and 13-cis-configurations as the major products. Tsukida et al (3) and Vecchi et al (4) have detailed these rearrangements for β-carotene. Chromatography with lime is the usual technique for separation; less complete separations are made with alumina. However, few commercial limes produce efficient separations, and their use requires unusual control of conditions. Such columns are not available commercially.

Reverse phase HPLC has not been widely successful for separation of the carotenes. Clean separation of α-carotene from β-carotene has been achieved with only a few such systems, and separation of cis- from trans-isomers has been demonstrated only for the β-carotene set of isomers. In tests with more than twenty commercial columns, only three showed this ability, the VYDAC TP-201 being the most effective (5,6). The usefulness of this column has been confirmed with different mobile phases; comparisons also were made with four other columns (7). This paper quantifies the analysis of cis-β-carotenes and extends the study to separation of cis-isomers of three other carotenoids.

Because of the tedium in current methodology, few workers have quantified cis-isomers in analysis of biological materials. Quantitative HPLC separations of cis-isomers on normal (lime) columns has been reported for a number of fruits and vegetables (8) but no such study has utilized commercially available reverse phase columns.

Only a few carotenoids occur naturally in the cis-form (2) e.g., prolycopene in tangerine tomatoes and cis-neoxanthin in chloroplasts; most are in the all-trans-configuration, and cis-isomers are generally considered to be artifacts attributable to isomerization during processing and analytical steps. The efficacy of the reverse phase system used in this study, together with a rapid method for extraction and solution preparation, has facilitated the reexamination of these relationships for β-carotene in some representative foods.

MATERIALS AND METHODS

The trans-pigments, β-carotene, α-carotene and lutein, were purified by open column chromatography and crystallization; lycopene by direct crystallization from an extract. All were tested for purity spectrophotometrically and by HPLC, the more sensitive test, prior to use in isomerization studies; purity was 98% or higher in each case. Original sources, before purification, were commercial for α- and β-carotenes, tomato for lycopene, marigold petal for lutein. Chloroform (Fisher) was certified ACS grade chemical. Methanol, iso-octane and hexane were HPLC grade. Sudan I, 1-(phenylazo)-2-naphthalenol (Aldrich), was recrystallized (9) before use as standard. Foods were from local stores or home garden.

The chromatograph consisted of a Spectra Physics 8700 Solvent Delivery System (helium-degassed), Beckman 165 Detector with spectral scan feature, and SP 4100 Computing Integrator, with a 5 μ VYDAC 201TP column (Cat. No. H5616, ANSPEC Co., Ann Arbor, MI 48107) preceded by an ODS guard column (Cat. No. 4102-010, Whatman Co., Clifton, NJ 07104). Mobile phase was 94:6 methanol-chloroform (MC), except as otherwise stated. In most cases, spectral scans were made during stop-flow of a few seconds' duration. Such scans showed reproducability of maxima within ± 2 nm. For the iodine-catalyzed light isomerization (1), 10 mL of iso-octane containing the pigment and iodine under nitrogen in a r.b. flask was placed 60 cm from a 2 x 40 watt fluorescent light source for a 5 to 8 minute period. In darkness, the solvent was removed immediately under vacuum. The residue was taken up in methanol with the aid of sufficient chloroform to dissolve it completely.

RESULTS AND DISCUSSION

Separation of cis-Isomers After Iodine-isomerization of trans-Carotenoids

cis-Isomers constituted nearly half of the components of the β-carotene reaction mixture. Spectral scans (Table 1) of the

TABLE 1

Properties of Major Isomers Separated by HPLC After Isomerization of trans-Carotenoids.

Starting Compound	Peak No.	% of Total Area	Absorbance Maxima, nm I	II	Q-ratio (II/cis)	Probable Identity
β-Carotene	1	52.7	477	453	<12	all-trans
	2	17.5	469	444	2.3	13-cis (neo B)
	3	24.7	473	448	8.5	9-cis (neo U)
α-Carotene	1	62.3	473	446	<12	all-trans
	2	8.1	466	441	3.7	13-cis (neo B)
	3	24.1	468	442	8.6	9-cis (neo U)
Lutein	1	8.7	465	439	2.0	13-cis* (neo-B)
	2	6.7	463	437	2.3	13'-cis* (neo-B)
	3	18.8	467	440	8.6	9-cis (neo U)
	4	63.8	473	447	<12	all-trans
Lycopene	1	3.5	484	450	6.5	
	2	12.7	497	467	5.3	
	3	29.4	501	474	10.7	
	4	47.6	501	473	11.4	
	5	2.0	495	467	1.8	
	6	4.6	496	469	1.8	

*These isomers may be in reverse order. Probable identities of all isomers are based on comparisons with published data (1-3).

numbered peaks (Figure 1-A) separated by stepwise HPLC (methanol for 5 min, then 94:6 MC), showed absorbances that were in good agreement with published data for the major β-carotene isomers. Zechmeister and coworkers (2) had designated the three main components as Neo-B, all-trans and Neo-U, in order of elution from the lime columns. Amounts were in the ratio of 25:48:22; two other entities totaled about 5% of the recovered components. Based partly on theoretical considerations of steric hindrance, Neo-U was believed to be 9-cis-β-carotene and Neo-B, incorrectly, to be 9,13'-di-cis-β-carotene.

Subsequent HPLC separations of similar iodine-isomerized mixtures by Vecchi et al (4) with an alumina column and by Tsukida et al (3) with a lime column produced similar distribution patterns

of components. When they isolated the cis-isomers and characterized them by NMR and spectral measurements, the predominant isomers were found to be 13-cis-β-carotene (neo B) and 9-cis-β-carotene (neo U). Height of the cis-peak, at 338 nm, reflected by the Q-ratio (absorbance at maximum/absorbance at cis-peak), clearly distinguishes 13-cis- from 9-cis-isomers. The mono-cis-isomer total was 40% of the recovered pigment. This included a minor (1.5%) third isomer, photolabile 15-cis-β-carotene with Q-ratio < 2.0 (3,10); the di-cis total was about 10% of the component mixture. The close agreement between properties observed in the above-mentioned studies and those of Table 1 clearly indicates that the numbered peaks in Figure 1 represent all-trans-, 13-cis- and 9-cis-β-carotenes, in the order of elution.

α-Carotene was iodine-isomerized similarly. The reaction mixture, subjected to the same HPLC mobile phase of methanol followed by 94:6 MC, yielded three major peaks (Figure 1-B), but the separations were less distinct and the latter two peaks were unusually broad. Omission of the methanol step in the HPLC sequence resulted in a single, broad, second peak. A small component that eluted just prior to the main peak was not characterized. Properties of the three numbered peaks were parallel to those of the β-carotene set: Peak 1, same as the parent trans-α-carotene; Peak 2, a large spectral shift, with a high cis-peak; and Peak 3, a smaller spectral shift, and a much lower cis-peak. The properties reported by Zechmeister (2) for the major cis-isomers of α-carotene, Neo-B and Neo-U, were similar to those in Table 1; three minor isomers were also described. The yield and properties of Neo B separated by Nash and Zscheile (11) also were similar. The data at hand support identity of the numbered peaks in Figure 1-B as all-trans-, 13-cis- and 9-cis-α-carotenes, in the order of elution.

Lutein isomers were separated with methanol as mobile phase. Three lesser peaks preceded elution of the parent trans-lutein peak (Figure 1-C). Spectral scans at Peaks 1 and 2 showed nearly identical properties, with an 8-10 nm spectral shift and low Q-ratios that are typical of 13-cis-carotenoids (Table 1). The scan

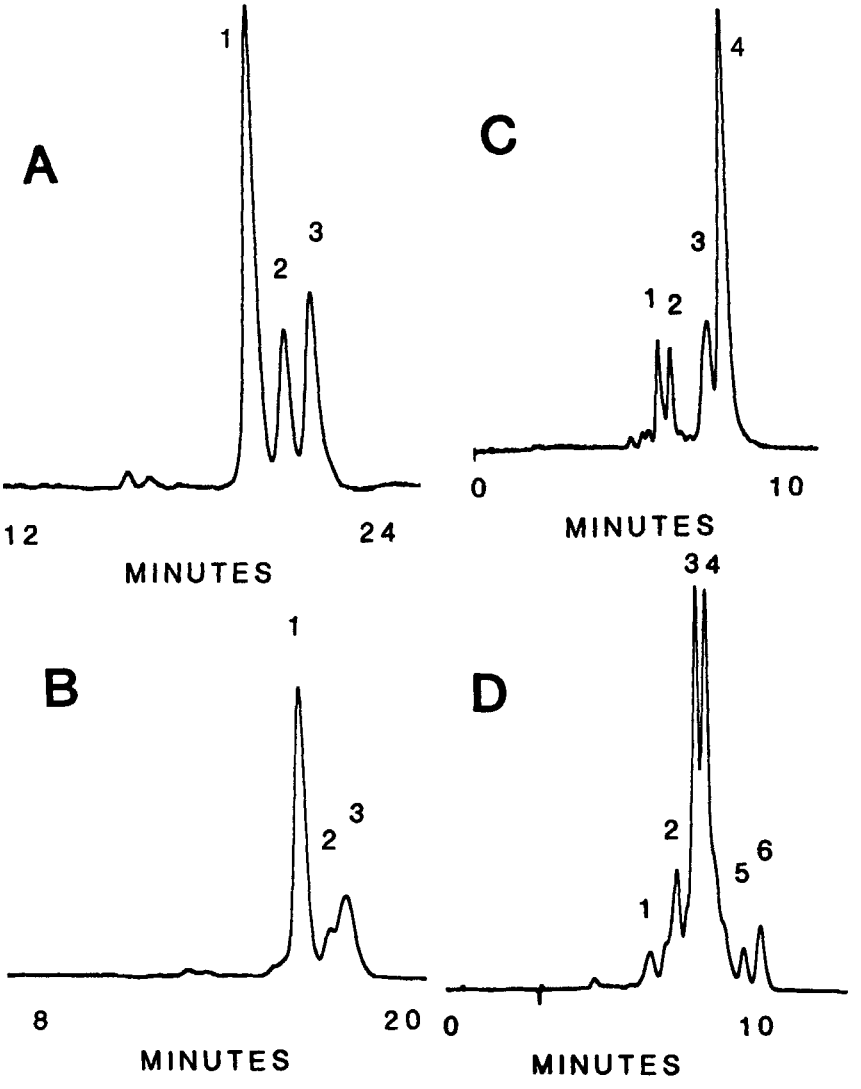

FIGURE 1. Separation of isomers after radiation. A. β-Carotene after 8 minutes of radiation. Mobile phase: methanol for 5 min, then 94:6 MC. No peaks prior to 12 min. 1) all-trans; 2) 13-cis; 3) 9-cis. B. α-Carotene after 5 minutes. Mobile phase: same as A. 1) all- trans; 2) 13-cis; 3) 9-cis. C. Lutein after 8 minutes. Mobile phase: methanol. 1 and 2) 13-cis; 3) 9-cis; 4) all-trans. D. Lycopene after 5 minutes. Mobile phase: 94:6 MC. Properties of numbered peaks are in Table 1.

for peak 3 showed 9-cis characteristics. Zechmeister (2) observed two major cis-components: Neo-A, with a high cis-peak, thought to be 13- or 15-cis-α-carotene), and Neo-B, with a low cis-peak, thought to be 9- or 9'-cis-α-carotene. It is possible that Peaks 1 and 2 reflect a separation of 13-cis- from 13'-cis-lutein, both of which are likely to be present. If so, presence of a mixture of 9-cis- and 9'-cis-luteins could explain the disproportionate width of Peak 3. The analogy might also extend to the broad Peaks 2 and 3 of Figure 1-B.

Lycopene isomers were separated with 80:20 MC, six as distinct peaks; four others appeared as inflections on Peaks 2, 3 and 4 (Figure 1-D). Spectral properties of the six are in Table 1. Zechmeister (2) described two cis-isomers, Neo A and Neo B, that accounted for 43% of the recovered pigment; both showed double cis-peaks. He also referred, as "unpublished", to isolation of Neo C and Neo D, with insufficient data to permit comparison with data in Table 1. The HPLC pattern for lycopene isomers is different from the above three in that components with low Q-ratios, typical of 13-cis- or 15-cis-isomers, are the first to elute. Peak 4 has properties consistent with those of the parent all-trans-compound (Table 1). All five cis-isomers showed double peaks, but the limited number of isomers observed by Zechmeister and the failure of his neo-fractions to match the Table 1 data suggest that separations on his lime columns were inferior. In the absence of such parallel data, probable identities of the peaks in Figure 1-D are not appropriate.

From the separations of components of the iodine-isomerization mixtures that have been attained (Table 1 and Figures 1) it appears that reverse phase HPLC can be a highly useful tool for separation of carotenoid cis-isomers both in studies of the isomerization reaction and in the analysis of natural products.

Application to Analysis of β-Carotene Isomers in Food Products

A representative group of root, leaf and fruit products, some fresh and some canned, was selected for this study. A 10- or

20-gram portion was blended with 200 mL MC (50+50) to produce a single-phase extract, and the insolubles were allowed a few minutes to settle. An aliquot (25 to 100 mL) of supernate placed in a separatory funnel was shaken with enough water to produce two phases, and after complete separation the hypophase was transferred to a second separator that contained 25 mL of 70% methanol (aqueous). The resultant single phase was broken by addition of a few drops of water, the hypophase transferred to a r.b. flask, 5 mL of ethanol added, and the extract reduced to dryness in a rotary evaporator. Samples that contained carotenol esters, e.g., apricot, peach, butternut squash, required a saponification step (6). To ester-free residues, cooled to <15 C, was added by pipet 1 to 5 mL of methanol and the flask then stoppered and rotated to dissolve all pigments. A portion (20 µL) was injected for HPLC analysis. The above procedure was conducted under low light intensity, and, from sample weighing to injection, was completed within a 60-minute period, exclusive of saponification. Details of chromatography, calculation and quantitation have been described elsewhere (6). In this study, Sudan I was used as external standard, and 2.79, 4.23 and 2.88 were the factors used for all-trans-, 13-cis- and 9-cis-β-carotenes, respectively, in calculations.

For best separations of the cis-β-carotenes, the stepwise methanol- 94:6 MC elution is preferred. A typical example of such separation is seen in Figure 2-A, for kale leaves taken from the greenhouse for immediate extraction. However, for routine carotene analysis, isocratic elution with 94:6 MC produces usable results. Such a one-step chromatogram obtained with an extract of canned carrot (Figure 2-B) shows the measureable cis-β-carotene peaks and also the cis-α-carotene peak that appears between trans-α- and trans-β-carotenes. For simplicity, α-carotene and carotenol data are not included in Table 2.

To ascertain whether cis-isomerization of β-carotene occurred during analysis, a solution of crystalline β-carotene was added as a spike to a 20-gram sample of canned pear. Analysis showed that the pear contained little carotene with no measurable cis-components and

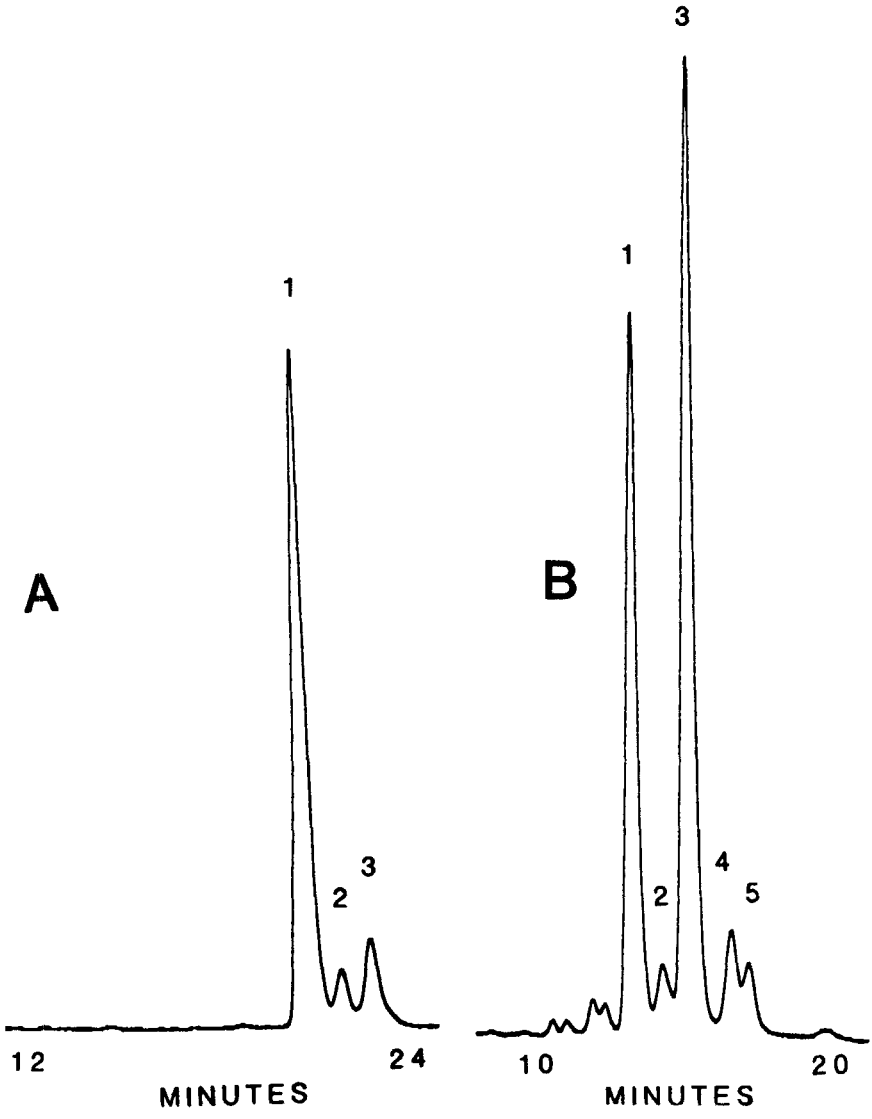

FIGURE 2. Separations of carotenes. A. Extract of fresh kale. Mobile phase: methanol for 5 min., then 94:6 MC. 1) all-trans-β-carotene; 2) 13-cis-β-carotene; 3) 9-cis-β-carotene. B. Extract of canned carrot. Mobile phase: 94:6 MC. 1) α-carotene; 2) cis-α-carotene; 3) β-carotene; 4) 13-cis-β-carotene; 5) 9-cis-β-carotene.

TABLE 2

β-Carotenes in Fresh and Canned Produce.

Product	Fresh or Can	Total β-Carotenes µg/100g	13-cis %	9-cis %	cis, % of Total
Pear	C	4	--	--	--
Pear + β-spike		1,887	tr	tr	tr
Carrot	F	9,936	1.2	0.4	1.6
Carrot	C	6,030	13.8	6.3	20.1
Sweet potato	F	5,029	2.1	0.8	2.9
Sweet potato (yam)	C	5,465	17.1	8.8	25.9
Kale	F	8,357	4.4	7.8	12.6
Spinach	F	8,220	3.2	7.4	12.4
B-nut squash	F	8,378	5.0	1.4	6.4
Apricot	C	2,402	11.8	6.2	18.0
Peach	C	433	23.6	6.0	29.6

that the spiked sample also had no measurable cis-β-carotene (Table 2).

The fresh, yellow root-vegetables, carrot and sweet potato, contained small, but measurable, amounts of cis-isomers, more 13-cis than 9-cis. In contrast, fresh greens, kale and spinach, had several-fold more cis-isomer, preponderantly 9-cis. Raw butternut squash was intermediate, with distribution similar to the roots. Canned root vegetables had about 10-fold as much total cis-isomer content as fresh.

Canned fruits, apricot and peach, had amounts and distribution of cis-isomers similar to those of canned root-vegetables. It is apparent that canning increases 13-cis-isomerization more than 9-cis-isomerization. This is in accord with the results of Sweeney and Marsh (8), who used non-commercial normal-phase columns in the only substantial study of β-isomers in foods. In general, the amounts of cis-isomers in Table 2 are lower in fresh produce, and higher in canned produce, than the amounts reported by Sweeney and Marsh. This difference may reflect the shorter analysis time and less exposure of the sample that are inherent in the Table 2 data.

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