

HPLC Separation of Geometric Carotene Isomers Using a Calcium Hydroxide Stationary Phase[†]

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HPLC methodology employing a calcium hydroxide stationary phase selective for separations of phytofluene, ζ -carotene, γ -carotene, α -carotene, and β -carotene geometric isomer sets was developed. The retention behavior of certain members of these isomer sets and the resulting impact of modifier selection and concentration was demonstrated. Pertinent modifications for specific analytical situations are recommended. Individual purified carotenoids were isomerized by iodine catalysis. Resolution of phytofluene isomers was achieved using hexane, that of α -carotenes using 0.1% *p*-methylanisole in hexane, that of β -carotenes using 2.0% *p*-methylanisole in hexane, that of ζ -carotenes using 1.4% benzyl ether in hexane, and that of γ -carotenes using 2.45% benzyl ether in hexane. Retention characteristics on calcium hydroxide and electronic absorption spectra allowed assignment of geometric configurations to some of the resolved peaks. These methods may be used, with modifications where necessary, to analyze isomeric sets of carotenes and to isolate individual isomers.

Keywords: *Phytofluene; ζ -carotene; γ -carotene; α -carotene; β -carotene; geometrical isomers*

INTRODUCTION

Carotenoids are uniquely functional polyene pigments ubiquitous in nature (Isler, 1971). The list of known naturally occurring carotenes (hydrocarbon carotenoids) and xanthophylls (oxygenated carotenoids) has grown to approximately 600, and this number is increased severalfold when known and theoretically possible geometric and optical isomers associated with these compounds are considered (Olson, 1992). The presence of certain carotenoids is mandatory in the plant kingdom for photoprotection and optimal photosynthetic function (Goodwin, 1980). In the animal kingdom, certain carotenoids serve as precursors to biologically active retinoids in humans and several other species (Goodwin, 1984). An increasing amount of evidence indicates that carotenoids have physicochemical properties which potentially may have, in humans and other mammals, biological functions independent of their role as precursors to vitamin A (Burton and Ingold, 1984; Di Mascio et al., 1989; Miki, 1991; Olson, 1992).

The differentiation of individual members of geometric carotenoid sets was made possible by the advent of chromatography, and adsorption chromatography has been the traditional choice for the separation of geometric carotenoid isomers using either column or thin layer chromatography (Zechmeister, 1950; Britton, 1985). The extensive contributions of Zechmeister and co-workers to the analysis of geometric carotenoid sets using column chromatography have been reviewed (1962) and should be consulted accordingly to obtain a fundamental knowledge of the occurrence and analysis of *cis-trans* isomeric carotenoids. Reversed-phase HPLC separations of geometric carotene isomers have recently been improved (Craft et al., 1990; Stahl et al., 1993), as have separations using supercritical fluid chromatog-

raphy (Schmitz et al., 1989; Aubert et al., 1991; Lesellier et al., 1991). However, normal-phase HPLC techniques using adsorptive stationary phases generally provide the greatest selectivity when optimal resolution of individual geometric isomer sets is desired (Craft et al., 1990; O'Neil et al., 1991). The excellent resolution of certain carotenoid isomer sets using a polymeric C₃₀ stationary phase (Emenhiser et al., 1995), developed by Sander et al. (1994), should be noted as a possible exception to this generalization. The elegant work of Tsukida et al. (1982) and Vecchi et al. (1981) demonstrated the exceptional abilities of calcium hydroxide and alumina adsorption supports used in conjunction with HPLC to resolve geometric isomers from the β -carotene set. The use of HPLC employing a calcium hydroxide stationary phase to resolve members of the neurosporene (Katayama et al., 1990), canthaxanthin (Hashimoto et al., 1988), and spirilloxanthin (Koyama et al., 1990) geometric isomers has also been reported.

The chromatographic pursuit of resolution and identification of individual geometric isomers of carotenoids is not an exercise in pedantry. The presence of 15-*cis*- β -carotene and other 15-*cis* carotenoids in the reaction centers of certain photosynthetic bacteria and their role in photoprotection have been established (Koyama, 1989; Koyama et al., 1990). Parry and Horgan (1992) have presented convincing evidence that 9'-*cis*-neoxanthin is an essential metabolic precursor in the biosynthetic pathway leading to abscisic acid in certain roots. *cis*-Carotenes have also been noted as essential cofactors during the desaturation and cyclization reactions in the carotenoid biosynthetic pathways of certain plant species (Beyer et al., 1989). In mammals, the importance of *cis-trans* carotenoid isomers is manifest in the significantly altered vitamin A activity of provitamin A carotenoids, such as β -carotene and γ -carotene, imparted by a change in geometric conformation (Zechmeister, 1962; Sweeney and Marsh, 1973).

Interest in the biological functions that individual geometric isomers of carotenoids have or potentially may have is increasing. Unfortunately, the analysis of

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[†] Paper FSR95-11 of the Journal Series of the North Carolina Agricultural Research Service, Raleigh, NC.

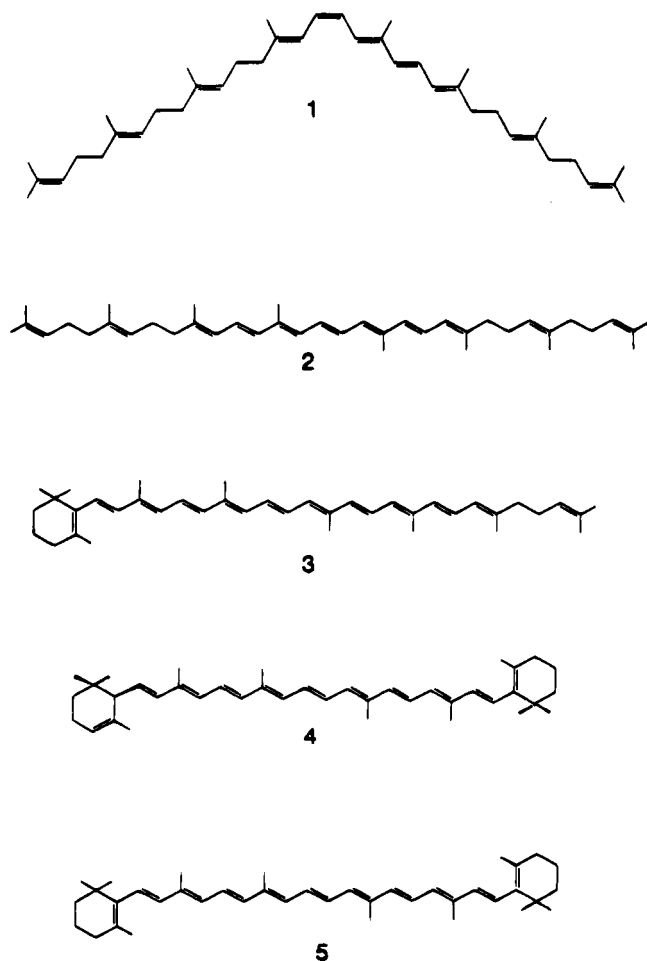


Figure 1. Predominant geometrical configurations of 1, 15-*cis*-phytofluene; 2, *all-trans*- ζ -carotene; 3, *all-trans*- γ -carotene; 4, *all-trans*- α -carotene; and 5, *all-trans*- β -carotene.

individual geometric carotenoid isomers present in certain sets can be extremely difficult, making confirmation of their presence and potential biological relevance equally difficult. The contributions of Zechmeister (1962) to this endeavor, though invaluable to this field of research, should be improved upon using more modern chromatographic techniques, especially HPLC. The purpose of the following research was to supplement the work of Tsukida et al. (1982) and Koyama et al. (1988, 1990) by demonstrating the utility of calcium hydroxide as a stationary support in conjunction with HPLC for the separation of various members of acyclic and cyclic geometric carotene isomer sets. The predominant geometrical configurations of these carotenes are shown in Figure 1.

EXPERIMENTAL PROCEDURES

Preparation of Carotene Isomers. Crystalline α -carotene was obtained from Sigma Chemical Co. (St. Louis, MO), and β -carotene was obtained from Hoffmann-La Roche (Nutley, NJ). Each compound was purified on deactivated alumina before further manipulation.

Processed carrot baby food and fresh tomatoes were extracted according to previously published procedures (Schmitz et al., 1989). Phytofluene, γ -carotene, and ζ -carotene isomers were isolated from these extracts using fully activated alumina weakened with 3% H_2O and an elution gradient employing acetone in hexane (Davies, 1976; Britton, 1985). Phytofluene and phytoene were eluted with 100% hexane, α - and β -carotenes were eluted with 99% hexane/1% acetone, and, finally, γ - and ζ -carotenes were eluted with 97% hexane/3% acetone.

Preparation of Geometric Carotene Isomers. α - and β -carotene standards were isomerized by catalysis with iodine in the presence of sunlight (Zechmeister, 1962). The procedure of Zechmeister was modified by adding approximately 25% of the iodine concentration originally recommended. Multiple geometric isomers of phytofluene, ζ -carotene and γ -carotene, were present in the processed carrot baby food due to thermal isomerization (Zechmeister, 1962; Chandler and Schwartz, 1987). Portions of these mixtures of carotene isomers were also subjected to photocatalytic isomerization in the presence of iodine as above, or, in the case of the phytofluene extract, isomerized only by exposure to sunlight for 30 min.

Preparation of Calcium Hydroxide HPLC Column. The analytical column used for this analysis was packed with calcium hydroxide. The stationary phase was generously provided by Professor Y. Koyama, Kwansei Gakuin University, Uegahara, Nishinomiya, Japan. Prior to packing, the $Ca(OH)_2$ was sieved through 500 mesh and equilibrated to 44% relative humidity in a K_2CO_3 saturated chamber for 48 h. Six grams of this calcium hydroxide was suspended in 50 mL of 0.5% acetone in hexane and sonicated for 10 min. Slurry packing of the column was then accomplished using a column packing reservoir (Scientific Systems, Inc., State College, PA) pressurized to 2600 psi using a Haskel air-driven fluid pump (Haskel, Inc., Burbank, CA). Approximately 100 mL of packing solvent was allowed to percolate through the column under packing pressure.

HPLC Instrumentation and Sample Analysis Conditions. The HPLC system consisted of a Model 510 pump controlled by a Model 680 automated gradient controller, a Model U6K injector, and a Model 990 photodiode array detector (Waters, Inc., Milford, MA) equipped with a Powermate SX/20 series computer (NEC Information Systems, Inc., Boxborough, MA) to obtain electronic absorption data. The chromatograms shown in this paper were obtained using an Anspec SM 95 UVIS detector (Linear, Ann Arbor, MI) coupled with a Shimadzu CR601 Chromatopac integrator (Kyoto, Japan). Column effluent was monitored at 325, 400, 440, and 455 nm for isomers of phytofluene, ζ -carotene, α - and β -carotenes, and γ -carotene, respectively.

Isocratic eluents consisting of hexane alone or hexane modified with either acetone, *p*-methylanisole, or benzyl ether were used at a flow rate of 0.7 mL/min for all separations. The individual elution solvents used for each separation are as follows: phytofluene, 100% hexane; α -carotene, 0.1% *p*-methylanisole in hexane; β -carotene, 2.0% *p*-methylanisole or 2.0% acetone in hexane; ζ -carotene, 1.4% benzyl ether in hexane; γ -carotene, 2.45% benzyl ether in hexane. Electronic absorption data in the UV region were obtained using an isocratic eluent of hexane modified with acetone because interference occurred in this region when *p*-methylanisole or benzyl ether was used. Selectivity of acetone toward carotene isomers was the same as that observed for *p*-methylanisole or benzyl ether. However, peak shape was improved when the *p*-methylanisole or benzyl ether modifiers were used. The concentration of acetone used to obtain these data was approximately equal in concentration to that of *p*-methylanisole and twice the concentration of benzyl ether in each developing solvent.

All samples were dried over sodium sulfate and redissolved in hexane prior to injection. Injection volumes were 10–20 μ L when using the fixed-wavelength detector and 200–300 μ L when the photodiode array detector was used.

Identification of Carotenoids and Their Geometric Isomers. The identities of the five individual carotenes were unambiguously assigned according to retention behavior on alumina, monomeric C_{18} , and calcium hydroxide supports, electronic absorption data obtained from photodiode array analysis, and molecular weight information obtained using fast atom bombardment mass spectrometry as previously described (Schmitz et al., 1992). Carotene isomers were tentatively identified by comparison of chromatographic retention behavior and electronic absorption spectra with those available in the literature (Zechmeister, 1962; Tsukida, 1992).

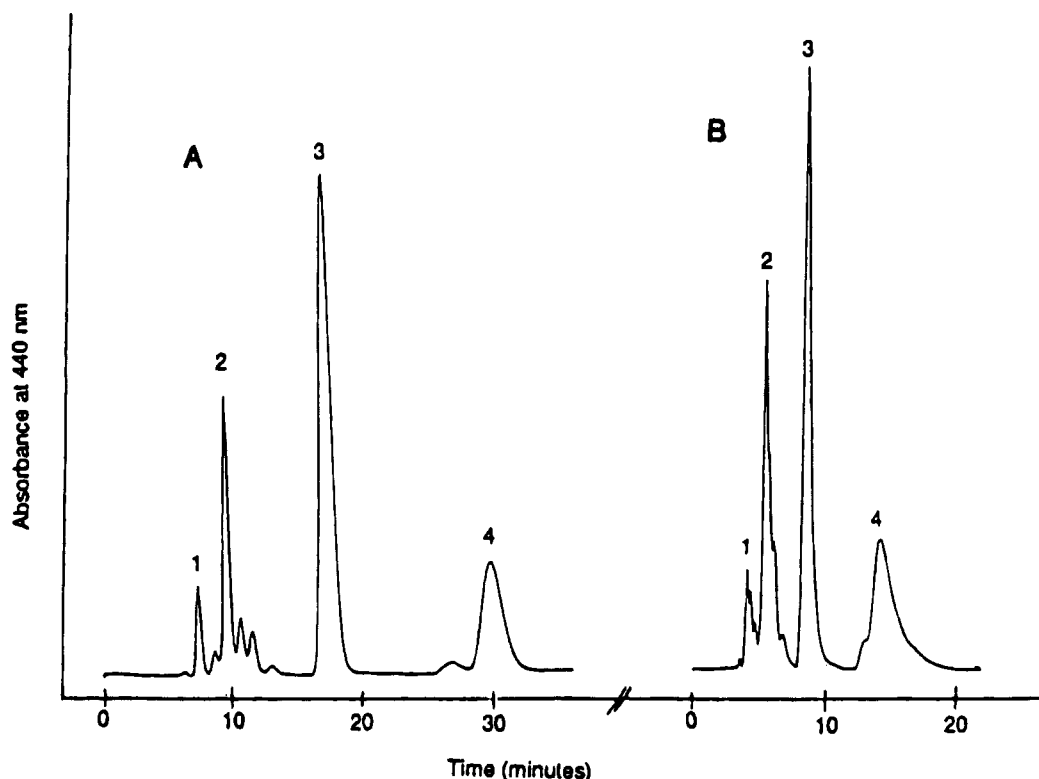


Figure 2. HPLC separation of an iodine isomerized β -carotene mixture using (A) 2% *p*-methylanisole in hexane and (B) 2.0% acetone in hexane. Identification of peaks: (1) 15-*cis*-, (2) 13-*cis*-, (3) *all-trans*-, and (4) 9-*cis*- β -carotene.

RESULTS AND DISCUSSION

Figure 2A shows the HPLC separation of geometric β -carotene isomers using a calcium hydroxide column in conjunction with a hexane developing solvent modified with 2.0% *p*-methylanisole. Excellent resolution of β -carotene isomers was achieved. The utility of calcium hydroxide for isomeric β -carotene separations has been appreciated for several decades (Zechmeister, 1962), and an extensive investigation in pursuit of optimization of column and thin layer chromatography using this stationary phase is reported by Bickoff (1948). The best contemporary analysis of geometric β -carotenes was reported by Tsukida et al. (1982), who employed HPLC in conjunction with a calcium hydroxide support and hexane mobile phase modified by acetone to obtain chromatographic resolution of a previously unattainable number of isomeric β -carotenes.

The choice of modifier to the primary developing solvent, usually hexane or petroleum ether for carotene separations, is apparently critical to the chromatographic results obtained during the analysis of β -carotene using calcium hydroxide supports. *p*-Methylanisole and anethole were identified as the modifiers of choice for the separation of neo-*B* (primarily 13-*cis*) and neo-*U* (primarily 9-*cis*) from *all-trans*- β -carotene using either column or thin layer chromatography (Bickoff, 1948). The results of the present study are in agreement with this finding. The use of an equal concentration of *p*-methylanisole (Figure 2A) resulted in greater peak retention and superior resolution of the geometric β -carotenes as well as decreased peak tailing of 9-*cis*- β -carotene, when compared to the use of acetone (Figure 2B) for separation of an identical β -carotenes mixture.

The separation of geometric α -carotene isomers using a mobile phase of hexane modified with 0.1% *p*-methylanisole is shown in Figure 3. The HPLC resolution of

at least two *cis*- α -carotenes has been achieved using a calcium hydroxide support and an eluting solvent of hexane modified with acetone (Chandler and Schwartz, 1987; Pettersson and Jonsson, 1990), while carotene fractionation using reversed-phase HPLC followed by TLC with calcium hydroxide completely resolved four α -carotene isomers (Schwartz and Patroni-Killam, 1985). The HPLC separation of geometric α -carotenes shown in Figure 3 compares favorably with the work of Zechmeister and Polgar (1944), and tentative peak identifications given for this figure are based on comparison of electronic absorption spectra with those of Zechmeister and Polgar (1944).

α -Carotene is an asymmetrical carotenoid (Figure 1), and thus it may assume substantially more *cis* configurations than its structural isomer, β -carotene (Zechmeister, 1962). The chromatographic result of this structural difference is illustrated in Figure 3 by the presence of two major peaks eluting after the *all-trans* form of α -carotene rather than the single major peak (9-*cis*- β -carotene) that elutes after *all-trans*- β -carotene (Figure 2A,B). The identities of the two peaks eluting after *all-trans*- α -carotene are presumably 9-*cis*- and 9'-*cis*- α -carotenes on the basis of adsorption affinity and electronic absorption data (Zechmeister, 1962).

The ability of calcium hydroxide to separate acyclic carotene geometric isomer sets has been demonstrated previously by Zechmeister (1962) and Hashimoto et al. (1988). However, a mixture of alumina/calcium hydroxide/Celite (3:1:1) or alumina was recommended by Petracek and Zechmeister (1952) for optimal resolution of 15-*cis*- and *all-trans*-phytofluenes using column chromatography. One additional unidentified isomer was reported by Wallace and Porter (1952) when a mixture of phytofluenes was eluted from ignited magnesium oxide/Super Cel with acetone in hexane. Beyer et al. (1985) successfully extended these applications by re-

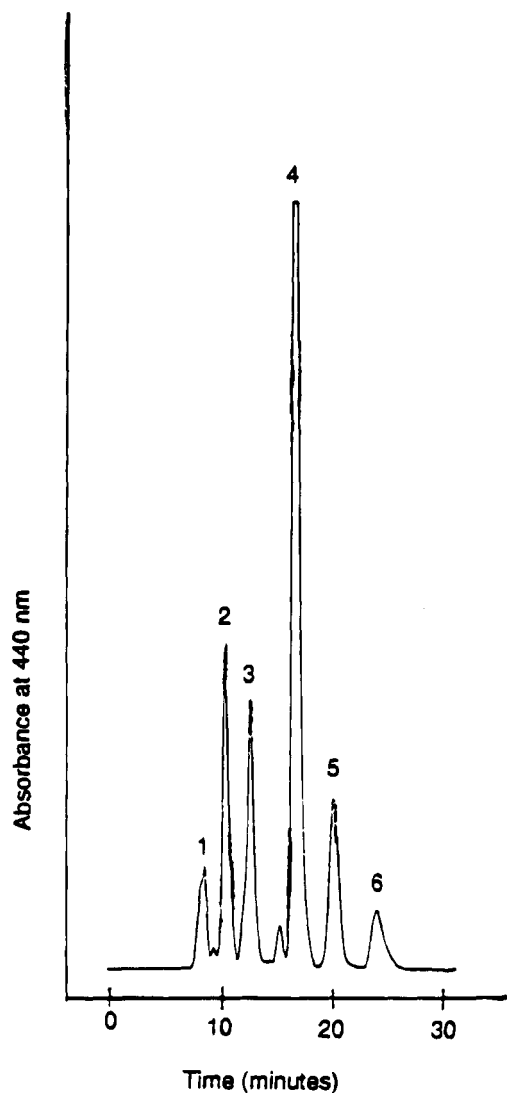


Figure 3. HPLC separation of an iodine isomerized α -carotene mixture using 0.1% *p*-methylanisole in hexane. Tentative identification of peaks: (1) 15-*cis*-, (2 and 3) either 13- or 13'-*cis*-, (4) *all-trans*-, and (5 and 6) either 9- or 9'-*cis*- α -carotenes.

solving 15-*cis*-, an unidentified *cis*-, and *all-trans*-phytofluenes on an alumina support with HPLC.

In the present study, calcium hydroxide was used as the stationary support in combination with an eluent of 100% hexane for the chromatographic separation of six geometric phytofluene isomers (Figure 4). The *all-trans* peak in isomerized mixtures of acyclic carotenes may be tentatively assigned according to the empirical observation that in most of these isomer sets, unlike the sets containing six-member ring moieties, the *all-trans* form is retained longest on calcium hydroxide (Zechmeister, 1962; Hashimoto et al., 1988). In addition, the electronic absorption spectra of the *cis* carotenoids are hypsochromically shifted relative to those of *all-trans* isomers, further supporting the identity of peak 6 as *all-trans*-phytofluene (Zechmeister, 1962).

ζ -Carotene (Figure 1) is composed of a more conjugated aliphatic system than phytofluene and therefore is retained longer on absorptive stationary phases (Davies, 1976). The two additional points of unsaturation are added in such a way that the compound is symmetrical. Thus, the amount of possible geometric conformations is minimized, with only 4-mono-*cis* forms being sterically unhindered (Pauling, 1939). As seen in Figure 5A, HPLC analysis of geometric ζ -carotenes

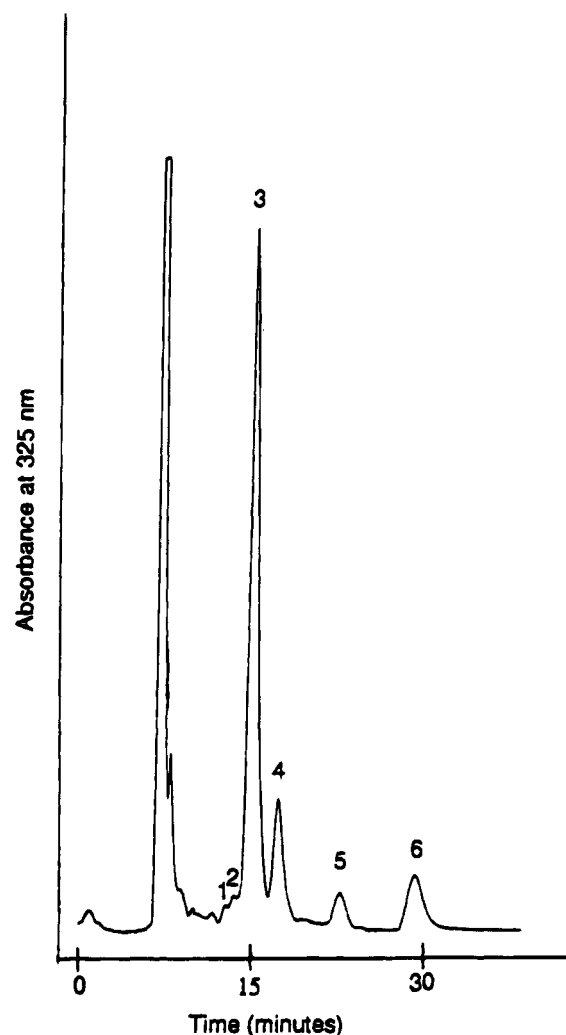


Figure 4. HPLC separation of a phytofluene mixture obtained from fresh tomato using 100% hexane following exposure to sunlight. Tentative identification of peaks: (1 and 2) unidentified *cis*-, (3) 15-*cis*-, (4 and 5) unidentified *cis*-, and (6) *all-trans*-phytofluene.

obtained from processed carrot baby food resulted in resolution of three isomers. This mixture was separated using an eluent of 1.4% benzyl ether in hexane. Use of 3% *p*-methylanisole, replacing benzyl ether, also resulted in resolution of three isomers; however, analysis time was extended by 30 min, and eluting bands were significantly wider (data not shown). Exposure of the ζ -carotenes mixture to sunlight in the presence of iodine resulted in the formation (subsequently resolved by HPLC) of three additional geometric ζ -carotene isomers, which are likely to be poly-*cis* compounds on the basis of the observed hypsochromic shift in comparison to that of the *all-trans* compound. A concomitant decrease of 15-*cis*- and increase of *all-trans*- ζ -carotene concentration upon development of the chromatogram with 1.45% benzyl ether (Figure 5B) was noted. Beyer et al. (1989) have previously separated geometric ζ -carotene isomers formed by illumination using both reversed-phase (monomeric C_{18}) and normal-phase (alumina) supports, with the alumina adsorptive phase providing resolution of 15-*cis*- and *all-trans*- ζ -carotene along with three other unidentified geometric isomers. On the basis of the retention time and bathochromic shift in the electronic absorption spectra relative to those of the other ζ -carotene isomers obtained during the present analysis, peak 6 was tentatively identified as *all-trans*- ζ -carotene. Peaks 4 and 5 are likely mono-*cis* isomers, while the

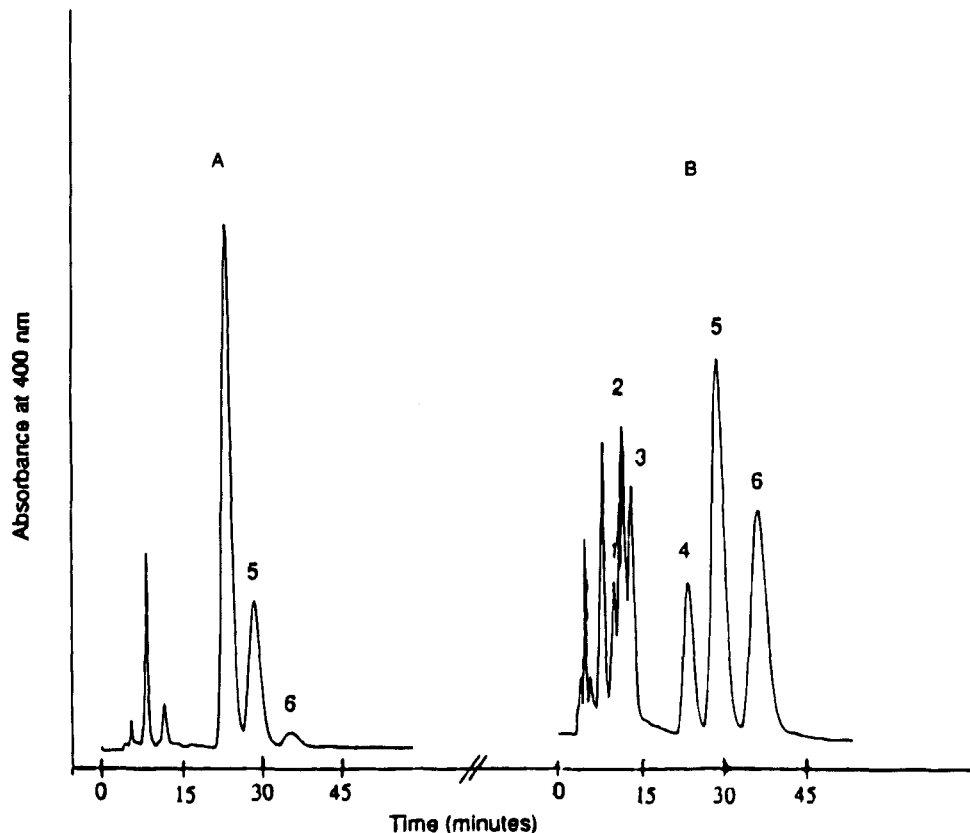


Figure 5. (A) HPLC separation of ζ -carotene mixture obtained from processed carrot baby food using 1.4% benzyl ether in hexane; (B) HPLC separation of the same mixture exposed to iodine and sunlight using the same chromatographic conditions. Tentative identification of peaks: (1–3) unidentified *cis*-, (4) 15-*cis*-, (5) an unidentified *cis*-, and (6) *all-trans*- ζ -carotene.

greater hypsochromic shift in the electronic absorption spectra of peaks 1–3 suggests they are poly-*cis*- ζ -carotenes (Zechmeister, 1962).

γ -Carotene is an asymmetrical molecule containing one cyclic and one acyclic end group (Figure 1). The asymmetrical nature of this compound, coupled with its extensive conjugation, allows for a number of geometric configurations that this compound may theoretically assume. Interestingly, certain members of the γ -carotene set, unlike any other provitamin A carotenoid, have vitamin A activity as great as or, in chicks, greater than that of the *all-trans* form (Zechmeister, 1962).

Zechmeister and Polgar (1945) indicated that chromatographic resolution of γ -carotene isomers using a calcium hydroxide support was largely unsatisfactory. Photocatalytic formation of geometric γ -carotene isomers catalyzed by iodine and subsequent chromatography of this mixture resolved four zones containing *cis* compounds. In addition to one zone containing the *all-trans* form, a zone exhibiting greater adsorption affinity than the *all-trans* compound was separated (Zechmeister and Polgar, 1945). In our laboratory, analysis of a mixture of γ -carotene isomers using an eluent consisting of 2.45% benzyl ether in hexane yielded the chromatogram shown in Figure 6. At least partial resolution of eight separate *cis*- γ -carotene peaks in addition to the *all-trans* peak was achieved with this method. The presence of a small *cis* fraction eluting after the *all-trans* compound was in agreement with the work of Zechmeister and Polgar (1945). Two peaks were observed to elute after the *all-trans* form (peaks 8 and 9). The tentative assignments of these two peaks are either 9-*cis*- or 9'-*cis*- γ -carotene on the basis of the observation that peripheral *cis* isomers elute after the *all-trans* compound in cyclic carotene sets (Zechmeister, 1962). Peaks 1–6 are unidentified as *cis*- γ -carotenes, and peak

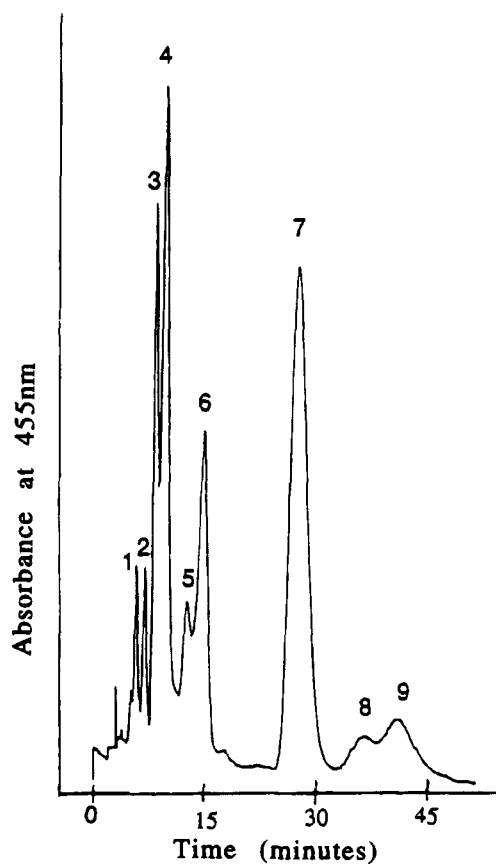


Figure 6. HPLC separation of an iodine isomerized γ -carotene mixture using 2.45% benzyl ether in hexane. Tentative identification of peaks: (1–6) unidentified *cis*-, (7) *all-trans*-, and (8 and 9) either 9- or 9'-*cis*- γ -carotenes.

7 is *all-trans-γ-carotene*. Although electronic absorption data in the visible region were useful for supporting the identity of these compounds as either the *all-trans-* or *cis-γ-carotenes*, interference by the relatively high levels of modifiers required to elute the compounds from the column interfered with the UV spectrum such that no information pertaining to their specific *cis* configurations could be obtained.

The analysis of *cis-γ-carotenes* (Figure 6) illustrates a fundamental point that must be considered when geometric isomer sets of highly conjugated carotenes are analyzed on calcium hydroxide. Due to significantly different absorptive behavior, the separation of centrally located mono-*cis-β-carotene* isomers requires substantially different developing solvents than those used to separate peripherally located mono-*cis* isomers of *β-carotene*. These peripheral mono-*cis* isomers are often difficult to elute from the column using the same eluent as that required for elution and resolution of other members of the set. Thus, a compromise in resolution of the earlier eluting peaks is sometimes required, in deference to the later eluting peaks, to separate these complex mixtures in a reasonable analysis time, as it was for the separation of *α-, β-, and γ-carotene* isomers in this paper. Another alternative is gradient elution; however, repeated sample analysis is difficult because of the sensitivity of the calcium hydroxide analysis to extremely small concentrations of modifier, thus significantly extending the required equilibrium time. Therefore, when detailed analysis of complex mixtures of geometric carotene isomers from the same set is desired, it is recommended that the investigator divide the chromatogram into two or three zones and apply elution solvents appropriate for optimal separation of the isomers present in each zone. Tsukida et al. (1982) successfully applied this technique to the resolution of certain geometric *β-carotenes*.

In summary, HPLC methodology employing a calcium hydroxide stationary phase selective for separation of acyclic and cyclic geometric carotenes containing 5, 7, or 11 aliphatic double bonds has been presented. The retention behaviors of certain *cis* isomers and the resulting impact of modifier selection and concentration have been considered. These HPLC methods, or the modifications referred to in the discussion of these chromatographic results, may be applied to the analysis of carotene isomer sets. These methods can also be used to obtain individual carotene isomers from biological or synthetic samples so that their confirmation may be unambiguously established by other physical methods.

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Received for review September 22, 1994. Accepted March 2, 1995.* Use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service or criticism of similar ones not mentioned.

JF940537U

* Abstract published in *Advance ACS Abstracts*, April 15, 1995.