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Capability of a polymeric C_{30} stationary phase to resolve *cis–trans* carotenoid isomers in reversed-phase liquid chromatography

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Abstract

A novel polymeric C_{30} stationary phase was tested for its ability to separate geometric isomers of six common carotenoids (lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene) prepared by photoisomerization of all-*trans* standards. Resolution and tentative identification of asymmetrical carotenoid isomers yielded the 13-*cis*, 13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis* isomers of both lutein and α-carotene, and the 15-*cis*, 13-*cis*/13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis* isomers of β-cryptoxanthin. Among symmetrical carotenoids, the 15-*cis*, 13-*cis*, all-*trans*, and 9-*cis* isomers of both zeaxanthin and β-carotene were resolved and tentatively identified, and nineteen geometric isomers of lycopene were separated. Separations were carried out using Vydac 201TP54 and Suplex pkb-100 stationary phases for comparison; in all cases, the C_{30} stationary phase gave superior resolution and produced unique separations.

1. Introduction

Certain carotenoids are metabolic precursors to vitamin A. Other forms of biological activity (e.g., singlet oxygen quenching) have also been demonstrated in vitro for some carotenoids, apparently independent of provitamin A activity [1–5]. This activity is believed to occur in vivo, and has been postulated to manifest itself in the significant inverse association between dietary intake of carotenoids and the incidence of certain types of cancer [6,7]. Among dietary sources of carotenoids, various geometric (*cis* and all-*trans*) isomers of carotenoids either occur naturally, or are formed during thermal processing [8–10]. With provitamin A carotenoids, the *cis*-isomeric forms are less efficiently converted to vitamin A than are their all-*trans* counterparts [11,12]. The presence and distribution of *cis*-carotenoids in biological tissues also varies [11,13,14]. Accurate nutritional assessment of foods, blood, and tissues is, therefore, dependent upon resolving and quantifying the geometric isomers of these provitamin A carotenoids. With respect to nutritional and other health-related aspects of carotenoid metabolism, the possibility that unique or altered physiological roles could be associated with *cis* versus

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all-*trans* carotenoids has not yet been rigorously addressed. To this end, the ability to accurately determine *cis–trans* carotenoid profiles of biological tissues is a prerequisite to acquiring a better understanding of the biological significance of *cis*-carotenoids.

Existing reversed-phase (RP) liquid chromatography (LC) methods are well suited for separating major carotenoids, but their ability to separate *cis–trans* isomers of a particular carotenoid is seldom adequate. In this regard, polymeric C_{18} stationary phases generally possess greater shape selectivity towards geometric carotenoid isomers than do monomeric C_{18} stationary phases [15], resulting in better performance for these separations. Traditionally, normal-phase liquid chromatography (NPLC) using a calcium hydroxide stationary phase has produced excellent separations of *cis–trans* carotenoid isomers, including sets of β-carotene [16–18], α-carotene [17], and several other carotenoids [19–21], but its use has been severely limited because these columns are not commercially available and their preparation is highly irreproducible. Other successful applications of NPLC to separations of carotenoid isomers include the use of an alumina stationary phase to separate β-carotene isomers [22] and a silica-based nitrile-bonded stationary phase for the separation of geometric isomers of lutein and zeaxanthin [23,24]. Argentation chromatography, where silver ions are incorporated into either the stationary phase or mobile phase, has been demonstrated to aid in LC separations of methyl ester derivatives of *cis* and *trans* fatty acids [25]; however, this approach has not yet been successfully applied to separations involving carotenoids or their geometric isomers.

The polymeric C_{30} stationary phase used in the present study was developed at NIST to optimize RPLC separations of carotenoids [26]. It was successfully demonstrated that this C_{30} stationary phase provides excellent resolution of all-*trans* carotenoids and possesses outstanding shape selectivity toward the predominant geometric isomers in a β-carotene sample prepared from *Dunaliella* algae (Betatene, Melbourne, Australia). In a separate report, the same type of stationary phase was used to separate the major carotenoids in an extract of human serum [27]. Our present objectives were: (1) to assess shape selectivity of the polymeric C_{30} stationary phase by determining its ability to separate geometric isomers of common carotenoids; and (2) to obtain comparative separations of the same isomer mixtures using RP stationary phases that are commercially available and commonly used for carotenoid chromatography.

2. Experimental[1]

2.1. Chemicals

The following all-*trans* carotenoid standards were used in this study: lutein (Kemin, Des Moines, IA, USA); zeaxanthin (Indofine, Belle Mead, NJ, USA); β-cryptoxanthin (Hoffmann-La Roche, Nutley, NJ, USA); lycopene (Sigma, St. Louis, MO, USA); α-carotene (Sigma); and β-carotene (Sigma). For the convenience of the reader, structures of these carotenoids are given in Fig. 1. HPLC solvents were methanol (certified ACS; Fisher Chemical, Fairlawn, NJ, USA) and methyl-*tert.*-butyl ether (MTBE) (HPLC grade; Fisher).

2.2. Stationary phases

Four RPLC stationary phases were tested using analytical scale (250 × 4.6 mm I.D.) columns. Of these stationary phases, two were prepared at NIST by polymeric synthesis of a C_{30} alkyl-bonded phase onto silica supports, without subsequent endcapping, according to the report on its development [26]. These polymeric C_{30} stationary phases were prepared using the same synthetic procedure, but with different diameter

[1] Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by North Carolina State University or the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

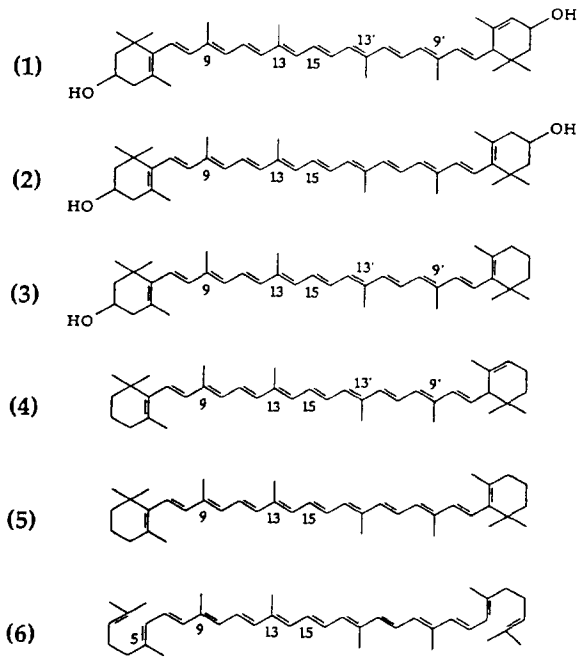

Fig. 1. Structures of the carotenoids analyzed in this report, shown in their all-*trans* configurations: 1 = lutein; 2 = zeaxanthin; 3 = β-cryptoxanthin; 4 = α-carotene; 5 = α-carotene; 6 = lycopene. Carbon numbers indicate positions where formation of *cis*-bonds is sterically unhindered.

silica particles (5 μm and 3 μm). The stationary phase containing 5-μm silica particles was used for most of the research presented here, and unless the particle size of the silica is otherwise indicated as 3 μm in diameter, the 5-μm stationary phase was used. The other two stationary phases were obtained in commercially manufactured columns: Suplex pkb-100 (Supelco, Bellefonte, PA, USA); and Vydac 201TP54 (Separations Group, Hesperia, CA, USA). These stationary phases were chosen from the many commercially available C_{18} columns because they are commonly used for carotenoid analyses and are known to exhibit selectivity toward geometric carotenoid isomers. Pertinent available data on the four stationary phases are given in Table 1. Guard columns were not used to preclude any possible pre-column effects on the resultant separations.

2.3. Instrumentation

The HPLC system used in this work consisted of a Waters (Milford, MA, USA) Model 501 solvent delivery system, a Waters Model U6K

Table 1
Physical properties of the stationary phases tested

Stationary phase	5-C_{30}[a]	Suplex pkb-100	Vydac 201TP54	3-C_{30}[a]
Bonded phase	C_{30}	–[b]	C_{18}	C_{30}
Synthesis	polymeric	–[b]	polymeric	polymeric
Endcapping	no	yes[c]	no	no
Silica				
–type	YMC S5-SIL200	–[b]	Vydac TP	YMC S3-SIL200
–particle shape	spherical	spherical	spherical	spherical
–particle diam. (μm)	5	5	5	3
–pore diam. (Å)[d]	200	100	300	200
–surface area (m^2/g)[d]	200	–[b]	90	200
Percent carbon	19.41	–[b]	8[d]	–[b]
Coverage (μmol/m^2)	3.64	–[b]	4.16	–[b]

[a] Ref. [26].
[b] Information is proprietary or unavailable.
[c] Reagent used for endcapping is proprietary.
[d] Nominal values provided by the purveyors.

injector, and an Anspec UV–Vis detector (Model SM 95; Linear Instruments, Reno, NV, USA). The detector was linked to a Dramen personal computer (Dramen, Raleigh, NC, USA) via a Dionex advanced computer interface (Model ACI-1; Dionex, Sunnyvale, CA, USA). Dionex AI-450 chromatography software (release 3.30; Dionex) was used to integrate the chromatograms. Electronic absorption spectra were obtained for some chromatographic peaks using a Waters Model 996 photodiode-array detector interfaced with a Gateway 2000 personal computer (Model 4DX2-66V; North Sioux City, SD, USA) equipped with Millennium 2010 chromatography software (LC Version 2.00; Millipore, Milford, MA, USA).

2.4. Sample preparation

All-*trans* carotenoid standards were purified of any oxidized carotenoid contaminants that may have formed during manufacture and storage of the standards using open alumina columns and acetone–hexane eluents. Briefly, acetone concentrations were adjusted to elute only the all-*trans* carotenoid, thereby removing any oxidized carotenoid contaminants which are preferentially retained on alumina. Purity of the all-*trans* carotenoids was assessed using the polymeric C_{30} column. Mobile phases of MTBE in methanol flowing at a rate of 1 ml/min were used for this assessment. Lutein, zeaxanthin, β-cryptoxanthin, and lycopene were of sufficient purity ($\geq 97\%$ all-*trans*) to obviate further preparative chromatography. Additional purification was necessary, however, to achieve adequate purity of the α-carotene and β-carotene standards. Removal of contaminating β-carotene and α-carotene, respectively, was achieved using a Vydac 201TP510 semi-preparatory column (250×10 mm I.D.; Separations Group, Hesperia, CA, USA), a mobile phase of 8% tetrahydrofuran and 0.05% triethylamine in methanol, and a flow-rate of 1.5 ml/min. This procedure gave α-carotene and β-carotene of high purity ($\geq 97\%$ all-*trans*). Each standard was photoisomerized into an equilibrium mixture of various geometric isomers by iodine catalysis according to the general procedure reported by Zechmeister [12]. Briefly, after dissolving each all-*trans* carotenoid in a suitable solvent (hexane when possible) and diluting it with hexane, iodine was added at a concentration of about 1% by weight of the carotenoid weight (determined spectrophotometrically using published extinction coefficients), and the solution was then exposed to ambient laboratory light for 1 h. After isomerization, each sample was dried over sodium sulfate, dried under a stream of nitrogen gas, and stored at −20°C until further use.

2.5. Chromatography

All separations on the polymeric C_{30} stationary phases were achieved isocratically using a binary mobile phase of MTBE in methanol, flowing at 1 ml/min. The concentration of MTBE in the mobile phase was adjusted to give optimal resolution of analytes under the other conditions of chromatography. Mobile-phase compositions for separations on the Suplex pkb-100 and Vydac 201TP54 stationary phases were selected to give optimal or near optimal resolution of each isomer set on the stationary phase being tested [28–30]. This approach was believed to provide a better comparison of column separation efficiency than would be possible using the same mobile phase, or same mobile-phase components, when testing different stationary phases and analytes. For all separations, the specific composition of the mobile phase used is given in the appropriate figure legend. When several stationary phases were tested comparatively for their ability to resolve a particular set of carotenoid isomers, the same isomer mixture was used for all chromatographic analyses, including photodiode-array detection.

All columns were used at ambient laboratory temperature (ca. 23°C). Injection solvents were MTBE in methanol mixtures for application to all four stationary phases. Injection volumes ranged from 2 to 25 μl. Column effluent was monitored at 453 nm during all separations, except for that of isomerized lycopene (460 nm), using a sensitivity setting absorbance units full

scale (AUFS) of 0.008. Electronic absorption spectra were obtained from 250 to 550 nm.

2.6. Peak identifications

For each set of geometric carotenoid isomers, all-*trans* configurations and *cis*-bond positions were tentatively assigned to chromatographic peaks according to characteristics of their electronic absorption spectra [12] and relative patterns of chromatographic retention [26].

3. Results and discussion

3.1. Asymmetrical carotenoids

With asymmetrical carotenoids (e.g., lutein, β-cryptoxanthin, and α-carotene), the number of theoretically possible geometric isomers is approximately two-fold greater than with those carotenoids having a plane of symmetry (e.g., zeaxanthin, lycopene, and β-carotene) [12]. We were interested in determining how well the C_{30} stationary phase could resolve the potentially complex isomerized mixtures of common asymmetrical carotenoids, and more specifically, whether mono-*cis* geometric isomers of asymmetrical carotenoids could be resolved where the *cis*-bonds are present at the same carbon number but at opposite ends of the molecule (e.g., 9-*cis* and 9′-*cis*). For these determinations, carotenoids were selected according to structural features that preclude a plane of symmetry as well as prevalence in human and food tissues. Isomeric mixtures of lutein, β-cryptoxanthin, and α-carotene were believed to serve adequately as probes for ascertaining shape selectivity of the polymeric C_{30} stationary phase.

Lutein

The separation of geometric lutein isomers on the C_{30} stationary phase is given in Fig. 2A. The C_{30} stationary phase completely resolved nine *cis–trans* lutein peaks. The 13-*cis*, 13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis* geometric forms of lutein were tentatively identified (Table 2), and the remaining four resolved peaks are unidentified *cis*-isomers of lutein. Unambiguous assignment of individual peak identities, including the 13-*cis* or 13′-*cis* and 9-*cis* or 9′-*cis* isomers, was not possible because NMR spectroscopy has not yet been applied to any peaks resolved on the C_{30} column. In Fig. 3, the absorption spectra for the 13-*cis* and 13′-*cis* isomers of lutein are given. When normalized and superimposed, these spectra are nearly indistinguishable, as are the absorption spectra for the 9-*cis* and 9′-*cis* isomers of lutein (not shown). The wavelengths of maximal absorbance and relative abundances of some of the resolved lutein peaks are given in Table 2. The 15-*cis* isomer of lutein was not resolved on the C_{30} stationary phase under the chromatographic conditions employed, but was believed

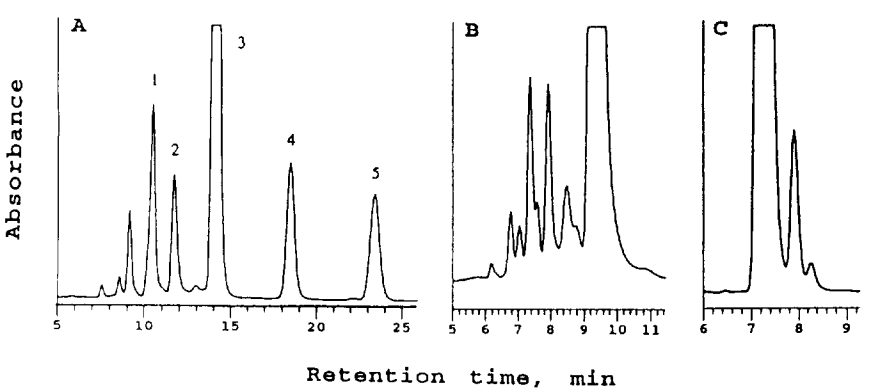

Fig. 2. RPLC separations of isomerized lutein. (A) C_{30} stationary phase; MTBE–methanol (5:95, v/v) mobile phase. (B) Vydac 201TP54; methanol mobile phase, 0.7 ml/min. (C) Suplex pkb-100; methanol mobile phase, 0.7 ml/min. Tentative peak identifications for (A) are given in Table 2.

Table 2
Wavelengths of maximum absorbance and relative abundances of certain geometric carotenoid isomers separated from iodine-isomerized mixtures on the C_{30} stationary phase

Carotenoid	Peak number[a]	Tentative identification	λ^{b}_{max} (nm)	Relative abundance[c] (%)
Lutein	1	13- or 13'-*cis*	435	10.2
	2	13- or 13'-*cis*	436	7.6
	3	all-*trans*	442	56.3
	4	9- or 9'-*cis*	438	10.3
	5	9- or 9'-*cis*	439	10.0
β-Cryptoxanthin	1	15-*cis*	444	3.6
	2	13- & 13'-*cis*	442	8.5
	3	all-*trans*	449	60.5
	4	9- or 9'-*cis*	444	13.0
	5	9- or 9'-*cis*	444	11.5
α-Carotene	1	13- or 13'-*cis*	438	9.4
	2	13- or 13'-*cis*	438	9.4
	3	all-*trans*	444	53.8
	4	9- or 9'-*cis*	441	11.0
	5	9- or 9'-*cis*	441	10.0
β-Carotene	1	15-*cis*	447	1.2
	2	13-*cis*	443	16.7
	3	all-*trans*	450	47.3
	4	9-*cis*	444	22.1
Zeaxanthin	1	15-*cis*	446	1.4
	2	13-*cis*	443	14.2
	3	all-*trans*	450	53.9
	4	9-*cis*	446	21.0
Lycopene	1	15-*cis*	466	1.9
	2	13-*cis*	465	7.8
	3	all-*trans*	472	13.3
	4	unidentified *cis*	472	23.2

[a] Numbers correspond to those given in Figs. 2A, 4A, 5A, 6A, 7, and 8.
[b] Obtained for the main absorption peak using photodiode-array spectrophotometry in the mobile phase (5–38% MTBE in methanol). Relative differences in λ_{max} of ca. 3 nm or less are generally insignificant when considered for assigning double bond geometries.
[c] Peak-area percent relative to total area of all peaks detected at the λ_{max} reported here for the all-*trans* forms, except for lycopene, which was monitored at 460 nm and integrated only for peaks eluting after 14 min. Measurements are based upon the assumption that absorptivities (ϵ) for geometrical isomers of each carotenoid are equal or nearly equal.

to elute in peak 1. This observation is based on the measured increase in the *cis*-peak effect (absorbance at ca. 330 nm) on a shoulder of peak 1 when multiple absorption spectra were obtained in this peak. Since absorbance in the *cis*-peak region is greatest with centrally located *cis*-bonds [12], the presence of the 15-*cis* isomer is suggested. In addition, when the same lutein mixture was separated on the 3-μm C_{30} stationary phase (not shown), a peak that could correspond to the 15-*cis* isomer was partially resolved at the same relative position in the chromatogram.

Using the Vydac 201TP54 column, nine geometric isomers of lutein were partially separated, but with poor resolution (Fig. 2B). On the Suplex pkb-100 stationary phase, only three peaks were separated from the same luteins mixture (Fig. 2C), and these peaks were not baseline-resolved. Neither the Vydac 201TP54 nor the Suplex pkb-100 stationary phase provide adequate retention of isomers of lutein, a rela-

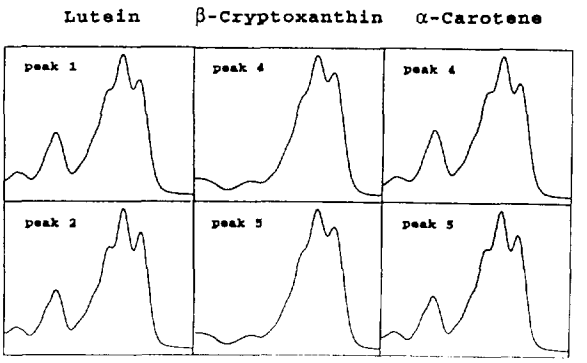

Fig. 3. UV–Vis absorption spectra for selected geometric carotenoid isomers: 13-*cis* and 13′-*cis* luteins (Fig. 2, peaks 1 and 2); 9-*cis* and 9′-*cis* β-cryptoxanthins (Fig. 4, peaks 4 and 5); and 9-*cis* and 9′-*cis* α-carotenes (Fig. 5, peaks 4 and 5).

tively polar carotenoid. Both the C_{30} and Vydac stationary phases, however, possess good selectivity toward these isomers, and this may be attributable to the presence of silanol activity, i.e., lack of endcapping (Table 1). Certain separations of oxygenated carotenoids have been shown to be aided by silanol activity in the stationary phase [26,31], but this has been recognized only for structural xanthophyll isomers (i.e., lutein and zeaxanthin; neoxanthin and antheraxanthin) and epimers (i.e., auroxanthins 2 and 3) and not for separations involving geometric carotenoid isomers. The advantage of using the C_{30} stationary phase for separations of lutein isomers is that this column combines a high degree of shape selectivity with adequate retention to a greater extent than either the Vydac 201TP54 or Suplex pkb-100 stationary phases.

There are several published reports of HPLC separations of geometric lutein isomers that compare favorably [32–35]. Five geometric isomers of lutein have been separated from an isomerized mixture using NPLC [32]. Similar results have been obtained using a C_{18} column in RPLC, by which four *cis–trans* luteins were resolved from an isomerized mixture and tentatively identified [35]. Excellent separations of lutein isomers have also been achieved using nitrile-bonded columns in NPLC [23,24]. Isomers of lutein were isolated from extracts of human plasma and unambiguously identified as all-*trans*, 9-*cis*, 9′-*cis*, and 13-*cis*/13′-*cis*.

β-Cryptoxanthin

Separations of geometric β-cryptoxanthin isomers obtained on the tested stationary phases are presented in Fig. 4. Using the C_{30} stationary phase and MTBE–methanol (8:92 v/v) as mobile phase, excellent resolution was achieved for most of the predominant β-cryptoxanthin isomers (Fig. 4A). The 15-*cis* and all-*trans* isomers were completely resolved, and the 9-*cis* and 9′-*cis* isomers were separated, but not fully resolved. Electronic absorption spectra for the 9-*cis* and 9′-*cis* isomeric pair are virtually identical (Fig. 3). Interestingly, the 13-*cis* and 13′-*cis* isomeric pair was resolved from all other major

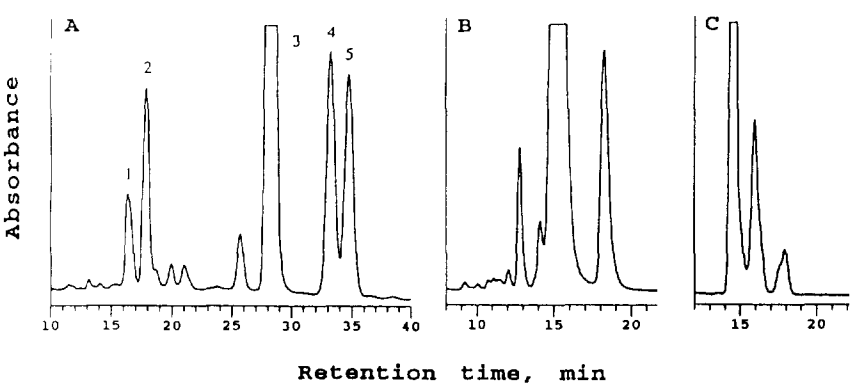

Fig. 4. RPLC separations of isomerized β-cryptoxanthin. (A) C_{30} stationary phase; MTBE–methanol (8:92, v/v) mobile phase. (B) Vydac 201TP54; acetonitrile–methanol (5:95, v/v) mobile phase, 1 ml/min. (C) Suplex pkb-100; methanol–acetonitrile–isopropanol–water (10:40:40:10, v/v) mobile phase, 0.7 ml/min. Tentative peak identifications for (A) are given in Table 2.

isomers, but these individual isomers were not separated from one another. Evidence for this observation includes analysis of absorption spectra for the resolved peaks, and a chromatogram of the same β-cryptoxanthins mixture developed on the 3-μm C_{30} stationary phase (not shown), where the 13-*cis*/13′-*cis* peak is partially separated into two peaks of almost equal abundance. In Table 2, wavelengths of maximal absorbance and percent composition of major peaks are provided. Several other, unidentified β-cryptoxanthin isomers were also separated. Other LC separations of geometrical β-cryptoxanthin isomers of this kind were not found in the literature for comparison.

Chromatography of the same isomerized mixture of β-cryptoxanthins on the Vydac 201TP54 stationary phase yielded separation of four *cis*-isomers from the predominant peak containing the all-*trans* form (Fig. 4B). When the β-cryptoxanthins mixture was applied to the Suplex pkb-100 stationary phase (Fig. 4C), three distinct peaks were separated, with the predominant peak containing the all-*trans* isomer and the other two peaks, each having a shoulder, containing *cis*-isomers.

α-Carotene

Comparative separations of an isomerized mixture of *cis–trans* α-carotenes are presented in Fig. 5. When α-carotene isomers were applied to the C_{30} column, the 13-*cis*, 13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis* isomers were at least partially resolved and are tentatively identified (Fig. 5A; Table 2). The absorption spectra for the 13-*cis*/13′-*cis* and 9-*cis*/9′-*cis* isomeric pairs are quite similar as can be seen in Fig. 3 for the latter pair. The remaining α-carotenes peaks separated are unidentified.

Six α-carotene isomers were separated on the Vydac 201TP54 stationary phase, but these peaks were poorly resolved (Fig. 5B). Fig. 5C is the chromatogram obtained for the same α-carotenes mixture when applied to the Suplex pkb-100 stationary phase. Under the chromatographic conditions employed, three *cis*-peaks and the all-*trans* isomer were nearly baseline-resolved.

Few HPLC separations of α-carotene isomers exist in the literature [17,35–37]. However, resolution of all-*trans*, 15-*cis*, 13-*cis*, 13′-*cis*, 9-*cis*, and 9′-*cis* α-carotenes from an iodine-isomerized mixture was recently achieved in our laboratory using a calcium hydroxide stationary phase [17].

It is interesting to note that, for the β,ε-carotenoids (lutein and α-carotene), the 15-*cis* isomers were unresolved on the C_{30} phase, while the 13-*cis*/13′-*cis* and 9-*cis*/9′-*cis* isomeric pairs were well separated. This is in contrast to the β,β-carotenoid (β-cryptoxanthin), for which the C_{30} separation resolved the 15-*cis* isomer but not the 13-*cis*/13′-*cis* isomeric pair.

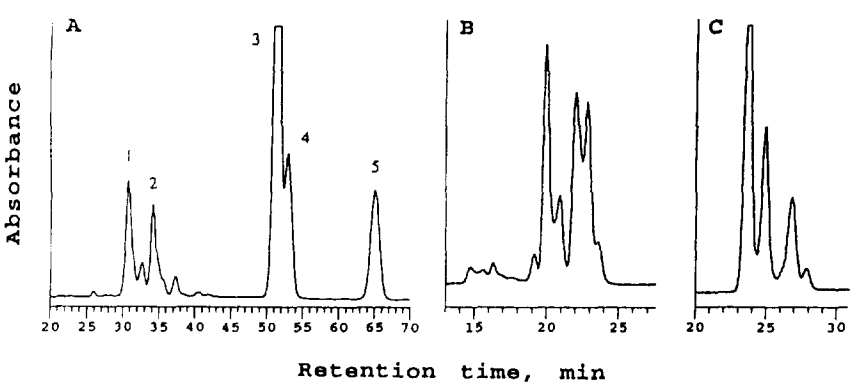

Fig. 5. RPLC separations of isomerized α-carotene. (A) C_{30} stationary phase; MTBE–methanol (9:91, v/v) mobile phase. (B) Vydac 201TP54; acetonitrile–methanol (10:90, v/v) mobile phase, 1 ml/min. (C) Suplex pkb-100; methanol–acetonitrile–isopropanol–water (10:40:40:10, v/v) mobile phase, 1 ml/min. Tentative peak identifications for (A) are given in Table 2.

3.2. Symmetrical carotenoids

Although isomerized mixtures of symmetrical carotenoids are generally less complex than are those of asymmetrical carotenoids, LC separations of either type of mixture are equally challenging. β-Carotene, lycopene, and zeaxanthin were selected for this study among the many known symmetrical carotenoids because of their prevalence in biological tissues and their structural features.

β-Carotene

Chromatographic separations of isomerized β-carotene on the C_{30}, Vydac 201TP54, and Suplex pkb-100 stationary phases are illustrated in Fig. 6. When chromatographed on the C_{30} stationary phase, excellent resolution of the tentatively identified 15-*cis*, 13-*cis*, all-*trans*, and 9-*cis* isomers was achieved (Fig. 6A). The wavelengths of maximal absorbance and relative abundances of these isomers are given in Table 2. Several other *cis*-isomers of unknown geometrical configuration were also separated from this mixture. The Vydac 201TP54 stationary phase was also tested for its ability to resolve the same geometric isomers of β-carotene (Fig. 6B). As with all isomer sets chromatographed on the Vydac stationary phase, several peaks containing *cis*-isomers were separated from all-*trans* β-carotene, but the resolution was not comparable to that achieved when using the C_{30} stationary phase. On the Suplex pkb-100 stationary phase, four peaks containing *cis*-isomers of β-carotene were nearly baseline-resolved from the predominant all-*trans* peak (Fig. 6C).

There have been numerous other separations of β-carotene isomers, and these have been recently reviewed [38]. Several normal-phase separations of geometric β-carotene isomers [16–18,22] have given comparable or favorable resolution with that which we achieved; however, no RP stationary phase has shown shape selectivity towards geometric isomers of β-carotene to the extent of the C_{30} phase used here. Interestingly, retention characteristics and elution order of β-carotene isomers are strikingly similar on calcium hydroxide and C_{30} stationary phases, despite the fundamental differences between their mechanisms of retention and selectivity. The same observation can be made for the α-carotene set [17], and perhaps for other sets of geometric carotenoid isomers as well. On calcium hydroxide, adsorption affinity of carotenoids is dependent upon the number and types of conjugated double bonds present, giving rise to stronger adsorption: (a) when conjugation is increased; (b) with acyclic versus cyclic carotenoids; and (c) with β,β-carotenoids as compared to β,ε-carotenoids [39]. The same general retention patterns are observed for carotenoids on the C_{30} stationary phase, although a different

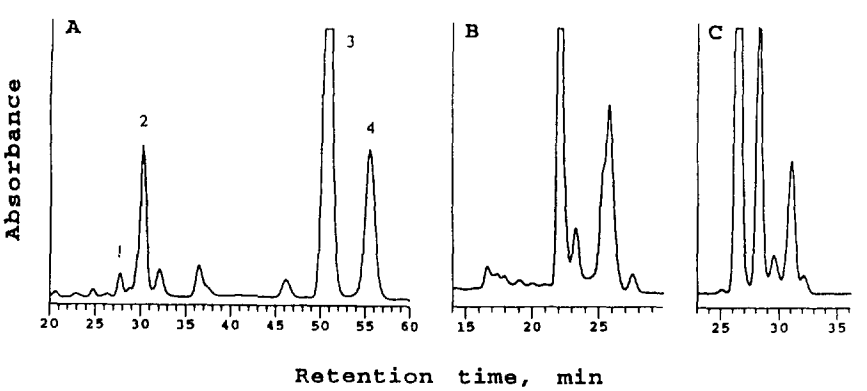

Fig. 6. RPLC separations of isomerized β-carotene. (A) C_{30} stationary phase; MTBE–methanol (11:89, v/v) mobile phase. (B) Vydac 201TP54; acetonitrile–methanol (10:90, v/v) mobile phase, 0.7 ml/min. (C) Suplex pkb-100; methanol–acetonitrile–isopropanol–water (10:40:40:10, v/v) mobile phase, 1 ml/min. Tentative peak identifications for (A) are given in Table 2.

retention mechanism has been proposed. Unlike the primary retention mechanism on C_{18} stationary phases (i.e., solvophobic interactions), the "slot model" was introduced, where solutes interact within "slots" of the C_{30}-bonded phase surface during retention [40]. This model could account for the excellent shape selectivity of the C_{30} polymeric phase toward geometric carotenoid isomers as well as the preferential retention of the acyclic lycopene, in a fashion similar to that observed for polyaromatic hydrocarbons of differing shapes [40]. Further insight into possible retention mechanisms can be gained through comparison of the relative dimensions of carotenoids and bonded-phase thicknesses. The length of all-*trans* β-carotene (as determined by molecular modeling) is ca. 29 Å, and the thickness of a C_{30} stationary phase estimated from small-angle neutron-scattering measurements is 25–30 Å [26]. By contrast, C_{18} stationary phases range in thickness from 17 to 21 Å. The enhanced selectivity exhibited by the C_{30} polymeric phase toward geometric carotenoid isomers has been attributed in part to the greater thickness of the stationary phase. More complete interaction of carotenoids is thought to be possible with a stationary phase with thickness comparable to the dimensions of the solute. By similar reasoning, it is hypothesized that because C_{18} stationary phases possess insufficient thickness to permit full penetration of carotenoid molecules, solute-bonded phase interactions do not occur at all points along the molecule, and poorer isomer separations result.

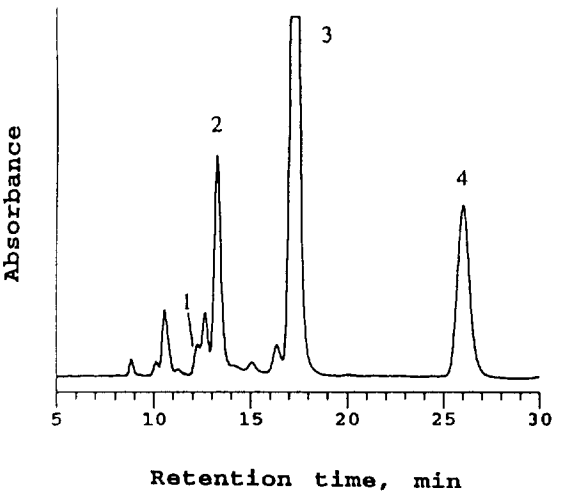

Fig. 7. C_{30} chromatogram of isomerized zeaxanthin, MTBE–methanol (5:95, v/v) mobile phase. Tentative peak identifications are given in Table 2.

Zeaxanthin

An isomeric mixture of geometric zeaxanthins was chromatographed only on the C_{30} stationary phase (Fig. 7). Among the predominant geometric isomers separated and tentatively identified are 15-*cis*, 13-*cis*, all-*trans*, and 9-*cis* zeaxanthins (Table 2). Several other unidentified zeaxanthin isomers were also separated.

There are few reports of HPLC separations of zeaxanthin isomers, and all deal with extracts of human plasma [23,24,33,34]. All-*trans* and 13-*cis* zeaxanthin were resolved using an Adsorbosphere-HS C_{18} column, and the 9-*cis* isomer was additionally separated from a corn meal extract [34]. A better separation of zeaxanthin isomers was obtained using NPLC with a nitrile-bonded silica-based column, on which the all-*trans*, 9-*cis*, 13-*cis*, and 15-*cis* geometric forms of zeaxanthin were resolved [23,24]. The geometrical configurations of these isomers were unambiguously identified using ^{1}H NMR.

Lycopene

An isomerized mixture of geometric lycopene isomers was also applied to the C_{30} stationary phase. The resultant chromatogram (Fig. 8) contains peaks of at least 18 *cis*-lycopene isomers plus the all-*trans* form. Peaks eluting before 14 min are presumed to be degradation (oxidation) products of lycopene formed during sample handling and chromatography. Because the absorption spectra of the two latest eluting peaks were indistinguishable, co-chromatography of a biological extract that contained numerous lycopene isomers and added all-*trans* lycopene was used to identify the all-*trans* isomer. Among the *cis*-lycopene isomers, only the 15-*cis* and 13-*cis* forms could be tentatively identified on the basis of electronic absorption spectra, because of

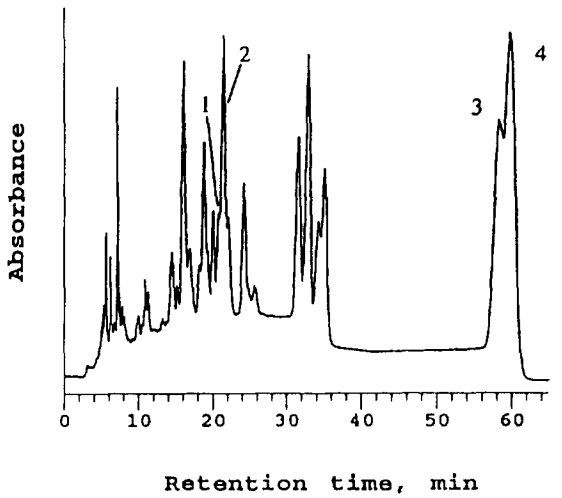

Fig. 8. C_{30} Chromatogram of isomerized lycopene. MTBE–methanol (38:62, v/v) mobile phase. All peaks eluting after 14 min are *cis*-lycopenes. Tentative peak identifications are given in Table 2.

remarkable similarities among these spectra for nearly all of the other *cis*-lycopene peaks. The wavelengths of maximal absorbance and relative abundances of several lycopene isomers are given in Table 2.

To our knowledge, separation of lycopene isomers has not been investigated using NPLC. There have been several reports in which a number of isomers of lycopene have been partially resolved from the all-*trans* configuration [14,34,35]. Six *cis-trans* lycopenes were separated from an isomerized standard using a C_{18} column [35], and four peaks containing *cis*-lycopenes were separated from all-*trans* lycopene in extracts of human plasma using a C_{18} column [34]. A Suplex pkb-100 column has previously given peaks corresponding to the all-*trans*, 15-*cis*, 13-*cis*, and 9-*cis* isomers of lycopene in human serum extracts [14]. Thus, many of the *cis*-lycopene peaks in Fig. 8 have not been previously observed, although numerous geometric lycopene isomers have been previously isolated using extensive open column chromatography [12]. Nonetheless, C_{30} column selectivity towards geometric isomers of lycopene is clearly unique in comparison to that of any other existing stationary phase.

3.3. C_{30} stationary phase prepared from 3-μm silica particles

Isomerized mixtures of all of the carotenoids included in this study were chromatographed on the 3-μm C_{30} stationary phase, in addition to the 5-μm C_{30} column. For isomerized lutein, the 3-μm C_{30} phase separated two peaks in addition to those resolved on the 5-μm C_{30} column, one eluting before peak 1 and the other after peak 3 of Fig. 2A. The 3-μm C_{30} separation of isomerized β-cryptoxanthin included partial separation of the 13-*cis* and 13′-*cis* isomers (peak 2 of Fig. 4A) as well as baseline resolution of the 9-*cis* and 9′-*cis* isomers (peaks 4 and 5 of Fig. 4A) as improvements over the corresponding 5-μm C_{30} separation. Similar improvements in resolution were also observed for the other carotenoid isomer sets.

4. Conclusions

The polymeric C_{30} RPLC stationary phase possesses outstanding shape selectivity toward geometric isomers of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene. This stationary phase also gives adequate retention for resolution of polar carotenoids. For all of the carotenoid isomer sets included in this work, *cis*-isomers were separated on the C_{30} phase that have not been previously separated on RP stationary phases. Assignment of peak identities using ^{1}H NMR spectroscopy is currently in progress for certain predominant geometric isomers of the carotenoids reported herein. As expected, the resolution of each isomer set was slightly enhanced when using the 3-μm C_{30} stationary phase as compared to that achieved with the 5-μm column. Shape discrimination and peak retention of *cis–trans* carotenoid isomers on the C_{30} stationary phases are superior to that available with existing, commercially available RPLC columns routinely used for carotenoid analysis.

The polymeric C_{30} stationary phase can be successfully applied to: (a) more accurate determinations of the provitamin A content and

geometric carotenoid isomer profiles of biological tissues; (b) analysis of *cis* versus all-*trans* carotenoids in studies of their comparative metabolic or physiological roles; and (c) purification and purity assessment of carotenoids.

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