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Isolation and structural elucidation of the predominant geometrical isomers of α-carotene

Curt Emenhiser[a], Gerhard Englert[b], Lane C. Sander[c], Bernard Ludwig[d], Steven J. Schwartz[a,*]

[a]*Department of Food Science, Box 7624, North Carolina State University, Raleigh, NC 27695-7624, USA*
[b]*Pharmaceutical Research, Building 65 - room 119, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland*
[c]*Analytical Chemistry Division, Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, MD 20899, USA*
[d]*Vitamins and Fine Chemicals Division, Building 90 - room 318, F. Hoffmann-La Roche Ltd., CH-4002 Basel, Switzerland*

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Abstract

The recent development and application of a polymeric C_{30} stationary phase have given unique separations of *cis–trans* carotenoid isomers in reversed-phase (RP) liquid chromatography (LC) owing to the exceptional shape selectivity of this stationary phase. In the present research, several geometrical isomers of α-carotene were at least partially resolved from a photo-isomerized mixture when chromatographed on a 3-μm polymeric C_{30} column. Double bond configurations of the five predominant α-carotene peaks, as isolated on a semi-preparative C_{30} column, were unambiguously assigned using ^1H nuclear magnetic resonance (NMR) spectroscopy, giving the following order of elution: 13-*cis*, 13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis* geometrical forms. Electronic absorption spectra for these isomers were in agreement with the identification of peaks. The α-carotene isomers separated and identified herein had not been previously resolved in RPLC. Confirmation of the structures of geometrical α-carotene isomers will aid further studies on the possible physiological roles of these compounds in biological tissues.

1. Introduction

α-Carotene is a prominent provitamin A carotenoid in human serum and tissues [1,2], crude palm oil [3], and carrots [4], and it is present in many other biological tissues at varying concentrations [4–7]. Among the carotenoids and other physiological components tested in vitro, α-carotene is an effective quencher of singlet oxygen [8,9]. The class of carotenoid compounds is also characterized by other anti-oxidant properties [10–12]. In addition, potent anti-carcinogenicity has been demonstrated for α-carotene in both mice and a human cell line [13–15]. With respect to provitamin A activity, α-carotene has about one-half the biological potency of β-carotene; however, *cis*-isomers of α-carotene are much less efficiently converted to vitamin A [16]. *cis*-α-Carotenes have been observed in several biological samples [3,17–20], although the chromatographic resolution of these particular isomers is usually not pursued with

* Corresponding author.

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rigor. Some studies have dealt with separations of *cis*- and *trans*-α-carotenes using photo-isomerized standards [20–22], but resolved peaks were only tentatively identified.

Recently, a polymeric C_{30} stationary phase was developed specifically to optimize RPLC separations of carotenoids [23]. The shape selectivity of this stationary phase toward geometrical carotenoid isomers is especially good, as has been demonstrated for several common carotenoids, including α-carotene [24]. Double-bond configurations of some chromatographic peaks could only be tentatively assigned, according to their electronic absorption spectra. However, proton NMR spectroscopy at high magnetic fields can be used to elucidate the double bond geometries of *cis-trans* carotenoids, facilitating identification of these isomers without ambiguity. Detailed information on this subject is available elsewhere [25–27]. NMR spectroscopy has been applied successfully to identify geometrical isomers of the following carotenoids: β-carotene [28–30], lycopene [31], lutein [32], zeaxanthin [32,33], canthaxanthin [26,34], neurosporene [35,36], fucoxanthin [37], spirilloxanthin [38], and astaxanthin diacetate [39].

The objectives of this research were: (1) to demonstrate the utility of a 3-μm polymeric C_{30} stationary phase for the separation and isolation of geometrical isomers of α-carotene, and (2) to unambiguously identify the predominant geometrical isomers of α-carotene in a photo-isomerized mixture resolved on the C_{30} column.

2. Experimental

2.1. Sample preparation

All-*trans*-α-carotene (Sigma, St. Louis, MO, USA)[1] was purified of any oxidized contaminants formed during manufacture and storage using an open column packed with neutral, deactivated alumina and a acetone–hexane (10:90) eluent. Greater than 97% purity of the all-*trans* standard was verified using the chromatographic conditions described below. The purified all-*trans*-α-carotene was photo-isomerized into an equilibrium mixture of various geometrical isomers by adding iodine catalyst at a concentration of about 2% (w/w) of the carotenoid weight (~0.1 mg/ml in hexane as determined spectrophotometrically at 442 nm using a specific extinction coefficient of 2800 [40]) and exposing the solution to ambient fluorescent laboratory light for 1 h [16]. The isomerized mixture was dried under a stream of nitrogen gas and stored at −20°C.

2.2. Instrumentation

Analytical (250 × 4.6 mm I.D.) and semi-preparative (250 × 10 mm I.D.) 3 μm polymeric C_{30} columns were prepared at NIST (Gaithersburg, MD, USA) as described previously [23]. Guard columns packed with the same stationary phase were used in-line during all separations. The HPLC system consisted of a Waters Model 501 solvent delivery system and Model U6K injector (Milford, MA, USA), an Anspec UV–Vis detector (Model SM 95; Linear Instruments, Reno, NV, USA), a Dionex advanced computer interface (Model ACI-1; Sunnyvale, CA, USA), and a Dramen personal computer (Raleigh, NC, USA). To determine the relative abundances of several α-carotene isomers, chromatographic peaks were integrated by area using Dionex AI-450 chromatography software (release 3.30). Electronic absorption spectra and absorption maxima of α-carotene peaks were obtained using a Waters Model 996 photodiode array detector that was linked to a Gateway 2000 personal computer (Model 4DX2-66V; North Sioux City, SD, USA) equipped with Millenium 2010 chromatography software (LC version 2.00; Millipore, Milford, MA, USA). Absorption maxima were also measured in several pure solvents using a double-beam UV–Vis spectrophotometer (Model UV-160A; Shimadzu, Kyoto, Japan).

[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.

2.3. Chromatographic analysis and peak isolation

A representative chromatogram of isomerized α-carotene, electronic absorption spectra, and purity assessments were obtained using the analytical scale C_{30} column and an isocratic mobile phase of methyl-*tert.*butyl-ether (MTBE)–methanol (11:89, v/v) at a flow-rate of 1 ml/min. Column back pressure was $1.38 \cdot 10^7$ Pa. Methanol and MTBE were certified A.C.S. and HPLC grades, respectively (Fisher Chemical, Fairlawn, NJ, USA). The representative chromatogram was obtained by injecting 8 μl of isomerized α-carotene dissolved in the mobile phase. The other separations were obtained using MTBE–methanol (50:50, v/v) as the injection solvent. For all chromatographic runs, column effluent was monitored at 450 nm and column temperatures were ambient laboratory temperature (~23°C). Electronic absorption spectra were measured from 250 to 550 nm in the LC mobile phase during C_{30} chromatography.

Five predominant α-carotene isomers were isolated for NMR spectroscopy using the semi-preparative C_{30} column, MTBE–methanol (15:85, v/v), and a flow-rate of 3.5 ml/min. Each peak of interest was collected into 2 ml of hexane, and 5 ml of deionized water were added to cause phase separation and partitioning of the isomer into the hexane epilayer. The hexane layer was withdrawn and several crystals of anhydrous sodium sulfate were added to remove any contaminating water. After pooling all fractions containing the same isomer, the hexane was evaporated to dryness under a stream of nitrogen, and the sample was held under reduced pressure (~6.5 Pa) for 10 min to remove any residual solvent. The purified isomers were stored under a headspace of nitrogen gas at −20°C or on dry ice during shipping. Exposure of the purified isomers to light was kept to an absolute minimum during all experimental procedures.

Absorption maxima for the same isomers purified separately from a single injection were determined in hexane (certified A.C.S.; Fisher), acetone (HPLC grade, UV cutoff 330 nm; Fisher), ethanol (absolute; Midwest Grain Products, Pekin, IL, USA), petroleum ether (spectrophotometric grade; Aldrich, Milwaukee, WI, USA), dichloromethane (Certified A.C.S.; Fisher), and MTBE by scanning from 250 to 350 nm and from 400 to 500 nm. With the spectrophotometer used, scanning over wavelength ranges of 100 nm or less gives values for absorption maxima with an accuracy of 0.1 nm.

2.4. ^1H NMR spectroscopy

The ^1H NMR (400 MHz) spectra were obtained on a Bruker ARX-400 spectrometer (Rheinstetten, Germany) with ASPECT station 1. All spectra were measured in C^2HCl_3 (100% deuterium quality) at ~25°C with a 5-mm reversed probe head with optimum sensitivity for protons. The internal standard was tetramethylsilane (TMS).

3. Results and discussion

3.1. C_{30} Separation of α-carotene isomers

As illustrated in Fig. 1, several geometrical isomers of α-carotene were separated from a photo-isomerized mixture on the 3-μm polymeric C_{30} stationary phase. A similar high de-

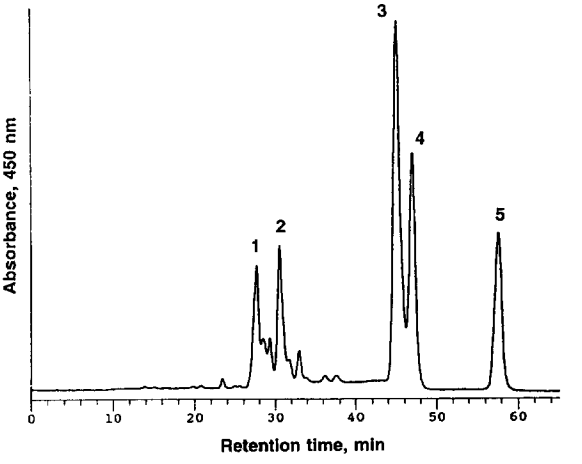

Fig. 1. Chromatographic separation of geometrical α-carotene isomers in a photo-isomerized mixture: 3-μm polymeric C_{30} stationary phase; MTBE–methanol (11:89); 1 ml/min; 450 nm. Double bond configurations were investigated for the numbered peaks.

gree of shape selectivity by a 5-μm polymeric C_{30} column has been demonstrated for geometrical isomers of β-carotene, lutein, β-cryptoxanthin, lycopene, and zeaxanthin, in addition to α-carotene [24]. As expected, resolution of isomerized mixtures of α-carotene and the other carotenoids was improved slightly when separated on the 3-μm C_{30} stationary phase as compared to the 5-μm C_{30} column. For separations of geometrical carotenoid isomers, the C_{30} stationary phase is clearly superior to existing RPLC columns that are commonly employed for this application [24]. Evident differences in the properties of C_{30} and C_{18} stationary phases are enhanced shape selectivity and increased retention on the C_{30} phase. It may be possible to improve upon these C_{30} separations of geometric carotenoid isomers by modifying column temperature, because this has been shown to significantly influence the separation selectivity of other polymerically synthesized reversed-phase columns toward carotenoids [41,42] and polyaromatic hydrocarbons [43]. Good separations of geometrical carotenoid isomers have also been achieved in normal-phase LC using calcium hydroxide, alumina, silica, and nitrile-bonded stationary phases [21,28,29,31,32,34,36,44,45]. α-Carotene, an asymmetrical carotenoid, was the subject of one such report [21], and peaks corresponding to the all-*trans*-, 15-*cis*-, 13-*cis*-, 13′-*cis*-, 9-*cis*-, and 9′-*cis*-isomers were resolved on a calcium hydroxide column. Unfortunately, these columns are not commercially available, and the reproducibility and reliability of self-packed columns is generally poor. In the previous reports on C_{30} and calcium hydroxide separations of geometrical α-carotene isomers [21,24], certain chromatographic peaks were only tentatively identified according to their electronic absorption spectra. Peaks that were believed to correspond to the 13-*cis*- and 13′-*cis*-isomers and the 9-*cis*- and 9′-*cis*-isomers of α-carotene, for example, could not be identified individually because the spectra for each isomeric pair could not be differentiated. In addition, for the C_{30} separation, relative retention characteristics of the various *cis*-α-carotene peaks could not be used to aid in peak identification because comparative C_{30} separations and authentic standards are currently unavailable. Therefore, elucidation of double bond geometries by ^{1}H NMR spectroscopy was pursued to unambiguously identify several geometrical isomers of α-carotene resolved on the C_{30} stationary phase.

3.2. Isolation of α-carotene peaks for NMR spectroscopy

Semi-preparative C_{30} chromatography was used to isolate approximately 75 μg of each of the numbered peaks in Fig. 1 for subsequent ^{1}H NMR spectroscopy. Assessment of the purity of these peaks gave the following results: peak 1, 65%; peak 2, 90%; peak 3, 94%; peak 4, 95%; and peak 5, 96%. Although it is known that *cis*-α-carotenes have lower absorptivities (ϵ) than the all-*trans* configuration [46], the individual molar absorptivity values for these isomers are not known. Therefore, these measurements are based on the assumption that their absorptivities are equal. From these analyses, the lack of greater purity could be attributed primarily to post-column re-isomerization and secondarily to difficulty isolating relatively large quantities of individual peaks. For example, purified peak 1 contained peak 3 (23%) and peak 5 (2%) as contaminants, as well as the two minor peaks eluting immediately after peak 1 (10%). The appearance of peaks 3 and 5 in the peak 1 isolate could have only occurred by re-isomerization, and this was believed to occur when multiple collections of the fraction were combined. Each fraction was dissolved several times in hexane as a result of this procedure, providing more favorable conditions for re-isomerization than would prevail if the fractions were held continuously in a dry state after the initial collection. To ascertain the stability of the α-carotene isomers, peaks 1–5 were collected individually from a single injection, immediately evaporated to dryness, and stored for 2 weeks at −20°C under a headspace of nitrogen gas. Under these conditions, all peaks maintained 95 ~ 100% purity (data not shown).

3.3. ^1H NMR spectroscopy of α-carotene isomers

Chemical shifts (δ in ppm) and important isomerization shifts (Δδ in ppm, where $\Delta\delta = \delta_{cis} - \delta_{trans}$) for α-carotene peaks 1–5 are given in Table 1. In general, isomerization shifts of $|\Delta\delta| > 0.02$ ppm are considered relevant for the identification of geometric carotenoid isomers [25]. For peak 3, assignment of the NMR signals was aided by one-dimensional TOCSY (total correlation spectroscopy) and one-dimensional ROESY (rotating-frame nuclear Overhauser effect spectroscopy) experiments. Similarly, one-dimensional TOCSY and homonuclear ^1H, ^1H COSY (correlation spectroscopy) two-dimensional experiments were performed to assign the NMR signals of peak 2. It should be mentioned that the NMR signals of peak 1 could not all be assigned unambiguously because the sample was not pure, but those signals required for the identification of its principle component were assigned without ambiguity.

Structures of the geometrical α-carotene isomers, isolated as peaks 1–5 on a polymeric C_{30} stationary phase and unambiguously identified by ^1H NMR spectroscopy, are shown in Fig. 2. The double bond geometries of these isomers were assigned according to the following details. By comparing the assigned chemical shifts of peak 3 to those obtained previously for an authentic standard, peak 3 was unambiguously identified as all-*trans* α-carotene. The chemical shifts observed for this isomer are in excellent agreement with published values [25,27]. Double bond configurations for the other α-carotene peaks were elucidated by determining characteristic isomerization shifts (Δδ). For peak 1, a downfield isomerization shift of $\Delta\delta = 0.53$ ppm is observed at C–H (12), along with other indicative shifts downfield at C–H (15) and C–H (10) and upfield at C–H (14) and C–H (15′). These isomerization shifts are typical of a *trans*-to-*cis* change in geometrical configuration at the 13 or 13′ position of carotenoids [25]. Since α-carotene is an asymmetrical carotenoid, a distinction must be made between *cis* bonds at the 13 and 13′ positions when these isomers are believed to be isolated individually. It is quite clear from the positions of the isomerization shifts for peak 1 that the presence of the 13-*cis*-isomer is confirmed. By the same reasoning, peak 2 was unambiguously identified as the 13′-*cis* geometrical form of α-carotene. In this case, isomerization shifts of the same magnitude were measured for each of the respective protons on the opposite end of the molecule (Table 1). For peak 4, an isomerization shift of $\Delta\delta = 0.53$ ppm was observed at C–H (8), and this shift was accompanied by characteristic shifts of smaller magnitude at C–H (10), C–H (11), and C–H (12). Isomerization shifts at these positions and magnitudes are characteristic of a *trans*-to-*cis* change in geometrical configuration at the 9 or 9′ position [25]. Based on the specific positions of these shifts, peak 4 is identified as 9-*cis*-α-carotene. The same characteristic isomerization shifts were measured for peak 5, but these shifts occurred at C–H (8′), C–H (10′), C–H (11′), and C–H (12′). Thus, peak 5 is the 9′-*cis*-isomer of α-carotene. An isomerization shift of $\Delta\delta = 0.06$ ppm observed for the proton at the 6′ position of peak 5, which was not observed for any of the other isomers, offers further evidence of the 9′-*cis* configuration. Differentiation between 9-*cis* and 9′-*cis* as well as 13-*cis* and 13′-*cis* is also reflected by minor changes of the signals of the respective end groups that are in relatively close proximity to the *cis* bond (Table 1).

Isomerization shifts of interior protons on the conjugated system of the *cis* carotenoid isomers, i.e., C–H (7) through C–H (15), are usually quite characteristic of specific double bond geometries, whereas those of the methyl protons are not [25]. Isomerization shifts of methyl protons, which are usually minor downfield shifts, may, however, be valuable for locating the position of *cis* bonds (Table 1).

3.4. Electronic absorption spectra of α-carotene isomers

Additional evidence for the identifications made by ^1H NMR spectroscopy can be gained from electronic absorption spectra and other

Table 1
^{1}H NMR data for the predominant α-carotene isomers isolated from a photo-isomerized mixture

Position and type of proton		Peak 3 (all-*trans*)	Peak 1 (13-*cis*)		Peak 2 (13′-*cis*)		Peak 4 (9-*cis*)		Peak 5 (9′-*cis*)	
		δ	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$	δ	$\Delta\delta$
2	C–H ax	~1.47	~1.47		~1.47		~1.49		~1.47	
2	C–H eq									
2′	C–H ax	~1.46	~1.46		~1.47		~1.46		~1.48	
2′	C–H eq	~1.18	~1.20		~1.19		~1.19		~1.21	~0.03
3	C–H ax	~1.61	~1.62		~1.61		~1.64	~0.03	~1.62	
3	C–H eq									
3′	C–H ax	~2.02	~2.02		~2.02		~2.01		~2.02	
3′	C–H eq									
4	C–H ax	~2.02	~2.03		~2.02		~2.05	~0.03	~2.02	
4	C–H eq									
4′	C–H	5.41	5.41		5.42		5.41		5.43	
6′	C–H (9.4 Hz)[a]	2.18	2.18		2.20		2.18		2.24	0.06
7	C–H	6.17	6.19		6.17		6.18		6.17	
7′	C–H	5.53	5.52		5.55		5.53		5.55	
8	C–H	6.14	6.15		6.13		6.67	0.53	6.13	
8′	C–H	6.11	6.11		6.12		6.11		6.63	0.52
10	C–H	6.15	6.19	0.04	6.15		6.05	−0.10	6.15	
10′	C–H	6.13	6.13		6.17	0.04	6.12		6.00	−0.13
11	C–H	6.65	6.65		6.64		6.75	0.10	6.65	
11′	C–H	6.61	6.60		6.62		6.61		6.76	0.15
12	C–H	6.35	6.88	0.53	6.36		6.29	−0.06	6.35	
12′	C–H	6.34	6.34		6.87	0.53	6.34		6.28	−0.06
14	C–H	~6.25	6.10	~−0.15	6.23		~6.24		~6.24	
14′	C–H		6.23		6.10	~−0.15				
15	C–H	~6.62	6.79	~0.17	6.55	~−0.07	~6.62		~6.62	
15′	C–H	6.55	~−0.07	6.79	~0.17					
16	CH_3	1.029	1.036		1.031		1.043		1.030	
17	CH_3									
16′	CH_3	0.904[b]	0.904[b]		0.911[b]		0.903[b]		0.926[b]	
17′	CH_3	0.822[b]	0.822[b]		0.828[b]		0.821[b]		0.880[b]	0.06
18	CH_3	1.718	1.726		1.719		1.756	0.04	1.718	
18′	CH_3	1.584	1.585		1.592		1.584		1.611	0.03
19	CH_3	1.972	1.977		1.970		1.959		1.972	
19′	CH_3	1.911	1.909		1.917		1.910		1.909	
20	CH_3	1.972	1.992	0.02	1.963		1.971		1.972	
20′	CH_3	1.962	1.952		1.981	0.02	1.959		1.986	0.02

[a] Coupling constant (J).
[b] For each peak, values may be interchanged.
δ in ppm; $\Delta\delta = \delta_{cis} - \delta_{trans}$ for all $|\Delta\delta| \leq 0.02$ ppm; measurements at 400 MHz in C^2HCl_3.

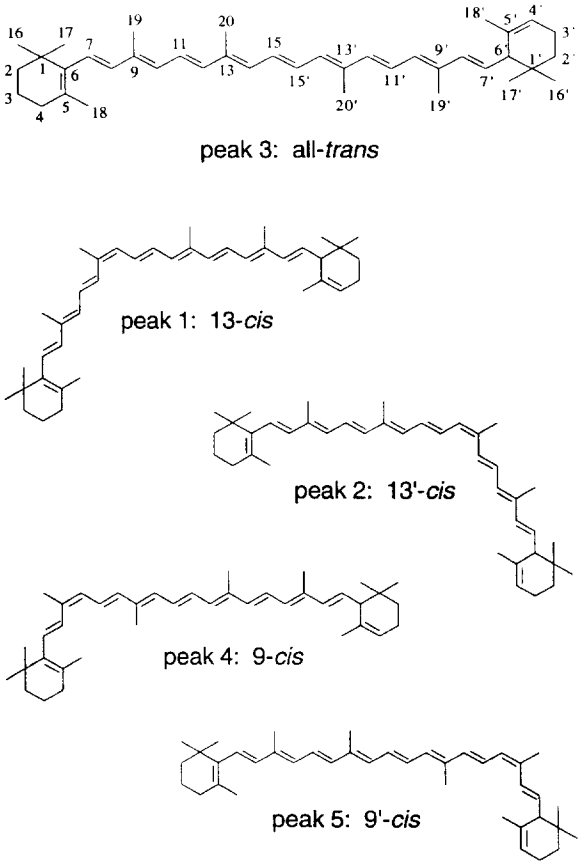

Fig. 2. Structures of the predominant geometrical isomers of α-carotene isolated on a polymeric C_{30} stationary phase and unambiguously identified by ^1H NMR spectroscopy.

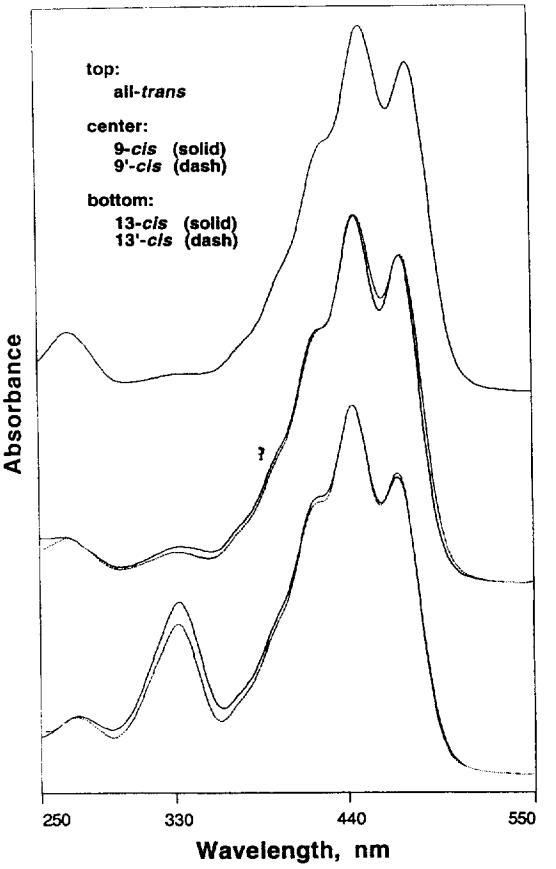

Fig. 3. Electronic absorption spectra of the all-*trans*, 9-*cis*, 9'-*cis*, 13-*cis*, and 13'-*cis* geometrical isomers of α-carotene obtained in the LC mobile phase [MTBE–methanol, 11:89] from 250 to 550 nm by photodiode array detection.

spectral characteristics obtained for each of the α-carotene isomers (Fig. 3; Tables 2 and 3). All of the spectra are characteristic of β,ϵ-carotenoids with respect to fine structure (~400 to 500 nm) [16]. The spectrum of all-*trans*-α-carotene (peak 3) has typical absorption maxima, and the practical absence of near-UV (~330 nm) absorbance is characteristic of this particular configuration. These properties are in good agreement with those published previously [40,46]. The 13-*cis*- (peak 1) and 13'-*cis*- (peak 2) isomers had identical absorption maxima in the LC mobile phase, and hypsochromic shifts of ~7 nm were detected in several pure organic solvents for their main absorption maxima, relative to that of all-*trans* (Tables 2 and 3). In general, a hypsochromic shift of about 5 nm is observed with the introduction of a *cis* bond in a carotenoid structure, but the specific magnitude of these shifts depends on the position of the *cis* bond. Because absorbance in the near-UV region generally increases as the position of the *cis* bond approaches the center of the conjugated system [16], rather strong absorbance in this region by the 13-*cis*- and 13'-*cis*-isomers was expected (Fig. 3). This absorbance is reflected in the ϵ_2/ϵ_1 values for these isomers [i.e., ratio of absorbances at the near-UV maxima (ϵ_2) and main absorbance maxima (ϵ_1)], which are greater than those of the all-*trans*- and 9-*cis*-isomers (Tables 2 and 3). The ϵ_2/ϵ_1 values of the 13-*cis*-and 13'-*cis*-α-carotene isomers are typical [16,28,32]. The

Table 2
Electronic absorption maxima and relative abundances of the predominant geometrical isomers of α-carotene in a photo-isomerized mixture

Isomer	Absorption maxima[a] (nm)				$[\epsilon_2/\epsilon_1]^b$	Relative abundance[c] (%)
all-*trans*	~336[d]	~424[d]	(446)	473	[0.052]	35
13-*cis*	332	416	(438)	465	[0.469]	9
13′-*cis*	332	416	(438)	465	[0.410]	10
9-*cis*	330	418	(441)	467	[0.102]	21
9′-*cis*	330	~421[d]	(441)	469	[0.086]	16

[a] Measured in the LC mobile phase [MTBE–methanol (11:89)] using a photodiode array detector. Values in parentheses represent the main absorption maxima.
[b] Ratio of absorption intensity (ϵ_2) at the near-UV maxima (330–336 nm) to absorption intensity (ϵ_1) at the main absorption. maximum (438–446 nm).
[c] Peak-area percent relative to total area of all peaks detected at 450 nm from a photo-isomerized mixture of α-carotenes. Measurements are based upon the assumption that absorptivities (ϵ) of the α-carotene isomers are equal.
[d] No maximum was detected by the photodiode array detector. Value represents the approximate center of the inflection point.

Table 3
Electronic absorption maxima of geometrical α-carotene isomers in pure organic solvents

Isomer	Absorption maxima[a] $[\epsilon_2/\epsilon_1]^b$	
	Hexane	Petroleum ether
all-*trans*	267, nd, 421, (445), 474 [——]	267, 328, 420, (443), 472 [0.047]
13-*cis*	267, 331, 415, (438), 465 [0.416]	271, 329, 413, (436), 463 [0.408]
13′-*cis*	270, 330, 415, (437), 465 [0.369]	268, 330, 413, (435), 463 [0.357]
9-*cis*	258, 330, 417, (440), 469 [0.091]	255, 328, 416, (439), 467 [0.101]
9′-*cis*	258, 328, 418, (441), 469 [0.095]	258, 328, 415, (439), 467 [0.100]
	MTBE	Ethanol
all-*trans*	268, nd, nd, (445), 474 [——]	267, nd, nd, (447), 474 [——]
13-*cis*	270, 331, nd, (438), 466 [0.426]	267, 331, nd, (439), 466 [0.443]
13′-*cis*	269, 331, nd, (438), 465 [0.380]	269, 331, nd, (438), 465 [0.386]
9-*cis*	259, 329, 420, (442), 470 [0.110]	258, 331, 420, (442), 470 [0.108]
9′-*cis*	266, 329, 420, (442), 470 [0.102]	259, 328, nd, (442), 470 [0.107]
	Dichloromethane	Acetone[c]
all-*trans*	271, nd, 432, (456), 484 [——]	330, nd, (448), 476 [0.095]
13-*cis*	273, 337, 428, (449), 476 [0.352]	332, nd, (440), 468 [0.433]
13′-*cis*	272, 337, 424, (448), 475 [0.331]	332, nd, (440), 467 [0.380]
9-*cis*	261, 339, 428, (450), 479 [0.109]	332, nd, (443), 471 [0.092]
9′-*cis*	262, 336, 429, (452), 480 [0.113]	332, nd, (444), 472 [0.087]

[a] Units are nm. Values in parentheses represent the main absorption maxima.
[b] Ratio of absorption intensity (ϵ_2) at the near-UV maxima (328–339 nm) to absorption intensity (ϵ_1) at the main absorption maximum (435–456 nm).
[c] Absorbance at ≤330 nm interfered with the detection of the overtone (~265 nm).
[nd] No maximum was detected by the spectrophotometer.

9-*cis*- (peak 4) and 9′-*cis* (peak 5)-isomers also have nearly indistinguishable absorption spectra, although subtle differences are apparent in their fine structures (Fig. 3).

Minor differences were also detected among their respective absorption maxima (Tables 2 and 3). A hypsochromic shift of ~5 nm was observed for their main absorption maxima in the solvents tested. These shifts are smaller than those observed for the 13-*cis*-isomers, in agreement with previously reported values for structurally similar carotenoids [32,36,45]. Small "*cis* peaks" are observed in absorption spectra of the 9-*cis*- and 9′-*cis*-isomers in the near-UV region, giving characteristic low values for ϵ_2/ϵ_1 [16,28,32].

Spectra of the 13-*cis* and 13′-*cis* isomers are difficult to distinguish, but a noticeable difference in absorbance exists in the near-UV region, giving respective ϵ_2/ϵ_1 ratios of 0.469 and 0.410 for these isomers in the LC mobile phase (Table 2). This difference could be caused by the presence of a co-eluting α-carotene isomer in either peak 1 or peak 2 during chromatography. No evidence was gained in this regard from the NMR analyses because small amounts of several known impurities were present in the peak 1 and peak 2 isolates. A similar observation can be made for the ϵ_2/ϵ_1 ratios of the 9-*cis* (0.102) and 9′-*cis* (0.086) isomers (Table 2), although the difference is smaller in this case. It is possible, and perhaps likely, that the 13-*cis*- and 13′-*cis*- and the 9-*cis*- and 9′-*cis*-isomeric pairs have slightly different, characteristic absorption spectra within each pair. The 13-*cis*- and 13′-*cis*-isomers of neurosporene have ϵ_2/ϵ_1 ratios of 0.57 and 0.25, respectively [36]. Similarly, the respective ϵ_2/ϵ_1 ratios of 13-*cis*- and 13′-*cis*-lutein are 0.489 and 0.421, while those of the 9-*cis*- and 9′-*cis*-isomers are 0.118 and 0.110, respectively [32]. These observations are possible only with asymmetrical carotenoids, and the evident trend is that a higher ϵ_2/ϵ_1 ratio is observed when the *cis* bond is positioned on the more highly conjugated end of the carotenoid molecule. These differences may be useful for tentative identifications of certain geometrical isomers when their unambiguous identification by NMR spectroscopy is not possible. It should be noted, however, that the ϵ_2/ϵ_1 ratios of α-carotene isomeric pairs vary with the type of solvent (Table 3).

3.5. Resolution and elution order of geometrical carotenoid isomers on the C_{30} stationary phase

The polymeric C_{30} stationary phase has been applied to separations of geometrical isomers of only several common carotenoids under very similar chromatographic conditions. However, several preliminary observations, that may have general implications, can be made about the resolution and elution order of these isomers on the C_{30} column. In a previous report based on tentative peak identifications [24], it was noted that the 15-*cis*-isomers of several β,β-carotenoids were separated while those of β,ϵ-carotenoids were not. Among several asymmetrical carotenoids, the 13-*cis*- and 13′-*cis*-isomers of a β,β-carotenoid co-eluted, while those of β,ϵ-carotenoids were resolved. The 9-*cis*- and 9′-*cis*-isomers of the same asymmetrical carotenoids were resolved, although there was clearly less selectivity toward the 9-*cis*- and 9′-*cis*-isomers of the β,β-carotenoid. Geometrical isomers of β-carotene and zeaxanthin eluted in the order: 15-*cis*, 13-*cis*, all-*trans*, and 9-*cis*. It is now known from the present research, that predominant α-carotene isomers elute in the order: 13-*cis*, 13′-*cis*, all-*trans*, 9-*cis*, and 9′-*cis*. A chromatographic profile similar to the one presented in Fig. 1 for α-carotene was obtained when photo-isomerized lutein was previously applied to the C_{30} stationary phase [24], and it is likely, but not confirmed, that the 13-*cis*- and 9-*cis*-isomers elute before their 13′-*cis* and 9′-*cis* counterparts.

The resolution of geometrical α-carotene isomers achieved with the C_{30} stationary phase allows a better assessment of the composition of a photo-isomerized mixture of these isomers. The relative abundances of the five α-carotene isomers isolated and identified herein, determined on the assumption of equal molar absorptivities, are given in Table 2. It is interesting to note that the all-*trans*-isomer represents only 35% of total α-carotene, while the combined contribution of the five predominant isomers is 91%. Because the photo-isomerized α-carotene

mixture used in the present research was at equilibrium, the relative stabilities of these isomers are generally indicated. Photo-isomerized carotenoid mixtures can be used to develop and verify LC methods and to aid in the identification of geometrical isomers. Knowing the isomeric composition of such a mixture can be useful for assessments of peak identity and purity.

4. Summary

The present research: (1) demostrates the high degree of shape selectivity by the polymeric C_{30} stationary phase toward geometrical isomers of α-carotene; (2) unambiguously confirms the double-bond configurations of five α-carotene isomers previously unresolved in RPLC; and (3) supplements the existing spectroscopic data on geometrical carotenoid isomers.

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