Separation and Identification of Cis/Trans Carotenoid Isomers

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To have a better assessment of major carotenoids, there is a need to systematically elucidate cis isomers of these polyenes. The combination of separation (Vydac and Zorbax C_{18} columns), spectral ratios (D_B/D_{II} and D_{II}/D_{III}), and spectral characteristics was used to help identify cis/trans isomers. The less lipophilic carotenoids are better separated by the Zorbax column and the more lipophilic carotenes by the Vydac column. The optimized isocratic mobile-phase composition for both columns was CH_3CN:CH_3OH:CH_2Cl_2 (80:18:2). One to four cis isomers were differentiated from their corresponding trans isomers in four acyclic (phytoene, phytofluene, ζ-carotene, and lycopene) and three cyclic (γ-carotene, β-carotene, and lutein) carotenoids. The D_B/D_{II} ratios were used to predict conjugated double bonds between 9 and 11 and the D_{II}/D_{III} ratios for conjugated double bonds between 3 and 7.

INTRODUCTION

Carotenoid pigments are widely distributed in nature and are found in many tissues (Simpson, 1983). Typically, carotenoids consist of eight isoprenoid units with varying end groups comprising the 40-carbon (C-40) skeletons. Hence, they are highly lipophilic. Carotenoids are further classified into two major groups: carotenes and xanthophylls. While carotenes are purely hydrocarbons, xanthophylls are usually oxygenated at the end groups and hence are more polar.

Spectral Characteristics. Absorption spectra of most carotenoids exhibit a main absorption band in the visible region. This band usually consists of three maxima or two maxima and a shoulder. In addition, minor bands appear at shorter wavelengths. It is well established (Tan and Soderstrom, 1989; Quackenbush, 1987) that cis double bonds in carotenoids cause characteristic hypsochromic spectral shifts with the appearance of the so-called "cis peak" in the near-visible region. Spectra of cis/trans mixtures exhibit a shift toward shorter wavelengths of a few nanometers, and the formation of a cis peak that appears at 330–350 nm. The molar absorptivity values of the main and cis peaks vary from isomer to isomer, and these qualitative characteristics allow for the identification of these carotenoids.

Recent HPLC Separation of Carotenoid Isomers. Various HPLC conditions have been developed. Matus et al. (1981) separated xanthophyll isomers of violaxanthin, lutein, auroxanthin, and mutatoxanthin using a Nucleosil 10-μm C_{18} column with a gradient mobile-phase system of CH_3COCH_3:H_2O. Tsukida et al. (1982) achieved good separations of mono-cis and di-cis isomers of β-carotene using a home-made lime column and a mobile phase of 0.1–2% CH_3COCH_3 in C_6H_{12}. Chandler and Schwartz (1987) succeeded in separating isomers of both α- and β-carotenes using a slurry-packed $Ca(OH)_2$ column and a mobile phase of CH_3COCH_3:C_6H_{12} (3:997 v/v). Bushway (1986) used a Vydac C_{18} (218TP54) column and a mobile phase of CH_3CN:CH_3OH:THF (40:56:4) to separate both polar and nonpolar carotenoids. Quackenbush (1987) used a Vydac C_{18} (201TP54) column to separate isomers of β-carotene using a gradient mobile-phase system of 100% CH_3OH for the first 5 min and CH_3OH:$CHCl_3$ (96:4) for the subsequent run.

The complexity of carotenoid mixtures in nature, as well as the variation in their polarity, makes it virtually impossible to separate all classes of carotenoids including their geometric isomers in a single chromatographic run. Most of the successful separations mentioned earlier used a home-made column of lime [$Ca(OH)_2$] or a gradient mobile phase system. Home-made columns may have irreproducible column efficiency, and these columns are not generally accessible to other researchers. Also, the use of gradient elution is not recommended in carotenoid analysis because of the poorer reproducibility resulting from long re-equilibration times between runs and the risk of solute precipitation in less lipophilic mobile phases causing the appearance of "ghost" peaks (Khachik et al., 1988). Better separation of α- and β-carotenes (including cis isomers of β-carotene) using C_{18} columns (e.g., Vydac 218TP54 and 201TP54 and Zorbax ODS) has been established (Bushway, 1985).

To have a better assessment of major carotenoids, it is important to have a handle on the cis isomeric forms of these major carotenoids (Sri Kantha et al., 1987; Simpson, 1983). Since scattered works have appeared in the literature, predominantly on the cis isomers of β-carotene, there is a need to systematically elucidate other major carotenoid cis isomers. In this paper, the analysis of cis isomers of complex carotenoid mixtures was conducted on carrot and palm oils.

EXPERIMENTAL PROCEDURES

Apparatus. The analytical instrument used was an HP 1090 L (Hewlett-Packard) liquid chromatograph equipped with a UV-vis diode array detector. This included a Rheodyne 7010 manual injection switching valve (20-μL sample loop), PV-5 ternary solvent delivery system, and a 4.5-μL flow cell (6 mm path length). The two analytical columns utilized in this research were a 25 × 0.46 cm Zorbax ODS column, containing 5 μm particle size (Du Pont, Wilmington, DE) with a 1.5 × 0.32 cm Du Pont Zorbax ODS guard column, and a 25 × 0.46 cm Vydac 218TP54 C_{18} column, containing 5 μm particle size (Vydac Separation Group, Hesperia, CA) with a 1.5 × 0.32 cm Vydac C_{18} guard column.

Materials and Methods. Carotenoid concentrates of crude palm oil (up to 20%) were prepared at Carotech Associates (Amherst, MA) and were diluted and dissolved in hexane prior to injection. Carrot oil carotenes (ca. 1.5% concentration) were obtained from Nutritional Research Associates Inc. (South Whitley, IN). Cis carotenoid isomers were derived from either palm or carrot oils. Chromatographic data were stored over a range of 250–550 nm, and chromatograms of various isomers were

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reconstructed at the wavelengths of their maximum absorbance for easier interpretation. Spectra of isomeric carotenoids were overlayed for identification and comparison purposes. Standards of α-, β-, ζ-, γ-carotene, lutein, lycopene, phytofluene, and phytoene were denoted by Hoffmann-La Roche (Nutley, NJ, and Basel, Switzerland). Twenty microliters of carotenoid standards in hexane were individually injected in each column for the mobile-phase system under study. Carotenoid identifications were performed through comparison with these external standards, other published reference spectral data, and/or chromatographic elution profiles.

RESULTS AND DISCUSSION

Chromatographic Columns. It is already established that the reduction of chain length of the bonded phases (e.g., use of hexyl- or octyl- instead of octadecyl-type materials) has an unfavorable effect on separation, since shorter chain stationary phases require higher water concentrations in the eluents to yield sufficient retention times. This higher water content requirement of the mobile phase is not suitable for the hydrophobic carotenoids, necessitating the use of nonaqueous reverse-phase systems (Nelis and De Leenheer, 1983). In this work, the less lipophilic carotenoids were separated by using the Zorbax column (e.g., for lutein cis isomers), and the more lipophilic carotenoids were separated by using the Vydac column (e.g., for *cis*-β-carotene isomers) (Taylor and Lanza, 1990). This selectivity may be due to the difference in functional groups in the stationary phase endcapping (Zorbax, trimethylsilane; Vydac, dimethylpropylsilane). The smaller pore size (or larger surface area; Zorbax 7 nm compared to Vydac 30 nm) and higher degree of carbon loading (Zorbax 20% C_{18} compared to Vydac 9% C_{18}) result in increased carotenoid retention times in the Zorbax column. Other explanations to the selectivity of the Vydac column via pore size (MacCrehan, 1990) and stationary phase functionality (Craft et al., 1990) have been forwarded. When the Vydac 218TP54 (C3 endcapped) column was used, the order of elution of β-carotene isomers was all-trans, 9-cis, and 13-cis (Quackenbush, 1987). However, when a Vydac 201TP54 (not endcapped) was used in the same experiment, the order of elution was found to be all-trans, 13 cis, and 9-cis.

Chromatographic sections of separated carotenoid isomers are shown in Figure 1. The less lipophilic carotenoids are better separated by the Zorbax column (Figure 1A–F) and the more lipophilic carotenoids by the Vydac column (Figure 1G). However, α-carotene closely retained with phytofluene and β-carotene, which makes the separation of α-carotene cis isomers, if present, difficult. Under the present separation conditions, the α-carotene isomers cannot be chromatographed.

Mobile-Phase Optimization. Mobile-phase optimization was performed on the Zorbax column according to a scheme that was then applied for the Vydac column. Various combinations of CH_3CN, CH_3OH, and CH_2Cl_2 were performed, and selection of the mobile-phase system was based on the resolution and the capacity factors of α- and β-carotenes (and isomers of β-carotene in the case of the Vydac column).

The first mobile-phase system (I of Table I) used in this study was a mixture of CH_3CN:CH_3OH:CH_2Cl_2 (60:20:20). A sample of palm oil carotenes was injected in the Zorbax and Vydac columns. While the Zorbax column showed higher retention times (capacity factor = 7.6 for β-carotene) than the Vydac column (capacity factor = 2.3 for β-carotene), the resolution of α- and β-carotenes did not exceed 0.7 for either column. Modification of the mobile-phase composition was then necessary to achieve better separations of these carotenes. Various mobile-phase compositions were explored, and some of these compositions are shown in Table I. High concentrations of CH_2Cl_2 (above 20%) in the mobile-phase system such as CH_3CN:CH_3OH:CH_2Cl_2 (60:4:36) did not prove useful. Peak shapes of both α- and β-carotenes showed a high degree of overlap and distortion due to insufficient partitioning time. Both α- and β-carotenes eluted in a period of 5 min, and the resolution was <0.5. Thus, the idea of using a high percentage of CH_2Cl_2 was ruled out.

While the concentration of CH_3CN was kept *constant* at 60%, CH_2Cl_2 concentration in the mobile phase was gradually decreased, and mobile-phase systems of higher concentrations of CH_3OH were performed. The resolution of α- and β-carotenes increased to 1.3 (Zorbax column) when a mobile-phase system of CH_3CN:CH_3OH:CH_2Cl_2 (60:36:4) was used. The same mobile-phase system (III) was able to separate three isomers of β-carotene on the Vydac column. These isomers were later identified as *all-trans*-, 9-*cis*-, and 13-*cis*-β-carotenes, respectively. Despite the separation of α- and β-carotenes on the Zorbax column, the down slope of α-carotene showed the coelution of phytofluene(s) when it was monitored at 348 nm. Because the phytofluene(s) coeluted closely with α-carotene, further decrease in the CH_2Cl_2 concentration did not improve the overall separation unless the CH_3CN concentration was *increased*.

Using a mobile-phase system of CH_3CN:CH_3OH:CH_2Cl_2 (80:18:2) resulted in complete separation of α- and β-carotenes. This mobile-phase system (IV) gave resolutions of 1.6 for the Zorbax column and 1.1 for the Vydac column. The two isomers of phytofluene eluted between α- and β-carotenes in the Zorbax column but before both carotenes in the Vydac column. Better separations of β-carotene isomers were also achieved on the Vydac column. Further decrease in the percentage of the CH_2Cl_2 as in systems V and VI (retention times exceeded 90 min in the case of the Zorbax column) did not affect any separation of β-carotenes. A similar decrease in CH_2Cl_2 in the Vydac column was not useful because moderately polar carotenes such as lycopene and γ-carotene would coelute with α-carotene.

This mobile-phase optimization led to the composition of CH_3CN:CH_3OH:CH_2Cl_2 (80:18:2) for both columns. The maximum number of cis isomers of carotenoids was elucidated by using this isocratic mobile-phase system in a reasonable time period.

Halogenated solvents have been reported to promote cis isomerization in conjugated polyenes such as retinyl palmitate and β-carotene (Mulry et al., 1983; Pesek et al., 1990). In this work, when no CH_2Cl_2 was used (system VI), no change of the 9-cis or 13-cis peaks as compared to the *all-trans*-β-carotene peak was observed. It is possible that the much shorter time of β-carotene exposure to CH_2Cl_2 *mobile phase* (<60 min; 1–2% concentration) as compared to the CH_2Cl_2 *extractant* (3–24 h; 100% concentration) (Pesek et al., 1990) is insignificant for isomerization to be detectable when CH_2Cl_2 is used as an HPLC solvent.

Identification Techniques. Using spectral information, Liaanen-Jensen (1962) defined the D_B/D_{II} value as the ratio of absorbance of the cis peak to the absorbance of the middle main absorption peak, while Inhoffen et al. (1951) described the reciprocal expression of D_B/D_{II} (Q value). An all-trans carotenoid was characterized as having a low D_B/D_{II} value or a high Q value, while the reverse was true for a cis carotenoid. These techniques were applied in this work to differentiate trans carotenoids from their

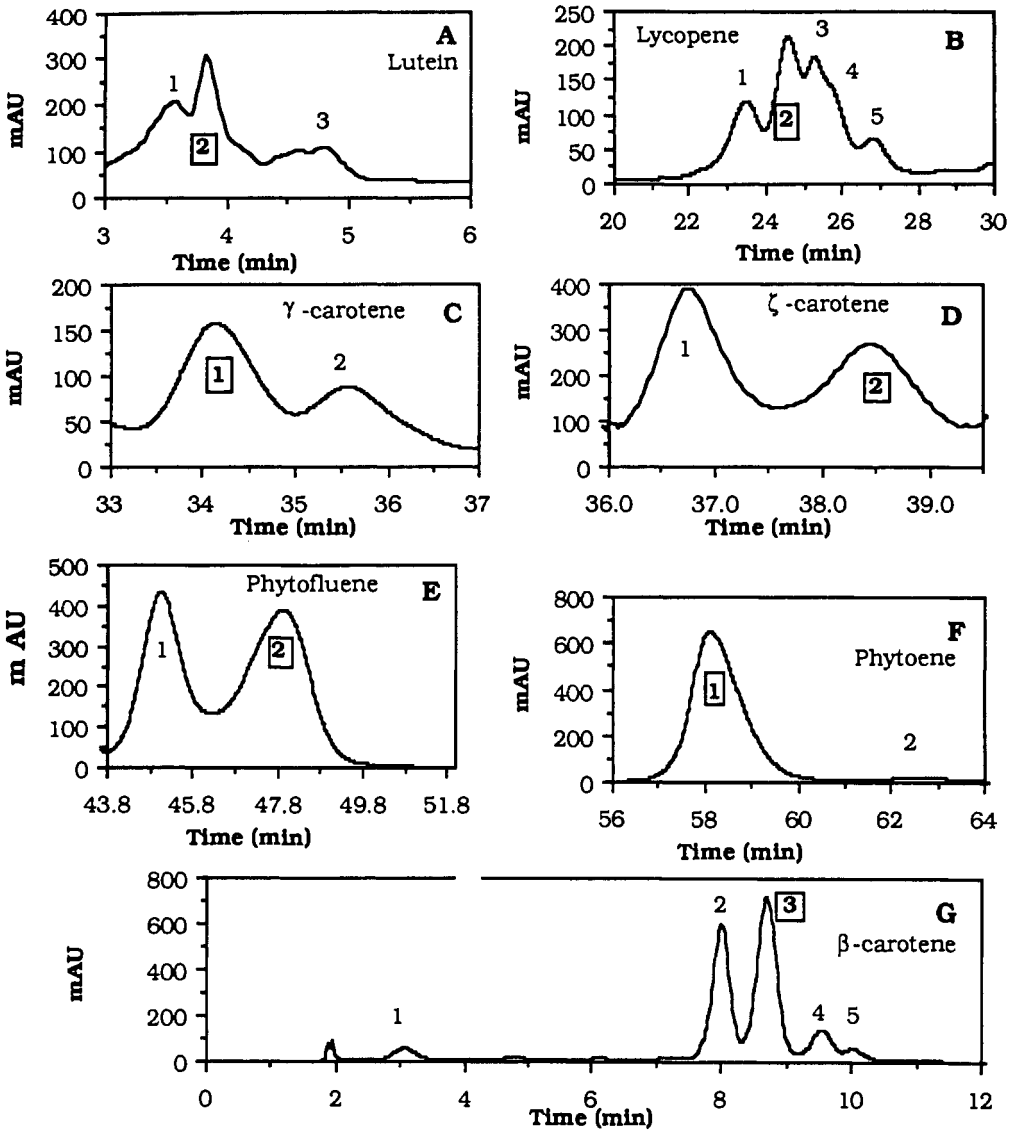

Figure 1. Chromatographic sections of carrot and palm carotenoids showing peaks at their corresponding λ_{max}. (A) (440 nm) 1, 13′-*cis*-lutein; 2, *trans*-lutein; 3, 13-*cis*-lutein. (B) (468 nm) 1, lycopene 1; 2, *trans*-lycopene; 3, lycopene 3; 4, lycopene 4; 5, lycopene 5. (C) (460 nm) 1, *trans*-γ-carotene; 2, *cis*-γ-carotene. (D) (378 nm) 1, *cis*-ζ-carotene; 2, *trans*-ζ-carotene. (E) (348 nm) 1, *cis*-phytofluene; 2, *trans*-phytofluene. (F) (285 nm) 1, *trans*-phytoene; 2, *cis*-phytoene. (G) (450 nm) 1, lutein; 2, α-carotene; 3, *all-trans*-β-carotene; 4, 9-*cis*-β-carotene; 5, 13-*cis*-β-carotene. Separations were performed in C_{18} reversed-phase columns (A–F, Zorbax; G, Vydac), and conditions are detailed in the text. Boxed numbers indicate trans isomers.

Table I. Data of Mobile-Phase Optimization[a]

	mobile-phase system				resolution			capacity factor	
column	no.	A	B	C	R_1	R_2	R_3	k_1	k_2
Zorbax	I	60	20	20	0.7	NS	NS	7.1	7.6
Zorbax	II	60	4	36	low	NS	NS	2.8	3.0
Zorbax	III	60	36	4	1.3	NS	NS	23	25
Zorbax	**IV**	**80**	**18**	**2**	**1.6**	**NS**	**NS**	**29**	**32**
Zorbax	V	95	4	1	2.2	NS	NS	32	36
Zorbax	VI	95	5	0	2.6	NS	NS	40	45
Vydac	I	60	20	20	0.7	NS	NS	2.1	2.3
Vydac	II	60	4	36	NS	NS	NS	1.9	2.1
Vydac	III	60	36	4	0.8	0.9	NS	2.3	2.5
Vydac	**IV**	**80**	**18**	**2**	**1.1**	**1.1**	**0.6**	**3.1**	**3.5**
Vydac	V	95	4	1	1.1	1.2	0.6	3.9	4.5
Vydac	VI	95	5	0	1.3	1.4	0.9	4.1	4.5

[a] Optimized isocratic mobile-phase composition is indicated in boldface. Other lettered abbreviations are summarized as follows: A, CH_3CN; B, CH_3OH; C, CH_2Cl_2; R_1, resolution of peaks of α- and *all-trans*-β-carotene; R_2, resolution of peaks of *all-trans*-β-carotene and first cis isomer; R_3, resolution of peaks of first and second cis isomers of β-carotene; k_1, capacity factor of α-carotene; k_2, capacity factor of β-carotene; NS, no separation (resolution < 0.5).

corresponding cis isomers, such as lutein, lycopene, and γ-, ζ-, and β-carotene.

Carotenoids of seven or fewer conjugated double bonds have their main absorption band in the 320–420-nm region, and their cis isomers do not have the usual cis peak. For these carotenoids, another term known as D_{II}/D_{III} was

Table II. Cis/Trans Absorption Ratio

carotene	% area	RT,[a] min	$Q = D_{II}/D_B$[b]	D_B/D_{II}[b]	D_{II}/D_{III}[b]
13′-*cis*-lutein	39	3.5	2.5 (2.3)	0.40	
trans-lutein	51	3.8	5.7 (<12)	0.17	
13-*cis*-lutein	10	4.8	2.0 (2.0)	0.49	
lycopene 1	23	23.2	4.2	0.24	
lycopene 2	33	24.8	4.9	0.20 (0.11)	
lycopene 3	18	25.2	3.7	0.27	
lycopene 4	18	25.8	3.9	0.26	
lycopene 5	9	26.8	3.4	0.29	
trans-γ-carotene	68	34.3	6.3	0.16	
cis-γ-carotene	32	35.2	4.9	0.20	
cis-ζ-carotene	57	36.8	4.3	0.23	1.1
trans-ζ-carotene	43	38.5	11.7	0.08	1.0
cis-phytofluene	71	45.0			1.2 (1.16)
trans-phytofluene	29	48.0			1.1 (1.05)
trans-β-carotene	63	8.8	12.7	0.08 (0.057)	
9-*cis*-β-carotene	30	9.6	8.2	0.12 (0.087)	
13-*cis*-β-carotene	7	10.0	4.1	0.24 (0.36)	
trans-phytoene	96	58.1			1.4
cis-phytoene	4	62.8			1.2

[a] Retention times of carotenoids using the Zorbax ODS column except for the β-carotene isomers for which the Vydac C_{18} column was used. [b] Numbers in parentheses correspond to available literature values (Tsukida et al., 1982; Tan and Soderstrom, 1989; Quackenbush, 1987; Ke et al., 1970).

used to differentiate between the cis and the trans isomers. The D_{II}/D_{III} term is defined as the ratio of the absorption of the second (middle main) and the third spectral peaks. Cis isomers of phytofluene and ζ-carotene have higher D_{II}/D_{III} ratios than their corresponding trans isomers (Table II). D_{II}/D_{III} values were taken from baseline to absorbance maximum.

Cis Isomer Identification. Isomers of lutein of carrot oil carotenoids (λ_{max} = 424, 444, 472 nm) eluted at 3.5, 3.8, and 4.8 min as shown in Figure 1A. The corresponding spectra of these peaks are shown in Figure 2A. The order of elution of lutein isomers was found to be 13′-*cis*, *all-trans*-, and 13-*cis*-lutein. The D_B/D_{II} ratio was found to be lowest for the peak at 3.8 min (Figure 1A), and this was assigned as the *all-trans*-lutein (0.17). The 13′-*cis*-lutein (0.40) and the 13-*cis*-lutein (0.49) had higher D_B/D_{II} ratios, respectively. The trends of these D_B/D_{II} values are in good agreement with the inverse of Q-ratio values used by Quackenbush (1987).

Five different isomers of lycopene (λ_{max} = 444, 468, 502 nm) were found to exist in palm oil (Figures 1B and 2B). Although the structural identity of the cis isomers could not be revealed (due to nonavailability of cis standards and insufficient spectral information in the literature for confirmation), it is clear that the second lycopene peak (RT = 24.8 min) is *trans*-lycopene, having the lowest D_B/D_{II} ratio and being the most abundant isomer (33% area). It is unclear if these isomeric lycopenes were naturally occurring or a function of the prior sterilization process of the palm fruits (in pressurized steam of about 150 °C). Lycopene in palm oil has been previously reported (Tan et al., 1986; Khachik and Beecher, 1987), but the occurrence of isomeric lycopenes in palm oil is shown here for the first time. The present method allows the identification of the *presence* of the lycopene isomers but not the *structure* of its cis isomers.

Carrot and palm oils showed the presence of *trans*- and *cis*-γ-carotene (λ_{max} = 460, 480, 492 nm; Figure 1C), and this has been indicated in earlier literature (Bauernfeind et al., 1981). Spectral information revealed that the predominant trans isomer (68% area) of D_B/D_{II} ratio of 0.16 eluted before the cis isomer (Figure 2C). It is interesting to note that γ-carotene is an exception of the generally accepted rule that cis isomers of carotenoids have lower provitamin A biopotency relative to that of their all-trans forms. In chicks, the cis isomer of γ-carotene has 51% of the potency of β-carotene, whereas the trans isomer has 42% (Bauernfeind et al., 1981).

The elution of ζ-carotene isomers followed γ-carotene, and these isomers were found in carrot and palm oils. The three-pronged spectra of ζ-carotene isomers are identical (λ_{max} = 378, 400, 424 nm) with absorptivity difference at the cis peak (Figures 1D and 2D). Two ratios, D_B/D_{II} and D_{II}/D_{III}, were used to differentiate between the cis and trans isomers. The more abundant cis isomer (57% area) had a D_B/D_{II} ratio of 0.23 and a D_{II}/D_{III} ratio of 1.1 as compared to the trans isomer (43% area) of lower D_B/D_{II} and D_{II}/D_{III} ratios (Table II). Unlike the usual plant occurrence, the cis isomer is more abundant than the trans isomer. This reverse abundance has been reported in pumpkin extracts (Khachik and Beecher, 1987).

Two isomers of phytofluene found only in carrot oil (λ_{max} = 330, 348, 368 nm) eluted between the peaks of α- and *all-trans*-β-carotene with retention times of 45.0 and 48.0 min (Figure 1E). The first isomer was identified as the cis isomer of D_{II}/D_{III} ratio 1.2, followed by the trans isomer of D_{II}/D_{III} ratio 1.1. Phytofluene showed more abundance of its cis (71%) isomer than of its trans (29%) isomer. It is probable that *cis*-phytofluene could be 15-*cis*-phytofluene. It was reported that the main phytofluene present in tomatoes was probably the 15-cis isomer, which showed rapid isomerization to the all-trans form on isolation, particularly if exposed to light (Weedon, 1971). The elution of *cis*-phytofluene before its corresponding trans isomer was also reported by Tan (1988).

Finally, two phytoene (λ_{max} = 276, 286, 296 nm) peaks found in both carrot and crude palm oil eluted at 58.1 and 62.8 min. The first predominant isomer (96% area) was assumed to be *trans*-phytoene. The spectra of the cis and trans isomers were identical. The utilization of D_{II}/D_{III} ratios to predict cis isomers of shorter conjugated polyenes (e.g., three conjugated double bonds) may not be useful because of the very low intensity of the spectrum of the cis isomer. However, the occurrence of *cis*-phy-

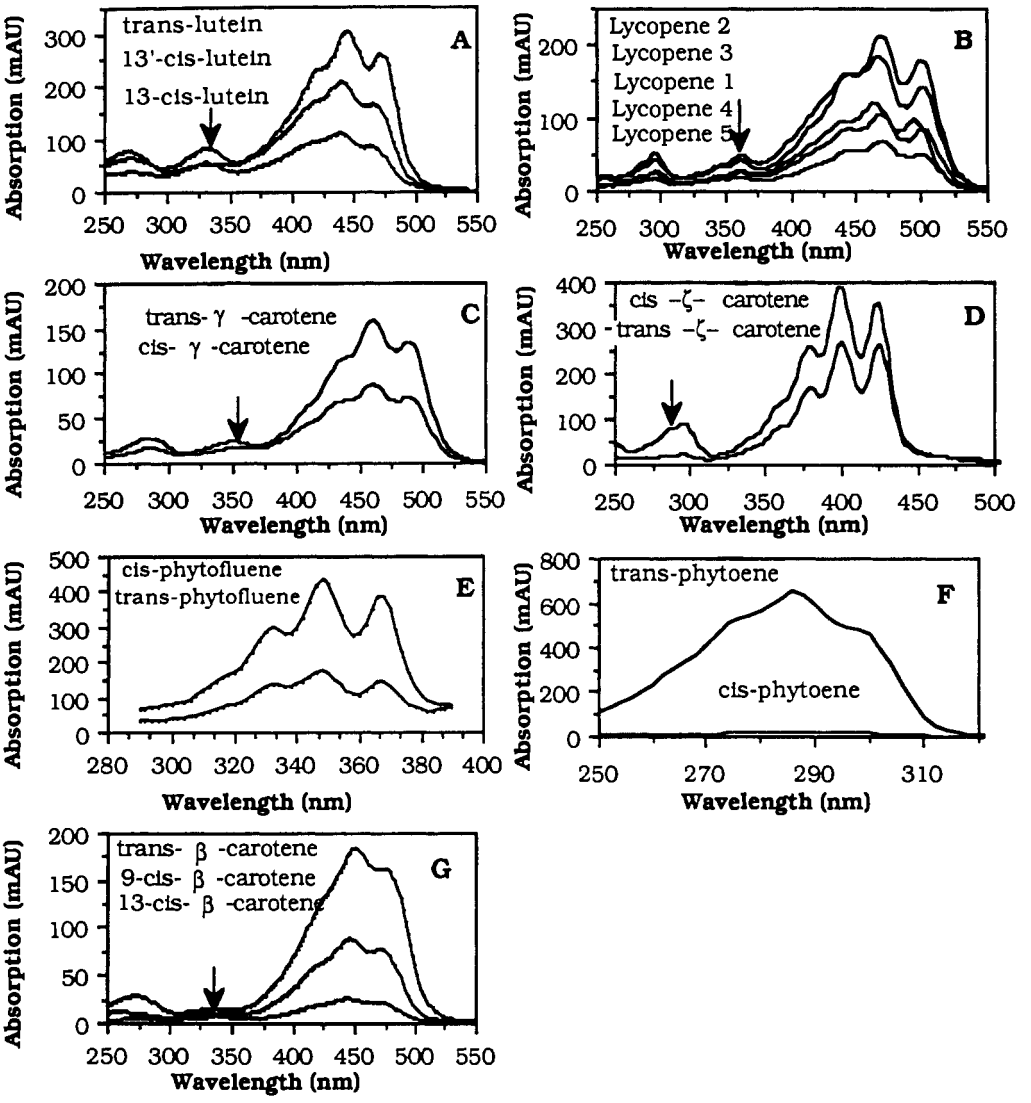

Figure 2. Spectral overlays of carotenoid isomers from carrot and palm oil samples. Arrows indicate the wavelength in the spectra where the cis peaks absorbed. Carotenoids are listed in descending order as in the spectra.

toene in carrots (15-cis isomer) has been established (Weedon, 1971).

Complete isomer separation of β-carotene was not achievable on the Zorbax ODS column. However, when the same sample of either carrot or palm oil was injected in the Vydac column, separation was possible in shorter retention times. The β-carotene eluted at 9 min (Figure 1G) as compared to 50 min on the Zorbax. The β-carotenes (λ_{max} = 450, 476 nm) resolved into three components, and at least three isomers of β-carotene were separated and identified. The first and most abundant isomer (63%) was identified as *trans*-β-carotene of D_B/D_{II} ratio 0.08, followed by 9-*cis*-β-carotene (30%) of D_B/D_{II} ratio 0.12, and finally the 13-*cis*-β-carotene had the highest D_B/D_{II} ratio of 0.24. The utilization of D_B/D_{II} ratios to identify cis isomers of β-carotenes is important. This technique aids in the unmistakable separation of the 13-cis isomer (D_B/D_{II} = 0.24) from the 15-cis isomer (D_B/D_{II} = 0.33).

Conclusion. This study shows the separation and identification of a series of carotenoid isomers using an isocratic mobile-phase system (CH_3CN:CH_3OH:CH_2Cl_2 80:18:2) in a reasonable time period. This mobile-phase system maximized the separation of *different* carotenoids (mainly by the use of the Zorbax column) and cis/trans carotenoids (including the use of the Vydac column). It may be generalized that when the carotenoid conjugated double bonds are *greater* than 7 (e.g. 9–11), the D_B/D_{II} ratio for a cis isomer will be larger than that for the corresponding trans isomer. Alternatively, when the carotenoid conjugated double bonds are *less* than 7 (e.g., 3–7), the D_{II}/D_{III} ratio for a cis isomer will be larger than that for the corresponding trans isomer. These ratios may be used in a complementary but not absolute manner for the spectral identification of cis/trans carotenoids.

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Registry No. *trans*-Lycopene, 502-65-8; 13′-*cis*-lutein, 79464-33-8; *all-trans*-lutein, 127-40-2; 13-*cis*-lutein, 32449-88-0; *trans*-γ-carotene, 472-93-5; *cis*-γ-carotene, 29558-18-7; *cis*-ζ-carotene, 52340-80-4; *trans*-ζ-carotene, 502-63-6; *trans*-phytofluene, 540-05-6; *cis*-phytofluene, 27664-65-9; *trans*-β-carotene, 7235-40-7; 9-*cis*-β-carotene, 13312-52-2; 13-*cis*-β-carotene, 6811-73-0; *trans*-phytoene, 540-04-5; *cis*-phytoene, 7699-28-7.