Separation of Geometrical Carotenoid Isomers in Biological Extracts Using a Polymeric C_{30} Column in Reversed-Phase Liquid Chromatography

Curt Emenhiser,[†] Nada Simunovic,[†] Lane C. Sander,[‡] and Steven J. Schwartz*[,§]

Department of Food Science, Box 7624, North Carolina State University, Raleigh, North Carolina 27695-7624, Analytical Chemistry Division, Chemical Science and Technology Laboratory, National Institute of Standards and Technology, Gaithersburg, Maryland 20899, and Department of Food Science and Technology, The Ohio State University, Columbus, Ohio 43210-1096

Application of a polymeric C_{30} reversed-phase (RP) liquid chromatography (LC) column for separations of geometrical carotenoid isomers extracted from biological sources is demonstrated. The relative retention characteristics of *all-trans* carotenoid standards on the C_{30} column is also shown under isocratic conditions. Carotenoids were extracted from human serum, raw and thermally processed carrots, a *Dunaliella* algae-derived preparation of *β*-carotene, and a poultry feed supplement containing marigold carotenoids. From extracts of plant and algal origin, geometrical isomers of *β*-carotene (*all-trans*, 15-*cis*, 13-*cis*, and 9-*cis*), α-carotene (*all-trans*, 13-*cis*, 13′-*cis*, and 9-*cis*), and lutein (*all-trans*, 13-*cis*, 13′-*cis*, 9-*cis*, and 9′-*cis*) were separated and tentatively identified, some of which were previously unresolved on other RPLC columns. Chromatography of serum carotenoid fractions resolved the prominent *all-trans* carotenoids and separated geometrical isomers of lutein, *β*-carotene, and lycopene (11 *cis*-lycopenes). Absolute recoveries of α-carotene and *β*-carotene from the C_{30} column were 64 and 52% in the LC mobile phase and 89 and 88% when 0.1% (v/v) triethylamine (TEA) was added to the mobile phase, respectively. TEA-mediated enhancements in recovery of 21−55% were observed for several other carotenoids.

Keywords: *Carotenoids; geometrical isomers; C_{30} column; biological extracts; HPLC*

INTRODUCTION

High-performance liquid chromatography (HPLC) is currently the method of choice for carotenoid analysis. The reversed-phase (RP) mode of LC, using columns packed with any of a number of various C_{18} stationary phases, is generally preferred for these analyses (Craft, 1992). The best selectivity toward geometrical carotenoid isomers, however, has traditionally been obtained in normal-phase LC using calcium hydroxide, alumina, or nitrile-bonded packing materials (Khachik et al., 1992a; O'Neil and Schwartz, 1992; Schmitz et al., 1995). A polymeric C_{30} stationary phase, recently developed for improved RPLC separations of carotenoids, may be an exception to these generalizations (Sander et al., 1994). Properties of the C_{30} column were tailored specifically for the analysis of carotenoids, resulting in adequate retention of polar carotenoids and excellent selectivity toward both polar and nonpolar carotenoids, including structural and geometrical isomers (Clinton et al., 1996; Craft, 1992; Emenhiser et al., 1995, 1996; Sander et al., 1994). The first published applications of the C_{30} stationary phase were separations of carotenoid standards and serum carotenoids obtained while the column was still in the developmental stage (Craft, 1992). Sander et al. (1994) achieved excellent separations of *all-trans* carotenoids from mixtures of standards as well as several extracts. These authors also demonstrated that the C_{30} stationary phase possesses a high degree of selectivity toward geometrical isomers of *β*-carotene. Shape selectivity of the C_{30} column has since been explored more extensively by Emenhiser et al. (1995), who separated geometrical isomers of six common carotenoids on the C_{30} column and, for comparison, two commercially available RP columns that are often used for these separations. Selectivity of the C_{30} phase toward these isomers was especially good and superior to that of the other stationary phases tested. Because the C_{30} column shows exceptional selectivity toward lycopene isomers (Emenhiser et al., 1995), it has subsequently been applied to the analysis of lycopene extracted from human prostate tissue (Clinton et al., 1996). Among RP columns, the C_{30} stationary phase is uniquely capable of resolving geometrical isomers of asymmetrical carotenoids in which *cis* bonds are present at the same carbon number but at opposite ends of the molecule. For example, the 13- *cis* and 13′-*cis* and the 9-*cis* and 9′-*cis* isomers of both lutein and α-carotene can be resolved by this column. The predominant geometrical isomers of α-carotene have been isolated from the C_{30} column, and their identities were unambiguously assigned using ^{1}H NMR (Emenhiser et al., 1996). These previous C_{30} carotenoid separations were obtained using either gradient programmed elution (Craft, 1992; Sander et al., 1994) or geometrical isomers of individual carotenoids under isocratic conditions of chromatography (Clinton et al., 1996; Emenhiser et al., 1995, 1996; Sander et al., 1994).

This research was undertaken to (1) determine the relative retention characteristics of *all-trans* carotenoid standards on the polymeric C_{30} column under isocratic conditions of chromatography, (2) demonstrate the utility of the C_{30} column for isocratic separations of geometrical carotenoid isomers extracted from biological

* Author to whom correspondence should be addressed [telephone (614) 292-2934; fax (614) 292-4233; e-mail schwartz.177@osu.edu].

[†] North Carolina State University.

[‡] National Institute of Standards and Technology.

[§] The Ohio State University.

S0021-8561(96)00104-5 CCC: $12.00

sources, and (3) assess recovery of several carotenoids from the C_{30} column and the influence of triethylamine (TEA), as a mobile phase additive, on carotenoid recovery.

EXPERIMENTAL PROCEDURES

Materials. *All-trans* standards of the following carotenoids were procured: lutein (Kemin Industries, Des Moines, IA), zeaxanthin (Indofine, Belle Meade, NJ), β-cryptoxanthin (a gift from Hoffmann-La Roche, Nutley, NJ), and α-carotene, β-carotene, and lycopene (Sigma Chemical Co., St. Louis, MO). These carotenoids were passed through open alumina columns using acetone−hexane eluents to remove oxidation products that may have formed during manufacture and storage and were used without further purification. Oro Glo Dry, a poultry feed supplement containing saponified marigold petal carotenoids, was a gift from Kemin Industries. A *Dunaliella* algae-derived β-carotene preparation, Betatene (4% β-carotene in soy oil), which is used as a natural colorant and nutritional supplement, was a gift from Betatene, Ltd., Melbourne, Australia. Carrots, obtained from a local grocery, were used either raw or after a thermal canning process (116 °C, 75 min) in a pilot scale retort. To promote isomerization of the carrot carotenoids, the thermal process was approximately twice as intensive in duration as that used for commercially canned carrots. Human blood was drawn from several healthy human volunteers into nonheparinized tubes, allowed to clot in ice, and centrifuged at 1500*g* for 15 min to obtain the serum.

The HPLC solvents, methanol and methyl *tert*-butyl ether (MTBE), were of Certified ACS and HPLC grade, respectively (Fisher Chemical, Fair Lawn, NJ).

Carotenoid Extractions. Carotenoids were obtained from Betatene by dissolving a small amount of the preparation in hexane. Oro Glo Dry was combined with $CaCO_3$ and deionized water, allowed to hydrate for 30 min, homogenized, and then extracted by successive incorporation and vacuum filtration of a 1:1 (v/v) mixture of acetone−hexane (2×). The filtrates were combined in a separatory funnel, and water was added to hasten phase separation of a hexane epilayer, which was withdrawn and saved. Raw and processed carrots were diced and homogenized in added $CaCO_3$ and deionized water. Carotenoids were extracted from the puréed carrot tissue by successive incorporation and vacuum filtration of methanol (1×) and 1:1 (v/v) acetone−hexane (2×). The filtrates were handled in the same manner as was done for extraction of Oro Glo Dry. Aliquots (2 mL) of pooled serum were deproteinated by incorporating 2 mL of ethanol, and carotenoids were extracted by incorporating 2 mL of hexane (2×) and withdrawing the hexane epilayer after phase separation. Individual serum extracts were passed through anhydrous sodium sulfate to remove any contaminating water and then fractionated using fully activated, neutral alumina Sep-Pak cartridges (type N; Millipore Corp., Milford, MA). To condition the alumina cartridges, 5 mL of hexane was passed through prior to use. Extracts were then loaded in ≤2 mL of hexane, and 5 mL of hexane was used to wash the sample. The α- and β-carotenes fraction was eluted using 3.5 mL of 10:90 (v/v) acetone−hexane, and all remaining carotenoids (xanthophylls and lycopene fraction) were eluted with 3.5 mL each of 30:70 (v/v) and 70:30 (v/v) acetone−hexane, followed by 14 mL of acetone. Fractions containing the same carotenoids were concentrated, combined, and evaporated to dryness under a stream of nitrogen gas. Extractions were carried out under subdued yellow light to prevent isomerization and photodegradative reactions. Extracts were stored at −20 °C under a headspace of nitrogen gas and used within 72 h.

Instrumentation. Chromatographic separations were achieved using an analytical scale (4.6 mm i.d. × 250 mm) 5 μm polymeric C_{30} RPLC column that was prepared at the National Institute of Standards and Technology (Gaithersburg, MD) according to the report on its development (Sander et al., 1994). This column is available commercially from YMC, Inc. (Wilmington, NC). A precolumn packed with the same stationary phase was used to protect the analytical column. The HPLC system consisted of a Waters Model 501 solvent delivery system and Model U6K injector (Milford, MA) and an Anspec UV−visible detector (Model SM 95; Linear Instruments, Reno, NV). The detector was linked to a Dramen personal computer (Dramen, Inc., Raleigh, NC) by a Dionex advanced computer interface (Model ACI-1; Dionex Corp., Sunnyvale, CA). Dionex AI-450 chromatography software (release 3.30) was used to store and integrate chromatograms. UV−visible absorption spectra were obtained for some chromatographic peaks using a Waters Model 996 photodiode array detector connected to a Gateway 2000 personal computer (Model 4DX2-66V; North Sioux City, SD) equipped with Millennium 2010 chromatography software (LC version 2.00; Millipore).

Chromatographic Analysis and Peak Identification. All separations were achieved isocratically using binary mobile phases of MTBE−methanol (3:97 to 38:62, v/v) and flow rates of 1 mL/min. The specific mobile phase composition used for each separation is given in the corresponding figure legend. Injection solvents were MTBE−methanol mixtures, usually of the same composition as the mobile phase. Injection volumes ranged from 5 to 25 μL. Column temperature was ambient laboratory temperature (~23 °C), and column effluent was monitored at 453 nm, except for lycopene (460 nm). UV−visible absorption spectra were obtained from 250 to 550 nm in the LC mobile phases during C_{30} chromatography.

Certain chromatographic peaks are tentatively identified by comparison to previous separations on polymeric C_{30} columns (Craft, 1992; Clinton et al., 1996; Emenhiser et al., 1995; Sander et al., 1994) and UV−visible absorption spectra. Assignment of several α-carotene isomers was further aided by their recent identification by ^{1}H NMR (Emenhiser et al., 1996). Additional evidence for the identification of peaks in fractions of human serum was gained by their relative retention characteristics on an alumina adsorptive phase. To ascertain whether peaks identified as *cis*-lycopene isomers in human serum could be oxygen addition products of lycopene, these peaks were collected and their molecular weights determined using electrospray mass spectrometry. The *all-trans* isomer of lycopene was identified in human serum by cochromatography of an authentic lycopene standard added to the lycopene fraction of a serum extract.

Recovery Determinations. Absolute recoveries of α- and β-carotene from the C_{30} column were determined using *all-trans* standards that were purified on open alumina columns, as described earlier, and assessed for purity (97% *all-trans*-α-carotene, 94% *all-trans*-β-carotene) on the polymeric C_{30} column. Standard solutions were prepared for each carotenoid by dissolving a quantity sufficient to give absorbance readings of about 1 absorbance unit (AU) when dissolved in 10 mL of hexane. Absorbance readings were obtained using a Bausch & Lomb UV−visible spectrometer (Spectronic 1001; Woburn, MA). Linearity of the detector response was verified using 1:10 serial dilutions of these solutions. The solutions were evaporated to dryness under nitrogen gas and reconstituted in 1 mL of 50:50 (v/v) MTBE−methanol. A Rheodyne injector (Model 7125; Rainin Instruments, Woburn, MA) equipped with a PEEK sample loop was used for all recovery measurements. The volume of the sample loop was determined to account for possible loop-to-loop variability. This was accomplished by comparing absorbance readings obtained from 20 μL each of the α- and β-carotene standards (dried and reconstituted in 1 mL of hexane) to that obtained when the contents of the completely filled loop were collected and treated in the same fashion. Four replications of this procedure gave a mean loop volume of 25.7 μL. Each carotenoid was chromatographed on the C_{30} column using 11:89 (v/v) MTBE−methanol, a flow rate of 1 mL/min, a sensitivity setting of 0.05 absorbance unit full scale (AUFS), and a wavelength setting of 450 nm. Peaks were collected into 2 mL of hexane, and 3 mL of water was added to cause phase separation of a hexane epilayer. The hexane layer was collected, and the aqueous layer was extracted twice with 1 mL of hexane to ensure a quantitative transfer of the collected carotenoid. Each sample was dried under nitrogen gas and redissolved in 1 mL of hexane, and absorbance was measured. Recovery was calculated using the ratios of initial absorbance to final absorbance. Values are reported as

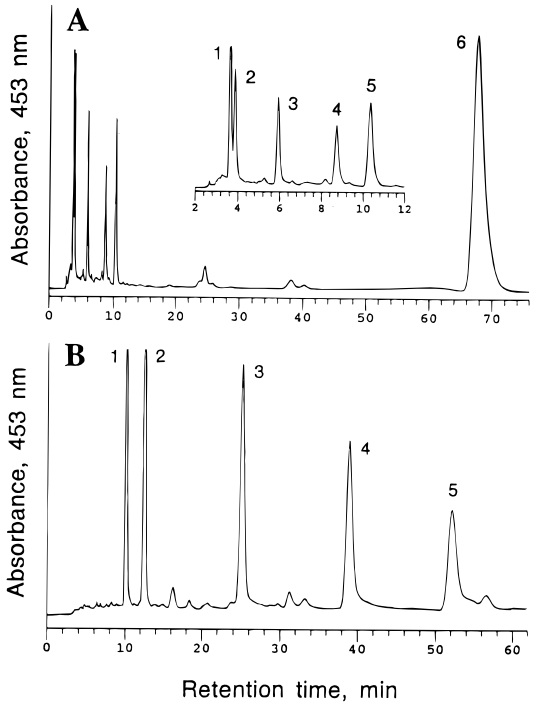

Figure 1. C_{30} chromatograms of *all-trans* carotenoid standards: (A) 38:62 (v/v) MTBE−methanol mobile phase (inset shows expanded view of the first 12 min of the chromatogram); (B) 11:89 (v/v) MTBE−methanol mobile phase. Lycopene was not detected in 120 min. Peak identifications: 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 4, α-carotene; 5, β-carotene; and 6, lycopene.

arithmetic means of duplicate analyses. The influence of adding 0.1% (v/v) TEA to the LC mobile phase on recovery of α- and β-carotene was determined using the same procedure. When the LC mobile phase was altered, the C_{30} column was equilibrated in the new mobile phase for 60 min at 1 mL/min before injection.

The influence of TEA, added to the LC mobile phase, on relative recoveries of several carotenoids in addition to α- and β-carotene was also assessed using the C_{30} column. A mixture containing authentic standards of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, and β-carotene was chromatographed in duplicate using 11:89 (v/v) MTBE−methanol as mobile phase with and without 0.1% (v/v) added TEA. Lycopene was tested separately using the same procedure but with a base mobile phase composition of 38:62 (v/v) MTBE−methanol. The percent change in recovery was calculated for each carotenoid from the ratio of mean integrated peak areas for the individual carotenoids.

RESULTS AND DISCUSSION

Carotenoid Standards. Before the polymeric C_{30} column was applied to separations of geometrical carotenoid isomers extracted from certain biological tissues, it was necessary to evaluate the C_{30} column for its ability to separate *all-trans* carotenoids under isocratic conditions of chromatography. A mixture containing *all-trans* standards of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene was separated on the C_{30} column using 38:62 (v/v) MTBE−methanol (Figure 1A). As this chromatogram illustrates, the acyclic carotenoid lycopene is much more strongly retained on the C_{30} stationary phase than the five bicyclic carotenoids. Although the analysis time is quite long (75 min), none of the bicyclic carotenoids were retained for more than 11 min. The inset in Figure 1A, which shows the early-eluting portion of the same chromatogram, demonstrates that a reasonably good separation of lutein and zeaxanthin was obtained, despite the use of a relatively strong mobile phase. The other carotenoids in this mixture were easily resolved. These chromatographic conditions could not, however, be expected to provide adequate retention for separation of multiple geometrical isomers of the bicyclic carotenoids. In an attempt to provide sufficient retention of the bicyclic carotenoids for subsequent application to separations of their geometrical isomers in biological extracts, the same carotenoid mixture was chromatographed with a weaker mobile phase (11:89, v/v, MTBE−methanol). The resultant chromatogram demonstrates that the bicyclic carotenoids were adequately retained and easily resolved (Figure 1B); however, lycopene was not detected in 120 min (not shown). For lycopene-containing mixtures or extracts, the ability to separate geometrical carotenoid isomers would likely require the use of gradient programmed elution or fractionation of the sample prior to C_{30} chromatography. Another possibility may be to use alternative mobile phases to alter the elution pattern of lycopene and other strongly retained carotenoids on the C_{30} column. For example, lycopene typically elutes after β-carotene on polymeric C_{18} columns when a methanol-based mobile phase is used (Epler et al., 1992). In contrast, when acetonitrile-based mobile phases are used with the same type of column, lycopene generally elutes after the xanthophylls but before most carotenes, including α- and β-carotene (Epler et al., 1992). If the same retention and elution patterns of carotenoids occur when methanol- and acetonitrile-based mobile phases are used with the polymeric C_{30} column, the use of acetonitrile-based mobile phases could be advantageous for separations involving lycopene-containing extracts. This possibility has not yet been tested. Separations of geometrical carotenoid isomers in samples that are devoid of strongly retained carotenoids, such as lycopene, should be possible under isocratic conditions, with the mobile phase strength adjusted to give a good separation in a reasonable analysis time. In the present research, fractionation of extracts was used, when necessary, in lieu of gradient programmed elution which requires column re-equilibration between each injection. Fractionation of carotenoid extracts can usually be accomplished with good results using alumina cartridges and acetone−hexane eluents.

Raw and Processed Carrot. C_{30} chromatography of carotenoids extracted from raw carrot gave the chromatogram in Figure 2A. Without sample fractionation and under isocratic conditions, the naturally occurring *all-trans* forms of lutein, α-carotene, and β-carotene were well separated. Several other carotenoids that eluted before lutein were also resolved, but their identification was not pursued. Carrots typically contain various hydrocarbon carotenoids in addition to α- and β-carotenes, including γ-carotene, ζ-carotene, neurosporene, phyofluene, and phytoene (Gross, 1991), some of which have wavelengths of maximum absorbance substantially lower than the 453 nm detector setting used in the present work (Davies, 1976). The ability of the C_{30} column to separate these carotenoids has not yet been determined.

After carrots from the same lot were canned and thermally processed, significant quantities of multiple geometrical isomers were generated (Figure 2B). The 13-*cis*, 13′-*cis*, *all-trans*, and 9-*cis* isomers of α-carotene were identified in the processed carrot extract, in addition to one other *cis*-α-carotene isomer. The 9′-*cis*

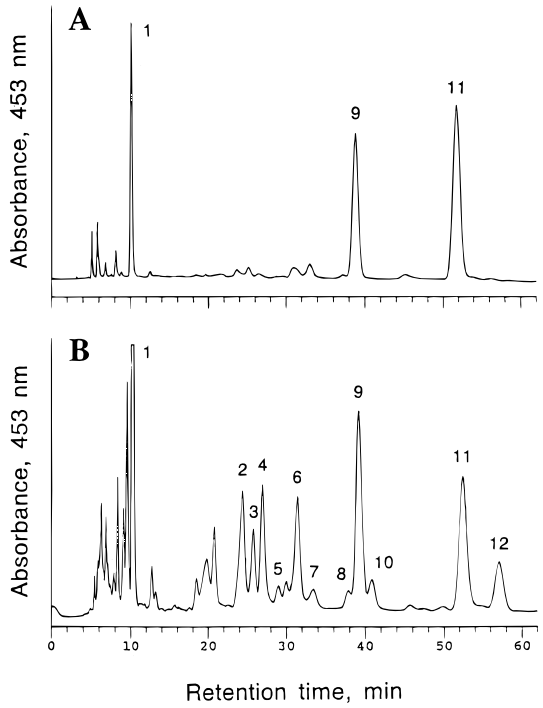

Figure 2. C_{30} chromatograms of carotenoids extracted from (A) raw and (B) thermally processed carrots, 11:89 (v/v) MTBE−methanol mobile phase. Tentative peak identifications: 1, *all-trans*-lutein; 2, 13-*cis*-α-carotene; 3, a *cis*-α-carotene isomer; 4, 13′-*cis*-α-carotene; 5, 15-*cis*-*β*-carotene; 6, 13-*cis*-*β*-carotene; 7−8, *cis*-*β*-carotene isomers; 9, *all-trans*-α-carotene; 10, 9-*cis*-α-carotene; 11, *all-trans*-*β*-carotene; and 12, 9-*cis*-*β*-carotene.

isomer of α-carotene is resolved on the C_{30} column (Emenhiser et al., 1995, 1996) and may have been present in the processed carrot extract, but this isomer has been shown to coelute with *all-trans*-*β*-carotene under the chromatographic conditions employed (unpublished observation). For *β*-carotene, the 15-*cis*, 13-*cis*, *all-trans*, and 9-*cis* isomers were separated and tentatively identified, along with two other *cis*-*β*-carotene isomers. Geometrical isomers of lutein also appeared to form during the thermal process, but resolution of the early-eluting peaks was compromised by the strength of the mobile phase required, thus preventing a tentative identification of any of these peaks. It was previously noted that the thermal process used for these carrots was more intensive than that used commercially. This would account for the unusually high degree of isomerization but would not likely influence the qualitative profile observed because it is well-known that commercial thermal processes cause isomerization of vegetable carotenoids (Chandler and Schwartz, 1988; Quackenbush, 1987; Sweeney and Marsh, 1971). The separations in Figure 2 are illustrative of several important considerations regarding the application of the C_{30} column to carotenoid separations. The simple carotenoid profile of Figure 2A became quite complex when geometrical isomers of carrot carotenoids, some having markedly different retention characteristics, were generated during thermal processing (Figure 2B). In applications where the *all-trans* carotenoid profile is complex and geometrical isomers are present, the high degree of selectivity toward geometrical isomers could actually compromise the overall separation and complicate the identification of peaks. This underscores the occasional necessity of using a gradient elution scheme or fractionating the sample prior to

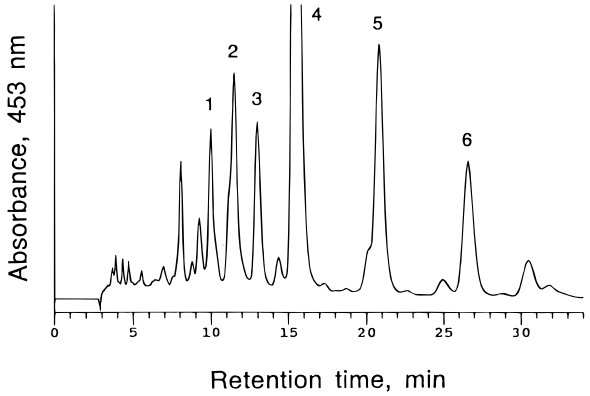

Figure 3. C_{30} chromatogram of saponified marigold carotenoids extracted from a poultry feed supplement, 3:97 (v/v) MTBE−methanol mobile phase. Tentative peak identifications: 1, a *cis*-lutein isomer; 2, 13-*cis*-lutein; 3, 13′-*cis*-lutein; 4, *all-trans*-lutein; 5, 9-*cis*-lutein; and 6, 9′-*cis*-lutein.

chromatography. There are also occasions when separation of the geometrical isomers of carotenoids is not desirable. However, when separation of these isomers is desirable or necessary, the C_{30} column possesses better retention and selectivity toward these isomers than existing RP columns, resulting in better overall performance.

Saponified Marigold Carotenoids. Saponified marigold petal carotenoids, which are incorporated into supplements added to poultry feeds, act as a pigmenting agent to enhance the color quality of poultry meat and eggs. As many as 17 different carotenoids have been previously identified in extracts of marigold petals, including lutein as the predominant pigment and zeaxanthin, α-cryptoxanthin, and *β*-cryptoxanthin among the many minor carotenoid constituents (Quackenbush and Miller, 1972). As illustrated in Figure 3, Oro Glo Dry contains lutein, in the *all-trans* geometrical configuration, as the predominant carotenoid component. Several other isomers of lutein were also separated and tentatively identified, including the 13-*cis*, 13′-*cis*, 9-*cis*, and 9′-*cis* geometrical configurations and an unidentified *cis* isomer. Discrimination of the 13-*cis* and 13′-*cis* and the 9-*cis* and 9′-*cis* isomers is based on the high degree of likelihood that lutein isomers elute from the C_{30} column in the same order as do the corresponding isomers of α-carotene (Emenhiser et al., 1996). The other chromatographic peaks are unidentified xanthophylls. It was possible to chromatograph these carotenoids isocratically without fractionation because marigold petals do not typically contain detectable concentrations of hydrocarbon carotenoids. A relatively weak mobile phase (3:97, v/v, MTBE−methanol) was used to promote retention of the analytes, as evidenced by a retention time of 15 min for *all-trans*-lutein. In general, lutein is poorly retained on commercially available C_{18} columns (Epler et al., 1992), but the combination of increased retention and excellent shape discrimination by the C_{30} column provides an effective means for separating geometrical isomers of this polar carotenoid by RPLC. This and previous C_{30} separations of polar carotenoids (Sander et al., 1994) and geometrical isomers of polar carotenoids (Emenhiser et al., 1995) suggest that the C_{30} column could be used successfully to analyze geometrical isomers of other polar carotenoids.

***Dunaliella* Algae Carotenoids (Betatene).** When an extract of Betatene was chromatographed on the C_{30} column, several geometrical isomers of *β*-carotene were

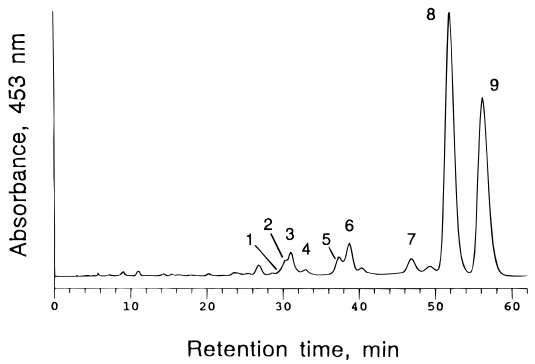

Figure 4. C_{30} chromatogram of *Dunaliella* algae carotenoids extracted from Betatene, 11:89 (v/v) MTBE−methanol mobile phase. Tentative peak identifications: 1, 15-*cis*-β-carotene; 2, a *cis*-β-carotene isomer; 3, 13-*cis*-β-carotene; 4−5, *cis*-β-carotene isomers; 6, *all-trans*-α-carotene; 7, a *cis*-β-carotene isomer; 8, *all-trans*-β-carotene; and 9, 9-*cis*-β-carotene.

separated and tentatively identified, including the 15-*cis*, 13-*cis*, *all-trans*, and 9-*cis* isomers (Figure 4). Several other *cis* isomers of β-carotene were separated from this extract, but *cis* bond positions could not be assigned according to their UV−visible absorption spectra. These unidentified isomers presumably are typical components of Betatene that coelute with the predominant isomers when separations are carried out with C_{18} stationary phases. A Betatene extract has been previously separated on a polymeric C_{30} column (Sander et al., 1994), but the conditions of this separation were different. These authors used 20:80 (v/v) MTBE−acetonitrile flowing at 2 mL/min with a column temperature of 3 °C. Resolution appears to be slightly better in the present work, presumably the result of more favorable conditions of chromatography. It is also worth mentioning that the column used to obtain the chromatogram in Figure 4 had been previously exposed to several hundred extracts of biological tissues over a 2 year period with only a slight loss in separating efficiency. When compared to previous separations of β-carotene isomers in *Dunaliella* algae-derived carotenoid preparations obtained using other RPLC columns (Mokady et al., 1990; Stahl et al., 1993), the polymeric C_{30} column provides better resolution. The comparative metabolic, physiological, and chemical properties of *all-trans*- vs 9-*cis*-β-carotene are currently receiving considerable attention (Gaziano et al., 1995; Levin and Mokady, 1994; Jiménez and Pick, 1993; Levin et al., 1994; Nagao and Olson, 1994; Stahl et al., 1993; Wang et al., 1994). The C_{30} column can provide these investigators with an effective means of analyzing or purifying these isomers. In a broader sense, geometrical isomers of β-carotene can now be analyzed with greater scrutiny in biological samples, including human tissues and foods, than was previously possible in RPLC.

Human Serum. Extracts of human serum were fractionated prior to C_{30} chromatography because serum is known to possess a complex mixture of carotenoids, including numerous polar carotenoids (Khachik et al., 1992b) and an abundance of lycopene predominantly in *cis* geometrical forms (Krinsky et al., 1990; Stahl et al., 1992). Fractionation of serum on alumina Sep-Pak cartridges gave two fractions, one containing α- and β-carotenes and the other containing xanthophylls and lycopene. When the latter fraction was chromatographed under conditions conducive to separating the xanthophylls, the separation shown in Figure 5A was obtained. The *all-trans* isomers of lutein, zeaxanthin,

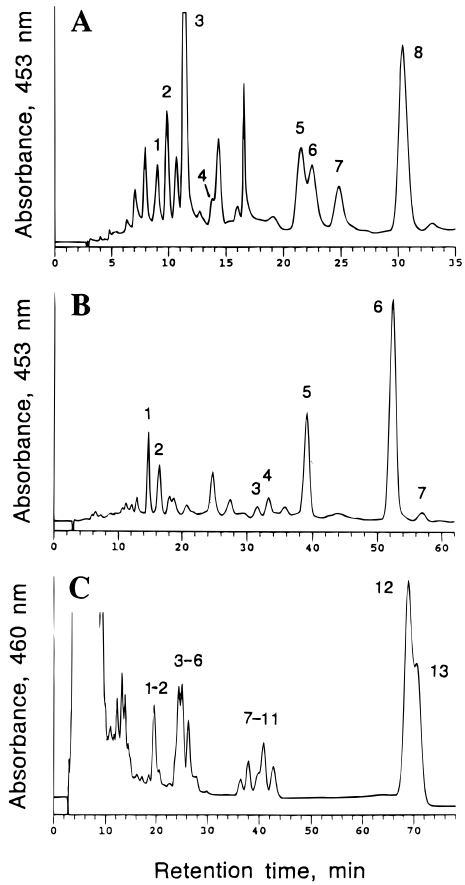

Figure 5. C_{30} chromatograms of carotenoids extracted from human serum: (A) xanthophylls fraction, 7:93 (v/v) MTBE−methanol mobile phase; (B) α- and β-carotenes fraction, 11:89 (v/v) MTBE−methanol mobile phase; (C) lycopene fraction, 38:62 (v/v) MTBE−methanol mobile phase. Tentative peak identifications: (A) 1, 13-*cis*-lutein; 2, 13′-*cis*-lutein; 3, *all-trans*-lutein; 4, zeaxanthin; 5−7, unidentified β,ϵ-carotenoids; and 8, β-cryptoxanthin; (B) 1−2, unidentified ζ-carotene isomers; 3, 15-*cis*-β-carotene; 4, 13-*cis*-β-carotene; 5, *all-trans*-α-carotene; 6, *all-trans*-β-carotene; and 7, 9-*cis*-β-carotene; and (C) 1−11 and 13, *cis*-lycopene isomers; and 12, *all-trans*-lycopene.

and β-cryptoxanthin were tentatively identified in this fraction. Other serum carotenoids were also separated, including several unidentified early-eluting carotenoids and three carotenoids eluting at 21.5, 22.5, and 25 min that could be identified only as β,ϵ-carotenoids. The 13-*cis* and 13′-*cis* isomers of lutein were resolved, but the 9-*cis* and 9′-*cis* lutein isomers, which are known to be present in human serum (Khachik et al., 1992a), were not identified. Figure 5B is the separation of the α- and β-carotenes fraction. In addition to the *all-trans* forms of α- and β-carotene, the 15-*cis* and 13-*cis* isomers of β-carotene were resolved and tentatively identified. The peak containing 15-*cis*-β-carotene is not pure, however, according to its UV−visible absorption spectrum. The 9-*cis* isomer of β-carotene was also detected, but the presence of this minor peak may be artifactual since it is not believed to be present in appreciable quantities in human serum (Stahl et al., 1993). The only other peaks in this fraction that were identified by UV−visible absorption spectra were peaks 1 and 2, which are believed to be isomers of ζ-carotene. The xanthophyll- and lycopene-containing fraction was also chromatographed under conditions that would give separation of lycopene and its geometrical isomers (Figure 5C). The 12 peaks eluting after 16 min are tentatively identified as geometrical isomers of lycopene. In addition to UV−

visible absorption spectra and retention characteristics on alumina, evidence for the identification of these peaks as lycopene isomers was gained by electrospray mass spectrometry, which confirmed a molecular weight of 536 for these peaks (data not shown). These results exclude the possibility that one or more of these peaks contained oxygen addition products of lycopene, unless they were present at concentrations below the sensitive limits of detection for electrospray mass spectrometry (van Breemen, 1995). Identification of the penultimate peak (peak 12) as *all-trans*-lycopene was made possible by cochromatography. Because of similarities between absorption spectra of the various lycopene peaks, tentative assignments of *cis* bond positions could not be made.

Recovery of Carotenoids. Complete chromatographic recovery is not normally observed for carotenoid compounds. Recoveries of α- and β-carotene from the C_{30} column were 64 and 52%, respectively. These values are significantly lower than those obtained for a mixture of carotenoids from polymeric C_{18} columns using either methanol- or acetonitrile-based mobile phases (Epler et al., 1992; Hart and Scott, 1995). Because carotenoid analytes are believed to interact to a greater extent with the polymeric C_{30} stationary phase as compared with polymeric C_{18} stationary phases (e.g., increased retention under similar chromatographic conditions), decreased carotenoid recovery might be expected with the C_{30} column. In addition, the lack of endcapping of the C_{30} column gives rise to unreacted silanol groups, which are thought to be at least partially responsible for on-column degradation of carotenoids. Recovery of carotenoids, however, can be improved considerably by the addition of TEA to the LC mobile phase (Epler et al., 1993; Hart and Scott, 1995). When 0.1% (v/v) TEA was incorporated into the mobile phase, recoveries of α- and β-carotene were improved to 89 and 88%, respectively. The mechanism by which TEA increases carotenoid recovery is not known, but one could hypothesize that TEA, a good base, either buffers acidic silanol groups or participates in hydrogen-bonding interactions with silanols that reduce carotenoid degradation. This is similar to the mechanism proposed by Handelman et al. (1992) for another mobile phase additive, ammonium acetate, which enhanced recovery of zeaxanthin (Handelman et al., 1992) and a mixture of carotenoids (Epler et al., 1992) when C_{18} columns were employed. However, there appear to be unique mechanisms for the enhancement of carotenoid recoveries by TEA and ammonium acetate because the simultaneous use of these mobile phase additives gives higher recoveries than those obtained when TEA or ammonium acetate is used individually (Epler et al., 1993). Another possible mechanism by which TEA enhances carotenoid recoveries was advanced by Epler et al. (1993). They suggested that TEA may interact with positively charged trace metals in the silica support, thereby preventing a potentially degradative interaction with carotenoid analytes. *N*,*N*-Diisopropylethylamine, which is structurally and chemically similar to TEA, has been incorporated into normal-phase LC mobile phases (Kamber and Pfander, 1984; Khachik et al., 1992a,b), but improvements in carotenoid recovery or peak resolution have not been demonstrated.

Recovery of other carotenoids was also enhanced by the addition of 0.1% (v/v) TEA to the LC mobile phase. Peak areas of lutein were increased by 26%, those of zeaxanthin by 42%, those of β-cryptoxanthin by 55%, and those of lycopene by 21%. By this same procedure, TEA increased the recovery of α-carotene by 47% and β-carotene by 64%. When α- and β-carotene were injected in more concentrated standard mixtures to obtain values for their absolute recovery from the C_{30} column, TEA affected relative increases in recovery of 39 and 69%, respectively. Although the absolute recoveries of lutein, zeaxanthin, β-cryptoxanthin, and lycopene from the C_{30} column were not determined in this study, an interesting observation can be made about the influence of TEA on relative enhancement in recovery for the various carotenoids tested. As part of an extensive evaluation of C_{18} column performance for carotenoid analysis (Epler et al., 1992), absolute recovery of carotenoids followed the trends β,β-carotenoids < β,ε-carotenoids, lutein < α-carotene, and zeaxanthin < β-cryptoxanthin < β-carotene. The reverse trend is observed for the influence of TEA on recovery enhancement with the C_{30} column; that is, recovery is enhanced the most where there is greatest opportunity for improvement. This trend, coupled with the observation that TEA increased absolute recoveries of α- and β-carotene to essentially equal values, suggests that factors other than carotenoid−silanol or carotenoid−metal cation interactions (e.g., column frits) are responsible for some of the on-column losses. Only 0.1% (v/v) TEA was tested in this work; thus, an optimal concentration of TEA has not been determined. When used at this concentration, TEA does cause some peak tailing, which can be problematic if a peak is obscured by the tail of another peak. The presence of 0.1% (v/v) TEA in LC mobile phases has also been noted to decrease carotenoid retention, and its use at 0.05% (v/v) has been recommended (Epler et al., 1993; Hart and Scott, 1995). Important benefits of TEA-mediated enhancements in carotenoid recovery would likely be realized when samples containing low concentrations of a carotenoid(s) are being analyzed. Because the various mobile phase additives discussed in this paper have not been tested in a comparative study, the advantages and disadvantages associated with their application to HPLC analysis of carotenoids are largely unknown.

ACKNOWLEDGMENT

We thank Richard van Breemen (University of Illinois−Chicago, Chicago, IL) for performing the electrospray mass spectrometric analysis of lycopene isomers. We gratefully acknowledge technical assistance provided by Ruth Watkins.

LITERATURE CITED

Chandler, L. A.; Schwartz, S. J. Isomerization and losses of *trans*-β-carotene in sweet potatoes as affected by processing treatments. *J. Agric. Food Chem.* **1988**, *36*, 129−133.

Clinton, S. K.; Emenhiser, C.; Schwartz, S. J.; Bostwick, D. G.; Williams, A. W.; Moore, B. J.; Erdman, J. W. *Cis*−*trans* lycopene isomers, carotenoids, and retinol in the human prostate. *Cancer Epidemiol., Biomarkers, Prev.* **1996**, *8*, 823−833.

Craft, N. E. Carotenoid reversed-phase high-performance liquid chromatography methods: reference compendium. *Methods Enzymol.* **1992**, *213*, 185−205.

Davies, B. H. Carotenoids. In *Chemistry and Biochemistry of Plant Pigments*; Goodwin, T. W., Ed.; Academic Press: New York, 1976; Vol. 2, Chapter 19.

Emenhiser, C.; Sander, L. C.; Schwartz, S. J. Capability of a polymeric C_{30} stationary phase to resolve *cis*−*trans* carotenoid isomers in reversed phase liquid chromatography. *J. Chromatogr. A* **1995**, *707*, 205−216.

Emenhiser, C.; Englert, G.; Sander, L. C.; Ludwig, B.; Schwartz, S. J. Isolation and structural elucidation of the predominant

geometrical isomers of α-carotene. *J. Chromatogr. A* **1996**, *719*, 333−343.
Epler, K. S.; Sander, L. C.; Ziegler, R. G.; Wise, S. A.; Craft, N. E. Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids. *J. Chromatogr.* **1992**, *595*, 89−101.
Epler, K. S.; Ziegler, R. G.; Craft, N. E. Liquid chromatographic method for the determination of carotenoids, retinoids and tocopherols in human serum and in food. *J. Chromatogr.* **1993**, *619*, 37−48.
Gaziano, J. M.; Johnson, E. J.; Russell, R. M.; Manson, J. E.; Stampfer, M. J.; Ridker, P. M.; Frei, B.; Hennekens, C. H.; Krinsky, N. I. Discrimination in adsorption or transport of β-carotene isomers after oral supplementation with either *all-trans*- or 9-*cis*-β-carotene. *Am. J. Clin. Nutr.* **1995**, *61*, 1248−1252.
Gross, J. Carotenoid distribution in vegetables. In *Pigments in Vegetables: Chlorophylls and Carotenoids*; Van Nostrand Reinhold: New York; pp 164−179.
Handelman, G. J.; Shen, B.; Krinsky, N. I. High resolution analysis of carotenoids in human plasma by high-performance liquid chromatography. *Methods Enzymol.* **1992**, *213*, 336−346.
Hart, D. J.; Scott, K. J. Development and evaluation of an HPLC method for the analysis of carotenoids in foods, and the measurement of the carotenoid content of vegetables and fruits commonly consumed in the UK. *Food Chem.* **1995**, *54*, 101−111.
Jiménez, C.; Pick, U. Differential reactivity of β-carotene isomers from *Dunaliella bardawil* toward oxygen radicals. *Plant Physiol.* **1993**, *101*, 385−390.
Kamber, M.; Pfander, H. Separation of carotenoids by high-performance liquid chromatography. III. 1,2-Epoxycarotenoids. *J. Chromatogr.* **1984**, *295*, 295−298.
Khachik, F.; Englert, G.; Daitch, C. E.; Beecher, G. R.; Tonucci, L. H.; Lusby, W. R. Isolation and structural elucidation of the geometrical isomers of lutein and zeaxanthin in extracts of human plasma. *Anal. Chem.* **1992a**, *64*, 153−166.
Khachik, F.; Beecher, G. R.; Goli, M. B.; Lusby, W. R.; Smith, J. C. Separation and identification of carotenoids and their oxidation products in the extracts of human plasma. *Anal. Chem.* **1992b**, *64*, 2111−2122.
Krinsky, N. I.; Russett, M. D.; Handelman, G. J.; Snodderly, D. M. Structural and geometric isomers of carotenoids in human plasma. *J. Nutr.* **1990**, *120*, 1654−1662.
Levin, G.; Mokady, S. Antioxidant activity of 9-*cis* compared to *all-trans* β-carotene *in vitro*. *Free Radical Biol. Med.* **1994**, *17*, 77−82.
Levin, G.; Ben-Amotz, A.; Mokady, S. Liver accumulation of soluble *all-trans* or 9-*cis* β-carotene in rats and chicks. *Comp. Biochem. Physiol.* **1994**, *107A*, 203−207.
Mokady, S.; Avron, M.; Ben-Amotz, A. Accumulation in chick livers of 9-*cis* versus *all-trans* β-carotene. *J. Nutr.* **1990**, *120*, 889−892.
Nagao, A.; Olson, J. A. Enzymatic formation of 9-*cis*, 13-*cis*, and *all-trans* retinals from isomers of β-carotene. *FASEB J.* **1994**, *8*, 968−973.
O'Neil, C. A.; Schwartz, S. J. Chromatographic analysis of *cis*/*trans* carotenoid isomers. *J. Chromatogr.* **1992**, *624*, 235−252.
Quackenbush, F. W. Reverse phase HPLC separation of *cis*- and *trans*-carotenoids and its application to food materials. *J. Liq. Chromatogr.* **1987**, *10*, 643−653.
Quackenbush, F. W.; Miller, S. L. Composition and analysis of the carotenoids in marigold petals. *J. Assoc. Off. Agric. Chem.* **1972**, *55*, 617−621.
Sander, L. C.; Epler Sharpless, K.; Craft, N. E.; Wise, S. A. Development of engineered stationary phases for separations of carotenoid isomers. *Anal. Chem.* **1994**, *66*, 1667−1674.
Schmitz, H. H.; Emenhiser, C.; Schwartz, S. J. HPLC separation of geometric carotene isomers using a calcium hydroxide stationary phase. *J. Agric. Food Chem.* **1995**, *43*, 1212−1218.
Stahl, W.; Schwarz, W.; Sundquist, A. R.; Sies, H. *Cis-trans* isomers of lycopene and β-carotene in human serum and tissues. *Arch. Biochem. Biophys.* **1992**, *294*, 173−177.
Stahl, W.; Schwarz, W.; Sies, H. Human serum concentrations of *all-trans* β- and α-carotene but not 9-*cis* β-carotene increase upon ingestion of a natural mixture obtained from *Dunaliella salina* (Betatene). *J. Nutr.* **1993**, *123*, 847−851.
Sweeney, J. P.; Marsh, A. C. Effect of processing on provitamin A in vegetables. *J. Am. Diet. Assoc.* **1971**, *59*, 238−243.
van Breemen, R. B. Electrospray liquid chromatography−mass spectrometry of carotenoids. *Anal. Chem.* **1995**, *67*, 2004−2009.
Wang, X-.D.; Krinsky, N. I.; Benotti, P. N.; Russell, R. M. Biosynthesis of 9-*cis*-retinoic acid from 9-*cis*-β-carotene in human intestinal mucosa *in vitro*. *Arch. Biochem. Biophys.* **1994**, *313*, 150−155.

Received for review February 15, 1996. Revised manuscript received September 3, 1996. Accepted September 3, 1996.[⊗] Certain commercial equipment, instruments, or materials are identified in this paper 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.

JF960104M

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.