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Note

Identification of *cis*-carotenoids in human sera analyzed by reversed-phase high-performance liquid chromatography with diode array detection

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An inverse relationship has been found between serum carotene levels and several types of cancer, including lung [1,2], cervical [3], and esophageal [4]. Additionally, β-carotene supplementation has been shown to be effective in reducing the number of precancerous cells in the oral mucosa of snuff users with a carotene-deficient diet [5]. The ability to accurately measure serum carotene levels is becoming more important as evidence for the inverse relationship between these levels and some forms of cancer increases. The use of high-performance liquid chromatographic (HPLC) analysis of serum carotenoids has largely replaced the less accurate spectrophotometric total carotene method, but concern still exists about the accuracy of these measurements. The serum carotene profile is complicated by the presence of a variety of major and minor carotenoids and the potential presence of geometric isomers of these species. A number of different HPLC methods for serum carotene analysis are available [6–12]; however, many of these methods measure only lycopene, α-carotene, and β-carotene [10–12], omitting lutein, its structural isomer zeaxanthin, and the provitamin A carotene cryptoxanthin, which have been shown to be a significant fraction of the total carotenes in serum from United States populations. Additionally, various *cis*-carotenoids have been found in food [13] and serum extracts [6,7,14].

cis-Carotenoids may be present in serum as a result of diet or as a result of the antioxidant activity of the carotenoids in the body [15]. Isomerization may also occur during sample preparation or analysis. The presence of *cis*-carotenoids in serum extracts may lead to an underestimation of the amount of carotene present

in the sample because of the lower absorbance of the *cis*-isomers at wavelengths usually monitored during carotene analysis.

EXPERIMENTAL*

Chromatography

The instrumentation consisted of a Hewlett-Packard 1090M liquid chromatograph (Hewlett-Packard, Avondale, PA, U.S.A.) equipped with a column heater, variable-injection volume autosampler and a diode array detector. The column was a Dynamax Microsorb C_{18}, 25 cm×4.6 mm, 5-μm column (Rainin Instruments, Woburn, MA, U.S.A.). The mobile phase was ethanol–acetonitrile (1:1, v/v) at a flow-rate of 1.0 ml/min and a column temperature of 40°C. Spectra were collected continuously over the 250- to 550-nm range. Chromatograms were recorded at 450 nm and 340 nm. Additionally closely eluting peaks were examined with the QuickRes software (Infometrix, Seattle, WA, U.S.A.), a program designed for use with the Hewlett-Packard ChemStation, which employs multivariate statistics to resolve components coeluting in a total-wavelength chromatogram collected with a diode array detector, and extracts the spectra of each component of peaks containing two components.

Serum samples

Specimens analyzed in this study were residual sera from specimens collected from healthy women between the ages of 50 and 60 years for other analyses prior to their participation in a study of the effects of dietary intervention on serum cholesterol levels.

Sample preparation

Fat-soluble compounds were extracted from human serum according to the following procedure. A mixture of 400 μl serum and 200 μl ethanol containing nonapreno-β-carotene as an internal standard was vortexed for 30 s. Then 200 μl of hexane were added, followed by vortex-mixing for 60 s. The phases were separated by centrifugation for 10 min at 1500 *g*. The hexane layer (150 μl) was removed and dried under nitrogen without heating. Ethanol (200 μl) was added to dissolve the residue. If the solution was turbid, the insoluble material was removed by centrifugation for 5 min at 1500 *g* at 4°C, followed by filtration of the supernatant through a 0.45-μm filter (No. SJHV004NS, Millipore, Bedford, MA, U.S.A.) into injection vials.

Chemicals

Standards of zeaxanthin and cryptoxanthin were provided by Hoffmann-La Roche (Nutley, NJ, U.S.A.). Standards of lycopene, α-carotene, and β-carotene were purchased from Sigma (St. Louis, MO, U.S.A.). Nonapreno-β-carotene used as the internal standard was contributed by Dr. Frederick Khachik (Nutrient

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*Use of trade names is for identification only and does not constitute endorsement by the Public Health Service or the U.S. Department of Health and Human Services.

Composition Laboratory, U.S. Department of Agriculture, Beltsville, MD, U.S.A.) HPLC-grade acetonitrile and hexane were obtained from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). Absolute ethanol, USP, was obtained from various sources. Standard solutions were prepared by dissolving small amounts of the standards in ethanol, injecting the solutions on the column, and collecting the middle third of the peak. The purified standard was dried under nitrogen, redissolved in ethanol, and stored under nitrogen at $-20\,^{\circ}C$. Purified standard solutions, four months old, which had been allowed to stand at room temperature overnight, were found to contain *cis*-isomers and were used to optimize the separation of the geometric isomers.

RESULTS AND DISCUSSION

Using reversed-phase HPLC with diode array detection and QuickRes software we have identified and separated *cis*- and *trans*-carotene in standard solutions and have examined serum samples for the presence of *cis*-carotenoids. In this work several different mobile phases were tried to optimize separation between the carotenoids and their geometric isomers. Using the Microsorb column, we achieved some separation between geometric isomers with all mobile phases, however, the best separation was obtained with a mobile phase containing 50% ethanol and 50% acetonitrile. This mobile phase also improved the peak height of the carotenoids relative to the height observed with the mobile phases previously used in our laboratory (methanol–ethanol, 8:2, with 100 μl/l diethylamine) and currently used by Stacewicz-Sapuntzakis [8] (methanol–acetonitrile–tetrahydrofuran, 50:45:5). With the standards, baseline separation was achieved between zeaxanthin and two *cis*-isomers and between β-carotene and a *cis*-isomer that has been identified by Bieri et al. [6] as 15,15′-*cis*-β-carotene. The other carotenes showed the *cis*-isomers as shoulders on the parent compound when observed at 450 nm and as separate peaks at 340 nm (Fig. 1).

Fourteen human serum samples were examined to determine if *cis*-carotenoids in addition to the previously noted 15,15′-*cis*-β-carotene were common in the samples. A typical chromatogram is shown in Fig. 2. The elution order is zeaxanthin/lutein, two unidentified peaks, the peak identified by Bieri et al. [6] as precryptoxanthin, cryptoxanthin, lycopene, α-carotene, all-*trans*-β-carotene and 15,15′-*cis*-β-carotene. The first unknown peak, which corresponds to the shoulder on the zeaxanthin/lutein peak observed by Bieri et al. [6], has a retention time similar to those of the *cis*-zeaxanthin isomers found in the standard but the spectrum of this peak does not contain the absorbance asociated with the central *cis*-isomers. *cis*-Isomers of lycopene and β-carotene were present in all sera. Some samples contained a *cis*-isomer of cryptoxanthin. The presence of these *cis*-isomers was readily detected from the chromatograms at 340 nm and confirmed by examination of the spectra of the peaks. Additionally, a significant amount of a compound with ultraviolet (UV) absorbance eluted where *cis*-α-carotene was expected but with a larger than expected peak height.

Analysis of the chromatograms with the QuickRes software also indicated the presence of a *cis*-isomer of cryptoxanthin eluting immediately after *trans*-cryp-

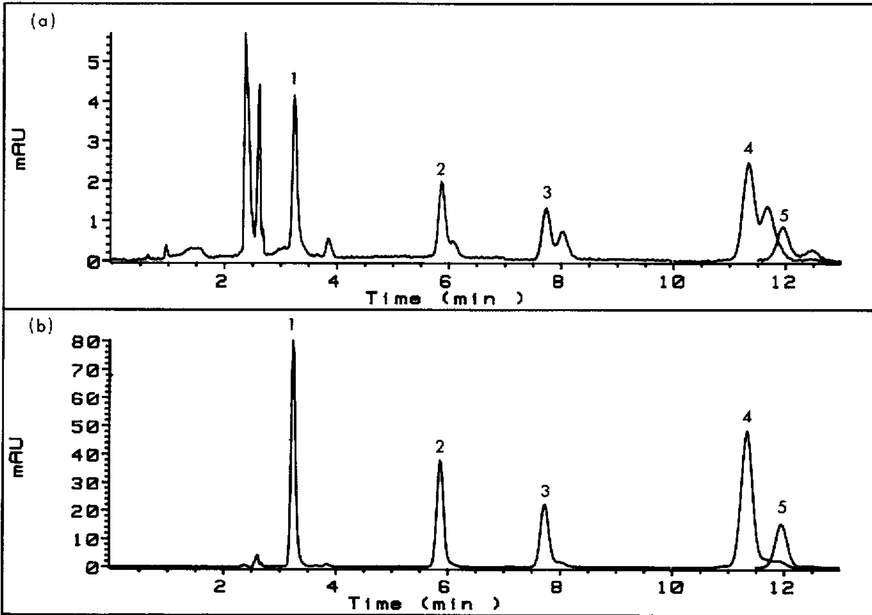

Fig. 1. Overlay of chromatograms of individual carotene standards containing *cis*- and *trans*-isomers at 340 nm (a) and 450 nm (b). Peaks: 1 = zeaxanthin (0.68 mg/l); 2 = cryptoxanthin (0.72 mg/l); 3 = lycopene (0.54 mg/l); 4 = α-carotene (0.89 mg/l); 5 = β-carotene (1.86 mg/l).

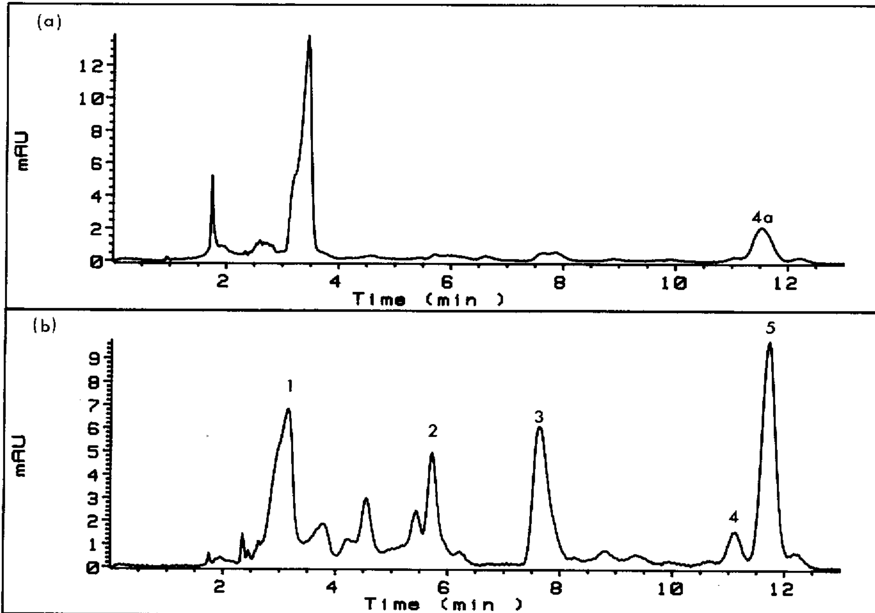

Fig. 2. Chromatograms at 340 nm (a) and 450 nm (b) of a human serum extract collected under conditions described in the text. Peaks: 1 = zeaxanthin and lutein; 2 = cryptoxanthin; 3 = lycopene; 4 = α-carotene; 4a = phytofluene; 5 = β-carotene.

toxanthin in some samples. QuickRes did not resolve the *cis*-component of the lycopene peak in six of the fourteen samples, but in those samples it did resolve, two *cis*-lycopene isomers were found: one eluting before *trans*-lycopene and one eluting after. Further examination of the chromatograms with QuickRes software to improve resolution of closely eluting compounds indicated that the material eluting where *cis*-α-carotene was expected had a spectrum with maxima at 331, 347, and 367 nm (Fig. 3). On the basis of the spectrum we have tentatively identified this compound as phytofluene, a colorless carotenoid found in tomatoes.

cis-Isomers in serum do not present a problem in quantitation of most serum carotenoids. The only *cis*-carotenoids observed in all of the samples are isomers of lycopene and β-carotene. These isomers are readily detected without the QuickRes software by using dual-wavelength detection at wavelengths corresponding to the region of maximum absorbance (450 nm) and the *cis* absorbance region (340 nm). Under the conditions used in this laboratory, 15,15′-*cis*-β-carotene separates from the all-*trans*-isomer and does not affect measurements of all-*trans*-isomer. The *cis*- and *trans*-lycopene isomers and the isomers of cryptoxanthin are not separated well enough to allow quantitation of the all-*trans*-isomer on the basis of peak area at 450 nm. The *cis*-isomers, however, make a negligible contribution to the height of the lycopene peak at that wavelength, thus peak height quantitation allows measurement of the *trans*-isomer only.

The formation of *cis*-isomers in standard solutions may have significant effect on carotenoid measurements based on peak height and peak area. In our laboratory a β-carotene standard solution stored under nitrogen at −20°C and lacking significant absorbance changes at 450 nm was found to contain sufficient amounts of the 15,15′-*cis*-isomer to result in errors of approximately 10% by peak-height quantitation. A similar situation was observed with lycopene. Increases in the concentration of standard solutions by evaporation of solvent can offset absorbance losses at the absorbance maximum of the *trans*-isomer that occur with

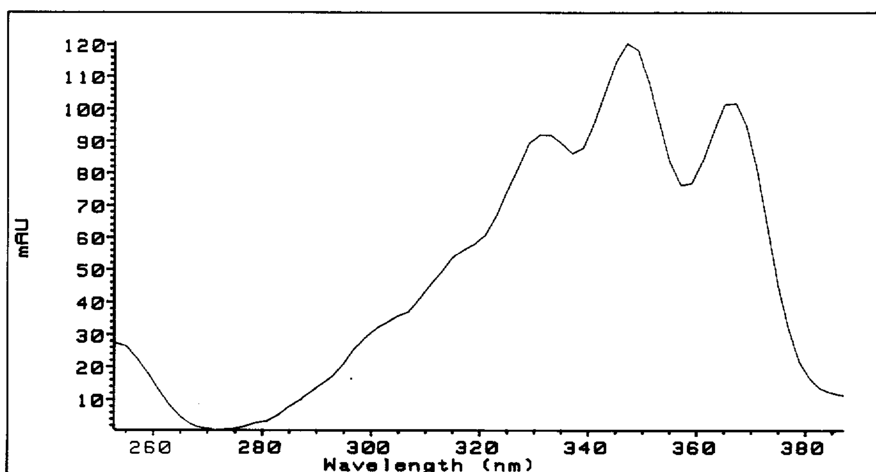

Fig. 3. Spectrum of the colorless compound eluting at 11.6 min, which has been tentatively identified as phytofluene.

isomerization. For this reason, it is important to monitor the ratio of the absorbance at the absorbance maximum of the all-*trans*-carotenoid to the maximum of the *cis* absorbance, especially for the more labile carotenoids.

While the QuickRes program was very useful in identifying the presence of phytofluene, it has been of limited use in examining carotenoid chromatograms because of the similarity of the spectra of the various carotenoids. The program may be of more use in applications where the coeluting materials have greater spectral differences. The program has a number of limitations. Because the diode array detector has a minimum bandwidth of 4 nm, components with very similar spectra such as zeaxanthin and lutein and α-carotene and β-carotene cannot be resolved. The software may not automatically detect the presence of the minor component of a two-component peak if it has a spectrum very similar to that of the major component and contributes less than 8–10% of the peak area. Additionally, the user cannot adjust a resolved spectrum to match the spectrum of a known component, which may affect the validity of quantitations of resolved components.

Our results demonstrate the presence in serum of three *cis*-carotenoids in addition to the previously noted 15,15′-*cis*-β-carotene: a *cis*-cryptoxanthin and two *cis*-isomers which elute near lycopene. These isomers are partially resolved from the all-*trans*-isomers at 450 nm and fully resolved in the chromatogram at 340 nm even on monomeric C_{18} columns. In the chromatogram at 450 nm quantitation on the basis of peak area may include a small error resulting from the presence of *cis*-isomers, but this error can be eliminated by using peak height as the basis of the quantitation.

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