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Concurrent liquid chromatographic separation and photodiode array detection of retinol, tocopherols, all-*trans*-α-carotene, all-*trans*-β-carotene and the mono-*cis* isomers of β-carotene in extracts of human plasma

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Abstract

In this report we describe the development of a method for the concurrent reversed-phase high-performance liquid chromatographic separation and photodiode array detection of human plasma retinol, tocopherols and carotenes. For a single sample injection, retinol, retinyl acetate, α-tocopherol, γ-tocopherol, α-tocopheryl acetate, all-*trans*-α-carotene and all-*trans*-β-carotene, as well as the mono-*cis* geometrical isomers of β-carotene were separated and detected. Analytical separations were performed at a subambient temperature (0°C) over a Suplex pKb-100 reversed-phase analytical column with an isocratic mobile phase of methanol–methyl *tert.*-butyl ether–water (80:20:5, v/v/v) at a flow-rate of 0.8 ml/min for 60 min. Standards and samples were reconstituted in ethanol, and typically, 50 μl was injected for analysis. By HPLC, compounds of interest were clearly resolved and detectable at the picomole level. © 1997 Elsevier Science B.V.

Keywords: Retinol; Tocopherols; Carotenes

1. Introduction

A liquid chromatographic method was required that concurrently could separate and detect retinol, retinyl acetate, α-tocopherol, γ-tocopherol and α-tocopheryl acetate as well as α-carotene, all-*trans*-β-carotene and the mono-*cis* geometrical isomers of β-carotene (Fig. 1). Gas chromatography (GC) can be used for the separation of retinoids and tocopherols, but the low analyte volatility and high rate of heat-induced isomerization precludes its use for the analysis of carotenoids. High-performance liquid chromatography (HPLC) is the preferred means of analysis since β-carotene is thermally labile, nonvolatile and easily isomerized by heat, ions and light. As antioxidants, carotenoids also are easily oxidized.

An abundance of literature exists detailing HPLC methods for the separation of retinoids, tocopherols, α-carotene and β-carotene isolated from plasma or serum [1–8]. Epler et al. [9] described a gradient reversed-phase method where retinol, retinyl palmitate and the carotenoids were separated by liquid chromatography (LC) and then measured with a programmable UV–Vis detector. Tocopherols were simultaneously measured with a fluorescence detec-

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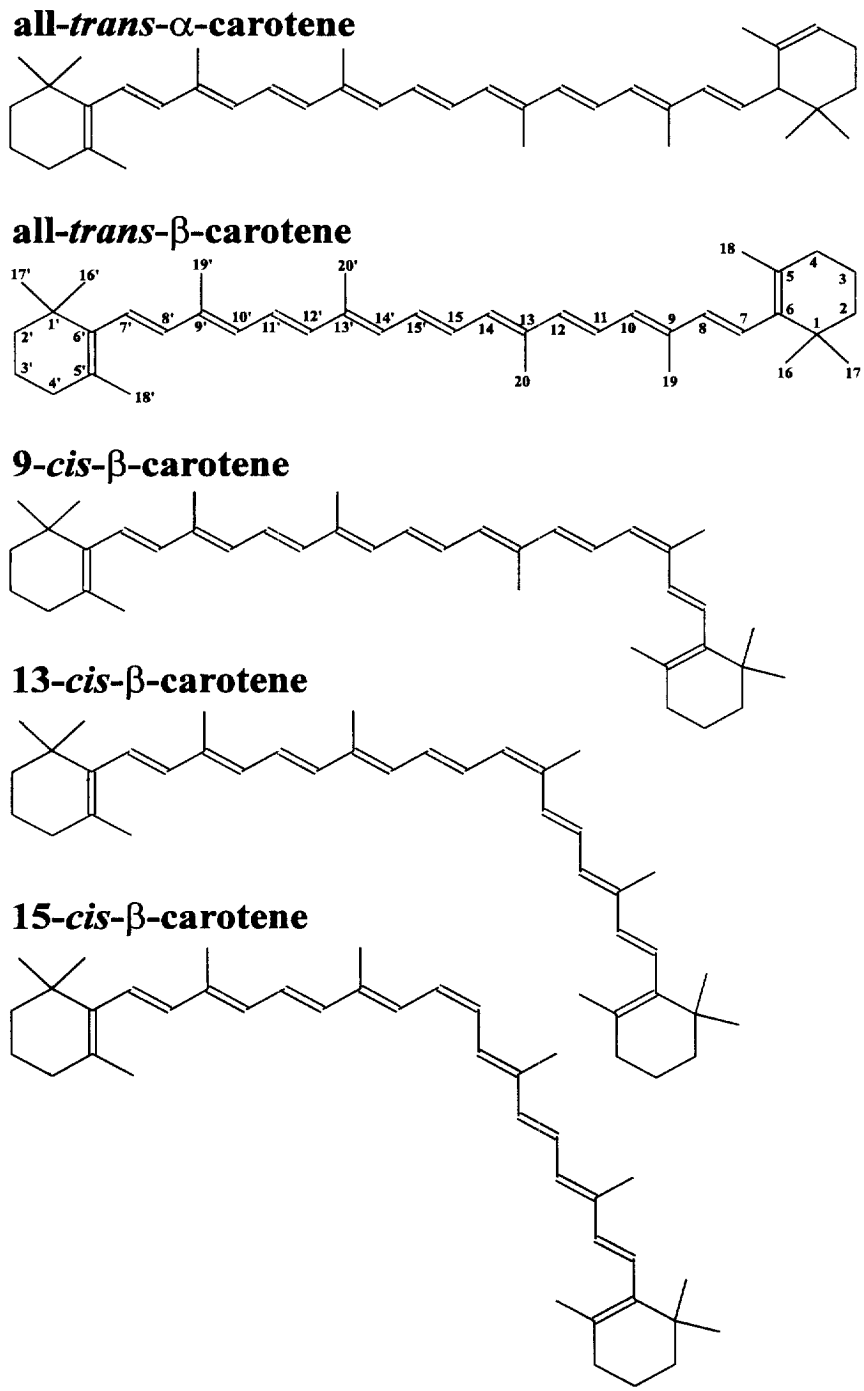

Fig. 1. Structures of all-*trans*-α-carotene, all-*trans*-β-carotene and the mono-*cis* isomers of β-carotene.

tor connected in-line to the UV–Vis detector. It was noted that the method gave resolution of the detected carotenoids, but the mono-*cis* isomers of β-carotene were not baseline resolved.

The increased perception that carotenoids exhibit positive health benefits has precipitated an abundance of literature detailing techniques for the analysis of these compounds. Craft et al. [10] reported that wide-pore, C_{18} polymerically-modified silica exhibited adequate selectivity to separate the geometrical isomers of β-carotene. Methanol and acetonitrile were investigated as primary mobile phase solvents with either water or ethyl acetate added as modifiers. A Vydac 201TP column ultimately was used with an isocratic mobile phase of methanol–water (97:3, v/v). In a later report, Craft et al. [11] described the optimization of an isocratic carotenoid separation using the Vydac 210TP column. It was determined that 5% tetrahydrofuran in methanol gave the best overall separation of a test mixture of seven carotenoids.

A C_{30} polymeric stationary phase has been specially engineered for the HPLC separation of polar and nonpolar geometric carotenoid isomers [12]. In collaboration with the National Institute of Standards and Technology, YMC (Wilmington, NC, USA) has made the phase commercially available in what has been termed the "Carotenoid Column". The C_{30} phase is reported to offer superior resolution over previously reported monomeric and polymeric C_{18} separations. To the best of our knowledge, no report exists detailing the use of the column for the concurrent analysis of carotenoids and other compounds (e.g., retinoids and tocopherols).

Stahl and coworkers [13,14] introduced the use of a Suplex pKb-100 reversed-phase analytical column with methanol–acetonitrile–2-propanol (54:44:2, v/v/v) as the mobile phase for the analysis of β-carotene geometrical isomers in biological matrices. Stahl et al. [15] then studied the uptake of all-*trans*-β-carotene and 9-*cis*-β-carotene from Betatene 20%, a commercial product (Henkel, LaGrange, IL, USA) derived from extracts of the alga *Dunaliella salina*. Betatene contains at 20% a mixture of β-carotene geometrical isomers (all-*trans*, 9-*cis*, 13-*cis* and 15-*cis*) along with small amounts of α-carotene in soybean oil.

This report describes the development of a method for the concurrent reversed-phase HPLC separation and photodiode array detection of human plasma retinol (328 nm), carotenes (452 nm) and tocopherols (292 nm). The described HPLC method provides the chromatographic separation and resolution so that $^2H_8$-labeled β-carotene and unlabeled β-carotene geometrical isomers can be detected and quantified by electron-capture chemical ionization in conjunction with selected anion monitoring mass spectrometry [16].

2. Experimental

2.1. Solvents and reagents

Methanol, ethanol, methyl *tert.*-butyl ether, hexane and toluene were purchased from Fisher Scientific (Springfield, NJ, USA) in HPLC-grade and were used as supplied. Water was double-distilled by means of an in-laboratory all-glass distillation apparatus. Butylated hydroxytoluene (BHT), mineral acids, bases and other chemicals were purchased from Sigma (St. Louis, MO, USA).

2.2. Standards

Carotene standards (α- and β-carotene), retinyl acetate, all-*rac*-α-tocopherol and all-*rac*-α-tocopheryl acetate were purchased from Sigma. Betatene 20% is a commercial product (Henkel) derived from extracts of the alga *Dunaliella salina* and contains at 20% a mixture of β-carotene geometrical isomers (all-*trans*, 9-*cis*, 13-*cis* and 15-*cis*) along with small amounts of α-carotene in soybean oil. Gelatin capsules containing Betatene 20% (each with 15 mg of *D. salina* β-carotene) were a gift from Henkel. Stock solutions of the carotenes (2 mg) and Betatene (10 mg of the oil-based product) were made by dissolving the compounds in 10.0 ml toluene–hexane (1:1, v/v) and stock solutions of retinyl acetate, all-*rac*-α-tocopherol and all-*rac*-α-tocopheryl acetate were made by weighing approximately 10 mg of each of the compounds into 10.0 ml ethanol. Stock solutions were stored in foil-wrapped glass tubes under a nitrogen headspace at −80°C.

Working standards were made fresh daily and

concentrations determined by absorption readings. For daily working standards, 100 μl aliquots of the stocks were transferred to 125×16 mm screw-capped tubes and dried under a clean stream of nitrogen at 40°C. The compounds subsequently were diluted in ethanol and concentrations determined by absorption readings. Concentrations were calculated using the Beer–Lambert Law with absorptivity coefficients (dL/g cm) in ethanol of 2592 at 452 nm for all-*trans* β-carotene [9], of 1565 at 328 nm for retinyl acetate [5], of 43.6 at 285 nm for α-tocopheryl acetate [17] and of 75.8 at 292 nm for α-tocopherol [9]. Standards were shielded from direct light and handled in foil-wrapped containers.

2.3. High-performance liquid chromatography

HPLC was performed on a Hewlett-Packard 1090L (Hewlett-Packard, Palo Alto, CA, USA) equipped with an auto-injector, a ternary solvent system, a 250 μl sample loop, a photodiode array detector and a MS-DOS based HP Chemstation data system. Chromatographic separations were carried out using a 250×4.6 mm Suplex pKb-100 reversed-phase analytical column, 5 μm particle size, 100 Å pore size (Supelco, Bellefonte, PA, USA) equipped with a 20×4.6 mm Supelguard column, 5 μm particle size, 100 Å pore size (Supelco). An isocratic mobile phase of methanol–methyl *tert.*-butyl ether–water (80:20:5, v/v/v) was used at a flow-rate of 0.8 ml/min for 60 min. Absorption spectra were recorded from 200 nm to 600 nm for all peaks monitored at 292 nm (tocopherols), 328 nm (retinoids) and 452 nm (carotenoids) using a photodiode array detector. Standards and samples were reconstituted in ethanol and typically, 50 μl was injected for analysis. The HPLC column was maintained at approximately 0°C by use of a laboratory-constructed ice bath and typical column pressures of 100 bar were noted. For storage, the column was left in mobile phase and placed in a refrigerator at 4°C.

2.4. Ice-bath

A 40 cm length of 9/16 in. I.D. polypropylene tubing was inserted length-wise through a 5 l polypropylene sterilizing tray (32×26×11 cm) approximately 4 cm from the tray's bottom (1 in.=2.54 cm). The tube was cemented to the tray and the tray was insulated with a suitable-sized polystyrene cooler with lid. Prior to HPLC operation, the chromatographic column, equipped with guard, was inserted through the tubing and the tray was packed with a mixture of chipped ice and water.

2.5. Quantification

Seven-point external standard curves (ranging in values of 3% to 300% for α-tocopherol and 2% to 200% for β-carotene of what is considered normal plasma values [18]) were constructed by adding 0.18, 0.90, 1.80, 4.50, 9.00, 13.40 and 17.90 μg of all-*rac*-α-tocopherol in ethanol to 4.44, 22.20, 44.40, 111.00, 222.00, 333.00 and 444.00 ng of all-*trans*-β-carotene in ethanol, respectively. Then to each standard, 83.7 ng of retinyl acetate in 100 μl of ethanol was added. The standards then were dried under a clean stream of nitrogen at 37°C. For injection, each standard was reconstituted in 200 μl of ethanol containing 6.1 μg of all-*rac*-α-tocopheryl acetate. The tocopheryl acetate was used as an injection standard to verify consistent HPLC injection volumes in both the external standards and the human plasma extracts. For HPLC analyses, 50 μl of each external standard solution was injected. Plasma concentrations of tocopherols and carotenes then were calculated using a linear regression (area vs. concentration) of the seven-point external standard curve and were adjusted by percent recovery of the added retinyl acetate internal standard. No corrections were made for the absorption differences of mono-*cis* isomers of β-carotene, where extinction coefficients may be as much as 30% lower than that of all-*trans*-β-carotene [14]. Plasma retinol concentrations were estimated based on the area of the retinyl acetate internal standard and were not corrected for any differences in absorptivity.

2.6. Carotenoid dosing and storage

A healthy, nonsmoking, nonmedicated human volunteer consumed a single 60 mg dose of Betatene 20% (four 15 mg capsules) with a high-fat breakfast. Approximately 10 ml of blood was collected each at −1, 0, 3, 6, 9, 12, 24, 48 and 72 h post-consumption with 100×16 mm Vacutainers (Becton Dickinson

Vacutainer Systems, Franklin Lakes, NJ, USA) containing 0.10 ml of a 15% EDTA (K_3) solution. Plasma was separated by centrifugation at 1500 rpm for 5 min and then stored at −80°C prior to extraction.

2.7. Plasma extraction

Extractions and sample handling were performed in subdued light to minimize isomerization and degradation of the analytes. For extraction of analytes, 500 μl plasma aliquots were transferred to 125×16 mm screw-capped culture tubes, and to each, 100 μl retinyl acetate in ethanol (837 ng/ml) was added. Plasma proteins then were precipitated by the addition of 400 μl ethanol. Samples then were vortexed for 30 s, overlaid with 2.0 ml hexane, vortexed an additional 30 s and subsequently spun at 1500 rpm for 5 min. The upper hexane layers were removed to clean tubes and saved for analysis. To the remaining lower layers, 2.0 ml hexane was added and the samples re-extracted as outlined above. The two upper hexane layers from each sample were pooled and evaporated to dryness under a clean stream of nitrogen at 40°C. The extracts then were reconstituted in 200 μl α-tocopheryl acetate in ethanol (30.28 μg/ml) and 50 μl was injected for HPLC analysis.

3. Results and discussion

In our hands, the chromatographic separation of Betatene as reported by Stahl and coworkers [13–15] gave similar retention times but with poorer resolution. Satisfactory resolution of β-carotene geometrical isomers (Fig. 2) was achieved over the Suplex pKb column by changing to an isocratic mobile phase of methanol–methyl *tert.*-butyl ether–water (80:20:5, v/v/v) at a flow-rate of 0.8 ml/min for 60 min and maintaining a column temperature of approximately 0°C by use of a laboratory-constructed ice bath. The ice bath was capable of maintaining an inside tubing temperature of 0–2°C for more than 30 h at room temperature.

Both all-*trans*-α-carotene and all-*trans*-β-carotene were identified by retention time comparisons with authentic standards. The geometrical isomers of β-

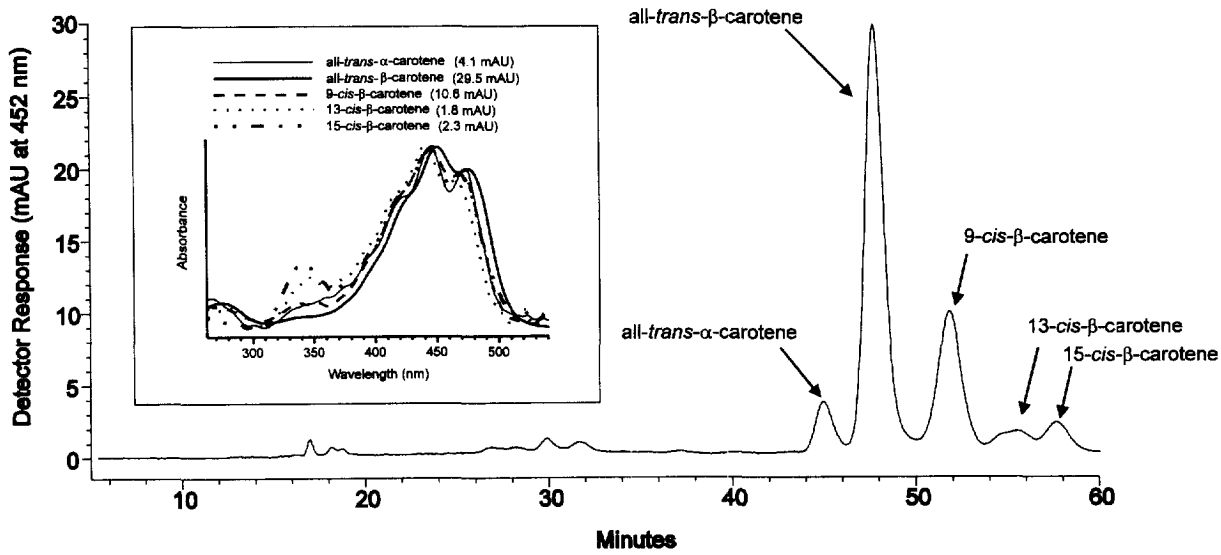

Fig. 2. Chromatogram of the carotene isomers contained in capsules of Betatene 20%. HPLC conditions: column, 250×4.6 mm Suplex pKb-100, 5 μm particle size, 100 Å pore size, equipped with a 20×4.6 mm Supelguard column, 5 μm particle size, 100 Å pore size (Supelco); detection, 452 nm; flow-rate, 0.8 ml/min; eluent, methanol–methyl *tert.*-butyl ether–water (80:20:5, v/v/v); column temperature, 0°C. Insert: absorption spectra of all-*trans*-α-carotene, all-*trans*-β-carotene and the mono-*cis* isomers of β-carotene. The spectra were captured on-line through the use of a photodiode array detector.

carotene were identified through HPLC generated UV–Vis absorption spectra (Fig. 2) by the rules compiled [19] from five different reports [20–24]. Additional confirmation of the mono-*cis* isomers was obtained by comparing HPLC peak percentages with the reported β-carotene *cis*-isomer composition of Betatene. We determined the composition of Betatene to be comprised of 4.6% all-*trans*-α-carotene, 57.6% all-*trans*-β-carotene, 28.0% 9-*cis*-β-carotene and 9.8% as other *cis*-isomers as compared to the reported values of 1%, 54%, 37% and 9%, respectively [15]. These differences most likely arise from algal crop and growth condition variations (Henkel sales literature).

Saponification of the plasma prior to neutral lipid extractions may have enhanced the recovery of analytes, but isomerization of all-*trans*-β-carotene, when treated with 0.2 *M* KOH in methanol at 37°C for 30 min, was observed in preliminary experiments (data not shown). Plasma extracts were not filtered prior to HPLC injection to minimize losses and degradation. It was found that approximately seventy unfiltered injections could be performed before there was a rise in column backpressure (to >150 bar) that warranted changing the guard column.

Typical liquid chromatographic separations of human plasma extracts (12 h post-consumption of 60 mg Betatene) are shown in Fig. 3A–C. Separation of the β-carotene geometrical isomers in Betatene is shown in Fig. 2. Chromatograms generated from the absorption at 292 nm (Fig. 3A) yielded typical retention times of 16.6 min for α-tocopheryl acetate ($k'=2.8$), 18.4 min for γ-tocopherol ($k'=3.2$) and 19.9 min for α-tocopherol ($k'=3.5$). Chromatograms generated from the absorption at 328 nm (Fig. 3B) yielded typical retention times of 6.7 min for retinol ($k'=0.6$) and 7.8 min for retinyl acetate ($k'=0.8$). And, chromatograms generated from the absorption at 452 nm (Fig. 3C) yielded typical retention times of 43.9 min for α-carotene ($k'=9.0$), 47.1 min for all-*trans*-β-carotene ($k'=9.7$), 51.8 min for 9-*cis*-β-carotene ($k'=10.8$), 55.6 min for 13-*cis*-β-carotene ($k'=11.6$) and 57.8 min for 15-*cis*-β-carotene ($k'=12.1$).

Prior to injecting samples, a seven-point external standard curve was injected (50 μl injected from a 200 μl standard volume) as well as a sample of Betatene. Then, one replicate of each sample was analyzed by HPLC (typically six samples, 50 μl injected). Following the injection of the first set of replicates, another sample of Betatene was injected to verify the chromatographic separation and retention times of the geometrical isomers of β-carotene. The second set of replicates then were injected followed by the injection of a second set of the seven-point external standard curve. With injection volumes of 50 μl, the all-*trans*-β-carotene external standard curves were linear (average $r^2=0.9963$, R.S.D. 0.20%) from 4.44 ng/200 μl to 444.00 ng/200 μl and the all-*rac*-α-tocopherol external standard curves were linear (average $r^2=0.9992$, R.S.D. 0.07%) from 0.18 μg/200 μl to 17.90 μg/200 μl for 22 standard curves generated during a three-month period.

The α-tocopheryl acetate was used to verify consistent HPLC injections between the external standards and plasma extracts. The retinyl acetate was used as a measure of quantitative recovery (average of 94.8%, $n=12$, R.S.D. 0.7%) when extracting plasma samples. It was assumed that the added retinyl acetate, the endogenous tocopherols and the endogenous carotenes were equally extractable, although it is doubtful that compounds of such diverse chemical structure possess similar partition coefficients.

Column recoveries of β-carotene, α-tocopherol, α-tocopheryl acetate and retinyl acetate were determined by comparison of the integrated absorption readings of injected standards with calculated expected area counts. Expected area counts were calculated by rearrangement of the Beer–Lambert Law:

$$\text{Expected area (mAu S)} = \frac{\text{absorptivity}\left(\frac{dL}{\text{g cm}}\right)\cdot\text{amount injected (g)}\cdot\text{flowcell pathlength (1 cm)}}{\text{flow-rate}\left(\frac{dL}{\text{S}}\right)}\cdot\left(\frac{1000\text{ mAu}}{1\text{ Au}}\right) \quad (1)$$

Average column percent recoveries ($n=7$) were 91.7% (R.S.D. 3.4%) for β-carotene, 124.1% (R.S.D. 4.6%) for α-tocopherol, 126.8% (R.S.D. 0.4%) for α-tocopheryl acetate and 100.3% (R.S.D. 3.2%) for retinyl acetate of the calculated expected areas. The differences between expected areas and measured

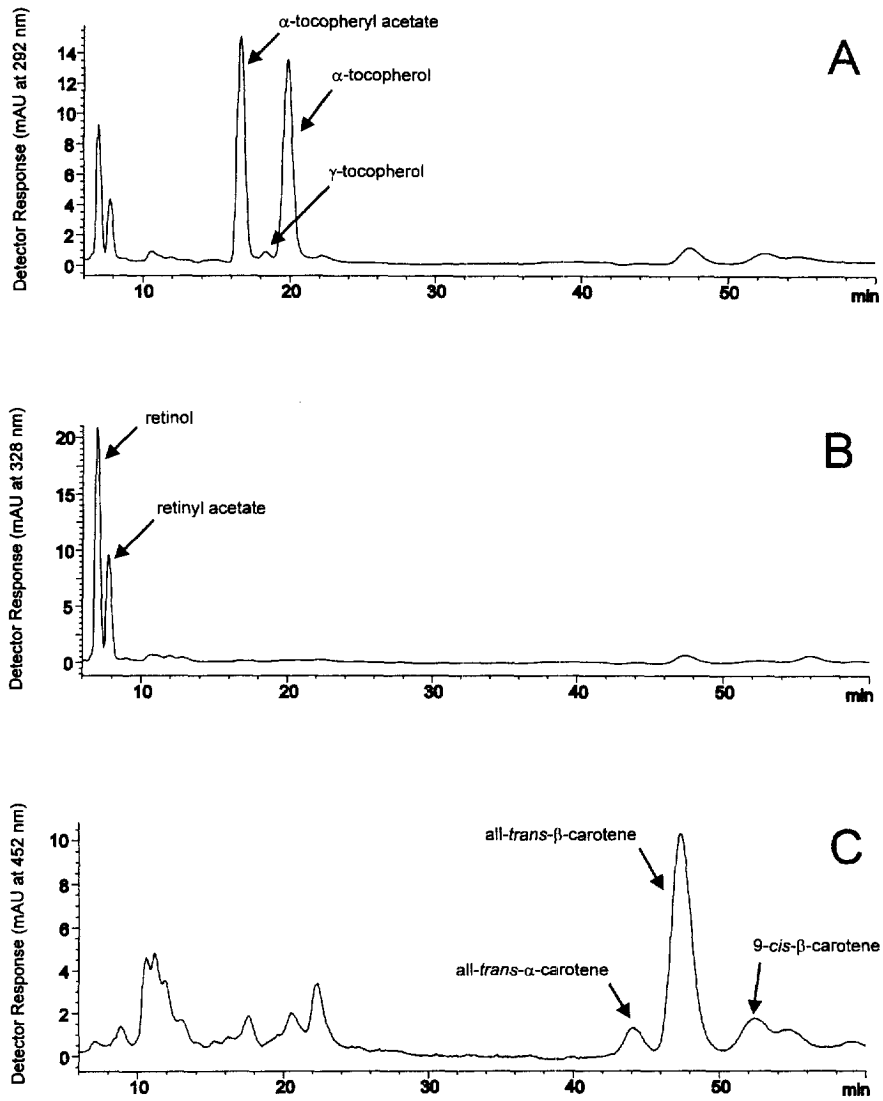

Fig. 3. Chromatograms of a human plasma extract (12 h post-consumption of 60 mg Betatene) concurrently generated from the absorptions at 292 nm (A), 328 nm (B) and 452 nm (C) with a photodiode array detector. HPLC conditions: see Fig. 2.

areas for the analytes might be explained by absorption differences of the compounds in ethanol as compared to the mobile phase.

Three capsules, containing Betatene 20% (manufacturer assayed to contain 15 mg algal-derived β-carotene in soybean oil), were sliced in half to provide a release of the contents and then each was added to 10.0 ml hexane–toluene (1:1, v/v) containing 5 mg BHT. Aliquots were diluted in ethanol and concentrations of the carotenes were calculated from spectrophotometric absorption readings. The aliquots also were analyzed by the described HPLC method. The concentration of β-carotene in the capsules was determined to be 14.61 mg ($n=6$, −2.63% error based on the nominal value of 15 mg) by spectrophotometric absorption readings. HPLC analyses confirmed the concentration to be 14.15 mg ($n=12$, R.S.D. 4.6%).

The described HPLC method provides chromatographic separation and resolution of α-carotene, all-

trans-β-carotene and the mono-*cis* geometrical isomers of β-carotene, as well as α-tocopherol, γ-tocopherol, α-tocopheryl acetate, retinol and retinyl acetate. Although photodiode array detection is satisfactory for the quantification of the plasma components, as well as identification of the carotene isomers, the specificity of detection can be increased by use of an in-line connected mass spectrometer.

Coupled with the sensitive and informative mass spectrometric detector, the described liquid chromatographic method could be used to provide answers concerning the uptake, transport and regulation of isotopically-labeled tocopherols, carotenoids and retinoids. For example, retinol, retinyl acetate, α-tocopherol, γ-tocopherol, α-tocopheryl acetate, α-carotene and the geometrical isomers of β-carotene can be separated and quantified by HPLC, and then, $^{2}H_{8}$-labeled and unlabeled β-carotene isomers can be detected and quantified by selected anion monitoring mass spectrometry [16].

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