Separation and Identification of Carotenoids and Their Oxidation Products in the Extracts of Human Plasma

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Eighteen carotenoids as well as vitamin A and two forms of vitamin E (γ- and α-tocopherol) have been separated from extracts of human plasma by high-performance liquid chromatography (HPLC) on reversed-phase and silica-based nitrile-bonded columns. In the order of chromatographic elution on a C_{18} reversed-phase column, the carotenoids were identified as (3*R*,3′*R*,6′*R*)-β,ϵ-carotene-3,3′-diol [(3*R*,3′*R*,6′*R*)-lutein], (3*R*,3′*R*)-β,β-carotene-3,3′-diol [(3*R*,3′*R*)-zeaxanthin], 5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene, 3-hydroxy-2′,3′-didehydro-β,ϵ-carotene, β,ϵ-caroten-3-ol, 3-hydroxy-β-carotene, ψ,ψ-carotene, 7,8-dihydro-ψ,ψ-carotene, β,ψ-carotene, 7,8,7′,8′-tetrahydro-ψ,ψ-carotene, β,ϵ-carotene, β,β-carotene, 7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene, and 7,8,11,12,7′,8′,-11′,12′-octahydro-ψ,ψ-carotene. The polar carotenoids, which eluted in the vicinity of lutein and were unresolved on the C_{18} column, have been separated on a nitrile-bonded column employing isocratic HPLC conditions. In the order of elution, the carotenoids were ϵ,ϵ-carotene-3,3′-dione, 3′-hydroxy-ϵ,ϵ-caroten-3-one, 5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene, 3-hydroxy-β,ϵ-caroten-3′-one, (*all-E*,3*R*,3′*R*,6′*R*)-lutein, (*all-E*,3*R*,3′*R*)-zeaxanthin, and (*all-E*,3*R*,3′*S*,6′*R*)-β,ϵ-carotene-3,3′-diol (3′-epilutein) followed by several geometrical isomers of lutein and zeaxanthin.

INTRODUCTION

In the past decade a number of foods and food constituents have been studied for their inhibitory effect on carcinogenesis.[1] Results of these studies have shown an inverse relationship between the consumption of certain fruits and vegetables and the risk of epithelial cancer.[2,3] Since carotenoids are among the micronutrients found in cancer-preventive foods, detailed qualitative and quantitative determination of these compounds, particularly in fruits and vegetables and in human plasma, have recently become increasingly important.

High-performance liquid chromatography (HPLC) has been employed as a powerful technique to quantify low levels and various forms of carotenoids in foods and human plasma. One of the original separations of carotenoids from an extract of human serum was reported by Nelis and De Leenheer[4] who developed nonaqueous reversed-phase chromatographic conditions on a Zorbax ODS column, with a mixture of acetonitrile (70%), dichloromethane (20%), and methanol (10%) as eluent. Under these conditions six carotenoids were separated and identified in an extract from human serum. Bieri et al.[5] employed this chromatographic procedure to separate carotenoids from extracts of human plasma on a C_{18} reversed-phase HPLC column. In addition to the six carotenoids reported by Nelis and De Leenheer, Bieri and coworkers reported the presence of two unidentified carotenoids in human plasma. Many other researchers have similarly developed nonaqueous reversed-phase HPLC conditions employing a variety of organic solvents and various HPLC columns.[6-11]

From our extensive studies on the distribution of carotenoids in common fruits and vegetables, it appeared that the number of available dietary carotenoids that may be absorbed, metabolized, and/or utilized by the human body is in excess of 40.[12] The objective of this research was to determine if human plasma contains carotenoids which were previously undetected. We describe the separation, identification, and quantification of 18 carotenoids as well as vitamin A and two forms of vitamin E (γ- and α-tocopherol) from extracts of human plasma. Among plasma carotenoids, we have separated and identified several ketocarotenoids which

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may be products of oxidation of naturaly occurring carotenoids.

EXPERIMENTAL SECTION

Chromatographic Procedures. HPLC System A. A Beckman Model 114M ternary solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Hewlett-Packard (HP) 1040A rapid-scanning UV/visible photodiode array detector. The data were stored and processed by a HP 9000/Series 300 (Chem-Station) computing system, in combination with a HP Model 9153B disk drive, color display monitor, Model 35741, and a Model 7470A plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at a rate of 12 spectra/min.

Column. Analytical separations with HPLC system A were carried out on a Microsorb (25-cm-length × 4.6-mm-i.d.) C_{18} (5-μm spherical particles) column (Rainin Instrument Co., Wouburn, MA), which was protected with a Brownlee guard cartridge (3-cm length × 4.6-mm i.d.) packed with spheri-5-C_{18} (5-μm particle size). The semipreparative separations were carried out on a Rainin stainless-steel (25-cm-length × 10-mm-i.d.) Microsorb C_{18} column (5-μm spherical particles).

Eluent A. Analytical and semipreparative separations with HPLC system A were achieved with a combination of isocratic and gradient chromatography. An isocratic mixture of acetonitrile (85%), methanol (10%), dichloromethane (2.5%), and hexane (2.5%) at time 0 was followed by a linear gradient beginning at time 10 min and completed at time 40 min. The final composition of the gradient mixture was acetonitrile (45%), dichloromethane (22.5%), hexane (22.5%), and methanol (10%). The column flow rate for analytical and semipreparative separations was 0.70 and 2 mL/min, respectively. At the end of the gradient, the column was reequilibrated for 15 min under the initial isocratic conditions. With this eluent the HPLC separations were monitored and optimized at five different wavelengths (470, 445, 400, 350, and 290 nm) simultaneously to ensure the detection of all components in the plasma extracts.

HPLC System B. A Beckman Model 114M single solvent delivery system equipped with a Beckman Model 421 controller was interfaced into a Waters 990 rapid-scanning UV/visible photodiode array detector. The data was stored and processed by means of a NEC APC IV computing system, which was operated with a color display monitor, Model APC-H431, and a Waters 990 plotter. The absorption spectra of the carotenoids were recorded between 200 and 600 nm at a rate of 12 spectra/min.

Column. Analytical separations with HPLC system B were carried out on a silica-based nitrile bonded (25-cm-length × 4.6-mm-i.d.; 5-μm spherical particle) column (Regis Chemical Co., Morton Grove, IL), which was protected with a Brownlee nitrile-bonded guard cartridge (3-cm length × 4.6-mm i.d.; 5-μm particle size). The semipreparative separations were carried out on a Regis stainless-steel (25-cm-length × 10-mm-i.d.) nitrile-bonded column (5-μm particle size).

Eluent B. Analytical and semipreparative separations with HPLC system B were achieved with an isocratic mixture of hexane (74.65%), dichloromethane (25.00%), methanol (0.25%), and *N,N*-diisopropylethylamine (0.10%). The column flow rates for analytical and semipreparative separations were 1 and 2.5 mL/min, respectively. For reproducible separations with this eluent, accurate composition of each solvent, particularly that of methanol, was maintained by preparing this HPLC eluent as needed. This is because of the volatility of hexane and dichloromethane which may result in gradual evaporation of these solvents when stored in loosely capped HPLC containers. The monitoring wavelengths with this eluent were 445 and 325 nm.

Apparatus. Absorption spectra of the carotenoids in various solvents were recorded on a Beckman DU-7 UV/visible spectrophotometer. Mass spectra were obtained from a Finnigan-MAT Model 4510 mass spectrometer (San Jose, CA) equipped with an INCOS data system and a direct-exposure probe which was heated by the application of current from 0 to 1000 mA at a rate of 50 mA/s. Desorption chemical ionization (DCI) spectra were obtained, employing ammonia and methane as the reagent gas at a source block temperature of 60 °C. Electron capture negative ionization (ECNI) mass spectra were produced by using methane as a buffer gas at an indicated pressure of 0.3 Torr. For negative ion spectra, the ionizing chamber was maintained at 60 °C and spectra were collected from m/z 45 to 650. Mass spectra were also obtained with an LC/MS system consisting of a Hewlett-Packard 1090L chromatograph (high-performance liquid chromatograph and a photodiode array detector) and a Hewlett-Packard Model 5989A mass spectrometer interconnected by a particle beam interface. A nitrile-bonded column and eluent B at a flow rate of 0.7 mL/min was used to separate analytes with this LC/MS system. The flow rate with the LC/MS interface system was slightly lower than the flow rate of 1.0 mL/min which was used in routine analysis of plasma with the nitrile-bonded column. Eluate from the HPLC was divided with a ratio of 1:3 with the lesser amount entering the particle beam interface which was operated at a desolvation temperature of 45 °C. Electron capture ionization was achieved using methane at a pressure of 0.85 Torr and a source temperature of 320 °C. Spectra were collected from m/z 100 to 700 using a scan cycle time of 1.5 s.

Reagents and Materials. The reference sample of ε,ε-carotene-3,3′-dione was synthesized according to the procedure described by Widmer et al.[13] 3′-Hydroxy-ε,ε-caroten-3-one, 3-hydroxy-β,ε-caroten-3′-one, and 2′,3′-anhydrolutein were prepared according to the procedures to be described in text. 3-Hydroxy-β,ε-caroten-3′-one was also prepared according to a published procedure.[14] (3*R*,3′*S*,6′*R*)-β,ε-Carotene-3,3′-diol (3′-epilutein) was prepared from natural lutein [(3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol] as published by Buchecker et al.[15] 5,6-Dihydroxy-5,6-dihydro-ψ,ψ-carotene was prepared from the reaction of lycopene with Micro-Cel C (synthetic calcium silicate, Manville Products Corp., Denver, CO) according to the method described by Ritacco et al.[16] A pure sample of this compound was also isolated and characterized from an extract of tomato paste.[17] Lutein was isolated from fruits and vegetables according to published procedures.[12] Reference samples of zeaxanthin and β,ψ-carotene (γ-carotene) were provided by Hoffmann-La Roche, Basel, Switzerland. 2′,3′-β,ε-Caroten-3-ol (α-cryptoxanthin) and 3-hydroxy-β-carotene (β-cryptoxanthin) were synthesized from condensation of (3*R*)-3-hydroxy-12′-apo-β-caroten-12′-al[18] with (α-ionylideneethyl)triphenylphosphonium chloride and (β-ionylideneethyl)triphenylphosphonium chloride, respectively.[19] {(2*E*,4*E*)-5-[(*R*)-4-Hydroxy-2,6,6-trimethylcyclohex-1-enyl]-3-methylpenta-2,4-dienyl}triphenylphosphonium chloride, a precursor for the synthesis of (3*R*)-3-hydroxy-12′-apo-β-caroten-12′-al, was provided by Hoffmann-La Roche, Basel, Switzerland.[20,21] Lycopene was isolated from extracts of tomato paste and raw tomatoes according to published procedures.[17,22] ξ-Carotene, phytofluene, and phytoene were isolated from the extracts of dried apricots and peaches by semipreparative TLC and HPLC.[23] Neurosporene and ξ-carotene were also synthesized according to the method of Davis et al.[24] The reference samples of (*all-E*)-α-carotene (Sigma, St. Louis, MO) and synthetic (*all-E*)-β-carotene (Aldrich Chemical Co., Milwaukee, WI) were further purified by preparative TLC (silica gel, petroleum ether/ethyl acetate, 49/1) and recrystallization (dichloromethane/methanol),

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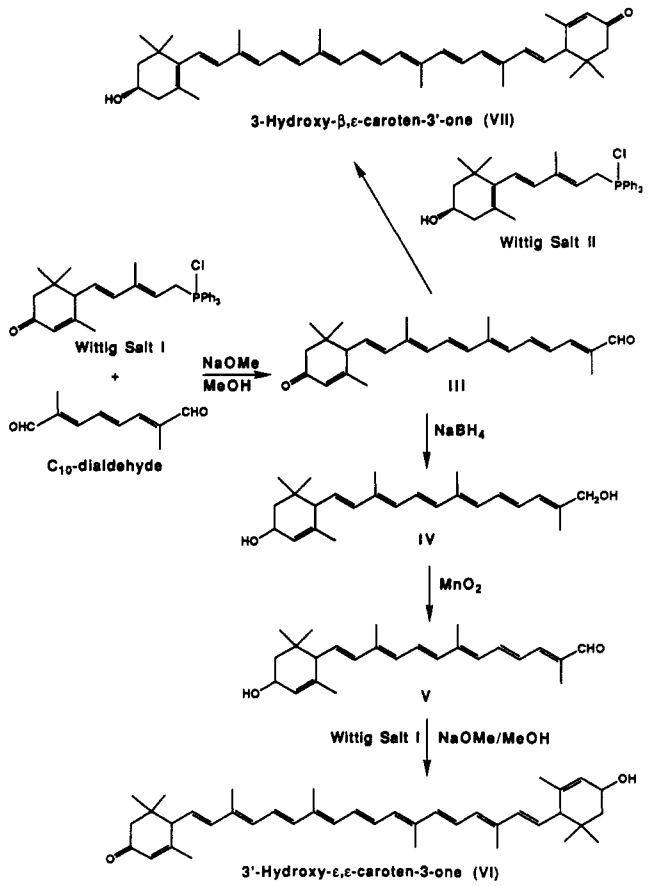

Figure 1. Chemical synthesis of (3′*RS*,6′*RS*,6*RS*)-3′-hydroxy-ε,ε-caroten-3-one and (3*R*,6′*RS*)-3-hydroxy-β,ε-caroten-3′-one.

respectively. Reference sample of cholesteryl oleate was obtained from Sigma Inc. (St. Louis, MO). Ethyl β-apo-8′-carotenoate (Fluka Chemical Co., New York, NY) was employed as one of the internal standards and was used without further purification. (3*R*)-8′-Apo-β-carotene-3,8′-diol (β-citraurinol), which was employed as a second internal standard, was prepared from the reduction of (3*R*)-3-hydroxy-8′-apo-β-caroten-8′-al (β-citraurin) according to the procedure reported by Pfander et al.[18] Retinol and γ- and α-tocopherol were obtained from Eastman Kodak Co. (Rochester, NY). The purities of the internal standards and the carotenoid reference samples were checked by HPLC as well as comparison of their absorptivity data in various solvents with those of published values.[25] When necessary, the samples were further purified by semipreparative TLC and HPLC. HPLC-grade solvents, methanol, acetonitrile, dichloromethane, hexane (Fisher Scientific, Pittsburgh, PA), and *N*,*N*-diisopropylethylamine (Aldrich Chemical Co., Milwaukee, WI) were used without further purification. Ethyl ether was stabilized by adding 1 ppm 2,6-di-*tert*-butyl-*p*-cresol as a peroxide inhibitor.

Synthesis of Several of the Plasma Carotenoids. 1. (3′*RS*,6′*RS*,6*RS*)-3′-Hydroxy-ε,ε-caroten-3-one. 3′-Hydroxy-ε,ε-caroten-3-one, with three asymmetric centers can exist as a mixture of eight diasteriomeric isomers. Among the six of the optically pure carotenoid end groups required for the synthesis of all of the configurational isomers of this compound, only a few have been prepared by elaborate synthetic methods.[26,27] Therefore in the present study a simple and routine procedure common for the synthesis of carotenoids has been employed which results in the formation of a mixture of diasteriomeric isomers of 3′-hydroxy-ε,ε-caroten-3-one according to the scheme shown in Figure 1. In addition to configurational isomers, the synthesis of carotenoids by Wittig type condensation (Figure 1) also resulted in a mixture of geometrical (*all-E* + *Z*) isomers for carotenoids and their precursors. No attempt was made to affect conversion of *Z*-carotenoids to their *all-E* counterparts by thermal isomerization.

Condensation of [(2′*E*,4′*E*)-5′-(2,6,6-trimethyl-4-oxo-2-cyclohexen-1-yl)-3′-methyl-2′,4′-pentadienyl]triphenylphosphonium chloride [Wittig salt I][13] with 2,7-dimethylocta-2,4,6-triene-1,8-dial [C_{10}-dialdehyde][28] gave 3-oxo-12′-apo-ε-caroten-12′-al (III), which was hydrogenated with sodium borohydride to yield 12′-apo-ε-carotene-3,12′-diol (IV). Selective oxidation of this compound with activated manganese dioxide produced 3-hydroxy-12′-apo-ε-caroten-12′-al (V), which on subsequent reaction with Wittig salt (I) gave the desired compound, 3′-hydroxy-ε,ε-caroten-3-one (VI). The detailed procedures for the synthesis of the intermediates III, IV, and V and the final product VI are described.

(a) 3-Oxo-12′-apo-ε-caroten-12′-al (III). A mixture of Wittig salt I (0.051 g, 0.20 mmol) and C_{10}-dialdehyde (0.033 g, 0.20 mmol) in dichloromethane (20 mL) was treated with NaOMe/MeOH (0.006 g of Na) at −20 °C under an atmosphere of N_2. C_{10}-Dialdehyde, HPLC (eluent A, HPLC system A) t_R = 5.732 min, λ_{max} = 326, 340 nm; product III, HPLC t_R = 7.229 min, λ_{max} = 410 nm in the HPLC solvents. The crude product was washed with water (3 × 30 mL), dried (Na_2SO_4), and concentrated. Preparative thin-layer chromatography (TLC) on *n* silica gel GF (Analtech, Newark, DE) plates [1000-μm thickness, petroleum ether/acetone (4/1)] gave 3-oxo-12′-apo-ε-caroten-12′-al (0.066 g, 0.18 mmol, 92%). UV/visible (nm): dichloromethane, λ_{max} = 414; acetone, λ_{max} = 405.5; ethanol λ_{max} = 411. Mass spectrum (MS) [ECNI, methane]: molecular anion peak at *m*/*z* = 364 (100). MS (DCI, methane): protonated molecular ions at *m*/*z* = 365 $(M + H)^+$, 393 $(M + C_2H_5)^+$, 405 $(M + C_3H_5)^+$.

(b) 12′-Apo-ε-carotene-3,12′-diol (IV). A solution of 3-oxo-12′-apo-ε-caroten-12′-al (0.050 g, 0.14 mmol) in 20 mL of tetrahydrofuran (THF) and 5 mL of water was stirred with sodium borohydride (0.006 g, 0.16 mmol) under an atmosphere of N_2 at 0 °C for 1.5 h. The product was partitioned between CH_2Cl_2 (30 mL) and water (25 mL). The organic layer was washed with water (3 × 30 mL), dried (Na_2SO_4), and concentrated to give 12′-apo-ε-carotene-3,12′-diol (0.048 g, 0.13 mmol; 93%). HPLC (eluent A) t_R = 6.836 min, λ_{max} = 356, 374, 394 nm in the HPLC solvents. UV/visible (nm): dichloromethane, λ_{max} = 395.5, 379.5, 400; acetone, λ_{max} = 355, 373.5, 395.5; ethanol, λ_{max} = 352.5, 371.5, 395. MS (ECNI, methane): molecular anion peak at *m*/*z* = 368 (100). MS (DCI, methane): protonated molecular ions at *m*/*z* = 369 [40, $(M + H)^+$], 351 [100, $(M + H - H_2O)^+$], 333 [10, $(M + H - 2H_2O)^+$].

(c) 3-Hydroxy-12′-apo-ε-caroten-12′-al (V). A solution of 12′-apo-ε-carotene-3,12′-diol (0.040 g, 0.11 mmol) in tetrahydrofuran (10 mL) was stirred in the presence of activated manganese dioxide (0.30 g, 3.45 mmol) under an atmosphere of N_2 at room temperature for 4 days. Diol (IV) [HPLC (eluent A) t_R = 6.836 min, λ_{max} = 356, 374, 394 nm in the HPLC solvents] (5% unreacted); product (V) [HPLC t_R = 7.647 min, λ_{max} = 412 nm in the HPLC solvents]. The product was filtered through Celite, and THF was removed on a rotary evaporator. Preparative TLC [*n* silica gel GF plates (1000-μm thickness), petroleum ether/acetone (2/1)] gave 3-hydroxy-12′-apo-ε-caroten-12′-al (0.034 g, 0.09 mmol, 82%). UV/visible (nm): dichloromethane, λ_{max} = 415.5; acetone, λ_{max} = 406.5, petroleum ether, λ_{max} = 401.5, 425; ethanol, λ_{max} = 412.5. Mass spectrum (DCI, ammonia): ammonium adduct ions at *m*/*z* 384 [100, $(M + NH_4)^+$] and 401 [30, $(M + NH_3 + NH_4)^+$].

(d) 3′-Hydroxy-ε,ε-caroten-3-one (VI). A solution of 3-hydroxy-12′-apo-ε-caroten-12′-al (V) (0.020 g, 0.05 mmol) and Wittig salt I (0.015 g, 0.06 mmol) in CH_2Cl_2 (20 mL) was treated with NaOMe/MeOH (0.0015 g of Na) at −20 °C under an atmosphere of N_2. The product was worked up as described in preparation of product III. The crude product contained 3-hydroxy-12′-apo-ε-caroten-12′-al [90%, HPLC (eluent A) t_R = 7.647 min, λ_{max} = 412 nm)] and product VI [10% (unreacted), HPLC t_R = 8.851 min, λ_{max} = 418, 440, 468 nm in the HPLC solvents]. Preparative TLC [*n* silica gel GF plates (1000-μm thickness), petroleum ether/acetone (4/1), R_f = 0.35] gave 3′-hydroxy-ε,ε-caroten-3-one (0.026

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g, 0.046 mmol, 92%). UV/visible (nm): chloroform, λ_{max} = (inflection 425), 448.5 ($E^{1\%}$ = 2685), 478; CH_2Cl_2, λ_{max} = (425.5–427), 447, 476.5; acetone, λ_{max} = (421–421.5), 440, 469; ethanol, λ_{max} = (420), 439, 468. MS (ECNI, methane): molecular anion peak at m/z = 566 (100) and an anion peak at m/z = 548 [40, (M – H_2O)$^-$].

2. (3*R*,6′*RS*)-3-Hydroxy-β,ε-caroten-3′-one. This compound was prepared from condensation of 3-oxo-12′-apo-ε-caroten-12′-al (III) and {(2*E*,4*E*)-5-[(*R*)-4-hydroxy-2,6,6-trimethylcyclohex-1-enyl]-3-methylpenta-2,4-dienyl}triphenylphosphonium chloride[20,21] (in this text referred to as Wittig salt II) according to the scheme outlined in Figure 1. In another experiment (3*R*,6′*R*)-3-hydroxy-β,ε-caroten-3′-one was prepared by partial synthesis from oxidation of (3*R*,3′*R*,6′*R*)-lutein with nickel peroxide according to the method reported by Liaaen-Jensen.[14]

A solution of 3-oxo-12′-apo-ε-caroten-12′-al (III) (0.022 g, 0.06 mmol) and Wittig salt (II) (0.033 g, 0.06 mmol) in CH_2Cl_2 (25 mL) was treated with NaOMe/MeOH (0.0016 g of Na) at −20 °C under an atmosphere of N_2. The product was worked up as described in preparation of product III. The crude product contained 3-oxo-12′-apo-ε-caroten-12′-al [10% (unreacted) HPLC (eluent A) t_R = 7.229 min, λ_{max} 410 nm] and product VII [90%, HPLC t_R = 11.526 min, λ_{max} = (inflection 425), 446, 474 nm in the HPLC solvents]. Preparative TLC [*n* silica gel GF plates (1000-μm thickness), petroleum ether/acetone (4/1)] gave (3*R*,6′*RS*)-3-hydroxy-β,ε-caroten-3′-one (0.031 g, 0.055 mmol, 92%). UV/visible (nm): acetone, λ_{max} = (inflection 424), 446, 474 [lit.[14] λ_{max} = (425), 449 ($E^{1\%}$ = 2425), 477]; hexane, λ_{max} = 267.5, 334.5, (422–422.5), 443, 471.5 [lit.[29] λ_{max} = 267, 334, (421), 445, 472]; CH_2Cl_2, λ_{max} = (430), 453, 481. MS (ECNI, methane): molecular anion peak at m/z = 566 (100).

A sample of 3-hydroxy-β,ε-caroten-3′-one prepared by partial synthesis from oxidation of lutein with nickel peroxide[14] was shown from its HPLC retention time, UV/visible absorption, and mass spectra to be identical with the synthetic compound described above.

3. (3*R*,3′*S*,6′*R*)-β,ε-Carotene-3,3′-diol (3′-Epilutein). This compound was prepared according to the general scheme outlined by Buchecker et al.[15] and Mayer.[26] A solution of (3*R*,6′*R*)-3-hydroxy-β,ε-caroten-3′-one [prepared from allylic oxidation of (3*R*,3′*R*,6′*R*)-lutein with nickel peroxide[14]] in tetrahydrofuran and water was reduced with sodium borohydride as described in preparation of product IV. Semipreparative HPLC (eluent B, HPLC system B) gave (3*R*,3′*R*,6′*R*)-lutein (HPLC t_R = 28.58 min) and (3*R*,3′*S*,6′*R*)-β,ε-carotene-3,3′-diol (3′-epilutein)/[HPLC t_R = 35.41 min] which were identified from their UV/visible absorption, NMR, mass, and CD (circular dichroism) spectra.[15,29]

4. 2′,3′-Anhydrolutein. 2′,3′-Anhydrolutein and 3,4-anhydrolutein were the major products from the reaction of lutein bispalmitate with phosphorus oxychloride, followed by alkaline hydrolysis.

A solution of lutein bispalmitate [0.031 g, 0.03 mmol][30] and phosphorus oxychloride (10 μL) in hexane (30 mL) was stirred at temperature under an atmosphere of N_2. According to HPLC (eluent A) of the crude product after 24 h, no lutein bispalmitate [t_R = 59.290 min, λ_{max} = (422), 448, 476 nm in the HPLC solvents] remained unreacted and products with HPLC retention times of 45.938 and 46.951 min were formed. The crude product was washed with water (4 × 40 mL), dried (Na_2SO_4), and concentrated. Preparative TLC [*n* silica gel GF plates (1000-μm thickness, petroleum ether/acetone (19/1)] gave a main yellow zone (R_f = 0.8) which was isolated and used without further purification in the next step.

An analytical sample of the above mixture of products was purified by preparative HPLC (eluent A) to give 2′,3′-anhydrolutein palmitate (70%) and 3,4-anhydrolutein palmitate (30%) [total yield of the isomeric mixture: 0.020 g, 0.025 mmol; 83%].

2′,3′-Anhydrolutein Palmitate. HPLC (eluent A) [t_R = 45.938 min, λ_{max} = (424), 446, 474 nm] showed the presence of about 25% of a central *Z*-isomer [t_R = 46.470 min, λ_{max} = 332 (intense *Z*-peak), (420), 440, 468 nm]. UV/visible (nm): hexane, λ_{max} = 330, (420), 440.5, 469; petroleum ether, λ_{max} = 330, (419), 439, 467; chloroform, λ_{max} = 338, (432), 452, 480.5; ethanol, λ_{max} = 330, (422), 442, 469.5. MS (ECNI, methane): molecular anion peak at m/z = 788 (100).

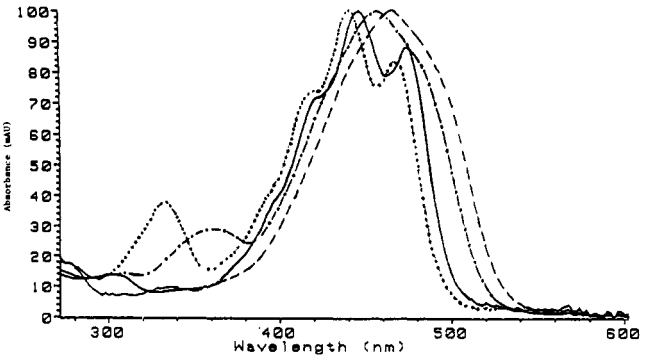

Figure 2. UV/visible absorption spectra of (*all-E*)-2′,3′-anhydrolutein [(—) λ_{max} = (424), 446, 474 nm], (*Z*)-2′,3′-anhydrolutein [(···) λ_{max} = (420), 440, 468 nm, *Z*-peak at 332 nm], (*all-E*)-3,4-anhydrolutein [(- - -) λ_{max} = 464 nm], and (*Z*)-3,4-anhydrolutein [(- · -) λ_{max} = 450–452 nm, *Z*-peak at 356 nm] monitored in the HPLC eluent A.

3,4-Anhydrolutein Palmitate. HPLC (eluent A) [t_R = 46.951 min, λ_{max} = 464 nm] showed the presence of about 15% of a central *Z*-isomer [t_R = 47.483 min, λ_{max} = 360 (intense cis peak), 456 nm]. UV/visible (nm): hexane, λ_{max} = 460; petroleum ether, λ_{max} = 460; chloroform, λ_{max} = 475; ethanol, λ_{max} = 465. MS (ECNI, methane): molecular anion peak at m/z = 788 (100).

Alkaline Hydrolysis. The isomeric mixture of 2′,3′-anhydrolutein palmitate and 3,4-anhydrolutein palmitate (0.020 g, 0.025 mmol) from above in tetrahydrofuran (15 mL) was treated with 10 mL of methanolic KOH (10%) under an atmosphere of N_2 for 2 h at room temperature. The product was partitioned into a 5% solution of sodium chloride (30 mL) and dichloromethane (30 mL), and the organic layer was removed. The organic layer was washed with water (3 × 30 mL), dried (Na_2SO_4), and concentrated to give an isomeric mixture of 2′,3′-anhydrolutein (70%) and 3,4-anhydrolutein (30%) [total yield: 0.0135 g, 0.025 mmol; 83% from lutein bispalmitate]. Purification by preparative HPLC (eluent A) gave the following:

2′,3′-Anhydrolutein. HPLC t_R = 22.577 min [λ_{max} = (424), 446, 474 nm] showed the presence of about 25% of a central *Z*-isomer (Figure 2) [t_R = 23.286 min, λ_{max} = 332 (intense *Z*-peak), (420), 440, 468 nm]. UV/visible (nm): hexane, λ_{max} = 330, (420), 440.5 ($E^{1\%}$ = 2460), 468 [lit.[30] λ_{max} = 331, (421), 442.5, 470.5]; petroleum ether, λ_{max} = 330, (419), 438.5, 467; chloroform, λ_{max} = 338, (432), 452, 479.5; ethanol, λ_{max} = 330, (422), 442, 468.5 [lit.[30] λ_{max} = 332, (422), 444, 471.5]. MS (ECNI, methane): molecular anion peak at m/z = 550 (100) and an anion peak at m/z = 532 [5, (M – H_2O)$^-$].

3,4-Anhydrolutein. HPLC t_R = 24.519 min (λ_{max} = 464 nm) showed the presence of about 15% of a central *Z*-isomer (see Figure 4) [t_R = 25.085 min, λ_{max} = 360 (intense *Z*-peak), 456 nm]. UV/visible (nm): hexane, λ_{max} = 460 [lit.[31] λ_{max} = 460]; petroleum ether, λ_{max} = 460 [lit. λ_{max} = 458–459;[32] λ_{max} = 460[33]]; chloroform, λ_{max} = 475 [lit.[33] λ_{max} = 475]; ethanol, λ_{max} = 465 [lit.[33] λ_{max} = 465]. MS (ECNI, methane): molecular anion peak at m/z = 550 (100) and an anion peak at m/z = 532 [15, (M – H_2O)$^-$].

Source of Human Plasma. Five healthy Caucasian males between the ages of 35 and 55 donated blood after fasting for the present study. These subjects had no history of chronic disease, were not taking any medication, were nonsmokers, and had no particular dietary patterns (e.g., vegetarianism). Blood samples were extracted and analyzed immediately after collection.

Extraction of Human Plasma. For a detailed HPLC analysis of plasma carotenoids, the volume of plasma needed for extraction is in the range of 1–3 mL. This is mainly due to the fact that

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the photodiode array detectors that were used to monitor the HPLC separation of the various classes of carotenoids at several wavelengths simultaneously are less sensitive than single wavelength detectors. Recently we have shown in our laboratory that modified photodiode array detectors can improve the sensitivity for the detection of carotenoids by HPLC (data will be published elsewhere). As a result, detailed carotenoid analysis of human plasma by HPLC may be obtained from extraction of 0.5 mL of plasma with such detectors.

All of the laboratory procedures were carried out under gold fluorescent lights and minimum exposure of the samples to air to avoid stereomutation and photodegradation of carotenoids. During collection, extraction, and handling of the blood samples, laboratory workers employed protective measures (i.e. gloves and eye glasses) to avoid contact of the samples with skin and eye. In a typical extraction, blood (5 mL) was collected in a 5-mL Vacutainer tube (Becton Dickenson Vacutainer Systems, Rutherford, NJ) containing 4.5 units of sodium or lithium heparin per milliliter of whole blood. The tube was centrifuged at 1200*g* for 10 min. The plasma (about 2.5 mL) was removed and transferred into a 20-mL culture tube. Ethanol (2.5 mL) and the two internal standards, ethyl β-apo-8′-carotenoate (0.045 μg) and (3*R*)-8′-apo-β-carotene-3,8′-diol (0.24 μg), were added; the tube was shaken vigorously for 20 s. The mixture was centrifuged for 5 min (1200*g*), treated with diethyl ether (5 mL), and shaken vigorously to break up the precipitated proteins into fine particles. The mixture was centrifuged for 5 min, the solution was removed, and the solid was further extracted with 2 × 5 mL of ether. The combined ethereal layers were washed with 5% NaCl solution (15 mL), dried over sodium sulfate, and evaporated to dryness on a rotary evaporator at 35 °C. The colored residue was dissolved in a small volume (1–2 mL) of dichloromethane and filtered through a 0.45-μm disposable filter assembly (Baxter, Scientific Products Division, McGaw Park, IL). The volume of the dichloromethane was reduced under a stream of nitrogen. The concentrated extract was transferred into a 1-mL graduated micro sample vial (Baxter, Scientific Products Division, McGaw Park, IL) and evaporated to dryness. HPLC solvents (eluent B) were added to the remaining oil until the total volume of the plasma extract was 100 μL. Samples (20 μL) were injected in duplicate for analysis on each of the HPLC systems A and B. Two consecutive extractions and analyses were carried out for each of the subjects according to the above procedure. Eluent B was found to be an appropriate injection solvent for the HPLC analyses of carotenoids in human plasma on both systems A and B and did not produce chromatographic artifacts.[34]

Large-Scale Extraction of Human Plasma. Four units of human plasma (ca. 880 mL, American Red Cross) were combined in a 2-L beaker and treated with 800 mL of ethanol, and the resulting white slurry was stirred at room temperature for 15 min. The white precipitate of proteins were collected on a Buchner funnel, and the ethanolic filtrate was saved for further workup. The precipitated protein were homogenized with tetrahydrofuran (800 mL) in a Waring blender immersed in an ice bath for 20 min, and the extract was filtered under suction. The solid materials were reextracted with tetrahydrofuran until the resulting filtrate was colorless. The filtrate was combined with the initial ethanolic filtrate from above, and the solvents were removed on a rotary evaporator under reduced pressure at 35 °C. The concentrated plasma extract was partitioned between dichloromethane (400 mL) and 400 mL of water (containing 5% NaCl). The water layer was washed with dichloromethane (2 × 100 mL), and the resulting organic layers were combined, dried (Na_2SO_4), and evaporated to dryness. The residue was dissolved in 40 mL of light petroleum ether and diethyl ether (1:1 v/v) and kept overnight under nitrogen at −20 °C. The precipitated lipid materials were removed by centrifugation, and this process was repeated twice to free the extract from the lipid materials. The solvents were removed, and the residue was dissolved in dichloromethane (10 mL) and filtered through a 0.45-μm disposable filter assembly (American Scientific Products, McGraw Park, IL). The volume of dichloromethane was reduced by a stream of nitrogen, and the concentrated plasma extract was used for isolation and purification of carotenoids by preparative thin-layer chromatography.

Isolation and Characterization of Carotenoids, Retinol, and Vitamin E in Human Plasma. The concentrated human plasma extract from above was chromatographed on C_{18} reversed-phase plates (20 × 20 cm, layer thickness 1000 μm; Whatman Chemical Separation Inc.) employing acetonitrile (65%), dichloromethane (12.5%), hexane (12.5%), and methanol (10%) as eluent. Three main bands were separated.

Band One. The first band (R_f = 0.65, 4-cm width) was shown by HPLC (eluent B) to consist of eight major and several minor components which were separated by semipreparative HPLC (eluent B). In the order of chromatographic elution (see Table I for HPLC peak identification) these components were characterized as follows.

Major Components of Band One: Retinol. UV Absorption maximum (nm): ethanol, λ_{max} = 325 [lit.[35] λ_{max} = 326–328]. MS (ECNI, methane): molecular anion peak at *m/z* = 286 (100).

ε,ε-Carotene-3,3′-dione (1). UV/visible (nm): chloroform, λ_{max} = 427, 452, 483 [lit.[13] λ_{max} = 405, 427, 452 ($E^{1\%}$ = 2670), 483]. MS (ECNI, methane): molecular anion peak at *m/z* = 564 (100).

3′-Hydroxy-ε,ε-caroten-3-one (2). UV/visible (nm): dichloromethane, λ_{max} = (426), 447, 476.5; acetone, λ_{max} = (421), 440, 469, MS (ECNI, methane): molecular anion peak at *m/z* = 566 (100) and an anion peak at *m/z* = 548 [40, $(M - H_2O)^-$].

5,6-Dihydroxy-5,6-dihydro-ψ,ψ-carotene (3 + 3′). HPLC (eluent A) showed the presence of a *Z*-isomer (see Table I). UV/visible (nm) absorption spectrum of the mixture (**3** + **3′**): petroleum ether, λ_{max} = 430, 455, 483 [lit.[16] λ_{max} = 430, 454, 483]; hexane, λ_{max} = 430, 455, 483 [lit.[36] λ_{max} = 431, 456 ($E^{1\%}$ = 2820), 488]. MS (ECNI, methane): molecular anion peak at *m/z* = 570 (35). MS [ECNI, deuteroammonia (ND_3)]: molecular anion peak at *m/z* = 572 indicated two exchangable hydrogens.

3-Hydroxy-β,ε-caroten-3′-one (4 + 4′). HPLC (eluent B) showed the presence of a *Z*-isomer (see Table I). UV/visible (nm) absorption spectrum of the mixture (**4** + **4′**): acetone, λ_{max} = (424), 446, 474 [lit.[14] λ_{max} = (425), 449, 447]; hexane, λ_{max} = 267, 334, (422), 443, 471 [lit.[29] λ_{max} = 267, 334, (421), 445, 472]. MS (ECNI, methane): molecular anion peak at *m/z* = 566 (100) and an ion peak at *m/z* = 548 [25, $(M - H_2O)^-$].

***(all-E,3R,3′R,6′R)*-β,ε-Carotene-3,3′-diol [*(all-E)*-Lutein, 5].** UV/visible (nm): hexane, λ_{max} = 422, 444, 473; petroleum ether, λ_{max} = 422, 444, 473; ethanol, λ_{max} = (423), 445, 474 in agreement with literature values.[25] MS (ECNI, methane): molecular anion peak at *m/z* = 568 (100) and an anion peak at *m/z* = 550 [20, $(M - H_2O)^-$]. ^1H NMR and CD spectra of this compound were identical with that of a sample of (3*R*,3′*R*,6′*R*)-lutein previously isolated from squash.[30]

***(all-E,3R,3′R)*-β,β-Carotene-3,3′-diol [*(all-E)*-Zeaxanthin, 6].** UV/visible (nm): hexane, λ_{max} = (430), 450.5, 476.5; ethanol, λ_{max} = 430), 451.5, 478 in agreement with literature values.[25] MS (ECNI, methane): molecular anion peak at *m/z* = 568 (100) as well as anion peaks at *m/z* = 550 [55, $(M - H_2O)^-$] and *m/z* = 532 [15, $(M - 2H_2O)^-$]. MS [ECNI, deuteroammonia (ND_3)]: molecular anion peak at *m/z* = 570 (70) indicated two exchangeable hydrogens.

***(all-E,3R,3′S,6R)*-β,ε-Carotene-3,3′-diol [*(all-E)*-3′-Epilutein, 7].** UV/visible (nm): hexane, λ_{max} = (422), 444, 473 [lit.[29] λ_{max} = 421, 445, 472]; ethanol, λ_{max} = (423), 445, 474; acetone, λ_{max} = (425), 447, 474; dichloromethane, λ_{max} = (430), 454.5, 483.5. MS (ECNI, methane): molecular anion peak at *m/z* = 568 (20) as well as anion peaks at *m/z* = 550 [100, $(M - H_2O)^-$] and *m/z* = 532 [60, $(M - 2H_2O)^-$]. ^1H NMR and CD spectra of this isolated fraction were identical with those of a synthetic sample of 3′-epilutein.

Minor Components of Band One: Geometrical Isomers of Lutein and Zeaxanthin. The geometrical isomers of lutein (compounds **5′**, **5″**, **5‴**) and zeaxanthin (compounds **6′**, **6″**, **6‴**)

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Table I. HPLC Peak Identification of Internal Standards and Carotenoids in an Extract from Human Plasma and Respective Wavelengths of Absorption Maxima Determined by Photodiode Array Detector in the Order of Elution with Eluents B and A

peak	plasma carotenoids[a,b]	absorption maxima (nm)[c]
	Eluent B	
1	ε,ε-carotene-3,3′-dione	420, 442, 472
2	3′-hydroxy-ε,ε-caroten-3-one	(422–424), 442, 472
3	(*all-E*)-5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene (5,6-dihydroxy-5,6-dihydrolycopene)	436, 460, 492
3′	(*Z*)-5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene	434, 458, 490
4	3-hydroxy-β,ε-caroten-3′-one	(422–424), 448, 476
4′	(*Z*)-3-hydroxy-β,ε-caroten-3-one	(418–420), 442, 470
5	(*all-E*,3*R*,3′*R*,6′*R*)-β,ε-carotene-3,3′-diol ((*all-E*)-lutein)	(422–424), 448, 476
6	(*all-E*,3*R*,3′*R*)-β,β-carotene-3,3′-diol ((*all-E*)-zeaxanthin)	(428), 454, 482
7	(*all-E*,3*R*,3′*S*,6′*R*)-β,ε-carotene-3,3′-diol ((*all-E*)-3′-epilutein)	(422–424), 448, 476
5′	(9*Z*)-lutein	334, 420, 442, 470
5″	(9′*Z*)-lutein	332, (420), 444, 472
19	(*all-E*,3*R*)-8′-apo-β-carotene-3,8′-diol (internal std)	(408), 427, 454
19′	(*Z*,3*R*)-8′-apo-β-carotene-3,8′-diol	(405), 424, 450
5‴	(13*Z*)-lutein + (13′*Z*)-lutein	334, (418), 440, 468
6′	(9*Z*)-zeaxanthin	340, (424), 450, 474
6″	(13*Z*)-zeaxanthin	338, (419), 446, 470
6‴	(15*Z*)-zeaxanthin	338, (426), 450, 478
	Eluent A	
8	(*all-E*)-3-hydroxy-2′,3′-didehydro-β,ε-carotene ((*all-E*)-2′,3′-anhydrolutein)	(424), 446, 474
8′	(*Z*)-2′,3′-anhydrolutein	332, (420), 440, 468
20	ethyl β-apo-8′-carotenoate (internal std)	444, (466)
9	β,ε-caroten-3-ol (α-cryptoxanthin)	(424), 446, 476
10	3-hydroxy-β-carotene (β-cryptoxanthin)	(428–430), 454, 480
11	(*all-E*)-ψ,ψ-carotene ((*all-E*)-lycopene)	446, 472–474, 502
11′	(*Z*)-ψ,ψ-carotene ((*Z*)-lycopene)	346, 360, 442, 468, 498
12	7,8-dihydro-ψ,ψ-carotene (neurosporene)	(420–422), 440, 468
13	β,ψ-carotene (γ-carotene)	(440), 462, 492
14	7,8,7′,8′-tetrahydro-ψ,ψ-carotene (ζ-carotene)	378, 400–402, 426
15	β,ε-carotene (α-carotene)	(428), 446–448, 474
16	(*all-E*)-β,β-carotene	(430), 454, 478
16′	(*Z*)-β,β-carotene	334, (422), 446, 474
17	(*all-E*)- or (*Z*)-7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene ((*all-E*)- or (*Z*)-phytofluene)	332–334, 350, 368
17′	(*Z*)- or (*all-E*)-phytofluene	332–334, 350, 368
18	7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene (phytoene)	(276), 286, (295)

[a] (*Z*)-Carotenoids have been designated the same number as their *all-E* isomers but distinguished from their *all-E* compounds by prime symbols. [b] Common names for certain carotenoids are shown in parentheses. [c] Values in parentheses represent points of inflection.

were identified from their absorption, mass, and ^{1}H NMR spectra.[37]

Band Two. The second band (R_f = 0.44, 2-cm width) was shown by HPLC (eluent A) to consist of eight major components which were separated by semipreparative HPLC (eluent A). In the order of chromatographic elution on a C_{18} reversed-phase column these components were characterized as follows.

Major Components of Band Two: (*all-E*)- + (*Z*)-3-Hydroxy-2′,3′-didehydro-β,ε-carotene [(*all-E*)- + (*Z*)-2′,3′-Anhydrolutein, 8 + 8′]. The UV/visible absorption spectrum was identical with that of synthetic 2′,3′-anhydrolutein. This compound was shown by HPLC (eluent A) to contain a *Z*-isomer (Figure 2 and Table I). MS (ECNI, methane): molecular anion peak at m/z = 550 (100).

β,ε-Caroten-3-ol (α-Cryptoxanthin) (9). UV/visible (nm): hexane, λ_{max} = 422, 445, 473 in agreement with literature values.[25] MS (ECNI, methane): molecular anion peak at m/z = 552 (100).

3-Hydroxy-β-carotene (β-Cryptoxanthin) (10). UV/visible (nm): hexane, λ_{max} = 425, 451, 483; petroleum ether, λ_{max} = (423), 451, 478 in agreement with literature values.[25] MS (ECNI, methane): molecular anion peak at m/z = 552 (100).

γ-Tocopherol. UV Absorption maximum (nm): ethanol, λ_{max} = 298 [lit.[38] λ_{max} = 298]. UV absorption spectrum in the HPLC solvents (eluent A) showed maximum at 292 nm. MS [desorption chemical ionization (DCI), methane]: protonated molecular ion at m/z = 417 $(M + H)^+$.

α-Tocopherol. UV absorption maximum (nm): ethanol, λ_{max} = 292 [lit.[38] λ_{max} = 292]. UV absorption spectrum in the HPLC solvents (eluent A) showed a maximum at 292 nm. MS (DCI, methane): protonated molecular ion at m/z = 431 $(M + H)^+$.

(*all-E*)- + (*Z*)-ψ,ψ-Carotene [(*all-E*)- + (*Z*)-Lycopene, 11 + 11′]. HPLC (eluent A) showed the presence of a *Z*-isomer (Table I). UV/visible (nm) of (*all-E*)- and (*Z*)-lycopene: petroleum ether, λ_{max} = 446, 472, 504; acetone, λ_{max} = 447, 474, 505 in agreement with the tabulated literature values.[25] MS (ECNI, methane): molecular anion peak at m/z = 536 (100).

7,8-Dihydro-ψ,ψ-carotene (Neurosporene) (12). UV/visible (nm): petroleum ether, λ_{max} = 416, 438, 468; hexane, λ_{max} = 416, 438, 468 in agreement with the literature values.[25] The HPLC retention time and absorption spectrum (Table I) of this compound were identical with those of synthetic neurosporene.[24] MS (ECNI, methane): molecular anion peak at m/z = 538 (70).

β,ψ-Carotene (γ-Carotene) (13). UV/visible (nm): petroleum ether, λ_{max} = 432, 460, 492. The HPLC retention time and absorption spectrum (Table I) of this compound were identical with those of an authentic sample of this compound.

Band Three. The third band (R_f = 0.30, 2-cm width) was shown by HPLC (eluent A) to consist of six major components which were separated by semipreparative HPLC (eluent A). In the order of chromatographic elution, these components were characterized as follows.

Major Components of Band Three: ζ-Carotene (14), α-Carotene (15), and (*all-E*)- + (*Z*)-β-Carotene (16 + 16′). The HPLC retention times and absorption spectra [determined by a photodiode array detector in the HPLC solvents (Table I)] of ζ-carotene and α- and β-carotene were identical with those of synthetic compounds. β-Carotene was shown by HPLC (eluent A) to contain a *Z*-isomer (16′).

(*all-E*)- + (*Z*)-Phytofluene (17 + 17′). This compound was shown by HPLC (eluent A) to contain a *Z*-isomer (17′). UV/visible (nm) absorption spectrum of the mixture of (*all-E*)- and (*Z*)-phytofluene: petroleum ether, λ_{max} = 332, 348, 366; hexane, λ_{max} = 332, 348, 366 in agreement with the tabulated literature values.[25] MS (ECNI, methane): molecular anion peak at m/z = 542 (20).

Phytoene (18) and Cholesteryl Oleate. The isolated phytoene fraction was shown by HPLC and mass spectrometry to contain substantial levels of cholesteryl oleate. The HPLC retention time and absorption spectrum of phytoene (Table I) were identical with those of a sample of phytoene previously isolated and characterized from an extract of peaches.[23] The mass spectrum (DCI, ammonia) of the mixture of phytoene and cholesteryl oleate contained peaks at m/z = 562 [65, $(M + NH_4)^+$)] for phytoene and 668 [100, $(M + NH_4)^+$] for cholesteryl oleate. The mass spectrum (DCI, methane) of the mixture of cholesteryl oleate and phytoene contained peaks at m/z = 545 [20, $(M + H)^+$] for phytoene and 651 [10, $(M + H)^+$] for cholesteryl oleate. The base peak appeared at 369 (100) due to the loss of oleic acid (282) from the protonated cholesteryl oleate. This fragmentation pattern is consistent with that of cholesteryl oleate reported in the literature.[39]

RESULTS AND DISCUSSION

In an earlier publication, we reported the presence of several ketocarotenoids in the extracts from human plasma.[12] The ketocarotenoids were isolated by removing the most polar fraction of human plasma (components that have retention times between 6 and 12 min on a C_{18} reversed-phase HPLC column employing eluent A) by preparative TLC. The TLC

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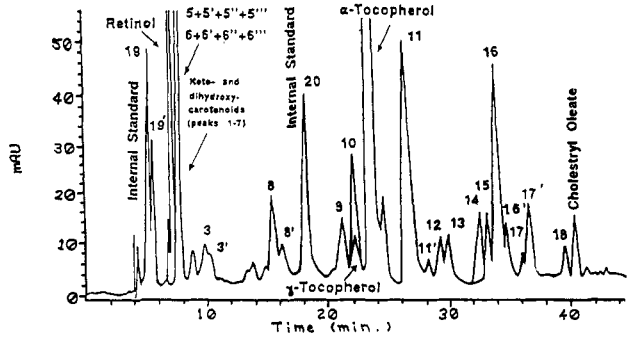

Figure 3. Carotenoid HPLC profile of an extract from human plasma on a C_{18} reversed-phase column employing HPLC system A (eluent A). HPLC peak for each carotenoid has been optimized at its λ_{max}. For peak identification, see Table I.

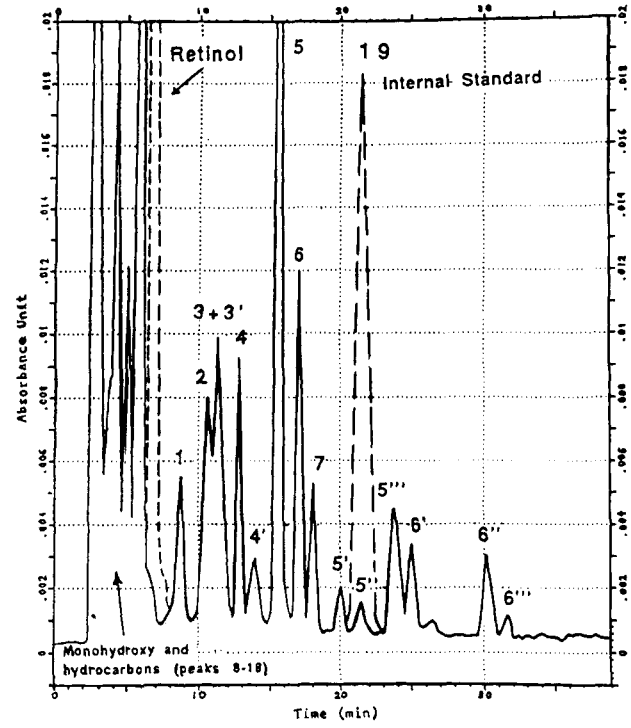

Figure 4. Carotenoid HPLC profile of an extract from human plasma on a silica-based nitrile-bonded column employing HPLC system B (eluent B). For peak identification, see Table I.

fraction of the concentrated extract was then evaluated by HPLC using a nitrile-bonded column. This approach is satisfactory for qualitative separation and identification of the polar oxygenated carotenoids found in human plasma. However, for routine carotenoid analyses of the extracts from human plasma by HPLC, where often numerous samples are to be examined within a reasonably short period of time, removal of the polar carotenoids by preparative TLC is inaccurate and time consuming. In the present report we have shown that extracts from human plasma can be directly examined on the nitrile-bonded column employing HPLC system B (eluent B) without the initial removal of the polar carotenoids. The chromatographic profiles of an extract of human plasma injected on a C_{18} reversed-phase column (HPLC system A, eluent A) and a nitrile-bonded column (HPLC system B, eluent B) are shown in Figures 3 and 4, respectively. Eighteeen carotenoids, as well as vitamin A and two forms of vitamin E (γ- and α-tocopherol), were shown to be present. The HPLC trace shown in Figure 3 has been simultaneously monitored at 470, 445, 400, 350, and 290 nm. This is due to the fact that depending on the nature of the chromophore involved, each of the plasma carotenoid show a main UV/visible absorption maximum at one of these monitored wavelengths (see Table I). However for maximum sensitivity and clarity of presentation, only the optimized HPLC signal due to each carotenoid at the appropriate wavelength has been shown in Figure 3. The chemical structures of these carotenoids are shown in Figure 5. In the following, the detailed qualitative and quantitative distribution of carotenoids in human plasma are described.

(A) Qualitative Distribution of Plasma Carotenoids. Owing to the low abundance of carotenoids, structural elucidation was based on the absorption and mass spectral data as well as comparison of the HPLC retention times of unknowns with those of pure synthetic compounds. Purified reference standards of specific plasma carotenoids in most cases were not available and were therefore prepared by partial or total synthesis. With the exception of (3*R*,3′*R*,6′*R*)-β,ϵ-carotene-3,3′-diol (lutein), (3*R*,3′*S*,6′*R*)-β,ϵ-carotene-3,3′-diol (3′-epilutein), and (3*R*,3′*R*)-β,β-carotene-3,3′-diol (zeaxanthin) whose absolute configurations were determined by comparison of their NMR, CD, and HPLC profiles with authentic reference compounds,[37] the absolute configurations of the other plasma carotenoids with chiral centers are not known at present. In the present report, several of these ketocarotenoids were prepared by nonstereospecific synthetic pathways in an attempt to facilitate the structural elucidation of unknown carotenoids in human plasma.

Major carotenoid components, isolated from extracts of human plasma, may be divided into three groups of compounds in the order of chromatographic elution on a C_{18} reversed-phase column. These are (1) keto- and dihydroxycarotenoids, (2) monohydroxycarotenoids, and (3) hydrocarbon carotenoids. Characterization of these various groups of carotenoids is discussed separately.

(1) Keto- and Dihydroxycarotenoids. The separation of plasma carotenoids on the nitrile-bonded column (Figure 4) resulted in coelution of nonpolar carotenoids within the first 6 min that were well separated on the C_{18} reversed-phase column (peaks 8–18, Figure 3). This was followed by the elution of retinol and carotenoids, which were identified as ϵ,ϵ-carotene-3,3′-dione (peak 1, Figure 4), 3′-hydroxy-ϵ,ϵ-caroten-3-one (peak 2), 5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene and its *Z*-isomer (peak 3 + 3′), (*all-E*)-3-hydroxy-β,ϵ-caroten-3′-one (peak 4) and its *Z*-isomer (peak 4′), lutein (peak 5), zeaxanthin (peak 6), and 3′-epilutein (peak 7). Also present were several geometrical isomers of lutein and zeaxanthin. There was some concern about the presence of ketocarotenoids in human plasma and the possibility that these compounds could be formed from oxidation of carotenoids from residual metal ions after collection of blood samples. When serum from several subjects was treated with ferric chloride for as long as 1 h, no significant change in the relative concentration of keto- and dihydroxycarotenoids was observed. In a separate experiment, plasma from the same subjects was obtained using ethylenediaminetetraacetic acid disodium salt (EDTA) which was expected to chelate cations, including ferric ions and other prooxidant ions, and thereby minimize the formation of ketocarotenoids. When extracts of these plasmas were analyzed by HPLC, the relative concentration of keto- to dihydroxycarotenoids were similar to that observed in extracts of serum for each subject. These results indicate that the appearance of ketocarotenoids is simply not the result of catalytic oxidation by Fe^{3+}/O_2 during sample preparation. Although all of the operations including extraction, TLC, and HPLC procedures were carried out under gold fluorescent lights and in the presence of an antioxidant, there may be some concern about light/O_2-induced oxidation of carotenoids during sample preparation. However, the formation of the ketocarotenoids requires oxidation of an allylic hydroxyl group as well as migration of a double bond in the β-ring of hydroxycaro-

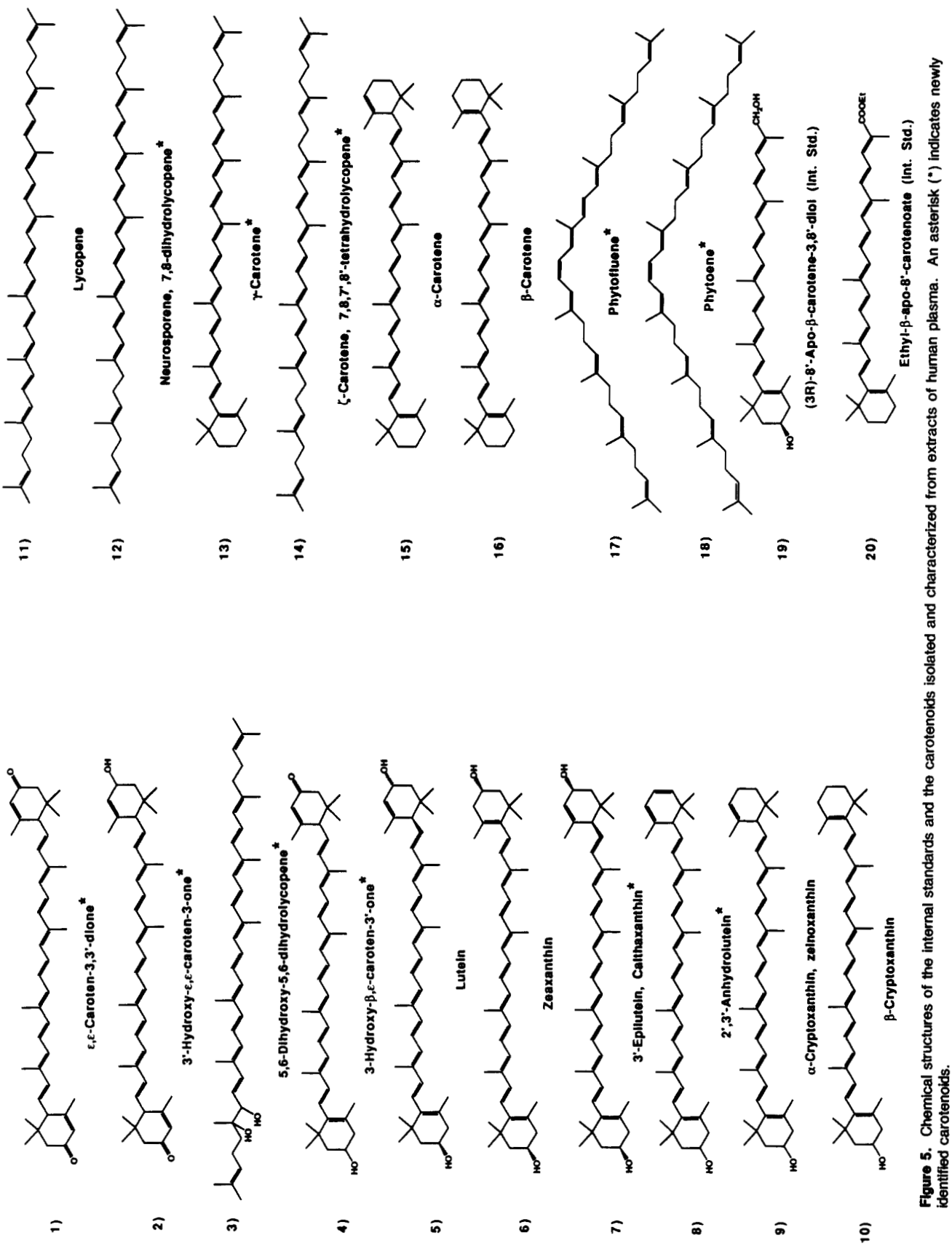

Figure 5. Chemical structures of the internal standards and the carotenoids isolated and characterized from extracts of human plasma. An asterisk (*) indicates newly identified carotenoids.

tenoids to form carotenoids with ε-rings. While allylic oxidation of hydroxycarotenoids can only be accompanied by strong oxidizing reagents (i.e. nickel peroxide) in organic solution to give ketocarotenoids, there is no precedence for any oxidation reaction involving double bond migration of a β-ring in a carotenoid to form an ε-ring. Furthermore, in the presence of light/O_2, carotenoids are expected to undergo oxidative cleavage to form smaller carotenoid fragments known as apocarotenals and carotenoid epoxides. The fact that none of these products were detected in human plasma suggests that the formation of ketocarotenoids is not the result of photodegradation of carotenoids through known mechanisms.

The presence of 5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene and its *Z*-isomer (peak 3+3′, Figures 3 and 4) in human plasma is rather interesting since we have recently shown that major dietary sources of this compound are tomato paste and concentrated tomato products.[17] In addition, concentrated tomato products also contain 5,6-epoxy-5,6-dihydro-ψ,ψ-carotene (lycopene 5,6-epoxide), which in strong acidic media, similar to that of the human digestive system, may undergo hydrolysis to form 5,6-dihydroxy-5,6-dihydro-ψ,ψ-carotene.

The presence of 3′-epilutein [(3*R*,3′*S*,6′*R*)-β,ε-carotene-3,3′-diol] in human plasma is also of particular interest. This carotenoid has been isolated from the petals of *Caltha palustris*.[29] Recently, 3′-epilutein has also been detected in anthers of flowers of several roses and those of peonies.[40] It is not clear, however, whether 3′-epilutein in human plasma is of dietary origin or whether this compound is a metabolite of lutein or lutein fatty acid esters.

(2) Monohydroxycarotenoids. The monohydroxycarotenoids in the extracts from human plasma are best separated by HPLC on a C_{18} reversed-phase column as shown in Figure 3. One of the monohydroxycarotenoids in the extracts from plasma is (*all-E*)-3-hydroxy-2′,3′-didehydro-β,ε-carotene (2′,3′-anhydrolutein, peak 8) and its *Z*-isomer (peak 8′). Dietary sources of 2′,3′-anhydrolutein are limited to one variety of squash;[41] therefore, its presence in the extracts from human plasma may be related to an enzymic dehydration of lutein or deesterification of lutein acyl esters in strong acidic media in the human digestive system.

Other monohydroxycarotenoids found in plasma are β,ε-caroten-3-ol (α-cryptoxanthin, peak 9) and 3-hydroxy-β-carotene (β-cryptoxanthin, peak 10). The two common forms of vitamin E in human plasma, γ- and α-tocopherol, have also been separated and identified along with the monohydroxycarotenoids. Other forms of vitamin E (i.e. δ- and β-tocopherol) are also expected to be present in human plasma; however, owing to their low concentration these were not detected. Although the HPLC peaks of β-cryptoxanthin and γ-tocopherol coelute, the absorption spectra of these compounds in the HPLC solvents with maxima at 450 and 292 nm, respectively, are sufficiently different to allow accurate determination of these two components in the extracts from human plasma. Among the two possible chemical structures for α-cryptoxanthin [β,ε-caroten-3-ol and β,ε-caroten-3′-ol],[42] we have shown that the 3-hydroxyl group in α-cryptoxanthin isolated from human plasma is substituted in the β rather than the ε end group.[12]

(3) Hydrocarbon Carotenoids. The hydrocarbon carotenoids separated from the extracts of human plasma were identified as ψ,ψ-carotene (lycopene), 7,8-dihydro-ψ,ψ-carotene (neurosporene), β,ψ-carotene (γ-carotene), 7,8,7′,8′-tetrahydro-ψ,ψ-carotene (ζ-carotene), β,ε-carotene (α-carotene), β,β-carotene, 7,8,11,12,7′,8′-hexahydro-ψ,ψ-carotene (phytofluene), and 7,8,11,12,7′,8′,11′,12′-octahydro-ψ,ψ-carotene (phytoene) (Figure 3). Along with the hydrocarbon carotenoids in extracts from human plasma, a noncarotenoid, namely cholesteryl oleate, was shown by HPLC to elute immediately after phytoene. Other esters of cholesterol, i.e. cholesteryl palmitate, were also shown by HPLC and mass spectrometry to the present in plasma; however, emphasis was placed on separation and detection of cholesteryl oleate because of its close elution to phytoene which could result in misidentification of this carotenoid. Detection of cholesteryl oleate by HPLC is mainly due to a weak absorption in the UV spectrum of this compound at the monitoring wavelength of 290 nm.

The presence of the hydrocarbon carotenoids in human plasma is not surprising considering they are abundant in common fruits and vegetables that are frequently consumed by humans.[12,23] Among the hydrocarbon carotenoids, dietary sources of neurosporene and γ-carotene include tomato paste and other concentrated tomato products.[17]

Confirmation of the Structure of Keto- and Hydroxycarotenoids by Liquid Chromatography/Mass Spectrometry. Further confirmation for the structures of the keto- and hydroxycarotenoids was obtained with HPLC/mass spectrometry interface. Since the HPLC system was equipped with a photodiode array detector, in addition to the molecular mass, this technique provided the absorption maxima of each of the plasma carotenoids as they eluted from the HPLC column. During the first 10 min of the HPLC separation of plasma carotenoids on the nitrile-bonded column (Figure 4), lipids, noncarotenoid impurities, as well as the nonpolar carotenoids (i.e. 2′,3′-anhydrolutein, α- and β-cryptoxanthin, hydrocarbon carotenoids) that are not well separated on the nitrile-bonded column are coeluted. This is then followed by the HPLC elution of keto- and hydroxycarotenoids of interest between time 10 and 40 min. The reconstructed total ion chromatogram (Figure 6, upper trace) obtained by mass spectrometry (MS) is difficult to correlate to the HPLC chromatogram of human plasma on the nitrile-bonded column (Figure 4). The predominant source of this difficulty is the difference in sensitivities of HPLC and MS for the same class or classes of compounds that are present in the extracts from human plasma. However, an ion chromatogram (Figure 6, lower trace) of molecular anions (*m/z* 564, 566, 568, 570) of the anticipated analytes can be readily correlated with the chromatogram of human plasma on the nitrile bonded column (Figure 4). Some of the spectra associated with Figure 6 (lower trace) are shown in Figure 7. The combination of absorption and mass spectra analyses of keto- and hydroxycarotenoids obtained with this HPLC/MS interface system clearly confirmed the structural assignments for these carotenoids.

(B) Quantitative Distribution of Carotenoids, Vitamin A, and Vitamin E in Human Plasma. The carotenoids, vitamin A (retinol), and vitamin E (γ- and α-tocopherol) in the extracts from human plasma were quantified from the HPLC response factors of the isolated or synthetic reference compounds at five or six different concentrations, employing HPLC systems A and B. The relative standard deviation for the calibration curves (i.e. area response at various concentrations) of each of the reference samples was less than 5%. To monitor the accuracy and reproducibility of the HPLC analysis (system A) with eluent A, a solution containing known concentrations of retinol, α-tocopherol, lutein, α- and β-cryptoxanthin, and α- and β-carotene was routinely analyzed. Similarly a solution of known concentration of lutein and zeaxanthin was monitored by HPLC system B with eluent B. From time to time samples of each individual component

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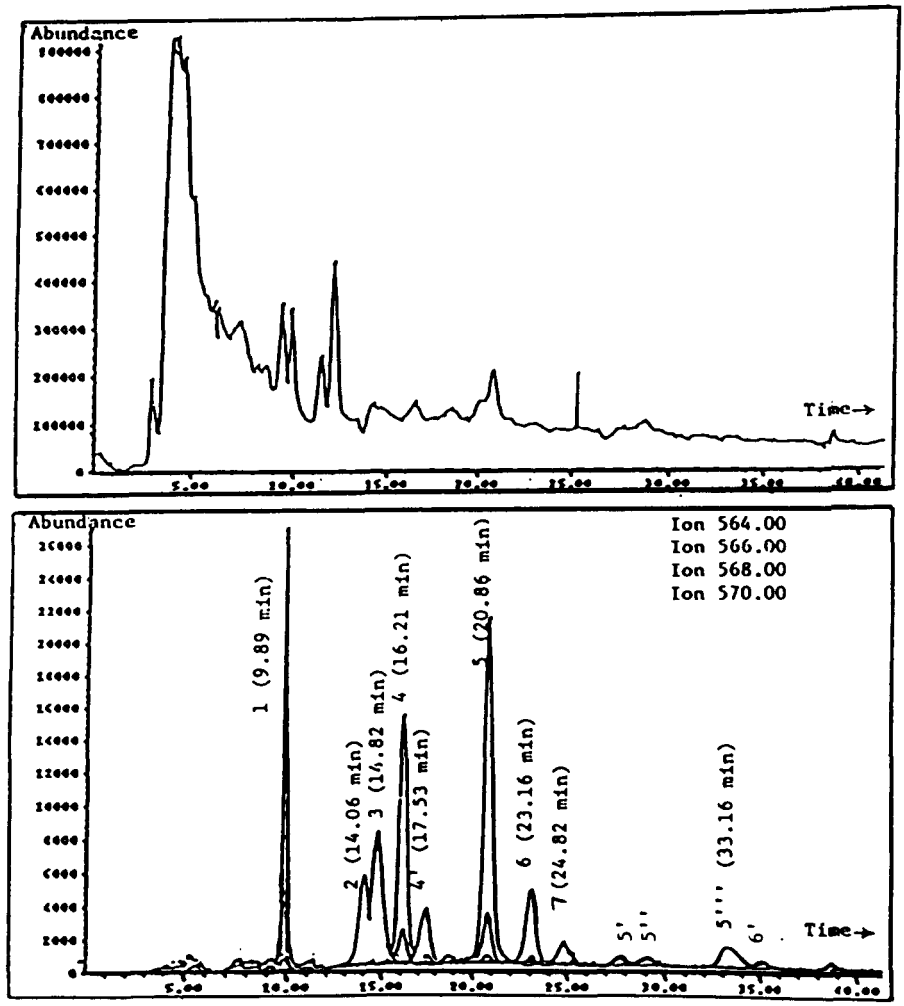

Figure 6. Total ion (upper trace) and selected molecular anion [*m/z* 564, 566, 568, 570] (lower trace) chromatograms of human plasma on a liquid chromatography/mass spectrometry system. For peak identification, see Table I. Conditions described in text.

were added to the plasma extract prior to HPLC analyses. The HPLC peak area of the external standard solutions and the added analytes were compared with those of the individual components determined from the calibration curves. These studies revealed that the recovery and the accuracy of the HPLC analyses with eluent A and B were greater than 95%. Furthermore, the recoveries of the two internal standards, (3*R*)-8′-apo-β-carotene-3,8′-diol and ethyl β-apo-8′-carotenoate from repeated extractions of plasma samples were in the range of 90–95%. These were determined by the HPLC peak area of these internal standards before and after extraction and workup procedure.

The HPLC peak area of *Z*-isomers of carotenoids, which were not resolved and appeared as a trailing shoulder on their *all-E* counterpart, were combined with the HPLC peak area of (*all-E*)-carotenoids. In the case of lutein and zeaxanthin, where the *Z*-isomers were well separated from their *all-E* counterparts, the HPLC peak area of these *Z*-isomers of carotenoids were reported separately from their *all-E* compounds. However, in all cases in quantification of carotenoids it has been assumed that the response factors of *Z*-isomers of carotenoids are reasonably close to that of their *all-E* carotenoids.

The quantitative distribution of carotenoids, retinol, and two forms of vitamin E (γ- and α-tocopherol) in human plasma are shown in Tables II and III, respectively. Since the concentration of individual carotenoids varied greatly among the subjects, the data in Table II have been expressed in terms of mean and range of values. This approach has also been used to present retinol and vitamin E concentrations in plasma (Table III). The data shown in Tables II and III should not be considered representative of the concentration of carotenoids, retinol, and vitamin E in large human populations, as these data were obtained at random from only five subjects with normal dietary patterns. The data does show that our HPLC procedures can be used for detection and quantification of specific carotenoids in human plasma that have not been previously reported. The level of carotenoids, vitamin A, and the two forms of vitamin E (γ- and α-tocopherol) in Tables II and III are expressed in micrograms per deciliter as well as SI units.[43] The plasma levels of carotenoids, retinol, and γ- and α-tocopherol presented in Tables II and III are consistent with the levels of these compounds reported in other human studies.[5,9,44] Relatively high concentrations of (*Z*)-luteins and (*Z*)-zeaxanthins with respect to their corresponding *all-E* counterparts in human plasma is particularly noticeable. The percentage of each of the geometrical isomers of lutein and zeaxanthin in plasma of each of the five subjects are shown in Table IV. These data indicate that approximately 29–42% of lutein in human plasma exists in various *Z* geometrical forms. In the case of zeaxanthin, as much as 55–64% exists as various *Z*-isomers. The *Z*-isomers of lutein and zeaxanthin in human plasma have also been reported by Krinsky et al.[44] The presence of fairly large amounts of (*Z*)-luteins and (*Z*)-zeaxanthins in human plasma is not likely to have been induced from stereoisomerization of their corresponding *all-E* counterparts as a result of extraction and sample preparation. (*all-E*)-

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(44) Krinsky, N.; Russett, M. D.; Handelman, G. J.; Snodderly, D. M. *J. Nutr.* **1990**, 1654–1662.

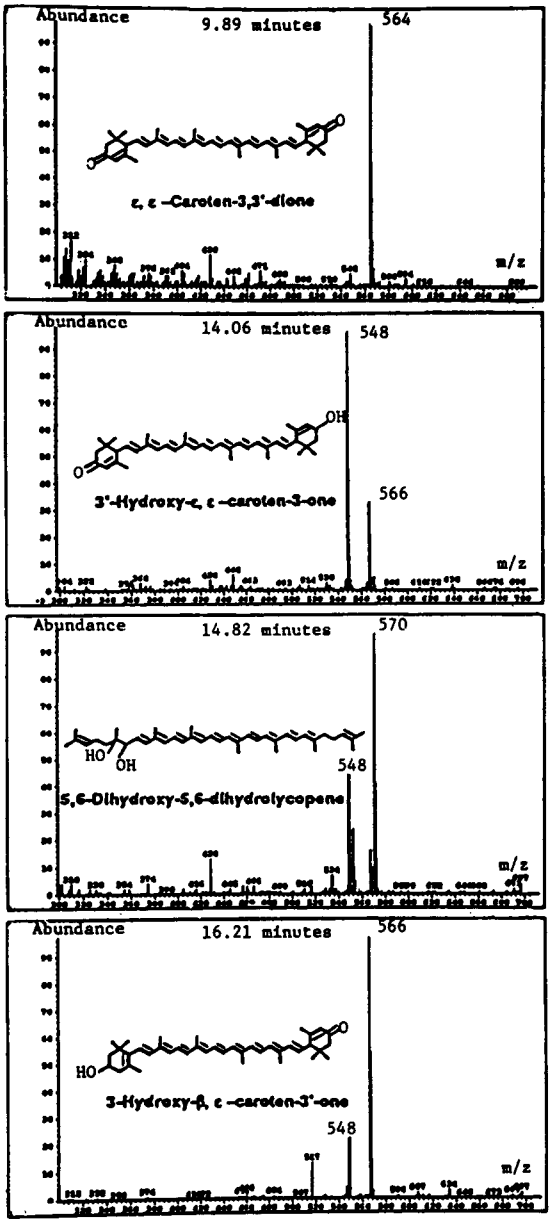

Figure 7. Mass spectra of several of the newly identified plasma carotenoids associated with the selected molecular anion chromatogram shown in Figure 6 (lower trace).

Lutein and (*all-E*)-zeaxanthin did not undergo such stereoisomerization when they were subjected to similar extraction and preparation conditions.

Selection of Internal Standards. In quantitative determination of the various classes of the naturally occurring carotenoids by HPLC, where extensive extraction and sample preparation techniques are involved, the use of an internal standard can greatly improve the accuracy of analytical data. In previous reports we have demonstrated the application of a number of internal standards in HPLC quantification of carotenoids isolated from fruits and vegetables.[17,19,23,41,45] In the present study, we have employed ethyl β-apo-8′-carotenoate and (3*R*)-8′-apo-β-carotene-3,8′-diol as internal standards for the quantification of plasma carotenoids by HPLC on reversed-phase and nitrile-bonded phase columns, respectively. Both of these internal standards are synthetic and as shown in Figures 3 and 4; their HPLC peaks do not result in a major interference with those of the plasma carotenoids. The synthesis of (3*R*)-8′-apo-β-carotene-3,8′-diol according to HPLC results in the formation of a mixture of *all-E*- (peak 19, Figure 3) and *Z*-isomers (peak 19′) for this compound. The ratio of these isomeric mixtures for this internal standard remains the same throughout the extraction procedure. However as it can be seen from Figure 4, the HPLC peak of (3*R*)-8′-apo-β-carotene-3,8′-diol (peaks 19 + 19′ coeluted) interferes with that of (9′*Z*)-lutein (peak 5″). Therefore in HPLC separations of plasma carotenoids where the evaluation of (9′*Z*)-lutein is of particular interest, the addition of this second internal standard may be omitted. We have examined many synthetic carotenoids as potential

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Table II. Quantitative Distribution of Carotenoids in Human Plasma[a]

carotenoids[b]	concentration mean/range (μg/dL)	concentration mean/range (nmol/L)
1	0.81/0.33–1.27	14.3/5.84–22.5
2	1.19/0.50–2.21	21.0/8.82–39.0
3 + 3′	4.14/3.73–4.81	72.5/65.3–84.3
4 + 4′	1.22/0.60–2.00	21.5/10.6–35.3
5	10.6/7.69–15.1	186.3/135.2–265.5
5′	1.71/1.59–1.88	30.1/28.0–33.1
5″	1.65/1.56–1.81	29.0/27.4–31.8
5‴	2.38/2.01–3.17	41.8/35.3–55.7
6	2.49/2.01–2.86	43.8/35.3–50.3
6′	1.29/1.14–1.48	22.7/20.0–26.0
6″	1.38/1.26–1.69	24.3/22.2–29.7
6‴	0.94/0.33–1.10	16.5/5.8–19.3
7	2.21/1.86–2.76	38.9/32.7–48.5
8 + 8′	8.27/6.76–10.2	150.2/122.7–185.2
9	4.34/2.14–7.20	78.5/38.7–130.2
10	14.7/8.25–20.5	266.1/149.2–370.8
11 + 11′	62.7/55.9–73.9	1167.5/1041.3–1376.5
12	6.7/4.3–8.1	124.3/79.8–150.3
13	5.5/3.8–7.5	102.4/70.8–139.7
14	12.8/7.79–15.0	236.7/144.0–277.3
15	6.96/4.36–10.3	129.7/81.2–191.9
16 + 16′	14.2/7.10–17.8	264.5/132.3–331.6
17 + 17′	16.1/11.7–26.5	295.9/215.5–488.1
18	1.34/0.88–1.71	24.6/16.5–31.4

[a] Mean and range of values for five subjects. [b] For identification of carotenoids see Table I.

Table III. Quantitative Distribution of Retinol[a] and α- and γ-Tocopherol[b] in Human Plasma[c]

	concentration (μg/dL)	concentration (μmol/L)
retinol	67.8/55.1–85.6	2.37/1.92–2.99
α-tocopherol	1681./1199.–2120.	39.0/27.8–49.2
γ-tocopherol	425./207.–644.	10.2/5.0–15.5

[a] Data were generated on HPLC system A (eluent A) at 325 nm. [b] Data were generated on HPLC system A (eluent A) at 290 nm. [c] Mean and range of values presented for five subjects.

Table IV. Percent Composition of Various Geometrical Isomers of Lutein and Zeaxanthin in Human Plasma from Five Subjects

carotenoid	% composition for each subject[a] 1	2	3	4	5	range
(*all-E*)-lutein	58	63	69	60	71	58–71
(9*Z*)-lutein	12	12	9	12	9	9–12
(9′*Z*)-lutein	13	11	8	12	8	8–13
(13*Z*)- + (13′*Z*)-lutein	17	14	14	16	12	12–17
(*all-E*)-zeaxanthin	43	41	40	36	45	36–45
(9*Z*)-xeaxanthin	26	21	20	21	18	18–26
(13*Z*)-zeaxanthin	25	21	24	24	20	20–25
(15*Z*)-zeaxanthin	6	17	16	19	17	6–19

[a] Calculated from the HPLC peak area in the extracts of plasma for each subject.

internal standards for the HPLC separation of plasma carotenoids on the nitrile-bonded column. (3*R*)-8′-Apo-β-carotene-3,8′-diol was found to be the most suitable. Most importantly, the solubility and chromatographic behavior of (3*R*)-8′-apo-β-carotene-3,8′-diol and ethyl β-apo-8′-carotenoate in the extracting solvents and the HPLC eluents A and B are similar to those of plasma carotenoids, resulting in recoveries of more than 90% for these internal standards. While ethyl β-apo-8′-carotenoate is commercially available, the elaborate synthesis of the second internal standard, namely, (3*R*)-8′-apo-β-carotene-3,8′-diol from its precursors has contributed to the lack of commercial availability for this compound.

CONCLUSION

The HPLC profile (system A, eluent A) of the human plasma described here and those of the common fruits and vegetables analyzed in our laboratory to date[12,23,41,45] were obtained under the same chromatographic conditions. As a result the correlation between these profiles reveals a much clearer picture of the absorption and metabolism of carotenoids in humans. One such interesting aspect is the finding that only selected classes of carotenoids make their way into the human blood stream. This has been clearly demonstrated in our laboratory by qualitative HPLC analyses (system A, eluent A) of plasma extracts obtained from more than several hundred subjects. For example, carotenoid epoxides such as neoxanthin, violaxanthin, and lutein epoxide which are abundant in our diets through consumption of green fruits and vegetables[45] were shown to be absent. The apparent absence of epoxycarotenoids in the extracts from human plasma suggests that the absorption and perhaps metabolism of these compounds is quite different than the hydroxycarotenoids and the hydrocarbon carotenoids routinely found in plasma. However it must be pointed out that human studies with carotenoids epoxides containing stable isotopes (i.e. ^{13}C) are needed in order to establish the metabolic pathways of these conpounds in humans. Another class of carotenoids that are absent in the extracts from human plasma are carotenol fatty acid esters, which are mostly found in yellow/orange fruits and vegetables.[23,41] However since the parent carotenoids of these esters (i.e. α- and β-cryptoxanthin, lutein, zeaxanthin) are present in plasma, it may be assumed that carotenol fatty acid esters that are of dietary origin undergo enzymic hydrolysis to regenerate their parent carotenoids according to similar mechanisms proposed for the enzymic hydrolysis of triglycerides. Perhaps the most interesting feature of the present study is the isolation and characterization of the ketocarotenoids from human plasma. These compounds have been recently isolated from hen's egg yolk by several investigators[29,46,47] Schiedt et al.[46] and Matsuno et al.[47] reported that these ketocarotenoids are oxidation products of lutein and zeaxanthin in hen's egg yolk and suggested possible metabolic pathways for their formation. Although the presence of ketocarotenoids in human plasma may suggest that these compounds are metabolic products of lutein, or perhaps even zeaxanthin, such assumptions may be premature at the present time. From our extensive studies of the distribution of carotenoids in common fruits and vegetables, it appears that the primary dietary source of these ketocarotenoids is egg yolk or egg products. It is doubtful, however, that eggs or the diet in general provides sufficient levels of ketocarotenoids to maintain them at the level observed in plasma. Based on the metabolic pathways proposed by Schiedt et al.[46] and Matsuno et al.,[47] possible pathways for the formation of ketocarotenoids in humans may also involve conversion of the 3-hydroxy β-rings of lutein and zeaxanthin to 3-hydroxy ε-rings which may be followed by allylic oxidation to form the corresponding ketocarotenoids. These types of reactions have been found to be quite common in the metabolism of carotenoids in animals.[48]

NOMENCLATURE

For convenience the trivial names of certain carotenoids have been used throughout this text. The correct systematic names[42] for these carotenoids have been presented in Table I. The terms *all-E-* and *Z*-isomers of carotenoids refer to all-trans and cis isomers of carotenoids, respectively. For in-chain geometrical isomers of carotenoids, the terms all-trans and cis, which have been used with the old nomenclature, are no longer appropriate.

ACKNOWLEDGMENT

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