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Synthetic carotenoids as internal standards for plasma micronutrient analyses by high-performance liquid chromatography

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ABSTRACT

A method is described for the synthesis of new carotenoids by transesterification of ethyl-β-apo-8'-carotenoate. The rate of transesterification was temperature-dependent with highest yields using primary alcohols. Reaction conditions were found avoiding *Z* isomerization. The structures of the new products were confirmed by ultraviolet, mass, ^{1}H and ^{13}C nuclear magnetic resonance spectroscopy. The synthetic carotenoids were used as internal standards for reversed-phase high-performance liquid chromatographic (HPLC) analyses of lipid phase plasma micronutrients. Retention times for the transesterified products increased as a function of alcohol chain length allowing a choice of synthetic carotenoids for use as internal standards for carotenoid quantitation without interference with the detection of analytes during HPLC analysis.

INTRODUCTION

Interest in human antioxidant status has increased considerably in recent years due to accumulated evidence that oxidative damage may play a key role in carcinogenesis [1]. Carotenoids and tocopherols, potent scavengers of reactive oxygen [2] and other radicals [3], have been linked in epidemiologic studies with decreased incidence rates for various types of cancer [4,5]. Many animal and *in vitro* studies have lent support to these observations by showing chemopreventive properties for various dietary carotenoids and α-tocopherol [6–8]. Carotenoids have also been found to modulate immune response [9], prevent atherosclerosis [10] and provide beneficial effects against other human diseases [11].

Epidemiologic investigations, assessing the relation between cancer risk and carotenoids as well as intervention trials using carotenoids require a fast, accurate and precise procedure for monitoring these blood components. In light of recent reports that multiple samples may be required for accurate characterization of individual levels of plasma micronutrients [12,13], it is essential that assay variability be minimized. For the successful separation of carotenoids HPLC was first used by Nelis and De Leenheer [14] and has since been developed as the most powerful tool for quantitation of carotenoids in blood and tissues [4,15–36]. Extraction of plasma, required prior to HPLC analyses, in combination with saponification [16,18] or protein denaturation by perchloric acid [17] is not recommended since the former leads to a total loss of α-tocopherol [19] and destruction of sensitive carotenoids [18], and the latter to epoxidation and isomerization of the carotenes [19]. Calibration curves obtained by

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adding standards to bovine serum albumin or to pooled human plasma are inappropriate since recoveries are very low in the former [20] and recoveries will always vary between individuals due to individual plasma protein composition. The latter fact and the risk of analyte loss during the multi-step extraction procedure [15] and during HPLC analysis requires the use of internal standards for precise carotenoid quantitation. Many investigators use tocol [12,15,21], α-tocopheryl acetate [21], retinyl acetate [22,23] or retinyl palmitate [24,25] as internal standards for this purpose while others use none [13,19,26–29].

An ideal internal standard should be a compound structurally related to the analyte and of similar polarity, but with a retention time that does not overlap with other peaks in the chromatogram. Few investigators use such an analogue as an internal standard for HPLC carotenoid analyses [30–34,36] since these analogues are either unavailable, too costly or co-elute with analytes [15].

We prepared such a carotenoid analogue in a fast one-step procedure which allows syntheses of a series of carotenoids suitable as internal standards for plasma carotenoid quantitation by HPLC without interference with the analytes. We also describe an extraction protocol with excellent extraction efficiencies for plasma retinol, tocopherols and carotenoids employing three internal standards, namely retinyl palmitate[a], tocol and one of the new synthetic carotenoids.

EXPERIMENTAL

Apparatus

HPLC analyses were carried out on a Beckman System Gold chromatograph using a Model 507 autosampler and a Model 168 dual-wavelength diode-array detector. Optical density readings were obtained fom a DU-62 spectrophotometer (Beckman). GC–MS measurements were performed on a Hewlett-Packard 5890 gas chromatograph with a 7673A autosampler and an HP 5971 mass-selective detector using 70 eV ionization energy. NMR data were obtained from an Omega GN 500 instrument (General Electric).

Chemicals

n-Butanol, isobutanol, methanol, tetrahydrofuran (THF), dioxane and all solvents used for HPLC and optical density readings were analytical grade or HPLC grade from Fisher (Fair Lawn, NJ, USA). Butylated hydroxytoluene (BHT), bis-tris-propane (BTP), *n*-pentanol, *n*-heptanol, isopentanol, all-*trans*-retinol, all-*trans*-retinyl palmitate, *dl*-α-tocopherol, (+)-γ-tocopherol, (+)-δ-tocopherol, lutein, all-*trans*-α-carotene (type V), all-*trans*-β-carotene (type I) and lycopene were purchased from Sigma (St. Louis, MO, USA). Tocol and β-cryptoxanthin were a gift from Hoffmann-La Roche (Basel, Switzerland). All-*trans*-ethyl-β-apo-8′-carotenoate was obtained from Fluka (Ronkonkoma, NY, USA), *n*-hexanol from Columbia Organic (Canden, SC, USA), 2-octanol, cinnamyl alcohol and 2-butanol from Eastman-Kodak (Rochester, NJ, USA), *d,l*-2-heptanol, phenethyl alcohol, 3-phenyl-1-propanol, 1-nonanol and cyclohexanol from Aldrich (Milwaukee, WI, USA), benzyl alcohol and 3-butyl alcohol from Baker (Phillipsburg, NJ, USA), phenol and potassium cyanide from Mallinckrodt (Paris, KY, USA) and 2-naphthol and sodium cyanide from Matheson-Coleman & Bell (Los Angeles, CA, USA).

Standard solutions and calibration curves

All procedures were carried out under subdued light. Carotenoid standards were dissolved in THF while retinol and the tocopherols were dissolved in hexane to prepare stock solutions. The purity of these solutions was checked by HPLC analysis with monitoring at the individual compound's absorption maximum. The purity (%) of the standard was calculated by dividing the peak area of the compound by all peak areas in the chromatogram and multiplying by 100 assuming that contaminants or by-products have the same light absorption properties as the standard. Compounds with less than 95% purity were discarded. The concentration of the stock solutions was

[a] Retinyl palmitate was recently replaced by retinyl laurate.

determined by optical density readings at the wavelength with maximum absorption (λ_{max}) after diluting the stock solutions to the appropriate concentration with the solvent given in the literature for the specific extinction coefficient [37–44]: *trans*-lutein in dioxane (λ_{max} = 453 nm; $E^{1\%}$ = 2672); β-cryptoxanthin in hexane (λ_{max} = 451 nm; $E^{1\%}$ = 2460); lycopene in hexane (λ_{max} = 474 nm; $E^{1\%}$ = 3470); α-carotene in hexane (λ_{max} = 451 nm; $E^{1\%}$ = 2554); β-carotone in hexane (λ_{max} = 451 nm; $E^{1\%}$ = 2590); retinol in ethanol (λ_{max} = 325 nm; $E^{1\%}$ = 1832); α-tocopherol in ethanol (λ_{max} = 292 nm; $E^{1\%}$ = 75.8); γ-tocopherol in ethanol (λ_{max} = 298 nm; $E^{1\%}$ = 91.4); δ-tocopherol in ethanol (λ_{max} = 298 nm; $E^{1\%}$ = 87.3). The final stock concentration of each individual standard was calculated using the optical density reading adjusted for the purity.

Calibration curves were obtained for each standard with high linearity ($r > 0.995$) by plotting the standard concentration as a function of the peak area obtained from an HPLC analysis with a 20-μl injection. For this purpose the stock solutions of the standards were diluted with the mobile phase to nine different concentrations starting with 25% of the lowest expected plasma concentration and ending with five times the highest expected plasma concentration. Each concentration was analyzed by triplicate injections.

Blood sampling

Overnight fasting blood was obtained from 22 healthy non-smokers aged 28–47 who were not on vitamin supplements or any other medication. Blood was drawn into vacutainers to prevent oxidation by air and was protected from light. The blood samples for plasma analyses contained EDTA or heparin and were put immediately on ice until centrifuged for 30 min at 4°C and 850 *g*. Serum was obtained by incubating the blood samples at room temperature for 30 min followed by centrifugation for 30 min at 4°C and 850 *g*. The supernatant plasma or serum was either worked up immediately or stored at −70°C.

Micronutrient extraction from plasma

All procedures were carried out under subdued light and all solvents and serum/plasma samples were kept on ice during work-up. A 0.5-ml volume of serum/plasma (fresh or refrigerator-thawed if kept at −70°C) was transferred into a 100 mm × 13 mm glass centrifuge tube and thoroughly vortex-mixed by hand for at least 1 min with 0.5 ml of ethanol containing 0.3 μg/ml *n*-butyl-β-apo-8′-carotenoate, 1.0 μg/ml retinyl palmitate, 6.0 μg/ml tocol and 250 μg/ml BHT. Each sample was extracted three times with 1.0 ml of hexane by vortex-mixing the mixture for at least 1 min, followed by centrifugation at 850 *g* for 5 min. The hexane was carefully removed, the combined hexane layers were evaporated at room temperature under a stream of nitrogen in amber vials and stored at −20°C until analyzed by HPLC. The dry extracts were reconstituted at room temperature in 200 μl of the mobile phase directly before injecting into the HPLC system via autosampler.

Chromatographic conditions

HPLC analyses were carried out on an Adsorbosphere C_{18} (10 mm × 4.6 mm I.D.; 5 μm particle size) direct-connect guard column (Alltech) coupled to a Spherex 5 C_{18} (250 mm × 4.6 mm I.D.; 5 μm particle size) reversed-phase column (Phenomenex). Elution was carried out isocratically with a mobile phase consisting of methanol–dichloromethane–acetonitrile (65:25:10) containing 0.025% BHT, to which 2 ml of 0.5 *M* BTP (pH 7.0) per liter were added at a flow-rate of 1.5 ml/min. Analytes were monitored at the wavelength of highest sensitivity with a dual-wavelength diode-array detector as follows: channel A = 450 nm (for carotenoids); channel B = 310 nm during retinol and retinol palmitate elution and 295 nm during elution of tocol and tocopherols.

GC separations were performed on an HP-1 (12 m × 0.20 mm I.D.; 0.33 μm film thickness) capillary column (Hewlett-Packard) at 280°C injector and detector temperature using a linear gradient of 70°C/min for the column temperature starting at 40°C and holding at 260°C for 20 min.

Transesterification reaction

All-*trans*-ethyl-β-apo-8′-carotenoate (30 mg) was dissolved in THF (200 μl). Sodium cyanide (50 mg) and 6.0 ml of the appropriate alcohol were added, and the mixture was stirred under a stream of nitrogen at 40–110 °C in an open amber vial until the conversion was complete. Aliquots were removed periodically and analyzed by HPLC to calculate the conversion rates and yields. The latter were also determined by optical density readings assuming that the molar extinction coefficients of the β-apo-8′-carotenoates were identical.

The crude products from the reaction mixture were used directly as internal standards and were purified for MS and NMR measurements by a small open column filled with C_{18} material using a methanol–water gradient after removal of the alcohol under reduced pressure.

RESULTS AND DISCUSSION

Internal standards

The analyses of standard reference serum [45]

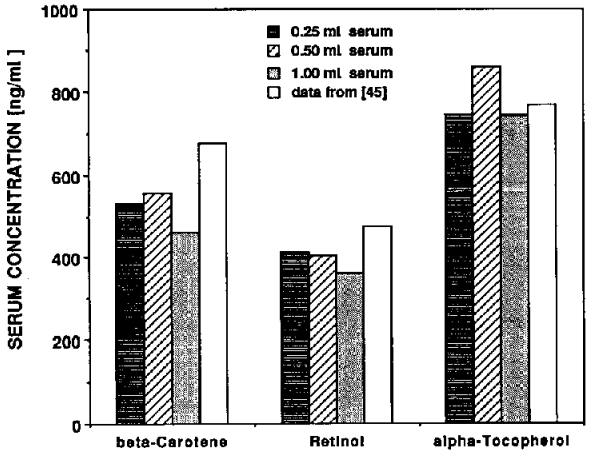


Fig. 1. Extraction efficiencies as a function of total serum volume extracted. The ethanol amount added equals the total serum amount in each extraction experiment (0.25, 0.5 and 1.0 ml of serum). The values shown were obtained from standard reference serum purchased from NIST [45] with medium micronutrient level and showed the same pattern as those obtained from low and high micronutrient levels (data not shown). Levels shown were not adjusted for internal standard recoveries and were therefore lower than the reported values [45]. Further extraction conditions as described in Experimental.

TABLE I

RECOVERY OF ANALYTES AND INTERNAL STANDARDS FROM SPIKED POOLED PLASMA SAMPLES ANALYZED IN TRIPLICATE

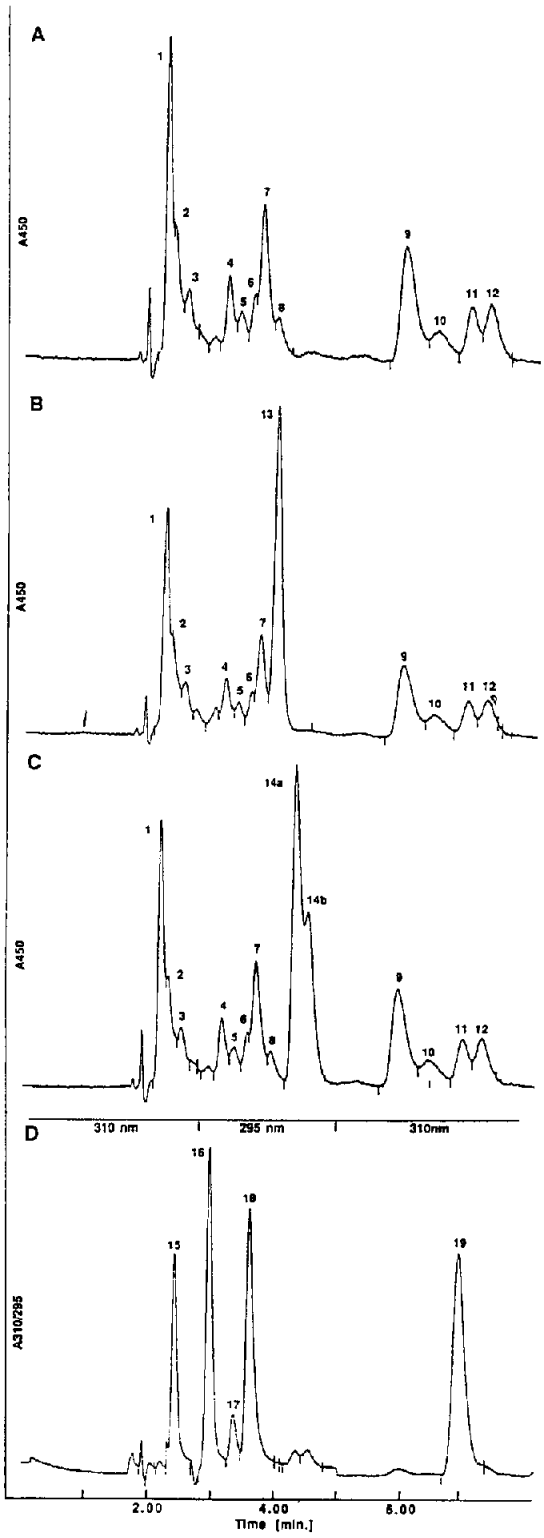
Compound	Amount added (μg)	Recovery (mean ± S.D.) (%)
Retinol	0.5	80 ± 3.7
Retinol palmitate	0.5	82 ± 2.4
γ-Tocopherol	10.0	97 ± 1.1
Tocol	3.0	98 ± 0.9
β-Carotene	0.5	93 ± 2.3
3a/b	0.3	95 ± 1.1

resulted in very similar levels for α-tocopherol, but 15–20% lower levels for β-carotene and retinol compared to the official values when we extracted and analyzed the standard serum without internal standards (Fig. 1). Spiking experiments with α-tocopherol, β-carotene and retinol shown in Table I demonstrated the absolute need of internal standards for each of the three individual analyte classes since each class showed different recoveries, probably due to differential binding to serum proteins. Additionally, internal standards correct for losses during the multi-step extraction procedure, for errors during the HPLC injection and for variabilities in the volume of the final extract due to non-volatile plasma lipids.

Tocol was used as internal standard for the tocopherols and retinyl palmitate for retinol. Initially ethyl-β-apo-8′-carotenoate (**1a**), an inexpensive, commercially available, synthetic carotenoid [46,47], was used as an internal standard for the carotenoids, but this compound was found to be unsuitable since it co-eluted with *cis*-cryptoxanthin in our HPLC system and appeared on the downhill slope of the β-cryptoxanthin peak (Fig. 2B) interfering with its integration.

Carotenoid synthesis

We concluded that a carotenoid was required with slightly lower polarity than **1a**, eluting later in an uncrowded area of the chromatogram. Therefore ethyl-β-apo-8′-carotenoate (**1a**) was

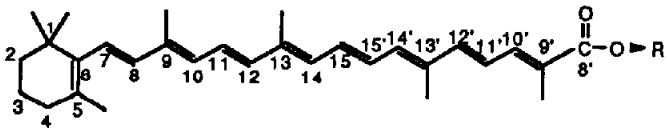


modified chemically by increasing the chain length of its alcohol unit via transesterification. Among the many recommended catalysts for transesterification [48] sodium or potassium cyanide [49] was found to be the most efficient with conversion rates of up to 99.95% and yields up to 72% (Table II). When the reaction was carried out between 70 and 120°C the products consisted of a mixture of *Z* (30%) and *E* (70%) isomers as indicated by the slightly longer retention of the *Z* isomer on reversed-phase material, its typical absorption at 330 nm and a negative shift in the maximum absorption by 4–5 nm compared to the *E* isomer (Table II) [42,43]. The *Z*/*E* ratio changed only for **3a/b** from 3:7 to 8:2 by storing the mixture in butanol or ethanol at −20°C for 2 weeks. The *E* isomer was obtained exclusively at reaction temperatures between 45 and 60°C and the complete conversion took 12 h at 20 Torr. Consequently, only the *E* isomer **2a** was obtained at various temperatures when **1a** was treated with methanol due to the low boiling point of the alcohol. No product was obtained at reaction temperatures below 40°C, and reaction temperatures over 130°C resulted in degradation of both the starting material and the product. Shorter conversion times were achieved by carrying out the reaction in an open vial in order to remove liberated ethanol, preventing competition with the de-

Fig. 2. HPLC analysis of micronutrients. HPLC of extracts from identical plasma samples (see Experimental for details): A and D = plasma spiked with only tocol and retinyl palmitate (A = carotenoid trace monitored at 450 nm; D = vitamin trace monitored at 310 nm during retinol and retinyl palmitate elution and at 295 nm during tocol and tocopherol elution); B = plasma spiked with tocol, retinyl palmitate and **1a** (carotenoid trace); C = plasma spiked with tocol, retinyl palmitate and **3a/b** (carotenoid trace). Peaks in the carotenoid trace (A–C): 1 = *trans*-lutein; 2 = *trans*-zeaxanthin; 3 = *cis*-lutein/*cis*-zeaxanthin; 4 = anhydrolutein; 5 = *cis*-anhydrolutein; 6 = α-cryptoxanthin; 7 = β-cryptoxanthin; 8 = *cis*-cryptoxanthin; 9 = lycopene (all isomers); 10 = dihydrolycopene; 11 = α-carotene; 12 = β-carotene; 13 = compound **1a** (internal standard); 14a+b = compound **3a+3b** (internal standard). Peaks in the vitamin trace (D): 15 = retinol; 16 = tocol (internal standard); 17 = γ-tocopherol; 18 = α-tocopherol; 19 = retinyl palmitate (internal standard).

TABLE II

TRANSESTERIFICATION: REACTION CONDITIONS, STRUCTURES AND RELEVANT DATA OF THE PRODUCTS OBTAINED FROM ETHYL-β-APO-8'-CAROTENOATE (**1a**)



All-*trans*-β-apo-8'-carotenoate

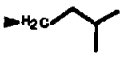
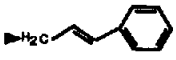
R	Structure	Alcohol[a]	Time[b]	Conversion[c] (%)	Yield[d,e] (%)	λ_{max}[f] (nm)	RRT[g]
C_2H_5	**1a** (*E*)					441–447	1.000[i]
	1b (*Z*)[h]		2	28.0	28.0	329, 441	1.020
CH_3	**2a** (*E*) = 100%)	Methanol	15	95.0	N.A.	444	0.981
	2b (*Z* = 0%)					328, 438	1.000
n-C_4H_9	**3a** (*E* = 68%)	*n*-Butanol	10	99.95	79.4	441 447	1.089
	3b (*Z* = 32%)					328, 438	1.132
	**4a** (*E* = 69%)	Isobutanol	14	85.5	35.0	444	1.073
	4b (*Z* = 31%)					329, 438	1.118
	**5a** (*E* = 68%)	*sec*-Butanol	30	69.4	19.4	444	0.970
	5b (*Z* = 32%)					329, 438	1.000
n-C_5H_{11}	**6a** (*E* = 69%)	*n*-Pentanol	11	99.6	68.0	444	1.146
	6b (*Z* = 21%)					329, 438	1.198
	**7a** (*E* = 72%)	Isopentanol	21	84.6	43.2	444	1.125
	7b (*Z* = 28%)					329, 438	1.183
n-C_6H_{13}	**8a** (*E* = 71%)	*n*-Hexanol	20	95.4	46.0	445	1.227
	8b (*Z* = 29%)					329, 437	1.308
	**9a** (*E* = 72%)	Cyclohexanol	30	95.4	3.0	446	1.181
	9b (*Z* = 28%)					329, 438	1.240
n-C_7H_{15}	**10a** (*E* = 70%)	*n*-Heptanol	15	96.1	48.3	446	1.320
	10b (*Z* = 30%)					329, 438	1.431
	**11a** (*E* = 72%)	*sec*-Heptanol	15	17.8	5.6	442	1.226
	11b (*Z* = 28%)					329, 439	1.278
	**12a** (*E* = 71%)	Methylcyclo-	35	17.8	3.3	444	1.229
	12b (*Z* = 29%)	hexanol				329, 437	1.278
	**13a** (*E* = 68%)	*sec*-Octanol	35	39.3	14.1	441–447	1.313
	13b (*Z* = 30.5%)					329, 437	1.402
n-C_9H_{19}	**14a** (*E* = 70%)	*n*-Nonanol	30	96.6	37.1	443–447	1.470
	14b (*Z* = 10%)					328, 438	1.581
	(*Z* = 20%)					329, 437	1.602

TABLE II *(continued)*

R	Structure	Alcohol[a]	Time[b]	Conversion[c] (%)	Yield[d,e] (%)	λ_{max}[f] (nm)	RRT[g]
	**15a** (*E* = 71%) **15b** (*Z* = 27%)	Phenylethyl alcohol	15	21.6	5.1	441–448 330, 439	0.966 1.003
	**16a** (*E* = 71%) **16b** (*Z* = 29%)	Phenylpropyl alcohol	21	53.2	21.3	443–445 329, 436	1.071 1.129
	**17a** (*E* = 81%) **17b** (*Z* = 19%)	Cinnamyl alcohol	25	99.96	40.0	444 329, 440	1.068 1.115

[a] The alcohol functioned as both reactant and solvent.
[b] Reaction time in hours at 110–120°C without refluxing.
[c] Determined by HPLC peak areas: product/(product + starting material after conversion) × 100.
[d] Determined by HPLC peak areas: product/(starting material before conversion) × 100.
[e] Determined by optical density.
[f] Absorption maximum in mobile phase in nanometers.
[g] HPLC retention times relative to **1a** (see Experimental for details).
[h] Heating of **1a** in toluene resulted in *Z* isomerization.
[i] $k' = 1.105$.

sired alcohol during transesterification. The time for the complete conversion decreased with higher temperatures and increased with longer or more-branched hydrocarbon chains in the reactant alcohol in the order primary alcohols << secondary alcohols (Fig. 3). No products were obtained with tertiary alcohols or aromatic alcohols like naphthol, phenol or benzyl alcohol. However **1a** was converted into **15a/b** (21%) with phenethyl alcohol and into **16a/b** (53%) with phenylpropyl alcohol. Therefore only aromatic alcohols with an aliphatic hydroxyl group located at least two carbon atoms distant to the aromatic ring will transesterify. Increasing the length of the hydrocarbon chain in the alcohol unit of the product resulted in longer retention times during reversed-phase HPLC whereas products with unsaturated or aromatic alcohol units showed shorter retention times than their saturated analogues (Table II).

When **1a** was heated in an alcohol without addition of the cyanide, no conversion took place, and **1a** started degrading after approximately 2 h at 110°C. So the cyanide not only acted as a catalyst but appeared to stabilize the carotenoids.

Structure elucidation

In order to confirm the conversion of **1a** to the proposed structures we obtained mass spectra as well as high-field ^1H and ^{13}C NMR spectra of representative products. The mass spectra showed a strong molecular peak for all measured compounds and ions for benzene, toluene and xylene as well as fragments created by loss of these aromatic compounds (Fig. 4 and Table III) typical for the "chain" fragmentation of carotenoids [50–52]. The peak at *m/z* 415 is formed by loss of an alkoxy radical from the molecular ion, characteristic for an apo-8'-carotenoate. The loss of 134 m.u. and the appearance of a peak at *m/z* 133 is presumably due to dimethyltropone and the peak at *m/z* 119 is due to methyltropone or trimethylbenzene [53].

The signals in the ^1H NMR spectra were assigned by NOESY and homonuclear COSY experiments (Table IV) and were in good agreement with reported values of analogous carotenoids obtained with low-field NMR spectroscopy [50,51]. The signals for the alcohol residues changed in the products as expected from a triplet and a quartet in the starting material **1a** to

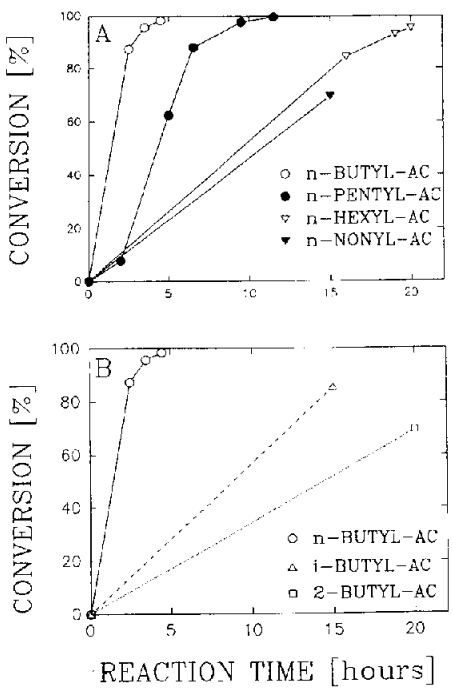


Fig. 3. Transesterification at 110°C of compound **1a** with various alcohols as a function of reaction time. (A) Conversion rate decreases with longer hydrocarbon chains in the alcohol. (B) Conversion rate is higher with primary than with secondary alcohols and decreases with more-branched hydrocarbon chains in the alcohol. n-butyl-AC = *n*-butyl-*β*-apo-8′-carotenoate (compound **3a/b**); n-pentyl-AC = *n*-pentyl-*β*-apo-8′-carotenoate (compound **6a/b**); n-hexyl-AC = *n*-hexyl-*β*-apo-8′-carotenoate (compound **8a/b**); n-nonyl-AC = *n*-nonyl-*β*-apo-8′-carotenoate (compound **14a/b**); i-butyl-AC = isobutyl-*β*-apo-8′-carotenoate (compound **4a/b**); 2-butyl-AC = 2-butyl-*β*-apo-8′-carotenoate (compound **5a/b**).

two triplets and two multiplets in **3a**, to two triplets and four multiplets in **8a** and to only one singlet in **2a**. Additional to the assigned signals for the all *E* isomers in Table IV the spectrum of the isomer mixture **3a/b** and **8a/b** showed doublets at 6.88 ppm (15.2 Hz), 7.16 ppm (15.0 Hz) and 7.32 ppm (11.0 Hz) with integrated proton amounts of 0.25, 0.15 and 0.40. Compared to all-*E* isomers apo-carotenoid *Z* isomers show a 0.5-ppm downfield shift for the proton positioned next to the isomerization center nearer to the cyclohexene ring [50,54–56]. Doublets with 15 Hz are caused in the measured molecule only by H-7, H-8, H-12 and H-12′. Therefore we assigned the signal at 6.88 ppm to H-12 of a 13-*Z* isomer and the signal at 7.16 ppm to H-12′ of a 13′-*Z* isomer since both signals are shifted downfield by 0.53 ppm relative to the all-*E* isomer. The doublet at 7.32 ppm is due to the H-10′ of both *Z* isomers. Consequently, the two *cis* isomers are either the 13-*Z* isomer and the 13′-*Z* isomer or the 13-*Z* isomer and the 13,13′-di-*Z* isomer with a *Z*/*E* ratio of 0.4:1 which is close to the ratio of 3:7 already detected by HPLC. These data confirmed our earlier suspicions about the presence of at least two *cis* isomers in the product mixture as evidenced by a shoulder in the HPLC peak of compound **14b**.

The umambiguous assignment of all the carbon signals in the obtained ^{13}C NMR spectra (Table V) was achieved by ^{13}C–^{1}H shift-correlated spectroscopy except for the quaternary carbons, which were identified with SFORD experiments. C-1 and C-8′ were easily assigned since they are the most shielded and deshielded quaternary carbons respectively. The residual six carbons were assigned by comparison with data reported for *β*-carotene [54,56,57] and methylazafrin, a methyl-apo-10′-carotenoate [58].

Application of the new internal standards for plasma micronutrient quantification

Several methods were examined for optimal extraction efficiency using standard serum samples obtained from the National Institute of Standards and Technology (NIST) containing three different micronutrient levels determined by seven independent laboratories [45]. As shown in Fig. 5 best extraction efficiency was achieved using 0.5 ml of serum with a serum-to-ethanol ratio of 1:1 except for *β*-cryptoxanthin. A correction factor for this compound was included in the response factor (see Experimental) taking into account that the applied procedure resulted in 77% of the optimum extraction efficiency for *β*-cryptoxanthin (data not shown). Vigorous vortex-mixing of the plasma with the ethanol–hexane mixture was essential for optimal extraction, probably due to the mechanical effect of freeing the plasma components from binding proteins as previously reported [59]. We found that extracting once or twice with hexane resulted in micro-

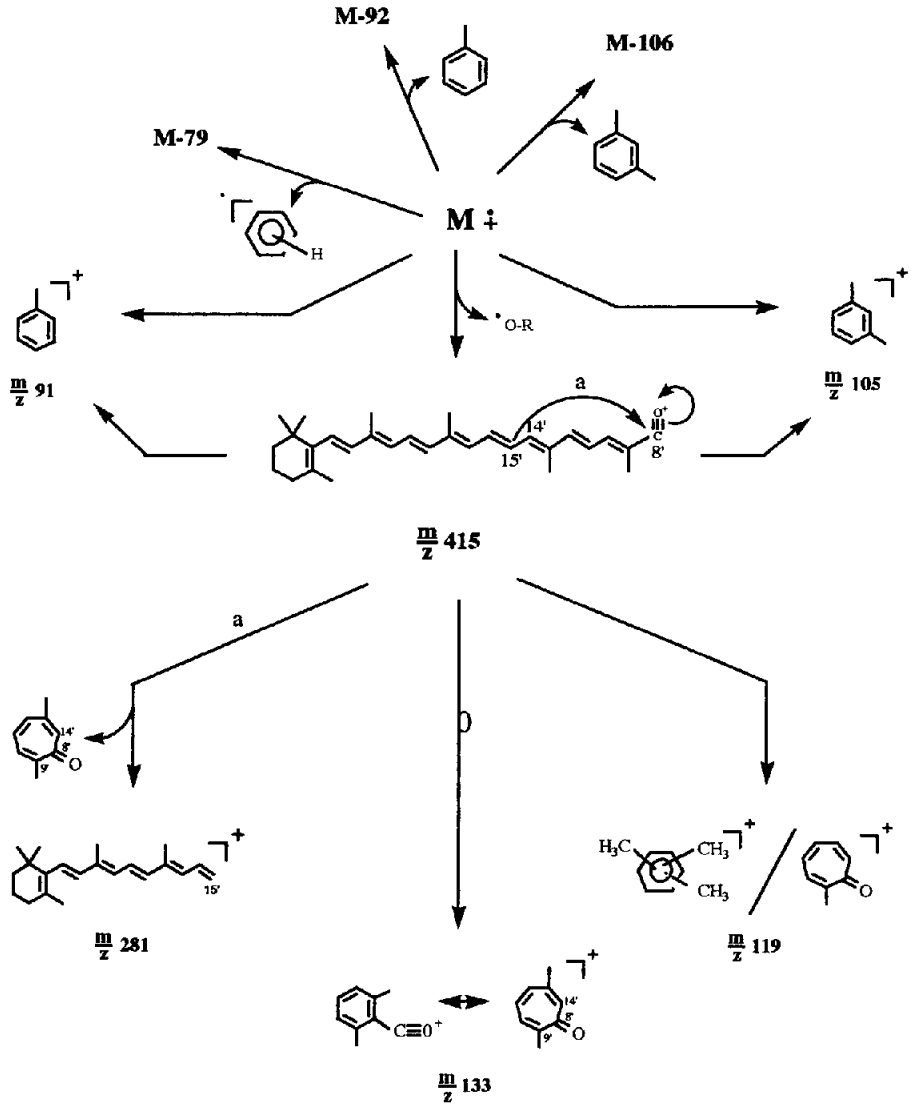


Fig. 4. Key fragments in the mass spectra of compounds **1a**, **2a**, **3a/b**, **6a/b** and **8a/b**.

nutrient recoveries of 88 or 96%, respectively, compared to three hexane extractions. The common technique of removing an aliquot of the hexane layer [15,26–28] led in our experiments to 130% recovery for the internal standards indicating possible evaporation and/or loss of hexane in the multiple layers of the sample [60]. The absolute amount of hexane did not affect the extraction efficiencies if volumes greater than 0.5 ml were used. We therefore extracted three times with 1.0 ml of hexane by vigorous, manual vortex-mixing followed by the careful removal of as much of the hexane layer as possible after centrifugation without removing any material from the other layers as this leads to lower extraction efficiencies.

BTP was added to the HPLC mobile phase to prevent carotenoid losses [35] due to acetonitrile [61] and to protect analytes against degradation caused by acids from chlorinated solvents or other impurities. Additionally, analytes were protected from oxidation by addition of BHT to the extraction solvent and mobile phase.

TABLE III

KEY FRAGMENTS AND THEIR INTENSITIES IN THE MASS SPECTRUM OF COMPOUNDS **1a**, **2a**, **3a/b**, **6a/b**, AND **8a/b**

The intensities are given in % relative to the base peak.

Compound	M^+	M−OR	M−79	M−92	M−106	*m/z* 281	*m/z* 133	*m/z* 119	*m/z* 105	*m/z* 91
1a	43; *m/z* 460	1.2; *m/z* 415	15; *m/z* 381	16; *m/z* 368	4.0; *m/z* 354	14	51	100	76	75
2a	4.0; *m/z* 446			3; *m/z* 354		33	40	100	57	46
3a/b	16; *m/z* 488		3; *m/z* 409			27	42	100	92	56
7a/b	16; *m/z* 502	0.2; *m/z* 415	0.3; *m/z* 423			48	58	100	90	57
9a/b	13 *m/z* 516	1.0 *m/z* 415				33	58	100	76	45

The individual plasma components were identified by comparing their absorption spectra with those obtained from standards as well as with literature values [37–44]. In this manner we were able to quantify (detection limits shown in Table VI) *trans*-lutein/*trans*-zeaxanthin, *cis*-lutein/*cis*-zeaxanthin, anhydrolutein, α-cryptoxanthin, β-cryptoxanthin, *cis*-cryptoxanthin, lycopene, dihydrolycopene, α-carotene, β-carotene, *cis*-carotene, retinol, δ-tocopherol, γ-tocopherol and α-tocopherol from serum or plasma simultanously by isocratic HPLC in less than 10 min (Fig. 2A–D).

HPLC methods with better resolution, in particular for minor carotenoid constituents or geometric carotenoid isomers [34,36], require elution with gradients or non-powerful mobile phases resulting in very long analysis times. However, as there is currently no evidence that these minor constituents possess any unique chemopreventive properties, no justification exists for the use of such a costly and time-consuming method in routine analyses for clinical and epidemiologic studies.

Compound **3a/b** appeared most promising as an internal standard for carotenoid quantitation since it eluted in our HPLC system in an uncrowded area of the chromatogram (Fig. 2C). Compound **3a/b** was prepared from **1a** at 110°C since the product is formed readily and the appearance of the **Z**–**E** mixture (3:7) in the HPLC chromatogram as a double peak does not interfere with its function as an internal standard. No further purification of this isomer mixture was necessary and the crude product was used directly as a stock solution to spike the ethanol designed to precipitate the plasma proteins. The *n*-butanol in the crude mixture used as transesterification reactant did not interfere with the extraction procedure nor with the HPLC analysis, and the cyanide prevented carotenoid degradation in the stock solution.

The accuracy of our method was determined by participating in the Round Robin test organized by the NIST [45]. Our results analyzing five serum samples deviated from the official values in average (data of mean deviation from 33 participating laboratories given in parantheses): *trans*-lutein/*trans*-zeaxanthin = 6.3% (16.3%); β-cryptoxanthin = 16.1% (16.1%); lycopene = 7.2% (23.7%); α-carotene = 17.6% (19.6%); β-carotene = 6.4% (13.7%); retinol = 5.5% (6.1%); γ-tocopherol = 6.3% (10.8%); α-tocopherol = 2.7% (8.2%).

We analyzed six plasma samples with a wide analyte concentration range in triplicate to deter-

TABLE IV

ASSIGNMENT OF ^1H NMR SIGNALS FROM ALL-*Z*-β-APO-8′-CAROTENOATES DISSOLVED IN C^2HCl_3

	2a[a]	**1a**[b,c]	**2a**[b–d]	**3a**[b–d]	**8a**[b–d]
H-2	1.47	1.469 (2H,m)	1.467	1.470	1.465
H-3	1.60	1.614 (2H,m)	1.616	1.674	1.612
H-4	2.02	2.004 (2H,m)	2.005	2.005	2.005
H-7	6.18	6.161 (3H,d,16)	6.164	6.162	6.157
H-8	6.14	6.127 (3H,d,16)	6.130	6.133	6.128
H-10	6.14	6.153 (3H,d,11.5)	6.153	6.153	6.147
H-11	6.71	6.686 (1H,dd,11.5,15)	6.690	6.688	6.682
H-12	6.35	6.354 (2H,d,15)	6.354	6.353	6.348
H-14	6.24	6.254 (1H,d,12)	6.256	6.255	6.250
H-15	N.A.[g]	6.710 (1H,dd,12,15)[e]	6.715[e]	6.710[e]	6.707[e]
H-15′	N.A.	6.616 (1H,dd,11,15)	6.612	6.617	6.610
H-14′	N.A.	6.359 (2H,d,11)	6.364	6.361	6.360
H-12′	6.53	6.617 (1H,d,15)	6.615	6.615	6.611
H-11′	N.A.	6.509 (1H,dd,11.3,15)	6.508	6.510	6.507
H-10′	7.28	7.289 (1H,dd,0.9,11.3)	7.293	7.283	7.278
CH_3-1,1	1.03	1.028 (6H,s)	1.029	1.029	1.023
CH_3-5	1.73	1.737 (3H,s)	1.718	1.717	1.710
CH_3-9	2.0	1.974 (2H,br.s)[f]	1.976[f]	1.975[f]	1.969[f]
CH_3-13	2.0	1.993 (2H,br.s)[f]	1.996[f]	1.990[f]	1.984[f]
CH_3-13′	2.0	1.993 (2H,br.s)[f]	1.996[f]	1.990[f]	1.984[f]
CH_3-9′	2.0	1.974 (2H,br.s)[f]	1.976[f]	1.975[f]	1.969[f]
COO-CH_2-α		4.216 (2H,q,7.1)		4.164 (2H,t,6.5)	4.146 (2H,t,6.6)
COO-CH_2-β				1.674 (2H,m)	1.677 (2H,m)
COO-CH_2-γ				1.414 (2H,m)	1.385 (6H,m)
COO-CH_2-δ					1.303 (6H,m)
COO-CH_2-ε					1.290 (6H,m)
COO-CH_3	3.76	1.315 (3H,t,7.1)	3.767 (3H,s)	0.963 (3H,t,7.3)	0.883 (3H,t,6.9)

[a] Data given in ref. 50 obtained at 220 MHz with TMS as internal standard (= 0.000 ppm).

[b] Our data obtained at 500 MHz with $CHCl_3$ as internal standard (= 7.260 ppm); assignments by homonuclear COSY and NOESY experiments.

[c] Values in parentheses: proton amount, multiplicity, coupling constant in Hz. (s = singlet; d = doublet; t = triplet; q = quadruplet; m = multiplet; br. = broad).

[d] Proton amount, multiplicity and coupling constants as described for **1a** if not noted otherwise.

[e] Assignment uncertain.

[f] Assignment interchangeable.

[g] N.A. = not assigned.

mine the precision of our procedure. The within-day coefficient of variation was found to be (between-day value in parentheses): *trans*-lutein/*trans*-zeaxanthin = 4.2% (0.9%); anhydrolutein = 4.3% (0.2%); α-cryptoxanthin = 1.8% (2.5%); β-cryptoxanthin = 3.4% (3.3%); lycopene = 2.9% (0.9%); α-carotene = 5.8% (9.2%); β-carotene = 3.2% (4.6%); retinol = 2.9% (0.2%); γ-tocopherol = 2.1% (2.0%); α-tocopherol = 3.0% (2.8%).

We analyzed the micronutrient levels of twenty-two individuals with this method in duplicate and our results compare favorably with similar studies as shown in Table VII.

TABLE V

ASSIGNMENT OF SIGNALS IN THE ^{13}C NMR SPECTRA OF COMPOUNDS **1a**, **2a**, **3a** AND **8a**

Assignments by COSY (^{13}C ^{1}H) and SFORD experiments. Signals obtained at 125 Mhz in C^2HCl_3 with $CHCl_3$ as internal standard set at 77.00 ppm. s = singlet; d = doublet; t = triplet; q = quadruplet.

	1a	2a	3a	8a
C-1	34.25s	34.27	34.27s	34.24
C-2	39.61t	39.64	39.64t	39.62
C-3	19.23t	19.24	19.27t	19.22
C-4	33.09t	33.11	33.11t	33.08
C-5	129.50s	129.51	129.50s	129.47
C-6	137.86s[a]	137.89[a]	137.89s[a]	137.69[a]
C-7	126.97d	127.01	126.99d	126.98
C-8	137.68d	137.68	137.68d	137.66
C-9	136.54s	136.58	136.56s	136.56
C-10	130.69d	130.69	130.70d	130.68
C-11	125.75d	125.80	125.76d	125.75
C-12	136.97d	136.97	136.97d	136.95
C-13	137.83s[a]	137.89[a]	137.83s[a]	137.81[a]
C-14	132.01d	132.04	132.01d	132.00
C-15	131.95d	132.00	131.94d	131.93
C-15′	129.34d	129.32	129.36d	129.34
C-14′	135.73d	135.85	135.72d	135.71
C-13′	126.16s	125.80	126.25s	126.22
C-12′	143.87d	144.07	143.88d	143.86
C-11′	123.16d	123.07	123.16d	123.15
C-10′	138.79d	139.10	138.76d	138.77
C-9′	135.41s	135.77	135.41s	135.38
C-8′	168.54s	169.01	168.63s	168.63
CH_3-1,1	28.96q	28.69	28.97q	28.94
CH_3-5	21.76q	21.75	21.76q	21.73
CH3-9	12.69q[a]	12.70[a]	12.70q[a]	12.67[a]
CH_3-13	12.77q[a]	12.77[a]	12.78q[a]	12.74[a]
CH_3-13′	12.83q[a]	12.84[a]	12.84q[a]	12.81[a]
CH_3-9′	12.87q[a]	12.87[a]	12.87q[a]	12.84[a]
COO-CH_2-α	60.48t		64.44t	64.73
COO-CH_2-β			30.32t	28.67
COO-CH_2-γ			19.27t	25.68
COO-CH_2-δ				31.43
COO-CH_2-ε				22.51
COO-CH_3	14.34q	51.77	13.76q	13.97

[a] Assignment interchangeable.

We also applied our method to compare the micronutrient levels from serum *versus* EDTA or heparin plasma from five individuals. No differences in the micronutrient levels were found after adjusting for internal standard recoveries.

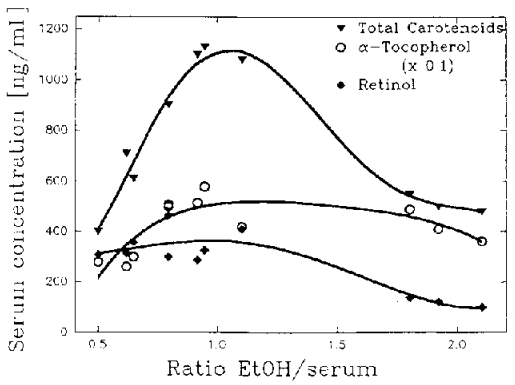


Fig. 5. Effect of ethanol on extraction efficiencies. Extraction efficiencies were determined as a function of the ethanol amount added to 1.0 ml of serum.

CONCLUSIONS

In this paper we have presented a fast, accurate and precise method for the simultaneous quantitation of twelve carotenoids, α-and γ-tocopherol and retinol from plasma or serum. This included an isolation procedure with maximum extraction efficiencies and a high-sensitivity HPLC system with a total analysis time of less than 10 min. We also showed the advantages in using an internal standard specific for each class of analyte, dem-

TABLE VI

DETECTION LIMITS OF A 20-μl HPLC INJECTION AT A SIGNAL-TO-NOISE RATIO OF 10

Compound	Detection limit (ng)
Retinol	0.9
α-Tocopherol	21.2
γ-Tocopherol	18.3
δ-Tocopherol	21.4
Lutein	0.5
β-Cryptoxanthin	0.6
Lycopene	0.6
α-Carotene	0.6
β-Carotene	0.7

TABLE VII

COMPARISON OF CAROTENOID, TOCOPHEROL AND RETINOL LEVELS IN HUMAN PLASMA AND SERUM BETWEEN THE PRESENTED AND PREVIOUS STUDIES

A = Present study; B = Schaefer-Ellinder and Walldius [25]; C = Cantilena *et al.* [13]; D = Palan *et al.* [4]; E = Ito *et al.* [27]; F = Krinsky *et al.* [34]; G = Stacewicz-Sapuntzakis *et al.* [22]. Values in parentheses are relative standard deviations (%). Previous studies represent results from healthy, non-smoking, non-medicated individuals. p = plasma; s = serum.

Analyte	Plasma or serum level (ng/ml)									
	A (p) (n = 22)	B (s) (n = 40)	C (s)		D (p) (n = 36)	E (s)		F (p) (n = 5)	G (s)	
			Diurnal (n = 33)	Seasonal (n = 29)		Male (n = 618)	Female n = 1196		Male (n = 55)	Female (n = 55)
trans-Lutein/ zeaxanthin	250 (30)		200	195				232	176 (46)	192 (47)
Anhydrolutein	80 (52)									
α-Cryptoxanthin	44 (41)							48		
β-Cryptoxanthin	145 (52)		95	112		190	336	117	85 (65)	100 (54)
Lycopene	306 (45)	227 (52)		340		187	246	203	212 (85)	188 (100)
α-Carotene	67 (62)	51 (86)	45	60		60	91	96	29 (79)	38 (63)
β-Carotene	261 (66)	280 (86)	130	200	287 (34)	183	339	338	168 (63)	237 (62)
Retinol	676 (22)	816 (19)			649 (24)	785	681		744 (28)	657 (28)
α-Tocopherol	10712 (27)	17000 (22)			6560 (41)	9920	10285			
γ-Tocopherol	2152 (49)	1880 (40)								

onstrating the usefulness of retinyl palmitate as internal standard for retinol analysis and tocol as internal standard for tocopherol analysis. We developed a fast, easy and inexpensive procedure for the synthesis of previously unavailable carotenoid analogues to be used as internal standards for carotenoid analyses. These synthetic carotenoids can be varied in polarity according to the chain length of the alcohol used as reactant during transesterification. In this way carotenoids can be custom-designed to function as internal standards without interfering with detection and quantitation of analytes during HPLC analyses.

In general the quantitation of lipophilic micronutrients with internal standards is critical because no procedure can mimic the binding of genuine analytes to their lipoproteins. Therefore the recoveries of standards or spiked materials will be greater than the recoveries of endogenous analytes. However, the approach described in this paper using three internal standards provides optimal accuracy for future epidemiologic and clinical studies.

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