An Isocratic Liquid Chromatographic Method with Diode-Array Detection for the Simultaneous Determination of μ-Tocopherol, Retinol, and Five Carotenoids in Human Serum

Sonia Gueguen[1], Bernard Herbeth[1], Gérard Siest[1], and **Pierre Leroy[2]**

[1]Inserm U525, Centre de Médecine Préventive, 2 rue du Doyen Jacques Parisot, 54500 Vandoeuvre-lès-Nancy, France and [2]Thiols and Cellular Functions, Faculté de Pharmacie, Université Henri Poincaré Nancy 1, 30 rue Lionnois, 54000 Nancy, France

Abstract

An isocratic high-performance liquid chromatography (HPLC) method for the simultaneous determination of α-tocopherol, retinol, and five carotenoids (lutein–zeaxanthin, β-cryptoxanthin, lycopene, and α- and β-carotene) in human serum is described. Serum samples are deproteinized with ethanol and extracted once with *n*-hexane. Resulting extracts are injected onto a C18 reversed-phase column eluted with methanol–acetonitrile–tetrahydrofuran (75:20:5, v/v/v), and full elution of all the analytes is realized isocratically within 20 min. The detection is operated using three channels of a diode-array spectrophotometer at 290, 325, and 450 nm for tocopherol, retinol, and the carotenoids, respectively. An internal standard is used for each channel, which improves precision. The choice of internal standards is discussed, as well as the extraction protocol and the need for adding an antioxidant during the extraction and chromatographic steps. The analytical recoveries for liposoluble vitamins and carotenoids are more than 85%. Intra-assay relative standard deviation (RSD) values (*n* = 20) for measured concentrations in serum range from 3.3% (retinol) to 9.5% (lycopene), and interassay RSDs (*n* = 5) range from 3.8% (α-tocopherol) to 13.7% (β-cryptoxanthin). The present method is used to quantitate the cited vitamins in healthy subjects (*n* = 168) from ages 9 to 55 years old.

Introduction

Retinol (vitamin A) and α-tocopherol (vitamin E) are nonenzymatic antioxidants (1). Vitamin A acts as a direct "scavenger" of reactive oxygen species (ROS) and is also thought to inhibit free radical synthesis via increasing the activity of detoxifying systems (2).

* Author to whom correspondence should be addressed: email pierre.leroy@pharma.uhp-nancy.fr.

Vitamin E protects unsaturated fatty acids located in both cell and organelle membranes against endo- and exogenous free radicals and ROS, which are involved in the initiation and extent of membrane damages caused by nonenzymatic lipid peroxidation (3,4). Carotenoids act as ROS and free radical scavengers (5), stimulants of immune response (6), and anticarcinogenic agents (7). Because of their wide variety of functions and biological roles, clinical interest in the evaluation of retinol, α-tocopherol, and carotenoids has increased in recent years owing to their role as antioxidants, which may be important in reducing the risk of numerous diseases including cancer (8–11), coronary heart disease (12,13), and diabetes mellitus (14–18).

Thus, rapid, simple, sensitive, and selective methods for the simultaneous determination of these antioxidants in biological fluids are needed. As a matter of fact, the measurement of an individual class of antioxidants such as thiols (19), hydrophilic, or liposoluble vitamins provides more information for the mechanistic evaluation of a clinical disease linked to oxidative stress than a total antioxidant status assay (20).

Numerous spectroscopic and separative methods have already been reported for the assay of retinol, α-tocopherol, and carotenoids in plasma or serum, and among them high-performance liquid chromatography (HPLC) is one of the most powerful analytical tools for this purpose (21–25).

Both normal-phase (26–28) and reversed-phase (29–35) HPLC conditions have been widely used. However, many of these methods include gradient elution (36–39), flow rate (34,36), wavelength time-programmation (36,40), a switching device between coupled columns (41,42), and the use of two different detectors in series (43,44). All of these approaches are time-consuming because of their long-equilibration period between each run and troublesome because of the hyphenated systems needed.

Indeed, the main difficulty for the simultaneous determination of liposoluble vitamins and carotenoids results from their different spectral characteristics (absorption maxima vary in the

range of 292 to 450 nm). This problem has been solved by using multichannel UV–vis spectrophotometric detectors (31,37,40, 45–47). More recently, a technique combining both isocratic elution in reversed-phase mode and diode-array detection was reported, providing selectivity between the three classes of liposoluble vitamins and thus a convenient way for their simultaneous measurements (32).

For all these methods, the preanalytical treatments, especially the extraction procedure relying upon either liquid–liquid (26–28,30–35,39,43,47,48) or solid–liquid (38,49,50) partition, are critical steps to obtain reliable data.

This study deals with some improvements of a previously reported method (32); the full validation of the optimized assay; and its use to quantitate retinol, α-tocopherol, lutein–zeaxanthin, β-cryptoxanthin, lycopene, and α- and β-carotene in healthy subjects.

Experimental

Chemicals, reagents, and standards

All solvents and reagents used were of analytical- or HPLC-grade. Ultrapure water was prepared using a Milli-Q system (Millipore Milford, MA). *Tert*-butylated hydroxytoluene (BHT) was purchased from Sigma-Aldrich (St. Quentin Fallavier, France).

All-*trans* retinol (henceforth simply referred to as retinol), retinol acetate, α-tocopherol, α-tocopherol acetate, and β-carotene standards were obtained from Fluka (Buchs, Switzerland). Zeaxanthin and β-cryptoxanthin were a generous gift from Hoffman-Laroche (Basle, Switzerland). Lycopene and echinenone were purchased from CaroteNature (Lupsingen, Switzerland). Stock solutions of retinol, α-tocopherol, and their corresponding internal standards (acetate form) were prepared in ethanol (EtOH) added with 0.01% (w/v) BHT. Carotenoids were prepared in tetrahydrofuran (THF) added with 0.01% BHT. Stock solutions were protected from light in ambered glass bottles, titrated by spectrophotometry using their specific absorbance (Table I), and stored under nitrogen at –80°C for up to 2 mo. The concentrations of stock solutions were 0.25–0.5 mg/mL for retinol and retinol acetate, 3–4 mg/mL for α-tocopherol and α-tocopherol acetate, and 0.1–0.2 mg/mL for carotenoids.

Daily working solutions for calibration curves were prepared by diluting stock solutions in EtOH containing 0.01% BHT. The ranges of tested concentrations are indicated in Table II. An internal standard mixture containing retinol acetate, α-tocopherol acetate, and echinenone was also prepared daily following a similar procedure (combining 100 µL of each stock solution of internal standard and diluting the volume to 20 mL with EtOH–0.01% BHT). All the operations were performed by handling solutions in darkness and ice.

The standards of β-carotene and zeaxanthin were used to quantitate α-carotene and both lutein and zeaxanthin, respectively.

Table I. Characteristics of Standards Used

Compounds	Molecular weight (g/mol)	Maximum wavelength (nm)	$A^{1\%}_{1\ cm}$*	ε ($mol^{-1}/L/cm^{-1}$)
Retinol	286.5	325	1835 (32,61)	52573
Retinol acetate	328.5	326	1550 (32,61)	50912
α-Tocopherol	430.7	292	75.8 (45)	3265
α-Tocopherol acetate	472.8	290	40 (32)	1891
Echinenone	550.9	458	2244 (Hoffmann-Laroche data source)	123622
Lutein–zeaxanthin	568.9	452	2765/2416 (45)	157301/137446
β-Cryptoxanthin	552.9	452	2486 (45)	137451
Lycopene	536.9	472	3450 (32,61)	185231
β-Carotene	536.9	450	2620 (35)	140667

* In EtOH as the solvent. Data references appear in the parentheses.

Blood collection and storage conditions

Blood was collected at the antecubital vein of 168 healthy control subjects from ages 9 to 55 years old (informed consent was obtained, and the research protocol was in agreement with the Helsinki Declaration) in a reclined position in dry tubes (Vacutainer Tube, Becton Dickinson, Grenoble, France). Blood samples were cen-

Table II. Equations of Calibration Curves and Values of LODs and LOQs*

	Equations of calibration curves					
	Concentration range (µmol/L)	Slope† (SD‡, *n* = 5)	Intercept (SD, *n* = 5)	Correlation coefficient†	LOD (µmol/L)	LOQ (µmol/L)
Retinol	0.45–7.50	0.16 (0.015)	0.021 (0.016)	0.998	0.45	0.66
α-Tocopherol	4.80–80.0	0.01 (0.000)	0.027 (0.008)	0.996	2.64	5.36
Lutein–zeaxanthin	0.10–1.90	0.35 (0.034)	0.024 (0.006)	0.997	0.06	0.11
β-Cryptoxanthin	0.09–1.50	0.34 (0.031)	0.022 (0.019)	0.996	0.03	0.09
Lycopene	0.12–1.90	0.24 (0.018)	0.018 (0.020)	0.997	0.03	0.08
β-Carotene	0.13–2.00	0.35 (0.019)	0.014 (0.006)	0.997	0.03	0.06

* Each calibration curve included six points, and each point was assayed in five replicates.
† Calculated by internal standardization: (standard peak area/internal standard peak area)/standard concentration.
‡ SD, standard deviation.

trifuged (1500 g for 15 min at 4°C) within 2 h after collection, and resulting serum samples were frozen in liquid nitrogen until HPLC analysis.

Serum sample treatment

All the handling operations were carried out in darkness. The serum samples were rapidly thawed at room temperature, homogenized, and 200 µL was transferred into a borosilicate glass tube kept on ice and 300 µL of the internal standard mixture added. After mixing with a vortex for 20 s, proteins were precipitated by adding 200 µL of EtOH–0.01% BHT, and the volume was diluted to 1 mL with ultrapure water. After mixing with an orbital shaker at 2500 rpm for 1 min, 2 mL of *n*-hexane–0.01% BHT was added. The samples were shaken for 1 min and centrifuged at 2700 g for 20 min at 4°C.

The organic layer was carefully transferred into a glass tube and evaporated to dryness under a stream of nitrogen at room temperature. The dried residue was redissolved in 25 µL of THF–0.01% BHT and vortexed for 30 s. A 75-µL amount of mobile phase was added, and the resulting mixture was vortexed for another 30 s. Samples were then transferred to 200-µL insert vials and placed into the HPLC autosampler.

HPLC system and operating conditions

The HPLC system consisted of an isocratic solvent delivery pump (Model Kontron Instruments 422), an autosampler equipped with a 20-µL injection loop, a cooling sample tray and a column oven (Model AS-300, ThermoQuest, Les Ulis, France), a UV–vis diode-array detector (Model Gold LC-168, Beckman Coulter, Fullerton, CA), and data-processing software (Gold New, Beckman).

A guard column (8- × 3-mm i.d.) packed with Nucleosil C18 (5 µm) (Macherey Nagel, Duren, Germany) and an analytical column (250- × 3-mm i.d.) packed with Nucleosil 100 C_{18} (5 µm) (Macherey Nagel) were eluted with a mobile phase consisting of a mixture of methanol–acetonitrile–tetrahydrofuran (75:20:5, v/v/v) containing 0.01% (w/v) BHT. The mobile phase was filtered through a 0.45-µm Nylon membrane and was used at a column temperature of 35°C and a flow rate of 0.6 mL/min. Three channels corresponding with different wavelength values were used to acquire data for the selective monitoring of α-tocopherol (290 nm), retinol (325 nm), and carotenoids (450 nm) and their respective internal standard. During analysis, the tray compartment containing sample vials was cooled at 5°C. After each working period (approximately 50 samples), it was necessary to rinse the column with methanol at a flow rate of 0.6 mL/min for 20 min to eliminate highly hydrophobic compounds and prevent the loss of column efficiency.

Calculation

The vitamin concentrations were determined from a standard curve of the peak-area ratio of the analyte–internal standard plotted against the concentration of analyte (expressed in micromoles per liter). A linear least-square regression analysis was performed for each analyte, and the standard curve was repeated if the correlation coefficient was below 0.990.

The detection limit (LOD) and the quantitation limit (LOQ) were expressed, respectively, as:

$$LOD = (a_0 + 3sa_0) / a_1 \quad \text{Eq. 1}$$

and

$$LOQ = (a_0 + 10sa_0) / a_1 \quad \text{Eq. 2}$$

where a_1 is the slope, a_0 the intercept, and sa_0 the standard deviation of the intercept (51).

Quality control

A human serum pool made with 1 mL of fresh serum from 100 healthy subjects and stored at –80°C was used for the routine quality control. Aliquots were extracted and analyzed according to the same procedure that was described previously. Evaluation of the method performance was assessed by comparing the results of the quality control with the means and relative standard deviations (RSDs) calculated using results from several preliminary runs (n = 20 per day for five days).

Results and Discussion

Optimization of sample treatment and HPLC technique

The basic method used in this study has been described by Talwar et al. (32). Some modifications relating to the internal standards, the sample preparation procedure, and the use of an antioxidant during both the extraction and chromatography processes have been made. We chose this method because it allows in a fast and easy way the simultaneous separation of the two classes of lipophilic vitamins (namely retinol, α-tocopherol, and carotenoids). Our main objective was to measure simultaneously lipophilic vitamins and carotenoids, which are the most abundant in human serum. Thus, the separation of the isomers of retinol, α-tocopherol, and carotenoids did not appear relevant for our present epidemiological studies.

In most methods, the use of an antioxidant during sample treatment was demonstrated to be necessary to prevent a significant loss in carotenoid contents, especially lycopene and β-carotene (32,37,39,40,47). Thus, we initially added 0.01% ascorbic acid to the organic solvents used for the standards preparation (EtOH and THF) and to the mobile phase, as indicated by Talwar et al. (32). After analyzing the same sample several times, we observed a decrease of the carotenoid concentrations, indicating degradation as a function of time. We tested another antioxidant (BHT) that is widely used in other methods (37,39,47) and added it to the mobile phase and all the solvents (EtOH, THF, and hexane) used for the standard and sample preparation. Indeed, hexane containing BHT efficiently protected the carotenoids from degradation during the evaporation of the extractive organic layers, and the addition of BHT to the mobile phase also prevented any loss of these analytes and probably helped increase the longevity of the column by neutralizing peroxides present in THF. Moreover, we observed that decreasing the evaporation temperature from 40°C to room temperature significantly increased carotenoid recoveries, as already noted by different authors (39,43).

Other parameters have to be optimized in order to provide the best conditions for the extraction of liposoluble vitamins and

carotenoids. The addition of ultrapure water to the deproteinized serum with EtOH has been noted to improve the recoveries of carotenoids and liposoluble vitamins (37,52). We tested several EtOH–water proportions in the 1:4 to 1:1 range (v/v) in order to obtain the highest recoveries, and we selected the 1:1 (v/v) proportion. Single and double extraction steps with *n*-hexane (an increase of the shaking period) were tested, but no significant improvement of recoveries was observed.

The method previously described (32) used two internal standards: retinol acetate as an internal standard for retinol, and α-tocopherol acetate as an internal standard for both α-tocopherol and carotenoid. We used a third internal standard (echinenone) for the quantitation of the carotenoids. Echinenone is a synthetic carotenoid and has a structure and chemical properties very similar to the naturally occurring carotenoids in serum. Thus, the use of echinenone is preferable to the use of retinol acetate and α-tocopherol acetate or tocol currently used in other methods (34,43,47), because it is detected at the same wavelength as the other carotenoids and is light- and temperature-sensitive as other carotenoids. Thus, the use of three internal standards allows for a better quality control and helps to correct analytical variations occurring for each liposoluble vitamin and carotenoid during the extraction and chromatography processes.

Because no loss of analytes was observed in serum extracts kept in darkness for at least 24 h at 5°C, as already reported (39), the automation of the technique was possible with a high throughput of samples (approximately 30 per day).

Several methods have been developed to measure the main

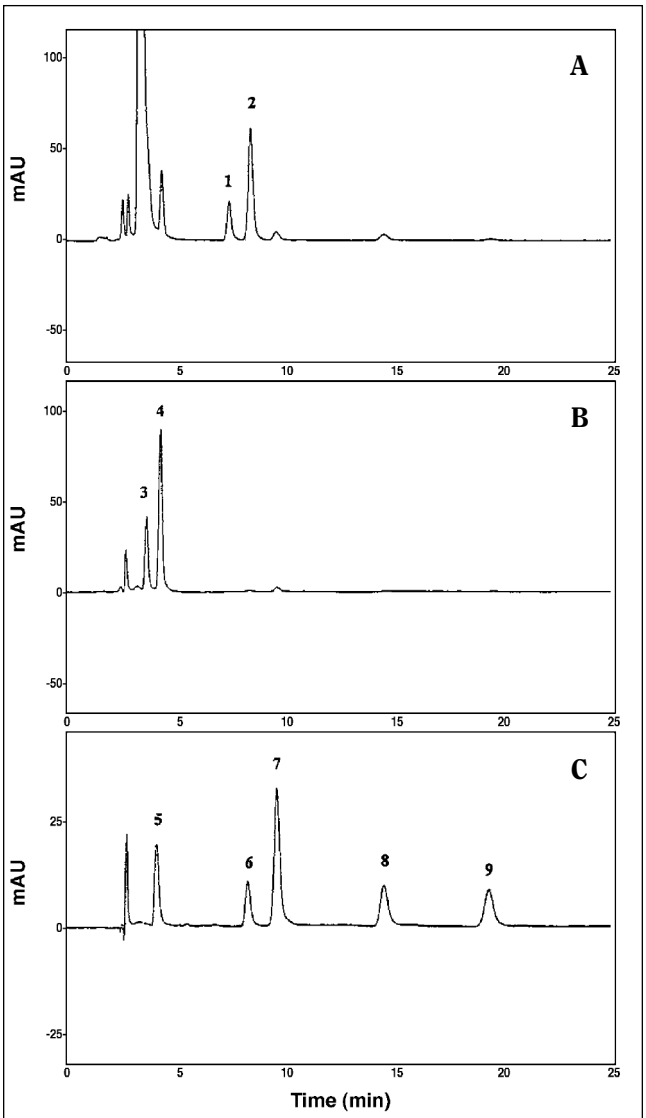

Figure 1. Typical chromatograms corresponding with a mixture of retinol, α-tocopherol, and carotenoid standards: (A) channel 1, diode-array detection at 290 nm for α-tocopherol and α-tocopherol acetate; (B) channel 2, diode-array detection at 325 nm for retinol and retinol acetate; and (C) channel 3, diode-array detection at 450 nm for carotenoids and echinenone. The peak numbers are as follows: (1) 26 µmol/L α-tocopherol, (2) α-tocopherol acetate (the internal standard), (3) 2.43 µmol/L retinol, (4) retinol acetate (internal standard), (5) 0.62 µmol/L lutein–zeaxanthin, (6) 0.49 µmol/L β-cryptoxanthin, (7) echinenone (internal standard), (8) 0.62 µmol/L lycopene, (9) α-carotene, and (10) 0.65 µmol/L β-carotene.

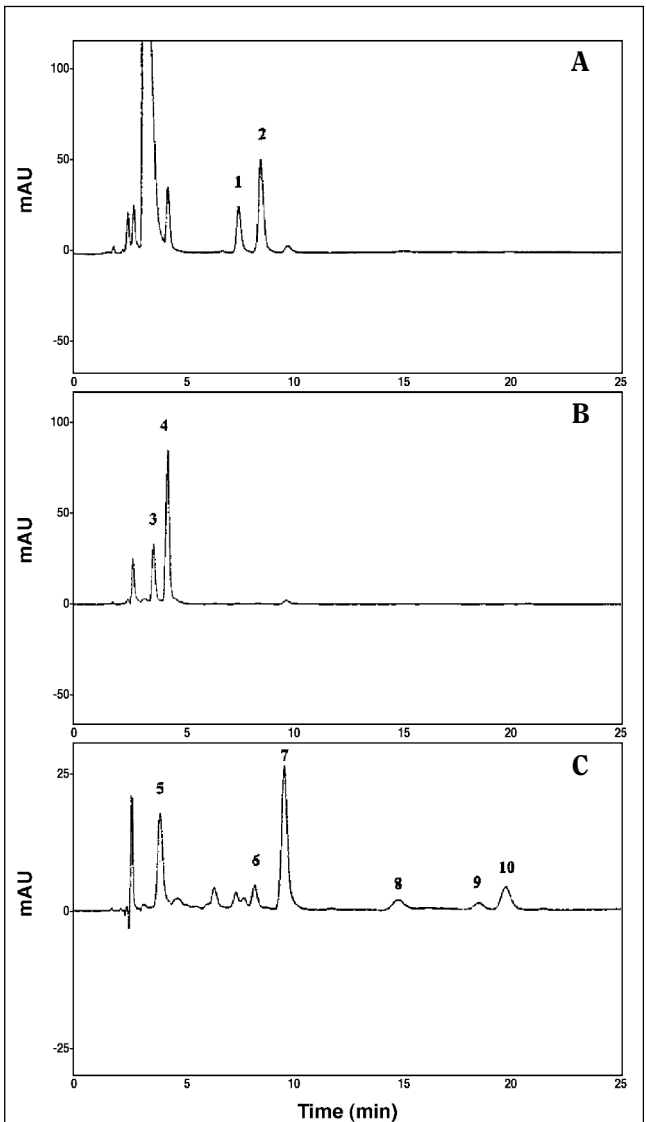

Figure 2. Typical chromatograms corresponding with an extract of a human serum sample: (A) channel 1, diode-array detection at 290 nm for α-tocopherol and α-tocopherol acetate; (B) channel 2, diode-array detection at 325 nm for retinol and retinol acetate; and (C) channel 3, diode-array detection at 450 nm for carotenoids and echinenone. Peak numbers are the same as Figure 1.

carotenoids present in serum in one run simultaneously with α-tocopherol and retinol (30–34,37,47). Most carotenoids are detected at 450 or 473 nm, but α-tocopherol and retinol can only be detected at 290 and 325 nm, respectively. Most of the previously mentioned methods therefore require the use of several detectors in series (43,44) and a multiwavelength detector either with simultaneous monitoring at different wavelengths (31,36,37,40,53) or a change in the detection wavelength during the run (30,32,44,47). The need for simultaneous detection at different wavelengths is illustrated by the retinol and lutein–zeaxanthin that elute within a 0.3-min interval and have to be detected at 325 and 450 nm, respectively. Typical chromatograms of a standard mixture and an extracted human serum are shown in Figures 1 and 2. The chromatograms revealed elution and baseline resolution between all the analytes of interest except for lutein and zeaxanthin, which are not separated by this method. The internal standard echinenone was eluted between β-cryptoxanthin and lycopene and thus did not interfere with the other carotenoids analyzed. Several additional carotenoids not identified as of yet appeared between the peak of lutein–zeaxanthin at 4 min and β-cryptoxanthin at 8 min. Before validation of the HPLC method, we have realized a selectivity study, and BHT has been analyzed with other analytes to see any potential chromatographic interference. BHT elutes with a short retention time (within 3 min) and is only detectable at 290 nm, thus no interference with vitamins was observed.

Assay validation and quality control of the HPLC method

The quantitation was achieved using the internal standardization mode. Data concerning linearity (the linearity range for each liposoluble vitamin was selected according to its physiological values), LOD, and LOQ are indicated in Table II (and precision in Table III).

The LOD and LOQ values agree with previous data in the literature (32). In order to calculate recoveries, a pooled serum was spiked with 20 µL of combined standards to provide the added concentrations of 0.7 µmol/L retinol, 8.7 µmol/L α-tocopherol, and 0.15 to 0.2 µmol/L carotenoids. The spiked serum samples (n = 5) were then extracted using a single extraction step with n-hexane. Recoveries found were 99.6% ± 11.1% for retinol, 91.2% ± 2.0% for retinol acetate, 109.4% ± 13.4% for α-tocopherol, 101.2% ± 3.0% for α-tocopherol acetate, 112.6% ± 22.2% for lutein–zeaxanthin, 104.3% ± 9.1% for β-cryptoxanthin, 109.4% ± 31.0% for lycopene, 85.1% ± 8.5% for β-carotene, and 95.6% ± 9.5% for echinenone. The different behaviors of carotenoids with regard to extraction using n-hexane has already been reported by Barua et al. (48). The calculated recoveries in this study are satisfactory and comparable with previously reported values (30,32,33).

In order to check the precision of the method, a human serum pool was analyzed 20 times during the same day to assess the repeatability. This operation was repeated 5 times over a period of one month to evaluate the interassay precision. The intra- and interassay variations calculated for each vitamin are shown in

Table III. Precision of the HPLC Assay of Liposoluble Vitamins and Carotenoids in Serum

	Within run		Between run	
Analyte	Concentration* (µmol/L)	%RSD	Concentration† (µmol/L)	%RSD
Retinol	1.90 (0.06)	3.3	2.1 (0.09)	4.4
α-Tocopherol	34.9 (1.31)	3.8	29.3 (1.1)	3.8
Lutein–zeaxanthin	0.65 (0.02)	3.8	0.51 (0.02)	4.5
β-Cryptoxanthin	0.13 (0.01)	7.8	0.10 (0.01)	13.7
Lycopene	0.53 (0.05)	9.5	0.28 (0.04)	12.5
α-Carotene	0.18 (0.02)	8.8	0.14 (0.02)	12.1
β-Carotene	0.57 (0.04)	6.7	0.52 (0.05)	9.1

* Mean (standard deviation), n = 20.
† Mean (standard deviation), n = 5.

Table IV. Concentrations of Retinol, α-Tocopherol, and Carotenoids in Millimoles per Liter Measured in the Serum of 168 Healthy Subjects from Ages 9 to 55 Years Old and a Comparison with Other Studies

	Present study*							
	Men		Women					
Compound	9–20 years old	21–55 years old	9–20 years old	21–55 years old	Sowell et al.*,† (32)	Talwar et al.*,‡ (37)	Steghens et al.*,§ (54)	Olmedilla et al.**,†† (31)
Retinol	1.37 (0.36)	2.18 (0.43)	1.36 (0.31)	1.86 (0.53)	2.00 (0.60)	1.84 (0.80)	1.71 (0.39)	1.91 (1.05–2.97)
α-Tocopherol	20.6 (4.08)	29.7 (8.16)	23.6 (10.9)	26.6 (6.38)	29.6 (7.60)	33.0 (6.67)	32.7 (7.40)	25.7 (13.9–47.0)
Lutein	0.42 (0.12)‡‡	0.43 (0.24)‡‡	0.49 (0.23)‡‡	0.52 (0.25)‡‡	0.26 (0.11)‡‡	0.71 (0.30)‡‡	0.24 (0.21)‡‡	0.36 (0.14–0.74)‡‡
Zeaxanthin	_‡‡	_‡‡	_‡‡	_‡‡	_‡‡	0.09 (0.05)	0.07 (0.04)	_‡‡
β-Cryptoxanthin	0.13 (0.08)	0.13 (0.11)	0.19 (0.14)	0.17 (0.12)	0.55 (0.11)	0.35 (0.27)	0.60 (0.47)	0.22 (0.05–0.52)
Lycopene	0.33 (0.16)	0.28 (0.16)	0.31 (0.16)	0.32 (0.22)	0.37 (0.18)	0.56 (0.43)	0.42 (0.24)	0.40 (0.11–0.80)
α-Carotene	0.08 (0.06)	0.10 (0.13)	0.13 (0.11)	0.14 (0.14)	0.07 (0.04)	0.36 (0.26)	0.07 (0.05)	0.08 (0.02–0.22)
β-Carotene	0.49 (0.43)	0.42 (0.29)	0.60 (0.37)	0.64 (0.72)	0.38 (0.20)	0.81 (0.45)	0.37 (0.23)	0.34 (0.07–0.88)

* Means (standard deviation).
† Concentrations in serum for men and women ranging from ages 19 to 62 years old, n = 111.
‡ Concentrations in serum for women ranging from ages 35 to 50 years old, n = 96.
§ Concentrations in serum for women ranging from ages 25 to 59 years old, n = 54.
** Concentrations in serum for men and women ranging from ages 4 to 93 years old, n = 3480.

Table III. The RSDs ranged from 3.3% (retinol) to 9.5% (lycopene) for intra-assay precision and 3.8% (α-tocopherol) to 13.7% (β-cryptoxanthin) for interassay precision. The RSD values obtained for some carotenoids were comparable with those reported for most of the other assays (16,31,32). However, the RSDs obtained for retinol and α-tocopherol were lower than those reported in these methods. This serum pool was then used for routine quality control.

Assay of liposoluble vitamins and carotenoids in a healthy population

The validated method was applied to the measurement of retinol, α-tocopherol, and five carotenoids in the serum of 168 healthy Caucasian subjects (Table IV). In comparison with previously published studies, including more than 25 subjects (31,32,37,54), the value ranges were comparable for most of the liposoluble vitamins and carotenoids measured except for lutein–zeaxanthin, which was higher than the value found by Talwar et al. (32) but similar to other studies (31,54). Similar results were demonstrated in a previous study by De Leehneer et al. (55). This fact can probably be explained by differences between the populations involved in the different studies. We can also notice that lutein–zeaxanthin and lycopene were in the serum in significant quantities, thus β-carotene could not be measured alone as a representative marker of the serum carotenoids. As a matter of fact, the carotenoids exhibited different distributions between subjects, tissues (56,57), and food (58). Moreover, their antioxidant capacities and functions may differ at the cellular level (59). More recently, an HPLC system coupling two different C18 columns has been reported for the separation of 13 carotenoids in plasma (60), but the overall run time for one sample reached 50 min, which limits the throughput, and thus no important additional epidemiological information was given.

Conclusion

The reported HPLC method devoted to the assay of liposoluble vitamins and carotenoids in serum permits the separation of the main carotenoids (lutein–zeaxanthin, β-cryptoxanthin, lycopene, α- and β-carotene, retinol, and α-tocopherol) within 20 min, which allows a high throughput of samples. The method was run for several months in the routine laboratory and has clearly proven its reliability. Because of its specificity and sensitivity for a great number of liposoluble vitamins corresponding with important serum antioxidant biomarkers, this method has an evident interest for nutritional and epidemiological studies and is now applied to various pathological groups such as alcoholic and Type I diabetic patients.

Acknowledgments

This project was supported in part by a grant from the Association de la Recherche sur le Cholesterol (ARCOL). The authors gratefully acknowledge the technical staff of the Center of Preventive Medicine for their kind participation. The authors thank the Société Francophone des Biofacteurs et Vitamines for giving them the opportunity to participate in an interlaboratory quality control.

References

1. E. Niki. Antioxidants in relation to lipid peroxidation. *Chem. Phys. Lipids* **44:** 227–53 (1987).
2. K. Satoh, Y. Ida, H. Sakagami, T. Tanaka, and S. Fujisawa. Effect of antioxidants on radical intensity and cytotoxic activity of eugenol. *Anticancer Res.* **18:** 1549–52 (1998).
3. L. Tesoriere, A. Bongiorno, R. Re, and M.A. Livrea. Reciprocal protective effects of all-trans retinol and alpha-tocopherol during lipid peroxidation in retinal membranes. *Biochem. Mol. Biol. Int.* **37:** 1–7 (1995).
4. L. Tesoriere, A. Bongiorno, A.M. Pintaudi, R. D'Anna, D. D'Arpa, and M.A. Livrea. Synergistic interactions between vitamin A and vitamin E against lipid peroxidation in phosphatidylcholine liposomes. *Arch. Biochem. Biophys.* **326:** 57–63 (1996).
5. G.W. Burton and K.U. Ingold. beta-Carotene: an unusual type of lipid antioxidant. *Science* **224:** 569–73 (1984).
6. A. Bendich and J.A. Olson. Biological actions of carotenoids. *FASEB J.* **3:** 1927–32 (1989).
7. N.I. Krinsky. Carotenoids and cancer in animal models. *J. Nutr.* **119:** 123–26 (1989).
8. R.M. Niles. Vitamin A and cancer. *Nutrition* **16:** 573–76 (2000).
9. D.C. McMillan, N. Sattar, D. Talwar, D.S. O'Reilly, and C.S. McArdle. Changes in micronutrient concentrations following anti-inflammatory treatment in patients with gastrointestinal cancer. *Nutrition* **16:** 425–28 (2000).
10. A.A. Botterweck, P.A. van den Brandt, and R.A. Goldbohm. Vitamins, carotenoids, dietary fiber, and the risk of gastric carcinoma: results from a prospective study after 6.3 years of follow-up. *Cancer* **88:** 737–48 (2000).
11. D.S. Michaud, D. Feskanich, E.B. Rimm, G.A. Colditz, F.E. Speizer, W.C. Willett, and E. Giovannucci. Intake of specific carotenoids and risk of lung cancer in 2 prospective US cohorts. *Am. J. Clin. Nutr.* **72:** 990–97 (2000).
12. E.S. Ford and W.H. Giles. Serum vitamins, carotenoids, and angina pectoris: findings from the National Health and Nutrition Examination Survey III. *Ann. Epidemiol.* **10:** 106–16 (2000).
13. R.W. Evans, B.J. Shaten, B.W. Day, and L.H. Kuller. Prospective association between lipid soluble antioxidants and coronary heart disease in men. The multiple risk factor intervention trial. *Am. J. Epidemiol.* **147:** 180–86 (1998).
14. F.S. Facchini, M.H. Humphreys, C.A. DoNascimento, F. Abbasi, and G.M. Reaven. Relation between insulin resistance and plasma concentrations of lipid hydroperoxides, carotenoids, and tocopherols. *Am. J. Clin. Nutr.* **72:** 776–79 (2000).
15. F. Granado, B. Olmedilla, E. Gil-Martinez, I. Blanco, I. Millan, and E. Rojas-Hidalgo. Carotenoids, retinol and tocopherols in patients with insulin-dependent diabetes mellitus and their immediate relatives. *Clin. Sci. (Colch.)* **94:** 189–95 (1998).
16. B. Olmedilla, F. Granado, E. Gil-Martinez, I. Blanco, and E. Rojas-Hidalgo. Reference values for retinol, tocopherol, and main carotenoids in serum of control and insulin-dependent diabetic Spanish subjects. *Clin. Chem.* **43:** 1066–71 (1997).
17. M.C. Polidori, P. Mecocci, W. Stahl, B. Parente, R. Cecchetti, A. Cherubini, P. Cao, H. Sies, and U. Senin. Plasma levels of lipophilic antioxidants in very old patients with type 2 diabetes. *Diabetes Metab. Res. Rev.* **16:** 15–19 (2000).
18. M.A. Abahusain, J. Wright, J.W. Dickerson, and E.B. de Vol. Retinol, alpha-tocopherol and carotenoids in diabetes. *Eur. J. Clin. Nutr.* **53:** 630–35 (1999).

19. J.F. Salazar, H. Schorr, W. Herrmann, B. Herbeth, G. Siest, and P. Leroy. Measurement of thiols in human plasma using liquid chromatography with precolumn derivatization and fluorescence detection. *J. Chromatogr. Sci.* **37:** 469–76 (1999).
20. G. Cao and R.L. Prior. Comparison of different analytical methods for assessing total antioxidant capacity of human serum. *Clin. Chem.* **44:** 1309–15 (1998).
21. T. Arnhold and H. Nau. "Vitamin A". In *Modern Analytical Methodologies in Fat- and Water-Soluble Vitamins.* John Wiley & Sons, Inc., New York, NY, 2000, pp. 3–49.
22. H.J. Nelis, E. D'Haese, and K. Vermis. "Vitamin E". In *Modern Chromatographic Analysis of Vitamins,* 3rd ed. Marcel Dekker, Inc. New York, NY, 2000, pp. 143–228.
23. R.R. Eitenmiller and W.O. Landen. "Simultaneous Determination of Fat-Soluble Vitamins in Food, Feed, and Serum". In *Modern Analytical Methodologies in Fat- and Water-Soluble Vitamins.* John Wiley & Sons, Inc, New York, 2000, pp. 171–221.
24. A.B. Barua, H.C. Furr, J.A. Olson, and R.B. van Breemen. "Vitamin A and Carotenoids". In *Modern Chromatographic Analysis of Vitamins,* 3rd ed. Marcel Dekker, Inc., New York, NY, 2000, 1–74.
25. R.R. Eitenmiller and W. Landen. *Vitamin Analysis for the Health and Food Sciences.* CRC Press, Boca Raton, FL, 1998, 1–501.
26. R. Ohmacht, G. Toth, and G. Voigt. Separation of serum carotenoids and vitamin A on chromsil-amino and -cyano phases by a bi-directional gradient elution technique. *J. Chromatogr.* **395:** 609–12 (1987).
27. G. Panfili, P. Manzi, and L. Pizzoferrato. High-performance liquid chromatographic method for the simultaneous determination of tocopherols, carotenes, and retinol and its geometric isomers in Italian cheeses. *Analyst* **119:** 1161–65 (1994).
28. R.B. McGeachin and C.A. Bailey. Determination of carotenoid pigments, retinol, and alpha-tocopherol in feeds, tissues, and blood serum by normal phase high performance liquid chromatography. *Poult. Sci.* **74:** 407–11 (1995).
29. P. Grolier. "Dosages des Caroténoïdes Sanguins". In *Le statut vitaminique.* G. Le Moël, A. Saverot-Dauvergne, T. Gousson, and J.L. Guéant, Eds. EM Int., Cachan., France, 1998, pp. 42–48.
30. Z. Zaman, P. Fielden, and P.G. Frost. Simultaneous determination of vitamins A and E and carotenoids in plasma by reversed-phase HPLC in elderly and younger subjects. *Clin. Chem.* **39:** 2229–34 (1993).
31. A.L. Sowell, D.L. Huff, P.R. Yeager, S.P. Caudill, and E.W. Gunter. Retinol, alpha-tocopherol, lutein/zeaxanthin, beta-cryptoxanthin, lycopene, alpha-carotene, trans-beta-carotene, and four retinyl esters in serum determined simultaneously by reversed-phase HPLC with multiwavelength detection. *Clin. Chem.* **40:** 411–16 (1994).
32. D. Talwar, T.K. Ha, J. Cooney, C. Brownlee, and D.S. O'Reilly. A routine method for the simultaneous measurement of retinol, alpha- tocopherol and five carotenoids in human plasma by reverse phase HPLC. *Clin. Chim. Acta* **270:** 85–100 (1998).
33. D. Hess, H.E. Keller, B. Oberlin, R. Bonfanti, and W. Schuep. Simultaneous determination of retinol, tocopherols, carotenes and lycopene in plasma by means of high-performance liquid chromatography on reversed phase. *Int. J. Vitam. Nutr. Res.* **61:** 232–38 (1991).
34. M.A. Abahusain, J. Wright, J.W. Dickerson, M.A. el Hazmi, and H.Y. Aboul Enein. Determination of retinol, alpha-tocopherol, alpha- and beta-carotene by direct extraction of human serum using high performance liquid chromatography. *Biomed. Chromatogr.* **12:** 89–93 (1998).
35. J.P. Steghens, B. Lyan, G. Le Moël, C. Galabert, V. Fayol, H. Faure, P. Grolier, N. Cheribi, F. Dubois, and F. Nabet. Measurement of carotenoids by high pressure liquid chromatography: from difficulties to solutions. *Ann. Biol. Clin. (Paris)* **58:** 327–35 (2000).
36. O. Sommerburg, L.Y. Zang, and F.J. van Kuijk. Simultaneous detection of carotenoids and vitamin E in human plasma. *J. Chromatogr. B Biomed. Sci. Appl.* **695:** 209–15 (1997).
37. J.P. Steghens, A.L. van Kappel, E. Riboli, and C. Collombel. Simultaneous measurement of seven carotenoids, retinol and alpha-tocopherol in serum by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Sci. Appl.* **694:** 71–81 (1997).
38. H.M. Arafa, F.M. Hamada, M.M. Elmazar, and H. Nau. Fully automated determination of selective retinoic acid receptor ligands in mouse plasma and tissue by reversed-phase liquid chromatography coupled on-line with solid-phase extraction. *J. Chromatogr. A* **729:** 125–36 (1996).
39. Q. Su, K.G. Rowley, and K. O'Dea. Stability of individual carotenoids, retinol and tocopherols in human plasma during exposure to light and after extraction. *J. Chromatogr. B Biomed. Sci. Appl.* **729:** 191–98 (1999).
40. D.W. Nierenberg and S.L. Nann. A method for determining concentrations of retinol, tocopherol, and five carotenoids in human plasma and tissue samples. *Am. J. Clin. Nutr.* **56:** 417–26 (1992).
41. A. Sobczak, B. Skop, and B. Kula. Simultaneous determination of serum retinol and alpha- and gamma-tocopherol levels in type II diabetic patients using high-performance liquid chromatography with fluorescence detection. *J. Chromatogr. B Biomed. Sci. Appl.* **730:** 265–71 (1999).
42. H. Moriyama, H. Yamasaki, S. Masumoto, K. Adachi, N. Katsura, and T. Onimaru. Rapid determination of vitamins A and E in serum with surfactant as a diluent by column-switching high-performance liquid chromatography. *J. Chromatogr. A* **798:** 125–30 (1998).
43. L. Yakushina and A. Taranova. Rapid HPLC simultaneous determination of fat-soluble vitamins, including carotenoids, in human serum. *J. Pharm. Biomed. Anal.* **13:** 715–18 (1995).
44. K.S. Epler, R.G. Ziegler, and N.E. Craft. Liquid chromatographic method for the determination of carotenoids, retinoids and tocopherols in human serum and in food. *J. Chromatogr.* **619:** 37–48 (1993).
45. R.W. Browne and D. Armstrong. Simultaneous determination of serum retinol, tocopherols, and carotenoids by HPLC. *Methods Mol. Biol.* **108:** 269–75 (1998).
46. J.R. Lane, L.W. Webb, and R.V. Acuff. Concurrent liquid chromatographic separation and photodiode array detection of retinol, tocopherols, all-trans-alpha-carotene, all-trans-beta-carotene and the mono-cis isomers of beta-carotene in extracts of human plasma. *J. Chromatogr. A* **787:** 111–18 (1997).
47. M.H. Bui. Simple determination of retinol, alpha-tocopherol and carotenoids (lutein, all-trans-lycopene, alpha- and beta-carotenes) in human plasma by isocratic liquid chromatography. *J. Chromatogr. B Biomed. Appl.* **654:** 129–33 (1994).
48. S. Barua, S. Tarannum, L. Nahar, and M. Mohiduzzaman. Retinol and alpha-tocopherol content in breast milk of Bangladeshi mothers under low socio-economic status. *Int. J. Food Sci. Nutr.* **48:** 13–18 (1997).
49. K.E. Savolainen, K.M. Pynnonen, S.P. Lapinjoki, and M.T. Vidgren. Determination of fat-soluble vitamins in a pharmaceutical dosage form by solid-phase extraction and reversed-phase liquid chromatography. *J. Pharm. Sci.* **77:** 802–803 (1988).
50. H. Berg, C. Turner, L. Dahlberg, and L. Mathiasson. Determination of food constituents based on SFE: applications to vitamins A and E in meat and milk. *J. Biochem. Biophys. Methods* **43:** 391–401 (2000).
51. M. Feinberg. *La Validation des Méthodes d'Analyse. Une Approche Chimiométrique de l'Assurance Qualité au Laboratoire.* Masson, Paris, France, 1996.
52. M. Jezequel-Cuer, G. Le Moël, J. Mounie, J. Peynet, C. Le Bizec, M.H. Vernet, Y. Artur, A. Laschi-Loquerie, and S. Troupel. Determination of serum or plasma alpha-tocopherol by high performance liquid chromatography: optimization of operative models. *Ann. Biol. Clin. (Paris)* **53:** 343–52 (1995).
53. J. Arnaud, I. Fortis, S. Blachier, D. Kia, and A. Favier. Simultaneous determination of retinol, alpha-tocopherol and beta-carotene in serum by isocratic high-performance liquid chromatography. *J. Chromatogr.* **572:** 103–16 (1991).
54. B. Olmedilla, F. Granado, I. Blanco, and E. Rojas-Hidalgo. Seasonal and sex-related variations in six serum carotenoids, retinol, and alpha-tocopherol. *Am. J. Clin. Nutr.* **60:** 106–10 (1994).

55. A.P. De Leenheer, H.J. Nelis, W.E. Lambert, and R.M. Bauwens. Chromatography of fat-soluble vitamins in clinical chemistry. *J. Chromatogr.* **429:** 3–58 (1988).
56. W. Stahl, W. Schwarz, A.R. Sundquist, and H. Sies. *cis-trans* Isomers of lycopene and beta-carotene in human serum and tissues. *Arch. Biochem. Biophys.* **294:** 173–77 (1992).
57. W. Stahl and H. Sies. Uptake of lycopene and its geometrical isomers is greater from heat-processed than from unprocessed tomato juice in humans. *J. Nutr.* **122:** 2161–66 (1992).
58. A.R. Mangels, J.M. Holden, G.R. Beecher, M.R. Forman, and E. Lanza. Carotenoid content of fruits and vegetables: an evaluation of analytic data. *J. Am. Diet. Assoc.* **93:** 284–96 (1993).
59. F. Khachik, G.R. Beecher, M.B. Goli, W.R. Lusby, and J.C. Smith. Separation and identification of carotenoids and their oxidation products in the extracts of human plasma. *Anal. Chem.* **64:** 2111–22 (1992).
60. B. Lyan, V. Azais-Braesco, N. Cardinault, V. Tyssandier, P. Borel, M.C. Alexandre-Gouabau, and P. Grolier. Simple method for clinical determination of 13 carotenoids in human plasma using an isocratic high-performance liquid chromatographic method. *J. Chromatogr. B Biomed. Sci. Appl.* **751:** 297–303 (2001).
61. *The Merck Index. An Encyclopedia of Chemicals, Drugs and Biologicals. Centennial Edition*, 11th ed. Merck & Co., Inc., Rahway, NY, 1989.

Manuscript accepted December 7, 2001.