Simultaneous Measurement of Three Tocopherols, All-*trans*-retinol, and Eight Carotenoids in Human Plasma by Isocratic Liquid Chromatography

Zhen Liu, Hyeon-Joo Lee, Francesca Garofalo, David J.A. Jenkins, and **Ahmed El-Sohemy***

Department of Nutritional Sciences, Faculty of Medicine, University of Toronto, 150 College Street, Toronto, Ontario M5S 3E2, Canada

Abstract

We describe a simplified isocratic HPLC method for the simultaneous determination of all-*trans*-retinol, 3 tocopherols (α-, δ-, and γ- tocopherol), and 8 carotenoids (lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, all-*trans*-lycopene, 5-*cis*-lycopne, α-carotene, and β-carotene) in human plasma, which uses a single C18 reversed-phase column (4.6 × 250 mm, 3 µm). A photodiode array detector was used to measure the UV–vis wavelength absorbance of retinol and the eight carotenoids, and fluorescence detection was used for the tocopherols. The linear ranges of the calibration curves for the calibration solutions injected into the HPLC column are 0.02–6.0 µg/mL for all-*trans*-retinol, β-cryptoxanthin and α-carotene; 0.01–3.0 µg/mL for δ-tocopherol, lutein, and lycopene; 0.08–24.0 µg/mL for γ-tocopherol; 0.3–90.0 µg/mL for α-tocopherol; 0.005–1.5 µg/mL for zeaxanthin and canthaxanthin; and 0.04–12.0 µg/mL for β-carotene.

Introduction

The concentrations of antioxidants such as tocopherols and carotenoids in the diet and plasma have been associated with a reduced risk of chronic diseases such as cancer and cardiovascular disease (1–7). The growing interest in these fat-soluble compounds has led to the development of a number of high-performance liquid chromatography (HPLC) methods for the simultaneous separation and quantitation of retinoids, tocopherols, and carotenoids from foods and biological samples such as plasma and tissues (8–25).

Of the existing HPLC methods, reversed-phase (RP) C18 and C30 columns have been widely used in order to separate and quantify retinoids, tocopherols and carotenoids. The polymeric C30 stationary phase provides superior resolution and unique separation that is specifically suited for the geometrical isomers of carotenoids (23,26,27). However, because of the increased analysis time and poor sensitivity using a C30 column, the majority of simultaneous determinations of these compounds are currently performed using a polymeric C18 stationary phase with 5 µm spherical particles packed in 250 mm columns (25). Because the isocratic mobile phase elution used in RP C18 columns did not provide good separation of lutein and zeaxanthin, as well as some carotene isomers, more complex methods such as a gradient mobile phase system, a column switch device, or a low temperature column chamber have been used to resolve these two xanthophylls. Lee et al. (15), used an isocratic elution with two monomeric C18 columns, which were maintained at 35°C and 4°C, respectively, to separate 14 vitamins and three internal standards. The complex column switch device and the low temperature column chamber required to accomplish this work may not be readily available in most laboratories.

Part of the difficulty in resolving the various carotenoids may lie in the small differences in polarity between the carotenoids. A method that increases the number of theoretical plates may be a strategy to help resolve these analytes (9). Columns packed with a smaller particle size such as 3 µm provide a higher number of theoretical plates compared with columns with 5 µm particles, and could provide a simple way to improve the resolution of the peaks without the use of the more complex gradient elutions and column switch devices. Few studies have used C18 stationary phase columns packed with 3 µm particles to separate carotenoids (9,28). Steghens, et al. (9) employed a 3 µm, C18 column with a step gradient elution for the separation of seven carotenoids (lutein zeaxanthin, canthaxanthin β-cryptoxanthin, lycopenes α-carotene, and β-carotene), retinol, α-tocopherol, and two internal standards (tocol and echinenone). Because of the lower sensitivity of UV detection and the baseline fluctuation caused by gradient elution, this method may not be suitable for measuring δ- and γ-tocopherol in plasma samples.

Previous studies have compared photodiode array (PDA) and fluorescence detectors to measure the absorbance of ultraviolet-visible (UV–vis) wavelength and fluorescent emission of absorbed light, respectively, of tocopherols (15,29). These studies have found that fluorescence detection was more sensitive than the UV absorbance for tocopherols.

In this paper, a simple, rapid, and highly sensitive isocratic HPLC method is described; in this method a polymeric octadecylsilane column (3 µm particles) and simultaneous detection by a PDA detector for UV absorbance of retinol and eight carotenoids and a fluorometric detector for tocopherols are used

*Author to whom correspondence should be addressed: email a.el.sohemy@utoronto.ca.

to measure the concentrations of all-*trans*-retinol, α-, δ-, and γ-tocopherols, and carotenoids (lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, all-*trans*-lycopene, 5-*cis*-lycopne, α-carotene, and β-carotene) in plasma with the use of three internal standards (retinyl acetate, α-tocopherol acetate, and echinenone).

Experimental

Reagents and chemicals

All-*trans*-retinol, retinyl acetate, α-, δ-, γ-tocopherol, α-tocopherol acetate, β-carotene, lycopene, butylated hydroxytoluene (BHT), and triethylamine were purchased from Sigma-Aldrich (St. Louis, MO). Echinenone, lutein, zeaxanthin, canthaxanthin, and α-carotene were purchased from CaroteNature (CH-4419, Lupsingen, Switzerland), and β-cryptoxanthin was from ChromaDex (Santa Ana, CA). Two levels of standard reference materials (SRM 968c) were acquired from the National Institute of Standards and Technology (NIST, Gaithersburg, MD). HPLC grade methanol, acetonitrile, and hexane were purchased from EMD Chemicals (Gibbstown, NJ). ACS grade anhydrous ethanol was from Commercial Alcohols Inc. (Brampton, ON, Canada).

Blood collection and storage

Blood for method validation was collected from healthy subjects aged 20–29 years old who were participants from the Toronto Nutrigenomics and Health Study. Blood samples were collected after a minimum 12 h overnight fast at LifeLabs Laboratories (Toronto, ON, Canada). Subjects were only permitted to consume water prior to the blood draw. All blood samples were collected from the antecubital vein and protected from light. Plasma samples were collected in a 7 mL sodium-heparin tube and centrifuged at 1500 *g* for 15 min at 4°C within 2 h after collection. An aliquot of 2 mL was placed in an amber transport vial, frozen immediately, and transported on dry ice to the University of Toronto and stored at a temperature of –80°C until analysis. All subjects provided written informed consent and the study protocol was approved by the Research Ethics Board at the University of Toronto.

Preparation of calibration solution of standards

Stock solutions of each standard were prepared individually with appropriate solvents. All-*trans*-retinol was dissolved in ethanol–0.0625% BHT; retinyl acetate (internal standard 1, IS1) was initially dissolved with 1 volume of acetone before the addition of 4 volumes of acetonitrile. Stock solutions of α-, δ-, and γ-tocopherols and α-tocopherol acetate (internal standard 2, IS2) were prepared in ethanol–0.0625% BHT. Stock solutions of the hydrocarbon carotenoids including α-carotene and β-carotene were prepared in hexane–0.005% BHT, and lycopene was first dissolved in 1 volume of chloroform followed by 4 volumes of hexane–0.005% BHT. Canthaxanthin was dissolved in tetrahydrofuran. Other carotenoids including lutein, zeaxanthin, β-cryptoxanthin, and echinenone (internal standard 3, IS3) were dissolved in ethanol–0.0625% BHT. Because the carotenoids and tocopherols are very unstable and easily degraded, the concentrations of individual calibration standard solutions were confirmed by measuring the absorption in a specific solvent and wavelength with a UV spectrometer and checking the purity by HPLC. If the measured concentrations dropped significantly, fresh new stock solutions of the individual calibration standard were prepared. Extinction coefficients used to determine concentrations of calibration standards are listed in Table I. Ethanol–0.0625% BHT was used to dissolve the working solution of mixed calibrators containing γ-, δ-, and α-tocopherol (1 mg/L, 8 mg/L and 30 mg/L, respectively), all-*trans*-retinol (2 mg/L), lutein and lycopene (1 mg/L of each), zeaxanthin and canthaxanthin (0.5 mg/L of each), β-cryptoxanthin and α-carotene (2 mg/L of each), and β-carotene (4 mg/L). Aliquots of the working solution were distributed into amber vials and stored at –70°C. Each time before use, a vial of the calibrator working solution was thawed to room temperature and sonicated for 5 min. Seven levels of different concentrations of calibrator mixture were prepared by diluting the calibrator working solution in ethanol–0.0625% BHT. The concentrations of the internal standards in the mixed internal standard solution in ethanol–0.0625% BHT were 0.25 mg/L for retinyl acetate (IS1), 50 mg/L for α-tocopherol acetate (IS2), and 0.2 mg/L for echinenone (IS3).

Sample preparation

Plasma samples were stored at –80°C and extracted on the day of HPLC analysis. All sampling procedures were performed under low ambient light conditions to minimize light-induced degradation of antioxidants. For extraction, a 200 µL aliquot of plasma, 100 µL of the mixed internal standard solution, and 100 µL of ethanol–BHT (0.0625%) were pipetted into an amber microfuge vial, and vortexed for 15 s for deproteinization (8). A volume of 1 mL of *n*-hexane–BHT (0.005%) was added to the mixture, which was then vortexed and shaken alternately for 5 min, and centrifuged for 3 min at 2000 *g*. The vial was placed on ice to improve phase separation after centrifugation and an aliquot of 900 µL of the supernatant was transferred to a 4 mL

Table I. Extinction Coefficient Used to Determine Concentration of Calibration Standards

Compound	Solvent	Wavelength (nm)	Extinction coefficient*	Reference
All-*trans*-retinol	Ethanol	325	1835	(13)
δ-Tocopherol	Ethanol	298	87.3	(37)
γ-Tocopherol	Ethanol	298	91.4	(37)
α-Tocopherol	Ethanol	292	75.8	(37)
Lutein	Ethanol	445	2550	(38)
Zeaxanthin	Ethanol	450	2480	(38)
Canthaxanthin	Petroleum	466	2200	(38)
β-Cryptoxanthin	Hexane	449	2386	(38)
all-trans-Lycopene	Petroleum	470	3450	(38)
α-Carotene	Hexane	445	2710	(38)
β-Carotene	Petroleum	450	2592	(38)
Retinyl acetate	Ethanol	326	1550	(13)
α-Tocopherol acetate	Ethanol	290	40	(13)
Echinenone	Petroleum	458	2158	(38)

* ($\varepsilon^{1\%}$1 cm)

amber glass vial with a screw cap. The extraction procedure was repeated twice with *n*-hexane-BHT (0.005%) and the supernatants were pooled into the amber glass vial. The extract was evaporated to dryness under a stream of nitrogen, reconstituted in 100 µL of ethanol–BHT (0.0625%) by vortexing for 3 min, and transferred into a microvolume HPLC vial insert. Samples were then placed into the HPLC autosampler compartment at 10°C before injection.

Chromatographic conditions

The HPLC system consisted of a Waters 2690 Alliance HPLC system (Milford, MA), a Waters 996 PDA detector and a Shimadzu RF 535 fluorescence detector (Kyoto, Japan), controlled by Waters Millennium[32] data management software, 4.0 edition. A Waters Spherisorb 3 µm ODS2, 4.6 × 250 mm analytical column was used for the separation of all components. The chromatographic separation was performed by isocratic elution with a mixture of acetonitrile and methanol (65:35, v/v) containing 0.065% of triethylamine at a flow rate of 1.5 mL/min. The temperatures of the sample chamber and column oven were 10°C and 30°C, respectively. The injection volume on the column was 10 µL. The PDA detection wavelength range was from 200 to 500 nm. The fluorescence detector parameters were as follows: excitation wavelength, 285 nm; emission wavelength, 325 nm; response, medium; sensitivity, high; and range, 1024.

The concentrations of δ-, γ-, α-tocopherol, and α-tocopherol acetate (IS1) were determined by fluorescence detection. The carotenoids lutein, zeaxanthin, canthaxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene, and echinenone (IS3) were quantified at 450 nm, and all-*trans*-retinol and retinyl acetate (IS2) were measured at 326 nm by PDA detection.

Table II. Reproducibility and Recovery

Compound	Detection	Added to plasma (µg/mL)	Mean plasma conc. (µg/mL, *n* = 12)	CV (%)		Recovery (%)
				Within-day	Between-day	
All-trans-retinol	PDA		0.376	1.86	5.34	
		0.02	0.400	1.72	5.59	117
		0.10	0.495	2.39	3.93	118
		1.00	1.411	1.57	3.00	103
δ-Tocopherol	Fluorescence		0.074	4.29	11.63	
		0.01	0.085	5.81	7.89	108
		0.05	0.121	5.56	4.71	94
		0.50	0.561	4.04	3.13	97
γ-Tocopherol	Fluorescence		1.129	2.66	4.19	
		0.08	1.180	5.45	5.24	64
		0.40	1.549	3.72	2.36	105
		4.00	5.279	4.39	3.13	103
α-Tocopherol	Fluorescence		8.699	7.78	4.90	
		0.3	8.796	8.26	5.46	32
		1.50	9.966	9.31	4.06	84
		15.0	23.310	3.69	4.66	97
Lutein	PDA		0.076	5.12	7.13	
		0.01	0.088	3.14	8.23	120
		0.05	0.130	3.86	5.07	107
		0.50	0.590	1.54	3.55	103
Zeaxanthin	PDA		0.022	5.13	12.15	
		0.005	0.028	1.79	9.16	116
		0.025	0.049	5.96	8.02	108
		0.250	0.259	4.55	10.71	95
Canthaxanthin	PDA		0.038	3.35	7.77	
		0.005	0.044	2.85	7.67	126
		0.025	0.064	3.97	8.82	106
		0.250	0.285	1.54	6.37	99
β-Cryptoxanthin	PDA		0.051	2.63	6.90	
		0.02	0.071	5.54	7.56	102
		0.10	0.150	4.90	3.68	100
		1.00	1.038	2.78	2.76	99
Lycopene	PDA		0.145	3.72	13.36	
		0.01	0.157	3.51	6.56	118
		0.05	0.188	3.33	11.05	86
		0.50	0.652	5.68	8.36	101
α-Carotene	PDA		0.058	4.93	8.60	
		0.02	0.075	3.80	5.05	85
		0.10	0.155	5.32	2.83	97
		1.00	1.003	2.15	2.80	94
β-Carotene	PDA		0.191	4.07	8.36	
		0.04	0.237	3.21	8.65	115
		0.20	0.387	5.29	6.27	98
		2.00	2.080	2.23	2.26	94

Calibration curves and quantification

External standard calibration curves were constructed by plotting the peak-area ratio of the analyte-internal standard against the concentration of the analyte. A linear least-squares regression analysis was performed for each analyte, and the final calibration curve for each analyte was the average based on ten-time replicates in which the correlation coefficient was above 0.99. Plasma concentrations of each analyte were quantified on the final calibration curves.

Recovery, reproducibility, and precision

For reproducibility and recovery studies, pooled human plasma samples were supplemented with different concentrations of the analytes of interest. Twelve sets of enriched samples were prepared to give the concentrations listed in Table II. Analyses were carried out within 24 h for within-day variation, and between-day variation was determined by repeating the same assay once a week for 6 consecutive weeks. Two lyophilized standard reference materials (SRM 968c) from NIST were processed to determine the accuracy of the method.

Results and Discussion

The six most common dietary carotenoids are lutein, zeaxanthin, lycopene, β-cryptoxanthin, α-carotene, and β-car-

otene, which reportedly account for more than 90% of the carotenoids present in human plasma (30). The critical issues for simultaneous analysis of these carotenoids along with the other antioxidants in human plasma include selectivity, sensitivity, and reliability. Figure 1 shows a set of overlaid chromatograms of the calibration standard mixture from different channels with PDA and fluorescence detection. All-*trans*-retinol and retinyl acetate (IS1) were detected at 326 nm and the seven carotenoids and echinenone (IS3) at 450 nm with PDA detection, and the tocopherols and α-tocopherol acetate (IS2) were measured with excitation wavelength 285 nm and emission wavelength 325 nm by fluorescence detection.

Although separation of lutein and zeaxanthin can be achieved by the use of polymeric C18 or C30 columns with a gradient elution (23), the retention of α-carotene, lycopene, and β-carotene tends to increase, resulting in decreased sensitivity. Because the smaller particle size of the column packing could provide a higher number of theoretical plates and, therefore, have higher resolution for separation, a polymeric C18 column packed with 3 µm spherical particles was used for testing with an isocratic elution method. With this approach, lutein and zeaxanthin were sufficiently resolved to enable their individual quantification.

To improve the resolution, ammonium acetate, acetic acid, and triethylamine have previously been used in the mobile phase (22,24). As a modifier, 0.065% triethylamine was added to the mobile phase, which improved the sharpness of each carotenoid peak. The use of an antioxidant such as BHT (9,22,25), or ascorbic acid (12) during sample treatment is necessary to prevent degradation of carotenoids, especially lycopene and β-carotene. Therefore, the antioxidant BHT was added to each solvent used for the standard and sample preparation. Hexane containing BHT protected the carotenoids from degradation during the evaporation of the organic layers. Echinenone is a synthetic carotenoid and has structural and chemical properties similar to the naturally occurring carotenoids in plasma. The use of three internal standards allowed better quality control and helped to correct analytical variations occurring for each antioxidant during the extraction and chromatography steps.

Figure 2 shows a set of chromatograms from a typical plasma sample. With the 3 µm C18 column used in this method, lutein and zeaxanthin were sufficiently separated under isocratic elution with a mixture of acetonitrile and methanol (65:35, v/v) containing 0.065% of triethylamine. As these results show, this approach enables the individual quantification of lutein and zeaxanthin. Figure 2 also shows that this method allows all analytes of interest to be well separated from each other and there is no interference with other compounds found in human plasma.

The geometric isomers of lycopene are not baseline-resolved (Figure 2), but are resolved sufficiently to separate and quantitate the all-*trans*-lycopene and 5-*cis*-lycopene from other *cis*-isomers. Because of the lack of standards, the peak assignment of *cis*-lycopene isomers is not certain, which may affect the determination of the ratio between lycopene isomers (31–34). Peak 7 (Figure 2) was tentatively assigned to 5-*cis*-lycopene because its peak area ratio to all-*trans*-lycopene is similar to previous studies (21, 22, 31, 32). The additional two *cis*-isomers of lycopene were also separated and are likely 9-*cis* and 13-*cis* lycopene according to their peak order by comparison to a previous report that used a similar C18 HPLC system (21). For quantitation, the calibration curve of all-*trans*-lycopene was used to calculate the contents of its geometric isomers, although it was reported that *cis*-isomers with a lower specific absorption than the all-*trans*-isomer, such as 9-*cis*- or 13-*cis*-lycopene were somewhat underestimated (33). The two *cis*-isomers of β-carotene were also resolved from their all-*trans* isomer by this method. Based on their peak order, they are likely 9-*cis*-β-carotene and 13-*cis*-β-carotene, respectively (21).

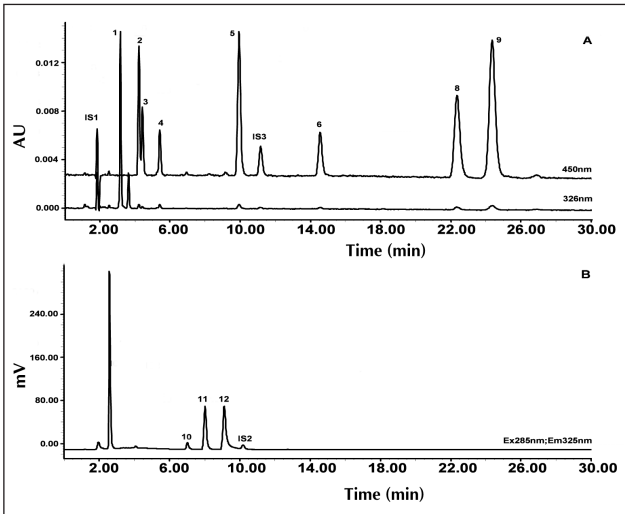

Figure 1. Typical chromatograms of calibration standards (A) as detected with PDA and (B) fluorescence detection. 1 = all-*trans*-retinol, 2 = lutein, 3 = zeaxanthin, 4 = canthaxanthin, 5 = β-cryptoxanthin, 6 = all-*trans*-lycopene, 8 = α-carotene, 9 = β-carotene, 10 = δ-tocopherol, 11 = γ-tocopherol, 12 = α-tocopherol, IS1= retinyl acetate, IS2 = α-tocopherol acetate, and IS3 = echinenone.

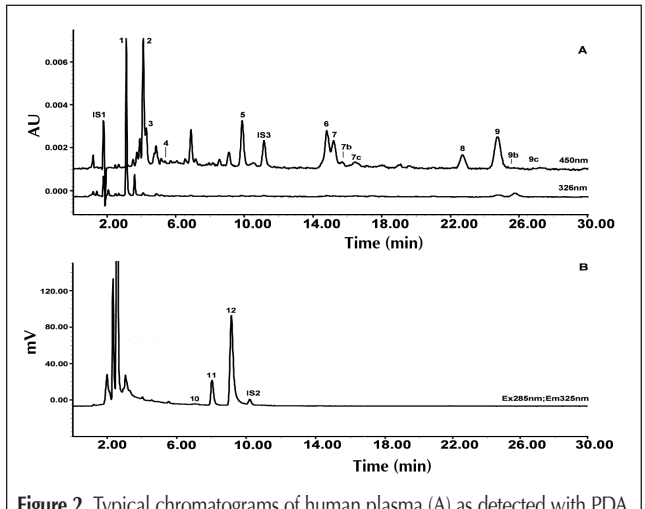

Figure 2. Typical chromatograms of human plasma (A) as detected with PDA and (B) fluorescence detection. 1 = all-*trans*-retinol, 2 = lutein, 3 = zeaxanthin, 4 = canthaxanthin, 5 = β-cryptoxanthin, 6 = all-*trans*-lycopene, 7a = 5-*cis*-lycopene, 7b = 9-*cis*-lycopene, 7c = 13-*cis*-lycopene, 8 = α-carotene, 9 = β-carotene, 9b = 9-*cis*-β-carotene, 9c = 13-*cis*-β-carotene, 10 = δ-tocopherol, 11 = γ-tocopherol, 12 = α-tocopherol, IS1 = retinyl acetate, IS2 = α-tocopherol acetate, and IS3 = echinenone.

Table III. Limits of Detection and Retention Time

Compound	Detection	Detection limit (µg/mL)	Retention time (min)
Retinyl acetate (IS1)	PDA		3.6
all-trans-Retinol	PDA	0.007	3.2
α-Tocopherol acetate (IS2)	Fluorescence		9.8
δ-Tocopherol	Fluorescence	0.01	6.8
γ-Tocopherol	Fluorescence	0.008	7.8
α-Tocopherol	Fluorescence	0.008	8.8
Lutein	PDA	0.01	4.2
Zeaxanthin	PDA	0.005	4.4
Canthaxanthin	PDA	0.005	5.4
β-Cryptoxanthin	PDA	0.01	9.9
Echinenone (IS3)	PDA		11.2
all-trans-Lycopene	PDA	0.01	14.5
5-cis-Lycopene	PDA	0.01	15.2
α-Carotene	PDA	0.01	22.4
β-Carotene	PDA	0.02	24.4

Besides those identified retinoids, carotenoids, and tocopherols, a few unknown peaks at wavelength 450 nm with PDA detection in human plasma were also observed. Some of these unknown peaks are well resolved from the other peaks and are present at relatively high concentrations. The identity of these peaks, however, remains to be determined. The retention times and detection limits of each analyte are summarized in Table III. The limit of detection was defined as the lowest concentration of an analyte in a standard solution that could be detected from zero with 95% confidence (n = 10).

Regression analysis of calibration data achieved good linearity over the concentration range tested. Slopes, intercepts, squared coefficients of correlation, and corresponding concentration ranges of calibration are shown in Table IV. Squared correlation coefficients (r^2) were > 0.99, indicating a linear relationship in the concentration ranges for all analytes. The day-to-day variations (n = 10) in slope and linearity were generally < 7% and < 0.1%, respectively.

The within-day precision, between-day variations, and recovery rates were tested by using the same human plasma sample enriched with different concentrations of the analytes of interest. The results of the within-day (n = 6) and between-day (n = 6) CV assays are shown in Table II, with CV generally < 10%. The recovery rates through the concentration range tested ranged from 85% to 120% for most of the analytes, indicating good reliability of this HPLC method for human plasma samples.

Table IV. Linearity and Ranges of Calibration (n = 10)

		Mean		CV (%)	
Compound	Calibration range (µg/mL)	Calibration curve*	r^2	Calibration curve*	R^2
all-trans-retinol	0.02–6.0	y = 0.2272x – 0.0113	0.9998	5.05	0.01
δ-Tocopherol	0.01–3.0	y = 0.374x – 0.0055	0.9998	5.04	0.02
γ-Tocopherol	0.08–24.0	y = 0.3841x – 0.0228	0.9997	6.88	0.02
α-Tocopherol	0.3–90.0	y = 1.0023x – 0.0799	0.9998	6.06	0.03
Lutein	0.01–3.0	y = 0.2238x + 0.0008	0.9997	4.45	0.03
Zeaxanthin	0.005–1.5	y = 0.2031x – 0.0075	0.9988	3.71	0.08
Canthaxanthin	0.005–1.5	y = 0.2703x – 0.0028	0.9996	3.23	0.04
β-Cryptoxanthin	0.02–6.0	y = 0.2267x + 0.0057	0.9996	4.32	0.03
Lycopene	0.01–3.0	y = 0.3254x – 0.0069	0.9982	6.75	0.17
α-Carotene	0.02–6.0	y = 0.1986x + 0.0074	0.9997	4.02	0.02
β-Carotene	0.04–12.0	y = 0.2241x + 0.0225	0.9996	4.12	0.03

* y = concentration (µg/mL); x = peak area ratio.

Two lyophilized standard reference material SRM 968c from the NIST were used to check the accuracy of the method. The measured concentration values of all-*trans*-retinol, δ-tocopherol, γ-tocopherol, α-tocopherol, and *trans*-β-carotene were compared with NIST certified concentration values (Table V). The concentration values of lutein, zeaxanthin, β-cryptoxanthin, all-*trans*-lycopene, total lycopene, and α-carotene along with the NIST reference concentration values are also presented. Our values are in very good agreement with NIST values.

Table V. Results (µg/mL) for the Standard Reference Material 968c from NIST

	Level 1		Level 2	
Analyte	Our result*	NIST†	Our result*	NIST†
all-*trans*-Retinol	0.844 ± 0.018	0.841 ± 0.027	0.486 ± 0.010	0.484 ± 0.012
δ-Tocopherol	0.129 ± 0.007	0.131 ± 0.018	0.600 ± 0.015	0.527 ± 0.071
γ-Tocopherol	3.76 ± 0.16	3.90 ± 0.13	1.61 ± 0.04	1.56 ± 0.10
α-Tocopherol	7.80 ± 0.48	7.47 ± 0.47	17.99 ± 0.63	16.79 ± 0.76
trans-β-Carotene	0.174 ± 0.010	0.157 ± 0.016	0.402 ± 0.008	0.391 ± 0.047
Lutein	0.047 ± 0.003	0.047 ± 0.007	0.078 ± 0.003	0.068 ± 0.007
Zeaxanthin	0.027 ± 0.002	0.026 ± 0.009	0.019 ± 0.001	0.019 ± 0.009
β-Cryptoxanthin	0.073 ± 0.003	0.072 ± 0.008	0.033 ± 0.001	0.030 ± 0.006
all-*trans*-Lycopene	0.175 ± 0.014	0.13 ± 0.03	0.230 ± 0.002	0.17 ± 0.03
total Lycopene	0.357 ± 0.034	0.34 ± 0.04	0.474 ± 0.010	0.45 ± 0.07
α-Carotene	0.020 ± 0.001	0.020 ± 0.006	0.095 ± 0.003	0.10 ± 0.02

* Mean ± SD (n = 4).
† Concentration certified or referred by NIST, mean ± the expanded uncertainty.

Plasma from 709 young subjects aged 20–29 years were chosen to compare values with those reported in various populations. Plasma concentrations of all-*trans*-retinol, three tocopherols, and eight carotenoids for these selected samples from women (n = 491) and men (n = 218) subjects are reported in Table VI. The results of plasma concentrations for individual analytes are in good agreement with previously reported values in various populations (9–13).

The plasma for which the retinol, tocopherol, and carotenoid concentrations

Table VI. Concentrations of Carotenoids, Tocopherols, and All-trans-retinol in Human Plasma

	Women (*n* = 491)				Men (*n* = 218)			
	Mean ± SD		Range		Mean ± SD		Range	
Analyte	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)	(µg/mL)	(µmol/L)
all-*trans*-retinol	0.554 ± 0.163	1.933 ± 0.569	0.056–1.189	0.197–4.150	0.563 ± 0.109	1.966 ± 0.379	0.269–1.032	0.939–3.603
δ-Tocopherol	0.096 ± 0.106	0.238 ± 0.263	0.013–1.568	0.033–3.893	0.122 ± 0.225	0.302 ± 0.558	0.023–2.699	0.058–6.702
γ-Tocopherol	1.56 ± 0.92	3.75 ± 2.21	0.27–6.92	0.65–16.61	1.80 ± 0.94	4.33 ± 2.25	0.32–5.92	0.77–14.21
α-Tocopherol	12.26 ± 3.85	28.47 ± 8.95	0.71–29.74	1.64–69.05	11.89 ± 4.11	27.61 ± 9.53	2.71–39.50	6.30–91.72
Lutein	0.227 ± 0.136	0.398 ± 0.239	0.053–1.573	0.093–2.766	0.194 ± 0.085	0.341 ± 0.149	0.036–0.500	0.064–0.879
Zeaxanthin	0.054 ± 0.040	0.095 ± 0.071	< D.L.*–0.688	< D.L.–1.210	0.050 ± 0.023	0.087 ± 0.040	0.009–0.138	0.01 –0.243
Canthaxanthin†	0.012 ± 0.020	0.021 ± 0.036	< D.L.–0.225	< D.L.–0.398	0.012 ± 0.023	0.020 ± 0.040	< D.L.–0.171	< D.L.–0.303
β-Cryptoxanthin	0.166 ± 0.122	0.301 ± 0.221	0.014–0.843	0.026–1.525	0.129 ± 0.092	0.234 ± 0.166	0.015–0.674	0.027–1.219
all-*trans*-Lycopene	0.546 ± 0.358	1.017 ± 0.667	< D.L.–1.928	< D.L.–3.592	0.590 ± 0.400	1.099 ± 0.745	0.058–1.878	0.108–3.498
5-cis-Lycopene‡	0.203 ± 0.100	0.366 ± 0.181	0.048–0.514	0.087–0.929	0.196 ± 0.105	0.355 ± 0.189	0.049–0.506	0.089–0.916
α-Carotene	0.102 ± 0.086	0.191 ± 0.161	0.011–0.738	0.021–1.375	0.066 ± 0.057	0.122 ± 0.107	< D.L.–0.381	<D.L.–0.710
β-Carotene	0.349 ± 0.274	0.650 ± 0.510	0.028–1.906	0.052–3.550	0.208 ± 0.156	0.388 ± 0.291	< D.L.–0.949	< D.L.–1.768

* D.L. = detection limit.
† *n* = 163 (women), n = 62 (men)
‡ *n* = 130 (women), n = 50 (men)

were reported had been stored for up to 3.5 years and had undergone only one freeze/thaw cycle prior to HPLC analysis. Although no stability studies were performed using the HPLC method described, retinol, α-tocopherol and β–carotene have previously been reported to be stable for at least 15 years when stored at temperatures at or below –70°C (35). Comstock et al. (36), have also reported no significant losses of retinol, α-carotene, β-carotene, cryptoxanthin, lutein, lycopene, α-tocopherol, and γ-tocopherol for up to 4 years when stored at a temperature of –70°C. Furthermore, stability studies on extracted samples have shown that extracted samples reconstituted in a solvent containing an antioxidant are stable in an autosampler for at least 24 h at a temperature of 10°C (12). In this method, extracted samples were reconstituted in a solvent (ethanol) containing the antioxidant BHT, placed in an autosampler at 10°C and injected into the HPLC column within 24 h. These previous stability studies suggest that the plasma analyzed using the HPLC method described did not undergo significant losses of retinoids, carotenoids, or tocopherols prior to HPLC analysis.

Conclusion

In summary, this method shows that all-*trans*-retinol, three tocopherols and most of the major carotenoids and their isomers in human plasma can be simultaneously separated and quantified by the use of a small particle size (3 µm) polymeric C18 chromatography column with isocratic elution. With this method, all analytes of interest are well resolved including the geometric isomers lutein and zeaxanthin and the different isomers of lycopene. The sample preparation procedure in this method provides excellent recoveries for all analytes, and the method is rapid, sensitive, reliable, and suitable for large-scale studies.

Acknowledgements

Funding for this research was provided by the Advanced Foods and Materials Network and the Canadian Institutes of Health Research. DJ holds a Canada Research Chair in Nutrition and Metabolism and AE holds a Canada Research Chair in Nutrigenomics.

References

1. S. Voutilainen, T. Nurmi, J. Mursu, and T.H. Rissanen. Carotenoids and cardiovascular health. *Am. J. Clin. Nutr.* **83:** 1265–1271 (2006).
2. R. Sato, K.J. Helzlsouer, A.J. Alberg, S.C. Hoffman, E.P. Norkus, and G.W. Comstock. Prospective study of carotenoids, tocopherols, and retinoid concentrations and the risk of breast cancer. *Cancer Epidemiol. Biomarkers. Prev.* **11:** 451–457 (2002).
3. S.C. Larsson, L. Bergkvist, I. Naslund, J. Rutegard, and A. Wolk. Vitamin A, retinol, and carotenoids and the risk of gastric cancer: a prospective cohort study. *Am. J. Clin. Nutr.* **85:** 497–503 (2007).
4. K. Ozasa, Y. Ito, K. Suzuki, Y. Watanabe, K. Hayashi, K. Mikami, M. Nakao, T. Miki, M. Mori, F. Sakauchi, M. Washio, T. Kubo, K. Wakai, and A. Tamakoshi. Serum carotenoids and other antioxidative substances associated with urothelial cancer risk in a nested case-control study in Japanese men. *J. Urol.* **173:** 1502–1506 (2005).
5. E. Giovannucci. A review of epidemiologic studies of tomatoes, lycopene, and prostate cancer. *Exp. Biol. Med. (Maywood)*. **227:** 852–859 (2002).
6. J.M. Yuan, R.K. Ross, Y.T. Gao, Y.H. Qu, X.D. Chu, and M.C. Yu. Prediagnostic levels of serum micronutrients in relation to risk of gastric cancer in Shanghai, China. *Cancer Epidemiol. Biomarkers Prev.* **13:** 1772–1780 (2004).
7. N.N. Maserejian, E. Giovannucci, B. Rosner, and K. Joshipura. Prospective study of vitamins C, E, and A and carotenoids and risk of oral premalignant lesions in men. *Int. J. Cancer*. **120:** 970–977 (2007).
8. Y. Gobel, C. Schaffer, and B. Koletzko. Simultaneous determination

of low plasma concentrations of retinol and tocopherols in preterm infants by a high-performance liquid chromatographic micromethod. *J. Chromatogr. B Biomed. Sci. Appl.* **688:** 57–62 (1997).
9. 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).
10. 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:** 2229–2234 (1993).
11. 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:** 411–416 (1994).
12. 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).
13. S. Gueguen, B. Herbeth, G. Siest, and P. Leroy. An isocratic liquid chromatographic method with diode-array detection for the simultaneous determination of alpha-tocopherol, retinol, and five carotenoids in human serum. *J. Chromatogr. Sci.* **40:** 69–76 (2002).
14. 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:** 129–133 (1994).
15. B.L. Lee, A. L. New and C. N. Ong. Simultaneous determination of tocotrienols, tocopherols, retinol, and major carotenoids in human plasma. *Clin. Chem.* **49:** 2056–2066 (2003).
16. B.S. Inbaraj, J.T. Chien, and B.H. Chen. Improved high performance liquid chromatographic method for determination of carotenoids in the microalga Chlorella pyrenoidosa. *J. Chromatogr., A.* **1102:** 193–199 (2006).
17. A.B. Barua. Improved normal-phase and reversed-phase gradient high-performance liquid chromatography procedures for the analysis of retinoids and carotenoids in human serum, plant and animal tissues. *J. Chromatogr., A.* **936:** 71–82 (2001).
18. A.B. Barua and J.A. Olson. Reversed-phase gradient high-performance liquid chromatographic procedure for simultaneous analysis of very polar to nonpolar retinoids, carotenoids and tocopherols in animal and plant samples. *J. Chromatogr. B Biomed. Sci. Appl.* **707:** 69–79 (1998).
19. J. Brown Thomas, M.C. Kline, L.M. Gill, J.H. Yen, D.L. Duewer, L.T. Sniegoski, and K.E. Sharpless. Preparation and value assignment of Standard Reference Material 968c Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum. *Clin. Chim. Acta.* **305:** 141–155 (2001).
20. K.S. Epler, L.C. Sander, R.G. Ziegler, S.A. Wise, and N.E. Craft. Evaluation of reversed-phase liquid chromatographic columns for recovery and selectivity of selected carotenoids. *J. Chromatogr.* **595:** 89–101 (1992).
21. W. Stahl, A.R. Sundquist, M. Hanusch, W. Schwarz, and H. Sies. Separation of beta-carotene and lycopene geometrical isomers in biological samples. *Clin. Chem.* **39:** 810–814 (1993).
22. 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:** 191–198 (1999).
23. K.E. Sharpless, J.B. Thomas, L.C. Sander, and S.A. Wise. Liquid chromatographic determination of carotenoids in human serum using an engineered C30 and a C18 stationary phase. *J. Chromatogr. B Biomed. Appl.* **678:** 187–195 (1996).
24. A.R. Weinmann, M.S. Oliveira, S.M. Jorge, and A.R. Martins. Simultaneous high-performance liquid chromatographic determination of retinol by fluorometry and of tocopherol by ultraviolet absorbance in the serum of newborns. *J. Chromatogr. B Biomed. Sci. Appl.* **729:** 231–236 (1999).
25. 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:** 209–215 (1997).
26. C. Emenhiser, L.C. Sander, and S.J. Schwartz. Capability of a polymeric C30 stationary phase to resolve cis-trans carotenoid isomers in reversed-phase liquid chromatography. *J. Chromatogr. A.* **707:** 205–216 (1995).
27. C. Emenhiser, G. Englert, L.C. Sander, B. Ludwig and S.J. Schwartz. Isolation and structural elucidation of the predominant geometrical isomers of a-carotene. *J. Chromatogr.* **719:** 333–343 (1996).
28. D.I. Thurnham, E. Smith, and P.S. Flora. Concurrent liquid-chromatographic assay of retinol, alpha-tocopherol, beta-carotene, alpha-carotene, lycopene, and beta-cryptoxanthin in plasma, with tocopherol acetate as internal standard. *Clin. Chem.* **34:** 377–381 (1988).
29. D. Hoehler, A.A. Frohlich, R.R. Marquardt, and H. Stelsovsky. Extraction of alpha-tocopherol from serum prior to reversed-phase liquid chromatograpy. *J. Agric. Food Chem.* **46:** 973–978 (1998).
30. 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).
31. 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:** 2161–2166 (1992).
32. 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:** 173–177 (1992).
33. J. Schierle, W. Bretzel, I. Buhler, N. Faccin, D. Hess, K. Steiner, and W. Schuep. Content and isomeric ratio of lycopene in food and human blood plasma. *Food Chemistry*. **59:** 459–465 (1997).
34. U. Hengartner, K. Bernhard, K. Mayer, G. Englert, and E. Glinz. Synthesis, isolation, and NMR-spectroscopic characterization of fourteen (Z)-isomers of lycopene and of some acetylenic didehydro- and tetradehydrolycopenes. *Helv. Chim. Acta.* **75:** 1848–1865 (1992).
35. G.W. Comstock, A.J. Alberg, and K.J. Helzlsouer. Reported effects of long-term freezer storage on concentrations of retinol, beta-carotene, and alpha-tocopherol in serum or plasma summarized. *Clin Chem*. **39:** 1075–1078 (1993).
36. G.W. Comstock, E.P. Norkus, S.C. Hoffman, M.W. Xu, and K.J. Helzlsouer. Stability of ascorbic acid, carotenoids, retinol, and tocopherols in plasma stored at –70 degrees C for 4 years. *Cancer Epidemiol Biomarkers Prev*. **4:** 505–507 (1995).
37. P. Schudel, H. Mayer, and O. Isler. Tocopherols. II. Chemistry. In *The Vitamins. Chemistry, Physiology, Pathology and Methods*. W.H. Sebrell, Jr. and R.S. Harris, Eds. Academic Press, New York, 1972, pp 168–218.
38. G. Britton, S. Liaaen-Jensen, and H. Pfander. *Carotenoids*, Vol. 1B: Spectroscope. Birkhauser, 1995.

Manuscript received September 13, 2009;
revision received December 18, 2009.