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An improved HPLC method for determination of carotenoids in human serum

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Abstract

An HPLC method was developed to determine the various carotenoids in human serum. A C-30 column and a mobile phase of 100% methanol (A) and 100% methylene chloride (B) with the following gradient elution were used: 90% A and 10% B in the beginning, maintained for 5 min, decreased to 78% A at 15 min, 62% A at 30 min, 52% A at 40 min, 41% A at 50 min, 38% A at 55 min, maintained for 3 min, and returned to 100% A at 65 min. A total of 21 carotenoids, including *all-trans* forms of lutein, zeaxanthin, α-cryptoxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene, as well as their 14 *cis*-isomers were resolved within 51 min at a flow rate of 1.0 mL/min and detection at 476 nm. *all-trans*-β-Carotene was found to be present in highest amount (256.3–864.2 ng/mL), followed by *all-trans*-lycopene (64.4–569.2 ng/mL), *all-trans*-lutein (137.9–450.3 ng/mL), *all-trans*-α-cryptoxanthin (55.7–188.2 ng/mL), *all-trans*-β-cryptoxanthin (43.1–134.5 ng/mL), *all-trans*-α-carotene (20.0–122.1 ng/mL) and *all-trans*-zeaxanthin (9.1–21.3 ng/mL). Similar trend was observed for *cis*-isomers of carotenoids.

Keywords: Carotenoids; Human serum; HPLC

1. Introduction

In the past decade a number of foods and food constituents have been studied for their inhibitory effect on carcinogenesis [1,2]. Results of these studies have shown an inverse relationship between the consumption of certain fruits and vegetables and the risk of epithelial cancer [3,4]. In human studies, numerous associations between a low carotenoid intake and increased risk for cancer, age-related macular degeneration, cataract, sunburn-induced skin damage or cardiovascular diseases have been observed [5,6]. Therefore, attention has been given to the uptake, distribution and metabolism of carotenoids after ingestion as supplements or as part of dietary yellow and green fruits and vegetables [7]. Interestingly, the serum in HIV-infected patients was found to have a lower level of lycopene [8]. Thus, it is possible to diminish the risk of chronic diseases by elevating the level of lycopene in human serum. In addition, the application of lutein in the treatment of age-related macular degeneration is well documented [6,9].

In view of the impact of these carotenoids to human health, a precise method for their simultaneous determination in human serum is important. High-performance liquid chromatography (HPLC) has been employed as a powerful technique to quantify low levels and various forms of carotenoids in foods and human serum. One of the original separations of carotenoids from an extract of human serum was reported by Neils and De Leenheer [10], who developed nonaqueous reversed-phase chromatographic conditions on a Zorbax ODS column, with a mixture of acetonitrile (70%), dichloromethane (20%), and methanol (10%) as eluent. Bieri et al. [11] employed the same chromatographic procedure to separate carotenoids from the extracts of human plasma on a C-18 column. However, the resolution of carotenoids and their *cis*-isomers in human serum remain inadequate.

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Emenhiser et al. [12] demonstrated that a C-30 column provided a better resolution of geometrical isomers of carotenoids than a C-18 column. As no information is available as to the contents of carotenoids and its *cis*-isomers in human plasma in Taiwan, in this study we tried to develop a gradient solvent system using a C-30 column to separate carotenoids and their geometrical isomers in human serum collected from the National Taiwan University Hospital. This paper focuses on modification of extraction and separation of carotenoids and their *cis*-isomers in human serum by HPLC.

2. Experimental

2.1. Materials

all-trans-Lutein and *all-trans*-β-carotene standards were obtained from Sigma Co. (St. Louis, MO, USA). *all-trans*-β-cryptoxanthin, *all-trans*-α-zeaxanthin and *all-trans*-lycopene standards were from Extrasynthese Co. (Geany, France). *all-trans*-α-Carotene standard was from Wako Co. (Japan). The HPLC-grade solvents, including methanol, methylene chloride, acetone and hexane were from Mallinckrodt Co. (Paris, KY, USA). Deionized water was prepared using Milli-Q water purification system (Millipore Co., Bedford, MA, USA). A YMC C-30 column (250 mm × 4.6 mm i.d., 5 μm particle size) with pore size 200 Å was from Waters Co. (Milford, MA, USA).

2.2. Instrumentation

The HPLC system is composed of a Phenomenex DG-440 degasser (Phenomenex Co., Torrance, CA, USA), a Rheodyne model 7161 injector (Rheodyne Co., CA, USA), an Agilent model 1100 UV–vis detector, a Jasco MD-915 photodiode-array detector (Tokyo, Japan), and a Borwin computer software system. The low-temperature incubator (model TL 520R) was from Seng-Long Co. (Taipei, Taiwan). The sonicator (model 2210 R-DTH) was from Branson Co. (Danbury, CT, USA).

2.3. Extraction of carotenoids from human serum

A total of 21 blood samples collected from National Taiwan University Hospital were centrifuged, placed in liquid nitrogen container, and transported to the laboratory immediately on the same day. This is only a preliminary experiment to analyse the carotenoid composition in serums of male adults in Taiwan. A 500 μL serum sample was poured into a 10 mL brown vial. Distilled water (1 mL) and 0.01% ascorbic acid (1 mL) were added to the vial for precipitation of protein and protection of carotenoids, followed by addition of 1 mL acetone. Hexane (3 mL) was then added for extraction of carotenoids and the mixture was shaken in a shaker at 200 rpm for 30 min, after which the hexane layer was collected and evaporated to dryness under nitrogen. The residue was dissolved in 100 μL of methanol/methylene chloride (55:45, v/v) and filtered through a 0.2 μm membrane filter for HPLC analysis. Twenty microliters was injected into HPLC. All the extraction procedure was performed under dimmed light and nitrogen gas was flushed into vials to avoid degradation and isomerization loss of carotenoids.

2.4. Recovery and reproducibility study

Pooled human serum was spiked with 50 μL of combined carotenoid standards so as to provide 50 ng/mL concentration of each standard. The spiked serum was then extracted adopting the method described above. After performing HPLC analysis, the recovery of each carotenoid was obtained by dividing the calculated concentration by the added concentration. Triplicate analyses were performed and the mean value was determined. The reproducibility of this method was carried out by performing six replicate analyses on the same day and on five different days. The intra-day relative standard deviations (R.S.D.) were 0.08–0.21% for retention times of individual carotenoid and 0.23–2.68% for standard concentrations, whereas the inter-day R.S.D. were 0.10–0.27% for retention times and 0.31–3.39% for standard concentrations.

2.5. HPLC analysis of carotenoids in human serum

Various binary and ternary solvent systems were compared with respect to the separation efficiency of lutein, zeaxanthin, α-crptoxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene and their *cis*-isomers. Two binary solvent systems in different proportions, including methanol/methylene chloride (99:1, 97:3 and 95:5, v/v) were used in isocratic condition. Methanol/isopropanol (99:1, v/v) (A) and methylene chloride (100%) (B) were used in the gradient condition. Likewise, two ternary solvent systems in different proportions, including *n*-butanol/acetonitrile/methylene chloride (30:70:10, v/v/v) and *iso*-butanol/acetonitrile/methylene chloride (25:70:5, 25:70:10 and 25:70:20, v/v/v) were used. The solvent strength of each mobile phase was carefully controlled by calculating the polarity index. Both C-18 and C-30 columns were used for comparison. The flow rate was 1.0 mL/min and column temperature was 25 °C with detection at 476 nm and sensitivity at 0.005 AUFS. The major carotenoids, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene in serum samples were identified by comparing the retention times and absorption spectra of unknown peaks with reference standards and cochromatography with added standards. No suitable internal standard was used for quantification because there are too many peaks present on the HPLC chromatogram. Instead, the various carotenoids were quantified using absolute calibration curves. Five concentrations ranging 0–4.0 μg/mL were prepared for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene. After injection into HPLC, the calibration curve of each carotenoid was made by plotting peak area against concentration. High correlation coefficients

(R^2) were found for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene, which reached to 0.9927, 0.9941, 0.9938, 0.9996, 0.9934 and 0.9995, respectively. The amounts of carotenoids were calculated from the following regression equations: $y = 270.64x - 32.479$ for lutein, $y = 423.3x - 4.1676$ for zeaxanthin, $y = 338.28x - 16.736$ for β-cryptoxanthin, $y = 200.24x - 4.2807$ for α-carotene, $y = 219.07x - 72.216$ for β-carotene and $y = 228.9x - 24.436$ for lycopene, where y denotes peak area and x denotes concentration. The *cis*-isomers of carotenoids were quantified using the standard curves of *all-trans*-carotenoids because of similarity in extinction coefficient [13,14]. Duplicate analyses were carried out and the data were expressed as mean ± standard deviation.

2.6. Isomerization of all-trans forms of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene standards

A concentration of 100 μg/mL of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene or lycopene standard was prepared separately in methylene chloride. Two milliliters of each standard solution was poured into a 10 mL glass vial and placed in an incubator for illumination at 25 °C for 24 h. All the vials were exposed to four fluorescent tubes (20 W each) with a distance of 30 cm and light intensity of 2000–3000 lx. After illumination, the standard solutions were dried under a stream of nitrogen. The residue was then dissolved in 500 μL sample solvent of methanol/methylene chloride (55:45, v/v) and filtered through a 0.2 μm membrane filter for HPLC analysis. Then the various *cis*-isomers were tentatively identified based on the spectral characteristics and *Q*-ratios as reported in the literature [15,16], and were compared with those unknown peaks in human serum samples for further identification. In addition, the isomerized extracts were also added to serum samples for cochromatography.

2.7. Determination of limits of detection and quantification

Both the limit of detection (LOD) and quantification (LOQ) were determined using a method described by International Conference on Harmonization [17]. Due to the difference in detector response, three concentrations 500, 1000 and 1500 ng/mL were prepared for *all-trans*-lutein, 40, 70 and 100 ng/mL for *all-trans*-zeaxanthin, 400, 500 and 600 ng/mL for *all-trans*-β-cryptoxanthin, 200, 300 and 400 ng/mL for *all-trans*-α-carotene, 1000, 2000 and 2500 ng/mL for *all-trans*-β-carotene and 1000, 1500 and 2000 ng/mL for *all-trans*-lycopene. Both LODs and LOQs were calculated based on a formula reported in the previous study [18]. The LODs and LOQs obtained were 5.0 and 15.0, 5.0 and 15.0, 3.0 and 9.0, 8.0 and 25.5, 127.0 and 386.0, as well as 511.0 and 1550.0 ng/mL, respectively, for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene. As the α-cryptoxanthin standard is not commercially available, both LOD and LOQ were assessed to be equivalent to β-cryptoxanthin standard.

3. Results and discussion

3.1. Extraction of carotenoids in human serum

The extraction method used in this study is simple and rapid, which requires about 35 min for complete extraction of carotenoids. In the beginning, ascorbic acid was added to the extraction solvent to precipitate protein, thereby protect carotenoids and minimize loss due to oxidation of carotenoids during extraction and subsequent evaporation. The extraction efficiency was validated by a high recovery of 93, 94, 89, 92, 91 and 94%, respectively, for lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene. The recoveries for lutein, β-carotene and lycopene were found to be higher than that reported by Tzeng et al. [19] who also used similar extraction solvents. The difference in the recovery may be due to the higher volume (1 mL for 500 μL of serum) of the antioxidant (ascorbic acid) added, as opposed to only 70 μL used for 200 μL serum by Tzeng et al. [19]. The addition of ascorbic acid, in fact, prevents significant carotenoid losses during extraction and subsequent evaporation. Talwar et al. [20] have reported that adding ascorbic acid prevented about 20% loss of carotenoid.

3.2. HPLC separation of carotenoids in human serum

According to a study by Chen and Chen [21], an appropriate sample solvent has to be selected carefully because it may change the polarity of mobile phase and thus affect the separation efficiency of carotenoids. For the present study, a sample solvent of methanol/methylene chloride (55:45) was used to dissolve the residue of extracted carotenoids. Two types of columns, C-18 and C-30, in combination with various binary and ternary solvent systems, were compared with respect to separation efficiency of various carotenoids in human serum. It was observed that the C-30 column could resolve more geometrical carotenoid isomers than C-18 column, which may be due to its characteristic shape selectivity [22,23]. Thus, a C-30 column was selected instead of a C-18 column for separation of complicated carotenoids in human serum. After various studies, a gradient mobile phase of methanol (100%) (A) and methylene chloride (100%) (B) was developed: 90% A and 10% B in the beginning, maintained for 5 min, decreased to 78% A at 15 min, 62% A at 30 min, 52% A at 40 min, 41% A at 50 min, 38% A at 55 min, maintained for 3 min, and returned to 90% A at 65 min. A total of 21 carotenoids were resolved within 51 min at a flow rate of 1.0 mL/min and detection at 476 nm. The injection volume was 20 μl.

Fig. 1 shows the HPLC chromatogram of carotenoids in human serum. With the exception of some peaks (1–5), most carotenoids were adequately resolved. Table 1 shows the

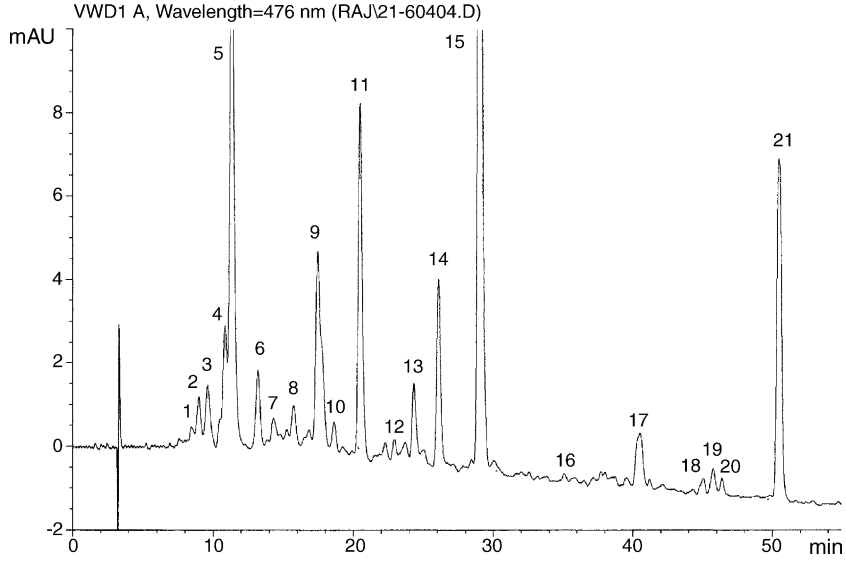

Fig. 1. HPLC chromatogram of carotenoids in human serum.

retention time (R_t), retention factor (k) and separation factor (α) of various carotenoids in human serum. The k values for all the peaks ranged from 1.58 to 14.25, indicating that a proper solvent strength was controlled. Dolan [24] reported that for separation of complicated components, the k values can be ranged between 0.5 and 20. The α values for all the peaks were greater than 1, implying that a good selectivity of mobile phase to sample components was achieved.

Table 1
Retention time (R_t), retention factor (k) and separation factor (α) of carotenoids in human serum

Peak no.	Compound	R_t (min)	k[a]	α[b]
1	*cis*-Lutein	8.54	1.58	1.09
2	9- or 9′-*cis*-Lutein	9.04	1.73	1.09
3	*cis*-Lutein	9.65	1.91	1.11
4	13- or 13′-*cis*-Lutein	10.91	2.29	1.19
5	*all-trans*-Lutein	11.39	2.43	1.06
6	*all-trans*-Zeaxanthin	13.26	3.01	1.23
7	9- or 9′-*cis*-Lutein	14.35	3.33	1.11
8	*cis*-β-Cryptoxanthin	15.79	3.77	1.13
9	*all-trans*-α-Cryptoxanthin	17.52	4.29	1.14
10	*cis*-β-Cryptoxanthin	18.72	4.65	1.09
11	*all-trans*-β-Cryptoxanthin	20.57	5.21	1.12
12	13- or 13′-*cis*-β-Carotene	24.39	6.36	1.22
13	15- or 15′-*cis*-β-Carotene	25.21	6.61	1.04
14	*all-trans*-α-Carotene	26.14	6.89	1.13
15	*all-trans*-β-Carotene	29.08	7.78	1.22
16	*cis*-Lycopene	38.09	10.49	1.04
17	13- or 13′-*cis*-Lycopene	40.58	11.25	1.07
18	15- or 15′-*cis*-Lycopene	45.18	12.64	1.12
19	9- or 9′-*cis*-Lycopene	45.89	12.85	1.02
20	5- or 5′-*cis*-Lycopene	46.49	13.03	1.01
21	*all-trans*-Lycopene	50.51	14.25	1.09

[a] Retention factor: $k = (t_R - t_0)/t_0$, where t_R denotes retention time of sample components and t_0 denotes retention time of sample solvent.
[b] Separation factor: $\alpha = (t_{R2} - t_0)/(t_{R1} - t_0)$, where both t_{R2} and t_{R1} denote two neighboring peaks.

Many previous studies on determination of carotenoids in human serum have either resolved only a few carotenoids or the separation time is lengthy. Talwar et al. [20] used an isocratic mobile phase of methanol/acetonitrile/tetrahydrofuran (75:20:5, v/v/v) containing 0.01% ascorbic acid for separation of lutein, lycopene, α-carotene, β-carotene and β-cryptoxanthin in human serum. However, no *cis*-isomers were detected. Hosotani and Kitagawa [25] employed a C-18 column with a quaternary mobile phase of acetonitrile/dichloromethane/methanol/1-octanol (90:15:10:0.1, v/v/v/v) for separation of only β-carotene in human serum. An HPLC method was developed by Lee et al. [26], who used a C-18 column and an isocratic system with a mixture of acetonitrile and methanol (65:35, v/v) for separation of β-cryptoxanthin, α-carotene, β-carotene, lutein, zeaxanthin, lycopene and canthaxanthin in human serum. However, no *cis*-isomers of carotenoids were resolved in human serum. Khachik et al. [2] developed a method to separate 34 carotenoids, including 13 *cis*-isomers in human serum, by employing two different eluents A and B, consisting of a mixture of quaternary solvents each. However, the retention time is lengthy (90 min) and the mobile phase used is too complicated.

Table 2 shows the identification data of the various carotenoids in human serum. Six *all-trans*-carotenoids, including lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene were identified based on the criteria as described above. The visible absorption spectrum (in-line) of peak 5 was 417, 441 and 465 nm, which was similar to that in a report by Liu et al. [15] and identical to the *all-trans*-lutein standard in this study. In addition, the retention behaviour of peak 5 was the same as *all-trans*-lutein standard. For confirmation, *all-trans*-lutein standard was added to the extract for cochromatography and peak 5 was found indeed to be *all-trans*-lutein. Following the same approach, peaks 6, 9, 11, 14,

Table 2
Tentative identification data for *all-trans* and *cis*-forms of carotenoids in human serum

Peak no.	Compound	λ (nm) (in-line)[a]			λ (nm) (reported)			*Q*-ratio[g] found	*Q*-ratio reported
1	*cis*-Lutein		435	465	419	422	466	0.23	0.21[b]
2	9- or 9′-*cis*-Lutein	417	429	459	417	440	470	0.23	0.22[b]
3	*cis*-Lutein	416	435	459	423	446	470	0.31	0.34[b]
4	13- or 13′-*cis*-Lutein	416	441	465	326	440	464	0.31	0.33[b]
5	*all-trans*-Lutein	417	441	465	426	448	472	0.06	0.06[b]
6	*all-trans*-Zeaxanthin	424	447	471	424	454	478	0.05	0.06[c]
7	9- or 9′-*cis*-Lutein		435	465	420	442	467	0.16	0.12[b]
8	*cis*-β-Cryptoxanthin	411	441	471	–[d]	–	–	0.45	–
9	*all-trans*-α-Cryptoxanthin	417	441	471	424[e]	446	476	0.06	–
10	*cis*-β-Cryptoxanthin	422	441	465	–		–	0.14	–
11	*all-trans*-β-Cryptoxanthin	422	447	471		446[b]	474	0.05	–
12	13- or 13′-*cis*-β-Carotene	422	441	465	423	441	465	0.33	0.31[c]
13	15- or 15′-*cis*-β-Carotene	422	453	470	421	443	470	0.41	0.43[c]
14	*all-trans*-α-Carotene	393	441	471	423	446	476	0.22	0.16[b]
15	*all-trans*-β-Carotene	426	453	477	426	454	478	0.08	0.08[b]
16	*cis*-Lycopene	446	459	483	446	458	488	0.20	0.20[f]
17	13- or 13′-*cis*-Lycopene	441	465	495	440	470	508	0.41	0.55[f]
18	15- or 15′-*cis*-Lycopene	441	465	495	446	470	500	0.67	0.61[f]
19	9- or 9′-*cis*-Lycopene	446	465	483	446	470	500	0.12	0.12[f]
20	5- or 5′-*cis*-Lycopene	411	459	483	434	464	494	0.10	0.10[f]
21	*all-trans*-Lycopene	447	471	507	452[f]	476	506	0.07	–

[a] A gradient mobile phase of methanol (100%) and methylene chloride (from 90:10, v/v to 35:65, v/v) was used.
[b] A mobile phase of methanol/isoproponal (99:1, v/v) and methylene chloride (from 100:0, v/v to 70:30, v/v) was used by Liu et al. [15].
[c] A mobile phase of methanol/isoproponal (99:1, v/v) and methylene chloride (from 100:0, v/v to 70:30, v/v) was used by Chen et al. [16].
[d] "–": data not available.
[e] A mobile phase of acetonitrile/methanol/methylene chloride/hexane (85:10:2.5:2.5, v/v/v/v) was used by Khachik et al. [2].
[f] A gradient mobile phase of 1-butanol/acetonitrile (30:70, v/v) and methylene chloride (from 99:1, v/v to 90:10, v/v) was used by Lin and Chen [13].
[g] *Q*-ratio is defined as the height ratio of the *cis*-peak to the main absorption peak.

15 and 21 were identified as *all-trans*-zeaxanthin, *all-trans*-β-cryptoxanthin, *all-trans*-α-carotene, *all-trans*-β-carotene and *all-trans*-lycopene, respectively. Peak 9 was tentatively identified as *all-trans*-α-cryptoxanthin because of absence of a commercial standard.

The tentative identification of *cis*-isomers of serum carotenoids was based on a hypsochromic shift (approximately 4–5 nm for mono-*cis* and longer for di-*cis* isomers) as compared to *all-trans*-carotenoids [13,27]. It has been reported that the presence of central *cis*-isomers, such as 13- or 15-*cis*-carotenoids, would result in a significant absorption in the ultraviolet region (320–380 nm) [27,28]. The ratio of height at *cis*-peak to the height at maximum absorption peak is defined as *Q*-ratio [29–31], which may also be used to identify the *cis*-isomers. In addition, *all-trans*-lutein, *all-trans*-zeaxanthin, *all-trans*-β-cryptoxanthin, *all-trans*-α-carotene, *all-trans*-β-carotene, and *all-trans*-lycopene standards were illuminated at 25° C for 24 h separately to facilitate formation of *cis*-isomers, which can be used for further identification. This illumination condition was selected to control the formation of *cis*-isomers of carotenoids, which may undergo further degradation after prolonged exposure to light. The various *cis*-isomers of carotenoids were then tentatively identified on the basis of criteria described in the method section. The illuminated standard solution was each added to the sample for cochromatography. Peaks 2, 4 and 7 were identified as 9- or 9′-*cis*-lutein, 13- or 13-*cis*-lutein, and 9- or 9′-*cis*-lutein, with the *Q*-ratios 0.23, 0.31 and 0.16, respectively. Both peaks 1 and 3 were tentatively identified as *cis*-isomers of lutein, however, no *cis*-position was assigned because there are no literature values available. Peaks 8 and 10 were identified as *cis*-isomers of β-cryptoxanthin. Peaks 12 and 13 were assigned as 13- or 13′- and 15- or 15′-*cis*-β-carotene based on the absorption wavelength and the *Q*-ratios [26]. Peaks 16–20 were tentatively identified as *cis*-, 13- or 13′-*cis*-, 15- or 15′-*cis*-, 9- or 9′-*cis*- and 5- or 5′-*cis*-lycopene, respectively, based on a hypsochromic shift of 12, 6, 6, 6 and 12 nm and the *Q*-ratios [13,18,32,33].

3.3. Concentration of carotenoids in human serum

The RP-HPLC method employed in this study enabled separation, identification and quantification of 21 carotenoids, including 14 *cis*-isomers. Tables 3–5 show the quantitative data of the various carotenoids in human serum samples. In most samples, β-carotene showed higher concentrations when compared to the other carotenoids. The concentration of the three major carotenoids in human serum followed the order: β-carotene > lycopene > lutein. This trend was similar to that reported by Sharpless et al. [34] and Tzeng et al. [19]. However, lycopene was shown to be present in higher amounts than β-carotene and lutein in the human serum of lactating mothers [2]. This difference may be caused by the diet pattern, as reported by Tzeng et al. [19]. The concentration levels of *all-trans* forms of β-carotene, lycopene and lutein ranged from 256.3 to 864.2 ng/mL

Table 3
Concentration (ng/mL)[a] of lutein and its *cis*-isomers, zeaxanthin and α-cryptoxanthin in human serum samples

Sample no.	Lutein						Zeaxanthin	α-Cryptoxanthin
	cis-Lutein	9- or 9′-*cis*	*cis*-Lutein	13- or 13′-*cis*	*all-trans*	9- or 9′-*cis*	*all-trans*	*all-trans*
1	42.3 ± 0.2	50.6 ± 0.1	42.9 ± 0.2	96.2 ± 0.3	283.8 ± 0.4	41.8 ± 0.1	11.6 ± 0.2	51.0 ± 0.1
2	40.5 ± 3.9	46.7 ± 1.1	42.8 ± 0.5	79.8 ± 0.5	254.0 ± 2.8	44.7 ± 0.1	10.5 ± 0.3	53.2 ± 1.2
3	29.4 ± 0.2	37.1 ± 0.5	36.9 ± 0.3	55.7 ± 0.8	137.9 ± 0.7	41.1 ± 0.8	12.9 ± 0.4	62.3 ± 0.6
4	29.6 ± 0.1	42.8 ± 2.0	48.8 ± 0.1	71.7 ± 0.3	250.7 ± 0.3	55.0 ± 0.7	18.1 ± 0.2	110.7 ± 0.1
5	32.7 ± 2.1	41.2 ± 0.3	52.7 ± 0.6	78.8 ± 0.7	276.6 ± 1.9	59.4 ± 0.5	16.9 ± 0.4	115.3 ± 2.1
6	ND[b]	38.9 ± 2.2	49.8 ± 1.2	66.4 ± 0.3	245.5 ± 3.3	56.1 ± 2.7	21.3 ± 0.4	132.2 ± 2.6
7	35.9 ± 0.8	42.4 ± 1.2	33.8 ± 1.1	67.2 ± 0.5	164.1 ± 0.2	64.2 ± 0.6	9.7 ± 1.8	62.3 ± 1.5
8	34.9 ± 0.4	42.1 ± 0.3	32.5 ± 0.2	66.3 ± 0.6	164.1 ± 1.8	60.2 ± 0.6	10.5 ± 0.2	58.6 ± 1.1
9	34.8 ± 1.4	44.9 ± 0.9	35.5 ± 0.6	60.0 ± 2.3	270.8 ± 0.1	60.1 ± 1.6	9.7 ± 1.5	43.1 ± 0.2
10	31.7 ± 0.1	46.4 ± 0.4	42.4 ± 0.2	47.1 ± 0.3	199.7 ± 2.7	46.3 ± 0.4	11.6 ± 0.9	87.6 ± 0.4
11	31.2 ± 0.1	42.6 ± 0.5	35.3 ± 1.5	45.0 ± 0.5	164.3 ± 2.4	58.0 ± 2.8	11.5 ± 0.2	64.2 ± 0.1
12	34.7 ± 3.3	44.3 ± 0.9	43.3 ± 1.4	46.4 ± 0.2	197.6 ± 0.1	42.1 ± 1.2	10.6 ± 0.6	62.9 ± 0.8
13	47.7 ± 0.7	61.6 ± 0.3	50.7 ± 0.8	140.6 ± 0.7	450.3 ± 1.4	62.2 ± 1.0	13.9 ± 1.3	87.6 ± 0.6
14	47.0 ± 6.9	62.6 ± 1.7	51.9 ± 4.8	121.1 ± 2.3	422.8 ± 2.4	60.2 ± 1.2	14.9 ± 1.8	79.5 ± 1.4
15	45.4 ± 4.1	62.6 ± 0.8	47.1 ± 0.5	105.9 ± 1.9	374.7 ± 1.4	59.7 ± 3.0	20.1 ± 0.3	72.7 ± 0.6
16	28.4 ± 0.3	34.6 ± 0.3	33.3 ± 0.4	53.5 ± 0.6	156.9 ± 0.7	54.1 ± 0.4	9.1 ± 0.1	134.5 ± 0.5
17	28.0 ± 0.2	32.7 ± 0.5	32.0 ± 0.2	50.8 ± 2.7	148.5 ± 2.2	38.8 ± 0.4	19.1 ± 1.7	98.7 ± 1.2
18	28.9 ± 0.1	37.4 ± 2.3	35.4 ± 0.1	57.4 ± 1.1	172.2 ± 0.6	58.4 ± 1.7	11.1 ± 1.1	89.0 ± 0.4
19	39.7 ± 4.1	54.7 ± 5.6	44.2 ± 4.8	83.6 ± 1.2	271.5 ± 2.6	46.8 ± 1.2	10.9 ± 0.5	95.3 ± 1.1
20	40.5 ± 0.4	64.9 ± 1.3	51.5 ± 2.3	93.5 ± 2.7	354.3 ± 2.3	51.8 ± 4.9	17.6 ± 3.3	89.9 ± 2.2
21	33.4 ± 0.2	46.7 ± 0.3	47.8 ± 0.5	70.2 ± 0.8	228.4 ± 0.2	48.7 ± 1.6	19.7 ± 1.1	119.5 ± 0.5

[a] Average of duplicate analyses ± standard deviation.
[b] ND: not detected.

(0.48–1.61 μmol/L), 64.4–569.6 ng/mL (0.12–1.06 μmol/L) and 137.9–450.3 ng/mL (0.24–0.79 μmol/L), respectively. The mean concentration (1.05 μmol/L) of *all-trans*-β-carotene was the same as that reported by Tzeng et al. [19], while the average concentrations of *all-trans* forms of lutein and lycopene were close to a report by Talwar et al. [20], who found that their levels in the human plasma were 0.26 and 0.37 μmol/L, respectively.

Although β-carotene has been the focus of attention because of its high provitamin A activity, the isolation and quantitation of the full profile of carotenoids in human serum will give fundamental knowledge to

Table 4
Concentration (ng/mL)[a] of α-carotene and isomers of β-cryptoxanthin and β-carotene in human serum samples

Sample no.	β-Cryptoxanthin			α-Carotene	β-Carotene		
	cis	*all-trans*	*cis*	*all-trans*	13- or 13′-*cis*	15- or 15′-*cis*	*all-trans*
1	22.1 ± 0.2	100.8 ± 0.1	22.6 ± 0.3	76.3 ± 0.2	95.6 ± 0.2	77.5 ± 0.1	460.9 ± 0.3
2	28.5 ± 0.5	122.5 ± 1.3	28.8 ± 0.2	78.5 ± 2.5	109.5 ± 0.9	83.1 ± 0.7	492.0 ± 2.1
3	34.3 ± 0.5	128.4 ± 2.2	31.0 ± 0.6	46.1 ± 1.5	100.1 ± 1.4	86.5 ± 2.1	361.4 ± 2.4
4	34.6 ± 0.9	97.9 ± 0.2	25.0 ± 0.5	86.3 ± 2.9	102.2 ± 0.1	75.5 ± 0.1	613.5 ± 1.2
5	42.9 ± 0.3	135.2 ± 3.7	43.7 ± 1.4	81.6 ± 0.7	121.3 ± 1.3	89.8 ± 0.4	676.8 ± 1.1
6	63.5 ± 0.6	188.2 ± 1.2	69.9 ± 0.6	67.8 ± 1.2	148.9 ± 1.4	116.2 ± 0.5	864.2 ± 1.2
7	17.9 ± 0.2	69.9 ± 2.0	16.6 ± 0.1	29.7 ± 0.5	90.7 ± 1.7	77.9 ± 0.5	395.9 ± 1.2
8	24.4 ± 1.2	87.0 ± 0.6	23.3 ± 0.8	28.1 ± 1.4	101.8 ± 3.3	76.1 ± 1.1	445.3 ± 1.8
9	25.9 ± 1.4	81.5 ± 0.5	20.9 ± 0.1	20.0 ± 0.5	97.9 ± 1.9	78.1 ± 0.9	350.3 ± 1.4
10	22.9 ± 0.2	55.7 ± 0.6	16.8 ± 0.1	39.2 ± 0.7	86.1 ± 3.1	74.4 ± 0.3	368.1 ± 1.4
11	27.1 ± 4.8	67.6 ± 1.8	20.4 ± 0.1	36.3 ± 0.3	86.1 ± 0.3	72.4 ± 0.2	321.5 ± 1.1
12	32.0 ± 0.2	87.8 ± 4.3	22.9 ± 2.3	55.5 ± 0.6	109.6 ± 3.3	79.7 ± 1.5	512.5 ± 3.4
13	19.4 ± 0.6	94.4 ± 1.5	18.3 ± 0.9	43.9 ± 0.2	89.9 ± 0.8	77.4 ± 0.5	593.5 ± 1.9
14	30.3 ± 1.6	137.4 ± 1.7	40.0 ± 3.1	42.6 ± 3.2	112.1 ± 2.4	81.2 ± 0.7	598.4 ± 2.1
15	51.7 ± 2.3	186.2 ± 2.5	43.6 ± 2.4	46.4 ± 0.1	118.8 ± 2.5	93.8 ± 2.6	709.1 ± 9.1
16	20.9 ± 0.5	68.1 ± 0.3	16.7 ± 0.1	51.5 ± 0.9	75.8 ± 0.8	72.5 ± 0.5	256.3 ± 0.1
17	31.9 ± 0.8	98.4 ± 3.8	32.1 ± 4.5	50.4 ± 0.1	83.1 ± 0.7	83.3 ± 0.1	301.5 ± 2.7
18	47.1 ± 0.2	150.4 ± 2.4	42.1 ± 0.3	44.7 ± 0.3	94.9 ± 1.8	88.4 ± 2.4	311.9 ± 1.1
19	27.8 ± 0.3	93.8 ± 1.8	25.9 ± 0.9	122.1 ± 1.1	100.9 ± 1.4	79.6 ± 3.5	544.8 ± 2.2
20	38.1 ± 5.2	123.2 ± 1.6	24.6 ± 2.2	115.1 ± 0.9	120.1 ± 2.3	77.5 ± 1.3	600.5 ± 2.3
21	37.7 ± 2.8	131.1 ± 0.7	31.4 ± 2.1	115.3 ± 0.9	117.7 ± 1.3	83.1 ± 0.7	644.6 ± 2.4

[a] Average of duplicate analyses ± standard deviation.

Table 5
Concentration (ng/mL)[a] of lycopene and its *cis*-isomers in human serum samples

Sample no.	Lycopene					
	cis	13- or 13′-*cis*	15- or 15′-*cis*	9- or 9′-*cis*	5- or 5′-*cis*	*all-trans*
1	23.3 ± 0.1	48.6 ± 0.3	28.6 ± 0.2	33.0 ± 0.2	28.1 ± 0.1	202.1 ± 0.3
2	26.3 ± 0.9	90.2 ± 2.3	36.7 ± 0.5	50.2 ± 1.3	23.4 ± 0.1	449.7 ± 1.8
3	26.5 ± 0.6	100.2 ± 0.9	41.8 ± 0.6	53.9 ± 0.3	27.8 ± 0.5	435.8 ± 2.9
4	23.5 ± 0.1	59.0 ± 1.1	29.9 ± 0.2	30.5 ± 0.5	27.6 ± 0.5	187.1 ± 3.2
5	28.8 ± 1.2	86.2 ± 2.9	30.1 ± 0.8	41.4 ± 0.5	29.5 ± 0.2	335.6 ± 1.6
6	29.8 ± 0.1	133.3 ± 1.3	30.9 ± 3.8	59.4 ± 1.6	36.7 ± 0.6	569.6 ± 1.3
7	23.5 ± 0.1	41.8 ± 1.2	28.1 ± 0.5	27.9 ± 0.6	28.4 ± 0.1	120.6 ± 1.9
8	24.4 ± 0.3	59.9 ± 1.8	30.3 ± 0.3	36.8 ± 0.6	25.6 ± 0.3	240.4 ± 0.9
9	23.8 ± 0.4	59.1 ± 0.9	29.7 ± 0.7	38.2 ± 1.6	24.8 ± 0.6	226.8 ± 1.3
10	23.9 ± 0.1	73.1 ± 6.1	28.6 ± 0.4	34.6 ± 2.1	29.5 ± 0.6	265.7 ± 3.4
11	24.8 ± 0.5	90.4 ± 1.3	30.4 ± 2.4	41.8 ± 0.4	26.2 ± 0.7	405.2 ± 1.6
12	26.7 ± 0.5	114.4 ± 2.3	40.6 ± 2.2	55.1 ± 1.2	28.4 ± 0.6	474.4 ± 3.8
13	26.3 ± 0.5	40.9 ± 0.6	26.6 ± 0.5	24.9 ± 0.1	24.3 ± 0.4	64.4 ± 0.3
14	28.5 ± 0.6	93.5 ± 3.3	31.7 ± 0.6	45.1 ± 0.5	23.9 ± 0.3	436.9 ± 1.7
15	31.8 ± 0.5	132.9 ± 0.2	48.6 ± 0.9	71.4 ± 0.6	29.3 ± 0.7	532.2 ± 1.2
16	27.2 ± 0.1	56.5 ± 0.8	23.8 ± 0.5	27.6 ± 0.5	23.6 ± 0.6	114.3 ± 3.5
17	27.0 ± 0.7	85.5 ± 2.7	31.5 ± 1.4	48.4 ± 0.4	23.3 ± 0.1	313.3 ± 3.6
18	30.3 ± 2.5	108.7 ± 0.5	45.7 ± 1.5	61.3 ± 1.1	24.8 ± 0.2	414.6 ± 2.2
19	23.9 ± 0.3	53.5 ± 4.5	28.9 ± 0.1	31.1 ± 0.5	24.5 ± 0.3	134.5 ± 1.4
20	24.9 ± 0.4	92.9 ± 1.4	31.3 ± 0.1	40.2 ± 0.9	24.1 ± 0.7	359 3 ± 1.2
21	23.9 ± 0.2	121.0 ± 2.4	37.7 ± 0.1	51.6 ± 2.6	26.2 ± 0.6	363.6 ± 2.2

[a] Average of duplicate analyses ± standard deviation.

our understanding of the role of the other provitamin A sources, like, β-cryptoxanthin and α-carotene as well as non-vitamin A active carotenoids. The concentration range of the other carotenoids, namely, zeaxanthin, α-cryptoxanthin and α-carotene were 9.1–21.3 ng/mL (0.02–0.04 μmol/L), 43.1–134.5 ng/mL (0.08–0.24 μmol/L) and 20.0–122.1 ng/mL (0.04–0.23 μmol/L), respectively, and their mean concentration levels were close to those reported by Khachik et al. [2], who have determined their contents as 0.04, 0.08 and 0.12 μmol/L.

The ability of an analytical method to accurately determine *cis–trans* carotenoid profiles of biological fluids is a prerequisite to acquiring a better understanding of the biological significance of *cis*-carotenoids. Apparently, C-30 columns, though takes longer time than C-18 column, provide good separation of carotenoids and their isomers, in particular. In this study, about five *cis*-isomers of lutein and lycopene each and two of β-cryptoxanthin and β-carotene each were identified and quantified. The *cis*-isomers make up about 52, 42, 30 and 52% (mean percentage) of the total lutein, β-cryptoxanthin, β-carotene and lycopene, respectively, in the human serum samples analyzed, which indicates that the *cis*-forms of lutein and lycopene also play an important biological role. The data analyzed in this study proves the hypothesis [35,36] that a high percentage of *cis*-lycopene is present in human serum and tissues because it may be better absorbed than *trans*-lycopene. This is probably because of lower tendency of *cis*-isomers to aggregate, enhanced solubility of *cis*-lycopene in bile acid micelles, and possibly preferential incorporation into chylomicrons [5]. It is also possible that lycopene exists in both human and animal tissues as ∼50% *cis*-lycopene because of existence of equilibrium between *trans*-lycopene and its *cis*-isomers. However, in the food matrix, this isomeric equilibrium does not occur. The *cis*-forms of β-cryptoxanthin also contribute significantly (42%) to their total contents in the human serum samples analyzed. But, the *cis*-isomers of β-carotene contributed less significantly, which may be due to the isomerization of 9-*cis*-β-carotene into *all-trans*-β-carotene during absorption in humans, as demonstrated by You et al. [37].

The in vitro antioxidant activity study conducted by Bohm et al. [38], using the Trolox equivalent antioxidant activity assay (TEAC), illustrate that the *cis*-isomers of lycopene had highest antioxidant activity (about two times higher than the activity of β-carotene) among the 17 geometrical isomers of α-carotene, β-carotene, lycopene and zeaxanthin. As β-carotene was shown to have a higher concentration than lycopene in this study, it is suggested that the lycopene level in the serum of the subjects could be elevated by advising them to consume lycopene-rich food products such as adequately processed tomatoes. Because, lycopene, though possesses no provitamin A activity, does exhibit other vital biological properties in comparison with β-carotene [39].

The foregoing study has demonstrated a simple, yet an improved HPLC method for determination of carotenoids and their isomers in human serum. The discussion on the results has clearly signified the importance of determination of *cis*-isomers of carotenoids in human serum so as to understand their bioavailability. A further study is warranted to improve/modify the method of determination in order to identify the various *cis*-isomers in human serum by HPLC-NMR.

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