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An improved high performance liquid chromatography–diode array detection–mass spectrometry method for determination of carotenoids and their precursors phytoene and phytofluene in human serum

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ABSTRACT

An improved high performance liquid chromatography–diode array detection–mass spectrometry method was developed for determination of various carotenoids and their precursors phytoene and phytofluene in human serum. A polymeric C30 column and mobile phase of (A) methanol/acetonitrile/water (84:14:4, v/v/v) and (B) dichloromethane (100%) were employed with the gradient condition of 100% A and 0% B initially, raised to 10% B at 4 min, 18% B at 12 min, 21% B at 17 min, 30% B at 20 min and maintained until 25 min and increased further to 39% B at 28 min, 60% B at 40 min and returned to 100% A and 0% B at 45 min. A total of 30 carotenoids, including 6 all-*trans* forms, 20 *cis*-isomers, 2 β-carotene epoxides, phytoene and phytofluene, were resolved within 45 min at a flow-rate of 1 mL/min, column temperature 25 °C and detection wavelengths 450, 348 and 286 nm. Identification of carotenoids was carried out by comparing retention behavior, absorption and mass spectral data with those of reference standards, isomerized standards and reported values. An internal standard parared was found appropriate for quantitation of all the carotenoids. The developed method provided high sensitivity with low detection and quantitation limits (2–14 and 6–43 ng/mL), high recovery (91–99%), and small intra-day and inter-day variations (0.14–6.01% and 0.31–7.28%). Application of the developed method to Taiwan subjects supplemented with carotenoid-rich capsules revealed β-carotene plus its *cis* isomers as well as epoxide derivatives to be present in largest amount (1069.8–2783.1 ng/mL) in serum, followed by lutein plus its *cis* isomers (511.6–2009.5 ng/mL), phytofluene plus its *cis* isomer (515.0–1765.0 ng/mL), lycopene plus its *cis* isomers (551.1–1455.1 ng/mL), β-cryptoxanthin plus its *cis* isomers (458.0–965.0 ng/mL), all-*trans*-zeaxanthin (110.0–177.0 ng/mL), phytoene (41.8–165.0 ng/mL) and all-*trans*-α-carotene (37.5–95.9 ng/mL).

1. Introduction

Carotenoids are pigmented molecules widely distributed in nature, with fruits and vegetables as major sources in human diet. Epidemiological studies have shown a relationship between high intake of fruits and vegetables and reduced risk of cancer, cardiovascular disease and immune deficiency [1–3]. Of the 600 carotenoids identified so far, about 40 are reported to be present in a typical human diet with only 20 being identified in human blood and tissues [1]. Nearly 90% of the carotenoids in both human diet and serum is composed of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene [1,4]. The beneficial effects of these carotenoids have been attributed to their antioxidant properties and tendency to mediate gap junction communication, regulate cell growth, modulate gene expression immune response and alter phase I and II drug metabolizing enzymes [1–3,5]. Nevertheless, α-carotene, β-carotene and β-cryptoxanthin being precursors of vitamin A should also play a significant role in the prevention of chronic diseases [1,6]. Thus, it is imperative to develop a suitable analytical technique for determination of absorption, distribution and metabolism in human serum or plasma and tissues.

Although crucial information on carotenoid status may be gained by tissue analysis, the circulating blood is often used for monitoring its level in human for bioavailability determination [7]. Accordingly, the liquid–liquid extraction method with hexane as solvent alone or in combination with some other solvents acetone or ethyl acetate is often used for purification of carotenoids from human serum [4,8]. Prior to extraction, a sample pretreatment is usually employed for precipitation of proteins with a

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polar solvent like ethanol [4,8]. High-performance liquid chromatography (HPLC) with normal or reversed-phase can be used for carotenoid analysis, with the latter being more frequently adopted owing to superior separation efficiency and commercial availability of various stationary phases and packed columns [7,9–12]. Ultraviolet–visible or diode-array detectors (DAD) are often coupled with HPLC, while electrochemical array and mass spectrometry (MS) used for high sensitivity in complex mixtures involving spectral interferences in DAD [7,9]. For ease of separation and simplicity, C18 columns with isocratic or gradient mode are preferred for separation of carotenoids [4,7,10–14]. But, they fail to resolve geometrical isomers and certain positional isomers, particularly lutein and zeaxanthin, inadequately. These shortcomings are often compromised for shorter analysis time and simple isocratic condition used. For instance, Thibeault et al. [10] employed a Thermo Spherisorb ODS2 column for simultaneous separation of 5 carotenoids, retinol and 3 tocopherols in human serum within 13 min. However, lutein and zeaxanthin were quantified together with no *cis*-carotenoids being resolved. In another study, a pair of Synergy Hydro-RP 80A columns connected in series was used by Karppi et al. [4] to resolve lutein and zeaxanthin, but no *cis*-isomers were separated. Separate quantification of lutein and zeaxanthin is essential to investigate their individual roles in age-related macular degeneration [7]. The polymeric C30 columns possess unique abilities to resolve *cis*/*trans*-carotenoids and are widely adopted for analysis in biological extracts [6,15–18]. In a study dealing with quantitation of *cis*- and *trans*-carotenoids in human blood of Taiwan subjects, Rajendran et al. [6] developed a HPLC method and separated 21 *cis* plus *trans* carotenoids within 51 min by using a polymeric C30 column and a gradient mobile phase. Yet, several peaks remained unidentified and the baseline drifted largely making quantitation more difficult. Thus, an improved HPLC method using a polymeric C30 column is indispensable for separation, identification and quantitation of various carotenoid isomers with better sensitivity and selectivity in human serum.

Furthermore, biosynthetic carotenoid precursors phytoene and phytofluene have been shown to accumulate in significant quantities in both human serum and tissues, including liver, lung, breast, skin and prostate [19–21]. Also, their antioxidant and anticarcinogenic activities have been demonstrated both *in vitro* and *in vivo* [19–21]. Consequently, the determination of their levels in biological samples and metabolites is vital. In early literature reports, Khachik et al. [12] separated several carotenoids and their metabolites in serum and milk of lactating mother by a C18 column and inadequate resolution of phytoene and phytofluene was shown due to co-elution with steryl esters. In another study, Porrini et al. [20] used a C18 column to analyze lutein, zeaxanthin, β-cryptoxanthin, α- and β-carotene with detection at 445 nm, lycopene at 472 nm, phytoene at 286 nm and phytofluene at 366 nm. However, the quantitation of phytoene and phytofluene was based on lycopene standard curve, which should be less accurate. The objective of this study was to develop a HPLC–DAD–MS method for determination of carotenoids and their biosynthetic precursors phytoene and phytofluene in human serum, with the method being validated by quality control parameters and testing with 6 blood samples collected from healthy subjects supplemented with carotenoid-rich capsules in an ongoing clinical trial study conducted at National Taiwan University Hospital.

2. Experimental

2.1. Materials

Carotenoids standards including all-*trans* forms of lutein, zeaxanthin, cryptoxanthin, α-carotene and β-carotene were procured from Sigma (St. Louis, MO, USA) and all-*trans*-lycopene from Extrasynthese Co (Genay, France). Phytoene and phytofluene standards were isolated from a reference standard mixture of phytoene and phytofluene provided by Health Ever Biotech Ltd. (Taiwan) by column chromatographic method described below. The internal standard parared and vitamin C were also purchased from Sigma. The HPLC-grade solvents methanol and acetonitrile were obtained from Merck (Darmstadt, Germany), while dichloromethane, hexane and ethyl acetate from Lab-Scan (Dublin, Ireland). Ethanol (95%) was purchased from Taiwan Tobacco and Wine Monopoly Bureau (Tainan, Taiwan). A Milli-Q water purification system from Millipore (Bedford, MA, USA) was used to prepare deionized water. For chromatographic separation by HPLC, a YMC polymeric C30 column (250 mm × 4.6 mm I.D., 5 μm particle size) from Waters (Milford, MA, USA) was used.

2.2. Instrumentation

The Agilent HPLC system (Palo Alto, CA, USA) consisted of an online degasser (Agilent G1379A), a column oven controller (Agilent G1316A), a photo-diode array detector (Agilent G1315B) and a single quadrupole mass spectrometer (Agilent 6130) with multi-ionization source (APCI/ESI). All the HPLC solvents and deionized water were sonicated by using a 2210R-DTH model sonicator from Branson (Danbury, CT, USA). A reciprocating shaker from Hsiangtai Co. (Taipei, Taiwan) was used for extraction of carotenoids from human serum. A high-speed centrifuge (model 5810) from Eppendorf (Hamburg, Germany) was employed to separate carotenoid extracts.

2.3. Preparation of phytoene and phytofluene standards

A column chromatographic method was developed to prepare phytoene and phytofluene standards from a reference standard mix containing phytoene and phytofluene. Initially, a glass column was packed with 70 g of MgO-Diatomaceous earth (1:1, w/w) and activated with 600 mL of hexane. Next, 1.5 mL of 1000 μg/mL reference standard mix dissolved in hexane was poured into the column, followed by eluting phytoene with 190 mL of 1% ethyl ether in hexane and phytofluene with 140 mL of 5% acetone in hexane. The eluates of phytoene and phytofluene were collected separately, evaporated to dryness and injected for identification and quantitation at 286 and 348 nm, respectively, by HPLC–DAD–MS. The purity of both phytoene and phytofluene was found to be >90%. In addition, the amounts of both phytoene and phytofluene were calculated.

2.4. Extraction of carotenoids from human serum

A method based on Rajendran et al. [6] was modified and used for extraction of carotenoids from human serum. A total of 10 blood samples from random healthy subjects not supplemented with carotenoid-rich capsules were collected from National Taiwan University Hospital and transported to the laboratory on the same day. Basic characteristics of gender, age, body weight and diet of healthy subjects are not provided here as this study is not epidemiological but intends to develop an improved method for determination of various *cis*- and *trans*-carotenoids in human serum. After pooling all the samples, 1 mL was poured into a 10-mL brown vial and 1 mL of 0.01% vitamin C in ethanol added for protein precipitation and carotenoid protection. Then, 1 mL of ethyl acetate and 3 mL of hexane were sequentially added, followed by vortexing (10 s), shaking (10 min at 200 rpm), centrifuging (20 min at 3000 rpm and 4 °C) and collecting the supernatant. To the residue, 3 mL of hexane was added and the procedure repeated three times for complete extraction of carotenoids. The supernatants from three replicate extracts were then pooled and evaporated under N_2. Next, the

residue was dissolved in 50 μL of dichloromethane, followed by mixing with 1 mL of internal standard parared for a final concentration of 1 μg/mL, filtering through a 0.2 μm nylon membrane filter (Critical Process Filtration Inc., Nashua, NH, USA) and injecting 20 μL into HPLC. The entire extraction procedure was carried out under dimmed light and nitrogen gas was flushed into the vials to prevent isomerization and/or degradation of carotenoids.

2.5. HPLC–DAD–MS analysis of carotenoids in human serum

A method based on Inbaraj et al. [22] was modified for separation of various carotenoids in human serum. By employing a binary solvent system of (A) methanol/acetonitrile/water (84:14:4, v/v/v) and (B) dichloromethane, different gradient conditions were evaluated for separation efficiency in terms of retention factor and selectivity (separation factor). The most suitable gradient condition was 100% A and 0% B initially, raised to 10% B at 4 min, 18% B at 12 min, 21% B at 17 min, 30% B at 20 min and maintained until 25 min, increased further to 39% B at 28 min, 60% B at 40 min and returned to 100% B in 45 min. The column temperature was maintained at 25 °C with flow rate at 1 mL/min and detection wavelengths for most carotenoids at 450 nm, phytoene at 286 nm and phytofluene at 348 nm. The purity of all the peaks was automatically determined by DAD, while the retention factor (k) calculated by using the formula $k = (t_R - t_0)/t_0$, where t_R and t_0 denote retention time of sample components and sample solvent, respectively. Based on the retention factor of two neighboring peaks (k_1 and k_2), the separation factor (α) was determined by using the formula, $\alpha = k_2/k_1$ [23].

The identification of carotenoids was performed by comparing retention time, absorption spectra and mass spectra of unknown peaks with those of reference standards and reported values in the literature. For detection by mass spectra, the positive-ion APCI mode was used with total ion current (TIC) scan ranging from 400 to 1200 m/z, vaporizer temperature at 230 °C, capillary voltage at 2000 V, charging voltage at 2000 V, fragmentor voltage at 200 V, corona current at 4 μA, drying gas flow at 7 L/min, drying gas temperature at 330 °C and nebulizer gas pressure at 10 psi. Additionally, the *cis* isomers of carotenoids were further identified by comparing both spectral characteristics and Q-ratios with the photoisomerized carotenoid standards and those reported in the literature.

2.6. Photoisomerization of carotenoids standards

One milligram of commercial standards, including all-*trans* forms of lutein, β-cryptoxanthin, β-carotene and lycopene, each dissolved in 10 mL of dichloromethane separately for a concentration of 100 μg/mL. A 1-mL volume of each standard was poured into a 10-mL glass vial separately and all the vials were illuminated for 6 h in a 25 °C incubator under 4 20-W fluorescent light tubes (FL20D-EX/18, Toalighting, Taoyuan, Taiwan) at a illumination distance of 30 cm and light intensity of 2500–3500 lx. After exposure time reached 6 h, the various standards were evaporated to dryness, dissolved in dichloromethane, filtered through a 0.2-μm nylon membrane filter and 20 μL injected into HPLC–DAD–MS for identification of *cis*-carotenoids through comparison of absorption and mass spectra of each peak in the isomerized standard chromatogram with those of unknown peaks in human serum.

2.7. Quantitation of carotenoids in human serum

The quantitation of carotenoids in human serum was carried out by incorporating an internal standard into the standard solution for standard curves preparation based on peak area ratio of standard to internal standard *versus* concentration ratio of standard to internal standard. Parared was chosen as an appropriate internal standard as its absorption maximum (488 nm) is similar to carotenoids and, more importantly, it did not interfere with separation of the other carotenoid peaks in the HPLC chromatogram. Seven concentrations of 0.5, 1, 5, 10, 15, 20 and 30 μg/mL each for all-*trans* forms of lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene as well as 0.1, 2, 5, 10, 15, 20 and 30 μg/mL for all-*trans* lycopene were prepared separately, and mixed with parared for a final concentration of 1 μg/mL. Since the absorption maximum for phytoene (286 nm) and phytofluene (348 nm) are different from parared, phytoene and phytofluene were quantified by preparing standard curves with 7 concentrations each at 0.1, 2, 5, 10, 15, 20 and 30 μg/mL without adding internal standard. After HPLC analysis, the data were subjected to linear regression analysis by a Microsoft Excel software and the regression equation for each carotenoid was obtained. Owing to the unavailability of commercial standards for *cis*-carotenoids, the quantitation of *cis*-isomers of lutein, β-cryptoxanthin, β-carotene and lycopene were based on the standard curves of their corresponding all-*trans* forms.

2.8. Determination of limits of detection and quantitation

According to a method described by International Conference on Harmonization [23], both limit of detection (LOD) and limit of quantitation (LOQ) were determined by preparing three different concentrations of 0.5, 1 and 5 μg/mL each for all-*trans*-forms of β-cryptoxanthin, α-carotene, β-carotene and lycopene, 0.3, 0.5 and 1 μg/mL for all-*trans*-lutein, 0.2, 0.5 and 1 μg/mL for all-*trans*-zeaxanthin and 0.5, 1 and 2 μg/mL for all-*trans* forms of phytoene and phytofluene. Then, each concentration of standard solution was injected into HPLC 3 times to obtain 3 calibration curves. From the mean of the three slopes (S) and standard deviation of the intercepts (σ), both LOD and LOQ were calculated using the formula, $3.3 \times \sigma/S$ and $10 \times \sigma/S$, respectively [23].

2.9. Determination of recovery and reproducibility

Recovery of carotenoid standards was determined by mixing two concentrations of 2 and 5 μg/mL each for all-*trans* forms of lutein, β-carotene, β-cryptoxanthin and lycopene, and 1 and 2 μg/mL each for all-*trans* forms of zeaxanthin and α-carotene with 1 μg/mL parared. Then, the standard-parared mixture was added to human serum for extraction and HPLC analysis. For phytoene and phytofluene, 1 and 2 μg/mL each were added directly to human serum for extraction and HPLC analysis. The recovery of each carotenoid standard was calculated based on the ratio of the standard concentration determined after HPLC analysis relative to that initially added before HPLC. It should be pointed out that the concentration of each carotenoid in human serum prior to spiking was subtracted during calculation of recovery. The percent recoveries of *cis*-isomers of carotenoids were considered equivalent to their all-*trans* counterparts, as the commercial standards are unavailable and the extinction coefficient of both all-*trans*- and *cis*-carotenoids are assumed to be similar.

Reproducibility of the developed method was determined based on the intra-day and inter-day variability by injecting a serum sample 3 times each in the morning, afternoon and evening on the same day for a total of 9 replicates for the former, while the latter was performed by injecting 3 times in a day and repeated for 3 consecutive days. The concentration of individual all-*trans*- and *cis*-carotenoids in each replicate was quantified based on the procedure described above. The intra-day and inter-day variability were then determined in terms of relative standard deviation (RSD, %) of carotenoid concentration within-a-day and between-days, respectively.

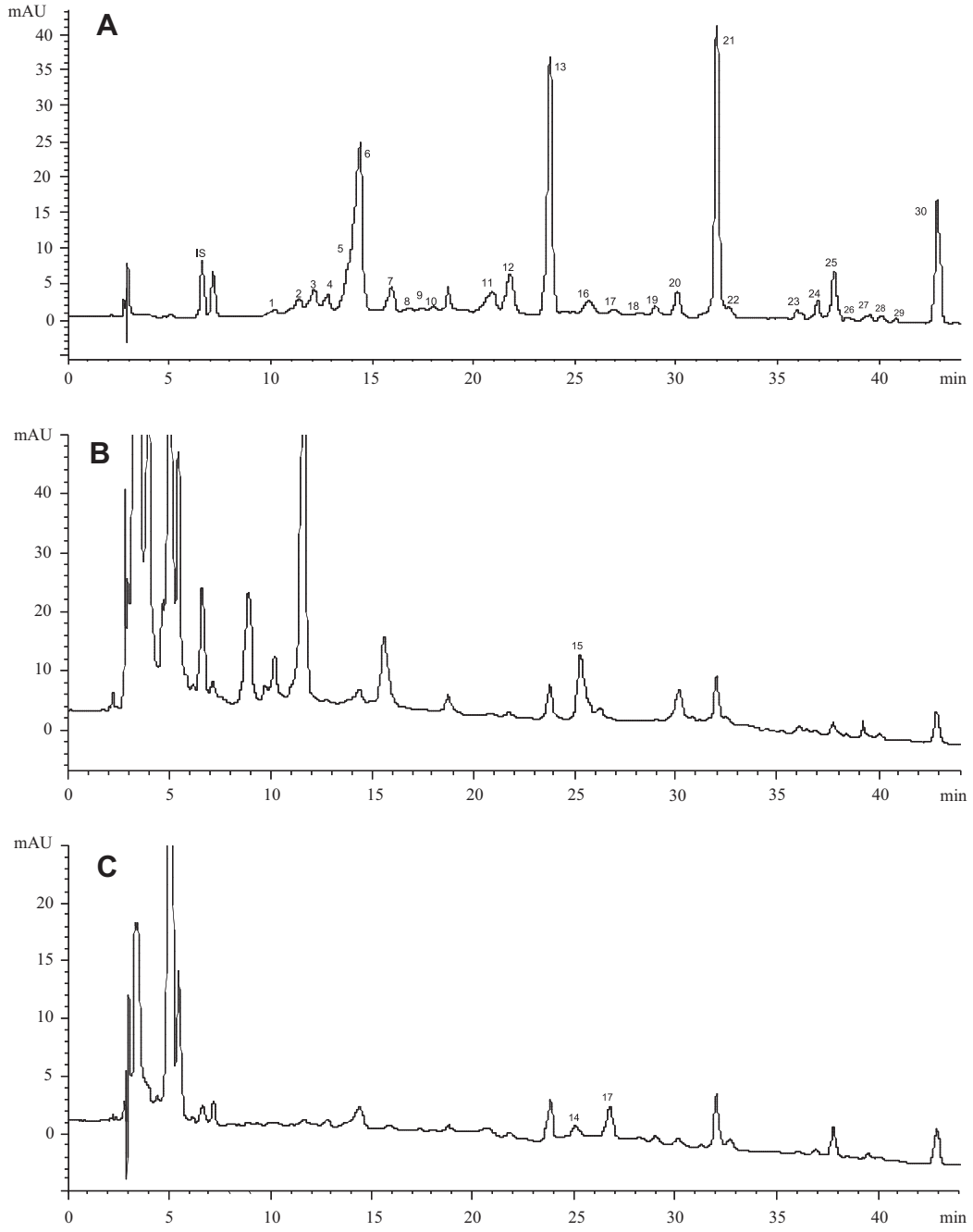

Fig. 1. HPLC chromatogram of carotenoids in human serum with DAD detection at multiple wavelengths 450 nm (A), 286 nm (B) and 348 nm (C). Chromatographic conditions are described in the text. Peaks: 1, *cis*-lutein; 2, *cis*-lutein; 3, *cis*-lutein; 4, 13- or 13′-*cis*-lutein; 5, 13- or 13′-*cis*-lutein; 6, all-*trans*-lutein; 7, all-*trans*-zeaxanthin; 8, 9- or 9′-*cis*-lutein; 9, *cis*-β-cryptoxanthin; 10, 9- or 9′-*cis*-lutein; 11, 13- or 13′-*cis*-β-cryptoxanthin; 12, 13- or 13′-*cis*-β-cryptoxanthin; 13, all-*trans*-β-cryptoxanthin; 14, *cis*-phytofluene; 15, phytoene; 16, β-carotene-5,6-epoxide; 17, phytofluene; 18, β-carotene-5,8-epoxide; 19, 13- or 13′-*cis*-β-carotene; 20, all-*trans*-α-carotene; 21, all-*trans*-β-carotene; 22, 9- or 9′-*cis*-β-carotene; 23, di-*cis*-lycopene; 24, di-*cis*-lycopene; 25, 13- or 13′-*cis*-lycopene; 26, 13- or 13′-*cis*-lycopene; 27, 9- or 9′-*cis*-lycopene; 28, 9- or 9′-*cis*-lycopene; 29, 5- or 5′-*cis*-lycopene and 30, all-*trans*-lycopene.

2.10. Method application to different serum samples

For application of the developed method to different serum samples, 6 blood samples were collected from the clinical trial subjects supplemented with carotenoid-rich capsules and analyzed in duplicate by adopting the same procedure shown above. As the clinical experiments are ongoing, the details of study scope, clinical trial subjects and capsules are not disclosed here. All the identified carotenoid compounds were quantified for each serum sample and reported.

3. Results and discussion

3.1. HPLC separation

Accordingly, it is vital that a suitable sample solvent should be selected as it may alter the polarity of mobile phase, which in turn affect the separation efficiency. In a previous study, a binary solvent mixture of methanol/dichloromethane (55:45, v/v) was shown to be appropriate for separation of carotenoids in human serum [6]. However, in this study, two different

Table 1
Retention time (t_R), retention factor (k), separation factor (α), peak purity and UV absorption data of all-*trans*- and *cis*-carotenoids in human serum.

Peak no.	t_R (min)	Compound[a]	k[b]	α[c]	λ (nm) (in-line)[d]	λ (nm) (reported)	Q-ratio found[k]	Q-ratio reported	Peak purity (%)
1	10.27	*cis*-Lutein	2.45	1.17	332, 412, 438, 466	322, 410, 440, 464[e]	0.32	0.31[e]	99.98
2	11.51	*cis*-Lutein	2.87	1.17	346, 420, 442, 472	332, 416, 440, 464[e]	0.11	0.16[e]	98.67
3	12.24	*cis*-Lutein	3.12	1.09	346, 418, 442, 470	338, 422, 446, 470[e]	0.19	0.28[e]	99.51
4	12.90	13- or 13′-*cis*-lutein	3.34	1.07	332, 416, 440, 468	332, 415, 440, 464[e]	0.44	0.41[e]	99.99
5	13.86	13- or 13′-*cis*-lutein	3.68	1.10	330, 418, 442, 470	332, 416, 440, 464[e]	0.42	0.39[e]	–
6	14.48	All-*trans*-lutein	3.87	1.05	332, 420, 446, 474	332, 422, 446, 470[e]	0.07	0.09[e]	95.71
7	15.97	All-*trans*-zeaxanthin	4.41	1.14	340, 428, 452, 478	–, 426, 450, 478[f]	0.07	0.06[f]	99.96
8	16.82	9-or 9′-*cis*-lutein	4.70	1.07	326, 414, 440, 466	332, 416, 440, 470[e]	0.15	0.13[e]	99.98
9	17.41	*cis*-β-Cryptoxanthin	4.90	1.04	334, 424, 448, 476	–, 411, 441, 471[g]	0.40	0.45[g]	99.99
10	18.01	9- or 9′-*cis*-lutein	5.11	1.04	336, 420, 442, 470	332, 421, 446, 470[e]	0.22	0.12[e]	99.99
11	20.94	13- or 13′-*cis*-β-cryptoxanthin	6.10	1.14	340, 424, 448, 472	336, 415, 443, 470[h]	0.36	0.48[h]	99.94
12	21.79	13- or 13′-*cis*-β-cryptoxanthin	6.39	1.05	332, 426, 452, 474	336, 415, 443, 470[h]	0.40	0.48[h]	99.37
13	23.78	All-*trans*-β-cryptoxanthin	7.06	1.10	340, 426, 454, 480	–, 428, 454, 480[f]	0.09	0.16[f]	99.81
14	25.01	*cis*-Phytofluene	7.48	1.06	–, 332, 348, 366	–, 330, 347, 366[h]	–	–	99.38
15	25.26	Phytoene	7.56	1.01	–, 276, 286, 300	–, 276, 286, 300[h]	–	–	99.69
16	25.46	β-Carotene-5,6-epoxide	7.70	1.02	338, 428, 452, 476	–, 420, 445, 471[h]	0.08	–	99.63
17	26.72	Phytofluene	8.05	1.05	–, 334, 350, 370	–, 330, 347, 366[h]	–	–	99.02
18	28.23	β-Carotene-5,8-epoxide	8.57	1.06	338, 406, 432, 456	–, 404, 428, 453[h]	0.07	–	98.18
19	28.98	13- or13′-*cis*-β-carotene	8.82	1.03	340, 420, 450, 474	342, 422, 448, 474[f]	0.46	0.43[f]	99.50
20	30.05	All-*trans*-α-carotene	9.19	1.04	342, 424, 448, 476	344, 426, 449, 476[e]	0.13	0.10[e]	99.65
21	32.09	All-*trans*-β-carotene	9.84	1.07	348, 430, 456, 482	–, 426, 456, 480[f]	0.10	0.09[f]	97.50
22	32.78	9- or 9′-*cis*-β-carotene	10.06	1.02	342, 430, 450, 476	344, 428, 452, 476[e]	0.16	0.20[e]	99.20
23	36.15	di-*cis*-Lycopene	11.19	1.11	366, 440, 462, 492	368, –, 458, 488[i]	0.19	0.20[i]	99.99
24	37.16	di-*cis*-Lycopene	11.53	1.03	364, 432, 460, 486	368, –, 458, 488[i]	0.17	0.20[i]	99.99
25	37.98	13- or 13′-*cis*-lycopene	11.81	1.02	364, 446, 470, 500	362, 446, 470, 500[j]	0.39	0.54[j]	99.98
26	38.67	13- or 13′-*cis*-lycopene	12.04	1.02	360, 446, 472, 498	362, 446, 470, 500[j]	0.35	0.54[j]	99.97
27	39.53	9- or 9′-*cis*-lycopene	12.40	1.03	366, 444, 472, 504	362, 446, 470, 500[i]	0.18	0.12[i]	99.80
28	40.23	9- or 9′-*cis*-lycopene	12.60	1.02	372, 442, 474, 502	362, 446, 470, 500[i]	0.16	0.12[i]	99.82
29	40.82	5- or 5′-*cis*-lycopene	12.84	1.02	376, 448, 476, 504	344, 446, 476, 506[i]	0.07	0.05[i]	99.99
30	42.94	All-*trans*-lycopene	13.52	1.05	368, 452, 478, 510	–, 452, 476, 506[i]	0.05	–	99.86

[a] Phytoene and phytofluene were detected at an absorption wavelength of 286 and 348 nm, respectively, while all the other carotenoids at 450 nm.
[b] Retention factor: $k = (t_R - t_0)/t_0$, where t_0 and t_R denotes retention time of sample components and sample solvent, respectively.
[c] Separation factor: $\alpha = (t_{R2} - t_0)/(t_{R1} - t_0)$, where both t_{R2} and t_{R1} denote two neighboring peaks.
[d] A gradient mobile phase of methanol/acetonitrile/water (84:14:4, v/v/v) and dichloromethane (from 100:0, v/v to 40:60, v/v) was used.
[e] A mobile phase of methanol–acetonitrile–water (84:14:2, v/v/v) and methylene chloride (from 100:0, v/v to 45:55, v/v) was used by Inbaraj et al. [22].
[f] A mobile phase of methanol–acetonitrile–water (81:14:5, v/v/v) and methylene chloride (from 84:16, v/v to 75:25, v/v) was used by Inbaraj et al. [24].
[g] A gradient mobile phase of methanol (100%) and methylene chloride (from 90:10, v/v to 35:65, v/v) was used by Rajendran et al. [6].
[h] A linear gradient mobile phase of methanol/methyl-tert-butyl ether (from 95:5 to 70:30 in 30 min, to 50:50 in 20 min and maintained for 35 min) was used by de Rosso and Mercadante [26].
[i] 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 [25].
[j] A mobile phase of 1-butanol–acetonitrile–methylene chloride (30:70:10, v/v/v) was used by Lee and Chen [27].
[k] Q-ratio is the height ratio of the *cis*-peak to the main absorption peak.

sample solvents, methanol/dichloromethane (50:50, v/v) and dichloromethane (100%), were evaluated and the latter was found appropriate as a better separation efficiency occurred when compared to the former under condition of the same mobile phase. Then, a HPLC gradient mobile phase system used previously for determination of carotenoids in microalga *Chlorella pyrenoidosa* by Inbaraj et al. [22] was adopted for separation of carotenoids in human serum. However, it failed to provide an adequate resolution because of difference in sample matrix and carotenoid variety. Therefore, after experimenting numerous gradient conditions, for adjustment of solvent strength, a new mobile phase was developed with a binary solvent system composed of (A) methanol/acetonitrile/water (84:14:4, v/v/v) and (B) dichloromethane (100%), with 100% A and 0% B initially, raised to 10% B at 4 min, 18% B at 12 min, 21% B at 17 min, 30% B at 20 min and maintained until 25 min, increased further to 39% B at 28 min, 60% B at 40 min and returned to the initial condition (100% A and 0% B) at 45 min. A total of 30 carotenoids, including all-*trans* forms and their *cis*-isomers, were resolved within 45 min at a flow rate of 1 mL/min and column temperature 25 °C by using a polymeric C30 column. Fig. 1 shows the HPLC chromatogram with detection at 3 wavelengths for most carotenoids at 450 nm (A), phytoene at 286 nm (B) and phytofluene at 348 nm (C). The retention time, retention factor (k), separation factor (α) and peak purity are summarized in Table 1. The k value for all the peaks ranged from 2.45 to 13.52, implying a proper solvent strength was maintained [6,22]. Also, the separation factor between neighboring peaks was >1 for all the peaks, signifying an acceptable selectivity of mobile phase to carotenoids was attained [6,22]. Except for peak 5, the purities of all the other peaks were greater than 95%, while most peaks were >99%.

3.2. Identification of carotenoids

The absorption spectral data of each peak and its corresponding Q-ratio in comparison with the reported ones are presented in Table 1 [6,22,24–27]. Q-ratio denotes the height ratio of the *cis*-peak relative to the main absorption peak. Peaks 6, 7, 13, 15, 17, 20, 21 and 30 were positively identified as all-*trans* forms of lutein, zeaxanthin, β-cryptoxanthin, phytoene, phytofluene α-carotene, β-carotene and lycopene, respectively, by comparing the retention time and absorption spectra with those of reference standards and reported values [6,22,24–26]. The tentative identification of *cis*-isomers in serum was based on a hypsochromic shift of approximately 2–6 nm as compared to their all-*trans* forms [6,27]. Thus, peak 14 was tentatively identified as *cis* isomer of phytofluene. To further ascertain the identity of *cis*-isomers, the retention behavior and absorption spectra of unknown peaks were compared with those of photoisomerized standards. Fig. 2 shows the HPLC chromatogram of illuminated carotenoid standards assigned with the same peak numbers as in Fig. 1 for comparative identification.

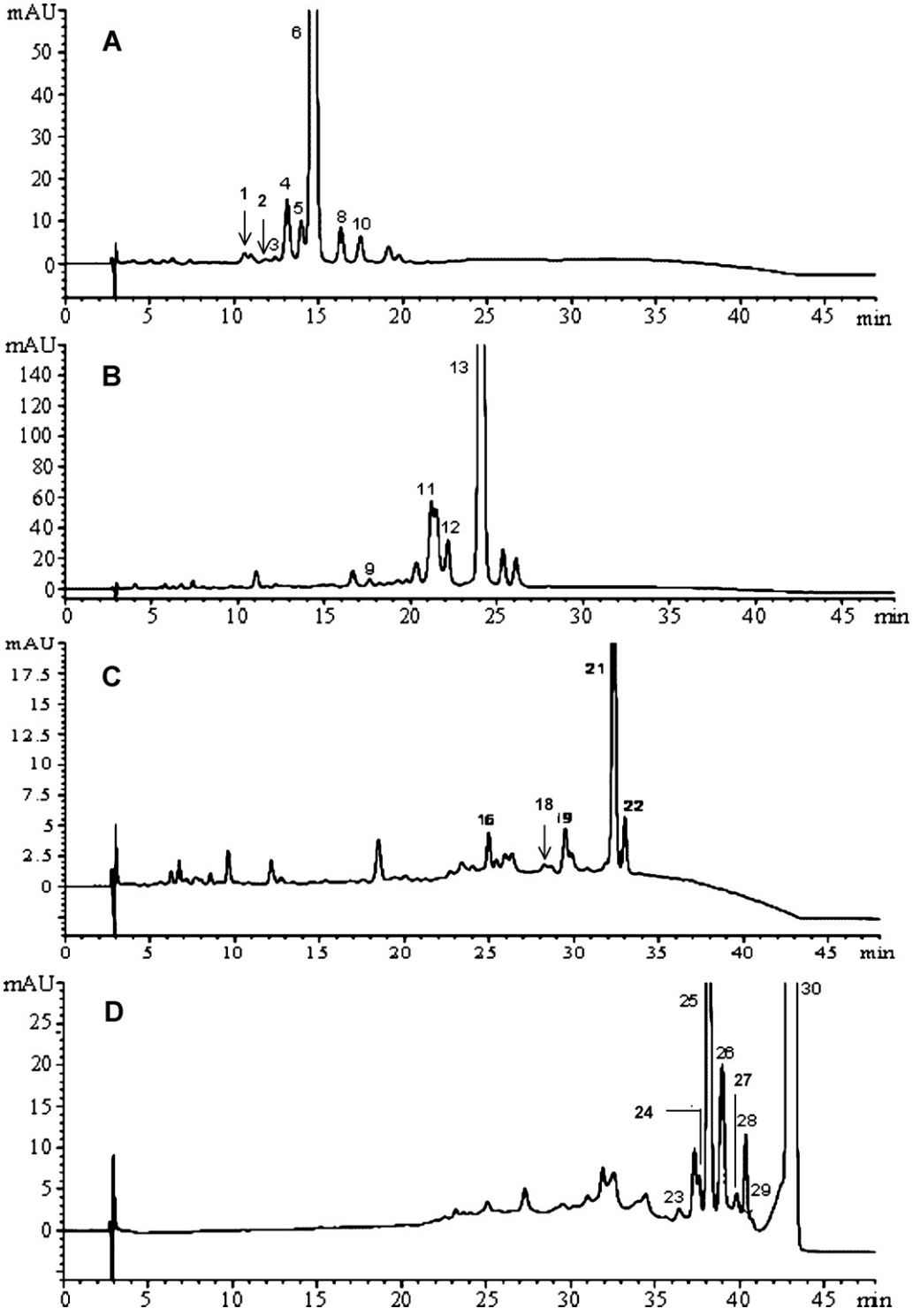

Fig. 2. HPLC–DAD chromatogram of carotenoid standards after illumination at 25 °C for 6 h with DAD detection at 450 nm. Chromatographic conditions are described in the text. (A) Lutein standard – peaks: 1, *cis*-lutein; 2, *cis*-lutein; 3, *cis*-lutein; 4, 13- or 13′-*cis*-lutein; 5, 13- or 13′-*cis*-lutein, 6, all-*trans*-lutein; 8, 9- or 9′-*cis*-lutein; 10, 9- or 9′-*cis*-lutein. (B) β-Cryptoxanthin standard – peaks: 9, *cis*-β-cryptoxanthin; 11, 13- or 13′-*cis*-β-cryptoxanthin; 12, 13- or 13′-*cis*-β-cryptoxanthin; 13, all-*trans*-β-cryptoxanthin. (C) β-Carotene standard – peaks: 16, β-carotene-5,6-epoxide; 18, β-carotene-5,8-epoxide; 19, 13- or 13′-*cis*-β-carotene; 21, all-*trans*-β-carotene; 22, 9- or 9′-*cis*-β-carotene. (D) Lycopene standard – peaks: 23, di-*cis*-lycopene; 24, di-*cis*-lycopene; 25, 13- or 13′-*cis*-lycopene; 26, 13- or 13′-*cis*-lycopene; 27, 9- or 9′-*cis*-lycopene; 28, 9- or 9′-*cis*-lycopene; 29, 5- or 5′-*cis*-lycopene; 30, all-*trans*-lycopene.

The peaks in Fig. 2 were identified based on absorption spectral characteristics and *Q*-ratios reported in the literature (Table 2) [6,22,24–27]. Consequently, the peaks 1–13 and 19–30 in Fig. 1 were found to possess similar retention time, spectral data and *Q*-ratios with the all-*trans*- and *cis*-carotenoid isomers identified in isomerized standards (Table 2). Additionally, the various illuminated standards were added to the sample extract separately and injected into HPLC for co-chromatography. Therefore, the peaks 1–5, 8–12, 19, 23–29 were identified as *cis*-isomers of carotenoids. The remaining 2 peaks 16 and 18 were assigned to β-carotene-5,6-epoxide and β-carotene-5,8-epoxide respectively, through comparison of absorption spectra of both human serum

Table 2
Identification data of all-*trans*- and *cis*-forms of lutein, β-cryptoxanthin, β-carotene, lycopene after illumination of all-*trans* standards at 25 °C for 6 h.

Peak no.	t_R (min)	Compound	λ (nm) (in-line)[a]	λ (nm) (reported)	Q-ratio found[b]	Q-ratio reported
Lutein isomers						
1	10.44	*cis*-Lutein	330, 416, 436, 464	332, 410, 434, 458[c]	0.32	0.31[c]
2	11.82	*cis*-Lutein	312, 416, 438, 470	332, 416, 440, 464[c]	0.15	0.16[c]
3	12.34	*cis*-Lutein	340, 414, 438, 470	338, 422, 446, 470[c]	0.11	0.28[c]
4	13.16	13- or 13′-*cis*-lutein	332, 418, 440, 468	332, 415, 440, 464[c]	0.50	0.41[c]
5	13.98	13- or 13′-*cis*-lutein	332, 416, 440, 468	332, 416, 440, 464[c]	0.47	0.39[c]
6	14.69	All-*trans*-lutein	334, 422, 446, 474	332, 422, 446, 470[c]	0.07	0.09[c]
8	16.53	9-or 9′-*cis*-lutein	334, 420, 442, 470	332, 416, 440, 470[c]	0.12	0.13[c]
10	17.67	9-or 9′-*cis*-lutein	342, 420, 442, 472	332, 421, 446, 470[c]	0.08	0.12[c]
β-Cryptoxanthin isomers						
9	17.54	*cis*-β-Cryptoxanthin	334, 422, 448, 476	–, 411, 441, 471[d]	0.26	0.45[d]
11	21.23	13- or 13′-*cis*-β-cryptoxanthin	340, 422, 448, 472	336, 415, 443, 470[e]	0.49	0.48[e]
12	22.07	13- or 13′-*cis*-β-cryptoxanthin	336, 424, 448, 476	336, 415, 443, 470[e]	0.38	0.48[e]
13	24.03	All-*trans*-β-cryptoxanthin	342, 428, 456, 482	–, 428, 454, 480[f]	0.07	0.16[f]
β-Carotene isomers						
16	25.17	β-Carotene-5,6-epoxide	338, 422, 450, 476	–, 420, 445, 471[e]	0.08	–
18	28.25	β-Carotene-5,8-epoxide	332, 406, 430, 456	–, 404, 428, 453[e]	0.07	–
19	29.28	13- or13′-*cis*-β-carotene	344, 420, 448, 472	342, 422, 448, 474[f]	0.41	0.43[f]
21	32.33	All-*trans*-β-carotene	346, 430, 456, 482	350, 430, 456, 480[e]	0.08	0.12[c]
22	32.98	9- or 9′-*cis*-β-carotene	346, 426, 452, 478	342, 426, 452, 478[f]	0.13	0.13[f]
Lycopene isomers						
23	36.31	di-*cis*-Lycopene	346, 436, 462, 494	368, –, 458, 488[g]	0.12	0.20[g]
24	37.49	di-*cis*-Lycopene	364, 436, 462, 492	368, –, 458, 488[g]	0.14	0.20[g]
25	38.08	13- or 13′-*cis*-lycopene	364, 444, 470, 502	362, 446, 470, 500[h]	0.45	0.54[h]
26	38.86	13- or 13′-*cis*-lycopene	364, 444, 470, 502	362, 446, 470, 500[h]	0.43	0.54[h]
27	39.71	9- or 9′-*cis*-lycopene	366, 450, 474, 504	362, 446, 470, 500[g]	0.12	0.12[g]
28	40.36	9- or 9′-*cis*-lycopene	366, 448, 472, 504	362, 446, 470, 500[g]	0.18	0.12[g]
29	40.73	5- or 5′-*cis*-lycopene	376, 448, 476, 504	344, 446, 476, 506[g]	0.08	0.05[g]
30	43.01	All-*trans*-lycopene	364, 452, 478, 510	–, 452, 476, 506[g]	0.04	–

[a] A gradient mobile phase of methanol/acetonitrile/water (84:14:4, v/v/v) and dichloromethane (from 100:0, v/v to 40:60, v/v) was used.
[b] Q-ratio is the height ratio of the *cis*-peak to the main absorption peak.
[c] A mobile phase of methanol–acetonitrile–water (84:14:2, v/v/v) and methylene chloride (from 100:0, v/v to 45:55, v/v) was used by Inbaraj et al. [22].
[d] A gradient mobile phase of methanol (100%) and methylene chloride (from 90:10, v/v to 35:65, v/v) was used by Rajendran et al. [6].
[e] A linear gradient mobile phase of methanol/methyl-tert-butyl ether (from 95:5 to 70:30 in 30 min, to 50:50 in 20 min and maintained for 35 min) was used by de Rosso and Mercadante [26].
[f] A mobile phase of methanol–acetonitrile–water (81:14:5, v/v/v) and methylene chloride (from 84:16, v/v to 75:25, v/v) was used by Inbaraj et al. [24].
[g] 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 [25].
[h] A mobile phase of 1-butanol–acetonitrile–methylene chloride (30:70:10, v/v/v) was used by Lee and Chen [27].

and isomerized standards chromatograms with reported values [26].

Further identification was carried out by comparing in-line LC–APCI-MS data of each peak with the reported values (Table 3) [14,26,28–32]. Lutein (peak 6) and zeaxanthin (peak 7) being positional isomers were identified based on the same $[M+H]^+$ value at 569.5 (m/z) and a fragment ion at 551.5 (m/z) due to loss of a water molecule [29,30]. Nevertheless, an additional loss of water molecule gave a fragment ion $[M+H-18-18]^+$ at m/z 533.4 for all-*trans*-lutein, but not for all-*trans*-zeaxanthin. Unlike all-*trans*-lutein, its *cis*-isomers (peaks 1–5, 8 and 10) did not generate $[M+H]^+$ ion as the parent ion, instead, $[M+H-18]^+$ was formed after loss of a water molecule [28]. It has been reported that lutein may undergo dehydration in the APCI vaporizer, leading to conversion into $[M+H-18]^+$ [33]. Peaks 13 and 9, 11, 12 were identified as all-*trans*- and *cis*-isomers of β-cryptoxanthin, respectively, based on the parent ion at m/z 553.4, with the former showing a fragment ion of $[M+H-18]^+$ at m/z 535.4 as well because of elimination of a water molecule. A similar mass spectral data was reported by de Rosso and Mercadante [26] for identification of β-cryptoxanthin isomers in dovyalis and tamarillo fruits. Likewise, two β-carotene epoxides (peaks 16 and 18) were tentatively identified by comparison of $[M+H]^+$ value at m/z 553.0 with the reported values [26,31]. Mass spectral identification of phytoene and phytofluene were based on comparison of their $[M+H]^+$ ion value at m/z 545.5 and 543.5, respectively, with the reported ones [14,26]. For α-carotene, β-carotene and lycopene, their all-*trans*- and *cis*-isomers gave the same $[M+H]^+$ ion value at m/z 537 [29,31,32]. Fang et al. [34] postulated that these isomeric carotenoids form abundant protonated molecules at m/z 537 with no significant fragmentation by using positive-ion APCI mode. However, with continuous-flow fast atom bombardment and APCI mode with LC–MS–MS, lycopene and its *cis* isomers could be distinguished from isomeric α-and β-carotenes based on the fragment ion at m/z 467 as a result of loss of acyclic isoprene group in lycopene [34].

3.3. Quantitation of carotenoids

As mentioned before, quantitation of carotenoids in human serum was performed by internal standard method. With the exception of phytoene and phytofluene, parared was incorporated as an internal standard into each carotenoid and a total of 8 calibration curves were prepared by plotting the peak area ratio of carotenoid to internal standard *versus* its concentration ratio. The linear regression equations obtained were $y = 0.9084x + 0.2654$ for all-*trans*-lutein (0.5–30 μg/mL, $R^2 = 0.998$), $y = 0.5960x + 0.1564$ for all-*trans*-zeaxanthin (0.5–30 μg/mL, $R^2 = 0.998$), $y = 2.6854x - 0.578$ for all-*trans*-β-cryptoxanthin (0.5–30 μg/mL, $R^2 = 0.999$), $y = 2.8614x - 0.3947$ for all-*trans*-α-carotene (0.5–30 μg/mL, $R^2 = 0.999$), $y = 1.0929x - 0.1947$ for all-*trans*-β-carotene (0.5–30 μg/mL, $R^2 = 0.999$), and $y = 1.9509x - 0.781$ ($R^2 = 0.998$) for all-*trans*-lycopene (0.1–30 μg/mL, $R^2 = 0.998$). For phytoene and phytofluene standards, the regression equations were $y = 2.3750x + 0.4715$ for the former (0.1–30 μg/mL, $R^2 = 0.998$) and $y = 1.0661x - 0.2959$ for the latter (0.1–30 μg/mL, $R^2 = 0.999$). For quantitation of *cis*-isomers, the regression equations of their corresponding all-*trans*-carotenoids were used.

Table 3
LC–APCI-MS data for tentative identification of all-*trans*- and *cis*-carotenoids in human serum.

Peak no.	Retention time (min)	Compound	*m*/*z* ratio	
			In-line	Reported
1	10.27	*cis*-Lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
2	11.51	*cis*-Lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
3	12.24	*cis*-Lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
4	12.90	13- or 13′-*cis*-lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
5	13.86	13- or 13′-*cis*-lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
6	14.48	All-*trans*-lutein	569.5 $[M+H]^+$	569.9 $[M+H]^+$
			551.5 $[M+H-18]^+$	551.4 $[M+H-18]^+$[b]
			533.4 $[M+H-18-18]^+$	533.4 $[M+H-18-18]^+$[b]
7	15.97	All-*trans*-zeaxanthin	569.5 $[M+H]^+$	569.7 $[M+H]^+$
			551.5 $[M+H-18]^+$	551.4 $[M+H-18]^+$[c]
8	16.82	9- or 9′-*cis*-lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
9	17.41	*cis*-β-Cryptoxanthin	553.4 $[M+H]^+$	553.4 $[M+H]^+$[b]
10	18.01	9- or 9′-*cis*-lutein	551.4 $[M+H-18]^+$	551.5 $[M+H-18]^+$[a]
11	20.94	13- or 13′-*cis*-β-cryptoxanthin	553.4 $[M+H]^+$	553.4 $[M+H]^+$[b]
12	21.79	13- or 13′-*cis*-β-cryptoxanthin	553.4 $[M+H]^+$	553.4 $[M+H]^+$[b]
13	23.78	All-*trans*-β-cryptoxanthin	553.4 $[M+H]^+$	553.4 $[M+H]^+$
			535.4 $[M+H-18]^+$	535.4 $[M+H-18]^+$[b]
14	25.01	*cis*-Phytofluene	543.5 $[M+H]^+$	543.5 $[M+H]^+$[d,e]
15	25.26	Phytoene	545.5 $[M+H]^+$	545.5 $[M+H]^+$[d,e]
16	25.46	β-Carotene-5,6-carotene	553.0 $[M+H]^+$	553.0 $[M+H]^+$[e]
17	26.72	Phytofluene	543.5 $[M+H]^+$	543.5 $[M+H]^+$[d,e]
18	28.23	β-Carotene-5,8-carotene	553.0 $[M+H]^+$	553.0 $[M+H]^+$[f]
19	28.98	13- or 13′-*cis*-β-carotene	537.4 $[M+H]^+$	537.0 $[M+H]^+$[b]
20	30.05	All-*trans*-α-carotene	537.5 $[M+H]^+$	537.0 $[M+H]^+$[f]
21	32.09	All-*trans*-β-carotene	537.4 $[M+H]^+$	537.0 $[M+H]^+$[b]
22	32.78	9- or 9′-*cis*-β-carotene	537.4 $[M+H]^+$	537.0 $[M+H]^+$[b]
23	36.15	di-*cis*-Lycopene	537.3 $[M+H]^+$	–
24	37.16	di-*cis*-Lycopene	537.3 $[M+H]^+$	–
25	37.98	13- or 13′-*cis*-lycopene	537.4 $[M+H]^+$	537.0 $[M+H]^+$[g]
26	38.67	13- or 13′-*cis*-lycopene	537.5 $[M+H]^+$	537.0 $[M+H]^+$[g]
27	39.53	9- or 9′-*cis*-lycopene	537.4 $[M+H]^+$	537.0 $[M+H]^+$[g]
28	40.23	9- or 9′-*cis*-lycopene	537.5 $[M+H]^+$	537.0 $[M+H]^+$[g]
29	40.82	5- or 5′-*cis*-lycopene	537.4 $[M+H]^+$	537.0 $[M+H]^+$[g]
30	42.94	All-*trans*-lycopene	537.5 $[M+H]^+$	537.0 $[M+H]^+$[g]

[a] Lakshminarayana et al. [28].
[b] de Faria et al. [29].
[c] Nakagawa et al. [30].
[d] Rivera et al. [14].
[e] de Rosso and Mercadante [26].
[f] Zepka and Mercadante [31].
[g] Richard et al. [32].

3.4. Method sensitivity, recovery and reproducibility

Sensitivity of the method was based on the limit of detection (LOD) and limit of quantitation (LOQ) determined by adopting the procedure described in the preceding section. The LOD and LOQ values obtained were 3 and 10 ng/mL for all-*trans*-lutein, 5 and 15 ng/mL for all-*trans*-zeaxanthin, 9 and 26 ng/mL for all-*trans*-β-cryptoxanthin, 9 and 29 ng/mL for all-*trans*-α-carotene, 14 and 43 ng/mL for all-*trans*-β-carotene, 2 and 6 ng/mL for all-*trans*-phytoene, 8 and 25 ng/mL for all-*trans*-phytofluene and 8 and 23 ng/mL for all-*trans*-lycopene. By employing a polymeric C30 column, comparable values were reported by Rajendran et al. [6] for most carotenoids with relatively higher LOD and LOQ being obtained for β-carotene (127 and 386 ng/mL) and lycopene (511 and 1550 ng/mL). In another study, Karppi et al. [4] reported a much higher LOD value of 10 ng/mL each for lutein, zeaxanthin and β-cryptoxanthin and 20 ng/mL each for α-carotene, β-carotene and lycopene for an isocratic RP-HPLC method using a C18 column.

For recovery, the respective mean value was 95, 91, 99, 98, 91 and 91% for all-*trans* forms of lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene at spiked concentrations of 2 and 5 μg/mL. At spiked concentrations of 1 and 2 μg/mL, the average percentage recovery of phytoene and phytofluene was 91 and 96%, respectively. The intra-day and inter-day variability of each carotenoid concentration in human serum as determined by HPLC–DAD was expressed in terms of % RSD in Table 4. The intra-day variability of different carotenoids analyzed within-a-day ranged from 0.14 to 6.01%, while the inter-day variability between-days ranged from 0.31 to 7.28%. The total amount of carotenoids in the pooled serum sample was 1185.5 ng/mL, with the content of all-*trans* plus *cis*-isomers of lycopene being present in largest amount (306.9 ng/mL), followed by all-*trans* plus *cis*-isomers of phytofluene (265.6 ng/mL), all-*trans* plus *cis*-isomers of β-carotene and its epoxide derivatives (221.8 ng/mL), all-*trans* plus *cis*-isomers of β-cryptoxanthin (167.7 ng/mL), all-*trans*-lutein (134.8 ng/mL), all-*trans*-α-carotene (38.0 ng/mL), all-*trans*-zeaxanthin (28.8 ng/mL) and phytoene (21.9 ng/mL). Several *cis*-isomers of lutein, β-carotene and lycopene were not quantified as their levels were lower than LOQ of their corresponding all-*trans*-carotenoids.

3.5. Method applicability to different serum samples

Applicability of the developed method was tested with 6 blood samples collected from clinical trial subjects supplemented with carotenoid-rich capsules. Each sample was analyzed in duplicate and the contents of all-*trans* and *cis*-carotenoids are presented in Table 5. The total concentration of carotenoids ranged from 3379.2 to 8677.5 ng/mL, which were approximately 3–7 folds higher than found in pooled serum (1185.5 ng/mL) from subjects not supplemented with any capsule. Among the carotenoids, β-carotene plus *cis* isomers and epoxide derivatives were present in largest amount (1069.8–2783.1 ng/mL),

Table 4
Content, intra-day and inter-day variability of all-*trans* plus *cis*-isomers of carotenoids in pooled human serum collected from 10 random healthy subjects not supplemented with carotenoid-rich capsules.

Peak no.	Compound	Intra-day variability[a]		Inter-day variability[a]	
		Mean ± SD	RSD (%)	Mean ± SD	RSD (%)
1	*cis*-Lutein	–[b]	–[b]	–[b]	–[b]
2	*cis*-Lutein	–[b]	–[b]	–	–
3	*cis*-Lutein	–[b]	–[b]	–[b]	–[b]
4	13- or 13′-*cis*-lutein	–[b]	–[b]	–[b]	–[b]
5	13- or 13′-*cis*-lutein	–[b]	–[b]	–[b]	–[b]
6	All-*trans*-lutein	134.8 ± 7.3	5.39	164.0 ± 7.1	4.28
7	All-*trans*-zeaxanthin	28.8 ± 1.3	4.61	30.9 ± 2.3	7.28
8	9- or 9′-*cis*-lutein	–[b]	–[b]	–[b]	–[b]
9	*cis*-β-Cryptoxanthin	27.7 ± 0.04	0.14	27.7 ± 0.1	0.31
10	9- or 9′-*cis*-lutein	–[b]	–[b]	–[b]	–[b]
11	13- or 13′-*cis*-β-cryptoxanthin	66.3 ± 4.0	6.01	68.3 ± 2.2	3.16
12	13- or 13′-*cis*-β-cryptoxanthin	34.1 ± 0.5	1.35	34.9 ± 0.4	1.03
13	All-*trans*-β-cryptoxanthin	39.6 ± 1.6	4.04	40.7 ± 0.8	2.04
14	*cis*-Phytofluene	84.5 ± 0.8	0.96	85.8 ± 3.2	3.73
15	Phytoene	21.9 ± 1.0	4.57	24.0 ± 1.7	7.06
16	β-Carotene-5,6-carotene	47.1 ± 0.6	1.32	47.4 ± 0.4	0.76
17	Phytofluene	181.1 ± 8.7	4.83	183.8 ± 3.6	1.97
18	β-Carotene-5,8-carotene	23.3 ± 0.5	1.97	23.4 ± 0.5	2.09
19	13- or 13′-*cis*-β-carotene	27.4 ± 1.0	3.71	27.8 ± 0.6	2.28
20	All-*trans*-α-carotene	38.0 ± 1.8	4.62	41.0 ± 0.4	1.03
21	All-*trans*-β-carotene	124.0 ± 4.5	3.60	135.0 ± 7.8	5.75
22	9- or 9′-*cis*-β-carotene	–[b]	–[b]	–[b]	–[b]
23	di-*cis*-Lycopene	56.3 ± 0.9	1.57	57.8 ± 1.2	2.10
24	di-*cis*-Lycopene	–[b]	–[b]	–[b]	–[b]
25	13- or 13′-*cis*-lycopene	59.2 ± 0.6	0.97	60.7 ± 1.1	1.75
26	13- or 13′-*cis*-lycopene	48.1 ± 0.9	1.76	48.3 ± 0.7	1.34
27	9- or 9′-*cis*-lycopene	–[b]	–[b]	–[b]	–[b]
28	9- or 9′-*cis*-lycopene	50.6 ± 0.3	0.67	51.5 ± 0.7	1.35
29	5- or 5′-*cis*-lycopene	–[b]	–[b]	–[b]	–[b]
30	All-*trans*-lycopene	92.7 ± 3.5	3.76	98.3 ± 2.2	2.19

[a] Mean of nine replicate analyses ($n = 9$).
[b] The amount is lower than the LOQ of corresponding all-*trans*-isomer.

Table 5
Concentration (ng/mL)[a] of all-*trans* plus *cis*-carotenoids in human serum collected from 6 clinical trial subjects in Taiwan supplemented with carotenoid-rich capsules.

Peak no.	Compound	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
1	*cis*-Lutein	–[b]	–[b]	–[b]	–[b]	10.5 ± 1.9	–[b]
2	*cis*-Lutein	63.0 ± 3.7	–[b]	–[b]	–[b]	75.6 ± 7.0	24.9 ± 0.4
3	*cis*-Lutein	76.2 ± 4.8	–[b]	34.5 ± 0.0	37.7 ± 0.1	53.4 ± 2.1	61.2 ± 4.5
4	13- or 13′-*cis*-lutein	43.5 ± 3.3	–[b]	32.2 ± 0.1	–[b]	28.5 ± 4.3	55.0 ± 5.1
5	13- or 13′-*cis*-lutein	61.5 ± 0.3	40.0 ± 0.5	65.6 ± 1.1	48.4 ± 0.6	95.5 ± 3.4	92.9 ± 4.2
6	All-*trans*-lutein	997 ± 24	429 ± 3	783 ± 16	642 ± 25	1636 ± 70	1271 ± 315
7	All-*trans*-zeaxanthin	177 ± 7	113 ± 15	110 ± 3	145 ± 9	151 ± 8	159 ± 16
8	9- or 9′-*cis*-lutein	55.9 ± 0.6	17.0 ± 0.2	11.4 ± 0.4	8.11 ± 0.07	9.02 ± 0.10	64.0 ± 0.3
9	*cis*-β-Cryptoxanthin	66.0 ± 0.8	29.4 ± 0.3	22.6 ± 0.02	69.4 ± 1.7	34.8 ± 0.5	58.4 ± 0.5
10	9- or 9′-*cis*-lutein	52.4 ± 0.2	25.6 ± 0.2	9.11 ± 1.50	84.2 ± 2.7	101 ± 4	10.3 ± 1.0
11	13- or 13′-*cis*-β-cryptoxanthin	482 ± 19	215 ± 7	318 ± 5	278 ± 12	244 ± 10	423 ± 27
12	13- or 13′-*cis*-β-cryptoxanthin	108 ± 5	42.5 ± 0.7	83.5 ± 4.3	61.3 ± 5.1	59.2 ± 0.3	114 ± 2
13	All-*trans*-β-cryptoxanthin	309 ± 13	190 ± 3	362 ± 16	184 ± 10	120 ± 4	198 ± 1
14	*cis*-Phytofluene	436 ± 18	146 ± 4	248 ± 7	320 ± 9	148 ± 2	211 ± 2
15	Phytoene	145 ± 3	47.6 ± 0.7	135 ± 1	165 ± 6	47.4 ± 0.2	41.8 ± 2.8
16	β-Carotene-5,6-epoxide	373 ± 29	165 ± 4	257 ± 9	311 ± 10	247 ± 7	312 ± 1
17	Phytofluene	1329 ± 79	369 ± 13	651 ± 2	1106 ± 32	387 ± 26	820 ± 2
18	β-Carotene-5,8-epoxide	33.1 ± 1.4	24.9 ± 1.2	37.4 ± 0.9	29.2 ± 0.4	34.4 ± 1.2	33.3 ± 0.9
19	13- or 13′-*cis*-β-carotene	133 ± 10	74.2 ± 1.6	150 ± 1	98.4 ± 2.6	122 ± 2	172 ± 1
20	All-*trans*-α-carotene	37.5 ± 3.8	48.2 ± 4.0	95.9 ± 1.0	39.1 ± 0.2	51.6 ± 3.0	74.1 ± 0.0
21	All-*trans*-β-carotene	2140 ± 38	772 ± 11	1579 ± 25	1244 ± 19	1647 ± 69	1674 ± 6
22	9- or 9′-*cis*-β-carotene	104 ± 3	33.7 ± 2.3	76.9 ± 3.1	59.9 ± 0.2	57.5 ± 0.3	44.3 ± 1.5
23	di-*cis*-Lycopene	142 ± 2	61.2 ± 1.0	90.9 ± 0.6	82.9 ± 3.5	57.8 ± 3.2	128 ± 2
24	di-*cis*-Lycopene	33.4 ± 0.7	26.1 ± 1.0	36.0 ± 0.2	26.2 ± 0.7	–[b]	–[b]
25	13- or 13′-*cis*-lycopene	262 ± 6	108 ± 3	180 ± 2	157 ± 2	94.1 ± 5.7	222 ± 4
26	13- or 13′-*cis*-lycopene	67.1 ± 0.0	35.0 ± 0.4	46.4 ± 0.8	59.8 ± 0.2	40.0 ± 1.3	65.6 ± 0.6
27	9- or 9′-*cis*-lycopene	46.5 ± 1.0	29.6 ± 0.2	35.8 ± 0.6	35.9 ± 1.6	–[b]	38.2 ± 0.1
28	9- or 9′-*cis*-lycopene	83.4 ± 0.6	42.0 ± 0.6	57.3 ± 2.8	67.1 ± 2.2	40.1 ± 1.5	69.0 ± 0.0
29	5- or 5′-*cis*-lycopene	38.7 ± 0.9	30.2 ± 0.4	24.7 ± 0.1	30.4 ± 0.3	26.1 ± 0.6	35.1 ± 0.8
30	All-*trans*-lycopene	782 ± 22	265 ± 3	621 ± 24	580 ± 15	293 ± 23	722 ± 10

[a] Mean of duplicate analyses ± standard deviation.
[b] The amount is lower than the LOQ of corresponding all-*trans* carotenoids.

followed by lutein plus its *cis* isomers (511.6–2009.5 ng/mL), phytofluene plus its *cis* isomer (515.0–1765.0 ng/mL), lycopene plus its *cis* isomers (551.1–1455.1 ng/mL), β-cryptoxanthin plus its *cis* isomers (458.0–965.0 ng/mL), all-*trans*-zeaxanthin (110.0–177.0 ng/mL), phytoene (41.8–165.0 ng/mL) and all-*trans*-α-carotene (37.5–95.9 ng/mL). Interestingly, the *cis*-isomers constituted a proportion of 16–26%, 54–75%, 5–11% and 43–56% of total lutein, β-cryptoxanthin, β-carotene and lycopene, respectively, which implies their significant role in biological activity. Obviously, unlike food matrix, the carotenoids in human serum are liable to be present in an isomeric equilibrium [6]. For instance, experiments with rats showed better absorption of *cis*-isomers of lycopene compared to all-*trans*-lycopene owing to their higher polarity and shorter molecular structure, resulting in lower tendency to crystallization, better solubility in lipids and preferential incorporation into chylomicrons [35–37]. Nevertheless, the *cis*-isomers of β-carotene contributed less significantly to its total content, probably due to shift in isomeric equilibrium to all-*trans* form [6,38]. Conversely, the level of *cis* isomers of β-cryptoxanthin (54–75%) dominated over all the other *cis*-carotenoids in human serum, which is different (42%) from that reported by Rajendran et al. [6]. This discrepancy may be ascribed to a more number of *cis*-β-cryptoxanthin isomers identified and quantified in this study. Such critical monitoring of *cis*/*trans* composition in provitamin A carotenoid like β-cryptoxanthin should provide a better understanding of their biological significance.

The literature is unanimous in reporting the efficiency of a polymeric C30 column over C18 column in resolving *cis*-isomers of carotenoids in fruits/vegetables and biological fluids/tissues [6,7,15–17,22,24–27]. However, there are still many studies using a C18 column for determination of major carotenoids like lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene and lycopene in human serum [4,11–13]. Commonly encountered drawbacks in these studies include poor resolution of lutein and zeaxanthin, which are often quantified together, and simultaneous elution of both all-*trans*- and *cis*-isomers of lycopene. In our study, lutein and zeaxanthin were resolved with the peak purity of each exceeding 99%. Additionally, all-*trans*-lycopene and its 7 *cis*-isomers were separated and quantified, which is crucial as *cis*-lycopene isomers constitute >50% of total lycopene in human blood and tissues [34,37]. Two β-carotene epoxides (peaks 16 and 18) were identified to be present in human serum. Though their role in prevention of chronic disease is lacking, two previous studies have shown their active role in promoting differentiation of NB4 cells and absorption in human [39,40]. In 2005, Rajendran et al. [6] reported a HPLC method employing a polymeric C30 column for separation of all-*trans*- plus *cis*-carotenoids. However, the method did not take the biosynthetic precursors of carotenoids such as phytoene and phytofluene into account. Moreover, several *cis*-isomers of major carotenoids remained unidentified and a total of only 21 carotenoids were resolved within 51 min [6], which should be inadequate when compared to 30 carotenoids separated within 45 min in our study. Determination of phytoene and phytofluene in this study requires a special mention as recent findings have provided an insight on their bioavailability and bioactivity in both *in vitro* and *in vivo* [18–21]. A polymeric C30 column was used by Campbell et al. [21] for separation of only phytoene and phytofluene. In a recent study, Rivera et al. [14] analyzed a mixture of 16 carotenoids including phytoene and phytofluene by LC–MS, but the method was not validated in real samples. Taken together, the method developed by a polymeric C30 column in this study provides a comprehensive determination of all-*trans*- and *cis*-carotenoids as well as phytoene and phytofluene in human serum.

4. Conclusion

All in all, an improved HPLC–DAD–MS method was developed with a polymeric C30 column and gradient mobile phase for separation of 30 *cis* plus *trans* carotenoids within 45 min. The method was sensitive with detection and quantitation limits ranging from 2–14 ng/mL and 6–43 ng/mL, respectively. Also, the recovery and reproducibility were excellent, with the former ranging from 91 to 99% and the relative standard deviation of intra-day and inter-day variability varying from 0.14 to 6.01% and 0.31 to 7.28%, respectively. Thus, the method developed in this study can be applied to determine the levels of *cis*/*trans*-carotenoids and their precursors phytoene and phytofluene in biological samples.

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