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# Simultaneous measurement of retinol, α-tocopherol and six carotenoids in human plasma by using an isocratic reversed-phase HPLC method

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ABSTRACT

A simple and sensitive isocratic reversed-phase high-performance liquid chromatography (HPLC) method for simultaneous determination of retinol, α-tocopherol and six carotenoids in human plasma was described. Sample preparation of the earlier published method was further developed by addition of ultrapure water, which enabled aqueous layer to freeze facilitating phase separation without pipetting thus also improving precision of the method. Developed method appeared to be less laborious and time consuming compared to the traditional extraction methods, which require removal of organic layer by pipetting. The recoveries (absolute and relative) were between 80% and 103%. The intra-assay CVs were 1.1–4.0% (normal level) and 3.3–9.0% (low level). Inter-assay CVs were 5.3–8.8%. Reference method for all these analytes was not available, but a comparison with another published method was carried out. The results of the comparison matched satisfactorily. The method is used routinely in our laboratory in a large population-based study.

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## 1. Introduction

Carotenoids are colourful compounds possessing yellow, orange and red pigments that are primarily synthesized in plants and micro-organisms. Nowadays, more than 600 carotenoids have been characterized [1] and ∼50 of these are consumed in the human diet [2]. About 12 carotenoids account for most of the dietary intake, and they are found in measurable levels in human blood and tissues. Most abundant carotenoids found in human plasma are lycopene, α-carotene and β-carotene, lutein, zeaxanthin and β-cryptoxanthin, which all quench effectively oxygen-derived free radicals and protect low-density lipoproteins (LDLs) against oxidation *in vitro*. Carotenoids have beneficial effects on human health such as reduction of the risk of chronic diseases (e.g. cardiovascular diseases and cancers) [3,4].

The analysis of carotenoid levels in serum or plasma has been of interest due to their antioxidant properties. Generally, sample preparation process includes precipitation of proteins with a polar solvent, such as ethanol containing internal standard, and extraction of retinol, tocopherols and carotenoids into a non-polar solvent [5]. Hexane alone or combined with other solvent seems to be the most often used solvent for extraction of carotenoids from biological fluids [5]. Numerous high-performance liquid chromatography (HPLC) methods have been developed for analysis of retinol, α-tocopherol and carotenoids in human serum and plasma. Reversed-phase HPLC on $C_{18}$ columns and isocratic or gradient elution have been the preferred mode for separating major carotenoids for quantitative analysis, but do not resolve well of their geometrical isomers [6–14]. The polymeric $C_{30}$ columns were developed specifically for separation of *cis–trans* isomers [15–18]. Methods based on $C_8$ stationary phases are rare [18]. Resolution of lutein and zeaxanthin remained inadequate in some reversed-phase HPLC methods [7,11,13]. The separation of lutein and zeaxanthin was suggested to be improved by connecting two analytical columns in series [12], or by using $C_{30}$ reversed-phase column, which had improved the resolution [16–18]. Multi-channel UV–vis spectrophotometric detectors and diode-array detectors (DAD) [6,7,9–14,18], fluorescence detectors [14], electrochemical detectors (ED) [8,16] and mass spectrometry (MS) [10,19] have been used with HPLC to analyze plasma retinol, tocopherols, tocotrienols, carotenoids and their isomers. There are some feasible normal-phase HPLC methods for carotenoid analysis. Khachik et al. [10] and McGeachin and Bailey [20] have determined carotenoids, retinol and α-tocopherol in feed, tissues, and blood serum by using normal phase HPLC.

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The aim of the present study was to develop a quick, simple and sensitive method for analyzing plasma levels of retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene in large population study. This HPLC method was compared with the HPLC method described by Olmedilla et al. [9]. Retinol, α-tocopherol and carotenoid levels in plasma and serum samples of 34 subjects were measured by the HPLC methods in the University of Kuopio (Finland) and in the Hospital Universitario Puerta de Hierro (Madrid, Spain). Stability of retinol, α-tocopherol and carotenoids at 4 °C after the extraction from plasma was also evaluated.

## 2. Experimental

### 2.1. Reagents

α-Tocopherol (vitamin E), *trans*-β-carotene, *trans*-lycopene, and α-tocopherol acetate were obtained from Sigma–Aldrich Co. (St. Louis, MO, USA). *Trans*-lutein, retinol (vitamin A) and *trans*-β-Apo-8′-carotenal were purchased from Fluka Co. (Buchs SG, Switzerland). *Trans*-α-carotene, *trans*-β-cryptoxanthin and *trans*-zeaxanthin were obtained from Carotenature GmbH (Lupsingen, Switzerland). Ultrapure water (resistivity ≥18 MΩ/cm) was prepared using a Milli-Q system (Millipore Co., Millford, MA, USA). Ethanol was purchased from Altia Co. (Helsinki, Finland). Sodium chloride (NaCl) was obtained from FF-Chemicals Co. (Yli-Ii, Finland). HPLC-grade acetonitrile and methanol and analytical-grade *n*-hexane were obtained from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Chloroform was purchased from Riedel-de-Haén Co. (Seelze, Germany). *Tert*-butylated hydroxytoluene (BHT) was obtained from Fluka Co. (Switzerland) and Euro-Trol Vitamin A/E quality control (levels 1 and 3) was purchased from Eurotrol B.V. (Vageningen, Netherlands).

### 2.2. Blood collection for method development, quality control and method comparison

A human lithium–heparin plasma pool for the quality control material and method development was collected from healthy volunteers. Aliquots of 0.5 mL of this pool were divided into Eppendorf tubes and stored at −70 °C. Every day, a vial of that quality control was thawed at room temperature and extracted and analyzed according to the same procedure as samples. There were two quality controls in every sequence, one at the beginning and one at the end of the assay.

Blood samples from male volunteers on non-supplemented diets were collected by venipuncture for method comparison. Lithium–heparin blood collection tubes (Venoject) were purchased from Terumo Europe N.V. (Leuven, Belgium). Tubes were kept on ice until centrifugation. Blood samples were centrifuged at 1500 × *g* for 10 min at 4 °C within 30 min after collection and plasma was separated immediately after centrifugation. Plasma was transferred to polypropylene tubes and stored in maximum for 18 months at −70 °C until analysis.

### 2.3. Calibration

#### 2.3.1. Preparation of chemical standard solutions

Preparation of stock solutions was modified from Thurnham et al. [6]. Stock solutions of α-tocopherol, which was an oily form and α-tocopherol acetate (Internal standard I) (1 mg/mL) were prepared in absolute ethanol–0.01% (w/v) BHT solution. Stock solutions of retinol (0.5 mg/mL), α-carotene (0.1 mg/mL), β-carotene (0.5 mg/mL), β-cryptoxanthin (0.1 mg/mL), lycopene (0.1 mg/mL) and β-Apo-8′-carotenal (1 mg/mL) (Internal standard II) were prepared in a solution containing acetonitrile–methanol–chloroform (18:7.5:74.5, v/v/v) and 0.01% (w/v) BHT. Stock solutions of lutein and zeaxanthin (0.1 mg/mL) were prepared in a solution containing ethanol–acetonitrile–methanol–chloroform (70:18:7.5:4.5, v/v/v/v) and 0.01% (w/v) BHT. Stock solutions in polypropylene tubes were protected from light by keeping them out from a direct sunlight and storing them in a box at −70 °C. Stock solutions were further diluted in the mobile phase containing 0.01% (w/v) BHT to provide working standards with approximate concentration of 1.75 μmol/L for retinol, 23.2 μmol/L for α-tocopherol and 0.18–0.90 μmol/L for carotenoids. The absorbances of working standard solutions were determined by using a Beckman Du 640i spectrophotometer (Beckman Coulter, Fullerton, CA, USA) and the concentration of the working standard was calculated from specific molar extinction coefficient. The molar extinction coefficients for all-*trans* retinol, α-tocopherol, β-cryptoxanthin, all-*trans* lycopene, α-carotene and β-carotene were obtained from Thurnham et al. [6]. Values for lutein and zeaxanthin were from Britton [21]. Concentrations for the standards were calculated from the absorbance instead of weighing because of chemical instability of carotenoids.

An internal standard mixture was prepared by combining the stock solution of α-tocopherol acetate and β-Apo-8′-carotenal dilution, and diluting to final volume with ethanol–0.01% (w/v) BHT solution. Internal standard mixture was protected from the light in the ambered glass bottle and stored at −20 °C. Concentrations of internal standards were not checked with spectrophotometer.

#### 2.3.2. Secondary calibrator

Pooled human Li–heparin plasma was collected from volunteers as described above. The pooled plasma was stored in 500 μL aliquots in Eppendorf tubes at −70 °C and used as a secondary calibrator. The values of the secondary calibrator were analyzed like samples, but calibrated with the extracted working standard mixture. Assigned values were mean of 12 measurements assayed against the working standard.

### 2.4. Sample preparation

Samples were prepared in a room that was protected from direct sunlight and heat. The sample preparation was modified from method described by Gueguen et al. [13]. The frozen Li–heparin plasma samples were thawed at room temperature, homogenized and 200 μL was pipetted into borosilicate glass tubes. 500 μL of ethanol–0.01% (w/v) BHT containing α-tocopherol acetate and β-Apo-8′-carotenal as internal standards was added. The volume was diluted to 1 mL with ultrapure water. After mixing with a vortex, 2 mL of hexane 0.01% (w/v) BHT solution was added to the samples and mixed. After extraction, 500 μL of ultrapure water was added into the samples in order to confirm complete aqueous layer freezing. The samples were centrifuged at 1500 × *g* for 5 min at 4 °C and frozen at −70 °C for 30 min. The samples were taken out of the freezer as batch. About 20 tubes in a batch can be decanted before the frozen aqueous layer began to defrost. Upper organic layer was decanted into a glass tube and evaporated to dryness using a Techne Sample Concentrator (Techne, Cambridge, UK) under a gentle stream of nitrogen at room temperature. The dried residue was reconstituted in 200 μL of the mobile phase and vortex mixed. Samples were transferred to insert vials and placed into the HPLC autosampler. The volume injected into the HPLC was 50 μL.

### 2.5. Instrumentation

HPLC system consisted of a Shimadzu system (Kyoto, Japan) equipped with an isocratic solvent delivery pump (LC-10ATvp),

a system controller (SCL-Avp), a cooled autosampler equipped with a 50 μL injection loop (SIL-10ADvp), a vacuum membrane degasser (DGU-14A) and a Beckman 168 diode-array detector (Beckman). Class WP software (version 6.1) from Shimadzu was used for instrument control, data acquisition and data processing. Chromatographic separations were performed on a pair of Synergy Hydro-RP 80A (150 mm × 4.6 mm), 4 μm columns coupled in series and a $C_{18}$ guard column (4 mm × 3 mm) (Phenomenex, Torrance, CA, USA). Coupling two columns in series to improve separation was first presented by Lyan et al. [12]. The mobile phase consisted of a mixture of acetonitrile–methanol–chloroform (60:25:15, v/v/v) containing 0.01% (w/v) BHT. The mobile phase used was modified from Thurnham et al. [6] and the composition was identical as described by Miller and Yang [22]. The mobile phase was filtered through a 0.20-μm polypropylene membrane before analysis. The flow rate was 1 mL/min (backpressure 70–80 bar) and the run time was 35 min. Two channels corresponding different wavelength values were used to collect data. α-Tocopherol, retinol and internal standard α-tocopherol acetate were monitored at 294 nm, although the sensitivity of the method for retinol may decrease. Carotenoids and internal standard β-Apo-8′-carotenal were monitored at 454 nm. The tray compartment containing sample vials was cooled at 4 °C during analysis.

### 2.6. Chromatographic repeatability and quantification

Ten consecutive injections of pure standard mixture from a single vial were done in order to study the stability of retention times and peak heights. The repeatability of the method was assessed with the coefficient of variation of intra-assay ($n = 10$) and inter-assay ($n = 107$) repeatability. Calibration curves for all compounds were constructed by graphing the ratio of peak heights of the secondary calibrator (or chemical standard) to peak heights of the internal standards versus concentration (μmol/L). Average calibration by 50% weighing was used for day-to-day calibration. Plasma levels of carotenoids were calculated by using a one-point non-weighed linear regression model ($ax + b$) after the linearity ranges of the analytes were checked. Analytes were identified by their retention times, and the *trans*-isomers were quantified, though also some of the *cis*-isomers were chromatographically resolved. The resolution values were calculated with the following formula: $R_S = (1/4)(\alpha - 1) \times \sqrt{N} \times k/(k + 1)$ where $N$ is plate number, $\alpha$ is separation factor and $k$ is retention factor [23].

### 2.7. Recovery study

In order to test the efficiency of the extraction method, the pooled Li–heparin plasma was spiked with 10, 20 and 30 μL of working standard mixture to provide for three added levels of analytes. The spiked plasma samples ($n = 3$) were extracted and analyzed with the method described above.

### 2.8. Stability of retinol, α-tocopherol and carotenoids after extraction

To study the stability of retinol, α-tocopherol and carotenoids after the extraction, pooled Li–heparin plasma ($n = 3$) were extracted, and placed on refrigerated autosampler (4 °C) in vials. The triplicate samples were analyzed immediately, and during the seven following days. The samples were not removed from the autosampler during the study.

### 2.9. Method comparison

Li–heparin plasma samples and serum samples from adult volunteers ($n = 34$) were analyzed both with the method presented in this paper and with the method described by Olmedilla et al. [9] in the Hospital Universitario Puerta de Hierro (Madrid, Spain). Paired samples *t*-test ($P < 0.05$) was used to statistically assess the differences of levels of analytes between the compared HPLC methods. SPSS software (version 14.0; SPSS, Inc., Chicago, IL, USA) was used for statistical analyses.

## 3. Results and discussion

### 3.1. Development of sample preparation method

Liquid–liquid extraction with hexane is the most often used method for purification of carotenoids from biological fluids since they are usually soluble in non-polar solvents [5]. At preliminary stage of the present work, the sample preparation process included precipitation of proteins with 1 mL of ethanol containing internal standards and thereafter single extraction with 5 mL of hexane [24]. Hexane layer separation turned out to be laborious and time-consuming, since separation was carried out by pipetting. More comfortable and faster sample preparation would provide for dozens of samples to be prepared on a workday that is desirable in large epidemiological population-based studies. Critical step of the extraction is just the phase separation. In this paper, it was clearly observed that after single extraction the addition of ultrapure water enabled aqueous layer below hexane phase to freeze accelerating phase separation without pipetting. Developed method appeared to be less laborious and time consuming compared to the traditional extraction methods, which required removal of organic layer by pipetting. Freezing method shortened the time spent for sample handling, recovered hexane layer completely and improved repeatability of the results. Extracts were also cleaner, because frozen aqueous layer prevents layers mixing during separation. Table 1 describes comparison data of intra-assay repeatability, when organic layer was removed by traditional way and by decanting after freezing.

### 3.2. Optimization of HPLC method of carotenoids

Representative chromatogram of extracted pure standard mixture is presented in Fig. 1. The large negative unknown disturbance after retinol peak seems to be consistent for plasma samples from

**Table 1**
Repeatability of plasma retinol, α-tocopherol and carotenoids by using the traditional extraction method and freezing method

| | Intra-assay[a] ($n = 10$) | | Intra-assay[b] ($n = 10$) | |
|---|---|---|---|---|
| Compound | Mean ± SD (μmol/L) | CV (%) | Mean ± SD (μmol/L) | CV (%) |
| Retinol | 2.30 ± 0.05 | 2.0 | 2.47 ± 0.04 | 1.7 |
| α-Tocopherol | 27.4 ± 0.36 | 1.3 | 31.7 ± 0.33 | 1.1 |
| α-Tocopherol acetate | ND | ND | ND | ND |
| Lutein | 0.27 ± 0.01 | 2.5 | 0.26 ± 0.01 | 2.6 |
| Zeaxanthin | 0.059 ± 0.005 | 8.8 | 0.065 ± 0.003 | 4.0 |
| β-Apo-8′-carotenal | ND | ND | ND | ND |
| β-Cryptoxanthin | 0.33 ± 0.014 | 4.3 | 0.27 ± 0.004 | 1.7 |
| Lycopene | 0.30 ± 0.011 | 3.8 | 0.24 ± 0.003 | 1.3 |
| α-Carotene | 0.19 ± 0.006 | 3.2 | 0.17 ± 0.003 | 2.1 |
| β-Carotene | 0.41 ± 0.01 | 3.5 | 0.60 ± 0.01 | 1.7 |

Intra-assay CVs were determined with the pooled Li–heparin plasma of several volunteers. ND, not determined.

[a] Hexane layer (5 mL) was separated by pipetting [24].

[b] Hexane layer (2 mL) was separated by decanting after freezing at −70 °C.

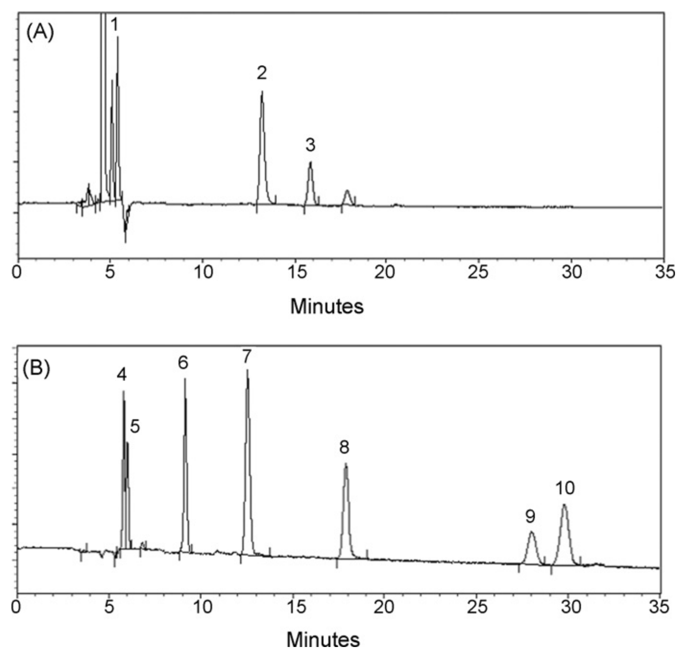


**Fig. 1.** Representative chromatogram of extracted pure standard mixture containing retinol, α-tocopherol and six carotenoids. (A) Channel 1 at 294 nm for detection of retinol (1,) α-tocopherol (2) and α-tocopherol acetate (internal standard I) (3); (B) channel 2 at 454 nm for detection of lutein (4), zeaxanthin (5), β-apo-8′-carotenal (internal standard II) (6), β-cryptoxanthin (7), lycopene (8), α-carotene (9) and β-carotene (10).

run to run. Its retention time was equal to lutein. All compounds of interest were resolved in a 35 min run and identified by comparing retention times of peaks relative to those of known pure standards. Retention times of retinol, α-tocopherol and carotenoids (5–30 min) remained stable during the sequence and the variation of the peak heights (547–6819 mV) was below 3.0% for the analytes. Zeaxanthin co-eluted with lutein, like in some other HPLC methods [7,11,13], when one Synergy Hydro-RP80A column was used. By connecting two Synergy Hydro-RP80A columns in series separation of lutein and zeaxanthin was possible. The single 250 mm analytical column was not investigated for this purpose. Low levels of zeaxanthin can be quantified in the presence of higher levels of lutein and vice versa by using peak height. Lycopene obtained from plasma appears to be composed of three broad peaks. There were *trans* and at least two *cis*-lycopene isomers [12] and these *cis*-isomers eluted after the *trans*-lycopene peak. Different isomers were partially resolved, but only *trans*-lycopene was quantified, because of the lack of pure *cis*-isomer standards. Butylated hydroxytoluene as an antioxidant was added to the extraction solvents and mobile phase in order to prevent significant degradation of carotenoids during sample preparation and HPLC analysis, because carotenoids tend to oxidize to colourless degradation products by oxygen and light [11,13]. BHT is widely used in several previous HPLC methods of carotenoids [6–10,13,14,16,17,19]. In the present method BHT eluted prior to retinol and they were well resolved. There was one peak in the chromatogram near the retinol peak in plasma, but they were well resolved. There were no other interfering peaks near the retinol peak in plasma. The use of single internal standard (α-tocopherol acetate) for retinol and α-tocopherol was based on the method of Thurnham et al. [6].

It was decided to use synthetic β-Apo-8′-carotenal as an internal standard for carotenoids, since it is chemically quite similar to the analytes [25] and it has also been successfully used before [26,27]. β-Apo-8′-carotenal eluted between zeaxanthin and β-cryptoxanthin and did not interfere with the other carotenoids. Carotenoids are very unstable compounds and concentrations of stock solutions tend to decrease due to oxidative degradation, or because carotenoids are able to crystallize in stock solutions when stored for several months at −20 °C [28]. The stability of carotenoids is possibly enhanced by their interactions with proteins and lipids in plasma [29]. Plasma standard was decided to use as secondary calibrator, since retinol, α-tocopherol and carotenoids have been reported to be stable in frozen serum from 5 months up to 4 years, depending on temperature [11,30–32]. Lunetta et al. [33] have also used human plasma as a secondary reference calibrator for quantification of retinol and β-carotene concentrations in plasma and tissue specimens.

### 3.3. Methodological parameters

The linearity of the assay was determined by single measurements of the compounds. The correlation coefficients were all >0.999. Table 2 summarizes the correlation coefficients of the curves of all compounds. The limits of detection (LOD) of analytes were determined by analyzing a series of diluted pure standard mixture. LOD value was the lowest concentration of the linear range of each analyte (ranged from 0.010 to 0.73 μmol/L), which also exceeded signal-to-noise ratio of 3:1. Concentrations above the LOD values were regarded quantifiable, although the variation was higher than determined for the method with the quality control samples (Table 2). The absolute and relative recoveries ranged from 80% to 103% (Table 3). The intra-assay CVs of the pooled Li–heparin plasma were 1.1–4.0% (normal level) and 3.3–9.0% (low level) ($n = 10$). The inter-assay CVs were obtained by analyzing quality control sample (plasma pool) duplicate for ∼3.5 months ($n = 107$). Inter-assay CVs were 5.3–8.8% (Table 3). The inter-assay CVs of Euro-Trol lyophilised QC material were obtained by analyzing duplicate for ∼2.5 months ($n = 71$). The inter-assay CVs were 6.7–15.2% (Level 1) and 6.5–9.2% (Level 3) (Table 4). Adjacent peaks have baseline separation if the resolution value is 1.5 for peaks of similar size [23]. Two critical peak-pairs in the run were lutein–zeaxanthin and *trans*-lycopene–*cis*-isomer (enlarged lutein–zeaxanthin peak-pairs from pure standard mixture and plasma are illustrated in Fig. 2). The resolution values were ∼1.0 for these peak-pairs, but there were no severe problems in their quantification, because peak height was used. Resolution values were calculated from four different assays for new and used columns

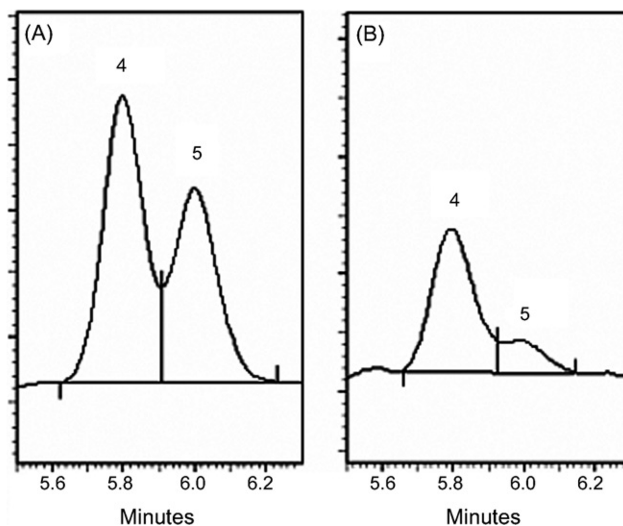


**Fig. 2.** Enlarged lutein–zeaxanthin peak-pairs obtained from (A) extracted pure standard mixture and (B) unknown plasma sample.

**Table 2**
Linearity range, repeatability of low levels and LOD of retinol, α-tocopherol and carotenoids

| Compound | Linearity range (μmol/L) | Correlation coefficient (r) | Intra-assay (low) (n = 10) | | Intra-assay (near to LOD) (n = 10) | | LOD (μmol/L) |
|---|---|---|---|---|---|---|---|
| | | | Mean ± SD (μmol/L) | CV (%) | Mean ± SD (μmol/L) | CV (%) | |
| Retinol | 0.04–21.8 | 0.9997 | 0.60 ± 0.02 | 3.5 | 0.07 ± 0.02 | 23.1 | 0.04 |
| α-Tocopherol | 0.73–232.2 | 0.9990 | 7.3 ± 0.33 | 4.5 | 0.74 ± 0.15 | 20.7 | 0.73 |
| α-Tocopherol acetate | ND | ND | ND | ND | ND | ND | ND |
| Lutein | 0.01–8.8 | 1.0000 | 0.084 ± 0.003 | 3.3 | 0.019 ± 0.002 | 7.8 | 0.01 |
| Zeaxanthin | 0.01–2.2 | 1.0000 | 0.020 ± 0.002 | 9.0 | 0.010 ± 0.001 | 12.9 | 0.01 |
| β-Apo-8′-carotenal | ND | ND | ND | ND | ND | ND | ND |
| β-Cryptoxanthin | 0.01–4.5 | 0.9999 | 0.057 ± 0.003 | 4.8 | 0.013 ± 0.002 | 11.8 | 0.01 |
| Lycopene | 0.02–9.3 | 0.9995 | 0.068 ± 0.004 | 5.3 | 0.025 ± 0.003 | 11.7 | 0.02 |
| α-Carotene | 0.02–5.8 | 0.9993 | 0.046 ± 0.003 | 5.7 | 0.024 ± 0.004 | 14.4 | 0.02 |
| β-Carotene | 0.02–11.6 | 0.9993 | 0.15 ± 0.006 | 3.7 | 0.030 ± 0.004 | 13.4 | 0.02 |

Intra-assay CVs were determined by diluting the pooled plasma of several volunteers. ND, not determined.

**Table 3**
Absolute and relative recoveries and method repeatability of plasma retinol, α-tocopherol and carotenoids

| Compound | Concentration[a] (μmol/L) | Absolute recovery (n = 9) | Relative recovery (n = 9) | Intra-assay (n = 10) | | Inter-assay (n = 107) | |
|---|---|---|---|---|---|---|---|
| | | Mean ± SD (%) | Mean ± SD (%) | Mean ± SD (μmol/L) | CV (%) | Mean ± SD (μmol/L) | CV (%) |
| Retinol | 0.76–2.27 | 90.0 ± 5.5 | 102.7 ± 1.3 | 2.47 ± 0.04 | 1.7 | 2.69 ± 0.19 | 6.9 |
| α-Tocopherol | 12.8–38.3 | 97.9 ± 1.0 | 100.8 ± 2.5 | 31.7 ± 0.33 | 1.1 | 32.0 ± 1.7 | 5.3 |
| α-Tocopherol acetate | 42.3 | 96.9 ± 3.0 | [b] | ND | ND | ND | ND |
| Lutein | 0.29–0.86 | 101.6 ± 12.3 | 100.3 ± 2.6 | 0.26 ± 0.01 | 2.6 | 0.28 ± 0.02 | 5.8 |
| Zeaxanthin | 0.13–0.38 | 90.1 ± 4.1 | 103.0 ± 2.9 | 0.065 ± 0.003 | 4.0 | 0.058 ± 0.004 | 7.6 |
| β-Apo-8′-carotenal | 0.48 | 97.2 ± 1.7 | [b] | ND | ND | ND | ND |
| β-Cryptoxanthin | 0.29–0.86 | 97.0 ± 1.8 | 94.2 ± 1.2 | 0.27 ± 0.004 | 1.7 | 0.27 ± 0.02 | 6.4 |
| Lycopene | 0.24–0.71 | 92.5 ± 3.7 | 97.7 ± 5.2 | 0.24 ± 0.003 | 1.3 | 0.26 ± 0.02 | 8.8 |
| α-Carotene | 0.10–0.30 | 85.8 ± 4.6 | 90.5 ± 3.0 | 0.17 ± 0.003 | 2.1 | 0.17 ± 0.01 | 7.7 |
| β-Carotene | 0.41–1.22 | 80.2 ± 2.2 | 86.8 ± 2.9 | 0.60 ± 0.01 | 1.7 | 0.65 ± 0.05 | 7.4 |

Intra-assay and inter-assay CVs were determined with the pooled plasma of several volunteers. ND, not determined.
[a] Concentration range of recovery experiment.
[b] Internal standard.

(used nearly 1 year and analyzed 1000–1500 samples). The differences in resolution values between new and used columns for the analytes were negligible, although the resolution of lutein and zeaxanthin decreased from 1.00 to 0.83.

### *3.4. Stability of stock solutions*

Stock solutions of retinol, α-tocopherol and carotenoids containing 0.01% (w/v) BHT were protected from light in polypropylene tubes and stored at −70 °C. Their levels were analyzed spectrophotometrically using full absorption spectra. The levels of retinol, α-tocopherol and carotenoids remained stable for 8 months storage time at −70 °C. It has been reported that carotenoids were stable in stock solutions from 3 days to 3 weeks depending on temperature [5,34,35].

### *3.5. Stability of retinol, α-tocopherol and carotenoids after extraction*

The stability of retinol, α-tocopherol and carotenoids extracted from pooled Li–heparin plasma were investigated during 1 week period on the autosampler refrigerated at 4 °C in order to find out how long storage period affects levels of analytes. The concentration of retinol increased during 1-week storage up to 16% (peak height increased ∼13%), which indicated that solvent evaporation occurred in the vial and retinol molecule was stable during the storage period. The peak height of internal standard decreased ∼7%, which indicated its degradation during the follow-up period. The concentration increase of retinol was not compensated by the internal standard. After 2 days, the levels of lycopene and β-carotene decreased 13.0 ± 2.7% and 11.0 ± 1.3% (mean ± SD). During time their levels decreased even more being after a week 29.0 ± 2.0% and 23.0 ± 9.0% lower than just after extraction, respectively. However, lycopene and β-carotene decomposed even more than percentage values show, because the effect of solvent evaporation was quite opposite. There was seen a slight trend of decrease of levels of α-tocopherol and other carotenoids. The results suggest that extracted samples can be stored for 3 days at 4 °C without significant decrease in the levels of carotenoids except for lycopene and β-carotene, which seemed to be the most sensitive carotenoids for degradation. In previous studies the stability of extracted samples in different storing conditions were also evaluated. Talwar et al. [11] have observed that carotenoids in extracted samples were stable on the autosampler 4–24 h depending on temperature or added antioxidant (ascorbic acid). Su et al. [36] have reported statistically significant changes in levels of carotenoids while storing extracted

**Table 4**
Inter-assay CVs of lyophilised Euro-Trol quality control (n = 71)

| Compound | Level 1 | | | Level 3 | | |
|---|---|---|---|---|---|---|
| | Range (μmol/L) | Mean ± SD (μmol/L) | CV (%) | Range (μmol/L) | Mean ± SD (μmol/L) | CV (%) |
| Retinol | 0.50–0.90 | 0.49 ± 0.05 | 10.2 | 3.10–5.30 | 3.80 ± 0.32 | 8.4 |
| α-Tocopherol | 6.3–9.7 | 8.5 ± 0.6 | 6.7 | 48.4–74.2 | 68.0 ± 4.4 | 6.5 |
| β-Carotene | 0.05–0.3 | 0.10 ± 0.02 | 15.2 | 0.10–4.90 | 1.16 ± 0.11 | 9.2 |

Five measurements of α-tocopherol were calculated by external calibration, because values were out of the reference range in both levels.

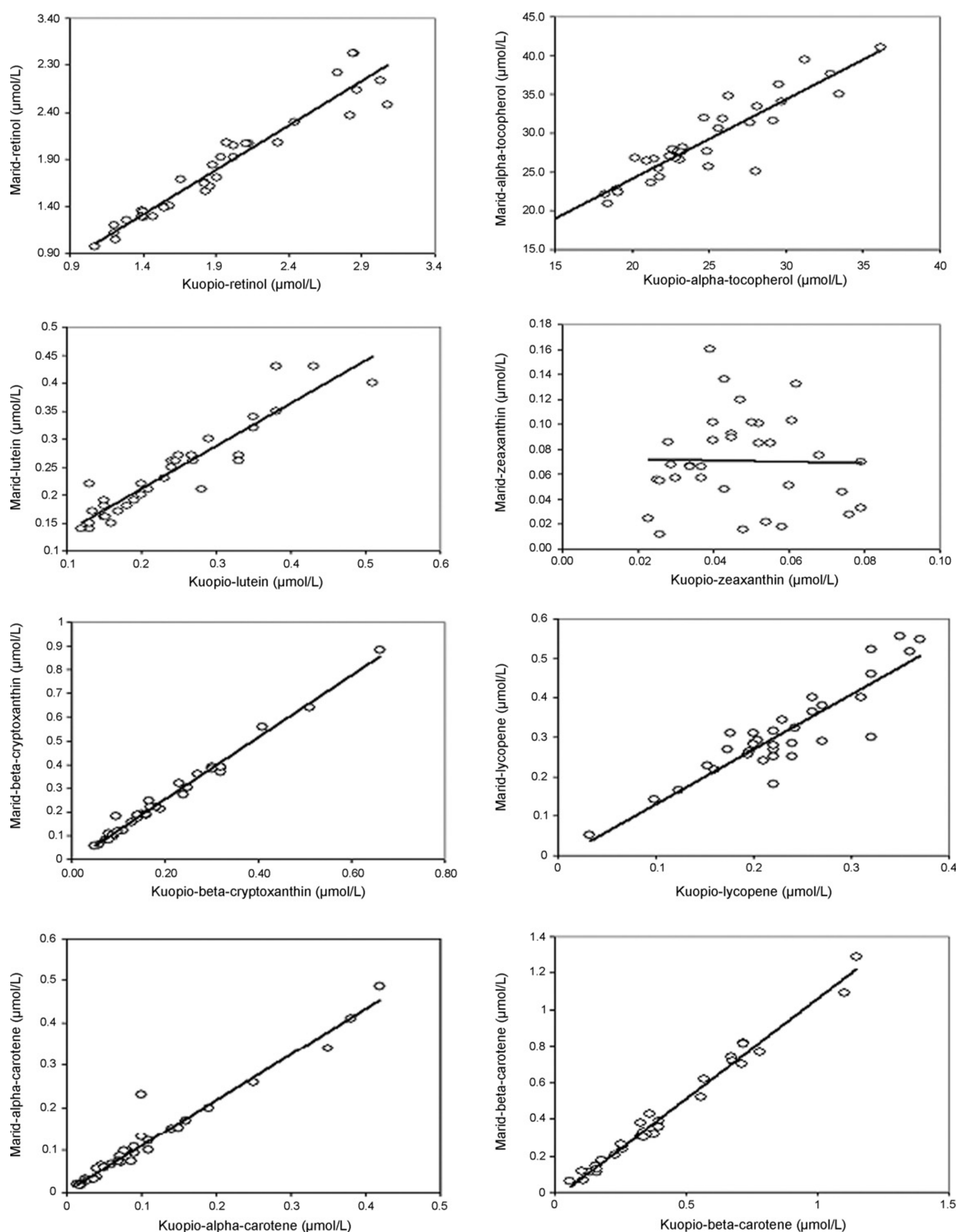


**Fig. 3.** The correlation plots of retinol, α-tocopherol, lutein, zeaxanthin, β-cryptoxanthin, lycopene, α-carotene and β-carotene between two compared HPLC methods in human plasma and serum samples ($n = 34$).

samples at different temperatures. Unlike others, Craft et al. [31] and Cavina et al. [37] have not observed any significant changes in the levels of retinol, tocopherol and carotenoids measured from extracted plasma left at room temperature for 18 and 24 h.

### 3.6. Method comparison

Plasma and serum samples from 34 adult volunteers were analyzed with two HPLC methods. Part of samples were analyzed first in Kuopio and then in Madrid and vice versa and there was a few days delay between measurements because of sample transport. The correlation coefficients ($r$) for the comparison between the two HPLC methods were high except for zeaxanthin indicating a strong linear relationship between the methods. The correlation coefficients were 0.9266 for retinol, 0.8367 for α-tocopherol, 0.8665 for lutein, 0.0007 for zeaxanthin, 0.9852 for β-cryptoxanthin, 0.8071 for lycopene, 0.9536 for α-carotene and 0.9836 for β-carotene. Correlation plots of each analytes are illustrated in Fig. 3. Low correlation of zeaxanthin between these two methods may be due to differences in the calibration, but unfortunately, calibrators were

**Table 5**
Concentrations of retinol, α-tocopherol and carotenoids in adult volunteers measured by two HPLC methods

| Compound | Kuopio (μmol/L) | Madrid [9] (μmol/L) | % Difference | *P*-value |
|---|---|---|---|---|
| Retinol | 1.83 ± 0.58 | 1.95 ± 0.59 | −5.9 | <0.001 |
| α-Tocopherol | 28.8 ± 5.4 | 24.6 ± 4.8 | 17.1 | <0.001 |
| Lutein | 0.24 ± 0.10 | 0.24 ± 0.08 | −0.3 | 0.926 |
| Zeaxanthin | 0.050 ± 0.02 | 0.070 ± 0.04 | −33.9 | <0.001 |
| β-Cryptoxanthin | 0.20 ± 0.14 | 0.25 ± 0.18 | −20.4 | 0.001 |
| Lycopene | 0.23 ± 0.07 | 0.31 ± 0.11 | −26.2 | <0.001 |
| α-Carotene | 0.11 ± 0.10 | 0.12 ± 0.11 | −9.8 | 0.019 |
| β-Carotene | 0.40 ± 0.28 | 0.40 ± 0.31 | −0.5 | 0.830 |

All values are mean ± SD ($n = 34$).

not compared. If the differences were exclusively due to a different sample pretreatment that would affect each analyte in a similar way. The percentage differences (Table 5) ranged from 0.3% to 34% for the results. The concentrations of retinol, α-tocopherol, zeaxanthin, β-cryptoxanthin, lycopene and α-carotene differed statistically significantly between two HPLC methods. However, the percentage difference for retinol (−5.9%) and α-carotene (−9.8%) may be considered to be acceptable. These methods were also compared with Bland–Altman bias plots according to which concentration differences between two HPLC methods were within 2 SD limits for all the analytes (results not shown). In addition, the physiological relevance of the results was similar, i.e. low values corresponded to low and high with high by using both methods. Because "golden standard" for all these analytes was not available, the results of the method comparison were overall satisfactory. Developed method is suitable for preparing and analyzing especially a multitude batch of samples, since the sample pretreatment is easy and quick to perform as compared with the method, which requires organic layer separation by traditional way.

## 4. Conclusion

A simple, repeatable and robust method for simultaneous measurement of retinol, α-tocopherol and six carotenoids in human plasma was developed, and compared with a previously published method. The correlation between methods for all compounds appeared to be good except for zeaxanthin. In sample purification the addition of ultrapure water enabled aqueous layer to freeze facilitating phase separation without pipetting, which saved time and improved precision. Reasonable run time and low variation of the results make this method suitable for routine measurement of retinol, α-tocopherol and carotenoids in large population studies.

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