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Stability of individual carotenoids, retinol and tocopherols in human plasma during exposure to light and after extraction

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Abstract

We have modified gradient HPLC procedures for simultaneous quantification of retinol, γ-tocopherol, α-tocopherol, lutein/zeaxanthin, β-cryptoxanthin, *trans*-lycopene, *cis*-lycopene, α-carotene and β-carotene in 200-μl aliquots of human plasma. The photosensitivity of these analytes in plasma exposed to fluorescent lighting for up to 72 h was investigated and most were stable under these conditions. The stability of these analytes held in darkness at −20°C, 4°C or room temperature for up to 48 h after extraction from plasma was also investigated. Variability in measurement of most analytes was greater at room temperature than at 4°C or −20°C. There were statistically significant variations in the measured concentrations of some analytes in samples kept cold. However, the magnitude of these variations was small and of little biological significance, particularly over the first 24 h.

Keywords: Carotenoids; Retinol; Tocopherol

1. Introduction

In the past decade, the analysis of carotenoids in food and human plasma has become increasingly important owing to the association of carotenoid-rich foods with protection against several types of human cancers [1–3] and cardiovascular disease [3,4]. High-performance liquid chromatography (HPLC) has been widely employed as the method for the separation of various classes of carotenoids, retinol and tocopherols in extracts from foods and human serum and plasma. One of the original separations of carotenoids from an extract of human serum was reported by Nelis and De Lenheer [5]. Six carotenoids were separated from extracts of human serum and the presence of two unidentified carotenoids was reported. Since then, several other researchers have developed similar nonaqueous, reversed-phase HPLC conditions employing a variety of organic solvents and various HPLC columns for the separation of carotenoids [6–19]. Few published methods separated lycopene isomers [18,19]. The stability of extracted antioxidant compounds, including the individual carotenoids, during standard laboratory processing and holding conditions has not been well documented.

Epidemiological studies designed to evaluate the association between serum or plasma levels of nutrients and risk of cancer or cardiovascular disease

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frequently require long intervals of storage time between blood collection and laboratory analysis. Furthermore, field studies may necessitate the use of sub-optimal storage conditions of biological samples. In addition, hypotheses are often generated after initial studies are complete or as new information becomes available. Thus the stability of samples under the relevant storage and processing conditions must be known prior to using such samples to test new hypotheses.

The aims of the present study were: (a) to modify an HPLC method for accurate determination and clear separation of individual lipid-soluble antioxidants (retinol, α-tocopherol, γ-tocopherol, β-carotene, α-carotene, β-cryptoxanthin, lutein/zeaxanthin and *trans*- and *cis*-lycopene) in human plasma; (b) to examine the stability of individual lipid-soluble antioxidants in plasma exposed to lighting conditions typical of an analytical laboratory; and (c) to examine the stability of individual carotenoids, retinol and tocopherol at three different temperatures after extraction from plasma.

2. Experimental

2.1. Chemicals

Except for lutein, zeaxanthin and β-cryptoxanthin which were gifts from Hoffman-LaRoche (Basel, Switzerland), pure (90–95% for lycopene, ≥95% for other antioxidants) compounds for peak identification were obtained from Sigma (Castle Hill, NSW, Australia). HPLC-grade solvents and other reagents were obtained locally from commercial suppliers (Mallinkrodt; Selby-Biolab, Melbourne, Australia). All solvents were prefiltered and degassed through a 0.45-μm Millipore filter (Milford, MA, USA) prior to use and thereafter at the start of each day.

2.2. Equipment

The chromatographic system used comprised the Waters Model 600 Controller gradient pump, 717 plus Autosampler and Model 440 programmable multiwavelength UV detector (Waters, Milford, MA, USA). Three channels were linked to a Delta Chromatography Data System (Digital Solutions, Brisbane, Australia), and the data was stored and processed by a 486 personal computer. Channel 1 monitored at 325 nm, channel 2 monitored at 450 nm and channel 3 monitored at 292 nm. A UV–Visible spectrophotometer (UV-1601, Shimadzu, Tokyo, Japan) was used to determine the concentrations of standard solutions.

2.3. Preparation of standard solutions

Stock solutions of lutein, zeaxanthin, β-cryptoxanthin, α-carotene and β-carotene, were prepared by dissolving 0.1–0.3 mg of compounds in hexane containing 0.01% butylated hydroxytoluene (BHT). Lycopene (0.1 mg) was dissolved in 1 ml of dichloromethane, which was then diluted with 10 ml hexane (0.01% BHT) in brown 15-ml bottles. Solutions of retinol, retinol acetate, α-tocopherol, γ-tocopherol and tocopherol acetate were dissolved in 96% ethanol. Concentrations of standard solutions were calculated from their absorbance with reference to published values of absorbtivity [20]. Concentrations were chosen to provide working ranges appropriate for human plasma. Lutein and zeaxanthin were used in the ratio 2:1 to mimic the ratio found in plasma [9] and their concentrations added for the construction of the standard curve. Retinol acetate and tocopherol acetate were used as internal standards [9,17]. All standard solutions were prepared under red light and stock solutions were stable under a nitrogen atmosphere at −20°C for at least one week.

2.4. Extraction of analytes from plasma

Aliquots (200 μl) of pooled plasma, kept on ice, were placed in 100×13 mm borosilicate glass tubes and extracted with 200 μl of 95% ethanol containing α-tocopherol acetate (200 μg/ml) and retinol acetate (570 ng/ml). After being vortexed for 45 s, they were extracted with hexane (1.0 ml, containing 0.01% BHT) and vortexed for 1 min. The phases were separated by centrifugation (1400 *g*, 10 min, 4°C). The upper organic phase was removed and evaporated in a glass test tube (as above) under a stream of nitrogen at room temperature. The residue was reconstituted in 30 μl of chloroform and vortexed for 40 s. Initial dissolution in chloroform was

necessary for efficient recovery of carotenoids. A 70-μl volume of acetonitrile–methanol (1:1) was added and vortexed for a further 40 s. Samples were then transferred to brown autosampler vials ready for injection.

2.5. Chromatography

After reference to several published methods [7–18] the following conditions were developed: solvent A consisted of methanol plus 0.05% ammonium acetate, solvent B was acetonitrile plus 0.1% triethylamine and solvent C was chloroform. Three linear gradient steps were programmed: from 0 to 5 min, solvent A remained at 50%, solvent B decreased from 50% to 44% and solvent C went from 0% to 6%; from 5 to 16 min, solvent A increased from 50% to 55%, solvent B decreased from 44% to 30%, and solvent C increased from 6% to 15%; and from 18 to 21 min, the solvent mix returned to 50% solvent A and 50% solvent B. Flow-rate was 1.4 ml/min throughout. Samples (50 μl) were injected onto a 250×4.6 mm Spherisorb ODS-2 reversed-phase C_{18} column (5 μm particle diameter) with a C_{18} biocompatible 10×4.3 mm guard cartridge (both from Activon, Melbourne, Australia). Retinol and retinol acetate were monitored at 325 nm, α- and γ-tocopherol and tocopherol acetate at 292 nm, and α- and β-carotene, β-cryptoxanthin, lutein/zeaxanthin and lycopene at 450 nm.

2.6. Photosensitivity of analytes in plasma

Aliquots (300 μl) of a pooled plasma sample were transferred to 2-ml screw cap polypropylene storage tubes and placed on a laboratory bench at room temperature (19–22°C) in a room of floor area 22 m^2. The room was lit by 12 fluorescent tubes each of 36 W, giving a total of 432 W. The lights were fitted with polycarbonate covers and were located approximately 2 m above the bench surface. There was minimal exposure to daylight. Triplicate determinations of analyte concentrations were made after exposure of plasma aliquots to these conditions for 0, 4, 24, 48 or 72 h. Samples were kept frozen at −20°C in darkness until analysis.

2.7. Stability of analytes after extraction from plasma

Stability of analytes after extraction was tested under three typical laboratory storage conditions: −20°C, 4°C and room temperature (19–22°C). A 5-ml volume of plasma was added to 20 ml hexane and 5 ml internal standard. The organic phase was extracted as described above. 0.8-ml aliquots of this extracted phase were dried under nitrogen and reconstituted as described above. Determinations were carried out in triplicate and injected after being held at −20°C, 4°C or room temperature for 4, 24 or 48 h.

2.8. Statistical analysis

Changes in concentration with time for each of the analytes were tested by one-way analysis of variance (ANOVA) using the Statistical Package for the Social Sciences 8.0 (SPSS, Chicago, IL, USA). In experiments examining the stability of analytes over time, separate analyses were conducted for each analyte and for the sum of the carotenoid concentrations (10 analyses in total). Hence differences were considered to be statistically significant at $P<0.005$ in order to account for multiple tests. Minimum detectable differences for time trend experiments were calculated for 80% power and 95% confidence using software available in the public domain [21].

3. Results and discussion

3.1. Chromatography

Typical chromatograms of extracted human plasma are shown in Fig. 1. The chromatograms reveal a clean baseline separation of all the analytes of interest. There were high separation efficiencies between retinol and retinol acetate (internal standard), and between α-tocopherol, γ-tocopherol and tocopherol acetate (internal standard). β-cryptoxanthin, and α- and β-carotene were also well resolved. This method also gave moderately good separation of *cis*- and *trans*-lycopene, unlike previous methods [5–19]. Lutein and zeaxanthin were not separated by

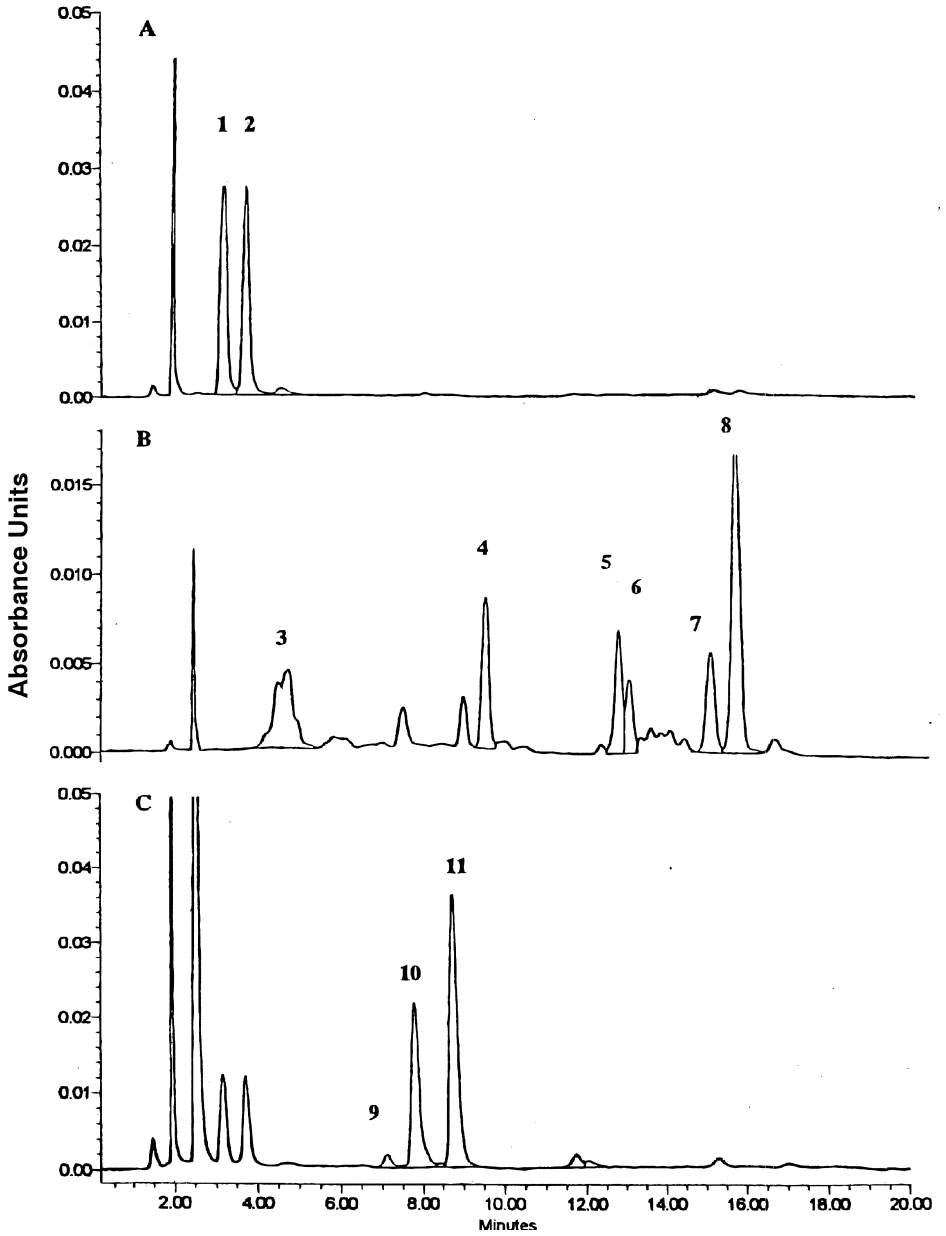

Fig. 1. Typical chromatograms of extracted human plasma analysed at (A) 325 nm, (B) 450 nm and (C) 292 nm. Peaks are as follows: 1=retinol; 2=retinol acetate (internal standard); 3=lutein/zeaxanthin; 4=β-cryptoxanthin; 5=*trans*-lycopene; 6=*cis*-lycopene; 7=α-carotene; 8=β-carotene; 9=γ-tocopherol; 10=α-tocopherol; 11=tocopherol acetate (internal standard).

this method. Several additional carotenoids not identified so far appeared between the peak of lutein/zeaxanthin at 6.0 min and the peak of β-cryptoxanthin at approximately 14 min.

3.2. Precision and accuracy of measurements

To assess the intra-batch precision of the method, a pooled plasma sample was analysed six times on

Table 1
Intra- and inter-batch variability of antioxidants in plasma

Analyte	Within run		Between run	
	Concentration[a]	RSD (%)	Concentration[b]	RSD (%)
Retinol	27.9 (0.7)	2.7	25.6 (1.5)	5.8
γ-Tocopherol	123 (6)	5.1	121 (8)	6.3
α-Tocopherol	878 (21)	2.4	950 (40)	4.2
β-Carotene	17.6 (0.3)	1.7	18.9 (1.1)	5.6
α-Carotene	5.0 (0.10)	2.0	6.0 (0.48)	8.1
β-Cryptoxanthin	3.7 (0.11)	2.9	3.9 (0.21)	5.5
Lutein/zeaxanthin	10.0 (0.5)	4.7	10.4 (0.8)	7.4
trans-Lycopene	8.1 (0.40)	5.0	8.9 (0.69)	7.8
cis-Lycopene	3.5 (0.25)	7.3	4.0 (0.37)	9.2
Total carotenoids[c]	47.8 (0.5)	1.0	52.1 (2.6)	5.0

[a] μg/dl, mean (SD), $n=6$.
[b] μg/dl, mean (SD), $n=8$.
[c] Sum of β-carotene, α-carotene, β-cryptoxanthin, lutein/zeaxanthin, *trans*-lycopene and *cis*-lycopene.

the same day. To assess between-run variability, a sample was extracted and analysed eight times over a period of two weeks. The intra- and inter-batch relative standard deviation (RSD) of each antioxidant is shown in Table 1. Intra-batch RSD was ≤5% for all analytes except γ-tocopherol and *cis*-lycopene. Inter-batch RSD was less than 10% for all analytes. These results compare favourably with between-run RSDs reported for other methods, which ranged from 1.4% for retinol [9] to 15.6% for β-cryptoxanthin [22]. Table 2 shows data obtained from the analysis of NIST standard reference material 968b. Results generally showed good agreement with certified values for retinol, α-tocopherol and β-carotene. Values for retinol in low and medium reference samples fell outside the 95% confidence interval (C.I.) (mean values −10% and +4% different from reference mean values, respectively), as did the measured value for α-tocopherol in the medium reference sample (11% above the reference mean value). All other measured values fell within the 95% C.I. of the reference mean value.

3.3. Photosensitivity of analytes in plasma

Table 3 shows the effect of exposure to lighting conditions typical of an analytical laboratory for up to 72 h on the concentrations of various lipid-soluble antioxidants in plasma. There were no significant changes in the measured concentrations of retinol, γ-tocopherol, β-cryptoxanthin, α-carotene, β-carotene, *cis*-lycopene or lutein/zeaxanthin at the

Table 2
Results of analysis of NIST standard reference material 968b[a]

Analyte	Concentration (mean, $n=2$, μg/dl)		
	Low	Medium	High
Retinol	27 *30 (28–31)*	49 *51 (50–53)*	85 *89 (80–98)*
α-Tocopherol	754 *707 (657–757)*	1120 *1011 (953–1069)*	1837 *1780 (1650–1910)*
β-Carotene	22.2 *23 (21–24)*	56.1 *57 (54–60)*	106 *113 (103–123)*

[a] Data in italics are means (95% confidence interval) of certified NIST reference values.

Table 3
Concentrations of lipid-soluble antioxidant compounds in plasma exposed to fluorescent lighting at room temperature

Analyte	Concentration [mean (SD), $n=3$, μg/dl]					ANOVA P
	Time (h)					
	0	4	24	48	72	
Retinol	28.0 (0.72)	27.3 (1.8)	28.0 (0.16)	28.4 (0.36)	27.7 (0.35)	0.68
α-Tocopherol	878 (32)	877 (9)	846 (24)	896 (35)	825 (7)	0.031
γ-Tocopherol	122 (10)	123 (3)	118 (2)	122 (9)	110 (3)	0.10
β-Carotene	17.5 (0.42)	17.7 (0.15)	17.5 (0.23)	17.2 (0.64)	17.2 (0.18)	0.38
α-Carotene	5.0 (0.07)	5.0 (0.14)	4.8 (0.21)	4.8 (0.21)	4.9 (0.09)	0.27
Cryptoxanthin	3.6 (0.04)	3.7 (0.15)	3.6 (0.23)	3.7 (0.12)	3.5 (0.04)	0.56
Lutein/zeaxanthin	10.2 (0.41)	9.8 (0.46)	9.5 (0.09)	10.4 (0.43)	10.0 (0.23)	0.054
trans-Lycopene	7.9 (0.44)	8.3 (0.33)	7.9 (0.32)	7.1 (0.69)	7.3 (0.12)	0.048
cis-Lycopene	3.4 (0.24)	3.6 (0.27)	3.2 (0.43)	3.0 (0.58)	3.4 (0.21)	0.42
Total carotenoids	47.6 (0.66)	48.0 (0.15)	46.5 (0.92)	46.2 (1.8)	46.2 (0.24)	0.12

level of $P<0.005$. Although there were variations of up to 6% in concentration of α-tocopherol which approached statistical significance ($P<0.05$), it is likely to be random methodological variation as it was not in a consistent direction and was small in magnitude. Variations in *trans*-lycopene concentration also approached statistical significance as measured concentrations were slightly lower after 48 and 72 h. The total carotenoid concentration (calculated as the sum of lutein/zeaxanthin, cryptoxanthin, *trans*-lycopene, *cis*-lycopene, α-carotene and β-carotene) did not vary significantly over time. The minimum detectable difference under these experimental conditions, calculated on the basis of 80% power and 95% confidence, was less than 7% of baseline values for all analytes except *cis*-lycopene (detectable difference 15%). Statistical power was therefore adequate to detect biologically-meaningful variations.

Thus the present study showed that most lipid-soluble antioxidant compounds are stable in plasma exposed to fluorescent lighting conditions typical of an analytical laboratory for up to 72 h at room temperature. This study examined stability in small volumes (300 μl) in standard 2-ml polypropylene storage vials. Hence the samples had a relatively large surface area-to-volume ratio, suggesting they were exposed to greater oxidative stress than would samples of a larger volume. In vivo, carotenoids are commonly located in membranes, where they constitute an integral part of the complex membrane structure, or in circulating lipid particles. Carotenoids associate with hydrophobic areas in proteins or with the lipid components of lipoproteins. The carotenoids are usually stabilised to a considerable degree by proteins and other molecules in their immediate vicinity [23]. Hence, their stability ex-vivo, at least in the short-term, is not unexpected. Commonly, carotenoids in vivo are much more stable than they are isolated and in organic solution [23].

3.4. Stability of analytes after extraction from plasma

To determine whether overnight, automated analysis was feasible, the stability of individual carotenoids, retinol and tocopherols extracted from a pooled plasma sample were investigated over 48 h at various temperatures. Table 4 shows the measured concentrations of antioxidant vitamins and individual carotenoids in extracts from plasma held in darkness at −20°C, 4°C or room temperature. At −20°C, there were statistically significant variations in the levels of β-carotene, α-carotene and lutein/zeaxanthin over time. For β- and α-carotene, these changes were small in magnitude and not consistently upwards or downwards in direction. They are likely to represent random fluctuations due to methodological variability. For lutein/zeaxanthin, the results suggest a downward trend in measured levels after 24 h. The change in total carotenoid concentration with time at −20°C approached statistical significance. The minimum detectable difference for this experiment was

Table 4
Concentrations of lipid-soluble antioxidant compounds extracted from plasma and held at −20°C, 4°C or room temperature (19–22°C)

Analyte	Concentration [mean (SD), $n=3$, μg/dl]				ANOVA *P*
	Time (h)				
	0	4	24	48	
−20°C					
Retinol	25.5 (0.21)	25.5 (0.12)	25.4 (0.39)	25.3 (0.16)	0.60
α-Tocopherol	749 (16)	774 (15)	752 (10)	753 (20)	0.28
γ-Tocopherol	106 (4)	105 (4)	96 (3)	102 (2)	0.020
β-Carotene	14.2 (0.16)	14.4 (0.15)	15.3 (0.25)	13.9 (0.23)	<0.001
α-Carotene	4.2 (0.03)	3.9 (0.19)	4.7 (0.28)	4.0 (0.05)	0.003
β-Cryptoxanthin	3.0 (0.11)	3.1 (0.15)	2.8 (0.13)	3.1 (0.03)	0.050
Lutein/zeaxanthin	9.0 (0.43)	8.3 (0.05)	7.8 (0.04)	8.2 (0.14)	0.001
trans-Lycopene	6.6 (0.34)	6.6 (0.19)	6.8 (0.60)	6.2 (0.23)	0.34
cis-Lycopene	2.8 (0.57)	2.8 (0.04)	3.1 (0.79)	2.3 (0.09)	0.27
Total carotenoids	39.8 (0.41)	39.1 (0.54)	40.5 (1.2)	37.6 (0.42)	0.007
4°C					
Retinol	25.5 (0.21)	25.2 (0.17)	25.1 (0.58)	25.5 (0.33)	0.44
α-Tocopherol	749 (16)	771(23)	763 (1)	789 (2)	0.045
γ-Tocopherol	106 (4)	113 (4)	96 (1)	106 (1)	0.020
β-Carotene	14.2 (0.16)	14.5 (0.63)	15.2 (0.21)	14.3 (0.07)	0.033
α-Carotene	4.2 (0.03)	3.9 (0.26)	4.5 (0.13)	4.0 (0.13)	0.006
β-Cryptoxanthin	3.0 (0.11)	3.1 (0.11)	2.9 (0.02)	3.2 (0.03)	0.009
Lutein/zeaxanthin	9.0 (0.43)	8.4 (0.16)	8.0 (0.09)	8.4 (0.20)	0.006
trans-Lycopene	6.6 (0.34)	6.2 (0.25)	7.0 (0.53)	6.2 (0.07)	0.075
cis-Lycopene	2.8 (0.57)	2.6 (0.48)	3.0 (0.15)	2.4 (0.03)	0.34
Total carotenoids	39.8 (0.41)	38.7 (0.60)	40.5 (0.68)	38.4 (0.41)	0.004
Room temperature (19–22°C)					
Retinol	25.5 (0.21)	26.1 (0.73)	25.9 (0.47)	26.5 (0.35)	0.16
α-Tocopherol	749 (16)	727 (24)	748 (22)	786 (47)	0.18
γ-Tocopherol	106 (4)	95 (2)	91 (4)	98 (2)	0.002
β-Carotene	14.2 (0.16)	14.0 (0.47)	14.5 (0.28)	13.9 (0.24)	0.14
α-Carotene	4.2 (0.03)	3.7 (0.14)	4.4 (0.44)	4.0 (0.13)	0.006
β-Cryptoxanthin	3.0 (0.11)	2.7 (0.52)	2.6 (0.14)	2.9 (0.07)	0.29
Lutein/zeaxanthin	9.0 (0.43)	7.6 (1.2)	7.9 (0.53)	8.4 (0.21)	0.13
trans-Lycopene	6.6 (0.34)	6.1 (0.72)	6.2 (0.52)	5.4 (0.43)	0.11
cis-Lycopene	2.8 (0.57)	2.2 (0.35)	3.1 (0.13)	1.9 (0.15)	0.014
Total carotenoids	39.8 (0.41)	36.3 (3.0)	38.8 (1.7)	36.6 (1.2)	0.12

≤8% of baseline values for all analytes except *cis*-lycopene (21%).

At 4°C, the total carotenoid concentration varied significantly with time but the magnitude of the absolute change was small (Table 4). Lutein/zeaxanthin concentrations again fell marginally at 24 and 48 h and this change approached statistical significance. It is not clear if this variation has a true chemical basis or merely represents random fluctuations due to methodological variability. In any case, these changes were small in magnitude and therefore of little biological significance in the interpretation of results. The minimum detectable difference for this experiment was ≤7% of baseline values for all analytes except *cis*-lycopene (18%).

At room temperature, there were statistically significant changes over time in concentration of γ-tocopherol (and, to a lesser extent, α-carotene), again likely representing random fluctuation in measurement. *cis*-Lycopene tended to decrease with time, but

this effect did not reach statistical significance ($P<0.05$). In general, the variability in measurement at room temperature was greater than in samples kept cold, as indicated by the greater standard deviation of samples kept at room temperature. The minimum detectable difference for this experiment was ≤12% of baseline values for all analytes except *cis*-lycopene (17%). Thus, storage of extracts at room temperature is not recommended.

The study by Craft et al. [19] determined the stability of individual carotenoids in extracted plasma left at room temperature for 0, 4, 12 and 24 h. They observed no significant changes in the measured concentrations of retinol, tocopherol, or individual carotenoids. For lycopene, an apparent downward trend was observed between 0 and 4 h, however this was not statistically significant. They did not report the stability of extracted analytes for periods longer than 24 h.

4. Conclusions

The present method for analysis of lipid-soluble antioxidants in plasma showed good reproducibility (RSDs<10% for most analytes) and accuracy (tested against NIST reference materials). The method described gives excellent recoveries (≥97% for all analytes; data not shown) and improved resolution of retinol, tocopherols and individual carotenoids. The separation of the isomers of lycopene is one of the important advantages of the method. Our review of the literature has found no other report that clearly separated *trans*- and *cis*-lycopene. Five unknown carotenoids were detected and well separated, but were not identified at this stage. Further studies of carotenoid metabolites are warranted.

The results of the present study indicate that even plasma samples which have not been kept under conditions considered to be optimal for the preservation of lipid-soluble antioxidants (up to at least 24 h at room temperature) may be suitable for inclusion in retrospective studies of the role of these compounds in the aetiology of disease. However, once extracted, samples should be kept in the dark, cold and analysed within 24 h if optimal accuracy in the determination of carotenoid levels is of interest.

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