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Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days: implications for epidemiological studies

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Background Biochemical measurement of fat-soluble vitamins would allow direct assessment in epidemiological studies of their association with disease. However, the perceived instability of these compounds and typically high cost of collection and analysis may make their measurement impractical, particularly in large-scale studies. Using a high performance liquid chromatography assay developed in-house, we have investigated the separate effects of temperature and light on the stability of vitamins in whole blood over several days.

Methods Multiple blood samples from 10 volunteers were stored at 20°C or 4°C and in dark or light conditions. Immediately after collection and 1, 2, 3, 4, and 7 days later, samples stored under each condition were centrifuged, and the plasma was aliquoted and stored at −80°C. Subsequently, all aliquots from each individual were analysed in one analytical run.

Results In whole blood stored under any of the four conditions for up to 7 days, concentrations of α-carotene, β-carotene, lutein, lycopene, retinol, and α-tocopherol changed by less than 8%, and cryptoxanthin and γ-tocopherol by less than 11%. Although significant temperature effects were observed for α-carotene, and α- and γ-tocopherol, and a significant effect of light was observed for α-carotene, cryptoxanthin, and lycopene, these analytes changed by less than 1% per day under all conditions.

Conclusions Concentrations of these fat-soluble vitamins change by only a few per cent in whole blood during storage at room temperature for several days. Hence, delayed separation of blood samples (which may be required for practical reasons in large-scale epidemiological studies) does not preclude reliable measurement of fat-soluble vitamins.

Keywords Vitamins, blood-based epidemiology, blood collection, stability, temperature, light

Analyses of vitamin levels in blood samples provide direct measures of exposure to dietary or supplemented vitamins, and would be preferable to indirect assessments based on self-reported dietary intake or compliance to study treatment.[1,2] Fat-soluble vitamins have typically been considered to be highly unstable in unseparated blood at ambient temperature and when exposed to light and oxygen. Consequently, some researchers who have included biochemical measurements in their studies have used complex blood collection and processing methods: for example, blood samples have been taken into red dye-coated tubes, placed immediately on ice, the plasma separated rapidly and aliquoted under subdued light into coloured vials placed on ice, and flushed with nitrogen before frozen storage.[3] This procedure may be prohibitively expensive and impractical for large-scale studies, particularly those involving blood collection at remote centres (e.g. in

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multiple clinics or in people's homes) and transport to a central laboratory, and so could preclude the measurement of vitamins in such studies.

Existing data on the stability of fat-soluble vitamins in whole blood are scarce and somewhat conflicting. Retinol has been reported to be stable, in the absence of oxygen, at room temperature in the dark for up to 24 hours.[4] β-carotene has been reported to be stable under chilled conditions in the dark for 48 hours and α-tocopherol to be stable at room temperature for 72 hours, although levels of these vitamins were found to decrease significantly in chilled whole blood during overnight transport.[5] We have recently established that the concentrations of certain analytes, such as lipids, change by less than 4% in whole blood stored at room temperature for several days.[6] These analytes may, therefore, be measured reliably in blood samples that were simply mailed to a central laboratory for separation, which would be particularly advantageous for large-scale studies. If fat-soluble vitamins were found to exhibit similar stability, then this simple and comparatively inexpensive blood collection method could enable their measurement in studies where it would not otherwise be considered feasible. Using a high-throughput, low-cost high performance liquid chromatography (HPLC) assay developed in-house, we have investigated the stability of α-carotene, β-carotene, cryptoxanthin, lutein, lycopene, retinol, α-tocopherol, and γ-tocopherol in whole blood stored (in the absence of oxygen) at room temperature or chilled, and in light or dark conditions.

Materials and methods

Subjects and procedures

Six 10 ml vacutainers of blood containing 0.12 ml preservative (15% potassium EDTA [Becton Dickinson UK Limited, Oxford, England]) were drawn from each of 10 healthy volunteers aged 25–60 years. Each vacutainer corresponded to a particular separation time-point (immediate or 1, 2, 3, 4, or 7 days post-collection), which was randomly assigned within each volunteer's set of vacutainers using a random number table. Immediately after collection, the vacutainers were mixed gently, and the contents of each was divided into four tubes of 2 ml aliquots whilst under nitrogen to avoid contact of the sample with oxygen. These tubes were sealed tightly. The four tubes for each time-point from each volunteer corresponded to four different storage conditions: room temperature dark, room temperature light, chilled dark, and chilled light. Four of the 2 ml tubes from each individual were centrifuged immediately (2000 g for 10 minutes at 4°C), and the plasma was aliquoted into 1.8 ml Nunc cryotubes (Nunc A/S, Roskilde, Denmark) and stored at −80°C. The remaining 20 tubes from each individual were stored under their allocated conditions. The room temperature samples were placed in the laboratory, which had a controlled temperature of 20°C, and the chilled samples were placed in a cold room at 4°C. The samples to be placed in the dark were wrapped in foil and covered by a box, and the samples to be placed in the light were situated beneath several lamps, providing intense lighting conditions. A light meter was used to ensure that the samples at room temperature and chilled were exposed to the same amount of light and this was checked daily. At each subsequent time-point, the assigned tube in each storage condition from each volunteer was retrieved and centrifuged, and the plasma aliquoted and stored at −80°C.

Biochemical analyses

Prior to analysis, the frozen samples were left to stand at room temperature to thaw, then inverted several times to mix. The aliquots from all four temperature/light conditions and all time-points for each volunteer were analysed together in one batch by the same technician, to avoid run-to-run variability, using an in-house method (Clark S, Youngman LD, Chukwurah B. Development of a high-throughput, low-cost and reliable HPLC assay for quantification of eight fat-soluble vitamins. Unpublished manuscript). The extraction procedure for the assay was as follows: 200 µl plasma was added to 250 µl distilled water and 50 µl internal standard (retinol acetate and tocopherol acetate in ethanol). 400 µl ethanol solution (containing 0.5 g/l butylated hydroxytoluene and 10 mmol/l lauryl sulphate) was added and the contents vortex-mixed for 5 minutes. 1 ml hexane was then added, the mixture centrifuged (4500 r.p.m. for 10 minutes at 4°C), and the top layer transferred to another tube containing 200 µl methanol. A further 1 ml hexane was added to the original tube for a second extraction step; the mixture was vortex-mixed and centrifuged, with the top layer removed and combined with the previous hexane layer from the first extraction step. This was then vacuum-dried and the residue re-dissolved in 50 µl dichloromethane and 150 µl mobile phase (0.05% tri-ethylamine in 45% acetonitrile, 45% methanol, 10% chloroform) for injection on the HPLC system.

Chromatographic separation was performed by reverse phase HPLC using a Waters system (Waters Ltd, Watford, UK) fitted with a photodiode array detector and a C18 column (Phenomenex, Macclesfield, UK). Chromatograms were extracted at 474 nm for lycopene, 446 nm for lutein, cryptoxanthin, α-carotene, and β-carotene, 325 nm for retinol and 292 nm for α-tocopherol and γ-tocopherol. Quantification of the vitamins was performed using Waters Millennium software. The intra-assay coefficient of variation (CV), derived from analysis of human plasma pools within each batch, was 11% for α-carotene at a level of 0.12 µmol/l, 4% for β-carotene at 0.85 µmol/l, 7% for cryptoxanthin at 0.12 µmol/l, 7% for lutein at 0.34 µmol/l, 5% for lycopene at 0.36 µmol/l, 3% for retinol at 2.28 µmol/l, 4% for α-tocopherol at 28.48 µmol/l, and 10% for γ-tocopherol at 1.11 µmol/l (n = 20 replicates). In-house reference intervals, established from analysis of 1400 individuals aged 40–79 years who were at increased risk of coronary heart disease, were comparable with those quoted for a similar published method[7] that had quantified all of the vitamins except γ-tocopherol (Table 1).

Data analysis

For each individual, the mean vitamin concentration from analysis of the four samples that had been separated and frozen immediately was used as the baseline 'fresh' value against which concentrations at future time-points were compared. The stability of a fat-soluble vitamin under each storage condition was determined by calculating the percentage change in concentration from this mean fresh value at each time-point for each individual, then calculating the mean percentage change (and standard error of the mean) at each time-point for all individuals.

Table 1 Assay values (μmol/l) in samples separated immediately after collection from 10 healthy volunteers aged 25–60 years (with reference intervals)

Vitamin	Mean	Standard deviation	Minimum	Maximum	Reference interval
α-carotene	0.20	0.15	0.09	0.61	0.03–0.26[a]
					0.02–0.22[b]
β-carotene	0.52	0.35	0.09	1.14	0.15–1.08[a]
					0.07–0.88[b]
cryptoxanthin	0.42	0.48	0.04	1.45	0.06–0.52[a]
					0.05–0.52[b]
lutein	0.40	0.21	0.19	0.90	0.10–0.56[a]
					0.14–0.74[b]
lycopene	0.55	0.20	0.33	0.95	0.07–0.90[a]
					0.11–0.80[b]
retinol	1.95	0.27	1.47	2.24	1.14–3.40[a]
					1.05–2.97[b]
α-tocopherol	26.10	4.43	18.41	33.82	17.73–51.58[a]
					13.9–47.0[b]
γ-tocopherol	1.59	0.66	0.71	2.37	0.90–4.97[a]

[a] Method-specific reference intervals established in-house from analysis of 1400 individuals aged 40–79 years who were at increased risk of coronary heart disease.

[b] Sowell AL *et al*. Serum concentrations in 3480 male and female subjects, aged 4–93 years.[7] No results available for γ-tocopherol.

As well as evaluating the stability under each of the four separate conditions, the data from these four groups were combined in order to allow simpler comparisons of room temperature versus chilled and of light versus dark conditions (provided that there was no significant interaction between temperature and light conditions for that vitamin). For example, the combined data for the room temperature dark and room temperature light conditions were compared with the combined data from chilled dark and chilled light conditions to enable consideration of room temperature versus chilled conditions.

All analyses were performed using log-linear regression, incorporating a term for individual subject. The regression analyses included tests for significant interaction between temperature and light conditions for each vitamin, for significant differences in vitamin concentrations between room temperature and chilled or dark and light conditions, and for significant trends over time at each temperature or light condition. *P*-values were based on t-tests for the addition into the model of terms for days (interval scaled), and days × storage condition (i.e. days × room temperature or chilled, and days × dark or light). Data analysis was performed using SAS (SAS Institute Inc, Cary, NC, USA).

Results

Table 1 shows the results obtained in samples separated immediately after collection from the healthy volunteers, along with reference intervals for each fat-soluble vitamin. One individual had particularly high concentrations of α-carotene, β-carotene, cryptoxanthin, and lutein, and another individual had a high concentration of cryptoxanthin. All other results were within, or close to, the in-house and published reference intervals.

Table 2 shows the mean percentage change in plasma vitamin concentrations at each time-point, as well as the mean percentage change per day, for samples stored under the four different conditions. Despite a statistically significant trend over time for some of the vitamin concentrations when stored under certain conditions, the change per day remained less than 1% for all of the vitamins under any condition (with the exception of α-carotene which decreased by 1.15% per day under chilled conditions in the light). The standard deviation of the individual vitamin concentrations did not differ appreciably between time-points (for example, the standard deviation of β-carotene concentrations in samples stored at room temperature in the light was 0.34, 0.33, 0.38, 0.35, 0.35, and 0.32 μmol/l in samples separated at 0, 1, 2, 3, 4, and 7 days after collection respectively).

Table 3(i) shows the mean percentage change in plasma vitamin concentrations at each time-point for samples stored at room temperature (20°C) and chilled (4°C), derived from the combined data for light and dark conditions. Figure 1 shows the summary estimate of the mean percentage change per day for each vitamin under each temperature condition. A small but statistically significant interaction between temperature and light effects was found for retinol ($P = 0.001$), so the data were not combined for this vitamin (and it is excluded from Table 3 and the Figures). For the other vitamins, the test for interaction between temperature and light effects yielded a *P*-value >0.2 (data not shown). There were statistically significant differences between the values in samples kept at 20°C versus 4°C for α- and γ-tocopherol ($P < 0.0001$), with smaller changes observed under chilled conditions than at room temperature. There was also a marginally statistically significant ($P = 0.02$) larger change in α-carotene concentration under chilled conditions than room temperature. Although statistically significant decreases in concentrations were observed over time at 20°C for β-carotene

Table 2 Percentage change in fat-soluble vitamin concentration over time in samples stored at 20°C (RT) or 4°C (CH), and in dark or light

		% change (SE) from immediately separated samples					% change per	
Vitamin	**Condition**	1 day	2 days	3 days	4 days	7 days	**day (95% CI)**	***P* trend**
α-carotene	RT dark	2.07 (2.39)	3.69 (2.67)	1.19 (3.31)	4.36 (1.84)	2.94 (2.48)	0.39 (−0.35, 1.13)	0.3
	RT light	2.72 (3.86)	1.28 (3.12)	2.61 (3.86)	1.43 (3.09)	−4.10 (4.25)	−0.48 (−1.21, 0.26)	0.2
	CH dark	0.43 (3.32)	0.81 (2.89)	0.60 (2.38)	−1.41 (4.10)	−0.82 (3.27)	−0.39 (−1.12, 0.35)	0.3
	CH light	0.53 (3.29)	0.88 (3.09)	−5.52 (2.28)	−4.74 (4.22)	−4.64 (2.24)	−1.15 (−1.87, −0.42)	0.002
β-carotene	RT dark	0.29 (1.47)	0.82 (1.74)	1.79 (2.28)	5.50 (2.88)	−4.95 (2.93)	−0.49 (−1.01, 0.04)	0.07
	RT light	1.90 (1.32)	3.52 (1.90)	0.38 (2.96)	3.33 (2.07)	−5.95 (3.23)	−0.69 (−1.21, −0.16)	0.01
	CH dark	0.17 (1.87)	−0.87 (1.36)	0.87 (1.31)	0.35 (2.71)	0.25 (3.21)	−0.35 (−0.88, 0.18)	0.2
	CH light	−0.40 (1.81)	2.69 (1.59)	0.76 (2.88)	−0.65 (3.34)	−3.00 (1.95)	−0.60 (−1.12, −0.08)	0.02
Cryptoxanthin	RT dark	2.19 (3.36)	6.67 (3.77)	4.55 (2.60)	8.28 (4.48)	2.12 (3.44)	0.25 (−0.49, 1.01)	0.5
	RT light	7.29 (2.68)	4.70 (2.74)	0.49 (4.45)	5.94 (1.94)	1.63 (3.97)	−0.06 (−0.80, 0.70)	0.9
	CH dark	2.72 (2.57)	1.52 (3.38)	5.69 (3.25)	4.22 (5.78)	9.70 (5.52)	0.55 (−0.20, 1.31)	0.2
	CH light	4.83 (3.67)	4.83 (1.81)	6.84 (4.57)	2.49 (3.52)	−4.84 (3.03)	−0.58 (−1.32, 0.16)	0.1
Lutein	RT dark	1.30 (1.24)	1.89 (1.63)	0.12 (1.79)	4.22 (2.07)	−6.47 (1.66)	−0.65 (−1.18, −0.11)	0.02
	RT light	1.69 (1.20)	3.03 (2.36)	1.22 (3.33)	2.99 (2.10)	−6.55 (2.91)	−0.70 (−1.24, −0.17)	0.01
	CH dark	0.37 (1.54)	−0.17 (1.48)	1.07 (1.53)	−0.41 (1.86)	5.67 (6.95)	0.07 (−0.47, 0.61)	0.8
	CH light	1.45 (1.60)	0.59 (2.58)	0.35 (1.83)	−1.58 (3.23)	−1.47 (1.49)	−0.51 (−1.05, 0.02)	0.06
Lycopene	RT dark	1.41 (1.71)	3.28 (1.65)	3.82 (2.12)	7.65 (1.79)	−0.59 (1.85)	0.15 (−0.32, 0.62)	0.5
	RT light	1.82 (1.87)	3.91 (2.27)	2.74 (2.57)	7.40 (1.91)	−4.22 (4.34)	−0.33 (−0.79, 0.14)	0.2
	CH dark	−0.28 (1.51)	−1.21 (1.63)	3.10 (1.36)	0.70 (2.45)	3.40 (4.36)	−0.05 (−0.52, 0.42)	0.8
	CH light	1.62 (1.50)	2.42 (1.71)	1.78 (1.80)	0.83 (2.52)	−2.70 (1.68)	−0.47 (−0.93, −0.00)	0.05
Retinol	RT dark	0.50 (0.91)	1.55 (1.22)	1.81 (1.48)	4.57 (1.66)	3.30 (1.43)	0.45 (0.11, 0.79)	0.01
	RT light	−0.15 (1.41)	0.33 (1.59)	−1.36 (2.09)	1.28 (1.48)	−6.63 (2.86)	−0.84 (−1.17, −0.50)	<0.0001
	CH dark	0.84 (1.09)	0.78 (0.83)	1.12 (1.10)	0.39 (2.21)	−0.33 (1.50)	−0.14 (−0.47, 0.20)	0.4
	CH light	1.67 (1.21)	0.19 (1.85)	−0.73 (1.27)	−2.06 (2.59)	−1.65 (1.37)	−0.45 (−0.78, −0.11)	0.009
α-tocopherol	RT dark	2.76 (1.32)	4.08 (1.50)	4.29 (1.82)	6.71 (2.12)	4.80 (1.36)	0.70 (0.32, 1.07)	0.0003
	RT light	3.67 (2.26)	4.63 (2.01)	3.39 (2.51)	7.41 (1.56)	3.34 (3.28)	0.54 (0.16, 0.91)	0.006
	CH dark	0.93 (1.17)	0.34 (1.28)	2.36 (1.55)	1.03 (2.47)	1.22 (1.92)	−0.09 (−0.47, 0.28)	0.6
	CH light	2.22 (1.32)	1.78 (1.18)	0.94 (1.74)	0.47 (2.75)	0.42 (1.48)	−0.19 (−0.56, 0.19)	0.3
γ-tocopherol	RT dark	0.50 (2.77)	3.78 (2.32)	7.54 (2.00)	7.01 (2.20)	6.99 (2.92)	0.92 (0.34, 1.50)	0.002
	RT light	2.28 (3.60)	6.88 (1.93)	5.66 (3.34)	10.49 (3.52)	1.22 (2.98)	0.56 (−0.02, 1.15)	0.06
	CH dark	5.07 (2.30)	2.48 (2.69)	1.95 (2.20)	−1.60 (3.26)	−1.28 (2.52)	−0.40 (−0.97, 0.18)	0.2
	CH light	1.74 (1.82)	−3.00 (2.74)	−2.88 (1.60)	−1.00 (2.01)	−0.81 (1.98)	−0.66 (−1.23, −0.08)	0.03

($P = 0.007$) and lutein ($P = 0.002$), and statistically significant increases were observed for α-tocopherol ($P < 0.0001$) and γ-tocopherol ($P = 0.002$), the magnitude of these changes remained less than 1% per day. At 4°C, statistically significant decreases in concentrations were observed over time for α-carotene ($P = 0.01$), β-carotene ($P = 0.03$), and γ-tocopherol ($P = 0.03$), but again these were less than 1% per day.

Table 3(ii) and Figure 2 show similar results for dark versus light conditions for each vitamin (except retinol), derived from the combined data for room temperature and chilled conditions. There were statistically significant differences between the values in samples kept under light versus dark conditions for α-carotene ($P = 0.01$), cryptoxanthin ($P = 0.03$), and lycopene ($P = 0.03$) and, with the exception of cryptoxanthin, the change was less under dark than light conditions. Although statistically significant decreases in concentrations were observed over time under light conditions for α-carotene ($P = 0.007$), β-carotene ($P = 0.003$), lutein ($P = 0.006$), and lycopene ($P = 0.04$), these were less than 1% per day. Under dark conditions, none of the vitamins exhibited statistically significant changes in concentration during 7 days of storage as whole blood.

Discussion

This study has yielded extensive information on the effect of storage conditions on the stability of α-carotene, β-carotene, cryptoxanthin, lutein, lycopene, retinol, α-tocopherol, and γ-tocopherol in whole blood samples. A sufficiently large number of samples were obtained from each individual to consider delays in plasma separation of up to 7 days after blood collection, and to

Table 3 Percentage change in fat-soluble vitamin concentration over time in samples stored at 20°C or 4°C, and in dark or light

Vitamin	Condition	% change (SE) from immediately separated samples					*P*-value trend	*P*-value
		1 day	2 days	3 days	4 days	7 days		
(i) Temperature								**20°C versus 4°C**
α-carotene	20°C	2.40 (1.68)	2.48 (1.74)	2.16 (2.75)	2.91 (2.13)	−0.58 (2.96)	0.9	
	4°C	0.48 (2.86)	0.64 (2.16)	−2.46 (1.07)	−4.85 (4.28)	−2.73 (2.49)	0.01	0.02
β-carotene	20°C	1.10 (0.96)	2.17 (1.40)	1.08 (1.74)	4.50 (1.91)	−5.45 (2.04)	0.007	
	4°C	−0.11 (1.51)	0.77 (0.90)	0.81 (1.90)	−1.32 (3.30)	−1.38 (1.62)	0.03	0.6
Cryptoxanthin	20°C	4.74 (2.01)	5.68 (2.79)	2.70 (2.16)	6.81 (2.56)	1.88 (2.70)	0.8	
	4°C	3.78 (2.56)	3.17 (1.94)	6.26 (2.66)	2.74 (3.96)	2.43 (3.04)	0.9	0.7
Lutein	20°C	1.50 (1.04)	2.46 (1.68)	0.74 (2.14)	3.52 (1.79)	−6.51 (1.78)	0.002	
	4°C	0.91 (1.44)	0.15 (1.36)	0.71 (1.29)	−1.22 (2.11)	2.10 (3.36)	0.3	0.05
Lycopene	20°C	1.62 (1.62)	3.60 (1.43)	3.18 (1.63)	7.29 (1.58)	−2.41 (2.63)	0.6	
	4°C	0.67 (1.32)	0.52 (1.29)	2.44 (1.13)	0.45 (2.32)	0.35 (2.86)	0.2	0.4
α-tocopherol	20°C	3.22 (1.46)	4.36 (1.14)	3.82 (1.46)	6.99 (1.62)	4.07 (1.80)	<0.0001	
	4°C	1.58 (1.06)	0.92 (1.01)	1.65 (1.46)	0.23 (2.54)	0.82 (1.63)	0.4	<0.0001
γ-tocopherol	20°C	1.39 (2.29)	5.33 (1.37)	6.86 (2.04)	8.58 (2.00)	4.10 (2.45)	0.002	
	4°C	3.41 (1.73)	−0.58 (1.83)	−0.47 (1.62)	−1.79 (2.44)	−1.05 (1.47)	0.03	<0.0001
(ii) Light								**Dark v Light**
α-carotene	Dark	1.25 (1.84)	2.25 (2.50)	0.67 (2.18)	1.63 (1.66)	1.06 (2.48)	1.0	
	Light	1.63 (2.49)	0.74 (2.35)	−1.45 (2.44)	−3.58 (4.21)	−4.37 (2.89)	0.007	0.01
β-carotene	Dark	0.23 (1.38)	−0.03 (1.35)	1.28 (1.38)	3.16 (2.22)	−2.35 (1.21)	0.05	
	Light	0.75 (1.16)	3.01 (1.53)	0.57 (2.10)	0.02 (3.12)	−4.47 (2.28)	0.003	0.3
Cryptoxanthin	Dark	2.45 (2.73)	4.09 (1.90)	4.56 (2.47)	6.04 (3.56)	5.91 (4.00)	0.2	
	Light	6.06 (2.54)	4.89 (1.91)	3.66 (2.06)	3.51 (2.58)	−1.60 (2.61)	0.3	0.03
Lutein	Dark	0.83 (1.34)	0.86 (1.48)	0.24 (1.22)	1.99 (1.81)	−0.40 (3.85)	0.2	
	Light	1.57 (1.19)	1.91 (2.07)	0.78 (2.26)	0.31 (1.80)	−4.01 (2.01)	0.006	0.2
Lycopene	Dark	0.57 (1.42)	1.04 (1.45)	3.06 (1.42)	4.28 (1.81)	1.40 (2.95)	0.8	
	Light	1.72 (1.49)	3.14 (1.82)	2.26 (1.56)	3.47 (2.16)	−3.46 (2.79)	0.04	0.03
α-tocopherol	Dark	1.85 (1.02)	2.21 (1.24)	2.85 (1.40)	4.12 (2.04)	3.01 (1.34)	0.06	
	Light	2.95 (1.47)	3.17 (1.50)	2.16 (1.56)	3.10 (2.20)	1.88 (2.25)	0.3	0.4
γ-tocopherol	Dark	2.78 (1.91)	3.13 (1.86)	4.33 (1.58)	3.13 (2.44)	2.85 (2.55)	0.3	
	Light	2.01 (1.96)	2.13 (1.96)	1.39 (1.99)	3.65 (2.42)	0.20 (1.45)	0.8	0.2

assess separately the effects of temperature and light conditions. We have investigated the stability of these fat-soluble vitamins in whole blood over a much longer period than previous studies, and found them to change by less than 8% over 7 days (except for cryptoxanthin and γ-tocopherol, which changed by about 10%), even in samples stored at room temperature in the light. Retinol was the only vitamin for which a possible interaction between temperature and light effects was observed. The most statistically significant effect of temperature was found for α- and γ-tocopherol, and the most statistically significant effect of light was found for α-carotene, but the percentage change in concentration per day remained less than about 1% for α- and γ-tocopherol even at 20°C, and less than about 1% for α-carotene even in the light. The chemical structure of these vitamins, comprising conjugated double-bond systems, has been cited as the reason for their susceptibility to oxygen, light, and heat, which can cause oxidative cleavage and isomerization.[8] However, their stability during storage as whole blood in vacutainers may be explained by the lack of contact with oxygen, the presence of natural antioxidants in blood,[4] and protection of the vitamins by their biological matrices.[8] The results from this study demonstrate that fat-soluble vitamins can be measured reliably in whole blood samples kept at room temperature in the light for at least a week before plasma separation.

There are few existing studies on the stability of fat-soluble vitamins in whole blood (with the most recent reported in 1996) and their results are somewhat conflicting. Only two of these previous studies evaluated the stability of fat-soluble vitamins at room temperature or beyond 24 hours, and none compared the effect of dark versus light conditions or considered time-points beyond 72 hours. Discrepancies between previous results and those obtained in the present study may be due to differences in sample collection methods, storage conditions, or analytical methods.

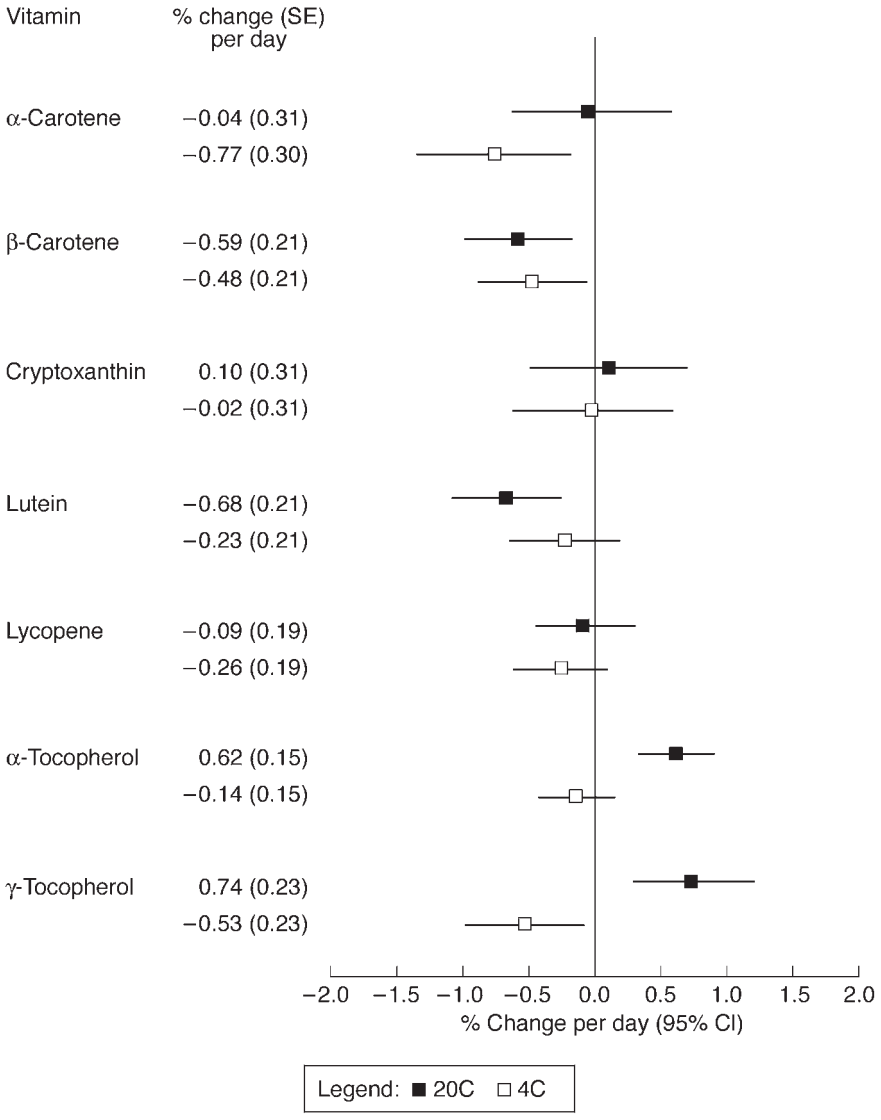

Figure 1 Percentage change per day for fat-soluble vitamins in whole blood samples stored at 20°C or 4°C for up to 7 days

In a study by Mejia *et al*. blood was obtained, using a syringe, from six individuals and aliquots were stored at 4°C.[4] Blood samples from a further six individuals were stored at 26–28°C. The samples were centrifuged and serum separated from the clot at 0, 2, 4, 6, 12, or 24 hours after collection, then analysed immediately for retinol. Retinol concentrations were quantified by UV inactivation, which quantifies retinol binding protein-bound retinol and retinyl esters together, rather than by HPLC. In a study by Peng *et al*., heparinized blood samples from two healthy individuals were stored on ice in the dark for 0, 1, 2, 4, and 24 hours before being centrifuged and the plasma analysed immediately for retinol by HPLC.[9] The results from these two studies may be subject to run-to-run variability, since samples were analysed immediately at each time-point, rather than being frozen and analysed in the same run. Both studies reported no significant change in retinol concentration over 24 hours.

Hankinson *et al*. investigated the stability of several vitamins in blood samples taken from 12 individuals into EDTA tubes and stored at room temperature (21°C) in ambient light for up to 72 hours, or placed in a Styrofoam mailer with a frozen gel pack (which maintained a temperature of 4°C for 20 hours) for up to 48 hours.[5] At each time-point, the blood samples were centrifuged, the plasma aliquoted and frozen at −80°C, and samples from all time-points were then analysed in one analytical run. They also compared the immediate separation and overnight transport of frozen plasma samples versus overnight transport of chilled whole blood samples in 16 individuals. Hankinson *et al*. found no significant change in retinol concentration in blood that was kept chilled for up to 48 hours, but a significant decrease of 5.0% per day (95% CI: −7.6%, −2.4%, $P = 0.001$) was detected at 21°C, compared with just 0.8% per day at 20°C under light conditions in the present study. Hankinson *et al*. found no significant difference in

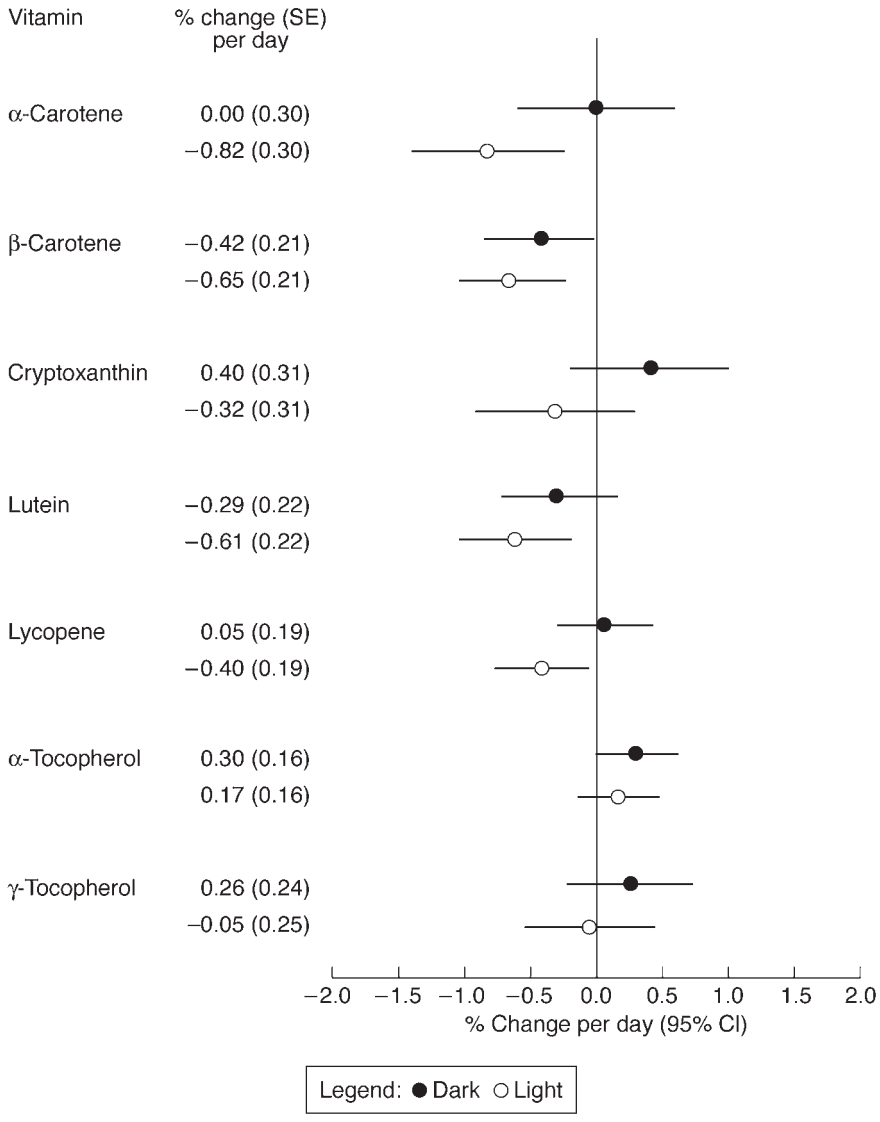

Figure 2 Percentage change per day for fat-soluble vitamins in whole blood samples stored under dark or light conditions for up to 7 days

retinol concentrations between samples transported overnight as frozen plasma, compared with samples transported overnight as chilled whole blood. They also found no significant change in α-tocopherol concentration in blood samples stored for up to 72 hours at 21°C or 48 hours chilled, although they did observe a significant decrease of 8.0% ($P = 0.01$) in blood samples that were transported chilled overnight compared with transport of frozen plasma. In the present study, a slight increase of just 0.6% per day ($P < 0.0001$) was observed in α-tocopherol at 20°C, and there was no significant change at 4°C. For γ-tocopherol, a slight increase of just 0.7% per day ($P = 0.002$) was observed at 20°C, and a slight decrease of 0.5% per day was observed at 4°C ($P = 0.03$). Hankinson *et al*. reported no significant change in β-carotene concentration in blood samples stored chilled for up to 48 hours, but did find significant decreases of 6.7% per day (95% CI: −11.8%, −1.6%, $P = 0.02$) in samples stored for up to 72 hours at 21°C, and of 17.2% ($P < 0.001$) in blood transported chilled overnight. In the present study, β-carotene was found to decrease slightly by 0.6% per day ($P = 0.007$) at 20°C and by 0.5% per day ($P = 0.03$) at 4°C. The discrepancy in results for stability of retinol and β-carotene at room temperature may be due to differences in lighting conditions, since Hankinson *et al*. stored samples in ambient light whereas samples in the present study were situated beneath lamps.

Key *et al*. evaluated the stability of various vitamins in blood samples that were drawn from 28 individuals into Safety-Monovettes containing citrate, and then stored in a refrigerator for 2, 6, or 24 hours before being centrifuged and the plasma aliquoted and frozen.[10] All samples from each individual were subsequently measured in the same analytical run. They reported a decrease in retinol concentration of 3.0% (95% CI: −5.5%, −0.4%, $P = 0.0273$) in blood samples stored at 4°C for 24 hours, but the confidence limits associated with this estimate are consistent with the results of the present study. Key *et al*. found no significant change in the concentration of α- or

γ-tocopherol in blood stored at 4°C for 24 hours, but found a significant decrease of 8.7% (95% CI: −14.9%, −2.1%, $P = 0.0123$) in α-carotene concentration, compared with a decrease of 0.4% per day observed under chilled dark conditions in the present study. In agreement with the present study, Key *et al*. found no significant change in the concentrations of β-carotene, cryptoxanthin, lutein, and lycopene.

The degree of change in concentrations may differ slightly in a real situation compared with a laboratory setting, as is suggested by some of the results from Hankinson *et al*.[5] It should be noted that 'room temperature' in the present study was defined as 20°C, which may not be realistic for studies in hotter climates (and further studies to examine the effects of higher temperatures on stability of vitamins and other analytes need to be conducted). However, since most of these vitamins were found to change by less than 8% over at least 7 days at 20°C, it might be supposed that any change at higher temperatures may remain in this range for at least 3 or 4 days (which might still be a feasible time-frame within which to transport whole blood samples to a central facility). We have speculated that some discrepancies in results between the study by Hankinson *et al*. and the present study may be due to differences in lighting conditions during storage at room temperature. If light conditions were shown to have a more deleterious effect on the stability of some fat-soluble vitamins, this should not affect the ability to measure them in mailed blood samples since they would be unlikely to be exposed to ambient light during transport. Moreover, recording the length of time from collection to separation of each sample might allow appropriate adjustment to be made in the analyses for the slight changes indicated by the present study in the measured concentrations of these fat-soluble vitamins.

In conclusion, we have shown that the concentration of many fat-soluble vitamins change by only a few per cent in whole blood stored at room temperature for up to 7 days, and may therefore be measured reliably in mailed blood samples or in other samples subject to delayed separation. These results are relevant for planning blood-based epidemiological studies and, with the availability of low-cost and reliable assays, could allow fat-soluble vitamin measurements to be included in studies from which they might previously have been excluded for practical reasons.

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KEY MESSAGES

- The perceived instability of fat-soluble vitamins has led to the general use of complex blood collection methods, which may be prohibitively expensive or impractical for large-scale epidemiological studies.
- The present study shows that blood concentrations of several fat-soluble vitamins (including β-carotene and α-tocopherol) may be measured reliably in whole blood stored at room temperature for up to 7 days.
- Fat-soluble vitamin measurements can be included in epidemiological studies from which they might previously have been excluded for practical reasons.

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