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# Analyte stability during the total testing process: studies of vitamins A, D and E by LC-MS/MS

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## Abstract

**Background:** There are limited evidence based studies demonstrating the stability of fat-soluble vitamins (FSV) measured in blood. This study aimed to examine the effects of light, temperature and time on vitamins A, D and E throughout the total testing process.

**Methods:** Four experiments were conducted. Three investigated the sample matrix, of whole blood, serum and the extracted sample, against the variables of temperature and light; and the fourth experiment investigated the sample during the extraction process against the variable of light. All samples were analysed via our simultaneous FSV method using liquid chromatography-tandem mass spectrometry technology. The allowable clinical percentage change was calculated based on biological variation and desirable method imprecision for each analyte. The total change limit was  $\pm 7.3\%$  for 25-OH-vitamin D3,  $\pm 11.8\%$  for retinol and  $\pm 10.8\%$  for  $\alpha$ -tocopherol.

**Results:** Vitamins D and E were stable in the investigated conditions (concentration changes  $< 4\%$ ) in the pre-analytical and analytical stages. Vitamin A showed photosensitivity in times  $> 48$  h with concentration changes of  $-6.8\%$  (blood) and  $-6.5\%$  (serum), both are within the allowable clinical percentage change. By contrast, the extracted retinol sample demonstrated a concentration

change of  $-18.4\%$  after 48 h of light exposure. However, vitamin A in the serum and extracted solution was stable for one month when stored at  $-20^\circ\text{C}$ .

**Conclusions:** Blood samples for vitamins D and E analyses can be processed in normal laboratory conditions of lighting and temperature. The required conditions for vitamin A analysis are similar when performed within 48 h. For longer-term storage, serum and vitamin A extracts should be stored at  $-20^\circ\text{C}$ .

**Keywords:** LC-MS/MS; light; 25-OH-vitamin D3; retinol;  $\alpha$ -tocopherol.

## Introduction

There are a plethora of clinical and translational research indications to measure fat-soluble vitamins (FSV), and each can be analysed routinely by chromatographic methods. Traditionally, vitamins A and E and more recently vitamin D have been measured simultaneously by chromatographic methods [1, 2]; hence the abbreviation FSV is used in the context of this manuscript to indicate vitamins A, D and E as vitamin K is infrequently measured directly. Currently, some FSV blood samples are treated as labile analytes, especially vitamin A (retinol). Therefore, a specific protocol for sample collection, transportation and storage is used to control several crucial factors, such as light exposure, temperature, storage conditions and time [3–5]. Although several factors are known to impact vitamin stability, precisely how they do so remains inconclusive [1].

Evidence based data of FSV stability across the total testing process is limited [1], and some studies' findings contradict each other. For instance, one study indicated that changes in whole blood retinol and  $\alpha$ -tocopherol at room temperature (RT) for 72 h decreased the levels of these vitamins [6], whilst another study reported that changes in whole blood retinol and  $\alpha$ -tocopherol at RT increased the levels of these vitamins [5]. Consequently, we conducted a systematic review of published studies, that focused on the stability of vitamins A, D and E, to fully explore the available peer reviewed literature. We examined the relevant English language articles ( $n=40$ ) [3, 5–43] and summarised the results (Supplemental Data, Appendix A).

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According to the systematic review, FSV stability in whole blood and serum has been intermittently studied over the past three decades. However, the available data does not cover stability of vitamins A, D and E simultaneously and not all factors that influence sample stability, especially during FSV extraction and post-extraction have been addressed. In addition, the methods applied did not include data to demonstrate full validation or acceptable peer reviewed comparison through external quality assurance (EQA) participation. This may partially explain some of the contradictory findings concerning FSV stability. Although two studies examined stability of vitamins A, D and E at RT for short time intervals [6]; and at  $-20^{\circ}\text{C}$  for a long storage time period [16], no study has simultaneously examined the influence of light, temperature and time on the stability of FSV across different matrices: whole blood, serum and vitamin extracts. Furthermore, no study has simultaneously investigated FSV in samples processed in ambient light at RT versus samples processed in subdued light at RT (Supplemental Data, Appendix A). More recently, liquid chromatography – tandem mass spectrometry (LC-MS/MS) has been used to evaluate the stability of plasma vitamins A and E for 48 h at “chill” temperature [7], and the stability of 25-hydroxy vitamin D3 (25-OHD3) for 24 h at RT and also for a few months at  $-20^{\circ}\text{C}$  [29]. However, there is no study that has used the advantages of LC-MS/MS technology to simultaneously examine the stability of vitamins A, D and E across the entire testing process; i.e. in various sample matrices under the influence of light, temperature and time.

This study aimed to simultaneously investigate the stability of 25-OHD3, retinol and  $\alpha$ -tocopherol: (1) in whole blood, (2) in serum, (3) during sample processing and (4) post-extraction processing (vitamin extracts) under the influence of light, temperature and storage time using LC-MS/MS for the vitamin quantification [2].

## Materials and methods

### Subjects

Three healthy volunteers (2 males and 1 female) aged (35–55 years) from our research group participated in this study. Each volunteer provided four separate blood samples. The whole blood samples were collected into four 10 mL vacutainer plain tubes (no thixotropic gel) wrapped in aluminium foil to protect from light. With the exception of experiment 1, all tubes collected were set aside to stand at RT in the dark (to allow for clotting) prior to centrifugation at 3500 rpm ( $\sim 2000\text{ g}$ ) for 20 min, followed by separation of the serum layer.

### Stability experiments

Samples of whole blood, serum and extracts were exposed to different conditions of light, temperature and time. To examine the effect of light, a group of samples was exposed to a light flux from a fluorescent lamp at 1 m distance, while another sample group was protected from the light by storing in a dark cupboard. Other storage conditions of different temperatures (RT  $23^{\circ}\text{C}\pm 2^{\circ}\text{C}$ ,  $4^{\circ}\text{C}\pm 2^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}\pm 3^{\circ}\text{C}$  and  $-80^{\circ}\text{C}\pm 3^{\circ}\text{C}$ ) over different time points (3 h, 6 h, 12 h, 24 h, 48 h, 1 week, 2 weeks and 1 month) were also examined throughout the experiments (Table 1). For each investigated condition, triplicate samples of every volunteer were used in the following experiments.

**Experiment 1:** This experiment aimed to investigate the stability of 25-OHD3, retinol and  $\alpha$ -tocopherol in whole blood. For this purpose, blood ( $\approx 20\text{ mL}$ ) was collected from each volunteer into three 10 mL plain tubes. Immediately, the blood was aliquoted (300  $\mu\text{L}$ ) into labelled polypropylene tubes wrapped in aluminium foil unless otherwise stated. All aliquots were kept standing for 90 min in the ambient dark RT condition for clotting. Later, the aliquots were categorised into four groups.





Group 1 aliquots were centrifuged for 20 min at 3500 rpm, and the serum was then transferred into new labelled polypropylene tubes prior to storing at  $-80^{\circ}\text{C}$ . This sample group was considered the control and was used to determine the baseline analyte concentration. Group 2 aliquots (not wrapped in aluminium foil) were exposed to fluorescent light at RT to examine the effects of the light at several time points: 3 h, 6 h, 12 h, 24 h, 48 h and 1 week. The group 3 aliquots and group 4 aliquots were protected from light and kept at RT and  $4^{\circ}\text{C}$ , respectively, to investigate the effects of temperature across times 3 h, 6 h, 12 h, 24 h, 48 h and 1 week. Triplicate aliquots from each volunteer sample were used at each investigated condition. After the aliquots were exposed to the target conditions, they were centrifuged for 20 min at 3500 rpm ( $\sim 2000\text{ g}$ ), and the serum was immediately transferred into new labelled polypropylene tubes prior to storing at  $-80^{\circ}\text{C}$  until they underwent analysis.

**Experiment 2:** This experiment aimed to examine the stability of the three FSV analytes in serum. The entire serum sample from each volunteer was collected in one glass bottle and then aliquoted into labelled polypropylene tubes wrapped in aluminium foil unless otherwise stated. These serum aliquots were divided into the five sample groups. Groups 1–3 were exposed to the conditions detailed in experiment 1. The sample groups 4 and 5 were protected from light and kept at  $4^{\circ}\text{C}$  and  $-20^{\circ}\text{C}$ , respectively at different storage times (3 h, 6 h, 12 h, 24 h, 48 h, 1 week, 2 weeks and 1 month) prior to storage at  $-80^{\circ}\text{C}$  until they were analysed.

**Experiment 3:** This experiment was designed to investigate the influence of light during the sample-processing (FSV extraction) on the stability of investigated analytes. The two groups (light and dark) consisted of the triple serum aliquots from each volunteer. The samples from the first group were processed under light exposure while the second group of samples were prepared under subdued light. Later, all samples from both groups were loaded into the LC-MS/MS in the same analytical batch.

**Experiment 4:** In this experiment the stability of the three analytes of interest post-extraction process (extracts) was explored.

**Table 1:** Experimental flowchart to investigate FSV stability.

Experiment	Sample processing	Investigated factors		
		Light	Temperature	Time
1	Whole blood ↓ 	Off → On	RT → 4 °C	3 h → 6 h → 12 h → 24 h → 48 h → 1 w
2	Serum ↓ 	Off → On	RT → 4 °C → -20 °C	3 h → 6 h → 12 h → 24 h → 48 h → 1 w → 2 w → 1 m
3	Extraction process ↓ 	Off → On	RT	NA
4	FSV extract ↓ 	Off → On	RT → 4 °C → -20 °C	3 h → 6 h → 12 h → 24 h → 48 h → 1 w → 2 w → 1 m

This Table shows the overall view of four experiments that were conducted to examine the stability of three FSV analytes, 25-OHD3, retinol and  $\alpha$ -tocopherol. Experiments 1, 2 and 4 examined the stability of FSV in whole blood, serum and analyte extracts, respectively, under the effect of light (off vs. on) and temperatures (RT, 4 °C vs. -20 °C) across several time points. The stability of FSV during the extraction process under the effect of light (subdued light at RT vs. light at RT) was explored in experiment 3. RT, room temperature; NA, not applicable.

All the serum samples from each volunteer were collected in one glass bottle and aliquoted into labelled glass tubes. These samples were processed according to our method's sample preparation, i.e. extraction protocol [2], and the entire extracts from all sample tubes were collected in one glass bottle prior to aliquoting into HPLC glass vials. These aliquots were categorised into five groups: group 2 (clear vials); groups 1 and 3–5 (amber vials). These sample groups were exposed to the same conditions detailed in experiment 2. The samples exposed to target conditions were immediately stored at -80 °C until analysis.

### Measurement procedure

Samples (100  $\mu$ L) were prepared using a routine liquid–liquid hexane extraction which incorporated tri-deuterated 25-OHD3 (25-OHD3-d3) and hexa-deuterated  $\alpha$ -tocopherol as the internal standards (Iso-Sciences LLC, PA, USA). A pursuit pentafluorophenyl (PFP) column (150 $\times$ 2 mm, 3  $\mu$ m) (Agilent Technology Inc., VIC, Australia), with matching guard column was used to identify the FSV including epimer of 25-OHD3 [6].

The LC–MS/MS (Agilent-1290 LC coupled with an Agilent-6490 Triple Quadrupole Mass Spectrometer, Agilent Technology Inc., VIC, Australia), method was established for the simultaneous quantification of 25-OHD3, its carbon position 3 epimer, retinol and  $\alpha$ -tocopherol. Electrospray ionisation (positive-mode) in association with multiple reaction monitoring (MRM) was utilised to quantify 25-OHD3 and its epimer (401>383), retinol (269>93) and  $\alpha$ -tocopherol (431>165). The 25-OHD3-d3 (404>386) was used as the internal standard for 25-OHD3 and retinol whilst hexa-deuterated  $\alpha$ -tocopherol (437>171) was the internal standard for  $\alpha$ -tocopherol [6].

Vitamin D was calibrated using a Recipe Calibrator set (Recipe, Munich, Germany) which is traceable to NIST-SRM972. Vitamins A and E were calibrated using the Bio-Rad Calibrator (Bio-Rad Laboratories, Munich, Germany) which is traceable to NIST-SRM968e.

Method imprecision for 25-OHD3 is 2.2%, 2.5% and 4.3% at 187  $\mu$ mol/L, 98  $\mu$ mol/L and 48 nmol/L, respectively; for retinol 3.5%, 3.7% and 5.9% at 3.5  $\mu$ mol/L, 0.5  $\mu$ mol/L and 0.2  $\mu$ mol/L, respectively; and for  $\alpha$ -tocopherol is 3.0%, 5.5% and 4.5% at 66  $\mu$ mol/L, 34  $\mu$ mol/L and 9  $\mu$ mol/L, respectively. The limit of quantitation (LOQ) was 3.4 nmol/L for 25-OHD3, 0.10  $\mu$ mol/L and 2  $\mu$ mol/L for retinol and  $\alpha$ -tocopherol, respectively. Independent ongoing peer review of this method is conducted through participation in the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) and it shows excellent method performance: imprecision (coefficient of variation, CV%) and inaccuracy (average bias) were 3.0% and 3.2 nmol/L, respectively, for 25-OHD3 based on the end-of-cycle report (cycle 41); 5.0% and 0.04  $\mu$ mol/L, respectively, for retinol; and 4.7% and 0.2  $\mu$ mol/L, respectively, for  $\alpha$ -tocopherol according to the end-of-cycle report (cycle 29) [2].

### Statistical analysis

For each subject, mean and standard deviation (SD) of the triplicate measurement of the analyte concentration at each time point was calculated. Then, the percentage concentration change of analyte in the tested samples compared to the control samples was determined. The average of mean, SD and percentage change at each time point was calculated for over all subjects.

Allowable clinical percentage change of the analyte during sample analysis was determined based on the total change limits (TCL)

as applied previously by Oddoze and colleagues for stability studies [44]. The TCL accounts for within-subject biological variation and method desirable imprecision. For each analyte TCL were calculated according to the following equation:

$$\text{TCL} = \sqrt{((2.77\text{CVa})^2 + (0.5\text{CVi})^2)}$$

In this equation the CVa represents the method imprecision and CVi is within-subject biological variation. “The factor 2.77 is derived from  $Z/\sqrt{2}$ , where  $Z=1.96$ , determined by the 95% of confidence interval value for bi-directional changes, and  $\sqrt{2}$  as we are comparing two results with the same CVa. We concluded that a mean percentage deviation  $>2.77$  CVa represents a probable difference in analyte concentration” [44]. “The imprecision of a method, for individual single and multipoint testing, should be equal or less than one-half of the average within-subject variation (CVi), and this should be the goal for short-term laboratory imprecision ( $\leq 0.5$  CVi)” [44].

The minimum method imprecision for 25-OHD3 (2.2%), retinol (3.5%) and  $\alpha$ -tocopherol (3.0%) was used as the TCL calculation for each analyte. The within-subject biological variations were taken from the Ricos Biological Variation database [45] for serum retinol (13.6%) and  $\alpha$ -tocopherol (13.8%); and from Stockl and colleagues’ work [46] for 25-OHD3 (8%). Consequently, the calculated TCL was  $\pm 7.3\%$  for 25-OHD3,  $\pm 11.8\%$  for retinol and  $\pm 10.8\%$  for  $\alpha$ -tocopherol. The statistically significant differences in analyte levels between tested samples and control samples were calculated using the two-way ANOVA (repeated measures) to check the effects of light, temperature and time in experiments 1, 2 and 4. In the third experiment, the independent t-test was used to compare the target analyte concentrations in samples processed under light versus samples processed in subdued light condition. The  $p < 0.05$  was considered as a statistically significant difference according to peer results. GraphPad Prism version 6 (GraphPad Prism Software Inc., CA, USA) was used for statistical analysis [47].

## Results

### Vitamin A

The percentage change of retinol levels in whole blood, serum and retinol extract (up to 48 h), as well as in samples processed under light were within the TCL ( $\pm 11.8\%$ ), whereas the extracted retinol exposed to light at RT for more than 48 h was greater than the TCL. Under the investigated conditions, whole blood and serum retinol level changes were between  $-6.5$  and  $4.1\%$ . Furthermore, the retinol concentration change seen in the extracted samples was  $\pm 3.3\%$  ( $p > 0.05$ ); except for those exposed to light at RT for 1 week which showed a significant decrease of  $18.4\%$  ( $p = 0.008$ ). In addition, there was a significant effect of temperature on the extracted retinol level ( $p = 0.048$ ). However, there was no significant difference in the concentration of retinol based on temperature ( $p > 0.05$ ) in whole blood and serum samples (Figure 1).

### Vitamin D

The stability of the 25-OHD3 was investigated and the concentration change from baseline under the investigated conditions were within the  $\pm 7.3\%$  of TCL. Specifically they were  $\pm 2.9\%$  in whole blood,  $\pm 3.9\%$  in serum,  $\pm 3.8\%$  in the extracts and  $2.5\%$  during processing of the sample in light compared to subdued light. Overall, there was no significant difference in concentration of 25-OHD3 based on light ( $p > 0.05$ ), temperature ( $p > 0.05$ ) and time ( $p > 0.05$ ) (Figure 2).

### Vitamin E

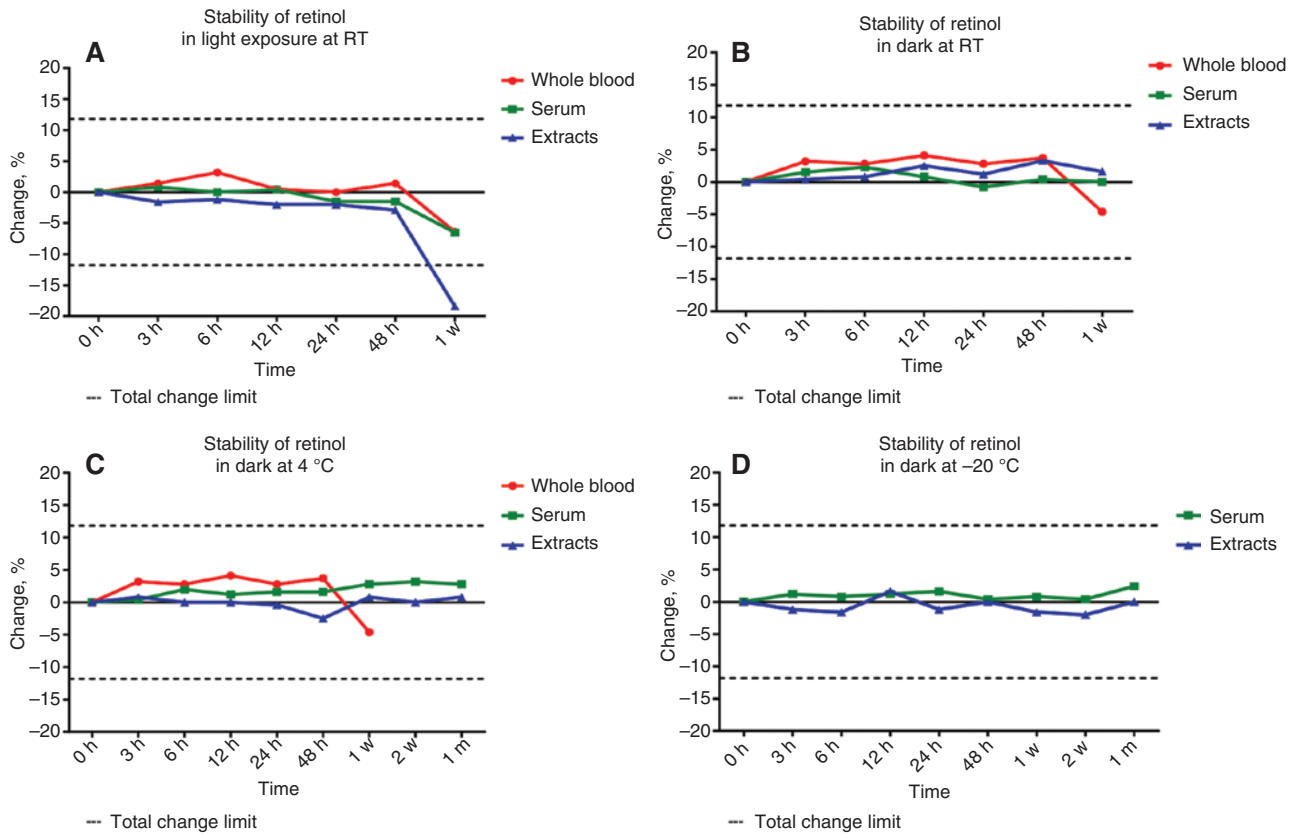
The stability of  $\alpha$ -tocopherol was investigated and the concentration change was  $\pm 3.2\%$  in whole blood, serum and extracts as well as during sample processing. These changes were within  $\pm 10.8\%$  of TCL for  $\alpha$ -tocopherol. We observed insignificant difference in concentrations of  $\alpha$ -tocopherol with light ( $p > 0.05$ ), temperature ( $p > 0.05$ ) and time ( $p > 0.05$ ) (Figure 3).

Additional details, in the form of tabulated summaries, of the results are available from the Supplemental Data, Appendix B.

## Discussion

This work successfully examined the stability of three FSV across the total testing process. We investigated their stability in whole blood, serum, during the liquid-liquid extraction process and the prepared extract. At each stage in the process we investigated the influence of light and temperature exposure over time; which was longer than reported in many previous studies. To the best of our knowledge, this work is the first study to explore simultaneously the stability of vitamins A, D and E utilising a precise LC-MS/MS method. In addition, the stability of each analyte is justified based on the calculation of acceptable clinical limits “TCL”, which reflect biological variation as well as method imprecision [44]. This valuable information provides evidence to help fill in some knowledge gaps related to vitamin analysis [1].

Recently, an evidence based guideline for the analysis of vitamins A and E across the total testing process was developed [1]; but equivalent laboratory evidence based guidelines for vitamin D do not exist. In this document, 13 substantial gaps were identified. Our study has made a significant contribution by providing evidence to fill in two of these gaps:



**Figure 1:** Stability of retinol.

These graphs show the stability of retinol in whole blood and serum under the influence of: light at RT (A), dark at RT (B), 4 °C (C) and -20 °C (D). Coloured lines show average of concentration change (%) of  $\alpha$ -tocopherol in samples, which were exposed to different conditions of light, temperature and time, from a baseline concentration in the control sample processed using standard ambient conditions. The red line represent whole blood samples while green and blue lines represent serum and sample extracts. Dashed lines represent  $\pm$  total change limits which are used to determine the acceptable clinical limits of analyte changes. The effect of light and RT on FSV stability were monitored only for 1 week, which is more than the expected time for sample storage at these conditions. Retinol concentration changes in the extracted retinol samples were  $\pm 3.3\%$  ( $p > 0.05$ ) except changes of those exposed to light at RT for 1 week that significantly decreased by 18.4% ( $p = 0.008$ ). In addition, there was significant effect of temperature on extracted retinol ( $p = 0.048$ ). However, there was no significant difference in concentrations of retinol based on temperature ( $p > 0.05$ ) in whole blood and serum samples.

Gap 1 – Pre-analytical “The stability of serum retinol,  $\beta$ -carotene and  $\alpha$ -tocopherol when stored at RT or 4 °C for short periods such as 1 to 2 weeks have not been conducted”. We can now state that retinol and  $\alpha$ -tocopherol are stable at RT and 4 °C for 1 week and 1 month, respectively, as changes for both are within the acceptable change limits.

Gap 2 – Analytical – sample preparation “Processing of the sample under normal laboratory lighting conditions”. Effects of light on retinol and  $\alpha$ -tocopherol were examined and their changes were in the acceptable limits.

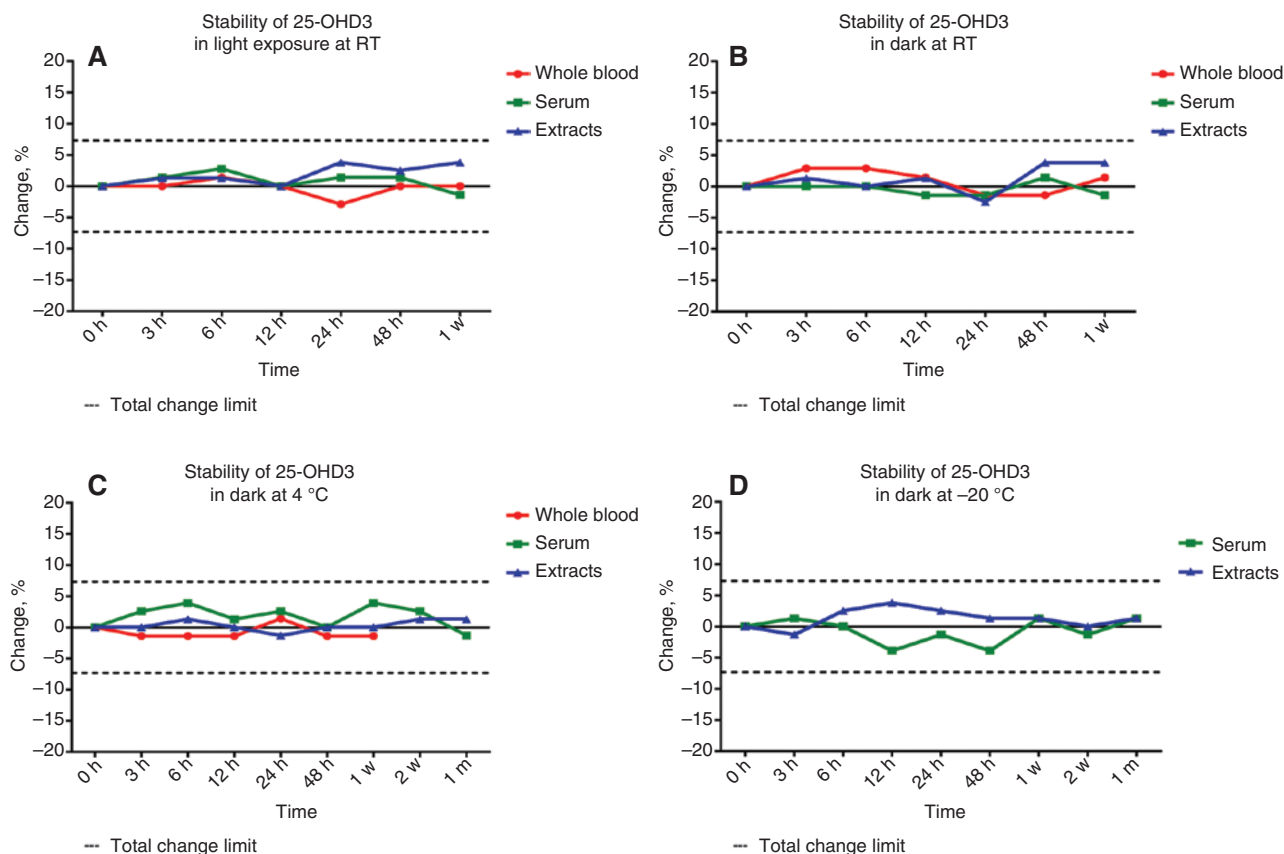
The systematic literature review highlighted 40 previous studies examining various aspects of vitamins A, D and/ or E stability. A number of these study findings were in

concordance with our stability results whilst others were not in agreement. From our four stability experiments we can now critically evaluate the previous literature using the evidence from our studies to support or refute prior findings in relation to light and temperature.

## Light

The light sensitivity of the FSV metabolites (25-OHD<sub>3</sub>, retinol and  $\alpha$ -tocopherol) was investigated in a number of studies [3, 5, 12, 15, 36, 40, 41].

**Vitamin A:** According to the current work, retinol can be reliably quantified in whole blood and serum samples exposed to light at RT for at least 1 week. Overall, retinol level changes were less than the TCL



**Figure 2:** Stability of 25-OHD3.

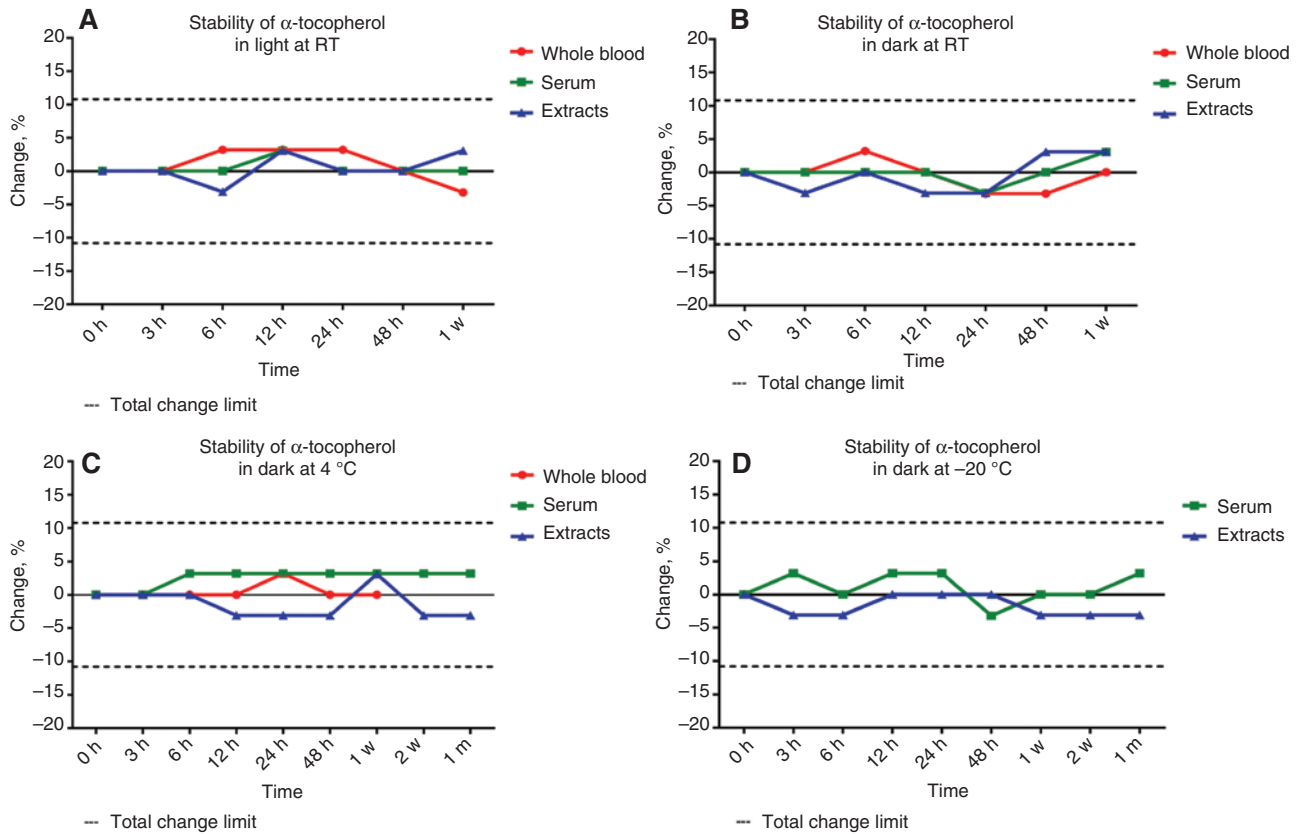
These graphs show the stability of 25-OHD3 in whole blood and serum under the influence of: light at RT (A), dark at RT (B), 4 °C (C) and -20 °C (D). Coloured lines show average of concentration change (%) of  $\alpha$ -tocopherol in samples, which were exposed to different conditions of light, temperature and time, from a baseline concentration in the control sample processed using standard ambient conditions. The red line represent whole blood samples while green and blue lines represent serum and sample extracts. Dashed lines represent  $\pm$  total change limits which are used to determine the acceptable clinical limits of analyte changes. The effect of light and RT on FSV stability were monitored only for 1 week, which is more than expected time for sample storage at these conditions. Overall, there were no significant difference in concentrations of 25-OHD3 based on light ( $p > 0.05$ ), temperature ( $p > 0.05$ ) and time ( $p > 0.05$ ).

( $\pm 11.8\%$ ). A limited number of studies investigated light impact on retinol levels in whole blood [3, 5] and serum/plasma [12, 15]. Our study results agreed with some previous observations that whole blood and serum/plasma retinol is stable in ambient light at RT for 48 h [3, 5, 12] and for 1 week [5].

It appears that retinol degradation by light is proportional to exposure time of the samples to light. Our results show that retinol levels in serum and extract exposed to light for 1 week decreased more than in those exposed to light for shorter times. However, a sharp degradation of retinol extracts (-18.4%) was observed after 1 week of light exposure compared to -6.5% in retinol whole blood and serum at the same condition. These retinol variations in different matrices may be due to the release of extracted retinol from its carriers. Retinol (molecular weight 286 Da) is

transported in the blood stream bound to the retinol binding protein ( $\approx 21$  kDa) that forms a larger complex with another protein called Transthyretin (TTR, 56 kDa). This complex ( $\approx 75$  kDa) is crucial for retinol protection and function [48, 49], and we speculate that extracted retinol might become more sensitive to light than retinol when bound to its transporters. Furthermore, our findings show no significant difference in retinol concentrations in the sample processed in ambient light at RT compared to those processed in subdued light. This is because complete sample processing usually takes  $< 3$  h; thus, light may have a limited effect on retinol degradation when exposed for a short time during sample processing.

**Vitamin D:** Our results show that 25-OHD3 in whole blood and serum was stable in ambient light at RT at least for 1 week. These results confirm the findings of



**Figure 3:** Stability of  $\alpha$ -tocopherol.

These graphs show the stability of  $\alpha$ -tocopherol in whole blood and serum under the influence of: light at RT (A), dark at RT (B), 4 °C (C) and -20 °C (D). Coloured lines show average of concentration change (%) of  $\alpha$ -tocopherol in samples, which were exposed to different conditions of light, temperature and time, from a baseline concentration in the control sample processed using standard ambient conditions. The red line represent whole blood samples while green and blue lines represent serum and sample extracts. Dashed lines represent  $\pm$  total change limits which are used to determine the acceptable clinical limits of analyte changes. The effect of light and RT on FSV stability were monitored only for 1 week, which is more than expected time for sample storage at these conditions. We observed insignificant difference in concentrations of  $\alpha$ -tocopherol based on light ( $p > 0.05$ ), temperature ( $p > 0.05$ ) and time ( $p > 0.05$ ).

previous studies, which reported that whole blood and serum 25-OHD3 was stable in ambient fluorescent light at RT [36, 50] and even in extreme exposure to direct sunlight for 4 h [40, 41]. Furthermore, we observed that light had no effect on 25-OHD3 levels during the extraction process as well as 25-OHD3 extracts stored at RT for at least 1 week. This indicates that extracted 25-OHD3 from human serum is stable under the investigated conditions, despite the fact that extracted 25-OHD3 is free from the vitamin D-binding protein.

**Vitamin E:** The  $\alpha$ -tocopherol in whole blood, serum and extract is stable for at least 1 week in light at RT based on the outcomes of the current study. This is in accordance with some previous findings regarding the stability of  $\alpha$ -tocopherol in whole blood for at least 1 week [5] and in serum for at least 48 h [12] under light at RT. Based on our results and previous study results,

$\alpha$ -tocopherol is not photo degraded, and is therefore not a photosensitive analyte when left for at least 1 week at RT under ambient light.

## Temperature

The thermostability of 25-OHD3, retinol and  $\alpha$ -tocopherol in whole blood, serum and extract was debatable because of limited or contradicted evidence (Supplemental Data, Appendix A).

**Vitamin A:** Our findings confirm that retinol can be reliably measured in whole blood samples stored at least for 1 week at RT or 4 °C. This supports some previous results of retinol change, including two studies that reported 3.3% at RT and -0.3% at 4 °C [5]. In contrast, other studies found whole blood retinol levels

decreased by -9.8% [6] and -15.5% [22] when whole blood samples were stored at RT for 72 h. Furthermore, this analyte in whole blood samples stored at chilled conditions for 48 h degraded by 8.7% [22]. This high degradation rate of retinol reported in these two studies may be due to method inter-run imprecision used in the analysis rather than retinol storage conditions.

Regarding serum retinol, our data shows that it is stable for at least 1 week at RT and for 1 month at 4 °C and -20 °C. Previously, it was reported that serum retinol was stable for 1–3 days at chilled conditions [6, 8, 22]. Furthermore, other studies found that serum retinol changes were -5% (1 week), -7% (2 weeks) and -17% (4 weeks) [13]. The results of these earlier studies, and also our current study, did not explore the mechanism of retinol degradation, hence we cannot determine if the decrease seen over time is due to a linear or exponential change.

We observed a greater percentage decrease in whole blood at 1 week of time in both the light exposed RT and dark 4 °C sample conditions. This may be related to the presence of blood cells contacted with the serum, which impact some analytes [44, 51]. The current work also investigated the impact of storage on extracted retinol for a longer time period; up to 1 week in the dark at RT, and up to 1 month in the dark at 4 °C and -20 °C. Our results show reliable stability of extracted retinol and this confirms previous findings related to stability of extracted retinol at RT, 4 °C and -20 °C for up to 48 h [12].

**Vitamin D:** Data from our study shows that 25-OHD3 is stable in whole blood, serum and extracts for at least 1 week at RT and for at least 1 month at 4 °C and -20 °C. Previous studies explored the stability of 25-OHD3 in whole blood for short times (up to 72 h) at RT, and they reported that 25-OHD3 was firmly stable [6, 36, 37, 43]. Other studies showed that 25-OHD3 in serum/plasma was stable at RT for 1–2 weeks [34, 36, 40] and for a couple of months to a few years at -20 °C [29, 34–36]. Further studies found that extracted 25-OHD3 was stable at RT for 3–7 days [29, 41].

**Vitamin E:** The current data demonstrates that  $\alpha$ -tocopherol is stable for at least 1 week at RT (in whole blood, serum and extract) and 1 month at 4 °C and -20 °C (in serum and extracts). Our results support and extend the previous findings for  $\alpha$ -tocopherol stability in whole blood, serum and extracts. According to previous studies, whole blood  $\alpha$ -tocopherol was stable for at least 48 h [6, 22] and for 1 week [5] at RT and chilled conditions. Two previous studies reported

that serum  $\alpha$ -tocopherol was stable for at least 24 h [8, 23], while another study found a change of <1% in serum stored at RT for 4 weeks [13]. Previous studies [12] reported that extracted  $\alpha$ -tocopherol was stable for at least 48 h at RT, 4 °C and -20 °C. Our results demonstrate that extracted  $\alpha$ -tocopherol is stable for a longer period of time, i.e. at least 1 week at RT and for 1 month at 4 °C and -20 °C.

Based on our studies, gaps of knowledge in relation to the sample handling of FSV across the total testing process have been addressed. With the results obtained in our four experiments, together with the information gathered from the literature, evidence based recommendations for appropriate sample handling of vitamins A, D and E, which support harmonisation efforts can now be made and implemented.

## Conclusions

Our results confirm that 25-OHD3, retinol and  $\alpha$ -tocopherol are stable in whole blood and serum samples with varying light and various temperatures (RT, 4 °C and -20 °C) and storage time. Whole blood and serum samples destined for FSV determination can be reliably processed in normal laboratory conditions of light and temperature during the pre-analytical stage. The measurements of extracted 25-OHD3 and  $\alpha$ -tocopherol can also be conducted under light at RT, while light protection for extracted retinol is recommended if analysis is performed more than 48 h after extraction.

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