A novel simultaneous quantification method for fat-soluble vitamins using liquid chromatography-tandem mass spectrometry for clinical applications

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Ali Abdulkarem Al Bahrani

BSc (Clinical Laboratory Sciences)
MSc (Medical Biotechnology)

School of Medical Sciences

College of Science, Engineering & Health

RMIT University

June 2015

Declaration

I certify that, except where due acknowledgement has been made, the work is of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Ali Abdulkarem Al Bahrani

15/06/2015

Dedication

To my wife for her faith and support throughout the PhD candidature journey

Acknowledgements

I would like to extend my sincerest thanks to my sponsor, General Administration of Medical Services-Ministry of Interior (Saudi Arabia) for offering me the opportunity to complete my PhD study. It is with sincere gratitude and pleasure that I acknowledge my senior supervisor Dr Ronda Greaves for her advice, support and immense knowledge throughout the research and writing of this thesis that would not have been completed without her help. I am extremely thankful to my supervisors Dr Peter Roche and Dr Victor Rotarou for their support, help, valuable guidance and encouragement. I would like to give special praise and recognition for Agilent Technologies, Australia for providing the Agilent LC-MS/MS 6490 system, as part of the RMIT-Agilent Clinical Biochemistry Mass Spectrometry Collaboration Laboratory, to complete this project. I would also offer my grateful thanks to Dr Chris Fouracre and MS Annabel Mitchell from Agilent Technologies for their input into the method development. I wish to acknowledge collaboration with the Sullivan Nicolaides Pathology-Sonic Healthcare (Brisbane, Queensland, Australia) on conducting the study investigated the status and correlation of vitamins D, A and E in two Australian populations at different latitudes. I also would to express gratitude to Ms Tamantha Harrower and A/Prof Greg Ward for their valuable helps in achieving the above study. I wish to acknowledge collaboration with the Barwon Infant Study (BIS) on conducting the study explored the validity of diluted umbilical cord blood plasma for analysis of fat-soluble vitamins. I extend special thanks to Dr Peter Vuillermin and Dr Fiona Collier from BIS for their valuable helps in achieving this study. I would like to thank Dr James Baglin for his guidance in the statistical analysis. I extend special recognitions to all my family members in Saudi Arabia especially my brothers and sisters and my friends in Australia for encouraging me during the doctorate program.

Table of the contents

List of Figures

List of Tables

List of Equations

Publications from this work

1. **Albahrani AA**, Rotarou V, Roche PJ, Greaves RF. (2013) Comparison of three commercial calibrators for alpha-tocopherol using liquid chromatography–tandem mass spectrometry. Clin Biochem. 2013;46(18):1884-8.

2. **Albahrani AA**, Collier F, Greaves RF, Ponsonby AL, Vuillermin P, Allen K, Ausimmune Investigator Group, Roche PJ, Clarke M. (2015) Vitamin D and A can be successfully measured by LC-MS/MS in cord blood diluted plasma, compared to serum Clin Biochem. 2015; in press (available online 25 April 2015: http://www.sciencedirect.com/science/article/pii/S0009912015001381).

Conference abstracts

3. **Albahrani AA.** (2015) Fat-soluble vitamin analysis using liquid chromatography-tandem mass spectrometry. Proceeding of Virtual Symposium on Applied Separation Science being held on May 25th-29th 2015 online.

4. **Albahrani AA**, Rotarou V, Roche PJ, Greaves RF. (2012) Comparison of commercial calibrators for alpha-tocopherol. Proceeding of AACB 50th Annual Scientific Conference 2012, Melbourne, Australia (Abstract) Clin Biochem Rev. 2012 Nov; 33(4): S1–S53

5. **Albahrani AA**, Rotarou V, Roche PJ, Greaves RF. (2012) A simultaneous fat soluble vitamin detection method using tandem mass spectrometry. Proceeding of College of Science, Engineering and Health Higher Degree by Research Student Conference 2012, RMIT University, Melbourne, Australia.

6. **Albahrani AA**, Rotarou V, Roche PJ, Greaves RF. (2013) Candidate reference method for quantification of fat soluble vitamins using chromatography-tandem mass spectrometry. Proceeding of AACB 51st Annual Scientific Conference 2013, Gold Coast, Australia (Abstract) Clin Biochem Rev. 2013;34(3):s20.

7. **Albahrani AA**, Rotarou V, Roche PJ, Greaves RF. (2014) Stability of blood fat-soluble vitamins under effects of light, temperature and time. Proceeding of College of Science, Engineering and Health Higher Degree by Research Student Conference 2014, RMIT University, Melbourne, Australia.

8. **Albahrani AA**, Collier F, Greaves RF, Ponsonby AL, Vuillermin P, Allen K, Ausimmune Investigator Group, Roche PJ, Clarke M. (2014) Vitamin D and A can be successfully measured by LC-MS/MS in cord blood diluted plasma, compared to serum. Proceeding of AACB 52nd Annual Scientific Conference 2014, Adelaide, Australia, (Abstract) Clin Biochem Rev. 2014;35(4):s44.

9. **Albahrani AA**, Rotarou V, Roche PJ, Greaves RF, Ward G Harrower T. (2014) Investigating the effect of age, gender and latitude on serum vitamin A, D and E levels measured by LC-MS/MS. Proceeding of AACB 52nd Annual Scientific

Conference 2014, Adelaide, Australia, (Abstract) Clin Biochem Rev. 2014;35(4):s44.

10. **Albahrani AA.** (2015) Fat-soluble vitamin analysis using liquid chromatography-tandem mass spectrometry. Virtual Symposium on Applied Separation Science being held on May 25th-29th 2015 online (Invited speaker). https://www.vsass.org

List of abbreviations

CLSI	The Clinical and Laboratory Standards Institute
CV	Coefficient of variation
Epi-25-OHD3	Epimer of 25-hydroxyvitamin D3
Epi-vit-D controls	In-house trilevel human serum epi-25-OHD3 controls (low, mid, high)
EQA	External Quality Assurance
FSVs	Fat-soluble vitamins
HPLC	High performance liquid chromatography
ISTD	Internal standard
1α,25-(OH)2D	1α,25-Dihydroxyvitamin D
1α,25-(OH)2D2	1α,25-Dihydroxyvitamin D2
1α,25-(OH)2D3	1α,25-Dihydroxyvitamin D3
25-OHD	25-Hydroxyvitamin D
25-OHD2	25-Hydroxyvitamin D2
25-OHD3	25-Hydroxyvitamin D3
25-OHD3-d3	Tri-deuterated hydroxyvitamin D3
LC	Liquid chromatography
LC-MS	Liquid chromatography-single mass spectrometry
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LLE	Liquid-liquid extraction
LoD	Limit of detection
LoQ	Limit of quantitation
MpA	Mobile phase A
MpB	Mobile phase B
PFP	Pentafluorophenyl
PSI	Pound per square inch
r	Correlation coefficient
RCPA	The Royal College of Pathologists of Australasia
RCPAQAP	RCPA Quality Assurance Programs
RIs	Reference intervals
S/N	Signal-to-noise ratio
SeraCon-DD	SeraCon vitamin D depleted diluent

SeraCon-DL	SeraCon II stripped delipidated serum
SMS	School of Medical Sciences
SPE	Solid phase extraction
MS/MS	Tandem mass spectrometry
TCL	Total change limits
α-Tocopherol-d6	Hexa-deuterated α-tocopherol
TEa	Allowable total error
UCB	Umbilical cord blood
VAD	Vitamin A deficiency
VDBP	Vitamin D binding protein
VDD	Vitamin D deficiency
VED	Vitamin E deficiency
Vit A/E controls	UTAK trilevel lyophilised vitamin A plus (low, mid, high)
Vit D controls	UTAK trilevel lyophilised vitamin D serum control (low, level 1, level 2)

Summary

The accurate and precise quantification of fat-soluble vitamins (FSVs), specifically vitamins A, D and E, has proved to be a challenge for clinical laboratories. This challenge is partially the result of the nature of FSV molecules and the limitations in techniques commonly used in their analysis. Consequently, there is a variation in the results obtained through different techniques or by different clinical laboratories. As a result, it has been difficult to reach agreement on the recommended levels or reference intervals of these vitamins. The current project encompassed the development of a state-of-the-art analytical method for the analysis of vitamins A (retinol), D (25-hydroxyvitamin D [25-OHD]) and E (α-tocopherol) in blood using liquid chromatography-tandem mass spectrometry (LC-MS/MS), which is a highly sensitive and specific quantification technique. Four studies were then performed to explore several scientific knowledge gaps related to vitamin measurement using the developed FSV quantification methods.

In this project, three methods were developed and validated using an in-house calibrator set, commercial calibrators and controls across two Agilent LC-MS/MS systems. The Agilent LC-MS/MS-6410 and LC-MS/MS-6490, which are the earliest and the latest (advanced) models of Agilent LC-MS/MS series, respectively, and which have different levels of analytical sensitivity, were used in the development of the quantification methods. This is important for checking the robustness of the FSV analysis using either the early model or advanced model of LC-MS/MS, which is not available for all clinical laboratories.

The first method (vitamin A/E method) was developed for the simultaneous quantification of only retinol and α-tocopherol using a C18 column, which does not have selectivity for adequate separation of epimers. The second method (FSV method-1) was for simultaneous quantification of five FSV analytes (25-OHD2, 25-OHD3, epimer of 25-OHD3, retinol and

α-tocopherol) using a pentafluorophenyl (PFP) column. Both methods were developed using the LC-MS/MS-6490 system. The third method (FSV method-2) was created based on transferring the second method to the LC-MS/MS-6410, which required further optimisation. This transference was conducted after full validation of the FSV method-1, and aimed to check the robustness of our FSV analysis, which involved not only the LC-MS/MS quantification method but also the sample-extraction protocol.

In each method, the samples were liquid-liquid extracted and injected into LC-MS/MS with ESI (positive mode) and multiple-reaction monitoring (MRM). Separation and quantification of 25-OHD3 from its epimer, and of 25-OHD2, retinol and α-tocopherol was achieved by the FSV methods 1 and 2. The reportable ranges were 4–200 nmol/L for 25-OHD3, 4–160 nmol/L for epi-25-OHD3 and 25-OHD2, 0.2–4.0 μmol/L for retinol and 6–72 μmol/L for α-tocopherol. Method validation experiments demonstrated that intra-run imprecisions (CV%) were <4.7% (25-OHD3), <6.5% (epimer of 25-OHD3), <8% (25-OHD2), <5.9% (retinol) and <5.5% (α-tocopherol) while inter-run imprecisions (CV%) were <7.8% (25-OHD3), <14.3% (epimer of 25-OHD3), <9.5% (25-OHD2), <8.6% (retinol) and <7.4% (α-tocopherol). The recoveries were between 87% and 112% for the investigated analytes. Based on reports from the external quality assurance program (the RCPAQAP), our simultaneous FSV methods displayed excellent imprecision (3.0%, 5.0% and 4.7% for 25-OHD3, retinol and α-tocopherol, respectively) and inaccuracy (average bias: 3.2 nmol/L, 0.04 μmol/L and 0.2 μmol/L for 25-OHD3, retinol and α-tocopherol, respectively). Consequently, simple LC-MS/MS methods were developed and validated for simultaneous quantification of the five FSV analytes, which were used to conduct four clinical studies.

The first study examined the effects of fluorescent light, temperature (RT, 4ºC, −20ºC) and storage time (up to 1 month) on FSV stability in whole blood, serum and extracts throughout

the sample-processing stages. All samples were analysed via the FSV method-1. The 25-OHD3 and α-tocopherol were stable under the investigated conditions (concentration changes <5.7%) in whole blood, serum and extracts. Retinol concentration changes in whole blood and serum (<7%) were within the total change limits (±11.8%) (the acceptable clinical limits) under the investigated conditions; conversely, degradation of extracted retinol was 18.1% after one week of light exposure. All investigated analytes in the serum and extracts were stable for up to one month when stored at −20ºC. Our results confirm that 25-OHD3, retinol and α-tocopherol are firmly stable in whole blood for up to week at RT and serum for up to one month at 4ºC and -20ºC under the investigated conditions. The measurements of extracts of 25-OHD3 and α-tocopherol can also be conducted under regular light at RT, while light protection for retinol extract is recommended if the analysis is postponed for more than 48 h.

The second study performed in this project investigated the deviation between the available commercial calibrators for α-tocopherol as an example of the trueness and traceability of the commercial calibrators. Three commercial single-level calibrators (Bio-Rad Laboratories, Chromsystems and RECIPE) were prepared in quintuplicate in conjunction with a seven-level in-house calibrator set for α-tocopherol. Samples were analysed by using both the vitamin A/E method and FSV method-1. The percentage observed difference for the commercial calibrators was calculated from the observed mean (±SE mean) against the given value of the calibrator: Bio-Rad (bias +1.3%, i.e. observed mean 43.6 µmol/L [±0.4] and expected 43.0 µmol/L), Chromsystems (bias +5.4%, i.e. observed mean 31.5 µmol/L [±0.3] and expected 29.9 µmol/L) and RECIPE (bias −8.9%, i.e. observed mean 51.4 µmol/L [±0.6] and expected 56.4 µmol/L). Our results demonstrated that the Bio-Rad calibrator closely agreed with the in-house calibrator set and that discordance between the commercial

calibrators was greater than the expected assay uncertainty. This lack of harmonisation means that results from different laboratories may not be comparable.

The third study aimed to ascertain the validity of umbilical cord blood (UCB) plasma diluted with RPMI 1640 medium samples for 25-OHD3 and its epimer, retinol and α-tocopherol analysis compared to UCB serum samples. Twenty UCB-matched samples of diluted plasma and serum were collected. The samples were analysed by FSV method-2 on two separate occasions. Our method demonstrated close agreement for 25-OHD3 in UCB serum versus diluted UCB plasma; the mean difference was 2.2 nmol/L (95% confidence interval [CI], −9.5 to 13.9]. Retinol was quantified in UCB serum and diluted UCB plasma; the mean difference between the results was −0.07 μmol/L (95% CI, −0.41 to 0.28). The results for epi-25-OHD3 and α-tocopherol in the diluted UCB plasma were below the limit of quantification and could not be compared with UCB serum. Accordingly, diluted UCB plasma can be used for the quantification of retinol and 25-OHD3 by LC-MS/MS. In contrast, measurement of 25-OHD3 epimer and α-tocopherol in diluted UCB plasma is not supported by this study.

The fourth study investigated the status and correlation of vitamins D, A and E in two Australian populations at different latitudes; Queensland and Victoria. De-identified serum samples were selected from routine samples delivered to Sullivan Nicolaides Pathology (Brisbane) from the Queensland (n=109) and Victoria (n=108) regions for a variety of clinical chemistry tests during the summer of 2013–2014. FSVs were analysed using our developed FSV method-2. There were no significant differences between genders in vitamins A (retinol) and E (α-Tocopherol) levels in Queensland and Victoria groups. Data of this study showed no significant effect of subjects' ages on retinol levels in QLD and VIC groups. In contrast, there was significant effect of age on α-tocopherol levels in QLD but not in VIC group. Significant differences in 25-OHD3 and retinol levels between the Queensland and

Victoria groups were observed ($p<0.003$). In the Queensland samples, the median levels were 73 nmol/L for 25-OHD3 and 3.2 nmol/L for its epimer, 2.1 μmol/L for retinol and 30 μmol/L for α-tocopherol. In contrast, the median levels in the Victorian samples were 63 nmol/L, 1.4 nmol/L, 1.9 μmol/L and 27 μmol/L, respectively. The prevalence of vitamin D deficiency (25-OHD3: <50 nmol/L) was higher in Victoria (39.8%) than in Queensland (11%). The correlation coefficients (R) were 0.4–0.6, 0.2–0.3 and 0.05–0.2 for the relationship of levels of 25-OHD3 with its epimer, retinol and α-tocopherol, respectively, in both Queensland and Victoria. Correlation of retinol levels with α-tocopherol levels was also observed (R=0.3–0.4) in the Queensland and Victoria samples. The conclusion from this study is that the two Australian populations at different latitudes have significant differences in vitamin D and A levels. Vitamin D deficiency is common in Victoria even in the summer season. This study shows that there is no strong correlation between blood levels of the investigated analytes in the two Australian populations but this does not exclude a potential correlation between the active forms of FSVs.

In summary, this translational clinical research introduced novel and precise simultaneous measurement methods for blood FSV analytes, which were applicability to clinical sampling trials. This thesis also provides the first study exploring the stability FSVs simultaneously in routine sample spectrum utilising a precise LC-MS/MS method. In addition, stability of each analytes is justified based on calculation of the acceptable clinical limits, which reflect biological variation as well as method imprecision. This project also highlighted problematic issue related to trueness and reliable traceability of commercial calibrators to high order references and that interrupts effort of method harmonisation and patient result comparability. Furthermore, the current thesis provides with first study validated the measurement of 25-OHD3 and retinol in UCB diluted plasma. Lastly, this thesis reports the first study exploring status of five FSV analytes in two Australian populations at different latitudes using

simultaneous measurement LC-MS/MS method. Furthermore, this is the first work examined the correlation between the blood FSV levels in two Australian populations.

Chapter 1

Literature review

Chapter 1 Literature review

1.1 Introduction

Fat-soluble vitamins, including vitamins A, D and E, are required for a wide variety of physiological functions. Over the past two decades, deficiencies of these vitamins have been associated with increased risk of cancer, type II diabetes mellitus and a number of immune system disorders (1, 2). In addition, there is increasing evidence of interactions between these vitamins, especially between vitamins A and D. As a result of this enhanced clinical association with disease, translational clinical research and laboratory requests for the vitamin measurements have significantly increased. These laboratory requests include measurements of 25-OHD (vitamin D), retinol (vitamin A) and α-tocopherol (vitamin E), which are the most common acceptable blood indicators for the assessment of body fat-soluble vitamin (FSV[1]) status. There are significant obstacles to precise FSV measurement in the blood. These obstacles include the physical and chemical properties of these metabolites, incomplete standardisation pillars for vitamin measurements and limitations in the techniques that are currently used for vitamin quantification. This literature review briefly emphasises metabolism, interactions and blood quantification of the three FSVs. Later in this Chapter, I present my PhD project's hypothesis and aims.

[1] The abbreviation 'FSV' is used to indicate vitamins A, D and E throughout this thesis

1.2 Fat-soluble vitamins

Vitamins are small organic compounds that are essential in very small amounts for diverse functions throughout the body; they are generally obtained from the diet. The thirteen known vitamins are divided into two classes, based on their relative solubility in water and fat. The fat-soluble vitamins include A, D, E and K; these are absorbed in the intestine in the presence of fat. Classical deficiencies of these vitamins can manifest clinically as night blindness (vitamin A), osteomalacia (vitamin D), increased oxidative cell stress (vitamin E) and haemorrhage (vitamin K).

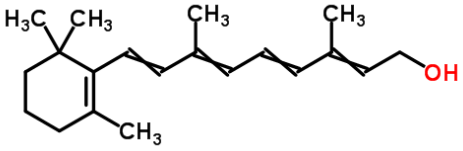
Recent studies have identified significant non-classical actions for FSVs, particularly vitamins A and D (2, 3). Deficiencies of the FSVs have been associated with serious health problems such as cancer, type II diabetes mellitus and a number of immune system disorders (1, 2). Laboratory requests for FSV measurement have significantly increased in the last decade as a result (4).

1.2.1 Vitamin A

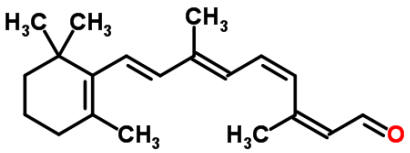
Vitamin A is essential for general physiological functions including vision, healthy epithelial tissue and infection resistance. The active forms of vitamin A are retinol, retinoic acid and retinal while the main liver storage form is retinyl palmitate (5), (Figure 1-1). Vitamin A has a hormonal role through retinoic acid, which is an active form of vitamin A; it works as an endocrine hormone and paracrine hormone. Retinoic acid is essential in embryonic stem cell differentiation and development, and in maintaining healthy structure and function in epithelial cells (6). In addition, retinoic acid may have a role in vitamin A metabolism in the liver (7). Furthermore, it is speculated that vitamin A plays roles in the regulation of macronutrient metabolisms, including carbohydrates, lipids and proteins (8). This vitamin

inhibits the growth of tumour cells *in vitro* (9) and plays a role in controlling cell division and differentiation, as well as cell apoptosis (10). Manna et al. (2000) found that all-trans-retinoic acid is involved in human lung cancer cell apoptosis (11).

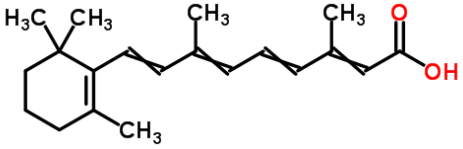
The term 'vitamin A' is used to refer to retinol and related compounds that exhibit the biological activity of retinol (12). The major source of this vitamin is dietary: either as preformed vitamin A (mainly as retinyl ester) from animal sources, or as provitamin A compounds (carotenoids, especially β-carotene) from pigmented vegetables and fruits (12, 13). Normally, 70–90% of vitamin A is absorbed in the gut in the presence of intestinal juice and bile salts; and the quantity of fat contributes to maximum absorption (14).



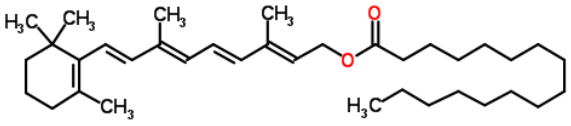
All-trans-Retinol (15)



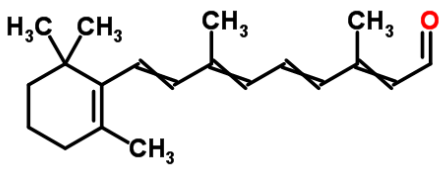
11-cis-Retinal (18)



Retinoic acid (16)



Retinyl palmitate (19)



All-trans-retinal (17)

Figure 1-1. Chemical structure of vitamin A and its derivatives.

In the intestine lumen, retinal esters are hydrolysed to retinol and free fatty acids by the brush-border retinyl ester hydrolase prior to taking up by enterocytes (small intestinal absorptive cells) (20). β-carotene is passively diffused into enterocytes; and the efficiency of β-carotene absorption is associated with the quantity of dietary fat intake (21). In enterocytes, the majority of β-carotene is symmetrically cleaved into retinal by 15,15'-monooxygenase, then converted to retinol by retinal reductase (22). Most retinol in enterocytes obtained from retinoid and carotenoid is esterified into retinyl ester by lecithin:retinol acyltransferase or acyl-CoA:retinol acyltransferase (8). Later, retinyl ester is incorporated along with chylomicrons that are secreted into the lymphatic system (23), (Figure 1-2). Small quantities of dietary retinoids are converted to retinoic acid, which is absorbed directly into blood circulation because it doesn't require a vehicle (12, 24).

About 70% of dietary retinoid is taken up by the liver, especially parenchymal cells, and then can be stored as retinyl palmitate in hepatic stellate cells (14). In parenchymal cells, retinyl ester is re-hydolysed into retinol by a number of enzymes, including retinyl ester hydrolases. Retinyl ester can be transferred to hepatic stellate cells where it is re-esterified and stored (14). The released retinol from the liver can be mediated to a variety of cells, where it is metabolised to other metabolites (such as retinal and retinoic acid) for different physiological functions (25), (Figure 1-2).

The hydrophobic nature of vitamin A means that it requires carriers for transportation. The extracellular transportation of vitamin A mainly occurs through binding with retinol-binding protein (RBP) and thyroxine binding-protein transthyretin (TTR). The retinol-RBP-TTR complex is not only essential for vitamin A solubility, but is also essential for vitamin A protection against oxidation and esterification (26, 27). Intracellular unesterified retinol is transported by binding with cellular RBP type I (CRBP-I) and cellular RBP type II (CRBP-

II). Other intracellular proteins, e.g. cellular retinoic acid-binding proteins (CRABP-I and CRABP-II), involve the transport of retinoic acid, and cellular retinal-binding protein (CRALBP) for retinal transportation (27).

The classic role of vitamin A in dim-light vision is well understood. Circulating retinol reaches the retinal pigments in the epithelial cells of the eye, where it is esterified to retinyl esters. By hydrolysis and isomerisation processes, retinyl esters are converted to 11-cis-retinol, then oxidised to 11-cis-retinal that binds with the protein opsin in the rods (i.e. the sensitive light cells that allow for dim-light vision) to form a complex called rhodopsin. When rhodopsin is exposed to a photon of light, 11-cis-retinal is isomerised to all-trans-retinal. All-trans-retinal is disassociated from the complex, and photochemical events are triggered; consequently, the brain deduces that a visual event has occurred (6).

Vitamin A also has a critical role in maintaining immunity. Vitamin A deficiency causes dryness and keratinisation in epithelial cells of the skin, the respiratory, gastrointestinal and urogenital tracts, all of which are initial preventative systems against infection. Furthermore, this deficiency disrupts neutrophil development, increases inflammatory cytokines released by macrophages, and decreases the number of natural killer cells and their lytic activity. These disruptions and changes lead to a decrease in the body's ability to eliminate infectious agents (2). As a result, communities that suffer from vitamin A deficiency may have a high infection prevalence (28).

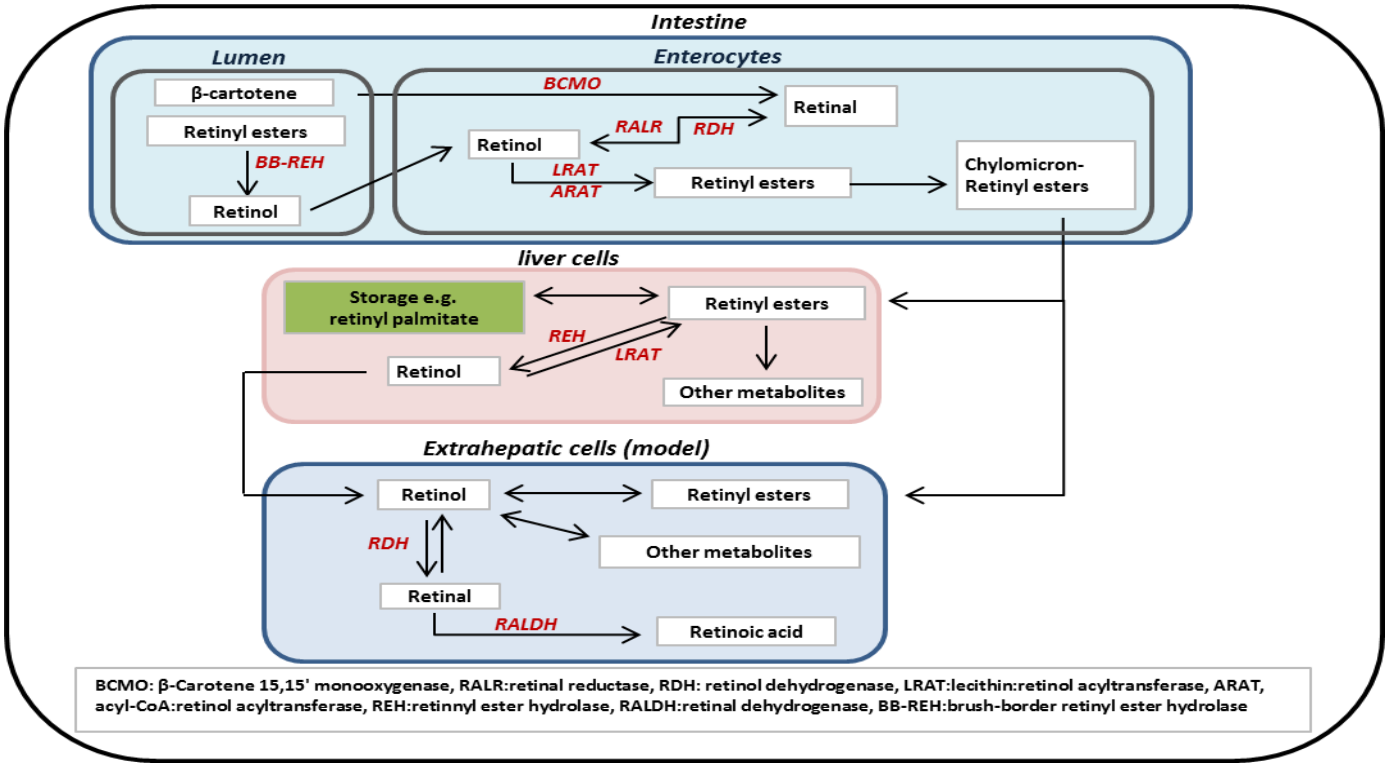


Figure 1-2. Vitamin A metabolism.

This is a general scheme for vitamin A metabolism. Dietary vitamin A (e.g., retinyl esters and β-carotene) is digested and absorbed through intestinal enterocytes by different mechanisms. In enterocytes, retinol is re-esterified to retinyl esters, which are packed with chylomicrons prior to secretion into the lymphatic system. Through blood circulation, retinyl esters are taken up by liver cells (parenchymal cells), in which retinyl esters are converted to retinol, which can be released to target organs or stored in the liver. Vitamin A is transported through binding with retinol-binding protein (RBP) and thyroxine binding-protein transthyretin (TTR) for extracellular transportation, while intracellular retinol is transported by binding with cellular RBPs (CRBPs).

Classically, vitamin A deficiency is associated with night blindness and xerophthalmia. More recently, this deficiency has been correlated with several health problems, including recurrent infections and cancer (6). The World Health Organization considers the high prevalence of vitamin A deficiency to be a serious public health problem in lower socioeconomic communities (29). About fifty per cent of preschool-aged children and pregnant mothers are at risk of vitamin A deficiency worldwide (29). The estimations of global vitamin A deficiency (<0.7 μmol/L serum retinol concentration) among preschool-aged children and pregnant women were 190 million and 19.1 million, respectively, between 1995 and 2005 (29). Most vitamin A–deficient patients suffer from chronically poor nutritional intakes that lead to the deficiency complications (29)

Biochemical markers

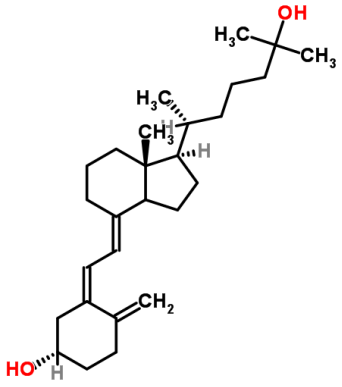
Blood retinol concentration is routinely used as a biochemical indicator for vitamin A status. The level of retinol in the blood is homeostatically regulated, and declines only when liver vitamin A storage is severely depleted. It is thus a valuable indicator of the depletion of liver vitamin A storage. In addition to retinol, other analytical tests, such as tests for β-carotene, accompanied with clinical symptoms, may be used for the diagnosis of individual vitamin A deficiency (28). Serum retinol is a reliable indicator to estimate the status of vitamin A in populations (28, 30).

1.2.2 Vitamin D

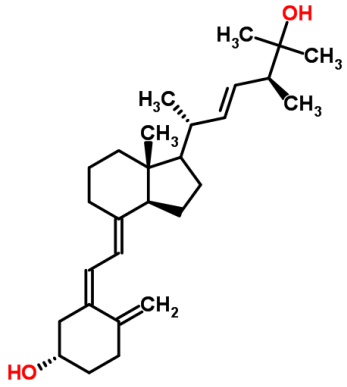
Vitamin D is a vitamin and a hormone that has been correlated with a number of significant health issues. While the most abundant form of the vitamin D is 25-hydroxyvitamin D (and its derivatives), the active form of vitamin D is 1,25-dihydroxyvitamin D3, (Figure 1-3). Historically, vitamin D was associated with the healing of rickets in children and with osteomalacia in adults (31). Low levels of vitamin D are linked with bone fractures (32) and other varieties of clinical manifestations (33). During the last two decades, a large numbers of studies have focussed on the biological roles of vitamin D. The optimum blood levels of vitamin D has been associated with the overall health of bone, skin and the cardiovascular and immune systems (3). Vitamin D has been correlated with preventive activities of cardiovascular disease and stroke (34). In addition, low levels of vitamin D were observed in patients with respiratory infections and HIV, which may relate to the role of vitamin D in immunity (35). Low levels of vitamin D and its metabolites may affect the development of breast cancer. The active form of vitamin D, (1,25-(OH)2D), and the vitamin D receptors (VDRs) have a regulatory effect on normal and breast cell growth and differentiation (36, 37). In addition, 1,25-(OH)2D has shown a role in TNF-α expression, which induces breast cancer cell apoptosis (38, 39).

Vitamin D deficiency is a public health problem in many countries (40). While it was previously thought that this deficiency was common only in countries that lacked a sunny climate for most of the year, progressive research findings have revealed that this deficiency is worldwide, with more prevalence in some ethnicities and in some geographical locations. Although Saudis enjoy a sunny climate most of the year, one study found that more than 87 of 834 healthy adult Saudi men (aged between 20–74 years) had vitamin D deficiency, especially older and obese men (41). Between 2005 and 2006, vitamin D deficiency was

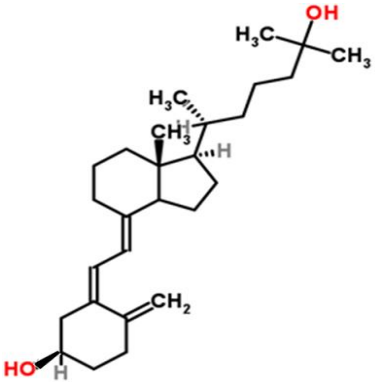
estimated among 12,862 U.S. adults (over 20 years old), with a prevalence rate of more than 41%, especially among African Americans and Hispanic Americans, with rates of 82.1% and 62.9%, respectively (42). According to the AusDiab study samples collected in 1999 and 2000 from 11,247 Australian adults (over 25 years old), vitamin D deficiency was estimated at 31 %, while vitamin D insufficiency was estimated at 73% (43). The 'Challenges' section below contains a discussion of the difficulty of determining what constitutes 'insufficient' versus 'deficient' vitamin levels.



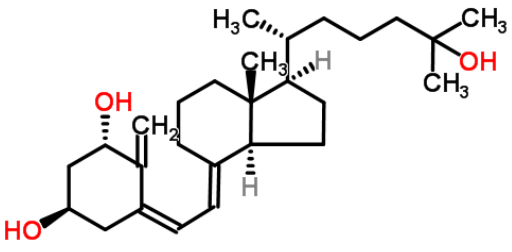
25-OHD3 (44)



25-OHD2 (45)



C3 epimer of 25-OHD3



1,25-dihydroxyvitamin D3 (46)

Figure 1-3. Chemical structure of some vitamin D metabolites.

Vitamin D metabolism

There are two main forms of vitamin D: ergocalciferol (also called vitamin D2) and cholecalciferol (also known as vitamin D3). Vitamin D2 is provided by plants that are subjected to ultraviolet irritation or dietary supplements. While a small quantity of vitamin D3 is obtained from food derived from animals, the greatest natural source of this vitamin is endogenous synthesis during sunlight exposure (47). This endogenous synthesis is influenced by skin colour, the period of sunlight exposure and the season (1, 47, 48).

The 7-dehydrocholesterol (pro-vitamin D3) is formed from the conversion of dietary cholesterol by mucosal dehydrogenase activity in the small intestine (49). The C9-C10 of pro-vitamin D3 is then broken down to form pre-vitamin D3 in the malpighian layer of the skin during exposure to ultraviolet radiation (UVR) between 280 nm and 315 nm wavelengths to synthesise endogenous vitamin D3. Unlike dietary sources, continuous exposure to UVR does not lead to vitamin D toxicity, because the excess amount of pre-vitamin D3 converts to lumisterol and tachysterol (50). This process is reversed when pre-vitamin D3 levels fall (49, 51). After that, pre-vitamin D3 is spontaneously isomerised to vitamin D3. Once synthesised, vitamin D circulates through the bloodstream by binding with vitamin D binding protein (DBP) to reach the liver (49, 51), (Figure 1-4).

In the liver, vitamin D is metabolised to 25-hydroxyvitamin D (25-OHD), also known as calcidiol, by a number of hepatic cytochrome P450 enzymes, especially CYP27A and CYP2R1 (51). After being formed, 25-OHD mediates through the blood stream to the kidneys for further hydroxylation. In the kidneys, CYP27B1 (25-hydroxyvitamin D-1α-hydroxylase) converts calcidiol to the biological active metabolite 1,25-dihydroxyvitamin D (1,25-(OH)2D), also known as calcitriol, (52). In addition, 24,25-dihydroxyvitamin (24,25-

(OH)2D) can be formed in the kidneys through the activity of CYP24A1 (25-OHD-24-hydroxylase) (49, 53), (Figure 1-4).

The 1,25-$(OH)_2$D has roles in many biological processes through binding with a vitamin D receptor (VDR), which is expressed by many cell types (54). Although its concentration in the bloodstream is estimated in picomolars, compared to nanomolars for 25-OHD, it stimulates intestinal calcium absorption, cell differentiation and insulin secretion. The interaction between 1,25-(OH)2D and VDR is essential for calcium absorption in the intestinal cells and for osteoblastogenesis (55). 1,25-$(OH)_2$D induces the maturation of preosteoclasts into osteoclasts, which have a role in maintaining calcium and phosphorus levels in the blood by removing them from the bone (52). Furthermore, by binding to VDR in some cells, 1,25-(OH)2D regulates the gene expression of upstream protein synthesis, such as osteocalcin and 24-hydroxylase, and downstream production effects, such as inflammatory markers (e.g., IL-2 and IL-12) (54). Although 1,25-(OH)2D is mainly synthesised in the kidneys under stimulation of parathyroid hormones (PTH), it causes the parathyroid gland to decrease its hormonal production and secretion (50).

When 1,25-$(OH)_2$D reaches a high level in the blood, it can trigger a negative feedback process to decrease its production and increase the synthesis of CYP24A1, which converts 1,25-$(OH)_2$D to an inactive form of calcitroic acid. Then calcitroic acid, which is water soluble, is eliminated into the bile (52). Many factors, such as serum phosphorus, calcium and fibroblast growth factor 23, have negative or positive effects on 1,25-(OH)2D synthesis in the kidneys (52).

Biochemical markers

25-OHD is considered to be the best biomarker to assess vitamin D status in the blood. This metabolite has several advantages as a biochemical indicator, including: 1) it reflects both

dietary and endogenous vitamin D; 2) it is an inactive metabolite, and is not tightly regulated; 3) the 25-OHD concentration is relatively high compared with other metabolites; for example, its concentration is 1,000 times more highly concentrated than 1,25-(OH)2D; and 4) the half-life of 25-OHD is relatively long (about three weeks). Compared to 25-OHD2 levels in the blood, 25-OHD3 is usually more abundant metabolites based on the origin of the vitamin D used during liver hydroxylation (48, 56).

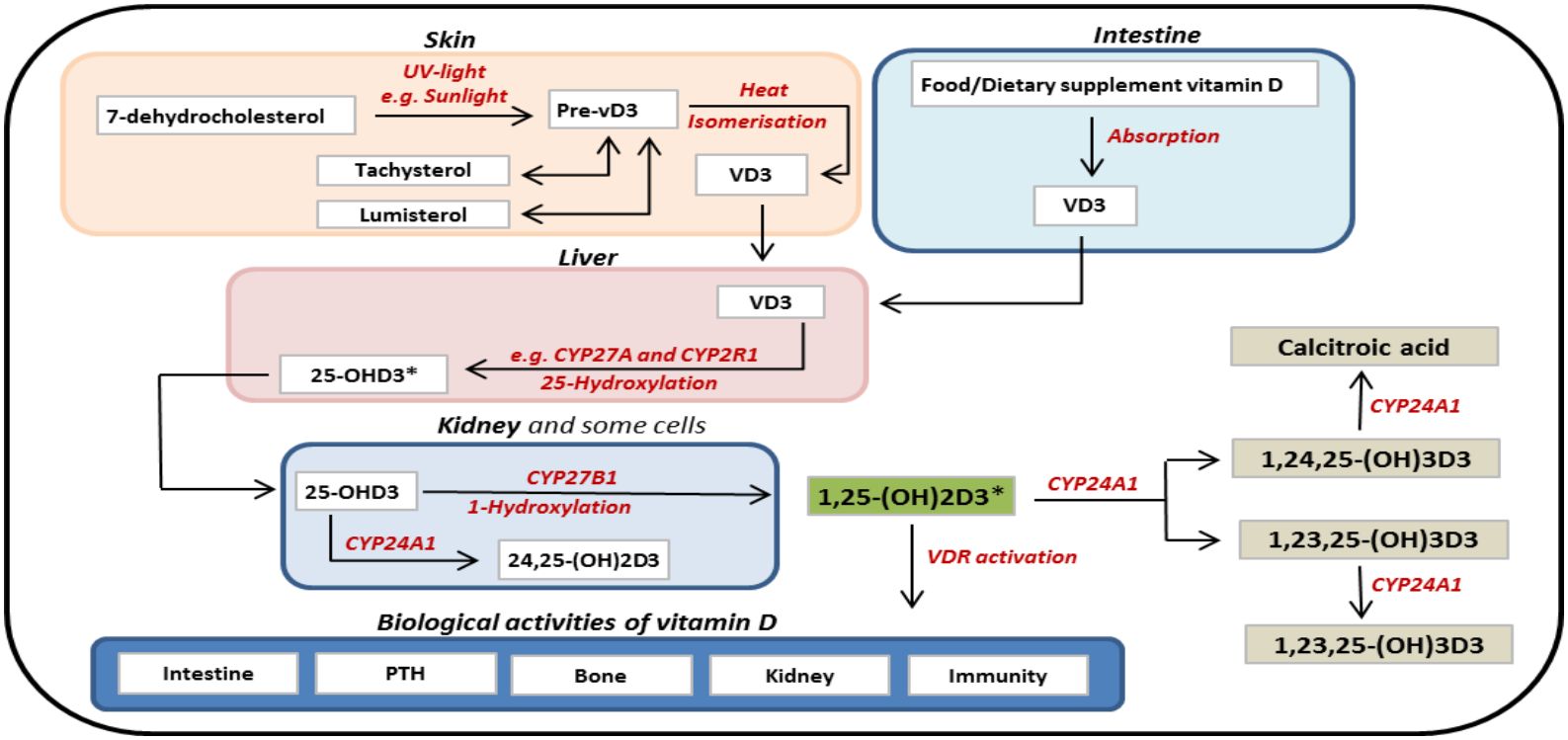


Figure 1-4. Vitamin D metabolism.

In the skin, 7-dehydrocholesterol is converted to pre-vitamin D3 under the effects of solar ultraviolet B radiation following isomarisation to vitamin D3 (VD3). Excess amounts of pre-vitamin D3 are converted to lumisterol and tachysterol to circumvent hypervitaminosis D. The VD3 is hydroxylated in the liver by cytochrome P450 enzymes (e.g., CYP27A and CYP2R1) to form 25-hydroxyvitamin D3 (25-OHD3), which is an inactive and storage form of vitamin D3. The 25-OHD3 is further hyroxylated systematically in the kidney (or locally in some cells) to 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) , which is the active form of vitamin D3), by CYP27B1. Most of the biological effects of vitamin D3 are conducted through binding 1,25-(OH)2D3 with a vitamin D receptor (VDR). The 1,25-(OH)2D3 levels might be down-regulated through its conversion to other metabolites such as calcitroic acid and 1,23,25-(OH)3D3 (52, 57). Vitamin D2 is metabolised through similar pathways.

*Although C3-epimers of 25-OHD3 and 1,25-(OH)2D3 were reported in human sera, the role of these metabolites is still not clear.

1.2.3 Vitamin E

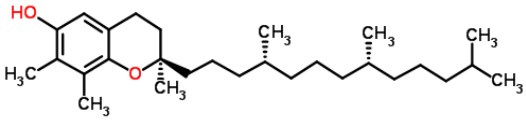
Vitamin E is a fat-soluble antioxidant metabolite and an essential dietary factor. Although the health importance of vitamin E is mostly related to its antioxidant properties, it has recently been correlated with non-antioxidant activities (58-61). Vitamin E is important for the normal morphology of erythrocytes and for slowing the aging process, since it is essential to eliminate reactive oxygen species (ROS), which are involved in cell destruction (62). Furthermore, this vitamin inhibits platelet aggregations, and therefore it may play a protective role against the atherosclerotic process and cardiovascular diseases (63-65). Proper vitamin E levels may also play a protective role against arthritis, cataracts and neurological and immunological disorders (62, 66).

Vitamin E has been associated with cancer prevention, since it is involved in a variety of biological activities, including anti-oxidation, anti-proliferation and anti-inflammation. Vitamin E plays a critical antioxidant role in protecting membrane polyunsaturated fatty acids and plasma lipoproteins from free radical attack (67). In addition, vitamin E, especially ɣ-tocopherol, plays a role in NO_2 detoxification (68). This vitamin has been linked with suppressive activates of syntheses of TNF, IL-1, IL-6 and IL-8 (61). In human breast cancer cell lines, vitamin E has shown significant apoptotic and growth inhibitory effects on the cancer cells (69, 70).

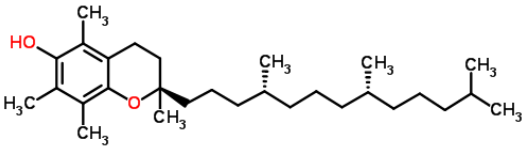
Vitamin E deficiency is rare in humans, since most food sources contain vitamin E; it is more likely to be due to genetic or malabsorption disorders, such as cystic fibrosis, chronic hepatitis and gastrointestinal disorders (58). Despite this, epidemiologically, vitamin E deficiency is more common in developing countries than in industrial countries due to inadequate vitamin intake and the high prevalence of infectious diseases that relate to oxidative stress processes, such as malaria and AIDS (66). This deficiency was estimated at

55.5% of Thai adults aged over 60 years (71). In other small-scale studies, the prevalence for vitamin E deficiency was estimated at 15.6% of Jordanian children and at 64% of urban Greeks aged over 65 years (72, 73).

Vitamin E naturally occurs in two groups: tocopherols and tocotrienols. Each have four isomers (α, β, ɣ and δ) based on the position and number of the methyl groups on the chromanol ring. In most cases, the main source of vitamin E is diets that contain excessive amounts of ɣ-tocopherol; however, α-tocopherol is the dominant form in the bloodstream and linked with many biological activities in humans and animals (68, 74), (Figure 1-5).



γ-Tocopherol (75)



α-Tocopherol (76)

Figure 1-5. Chemical structure of γ-tocopherol and α-tocopherol.

Vitamin E in humans is absorbed in the small intestine, which is enhanced in the presence of dietary fat and the food matrix (77). Intestinal absorption of vitamin E requires mixing the vitamin with micelles under the effects of biliary and pancreatic secretions for vitamin E solubility. Consequently, enterocytes passively absorb micelles, which integrate with chylomicrons, and secrete into the lymph system (63, 68, 74). Chylomicrons are enriched with cholesterol and different types of vitamin E, such as α-tocopherol and γ-tocopherol (78). In the circulatory system, chylomicrons are hydrolysed by lipoprotein lipase to mediate vitamin E to some target tissues, such as brain and muscle tissues. As a result of chylomicron hydrolysis, chylomicron remnants, which still contain vitamin E, are formed (63).

Vitamin E is mediated to the liver where only α-tocopherol is re-secreted into blood circulation, which is facilitated by the hepatic α-tocopherol transfer protein (α-TTP). This protein maintains the concentration of α-tocopherol in the blood; therefore, α-TTP gene defects are associated with vitamin E deficiency (68, 74). Blood α-tocopherol is transferred by lipoproteins such as very low-density lipoprotein (VLDL) and low-density lipoproteins (LDLs) to target tissues (79), (Figure 1-6).

Biochemical markers

The blood level of α-tocopherol is commonly used as an indicator of vitamin E status. It is speculated that α-tocopherol varies according to gender, age and the blood level of lipids, especially cholesterol. Therefore, the ratio of α-tocopherol to total cholesterol has also been suggested as a biomarker (80). Alpha-tocopherol concentration in the plasma does not reflect vitamin E intake, since α-TTP selectively re-secretes α-tocopherol to the blood (68). Practically speaking, blood α-tocopherol is an acceptable indicator of vitamin E status (66, 81).

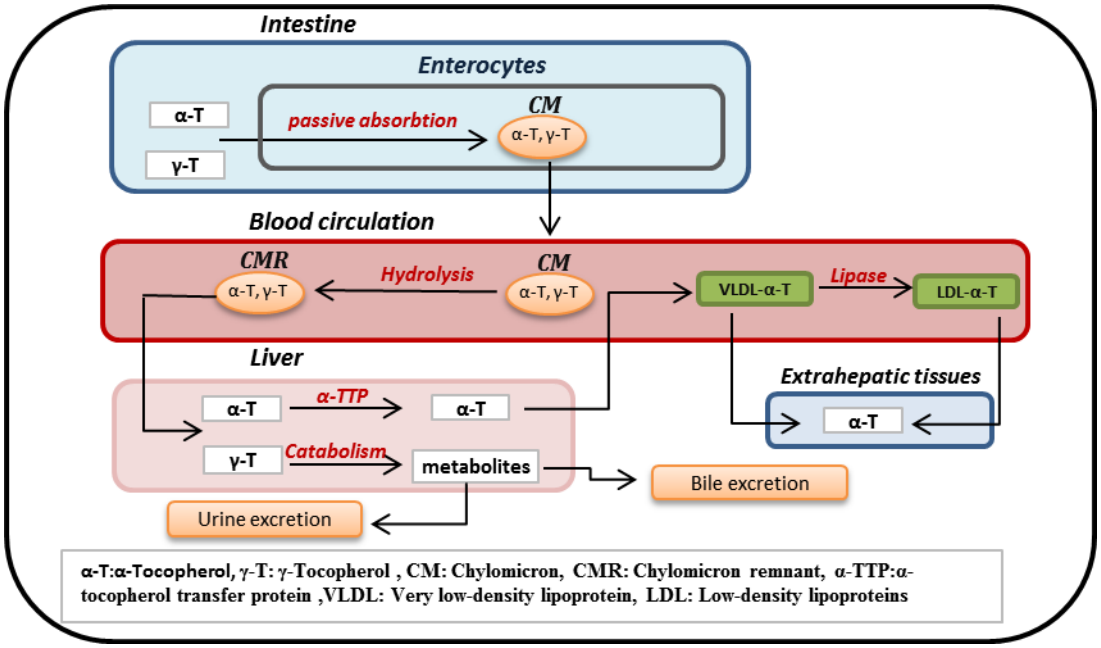


Figure 1-6. Vitamin E metabolism.

This is a general scheme for vitamin E metabolism. Dietary vitamin E (mainly γ-tocopherol and α-tocopherol) is absorbed through intestinal enterocytes. In enterocytes, γ-tocopherol and α-tocopherol and other vitamin E forms are packed with chylomicrons prior to secretion into the lymphatic system. Through blood circulation, chylomicrons are hydrolysed and chylomicrons remnants are formed. γ-Tocopherol and α-tocopherol are taken up by liver cells, although only α-tocopherol is re-secreted to the bloodstream because of the selective binding of α-tocopherol transfer protein α-tocopherol. Blood α-tocopherol is transferred to target tissues by lipoproteins such as very low-density lipoprotein (VLDL) and low-density lipoproteins (LDLs).

1.2.4 Fat-soluble vitamin interaction

The interference of vitamin A in vitamin D functions has been observed in animals and humans (82, 83). In rats, high vitamin A intake attenuated the toxicity of hypervitaminosis D (82). Based on a nested case-control study, Jenab and colleagues found that the blood level of 25-OHD3 was inversely associated with colorectal cancer among individuals who had lower retinol intake (84). Vitamin D deficiency (<50nmol/L) and high level of retinol (>2.8umol/L) have been associated with a high risk of osteoporotic fractures (85).

There is evidence that a supplement of one FSV has an impact on other FSV levels in blood. Vitamin D3 supplementation (800 IU/D for 6 months) alone or with calcium (2 g/d for 6 months) significantly increased 25-OHD3 levels by 48% and decreased α-tocopherol by 14%. Serum 25-OHD2 level decreased under the effects of vitamin D3 supplementation, however, by 48% (statistically insignificant results). Vitamin D3 supplementation, however, had no constant effects on retinol level among 85 study subjects (86).

Although FSVs are absorbed in the small intestine through different mechanisms, the absorption efficiency of one of them could be interfered with by other FSV vitamins (87). Based on experiments conducted in an *in vitro* cell line culture (Caco-2 TC7[2]), Groncalves and colleagues found that vitamin E significantly improved the absorbance of vitamin A but it significantly decreased the absorbance of vitamin D. In contrast, both vitamins A and D have negative effects on the absorbance of vitamin E. Furthermore, it was reported that vitamin A reduced both vitamin D and E uptake significantly (87). It was hypothesised that during the absorption process in the intestine, vitamin E serves as an antioxidant and protects

[2] Caco-2 TC7 is a cell line derived from colon carcinoma, but under specific conditions this type of cell resembles the enterocytes that line the small intestine. Therefore, it is mostly used to study compound absorbance in humans.

vitamin A when there is concomitant consumption of vitamins A and E. As a result, vitamin A increases at the expense of vitamin E absorption (87).

Whilst vitamins A and E have been routinely measured together, it is actually the interaction of vitamins A and D at molecular levels that is currently generating research interest in their regulatory roles in gene expression. The active form of vitamin D forms a complex with vitamin D receptor (VDR) to form heterodimer with retinoid X receptor (RXR), which triggers the gene expression process. Also, the regulation of gene expression by the active form of vitamin A requires forming heterodimer with RXR. It is worth mentioning that several nuclear receptors, including thyroid hormone receptors, can form heterodimer with RXR. High doses of vitamin A may attenuate the formation of heterodimer of vitamin D receptor and RXR. In an *in vitro* study, it was found that the heteromeric interaction of VDR and RXR was influenced by the presence of 1,25-(OH)2-D3 (the active form of vitamin D3) and inhibited by high concentrations of retinoid (88).

1.3 Quantification of blood fat-soluble vitamins

Clinical laboratory requests for FSV measurement, especially for vitamin D (25-OHD), have risen dramatically during the last decade. For example, in Australia, the number of laboratory requests for vitamin D measurement increased from 23,000 in 2000 to 2.2 million in 2010 (89). This increase is due to research findings that have found associations between FSV deficiencies and health problems. This demand for FSV analysis has highlighted the limitations of current FSV quantification methods, especially for vitamin D, and the limited success of standardisation with efforts of FSV measurement.

1.3.1 Challenges

The accurate and precise quantification of FSVs has proved to be a significant challenge for clinical laboratories. This challenge is the result of the nature of FSV molecules and their metabolites, the availability of acceptable reference materials, reference measurement procedures (reference method) and reference laboratories. Consequently, there is significant variation in the results obtained, either by different techniques or by different clinical laboratories using the same diagnostic techniques (89-91). As a result, it has been difficult to reach agreement on the recommended level of vitamins and their metabolites for healthy people. The definitions of vitamin insufficiency, deficiency and severe deficiency therefore remain unclear. There has been much debate as to whether these analytical techniques are accurate and precise enough to diagnose and monitor the pathologies associated with FSV deficiency.

Properties of fat soluble vitamins

The chemical and physical properties of FSV molecules and their metabolites are sources of analytical challenges in clinical laboratories. FSVs are small molecules (less than 500 Da),

and each vitamin has several active and inactive metabolites. The blood concentration of FSVs is relatively low and varied, ranged from nmol/L level for vitamin D to μmol/L level for vitamins A and E (92, 93). Adding to the challenge is the fact that the metabolite concentrations of one vitamin may also vary; for example, the 1,25-(OH)2D3 concentration is 1,000 times less concentrated than 25-OHD3 concentration. Consequently, the specificity and sensitivity of techniques used in detection of these metabolites are critical for accurate and precise measurement. Another problem related to this issue is that the majority of these vitamin metabolites are hydrophobic compounds and are mediated in the blood by binding with relatively large proteins (for instance, VDBP is around 50 kDa). The dissociation of a vitamin from its binding protein is therefore essential before vitamin measurement. This step may significantly contribute to the technique's sensitivity and specificity (89, 94).

Stability

The stability of FSV in blood, especially vitamin A (retinol) and vitamin E (α-tocopherol), represents a gap in our knowledge. Although several factors, such as sample storage and transportation, are known to have an impact on vitamin stability, precisely how they do so remains inconclusive. Currently, FSVs in blood samples are treated as labile analytes, especially in the cases of retinol and α-tocopherol. As a result, a specific protocol for sample collection, transport and storage is used to control several crucial factors, such as light exposure, temperature, storage conditions and time (95-97).

The data on FSV stability is limited, and some studies' results contradict each other. For instance, one study indicated that changes in whole blood retinol and α-tocopherol at room temperature (RT) for 72h were -9.8% and -1.0%, respectively (98). Another study reported that changes in whole blood retinol and α-tocopherol at RT after 1 week were 1.8% and 4.8%, respectively (97). Because of the limited availability of FSV stability data, every

clinical laboratory has its own procedure for dealing with blood samples, from patient sample collection to sample analysis. This contributes to the variation in results obtained by clinical laboratories.

Standardisation

The standardisation of metabolite analysis, including FSVs, in laboratory medicine has become critical for clinical decisions and for health care improvement. Analytical results from patient samples are essential for clinical decisions, which usually need to compare previous results with current analyses, either for diagnosis or for treatment. In practice, there are variations in the results obtained, even using the same measurement technique for the same patient sample (66, 91). This poor comparison between analytical results is in part due to the limited succeeded efforts of standardisation (99), which not only affects daily patient care but also affects population-based vitamin deficiency assessment and the determination of common reference intervals. In addition, the limited success of standardisation may cause misinterpretations or contradictions of research results performed in different geographical places and/or at different times. Standardisation thus would help significantly in considering analytical results regardless of time, location or the measurement system used to obtain the results (100).

The standardisation of analyte measurement relies on five main mainstays: 1) reference measurement procedure (RMP) (reference method), 2) reference materials, 3) reference laboratories, 4) reference intervals and 5) external quality programs. The RMP is the procedure used to assign and certify value to a reference material as a primary calibrator (pure analyte) or as a secondary calibrator (analyte in human clinical samples). In industrial contexts, this certified reference material can be used to assign values to a commercial calibrator. Routine laboratory medicine later uses validated commercial calibrators to

measure analytes in human samples. Analytical results obtained from routine laboratory tests are therefore traceable to certified material (99, 101). Although standardisation efforts made, mainstays required for standardisation of FSV measurement are still in completed.

At the beginning of this project, the majority of FSVs had neither RMPs nor reference materials based on the Joint Committee for Traceability in Laboratory Medicine (JCTLM) listings. The National Institute of Standards and Technology (NIST) has introduced standard reference materials (SRM 968) for FSV, and SRM 972 and SRM 2972 for 25-OHD2 and 25-OHD3, since 1989 and 2009, respectively (94, 102). None of them, however, was recognised as a reference material by JCTLM at the beginning of this project. More recently, the isotope dilution liquid chromatography-mass spectrometry method has been recognised as an RMP for 25-OHD2 and 25-OHD3 by JCTLM (103). However, there are no RMPs for vitamins A and E that have been recognised as reaching the JCTLM requirements (103). The JCTLM database previously recognised NIST SRM 972 for vitamin D (104) and recently SRM 968e for vitamins A and E (105) as reference materials.

Individual commercial calibrators for FSVs are currently available from a limited number of manufacturers. These commercial calibrators are traceable to available NIST reference materials for FSVs. Theoretically, the availability of commercial calibrators is crucial for minimising variation in patient results, as most clinical laboratories use commercial calibrators, which are traceable to high-order references. However, these manufacturers do not provide their customers with details of the method performance used in the traceability process and how the calibrator matrices are prepared. Both method performance and matrix treatment can affect the patient results obtained using these calibrators (106-112). Questions have therefore been raised regarding the trueness and traceability of commercial calibrators.

1.3.2 Current platforms used in analysis

Immunoassay and high performance liquid chromatography (HPLC) are the most common laboratory diagnostic tools used in blood FSV measurement. For vitamin D measurement, automated immunoassays from different manufactures are the dominant systems in clinical laboratories, despite the fact that HPLC and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are more accurate (90). The automated systems have a higher throughput, faster turnaround time and easier operating and troubleshooting abilities. In contrast, unsatisfactory accuracy and precision have been reported in various automated immunoassays (56). In addition, most immunoassays are unable to determine concentrations of 25-OHD2 and 25-OHD3 individually (89). This problem may be related to the specificity and sensitivity of antibodies targeting a small molecule (a vitamin) binding with a large molecule (a vitamin-binding protein) in the presence of serum matrix interferences. While HPLC is widely used to detect other FSVs, it is unable to detect coeluted compounds, which escape from the chromatography at the same time. Liquid chromatography coupled with tandem mass spectrometry quantification can precisely quantify each of these vitamins, including the separation of epimer of 25-OHD3, which recent studies have highlighted.

Liquid chromatography mass spectrometry was introduced to clinical chemistry as an emerging technique in the late 1990s. It has recently been considered to be a stronger competitive technique over other methods such as immunoassay (89). The LC-MS system is based on coupling liquid chromatography (LC), an eluting power used for physical analyte separation, with mass spectrometry (MS), which is a highly metabolite-selective detector. More recently, large- and medium-sized clinical laboratories have used the LC-MS system for drug monitoring, newborn screening and endocrinology and metabolism (113).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) uses multiple mass quadrupole spectrometers (114). In practice, LC separates analytes from the sample matrix, which will then be charged through the ionisation process before undergoing MS. In the MS, charged molecules (precursor ions, also called 'parent ions') will be detected according to their mass-to-charge ratio (m/z) in the first quadrupole (Q1). The precursor ions are fragmented in the collision cell (Q2) by the collision between these ions and pure gas (e.g., nitrogen gas). For further selection, fragmented molecules (product ions) can be detected by the second quadrupole (Q3). The effectiveness of the high sensitivity and selectivity of LC-MS/MS is based on its ability to measure concentrations of the most sensitive transition ions (the transition from precursor ion to its product ion), which are proportional to the concentration of the target compound in the sample (113), (Figure 1-7).

With these advances in technology, LC-MS/MS can now be used to create highly specific and sensitive methods to simultaneously measure the majority of FSVs in one patient sample. This should bring with it an improvement in patient management and outcomes, as well as providing more opportunity to understand the relationships between blood vitamin levels. Despite the advantages of the LC-MS/MS, several variables affect LC separation, such as mobile phase (composition, flow rate and gradient and isocratic time) and column (size, type, temperature and pressure). Other influences affect MS analysis, such as the voltages applied in quadrupoles and the collision cell. As a result, LC-MS/MS system parameters have to be optimised to detect the most sensitive transition for each analyte (115). Furthermore, many method validation processes and method performance criteria must be considered during the course of method development (81, 116-118).

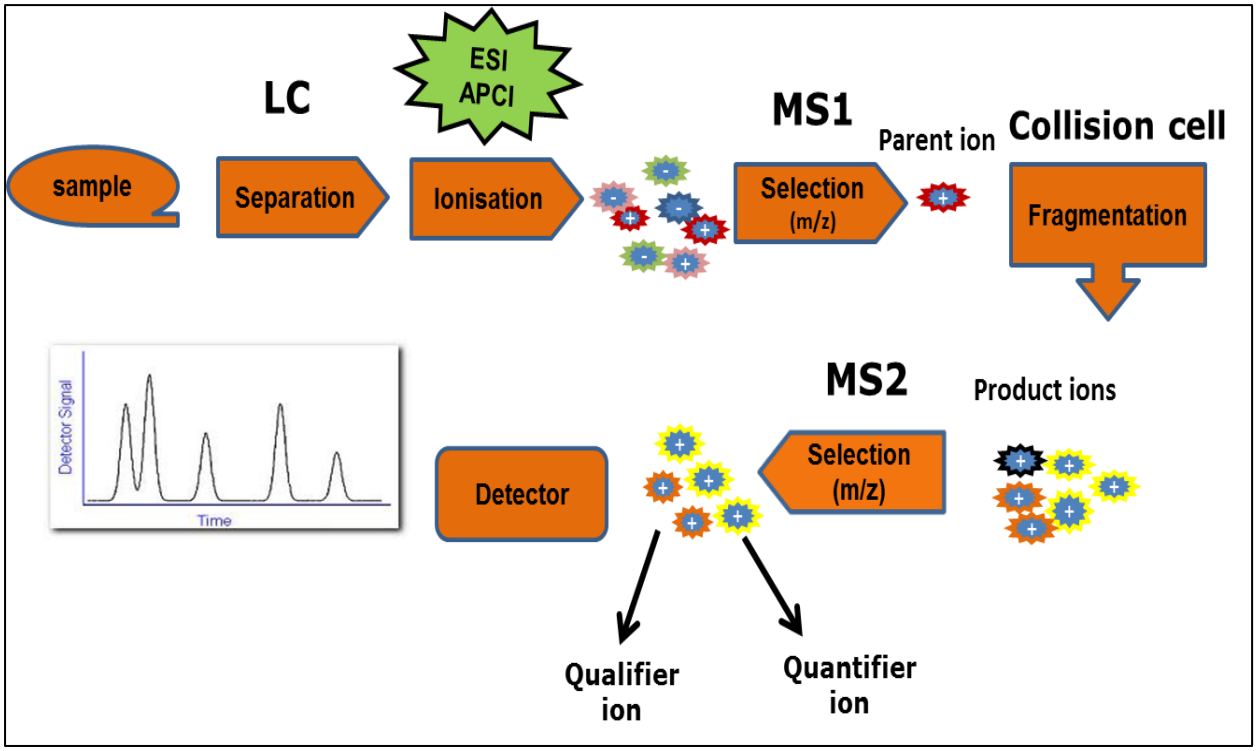


Figure 1-7. Schematic of liquid chromatography-tandem mass spectrometry.

LC separate analytes, from sample matrix, that will then be charged through the ionisation process before LC separate analytes, from sample matrix, that will then be charged through the ionisation process before undergoing MS. In the MS, charged molecules (parent ions) will be detected according to their mass-to-charge ratio (m/z) in the first quadrupole (Q1). The parent ions are fragmented in the collision cell (Q2) by the collision between these ions and high purity gas (e.g., nitrogen gas). For further selection, fragmented molecules (product ions) can be detected by the second quadrupole (Q3).

1.4 Current project

The broad aim of this project is to contribute the efforts of standardisation of FSV measurement and exploring and cover some knowledge gaps related to FSV. The development and full validation of a highly sensitive and precise method for simultaneous FSV quantification using the advanced technology of LC-MS/MS is a key to FSV measurement standardisation. Such a precise simultaneous measurement method using one patient sample helps to minimise possible results variation in investigated analytes that could be observed when two methods / techniques and samples are utilised. In addition, the developed method helps to investigate some knowledge gaps surrounding FSV measurement, stability and interaction.

Therefore, the hypothesis of the thesis is that blood fat-soluble vitamin levels and interactions can be effectively evaluated by a robust, simultaneous quantification method. Accordingly, the specific aims of this thesis are: 1) to develop and validate a simultaneous measurement method for FSV using LC-MS/MS and apply for clinical studies as part of standardisation efforts for FSV measurements; 2) to determine the influence of light, temperatures and time pre-analytically on blood FSV stability; 3) to investigate the trueness and traceability of commercial α-tocopherol calibrators as examples of commercial calibrators; 4) to examine the influence of diluent (RPMI-1640) on cord blood FSV measurement; and 5) to study the status and correlation of FSV in two Australian populations located at different latitudes (Queensland and Victoria).

Chapter 2

General Materials and Methods

Chapter 2 General Materials and Methods

This chapter describes general equipment, tools and chemicals used in this project. This also includes the procedures utilised for preparing reagent stock and samples for the general studies conducted in the current project. An additional, methodology section is included in each chapter to detail specific material and procedures used for the study.

2.1 Equipment and tools

A variety of general equipment and tools were utilised for this work. These included; Agilent LC-MS/MS 6490 system (Agilent 1290 infinity LC and Agilent 6490 triple quadrupole mass spectrometer) and Agilent LC-MS/MS 6410 system (Agilent 1200 infinity LC and Agilent 6410 triple quadrupole mass spectrometer) from Agilent Technologies (VIC, Australia); Heraeus centrifuge Multifuge 1 S-R from Thermo Scientific (VIC, Australia); Dry block heater, 3x4 nozzle gas manifold, roller mixer and vortex mixer from Ratek (VIC, Australia); temperature adjustable laboratory oven from Thermoline scientific (NSW, Australia); a set of pipettes from Thermo Scientific Finnpipette (VIC, Australia); water purification system (Milli-Q Direct 8) from Merck Millipore (VIC, Australia); electronic balance (Mettler AT261 Deltarange®) from Mettler (VIC, Australia); and glassware from Schott-Duran (NSW, Australia).

The columns used are: Pursuit Pentafluorophenyl (PFP) column (150 mm × 2 mm× 3 µm, Cat no. A3051150X020), MetaGuard 2.0 mm Pursuit 3u PFP (Cat no. A3051MG2), Varian Pursuit XRS C18 column (20 mm × 2 mm× 3 µm, Cat no. A6001020X020), Varian MetaGuard 2.0 mm Pursuit XRs 3 µm C18 (Cat no. A6001-MG2), amber vials (screw cap) (Cat no. 5183-2081) and flat bottom glass inserts (400 µL, Cat no. 5181-3377) were purchased from Agilent Technologies (VIC, Australia). KIMAX glass tubes (13 × 100 mm,

Cat no. 45066A-13100) were supplied from Pacific laboratory products (Melbourne, Australia). Microcentrifuge propylene tubes (1.5mL, Cat no. 72.690.001) were supplied from Sarstedt (Adelaide, Australia). Glass Pasteur pipettes (Cat no. NAAU92501.01) were purchased from Merck (VIC, Australia).

2.2 Reagents and chemicals

The reagents and chemicals used in this project were purchased from different vendors and manufacturers. The following were purchased from Merck (VIC, Australia): ethanol gradient grade for liquid chromatography (Chemical Abstracts Service number [CAS No] 64-17-5, Cat no. 1.11727.1000), hexane for liquid chromatography (CAS No. 110-54-3, Cat no. 1.04391.2500), 2-Propanol for liquid chromatography (CAS No. 67-63-0, Cat no. 1.01040.2500), methanol for liquid chromatography (CAS No. 67-56-1, Cat no. 1.06018.4000), nitric acid 65% suprapur (CAS No. 7697-37-2, Cat no. 1.00441.1000).

Formic acid (purity ≈98%, CAS No. 64-18-6, Cat no. 94318), retinol (purity ≥95%, CAS No. 68-26-8, Cat no. R7632) and α-tocopherol (purity ≥96%, CAS No. 1019-41-0, Cat no. T3251) were obtained from Sigma-Aldrich (VIC, Australia). 25-Hydroxyvitamin D2 (purity ≥98%, CAS No. 21343-40-8, Cat no. S4176UNL), 25-hydroxyvitamin D3 (purity ≥98%, CAS No. 19356-17-3, Cat no. S4163UNL), 3-epi-25-hydroxyvitamin D3, (purity ≥98%, CAS No. 73809-05-9, Cat no. S7004), 25-hydroxyvitamin D3-[$^{2}H_{3}$], (purity ≥98%, CAS No. 140710-94-7, Cat no. S4163), α-tocopherol-[$^{2}H_{6}$] (purity ≥98%, CAS No. 113892-08-3, Cat no. 10097) and retinol-[$^{2}H_{5}$] (purity ≥98%, Cat no. LN8-2011-256A1) were purchased from IsoSciences (PA, USA). Retinol acetate (CAS No. 127-47-9, Cat no. V676000) was sourced from TRC (Ontario, Canada). The nitrogen gas cylinder (N5.0 [Purity ≥ 99.999%], CAS No. 7727-37-9) was supplied by Coregas (VIC, Australia).

Lyophilised calibrators and controls as well as frozen matrix material were utilised in the project. Lyophilised vitamins A and E calibrator (Cat no. 195-5878) used was obtained from Bio-Rad Laboratories, (Munich, Germany). Lyophilised serum calibrator set for 25-hydroxyvitaminD2/D3 (Cat no. MS7013) and for vitamins A and E (Cat no. 22013) were purchased from RECIPE (Munich, Germany) supplied by PM separations, QLD, Australia. Furthermore, lyophilised serum calibrator for vitamins A and E (Cat no. 34.004) was sourced from Chromsystems Diagnostics (Munich, Germany). Serum quality control (lyophilised) sets for vitamin A and E (low-mid-high, Cat no. 8898-8900) and for vitamin D (low-mid-high, Cat no. 10060- 10062) were procured from UTAK Laboratories Inc (CA, USA). SeraCon II stripped delipidated serum (Cat no. 22011-100) and SeraCon Vitamin D depleted diluent (generated from human plasma, Cat no. 502079-100) were from SeraCare Life Sciences (MA, USA) and were generously donated from Abacus (ALS) (QLD, Australia).

2.3 Safety

The biological specimens, chemicals and medical waste were handled based on the laboratory safety procedures. The specimens used included unknown human samples, calibrators, quality controls and external quality assurance material (from The Royal College of Pathologists of Australasia Quality Assurance Programs [RCPAQAP]). These biological samples were considered to be potentially infectious agents; therefore, they were handled according to the School of Medical Sciences (SMS) laboratory safety procedures that are available online and as hardcopies in the liquid chromatography-tandem mass spectrometry (LC-MS/MS) laboratory. Chemicals were handled safely according to material safety data sheet (MSDS) procedures and risk assessments, which were created at the commitment of the project, are available as hardcopies in the MSDS file in the LC-MS/MS laboratory. Medical wastes (including biological and chemical waste) were discarded according to the laboratory waste disposal management strategy procedure of the SMS (119).

2.4 Glassware cleaning

Glassware was maintained clean and regularly washed according to the mass spectrometry and good laboratory practice requirements. All glassware was cupped and stored in the allocated cupboard after being cleaned.

Bottles were washed with a sufficient amount of Milli-Q water prior to drying them in a lab oven at 50ºC to avoid diluting the nitric acid in the next step. Later, under a fume hood, a amount of 65% nitric acid was poured into the bottles using a funnel, and then the bottles were capped and rolled for at least 10 min to ensure the nitric acid covered the entire internal surface of each bottle. The nitric acid was then poured back into the nitric acid bottle for further use, and the bottles were rinsed with Milli-Q water five times, then filled with Milli-Q water and left overnight. They were then rinsed with Milli-Q water two times prior drying them in the oven at 50ºC.

KIMAX screw cup culture glass tubes were extensively used in this project for sample preparation. Two glass tube sets were used in every sample preparation batch; a set for protein precipitation and liquid/liquid extraction and another set for organic layer evaporation. The first tube set was washed with Milli-Q water until all specimen residues were gone. A glassware brush was used when required, and the tubes were then dried in the lab oven at 50ºC. The second tube set was rinsed with a small volume of methanol then dried. Under the fume hood, both tube sets were treated with amount of 65% nitric acid, and the tubes were left to stand for 10 min. The nitric acid was then poured back into the nitric acid bottle for further use. The tubes were rinsed with Milli-Q water five times, then filled with Milli-Q water and left overnight. They were then rinsed with Milli-Q water two times and dried using the lab oven at 50ºC.

2.5 Chemical preparation

2.5.1 Preparation of deoxygenating solvents

Solvent deoxygenating is a process of substituting dissolved oxygen in the solvent with nitrogen gas to minimise the potential oxidisation of labile compounds when dissolving in the solvent. Deoxygenated ethanol and methanol were used to prepare vitamins A, D and E stocks as well as internal standard stock solutions. The solvents were deoxygenated as follows:

A total of 250 mL of ethanol\methanol was poured into a glass bottle (500 mL) under a fume hood. A Pasteur pipette (250 mm) was connected to the nitrogen gas cylinder using a hose with a 0.25 µm filter; and then the nitrogen gas cylinder valve and gas regulator valve were carefully opened at a low flow rate. Later, the Pasteur pipette was immersed in the solvent for at least 10 min while gently moving the pipette. After that, the cylinder and regulator valves were closed; and the glass Pasteur pipette was taken off prior to tightly capping the solvent bottle.

2.5.2 Preparation of solutions for liquid chromatography

Needle wash solution

Needle wash solution was used to wash the auto-sampler needle prior to each sample injection to minimise the potential carryover. The needle wash solution consisted of 40% water, 10% 2-propanol and 50% methanol. The solution was prepared as follows:

A total of 400 mL of Milli-Q water was mixed with 100 mL of 2-propanol in the graduated cylinder (1 L). Then 500 mL of methanol was gradually added followed by mixing. The prepared mixture was poured into a labelled clean brown glass bottle. This solution was made fortnightly.

Mobile phases

The principle of reversed-phase chromatography with two mobile phases was utilised for the target analyte separations. A half litre of mobile phase A (MpA) and one litre of mobile phase B (MpB) were prepared weekly. Based on our observation, analyte peak resolutions were negatively affected by mobile phase age and weekly preparation was a suitable time interval. Two labelled bottles were reserved for each mobile phase and washed weekly with Milli-Q water and monthly with 65% nitric acid, as detailed in section 2.4.

The hydrophilic MpA consisted of 0.1% formic acid (to enhance molecule ionisation) and 2% methanol (to minimise the potential of microbial contamination) in Milli-Q water. This mobile phase was prepared by adding 250 mL of Milli-Q water into the graduated cylinder (1 L) before adding 500 μL of formic acid and then 10 mL of methanol. After mixing this solution, the graduated cylinder was filled to 500 mL with Milli-Q water and then mixed again. Later, the mixture was transferred into a labelled glass bottle (500 mL).

MpB consisted of 0.1% formic acid in methanol. It was prepared by adding 500 mL of methanol into the graduated cylinder (2 L) prior to adding 1 mL of formic acid and mixing. Later, the graduated cylinder was filled to 1 L of methanol, followed by a mixing. The mixture was then transferred into the labelled glass bottle (1 L).

2.5.3 Reagent preparation

All reagents were prepared in amber vials or in glass bottles wrapped with aluminium foils under the fume hood in subdued light. For more accuracy, all reagent stocks were gravimetrically prepared using calibrated electronic balance[3] based on the below equation unless otherwise stated

$$\boldsymbol{Mass\ (g) = Volume\ (L)\ \times Density\ (\frac{g}{L})}$$

These stocks were used to prepare the FSV mixture, the in-house calibrator sets and the spiked serum unless otherwise stated.

25-Hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 stock solutions

The 25-hydroxyvitamin D2 (12.1 μmol/L), 25-hydroxyvitamin D3 (12.5 μmol/L) and 3-epi-25-hydroxyvitamin D3 (253.3 μmol/L) were supplied in 1mL of ethanol in sealed glass ampoules (stock 1). The ampoule content was transferred into an amber vial which was tightly cupped and stored at -80ºC. 3-Epi-25-hydroxyvitamin D3 Stock 2 (10.0 μmol/L) was prepared by diluting stock 1 with deoxygenated methanol.

25-Hydroxyvitamin D3-[$^2H_3$] stock solution

The tri-deuterated 25-hydroxyvitamin D3 (25-hydroxyvitamin D3-d3) was supplied in sealed glass ampoule (1mL) with 247.8 μmol/L in ethanol (stock 1). This stock was transferred into a glass bottle containing 49 mL of deoxygenated methanol to prepare 50 mL of 25-hydroxyvitamin D3-d3 at a concentration of 5.0 μmol/L (stock 2). Fifty mL of stock 3 was

[3] Electronic balance and pipettes, which were used in the project, were calibrated and periodically checked every six months. The balance was checked periodically using set of certified weights.

prepared by diluting stock 2 with the deoxygenated methanol at a concentration of 330 nmol/L. This stock was used as the vitamins D and A internal standard working solution.

Retinol stock solution

A calculated mass of retinol crystals was dissolved into the deoxygenated ethanol at a 1.0 mmol/L concentration to prepare stock 1. Stocks 2 and 3 (100.0 μmol/L and 10.0 μmol/L respectively) were prepared by diluting stock 1 with deoxygenated methanol.

Retinol-[$^{2}H_{5}$] solution

Deuterated retinol crystals were dissolved into deoxygenated ethanol at a 3.4 mmol/L concentration for stock 1 preparation. Aliquots from stock 1 were diluted with de-oxygenated methanol to generate stocks 2 and 3 with 20.0 μmol/L and 2.0 μmol/L concentrations respectively. Stock 3 was used as the potential isotopic retinol internal standard working solution; however, it was not stable as detailed in the Chapter 3.

Retinol acetate

Retinol acetate crystals were dissolved into the deoxygenated ethanol at a 60.9 mmol/L concentration for stock 1. Stocks 2 and 3 (600.0 μmol/L and 3.0 μmol/L, respectively) were prepared by diluting stock 1 with the deoxygenated methanol. Retinol acetate was commonly used as a retinol internal standard in HPLC methods. We tested it as a potential internal standard in the FSV quantification method using LC-MS/MS in place of the problematic "deuterated" retinol internal standard, but it was not suitable choice as predicated. This detailed in Chapter 3.

α-Tocopherol stock preparation

The α-tocopherol (viscous liquid) was dissolved into the deoxygenated ethanol at 3.0 mmol/L for stock 1. The α-tocopherol stock 2 (100.0 μmol/L) was prepared by diluting stock 1 with deoxygenated methanol.

α-Tocopherol-[$^{2}H_{6}$] stock preparation

The initial content of α-tocopherol-[$^{2}H_{6}$] (α-tocopherol-d6) vial (2.0 mg) was dissolved in deoxygenated methanol and transferred into a glass bottle, which was then adjusted to 100 mL of deoxygenated methanol for a concentration of 46.0 μmol/L. This stock was used as the α-tocopherol internal standard working solution.

Fat-soluble vitamin mixture in methanol

Deoxygenated methanol (15 mL) was spiked with entire FSVs to use as the initial quantity controls for the analyte peak and LC-MS/MS system performance prior to running a sample batch. This mixture was prepared by diluting each stock solution with deoxygenated methanol to obtain a mixture of FSV with concentrations of 150.0 nmol/L for 25-OHD2 and 25-OHD3, 100.0 nmol/L for epi-25-OHD3, 60.0 nmol/L for 25-OHD3-d3, 1.5 μmol/L for retinol and 10.0 μmol/L for both α-tocopherol and α-tocopherol-d6. This mixture was stirred and aliquoted (150 μL) in labelled amber vials and stored at -80°C.

2.5.4 Calibrators

Calibrator set for the entire FSV analytes are not commercially available. However, separate commercial sets for vitamin D analytes and for vitamins A and E are accessible from at least three manufactures (Bio-Rad, Chromsystems and RECIPE). Consequently, generating an in-house calibrator set for these vitamins (25-hydroxyvitamin D analytes, retinol and α-tocopherol) had become worthy for cost and time efficiency. The two matrix materials, SeraCon II stripped delipidated serum (SeraCon-DL) and SeraCon vitamin D–depleted diluent (SeraCon-DD), were investigated. While the SeraCon-DD was free from all target FSV analytes, the SeraCon-DL contained endogenous 25-OHD3. Thus, the SeraCon-DL was used to prepare in-house calibrator for only vitamins A and E; and the SeraCon-DD was utilised for the entire FSV in-house calibrator set. The evaluation of these two sets is detailed in the Chapter 3.

In-house vitamins A and E calibrator set preparation

A seven calibrator level set containing of retinol and α-tocopherol was prepared using the SeraCon-DD, which had no retinol or α-tocopherol content based on our laboratory check. The SeraCon-DL was spiked with retinol stock 2 and α-tocopherol stock 1 to prepare pre-calibrator level 7 for retinol at a concentration of 8.0 μmol/L and another pre-calibrator level 7 for α-tocopherol at a concentration of 100.0 μmol/L.

These pre-calibrator levels were then gently mixed for 1 h and then kept on crushed ice in the refrigerator overnight. The next day, the two pre-calibrator levels were left for 30 min at room temperature (RT) prior to mixing. The pre-calibrator levels were then diluted with SeraCon-DL to create the other six pre-calibrator levels for retinol (0.4, 1.6, 2.8, 4, 5.2, 6.4, 8.0 μmol/L for levels 1 to 6, respectively) and for α-tocopherol (12.0, 24.0, 40.0, 56.0, 70.0,

86.0, 100.0 μmol/L for levels 1 to 6, respectively). All pre-calibrator levels were gently mixed using the roller mixer for 1 h prior to storing them at 4°C in the refrigerator on crushed ice overnight. The next day, the pre-calibrators were kept at RT and then mixed for 30 min using a roller mixer.

Later, every pre-calibrator level of retinol was mixed with an equivalent pre-calibrator level of α-tocopherol to form a combined calibrator level. Each combined calibrator contained retinol and α-tocopherol analytes at different concentrations (retinol: 0.2, 0.8, 1.4, 2.0, 2.6, 3.2 and 4.0 μmol/L; α-tocopherol: 6.0, 12.0, 20.0, 28.0, 35.0, 43.0 and 50.0 μmol/L). The seven calibrator levels were mixed gently for 1 h using the roller mixer. Every single calibrator level was then aliquoted (110 μL) into labelled polypropylene tubes (1.5 mL) and stored at -80°C. This was used for vitamin A/E method.

In-house FSV calibrator set preparation

A seven calibrator level set for the five FSV analytes, including 25-OHD2, 25-OHD3, epi-25-OHD3, retinol and α-tocopherol, was prepared. The SeraCon-DD was spiked with the five FVS analytes to prepare calibrator 1 (cal-1; at a concentration of 4 nmol/L for 25-OHD3, epi-25-OHD3 and 25-OHD2 and 0.1 μmol/L and 6 μmol/L for retinol and α-tocopherol respectively). The calibrator 7 (cal-7; a concentration of 200.0 nmol/L for 25-OHD3, 160.0 nmol/L for epi-25-OHD3 and 25-OHD2, 4.0 μmol/L and 71.5 μmol/L for retinol and α-tocopherol respectively) was made. The two calibrators were gently mixed using a roller mixer for 1 h prior to storing them at 4°C on crushed ice overnight. The next day, they were kept at RT and then mixed for 30 min.

The other five calibrator levels were generated by mixing cal-1 and cal-7 in different proportions. The final level concentrations were 4.0, 9.9, 23.6, 62.8, 102.0, 160.8, and 200.0 nmo/L for of 25-OHD3; 4.0, 8.7, 19.6, 50.8, 82.0, 128.8 and 160.0 nmol/L for the epi-25-

OHD3 and 25-OHD2; 0.1, 0.2, 0.5, 1.3, 2.1, 3.2, and 4.0 μmol/L for the retinol; and 6.0, 8.0, 12.5, 25.6, 38.7, 58.4 and 71.5 μmol/L for the α-tocopherol. Later, each calibrator level was mixed and aliquoted (110 μL) at labelled polypropylene tubes (1.5 mL) and stored at -80°C.

Commercial calibrators

The RECIPE calibrator set (four levels) for 25-OHD2 and 25-OHD3 was utilised in the current project. This set was multi-levels and traceable to the National Institute of Standards and Technology-standard reference material 972 (NIST-SRM972) (120). It was prepared according to the manufacturer's instructions and then aliquoted (110 μL) at labelled polypropylene microcentrifuge tubes and stored at -80°C.

Three commercial calibrators (single level) for retinol and α-tocopherol were used. These included Bio-Rad calibrator (traceable to the NIST-SRM968e (121)), Chromsystems (traceable to the NIST-SRM968e (121)) and RECIPE (traceable to the NIST-SRM968d (122)). They were prepared according to the manufacturer's instructions and then aliquoted (250 μL) at polypropylene microcentrifuge tubes and then stored at -80 °C. During the sample preparation, the calibrator was diluted using Milli-Q water (0%, 30%, 60% and 80% for calibrator levels 4, 3, 2 and 1, respectively).

2.5.5 Controls

Two commercial quality control (QC) sets (for 25-OHD2 and 25-OHD3; and for retinol and α-tocopherol) from UTAK were used. In addition, an in-house QC set was prepared for epi-25-OHD3 because of the unavailable of a commercial set. These QCs were simultaneously prepared with unknown samples in the beginning and in the end of the batch. Results of the controls were used to monitor intra-run and inter-run method performance.

UTAK lyophilised QC set (tri-levels: low, mid, high) for retinol and α-tocopherol [Vit A/E control] and for 25-OHD2 and 25-OHD3 [Vit-D control] were used in this project. The UTAK three level QCs were reconstituted according to the manufacturer's instructions. Aliquots (110 μL) were prepared using polypropylene microcentrifuge tubes and stored at -80°C.

The in-house tri-level QC set for epi-25-OHD3 [epi-Vit -D control] was generated as follows. Human serum, containing no endogenous 25-OHD3 epimer, was spiked with epi-25-OHD3 (135 nmo/L) to prepare a high control level (QC high). This QC high level was gently mixed using the roller mixer for 1 h before storing at 4ºC on crushed ice overnight. The next day, it was restored to RT for equilibration and then mixed for 30 min. Mid and low control levels with concentrations of 45 and 9.5 nmol/L respectively, were prepared by diluting the QC high level using non-spiked serum, which were obtained from human research team volunteers. The three QC levels were mixed gently for 1 h. Later, each control level was aliquoted (110 μL) into labelled polypropylene tubes (1.5 mL) and then frozen at -80ºC.

2.6 Sample preparation

Sample preparation processes were investigated and optimised during the course of development and validation of the FSV quantification method. Details of this optimisation processes are provided in the Chapter 3. The following protocol is a final version of the sample preparation procedure which was used in the project's studies unless otherwise stated.

The biological samples used in the work were serum or plasma origin, including unknown human samples, calibrators, controls and external quality assurance material. All samples were left at RT until they were completely thawed and then inverted several times and mixed for five minutes before they were processed. The internal standards and aliquot of FSV solution were also left at RT and mixed before they were used. Fresh Milli-Q water was collected from the Milli-Q water system according to the manufacturer's instructions. The QCs (9 levels overall) were each prepared and included at the beginning and end of each batch. All sample preparation processes except for specific stability study experiments were conducted under subdued light.

Procedure: 100 µL of sample was placed in a Kimax glass tube, 100 µL of Milli-Q water was added, and then the mixture was vortexed for 10 sec. Later, 200 µL of methanol containing the tri-deuterated 25-OHD3 (330 nmol/L) and hexa-deuterated α-tocopherol (46 µmol/L) was added, followed by vortexing for 10 sec. It was then left at RT for 10 min under subdued light. After that, 1.5 mL of hexane was added and vortexed extensively prior to centrifugation at 3,000 rpm for 5 min. About 800 µL of the organic layer was transferred into a new glass tube and then dried under nitrogen gas at RT. Later, the sample was reconstituted in 250 µL methanol and vortexed for 20 sec. Finally, 1 µL (for vitamin A/E method) and 8 µL (for FSV method-1 and FSV method-2) of the sample was injected into LC-MS/MS system (for both Agilent LC-MS/MS-6490 and LC-MS/MS-6410).

2.7 Specification of the LC-MS/MS systems

Performance specifications of the two LC-MS/MS systems used in the current project are summarised in the following Table 2-1, Table 2-2.

Table 2-1. Performance specifications of Agilent LC-1200 and Agilent LC-1290.

Specification*		Agilent LC-1200 (123)	Agilent LC-1290 (124-126)
Pump	Hydraulic system	Dual plunger in series pump with proprietary servo-controlled variable stroke drive, floating plungers and passive inlet valve	Two dual pistons in series, pumps with proprietary servo-controlled variable stroke design and smooth motion control
	Settable flow range	0.001 – 10 mL/min, in 0.001 mL/ min increments	0.001 – 5 mL/min, in 0.001 mL/min increments
	Flow precision	<0.07% RSD	≤0.07 % RSD
	Flow accuracy	± 1%	±1%
	Pressure operating range	0– 60 MPa (0–600 bar) up to 5 mL/min 0–40 MPa (0–400 bar) up to 5 mL/min 0–20 MPa (0–200 bar) up to 10 mL/min	Binary Pump: up to 120 MPa (1200 bar) up to 2 mL/min, 80 MPa (800 bar) at 5 mL/min Binary Pump VL: up to 105 MPa (1050 bar) up to 2 mL/min
	Pressure pulsation	< 2 % amplitude	< 1 % amplitude
	Gradient formation	Low pressure dual mixing	High pressure binary mixing
	Delay volume	600 – 900 µL	JetWeaver V35: < 45 µL JetWeaver V100: < 75 µL
	Composition range	0 – 95 % or 5 – 100 %	Settable range: 0 - 100 %
	Composition precision	< 0.2 % RSD or < 0.04 min SD	< 0.15 % RSD or 0.01 min SD
Autosampler	Injection range	0.1–100 µL in 0.1 µL increments	0.1–20 µL in 0.1 µL increments
	Injection precision	< 0.25% from 5–100 µL, < 1% from 1–5 µL	<0.25% from 5–40 µL <0.5% from 2–5 µL <0.7% from 0.5–2 µL
	Sample viscosity range	0.2–50 cp	0.2–5 cp
	Injection cycle time	Typically 50 s depending on draw speed and injection volume	Typically < 21 s depending on draw speed and injection volume
	Carryover	Typically < 0.1%	Typically < 0.004 %
Column Oven	Temperature range	5ºC above ambient to 80ºC	10ºC below ambient to 100ºC
	Temperature stability	± 0.15ºC	± 0.05ºC
	Temperature accuracy	± 0.8ºC	± 0.8ºC
	Column capacity	One 250 mm column	2 columns of 300 mm with individual solvent heating
	Internal volume	6 µL	3 µL left heat exchanger 6 µL right heat exchanger

*These performance specifications are based on data analysed by Agilent technologies.

Table 2-2 Performance specifications of Agilent MS/MS-6410 and Agilent MS/MS-6490.

Specification*	Agilent MS/MS- 6410 (127)	Agilent MS/MS-6490 (128)
Sensitivity: 1pg reserpine# quantifying on m/z 609→195 using ESI positive mode	S/N > 6,000:1	S/N > 50,000:1
Sensitivity: 1pg chloramphenicol# quantifying on m/z 321→152 using ESI negative mode	S/N > 2,000:1	S/N > 50,000:1
Linear dynamic range	$> 6.0 \times 10^6$	$> 6.0 \times 10^6$
Mass range	5–2,250 Da	5–1,400 Da
Maximum scan rate	12,500 Da/sec	12,500 Da/sec
Minimum MRM dwell time	1 ms	1 ms
MRM transitions	450 per time segment, > 40,000 ion transitions per method	500 per time segment, > 40,000 ion transitions per method
Polarity switching (from positive to negative)	30 ms	30 ms
Collision cell ion clearance	< 1 ms	< 1 ms
Agilent Jet stream technology$	no	yes
Agilent iFnnel technology$	no	yes

*These performance specifications are based on data analysed by Agilent technologies.
Agilent technologies use reserpine and chloramphenicol compounds to compare their MS/MS systems.
$"Jet Stream sample introduction, providing high-efficiency ESI ion generation and focusing; a hexabore capillary; and a unique dual-stage ion funnel assembly. Together, these technologies reduce neutrals and increase ion sampling to dramatically improve overall signal within the system, delivering significant increases in sensitivity compared with conventional instruments." (129)

2.8 Operation of the LC-MS/MS system

The LC-MS/MS system was operated using Agilent MassHunter data acquisition software (version B.4.01) according to the general operation manual (perpetrated by LC-MS/MS research group) for the Agilent LC-MS/MS 1200/6400 that was available as a hardcopy in the LC-MS/MS laboratory.

2.9 Data analysis

The data were analysed using the Agilent MassHunter quantitative analysis software (version B.05.00/B5.0.291.0) and qualitative analysis software (version B.04.00) according to the general operation manual for the Agilent LC-MS/MS 1200/6400 that was available as a hardcopy in the LC-MS/MS laboratory. All data were stored in the main computer of the LC-MS/MS laboratory and backed up weekly on an external memory drive.

Chapter 3

Development of the simultaneous fat-soluble vitamin quantification method using liquid chromatography-tandem mass spectrometry

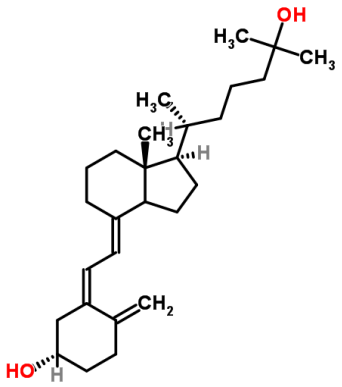
Chapter 3 Development of the simultaneous fat-soluble vitamin quantification method using liquid chromatography-tandem mass spectrometry

3.1 Introduction

The accurate and precise quantification of fat-soluble vitamins (FSV) has proven to be a significant challenge for clinical laboratories (130). This challenge is the result of the nature of FSV molecules and their metabolites, the limitations of measurement assays and standardisation of vitamin analysis. Consequently, there is a significant variation in the results obtained both by different assays and different clinical laboratories even using the same diagnostic techniques (89-91). As a result, it has been difficult to reach an agreement on the recommended levels and reference intervals of these vitamins and their metabolites for healthy people (discussed in Chapter 7). Consequently, debate has ignited as to whether conventional analytical techniques are accurate and precise enough to diagnose and monitor the pathologies associated with FSV deficiencies.

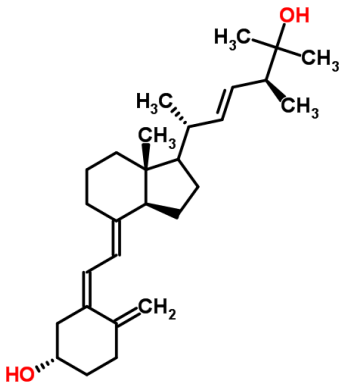
The chemical and physical properties of FSV molecules and their metabolites represent analytical challenges in clinical laboratories (Figure 1-3) (130). FSV metabolites, including 25-OHD, retinol and α-tocopherol, are hydrophobic compounds that are mediated in the blood by binding with relatively large proteins (i.e., VDBP is 50 kDa) (130). The dissociation of a vitamin from its binding protein is essential before vitamin measurement, and variation in the process aiming to complete dissociation may significantly contribute to the sensitivity and specificity of an analytical assay (89, 94). In addition, the precise and simultaneous measurement of FSVs is an even more complicated task, as these vitamins and their

metabolites are present in the blood in different concentration levels, as well as bind to different binding proteins.



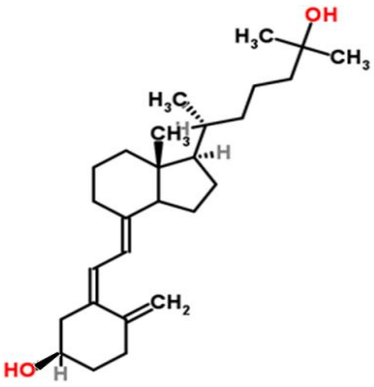
25-Hydroxyvitamin D3 [25-OHD3]

(C27H44O2, 400 .64 Da)(44)



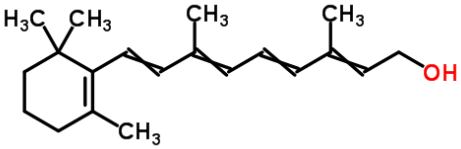
25-Hydroxyvitamin D2 [25-OHD2]

(C28H44O2, 412.64 Da) (45)



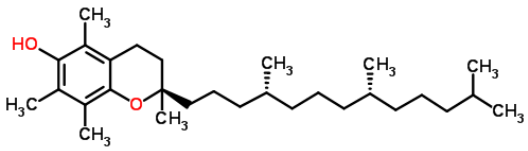
C3 25-Hydroxyvitamin D3 [Epi-25-OHD3]

(C27H44O2, 400 .64 Da)



Retinol

(C20H30O, 286.45 Da) (15)



α-Tocopherol

(C29H50O2, 430.71 Da) (76)

Figure 3-1. Chemical structure of investigated FSV.

These molecules are highly hydrophobic molecules that are transported in the blood with bind strongly with proteins through hydrophobic interaction. Destruction of this binding is an essential step for accurate and precise FSV measurement. In addition, these compounds are small molecules (less than 500 Da) with only one or two hydroxyl groups, and therefore present greater difficulty in the ionisation process, which is a critical step for mass spectrometry detection.

Currently, the most routine analytical assays utilised for FSV measurement are the immunoassay technique (IA) for 25-OHD2 and 25-OHD3; and the high-performance liquid chromatography technique (HPLC) for retinol and α-tocopherol. In general, commercial immunoassays (automated platforms) are most commonly used for total 25-OHD quantification and are available from several manufacturers, such as DiaSorin, Immunodiagnostic Systems, Abbott, Roche and Siemens (90, 131).

Several previous studies have observed significant variation in the performance of automated immunoassays, particularly in samples with low levels of 25-OHD (89, 132, 133). This may relate to the challenges involved in disassociating the FSV analytes from their binding proteins, as well as in generating specific antibodies to capture 25-hydroxylated vitamin D analytes during blood sample preparation (132). A further limitation of IAs is their incapability to detect the epimer of 25-OHD3 (epi-25-OHD3), which has been quantified in child and adult samples (134-141).

In clinical practice, blood retinol and α-tocopherol are routinely measured concurrently based on HPLC chromatographic technique facility (81). However, inter-laboratory discrepancies in these vitamin results have been reported (91). The majority of HPLC instruments used for retinol and α-tocopherol measurements are coupled with UV/Vis or photodiode array (PDA) detectors (142), which are adequate for these analytes but less sensitive compared to mass spectrometers. Therefore, quantification of the epi-25-OHD3 using HPLC with traditional detectors is also a challenge because of the low concentration levels of epi-25-OHD3 (143).

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) represents a highly specificity and sensitivity technique that is superior to IAs and HPLC (113, 114). This technology quantifies analytes based on the strengths of the chromatography technique for analyte separation and the tandem mass spectrometry technique for high sensitive and

selective analyte detection. The use of LC-MS/MS in clinical laboratories has increased over the last decade, especially for the measurement of low molecular weight analytes (114). Consequently, LC-MS/MS is the optimal analytical platform for the precise quantification of 25-OHD3 and its epimer, as well as other vitamin D analytes (144). A few LC-MS/MS methods quantifying blood 25-OHD2, 25-OHD3, epi-25-OHD3 (134, 141, 145, 146), retinol and α-tocopherol (147) have been published but none of these methods quantified all these analytes together.

The development of a simultaneous measurement method for most FSV analytes in the blood is a challenge due to the discrepancies between their structures and concentrations. Few published methods have simultaneously measured FSV analytes (148, 149). Priego-Capote and colleagues (148) developed a method for quantifying FSVs using LC-MS/MS, however, they did not use isotopic internal standards, which are crucial for highly precise methods. In addition, this method required a large serum sample volume (1000 μL), which is not suitable for routine laboratory work. In contrast, Midttun and colleagues (149) developed a LC-MS/MS method for FSV measurement using isotopic internal standards and small sample sizes (50 μL). However, neither of these studies quantified epi-25-OHD3 (which is a recognised interferent for the accurate determination of 25-OHD3), and each offered limited details as to their method validation and independent quality assessment through on external quality assurance (EQA) program.

Therefore, the present work aimed to establish a precise and simultaneous sample preparation and quantification for the five FSV analytes (25-OHD3, epi-25-OHD3, 25-OHD2, retinol and α-tocopherol) utilising LC-MS/MS technology. This procedure included development of a simple and cost-effective sample preparation protocol, and a simultaneous FSV quantification method using two Agilent LC-MS/MS systems. The Agilent LC-MS/MS-6410 and LC-

MS/MS-6490, which are the earliest and latest models of Agilent LC-MS/MS systems respectively and have different levels of analytical sensitivity, were used in the development of these methods. This issue is important to check robustness of the FSV analysis either using early model of LC-MS/MS or advanced LC-MS/MS which are not available for all clinical laboratories. The developed methods were then used to explore several clinical issues related to simultaneous blood FSV analysis in four clinical studies, which are discussed to prove clinical activity in the next chapters.

3.2 Methodology and results

Developments of simultaneous FSV extraction and quantification encountered several challenges in terms of calibration materials, internal standards and interference. This section will discuss the development of the simultaneous FSV measurement procedures, including sample extraction protocol and chromatographic-tandem mass spectrometry quantification. Methodology and results of the current work are detailed together here for greater clarity and coherency.

The FSV extraction protocol and analysis were developed in the following three stages using two LC-MS/MS systems. These stages included: 1) optimising the LC-MS/MS system for chromatographic separation and mass spectrometric detection using pure FSV in a methanolic solution, 2) developing a protocol for serum FSV extraction and progressively checking optimal analyte recoveries based on the first stage, and 3) improving both LC-MS/MS optimisation and serum FSV extraction protocol in parallel.

In this work, three methods were developed and validated as follows. The first method was developed for the simultaneous quantification of vitamins A (retinol) and E (α-tocopherol) [**vitamin A/E method**] using the Agilent LC-MS/MS 6490 system equipped with a C18 column. It was initially proposed to extend this analytical assay to include vitamin D analytes, however, this did not happen due to unsatisfactory chromatographic resolution. The second method developed was a simultaneous FSV quantification method (**FSV method-1**) for the quantification of five FSV analytes, including 25-OHD3, epi-25-OHD3, 25-OHD2, retinol and α-tocopherol. This method was developed using the Agilent LC-MS/MS 6490 system equipped with a pentafluorophenyl (PFP) column. The third method (**FSV method-2**) was created based on transferring the FSV method-1 to Agilent LC-MS/MS 6410 which required further optimisation. The Agilent LC-MS/MS 6410 is an earlier model of the Agilent

LC-MS/MS series and less sensitive compared to the LC-MS/MS 6490. This transference of method was conducted after full validation of the FSV method-1; and aimed to check the robustness of our FSV analysis which involved the LC-MS/MS quantification method and the sample extraction protocol.

The following sub-sections detail the optimisation of the LC-MS/MS systems and the sample preparation protocol. In addition, they detail the challenges faced in terms of internal standards and calibrators over the course of method development. Later in this chapter, the method validation and analytical performance will be presented.

3.2.1 LC-MS/MS system optimisation

This phase of the study aimed to explore the optimal conditions for the liquid chromatography (LC) and tandem mass spectrometer (MS/MS), which together represent the main integrated instruments of LC-MS/MS, using a mixture of FSV in methanol. For chromatographic analyte separation, several LC parameters were optimised, including column temperature, flow rate, injection volume, mobile phase gradient profile, and total method running time, using the C18 and PFP columns.

Two liquid chromatography instruments (LC-1290 and LC-1200), coupled to the MS/MS-6490 and MS/MS-6410 respectively, were optimised. In both instruments, different gradient mobile phase compositions, flow rates and column oven temperatures were investigated for the best target peak resolution. LC-1290 was optimised for the vitamin A/E method using the C18 column, which successfully separated retinol and α-tocopherol in the serum samples (Figure 3-2, Table 3-1). The C18 column could not separate the epi-25-OHD3 under the trialled conditions. Hence, for the extended methods (FSV methods 1 and 2), the LC-1290 and LC-1200 were adjusted using a PFP column whereby the three 25-hydroxylated vitamin

D analytes (25-OHD2, 25-OHD3 and epi-25-OHD3), retinol and α-tocopherol in the serum sample were separated (Figure 3-3, Figure 3-4, Table 3-1).

Optimisation of the LC systems was accompanied by an exploration of the optimal conditions for both the MS/MS-6490 and MS/MS-6410 as integrated parts of equipment on the LC-MS/MS 6490 and 6410 systems respectively. Both instruments were operated in positive electrospray ionisation mode with multiple reaction monitoring (MRM) to quantify the five proposed analytes. For greater specificity, two transition ions were utilised for each analyte: a quantifier transition ion (the most abundant ions) and a qualifier transition ion (the second most abundant ion). While the quantifier ion was used to calculate analyte concentration, both the quantifier and qualifier transition ions and their ratios were progressively monitored for each sample as part of quality control for specific target analyte detection. Other MS/MS parameters were also adjusted, including gas temperature, gas flow rate, nebuliser pressure and capillary voltage. Additional parameters on the MS/MS 6490, such as sheath gas temperature, sheath gas flow rate and nozzle voltage, were also adjusted. Each of these LC-MS/MS parameters were optimised using a mixture of FSV in methanolic solution and serum samples (Table 3-2).

In conjunction with the optimisation of the LC-MS/MS, the serum sample preparation protocol was developed for optimal simultaneous FSV extraction (discussed in section 3.2.2).

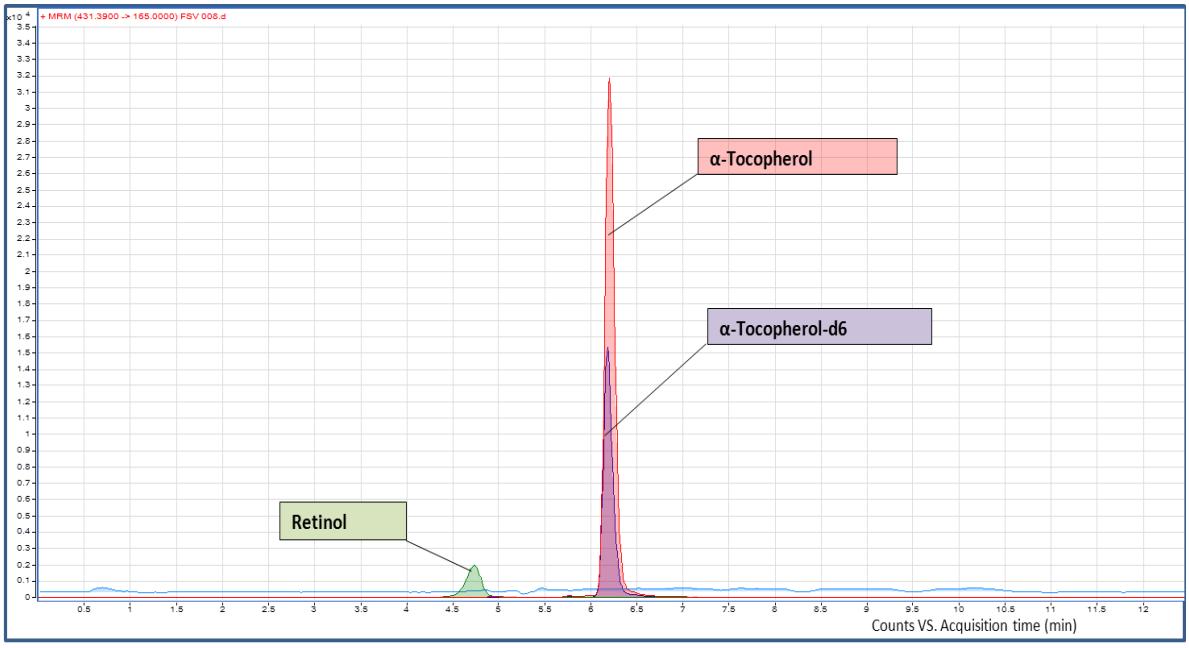


Figure 3-2. Retinol and α-tocopherol chromatogram using the vitamin A/E method.

The chromatogram demonstrates the separation of retinol, α-tocopherol and hexa-deuterated α-tocopherol in human serum using the vitamin A/E method (column C18 equipped to the Agilent LC-MS/MS 6490 system).

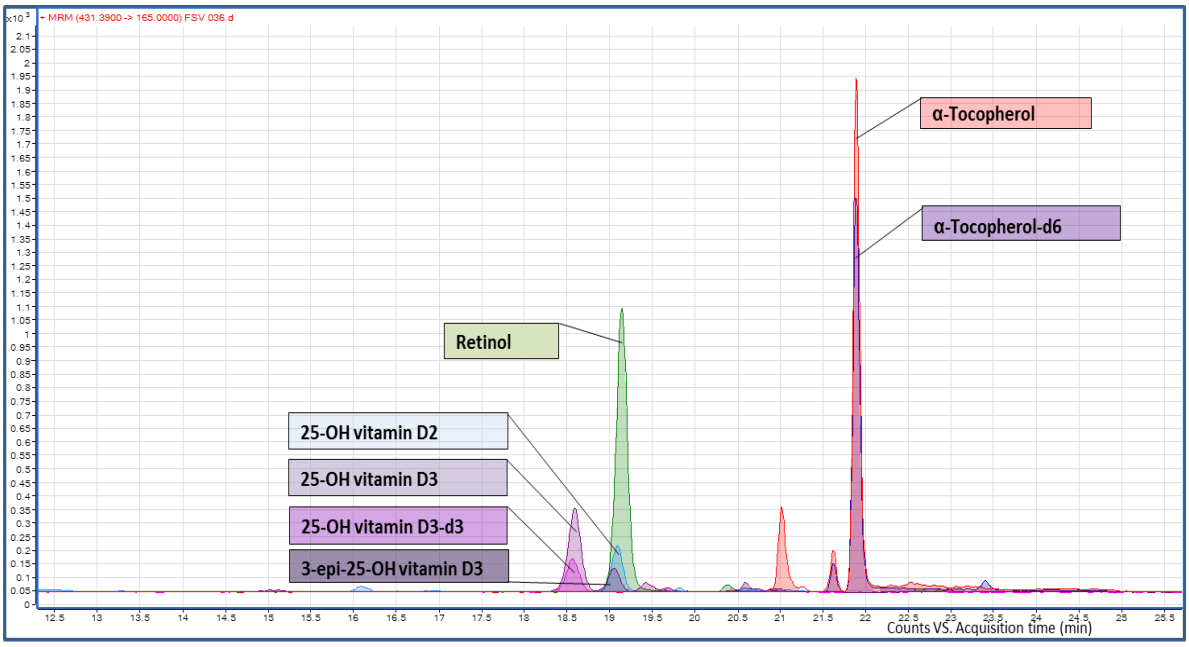


Figure 3-3. Fat-soluble vitamin chromatogram using the FSV method-1.

The chromatogram demonstrates the identification of 25-OHD2, 25-OHD3, epi-25-OHD3, 25-OHD3-d3, retinol and α-tocopherol in a human serum sample spiked with epi-25-OHD3 and 25-OHD2. These metabolites were identified using FVS method 1 (PFP column utilised with the Agilent LC-MS/MS 6490 system).

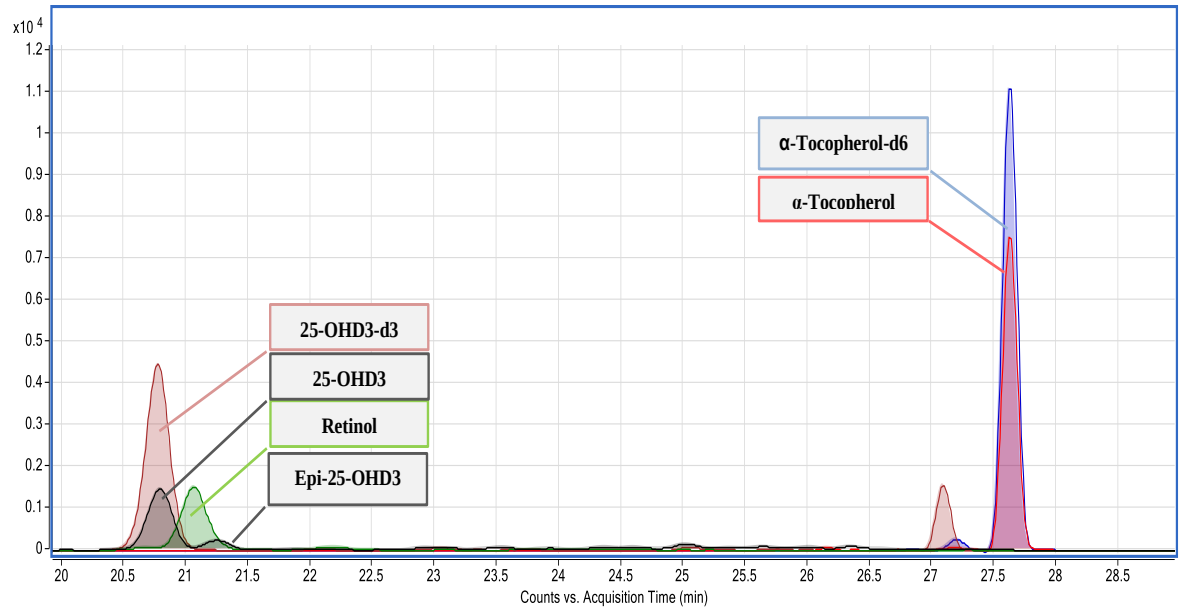


Figure 3-4. Fat-soluble vitamin chromatogram using the FSV method-2.

The chromatogram demonstrates the identification of 25-OHD3, epi-25-OHD3, 25-OHD3-d3, retinol and α-tocopherol in a human serum sample with no 25-OHD2 analytes. FSV anlaytes were identified using a column PFP equipped to the Agilent LC-MS/MS 6410 system.

Table 3-1. Final liquid chromatography conditions .

This table demonstrates the condition of the Agilent LC-1290 (integrated to the MS/MS-6490) and LC-1200 (integrated to the MS/MS-6410) for the vitamin A/E method and FSV methods 1 and 2. These three methods included the same mobile phases, yet with different columns, gradient elution profiles, injection volumes and running times. Column flushing and equilibrating from one sample analysis to the next were taken into consideration in terms of mobile gradient elution profiles and running times.

Method	Vitamin A/E			FSV method-1			FSV method-2		
Liquid chromatography	LC-1290						LC-1200		
Analytical column	Pursuit C18 column (20 × 2 mm)			Pursuit PFP (150 × 2 mm)					
Guard column	Pursuit XRs 3 µm C18			Pursuit 3u PFP					
Column temperature	45ºC			45ºC					
Mobile phases A (MpA)	0.1% formic acid and 2% methanol in milli-Q water								
Mobile phases B (MpB)	0.1% Formic acid in methanol								
Flow rate	0.2 mL/min								
Injection volume	1 µL			8 µL					
Total running time	12.5 mins			42 mins			45 mins		
Mobile phase gradient profile	Time (min)	MpA (%)	MpB (%)	Time (min)	MpA (%)	MpB (%)	Time (min)	MpA (%)	MpB (%)
	0.0	20	80	0.0	65	35	0.0	60	40
	3.5	20	80	5.5	30	70	7.5	30	70
	4.0	0	100	16	22	78	18.0	22	78
	9.0	0	100	18	4	96	23.0	4	96
	9.5	20	80	19.5	0	100	24.5	0	100
	12.5	20	80	34	0	100	36.0	0	100
				36	45	55	36.1	45	55
				37	65	35	38	60	40
				42	65	35	45	60	40

Table 3-2. Final tandem mass spectrometry conditions.

This table demonstrates the conditions of the Agilent MS/MS-6490 and MS/MS-6410 for detection using three methods. The vitamin A/E method successfully separated vitamins A and E, but not vitamin D analytes. The extended methods (methods 1 and 2) were capable of quantifying the three 25-hydroxlated vitamin D analytes, plus retinol and α-tocopherol. Deuterated 25-OHD3 was used as the ISTD to quantify target vitamin D metabolites, and deuterated α-tocopherol was used as the ISTD to quantify α-tocopherol. Due to the challenge of finding a typical retinol ISTD (to be discussed later in the chapter), the deuterated 25-OHD3 was taken as the best available option for retinol quantification, as it eluted in close retention time to retinol (81).

Method	Vitamin A/E method and FSV method-1					FSV method-2				
MS/MS	6490					6410				
Ionisation	Electrospray ionisation / Positive									
Analytical mode	Multiple reaction monitoring									
Gas temperature	300ºC					250°C				
Gas flow rate	11 L/min					6 L/min				
Nebuliser pressure	40 psi					50 psi				
Sheath gas temperature	275ºC					NA				
Sheath gas flow	7 L/min					NA				
Capillary voltage	3500 positive					5500 positive				
Nozzle voltage	2000 v					NA				
Multiple reaction monitoring (MRM)	Analyte	Prec Ion	Prod Ion	Dwell	CE	CAV	Prec Ion	Prod Ion	Dwell	CE	Frag. volt
	25-OHD3* Epi-25-OHD3*	401	383	50	4	8	401	383	50	4	135
	25-OHD3# Epi-25-OHD3#	401	365	50	12	8	401	365	50	12	135
	25-OHD3-d3	404	386	50	4	8	404	386	50	4	135
	25-OHD2*	413	395	50	4	7	413	395	50	4	135
	25-OHD2#	413	159	50	28	7	413	159	50	28	135
	Retinol *	269	93	50	25	8	269	93	50	25	135
	Retinol #	269	213	50	15	8	269	213	50	15	135
	α-Tocopherol*	431	165	50	28	5	431	165	50	28	135
	α-Tocopherol#	431	137	50	36	5	431	137	50	36	135
	α-Tocopherol-d6	437	171	50	20	5	437	171	50	20	135
	Phospholipids	104	104	50	0	0	104	104	50	0	0
	Phospholipids	184	184	50	0	0	184	184	50	0	0

*Quantifier ion, #qualifier ion, Prec: precursor ion, Prod: product ion, Dwell: time (s) spent to scan for each m/z value (mass-to-charge ratio of target molecule), CE: collision energy (voltage), CAV: cell accelerator voltage, NA: not applicable. Phospholipid transition ions (104→104 and 184→184) were examined based on Honour's recommendations (116)

3.2.2 Sample preparation protocol development

The development of the sample preparation protocol aimed to explore simultaneous serum FSV extraction and optimal analyte recovery. Two extraction techniques including protein precipitation extraction (PPE) and liquid-liquid extraction (LLE) are commonly used in FSV extraction. PPE is used to destruct and precipitate serum proteins using an organic solvent (e.g., methanol, ethanol or acetonitrile), which liberates the FSVs from their binding proteins. However, this technique may cause matrix interference due to co-extracting molecules such as phospholipids. Hence, further sample clean-up may be required. By using LLE, compounds are separated based on their solubility in various solvents. The liberated FSVs (hydrophobic compounds) during PPE are isolated from the aqueous phase by adding an organic solvent (e.g. hexane), which dissolves the FSVs but is immiscible with water. The organic layer formed post the LLE process is then transferred into a new tube and evaporated commonly using nitrogen gas (Purity ≥ 99.999%). The FSVs are then reconstituted using solvent appropriate for chromatographic mass spectrometry assays (130).

To develop the final sample preparation protocol, we used a simple extraction protocol consisting of both PPE and LLE and was utilised in the experiments discussed in this chapter (unless otherwise stated). In this simple protocol, the pooled human serum sample (100 µL), followed by Milli-Q water (100 µL or as stated in the experiment), was placed in a glass tube (or as stated in the experiment). The mixture was then vortexed for 10 s. Next, 200 µL of methanol containing the tri-deuterated 25-OHD3 (330 nmol/L), hexa-deuterated α-tocopherol (46 µmol/L) and other ISTD (if required in the experiment) were added, followed by vortexing for 10 s. The mixture was left at RT for 10 min under subdued light. Next, the hexane (1.5 mL or as stated in the experiment) was added and vortexed for 3 min prior to centrifugation for 5 min. The organic layer was transferred into a new tube and then dried

under nitrogen gas at RT. Later, the sample was reconstituted in 250 µL of methanol and vortexed for 20 s. Finally, the samples were loaded into the LC-MS/MS system. This simple protocol was later updated to the final sample preparation protocol based on several experiments detailed in the following sections.

These experiments were designed to explore several factors affecting the efficiency of analyte extraction. These factors included, but were not limited to, the effects of adding Milli-Q water prior to sample extraction, increasing the organic solvent volume, using different proportions of hexane-to-2-propanol volume and the number of LLEs used (single versus double extractions). Furthermore, the suitability of using polypropylene tubes versus glass tubes in the sample preparation was also investigated. These experiments were designed and conducted on different occasions using two sets of pool human serum as discussed in the following sub-sections.

Liquid-liquid extraction (LLE)

Hexane is widely used in the LLE of retinol and α-tocopherol whilst a mixture of hexane with a small proportion of 2-propanol for 25-OHD analyte extraction has reported (130). In this work, the ratios of hexane volume (H) to 2-propanol volume (P) were explored to determine the optimal ratio for serum FSV extraction. These included H:P (1 mL): 75:25, 80:20, 85:15, 90:10, 95:05, 99:01 and 100:00. Pooled human serum in triplicate samples were extracted for each proportion and each analyte peak area was then calculated and compared.

It was found that 100% hexane was best suited for 25-OHD analytes and retinol extraction, while a mixture of hexane (75%) and 2-propanol (25%) was best for α-tocopherol recovery (Figure 3-5). Due to the low concentrations of 25-OHD analytes compared to other target vitamins in blood, absolute hexane was considered best to use in the LLE of the final sample preparation protocol.

After considering absolute hexane in LLE, it was important to determine the optimal hexane volume and number of LLEs required for maximum FSV extraction efficiency. Therefore, hexane volumes (1.0, 1.5 and 2.0 mL) and the question of single versus double LLEs were examined. For these purposes, pooled human serum in triplicate samples were prepared to investigate each hexane volume in single LLEs, and another triplicate samples were prepared for double LLEs. Later, the mean analyte peak areas were calculated and compared.

It was observed that using 1.5 mL of hexane improved the recoveries of 25-OHD analytes and retinol, while 2 ml of hexane was a better fit for α-tocopherol recovery. In addition, it was found that double LLEs did not improve recoveries of 25-OHD analytes and retinol, and had a negative effect on α-tocopherol recovery (Figure 3-6). Thus, single LLEs with 1.5 mL of hexane were considered for the final sample preparation protocol.

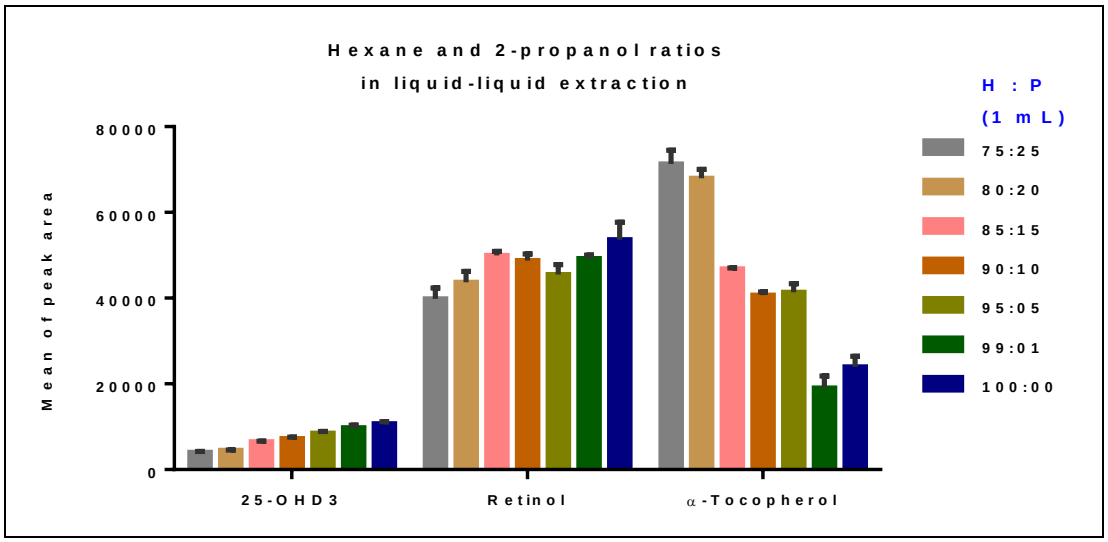


Figure 3-5. Fat-soluble vitamins extracted with different hexane and 2-propanol ratios.

The bar graph demonstrates FSV recoveries (peak areas) in pooled human serum in triplicate samples using a range of hexane (H) to 2-propanol (P) ratios. Absolute hexane is shown as better suited for 25-OHD analytes and retinol extraction, whereas a mixture of hexane and 2-propanol (H:P 75:25) is best for α-tocopherol recovery. For the final sample preparation procedure, 100% hexane was used due to low concentrations of 25-OHD analytes compared to other target vitamins. Error bars represent result standard deviations. This experiment was done using LC-MSMS-6490

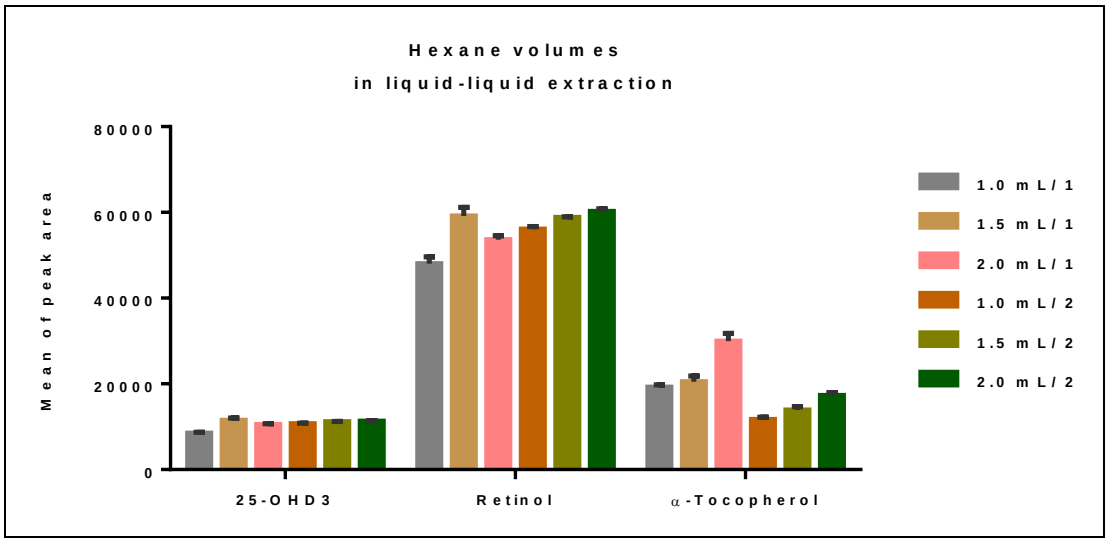


Figure 3-6. Fat-soluble vitamin extraction using different hexane volumes.

The bar graph demonstrates the efficiency of FSV extraction from triple serum samples using different hexane volumes (1.0, 1.5, 2.0 mL) and either single or double liquid-liquid extraction (LLE). Using 1.5 mL of hexane improved the recoveries of 25-OHD analytes and retinol, while 2 ml of hexane was found to be better for α-tocopherol recovery. Double LLEs did not improve the recoveries of 25-OHD analytes and retinol, and had a negative effect on α-tocopherol recovery. Therefore, single LLEs with 1.5 mL of hexane were considered for the final sample preparation procedure. Error bars represent result standard deviations. This experiment was done using LC-MSMS-6490

Effect of aqueous phase volume on fat-soluble vitamin recoveries

The effect of adding water to serum immediately prior to sample preparation represents part of the current knowledge gap concerning FSV extraction (81). The ratio of total aqueous phase volume to alcoholic phase volume has also been controversial. Therefore, we investigated the effect of adding different Milli-Q water volumes (0, 25 and 100 µL) to pooled human serum in triplicate as part of the FSV extraction process. Five serum samples were prepared to test each water volume. The mean analyte peak area obtained from the sample analyses were calculated and compared. It was observed that recoveries of FSV improved by adding 100 µL of Milli-Q water to the serum samples prior to sample preparation (Figure 3-7).

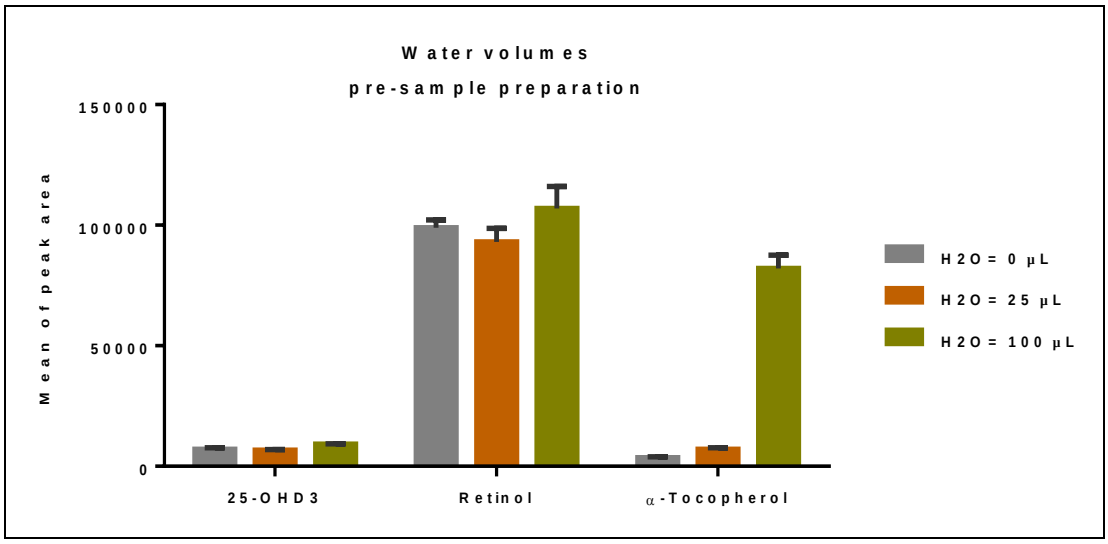


Figure 3-7. Effect of aqueous phase on fat-soluble vitamin recoveries.

Graph shows effect of adding Milli-Q water to triple serum samples of different volumes (0, 25 and 100 µL) prior to sample preparation. Recoveries of FSV were found to improve by adding 100 µL of Milli-Q water to serum samples prior to sample preparation. Error bars represent result standard deviations. This experiment was done using LC-MSMS-6490.

Effect of tube type on fat-soluble vitamin recoveries

While polypropylene tubes are widely used in laboratories, it has been reported that certain polypropylene tube brands may cause ion suppression and enhancement in mass spectrometry methods (150). Furthermore, the extent of the effect of organic solvents used during sample preparation on FSV extraction, especially with a high sensitive technology such as LC-MS/MS, is unclear. Hence, as part of the sample preparation procedure development, two commercial brands of polypropylene tubes (Biocentrix and Sarstedt) were compared with glass tubes (KIMAX) in terms of FSV recovery. It was found that using glass tubes improved the recoveries of retinol and α-tocopherol and decreased the ion enhancement of 25-OHD3 (Figure 3-8). Therefore, glass tubes were used in the final sample protocol.

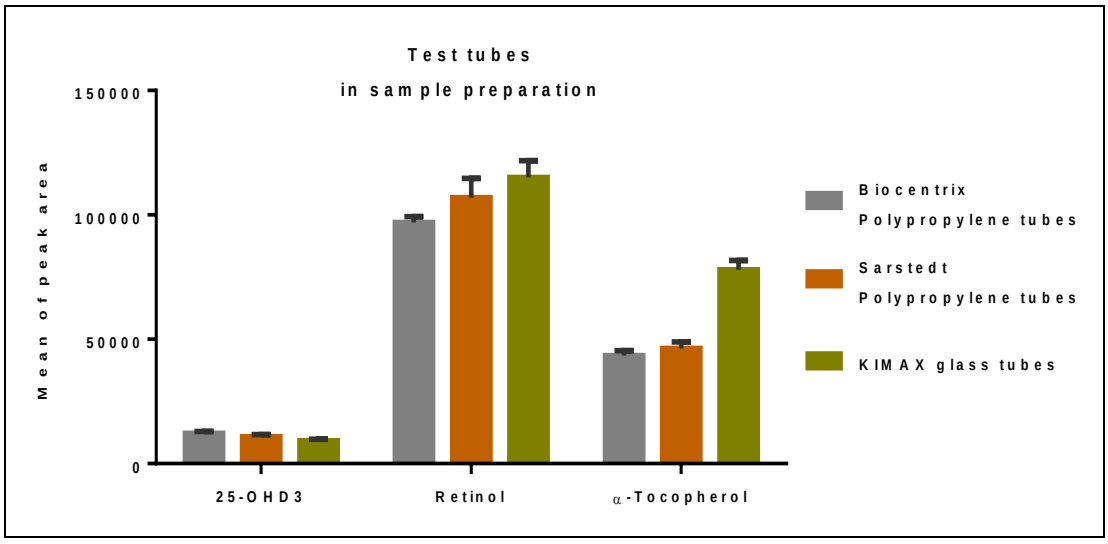


Figure 3-8. Effect of tube types on fat-soluble vitamin recoveries.

Graph shows FSV peak areas of triple serum samples using different polypropylene tubes versus glass tubes, demonstrating, in support of previous findings (150), that glass tubes improved the recoveries of retinol and α-tocopherol and decreased the ion enhancement of 25-OHD3. Error bars represent result standard deviations. This experiment was done using LC-MSMS-6490.

3.2.3 Internal standards

The isotopic internal standard (ISTD) approach in chromatography-mass spectrometry methods is strongly recommended (81). The ISTD is usually added to a specimen in the earliest stage of sample preparation to correct for the amount of analyte lost during analysis. The ISTD should have similar reactivity behaviour to the target analyte, and should not be in authentic samples. Isotopic deuterium or carbon-13-labelled compounds are recommended as ISTDs (116), however, it is difficult to find isotopic compounds commercially at a reasonable cost and stable within a satisfactory time. In this project, several potential ISTDs were examined, with successful ISTDs later used in the validation of the developed methods.

Retinol internal standard

Finding an optimal retinol internal standard proved to be a significant challenge for this project. Deuterated retinol is suggested as an ISTD for retinol measurement, as it has a similar chemical structure to retinol and reactivity during sample preparation and analysis. The main challenge of deuterated retinol, however, is its instability and supply at a reasonable price (81). In this study, three potential ISTDs for retinol were investigated, including deuterated retinol, retinol acetate and tri-deuterated 25-OHD3 (25-OHD3-d3).

The penta-deuterated retinol (retinol-d5), from Isoscience[4], was tested as a retinol ISTD (transition ion 274→98 was chosen as the best response obtained for the retinol-d5). The retinol-d5 (2 μmol/L) was prepared in methanol containing an equivalent concentration of retinol and then injected into the LC-MS/MS 6490 using the vitamin A/E method. The responses (analyte peak areas) of the two compounds were then compared, revealing the

[4] Isoscience no longer produces deuterated retinol

analyte peak response of retinol-d5 to be much lower than the response obtained from the equivalent retinol concentration (Figure 3-9). This indicated the instability of retinol-d5, and hence, it could not be used for retinol quantification. During method development started in 2011, seeking out deuterated retinol from other sources was found to be highly difficult in terms of cost and availability. As a result, other potential retinol ISTDs were investigated.

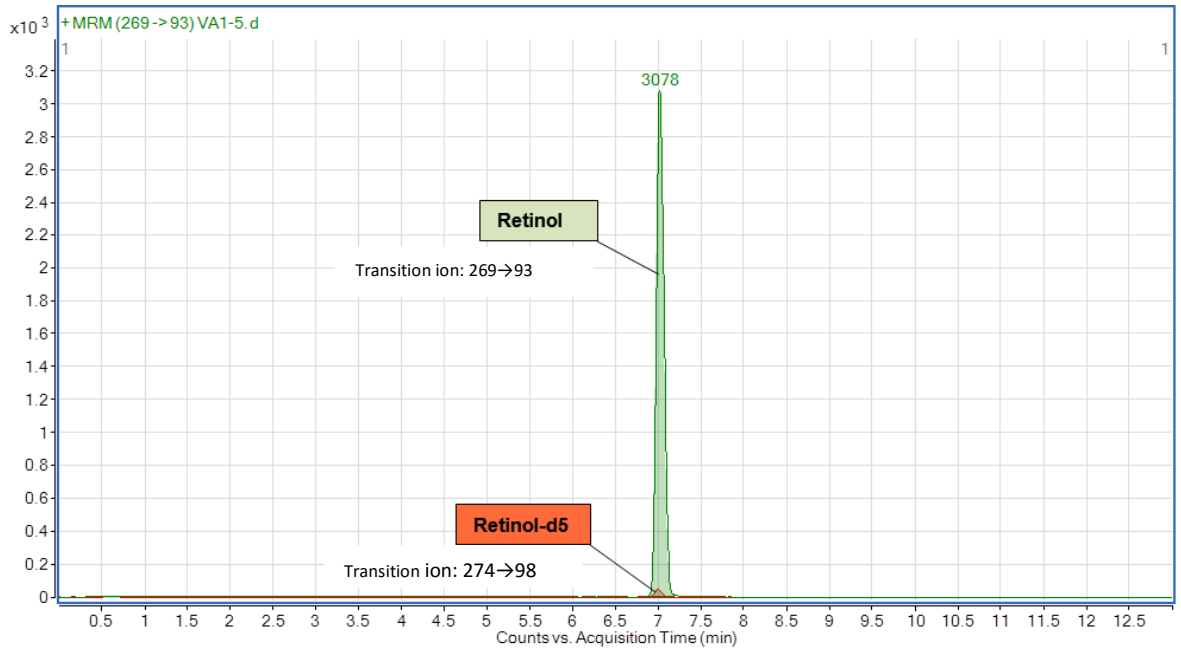


Figure 3-9. Responses of retinol and deuterated retinol.

Chromatogram shows equivalent retinol and deuterated retinol concentration (2 μmol/L) in a methanol sample. The peak response (area) of deuterated retinol (retinol-d5, transition 274→98 for the best response) was significantly lower than the retinol peak response (area), indicating the instability of retinol-d5. Therefore, the investigated deuterated retinol could not be used as the retinol internal standard. This experiment was done using LC-MSMS-6490.

Retinol acetate and 25-OHD3-d3 were prepared and investigated as potential retinol ISTDs. Retinol acetate is a classic ISTD utilised for retinol quantification as both are monitored at the same wavelength in HPLC methods, therefore, it was expected that retinol acetate could be inappropriate ISTD for mass-spectrometry methods. The 25-OHD-d3 has a closer retention time to retinol than retinol acetate. The LC-MS/MS system was optimised to separate retinol, retinol acetate and 25-OHD3-d3 (Figure 3-10).

Two retinol standard curves using the in-house vitamin A and E calibrator set[5] were created using retinol acetate and 25-OHD3-d3 as ISTDs. The linearity of the standard curve and results of the vitamin A/E controls were found to be affected by the ISTD used. The linearity of standard curve was better when 25-OHD3-d3 used as retinol ISTD (r^2=0.999) compared with retinol acetate (r^2=0.991) (Figure 3-11). We also observed that retinol concentrations in the commercial controls were higher than the expected manufacturer's assign values (low=0.5, mid=1.5 and high=4.0 µmol/L). When the retinol acetate applied as ISTD, the retinol results of the controls were 1.2, 5.9, 13.8 µmol/L, respectively, compared to 0.47, 1.7 and 4.1 µmol/L using 25-OHD3-d3 as ISTD. This experiment was repeated several times and the same findings were observed. We inferred that retinol acetate was not an appropriate ISTD to the difference in RT and resulted ion alteration variations. This is due to the possible difference of reactivity between retinol and retinol acetate during sample analysis using LC-MS/MS methods (151).

[5] The in-house vitamin A and E calibrator set preparation and validation will be discussed later in this chapter.

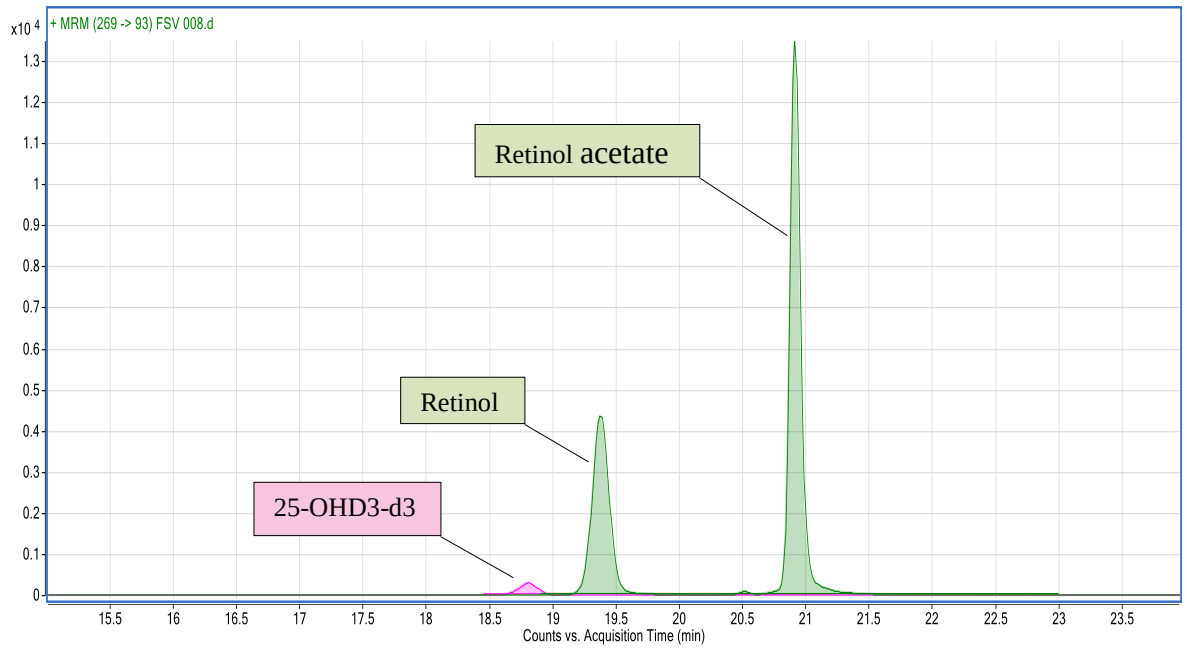
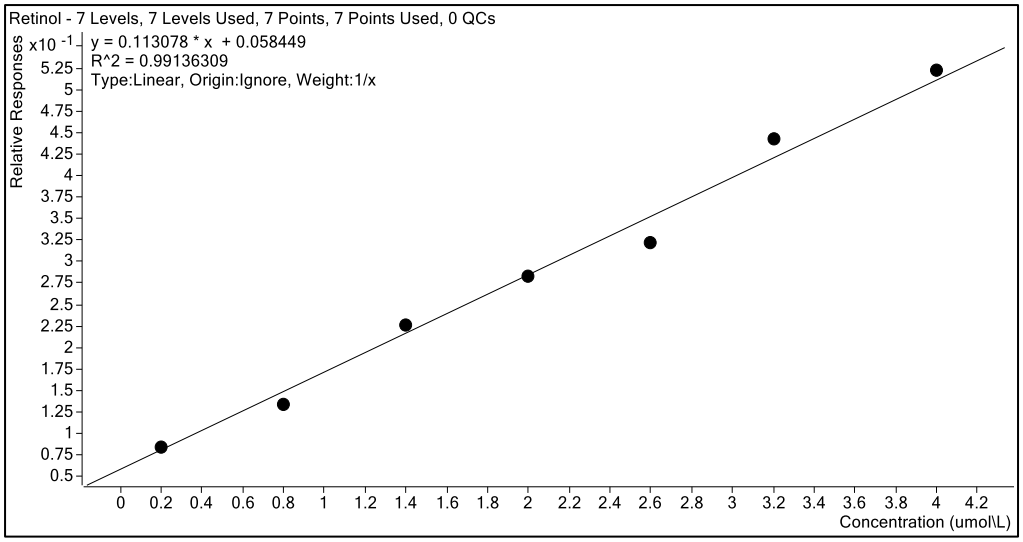
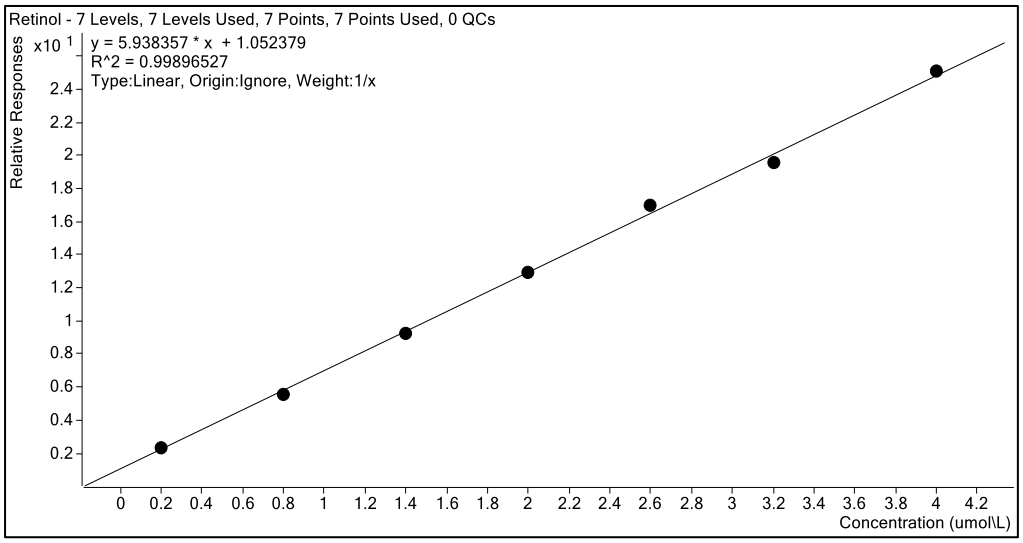


Figure 3-10. Chromatographic separation of retinol, retinol acetate and deuterated 25-hydroxyvitamin D3.

Retinol acetate, retinol and 25-OHD-d3 were successfully separated using the LC-MS/MS-6490 for the comparison of retinol acetate and 25-OHD3-d3 as potential retinol ISTDs. This experiment was done using LC-MSMS-6490.



a) Standard curve for retinol using retinol acetate as the internal standard



b) Standard curve for retinol using 25-OHD3-d3 as internal standard

Figure 3-11. Standard curves for retinol using different internals standards.

Retinal acetate and 25-OHD3-d3 were investigated as potential retinol ISTDs. Two standard curves were created using a set of in-house vitamin A and E calibrators. Standard curves were generated using a) retinol acetate and b) 25-OHD3-d3 as ISTDs. The linearity of standard curve was observed to be affected by the ISTD used. In addition, the retinol concentrations in commercial control levels were found to have higher values than accepted ones when retinol acetate was used as the ISTD compared to 25-OHD3-d3, which could be an indicator of the different reactive behaviours of retinol and retinol acetate. This experiment was done using LC-MSMS-6490.

Vitamin D analyte internal standard

The 25-OHD3-d3 was also investigated as a potential internal standard for the quantification of the three target vitamin analytes (25-OHD3, epi-25-OHD3 and 25-OHD2). The 25-OHD3-d3 was successfully used as an ISTD for the quantification of the three target vitamin D analytes (25-OHD3, epi-25-OHD3 and 25-OHD2) based on analysis of these analytes in the quality controls, linearity of standard curves and method validation (to be discussed later in the chapter).

α-Tocopherol internal standard

Hexa-deuterated α-tocopherol (α-tocopherol-d6) was investigated as a potential internal standard for α-tocopherol quantification. α-Tocopherol-d6 was successfully used as an ISTD for the quantification of α-tocopherol based on analyte quantification in the quality controls, linearity of standard curves, and method validation (to be discussed later in the chapter).

Final sample preparation protocol

The final sample preparation protocol was developed based on the development course detailed in this chapter. Concisely put, the samples were prepared as follows:

1) First, 100 µL of the sample was placed in a glass tube, 100 µL of Milli-Q water was added, and then the mixture was vortexed.
2) Following this, 200 µL of methanol, which contained tri-deuterated 25-OHD3 and hexa-deuterated α-tocopherol, was added, followed by vortexing.
3) This was then left at RT for 10 min under subdued light.
4) Thereafter, 1.5 mL of hexane was added and vortexed extensively prior to centrifugation.
5) The organic layer was transferred into a new glass tube and then dried under nitrogen gas at RT.
6) Later, the sample was reconstituted in 250 µL of methanol and then vortexed.
7) Finally, the samples were loaded into the LC-MS/MS system.

3.2.4 Calibrators

Calibrators are crucial elements in sample analysis for trueness and usually prepared in each batch to generate standard curves. Sample results are calculated based on these standard curves; therefore, calibrators should be traceable to the appropriate reference material. There is currently no commercial calibrator for entire FSV analytes, although separate commercial calibrators for vitamin D (25-OHD2 and 25-OHD3) and for vitamins A and E (retinol and α-tocopherol) are available from different manufacturers.

The calibrator choices in the current project were either to use separate commercial calibrators or to generate an in-house calibrator set for the entire FSV set in a cost effective and timely manner. A good calibrator choice was determined based on the linearity of the calibrator levels, the inaccuracy and imprecision of quality control results and external quality assurance monitoring. The following two sub-sections discuss the investigation of the two in-house calibrators, as well as two commercial calibrators for vitamin D analytes (RECIPE) and for vitamins A and E (Bio-Rad).

3.2.4.1 In-house calibrators

The key challenge in generation of an in-house calibrator set was finding a matrix that originated from human serum/plasma, free from endogenous FSVs and commutable to authentic human serum samples. In this project, two matrixes were investigated, SeraCon II stripped delipidated serum (SeraCon-DL) and SeraCon vitamin D depleted diluent (Sercon-DD). The SeraCon-DL matrix was free from endogenous retinol and α-tocopherol, but contained endogenous 25-OHD3, while the Sercone-DD matrix was free from endogenous target FSVs, based on the manufacturer check.

Therefore, SeraCon-DL matrix was used to prepare a set of seven calibrator levels for retinol (ranged 0.2-4.0 μmol/L) and α-tocopherol (ranged 6-50 μmol/L), as follows. The SeraCon-DL matrix was spiked with retinol stock 2 and α-tocopherol stock 1 to prepare pre-calibrator level 7 for retinol at a concentration of 8 μmol/L and another pre-calibrator level 7 for α-tocopherol at a concentration of 100 μmol/L.

These pre-calibrator levels were then gently mixed for 1 h and then kept on crushed ice in the refrigerator overnight. The next day, the two pre-calibrator levels were left for 30 min at room temperature (RT) prior to mixing. They were then diluted with SeraCon-DL to create the other six pre-calibrator levels for retinol (0.4, 1.6, 2.8, 4, 5.2, 6.4, 8 μmol/L for levels 1 to 6, respectively) and for α-tocopherol (12, 24, 40, 56, 70, 86, 100 μmol/L for levels 1 to 6, respectively). All pre-calibrator levels were gently mixed using the roller mixer for 1 h, prior to storing them at 4ºC in the refrigerator on crushed ice overnight. The next day, the pre-calibrators were kept at RT and then mixed for 30 min using a roller mixer.

Later, every pre-calibrator level of retinol was mixed with an equivalent pre-calibrator level of α-tocopherol to form a combined calibrator level. Each combined calibrator contained retinol and α-tocopherol analytes at different concentrations (retinol: 0.2, 0.8, 1.4, 2, 2.6, 3.2 and 4 μmol/L; α-tocopherol: 6, 12, 20, 28, 35, 43 and 50 μmol/L). The seven calibrator levels were mixed gently for 1 h using the roller mixer. Every single calibrator level was then aliquoted (110 μL) into a labelled polypropylene tube (1.5 mL) and stored at -80°C.

Standard curves for both analytes were generated and showed consistent linearity (r^2>0.999) (Figure 3-12). Quality control results were successfully monitored using a Levey–Jennings chart with respect to manufacturer-assigned values and Westgard rules through the project. In addition, this calibrator set was used to report vitamin A and E results of the external quality assurance samples to the Royal College of Pathologists of Australasia Quality Assurance

Programs (RCPAQAP). Based on RCPAQAP reports (cycle 27, 2013), our method imprecision was 9.3% and 5.9% for vitamin A and E, respectively (Figure 3-13). It is worthwhile to mention that the imprecisions were later improved at the end of method development and validation, as detailed later in this chapter.

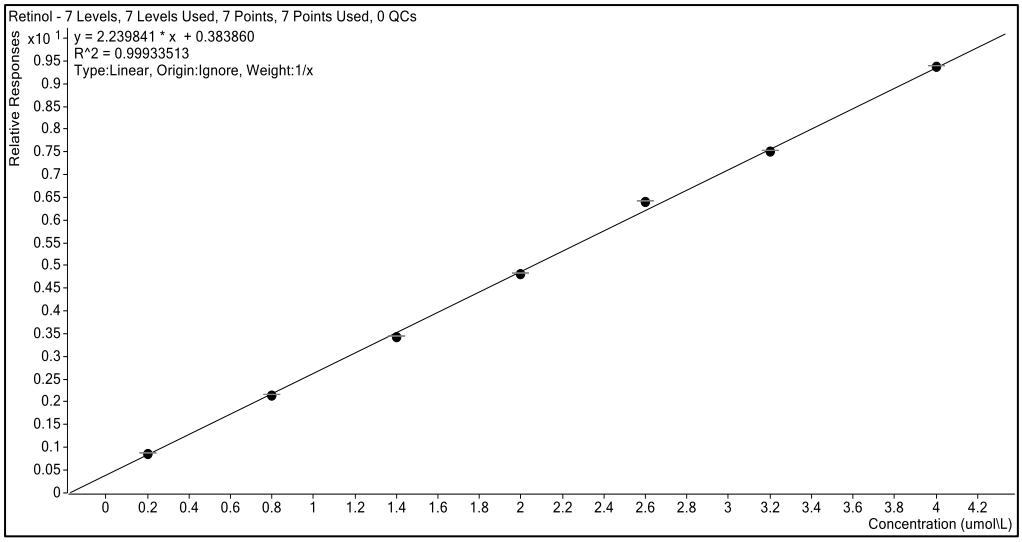
Another set of seven calibrator levels was also prepared for the three 25-OHD analytes (25-OHD3, epi-25-OHD3 and 25-OHD2), retinol and α-tocopherol using SeraCon-DD matrix. This matrix was spiked with the five FVS analytes to prepare calibrator 1 (cal-1: a concentration of 4.0 nmol/L for 25-OHD3, epi-25-OHD3 and 25-OHD2; and 0.10 μmol/L and 6.0 μmol/L for retinol and α-tocopherol, respectively). The calibrator 7 (cal-7: a concentration of 200.0 nmol/L for 25-OHD3, 160.0 nmol/L for epi-25-OHD3 and 25-OHD2, 4.0 μmol/L and 71.5 μmol/L for retinol and α-tocopherol respectively) was made. The two calibrators were gently mixed using a roller mixer for 1 h prior storing them at 4ºC on crushed ice overnight. The next day, they were kept at RT and then mixed for 30 min.

The other five calibrator levels were generated by mixing cal-1 and cal-7 in different proportions. The final level concentrations were 4.0, 9.9, 23.6, 62.8, 102.0, 160.8, and 200.0 nmo/L for 25-OHD3; 4.0, 8.7, 19.6, 50.8, 82.0, 128.8 and 160.0 nmo/L for the epi-25-OHD3 and 25-OHD2; 0.1, 0.2, 0.5, 1.3, 2.1, 3.2, 4.0 μmol/L for the retinol; and 6.0, 8.0, 12.5, 25.6, 38.7, 58.4 and 71.5 μmol/L for the α-tocopherol. Later, each calibrator level was mixed and aliquoted (110 μL) into labelled polypropylene tubes (1.5 mL) and stored at -80ºC.

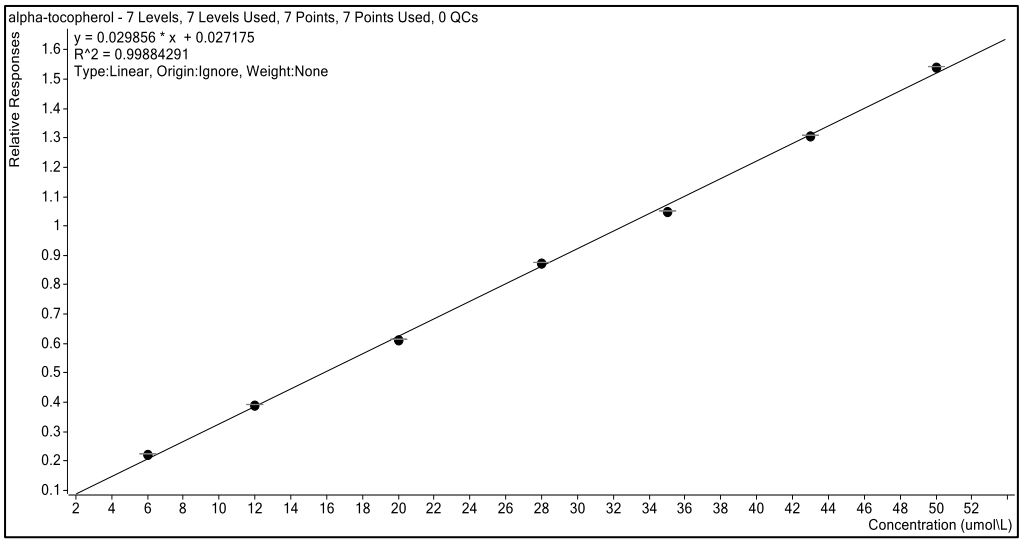
The in-house FSV calibrator set was investigated in eight batches on non-consecutive days. Standard curves were linear (r^2 >0.992) for all analytes. However, the commercial control results for retinol were not consistent with the manufacturer-assigned values for retinol (Table 3-3). This was an indication of unsuitability of the SeraCon-DD matrix for the entire

FSV in-house calibrator preparation. In other words, this in-house calibrator was not commutable with routine samples for retinol, and it did not fit our purpose.

To summarise, we initially aimed to generate a single in-house calibrator set for all the FSVs. However, our trials were not successful because of matrices inappropriate for this purpose. Consequently, the separate commercial calibrator sets for vitamin D analytes (from RECIPE) and for vitamins A and E (from Bio-Rad) were examined as the alternative.



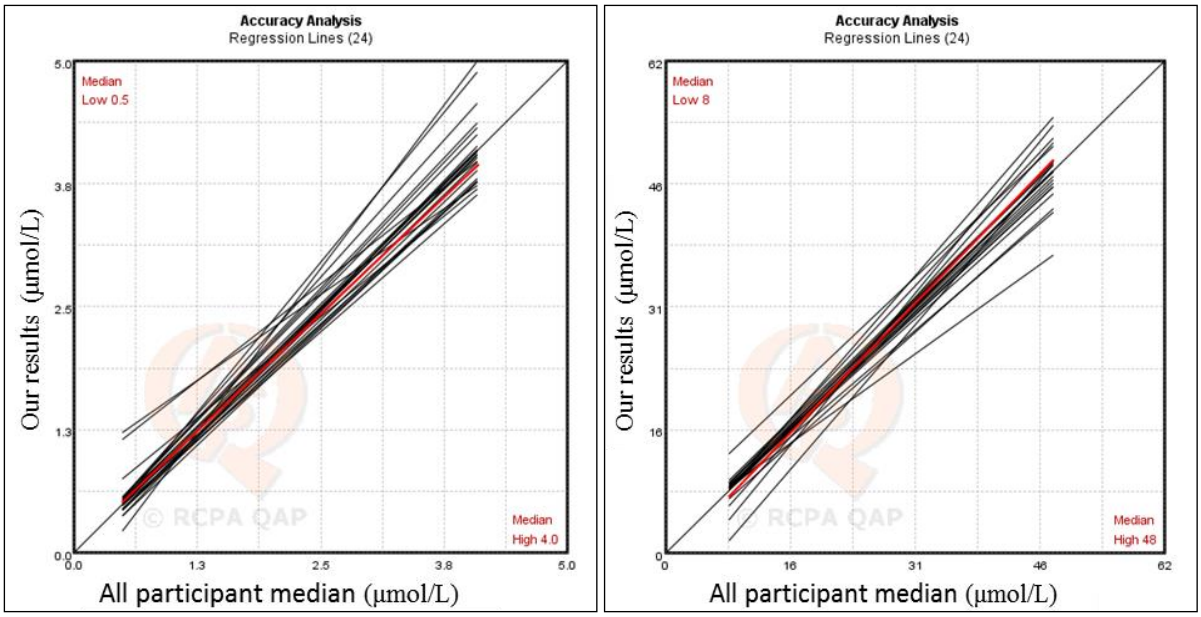
a) Retinol standard curve using in-house vitamin A and E calibrators



b) α-tocopherol standard curve using the in-house vitamin A and E calibrator set

Figure 3-12. Standard curves for retinol and α-tocopherol using in-house vitamin A and E calibrator set.

In-house vitamin A and E calibrator set was prepared from sercon-DL matrix that was free from endogenous vitamins A and E. Standard curves were created using the in-house calibrator set for a) retinol and b) α.tocopherol. Seven concentration points were plotted in the standard curves with linearity (r^2 >0. 999) using LC-MS/MS-6490.



a) Retinol b) α-Tocopherol

Figure 3-13. External quality assurance for vitamin A and E.

These graphs were obtained from RCPAQAP end of cycle 27 reports for vitamins A (retinol) and E (α-tocopherol) based on analysis of RCPA samples using the in-house calibrator for vitamin A and E. These graphs depict the regression line of our LC–MS/MS method as a red line compared to the all participant regression lines (n = 24) for a) vitamin A and b) vitamin E. Our method imprecision was 9.3% and 5.9% for vitamin A and E respectively.

Table 3-3. Monitoring of FSV controls results using in-house FSV calibrator set.

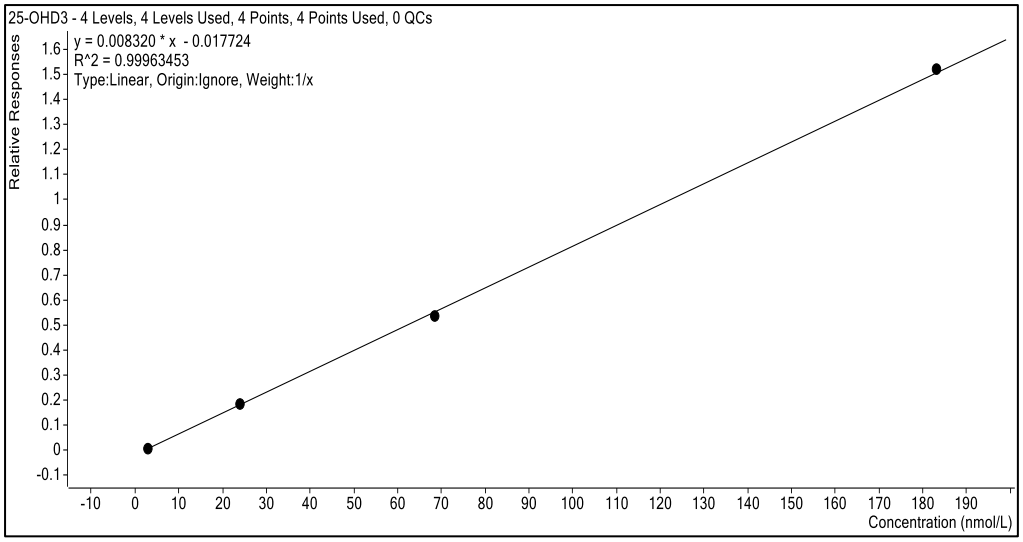
The in-house FSV calibrator set was prepared using SeraCon-DD matrix, which was free of endogenous FSVs, and examined by monitoring the FSV results of commercial controls as part of our calibrator set evaluation. This table demonstrates manufacturer-assigned values versus mean results of 25-OHD3, 25-OHD2, retinol and α-tocopherol obtained from analysis of eight control batches on non-consecutive days. The control results for retinol were not consistent with manufacturer-assigned values. In addition, the analyte imprecision results, especially for retinol, were high. This indicates the inappropriateness of a SeraCon-DD matrix for all vitamins.

	25-OHD3			25-OHD2			Retinol			α-Tocopherol		
	nmol/L			nmol/L			µmol/L			µmol/L		
Control	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3	QC1	QC2	QC3
Assign value	29	78	175	30	75	180	0.5	1.6	4.0	7	24	55
Mean	29	84	175	27	84	178	0.8	2.2	4.6	8	25	55
SD	2.3	2.9	6.6	2.8	2.5	13.8	0.1	0.2	0.6	1.1	1.5	2.8
CV%	7.9	3.5	3.8	10.5	3	7.8	11.9	11.4	12.9	13.6	6.1	5.1

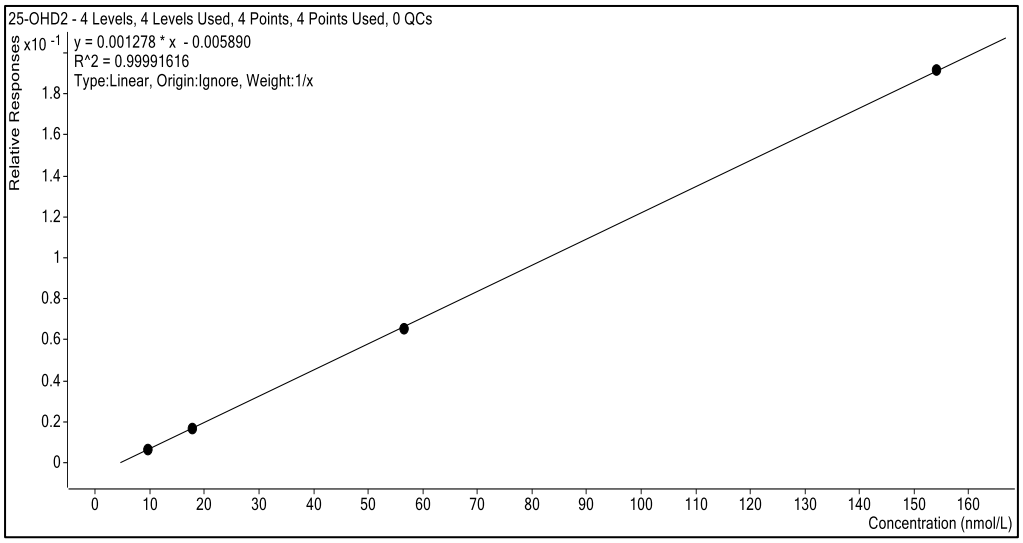
3.2.4.2 Commercial calibrators

Two commercial calibrators were investigated. A multilevel calibrator set for 25-OHD2 and 25-OHD3 (traceable to SRM 972, from RECIPE) was explored. Also, a uni-level calibrator for vitamins A and E (traceable to SRM 968e, from Bio-Rad) was tested.

Standard curves, which showed excellent linearity (r^2>0.999), were generated for analysis of 25-OHD analytes (Figure 3-14) and for retinol and α-tocopherol (Figure 3-15). Quality control results were successfully monitored using a Levey–Jennings chart with respect to the manufacturer-assigned values and Westgard rules (152). In addition, EQA reports showed excellent method performance was achieved using these calibrators. More details are discussed in the method validation section of this chapter. Therefore, these calibrators were used to conduct the studies detailed in the next chapters.



a) Standard curve used for 25-OHD3 and its epimer

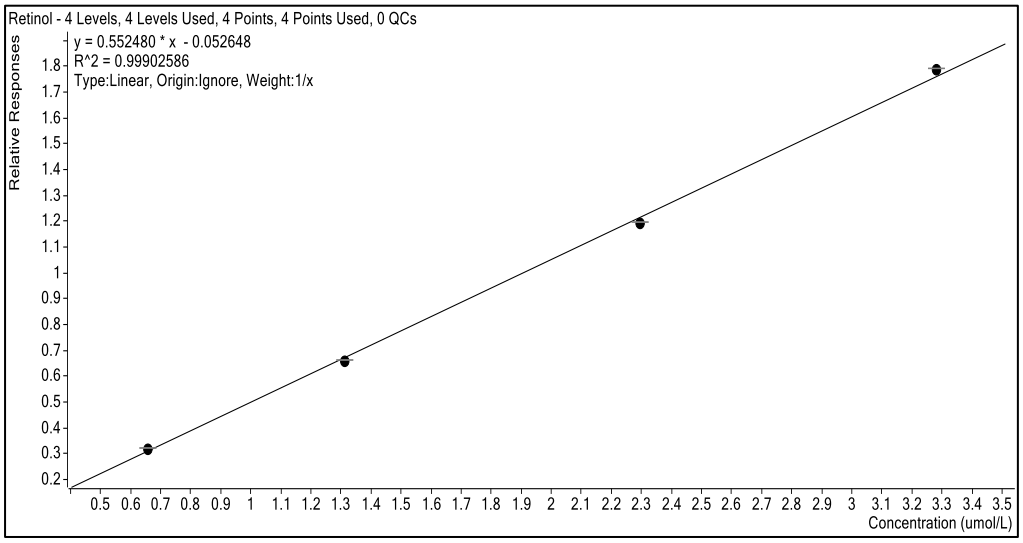


b) Standard curve used for 25-OHD2

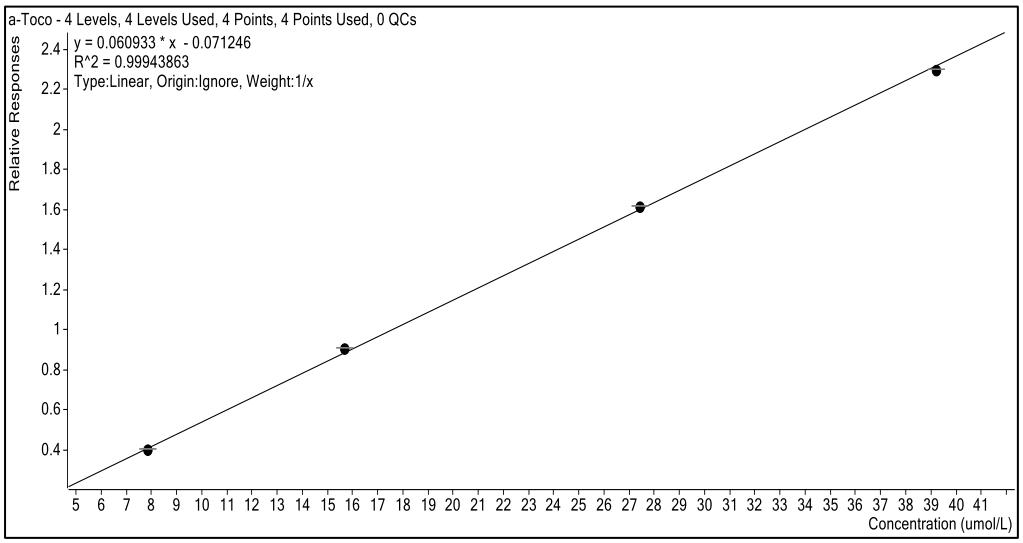
Figure 3-14. Typical standard curves for 25-hydroxyvitamin D analytes.

Typical standard curves were generated for a) 25-OHD2 and b) 25-OHD3 using a commercial calibrator from Recipe, which was traceable to NIST SRM-972. The standard curves showed excellent linearity (r^2 >0. 999) using four concentration points.

Vitamin A and E calibrator (Bio-Rad)



a) Retinol standard curve



b) α-tocopherol standard curve

Figure 3-15. Typical standard curves for retinol and α-tocopherol.

Typical standard curves were generated for a) retinol and b) α-tocopherol using dilutions of commercial calibrator from Bio-Rad, which was traceable to NIST SRM-968e. The standard curves showed excellent linearity (r^2 >0. 999) using the four concentration points.

3.2.5 Method validation studies

Method validation is an essential process aimed of providing objective evidence of method suitability for its intended use. In the current study, the FSV quantification methods were validated by using two Agilent LC-MS/MS systems. Method linearity, imprecisions, sensitivity, recovery, ion suppression and carryover were examined. All experiments of the validation process were conducted based on the final sample protocol and analysis as detailed in Chapter 2, sections 2.6 –2.8.

3.2.5.1 Standard curves

Method standard curve were generated for each run batch to quantify target analytes in blank (without ISTDs), blank-ISTDs (with ISTDs), quality controls and unknown samples. Standard curves for 25-OHD2 and 25-OHD3 were created using the RECIPE calibrator for 25-OHD2 and 25-OHD3, as mentioned earlier. Due to the unavailability of a commercial calibrator for epi-25-OHD3, the standard curve of the 25-OHD3 was also used for quantification of epi-25-OHD3, as both have similar physical and chemical properties. The standard curves for retinol and α-tocopherol were made using a Bio-Rad calibrator. The correlation coefficient (r^2) was > 0.999 for each calibration curve used in FSV quantification. The curve plots were not forced to origin and calculated using 1/x weighting (Figure 3-14, Figure 3-15) (116).

3.2.5.2 Reportable range

Reportable range is the ability of a measurement assay within a specific analyte concentration range to generate results that are directly proportional to the analyte concentration in specific samples (153). For this study, seven concentration levels of each analyte, which covered more than the common clinical FSV concentration ranges, using samples prepared for in-

house calibrators against the Recipe and Bio-Rad calibrators were used to determine the reportable range. The analytical linear ranges (at least) were 4–200 nmol/L for 25-OHD3, 4–160 nmol/L for epi-25-OHD3 and 25-OHD2, 0.2–4.0 μmol/L for retinol and 6–72 μmol/L for α-tocopherol (Figure 3-16, Figure 3-17).

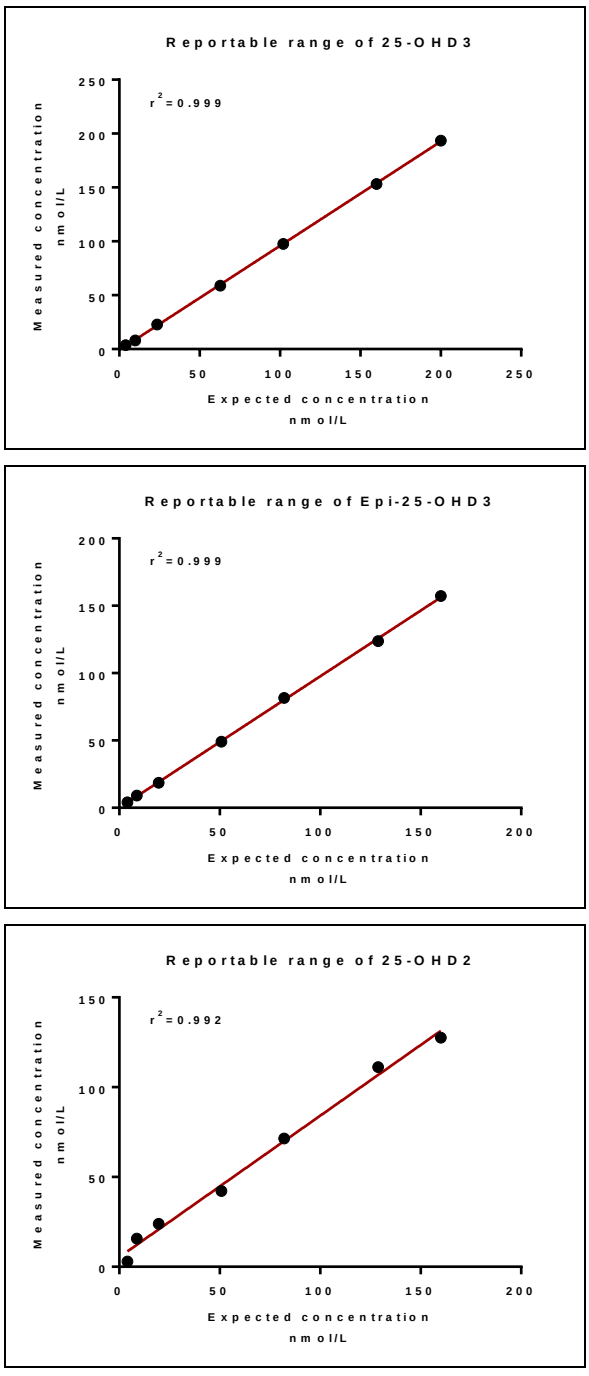


Figure 3-16. Reportable range of 25-OHD analytes in FSV methods.

These graphs show Reportable range of the 25-OHD3 (4-200 nmo/L, r^2=0.999) and its epimer (4-160 nmo/L, r^2=0.999) and 25-OHD2(4-160 nmo/L, r^2=0.992).

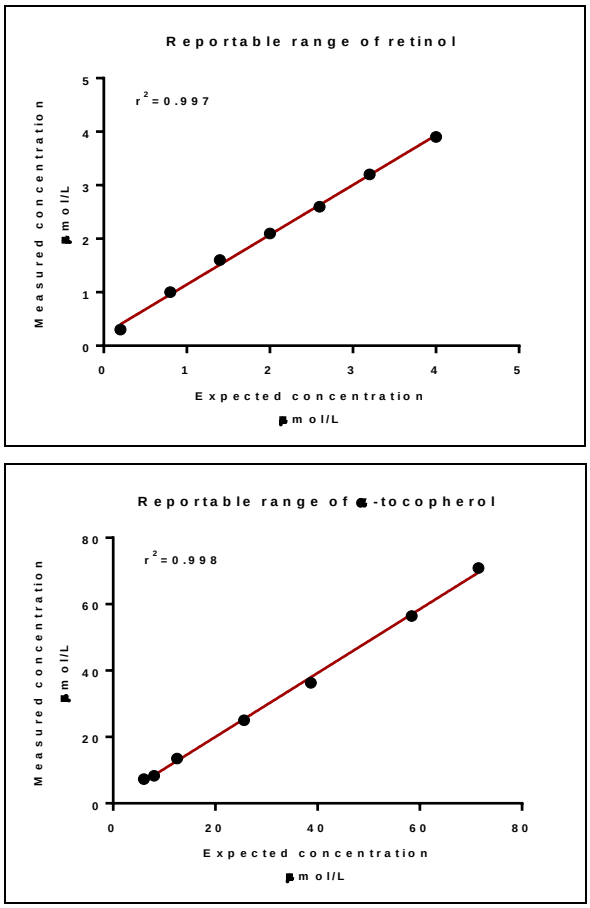


Figure 3-17. Reportable range of retinol and α-tocopherol in FSV methods.

These graphs show Reportable range of the retinol (0.1-4.0 μmo/L, r^2=0.997) and α-tocopherol (6-72 μmo/L, r^2=0.998).

3.2.5.3 Imprecision

Imprecision is an essential element of the method validation and indicates closeness of agreement among the analyte results when samples are repeatedly analysed using a specific analytical method. The following study aimed to determine the method intra-run imprecision (repeatability within-run) and inter-run imprecisions (repeatability between-runs) for each investigated analyte using the developed methods.

For the vitamin A/E method, the intra-imprecision study was conducted using RCPAQAP material (n=15; vitamin program, cycle 26, samples 26.06 and 26.09). These samples were prepared based on the final sample preparation protocol and sample analysis (see Chapter 2, sections 2.7, 2.8 and 2.9). It was found that the intra-run imprecision for α-tocopherol was 4.8% at 34 μmol/L and 3.0% at 51 μmol/L, while the inter-run imprecision was 8.1% at 22 μmol/L and 5.8% at 53 μmol/L. In contrast, because of using retinol acetate as ISTD, the retinol imprecision was not satisfied (<17.5%). However, this was improved after using 25-OHD3-d3, as revealed by the RCPAQAP end-of-cycle report (over all imprecision: 9.3% for vitamin A and 5.9% for vitamin E) (Figure 3-13).

The intra-run imprecisions for FSV method-1 and 2 were determined using serum samples spiked with FSVs and the three control, respectively. The inter-run imprecision of the FSV methods 1 and 2 was determined based on the cumulative data generated from running three control sets with every batch for at least 20 days, unless otherwise mentioned. All the samples were prepared and analysed according to the final sample preparation protocol and sample analysis (see Chapter 2, sections 2.7, 2.8 and 2.9). The imprecision results for the two methods were detailed in Table 3-4 and Table 3-5.

Table 3-4. Analyte imprecision for FSV method-1.

This table shows intra-run and inter-run imprecision of the five FSV analytes using FSV method-1 utilising Agilent LC-MS/MS 6490.

Compound	Intra-run imprecision			Inter-run imprecision		
	n=15			n¶=15		
	Low	Mid	High	Low	Mid	High
25-OHD3 CV% (mean nmol/L)	4.3 (48)	2.5 (98)	2.2 (187)	7.8 (27)	7.2 (77)	4.5 (167)
3-epi-25-OHD3 CV% (mean nmol/L)	5.4 (40)	2.5 (78)	2.0 (152)	14.3 (16)	8.0 (50)	7.8 (148)
25-OHD2 CV% (mean nmol/L)	4.2 (50)	4.2 (80)	2.2 (160)	8.1 (26)	6.8 (71)	5.5 (148)
Retinol CV% (mean nmol/L)	5.9 (0.2)	3.7 (0.5)	3.5 (3.50)	8.6 (0.5)	6.7 (1.7)	6.9 (3.7)
α-Tocopherol CV% (mean nmol/L)	4.5 (9)	5.5 (34)	3.0 (66)	7.1 (6)	5.8 (23)	5.2 (54)

¶ Samples were analysed in one replicate a day over 15 non-consecutive days for all metabolites.

Table 3-5. Analyte imprecision for FSV method-2.

This table shows intra-run and inter-run imprecision of the five FSV analytes using FSV method-1 utilising Agilent LC-MS/MS 6410.

Compound	Intra-run imprecision			Inter-run imprecision		
	n=15			n=30		
	Low	Mid	High	Low	Mid	High
25-OHD3 CV% (mean nmol/L)	4.7 (25)	3.14 (68)	2.64 (150)	3.3 (26)	3.1 (73)	3.1 (162)
3-epi-25-OHD3 CV% (mean nmol/L)	6.5 (11)	4.4 (46)	3.2 (142)	5.2 (11.6)	4.8 (46)	3.9 (141)
25-OHD2 CV% (mean nmol/L)	8.0 (20)	4.9 (57)	3.9 (128)	9.5 (22.6)	7.6 (65)	5.4 (141)
Retinol CV% (mean nmol/L)	4.7 (.51)	3.8 (1.67)	2.9 (3.39)	5.4 (0.51)	5.3 (1.54)	4.7 (3.41)
α-Tocopherol CV% (mean nmol/L)	5.5 (5)	4 (21)	4.4 (54)	7.4 (6)	6.2 (22)	5.8 (52)

3.2.5.4 Recovery

The recovery study purposed to evaluate the amount of analyte recovered post-sample preparation. Spiked and non-spiked human serums were used this study. The serum samples used were prepared as follows. The serum was completely thawed, equilibrated to RT, and then mixed for 30 min. The serum was then spiked with FSV at concentrations of 140 nmol/L for both 25-OHD2 and epi-25-OHD3, 130 nmol/L for 25-OHD3, 0.5 μmol/L for retinol and 10 μmol/L for α-tocopherol. The spiked and non-spiked serums were gently mixed using a roller mixer for 1 h. They were then stored in the refrigerator overnight at 4°C on crushed ice. The next day, the samples were equilibrated to RT and mixed for 30 min prior to preparation and loading into the LC-MS/MS. The recovery results were 87% for 25-OHD3, 94.1% for epi-25-OHD3, 112.9% for 25-OHD2, 91% and 100.9% for α-tocopherol.

The analyte recovery percentage (R%) was determined by calculating the concentration difference between the analytes measured in the spiked and non-spiked serums divided by the concentration added to the spiked serum:

Equation 3-1. Calculation of analyte recovery

$$\text{Recovery (R\%)} = \frac{Measured\ concentration\ - \text{endogenous concentration}}{\text{Concentration added}} \times 100$$

3.2.5.5 Sensitivity

The sensitivity of the analyte measurement by LC-MS/MS was evaluated by calculating the limit of detection (LoD) and limit of quantification (LoQ). According to the Clinical and Laboratory Standards Institute (CLSI), LoD is defined as the "lowest amount of analyte in a sample that can be detected with (stated) probability, although perhaps not quantified as an exact value" (118). LoQ is defined as the "lowest amount of analyte in a sample that can be quantitatively determined with stated acceptable precision and trueness, under stated

experimental conditions" (118). In the current work, LoD was considered at the lowest analyte concentration at which the analyte peak was detected at a signal-to-noise ratio (S/N) ≥ 3. That concentration was more than 3SD of analyte concentration in blank. The LoQ was considered at the lowest analyte concentration at which the analyte peak was detected at S/N ≥10, and method imprecision and accuracy were at < 20% and 80–120%, respectively (116).

To determine the LoD and LoQ, three sets of samples with different FSV analyte concentrations were prepared according to Honour's recommendations with minor modifications (116). The first sample set was prepared to determine the LoD and LoQ of 25-OHD2 and 25-OHD3 using the low level of vit D control, which was diluted with Milli-Q water at different diluting factors (1/2, 1/4, 1/8 and 1/16) and then mixed. The second sample set was used to test the method sensitivity of the epi-25-OHD3 analysis. This sample set was prepared by diluting the low level of vit epi-D with Milli-Q water in two diluting factors (1/3 and 1/5) and then mixing. The third sample set was prepared to determine the method's sensitivity to retinol and α-tocopherol analysis using low level of the vitamin A/E controls, which was diluted with Milli-Q water in different diluting factors (1/2, 1/4 and 1/8) and then mixed.

All controls and diluting samples were mixed using a roller mixer for 30 min prior to conducting the sensitivity studies. Each low control level sample, diluted sample and blank were prepared in triplicate according to the final sample preparation protocol and sample analysis (see Chapter 2, sections 2.7, 2.8 and 2.9). The determined LoD and LoQ for each analyte are illustrated in detail in Table 3-6.

Table 3-6. Method sensitivity using Agilent 6490 and Agilent 6410.

Compound	Agilent 6490		Agilent 6410	
	LoD†	LoQ††	LoD†	LoQ††
25-OHD3 (nmol/L)	1.7	3.4	2	3.5
3-epi-25-OHD3 (nmol/L)	1.9	3.8	2	3.5
25-OHD2 (nmol/L)	1.6	3.3	2.5	5
Retinol (µmol/L)	0.1	0.1	0.1	0.16
α-Tocopherol (µmol/L)	1	2	1	3

† LoD: Limit of Detection, †† LoQ: Limit of Quantitation

3.2.5.6 Ion suppression

Phospholipids are encountered as one of the causes of ion suppression in chromatographic-mass spectrometry methods, because of their obstruction of the efficiency of ionisation. Phospholipids are endogenous biological matrices, and they should be minimised through sample clean-up. Therefore, potential ion suppression from phospholipids was progressively monitored through two transition ions (104→104 and 184→184) (116). It was found there was no chromatographic effect on FSV peaks (Figure 3-18).

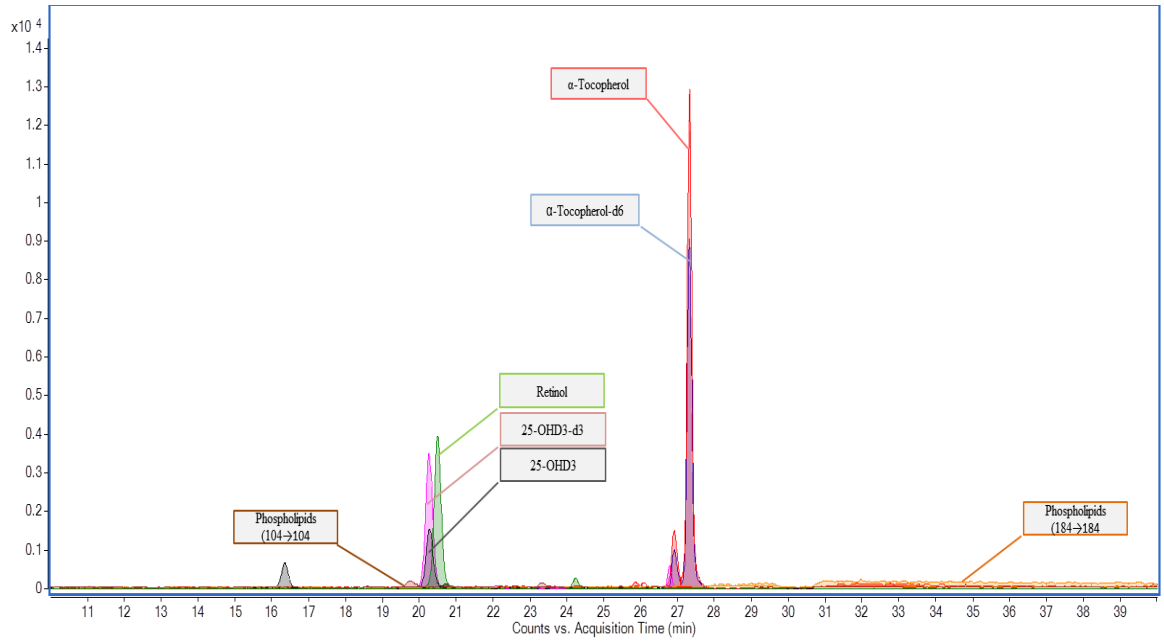


Figure 3-18. Effect of phospholipids on FSV separation.

Phospholipids are considered a potential cause of ion suppression, whereby they impede the efficiency of ionisation. Therefore, ion suppression from phospholipids was progressively monitored through two transition ions (104→104 and 184→184). This chromatogram shows that there was no chromatographic effect on FSV peaks.

3.2.5.7 Carryover

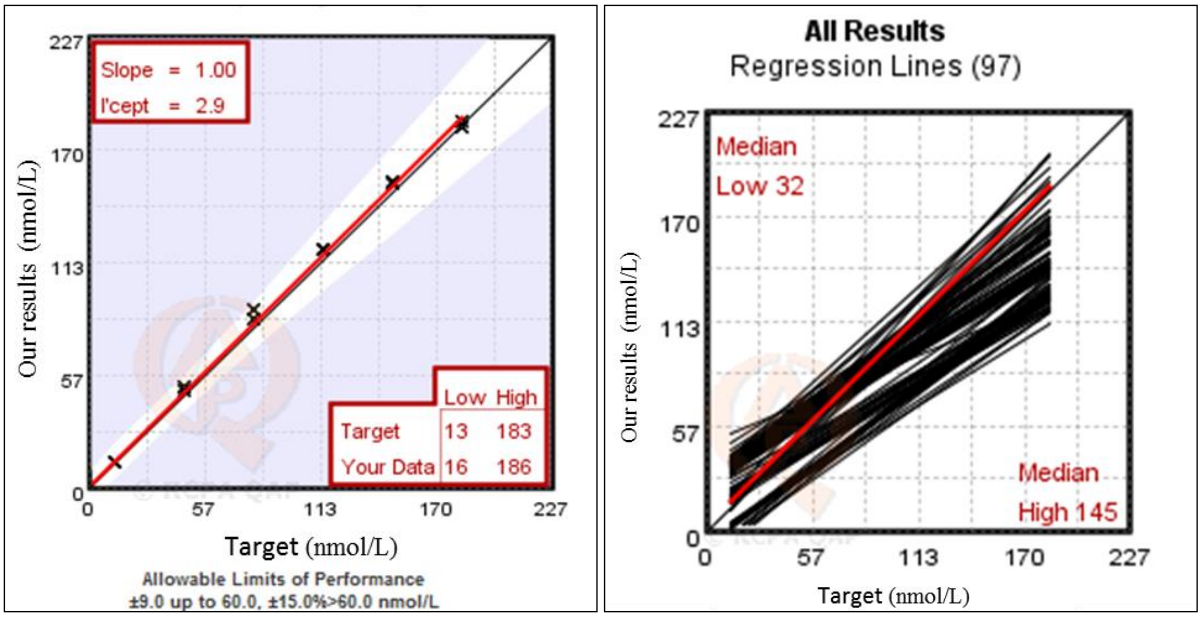
This study aimed to investigate the potential impact of a previous sample analysis on subsequent sample results, because carryover influences method imprecision and inaccuracy. Based on the Honour recommendations (116), carryover was assessed using samples with low and high FSV concentration levels. The SeraCon-DD and human serum spiked with 25-OHD2, 25-OHD3 and epi-25-OHD3 were used as the samples with low and high FSV content, respectively.

These samples were prepared according to the final sample preparation protocol. The low-level sample was injected 10 times sequentially; then the high and low samples were alternately injected 10 times. The carryover was evaluated based on the mean difference between concentration obtained from the sequential and alternate injections. The mean analyte concentration of the sequential and alternate injections and the percentage difference the two injection groups were calculated. It was observed that carryover was less than 0.55% over all investigated analytes (25-OHD2 and epi-25-OHD3 (0.1%), 25-OHD3 (0.55%), retinol (0.01%) and α-tocopherol (0.13%)).

3.2.6 External quality assurance

The performance of simultaneous FSV quantification methods was progressively evaluated. Our laboratory participated in two external quality assurance (EQA) programs since beginning of method development. The two programs (Endocrine and Vitamins programs) were offered by RCPAQAP.

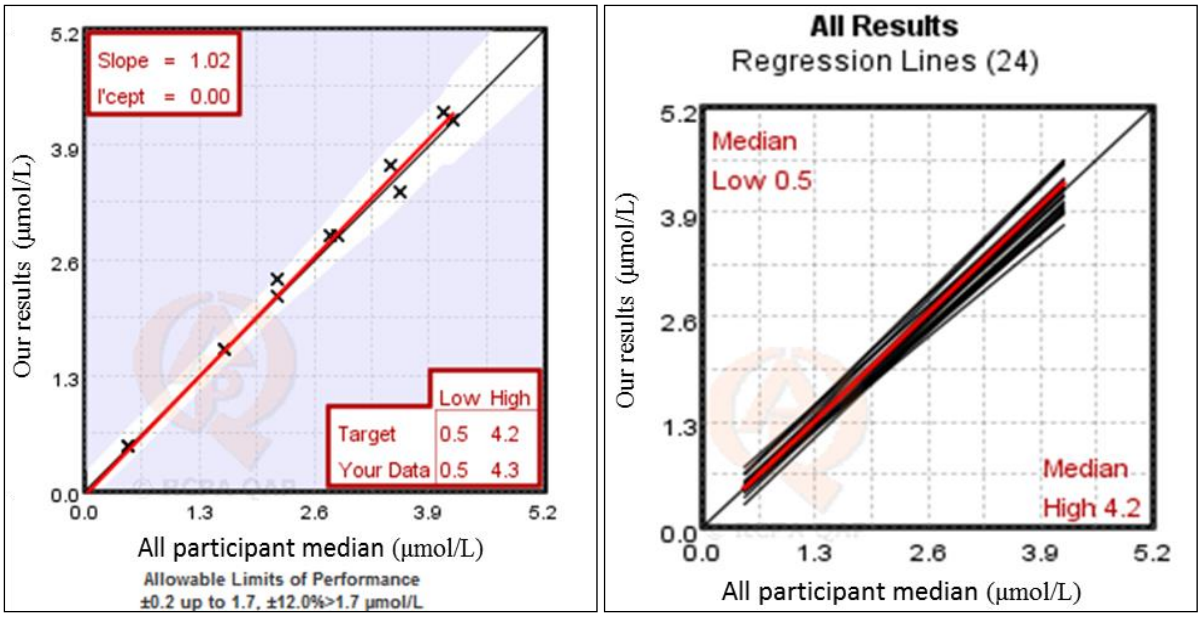
Our laboratory joined the Endocrine program (for 25-OHD3 and other 17 analytes) and Vitamin program (for retinol, α-tocopherol and 3 other analytes) from RCPAQAP. The last RCPAQAP reports showed good method performance, which reflects the final sample preparation protocol and FSV methods 1 and 2 performances. The simultaneous FSV method 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, 2014); 5.0% and 0.04 μmol/L, respectively, for retinol; and 4.7% and 0.2 μmol/L, respectively, for α-tocopherol according to the end-of-cycle report (cycle 29, 2013) (Figure 3-19, Figure 3-20, Figure 3-21).



a) b)

Figure 3-19. Typical external quality assurance report for 25-OHD.

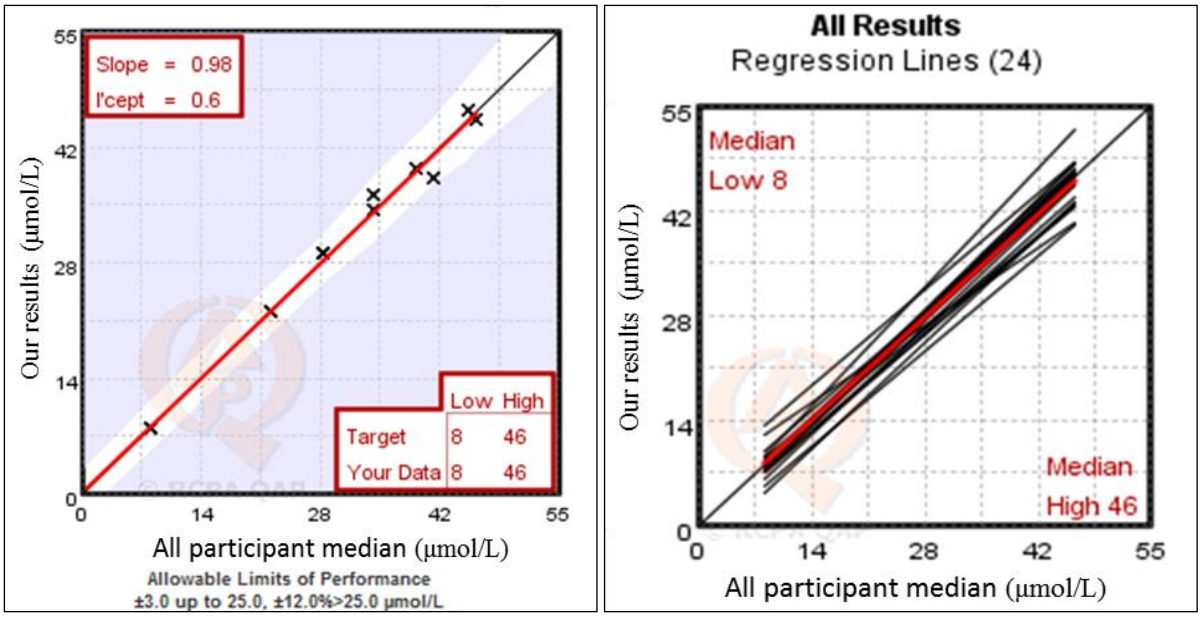
a) 41 RCPAQAP end-of-cycle report for 25-OHD3 (Vitamin D3) shows results obtained by our laboratory compared with target values. Results within the white area are within allowable limit of performance; b) 41 RCPAQAP end-of- cycle report for 25-OHD3 demonstrates the regression line of our LC–MS/MS method, the red line, compared to all participant regression lines (n = 97). The other 96 laboratories measured vitamin D3 by immunoassay and LC-MS/MS platforms.



a) b)

Figure 3-20. Typical external quality assurance report for retinol.

a) 29 RCPAQAP end-of- cycle report for vitamin A (retinol) shows results obtained by our laboratory compared with all participants' median. Results within the white area are within allowable limit of performance; b) 29 RCPAQAP end-of- cycle report for vitamin A demonstrates the regression line of our LC–MS/MS method, the red line, compared to all participants' regression lines (n = 24). The other 23 laboratories' reports measured vitamin A by HPLC with UV/Vis or PDA detection.



a) b)

Figure 3-21. Typical external quality assurance report for α-tocopherol.

a) 29 RCPAQAP end-of- cycle report for α-tocopherol (vitamin E) show results obtained by our laboratory compared with all participant median. Results within the white area are within allowable limit of performance; b: 29 RCPAQAP end-of- cycle report for vitamin E demonstrate the regression line of our LC–MS/MS method, the red line, compared to all participants' regression lines (n = 24). The other 23 laboratories' reports measured vitamin E by HPLC with UV/Vis or PDA detection.

3.3 Discussion

In this study, we introduced the precise simultaneous fat-soluble vitamin (FSV) measurement procedure for quantification of the five FSV analytes. This procedure entailed development of the sample preparation protocol for FSV extraction from serum and the establishment of the simultaneous FSV quantification methods using two LC-MS/MS systems. Several technical issues in the simultaneous FSV measurement procedure were explored during development. Furthermore, this procedure was independently evaluated by participation in two RCPAQAP programs, including the Endocrine and Vitamin programs. The current section discusses challenges and results of the overall procedure in the development process.

3.3.1 Sample preparation

Serum sample preparation for LC-MS/MS analysis is not done just to extract the FSV from blood but also to reduce the matrix effect. For example, endogenous protein and phospholipids affect the quality of analyte chromatographic separation, ionisation and mass spectrometry quantification (113). Hence, the performance of the LC-MS/MS method is strongly influenced by the extent of sample clean-up. In this work, PPE and LLE were used in the sample preparation. The PPE is useful for eliminating sample proteins and to liberate the hydrophobic vitamins from their binding proteins. The LLE is required for further sample cleaning-up by separating the compounds based on their solubility in a specific organic solvent (154). Both techniques are commonly used for human serum FSV extraction prior to chromatographic analysis (142, 155) (Table 3-7, Table 3-8, Table 3-9).

Several organic solvents are classically used for PPE and LLE in HPLC methods; nonetheless, not all organic solvents are compatible with the LC-MS/MS systems. Acetonitrile, ethanol and methanol are water-miscible with close polarity to each other and

are routinely utilised for serum protein precipitation (130, 155). During the development stage, we chose the organic solvents that are compatible with LC-MS/MS analysis. Methanol was used in the PPE process because it is the same solvent that was used in preparation of FSV stocks including deuterated ISTDs, and miscible with hexane (for LLE) as well as it is compatible with mobile phases. For LLE of interesting vitamins, hexane is a common extraction solvent (130, 142) because it is water-immiscible and lighter than both water and methanol. The mixture of hexane with another organic solvent, such as 2-propanol, has been used for vitamin D extraction (138, 145, 146). This 2-propanol is miscible with water, methanol and hexane, and it is lighter than water. These physical properties of these solvents may improve FSV extraction from serum. However, our experiment showed better extraction for 25-OHD analytes and retinol (but not for α-tocopherol) with the absolute hexane compared to the mixture with 2-propanol. Accordingly, hexane was used as an extraction solvent because of its better recovery in retinol and low levels of 25-hyroxylated vitamin D analytes.

The current study also investigated the effect of adding water to the serum prior to sample preparation to explore some knowledge gaps (81). We found that the FSV extraction was improved with water added to the serum sample. Potentially, the addition of equivalent volumes of the aqueous phase and organic phase during the early sample preparation stage may offer a better opportunity to extract liberated hydrophilic analytes into the hexane layer during the vortexing of the sample.

Internal standard

Internal standards (ISTDs) are utilised for the accuracy and precision of analytical methods. One of advantages of mass spectrometry technology is that compounds and their isotopes (ISTDs) are distinguished based on their mass to charge ratio differences (81, 116, 155). This

allows elution of interested compound and its isotope to be at the same the retention time, which is benefit for analyte quantification using mass spectrometry. However, one practical challenge of the ISTD choice is the availability of matched stable isotopes at a reasonable price (81, 116, 155).

In the current work, stable isotopic-labelled ISTDs were successfully utilised, including the 25-OHD3-d3 (for quantification of the three 25-hydroxylated vitamin D analytes) and α-tocopherol-d6 (for quantification of α-tocopherol). For retinol quantification, retinol-d5 and retinol acetate were investigated as potential retinol ISTD but they did not work well because of their instability and different reaction behaviour respectively. The 25-OHD3-d3 was found that it is an optimal alternative ISTD because it had the closest retention time to retinol (81). This work introduced 25-OHD3-d3 as a reasonable and effective solution for retinol quantification with precise results.

The number of deuterated sites is important for method specificity. Triplet to hexa-deuterated ISTD is recommended but not di-deuterated ISTD, which may be naturally present in samples and that leads to false increasing in deuterated ISTD detection (156). In the present study, the tri-deuterated 25-OHD3 (transition ions: 404→386) was chosen instead of hexa-deuterated 25-OHD3 to avoid potential interferences. Transition ions 407→389 and 407→159 used for hexa-deuterated 25-OHD3 might be interfered with isobars that could be formed during the fragmentation process (138, 150). Since the inception of this study some LC-MS/MS advocates have critiqued the use of the higher isotopic ISTDs as their chromatographic behaviour may be slightly different to the analyte of interest. We did not find this to be an issue in α-tocopherol-d6 for vitamin E quantification based on the method performance evaluation including the external quality assurance reports.

Traditional ISTDs used in HPLC methods are usually chosen partially based on the criterion that ISTDs and target analytes are detected at the same emission wavelength, therefore, they are not necessarily recommended ISTDs for LC-MS/MS methods. Non-isotope ISTDs for quantification of hydroxylated vitamin D analytes were utilised by few methods (138, 157, 158). These ISTD types are not recommended for chromatographic-mass spectrometry methods, as they are co-eluted at significantly different retention times (155). Major discrepancies in the retention time and chemical structure of analyte and its matched non-isotope ISTD reflect their different physicochemical behaviours during sample analysis. This problem was observed when we explored retinol acetate (classic retinol ISTD in HPLC methods) as ISTD for retinol as expected.

Calibration

Choosing an appropriate matrix matched calibrator is a crucial part of method development (discussed in Chapter 5). In the current work, we initially aimed to generate an in-house calibrator set for the five target FSV analytes and then tracing them to available reference material from the National Institute of Standards and Technology (NIST). This process allows generating calibrator set for the target five FSV analytes with wide range of analyte concentration levels that are not commercially available. Also, it significantly reduces calibration costs by dispensing with two separate commercial calibrators (a set for vitamin D analytes not including epi-25-OHD3 and another set for retinol and α-tocopherol). However, the main issue was to find a suitable matrix that was free from endogenous FSV.

For this purpose, we gravimetrically prepared in-house calibrator sets using pure chemical of FSV with two matrices: SeraCon-DL and SeraCon-DD, however, neither were suitable for the entire FSVs. SeraCon-DL contained endogenous 25-OHD3 but almost free from retinol and α-tocopherol. Hence, SeraCon-DL was suitable matrix to generate in-house calibrator set

for retinol and α-tocopherol but not for 25-OHD3. It seems that stripped delipidation process of the SeraCon-DL was not enough to eliminate endogenous 25-OHD3 of serum as it is proved to be difficult task (159).

While the Sercone-DD matrix was free from endogenous target FSVs, the in-house calibrator set prepared using the Sercone-DD matrix did succeed in validation processes. By using this in-house calibrator set, retinol results of the commercial controls were not consistent with the manufacturer-assigned values for retinol. This was an indication of unsuitability of the SeraCon-DD matrix for the entire FSV in-house calibrator preparation. In other words, this in-house calibrator was not commutable with routine samples for retinol, and it did not fit our purpose. The extensive process of vitamin D depletion conducted in Sercone-DD matrix may have affected retinol-binding proteins, which is required for the stability of spiked retinol. Since the combined prepared in-house calibrator set for FSVs was unsuccessful, we used two commercial calibrators from RECIPE (for 25-OHD2, 25-OHD3 and epi-25-OHD3) and from Bio-Rad (for retinol and α-tocopherol).

3.3.2 Liquid chromatography-mass spectrometry

The LC-MS/MS separation technology is considered to be highly sensitive because of its integrated powers of liquid chromatography and mass spectrometry. However, simultaneous FSV quantification using LC-MS/MS encounters several challenges, including the selection of suitable column and ionisation type, especially with non-polar or weak polarity molecules and interferences (e.g. isobars).

Columns

The C18 columns are classically used for the chromatographic separation of FSV, especially for vitamins A and E (142, 148, 149, 160, 161). However, this column does not have

selectivity for adequate separation of epimers. The epimer of 25-OHD3,which is present in the majority of human serum samples (134-141), has become important to be quantified to avoid misleading of vitamin D results. Alternative column types, such as Chiral, cyanopropyl (CN) and particularly pentafluorophenyl (PFP) have been used in several recently published methods demonstrating ability to resolve epi-25-OHD3 from 25-OHD3 (134, 138, 141, 145, 146, 162, 163). By using PFP column, analyte separation takes longer compared with the C18 column (164). Generally, a longer run time improves analyte signal intensity and increases S/N although both sample clean-up and chromatographic optimisation are crucial to improve method sensitivity (114).

In this study, we investigated C18 and PFP columns; the latter demonstrated better chromatographic resolution for the entire FSVs. Although retinol and α-tocopherol were successfully resolved with a low injection volume (1 μL) using the C18 column, 25-OHD2 and 25-OHD3 were not sufficiently resolved even with higher injection volume, which compromised the resolution of the retinol and α-tocopherol peaks. This may be due to selection of a short C18 column (20 mm) that had insufficient power to eliminate ion suppression (as discussed later). We did not test a longer C18 column, as it was unlikely to separate the isomer of 25-OHD3 (164). Though 25-OHD2 and 25-OHD3 are co-eluted earlier compared to the PFP and CN columns (164), the C18 column is readily contaminated, and this increases baseline noise (165). We found that the C18 column used in our vitamin A/E method had a shorter life (≈ 1000 injections) compared with the PFP column used in our FSV methods 1 and 2 (>3000 injections).

The flow rate of mobile phases impacts on peak resolution and ionisation, and both influence method sensitivity. A low flow rate (≤ 0.2 mL/min) improves molecule ionisation and columns with 1.0 to 2.1 mm diameter are suitable for this purpose (113). Using capillary

tubes in the LC system has been suggested for improving chromatographic resolution and sensitivity (113). In this study, we found that 0.2 mL/min was the optimal flow rate for symmetrical peaks resolution of analytes using the PFP column (2.1 mm diameter). In addition, we minimised the dead volume by using short capillary tubes with a small internal diameter (in the LC system as much as practicable.

Ionisation

The ionisation process is critical for the specificity and sensitivity of mass spectrometry analysis. This process converts molecules [M] to charged ions, and the molecule [M] usually accepts a proton $[M+H]^+$ or loses a proton $[M-H]^-$. The ionisation process can be influenced by ion suppression or ion enhancement. The mobile phase buffer or any other additives, which are used to improve chromatography, may interfere with the ionisation process and cause ion suppression or ion enhancement (166).

Two types of ionisation, which are commonly used in ion sources for biological samples (167), include electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) (151). Both are considered as a soft ionisation as low electrical energy is applied to molecules during the ionisation process to avert excessive molecule fragmentation (113). We developed our LC-MS/MS methods using positive ESI, which is more commonly used for vitamin D analyte quantification (155), especially in recently published studies (Table 3-9). The APCI may contribute conversion of some vitamin D metabolites, such as 25-hydroxyvitamin D3 sulphate[6] to 25-OHD3 (169). This conversion was overcome by using ESI (138).

[6] 25-hydroxyvitamin D3 sulphate is a blood circulating metabolite (168)

Interferences

Quantification of FSVs using LC-MS/MS methods encounter potential interferences, such as isobars and epimers (155). Isobars and epimers are compounds that form the same m/z of the target compound during the ionisation and fragmentation process. For 25-OHD3, 1-OHD3 and 7α-hydroxy-4-cholesten-3-one (endogenous and therapeutic agent isobars, respectively) as well as the epimer of 25-OHD3 were reported as possible interferences (138). These Isobars and isomers could cause chromatographic overlap, especially in short chromatographic run times (170). In our FSV methods, we utilised the PFP column, which is capable of separating 25-OHD3 and its epimer as well as 25-OHD2, retinol and α-tocopherol. Furthermore, the potential isobars (1-OHD3 and 7α-hydroxy-4-cholesten-3-one) for 25-OHD3, were chromatographically excluded, as they are co-eluted of different retention times (138).

Another potential source of interference in the mass spectrometry method was the tubes used in the sample preparation. We observed that one brand of polypropylene tube (Biocentrix) showed higher responses of 25-OHD3 (ion enhancement) compared to the other investigated brand of polypropylene tubes (Sarstedt) and glass tubes by 12% and 25% respectively. In contrast, the first polypropylene brand showed lower responses (ion suppression) of retinol by 15% and 7% compared with other polypropylene brand and glass tubes respectively. Furthermore, α-tocopherol responses were lower when Biocentrix tubes (-44%) and Sarstedt tubes (-40%) were used compared to glass tubes. Chen and colleagues examined two brands of polypropylene tubes and two types of glass tubes. They found that one polypropylene tube brand caused ion enhancement when using transition ions 401→383, while the other polypropylene tube brand and the investigated glass tubes showed no interference (150). The ion suppression and enhancement affect the linearity of the standard curves. Clare and

colleagues reported that quantification of 25-OHD3 and 25-OHD2 using transition ions 401→383 and 413→395 respectively, showed unsatisfactory analyte chromatography and imprecision (145), but they did not state the type of tubes used in their method. Furthermore, it was recently reported that there was no significant difference between mean 25-OHD3 results obtained utilising water loss transition ions versus alternative transition ions used for 25-OHD3 quantification across 65 laboratories (171).

Phospholipids

Phospholipids could be a source of interference (ion suppression) due to their effect on ionisation efficiency. An accumulation of phospholipids on the chromatographic columns reduces column lifespans and may cause changes in retention time. Optimal sample clean-up should minimise the phospholipid content in the analysed specimen. Furthermore, sample clean-up and chromatographic optimisation are crucial for improving detection and sensitivity (114). Therefore, it is strongly recommended to check the transition ions of phospholipids (104→104 and 184→184) in prepared samples for analysis (116). In the current method, these transition ions were monitored in prepared serum samples (cleaned-up samples), and there were no coeluted phospholipids with our interested analytes. This is an indication of optimal sample extraction..

3.3.3 Method performance

Our FSV methods showed good performance, including sensitivity, imprecision and reportable ranges for the five analytes. These were compared with peer method groups by participating in quality assessment programs (RCPAQAP) for 25-OHD3, retinol and α-tocopherol. Based on the RCPAQAP reports, our simultaneous FSV methods displayed excellent imprecision (3.0%, 5.0% and 4.7% for 25-OHD3, retinol and α-tocopherol,

respectively) and inaccuracy (average bias, 3.2 nmol/L, 0.04 μmol/L and 0.2 μmol/L for 25-OHD3, retinol and α-tocopherol, respectively).

There are previously published methods simultaneous quantified vitamins D, A and E (148, 149). Comparing performance of our method with these published methods is demanding because of limited data provided of these published methods. Priego-Capote and colleagues (2007) created a detection method for 25-OHD3, 25-OHD2, retinol and α-tocopherol as well as six other metabolites (148). However, Priego-Capote and colleagues did not utilise isotope-labelled ISTD and qualitative transition ions in their method. This raised doubts about their method's reported performance. Furthermore, they used a serum sample volume of 1000 μL, which is a large sample volume, especially for compromised patients. They provided a limited amount of data for validating the method. Conversely, Midttun and colleagues (2011) developed a method that utilised isotope-labelled ISTD and qualitative transition ions with a relatively small sample volume (50 μL) to quantify 25-OHD3, 25-OHD2, retinol and α-tocopherol (149). However, none of these methods resolved the epimer of 25-OHD3, which has become a more important issue in clinical laboratories; both method were not independently evaluated EQA programs (Table 3-7).

In the literature, several published methods that measured retinol and α-tocopherol simultaneously are available, though they are relatively older methods (Table 3-8). Andreoli and colleagues (2004) developed a method quantifying retinol, α-tocopherol and β-carotene utilising LC-MS/MS (147) but isotope-labelled ISTD was not used in this method. Other methods that quantified retinol and α-tocopherol using liquid chromatography techniques were published in 2009 (160, 161). However, full validation and performance data of these three methods were not provided, which makes it difficult to reliably compare the methods. Our methods generally showed better performance than their methods (Table 3-8).

Throughout the last decade, LC-MS/MS methods for the quantification of 25-OHD2 and 25-OHD3 in blood used in clinical laboratories have been growing. However, only 5% of the 65 laboratories, whose staff members participated in questionnaires, could distinguish epimer 25-OHD3 from 25-OHD3 (171). Unlike published methods for retinol and α-tocopherol, additional LC-MS/MS methods for 25-OHD2 and 25-OHD3 measurements were recently available with sufficient data of method validation and performance. Even though our method quantified five FSV analytes simultaneously, the these methods showed good performance compared with most recent published methods for 25-OHD analyte quantification, as shown in Table 3-9.

Table 3-7. Specification and performance of published blood FSV measurement methods.

		Published methods			
Reference no. , year		FSV method-1, (2015)	FSV method-2 , (2015)	(149), 2011	(148), 2007
Platform		LC-MS/MS Agilent 6490	LC-MS/MS Agilent 6410	LC-MS/MS	LC-MS/MS
Analyte		25-OHD2 25-OHD3 Epi-25-OHD3 Retinol α-Tocopherol	25-OHD2 25-OHD3 Epi-25-OHD3 Retinol α-Tocopherol	25-OHD2 25-OHD3 Retinol α-Tocopherol	25-OHD2, 25-OHD3, Retinol, α-Tocopherol & other 6 metabolites
Sample volume (µL)		100	100	50	1000
Sample preparation		PPE, LLE	PPE, LLE	LLE	LLE
Calibrators (matrix)		Recipe (Vit D) Bio-Rad (vit A and E	Recipe (Vit D) Bio-Rad (vit A and E	IH (spiked sera)	IH (spiked sera)
Column (size, mm)		PFP (2.0×150)	PFP (2.0×150)	C18 (4.6×50)	C18 (4.6×150)
Ionisation		ESI +	ESI +	ESI +	ESI +
25-OHD2	Quant ions	413→395	413→395	413→395	413→395
	LoQ (nmol/L)	3.3	5	6.6	1
	Intra-CV (mean nmol/L, n)	4.2 (50, n=15)	4.9 (57, n=15)	4.3 (113, n=24)	3.17 (ns, n=ns)
	Inter-CV (mean nmol/L, n)	6.8 (71, n=15)	7.6 (65, n=30)	4.6 (113, n=19)	4.5 (ns, n=ns)
25-OHD3	Quant ions	401→383	401→383	401→383	383→159
	LoQ (nmol/L)	3.4	3.5	6.6	1.5
	Intra-CV (mean nmol/L, n)	2.5 (98, n=15)	3.1 (68, n=15)	4.6 (57, n=24)	6.4 (ns, n=ns)
	Inter-CV (mean nmol/L, n)	7.2 (77,15)	3.1 (73, n=30)	8.2 (57, n=19)	11.5 (ns, n=7)
Epi-25-OHD3	Quant ions	401→383	401→383	NQ	NQ
	LoQ (nmol/L)	3.8	3.5	-	-
	Intra-CV (mean nmol/L, n)	5.4 (40, n=15)	4.4 (46, n=15)	-	-
	Inter-CV (mean nmol/L, n)	8.0 (50, n=15)	4.8 (46, n=30)	-	-
Retinol	Quant ions	269→93	269→93	269→93	269→93
	LoQ (µmol/L)	0.10	0.16	0.2	0.17
	Intra-CV (mean µmol/L, n)	3.7 (0.5, n=15)	4.7 (0.51, n=15)	3.8 (2.19, n=24)	3.17 (ns, n=ns)
	Inter-CV (mean µmol/L, n)	8.6 (0.5, n=15)	5.4 (0.51, n=30)	6.2 (2.19, n=19)	5.1 (ns, n=7)
α-Tocopherol	Quant ions	433→167	433→167	433→167	431→165
	LoQ (µmol/L)	2	3	NS	1
	Intra-CV (mean µmol/L, n)	5.5 (34, n=15)	4.0 (21, n=15)	2.4 (38, n=24)	4.3 (ns, n=ns)
	Inter-CV (mean µmol/L, n)	5.8 (23, n=15)	6.2 (22, n=30)	5.3 (38, n=19)	6.1 (ns, n=7)
Comment				- LoQ was considered as two times of LOD	- No deuterated ISTD - LOQs was calculated based on a minimal value of S/N ratio of 10

IH: In-house calibrators, NS: not stated, NQ: not quantified by method, ND: not determined

Table 3-8. Specification and performance of published blood retinol and α-tocopherol measurement methods.

		Published methods		
Reference no,, year		(147), 2004	(161), 2009	(160), 2009
Platform		LC-MS/MS	HPLC	UHPLC
Analyte		Retinol, α-Tocopherol ß-Carotene	Retinol α-Tocopherol	Retinol, α-tocopherol ß-Carotene and CoQ10
Sample volume (µL)		60	200	500
Sample preparation		PPE	LLE	PT, LLE
Calibrators (matrix)		IH (spiked sera)	IH (Organic solvent)	IH (Organic solvent)
Column (size, mm)		C8 (4.6×150)	C18 (4×200)	C18 (2.1 × 50)
Ionisation		APCI+	NA	NA
Retinol	Quant ions	269→93	NA	NA
	LoQ µmol/L	NS	0.17	0.08
	Intra-CV (mean µmol/L, n)	3.9	2.7 (1.9, n=10)	2.1 (2.4, n=10)
	Inter-CV (mean µmol/L, n)	5.7	4.6 (1.9, n=24)	6.1 (2.5, n=20)
α-Tocopherol	Quant ions	430→165	NA	NA
	LoQ µmol/L	NS	2.2	1
	Intra-CV (mean µmol/L, n)	2.1	2.9 (9, n=10)	2.3 (31, n=10)
	Inter-CV (mean µmol/L, n)	4.5	3.2 (10,n=25)	6.7 (31, n=20)
Comment		-No deuterated ISTD used		- LOQ= 10×(standard error for the y estimate)/ slope)

IH: In-house calibrators, NS: not stated, NA: Not applicable, NQ: not quantified by method, ND: not determined

Table 3-9. Specification and performance of a number of published vitamin D measurement methods.

		Published methods				
Reference no. , year		(172), 2014	(145), 2013	(173), 2013	(173), 2013	(146), 2012
Platform		LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-HR/MS	LC-MS/MS
Analyte		25-OHD2 25-OHD3	25-OHD2 25-OHD3 Epi-25OHD3	25-OHD2 25-OHD3	25-OHD2 25-OHD3	25-OHD2 25-OHD3 Epi-25-OHD3 other 5 metabolites
Sample vol (µL)		140	50	150	150	1000
Sample prep		LLE	LLE	PP,SPE	PP,SPE	LLE
Calibrators (matrix)		IH (plasma)	Chromsystems	Chromsystems	Chromsystems	IH (saline with human albumin)
Column (size, mm)		Phenyl (2.1×50)	PFP (3×50) & PFP (3×150)	CN (2.1×100)	CN (2.1×100)	Chiral (2×150) & C18 (2.1× 100)
Ionisation		ESI	ESI $^{+}$	ESI $^{+}$	ESI $^{+}$	ESI $^{+}$
25-OHD2	Quant ions	413→355	413→83	413→395	413→395	413→395
	LoQ nmol/L	NS	ND	15.5	5	NS
	Intra-CV % (nmol/L, n)	8.5 (65-90*, ns)	2.1 (36.3,10)	ND	ND	2.7 (40, NS)
	Inter-CV % (nmol/L, n)	1.7 (65-90*, n=5)	7.0 (75, 28)	3.5 (44,10)	3.4 (45, 10)	3.9 (40, NS)
25-OHD3	Quant ions	401→159	401→159	401→383	401→383	401→383
	LoQ nmol/L	6	2	8.5	4	NS
	Intra-CV % (nmol/L, n)	4.6 (70-95*, NS)	1.1 (45,10)	2.0 (43, 6)	2.0 (42, 6)	2.7 (40, NS)
	Inter-CV (nmol/L, n)	9.1 (70-95*, 5)	4.9 (73, 28)	4.6 (41, 10)	4.7 (42, 10)	3.7 (40, NS)
Epi-25-OHD3	Quant ions		401→159	NA	NA	401→383
	LoQ nmol/L		2	NA	NA	NS
	Intra-CV % (nmol/L, n)		5.3 (6.8, 10)	NA	NA	3.1 (40, NS)
	Inter-CV % (nmol/L, n)		NS	NA	NA	10.9 (40, NS)
Comment		* Manufacture ranges of controls				

IH: In-house calibrators, NS: not stated, NQ: not quantified by method, ND: not determined, ESI: Electrospray ionisation, APCI: Atmospheric Pressure Chemical Ionization.
a:LoQ calculated as double of LoD, b:manufacture ranges.

Continue Table 3-9.

		Published methods			
Reference no. , year		(134), 2012	(141), 2011	(162), 2011	(138), 2011
Platform		LC-MS/MS	LC-MS/MS	LC-MS/MS	LC-MS/MS
Analyte		25-OHD2 25-OHD3 Epi-25OHD3 24,25-(OH)2-D3)	25-OHD2 25-OHD3 Epi-25OHD3	25-OHD3 Epi-OHD3	25-OHD2, 25-OHD3, Epi-25-OHD3, 2 isobars
Sample volume (μL)		200	250	250	NS
Sample preparation		PP	PP & SPE	LLE	LLE
Calibrators (matrix)		IH (organic solvent)	Chromsystems	IH traceable to SRM2972	Chromsystems
Column (size, mm)		PFP (150×4.6)	PFP (2.1×100)	C4 (2.1×50) CN (2.1×250)	C18 ((2.1×100) Chiral (2×150)
Ionisation		APCI	ESI^+	ESI^+	ESI^+
25-OHD2	Quant ions	413→159	413→159	413→159	413→377
	LoQ nmol/L	3.9 in ethanol	2	1.5	NS
	Intra-CV (nmol/L, n)	<5, (NS, 10)	NS	2 (64, 10)	9.1 (63, NS)
	Inter-CV ,(nmol/L, n)	<4 (NS, 10)	NS	1.1 (64, 10)	8.6 (63, NS)
25-OHD3	Quant ions	401→159	401→159	401→159	401→383
	LoQ nmol/L	4 in ethanol	3.5	1.7	NS
	Intra-CV (nmol/L, n)	<5 (NS, 10) in solvent	4.2 (39, 4)	2.2 (46, 10)	4.6 (63, NS)
	Inter-CV (nmol/L, n)	<4 (NS, 10) in solvent		0 (46,10)	6.7 (63, NS)
Epi-25-OHD3	Quant ions	401→159	401→159	401→159	401→365
	LoQ nmol/L	2 in ethanol	NS	NS	NS
	Intra-CV (nmol/L, n)	<5 (NS,10) in solvent	NS	NS	NS
	Inter-CV (nmol/L, n)	<4 (NS, 10) in solvent	NS	NS	NS
Comment					

IH: In-house calibrators, NS: not stated, NQ: not quantified by method, ND: not determined, ESI: Electrospray ionisation, APCI: Atmospheric Pressure Chemical Ionization.
a:LoQ calculated as double of LoD, b: manufacture ranges.

3.4 Conclusions

In the current work, an efficient, precise method for the simultaneous quantification of five analytes (25-OHD3 and its epimer, 25-OHD2, retinol and α-tocopherol) was developed and validated. This quantification included robust, simple, inexpensive sample preparation as well as quantification methods using two modes of LC-MS/MS systems with different capacities for analytical sensitivity. These methods showed excellent performance, including sensitivity, imprecision and peer reviewed methods, as displayed by the RCPAQAP reports for 25-OHD3, retinol and α-tocopherol. In addition, commercial calibrators and controls that are commonly used in routine clinical laboratories for FSV measurement were utilised. The robust simple sample preparation and LC-MS/MS quantification methods utilised commercial calibrators and controls help routine clinical biochemistry laboratories to reproduce sample preparation and LC-MS/MS methods for reference or routine FSV analysis.

Chapter 4

Stability of fat-soluble vitamins

Chapter 4 Stability of fat-soluble vitamins

4.1 Introduction

The stability of fat-soluble vitamins (FSVs) in blood, especially vitamin A (retinol) and vitamin E (α-tocopherol), represents a gap in our knowledge. Although several factors are known to impact vitamin stability, such as sample storage and transportation, precisely how they do so remains inconclusive. The sample analysis journey starts with collecting a patient's blood sample, which usually occurs in a primary healthcare facility or an inpatient ward. Then, the sample is transported to a centralised laboratory. Transportation and storage conditions (e.g. temperature, light and time) might enhance redundancies in the in vitro reactions or cause red blood cell damage, called "haemolysis". Haemolysed blood samples influence analyte measurement results, especially for analytes like enzymes (174) and electrolytes (175). In primary healthcare, pre-transportation centrifugation is an improper practice because it increases the probability of a mismatch between the whole blood sample and the serum/plasma samples as well as increases the risk of infection and pre-analytical timeframes (176). The stability of analytes in blood and serum/plasma provides valuable information. It is required to prepare suitable protocols for blood collection, transportation and storage. In turn, these samples are used in clinical studies, laboratory diagnostics and long-term sample storage, such as bio-bank samples.

Currently, FSVs in blood samples are treated as labile analytes, especially in the cases of retinol and α-tocopherol. Therefore, a specific protocol for sample collection, transport and storage is used to control several crucial factors, such as light exposure, temperature, storage conditions and time (95-97). For example, blood samples are usually collected in amber tubes or tubes wrapped with aluminium foil, kept in subdued light conditions and transported on ice

and in insulated containers to centralised laboratories. The serum/plasma is obtained as soon as possible to avoid any potential negative impacts from delayed separation prior to being stored at -20ºC or -80ºC until the time of analysis.

Data of FSV stability is limited, and some studies' results contradict each other. For instance, a study indicated that changes in whole blood retinol and α-tocopherol at room temperature (RT) for 72h were -9.8% and -1.0%, respectively (98). Another study reported that changes in whole blood retinol and α-tocopherol at RT after for 1 week were 1.8% and 4.8%, respectively (97).

4.1.1 Systematic literature review for FSV stability

Consequently, a systematic review was conducted using published studies that focus on the stability of vitamins A, D and E to explore this knowledge gap and to design an experimental study. For this systematic review, PubMed and Scopus databases were used to identify the English language studies published between January 1980 and January 2015. The keyword search included the terms "vitamin A", "retinol", "vitamin D", "calcifediol", "calcidiol", "cholecalciferol", "25-hydroxyvitamin D", "vitamin E" and "tocopherol". Articles relevant to FSV stability were retrieved by reviewing article titles and then abstracts/full articles (Figure 4-1). In addition, reference lists of relevant articles were investigated for any missing studies.

The relevant articles were examined and their results were summarised (Table 4-1, Table 4-2). 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 all factors that influence sample stability, especially during FSV extraction and post-extraction. Furthermore, some results are contradictory. In addition, most of the results were

based on methods that were not fully validated and not subject to peer reviewed comparisons through external quality assurance program (EQA) participation. This may partially explain some of the contradictory results concerning FSV stability. Although light, temperature and time are potentially impact the stability of FSV analytes, no studies have simultaneously examined those factors in different matrices: in the form of whole blood, serum and extracts. In addition, no studies have compared FSV levels in samples processed in ambient light at RT with FSV levels in samples processed in subdued light at RT.

Based on the available data in the literature, 25-OHD, frozen serum/plasma (at -70°C to -20°C) for retinol and α-tocopherol analysis remains stable for 1 to 5 years (177, 178). This study examined frozen conditions for 1 month to obtain reliable comparisons between storage conditions at different temperatures.

In previous studies, the stability of retinol in the whole blood and serum sample remains inconclusive. Two studies reported that changes in whole blood retinol samples at RT were between -15% and -10% for 3 days of storage (98, 179), while another study reported that the change was 3% for 7 days of storage (97). In addition, changes in whole blood retinol samples at chilled temperatures were 3% (98) and -2% (97) after the samples had been stored for 3 days and 7 days, respectively. For serum/plasma samples, retinol concentration changes at RT were 0% for 1 week of storage (180), -7% for 2 weeks of storage and -17% for 4 weeks of storage (177). Other studies monitored the stability of retinol in chilled temperatures for up to two days and found minor changes in retinol concentrations (181, 182). The stability of the extracted retinol was reported in only a single study, which found that concentration changes were -4% at RT and 0% at a chilled temperature after 2 days of storage (183).

The effects of fluorescent light on retinol concentration in whole blood samples were -2% (95) at RT after 2 days. Another study reported that the changes were -7% and -2% (97) at

RT and chilled temperatures after 1 week, respectively. In contrast, changes in plasma retinol under the same conditions were reportedly 1% at RT after 2 days (183).

Consequently, the stability of retinol in different matrices remains uncertain, and there are contradictory results concerning retinol stability in whole blood at different temperatures and times. Furthermore, retinol stability in the serum/plasma was not monitored for longer periods of time. No studies have investigated potential factors (matrix, temperatures, light and storage times) simultaneously to minimise impacts on the final results of retinol stability.

It seems that α-tocopherol is more stable than retinol. Concentration changes in whole blood α-tocopherol were about ±5% at RT and chilled temperatures for up to 1 week of storage (97, 98, 184). Serum α-tocopherol was observed to be stable for at least 1 week at RT (177, 180) and for at least 2 days at chilled temperatures (181, 182) with less than 1% concentration change. Furthermore, the change was 5% in extracted α-tocopherol samples stored at RT or chilled temperatures for 2 days (183). Under the effect of light, concentration changes of whole blood α-tocopherol was less than 3% at RT and chilled temperature for up to 1 week of storage (97). Alternately, the change in plasma α-tocopherol was -6% at RT for 2 days of monitoring. Although available data concerning α-tocopherol stability is more consistent than for retinol stability, the potential factors affecting α-tocopherol stability in whole blood, serum/plasma and extract were not investigated concurrently for long periods of time using the same analytical method.

Only 4 studies examined the stability of 25-OHD in whole blood samples stored at RT for a short time (up to 3 days); the concentration changes were around -4% (98, 185-187). No studies have explored the stability of whole blood 25-OHD in chilled temperatures. Three studies monitored the stability of serum/plasma 25-OHD at RT and chilled temperatures for 24 hours or less (with concentration changes of ±5% at RT and ±9% at chilled temperatures)

(154, 188, 189). Two other studies verified the sample's stability for 1 week (with concentration changes of -8% at RT and also -2% to -6% at chilled temperatures) (98, 159) and 2 weeks (with concentration changes of -8% at RT and 3.1% at chilled temperatures) (98). Extracted 25-OHD was stable for 1 day with concentration changes of 4% (154). Based on the published data available, 25-OHD was stable under the effects of artificial light at RT for up to 2 days with a -2% concentration change (159) in the whole blood samples. Under the same conditions, serum/plasma 25-OHD was reportedly stable from 1 day (159) to 1 week of storage (190). Although most of the 25-OHD stability studies were conducted over the past 10 years, none of them examined 25-OHD stability in whole blood samples at RT or chilled temperatures for longer periods of time, such as a week, which is the worst case scenario for storage conditions prior to centrifugation for technical or logistic reasons. Furthermore, like retinol and α-tocopherol, no studies have simultaneously explored the effects of different matrices on 25-OHD stability.

Given the knowledge gaps identified through the systematic review, this study aimed to simultaneously investigate the stability of 25-OHD3, retinol and α-tocopherol in whole blood samples prior to centrifugation under the effects of light, time (up to 1 week prior to centrifugation) and temperature (RT, 4ºC). The current study aimed to explore the stability of the 3 vitamins in the serum and extract samples under the influence of light at RT (for 1 week) and subdued light at RT (for 1 week) at 4ºC, -20ºC and -80ºC for up to 1 month. Monitoring the effect of light and RT for one week was chosen because it is the worst situation in which to keep samples, either for diagnostic purposes or for intervention studies, specifically at RT in ambient artificial light or subdued light during sample collection.

In the stability study experiments, the analyses of the 3 vitamins (25-OHD3, retinol and α-tocopherol) were conducted using our precise simultaneous quantification method, which utilised LC-MS/MS.

Figure 4-1. Literature search scheme for FSV stability.

This scheme shows the literature strategy used to identify published studies focused on FSV stability between 1980 and early 2015 in English language (adapted from (81)).

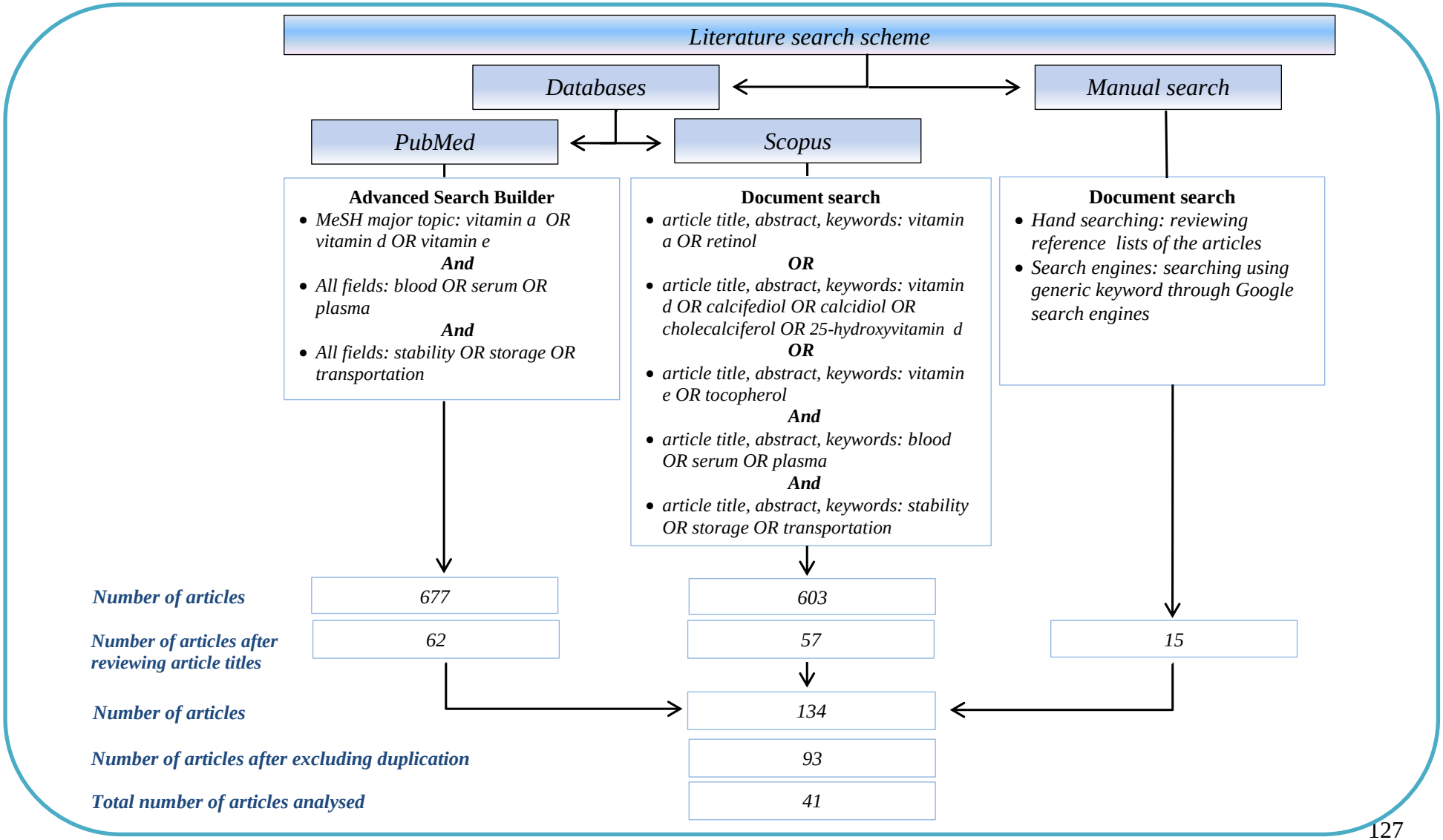


Table 4-1. Previous studies descripting the stability of 25-OHD in whole blood, plasma and serum.

This table chronologically summarise published studies that investigated the stability of 25-OHD under different conditions between 1980 and 2014. It is noted that most studies focused on the stability of these analytes either in whole blood or in serum.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
25-OHD3	Serum, extract	25	LC-M/SMS	Time: up to 265 d T: RT, -20ºC Freeze/thaw: 4 cycles	**Analyte** / **Condition** / **24 h (%)** / **72 h (%)** / **265 d (%)** Serum 25-OHD3 / RT / -1.9 / - / - -20ºC / - / - / -6.4 25-OHD3 extract / RT / - / 4.0 / - • 25-OHD3 changes between heparin plasma compared to serum was 3.4 • 25-OHD3 changes between EDTA plasma compared to serum was -3.8 • 25-OHD3 level changes for 4 cycles of serum freezing and thawing was 9.9%	2014	(154)
25-OHD3	Serum	19	IA	Time: mean 73 d Tubes: SST vs. plain T: -20°C	• Good correlation was observed between levels of 25-OHD3 measured in gel tubes (SST) and gel-free tubes (r>0.990, bias -1.5 nmol/L).	2014	(191)
25-OHD	Whole blood	30	RIA	Time: up to 24 h T: 15°C, 25°C, 35°C	• It was stable under investigated conditions and changes (%) were from -4.1% to 1.1%.	2014	(185)
25-OHD	Serum, plasma	15	IA	Time: up to 3 m T: RT, 4°C, -20°C,-80°C	**Analyte** / **Condition** / **4 h (%)** / **24 h (%)** / **7 d (%)** / **3 m (%)** Serum 25-OHD3 / RT / 4.4 / - / - / - 4ºC / - / -9.1 / - / - -20ºC / - / 4.3 / 0.0 / - -80ºC / - / - / - / 8.0 Plasma 25-OHD3 / RT / 4.5 / - / - / - 4ºC / - / 8.4 / - / - -20ºC / - / 0.5 / 9.6 / - -80ºC / - / - / - / -1.4	2013	(188)

Continue table. 4-1.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
25-OHD	Serum, plasma	10-22	IA	Time: up to 4 m T: -20ºC Light: sunlight Freeze/thaw: 2-5 cycles Matrix: serum vs. heparinised plasma	**Analyte** / **Condition** / **3 d (%)** / **1 w (%)** / **2 w (%)** / **3 w (%)** / **1 m (%)** / **4 m (%)** Serum 25-OHD3 / -20ºC / 1.5 / -2.2 / -4.2 / -3.3 / -7 / -15.3 • No significant effect of sunlight exposure for 4 h was observed. • No significant differences were observed in samples collected in tubes with gel compared with those without gel. • No significant effect of freezing and thawing cycles (2-5) was observed. • A significant differences were found in 25-OHD levels in heparinised plasma compared with serum samples.	2012	(192)
25-OHD3	Plasma	16	LC-MS/MS	Time: up to 4 w T: RT, 4ºC, -20ºC Freeze/thaw: 5 cycles	• Plasma 25-OHD3 showed good stability up to 4 weeks at RT, 4◦C and -20◦C (data not showed in the article). • No significant difference was found between concentrations of analyte measured in plasma and serum samples. • No degradation was observed in this analyte after 5 cycles of plasma freezing and thawing.	2010	(193)
25-OHD	Serum	402	RIA	Time: 6-24 y T: -25ºC	• Long-term storage had no effect on serum 25-OHD levels	2010	(194)
25-OHD3	Whole blood, serum	8	IA	Time: up to 1 m T: RT, CT (4ºC -11ºC), -20ºC Light: light/dark Freeze/thaw: 4 cycles	**Analyte** / **Condition** / **24 h (%)** / **72 h (%)** / **1 w (%)** / **2 m (%)** Blood 25-OHD3 / Light at RT / - / -2.3 / - / - Serum 25-OHD3 / Light at RT / -3.4 / - / -8.5 / - Dark at RT / - / -4.5 / -8.1 / - Dark at chill / - / - / -1.8 / - Dark at -20ºC / - / - / - / -4.0 • 25-OHD3 level changes for 4 cycles of serum freezing and thawing was 2.6%.	2009	(159)
25-OHD3	Whole blood	11	IA	Time: 24 h T: RT	**Analyte** / **Condition** / **24 h (%)** Blood 25-OHD3 / RT / -3.9	2009	(187)
25-OHD3	Serum	6	IA	Time: 12 m T: -80ºC Tubes: without gel vs. gel (SST)	**Analyte** / **Condition** / **12 m (%)** Serum 25-OHD3 / SST tube at -80ºC / -7.0	2009	(195)

Continue table. 4-1.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
25-OHD3	Plasma	40	HPLC	Time: up to 12 h T: 4ºC	**Analyte** **Condition** **12 h (%)** Serum 25-OHD3 4ºC -4.0	2009	(189)
25-OHD	Whole blood, Serum	35	RIA	Time: up to 2 w T:11ºC, 32ºC	**Analyte** **Condition** **24 h (%)** **48 h (%)** **72 h (%)** **1 w (%)** **2 w (%)** Blood 25-OHD 32ºC -2.8 -0.8 -2.0 - - Serum 25-OHD 11ºC - -0.9 - 6.5 3.1	2008	(98)
25-OHD3	Spiked serum	1	LC-MS	Time: 8 d Light: artificial light, sunlight, UV Place: outdoor, indoor Freeze/thaw: 5 cycles	• Only samples exposed to direct sunlight had significantly lower 25-OHD levels compared with samples exposed to artificial light at RT for up to 8d. • No significant concentration differences were observed in 25-OHD levels after 5 cycles of serum freezing and thawing.	2008	(190)
25-OHD3	Spiked serum, extract	pooled serum	LC-MS	Light: sunlight Freeze/thaw: 4 cycles	• Serum 25-OHD3 was stable in sunlight at RT for at least 6 h. • Extracted analyte from serum remained stable for at least 1 w at 4ºC • No significant concentration differences were observed in samples after 4 cycles of serum freezing and thawing cycles.	2008	(150)
25-OHD	Serum	20	RIA	Freeze/thaw: 4 cycles	• No significant effect on serum 25-OHD concentrations was observed after 3 cycles of serum freezing and thawing.	2005	(196)
25-OHD	Plasma	55	IA	Time: up to 4 y T: -20ºC	• Plasma 25-OHD results showed no decline during 4 y of storage	1995	(197)
25-OHD	Whole blood, serum, plasma	1	NS	Time: up to72 h T: RT	**Analyte** **Condition** **24 h (%)** **72 h (%)** Blood 25-OHD RT 4.8 0.0 • No significant differences in 25-OHD levels were reported in plasma (heparin, EDTA) samples compared with serum samples.	1981	(186)

d: day, G-LC: Gas-liquid Chromatography, h: hour, HPLC: High performance liquid chromatography, IA: immunoassay, LC-MS: liquid chromatography-single mass spectrometry, LC-MS/MS: liquid chromatography-tandem mass spectrometry, m: month, NS: not stated, RIA: radioimmunoassay, RT: room temperature, SST: serum separator tubes, T: temperature, TFA method: Trifluoroacetic acid colorimetric method, α-Toco: α-Tocopherol, w: week, y: year.

Table 4-2. Previous studies descripting the stability of retinol and α-tocopherol in whole blood and plasma/serum.

This table summarise chronologically published studies that investigated the stability of retinol and α-tocopherol under different conditions between 1980 and 2014. It is noted that most studies focused on the stability of these analytes either in whole blood or in serum.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
Retinol α-Toco	Whole blood	18	HPLC	Time: up to 48 h T: RT Light	**Analyte** **Condition** **24h (%)** **48 h (%)** Blood Retinol Light at RT -1.2 -1.5 Blood α-toco Light at RT 1.3 1.3	2014	(95)
Retinol α-Toco	Plasma	12	LC-MS/MS	Time: up to 48 h T: stored with ice	• Both anlaytes were stables and level changes were <1% per hour during investigated storage times.	2014	(181)
Retinol α-Toco	Spiked serum	NS	HPLC	Time: 24 h T: 30ºC, 4ºC, -20ºC	**Analyte** **Condition** **24h (%)** Serum retinol 30ºC -2.2 4ºC -0.1 -20ºC -0.1 Serum α-toco 30ºC -1.6 4ºC 0.0 -20ºC 0.0	2010	(182)
Retinol α-Toco	Whole blood, serum	35	HPLC	Time: up to 72 h T:11ºC, 32ºC	**Analyte** **Condition** **24h (%)** **48 h (%)** **72 h (%)** Blood retinol 32ºC -0.5 -2.3 -9.8 Serum retinol 11ºC 0.0 1.4 2.8 Blood α-toco 32ºC 3 2.8 -1.0 Serum α-toco 11ºC 0.7 1.4 3.2	2008	(98)
Retinol	Whole blood	41	HPLC	Time: up to 96 h T: RT	**Analyte** **Condition** **24h (%)** **96 h (%)** Blood retinol RT 1.7 1.1	2005	(198)
α-Toco	Whole blood	40	HPLC	Time: up to 144 h T: chill (4ºC-10ºC)	**Analyte** **Condition** **32h (%)** **72 h (%)** **144 h (%)** Blood α-toco CT 0.2 1.0 -0.3	2005	(199)
Retinol α-Toco	Lyophilized serum	Frozen pool serum	HPLC	Time: up to 12 m T: -20ºC	**Analyte** **Condition** **12 m (%)** Lyophilized serum retinol -20ºC -0.7 Lyophilized serum α-toco -20ºC -2.8	2004	(200)

Continue table 4-2.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
Retinol α-Toco	Whole blood	10	HPLC	Times: up to 7 d T.: RT, 4ºC Light	**Analyte** **Condition** **24h (%)** **48 h (%)** **72 h (%)** **96 h (%)** **1 w (%)** Blood retinol Dark at RT 0.5 1.6 1.8 4.6 3.3 Light at RT -0.2 0.3 -1.4 1.3 -6.6 Dark at 4ºC 0.8 0.8 1.1 0.4 -0.3 Light at 4ºC 1.7 0.2 -0.7 -2.1 -1.7 Blood α-toco Dark at RT 2.8 4.1 4.3 6.7 4.8 Light at RT 3.7 4.6 3.4 7.4 3.3 Dark at 4ºC 0.9 0.3 2.4 1.0 1.2 Light at 4ºC 2.2 1.8 0.9 0.5 0.4	2004	(97)
Retinol α-Toco	Pooled plasma, extract	NS	HPLC	Time: up to 48 h T: RT, 4ºC, -20ºC Light	**Analyte** **Condition** **24h (%)** **48 h (%)** **72 h (%)** Plasma retinol Light at RT 0.0 1.4 -1.1 Retinol extract RT 1.6 3.9 - 4ºC -1.6 0.0 - -20ºC -0.4 -0.8 - Plasma α-toco Light at RT -3.6 2.1 -6.0 α-Toco extract RT -0.1 4.9 - 4ºC 1.9 5.3 - -20ºC 0.4 0.5 -	1999	(183)
Retinol α-Toco	Pooled serum	NS	HPLC	Time: over 10 y T: RT, -25ºC, -80ºC Freeze/thaw: 5 cycles	**Analyte** **Condition** **1 w (%)** **2 w (%)** **4 w (%)** Serum retinol RT -5 -7 -17 Serum α-toco RT <1 <1 <1 • Serum retinol was stable for 3 years and 5 years at -25ºC and -80ºC, respectively, and α-tocopherol was stable for 5 years at -25ºC and -80ºC. • Both were stable for at least 5 cycles of serum freezing and thawing	1998	(177)
Retinol α-Toco	Plasma, extracts	7	HPLC	Time: up to 1 y T: -70ºC	• Plasma retinol and α-tocopherol were stable for at least 12 m at -70ºC. • Retinol and α-toco extracts were stable for 16 h at RT and 24 h at 10ºC.	1998	(178)
Retinol α-Toco	Plasma	28	HPLC	Time: 24 h T: RT	**Analyte** **Condition** **24h (%)** Plasma retinol RT -3.0 Plasma α-toco RT -2.1	1996	(201)

Continue table 4-2.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
Retinol α-Toco	Plasma	55	HPLC	Time: up to 1 y T: -20ºC	• Plasma retinol concentration showed significant decreases after 1 y of storage • Plasma α-toco concentration showed significant decreases after 6 m of storage	1995	(197)
Retinol α-Toco	Pooled plasma	NS	HPLC	Time: up to 4 y T: -70ºC	• Results showed no significant losses in the plasma retinol concentrations in storage at -70ºC up to 4 y. • Results showed no significant losses in the plasma α-toco concentrations in storage at -70ºC up to 4 y.	1995	(202)
α-Toco	Plasma	17	HPLC	Sample processing in ambient dark/light, air/flushing with nitrogen	**Analyte** **Condition** **(%)** Plasma α-toco air/light 0% Plasma α-toco air/dark 0% Plasma α-toco nitrogen/dark 0%	1995	(203)
Retinol α-Toco	Pooled plasma	NS	HPLC	Time: up to 1 w T: RT Freeze/thaw: 4 cycles	**Analyte** **Condition** **24 h (%)** **1 w (%)** Plasma retinol RT -0.6 -0.3 Plasma α-toco RT 1.2 -0.7 • No significant changes were reported in concentrations after 4 cycles of plasma freezing and thawing.	1993	(180)
Retinol α-Toco	Serum	23	HPLC	Time: 5 y T: -70ºC	• No significant differences were observed in serum retinol levels during 5 y of storage (r^2= 0.895). • No significant differences were observed in serum α-toco levels during 5 y of storage (r^2= 0. 978).	1993	(204)
α-Toco	Plasma	24	HPLC	Time: 12 m T: -70ºC	• Plasma α-toco was stable for at least 6 w at -20ºC	1991	(184)
Retinol α-Toco	Whole blood, plasma	17	HPLC	Time: up to 72 h T: RT, 9ºC	**Analyte** **Condition** **24h (%)** **48 h (%)** **72 h (%)** Blood retinol RT -7.1 -11.1 -15.5 9ºC -6.9 -8.7 - Plasma retinol 9ºC -1.0 - - Blood α-toco RT 2.0 -2.0 -5.0 9ºC -3.0 -2.0 - Plasma α-toco 9ºC -8.0 - -	1989	(179)

Continue table 4-2.

Analyte	Matrix	Subject	Technique	Monitoring conditions	Changes from typical analyte analysis	Year	Ref
Retinol α-Toco	Plasma	NS	HPLC	Time: 24 h T: RT	**Analyte** **Condition** **24h (%)** Plasma retinol RT 0.0 Plasma α-toco RT -6.1	1988	(205)
Retinol α-Toco	Pooled plasma	NS	HPLC	Time: 12 m T:-20ºC	• No significant differences were found in plasma retinol levels during up to 12 m of storage at -20ºC based on 102 measurements. • No significant differences were found in plasma α-toco levels during up to 12 m of storage at -20ºC based on 102 measurements.	1988	(206)
Retinol α-Toco	Serum	238	HPLC	Time: up to 1 year T: -20ºC	• No degradation was observed in serum retinol levels after 6-13 m of storage (r^2= 0.912). • No degradation was observed in serum α-toco levels after 6-13 m of storage (r^2= 0.621).	1988	(207)
Retinol	Whole blood	2	HPLC	Time: up to 24 h T: ice (0-2ºC)	**Analyte** **Condition** **24h (%)** Blood retinol Dark/ ice: subject 1 0.5 Dark/ ice: subject 2 -4.8	1987	(208)
Retinol	Serum	10-42	• TFA method: for fresh samples HPLC: for stored samples	Time: up to 8 y T: -20ºC	**Analyte** **Condition** **5y (%)** **6y (%)** **7 y (%)** **8 y (%)** Serum retinol -20ºC -6.8 11.1 0.0 -2.9 • The r^2 were 0.518 (5y), 0.592 (6y), 0.372 (7y) and 0.490 (8y).	1985	(209)
Retinol	Whole blood	6	UV inactivation method	Time: up to 24 h T: RT, 4ºC	**Analyte** **Condition** **24h (%)** Blood retinol RT -0.5 4ºC 0.2	1983	(210)

d: day, G-LC: Gas-liquid Chromatography, h: hour, HPLC: High performance liquid chromatography, IA: immunoassay, LC-MS: liquid chromatography-single mass spectrometry, LC-MS/MS: liquid chromatography-tandem mass spectrometry, m: month, NS: not stated, RIA: radioimmunoassay, RT: room temperature, SST: serum separator tubes, T: temperature, TFA method: Trifluoroacetic acid colorimetric method, α-Toco: α-Tocopherol, w: week, y: year.

4.2 Methodology

This section describes the subjects, experiments and statistical analysis used in FSV stability study. During sample transportation and process, several factors may impact the FSV stability, such as light, temperature and extended time prior to sample analysis; and these could influence the interpretation of results. To explore the effects of these factors on the stability of three FSV analytes (25-OHD3, retinol and α-tocopherol), four experiments were designed and conducted, as detailed in the next sub-sections (Table 4-3).

4.2.1 Subjects

Three volunteers (2 males and 1 female) aged (35-55 years) from our research group were recruited for this study. Each volunteer provided four separate blood samples (10-40 mL) at different occasions in the School of Medical Sciences' phlebotomy facility. The whole blood samples were collected in 10 mL vacutainer (BD) plain tubes (no anticoagulant) wrapped in aluminium foil to protected them from the light. All samples were processed according to the FSV extraction protocol detailed in Chapter 2 (sections 2.6–2.8) and quantified on Agilent LC-MS/MS 6490 using the FSV method 1 as detailed in Chapter 3.

4.2.2 Investigated conditions

Samples (whole blood, serum, 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 fluorescent lamp from one meter distance while another sample group was protected from the light through storing in dark cupboards. Other storage conditions of different temperatures: room temperature (RT) 23ºC ±2ºC, 4ºC ±2ºC, -20ºC ±3 and -80ºC ±3

over different time points: 3h, 6h, 12h, 24h, 48h, 1 week and 1 month were also examined throughout the experiments.

Experiment 1:

This experiment aimed to investigate the stability of 25-OHD3, retinol and α-tocopherol in whole blood. For this purpose, blood (≈ 20 mL) was collected from each volunteer into three 10 mL plain tubes. Immediately, the blood was aliquoted (300 μL) into labelled polypropylene tubes (1.5 mL) wrapped in aluminium foil unless otherwise stated. All aliquots were kept standing for 90 min at ambient dark RT condition for clotting. Later, the aliquots were categorised into four groups.

Group 1: aliquots (n=12) were centrifuged for 20 min at 3500 rpm, and the serum was then transferred into new labelled polypropylene tubes (1.5 mL) prior to storing at -80ºC. This sample group was considered the control and was used to determine the baseline analyte concentration. Group 2: aliquots (n=18, not wrapped in aluminium foil) were exposed to florescent light at RT to examine the effects of the light for several time points: 3h, 6h, 12h, 24h, 48h and 1 week. The group 3 aliquots (n=18) and group 4 aliquots (n=18) were protected from light and kept at RT and 4ºC, respectively, to investigate the effects of temperature across times 3h, 6h , 12h, 24h, 48h 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, and the serum was immediately transferred into new labelled polypropylene tubes (1.5 mL) prior to storing at -80ºC until they underwent analysis.

Experiment 2:

This experiment aimed to examine the stability of the three FSV analytes in serum. Thus, the whole blood samples (≈ 40 mL) were collected from each volunteer into four 10 mL plain tubes wrapped in aluminium foil. These tubes were kept to stand for 90 min at ambient dark RT for clotting prior to centrifugation at 3500 rpm for 20 min. The entire serum sample from each volunteer was collected in 1 glass bottle and then aliquoted (150 µL) into labelled polypropylene tubes (1.5 mL) wrapped in aluminium foil unless otherwise stated. These serum aliquots were divided into the five sample groups (n=18 for groups 1–3, n=24 for groups 4–5). 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 4ºC and -20ºC, respectively at different storage times (3h, 6h , 12h, 24 h, 48h 1 week, 2 weeks and 1 month) prior storage at -80ºC until they were analysed.

Experiment 3:

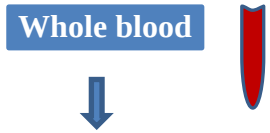
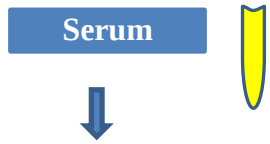
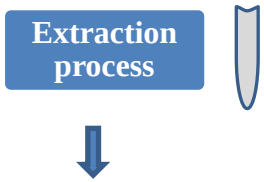
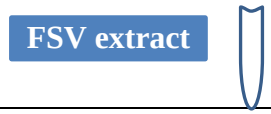
This experiment was designed to investigate the influence of light during the sample-processing (FSV extraction) on the analytes of interest. A blood sample (≈ 10 mL) was collected from each volunteer into 10 mL plain tube wrapped in aluminium foil. Later, this tube was kept for 90 min at ambient dark RT for clotting prior centrifugation at 3500 rpm for 20 min to separate serum. The two groups consist of the triple serum aliquots from each volunteer. The samples from the first group were processed under light exposure while the second group samples were prepared under subdued light. Later, entire samples from both groups were loaded into the LC-MS/MS in the same analytical batch.

Experiment 4:

This experiment explored the stability of the three analytes of interest post-extraction process (extracts). Thus, whole blood (≈ 25 mL) from each volunteer was collected into four 10 mL plain tubes wrapped in aluminium foil and kept for 90 min at ambient dark RT conditions for clotting. Later, they were centrifuged at 3500 rpm for 20 min. All the serum samples from each volunteer were collected in 1 glass bottle and aliquoted (100 µL) into 70 labelled glass tubes. These samples were processed based on extraction protocol, and the entire extracts from all sample tubes were collected in 1 glass bottle prior aliquot into 18 HPLC transparent glass vials and 87 HPLC amber glass vials containing 150 µL. These aliquots were categorised into five groups: group 1 (n=18 transparent vials); group 2 (n=18 amber vials) and group 3 and 4 (n=24 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 -80ºC until they were analysed.

Table 4-3. Experimental flowchart to investigate FSV stability.

This table shows overall view of four experiments that were conducted to examine the stability of three FSV analytes (25-OHD3, retinol and α-tocopherol) in the pre-analytical stage. Experiments 1, 2 and 4 examined the stability of FSVs in whole blood, serum and analyte extracts, respectively, under the effect of light (off vs. on) and temperatures (RT, 4°C and -20°C vs -80°C) across several time points. The stability of FSV during the extraction process under the effect of light (subdued light vs light RT) was explored in experiment 3.

Experiment	Sample processing	Investigated factors		
		Light	Temperature	Time
1		Off, On	RT, 4°C	3h, 6h, 12h, 24h, 48h, 1w
2		Off, On	RT, 4°C, -20°C	3h, 6h, 12h, 24h, 48h, 1w, 2w, 1m
3		Off, On	RT	NA
4		Off, On	RT, 4°C, -20°C	3h, 6h, 12h, 24h, 48h, 1w, 2w, 1m

RT: room temperature, NA: not applicable.

4.2.3 Statistical analysis

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

Allowable clinical percentage changes of analyte during sample analysis was determined based on the total change limits (TCL) (175). The TCL account within-subject biological variations and method desirable imprecision. For each analyte TCL were calculated according to the following equation:

Equation 4-1. Calculation of total change limits

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

The CVa is method imprecision and CVb is within-subject biological variations. "The factor 2.77 is derived from Z√2, where Z=1.96, determined by the 95% of confidence interval value for bi-directional changes, and √2 as we are comparing two results with the same CVa. We concluded that a mean percentage deviation greater than 2.77 CVa represents a probable difference in analyte concentration" (175). "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 (CVb), and this should be the goal for short-term laboratory imprecision (≤0.5 CVb)" (175).

In the current work, we used the minimum method imprecision, 25-OHD3 (2.2%), retinol (3.5%) and α-tocopherol (3.0%) in the TCL calculation for each analyte. The within-subject biological variations were taken from the Ricos Biological Variation database (211) for serum retinol (13.6%) and α-tocopherol (13.8%); and from Stockl and colleagues' work

(212) for 25-OHD3 (8%). Consequently, the calculated TCL was 7.3% for 25-OHD3, 11.8% for retinol and 10.8% for α-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. The independent t-test was used to compare the target analyte concentrations in samples processed under light versus samples processed in subdued light conditions. The $p < 0.05$ was considered as a statistically significant difference according to peer results. The GraphPad Prism version 6 (GraphPad Prism Software Inc., CA, USA) was used for statistical analysis (213).

4.3 Results

4.3.1 Stability of 25-OHD3

The stability of the 25-OHD3 was investigated in three matrices (whole blood, serum and analyte extract) under the effects of light, temperature and storing time. The concentration changes from baseline concentration under investigated conditions were within ±7.3% of TCL for 25-OHD3. They were ±2.9% in the whole blood, ±3.9% serum and ±3.8% extracts and 2.5% in processing sample in light compared to samples processed in subdued light. We observed insignificant differences in concentrations of 25-OHD3 in factors of light, temperature and time (Table 4-4, Table 4-5, Table 4-6, Table 4-7, Figure 4-2, Figure 4-3).

Table 4-4. Stability of 25-OHD3 in whole blood.

This table shows the percentage changes in whole blood 25-OHD3 levels under the effects of light, temperature and time. The examined whole blood samples were exposed to different conditions prior to serum obtained and stored at -80ºC while the serum from whole blood control samples was immediately obtained and stored at -80ºC until analysis. Results show that 25-OHD3 was stable in whole blood under the effect of investigated conditions, and percentage changes (±2.9%) were within ±7.3% of TCL for 25-OHD3. There was no significant difference in the 25-OHD3 levels under the effect of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	69 (0.5)	0.0	69 (0.5)	0.0	69 (0.5)	0.0
3h	69 (1.0)	0.0	71 (0.7)	2.9	68 (0.9)	-1.4
6h	70 (0.8)	1.4	71 (0.6)	2.9	68 (1.0)	-1.4
12h	69 (1.4)	0.0	70 (1.1)	1.4	68 (3.0)	-1.4
24h	67 (1.0)	-2.9	68 (0.4)	-1.4	70 (1.3)	1.4
48h	69 (1.3)	0.0	68 (0.7)	-1.4	68 (1.5)	-1.4
1w	69 (0.5)	0.0	70 (2.4)	1.4	68 (0.4)	-1.4
Effect of storage time	***p*=0.789**					
Effect of temperature	**NA**	**NA**	***p*=0.428**			
Effect of light	***p*=0.221**				**NA**	**NA**

h: hour/s, w: week, RT: room temperature, NA: not applicable

Table 4-5. Stability of 25-OHD3 in serum.

This table shows the percentage changes in concentrations of serum 25-OHD3 samples under the effect of light and temperature across several time points compared to the baseline concentration of the serum control samples. The examined serum samples were exposed to different conditions prior to storing at -80ºC, while the serum control samples were immediately stored at -80ºC until analysis. The results show that 25-OHD3 was stable in serum under the effects of the investigated conditions, and percentage changes (±3.90%) were within ±7.3% of TCL for 25-OHD3. There was no significant difference in the 25-OHD3 levels under the effects of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)		-20ºC (Dark)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	72 (0.8)	0.0	72 (0.8)	0.0	77 (0.6)	0.0	77 (0.6)	0.0
3h	73 (1.0)	1.4	72 (1.4)	0.0	79 (1.3)	2.6	78 (0.8)	1.3
6h	74 (1.4)	2.8	72 (1.7)	0.0	80 (1.7)	3.9	77 (2.0)	0.0
12h	72 (0.9)	0.0	71 (0.8)	-1.4	78 (1.8)	1.3	74 (1.7)	-3.9
24h	73 (1.6)	1.4	71 (1.7)	-1.4	79 (2.6)	2.6	76 (2.0)	-1.3
48h	73 (2.1)	1.4	73 (1.8)	1.4	77 (2.7)	0.0	74 (1.9)	-3.9
1w	71 (0.7)	-1.4	71 (0.8)	-1.4	80 (1.2)	3.9	78 (1.7)	1.3
2w	NA	NA	NA	NA	79 (1.2)	2.6	76 (2.8)	-1.3
1m	NA	NA	NA	NA	76 (1.9)	-1.3	78 (2.3)	1.3
Effect of storage time	***p*= 0.118**							
Effect of temperature	**NA**		***p*= 0.199**					
Effect of light	***p*= 0.167**				**NA**			

h: hour/s, w: week/s, m:mounth, NA: not applicable

Table 4-6. Stability of 25-OHD3 during sample processing.

This table shows 25-OHD3 concentration changes in samples processed under subdued light compared to samples processed in regular light at RT.

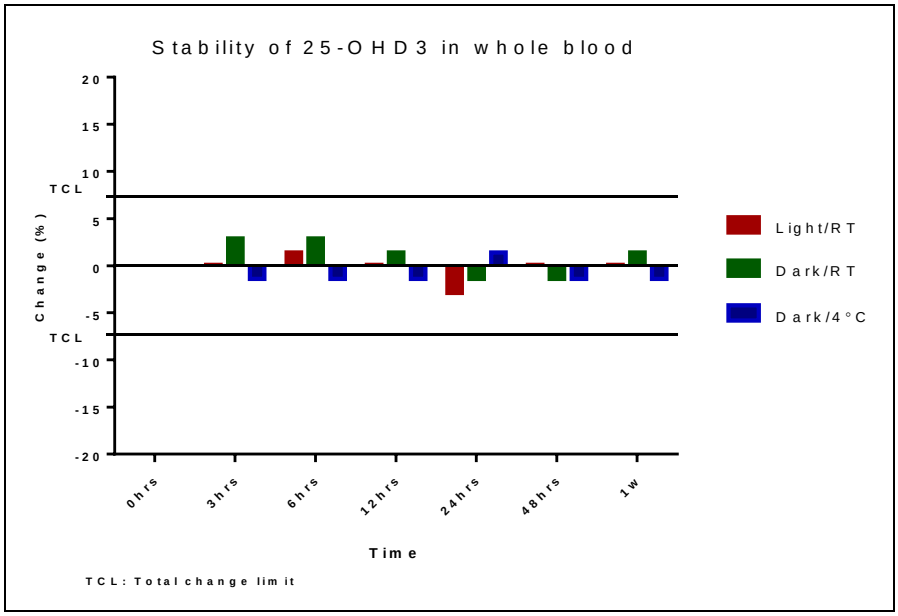
	Subdued light at RT nmol/L	Light at RT nmol/L
Mean (SD)	80 (1.5)	79 (2.3)
Mean change (%)	-2.5	
p value	0.967	

Table 4-7. Stability of extracted 25-OHD3.

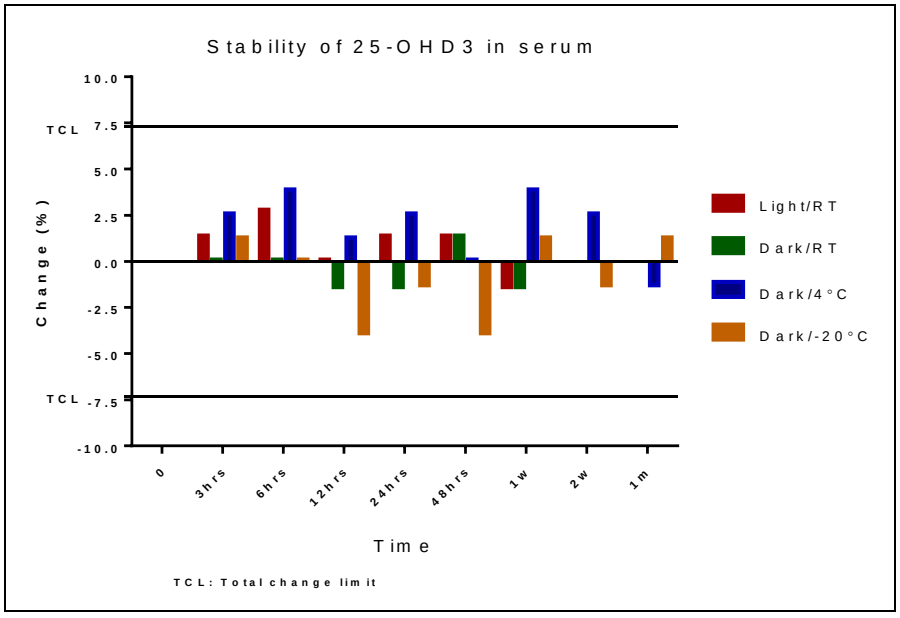
This table shows the percentage changes in concentrations of extracted 25-OHD3 from serum samples under the effects of light and temperature across several time points compared to baseline concentration of control samples. The extracted 25-OHD3 samples were exposed to different conditions prior to storing in -80ºC while the extracted 25-OHD3 control samples were immediately stored at -80ºC. Results show that extracted 25-OHD3 was stable under the effect of the investigated conditions, and percentage changes (±3.8%) were within ±7.3% of TCL for 25-OHD3. There was no significant difference in the 25-OHD3 levels under the effect of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)		-20ºC (Dark)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	79 (3.0)	0.0	79 (3.0)	0.0	79 (3.0)	0.0	79 (3.0)	0.0
3h	80 (2.3)	1.3	80 (3.7)	1.3	79 (4.7)	0.0	78 (2.2)	-1.3
6h	80 (3.1)	1.3	79 (4.3)	0.0	80 (3.9)	1.3	81 (3.7)	2.5
12h	79 (2.5)	0.0	80 (4.8)	1.3	79 (3.1)	0.0	82 (3.4)	3.8
24h	82 (3.1)	3.8	77 (2.4)	-2.5	78 (2.3)	-1.3	81 (3.9)	2.5
48h	81 (2.1)	2.5	82 (4.2)	3.8	79 (4.5)	0.0	80 (3.2)	1.3
1w	82 (2.9)	3.8	82 (4.4)	3.8	79 (3.7)	0.0	80 (3.6)	1.3
2w	NA	NA	NA	NA	80 (3.9)	1.3	79 (3.2)	0.0
1m	NA	NA	NA	NA	80 (2.8)	1.3	80 (3.0)	1.3
Effect of storage time	***p*=0.056**							
Effect of temperature	**NA**		***p*=0.066**					
Effect of light	***p*=0.183**				**NA**			

h: hour/s, w: week/s, m:mounth, NA: not applicable



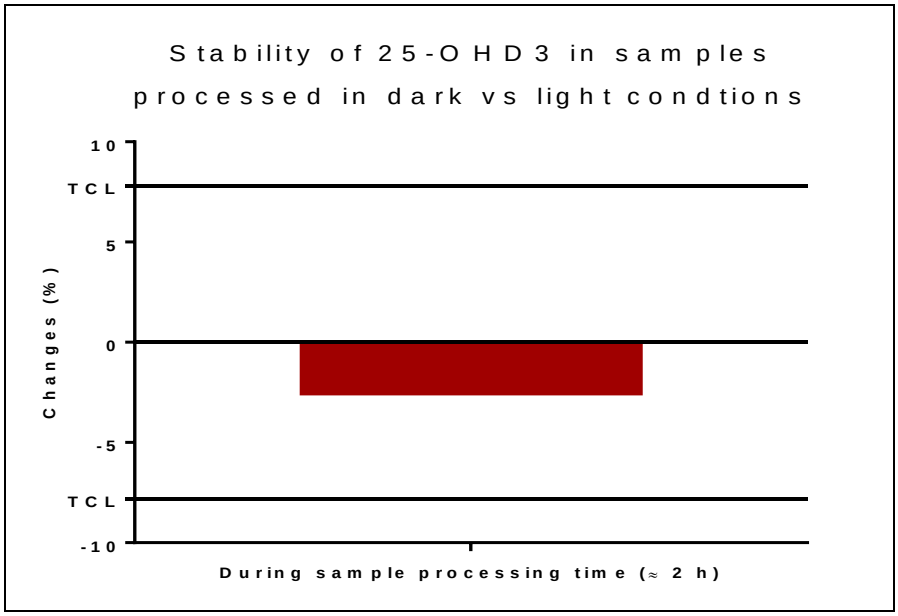
a)



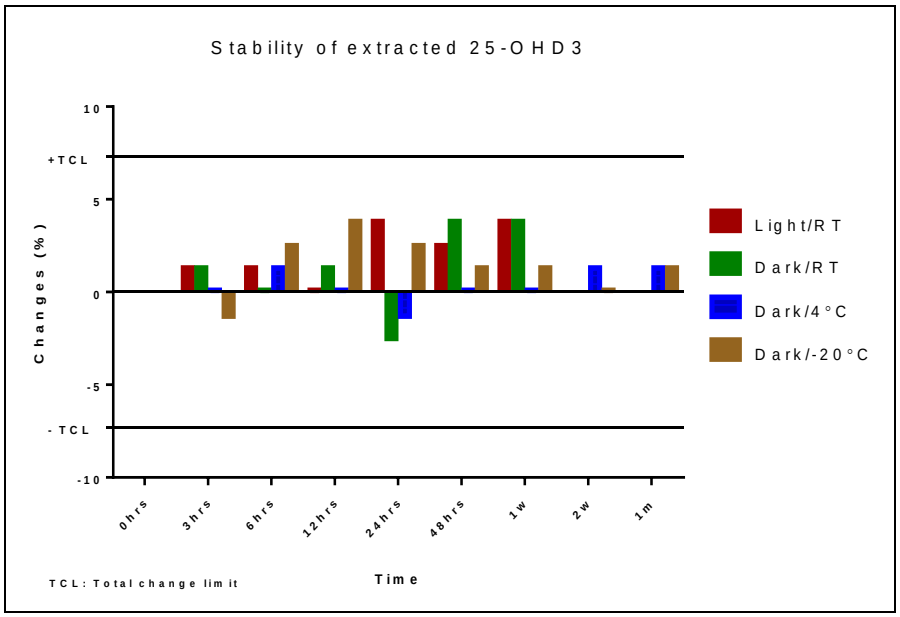
b)

Figure 4-2. Stability of 25-OHD3 in unprocessed samples.

These graphs show the stability of 25-OHD3 in a) whole blood and b) serum under different conditions. Bar graphs show average of concentration changes (%) of 25-OHD3 in samples, which were exposed to different conditions of light, temperature and time, from a baseline concentration in control samples processed using optimal conditions. The red bars represent samples exposed to light at RT while green, blue and brown bars represent samples protected from the light at RT, 4ºC and -20ºC, respectively. Solid lines represent ± total change limits (TCL) which are used to determine the acceptable clinical limits of analyte changes. Effects 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.



a)



b)

Figure 4-3. Stability of 25-OHD3 samples during processing.

These graphs show the stability of 25-OHD3 during sample processing under different conditions. Bar graph (a) displays average of 25-OHD3 concentration changes (%) in samples processed in subdued light versus to samples processed in light RT. Bar graphs (b) show average of concentration changes (%) of extracted 25-OHD3, which were exposed to different conditions of light, temperature and time, from baseline concentration in control samples processed using optimal conditions. The red bars represent samples exposed to light at RT while green, blue and brown bars represent samples protected from the light at RT, 4ºC and -20ºC, respectively. Solid lines represent ± total change limits (TCL) which are used to determine the acceptable clinical limits of analyte changes. Effects 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.

4.3.2 Stability of retinol

Data of retinol stability under investigated conditions show that percentage changes of retinol levels in whole blood, serum and retinol extract as well as in samples processed in lighted condition were within TCL (±11.8%) except the changes of extracted retinol exposed to light at RT for more than 48h (Table 4-7, Figure 4-5). Under investigated conditions, whole blood and serum retinol level changes were between -6.5 and 4.11% (Table 4-8, Table 4-9, Table 4-10, Figure 4-4, Figure 4-5). Furthermore, retinol concentration changes in the extracted retinol samples were ±3.3% except changes of those exposed to light at RT for one week that significantly decreased by 18.4%.

Table 4-8. Stability of retinol in whole blood.

This table shows percentage changes in whole blood retinol levels under effect of light, temperature and time. The tested whole blood samples were exposed to different conditions prior to serum obtained and stored at -80ºC while serum of control samples was immediately obtained and stored at -80ºC until analysis. Data show that retinol was stable in whole blood under effect of investigated conditions and percentage changes (-6.4 to 4.1%) were within ±11.8% of retinol TCL. There was no significant difference in the retinol levels in the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	2.18 (0.06)	0.0	2.18 (0.06)	0.0	2.18 (0.06)	0.0
3h	2.21 (0.05)	1.4	2.25 (0.02)	3.2	2.19 (0.04)	0.5
6h	2.25 (0.02)	3.2	2.24 (0.02)	2.8	2.21 (0.02)	1.4
12h	2.19 (0.03)	0.5	2.27 (0.02)	4.1	2.19 (0.02)	0.5
24h	2.18 (0.04)	0.0	2.24 (0.03)	2.8	2.23 (0.03)	2.3
48h	2.21 (0.03)	1.4	2.26 (0.05)	3.7	2.20 (0.05)	0.9
1w	2.04 (0.19)	-6.4	2.08 (0.19)	-4.6	2.10 (0.19)	-3.7
Effect of storage time	***p*=0.124**					
Effect of temperature	**NA**		***p*=0.437**			
Effect of light	***p*=0.359**				**NA**	

h: hour/s, w: week/s, m:mounth, NA: not applicable

Table 4-9. Stability of retinol in serum.

This table shows percentage changes in concentrations of serum retinol under effect of light and temperature across several time points compared to baseline concentration of serum control. The examined serum samples were exposed to different conditions prior to storing in -80ºC while serum control samples were immediately stored at -80ºC until analysis. Results show that retinol was stable in serum under the effects of the investigated conditions, and percentage changes (-6.5 to 2.8%) were within ±11.8% of retinol TCL. There was no significant difference in the retinol levels under effect of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)		-20ºC (Dark)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	2.61 (0.07)	0.0	2.61 (0.07)	0.0	2.53 (0.03)	0.0	2.52 (0.03)	0.0
3h	2.63 (0.02)	0.8	2.65 (0.09)	1.5	2.54 (0.01)	0.4	2.55 (0.02)	1.2
6h	2.61 (0.04)	0.0	2.67 (0.03)	2.3	2.58 (0.05)	2.0	2.54 (0.04)	0.8
12h	2.62 (0.04)	0.4	2.63 (0.04)	0.8	2.56 (0.03)	1.2	2.55 (0.01)	1.2
24h	2.57 (0.05)	-1.5	2.59 (0.06)	-0.8	2.57 (0.05)	1.6	2.56 (0.01)	1.6
48h	2.57 (0.05)	-1.5	2.62 (0.07)	0.4	2.57 (0.02)	1.6	2.53 (0.03)	0.4
1w	2.44 (0.03)	-6.5	2.61 (0.05)	0.0	2.6 (0.09)	2.8	2.54 (0.06)	0.8
2w	NA	NA	NA	NA	2.61 (0.05)	3.2	2.53 (0.06)	0.4
1m	NA	NA	NA	NA	2.6 (0.05)	2.8	2.58 (0.03)	2.4
Effect of storage time	***p*= 0.230**							
Effect of temperature	**NA**		***p*=0.198**					
Effect of light	***p*=0.602**				**NA**			

h: hour/s, w: week/s, m:mounth, NA: not applicable

Table 4-10. Stability of retinol during sample processing.

This table shows retinol concentration changes in samples processed under subdued light compared to samples processed in regular light at RT.

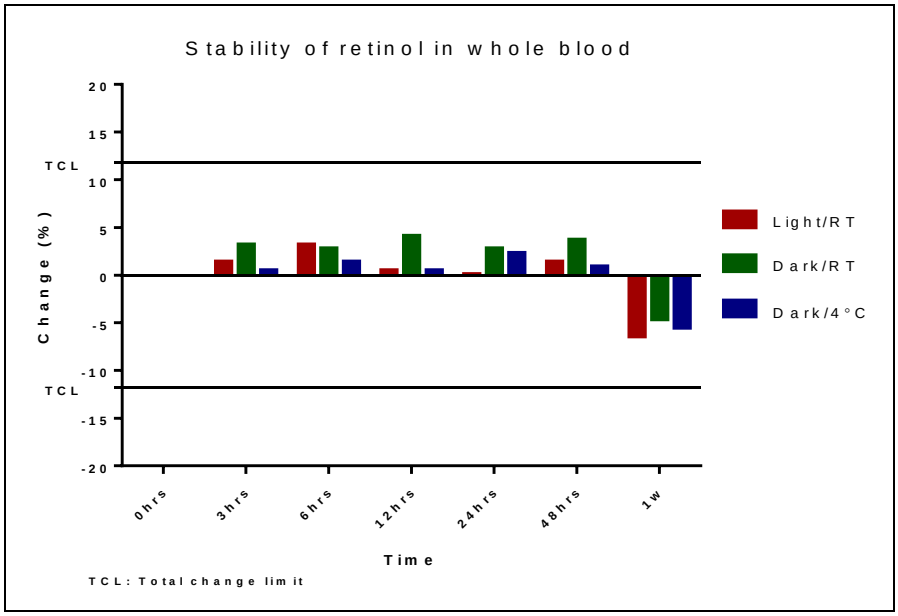
	Subdued light at RT nmol/L	Light at RT nmol/L
Mean (SD)	2.23 (0.06)	2.22 (0.07)
Change (%)	-0.4	
p value	0.938	

Table 4-11. Stability of extracted retinol.

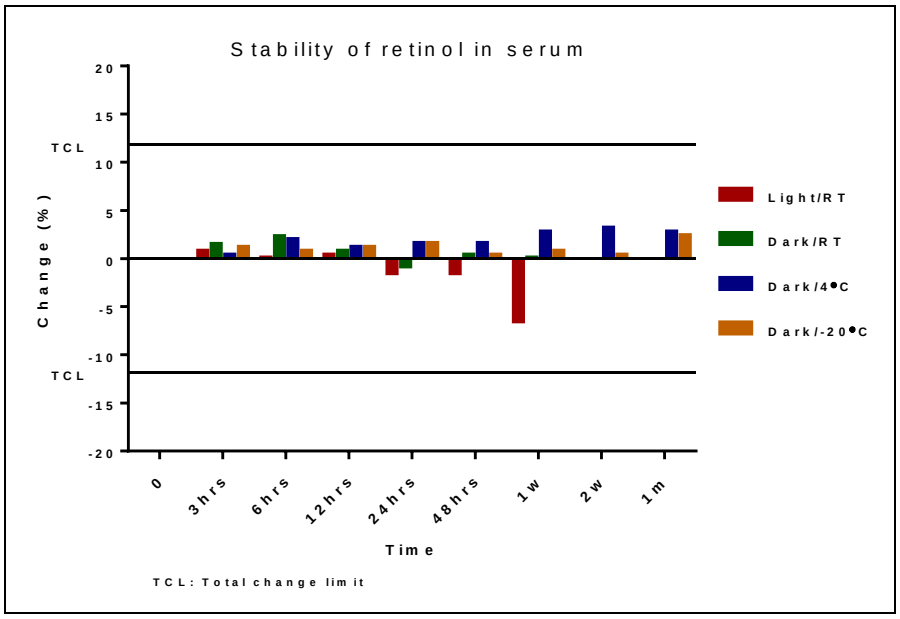
This table shows percentage changes in concentrations of extracted retinol from serum samples under effect of light and temperature across several time points compared to baseline concentration. The extracted retinol samples were exposed to different conditions prior to storing in -80ºC while the extracted retinol control samples were immediately stored at -80ºC. Results show that extracted retinol in the samples protected from the light was stable in investigated conditions, and percentage changes (-2.5 to 3.3) were within ±11.8% of retinol TCL. In contrast, extracted retinol exposed to light was stable at least for 48h at RT.

	RT (Light)		RT (Dark/)		4ºC (Dark/)		-20ºC (Dark)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	2.44 (0.07)	0.0	2.44 (0.07)	0.0	2.44 (0.07)	0.0	2.44 (0.07)	0.0
3h	2.40 (0.07)	-1.6	2.45 (0.08)	0.4	2.46 (0.08)	0.8	2.41 (0.13)	-1.2
6h	2.41 (0.08)	-1.2	2.46 (0.13)	0.8	2.44 (0.08)	0.0	2.40 (0.12)	-1.6
12h	2.39 (0.08)	-2.0	2.5 (0.16)	2.5	2.44 (0.13)	0.0	2.48 (0.08)	1.6
24h	2.39 (0.08)	-2.0	2.47 (0.14)	1.2	2.43 (0.07)	-0.4	2.41 (0.09)	-1.2
48h	2.37 (0.12)	-2.9	2.52 (0.12)	3.3	2.38 (0.11)	-2.5	2.44 (0.11)	0.0
1w	1.99 (0.09)	-18.4	2.48 (0.10)	1.6	2.46 (0.08)	0.8	2.40 (0.09)	-1.6
2w	NA	NA	NA	NA	2.44 (0.08)	0.0	2.39 (0.11)	-2.0
1m	NA	NA	NA	NA	2.46 (0.08)	0.8	2.44 (0.13)	0.0
Effect of storage time	***p*=0.907**							
Effect of temperature	**NA**		***p*=0.048**					
Effect of light	***p*=0.008**				**NA**			

h: hour/s, w: week/s, m:mounth, NA: not applicable



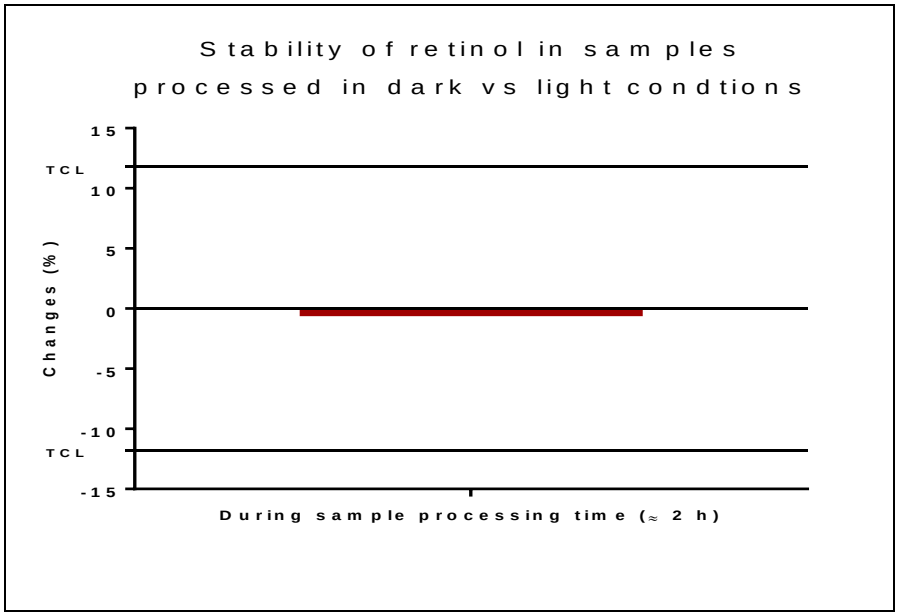
a)



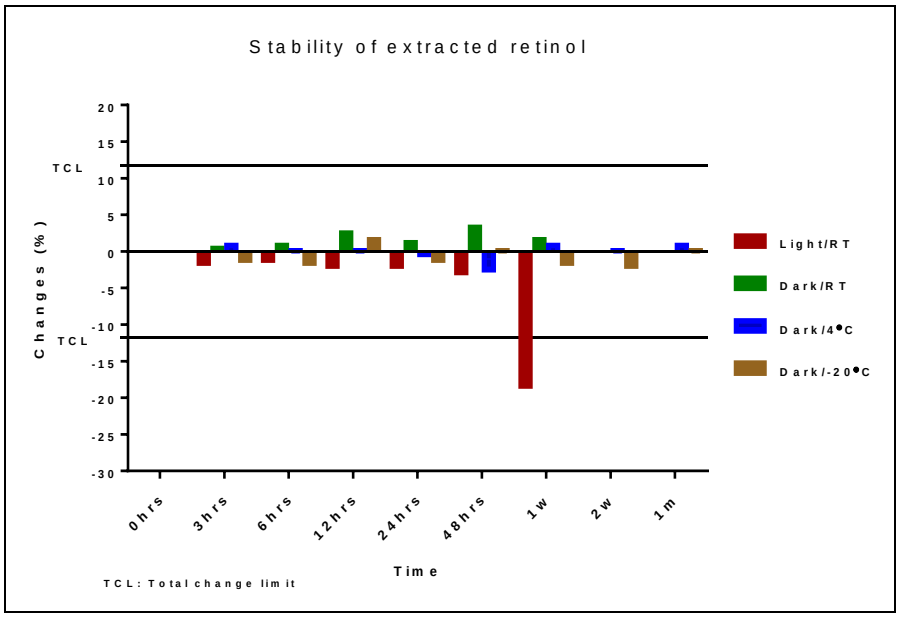
b)

Figure 4-4. Stability of retinol in unprocessed samples.

These graphs show the stability of retinol in a) whole blood and b) serum under different conditions. Bar graphs show average of concentration changes (%) of retinol in samples, which were exposed to different conditions of light, temperature and time, from a baseline concentration in control sample processed using optimal conditions. The red bars represent samples exposed to light at RT while green, blue and brown bars represent samples protected from the light at RT, 4ºC and -20ºC, respectively. Solid lines represent ± total change limits which are used to determine the acceptable clinical limits of analyte changes. Effects 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.



a)



b)

Figure 4-5. Stability of retinol samples during processing.

These graphs show the stability of retinol during sample processing under different conditions. Bar graph (a) displays average of 25-OHD3 concentration changes (%) in samples processed in subdued light versus to samples processed in light RT. Bar graphs (b) show average of concentration changes (%) of extracted retinol, which were exposed to different conditions of light, temperature and time, from a baseline concentration in control sample processed using optimal conditions. The red bars represent samples exposed to light at RT while green, blue and brown bars represent samples protected from the light at RT, 4ºC and -20ºC, respectively. Solid lines represent ± total change limits which are used to determine the acceptable clinical limits of analyte changes. Effects 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.

4.3.3 Stability of α-tocopherol

The stability of α-tocopherol was investigated at different matrixes under different conditions. The concentration changes of α-tocopherol were ±3.2% in whole blood, serum and extracts as well as in sample processed under investigated conditions. These changes were within ±10.8% of TCL for α-tocopherol. We observed insignificant difference in concentrations of α-tocopherol in factors of light, temperature and time Table 4-12, Table 4-13, Table 4-14, Table 4-15, Figure 4-6, Figure 4-7).

Table 4-12. Stability of α-tocopherol in whole blood.

This table shows percentage changes in whole blood α-tocopherol levels under effect of light, temperature and time. The examined whole blood samples were exposed to different conditions prior to serum obtained and stored at -80ºC while serum from whole blood control samples was immediately obtained and stored at -80ºC until analysis. Results show that α-tocopherol was stable in whole blood under effect of investigated conditions and percentage changes (±3.2%) were within ±10.8% of TCL forα-tocopherol. There was no significant difference in the α-tocopherol levels under effect of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	31 (1.3)	0.0	31 (1.3)	0.0	31 (1.3)	0.0
3h	31 (1.1)	0.0	31 (0.9)	0.0	31 (0.8)	0.0
6h	32 (0.9)	3.2	32 (1.2)	3.2	31 (0.6)	0.0
12h	32 (0.4)	3.2	31 (1.2)	0.0	31 (0.4)	0.0
24h	32 (0.5)	3.2	30 (0.5)	-3.2	32 (0.7)	3.2
48h	31 (0.3)	0.0	30 (1.4)	-3.2	31 (0.8)	0.0
1w	30 (0.3)	-3.2	31 (0.7)	0.0	31 (1.2)	0.0
Effect of time	***p*=0.567**					
Effect of temperature	**NA**		***p*=0.133**			
Effect of light	***p*=0.242**				**NA**	

h: hour/s, w: week/s, m:mounth, NA: not applicable

Table 4-13. Stability of α-tocopherol in serum.

This table shows percentage changes in concentrations of serum α-tocopherol samples under effect of light and temperature across several time points compared to baseline concentration of serum control samples. The examined serum samples were exposed to different conditions prior to storing in -80ºC while serum control samples were immediately stored at -80ºC until analysis. Results show that α-tocopherol was stable in serum under effect of investigated conditions and percentage changes (±3.2%) were within ±10.8% of α-tocopherol. There was no significant difference in the α-tocopherol levels under effect of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)		-20ºC (Dark)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	32 (0.6)	0.0	32 (0.6)	0.0	31 (0.9)	0.0	31 (0.9)	0.0
3h	32 (1.5)	0.0	32.5 (1.2)	0.0	31 (0.7)	0.0	32 (2.8)	3.2
6h	32 (2.0)	0.0	32.5 (0.7)	0.0	32 (1.2)	3.2	31 (1.0)	0.0
12h	33 (1.7)	3.1	31.6 (1.1)	0.0	32 (0.5)	3.2	32 (1.0)	3.2
24h	32 (1.2)	0.0	31.4 (0.8)	-3.1	32 (0.5)	3.2	32 (1.7)	3.2
48h	32 (1.6)	0.0	32.5 (0.8)	0.0	32 (0.6)	3.2	30 (1.1)	-3.2
1w	32 (0.6)	0.0	33 (1.3)	3.1	32 (1.0)	3.2	31.4 (0.6)	0.0
2w	NA	NA	NA	NA	32 (1.5)	3.2	31 (0.8)	0.0
1m	NA	NA	NA	NA	32 (1.9)	3.2	32 (0.7)	3.2
Effect of time	***p*=0.509**							
Effect of temperature	**NA**		***p*=0.745**					
Effect of light	***p*=0.827**				**NA**			

h: hour/s, w: week/s, m:mounth, NA: not applicable

Table 4-14. Stability of α-tocopherol during sample processing.

This table shows α-tocopherol concentration changes in samples processed under subdued light compared to samples processed in regular light at RT.

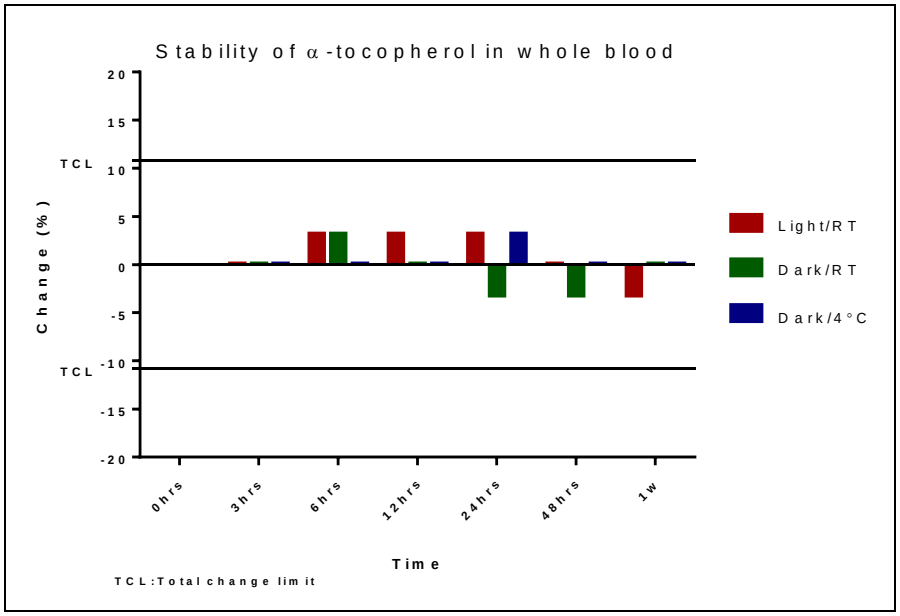
	Subdued light at RT nmol/L	Light at RT nmol/L
Mean (SD)	40 (0.9)	40 (1.5)
Change (%)	0.0	
p value	0.956	

Table 4-15. Stability of extracted α-tocopherol.

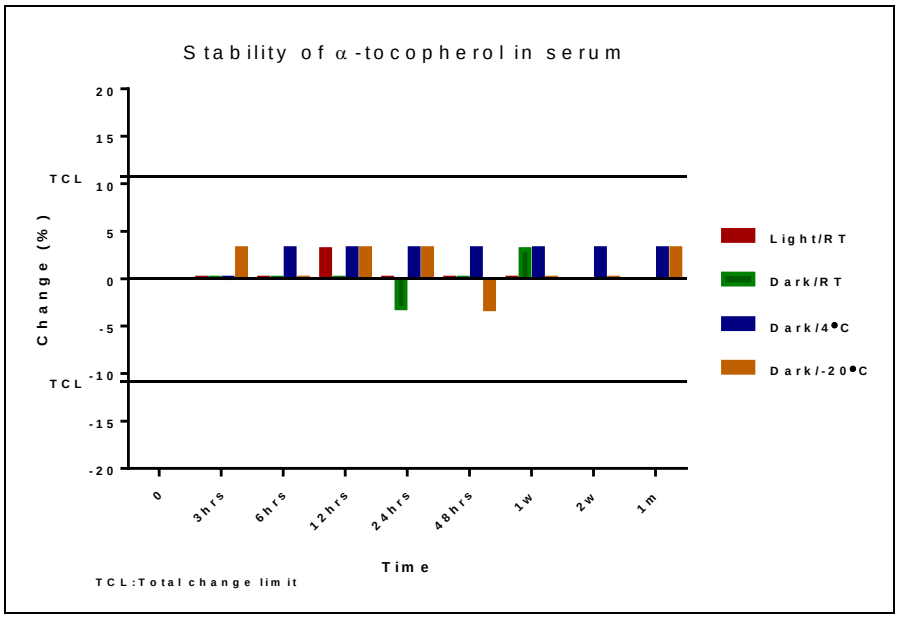
This table shows percentage changes in concentrations of extracted α-tocopherol from serum samples under effect of light and temperature across several time points compared to baseline concentration of control samples. The extracted α-tocopherol samples were exposed to different conditions prior to storing in -80ºC while the extracted α-tocopherol control samples were immediately stored at -80ºC. Results show that extracted α-tocopherol was stable in investigated conditions and percentage changes (±3.1%) were within ±10.8% of TCL for α-tocopherol. There was no significant difference in the α-tocopherol levels under effect of the investigated conditions.

	RT (Light)		RT (Dark/)		4ºC (Dark/)		-20ºC (Dark)	
Time	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %	Mean (SD) nmol/L	Mean change %
Baseline concentration	32 (1.2)	0.0	32 (1.2)	0.0	32 (1.2)	0.0	32 (1.2)	0.0
3h	32 (1.1)	0.0	31 (1.8)	-3.1	32 (2.4)	0.0	31 (2.9)	-3.1
6h	31 (2.2)	-3.1	32 (2.8)	0.0	32 (3.1)	0.0	31 (1.9)	-3.1
12h	33 (1.7)	3.1	31 (1.0)	-3.1	31 (2.8)	-3.1	32 (1.8)	0.0
24h	32 (2.2)	0.0	31 (2.6)	-3.1	31 (2.0)	-3.1	32 (3.0)	0.0
48h	32 (1.0)	0.0	33 (1.9)	3.1	31 (2.0)	-3.1	32 (2.9)	0.0
1w	33 (2.5)	3.1	33 (3.3)	3.1	33 (1.6)	3.1	31 (2.2)	-3.1
2w	NA	NA	NA	NA	31 (01.8)	-3.1	31 (1.7)	-3.1
1m	NA	NA	NA	NA	31 (2.8)	-3.1	31 (2.3)	-3.1
Effect of time	***p*=0.775**							
Effect of temperature	**NA**		***p*=0.776**					
Effect of light	***p*=0.335**				**NA**			

h: hour/s, w: week/s, m:mounth, NA: not applicable



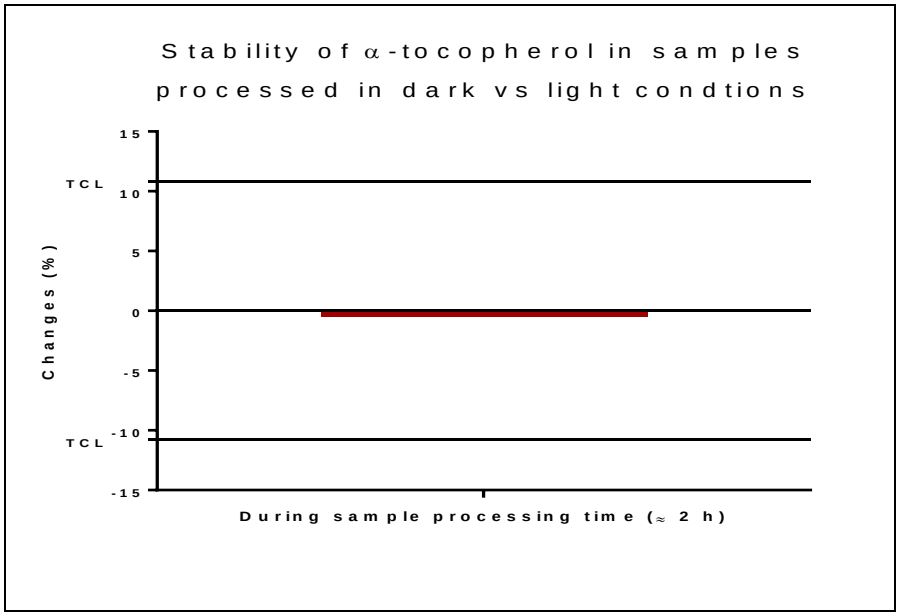
a)



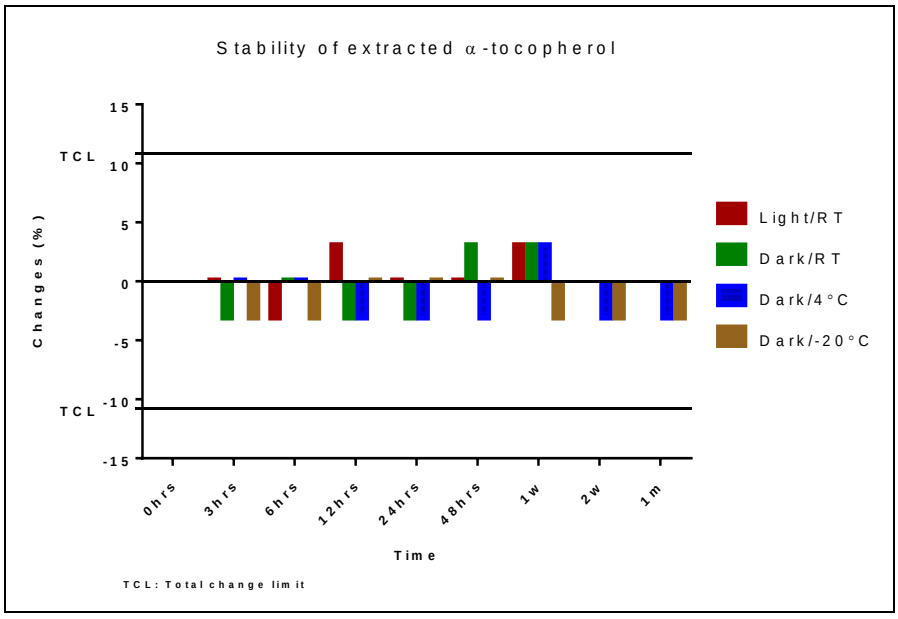
b)

Figure 4-6. Stability of α-tocopherol in unprocessed samples.

These graphs show the stability α-tocopherol in a) whole blood and b) serum under different conditions. Bar graphs show average of concentration changes (%) of α-tocopherol in samples, which were exposed to different conditions of light, temperature and time, from a baseline concentration in control sample processed using optimal conditions. The red bars represent samples exposed to light at RT while green, blue and brown bars represent samples protected from the light at RT, 4ºC and -20ºC, respectively. Solid lines represent ± total change limits which are used to determine the acceptable clinical limits of analyte changes. Effects 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.



a)



b)

Figure 4-7. Stability of α-tocopherol samples during processing.

These graphs show the stability of α-tocopherol during sample processing under different conditions. Bar graph (a) displays average of α-tocopherol concentration changes (%) in samples processed in subdued light versus to samples processed in light RT. Bar graphs (b) show average of concentration changes (%) of α-tocopherol, which were exposed to different conditions of light, temperature and time, from a baseline concentration in control sample processed using optimal conditions. The red bars represent samples exposed to light at RT while green, blue and brown bars represent samples protected from the light at RT, 4ºC and -20ºC, respectively. Solid lines represent ± total change limits which are used to determine the acceptable clinical limits of analyte changes. Effects 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.

4.4 Discussion

Evidence of FSV stability across the pre-analytical and analytical stages is valuable information for fill some knowledge gaps as part of standardisation efforts for FSV analysis. Furthermore, it is important to avoid clinical result misinterpretation and for development of suitable protocols for sample collection, transportation and storage for routine patient diagnostic study and for large-scale studies. This work examined the stability of FSVs in whole blood and serum as well as during and post-extraction process (extract) under the influence of light and temperature over longer periods of storage time than most previous studies investigated. This valuable information helps to fill some knowledge gaps in vitamin analysis (81). To the best of our knowledge, this work is the first study exploring the stability FSVs simultaneously utilising a precise LC-MS/MS method. In addition, stability of each analytes is justified based on calculation of the acceptable clinical limits "TCL", which reflect biological variation as well as method imprecision (175).

The photosensitivity of the FSV analytes (25-OHD3, retinol and α-tocopherol) was investigated by a number of studies (95, 97, 150, 159, 190). 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 confirmed the some previous studies, which reported that whole blood and serum 25-OHD was stable in ambient fluorescent light at RT (159) and even in extreme exposure to sunlight for 4 h (150, 190). 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 liberates from vitamin D-binding proteins which protect the 25-OHD3, unlike 25-OHD3 in whole blood and serum.

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 TCL (11.8%). A limited number of studies investigated light impact on retinol levels in whole blood (95, 97) and serum/plasma (183, 201). Our study results agreed with some previous observations that whole blood and serum/plasma retinol is stable in ambient light at RT for 48h (95, 97, 183) and for 1 week (97).

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 due to the liberty of extracted retinol from its carriers. Retinol (286 Da) is transported in blood stream through a bound with retinol binding protein (≈ 21 kDa) that forms a larger complex with another protein called Transthyretin (TTR, 56 kDa). This complex (≈75 kDa) is crucial for retinol protection and functions (5, 214), and extracted retinol might become more sensitive to light than retinol bound with its transporters. Furthermore, our findings show no significant difference in retinol concentrations in the sample processed in ambient light RT compared to those processed in subdued light. This is because complete sample processing usually takes less than three hours; thus, light may have a limited effect on retinol concentrations during short time of exposure such a time required for sample processing.

The α-tocopherol in whole blood, serum and extract is stable for at least 1 week in ambient light at RT based on the current study outcomes. This is in accordance with some previous findings regarding the stability of α-tocopherol in whole blood for at least 1 week (97) and in

serum for at least 48h (183) under light at RT. Based on our results and previous study results, α-tocopherol is not a photosensitive analyte for at least 1 week at RT.

The thermostability of 25-OHD3, retinol and α-tocopherol in whole blood, serum and extract was debatable because of limited or contradicted evidence (Table 4-1, Table 4-2). Data from our study show that 25-OHD3 was stable in whole blood, serum and extract for at least 1 week at RT and for at least 1 month at 4ºC and -20ºC with acceptable concentration changes. Previous studies explored the stability of 25-OHD in whole blood for short times (up to 72h) at RT, and they reported that 25-OHD was firmly stable (98, 159, 186, 187). Other studies showed 25-OHD in serum/plasma was stable at RT for 1–2 weeks (159, 190, 193) and for couple of months to a few years at -20ºC (154, 159, 193, 194). Further studies found that extracted 25-OHD was stable at RT for 3–7 days (150, 154).

In the current study, we also observed that retinol can be reliably measured in whole blood samples stored at least for 1 week in RT or 4ºC, and that supports some previous results of retinol changes 3.3% at RT and -0.3% at 4ºC (97). In contrast, other studies found whole blood retinol levels decreased by -9.8% (98) and -15.5% (179) when whole blood samples stored at RT for 72h. Furthermore, this analyte in whole blood samples stored at chilled conditions for 48h degraded by 8.7% (179). High degradation rate for retinol reported in these two studies may be due to method inter-run imprecisions used in the analysis rather than retinol storage conditions. Regarding serum retinol, our data shows that serum retinol was also stable at least for 1 week at RT and for 1 month at 4ºC and -20ºC. Previously, it was reported that serum retinol was stable for 1–3 days at chilled conditions (98, 179, 182). Furthermore, Other studies found that serum retinol changes were -5% (1 week), -7% (2 weeks) and -17% (4 weeks) (177). These results highlight whether RT has linear effect over long period of time, such of the question that our study could not answer.

Although changes in the trends of retinol in whole blood and serum were slightly decreasing (within retinol TCL ±11.8%), it seems that whole blood retinol is more affected by the time of pre-centrifugation rather than the effects of light and temperature (RT and 4ºC). We observed a higher decreasing percentage in 1 week of time in both lighted RT and dark 4ºC conditions. This may be related to the presence of blood cells contacted with the serum, which impact some analytes (174, 175). The current work also investigated the impact of storage extracted retinol for a longer time period: up to 1 week at dark RT, and up to 1 month at dark 4ºC and -20ºC. Our results show reliable stability of extracted retinol and that confirm previous findings related stability extracted retinol at RT, 4ºC and -20ºC for up to 48h (183).

The current data demonstrates that α-tocopherol was stable for at least 1 week at RT (in whole blood, serum and extract) and 1 month at 4ºC and -20ºC (in serum and extract). According to previous studies, whole blood α-tocopherol was stable for at least 48h (98, 179) and for 1 week (97) at RT and chilled conditions. It was reported that serum α-tocopherol was for at least 24h (182, 205) while other study found changes in serum stored at RT for 4 weeks was less than 1% (177). Previous studies (183) reported that extracted α-tocopherol was stable for at least 48h at RT, 4ºC and -20ºC. The current work examined its stability for longer time periods. Our results show that extracted α-tocopherol was also stable for at least 1 week at RT and for 1 month at 4ºC and -20ºC.

Other issues have been accounted as potential factors influencing FSVs, including repeating freezing-thawing cycle, nature of matrix (serum vs plasma) and type blood collection tubes (plain tubes vs serum separator tube). It was reported that repeating freezing-thawing cycle for 3–5 times had no effect on serum levels of retinol and α-tocopherol (177) as well as serum 25-OHD levels (150, 154, 159, 190, 193).

Serum and plasma samples are commonly used in clinical chemistry, however, the question is whether the stability of target FSVs analytes are varied based on serum compared to plasma. Previous studies observed no significant difference in 25-OHD3 in plasma compared to serum (154, 186, 193). Furthermore, they found no difference in serum 25-OHD3 concentration between serum separator tubes and plain tubes even in long-term storage (191, 195). These results could be true regarding their effect on retinol and α-tocopherol concentrations in serum and plasma.

4.5 Conclusions

Our results confirm that 25-OHD3, retinol and α-tocopherol are firmly stable in whole blood and serum samples under the investigated conditions. Whole blood and serum samples destined for FSVs interrogation can be reliably processed in normal laboratory conditions of light and temperature during the pre-analytical stage. The measurements of extracted 25-OHD3 and α-tocopherol can also be conducted under light at RT, while light protection for extracted retinol is recommended if the analysis requires more than 48 h.

Chapter 5

Traceability of commercial calibrators to reference material: commercial α-tocopherol calibrators' example

Chapter 5 Traceability of commercial calibrators to reference material: α-tocopherol calibrators' example

5.1 Introduction

Blood vitamin E refers to fat-soluble antioxidant metabolites, including α-tocopherol and γ-tocopherol, where α-tocopherol is a predominant form in the blood and commonly used as an indicator of vitamin E status. Most clinical laboratories use commercial calibrators for blood α-tocopherol measurement in patient samples (215). For a reliable patient results comparison regardless of time, location or method applied, these results should be traceable to high-order references. These references consist of three pillars, including reference measurement procedures, standard reference materials and reference laboratories, which are essential for measurement traceability implementation and method harmonisation (100, 117).

Both reference measurement procedures and reference materials contribute to the measurement traceability of an analyte. Reference measurement procedures are used to assign and certify a value to a reference material as a primary calibrator (pure analyte) or as a secondary calibrator (analyte in human samples) (99). Industrially, this certified reference material can be used to assign values to a commercial calibrator. Later, routine laboratory medicine uses validated commercial calibrators in measuring analytes in human samples. Hence, analytical results obtained from routine laboratory tests are traceable to certified materials.

The National Institute of Standards and Technology (NIST) introduced a number of standard materials for several analytes as part of efforts for traceability and standardisation in laboratory medicine. Since 1989, the NIST has released standard reference materials (SRM) for fat-soluble vitamins (SRM 968), including α-tocopherol, in human serum (102). The

available commercial calibrators for vitamins A and E are produced and theoretically traceable to a specific SRM 968 version. However, the accurate traceability chain (from the standard reference material to the commercial calibrators) could be affected by a number of factors, such as the matrix, manufacturing procedures and method employed to assign their values (216-218). Furthermore, patient results are influenced by calibrators used in the sample analysis (219-221). As such, an unacceptable agreement between calibrators is potentially a significant obstacle to method harmonisation (106, 117).

The current study aimed to explore the extent of the variation among three commercial calibrators for vitamin E (α-tocopherol) usually used in a clinical chemistry laboratory for a serum/plasma α-tocopherol analysis using two LC-MS/MS methods. This exploring introduced an example of how different commercial calibrators could impact on patient result interpretation.

5.2 Methodology

Three commercial single-level calibrators were sourced simultaneously from the current available stock. These calibrators include: Bio-Rad (traceable to NIST SRM 968e), Chromsystems (traceable to NIST SRM 968e) and RECIPE (traceable to NIST SRM968d). They were prepared in quintuplicate in conjunction with a seven-level in-house vitamins A and E calibrator set for an analysis of α-tocopherol. Hexa-deuterated α-tocopherol was used as the internal standard, and the in-house calibrators were used to create a standard curve to which the three commercial calibrators were compared. Preparation of the in-house calibrator set and commercial calibrators as well as internal standard was detailed in Chapter 2, sections 2.5.3 and 2.5.4.

All samples were prepared based on the sample preparation protocol detailed in Chapter 2, section 2.6. Samples were prepared briefly as follows: sample and Milli-Q water were added to a glass tube and vortexed. Next, methanol containing the deuterated internal standard was added to this mixture, vortexed and equilibrated room temperature. Analytes were extracted by adding hexane to the mixture and then vortexed extensively prior to centrifugation at room temperature.The organic layer was transferred to a new glass tube and dried under a stream of nitrogen gas at room temperature and subsequently reconstituted in methanol. This sample preparation procedure was conducted under subdued light. The reconstituted sample (1 μL) was injected onto the Agilent LC–MS/MS-6490 system.

In this study, two methods—the vitamin A/E method (first method) and the FSV method 1 (second method)—were employed. Both methods were detailed in Chapter 3. The following is a brief description of the two methods.

The first method

The chromatographic separation of analytes was performed using a Pursuit (R) XRs C18 column with matched guard column. The mobile phases were 0.1% formic acid in Milli-Q water containing 2% methanol (mobile phase A) and 0.1% formic acid in methanol (mobile phase B). A constant flow rate of 0.2 mL/min and a gradient profile from 80% to 100% of mobile phase B were employed. This method was used to quantify α-tocopherol and its hexa-deuterated internal standard plus retinol, as detailed in Chapter 3 (method A/E).

The second method

Analytes were separated using a Pursuit pentafluorophenyl (PFP) column, which provides a highly efficient separation of stereometric isomers, along with matched guard column. The flow rate and mobile phases were the same as used in the first method. A gradient profile from 35% to 100% of mobile phase B was employed. This method was developed to quantify α-tocopherol in addition to 25-OHD2, 25-OHD3 and its epimer and retinol, as described in Chapter 3 (FSV method-1).

The electrospray ionization technique (positive mode) was used in both methods. Multiple reaction monitoring (MRM) was utilized to quantify α-tocopherol (quantifier, 431 → 165 and qualifier, 431 → 137) and hexa-deuterated α-tocopherol (quantifier, 437 → 171).

Statistical analysis

The percentage bias of the results of each commercial calibrator was calculated according to Equation 5-1.

Equation 5-1. Percentage bias calculation.

$$Bias\ (\%) = \frac{Mean\ observed\ concentration - \ manufacturer\ \ nominal\ concentration}{manufacturer\ \ nominal\ concentration} \times 100$$

Bland–Altman difference plots were created to demonstrate the results graphically using GraphPad Prism version 6 (GraphPad Prism Software Inc., CA, USA). The standard error of the mean (SEM) of each commercial calibrator result was calculated based on Equation 5-2 using Microsoft Excel. The expanded imprecision (uncertainty), calculated as two times the within run coefficient of variation, was ±6% at the 95% confidence interval across the assay range (222).

Equation 5-2. Standard error of the mean calculation.

$$\boldsymbol{Standard\ error\ of\ the\ mean} = \frac{\boldsymbol{SD}}{\sqrt{\boldsymbol{n}}}$$

SD: the standard deviation of the calibrator replicates, n: the number of sample replicates (n=5)

5.3 Results

The current study results show that biases of the three commercial calibrators ranged from -9.8% to +5.4% across the two methods, with a consideration of employing the in-house calibration curve as the point of nominal zero bias (Table 5-1, Figure 5-1, Figure 5-2). The Bio-Rad calibrator bias across the two methods was +1.4, whereas the analytical biases of the Chromsystems calibrator at +5.4% and +5.0%, as based on the first and second methods, respectively. The biases of the RECIPE calibrator were greater, observed as -8.9% and -9.8% with the first and second methods, respectively. The overall discrepancy among the commercial calibrators was greater than the expected measurement uncertainty.

Table 5-1. Commercial calibrator bias comparison.

Expected and observed concentrations of α-tocopherol in the three commercial calibrators based on an in-house calibrator set.

Calibrator	Expected Concentration µmol/L	First Method		Second Method	
		Mean observed concentration (+/- SE mean) µmol/L	Bias %	Mean observed concentration (+/- SE mean) µmol/L	Bias %
Bio-Rad[a]	43.0	43.6 (±0.4)	+1.4	43.7 (±0.3)	+1.4
Chromsystems[a]	29.9	31.5 (±0.3)	+5.4	31.4 (±0.3)	+5.0
RECIPE[b]	56.4	51.4 (±0.6)	-8.9	50.9 (±0.5)	-9.8

a Secondary calibrator traceable to NIST SRM 968e.

b Secondary calibrator traceable to NIST SRM 968d.

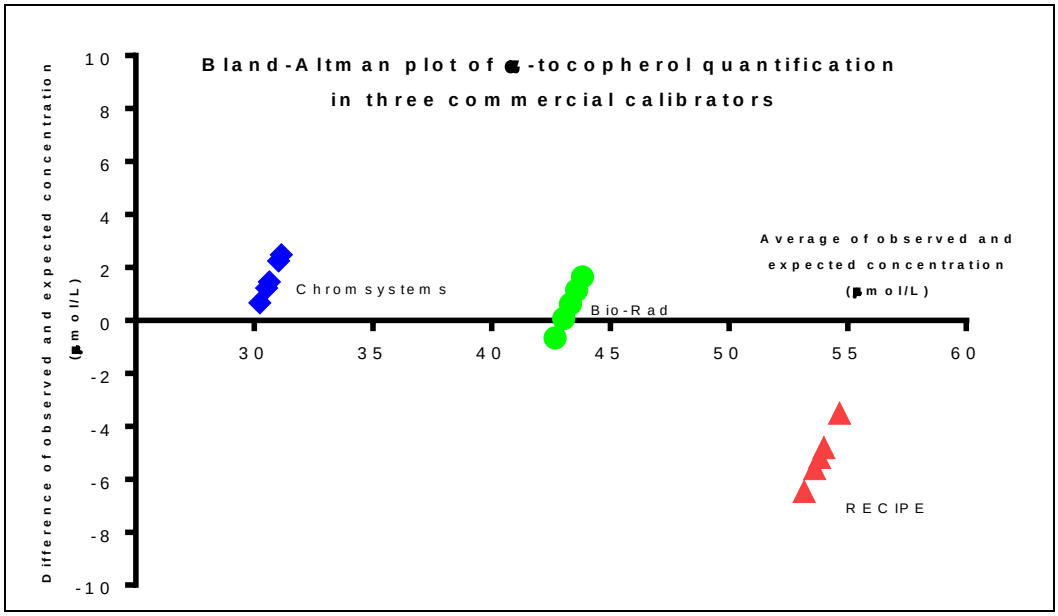


Figure 5-1. Bland-Altman plot of results obtained by the first method.

Bland–Altman difference plot showing the extent of agreement between expected and observed concentrations of α-tocopherol in three commercial calibrators using the first method. The difference between the expected and the observed concentration (y-axis) is plotted against the average concentration for each calibrator (x-axis).

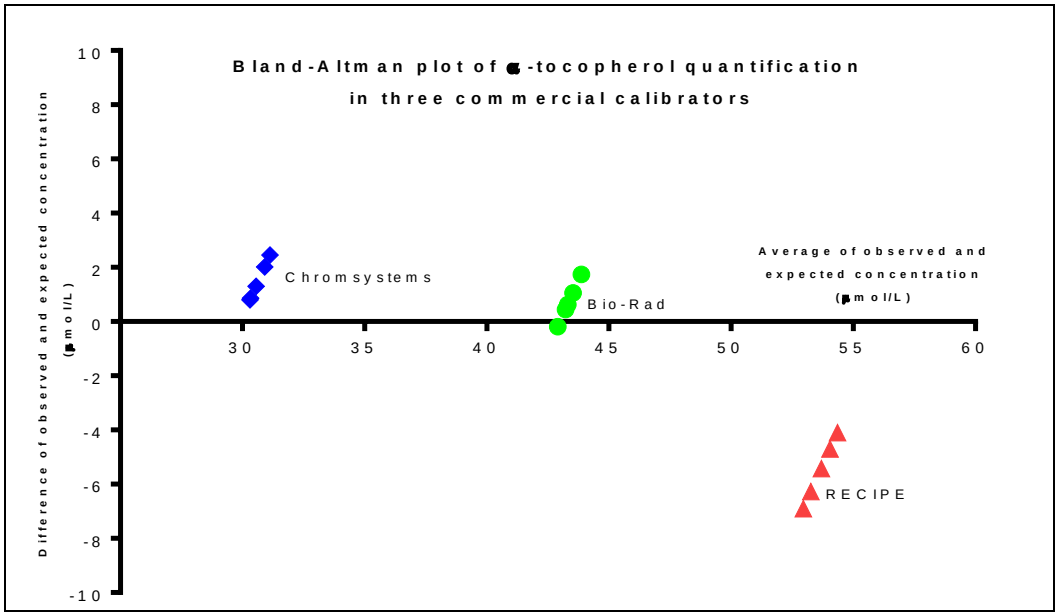


Figure 5-2. Bland-Altman plot of results obtained by the second method.

Bland–Altman difference plot showing the extent of agreement between expected and observed concentrations of α-tocopherol in three commercial calibrators using the second method. The difference between the expected and the observed concentration (y-axis) is plotted against the average concentration for each calibrator (x-axis).

5.4 Discussion

In the current study, the investigated three commercial calibrators for serum/plasma α-tocopherol showed discrepancies between observed concentrations versions manufacturer expected concentrations, although they were traceable to the same reference standard material version. The manufacturers of the reference standard materials and commercial calibrators, as well as routine clinical laboratories, employ a liquid chromatography platform (142, 223). To our knowledge, this report is the first comparing commercial calibrators for α-tocopherol utilising LC–MS/MS as the reference point.

It is well recognised that correct and consistent calibrator assignment is important for accurate result interpretations and medical decisions (224). Calibrator discrepancies used in the analytical stage contribute to systematic errors, in terms of traceability to a reference material and their lot-to-lot variation (225). It has been reported that traceability to common calibrators has improved inter-method variations (imprecision) (219, 220, 226). The method agreement of vitamin E assay by laboratories participating in an external quality assurance program has been monitored by RCPAQAP as part of efforts towards the harmonisation and standardisation of analyses (142, 215). This is important to ensure reliable patient results comparisons.

Commercial manufacturers validated the three commercial calibrators using HPLC platform. HPLC systems are routinely used for vitamin E analysis along with UV/Vis detection (142). Although tandem mass spectrometry was utilised in the current study, which is higher in sensitivity compared to traditional detectors used in the HPLC, this is not a solid explanation for the observed deviations in the three calibrators. Owens and colleagues recently concluded, based on their effort in testosterone standardisation, using different methodologies was a

source of inter-laboratory variations (216). Potentially the same could be true for differences in methodologies among manufacturers of commercial calibrators.

Commutability of the reference materials or commercial calibrators is essential for method harmonisation. Commutability is expressed as a mathematical relationship between the results of the reference materials and authentic clinical samples using different measurement procedures (108). Hence, commutability reflects the extent of similar behaviours between clinical samples and reference materials (or commercial calibrators) during analyses. Such a "matrix effect" can occur due to changes in the matrix condition due to a supplement added or the production process during calibrator manufacturing (106, 108). Different matrices in calibrators could decrease assay imprecision and inaccuracy (150). For example, two levels of NIST SRM-972 for vitamin D analytes were non-commutable for all routine analytical methodologies because these levels contain non-human serum and exogenous analytes (227). Furthermore, Cattozzo and colleagues reported that patient results obtained using commutable calibrators were contradicted by results generated using non-commutable calibrators (107). The extent of commutability cannot be excluded here as a potential source of the discrepancy observed among the three commercial vitamin E calibrators.

The present study investigated the three commercial calibrators, which are traceable to NIST SRM 968. Both the Bio-Rad calibrator and the Chromsystems calibrator were traceable to SRM 968e, while the RECIPE calibrator was traceable to SRM 968d. In fact, NIST SRM 968d is a single concentration level (13.77 μmol/L), whereas the newer SRM 968e has been released in three concentration levels (15.2 μmol/L, 23.98 μmol/L and 45.0 μmol/L) of vitamin E (102, 121, 122). Compared to the other two calibrators, the RECIPE calibrator had the highest concentration of α-tocopherol (56.4 μmol/L), which interestingly was traceable to the single-level SRM with the lowest assigned value (NIST SRM 968d: 13.77 μmol/L).

Potentially, this could be a source of the discrepancy observed in the RECIPE calibrator. This may be coincidental and this point could not be confirmed or refuted, as there was no stock of the NIST SRM 968d available to purchase for comparison studies at the time the experiments were performed.

The Joint Committee for Traceability in Laboratory Medicine (JCTLM) provides a database of certified reference material (CRM). There was no listing on this database for a CRM for α-tocopherol when this study was conducted (228). However, the JCTLM database recently recognised NIST SRM 968e as CRM for vitamin E (105). The current study supports importance of concerted efforts by analytical parties, including the manufacturers of commercial calibrators, to evaluate rigorously traceability chain from CRM to commercial calibrators to ensure patient results are precisely comparable regardless of laboratories analysed patient samples.

5.5 Conclusions

Calibrators are potentially a significant obstacle to reliable comparison of patient results and epidemiologic study results. The traceability of measurements and the method harmonisation process can be interrupted due to the unacceptable agreement among commercial calibrators. The limited success efforts of harmonisation encumber the comparability of results obtained from different laboratories. Hence, efforts are required by all parties to address this issue in terms of value assignment, allowable error of calibrator assignment and inclusion of mass selective detection-based reference methods.

This study, which was published in the Clinical Biochemistry journal in 2013 (229), highlighted the issue related to calibrators in terms of the chain of the traceability from CRM to the commercial calibrators. Although the commercial calibrator manufacturers mentioned that their calibrators were traceable to CRM version, the manufacturers did not provide evidence of how traceability process was achieved. In addition, they did not mention about the other factors affecting the traceability chain and the commutability of commercial calibrators, such as matrix and production processes and method of determination. Until all of these problems related to the traceability chain are resolved, there is no guarantee of trueness of measurement results.

Chapter 6

Influence of cell culture media dilution of cord blood on vitamins A, D and E measurements

Chapter 6 Influence of cell culture media dilution of cord blood on vitamins A, D and E measurements

6.1 Introduction

Vitamins A, D and E are associated with a wide range of physiological roles in the body. Vitamin A has roles in various health outcomes including vision, healthy epithelial tissue and infection resistance (14). Vitamin D is both a vitamin and a hormone and has been correlated with a number of significant health issues including the overall bone health, the cardiovascular system, skin and the immune system (3). Vitamin E has powerful antioxidant activities which are essential for the protection of cellular structures and functions (58). These three vitamins are associated with both gene regulation and preventive roles in a wide range of health issues, such as cardiovascular disease and cancer (14, 58, 230, 231).

A growing number of studies have examined the potential importance of vitamin D levels, and to a lesser extent vitamins A and E levels, during the early stages of life. Vitamin D status in early life is correlated with numerous health outcomes, such as bone mineralisation, central nervous system disorders and autoimmune disorders (232). Adequate levels of vitamin A are correlated with preventive roles in childhood blindness, morbidity, and mortality, especially in populations at high risk for vitamin A deficiencies (29, 233). Both vitamins A and D have been correlated with food allergies in children (234). Furthermore, neonates, especially those with very low birth weights, are at high risk of vitamin A, D and E deficiencies, particularly in developing countries (235).

Umbilical cord blood (UCB) is an appropriate sample choice to assess vitamin status in neonates because limited venous blood samples can safely be obtained. UCB has been utilised to investigate the correlation of vitamin D with neonatal health problems (236).

Sufficient 25-hydroxyvitamin D (25-OHD) levels in the UCB may play a role in the enhancement of the neonatal innate immune response that minimises microbial infections (237). Camargo and colleagues (2011) examined 922 UCB samples and reported a significant inverse association between the UCB 25-OHD3 levels and the risk of respiratory infection and wheezing in early childhood (238). A vitamin D deficiency (25-OHD3 < 50 nmol/L) in UCB has been linked to an increased risk of respiratory syncytial viral infections and an increased occurrence of eczema in neonates during the first year of life (239, 240). The risk of food allergies in the first two years of life has also been linked with the maternal and UCB vitamin D status (241).

The avoidance of vitamin A deficiency is vital in preventing critical health problems in infants. Based on animal and human studies, a maternal vitamin A deficiency has been linked to a decreased kidney size and a reduced number of nephrons in neonates (242, 243). Low retinol cord blood (< 0.7 µmol/L) was correlated with low infant birth weights (2.5–3.0 kg) among 313 investigated babies (244).

The feasibility of vitamin measurements in UCB prepared for various research studies is worthwhile investigating. UCB is used as a source of stem cells, which can be used in a wide range of regenerative medical disciplines (245). Additionally, plasma UCB can be diluted with a cell culture media (RPMI-1640) to maintain and isolate UCB mononuclear cells for immunological studies. Because of the limited UCB available for a variety of studies that include diagnostic and therapy issues, concerns are raised whether it is feasible to measure vitamin D, as well as vitamins A and E, in the diluted plasma. This information introduces valuable data for using a limited UCB volume in large-scale epidemiological projects.

Measurements of vitamin D (25-OHD3) in serum/plasma is most commonly conducted by an immunoassay. However, this technique cannot identify the epimer of 25-OHD3, which is found in high levels in children. Liquid chromatography coupled with a tandem mass

spectrometry (LC-MS/MS) platform provides a highly selective quantification technique; therefore, it is the optimal analytical approach for 25-OHD3 as the epi-25-OHD3 (143). Additionally, the quantification of serum retinol and α-tocopherol using the LC-MS/MS platform has been reported in other studies (142, 234).

The aim of this study was to validate the measurement of 25-OHD3 and its epimer (epi-25-OHD3), retinol, and α-tocopherol by using our simultaneous quantification LC-MS/MS method for FSVs. Another aim was to compare the vitamin D results obtained by our laboratory (Laboratory A) versus another laboratory (Laboratory B) which also used the LC-MS/MS method for quantification of only vitamin D.

6.2 Methodology

Subjects:

Serum UCB samples (n = 20) and matched diluted UCB plasma samples (n = 20) were randomly selected from participants recruited as part of the Barwon Infant Study (BIS). The BIS study is a birth cohort study conducted in population of south-eastern Australia, and it was designed to investigate the early life origins of immune dysregulation in the modern environment. The project was approved by the Barwon Health Human Research Ethics Committee (10/24) and written informed consent was obtained prior to UCB collection and storage.

Sample collection:

The primary aim in the UCB collection was to isolate a large number of viable mononuclear cells (MNCs) that could be cryopreserved for future immune studies. For this purpose, UCB was collected using a 50 mL syringe inserted into the umbilical cord vein. An adequate volume of UCB was then added to a sterile tube containing exactly 20 mL of sterile transport medium (RPMI-1640 with 10 IU/mL preservative-free heparin [DBL Heparin Injection BP; porcine mucous; 5,000 IU/5 mL]), and the remaining blood was added to serum collection tubes. Serum was collected and aliquoted following centrifugation at 2700xg for 10 min at 20ºC. For diluted UCB plasma samples, a proportional UCB volume to diluent volume (anti-coagulant and RPMI-1600 volume) was accurately measured prior to centrifugation at 2700xg for 10 min at 20ºC. Once the blood cells were pelleted and isolated, the diluted plasma volume was estimated (Equation 6-1) and dilution factor was calculated (Equation 6-2). Depending on the volume of the UCB collected, samples dilution percentage

ranged from 0.26% to 0.43% (mean ± SEM: 0.32 ± 0.01) of neat plasma. The diluted plasma samples were then aliquoted and stored with the matched serum samples in a -80ºC freezer.

Equation 6-1. Estimation of diluted UCB plasma.

Diluted plasma = total volume of anticoagulated diluted UCB – volume of pelleted blood cells

Equation 6-2. Calculation of dilution factor.

$$\boldsymbol{Dilution\ factor = \frac{Diluted\ plasma\ volume - 20}{Diluted\ plasma\ volume}}$$

Experimental:

Twenty UCB serum and 20 diluted plasma de-identified samples were thawed and 150 uL aliquots were delivered in a Styrofoam container to both Laboratory A (lab A) [LC-MS/MS laboratory, Clinical Biochemistry Mass Spectrometry Laboratory, RMIT University, VIC, Australia] and to laboratory B (lab B) [UWA Centre for Metabolomics, Metabolomics Australia, University of Western Australia, WA, Australia]. They were analysed in two non-consecutive runs (R1 and R2) in random order to consider the between-run effects in the two laboratory results comparison. They were also analysed to ascertain if the diluted plasma samples provided an accurate measure of 25-OHD3 compared to serum.

The samples were analysed by two LC-MS/MS methods on two separate occasions. Samples were analysed in lab A using the FSV method 2, which is detailed in Chapter 3, and the sample preparation is described in Chapter 2, Section 2.6–2.8. The samples in lab B were analysed using the LC-MS/MS method that utilised Agilent-6460 coupled to a 2-dimensional 1290 UPLC system to quantify 25-OHD3 and it epimer. The analyses were performed using

50 µL of serum with a run time of 8 mins. Standard curves were created based on a Chromsystems Calibrator set (Chromsystems, Munich, Germany), which has been reported to be traceable to NIST-SRM972. The imprecision of the method for 25-OHD3 at 75 nmol/L and 18 nmol/L was 0.5% and 2.2%, respectively. The limit of quantification (LOQ) for 25-OHD3 and its epimer was 2 nmol/L (145).

Statistical analysis

Passing-Bablok regression and Bland-Altman plots were used to compare the results of vitamin measurements in UCB serum and diluted UCB plasma. The non-parametric Spearman correlation coefficient (r) was used to examine the correlation of the group of results. A p-value was calculated using the non-parametric comparison Mann-Whitney test (two-tailed test), and $p < 0.05$ was considered a statistically significant difference between peer results. Percentages of mean differences were calculated based on the average percentage differences of the overall peer results. All statistical calculations and comparison plots were conducted using XLSTAT software (246).

Allowable total error (TEa) for vitamin D was calculated based on Equation 1. The TEa% for vitamins A and E was taken from the Ricos Biological Variation database (211).

Equation 6-3. Calculation of allowable total error.

$$TEa\ \% = Z \times X \times CVw + B$$

Bias can be calculated from:

$$B = 0.25 \times [CVw2 + CVg2\ ]1/2$$

From reference (212)

$$CVw = 8\%\ and\ CVg = 20\%$$

Then

$$B = 0.25\ x\ [8\text{^}2 + 20\text{^}2]\text{^}1/2 = 5.4\%$$

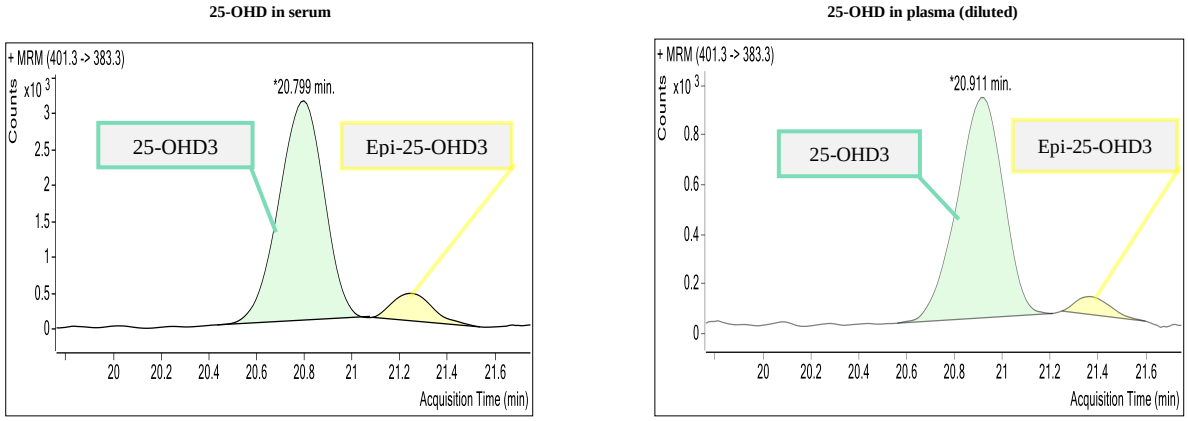
Hence the allowable total error for 25-OHD3 is

$$TEa\% = 1.65\ x\ 0.5\ x\ 8 + 5.4 = 12\%$$

TEa - Allowable total error, CVw - Within subject biological variation, CVg - Between subject biological variations

6.3 Results

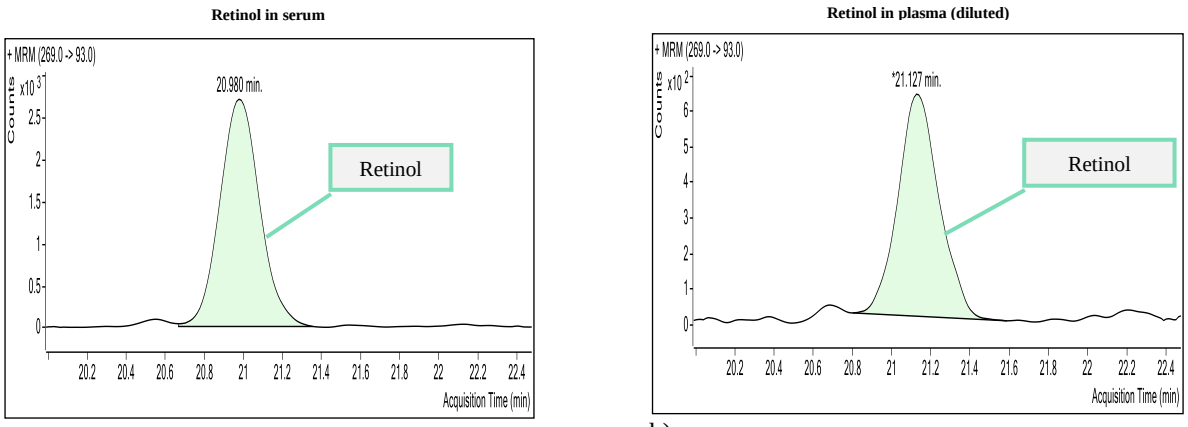
Two groups of UCB samples (serum and diluted plasma samples) were analysed for simultaneous quantification of 25-OHD3 and its epimer, retinol and α-tocopherol on two consecutive occasions using our LC-MS/MS method (Figure 6-1, Figure 6-2, Figure 6-3). These samples were also analysed for 25-OHD3 and its epimer using the lab B LC-MS/MS method. Statistical description of the vitamin results are shown in Table 6-1, Table 6-2.



a) b)

Figure 6-1. Chromatogram for 25-OHD3 in UCB.

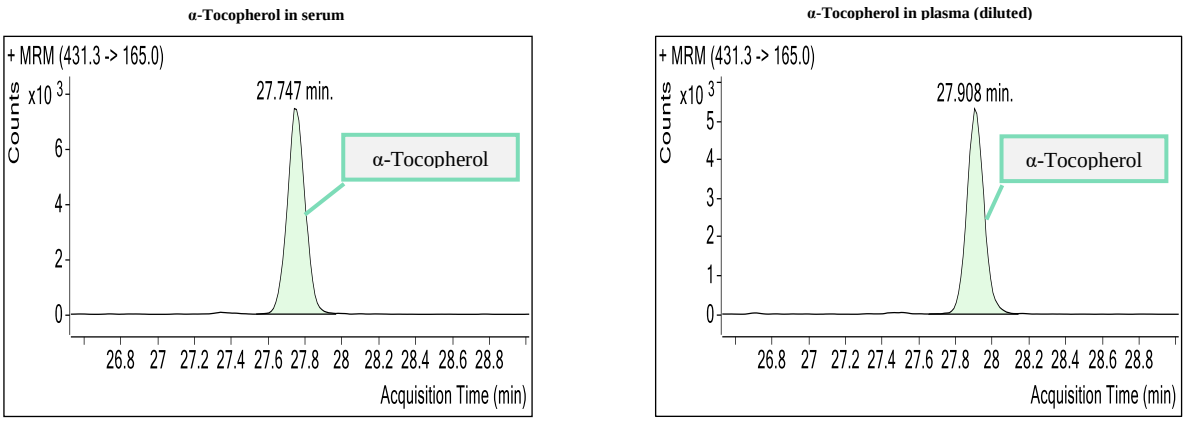
These chromatograms show separation of 25-OHD3 and epi-25-OHD3 in a) UCB serum and b) diluted UCB plasma samples, from the same subject.



a) b)

Figure 6-2. Chromatogram for retinol in UCB.

These chromatograms show separation of retinol in a) UCB serum and b) diluted UCB plasma samples, from the same subject.



a) b)

Figure 6-3. Chromatogram for α-tocopherol in UCB.

These chromatograms show separation of α-tocopherol in a) UCB serum and b) diluted UCB plasma samples, from the same subject.

Table 6-1. Statistical description of vitamin results in UCB serum and plasma samples obtained by lab A.

This table shows a summary of vitamin results in UCB serum and diluted plasma samples obtained by lab A. Statistical description was calculated based on results of matched UCB serum and diluted plasma samples, which were greater than the method LoQ (3.5 nmol/L for both 25-OHD3 and its epimer, and 0.16 μmol/L and 3 μmol/L for retinol and α-tocopherol, respectively).

Laboratory	Analyte	n	Serum			Plasma			*P* value
			Median	Min	Max	Median	Min	Max	
lab A	25-OHD3 (nmol/L)	20	48	26	96	48	28	96	0.531
	Epi-25-OHD3 (nmol/L)	12	6.0	4.1	12.9	NQ			NA
	Retinol (μmol/L)	13	0.84	0.63	1.16	0.82	0.48	1.21	0.223
	α-Tocopherol (μmol/L	20	7	4	14	NQ			NA

NQ: not quantified because low results (less than LoQ). NA: not applicable

Table 6-2. Statistical description of vitamin results in UCB serum and plasma samples obtained by lab B.

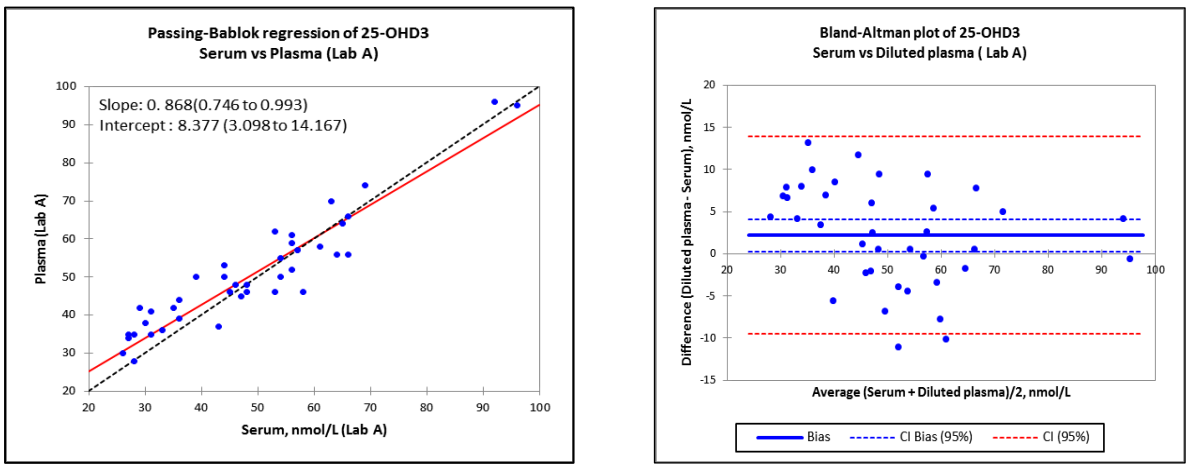
This table shows a summary of vitamin results in UCB serum and diluted samples obtained by lab B. Statistical description was calculated based on results of matched UCB serum and diluted plasma samples, which were greater than method LoQ (2.0 nmol/L for both 25-OHD3 and its epimer).

Analyte	n	Serum			Plasma			*P* value
		Median	Min	Max	Median	Min	Max	
25-OHD3 (nmol/L)	20	48	27	108	43	21	97	0.205
Epi-25-OHD3 (nmol/L)	14	4.3	2.9	13.4	NQ			NA

Vitamin D (25-OHD3)

The study method (lab A) shows a close relationship between 25-OHD3 results in serum when compared with diluted plasma (r = 0.914, *p* = 0.532 for lab A; r = 0.904, *p* = 0.205 for lab B) with a mean difference of 2.2 nmol/L (6.6%) [95% CI, -9.5 to13.9] (Figure 6-4). In addition, the lab B method demonstrates a close correlation of 25-OHD3 results obtained from the same samples (r = 0.904, *p* = 0.205) with a mean differences of 4.1 nmol/L (-8.5%) [95% CI, -14.5 to 6.1] (Figure 6-5).

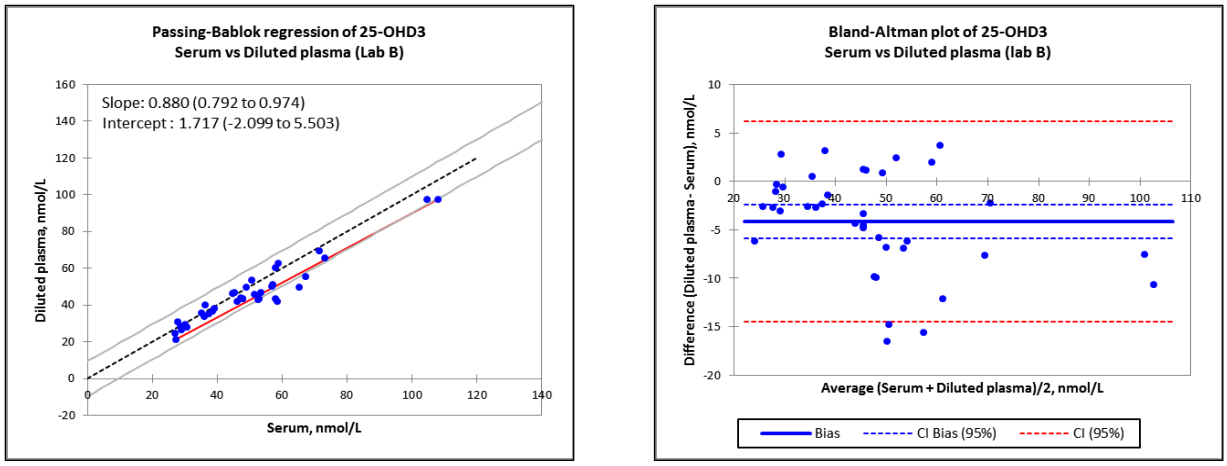
The results of 25-OHD3 obtained by the two laboratories were compared (Figure 6-6). There is close agreement as shown by the Passing-Bablok regression and Bland-Altman plots (Figure 6-7). Results obtained by lab A compared with lab B (r = 0.983, *p* = 0.703) with a mean differences of 0.14 nmol/L (-4.42%) [95% confidence interval (95% CI), -6.8 to 7.1] (Figure 6-6, Figure 6-7).



a) b)

Figure 6-4. Results of 25-OHD3 in UCB serum and diluted UCB plasma obtained by lab A.

Passing-Bablok regression plot a) and Bland-Altman plot b) demonstrate the agreement in 25-OHD3 results obtained from UCB serum and diluted UCB plasma.



a) b)

Figure 6-5. Results of 25-OHD3 in UCB serum and diluted UCB plasma obtained by lab B.

Passing-Bablok regression plot a) and Bland-Altman plot b) demonstrate the agreement in 25-OHD3 results obtained from UCB serum and diluted UCB plasma.

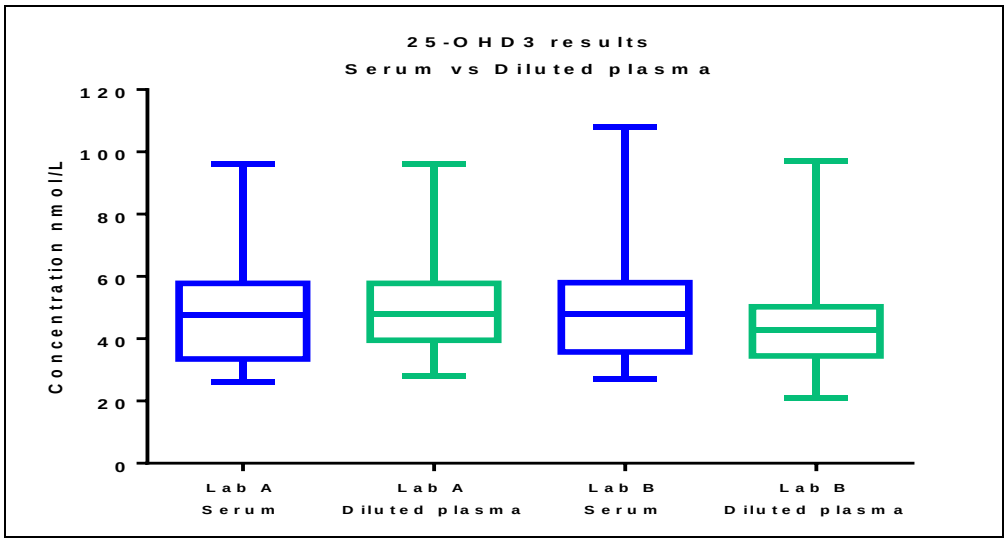
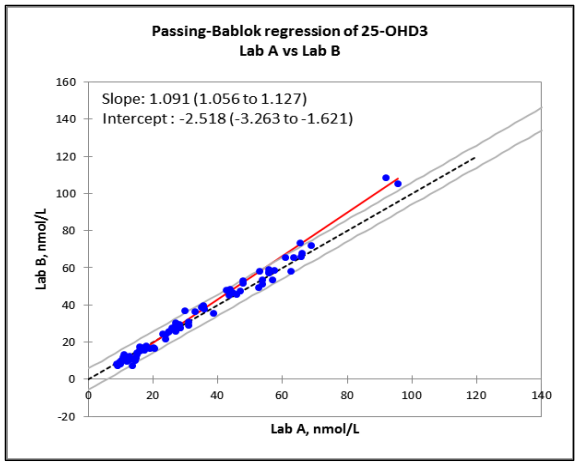
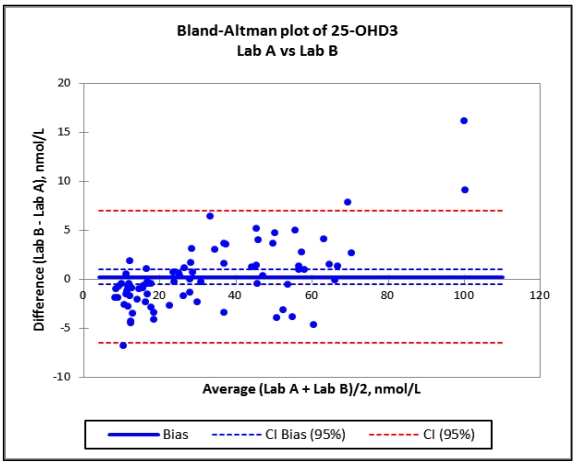


Figure 6-6. Overall 25-OHD3 results obtained by lab A and lab B.

Box plot demonstrates the minimum, first quartile, median, third quartile, and maximum of 25-OHD3 results of UCB serum versus diluted UCB plasma obtained by laboratories A and B. The method LoQ is 3.5 nmol/L (lab A) and 2.0 nmol/L (lab B) for both 25-OHD3.



a)



b)

Figure 6-7 .Results of 25-OHD3 in UCB serum and diluted UCB plasma obtained by lab A versus lab B.

Passing-Bablok regression plots a) and Bland-Altman plot b) demonstrate the agreement in 25-OHD3 results obtained from lab A compared with lab B for all the samples analysed i.e. both serum and diluted plasma.

Epi-Vitamin D (Epi-25-OHD3)

Epi-25-OHD3 was analysed in UCB serum and diluted plasma samples by the two laboratories. Epi-25-OHD3 was detected in all serum samples, however, 40% (lab A) and 30% (lab B) of serum results and all diluted plasma results were below the limit of quantification (LoQ); lab A LoQ is 3.5 nmol/L and lab B LoQ is 2.0 nmol/L. Serum epi-25-OHD3 results above the LoQ obtained by the two laboratories were well correlated (r = 0.869) with a mean difference -0.76 nmol/L (-16.5%) [95% CI, -2.3 to 0.77] (Figure 6-8).

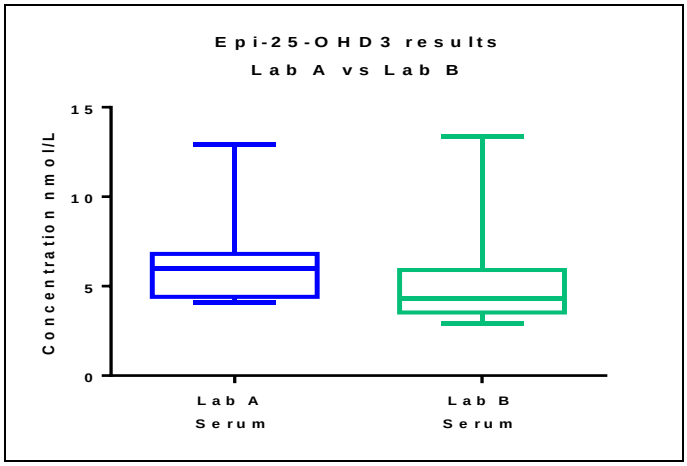
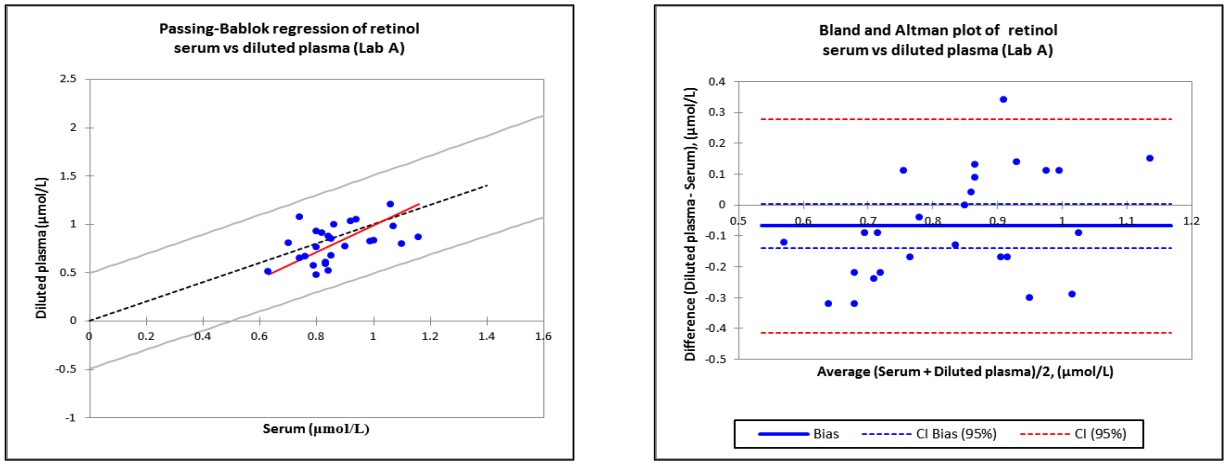


Figure 6-8. Overall epi-25-OHD3 results in serum obtained by lab A and lab B.

Box plot demonstrates the minimum, first quartile, median, third quartile, and maximum of epi-25-OHD3 results of UCB serum obtained by laboratories A and B. Only results above LoQ methods (3.5 nmol/L for lab A and 2.0 nmol/L for lab B) were plotted.

Retinol

Retinol was measured in UCB serum and diluted plasma samples using the lab A method. Retinol was quantifiable in all serum samples and 65% of diluted plasma samples, with 35% of diluted plasma results below the method LoQ. Quantified results of retinol in serum and diluted plasma samples demonstrated a mean difference of -0.07 µmol/L [95% CI, -0.41 to 0.28) representing a mean change of -9.9% across the analytical runs. Results of UCB serum compared with diluted UCB plasma showed a moderate correlation (r=0.45, p = 0.224) (Figure 6-9, Figure 6-10).



a) b)

Figure 6-9. Retinol results in UCB serum and diluted UCB plasma obtained by lab A.

Passing-Bablok regression plots a) and Bland-Altman plot b) demonstrate the agreement in retinol results obtained from UCB serum and diluted UCB plasma. Results above method LoQ (0.16 µmol/L) were only plotted in the graphs.

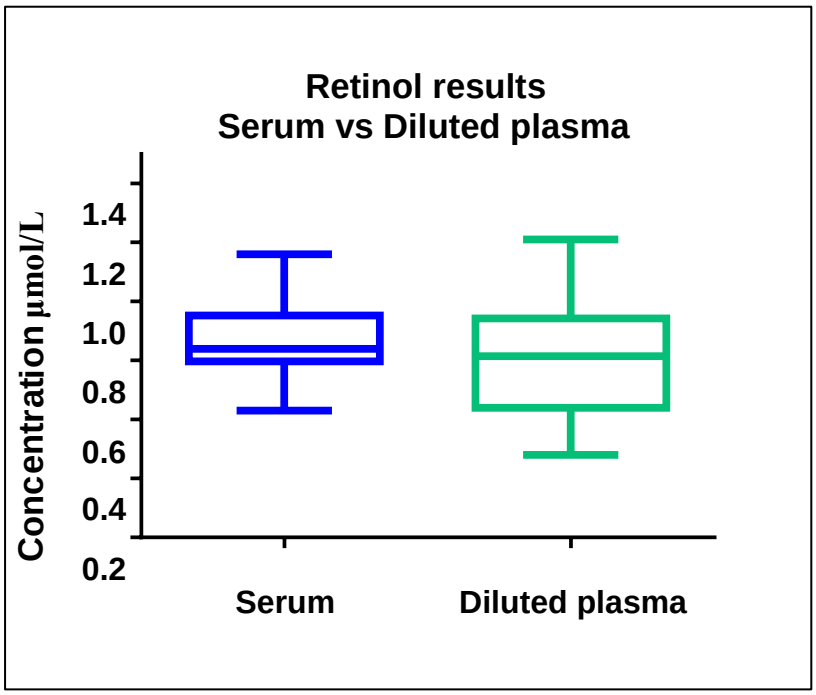


Figure 6-10. Overall results of retinol in UCB serum and diluted UCB plasma.

Box plot demonstrates the minimum, first quartile, median, third quartile, and maximum of retinol results of UCB serum versus diluted UCB plasma obtained by laboratory A. The box plot was created based the results, which were greater than method LoQ (0.16 µmol/L).

α-Tocopherol

α-Tocopherol levels were quantified in both serum and diluted plasma samples; however, only the serum results were above the LoQ (3 µmol/L) and hence a reliable comparison could not be made (Figure 6-11).

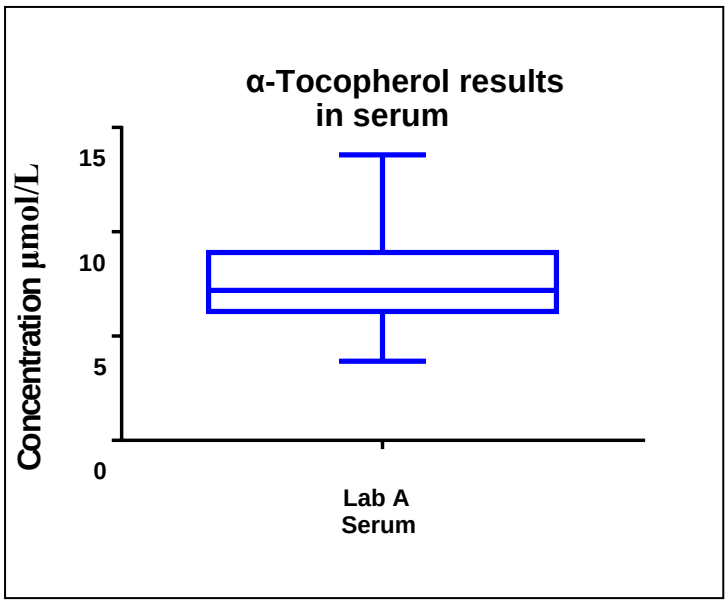


Figure 6-11. Overall results of α-tocopherol in UCB serum.

Box plot demonstrates the minimum, first quartile, median, third quartile, and maximum of α-tocopherol results of UCB serum obtained by laboratory A. The box plot was created based the results, which were greater than method LoQ (3µmol/L).

6.4 Discussion

This study examined the influence of cell culture media dilution of UCB plasma compared with undiluted UCB serum on 25-OHD3 and its epimer, retinol and α-tocopherol measurements by LC-MS/MS. This study is the first to report on the utility of UCB diluted plasma for the quantification of 25-OHD3 and retinol. In addition, this study demonstrates the sensitivity challenges of measuring very low levels of epi-25-OHD3 and α-tocopherol as a consequence of UCB plasma dilution.

Accurate results of a number of blood analytes is sample matrix dependent. For example, serum and plasma are commonly utilised to quantify a wide range of analytes, however, they are not completely equivalent biological matrices because of the blood clotting process (247). Based on recent evidence-based recommendations, both serum and plasma can be used for retinol and α-tocopherol quantification (81). Although similar recommendations are not currently in place for 25-OHD, previous studies reported no significant difference in 25-OHD results in plasma compared with serum (154, 186, 193). Our results support reliable quantification of 25-OHD3 and retinol in plasma matrix diluted with cell culture media RPMI-1640.

The current work demonstrates reliability of 25-OHD3 measurement in diluted UCB plasma and UCB serum. Both laboratories reported a close agreement between 25-OHD3 results obtained from diluted UCB plasma (with dilution range up to 43%) and those from UCB serum, even though they used different commercial calibrators, with a mean difference of 4.4%. Laboratories A and B reported 6.6% and 8.5% mean difference in results of serum UCB versus diluted UCB plasma, respectively, and these percentage differences are within the limit for TEa% for 25-OHD3 (12.0%).

Over-estimation of 25-OHD3 levels has been recently highlighted due to the presence of the epimer of 25-OHD3 in both paediatric and adult samples. Chromatographic separation and high mass selective detection were used to detect epi-25-OHD3 in 90% of adults (n=156) and 93% of children (n=58) (136). In the present study, the epi-25-OHD3 was detected in all serum UCB samples, of which 60% and 70% of samples had levels higher than the LoQ of the Labs A and B methods, respectively.

Measurement of epi-25-OHD3 is still a challenge even using the LC-MS/MS platform regardless of sample matrix. In addition to the incomplete elucidation of its physiological roles, biological variation data for epi-25-OHD3 is not currently in place because of a recent quantification attention in blood samples. Therefore, the allowable TEa% could not be calculated. Furthermore, a commercial calibrator for epi-25-OHD3 is currently not available and, accordingly, commercial calibrators from Recipe (lab A) and Chromsystems (lab B) were used to generate 25-OHD3 standard curves that were then applied for the quantification of epi-25-OHD3. Further confounding the quantitation is that most of the UCB serum samples had epi-25-OHD3 levels close to the method LoQ levels (3.5 nmol/L and 2 nmol/L for Labs A and B respectively). All these factors could be sources of variation in the two laboratory results.

Low levels (<0.7 umol/L) of retinol in UCB serum have been linked to low birth weight (244). This study introduced the first comparison of retinol UCB results in serum versus diluted plasma results using the LC-MS/MS methodology. The mean difference between retinol results in UCB serum and diluted UCB plasma was about 10%, which is less than TEa% (17.1%) for serum retinol measurement (211). However, retinol UCB results in the two groups showed a moderate correlation (correlation coefficient (r) =0.451). This

observation may be linked to reported unexplained biological variation differences in serum (13.6%) versus plasma (6.2%) for retinol analysis (81, 248, 249).

In the current study, α-tocopherol (vitamin E) levels in the UCB serum group were between 4 and 14 μmol/L, while its levels in all diluted UCB plasma were below the method LoQ (3 μmol/L). Consequently, reliable comparison between the two groups could not be achieved. Our observation of the low levels of vitamin E in UCB is consistent with previous study findings. For example, Didenco and colleagues found that UCB α-tocopherol levels were significantly lower than the maternal blood level by 80%, on average, and this is potentially related to the selective transfer of α-tocopherol to the placenta (250).

6.5 Conclusions

This study, which was published in the Clinical Biochemistry journal in 2015 (251), introduced an evidence of reliability of using dilution of UCB plasma with cell culture media (RPMI 1600) for quantification of 25-OHD3 and retinol using the LC-MS/MS method. The 25-OHD3, epi-25-OHD3, retinol and α-tocopherol levels were successfully measured in the UCB serum. In contrast, analysis of the epimer of 25-OHD3 and α-tocopherol in diluted UCB plasma is not supported by this study due to limitations in analytical sensitivity for quantification. This limitation could potentially be addressed in the future through the use of increased sample volume.

Chapter 7

Fat-soluble vitamins: status and correlation in two Australian populations

Chapter 7 Fat-soluble vitamins: status and correlation in two Australian populations

7.1 Introduction

The roles of fat-soluble vitamins (FSVs) D, A and E, particularly vitamins D and A (2, 3), have been identified in several non-classical physiological functions. Deficiencies of FSV have been associated with increased risk of cancer, type 2 diabetes mellitus and a number of immune system disorders (1, 2). As a result of this enhanced clinical association with diseases, population-based studies and translational clinical research activities, especially regarding vitamin D, have significantly increased in the last decade.

Several national population-based studies around the world have reported a prevalence of vitamin D deficiency (VDD), however, the estimations vary across different countries. For example, VDD was estimated at 41% of the population in the USA (42), 20% in Canada (252), 47% in the UK (in winter) (253), 48% in New Zealand (254) and 31% in Australia (255). Deficiencies of vitamin A and E are present in developing countries due to malnutrition, and in developed countries due to absorption defects in the intestine (29, 66, 256). Generally, there have been studied vitamin A and E deficiencies in children with limited data available on adults (29, 66), and no agreements on the status of vitamins A and E among children or adults in developed and developing countries (160, 257).

Other problematic issues related to FSV are the limited agreement on reference intervals (RIs) and the extent of interaction between blood vitamin levels. Although it is well known that various environmental, genetic and lifestyle factors have essential impacts on FSV statuses, inappropriate standardisation of the assays may affect results obtained by different laboratories (as discussed in the Chapter 3). These may impact the overall estimation of

vitamin statuses and RIs for various populations. This is further confounded by current debate regarding the selection of population-based RIs versus recommended levels for health.

The current work aimed to create a snapshot of the current status and correlation of vitamin D (25-OHD2, 25-OHD3 and epi-25-OHD3), vitamin A (retinol) and vitamin E (α-tocopherol) in the blood across two Australian populations at different latitudes; Queensland (QLD, latitudes between 10º S and 28ºS) and Victoria (VIC, latitudes between 34º S and 38º S). In addition, RIs were estimated for vitamins A and E using the precise simultaneous LC-MS/MS quantification method.

The novelty of this work is that it examines the status and correlation of five blood vitamin metabolites in two populations living at different latitudes. Simultaneous analysis of these vitamins eliminated any variations that might be observed when using different quantification techniques or methods. Further, this work introduces data about the status of 25-OHD3 epimer in two Australian populations, which was not previously available.

7.2 Methodology

7.2.1 Subjects

De-identified serum samples were selected from samples delivered daily for a variety of clinical chemistry tests to Sonic Healthcare Australia (Sullivan Nicolaides Pathology, Brisbane (Queensland) and Melbourne Pathology, Melbourne (Victoria), Australia) from several regions of Queensland and Victoria states. These samples were selected from individual outpatients with no diagnosed diseases, referred by general practice facilities either from Queensland (QLD) or Victoria (VIC). Further selection criteria were made based on the age and gender of the subjects (Table 7-1). Chosen serum samples were stored at -20ºC until they were analysed in the clinical LC-MS/MS Laboratory, Clinical Chemistry department, at RMIT University.

The serum samples were collected during the summer season (December, 2013 - February, 2014) from two Australian states (QLD n=109, VIC n=108) as detailed in Table 7-1. Samples were prepared and analysed with the simultaneous FSV quantification method (FSV method-2) using the Agilent 6410 LC-MS/MS system as described in Chapters 2 and 3.

Table 7-1. Subject age distribution.

This table shows age of the subjects in QLD and VIC groups.

State	Gender	Age group 1 (18 - 39 years)	Age group 2 (40 - 60 years)	Age group 3 (Over 60 years)	Total
QLD	Men	19	20	17	56
	Women	18	19	16	53
	Total	37	39	33	109
VIC	Men	18	19	18	55
	Women	20	17	16	53
	Total	38	36	34	108

7.3 Statistical analysis

Normality of data distribution for groups and subgroups was investigated using the Shapiro-Wilk test, where normal data distribution is considered when *p* value is greater than 0.05. Normality was also visually examined using box plots, where normal data distribution is considered when two sides of box plot are approximately symmetric around the median level. Based on data distribution, parametric tests (for normal distribution) or non-parametric tests (for non-normal distribution) were used in statistical calculation. However, when the normal distribution of data was not firmly observed across groups or subgroups, both parametric tests and non-parametric tests were used to increase the reliability of statistical analysis.

Prevalence (%) of a vitamin deficiency was calculated by dividing the number of vitamin-deficient cases by the total number of cases, multiplied by 100. Vitamin D status was described using two thresholds: 25-OHD3 <50 nmol/L for vitamin D deficiency and 25-OHD3 <75 nmol/L for vitamin D insufficiency (255, 258). As there are no agreement in recommendations for adequate levels of vitamin A and E, in this study retinol less than 0.7 μmol/L (29) and α-tocopherol less than 12 μmol/L (259) were used as thresholds for vitamin A and E deficiencies, respectively.

Effects of gender, age and geographical position on blood vitamin results were also statistically examined using parametric tests or non-parametric tests according to normality of data distribution. If partial inconsistency was found between results of the Shapiro-Wilk test and box plots used in investigating the normality of data distribution, both parametric and non-parametric statistical tests were utilised to increase confidence of statistical analysis. An independent t-test, one-way ANOVA and multiple comparisons tests (Bonferroni test and Dunnett's T3 test) were used as parametric tests. Mann-Whitney and Kruskal-Wallis tests

were used as non-parametric tests. Statistical data description and normality, parametric tests and non-parametric tests were conducted using IBM SPSS Version 22 (260).

The RIs for vitamins A and E were estimated. Outlying values (outliers) were statistically determined using the Tukey test for each group and subgroup according to the Clinical and Laboratory Standards Institute (CLSI, USA) Guidelines C28-A3 (CLSI 2008). "Robust method" was used to calculate the 95^{th} percentile reference based on CLSI Guidelines C28-A3 (CLSI 2008). This calculation method is recommended by CLSI for a sample size of less than 120 (261). The RIs were calculated using MedCalc Statistical Software version 13.1.0 (262).

Correlations between vitamins D, A and E were investigated. Due to irregular observation in normality of data distribution among groups and subgroups, as shown later, both Pearson's test (parametric correlation test) and Spearman's test (non-parametric correlation test) were used to investigate correlations of vitamin D levels with vitamin A and E levels. Correlations were explored based on: correlation trend (+/-); correlation strength (correlation coefficient (r): small, ±0.1 to ±0.29; medium, ±0.3 to ±0.49; large, ±0.5 to ±1.0) (263); and correlation statistical significance (significant: $p<0.05$). Statistical correlation tests were conducted using IBM SPSS Version 22 (260).

7.4 Results

7.4.1 Statistical description of data

The subjects' ages and FSV levels in groups and subgroups are statistically described in Table 7-2, Table 7-3.

Table 7-2. Statistical description of subjects' ages.

State	Description		Mean	Median	SD	Minimum	Maximum
			(years)				
QLD	Total (n=109)		51	49	21.1	18	99
	Gender	Men (n=56)	50	49	20.1	18	99
		Women (n=53)	52	49	22.2	18	97
	Age (years)	18-39 (n= 37)	28.3	28	6.4	18	39
		40-60 (n= 39)	50	50	6.1	40	60
		> 60 (n= 33)	77.4	76	9.9	61	99
VIC	Total (n=108)		48	47	18.9	18	93
	Gender	Men (n=55)	47.9	50	19.1	18	82
		Women (n=53)	48.1	44	18.8	20	93
	Age (years)	18-39 (n= 38)	27.6	29	6.3	18	39
		40-60 (n= 36)	48	48	5.8	40	59
		> 60 (n= 34)	70.8	71	7.7	61	93

SD: Standard deviation

Table 7-3. Statistical description of serum FSV levels.

State	Description		25-OHD3 nmol/L	Epi-25-OHD3 nmol/L	Epi- 25-OHD3 (%)*	Retinol µmol/L	α-Tocopherol µmol/L
QLD	Total (n=109)	Mean	73.5	3.1	3.9	2.14	30.2
		Median	73	3.2	4.2	2.12	30
		SD	20.9	2.6	2.8	0.54	7.5
		Minimum	27	0	0	0.69	15
		Maximum	126	11.9	10.7	3.66	51
	Men (n=56)	Mean	73.3	3.8	4.7	2.2	29.2
		Median	73	3.7	4.5	2.17	29
		SD	22.2	2.5	2.6	0.52	7.3
		Minimum	27	0	0	1.01	15
		Maximum	126	11.9	9.9	3.29	51
	Women (n=53)	Mean	73.7	2.4	3.0	2.1	31.3
		Median	73	2.6	3.5	2.0	31
		SD	19.5	2.4	2.8	0.56	7.7
		Minimum	27	0	0	0.69	17
		Maximum	121	10.4	10.7	3.66	46
VIC	Total (n=108)	Mean	64.5	1.8	2.5	1.91	28.9
		Median	63	1.4	2.4	1.85	27
		SD	23.4	2.0	2.6	0.62	9.1
		Minimum	17	0	0	0.62	10
		Maximum	127	9.0	11.7	3.63	62
	Men (n=55)	Mean	64.4	2.0	3.0	2.01	28
		Median	64	1.8	3.1	1.94	26
		SD	18.8	1.8	2.4	0.69	8.9
		Minimum	30	0	0	0.62	15
		Maximum	102	6.8	9.3	3.63	62
	Women (n=53)	Mean	64.6	1.5	1.96	1.81	29.8
		Median	58	0	0	1.75	29
		SD	27.6	2.3	2.8	0.53	9.4
		Minimum	17	0	0	0.88	10
		Maximum	127	9.0	11.7	3.05	59

SD: Standard deviation, * Percentage of epimer of 25-OHD3 to total 25-OHD3 concentration.

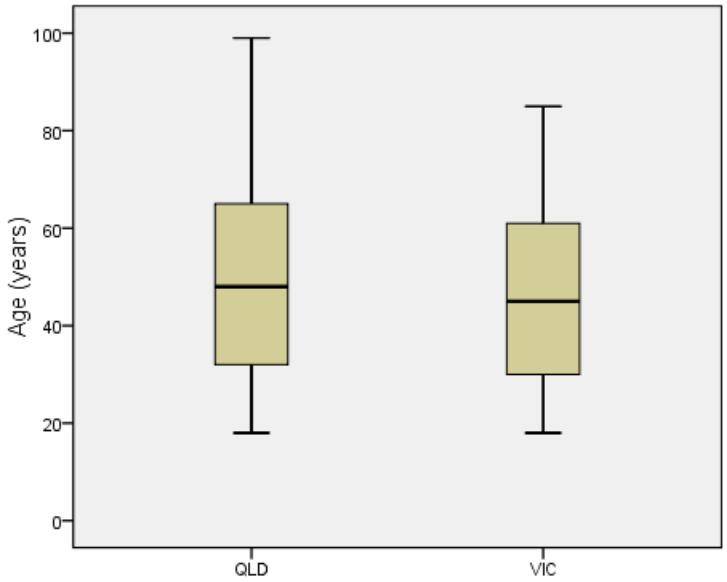
7.4.2 Data distribution

Two hundred and seventeen de-identified samples from two Australian states, QLD (n=109) and VIC (n=108), were analysed. Distributions of age and FSV levels among the two groups and their subgroups were analysed. We found inconsistent normal distributions in the groups and subgroups based on Shapiro-Wilk test and box-plots (Table 7-4, Figure 7-1, Figure 7-2, Figure 7-3, Figure 7-4, Figure 7-5).

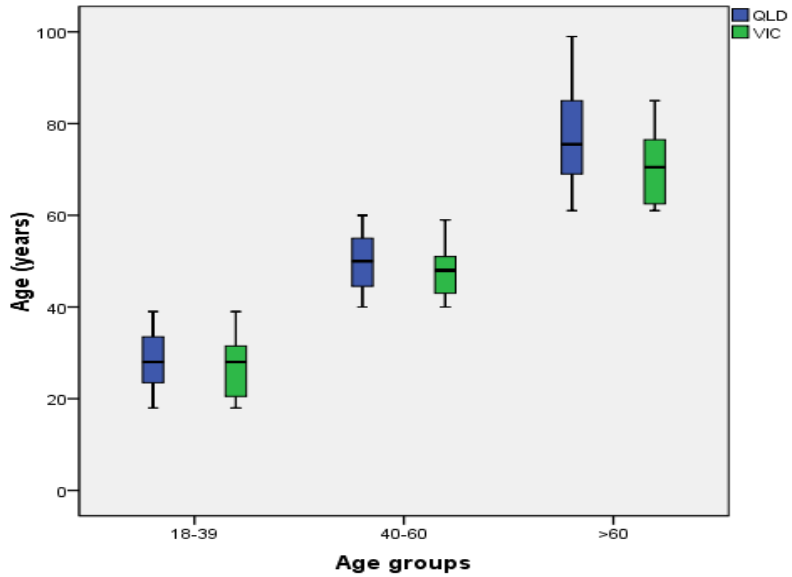
Table 7-4. Normality testing for data distribution.

Normality of data distribution of subjects' age, gender and FSV levels was tested using a Shapiro-Wilk test (p >0.05 indicates normal data distribution).

State	Description		Age	25-OHD3	Epi-25-OHD3	Retinol	α-Tocopherol
			p value				
QLD	Subjects		0.003	0.655	<0.001	0.564	0.103
	Gender	Men	0.173	0.666	0.012	0.915	0.243
		Women	0.019	0.954	<0.001	0.145	0.156
	Age (years)	18 – 39	0.087	0.625	0.024	0.671	0.214
		40 – 60	0.104	0.881	0.002	0.179	0.143
		Over 60	0.642	0.195	0.004	0.718	0.544
VIC	Subjects		0.005	0.335	<0.001	0.241	<0.001
	Gender	Men	0.019	0.43	0.001	0.839	<0.001
		Women	0.013	0.12	<0.001	0.05	0.061
	Age (years)	18 – 39	0.027	0.718	<0.001	0.457	0.012
		40 – 60	0.074	0.146	<0.001	0.574	0.044
		Over 60	0.064	0.038	0.001	0.73	0.002



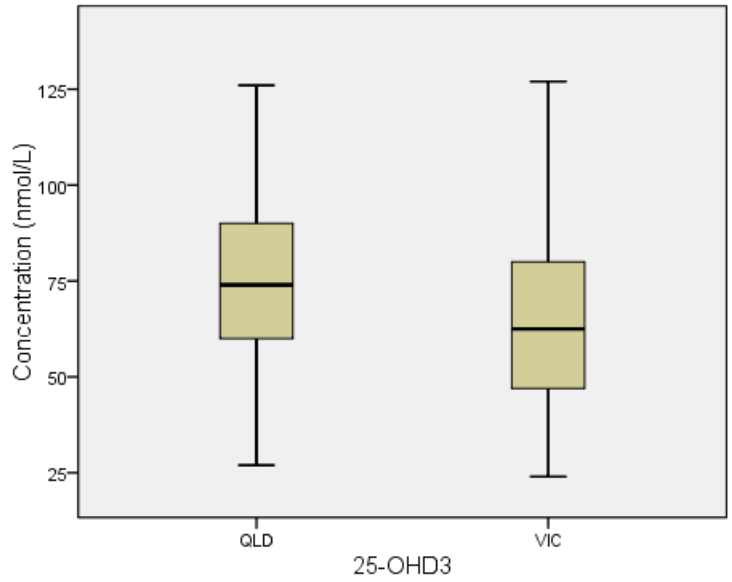
a)



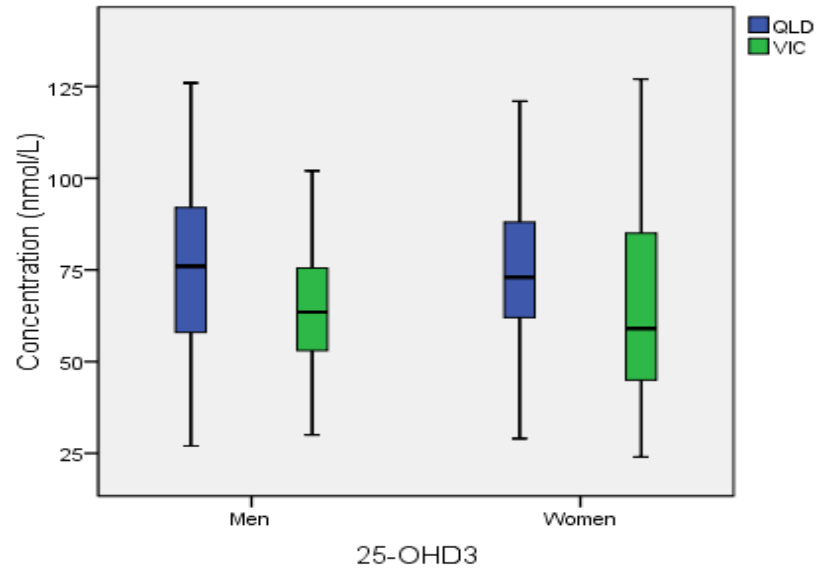
b)

Figure 7-1. Distribution of subjects' ages.

These box plots demonstrate the minimum, first quartile, median, third quartile, and maximum subjects' ages in the QLD and VIC samples: a) this box plot shows overall age distribution among QLD and VIC groups, b) this box plot demonstrates age distribution across age groups of the two states. These plots show inconsistency in normality between groups and subgroups.



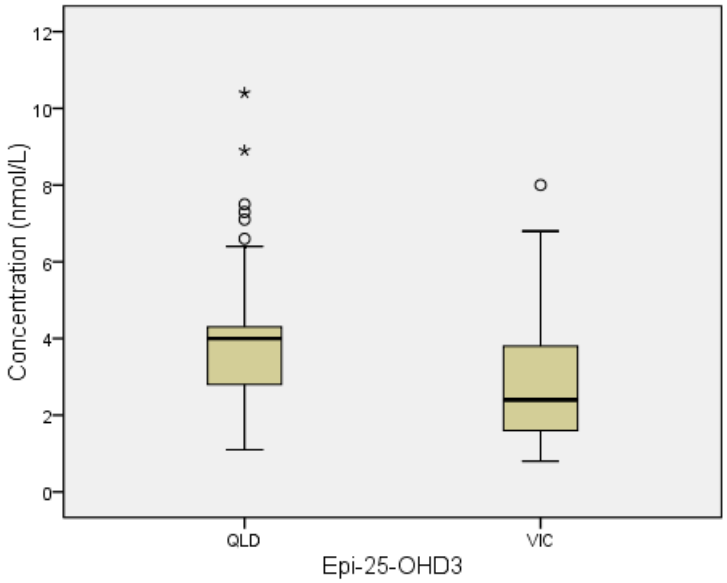
a)



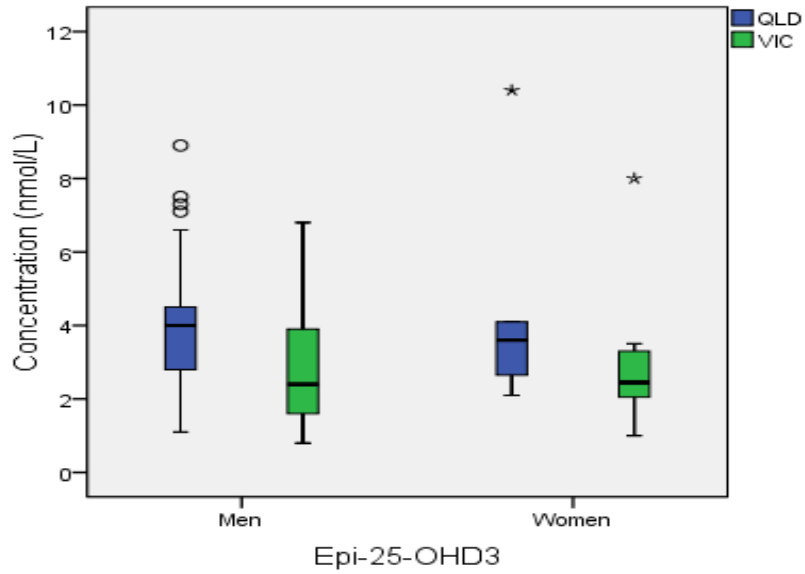
b)

Figure 7-2. Distribution of 25OHD3 levels.

These box plots demonstrate the minimum, first quartile, median, third quartile, and maximum 25-OHD3 levels: a) overall levels, and b) levels based on gender. These plots show inconsistent normal distribution of 25-OHD3 levels across QLD and VIC groups and subgroups.



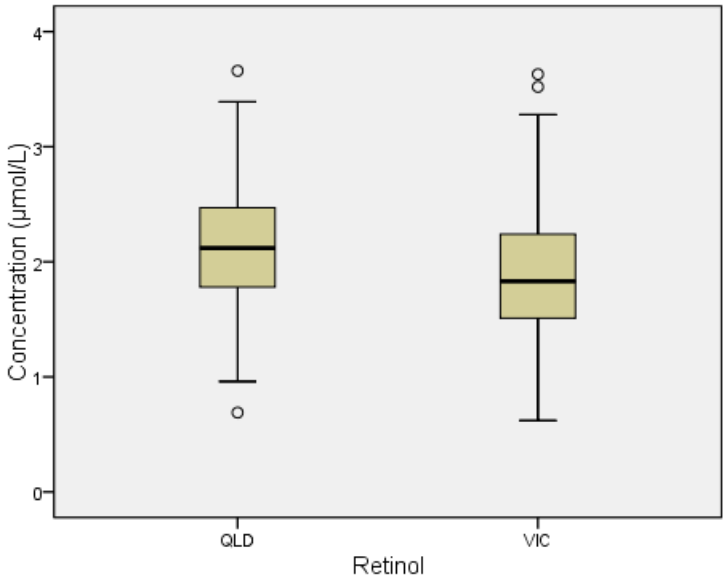
a)



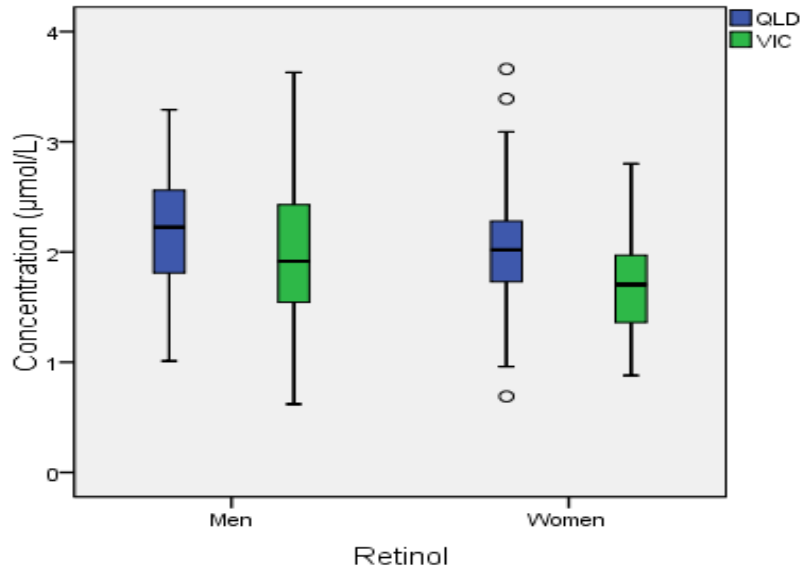
b)

Figure 7-3. Distribution of epi-25-OHD3 levels.

These box plots demonstrate the minimum, first quartile, median, third quartile, and maximum epi-25-OHD3 levels: a) overall levels, and b) levels based on gender. These plots show inconsistent normal distribution of epi-25-OHD3 levels across QLD and VIC groups and subgroups. Circles indicate outliers and stars indicate extreme outliers.



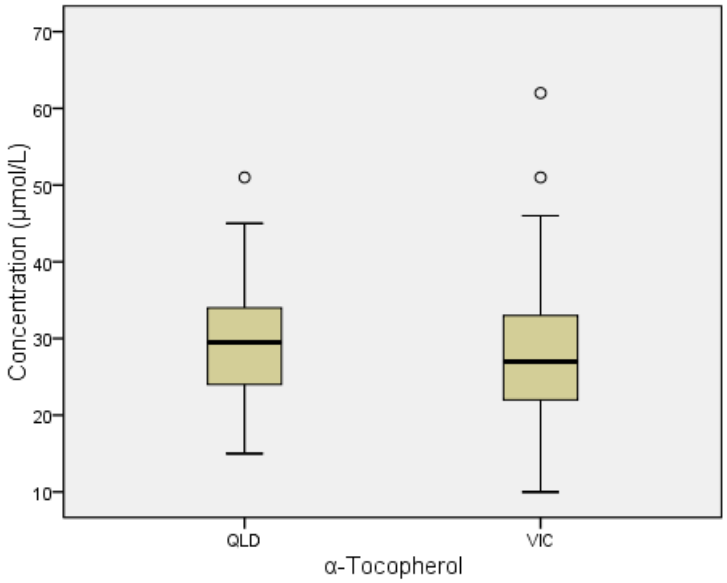
a)



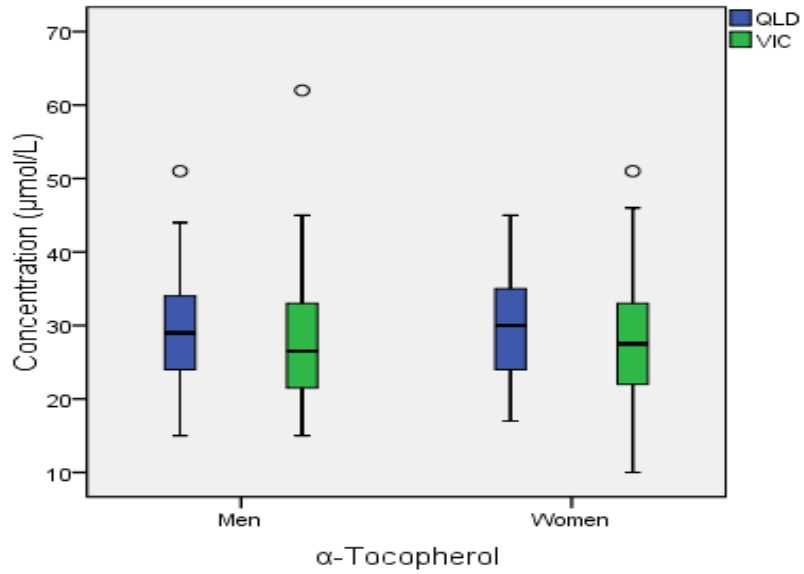
b)

Figure 7-4. Distribution of retinol levels.

These box plots demonstrate the minimum, first quartile, median, third quartile, and maximum retinol levels: a) overall retinol levels and b) retinol levels based on gender. These plots show inconsistent normal distribution of retinol levels across QLD and VIC groups and subgroups. Circles indicate outliers.



a)



b)

Figure 7-5. Distribution of α-tocopherol levels.

These box plots demonstrate the minimum, first quartile, median, third quartile, and maximum of α-tocopherol levels for a) overall results and b) results based on gender among QLD and VIC samples. These plots show an inconsistency in normality between groups and subgroups. Circles indicate outliers.

7.4.3 Effect of gender on blood FSV levels

The effect of gender on blood FSV levels was statistically analysed. I used an independent t-test (parametric test) and Mann-Whitney test (non-parametric test) for this analysis after inconsistent normal data distribution was observed, as shown earlier. Based on statistical analysis there was no significant gender effect on FSV levels among QLD and VIC groups (Table 7-5). Consequently, gender subgroups were not required for RIs calculation of vitamin A and E in QLD and VIC groups (current chapter, section 7.4.7).

Table 7-5. Effect of gender on blood FSV levels.

Table shows no significant gender effect on levels of blood 25-OHD3, retinol and α-Tocopherol in QLD and VIC groups.

Statistical test	QLD			VIC		
	25-OHD3	Retinol	α-Tocopherol	25-OHD3	Retinol	α-Tocopherol
	p value					
Independent t-test	0.907	0.296	0.072	0.961	0.208	0.356
Mann-Whitney test	0.894	0.225	0.122	0.796	0.133	0.261

7.4.4 Effect of age on blood FSV levels

The effect of age on blood FSV levels was statistically investigated using parametric (one way ANOVA and multiple comparisons) and non-parametric (Kruskal-Wallis) tests due to inconstant normal distribution among age groups. There was no significant effect of age on retinol levels of QLD and VIC groups. No statistically significant impact of age on blood levels of 25-OHD3 was observed in the QLD group, but was found in the VIC group. In contrast, there was a significant effect of age on levels of α-tocopherol in the QLD group but not in the VIC group (Table 7-6). Consequently, age groups were not required for RIs calculation of retinol (vitamin A) in the QLD and VIC groups, while they were required for α-tocopherol (vitamin E) RIs estimation in the QLD group (shown later in section 7.4.7).

Table 7-6. Effect of age on FSV levels.

Data show no significant differences in retinol (vitamin A) levels across age groups in QLD and VIC samples. In contrast, there are significant differences in 25-OHD3 (vitamin D) and α-tocopherol (vitamin E) among age groups in the VIC and QLD samples, respectively.

Statistical test		Age group comparison	QLD			VIC		
			25-OHD3	Retinol	α-Tocopherol	25-OHD3	Retinol	α-Tocopherol
			p value					
One way ANOVA (Parametric test)		1-3	0.457	0.452	<0.001	0.001	0.153	0.284
Multiple comparisons (Parametric test)	Bonferrroni	1 and 2 1and 3 2 and 3	1.000 0.636 1.000	0.666 1.000 1.000	0.113 <0.001 0.060	1.000 0.006 0.003	1.00 0.451 0.187	0.895 0.365 1.000
	Dunnett T3	1 and 2 1and 3 2 and 3	0.900 0.555 0.872	0.527 0.784 0.983	0.093 <0.001 0.076	0.989 0.008 0.008	0.931 0.441 0.255	0.667 0.309 0.926
Kruskal Wallis test (non-parametric test)		1-3	0.336	0.420	<0.001	0.02	0.218	0.314

7.4.5 Effect of latitude on blood FSV levels

The effect of latitude on blood FSV levels among QLD and VIC groups was statistically investigated using parametric (independent t-test) and non-parametric (Mann-Whitney) tests due to inconsistent normal data distribution observed across investigated groups and sub-groups. Significant differences were found in vitamins D and A levels across the QLD and VIC groups. In contrast, no statistically significant differences in vitamin E levels between QLD group and VIC groups were found based on an independent t-test (p=0.069), which contradicted with the Mann-Whitney test result (p=0.043) (Table 7-7). Therefore, geographical regions (QLD and VIC) were considered when RIs of vitamin A and E were estimated later in the current chapter, section 7.4.7.

Table 7-7. Effect of latitude on FSV levels.

Table shows highly significant differences in levels of 25-OHD3 (vitamins D) and retinol (vitamin A) among QLD and VIC groups. Parametric and non-parametric tests revealed differences of inconsistent significance in α-tocopherol levels between the QLD and VIC groups.

Statistical test	25-OHD3	Retinol	α-Tocopherol
	p value		
Independent t-test (Parametric test)	0.003	<0.001	0.069
Mann-Whitney test (non-parametric test)	0.003	<0.001	0.043

7.4.6 Fat soluble vitamin status

Vitamin D status was estimated across men and women in the QLD and VIC groups. It was found that the prevalence of vitamin D deficiency (VDD) (25-OHD3 <50 nmol/L) in the VIC group was higher than the prevalence observed in the QLD group. About one third (27.8%, n=30) of the VIC group had VDD, compared to 11% (n=12) of the QLD group. When prevalence of VDD was estimated based on total 25-OHD levels (25-OHD2, 25-OHD3 and epi-25-OHD3), VDD % did not change in the QLD group but increased to 28.7% in the VIC group. Only one vitamin D deficient case (female, VIC) from the total samples analysed could still be deemed vitamin D insufficient when total 25-OHD levels were considered (0 nmol/L 25-OHD2 + 47 nmol/L 25-OHD3 + 3 nmol/L epi-25-OHD3 = 50 nmol/L). Furthermore, 2.8% (n=3) of subjects in the VIC group had 25-OHD3 level less than 25 nmol/L, while no subject had less than 25 nmol of 25-OHD3 in the QLD group. Women had more VDD (34.5%, n=19) than men (20.8%, n=11) in the VIC group; in contrast, there were slightly more men with VDD (12.5%, n=7) than women with VDD (9.4%, n=5) in the QLD group (Table 7-8, Figure 7-6).

Epi-25-OHD3 was detected in 75.3% (n=82) of samples (ranged 1.0-11.9 nmol/L) in the QLD group compared to 57.4% (n=62) of VIC samples (ranged 1-10.2 nmol/L), (Table 7-9, Figure 7-7). Percentages of epi-25-OHD3 median levels and total 25-OHD3 median levels were 4.2% (ranged 0-10.7%) and 2.4% (ranged 0-11.7%) among QLD and VIC groups, respectively. 25-OHD2 was quantified in some QLD samples, but only 10% of samples (n=11) contained 25-OHD2 above the method limit of quantification (LoQ ≥5 nmol/L), and it was not quantified in the VIC samples.

The majority of subjects in the QLD and VIC groups had no vitamin A deficiency (VAD) (retinol <0.7 µmol/L). Only one subject (0.9%) had VAD in each investigated group

(Table 7-10, Figure 7-8). Vitamin E deficiency (VED) (α-tocopherol <12 µmol/L) was found in one subject (0.9%) in the VIC group (Table 7-11, Figure 7-9).

Table 7-8. Status of vitamin D3 (25-OHD3).

Three thresholds were used to define vitamin D status including: vitamin D deficiency (25-OHD3 <50 nmol/L); vitamin D insufficiency (25-OHD3 ≥50 <75 nmol/L); and vitamin D sufficiency (25-OHD3 >75 nmol/L).

		25-OHD3/QLD			25-OHD3/VIC		
		< 50 nmol/L	≥ 50 < 75 nmol/L	≥ 75 nmol/L	< 50 nmol/L	≥50 < 75 nmol/L	≥ 75 nmol/L
Men	n	7	22	27	11	29	15
	%	12.5	39.3	48.2	20.0	52.7	27.3
Women	n	5	23	25	19	14	20
	%	9.4	43.4	47.2	35.8	26.4	37.7
Total	n	12	45	52	30	43	35
	%	11.0	41.3	47.7	27.8	39.8	32.4

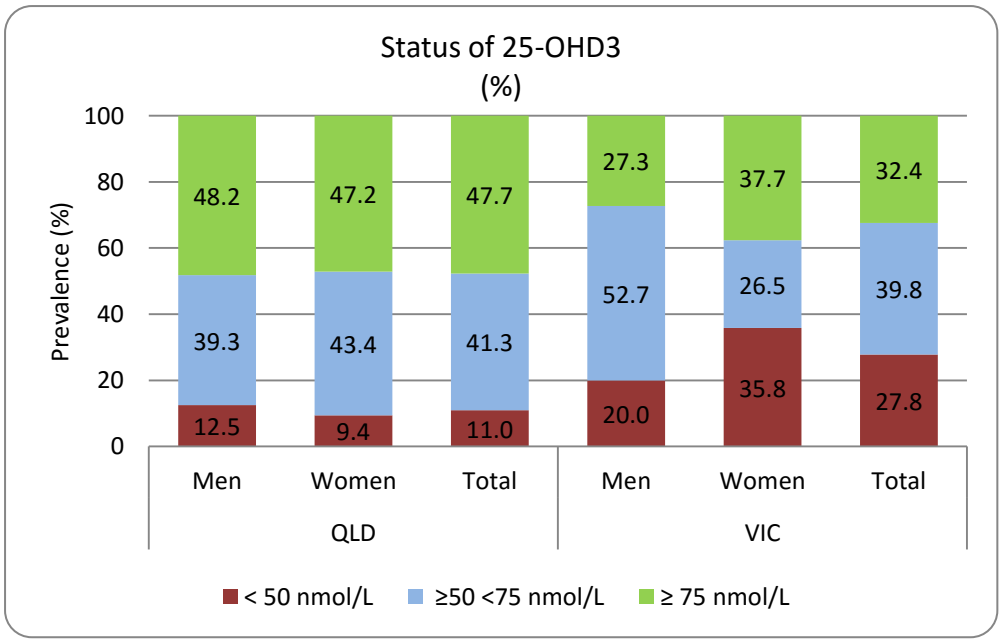


Figure 7-6. Status of vitamin D3 (25-OHD3).

Three thresholds were used to define vitamin D status including: vitamin D deficiency (25-OHD3 <50 nmol/L); vitamin D insufficient (25-OHD3 ≥50 <75 nmol/L); and vitamin D sufficient (25-OHD3 >75 nmol/L).

Table 7-9. Status of vitamin D3 epimer (epi-25-OHD3).

This table demonstrates vitamin D3 epimer (epi-25-OHD3) status in the QLD group compared to the VIC group. The method limit of quantification is ≥3.5 nmol/L.

		Epi-25-OHD3 /QLD			Epi-25-OHD3 /VIC		
		Not detected	≥1 < 3.5 nmol/L	≥ 3.5 nmol/L	Not detected	≥1 < 3.5 nmol/L	≥ 3.5 nmol/L
Men	n	6	20	30	14	29	12
	%	10.7	35.7	53.6	25.5	52.7	21.8
Women	n	21	15	17	32	11	10
	%	39.6	28.3	32.1	60.4	20.8	18.9
Men and Women	n	27	35	47	46	40	22
	%	24.8	32.1	43.1	42.6	37.0	20.4

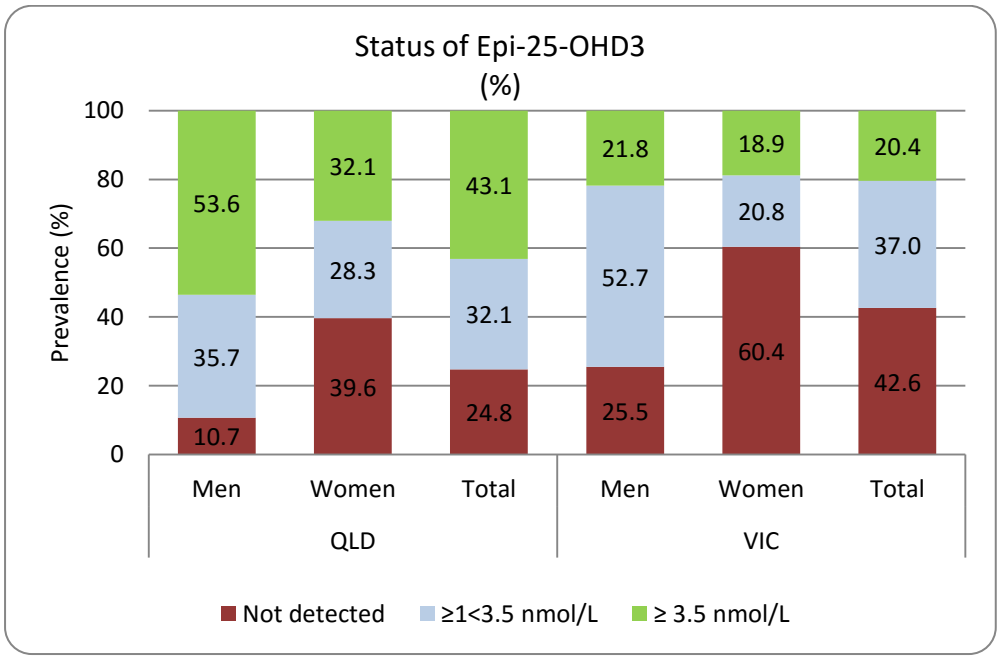


Figure 7-7. Status of vitamin D3 epimer (epi-25-OHD3).

This chart demonstrates vitamin D3 epimer (epi-25-OHD3) status in the QLD group compared with the VIC group. The method limit of quantification is ≥3.5 nmol/L.

Table 7-10. Status of vitamin A (retinol).

This table shows vitamin A (retinol) status. A threshold of less than 0.7 µmol/L of retinol was used to indicate vitamin A deficiency.

		Retinol /QLD		Retinol /VIC	
		< 0.70 µmol/L	≥ 0.70 µmol/L	< 0.70 µmol/L	≥ 0.70 µmol/L
Men	n	0	56	1	54
	%	0	100	1.8	98.2
Women	n	1	52	0	53
	%	1.9	98.1	0	100
Total	n	1	108	1	107
	%	0.9	99.1	0.9	99.1

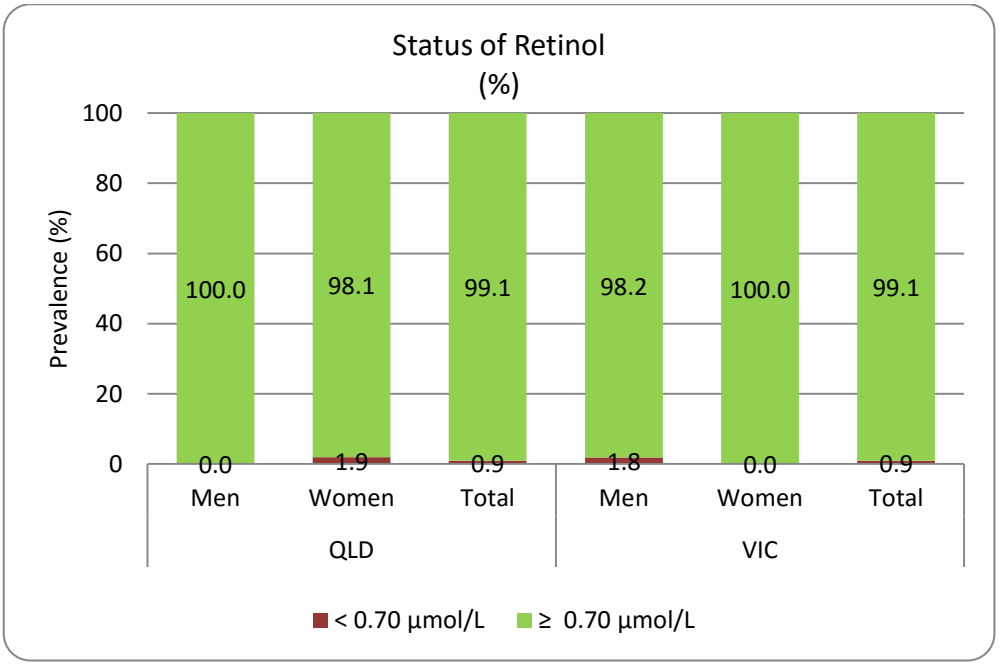


Figure 7-8. Status of vitamin A (retinol).

Chart demonstrates vitamin A (retinol) status. A threshold of less than 0.7 µmol/L of retinol was used to indicate vitamin A deficiency.

Table 7-11. Status of vitamin E (α-tocopherol).

Table demonstrates α-tocopherol status. A threshold of less than 12 µmol/L of α-tocopherol was used to indicate vitamin E deficiency.

		α-Tocopherol /QLD		α-Tocopherol /VIC	
		< 12 µmol/L	≥ 12 µmol/L	< 12 µmol/L	≥ 12 µmol/L
Men	n	0	56	0	55
	%	0	100	0	100
Women	n	0	53	1	52
	%	0	100	1.9	98.1
Total	n	0	109	1	107
	%	0	100	0.9	99.1

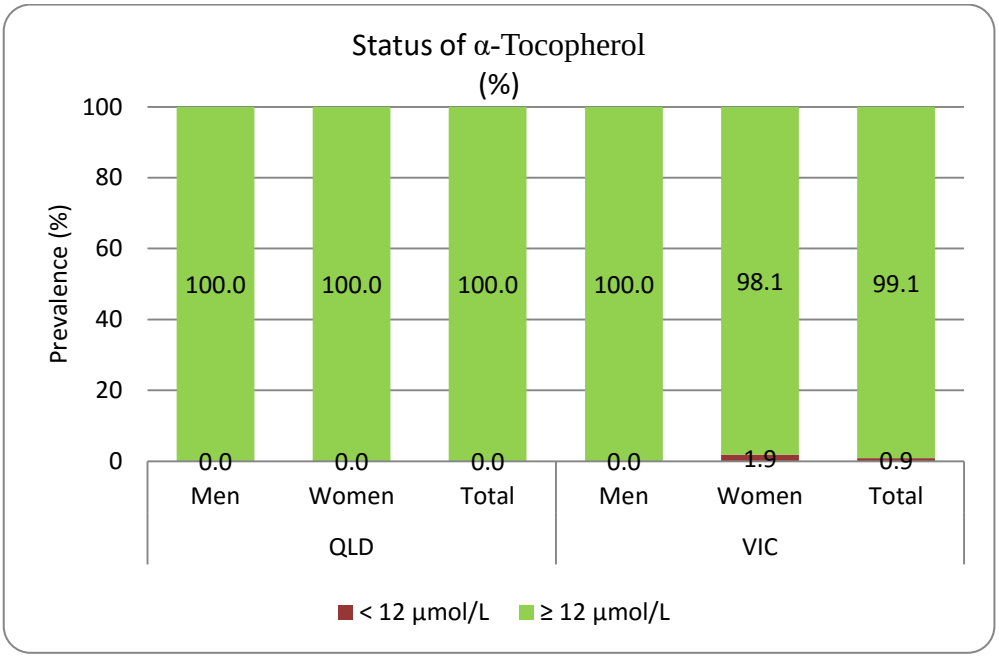


Figure 7-9. Status of α-tocopherol.

Chart demonstrates α-tocopherol status. A threshold of less than 12 µmol/L of α-tocopherol was used to indicate vitamin E deficiency.

7.4.7 Reference intervals for vitamins A and E

RIs were estimated for vitamin A (retinol) and vitamin E (α-tocopherol) in two Australian populations at different latitudes: Queensland (QLD) and Victoria (VIC). For this purpose, outlying results of vitamins A and E were statistically determined using the Tukey test based on the recommendations from the CLSI Guidelines C28-A3 (CLSI 2008) (261).

According to statistical analysis investigating the effect of gender and age groups on vitamin A and E levels conducted earlier in this chapter (sections 7.4.3-7.4.5), RIs for vitamin A were estimated irrespective of gender and age for the QLD and VIC groups. In contrast, RIs for vitamin E in the QLD group were evaluated with consideration of age but not gender. For the VIC group, RIs of vitamin E were calculated regardless of gender and age (Table 7-12).

Table 7-12. Estimation of reference intervals for blood vitamins A and E.

Reference intervals for vitamins A (retinol) and E (α-tocopherol) in blood were assessed based on the "robust method", which is recommended by the Clinical and Laboratory Standards Institute Guidelines C28-A3.

Vitamin	State	Age group (years)	n	Lower 2.5%	Upper 97.5%
Retinol	QLD	18 – over 60	107	1.12 (0.98 –1.25)	3.14 (2.98 – 3.29)
	VIC	18 – over 60	106	0.68 (0.54 – 0.83)	3.03 (2.85 – 3.21)
α-Tocopherol	QLD	18 – 39	37	13 (10 – 16)	39 (36 – 42)
		40 – 60	38	16 (13 – 19)	44 (41 – 47)
		over 60	33	19 (15 – 23)	49 (45 – 52)
	VIC	18 – over 60	105	12 (10 – 14)	43 (40 – 46)

7.4.8 Correlations of fat-soluble vitamin levels

Correlations between FSV levels in the QLD and VIC groups were statistically investigated. Due to inconsistent data distribution across groups and subgroups as showed in earlier sections of this chapter, Pearson (parametric) and Spearman (non-parametric) tests were used in the analysis to provide more confidence in the statistical analysis. Both tests show close results over all investigated correlations, showing a significant positive medium correlation between levels of 25-OHD3 and its epimer in both QLD and VIC groups (Table 7-13, Figure 7-10).

A small correlation between levels of 25-OHD3 and retinol was observed in both QLD and VIC groups (Table 7-13, Figure 7-11). Furthermore, correlations of retinol levels with lower (<50 nmol/L, vitamin D deficiency threshold) and higher (>50 nmol/L, vitamin D insufficiency threshold) levels of 25-OHD3 were also investigated to identify the potential effect of retinol level on vitamin D. However, results did not support the potential effect of retinol on 25-OHD3 levels in QLD and VIC groups (Table 7-13, Figure 7-11).

Possible correlation was examined between levels of 25-OHD3 and α-tocopherol. There was no correlation was found in the QLD group, while a small correlation was observed in the VIC group. Addition statistical investigation was conducted on correlations between α-tocopherol and vitamin D levels of deficiency and insufficiency. A small correlation was found between vitamins E and D levels in both QLD and VIC groups (Table 7-13, Figure 7-12).

Possible correlation between retinol and α-tocopherol was also investigated in the QLD and VIC groups. Small to medium correlations were found between these two blood metabolites in both QLD and VIC samples (Table 7-13, Figure 7-13).

Table 7-13. Correlation between blood FSV levels in QLD and VIC populations.

Correlation between blood FSV levels was statistically examined using Pearson and Spearman tests. Extent of correlation was justified based on trend of correlation (+/-); strength of correlation (correlation coefficient (r): small correlation (±0.1 - ±0.29), medium correlation (±0.3 - ±0.49) and large correlation (±0.5 - ±1.0)); and statistical significance (p <0.05 was considered as significant) (263).

	QLD		VIC	
Correlation	Correlation coefficient (r)	*p* value	Correlation coefficient (r)	*p* value
25-OHD3 and Epi-25-OHD3				
Pearson test	0.401	<0.001	0.388	<0.001
Spearman test	0.359	<0.001	0.344	<0.001
25-OHD3 and Epi-25-OHD3*				
Pearson test	0.467	<0.001	0.586	<0.001
Spearman test	0.449	<0.001	0.571	<0.001
25-OHD3 and Retinol				
Pearson test	0.175	0.069	0.299	0.002
Spearman test	0.206	0.032	0.324	0.001
25-OHD3 (<50nmol/L) [≠] and Retinol				
Pearson test	-0.052	0.871	0.134	0.481
Spearman test	-0.018	0.957	0.120	0.526
25-OHD3 (≥50nmol/L) [$] and Retinol				
Pearson test	0.160	0.117	0.137	0.232
Spearman test	0.193	0.058	0.134	0.243
25-OHD3 and α-Tocopherol				
Pearson test	0.011	0.908	0.237	0.013
Spearman test	0.044	0.648	0.279	0.003
25-OHD3 (<50nmol/L) [≠] and α-Tocopherol				
Pearson test	-0.111	0.732	0.075	0.693
Spearman test	-0.039	0.905	0.022	0.907
25-OHD3 (≥50nmol/L) [$] and α-Tocopherol				
Pearson test	-0.030	0.768	0.276	0.015
Spearman test	0.018	0.864	0.242	0.033
Retinol and α-Tocopherol				
Pearson test	0.266	0.005	0.371	<0.001
Spearman test	0.297	0.002	0.418	<0.001

* Correlation between the 25-OHD3 and its epimer based on samples in which epi-25-OHD3 detected.

[≠] In individuals with vitamin D deficiency (25-OHD3 <50nmol/L)

[$] In individuals with non-vitamin D deficiency (25-OHD3 ≥50nmol/L)

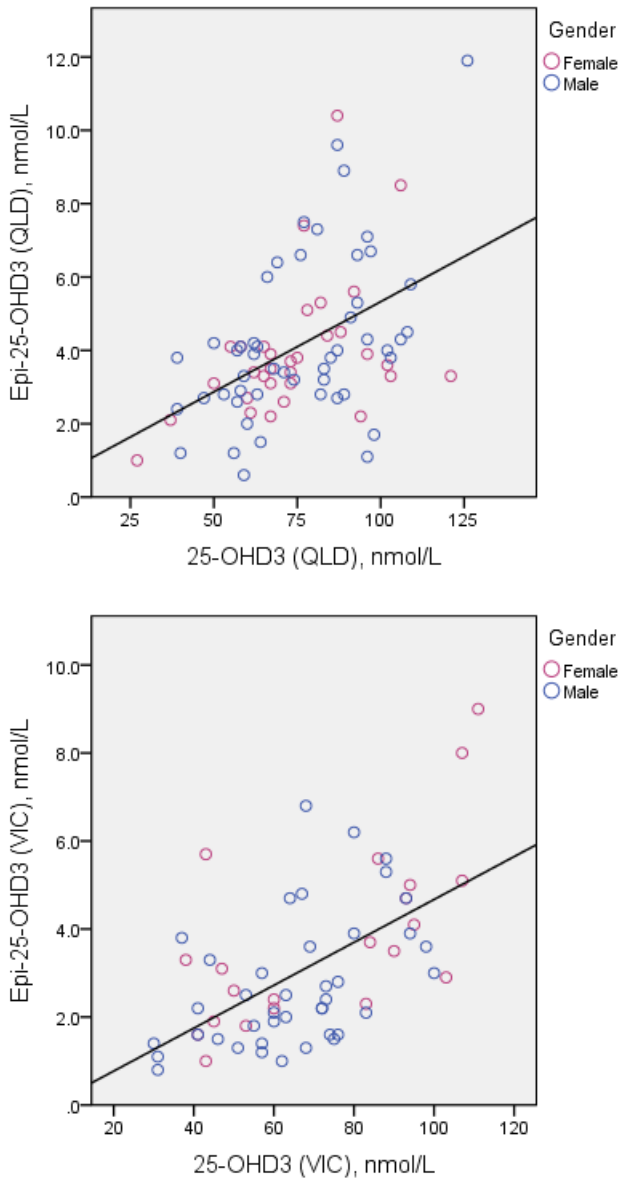


Figure 7-10. Scatter plot of 25-OHD3 versus epimer of 25-OHD3 levels.

This plot demonstrates the correlation between results of 25-OHD3 and epi-25-OHD3 in both QLD (r =0.359, *p* <0.001) and VIC (r=0.344, *p* <0.001) groups. The linear regression line plotted shows the relationship between the investigated vitamin results.

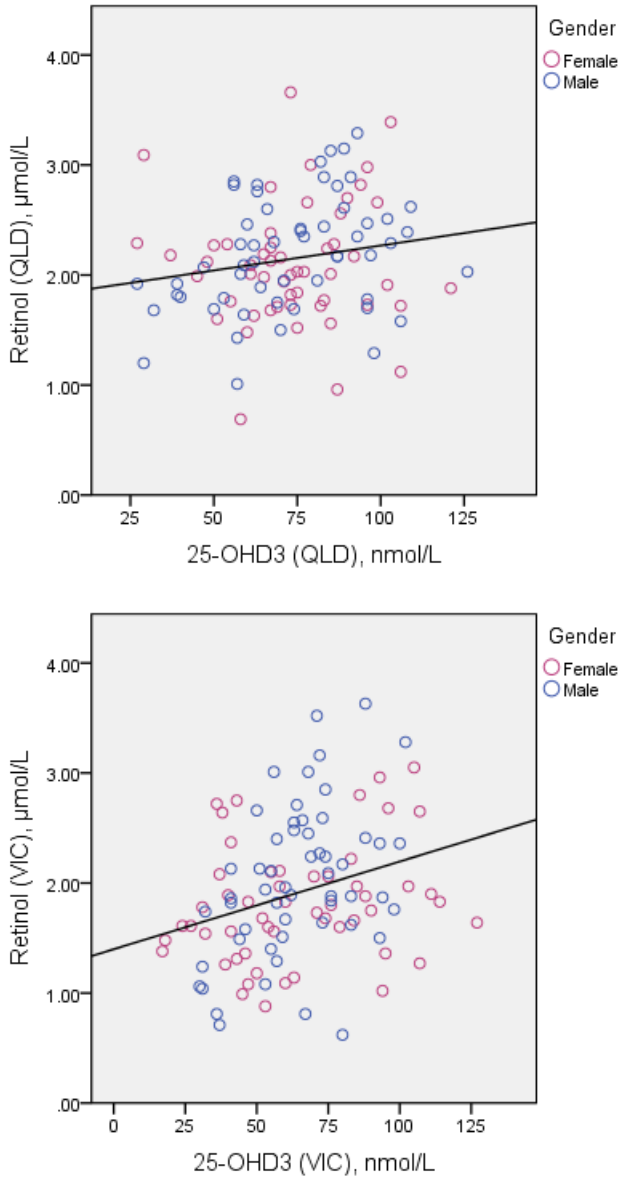


Figure 7-11. Scatter plot of 25-OHD3 versus retinol levels.

This plot demonstrates the correlation between results of 25-OHD3 and retinol in both QLD (r=0.206, *p*= 0.032) and VIC (r=0.324, *p*= 0.001) groups. The linear regression line plotted shows the relationship between the investigated vitamin results.

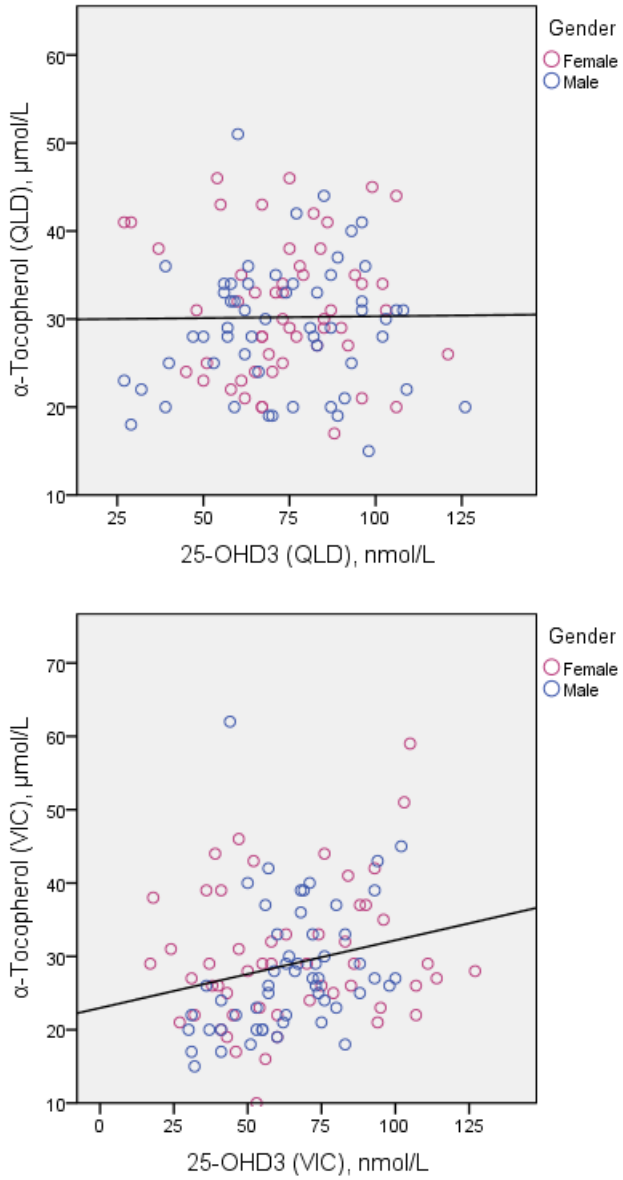


Figure 7-12. Scatter plot of 25-OHD3 versus α-tocopherol levels.

This plot demonstrates the correlation between levels of 25-OHD3 and α-tocopherol in both QLD (r=0.044, *p*= 0.648) and VIC (r=0.279, *p*= 0.003) groups. The linear regression line plotted shows the relationship between the investigated vitamin results.

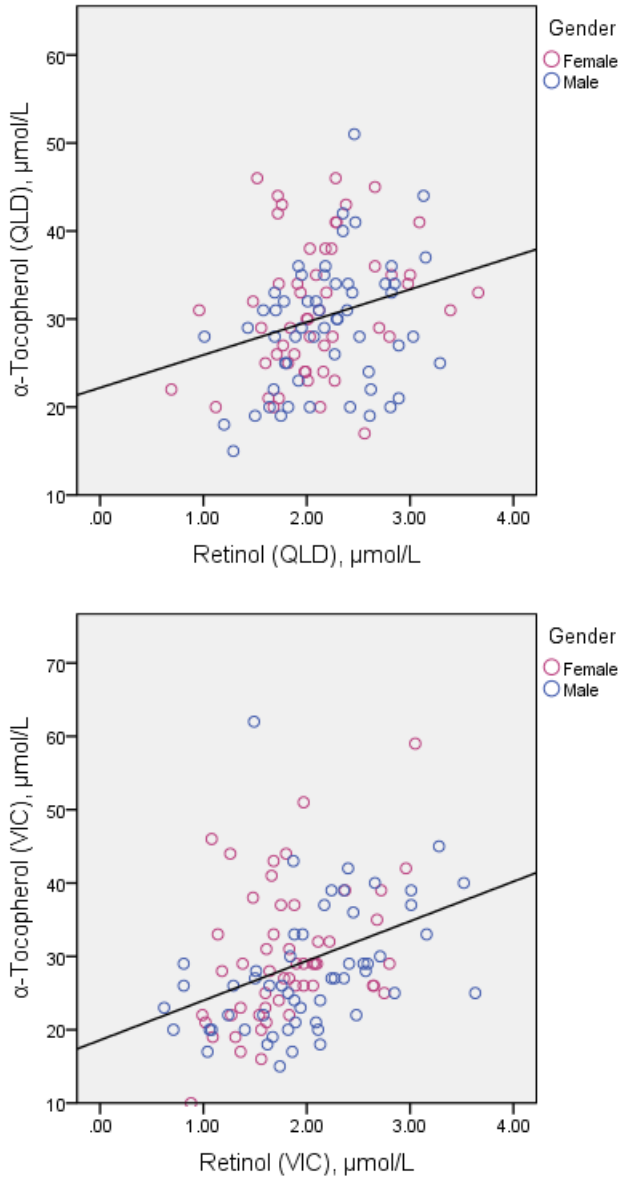


Figure 7-13. Scatter plot of retinol versus α-tocopherol levels.

This plot demonstrates the correlation between levels of retinol and α-tocopherol in both QLD (r=0.297, *p*= 0.002) and VIC (r=0.418, *p* <0.001) groups. The linear regression line plotted shows the relationship between the investigated vitamin results.

7.5 Discussion

This study investigated status and correlation of five FSV metabolites in two Australian populations at different latitudes using precise simultaneous LC-MS/MS quantification methods. While the effect of latitude on vitamin D status has been previously established (40, 264), it is doubtful whether latitude has impact on levels of vitamins A and E (265). The current study investigated the status of three vitamin D metabolites in addition to vitamins A and E in two Australian states, QLD (latitude between 10º S and 28º S) and VIC (latitude between 34º S and 38º S). This study investigated correlations between vitamins in the two populations.

Vitamin D deficiency

Accurate estimation of VDD prevalence has become more complicated due to several factors, including the controversy surrounding the cut-off level of vitamin D deficiency and sufficiency (258, 266). The Institute of Medicine (IOM) proposed 50 nmol/L (20 ng/mL) as the optimal level of serum 25-OHD3 for skeletal health (266). In contrast, the Endocrine Society Clinical Practice Guidelines suggested 75 nmol/L of 25-OHD as the optimal level for vitamin D, and less than 50 nmol/L as the threshold of VDD (258). A nationally based Australian study of the prevalence of VDD used thresholds of less than 50 nmol/L and less than 75 nmol/L of blood 25-OHD3 for vitamin D deficiency and insufficiency, respectively (255).

Our study found that the prevalence of VDD (25-OHD3 <50 nmol/L) in the VIC group was around three times higher than in the QLD group. The vitamin D status variation among VIC and QLD populations is consistent with the fact that people in VIC generally have less sunlight exposure for the most of the year, which is essential for endogenous vitamin D

synthesis, compared with people residing in QLD (255, 267, 268). The Australian national population-based study reported that prevalence of VDD in southern Australian regions (latitude > 35º S) was more than three times higher than the prevalence in central to northern Australian regions (latitude <30º S) during the summer-autumn period (255). VDD was also more common in southern Australian regions in winter-spring (255). In contrast, another study showed that VDD prevalence in Geelong (a city in south VIC, latitude 38º S) was lower than in southeast QLD (latitude 27º S) in the summer and winter seasons (268). This observation is not expected and is inconsistent with the latitude difference between Geelong and southeast QLD.

Significantly low vitamin D levels (25-OHD <25 nom/L) are associated with symptoms of osteomalacia and osteoporosis (266). Therefore, it is important to identify the percentage of cases with less than 25 nmol/L of 25-OHD3 in both QLD and VIC groups. Our results showed that 5.8% of the VIC group had less than 25 nmol/L of 25-OHD3, compared to 0.8% found in the QLD group. As a matter of note, the national study found that 4% of the overall Australian population had less than 25 nmol/L of 25-OHD3 (255).

VDD is generally more common in women than men. In this study, women in the VIC groups had a higher prevalence of VDD (34.5%) than men (20.8%), while in the QLD group, the prevalence of VDD in men (12.5%) was slightly higher than in women (9.4%). Previously, it was documented that the prevalence of VDD in southern Australia populations was higher in women than in men (255). These results are consistent with our result findings in the VIC group. Average of vitamin D level in men and women were very close in QLD group and that is consistent with findings of several population-based studies (42, 253, 254, 269).

Epimer of 25-OHD3 has recently been highlighted by a number of studies that used LC-MS/MS methods, which made possible to measure the epi-25-OHD3 in blood samples (270).

The epi-25-OHD3 has been identified in children and adults (134-141). In this work, epi-25-OHD3 was detected in 75.2% and 57.4% of the investigated QLD and VIC samples, respectively. The proportion of epi-25-OHD3 median level to the total 25-OHD3 median level (% epi-25-OHD3) was 4.2% (QLD group) and 2.4% (VIC group). Based on previous studies, 25-OHD3 epimer was detected in 43–100% of children and adults (136, 270), and it formed 0–16% of total serum 25-OHD level (136, 138, 271). A higher percentage of epi-25-OHD3 was significantly associated with the summer season and with vitamin D supplements, but not with food intake (271). Although the clinical role of epi-25-OHD3 is still not clear, one study recently reported that percentage of epi-25-OHD3 levels in patient groups (diabetes, rheumatoid arthritis and Alzheimer disease groups) were higher than those in healthy people (47.4% and 27.2 %, respectively) (272). Interestingly, we found that epimer of 25-OHD3 was detected in the men more than women in both QLD and VIC populations. This invites the question whether this observation relates to only healthy people or extends to patients as well. This accentuates the importance of exploring the roles of epi-25-OHD3 in pathophysiological functions.

Vitamin D2 forms a minor proportion of total vitamin D in the body. Our results are consistent with the expected low levels of 25-OHD2. Only 10% (n=12) of the QLD samples contained 25-OHD2 (ranged 5-8 nmol/L), while it was not quantified in the VIC sample group. Vitamin D2 is sourced from a limited number of natural foods, such as sun-dried mushrooms, and from fortified foods, which are not commonly available in Australia (273). Furthermore, serum 25-OHD2 level is negatively affected by the increase of vitamin D3 intake (86).

Vitamin A and E deficiencies

Vitamin A deficiency (VAD) is a problematic public health issue, especially due to malnutrition in developing counties (29). VAD occurs in developed countries as secondary malnutrition resulting from gastrointestinal disorders such as celiac disease (256). Furthermore, chronic diseases that affect vitamin A absorption or liver storage could be a cause of VAD (274-276). As a result, about 50% of preschool-aged children and pregnant mothers are at risk of VAD worldwide (29). Between 1995 and 2005, the estimates for global VAD (serum retinol <0.7 μmol/L, based on World Health Organisation (WHO) recommendations) among preschool-aged children and pregnant women were 190 million and 19.1 million, respectively (29).

Vitamin E deficiency (VED) is rare in humans, as most food sources contain vitamin E. VED is commonly caused by malabsorption disorders such as cystic fibrosis, chronic hepatitis and gastrointestinal disorders (256). However, this deficiency is more common in developing countries than in industrial countries, due to inadequate vitamin intake and high prevalence of infectious diseases that relate to oxidative stress processes, such as malaria and AIDS (66). To evaluate vitamin E status, MOI suggests plasma α-tocopherol levels of 12 μmol/L as the threshold of vitamin E adequacy (259). This α-tocopherol level is used to determine sufficient vitamin E intake, and is also linked to a normal *in vitro* hydrogen peroxide induced haemolysis (259).

The status of vitamins A and E is affected by dietary intake and possibly other controversial factors, including gender, age and season. Our results showed no significant gender effect on vitamin A and E levels, which is consistent with several previous studies (257, 277-279). In contrast, however, other studies reported that vitamin A and E levels were influenced by gender (248, 280). Overall, the median levels of vitamins A and E in both QLD and VIC

groups were observed at adequate levels. These results were not unexpected, as vitamin A and E deficiencies are not common in well-nourished populations (278).

The current study showed that median levels of vitamins A and E in the VIC group were slightly lower than those in the QLD group. The slight discrepancies in the median results between the two investigated groups may relate to variances in dietary patterns rather than latitudes or seasons. Olmedilla and colleagues indicated that seasons had no significant effect on vitamin A and E levels (248). In fact, food purchasing behaviours across seasons, which are influenced by socioeconomic status and the food basket market, might have more impact on vitamin consumption (281-285). Furthermore, people's educational and socioeconomic levels are associated with their food choices and consumption (286). These indicate how dietary lifestyle patterns are affected by food availability at certain times and socioeconomic levels rather than the season or latitude themselves.

Queensland and Victoria are multicultural communities that have a variety of dietary and lifestyle behaviours, and the population structure in QLD and VIC is likewise varied. Based on the 2011 census, the four main ancestries (English, Australian, Irish and Scottish) formed 95.2% and 78.2% of total people ancestries in QLD and VIC, respectively (287, 288). The percentage of people who speak languages other than English at home was higher in VIC (23.1%) than in QLD (9.8%) (288, 289). Furthermore, people who were born in overseas formed 26.2% of the total VIC population compared with 20.5% of total QLD residents (288, 289). In addition, between 2007 and 2013, 28.6% and 12.8% of total refugees and humanitarian entrants to Australia were welcomed by VIC and QLD, respectively (290). Therefore, a potentially greater variety of dietary patterns and lifestyles in Victorian people compared with Queensland residents might relate to the diversity in population structures in

VIC versus QLD. However, this could not be confirmed in our study as dietary and lifestyle data were unavailable; this is a limitation to the current study.

Reference intervals

The establishment of RIs for FSVs is challenged by several difficulties, including disagreement on defining optimal levels for FSVs. Three main factors affect the establishment of appropriate RIs for vitamin D. Firstly, there is debate as to whether the basis of skeletal health is enough to define optimal levels for vitamin D, or if other health demands, such as parathyroid gland functions, should be considered in defining the optimal vitamin D level (21). Secondly, endogenous synthesis of vitamin D is influenced by several factors such as race, lifestyle, seasons and latitudes (10), and VDD is a global health problem (22). Thirdly, although standardisation efforts in measurement of FSVs have been made, there are still significant knowledge gaps as discussed in Chapter 3. As a result, it is difficult to establish appropriate RIs for vitamin D.

Vitamin A and E deficiencies in adults are uncommon, especially in developed countries or well-nourished communities, and they are more related to serious health problems such as cystic fibrosis. Several studies demonstrated average levels of vitamins A and E in certain populations (23-25), however, there is a limited number of studies establishing 95th percentile RIs for vitamins A and E in adults (26, 27) (Table 7-14). The serious issue here that these published RIs were established based on different methods and calibrators, which affected the final results (28, 29). Hence, finding reliable comparisons between published RIs is challenging.

Consequently, there is disagreement between laboratories regarding RIs of vitamins A and E (81, 142). In the current study, 95^{th} percentile RIs were calculated for vitamins A and E in the QLD and VIC groups. It showed that the calculated upper limits of RIs of vitamins A and E

in QLD and VIC groups were close; in contrast, the calculated lower limits of vitamins A and E were lower in the VIC group than those in the QLD group. These lower limits were close to the suggested borders of vitamin A and vitamin E deficiencies (<0.70 µmol/L and 12 µmol/L, respectively). These lower limits might reflect the diversity of Queensland and Victorian populations in dietary patterns and lifestyles. The calculated RIs for vitamin A based on investigated VIC population may not be appropriate RIs.

A limited number of studies established 95th percentile RIs for vitamins A and E in adults (92, 160, 256, 257, 277, 291, 292). Some of these studies reported lower limits of RIs close to the suggested deficiency borders of vitamin A (256, 277, 292) and vitamin E (160, 257, 277, 291). Comparing our calculated ranges with those in previous studies is difficult, however, as these studies determined RIs using different methods and calibrators that had an impact on their results (219, 220, 229). Recommended ranges for health should be used until appropriate RIs are established. This, however, cannot be completely adequate until traceability of chain is validated as discussed in Chapter 5.

Table 7-14. Summary of published reference intervals for blood vitamins A and E in adolescents and adults.

Table demonstrates published 95th percentile reference intervals for vitamins A and E. These reference intervals were established using the HPLC platform with commercial and in-house calibrators of different matrix types.

Analyte	Country, year, references	n, specimen	Gender	Age range	Calibrator	Imprecision CV % (at µmol/L)	Lower limit	Upper limit
				year			µmol/L	µmol/L
Vitamin A (Retinol)	Europe 2011 (292)	444 serum	M	13–18	NS	2.9% (NS)[#]	0.69[$]	2.20
	USA 2009 (257)	517 Serum	M + F	13–17	In-house calibrator in ethanol	9% (at 1.05)[*]	1.12	2.62
	Canada 1988 (277)	24 Serum	M + F	13–19	NS	NS	0.90	2.50
	Canada 2014 (256)	71 Serum	M + F	11–<16	Chromsystems (traceable to NIST 968e)	3.8% (at 2.11)[*]	0.9	1.9
		50 Serum	M + F	16–<19			1.0	2.6
	Spain 1997 (92)	210 Serum	M	5–79	NS	NS	1.13	2.63
		240 Serum	F				1.01	2.44
	USA 2009 (160)	160 serum	M + F	>13	In-house calibrator in ethanol	6.1% (NS)[*]	1.05	5.03
Vitamin E (α-Tocopherol)	Europe 2011 (292)	444 Serum	M	13–18	NS	2.9% (NS)[#]	14	32
	USA 2009 (257)	517 Serum	M + F	7–17	In-house calibrator in ethanol	9% (at 1.05)[*]	11	30
	Canada 1988 (277)	24 Serum	M + F	13–19	NS	NS	13	24
	Canada 2014 (256)	245 Serum	M + F	1–<19	Chromsystems (traceable to NIST 968e)	2.99% (at 26.9)[*]	14.5	33
	Spain 1997 (92)	210 Serum	M	5–79	NS	NS	18	46
		210 Serum	M				18	46
	UK 1997 (291)	4943 Plasma	M	35–55	In-house calibrator in hexane	10.5% (NS)[*]	11	52
		2234 Plasma	F				11	51
	USA 2009 (160)	160 serum	M +F	> 13	In-house calibrator in ethanol	6.7 (NS)[*]	10	70

NS: not stated, [#] no details,[*] Inter-assay imprecision, [$] 0.7 µmol/L is considered as vitamin A deficiency.

Vitamin Interaction

Interaction between vitamins, especially between vitamins A and D (82, 83, 293), has been reported. Interference of vitamin A with vitamin D function has been observed in animals and humans (82, 83). In rats, high vitamin A intake attenuated toxicity of hypervitaminosis D (82). VDD (<50 nmol/L) and a high level of retinol (>2.8 μmol/L) have been associated with high risk of osteoporotic fractures (85). Association between vitamins A and E has also been suggested (294, 295), and correlation between levels of serum retinol and α-tocopherol in humans was also reported (277). Dietary vitamin A was linked with blood α-tocopherol levels, while vitamin E intake had no significant effect on blood retinol level (294, 295). The current work is the first study investigated interactions of these vitamins using simultaneous analysis method which is important to minimise misdirection of vitamin interactions resulting from the use of several quantification methods.

Correlations between levels of 25-OHD3 and its epimer have been reported in several studies (135, 136, 296). In the current study, a medium correlation between 25-OHD3 and its epimer was observed in the QLD and VIC groups, and epi-25-OHD3 levels were not associated with age in adults. In addition, our study investigated correlations between blood levels of 25-OHD3 and retinol, which both are non-active form analytes. We found a small correlation between the levels of these two inactive analytes in both the VIC and QLD groups. However, this does not rule out a possible association between the active forms of vitamin D (1α,25-dihydroxyvitamin D (1α,25-(OH)2D3)) and vitamin A (retinoic acid).

At the molecular level, retinoic acid might interfere with function of 1α,25-(OH)2D3, where both have regulatory roles in gene expression. 1α,25-(OH)2D3 forms a complex with vitamin D receptors (VDR) to create heterodimers with the retinoid X receptor (RXR). The heterodimer of the 1α,25-(OH)2D3-VDR complex and RXR triggers the gene expression

process. On the other hand, retinoic acid binds with retinoic acid receptors (RAR) to form the retinoic acid-RAR complex. This complex also requires forming heterodimers with RXR to facilitate gene expression. It is worth mentioning that several nuclear receptors, including thyroid hormone receptors, can form heterodimers with RXR. Therefore, intracellular vitamin D function could be affected by competitive metabolites forming heterodimers with RXR. High doses of vitamin A may attenuate the formation of the heterodimer 1α,25-(OH)2D3-VDR complex with RXR. In *in vitro* studies, it has been found that the heteromeric interaction of the 1α,25-(OH)2D3-VDR complex with RXR was influenced by the presence of 1α,25-(OH)2-D3 and inhibited by high concentrations of retinoic acid (88).

In the current work, possible correlation between 25-OHD3 levels and α-tocopherol levels among the QLD and VIC groups was investigated. Our findings showed a small correlation between the two vitamin levels, which agrees with previous research (297). It was documented that vitamin D3 supplements (800 IU/D for 6 months) alone or with calcium (2 g/d for 6 months) significantly decreased α-tocopherol by 14% (86). However, there is little in the literature to support or deny this finding.

Associations between vitamin A and vitamin E have been reported (294, 295). In this study, there was a significant positive medium correlation between the levels of retinol and α-tocopherol in the QLD and VIC groups. These findings are consistent with those of a previous study, which reported a positive correlation between serum retinol and α-tocopherol in humans (277). However, in animal studies, high dietary vitamin A has been linked to a decrease in plasma α-tocopherol levels (294, 295). In contrast, varying levels of vitamin E intake had no significant effect on plasma retinol (295), whereas high levels of vitamin E intake interfere with the utilisation of beta-carotene (a vitamin A precursor) to retinol (298). Further studies are needed to explore association between vitamins A and E.

7.6 Conclusions

In conclusion, this study has determined that there is no solid correlation between the levels of the investigated biomarkers of FSVs in serum. The measurement of the non-active forms of vitamin D (25-OHD3) and vitamin A (retinol) may not reflect the hypothesised correlation between the active forms of these vitamins (1,25-(OH)2D3 and retinoic acid) at the level of the gene regulation. The difference in levels of vitamins did not vary across the adult populations based on gender. As anticipated, VDD is common even in the summer season in QLD and VIC groups, while deficiencies of vitamins A and E are not common in either state. There were, however, a proportion of participants in each cohort that were deficient as determined by the current list of recommended levels.

Based on the current study using population, which was expected to be representative, we provide the following recommendations:

1. No age stratification for vitamin A in adults, but age stratification may be considered further for vitamins D and E.
2. No gender stratification for vitamins A, E and D in adults.
3. We suggest using recommended vitamin ranges for health rather than RIs.

These recommendations fill some important knowledge gaps related to these vitamins. The appropriate recommended levels, however, cannot be completely established until chain of traceability is fully validated (discussed in Chapter 5).

Chapter 8

General discussion and conclusions

Chapter 8 General discussion and conclusions

8.1 General discussion

Standardisation initiatives for the measurement of FSVs were first successfully introduced in the late 1980s. The National Institute of Standards and Technology (NIST) produced the standard reference material SRM 968 in 1989, and in 2009 added SRM 972 to support the standardisation of FSV measurement (94, 102). Further important efforts for the standardisation of clinical analyte measurement came through establishing The Joint Committee for Traceability in Laboratory Medicine (JCTLM) in 2002. The JCTLM was formed as a result of collaboration between the International Committee for Weights and Measures (CIPM), the International Federation for Clinical Chemistry and Laboratory Medicine (IFCC) and the International Laboratory Accreditation Cooperation (ILAC). The JCTLM plays a significant role in promoting the standardisation of analyte measurements through identifying appropriate reference materials, measurement procedures and laboratories (299). Recently, the isotope dilution liquid chromatography-mass spectrometry method has been recognised by JCTLM as a reference measurement procedure (RMP) for 25-OHD2 and 25-OHD3, although not for the epimer of 25-OHD3 (103). There are no RMPs for vitamins A and E recognised as meeting the JCTLM requirements (103).

Despite the fact that efforts have been made to standardise FSV measurements, many knowledge gaps remain. Until now, there have been no RMPs for simultaneous measurement of FSVs in blood as discussed in Chapter 3. In addition, there was limited data regarding the stability of FSVs in routine samples, including whole blood and serum (Chapter 4), traceability from reference material to commercial calibrators (Chapter 5) and commutability of using different biological matrices, such as diluted cord blood with cell culture media, for

FSV measurements (Chapter 6). Furthermore, the current status, appropriate RIs and correlation of blood FSV levels were part of the knowledge gaps (Chapter 7). The main aim of the current project is to be a part of standardisation efforts for FSV measurement through the introduction of a candidate reference quantification method applicable to a variety of clinical studies.

In this project, an efficient and precise method for the simultaneous quantification of five analytes (25-OHD3 and its epimer, 25-OHD2, retinol and α-tocopherol) was developed and validated using LC-MS/MS technology (Chapter 3). This method uses a robust, simple sample preparation with commercial calibrators and controls, and can be easily reproduced by clinical laboratories. Simultaneous measurement of the analytes using one patient sample reduces the potential result variations that could be observed, compared with using different techniques under different analytical conditions. This method was utilised in four clinical studies to explore existing knowledge gaps (Chapters 4-7).

The knowledge gaps in FSV stability were identified through performing a systematic review of published data (Chapter 4). Accordingly, the stability of the three FSVs in whole blood, serum and analyte extracts under the influence of light and temperatures across different storage times was explored. The importance of this study is in simultaneously investigating the stability of FSVs in routine samples, utilising a precise LC-MS/MS method. In addition, the stability of each analyte was justified based on the calculation of acceptable clinical limits (i.e. TCL), which reflect biological variation and method imprecision (175). This work confirms that blood and serum samples designated for FSV measurement can be reliably processed without further precautions in normal laboratory conditions, including lighting and ambient temperature, during the pre-analytical stage. This data is useful to decrease the cost of sample transportation and storage, especially for large-scale studies.

The traceability of commercial calibrators to reference materials was explored using three commercial calibrators for vitamin E (α-tocopherol), as an example of extent of traceability of commercial calibrators to reference material (Chapter 5). It is well recognised that a correct and consistent calibrator assignment is important for accurate interpretation of results and medical decisions (224). Although the three commercial calibrators used in the current study were traceable to the same reference material (SRM 968), we found discrepancies between the observed concentrations and manufacturers' expected concentrations. This exploration raises a problematic issue related to the trueness and traceability of commercial calibrators, and how different commercial calibrators could affect interpretation of patient results. This study also highlights the importance of standardising all parts of a traceability chain.

This project examined the validity of measurement of 25-OHD3 and its epimer (epi-25-OHD3), retinol and α-tocopherol in umbilical cord blood (UCB) plasma with cell culture media (RPMI 1640) (Chapter 6). UCB has been utilised to investigate the correlation of vitamins with neonatal health problems (236). The emphasis of this study is that UCB is an appropriate sample choice to assess vitamin status in neonates as the number of venous blood samples that can safely be obtained is limited. Our results indicate that dilution of UCB diluted plasma is a reliable matrix for the quantification of 25-OHD3 and retinol using the LC-MS/MS method.

The last study in this project focused on the status and correlation of FSVs in two Australian populations at different latitudes (Chapter 7). Vitamins A, D and E are routinely measured in the clinical laboratory; however, controversy exists in relation to the appropriate standardisation of the assays, which affects the interpretation of results. This is further confounded by current debate regarding the selection of population-based RIs versus

recommended levels for health. As a starting point to address this gap in knowledge, we created a snapshot of the current status and correlation of vitamin D (25-OHD2, 25-OHD3 and epi-25-OHD3), vitamin A (retinol) and vitamin E (α-tocopherol) in blood across two Australian populations at different latitudes: Queensland (QLD) and Victoria (VIC), using simultaneous measurement LC-MS/MS method.

A significant difference in 25-OHD3 and retinol levels, but not α-tocopherol, was observed between latitudes. While the effect of latitude location on vitamin D status is well known, our finding of a low vitamin A level in the VIC population may be related to a greater variety of dietary patterns and lifestyles in VIC people compared with QLD residents (287, 288). Our results also show low epi-25-OHD3 levels in the VIC group compared to the QLD group. The epimer of 25-OHD3 was detected in men more than in women in both the QLD and VIC populations. This raises the question of whether this observation is more likely seen in healthy people or in both healthy people and patients, which accentuates the importance of exploring the roles of epi-25-OHD3 in pathophysiological functions. The current study also found no gender difference in the studied vitamins. The effect of age stratification was not constant across the two latitudes, and consistent RIs could not be applied to the VIC and QLD populations. Finally, while the interaction of vitamins A and D has been observed in animals and humans (82, 83), only a small correlation between levels of the investigated biomarkers of FSV was observed.

8.2 Conclusions

This thesis contributes to standardisation efforts for FSV measurement by providing a novel and precise simultaneous method of quantifying FSVs using LC-MS/MS technology, and using it to fill existing knowledge gaps. This method is fully validated and easily reproducible by clinical laboratories. We confirm that FSVs are stable enough to be analysed

under normal laboratory conditions for at least 48 h, and serum FSVs can be kept at -20°C for at least one month and 25-OHD3 and retinol can be successfully measured by LC-MS/MS in UCB diluted plasma with RPMI-1640. In addition we recommend no gender stratification for vitamins A, D and E in adults, and appropriate levels for vitamins A and E are still need to be determined. We also suggest that vitamin A levels be reviewed when moderate to severe vitamin D deficiency is suspected. Finally, this thesis highlights that further collaboration efforts are required by all parties to improve the metrological traceability chain, which is essential for reliable comparison of results.

8.3 Future directions

Standardisation of measurement relies on main five pillars, including availability of reference material, reference measurement procedures, reference measurement laboratories, reference intervals and external quality assurance programs. Three of these pillars of standardisation of FSV measurements have not yet been completed. This thesis demonstrates the necessity for continued standardisation efforts of FSV measurement by all parties, including clinical institutes, for measurement standardisation, and manufacturers of *in vitro* diagnostic medical devices, for implementation of traceability requirements. The simultaneous FSV quantification method developed in the current project will be published as an essential step prior to submission for recognition as a reference measurement procedure by JCTLM and this may cover pillar of reference measurement procedure for FSV measurement.

Studies undertaken in this thesis demonstrate that the correlation of FSVs, including active forms (e.g. 1α,25-dihydroxyvitamin D3 and retinoic acid), in healthy and patient samples should be investigated further. Correlations of other FSV analyte levels, such as 24,25-dihydroxyvitamin D3, ß-carotene and γ-tocopherol, are also worthy of study. Therefore, it is recommended to extend the current simultaneous quantification method to include 1α,25-

dihydroxyvitamin D3, 24,25-dihydroxyvitamin D3, retinoic acid, ß-carotene and γ-tocopherol. Such a method would be helpful for a better understanding of patterns of vitamin interaction in healthy people and patients.

In this thesis, epi-25-OHD3 was identified in men more than in women in both QLD and VIC samples, though the median level of the epimer in the QLD samples was higher than in the VIC samples. Epi-25-OHD3 levels have been associated with certain diseases (143) using a small sample size, although its role is not well understood. Further studies exploring epi-25-OHD3 status in men versus women in healthy and patient samples are recommended.

Finally, this research reveals a problematic issue related to the trueness and traceability of commercial calibrators. It is recommended to explore the effects of using different commercial calibrators for FSVs on patient results, which will support efforts in the harmonisation of measurement methods. This will further support quality of measurement which in turn will aid clinical diagnostic and research initiatives in the future.

References

References

1. Holick MF. Sunlight and vitamin D for bone health and prevention of autoimmune diseases, cancers, and cardiovascular disease. Am J Clin Nutr. 2004;80(6 Suppl):1678S-88S.

2. Stephensen CB. Vitamin A, infection, and immune function. Annu Rev Nutr. 2001;21:167-92.

3. Plum LA, DeLuca HF. Vitamin D, disease and therapeutic opportunities. Nat Rev Drug Discov. 2010;9(12):941-55.

4. Herrmann M. The measurement of 25-hydroxy vitamin D - an analytical challenge. Clin Chem Lab Med. 2012;50(11):1873-5.

5. Ross C. Vitamin A and Carotenoids. In: Shils ME, Shike M, editors. Modern nutrition in health and disease. 10th ed. Philadelphia: Lippincott Williams & Wilkins; 2006. p. 305-27.

6. Barua A, Stacewicz-Sapuntzakis M, Furr H. Vitamin A - Retinol. In: Herrmann W, Obeid R, editors. Vitamins in the prevention of human diseases. Berlin ; New York: Walter de Gruyter; 2011. p. 7-39.

7. Ross AC, Ternus ME. Vitamin A as a hormone: recent advances in understanding the actions of retinol, retinoic acid, and beta carotene. J Am Diet Assoc. 1993;93(11):1285-90; quiz 91-2.

8. Chen W, Chen G. The Roles of Vitamin A in the regulation of carbohydrate, lipid, and protein metabolism. J Clin Med. 2014;3(2):453-79.

9. Niles RM. Vitamin A and cancer. Nutrition. 2000;16(7-8):573-6.

10. Kim KN, Pie JE, Park JH, Park YH, Kim HW, Kim MK. Retinoic acid and ascorbic acid act synergistically in inhibiting human breast cancer cell proliferation. J Nutr Biochem. 2006;17(7):454-62.

11. Manna SK, Aggarwal BB. All-trans-retinoic acid upregulates TNF receptors and potentiates TNF-induced activation of nuclear factors-*k*B, activated protein-1 and apoptosis in human lung cancer cells. Oncogene. 2000;19(17):2110-9.

12. Ross C. Vitamin A. In: Coates PM, Blackman MR, Cragg GM, Levine M, Moss J, White JD, editors. Encyclopedia of dietary supplements. New York: Marcel Dekker; 2005. p. 713.

13. Blomhoff R, Green MH, Norum KR. Vitamin A: physiological and biochemical processing. Annu Rev Nutr. 1992;12:37-57.

14. D'Ambrosio DN, Clugston RD, Blaner WS. Vitamin A Metabolism: An Update. Nutrients. 2011;3(1):63-103.

15. CSID:1042. All-trans-retinol UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.1042.html.

16. CSID:5337. Retinoic acid UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.5337.html.

17. CSID:1041. All-trans-retinal UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.1041.html.

18. CSID:10370522. 11-cis-Retinal UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.10370522.html.

19. CSID:4444162. Retinyl palmitate UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.4444162.html.

20. Rigtrup KM, Ong DE. A retinyl ester hydrolase activity intrinsic to the brush border membrane of rat small intestine. Biochemistry. 1992;31(11):2920-6.

21. Erdman JW, Bierer TL, Gugger ET. Absorption and transport of carotenoids. Ann N Y Acad Sci. 1993;691(1):76-85.

22. Reboul E. Absorption of vitamin A and carotenoids by the enterocyte: focus on transport proteins. Nutrients. 2013;5(9):3563-81.

23. Nayak N, Harrison EH, Hussain MM. Retinyl ester secretion by intestinal cells: a specific and regulated process dependent on assembly and secretion of chylomicrons. J Lipid Res. 2001;42(2):272-80.

24. Schneider HG. Vitamins. In: Kaplan LA, Pesce AJ, editors. Clinical Chemistry Theory Analysis Correlation. 5th ed: Mosby Elsevier; 2010. p. 822-53.

25. O'Byrne SM, Blaner WS. Retinol and retinyl esters: biochemistry and physiology Thematic Review Series: Fat-Soluble Vitamins: Vitamin A. J Lipid Res. 2013;54(7):1731-43.

26. Khillan JS. Vitamin A/Retinol and Maintenance of Pluripotency of Stem Cells. Nutrients. 2014;6(3):1209-22.

27. Blomhoff R, Green MH, Green JB, Berg T, Norum KR. Vitamin A metabolism: new perspectives on absorption, transport, and storage. Physiol Rev. 1991;71(4):951-90.

28. de Pee S, Dary O. Biochemical indicators of vitamin A deficiency: serum retinol and serum retinol binding protein. J Nutr. 2002;132(9 Suppl):2895S-901S.

29. WHO. Global prevalence of vitamin A deficiency in populations at risk 1995–2005. WHO global database on vitamin A deficiency. Geneva: World Health Organization; 2009.

30. Wasantwisut E. Recommendations for monitoring and evaluating vitamin A programs: outcome indicators. J Nutr. 2002;132(9 Suppl):2940S-2S.

31. Holick MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest. 2006;116(8):2062-72.

32. Cranney A, Horsley T, O'Donnell S, Weiler H, Puil L, Ooi D, et al. Effectiveness and safety of vitamin D in relation to bone health: US Department of Health and Human Services, Public Health Service, Agency for Healthcare Research and Quality; 2007. 235 p.

33. Haroon M, Regan MJ. Vitamin D deficiency: the time to ignore it has passed. Int J Rheum Dis. 2010;13(4):318-23.

34. McGreevy C, Williams D. New insights about vitamin d and cardiovascular disease: a narrative review. Ann Intern Med. 2011;155(12):820-6.

35. Beard JA, Bearden A, Striker R. Vitamin D and the anti-viral state. J Clin Virol. 2011;50(3):194-200.

36. Welsh J. Vitamin D and breast cancer: insights from animal models. Am J Clin Nutr. 2004;80(6 Suppl):1721S-4S.

37. Van den Bemd GJ, Pols HA, van Leeuwen JP. Anti-tumor effects of 1,25-dihydroxyvitamin D3 and vitamin D analogs. Curr Pharm Des. 2000;6(7):717-32.

38. Rocker D, Ravid A, Liberman UA, Garach-Jehoshua O, Koren R. 1,25-Dihydroxyvitamin D3 potentiates the cytotoxic effect of TNF on human breast cancer cells. Mol Cell Endocrinol. 1994;106(1-2):157-62.

39. Mathiasen IS, Hansen CM, Foghsgaard L, Jaattela M. Sensitization to TNF-induced apoptosis by 1,25-dihydroxy vitamin D3 involves up-regulation of the TNF receptor 1 and cathepsin B. Int J Cancer. 2001;93(2):224-31.

40. Mithal A, Wahl DA, Bonjour JP, Burckhardt P, Dawson-Hughes B, Eisman JA, et al. Global vitamin D status and determinants of hypovitaminosis D. Osteoporos Int. 2009;20(11):1807-20.

41. Ardawi MS, Sibiany AM, Bakhsh TM, Qari MH, Maimani AA. High prevalence of vitamin D deficiency among healthy Saudi Arabian men: relationship to bone mineral density, parathyroid hormone, bone turnover markers, and lifestyle factors. Osteoporos Int. 2012;23(2):675-86.

42. Forrest KY, Stuhldreher WL. Prevalence and correlates of vitamin D deficiency in US adults. Nutr Res. 2011;31(1):48-54.

43. Daly RM, Gagnon C, Lu ZX, Magliano DJ, Dunstan DW, Sikaris KA, et al. Prevalence of vitamin D deficiency and its determinants in Australian adults aged 25 years and older: a national, population-based study. Clin Endocrinol (Oxf). 2011.

44. CSID:4444103. 25-Hydroxyvitamin D3 UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.4444103.html.

45. CSID:17216121. 25-Hydroxyvitamin D2 UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.17216121.html.

46. CSID:4444108. 1,25-Dihydroxyvitamin D3 UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.4444108.html.

47. Amiot-Carlin MJ. Digestion and absorption of lipophilic food micronutrients. In: McClements DJ, Decker EA, editors. Designing functional foods: Measuring and controlling food structure breakdown and nutrient absorption: Elsevier; 2009.

48. Wolpowitz D, Gilchrest BA. The vitamin D questions: how much do you need and how should you get it? J Am Acad Dermatol. 2006;54(2):301-17.

49. Itani O, Tsang R. Hormone Physiology. In: Kaplan LA, Pesce AJ, editors. Clinical Chemistry Theory Analysis Correlation: Mosby Elsevier; 2010. p. 622-5.

50. Kochupillai N. The physiology of vitamin D : current concepts. Indian J Med Res. 2008;127(3):256-62.

51. Bikle D. Nonclassic Actions of Vitamin D. J Clin Endocrinol Metab. 2009;94(1):26-34.

52. Holick MF. Vitamin D deficiency. N Engl J Med. 2007;357(3):266-81.

53. Eitenmiller R, Ye L, Landen J. Vitamin D. 2008 [cited 18/05/2015]. In: Vitamin Analysis for the Health and Food Sciences [Internet]. 2nd ed: CRC Press; 2008. p. 83-117. [accessed 18/05/2015]. Available from: http://dx.doi.org/10.1201/9781420009750.ch2.

54. Lips P. Vitamin D physiology. Prog Biophys Mol Biol. 2006;92(1):4-8.

55. Panda DK, Miao D, Bolivar I, Li J, Huo R, Hendy GN, et al. Inactivation of the 25-hydroxyvitamin D 1alpha-hydroxylase and vitamin D receptor demonstrates independent and interdependent effects of calcium and vitamin D on skeletal and mineral homeostasis. J Biol Chem. 2004;279(16):16754-66.

56. M H, P W. Measurement of vitamin D. In: W. H, R. O, editors. Vitamins in the prevention of human disease. 1st ed. Berlin/ New York: Walter de Gruyter GmbH & Co KG; 2011. p. 395-409.

57. Jones G. Metabolism and biomarkers of vitamin D. Scand J Clin Lab Invest Suppl. 2012;243:7-13.

58. Traber MG. Vitamin E- Alpha-tocopherol. In: Herrmann W, Obeid R, editors. Vitamins in the prevention of human diseases. Berlin ; New York: Walter de Gruyter; 2011. p. 457-82.

59. Cook-Mills JM, Avila PC. Vitamin E and D regulation of allergic asthma immunopathogenesis. Int Immunopharmacol. 2014;23(1):364-72.

60. Wassall HJ, Devereux G, Seaton A, Barker RN. Complex Effects of Vitamin E and Vitamin C Supplementation on in Vitro Neonatal Mononuclear Cell Responses to Allergens. Nutrients. 2013;5(9):3337-51.

61. Nazrun AS, Norazlina M, Norliza M, Nirwana SI. The anti-inflammatory role of vitamin e in prevention of osteoporosis. Adv Pharmacol Sci. 2012;2012:142702.

62. Packer L. Protective role of vitamin E in biological systems. Am J Clin Nutr. 1991;53(4 Suppl):1050S-5S.

63. Clarke MW, Burnett JR, Croft KD. Vitamin E in human health and disease. Crit Rev Clin Lab Sci. 2008;45(5):417-50.

64. Alkhenizan A, Hafez K. The role of vitamin E in the prevention of cancer: a meta-analysis of randomized controlled trials. Ann Saudi Med. 2007;27(6):409-14.

65. Kline K, Lawson KA, Yu W, Sanders BG. Vitamin E and breast cancer prevention: current status and future potential. J Mammary Gland Biol Neoplasia. 2003;8(1):91-102.

66. Dror DK, Allen LH. Vitamin E deficiency in developing countries. Food Nutr Bull. 2011;32(2):124-43.

67. Burton GW, Traber MG. Vitamin E: antioxidant activity, biokinetics, and bioavailability. Annu Rev Nutr. 1990;10:357-82.

68. Traber MG. Vitamin E. In: Coates PM, Blackman MR, Cragg GM, Levine M, Moss J, White JD, editors. Encyclopedia of dietary supplements. New York: Marcel Dekker; 2005. p. 757-69.

69. Pierpaoli E, Viola V, Pilolli F, Piroddi M, Galli F, Provinciali M. Gamma- and delta-tocotrienols exert a more potent anticancer effect than alpha-tocopheryl succinate on breast cancer cell lines irrespective of HER-2/neu expression. Life Sci. 2010;86(17-18):668-75.

70. Comitato R, Nesaretnam K, Leoni G, Ambra R, Canali R, Bolli A, et al. A novel mechanism of natural vitamin E tocotrienol activity: involvement of ERbeta signal transduction. Am J Physiol Endocrinol Metab. 2009;297(2):E427-37.

71. Assantachai P, Lekhakula S. Epidemiological survey of vitamin deficiencies in older Thai adults: implications for national policy planning. Public Health Nutr. 2007;10(1):65-70.

72. Washi SA, Ageib MB. Poor diet quality and food habits are related to impaired nutritional status in 13- to 18-year-old adolescents in Jeddah. Nutr Res. 2010;30(8):527-34.

73. Leotsinidis M, Alexopoulos A, Schinas V, Kardara M, Kondakis X. Plasma retinol and tocopherol levels in Greek elderly population from an urban and a rural area: associations with the dietary habits. Eur J Epidemiol. 2000;16(11):1009-16.

74. Eggermont E. Recent advances in vitamin E metabolism and deficiency. Eur J Pediatr. 2006;165(7):429-34.

75. CSID:83708. γ-Tocopherol UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.83708.html.

76. CSID:14265. α-Tocopherol UK: Royal Society of Chemistry; [accessed 30/04/2015]. Available at: http://www.chemspider.com/Chemical-Structure.14265.html.

77. Jeanes YM, Hall WL, Ellard S, Lee E, Lodge JK. The absorption of vitamin E is influenced by the amount of fat in a meal and the food matrix. Br J Nutr. 2004;92(04):575-9.

78. Kayden HJ, Traber MG. Absorption, lipoprotein transport, and regulation of plasma concentrations of vitamin E in humans. J Lipid Res. 1993;34:343-.

79. Mardones P, Rigotti A. Cellular mechanisms of vitamin E uptake: relevance in α-tocopherol metabolism and potential implications for disease. J Nutr Biochem. 2004;15(5):252-60.

80. Morrissey PA, Sheehy PJ. Optimal nutrition: vitamin E. Proc Nutr Soc. 1999;58(2):459-68.

81. Greaves RF, Woollard GA, Hoad K, Walmsley TA, Johansson LA, Briscoe S, et al. Laboratory Medicine Best Practice Guideline: Vitamins A, E and the Carotenoids in blood. Clin Biochem Rev. 2014;35(2):85-118.

82. Rohde CM, Manatt M, Clagett-Dame M, DeLuca HF. Vitamin A antagonizes the action of vitamin D in rats. J Nutr. 1999;129(12):2246-50.

83. Johansson S, Melhus H. Vitamin A antagonizes calcium response to vitamin D in man. J Bone Miner Res. 2001;16(10):1899-905.

84. Jenab M, Bueno-de-Mesquita HB, Ferrari P, van Duijnhoven FJ, Norat T, Pischon T, et al. Association between pre-diagnostic circulating vitamin D concentration and risk of colorectal cancer in European populations: a nested case-control study. BMJ. 2010;340:b5500.

85. Mata-Granados JM, Cuenca-Acevedo R, Luque de Castro MD, Sosa M, Quesada-Gomez JM. Vitamin D deficiency and high serum levels of vitamin A increase the risk of osteoporosis evaluated by Quantitative Ultrasound Measurements (QUS) in postmenopausal Spanish women. Clin Biochem. 2010;43(13-14):1064-8.

86. Chai W, Bostick RM, Ahearn TU, Franke AA, Custer LJ, Cooney RV. Effects of vitamin D3 and calcium supplementation on serum levels of tocopherols, retinol, and specific vitamin D metabolites. Nutr Cancer. 2012;64(1):57-64.

87. Goncalves A, Roi S, Nowicki M, Dhaussy A, Huertas A, Amiot M-J, et al. Fat-soluble vitamin intestinal absorption: Absorption sites in the intestine and interactions for absorption. Food Chem. 2015;172:155-60.

88. MacDonald P, Dowd D, Nakajima S, Galligan M, Reeder M, Haussler C, et al. Retinoid X receptors stimulate and 9-cis retinoic acid inhibits 1, 25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. Mol Cell Biol. 1993;13(9):5907-17.

89. Farrell C-JL, Martin S, McWhinney B, Straub I, Williams P, Herrmann M. State-of-the-Art Vitamin D Assays: A Comparison of Automated Immunoassays with Liquid Chromatography–Tandem Mass Spectrometry Methods. Clin Chem. 2012;58(3):531-42.

90. Wallace AM, Gibson S, de la Hunty A, Lamberg-Allardt C, Ashwell M. Measurement of 25-hydroxyvitamin D in the clinical laboratory: current procedures, performance characteristics and limitations. Steroids. 2010;75(7):477-88.

91. Hulshof PJ, Brouwer JT, Burema J, West CE. Bias and random error in retinol measurements of laboratories in countries with populations with mild to severe vitamin A deficiency. Clin Chem. 2002;48(11):2061-3.

92. Olmedilla B, Granado F, Gil-Martinez E, Blanco I, Rojas-Hidalgo E. Reference values for retinol, tocopherol, and main carotenoids in serum of control and insulin-dependent diabetic Spanish subjects. Clin Chem. 1997;43(6 Pt 1):1066-71.

93. Sadowski JA, Hood SJ, Dallal GE, Garry PJ. Phylloquinone in plasma from elderly and young adults: factors influencing its concentration. Am J Clin Nutr. 1989;50(1):100-8.

94. Carter GD. 25-Hydroxyvitamin D: a difficult Analyte. Clin Chem. 2012;58(3):486-8.

95. Cuerq C, Peretti N, Chikh K, Mialon A, Guillaumont M, Drai J, et al. Overview of the in vitro stability of commonly measured vitamins and carotenoids in whole blood. Ann Clin Biochem. 2015;52(Pt 2):259-69.

96. Greaves RF. Vitamin A – Serum Vitamin A Analysis. In: Preedy VR, editor. Vitamin A and Carotenoids: Chemistry, Analysis, Function and Effects: Royal Society of Chemistry; 2012.

97. Clark S, Youngman LD, Chukwurah B, Palmer A, Parish S, Peto R, et al. Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days: implications for epidemiological studies. Int J Epidemiol. 2004;33(3):518-25.

98. Drammeh BS, Schleicher RL, Pfeiffer CM, Jain RB, Zhang M, Nguyen PH. Effects of delayed sample processing and freezing on serum concentrations of selected nutritional indicators. Clin Chem. 2008;54(11):1883-91.

99. Panteghini M, Forest JC. Standardization in laboratory medicine: new challenges. Clin Chim Acta. 2005;355(1-2):1-12.

100. Vesper HW, Thienpont LM. Traceability in laboratory medicine. Clin Chem. 2009;55(6):1067-75.

101. Panteghini M. Traceability as a unique tool to improve standardization in laboratory medicine. Clin Biochem. 2009;42(4-5):236-40.

102. Thomas JB, Duewer DL, Mugenya IO, Phinney KW, Sander LC, Sharpless KE, et al. Preparation and value assignment of standard reference material 968e fat-soluble vitamins, carotenoids, and cholesterol in human serum. Anal Bioanal Chem. 2012;402(2):749-62.

103. JCTLM Database. Reference measurement methods/procedures France: Bureau International des Poids et Mesures; 2013 [accessed 18/03/2013]. Available at: http://www.bipm.org/jctlm/.

104. JCTLM Database. List of higher-order reference materials: SRM 972, Vitamin D in Human Serum France: Bureau International des Poids et Mesures; 2015 [accessed 25/01/2015]. Available at: http://www.bipm.org/jctlm/viewResults.do?type=isRM&searchString=vitamin&searchStringIUPAC=&searchStringMixed=&analyteCategory=&matrixCategory=&sortBy=Analyte_Name&status=0&id=C7RM16R&id=C7RM17R&id=C7RM18R&.x=26&.y=12.

105. JCTLM Database. List of higher-order reference materials:SRM 968e, Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum France: Bureau International des Poids et Mesures; 2015 [accessed 23/09/2015]. Available at: http://www.bipm.org/jctlm/viewResults.do?type=isRM&searchString=retinol&searchStringIUPAC=&searchStringMixed=&analyteCategory=&matrixCategory=&sortBy=Analyte_Name&status=P&id=C10RM41&.x=48&.y=12.

106. Vesper HW, Miller WG, Myers GL. Reference materials and commutability. Clin Biochem Rev. 2007;28(4):139-47.

107. Cattozzo G, Franzini C, Melzi d'Eril GM. Commutability of calibration and control materials for serum lipase. Clin Chem. 2001;47(12):2108-13.

108. Miller WG, Myers GL, Rej R. Why commutability matters. Clin Chem. 2006;52(4):553-4.

109. Rami L, Roura M, Canalias F. Evaluation of commutability of several materials for harmonization alkaline phosphatase catalytic concentration measurements. Clin Chim Acta. 2012;413(15):1249-54.

110. Ricós C, Juvany R, Alvarez V, Jiménez CV, Perich C, Minchinela J, et al. Commutability between stabilized materials and fresh human serum to improve laboratory performance. Clin Chim Acta. 1997;263(2):225-38.

111. Ricos C, Juvany R, Simon M, Hernández A, Alvarez V, Jiménez C, et al. Commutability and traceability: their repercussions on analytical bias and inaccuracy. Clin Chim Acta. 1999;280(1):135-45.

112. Clark P, Kricka LJ, Whitehead TP. Matrix effects in clinical analysis: commutability of control materials between the Ektachem, Beckman and SMA 1260 glucose and urea methods. Clin Chim Acta. 1981;113(3):293-303.

113. Pitt JJ. Principles and applications of liquid chromatography-mass spectrometry in clinical biochemistry. Clin Biochem Rev. 2009;30(1):19-34.

114. Grebe SK, Singh RJ. LC-MS/MS in the Clinical Laboratory - Where to From Here? Clin Biochem Rev. 2011;32(1):5-31.

115. Ardrey RE. Liquid chromatography-mass spectrometry : an introduction. New York: Wiley; J.; 2003. xviii, 276 p. p.

116. Honour JW. Development and validation of a quantitative assay based on tandem mass spectrometry. Ann Clin Biochem. 2011;48(2):97-111.

117. Greaves RF. A guide to harmonisation and standardisation of measurands determined by liquid chromatography - tandem mass spectrometry in routine clinical biochemistry. Clin Biochem Rev. 2012;33(4):123-32.

118. Chace DH, Barr JR, Duncan MW, Matern D, Morris MR, Palmer-Toy DE, et al. Mass Spectrometry in the Clinical Laboratory: General Principles and Guidance; Approved Guideline.: Clinical and Laboratory Standards Institute; 2007. 1-97 p.

119. Policies and procedures: Laboratory waste disposal management strategy procedure Melbourne: RMIT Univesity; 2014 [accessed 22/05/2014]. Available at: http://www.rmit.edu.au/browse/Staff%2FAdministration%2FPolicies%20and%20procedures%2FHealth%20and%20safety%2FHealth%20and%20biosciences%20safety%2FLaboratory%20waste%20disposal%20strategy%20procedure/.

120. Certificate of Analysis, Standard Reference Material 972 for Vitamin D in Human Serum. Gaithersburg, MD, USA: National Institute of Standards and Technology, 2009.

121. Certificate of Analysis, Standard Reference Material 968e for Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum. Gaithersburg, MD, USA: National Institute of Standards and Technology, 2012.

122. Certificate of Analysis, Standard Reference Material 968d for Fat-Soluble Vitamins, Carotenoids, and Cholesterol in Human Serum. Gaithersburg, MD, USA: National Institute of Standards and Technology, 2008.

123. Agilent Technologies. Agilent 1220 Infinity Mobile LC Solution United States November 01, 2013 5991-0529EN [accessed 18/05/2015]. Available at: http://www.chem.agilent.com/Library/datasheets/Public/5991-0529EN.pdf.

124. Agilent Technologies. Agilent 1290 Infinity Thermostatted Column Compartment United States November 01, 2013 5990-6124EN [accessed 18/05/2015]. Available at: http://www.chem.agilent.com/Library/datasheets/Public/5990-6124EN.pdf.

125. Agilent Technologies. Agilent 1290 Infinity Autosampler United States July 01, 2010 5990-6123EN [accessed 18/05/2015]. Available at: http://www.chem.agilent.com/Library/datasheets/Public/5990-6123EN.pdf.

126. Agilent Technologies. LC pumps and vacuum degassers-1290 Infinity Binary Pump United States: Agilent Technologies; 2015 [accessed 18/5/2015]. Available at: http://www.chem.agilent.com/en-US/products-services/Instruments-Systems/Liquid-Chromatography/1290-Infinity-Binary-Pump/Pages/default.aspx.

127. Agilent Technologies. Agilent 6420 Triple Quadrupole LC/MS with Dynamic and Triggered MRM- Data sheet United States September 21, 2014 5990-8336EN [accessed 17/05/2015].

128. Agilent Technologies. Agilent 6490 Triple Quadrupole LC/MS with iFunnel Technology- Data sheet United States September 21, 2014 5990-8336EN [accessed 17/05/2015]. Available at: www.agilent.com/chem/qqq.

129. Agilent Technologies. Agilent 6400 Series Triple Quadrupole LC/MS Systems-Superior sensitivity with proven performance United States February 10, 2014 5990-9758EN [accessed 18/05/2015]. Available at: http://www.alphachrom.hr/upload_data/site_files/5990-9758en_hi.pdf.

130. Luque-Garcia JL, Luque de Castro MD. Extraction of fat-soluble vitamins. J Chromatogr A. 2001;935(1-2):3-11.

131. Babić N. Analytical methods and performance of the immunoassay methods for determination of vitamin d in comparison to mass spectrometry. J Med Biochem. 2012;31(4):333-8.

132. Herrmann M, Harwood T, Gaston-Parry O, Kouzios D, Wong T, Lih A, et al. A new quantitative LC tandem mass spectrometry assay for serum 25-hydroxy vitamin D. Steroids. 2010;75(13):1106-12.

133. Lai JKC, Lucas RM, Banks E, Ponsonby AL, Ausimmune Investigator G. Variability in vitamin D assays impairs clinical assessment of vitamin D status. Intern Med J. 2012;42(1):43-50.

134. Baecher S, Leinenbach A, Wright JA, Pongratz S, Kobold U, Thiele R. Simultaneous quantification of four vitamin D metabolites in human serum using high performance liquid chromatography tandem mass spectrometry for vitamin D profiling. Clin Biochem. 2012;45(16-17):1491-6.

135. Engelman CD, Bo R, Zuelsdorff M, Steltenpohl H, Kirby T, Nieto FJ. Epidemiologic study of the C-3 epimer of 25-hydroxyvitamin D3 in a population-based sample. Clin Nutr. 2014;33(3):421-5.

136. Keevil B. Does the presence of 3-epi-25OHD3 affect the routine measurement of vitamin D using liquid chromatography tandem mass spectrometry? Clin Chem Lab Med. 2012;50(1):181-3.

137. Schleicher RL, Encisco SE, Chaudhary-Webb M, Paliakov E, McCoy LF, Pfeiffer CM. Isotope dilution ultra performance liquid chromatography-tandem mass spectrometry method for simultaneous measurement of 25-hydroxyvitamin D2, 25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 in human serum. Clin Chim Acta. 2011;412(17-18):1594-9.

138. Shah I, James R, Barker J, Petroczi A, Naughton DP. Misleading measures in Vitamin D analysis: a novel LC-MS/MS assay to account for epimers and isobars. Nutr J. 2011;10:46.

139. Singh RJ, Taylor RL, Reddy GS, Grebe SK. C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status. J Clin Endocrinol Metab. 2006;91(8):3055-61.

140. Stepman H, Vanderroost A, Stöckl D, Thienpont LM. Full-scan mass spectral evidence for 3-epi-25-hydroxyvitamin D3 in serum of infants and adults. Clin Chem Lab Med. 2011;49(2):253-6.

141. Van den Ouweland JM, Beijers AM, van Daal H. Fast separation of 25-hydroxyvitamin D3 from 3-epi-25-hydroxyvitamin D3 in human serum by liquid chromatography-tandem mass spectrometry: variable prevalence of 3-epi-25-hydroxyvitamin D3 in infants, children, and adults. Clin Chem. 2011;57(11):1618-9.

142. Greaves R, Jolly L, Woollard G, Hoad K. Serum vitamin A and E analysis: comparison of methods between laboratories enrolled in an external quality assurance programme. Ann Clin Biochem. 2010;47(Pt 1):78-80.

143. Shah I, Petroczi A, Naughton DP. Exploring the Role of Vitamin D in Type 1 Diabetes, Rheumatoid Arthritis, and Alzheimer Disease: New Insights From Accurate Analysis of 10 Forms. J Clin Endocrinol Metab. 2014;99(3):808-16.

144. Van den Ouweland JM, Vogeser M, Bächer S. Vitamin D and metabolites measurement by tandem mass spectrometry. Rev Endocr Metab Disord. 2013:1-26.

145. Clarke MW, Tuckey RC, Gorman S, Holt B, Hart PH. Optimized 25-hydroxyvitamin D analysis using liquid–liquid extraction with 2D separation with LC/MS/MS detection,

provides superior precision compared to conventional assays. Metabolomics. 2013;9(5):1031-40.

146. Shah I, Petroczi A, Naughton DP. Method for simultaneous analysis of eight analogues of vitamin D using liquid chromatography tandem mass spectrometry. Chem Cent J. 2012;6(1):112.

147. Andreoli R, Manini P, Poli D, Bergamaschi E, Mutti A, Niessen WM. Development of a simplified method for the simultaneous determination of retinol, alpha-tocopherol, and beta-carotene in serum by liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization. Anal Bioanal Chem. 2004;378(4):987-94.

148. Priego Capote F, Jimenez JR, Granados JM, de Castro MD. Identification and determination of fat-soluble vitamins and metabolites in human serum by liquid chromatography/triple quadrupole mass spectrometry with multiple reaction monitoring. Rapid Commun Mass Spectrom. 2007;21(11):1745-54.

149. Midttun O, Ueland PM. Determination of vitamins A, D and E in a small volume of human plasma by a high-throughput method based on liquid chromatography/tandem mass spectrometry. Rapid Commun Mass Spectrom. 2011;25(14):1942-8.

150. Chen H, McCoy LF, Schleicher RL, Pfeiffer CM. Measurement of 25-hydroxyvitamin D3 (25OHD3) and 25-hydroxyvitamin D2 (25OHD2) in human serum using liquid chromatography-tandem mass spectrometry and its comparison to a radioimmunoassay method. Clin Chim Acta. 2008;391(1-2):6-12.

151. Van den Ouweland JM, Kema IP. The role of liquid chromatography–tandem mass spectrometry in the clinical laboratory. J Chromatogr B. 2012;883:18-32.

152. Westgard JO, Barry PL. Basic method validation. 3rd ed. Madison, WI: Westgard QC; 2008. 320 p. p.

153. Tholen DW, Kroll M, Astles JR, Caffo AL, Happe T, Krouwer J, et al. Evaluation of the Linearity of Quantitative Measurement Procedures: A Statistical Approach, Approved Guideline: National Committee for Clinical Laboratory Standards; 2003.

154. Zhang SW, Jian W, Sullivan S, Sankaran B, Edom RW, Weng N, et al. Development and validation of an LC–MS/MS based method for quantification of 25 hydroxyvitamin D2 and 25 hydroxyvitamin D3 in human serum and plasma. J Chromatogr B. 2014;961:62-70.

155. El-Khoury JM, Reineks EZ, Wang S. Progress of liquid chromatography-mass spectrometry in measurement of vitamin D metabolites and analogues. Clin Biochem. 2011;44(1):66-76.

156. Duxbury K, Owen L, Gillingwater S, Keevil B. Naturally occurring isotopes of an analyte can interfere with doubly deuterated internal standard measurement. Ann Clin Biochem. 2008;45(2):210-2.

157. Okano T, Mizuno N, Shida S, Takahashi N, Kobayashi T, Kuroda E, et al. A method for simultaneous determination of 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 in human plasma by using two steps of high-performance liquid chromatography. J Nutr Sci Vitaminol. 1980;27(1):43-54.

158. Higashi T, Yamaguchi A, Shimada K. Application of 4-(4-nitrophenyl)-1, 2, 4-triazoline-3, 5-dione to analysis of 25-hydroxyvitamin D3 in human plasma by liquid chromatography/electron capture atmospheric pressure chemical ionization-mass spectrometry. Anal Sci. 2003;19(6):941-3.

159. Wielders JP, Wijnberg FA. Preanalytical stability of 25(OH)-vitamin D3 in human blood or serum at room temperature: solid as a rock. Clin Chem. 2009;55(8):1584-5.

160. Paliakov EM, Crow BS, Bishop MJ, Norton D, George J, Bralley JA. Rapid quantitative determination of fat-soluble vitamins and coenzyme Q-10 in human serum by reversed phase ultra-high pressure liquid chromatography with UV detection. J Chromatogr B. 2009;877(1):89-94.

161. Semeraro A, Altieri I, Patriarca M, Menditto A. Evaluation of uncertainty of measurement from method validation data: An application to the simultaneous determination of retinol and α-tocopherol in human serum by HPLC. J Chromatogr B. 2009;877(11):1209-15.

162. Stepman HC, Vanderroost A, Van Uytfanghe K, Thienpont LM. Candidate reference measurement procedures for serum 25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 by using isotope-dilution liquid chromatography-tandem mass spectrometry. Clin Chem. 2011;57(3):441-8.

163. van den Ouweland JM, Beijers AM, van Daal H. Overestimation of 25-hydroxyvitamin D3 by increased ionisation efficiency of 3-epi-25-hydroxyvitamin D3 in LC-MS/MS methods not separating both metabolites as determined by an LC-MS/MS method for separate quantification of 25-hydroxyvitamin D3, 3-epi-25-hydroxyvitamin D3 and 25-hydroxyvitamin D2 in human serum. J Chromatogr B. 2014;967:195-202.

164. Kobold U. Approaches to measurement of vitamin D concentrations - mass spectrometry. Scand J Clin Lab Invest Suppl. 2012;243:54-9.

165. Bedner M, Phinney KW. Development and comparison of three liquid chromatography–atmospheric pressure chemical ionization/mass spectrometry methods for determining vitamin D metabolites in human serum. J Chromatogr A. 2012;1240:132-9.

166. Agilent Technologies. Ionisation sources and Jet Stream Technology; Course number R1893A. Agilent 6400 Series QQQ LC/MS Techniques and Operation. Volume 1. The USA: Agilent Technologies; 2010. p. 67-122.

167. Wu AH, French D. Implementation of liquid chromatography/mass spectrometry into the clinical laboratory. Clin Chim Acta. 2013;420:4-10.

168. Shimada K, Mitamura K, Higashi T. Determination of Vitamin D3 Metabolites Using High-Performance Liquid Chromatography or Immunoaffinity Chromatography. J Chin Chem Soc. 2000;47(2):285-9.

169. Vogeser M. Quantification of circulating 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry. J Steroid Biochem Mol Biol. 2010;121(3):565-73.

170. Farrell C-J, Herrmann M. Determination of vitamin D and its metabolites. Best Pract Res Clin Endocrinol Metab. 2013;27(5):675-88.

171. Couchman L, Benton CM, Moniz CF. Variability in the analysis of 25-hydroxyvitamin D by liquid chromatography–tandem mass spectrometry: The devil is in the detail. Clin Chim Acta. 2012;413(15):1239-43.

172. Sandhu JK, Auluck J, Ng LL, Jones DJ. Improved analysis of vitamin D metabolites in plasma using liquid chromatography tandem mass spectrometry, and its application to cardiovascular research. Biomed Chromatogr. 2014;28(6):913-7.

173. Bruce SJ, Rochat B, Beguin A, Pesse B, Guessous I, Boulat O, et al. Analysis and quantification of vitamin D metabolites in serum by ultra-performance liquid chromatography coupled to tandem mass spectrometry and high-resolution mass spectrometry - a method comparison and validation. Rapid Commun Mass Spectrom. 2013;27(1):200-6.

174. Clark S, Youngman LD, Palmer A, Parish S, Peto R, Collins R. Stability of plasma analytes after delayed separation of whole blood: implications for epidemiological studies. Int J Epidemiol. 2003;32(1):125-30.

175. Oddoze C, Lombard E, Portugal H. Stability study of 81 analytes in human whole blood, in serum and in plasma. Clin Biochem. 2012;45(6):464-9.

176. Jensen EA, Stahl M, Brandslund I, Grinsted P. Stability of heparin blood samples during transport based on defined pre-analytical quality goals. Clin Chem Lab Med. 2008;46(2):225-34.

177. Brown Thomas J, Duewer DL, Kline MC, Sharpless KE. The stability of retinol, alpha-tocopherol, trans-lycopene, and trans-beta-carotene in liquid-frozen and lyophilized serum. Clin Chim Acta. 1998;276(1):75-87.

178. Talwar D, Ha TK, Cooney J, Brownlee C, St JO'Reilly D. A routine method for the simultaneous measurement of retinol, α-tocopherol and five carotenoids in human plasma by reverse phase HPLC. Clin Chim Acta. 1998;270(2):85-100.

179. Hankinson S, London S, Chute C, Barbieri R, Jones L, Kaplan L, et al. Effect of transport conditions on the stability of biochemical markers in blood. Clin Chem. 1989;35(12):2313-6.

180. Zaman Z, Fielden P, Frost PG. Simultaneous determination of vitamins A and E and carotenoids in plasma by reversed-phase HPLC in elderly and younger subjects. Clin Chem. 1993;39(11):2229-34.

181. Midttun O, Townsend MK, Nygard O, Tworoger SS, Brennan P, Johansson M, et al. Most blood biomarkers related to vitamin status, one-carbon metabolism, and the kynurenine pathway show adequate preanalytical stability and within-person reproducibility to allow assessment of exposure or nutritional status in healthy women and cardiovascular patients. J Nutr. 2014;144(5):784-90.

182. Khan MI, Khan A, Iqbal Z, Ahmad L, Shah Y. Optimization and validation of RP-LC/UV–VIS detection method for simultaneous determination of fat-soluble anti-oxidant

vitamins, all-trans-retinol and α-tocopherol in human serum: effect of experimental parameters. Chromatographia. 2010;71(7):577-86.

183. Su Q, Rowley KG, O'Dea K. Stability of individual carotenoids, retinol and tocopherols in human plasma during exposure to light and after extraction. J Chromatogr B Biomed Sci Appl. 1999;729(1):191-8.

184. Clevidence BA, Ballard-Barbash R. Tocopherol contents of lipoproteins from frozen plasma separated by affinity chromatography. Lipids. 1991;26(9):723-8.

185. Tanner M, Kent N, Smith B, Fletcher S, Lewer M. Stability of common biochemical analytes in serum gel tubes subjected to various storage temperatures and times pre-centrifugation. Ann Clin Biochem. 2008;45(Pt 4):375-9.

186. Lissner D, Mason R, Posen S. Stability of vitamin D metabolites in human blood serum and plasma. Clin Chem. 1981;27(5):773-4.

187. Zwart SR, Wolf M, Rogers A, Rodgers S, Gillman PL, Hitchcox K, et al. Stability of analytes related to clinical chemistry and bone metabolism in blood specimens after delayed processing. Clin Biochem. 2009;42(9):907-10.

188. Colak A, Toprak B, Dogan N, Ustuner F. Effect of sample type, centrifugation and storage conditions on vitamin D concentration. Biochemia medica. 2013;23(3):321-5.

189. Kand'ar R, Zakova P. Determination of 25-hydroxyvitamin D3 in human plasma using HPLC with UV detection based on SPE sample preparation. J Sep Sci. 2009;32(17):2953-7.

190. Lewis JG, Elder PA. Serum 25-OH vitamin D2 and D3 are stable under exaggerated conditions. Clin Chem. 2008;54(11):1931-2.

191. Hayden Y, Pillay T, Marx G, de Lange W, Kuyl JM. Pre-analytical stability of 25 (OH)-vitamin D in primary collection tubes. Clin Chem Lab Med. 2015;53(3):e55-7.

192. Bianchi S, Maffei S, Prontera C, Battaglia D, Vassalle C. Preanalytical, analytical (DiaSorin LIAISON) and clinical variables potentially affecting the 25-OH vitamin D estimation. Clin Biochem. 2012;45(18):1652-7.

193. Kushnir MM, Ray JA, Rockwood AL, Roberts WL, La'ulu SL, Whittington JE, et al. Rapid analysis of 25-hydroxyvitamin D2 and D3 by liquid chromatography-tandem mass spectrometry and association of vitamin D and parathyroid hormone concentrations in healthy adults. Am J Clin Pathol. 2010;134(1):148-56.

194. Agborsangaya C, Toriola AT, Grankvist K, Surcel HM, Holl K, Parkkila S, et al. The effects of storage time and sampling season on the stability of serum 25-hydroxy vitamin D and androstenedione. Nutr Cancer. 2010;62(1):51-7.

195. Mathew G, Zwart SR, Smith SM. Stability of blood analytes after storage in BD SST™ tubes for 12 mo. Clin Biochem. 2009;42(16):1732-4.

196. Antoniucci DM, Black DM, Sellmeyer DE. Serum 25-hydroxyvitamin D is unaffected by multiple freeze-thaw cycles. Clin Chem. 2005;51(1):258-61.

197. Ocké MC, Schrijver J, Obermann-De Boer GL, Bloemberg BPM, Haenen GRMM, Kromhout D. Stability of blood (pro)vitamins during four years of storage at -20 °C: Consequences for epidemiologic research. J Clin Epidemio. 1995;48(8):1077-85.

198. van Eijsden M, van der Wal MF, Hornstra G, Bonsel GJ. Can whole-blood samples be stored over 24 hours without compromising stability of C-reactive protein, retinol, ferritin, folic acid, and fatty acids in epidemiologic research? Clin Chem. 2005;51(1):230-2.

199. Kristal AR, King IB, Albanes D, Pollak MN, Stanzyk FZ, Santella RM, et al. Centralized blood processing for the selenium and vitamin E cancer prevention trial: effects of delayed processing on carotenoids, tocopherols, insulin-like growth factor-I, insulin-like growth factor binding protein 3, steroid hormones, and lymphocyte viability. Cancer Epidemiol Biomarkers Prev. 2005;14(3):727-30.

200. Ihara H, Hashizume N, Matsubayashi T, Futaki K, Yoshida M, Sagawa N, et al. Stability of fat-soluble and water-soluble vitamins in artificially prepared, vitamin-enriched, lyophilized serum. J Clin Lab Anal. 2004;18(4):240-6.

201. Key T, Oakes S, Davey G, Moore J, Edmond LM, McLoone UJ, et al. Stability of vitamins A, C, and E, carotenoids, lipids, and testosterone in whole blood stored at 4 degrees C for 6 and 24 hours before separation of serum and plasma. Cancer Epidemiol Biomarkers Prev. 1996;5(10):811-4.

202. Comstock GW, Norkus EP, Hoffman SC, Xu MW, Helzlsouer KJ. Stability of ascorbic acid, carotenoids, retinol, and tocopherols in plasma stored at -70°C for 4 years. Cancer Epidemiology Biomarkers and Prevention. 1995;4(5):505-7.

203. Gross MD, Prouty CB, Jacobs DR, Jr. Stability of carotenoids and alpha-tocopherol during blood collection and processing procedures. Clin Chem. 1995;41(6 Pt 1):943-4.

204. Edmonds BK, Nierenberg DW. Serum concentrations of retinol, d-alpha-tocopherol and beta-carotene: effects of storage at -70 degrees C for five years. J Chromatogr. 1993;614(1):169-74.

205. Craft NE, Brown ED, Smith J. Effects of storage and handling conditions on concentrations of individual carotenoids, retinol, and tocopherol in plasma. Clin Chem. 1988;34(1):44-8.

206. Thurnham DI, Flora PS. Stability of individual carotenoids, retinol, and tocopherol in stored plasma. Clin Chem. 1988;34(9):1947.

207. Gunter EW, Driskell WJ, Yeager PR. Stability of vitamin E in long-term stored serum. Clin Chim Acta. 1988;175(3):329-35.

208. Peng Y-M, Xu M-J, Alberts DS. Analysis and stability of retinol in plasma. J Natl Cancer Inst. 1987;78(1):95-9.

209. Driskell WJ, Lackey AD, Hewett JS, Bashor MM. Stability of vitamin A in frozen sera. Clin Chem. 1985;31(6):871-2.

210. Mejia L, Arroyave G. Determination of vitamin A in blood. Some practical considerations on the time of collection of the specimens and the stability of the vitamin. Am J Clin Nutr. 1983;37(1):147-51.

211. Ricos C, Alvarez V, Cava F, Garcia-Lario J, Hernandez A, Jimenez C, et al. Current databases on biological variation: pros, cons and progress. Scandinavian journal of clinical & laboratory investigation. 1999;59(7):491-500. [accessed 25/01/2015]. Available at: http://www.westgard.com/biodatabase1.htm.

212. Stockl D, Sluss PM, Thienpont LM. Specifications for trueness and precision of a reference measurement system for serum/plasma 25-hydroxyvitamin D analysis. Clin Chim Acta. 2009;408(1-2):8-13.

213. The GraphPad Prism version 6 (GraphPad Prism Software Inc., CA, USA), www.graphpad.com).

214. Monaco HL. The Transthyretin—Retinol-Binding Protein Complex. Recent Advances in Transthyretin Evolution, Structure and Biological Functions: Springer; 2009. p. 123-42.

215. Greaves RF, Hoad KE, Woollard GA, Walmsley TA, Briscoe S, Johnson LA, et al. External quality assurance target setting with NIST SRM 968d material: performance in the 2010 Royal College of Pathologists of Australasia Quality Assurance Program with retinol, α-tocopherol and ß-carotene. Ann Clin Biochem. 2011;48(Pt 5):480-2.

216. Owen L, MacDonald P, Keevil B. Is calibration the cause of variation in liquid chromatography tandem mass spectrometry testosterone measurement? Ann Clin Biochem. 2013;50(Pt 4):368-70.

217. Saibaba K, Rao PS, Ramana G, Kumar E, Tripathi R. Analytical bias due to calibrator matrix effects. Indian J Clin Biochem. 1995;10(2):112-5.

218. Lo S, Jendrzejczak B, Doumas BT. Bovine Serum–Based Bilirubin Calibrators Are Inappropriate for Some Diazo Methods. Clin Chem. 2010;56(5):869-72.

219. Carter GD, Jones JC. Use of a common standard improves the performance of liquid chromatography-tandem mass spectrometry methods for serum 25-hydroxyvitamin-D. Ann Clin Biochem. 2009;46(1):79-81.

220. Yates AM, Bowron A, Calton L, Heynes J, Field H, Rainbow S, et al. Interlaboratory variation in 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 is significantly improved if common calibration material is used. Clin Chem. 2008;54(12):2082-4.

221. Lippi G, Cascio CL, Ruzzenente O, Brentegani C, Guidi G. Significant reduction of the bias among commercial immunoassays for lipoprotein (a) after use of a uniform calibrator. Clin Chim Acta. 1996;256(2):125-34.

222. White GH. Basics of estimating measurement uncertainty. Clin Biochem Rev. 2008;29 Suppl 1:S53-60.

223. RCPA Quality Assurance Programs. Chemical Pathology 2013. RCPAQAP; 2013 [accessed 18/09/2013]. Available at: http://www.rcpaqap.com.au/chempath/.

224. Gallaher MP, Mobley LR, Klee GG, Schryver P. The impact of calibration error in medical decision making. Washington: National Institute of Standards and Technology, 2004.

225. Panteghini M. Traceability, reference systems and result comparability. Clin Biochem Rev. 2007;28(3):97-104.

226. Roth HJ, Schmidt-Gayk H, Weber H, Niederau C. Accuracy and clinical implications of seven 25-hydroxyvitamin D methods compared with liquid chromatography–tandem mass spectrometry as a reference. Ann Clin Biochem. 2008;45(2):153-9.

227. Horst RL. Exogenous versus endogenous recovery of 25-hydroxyvitamins D2 and D3 in human samples using high-performance liquid chromatography and the DiaSorin LIAISON Total-D Assay. J Steroid Biochem Mol Biol. 2010;121(1):180-2.

228. JCTLM Database. List of higher-order reference materials France: Bureau International des Poids et Mesures; 2013 [accessed 18/03/2013]. Available at: http://www.bipm.org.

229. Albahrani AA, Rotarou V, Roche PJ, Greaves RF. Comparison of three commercial calibrators for alpha-tocopherol using liquid chromatography–tandem mass spectrometry. Clin Biochem. 2013;46(18):1884-8.

230. Brazionis L, Walker KZ, Itsiopoulos C, O'Dea K. Plasma retinol: a novel marker for cardiovascular disease mortality in Australian adults. Nutr Metab Cardiovasc Dis. 2012;22(10):914-20.

231. Grober U, Spitz J, Reichrath J, Kisters K, Holick MF. Vitamin D: Update 2013: From rickets prophylaxis to general preventive healthcare. Dermatoendocrinol. 2013;5(3):331-47.

232. Lucas RM, Ponsonby AL, Pasco JA, Morley R. Future health implications of prenatal and early-life vitamin D status. Nutrition reviews. 2008;66(12):710-20.

233. Darlow BA, Graham PJ. Vitamin A supplementation to prevent mortality and short- and long-term morbidity in very low birthweight infants. The Cochrane database of systematic reviews. 2011(10):Cd000501.

234. Zakariaeeabkoo R, Allen KJ, Koplin JJ, Vuillermin P, Greaves RF. Are vitamins A and D important in the development of food allergy and how are they best measured? Clin Biochem. 2014;47(9):804-11.

235. Fares S, Sethom MM, Khouaja-Mokrani C, Jabnoun S, Feki M, Kaabachi N. Vitamin A, E, and D Deficiencies in Tunisian Very Low Birth Weight Neonates: Prevalence and Risk Factors. Pediatr Neonatol. 2014;55(3):196-201.

236. Zoratti EM, Wegienka G. Vitamin D concentration in newborn cord blood is correlated with maternal intake of supplemental vitamin D, and lower levels are associated with increased risk of the infant developing eczema. Evid Based Nurs. 2013;16(4):105-6.

237. Walker VP, Zhang X, Rastegar I, Liu PT, Hollis BW, Adams JS, et al. Cord blood vitamin D status impacts innate immune responses. J Clin Endocrinol Metab. 2011;96(6):1835-43.

238. Camargo CA, Ingham T, Wickens K, Thadhani R, Silvers KM, Epton MJ, et al. Cord-blood 25-hydroxyvitamin D levels and risk of respiratory infection, wheezing, and asthma. Pediatrics. 2011;127(1):e180-e7.

239. Jones AP, Palmer D, Zhang G, Prescott SL. Cord blood 25-hydroxyvitamin D3 and allergic disease during infancy. Pediatrics. 2012;130(5):e1128-e35.

240. Belderbos ME, Houben ML, Wilbrink B, Lentjes E, Bloemen EM, Kimpen JL, et al. Cord blood vitamin D deficiency is associated with respiratory syncytial virus bronchiolitis. Pediatrics. 2011;127(6):e1513-e20.

241. Weisse K, Winkler S, Hirche F, Herberth G, Hinz D, Bauer M, et al. Maternal and newborn vitamin D status and its impact on food allergy development in the German LINA cohort study. Allergy. 2013;68(2):220-8.

242. Gilbert T, Merlet-Bénichou C. Retinoids and nephron mass control. Pediatr Nephrol. 2000;14(12):1137-44.

243. El-Khashab EK, Hamdy AM, Maher KM, Fouad MA, Abbas GZ. Effect of maternal vitamin A deficiency during pregnancy on neonatal kidney size. J Perinat Med. 2013;41(2):199-203.

244. Gazala E, Sarov B, Hershkovitz E, Edvardson S, Sklan D, Katz M, et al. Retinol concentration in maternal and cord serum: its relation to birth weight in healthy mother–infant pairs. Early Hum Dev. 2003;71(1):19-28.

245. Harris DT, Badowski M, Ahmad N, Gaballa MA. The potential of cord blood stem cells for use in regenerative medicine. Expert opinion on biological therapy. 2007;7(9):1311-22.

246. XLSTAT software (version 2014.1.07; Addinsoft SARL, www.xlstat.com).

247. Sapan CV, Lundblad RL. Considerations regarding the use of blood samples in the proteomic identification of biomarkers for cancer diagnosis. Cancer Genomics Proteomics. 2006;3(3-4):227-30.

248. Olmedilla B, Granado F, Blanco I, Rojas-Hidalgo E. Seasonal and sex-related variations in six serum carotenoids, retinol, and alpha-tocopherol. Am J Clin Nutr. 1994;60(1):106-10.

249. Talwar DK, Azharuddin MK, Williamson C, Teoh YP, McMillan DC, O'Reilly DSJ. Biological variation of vitamins in blood of healthy individuals. Clin Chem. 2005;51(11):2145-50.

250. Kiely M, Cogan PF, Kearney PJ, Morrissey PA. Concentrations of tocopherols and carotenoids in maternal and cord blood plasma. Eur J Clin Nutr. 1999;53(9):711-5.

251. Albarhani AA, Collier F, Greaves RF, Ponsonby A-L, Allen KJ, Vuillermin PJ, et al. Vitamin D and A can be successfully measured by LC-MS/MS in cord blood diluted plasma. Clin Biochem. 2015; Doi: 10.1016/j.clinbiochem.2015.04.014. [Epub ahead of print].

252. Greene-Finestone L, Berger C, de, De Groh M, Hanley D, Hidiroglou N, Sarafin K, et al. 25-Hydroxyvitamin D in Canadian adults: biological, environmental, and behavioral correlates. Osteoporos Int. 2011;22(5):1389-99.

253. Hyppönen E, Power C. Hypovitaminosis D in British adults at age 45 y: nationwide cohort study of dietary and lifestyle predictors. Am J Clin Nutr. 2007;85(3):860-8.

254. Rockell J, Skeaff C, Williams S, Green T. Serum 25-hydroxyvitamin D concentrations of New Zealanders aged 15 years and older. Osteoporos Int. 2006;17(9):1382-9.

255. Daly RM, Gagnon C, Lu ZX, Magliano DJ, Dunstan DW, Sikaris KA, et al. Prevalence of vitamin D deficiency and its determinants in Australian adults aged 25 years and older: a national, population-based study. Clin Endocrinol (Oxf). 2012;77(1):26-35.

256. Raizman JE, Cohen AH, Teodoro-Morrison T, Wan B, Khun-Chen M, Wilkenson C, et al. Pediatric reference value distributions for vitamins A and E in the CALIPER cohort and establishment of age-stratified reference intervals. Clin Biochem. 2014;47(9): 812–5.

257. Johnson-Davis KL, Moore SJ, Owen WE, Cutler JM, Frank EL. A rapid HPLC method used to establish pediatric reference intervals for vitamins A and E. Clin Chim Acta. 2009;405(1-2):35-8.

258. Holick MF, Binkley NC, Bischoff-Ferrari HA, Gordon CM, Hanley DA, Heaney RP, et al. Evaluation, treatment, and prevention of vitamin D deficiency: an Endocrine Society clinical practice guideline. J Clin Endocrinol Metab. 2011;96(7):1911-30.

259. Panel on dietary antioxidants and related Compounds IOM. Vitamin E. Dietary Reference Intakes for Vitamin C, Vitamin E, Selenium, and Carotenoids: National Academies Press; 2000. p. 186-283.

260. IBM Corp Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

261. Horowitz GL. Defining, establishing, and verifying reference intervals in the clinical laboratory: approved guideline: Clinical and Laboratory Standards Institute; 2008.

262. MedCalc Statistical Software version 13.1.0 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2014).

263. Pallant J. SPSS survival manual: A step by step guide to data analysis using SPSS. 4 ed: McGraw-Hill International; 2010.

264. Nowson CA, McGrath JJ, Ebeling PR, Haikerwal A, Daly RM, Sanders KM, et al. Vitamin D and health in adults in Australia and New Zealand: a position statement. Med J Aust. 2012;196(11):686-7.

265. Woodside J, Young I, Gilchrist S, Vioque J, Chakravarthy U, de Jong P, et al. Factors associated with serum/plasma concentrations of vitamins A, C, E and carotenoids in older people throughout Europe: the EUREYE study. Eur J Nutr. 2013;52(5):1493-501.

266. Ross AC, Manson JE, Abrams SA, Aloia JF, Brannon PM, Clinton SK, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab. 2011;96(1):53-8.

267. Ross AC, Taylor CL, Yaktine AL, Del Valle HB. Dietary reference intakes for calcium and vitamin D: National Academies Press; 2011.

268. Van der Mei IA, Ponsonby A-L, Engelsen O, Pasco JA, McGrath JJ, Eyles DW, et al. The high prevalence of vitamin D insufficiency across Australian populations is only partly explained by season and latitude. Environ Health Perspect. 2007;115(8):1132.

269. Langlois K, Greene-Finestone L, Little J, Hidiroglou N, Whiting S. Vitamin D status of Canadians as measured in the 2007 to 2009 Canadian Health Measures Survey. Health Rep. 2010;21(1):47-55.

270. Bailey D, Veljkovic K, Yazdanpanah M, Adeli K. Analytical measurement and clinical relevance of vitamin D3 C3-epimer. Clin Biochem. 2013;46(3):190-6.

271. Karras SN, Shah I, Petroczi A, Goulis DG, Bili H, Papadopoulou F, et al. An observational study reveals that neonatal vitamin D is primarily determined by maternal contributions: implications of a new assay on the roles of vitamin D forms. Nutr J. 2013;12(1):77.

272. Shah I, Petroczi A, Naughton DP. Exploring the Role of Vitamin D in Type 1 Diabetes, Rheumatoid Arthritis and Alzheimer's Disease: New Insights from Accurate Analysis of Ten Forms. J Clin Endocrinol Metab. 2014:jc20132872.

273. Nowson CA, Margerison C. Vitamin D intake and vitamin D status of Australians. Med J Aust. 2002;177(3):149-52.

274. Basu TK, Donaldson D. Intestinal absorption in health and disease: micronutrients. Best Pract Res Clin Gastroenterol. 2003;17(6):957-79.

275. Newsome P, Beldon I, Moussa Y, Delahooke T, Poulopoulos G, Hayes P, et al. Low serum retinol levels are associated with hepatocellular carcinoma in patients with chronic liver disease. Aliment Pharmacol Ther. 2000;14(10):1295-301.

276. Davey GK, Spencer EA, Appleby PN, Allen NE, Knox KH, Key TJ. EPIC–Oxford: lifestyle characteristics and nutrient intakes in a cohort of 33 883 meat-eaters and 31 546 non meat-eaters in the UK. Public Health Nutr. 2003;6(03):259-68.

277. Lockitch G, Halstead A, Wadsworth L, Quigley G, Reston L, Jacobson B. Age-and sex-specific pediatric reference intervals and correlations for zinc, copper, selenium, iron, vitamins A and E, and related proteins. Clin Chem. 1988;34(8):1625-8.

278. Olmedilla B, Granado F, Southon S, Wright AJ, Blanco I, Gil-Martinez E, et al. Serum concentrations of carotenoids and vitamins A, E, and C in control subjects from five European countries. Br J Nutr. 2001;85(02):227-38.

279. Winklhofer-Roob BM, van't Hof MA, Shmerling DH. Reference values for plasma concentrations of vitamin E and A and carotenoids in a Swiss population from infancy to adulthood, adjusted for seasonal influences. Clin Chem. 1997;43(1):146-53.

280. Lindblad BS, Patel M, Hamadeh M, Helmy N, Ahmad I, Dawodu A, et al. Age and sex are important factors in determining normal retinol levels. J Trop Pediatr. 1998;44(2):96-9.

281. Van Dokkum W, De Vos RH, Schrijver J. Retinol, total carotenoids,. beta.-carotene, and tocopherols in total diets of male adolescents in the Netherlands. J Agric Food Chem. 1990;38(1):211-6.

282. Nana CP, Brouwer ID, Zagre NM, Kok FJ, Traore AS. Community assessment of availability, consumption, and cultural acceptability of food sources of (pro)vitamin A: toward the development of a dietary intervention among preschool children in rural Burkina Faso. Food Nutr Bull. 2005;26(4):356-65.

283. Mansour A, Ahadi Z, Qorbani M, Hosseini S. Association between dietary intake and seasonal variations in postmenopausal women. J Diabetes Metab Disord. 2014;13(1):52.

284. Ziegler R, Wilcox H, Mason T, Bill J, Virgo P. Seasonal variation in intake of carotenoids and vegetables and fruits among white men in New Jersey. Am J Clin Nutr. 1987;45(1):107-14.

285. Turrell G, Hewitt B, Patterson C, Oldenburg B, Gould T. Socioeconomic differences in food purchasing behaviour and suggested implications for diet-related health promotion. J Hum Nutr Diet. 2002;15(5):355-64.

286. Mishra G, McNaughton S, Ball K, Brown W, Giles G, Dobson A. Major dietary patterns of young and middle aged women: results from a prospective Australian cohort study. Eur J Clin Nutr. 2010;64(10):1125-33.

287. Victoria Ancestry Australia: .id the population experts; 2014 [accessed 30/07/2014]. Available at: http://profile.id.com.au/australia/ancestry?WebID=110.

288. Diversity in Queensland (Census 2011) Australia: the Queensland Government Statistician's Office (QGSO); 2012 [accessed 30/07/2014]. Available at: http://www.qgso.qld.gov.au/products/bulletins/diversity-qld-c11/diversity-qld-c11.pdf.

289. Victoria's Diverse population 2011 census Australia: Victorian Multicultural Commission; 2013 [accessed 30/07/2014]. Available at: http://www.multicultural.vic.gov.au/images/stories/documents/2013/pop_diversity_vic_brochure_2013_web.pdf.

290. Statistics on Australia's current Refugee and Humanitarian Program Australia: The Refugee Council of Australia (RCOA) 2014 [accessed 30/06/2014]. Available at: http://www.refugeecouncil.org.au/r/stat-rhp.php

291. Armstrong NC, Paganga G, Brunner E, Miller NJ, Nanchahal K, Shipleyb M, et al. Reference values for α-tocopherol and β-carotene in the Whitehall II study. Free Radic Res. 1997;27(2):207-19.

292. Breidenassel C, Valtuena J, Gonzalez-Gross M, Benser J, Spinneker A, Moreno LA, et al. Antioxidant vitamin status (A, E, C, and beta-carotene) in European adolescents-the HELENA Study. Int J Vitam Nutr Res. 2011;81(4):245-55.

293. Rohde CM, DeLuca HF. All-trans retinoic acid antagonizes the action of calciferol and its active metabolite, 1, 25-dihydroxycholecalciferol, in rats. J Nutr. 2005;135(7):1647-52.

294. Schelling GT, Roeder RA, Garber MJ, Pumfrey WM. Bioavailability and interaction of vitamin A and vitamin E in ruminants. J Nutr. 1995;125(6 Suppl):1799S-803S.

295. Sklan D, Donoghue S. Vitamin E response to high dietary vitamin A in the chick. J Nutr. 1982;112(4):759-65.

296. Lensmeyer G, Poquette M, Wiebe D, Binkley N. The C-3 epimer of 25-hydroxyvitamin D3 is present in adult serum. J Clin Endocrinol Metab. 2012;97(1):163-8.

297. Cooney RV, Franke AA, Wilkens LR, Gill J, Kolonel LN. Elevated plasma gamma-tocopherol and decreased alpha-tocopherol in men are associated with inflammatory markers and decreased plasma 25-OH vitamin D. Nutr Cancer. 2008;60 Suppl 1:21-9.

298. Bieri JG. Effect of excessive vitamins C and E on vitamin A status. Am J Clin Nutr. 1973;26(4):382-3.

299. Armbruster D, Miller RR. The Joint Committee for Traceability in Laboratory Medicine (JCTLM): a global approach to promote the standardisation of clinical laboratory test results. Clin Biochem Rev. 2007;28(3):105.