Development and Application of LC-MS/MS Methodology to Characterise Plasma Fat-Soluble Vitamer Profiles in Australian Children and Adults

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Abstract

Vitamers are organic substances generally classified as either fat-soluble or water-soluble. Fat-soluble vitamers (FSV; include A, D, E and K) are biochemically diverse micronutrients with fundamentally different biological activities. The biochemical, medical and nutritional value of FSV are well documented. FSV play integral roles in a number of physiological processes that include immune function, bone health, vision, and coagulation. Suboptimal FSV concentrations are a significant risk factor for cardiometabolic complications, type II diabetes mellitus, cancer, and several immune system disorders, amongst others. There is increasing evidence that vitamer-specific interactions (*e.g.*, between A, D and K group vitamers) may result in deficiencies, or hypervitaminosis, which can affect their bioavailability and metabolism.

FSV concentrations in biological fluids (*e.g.*, plasma and serum) represent the intersection of exogenous and endogenous factors and can help predict future health and disease outcomes. Over the past two decades, there has been a significant increase in translational clinical research and laboratory requests for measurements of FSV concentrations. Recent advances in analytical chemistry, including sophisticated and high-accuracy analytical platforms (*e.g.*, orbitrap mass analyser, ultra-high performance liquid chromatography UHPLC), analytical column technology (*e.g.*, sub-2 µm particle columns), and liquid handling automation techniques have enabled the high throughput identification, detection, and quantification of FSV in biological matrixes.

In this thesis, I have developed and validated an automated robotic LC-MS/MS method for the multiplexed quantitation of 11 plasma FSV including all four major FSV groups (*i.e.*, A group vitamers; retinol, retinoic acid and retinyl palmitate, D group vitamers 25 hydroxyvitamin D3 [25-OH-D3] and 1-α-25-dihydroxy-D3 [1-α-25-(OH)2-D3, E group vitamers α-tocopherol, γ-tocopherol and α- tocotrienol and K group vitamers phylloquinone [K1], Menatetrenone [MK-4], and menaquinone-7 [MK-7]). This method quantifies these vitamers at their respective

physiological concentrations and at levels that indicate clinical deficiencies (*e.g.*, α-tocopherol).

The method I developed was used to characterise FSV profiles in plasma samples from the CheckPoint study of the Longitudinal Study of Australian Children's (LSAC). I observed a strong vitamer-specific parent-child concordance for all FSV tested. My results also highlight that age, BMI, and sex are significant contributors to FSV concentrations in the family setting. Other exogenous factors: physical activity, supplement intake, healthy food consumption, sun exposure and sunscreen use and endogenous factors; volumetric body fat distribution, the bioavailability of the vitamers, differential inter-conversions between metabolically active vitamers, hereditary contributions and inflammatory conditions also appear to affect the epidemiological distributions of FSV concentrations. In conclusion, family, age, sex, and BMI are important parameters characterising FSV plasma levels in a population setting. The associated endogenous and exogenous factors, however, need to be further validated in future studies to fully elucidate how the FSV profile changes in the epidemiological context and to predict the future health and disease outcomes of an individual.

This thesis is dedicated to

Manel Kariyawasam, Chandrapala Arachchige,

Mothila Arachchige and Nirodhi Rajapakse

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PhD taught me a great life lesson, and that is to use failures and rejections as fuel to keep going.

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"Scientists have become the bearers of the torch of discovery in our quest for knowledge."

-Stephen Hawking

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Glossary

ACN	Acetonitrile
APCI	Atmospheric Pressure Chemical Ionisation
BHT	Butylated hydroxytoluene
BMI	Body mass index
BSA	bovine serum albumin
CE	Collusion energy
EDTA	Ethylene diamine tetraacetic acid
ESI	Electrospray ionisation
FSV	Fat-Soluble Vitamers
HPLC	High pressure liquid chromatography
IDL	Instrument detection limit
IPA	2-propanol
IQL	Instrument quantitation limit
IS	Internal standards
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LSAC	Longitudinal study of Australian children
MK	Menaquinone

MP	Mobile Phase
MS/MS	Tandem mass spectrometry
PBS	phosphate-buffered saline
PPT	Phospholipid removal plates
PRM	Parallel reaction monitoring
QC	Quality control
SLE	Supported liquid extraction
SP	Stationary Phase
SPE	Solid phase extraction
UHPLC	Ultra-high-pressure liquid chromatography
UV	Ultraviolet

Co-Authorship Forms

Chapter 1

General Introduction

1.1 Overview of Fat-Soluble Vitamers

Sections 1.1 was modified from G.R.P. Arachchige, E.B. Thorstensen, M. Coe, E.J. McKenzie, J.M. O'Sullivan, C.J. Pook, LC-MS/MS quantification of fat-soluble vitamers – A systematic review, Anal. Biochem. 613 (2021). https://doi.org/10.1016/j.ab.2020.113980 [1].

An accessory food substance named "Fat-soluble A," which was initially believed to be one vitamin, was first discovered in 1913 by McCollum and Davis [2]. Subsequent advances in the field lead to the discovery of other groups of vitamins [2]. Vitamins can be divided into two groups according to their solubility: Water Soluble Vitamins (C, B (B1, B2, B3, B5, B6, B7, B9, and B12) and choline) [3]; and Fat-Soluble Vitamins (A, D, E, and K) [4–6]. Every vitamin is a family of related molecules with similar molecular structures. The members of a vitamin family are termed as vitamers [7]. The related forms of Fat-Soluble Vitamers (FSV) are A group vitamers: retinol, retinoic acid, retinyl palmitate, retinyl esters, and retinal, D group vitamers 25-Hydroxy Vitamin D_2[25-OH-D_2], 25-Hydroxy Vitamin D_3 [25-OH-D_3], 1 alpha, 25-dihydroxyvitamin D_3 [1α-25$(OH)_2D_3$]), E group vitamers α, β, γ, δ tocopherols and α, β, γ, δ tocotrienols and K group vitamers Phylloquinone [K1], and Menaquinone 4-14 [MK-4 to MK-14] [6,8].

FSV and their metabolites have vital roles in key physiological functions of the human body. For example, vitamer A impacts the development and function of vision [9], D group vitamers are important in bone and cardiac health [10,11], vitamer E is important in reducing oxidative stress [12,13], and vitamer K in blood clotting and cardiac health [14]. Inadequate or excess dietary intake of FSV is a significant risk factor for lifestyle-related diseases. Considering the roles FSV play in human health and disease, there has been an increasing demand for an accurate and sensitive analytical method for quantifying these vitamers. Unfortunately, currently available methods (*i.e.*, immunoassays, gas chromatography, and even liquid-chromatography mass spectrometry (LC-MS)) are limited to the range of fat-soluble vitamers quantified, subject to non-target analyte interferences, time-consuming, and lack sensitivity [15]. Arguably, improvements in the sensitivity and selectivity of LC-MS/MS technology are the key to developing a multiplexed bioanalytical panel to quantify a wide range of FSV in plasma or serum [16]. The terminology associated with mass spectrometry techniques is complex, and Table 1-1 contains a list of the key terms that will be used throughout this thesis.

Table 1-1: List of LC-MS Terms and Definitions

Term	Definition
Deproteinization	Disruption of the tertiary structure of dissolved proteins, causing them to precipitate.
Liquid-Liquid Extraction (LLE)	Addition of a non-miscible solvent to a sample to separate target compounds and matrix constituents into different partitions.
Antioxidants	A compound added to Prevent oxidation of FSV during storage, extraction, and analysis.
Matrix effect	Interference with chromatography, ionisation, and detection of targets by compounds co-extracted from the sample (*e.g.*, small proteins, phospholipids).
Stationary Phase (SP)	A particulate or polymer substrate is often enhanced with a sorbent coating that selectively binds target compounds.
Mobile Phase (MP)	A liquid that flows through the SP and carries the analytes of interest with it.
Solid Phase Extraction (SPE)	Selective extraction and concentration of target compounds from complex matrices by the passage of liquid samples through a bed of SP.
Liquid Chromatography (LC)	Separation of individual compounds from a liquid mixture via analyte interactions with the SP and liquid MP.
Isocratic Elution	The mobile phase is held at a constant composition throughout the chromatographic run.
Gradient Elution	The mobile phase composition changes throughout the chromatographic run.
Mass Spectrometry (MS)	Quantification of ions separated by their mass-to-charge ratio using electromagnetic fields.
Electrospray Ionisation (ESI)	A high voltage applied to an aerosol generated from the nebulization of a solvent flow causes dissolved target compounds to ionize.
Atmospheric Pressure Chemical Ionisation (APCI)	APCI is similar to ESI, but target compounds in the aerosol are ionized using a corona discharge instead of high voltage.
External Standards	A set of calibration standards made up of known target compounds that are run before and after analysis of the samples to enable quantitation of unknowns.
Internal Standards	Addition of a substance with similar properties to the target compounds but otherwise unique to the analysis to improve the accuracy of the quantitation.
Limit of Quantification (LoQ)	The level at which an analytical method detects and quantifies an analyte signal from the noise with an acceptable level of trueness, repeatability, and precision.
Traceability	The ability to continuously compare or relate the result parameters of a measurement to a national or International standard reference materials.

In this introduction, I will: introduce FSV and associated health outcomes, systematically review the strengths and weaknesses of the currently available LC-MS/MS methods in FSV quantification and discuss the importance of large cohort studies in understanding the global prevalence of FSV.

1.1.1 A Group Vitamers

A group vitamers (Figure 1-1: vitamin A are vital to a number of biological functions, including immunity, maintenance of cells, vision, epithelial cell integrity, growth, reproduction, red cell production, and pulmonary function [17,18]. There are two main forms of vitamer A; retinoids (*e.g.*, retinol) and provitamin A (carotenoids) [17]. Retinoids are predominately acquired from dietary sources such as milk, liver, eggs, and cheese, whereas carotenoids are common in fruits and vegetables, including ripe mangos and carrots [19]. Approximately half of the provitamin A (carotenoids) gets converted into retinol in the intestine,[20] and ~70 - 90% of the retinol gets absorbed into the body through the intestine. Of the unmetabolized fraction (β-carotene), only 3% gets absorbed into the body [21]. The absorption of retinol into enterocytes is initiated when the retinol is emulsified by bile acids and incorporated into micelles. Within enterocytes, the retinol is esterified and packed into ultra-low-density lipoproteins (ULDL), also known as chylomicrons. These chylomicrons are then transported to the liver via the lymphatic system, where the retinol is further metabolized into other forms of retinoids. When the body needs A group vitamers, retinyl esters in the liver are hydrolysed and transported into the target tissues as retinol, bound to plasma retinol-binding protein (RBP) [22,23].

Deficiencies and toxicity associated with A group vitamers

Vitamer A deficiency is recognized by the World Health Organization (WHO) as one of the three (iodine, iron, and vitamer A) main nutrient deficiencies [9]. Retinol is the active form of the A group vitamers [21] and is present at between 300-720 ng/mL [24] within the circulatory system. Vitamer A deficiency is defined as plasma or serum retinol levels <196 ng/mL in children and adults and severe deficiency at <98 ng/mL[25]. Most of these vitamer A deficiency symptoms are treatable; however, once corneal scarring occurs, the damage becomes untreatable and can result in permanent blindness [26].

Excessive vitamer A concentrations are associated with many toxicities [27]. Plasma retinol concentrations >0.95 µg/mL are considered toxic [28,29]. However, there is no consensus on what concentration constitutes an excess of plasma retinol [27]. The most common clinical symptoms of vitamer A toxicity include photophobia, pseudotumor cerebri, ataxia, alopecia, conjunctivitis, muscle and bone pain, hepatotoxicity, hyperlipidemia, and cheilitis [22,30,31].

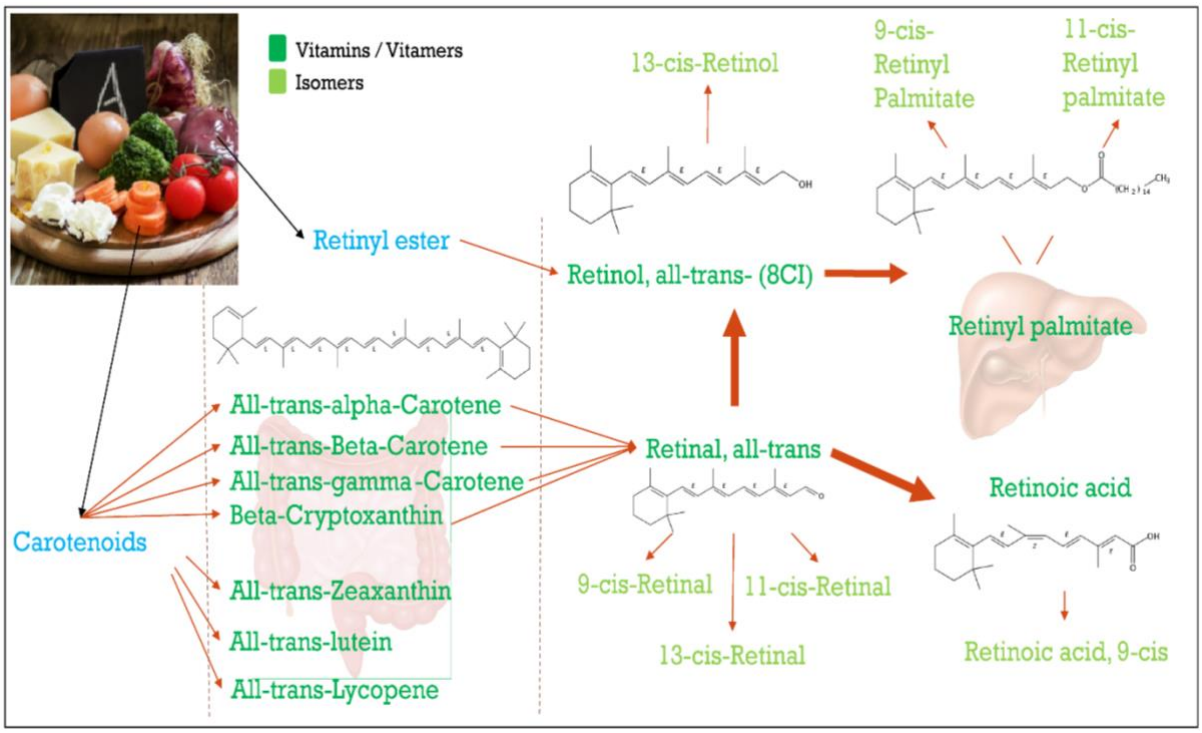

Figure 1-1: vitamin A vitamer

1.1.2 D Group vitamers

Vitamer D is a prohormone that belongs to the family of secosteroid homologs [22]. The most important forms of vitamer D in human nutrition are vitamin D2 (ergocalciferol) and vitamin D3 (cholecalciferol) (Figure 1-2). Approximately 80% of the daily requirement for vitamer D (*i.e.*, D_3) is produced in the skin via conversation of 7-dihydro cholesterol by ultraviolet light from the sun [32]. Dietary sources, including fatty fish, liver oils, and fortified foods, provide the remaining 20% (*i.e.*, D_2) [22]. Vitamer D is absorbed by the skin, bound to Vitamin D binding protein (DBP) in the circulation, and transported to the liver. The dietary vitamer D is emulsified by bile acids and formed into micelles within the enterocytes in the duodenum and

distal small intestine. Upon absorption through the small intestine, DBP is subsequently transferred to the liver through the lymphatic system [33]. Vitamer D is hydrolysed into 25-OH-D_2 / 25-OH-D_3 in the liver and to its active form 1α- 25-$(OH)_2$-D_2 / 1α- 25-$(OH)_2$-D_3 in the kidneys [10,34]. The biological importance of vitamer D in bone metabolism, immune function, type 1 diabetes, autoimmune disease, cancer treatment and prevention, and heart disease has been reviewed in [10,11].

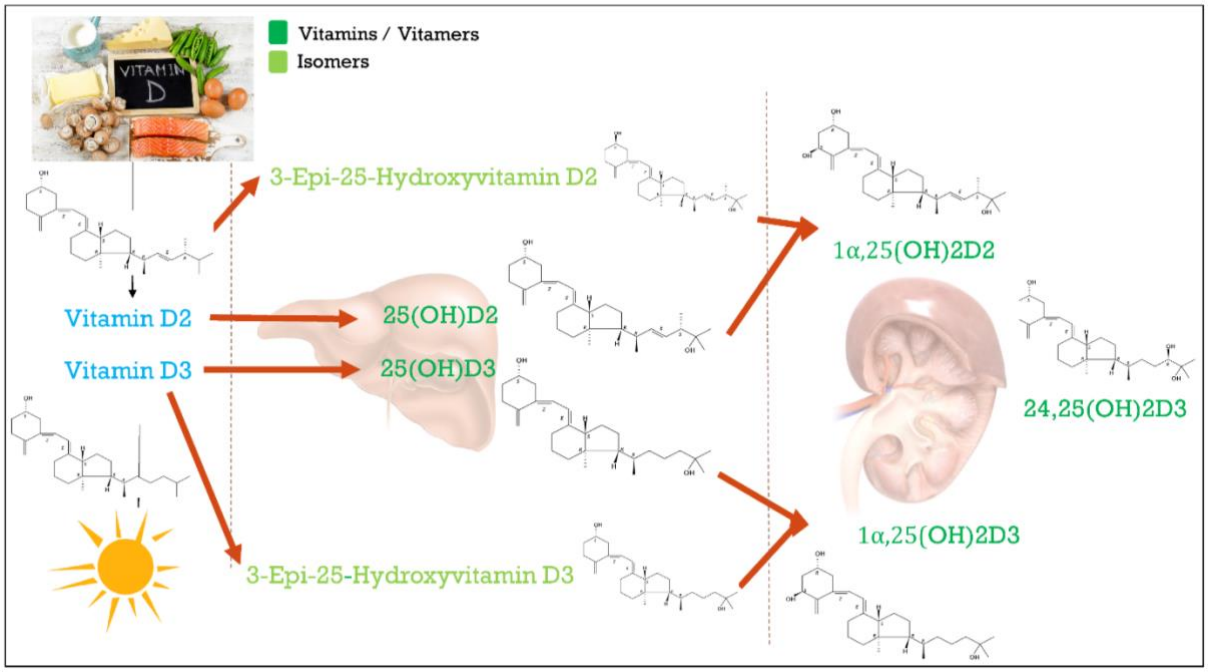

Figure 1-2: vitamin D vitamers.

Deficiencies and toxicity associated with D group vitamers

In clinical settings, vitamer D status is commonly expressed as the circulatory concentration of 25-OH-D_3. The physiological concentration of 25-OH-D_3 ranges from 64-165 ng/mL [35–37]. In the developed world, vitamer D deficiency is recognized as a significant public health issue, mainly due to an inactive indoor lifestyle and less sunlight exposure [22]. 25-OH-D_3 deficiency is defined as plasma concentrations below 20 ng/mL [10]. Vitamer D deficiency is associated with poor health outcomes, including osteoporosis and muscle weakness, growth retardation, osteomalacia in adults, aggravations of osteopenia, skeletal deformities, and rickets in children [38–40].

Supplementation abuse is the leading cause of Vitamer D toxicity. Dietary intake or skin-produced vitamer D has not been observed to cause toxicity [41]. It is recommended not to exceed the vitamer D3 supplement intake of 10,000 (250 µg) international units (IU) [42], and circulating vitamer D3 levels >100 ng/mL or >250 nmol/L are considered toxic [10,42]. Clinically, excess vitamer D has been associated with: nephrolithiasis, hypercalcemia fatigue, weakness, diarrhoea, anorexia, confusion, headache, psychosis, hypercalciuria, and tremor [42–44]. Despite being rare, severe vitamer D intoxication can cause kidney stones or even acute renal failure [45].

1.1.3 E Group Vitamers

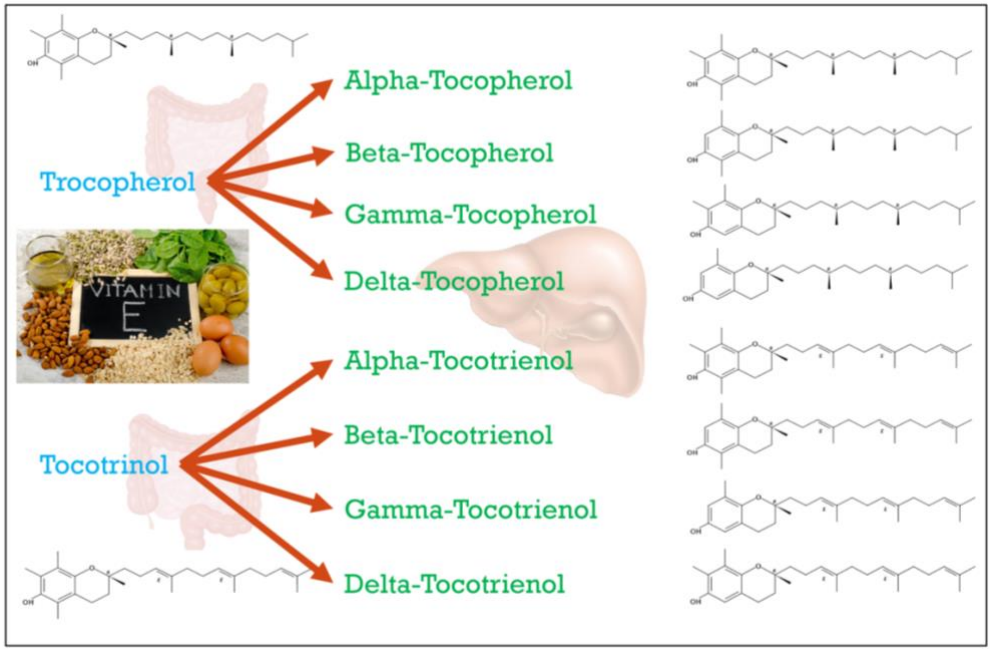

Figure 1-3: Vitamin E vitamers.

The vitamer E family includes both tocopherols (α, β, γ, δ) and tocotrienol (α, β, γ, δ), with α-tocopherol being the most abundant form in the circulation (Figure 1-3) [8]. E group vitamers are antioxidants and have the free radical scavenging ability. The biological importance of vitamer E has been identified in immune function [12,13], cell regulation, cell proliferation, and gene function [46–48]. The daily requirement for Vitamer E is mainly fulfilled by dietary sources, including vegetable oils, dairy products, cereals, and eggs [22]. Vitamer E is absorbed by passive diffusion across the intestinal wall and is transported to the liver by the lymphatic system via low-density β-lipoproteins [49].

Deficiencies and toxicity associated with E group vitamers

Normal physiological concentrations of α-tocopherol and α- tocotrienol range between 3000-13000 ng/mL [50–53] and 0.9 – 30 ng/mL [54,55], respectively. These values may vary for patients with cystic fibrosis [56], cholestasis [13,57], cholestatic liver disease [58], or short bowel syndrome [59]. Symptoms of vitamer E deficiency include anaemia, neurological deficits, ataxia, altered reflexes, muscle weakness, strabismus, visual defects, dementia, cardiac arrhythmias, and blindness [22].

Vitamer E toxicity is rare [60]. Nevertheless, a daily intake of >1000 mg can cause similar symptoms to Vitamer E deficiency (anaemic symptoms and fatigue) [22]. Chronic overconsumption of vitamer E has been shown to impair vitamer K activity, resulting in defective blood coagulation and poor vitamer A storage [61]. Despite not being reported among healthy infants, in preterm babies, over-supplementation of vitamer E increases the risk of retinal haemorrhage and necrotizing enterocolitis (NEC) [62].

1.1.4 K Group Vitamers

There are two dominant forms of K group vitamers in the human body K1 (phylloquinone) and K2 (Menaquinone)] (Figure 1-4). Phylloquinone is predominantly found in leafy vegetables, fruits, soybean oil, cow's milk, seeds and accounts for 90% of the western dietary intake of vitamer K [63]. Menaquinone (vitamer K_2) is predominately produced by the gut microbiota except for menaquinone-4 (MK-4), which is formed by the enzymatic conversion of dietary vitamer K phylloquinone (vitamer K_1) [64–66]. Vitamer K2 is present in a few food items, including Fermented food and drinks. For example, fermented soya beans (Nitto diet) are a traditional Japanese food that is rich in vitamer K2 (predominantly MK-7); in this instance, it is produced by *Bacillus subtilis natto* [67]. *Propionic acid bacteria* (produce predominantly MK-10) and *lactic acid bacteria* (produce predominantly MK-8 and MK-9) are enriched in cheese and curd cheese and also contribute to vitamer K2 intake [68].

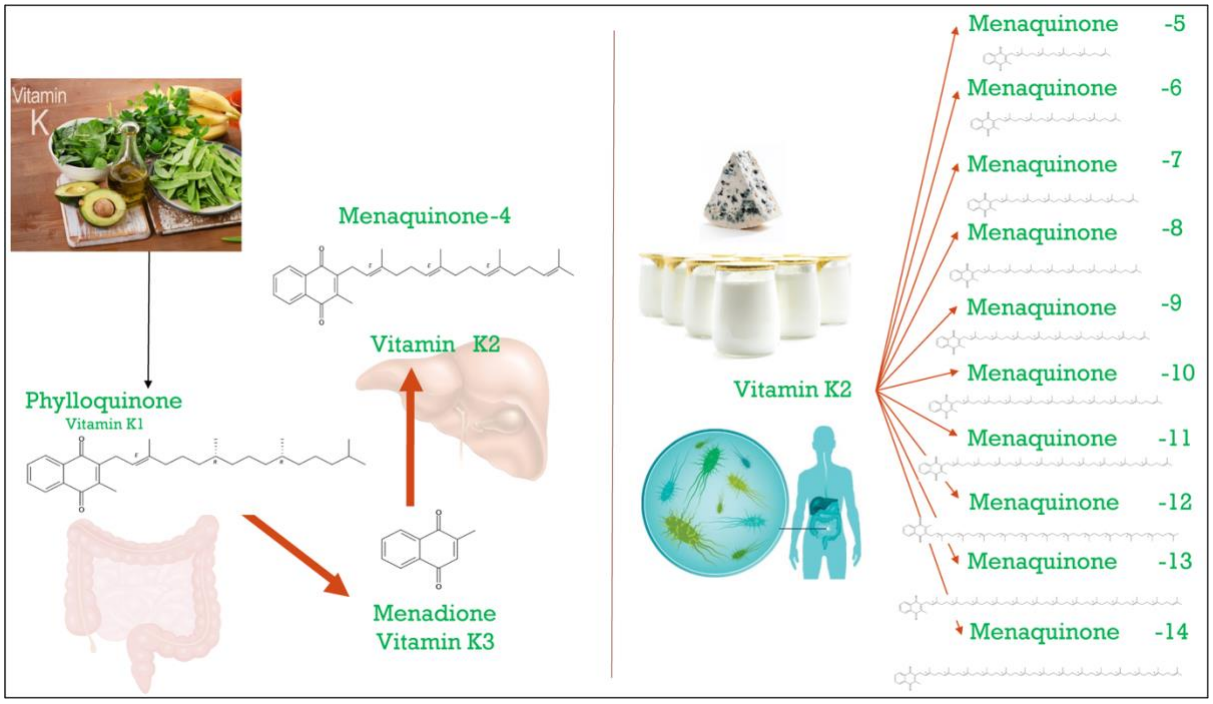

Figure 1-4: Vitamin K vitamers.

After intestinal absorption, K group vitamers are fused into triglyceride-rich lipoproteins and transported to the liver. A large portion of K1 is retained in the liver to be used for clotting factor synthesis. Vitamer K2 is incorporated into low-density lipoproteins and released into circulation for later use in tissue-specific biological activities [69]. The primary biological importance of K group vitamers stems from their roles in promoting blood clotting by activating coagulation factors 2, 7, 9, and 10 [63]. Vitamer K also acts as a cofactor in the gamma-carboxylation of matrix Gla-protein (MGP), which inhibits arterial calcification, affects bone metabolism, calcium homeostasis, and regulates cell growth [14].

Deficiencies and toxicity associated with K group vitamers

The physiological concentration of vitamer K1 typically ranges from 0.018 – 4 ng/mL [70,71], vitamer K2; MK-4 0.56 – 2.2 ng/mL [70,72] and MK-7 0.11 - 2.2 ng/mL [70,72,73]. Long-term deficiencies of K group vitamers can cause bleeding due to impaired blood clotting, which can be life-threatening, especially for older adults [74]. Poor vitamer K status was found to be associated with cardiovascular complications, including coronary artery calcification [75–79], aortic mild or severe calcification [80], and aortic valve calcification [81]. Vitamer K insufficiencies are also associated with osteoporosis, fracture risk, and low bone mass due to calcium imbalance [82].

Long-term supplementation with vitamer K has been associated with low bone mass, accelerated bone loss, arterial calcification, valvular calcification, and some isolated cases of thrombocytopenia and hemolytic anaemia [83]. Excess short-term intake of K group vitamers has not been observed to cause any health complications [63].

1.2 Fat-soluble vitamer Quantification

There is currently no standardized method that is routinely applied to quantify all four groups (A, D, E, and K) of FSV. Yet, sensitive and accurate FSV quantification is vital to understanding human nutrition, the pathology of vitamin deficiencies, and monitoring supplementation to avoid over-supplementation hazards [84].

Quantification of vitamers in human tissues for research purposes is commonly carried out using Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) [15]. However, complex matrices such as plasma and urine present analytical challenges due to non-specific associations between the vitamers and plasma proteins (*e.g.,* albumin, globulin, and other abundant metabolites). To minimise the impact of non-specific associations between the analytes and matrix, many researchers have implemented deproteinisation techniques and long chromatographic run-times for FSV LC-MS/MS analysis [85,86].

Sample instability is a crucial factor affecting the accuracy of FSV quantification. For example, vitamers A and D are sensitive to light, oxidation, heat, and acidic pH. However, sensitivity can vary even within a subgroup, as illustrated by the fact that xanthophyll A group vitamers are sensitive to both acidic and alkaline pH. E group vitamers are relatively robust to high temperatures, acid, and alkaline pH, but still sensitive to light and oxidation. Vitamer K has the most heightened sensitivity to light, acids, and alkaline pH but is stable when exposed to oxygen and heat. Therefore, it is imperative that amber glassware, subdued light, and/or aluminium foil wrapping of tubes are used to protect light-sensitive FSV [87,88]. Moreover, adding antioxidants to samples during collection or at the beginning of the sample preparation procedure can help to stabilize the FSV [84,89].

1.2.1 FSV Extraction in serum and plasma

Sections 1.2.1, 1.2.2, 1.2.3, and 1.2.4 were modified from Arachchige *et al*., 2021 [1] and discuss qualitative comparisons of 13 studies that quantified and validated fat-soluble vitamers in plasma and serum using LC-MS/MS (

Figure 1-5). The systematic review methods have been attached in Appendix 2 and Arachchige *et al*., 2021 [1].

FSV are labile to UV, light, oxidation, heat, acidic pH, and storage conditions. Therefore, it is crucial to take precautions to prevent FSV degradation during sample collection, extraction, and analysis [4]. The time gap between sample collection, storage, and exposure to the conditions above are essential. Only a few studies [16,90,91] reported the required details (Table 1-2), suggesting that many of the studies have not considered the stability of the FSV as an important aspect. Alternatively, the studies failed to report FSV stability is a critical gap in the literature that needs to be addressed in future studies.

Deproteinization-based sample clean-up exploits the high solubility of polar analytes in aqueous-organic solvent solutions and the reduced solubility of proteins [92]. Polson *et al*. [92] demonstrated that using a plasma-to-solvent ratio of 1:2.5, methanol removed 94% of protein, and acetonitrile removed >97% of the protein. In support, Le *et al*., 2018 evident that deproteinisation solvents methanol (100%) compared to acetonitrile (60%) yielded better isometric separation of D group vitamers without overlapping peaks [93]. Protein removal using ethanol extraction was reported to be 88.6% efficient [94]. Although acetonitrile is better at protein removal, ethanol [16,84,89,90,95] and methanol [91,96–98] are cheaper and more commonly used to prepare FSV and isotopically labelled standard stock solutions. Ethanol and methanol are also compatible with a broader range of mobile-phase solvents [99]. Ethanol typically extracts a more comprehensive range of analytes than methanol and is safer to use [16,90]

Table 1-2: Article quality grading system used in this systematic review of LC-MS/MS quantification of fat-soluble vitamer.
Green indicates the factor listed on the left has been adequately described in that study; yellow represents partial fulfilment, and red indicates that the factor was not fulfilled. Adapted from [100–102].

Factors	**Midttun, *et al.*, 2011** [84]	**Midttun, et *al.*, 2016** [89]	**Yu, et *al.*, 2016** [96]	**Zhang*et al.*, 2018** [95]	**Hrvolovÿ *et al.*, 2016** [90]	**Abro *et al.*, 2014** [103]	**Andreoli *et al.*, 2004 [16]**	**Albahrani *et al.*, 2016** [99]	**Capote*et al.*, 2007** [97]	**Konieczna *et al.*, 2016** [98]	**Khaksari et *al.*, 2017** [91]	**Hinchliffe *et al.*, 2016**[104]	**Le *et al.*, 2018** [93]
Sample matrix													
Sample collection													
The time between sampling and storage													
Simple collection process													
QA criteria													
Stability													
Internal and external standard													
Calibration matrix													
Calibration curve details													
LC parameters													
Mobile phases													
MS parameters													
Precursor and product ions													
Method validation													
Matrix effect													
LOD and LOQ													
Statistical analysis described													
chromatography software													
Example chromatogram													
Limitations described													

Similar to deproteinization, Solid Phase Extraction (SPE) and Liquid-Liquid Extraction (LLE) methods also use organic solvents to separate FSV from plasma matrix components (*i.e.*, vitamins, amino acids, and glucose [15]). LLE is one of the most commonly used methods in vitamin and metabolomics studies due to its high extraction efficiency and simultaneous clean-up [15]. LLE is widely used to extract FSV from matrixes that do not require grinding and sonicating [8]. LLE using chloroform and methanol [84,89] can be used to recover a wide range of lipids and vitamins [96,105]. However, chloroform is a known carcinogen unsuitable for routine use [95,96,106].

Multiple extractions of the same sample are often required to obtain the desired analyte concentrations by LLE [107]. Of the studies that have used hexane for LLE [90,91,96–99], Konieczna *et al*. and Khaksari *et al*. had to extract two or three times with hexane to achieve complete extraction of the analytes [91,98]. It can be argued that the use of hexane instead of chloroform is due to its cost-effectiveness, safe use, simplicity, and robustness [96]. Hexane, however, is water-immiscible [90] and highly nonpolar when compared to other commonly used extraction solvents (*e.g.*, ethanol, methanol, acetonitrile) [108]. Unfortunately, this makes hexane by itself an inappropriate choice for mid-polarity analytes, which include most of the FSV.

Both α-tocopherol [109] and all-*trans*-retinol [110] are prone to degradation during sample extraction, clean-up, and analysis. Midttun *et al*. [89] and Karppi *et al*. [111] reported that the addition of BHT [84,89,90] prevents sample and internal standard degradation during sample preparation. As none of the studies reported the extraction recoveries, with or without the addition of BHT, its impact on extraction efficiency cannot be evaluated. Moreover, no sensitivity difference was observed between the published methods developed with or without BHT [16,90,91,93,95–99,103,104].

Solid Phase Extraction (SPE) [95,103,104,106] requires less sample volume than LLE, typically only requiring ~100 µl of plasma [95,104,112] (5). However, some LLE methods have been developed that use lower plasma/serum sample volumes than SPE [16,84,89,93]. Reducing sample volume is always desirable as it reduces the amount of blood that needs to be drawn from the individuals (Appendix 1.1) and facilitates multiple analyses on the same sample [113]. Despite this, LLE seems to yield better sensitivity for FSV quantification and is quicker, cheaper, and simpler [90,91,96–99].

Although LLE and SPE are much cleaner when compared to deproteinisation and direct analysis [114,115], Both techniques are very labour-intensive and use a lot of solvents [15]. These obstacles, however, can be overcome with the use of a single solvent ‘‘dilute-and-shoot’ extraction method. The dilute-and-shoot method uses an organic solvent to simultaneously deproteinise [116] the sample and as a matrix to inject directly into the LC-MS/MS for analysis [93]. The simplicity and speed of the extraction technique can be advantageous for analyzing large sample numbers (*e.g.*, cohort studies, epidemiological studies) [117], and it is much easier to automate [118,119]. However, due to the lack of clean-up, matrix effects can be more pronounced, complicating the analysis [120]. Additionally, studies usually do not report their extraction recoveries using each extraction technique, which prevents direct comparison and evaluation of the different extraction methods and their effects on method sensitivity.

Across several methods developed to quantify FSV, the number of vitamers in a panel ranged from 2 to 16. Some of the studies have only focused on separating the most abundant vitamers of each vitamin group, which are in high circulatory concentrations [84,91,95,96,103,104], while other studies reported on the separation and quantification of isomeric compounds [16,89,90,93,97–99]. Arachchige *et al.*, 2021 [1] identified that the meaning of the term “sensitivity” in an FSV method is its ability to accurately quantify FSV at their physiological levels. Therefore, being able to separate the isomeric vitamers allows better sensitivity for each vitamer (Appendix 1.1).

1.2.2 Chromatographic separation of the FSV

Micellar electrokinetic chromatography [121], fluorescence spectrometry [122], immunoassays [99], supercritical fluid chromatography [8,123], and HPLC [84,89,103] have all been applied to FSV quantification. All aforementioned technologies, except for HPLC, are limited to either one or a limited panel of vitamers [99] or suffer from a lack of sensitivity and efficiency [8,124]. By contrast, HPLC provides better sensitivity, a broader dynamic range [125], and separation of the analytes within a shorter period [126]. The inherent advantages in sensitivity and wide linear ranges [8], [127,128] associated with HPLC make it the most widely used method in vitamin studies (Appendix 1.2) [16,84,90,97–99,103].

The choice of the analytical column is an essential aspect of any analytical method. At high pressures, certain mobile phases can induce a significant level of frictional heating [129]. Columns with an internal diameter of 3.0-4.6 mm are commonly used in HPLC [130].

However, these columns have higher flow rates and longer retention rates [16,84,90,97,98,103] (Appendix 1.2) and the side-effect of an increase in frictional heating, leading to decreased uniformity of the flow [131]. The use of 2.1 mm diameter columns in FSV HPLC [91,96] has resulted in shorter chromatographic run-times with slower flow rates and reduced frictional heating [131]. Slower flow rates [91,99] can significantly improve the sensitivity of an assay by increasing the ionisation efficiency [132].

Column length, particle size, retention time, and packing material are essential in high throughput analyses because of their effect on the resolution [133]. The chromatographic resolution is a vital HPLC performance indicator calculated by dividing the difference in peak retention times by the average peak width. Retention measures the length of time that compounds reside in the stationary phase (column) and the retention time of the last peak defines the total duration of the chromatographic run. Retention times are not only specific to the structure of the specific analyte but also on factors such as the flow rate of the mobile phase, the nature of the mobile and stationary phases and column dimensions [134]. Since 2010, there has been an increasing interest in using semi-porous particles [8,84,89,91,93,96,98,99,104] with a 2-3µm diameter as they provide better peak shape, separation efficiency, and significantly lower backpressures when compared to the fully porous particles [16,90,97,103]. However, small particle sizes and longer-length columns suffer from higher backpressure [135]. Shorter columns with a smaller pore size (<2 µm) and a high-pressure pump system (UHPLC) produce better chromatographic separation with a better signal-to-noise ratio compared to the larger particle size columns [135,136]. This effect can be seen when comparing the studies by Albahrani *et al*. and Zhang *et al*. [95,99] (Appendix 1.2). Compared to the separation of the parent vitamer, the chromatographic separation of isomer and epimers need longer runtimes. Longer length custom columns facilitate more active sites for the FSV to bind into, *e.g.*, vitamin D isomers in [99].

Despite proving difficult, it is desirable to develop a sensitive FSV quantification method to be able to chromatographically separate epimers and isomers. For example, total vitamin D represents a combined value of 25-hydroxy vitamin D and 3-epi-25-hydroxy vitamin D. However, due to analytical difficulties and convenience, 25-hydroxy vitamin D concentration is measured as an individual's total Vitamin D status in current clinical settings [137]. Although the vitamin activity is similar, the proportion of 3-epi-25-hydroxy vitamin D in a significant number of infants and young adults (up to one year) is accountable for up to 60% of the total 25-hydroxy vitamin D [138]. Therefore, the inability of an FSV method to individually separate

and quantify 3-epi-25-hydroxy vitamin D from 25-hydroxy vitamin D can lead to over estimation of vitamin D status (false positive hypervitaminosis) [139].

The highly nonpolar K group vitamers are typically the last to appear in chromatographic runs using C18, Phenyl-hexyl, PFP, and 18e columns (Appendix 1.2). Therefore, including K group vitamers in an FSV panel extends the analytical run time irrespective of the column type [91,97]. Plasma and serum samples degrade columns over time due to incomplete deproteinisation [140]. The use of a guard column can extend the operating life of the analytical column by preventing plasma and serum-derived impurities, reaching the analytical column [141]. Only four studies [91,95,99,104] have used guard columns in FSV methods.

Mobile phase solvents modulate analyte retention times. Many of the published FSV methods used either methanol or acetonitrile as the solvent component of the mobile phase (Appendix 1.2). The use of acetonitrile as the mobile phase solvent improves the ionizing efficiency of ESI when compared to methanol [142]. Notably, acetonitrile mobile phases also result in sharper peak shapes and better sensitivity compared to methanol [143]. Therefore, acetonitrile is preferred in the mobile phase solution.

FSV, in general, is pH labile; therefore, it is essential to have mobile phase solvents that do not change the pH of the solution significantly [4]. Different additives are added to the mobile phases to overcome this issue, such as acidic and basic modifiers [144]. Acidic modifiers significantly affect the ionisation efficiency of the analytes in the MS by modifying the mobile phase pH and reinforcing the chromatographic stability of the analytes [145]. Formic acid is a facilitator of positive ionisation by modulating the pH of the mobile phase to acidic pH, which has been used by many studies [89,93,95,96,98,99,103,104]. The addition of ammonium formate greatly enhances the ionic strength of the mobile phase with a minimal increase in the pH level (2.7 to 3.3) [146]. The ammonium ions act as a base buffering the pH increase from the acidic modifiers; and have been discussed previously [84,91,95,97,98]. Better chromatographic separations and analytical sensitivity were observed in the methods developed using ammonium formate and formic acid as a mix [95,98].

Both isocratic and gradient elution modes are essential in HPLC to optimize the retention of the analytes of interest [147]. Isocratic HPLC is an approach that is designed to supply an unchanged mobile phase composition during the separation of the analytes of interest [148]. However, isocratic HPLC has inherent complications. Some of the complications include poor resolution of early eluting peaks, wider later eluting peaks due to peak dispersion, long analysis

time, and carry-over [127,128]. Some of these issues can be overcome by the use of gradient HPLC [16,84,89–91,93,95–99,103,104]. However, running a gradient elution method and transferring it between columns and instruments requires extra effort compared to the isocratic elution mode [149].

Liquid chromatography enables the separation of compounds based on retention time; however, it does not provide much information about the compounds, and co-eluting compounds cannot be differentiated. Parallelization of liquid chromatography with mass spectrometry enables both the detection and quantification of co-eluting analytes of interest.

1.2.3 Mass spectrometric detection of the FSV

Three main types of Mass spectrometry (MS) discussed in this introduction are: triple quadrupole [16,84,89,95,97–99,104], ion trap [91,103] and hybrid triple quadrupole/linear ion trap [90,93,96] (Appendix 1.3). While the selection of the mass analyzer should be primarily based on specific analytical needs, in reality, instrument availability is the strongest determinant.

In general, the ion trap is the most versatile mass spectrometer (these include orbitraps), while triple quadrupole instruments are typically used for targeted analysis [150]. By comparison, orbitraps produce a better mass resolution, mass accuracy and provide more mass spectrometric information for the analytes of interest [151]. Another advantage of using orbitrap and triple quadrupole mass analyzers is their ability to detect analyte decomposition [152], which is extremely important in vitamin quantification due to their low concentration in plasma, as discussed in several studies [84,89,91,93,95,98,99,153].

Atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) are the most frequently used ionisation techniques for vitamin analysis in research laboratories and clinical settings [154]. ESI is the most commonly used ionisation method for FSV analysis [84,89,91,93,95–99,104,153] (Appendix 1.3).

The efficiency of using an ionisation source is highly dependent on the analyte characteristics and matrix effects [153]. The reduction in ionisation efficiency resulting from the matrix effect is one of the most important factors that need to be considered during FSV analysis. Trufelli *et al*. [155] reported that ESI is much more vulnerable to matrix effects when compared to atmospheric pressure photoionisation (APPI) and APCI sources. For vitamin D studies in

human plasma, it has been claimed that ESI is not successful in protonating the molecules, whereas APCI provides sensitive detection [90,98,156]. Consistent with this, Abro *et al*. [103] developed a rapid, sensitive, and simple method using APCI to quantify vitamins D3, D2, E, and K1. However, most of the previous studies [87,92,94,96,98,100–102,107] support the argument that ESI could successfully ionize D group vitamers.

Andreoli *et al*. [16] successfully ionized 16 FSV using APCI-MS, by far the highest number of vitamers any study has quantified in a single analysis. In APCI, the solvents transferred into the mass spectrometer are significantly less compared to ESI [157]. By contrast, Zhang *et al*. [95] argue that despite APCI being more commonly used in vitamin studies, ESI can provide a ten times higher response. My systematically accumulated evidence supports the conclusion that ESI provides better ionisation responses than APCI. Moreover, the number of studies that used ESI as an ionisation source in FSV quantification is high compared to APCI [84,89,91,93,95,97–99,104]. This may be because: 1) ESI is the most versatile source, and 2) few labs purchase an APCI source.

1.2.4 Method validation and quality assurance

Method validation demonstrates that a method is sensitive, reproducible, and robust [158]. Linearity, range, specificity, accuracy, precision, stability, and Limit of Quantitation (LoQ) are the primary method validation criteria in LC-MS/MS (Table 1-2).

Sample stability is one of the main factors affecting method validation and reproducibility for the accurate quantification of FSV. Many previous studies [16,84,89–91,93,95,97–99,103] reported their developed methods' linearity, range, accuracy, and precision. However, only a few studies [93,104] investigated the stability of FSV during sample preparation and LC/MS analysis [4] (Table 1-2). This limits my ability to comment on the impact of FSV stability on method sensitivity.

Analytical inaccuracies are common among underdeveloped and well-established methods. One of the improvements to address this analytical challenge is the introduction of the method traceability concept [159]. In FSV methods, both internal and external standards [160] and secondary quality assurance calibrators are used to assess assay precision. The addition of internal standards can compensate for the matrix-induced ion suppression in both precision and accuracy calculations [96]. The majority of the previous FSV methods [84,89,91,93,95,96,99,104] reported the use of isotopically labelled internal standards for all

the vitamers quantified. Several others stated only a partial use (Same IS for two different vitamin groups) or data absent (Table 1-2). The omission of information on critical method development steps, such as internal standards, is a serious disadvantage that limits the ability of other researchers to replicate work. NIST SRMs were used by four studies [89,93,96,104], while Albahrani *et al*.[99] used inter-laboratory testing Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) and Joint Committee for Traceability in Laboratory Medicine (JCTLM). Though these calibrators are important in the quality assurance process, current FSV coverage of these standardized reference materials is limited to a small number of vitamers, exclusive of K group vitamers.

Limits of quantitation (LoQ) is a performance parameter associated with the ability of the method to detect noise from the signal leading to the quantification of low analyte levels in the sample. In FSV studies, the analytical utility of a method is its ability to detect and quantify the analytes of interest at their physiological levels, the upper limit (hypervitaminosis), lower limit (deficiencies), and separate the isomers. Not all the studies that reported LoQ values successfully managed to detect FSV at physiological levels [84,89,90]. Some vitamers, *e.g.*,1-α-25-dihydroxy-D_3 (30.3 pg/mL) [97,98,161] (

Figure 1-5), are present at concentrations below the LoQ for most approaches. In essence, failure to detect these vitamers reflects a lack of sensitivity in the developed method.

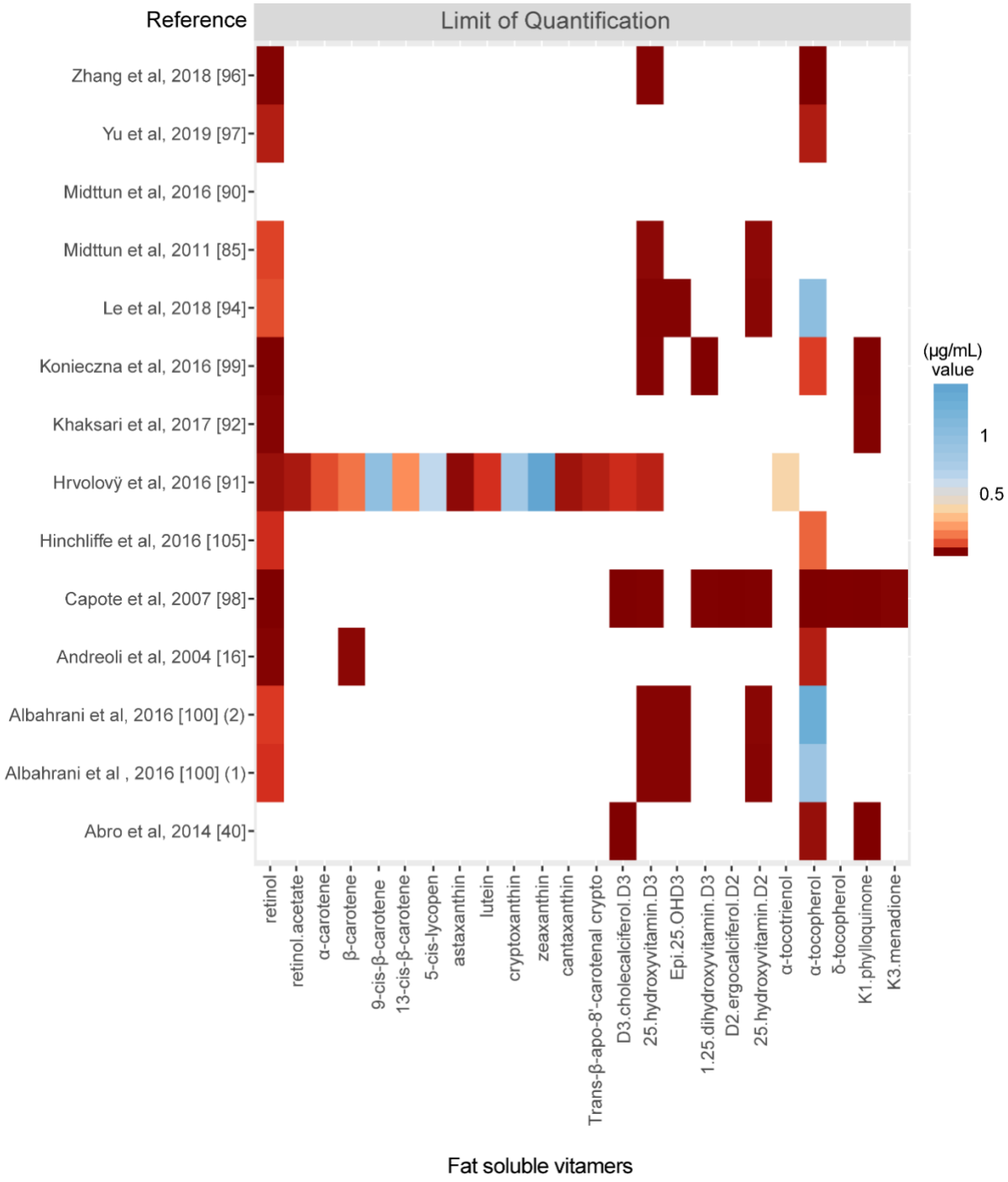

Figure 1-5: A heat map of the Limit of Quantification achieved for each Fat-Soluble Vitamer quantified in the studies (Arachchige *et al.*, 2021) [1]. The colour scale indicates the LoQ value in µg/mL with lower LoQs as red colours and higher values as blue

1.3 The global epidemiology of Fat-soluble vitamers

Vitamer A deficiency (VAD) is a global public health problem caused by ecological (*e.g.*, arid and infertile land or excessive rainfall), economic (*e.g.*, poverty, unemployment, low-wage jobs or high death rates among infants), and social factors (*e.g.*, limited access to health and social services or being under-educated) [162]. In addition, host factors also greatly influence the prevalence of VAD, including age [163], sex [164], feeding practices, and disease patterns [165]. In developing countries, vitamer A deficiency is the leading cause of night blindness among pregnant women (Southeast Asia (17%), East Asia (22%), Africa (14%), and Eastern Mediterranean (16%) total of 19 million) and permanent blindness among children (age 1-6 years) [9]. According to the global data on VAD, approximately 2.8 million preschool children are at risk of blindness, and over 251 million others live with seriously compromised health [162]. The mortality rate in children under 5 years (≥50 deaths per 1,000 live births) was identified globally as the surrogate indicator of VAD. Sub-Saharan African countries have the highest VAD-associated global mortality rates per continent (>100 deaths per 1,000 live births) [165].

Similar to vitamer A deficiency, vitamer D deficiency (VDD) is a globally widespread health concern. VDD (*i.e.*, < 12 ng/mL) has been detected in 13% (Europe) [166], 7.4% (Canada) [167], 5.9% (United States) [168] and >20% of the population in Tunisia, Afghanistan, India and Pakistan [169]. The prevalence of VDD varies with age (lower circulatory concentration in children and elderly), ethnicity (VDD high in dark-skinned individuals compared to European Caucasians), region (individuals who live in high or low latitudes have different sun exposure), and chronic medical conditions (*e.g.*, haemodialysis, renal transplant patients and patients with liver disease, or after liver transplantation) [39,166,169,170]. Vitamer D deficiency has been reported in New Zealand and Australia [171]. For example, studies in Tasmania have shown that VDD (plasma 25(OH)D levels <20.03 ng/mL) occurs in ≤ 10% of 8-year-olds and 68% of 16–18-year-old adults [172,173] during winter and spring. VDD has also been detected among 78% of young children in New Zealand during winter [174].

Vitamer E deficiency (VED) is more common among children when compared to the other age groups. VED is mainly due to underconsumption. Other risk factors, including age, obesity, and sex, have also been observed to influence VED [175]. Deficiencies in vitamers A and E are not a major concern in New Zealand and Australia [162]. By contrast, the prevalence of

VED ranges from 20% to 90% in Asia, Africa, the Middle East, and South America. For example, a study conducted in Jordan found that among 262 children at the age of 0.5 to 5.5 years, 17.1% - 89.2% are VED [176]. In this study, the cut-off for the VED was set at serum α-tocopherol < 11.6 μmol/L [176]. Similar VED results were reported among 131 Korean children aged 2 to 6 years with plasma α-tocopherol levels < 12 μmol/L in 67% [177].

Vitamer K bleeding deficiency (VKBD) primarily affects new-born's; however, some symptoms (*e.g.*, haemorrhages, short gut syndrome, gut microbiome overgrowth, and genetic conditions can occur beyond the neonatal period. VKBD is associated with long-term mobility and mortality in infants due to heavy bleeding. China and India have the highest neonatal mortality due to VKBD [178]. The risk of bleeding in infants who were not supplemented with vitamer K is 1,700 per 100,000 infants, whereas when vitamer K prophylaxis was instituted, this risk rate falls 1 per 100,000 infants [179]. No associations have been identified between VKBD and race, sex, or age [178].

1.3.1 The Growing Up in Australia's Longitudinal Study of Australian Children (LSAC)'s Child Health Checkpoint Study

The Longitudinal Study of Australian Children (LSAC)'s Child Health Checkpoint Study is the most extensive and only nationally representative children's cohort study performed in Australia. LSAC was approved by the Australian Institute of Family Studies Ethics Committee, The Royal Children's Hospital (Melbourne, Australia), and the Human Rights Committee (33225D) [117]. The work undertaken in this thesis was primarily based on the LSAC Child Health Checkpoint Study.

The Growing Up in Australia's LSAC cross-sectional cohort study consists of 10,000 children and their parent samples. The samples from these parent-child dyads were collected by trained professionals in popup centres across all major cities in Australia (*i.e.*, South Wales, Victoria, Queensland, South Australia, West Australia, Northern Territory, Tasmania, and Australian Capital Territory [180,181]. The LSAC population-based study initially recruited 5107 babies in 2004 at birth to 1 year of age (Wave 1, n=5107) and subsequently followed up every two years until 2014 (participant rate 74% at Wave 6 in 2014, n=3764)[180].

The LSAC Child Health Checkpoint is a single cross-sectional segment of LSAC's B cohort between waves 6 and 7 (hereafter named wave 6.5). In wave 6.5, bio specimens and physical

health data of 11–12-year-old children were collected. The main aim of the Child Health Checkpoint study was for the policymakers and researchers to acquire an in-depth understanding of the health status of Australian children at adolescence or the "checkpoint" between adulthood and childhood and implement new strategies to improve future health outcomes [117,180]. In Wave 6 of the LSAC study, 3513 families agreed to participate in the Checkpoint study. Consents were taken from the parents and caregivers for 1874 children's bio specimens to be collected for research purposes [117].

1.4 Hypothesis

I hypothesize that plasma Fat-soluble vitamers (FSV) circulatory concentrations are age, sex, and BMI specific. However, there should also be correlations between parent and child dyads from the same family that reflects shared environments and genetics.

1.5 Objectives

My objective is to develop an optimized bioanalytical panel to quantify fat-soluble vitamers (FSV) in plasma and apply that method to characterize the epidemiology of these FSV across parent and child dyads in the Checkpoint study of Australian Children and adults.

Objective 1: Develop, validate, and automate a multiplexed bioanalytical assay to quantify Vitamin A, D, E, K and their metabolic products in plasma.

Objective 2: Characterize the population-level distribution of these FSV within the Checkpoint study of Australian Children and adults.

1.6 Thesis Outline

I have organized my thesis as follows.

1 Chapter two describes method optimization steps that were undertaken to develop a high throughput bioanalytical assay using liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS).

2 Chapter three details the semi-automated methodology that was developed and validated to quantify 11 A, D E, and K group vitamers in lithium heparin plasma using liquid chromatography coupled with tandem mass spectrometry (LC/MS-MS).

3 Chapter four investigates the associations between familial effect, generation effect, age, BMI, and Sex with fat-soluble vitamer concentrations in Australian parent and child dyads.

4 Chapter five discusses the findings of the chapters mentioned above.

Chapter 2

LC-MS/MS Method Optimization

2.1 Background

Method optimization is an integral part of any analytical assay development. The objective of the method optimization is to minimise in-built errors in measurement, eliminate unnecessary steps, avoid duplicate work, and enable process automation to save time. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) is commonly used to identify and quantify analytes in trace amounts with high accuracy, sensitivity, and specificity. An optimized LC-MS/MS method encompasses three components: sample preparation, chromatography, and tandem mass spectrometry. These components individually address aspects of the chemical and physical properties of the target and matrix. The challenges that are inherent in developing these methods derive from the fact that each analyte has its own unique chemical structure and, consequently, individual chemical and physical properties (*e.g.*, molecular weight, polarity, molecular size, volatility, stability, solubility, and many more) [182]. Robust methodological operating processes are critical in these assays as the complexity of the analytes means that any methodological variation affects the final sensitivity [183].

2.1.1 FSV Stability

Fat-Soluble Vitamers (FSV) include the A, D, E, and K group vitamins and their metabolic products. FSV are particularly difficult to analyze as they are labile to pH, heat, oxygen, UV light to varying degrees [1,184], sample drying [90], and freeze-thaw cycles [185]. Evidence-based studies into the stability of FSV during storage are scarce and contradictory [88]. However, FSV degradation is faster when the FSV are extracted in organic solvents compared to their stability in the original biological matrix (Blood, plasma, serum) [186]. The available evidence is also restricted to the main circulatory forms or a limited range of vitamers (*i.e.*, retinol, 25-OH-D_3, α-tocopherol, γ-tocopherol) [88,187]. Variable degradation of FSV at any stage during analysis (*e.g.*, sample collection, storage, preparation, or analysis using LC-MS/MS) results in poor recoveries and quality control issues [187].

2.1.2 Matrix effect

The matrix effect is a phenomenon caused by co-eluting non-specific matrix constituents that act to minimise the ionisation efficiency of the analytes of interest and thus reduce signal intensity [188]. Sample matrices (*e.g.*, serum, plasma, and urine) are complex. The matrix effect in plasma could be due to lipids (mainly phospholipids), steroids, small proteins, salts, and other metabolites [189]. Over the years, several approaches have been introduced to minimise the matrix effect and improve recoveries. These approaches include optimizing cleanup, adjusting MS parameters and chromatographic conditions, and the use of isotope-labelled internal standards [190]. Sample preparation or cleanup is one of the key steps used in LC-MS/MS to combat the matrix effect and the most challenging part of the LC-MS/MS bioanalytical workflow. The main aim of the sample preparation process is to reduce matrix interference and improve recoveries while maintaining high throughput [191].

Deproteinization, liquid-liquid extraction (LLE), solid-phase extraction (SPE), and Supported Liquid Extraction (SLE) techniques predominate in bioanalytical sample preparation. In deproteinization, an organic solvent (*e.g.*, methanol, acetonitrile, or 2-propanol) is added to serum, plasma, or whole blood matrix to denature and precipitate protein dissolved in the sample [92]. LLE works by adding two immiscible organic solvents into an aqueous sample, one to initiate the deproteinisation of the analytes and the other to trigger biphasic separation (*e.g.*, hexane, iso octane, and chloroform) of the mixture [84,89]. LLE is the most commonly used sample preparation method in FSV analysis due to its ability to reduce matrix effects and effectively isolate analytes of interest [84,89,90,192]. SPE separates analytes using a solid media mounted on a sorbent material in the form of a cartridge (physical or chemical adsorption interaction principal). When the samples pass through the sorbent material analytes, they are retained by association with the solid media before being eluted using an organic solvent [193]. Although SPE is typically much cleaner than LLE, it's unsuitable for extracting analytes at lower concentrations due to poor recoveries and reproducibility issues [114]. In SLE, the sample directly flows into the extraction bed within the cartridge. The entire sample (both analytes of interest and matrix components) is retained in the extraction bed. An organic solvent is added onto the extraction bed to selectively elute the analytes of interest, leaving the matrix constituents in the media [194]. For example, the Phree® phospholipid removal plates are designed to use the principle of SLE to quickly clean plasma proteins and phospholipids and

collect the eluents to the collection plate [190]. Notably, Phree® plates enable the one-step removal of phospholipids and other non-specific matrix constituents.

In addition to the non-specific analytes that impact ion enhancement, plasma type (*e.g.*, Lithium heparin, ethylene diamine tetraacetic acid (EDTA), and citrate) is a major factor influencing the matrix effect [195]. The LC-MS/MS analysis of FSV in plasma is limited to a small blood sample volume. Therefore, the correct choice of anticoagulant is imperative. Currently, there is no consensus on which type of anticoagulant should be used in FSV analysis. However, anticoagulants are known to impact LC-MS/MS methods at concentrations seen in collected blood [196]. According to previously published FSV quantification methods, Heparin-based plasma provides better sensitivity with reduced matrix effect when compared to EDTA plasma [29]. This is consistent with observations that lower concentrations of FSV (β-carotene and retinol) are observed in EDTA-treated plasma samples due to oxidation and degradation of these analytes introduced by EDTA [197].

Another factor contributing to the matrix effect in LC-MS/MS analysis of FSV is the leaching of plasticizers into the sample extracts [104]. Yao *et al*., 2016 tested the effect of the packing material (glass or plastic) on the LC-MS/MS quantification of retinyl palmitate and identified highly variable retinyl palmitate concentrations even within the same batch of plastic consumables. Yao *et al*., 2016 suggested using glass vials and glass pipettes for the best results [198]. As an alternative to the use of glass vials and pipettes, Hinchliffe, Rudge, and Reed, 2016 proposed that glass inserts placed inside polypropylene deep well 96 plates could minimise the occurrence of plasticizer contamination in sample extracts for retinol and α-tocopherol quantification [104].

2.1.3 Liquid chromatography and mass spectrometry

Since 2010, High-Pressure Liquid Chromatography (HPLC) has been the most commonly used method in vitamer analysis [126,199,200]. HPLC can quickly provide functional analysis, purification, identification, and separation of the analytes [126]. Swartz (2005) [131] reported that smaller particle size columns that could handle faster flow rates and higher back pressures were major requirements for advancing the drug development industry. This was achieved by the transition from HPLC to ultra-high pressure liquid chromatography (UHPLC), which allowed the analytical platforms to handle higher backpressures and use small particle-size columns with better separation ability. However, the choice of a combination of stationary

(analytical column) and a mobile phase (solvents) remains a crucial factor in ensuring that finely resolved peaks are achieved [201].

Coupling the LC and MS methods into one analytical approach requires target molecules to be converted to ions, either by hard ionisation techniques, such as electron ionisation, which causes target ions to fragment or by soft ionisation, which generates intact parent ions that can then be fragmented using Collision Induced Dissociation (CID) [155]. Electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) are the two main soft ionisation methods used in mass spectrometry [155]. ESI is distinguished for producing both negative and positive ions by optimizing the solvents and conditions and is most suitable for polar smaller molecular weight compounds [202]. At the same time, APCI is ideal for heat-stable, volatile, low to moderate polarity compounds [203].

LC-MS/MS is the ideal analytical platform for the quantitative analysis of FSV and other metabolites as it has significantly improved sensitivity and specificity when compared to Gas chromatography-mass spectrometry (GC-MS) and immunoassays [204]. Notably, analytes for LC-MS/MS do not require derivatisation, which reduces the risk of chemical interference with the analytes of interest [182]. Moreover, LC-MS/MS is preferable for thermally unstable and non-volatile molecules (*e.g.*, steroids, lipids, and vitamins [88]) as the high temperatures (>300°C) are used in the GC-MS oven and injection port can result in thermal degradation of injected molecules [205].

2.1.4 Liquid handing

Large-scale analyses of FSV require the handling and preparation of a large number of samples [206]. This introduces numerous aspects where the 'human factor' can accidentally introduce variation in the methodology. For example, this includes the highly repetitive manual pipetting required for sample preparation. The introduction of the liquid handling robot was one of the significant technological advances in the 1990s to make High-Throughput screening a reality by enabling a large number of samples to be efficiently processed or tested in a concise period of time [207]. Automated liquid handlers reduce human error, the occurrence of repetitive stress injuries, protect against hazardous or infectious samples, increase throughput, and improve reproducibility [207,208].

2.1.5 Method validation

LC-MS/MS based methods are notorious for their complexity, mainly due to advanced instrumentation and complex sample matrices (*e.g.*, plasma lipid fraction contains cholesterol, triglycerides, phospholipids, vitamins, and apoproteins across a wide range of concentrations) [209,210]. It is essential to prove that the results obtained from an LC-MS/MS-based method are reliable and reproducible. As such, method validation is an indispensable component of LC-MS/MS-based biochemical analysis which provides precise information on how the assay performs [211]. Different regulation bodies have published a number of validation guidelines, *i.e.*, the European Medical Agency (EMA) [212], Food and Drug Administration Centre for Veterinary Medicine (FDA) [211], Australian pesticides and veterinary medicines authority (APVMA) [213], United Nations Office on Drugs and Crime (UNODC) [214] and Association of Official Agricultural Chemists (AOAC) [209]. Despite a lack of consensus on which guidelines to follow, the general sequence of operations and decisions applies to LC-MS/MS-based method development, including optimization of stability, selectivity, linearity, precision, recovery, matrix effect, Instrument Detection Limit (IDL), Instrument Quantitation Limit and robustness [212]. If the method's performance does not meet the standards set by the validation guidelines, changes should be made to optimize the method parameters to achieve satisfactory results [213].

No standardized LC-MS/MS methodology exists to separate, detect, and quantify the wide range of chemically diverse FSV of biological relevance. The purpose of this chapter is to describe the process I followed to obtain the optimized method parameters that are presented in the FSV robotic method published in Analytica Chimica Acta on 9th October 2021 (G.R.P. Arachchige, E.B. Thorstensen, M. Coe, J.M. O'Sullivan, C.J. Pook, Absolute quantification of eleven A, D, E, and K vitamers in human plasma using automated extraction and UHPLC-Orbitrap MS, Analytica Chimica Acta, Volume 1181, 2021, 338877, ISSN 0003-2670, https://doi.org/10.1016/j.aca.2021.338877. The process of FSV LC-MS/MS quantification method optimization incorporated aspects of analytical stability, matrix effect, sample preparation, liquid chromatography, and tandem mass spectrometry to ensure the accurate artefact-free quantification of 11 FSV. The number of FSV included in different experiments varies due to internal and external standard availability, the stage of the method developmental process and the nature of the experiments. For *e.g.*, I noticed that the FSV that are at lower physiological concentrations within plasma were absent (e.g., K1, MK-4, MK-7, 1α-25-(OH)2-

D3, and retinyl palmitate) when using pooled unspiked plasma. Therefore, these were excluded from specific experiments.

2.2 Methods

2.2.1 Ethical approval and funding

This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Auckland Health Research Ethics Committee of the University of Auckland (project 3408 (review reference AH3408)). The present study was funded by the MBIE Catalyst grant (The New Zealand-Australia Life Course Collaboration on Genes, Environment, Nutrition, and Obesity (GENO); UOAX1611; to JOS).

2.2.2 Storage conditions optimization

Sample Collection: Peripheral blood samples from 7 volunteers were collected in 5 mL lithium heparin tubes, de-identified, and centrifuged (1600 RCF, 10 min at 4°C); the resulting plasma (2.5 mL) was pooled and transferred to 1.5 mL microcentrifuge tubes and stored (-80°C) until use in FSV assays.

Stock preparation: stock solutions of all-*trans*-retinol, retinoic acid, 25-OH-D_3, 1-α-25$(OH)_2$-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, K1, MK-4, MK-7 (Table 2-1), and isotopically-labelled internal standards 100 ng/mL of ($[^2H]_8$ Retinol, $[^2H]_7$ 25-OH-D_3, $[^{13}C]_6$ α-tocopherol and $[^{13}C]_6$ phylloquinone were prepared on ice in ethanol.

Table 2-1: Concentrations (ng/mL) of the standard curve

Compound	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
retinol	1600	800	400	200	100	50	25
retinoic acid	115	57.5	28.75	14.38	7.19	3.59	1.80
25-OH-D_3	364.35	182.18	91.09	45.54	22.77	11.39	5.69
1-α-25$(OH)_2$-D_3	165	82.5	41.25	20.63	10.31	5.16	2.58
α-tocopherol	14000	7000	3500	1750	875	437.5	218.75
γ-tocopherol	2000	1000	500	250	125	62.5	31.25
α-tocotrienol	100	50	25	12.5	6.25	3.13	1.56
K1	20	10	5	2.5	1.25	0.63	0.31
MK-4	20	10	5	2.5	1.25	0.63	0.31
MK-7	30	15	7.5	3.75	1.88	0.94	0.47

Abbreviations: Std: Standards and Std1: Original Stock concentrations

The calibration standards containing 10 FSV were prepared as a mixture with concentrations ranging from 0.32-14,000 ng/mL (Table 2-1) in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) 4% (w/v). The calibrator mixture was serially diluted (2 fold) to achieve the desired concentrations. Acetonitrile was used as a solvent blank. Each heparin plasma sample was spiked with physiologically relevant concentrations of these vitamers (Table 2-2).

Table 2-2: Vitamer concentrations that were spiked into the plasma samples

Compound	Spiked Concentrations (ng/mL)
retinol	100
retinoic acid	7.19
25-OH-D_3	22.77
1-α-25$(OH)_2$-D_3	10.31
α-tocopherol	875
γ-tocopherol	125
α-tocotrienol	6.25
K1	1.25
MK-4	1.25
MK-7	1.88

Liquid handling: The Eppendorf EpMotion liquid handling robot (EpMotion 5075vt, Germany), integrated with a vacuum manifold and thermal mixer, was used for liquid handling procedures such as pipetting (different volumes using 50, 300, and 1000 µL pipettes), vacuum extraction, thermostating samples (*e.g.*, 4°C), and sample mixing at a certain speed [208].

FSV stability at -80°C storage over six months: My objective was to evaluate the stability of FSV in the dark at -80°C over six months period. The heparin plasma samples (n=96, 4.8 mL) were prepared and stored as follows: plasma samples (n=24, 1.2mL) spiked with the representative physiological concentrations of the FSV (Table 2-2), samples without treatment as controls (n=24, 1.2mL), non-spiked plasma samples with BHT [(butylated hydroxytoluene (BHT, 99%), (1 g/L), (n=24, 1.2mL)] and 24 spiked samples with BHT [(1 g/L), (n=24, 1.2mL)]. All samples were stored at -80°C and defrosted immediately before extraction (samples were only defrosted once).

FSV stability in solvent extracts at 4°C over 7 days: This experiment was designed to determine the stability of the FSV, extracted in solvents and stored at 4°C over 7 days in the dark. Two sets of heparin plasma samples (n=54, 2.7mL) were spiked with physiological

concentrations of the 9 FSV (3 replicates of each vitamer) (Table 2-2). Blank heparin plasma was used as controls (n=3). Upon extraction, the first sample set (n=27,1.35mL) was analysed on the same day, and the second sample set (n=27,1.35mL) was stored at 4°C in the dark for 7 days before analysis.

Sample preparation: The working calibrators, spiked plasma, and control plasma (50 µL) were transferred into glass-coated 96 well microplates (1st plate), IS, and 200µL of acetonitrile were added to initiate the deproteinization. Samples were mixed for 15 min, 800 rpm (linear) at 4 °C. Hexane (400µL) was added to initiate the biphasic separation. The solution was then mixed (10 min at 800 rpm (linear), 15 °C), and the plate was centrifuged (2000 RCF, 5 min, 15 °C). The supernatant (580uL) was transferred to a new glass-coated 96-well microplate (2nd plate). 300uL of hexane was added to the 1st plate, and the mixing and centrifugation were performed as above. 300uL of supernatant was transferred to the 2nd plate. The samples in the 2nd plate were dried with N_2 (one hour, 20 °C. Extracts were suspended in 100 µL of 2-propanol. The samples were transferred to a Phree® phospholipid removal plate, with the 2nd plate reused as the collection plate. The Phree® plate was centrifuged (2000 RCF, 5 min, 15 °C), and a vacuum was applied (10 psi, 10 min; repeated twice).

The resulting eluents in the 2nd plate were covered with a sealing mat, and 15 µL injection volumes of blanks, calibrators, and plasma spikes were analysed by LC-MS/MS. Sample introduction to the LC-MS/MS was performed using a PAL autosampler with refrigerated sample trays (CTC Analytics, Thermo) at 10 °C and a HotDog 5090 column oven (Thermo). Kinetex C18 100 Å (100 x 2.1 mm) 1.7 µm analytical column (Phenomenex; Auckland, New Zealand) fitted with a KrudKatcher Ultra HPLC in-line filter (Phenomenex) connected to an Accela 1250 UHPLC pump (Thermo Fisher Scientific, Austin, Texas USA) were used for sample separation. Q-Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Germany) equipped with a heated electrospray ionisation source was used as the detector. A more detailed version of this LC-MS/MS method was published in Arachchige *et al*. 2021 [184] (Thesis Chapter 3).

Stability difference statistics: The stability difference is calculated as an absolute difference**,** as a mean percentage deviation of results from time (T0) and after a defined period of time (Tx).

$$\text{Mean percentage deviation} = \frac{\text{Tx} - \text{T0}}{\text{T0}} \times 100$$

The statistical significance in the aforementioned conditions was calculated using Wilcoxon non-parametric signed-rank test. A *p*-value of >0.05 was considered statistically significant—R Version 3.6.1 (https://www.r-project.org/ was used for the statistical analysis and graphing.

2.2.3 Solvent extraction optimization

The sample collection, stock preparation, and liquid handing steps as detailed in section 2.2.2 Storage conditions optimization. The type and number of solvent combinations used in these experiments are primarily based on previously published literature as well as solvent and standard availability.

Single solvent extraction. The objective of this experiment was to test the single solvent extraction efficiency of 10 FSV from heparin plasma. Heparin plasma samples (45 µL) were spiked with 5 µL of external standards at physiological concentrations (Table 2-2) to make a total volume of 50 µL. Internal standards (IS, 5 µL) were added to calibrators (n=7), spiked plasma, and plasma blanks (n=3) in a glass-coated 96-well plate (1st plate). Each solvent (*e.g.*, pentanol, ethanol, methanol, acetone, acetonitrile, 2-propanol, ethyl acetate, and chloroform) was tested at 3 different plasma: solvent ratios (1:2, 1:3 and 1:4, Table 2-6). The samples in the 1st plate were then mixed (15 min, 800 rpm (linear), 4 °C) and centrifuged (5 min, 2000 RCF, 4 °C). The resultant supernatant was carefully transferred to a new glass-coated 96-well plate (2nd plate) without disrupting the protein pellet. Samples were then analysed using LC-MS/MS (as outlined in section 2.2.2 Storage conditions optimization).

Liquid-liquid extraction. The objective of this experiment was to test the plasma FSV extraction efficiencies of two different LLE methods (deproteinisation solvents with isooctane: chloroform (3:1, v: v) and hexane). As per Table 2-6, deproteinisation solvents ethanol, methanol, and 2-propanol were added to make plasma: solvent ratios of 1:3 and 1:4. IS mix (5 µL) was added to the 50µL calibrators (n=7), spiked plasma, and plasma blanks (n=3) in a glass-coated 96 well plate (1st plate). Liquid-liquid extraction was performed using plasma: deproteinisation solvent: using isooctane: chloroform ratios of 1:2:6. Similarly, biphasic separation using hexane was tested using plasma: solvent ratios of 1:2:4, 1:3:6, and 1:4:8. The content in the 1st plate was mixed (15 min at 800 rpm [linear], 15 °C), and the plate was centrifuged (2000 RCF, 10 min, 4 °C). The supernatant was transferred to a new plate (2nd plate). To initiate the 2X hexane extraction 300µL of hexane was added to the 1st plate, and the mixing and centrifugation steps were performed as outlined above. The supernatant was

transferred to the second plate. 2nd plate was N_2 dried for 45 min at 20 °C. The extracts were re-suspended in 100 µL of 2-propanol. The resultant suspension in the 2nd plate was covered with a sealing mat and analysed using LC-MS/MS (Similar to section 2.2.2 Storage conditions optimization).

The extraction efficiencies were calculated as percentage recovery values according to the EMA guidelines [212] (% Recovery values 70-130 are considered as good recoveries, <70 under-recoveries, and >130 over recoveries).

2.2.4 Effect of sample matrix constituents (EDTA vs Heparin plasma)

The objective of this experiment was to compare the anticoagulant (EDTA vs heparin plasma) effect on the peak areas of 11 different FSV. Heparin sample collection, stock preparation, and liquid handling robot optimization were as described in section 2.2.2 Storage conditions optimization. Pooled EDTA plasma was purchased from the New Zealand Blood Service and stored at -80°C.

Sample extraction: A Few modifications were made to section 2.2.2 Storage conditions optimization (sample extraction, experiment 2) to investigate FSV concentrations in heparin and EDTA plasma. Thirty aliquots of each plasma type (EDTA and heparin) were spiked with the physiological concentration of the FSV (Table 2-2). The isotopically-labelled internal standards 100 ng/mL of ($[^2H]_8$ Retinol, $[^2H]_7$ 25-OH-D_3, $[^{13}C]_6$ α-tocopherol and $[^{13}C]_6$ phylloquinone) and 200µL of acetonitrile were added to the 50µL calibrators (n=7), spiked plasma (n=60), and plasma blanks (n=10) in a glass-coated 96 well plate (1st plate). The plate was then mixed (15 min, 800 rpm (linear) at 4 °C) before adding 400 µL of hexane to initiate the biphasic separation. The solution was then mixed (10 min at 800 rpm (linear), 15 °C), and the plate was centrifuged (2000 RCF, 5 min, 15 °C). The supernatant (580 µL) was transferred to a new plate (2nd plate). 300µL of hexane was added to the 1st plate, mixed, and centrifuged as mentioned above. 300µL of supernatant was transferred to the second plate and N_2 dried (30min, 20 °C). Extracts were re-suspended in 100µL of 2-propanol. The re-suspended samples were transferred to a Phree® phospholipid removal plate, with the 2nd plate reused as the collection plate. Phree® plate was centrifuged (2000 RCF, 5 min, 15 °C), and a vacuum was applied (10 psi, 10 min; repeated twice). The Phree® filtered samples in the 2nd plate were covered with a sealing mat and analysed using LC-MS/MS. The EDTA and heparin median peak area differences and level of significance were calculated using Wilcoxon non-parametric

signed-rank test. A *p*-value of >0.05 was considered statistically significant. R Version 3.6.1 (https://www.r-project.org/ was used for the statistical analysis and graphing.

2.3 Results

2.3.1 FSV stability at different storage conditions

FSV stability at different storage conditions was tested using three experimental procedures. These include a) unspiked heparin plasma FSV stability at -80°C in the dark for six months with or without BHT, b) spiked heparin plasma FSV stability at -80°C in the dark for six months with or without BHT and c). FSV stability in heparin plasma extracts at 4°C in the dark for a week.

For the unspiked plasma stored for six months at -80°C in the dark, a low mean percentage change was observed in A group vitamers (retinol -0.4% (*P*=0.87) and retinoic acid -6.8% (*P*=0.1). By comparison, E (α-tocopherol -22.1% (*P*=0.05), γ-tocopherol -58.2% (*P*=0.004) and α-tocotrienol 60.1% (*P*=0.011)) and D (25-OH-D_3 -30.1% (*P*=0.033)) group vitamers had high mean percentage variation over the same period. The addition of BHT to the plasma did not significantly affect the FSV stability after six months (Table 2-3 and Figure 2-1).

The mean percentage change over six months at -80°C in the dark was also calculated using the heparin plasma spiked recoveries (Table 2-4 and Figure 2-2). At the 6-month mark, high mean percentage changes were detected for retinoic acid -61.5% (*P*=0.003), γ-tocopherol -36.2% (*P*=0.012), α-tocotrienol -26.4% (*P*=0.002) and MK-7 -57.8% (*P*=0.029). By contrast, the mean percentage change was low in retinol -12.8% (*P*=0.23), 25-OH-D_3 -0.6% (*P*=0.83), α-tocopherol 0.2% (*P*=0.99), K1 -11.8% (*P*=0.23) and MK-4 -17% (*P*=0.29). Similar to the spiked plasma, a rapid degradation was observed in the BHT-treated spiked plasma retinoic acid -48% and MK-7 54.4% after the 2nd month (Figure 2-2). In comparison to Spiked plasma, only BHT-treated 25-OH-D_3 3.8% (*P*=0.04) and γ-tocopherol -1% (*P*=0.02) showed significant improvement in the mean percentage change at six months.

Finally, the stability of nine FSV was measured following extraction in solvents (acetonitrile and hexane, 1:2) and stored at 4°C for seven days. Significant degradation of retinol, retinoic acid, 25-OH-D_3. α-tocotrienol, γ-tocopherol, α-tocopherol, MK-4, and K1 were observed with high mean percentage deviations (Figure 2-3). Notably, no significant change was detected for MK-7 (p = 0.1524) over the seven days (Table 2-5).

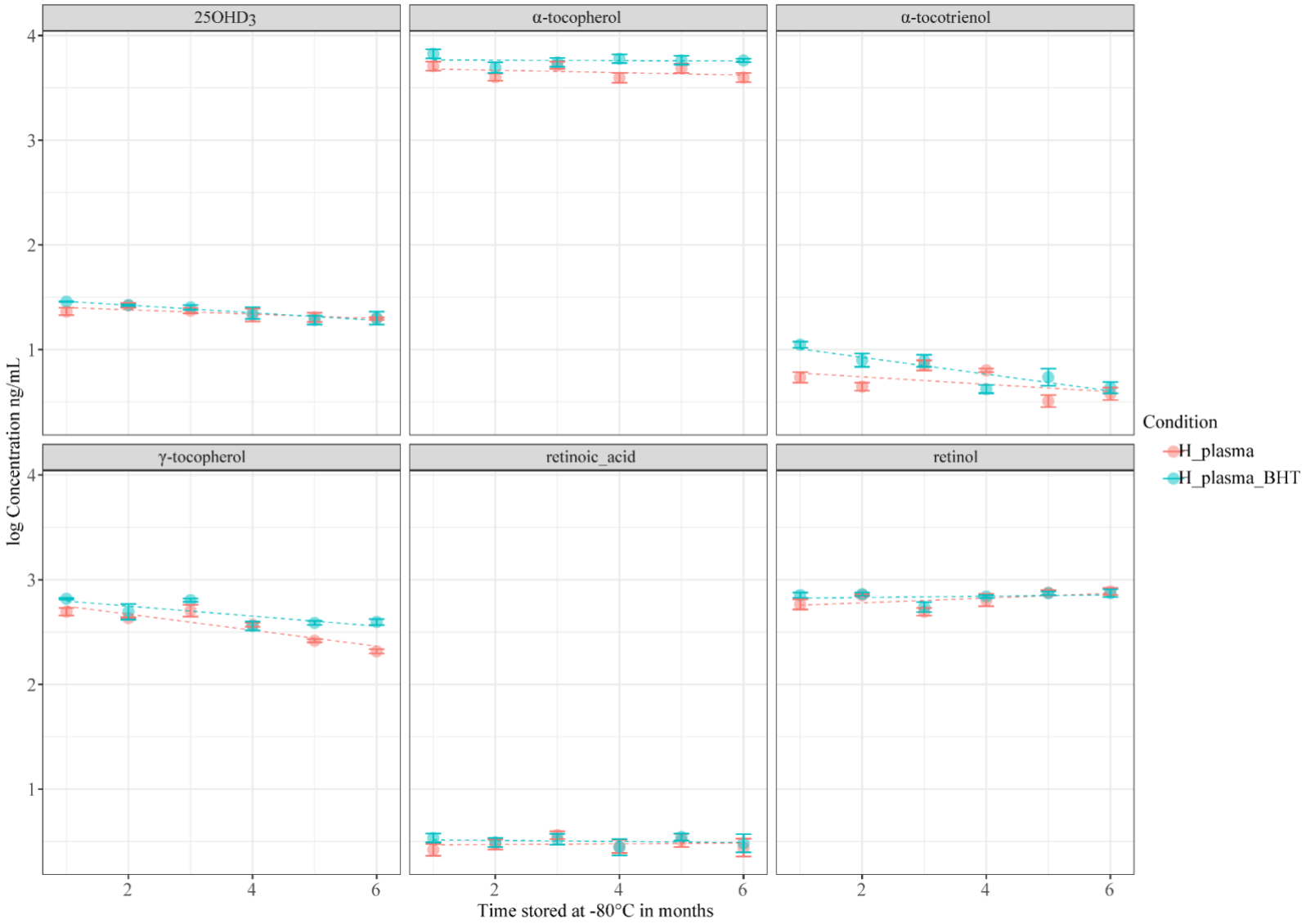

Figure 2-1: 6-month stability of the plasma FSV at −80 °C

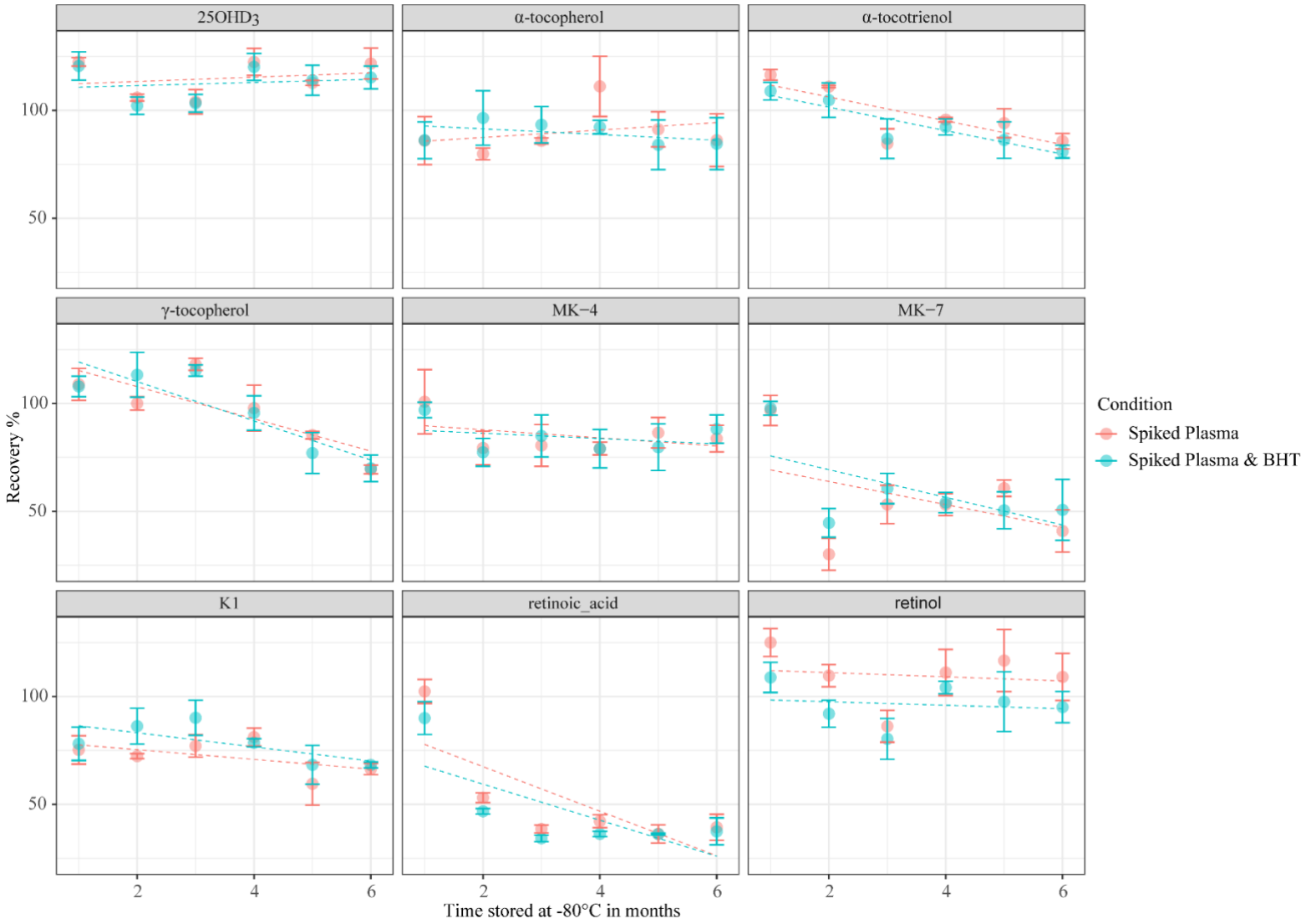

Figure 2-2: 6-month stability of the spiked plasma FSV at −80 °C

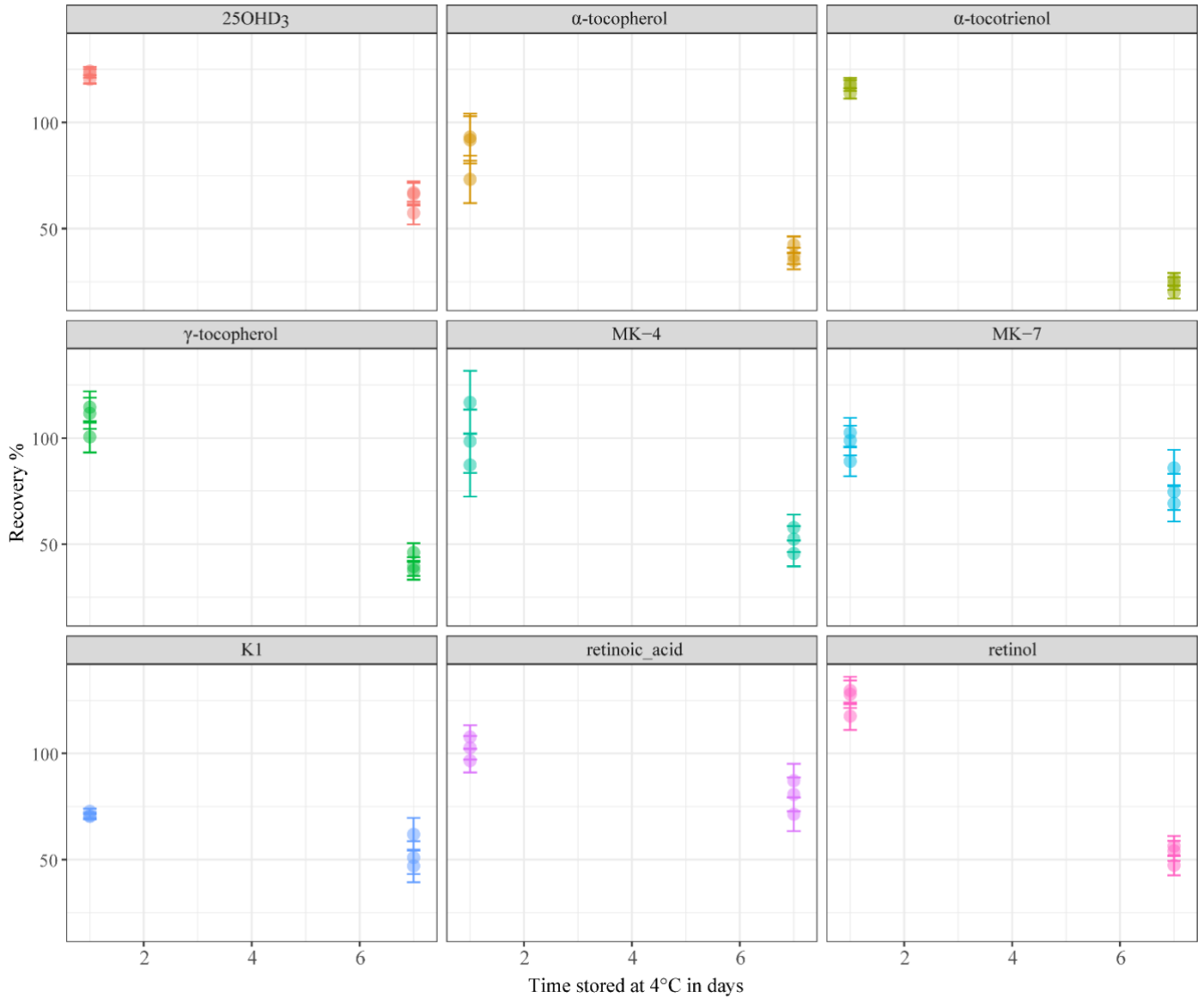

Figure 2-3: Seven-day plasma FSV stability at 4°C in organic solvents

Table 2-3: FSV Stability over six months -80°C in heparin plasma and heparin plasma with BHT

Vitamer	Condition	Mean % deviation					Month-6 *P* value	Plasma vs Plasma/BHT *P* value
		Month-2	Month-3	Month-4	Month-5	Month-6		
retinol	Plasma	-0.4	-12.3	-10.2	2.9	-0.4	0.872	0.334
retinol	Plasma-BHT	1.9	-22.9	-3.2	4.1	4.5	0.171	
retinoic acid	Plasma	12.4	37.7	8.2	23.3	-6.8	0.526	0.065
retinoic acid	Plasma-BHT	-9.5	-2.8	-17.9	2.0	-19.9	0.098	
25-OH-D_3	Plasma	14.4	1.7	-7.4	-11.9	-14.7	0.092	0.842
25-OH-D_3	Plasma-BHT	-7.5	-11.8	-21.7	-33.2	-30.1	0.033	
α-tocotrienol	Plasma	-18.6	30.1	16.2	-40.6	-30.2	0.008	0.444
α-tocotrienol	Plasma-BHT	-28.4	-29.4	-62.3	-50.5	-61.0	0.011	
γ-tocopherol	Plasma	-13.2	2.8	-24.9	-47.3	-58.2	0.004	0.009
γ-tocopherol	Plasma-BHT	-24.1	-2.9	-44.9	-41.1	-39.9	0.001	
α-tocopherol	Plasma	-21.4	1.8	-22.6	-4.4	-22.1	0.050	0.034
α-tocopherol	Plasma-BHT	-26.1	-17.2	-10.3	-13.3	-13.9	0.076	

Table 2-4: FSV spiked heparin plasma and spiked heparin plasma with BHT stability over six months at -80°C

Vitamer	Mean % deviation					Month-6 *P* value	Month-6 (%) deviation Plasma vs Plasma/BHT	*P* value
	Condition	Month-2	Month-3	Month-4	Month-5	Month-6			
retinol	Spiked Plasma	-12.3	-31.1	-11.1	-6.7	-12.8	0.228	-0.1	0.050
retinol	Spiked Plasma-BHT	-15.5	-26.2	-4.3	-10.3	-12.6	0.235		
retinoic acid	Spiked Plasma	-48.2	-62.3	-58.8	-64.5	-61.5	0.003	-3.2	0.756
retinoic acid	Spiked Plasma-BHT	-48.0	-61.9	-59.7	-59.7	-58.4	0.021		
25-OH-D_3	Spiked Plasma	-13.5	-15.1	0.03	-8.0	-0.6	0.830	3.8	0.040
25-OH-D_3	Spiked Plasma-BHT	-15.2	-14.3	-0.3	-5.5	-4.4	0.486		
α-tocotrienol	Spiked Plasma	-4.7	-27.4	-17.8	-19.2	-26.4	0.002	-0.6	0.296
α-tocotrienol	Spiked Plasma-BHT	-3.8	-20.3	-15.3	-20.8	-25.8	0.018		
γ-tocopherol	Spiked Plasma	-8.1	8.5	-10.1	-21.6	-36.2	0.012	-1	0.020
γ-tocopherol	Spiked Plasma-BHT	5.0	6.8	-11.4	-28.6	-35.2	0.005		
α-tocopherol	Spiked Plasma	-7.1	-0.1	29.2	6.1	0.2	0.990	2	0.794
α-tocopherol	Spiked Plasma-BHT	12	8.32	7.11	-2.43	-1.82	0.846		
K1	Spiked Plasma	-3.89	2.44	7.89	-20.88	-11.8	0.231	0.8	0.473
K1	Spiked Plasma-BHT	10.4	15.4	0.6	-12.5	-12.6	0.194		
MK-4	Spiked Plasma	-21.3	-20.1	-21.5	-14.3	-17.0	0.293	-7.9	0.563
MK-4	Spiked Plasma-BHT	-20.2	-12.4	-18.5	-17.8	-9.1	0.271		
MK-7	Spiked Plasma	-68.9	-45.0	-45.1	-37.3	-57.8	0.029	-9.6	0.455
MK-7	Spiked Plasma-BHT	-54.4	-38.0	-44.7	-48.4	-48.2	0.026		

Table 2-5: Plasma FSV stability at 4°C in organic solvents over Seven-days

Vitamer	**Mean % deviation**	***P* value**
retinol	-57.9	0.0002
retinoic acid	-22	0.0241
25-OH-D_3	-48.2	0.0011
α-tocotrienol	-79.9	0.0009
γ-tocopherol	-62.3	0.0030
α-tocopherol	-55.8	0.0163
K1	-25.2	0.0401
MK-4	-48.5	0.0463
MK-7	-20.9	0.1524

2.3.2 Solvent extraction to reduce matrix effect.

Single solvent extraction

This one-step "dilute and shoot" method was tested using several organic solvents (*i.e.*, pentanol, ethanol, methanol, acetone, acetonitrile, 2-propanol, ethyl acetate, and chloroform) at plasma: solvent ratios of 1:2, 1:3, and 1:4 (Table 2-6).

Comparatively, the best single solvent recoveries were obtained using acetonitrile plasma: solvent ratio of 1:4 (% recovery (CV); retinol 104.5(2); 25-OH-D_3 80(10); γ-tocopherol 84.5(7); α-tocotrienol 118.2(11) and K1 113.2(12) (Figure 2-4). All the other tests, solvents, or ratios used, resulted in either under or over-recoveries (Table 2-6). Across all the solvents experimented with, the E and K group vitamers consistently had the worst extraction recoveries, suggesting that the vitamers in these two groups are susceptible to ion suppression or enhancement under these conditions (Table 2-6 and Figure 2-4).

Liquid-liquid extraction with Isooctane/chloroform

Isooctane chloroform at 3:1(v:v) with ethanol was previously shown to result in good recoveries for all-trans-retinol 25-OH-D_2, 25-OH-D_3, and α-tocopherol [84], γ-tocopherol and K1 [89] when analyzing the top layer of the biphasic separation. I investigated this method's applicability to the current vitamers panel (Table 2-6). Isooctane/chloroform with ethanol (2:6) resulted in % recoveries (CV) within the allowable range for retinol 108.0(12.3); retinoic acid 104.5(9.3), 1-α-25-$(OH)_2$-D_3 84.7(5.9), and K1 79.5(3.8), but not for 25-OH-D_3, α-tocopherol, γ-Tocopherol, α-tocotrienol, MK-4 and MK-7 (Figure 2-4).

Irrespective of the modifications made to the deproteinisation solvent and ratios as per Table 2-6, the best extraction (Plasma: Acetonitrile: isooctane/ chloroform (1:3:6)) only recovered six of the vitamers within the acceptable range (*i.e.*, % recovery (CV); retinol 103.8(5.0); retinoic acid 82.5(12.2), 25-OH-D_3 74.7(13.1), 1-α-25-dihydroxy-D_3 130.2(13.0), α-tocopherol 127.2(3.7) and K1 84.2(8.4)) when extracting and analyzing the top layer of the solvent mix. A separate analysis of the bottom layer of the biphasic separation enabled recovery of FSV, %recoveries (CV); retinoic acid 120.3(12.9), 25-OH-D_3 110.4 (12.1), and K1 70.2(13.8)). Combining the top and bottom solvent layers only improved the %recoveries (CV) of retinol 112.6(2.5), retinoic acid 68.0(11.4), and K1 83.3(9.2) (Table 2-6). However, this method was

deemed inadequate for the present analysis due to the limited range of vitamers that could be quantified with acceptable recoveries.

Hexane Extraction

Hexane is one of the most commonly used solvents in FSV analysis [90,91,96–99]. Hexane use also relies on the principle of biphasic separation. The majority of the FSV quantification studies that used hexane also used methanol as the deproteinisation solvent [91,96,98,99].

I tested several deproteinisation solvents (IPA, methanol, and acetonitrile) with hexane at plasma: solvent ratios of 1:2:4, 1:2:6, and 1:4:8 (Figure 2-4). The current study observed poor recoveries for methanol and IPA combined with hexane for the E and K group vitamers. By comparison, acetonitrile and hexane yielded better recoveries irrespective of the solvent ratios tested (Table 2-6).

I investigated the impact of extracting the individual layers and combined layers following biphasic hexane extraction. For some FSV, bottom layer extraction (recovery % (CV), 1-α-25-dihydroxy-D_3 82.9(11.2), γ-tocopherol 126.8(6.0), and α-tocotrienol 113.5(1.6)) produced improved recoveries (Table 2-6). For retinoic acid 79.4(14.7), acceptable recoveries were obtained using the top layer following the biphasic separation. Combining both the top and bottom layers resulted in improved recoveries for all 10 FSV (Table 2-6).

Several studies have reported that double or triple-hexane extraction can yield better efficiency when compared to single-hexane extraction [91,98]. Similarly, in the present, I observed much-improved recoveries using plasma: acetonitrile: 2 x hexane ratio of 1:4:8 for all 11 FSV tested (recovery % (CV), retinol 106.4(6.4), retinoic acid 81.2 (6.0), 25-OH-D_3 84.2(1.5), 1-α-25-dihydroxy-D_3 81.4(6.9), α-tocopherol 107.6(3.3), γ-tocopherol 101.1 (7.2), α-tocotrienol 104.9(9.3), K1 82.7(10.9), MK-4 81.4(10.1), MK-7 88.3(12.3)) (Figure 2-4).

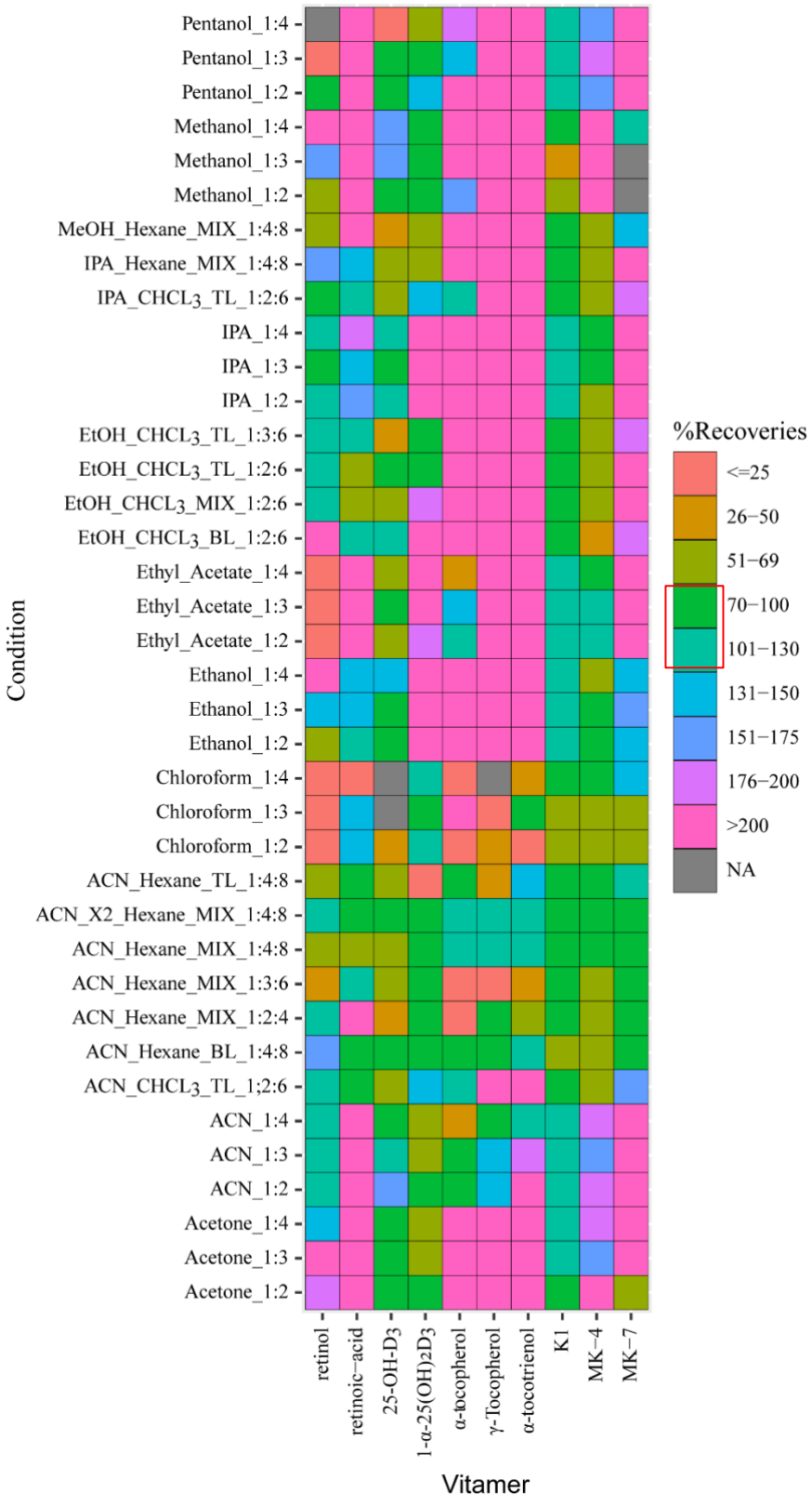

Figure 2-4: Plasma FSV recoveries using different extraction methods.

Abbreviations: TL, Top Layer; BL, Bottom Layer; MIX, Combination of both top and bottom layers; X2, Two times extraction using hexane; IPA, 2-propanol; ACN, Acetonitrile; MeOH, Methanol. *i.e.*, The red box marked the acceptable range (70-130% recovery) according to the EMA guidelines [212]

Table 2-6: FSV extraction efficiency (% recoveries) using a variety of organic solvents at different ratios.

Condition	Plasma: Solvent	retinol	retinoic acid	25-OH-D_3	1-α-25$(OH)_2$-D_3	α-tocopherol	γ-Tocopherol	α-tocotrienol	K1	MK-4	MK-7
Pentanol	1:02	85.8	639.6	81.7	140.3	236.6	471.2	384.8	118	166.1	515.1
Pentanol	1:03	N/A	643.4	71.3	78.9	141.7	405.5	361.1	123	192.4	355
Pentanol	1:04	10.9	464.4	7.5	61.3	194.2	254.1	288.6	107.7	172.5	273.2
Methanol	1:02	63.7	211.2	93.9	96.8	166.5	364.8	474.6	69	331.4	20.4
Methanol	1:03	170.7	300.6	164.6	82.1	1079.6	1092.6	1115.7	39.6	519.4	14.4
Methanol	1:04	215.9	265.3	171.6	73.5	583.2	421.8	330.4	82.5	211.8	113.2
IPA	1:02	120.4	153.1	115.9	235.4	876.8	684.8	568	112.7	69.4	277.9
IPA	1:03	78.3	149.1	85.5	241.5	458.5	314.4	307.1	107	77.7	268.2
IPA	1:04	112.3	200	127.9	248.6	1457.3	892.2	724.6	123.6	80.8	340.8
ACN	1:02	115.5	405.7	156.1	78.2	94.6	142.8	282.5	119.9	176.2	271.4
ACN	1:03	123.8	430.5	127.5	58.5	78.2	140.8	189.5	118.1	173.1	282.8
ACN	1:04	104.5	317	80	55.1	41.9	84.5	118.2	113.2	181.3	277.3
Acetone	1:02	185.8	290.4	93	81.1	893.8	799.5	862.8	98	264.2	68.2
Acetone	1:03	250.1	276.9	93	55.1	1487	709.8	874.3	109	171.1	274.6
Acetone	1:04	133.4	255	91.6	59	1375.8	743.2	818.5	118.8	190.2	420.6
Ethyl Acetate	1:02	N/A	349.7	54.4	193.2	116.9	501.9	320.4	100.6	103.3	257.3
Ethyl Acetate	1:03	N/A	444.1	74.6	215.9	147.4	568.3	244.5	116.4	126	331.2
Ethyl Acetate	1:04	N/A	435.8	66.6	249.2	37.1	415.3	329	109.2	94.4	374.1
Ethanol	1:02	57.3	129.3	86.5	247.6	609.2	594.3	417.5	115.5	74.6	143.1

Ethanol	1:03	102.9	137.4	93.3	252.4	664.5	512	450.6	115.1	77.8	173.3
Ethanol	1:04	407	136.9	134	253.9	1441.2	1350.2	714.6	122.1	68.4	142.4
Chloroform	1:02	N/A	137.2	25	119.3	N/A	42.1	N/A	50.7	57.4	51.4
Chloroform	1:03	N/A	130.2	24.8	88.5	243.7	7	72.2	50.9	54.7	59.5
Chloroform	1:04	N/A	N/A	22	103.7	N/A	21.6	39	88.7	83.9	135.3
MeOH/Hexane MIX	1:04:08	63.6	298.4	33.9	69.1	848.3	404.3	225.1	75.3	55.7	137.5
IPA/Hexane MIX	1:04:08	166.3	142.2	56.4	64.4	1260.6	1187.7	588	79.7	57.9	201
ACN/Hexane TL	1:04:08	51	79.4	61	N/A	80.6	37.1	134.1	81.3	74.6	106.6
ACN/Hexane MIX	1:04:08	161.8	84.4	70.2	96.7	73.8	98.3	110.5	51.9	54	76.1
ACN/Hexane BL	1:04:08	61.2	69.2	56.4	82.9	126.8	110.1	113.5	81.1	83.3	90
ACN/ X2 Hexane MIX	1:04:08	106.4	81.2	84.2	81.4	107.6	101.1	104.9	82.7	81.4	88.3
ACN/Hexane MIX	1:03:06	42.7	123.5	50.2	99.6	N/A	N/A	30.8	76.7	62.5	95.1
ACN/Hexane MIX	1:02:04	107.7	202.7	45.6	84.8	N/A	78.7	52.8	72.8	57.9	96.5
ACN/iso/CHCL3 TL	1:03:06	103.8	82.5	74.7	130.2	127.2	380.7	264.6	84.2	66.5	160.6
IPA/iso/CHCL3 TL	1:02:06	85.7	114.1	57.3	137.7	112.7	320.8	261.5	80.4	56.6	185.2
EtOH/iso/CHCL3 TL	1:03:06	108	104.5	44.9	84.7	926.2	709.7	330.5	79.5	67.5	181.4
EtOH/iso/CHCL3 TL	1:02:06	111	67.9	74.8	83.8	887.1	846.3	435.6	90.8	66.2	216.7
ACN/iso/CHCL3 MIX	1:02:06	112.6	68	64.4	184.8	1145.9	1040.5	485	83.3	52.8	213.8
ACN/iso/CHCL3 BL	1:02:06	556.9	120.3	110.4	407.6	1732.7	1153.8	416.8	70.2	48.5	182.3

Abbreviations: TL, Top Layer; BL, Bottom Layer; MIX, Combination of both top and bottom layers; X2, Two times extraction using hexane**;** IPA, 2-propanol; ACN, Acetonitrile; MeOH, Methanol

2.3.3 Use of Phree® phospholipid removal plates to reduce matrix effect

Phospholipid removal plates (PPT) enable one-step removal of the phospholipids and other matrix components using SLE principles. In the present method, the single-step protein and phospholipid removal using Phree® plates, as suggested by the manufacturers, was unsuccessful. I observed that several FSV that are at lower physiological concentrations within plasma were absent (*e.g.,* K1, MK-4, MK-7, 1α-25-$(OH)_2$-D_3, and retinoic acid) following extraction with acetonitrile and hexane (1:3). The remaining FSV had partial recoveries (n=30, retinol (63%), 25-OH-D_3 (55%), α-tocopherol (61%), γ-tocopherol (47%) and α-tocotrienol (59%)). I further tested the application of Phree® plates as a secondary cleanup method followed by deproteinisation and LLE. This approach successfully recovered all 11 FSV tested (Table 2-7).

Table 2-7: Relative matrix effect difference with or without Phree® phospholipid removal plates.

Analyte	**Relative Matrix effect [Iso labelled IS] (n=10)**	**CV%**	**Relative Matrix effect [Iso labelled IS+ Phree® plates} (n=10)**	**CV%**
retinol	47.1	25.1	104.2	2.9
retinoic acid	54.6	17.3	100.9	1.9
retinyl Palmitate	62.6	43.6	87.1	10.2
25-OH-D_3	108.3	2	98.1	5.1
1-α-25$(OH)_2$-D_3	114	4.6	101.3	2.0
α-tocopherol	149.6	14.1	125.4	5.1
γ-tocopherol	318.4	17.1	128.7	7.0
α-tocotrienol	425.1	16.5	102.7	2.2
K1	99.3	7	94.7	5.8
MK-4	85.8	11.7	99.1	10.8
MK-7	149.2	25.7	114.7	5.0

2.3.4 Anticoagulant (heparin and EDTA) influence on FSV quantification

The effect of anticoagulant use in FSV quantification was tested using EDTA and heparin plasma. There is a positive correlation between FSV peak areas and the anticoagulant choice (Figure 2-5 and Table 2-8). Significantly higher peak areas of retinol (p =1.10e-05), retinyl palmitate (p =1.10e-05), 25-OH-D_3 (p =1.10e-05), 1α-25-$(OH)_2$-D_3 (p =4.30e-05), α-tocopherol (p =1.10e-05), γ-tocopherol (p =1.10e-05), α-tocotrienol ((p =0.00105), K1 ((p =0.0426), MK-4 (0.02323), and MK-7 (0.00021) were observed using heparin plasma when compared to EDTA plasma. Retinoic acid (p =1.10e-05) was the only vitamer that showed a better recovery from EDTA than heparin-treated plasma. I concluded that the use of heparin-treated plasma in FSV analysis provides much higher peak areas for a broader range of vitamers than the EDTA-treated plasma.

Table 2-8: Effect of EDTA or heparin-treatment on FSV extraction from plasma

Compound	**EDTA** **n=10**	**Heparin** **n=10**	***p*-value**
retinol	22579190.06	69756925.86	1.10e-05
retinoic acid	4710058.25	1096497.80	1.10e-05
retinyl palmitate	2075732.51	4766115.25	1.10e-05
25-OH-D_3	7923294.00	18830450.24	1.10e-05
1-α-25$(OH)_2$-D_3	543278.74	818608.75	4.30e-05
α-tocopherol	40957665.88	1142301767.40	1.10e-05
γ-tocopherol	7951161.98	21410171.52	1.10e-05
α-tocotrienol	113600341.28	140612712.86	1.05e-03
K1	862804.57	1357045.85	4.33e-02
MK-4	1236923.06	2181105.21	2.32e-02
MK-7	207861.00	702623.85	2.10e-04

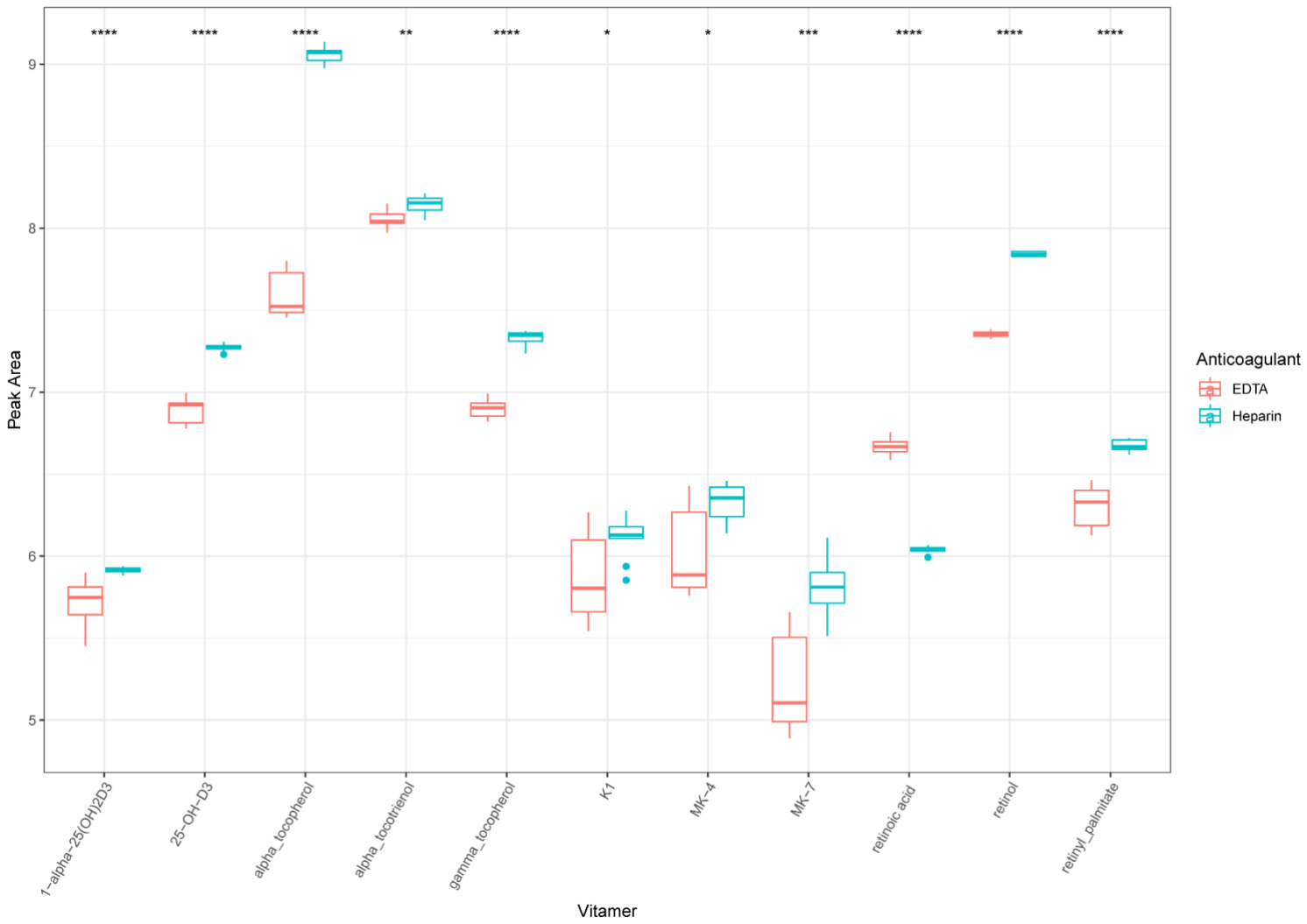

Figure 2-5: Comparison of FSV (n=10) concentrations in plasma collected in heparin and EDTA vacutainers. Significant codes 0, '****' ≤0.0001, '***' ≤0.001, '**' ≤0.01, '*' ≤0.05, '·' 0.1, ' ' 1.

2.4 Discussion

Method optimization is crucial for developing a sensitive, robust, and reproducible LC-MS/MS assay. In the present attempt at method optimization, attention was paid to FSV stability, matrix effect, anticoagulant use for plasma collection, sample preparation, liquid chromatography, and mass spectrometry.

2.4.1 FSV Stability

FSV degradation during storage, sample processing, and analysis has been discussed in previous studies [84,88,89]. However, experimenting on the physical properties of individual vitamers and exploring the cause of instability is time-consuming, labour-intensive, and, consequently, often neglected. Moreover, those studies that report on FSV stability are contradictory and limited in the range of quantified FSV [88,215].

My findings confirm that plasma retinol, retinoic acid, 25-OH-D_3, and α-tocopherol are stable for up to six months at -80°C while γ-tocopherol and α-tocotrienol are subject to some degradation over the same time-frame (Table 2-3 and Figure 2-1). I further investigated this phenomenon using recoveries of spiked FSV standards in heparin plasma. Retinol, 25-OH-D_3, and α-tocopherol, K1 and MK4 were stable for six months at -80°C, and degradation was observed for retinoic acid, γ-tocopherol, α-tocotrienol, and MK7 (Table 2-4 and Figure 2-2). Consistent with my results, Peng *et al.*, 1987 demonstrated that plasma retinol is stable for one year at -20°C [216]. On the contrary, Ocké *et al.*, 1995 demonstrated a dramatic decrease in plasma retinol (20-30%) and α-tocopherol (50%) concentrations after 12 months of storage at -20°C [217]. My study period is limited to 6 months; therefore, a direct comparison cannot be made with Ocké *et al.*'s outcome. Again, consistent with my observations, plasma retinoic acid has been demonstrated to be stable for 90 days at −20 °C (3.6%)) and 180 days at −80 °C (4.2%) storage [218]. Previous studies that explored the stability of 25-OH-D_3 reported that, in serum/plasma, it is stable for up to 1-2 weeks at RT and a few months to a few years at -20°C [219,220]. By contrast, Colak *et al.*, 2013 demonstrated that 25-OH-D_3 was stable in both serum and plasma for seven days at −20 °C and up to three months at −80 °C [221]. My results are consistent with this, as I observed a mean change of -11.81% (P = 0.059) over six months. However, 1 observed degradation of -30.053% (P = 0.033) after 6 months of storage at −80 °C (Table 2-3).

Butylated hydroxytoluene (BHT) is an antioxidant reported to protect analytes of interest from oxidative damage [222]. Several studies discussed the importance of butylated hydroxytoluene (BHT) in stabilizing the plasma A and E group vitamers during sample extraction [84,89,222]. In the present method, BHT was tested for its ability to stabilize plasma FSV during −80 °C storage. Compared to the untreated plasma, no significant improvement in the six-month FSV stability was observed in the BHT-treated plasma samples. Unfortunately, none of the previous studies investigated the effects of BHT addition on plasma FSV stability during storage. Therefore, comparisons or my results to existing findings could not be made. However, my study provides the first comprehensive overview of plasma FSV stability during storage.

When extracted in solvents, the FSV are much more labile than when they are present in whole blood, plasma, or serum [187]. Sample stability data after extraction is limited to a small number of FSV in the literature [93,223]. My results show that except for MK-7, all the other FSV in solvents degraded after seven days at 4°C (Table 2-5 and Figure 2-3). This is consistent with the previous stability result for retinol extracts, which indicates that it is only stable for 48h at 4°C [224]. Another study also reported that deproteinised retinol supernatant has a maximum stability time of 48h at 4°C in the autosampler [93]. Several other studies stated that the whole blood and plasma retinol in extracted solvents only lasted 72h before degradation at RT or cooled [225], while α-tocopherol degraded within 48h at RT [225] and within one week at -20°C [187]. Two studies found that 25-OH-D_3 extracts were stable for 3-7 days at RT; however, they did not report the level of degradation [156,226]. Although most of the previously published results agree with my findings, the stability data after extraction within the published literature is limited to a small subset of FSV. Therefore, future studies should address this gap in knowledge.

In the present study, I did not experiment with the effect of photosensitivity on FSV quantification. My choice of light protection measures (aluminium wrapping, amber glass vials, and subdued sunlight) was based on previously published results [87,88]. Several studies reported that the vitamin D metabolites α-tocopherol, and α-tocotrienol, are subject to light degradation and, therefore, recommended to minimise light exposure during extraction [227–229]. Evans *et al*., 2010 observed Light-induced degradation of retinoid extracts just after ~10 min exposure to white light (regular room light). They recommended using yellow light in a dark room to minimise this effect [230]. Vitamin K is highly light-sensitive and degrades at a rate of 20% per day by light exposure when present in serum [215]. AlBahrani *et al*., 2016 [88] reported no difference between subdued sunlight and ambient sunlight for retinol, 25-OH-D_3**,**

and α-tocopherol during their analysis. However, this result is not a good representation of 25-OH-D_3 photosensitivity as their sample processing time was only three hours.

The effect of freeze-thaw cycles on FSV solubility in the plasma matrix was discussed in several studies [231–233]. Hooster *et al.*, 2000 [231] reported 50% degradation of vitamin E after three freeze-thaw cycles. By contrast, Greaves *et al.*, 2014 [232] observed that vitamins A, E, and β-carotene remained stable during freeze-thaw. However, the number of freeze-thaw cycles was not mentioned in this study. Another study reported that the plasma lipophilic vitamins, 25-hydroxy-vitamin D, retinol, β- carotene, and α-tocopherol were stable during three freeze-thaw cycles when stored at -80°C [233]. However, none of the studies researched the stability of K group vitamers during the freeze-thaw cycles. Hence, I limited the number of freeze-thaw cycles to one in the current method to minimise the freeze-thaw degradation of the selected FSV.

Extraction solvents such as hexane, isooctane, and chloroform are water-immiscible and incompatible with most mobile phase solvents (*e.g.*, MilliQ water, methanol, ethanol, and IPA) [90]. Therefore, sample extracts in these solvents required drying before injecting into the LC/MS. The two most commonly used sample drying methods include vacuum concentrated-[234] and nitrogen-drying [90]. The speed vac vacuum concentrator uses the balance between vacuum, centrifugation, and heat to dry samples. One drawback of this method is that it is unsuitable for heat-labile analytes such as vitamins[235]. On the contrary, N_2 drying is oxygen-free, minimises oxidative damage, and quickly reduces samples to the endpoint volume or complete dryness. N_2 drying has been used in several previously published FSV quantification methods [90,91,97–99]. I also used N_2 to dry the FSV sample extracts, which overcame the issues with temperature lability and oxidative damage.

2.4.2 Sample matrix

The plasma matrix is complex and can interfere with the ionisation of the analytes of interest if correct measures are not in place. During method development, several techniques were tested to reduce this matrix effect, including the appropriate use of anticoagulant, reducing interference with cryoprecipitate, use of internal standards (IS), the addition of divert valve, avoidance of plastic consumables, and application of Phree® phospholipid removal plates.

The plasma or serum sample matrices commonly used in FSV quantification [84,89,91,98,99,236] are obtained from the whole blood by collecting in vacutainers containing anticoagulants (*e.g.*, EDTA, heparin, or citrate). The interactions between FSV and anticoagulants can impact the recoveries and the final sensitivity of an analytical method [237]. My results identified a significant difference between FSV peak areas in heparin and EDTA plasma. The plasma peak area difference was significantly higher for retinol, retinyl palmitate, 25-OH-D_3, 1α-25-$(OH)_2$-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, K1, MK-4, and MK-7. Several other studies [226,238] reported that the retinol, β carotene, and 25-OH-D_3 recoveries were higher from heparin plasma than EDTA. Yichao Huang *et al.*, 2016 [29] also reported significantly high vitamer concentrations of retinol (p <0.0001) and 25-OH-D_3 (p =0.03) in lithium heparin plasma when compared to EDTA plasma which is consistent with my observations. The lower retinol levels could be due to the dose-dependent isomerization of retinoids in EDTA plasma, causing retinol degradation [239]. However, in contrast to my findings, Bernstein *et al.*, 1987 found that the plasma concentrations of β-carotene (p <0.0001), lycopene (p <0.0001), γ-tocopherol (p <0.0004), and α-tocopherol (p <0.0001) were substantially lower in lithium heparin when compared to EDTA plasma. Three studies reported no association between α and β carotin, retinol, lycopene, and 25-OH-D_3 [240–242]. Therefore, it is clear that the associations between different anticoagulant use and FSV recoveries from plasma are inconsistent [226,238,240–242]. Moreover, the previous studies investigating this association are limited to the range of vitamers tested. One can only speculate as to why these FSV concentrations vary in different anticoagulants. Therefore, the effects of anticoagulant use on vitamer-specific concentrations need to be further addressed in future studies.

The formation of cryoprecipitate (fibrinogen, factor XIII, von Willebrand factor (vWF), factor VIII (antihemophilic factor), and other proteins [243]) is comparatively high in heparin plasma when compared to EDTA. This was also observed during my method development. As also discussed in the literature [244], one of the main obstacles faced in the present method development was the cryoprecipitate blocking the pipette tips while extracting and dispensing plasma leading to inaccurate quantitative transfers. Previous studies provide evidence that slow thawing at low-temperature 8-10 °C promotes the aggregation of cryoprecipitate. By contrast, water bath temperatures at 15-25 °C minimise the formation of cryoprecipitate, and incubation at >30°C eliminates the formation of cryoprecipitate. Unfortunately, as mentioned previously, the FSV are heat-labile [88], and conventional water bath temperatures >30°C can cause heat

degradation. Considering these factors, water bath temperatures at 20°C were selected to freeze-thaw the samples in my method, which greatly reduced the cryoprecipitate formation.

The internal standards (IS) have similar properties to the target compounds and act as references to improve the accuracy of the quantitation [245]. One of the major drawbacks of using the deuterated IS is the loss of deuterium over the course of the analytical run. The deuterium exchange with protons and the IS can transform into the unlabelled native analyte [101]. This phenomenon has been observed to cause unrealistic, irreproducible, and inaccurate results, consistent with my observations [246]. Carbon-13, on the contrary, has a high isotopic purity (99.5%) and is chemically stable; there can be no loss of 13C via exchange with 12C [247]. Therefore, higher deuterated and 13C labelled IS that best represent the chemical properties of these analytes were used as reference material in the present analysis (*e.g.*, $[^{2}H]_{8}$ Retinol, $[^{2}H]_{7}$ 25-OH-D_{3}, $^{13}C]_{6}$ α-tocopherol, and $[^{13}C]_{6}$ phylloquinone). However, the addition of 13C labelled IS solely was insufficient to control for ion suppression caused by the matrix effect (Table 2-7).

The role of a divert valve is to direct the column eluent phase to waste, thus removing the unwanted matrix components while enabling the compounds of interest to be directed into the mass analyser [248]. Plasma constituents such as phospholipids, triglycerides, and small plasma proteins can co-elute with the analytes of interest, resulting in reduced analytical sensitivity [249]. Thus, in the method I developed, the divert valve was used at the beginning and the end of the analytical run to minimise matrix interference. Additionally, the analytical runs that were performed without using the divert valve had significantly reduced numbers of samples (>50) and an increased loss of sensitivity associated with contamination of the ion source. By contrast, using the divert valve to remove waste increased the number of plasma samples that could be analysed, before the ion source interface needed to be cleaned, to <100.

Plastic consumables (*e.g.*, pipette tips, flasks, centrifuge tubes, and 96 well plates) are commonly used in laboratories because they are convenient, safe, and cost-efficient. It is well documented that the surface binding of FSV to plastic consumables is much greater than to glass [250,251]. When extracted in organic solvents, all FSV have a surface binding affinity for plastic [252–254], with the exception of vitamer K1, which binds to both glass and plastic, leading to low recoveries [215]. Therefore, I ensured that the developed method used glass-coated plates, glass inserts, and amber glass vials in attempt to minimise this issue.

As also mentioned previously, the highly ionic nature of phospholipids makes them one of the major causes of ion suppression during FSV analysis [255]. My method incorporated Phree® plate extraction as a secondary cleanup method followed by deproteinisation and LLE to minimise analyte loss, the matrix effect (Table 2-7) and improve the recoveries.

2.4.3 Solvent Extraction

Solvent extraction is the most widely used technique in FSV method development [90,91,96–99]. This section will discuss two of the main solvent extraction methods, including single solvent extraction (dilute and shoot method) and liquid-liquid extraction (LLE).

Single solvent extraction is an attractive approach for FSV quantification as it reduces: the labour required to perform the method, solvent use, and time [93]. However, in the present method, irrespective of the solvents or ratios tested, the dilute and shoot method's overall recoveries were limited to a narrow range of analytes and significantly compromised by the plasma-induced matrix effect compared to recoveries from LLE (Figure 2-4 and Table 2-6). Le *et al*., 2018's one-step acetonitrile extraction successfully recovered retinol, 25-OH-D_2, 25-OH-D_3, epi-25(OH)D_3, and α-tocopherol [93]. Another study reported the acceptable recoveries of retinol (93.6%), α-tocopherol (101%), and γ-tocopherol (101 %) using ethanol extraction [256]. In comparison, good recoveries (CV) were only observed for retinol 102.9(5.4) and 25-OH-D_3 127.5 (3.4), using acetonitrile and Ethanol extraction in the present study. The recoveries of α-tocopherol 664.48 (0.74) and γ-tocopherol (CV) 512.02 (13.51) were severely compromised by the matrix effect (Table 2-6 and Figure 2-4). The organic solvents (pentanol, methanol, acetone, 2-propanol, ethyl acetate, and chloroform) that were experimented within this study are commonly used as solvent combinations in LLE. My observations indicate that these solvents should not be used in single solvent extraction methods when analyzing multiple FSV in complex matrixes.

The liquid-liquid extraction (LLE) technique relies on the biphasic separation of organic solvents is often used in FSV methods due to its higher recoveries, purity, and reduced matrix effect. Midttun *et al.*, 2011 and 2016 [84,89] used a methanol protein crush followed by LLE using isooctane and chloroform 3:1 to successfully extract (recovery %) all-trans-retinol (100.6), 25-OH-D_2 (99.0), 25-OH-D_3 (103.2) and α-tocopherol (105) [84], γ-tocopherol (94.3) and K1 (91.3) from EDTA plasma [89]. In their method, the top layer of the biphasic separation was collected, dried with N_2, and injected into the LC/MS. Midttun *et al*.'s method was tested

in the present method optimization. However, I observed poor recoveries irrespective of the deproteinisation solvents, ratios, or the number of layers that were assessed (Figure 2-4 and Table 2-6). It is possible that the poor recoveries could be due to the wide range of FSV concentrations in the current panel (0.050 ng to 14000 ng). Notably, the poor extraction recoveries primarily affected the E and K group vitamers (*e.g.*, α-tocopherol, γ-tocopherol, α-tocotrienol, MK-4, MK-7). Therefore, I decided not to proceed with Midttun *et al.*'s method as the recoveries were insufficient for the current panel of FSV.

Liquid-liquid extraction using plasma: acetonitrile: 2x hexane ratio of (1:4:8) resulted in the best-combined extraction efficiency for the selected FSV. The majority of published studies used the basic principles of deproteinisation followed by one-time hexane extraction [90,91,96–99]. My results indicate that 2x hexane extraction provides significantly better recoveries than single hexane extraction (Table 2-6 and Figure 2-4). Consistent with this, Konieczna *et al*., 2016 [98] used a serum: methanol: 2x hexane ratio of 1:4:4 and successfully quantified retinol, α-tocopherol, 25-OH-D_3, 1α-25$(OH)_2D_3$ at physiological concentrations. Khaksari *et al*., 2017 [91] used plasma: methanol: 3x hexane at a ratio of 2:2:5 in their extraction method and reliably quantified retinol and α-tocopherol.

2.4.4 Robotic automation

The extraction of the analytes from the dissolved matrix is one of the key steps in LC-MS/MS sample processing. Usually, liquid handing steps are performed using manual pipetting. Conventional manual pipetting is labour-intensive, time-consuming, and error-prone [206]. The application of liquid handing robots during sample preparation provides solutions for fully automated workflows and minimises the aforementioned complications. Therefore, the Eppendorf EpMotion liquid handling robot was used to perform all the liquid handling tasks.

Several critical changes were made to the standard EpMotion liquid handling process to maximize the extraction of the analytes of interest. For example, the liquid handling robot was optimized during the extraction process to aspirate 580 µL of the supernatant from 650 µL total volume without disrupting the protein pellet at the bottom. This was achieved by changing three crucial settings: aspiration speed (0.5 µl/s), aspiration delay (0.8ms), and immersion depth (3 mm from the bottom of the well). While dispensing the extracted supernatant, dispensing speed (1.5 µl/s) and dispensing delay (0.8ms) were controlled to minimise the splash contamination using epBlue Client version 40.6.2.6 software.

To hold the liquid in the pipette tips, EpMotion uses the air displacement pipetting technique, which uses a vacuum to hold the liquid. However, when this pressure is applied to volatile liquids such as hexane, they vaporize, increasing the pressure inside the pipette tips, forcing the liquid out of the pipette tips [257,258]. The density of the volatile organic solvents [*e.g.*, hexane (655 kg/m^3, acetonitrile (786 kg/m^3), methanol (792 kg/m^3), ethanol (789 kg/m^3), 2-propanol (786 kg/m^3)] used in this method varies significantly. This posed a new challenge as the standard robotic setup did not have a setting for the liquid handling of these solvents. Low-density volatile liquids (*e.g.*, hexane) started dripping off from the pipette tips during transfers leading to cross-contamination. Pre-wetting (3x for hexane and 2x for the other solvents) the pipette tips before aspirating the solvents eradicated this problem.

2.4.5 LC-MS/MS

The final analytical sensitivity of an FSV method primarily lies in the correct choice of analytical platforms and optimization of its parameters [259]. Liquid chromatography and tandem mass spectrometry were used in the current method development.

The most critical components in the liquid chromatography methodology are the analytical platform, mobile phase, mobile phase solvents, and stationary phase (analytical column) [259]. The introduction of the HPLC technique has revolutionized the FSV quantification process compared to previously used methods such as gas chromatography, thin-layer chromatography, and immunoassays [126,199,200]. However, the transition from the HPLC to UHPLC reduced dwell time by reducing the number of points scanned in a peak without compromising the sensitivity and narrower peaks [259]. Therefore, I used UHPLC as my method's analytical platform of choice.

The Agilent InfinityLab Poroshell 120 (100 x 2.1 mm) 2.7 µm analytical column was previously used in FSV (α-tocopherol and 25-OH-D_3) analysis and is renowned for its ability to retain nonpolar analytes [201]. Another study also reported using Poroshell 120 EC-C18 (4.6 x 100 mm, 2.7 µm) column to quantify vitamin A palmitate, A acetate, E succinate, and D [260]. Due to poor separation, the Poroshell 120 column has not been used to quantify K group vitamers. However, my method's application of the Phenomenex Kinetex C18 100 Å (100x 2.1 mm, 1.7µm) column enabled all 11 FSV targets, including three K group vitamers (K1, MK-4, and MK7) to be successfully chromatographically resolved. Notably, two other studies also

quantified Vitamin D3, MK-7, and MK-4 using Phenomenex Kinetex 2.6 C18 (100 × 2.1 mm, 2 μm) and Kinetex C18 5um, 2.1 mm x 15 cm columns [201,261].

The addition of the mobile phase additives stabilizes the solution's pH balance, improving the ionisation and sensitivity of the analytes of interest [262]. In the present method, the mobile phases (A): 5 mM ammonium formate, 0.1% formic acid in Di H_2O, (B): 0.1% formic acid in methanol, and (C) 100% 2-propanol were used to achieve the best resolution. In methanol or acetonitrile, formic acid 0.1% and 5-10 ammonium formate are the most commonly used mobile phase additives in published FSV methods [98,103,104]. I discovered that the elution of highly non-polar FSV (*e.g.,* K1, retinyl palmitate, and MK-7) required the addition of the third, less polar mobile phase ramped to a high percentage for a prolonged period.

APCI is commonly used to ionize mid to nonpolar analytes [90,98,156] and ESI for more polar compounds. However, ESI [84,89,91,93,95,97–99,104] is the most commonly used ionisation source in FSV analysis. Not all FSV are nonpolar. Therefore, it can be argued that the choice of ESI is due to its ability to ionize a broader range of FSV, covering both polar and nonpolar analytes. By contrast, APCI is more suitable for nonpolar analytes. I observed that ESI ionized a more comprehensive range of FSV while APCI was limited to retinol, 25-OH-D_3, α-tocopherol, γ-tocopherol, α-tocopherol, K1, and K3. The choice of positive or negative ionisation using ESI depends on solvent composition, mobile phase additives, analyte concentration, pH, and flow rate [262]. In the method I developed, both positive and negative ionisations were tested for the selected FSV. The majority of the FSV ionized better using the ESI-positive mode. Of the 11 FSV in the present panel, ten were highly responsive for the protonated positive ion $[M+H]^+$, while MK-7 favoured protonated NH_4 ion $[M+NH_4]^+$.

2.5 Conclusion

A finely optimized method is the secret of developing a sensitive, robust, and accurate LC-MS/MS method. The chemical and physical properties of the FSV vary from one analyte to another and the sample matrix. Such complexity triggers new challenges that must be understood and addressed before an analytical method can be fully validated. During the method development process, the majority of the complexity issues were related to vitamin stability, including storage conditions, photosensitivity, use of plastic consumables, N_2 drying, freeze-thaw cycles, and the remainder were due to matrix complexity, including matrix type and anticoagulant use. The analytical optimization was mainly addressed in three sections; sample preparation, liquid chromatography, and mass spectrometry. During sample preparation, robotic automation was optimized to improve the fluid handling process. The extraction techniques, liquid-liquid extraction, solid-phase extraction followed by deproteinization, and single solvent extraction using various organic solvents and ratios, were tested. Liquid-liquid extraction using Hexane/acetonitrile at a 4:1 ratio was identified to produce the best recoveries for the selected vitamers. The liquid chromatography was optimized using a Phenomenex Kinetex C18 100 Å (100x 2.1 mm) 1.7µm column with gradient elution using A): 5 mM ammonium formate, 0.1% formic acid in Di H_2O, (B): 0.1% formic acid in methanol and (C) 100% 2-propanol. The orbitrap mass analyser using an ESI probe in positive ion mode provided the best detection.

Chapter 3

Absolute Quantification of Eleven A, D, E and K Vitamers in Human Plasma Using Automated Extraction and LC-MS/MS

3.1 Background

Fat-Soluble Vitamers [FSV] (*e.g.*, A, D, E, and K) are a large family of essential macronutrients involved in vital physiological functions such as growth, development, maintenance of blood glucose levels, protein metabolism, and regulation of cell growth [99,263,264]. FSV are not synthesised by human cells; they must be obtained from the diet [263], the gut microbiome [265], or by synthesis with Ultraviolet B (UVB) light exposure in the skin [266]. Circulating concentrations of FSV are influenced by several factors, including micronutrient bioavailability, body composition, dietary intake and supplementation, and disease status. Deficiency or excess supplementation of FSV is a significant risk factor for lifestyle-related diseases such as cardiovascular disease [267], type-2 diabetes Mellitus, cancer, and metabolic complications [96,98].

Therefore, there is a need to develop robust, reliable, high throughput methods to quantify circulating FSV [88]. Such methods are essential in large-scale cohort studies designed to understand the metabolic interactions between vitamers and other metabolic biomarkers. Generally, there is a 1000-fold variance in endogenous concentrations between low abundance FSV and those present at much higher levels, *e.g.*, α-tocopherol (5000-13000 ng/mL) to MK-7 (0.4-2.2 ng/mL). Circulating FSV concentrations are lower in children, especially infants (2 months to 1 year of age). These variations in circulating FSV concentrations make it challenging to develop a single method that provides precise measurements across the clinically relevant range of concentrations [268].

Analytical techniques for quantifying FSV in biological material have traditionally relied on immunoassays and gas chromatography. Unfortunately, these methods are subject to non-target analyte interferences, are time-consuming, and have lower sensitivity than LC-ESI-MS/MS techniques [269]. The advent of isotope dilution liquid chromatography-tandem mass spectrometry [LC-ESI-MS/MS] has revolutionised analytical detection methods and rapidly become the gold standard for FSV quantification [96]. Unfortunately, current analytical methods using LC-ESI-MS/MS are restricted to a limited number of vitamers or suffer from poor sensitivity [270].

Method development for FSV quantification is made challenging by compound instability, bioavailability, low circulating concentrations, biochemical heterogeneity, matrix effects, and a lack of suitable stable isotopically-labelled standards for chromatographic analysis (Figure 3-1) [87,88].

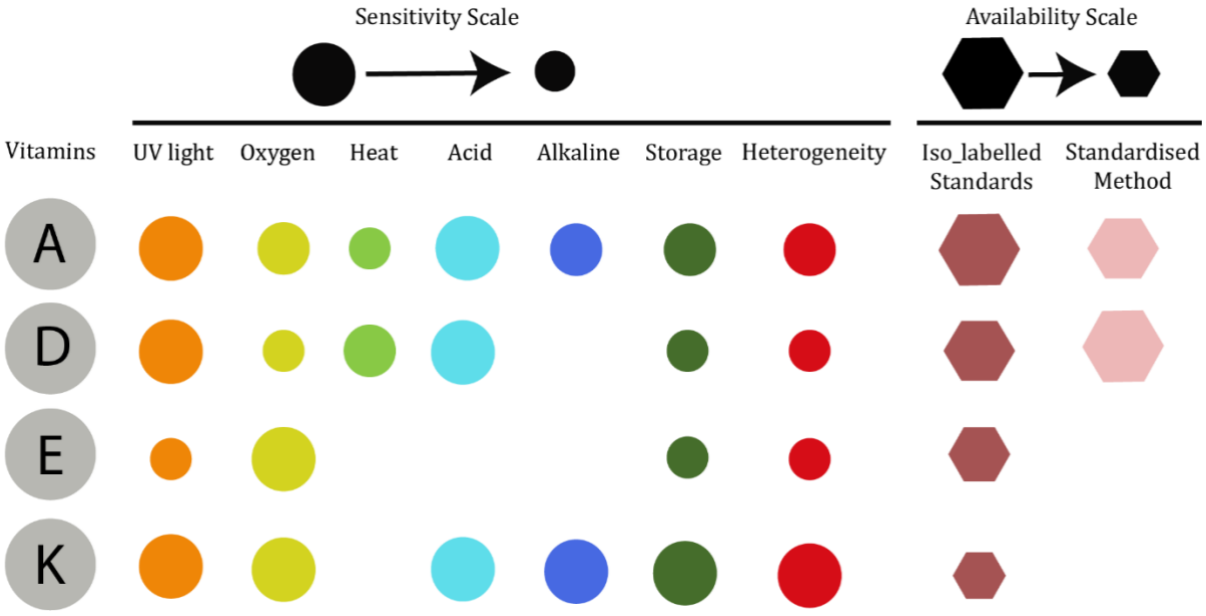

Figure 3-1: Analytical complications of FSV.
Both A and D group vitamers are sensitive to light, oxygen, heat, and acidic pH [88]. Xanthophyll's (metabolites of vitamin A) are also sensitive to alkaline pH. E group vitamers are light and oxygen labile but stable at high temperatures and acidic and alkaline pH. K group vitamers have the most heightened sensitivity to light, acid, and alkaline pH but are stable in the presence of heat [70,88,187,271,272].

Chapter 3 was modified from G.R.P. Arachchige, E.B. Thorstensen, M. Coe, J.M. O'Sullivan, C.J. Pook, Absolute quantification of eleven A, D, E and K vitamers in human plasma using automated extraction and UHPLC-Orbitrap MS, Anal. Chim. Acta. 1181 (2021) 338877. https://doi.org/10.1016/j.aca.2021.338877 [184]. I have developed an automated, hybrid sample preparation method that uses ultra-high-performance liquid chromatography coupled with tandem mass spectrometry [LC-ESI-MS/MS] to quantify 11 FSV in plasma. This rapid, versatile method enables quantification with high sensitivity and specificity following the separation of parent vitamins and their metabolic products from a plasma matrix without derivatisation.

3.2 Material and methods

3.2.1 Ethical approval and funding

The study was conducted according to the guidelines of the declaration of Helsinki, and approved by the Auckland Health Research Ethics Committee of the University of Auckland (project 3408 (review reference AH3408)). This study was funded by the MBIE Catalyst grant (The New Zealand-Australia Life Course Collaboration on Genes, Environment, Nutrition, and Obesity (GENO); UOAX1611; to JOS).

3.2.2 Materials, chemicals, and reagents

All-*trans* retinol, retinyl palmitate, retinoic acid, α-tocopherol, γ-tocopherol, K1, and MK-4 were purchased from BGD synthesis (Wellington, New Zealand). MK-7 was purchased from Sigma Aldrich PTY LTD (Castle Hill, Australia). α-tocotrienol was purchased from eMolecules (La Jolla, USA). $[^{2}H]_{8}$ Retinol, 25-OH-D_3, 3-epi-25OH-D_3, 1-α-25$(OH)_2$-D_3 were purchased from Cambridge Isotope Laboratories, Inc (Andover, USA). $[^{13}C]_{6}$ α-tocopherol and $[^{13}C]_{6}$ phylloquinone standards were purchased from Isosciences (Ambler, USA). $[^{2}H]_{7}$ 25-OH-D_3 was purchased from PM Separations NZ Ltd (Auckland, New Zealand). Phree® phospholipid removal plates were purchased from Phenomenex (Auckland, NZ). Glass-coated 96 well 1mL microplates were purchased from Thermo Fisher Scientific NZ Ltd. Acetonitrile (Optima® LC/MS grade) and isopropanol (100% pure, LC/MS Grade) were purchased from Fisher Scientific (USA). Ethanol (100% HPLC grade) and methanol (Hypergrade for LC/MS) were supplied by Merck (USA). The HPLC grade, 98% pure hexane purchased from Scharlab (Barcelona, España). The bovine albumin low-fat acid powder was purchased from MP Biomedicals (New Zealand)

3.2.3 Sample collection and preparation

Stock solutions of all-*trans*-retinol, retinyl palmitate, retinoic acid, 25-OH-D_3, 1-α-25$(OH)_2$-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, K1, MK-4, MK-7, and isotopically-labelled internal standards were prepared on ice in ethanol and stored (-80°C) until use. Precautions such as the use of amber glassware, subdued light, and aluminium foil wrapping of tubes were taken to protect the stocks from UV damage at all stages.

The working calibrators containing 11 FSV were prepared as a mixture with concentrations ranging from 0.31-14000 ng/mL (Table 3-1) in phosphate-buffered saline (PBS) containing bovine serum albumin (BSA) 4% (w/v). The calibrator mixture was serially diluted to achieve the desired concentrations. Acetonitrile was used as the blank calibrator. The internal standards: 4000 ng/mL of ($[^{2}H]_{8}$ Retinol, $[^{2}H]_{7}$ 25-OH-D_3, $[^{13}C]_{6}$ α-tocopherol and $[^{13}C]_{6}$ phylloquinone, were prepared as a mix in 200 µL acetonitrile deproteinisation solution.

Table 3-1: Concentrations (ng/mL) of the standard curve

Compound	Physiological conc	Std 1	Std 2	Std 3	Std 4	Std 5	Std 6	Std 7
retinol	630	1600	800	400	200	100	50	25
retinoic acid	3	115	57.5	28.75	14.38	7.19	3.59	1.80
retinyl Palmitate	200	800	400	200	100	50	25	12.5
25-OH-D_3	64	364.35	182.18	91.09	45.54	22.77	11.39	5.69
1-α-25$(OH)_2$-D3	0.06	165	82.5	41.25	20.63	10.31	5.16	2.58
α-tocopherol	5000	14000	7000	3500	1750	875	437.5	218.75
γ-tocopherol	510	2000	1000	500	250	125	62.5	31.25
α-tocotrienol	27	100	50	25	12.5	6.25	3.13	1.56
K1	4	20	10	5	2.5	1.25	0.63	0.31
MK-4	2.2	20	10	5	2.5	1.25	0.63	0.31
MK-7	2.2	30	15	7.5	3.75	1.88	0.94	0.47

Abbreviations: conc, Concentration; Std, Standard

Table 3-2: Spiked concentrations to prepare the QCs

Compound	High (µg/mL)	Mid (µg/mL)	Low (µg/mL)
retinol	1.60	0.40	0.10
retinoic acid	0.12	0.03	0.01
retinyl Palmitate	0.80	0.20	0.05
25-OH-D_3	0.36	0.09	0.02
1-α-25$(OH)_2$-D3	0.17	0.04	0.01
α-tocopherol	14.0	3.50	0.88
γ-tocopherol	2.00	0.50	0.13
α-tocotrienol	0.10	0.03	0.02
K1	0.02	0.01	0.00
MK-4	0.02	0.01	0.00
MK-7	0.03	0.01	0.00

Peripheral blood samples from Liggins Institute volunteers were collected in lithium heparin tubes, de-identified, and centrifuged (1600 RCF, 10 min at 4°C); the resulting plasma was pooled and transferred to 1.5 mL microcentrifuge tubes and stored (-80°C) until use as quality controls in FSV assays. Quality control samples were spiked with low, medium, and high concentrations of each vitamer (Table 3-2) to cover the physiological range; three replicates of each were used per assay.

All additions, mixing, supernatant removal, and vacuum extraction steps of the sample preparation procedure were carried out on an epMotion 5075 liquid handling robot (Eppendorf epMotion® 5075t, New Zealand). Briefly, appropriate concentrations (Table 3-1) of the calibration standards (all-*trans*-retinol, retinyl palmitate, retinoic acid, 25-OH-D_3, 1-α-25$(OH)_2$-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, K1, MK-4, and MK-7) were generated to make the calibration curves. Standards, QCs, and unknown plasma (45 µL) were transferred into glass-coated 96 well microplates. Deproteinisation was initiated by the addition of 200 µL acetonitrile containing the internal standards, followed by mixing (15 min, 800 rpm (linear) at 4 °C). To initiate biphasic separation, hexane (400 µL) was added to each well and then mixed (10 min at 800 rpm (linear), 15 °C). The plate was centrifuged (2000 RCF, 5 min, 15 °C), and the hexane and acetonitrile layers (580 µL) were transferred into a new glass-coated 96-well microplate (2nd Plate). The remaining protein pellet was washed a second time (300 µL of hexane) to complete the extraction procedure, and that supernatant (300 µL) was also transferred into the 2nd plate.

The 2nd plate was N_2 dried (30min, 15 °C), and the extracts were re-suspended in 100 µL isopropanol. The sample was transferred into a Phree® phospholipid removal plate, with the 2nd plate reused as a collection vessel. The Phree® plate and 2nd collection plate were shaken (1200 rpm (linear), 15 °C , 10 min), centrifuged (5000 RCF, 5 min, 15 °C), and a vacuum applied (10 psi, 10 min; repeated twice). The collection plate was covered with a sealing mat (Eppendorf, NZ), and the resultant elutants were analysed for FSV (Figure 3-2).

Analysis of the FSV/IPA solution was carried out using a Q-Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Germany) equipped with a heated electrospray ionisation source. The UHPLC system for sample introduction and separation consisted of an Accela 1250 UHPLC pump (Thermo Fisher Scientific, Austin, Texas USA), a PAL autosampler with refrigerated sample trays (CTC Analytics, Thermo), and a HotDog 5090 column oven (Thermo)

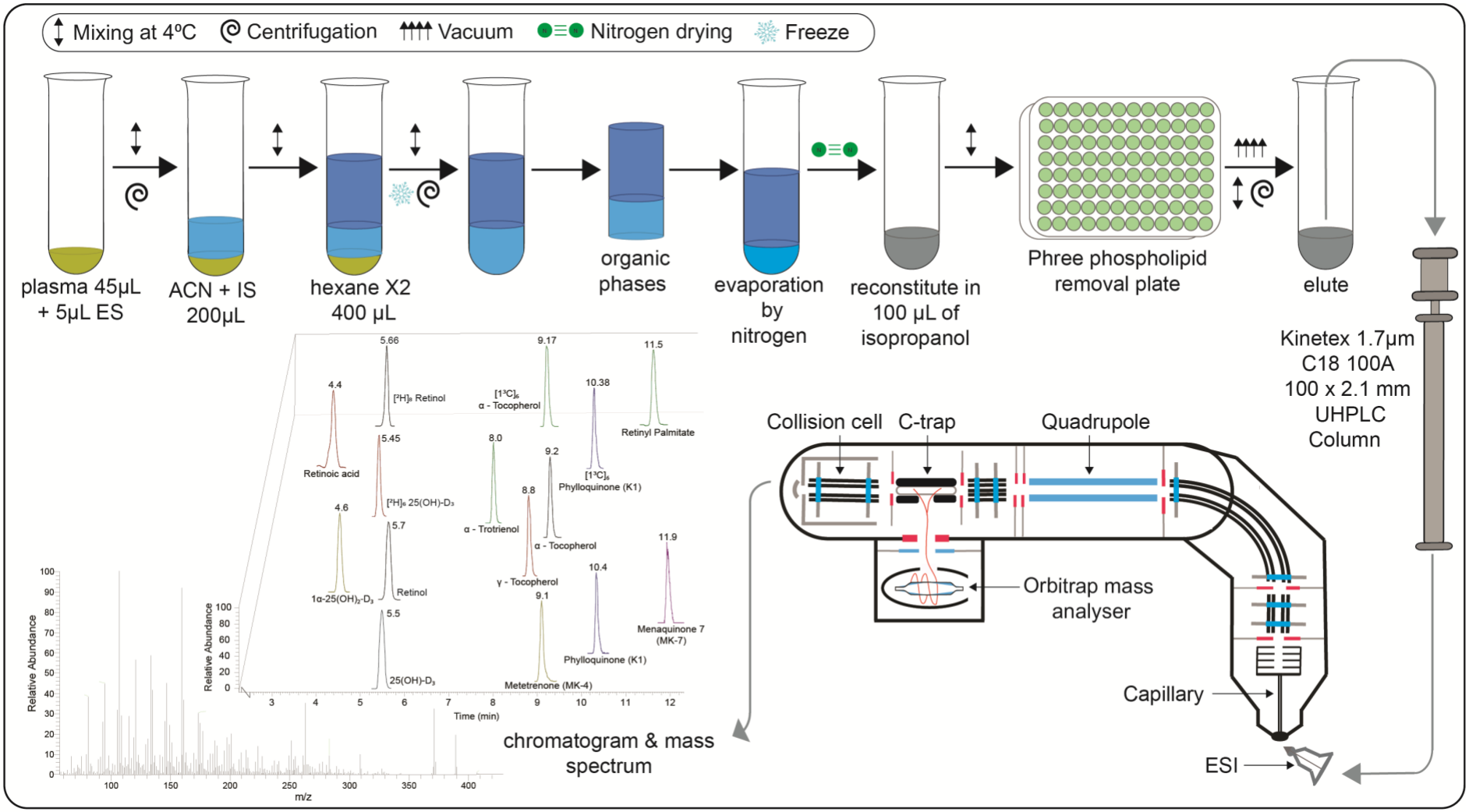

Figure 3-2: Workflow for sample preparation and LC-MS/MS analysis

3.2.4 LC-ESI-MS/MS conditions

Processed samples were kept in Glass coated 96 well plates (10 °C, in the dark) in the PAL autosampler; a 15 µL injection volume was used for all blanks, standards, QCs, and unknowns. Separation was carried out on Kinetex C18 100 Å (100 x 2.1 mm) 1.7 µm analytical column (Phenomenex; Auckland, New Zealand) that was fitted with a KrudKatcher Ultra HPLC in-line filter (Phenomenex). The column compartment was maintained at 30 °C, and the flow at 0.3 mL/min across the total analytical runtime (19 min). An optimised gradient elution system was used to achieve the best FSV separation (Figure 3-3).

Time (min)	Flow mL/min)	A%	B%	C%
0.01	0.3	95	5	
1.5	0.3	10	90	
5.5	0.3	2	88	10
12.5	0.3	2	18	80
13	0.3	2		98
14.5	0.3	95	5	
19	0.3	95	5	

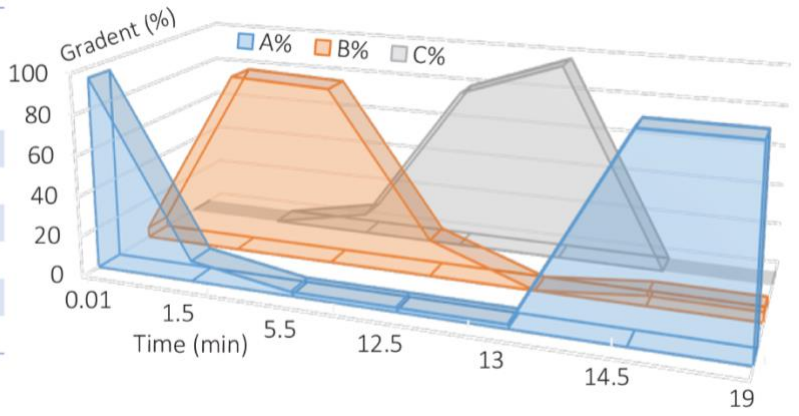

Figure 3-3: Gradient elution. The mobile phase consisted of three solvents; (A) 5mM Ammonium formate mixed with 0.1% (V/V) formic acid in Milli-Q water, (B) 0.1% (V/V) formic acid in methanol, and (C) 2-Propanol.

The MS capillary temperature and spray voltages were 263 °C and 3.5 KV, respectively. Optimum signal strength was attained with an ion source temperature of 425 °C; nitrogen was used as the collision, sweep, and aux gases. Ions were detected using multiple reaction monitoring (MRM) in positive ion mode. The S lens Rf level was at 50, the AGC target was at 1e6, and the resolution was at 17500. The precursor ions (m/z), product ions (m/z), analyte-specific retention times, and optimised collision energies are summarised in (Table 3-3).

Chromatographic, MS data collection, and processing were performed using Xcalibur™ Software (Thermo, Version 4.2.47). Plasma concentrations of each FSV were calculated by comparing the peak: internal standard area ratio with those generated from the relevant standard curve.

Table 3-3: Instrument parameters and retention times

Analyte	Parent ion (m/z)	CE (eV)	t_r (min)	Product ions(m/z)	
Calibration standards					
retinol	269.2264	25	5.66	93.0702	95.0858
retinoic acid	301.2162	36	4.42	165.0913	137.0964
retinyl palmitate	269.2273	27	11.49	93.0701	81.0701
25-OH-D_3	401.4319	37	5.45	107.0858	121.1016
1-α-25-dihydroxy-D_3	399.3257	30	4.56	227.1431	107.0857
α-tocopherol	431.3846	41	9.17	165.0914	69.0701
γ-tocopherol	416.3290	41	8.87	151.0757	152.0789
α-tocotrienol	425.3414	25	8.01	165.0914	205.1227
phylloquinone [K1]	451.3571	32	10.37	187.0758	185.0967
menatetrenone [MK-4]	445.3101	27	9.09	187.0757	81.0701
menaquinone [MK-7]	666.5250	45	11.94	187.0758	81.0701
Internal Standards					
$[^2H]_8$ retinol	277.2766	30	5.65	277.278	98.1016
$[^2H]_6$ 25-OH-D_3	407.3791	30	5.43	107.0859	159.1173
$^{13}C_6$ α-tocopherol	437.4260	32	9.17	171.1293	
$^{13}C_6$ phylloquinone	458.4010	32	10.37	194.1199	

Abbreviations: t_r, retention time; CE, Collision Energy

3.2.5 Matrix effects

The matrix effects were calculated using ten aliquots of spiked and blank plasma utilising the formula below.

$$\text{Matrix effect (\%)} = \frac{\text{Spiked}\,\frac{\text{PBS}}{\text{BSA 4\%}} - (\text{Spiked plasma area} - \text{Blank plasma area})}{\text{Spiked}\,\frac{\text{PBS}}{\text{BSA 4\%}}} \times 100$$

3.2.6 Stability

The 11 vitamers' plasma stability was quantified and tested using spiked pooled plasma over 29 hours (to reflect the complete assay run time), with one injection every hour. This test was repeated over three days.

3.2.7 Linearity

The calibration curves' linearity was measured using seven calibration standards in PBS/BSA (4% w/v), having the average physiological concentrations of each FSV as the mid-point.

3.2.8 Recovery and precision

Inter-day recovery and precision were calculated using thirty aliquots of spiked plasma analysed in three different batches, run over three consecutive days. Unspiked heparinised plasma was used as the blank. Three different concentrations of each vitamer were selected (average endogenous plasma concentrations were chosen as the mid-point) to estimate the measurement recovery of the 11 FSV.

$$\text{Accuracy } (\%) = \frac{\text{Measured concentration} - \text{Physiological concentrations(QCs)}}{\text{Supplementary concentration}} \times 100$$

3.2.9 Instrument Detection Limit [IDL] and Instrument Quantitation Limit [IQL]

The IDL's (method detection limit or Limit of Detection [LoD]) were determined by analysing the standard deviations of ten aliquots of spiked heparinised pooled plasma containing the lowest physiologically observed concentration of the 11 FSV (Table 3-1 an Table 3-2)**.** Signal-to-noise (S/N) ratios were calculated (Xcalibur™ software), and the IDL determined as the lowest concentration of the FSV that can be distinguished from noise by the instrument (IDL = Standard deviation (SD) of the lowest concentrations x $t_{0.99}$ (t = one-tailed t-statistic at the 99% confidence level for n-1 replicates)).

The IQL (method quantitation limit or Limit of Quantitation [LoQ]) is defined as the concentration at which an analytical method detects and quantifies an analyte signal from the noise with an acceptable level of trueness, repeatability, and precision. The IQL is typically calculated as IQL = IDL x 3.

3.3 Results and Discussion

We developed a novel hybrid sample preparation method to extract and quantify 11 FSV from heparinised plasma volumes of 45 µL. Sample handing can be manual or automated, and analysis was performed using LC-ESI-MS/MS. This assay's chromatographic runtime is 19 min, which allows a throughput of 74 samples within 30.4 hours.

3.3.1 Sample stability, processing, and internal standards.

FSV are susceptible to many biological and analytical factors. As such, FSV can degrade during sample preparation and analysis if appropriate measures aren't put in place in advance [87,88] (Figure 3-1). Therefore, precautions were taken during sample preparation that included low actinic amber glassware for standard preparation [273,274], minimal UV exposure, and the use of aluminium foil-wrapped centrifuge tubes [275]. Both samples and standards were stored at -80 °C and kept on ice throughout the process [84,89].

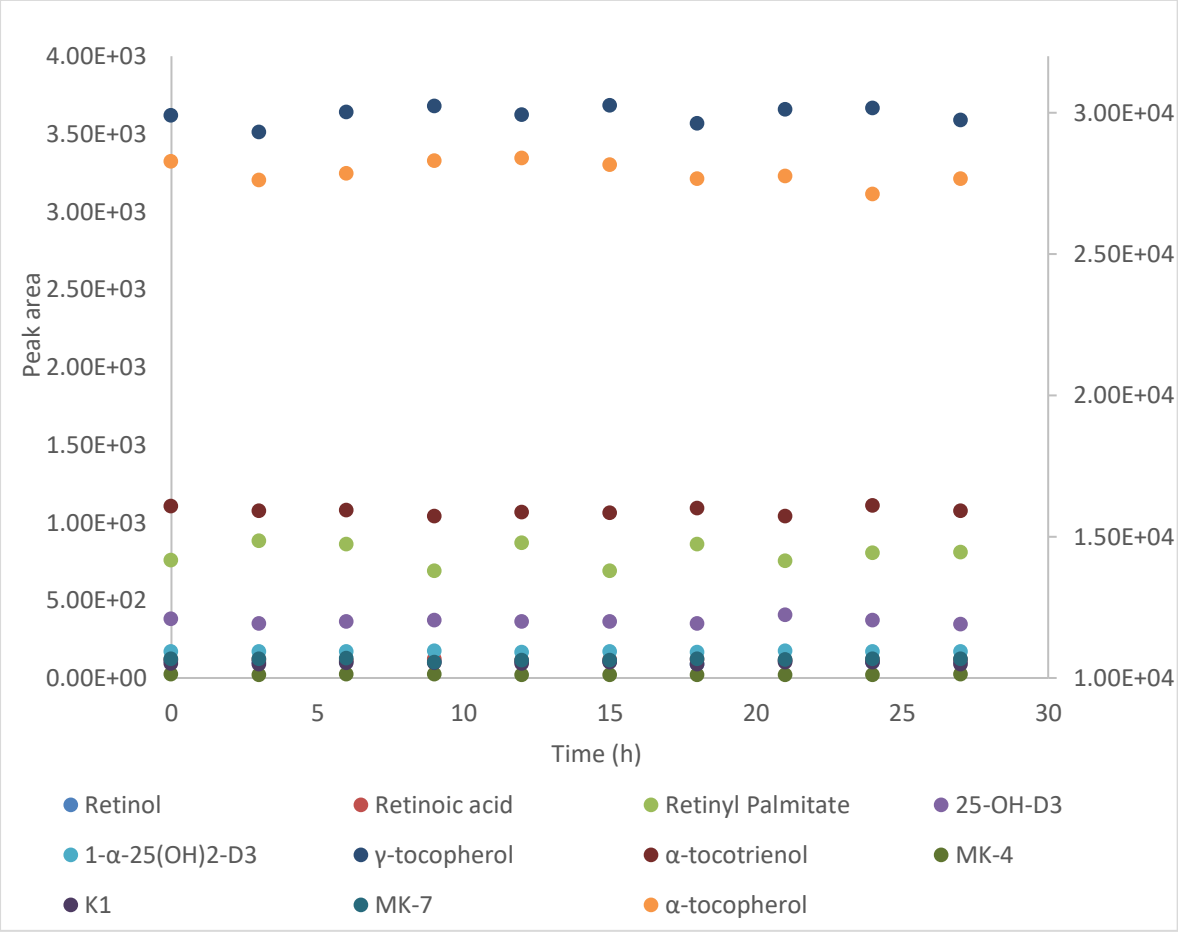

Figure 3-4: Stability of 11 FSV over the 29 hours.

Konieczna *et al*. 2016 [98] evaluated the stability of FSV processed using serum during freeze-thaw cycles and established that the FSV concentrations in the spikes are stable up to 24 hours at 4 °C in the autosampler. In the present study, calibration mixes and QCs were prepared fresh on the day of the experiment; to limit freeze-thaw cycles to one. I tested and confirmed that the hexane/acetonitrile Phree® plate processed FSV were stable for the time to analyse a whole plate (Figure 3-4).

All FSV, particularly α-tocopherol [276] and all-*trans*-retinol [110], are susceptible to oxidation and can degrade during the sample preparation and analysis. The addition of butylated hydroxytoluene [BHT] to the sample mix has been reported to minimise oxidative degradation [111]. Several FSV methods used BHT as an antioxidant [84,89,90]; however, none of these studies reported the extraction recoveries with or without BHT. Therefore, the pros and cons of adding BHT cannot be established. In the present study, both E and K vitamer standard stocks contain BHT; therefore, additional BHT was not added during sample preparation.

The LLE starts with the addition of immiscible organic solvents, which disrupt the intermolecular interactions of the proteins [8]. The denatured proteins then aggregate and fall out of the solution. Centrifugation can be used to separate and extract the particulate-free supernatant. In the present method, plasma deproteinisation was trialled using; solvent (Extraction efficiency), methanol (60%), ethanol (60%), isopropanol (>85%), ethyl acetate (70%), and chloroform (70%). For the vitamers, which are at very low concentrations in plasma (Table 3-1), the extraction efficiency using the aforementioned organic solvents was poor. The best extraction efficiency was obtained using acetonitrile (<90%) at a plasma-to-solvent ratio of 1:4, consistent with published observations [277,278]. In their study, Le, 2018. [93] reported that acetonitrile could promote the best deproteinisation efficiencies at plasma-to-solvent ratios (1:1 and 3:2), while methanol, ethanol, and isopropanol failed. Acetonitrile (polarity index of 0.46) is a mid-polarity solvent, less polar than ethanol and methanol [279]. The majority of the FSV are moderately polar, with A group vitamers retinoic acid [280]) being more polar, MK-7 is highly non-polar. Therefore, for a multianalyte panel of FSV vitamers with mixed polarities, isopropanol is predicted to be the most suitable reconstituting solvent. Polson *et al*. [92] showed that a plasma-to-solvent ratio of <1:2.5 is required to achieve reliable deproteinisation, irrespective of the organic solvent used. Moreover, at a ratio of <1:2.5, methanol and acetonitrile removed 94% and >97% of the protein, respectively. In this method, I maintained the plasma-to-acetonitrile ratio at 1:4.

Table 3-4: Inter day and intraday recoveries

Analyte	Day 1 - Recovery %			Day2 - Recovery %			Day 3 - Recovery%			Average bias			CV%		
	High	**Mid**	**Low**	**High**	**Mid**	**Low**	**High**	**Mid**	**Low**	**High**	**Mid**	**Low**	**High**	**Mid**	**Low**
retinol	102.5	116.1	117.9	95.9	106.8	109.0	101.0	105.0	107.6	99.8	109.3	111.5	3.5	5.5	5.0
retinoic acid	98.1	96.8	110.7	98.3	92.7	103.1	98.8	94.6	103.1	98.4	94.7	105.7	0.4	2.2	4.1
retinyl Palmitate	99.5	100.2	111.5	83.5	91.4	111.0	99.6	93.9	99.8	94.2	95.2	107.4	9.8	4.8	6.2
25-OH-D_3	96.5	89.0	77.1	99.1	101.1	81.5	103.1	98.5	85.1	99.5	96.2	81.2	3.3	6.6	5.0
1-α-25$(OH)_2$-D3	101.5	104.4	109.8	102.8	104.1	106.5	102.4	107.8	126.0	102.2	105.5	114.1	0.6	1.9	9.2
α-tocopherol	117.2	86.8	0.0	110.5	81.8	0.0	107.3	101.5	0.0	111.7	90.0	0.0	4.5	11.4	0.0
γ-tocopherol	113.3	104.9	97.6	127.8	115.8	119.8	120.4	100.9	93.3	120.5	107.2	103.6	6.0	7.2	13.7
α-tocotrienol	105.6	98.8	98.4	102.9	104.4	101.2	103.1	101.4	84.7	103.8	101.5	94.8	1.4	2.8	9.3
K1	93.1	87.5	81.8	95.7	89.7	87.7	92.0	88.7	88.6	93.6	88.6	86.0	2.0	1.2	4.3
MK-4	97.6	99.2	101.5	104.3	91.4	123.0	108.6	103.0	93.1	103.5	97.9	105.9	5.4	6.0	14.5
MK-7	115.6	93.4	77.1	93.0	86.2	79.2	107.1	89.7	85.1	105.2	89.8	80.5	10.9	4.0	5.2

The current method is based on a hybrid sample preparation method that involves both liquid-liquid extraction [LLE] and a phospholipid removal step using Phree® plates. LLE is one of the most commonly used extraction methods in vitamin and metabolomic studies [15]. LLE is based on the principle that the addition of a non-miscible solvent can facilitate the separation of target compounds and matrix constituents. Two of the most commonly used extraction solvents in LLE are hexane [90,91,96–99] and chloroform [84,89]. The use of hexane can be justified due to its cost-effectiveness, safety (*e.g.,* [8,96]), and robustness compared to chloroform. Hexane is water-immiscible and, therefore, suitable for most of the FSV [90]. I achieved high recovery rates using a multi-stage hexane extraction approach (Table 3-4) as described previously [91,98]. Application of a double hexane extraction step in my method yielded 80-90% recovery and only 60-70% using single hexane extraction.

In LLE, the solvent transfer step is critical, as incomplete transfers minimise the recovery of the solvents leading to poor sensitivity [281]. The current method used an extra step of 20 min freeze down of the protein pellet [91,98] to address this issue. Once the protein pellet is frozen, it adheres thoroughly to the bottom of the 96-well plate, allowing a higher volume (580 µL of 650 µL) of supernatant solvent to be collected. Moreover, the current method uses glass syringes (PAL autosampler), glass Pasteur pipettes to transfer the standards and samples, and Glass coated 96 well plates throughout the analysis. Using low-actinic glassware instead of plastic materials can significantly reduce sample loss [110,273].

Hexane is immiscible with other organic solvents such as methanol, ethanol, acetonitrile, isopropanol, and ethyl acetate [282]. The mobile phases of the current FSV method consist of methanol and water. In LC-MS/MS, it is extremely important for the mobile phase solvents to be miscible with extraction solvents. Therefore, FSV methods developed using both hexane and chloroform require additional clean-up steps of nitrogen drying and reconstitution in an organic solvent before injection into an LC-MS/MS, as mentioned in the literature [84,89,90,99].

Even with such an extensive, solvent-based sample preparation method, there was a noticeable matrix effect (Table 3-5 and Table 3-6). Matrix effects are characterised by analytes of interest co-eluting with the other matrix components (*e.g.,* phospholipids and small proteins). This co-elution causes interference with the chromatography and ion suppression, leading to poor recoveries and sensitivity [84,153]. In the absence of the Phree® phospholipid removal plate, the absolute matrix effect based on the analyte areas was high for E, K, and A group vitamers

and relatively low for D group vitamers (Table 3-5). This matrix effect was noticeable for E and K group vitamers even with the isotopically labelled IS (Table 3-6). However, the addition of the Phree® protein removal step significantly reduced the matrix effect for the E, K, and A group vitamers (Table 3-6) [191]. The sensitivity of an FSV method is dependent on the degree of matrix effects. It is crucial to take the necessary steps to minimise them when working with the matrices such as plasma.

Table 3-5: Matrix effect without Phree® Phospholipid removal plates

Analyte	**Analyte/ ISTD area ratio**	
	Relative Matrix effect (n=10)	**CV%**
retinol	47.1	25.1
retinoic acid	54.6	17.3
retinyl Palmitate	62.6	43.6
25-OH-D_3	108.3	2.0
1-α-25$(OH)_2$-D3	114.0	4.6
α-tocopherol	149.6	14.1
γ-tocopherol	318.4	17.1
α-tocotrienol	425.1	16.5
K1	99.3	7.0
MK-4	85.8	11.7
MK-7	149.2	25.7

Table 3-6: Matrix effect and relative matrix effect

	Absolute matrix effect[a]		**Relative matrix effect[b]**	
Analyte	**Mean % (n=10)**	**CV%**	**Mean % (n=10)**	**CV%**
retinol	140.0	15.2	104.2	2.9
retinoic acid	121.8	13.6	100.9	1.9
retinyl Palmitate	111.3	12.5	87.1	10.2
25-OH-D_3	102.0	5.6	98.1	5.1
1-α-25$(OH)_2$-D3	105.9	5.9	101.3	2.0
α-tocopherol	168.3	36.9	125.4	5.1
γ-tocopherol	165.1	37.5	128.7	7.0
α-tocotrienol	126.1	31.4	102.7	2.2
K1	161.5	47.7	94.7	5.8
MK-4	157.1	37.6	99.1	10.8
MK-7	204.1	49.2	114.7	5.0

[a] Matrix effect: based on analyte areas

[b] Relative Matrix effect: Matrix effect based on the internal standard area

3.3.2 Analytical platform LC-ESI-MS/MS

I used liquid chromatography coupled with Q-Exactive to quantify the FSV in plasma in this method. Both instruments are designed to improve efficiency, reliability, sensitivity and deliver high throughput analysis. In UHPLC [135,136], the ultra-high pressure pump systems allow users to use shorter, narrower columns with smaller particle sizes (<2 µm) which yield better signal-to-noise ratios and chromatographic separation compared to the commonly used larger diameter, longer HPLC columns with larger pore sizes (2-5µm) [84,89,91,93,96,98,99,104]. The present method was developed using a Kinetex C18 100Å (100 x 2.1 mm) 1.7 µm analytical column at a 0.3 mL/min flow rate. During the method development, I also tested the Kinetex C18 100Å (150 x 2.1 mm x 1.7 µm) and Agilent Infinity Lab Poroshell 120 PFP (150 x 2.1 mm x 2.7 µm) column. Neither could achieve the anticipated peak resolution. One of the most crucial mass spectrometry tasks is molecular ionisation; the ionisation efficiency is highly dependent on the nature of the analyte and matrix effect [153]. In their study, Zhang *et al*. [95] reported that overall ionisation efficiency using ESI is ten times higher compared to atmospheric pressure chemical ionisation (APCI). In this study, both ESI and APCI ionisation sources were tested. There was a significantly higher signal produced by ESI for the selected FSV compared to the APCI; similar observations have been previously reported [84,89,93,95,97–99,104].

The chromatographic separation of the FSV is based purely on their polarity. Of the FSV quantified by my method, the earliest to elute is the more polar Retinoic acid (4.4 min), while the highly non-polar MK-7 elutes at the end of the analytical run (11.9 min). Vitamers retinal, 3-epi-25(OH)-D_3, and menadione [K3] were also included in pilot analyses. One of the downsides of developing a multianalyte FSV method is that chromatographic resolution is often compromised, leading to poor ionisation and reduced method sensitivity. Therefore, such vitamers can only be accurately quantified by methods optimised to target those specific analytes. The present method's run time is 19 min, and all the analytes of interest are resolved in 12 min. While all the other peaks are satisfactorily resolved, α-tocopherol and MK-4 elute at 8.91 and 8.89 min, respectively. While elution in close proximity can reduce the assay's sensitivity, in this case, the resolution achieved is satisfactory to quantify both vitamers at their physiological concentrations. Therefore, I did not extend the runtime to increase the resolution of α-tocopherol and MK-4.

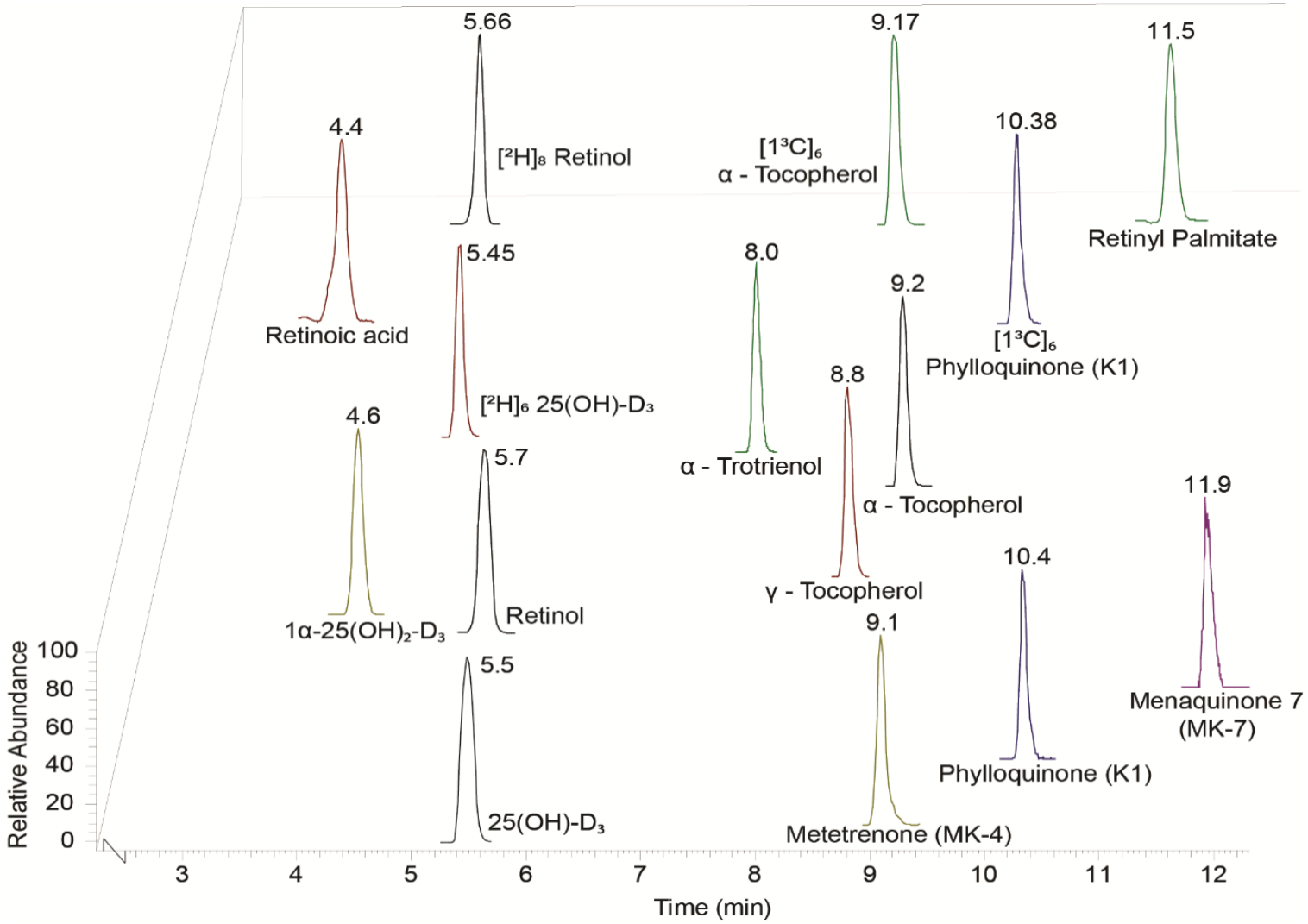

Figure 3-5: Chromatographic separation of 11 FSV from healthy human plasma spiked with IS in PRM (parallel reaction monitoring) mode using LC-ESI-MS/MS.

The quantification of 1-α-25(OH)$_2$-D$_3$ is challenging as, despite being the active form of vitamin D, it is typically present in very low plasma concentrations[283]. The detection limit (2.7 ng/mL) of 1-α-25(OH)$_2$-D$_3$, using my method, is higher than endogenous circulating concentrations (40-61 pg/mL). Notably, a higher plasma percentage of 1-α-25(OH)$_2$-D$_3$ other D group vitamers is reported among individuals with celiac disease [284], Williams syndrome [285], non-infectious diseases; lymphoma [286], hypothyroidism [287], Klinefelter syndrome [288], and infectious diseases; Tuberculosis [289] and sarcoidosis [290] compared to the controls (endogenous concentration). Therefore, my limitation in measuring endogenous 1-α-25(OH)$_2$-D$_3$ is of value in assessing the presence of some of these conditions.

Quantification of the main circulatory form 25-OH-D$_3$ in plasma or serum is the standard measure of vitamin D status in humans. However, not measuring 3-epi-25-OH-D$_3$ (55% of the vitamin D status in infants [291]) results in an underestimate of 25-OH-D$_3$ endogenous concentrations [292]. The present assay successfully quantified 25-OH-D$_3$. However, consistent with earlier observations [89], I was unable to chromatographically separate 25-OH-D$_3$ from its epimeric form 3-epi-25-OH-D$_3$. In infants, higher endogenous concentrations of 25-OH-D$_3$ have been reported by Singh and colleagues due to elevated levels of 3-epi-25-OH-D$_3$, emphasising the importance of separating the epimer [138].

The ability to separate and quantify three K group vitamers is a key advance of this FSV method. These K group vitamers have not previously been resolved in a multianalyte panel. These vitamers' endogenous plasma concentrations (K1, MK-4, and MK-7) (Table 3-1 and Table 3-7) are very low. However, using the highly sensitive, present robust method, these vitamers can be accurately quantified with excellent reproducibility. Previously developed, multi-analyte panel methods that incorporated K group vitamers required much longer analytical runtimes or were limited to one or two K group vitamers (phylloquinone (K1)) [84,89,91,95,97,98,103]. K1 plays an important role in blood clotting, and MK-4 and MK-7 are integral in bone calcium metabolism and γ-carboxylation of osteocalcin [293]. Additionally, K group vitamers promote the γ-carboxylation of matrix gla protein (MGP) in the vascular smooth muscle cells, which bind on to Ca^{2+} and act as an inhibitor of vascular calcification. Therefore the accurate determination of these individual vitamers is clinically important.

3.3.3 Performance parameters (Linearity, range, precision, recovery, and matrix effect)

There is no standardised method for analysing FSV using LC-ESI-MS/MS to date. In this method, I successfully validated my method according to the European Medicines Agency (EMA) guidelines (https://www.ema.europa.eu/en/documents/scientific-guideline/ guideline-bioanalytical-method-validation_en.pdf). Specifically, linearity, range, instrument detection limit (IDL), instrument quantitation limit [IQL], interday and intraday precision (<15%), and recovery (70-140%) were included as validation criteria (acceptance limits).

Four internals standards (IS) ([^{2}H]$_{8}$ retinol, [^{2}H]$_{6}$ 25-OH-D$_{3}$, [^{13}C]$_{6}$ α-tocopherol, and [^{13}C]$_{6}$ K1), one for each vitamer group, were used during method validation. Internal standard stability was assessed using peak areas and peak height. No degradation was observed in 3 repeated analyses of a set of 10 samples over a 24h period (Figure 3-4). Chromatographic separation of the 11 FSV was reproducibly achieved (Figure 3-5). Retention times between analytes and their isotopically labelled standards [^{2}H]$_{8}$ Retinol, [^{2}H]$_{6}$ 25-OH-D$_{3}$, [^{2}H]$_{6}$ α-tocopherol, and [^{2}H]$_{7}$ K1 were >0.02 min which is well within the validation guidelines set by FDA, EMA, and SANTE/SANCO (https://www.eurl-pesticides.eu/docs/public/tmplt_ article.asp CntID=727). I conclude that the method I developed met the criteria for method validation.

The isolation of the matrix effects meant that 11 FSV standard curves are linear with correlation coefficients [r^{2}] ranging from 0.9987 to 0.9999 (Table 3-7). Notably, these seven-point calibration curves cover each analyte's physiological plasma concentration ranges.

Interday and intraday precisions (CV %) values were both within the EMA guidelines. Precision for all the FSV was ≤ 13.9%. The intraday precision ranged from 0.9-13.9%, and the interday precision ranged from 1.1- 11.2%. K group vitamers (K1 (11.8%), MK-4 (11.6%), and MK-7 (13.9%) had high CV% when compared to the A, D, and E group vitamers (Table 3-8). This is consistent with observations from Konieczna *et al*. [98], who obtained similar results for vitamin K1.

In clinical settings, it is crucial to identify deficiencies and hypervitaminosis of these vitamers [270]. Therefore, in this method, a particular focus was given to cover those concentrations when selecting the measuring range. The choice of anticoagulant can play a role in analyte

recovery; therefore, I tested FSV recoveries using both EDTA and heparinised plasma. The sensitivity of retinoic acid, retinyl palmitate, K1, MK-4, and MK-7 were ~40% lower when using EDTA plasma compared with lithium heparin plasma. Therefore, lithium heparin plasma was used in this analysis and for developing QC material. Recovery values for each vitamer were calculated using three external standard concentrations (low, mid, and high) spiked into healthy human lithium heparin plasma (Table 3-2). Table 3-4 summarises the interday and intraday mean recovery values for the 11 FSV. Recovery percentages ranged from 80.5% to 120.5% and were within the validation guidelines set by EMA. Overall, K group vitamers consistently had the lowest recoveries, while E group vitamers had the highest recoveries. Konieczna *et al*. [98] also reported low recovery values for vitamin K1. A high matrix effect on the K group vitamers could explain their low recoveries, and high precision values (CV%), as ion suppression can considerably reduce the sensitivity of an analytical method [84,153] (Table 3-5 and Table 3-6).

Instrument detection limit [IDL] and instrument quantitation limits [IQL] are key performance parameters of any analytical method [89]. Two studies [84,89] previously reported the limit of detection [LOD] of several FSV (retinol, 25-OH-D_3, 25-OH-D_2, α-tocopherol, γ-tocopherol, and K1) spiked into 4% albumin in phosphate-buffered saline (PBS). Compared to 4% albumin PBS, plasma contains more phospholipids and small proteins, which can interfere with the analysis [84,99,153]. Therefore, LOD values obtained using 4% albumin/PBS are not necessarily directly comparable to those in plasma [99].

The primary purpose of the current method is to quantify the FSV at deficient, physiological, and excess levels so as to be clinically relevant. Though the requirement is measuring clinically relevant values of these FSV, the IQL levels in some previously published methods are not within the physiological ranges [8,16,89,90,98]. The method I have developed reliably quantitates retinol [88–90], retinoic acid [294], retinyl palmitate [294], 25-OH-D_3 [37,84,89], α-tocopherol [84,89,91], γ-tocopherol [89], α-tocotrienol [54], K1 [70,89], MK-4 [70], and MK-7 [70] at their respective physiological levels (Table 3-7). Notably, only the most abundant FSV in the circulation have defined values for deficiencies (*e.g.*, retinol; >200-280 ng/mL, 25-OH-D_3; >20 ng/mL [35], and α-tocopherol >5000 ng/mL [51]. My method can quantify retinol at 64 ng/mL, 25-OH-D_3 at 10 ng/mL, and α-tocopherol at 3000 ng/mL, below the clinical deficiencies.

Table 3-7: Performance parameters of the FSV method

Analyte	Physiological conc (ng/mL)	IDL (ng/mL)	IQL (ng/mL)	Range (ng/mL)	Linearity r^2
retinol	300-720 [24,295]	21.4	64.1	25-1600	0.9999
retinoic acid	2.4-4.5 [294,296]	1.2	3.6	1.7-115	0.9998
retinyl palmitate	131-525 [294,297]	9.7	29.2	12.5-800	0.9996
25-OH-D_3	30.1-104.2 [35–37]	3.4	10.2	5.7-365	0.9998
1-α-25$(OH)_2$-D3	0.033-0.085 [37]	2.7	8.2	2.5-165	0.9998
α-tocopherol	3000-13000 [50–53]	1004.6	3013.9	219-14000	0.9993
γ-tocopherol	190-510 [51,52]	42.8	128.4	31-2000	0.9993
α-tocotrienol	0.9 – 30 [54,55]	1.2	3.7	4.7-300	0.9995
K1	0.018 – 4 [70,71]	0.2	0.7	0.312-20	0.9991
MK-4	0.56 – 2.2 [70,72]	0.5	1.6	0.312-20	0.9997
MK-7	0.11 - 2.2 [70,72,73]	0.4	1.3	0.468-30	0.9989

Processed in 4% PBS/BSA, IDL; Instrument detection limit, IQL Instrument quantitation limit

Table 3-8: Inter day and Intraday precision of 11 FSV spiked into heparin plasma

Analyte	Intraday precision (CV %)									Inter day precision (CV %)		
	Day 1 (n=10)			Day 2 (n=10)			Day 3 (n=10)					
	High	Mid	Low	High	Mid	Low	High	Mid	Low	High	Mid	Low
retinol	4.6	2.9	1.0	3.0	3.1	2.1	4.3	3.5	1.4	4.0	3.2	1.5
retinoic acid	2.0	2.6	2.9	5.0	5.1	9.8	2.6	8.3	7.4	3.2	5.3	6.7
retinyl palmitate	9.1	10.1	5.0	9.4	5.6	7.8	6.2	4.8	9.4	8.3	6.8	7.4
25-OH-D_3	4.9	4.8	4.3	1.3	5.0	8.6	3.1	5.4	7.7	3.1	5.1	6.8
1-α-25$(OH)_2$-D3	2.0	5.6	7.3	1.9	9.1	11.2	7.5	6.9	7.7	3.8	7.2	8.7
α-tocopherol	6.3	2.3	12.8	2.9	2.6	9.0	5.6	3.0	10.2	5.0	2.6	10.7
γ-tocopherol	1.5	2.6	1.0	5.8	2.4	1.4	2.8	2.5	0.9	3.4	2.5	1.1
α-tocotrienol	2.2	3.4	7.5	3.0	4.6	6.0	2.4	5.0	5.9	2.5	4.3	6.5
K1	5.7	7.0	8.1	4.9	11.8	9.4	7.9	11.1	11.8	6.2	10.0	9.8
MK-4	11.1	9.6	8.9	9.2	11.6	13.0	11.2	11.3	4.3	10.5	10.8	8.7
MK-7	7.7	7.9	11.3	13.7	9.3	6.2	12.1	13.9	12.2	11.2	10.4	9.9

3.4 Conclusion

I have successfully combined an automated liquid-liquid extraction with phospholipid removal to develop and validate a robust, high throughput LC-ESI-MS/MS assay to quantify 11 fat-soluble vitamers in 45 µL of plasma, including retinol, retinoic acid, retinyl palmitate, 25 hydroxyvitamin D3 [25-OH-D3], 1-α-25-dihydroxy-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, phylloquinone [K1], Menatetrenone [MK-4], and menaquinone-7 [MK-7]. Besides 1-α-25-dihydroxy-D3, my method is sensitive enough to detect and quantify the physiological concentrations and, in the case of retinol (IQL, 64 ng/mL), 25-OH-D3 (10 ng/mL), and α-tocopherol (3000 ng/mL), clinical deficiencies of these vitamers. The developed method is currently being implemented in our laboratory to analyse clinical samples from the LSAC (Longitudinal Study of Australian Children) CheckPoint child/parent dyads and can be reliably used to analyse large cohort studies with limited sample volumes. Compared to the previous methods, for the first time, this unique multi-analyte panel can quantify a broader range of compounds covering all four FSV groups. My automated method will assist in the understanding of the complex interaction between these compounds and their possible role in health and disease.

Chapter 4

Fat-Soluble Vitamers: Parent-Child Concordance and Population Epidemiology in the Checkpoint Study of Australian Children and Their Parents

4.1 Background

Fat-soluble vitamers (FSV) A, D, E, and K are micronutrients that are indispensable to growth, reproduction and the sustenance of optimum health at all stages of life. Deficiencies in or a suboptimal FSV status can result in adverse health effects. As such, FSV deficiencies are considered a global public health concern [298]. A key public health nutrition challenge is to provide a sufficient level of micronutrients to the majority of the population while preventing over-consumers from exceeding the tolerable upper intake level [299]. FSV deficiency symptoms include night blindness among pregnant women and permanent blindness in children aged 1-6 years from vitamin A deficiency [9], osteomalacia in adults and rickets in children from vitamin D deficiency [38–40], neurological and visual deficits from vitamin E deficiency [22], and impaired blood clotting [74] and cardiovascular complications [80] from vitamin K deficiency. The causes of these deficiencies are widely recognised to depend on the society in which they occur. For example, most of the FSV deficiencies that have been presented in high-income countries over the last few decades are due to low activity levels and a shift towards more energy-dense and nutrient-poor diets [300]. By contrast, micronutrient deficiencies in the developing world are mainly due to malnutrition through food shortages [301].

Over the last decade, many physicians and researchers in high-income countries have raised increasing health concerns over the use of dietary supplements and indiscriminate food intake. The consumption of supplements together with fortified foods and whole foods may exceed safe upper limits of micronutrients, including FSV, potentially leading to a toxic accumulation of these micronutrients [302]. For example, excessive FSV intake levels have been associated with a wide range of toxicities, including seizures, headache, blurred vision from vitamin A [22,30,31], kidney stones, nephrolithiasis and hypercalcemia fatigue from vitamin D [42–44], rare symptoms of anaemic symptoms and fatigue from vitamin E [22] and low bone mass, and accelerated bone loss from vitamin K [83].

The association of age, sex, and BMI with circulatory concentrations of FSV has been discussed in a number of epidemiological studies [303–305]. More than 60% of the American population, 67% of Australians aged 18 over [306], 31.2% of adults aged 15 years and over [307], and 38.8% of women, 30.7% of men and in India [308] are overweight and obese, and these conditions that have been shown to alter nutrient absorption or metabolism, including

that of the FSV [305,309,310]. Obese children and adolescents have a higher risk of having low FSV concentrations (*e.g.*, 25-hydroxyvitamin D and α-tocopherol) when compared to individuals with adequate weight. This has been hypothesised to be due to a combination of higher deposition in adipose tissue and/or lower intake of these nutrients [310–312]. Globally, obesity is more prevalent (~ 70%) among women than men [313]. Dietary [314], supplement intake [315], location of body deposition [316], and body fat distribution [317] have been associated with vitamer-specific levels in males and females. Physiologically, the bioavailability of the nutrients decreases with age [318], mainly due to impaired digestion, malabsorption from the gastrointestinal tract, and chronic diseases [319].

Population-level dietary intake data are required to evaluate potential problems regarding dietary intake and formulate appropriate public health and food safety recommendations for effective supplementation or fortification policy development [320]. Most current epidemiologic studies focus on low-cost, easy-to-administer food frequency questionnaires (FFQs) to semi-qualitatively assess FSV intake [321]. However, the validity of FFQ studies for the assessment of food and nutrient intakes in young children are unclear, and self-reporting methods of food intake are subject to errors [322]. In addition, FFQs generally focus on foods with fat-soluble vitamin activity but do not distinguish between vitamers [323] (*e.g.,* Vitamin A vitamers: retinol, retinoic acid, retinyl palmitate, vitamin D vitamers: 25-OH-D_3, 1-α-25-$(OH)_2$-D_3, vitamin E vitamers: α, β, γ, δ tocopherols and tocotrienols and vitamin K vitamers: K1, MK-4, and MK-7). By contrast, the quantitative analysis of FSV levels enables identifying and quantifying the circulatory concentrations of different vitamers with higher accuracy and specificity, creating an opportunity to improve dietary assessment using objective biomarkers [6,324]. Quantitative analyses of FSV were previously performed using immunoassays due to their turnaround time, throughput, and ease of troubleshooting and operating [6]. However, a lack of specificity and poor sensitivity, bias, and imprecision have been reported in a number of automated immunoassay platforms when compared to chromatography-based assays [325]. As a result, I developed a high throughput, robust LC-MS/MS method to quantify FSV with high specificity and sensitivity [184].

Current data on population-level circulatory concentrations FSV are scarce and often limited by the range of analysed vitamers and small sample size [1]. My objective was to analyse the population-level relationships between generation, sex, age, and BMI with a wide range of FSV, using the Child Health CheckPoint study of the Longitudinal Study of Australian Children's (LSAC).

4.2 Methods

4.2.1 Ethical approval and consent

The work undertaken in this chapter is based on the B cohort nested between waves 6 and 7 (Child Health CheckPoint) of the Longitudinal Study of Australian Children's (LSAC) [117,180]. The study was approved by the Australian Institute of family studies Ethics Committee and Royal Children's Hospital (Melbourne, Australia) Human Research Committee (33225D). Consent was obtained from the parents or caregivers for themselves and their children to participate in the biological assessment and collection of their blood samples for this study [117].

4.2.2 Sample collection

Venus Blood samples (28mL) were collected from fasting parents (mean (SD) fasting time of 4.4 (2.1) hours) and semi-fasted children (fasting time 3.4 (2.4) hours) using single venepuncture. Blood was processed into 0.5 mL aliquots. Up to 6 EDTA plasma, 6 lithium heparin plasma, and 6 serum were extracted per participant and processed within ~1 hour (range 1 minute to 3.8 hours) before storage at -80 °C [117]. A total of 2,490 lithium heparin plasma were shipped to Liggins Institute, the University of Auckland, on dry ice in thermosafe boxes. Upon arrival, samples were stored at -80 °C until further use.

4.2.3 Sample randomization

Prior to the LC-MS/MS analysis, heparin plasma samples were randomised on dry ice onto 34 different Fluidex 1mL 96 well plates (Phenomenex) with 74 samples per plate, similar to Stephanie *et al*. 2020 [326]. During the randomisation, the parent-child pairs (1121 pairs) were kept together on the same plate and stored at 80 °C.

4.2.4 Sample preparation

The FSV stock and calibration curve standards preparation was performed as described in Chapter 2, section 2.2.2 Storage conditions optimization. Three quality controls (QC's) were prepared using pooled lithium heparin plasma (Chapter 2, section 2.2.2 Storage conditions

optimization). The QC's were spiked with three different concentrations of the 10 FSV external standards (Table 4-1). These values represent below physiological, physiological and above the physiological concentration of circulating FSV, as mentioned in Arachchige *et al*., 2021 [184].

Table 4-1: External standard concentrations spiked into heparin plasma to make QC's

Compound	QC 1	QC 2	QC 3
retinol	1600	400	100
retinoic acid	115	28.75	7.19
25-OH-D_3	364.35	91.09	22.77
1-α-25$(OH)_2$-D_3	165	41.25	10.31
α-tocopherol	14000	3500	875
γ-tocopherol	2000	500	125
α-tocotrienol	100	25	6.25
K1	20	5	1.25
MK-4	20	5	1.25
MK-7	30	7.5	1.88

4.2.5 Liquid handing robot automation

The Eppendorf EpMotion liquid handling robot, with a built-in vacuum manifold (EpMotion 5075vt, Germany) and thermal mixer, was used in the FSV sample analysis. The automated extraction of the FSV from plasma was programmed using epBlue Client version 40.6.2.6 software. All liquid handling procedures such as pipetting (different volumes using 50, 300, and 1000 µL pipettes), vacuum extraction, liquid dispensing, holding samples at a specified temperature (4°C), sample mixing at a certain speed (800 RPM) were performed using the Eppendorf robot. The protocol was optimised to extract the standard mix, plasma samples, blank, and QCs. The robot deck consisted of serially diluted calibration standards using ethanol (for the 7-point calibration curve), three different QC mixes, acetonitrile mixed with the internal standards, hexane, and isopropanol. The plasma samples were loaded on the EpMotion immediately after thawing in a 20°C water bath (Appendix 3).

4.2.6 LC-MS/MS analysis of FSV

All FSV were measured using an Accela 1250 UHPLC pump (Thermo Fisher Scientific, Austin, Texas USA) coupled with Q-Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Germany) equipped with a heated electrospray ionisation source. The LC-MS/MS analysis and validation criteria have been described elsewhere [184]. In brief, working calibrators, QC's, and control plasma (50 µL) were transferred to 3 different locations in a glass-coated 96-well microplate (1st plate). 50 µL of The LSAC lithium heparin plasma samples (n=74) were then transferred to the same plate. Deproteinisation was initiated by adding 200µL of acetonitrile and internal standard mix to each well. The samples were then mixed for 15 min, 800 rpm (linear) at 4 °C in a thermomixer before adding 400µL of hexane to initiate the biphasic separation. The solution was then mixed (15 min at 800 rpm (linear), 4 °C), and the plate was centrifuged (2000 RCF, 10 min, 4 °C). The resultant supernatant of ~580 µL was transferred to a new plate (2nd plate). To initiate the double hexane extraction, 300uL of hexane was again added to the 1st plate, the mixing and centrifugation were performed as above. The resultant supernatant of ~ 300uL was transferred to the 2nd plate. The samples in the second plate were N_2 dried for 1-2 hours at 20 °C until the supernatant was thoroughly dried. The extracts were resuspended in 100 µL of 2-propanol. The samples were transferred to a Phree® phospholipid removal plate, re-using the 2nd plate as the collection plate. The Phree® plate was centrifuged (2000 RCF, 5 min, 15 °C), and a vacuum was applied (10 psi, 10 min; repeated twice). The resulting eluents in the 2nd plate was covered with a sealing mat and placed in the PAL autosampler. Sample introduction to the analytical column was performed using a PAL autosampler with refrigerated sample trays (CTC Analytics, Thermo) and a HotDog 5090 column oven (Thermo). A Kinetex C18 100 Å (100 x 2.1 mm) 1.7 µm analytical column (Phenomenex; Auckland, New Zealand) fitted with a KrudKatcher Ultra HPLC in-line filter (Phenomenex) was used to chromatographically separate the compounds. A flow of 300 mL/min starting at 5% methanol and 95% 5mM ammonium formate, and 0.1% formic acid in MilliQ® H_2O was applied to the column; analytes of interest were eluted using an increasing isopropanol gradient. The sample injection volume was 15 uL, and the runtime was 19 minutes. According to the EMA guidelines [212], all quality controls had acceptable recoveries and reproducibilities.

4.2.7 Statistical analysis

All statistical analyses were performed using R Version 3.6.1 (https://www.r-project.org/).. FSV α-tocopherol in plates 1 and 7 fell outside the 2 standard deviation range; therefore, these plates were removed from the study to minimise the technical plate effect. The QC data can be accessed through https://auckland.figshare.com/articles/dataset/Child_CheckPoint_the_LSAC_cohort_study_quality_control_QC_data_of_FSV_analysis_using_LC-MS_MS/19333520/2. The reported FSV included retinol, retinoic acid, retinyl palmitate, 25-OH-D3, 1-α-25$(OH)_2$-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, K1, MK-4, and MK-7. The plasma concentrations of retinyl palmitate, 1-α-25$(OH)_2$-D_3, MK-4, and MK-7, were below the instrument detection limit for most of my plates. Therefore, these FSV were excluded from the study.

The effects of generation (Parent vs child), Sex (male vs female), and family (shared gene-environment) were tested using two sets of mix models using the *lme4* package in R. The log-likelihood of the mix models were compared for generation and sex (fixed effect) and family (random effect). If the interactions between sex and generation were insignificant, model 1 tested the additive effects of Generation (For both parents and children) and sex (male and female) on FSV concentrations. If the interaction between sex and generation were significant, model 2 tested the relationships of FSV concentrations in Female child vs Male Child, Female child vs Female Parent, Male Parent vs Female Parent, and Male Parent vs Male Child. The familial concordance within parent-child dyads was also tested using Pearson's correlations in R.

Two sets of linear models, age and BMI, were fitted for each variable in parents and children separately on a continuous scale. Due to the narrow age distribution in children (11-12 years), I only tested for age-specific differences in parents (28-71 years), while BMI was tested for children and parents. The gender-specific effects of BMI were also tested for the selected groups of vitamers. Subjects with FSV concentrations beyond 3 standard deviations (SD) of the sex, age, and BMI-specific means were considered outliers and removed from the study. The R scripts used for this analysis can be accessed through https://auckland.figshare.com/articles/software/R_Scripts_Characterising_Fat-Soluble Vitamer_profiles_in_Child_CheckPoint_of_the_LSAC_cohort_study/19333607/4.

4.3 Results

The LSAC checkpoint cohort consists of 1121 parent-child pairs (2,490 participants), 1,166 children (49% male), and 1,324 parents (87% females, predominantly the biological mothers of children).

4.3.1 FSV profiles are concordant between children and their parents

All FSV concentrations show a significant concordance between children and parents from the same family (Figure 4-1). According to Pearson's correlation, the observed familial effects between parent-child dyads were: retinoic acid (coefficient correlation R= 0.57); 25-OH-D_3 (R=0.35); α-tocopherol (R=0.38); γ-tocopherol (R= 0.36); α-tocotrienol (R=0.38); and retinol (R=0.18).

4.3.2 FSV profiles from the same family are generation and sex-dependent

Sex-specific interaction effects were identified for 25-OH-D_3 and γ-tocopherol (Table 4-2 and Figure 4-2). The 25-OH-D_3 concentrations were significantly high (p =3.31e-03) in males (child-male and parent-male) when compared to females (child-female and parent-female). The opposite effect was observed for γ-tocopherol (p =0.019). There was no observed sex effect for α-tocotrienol (p =0.398).

A significant family effect was identified for 25-OH-D_3, α-tocotrienol, and γ-tocopherol (Table 4-2 and Figure 4-2). Compared to parents, the children had significantly higher 25-OH-D3 (p =2.11e-10) and α-tocotrienol (p =0.047) concentrations. By contrast, the γ-tocopherol concentration (p =7.91e-12) was higher in parents than in children. The concentrations of retinol, retinoic acid, and α-tocopherol were significantly higher in both male and female parents than their children in both sexes (Table 4-2 and Figure 4-2). Across the parent population, significantly high concentrations of retinol (p <2e-16) and retinoic acid (p =1.70e-04) were observed in parent males and α-tocopherol in females (p =2.05e-04).

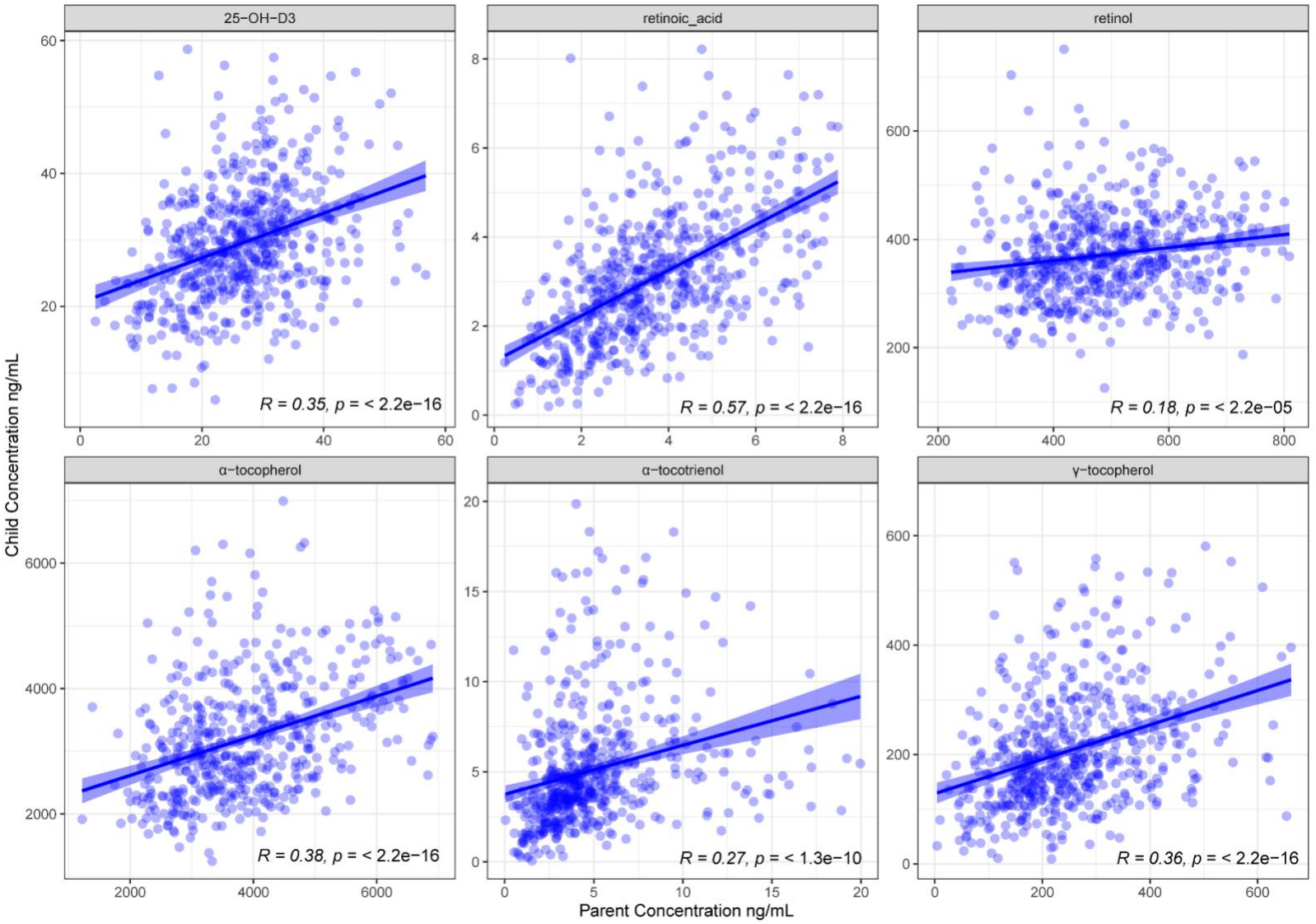

Figure 4-1: Scatter plot and trend line to illustrate Parent-child concordance of FSV concentrations

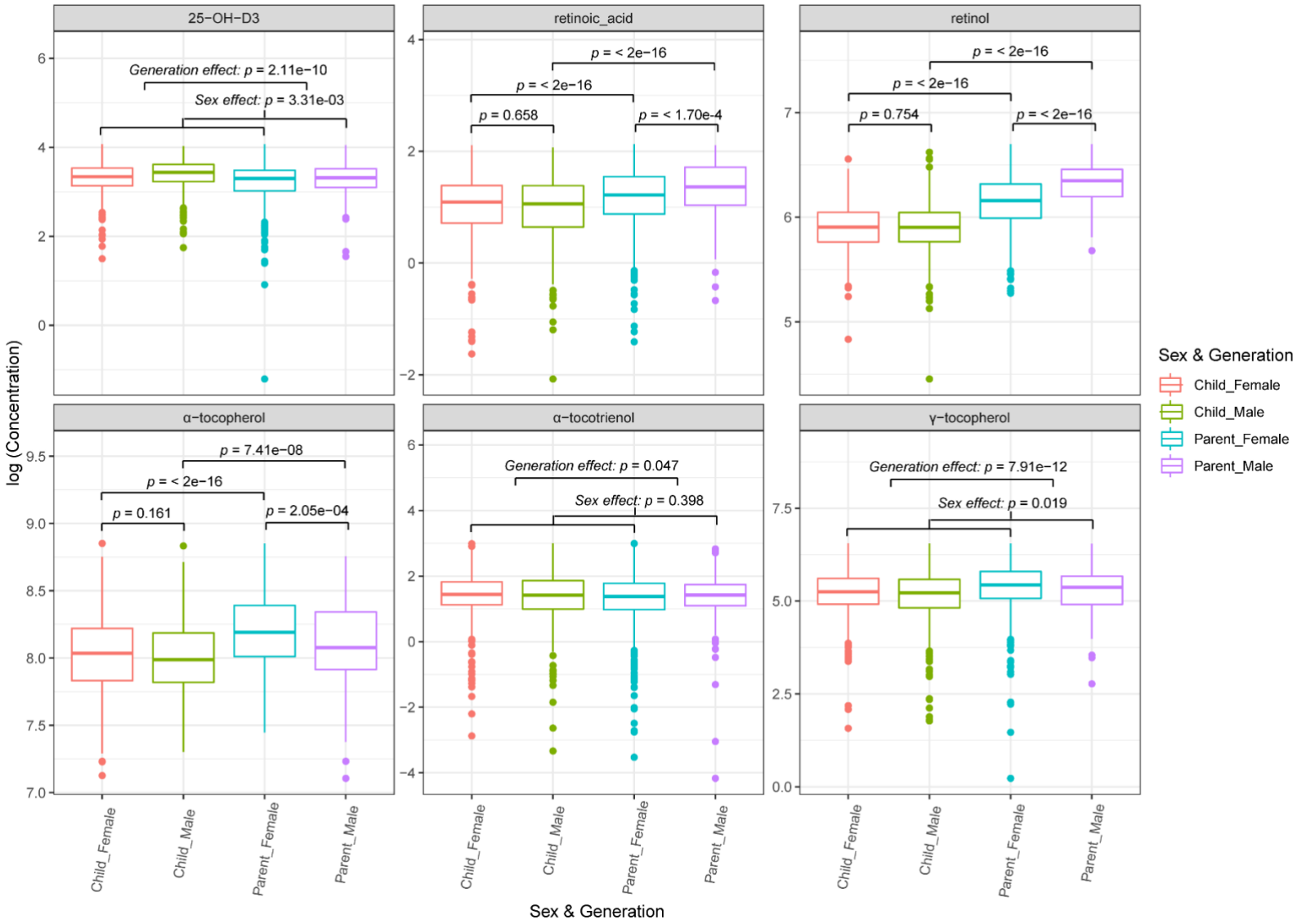

Figure 4-2: Box plots of sex and generation effects on FSV concentrations

Table 4-2: Mix model results of sex and generation effects on FSV concentrations

Vitamer	Condition	*p*-value	Estimate
retinol	Model 1 (Baseline Female Child, Intercept; Estimate: 5.904, *p* = <2e-16)		
	Male Child vs Female Child	7.54e-01	-4.40e-03
	Female Child vs Female Parent	<2e-16	2.62e-01
	Interaction between Sex vs Generation	9.13e-16	1.94e-01
	Model 2 (Baseline Male Parent , Intercept; Estimate: 6.355, *p* = <2e-16)		
	Male Parent vs Female Parent	<2e-16	-1.89e-01
	Male Child vs Male Parent	<2e-16	-2.15e+02
	Interaction between Sex vs Generation	9.13e-16	1.94e-01
retinoic acid	Model 1 (Baseline Female Child, Intercept; Estimate: 1.022, *p* = <2e-16)		
	Male Child vs Female Child	6.58e-01	-1.15e-02
	Female Child vs Female Parent	<2e-16	1.67e-01
	Interaction between Sex vs Generation	7.35e-04	1.51e-01
	Model 2 (Baseline Male Parent , Intercept; Estimate: 1.32868, *p* = <2e-16)		
	Male Parent vs Female Parent	1.70e-04	-1.39e-01
	Male Child vs Male Parent	<2e-16	-3.18e-01
	Interaction between Sex vs Generation	7.35e-04	1.51e-01
25-OH-D_3	Model 1 (Baseline Female Child, Intercept; Estimate: 3.334, *p* = <2e-16)		
	Male vs Female	1.31e-03	6.61e-02
	Child vs Parent	2.11e-10	-1.02e-01
	Interaction between Sex vs Generation	8.29e-01	-7.58e-03
α-tocopherol	Model 1 (Baseline Female Child, Intercept; Estimate: 8.025, *p* = <2e-16)		
	Male Child vs Female Child	1.61e-01	-2.43e-02
	Female Child vs Female Parent	<2e-16	2.02e-01
	Interaction between Sex vs Generation	2.53e-02	-6.63e-02
	Model 2(Baseline Male Parent , Intercept; Estimate: 8.137, *p* = <2e-16)		
	Male Parent vs Female Parent	2.05e-04	9.06e-02
	Male Child vs Male Parent	7.41e-08	-1.36e-01
	Interaction between Sex vs Generation	2.53e-02	-6.63e-02
γ-tocopherol	Model 1 (Baseline Female Child, Intercept; Estimate: 5.231, *p* = <2e-16)		
	Male vs Female	1.92e-02	-8.04e-02
	Child vs Parent	7.91e-12	1.83e-01
	Interaction between Sex vs Generation	7.72e-01	-1.71e-02
α-tocotrienol	Model 1 (Baseline Female Child, Intercept; Estimate: 1.406, *p* = <2e-16)		
	Male vs Female	3.98e-01	3.68e-02
	Child vs Parent	4.74e-02	-6.73e-02
	Interaction between Sex vs Generation	9.59e-01	3.87e-03

i.e., Estimate: magnitude of change

4.3.3 FSV concentrations are age-dependent

Among parents, there was a detectable statistically significant association between age and increasing concentrations of retinol (p = 2.07e-05) and α-tocopherol (p = 1.43e-2). By contrast, there was no detectable statistically significant correlation between retinoic acid, 25-OH-D_3, γ-tocopherol or α-tocotrienol and age (Table 4-3).

Table 4-3: Linear model results of age effect on FSV concentrations in Parents

Vitamer	Condition	Estimate	Std, Error	*p*-value	Interpretation
retinol	Parent	3.0446	0.7126	<2.07e-05	***
retinoic acid	Parent	0.0122	0.0099	0.2160	Non Significant
25-OH-D_3	Parent	0.0259	0.0571	0.6500	Non Significant
α-tocopherol	Parent	18.8630	7.6860	0.0143	*
γ-tocopherol	Parent	0.3967	0.8895	0.6560	Non Significant
α-tocotrienol	Parent	0.0082	0.0302	0.7845	Non Significant

i.e., Estimate: magnitude of change

4.3.4 FSV concentrations are BMI dependent

The majority of the FSV, irrespective of sex, exhibit an association with BMI. BMI positively associated with the parent FSV concentrations of retinoic acid (p =2.92e-03), α-tocotrienol (p =4.48e-03), γ-tocopherol (p =3.74e-13). Only 25-OH-D3 (p =2.94e-11) was negatively associated with increasing BMI in parents. Plasma α-tocopherol concentration was not associated with BMI (Table 4-4).

In children, concentrations of retinoic acid (p = 1.96e-03) and γ-tocopherol (p =1.26e-04) positively and 25-OH-D_3 (p =2.31e-04) and retinol (p =2.34e-05) negatively associated with accumulative BMI. The plasma concentration of α-tocopherol and α-tocotrienol was not associated with BMI (Table 4-4).

BMI in males was positively associated with plasma concentrations for retinol (p =<2e-16, R=0.27), retinoic acid (p =1.33e-12), α-tocopherol (p =6.33e-07) and γ-tocopherol (p =9.16e-

07) and negatively with 25-OH-D_3 (p =3.87e-08) (Table 4-4). Plasma α-tocotrienol concentrations did not show any relationship with increasing BMI. In females, plasma concentrations for retinol (p =<2e-16), retinoic acid (p =2.88e-10), α-tocopherol (p =3.37e-13), γ-tocopherol (p =<2e-16) and α-tocotrienol (p =0.0409) were positively and 25-OH-D_3 (p =1.53e-13) negatively associated with increasing BMI (Table 4-4).

Table 4-4: BMI effect on FSV concentrations in male and female parents and their children

Vitamer	Condition	Estimate	Std. Error	*p*-value	Interpretation
retinol	Child	3.170e+00	7.464e-01	2.340e-05	***
	Parent	-1.009e+00	6.038e-01	9.500e-02	Non Significant
	Male	1.283e+01	7.693e-01	<2e-16	***
	Female	4.521e+00	4.437e-01	<2e-16	***
retinoic acid	Child	4.330e-02	1.396e-02	1.960e-03	**
	Parent	2.476e-02	8.305e-03	2.920e-03	**
	Male	8.394e-02	1.163e-02	1.330e-12	***
	Female	3.873e-02	3.873e-02	2.880e-10	***
25-OH-D3	Child	-3.119e-01	8.442e-02	2.310e-04	***
	Parent	-3.172e-01	4.730e-02	2.940e-11	***
	Male	-3.525e-01	6.345e-02	3.870e-08	***
	Female	-2.698e-01	3.624e-02	1.530e-13	***
α-tocopherol	Child	1.351e+01	8.857e+00	1.280e-01	Non Significant
	Parent	7.987e+00	6.503e+00	2.200e-01	Non Significant
	Male	3.644e+01	7.242e+00	6.330e-07	***
	Female	3.487e+01	4.749e+00	3.370e-13	***
γ-tocopherol	Child	5.336e+00	1.387e+00	1.260e-04	***
	Parent	5.386e+00	7.338e-01	3.740e-13	***
	Male	4.833e+00	9.760e-01	9.160e-07	***
	Female	4.948e+00	5.856e-01	<2e-16	***
α-tocotrienol	Child	6.116e-02	4.615e-02	1.850e-01	Non Significant
	Parent	7.168e-02	2.517e-02	4.480e-03	**
	Male	-5.706e-02	3.260e-02	8.050e-02	Non Significant
	Female	4.094e-02	2.001e-02	4.090e-02	*

i.e., Estimate: magnitude of change

4.4 Discussion

I have measured the plasma FSV concentrations within the LSAC cohort. In so doing, I have identified sex, family, age, and BMI as factors that are statistically associated with plasma FSV concentrations. Strong familial concordance was observed for all the FSV tested and supported a shared gene-environment contribution to FSV plasma concentrations. This is the first report of an interaction between sex, generation and plasma FSV concentrations.

4.4.1 Family concordance of FSV

Studies profiling FSV simultaneously in parent-child dyads reveal the shared gene-environment contributions to circulating FSV concentrations. I observed a highly statistically significant familial concordance for all FSV concentrations (Figure 4-1). The gene-environment interaction has been associated with parental feeding behaviour as well as child and adolescent nutrition [327]. This is consistent with Familial, twin, [328,329] and Genome-wide meta-analyses [327]; studies on the gene and environmental influences in childhood obesity which have provided evidence that BMI is highly heritable in both late childhood and adulthood (~70%). Within a shared family environment, parents' genetic predisposition to be of a lower or higher BMI may be reflected in their own feeding practices and the feeding practices of their children with whom they share 50% of their DNA [330]. The home environment provides 63%–65% of early adolescents' daily energy and nutrient intake [331,332], making the family and home environment an important target to improve dietary quality and prevent obesity. Parenting practices also have been identified regarding early adolescence physical activity, including providing support for physical activity, modelling, and establishing rules or expectations for physical activity in the home environment [333]. I contend that the parent-child concordance in FSV levels likely reflects common dietary, metabolic, gene, and environmental determinants and is important to be addressed in future studies.

4.4.2 FSV concentrations are associated with age

The generation effect on plasma 25-OH-D_3 concentrations was statistically significant. My results indicate that the concentrations of 25-OH-D_3 are higher (p =2.11e-10) in children compared to their biological parents (Table 4-3). Approximately 80% of vitamin D is

synthesised in the skin via bioconversion of provitamin D_3 to previtamin D_3 [334]. Photosynthesis of D group vitamers varies widely due to many biological and environmental factors. (*e.g.*, skin pigmentation, latitude, seasonal effect, duration of sun exposure, time of day, atmosphere composition, clothing, and sunscreen use [335]. Young adults at puberty (girls from age 8 to 13 years and boys from age 10 to 15 years) are more active and prefer to be involved in more outdoor activities (84.7%) than adults [336,337]. A study using the Australian Health Survey (AHS), a nationally representative sample of Australian adolescents (age 12–17; n = 692) and young adults (age 18–24; n = 400), found that a higher proportion of young adults (32%) than adolescents (17%) were deficient in vitamin D [338]. Another study reported that 80% of adolescents spent most weekends in the sun, and only 9% used sunscreen, while 33% never did [339]. Therefore, I speculate that the longer duration of sun exposure and limited sunscreen use provide adolescents with an added advantage in producing more vitamin D than parents. Malacova *et al.*, 2019 observed that vitamin D deficiency is associated with adult age (*e.g.*, 29% in age 25–34, 23% in age 35–44, 21% in age 45–54, 17% in age 55–64, 15% in age 65–74, and 19% in those aged ≥ 75 years) [340]. My results did not support this (p =0.65) for plasma 25-OH-D_3 concentrations for the parents (Table 4-3). I speculate that the difference may result from the LSAC participants having had an added geographical advantage of adequate sun exposure throughout the year [61], enabling them to produce sufficient vitamin D irrespective of their age; however, this needs to be validated in future studies.

A study of four British National Diet and Nutrition Surveys using children 1.5-4.5 years, young children to adolescence 4.0-18.0 years, adults 19.0-64.0 years, and adults > 65.0 years identified that absolute plasma concentrations and the ratio of α and γ-tocopherol declined with age [341]. I observed increased levels of α-tocopherol and no association of γ-tocopherol with age in Australian parents (Table 4-3). Indeed the generation effect shows significantly higher α and γ -tocopherol plasma concentrations in parents than in children. It is difficult to interpret the biological significance of the circulating vitamer E concentrations with age, as plasma lipid concentrations also increase with age and higher lipid concentrations. Therefore, as the carrier protein concentrations for α-tocopherol increase, there is a capacity for higher circulating α-tocopherol concentrations. However, abnormal lipoprotein metabolism does not necessarily increase α-tocopherol delivery to tissues [342]. Instead, both α and γ-tocopherol concentrations have been shown to be directly correlated with 'healthy' nutritional choices (*e.g.*, dietary fibre, intrinsic sugars, fresh fruits, and fruit juices, polyunsaturated fats, and food supplements) and inversely with 'unhealthy' choices (monounsaturated fats, non-polyunsaturated fats, and

extrinsic sugars) [341]. Eating patterns for many early adolescents (10–14 years) are generally associated with energy-rich and nutrient-poor diets [343]. Adolescent diets are high in sodium [344,345] and calories from added sugars [346] and low in fruit, vegetables [347], whole grains and fibre [348]. The mechanisms underlying these relationships are obscure; however, the high α-tocopherol levels observed in the current study may reveal better dietary choices and supplement intake in parents than adolescents. These assumptions need to be further explored using prospective cohort studies.

I observed a gradual increase in plasma retinol concentrations with age. Notably, there was no association between plasma retinoic acid concentrations and age (Table 4-3). The generational effect for the plasma retinol concentration was significantly higher in parents than in children. This is consistent with observations by Stephensen *et al*. 2000, who reported that serum retinol concentration increases between 27-69 years of age, and the mean values are 2 fold higher in males than females [349]. Similarly, Yang *et al*., 2015 observed that the average plasma retinol concentration was significantly higher (p <0.001) in adolescents than in children [350]. Therefore, the finding that plasma retinol concentration increases with age and generation is reproducible between cohorts. Western diets have an abundance of pre-formed vitamin A-containing foods (*e.g.,* organ eggs, fish oil, meat, beef, as well as fortified foods like non-fat milk, breakfast cereal, margarine, and some snack foods. Additionally, the use of multivitamins in the forms of retinyl palmitate or retinol [351] is increasingly becoming popular among the older population due to their claims to be beneficial for many age-related conditions (*e.g.,* inflammatory conditions [352] and immunity [353]. In view of the fact, the dietary and supplemental abundance of vitamin A in western diets may account for its liver accumulation and high blood concentrations over a period of one's lifetime. This seems the best current explanation of this phenomenon.

4.4.3 Sex effect on FSV concentration

In the present study, I observed higher concentrations of 25-OH-D_3 in males than females (p =3.31e-03) in both children and parents (Figure 4-2). Consistent with this, Muscogiuri *et al*., 2019 showed that irrespective of the BMI, 25(OH)D concentrations were significantly lower (p =0.01) in males compared to females [354]. Similarly, a cross-sectional study of 302 participants (176 men and 126 women) performed in China stated that 25(OH)D levels in women (38.40±12.37 nmol/l, age 46.48±15.06) were significantly lower than measured in men

(43.49±14.78 nmol/l) (p <0.01) [355]. Moore *et al*., 2004 reported that in the USA, adolescent females were approximately half as likely as males to meet their dietary reference intakes of vitamin D when compared to adolescent males of the same age group. It remains possible that these differences reflect lower vitamin D intakes in females. For example, Moore *et al*. 2004 estimate that only 50% of girls aged 9–13 and 32% of 14–18-year-olds, respectively, have adequate intake value for vitamin D [314] and suboptimal female vitamin D intakes have also been confirmed in several other studies [356–358]. However, I was unable to measure or accurately estimate these intakes in the LSC cohort. Therefore, future cohort studies need to investigate the link between dietary intake and sex-related FSV concentrations.

The α-tocopherol and γ-tocopherol plasma concentrations were significantly higher in children and parent females within the LSAC cohort. The α-tocotrienol concentrations, however, did not differ between genders (Figure 4-2). Talegawkar *et al*., 2007 also observed that regardless of supplement usage, women (age 61.5 ± 0.61) had significantly higher serum α-tocotrienol (0.06 ± 0.04) and γ-tocopherol (−0.16 ± 0.06) concentrations when compared to men (age 60.2 ± 0.76) [315]. This may reflect a biological difference in the fat deposits in which men and women store FSV. Specifically, FSV are generally stored in the liver and adipose (fat) tissue (90% of the total vitamers E and A), more specifically in the lipid droplets of adipocytes [359–361]. Women typically have ~10% higher body fat than men [362] and store fat in the gluteal–femoral region, while men have more body fat in the visceral (abdominal) depot [316]. Gluteal-femoral adipose deposits in women may provide a safer lipid reservoir for excess energy, or they may directly regulate systemic metabolism through the release of adipokines or metabolic products [317]. Although the underlying mechanisms' relationship is fully elucidated, it can be assumed that the higher FSV concentrations, including α-tocopherol and γ-tocopherol in females, are associated with regional fat deposits and their metabolism. These associations, however, need to be further investigated in future studies.

I did not observe gender-specific variations in children's plasma retinol and retinoic acid concentrations. However, the parent concentrations were significantly higher in males compared to females (Figure 4-2). This is consistent with observations by Stephensen *et al*. 2000, who also reported that the serum retinol mean values are 2 fold higher in males than females (aged 20–59) [349]. Similarly, Söderlund *et al*., 2002 observed that females (aged 22 - 63 years) had lower serum retinoic acid concentrations (4.5 nmol/L vs 5.5 nmol/L) and lower serum retinol concentrations (2.1 µmol/L vs 2.5 µmol/L) than males (aged 21 - 54 years)

irrespective of their food intake [363]. There are also several other studies, primarily in children and the elderly, where gender differences have not been found [364–366]. The sex-associated biological variations in A group vitamers need to be further investigated across different gender reference intervals to better understand the between-subject variation of these vitamers.

4.4.4 BMI effect on FSV concentration

In the present analysis, BMI increase in both parents and children was negatively associated with 25-OH-D_3 concentrations irrespective of the gender difference (Table 4-4). My data confirm previous literature showing the prevalence of depleted vitamin D levels with increased BMI [312,367,368]. Consistent with my findings, Zoya *et al.*, 2009 reported that BMI-associated vitamin D levels significantly decreased (p <0.01) for both sexes and both age groups (<50 years; age 8-49 and ≥50 years) [369]. Several studies exploring gender differences in the prevalence of vitamin D deficiency found that obese men have significantly higher odds of developing vitamin D deficiency than women [369,370]. The volumetric distribution of body fat may also play a role in the bioavailability of D group vitamers. Thus, subcutaneous fat deposits may store more vitamin D synthesised in the skin than dietary sources or supplements [311]. In agreement, one study reported lower vitamin D levels in obese subjects are caused by decreased bioavailability (57% lower in obese than non-obese individuals after one UV-B exposure) [311].

Obesity is more strongly associated with abdominal fat or adipose deposition, which plays a crucial role in many health risks associated with overweight and obese individuals [371]. It is postulated that high BMI individuals have persistent adipose tissue inflammation and the accumulation of proinflammatory cytokines [310]. Proinflammatory cytokines suppress the hepatic production of many carrier proteins necessary for the transportation of micronutrients and promote the sequestration of some micronutrients, including vitamins, to the liver and other organs [372]. The previous literature shows that, in the presence of inflammation, the levels of retinol [373], vitamin E [341,374], and vitamin D [375] are significantly lower. I, however, only observed decreased retinol concentrations in children but not in parents with increasing BMI. As opposed to the previous literature, both retinoic acid and γ-tocopherol concentrations significantly increased for parents and children. The BMI change only increased the α-tocotrienol concentrations of the parents but not their children (Table 4-4). Supplementation with α and γ-tocopherol has been identified to reduce inflammation, oxidative stress, and

adipose tissue fibrosis and improve metabolic profile in obesity [376]. Therefore, it can be speculated that these high vitamin E concentrations observed in the present study could be due to supplement intake. Additionally, the vast majority of both the adults (72%) and children (81%) population in the current study were below the obese range (BMI of 30 to < 35) [377]. Therefore, the low FSV concentrations seen in obese individuals cannot be directly applied to the present study.

Limitations

This study has a number of limitations. Some of the FSV measured in this method were present at low circulatory concentrations in plasma and subject to degradation when stored for an extended period at -80°C [217,218,221]. My analysis and discussion excluded the FSV measures below the instrument detection limit, including all K group vitamers (*e.g.*, K1, MK-4, and MK-7), retinyl palmitate, and the active form of vitamin D: 1-α-25$(OH)_2$-D_3. Therefore, my final panel of vitamer measures does not fully reflect the comprehensive FSV profile that was used in the measurements.

Despite the magnitude of the sample (n = 2490 subjects), my study design was cross-sectional and limited to a single semi-fasted plasma sample from the participants. In the adult subgroup, males were underrepresented (1:10 ratio of males to females) compared to the 1:1 male-to-female ratio in children, which limited my ability to examine the effect of age and identify sex-specific differences in adult males. Additionally, the narrow age distribution in children (11-12 years) limited my exploration of the age-associated variation in FSV concentrations. The current study is the first of its kind to investigate the generation and gender-specific effects on FSV concentrations. Unfortunately, this limited my ability to validate my findings directly with the previous literature.

My cohort's socio-economic status is relatively higher than seen across the general Australian population (SEIFA score for over 78% of the present population scored in the middle to least disadvantaged compared to ~62% in the general Australian population [378]). Finally, I was unable to include measures of physical activity status, which could have supported some of my conclusions. This limitation was due to these variables not being included in the B cohort of Child Health CheckPoint of the Longitudinal Study of Australian Children's (LSAC).

4.5 Conclusion

I have characterised FSV profiles in the CheckPoint study of the Longitudinal Study of Australian Children's (LSAC). The parent-child concordance was significant for all the FSV tested and tended to differ by vitamer, which may reflect the influence of dietary intake, differential inter-conversions between metabolically active vitamers, and hereditary contributions. My results also highlight that age, BMI, and sex in a family setting are significant contributors to FSV concentrations. It remains possible that other factors such as physical activity, supplement intake, healthy food consumption, volumetric body fat distribution, the bioavailability of the vitamers, inflammatory conditions, sun exposure, and sunscreen may explain the epidemiological distributions of FSV concentrations. Future studies should evaluate associations between FSV profiles and metabolic outcomes in healthy vs diseased subjects to better characterise 'normality' in micronutrient profiles.

Chapter 5

General Discussion

5.1 Summary of findings

This thesis describes the development and validation of an automated, multiplexed, bioanalytical method using LC-MS/MS to quantify 11 Fat-Soluble Vitamers (FSV; A, D, E, and K group vitamers) in plasma [184]. The developed method was applied to characterise the population-level distribution of FSV using child/parent dyads of the CheckPoint cohort from the Longitudinal Study of Australian Children (LSAC).

FSV are essential micronutrients for healthy development, growth, metabolism, and cell regulation [6]. Suboptimal circulatory FSV levels can result in a number of health complications such as cardiovascular diseases, cancer, metabolic complications, and type-2 diabetes Mellitus [96,98]. The biological significance of FSV reinforced the importance of developing a reliable and reproducible method in FSV quantification. Quantification of vitamers in biological fluids is commonly performed using Liquid Chromatography coupled with Tandem Mass Spectrometry (LC-MS/MS) [15,84,89,98,99]. However, due to pre-analytical challenges (*e.g.,* FSV sensitivity to light, oxygen, heat, pH, chemical heterogeneity, reference standard availability, low circulatory concentrations [70,88,187,272]) as well as LC-MS/MS method complexity [84,89,98], developing an assay for the simultaneous quantification of multiple FSV at physiological concentrations has been challenging. When systematically investigating previous FSV quantification methods [1], I identified that sample extraction techniques, column technology, mobile phase solvents, ionisation, and method validation steps to be the most important factors in the LC-MS/MS workflow. I concluded that co-opting the best procedures and parameters from these methods is the key to developing a sensitive, robust method for FSV quantification.

Chapter two investigated the importance of optimising analytical and pre-analytical factors in LC-MS/MS method development. Method optimisation is a fundamental component of any analytical assay development. Optimisation eliminates unnecessary steps, avoids duplicate work by minimising in-built errors in measurement, and enables process automation [182]. During the present method optimisation, I identified that both pre-analytical factors such as stability (*e.g.,* storage conditions, photosensitivity, use of plastic consumables, N_2 drying, freeze-thaw cycles) and matrix complexity (*e.g.,* matrix type and anticoagulant use) as well as analytical factors, such as sample preparation, liquid chromatography, mass spectrometry, and

robotic automation are crucial in developing a sensitive, robust, and accurate LC-MS/MS method.

Chapter three described the development, validation, and robotic automation of an analytical assay to quantitate 11 FSV in heparin-treated plasma. The method I developed reported the quantification of a comprehensive range of vitamers covering all four FSV groups (*e.g.*, A group vitamers retinol, retinoic acid, and retinyl palmitate, D group vitamers 25-OH-D_3 and1α-25-$(OH)_2D_3$, E group vitamers α-tocopherol, γ-tocopherol and α-tocotrienol and K group vitamers K1, MK-4 and MK-7). The matrix effect caused by co-eluting matrix components (*e.g.*, phospholipids and small proteins) [191] with the analytes of interest was previously identified as one of the critical factors leading to poor recoveries and accuracy [84,153]. I included the use of Phree® phospholipid removal plates as an additional purification step at the end of the extraction process to reduce matrix effects, improving precision, recoveries, and the method's final sensitivity. Robotic automation of the liquid handling process made the present method more applicable for reproducible and robust analyses of large cohorts than labour-intensive, time-consuming, and error-prone manual pipetting methods [39]. I demonstrated the method's applicability to large cohort samples by analysing clinical samples from the LSAC (Longitudinal Study of Australian Children) CheckPoint child/parent dyads.

Chapter four: I characterised the parent and child concordance, age, sex, and BMI specificity of plasma FSV in CheckPoint child/parent dyads of the Longitudinal Study of Australian Children's (LSAC). Parents and children from the same family showed vitamer-specific concordant levels of plasma FSV. The strongest familial effects between parent-child dyads were observed for retinoic acid (coefficient correlation R= 0.57, p <2.2e-16) and weakest for retinol (R=0.18, p <2.2e-05). The familial concordance highlights the existence of a potential environmental (e.g. dietary intake) or shared gene environmental interaction contribution to FSV profiles [331,332]. The majority of FSV concentrations were markedly different between children and their parents, except for α-tocotrienol (p = 0.047). Retinol, retinoic acid, α-tocopherol, and γ-tocopherol concentrations were higher in parents when compared to children. By contrast, 25-OH-D_3 was observed at higher concentrations in children (p = 2.11e-10). The higher Vitamin D levels measured in children's plasma could potentially be due to higher sun exposure [336,337] and limited sunscreen use [339] as vitamin D is predominantly (~80%) produced in the skin via conversion of 7 dihydrocholesterol in response to sun UV light [334].

Retinol (p = 2.07e-05) and α-tocopherol (p = 0.0143) concentrations showed an association with age in parents. This is consistent with published observations of increased retinol levels with age [349,350]. By contrast, Bates *et al*., 2004 reported that both α and γ-tocopherol declined with age [341]. However, it has been previously reported that α-tocopherol carrier protein concentrations increase with age in the circulation, increasing the circulatory α-tocopherol concentration [342]. Moreover, dietary vitamin E requirement is primarily based on healthy diet choices and supplement intake. While I did not have access to this data for the children or parents of LSAC, previous studies have demonstrated that parents tend to acquire the required dietary intake and supplementation [341] compared to children [343]. Therefore, I speculate that the age-associated increase in α tocopherol concentrations I identified may be due to dietary choices, supplement intake, and increased carrier protein concentrations.

The plasma FSV concentrations I measured differed in males and females, except for α-tocotrienol (p = 0.398). The 25-OH-D_3 concentrations were higher in adult and child males (p =3.31e-03), while adult and child females (p =0.019) had higher γ-tocopherol concentrations. The α-tocopherol concentrations were higher in adult females (p =2.05e-04) but not in children. The A group vitamers retinol (p =<2 e-16) and retinoic acid (p =<1.7e-04) were higher in adult males than females but didn't vary across the child population. Several studies have reported that female adults and children are less likely to fulfil the dietary requirement of vitamin D compared to their male counterparts [314,356–358]. Therefore, it can be argued that dietary intake may have a crucial impact on gender-specific vitamer differences. FSV are lipid-soluble and mainly stored in the liver and adipose (fat) tissue [359–361]. Biologically, females store ~10% higher body fat, more specifically in their gluteal–femoral region than men [362]. Therefore, the additional fat deposits may explain the higher α and γ-tocopherol concentrations in females; however, they don't necessarily explain the lower A group vitamer concentrations in females. Similar to my findings, Stephensen *et al*. 2000 [349] and Söderlund *et al*., 2002 [363] reported higher retinol retinoic acid concentrations in males than females. Söderlund and several other studies further explained that although the dietary intake doesn't directly explain these vitamer differences, variation in seasonal food consumption may have an influence on these concentrations [379,380] since the intake of fresh fruits and vegetables is higher in the summer compared to winter [363]. In essence, it can be speculated that dietary intake [314], seasonal variation [363] and body fat deposition [317] may have played a role in sex-associated FSV differences.

The plasma FSV concentrations I measured were positively or negatively associated with an increase in BMI. I tested for both generation and sex-specific effects of BMI on FSV concentrations. In both parents (A, p =2.94 e-11) and children (C, p =2.31 e-04), with increasing BMI, decreased levels of 25-OH-D_3 and increased levels of retinoic acid (A: p =2.92 e-03, C: p =1.96 e-03) and γ-tocopherol (A: p =3.74 e-13, C: p =1.26 e-04) were observed, irrespective of the participant's sex. Both males (M) and females (F) had increased levels of α-tocopherol (M: p =6.33 e-07, F: p =3.37 e-13) and retinol (M: p <2 e-16, F: p <2 e-16) correlated with increasing BMI. BMI was negatively associated with retinol concentration in children (p = 2.34 e-05), while no association was observed for parents. On the contrary, BMI was positively associated with observed α-tocotrienol levels in parents (p =4.48 e-03), while child concentrations and gender-specific effects were not detected. Previous studies show that in overweight and obese individuals, volumetric distribution of body fat [311], persistent adipose tissue inflammation [310], and supplement intake [376] can affect circulatory FSV concentrations. I contend that this may explain the present results. Future studies need to further investigate the aforementioned associations to fully understand the relationship between changing BMI and FSV profiles.

5.2 Limitations

5.2.1 Limitations of the FSV quantification panel I developed

The method that I developed and validated consists of 11 vitamers representing all four major fat-soluble vitamin groups (A, D, E, and K). This is a relatively comprehensive FSV quantification method. However, some of the biologically important vitamers had to be excluded from the current method as their accepted levels in plasma were below the methods quantification limit (*e.g.,* 1-α-25(OH)2-D_3 (0.033-0.085 ng/mL [61])), or there was limited availability of the reference standards (*e.g.*, menaquinone 5, 6, 8, 9, 10 [381,382]), or chromatographic separation of the isomers was unable to be achieved using the current method (*e.g.,* 3-epi-25OH-D_3 [138,383]). Given the biological importance, future FSV methods could accumulate much more information and expand the existing knowledge by including the aforementioned vitamers in their panels.

FSV deficiencies can cause a number of adverse health effects, including night blindness [4], osteomalacia, rickets [5–7], neurological and visual deficits [8], impaired blood clotting [9],

and cardiovascular complications [10]. Therefore, it's important for an FSV method to be able to quantify not only the typical physiological concentrations observed across the population but also the suboptimal values of these vitamers. Although my method can reliably quantify 11 FSV at physiological concentrations, only retinol, 25-OH-D3, and α-tocopherol can be measured at clinically deficient levels. Therefore, I believe the future method development should consider not only quantifying the physiological concentrations of these FSV but also the clinical deficiencies and hypervitaminosis.

The current method's processing and analysis time for 96 samples was ~40h. This represents a significant limitation for the clinical application of this method. Specifically, a short turnaround time is essential during large cohort analysis and routine application in clinical settings [384]. Therefore, a fully automated sample extraction method with fewer steps and faster chromatography with alternative column choice that might improve the current turnaround time would be beneficial for the future applications of this method.

5.2.2 Limitations within the LSAC CheckPoint study design

There were several limitations in the present study that resulted directly from the LSAC study design. Although my research includes a large sample size (n=2490) representing both parents and their children, the study design was cross-sectional, and the plasma was only collected from the subjects at a single time point. Several longitudinal studies have noted the importance of collecting samples multiple times (2-5) points [385,386]. Comparing the results from longitudinal studies that collect samples at multiple time points can be vital in validating my outcomes as well as identifying the FSV profiles that have significant health outcomes at later time points.

The checkpoints child population only represents early adolescents (age 11-12 years), which is a narrow age range. Therefore, conducting CheckPoint studies at later stages of life (*e.g.*, middle to late adolescence (ages14-17), early adulthood (ages 17-22), and adulthood (ages 22-28)) would have allowed us to establish and compare the populational level FSV reference ranges between several developmental stages of life, investigate if the familial concordance of the measured FSV remains the same, or changes when early adolescents reach adulthood and no longer live with their parents; and predict future metabolic outcomes based on the changes in the FSV profiles.

5.2.3 Exploration of a wider Australian population

The population tested in the LSAC checkpoint was limited by unbalanced adult sex ratios compared to the general Australian population. The existence of a noticeable sex imbalance in the adult subgroup of the present cohort (males to females ratio of 1:10), when compared to the general Australian population (ratio of 1:1, 98.2 males per 100 females) [52], was a significant limitation of my study. A well-balanced sex distribution among the adult subgroups would have enhanced our understanding of the population-level characterisation of gender-specific FSV profiles. However, this is practically difficult to achieve in large cohort studies [387].

Compared to the broader Australian population, most of my cohort subjects (~78%) belonged to the middlemost advantaged socio-economic category according to the SEIFA scores. Among the general population, advantaged and disadvantaged individuals are spread throughout Australia. The top 20% of the advantaged individuals are clustered around capital cities and coastal regions, while the most disadvantaged live in regional and rural areas [388]. Therefore, including samples from a wider Australian population representing both advantaged and disadvantaged subjects would have provided a better representative populational level characterisation of the FSV profile.

5.2.4 Limitations in sample collection protocols

In the CheckPoint study, the samples from the participants were collected at a semi-fasted state, and only a single time point was measured. In metabolic studies, a single-time point analysis at a semi-fasted state does not account for biological fluctuations over time or fast/fed state changes [61,303]. In contrast, the inclusion of multi-time point measures would have reduced subject-to-subject and within-subject variation and allowed biological and technical variability analysis to provide reproducible and reliable results [389].

The collection of additional samples that may inform on physiological measurements such as chylomicrons [390], and low-density lipoprotein (VLDL) [391], would have improved my ability to further investigate the effects of age [390,392] and sex [393] on plasma FSV profiles. The sex effect in the recovery of ingested fat-containing plasma fractions of chylomicrons and VLDL was investigated by Nicolas *et al.*, 2006. They found that the ingested tracer-labelled fat in the chylomicron fraction was higher in men compared to women (p <0.05), and the

difference was insignificant for VLDL [393]. It can be presumed that the FSV dissolved in ingested fat behave the same. However, the relationship needs to be further addressed in future studies as this was one of the few studies investigating the sex-associated differences in ingested fat-containing plasma fractions of chylomicrons and VLDL.

Relas *et al*., 2000 discovered that the postprandial clearance of VLDL measured by retinyl palmitate in plasma was higher ($p < .01$) in older (78–79 years of age) subjects than in young (22–25 years of age) [394]. On the contrary, Cardinault *et al*., 2003 [392], in their research on the postprandial chylomicron carotenoid responses in young (age 20-35) and older (age 60-75) subjects, did not find evidence of an age effect. Borel *et al*., 1997 [395] observed significantly higher fasted chylomicron alpha-tocopherol postprandial concentrations in the elderly ((age 64-72), 33 +/- 2 µmol L^{-1}) than in the young ((age 20-30), 22 +/- 2 µmol L^{-1}). Interestingly, when the subjects were supplemented with vitamin E (432 or 937 IU as d1-α-tocopherol acetate), Borel *et al*. noticed significantly lower chylomicron alpha-tocopherol concentrations in the elderly than in the young subjects. These disparities highlight the importance of further investigating chylomicrons and VLDL in future FSV studies.

5.2.5 Targeted metabolomics approach in FSV quantification

This thesis describes the method development, validation, robotic automation, and a targeted metabolomics approach to quantify plasma FSV in CheckPoint child/parent dyads of the Longitudinal Study of Australian Children's (LSAC). Metabolomics approaches can be targeted or untargeted [396]. Targeted metabolomics is a valuable tool for quantitative analysis in epidemiological, clinical, and hypothesis-driven research [397]. Targeted approaches require previous knowledge of analytes of interest-based on metabolite-specific signals; however, they do not cover the full range of metabolites [398]. By contrast, non-targeted metabolomics approaches are typically employed in hypothesis-generating studies (*e.g.*, biomarker discovery). These non-targeted approaches have the potential to determine novel biomarkers and provide information on a wide range of metabolites where comprehensive metabolite identification and quantification is generally not the goal [399]. Combining these targeted and un-targeted approaches enables the discovery and quantification of interrelated metabolites of interest [396]. Therefore, conducting an untargeted metabolomics approach prior to the targeted metabolomics approach would have broadened the current FSV range through the identification of uncommon FSV forms upregulated by a particular pathology or lifestyle.

Furthermore, it would have allowed me to draw connections between metabolites of interest and other non-FSV biomarkers of health and pathology.

5.3 Future directions

5.3.1 Concerning FSV quantification methods

The present method demonstrates the capability of LC-MS/MS for quantifying 11 FSV covering all four vitamer groups using a low volume of plasma (45 µL). Simultaneous measurement of a wide range of FSV in one assay is vital for cost and time efficiency and acquiring a more comprehensive FSV profile using a limited sample volume. This applies in infant studies where sample volumes are minimal [29,89,236]. However, previous methods measuring vitamins A, D, E, and K in one assay either require higher sample volumes (plasma and serum), are limited to the range of vitamers or are absent, specially for infant and early childhood studies [84,89,91,95,97,192,400]. This highlights the importance of developing future methods sensitive enough to quantify all four FSV groups in one panel using a smaller plasma or serum volume. The development of a likely method will greatly enhance my understanding of the full FSV profile of the targeted individuals (*e.g.,* infants and young children). Khaksari *et al*., 2017 and 2018 [91] explored the option of using alternative biological fluids (*e.g.,* tears) in FSV quantification. Using tears to ascertain vitamin concentrations specially in young children and infants, is an attractive option due to the less-invasive nature of sample collection and simple preparation; however, it can be limited by the range of FSV measured [91,192,400]. Therefore, future FSV quantification methods should also explore the viability of using other biological fluids, such as tears, urine, milk, and lymph, as a suitable substitute for blood samples.

Measuring both FSV and water-soluble vitamers (WSV) together in one assay is another approach that can further enhance the analytical range, reduce required sample volume, labour and associated costs. Unfortunately, due to the complexity of the method, previous studies [89,91] that have tested this approach were limited to fewer FSV and WSV. Therefore, future method development exploring the applicability of this hybrid method to include both FSV and WSV can significantly enhance the power of an FSV method to characterise a broader range of vitamers in one assay.

My systematic review of previously published FSV methods and method optimisation identified several important pre-analytical and analytical parameters in method optimisation and multi-analyte FSV method development. These include the importance of investigating and/or optimising pre-analytical determinants (*e.g.*, storage conditions, photosensitivity, use of plastic consumables, N_2 drying, freeze-thaw cycles, and matrix complexity due to matrix type and anticoagulant use) and evaluating the strengths and weaknesses of previous and present LC-MS/MS analytical procedures (*e.g.,* extraction methods, parameters in chromatographic separation and detection. There are a number of emerging technologies (*e.g.*, micro-sampling [401], supercritical fluid chromatography, supercritical fluid extraction [123], matrix-assisted laser desorption/ionisation (MALDI)-MS [402], nano-liquid chromatography [403] and ion mobility mass spectrometry (IM-MS) [404]) that may have applications in FSV quantification. For example, micro-sampling techniques (*e.g.*, neonatal dried blood spot analysis) have been used to identify an infant's nutritional status and the requirement for supplementation. This technique uses approximately 3 µL of whole blood, which is a significant advantage as no additional blood drawing is needed [401].

The use of supercritical fluids is an interesting alternative to the method of FSV extraction using organic solvent extraction that I used. Potentially, the use of supercritical fluids will provide better sensitivity, sample throughput, and shorter extraction times. Supercritical fluid chromatography has a higher mass transfer rate and therefore permits the separation of analytes at a wide range of polarities and molecular masses faster than LC [123]. Nano-liquid chromatography has been identified to be advantageous due to its lower consumption of mobile phase solvents, reducing expenses due to both waste and solvents [403]. The quantitative ability using MALDI-MS has been identified to be x100 faster throughput than ESI-based LC/MS-MS with less sample volume and no organic solvent use [402]. Despite these technologies offering advantages over the method I described in Chapter 3, they generally also possess inherent disadvantages, which will likely prevent their widespread adoption in this field. For example, due to the limited sample volumes used in micro-sampling, MALDI-MS, as well as nano-liquid chromatography, have limited coverage and the quantification ability for FSV at very low circulatory concentrations [401–403]. The exception to this is LC/IM-MS, which is entirely complementary to existing LC-MS/MS and has been identified to successfully resolve epimers and isomers of D group vitamers [404]. However, to date, this technique also has limited analytical coverage (mainly D group vitamers). Therefore, due to the limited number of applications of these technologies to date, it’s difficult to perform reproducible, robust

comparisons. Future methods that include these techniques will provide critical information for specialist applications of FSV quantification.

5.3.2 Importance of familial concordance

As discussed in Chapter 4, the family was a vital contributor to the circulatory FSV concentrations in parent and child dyads. This highlights a likely dietary, behavioural, and shared gene environmental contribution to the FSV profiles. A multi-omics approach, combining metabolomics, proteomics, genomics, transcriptomics, and interactomics within a family setting, would provide great insight into [405] a multilayer characterisation of the underlying biomarkers, their involvement in circulatory concentrations of FSV and understanding their relationships with health and disease phenotypes [406].

The shared family environment's effect on plasma FSV levels also pinpoints the importance of family in the context of raising awareness and improving early adolescent lifestyle. Previous studies have reported that adolescents' dietary intake reflects parental feeding practices in a shared gene-environment. For example, obese or overweight parents would potentially pass their nutritional habits onto children with whom they share 50% of the DNA resulting in the children being overweight and obese [330]. In addition to the close family environment, previous work has identified a strong association between adolescents' dietary patterns and activity levels with their close friends in both home and school environments [407,408]. It would be interesting to investigate how this peer influence on physical activity and dietary patterns is also reflected in adolescents' FSV profiles. An intervention program comparing peer influence versus family setting to explore both subjective (*e.g.,* diet and activity level) and objective (*e.g.,* FSV) profile changes may uncover areas that need to be improved for the healthier transition from adolescents to adulthood. However, developing such targeted interventions requires extensive further research, including longitudinal and qualitative studies, to better understand the mechanisms by which parents and peers may influence adolescent eating and lifestyle behaviours.

5.3.3 Age, sex and BMI-specific effects on FSV profile

FSV concentrations exhibited vitamer-specific age (in parents), sex, and BMI effects in the present study. Therefore, future association studies between FSV, health, and disease outcomes should consider participants' age, sex, and BMI as important factors. This can be achieved by developing longitudinal FSV profile trajectories in prospective studies of health and disease varied by age, sex, and BMI. These studies could be similar to the vitamin D trajectories that are used to monitor the development of food sensitisation from birth to early childhood [409] and healthy ageing in adults [410]. Aside from demographics and BMI, several other factors also have been identified to affect the levels of circulating FSV, including individuals' baseline health [411], physical activity level [336,337], dietary intake [341], supplement intake [320,351], medication [412], socioeconomic status [411], smoking [413], alcohol consumption [414], skin pigmentation, latitude, seasonal effect, duration of sun exposure, atmosphere composition, clothing, and sunscreen use [335]. Therefore, multifactorial algorithms need to be developed to describe these longitudinal trajectories by incorporating additional social, biological and environmental factors. Additionally, a combination of the longitudinal trajectories for FSV profiling associated risk factors with epidemiology risk models in a time series would allow the investigation of long-term health events, such as cancer, cardiovascular diseases, and mortality [415].

Combining results from several studies has inherent limitations, such as differences in study design, purpose, and study parameters (e.g., methodologies, sample handling, user expertise, data analysis, and data processing). Standardisation of the LC-MS/MS methodologies is crucial in developing longitudinal trajectories. In Chapter 2, I described how robotic automation of the liquid handling process can minimise human error in manual pipetting and can help in the standardisation of the FSV extraction method. Further, automation of the LC-MS/MS data handling and interpretation process could also be beneficial for standardisation as it creates consistency across different studies and facilitates making reliable data-driven conclusions. For example, the semi-automated WEKA and Pipeline Pilot (Pipeline Pilot version 8.5.0.200, BIOVIA, San Diego, CA) developed by AstraZeneca to determine it's compound collection, limits manual review to 36.4% and enhanced the threshold of 90% confidence in predictions [416].

5.4 Conclusions

In this thesis, I report the successful development and validation of an automated method for measuring 11 A, D, E, and K vitamers in heparin plasma using LC-MS/MS. I investigated the importance of pre-analytical and analytical factors on final assay sensitivity using published literature and experimental procedures during the method development and optimisation. I identified several imperative parameters in LC-MS/MS method sensitivity, including pre-analytical (*e.g.*, experimental; storage conditions and matrix complexity due to matrix type and anticoagulant use, published literature; photosensitivity, use of plastic consumables, N_2 drying and freeze-thaw cycles) and analytical (*e.g.*, experimental; sample preparation, liquid chromatography, and mass spectrometry) conditions. The developed method was applied to CheckPoint parent and child dyads of the Longitudinal Study of Australian Children (LSAC) to characterise population-level FSV profiles and their variation by family, age, BMI, and sex. I identified a strong concordance in the circulatory concentrations of FSV between parents and children of the same family and marked differences by age, BMI, and sex. In the future, this vitamer panel can be applied to better characterise FSV profiles at population-level settings, expand the pre-existing predictive models of health and disease settings; and generate multifactorial, clinically relevant longitudinal nutrition trajectories of health and disease outcomes.

Appendices

Appendix 1: Supplementary tables of Chapter 1

Appendix 1.1: Supplementary Table 1: Deproteinization, SPE, and LLE methods

Author, year		**(Midttun & Ueland, 2011)**	**(Midttun *et al.*, 2016)**	**(Yu *et al.*, 2019)**	**(Hrvolová *et al.*, 2016)**	**(Andreoli *et al.*, 2004)**	**(Albahrani, Rotarou, Roche, & Greaves, 2016)**	**(Capote *et al.*, 2007)**	**(Konieczna, *et al.*, 2016)**	**(Khaksari, Mazzoleni, Ruan, Kennedy, & Minerick, 2017)**	**(Le, Yuan, Zhang, Wang, & Li, 2018)**	**Author, year**		**(Zhang *et al.*, 2018)**	**(Abro *et al.*, 2014)**	**(Hinchliffe, Rudge, & Reed, 2015)**
Plasma	Medium	EDTA	EDTA	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Plasma	Volume (µl)	100	200	N/A
	Volume (µl)	50	50	N/A	200	N/A	N/A	N/A	N/A	100	60	Serum	Volume (µl)	N/A	N/A	100
Serum	Volume (µl)	N/A	N/A	50	N/A	60	100	1000	200	N/A	N/A	Solvent 1		EtOH	Acetonitrile	35:65 (v/v) propan-2-ol:water containing 0.25 mol/L sodium hydroxide
Solvent 1		EtOH	EtOH	MeOH	EtOH	EtOH	Water	MeOH	MeOH	MeOH	ACN	Volume (µl)		300	200	100

Volume (µl)	100	100	200	200	240	100	3000	800	800	90	Rigorous mix	50s	N/A	1 min
Rigorous mix	N/A	N/A	N/A	N/A	N/A	10s	1 min	30s	N/A	1 min	Sonic ation	1min	N/A	N/A
Centrifuge	N/A	N/A	N/A	N/A	N/A	N/A	N/A	10 min 16770g 4C	5min 13000 rpm	12,000 g, 2 min at 4 °C	Gentle mix	5min	1min	N/A
Solvent 2	BHT(1g /L)	N/A	0.1mol /L ZnSO4	N/A	Ethyl acetate	MeOH	N/A	N/A	N/A	N/A	Incubation	30 min/4C	N/A	5min gravity flow
Volume (µl)	N/A	N/A	100	N/A	240	200	N/A	N/A	N/A	N/A	Centrifuge	12000 RPM 4C/10 min	5min 1900g	N/A
Rigorous mix	15s	20s	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Supernant Volume (µl)	300	N/A	N/A
Sonic ation	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Solvent 2	water	MeOH	N/A
Gentle mix	5min	N/A	N/A	N/A	N/A	10s	N/A	N/A	Yes / Details N/A	N/A	Volume (µl)	300	680	N/A
Incubation	N/A	N/A	N/A	N/A	N/A	RT / 10min	4c /5min	N/A	4c /10min	N/A	SPE plate	HBL µElution Plate	Lichrolut RP-18E 200mg, 3ml	Isolute Supported Liquid Extraction (SLE) 96-well plate (Biotage,
Solvent 3	isooctane CHCl3 (3:1)	isooctane CHCl3 (3:1)	Hexane	Hexane	N/A	Hexane	Hexane	Hexane x 2	Hexane x 3	N/A	SPE plate Pre-treatment	MeOH: water 50:50 V: V	MeOH :EtOH: water (V:V:V)	N/A

Solvent 2	N/A	BHT(1g /L)	N/A	BHT(0.1g /L)	N/A	N/A	N/A	N/A	N/A	N/A	Volume (µl)	600	3000	N/A
Volume (µl)	300	300	500	500	N/A	1500	3000	800	1000	N/A	Incubation	N/A	N/A	5min gravity flow
Gentle mix	5min	5min	1500 rpm 5 min	1 min	N/A	Yes / Details N/A	N/A	N/A	N/A	N/A	organic phase (µl)	600	N/A	N/A
Centrifuge	3 min, 4000g, 4C	5 min, 4000g, 4C	10 min, 1200g	5 min, 2070g 4C	4min 11000 rpm	5 min 3000 rpm	4500 rpm	N/A	N/A	N/A	Wash	20% MeOH /water	10% MeOH	N/A
organic phase (µl)	200	200	400	N/A	N/A	800	N/A	N/A	N/A	N/A	Volume (µl)	600	3000	N/A
Drying	Nitrogen 23C	Nitrogen 35C	Nitrogen 35C	Nitrogen 25C	N/A	Nitrogen RT	Nitrogen	Nitrogen 45C	Nitrogen	N/A	Drying	Vacuum	N/A	N/A
Reconstitute Solvent 4	MeOH	MeOH	Methanol /water (70:30)	MeOH	N/A	MeOH	MeOH	MeOH	MeOH	N/A	Elution	Isopropanol /ethyl acetate (80:20)	MeOH	90:10 (v/v) hexane:propan-2-ol
Volume (µl)	50	100	300	100	N/A	250	300	100	150	100	Volume (µl)	600	1000	750
Solvent 5	BHT(1g /L)	BHT(1g /L)	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	Collection	96 well plate	N/A	2mL 96-deep-well polypropylene plate
Rigorous mix	20s	20s	N/A	N/A	N/A	20s	N/A	N/A	N/A	N/A	Drying	Nitrogen 35C	N/A	SPE-Dry 96
											Incubation	N/A	N/A	5min gravity flow
											Reconstitute	Methanol	N/A	80:20 (v/v) methanol:water containing 2 mmol/L ammonium

				acetate and 0.1% (v/v) formic acid
	Volume (µl)	100	N/A	200
	Rigorous mix	5min	N/A	2 min
	Centrifuge	N/A	N/A	5 min at 8000 g

Appendix 1.2: Supplementary Table 2: Liquid chromatography parameters

Author, year	**(Midttun & Ueland, 2011)**	**(Midttun *et al.*, 2016)**	**(Yu *et al.*, 2019)**	**(Zhang *et al.*, 2018)**	**(Hrvolová *et al.*, 2016)**	**(Abro *et al.*, 2014)**	**(Andreoli *et al.*, 2004)**	**(Albahrani, Rotarou, Roche, & Greaves, 2016)**	**(Capote *et al.*, 2007)**	**(Konieczna, *et al.*, 2016)**	**(Khaksari, Mazzoleni, Ruan, Kennedy, & Minerick, 2017)**	**(Hinchliffe, Rudge, & Reed, 2015)**	**(Le, Yuan, Zhang, Wang, & Li, 2018)**
Analytical Platform	Agilent series 1100 (HPLC)	Agilent 1290 Infinity (UHPLC)	Waters ACQUITY UHPLC system	Waters iclass-Xevo TQ-S UPLC	Agilent 1100 HPLC system	LCQ Advantage Max HPLC	Perkin–Elmer series 200 binary system HPLC	Agilent 1290 Infinity UHPLC Agilent 1200 Infinity HPLC	Agilent 1200 Series HPLC	Agilent 1260 Infinity system HPLC	Accela LC (UHPLC)	Waters ACQUITY UHPLC system	Ekspert ultraLC 100-XL system (UHPLC)
Analyte	all-*trans*-retinol 25-hydroxyvitamin D3 25-hydroxyvitamin D2 α-tocopherol	Retinyl palmitate β-carotene D3, vitamin D2 (ergocalciferol D2) 25(OH) D3 25-hydroxyvitamin D2	all-*trans* retinol α-tocopherol	Retinol 25-hydroxy vitamin D3 α-tocopherol Vitamin K1	retinol 25-hydroxycholecalciferol retinol acetate α-tocotrienol cholecalciferol astaxanthin lutein zeaxanthin canthaxanthin E-β-apo-8'-carotenal cryptoxant	vitamin D3 vitamin E vitamin K1	*trans*-β-Carotene all-*trans*-Retinol retinyl acetate retinyl palmitate α-tocopherol α-tocopherol acetate	Retinol 25-OHD3 Epi-25-OHD3 25-OHD2 α-tocopherol	A (all-*trans*-retinol) D2 (ergocalciferol) D3 (cholecalciferol) 25-hydroxy vitamin D3 1,25-dihydroxyvitamin D3	all-*trans*-retinol retinol acetate 25-hydroxyvitamin D3 1,25 dihydroxy vitaminD3 α-tocopherol K1(phylloquinone)	Retinol 25(OH)D3 D3-Cholecalciferol α-Tocopherol Phylloquinone	all-*trans*-retinol α-tocopherol	all-*trans*-retinol 25-hydroxyvitamin D3 Epi-25-OHD3 25-hydroxyvitamin D2 α-tocopherol

		Tocopherol (-Toc) PK MK-4 MK-7			hin 13-Z-β-carotene α-carotene β-carotene 9-Z-β-carotene 5-Z-lycopene				25-hydroxyvitamin D2 E (α-tocopherol) δ-tocopherol K1 (phylloquinone) K3 (menadione)				
Standards	2H6-all-*trans* retinol 2H6-25-hydroxyvitamin D3 2H9-α-tocopherol	d6-retinyl acetate d6- -carotene d7-D3 d6-25(OH)D3 d6- -Toc [18O2]-PK [18O2]-MK-4 [18O2]-MK-7	[2H]6-Retinol [2H]6-α-tocopherol	2H4-retinol-d4 2H6-25-hydroxyvitamin D3-d6 2H9-α-tocopherol-d9 2H7-vitamin K1-d7	n.d.	Vitamin-D2	n.d.	25-hydroxyvitamin D3-[2H3](D3) α-tocopherol-[2H6](D6) retinol-[2H5](D5)		retinol acetate	α-tocopherol-D6 Retinol-D5 Phylloquinone-D7	d5-retinol d6- α-tocopherol	25(OH)D2-d3 25(OH)D3-d6 α-tocopherol-d6
Injecting volu	20	10	10	6	20	25	20	8	10	10	25	7.5	15

me (ul)													
Column size, mm	Ascentis Express C18 column (50 × 4.6 mm, particle size 2.7 µm)	Poroshell 120 (50 × 4.6 mm, particle size 2.7 µm) stable-bond C18 column	Waters ACQUITY UPLC BEH Phenyl column (2.1 mm × 100 mm, 1.7 um)	Phenyl-Hexyl analytical column (ACQUITY UPLC® CSH™, 1.7 µm, 2.1×50 mm) Phenyl-Hexyl VanGuard™ Pre-column C18 (ACQUITY UPLC® CSH™, 1.7 µm, 2.1×50 mm)	YMC Carotenoid S-5 µm, C30, 250 × 4.6 mm(Waters, Milford, MA, USA)	RP-18e column, C18, (100 x4.6 mm)	Supelcosil LC-8-DB column (150mm ×4.6-mm I.D., 3 µm	(PFP 150 mm x 2 mm x 3 µm) Meta-Guard (2.0 mm Pursuit 3u PFP	Zorbax Eclipse XDB-C18 analytical column (4.6mm x 150mm, 5 µm particle size; Agilent)	core-shell column − Poroshell120 EC - C 18 column (100 × 3.0 mm, 2.7 µm particle size)	2.1 x 150 mm, reverse-phase column 3µm C18 silica 100 _A pore size connected to a 2.1 x 10 mm guard column	Kinetex Biphenyl 2.6 µm, 50mmx2.1mm analytical security guard ULTRA UHPLC Biphenyl filte	2.6 µm PFP 100 Å (100 × 3 mm)

Mobile phase	A 2.5mM ammonium formate in MeOH B 80% MeOH, 20% H2O	A 80% methanol 20% water B 0.1% Formic acid in methanol	A contained water with 0.1% formic acid B consisted of methanol	A: 0.1% formic acid aqueous B: 5mM ammonium formate and 0.1% formic acid in acetonitrile	A: MeOH, 2-aminoacridone(AmAc) at a concentration of 0.7 g/L and 0.1% of acetic acid B: MTBE and MeOH (80:20, v/v), AMAC at a concentration of 0.7 g/L and 0.1% of acetic acid	A: 95% methanol and 0.1% of formic acid B: Methanol	A: methanol/ dichloromethane mixture (95:5, v/v)	A: 0.1% formic acid and 2% methanol in milli-Q water B: 0.1% Formic acid in methanol	A: 5mM ammonium formate in acetonitrile/water 90:10 (v/v) B: 5mM ammonium formate in 100% methanol	A: acetonitrile mixed with 0.1% formic acid and5 mM ammonium formate (90:10, v/v) B: methanol mixed with 0.1% formic acid and 5 mM ammonium formate.	A 9:1 (v/v) ACN/water and 5 mM ammonium formate	A: 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid in water B: 2 mmol/L ammonium acetate and 0.1% (v/v) formic acid	A: 50% methanol in Water with 0.2% formic acid B: methanol with 0.2% formic acid
Flow rate	1.1 mL/min	1.6 mL/min	0.4 mL/min	0.4 mL/min	0.6 mL/min	1.0 mL/min	0.80 mL/min	0.2 mL/min	1.0 mL/min	0.5 mL/min	0.2 mL/min	0.4 mL/min	0.35 mL/min
Gradient	0–4.0 2.5mM ammonium formate in MeOH; 4.1–5.0 80% MeOH, 20% H2O	0−0.1 80% methanol 20% water 1.0−3.30 2.5 mM ammonium formate in	0–0.5 min, 60% B 1.5–2.0 min, 90% B 2.01–3.0 min, 100	solvent B 0 min 30% 0–5 min, 30% to 75% 6 min, 75% 6.1 min, 100%,	(t (min), %A): (0.0, 90%); (10.0, 75%) (20.0, 50%) (25.0, 30%) (35.0, 10%) (37.0, 6%) (39.0, 90%) (50.0, 90%)	95 to 99% over 10 min 99% methanol 2 min 95% methanol	Isocratic	LC-1290 (t (min), %A, B%): (0.0, 65, 35): (5.5, 30, 70): (16, 22, 78): (18, 4, 96): (19.5, 0,	(t (min)): 0.0-2.0, A isocratic 2.0-5.0, B linear 23-27, A	0–2 min − 0% B, 2–7 min − 0–100% B, 7–18 min − 100% B, 18–18.1 min − 100–0% equilibration process: 18.1–25	0 min, 100% A; 1 min, 100% A; 6 min, 0% A; 25 min, 0%	30:70 (v/v) mobile phase A:B 1.5 min 70% to 100% mobile phase B 1 min holding at 100% mobile phase B	0-2.5 min 40% B, 2.5-6 min 40-100% B, 6-10 min 100% B, 10-10.1 min 100-40% B, 10.1-15 min 40% B

	methanol 3.35−4.5 min, 80% methanol/20% water.	% B 3.01–4.5 min, 60% B	8 min, 30% 12 min 30%	3 min	100): (34, 0, 100): (36, 45, 45): (37, 65, 35): (42,65, 35) LC-1200 (t (min), %A,B %): (0.0, 60, 40): (7.5, 30, 70): (18, 22, 78): (23, 4, 96): (24.5, 0, 100): (36, 0, 100): (36.1, 45, 55): (38, 60, 40): (45,60, 40)	min − 0% B

Appendix 1.3: Supplementary Table 3: Mass spectrometry details

Publication	Analytical Platform	Ionisation	Precursor (m/z)	Product (m/z)	Declustering potential (V)	Entrance potential (V)	Exit potential (V)	Collision energy (V)	RT (min)	LoQ (ug/ml)	LoD (ug/mL)
(Midttun & Ueland, 2011)	MS/MS API 4000 triple-quad	ESI+	all-*trans*-retinol								
			269.1	93.2	60	n.d.	5	30	1.73	0.05729	0.028645
			25-hydroxyvitamin D2								
			413.5	395.4	50	n.d.	10	14	1.61	0.002722	0.001361
			25-hydroxyvitamin D3								
			401.4	383.2	30	n.d.	10	10	1.59	0.002644	0.001322
			401.4	257	30	n.d.	15	21	1.59	n.d.	n.d.
			α-tocopherol								
			433.4	167.4	30	n.d.	15	30	3.01	n.d.	n.d.
			433.4	137.4	30	n.d.	15	50	3.01	n.d.	n.d.
(Midttun *et al*., 2016)	MS/MS Agilent 7890B coupled to 7010	ESI+	all-*trans*-retinol								
			270.1	94.2	30	12	n.d.	30	1.3	n.d.	n.d.
			25-hydroxyvitamin D2								
			413.5	355.5	40	12	n.d.	15	1.3	n.d.	0.01032
			25-hydroxyvitamin D3								
			401.4	365.4	50	12	n.d.	20	1.28	n.d.	0.00681
			α-tocopherol								
			431.4	137.4	40	12	n.d.	60	2.04	n.d.	n.d.
			γ-tocopherol								
			417.5	151	30	14	n.d.	30	1.9	n.d.	n.d.
			K1 (phylloquinone)								

			451.2	187.2	40	12	n.d.	30	2.78	n.d.	0.000148
(Yu *et al*., 2019)	MS/MS AB Sciex 4000 QTrap system	ESI+	all-*trans*-retinol								
			269.2	95.2	40	n.d.	14	14	1.96	0.00972	n.d.
			269.2	213.2	40	n.d.	14	16	1.96		
			α-tocopherol								
			431.5	165	54	n.d.	14	24	2.57	0.0092	n.d.
			431.5	137	54	n.d.	14	57	1.96		
(Zhang *et al*., 2018)	MS/MS Waters iclass-Xevo TQ-S	ESI+	Retinol								
			269.1	93.04	18	n.d.	n.d.	26	5.11	0.001	0.00215
				213	n.d.	n.d.	n.d.	12			
			25-hydroxy vitamin D3								
			383.29	107.08	20	n.d.	n.d.	22	4.73	0.0012	0.0004
				257.04	n.d.	n.d.	n.d.	22			
			α-tocopherol								
			431.26	136.99	2	n.d.	n.d.	46	7.19	0.00004	0.00001
				165.09	n.d.	n.d.	n.d.	30			
			Vitamin K1								
			n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
(Hrvolová *et al*., 2016)	MS/MS QTRAP4000 (DAD detector)	APCI	retinol								
			269	181	35	10	15	14	5	0.005	0.002
			25-hydroxycholecalciferol								
			383	365	58	10	15	17	6.45	0.011	0.003
			retinol acetate								
			329	269	41	10	15	18	7.34	0.008	0.002

		α-tocotrienol									
		411	165	181	10	15	57	9.03	0.376	0.113	
		cholecalciferol D3									
		385	367	60	10	15	24	10.45	0.018	0.005	
		astaxanthin									
		597	147	84	10	15	40	11.69	0.003	0.001	
		lutein									
		551	429	102	10	15	26	12.98	0.028	0.008	
		zeaxanthin									
		568	476	85	10	15	25	14.4	1.406	0.422	
		canthaxanthin									
		565	363	70	10	15	15	16.47	0.006	0.002	
		E-β-apo-8'-carotenal									
		417	325	70	10	15	14	17.16	0.01	0.003	
		cryptoxanthin									
		553	535	94	10	15	20	20.71	0.812	0.244	
		13-Z-β-carotene									
		536	444	48	10	15	24	25.03	0.187	0.056	
		α-carotene									
		536	444	120	10	15	24	25.85	0.073	0.022	
		β-carotene									
		537	413	85	10	15	28	27.42	0.138	0.041	
		9-Z-β-carotene									
		537	413	75	10	15	30	28.13	0.975	0.293	
		5-Z-lycopene									
		537	413	87	10	15	23	37.65	0.631	0.189	
(Abro *et al*., 2014)	MS/MS Ion trap APCI	D3									

			385.23	367.27					6.58	0.00033	0.0001
			E								
			473.47	328.23					9.36	0.00455	0.00136
			K1								
			451.41	369.41					11.68	0.00017	0.000052
(Andreoli *et al*., 2004)	MS/MS Sciex API 365 triple-quadrupole mass spectrometer	APCI	*trans*-β-Carotene								
			537	177	n.d.	n.d.	n.d.	21	6.2	0.0023	0.001
			all-*trans*-Retinol								
			269	213	n.d.	n.d.	n.d.	17	1.2	0.0011	0.0002
			retinyl acetate								
			269	213	n.d.	n.d.	n.d.	17	1.5	n.d.	n.d.
			retinyl palmitate								
			269	213	n.d.	n.d.	n.d.	17	5.9	n.d.	n.d.
			α-tocopherol								
			430	165	n.d.	n.d.	n.d.	29	2.7	0.0099	0.0005
			α-tocopherol acetate								
			473	207	n.d.	n.d.	n.d.	29	3.1	n.d.	n.d.
(Albahrani, Rotarou, Roche, & Greaves, 2016)	MS/MS Agilent 6490 triple quad (1) Agilent 6410 triple quad(2)	ESI+	Retinol								
			269	93	n.d.	n.d.	n.d.	25	20-21	0.028	0.028
			269	93	n.d.	n.d.	n.d.	n.d.	n.d.	0.045	0.028
			25-OHD3								
			401	383	n.d.	n.d.	n.d.	4	20-21	0.0014	0.0006
			401	383	n.d.	n.d.	n.d.	n.d.	n.d.	0.0014	0.0008
			Epi-25-OHD3								
			401	383	n.d.	n.d.	n.d.	n.d.	n.d.	0.0015	0.0007
			401	383	n.d.	n.d.	n.d.	n.d.	n.d.	0.0014	0.0008

		25-OHD2								
		413	395	n.d.	n.d.	n.d.	4	n.d.	0.0014	0.0006
		413	395	n.d.	n.d.	n.d.	n.d.	n.d.	0.002	0.001
		α-tocopherol								
		431	165	n.d.	n.d.	n.d.	28	27-28	0.8614	0.43071
		431	165	n.d.	n.d.	n.d.	n.d.	n.d.	1.2921	0.43071
(Capote *et al*., 2007)		(all-*trans*-retinol)								
		269.3	93.1	n.d.	n.d.		20	5.648	0.00005	0.000015
		D3 (cholecalciferol)								
		397.4	158.9	n.d.	n.d.		20	13.724	0.0003	0.00009
		25-hydroxyvitamin D3								
		383.3	121.3	n.d.	n.d.		20	6.09	0.0006	0.00018
		1,25-dihydroxyvitamin D3								
		399.4	226.9	n.d.	n.d.		20	3.371	0.0005	0.00015
		D2 (ergocalciferol)								
		397.4	158.9	n.d.	n.d.		20	13.308	0.0002	0.00006
		25-hydroxyvitamin D2								
		413.3	395.3	n.d.	n.d.		12.5	6.586	0.0004	0.00012
		α-tocopherol								
		431.4	165	n.d.	n.d.		15	16.095	0.00004	0.000012
		δ-tocopherol								
		403.4	137	n.d.	n.d.		15	12.898	0.00015	0.000045
		K1 (phylloquinone)								
		451.4	187	n.d.	n.d.		20	26.924	0.0001	0.00003
		K3 (menadione)								
		173	135.1	n.d.	n.d.		20	1.825	0.001	0.0003
	MS/MS Agilent 6410 triple quadrupole	ESI+								

(Konieczna, 2016)	MS/MS Agilent 6120 mass detector with UV detector	ESI+	A (all-*trans*-retinol)								
			269							0.00005	0.000015
			25-hydroxyvitamin D3								
			383							0.0012	0.0036
			1,25-dihydroxyvitamin D3								
			399							0.0005	0.00015
			E (α-tocopherol)								
			431							0.05	0.015
			K1 (phylloquinone)								
			451							0.00033	0.0001
(Khaksari, Mazzoleni, Ruan, Kennedy, & Minerick, 2017)	MS/MS LCQ Fleet MS	ESI+	A (retinol)								
			269	213				25	7.5	0.001317	0.000401
			25-hydroxyvitamin D3								
			401	383				16	7.91		
			Vitamin D3								
			385	367				22	13.56		
			E (α-tocopherol)								
			429	165				27	15.41		
			K1 (phylloquinone)								
			451	187				25	20.81	0.000602	0.00018
(Hinchliffe, Rudge, & Reed, 2015)	Waters TQD tandem mass spectrometer	ESI+	Retinol								
			269	93.15	n.d.	n.d.	n.d.	25	1.5	0.0196	0.014
			269	81.1	n.d.	n.d.	n.d.	25	1.5		
			α-tocopherol								
			431	165	n.d.	n.d.	n.d.	28	2	0.112	0.0775
			431	165	n.d.	n.d.	n.d.	n.d.	2		

(Le, Yuan, Zhang, Wang, & Li, 2018)	AB SCIEX 4500 QTRAP mass spectrometer	ESI+	all-*trans*-retinol						
			269.2	93.1	60		29.2	6.4	0.075
			269.2	213.1	60		18.8	6.4	
			25-hydroxyvitamin D2						
			413.2	395.4	90		13	6.3	0.002
			413.2	159.1	90		35	6.3	
			25-hydroxyvitamin D3						
			401.2	365.3	100		15	6.2	0.001
			4.1.2	383.3	100		13	6.2	
			Epi-25-OHD3						
			401.2	365.3	100		15	6.25	0.001
			401.2	383.3	100		13	6.25	
			α-tocopherol						
			431.4	165	100		28	8.4	1
			431.4	137	90		58	8.4	

Appendix 2: Supplementary methods of chapter 1 systematic review

G.R.P. Arachchige, E.B. Thorstensen, M. Coe, E.J. McKenzie, J.M. O'Sullivan, C.J. Pook, LC-MS/MS quantification of fat-soluble vitamers – A systematic review, Anal. Biochem. 613 (2021). https://doi.org/10.1016/j.ab.2020.113980 [1].

The objective of the study

This study aimed to review methods developed for multi-analyte vitamer panels. Therefore, primary literature on analytical method development for the quantification of fat-soluble vitamers was included in this study. The study comprises articles on circulatory forms of FSV, A, D, E, and K in human and non-human mammals. Nearly all FSV quantification methods are limited to either single or a limited number of vitamers apart from LC-MS/MS techniques.

Database search strategy

I searched the online databases (see below) using a mixture of free-text terms. Searching started on the 4th of July 2018 and lasted until the 16th of September 2018. Keywords, search terms, and the search algorithm is presented (Tables 1 and 2).

Database selection was determined by the coverage of articles in the fields of biochemistry and analytical chemistry. BIOSIS, EMBASE, and MEDLINE primarily focus on clinical research, not analytical chemistry. Moreover, the search subject heading terms (MESH) in these three databases did not cover terms that were appropriate for this review. Therefore, BIOSIS, EMBASE, and MEDLINE were not included in this study.

The most relevant databases for this systematic review had significantly different search algorithms. Therefore, I modified my search strategies for each database (see below). Based on the initial evaluation, I determined that the most relevant databases were SciFinder, Scopus, and Google. I did not restrict database results by date of publication. Web of Science, Science Direct and Reaxys were considered but are fully covered by Scopus and SciFinder.

SciFinder

SciFinder is the most comprehensive chemical literature database available, produced by Chemical Abstracts Service. SciFinder coverage was from 1907 – the present (www.sso.cas.org/as/E5VS4/resume /as/authorization.ping). SciFinder fully covers PubMed, Reaxys, Compendex, INSPEC, and Web of Science.

The search strategy started with entering the chemical names (Table 2) into the search box using the 'substrate identifier' function. The chemical names with two parts were either connected with a hyphen or inverted commas within brackets to avoid the complex chemical names being searched as separate terms like beta-carotene or α-tocopherol. Substrate identifier results were then copied into the structural similarity search to include all synonyms for each vitamer. The resulting reference list, linked with the structural similarity search, was then further refined with additional key terms specified in Tables 1 and 2. The resultant articles were further refined to the 'analytical chemistry' category provided by SciFinder. My SciFinder results returned articles from 1964 - 2018. Wildcard symbols were not required as SciFinder auto-truncates. While I included abbreviations and the full term, *e.g.,* ("liquid chromatography") or ("LC"), SciFinder can detect and translate some abbreviations.

Scopus

Scopus is a commercial database for peer-reviewed literature produced by Elsevier (www.Elsevier .com/solutions/Scopus). It covers articles from 1966 to 2019 and fully covers Web of Science, MEDLINE, EMBASE, and ScienceDirect.

My search for Scopus used the title, abstract, keywords, chemical name, and CAS number. The resultant articles were then further refined with additional key terms (Tables 1, 2). Unlike SciFinder, I had to use search phrases, proximity operators, and wild cards in Scopus. The Scopus search algorithm only provided the option to narrow down the articles to "Biochemistry, Genetics, and Molecular Biology" and "Chemistry" but did not offer an analytical chemistry category. Even with the addition of the key terms 'analytical' and 'analytical chemistry,' I found the resultant articles were not specific enough, leading us to add the terms "vitamin" or "vitamins" to obtain more relevant results.

Google

Google is a web search algorithm developed by Larry Page and Sergey Brin [417]. Google was used to search for commercial analytical methods developed by instrument vendors. This search was included because instrument vendors sometimes develop new methods before the academic literature is published. Google Scholar was not used in this review due to its lack of comprehensive coverage, less specific search algorithms, and less accurate citation tracker (www.uit.stanford .edu/service/gsuite).

Table 1: Key words used for Scopus, Scifinder and Google searches

Scopus search final

Vitamin A 2216	((((TITLE-ABS-KEY ("beta carotene") OR SRCTITLE ("beta carotene") OR CHEMNAME ("beta carotene") OR CASREGNUMBER (7235-40-7))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("alpha Carotene") OR SRCTITLE ("alpha Carotene") OR CHEMNAME ("alpha Carotene") OR CASREGNUMBER (432-70-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*cryptoxanthin) OR SRCTITLE (*cryptoxanthin) OR CHEMNAME (*cryptoxanthin) OR CASREGNUMBER (472-70-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*lutein) OR SRCTITLE (*lutein) OR CHEMNAME (*lutein) OR CASREGNUMBER (127-40-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR ((((TITLE-ABS-KEY (*zeaxanthin) OR SRCTITLE (*zeaxanthin) OR CHEMNAME (*zeaxanthin) OR CASREGNUMBER (144-68-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*lycopene) OR SRCTITLE (*lycopene) OR CHEMNAME (*lycopene) OR CASREGNUMBER (502-65-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*retinol) OR SRCTITLE (*retinol) OR CHEMNAME (*retinol) OR CASREGNUMBER (68-26-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*retinoic-acid) OR SRCTITLE (*retinoic-acid) OR CHEMNAME (*retinoic-acid) OR CASREGNUMBER (302-79-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR ((((TITLE-ABS-KEY (*retinal) OR SRCTITLE (*retinal) OR CHEMNAME (*retinal) OR CASREGNUMBER (116-31-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*palmitate) OR SRCTITLE (*palmitate) OR CHEMNAME (*palmitate) OR CASREGNUMBER (79-81-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (retinal*) OR SRCTITLE (retinal*) OR CHEMNAME (retinal*) OR CASREGNUMBER (116-31-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) AND (LIMIT-TO (SUBJAREA , "BIOC ") OR LIMIT-TO (SUBJAREA , "CHEM ") OR LIMIT-TO (SUBJAREA , " CHEM "))
Vitamin D 860	((((TITLE-ABS-KEY (*25-hydroxyvitamin-d3) OR SRCTITLE (*25-hydroxyvitamin-d3) OR CHEMNAME (*25-hydroxyvitamin-d3) OR CASREGNUMBER (19356-17-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Vitamin D3") OR

	SRCTITLE ("Vitamin D3") OR CHEMNAME ("Vitamin D3") OR CASREGNUMBER (67-97-0))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("3-Epi-25-Hydroxyvitamin D3") OR SRCTITLE ("3-Epi-25-Hydroxyvitamin D3") OR CHEMNAME ("3-Epi-25-Hydroxyvitamin D3") OR CASREGNUMBER (73809-05-9))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("1α-25-dihydroxyvitamin-D3") OR SRCTITLE ("1α-25-dihydroxyvitamin-D3") OR CHEMNAME ("1α-25-dihydroxyvitamin-D3") OR CASREGNUMBER (32222-06-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR ((((TITLE-ABS-KEY ("24,25-dihydroxyvitamin D3") OR SRCTITLE ("24,25-dihydroxyvitamin D3") OR CHEMNAME ("24,25-dihydroxyvitamin D3") OR CASREGNUMBER (55721-11-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*25-dihydroxyvitamin-d3) OR SRCTITLE (*25-dihydroxyvitamin-d3) OR CHEMNAME (*25-dihydroxyvitamin-d3) OR CASREGNUMBER (32222-06-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("VITAMIN D2") OR SRCTITLE ("VITAMIN D2") OR CHEMNAME ("VITAMIN D2") OR CASREGNUMBER (50-14-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("25-HYDROXYVITAMIN D2") OR SRCTITLE ("25-HYDROXYVITAMIN D2") OR CHEMNAME ("25-HYDROXYVITAMIN D2") OR CASREGNUMBER (21343-40-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR ((((TITLE-ABS-KEY (*25-hydroxy-vitamin-d2) OR SRCTITLE (*25-hydroxy-vitamin-d2) OR CHEMNAME (*25-hydroxy-vitamin-d2) OR CASREGNUMBER (908126-48-7))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*25-dihydroxyvitamin-d2) OR SRCTITLE (*25-dihydroxyvitamin-d2) OR CHEMNAME (*25-dihydroxyvitamin-d2) OR CASREGNUMBER (60133-18-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) AND (LIMIT-TO (SUBJAREA , "BIOC ") OR LIMIT-TO (SUBJAREA , "CHEM ") OR LIMIT-TO (SUBJAREA , " CHEM "))
Vitamin E 1200	(((((TITLE-ABS-KEY (*tocopherol) OR SRCTITLE (*tocopherol) OR CHEMNAME (*tocopherol) OR CASREGNUMBER (59-02-9))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR (((TITLE-ABS-KEY ("Alpha-Tocopherol") OR SRCTITLE ("Alpha-Tocopherol") OR CHEMNAME ("Alpha-Tocopherol") OR CASREGNUMBER (59-02-9))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("BETA-TOCOPHEROL") OR SRCTITLE ("BETA-TOCOPHEROL") OR CHEMNAME ("BETA-TOCOPHEROL") OR CASREGNUMBER (16698-35-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Gamma-Tocopherol") OR SRCTITLE ("Gamma-Tocopherol") OR CHEMNAME ("Gamma-Tocopherol") OR CASREGNUMBER (54-28-4)))

	AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Delta-Tocopherol") OR SRCTITLE ("Delta-Tocopherol") OR CHEMNAME ("Delta-Tocopherol") OR CASREGNUMBER (19-13-1))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR ((((TITLE-ABS-KEY (*tocotrienol) OR SRCTITLE (*tocotrienol) OR CHEMNAME (*tocotrienol))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Alpha-Tocotrienol") OR SRCTITLE ("Alpha-Tocotrienol") OR CHEMNAME ("Alpha-Tocotrienol") OR CASREGNUMBER (58864-81-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Beta-Tocotrienol") OR SRCTITLE ("Beta-Tocotrienol") OR CHEMNAME ("Beta-Tocotrienol") OR CASREGNUMBER (490-23-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Gamma-Tocotrienol") OR SRCTITLE ("Gamma-Tocotrienol") OR CHEMNAME ("Gamma-Tocotrienol") OR CASREGNUMBER (14101-61-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("Delta-Tocotrienol") OR SRCTITLE ("Delta-Tocotrienol") OR CHEMNAME ("Delta-Tocotrienol") OR CASREGNUMBER (25612-59-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) AND (LIMIT-TO (SUBJAREA , "BIOC ") OR LIMIT-TO (SUBJAREA , " CHEM ") OR LIMIT-TO (SUBJAREA , "CHEM "))
Vitamin K 203	((((TITLE-ABS-KEY (*phylloquinone) OR SRCTITLE (*phylloquinone) OR CHEMNAME (*phylloquinone) OR CASREGNUMBER (84-80-0))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*vitamin-k-1) OR SRCTITLE (*vitamin-k-1) OR CHEMNAME (*vitamin-k-1) OR CASREGNUMBER (84-80-0))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (vitamin-k1*) OR SRCTITLE (vitamin-k1*) OR CHEMNAME (vitamin-k1*) OR CASREGNUMBER (572-96-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR ((((TITLE-ABS-KEY (menaquinone*) OR SRCTITLE (menaquinone*) OR CHEMNAME (menaquinone*) OR CASREGNUMBER (2124-57-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("vitamin-k-2") OR SRCTITLE ("vitamin-k-2") OR CHEMNAME ("vitamin-k-2") OR CASREGNUMBER (2124-57-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*menadione) OR SRCTITLE (*menadione) OR CHEMNAME (*menadione) OR CASREGNUMBER (863-61-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY ("vitamin-k-3") OR SRCTITLE ("vitamin-k-3") OR CHEMNAME ("vitamin-k-3") OR CASREGNUMBER (863-61-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")

	) AND ("blood" OR "serum" OR "plasma "))) AND (LIMIT-TO (SUBJAREA , "BIOC ") OR LIMIT-TO (SUBJAREA , "CHEM "))
Final A, D, E and K 1,507	((((((TITLE-ABS-KEY("beta carotene") OR SRCTITLE("beta carotene") OR CHEMNAME("beta carotene") OR CASREGNUMBER(7235-40-7))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY ("alpha Carotene") OR SRCTITLE ("alpha Carotene") OR CHEMNAME ("alpha Carotene") OR CASREGNUMBER (432-70-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*Cryptoxanthin) OR SRCTITLE (*Cryptoxanthin) OR CHEMNAME (*Cryptoxanthin) OR CASREGNUMBER (472-70-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY(*lutein) OR SRCTITLE(*lutein) OR CHEMNAME(*lutein) OR CASREGNUMBER(127-40-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))) OR ((((TITLE-ABS-KEY(*Zeaxanthin) OR SRCTITLE(*Zeaxanthin) OR CHEMNAME(*Zeaxanthin) OR CASREGNUMBER(144-68-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*Lycopene) OR SRCTITLE(*Lycopene) OR CHEMNAME(*Lycopene) OR CASREGNUMBER(502-65-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*Retinol) OR SRCTITLE(*Retinol) OR CHEMNAME(*Retinol) OR CASREGNUMBER(68-26-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*Retinoic-acid) OR SRCTITLE(*Retinoic-acid) OR CHEMNAME(*Retinoic-acid) OR CASREGNUMBER(302-79-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))) OR ((((TITLE-ABS-KEY(*Retinal) OR SRCTITLE(*Retinal) OR CHEMNAME(*Retinal) OR CASREGNUMBER(116-31-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*PALMITATE) OR SRCTITLE(*PALMITATE) OR CHEMNAME(*PALMITATE) OR CASREGNUMBER(79-81-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(Retinal*) OR SRCTITLE(Retinal*) OR CHEMNAME(Retinal*) OR CASREGNUMBER(116-31-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))))) OR (((((TITLE-ABS-KEY(*25-hydroxyvitamin-D3) OR SRCTITLE(*25-hydroxyvitamin-D3) OR CHEMNAME(*25-hydroxyvitamin-D3) OR CASREGNUMBER(19356-17-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("Vitamin D3") OR SRCTITLE("Vitamin D3") OR CHEMNAME("Vitamin D3") OR CASREGNUMBER(67-97-0))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("3-Epi-25-Hydroxyvitamin D3") OR SRCTITLE("3-Epi-25-Hydroxyvitamin D3") OR CHEMNAME("3-Epi-25-Hydroxyvitamin D3") OR CASREGNUMBER(73809-05-9))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("1α-25-dihydroxyvitamin-D3") OR SRCTITLE("1α-25-dihydroxyvitamin-D3") OR CHEMNAME("1α-25-dihydroxyvitamin-D3") OR CASREGNUMBER(32222-

06-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))) OR ((((TITLE-ABS-KEY("24,25-dihydroxyvitamin D3") OR SRCTITLE("24,25-dihydroxyvitamin D3") OR CHEMNAME("24,25-dihydroxyvitamin D3") OR CASREGNUMBER(55721-11-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*25-dihydroxyvitamin-D3) OR SRCTITLE(*25-dihydroxyvitamin-D3) OR CHEMNAME(*25-dihydroxyvitamin-D3) OR CASREGNUMBER(32222-06-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("VITAMIN D2") OR SRCTITLE("VITAMIN D2") OR CHEMNAME("VITAMIN D2") OR CASREGNUMBER(50-14-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("25-HYDROXYVITAMIN D2") OR SRCTITLE("25-HYDROXYVITAMIN D2") OR CHEMNAME("25-HYDROXYVITAMIN D2") OR CASREGNUMBER(21343-40-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))) OR ((((TITLE-ABS-KEY (*25-Hydroxy-Vitamin-D2) OR SRCTITLE (*25-Hydroxy-Vitamin-D2) OR CHEMNAME (*25-Hydroxy-Vitamin-D2) OR CASREGNUMBER (908126-48-7))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY (*25-Dihydroxyvitamin-D2) OR SRCTITLE (*25-Dihydroxyvitamin-D2) OR CHEMNAME (*25-Dihydroxyvitamin-D2) OR CASREGNUMBER (60133-18-8))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")))) OR ((((((TITLE-ABS-KEY (*Tocopherol) OR SRCTITLE (*Tocopherol) OR CHEMNAME (*Tocopherol) OR CASREGNUMBER (59-02-9))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma "))) OR (((TITLE-ABS-KEY("Alpha-Tocopherol") OR SRCTITLE("Alpha-Tocopherol") OR CHEMNAME("Alpha-Tocopherol") OR CASREGNUMBER(59-02-9))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("BETA-TOCOPHEROL") OR SRCTITLE("BETA-TOCOPHEROL") OR CHEMNAME("BETA-TOCOPHEROL") OR CASREGNUMBER(16698-35-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("Gamma-Tocopherol") OR SRCTITLE("Gamma-Tocopherol") OR CHEMNAME("Gamma-Tocopherol") OR CASREGNUMBER(54-28-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("Delta-Tocopherol") OR SRCTITLE("Delta-Tocopherol") OR CHEMNAME("Delta-Tocopherol") OR CASREGNUMBER(19-13-1))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))) OR ((((TITLE-ABS-KEY(*Tocotrienol) OR SRCTITLE(*Tocotrienol) OR CHEMNAME(*Tocotrienol))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("Alpha-Tocotrienol") OR SRCTITLE("Alpha-Tocotrienol") OR CHEMNAME("Alpha-Tocotrienol") OR CASREGNUMBER(58864-81-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY ("Beta-Tocotrienol") OR SRCTITLE ("Beta-Tocotrienol") OR CHEMNAME ("Beta-Tocotrienol") OR CASREGNUMBER (490-23-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" OR "MS")) AND ("blood" OR "serum" OR "plasma ")) OR (((TITLE-ABS-KEY("Gamma-Tocotrienol") OR SRCTITLE("Gamma-Tocotrienol")

	OR CHEMNAME("Gamma-Tocotrienol") OR CASREGNUMBER(14101-61-2))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("Delta-Tocotrienol") OR SRCTITLE("Delta-Tocotrienol") OR CHEMNAME("Delta-Tocotrienol") OR CASREGNUMBER(25612-59-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))))) OR (((((TITLE-ABS-KEY(*Phylloquinone) OR SRCTITLE(*Phylloquinone) OR CHEMNAME(*Phylloquinone) OR CASREGNUMBER(84-80-0))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*vitamin-k-1) OR SRCTITLE(*vitamin-k-1) OR CHEMNAME(*vitamin-k-1) OR CASREGNUMBER(84-80-0))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(vitamin-k1*) OR SRCTITLE(vitamin-k1*) OR CHEMNAME(vitamin-k1*) OR CASREGNUMBER(572-96-3))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))) OR ((((TITLE-ABS-KEY(Menaquinone*) OR SRCTITLE(Menaquinone*) OR CHEMNAME(Menaquinone*) OR CASREGNUMBER(2124-57-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("vitamin-k-2") OR SRCTITLE("vitamin-k-2") OR CHEMNAME("vitamin-k-2") OR CASREGNUMBER(2124-57-4))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY(*Menadione) OR SRCTITLE(*Menadione) OR CHEMNAME(*Menadione) OR CASREGNUMBER(863-61-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma ")) OR (((TITLE-ABS-KEY("vitamin-k-3") OR SRCTITLE("vitamin-k-3") OR CHEMNAME("vitamin-k-3") OR CASREGNUMBER(863-61-6))) AND ((("liquid chromatography" OR "LC")) AND ("mass spectrometry" or "MS")) AND ("blood" or "serum" or "plasma "))))) AND ((ANALYTICAL OR ANALYTICAL CHEMISTRY)) AND (VITAMIN OR VITAMINS) AND (LIMIT-TO (SUBJAREA,"BIOC") OR LIMIT-TO (SUBJAREA,"CHEM")) AND (EXCLUDE (DOCTYPE,"re") OR EXCLUDE (DOCTYPE,"cp") OR EXCLUDE (DOCTYPE,"le") OR EXCLUDE (DOCTYPE,"sh") OR EXCLUDE (DOCTYPE,"no")) AND (EXCLUDE (LANGUAGE,"Chinese") OR EXCLUDE (LANGUAGE,"Japanese") OR EXCLUDE (LANGUAGE,"Czech") OR EXCLUDE (LANGUAGE,"French") OR EXCLUDE (LANGUAGE,"Korean") OR EXCLUDE (LANGUAGE,"Portuguese") OR EXCLUDE (LANGUAGE,"Spanish") OR EXCLUDE (LANGUAGE,"Dutch") OR EXCLUDE (LANGUAGE,"Turkish")) AND (EXCLUDE (LANGUAGE,"German"))
Scifinder search Vitamin A 275	Combine Reference Answer Sets "Combine: Include all answers that appear in: Retinyl_palmitate_stru_simil_, 3,4-Didehydro-Retinoic_stru_sim, Retinal_stru_simil_Analy, Retinoic acid_stru_simil_Analy, Lycopene_stru_simil_Analy, retinol_stru_simil_Analy, Zeaxanthin_stru_simil_Analy, lutein_stru_simil_Analy, Beta_Cryptoxanthin_stru_simil_Analy, beta_carotene_stru_simil_Analy and alpha_carotene_stru_simil_Analy" (22953) > refine ""mass spectrometry" or "MS"" (5417) > refine ""liquid chromatography" or "LC"" (1478) > refine ""liquid chromatography" or "LC"" (1478) > refine ""blood" or "plasma" or "serum"" (275)

Vitamin D 1601	Combine Reference Answer Sets "Combine: Include all answers that appear in: 3_Epi_25_Hy_D2_stru_simil_Analy_, 3_Epi_25_Hy_D3_stru_simil_Analy_mas, 1α,25-dihy_vi_D2_stru_simi, 25-HY_Vit_D2_stru_simil_Analy_ma, Vit_D2_stru_simil_Analy_mass_spec, 24,25-Dihydroxy_stru_simil_Analy_ma, 1α,25-dihy_vi_D3_stru_simil_Analy_, Vit_D3_stru_simil_Analy_mass_spec and 25_hy_vi_D3_stru_simil_Ana_ma" (12067) > refine ""mass spectrometry" or "MS"" (7063) > refine ""Liquid chromatography" or "LC"" (3400) > refine ""Blood" or "plasma" or "serum"" (1601)
Vitamin E 391	Combine Reference Answer Sets "Combine: Include all answers that appear in: gamma_Tocotrienol_stru_simi, delta_Tocotrienol_stru_simi, beta_Tocotrienol_stru_simi, Alpha_Tocotrienol_stru_simi, Delta_Tocopherol_stru_simi, Gamma_Tocopherol_stru_simi, Beta_Tocopherol_stru_simil and Alpha_Tocopherol_stru_simil_A" (10011) > refine ""mass spectrometry" or "MS"" (2583) > refine ""Liquid chromatography" or "LC"" (1183) > refine ""Blood" or "plasma" or "serum"" (391)
Vitamin K 151	Combine Reference Answer Sets "Combine: Include all answers that appear in: Minadione_vita_K3_stru_simi, meanquinone_vita_K2_stru_simi and Phylloquinone_vita_K1_stru_simi" (10806) > refine ""mass spectrometry" or "MS"" (1293) > refine ""Liquid chromatography" or "LC"" (690) > refine ""Blood" or "plasma" or "serum"" (151)
All A,D E and K 1909	Combine Reference Answer Sets "Combine: Include all answers that appear in: All_Vita_K_liq_chro_LC_mas_spec_MS_Blo_Pla_ ser, All_Vita_E_liq_chro_LC_mas_spec_MS_Blo_Pla_ ser, All_Vita_D_liq_chro_LC_mas_spec_MS_Blo_Pla_ ser and All_Vita_A_liq_chro_LC_mas_spec_MS_Blo_Pla_ ser" (2257) > refine "Book Journal Report" (2004) > refine "English" (1909)
Duplicates removed 1587	"All_English" (1909) > remove 322 references (1587)

Table 2: Compound list

Vitamin A,D,E,K and their metabolised	Beta-carotene, alpha-Carotene, Cryptoxanthin, lutein, Zeaxanthin, Lycopene, Retinol, Retinoic-acid, Retinal, Pamitate, 25-hydroxyvitamin-D3, Vitamin D3, 3-Epi-25-Hydroxyvitamin-D3, 1α-25-dihydroxyvitamin-D3, 24,25-dihydroxyvitamin D3, VITAMIN D2, 25-HYDROXYVITAMIN D2, Tocopherol, Alpha-Tocopherol, Beta-Tocopherol, Gamma-Tocopherol, Delta-Tocopherol, Tocotrienol, Alpha-Tocotrienol, Beta-Tocotrienol, Gamma-Tocotrienol, Delta-Tocotrienol, Phylloquinone, Vitamin-k-1, Menaquinone, Vitamin-k-2, Menadione, Vitamin-k-3

Inclusion and Exclusion criteria

The main aim of this systematic review was to look at all literature available on LC-MS/MS methods for fat-soluble vitamer analysis. I included articles from 2004 to 2019, covering methods developed from the very first discovery of LC-MS/MS to the present day.

Table 3: Inclusion and exclusion criteria for this systematic review of LC-MS/MS quantification of fat-soluble vitamer.

Inclusion criteria	Exclusion criteria
• FSV alone or in combination with other vitamers • Blood, serum, plasma • Blood, serum, plasma with other body fluids. • Animals • LC-MS, HPLC-MS, UHPLC-MS • Two or more FSV groups (*e.g.*, A and D) • Articles in analytical chemistry • Primary articles, book series, books and trade publications • English language	• LC only • MS only • No biological fluids (blood, serum, or plasma) • Non-vitamer studies • Only one FSV group (*e.g.*, A only) • Conference proceedings, reviews, editorials • Non-English language

Furthermore, I included studies on all animal species without any restriction as there were very few articles for humans, particularly for K group vitamers. I included articles from all countries written in the English language. Although I was only searching for articles which focused on FSV, I included articles that contained methods for both fat and water-soluble vitamers. I primarily looked at the methods for three different matrices, including blood, plasma, and serum, as the main transportation medium of FSV is blood with carrier proteins before they are stored in the liver [418]. Due to the low number of available articles in human studies, I did not restrict the age groups of the individuals the blood that had been drawn from, and I included articles that report on blood along with other biological fluids. All selected articles were separated based on the inclusion and exclusion criteria (Table 3).

Data collection, extraction, and analysis

For the assessment of published methods in FSV quantification, formal meta-analysis criteria were not used due to a lack of evidence in experimental design (*e.g.*, method validation and some LC-MS/MS parameters) and heterogeneity in the published methods. Instead, detailed descriptive qualitative analyses were carried out using the extracted data.

Covidence systematic review management software was used to aid two researchers' independent evaluation of literature (Githal Arachchige and Elizabeth McKenzie). All applicable studies with full-text articles were verified using the inclusion and exclusion criteria (Table 3). Conflicting reports were resolved through discussion and/or consultation with a third reviewer (Figure 1). Only articles that described methods related to two or more vitamin groups were included in the final Covidence selection.

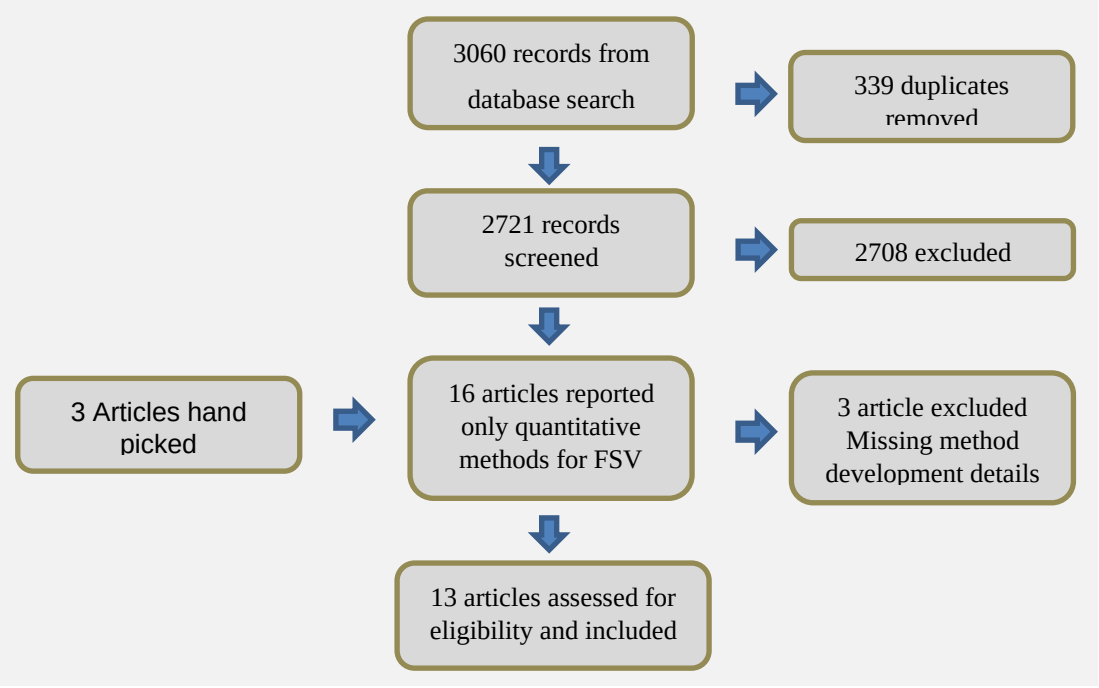

Figure 1: Block diagram of the article selection process used in this systematic review. The process was carried out using Covidence software.

The articles that were included in the systematic review were assessed for comprehensiveness and quality using a grading system adapted from references [100], [101]**,** and [102]. The details assessed included whether the sample matrix was described, whether the sample collection method was reported (time between sampling and storage, sample collection process), what quality assurance criteria were reported (stability of the samples, internal and external standards, the internal and external standard used, calibration matrix, calibration curve use/number points in the calibration curves), specifics of the sample extraction method (deproteinization, extraction solvents, extraction method), liquid chromatographic parameters (column/dimensions, mobile phases, gradient, retention times, flowrate), mass spectrometry parameters (ionisation type, precursor and product ions), whether the method was validated (linearity, range, precision, accuracy, matrix effect, limit of detection (LOD) and limit of quantitation (LOQ)), description of data processing and statistical analysis (chromatographic software, example chromatogram of peaks) and whether limitations were described.

Appendix 3: Eppendorf liquid handling robot setup of chapter 4

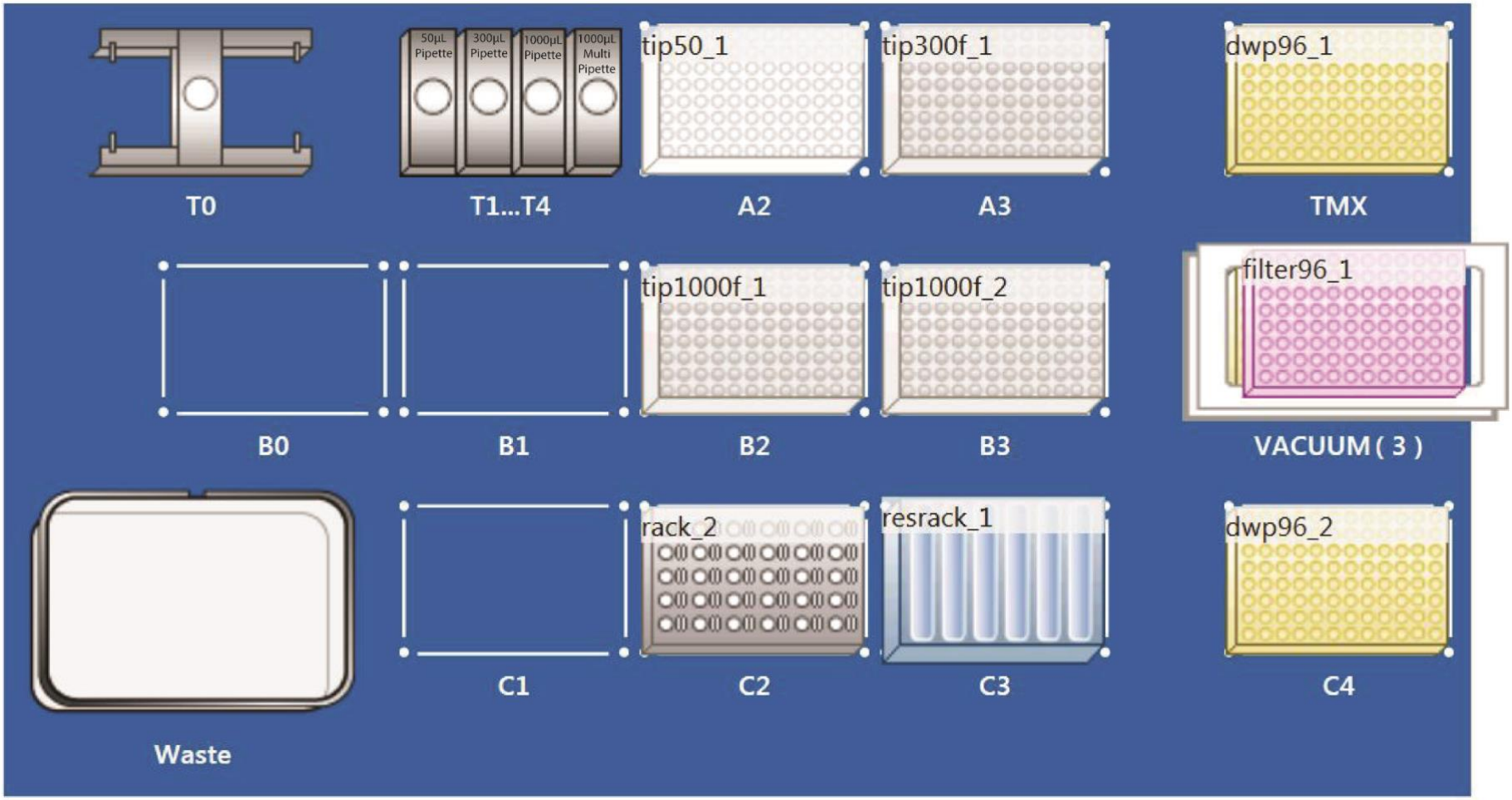

Figure 1: The Eppendorf EpMotion liquid handling robot setup.

I.e., T0; Plate mover, T1-T4; Automated pipettor, A2, A3, B2 and B3; Filtered pipette tips, dwp96: 96 Deep well plates, Vacuum; vacuum manifold, resrack; reservoirs (3mL and 10mL), rack2; 2mL 2 ml Eppendorf tube holder

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