Analytica Chimica Acta 1181 (2021) 338877

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Absolute quantification of eleven A, D, E and K vitamers in human plasma using automated extraction and UHPLC-Orbitrap MS

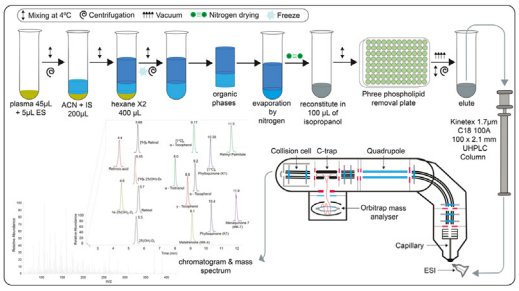
G.R.P. Arachchige, E.B. Thorstensen, M. Coe, J.M. O'Sullivan**, C.J. Pook*

The Liggins Institute, The University of Auckland, Private Bag 92019, Auckland, 1142, New Zealand

HIGHLIGHTS

- Fully validated LC-ESI-MS/MS assay to quantify 11 Fat-Soluble Vitamers [FSV] in plasma.
- A novel automated liquid-liquid extraction method including phospholipid removal.
- Precision, linearity, accuracy and range, all comply with the EMA guidelines.
- Quantifies FSV at physiological concentrations or clinical deficiency levels.
- Suitable for application to large cohort studies with limited sample volumes.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 19 May 2021
Received in revised form
8 July 2021
Accepted 22 July 2021
Available online 28 July 2021

Keywords:
Liquid-liquid extraction [LLE]
Ultra high-performance liquid chromatography [UHPLC]
Mass spectrometry [MS]
Phree® phospholipid removal plates [Phree®]
Fat soluble vitamers [FSV]

ABSTRACT

Fat-Soluble Vitamers [FSV] deficiencies and hypervitaminosis are associated with lifestyle diseases such as cardiovascular disease, diabetes, and cancer. Quantification of FSV and their metabolites in plasma has proved to be one of the most demanding analytical chemistry challenges. Current FSV quantification methods are compromises between breadth of coverage and sensitivity across the physiological range. Here, we developed and validated a sensitive, robust, semi-automated method using liquid-liquid extraction coupled with LC-ESI-MS/MS to quantify 11 FSV across their physiological concentrations in plasma. The addition of Phree® phospholipid removal plates as the last step in the extraction process reduced matrix effects, improving precision, recoveries, and the method's final sensitivity. This method can detect and quantify: retinol, retinoic acid, retinyl palmitate, 25 hydroxyvitamin D_3 [25-OH-D_3], 1-α-25-dihydroxy-D_3, α-tocopherol, γ-tocopherol, α-tocotrienol, phylloquinone [K1], Menatetrenone [MK-4], and menaquinone-7 [MK-7].The Instrument Quantitation Limit [IQL]s for retinol (64.1 ng/mL), 25-OH-D_3 (10.2 ng/mL), and α-tocopherol (3000 ng/mL) can detect clinical deficiencies. Our automated method will assist in the understanding of the complex interaction between these compounds and their possible role in health and disease.

* Corresponding author.
** Corresponding author.
E-mail addresses: g.arachchige@auckland.ac.nz (G.R.P. Arachchige), e.thorstensen@auckland.ac.nz (E.B. Thorstensen), m.coe@auckland.ac.nz (M. Coe), justin.osullivan@auckland.ac.nz (J.M. O'Sullivan), chris.pook@auckland.ac.nz (C.J. Pook).

https://doi.org/10.1016/j.aca.2021.338877
0003-2670/

1. Introduction

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 [1]. FSV are not synthesized by human cells; they must be obtained from the diet, the gut microbiome, or by synthesis with Ultraviolet B (UVB) light exposure in the skin. 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, type-2-diabetes mellitus, cancer, and metabolic complications [2,3].

Therefore, there is a need to develop robust, reliable, high throughput methods to quantify circulating FSV [4]. 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 (3000–13000 ng/mL) to MK-7 (0.4–2.2 ng/mL). Circulating FSV concentrations are lower in children, especially infants (2 months–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.

Analytical techniques for the quantification of 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. The advent of isotope dilution liquid chromatography-tandem mass spectrometry [LC-ESI-MS/MS] has revolutionized analytical detection methods and rapidly become the gold standard for FSV quantification [2]. Unfortunately, current analytical methods using LC-ESI-MS/MS are restricted to a limited number of vitamers or suffer from poor sensitivity [5].

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 (Fig. 1) [4].

We 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 derivatization.

2. Material and methods

2.1. Materials, chemicals, and reagents

All-*trans* retinol, retinyl palmitate, retinoic acid, α-tocopherol, γ-tocopherol, K1, 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). $[^2H]_8$ 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). $[^2H]_7$ 25-OH-D_3 was purchased from PM Separations NZ Ltd (Auckland, New Zealand). Phree® phospholipid removal plates purchased from Phenomenex (Auckland, NZ). Glass coated 96 well 1 mL 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), methanol (Hypergrade for LC/MS) 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 the MP Biomedicals, (New Zealand).

2.2. 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 to 14000 ng/mL in

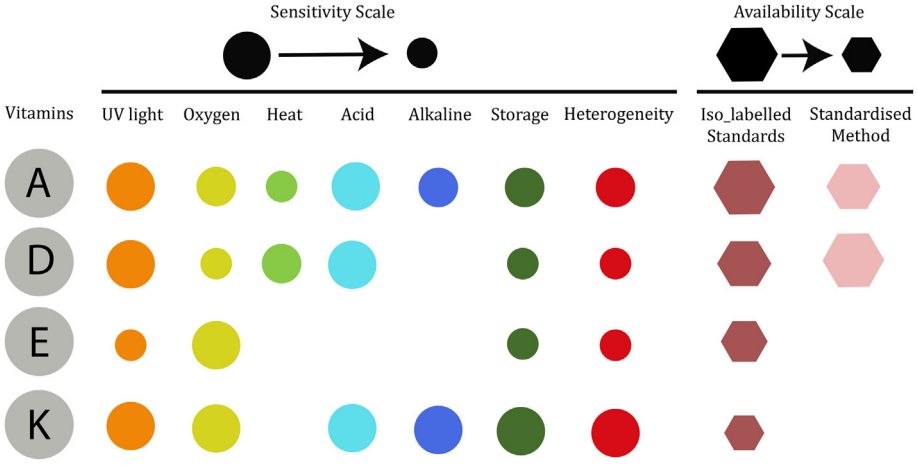


Fig. 1. Analytical complications of FSV. Both A and D group vitamers are sensitive to light, oxygen, heat, and acidic pH [4]. Xanthophylls (metabolites of vitamin A) are also sensitive to alkaline pH. E group vitamers are light and oxygen labile, however stable at high temperatures and in acidic and alkaline pH. K group vitamers have the most heightened sensitivity to light, acid and alkaline pH but are stable in the presence of heat [4,6–8].

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	

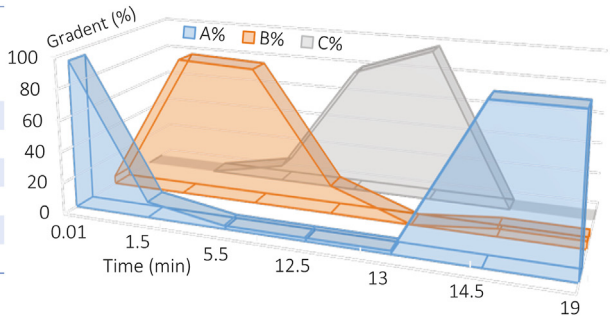


Fig. 2. Gradient elution. The mobile phase consisted of three solvents; (A) 5 mM Ammonium formate mixed with 0.1% (V/V) formic acid in Milli-Q water, (B) 0.1% (V/V) formic acid in methanol, and (C) 2-Propanol.

phosphate buffered saline (PBS) containing bovine serum albumin (BSA) 4% (w/v). The calibrator mixture was serially diluted to achieve the desired concentrations (Supplementary Table 1). Acetonitrile was used as the blank calibrator. The internal standards: 4000 ng/mL of ($[^2H]_8$ Retinol, $[^2H]_7$ 25-OH-D_3, $[^{13}C]_6$ α-tocopherol and $[^{13}C]_6$ phylloquinone, were prepared as a mix in 200 μL acetonitrile deproteinization solution.

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 (Supplementary Table 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 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. Deproteinization was initiated by the addition of 200 μL acetonitrile [ACN] 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 ACN 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) also transferred into the 2nd plate.

The 2nd plate was N_2 dried (30min, 15 °C) and the extracts 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 (800 rpm (linear), 15 °C, 10 min), centrifuged (2000 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.

Analysis of the FSV/IPA solution was carried using a Q-Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Germany) equipped with a heated electrospray ionization 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).

2.3. 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 × 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^{-1} across the total analytical runtime (19 min). An optimized gradient elution system was used to achieve the best FSV separation (Fig. 2).

The MS capillary temperature and spray voltage were 263 °C and 3.5 KW 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 optimized collision energies are summarised in (Table 1).

Chromatographic, MS data collection, and processing were performed using Xcalibur™ Software (Thermo, Version 4.2.47). Plasma concentrations of each of the 11 FSV were calculated by comparing the peak: internal standard area ratio with those generated from the relevant standard curve.

2.4. Matrix effects

The matrix effects were calculated using ten aliquots of spiked and blank plasma (Supplementary Table 3) utilising the formula below.

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

2.5. Stability

The 11 vitamers' plasma stability was quantified and tested using spiked pooled plasma over a 29 h period (to reflect the complete assay run time), one injection every hour. This test was repeated over three days (Supplementary Fig. 1).

Table 1
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.

Table 2
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 [35,36]	21.4	64.1	25–1600	0.9999
retinoic acid	2.4–4.5 [37]	1.2	3.6	1.7–115	0.9998
retinyl palmitate	131-525 [38]	9.7	29.2	12.5–800	0.9996
25-OH-D_3	30.1–104.2 [13]	3.4	10.2	5.7–365	0.9998
1-α-25$(OH)_2$-D3	0.033–0.085 [39]	2.7	8.2	2.5–165	0.9998
α-tocopherol	3000-13000 [40,41]	1005	3014	219–14000	0.9993
γ-tocopherol	190-510 [41]	42.8	128.4	31–2000	0.9993
α-tocotrienol	0.9–30	1.2	3.7	4.7–300	0.9995
K1	0.018–4 [8,42]	0.2	0.7	0.312–20	0.9991
MK-4	0.56–2.2 [8]	0.5	1.6	0.312–20	0.9997
MK-7	0.11–2.2 [8]	0.4	1.3	0.468–30	0.9989

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

2.6. Linearity

The calibration curves' linearity were measured using seven calibration standards in PBS/BSA (4% w/v), having the average physiological concentrations of each FSV as the mid-point. The coefficient of correlation [r^2] values ranged from 0.9987 to 0.9999 and demonstrated sufficient linearity for the quantification of 11 FSV (Table 2).

2.7. 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 heparinized 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's (Supplementary Table 2).

2.8. Instrument detection limit [IDL] and instrument quantitation limit [IQL]

The IDL's (method detection limit or Limit of Detection [LoD]) were determined by analyzing the standard deviations of ten aliquots of spiked heparinized pooled plasma, containing the lowest physiologically observed concentration of the 11 FSV (Table 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.

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

3. Results and discussion

We developed a novel hybrid sample preparation method to extract and quantify 11 FSV's from heparinized 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 h.

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 [4] (Fig. 1). Therefore, precautions were taken during sample preparation that included low actinic amber glassware for standard preparation [9,10], minimal UV exposure, and the use of aluminum foil wrapped centrifuge tubes [11]. Both samples and standards were stored at −80 °C and kept on ice throughout the process [12,13].

Konieczna et al., 2016 [3] 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 h 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. We tested and confirmed that the hexane/acetonitrile Phree® plate processed FSV are stable, in the length of time to analyze a whole plate (Supplementary Fig. 1).

All FSV's, particularly α-tocopherol and all-*trans*-retinol [14], 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 minimize oxidative degradation [15]. Several FSV methods used BHT as an antioxidant [12,13,16]; 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 disrupts the intermolecular interactions of the proteins [17]. 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 deproteinization 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 2), the extraction efficiency using the aforementioned organic solvents was poor. The best extraction efficiency was obtained using ACN (<90%) at a plasma to the solvent ratio of 1:4, consistent with published observations [18]. In their study, Le, 2018 [19]. reported that ACN could promote the best deproteinization 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. The majority of the FSV are moderately polar, with A group vitamers retinoic acid [20]) 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 most suitable reconstituting solvent. Polson et al., 2003 showed that irrespective of the organic solvent used, a plasma to solvent ratio of <1:2.5 is required to achieve reliable deproteinization. Moreover, at a ratio of <1:2.5, methanol and acetonitrile removed 94% and >97% of the protein, respectively. In this method, we maintained the plasma to ACN ratio at 1:4.

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. 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 [1–3,16,21,22] and chloroform [12,13]. The use of hexane can be justified due to its cost-effectiveness, safety (e.g. Refs. [2,17]), and robustness compared to chloroform. Hexane is water-immiscible and, therefore, suitable for most of the FSV [16]. We achieved high recovery rates using a multi-stage hexane extraction approach (Supplementary Table 4) as described previously [3,22]. Application of a double hexane extraction step in our method yielded 80–90% recovery and only 60–70% using single hexane extraction.

In LLE, the solvent transfer step is critical, as incomplete transfers minimize the recovery of the solvents leading to poor sensitivity. The current method used an extra step of 20 min freeze down of the protein pellet [3,22] to address this issue. Once the protein pellet is frozen it adheres thoroughly to the bottom of the96 well plate, allowing 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. The use of low actinic glassware instead of plastic materials can reduce the sample loss significantly [9,14].

Hexane is immiscible with other organic solvents such as methanol, ethanol, acetonitrile, iso-propanol and ethyl acetate. The mobile phases of the current FSV method consists 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 a LC-MS/MS, as mentioned in the literature [1,12,13,16].

Even with such an extensive, solvent-based sample preparation method there was a noticeable matrix effect (Supplementary Table 5). Matrix effects are characterized 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 [13,23]. 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 . This matrix effect was noticeable for E and K group vitamers even with the isotopically labelled IS. However, the addition of the Phree® protein removal step significantly reduced the matrix effect for the E, K, and A group vitamers (Supplementary Table 5) [24]. The sensitivity of an FSV method is dependent on the degree of matrix effects. It is crucial to take the necessary steps to minimize them when working with the matrices such as plasma.

3.2. Analytical platform LC-ESI-MS/MS

In this method, we used liquid chromatography coupled with Q-Exactive to quantify the FSV's in plasma. Both instruments are designed to improve efficiency, reliability, sensitivity and deliver high throughput analysis. In UHPLC [25] 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 (2–5 μm) [1–3,12,13,19,22,26]. The present method was developed using a Kinetex C18 100 Å (100 × 2.1 mm) 1.7 μm analytical column at a flow rate of 0.3 mL/min. During the method development, we also tested Kinetex EVO C18 100 Å (150 × 2.1 mm x 1.7 μm) and

Agilent Infinity Lab Poroshell 120 PFP (150 × 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 ionization, the ionization efficiency is highly dependent on the nature of the analyte and matrix effect [23]. In their study, Zhang et al. [27] reported that overall ionization efficiency using ESI is ten times higher compared to atmospheric pressure chemical ionization (APCI). In this study, both ESI and APCI ionization 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 [1,3,12,13,19,21,26,27].

The chromatographic separation of the FSV is based purely on their polarity. Of the FSV quantified by our 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 in developing a multianalyte FSV method is that chromatographic resolution is often compromised, leading to poor ionization and reduced method sensitivity. Therefore, such vitamers can only be accurately quantified by methods optimized to target those specific analytes. The present method's run time is 19 min, and all the analytes of interest are resolved in 12min. 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, we did not extend the runtime to increase the resolution of α-tocopherol and MK-4.

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 [28]. The detection limit (2.7 ng/mL) of 1-α-25$(OH)_2$-D_3, using our method, is higher than endogenous circulating concentrations (40–61 pg/mL). Notably, higher plasma percentage of 1-α-25$(OH)_2$-D_3 other D group vitamers is reported among individuals with celiac disease, Williams syndrome [29], non-infectious diseases; lymphoma, hypothyroidism, Klinefelter syndrome, and infectious diseases; Tuberculosis and sarcoidosis compared to the controls (endogenous concentration). Therefore, our limitations 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 [30]) results in an underestimate of 25-OH-D_3 endogenous concentrations [31]. The present assay successfully quantified 25-OH-D_3. However, consistent with earlier observations [12], we were 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, emphasizing the importance of separating the epimer [32].

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 2) 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, required much longer analytical run-times, or were limited to one or two K group vitamers (phylloquinone (K1)) [3,12,13,21,22,27,33]. K1 plays an important role in blood clotting and MK-4 and MK-7 are integral in bone calcium metabolism and gamma-carboxylation of osteocalcin. Additionally, K group vitamers, promote the gamma-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. Performance parameters (linearity, range, precision, recovery, and matrix effect)

There is no standardized method for analyzing FSV using LC-ESI-MS/MS to date. In this method we successfully validated our 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%), recoveries (70–140%) were included as validation criteria (acceptance limits).

Four internals standards (IS) ($[^2H]_8$ retinol, $[^2H]_6$ 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 hight. No degradation was observed in 3 repeated analyses of a set of 10 samples over a 24 h period (Supplementary Fig. 1). Chromatographic separation of the 11 FSV was reproducibly achieved (Fig. 3 and Table 1). Retention times between analytes, and their isotopically labelled standards $[^2H]_8$ Retinol, $[^2H]_6$ 25-OH-D_3, $[^2H]_6$ α-tocopherol, and $[^2H]_7$ 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). We conclude that the method we developed met the criteria for method validation.

The matrix effects' isolation meant that 11 FSV standard curves are linear with the correlation coefficients [r^2] ranging from 0.9987 to 0.9999 (Table 2). 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 to 13.9%, and interday precision ranged from 1.1 to 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). This is consistent with observations from Konieczna et al. [3], who obtained similar results for vitamin K1.

In clinical settings, it is crucial to identify deficiencies and hypervitaminosis of these vitamers [5]. Therefore, in this method, a particular focus was given to cover those concentrations when selecting the measuring range. The choice of anti-coagulant can play a role in analyte recovery, therefore we tested FSV recoveries using both EDTA and heparinized 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 (Supplementary Table 2). Supplementary Table 4 summarizes 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. [3] 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 [13,23] (Supplementary Table 3).

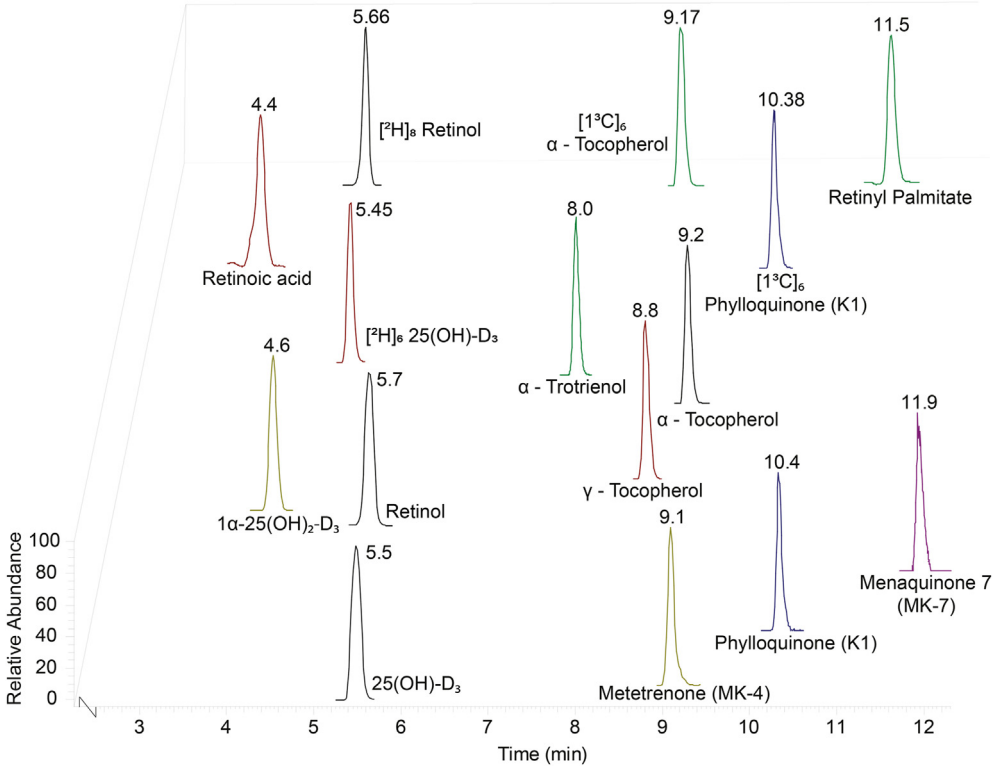


Fig. 3. Chromatographic separation of 11 Fat-Soluble Vitamers from healthy human plasma spiked with IS in PRM (Parallel reaction monitoring) mode using LC-ESI-MS/MS.

Table 3
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

Instrument detection [IDL] and instrument quantitation limits [IQL] are key performance parameters of any analytical method [12]. Two studies [12,13] 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 [1,13,23]. Therefore, LOD values obtained using 4% albumin/PBS are not necessarily directly comparable to those in plasma [1].

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 [3,12,16,17,34]. The method we have developed reliably quantitates retinol [4,12,16], retinoic acid, retinyl palmitate, 25-OH-D_3 [12,13], α-tocopherol [12,13,22], γ-tocopherol [12], α-tocotrienol, K1 [8,12], MK-4 [8], and MK-7 [8] at their respective physiological levels (Table 2). 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, and α-tocopherol >3000 ng/mL. Our 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.

4. Conclusion

We 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. Besides 1-α-25-dihydroxy-D3, our method is sensitive enough to detect and quantify the physiological concentrations and, in the case of retinol, 25-OH-D3, and α-tocopherol, clinical deficiencies of these vitamers. The

developed method is currently being implemented in our laboratory to analyze clinical samples from the LSAC (Longitudinal Study of Australian Children) CheckPoint child/parent dyads and can be reliably used to analyze 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.

Funding

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).

Institutional review board statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Auckland Health Research Ethics Committee of University of Auckland (project 3408 (review reference AH3408)).

CRediT authorship contribution statement

G.R.P. Arachchige: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft. **E.B. Thorstensen:** Software, Validation, Data curation, Writing – review & editing, Visualization. **M. Coe:** Software, Validation, Investigation, Visualization. **J.M. O'Sullivan:** Conceptualization, Resources, Writing – review & editing, Supervision, Project administration, Funding acquisition. **C.J. Pook:** Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Supervision, All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

The authors wish to thank E.J. McKenzie for her support in method development.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2021.338877.

References

[1] A.A. Albahrani, V. Rotarou, P.J. Roche, R.F. Greaves, A simultaneous quantitative method for vitamins A, D and E in human serum using liquid chromatography-tandem mass spectrometry,, J. Steroid Biochem. Mol. Biol. 159 (2016) 41–53.
[2] S. Yu, Y. Yin, D. Wang, S. Xie, Q. Cheng, X. Cheng, L. Qiu, Development and validation of a simple isotope dilution-liquid chromatography-tandem mass spectrometry method for detecting vitamins A and E in serum and amniotic fluid, Int. J. Mass Spectrom. 435 (2019) 118–123.
[3] L. Konieczna, K. Kaźmierska, A. Roszkowska, A. Szlagatys-Sidorkiewicz, T. Bączek, The LC–MS method for the simultaneous analysis of selected fat-soluble vitamins and their metabolites in serum samples obtained from pediatric patients with cystic fibrosis,, J. Pharmaceut. Biomed. Anal. 124 (2016) 374–381.
[4] A.A. AlBahrani, V. Rotarou, P.J. Roche, R.F. Greaves, Analyte stability during the total testing process: studies of vitamins A, D and e by LC-MS/MS, Clin. Chem. Lab. Med. 54 (2016) 1609–1618.
[5] J. Jakobsen, A. Melse-Boonstra, M. Rychlik, Challenges to quantify total vitamin Activity: how to combine the contribution of diverse vitamers? Curr. Dev. Nutr 3 (2019).
[6] C. Cuerq, N. Peretti, K. Chikh, A. Mialon, M. Guillaumont, J. Drai, E. Blond, Overview of the in vitro stability of commonly measured vitamins and carotenoids in whole blood, Ann. Clin. Biochem. 52 (2015) 259–269.
[7] S. Clark, Effect of temperature and light on the stability of fat-soluble vitamins in whole blood over several days: implications for epidemiological studies, Int. J. Epidemiol. 33 (2004) 518–525.
[8] E. Klapkova, J. Cepova, K. Dunovska, R. Prusa, Determination of vitamins K 1, MK-4, and MK-7 in human serum of postmenopausal women by HPLC with fluorescence detection, J. Clin. Lab. Anal. 32 (2018) e22381.
[9] N. Tsugawa, Y. Suhara, M. Kamao, T. Okano, Determination of 25-hydroxyvitamin D in human plasma using high-performance liquid chromatography-tandem mass spectrometry, Anal. Chem. 77 (2005) 3001–3007.
[10] Z. Maunsell, D.J. Wright, S.J. Rainbow, Routine isotope-dilution liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of the 25-hydroxy metabolites of vitamins D2 and D3, Clin. Chem. 51 (2005) 1683–1690.
[11] P. Worsfold, C.F. Poole, A. Townshend, M. Miro, Vitamins|Overview, in: Encycl. Anal. Sci., third ed. vol. 2019, Elsevier, Amsterdam, 2019, p. 164.
[12] Ø. Midttun, A. McCann, O. Aarseth, M. Krokeide, G. Kvalheim, K. Meyer, P.M. Ueland, Combined measurement of 6 fat-soluble vitamins and 26 water-soluble functional vitamin markers and amino acids in 50 μL of serum or plasma by high-throughput mass spectrometry, Anal. Chem. 88 (2016) 10427–10436.
[13] Ø. Midttun, P.M. Ueland, Determination of vitamins A, D and e in a small volume of human plasma by a high-throughput method based on liquid chromatography/tandem mass spectrometry, Rapid Commun, Mass Spectrom. 25 (2011) 1942–1948.
[14] M.A. Kane, J.L. Napoli, Quantification of endogenous retinoids, Methods Mol. Biol. 652 (2010) 1–54.
[15] J. Karppi, T. Nurmi, B. Olmedilla-Alonso, F. Granado-Lorencio, K. Nyyssönen, Simultaneous measurement of retinol, α-tocopherol and six carotenoids in human plasma by using an isocratic reversed-phase HPLC method, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 867 (2008) 226–232.
[16] B. Hrvolová, M. Martínez-Huélamo, M. Colmán-Martínez, S. Hurtado-Barroso, R. Lamuela-Raventós, J. Kalina, Development of an advanced HPLC–MS/MS method for the determination of carotenoids and fat-soluble vitamins in human plasma, Int. J. Mol. Sci. 17 (2016) 1719.
[17] Y. Zhang, W. Zhou, J. Yan, M. Liu, Y. Zhou, X. Shen, Y. Ma, X. Feng, J. Yang, G. Li, A review of the extraction and determination methods of thirteen essential vitamins to the human body: an update from 2010,, Molecules 23 (2018) 1484.
[18] A.A. Redhwi, D.C. Anderson, G.N. Smith, A simple method for the isolation of vitamin D metabolites from plasma extracts,, Steroids 39 (1982) 149–154.
[19] J. Le, T.-F. Yuan, Y. Zhang, S.-T. Wang, Y. Li, New LC-MS/MS method with single-step pretreatment analyzes fat-soluble vitamins in plasma and amniotic fluid, J. Lipid Res. 59 (2018) 1783–1790.
[20] E.Z. Szuts, F.I. Harosi, Solubility of retinoids in water, Arch. Biochem. Biophys. 287 (1991) 297–304.
[21] F. Priego Capote, J.R. Jiménez, J.M.M. Granados, M.D.L. de Castro, Identification and determination of fat-soluble vitamins and metabolites in human serum by liquid chromatography/triple quadrupole mass spectrometry with multiple reaction monitoring, Rapid Commun, Mass Spectrom. 21 (2007) 1745–1754.
[22] M. Khaksari, L.R. Mazzoleni, C. Ruan, R.T. Kennedy, A.R. Minerick, Determination of water-soluble and fat-soluble vitamins in tears and blood serum of infants and parents by liquid chromatography/mass spectrometry, Exp. Eye Res. 155 (2017) 54–63.
[23] J. Adamec, A. Jannasch, J. Huang, E. Hohman, J.C. Fleet, M. Peacock, M.G. Ferruzzi, B. Martin, C.M. Weaver, Development and optimization of an LC-MS/MS-based method for simultaneous quantification of vitamin D2, vitamin D3, 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3, J. Separ. Sci. 34 (2011) 11–20.
[24] H. Guo, J. Wang, Y. Wu, W. Liu, J. Bu, Q. Zhao, Sensitive and simultaneous determination of nine anticoagulant rodenticides in human blood by UPLC–MS-MS with phospholipid removal pretreatment, J. Anal. Toxicol. 42 (2018) 459–466.
[25] P.A. Aronov, L.M. Hall, K. Dettmer, C.B. Stephensen, B.D. Hammock, Metabolic profiling of major vitamin D metabolites using Diels-Alder derivatization and ultra-performance liquid chromatography-tandem mass spectrometry, Anal. Bioanal. Chem. 391 (2008) 1917–1930.
[26] E. Hinchliffe, J. Rudge, P. Reed, A novel high-throughput method for supported liquid extraction of retinol and alpha-tocopherol from human serum and simultaneous quantitation by liquid chromatography tandem mass spectrometry,, Ann. Clin. Biochem. 53 (2015) 434–445.
[27] H. Zhang, L. Quan, P. Pei, Y. Lin, C. Feng, H. Guan, F. Wang, T. Zhang, J. Wu, J. Huo, Simultaneous determination of Vitamin A, 25-hydroxyl vitamin D 3 α-tocopherol in small biological fluids by liquid chromatography-tandem mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1079 (2018) 1–8.
[28] S.R. Chalkley, J. Richmond, D. Barltrop, Measurement of vitamin D3

metabolites in smelter workers exposed to lead and cadmium, Occup. Environ. Med. 55 (1998) 446–452.
[29] M. Garabédian, E. Jacqz, H. Guillozo, R. Grimberg, M. Guillot, M.-F. Gagnadoux, M. Broyer, G. Lenoir, S. Balsan, Elevated plasma 1,25-dihydroxyvitamin D concentrations in infants with hypercalcemia and an Elfin Facies, N. Engl. J. Med. 312 (1985) 948–952.
[30] N. Ooms, H. van Daal, A.M. Beijers, G.P.J.M. Gerrits, B.A. Semmekrot, J.M. van den Ouweland, Time-course analysis of 3-epi-25-hydroxyvitamin D3 shows markedly elevated levels in early life, particularly from vitamin D supplementation in preterm infants, Pediatr. Res. 79 (2016) 647–653.
[31] F. Aghajafari, C.J. Field, D. Rabi, B.J. Kaplan, J.A. Maggiore, M. O'Beirne, D.A. Hanley, M. Eliasziw, D. Dewey, S. Ross, Plasma 3-epi-25-hydroxycholecalciferol can alter the assessment of vitamin D status using the current reference ranges for pregnant women and their newborns, J. Nutr. 146 (2016) 70–75.
[32] R.J. Singh, R.L. Taylor, G.S. Reddy, S.K.G. Grebe, C-3 epimers can account for a significant proportion of total circulating 25-hydroxyvitamin D in infants, complicating accurate measurement and interpretation of vitamin D status, J. Clin. Endocrinol. Metab. 91 (2006) 3055–3061.
[33] K. Abro, N. Memon, M.I. Bhanger, S. Abro, S. Perveen, A.H. Lagharì, Determination of vitamins E, D3, and K1 in plasma by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry utilizing a monolithic column, Anal. Lett. 47 (2014) 14–24.
[34] R. Andreoli, P. Manini, D. Poli, E. Bergamaschi, A. Mutti, W.M. a Niessen, Development of a simplified method for the simultaneous determination of retinol, alpha-tocopherol, and beta-carotene in serum by liquid chromatography-tandem mass spectrometry with atmospheric pressure chemical ionization, Anal. Bioanal. Chem. 378 (2004) 987–994.
[35] M.C. Álvarez Uribe, R.M. Uscátegui Peñuela, C. López Bedoya, C.M. Baracaldo Barrera, L. Castro, V. Noy Ballesteros, Plasma retinol concentration according to pubertal maturation in school children and adolescents of Medellín, Colombia, Eur, J. Clin. Nutr. 58 (2004) 456–461.
[36] A. Maqbool, R.C. Graham-Maar, J.I. Schall, B.S. Zemel, V.A. Stallings, Vitamin A intake and elevated serum retinol levels in children and young adults with cystic fibrosis, J. Cyst. Fibros. 7 (2008) 137–141.
[37] G.A. Fex, K. Larsson, I. Nilsson-Ehle, Serum concentrations of all-trans and 13-cis retinoic acid and retinol are closely correlated, J. Nutr. Biochem. 7 (1996) 162–165.
[38] D. V Reinersdorff, E. Bush, D.J. Liberato, Plasma kinetics of vitamin A in humans after a single oral dose of [8,9,19-13C]retinyl palmitate, J. Lipid Res. 37 (1996) 1875–1885.
[39] C. Jenkinson, A.E. Taylor, Z.K. Hassan-Smith, J.S. Adams, P.M. Stewart, M. Hewison, B.G. Keevil, High throughput LC-MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 1014 (2016) 56–63.
[40] C. Cuerq, L. Restier, J. Drai, E. Blond, A. Roux, S. Charriere, M.C. Michalski, M. Di Filippo, E. Levy, A. Lachaux, N. Peretti, Establishment of reference values of α-tocopherol in plasma, red blood cells and adipose tissue in healthy children to improve the management of chylomicron retention disease, a rare genetic hypocholesterolemia, Orphanet J. Rare Dis. 11 (2016) 114.
[41] Q. Jiang, S. Christen, M.K. Shigenaga, B.N. Ames, γ-Tocopherol, the major form of vitamin E in the US diet, deserves more attention, Am, J. Clin. Nutr. 74 (2001) 714–722.
[42] C.W. Thane, L.Y. Wang, W.A. Coward, Plasma phylloquinone (vitamin K1) concentration and its relationship to intake in British adults aged 19-64 years, Br. J. Nutr. 96 (2006) 1116–1124.