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# High throughput LC-MS/MS method for the simultaneous analysis of multiple vitamin D analytes in serum

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## Abstract

Recent studies suggest that vitamin D-deficiency is linked to increased risk of common human health problems. To define vitamin D ‘status’ most routine analytical methods quantify one particular vitamin D metabolite, 25-hydroxyvitamin D3 (25OHD3). However, vitamin D is characterized by complex metabolic pathways, and simultaneous measurement of multiple vitamin D metabolites may provide a more accurate interpretation of vitamin D status.

To address this we developed a high-throughput liquid chromatography-tandem mass spectrometry (LC-MS/MS) method to analyse multiple vitamin D analytes, with particular emphasis on the separation of epimer metabolites. A supportive liquid-liquid extraction (SLE) and LC-MS/MS method was developed to quantify 10 vitamin D metabolites as well as separation of

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an interfering 7α-hydroxy-4-cholesten-3-one (7αC4) isobar (precursor of bile acid), and validated by analysis of human serum samples.

In a cohort of 116 healthy subjects, circulating concentrations of 25-hydroxyvitamin D3 (25OHD3), 3-epi-25-hydroxyvitamin D3 (3-epi-25OHD3), 24,25-dihydroxyvitamin D3 (24*R*, $25(OH)_2D3$), 1,25-dihydroxyvitamin D3 ($1\alpha,25(OH)_2D3$), and 25-hydroxyvitamin D2 (25OHD2) were quantifiable using 220 μl of serum, with 25OHD3 and 24*R*,$25(OH)_2D3$ showing significant seasonal variations.

This high-throughput LC-MS/MS method provides a novel strategy for assessing the impact of vitamin D on human health and disease.

**Keywords**

LC-MS/MS; Vitamin D; Method validation; Chiral separation; Serum analysis

## 1. Introduction

In the last decade, studies of the prevalence of vitamin D deficiency and its clinical implications have increased demand for laboratory testing to determine vitamin D ‘status’ [1]. The most common approach to this has been the measurement of serum levels of 25-hydroxyvitamin D3 (25OHD3). Compared to other vitamin D metabolites, 25OHD3 levels are significantly higher in serum and it has a relatively long serum half-life, making it an ideal marker for monitoring short and long-term changes in vitamin D status [1, 2]. Analytical methods detecting only 25OHD3 metabolite can be automated, allowing laboratories with large sample demands to perform high throughput measurements of vitamin D [2].

Vitamin D occurs as two forms, D3 and D2, with approximately 95% of 25OHD circulating as the D3 form. Without dietary supplementation, only a small proportion of 25OHD is comprised of the D2 form [3, 4]. Contradictory data exists as to whether the D2 form, through supplementation, has less or equal effectiveness at maintaining vitamin D status and action in comparison to the D3 form [3]. The biologically active vitamin D metabolite, $1\alpha,25(OH)_2D$, is formed by hydroxylation of 25OHD in the kidney [5]. The $1\alpha,25(OH)_2D3$ metabolite is present in serum at low picomolar concentration ranges with a short half life time of 4 hours. These low concentration ranges have proved challenging for the development of analytical methods that can accurately measure this metabolite [6], particularly using less sensitive older generation LC-MS/MS instruments. Chiral metabolites $23R,25(OH)_2D3$ and $24R,25(OH)_2D3$ can also be converted from 25OHD3, however unlike $1\alpha,25(OH)_2D3$, these chiral metabolites are thought to be non-active [5].

Epimerisation of 25OHD2 and 25OHD3 form the C3-epimers, 3-epi-25OHD2 and 3-epi-25OHD3 respectively [1]. The site of epimerisation occurs at the third carbon atom of 25OHD, altering the position of the hydroxyl group at this site. The structures of 25OHD and 3-epi-25OHD are identical apart from the position of epimerisation [7]. 3-Epi-25OHD metabolites are hydroxylated to form 3-epi-$1\alpha,25(OH)_2D$, in the same manner of 25OHD is hydroxylated to form $1\alpha,25(OH)_2D$. The physiological role of 3-epi-$1\alpha,25(OH)_2D$ remains

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unclear, although it is capable of binding to the vitamin D receptor with reduced physiological effect [8, 9]. 3-Epi-1α,25$(OH)_2$D does not raise calcium levels, whereas it has been shown to suppress parathyroid hormone in rats, as effectively as the 1α,25$(OH)_2$D form [1, 8, 10, 11].

Common techniques for quantifying vitamin D metabolites are immunoassays (IA), high performance liquid chromatography (HPLC) and liquid chromatography tandem-mass spectrometry (LC-MS/MS) [2, 12]. A comparison between IA and LC-MS/MS methods revealed variations in the accuracy between the two methods owing to matrix effects and binding specificity [6, 13]. Other limitations that have been observed for IA include variability between IA batches and deviation of analyte concentrations over a linear calibration range. Several IA methods are unable to distinguish between the two main forms of vitamin D (vitamin D3 and vitamin D2) and can be influenced by the variability in vitamin D binding protein, thereby reducing selectivity [6, 14-16]. Cross reactivity of 25OHD3 with 24,25$(OH)_2$D3 occurs IA, this analyte can be present endogenously at low nanomolar concentration ranges [16]. LC-MS/MS has superior selectivity to IA, due to differentiation of analytes via chromatographic separation and differences in mass transitions.

In a clinical setting LC-MS/MS is considered the most efficient reference method for measuring vitamin D 'status', being the most accurate analytical technique, particularly for quantification of multiple vitamin D analytes [6, 17]. Recent reports have highlighted the importance of accurate LC-MS/MS methods, and some scientific journals now only accept a fully validated assays for the analysis of steroids and sterols such as vitamin D [16, 18]. Sample preparation is required for LC-MS/MS analysis, to avoid matrix effects and concentrate sample if required, whereas in IA samples are analysed directly without any pre-processing meaning sample throughput is higher in IA which has an associated cost benefit [12].

Several techniques exist for preparing samples for LC-MS/MS analysis. A common approach is by liquid-liquid extraction (LLE) as this is considered a routine and inexpensive approach. However LLE can be time consuming, particularly for large sample batches as it can be difficult to automate. Supported liquid-liquid extraction (SLE) is a recently developed sample preparation technique that reduces sample preparation time compared to LLE. Both of these extraction techniques can be effective at removing matrix effects and avoiding extraction of any ionised compounds, particularly phospholipids that are associated with protein precipitation for vitamin D analysis [19]. As LLE is more time consuming, SLE is likely to be the preferred method of choice in the development of a high throughput assay for vitamin D LC-MS/MS analysis. The SLE method described here provides a more efficient protocol over LLE and protein precipitation methods, improving the extraction efficiency of protein precipitation and reducing sample preparation time over LLE without compromising analyte recovery.

Here we developed an LC-MS/MS assay capable of quantifying multiple vitamin D metabolites reflecting the principal vitamin D metabolic pathways. SLE for vitamin D was

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optimised as an alternative approach to other modes of sample extraction, and the optimised and validated methodology was applied to serum samples from healthy donors.

## 2. Materials and methods

### 2.1. Chemicals

Reference standards for vitamin D metabolites (**Table 1**), formic acid, isopropanol, LC-MS grade ethyl acetate and methyl tert-butyl ether (MTBE) were purchased from Sigma Aldrich (Poole, UK). 7αC4 reference standard was purchased from LGC standards (Teddington, UK). A Lux cellulose-3 chiral column (100 mm, 2 mm, 3μm) and 96 well SLE plates were purchased from Phenomenex (Macclesfield, UK). A 2μm inline filter was purchased from Waters Corporation (Manchester, UK). Vitamin D depleted charcoal stripped serum was purchased from Golden West Biologicals Inc. (Temecula, US). LC-MS grade water was purchased from Fisher Scientific (Leicestershire, UK) and LC-MS grade methanol was purchased from Greyhound Chromatography (Merseyside, UK). External vitamin D calibrators and quality controls were purchased from Chromsystems (Am Haag, Germany).

### 2.2. Preparation of standard solutions

Vitamin D standards were purchased as stock solutions in ethanol. These were diluted in methanol to prepare standard curves ranging between 5-100 ng/mL and quality controls. All solutions were stored at −20 °C in amber salinized vials. Internal standards (1α,25$(OH)_2$D3-d3, 25OHD3-d3 and 3-epi-25OHD3-d3) were diluted to prepare a working solution, combining all the internal standards in methanol.

### 2.3. Sample Collection

116 healthy volunteers (79 women, 37 men) between 20 and 74 years of age and with a body mass index (BMI) between 20 and 30 kg/$m^2$ were recruited from the local population (Birmingham, UK) (Z. H-S). Exclusion criteria included pregnancy and medical history of diabetes mellitus, ischemic heart disease, cerebrovascular disease, severe respiratory disease, and epilepsy. Patients arrived fasted at 8:30 am at the National Institute for Health Research (NIHR)-Wellcome Trust Clinical Research Facility, Queen Elizabeth Hospital Birmingham. Venous blood was obtained via a peripheral cannula between 9 and 11am.

### 2.4. Ethical approval

Ethical approval was obtained from the Coventry and Warwickshire Research Ethics Committee (REC reference no. 07/H1211/168) and NRES Committee West Midlands (REC reference no. for CHHIP 14/WM/1146). The protocol was approved by the Scientific Committee of the NIHR-Wellcome Trust Clinical Research Facility at Queen Elizabeth Hospital Birmingham. The study visits were carried out between October 2010 and March 2013. Volunteers were given written and verbal information on the study, and written informed consent was obtained. After study completion, they received travel expenses, and clinically relevant results were passed on to general practitioners.

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### 2.5. Serum extraction

Vitamin D analytes were extracted from 220 µL of serum. Firstly 20 µL of internal standard was added containing 3-epi-25OHD3-d3 (100 ng/mL), 25OHD3-d3 (100ng/mL) and 1α, 25(OH)$_2$D3-d3 (50 ng/mL) in methanol/water (50/50%), the final internal standards in solution were 16,16 and 8ng/mL respectively. Secondly proteins were precipitated using 80 µL of methanol, 50 µL isopropanol and 80 µL of water. The solution was then vortexed at high speed for 30 seconds and left for 7 minutes, followed by centrifugation at 7,516 × g for 5 minutes. Finally the supernatant was transferred onto the SLE plate, where the samples were completely absorbed into the SLE sorbent by applying a vacuum (5 Hg) for 10 seconds and left for 6 minutes. Vitamin D metabolites were extracted from the SLE wells by applying two 800 µL volumes of MTBE/ethyl acetate (90/10%), eluting under gravity initially, followed by applying a vacuum (5 Hg) to completely remove the final volume. The elution solvent was evaporated under nitrogen at 50 °C after each 800 µL addition. Samples were reconstituted in 125 µL water/methanol (50/50%) for LC-MS/MS analysis.

### 2.6. LC-MS/MS analysis

The LC-MS/MS system used was an ACQUITY ultra performance liquid chromatography (uPLC) coupled to a Waters Xevo TQ-S mass spectrometer (Waters, Manchester, UK). Ionisation was performed in electrospray ionisation (ESI) mode and the mass spectrometer was operated in positive ion mode. Multiple reaction monitoring (MRM) mode was used to monitor and quantify vitamin D analytes. The capillary voltage was 3.88 KV and the desolvation temperature was 500 °C. The full mass spectrometry conditions (MRM transitions, cone voltage and collision energies) for each analyte are displayed in the **Table 1**.

Chromatography separation was carried out using a Lux Cellulose-3 chiral column (100 mm, 2 mm, 3 µm), which was maintained at 60 °C in a column oven. A 0.2 µm inline filter was added before the column to prevent blocking of the column and contamination. The mobile phase was methanol/water/0.1% formic acid at a flow rate of 330 µL/min. The mobile phase gradient is described is displayed in **Supplementary Table 1**. The total run time was 8 minutes per sample.

### 2.7. Method validation

Vitamin D depleted charcoal stripped serum, certified for vitamin D LC-MS/MS applications, was used as a negative control matrix. Known concentrations of vitamin D metabolites and internal standards were added to 220 µL charcoal stripped serum to prepare calibration and quality control (QC) standards, extracted using the same method as unknown samples. Method validation was carried out following US Food and Drug Administration (FDA) guidelines [20]. Method validation parameters assessed for this method were accuracy, precision, lower limit of quantification (LLOQ), lower limit of detection (LLOD), linearity, extraction recovery and selectivity.

The accuracy and precision of the method was measured using replicates (N=6) at four concentration ranges to compare mean calculated values with nominal concentrations. Accuracy and precision was performed intra-day on the same day and inter-day with 6

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replicates measured on three consecutive days. Accuracy was assessed by comparing QC concentrations to the nominal value. Precision was characterised by the relative standard deviation (RSD%) of the QC's. Acceptable variation for accuracy and precision was set at 15%, and at 20% at the LLOQ. A calibration curve was produced for each compound by plotting known concentrations against the ratio of analyte peak area/internal standard.

LLOD and LLOQ were determined by initially running known concentration standards of each metabolite, diluting concentrations and observing the peak signal with the corresponding signal to noise ratio (S/N). LLOD was the lowest concentration to produce a signal with S/N greater than 3:1. LLOQ was identified as the lowest concentration to produce a signal with S/N greater than 10:1 and was within FDA guidelines for accuracy and precision. Linearity of each compound was monitored over a calibration range, analysed using Waters Target Lynx quantitative software.

Extraction recoveries of each analyte was determined by comparing the peak area of extracted QC's with the peak area of standard solutions at the same concentrations in water and methanol solution (50/50%). The method selectivity was observed by extracting an un-spiked charcoal stripped serum sample and confirming that no interfering signals were detected in the sample, particularly at the expected retention times of the vitamin D metabolites. Blank charcoal stripped serum was then extracted and spiked with water/methanol (50/50%) containing known vitamin D and internal standards concentrations. The concentrations were compared with standards at the same concentration in water and methanol solution (50/50%).

### 2.8. Statistical analysis

Statistical analysis was conducted using SPSS v22. The relationship between vitamin D metabolites was determined using Spearman one-tailed correlation coefficient. Group differences were tested using independent t-test and one-way ANOVA, the significance was set at $P<0.05$. The chromatography resolution factor was calculated as shown in **Equation 1** [21].

$$R_s = \frac{t_2 - t_1}{\frac{1}{2} W_{1_{+W_2}}} \quad (1)$$

## 3. Results

### 3.1. Method development

The optimised mass spectrometry method detected and quantified 12 metabolites of vitamin D, four vitamin D deuterated internal standards, and the isobar 7αC4 in an unextracted standard solution prepared in water and methanol (50/50%). Following SLE extraction of charcoal stripped serum the vitamin D2 and D3 analogues and vitamin D2-d3 internal standard were retained on the SLE columns and could not be quantified. The final extraction and LC-MS/MS method was therefore capable of detecting 10 vitamin D metabolites, along with three internal standards and 7αC4.

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Further optimization was carried out to achieve maximum resolution of analytes, with the same MRM transitions; 25OHD3 and 3-epi-25OHD3, along with 1α,25$(OH)_2$D3 and 24R,25$(OH)_2$D3. Baseline chromatography separation of 25OHD3 and 25OHD2 with their respective 3-epi-25OHD compounds was achieved. The resolution factor between 25OHD and 3-epi-25OHD was 3.81 and 4.71 for the D3 and D2 forms respectively. The achieved resolution was above the required 1.5 for accurate integration [21]. The method also achieved baseline separation of active 1α,25$(OH)_2$D3 with non-active chiral metabolites. Separation of 25OHD2 and 24OHD2 allowed quantification of these compounds separately, even though it was not possible to completely separate these metabolites at baseline. The resolution factor between 24OHD2 and 25OHD2 was 0.76. A chromatogram of the vitamin D metabolites extracted from a spiked charcoal stripped serum is shown in **Figure 1**. Additional details of the method development are described in the **Supplementary material**.

### 3.2. Method validation

The inter-day and intra-day validation results for accuracy and precision were within the FDA guideline limits for each metabolite. Accuracy and precision results along with the LLOQ values are displayed in **Table 2**. The method showed good linearity for all compounds, the determination coefficient ($R^2$) values were >0.98, apart from 3-epi-25OHD2 which had an $R^2$ value of 0.952. Extraction recovery and selectivity results are displayed in **Supplementary material Table 2**. Recovery values were between 63.05-90.44%, with variation in extraction efficiency between the D2 and D3 metabolites. The selectivity results to determine matrix effects confirmed that there were no interfering peaks from the matrix enhancing or suppressing the concentrations of any compounds.

Cross validation with external Chromsystem calibrators and QC's of 25OHD3 and 25OHD2 were performed (**Table 3**). The calibration curves were within the accepted range for each calibration point, apart from the lowest 25OHD3 concentration. All QC points where within the Chromsystems acceptable concentration ranges.

### 3.3. Application of method to donor serum analysis

This method was applied to 116 serum samples from healthy humans (37 male, 79 female). Measurement of 25OHD3, 3-epi-25OHD3 and 24R,25$(OH)_2$D3 was achieved in all samples. 1α,25$(OH)_2$D3 and 25OHD2 were quantifiable in 42 and 53 samples respectively (**Table 4**). A further 74 samples had detectable levels of 1α,25$(OH)_2$D3 and 63 had detectable 25OHD2, however these levels were below the LLOQ. Seasonal variation in concentrations of the five vitamin D metabolites quantified in the human serum samples is shown in **Figure 2**. Data for serum analyses revealed correlation between concentrations of some vitamin D metabolites, including 25OHD3 levels and 3-epi-25OHD3 ($r = 0.687$; $p = <0.001$), and 24R,25$(OH)_2$D3 and 25OHD3 ($r= 0.916$; $p = <0.001$) (**Supplementary material Figure 2**). The levels of 25OHD3 and 1α,25$(OH)_2$D3 ($r = 0.212$; $p = 0.076$) and 24R,25$(OH)_2$D3 and 1α,25$(OH)_2$D3 showed a significant linear correlation, which would be expected for a functional vitamin D pathway. Individual serum concentrations of vitamin D metabolites are displayed in **Supplementary material Table 4.**

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## 4. Conclusions

In the current report we describe a novel optimised and validated high throughput (8 mins/sample) LC-MS/MS method for analysis of multiple vitamin D metabolites. The endogenous reference ranges of 25OHD3, 3-epi25OHD3, 24R,25(OH)$_2$D3 and 1α, 25(OH)$_2$D3 from these samples correlated well with previous literature [22-25]. In the human serum validation studies 25OHD3 and 24R,25(OH)$_2$D3 were quantified in all samples, with the cohort displaying a strong correlation of 25OHD3 with 24R,25(OH)$_2$D3. Thus the method we describe here could be applied to assessment of the vitamin D pathway forming inactive 24R,25(OH)$_2$D3 catalysed by the enzyme 24-hydroxylase (*CYP24A1*) [24-26]. Mutations in *CYP24A1*, leading to inhibition oh 24-hydroxylase are associated with hypercalcemia and increased concentrations of the active vitamin D3 form [24, 27], whereas increased expression of *CYP24A1* can lead to reduced levels of active vitamin D3 [28]. The application of this method could be used as an approach to identifying alterations in *CYP24A1*, where changes in correlation between 25OHD3 and 24R,25(OH)$_2$D3 are outside a control range.

A key objective of this method was to assess the relative contribution of C3-epimer forms of vitamin D to the circulating concentrations of vitamin D metabolites. The MRM transitions of the C3-epimers are identical to their respective 25OHD isoforms, which could results in the interference of 25OHD3. Overestimation of 25OHD3 has occurred when 3-epi-25OHD3 has not been separated from 25OHD3 and the endogenous levels of 3-epi-25OHD3 are above the limits of quantification for the method [1, 29]. Levels of 3-epi-25OHD3 have been reported to be high in serum from new-borns and infants (<1 year old) [1, 6, 29, 30], but it is unclear whether this is enough to cause interference with 25OHD when the epimer is not chromatographically separated [8, 31]. Owing to the conflicting findings of 3-epi-25OHD3 levels, it was recommended that 3-epi-25OHD3 should be separated when quantified along with 25OHD3 [32, 33]. The resolution we achieved for the separation of 3-epi-25OHD3 and 25OHD3 improved upon reported separations of these metabolites using a pentafluorophenyl (PFP) column [30, 32, 34]. This highlights the importance of separating these two metabolites. Finally, the new LC-MS/MS method further enhances the accuracy of 25OHD3 measurements by separating the endogenous isobar 7αC4 from both 25OHD3 and 3-epi-25OHD3.

Levels of 24OHD2 were below the limits of detection in all samples measured. This metabolite did not interfere with 25OHD2 quantitation although baseline separation of 25OHD2 from 24OHD2 could not be achieved. In data presented here, there was a trend towards higher levels of vitamin D analytes in samples where 25OHD2 was not quantifiable, with 3-epi-25OHD3 being significantly higher ($p = 0.010$). This effect of D2 could be particularly important when monitoring supplementation with this specific form of vitamin D. In the USA, vitamin D2 is the primary form of vitamin D used for higher dose therapy [3, 35], whereas both D2 and D3 are used in the UK and Europe [36]. Thus, analysis of multiple vitamin D metabolites as detailed in the current study would be important to identify metabolism after supplements are given and correlate any positive (or negative) health effects of these may be particularly important in patients where vitamin D2 is used as a supplement.

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The human cohort used to validate analysis of multiple vitamin D analytes was relatively vitamin D-deficient, based on current parameters defined by the Institute of Medicine in the USA [37], with mean serum 25OHD3 levels less than 40 nM (15.750 ± 8.704 ng/ml). Despite this it was possible to quantify low abundance analytes such as 1α,$25(OH)_2D3$ and 25OHD2 in approximately 50% of the samples. It therefore seems likely that studies of more vitamin D-replete cohorts will enable a broader range of analyte quantification. Although an application to serum samples is described here this method could also be useful for other settings including analysis of other biological fluids such as synovial fluid, where levels of some vitamin D metabolites such as 1α,$25(OH)_2D3$ may be higher than in serum [38]. The functional importance of the other vitamin D metabolites included in the current analytical protocol is less clear at present, but these metabolites may become more relevant with changing trends in vitamin D research. Notably, the increasing interest in placebo-controlled supplementation trials using vitamin D3 or vitamin D2 means that it may be important to include analysis of less well characterised D2 metabolites such as 24OHD2, 1α,$25(OH)_2D2$ and 3-epi-25OHD2 alongside more conventional D3 metabolites. Other vitamin D metabolites such as 23,$25(OH)_2D3$ appear to play a central role in the catabolism of vitamin D [39] and may therefore provide systemic insight into the regulation of vitamin D metabolism and function. The new LC-MS/MS protocol may also be useful for cell culture experiments to elucidate vitamin D metabolism pathways for example 25OHD is frequently used as a substrate for analysis of localised, tissue-specific metabolism of 25OHD3 by cells such as macrophages [40].

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- High throughput LC-MS/MS method to measure multiple vitamin D metabolites in serum.
- SLE is an effective sample preparation method for multiple vitamin D metabolites.
- Chiral column separation improves resolution between identical mass to charges.
- Routine serum analysis shows correlations between active and inactive metabolites.

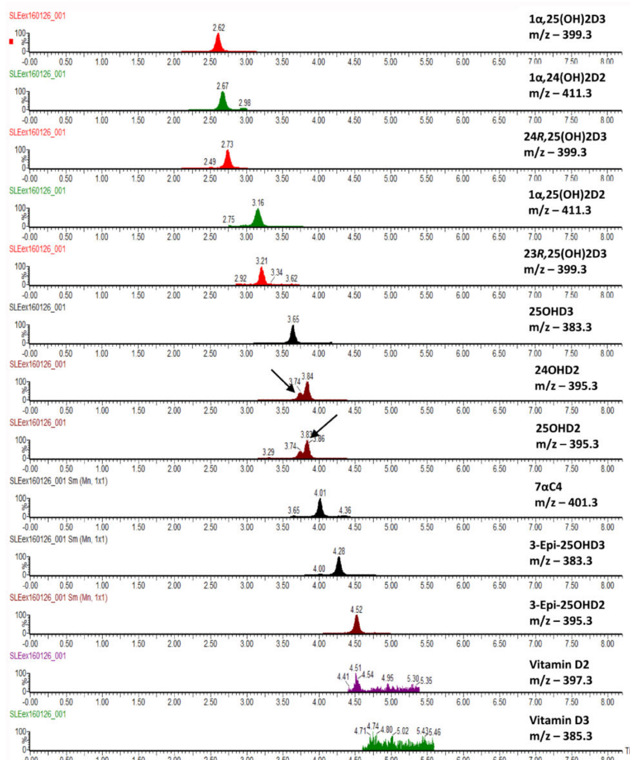


**Figure 1. Chromatogram of vitamin D analytes extracted from a spiked serum quality control standard**

All vitamin D metabolites could be quantified except vitamin D2, vitamin D and vitamin D2-d3 which could not be quantified from the SLE extraction.



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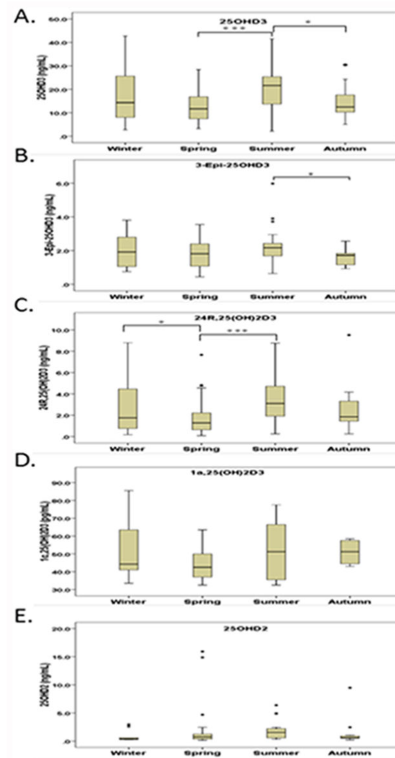


**Figure 2. Seasonal variations in vitamin D metabolites**

Circulating concentrations of 25OHD3, 3-epi-25OHD3, 24R,25$(OH)_2$D3, 1α,25$(OH)_2$D3 and 25OHD2 in serum samples from 116 healthy donors. Season was based on the date of sample collection broken down in the following date ranges; Winter-21/12-20/03; Spring-21/03-20/06; Summer-21/06-20/09; Autumn-21/09-20/12.

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**Table 1**

MRM transitions, collision energies and cone voltages of vitamin D metabolites.

| Compound | Abbrev. | MRM transitions | Collision energy (eV) | Cone voltage (V) |
|---|---|---|---|---|
| 25-hydroxyvitaminD3 | 25OHD3 | 383.2 > 91.0<br>383.2 > 107.0 | 52<br>32 | 28<br>28 |
| 3-Epi-25-hydroxyvitaminD3 | 3-Epi25OHD3 | 383.2 > 95.4<br>383.2 > 107.0 | 36<br>32 | 26<br>26 |
| 1α,25-dihydroxyvitaminD3 | 1α,25$(OH)_2$D3 | 399.2 > 105.1<br>399.2 > 151.1 | 46<br>24 | 22<br>22 |
| 23R,25-dihydroxyvitaminD3 | 23R,25$(OH)_2$D3 | 417.4 > 325.3<br>417.4 > 343.3 | 12<br>10 | 12<br>12 |
| 24R,25-dihydroxyvitaminD3 | 24R,25$(OH)_2$D3 | 417.4 > 121.1<br>417.4 > 381.4 | 14<br>10 | 14<br>14 |
| 25-hydroxyvitaminD2 | 25OHD2 | 395.3 > 91.0<br>395.3 > 119.0 | 54<br>22 | 26<br>26 |
| 24-hydroxyvitaminD2 | 24OHD2 | 395.3 > 340.9<br>395.3 > 119.0 | 36<br>26 | 66<br>30 |
| 3-Epi-25hydroxyvitaminD2 | 3-Epi-25OHD2 | 395.3 > 91.0<br>395.3 > 119.0 | 50<br>26 | 26<br>26 |
| 1α,25- dihydroxyvitaminD2 | 1α,25$(OH)_2$D2 | 411.3 > 133.0<br>411.3 > 151.0 | 30<br>22 | 26<br>26 |
| 1α,24- dihydroxyvitaminD2 | 1α,24$(OH)_2$D2 | 411.3 > 133.0<br>411.3 > 151.0 | 30<br>20 | 26<br>26 |
| Ergocalciferol | Vitamin D2 | 397.4 > 69.0<br>397.4 > 107.1 | 22<br>28 | 16<br>16 |
| Cholecalciferol | Vitamin D3 | 385.4 > 107.0<br>385.4 > 259.3 | 30<br>16 | 20<br>20 |
| 7α-hydroxy-4-cholesten-3 -one | 7αC4 | 401.4 > 97.0<br>401.4 > 117.1 | 26<br>24 | 34<br>32 |
| 1α,25-dihydroxyvitaminD3-d3 | 1α,25$(OH)_2$D3-d3 | 402.4 > 138.0<br>402.4 > 154.1 | 18<br>20 | 22<br>22 |
| 3-Epi-hydroxyvitamin-d3 | 3-Epi-25OHD3-d3 | 404.4 > 107.2<br>404.4 > 109.4<br>404.4 > 368.4 | 40<br>22<br>12 | 40<br>22<br>12 |
| 25-hydroxyvitaminD3 | 25OHD3-d3 | 386.4 > 95.1<br>386.4 > 109.3 | 26<br>24 | 26<br>26 |
| Ergocalciferol-d3 | Vitamin D2-d3 | 400.3 > 109.8<br>400.3 > 69.02<br>400.3 > 83.02 | 24<br>30<br>22 | 16<br>16<br>16 |

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**Table 2**

Summary of accuracy and precision data for each vitamin D metabolite.

| Compound | Concentration (ng/mL) | Level | Precision (% RSD) | | |
|---|---|---|---|---|---|
| | | | Intra-day | Inter-day | Accuracy (%) |
| | 0.500 | Low | 7.61 | 9.20 | 100.15 |
| 25OHD3 | 5.00 | Medium | 10.68 | 7.45 | 91.34 |
| | 20.00 | High | 4.18 | 4.93 | 106.84 |
| | 0.188 | Low | 8.38 | 11.61 | 103.83 |
| 3-Epi-25OHD3 | 1.875 | Medium | 8.41 | 6.10 | 111.74 |
| | 7.500 | High | 3.20 | 5.25 | 97.45 |
| | 0.061 | Low | 9.64 | 8.20 | 111.80 |
| 1α,25$(OH)_2$D3 | 0.750 | Medium | 3.05 | 5.30 | 99.33 |
| | 2.000 | High | 4.29 | 4.20 | 99.96 |
| | 1.00 | Low | 6.46 | 9.62 | 98.02 |
| 23R,25$(OH)_2$D3 | 4.00 | Medium | 9.31 | 9.17 | 98.56 |
| | 8.00 | High | 4.49 | 10.85 | 96.71 |
| | 0.500 | Low | 6.19 | 8.13 | 98.62 |
| 24R,25$(OH)_2$D3 | 4.00 | Medium | 7.22 | 8.26 | 98.47 |
| | 8.00 | High | 6.08 | 8.48 | 100.40 |
| | 1.000 | Low | 10.59 | 9.58 | 96.47 |
| 25OHD2 | 2.500 | Medium | 9.52 | 8.38 | 92.91 |
| | 10.00 | High | 14.19 | 9.80 | 99.13 |
| | 0.625 | Low | 9.26 | 7.07 | 99.11 |
| 24OHD2 | 1.000 | Medium | 7.46 | 6.86 | 108.03 |
| | 2.500 | High | 3.37 | 5.65 | 90.77 |
| | 0.500 | Low | 11.11 | 8.20 | 114.80 |
| 3-Epi-25OHD2 | 1.875 | Medium | 3.75 | 6.62 | 110.89 |
| | 7.500 | High | 0.69 | 3.15 | 100.98 |
| | 0.075 | Low | 10.44 | 7.88 | 90.44 |
| 1α,25$(OH)_2$D2 | 0.750 | Medium | 4.08 | 7.60 | 93.19 |
| | 2.000 | High | 5.34 | 3.63 | 102.87 |
| | 0.0630 | Low | 8.45 | 10.91 | 96.30 |
| 1α,25$(OH)_2$D2 | 0.625 | Medium | 4.24 | 5.96 | 102.41 |
| | 2.000 | High | 4.10 | 5.20 | 109.39 |
| | 0.375 | Low | 5.54 | 8.00 | 106.13 |
| 7αC4 | 3.750 | Medium | 9.81 | 8.71 | 96.07 |
| | 15.00 | High | 7.85 | 8.20 | 98.55 |

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**Table 3**

Chromsystems external calibrators.

| 25OHD3 | | | 25OHD2 | | |
|---|---|---|---|---|---|
| **Chromsystems concentration (ng/mL)** | **Measured Concentration (ng/mL)** | **%DEV** | **Chromsystems concentration (ng/mL)** | **Measured Concentration (ng/mL)** | **%DEV** |
| 4.50 | 6.245 | 38.8 | n.d | 1.98 | |
| 9.70 | 8.967 | −7.6 | 5.00 | 5.03 | 2.8 |
| 18.50 | 19.176 | 3.7 | 14.30 | 14.69 | 2.7 |
| 32.90 | 30.959 | −5.9 | 28.60 | 27.75 | −3.0 |
| 65.60 | 67.321 | 2.6 | 57.10 | 65.56 | 14.8 |
| 102.00 | 101.078 | −0.9 | 98.60 | 106.90 | 8.4 |
| 140.00 | 146.574 | 4.7 | 140.00 | 158.06 | 12.9 |
| QC (acceptable range 36.0-54.1 ng/mL) | | | QC (acceptable range 29.6-44.4 ng/mL) | | |
| 45.1 | 42.772 | | 37.0 | 37.25 | |
| 45.1 | 48.372 | | 37.0 | 40.99 | |
| 45.1 | 47.769 | | 37.0 | 38.38 | |
| 45.1 | 44.178 | | 37.0 | 33.37 | |
| 45.1 | 47.463 | | 37.0 | 35.36 | |
| | %CV = 4.8 | | | %CV = 12.3 | |

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## Table 4

Concentrations of serum vitamin D metabolites in serum

| **Serum** | | | |
| --- | --- | --- | --- |
| **Compound** | **N =** | **Mean ±SD (ng/mL)** | **Range (ng/mL)** |
| 25OHD3 | 116 | 15.750 ±8.704 | 2.166-42.706 |
| 3-Epi-25OHD3 | 116 | 1.913 ±0.862 | 0.445-5.977 |
| 24R,24$(OH)_2$D3 | 116 | 2.499 ±2.184 | 0.084-9.514 |
| 25OHD2 | 53 | 1.795 ±2.979 | 0.157-15.921 |
| Compound | N = | Mean ±SD (pg/mL) | Range (pg/mL) |
| 1α,25(OH)2D3 | 42 | 47.957 ±12.855 | 32.514-85.389 |