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# The validation of an LC-MS/MS method for the quantification of vitamin D metabolites in human milk and their biological variability in Gambian women

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ABSTRACT

Vitamin D is required for healthy growth and development, but data on human milk vitamin D content is limited. We describe a liquid chromatography tandem mass spectrometry (LC-MS/MS) method for the analysis of vitamin D metabolites in human milk, and its application in samples collected on two consecutive days from women in rural Gambia. Vitamin D compounds were extracted from 1 mL of milk by liquid-liquid extraction and derivatised with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) prior to analysis by LC-MS/MS. The limit of quantification was 0.05 nmol/L for vitamin $D_2$, 0.025 nmol/L for vitamin $D_3$ and 0.1 nmol/L for 25(OH)$D_2$ and 25(OH)$D_3$. Within- and between-day imprecision was <12 % for all analytes except vitamin $D_2$ (14 %).

From all data combined, geometric mean (-/+ 1 SD) vitamin $D_3$ concentration was 0.94 (0.43, 1.80) nmol/L and for 25(OH)$D_3$ 0.32 (0.23, 0.42) nmol/L. The within-person (intra-individual) coefficient of variation (%CV) was 32 % and 12 % for vitamin $D_3$ and 25(OH)$D_3$, respectively. Between-person (inter-individual) %CVs were 89 % and 34 % for vitamin $D_3$ and 25(OH)$D_3$, respectively. There was no significant association between vitamin D metabolite concentrations and milk fat (creamatocrit). Mean vitamin D content of human milk as ARA averaged 42 IU/L with 25(OH)$D_3$ responsible for around two-thirds of the biological activity. In conclusion, this work describes a reliable LC-MS/MS method for quantification of vitamin D and 25(OH)D in low volumes of human milk providing a platform for future work. This study contributes to current understanding of variability of milk vitamin D content.

## 1. Introduction

Vitamin D is essential for bone development and vitamin D status may also be related to other health effects [1,2]. Global recommendations state that infants under six months of age should be exclusively breastfed, but information on human milk vitamin D (referring to all metabolic forms) content and its forms is limited [3,4]. This is partly due to the difficulties of extracting vitamin D compounds from the complex milk matrix [5–7]. Liquid chromatography tandem mass spectrometry (LC-MS/MS) methods can provide the specificity and sensitivity required for milk vitamin D analysis and have been applied in observational studies [8–11] and randomised controlled trials [12,13]. However, the apparently low concentration of vitamin D in human milk and analytical methods with poor sensitivity [14,15] have led to the requirement for laborious sample extraction protocols and relatively large sample volumes of milk [9]. Other studies have not measured the primary forms of milk vitamin D [16,17] and the relative contribution of vitamin D forms in milk remains unclear.

Variation in the concentration of human milk vitamin D in its various forms is related to many factors, including methodological or pre-analytical factors (e.g. stage of feed, method of collection, storage) [18] and analytical variation and differences within and between individuals. Standardised specimen collection, processing and storage protocols together with a precise analytical method can minimise variation due to pre-analytical and analytical factors. The remaining within- and between-person variation together comprise the biological variation

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[19] and will include temporal effects, e.g. stage of lactation, diurnal rhythm, milk volume and macronutrient composition [18]. An appreciation and understanding of biological variation is important since such information can be used to set analytical quality goals, interpret change within individuals and populations and estimate variation in relation to setting nutrient reference values [19–22]. Some studies have investigated longitudinal changes in the content of vitamin D compounds in milk content over several months [9,10,16], but there are no data on intra-individual variation over a shorter duration.

In this report, we describe the development and validation of an LC-MS/MS method for the analysis of 25-hydroxyvitamin D (25(OH)D) and vitamin $D_{2/3}$ (ergocalciferol/ cholecalciferol) in human milk. We subsequently apply the method to the assessment of the concentrations of these vitamin D compounds in human milk samples collected from women in rural Gambia, to assess variation within and between person and correlation with milk fat concentration.

## 2. Methods

### 2.1. Study population and sample collection

Milk samples were collected between April and August 2013 from women living in and around Keneba, a rural village in the West Kiang region of The Gambia [23,24]. Ethical permission for the study was obtained from the joint Medical Research Council (MRC) Gambia Unit-Gambian Government Ethics Committee and written, informed consent was obtained from all participants. Milk samples were collected by manual expression in the morning on two consecutive days at (mean (SD)) 14 (2) weeks postpartum. Samples were stored initially at −20 °C before being shipped on dry ice to the UK. Samples were subsequently stored at −70 °C until analysis and were not used or thawed until the extraction.

### 2.2. Analysis

Due to the complex matrix of human milk, rigorous extraction procedures are required to liberate and extract vitamin D compounds for analysis [25]. The extraction of milk ergocalciferol (vitamin $D_2$), cholecalciferol (vitamin $D_3$), 25-hydroxyergocalciferol (25(OH)$D_2$) and 25-hydroxycholecalciferol (25(OH)$D_3$) was adapted from a previously published method [26]. Quantification was performed by LC-MS/MS using isotope-labelled internal standards for each analyte. Concentrations were calculated from the ratio of analyte-to-internal standard peak area compared with that of a calibration curve.

### 2.3. Chemicals

Ethanol (>99.8 %), formic acid and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) were purchased from Sigma (Sigma-Aldrich, Gillingham, Dorset, UK). Methanol (LC-MS grade) and *n*-hexane (97 %) were purchased from VWR (VWR Chemicals, Lutterworth, Leicestershire, UK). Isooctane (99.5 %), ethyl acetate (99.5+%) and acetone were purchased from Thermo Fisher (Thermo Fisher Scientific, Loughborough, Leicestershire, UK). Sodium sulphate anhydrous was purchased from LP (LP Chemicals, Winsford, Cheshire UK).

Vitamin D compounds as calibration standards (vitamin $D_2$; vitamin $D_3$; 25(OH)$D_2$; 25(OH)$D_3$) and matched tri-deuterated compounds as internal standards were purchased from Sigma (Sigma-Aldrich, Gillingham, Dorset, UK).

### 2.4. Sample preparation

All work was carried out under yellow light to minimise the risk of light degradation of analytes. Samples were defrosted and mixed thoroughly to ensure a homogenous sample. To 1 mL of sample or quality control (QC) material in a 16 ×100 mm borosilicate glass tube was added 900 μL ethanol, to precipitate proteins, and 100 μL internal standard solution. Samples were mixed for 10 minutes on a multitube vortex mixer using the pulse mode, followed by liquid-liquid extraction performed with the addition of 2.5 mL hexane:ethyl acetate (9:1) for 10 minutes. After centrifugation at 3400 *g* and 4 °C for 10 minutes ~2 mL of the upper solvent layer was transferred into fresh 12 ×75 mm borosilicate glass tubes. Samples were kept in an ice bath during this phase to minimize sample degradation. The liquid-liquid extraction was then repeated with a further volume of 2.5 mL hexane:ethyl acetate and the supernatant combined with that from the first extraction. Samples were dried down under vacuum (~50 minutes) using a SpeedVac concentrator (Thermo Scientific, UK). After drying, samples were reconstituted with 1 mL isooctane and~100 mg sodium sulphate anhydrous for 10 minutes to remove any residual aqueous phase. After centrifugation at 3400 *g* and 4°C for 10 minutes the isooctane was transferred to a clean 12 ×75 mm borosilicate glass tube and derivatised for 1 hour with 25 μL PTAD (10 mg/mL in acetone). Vitamin D metabolites were subsequently back extracted by addition of 200 μL acetonitrile:water (8:2) for 5 minutes, centrifugation at 3400 *g* and 4 °C for 10 minutes and subsequent transfer of 150 μL of the acetonitrile:water layer into an amber HPLC vial with 200 μL insert for analysis.

### 2.5. Standards

All standards and internal standards were dissolved separately in ethanol at ~100 μg/mL and stored at −70 °C to generate stock solutions. The exact concentration of the stock standard solutions was calculated using absorbance at 264 nm and molar absorption coefficients (vitamin $D_2$: 18,900 L/mol*cm; vitamin $D_3$: 18,300 L/mol*cm; 25(OH)$D_2$ and 25(OH)$D_3$: 18,200 L/mol*cm). Working standards were prepared in ethanol to give ranges of 0.1–100 nmol/L for all analytes. Internal standards were prepared using 10 μL of each stock in 100 mL ethanol to give a final concentration of approximately 25 nmol/L. Standards and internal standards (100 μL) were dried down under vacuum (~20 minutes) using a SpeedVac concentrator, reconstituted in 1 mL isooctane and derivatised with 25 μL PTAD alongside the samples to give a standard curve equivalent to 0.01–10 nmol/L. Due to the inability to obtain milk with known very low vitamin D levels in which to prepare matrix matched standards, solvent standards were used which did not require full extraction alongside the samples.

### 2.6. Quality control (QC) material

Three QC materials were used; an in-house pooled human milk prepared from residual human milk samples, a commercially obtained single donor human milk sample (BioIVT, West Sussex, UK) and reconstituted milk powder, reference material ERM-BD600 (European Commission Joint Research Centre, Geel, Belgium). Separate aliquots were prepared, stored at −70 °C and removed from the freezer for analysis in duplicate with each batch.

### 2.7. LC-MS/MS

The LC-MS/MS system consisted of a Waters Acquity UPLC and AB Sciex 5500 QTrap mass spectrometer fitted with an electrospray ionization (ESI) probe (AB Sciex UK Ltd, Macclesfield, UK). Calibrators and samples were injected (10 μL) onto a reversed-phase column (Waters Cortecs C18+ 1.6 μm 2.1 ×150 mm column with an in-line filter) (Waters Ltd, Wilmslow, UK). Chromatographic separation was performed under a gradient using (A) methanol with 0.1 % formic acid and (B) and water with 0.1 % formic acid. The flow rate was 200 μL/min, column temperature 45 °C and run time of 17 min (0 – 5 min 80 % A, 5.1 min 90 % A, 10 min 94 % A, 10.1 – 12 min 100 % A, 12.1 – 17 min 80 % A). The MS was operated in positive ionization mode using the parameters shown in Table 1. Analyte peak area to internal standard peak area ratio was compared to that of a calibration curve to determine

**Table 1**
Mass spectrometry parameters for each analyte. Abbreviations: 25(OH)$D_2$, 25-hydroxyvtiamin $D_2$, 25(OH)$D_3$, 25-hydroxyvitamin $D_3$; d3, deuterated (internal standard); DP, de-clustering potential.

| Analyte | Transition (*m/z*) | DP | Collision energy | Collision cell exit potential | Entrance potential | Dwell time (msec) |
|---|---|---|---|---|---|---|
| Vitamin $D_2$ | 572.4>298.2 / 280.2 | 90 | 33 / 33 | 10 / 31 | 14 / 9 | 165 |
| Vitamin $D_3$ | 560.4>298.2 / 280.2 | 80 | 25 / 39 | 18 / 26 | 6 / 8 | 165 |
| d3-vitamin $D_2$ | 575.4>301.2 | 120 | 23 | 25 | 10 | 165 |
| d3-vitamin $D_3$ | 563.4>301.2 | 90 | 20 | 19 | 12 | 165 |
| 25(OH)$D_2$ | 570.4>298.2 / 280.2 | 80 | 21 / 40 | 23 / 20 | 6 / 11 | 165 |
| 25(OH)$D_3$ | 558.4>298.2 / 280.2 | 89 | 23 / 39 | 20 / 22 | 11 / 9 | 165 |
| d3–25(OH)$D_2$ | 573.4>301.2 | 80 | 21 | 23 | 6 | 165 |
| d3–25(OH)$D_3$ | 561.4>301.2 | 120 | 23 | 21 | 10 | 165 |

analyte concentration. Samples were analysed over two analytical runs and samples from the same participant were measured on the same analytical run. An example chromatogram of a human milk sample is shown in Fig. 1. Retention times for vitamin $D_3$ and 25(OH)$D_3$ were around 10 and 5 minutes, respectively.

### 2.8. Method validation

Method validation was performed with (i) pooled human milk from residual samples and (ii) unhomogenised, full-fat pasteurised Jersey cow milk from a local supermarket. The limit of quantification (LOQ) and limit of detection (LOD) were calculated using solvent solutions and a signal to noise ratio of 10 and 3, respectively. Intra-assay coefficient of variation (%CV) was calculated from 10 replicates in human milk for vitamin $D_3$ and 25(OH)$D_3$, and in cow milk for vitamin $D_3$, 25(OH)$D_3$ and 25(OH)$D_2$. The inter-assay %CV for vitamin $D_3$ and 25(OH)$D_3$ was calculated using 3 replicates of 10 human milk samples. None of these samples had measurable levels of vitamin $D_2$ or 25(OH)$D_2$. Spiked recovery was measured in human milk by adding 10 μL of an ethanolic solution containing vitamin D metabolites to 10 mL of milk to give concentrations in the milk samples of 0.1, 0.5, 1.0 and 5.0 nmol/L. Each of the spiked samples were analysed 5 times. Extraction efficiency was calculated from the difference in peak area of stable isotope labelled internal standard between samples and solvent standards. As an indicator of consensus with other methods, 10 replicates of milk powder reference material ERM-BD600 was measured prepared as a 10 mg/mL solution. This material does not have a certified value for vitamin $D_3$ but an 'informative' range of 1.41 – 6.64 nmol/L (0.57 – 2.69 ng/mL).

### 2.9. Calculations

Where several %CVs were available, e.g. for different participants or levels of QC material, average %CVs were calculated using the equation:

$$average\%CV = \sqrt{\left(\frac{(n^a-1)\quad x\quad CV^a + (n^b-1)\quad x\quad CV^b \ldots}{(n^a-1)+(\quad n^b-1)\ldots}\right)}$$

### 2.10. Milk fat content

After collection and prior to freezing, milk fat was measured by the creamatocrit method as described elsewhere [27,28].

### 2.11. Statistical analysis

Statistical analysis was performed in Datadesk 6.3 (Data Description Inc). Descriptive statistics for participant characteristics are presented as means (standard deviations (SD)). Concentrations of vitamin $D_3$, 25(OH)$D_3$, and milk fat were skewed and were transformed to natural logarithms for statistical testing and regression analysis. Their summary data are presented as geometric means (-1SD, +1 SD] calculated by back-transformation of the logged summary data. Time point comparisons were initially performed with paired t-tests and relationships between variables performed with multiple regression, including where specified, maternal participant identification number (maternal ID), time point and milk fat concentration. Anti-rachitic activity (ARA) in units IU/L is used to express vitamin D activity and takes into account the potential higher biological activity of 25(OH)D compared with vitamin D assigning a value of 1 IU/L for each 25 pg/mL of vitamin $D_3$ and 5 pg/

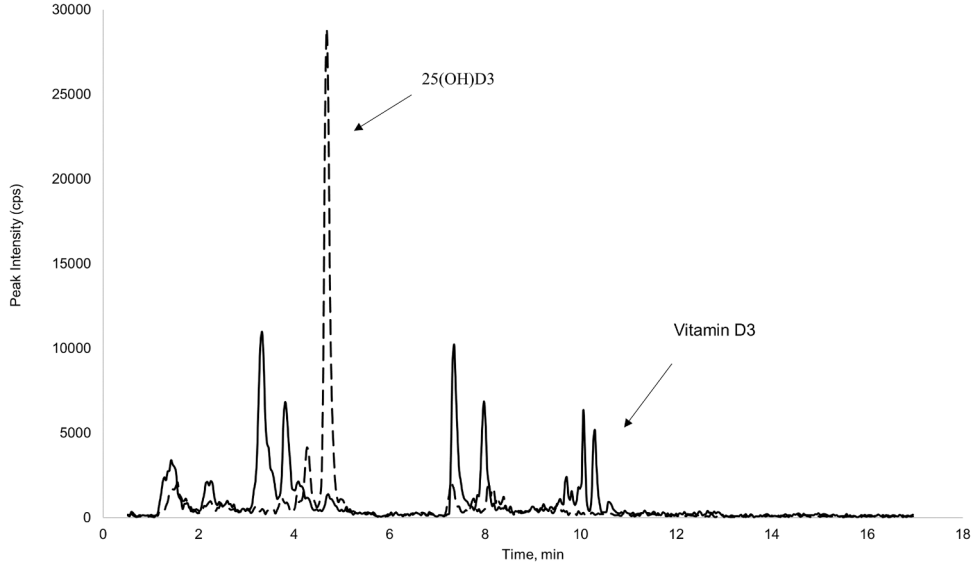


**Fig. 1.** Example chromatogram showing traces for vitamin $D_3$ (solid line) and 25(OH)$D_3$ (dashed line) from a human milk sample. Concentrations were 0.53 and 0.45 nmol/L for vitamin $D_3$ and 25(OH)$D_3$, respectively.

mL of 25(OH)$D_3$. To convert concentration data to international units (IU) the following conversions were performed: nmol/L to ng/mL (μg/L) divide by 2.59 for vitamin $D_3$ and 2.49 for 25(OH)$D_3$. The ng/mL concentrations can be then multiplied by 1000 to derive ng/L (pg/mL) and the IU/L value calculated by dividing the ng/L (pg/mL) vitamin $D_3$ concentration by 5 and the 25(OH)$D_3$ concentration by 25.

## 3. Results

### 3.1. Method validation

Of the three milks used for validation purposes, 25(OH)$D_2$ was detected only in the cow milk. Sensitivity limits, imprecision and recovery are summarised in Table 2. Average analytical variation was 7.2 % for vitamin $D_3$ and 6.2 % for 25(OH)$D_3$. Extraction efficiency between different milk samples was highly variable with %CV for vitamin $D_3$ and 25(OH)$D_3$ of 59 % and 21 %, respectively. This contrasts with the %CVs from the same pooled milk sample extracted 10 times which were 18 % and 2 % for vitamin $D_3$ and 25(OH)$D_3$, respectively. From 10 replicates, the mean (SD) vitamin $D_3$ concentration of reference milk powder ERM-BD600 was 3.2 (0.08) nmol/L (1.3 (0.39) ng/mL) and the %CV was 2.6 %. For the QC material, nominal vitamin D concentrations for pooled human milk were 1.18 and 0.31, for vitamin $D_3$ and 25(OH)$D_3$, respectively. For the single donor sample, concentrations were 0.57, 4.18 and 0.40 nmol/L for vitamin $D_2$, vitamin $D_3$ and 25(OH)$D_3$, respectively.

### 3.2. Milk vitamin D in Gambian women

Data were analysed from 23 Gambian women who had provided milk specimens on two consecutive days. Participant characteristics are shown in Table 3. No vitamin $D_2$ or 25(OH)$D_2$ was detected in any specimen. All vitamin $D_3$ and 25(OH)$D_3$ concentrations were above the LOQ.

Biochemical data are shown in Table 4 and Fig. 2 for Days 1 and 2. By paired t-test there were no significant differences between Day 1 and Day 2. The geometric means (-1SD, +1 SD) of Day 1 and 2 were 0.94 (0.43, 1.80) and 0.32 (0.23, 0.42) for vitamin $D_3$ and 25(OH)$D_3$, respectively. Mean within-person (intra-individual) coefficient of variation (%CV) was 32 % and 12 % for vitamin $D_3$ and 25(OH)$D_3$, respectively. Vitamin $D_3$ had a wider range of concentrations (0.17 – 5.06 nmol/L) than 25(OH)$D_3$ (0.14 – 0.70 nmol/L) across all time points (Fig. 3). Between-person (inter-individual) %CVs were 89 % and 34 % for vitamin $D_3$ and 25(OH)$D_3$, respectively.

Fig. 3(**B**) shows the contribution of the vitamin D forms to ARA in each of the 23 participants. Consistent with the wide variation in vitamin $D_3$ concentration the relative contribution of the two forms varied between individuals (Fig. 3) and 25(OH)$D_3$ contributed around two-thirds of the total ARA. We observed a significant positive relationship between 25(OH)$D_3$ and vitamin $D_3$ concentrations (Fig. 4, Table 5). In regression models including maternal ID and day, maternal ID ($P \leq 0.0001$) was the strongest predictor of both vitamin $D_3$ and 25(OH)$D_3$ concentration. There were small, non-significant percent differences between days (Day 2 was 2 % and 8 % lower than Day 1 for vitamin $D_3$ and 25(OH)$D_3$, respectively). Milk fat content ranged between 21 to 83 g/L and 16 to 52 g/L at Days 1 and 2, respectively. Within-person variation was 25 % and between-person variation of Day 1, Day 2 and the means were 35 %, 28 % and 24 %, respectively. The effect of fat on vitamin D milk concentrations was inconsistent between days; using mean values, fat was a significant predictor of 25(OH)$D_3$, but not vitamin $D_3$. Fat concentration attenuated the strength of the relationship between vitamin D metabolites, but as for the unadjusted model, the relationship between 25(OH)$D_3$ and vitamin $D_3$ remained

**Table 3**
Maternal participant characteristics and infant age (*n* 23).

| Characteristic | Mean (SD) | Minimum | Maximum |
|---|---|---|---|
| Age, y | 26 (7) | 17 | 36 |
| Height, m | 163 (4) | 155 | 169 |
| Weight, kg | 58 (7) | 48 | 80 |
| BMI, kg/m$^2$ | 21.8 (2.6) | 18.5 | 29.2 |
| Parity, *n* | 2 [median] | 1 | 9 |
| Infant age, weeks | 14 (2) | 11 | 16 |

**Table 4**
Biochemical data for human milk on consecutive days. Data are geometric means (-1SD, +1 SD) obtained from back transformation of summary statistics in natural logarithms. P value is the significance by paired t-test of difference between the two time points.

| | Day 1 *n* 23 | Day 2 *n* 23 | Mean of Day 1 and Day 2 | |
|---|---|---|---|---|
| Analyte, units | Geometric mean (-1SD, +1 SD) | | | *P* |
| Vitamin $D_3$, nmol/L | 0.91 (0.41, 1.94) | 0.89 (0.35, 2.19) | 0.94 (0.43, 1.80) | 0.87 |
| 25(OH)$D_3$, nmol/L | 0.33 (0.23, 0.43) | 0.30 (0.21, 0.44) | 0.32 (0.23, 0.42) | 0.17 |
| Vitamin $D_3$, IU/L | 14.0 (6.3, 29.9) | 13.7 (5.4, 33.8) | 14.7 (6.7, 27.6) | 0.87 |
| 25(OH)$D_3$, IU/L | 26.3 (18.5, 34.3) | 24.2 (17.2, 34.9) | 25.5 (18.7, 33.5) | 0.17 |
| ARA, IU/L | 43 (28, 56) | 40 (24, 58) | 42 (27, 62) | 0.44 |
| Fat, g/L | 39 (28, 59) | 33 (24, 50) | 37 (29, 49) | 0.07 |

**Table 2**
Method performance characteristics. For imprecision, superscripts indicate whether analysis was performed in human milk or cow milk. Spiked recovery experiments were performed with human milk.

| Vitamin D metabolite | LOD, nmol/L | LOQ, nmol/L | Intra-assay CV% | Inter-assay CV% | Spiked recovery, %: Level: Mean (SD) |
|---|---|---|---|---|---|
| 25(OH)$D_2$ | 0.025 | 0.1 | 13.6$^{cow}$ | Insufficient sample to calculate | 0.1 nmol/L: ND<br>0.5 nmol/L: 89 (5)<br>1.0 nmol/L: 84 (2)<br>5.0 nmol/L:73 (6) |
| 25(OH)$D_3$ | 0.025 | 0.1 | 8.6$^{human}$<br>11.2$^{cow}$ | 5.5 (3.0) | 0.1 nmol/L: 108 (37)<br>0.5 nmol/L: 104 (3)<br>1.0 nmol/L: 113 (7)<br>5.0 nmol/L: 114 (5) |
| Vitamin $D_2$ | 0.01 | 0.05 | No samples with vitamin $D_2$ | No samples with vitamin $D_2$ | 0.1 nmol/L: 86 (5)<br>0.5 nmol/L: 91 (4)<br>1.0 nmol/L:89 (4)<br>5.0 nmol/L:93 (2) |
| Vitamin $D_3$ | 0.01 | 0.025 | 4.1$^{human}$<br>9.6$^{cow}$ | 7.1 (2.0) | 0.1 nmol/L: 79 (6)<br>0.5 nmol/L: 90 (4)<br>1.0 nmol/L:88 (4)<br>5.0 nmol/L: 94 (3) |

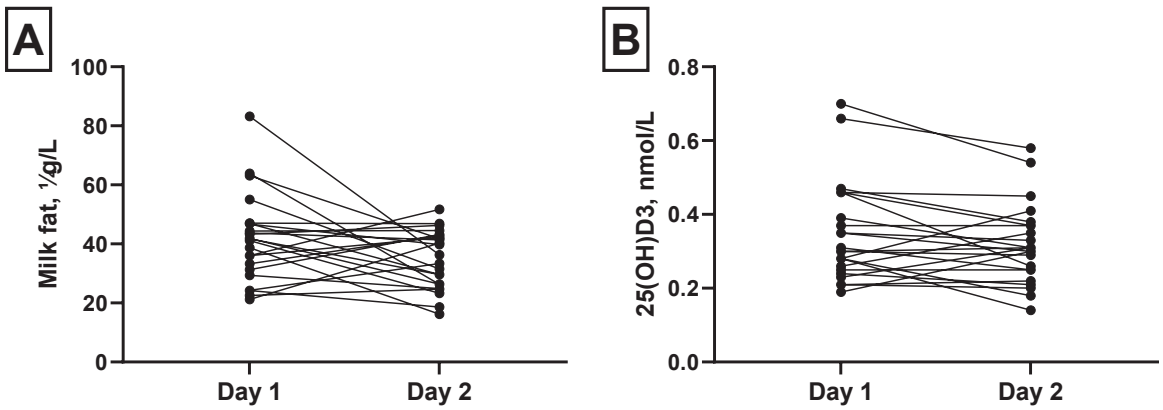


**Fig. 2.** Vitamin $D_3$ (A) and 25-hydroxyvitamin $D_3$ (B) concentrations in milk samples collected on consecutive days.

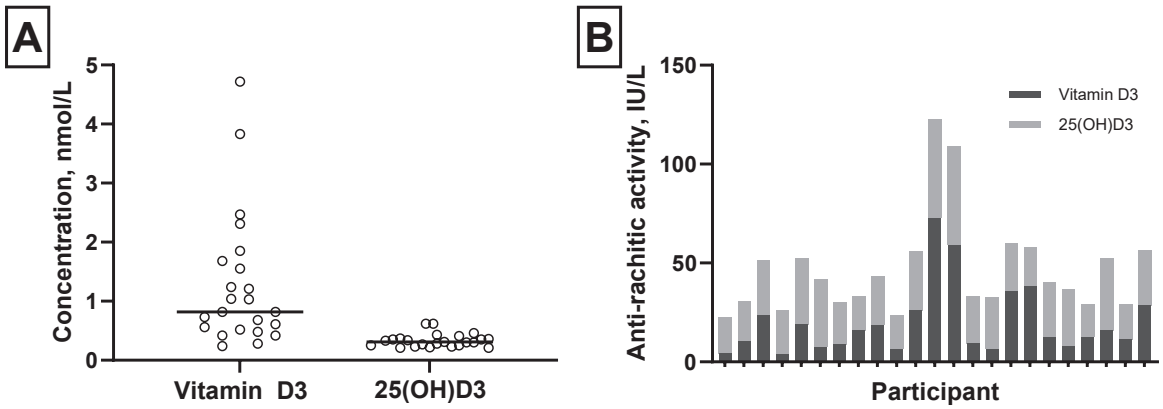


**Fig. 3.** Mean concentration and anti-rachitic activity (ARA) of Day 1 and Day 2 for each participant. A: Distribution of vitamin $D_3$ and 25(OH)$D_3$ concentrations. B: ARA calculated assuming 5 pg/mL and 25 pg/mL (ng/L) is equal to 1 IU/L for vitamin $D_3$ and 25(OH)$D_3$, respectively.

significant for Day 2 and the mean values (Fig. 3, Table 5).

For 25(OH)$D_3$, when milk fat was additionally included in the model including maternal ID and day, the non-significant effect of day (P=0.5) was further reduced from 8 % to 4 % with maternal ID the major contributor (P=0.0004) to variance and a borderline significant effect of fat (P=0.06). Similarly for vitamin $D_3$, maternal ID was the major predictor (P=0.0001), day was non-significant (P=0.7) and fat, whilst not a significant predictor in the combined model (P=0.17), its inclusion increased the difference between days from –2 % to +5 %.

## 4. Discussion

We have described and validated an LC-MS/MS method for the measurement of vitamin D and 25(OH)D in human milk and demonstrated acceptable recovery and precision for all measured metabolites. To test the utility of the assay, the method was applied to the study of vitamin D content of milk from rural Gambian women. We observed generally low concentrations of vitamin $D_3$ and 25(OH)$D_3$. Between women, variation in vitamin $D_3$ concentration was greater than for 25(OH)$D_3$, but for both vitamin D forms between-person variation was greater than within-person variation across two days, indicating that a single sample can provide a characteristic measure of an individual's milk vitamin D content at that time.

The selectivity and sensitivity of LC-MS/MS are well-suited to the measurement of the low concentrations of vitamin D metabolites in human milk. Studies applying LC-MS/MS to measure vitamin D metabolites in human milk were recently reviewed, and limitations related to the choice of metabolite, sensitivity, insufficient method detail, the requirements of often high sample volume and limited subsequent application were highlighted [5]. The unique and variable characteristics of the human milk matrix mean that extraction methods optimised for recovery of 25(OH)D from serum or plasma are unlikely to be suitable for vitamin D compounds in milk. We measured the two major vitamin D compounds in human milk although the method was also optimised for vitamin $D_2$ and 25(OH)$D_2$, although these metabolites were not detected in any of the samples tested. The method used 1 mL of milk and therefore is practical for application in larger studies. The extraction method used in this study was based on the method reported by Oberson *et al.*, for human milk [26], but we used LC-MS/MS rather than supercritical fluid chromatography (SFC) tandem mass spectrometry (SFC-MS/MS) and a larger sample volume. Of the complementary chromatography techniques, SFC offers potential advantages from the lower use of organic solvents and better chromatographic separation, but it is a relatively new technique and consequently the availability of equipment and published methods are limited compared to vitamin D analysis by LC-MS/MS, a common and readily available analytical platform in many laboratories [7,29–31]. Sensitivity was calculated with solutions of vitamin D standards in solvent. In practise, the limit of quantitation in milk was 0.1 nmol/L, providing adequate sensitivity and biological relevance to characterise vitamin D metabolites in milk. The performance characteristics of our method were similar to those reported with SFC-MS/MS, including recovery, precision and sensitivity. However, we observed highly variable extraction efficiency between different milk samples, which was higher for vitamin $D_3$ than 25(OH)$D_3$. This highlights the importance of using isotope labelled internal standards to correct for variable vitamin D metabolite recovery in milk samples.

Our study provides the first data on short-term intra-individual

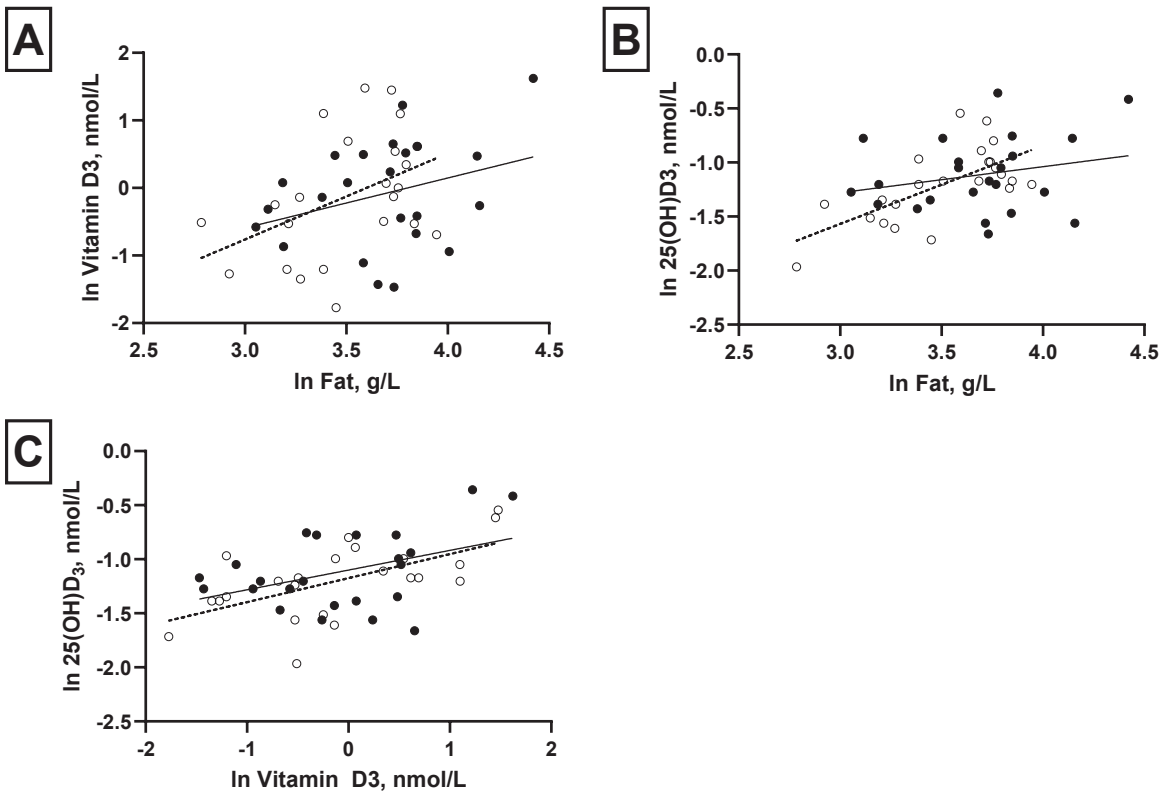


**Fig. 4.** Relationships between vitamin D metabolites and with fat content in milk for Day 1 (solid symbols and line) and Day 2 (open symbols and dashed line). A: vitamin D3 and fat; B: 25-hydroxyvitamin D3 (25(OH)D3) and fat; C: 25-hydrovitamin D3 and vitamin D3. All concentrations are expressed as natural logarithms.

**Table 5**
Regression statistics for relationships between 25(OH)$D_3$, vitamin $D_3$ and fat. $^{fat}$ superscript indicates milk fat concentration is included in the model.

| Time point | *y* - variable | *x* - variable | Slope (SE) | Intercept (SE) | *P* |
|---|---|---|---|---|---|
| Day 1 | 25(OH)$D_3$ | Vitamin $D_3$ | 0.18 (0.09) | −1.10 (0.07) | 0.049 |
| | 25(OH) $D_3^{fat}$ | Vitamin $D_3$ | 0.17 (0.09) | −1.54 (0.81) | 0.09 |
| | 25(OH)$D_3$ | Fat | 0.24 (0.22) | −2.00 (0.80) | 0.28 |
| | Vitamin $D_3$ | Fat | 0.74 (0.48) | −2.81 (1.78) | 0.14 |
| Day 2 | 25(OH)$D_3$ | Vitamin $D_3$ | 0.22 (0.06) | −1.17 (0.06) | 0.002 |
| | 25(OH) $D_3^{fat}$ | Vitamin $D_3$ | 0.14 (0.06) | −3.09 (0.62) | 0.03 |
| | 25(OH)$D_3$ | Fat | 0.72 (0.18) | −3.74 (0.62) | <0.0001 |
| | Vitamin $D_3$ | Fat | 1.27 (0.58) | −4.59 (2.04) | 0.04 |
| Mean | 25(OH)$D_3$ | Vitamin $D_3$ | 0.21 (0.07) | −1.13 (0.06) | 0.008 |
| | 25(OH) $D_3^{fat}$ | Vitamin $D_3$ | 0.17 (0.08) | −2.36 (0.085) | 0.01 |
| | 25(OH)$D_3$ | Fat | 0.53 (0.24) | −3.07 (0.86) | 0.04 |
| | Vitamin $D_3$ | Fat | 1.13 (0.63) | −4.13 (2.28) | 0.08 |

variation in human milk vitamin D concentrations. We saw no significant difference in vitamin D samples collected on two consecutive days even after correcting for fat content, which suggests that within an individual a single milk sample is characteristic of human milk vitamin D concentration, at least over days or weeks. Person was the strongest predictor of vitamin D concentration in milk. These data provide basic information on milk vitamin D variability useful for study design, sample size collections and for assessing analytical performance. For other fat-soluble vitamins in milk, between-person variance has also been reported as the major component of total variance [32]. The evidence for longer-term changes in milk vitamin D compounds is mixed with a recent study observing an increase in vitamin $D_3$ and 25(OH)$D_3$ over 12 months lactation [33], whereas other studies have seen no change over nine months [9,10].

By concentration, vitamin $D_3$ content was greater and more variable than 25(OH)$D_3$ indicating that 25(OH)$D_3$ concentration does not vary to the same extent as vitamin $D_3$. This likely reflects the relative stability of 25(OH)$D_3$ in maternal circulation in comparison to vitamin $D_3$ that can change more markedly over a short time frame due to dietary supplementation and UVB exposure [5].

Milk fat content in this cohort was similar to earlier studies conducted in The Gambia [34,35] and consistent with published mean of 35 – 40 g/L [36]. Milk fat is variable across and between feeds, across a day, across lactation stage and between women and season [34,35,37,38]. In our study, fat concentration was inconsistently associated with vitamin D metabolite concentrations. In combined regression models including maternal ID and day, the impact of fat was not significant but nevertheless did influence metabolite concentrations by around 5 %, and highlights the potential importance of fat concentration as a factor affecting vitamin D concentration in milk. Some, but not all studies [39], have observed similar findings for 25(OH)$D_3$, but not vitamin $D_3$ [9]. Hind milk (with higher fat content) repotedly had a higher concentration of 25(OH)D [10,40], and vitamin D [10] than foremilk. Overall, these data highlight the need to use standardised collection techniques to compensate for factors such stage of feed and time of day and/or the relevance of correcting for milk fat content.

As with vitamin D metabolites in food, vitamin D content of milk can be expressed as biological activity or anti-rachitic activity (ARA). In this calculation, 25(OH)D is assigned a higher biological potency (due to greater bioavailability) than vitamin D in a ratio of 5:1. Therefore, as shown in Fig. 2, and despite its lower concentration, 25(OH)$D_3$ contributed on average around two-thirds of total vitamin D biological

activity. However, as demonstrated in maternal vitamin D supplementation and UVB exposure experiments milk 25(OH)$D_3$ is less amenable to changes in concentration than milk vitamin $D_3$ [41].

In the current study, average milk 25(OH)$D_3$ concentration was similar to the majority of other reports in different populations using LC-MS/MS. Vitamin $D_3$ concentrations were generally higher than 25(OH)$D_3$ but the range was consistent with countries of similar latitude [5,8–11]. Stoutjesdjik *et al.* measured milk vitamin D content in women from 10 different populations across Europe, Asia and Africa and found higher vitamin $D_3$ concentrations in some populations, including Tanzanian groups [11]. The Gambia lies at latitude 13°N [23] with year-round availability of UVB sunshine and hence high potential supply of vitamin D; women in this rural region have higher vitamin D status compared with women in European countries [42,43]. Vitamin $D_3$ as the predominant form of vitamin D in milk in terms of concentration is consistent with observations in Gambian and Tanzanian women that vitamin D status measured by 25(OH)D concentration in serum is lower in lactation than non-lactating women [43,44], potentially due to transfer of vitamin $D_3$ into milk. On average, milk vitamin D content was 47 IU/L; assuming a milk intake of 780 mL [45], this would supply 37 IU/d to the infant, only approximately one-tenth of recommendation for infants. This is consistent with evidence from other studies [10,11,46,47].

The aims of this work were to develop a reliable method for the quantification of vitamin D metabolites in human milk samples and apply this in an exploratory study to assess variation in vitamin D metabolite concentration in a small cohort of Gambian women. It is unknown if variability would be different at different stages of lactation and more rigorous determination of biological variation requires a larger sample size and more regular measurements within an individual [19]. Whilst the analytical method was calibrated against pure standards measured by spectroscopy, no matrix-matched materials or reference methods are available to confirm accuracy. Powdered milk or infant formula are available as reference materials [5], but extraction of vitamin D metabolites will not be representative of liquid human milk. The use of reference materials to assess the accuracy of serum 25(OH)$D_3$ assays is well-established, but these reference materials would not be appropriate to assess milk vitamin D concentrations due to the different matrix. Standard Reference Material (SRM) whole milk powder from the National Institute of Science and Technology (NIST 1549a) can be purchased with values for both 25(OH)$D_3$ and vitamin $D_3$ but this was not available to us due to the requirement to obtain an agricultural import licence from the UK government. Rather, we used a European Reference Material (ERM) whole milk powder to assess accuracy of vitamin $D_3$ only, but this material only carries an "informative" range due to variation in results from the 16 laboratories contracted to measure vitamin D. Although our method measured the two major forms of vitamin D in human milk, we did not assess sulfated forms. The potential of these water-soluble conjugated forms to contribute to vitamin D status has been a matter of debate for decades [5,48]. Whilst questions remain around the biological activity of the sulfated forms, this is an emerging area and recent advances in analytical methods have led to the suggestion that sulfated vitamin D metabolites may indeed contribute total milk vitamin D content at a level similar to the lipid soluble metabolites [48–50]. However, further work and larger sample sizes are required to demonstrate the biological relevance of sulfated forms.

## 5. Conclusions

We have described an LC-MS/MS method for the reliable and reproducible measurement of 25(OH)D and vitamin D in low volumes of human milk samples that may be applied in larger studies. We demonstrate high levels of between-person variability and the contribution of milk fat concentration as a predictor of milk vitamin D content. In this population with unrestricted availability of UVB, vitamin $D_3$ was quantitatively the predominant vitamin D metabolite in milk, but overall 25(OH)$D_3$ was responsible for two-thirds of the biological activity. This study contributes to current understanding of milk vitamin D content and the method provides a platform for future work.

## CRediT authorship contribution statement

**Kerry Jones:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Formal analysis, Conceptualization. **Ann Prentice:** Writing – review & editing, Resources, Formal analysis, Data curation, Conceptualization. **Albert Koulman:** Writing – review & editing, Methodology, Conceptualization. **Georgia Billing:** Writing – review & editing, Resources, Conceptualization. **Sarah Meadows:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization.

## 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

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## Data availability

Data will be made available on request.

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