

Article

Measuring Vitamin D₃ Metabolic Status, Comparison between Vitamin D Deficient and Sufficient Individuals

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Citation: Castillo-Peinado, L.d.I.S.; Calderón-Santiago, M.; Herrera-Martínez, A.D.; León-Idougourram, S.; Gálvez-Moreno, M.A.; Sánchez-Cano, R.L.; Bouillon, R.; Quesada-Gómez, J.M.; Priego-Capote, F. Measuring Vitamin D₃ Metabolic Status, Comparison between Vitamin D Deficient and Sufficient Individuals. *Separations* **2022**, *9*, 141. <https://doi.org/10.3390/separations9060141>

Academic Editors: Slawomir Nefke, Zygryd Witkiewicz and Eduardo Sommella

Received: 3 May 2022

Accepted: 31 May 2022

Published: 3 June 2022

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Abstract: The main branch of vitamin D₃ metabolism involves several hydroxylation reactions to obtain mono-, di- and trihydroxylated metabolites, including the circulating and active forms—25(OH)D₃ and 1,25(OH)₂D₃, respectively. However, most clinical trials strictly target the determination of 25(OH)D₃ to offer a view of the metabolic status of vitamin D₃. Due to the growing interest in expanding this restricted view, we have developed a method for measuring vitamin D₃ metabolism by determination of vitamin D₃, 25(OH)D₃, 24,25(OH)₂D₃, 1,25(OH)₂D₃ and 1,24,25(OH)₃D₃ in human plasma. The method was based on SPE–LC–MS/MS with a large volume injection of human plasma (240 µL). Detection of di- and trihydroxymetabolites, found at the picogram per milliliter level, was attained by the combined action of high preconcentration and clean-up effects. The method allows obtaining information about ratios such as the known vitamin D metabolite ratio (24,25(OH)₂D₃/25(OH)D₃), which can provide complementary views of vitamin D₃ metabolic status. The method was applied to a cohort of obese patients and a reference cohort of healthy volunteers to find metabolic correlations between target analytes as well as differences as a function of vitamin D levels within and between cohorts.

Keywords: vitamin D deficiency; vitamin D₃; 1,25-dihydroxyvitamin D₃; 24,25-dihydroxyvitamin D₃; 1,24,25-trihydroxyvitamin D₃; SPE–LC–MS/MS; plasma

1. Introduction

Vitamin D status is a worldwide issue assessed in detail by several research studies due to its association with human health conditions [1]. This parameter is estimated by the quantitation of 25-hydroxyvitamin D₃ (25(OH)D₃) since it is the most abundant vitamin D circulating form in the human body and is mainly produced by hepatic *CYP2R1* [2]. Nevertheless, the reliability of this indicator for vitamin D status has been questioned as it may not reflect the complex vitamin D metabolism. Several external factors influence serum and plasma 25(OH)D₃ levels, such as the sampling period of the year, geographical

location where individuals live, ethnicity and exposure to sunlight [3]. Moreover, endogenous factors such as obesity, starvation, diabetes and glucocorticoids have a major influence on *CYP2R1* activity [4]. Consequently, several studies have suggested that the strong relation between the primary product of 25(OH)D₃ catabolism, 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃), and 25(OH)D₃ per se represents a more realistic marker for vitamin D status, which is generally named the vitamin D metabolite ratio (VMR) [5–8]. This recommendation arose from the fact that this parameter overcomes the significant differences induced by factors such as those mentioned earlier and allows an improved description of vitamin D status compared to just the measurement of 25(OH)D₃ [6]. Since 24,25(OH)₂D₃ is the conversion product of 25(OH)D₃ upon *CYP24A1* expression, VMR is frequently used for studies of chronic kidney disease, idiopathic infantile hypercalcemia and cancer, among others, which are associated with this particular enzyme activity and vitamin D deficiency [6,7,9,10]. It is worth noting that, apart from implying the inactivation of 25(OH)D₃, some biological functions of 24,25(OH)₂D₃ such as its important contribution to bone fracture healing have been elucidated [11,12].

Analogously, the activation of 25(OH)D₃ through C1-hydroxylation by *CYP27B1* produces the most active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). This metabolite is also further metabolized by *CYP24A1*, generating 1,24,25-trihydroxyvitamin D₃ (1,24,25(OH)₃D₃). *CYP24A1* is strongly regulated by 1,25(OH)₂D₃ and is thus an important regulator of the activity of the vitamin D endocrine system [2,9,13,14]. Although the *CYP24* enzyme catalyzes C24- and C23-hydroxylation reactions, it has been demonstrated that the ratio of C23- and C24-hydroxylation products for 25(OH)D₃ and 1,25(OH)₂D₃ is 1:4, allowing the conclusion that C24-hydroxylation is the predominant catabolic pathway occurring in vitamin D metabolism [15]. In addition, Kaufmann et al., reported a continuous formation of 1,24,25(OH)₃D₃ in absence of the *CYP24A1* enzyme in 24,25(OH)₂D₃-treated mice, suggesting a C1-hydroxylation of 24,25(OH)₂D₃ due to the contribution of *CYP27B1* [14]. In vitro, 1,24,25(OH)₃D₃ is a poor VDR agonist, and in vivo, no biological functions of 1,24,25(OH)₃D₃ have been found other than its participation as an intermediate in the inactivation pathway of 1,25(OH)₂D₃ ending in its primary excretory catabolite, calcitroic acid. Nevertheless, it could be interesting to study this metabolite in greater detail as a potential indicator of overall vitamin D metabolism and its correlation with its precursors, 24,25(OH)₂D₃ and 1,25(OH)₂D₃ [14]. The determination of 1,24,25(OH)₃D₃ would indeed allow providing more details about the catabolism of the most relevant forms of vitamin D, the major circulating and active forms.

Due to the fact that these metabolites are found at low concentration levels (nanograms per milliliter or picograms per milliliter), liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS) is the most important analytical platform employed in their determination by virtue of its high sensitivity and precision [16]. However, it usually comes along with a previous sample preparation step, allowing analyte preconcentration and clean-up of potential interferences. The aim of this study is to propose an analytical method based on online solid-phase extraction (SPE) coupled to LC–MS/MS for evaluation of vitamin D₃ metabolic status by combined measurements of vitamin D₃ (substrate), 25(OH)D₃ (circulating metabolite), 1,25(OH)₂D₃ (active metabolite), 24,25(OH)₂D₃ (metabolic regulator) and 1,24,25(OH)₃D₃ (inactive metabolite). Hence, metabolite ratios may be estimated to enhance our knowledge of physiological and metabolic changes in vitamin D deficient patients as well as healthy control individuals, and this method allows defining levels of vitamin D status more accurately.

2. Materials and Methods

2.1. Chemicals and Reagents

Mass spectrometry grade ammonium formate and formic acid (FA) were acquired from Sigma (Sigma–Aldrich, St. Louis, MO, USA) as ionization and sorbent activation agents; methanol (MeOH) and acetonitrile (ACN) from Scharlab (Barcelona, Spain) and

deionized water (18 mΩ cm) from a Millipore Milli-Q water purification system were employed for the preparation of chromatographic mobile phases and SPE solutions.

Analytical standards of vitamin D₃, 25-hydroxyvitamin D₃ (25(OH)D₃), 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and 24,25-dihydroxyvitamin D₃ (24,25(OH)₂D₃) and their deuterated internal standards (ISs), vitamin D₃-d₃, 25(OH)D₃-d₃, 1,25(OH)₂D₃-d₃ and 24,25(OH)₂D₃-d₆, were purchased from Sigma–Aldrich. 1,24,25-Trihydroxyvitamin D₃ (1,24,25(OH)₃D₃) analytical standard was obtained from Quimigen S.L. (Madrid, Spain) but no IS for this analyte was available. According to endogenous concentration ranges of these metabolites found in plasma samples, a multistandard working solution was prepared, vitamin D₃ at 1.5 µg mL⁻¹, 25(OH)D₃ at 15 µg mL⁻¹, 24,25(OH)₂D₃ at 0.5 µg mL⁻¹, 1,25(OH)₂D₃ at 25 ng mL⁻¹ and 1,24,25(OH)₃D₃ at 50 ng mL⁻¹. Since the presence of matrix effects has been proven in LC–MS/MS analyses of vitamin D₃ and its metabolites in plasma samples [2,17], a working solution of ISs was used, in order to correct variations in results due to this phenomenon, at the following concentrations: vitamin D₃-d₃ and 25(OH)D₃-d₃ at 625 ng mL⁻¹, 24,25(OH)₂D₃-d₆ at 125 ng mL⁻¹ and 1,25(OH)₂D₃-d₃ at 7.5 ng mL⁻¹.

2.2. Instruments and Apparatus

Plasma separation from whole blood was conducted by centrifugation in a Digtor 21 centrifuge (Orto Alresa, Madrid, Spain). Homogenization, centrifugation and filtration of common plasma pools were carried out with a vortex shaker from IKA (Wilmington, NC, USA), a Sorvall Legend Micro 21R microcentrifuge from Thermo Fisher Scientific (Waltham, MA, USA) and 0.2 µm nylon syringe filters (Agilent Technologies, Palo Alto, CA, USA), respectively.

SPE sample processing was conducted using a Symbiosis system (Spark Holland, Emmen, the Netherlands), which incorporates three modules: (i) an autosampler (Reliance) including a sample loop of 0.2 mL, (ii) two high-pressure syringe dispensers (HPDs) for solvent delivery and (iii) an automatic cartridge exchange unit (ACE) in which Hysphere C8 packed cartridges (8 µm, 10 × 2 mm) were employed as a sorbent (SIA Engineers, Barcelona, Spain). A stainless steel tube (1.0 mm i.d.) of 1 mL volume capacity was used to connect Reliance and ACE units allowing appropriate mixing efficiency of the sample and the loading solvent. Symbiosis modules and the LC–MS/MS system were linked by PEEK tubing of 0.25 mm i.d. (VICI, Houston, TX, USA).

Separation of analytes was performed through reversed-phase liquid chromatography (RP-LC) with an Agilent 1200 Series LC system (Palo Alto, CA, USA) equipped with a Poroshell 120 EC-C18 analytical column (2.7 µm particle size, 50.0 × 4.6 mm i.d.) preceded by a C18 guard column (2.7 µm particle size, 5.0 × 4.6 mm i.d.), both maintained at 15 °C during analysis and acquired from Agilent Technologies (Palo Alto, CA, USA).

Mass spectrometric detection was executed in multiple reaction monitoring (MRM) mode using an Agilent (Palo Alto, CA, USA) 6410 triple quadrupole (QqQ) mass spectrometer with electrospray ionization in positive polarity mode (ESI+).

2.3. Plasma Sampling and Storage

Blood samples from 40 patients with obesity and 90 healthy volunteers were extracted into adequate plastic plasma Vacutainer tubes (Becton Dickinson, East Rutherford, NJ, USA) in compliance with the World Medical Association Declaration of Helsinki (2004) guidelines. The general and clinical characteristics of the two cohorts are summarized in Table 1. The Ethics Committee of the Reina Sofia University Hospital (Cordoba, Spain) approved the study, and written informed consent was signed by every individual before inclusion in the study. Blood tubes were centrifugated at 4000× g for 20 min. Then, plasma samples were stored at –80 °C until analysis. A plasma pool prepared from nine volunteer donors was used for the development of the method.

Table 1. Characteristics of the evaluated individuals.

Cohorts		Obese Patients				Control Individuals				Obese Patients vs. Control Individuals
Characteristic	Total (n = 40)	Severe deficiency group (n = 16)	Deficiency group (n = 24)	^o p	Total (n = 90)	Severe deficiency group (n = 20)	Deficiency group (n = 40)	Sufficiency group (n = 30)	^o p	[†] p
Gender (%)	Female	55.0 (22/40)	50.0 (8/16)	58.3 (14/24)	0.52	71.1 (64/90)	55.0 (11/20)	72.5 (29/40)	0.16	0.11
	Male	45.0 (18/40)	50.0 (8/16)	41.7 (10/24)		28.9 (26/90)	45.0 (9/20)	27.5 (11/40)		
Age (years old) *	45.2 ± 10.6	44.0 ± 2.4	45.5 ± 2.0	0.83	26.8 ± 2.5	26.6 ± 2.5	26.9 ± 2.6	26.9 ± 2.2	0.71	<0.05
BMI (kg m ⁻²) *	46.1 ± 5.9	49.5 ± 3.1	45.4 ± 0.9	0.27						
Presence of metabolic or mechanic comorbidities (%)	70.0 (28/40)	87.5 (14/16)	66.7 (16/24)	0.25						

* Quantitative variables are presented as mean ± SD. ^ Metabolic comorbidities, hypertension, type 2 diabetes, dyslipidemia; mechanic comorbidities, sleep-obstructive apnea. ^o p represents the p-value of comparison between patients' distribution with 25(OH)D₃ < 12 ng mL⁻¹, 12–20 ng mL⁻¹ and >20 ng mL⁻¹ levels, within each cohort. [†] p represents the p-value of comparison between patients' distribution from the two studied cohorts.

2.4. Online SPE–LC–MS/MS Determination

The sample preparation step consisted of thawing plasma samples at room temperature, centrifuging (4 °C) at 20,000× g for 10 min and sterilizing the resulting supernatant fraction by filtration. Then, aliquots of 240 µL were pretreated by adding 10 µL of IS working solution to each one followed by SPE after shaking in a vortex shaker for 2 min. Regarding the SPE stage, activation of sorbent was achieved by adding 6 mL of MeOH, and conditioning and equilibration of cartridges was achieved by adding 8 mL of the loading sample solvent mix as recommended by the manufacturer. However, different loading sample and interferent clean-up volumes and solvent mixes were evaluated for appropriate process performance. Finally, elution of retained metabolites was achieved with LC mobile phases for 5 min.

The composition of LC mobile phases was 5 mM of ammonium formate in water (phase A) and 5 mM of ammonium formate in MeOH (phase B). The chromatographic gradient was programmed with a constant flow rate of 0.5 mL min⁻¹, from 85% of phase B, maintained during the initial 2 min, up to 100% of phase B in the next 5 min, conditions that were kept constant for the final 7 min of the chromatography. Furthermore, a post-run of 10 additional min was set to re-establish and equilibrate the initial conditions for the consequent run.

Chromatograph–detector interface parameters were set to 350 °C and 9 L min⁻¹ of drying gas (N₂), a nebulizer pressure of 50 psi and 4500 V of capillary voltage. All metabolite detection parameters were studied by direct infusion of individual standard solutions at a concentration of 1 µg mL⁻¹.

2.5. Method Validation

The assessment of the analytical features of the proposed method was performed according to the Center for Drug Evaluation and Research (CDER) guidelines [18]. Thus, linearity, sensitivity, accuracy, precision and extraction efficiency were characterized. The applicability of the proposed method was also determined for further use in clinical studies.

2.6. Data Treatment

Acquisition and processing of data were performed with MassHunter software (V-B.05, Agilent Technologies, Palo Alto, CA, USA). For data treatment and statistical analysis (e.g., calibration curves and box and whisker plots), Microsoft Excel V 16.30 (Microsoft, Redmond, WA, USA), R V 3.0 (R Core Team, Vienna, Austria) with RStudio V 1.3.959 (RStudio Team, Boston, MA, USA) and Metaboanalyst 5.0 online software were employed (<https://www.metaboanalyst.ca/docs/About.xhtml> (accessed on 1 March 2022)).

3. Results

3.1. Optimization of MS/MS Detection of Target Metabolites

Since ionization efficiency and fragmentation of metabolites mainly depend on their chemical structures, an evaluation of the optimum parameters for target metabolite detection was accomplished by direct infusion of standard solutions. A study of the most sensitive precursor ions, with the analysis conditions described previously (Section 2.4), was performed in scan mode. As a result, Table S1 shows the most sensitive adducts for analytes and ISs. Thus, $[M + H - H_2O]^+$ was the preferred precursor ion for hydroxylated and dihydroxylated metabolites while $[M + H]^+$ was the most intense ion for vitamin D₃. For the particular case of 1,24,25(OH)₃D₃, 397.3 *m/z* was the precursor ion providing the highest analytical signal, which corresponds to its $[M + H - 2H_2O]^+$ adduct. Fragmentation patterns were assessed to elucidate the most abundant fragments for the selection of transitions. In the case of the trihydroxylated metabolite, preferred product ions were detected at 121.1, 107.1 and 71.1 *m/z* when applying collision energies of 20, 30 and 40 eV, respectively. Chromatograms obtained by analysis of standard solutions in MRM mode proved that the quantitation transitions providing the highest signal intensity were those shown in Table S1. Optimum separation was attained in 12 min and 1,24,25(OH)₃D₃ eluted at 2.8 min.

3.2. Evaluation of Adequate SPE Conditions

SPE sample preparation was studied by testing two critical steps, namely sample loading and interferent clean-up. The evaluated parameters were the volume and composition of the solutions used in both steps. Particularly, the solution composition was varied from 10 to 25% ACN in water (*v/v*), acidified with 0.7% of FA, for sample loading, and from 0 to 30% ACN in water (*v/v*) for interferent clean-up. The concentration of ACN was slightly higher in the washing step since proteins were partially eluted in the loading step and, therefore, the risk of precipitation in the cartridge and connection tubes is reduced. On the other hand, volumes in both steps were tested from 0 to 3.5 mL. Considering that 1,24,25(OH)₃D₃ and 1,25(OH)₂D₃ are the target metabolites normally found at the lowest concentration levels (picogram per milliliter) in plasma samples, we based SPE evaluation on results obtained for these two analytes. As Figure 1 shows, globally optimum analytical responses were found using 3 mL 25% ACN in water acidified with 0.7% of FA for sample loading and 2 mL 30% ACN in water for interferent clean-up.

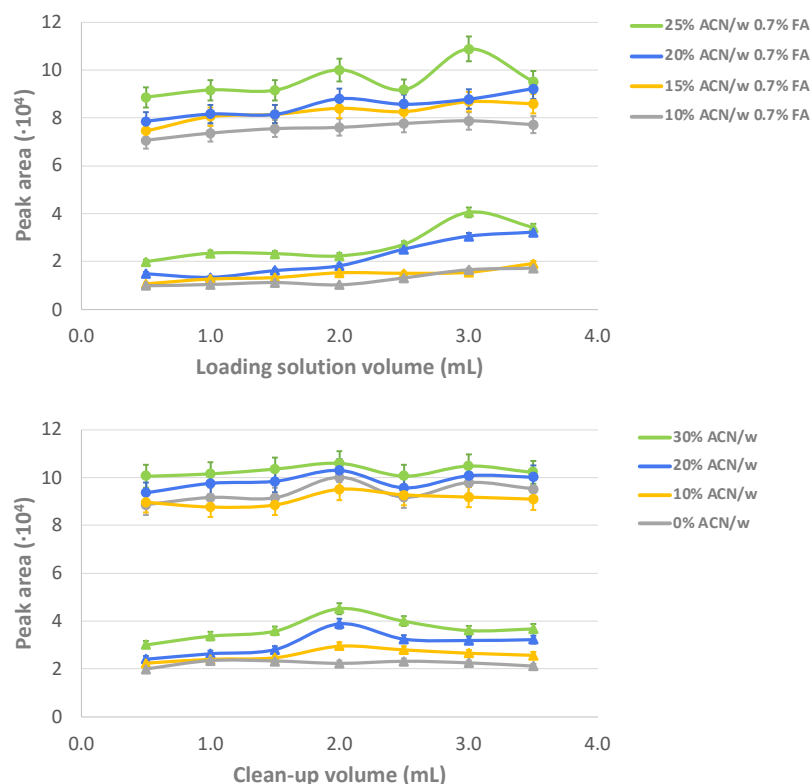


Figure 1. Effect of the composition and volume of the solutions used for sample loading and clean-up in SPE. The evaluation is based on the response of the two metabolites found at lowest concentration in plasma, (\blacktriangle) 1,24,25(OH)₃D₃ and (\bullet) 1,25(OH)₂D₃.

3.3. Analytical Features of the Proposed Method

3.3.1. Best-Matched IS (B-MIS) Evaluation, Linearity, and Sensitivity

Since isotope-labeled ISs were not available for 1,24,25(OH)₃D₃, the two ISs having the most similar structures, 1,25(OH)₂D₃-d₃ and 24,25(OH)₂D₃-d₆, were compared to select the most convenient for quantitation of the trihydroxylated metabolite. The fit of calibration models was considered to select the B-MIS providing the most suited correction of variability sources due to matrix effects and possible interferences [19]. Calibration curves were constructed by analysis of plasma pool aliquots spiked at variable concentrations with target analytes. Table S2 shows a satisfactory linearity since all coefficients of determination (R²) were above 0.99. In the case of 1,24,25(OH)₃D₃, Figure 2 shows the calibration models considering both IS candidates. Thus, linearity was better with the application of 1,25(OH)₂D₃-d₃ (R² = 0.9964), as compared to 24,25(OH)₂D₃-d₆ (R² = 0.9537). Furthermore, mean variability (expressed as %RSD) considering all calibration levels was 9.0 and 16.2% when utilizing 1,25(OH)₂D₃-d₃ and 24,25(OH)₂D₃-d₆, respectively. With these premises, 1,25(OH)₂D₃-d₃ was considered the B-MIS for quantitation of 1,24,25(OH)₃D₃ in plasma samples. This result can be explained since 1,25(OH)₂D₃ is the main structural precursor for the production of 1,24,25(OH)₃D₃ [15].

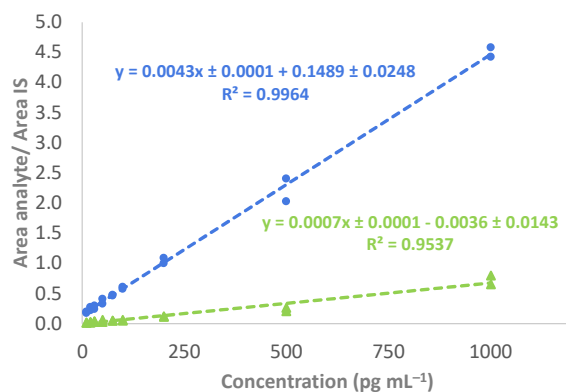


Figure 2. Calibration curves and linearity for determination of 1,24,25(OH)₃D₃ employing 1,25(OH)₂D₃-d₃ (●) and 24,25(OH)₂D₃-d₆ (▲) as ISs.

Limits of detection (LODs) and quantitation (LOQs) were evaluated as parameters representing the sensitivity of the method. These were estimated for each analyte by spreading calibration ranges down to low concentration levels. Prediction of LODs and LOQs was achieved by 3- and 10-times signal above background noise, respectively. However, confirmation of LOD and LOQ estimation was made by analyzing plasma aliquots spiked at the lowest concentration levels of each calibration range. LODs and LOQs reached for vitamin D₃ metabolites were below 3 and 10 pg mL⁻¹ (Supplementary Materials Table S2), respectively, which are comparable to previous studies [16,20,21]. Particularly, it is worth noting that scant information is available in the literature about the trihydroxylated metabolite of vitamin D₃. Kaufmann et al., determined 1,24,25(OH)₃D₃ in mouse serum and found an LOD of 4 pg mL⁻¹ [14], which is above the LOD presented in our research.

3.3.2. Extraction Efficiency

Extraction efficiency (%EE) was assessed by a partial modification of the SPE configuration by on-line insertion of a second cartridge. Thereby, the loaded sample flowed through both cartridges and the fraction independently eluted from each cartridge was sequentially analyzed by LC–MS/MS to estimate the retention/elution capability of the sorbent. The following equation was used:

$$\%EE = (\text{concentration retained in cartridge 1}) / ((\text{concentration retained in cartridge 1} + \text{concentration retained in cartridge 2})) \times 100$$

This equation was calculated by measuring the concentration of four quality control samples (QCs) that were analyzed in triplicate. The set of QC_b consisted of non-spiked aliquots of the pool, whereas QC_l, QC_m and QC_h were pool aliquots spiked with the target analytes at low, medium and high concentration levels. Particularly, QC_l were pool aliquots spiked at 0.3 ng mL⁻¹ for vitamin D₃, 3 ng mL⁻¹ for 25(OH)D₃, 0.1 ng mL⁻¹ for 24,25(OH)₂D₃, 5 pg mL⁻¹ for 1,25(OH)₂D₃ and 10 pg mL⁻¹ for 1,24,25(OH)₃D₃. QC_m and QC_h were spiked at concentrations 10 and 60 times higher than those used for QC_l, respectively. Results of extraction efficiency, listed in Table 2, revealed a quantitative extraction of all metabolites in the first cartridge from 90.3 to 100.0% with the same performance at different concentration levels. On the other hand, residual retention was found in the second cartridge.

Table 2. Extraction efficiency, accuracy and precision assessment of the method proposed for quantitation of vitamin D3 and metabolites.

Analyte	Concentration Level	Extraction Efficiency	Accuracy (%)	Within-Day Variability	Between-Days Variability
		(%)		(RSD, %)	(RSD, %)
1,24,25(OH) ₂ D ₃	QCb	99.7 ± 0.5	100.0 ± 14.0	6.9	7.2
	QCl	92.9 ± 2.0	103.3 ± 9.0	7.2	7.7
	QCm	97.4 ± 2.6	98.3 ± 7.0	6.3	6.6
	QCh	98.9 ± 1.0	99.1 ± 4.0	5.1	5.8
1,25(OH) ₂ D ₃	QCb	95.8 ± 7.4	102.6 ± 10.0	9.0	11.7
	QCl	90.3 ± 1.3	111.4 ± 8.0	7.8	8.1
	QCm	94.6 ± 1.9	99.7 ± 8.0	6.4	6.7
	QCh	98.2 ± 0.2	99.0 ± 5.0	4.7	6.2
24,25(OH) ₂ D ₃	QCb	98.7 ± 2.3	90.4 ± 5.0	5.1	8.1
	QCl	97.8 ± 2.0	90.0 ± 4.0	10.7	10.8
	QCm	95.0 ± 4.4	92.4 ± 3.0	6.8	7.3
	QCh	100.0 ± 0.0	95.2 ± 6.0	5.0	5.5
25(OH)D ₃	QCb	96.0 ± 0.8	96.6 ± 3.0	4.7	5.3
	QCl	98.9 ± 1.4	105.8 ± 8.0	6.9	8.1
	QCm	99.2 ± 1.4	97.0 ± 7.0	6.1	6.2
	QCh	99.0 ± 1.7	100.2 ± 6.0	4.6	5.1
Vitamin D ₃	QCb	99.8 ± 0.1	102.6 ± 9.0	5.1	7.6
	QCl	97.6 ± 3.7	99.7 ± 7.0	9.3	9.8
	QCm	98.4 ± 2.2	100.6 ± 8.0	7.3	7.7
	QCh	99.6 ± 0.6	103.1 ± 5.0	3.7	5.2

3.3.3. Accuracy and Precision Assessment

The accuracy was evaluated with the set of QCs. Each QC was analyzed in triplicate and results were expressed as percentages. The acceptance cut-off was set at 15% RSD of nominal concentration values for each QC, although this cut-off can be increased up to 20% RSD at levels close to LOQs (QCl) [18]. Table 2 shows accuracy mean results per analyte for each concentration level. Mean results were in a range between 90.0 and 111.4%, which perfectly fits the adopted criteria [18].

Method precision was also defined with QCs at the four concentration levels, which were analyzed for five consecutive days. Each day, samples were analyzed in triplicate. Within-day variability and between-days variability were estimated by calculating % RSD mean values of results obtained on each day ($n = 3$) and after the full experiment ($n = 5$), respectively. Within-day variability ranged from 3.7 to 10.7%, while this range was slightly increased for between-days variability, from 5.1 to 11.7%. Considering the guidelines for methods validation used as a reference [18], precision estimated with the applied experimental plan was acceptable.

3.4. Application of the Proposed Method

Analysis of the selected cohorts was performed with the proposed method to show its applicability. Previous studies have defined vitamin D metabolic levels according to 25(OH)D₃ concentration. Thus, severe vitamin D deficiency is pointed out for 25(OH)D₃ concentrations below 12 ng mL⁻¹, deficiency is related to levels of this circulating metabolite between 12 and 20 ng mL⁻¹ and sufficiency is considered above 20 ng mL⁻¹ [4,22,23]. Table 3 shows concentration levels in the two cohorts and the distribution of cases according to this categorization.

Table 3. Concentration of target metabolites detected in the studied cohorts.

Cohort	Obese Patients				Control Individuals						Obese Patients vs. Control Individuals
	Total Group Levels	Severe Deficiency Group Levels ¹	Deficiency Group Levels ²	Mann–Whitney U Test (<i>p</i> -Value) [*]	Total Group Levels	Severe Deficiency Group Levels ¹	Deficiency Group Levels ²	Sufficiency Group Levels ³	Kruskal–Wallis (<i>p</i> -Value) [^]	Significant Pairwise Comparisons (Wilcoxon Rank)	Mann–Whitney U Test (<i>p</i> -Value) [*]
1,24,25(OH) ₃ D ₃ (pg mL ⁻¹)	56.5 ± 113.9	23.9 ± 50.8	78.2 ± 138.2	>0.05	564.8 ± 236.0	581.5 ± 254.5	587.9 ± 229.5	522.8 ± 234.2	>0.05	Not applicable	<0.0001
24,25(OH) ₂ D ₃ (ng mL ⁻¹)	1.5 ± 1.0	1.0 ± 0.7	1.9 ± 1.0	0.002	1.4 ± 2.3	0.2 ± 0.6	0.5 ± 0.9	3.2 ± 3.1	<0.0001	Severe Deficiency vs. Sufficiency <0.0001	0.0005
1,25(OH) ₂ D ₃ (pg mL ⁻¹)	38.1 ± 39.2	37.0 ± 30.4	38.8 ± 44.7	>0.05	93.0 ± 78.8	96.2 ± 76.6	88.2 ± 89.2	97.2 ± 66.7	> 0.05	Not applicable	<0.0001
25(OH)D ₃ (ng mL ⁻¹)	13.0 ± 4.8	8.2 ± 2.5	16.1 ± 3.1	3.2·10 ⁻¹¹	19.7 ± 11.5	9.2 ± 2.1	15.3 ± 2.3	32.4 ± 11.4	<0.0001	Severe Deficiency vs. Sufficiency <0.0001	0.001
Vitamin D ₃ (ng mL ⁻¹)	2.9 ± 1.9	2.5 ± 1.3	3.1 ± 2.1	>0.05	2.7 ± 1.3	1.8 ± 1.0	2.8 ± 1.0	3.2 ± 1.5	0.0005	Severe Deficiency vs. Sufficiency 0.002	>0.05
										Severe Deficiency vs. Deficiency 0.001	

^{*} Mann–Whitney U test was used to compare levels between obese patients and control individuals, as well as between the two groups of obese individuals according to vitamin D status. [^] Kruskal–Wallis test was used to compare levels among the three groups of control individuals according to vitamin D status. ¹ Participants with 25(OH)₂D₃ serum levels below 12 ng mL⁻¹. ² Participants with 25(OH)₂D₃ serum levels between 12 and 20 ng mL⁻¹. ³ Participants with 25(OH)₂D₃ serum levels above 20 ng mL⁻¹.

In the cohort of obese patients, 40% of individuals presented severe vitamin D deficiency. This percentage was lower for the control cohort since only 22.2% of individuals reported severe vitamin D deficiency. Complementarily, 44.4% of control individuals were classified as vitamin D deficient individuals, and a third of the total cohort provided sufficient vitamin D levels. Comparison between the two cohorts was carried out with a non-parametric Mann–Whitney U test for independent samples (95% confidence level). Consequently, significant differences (p -value < 0.01) were revealed in the levels of all hydroxylated metabolites, whereas no differences were found for vitamin D₃ (Figure 3). As expected, 25(OH)D₃ levels were higher in the control cohort as compared to the obese cohort. The cohort of obese patients was characterized by a predominance of metabolic or mechanic comorbidities since 70% of obese individuals were diagnosed with hypertension, type 2 diabetes, dyslipidemia and sleep-obstructive apnea. 1,25(OH)₂D₃ concentration was also significantly higher in the control group, as was the concentration of 1,24,25(OH)₃D₃. On the other hand, the other dihydroxylated metabolite, 24,25(OH)₂D₃, was found at a higher concentration in obese patients as compared to control individuals. This result was also evident for ratios between the concentrations of the dihydroxylated metabolites and the concentration of 25(OH)D₃. The activation of 25(OH)D₃, through the 1,25(OH)₂D₃/25(OH)D₃ ratio, is clearly favored in control individuals, while the conversion to 24,25(OH)₂D₃ is enhanced in obese individuals. This fact could support an increased vitamin D catabolism in obese patients, as has been proposed by other authors [24].

Concerning the groups defined by vitamin D levels in the two cohorts, no significant differences were found in the groups as a function of age, sex or body mass index (BMI) (Table 1). No significant differences for comorbidities in the obese cohort were detected in the severe deficiency versus deficiency groups. According to the categorization, significant differences found in 25(OH)D₃ levels were evident. As expected, significance for 24,25(OH)₂D₃ supports its modulation role in vitamin D₃ metabolism and a formation strictly dependent on 25(OH)D₃ levels (Figure 4). In both cohorts, levels were lower for the severe vitamin D deficiency group. Furthermore, this was supported by two statistically significant correlations (p -value = 0.0004, $\rho > 0.5$ and p -value < 0.0001 , $\rho = 0.82$; Table S3 and Figure S1) in the two cohorts corresponding to 25(OH)D₃ versus 24,25(OH)₂D₃ and 24,25(OH)₂D₃ versus 24,25(OH)₂D₃/25(OH)D₃ ratio, respectively. From a clinical perspective, interpretation of these values suggests a moderate association between 24,25(OH)₂D₃ and the major circulating form of vitamin D₃ [25], which confirms the strength of VMR as a parameter to describe vitamin D status [5,6]. Contrarily, no significant differences were obtained when comparing 1,24,25(OH)₃D₃ and 1,25(OH)₂D₃ concentration levels in the two cohorts according to vitamin D metabolic status. The concentration of 1,25(OH)₂D₃ seems not to be influenced by 25(OH)D₃ concentration when compared among the groups defined by vitamin D metabolic status. However, we found a strong significant correlation of 1,25(OH)₂D₃ levels with the 1,25(OH)₂D₃/25(OH)D₃ ratio (p -value < 0.05 , $\rho = 0.89$) that represents the *CYP27B1* activity regulating the formation of the active form. The 1,25(OH)₂D₃/25(OH)D₃ ratio was also significantly different in the two cohorts when comparing groups organized by vitamin D levels, being higher for individuals with severe vitamin D deficiency.

Furthermore, no significant differences in the levels of the trihydroxylated metabolite between groups suggest that 1,24,25(OH)₃D₃ is not associated with 25(OH)D₃ concentration levels. Nevertheless, a negative correlation between 1,25(OH)₂D₃ and the 1,24,25(OH)₃D₃/1,25(OH)₂D₃ ratio (p -value < 0.0001 , $\rho = -0.63$) determines its inactivation due to the contribution of *CYP24A1*. The absence of significance can be explained by the involvement of other parameters such as calcium and phosphate levels as well as parathyroid hormone (PTH) and fibroblast growth factor 23 (FGF23) activity [4,26].

For the control cohort, significant differences in the concentration of vitamin D₃ among the three groups were also found for the vitamin D level (Figure 4), and the highest content was found in vitamin D sufficient individuals. This does not occur in the obese

cohorts, where vitamin D₃ levels do not contribute to explaining vitamin D metabolic status.

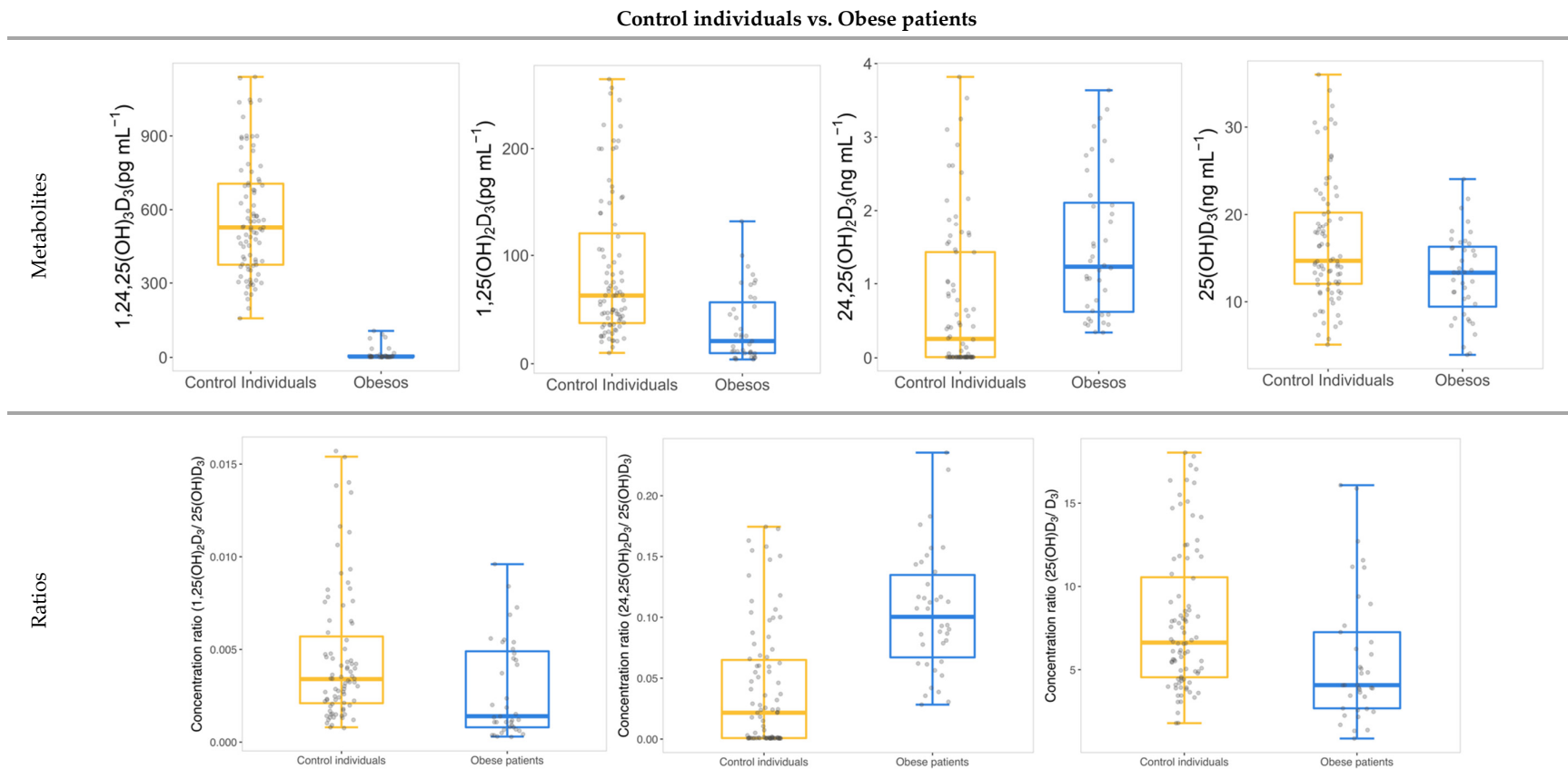


Figure 3. Box and whisker plots showing significant differences in concentration levels and ratios between obese patients and control individuals.

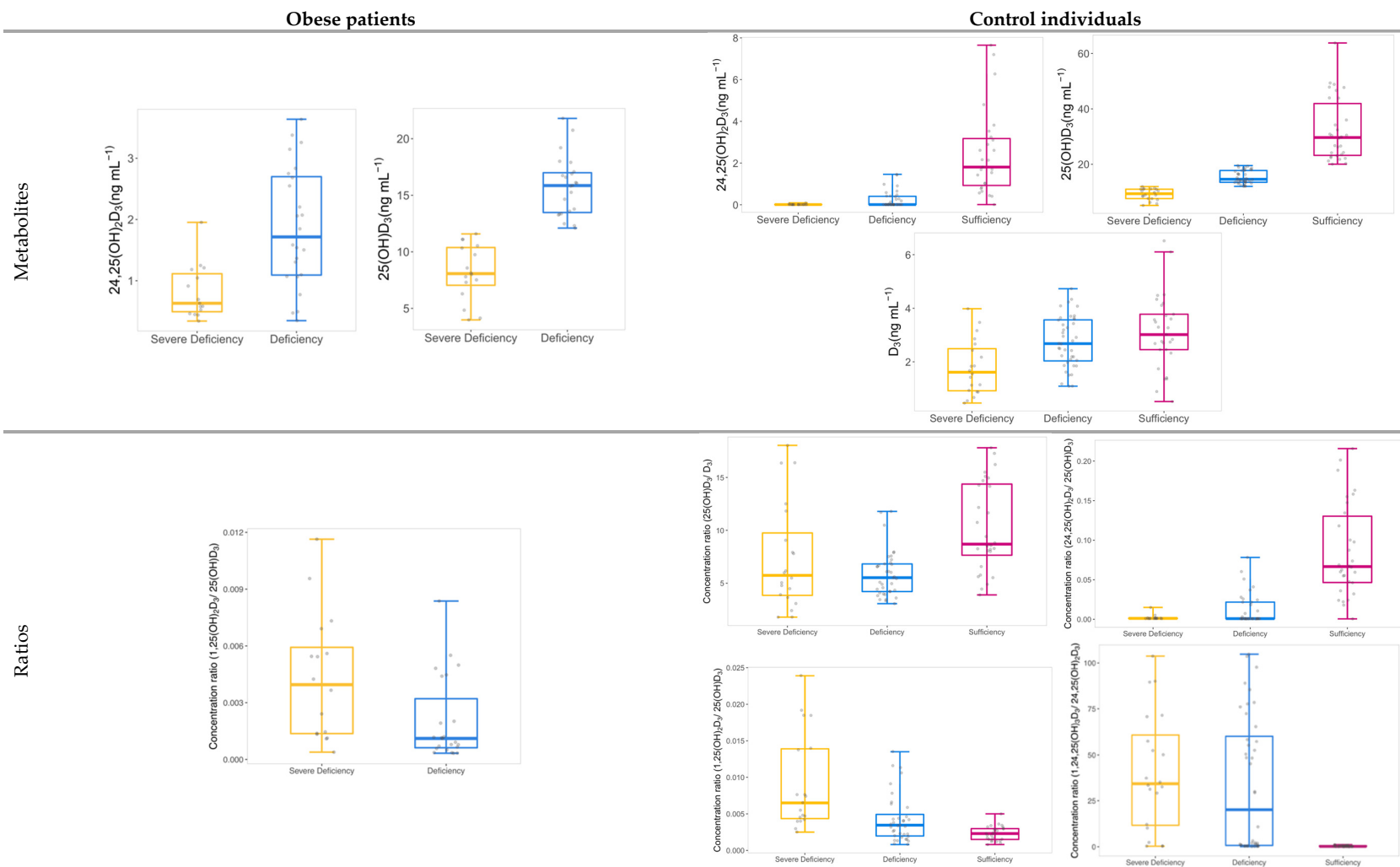


Figure 4. Box and whisker plots showing significant differences in concentration levels and ratios between vitamin D groups within both studied cohorts.

In the control cohort, metabolic differences were also detected in the three groups when comparing the values for ratios $25(\text{OH})\text{D}_3/\text{D}_3$, $1,24,25(\text{OH})_3\text{D}_3/24,25(\text{OH})_2\text{D}_3$ and $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$. The $24,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ ratio reflects the formation of $24,25(\text{OH})_2\text{D}_3$, which seems to be a direct indicator of the vitamin D level. On the other hand, the $1,24,25(\text{OH})_3\text{D}_3/24,25(\text{OH})_2\text{D}_3$ ratio was higher for severe vitamin D deficiency individuals as compared to deficiency and sufficiency controls. Therefore, there seem to be clear differences in the second hydroxylation step for activation of the circulating form, $25(\text{OH})\text{D}_3$, as a function of vitamin D status. Conversion to $1,25(\text{OH})_2\text{D}_3$ or $24,25(\text{OH})_2\text{D}_3$ seems to be influenced by vitamin D status. Additionally, the correlation between $1,25(\text{OH})_2\text{D}_3/25(\text{OH})\text{D}_3$ and $1,24,25(\text{OH})_3\text{D}_3/1,25(\text{OH})_2\text{D}_3$ (p -value < 0.0001 , $\rho = -0.70$) was also negative, which represents that an increase or decrease in the formation of the active form would be associated with the contrary effect on its conversion to $1,24,25(\text{OH})_3\text{D}_3$.

4. Conclusions

Determination of vitamin D metabolic status is a current worldwide challenge since many controversies exist regarding its clinical assessment. Addressing vitamin D status as a function of $25(\text{OH})\text{D}_3$ concentration levels in plasma is being questioned because several factors have been proven to influence this physiological condition. Therefore, a method was proposed in this study to gather more information about vitamin D metabolism by online SPE–LC–MS/MS quantitation of vitamin D_3 , 25OHD_3 , $24,25(\text{OH})_2\text{D}_3$, $1,25(\text{OH})_2\text{D}_3$ and $1,24,25(\text{OH})_3\text{D}_3$ in human plasma. Results demonstrated that the proposed analytical method was successfully developed and validated according to elected guidelines as reference. Application of the method to the selected cohorts resulted in significant differences in $25(\text{OH})\text{D}_3$, $24,25(\text{OH})_2\text{D}_3$ and vitamin D_3 concentration levels, according to a categorization of individuals based on severe vitamin D deficient, deficient, and sufficient groups. Furthermore, significant differences were found for all vitamin D hydroxylated metabolites when comparing both cohorts, which could help to elucidate vitamin D metabolism in obese patients. Additionally, this study revealed intense correlations among vitamin D_3 metabolites and metabolic ratios, which may lead to a better understanding of vitamin D status.

Supplementary Materials: The following supporting information can be downloaded at, <https://www.mdpi.com/article/10.3390/separations9060141/s1>, Figure S1. Plots of significant correlations between metabolites and ratios involved in vitamin D_3 metabolism; Table S1. MRM parameters for detection of target metabolites and ISs; Table S2. Calibration models for determination of target metabolites in plasma; Table S3. Spearman correlation results between metabolites and ratios involved in vitamin D_3 metabolism.

Author Contributions: Conceptualization, L.d.I.S.C.-P., R.B., J.M.Q.-G. and F.P.-C.; methodology, L.d.I.S.C.-P., M.C.-S. and F.P.-C.; validation, L.d.I.S.C.-P., M.C.-S., J.M.Q.-G. and F.P.-C.; formal analysis, L.d.I.S.C.-P., M.C.-S., J.M.Q.-G. and F.P.-C.; investigation, L.d.I.S.C.-P., M.C.-S. and F.P.-C.; resources, A.D.H.-M., S.L.-I., M.Á.G.-M. and R.L.S.-C.; writing—original draft preparation, L.d.I.S.C.-P., M.C.-S., R.B., J.M.Q.-G. and F.P.-C.; writing—review and editing, L.d.I.S.C.-P., M.C.-S., A.D.H.-M., S.L.-I., M.Á.G.-M., R.L.S.-C., R.B., J.M.Q.-G. and F.P.-C.; funding acquisition, F.P.-C. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded jointly by the Spanish Ministerio de Ciencia e Innovación (PID2019-111373RB-I00 project), Junta de Andalucía (PI-0038/2019, bio-0139 project) and European Regional Development Fund/European Social Fund (“Investing in your future”). Consortium for Biomedical Research in Frailty and Healthy Ageing (CIBERFES) is an initiative of Carlos III Institute of Health.

Institutional Review Board Statement: The study was conducted in accordance with Declaration of Helsinki and, national and international guidelines. It was also approved by Ethics Committee of Reina Sofia University Hospital from Cordoba, Spain.

Informed Consent Statement: Informed consent was obtained from all subjects involved in this study.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

ACE, automatic cartridge exchange; BMI, body mass index; EE, extraction efficiency; ESI, electrospray ionization; FGF23, fibroblast growth factor 23; HPD, high-pressure syringe dispenser; IS, internal standard; LC–MS/MS, liquid chromatography coupled to tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; MRM, multiple reaction monitoring; PTH, parathyroid hormone; QC, quality control; QqQ, triple quadrupole mass spectrometer; ρ , Spearman's correlation coefficient rho; R^2 , coefficient of determination; RSD, relative standard deviation; SPE, solid-phase extraction; VMR, vitamin D metabolite ratio.

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