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REVIEW ARTICLE

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Analysis of vitamin D metabolic markers by mass spectrometry: Recent progress regarding the "gold standard" method and integration into clinical practice

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Abstract

Liquid chromatography/tandem mass spectrometry is firmly established today as the gold standard technique for analysis of vitamin D, both for vitamin D status assessments as well as for measuring complex and intricate vitamin D metabolic fingerprints. While the actual mass spectrometry technology has seen only incremental performance increases in recent years, there have been major, very impactful changes in the front- and back-end of MS-based vitamin D assays; for example, the extension to new types of biological sample matrices analyzed for an increasing number of different vitamin D metabolites, novel sample preparation techniques, new powerful chemical derivatization reagents, as well the continued integration of high resolution mass spectrometers into clinical laboratories, replacing established triple-quadrupole instruments. At the same time, the sustainability of mass spectrometry operation in the vitamin D field is now firmly established through proven analytical harmonization and standardization programs. The present review summarizes the most important of these recent developments.

KEYWORDS

25-hydroxyvitamin D_3, chemical derivatization, epimers, free vitamin D, HRMS, LC-MS/MS, sample preparation, vitamin D metabolites, vitamin D standardization

1 | INTRODUCTION

Beginning in the early evolution, organisms such as phytoplankton and zooplankton produced vitamin D during sunlight exposure, a possible function to protect them from damage from solar ultraviolet radiation (Holick, 2003). The sun remains an important source of vitamin D until today. Vitamin D plays a fundamental role in calcium homeostasis (Gallieni et al., 2009; Gil et al., 2018; Khammissa et al., 2018) and is essential for development and maintenance of the mineralized skeleton. Beyond its established influence on bone health, vitamin D deficiency has been associated with other conditions such as autoimmune diseases (Murdaca et al., 2019), cancers and cardiovascular diseases as well as chronic pain (Baute et al., 2019; Martin & Reid, 2017). Furthermore, vitamin D has come to the limelight again during the COVID-19 pandemic, given its reported effects on viral infection, particularly those affecting the respiratory tract (Ali, 2020; Bergman, 2020; Grant et al., 2020; Ilie et al., 2020).

There are many guidelines and suggestions for cut-offs representing vitamin D deficiency as well as discrepancies regarding optimal values of serum 25-hydroxyvitamin D (25(OH)D) concentrations, which are summarized in Table 1 (Holick et al., 2011; Patseadou & Haller, 2020; Pludowski et al., 2017; Scientific Advisory Committee on Nutrition, 2016; The National Academies Press, 2011). Measurement of vitamin D status is important to monitor levels of vitamin D in individuals and populations as a large proportion of people develop vitamin D deficiency (Cashman et al., 2016). Vitamin D status assessment measures the total 25(OH)D concentrations circulating in blood, that is, the sum of 25(OH)D_3 and 25(OH)D_2 concentrations. Accurate assessment of vitamin D status is vital and initiatives such as the Vitamin D Standardization Program (VDSP) standardize measurements of total 25(OH)D among different laboratories (Durazo-Arvizu et al., 2017). Importantly, many additional vitamin D metabolites can be quantitatively measured by mass spectrometry-based methods (Volmer & Stokes, 2016). These profiling and fingerprinting methods are still mostly used as research tools and have not been widely adopted by the clinical community. In the future, however, additional metabolites will likely be included in the routine assessment of vitamin D status using advanced status markers (Hollis & Wagner, 2013) or as functional markers in studies of diseases related to vitamin D deficiency. A number of reviews have highlighted the mass spectrometric measurement of multiple vitamin D metabolites (Altieri et al., 2020; van den Ouweland et al., 2013; van den Ouweland, 2016; Müller & Volmer, 2015; Volmer & Stokes, 2016; Volmer et al., 2015). Furthermore, the determination of non-protein-bound, free 25(OH)D_3 levels has been of great interest in recent years (Tsuprykov et al., 2018), as a more meaningful marker for vitamin D status and other phenomena such as the "vitamin D paradox in Black Americans" (Brown et al., 2018).

We have previously reported on the status of mass spectrometry as the gold standard method for vitamin D measurement in this journal (Volmer et al., 2015). Since then, the field has expanded significantly, particularly in terms of the application range of mass spectrometry to vitamin D analysis, including novel sample matrices, new chemical derivatization techniques, improved method standardization efforts and wider application of high resolution mass spectrometry. Thus, this present review complements the previous review and adds entirely new topics that were either in an infant state at the time of the previous report or simply technically not possible only a few years ago. The paper will mainly focus on recent studies of human samples and their implementation in nutritional, clinical, and epidemiological studies, with occasional references to animal studies.

There is an extensive body of work on the analysis of vitamin D in plants and food matrices, which is outside the scope of this article. The present authors refer interested readers to excellent review articles on this subject (Dimartino, 2007; Gomes et al., 2013 Jäpelt & Jakobsen, 2013; Kasalová et al., 2015; Socas-Rodríguez et al., 2020).

2 | THE VITAMIN D METABOLOME

Ergosterol and 7-dehydrocholesterol are pro-vitamins of ergocalciferol (=vitamin D_2) and cholecalciferol (=vitamin D_3). Humans can synthesize vitamin D_3 from 7-dehydrocholesterol when they are exposed to UVB radiation (290–315 nm). First, provitamin D_3 is formed, which rapidly undergoes rearrangement of its double bonds and forms vitamin D_3, which is the thermodynamically stable product (Holick, 2004). On the contrary, humans cannot synthesize vitamin D_2, which is synthesized by mushrooms and plants after UVB action on ergosterol (Horst et al., 2005). Both vitamins D_2 and

TABLE 1 Summary of cut-offs that represent various serum 25(OH)D values for vitamin D status as proposed by different societies and agencies

Vitamin D cut-off	NAM/NIH (ng/ml; The National Academies Press, 2011)	ES (Holick et al., 2011)	ESE (ng/ml; Pludowski et al., 2018)	SACN (ng/ml; Scientific Advisory Committee on Nutrition, 2016)
Deficiency	<12	<20 ng/ml	<20	<10
Insufficiency	<20	<30 g/ml	<30	
Sufficiency	≥20	30–100 ng/ml	30–50	≥10
Toxicity	>100			≥120

Abbreviations: ES, Endocrine Society, USA, UK; ESE, European Society of Endocrinology; NAM, National Academy of Medicine (former Institute of Medicine, IOM), USA; NIH, National Institute of Health, USA; SACN, Scientific Advisory Committee on Nutrition, UK.

D_3 can be obtained from the diet (e.g., fatty fish such as salmon and sardines, eggs and mushrooms), fortified foods (such as vitamin D-fortified breakfast cereals) and supplements (Dunlop et al., 2021; Ložnjak Švarc et al., 2021). UV radiation is essential not only for the synthesis of vitamin D_3 but also for its inactivation (Herrmann et al., 2017). Otherwise, extended UV exposure would lead to vitamin D_3 intoxication. The UV degradation process provides a mechanism of protection to the organism. Provitamin D_3 and vitamin D_3 are converted into biologically inactive species by further UV radiation, if they have not reached the circulation. Important photodegradation products are lumisterol and tachysterol.

Holick summarized several factors that alter the cutaneous production of vitamin D_3 (Holick, 2004). For example, the age of a person influences the amount of 7-dehydroxycholisterol in the epidermis. If a 70 and a 20 year old person are exposed to the same amount of sunlight, the old person will produce only 25% of the amount of vitamin D_3 as compared to the young person. The use of sunscreens reduces the capacity of the skin to produce vitamin D_3. Melanin is a natural sunscreen, which absorbs UVB radiation; that is, people with increased melanin pigmentation need longer exposure to sunlight. Also, the season, the time of the day and the latitude have drastic effects on vitamin D_3 cutaneous synthesis. Finally, as vitamin D is a lipophilic molecule, it is stored in body fat and therefore obese individuals face an increased risk of vitamin D deficiency, since their adipose tissue is potentially an irreversible sink for vitamin D.

Vitamin D_3 can be considered a pro-hormone and its active metabolite $1,25(OH)_2D_3$ (=calcitriol) as a hormone (Gallieni et al., 2009), because of (1) their endogenous synthesis, (2) the existence of a specific receptor for the metabolite, (3) their action in many tissues and organs, not only at the place of synthesis, and (4) the generation of specific biological responses after calcitriol–receptor binding.

Vitamin D metabolism, in simple terms, consists of three main steps; 25-hydroxylation, 1α-hydroxylation and 24-hydroxylation (Bikle, 2014). The hydroxylations are performed by cytochrome P450 mixed-function oxidases (CYP). The most important enzyme for 25-hydroxylation is CYP2R1, while CYP27B1 is the single enzyme responsible for the subsequent 1α-hydroxylation in the kidneys. CYP24A1 performs the catabolism of 25(OH)D and $1,25(OH)_2D$. While $24,25(OH)_2D$ and $1,24,25(OH)_3D$ derive from this catabolic pathway, some data suggest that they are also biological active species (Bikle, 2014).

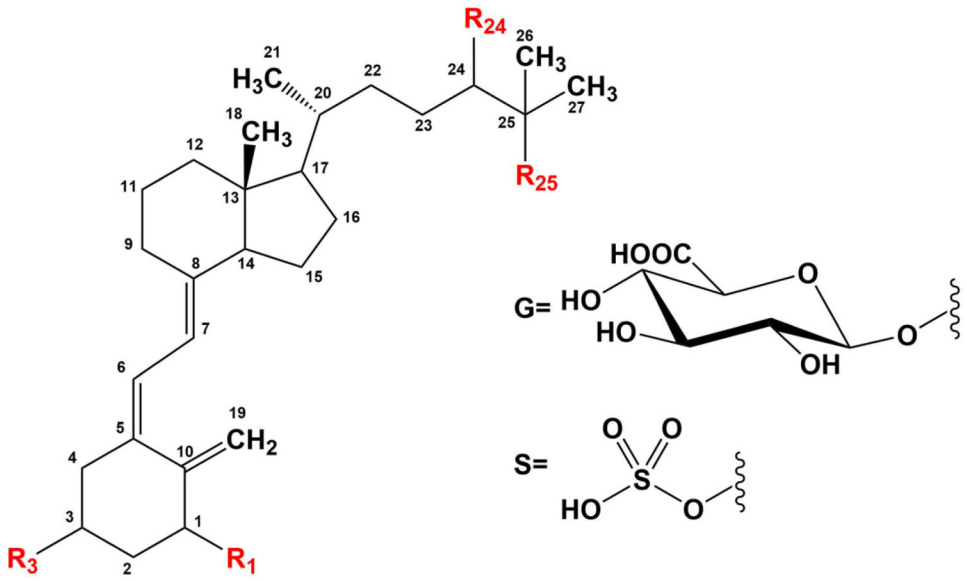
Other important enzymes include CYP27A1, CYP3A4, CYP2R1, CYP2C11, CYP2J1, and CYP2D25, which exhibit 25-hydroxylase activity. CYP27A1 hydroxylates vitamin D_3 at C-25 and vitamin D_2 at C-24. CYP2R1 is a liver microsomal CYP and hydroxylates both vitamin D_3 and D_2 at C-25. CYP24A1 catabolises $1α,25(OH)_2D_3$ and $25(OH)D_3$ to form 23- and 24-hydroxylated metabolites. CYP3A4 inactivates $25(OH)D_3$, to give $4α,25(OH)_2D_3$ and $4β,25(OH)_2D_3$. CYP11A1 hydroxylates vitamin D_3 to form $20(OH)D_3$, $22(OH)D_3$ and $17(OH)D_3$. These oxidations are phase I metabolic products. There is also phase II metabolism; namely, $25(OH)D_3$-glucuronide is formed after glucuronidation of the $25(OH)D_3$ metabolite. Two glucuronide conjugates can be formed at C-3 and C-25. The main enzymes for $25(OH)D_3$ glucuronidation are UGT1A4 and UGTA3, which are UDP-glucuronosyltransferases. Other vitamin D_3 metabolites as well as vitamin D_2 metabolites can also undergo glucuronidation. Sulfotransferase enzymes also induce phase II metabolism of vitamin D compounds; SULT2A1 is the major sulphating enzyme of $25(OH)D_3$ and forms the major circulating sulphation metabolite, which is $25(OH)D_3$-3-O-sulfate.

The chemical structures of vitamin D_3, its main oxidative metabolites as well as phase II metabolites are given in Figure 1.

Three extensive reviews on vitamin D metabolism and catabolism by Tuckey et al. (2018), Jenkinson (2019), and Bikle and Christakos (2020) are very informative in this context.

3 | BIOLOGICAL SAMPLES FOR VITAMIN D ANALYSIS BEYOND SERUM/PLASMA

Most studies on vitamin D metabolites focus on the levels of these compounds in serum and plasma matrices, primarily because the vitamin D status marker is assessed from blood. The first step in this process is blood sampling and storage of the blood in appropriate collection tubes, followed by separation of its components. Subsequently, usually 20–500 μl of serum or plasma (Jenkinson, Taylor, Hassan-Smith, et al., 2016; Satoh et al., 2016; Sofiah et al., 2018; Wang et al., 2015; Yu et al., 2019a) are used to determine levels of $25(OH)D_3$ and other metabolites. Typically, whole blood is obtained intravenously (Lei & Prow, 2019), which is an invasive method and requires trained staff, specialized collection tubes and suitable storage and shipment conditions. Moreover, in some cases, for example, sampling from new-borns or people with damaged veins, this procedure cannot be applied. ***Dried blood spots*** **(DBS)** can overcome some of the problems and have become somewhat of a "trend," as sampling is minimally invasive and can be performed by almost anyone with minimal training (Lei & Prow, 2019). Samples can even be taken from subjects, who are geographically far away from the



Compound	R_1	R_3	R_{24}	R_{25}
D_3	H	OH	H	H
25(OH)D_3	H	OH	H	OH
1,25(OH)$_2D_3$	OH	OH	H	OH
24,25(OH)$_2D_3$	H	OH	OH	OH
D_3-3G	H	G	H	H
25(OH)D_3-3G	H	G	H	OH
25(OH)D_3-25G	H	OH	H	G
1,25(OH)$_2D_3$-1G	G	OH	H	OH
1,25(OH)$_2D_3$-3G	OH	G	H	OH
1,25(OH)$_2D_3$-25G	OH	OH	H	G
24,25(OH)$_2D_3$-3G	H	G	OH	OH
24,25(OH)$_2D_3$-24G	H	OH	G	OH
24,25(OH)$_2D_3$-25G	H	OH	OH	G
D_3-3S	H	S	H	H
25(OH)D_3-3S	H	S	H	OH
25(OH)D_3-25S	H	OH	H	S
1,25(OH)$_2D_3$-1S	S	OH	H	OH
1,25(OH)$_2D_3$-3S	OH	S	H	OH
1,25(OH)$_2D_3$-25S	OH	OH	H	S
24,25(OH)$_2D_3$-3S	H	S	OH	OH
24,25(OH)$_2D_3$-24S	H	OH	S	OH
24,25(OH)$_2D_3$-25S	H	OH	OH	S

FIGURE 1 Chemical structures of vitamin D_3 metabolites [Color figure can be viewed at wileyonlinelibrary.com]

laboratory, as samples can be shipped by regular mail at ambient conditions. Thus, it is a useful tool for epidemiological studies over extended periods of time or for athletes' testing (Freeman et al., 2018).

In 2009, Eyles and coworkers published an assay for determination of 25(OH)D_3 from DBS, which remains the most sensitive method today, since the authors used only 3.28 μl of whole blood (Eyles et al., 2009). The authors applied LC-MS/MS with electrospray ionization in positive ion mode after derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) to quantify 25(OH)D_2 and 25(OH)D_3. The calibration routine was performed using standard addition from whole blood samples. The assay correlated with a commercial radioimmunoassay RIA for plasma 25(OH)D_3 ($r^2 = 0.95$). Finally, the assay was applied to neonatal clinical samples, where the mean

concentration was 20.8 nmol/L for 25(OH)D_3 and 2.6 nmol/L for 25(OH)D_2. Subsequently, the same team established the utility of neonatal DBS for 25(OH)D_3 status (Eyles et al., 2010), showing strong correlation of 25(OH)D_3 levels between cord blood and DBS. Moreover, the authors demonstrated that seasonal variation of 25(OH)D_3 can be shown using the DBS assay. The method was also used in a cohort study consisting of 62 participants (Heath et al., 2014). Plasma samples and DBS from adults were kept for 19 years; nevertheless 25(OH)D_3 concentrations in both types of samples were highly correlated. Recently, the authors proposed changes to improve their method (Kvaskoff et al., 2016), providing shorter analysis times, lower limits of quantitation, reduced matrix interferences and less ion suppression. The limitation of the method was the lack of separation of the C-3-epimers, which was accepted as otherwise the analysis times would have increased threefold, with negative implications on throughput levels when applied in large clinical studies.

Hoeller et al. (2016) conducted a pan-European randomized controlled trial using DBS and triple quadrupole LC-MS/MS to determine 25(OH)D_3 status during a one-year period in more than 220 participants from clinical centers in seven different countries. Participants performed the sampling on their own after been given written and online video instructions. Correction factors were applied for each sex, based on reference hematocrit ranges from the clinical centers.

Müller et al. (2016) developed an LC-MS/MS method for determination of 3α-25(OH)D_3 and 3β-25(OH)D_3 epimers in DBS for the first time. The authors did not use standard addition for calibration as commonly used for DBS, but rather applied a novel artificial vitamin D-free whole blood for calibration. Initially, Higashi et al. (2011) had suggested the addition of albumin/saline solution to the red blood cell component to mimic whole blood, which was lacking some components of whole blood abd could thus confound the results. Müller et al.'s (2016) method used antibody-purified vitamin D-free plasma, which was added to red blood cells, to establish a vitamin-free whole blood. The authors demonstrated improved lower limits of quantitation and precision as compared to the standard addition method; the methods also provided separation of individual quantification of the two epimers. Finally, 3α-25(OH)D_3 and 3β-25(OH)D_3 concentrations in DBS and in serum were found to be in good agreement.

A comparison of concentrations between DBS and serum was also performed by Jensen et al. The authors used 2D-LC with MS/MS to determine 25(OH)D_3 in adults' and neonates' DBS. 2D-LC allowed injection of larger sample volumes with higher sensitivity. Both columns were C-18 phases and did not allow separation of 3α-25(OH)D_3 and 3β-25(OH)D_3, which was given as possible reason for the only moderate correlation between neonatal DBS and cord blood. The proposed assay was therefore not applicable to neonatal DBS, but only to adult DBS samples. The adult DBS correlated with serum concentrations ($r = 0.94$) (Jensen et al., 2018).

Moreover, the quantification of multiple vitamin D metabolites (specifically, 25(OH)D_3, 25(OH)D_2, 3α-25(OH)D_3 and 24,25$(OH)_2D_3$) were determined in DBS by Rola et al. using LC-MS/MS in positive ion ESI MRM mode (Rola et al., 2021). Derivatization was performed using 4-(4′-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) and a pentafluorophenyl column enabled separation of 3α-25(OH)D_3 and 3β-25(OH)D_3. The mean hematocrit value was used to determine the compounds' concentrations (serum [ng/ml] = DBS [ng/ml]/(1-Hematocric fraction)). Validation was extensive, including the influence of the size of the spot and the location where the disk was cut from the DBS. Zakaria et al. (2019) suggested a candidate reference method for determination of 25(OH)D_3 in DBS, which is traceable to NIST-972a standard reference material (SRM). Samples were obtained from children and adults. The authors did not determine 3α-25(OH)D_3 or 25(OH)D_2, as the compounds were undetectable. Supported liquid extraction (SLE) and derivatization with Amplifex reagent was performed using positive ion ESI and MRM mode. The measurements from all DBS correlated with their matching plasma samples ($r = 0.86$). A comparative study of DBS and their matching serum samples from 98 women (who were healthy or were diagnosed with Hashimoto's thyroiditis) showed that DBS can be a suitable alternative to serum, simplifying the sample collection process (Rola et al., 2021). Recently, Binks et al. (2021) presented an LC-MS/MS method with PTAD derivatization to determine 25(OH)D_3, 3α-25(OH)D_3 and 25(OH)D_2 in DBS. The authors underlined the difficulty of using DBS samples in quantitative applications, since the hematocrit correction was sometimes not able to reflect the real bias between DBS samples and plasma samples.

Urine: Another useful biological matrix for clinical analysis is urine. Ogawa et al. (2014) developed an electrospray LC-MS/MS method with isotope-coded derivatization to determine 25(OH)D_3 and 24,25$(OH)_2D_3$ in urine samples. No isotope-labeled internal standards for vitamin D were used, as DAPTAD agent was included for derivatization of urine samples and *d*-DAPTAD ($^2H_4$-DAPTAD) for the compounds' standard solutions. Enzymatic hydrolyzation with β-glucuronidase, SPE and derivatization were applied before MS analysis. The C-3 epimers were readily separated. In a clinical study, urine samples were collected on Day 1 and 7 days after oral administration of vitamin D_3. Vitamin D levels went up significantly; moreover, concentrations of 24,25$(OH)_2D_3$

were three times higher than $25(OH)D_3$ before administration of vitamin D_3. Urine analysis is also an option for studying pathological conditions. Tamblyn et al. (2018) analyzed $25(OH)D_3$ and $24,25(OH)_2D_3$ in urine by LC-MS/MS. This was the first report of studying vitamin D_3 metabolites in urine of pregnant women. The authors compared urinary and circulating vitamin D_3 levels to evaluate the pregnancy outcome and to investigate whether urine analysis could indicate predisposition indicators for development of pre-eclampsia. The measurements showed reduced concentrations of $25(OH)D_3$ and $24,25(OH)_2D_3$ in women who developed pre-eclampsia in comparison to those who did not, demonstrating that urinary metabolites could potentially give additional insight into kidney function and dysregulation of vitamin D_3 in pre-eclampsia.

In clinical practice, concentrations of compounds in urine cannot be easily evaluated if the corresponding concentrations in blood are unknown. Sometimes, however, urine analysis alone can provide useful information on metabolism. For example, Yoshimura et al. (2019) investigated conjugation positions of monoglucuronides of vitamin D metabolites. The results of this study are summarized further below (see section on glucorinated vitamin D_3 metabolites below).

***Breast milk*:** Breast milk is a source of vitamin D for infants, even though it usually does not provide them with their full vitamin D requirements. Human milk is nevertheless a matrix of interest for vitamin D determinations. In one study, an LC-MS/MS methodology was used in combination with PTAD derivatization to determine vitamin D_2, vitamin D_3, $25(OH)D_2$, $25(OH)D_3$, $1,25(OH)_2D_2$, $1,25(OH)_2D_3$, $24,25(OH)_2D_2$ and $24,25(OH)_2D_3$ in human, cow, mare, goat and sheep milk (Gomes et al., 2015). In addition to fat-soluble metabolites, water-soluble conjugates are also present in biological samples and their determination could potentially add valuable information to study vitamin D metabolism. Gomes et al. developed an LC-MS/MS method using negative ion ESI mode to determine sulphated vitamin D metabolites such as D_2-S, D_3-S, $25(OH)D_2$-S and $25(OH)D_3$-S in human serum and milk (Gomes et al., 2016). All metabolites were also present in human serum and only $25(OH)D_2$-S was not detected in breast milk. A recent publication described the first use of supercritical fluid chromatography (SFC) and MS/MS for analysis of vitamin D in human milk (Oberson et al., 2020). Cholecalciferol, ergocalciferol and their 25-hydroxy metabolites were separated on a fluorophenyl column and detected using APCI in MRM mode after PTAD derivatization. Only 1 ml of milk was used in that study as compared to 4 ml for the methods described above.

***Saliva*:** MS has also been used for measurement of $25(OH)D_3$ in saliva. Collection of saliva samples is easy and can be performed even by laypersons in a noninvasive manner. Higashi et al. (2008) correlated serum and salivary $25(OH)D_3$ levels and investigated whether there was any change in salivary concentrations after oral administration of vitamin D_3. Their results showed that salivary concentrations of 25(OH)D were more than 1000-fold lower than in serum, thus requiring chemical derivatization to reach the required limits of quantification. There was a significant increase in salivary $25(OH)D_3$ in all volunteers after vitamin D_3 administration (400 IU or 10 μg daily for 10 d). Subsequently, Higashi et al. (2013) investigated the effect of stimulated saliva with gum-chewing on $25(OH)D_3$ levels. This alternative collection protocol resulted in overestimation of $25(OH)D_3$ levels in all saliva samples. The opposite observation was made by Clarke et al. (2019), who developed an LC-MS/MS method for determination of $25(OH)D_3$ and $25(OH)D_2$ in saliva using PTAD for derivatization. Clarke et al.'s contribution was significant as they clearly identified the challenges of measuring vitamin D in saliva and made several suggestions to improve accuracy. The authors concluded that the passive method of sampling led to greater saliva concentrations of vitamin D than stimulated saliva. Also, storage temperature had no significant effect on salivary $25(OH)D_3$ levels; the flow rate of sampling and the time of day of collection caused little variation in saliva levels of $25(OH)D_3$. Moreover, the authors conducted tests regarding the mucin content of saliva and presence of vitamin D binding protein as potential factors of variation of $25(OH)D_3$ concentration and suggest that their method could be applied in cases where acquisition of blood samples is difficult such as field-based research sampling.

***Cerebrospinal fluid (CSF)*:** Another alternative matrix is CSF, the analysis of which could further contribute to the understanding of vitamin D function. Holmøy et al. (2009) developed an LC-MS method using APCI for determination of $25(OH)D_2$ and $25(OH)D_3$ in serum and CSF. Their results did not support the theory of active transport of 25(OH)D to CSF. The authors suggested that serum levels and the integrity of the blood-brain barrier are responsible for CSF levels of $25(OH)D_3$. An LC-MS/MS method for $25(OH)D_3$ and $24,25(OH)_2D_3$ in serum and CSF using PTAD as derivatization reagent was reported by He et al. (2016). In their report, $25(OH)D_3$ and $24,25(OH)_2D_3$ mean concentrations in CSF were 2.76 and 0.881 ng/ml, respectively.

***Human tears and aqueous humor*:** Vitamin D has a potential role in eye health, leading to a recent increase in analytical studies related to the eye. Lai et al. (2019) used an electrochemiluminescence method to determine

vitamin D in human tears. The mean level of vitamin D was higher in tears than in blood. Ocular barrier epithelial cells are able to convert $25(OH)D_3$ to $1,25(OH)_2D_3$, which could be a protective molecule for the eye and play a role in immune regulation (Alsalem et al., 2014). Moreover, an LC-MS/MS method was developed to determine $24,25(OH)_2D_3$, $25(OH)D_3$, and $25(OH)D_2$ in human aqueous humor after Amplifex derivatization (Fabregat-Cabello et al., 2019), which was the first reported method for determination of vitamin D metabolites in this matrix. All samples showed measurable levels of $24,25(OH)_2D_3$ and/or $25(OH)D_3$, but no $25(OH)D_2$ was detected. Lu et al. (2015) measured vitamin D_3 and D_2 as well as their metabolites in mouse tear fluid using LC-MS/MS. The $25(OH)D_3$ ocular pharmacokinetics after supplementation in rabbits was investigated by Kumar et al. (2018) using an LC-MS/MS method for $25(OH)D_3$ in serum and ocular fluid. The authors observed the maximum concentration in aqueous humor with a 14 h delay compared to serum.

Hair: An interesting biological matrix for analysis of endogenous and exogenous substances is hair. Not only is the sampling simple and noninvasive, but the hair also keeps a "memory" of the exposure of a person to different compounds (Cooper et al., 2012). To date, only a single example for hair analysis of $25(OH)D_3$ using LC-MS/MS has been published (Zgaga et al., 2019). The authors reported median concentrations of $25(OH)D_3$ of 28.7 pg/mg. While hair analysis is unlikely to replace blood-based analysis, it has the potential to be a useful tool to visualize, for example, seasonal variations of $25(OH)D_3$ levels of individuals and oral intake variations, without requiring multiple blood samples at different time points to be taken. As shown for other analytes, vitamin D metabolites are likely to be very stable in the hair matrix at room temperature over extended periods of time, therefore allowing easy storage and shipment of samples. Of course, hair samples of sufficient length must be available from the test subjects, thus limiting the general applicability of a hair analysis approach. In addition, calibration is more difficult and no certified reference materials are available.

Adipose tissue: Fat tissue plays the role of a storage reservoir of the lipophilic vitamin D_3, Blum et al. (2008) developed an LC-MS method to measure vitamin D_3 in serum and subcutaneous fat tissue from obese adults that underwent gastric bypass surgery. The mean serum concentration of vitamin D_3 was 7.78 ± 3.99 nmol/L and the mean concentration in fat tissue samples was 102.8 ± 42.0 nmol/kg with a range of values among the subjects from 28.1 to 186.6 nmol/kg. The authors obtained the data using a C-30 stationary phase and APCI in positive ion SIM mode (m/z 358 for vitamin D_3). They concluded that there is a positive association between serum and fat tissue concentrations of vitamin D_3 in obese individuals. Picollo et al. developed an APCI MRM method for $25(OH)D_3$ in subcutaneous white adipose tissue in overweight and obese adults before and after controlled weight loss (6% body weight, 13% fat loss) (Piccolo et al., 2013). Serum samples were analyzed by radioimmunoassay. Their results showed that $25(OH)D_3$ can be released from adipose tissue, but this release contributes little to serum $25(OH)D_3$ concentration. The authors suggested that a much greater loss of body weight or fat tissue (i.e., ≥15% loss) is required to observe a noticeable contribution to serum 25(OH)D levels. This suggestion agrees with Mason et al. (2011), who concluded that a greater degree of weight loss is associated with increased serum 25(OH)D concentration. Another interesting observation has been made recently by Marques-Pamies et al. (2021), who compared $25(OH)D_3$ and free $25(OH)D_3$ levels after bariatric surgery. One year after the surgery, free $25(OH)D_3$ showed significantly higher concentrations in patients that achieved a body mass index <35 kg/m^2 than the protein-bound metabolite, suggesting that the free vitamin D metabolite is more lipophilic. Malmberg et al. (2014) developed a time of flight secondary ion MS (TOF-SIMS) imaging method for localization of vitamin D species (vitamin D_3, $25(OH)D_3$, $1,25(OH)_2D_3$) in the adipocytes and to determine these species semi-quantitatively in adipose tissue of obese and lean people. The authors showed that the distribution of vitamin D_3, $25(OH)D_3$, $1,25(OH)_2D_3$ was similar in both groups.

While this review primarily focuses on human samples, we will nevertheless refer to some interesting adipose and soft tissue work from animals, as it adds further insight into soft tissue analysis of vitamin D. Bonnet et al. developed an LC-HRMS method to measure cholecalciferol, $25(OH)D_3$ and $1,25(OH)_2D_3$ in mice adipose tissue and plasma after derivatization with Amplifex (Bonnet et al., 2019). The authors used ESI parallel reaction monitoring (PRM), quantifying for the first time $1,25(OH)_2D_3$ among the other two metabolites in mouse adipose tissue. Höller et al. presented an LC-MS method for $25(OH)D_3$ in different swine tissues after supplementation using APCI in positive ion SIM mode (Höller et al., 2010). The highest concentration was observed in the skin (24.8 ± 3.5 ng/g), followed by kidney (14.2 ± 1.5 ng/g), liver (11.4 ± 0.1 ng/g) and muscle (5.7 ± 0.6 ng/g). In the spleen, abdominal fat and subcutaneous fat, $25(OH)D_3$ was not detectable with their method. Another interesting approach to determine the tissue distribution of vitamin

D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 soft tissues of rats was shown by Lipkie et al. (2013). Samples such as liver, epididymal fat, and gastrocnemius muscles were analyzed using LC-MS/MS after derivatization with PTAD and ESI positive ion SRM. Burild et al. developed an LC-MS/MS method to measure vitamin D_3 and 25(OH)D_3 in porcine fat and liver using 0.2–1 g of tissue (Burild et al., 2014).

In conclusion, blood-related matrices dominate clinical analyses of vitamin D. Nevertheless, alternative biological specimens should not be excluded, as vitamin D compounds can clearly be quantified in these matrices (Table 2). Concentration data in these compartments may be useful to further understand the metabolism and biological function of vitamin D both under physiological and pathological conditions.

4 | PROGRESS IN SAMPLE PREPARATION TECHNIQUES FOR MS ANALYSIS OF VITAMIN D

Sample preparation protocols for vitamin D compounds from complex biological samples ideally exhibit the following trademarks: they eliminate or reduce the presence of endogenous or exogenous substances that interfere with vitamin D measurements; are able to quantitatively measure the very low endogenous concentrations of vitamin D and its analogues; avoid issues with stability and isomerization reactions of vitamin D compounds; increase the low ionization efficiency of vitamin D compounds, in particular for low abundant vitamin D metabolites, for example, via chemical derivatization; and they provide economical high throughput analyses.

TABLE 2 Main features of MS-based analytical assays utilizing of unconventional sample matrices[a]

Sample	Sample quantity	Analytical method	Vitamin D metabolites	Reported concentration values
DBS	2.65–50 μl	LC-ESI-MS/MS	25(OH)D_3	ng/ml range
Urine	700–1000 μl	LC-ESI-MS/MS	25(OH)D_3	pg/ml range
			24,25$(OH)_2D_3$	pg/ml range
Breast milk	1000–4000 μl	LC-ESI-MS/MS	D_3	pg/ml range
			D_2	pg/ml range
			25(OH)D_3	pg/ml range
			25(OH)D_2	pg/ml range
			1,25$(OH)_2D_3$	pg/ml range
			1,25$(OH)_2D_2$	pg/ml range
		SFC-APCI-MS/MS	24,25$(OH)_2D_3$	pg/ml range
			24,25$(OH)_2D_2$	pg/ml range
			D_2-S	pg/ml range
			D_3-S	pg/ml range
			25(OH)D_3-S	pg/ml range
Saliva	1000 μl	LC-ESI-MS/MS	25(OH)D_3	pg/ml range
CSF	200–400 μl	LC-ESI-MS/MS	25(OH)D_3	pg/nl–ng/ml range
			24,25$(OH)_2D_3$	pg/nl–ng/ml range
Tears/Aqueous humor	50 μl	LC-ESI-MS/MS	D_3	ng/ml range
			25(OH)D_3	pg/ml range
			24,25$(OH)_2D_3$	pg//ml range
Hair	20–75 mg	LC-APCI-MS/MS	25(OH)D_3	pg/mg range
Adipose tissue	100–500 mg	LC-APCI-MS	D_3 25(OH)D_3	nmol/kg range
		LC-APCI-MS/MS		nmol/kg range
		TOF-SIMS		

[a]Taken from a selection of references listed in Section 3.

Protein precipitation is used extensively as first step of purification, to remove proteins from the sample after release of the protein-bound vitamin D molecules. The most frequently used organic solvent for protein precipitation is acetonitrile but methanol, ethanol, isopropanol or mixed solvents have also been used (Yin et al., 2019). Biological samples such as serum (Jenkinson, Taylor, Hassan-Smith, et al., 2016; Sofiah et al., 2018; Wang et al., 2015; Yu et al., 2019a), plasma (Gao et al., 2017; Higashi et al., 2014; Petruzziello et al., 2017), urine (Ogawa et al., 2014; Yoshimura et al., 2019), CSF (He et al., 2016), saliva (Higashi et al., 2008), and others are usually pretreated by protein precipitation before subsequent preparation steps. Saponification is typically used to remove lipids from food samples. Extra caution should be taken when thermally assisted saponification is used due to thermal isomerization of vitamin D to previtamin D (Yin et al., 2019). Abu et al. optimized a saponification method for serum/plasma and compared their method with protein precipitation for the first time (Abu et al., 2016), demonstrating improved performance over protein precipitation.

To enhance sensitivity and selectivity of the assay, additional clean-up steps are often required. Moreover, as vitamin D is present in biological samples at very low concentrations (ng/ml, pg/ml), a preconcentration step is often necessary. Liquid-liquid extraction (LLE) (Chin et al., 2018; Fabregat-Cabello et al., 2019; Jumaah et al., 2016; Le et al., 2019; Oberson et al., 2020; Tai & Nelson, 2015; Wang et al., 2015; Yu et al., 2019a) and solid phase extraction (SPE) (Gao et al., 2017; Higashi et al., 2011, 2014; Ogawa et al., 2014; Yoshimura et al., 2019) or online SPE (Kassim et al., 2018) are the most commonly used methods among the many published studies for vitamin D and its analogues. During the last 5 years, improved sample preparation has been applied for vitamin D, such as supported liquid extraction (SLE) (Geib et al., 2016; Jenkinson, Taylor, Hassan-Smith, et al., 2016; Petruzziello et al., 2017; Satoh et al., 2016; Tang et al., 2017) and hollow fiber-based liquid phase microextraction (Saber-Tehrani et al., 2014). Geib et al. developed a simple method based on commercial 96-well microextraction plates and commercial calibrators and controls (Geib et al., 2015), providing a cost-effective, simple pipetting workflow. The aim of the study was to suggest an easy-to-perform method, that could be quickly established and readily transferred from one laboratory to another. Other alternative sample preparation methods for nonbiological samples such as pharmaceuticals, milk, yogurt, cereal, and so on are magnetic SPE (Hu et al., 2010; Jiao, Jiao, et al., 2016; Jiao, Zhang, et al., 2016; Momenbeik & Yazdani, 2015), SLE (Jenkinson et al., 2018), dispersive liquid–liquid microextraction (DLLME) (Kamankesh et al., 2017, 2018) and dissolved carbon dioxide flotation microextraction (Shahdousti & Aghamohammadi, 2018).

In a very recent study, Castillo-Peinado et al. (2021) examined lyophilization as an alternative approach for serum and plasma samples storage before analysis. The concentrations of vitamin D_3, 25(OH)D_3, 24,25$(OH)_2D_3$, and 1,25$(OH)_2D_3$ showed no significant differences after lyophilization of the samples. Additionally, the stability of the above analytes in the lyophilized samples were evaluated after 3, 6 and 9 months of storage. The metabolites were found to be stable. These results agree with the observation of Mena-Bravo et al. (2019), who examined the stability of the mentioned metabolites in lyophilized serum samples in the period of 2 months (Mena-Bravo et al., 2019). The only difference concerns the concentration of vitamin D_3, which was affected after 1 month according to Mena-Bravo et al. As a result, lyophilization could be an alternative to the traditional samples' storage at −80°C, since the lyophilized samples can be stored at room temperature, requiring less space and energy consumption, provide easier shipment and allow for making stable reference materials.

5 | RECENT INSTRUMENTAL PROGRESS

5.1 | Quantitative LC-MS assays

Most assays for vitamin D metabolites fall into one of two categories: immunoassays or chromatography-based methods in combination with electrospray ionization (ESI) mass spectrometry, usually tandem mass spectrometry implemented on triple quadrupole instruments. Immunoassays continue to dominate 25(OH)D measurements in the clinic, because they are readily automated at very high throughput levels thus keeping cost low; they are also readily performed by non-specialized staff. They exhibit serious disadvantages, however, such as limited sensitivity and working range, cross-reactivity, lack of adequate standardizations and interferences (van den Ouweland, 2016). While LC-MS/MS assays require elaborate optimization steps and sometimes specialized staff, they are more accurate and sensitive, allowing numerous vitamin D species to be determined simultaneously. LC-MS/MS is therefore often referred to as the "gold standard" method. Generally, to develop an LC-MS/MS method, parameters such as sample preparation, chemical derivatization (if desired or required), calibration model and internal standards, chromatographic column and gradient composition, ionization mode and selection of a specific precursor/product ion transition must be optimized. Table 3 summarizes important recent LC-MS/MS assays for several vitamin D

TABLE 3 Selected LC-MS/MS methods on QqQ instruments for quantification of vitamin D metabolites in biological samples

Vitamin D species	Internal standard	Chemical derivatization	C-3 epimer separated?	Sample/Volume/ Preparation	LC column/ Ionization	Acquisition mode MRM transitions (*m/z*)	References
$25(OH)D_2$ $25(OH)D_3$	$25(OH)D_2$-d_3 $25(OH)D_3$-d_6	No	Yes	Serum/0.25-1 g/LLE	PFP/$APCI^+$	$25(OH)D_2$ 395.3 → 377.3, 209.1 $25(OH)D_3$ 383.3 → 365.1, 105 $25(OH)D_2$-d3 398.3 → 380.3 $25(OH)D_3$-d6 389.3 → 371.1	Mineva et al. (2015)
$1\alpha,25(OH)_2D_3$ $1\alpha,25(OH)_2D_2$	$1\alpha,25(OH)_2D_3$-d6 $1\alpha,25(OH)_2D_2$-d6	Amplifex Diene reagent	No	Serum/500 μL/SPE	C18/ESI^+	$1\alpha,25(OH)_2D_3$ 748.6 → 689.5 $1\alpha,25(OH)_2D_2$ 754.6 → 695.5 $1\alpha,25(OH)_2D_3$-d6 760.6 → 701.6 $1\alpha,25(OH)_2D_2$-d6 766.6 → 707.5	Chan and Kaleta (2015)
(24 R),$25(OH)_2D_3$	(24 R),$25(OH)_2D_3$-d6	No	No	Serum/2 g/LLE	C18/$APCI^+$	(24 R),$25(OH)_2D_3$ 417 → 381 (24 R),$25(OH)_2D_3$-d6 423 → 387	Tai and Nelson (2015)
$25(OH)D_2$ $25(OH)D_3$	$25(OH)D_2$-d3 $25(OH)D_3$-d3	No	Yes	Serum/50 μL/LLE	PFP/ESI^+	$25(OH)D_2$ 413.3 → 395.4, 159.1 $25(OH)D_3$ 401.3 → 383.4, 159.1 $25(OH)D_2$-d3 416.3 → 398.4 $25(OH)D_3$-d3 404.3 → 386.4	Wang et al. (2015)
3β-$25(OH)D_3$ 3α-$25(OH)D_3$ $1\alpha,25(OH)_2D_3$ 23 R,$25(OH)_2D_3$ 24 R,$25(OH)_2D_3$ 3β-$25(OH)D_2$ $24(OH)D_2$ 3α-$25(OH)D_2$ $1\alpha,25(OH)_2D_2$ $1\alpha,24(OH)_2D_2$ D_2 D_3 7α-Hydroxy-4-cholesten-3-one	$1\alpha,25(OH)_2D_3$-d3 3α-$25(OH)D_3$-d3 3β-$25(OH)D_3$-d3 D_2-d3	No	Yes	Serum/220 μL/SLE	Chiral/ESI^+	3β-$25(OH)D_3$ 383.2 → 91, 107 3α-$25(OH)D_3$ 383.2 → 95.4, 107 $1\alpha,25(OH)_2D_3$ 399.2 → 105.1, 151.1 23 R,$25(OH)_2D_3$ 417.4 → 325.3, 343.3 24 R,$25(OH)_2D_3$ 417.4 → 121.1, 381.4 3β-$25(OH)D_2$ 395.3 → 91, 119 $24(OH)D_2$ 395.3 → 340.9, 119 3α-$25(OH)D_2$ 395.3 → 91, 119 $1\alpha,25(OH)_2D_2$ 411.3 → 133, 151 $1\alpha,24(OH)_2D_2$ 411.3 → 133, 151 D_2 397.4 → 69, 107.1 D_3 385.4 → 107, 259.3 7α-Hydroxy-4-cholesten-3-one 401.4 → 97, 117.1 $1\alpha,25(OH)_2D_3$-d3 402.4 → 138, 154.1 3α-$25(OH)D_3$-d3 404.4 → 107.2, 109.4, 368.4	Jenkinson, Taylor, Hassan-Smith, et al. (2016)

TABLE 3 (Continued)

Vitamin D species	Internal standard	Chemical derivatization	C-3 epimer separated?	Sample/Volume/ Preparation	LC column/ Ionization	Acquisition mode MRM transitions (*m/z*)	References
						3β-25(OH)D_3-d3 386.4 → 95.1, 109.3 D_2-d3 400.3 → 109.8, 69, 83	
1α,25(OH)$_2$$D_3$ 1α,25(OH)$_2$$D_2$	1α,25(OH)$_2$$D_3$-d3	No	No	Serum/500 μL/LLE	C18/ESI$^+$	1α,25(OH)$_2$$D_3$ 423.1 → 369 1α,25(OH)$_2$$D_2$ 435.1 → 381 1α,25(OH)$_2$$D_3$-d3 426.1 → 372.1	Fang et al. (2016)
3β-25(OH)D_3 3α-25(OH)D_3 25(OH)D_2 24,25(OH)$_2$$D_3$	3β-25(OH)D_3-d6 3α-25(OH)D_3-d6 25(OH)D_2-$^{13}C_3$ 24,25(OH)$_2$$D_3$-d6	DAPTAD	Yes	Serum/220 μL/SLE	C18/ESI$^+$	3β-25(OH)D_3 619.5 → 341.2 3α-25(OH)D_3 619.5 → 341.2 25(OH)D_2 631.45 → 341.2 24,25(OH)$_2$$D_3$ 635.44 → 341.2 3β-25(OH)D_3-d6 625.5 → 341.2 3α-25OHD_3-d6 625.5 → 341.2 25(OH)D_2-$^{13}C_3$ 634.45 → 341.2 24,25(OH)$_2$$D_3$-d6 641.45 → 341.2	Satoh et al. (2016)
25(OH)D_3 25(OH)D_2 24,25(OH)$_2$$D_3$ 24,25(OH)$_2$$D_2$	25(OH)D_3-[$^2H_6$] 24 R,25(OH)$_2$$D_3$-[$^2H_3$]	PTAD	No	Serum/100 μL/SLE	C18/ESI$^+$	25(OH)D_3 607 → 298 25(OH)D_2 619 → 298 24,25(OH)$_2$$D_3$ 623 → 298 24,25(OH)$_2$$D_2$ 635 → 298 25(OH)D_3-[$^2H_6$] 613 → 298 24 R,25(OH)$_2$$D_3$-[$^2H_3$] 626 → 316	Tang et al. (2017)
1α,25(OH)$_2$$D_3$ 1α,25(OH)$_2$$D_2$	1α,25(OH)$_2$$D_3$-d6 1α,25(OH)$_2$$D_2$-d6	DAPTAD	No	Serum/200 μL/SLE	C18/ESI$^+$	1α,25(OH)$_2$$D_3$ 635.45 → 357.24 1α,25(OH)$_2$$D_2$ 647.45 → 357.24 1α,25(OH)$_2$$D_3$-d6 641.45 → 357.24 1α,25(OH)$_2$$D_2$-d6 653.45 → 357.24	Ishige et al. (2017)
25OHD_2 3β-25(OH)D_3 3α-25(OH)D_3	25(OH)D_2-d3(6,19,19) 25(OH)D_3-d3(6,19,19)	No	Yes	DBS/Extraction	PFP/ESI$^+$	25(OH)D_2 413.3 → 395.3, 337.3 3β-25(OH)D_3 401.3 → 383.3, 365.3 3α-25(OH)D_3 401.3 → 383.1, 365.1 25(OH)D_2-d3 416.3 → 398.3, 358.3 25(OH)D_3-d3 404.3 → 386.3, 368.3	Makowski et al,(2017)
3α-25(OH)D_3 3β-25(OH)D_3	d6-3α-25(OH)D_3 d6-3β-25(OH)D_3	Amplifex	Yes	DBS/corresponding to 4.8 μL of whole blood/Extraction	PFP/ESI$^+$	3α-25(OH)D_3 732.2 → 673.5 3β-25(OH)D_3 732.2 → 673.5	Müller, Stokes, & Volmer (2016)
24,25(OH)$_2$D 3β-25(OH)D_3 3β-25(OH)D_2	$^2H_6$-3β-25(OH)D_3 $^2H_3$-25(OH)D_2 $^2H_6$-3α-25(OH)D_3	No	Yes	Serum/100 μL/ Protein precipitation	PFP/APCI$^+$	24,25(OH)$_2$$D_3$ 381.2 → 363.25, 295.25 3β-25(OH)D_3 383.2 → 365.2, 257.1	Fabregat-Cabello et al. (2017)

(Continues)

TABLE 3 (Continued)

Vitamin D species	Internal standard	Chemical derivatization	C-3 epimer separated?	Sample/Volume/ Preparation	LC column/ Ionization	Acquisition mode MRM transitions (*m/z*)	References
3α-25(OH)D_3 3α-25(OH)D_2	$^2H_6$-24,25$(OH)_2D_3$					3β-25(OH)D_2 395.2 → 377.3, 209 3α-25(OH)D_3 383.2 → 365.2, 257.1 3α-25(OH)D_2 395.2 → 377.3, 209 $^2H_6$-3β-25(OH)D_3 389.2 → 371.2, 263.1 $^2H_3$-25(OH)D_2 398.2 → 380.3, 212 $^2H_6$-3α-25(OH)D_3 389.2 → 371.2, 263.1 $^2H_6$-24,25$(OH)_2D_3$ 387.2 → 369.25, 295.25	
25(OH)D_3	25(OH)D_3-d3	No	No	Serum/100 μL/SPE	C18/APCI$^+$	25(OH)D_3 383.4 → 365.4 25(OH)D_3-d3 395.4 → 377.3	(Okabe et al. (2018)
25(OH)D_2 25(OH)D_3 24,25$(OH)_2D_3$ 24,25$(OH)_2D_2$	25(OH)D_2-d3 25(OH)D_3-d3 24,25$(OH)_2D_3$-d6	No	Yes	Serum/200 μL/LLE	PFP/ESI$^+$	25(OH)D_2 413.4 → 355.4, 395.5 25(OH)D_3 401.4 → 365.4, 383.4 24,25$(OH)_2D_3$ 417.3 → 381.3, 159.1 24,25$(OH)_2D_2$ 429.3 → 271.2, 393.4 25(OH)D_2-d3 416.3 → 358.3 25(OH)D_3-d3 404.3 → 368.3 24,25$(OH)_2D_3$-d6 423.3 → 387.3	Yu et al. (2019b)
3β-25(OH)D_2 3β-25(OH)D_3 3α-25(OH)D_2 3α-25(OH)D_3	25(OH)D_2-d2 25(OH)D_3-d3	No	Yes	Serum/200 μL/LLE	PFP/ESI$^+$	3β-25(OH)D_2 395.3 → 119.4, 377.3 3β-25(OH)D_3 383.3 → 365.1, 257.2 3α-25(OH)D_2 395.3 → 377.3 3α-25(OH)D_3 383.3 → 257.2 25(OH)D_2-d2 398.3 → 380.3 25(OH)D_3-d3 386.3 → 368.1	Cai et al. (2020)
1α,25$(OH)_2D_3$ 3α-25(OH)D_3 24 R,25$(OH)_2D_3$ 3β-25(OH)D_3 23 R,25$(OH)_2D_3$ D_3 3α-25(OH)D_2 1α,25$(OH)_2D_2$ 3β-25(OH)D_2 D_2	1α,25$(OH)_2D_3$-d6 1α,25$(OH)_2D_2$-d6 24 R,25$(OH)_2D_3$-d6 3α-25OHD_3-d3 25OHD_3-d6 25OHD_2-d6 12-[(Cyclohexyl carbamoyl)amino] dodecanoic acid	PTAD	Yes	Plasma/200 μL/ Extraction in 96-well plates	C18/ESI$^+$	1α,25$(OH)_2D_3$ 574.36 → 314.1 3α-25(OH)D_3 558.37 → 298.1 24 R,25$(OH)_2D_3$ 574.36 → 298.1 3β-25(OH)D_3 558.37 → 298.1 23 R,25$(OH)_2D_3$ 592.37 → 298.1 D_3 560.38 → 298.1 3α-25(OH)D_2 570.37 → 298.1 1α,25$(OH)_2D_2$ 586.36 → 314.1 3β-25(OH)D_2 570.37 → 298.1	DeFelice et al. (2020)

TABLE 3 (Continued)

Vitamin D species	Internal standard	Chemical derivatization	C-3 epimer separated?	Sample/Volume/ Preparation	LC column/ Ionization	Acquisition mode MRM transitions (*m/z*)	References
						D_2 572.38 → 298.1 1α,25$(OH)_2D_3$-d6 580.41 → 314.1 1α,25$(OH)_2D_2$-d6 592.41 → 314.1 24 R,25$(OH)_2D_3$-d6 580.41 → 298.1 3α-25OHD_3-d3 561.39 → 301.1 25OHD_3-d6 564.42 → 298.1 25OHD_2-d6 576.42 → 298.1 CUDA 341.3 → 216.2	

metabolites, published during the past 5 years. Please note that this list is not exhaustive and features a selection by the present authors; also note that additional studies are mentioned throughout this review. We refer the reader to the first installment of this review (Volmer et al., 2015) and other publications (Altieri et al., 2020; Makris et al., 2020; van den Ouweland et al., 2013; van den Ouweland, 2016), where many more excellent LC-MS/MS assays for vitamin D metabolites are summarized. Gas chromatography (GC) has also been employed for vitamin D measurement (Lehner et al., 2021; Yang et al., 2019), but this separation technique is out of the scope of this review.

In recent years, two-dimensional LC separations have been increasingly applied to vitamin D analysis before MS detection, to resolve overlapping signals in the first dimension. Mena-Bravo et al. developed a 2D-LC method coupled to MS/MS for determination of vitamin D_3, vitamin D_2, 3β-25$(OH)D_3$, 25$(OH)D_2$, 3α-25$(OH)D_3$, 1,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, and 24,25$(OH)_2D_3$ in serum (Mena-Bravo et al., 2016). The authors used on-line SPE and two separation columns; pentafluorophenyl (PFP) and C-18, followed by ESI in positive ion mode and SRM. The monohydroxy metabolites passed through the PFP column to achieve C-3 epimer separation and were directed straight to the MS. All other analytes interacted with both columns, giving increased detection sensitivity. Dirks et al. developed a 2D-LC-MS/MS method, combining C-4 and C-18 stationary phases for determination of 1,25$(OH)_2D$ in serum (Dirks et al., 2016). Immuno-extraction and PTAD derivatization were applied to samples before 2D-LC-MS/MS analysis. This was the first establishment of reference values for 1,25$(OH)_2D_3$ and 1,25$(OH)_2D_2$ (1,25$(OH)_2D_3$, 59-159 pmol/L (25-66 pg/ml); 1,25$(OH)_2D_2$, < 17 pmol/L (< 7.2 pg/ml).

SFC has also been frequently applied to vitamin D measurements. Jumaah et al. developed a SFC-MS method for separation and identification of vitamin D_3, vitamin D_2, 25$(OH)D_3$, 25$(OH)D_2$, 1$(OH)D_3$, 1$(OH)D_2$, 1,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, and 24,25$(OH)_2D_3$ in plasma (Jumaah et al., 2016). The authors chose a 1-AA column for separation and positive ion ESI over APCI as the last one was mass flow dependent. Analysis time was <8 min; the method was partially validated and isotopically labeled internal standards were not used. Another interesting application of SFC has been described by Liu et al. Their SFC-MS/MS method was developed to determine vitamin D_3, vitamin D_2, 3β-25$(OH)D_3$, 3β-25$(OH)D_2$, 3α-25$(OH)D_3$, 3α-25$(OH)D_2$, 1,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, 24,25$(OH)_2D_3$ and 24,25$(OH)_2D_2$ in serum (Liu et al., 2019). For separation a PFP column was selected, and positive ion ESI was chosen as ionization mode, because it showed sixfold higher detection sensitivity than APCI. The method was validated and compared to a reference LC-MS/MS method. SFC-MS was faster (10 min) and provided lower LOQ than the LC method. Moreover, Blant-Altman analysis

showed no concentration-dependent differences between the two methods. The LOQ for $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$, was insufficient for quantification in serum samples however. Recently, SFC-MS/MS was applied to vitamin D_3, vitamin D_2, 3β-$25(OH)D_3$, 3β-$25(OH)D_2$, 3α-$25(OH)D_3$, 3α-$25(OH)D_2$, $1,25(OH)_2D_3$, $1,25(OH)_2D_2$, $24,25(OH)_2D_3$ and $24,25(OH)_2D_2$ in human milk (Oberson et al., 2020). In contrast to the previous two methods, derivatization with PTAD was performed before analysis, to improve detection sensitivity.

One major difference between SFC-MS and LC-MS is the elution time and order for vitamin D compounds. The elution order in SFC is affected by the number of hydroxyl groups (Liu et al., 2019). Increasing the number of hydroxyl groups leads to longer retention times as a result of hydrogen bonding and dipole-dipole interactions between hydroxyl groups and the stationary phase. The elution order in reversed-phase LC is the opposite because separation is based on the polarity (Figure 2) (Jenkinson et al., 2018). The elution order under SFC conditions is also affected by the structural location of the hydroxyl groups. For example, $1,25(OH)_2D_3$/$1,25(OH)_2D_2$ are retained longer than $24,25(OH)_2D_3$/$24,25(OH)_2D_2$. One additional observation is that the elution order of 3β-$25(OH)D_3$/$25(OH)D_2$, $24(OH)D_2$ and 3α-$25(OH)D_3$ was the same in both SFC and LC.

5.2 | Ionization techniques for vitamin D compounds

5.2.1 | Electrospray ionization (ESI)

ESI is the most widely used ionization technique in LC-MS today, since it offers broad compatibility between LC and MS for analysis of biological molecules (Hagenhoff & Hayen, 2018). Most of the methods summarized in the previous Section 5.1 utilize ESI to ionize vitamin D species, with or without derivatization. As ESI is a soft ionization technique, the ions generated from vitamin D metabolites are usually combinations of $[M + H]^+$, $[M + H\text{-}H_2O]^+$ and $[M + H\text{-}2H_2O]^+$ ions at various ratios, depending on the experimental conditions. More details on ESI analyses of vitamin D compounds have been provided in the previous section.

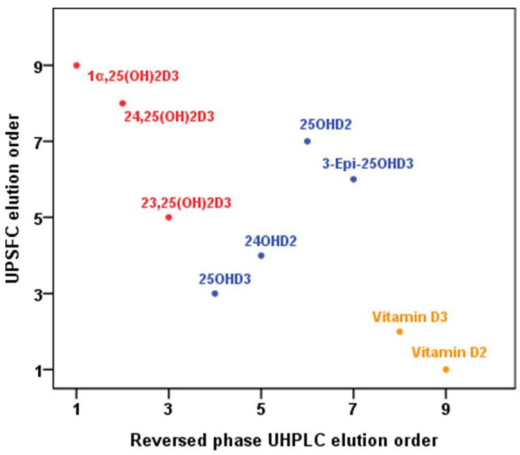


FIGURE 2 Elution order of vitamin D analytes in SFC and LC system (Reprinted with permission by Elsevier; Jenkinson et al., 2018) [Color figure can be viewed at wileyonlinelibrary.com]

5.2.2 | Atmospheric pressure chemical ionization (APCI)

The efficiency of protonation of vitamin D metabolites under ESI conditions is generally low and the sensitivity obtained is often insufficient for the very low abundant species. One approach to overcome this limitation is the use of chemical derivatization, which will be explained in more detail in Section 6. Another option is the application of alternative ionization techniques such as APCI. Interestingly, APCI was used more often in the past, but it is still occasionally applied in vitamin D analysis. Viñas et al. (2013) measured vitamin D_2 and D_3 in food samples using LC-APCI-MS. The maximum sensitivity for vitamin D compounds was obtained in positive ionization mode, where the protonated molecules of vitamin D_2 (*m/z* 397) and vitamin D_3 (*m/z* 385) were observed as base peaks. The dehydrated ions at *m/z* 379 and 367 were also observed. The limit of detection (LOD) was 4 ng/ml for vitamin D_3 and 3.1 ng/ml for vitamin D_2, the limit of quantification was 13 and 10 ng/ml, respectively. Strobel et al. (2013) developed an LC-ion trap-MS/MS method to measure vitamin D_2, vitamin D_3, $25(OH)D_2$ and $25(OH)D_3$ in meat samples. They used APCI in positive ion mode and were able to obtain excellent analytical figures of merit. In particular, the LOD for vitamin D and 25(OH)D was 0.03 μg/100 g and the LOQ was 0.05 μg/ml.

An LC electron capture APCI-MS method was developed by Higashi et al. (2003) for plasma samples to measure $25(OH)D_3$. The authors used a derivatization reagent, namely 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (MW = 220.144 g/mol) and APCI was performed in negative ion mode. After derivatization, two isomers in position 6 were observed (6S:6R, 7:1) with a molecular anion at *m/z* 620, without observing the deprotonated molecule. In addition, the anion at *m/z* 651 $[M\text{-}H + O_2]^-$ was seen in the spectrum. Using an orbitrap mass analyzer equipped with an APCI source, Adela et al. measured serum vitamin D_3, D_2, $25(OH)D_3$, $25(OH)D_2$, $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$ (Adela et al., 2017) using the following ions for quantification: vitamin D_3 *m/z* 385 $[M + H]^+$, vitamin D_2 *m/z* 379 $[M + H\text{-}H_2O]^+$, $25(OH)D_3$ *m/z* 383 $[M + H\text{-}H_2O]^+$, $25(OH)D_2$ m/z

395 $[M + H\text{-}H_2O]^+$, $1,25(OH)_2D_3$ m/z 399 $[M + H\text{-}H_2O]^+$, and $1,25(OH)_2D_2$ *m/z* 411 $[M + H\text{-}H_2O]^+$. The authors obtained LODs for vitamin D_3, $25(OH)D_3$ and $1,25(OH)_2D_3$ of 0.8, 0.8, and 2 ng/ml, respectively, whereas for vitamin D_2, $25(OH)D_2$ and $1,25(OH)_2D_2$, LOD was 0.8, 0.8, and 2 ng/ml, respectively. The LOQs for vitamin D_3, $25(OH)D_3$ and vitamin D_2 were 3 ng/ml and for $1,25(OH)_2D_3$ and $25(OH)D_2$ and $1,25(OH)_2D_2$ they were 5 ng/ml (Adela et al., 2016). A very recent application of positive ion APCI was presented by Román-Hidalgo et al. (2021), for vitamin D in mushrooms.

5.2.3 | Atmospheric pressure photoionization (APPI)

APPI is another option for the ionization of vitamin D metabolites. APPI often delivers improved ionization efficiency for compounds that do not respond well under ESI (Parr et al., 2018). Since our previous review (Volmer et al., 2015), where we described the LC-APPI-MS/MS method for vitamin D_3, D_2, $25(OH)D_3$ and $25(OH)D_2$ in serum by Adamec et al. (2011), unfortunately, to the present authors' knowledge, no further APPI assay has appeared in the literature. As a reminder, the comparison of APCI and APPI showed 8-11×- and 16-17× improved signal intensities for APPI versus APCI of $25(OH)D_3$ and $25(OH)D_2$, respectively. Signal intensities for vitamin D_2 and vitamin D_3 improved 4×–to 7× over APCI. APPI does generally show good results for compounds with structural similarities to vitamin D, such as oxysterols (Ahonen et al., 2014), further demonstrating the potential of this technique for vitamin D analysis.

5.2.4 | Matrix-assisted laser desorption/ionization (MALDI)

MALDI mass spectrometry has shown great promise in the high throughput quantitative analysis of low molecular weight compounds such as pharmaceutical drugs, because of its rapid speed of measurement (Volmer et al., 2007). Its application to analysis of vitamin D compounds is therefore equally interesting.

MALDI was recently applied to the identification of vitamin D epimers. Stand-alone MS analysis of the epimers is very challenging since the isomers exhibit almost identical product ion spectra under collision induced dissociation (CID). Chromatographic separation is therefore usually inevitable before MS detection. Our group developed a MALDI-CID method to distinguish $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ without the need for chromatographic separation or additional sample preparation steps (Qi et al., 2017). The method utilized ion activation of reactive analyte/MALDI matrix adducts to generate more structure informative product ions compared to product ions derived from ESI-CID. In particular, 1,5-diaminonaphthalene (1,5-DAN) adducts resulted in specific cleavages at C-6 and C-7 of the secosteroid's backbone structures enabling the differentiation of the two analytes. We observed $[M + H]^+$ at *m/z* 417 and $[M + 1,5\text{-}DAN]^{+\cdot}$ at *m/z* 574 as precursor ions for both analytes. In the next step, CID was applied to $[M + 1,5\text{-}DAN]^{+\cdot}$ resulting in the loss of the neutral $[1,5\text{-}DAN\text{-}H]^{\cdot}$, creating mainly $[M\text{-}H]^+$ and $[M]^{+\cdot}$ product ions. Closer inspection of the spectra revealed two unique, isomer-specific product ions, namely *m/z* 297 and *m/z* 433 for $1,25(OH)_2D_3$ and *m/z* 281 and *m/z* 449 for $24,25(OH)_2D_3$. Subsequently, we presented a quantitative MALDI-MS method as an alternative to conventional LC-MS/MS method for $25(OH)D_3$ in serum after derivatization with Amplifex reagent (Qi et al., 2018). Our method implemented an automated MS imaging step for each MALDI spot, to locate areas of high intensity and to avoid MALDI sweet spot phenomena. Moreover, we compared the results with an established LC-MS/MS method. No statistically significant differences were observed between the two methods. To our knowledge, this assay has been the first MALDI-MS method quantifying vitamin D species by MALDI, offering the advantages of generating more data in a smaller time frame than the LC-MS/MS method, being free of organic solvents and consuming smaller volumes of sample.

Recently, Ahn et al. (2020) developed a MALDI-TOF-MS method to quantitatively determine $25(OH)D_3$ in human serum and breast adenocarcinoma cells. Derivatization using SecoSET reagent (a permanently positively-charged dienophile) was performed before MALDI-MS analysis. The precursor ion for $25(OH)D_3$ was observed at *m/z* 605 and its fragments at *m/z* 107, 149, and 207. The method showed sensitivity levels down to the femtomolar level.

An interesting imaging method was presented by Smith et al. (2020). Even though it was not applied to human samples, it is worth mentioning here, as this method was the first on-tissue chemical derivatization-MALDI-MS imaging method for mapping the spatial distribution of vitamin D metabolites. The authors examined several derivatization reagents, deposition techniques, reaction conditions and ionization techniques such as MALDI and desorption electrospray ionization (DESI). The method was applied to murine kidney sections. PTAD, DMEQ-TAD and Amplifex were investigated under MALDI and DESI conditions. PTAD displayed no fingerprint signals under both ionization techniques in contrast to DMEQ-TAD and Amplifex.

Amplifex showed higher signal intensities than DMEQ-TAD for both ionization techniques, with MADLI-Amplifex being the optimum for further investigations. $25(OH)D_3$ and $1,25(OH)_2D_3$ were detected in the kidney sections showing differences in their special distribution.

In summary, MALDI-MS can be a useful alternative ionization technique to ESI, since it provides more data in a much shorter timeframe than LC-ESI-MS/MS. Additionally, it can be used for MS imaging applications of various biological samples, potentially revealing more biochemical information on the role of vitamin D in disease.

5.2.5 | Other ionization techniques

While ESI, APCI, and APPI are the most robust atmospheric pressure ionization techniques for LC-MS (Hayen et al., 2009), another alternative technique is dielectric barrier discharge ionization (DBDI), which offers efficient ionization of analytes over a wide range of polarities. In DBDI, a plasma is generated at atmospheric pressure and low temperature (Guo et al., 2015). An interesting comparison was carried out between DBDI, ESI, and APCI by Hagenhoff et al. for the determination of vitamin D species in human serum (Hagenhoff & Hayen, 2018). The ion formation, matrix effect and ionization efficiency were investigated for all three ionization techniques. The data were acquired in positive ion mode. DBDI and APCI kept all the parameters identical and they differed only in the ionization source (helium plasma vs. corona needle). The source housing was the same for ESI, but the gas flows were optimized. In DBDI and APCI, $[M + H\text{-}H_2O]^+$ were the main fragment ions for $25(OH)D_3$ and $25(OH)D_2$, while $[M + H\text{-}2H_2O]^+$ were also seen for $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$. Ammonium and sodium adduct species were also detected. In contrast to DBDI and APCI, ESI generated mainly intact molecule-ion adducts such as $[M + H]^+$, $[M + NH_4]^+$ and $[M + Na]^+$. The $[M + H]^+$ ion was the main ion seen for $25(OH)D_3$ and $25(OH)D_2$, while $[M + NH_4]^+$ dominated the spectra of $1,25(OH)_2D_3$ and $1,25(OH)_2D_2$. With regard to matrix effects, the preferable ionization technique was APCI and, with some limitations, DBDI. Finally, compared to ESI, APCI and DBDI were the most sensitive ionization techniques with respect to LOD.

5.3 | High resolution mass spectrometry assays for vitamin D

In high resolution mass spectrometry (HRMS), the accurate molecular weight of an unknown compound can be determined to several decimal places (Pleil & Isaacs, 2016). That accuracy often allows the molecular formula to be narrowed down to only a few possibilities. Nevertheless, low resolution mass spectrometers are still dominating most analytical fields because they are less expensive and easier to operate. Obviously, the disadvantage of LRMS instruments is that compounds with the same nominal mass but different exact mass appear to be identical in the LRMS experiments. In analytical chemistry, high resolution instruments (time-of-flight, Fourier transform ion cyclotron resonance and orbitrap mass analyzers) become more and more popular and many metabolomics applications rely on them (Marshall & Hendrickson, 2008). These applications still almost always require chromatographic separation, however, because of the presence of multiple isobaric and isomeric species in complex biological samples. For example, during the measurement of the total vitamin D level, interferents such as the $3\alpha\text{-}25OHD_3$ epimer and 1-α-hydroxyvitamin-D_3 and 7-α-hydroxy-4-cholesten-3-one isobars led to overestimations on the order of 14%–55% (Shah et al., 2011).

In our previous vitamin D review (Volmer et al., 2015), there was just one example for a quantitative HRMS assay using orbitrap-MS (Bruce et al., 2013). Since then, a number of excellent HRMS assays have been reported for vitamin D metabolites. Our group developed a Fourier transform ion cyclotron resonance (FTICR) HRMS method using ESI to characterize coextracted isobaric components of 25(OH)D from human serum (Qi et al., 2015). The method revealed multiple isobaric compounds of endogenous and exogenous sources, proving that HRMS methods are required, in particular if no appropriate LC preseparation is performed. In that study, we were also able to use differential ion mobility spectrometry (DMS) after the LC separation to further separate coeluting isobars to allow a low resolution instrument to be used for accurate analysis of $25(OH)D_3$ in serum. Ahonen et al. developed a UHPLC-APPI-HRMS method (using a quadrupole-time of flight MS) as confirmatory method to a UHPLC-APPI–MS/MS method which was used to quantify hydroxysterols and vitamin D compounds in human neuroblastoma cell line samples (Ahonen et al., 2014). For vitamin D_3 and D_2, $[M + H]^+$ was used as precursor ion, while $[M + H\text{-}H_2O]^+$ was used for $25(OH)D_3$ and $1,25(OH)_2D_3$. The comparison of LODs obtained by HRMS and MS/MS showed that UHPLC-APPI–MS/MS provided slightly better sensitivity for some analytes. Another assay for $25(OH)D_3$ and $25(OH)D_2$ on an orbitrap-MS was combined with an enzymatic derivatization to increase the sensitivity of the method (Abdel-Khalik et al., 2014).

Liebisch et al. developed a hybrid quadrupole-orbitrap MS method to determine $3\beta\text{-}25(OH)D_3$,

3β-25(OH)D_2, 3α-25(OH)D_3 and 3α-25(OH)D_2 in serum (Liebisch & Matysik, 2015). A PFP separation column separated all analytes and the $[M + H\text{-}H_2O]^+$ ions were selected after ESI. Resolution was set to 35,000. The authors managed to separate isobaric ions at *m/z* 383 derived from 25(OH)D_3 (*m/z* 383.33139) and d_6-25(OH)D_2 (*m/z* 383.35848). In addition, their method showed specificity, as the extracted ion chromatograms contained no baseline noise. Therefore, the LOQs were determined by serial dilutions of control and calibrator samples and were found to be below 10 nM for all analytes. Raml et al. (2015) presented an HRMS method for the quantification of 24,25$(OH)_2D_3$, 3β-25(OH)D_3, 3α-25(OH)D_3, 25(OH)D_2, D_3 and D_2 in serum. APCI was applied and the resolution was set to 60000 on the orbitrap-MS system. The advantage of this method was the separation of the $[M + H\text{-}H_2O]^+$ ion with *m/z* 401.3685 of the d_6-25(OH)D_2 from the $[M + H]^+$ ion at *m/z* 401.3414 for 25(OH)D_3. Geib et al. compared two LC-MS/MS systems (QqQ LRMS vs. QqTOF HRMS) assays for the determination of 25(OH)D_3 in serum (Geib et al., 2016). The two assays were performed under identical experimental conditions, used virtually identical MS front-ends and differed only in the mass analyzers. Both assays exhibited high correlation and allowed the quantification of 25(OH)D_3 resolving the $[M + H\text{–}H_2O]^+$ product ion free of isobaric interferences. LODs, accuracy and inter-day precision were similar for both assays. There was a slight difference between the intra-day precision, since the coefficients of variation for LRMS were lower than for HRMS. A UHPLC-APCI-HRMS (orbitrap) method was developed by Adela et al. to quantify vitamin D metabolites in serum from type 2 diabetes patients in India (Adela et al., 2016). The following ions were used for quantification: $[M + H]^+$ at *m/z* 385.34649 for vitamin D_3, $[M + H\text{-}H_2O]^+$ at *m/z* 383.33084 for 25(OH)D_3, $[M + H\text{-}H_2O]^+$ at *m/z* 399.32576 for 1,25$(OH)_2D_3$, $[M + H\text{-}H_2O]^+$ at *m/z* 379.33593 for vitamin D_2, $[M + H\text{-}H_2O]^+$ at *m/z* 395.33084 for 25(OH)D_2, $[M + H\text{-}H_2O]^+$ at *m/z* 411.32576 for 1,25$(OH)_2D_2$ and $[M + H\text{-}H_2O]^+$ at *m/z* 381.31519 for the internal standard (dihydrotachysterol). The same method was applied to serum samples from pregnant women with hypertensive disorders (Adela et al., 2017). An LC-HRMS/MS method was developed by Bonnet et al. to determine cholecalciferol, 25(OH)D_3 and 1,25$(OH)_2D_3$ in mouse's adipose tissue and plasma after derivatization with Amplifex reagent (Bonnet et al., 2019). Their system combined quadrupole precursor ion selection with high resolution orbitrap MS detection. The authors used a C-18 column, ESI and PRM, while the resolving power was set to 35,000. The LOQ for the derivatized vitamin D_3, 25(OH)D_3 and 1,25$(OH)_2D_3$ was 0.78, 0.19, and 0.02 ng/ml, respectively. Their final results agreed with their hypothesis that in obese individuals the adipose tissue plays the role of a vitamin D storage site (Carrelli et al., 2017). Another HR method has been presented by Oranzi et al. (2019), quantifying 25(OH)D_3 and 25(OH)D_2 in serum without epi-interferences. The authors used liquid chromatography and ion mobility QTOF with ESI in positive ion mode. Sodiated adducts of 25(OH)D_3 (*m/z* 423.3239), 25(OH)D_2 (*m/z* 435.3239), d_6-25(OH)D_3 (*m/z* 429.3600), and d_3-25(OH)D_2 (*m/z* 438.3424) using a mass window of ±0.01 u and IMS resolving power of 50 were used. For quantitation, only the accurate masses of the mobility selected conformers were used, since the fragmentation of the sodiated adducts $[M + Na]^+$ was very poor. This method is a good example of how HRMS methods combined with IMS can reduce the duration of the time-consuming chromatographic separation, but still provide accurate results in the presence of epimeric and isobaric interferences.

Recently, Arachchige et al. presented a UHPLC-Orbitrap MS method to quantify 11 fat-soluble vitamins (A, D, E and K) in 45 µl of human plasma without derivatization and total analysis time of 19 min (Arachchige et al., 2021). They used a C-18 column and MRM in positive ion mode. Both ESI and APCI were tested, with ESI providing significantly higher signals. The working range for total 25(OH)D_3 was 5.7–365 ng/ml, for 1α,25$(OH)_2D_3$ it was 2.5-165 ng/ml with very good linearity ($r^2 = 0.9998$). The method can be used to quantify 1α,25$(OH)_2D_3$ in clinical samples at higher concentration levels as compared to the physiological levels (40–61 pg/ml) (Celiac Disease, Williams Syndrome, etc.). A similar method was presented by Liu et al., who developed a UHPLC-Orbitrap-MS method to quantify four fat-soluble vitamins (vitamin A, 25(OH)D_2, 25(OH)D_3 and vitamin E) in children's plasma using a C-18 column and positive ion ESI (Liu et al., 2021). According to the authors, this was the first method that quantifies fat-soluble vitamins based on a benchtop Orbitrap system operated in PRM mode. During PRM, a complete mass spectrum of all product ions is generated in a single run using the accurate masses of the precursor ion, allowing specific, reliable mass analysis. The LLOD for 25(OH)D_2 was 0.015 ng/ml and for 25(OH)D_3 it was 0.011 ng/ml; linearity was $r^2 = 0.9975$ and $r^2 = 0.9976$, respectively.

Unfortunately, mass analyzers with high mass resolving power are still not routinely used in clinical practice, because they continue to require expert knowledge and are more expensive to maintain. Nevertheless, if one want results free of systematic errors, HRMS is the method of choice, since one never knows in advance whether interferences from isobars are present or not in the sample. For this reason, there is no doubt that HRMS will very soon be routinely implemented in clinical

laboratories for quantitative vitamin D analysis. For a more thorough discussion of isobaric and isomeric interferences of 25(OH)D and their impact on systematic errors and accuracy of determination, the interested reader is referred to part 1 of this review (Volmer et al., 2015) as well as references Volmer and Stokes (2021) and Qi et al. (2015).

6 | CHEMICAL DERIVATIZATION OF VITAMIN D COMPOUNDS

Due to the nonpolar character of vitamin D and its metabolites—resulting in insufficient ionization and nonspecific product ions—chemical derivatization is often used for low abundant vitamin D metabolites such as $1,25(OH)_2D_3$. Chemical derivatization of vitamin D metabolites aims to improve their detection limits by increasing their ionization efficiency, shifting the mass to higher *m/z* values with less isobaric noise and by providing specific fragmentation patterns for MS/MS. Vitamin D and its metabolites offer two major possibilities for chemical derivatization: (1) the hydroxyl group at C-3 and other hydroxyl groups as well as (2) the *cis* diene moiety. The diene moiety is mostly used for derivatization because of the higher specificity. In this case, Diels–Alder reagents are utilized, where most studies implemented Cookson-type reagents (4-substituted 1,2,4-triazoline-3,5-dione) such as 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Ding et al., 2010; Lyu et al., 2020), 4-(4′-dimethylaminopheyl)-1,2,4-triazoline-3,5-dione (DAPTAD) (Ogawa et al., 2013), (14-(4-(dimethylamino)phenyl)-9-phenyl-9,10-dihydro-9,10[1,2]epitriazoloanthracene-3,15-dione (DAP-PA), which is a stabilized version of DAPTAD by 9-phenylanthracene, activated through retro-Diels–Alder reaction in situ by heating it up to 80°C (Figure 3A) (Seki et al., 2020), 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl) ethyl]-1,2,4-triazoline-3,5-dione (DMEQTAD) (Ogawa et al., 2014), 4-[4-(6-methoxy-2,2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione (MBO-TAD) (Ogawa et al., 2013), 4-ferrocenylmethyl-1,2,4-triazoline-3,5-dione (FMTAD) (Murao et al., 2005), 4-(4-nitrophenyl)-1,2,4-triazoline-3,5-dione (NPTAD) (Ogawa et al., 2013), 4-(4-diethylaminophenyl)-1,2,4-triazoline-3,5-dione (DEAPTAD), 4-(6-quinolyl)-1,2,4-triazoline-3,5-dione (QTAD) (Ogawa et al., 2016), SecoSET (Ahn et al., 2020; Kim et al., 2013), or commercial Amplifex Diene reagent (Hedman et al., 2014).

The ionization efficiency is increased by introducing ionizable groups such as amines to the analytes. In case of PTAD, the addition of methylamine and formic acid to the LC solvent led to better results in the ionization efficiency compared to the addition of neat formic acid (Ding et al., 2010; Higashi et al., 2008). SecoSET and Amplifex introduce a permanently charged quaternary ammonium group. Amplifex results in specific fragments, where the neutral loss of trimethylamine (59 u) can be observed, while all the other Cookson-type reagents mentioned above, apart from SecoSET and FMTAD, use the cleavage between C-6 and C-7 as product ion in MS/MS experiments (Ogawa et al., 2013) (Figure 3B). In addition to Cookson-type reagents, two other reagents have been used for Diels–Alder reactions. Müller et al. used 12-(maleimidyl)

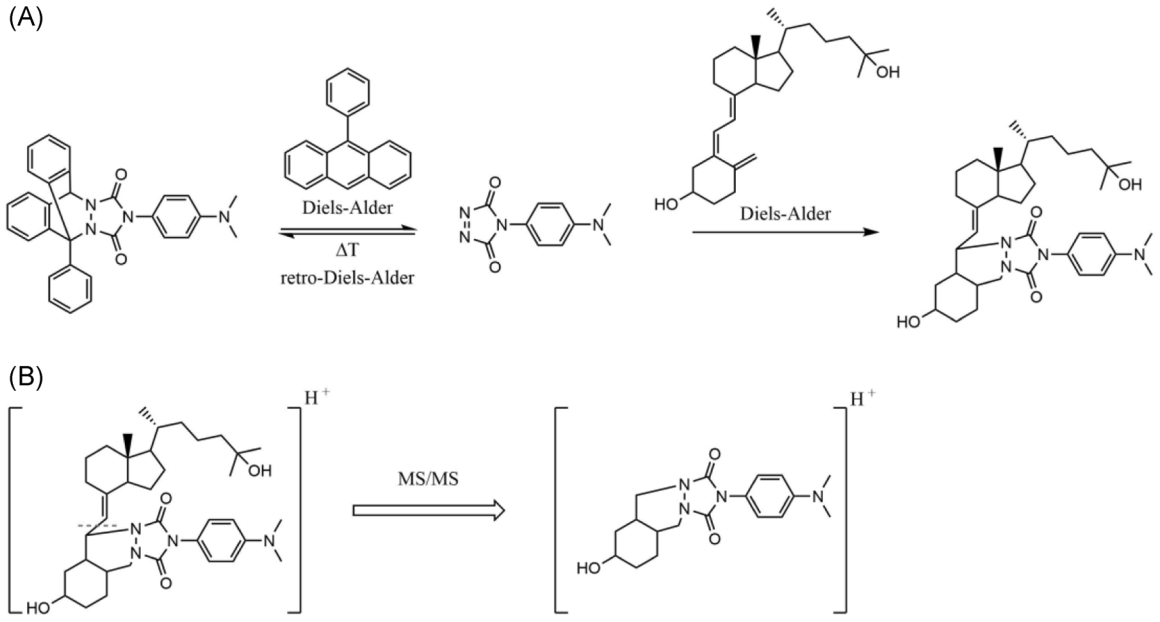


FIGURE 3 (A) in situ activation of DAPTAD out of DAP-PA by retro-Diels–Alder and (B) C-6-C-7 cleavage. *Source*: Reprinted with permission from Jon Wiley & Sons; Ogawa et al. (2013) and Seki et al. (2020)

dodecyl-tri-n-butylphosphonium bromide (MDPB), which utilizes a phosphonium cation to add a permanently charged group (Müller et al., 2017). Interestingly, this reagent was stable under aqueous conditions, which allowed the reaction to take place without prior extraction or drying in extracellular liquid. Wan et al. (2017) used 2-nitrosopyridine (PyrNO) instead of a Cookson-type reagent to access the diene group by hetero Diels–Alder reaction. Moreover, they compared their method with a PTAD method and demonstrated that PyrNO provided better ionization efficiency than PTAD, as PyrNO derivatives were more likely to capture a proton than PTAD analogues.

Ogawa et al. (2014) presented an alternative to the commonly used stable isotope-labeled internal standards by combining derivatization and isotope-labeling of the compounds of interest. They derivatized the vitamin D metabolites in urine sample extracts using DAPTAD and used isotope (^{2}H)-coded DAPTAD to derivatize the standard solutions that were added to the urine samples as internal standards before analysis. The matrix effects and the ionization process were expected to be identical for the derivatives since their elution time was almost the same (d-DAPTAD exhibited slightly earlier elution times [0.1 min]) and peak areas were equal at the same concentration for both.

Also, Ogawa et al. (2017) used DAPTAD isotopologues to quantify 25(OH)D_3 in plasma to achieve higher throughput by reducing the analysis time to 1/3 of that of the original assay (Ogawa et al., 2017). Three aliquots of the same plasma sample were derivatized with DAPTAD, $^2H_3$-DAPTAD and $^2H_6$-DAPTAD, they were then combined and analysed within the same run. The measured concentrations of 25(OH)D_3 showed similar values for the same sample, for all isotopologues used. Subsequently, the authors showed the applicability of their method in measuring 25(OH)D_3 in three different plasma samples within a single LC run, which correlated ($r^2 = 0.968$) to the respective measured concentrations from three separate injections. ^{2}H-coded compounds exhibited weaker hydrophobic interactions with the stationary phase of the reversed-phase LC. As a result, $^2H_6$-DAPTAD derivatives were eluted slightly earlier than $^2H_3$-DAPTAD derivatives and $^2H_3$-DAPTAD derivatives eluted earlier than DAPTAD derivatives.

Diels–Alder reagents attack at the *cis*-diene moiety of vitamin D compounds from the α- and β-sides and the resulting reaction products are therefore a mixture of two stereoisomers (6S and 6R) with 6S being the major one. The ratio for 25(OH)D_3 using PTAD is 4:1 (6S:6R) and for DAPTAD is 5:1 (6S:6R), according to the majority of studies in the literature (Higashi et al., 2008; Ogawa et al., 2013; Rola et al., 2019). However, Müller et al. (2016) demonstrated a temperature dependent ratio for Amplifex and Shimidzu et al. (1993) showed analyte and solvent dependence of the ratio for PTAD and DMEQTAD.

An alternative approach to derivatize vitamin D species is the derivatization of the hydroxyl groups. Le et al. used isonicotinoyl chloride (INC), which attacked vitamin D metabolites at the C-3 hydroxyl group through an acylation reaction (Le et al., 2019). Their method overcame some limitations of the Diels–Alder approach. There was no formation of diastereoisomers and acylation reaction could be completed rapidly at room temperature without long incubation times or heating processes. Abdel-Khalik et al. proposed an enzyme-assisted derivatization with cholesterol oxidase and Girard P (GP) reagent for the quantification of 25(OH)D_3 in serum (Figure 4A) (Abdel-Khalik et al., 2014). This type of derivatization provides advantages including enhanced ionization and characteristic fragmentation patterns (loss of pyridine and water; Figure 4B). The products of 3α-25(OH)D_3 and 3β-25(OH)D_3 after oxidation and GP derivatization were identical. However, the authors found that less than 10% of 3α-25(OH)D_3 was oxidized by cholesterol oxidase thus it would result in less than 1% overestimation of 25(OH)D_3.

Higashi et al. combined the two derivatization sides of 25(OH)D_3; the hydroxyl group at C-3 and the *cis*-diene moiety. They used PTAD derivatization to increase the ionization efficiency and to provide specific fragmentation reactions. Then, acetylation of the C-3 hydroxyl group was performed using acetic anhydride, to improve the separation of 3α-25(OH)D_3 and 3β-25(OH)D_3.

In conclusion, the main reason for chemical derivatization is to increase the sensitivity of analysis of vitamin D compounds, therefore many studies compare their methods to underivatized vitamin D metabolites or to PTAD derivatives. Unfortunately, the studies reported in the literature have provided widely varying enhancements factors for 25(OH)D_3 PTAD derivatives, varying between 15 and 200-fold increase over underivatized 25(OH)D_3. These different values are likely due to differences in the LC methods used, such as stationary phase, mobile phase, additives, ion suppression and peak widths, which all have a major impact on signal to noise ratios and peak areas.

7 | MS METHODS FOR DISCOVERY AND ANALYSIS OF METABOLIC SPECIES BEYOND THE USUAL VITAMIN D FINGERPRINTS

One aim of this review is to highlight the analysis of lesser common vitamin D species, and those that go beyond the usual vitamin metabolic fingerprint

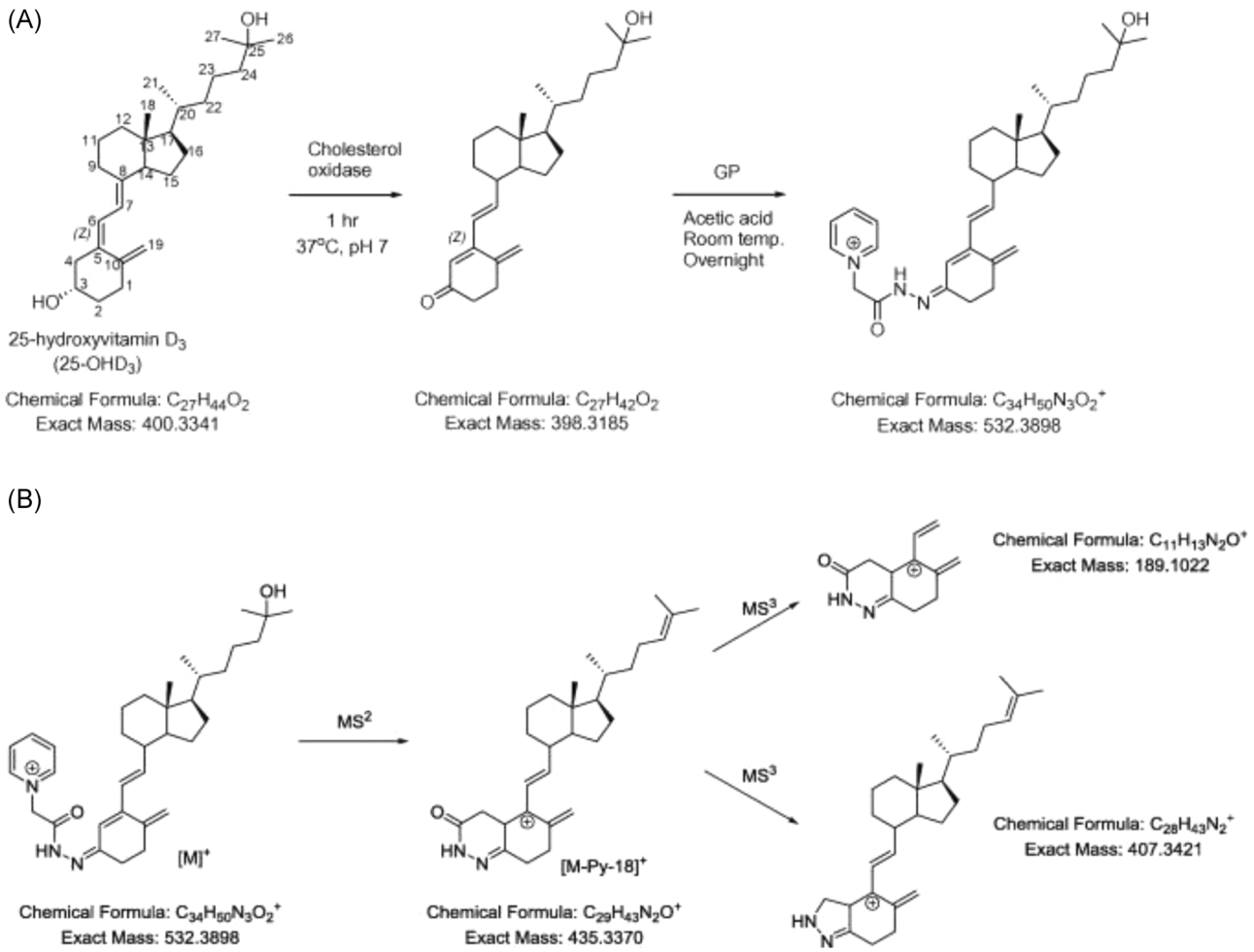


FIGURE 4 (A) An enzyme-assisted derivatization method for 25(OH)D_3. (B) Fragmentation pathway for 25(OH)D_3. *Source*: Reprinted with permission from Abdel-Khalik et al. (2014), under the CC BY 3.0 license

(i.e., beyond those shown in Table 3). Studying these lesser popular metabolites is important as it can improve our understanding of vitamin D metabolism and discovery of new biomarkers. Tuckey et al. (2018) compiled a comprehensive list of expected hydroxylated vitamin D molecules and conjugates in human serum from the existing knowledge of the enzymatic reactions and pathways of vitamin D. Several of these described compounds have already been measured in human serum, for example 23,25(OH)$_2$D$_3$ using LC-MS/MS (Shah et al., 2014), 25,26(OH)$_2$D$_3$ using a competitive binding assay, 4α,25(OH)$_2$D$_3$ and 4β,25(OH)$_2$D$_3$ by LC-MS/MS (Wang et al., 2012) and 1β,25(OH)$_2$D$_3$ and conjugated forms of vitamin D. However, much more comprehensive and sufficiently sensitive analytical techniques are needed to systematically assess the full spectrum of generated vitamin D metabolites.

7.1 | Free vitamin D

The circulating form of vitamin D (25(OH)D) is mostly tightly bound to vitamin D binding protein (DBP), up to 85%–90% of the total levels (Bikle & Christakos, 2020). DBP is a multifunctional protein that is produced in the liver and circulates in the plasma. The human DBP is 458 amino acids long and consists of three domains (Figure 5) (Bouillon et al., 2020). DBP's encoding gene has generic polymorphisms that could lead to changes in its carrying capacity and binding affinity to 25(OH)D. The most common genetic variants are found in domain III and few others in domain II. In domain I, there is a single binding side for vitamin D metabolites. The genotype of this gene should be taken into consideration when free 25(OH)D is calculated using equations which include the measurement of the DBP. Albumin is a weaker carrier than DBP and approximately 10-15% of

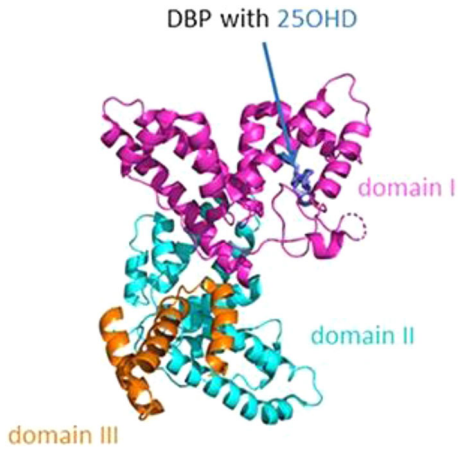


FIGURE 5 Structure of human DBP in combination with 25(OH)D. *Source*: Reprinted with permission from Bouillon et al. (2020) under the CC BY 4.0 license [Color figure can be viewed at wileyonlinelibrary.com]

serum 25(OH)D is bound to it (Bouillon et al., 2020). Finally, the concentration of free 25(OH)D is less than 0.1% of the total concentration of 25(OH)D (Bikle et al., 2017). The bioavailable 25(OH)D is the sum of the loosely bound and the free fraction. Until now, the best biomarker for the determination of vitamin D status is believed to be the total serum 25(OH)D, but more recently it has been suggested that free or bioavailable 25(OH)D are potentially better markers. The concentration of free 25(OH)D is usually estimated by calculations, which include the measurement of DBP, albumin and total 25(OH)D. Recent technological development has allowed the direct measurement of free 25(OH)D by enzyme-linked immunosorbent assay (Banerjee et al., 2020; Carlsson et al., 2018; Heureux et al., 2017; Peris et al., 2017).

Bikle et al. (1984) presented a centrifugal ultrafiltration assay for the measurement of free $1,25(OH)_2D$ and 25(OH)D in serum. They used a mathematical calculation to estimate the free metabolite concentration. This calculation relied on the accurate measurement of DBP and albumin and provides an approximation of the free metabolite concentration; the assay was not proven to be valid because of variations in measurements of the proteins. The author's data suggested that free $1,25(OH)_2D$ levels are maintained in patients with liver disease and lower DBP levels and that during pregnancy, free $1,25(OH)_2D$ levels increase despite the increase of DBP. Thirty years later, the authors compared their method with an immunoassay for free 25(OH)D measurement and for both assays the results agreed for subjects with liver disease (Bikle et al., 2017). The results were quite different with respect to the direct measurement of free 25(OH)D due to the different measurement techniques for DBP.

Powe et al. (2013) compared African Americans and Caucasians and reported lower levels of total 25(OH)D for African Americans, but similar concentrations of bioavailable 25(OH)D as the levels of DBP were equally lower. Lee et al. (2019) correlated the 25(OH)D level in serum with the 25(OH)D level in CSF. Moreover, they determined total, bioavailable and free 25(OH)D in serum considering the genotypes of the DBP gene. Total 25(OH)D was measured in serum and CSF using an electrochemiluminescence binding assay. DBP was measured using ELISA, bioavailable and free 25(OH)D were calculated. Mean serum total, bioavailable and free 25(OH)D concentrations were 25.72, 4.01, and 11.15 pg/ml, respectively. Mean CSF total 25(OH)D concentration was 37.14 ng/ml. The results showed that there was a weakly positive but significant correlation between total 25(OH)D levels in CSF and serum total, bioavailable and free 25(OH)D levels.

Comparing ELISA assays and methods using calculations of free 25(OH)D, the former is the better choice (Peris et al., 2017), because measurement of DBP using monoclonal antibodies versus polyclonal antibodies can lead to variations, especially in multicultural populations. To solve this problems, LC-MS/MS is a possibility because an appropriate sample preparation could potentially allow the separation of free 25(OH)D from the bound fraction. Wang et al. (2020) presented an LC-MS/MS method to determine free 25(OH)D in serum. Ultra-filtration tubes were used to separate the compound from the matrix followed by PTAD derivatization. The median free 25(OH)D concentration was 2.65 pg/ml, which was narrower than reported values by ELISA. This observation could be explained by differences in the methodology and the examined populations. Finally, the results showed positive correlation between free 25(OH)D and total 25(OH)D, $25(OH)D_3$, $1,25(OH)_2D_3$, and $24,25(OH)_2D$ and negative correlation with $25(OH)D/24,25(OH)_2D$.

7.2 | Vitamin D epimers

Epimerization of vitamin D is the nonreversible conversion of the hydroxyl group at C-3 from the 3β to the 3α orientation (Figure 6). $3β\text{-}25(OH)D_3$, $3β\text{-}1α,25(OH)_2D_3$, and $3β\text{-}24R,25(OH)_2D_3$ can all be epimerized to $3α\text{-}25(OH)D_3$, $3α\text{-}1α,25(OH)_2D_3$, and $3α\text{-}24R,25(OH)_2D_3$, respectively (Jenkinson, 2019). Following epimerization, the epimers can be metabolized further following the same hydroxylation pathways of $25(OH)D_3$ using the same enzymes as in the standard pathway (Figure 7).

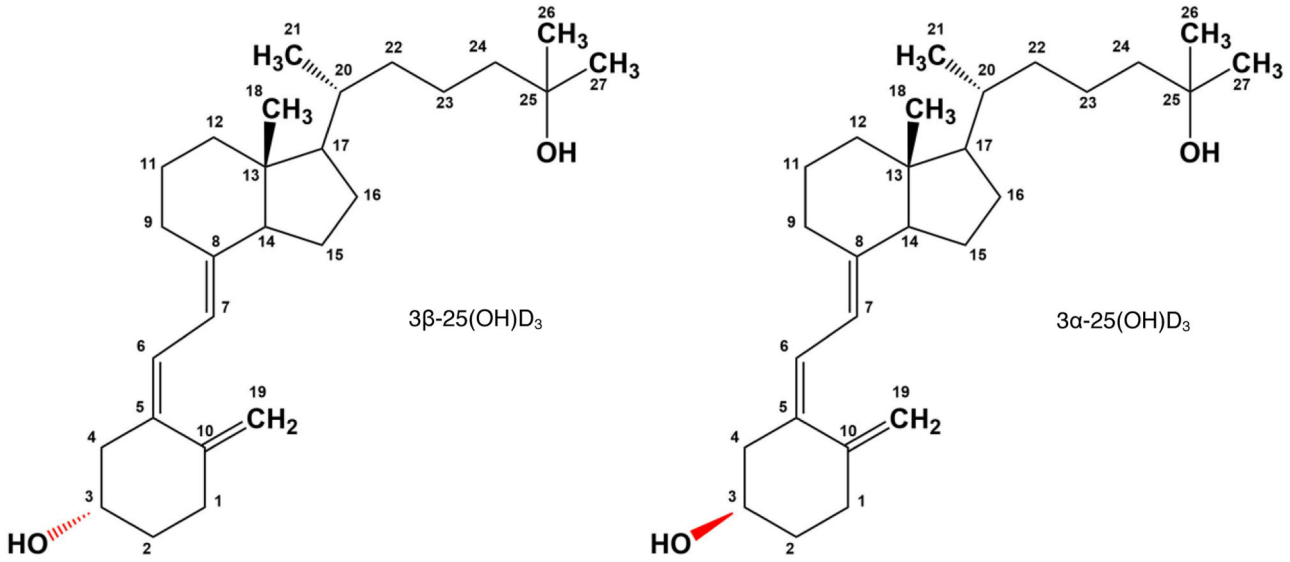


FIGURE 6 3β-25(OH)D_3 and 3α-25(OH)D_3 epimers

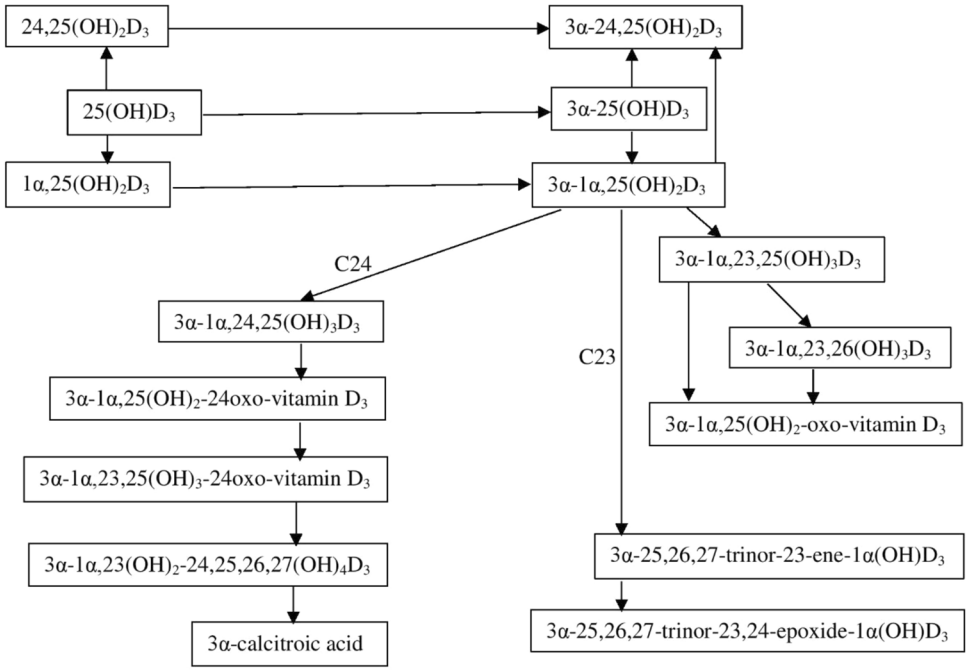


FIGURE 7 Pathway following epimerization of 3β-25(OH)D_3, 3β-1α,25(OH)$_2D_3$, and 3β-24,25(OH)$_2D_3$. The epimerization pathway undergoes CYP24A1 metabolism. *Source*: Reprinted with permission by John Wiley & Sons; Jenkinson (2019) [Color figure can be viewed at wileyonlinelibrary.com]

The C-3 epimerization pathway appears to be cell-type specific and occurs in extrarenal tissue (Bailey et al., 2013). The affinity of 3α-25(OH)D_3 and 3α-1α,25(OH)$_2D_3$ to DBP and VDR is significantly lower compared to the 3β forms, thus reducing the transcriptional activity and most of the biologic effects (Bikle, 2014; Herrmann et al., 2017). Nevertheless, 3α-1α,25(OH)$_2D_3$ induces gene expression, possesses some antiproliferative activity and differentiation activity and is capable of suppressing parathyroid hormone secretion but only in lower levels than the 3β form (Bailey et al., 2013).

In 2013, Bailey et al. (2013) highlighted publications that describe quantification of 3α-25(OH)D_3 in different populations. Several LC-MS/MS methods were developed for determination of the epimers, with emphasis on the chromatographic separation step before MS. C-18, CN,

and PFP columns were mostly used. Finally, the authors evaluated the consequences of reporting or not reporting the 3α epimer in clinical applications concerning different age groups. Another interesting and more resent review was published by Al-Zohily et al. (2020) describing the epimers' formation, metabolism, function and techniques for their measurement.

Yazdanpanah et al. (2013) described an LC-MS/MS method using a PFP column to quantify 3β-25(OH)D_3, 3β-25(OH)D_2, 3α-25(OH)D_3 and 3α-25(OH)D_2 in serum samples from infants and children. Within the first year of life, 3β-25(OH)D_3 levels appear to increase while 3α-25(OH)D_3 levels remained constant but dropped abruptly at the age of 1-year old, continuing to fall gradually throughout childhood. The authors examined liquid vitamin D_3 supplements that were potentially prescribed to breast-fed infants, to explain the increased 3α-25(OH)D_3 levels in this age group. The authors concluded that the supplements were not an exogenous source of 3α-25(OH)D_3. Importantly, even though 3α-25(OH)D_3 concentrations may not be significant in some cases, infants may be falsely misdiagnosed as vitamin D sufficient and then not provided with the recommended supplementation.

As mentioned before, the key for the measurement of epimers is the chromatographic separation and therefore the proper choice of a suitable stationary phase is essential. Rola et al. (2019) tested four different stationary phases, including cholesteryl, pentafluorophenyl, pentabromophenyl and biphenyl groups. The cholesterol stationary phase did not separate the epimers, while the other columns provided adequate resolving power. The total run time was shorter for the pentafluorophenyl column when compared to the biphenyl column.

In Section 8, we will describe the vitamin D standardization program, where a part of the standardization procedure requires the use of SRMs. One of these materials (SRM 972, human serum) developed by the National Institute of Standards and Technology (NIST) in cooperation with the National Institutes of Health's Office of Dietary Supplements (Phinney et al., 2012) contains 25(OH)D_2, 3β-25(OH)D_3, and 3α-25(OH)D_3 at different levels. This SRM provides a mean to ensure measurement accuracy and comparability and emphasizes the need to measure both 25(OH)D_3 epimers in adult or pediatric samples.

In conclusion, to accurately assess vitamin D status, both epimers should be measured individually, especially when assessing infants' samples. The higher concentrations of 3α-25(OH)D_3 in infants' samples have been suggested to result from the immaturity of vitamin D metabolism at this young age, as it is especially predominant in premature infants (Singh et al., 2006). Alternatively, van den Ouweland and coworkers proposed that the high 3α-25(OH)D_3 levels may indicate a mechanism to avoid high vitamin D exposure (and corresponding elevated levels of 25(OH)D_3) in preterm infants (Ooms et al., 2016). For adult samples, it has been argued that the cost/benefit ratio of routinely measuring vitamin D status may not justify the development of special chromatographic methods (Bikle, 2014).

7.2.1 | Response behavior of vitamin D epimers

Differences of ionization efficiency between the epimers is another factor that must be considered since overestimation of concentrations can occur if quantifications are performed using internal standardization based on the 3β-25(OH)D_3 isotope standard. Van den Ouweland et al. compared the absolute MS responses from different concentrations of 3β-25(OH)D_3 and 3α-25(OH)D_3 (Ouweland et al., 2014) and showed a 30%–40% higher SRM signal for 3α-25(OH)D_3. Independently, Flynn et al. (2014) also observed higher intensity for 3α-25(OH)D_3 in comparison to 3β-25(OH)D_3. Recently, our group attributed the higher ionization efficiency of the 3α epimer to a kinetically controlled protonation process (Schorr et al., 2021). The proposed mechanism of this process suggests that ionization initially takes place at the hydroxyl group with subsequent proton transfer to a basic carbon atom. The barrier for this transfer differs between the epimers and is in direct competition with H_2O elimination from the protonated hydroxyl group.

These findings underscore the need for individual calibration curves and dedicated internal isotope standards for each vitamin D epimer. This strategy should be followed even if the epimers are chromatographically separated before MS detection. We have described the application of stable isotope standards for both epimers to accurately quantify these epimers in patients with chronic liver diseases (Stokes & Volmer, 2016). Alternatively, derivatization can be used to introduce a permanently charged group to both epimers to generate derivatives with equal ionization efficiency (Müller, Stokes, & Volmer, 2016; Müller, Stokes, Lammert, et al., 2016). This method equalizes the response factors of the epimers but requires an additional sample preparation step.

7.2.2 | Vitamin D epimers and ion mobility spectrometry (IMS)

IMS is an interesting supplementary technique to separate vitamin D epimers. IMS can provide several

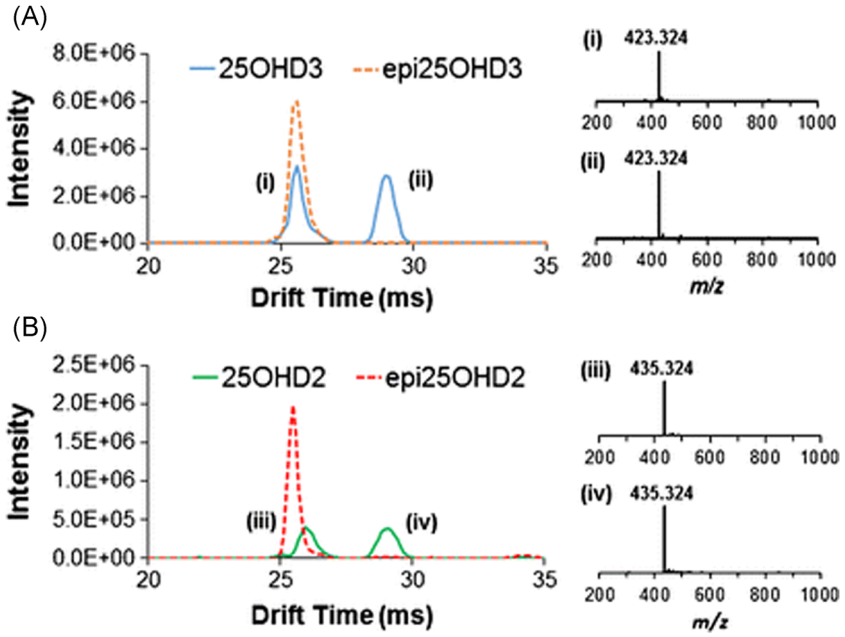


FIGURE 8 Overlay of sodiated monomer drift spectra for (A) 3β-25(OH)D_3 and 3α-25(OH)D_3, and (B) 3β-25(OH)D_2 and 3α-25(OH)D_2. Inset: mass spectra collected for (i) 3β-25(OH)D_3 at 26.0 ms, (ii) 3β-25(OH)D_3 at 29.0 ms, (iii) 3β-25(OH)D_2 at 26.0 ms, and (iv) 3β-25(OH)D_2 at 29.0 ms. *Source*: Reprinted with permission from Chouinard et al. (2017); Copyright 2021 American Chemical Society [Color figure can be viewed at wileyonlinelibrary.com]

individual mobility spectra across a single chromatographic peak. Chouinard et al. investigated the differences of the gas phase conformations of 3β-25(OH)D_3 and 3α-25(OH)D_3, and 3β-25(OH)D_2 and 3α-25(OH)D_2 utilizing IMS-MS (Chouinard et al., 2017). The authors compared theoretical and experimental cross-sections to determine gas phase structures. Ion mobility-QTOF was used with an ESI source in positive ion mode. The sodiated monomer $[M + Na]^+$ at *m/z* 423.324 for the D_3 epimers and at *m/z* 435.324 for D_2 epimers were the major ion species in the spectra. Additional ions such as $[2M + Na]^+$ and $[3M + 2Na]^+$ were seen at low intensities. Both 3β-25(OH)D_3 and 3β-25(OH)D_2 showed slightly lower signal intensity than their 3α-species, in agreement with the literature (Ouweland et al., 2014; Schorr et al., 2021). As shown in Figure 8, 3β-25(OH)D_3 exhibited two major drift peaks (25.99 and 29.47 ms) in contrast to 3α-25(OH)D_3 which exhibited only one signal (25.78 ms). The mass spectrum showed a single peak at *m/z* 423.324 for D_3 epimers and at *m/z* 435.324 for D_2 epimers, proving that only the sodiated monomer contributed to the ion mobility separation (Figure 8).

Chouinard et al. (2017) also performed theoretical modeling as well as experimental investigations of the "open" conformer. In Figure 9, the 3D structures of the most stable conformers are shown. In the "closed" conformation, the C-3 and C-25 hydroxyl groups interact with one sodium ion and the carbon chain is bent. In the "open" conformation, one of the two hydroxyl groups interact with the sodium ion. For 3β-25(OH)D_3 the most stable structure is the "closed" conformation and the most stable of the "open" conformations occurs when C-3 hydroxyl group interacts with Na^+. The same applies

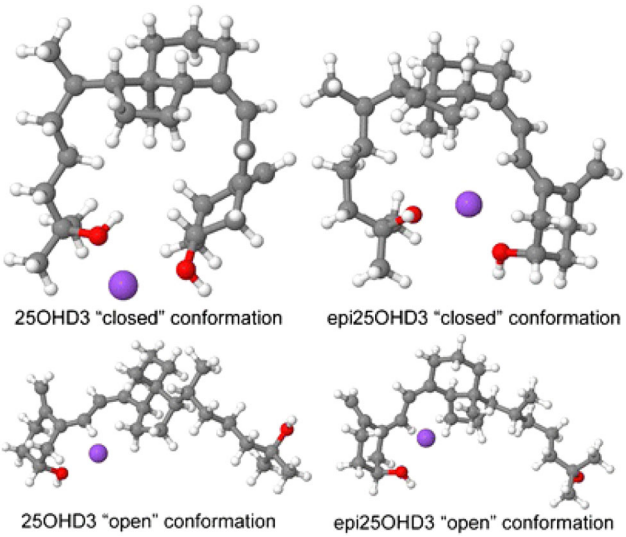


FIGURE 9 Structures of the most stable "closed" and "open" conformations of 3β-25(OH)D_3 and 3α-25(OH)D_3. (Reprinted with permission from Chouinard et al. (2017); Copyright 2021 American Chemical Society [Color figure can be viewed at wileyonlinelibrary.com]

for 3α-25(OH)D_3, with the difference that no "open" conformation occurs with the C-25 hydroxyl group interacting with Na^+, only for the C-3 position. Nevertheless, the 3α-25(OH)D_3 is found only in the "closed" conformation, because of the large energy difference between the two conformers, which favors only the "closed" formation.

Similarly, Oranzi et al. (2019) developed an LC-IMS-MS to separate 3β-25(OH)D_3 and 3β-25(OH)D_2 from 3α-25(OH)D_3 and 3α-25(OH)D_2. The separation

of the epimers was not achieved during the chromatographic separation but was based on the difference in collision cross sections in the IMS separation. The authors showed that sodium adducts of the 3β metabolites can adopt either a compact-closed or extended-open conformation in the gas phase, while the 3α epimers adopt only the compact conformation. The extended conformer of 3β species was detected as a drift peak, which was resolved from the compact conformer and thus free of interferences from the epimers (Figure 10). The authors suggest that the intensity ratio of the two conformers is a result of the instrument settings and ion flux within the mass spectrometer, where the extended conformer converts to the compact one. Another interesting observation was seen when APCI was used: the protonated molecules $[M + H]^+$ were formed in contrast to ESI, which favored the formation of $[M + Na]^+$ ions. The protonated molecules were found to form only one conformer structure that did not provide separation by IMS-MS.

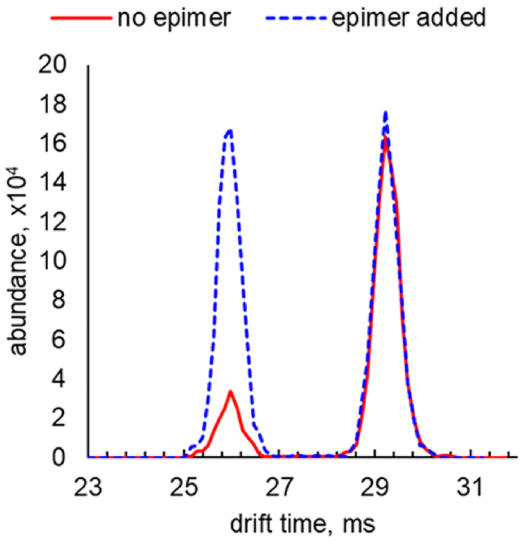
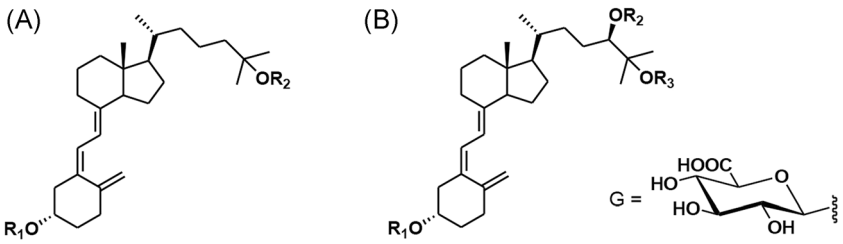


FIGURE 10 Extracted ion drift time spectrum (*m/z* 423.3239 and RT 0.75–0.85 min) of the chromatographic peak containing 3β-25(OH)D_3 and 3α-25(OH)D_3. The drift peak at 25 ms, represents the closed conformer, containing signal from both epimers; however, the peak at 29 ms is unique to 3β-25(OH)D_3 and shows the same intensity for both samples (red trace: sample containing only 3β-25(OH)D_3, blue trace: sample containing 3β-25(OH)D_3 and 3α-25(OH)D_3). *Source*: Reprinted with permission from Oranzi et al. (2019); Copyright 2021 American Chemical Society [Color figure can be viewed at wileyonlinelibrary.com]

7.3 | Glucuronidated vitamin D3 metabolites

The biological role of glucuronidated conjugates is still not fully understood. One hypothesis suggests that they could serve as a reservoir for 25(OH)D_3 through deconjugation to 25(OH)D_3 (Huynh et al., 2021). To provide a more comprehensive assessment of vitamin D status, measurement of glucuronidated metabolites could provide further information for health and disease. Wang et al. (2014) described the identification of three monoglucuronide conjugates of 25(OH)D_3 and their formation by UGT1A4 and UGT1A3. 25(OH)D_3-25-glucuronide, 25(OH)D_3-3-glucuronide and 5,6-trans-25(OH)D_3-25-glucuronide are produced in the human liver, but only 25(OH)D_3-3-glucuronide was detected in both human plasma and bile.

Yoshimura et al. (2019) identified the conjugation position of urinary glucuronidated vitamin D_3 metabolites by mass spectrometry to contribute to further understanding vitamin D metabolism. Previous studies confirmed the presence of glucuronidated 24,25$(OH)_2D_3$ and 25(OH)D_3 in urine, but the conjugation positions remain unknown due to enzymatic pretreatment of samples before analysis (Higashi et al., 2002; Ogawa et al., 2014). The chemical structures of 24,25$(OH)_2D_3$, 25(OH)D_3 and their monoglucuronides are given in Figure 11. Yoshimura



25(OH)D_3: $R_1 = R_2 = H$
25(OH)D_3-3G: R_1 = G, $R_2 = H$
25(OH)D_3-25G: $R_1 = H$, R_2 = G

24,25$(OH)_2D_3$: $R_1 = R_2 = R_3 = H$
24,25$(OH)_2D_3$-3G: R_1 = G, $R_2 = R_3 = H$
24,25$(OH)_2D_3$-24G: $R_1 = H$, R_2 = G, $R_3 = H$
24,25$(OH)_2D_3$-25G: $R_1 = R_2 = H$, R_3 = G

FIGURE 11 Chemical structures of 24,25$(OH)_2D_3$, 25(OH)D_3 and their monoglucuronides. *Source*: Reprinted with permission, Yoshimura et al. (2019)

et al.'s LC-MS/MS method included a C-18 column, positive ion ESI and MRM for the DAPTAD-derivatized glucuronides (Yoshimura et al., 2019). The MRM transitions were as follows: *m/z* 795.5 → 341.2 and 517.1 for 25(OH)D_3-3G and -25G, *m/z* 811.5 → 341.3 and 517.0 for 24,25$(OH)_2D_3$-3G, 24G and −25G, *m/z* 610.5 → 381.1 for intact 24,25$(OH)_2D_3$-24G. Finally, 25(OH)D_3-3G and 24,25$(OH)_2D_3$-24G were identified as urinary metabolites. Noteworthy is the fact that the 3-glucuronide metabolite was present for 25(OH)D_3 unlike for 24,25$(OH)_2D_3$. These results demonstrate that the selective glucuronidation in C-24 position is more likely to occur, where a secondary hydroxy group is present on the side chain rather than in C-3 position.

7.4 | Sulphated vitamin D3 metabolites

The major circulating form of vitamin D is 25(OH)D_3-sulfate (S) and its levels may exceed those of 25(OH)D_3. Moreover, it has been suggested that the sulphated form is a storage form of the non-sulphated compound (Higashi et al., 2014). Therefore, the quantification of this metabolite in biological samples could be helpful for further assessment of vitamin D status.

Higashi and coworkers presented an LC-MS/MS method to determine 25(OH)D_3-S in 20 μl of infant serum after SPE (Higashi et al., 2014). Due to the strongly acidic sulfate group, the negative ion mode was chosen and the MRM transitions; for 25(OH)D_3-S were *m/z* 479.1 → 96.6 and for $^2H_3$-25(OH)D_3-S *m/z* 482.1 → 96.6. 25(OH)D_3-S concentrations of the infant plasma ranged from 3.9 to 58.2 ng/ml. These concentrations were similar or higher than those of adults, unlike the 25(OH)D_3 concentrations. These results suggest that 25(OH)D_3-S could be a storage form of vitamin D_3 in infants and that premature infants with lower levels of 25(OH)D_3-S could be suspected of rickets. Higashi et al. (2016) also presented a method for the simultaneous determination of 25(OH)D_3-S and 3β-25(OH)D_3 in new-born's plasma with the separation of 3α-25(OH)D_3. DAPTAD derivatization was performed before analysis and positive ESI was applied to achieve the simultaneous determination of the compounds because negative ESI allowed only the determination of 25(OH)D_3-S. MRM was applied and the transitions for the derivatized metabolites were as follows: 25(OH)D_3 *m/z* 619.6 → 341.3, $^2H_3$-25(OH)D_3 *m/z* 622.7 → 344.2, 25(OH)D_3-S *m/z* 699.6 → 421.2 and $^2H_6$-25(OH)D_3-S *m/z* 705.6 → 421.2.

Recently, Jenkinson et al. (2021) described a novel assay that permits simultaneous analysis of conjugated and unconjugated 25(OH)D species in a single LC-MS/MS assay after an enzymatic hydrolysis step to release the conjugated phase II metabolites (glucoronides and sulfates) into the sample extract. The authors point out that the inclusion of the conjugated forms (especially the sulphated metabolite) may provide an improved status marker.

25(OH)D_3-S, D_3-S and D_2-S are some of the sulphated metabolites that have been determined in human milk after PTAD derivatization (F. P. Gomes et al., 2016). Kassim et al. (2018) developed a method for the simultaneous determination of both lipophilic and hydrophilic vitamin D compounds after online-SPE in serum (500 μl). A PFP column was used and MRM scan mode. In a single run, positive ion ESI mode was used for D_2, D_3, 25(OH)D_2, 25(OH)D_3, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, and 1,25$(OH)_2D_3$ and negative ESI mode for D_2-S, D_3-S, 25(OH)D_3-S and 25(OH)D_2-S. According to the authors, this was the first report, where a single analysis procedure simultaneously quantified 12 vitamin D compounds with different lipophilicities.

Finally, two methods combined the determination of conjugates from both sulphation and glucuronidation. The first method combined the determination of 25(OH)D_3-S and 25(OH)D_3-G DAPTAD derivatives in plasma and serum, which according to the authors was the first validated LC-MS/MS method for their simultaneous determination (Gao et al., 2017). ESI in positive ion mode was applied and the MRM transitions are shown in Figure 12. The method was accurate, reproducible and robust as well as applicable to clinical studies. The second method was developed by Huynh et al., who measured 3-sulfate and 3-glucuronide 25(OH)D_3 in serum without the need for derivatization (Huynh et al., 2021). Separation was performed on a C-18 column and negative ion ESI was applied. The optimum MRM transitions were: *m/z* 479.4 → 79.8, 96.9, 122.9 for 25(OH)D_3-S and *m/z* 575.4 → 74.9, 84.9, 122.9 for 25(OH)D_3-G. LLOD was determined to be 0.063 ng/ml for 25(OH)D_3-S and 0.125 ng/ml for 25(OH)D_3-G. Signal/noise ratios were >3. LLOQs were 0.125 ng/ml for 25(OH)D_3-S and 0.250 ng/ml for 25(OH)D_3-G with signal/noise ratios >10.

7.5 | 25,26$(OH)_2D_3$

Another vitamin D catabolite is 25,26$(OH)_2D_3$. Very little is known about this compound, since most studies describing this compound date back to 1980s, when the analytical instrumentation was not sufficiently advanced for proper analysis of this molecule. Early studies suggest that this metabolite is only active in the intestine and has no effect on bone mineral mobilization and rats' rickets (Suda et al., 1970). After oral administration, no elevation in serum calcium concentration was observed. However,

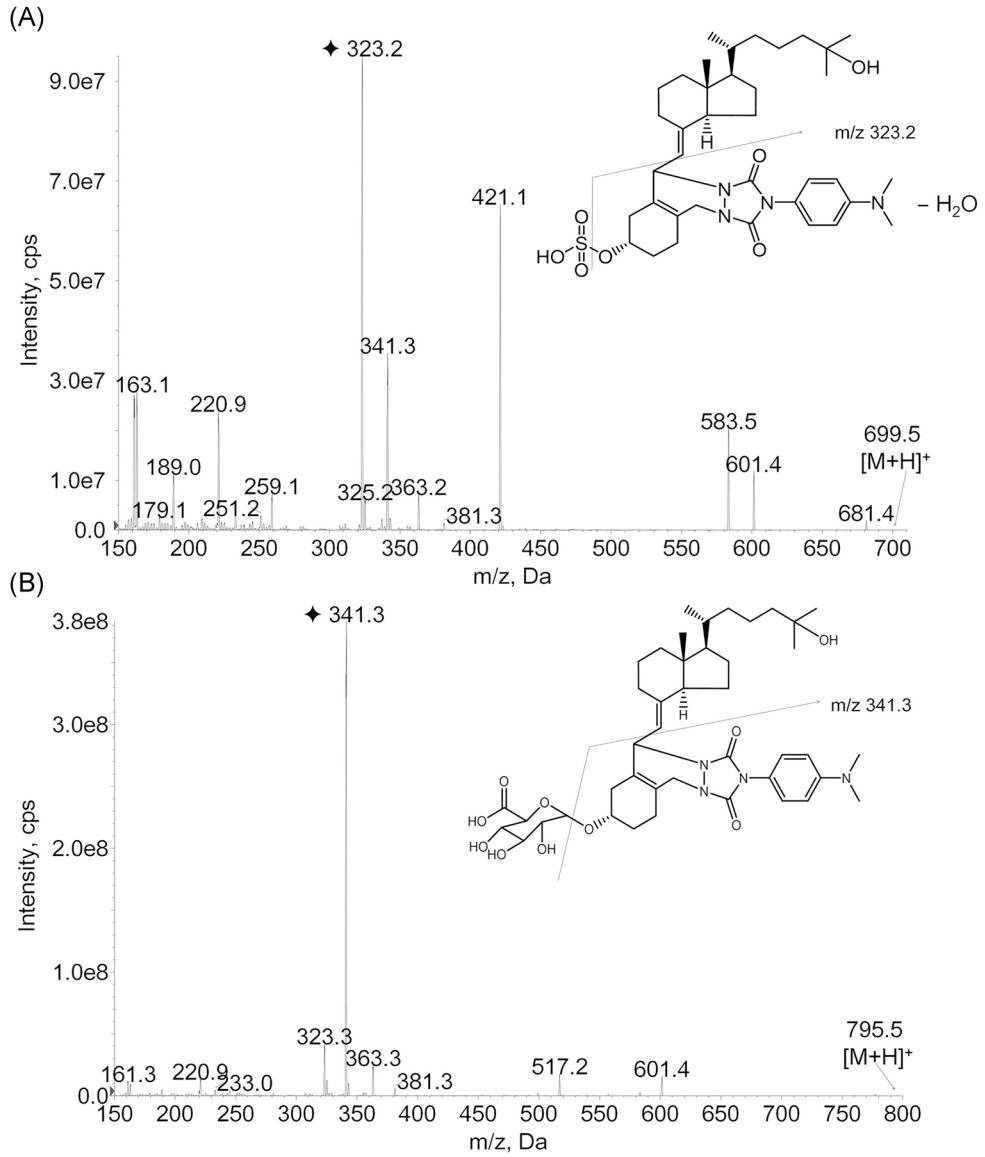


FIGURE 12 (A) Product ion spectra of DAPTAD-25(OH)D_3-3-sulfate (*m/z* 699.5 → 323.2) and (B) DAPTAD-25(OH)D_3-3-glucuronide (*m/z* 795.5 → 341.3). *Source*: Reprinted with permission by John Wiley & Sons; Gao et al. (2017)

intestinal calcium transport was increased and 25,26(OH)$_2$$D_3$ activity was comparable to one-half of the activity of 25(OH)D_3 in the stimulation of intestinal calcium transport (Suda et al., 1970). It is therefore important to assess the biological role of this compound, as it could be an active and functional metabolite in the tissue where it is generated. Alternatively, it could be just an intermediate of the vitamin D_3 inactivation path. Care et al. (1978) reported a significant reduction in the rate of secretion of parathyroid hormone in goats after 25,26(OH)$_2$D perfusion, possibly as a result of increased flux of calcium ions into parathyroid chief cells. Miravet et al. (1976) studied the biological activity of the compound in vitamin D deficient rats. A considerable increase in calcium absorption was observed in rats following a calcium deficient diet after intravenous injection (after 48 h). This finding is in contrast to the rats that followed a normal calcium diet, where the calcium absorption was just slightly increased after 24 h. Nephrectomised rats showed no intestinal absorption. 25,26(OH)$_2$$D_3$ had no antirachitic activity after oral administration in phosphorus and vitamin D deprived rats. However, antirachitic activity and increases in the calcium content of bone ash was observed after intravenous administration. The aforementioned results agree with the observations of Lam et al. (1975). Regarding the two orientations of the C-25 hydroxyl group, Bikle et al. (1984) showed that both 25(S) and 25(R),26(OH)$_2$$D_3$ are produced in vitro, but it is not clear which configuration predominates.

Recently, Zelzer et al. (2020) presented an LC-MS/MS method for determination of 25(OH)D_3, 25(OH)D_2, 24,25(OH)$_2D_3$ and 25,26(OH)$_2D_3$ in serum samples after PTAD derivatization. ESI in positive ion mode was used. The precursor ion for 25,26(OH)$_2D_3$ was *m/z* 574.1 and the product ion for all the analytes was *m/z* 298. 25,26(OH)$_2D_3$ (6.06 min) eluted before 24,25(OH)$_2D_3$ (6.24 min). The concentration of 25,26(OH)$_2D_3$ in four serum samples ranged from 4 to 11.8 nmol/L. Importantly, if chromatographic separation between 24,25(OH)$_2D_3$ and 25,26(OH)$_2D_3$ is not achieved, then there will be an overestimation in the vitamin D metabolite ratio (VMR) (24,25(OH)$_2D_3$/25(OH)D_3*100), especially in patients with 24-hydroxylase deficiency.

When 25,26(OH)$_2$D concentrations were determined in plasma samples using a competitive protein binding assay, concentrations ranged from 0.13 to 0.84 ng/ml throughout infancy and 0.21 to 0.80 ng/ml in adults (Markestadf, 1983). Another approach using GC-MS to measure this analyte in plasma samples after derivatization was described by Coldwell et al. The concentrations from healthy volunteers ranged from 0.05 to 0.79 ng/ml; from 0.30 to 1.30 ng/ml in volunteers after sun exposure (Coldwell et al., 1985).

Another interesting contribution was presented from Fraher et al. (1980), who measured this metabolite's concentration in human serum samples of different groups using radioimmunoassay. The mean concentration of 18 healthy subjects was 587 pmol/L; for seven patients with osteomalacia, the levels were very low or even undetectable (<96–231 pmol/L). Moreover, the group investigated the renal importance in the production of the metabolite. They determined the concentration in the serum of anephric patients (patients without functioning kidneys). The subjects exhibited detectable concentrations of the analyte and there was a strong correlation with the circulating 25(OH)D_3 levels. However, when compared to the 25,26(OH)$_2D_3$ concentrations from healthy subjects for a given 25(OH)D_3 level, the anephric patients showed lower concentrations, suggesting that the kidney potentially contributes partially to the total circulating concentration of this metabolite. The authors concluded that extrarenal sites for 26-hydroxylation of the precursor molecule (25(OH)D_3) exist in man. Finally, they confirmed a strong correlation between the concentrations of the dihydroxylated metabolite and the precursor.

The chromatographic separation of 25,26(OH)$_2D_3$ from other vitamin D metabolites, in particular 24,25(OH)$_2D_3$ is crucial, since interferences have been observed (Kaufmann et al., 2017; Ketha et al., 2016). Determination of these two compounds could potentially provide an alternative to expensive genetic tests detecting CYP24A1 mutations and could be a helpful screening tool. Kaufmann et al. suggested an explanation of the detectable levels of 24,25(OH)$_2D_3$ in patients with idiopathic infantile hypercalcemia (IIH) (Kaufmann et al., 2017), including the demonstration that 25,26(OH)$_2D_3$ interferes in the chromatographic separation of 24,25(OH)$_2D_3$. After derivatization using DMEQ-TAD, the authors determined that on average 80% of the supposed 24,25(OH)$_2D_3$ levels originated from 25,26(OH)$_2D_3$ in patients with IIH.

8 | PROGRESS IN VITAMIN D STANDARDIZATION PROGRAMS

In the past, there was no internationally recognized procedure or reference material to ensure that every laboratory obtains the same results for measuring the total 25(OH)D level, regardless of the assay that was used (Binkley & Carter, 2017). Vitamin D standardization programs are important so that results from different laboratories can be used to determine accurate biological requirements and assess potential links to disease (Brooks & Sempos, 2017). At this point, it is important to mention that standardization is not the same as harmonization. Standardization takes harmonization a step further and requires not only agreement between the measured values among the different laboratories but also agreement to the "true" value (Binkley & Carter, 2017). To establish consensus between clinical and public health guidelines for vitamin D, the VDSP has been operational since 2010. It is now possible to standardize 25(OH)D measurements in current and future measurement systems, due to the collaboration of the National Institute for Standards and Technology (NIST) and their reference methods, the University of Ghent, the Centers for Disease Control and Prevention (CDC) and the SRM program from NIST (Binkley, Dawson-Hughes, Durazo-Arvizu, et al., 2017; Wise, Tai, et al., 2017). Moreover, there are other initiatives such as the CDC's Vitamin D Standardization Certification Program (VDSCP), the College of American Pathologists Accuracy-Based Vitamin D Survey and the Vitamin D External Quality Assessment Scheme (DEQAS) as well as statistical criteria for assessing traceability developed by Ghent University (Sempos et al., 2017).

SRMs from NIST are either 25(OH)D calibration solutions (2972a) or pooled serum materials (972a, 2973; Tai et al., 2017). CDC's VDSCP is a process to assess 25(OH)D concentrations, including accuracy and precision. Assay manufacturers and commercial, clinical and research laboratories can take part in this program. As well, the College of American Pathologists Accuracy-Based Vitamin D Survey and the DEQAS are used for

proficiency testing and/or external quality assessment. The VDSP coordinated the first study (2012) to assess the commutability of NIST's SRM, DEQAS and College of American Pathologists materials (Phinney et al., 2017). The majority of the test materials were found to be commutable with both immunoassays and LC-MS/MS methods. Also, the VDSP coordinated an interlaboratory study between 15 different laboratories to assess the comparability of measurements of total 25(OH)D in serum samples (Wise, Phinney, et al., 2017). The recommended assay performance limits were set by VDSP and were as follows: CVs of ≤10% and biases ≤5%. Nearly all LC-MS/MS methods met the criteria, whereas only 50% of the immunoassays achieved CV of ≤10% and three out of eight of them achieved bias ≤5%.

Previous measurements of 25(OH)D from national surveys or epidemiological studies can be retroactively standardized to the gold standard reference measurement procedure, if serum samples are still available and have been stored properly. In order for a method to be standardized for the measurement of total 25(OH)D, it needs to be accurate and traceable to the appropriate reference methods and reference materials. In general there are five steps in a VDSP protocol (Binkley, Dawson-Hughes, Durazo-Arvizu, et al., 2017): (1) estimate the number of the serum samples that need to be re-measured; (2) use the VDSP's sampling approach (Tian et al., 2014); (3) use a certified method that is traceable to the gold standard reference measurement procedure assay to remeasure the 25(OH)D concentration of the above samples; (4) develop a mathematical model to convert the initial 25(OH)D value to the "new-true" value; and (5) apply the model to the whole data set. Sempos et al. has described the five steps mentioned above in detail for both clinical and research laboratories, regardless of the chosen assay type (Durazo-Arvizu et al., 2017; Sempos et al., 2017).

Even though the possibility of standardization of 25(OH)D assays exists, it seems that even today many commercially available assays do not meet the criteria of precision and bias as set out in the VDSP. There is still difficulty in the clinical evaluation and classification of the patients based on their vitamin D status (sufficient, insufficient, deficient, or severely deficient). Bjerg et al. in 2017 evaluated the accuracy of seven different assays for 25(OH)D measurement using the SRM 972a from NIST (Bjerg et al., 2019). All assays achieved precision below VDSP requirement (CVs of ≤10%), but only two of them achieved the required bias (bias ≤ 5%) when measuring the SRM. Moreover, clinically relevant differences were detected among the assays, especially in the deficiency group. That is, even 8 years after the initiation of the VDSP, there are still many assays, which do not achieve the requirements, with potential clinical consequences for the patients.

9 | COMPLEX METABOLIC BIOMARKERS AND ADVANCED CLINICAL APPLICATIONS

Beyond the established role of vitamin D on bone health, pleiotropic effects of this vitamin have emerged, associating it with numerous health conditions and diseases. For example, the vitamin D status marker, serum 25(OH)D has been linked to cancer, cardiovascular diseases, liver diseases, metabolic-related conditions such as diabetes and to overall mortality (Baute et al., 2019; Martin & Reid, 2017; Stokes & Lammert, 2016; Stokes et al., 2013). Given the emergence of other vitamin D metabolites with reported biological properties (e.g., free 25(OH)D and 24,25$(OH)_2$D) as already mentioned in this review, there is an increasing need to capture the vitamin D metabolic phenotype (i.e., the full set of vitamin D metabolites) and associate it with specific disease phenotypes at a given time point as well as with patterns of change in this metabolic phenotype longitudinally. Such an approach might help us to better understand the biological role of vitamin D in health-related conditions that go beyond its classic functions in bone health. This type of data might also help to make sense of the discrepant findings, which are often reported in the literature regarding vitamin D and its health-related associations.

Having reliable analytical tools such as those used in mass spectrometry provide invaluable advantages, because the accurate assessment of the vitamin D metabolites can have significant implications for clinical decision making—an area which is already fraught with discrepancies in terms of clinical recommendations. For example, various concentrations of 25(OH)D have been recommended as representative of optimal vitamin D status. The Institute of Medicine recommends values ≥20 ng/ml which is based on research regarding bone health (Ross et al., 2011). Conversely, the Endocrine Society (Holick et al., 2011) advocate values of 30 ng/ml and above. Others have gone even further and suggested optimal values for what constitutes an adequate vitamin D status might indeed depend on the disease in question (Spedding et al., 2013). Therefore, vitamin D supplementation regimes should ideally take these factors into account, whether in the clinical or the research setting.

Regular monitoring of circulating vitamin D in serum is also recommended during replacement interventions. This is because unlike water-soluble vitamins, ingestion of fat-soluble vitamins (such as vitamin D) can lead to a

build-up in the body stores and this can be toxic at higher levels (e.g., >150 ng/ml) (Alshahrani & Aljohani, 2013). For this reason, any patient undergoing vitamin D replacement therapy needs regular blood work (e.g., at baseline and after 3 and 6 months of beginning treatment) to monitor 25(OH)D and essential parameters related to vitamin D metabolism such as calcium, phosphate and parathyroid hormone (PTH) concentrations. Measuring serum calcium and phosphate concentrations helps when attempting to determine whether indications of toxicity are present (Holick, 2007). Moreover, given the known inverse association of low vitamin D status (25(OH)D) with PTH, patients should be checked for secondary hyperparathyroidism when vitamin D status is low (Sahota et al., 2004). Conversely, PTH levels that return to within the normal range indicate that sufficient vitamin D concentrations have been biochemically attained (Holick et al., 2011; Sahota et al., 2004) and might even represent a treat-to-target approach in certain circumstances.

Vitamin D testing can be cumbersome both from the point of view of the patient who requires regular testing but also because of the resources that are required. There are also very few gold-standard methods available for quantifying other vitamin D metabolites. Thus, there is a need for cost-effective analytical approaches to quantify the vitamin D metabolic phenotype which are also valid and reliable. From all of the biological samples discussed in this review, dried blood spots hold great potential from a practical point of view because sampling for vitamin D status would become much easier to implement. Patients would be able to collect the sample themselves in the comfort of their own homes using the simple skin prick method. Furthermore, they could send the sample in the post, therefore circumventing the need for attending any in person testing. The resources required for this process would also be kept to a minimum. However, given the above-mentioned reasons for measuring multiple parameters related to vitamin D metabolism, such as PTH and calcium, such methods ideally need to be expanded to capture all the required information that is needed to maintain the safety-related monitoring aspects of vitamin D supplementation.

10 | CONCLUSIONS

Significant progress has been made in the development of mass spectral methods for vitamin D in the last 5 years. Today LC-MS/MS is firmly established as the gold standard technique, for simple target analysis of 25(OH)D_3—as needed for vitamin D status or clinical assessments—but also for measuring complex and intricate vitamin D metabolic fingerprints. LC-MS/MS, in the hands of experienced users, can readily separate the various vitamin D isomers and isobaric species, eliminate interferences and enhance the sensitivity to allow quantitative analysis of very low abundant species. Recent advances in sample preparation techniques have further contributed to the success. Importantly, as physiological levels of vitamin D compounds are often only on the order of only a few picomoles, chemical means to improve their detection ability are required. For this reason, there is a renewed interest in the development of novel derivatization reagents for vitamin D, as seen in the developments during last few years.

Analysis of vitamin D levels is still primarily conducted in serum or plasma samples but has also been explored in nonconventional biological samples, where it has demonstrated promise, both for reasons of analytical practicality and for improving the understanding of vitamin D metabolism in health and disease. Routinely investigated vitamin D compounds today mainly comprise vitamin D, 25(OH)D, 1,25$(OH)_2$D, 24,25$(OH)_2$D as well as the corresponding C-3 epimers, but in the future, there are likely to be other metabolites "pulled-out" from the sample's background because of improved mass spectral methodologies that increase sensitivity and specificity. Furthermore, it is now only a matter of time until routine HRMS assays replace many of the established triple-quadrupole MS methods in clinical laboratories as they provide higher specificity and accuracy with comparable sensitivity to LRMS assays. Finally, as shown in this review, successful harmonization and standardization of mass analytical procedures has strongly improved reliability and comparability of vitamin D assays on a global scale.

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