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Abstract: The constantly increasing requests for the measurement of serum 25-hydroxyvitamin D over the last years has led reagent manufacturers to market different automated and semi-automated methods, that being unfortunately not fully harmonized, yield different results. Liquid chromatography coupled to tandem mass spectrometry (LC/MS2) has more recently been introduced. This approach allows the distinction between the two forms of 25-hydroxyvitamin D and to measure other metabolites. This approach also requires harmonization to curtail the differences between the different analytical methods. To meet this requirement, the American National Institutes of Health (NIH), the CDC (Centre for Disease Control and Prevention) in Atlanta, the NIST (National Institute of Standards and Technology) and the vitamin D Reference laboratory of Ghent University have pooled their expertise to develop a standardization program.
This article reviews the main elements and the difficulties of the automated and semi-automated methods for 25-hydroxyvitamin D, from sample preparation to the analytical phase, as well as those related to mass spectrometry. It also addresses the issues related to the clinical decision thresholds and the possibility of measurements in different biological liquids.

Measurement of circulating 25-hydroxyvitamin D: A historical review

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Keywords: Vitamin D, 25-hydroxycholecalciferol, 25-hydroxyergocalciferol, 25OHD, HPLC, mass spectrometry, immunoassays.

Abstract

The constantly increasing requests for the measurement of serum 25-hydroxyvitamin D over the last years has led reagent manufacturers to market different automated and semi-automated methods, that being unfortunately not fully harmonized, yield different results. Liquid chromatography coupled to tandem mass spectrometry (LC/MS^2) has more recently been introduced. This approach allows the distinction between the two forms of 25-hydroxyvitamin D and to measure other metabolites. This approach also requires harmonization to curtail the differences between the different analytical methods. To meet this requirement, the American National Institutes of Health (NIH), the CDC (Centre for Disease Control and Prevention) in Atlanta, the NIST (National Institute of Standards and Technology) and the vitamin D Reference laboratory of Ghent University have pooled their expertise to develop a standardization program.

This article reviews the main elements and the difficulties of the automated and semi-automated methods for 25-hydroxyvitamin D, from sample preparation to the analytical phase, as well as those related to mass spectrometry. It also addresses the issues related to the clinical decision thresholds and the possibility of measurements in different biological liquids.

Introduction

The role of cholecalciferol or vitamin D_3 in growth and bone metabolism is well established [1]. Its effects in the prevention and treatment of diseases as varied as diabetes, multiple sclerosis and cancer have also been reported, but are still matter of debate [2-6]. Both the Institute of Medicine (IoM) [7] and the Agency for Healthcare Research and Quality (AHRQ) [8] have published extensive documents dampening the optimism aroused by these reports. The AHRQ report [8] makes the case that studies (observational, randomised controlled interventions) and systematic reviews or meta-analyses based on those, involved different types of assays that, except for the most recently published, did not use appropriate reference material. It also shows, as a series of bubble plots, that there was an important variation in responses to vitamin D supplementation (Figure 1). This apparent variation is multifactorial. The individual response to sun exposure and the formulation of the vitamin D supplement are parts of the equation. However, inter-laboratory variations also contribute to this observation as they hinder comparison between results. Indeed, the inter-laboratory differences between the mean serum 25OHD values, that reached almost 32%, according to a DEQAS survey in 1994, could have, in those years, possibly lead to misclassification of patients in terms of vitamin D nutritional status, despite the fact that their ranking might have been similar. Since then, the standardisation process has improved, and in 2009, the inter-laboratory imprecision had dramatically decreased [9], and thus if similar experiments were conducted today, the dose-response relationship might be tighter. In any case, these limitations restrain the conclusions of past epidemiological studies on the circulating 25OHD concentrations required for optimal health status.

As it has often been mentioned, the number requests for the measurement of circulating 25-hydroxyvitamin D (25OHD), the accepted biomarker for the vitamin D nutritional status [10,11],

has constantly increased over the last 3 decades, imposing structural and financial burdens on laboratory facilities and public funding. The Ontario Health Technology Advisory Committee (OHTAC) has reported that, the volume of laboratory vitamin D tests had increased from approximately 30,000 in 2004 to over 730,000 in 2009 [12]. Similar observations were made worldwide. This increased request load has lead most of the clinical laboratories to abandon manual binding-protein assays and radio-immunological assays (RIAs), the methods mostly utilised clinical laboratories in the 1980s and early 1990s, in favour of automated competitive binding-protein assays (CBPA), enzyme-linked immunoassays (ELISAs) or chemiluminescent immunoassays (CLIA). Techniques based on high-performance liquid chromatography (HPLC), coupled or not to simple or tandem mass spectrometry, while more exact, are still the privilege of specialised and research laboratories.

The variety of circulating vitamin D metabolites and the complex nature of the matrix makes the measurement of 25OHD difficult, despite the technological advances. Many important issues have still to be resolved to obtain an accurate measure of serum 25OHD concentration. Each phase of the process will be reviewed in order to provide clinical laboratories with information on the difficulties they have to face.

The sample preparation phase

In order to understand the problems related to the recovery of 25OHD during the extraction procedures, one must have some knowledge of the physiological processes involved in its transport. Due to their lipophilicity, vitamins D_3 and D_2, as well as their respective hydroxylated metabolites (ligands), must be transported by amphoteric carriers. Although vitamin D binding-protein (DBP) is their predominant transporter, albumin and lipoproteins are also important components. Whereas vitamin D synthesised in the skin is preferentially transported by DBP to be hydroxylated in the liver, lymphatic chylomicrons and lipoproteins mediate its transport and

hepatic uptake [13-16].

Each ligand-vitamin D-carrier complex possesses its own affinity constant. For example 25OHD binds DBP with high affinity (**K**a = $5X10^{-8}$ M), whereas $1,25(OH)_2D$, the hormonal form of vitamin D, exhibits a lower affinity (**K**a=$4X10^{-7}$ M) [17,18]. In both cases the carrier being in large excess (<5%) of the DBP sites are occupied), the free concentrations of the metabolites are thus extremely low. The other transporters have similar kinetics at however different orders of magnitude. It becomes apparent that the dissociation of 25OHD from the collection of the carriers must be highly efficient in order to obtain an accurate total quantitation. The problem is not so much for protein-binding assays, radio-immunoassays, high performance liquid chromatography, coupled or not to mass spectrometry, that all require an organic extraction step destroying the binding capacity of the carriers, but for automated non-extracting sample assays for which organic solvents are not compatible, and in which alternative releasing agents with proprietary protection are used instead. Since the serum concentration of DBP varies with physiological and pathological conditions, such as pregnancy, oestrogen therapy or renal failure [19-21], the efficiency of the dissociation and on competition kinetics involved in methods relying on pH changes or blocking agents that liberate the 25OHD from its carrier protein could be affected. In support of this hypothesis, several reports have highlighted the inaccuracy of total 25OHD measurement by automated immunoassays and competitive binding-protein assays performed in populations with different levels of DBP [22-25]. Addition of $25OHD_3$ and $25OHD_2$ to serum or plasma samples is customary in evaluating their recovery in the on-line dissociation step from the binding components. The validity of such *in vitro* recovery experiments is founded on the acceptance that exogenous and endogenous vitamin D metabolites fully equilibrate with and bind equally to serum components such as binding proteins. In practice, this may not occur. The rise in serum pH during storage, decreasing the affinity of binding proteins for Vitamin D metabolites,

might stimulate the sequestration of exogenous 25-OHD by other serum components, such as lipids or lipoproteins. Carter et al. [26] and Horst [27] have reported this artefact showing an under-recovery of exogenously added 25OHD in automated assays. This has been extended to methods based on HPLC-tandem-mass spectrometry, when Lankes et al. [28] have shown that the recovery of 25OHD was affected by suboptimal extraction conditions. These observations, that elude complete understanding, question the present process of recovery experiments, and warrant caution in interpreting published data.

The analytical phase

Supplements currently provide 2 forms of vitamin D: vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). It is therefore essential that the analytical methods be able to measure the 2 forms equally in order to avoid an underestimation of the circulating 25(OHD in vitamin D_2 supplemented individuals [29-32]. On the other hand, they must be able to distinguish the 25(OH)D-C3-epimer and the 24,25$(OH)_2$D, present in different proportions and thus lead to an overestimation of circulating 25OHD. This is particularly important for samples from infants under the age of 1 year [33] in which the C3-epimer may constitute the major proportion of the toal 25OHD. A number of assays have been published and marketed, certain of which claim to achieve these goals. The following paragraphs address their characteristics.

Binding-protein assays and immunoassays

Table 1a summarizes some of the characteristics of the Binding-protein assays and immunoassays. A limited number of protein-binding assays were reported and used clinically between 1971 and 1980 (Table 1a). Haddad et al. [34] reported first a manual competitive binding-protein assay for the measurement of serum 25OHD. The method was based on the displacement of ^{3}H-labelled 25OHD_3 from post-microsomal kidney supernatants of rachitic rats

by human serum ether extracts followed by chromatography on silicic acid columns. The authors suggested that the crude binding-protein assay recognized equally $25OHD_3$ and $25OHD_2$. The assay analytical sensitivity was 10 nmol/L. Almost 10 years later, Delvin et al. [35] published a simplified protein-binding assay using a commercially available bovine □-globulin enriched fraction (Cohn fraction IV). The serum samples, spiked with purified ^{3}H-$25OHD_3$, for recovery calculation purposes, were chromatographed on silicic acid columns after lipoprotein precipitation with heparin/$MnCl_2$. The analytical sensitivity was 5 nmol/L. Although both $25OHD_3$ and $25OHD_2$ were equally recognized, contrary to the rat kidney extracts, the □-globulin fraction did not show affinity for $24,25(OH)_2D$. These assays requiring chromatographic purification on silicic acid and Sephadex LH-20 column after organic extraction were time-consuming and could not be implemented in routine clinical laboratories. In 1984, Bouillon et al. [36] described a non-chromatographic direct assay for 25OHD using rachitic rat serum as the source of DBP, after extraction with ethylacetate and cyclohexane. It measured $25OHD_3$ and $25OHD_2$ equally and exhibited a 100% cross-reactivity for $24,25(OH)_2D$. Parviainen et al. [37] published in 1981, a method based on both HPLC separation of vitamin D metabolites and their subsequent measurement by competitive binding-protein for 25OHD and $24,25(OH)_2D$ or vitamin D-receptor assay for $1\alpha,25(OH)_2D$. Although the recovery of the labelled metabolites was relatively low, the precision was below 10% for 25OHD. This method proved to be time-consuming and hence was not applied for routine purposes by other groups. Although the above assays exhibited clinically acceptable analytical sensitivity and imprecision, they soon became obsolete with the development of polyclonal antibodies directed against 25OHD that lead to radio-immunoassays (RIAs), and the with the simplification of High-Performance Liquid Chromatography (HPLC) equipment that allowed their introduction in clinical laboratories.

Radioimmunoassays

RIAs, developed early in the 1980s, constitute the next generation of assay methods. In 1984, Bouillon et al. [36] described a simplified non-chromatographic RIA, based on the production of rabbit polyclonal antibodies directed against BSA-25OHD$_3$-hemisuccinate conjugate and the competition of the serum-extracted 25OHD for [26(27)-methyl-^{3}H]-25-hydroxyvitaminD$_3$ as tracer. Although the assay was analytically as sensitive as the binding-protein assay, the 2 anti-sera produced had widely different characteristics in terms of specificity, the cross-reactivity varying between 0 and 11% for 25OHD$_2$ and 40 to 270% for 1α,25(OH)$_2$D$_3$. The second, developed by Hummer et al. [38], required a preliminary chromatography step, and neither measured 25OHD$_2$ decreasing its usefulness in assessing total vitamin D nutritional status, in the context of where vitamin D$_2$ was widely used as dietary supplement. The next year, Hollis et al. [39] described and validated a non-chromatographic radioimmunoassay based on an anti-serum raised against the 23,24,25,26,27-pentanor-C-(22)-carboxylic acid vitamin D-BSA conjugate. [26,27-methyl-^{3}H]-25-hydroxyvitaminD$_3$ was also used as tracer. Although the antibody had little affinity for both 1α,25(OH)$_2$D$_3$ and 1α,25(OH)$_2$D$_2$ (5%) or for vitamin D$_3$ or D$_2$ (10%), it had a 100% cross-reactivity for 25OHD$_2$ and the other known vitamin D metabolites. Of concern, in this assay, is the radically different recovery of labelled 25OHD$_3$ depending whether the tracer was added to the sample before or after the addition of acetonitrile. In order to obtain a quantitative recovery, the tracer had to be added after the addition of acetonitrile. If it was added to the native sample and equilibrated before the extraction step, then the recovery dropped to 53%. One may therefore question whether the endogenous 25OHD was quantitatively recovered.

To further confuse matters, in the above-mentioned assays, when recovery was monitored, only ^{3}H-25OHD$_3$ was used. Under those conditions, as Stryd et al. [40] had emphasized as soon as

1978, total 25OHD could be underestimated since the recovery of the 2 vitamin D isomers may not necessarily be identical in the extraction processes.

Eight years later Hollis et al. [41] described a radioimmunoassay based on goat anti 23,24,25,26,27-pentanor-C(22)-carboxylic acid of vitamin D-BSA conjugate and ^{125}I-vitamin D-23,24,25,26,27-pentanor-C(22)-carboxylic-amide-3-aminopropyl as the tracer. As in the former assay [39] this antibody had little affinity for both 1α,25$(OH)_2D_3$ and 1_a,25$(OH)_2D_2$ (2.5%) or for vitamin D_3 or D_2 (<1%), and had a 100% cross-reactivity for 25OHD_2 and the other vitamin D metabolites. Despite the fact that collectively these metabolites account for a small percentage, the assays probably did over-quantify the "true" 25OHD concentration. Nevertheless this RIA gave a better estimate of the total vitamin D status as both 25OHD_3 and 25OHD_2 could be quantified equally, on the proviso that 25OHD was quantitatively recovered during the extraction procedure. This assay is probably the one that led to the 1st commercial radioimmunoassay for the measurement of 25OHD marked by DiaSorin (Stillwater, MN, USA).

Table 1b summarizes the characteristics of the marketed radioimmunoassays and automated non-radioactive immunoassays. It can be appreciated that the 2 RIAs differ in their performance claimed by the respective manufacturers. The DiaSorin assay measures 25OHD_2 and 25OH_3 equally whereas the IDS RIA underestimates 25OHD_2 by 25%. The different affinity of the antibodies may be due to the difference in the vitamin D analogue used to raise the polyclonal antibodies. DiaSorin using as the hapten a vitamin D analogue that lacked the side-chain while retaining the open B-ring *cis*-triene structure common to both vitamins D_2 and D_3 ensured that the antibodies would only recognize this structure. It should be noted that neither assay kit is standardised with reference material, thereby diminishing their accuracy. In both cases the lower limit of detection is in the range of 3 nmol/L, although there are no independent data to support

this claim. The assays also differ in their imprecision, DiaSorin reporting an intra-assay CV of 11.7% at 21.5 nmol/L and IDS a CV of 5.3% at 26 nmol/L. Although DiaSorin and IDS claimed 100% 25OHD recovery from spiked samples, a 2005 DEQAS survey reported, for the DiaSorin assay, a mean recovery of 82% and 83% for exogenous $25OHD_3$ and $25OHD_2$ respectively [26]. In the case of the IDS RIA kit, the recoveries were 45% and 25% for $25OHD_3$ and $25OHD_2$ respectively. Both methods used an acetonitrile extraction of vitamin D metabolites. Addition of NaOH in the initial denaturation-extraction procedure of the IDS RIA has been suggested as the source of the difference. This hypothesis can be dismissed as both the DiaSorin and IDS assays gave similar results for the specimen containing only endogenous vitamin D. The discrepancy can be explained at least in part by the lower affinity of the IDS primary antibody for $25OHD_2$ [42]. On the other hand, Glendenning et al. [43] have reported that the DiaSorin RIA overestimates total 25OHD within the range of 40-60 nmol/L when compared to a HPLC method.

Automated Immunoassays

Radioimmunoassays gradually gave way to automated enzyme-linked immunoassays (EIAs), chemiluminescent immunoassays (CLIAs), or competitive binding-protein assays (CBPAs). Characteristics of the direct automated methods found in the manufacturers' information inserts are summarised in table 1b. As can been appreciated, according to the manufacturers' respective inserts, 5 out of 6 automated CLIA-based assays methods measured $25OHD_2$ and $25OHD_3$ equivalently (IDS, DiaSorin, Advia Centaur, Vitros, Beckman) whereas the IDS EIA assay underestimated $25OHD_2$ by 25%, the Abbott CLIA by 18% and Roche ECL by 8%. However in the case of the Advia Centaur, Le Goff et al. [44] using native clinical samples reported a 30% mean overestimation (4–59%) of $25OHD_2$. These assays exhibited, when reported, variable cross-reactivity for $24,25(OH)_2D$ (0% for Beckman to 149% for Roche) and C3-epi-$25OHD_3$ (1% for the IDS CLIA assay to 91% for the Roche CBP assay). Interestingly, van den Ouweland et al.

[45] demonstrated recently, that when present endogenously, C3-epi-25OHD$_3$ is not recognized in the Roche CPB assay and warrant caution in interpreting recovery data. All assays have satisfactory precision, although defined at variable concentrations. It is interesting to note that 4 out of 8 automated assays were directly or indirectly standardized against a National Institute of Standards and Technology (NIST) Standard Reference Material, however none do provide information on recovery of exogenous 25OHD$_3$ or 25OHD$_2$. Automated immunoassays, as well as competitive binding protein assays, are based on delicate non-denaturing conditions to free 25OHD from DBP and other serum binding components to allow its binding either to the kit antibodies or DBP. This step, sensitive to matrix effects, may yield varying results [46,47].

The performance of different commercial assays has recently been reported in independent investigations. Su et al. [48] have reported in a comparative study in which serum samples contained increasing 25OHD$_2$/25OHD$_3$ ratios that a CBPA exhibited a positive bias when samples contained only 25OHD$_3$ and negative biases as the 25OHD$_2$/25OHD$_3$ ratios increased, compared to a LC-MS/MS method (10.8%, -23.6%, -38.4%). As the DBP in all likelihood recognises the 25OHD isomers equally, the bias could be explained by the inefficient recovery of 25OHD$_2$. Holmes et al. [49], compared total 25OHD results in 163 clinical specimens obtained by 3 direct immunoassays, (DiaSorin Liaison assay, Siemens Centaur, Abbott Architect), to those obtained after extraction and followed by LC/MS^2 and RIA. Their data revealed high degrees of random variability and bias relative to LC/MS^2 and RIA results. Importantly, the magnitude of the biases and random errors exceeded the criterion for the total allowable error of a 25OHD test [50] in almost ½ of the clinical specimens and led to misclassify an appreciable number of study patients as vitamin D deficient. Cavalier et al. [51] also reported a concordance between methods varying between 65 to 82% when comparing 6 automated platforms to the NIST/NIH Vitamin D

Standardization Program (VDSP)-accredited LC/MS^2 method. As Sempos et al. [52] have stressed, this inter-assay variability could lead to misleading conclusions in epidemiological studies aiming at evaluating the vitamin D status and to limiting the comparability between national surveys.

High performance Liquid Chromatography

Table 2 lists the different HPLC methods published the last 35 years. Eisman et al. [53] published the 1st HPLC method for the measurement of 25OHD in 1978, followed within a year by Gilbertson et al. [54] and Jones [55]. Variants of these initial methods have been published until very recently [56-69]. As can be appreciated, although the HPLC-based methods were able to separate $25OHD_2$ from $25OHD_3$, the authors used either a single in-house or commercial labelled $25OHD_3$ internal standard or even surrogate molecules (retinyl acetate, docecanophenone, derivatised 25-hydroxydehydrocholesterol, 1α-OHD) to monitor the recovery of 25OHD, although reporting in most case concentrations for both isomers. However, Stryd et al. [70], as early as 1978, questioned the accepted notion that $25OHD_2$ and $25OHD_3$ behaved identically during the extraction and chromatographic procedures, and therefore held that using the recovery of the tracer ^{3}H-$25OHD_3$ to calculate the concentration of the 2 isomers was an error. This led them to report values only for $25OHD_3$ contrary to others. This premise can be extended to the proxy tracers. Among variants reported, Shimada et al. [60] used 2 internal standards: $25OHD_2$ (IS_1) and derivatised 25-hydroxy-7-dehydrocholesterol (IS_2) to assess $25OHD_3$ recovery. However the methodology used requires clarification. To start with, they added the 1st internal standard after precipitation of plasma proteins with ethanol, thereby removing an important step that could lead to misinterpretation. They also performed experiments to evaluate the "absolute" recovery of $25OHD_3$. For this part, they added $25OHD_3$ standards to 7% buffered Bovine Serum

Albumin together with the IS_1 and performed the extraction. They then added the IS_2 after the HPLC process they calculated the peak-height ratios between the $25OHD_3$, the IS_1 and IS_2. It is difficult to conceive how this manoeuvre allows the accurate assessment of the endogenous 25OHD. Some investigators have proposed a coulometric electrochemical detection system [61,69] based on the oxidation potential of the conjugated-diene structure of vitamin D metabolites to quantitate 25OHD after the HPLC step. Although this detection method is as efficient as methods based on UV, it is not widely adopted by clinical laboratories. This may be due to the demanding maintenance of the detectors. The recovery studies vary in their structure (labelled or not-labelled tracer, 25OHD or surrogate molecules). Hence it is difficult to assess accurately the performance of the methods. Also, precision data vary in terms of the concentrations at which the experiments were performed. The accuracy of the methods described is ill-defined, as in most cases no calibrator traceable to a standard reference material was available. Hymøller et al. [68] have shown that their method yielded results within acceptable boundaries for $25OHD_2$ and $25OHD_3$ for the National Institute of Standards and Technology (NIST) standard reference material 972.

Mass spectrometry

Watson et al. [71] described in 1991 an on-line HPLC-Thermospray (TSP) mass spectrometry method for vitamin D_2, vitamin D_3, and their respective mono- and di-hydroxylated metabolites. However, at that stage, they reported a superior precision for UV absorbance than for TSP, which they attributed to the inherent instability of the TSP ion beam. Since Vogeser [72] and van den Ouweland et al. [73] have published extensive reviews on the subject, a summary is presented in table 3 that highlights, in a chronological order, the methodology and performance characteristics of published methods since 2001 [74-97]. The methods fall into two categories, those involving

derivatisation of the vitamin D metabolites, and those based on analysis of the native compounds. Higashi et al. [74,75], Ding et al. [83] and Kaufmann et al. [97] have developed methods for the measurement of 25OHD by atmospheric pressure chemical ionisation in the positive mode ($ACPI^+$) LC/MS^2 following derivatisation by the Diels-Adler reaction with Cookson-like reagents. The addition of a nitrophenyl group to the conjugated-diene portion of the secosteroids, increases the ionisation efficiency relative to the native metabolites, and the analytical sensitivity by moving molecular masses of the parent ions to a region where there is reduced background noise thereby increasing the signal/noise ratio. Although sensitive and specific, these labour-intensive methods are not transposable for routine analysis in clinical laboratories. They however are useful for vitamin D metabolite profiling as shown recently by Kaufmann et al. [97].

Three candidate reference methods have been proposed in the last 10 years. In 2004, Vogeser et al. [76] published the 1st candidate reference method for the measurement of $25OHD_3$ by stable isotope-dilution LC/MS^2 applicable to clinical laboratory practice. Their method involved a protein denaturation process to release the bound vitamin D metabolites, and on-line solid-phase extraction before the reverse-phase HPLC coupled to MS^2 with the detector set in the electrospray atmospheric pressure ionisation in the positive mode. In 2010 and 2011, Tai et al. [85] and Stepman et al. [86] proposed each a candidate method that differed from that of Voseger et al. [75] and from each other in a number of ways. Whereas Voseger et al. [76] utilised a $25OHD_3$ internal standard containing 3 Deuterium and 1 ^{13}C atom, Tai et al. [85] used tri-deuterated $25OHD_3$ and $25OHD_2$, and Stepman et al. [86] hexa-deuterated hydroxylated vitamins D_2 and D_3. Differences lied also in the sample volume (200 µl to 2 ml), sample preparation (liquid-liquid or solid-phase extraction), HPLC conditions and detection process [$APCI^+$ or ESI^+ and multiple reaction monitoring (MRM) or single reaction monitoring (SRM)]. Despite their

differences, the IFCC Joint Committee for Traceability in Laboratory Medicine (JCTLM) recognized Tai's et al. [85] and Stepman's et al. [86] as reference method procedures (RMP). Furthermore, the National institute of Standards and technology (NIST) has used Tai's et al. [85] candidate RMP to certify the concentrations of $25(OH)D_3$ and $25(OH)D_2$ in their Standard Reference Material for Vitamin D in human serum to validate the accuracy for the methods used in clinical laboratories. The other tandem-mass spectrometry methods published in the last 10 years all have quantitation limits below 10 nmol/L well below the concentration considered as severe hypovitaminosis (25 nmol/L) [77-82, 84, 87-96].

The TMS approach has gained ground over the last 10 years, and according to the October 2013 Vitamin **D** **E**xternal **Q**uality **A**ssessment **S**cheme (DEQAS, www.deqas.org), 25% of the participants reported using such a method. Mass spectrometry methods have the advantage of being able to measure all species of the 25-hydroxylated vitamin D, including the di-hydroxylated moieties. Furthermore this physical method is not bound to the conditions imposed by the manufacturers, although commercial "turn-key" tandem-mass spectrometry methods are now available. Gervasoni et al. [95] have recently reported a comparison between 2 such methods. Although both methods are suitable for routine, they make the point that, in their hands, the Chromsytems kit does not allow quantitation of $25OHD_2$ and that the Perkin-Elmer kit without derivatisation does not guarantee acceptable performance.

Problems related to LC-TMS

The development of refined informatics coupled to the simplified TMS equipment have led users to underestimate the complexity of the analytical processes involved in the quantitation of vitamin D metabolites and hence to undervalue limitations that may compromise the dependability of the data. The sample preparation, including the protein denaturation, the

extraction, the chromatography, although important, have been overlooked because of the preconceived perception that the high selectivity of the mass spectrometer detectors could cover for the lack in the preparatory steps. However this misconception has vanished with time when it was realised that isobaric compounds co-eluting with the vitamin D metabolites could affect precision, accuracy and sensitivity of the method [72,100,101]. Therefore minimal HPLC separation of the target metabolites with retention times close to the column dead volume should be avoided as it may lead to ion suppression by co-eluting substances [100,101].

The example of 1α-OHD and 7α-OH-4-cholestene-dione (a marker of bile acid mal-absorption) as being potential interfering substances in the TMS analysis, but resolved by the HPLC step illustrates this point [78]. At the level of the quantification of the two forms of 25OHD, and of their respective C3-epimer, the methods described so far make use of the same protonated molecular ions [H^+25OHD_3 (m/z 401), H^+25OHD_2 (m/z 413)] but of different transition ions, which efficacy of formation is instrument- and energy-dependent [73]. The use of specific qualifier and quantifier TMS transition ions, instead of the often-applied water-lost ions, also reduces specificity problems [102]. This is exemplified when using $^2H_6$-25OHD_2 for 25OHD_2 analysis and water loss is monitored. Under these circumstances, HPLC resolution of 25OHD_2 and 25OHD_3 is compulsory as the signal contribution from the internal standard to 25OHD_3 takes place when the water loss from $^2H_6$-25OHD_2 yields the same transition ion as the 25OHD_3 parent molecule. Hence no further selectivity is gained from monitoring a second water loss for the daughter ion. [80,82]. Knox et al. [103], recognising that the purification steps are time-consuming in the perspective of clinical laboratories, proposed a procedure that involves protein precipitation with Methanol and a robotised 6-step solid-phase extraction, that could handle up to 300 samples per day. This procedure should yield cleaner extracts before injection on the HPLC-

TMS instrument, decrease background noise and increase sensitivity.

As specific as LC-TMS may be for the measurement of vitamin D metabolites, precision and accuracy depend on a strict standardisation procedure. This aspect has been Achilles' heel of this field until recently, when SRM was widely made available by the NIST, and weakens the threshold definition for vitamin D nutritional status. However there are other elements to the inaccuracy of measured 25OHD concentrations. One of these is the C3-epimer of $25OHD_3$ present in high concentration in infants' serum [104] and later, to a lesser extent, in adults [92]. This is particularly true for methods that do not separate this metabolite. As there are diverging opinions on the biological action of C3-epi-$25OHD_3$ [105,106] the question of reporting its concentration remains. Whatever the answer is, it should be quantified for further potential clinical evaluation. The observed coefficients of variation in a 2013 DEQAS survey varying between 11 to 25% for all tested laboratory methods (437 participants) and between 9.7 to 11.3% for TMS-based methods (147 laboratories), illustrate the between laboratory and laboratory imprecision. However the lack of a RMP and/or RSM prohibited the evaluation of the accuracy. These steps having been solved [85,86], the NIST has produced the SRM 972, consisting of 4 vials of frozen human serum containing 4 different certified $25(OH)D_3$ et $25(OH)D_2$ concentrations and one of C3-epi-$25(OH)D_3$ [107] and $25(OH)D_3$ et $25(OH)D_2$ ethanol calibrators [108]. The introduction of these certified reference and calibration materials will improve the analytical performance of all methods, as Cavalier et al. [109] have shown for automated methods. The precision issue being resolved, accuracy remains. Carter et al. [110] have reported in a detailed study of analytical performance of the laboratories using LC-TMS, an 11% positive bias with respect to the RMP and suggested that it was due to the inclusion of the C3-epimer, that most laboratories could not separate from $25OHD_3$.

The consortium uniting of the Office of Dietary Supplements of the American National Institutes of Health (NIH), the Centre for Disease Control and Prevention (CDC) in Atlanta, the NIST and the Ghent University vitamin D Reference Laboratory, has recently initiated a fee-based 3-step standardisation program consisting of 1) the calibration and validation of the $25OHD_3$ et $25OHD_2$ concentrations in 40 serum samples measured by LC-TMS in the Ghent laboratory [52]; 2) the verification of the efficacy of the calibration by the blind analysis of 10 samples every 3 months; and 3) the method comparison and bias estimation according to the Clinical Laboratory Standardization Institute (CLSI) guidelines [111]. The laboratory is accredited if the observed bias is ± 5,0 % and the imprecision ≤ 10% after 4 cycles (1 year). At the present time only 5 laboratories have accreditation label [http://ods.od.nih.gov/Research/vdsp.aspx].

Conclusions

The different serum 25OHD values obtained through the years with different methods may have lead to misclassification of patients in terms of the vitamin D nutritional status. The historical thresholds defining vitamin D sufficiency, insufficiency and deficiency, upon which a supplementation decision was taken, are hence to be interpreted cautiously. Cavalier [112] has made the point that for assuring the "optimal" serum 25OHD concentration at 75 nmol/L, the measured value could vary from 50 to 100 nmol/L and that the threshold should be method-specific. For example, the Diasorin™ method yielding generally lower values that those obtained by LC-TMS, the deficiency and insufficiency thresholds should be re-evaluated. However clinicians will slowly adopt this modification. The C3-epi-$25OHD_3$ present in high concentration in infants' serum and to a lesser extent in adults, remains an issue as there are diverging opinions on the biological action of C3-epi-1α,25$(OH)_2D_3$ [113,114]. Whatever the answer is, it should be quantified for further potential clinical evaluation.

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Table

Table 1a: Characteristics for in-house manual competitive binding-protein and radioimmunological 25OHD assays.

In- house and commercial manual assays							
Reference	Extraction & purification procedures	Vehicle for assay solubilisation	Assay principle	Equivalence for $25OHD_2/25OHD_3$ Cross-reactivity C3-epi/$24,25(OH)_2D$	Traceability Recovery	LOQ/(LOD) nmol/L	Precision Intra-assay *Inter-assay CV*
Haddad et al. [34]	Plasma 1 ml diethyl ether Silicic acid chromatography	Absolute ethanol	Rachitic rat kidney extracts Competitive protein-binding ^{3}H-25OHD as tracer	Equivalence: NR cross-reaction: NR	Traceability NR $25OHD_3$ 64.1 ± 10.9%	NR/(10)	14% at 40 nmol/L *NR*
Delvin et al. [35]	Serum 500 µl Lipoprotein precipitation ($NaHep/MnCl_2$) diethyl ether Silicic acid chromatography	Absolute ethanol	Bovine □-globulin Competitive protein-binding ^{3}H-25OHD as tracer	Equivalence: 74%/100% No cross-reactivity for $24,25(OH)_2D$	Traceability NR $25OHD_3$ 90 ± 1.6%	NR	8.9% at 54 nmol/l *8.4% at 37 nmol/L*
Bouillon et al. [36]	Serum 100 µl EtAc/cycloHexane (1:1 v/v)	Absolute ethanol	Rachitic rat serum Competitive protein-binding ^{3}H-25OHD as tracer	Equivalence: Yes 100% cross-reactivity for $24,25(OH)_2D$	Traceability NR $25OHD_3$ 107 ± 8.9%	NR/(2.5)	5.6% at 45 nmol/L *NR*
Hummer et al. [38]	Serum 500 µl MeCN SPE	Absolute ethanol	RIA ^{3}H-$25OHD_3$ as tracer	Equivalence: 2.2%/100% 10% cross-reactivity for $24,25(OH)_2D$	Traceability NR $25OHD_3$ 93.7-115.1%	NR/(4.3)	4.5% at 54 nmol/l *10.4% at 32 nmol/L*
Hollis et al. [39]	Plasma 25 µl MeCN	Absolute ethanol	RIA ^{3}H-$25OHD_3$ as tracer	Equivalence: Yes 100% cross-reactivity for $24,25(OH)_2D$	Traceability NR $25OHD_3$ 108 ± 18%	NR/(7.5)	< 13%[a] *NR*
Hollis et al. [41]	Plasma/Serum 25 µl MeCN	Absolute ethanol	RIA ^{125}I-CC Derivative	Equivalence: Yes 100% cross-reactivity for $24,25(OH)_2D$	Traceability NR $25OHD_3$ 97 ± 10%	NR/(7.0)	5.6% at 23 nmol/L *15.9% at 23 nmol/L*

Table 1b: Characteristics for manual and automated commercial 25OHD assays according to insertrs

Platform Vendor	Extraction & purification procedures	Assay principle	Equivalence $25OHD_2/25OHD_3$ Cross-reactivity (C3-epi/24,25$(OH)_2$D	Traceability Recovery (%)	LOQ/(LOD) nmol/L	Precision Intra-assay *Inter-assay* CV %
DiaSorin	S/P Acetonitrile	RIA ^{125}I-CC Derivative Goat polyclonal Ab	Equivalence: Yes Cross-reactivity: Yes NR/100%	Calibrators traceable to a pure preparation of the 25OHD Ag calculated by spectrophotometry	6.25*/(4.0)	11.7% at 21.5nmol/L *9.4% at 21.5nmol/L*
Immuno Diagnostics Ltd	S/P 50 µl NaOH Acetonitrile	RIA ^{125}I-25OHD	Equivalence: 75%/100% Cross-reactivity: NR/≥100%	Calibrators standardised by UV quantitation 89-102 at 20 nmol/L	NR/(3.0)	5.3% at 26 nmol/L *8.2% at 20 nmol/L*
Immuno Diagnostic Systems Ltd	S/P 25 µl 2-step procedure w/o extraction	EIA Immobilised anti-25OHD sheep polyclonal Ab, 25OHD-labelled with biotin HRP/TMB	Equivalence: 75%/≥100% Cross-reactivity NR/≥100%	Calibrators standardised by UV quantitation. 97-105	NR/(5.0)	5.3% at 39 nmol/L *4.6% at 40 nmol/L*
Immuno Diagnostic Systems Ltd	S 10 µl 2-step procedure Denaturation DBP + NaOH	CLIA Acridinium-labeled anti-25OHD sheep polyclonal Ab	Equivalence: Yes Cross-reactivity: 1%/NR	Calibrators standardised to ID-LC-/MS/MS) 25OHD RMP; traceable to the NIST SRM 2972 Recovery not reported	17.5/(6.0)	6.2% at 30 nmol/L *11.6% at 30 nmol/L*
DiaSorin Liaison Total		CLIA HRP - Isoluminol derivative	Equivalence: Yes Cross-reactivity: 1.3%/NR	Calibrators traceable to UV spectrophotometric	10.0/(NR)	3.8% at 20 nmol/L *12.2% at*

DiaSorin				analysis.		*nmol/L*
Advia Centaur Siemens	S/P 20 µl Buffered releasing agent	CLIA Acridinium-labeled mouse mAb Fluorescein vitamin D analog Anti-fluorescein mAb PMP 1-anilinonaphthalene-8-sulfonic	Equivalence: Yes 104%/100% Cross-reactivity: 1.1%/NR	Calibrators standardised to ID-LC-/MS/MS) 25OHD RMP; traceable to the NIST SRM 2972 Recovery not reported	10.5(8.0)	4.7% at 34 nmol/L *11.9% at 34 nmol/L*
Architect 1 Abbott	S/P 60 µl 2 step procedure EtOH/triethanol amine /ANSA	CLIA Sheep polyclonal Ab-anti-25OHD Acridinium-labeled biotinylated anti-biotin IgG complex	Equivalence: 82%/100% Cross-reactivity: 2.7%/112%	NR No mention of traceability Recovery not reported	20 (7.8)	3.1% at 58 nmol/L *4.0% at 58 nmol/L*
Roche Elecsys Roche Diagnostics	S/P 15 µl 2 step procedure Dithiothreitol pH 5.5 Then NaOH	ECL CBPA Ruthenium	Equivalence: 92%/100% Cross-reactivity: 91%/149%	Standardized against in house LC- MS/MS standardized to the NIST standard Recovery not reported	10 (7.5)	*7.8% at 17 nmol/L*
Vitros 5600 Vitros	S 60 µl 1 step procedure Acid pH	CLIA Sheep mcAB-anti-25OHD Horseradish peroxidase - Luminol	Equivalence: Yes Cross-reactivity: Yes 37.4%/34.3%	In house reference calibrators Correlation to LC/MS/MS Recovery not reported	32 (21.6)	7.4% at 56 nmol/L *14.0% at 56 nmol/L*
Beckman Dxi Beckman-Coulter	S/P 30 µl 1 step procedure Tris buffered saline	CLIA Sheep mcAB-anti-25OHD 25OHD analogue AP-conjugate Lumi-Phos* 530	Equivalence: Yes Cross-reactivity: 65%/0%	Calibrators standardised to ID-LC-/MS/MS) 25OHD RMP; traceable to the NIST SRM 2972 Recovery not reported	11 (3.7)	4.6% at 39 nmol/L *8.1% at 39 nmol/L*

Unless otherwise specified, the characteristics of the commercial assays are derived from the information given in the respective inserts. [a]Concentration tested not reported. Recovery refers to the % of the exogenously added $25OHD_3$ (nmol/L) before extraction recovered at completion of the assay. RIA:RadioImmnunoAssay; EIA: Enzyme-Linked ImmunoAssay; CLIA: ChemiLuminescent ImmunoAssay, CBPA: Competitive Binding-Protein Assay.
S: Serum; P: Plasma; LOQ: Lower limit of Quantification defined as a measure with a CV <20%; LOD: Lower limit of Detection defined as the lowest concentration that can be defined with a confidence of 95%; NR: Not reported; CV: coefficient of variation at the lowest concentration tested.
EtOH: Ethanol; ^{3}H-$25OHD_2$: [23,24(n)-^{3}H]-25-hydroxyvitamin D_3 or [26(27)-methyl-^{3}H]-25-hydroxyvitamin D_3; ^{125}I-CC: vitamin D-23,24,25,26,27-pentanor-C(22)-carboxylic-amide-3-aminopropyl; ANSA: 8-anilino-1-naphthalene sulfonic acid; IgG:Immunoglobulin G; mcAB: monoclonal antibody; BSA: Bovine Serum Albumin; AP: alkaline phosphatase; Lumi-Phos* 530: Trademark of Lumigen Inc. (Southfield, MI); ID-LC-/MS/MS: isotope dilution-

liquid chromatography/tandem mass spectrometry; RMP: Reference Method Procedure; NIST: National Institute of Standards and Technology; SRM: Standard Reference Material. *Personal communication (E Cavalier)

Table

Table 2: Physical separation and detection methods

Reference	Sample volume Extraction procedure Chromatographic procedure Detection Wavelength	Internal standards Analyte measured	Recovery	LOQ nmol/L	Precision Intra-assay CV *Intra-assay CV*
Eisman et al. [51]	Plasma 4 ml Extraction: MeOH:$CHCl_3$ (50:50 v/v) Pre-treatment: Sephadex LH-20 SkellySolve B: $CHCl_3$ (50:50 v/v) SkellySolve B: $CHCl_3$:MeOH (18:2:1 v/v) HPLC: Porasil silicic acid column 2-propanol:Hexane (2.5 :97.5 v/v) Detection : 254 nm	In-house IS [26,27]^{3}H-25OHD$_3$ [3α]^{3}H-25OHD$_2$ 25OHD$_2$ 25OHD$_3$	^{3}H-25OHD$_3$: 72.2 ± 10%	NR	NR
Gilbertson et al. [52]	Serum 1 ml Extraction: CHCl2:MeOH (2:1 v/v) Pre-treatment: silicic acid CH_2Cl_2:EtOH (98:2 v/v) then n-hexane HPLC: Porasil silicic acid column EtOH:Hexane (5:95 v/v) Detector: 254 nm	Commercial IS [24,25]^{3}H-25OHD$_3$ 25OHD$_3$	^{3}H-25OHD$_3$: 60.8 ± 14.4%	NR	25OHD$_3$ 5.2% at 28 nml/L *5.5% at 28 nml/L*
Jones [53]	Plasma or serum 2 ml Extraction: MeOH:$CHCl_3$ (2:1v/v) 2-propanol:Hexane (4.5 :95.5 v/v) HPLC: Zorbax-SIL MeOH:H_2O (98.5:1.5 v/v) followed by MeOH:H_2O (91.0:9.0 v/v) Zorbax-ODS MeOH:H_2O (98.5:1.5 v/v) Detection : 254 nm	Commercial IS [26,27]^{3}H-25OHD$_3$ In-house IS [3α]^{3}H-25OHD$_2$ 25OHD$_2$ 25OHD$_3$	^{3}H-25OHD$_3$: 68.8 ± 6.5%	NR	25OHD$_3$ 9.0% at 30 nmol/L *16% at 30 nmol/L*
Dabec [54]	Plasma 0.5 – 3.0 ml Pre-treatment: SPE: Sep-pak C18 MeOH:H_2O (69:31 then 80:20 v/v) Silicic acid HPLC n-hexane-propane-2-ol (100:2.4 v/v) Detection: 254 nm	Commercial IS [23,24]^{3}H-25OHD$_3$ 25OHD$_3$ 25OHD$_2$	^{3}H-25OHD$_3$: 93%	NR	25OHD$_3$: 5% 25OHD$_2$: 5% Concentrations not mentioned

Turnbull [55]	Plasma 2.0 – 3.0 ml Extraction: MeCN Pre-treatment: SPE: Sep-pak C18 MeOH:H_2O (70:30 v/v) then MeCN Derivatisation to Isotachysterols Zorbax-Sil n-hexane-propane-2-ol (95:5 v/v) Detection: 301 nm	Commercial IS [23,24]^{3}H-25OHD$_3$ 25OHD$_3$ 25OHD$_2$	^{3}H-25OHD$_3$: 54.9 ± 2.5%	NR	25OHD$_3$: 5.9% at 57 nmol/L 25OHD$_2$: 6.8% at 14 nmol/L *25OHD$_3$: 8.0% at* *62 nmol/L* *25OHD$_2$: 7.1% at* *16 nmol/L*
Loo [56]	Plasma 1.0 ml PP: MeOH Extraction: n-hexane 1st HPLC: Li-Chrosorb-Si n-hexane-EtOH (90:10 v/v) 2nd HPLC: Ultraspher-Octyl C-8 MeCN:H2O (80:20 v/v) Detection 254 nm	Commercial IS [26,27]^{3}H-25OHD$_3$ 25OHD$_3$ 25OHD$_2$	^{3}H-25OHD$_3$: 74.7 ± 3.4%	NR	NR
Norris [57]	Plasma/Serum 2.0 ml PP: MeOH Pre-treatment: SPE: Sep-pak C18 (MeOH) 1st HPLC: Li-Chrosorb-Si n-hexane-propane-2-ol (91:9 v/v) 2nd HPLC: Spherisorb-ODS MeOH:H_2O (88:12 v/v) Detection 285 nm	Commercial IS [23,24]^{3}H-25OHD$_3$ 25OHD$_3$ 25OHD$_2$	^{3}H-25OHD$_3$: 54.9 ± 2.5%	25OHD$_3$: 7.5 25OHD$_2$: 7.5	*25OHD$_3$: 7.3% at* *28 nmol/L* *25OHD$_2$: 6.4% at* *16 nmol/L*
Shimada [58]	500µl Plasma PP: EtOH Extraction: EtOH/KOH followed by Et_2O Pre-treatment: Silicic acid column n-hexane-propane-2-ol (98.5:1.5 v/v) n-hexane-propane-2-ol (84:16 v/v) HPLC:J'sphere ODS-HS0 MeCN:H_2O (70:30 v/v) Detection 265 nm	In-house IS 25OHD$_2$ MBPTD-25OHdC 25OHD$_3$	25OHD$_2$ 55.2 ± 3.3% 25OHD$_3$: 59.3 ± 4.2%	12.5	4.0% at 43.6 nmol/L (Average of 4 determinations) *8.2% at* *65.0 nmol/L* *(Average of 4* *determinations)*

Masuda [59]	100µl Plasma Extraction $MeCl_2$/MeOH HPLC: Nucleosil 5-C_{18} column MeCN:MeOH (95:5 v/v)/$HClO_4$ Detection: ECD at +0.60 V	IS: NR $25OHD_3$	$25OHD_3$: 81.5 ± 5.8%,	NR	5.3% at 76 nmol/L *9.7% at* *76 nmol/L*
Alvarez [60]	500µl Plasma PP: EtOH Extraction: n-Hexane/$MeCl_2$ HPLC: Lichrospher 100 RP-18 MeCN:MeOH:H_2O (90:4:6 v/v) Gradient to MeCN:MeOH (40:60 v/v) Detection 267 nm	Commercial IS 1α-OHD_3 $25OHD_2$ $25OHD_3$	1α-OHD_3 93.0 ± 7.9% $25OHD_2$: 81.5 ± 4.7% $25OHD_3$: 88.0 ± 5.1%	$25OHD_2$: 12.5 $25OHD_3$: 12.5	$25OHD_2$: 6.1% at 15 nmol/L $25OHD_3$: 7.7% at 22.5 nmol/L *$25OHD_2$: 10.8%* *at 15 nmol/L* *$25OHD_3$: 11.8%* *at 22.5 nmol/L*
Brunetto [61]	1ml Plasma Extraction: EtOH:MeCN HPLC: Spherisorb C18, Gradient: MeCN:phosphate buffer pH6.5 (20:80 v/v) to MeOH :MeCN :THF (65:20:15 v/v) Detection: 265 nm	No IS $25OHD_3$	Spiked sample $25OHD_3$: 91% at 20 nmol	$25OHD_3$: 7.5	$25OHD_3$: 2% at 17.5 nmol/L *$25OHD_3$: 2%* *at 17.5 nmol/L*
Quesada [62]	1ml Serum PP: EtOH Extraction: n-hexane:$MeCl_2$ HPLC: Ultrabase C18 column Gradient from MeOH:H_20 (90:10 v/v) to MeOH:propane-2-ol (90:10 v/v) Detection : 265 nm	Commercial IS Retinyl acetate $25OHD_3$	NR	$25OHD_3$: 0.75	$25OHD_3$: 4.3% Concentration: NR *$25OHD_3$: 9.2%* *Concentration: NR*
Lensmeyer [63]	Serum 1 ml PP (MeCN) Extraction: HPLC: SB-CN column MeOH :H_2O (67:33 v/v) Detection: 275 nm	Commercial IS Laurophenone (dodecanophenone) $25OHD_3$ $25OHD_2$	Exogenous $25OHD_2$: 101.2 ± 9.4% (8 – 253 nmol/L) $25OHD_3$: 95.1 ± 7.6% (11 – 260 nmol/L)	$25OHD_2$: 12.5 $25OHD_3$: 12.5	*$25OHD_2$: 13% at* *11.0 nmol/L* *$25OHD_3$: 8.5% at* *28.9 nmol/L*

Granado-Lorencio [64]	1 ml Serum PP: EtOH Extraction: n-hexane:$MeCl_2$ HPLC: Spheri-5-ODS column Gradient from MeCN:MeOH (85:15 v/v) to MeCN:$MeCl_2$:MeOH (70:20:10 v/v/v) Detection: 267 nm	Commercial IS Retinyl acetate 25OHD (No distinction between $25OHD_3$ and $25OHD_2$)	25OHD: >85% (No details given)	NR	<10% Concentration: NR *<10%* *Concentration: NR*
Kand'ár [65]	500µl Plasma PP: EtOH Extraction: SPE Discovery DSC-18 MeOH:H_2O (2:3 v/v), MeOH. HPLC: Purospher STAR-RP-18e MeOH/H_2O (95:5 v/v) Detection: 265nm	Commercial IS Retinyl acetate $25OHD_3$	Spiked samples $25OHD_3$: 96.9 ± 7.6% from 5 to 100 nm/L	10 nmol/L (2.5 nmol/L)	$25OHD_3$: 5.3% at 57 nmol/L *$25OHD_3$: 8.7% at 67 nmol/L*
Hymøller [66]	1.5 ml Plasma Saponification: MeOH/KOH/ASC Extraction: heptane HPLC: YMC-C_{30} RP column Gradient: H_2O:EtOH (95:5 v/v), H_2O:EtOH (60:40 v/v); H_2O:EtOH (10:90 v/v) Detection: 265 nm	Commercial IS 1α□OHD_3 $25OHD_2$ $25OHD_3$	$25OHD_2$: 101.0% at 75 nmol/L $25OHD_3$: 100.3% at 75 nmol/L	1.3 nmol/L (Metabolite not specified)	*$25OHD_2$:0.2% at 150 nmol/L* *$25OHD_3$: 0.6% at 150 nmol/L*
Nurmi [67]	500 µl Serum PP: MeOH:propane-2-ol (80:20 v/v) Extraction : n-hexane HPLC: Supelco Discovery HS F5 Gradient: 60mM $NaClO_4$/$HClO_4$/MeOH/MeCN (30:50:20 v/v/v), $NaClO_4$/$HClO_4$/MeCN, (10:90 v/v) Detection: CEAD 630 mV	No IS $25OHD_2$ $25OHD_3$	$25OHD_2$: 72% at 24 nmol/L $25OHD_3$: 61% at 24 nmol/L	$25OHD_2$: 12 $25OHD_3$: 12	*$25OHD_3$: 6.2% at 27.5 nmol/L*

PP: Protein Precipitation; SPE: Solid Phase Extraction; LLE: Liquid-Liquid Extraction; OLTFE: On line turboflow extraction; ECD: Electrochemical Detection; CEAD: Coulometric Electrode Array Detector.

25OHdC: 25-Hydroxy-7-dehydrocholesterol; 1α-OHD_3 : 1-alpha-hydroxyvitamin D_3; MBPTD: 4-[4-(6-methoxy-2-benzoxazolyl)phenyl]-1,2,4-triazoline-3,5-dione;

$MeNH_2$: Methyl Amine; MeOH: Methanol; EtOH: Ethanol; NH_4Ac: Ammonium acetate; MeCN: Acetonitrile: Et_2O: diethyl-ether; KOH: Potassium hydroxide; $MeCl_2$: Dichloromethane; $HClO_4$: Perchloric acid; THF: Tetrahydrofuran; ASC: 20% Ascorbic acid water solution;

IS: Internal Standard; NR: Not reported; #: Spiked samples with 25 nmol/L of each of the 2 metabolites; †: % recovery ± SD for the 2 deuterated compounds at a 50 fmol/□l fortification level. ††: Expressed as percent recovery of the NIST-certified values;

Table

Table 3: Mass spectrometric methods applicable to clinical laboratories

Ref	Sample volume Extraction procedure Chromatographic procedure Ionisation Mode of monitoring	Internal standards Analyte measured Acquisition settings m/z	*Recovery	LOQ nmol/L *LOD nmol/L*	Precision (CV) Intra-assay *Inter-assay*
Higashi 2001 [72]	Plasma 20 µl PP: MeCN Extraction: LLE (AcOEt) Derivatisation (DMEQ-TAD) HPLC: J'sphere ODS H-80 MeCN/H_2O (3/2 v/v) TMS: $APCI^+$ SIM	In-house IS: $25OHD_4$: 760.1 $25OHD_3$: 746.1 $25OHD_2$: 758.1	$25OHD_3$: 98.8 – 109.8% (12.5 nmol/L) $25OHD_2$: 101.1 – 104.2% (12.5 nmol/L)	$25OHD_3$: 7.5 $25OHD_2$: 7.5 *$25OHD_3$: 1.3*	$25OHD_3$: 3.24% at 21.9 nmol/L $25OHD_2$: 3.17% at 12.5 nmol/L
Higashi 2003 [73]	Plasma 20 µl PP: MeCN Extraction: LLE (AcOEt) Derivatisation (NPTAD) HPLC: J'sphere ODS H-80 MeOH/H_2O (7/1 v/v) TMS: $APCI^-$ SIM	In-house IS: $25OHD_4$: 634.2 $25OHD_3$: 620.2	Analytical recovery: NR	$25OHD_3$: 7.5 *$25OHD_3$: 1.3*	$25OHD_3$: 8.2% at 7.5 nmol/L
Vogeser 2004 [74]	Serum 200 µl NaOH, PP: MeCN Extraction: on-line SPE: Oasis HLB® HPLC: LiCrospher® 100 RP-18 MeOH/NH_4Ac:0.5mM (90/10 v/v) TMS: ESI^+	In-house IS: $^2H_3$,$^{13}C_1$-$25OHD_3$: 405 > 159 $25OHD_3$: 401 > 159	$25OHD_3$: 91 ± 1.6% IS (325 nmol/L) injected into TMS/IS Extracted+TMS	NR	$25OHD_3$: 12% at 14.5 nmol/L
Tsugawa 2005 [75]	Serum 100 µl PP: MeOH Extraction: SPE: Bond-Elute C18® HPLC: CapCell PAK C-18 UG120® MeOH/H_2O (95/5 v/v) TMS: $APCI^+$ MRM	In-house IS: $^2H_6$- $25OHD_3$: 407 > 263 $25OHD_3$: 401 > 257 $25OHD_2$: 413 > 255	$25OHD_3$: 103.8% (50 nmol/L) $25OHD_2$: 98.8% 7.5 nmol/L)	*$25OHD_3$: 2.5* *$25OHD_2$: 2.5*	$25OHD_3$: 5.7% at 50 nmol/L $25OHD_2$: 4.5% at 7.5 nmol/L *$25OHD_3$: 2.5%* *at 47.5 nmol/L* *$25OHD_2$: 5.1%* *at 8.0 nmol/L*

Maunsell 2005 [76]	Serum 100 µl PP: MeOH:Propanol (80:20 v/v) Extraction: LLE: n-Hexane HPLC: BDS C8® ThermoHypersil MeOH > H_2O+0.05% CHO_2H Gradient TMS: ESI^+ MRM	In-house IS: $^2H_6$-25OHD$_3$: 407.2 > 389.4 25OHD$_3$: 401.8 > 383.5 25OHD$_2$: 413.5 > 395.4	25OHD$_3$: 91 – 110 % at 128 - 256 nmol/L 25OHD$_2$: 94 - 108% at 158 - 317 nmol/L	25OHD$_3$: < 4.0 25OHD$_2$: < 5.0	25OHD$_3$: 6.2% at 16 nmol/L *25OHD$_3$: 5.1%* *at* *55 nmol/L* *25OHD$_2$: 9.5%* *at* *52 nmol/L*
Chen 20008 [77]	Serum 200 µl PP: MeCN Extraction: SPE: Oasis HLB® MeOH/H_2O (30/70 v/v); MeCN/MeOH (50/50 v/v) HPLC: SupelCosil®LC-18-DB EtOH:H_2O (83:17 v/v) TMS: $APCI^+$ MRM	Commercial IS: $^2H_6$-25OHD$_3$: 407.7 > 389.7 25OHD$_3$: 401.4 > 383.4 25OHD$_2$: 413.4 > 395.4	25OHD$_3$: 99 ± 2 % at 34.2 – 132.8 nmol/L 25OHD$_2$: 95 ± 0.8% at 32.2 – 115.5 nmol/L	25OHD$_3$: 4.0 25OHD$_2$: 15.5 *25OHD$_3$: 1.2* *25OHD$_2$: 4.6*	25OHD$_3$: 6.2% at 34 nmol/L 25OHD$_2$: 8.7% at 23 nmol/L *25OHD$_3$: 11%* *at* *34 nmol/L* *25OHD$_2$: 16%* *at* *23 nmol/L*
Bunch 2009 [78]	Serum 100 µl PP: MeOH Extraction: OLTFE HPLC: Hypersil Gold aQ® MeOH/H_2O (95/5 v/v) TMS: $APCI^+$ MRM	In-house IS $^2H_6$-25OHD$_3$: 407.2 > 389.4 25OHD$_2$: 413.5 > 395.4 25OHD$_3$: 401.8 > 383.5		25OHD$_3$: 3.0 25OHD$_2$: 4.6	
Hojskov 2010 [79]	Serum 100 µl PP: MeCN Extraction: automated LLE: 96-well	Commercial IS $^2H_6$-25OHD$_3$: 407.4 > 371.4 25OHD$_3$:	NR	25OHD$_3$: <10 25OHD$_2$: <10	*25OHD$_3$: 9.4%* *at 32 nmol/L* *25OHD$_2$: 8.6%* *at23.4 nmol/L*

	Isolute HM-N plate®/diatomaceous earth; Heptane HPLC: Synergi MAX-RP® MeOH/2.0mM NH_4Ac (85/15 v/v) TMS: $APCI^+$ MRM	401.4 > 365.2 $25OHD_2$: 413.4 > 395.4			
Hermann 2010 [80]	Serum 100 µl PP: MeCN HPLC: Supelcosil LC-8® H_2O >MeOH >H_2O/MeOH (98/2 v/v) > Toluene $APPI^+$ MRM	Commercial IS $^2H_6$-$25OHD_3$: 389 > 371 $^2H_6$-$25OHD_2$ 401 > 383 $25OHD_3$: 395 > 377 $25OHD_2$: 413.5 > 395.4	108 – 113% Expressed as total 25OHD added (45 - 90 nmol/L)	$25OHD_3$: 1.3 $25OHD_2$: 1.3	25OHD: 5.7% at 17 nmol/L *25OHD: 8.7% at 17 nmol/L*
Ding 2010 [81]	Serum 200 µl PP: MeCN Extraction: SPE Oasis HLB® MeCN; EtOAc Derivatisation (PTAD)/MeCN HPLC: ACQUITY BEH C18® 0.1% CHO_2H /H_2O/$MeNH_2$; CHO_2H /MeOH gradient TMS: ESI^+ MRM	Commercial IS $^2H_6$-$25OHD_3$: 613 > 298 $^2H_6$-$25OHD_2$ 625 > 298 $25OHD_3$: 607 > 298 $25OHD_2$: 619 > 298	$^2H_6$-$25OHD_3$: 84.9 ± 2.4%† $^2H_6$-$25OHD_2$: 79.3 ± 14.4%†	#$25OHD_3$: 0.025 #$25OHD_2$: 0.025	#$25OHD_3$: 3.8% at 0.025 nmol/L #$25OHD_2$: 1.6% at 0.025 nmol/L
Van den Ouweland 2010 [82]	Serum 250 µl PP: NaOH-MeCN/MeOH (9/1 v/v) SPE: Strata C18-E® H_2O-MeOH/H_2O (60/40 v/v)-MeOH HPLC: ACQUITY UPLC BEH C18® 0.1% CHO_2H /2 mM NH_4Ac; MeOH/CHO_2H (99.7:0.3 v/v) gradient TMS: AP-ESI^+ SRM	Commercial IS $^2H_6$-$25OHD_3$: 407.5 > 159.2 $25OHD_3$: 401.5 > 159.2 $25OHD_2$: 413.4 > 83.1	$25OHD_3$: 94.9-106.9% at 49.9 – 99.9 nmol/L $25OHD_2$: 82.7-100.3% at 54.3 – 108.6 nmol/L	$25OHD_3$: 3.5 $25OHD_2$: 2.0 *$25OHD_3$: 1.5* *$25OHD_2$: 1.2*	$25OHD_3$: 2.7% at 64.9 nmol/L $25OHD_2$: 4.2% at 33.3 nmol/L *$25OHD_3$: 6.0% at 64.9 nmol/L* *$25OHD_2$: 3.8% at 33.3 nmol/L*

Tai 2010 [83]	Serum 2g pH adjusted to 9.8 (Na_2CO_3) LLE Extraction: n-hexane/EtAc (50/50 v/v) Residue dissolved in MeOH HPLC: Zorbax CB-CN column H_2O/MeOH (34/66 v/v) TMS: $APCI^+$ MRM	Commercial IS $^2H_3$-25OHD$_3$ 404 > 386 $^2H_3$-25OHD$_2$ 416 > 398 25OHD$_3$ C3-epi-25OHD$_3$: 401 > 383 25OHD$_2$ C3-epi-25OHD$_2$: 413 > 395 Stds traceable to NIST	25OHD$_3$: 100.0 – 10% 25OHD$_2$: 98.0 – 100.1%	*25OHD$_3$: 0.15 ng/g* *25OHD$_2$: 0.15 ng/g*	25OHD$_3$: 0.4% at 6.31 ng/g 25OHD$_2$: 0.9% 0.86 ng/g *25OHD$_3$: 0.6%* *at 6.31 ng/g* *25OHD$_2$: 0.86%* *0.86 ng/g*
Stepman 2011 [84]	Serum 250 µl Extraction: LLE: NaOH/n-hexane Sephadex LH-20 chromatography MeOH/$CHCl_3$/cC6H14 (1/4/8, v/v/v) 2-dimensional UPLC Chromatography 1:Acquity BEH 300 C4® column 2: Acquity BEH C18® column-25OHD$_2$ 2: Zorbax SB-CN® column-25OHD$_3$ Step gradients MeOH/H_2O/ CHO_2H (50/50/0.025) MeOH/H_2O/ CHO_2H (95/5/0.025) TMS: ESI^+ SIM	Commercial IS $^2H_6$-25OHD$_3$ 407.3 > 159.3 $^2H_6$-25OHD$_2$ 419.4 > 159.4 25OHD$_3$: 401.3 > 159.3 25OHD$_2$: 413.4 > 159.4 C3-epi-25OHD$_3$ 401.3 > 159.3 Stds Traceable to NIST	25OHD$_3$: 71% ± 4%†† 25OHD$_2$: 70% ± 8%††	25OHD$_3$: 1.12 ± 0.05 25OHD$_2$: 1.22 ± 0.05	25OHD$_3$: 1.4% at 30.8 nmol/L 25OHD$_2$: 2.0% at 64.1 nmol/L *25OHD$_3$: 1.7%* *at 30.8 nmol/L* *25OHD$_2$: 1.1%* *at 64.1 nmol/L*
Adamec 2011 [85]	Serum 100 µl Extraction: LLE: Acetone HPLC: ACE3C8® column Gradient: H_2O/MeOH+1% toluene TMS: APPI+ MRM	Commercial IS $^2H_6$-25OHD$_3$ 407.3 > 263.3 $^2H_6$-25OHD$_2$ 419.3 > 401.2 25OHD$_3$:	25OHD$_3$: NR 25OHD$_2$: NR	25OHD$_3$: 2.0 25OHD$_2$: 2.0	25OHD$_3$: 3.7% at 5 nmol/L 25OHD$_2$: 16.7% at 5.0 nmol/L *25OHD$_3$: 15.4%* *at 5.0 nmol/L*

		401.2 > 257.2 $25OHD_2$: 413.3 > 337.2 Stds traceable to NIST			*$25OHD_2$: 14.0% at 5.0 nmol/L*
Wang 2011 [86]	Plasma 1 ml PP: MeCN LLE: EtOAc Derivatisation: PTAD HPLC: Hypersil Gold® column MeCN/H_2O+0.1% CHO_2H gradient (40/60; 60/40; 90/10, 40/60 v/v) TMS: ESI^+ MRM	Commercial IS $^2H_6$-$25OHD_3$ 564 > 298 $25OHD_3$: 558 > 298	$25OHD_3$: 73% ± 2% (BSA matrix)	$25OHD_3$: 0.125 *$25OHD_3$: 0.01*	$25OHD_3$: 2.1% at 25 nmol/L *$25OHD_3$: 7.0% at 25.0 nmol/L*
Bogusz 2011 [87]	Serum 100 µl PP: MeOH/MeCN/0.05 M $ZnSO_4$ (6.5/1/2 v/v/v) HPLC: Kinetex C18 NH_4CHO_2H/MeOH Gradient (70/30; 90/10; 70/30) TMS: $APCI^+$ MRM	Commercial IS $^2H_6$-$25OHD_3$ 389 > 371 389 > 211 $^2H_6$-$25OHD_2$ 401> 383 401 > 209 $25OHD_3$: 383 > 365 383 > 211 $25OHD_2$: 395 > 209 395 > 269 Stds traceable to NIST	$25OHD_3$: 98% $25OHD_2$: 97%	$25OHD_3$: 3.0 $25OHD_2$: 1.5 *$25OHD_3$: 1.5* *$25OHD_2$: 0.5*	$25OHD_3$: 3% at 41.7 nmol/L $25OHD_2$: 4% at 42.1 nmol/L
Baecher 2012 [88]	Serum 200 µl PP: MeCN On-line SPE: LiChrospher® column MeOH/H_2O (5/95 v/v) HPLC: Kinetex® PFP column MeOH/0.5mM NH_4Ac (75/25 v/v) TMS: $APCI^+$ MRM	Commercial IS $^2H_6$-$25OHD_3$ 407.3 > 263.2 407.3 > 159.2 $25OHD_3$: 401.3 > 257.2	$25OHD_3$: NR $25OHD_2$: NR C3-epi$25OHD_3$ 95.5% at 5.05 nmol/L	$25OHD_3$: 4.0 $25OHD_2$: 3.9 C3-epi$25OHD_3$: 2.0	$25OHD_3$: 3.1% at 39.8 nmol/L $25OHD_2$: 4.9% at 27.5 nmol/L C3-epi$25OHD_3$: 4.2% at 20.1 nmol/L

		401.3 > 159.2 $25OHD_2$: 413.4 > 159.2 C3-epi$25OHD_3$ 401.3 > 257.2 401.3 > 159.2 NIST SRM 2972 (levels 1-4) used for comparison			*$25OHD_3$: 3.8% at 39.8 nmol/L* *$25OHD_2$: 3.4% at 27.5 nmol/L* *C3-epi$25OHD_3$: 3.4% at 20.1 nmol/L*
Farrell 2012 [89]	Serum 150 µl PP: 2.0 M $ZnSO_4$/MeOH TMS: 0.2M/MeOH SPE:Oasis µElutionHLB plate MeOH/H_2O (60/40 v/v) 2 mM NH_4Ac + 0.1% CHO_2H / MeOH/2 mM NH_4Ac + 0.1% CHO_2H (27/73 v/v) UPLC: ACQUITY BEH C8® 2 mM NH_4Ac + 0.1% CHO_2H / MeOH/2 mM NH_4Ac + 0.1% CHO_2H Gradient from (27/73 v/v) to (98/2 v/v) ESI^+ MRM	Commercial IS $^2H_6$-$25OHD_3$ 407.3 > 159.1 $^2H_3$-$25OHD_2$ 416.3 > 398.3 $25OHD_3$: 401.3 > 383.5 401.3 > 159.1 $25OHD_2$: 413.3 > 83.1 413.3 > 395.3	$25OHD_3$: NR $25OHD_2$: NR	$25OHD_3$: 2.0 $25OHD_2$: 2.0 *$25OHD_3$: 0.5* *$25OHD_2$: 0.5*	25OHD: 1.6% at 79 nmol/L *25OHD: 2.0% at 79 nmol/L*
Lensmeyer 2012 [90]	Serum 300 µl PP: MeCN/2 mM $ZnSO_4$ (87/13 v/v)/MeOH Extraction: SPE Strata C18E® MeCN/H_2O (45/55 v/v) Acetone/MeCN (20/80 v/v) HPLC: Zorbax cyanopropyl column MeOH/H_2O (67/33 v/v) TMS: $APCI^+$ MRM	IS: NR $25OHD_3$: 383.3 > 211.1 $25OHD_2$: 395.3 > 209.1 C3-epi$25OHD_3$ 383.3 > 211.1	NR	NR	NR
Thibault 2012 [91]	Serum 200µl PP: MeCN On-line SPE: X-Terra C18	Commercial IS [$^2H_6$]25-OHD_3 407.5 > 371.3		$25OHD_3$: 4 $25OHD_2$: 3	$25OHD_3$: 3.4% at 59.8 nmol/L $25OHD_2$: 1.8%

	MeOH/0.1% CHO_2H + 2mM NH_4Ac in H_2O (98/2 v/v)/ 0.1% CHO_2H + 2mM NH_4Ac in H_2O (68/32 v/v) HPLC: Sunfire C18 MeOH/0.1% CHO_2H + 2mM NH_4Ac in H_2O (98/2 v/v)/ 0.1% CHO_2H + 2mM NH_4Ac in H_2O (85/15 v/v) TMS: ESI^+ MRM	$[^2H_6]$25-OHD_2 419.4 > 355.2 $25OHD_3$: 401.4 > 365.3 $25OHD_2$: 413.4 > 355.3			at 99.5 nmol/L *$25OHD_3$: 5.9% at 66.7 nmol/L* *$25OHD_2$: 5.9% at 101.3 nmol/L*
Strathmann 2012 [92]	Serum 200µl Extraction: 1M NaOH/n-heptane HPLC: XTerra MS C8 + Restek columns NH_4Ac/0.1% CHO_2H in MeOH/H_20 (95/5 v/v) TMS: $APCI^+$ MRM	Commercial IS $[^2H_6]$25-OHD_3 407.3 > 371.3 $[^2H_6]$25-OHD_2 419.4 > 355.2 $25OHD_3$: 401.3 > 355.3 $25OHD_2$: 413.4 > 355.3 Stds traceable to NIST	$25OHD_3$: 80 - 116% (23.4 nmol/L) $25OHD_2$: 94 – 115% (23.4 nmol/L)	$25OHD_3$: 1.95 $25OHD_2$: 0.6	$25OHD_3$: 2.9% at 58 nmol/L $25OHD_2$: 2.8% at 85 nmol/L *$25OHD_3$: 9.6% at 63 nmol/L* *$25OHD_2$: 6.2% at 95 nmol/L*
Mochizuki 2013 [93]	Serum or plasma 25µl PP: MeCN 2-dimension HPLC: SPE: Turboflow XL C18-P® column Step gradient 0.1% CHO_2H; MeCN/propanol-2ol/acetone (44/40/20 v/v/v); MeOH/0.1% CHO_2H HPLC: Hypersil Gold® column 0.1% CHO_2H; MeOH/0.1% CHO_2H TMS: $APCI^+$ SRM	Commercial IS $[^2H_6]$25-OHD_3 389.3 > 263.2 $25OHD_3$: 383.3 > 365.2 $25OHD_2$: 395.3 > 377.4 Stds traceable to NIST	$25OHD_3$: 102.6 – 106% (36.9 – 59.8 nmol/L) $25OHD_2$: NR	$25OHD_3$: 2.2 $25OHD_2$: 3.5 *$25OHD_3$: 0.8* *$25OHD_2$:2.2*	$25OHD_3$: 5.2% at 18 nmol/L $25OHD_2$: 10.6% at 18 nmol/L *$25OHD_3$: 7.2% at 18 nmol/L* *$25OHD_2$: NR*
Zhang 2014 [94]	Serum 200µl PP: MeOH Extraction: n-heptane HPLC: Zorbax SB-C18 Step Gradient: 2 mM NH4Ac/0.1% CHO_2H - H_2O;	Commercial IS $[^2H_3]$-$25OHD_3$ 404.3 > 368.2 $[^2H_3]$-$25OHD_2$ 416.3 > 358.2	$25OHD_3$: ≥ 62% (125 - 200 nmol/L) $25OHD_2$: ≥ 72% (18 - 200 nmol/L)	$25OHD_3$: 6.2 $25OHD_2$: 6.2 *$25OHD_3$: NR* *$25OHD_2$: NR*	$25OHD_3$: 2.2% at 18 nmol/L $25OHD_2$: 2.1% at 18 nmol/L *$25OHD_3$: 4.4%*

	2 mM NH4Ac/0.1% CHO_2H - MeOH TMS: ESI^+ MRM	$25OHD_3$: 401.3 > 365.2 $25OHD_2$: 413.3 > 355.2			*at 18 nmol/L* *$25OHD_2$: 5.0* *at 18 nmol/L*
Kaufmann 2014 [95]	Serum 100 µl PP: 0.1M HCl/0.2M $ZnSO_4$/MeOH Extraction: n-hexane/t-butyl ether (1/1 v/v) Derivatisation (DMEQ-TAD)/ AcOEt UPLC: BEH-Phenyl column MeOH/H_2O gradient TMS: ESI^+ MRM	Commercial IS $^2H_3$-$25OHD_3$: 613 > 298 $^2H_3$-$25OHD_2$ 625 > 298 $25OHD_3$: 746.6 > 468 $25OHD_2$: 758.6 > 468	$25OHD_3$: NR $25OHD_2$: NR	$25OHD_3$: 0.25 $25OHD_2$: 0.25 *$25OHD_3$: 0.10* *$25OHD_2$: 0.10*	$25OHD_3$: 3-4% at 55 nmol/L $25OHD_2$: 3-4% at 83 nmol/L $25OHD_3$: 4-7% at 55 nmol/L $25OHD_2$: 4-7% at 83 nmol/L

HPLC: High Performance Liquid Chromatography; UPLC: UlLC: Performance Liquid Chromatography; MS: Mass Spectrometry; TMS: Tandem-Mass Spectrometry; AP: Atmospheric Pressure; ESI: Electron Spray Ionisation; APCI: Atmospheric Pressure Chemical Ionisation; APPI: Atmospheric Pressure Photo-Ionisation; ID: Isotope Dilution; MRM: Multiple Reaction Monitoring; SRM: Selected Reaction Monitoring; PP: Protein Precipitation; SPE: Solid Phase Extraction; LLE: Liquid/Liquid Extraction; OLTFE: On-line turboflow extraction;

DMEQ-TAD: 4-[2-(6,7-dimethoxy-4-methyl-3-oxo-3,4-dihydroquinoxalyl)ethyl]-1,2,4-triazoline-3,5-dione; NPTAD: 4-(4-Nitrophenyl)-1,2,4-triazoline-3,5-dione; PTAD: 4-phenyl-1,2,4-triazoline-3,5-dione; EAD: enzyme-assisted derivatisation; GP: Girard Reagent P reagent (1-(carboxymethyl)pyridinium chloride hydrazide); 25OHdC: 25-Hydroxy-7-dehydrocholesterol; 1α-OHD_3 : 1-alpha-hydroxyvitamin D_3

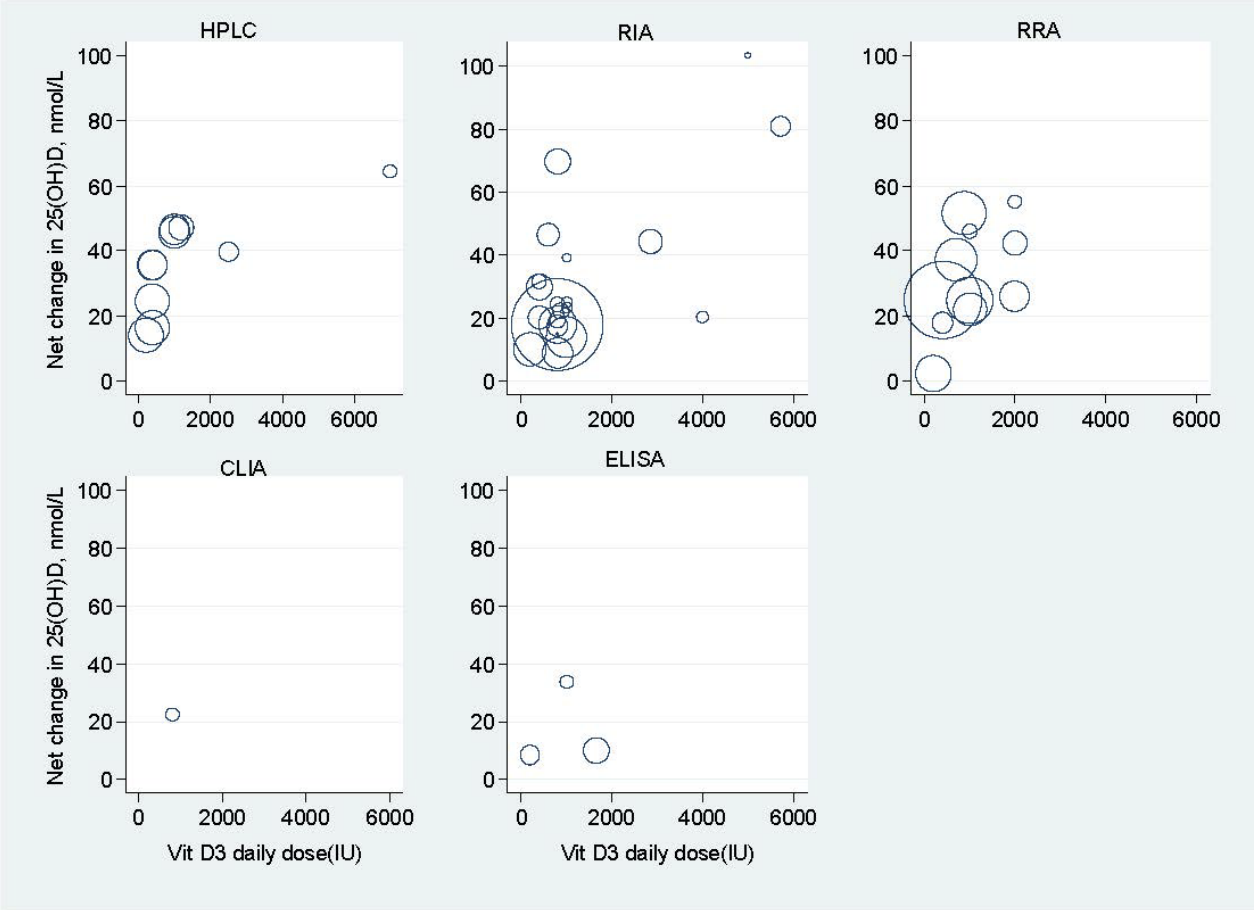
AcOEt: Ethyl acetate; $MeNH_2$: Methyl Amine; MeOH: Methanol; EtOH: Ethanol; NH_4Ac: Ammonium acetate; MeCN: Acetonitrile: Et_2O: diethyl-ether; KOH: Potassium hydroxide; $MeCl_2$: Dichloromethane; $HClO_4$: Perchloric acid; THF: Tetrahydrofuran; ASC: 20% Ascorbic acid water solution; CHO_2H: Formic acid;

IS: Internal Standard; NR: Not reported; NIST: National Institute of Standards and Technology (Gaithersburg, USA); SRM: Standard Reference Material; Levels 1-4: level 1: human serum; level 2: human serum diluted with horse serum to achieve a lower 25(OH)Dx concentration; level 3: human serum fortified with $25(OH)D_2$; and level 4: human serum fortified with 3-epi-$25(OH)D_3$;

*Recovery: Exogenously added vitamin D metabolite; †: % recovery ± SD for the 2 deuterated compounds at a 50 fmol/□l fortification level. ††: Expressed as % recovery of the NIST-certified values;

Figure

Figure 1. Relationship between doses of vitamin D_3 supplementation and net changes in serum 25OHD concentrations in RCTs by assay type.



Legends: Each empty circle represents one study. The area of the circle is proportional to the inverse of the within-study variances. The larger the bubble is, the larger the sample size and the smaller the standard error of the changes in 25OHD. Reprinted with permission from *Vitamin D and Calcium: A Systematic Review of Health Outcomes (Update)* Newberry et al. 2014 [8]