Development of an assay for vitamin D in biological samples

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Abstract

The role of vitamin D in bone health has been well established. Recent studies have provided a greater focus on the ways in which vitamin D and its metabolites have additional functions in the body that subsequently influence conditions such as common cancers, autoimmune diseases, cardiovascular diseases, depression and schizophrenia. Vitamin D is known to have more than 50 metabolites. The major circulating form in man is 25(OH)D₃; having a long half-life, this compound is considered the principal biomarker therefore the best indicator of vitamin D status. Measuring 25(OH)D₃ will, however, only provide an incomplete picture of vitamin D status that may or may not correlate with functions in disease. The 1,25-dihydroxyvitamin D metabolites (1,25(OH)₂D₃ and 1,25(OH)₂D₂) are the most active forms that are responsible for bone health; evidence has now accumulated indicating its role in brain development. The 24,25-dihydroxyvitamin D metabolites (24,25(OH)₂D₃ and 24,25(OH)₂D₂) are responsible for bone fracture repair. The biological roles of vitamin D-sulfate compounds (D₃-S, D₂-S, 25(OH)D₃-S and 25(OH)D₂-S) are unclear to date, probably due to the lack of sufficiently sensitive assay methods to study them. It has been suggested that sulfated compounds are the storage forms of the non-conjugated metabolites, and may have similar potencies.

Considering the above information, and given the diverse biological roles of vitamin D, it is important to accurately quantify as many forms as possible. Although widely used, immunoassay-based methods are not suitable for this purpose because of their inability to differentiate between some of these compounds. Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) has been the method of choice in recent years because of its high selectivity and sensitivity.

This thesis describes the development and validation of an LC-MS/MS method for the quantitative analysis of 12 vitamin D compounds, specifically: D₂, D₃, 25(OH)D₂, 25(OH)D₃, 24,25(OH)₂D₂, 24,25(OH)₂D₃, 1,25(OH)₂D₂, 1,25(OH)₂D₃, D₃-S, D₂-S, 25(OH)D₃-S and 25(OH)D₂-S in biological fluids. During the initial method development, samples of human plasma and serum were compared. It was found that serum yielded higher concentrations of vitamin D compounds. Protein precipitation was compared with saponification method for extraction of vitamin D from biological fluids and found to be more effective. Vitamin D metabolites are known to have low ionisation efficiencies therefore produce weak signals in
MS detector. Pre-column derivatisation procedure using 4-phenyl-1,2,4-triazoline-3,5-dione, (PTAD) was attempted to enhance the ionisation efficiencies of 12 vitamin D compounds. However, this method failed to detect the sulfated compounds, except for traces of 25(OH)D$_3$-S and the derivatisation procedure proved unsuitable for sulfated compounds.

The principal analytical challenge was that of extracting and chromatographing vitamin D compounds with very different lipophilicities in a single analysis procedure. The aim was therefore to overcome this challenge, and to simplify the method to be suitable for routine analysis. The compounds were extracted using online solid phase extraction (SPE) following serum protein precipitation with acetonitrile (without derivatisation). Pentafluorophenyl (PFP) stationary phase, used as the guard column / SPE trapping device, and as the analytical column enabled the extraction and separation of all 12 compounds in a single procedure. Both the extraction step and the separation step were optimised including the chromatographic separation of 25(OH)D$_3$ from the inactive isomer 3-epi-25(OH)D$_3$. The effects of mobile phase pH on separation and the effects of precipitant solvent on the protein precipitation efficiency were also investigated.

Detection limits for all 12 compounds were in the picomole range when using a 500 µL sample volume. Recovery percentages ranged from 92% to 99%. A stable isotope labelled-internal standard (SIL-IS) for each of the 12 vitamin D compounds was used as the co-eluting internal standard to correct for matrix effects in the MS detector as well as to correct for the procedural errors during sample extraction and clean up. In order to demonstrate the applicability of the proposed method, the validated method was then applied to assay for vitamin D compounds in mouse brain samples and donations of human serum samples collected from volunteers.

Although the method developed and validated was simple, sensitive and accurate and amenable for the routine assay of 12 vitamin D compounds in serum, it was expensive to develop and implement because of the SIL-IS compounds required for each of the 12 vitamin D compounds. A standard addition method of calibration, instead of using SIL-IS, was proposed to overcome the matrix effects in MS detection. Since the conventional standard addition procedure does not address procedural errors, an additional internal standard (not co-eluting) was included in the proposed method. The accuracy of this improved standard
addition method was validated through the determination of recovery, and by comparison with existing method SIL-IS method. Recoveries determined on human serum samples showed that the proposed method of standard addition yielded more accurate results than internal standardisation using SIL-IS. The precision of the proposed method of standard addition also proved superior to the conventional method of standard addition.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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Peer-reviewed papers:

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Conference abstracts:


Publications included in this thesis


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Contributions by others to the thesis

Professor Nick Shaw (principal supervisor) and Dr Amita K. Hewavitharana (associate supervisor) supported me throughout my PhD with design and supervision of my projects and was the prevalent contact person for the interpretation of obtained data. Dr Karen Whitfield (associate supervisor) supported me during my PhD with writing and editing support. Writing of the thesis was conducted by the candidate with regular feedback from Professor Nick Shaw, Dr Amita K. Hewavitharana and Dr Karen Whitfield. They also contributed to the preparation of the manuscripts. Dr Virginie Lam and Calvin Chye were involved in the experimental process and data analysis for Chapter 5.

Statement of parts of the thesis submitted to qualify for the award of another degree

None.
**Research Involving Human or Animal Subjects**

Ethical approvals were obtained from:

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Dedications

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<td>°C</td>
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<td>D(_3)-S</td>
<td>Vitamin (D_3)-sulfate</td>
</tr>
<tr>
<td>D(_2)-S</td>
<td>Vitamin (D_2)-sulfate</td>
</tr>
<tr>
<td>DBP</td>
<td>Vitamin D-Binding Protein</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
</tr>
<tr>
<td>DP</td>
<td>Declustering Potential</td>
</tr>
<tr>
<td>EtOH</td>
<td>Ethanol</td>
</tr>
<tr>
<td>ESI</td>
<td>Electrospray ionization</td>
</tr>
<tr>
<td>FA</td>
<td>Formic acid</td>
</tr>
<tr>
<td>FD</td>
<td>Freeze drying</td>
</tr>
<tr>
<td>FSV</td>
<td>Fat-soluble vitamin</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>Water</td>
</tr>
<tr>
<td>IS</td>
<td>Internal standard</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>IUPAC</td>
<td>Union of pure and applied chemistry</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LLE</td>
<td>Liquid – liquid extraction</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography- tandem mass spectrometry</td>
</tr>
<tr>
<td>LOD</td>
<td>Limit of detection</td>
</tr>
<tr>
<td>LOQ</td>
<td>Limit of quantitation</td>
</tr>
<tr>
<td>m/z</td>
<td>Mass/charge</td>
</tr>
<tr>
<td>MRM</td>
<td>Multiple reaction monitoring</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
</tr>
<tr>
<td>PFP</td>
<td>Pentafluorophenyl</td>
</tr>
<tr>
<td>PP</td>
<td>Protein precipitation</td>
</tr>
<tr>
<td>PTAD</td>
<td>4-phenyl-1, 2, 4-triazoline-3, 5-dione</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>SIL–IS</td>
<td>Stable isotope labelled- internal standard</td>
</tr>
<tr>
<td>SN</td>
<td>Saponification</td>
</tr>
<tr>
<td>S/N</td>
<td>Ratio of signal/noise</td>
</tr>
<tr>
<td>SPE</td>
<td>Solid phase extraction</td>
</tr>
<tr>
<td>TIC</td>
<td>Total ion current</td>
</tr>
<tr>
<td>UQ</td>
<td>University of Queensland</td>
</tr>
<tr>
<td>UV-B</td>
<td>Ultraviolet-B</td>
</tr>
<tr>
<td>VDR</td>
<td>Vitamin D receptor</td>
</tr>
</tbody>
</table>
CHAPTER 1: LITERATURE REVIEW

1.1 Foreword

Chapter 1 introduces the topic of vitamin D and its metabolism. This chapter also discusses the literature related to current research on analytical methods used to quantify vitamin D metabolites. The research hypothesis and aims of this thesis are also included. In this chapter, the general term of ‘vitamin D’ without a subscript refers to both the D2 and D3 metabolites as well as their hydroxylated metabolites, unless each substance was assigned by name. The topics are divided into main themes as shown below.

1.2 Introduction

Vitamins are a broad group of organic compounds that are minor, but essential, constituents of food diet, required for the normal growth, self-maintenance and functioning of both human and animal bodies. They have been categorised based on their solubility as being water-soluble (which refers to vitamins B and C), as opposed to fat-soluble vitamins (FSVs) (particularly A, D, E and K) (1). Vitamin D is not a vitamin by strict definition because it can be produced by exposure of the skin to sunlight (2-4). Consequently, it is commonly regarded as the “sunshine vitamin”. In the case of humans, when sunlight is sufficient, a dietary requirement of vitamin D is not necessary. However, nutritional intake of vitamin D is required when sunlight is insufficient to meet daily needs. Vitamin D is comprised of six distinct compounds D2–D7, but only two compounds, D2 (ergocalciferol) and D3 (cholecalciferol) and their metabolites circulate in human biological fluids (5). In contrast to the other known vitamins crucial to human health, vitamin D is unusual due to the variety of sources available (6). Vitamin D2 is sourced from the UV irradiation of ergosterol (5), which is a steroid found in some plants but mostly in fungi such as shiitake mushrooms. Meanwhile, vitamin D3 is synthesised in human skin during skin exposure to solar radiation at 290-315 nm wavelength range from 7-dehydrocholesterol (5, 7). Figure 1.1 shows the chemical structure and numbering of 7-dehydrocholesterol (8) as a parent compound of vitamin D.
Figure 1.1: Chemical structure of 7-dehydrocholesterol. [Adapted from reference (8)]

Figure 1.2: Chemical structures of D$_2$ and D$_3$. [Adapted from reference (11)]
During a heat-dependent process, pre-vitamin D₃ (which is located in the skin) undergoes a rapid rearrangement of its double bonds to form vitamin D₃ (9). Cleavage of the B ring of 7-dehydrocholesterol at the 9, 10-carbon bond as shown in Figure 1.2 yields pre-vitamin D₃. Excessive exposure to sunlight degrades pre-vitamin D₃ and vitamin D₃ into inactive photoproducts (5). Hence, too much exposure to sunlight does not result in vitamin D₃ intoxication (10). The structures of vitamin D₂ and vitamin D₃ differ only at the C-17 side chain, which in vitamin D₂ has a double bond and an additional methyl group at C-24 as shown in Figure 1.2 (11).

Vitamin D compounds in the circulation are bound to the vitamin D binding protein (DBP)(12) and are also incorporated into chylomicrons which are transported via the lymphatic system into the venous circulation and then to the liver(13). A first oxidation step occurs in the liver, converting vitamin D₃ and D₂ into 25-hydroxyvitamin D₃ [25(OH)D₃] and 25-hydroxyvitamin D₂ [25(OH)D₂], respectively. A further oxidation reaction takes place in the kidney and converts 25(OH)D₃ and 25(OH)D₂ to form the active metabolite either into 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] or 1,25-dihydroxyvitamin D₂ [1,25(OH)₂D₂], respectively (14, 15). Alternatively, 25(OH)D₃ and 25(OH)D₂ are thought to be catabolised into 24,25-dihydroxyvitamin D₃ [24,25(OH)₂D₃] and 24,25-dihydroxyvitamin D₂ [24,25(OH)₂D₂], respectively, and other oxidative metabolites followed by conjugation (16). 25(OH)D₃ is the major circulating metabolite of vitamin D₃ and its 3-sulfate metabolite is postulated be a storage form (17). The biosynthesis and metabolism of D₃ in humans are shown in the schematic illustrated in Figure 1.3 (18).
Figure 1.3: Biosynthesis and metabolism of D₃ in man. [Modified from references (18, 19)]
Epimers are compounds that have the same molecular formula but different stereochemistries. The structures of 25(OH)D₃ and their respective 3-epimers differ only in the OH position on C-3. Initial research showed that the 3-epi-25(OH)D₃ occurs only in infants and not adults (20). However, a study has also reported that the C-3 epimer was detected in adult serum in all samples, although the concentration was not listed (21). Collective measurement of the 3-epimers and 25(OH)D may contribute to an overestimation of the vitamin D forms and thereby adversely affect the outcome of clinical studies (22). Therefore, chromatographic separation of 25(OH)D₃ from its 3-epi-25(OH)D₃ isomer is necessary to enable quantification of each of these compounds, using liquid chromatography-mass spectrometry (LC-MS), since they have the same mass transitions. Although the biological function of 3-epi-25-hydroxyvitamin D₃ (3-epi-25(OH)D₃) remains unknown, the first LC–MS method employing MS/MS detection for accurately capturing and separating the 3α epimer from the main 3β epimer and the subsequent quantification of 25(OH)D₃ was described in 2017 (23). This had the effect of, avoiding positive bias in vitamin D status analyses. However, the biological effects of 3-epimer of 1,25 dihydroxyvitamin D₃ [3-epi-1,25 (OH)₂D₃], appear to be variable and it has been reported that this epimer is relatively inactive in vitro (24). It does not increase serum calcium but does suppress circulating parathyroid hormone (PTH) concentration in rats (25). Figure 1.4 shows the possible pathways of epimerisation for 25(OH)D₃ and 1,25(OH)₂D₃ (26).
Figure 1.4: Epimerisation routes for 25-hydroxyvitamin D₃ and 1,25-dihydroxyvitamin D₃. The same reaction applies to 25(OH)D₂ and 1,25(OH)₂D₂. Both 25(OH)D and 1,25(OH)₂D can be epimerized at the carbon number 3 (C-3 position; epimeric centre). Epimers are non-superimposable (or non-mirror images) that only differ in the configuration at one carbon atom. 3-Epi-25(OH)D can be converted to 3-epi-1,25-(OH)D by 1-hydroxylase.

[Adapted from reference (26)]

1.3 Chemistry and biological functions

Vitamin D regulates the calcium and phosphorus levels in the blood by promoting their absorption from food in the intestines, and by stimulating reabsorption of calcium in the kidneys, thereby allowing normal mineralisation of bone. It is also essential for bone growth and bone re-modelling thus preventing rickets. Rickets is a condition associated with bone-deformity due to inadequate mineralisation in growing bones (27, 28). It is an example of
known extreme vitamin D deficiency and can be prevented with adequate nutritional intake of vitamin D (27).

Vitamin D is not only important for controlling bone health in children and adults, but also reportedly for biomedical advantages. These include reducing the risk of chronic diseases such as autoimmune diseases (29), common cancers (29-31), and multiple sclerosis (32). One meta-analysis of epidemiological studies has demonstrated a positive correlation between the intake of vitamin D and colorectal cancer prevention (33). The colon cancer risk was reduced by 50% with an intake of an additional 1000-2000 international units per day (25-50 µg)(33). Most studies examining the relationships between vitamin D and disease or health focus on the main 25-hydroxyvitamin D₃[25(OH)D₃] metabolite, thus potentially overlooking the contributions and biological functions of other vitamin D metabolites, the crucial roles of several of these which have been previously demonstrated (34). Considering the above information and the respective studies recognising the role of vitamin D in the prevention of these diseases, an increased interest has been generated in quantifying vitamin D levels in human serum or plasma. Therefore, advances in our understanding of the relationship between vitamin D status and health are subject to our capacity to measure the precise vitamin D status.

In the following section, each vitamin D compound and their biological functions were described and LC/MS methodologies for quantitative analysis of vitamin D compounds was discussed including  D₃,  D₂,  25(OH)D₃,  25(OH)D₂,  1,25(OH)₂D₃,  1,25(OH)₂D₂,  24,25(OH)₂D₃, 24,25(OH)₂D₂ as well as conjugation products such as sulfate metabolites (D₃-S, D₂-S, 25(OH)D₃-S, 25(OH)D₂-S).

1.3.1  D₃ and D₂

As mentioned above, vitamin D occurs in man as two forms: D₃ and D₂. Both vitamins D₂ and D₃ are biologically inactive and have half-lives of approximately 24 h. Accordingly, the measurements of these compounds in serum do not reflect the physiologically relevant levels of vitamin D in the body (35). However, the requirement for vitamin D is normally met by its synthesis in the skin. The vitally important vitamin D₃ and D₂ synthesis is induced by natural UV irradiation in the epidermis. Therefore, both D₃ and D₂ play an important role in the regulation of the systemic calcium and phosphorus from the gastrointestinal tract and in the
treatment of rickets (36, 37). Non-metabolised vitamin D$_3$ and D$_2$ are stored in adipose tissue and skeletal muscle, then released during period of vitamin D$_3$ or D$_2$ deprivation for use in bone metabolism (11).

Vitamin D$_3$ and D$_2$ compounds, as precursor compounds have rarely been measured except for a few studies concerned with breastfeeding mothers (38). In animal studies, the potency of vitamin D$_3$ was found to be lower than vitamin D$_2$ when the animal is nocturnal and/or when its diet is mainly plant-based (11). In monkeys, the concentrations of serum 25(OH)D$_3$ increased two-fold to three-fold after intake of vitamin D$_3$ compared to that generated by similar amounts of vitamin D$_2$(39). Similarly, in rats vitamin D$_3$ was found to be more effective (40, 41). Meanwhile, studies conducted on birds demonstrated vitamin D$_2$ to be only one-tenth as effective as vitamin D$_3$ at increasing plasma concentrations of 25(OH)D$_3$ (42).

Although, in humans, vitamins D$_2$ and D$_3$ are comparable in their metabolism and the potency of their metabolites (43), there has been debate over the effectiveness of vitamin D$_2$ compared to D$_3$. Some studies report that D$_2$ is less effective than D$_3$ at raising the 25(OH)D$_3$ or 25(OH)D$_2$ concentrations in blood (43-45). These differences have been largely explained on the basis of the relative binding affinity of vitamin D and its metabolites to the plasma vitamin D binding protein (DBP). This difference in the binding ability is potentially explained by the presence of a methyl group at carbon 24 in the D$_2$ molecule as shown in Figure 1.2 (46).

1.3.2 25(OH)D$_3$ and 25(OH)D$_2$

Although, there is no universal agreement, vitamin D nutritional status is defined by most experts with reference to the 25(OH)D [25(OH)D$_3$ and 25(OH)D$_2$] concentration in serum or plasma (31, 47). However, 25(OH)D is considered to be the best biomarker of vitamin D status because this compound has been found at higher concentration in serum and has a three week half-life which allows a more robust estimate of vitamin D status from both dietary intake and UV irradiation (35). As 25(OH)D is converted to the active form in the kidney, it is a poor marker for vitamin D status in kidney disease patients (48). A low level of 25(OH)D may be related with different cardiovascular risk factors, such as: hyperglycaemia; insulin resistance, Type 2 diabetes, dyslipidaemia and high blood pressure (49-52).
Most experts agree that 25(OH)D concentration above 30 ng/mL (75 nmol/L) are considered to be normal and sufficient (53-57), whereas a concentration of 21–29 ng/mL (51–74 nmol/L) is considered to indicate insufficiency and below 20 ng/mL (50 nmol/L) (58-60) is an indication of vitamin D deficiency. Severe deficiency leads to rickets in children and osteomalacia in adults in much the same way as calcium deficiency (5). Contrary to the case with hypovitaminosis, vitamin D intoxication is observed when serum levels of 25(OH)D$_3$ are greater than 150 ng/mL (374 nmol/L) (5) and are associated with hypercalcemia and hyperphosphatemia (57, 61-64). With the use of such definitions, it has been estimated that one billion people worldwide have either a deficiency or in insufficiency of vitamin D(65).

1.3.3 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$

1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ are biologically active forms of vitamin D converted from 25(OH)D$_3$ and 25(OH)D$_2$ in the liver. Although a number of research papers have reported that serum levels of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ do not reflect the body’s storage and are not useful for determining vitamin D status, one of the functions of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ is to maintain the content of calcium and phosphorus in the blood (8). 1,25(OH)$_2$D stimulates the absorption of dietary calcium through the intestines and participates in the incorporation of calcium into the skeleton (66). It was discovered, that when the presence of 1,25(OH)$_2$D, it will increase the absorption of renal calcium and of intestinal calcium and phosphorus (29, 63)

The respective levels of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ are not adequate to evaluate the nutritional status of vitamin D due to their short half-life in serum of only 4 h (35). Furthermore, in routine analysis, 1,25(OH)$_2$D$_3$ is a challenging analyte since its concentration in human serum is extremely low at picogram levels [normal range: 25-70pg/mL (65-182 pmol/L)] (11).The first assay technique for 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$, reported by Brumbaugh et al. (67) in 1974, utilised the chick intestinal cytosol-chromatin receptor system. The measurement of 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$ levels in serum are useful in evaluating patients with chronic renal failure (67, 68), hypoparathyroidism, hyperphosphatemia, hypomagnesaemia, rickets granulomatous diseases or lymphoma (68, 69) and in the treatment of psoriasis (70).
1.3.4 24,25(OH)_2D_3 and 24,25(OH)_2D_2

The serum concentrations of 24,25-dihydroxyvitamin D3 [24,25(OH)_2D_3] are also of potential interest when attempting to understand the *in vivo* kinetics of 25(OH)D_3 in serum. The metabolites 24,25(OH)_2D_3 and 24,25(OH)_2D_2 were originally considered to be inactive and serving little purpose other than redirecting the metabolism of 25(OH)D_3 and 25(OH)D_2 respectively, but were later found to play a crucial role in healing processes in bone formation (71) and fracture repair (11). Furthermore, incidence of increased bone volume was reported (72). It has been argued that the concentrations of 24,25(OH)_2D_3 and 24,25(OH)_2D_2 in the circulation have the potential to be both clinically valuable indicators but also potential nuisances as analytical interferences at the same time, thereby underlining the significance of their definitive measurement in plasma or serum (73). The normal plasma/serum concentration of 24,25(OH)_2D_3 in humans ranges are approximately 1-4 ng/mL (19).

Attempts to increase the ionisation efficiency for mass spectrometry have been reported in several studies as part of methods for multiple vitamin D compounds (19, 74). However, in a recent study in 2017, Dowling et al. (75) improved the accuracy of measuring both 3-epi-25(OH)D_3 and 24R,25(OH)_2D_3 in serum without the use of a derivatisation agent. The most common method used to measure vitamin D, immunoassay, is not capable of differentiating between 24,25(OH)_2D_3 and 25(OH)D_3 (76).

1.3.5 25(OH)D_3-Sulfate, 25(OH)D_2-Sulfate, D_3-Sulfate and D_2-Sulfate

In 1985, one study first identified the existence of 25-hydroxyvitamin D3-sulfate [25(OH)D_3S] in human plasma (77). Although the biological role of 25(OH)D_3-S is still not fully understood, there is an assumption that it might be a storage form of vitamin D_3 (78, 79). The circulating levels of this metabolite were found to be similar to or exceeding that of the major circulating form (25(OH)D_3) in adults and infants, respectively (77, 80). Therefore, it has been suggested that conjugation of vitamin D with sulphate plays an important role in vitamin D metabolism. It is also considered that the quantification of 25(OH)D_3-S in plasma/serum was viewed as being important in researching its significance in pregnant women and newborns as well as for its assessment of vitamin D status, especially in infants (78).

Sasashi *et al.* suggested that vitamin D_2-S has approximately the same antirachitic potency as vitamin D_2 (81). Miravet *et al.* further reported potent biological activity of the vitamin D_3-
Sand D₃ (82). However, the results from a study by Cancela et al. shows that vitamin D₃-S is clearly less active than the same dose of free vitamin D₃ in promoting normal mineral homeostasis and bone mineralisation in rats during the lactation period (83). In 2015, a method using LC with MS detection has been reported for the quantification of four sulfated forms of vitamin D: vitamins D₂ and D₃-sulfate (D₂-S and D₃-S) and 25-hydroxyvitamin D₂ and 25-hydroxyvitamin D₃-sulfate (25(OH)D₂-S and 25(OH)D₃-S) in human serum and breastmilk (84). **Figure 1.5** shows the chemical structures for vitamin D-sulfate.

![Chemical structures of 25(OH)D₃-S, 25(OH)D₂-S, D₃-S and D₂-S. The sulfates are shown as the sulfate anion](image)

**Figure 1.5**: Chemical structures of 25(OH)D₃-S, 25(OH)D₂-S, D₃-S and D₂-S. The sulfates are shown as the sulfate anion

### 1.4 Sources of vitamin D

One of the main sources of vitamin D for most humans is sun exposure (62, 85). Circulating vitamin D₃ levels are largely dependent on the action of UV light. As mentioned in the introduction of vitamin D above, people who are exposed to sunlight every day have never had to be concerned about vitamin D intoxication because once pre-vitamin D₃ and vitamin D₃ are made in the skin they can absorb UVB and UVA radiation. This results in their
conversion to a wide variety of photoproducts that have little, if any, effect on calcium metabolism (86, 87). This is the likely explanation for no reported cases of vitamin D intoxication due to excessive exposure to either sunlight (88) or UVB radiation from artificial sources such as tanning beds (87). However, the total of the vitamin biosynthesised for each day is affected by many factors, including: ultraviolet radiation (sunlight) exposure on the skin, the season of the year, clothing, and skin melanin pigments.

Humans also obtain vitamin D from their diet and dietary supplements. Vitamin D(D represents either D_2 or D_3) is derived from fortified foods, tuna, sardines, salmon, mackerel, egg yolk, cod liver oil, mushrooms and fish ingested from one’s diet as shown in Table 1.1 (5). A naturally oily fish such as salmon contains significant amounts of vitamin D_3. It is an excellent source of vitamin D_3 that could prevent vitamin D deficiency (63).

There are relatively few sources of vitamin D_2, and these are mainly found in plants. D_2 is produced through the ultraviolet irradiation of ergosterol in plants and is also found in foods such as shiitake, wild mushrooms and egg yolks. In many countries, vitamin D_2 (ergocalciferol) is used for the purpose of fortification; this is derived from plant sources (89). In addition, supplements are another source of vitamin D in the body which could help those at risk of vitamin D deficiency due to insufficient sun exposure and/or poor dietary intake. Vitamin D_3 is the form mostly used in supplements; less frequently for vitamin D_2. Both can be accessed as over-the-counter vitamin D supplements. It is very important to have early identification for those individuals who are vitamin D deficient so that adequate supplementation can be prescribed as early as possible. However, studied by Saande et al.(90) demonstrated that dietary consumption of whole eggs may be more effective than supplemental cholecalciferol in maintaining normal circulating 25(OH)D concentrations.

Metabolically, hypervitaminosis D is characterised by high levels of 25-hydroxyvitamin D_3 and 25-hydroxyvitamin D_2 in serum (61). Toxicity (hypervitaminosis D) caused by over-fortification of food is unusual; it does exist, however, and has been reported as accidental vitamin D intoxication (61, 91). A previous study discovered a case of 35 hypervitaminotic patients having hypercalcaemia resulting from chronic ingestion of accidentally over-fortified milk (61). However, hypervitaminosis D could also occur in patients taking prescribed vitamin D supplements (92); increasing age was related to the development of hypervitaminosis D. It was predicted that connection to age-related decrease in renal function
may be relevant (93). The consequences of this case also highlight the importance of observing carefully the limits of vitamin D supplementation. Prolonged hypervitaminosis D can cause calcium deposition in the soft tissues (especially the kidneys and heart); this may affect the central nervous system and in severe cases can result in death (94-97).
Table 1.1: Dietary sources of Vitamin D<sub>2</sub> and D<sub>3</sub>(5)

<table>
<thead>
<tr>
<th>Source</th>
<th>Vitamin D Content*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fortified milk, usually vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 100 IU/8 oz (2.5 μg/8 oz)</td>
</tr>
<tr>
<td>Fortified orange juice, vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 100 IU/8 oz (2.5 μg/8 oz)</td>
</tr>
<tr>
<td>Infant formulas, vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 100 IU/8 oz (2.5 μg/8 oz)</td>
</tr>
<tr>
<td>Fortified yogurts, usually vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 100 IU/8 oz (2.5 μg/8 oz)</td>
</tr>
<tr>
<td>Fortified butter, usually vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 50 IU/3.5 oz (1.3 μg/3.5 oz)</td>
</tr>
<tr>
<td>Fortified margarine, usually vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 430 IU/3.5 oz (10.8 μg/3.5 oz)</td>
</tr>
<tr>
<td>Fortified cheeses, usually vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 100 IU/3 oz (2.5 μg/3 oz)</td>
</tr>
<tr>
<td>Fortified breakfast cereals, usually vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 100 IU/serving (2.5 μg/serving)</td>
</tr>
<tr>
<td>Egg yolk, vitamin D&lt;sub&gt;3&lt;/sub&gt; or D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>About 20 IU/yolk (0.5 μg/yolk)</td>
</tr>
<tr>
<td>Tuna, canned, vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 230 IU/3.5 oz (5.8 μg/3.6 oz)</td>
</tr>
<tr>
<td>Mackerel, canned, vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 250 IU/3.5 oz (6.3 μg/3.5 oz)</td>
</tr>
<tr>
<td>Sardines, canned, vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 300 IU/3.5 oz (7.5 μg/3.5 oz)</td>
</tr>
<tr>
<td>Salmon, farmed, vitamin D&lt;sub&gt;3&lt;/sub&gt;orD&lt;sub&gt;2&lt;/sub&gt;</td>
<td>About 100–250 IU/3.5 oz (2.5- 6.3 μg/3.5 oz)</td>
</tr>
<tr>
<td>Salmon, wild, vitamin D&lt;sub&gt;3&lt;/sub&gt;</td>
<td>About 600–1000 IU/3.5 oz (15- 25 μg/3.5 oz)</td>
</tr>
<tr>
<td>Shiitake mushrooms, sun-dried, vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>About 1,600 IU/3.5 oz (40 μg/3.5 oz)</td>
</tr>
<tr>
<td>Shiitake mushrooms, fresh, vitamin D&lt;sub&gt;2&lt;/sub&gt;</td>
<td>About 100 IU/3.5 oz (2.5 μg/3.5 oz)</td>
</tr>
</tbody>
</table>

*IU denotes international unit, which equals 0.025 μg or 65pmol. The unit definition of the active metabolite calcitriol was set to be equivalent in terms molar to that of the parent vitamin D<sub>3</sub>(2).
1.5 Causes of vitamin D deficiency

It is widely recognised that, the major source of vitamin D for humans is exposure to sunlight. However, sun exposure also causes 99% of non-melanoma skin cancers and 95% of melanoma cases in Australia (98). Thus, a balance of sunshine is required to allow sufficient sun exposure so as to maintain adequate vitamin D₃ production but to minimise the risk of skin cancer. No paediatric data from Australian or New Zealand are available concerning the duration of UVB radiation exposure required to maintain adequate levels of vitamin D (99).

Populations at high latitudes, together with dark-skinned persons, veiled persons and pregnant women also have a high risk of vitamin D deficiency (99). Anything that interferes with the penetration of UVB radiation into the skin will affect the cutaneous synthesis of vitamin D₃ (86). A sunscreen prevents UV-B and some UV-A light from entering the skin and sunscreen with a sun protection factor SPF 8 can prevent and decrease vitamin D₃ synthetic capacity by 95%, while SPF 15 can decrease it by 98% (100). Differences in vitamin D levels related to season are also highly significant; there is less occurrence of vitamin D₃ synthesis during the winter time as well as in the early morning and late afternoon (101). The amount of human body that is exposed to the sun is associated with marked differences in vitamin D synthesis (102). At least 20% of the skin should be uncovered to UV-B in order for blood vitamin D concentrations to increase (103). In Saudi Arabia, women and children wear traditional clothing as their routine, and therefore, are at greatest risk of vitamin D deficiency (103). The practice of purdah, whereby all skin is covered and prevented from being exposed to sunlight, places those who practice it at a high risk of vitamin D deficiency. This explains why, in the sunniest areas of the world, vitamin D deficiency is very common in both children and adults (104-106).

Melanin is extremely efficient in absorbing UVB radiation, and, thus, increased skin pigmentation markedly reduces vitamin D₃ synthesis (107). Overall, an Asian Indian person requires a longer duration of sun exposure a day than light-skinned person for a similar response (three-fold; compared to a black person at six to ten-fold) to achieve equivalent vitamin D concentrations (107, 108). Mothers who were experiencing vitamin D deficiency through pregnancy and exclusively breastfed dark-skinned infants appear to be at particularly high risk of vitamin D deficiency and, additionally, their infants (103).
There are several factors might be related to low levels of vitamin D amongst the elderly; they frequently do not go out into the sunshine for sufficient periods and aging also lowers both the amount of 7-dehydrocholesterol in the skin and therefore their the capacity for vitamin D production (109, 110). In addition, vitamin D deficiency can also be caused by several factors including (a) deprivation of sunlight, (b) avoidance of dairy products, due either to low socioeconomic status or intolerance due to lactase deficiency, and (c) intestinal malabsorption of lipophilic vitamin D (110).

A low intake in food may be from individual choice or from necessity in societies too poor to afford foods containing vitamin D. A reduced intake of fortified milk is common among young women of motherhood age, which results in decreased vitamin D concentrations in the blood (103). Vitamin D is fat-soluble and therefore it is readily taken up by fat cells. Hence, obesity has been linked with vitamin D deficiency, and it is believed to be due to the sequestration of vitamin D by the large body fat pool (111, 112). Medications including anti-seizure medications, glucocorticoids and fat malabsorption are also common causes of deficiency (113).

1.6 Consequences of vitamin D deficiency

A study by Holick (65) found that the burning of coal caused severe pollution in northern Europe and was the trigger point whereby people started to become concerned about vitamin D deficiency and its health consequences in humans. In addition, industrial structures and houses in close proximity insulate children from sun exposure, resulting in the bone-deforming disease commonly known as rickets (65). In addition to skeletal effects, evidence also suggests that vitamin D deficiency can be related to the non-skeletal disorders mentioned below.

1.6.1 Skeletal

Vitamin D deficiency causes muscle weakness has been associated with vitamin D deficiency (31, 62, 114). Skeletal muscles have a vitamin D receptor and require vitamin D for maximum function (62, 115, 116). In adults, it can cause a skeletal mineralisation defect; the non-mineralised osteoid provides little structural support for the periosteal covering. As a result, patients with osteomalacia often complain of global bone discomfort along with aches and pains in their joints and muscles (117). In addition, the effects of rickets in young women
may mean that they cannot deliver babies vaginally because of a deformed pelvis and consequently often need to have caesarean sections to deliver children (86).

1.6.2 Non-skeletal

The vitamin D receptor (VDR) is also present in many body tissues (118, 119), suggesting that vitamin D has non-skeletal actions (60). Low levels of 25(OH)D$_3$ may be related with different risk factors, such as: various cancers (33, 120, 121), Type 1 (122) and Type II diabetes (123, 124), multiple sclerosis (125-127) and melanoma (128). An optimal level of vitamin D may also have a positive effect on decreasing the risk of specifically: obesity, wheezing, allergy asthma, mental disease, depression (129), schizophrenia, infections (pulmonary and urinary), kidney failure and hypertension, respectively (35). **Figure 1.6** shows a schematic representation of the major causes of vitamin D deficiency and potential health consequences, including: adult onset diabetes mellitus (AODM); coronary heart disease (CHD); forced expiratory volume in 1s (FEV$_1$); highly active antiretroviral therapy (HAART); high blood pressure (HBP); multiple sclerosis (MS); rheumatoid arthritis (RA); tuberculosis (TB); as well as urinary tract infection (URI) (130).
Figure 1.6: A schematic representation of the major causes of vitamin D deficiency. AODM, adult onset diabetes mellitus; CHD, coronary heart disease; FEV$_1$, forced expiratory volume in 1s; HAART, highly active antiretroviral therapy; HBP, high blood pressure; MS, multiple sclerosis; RA, rheumatoid arthritis; TB, tuberculosis; URI, urinary tract infection.

[Adapted from reference (130)]
1.7 Treatment of vitamin D deficiency

Most experts have suggested that the daily maintenance dose of vitamin D varies by age; children and adults alike require 800–1000 IU (20-25 µg) of vitamin D₃ to satisfy the body’s vitamin D requirement, when sunlight is unable to provide it (31, 62, 131, 132). Only few natural dietary sources contain vitamin D, hence it is important to obtain vitamin D replacement from supplements (133). Thus, taking a vitamin D supplement and taking advantage of the beneficial effect of sun exposure will help guarantee vitamin D sufficiency for maximising bone health and possibly for other health benefits (134).

When a person is vitamin D deficient, 50 000 IU/week of vitamin D for eight weeks is often effective (5, 58) compared to 1000 IU/day. Higher doses may be required for patients who are severely deficient or with intestinal fat malabsorption syndrome and obesity to sustain serum level of 25(OH)D₃ > 30 ng/mL (75 nmol/L) (5, 112).

To correct vitamin D deficiency in infants and children, vitamin D supplementation should be administered as follows: 200 IU/day (5 µg/day) for infants and children of all ages. However, there are also higher levels, such as: 1000 IU/day (25 µg/day) for children 0–1 yr, 2000 IU/day (50 µg/day) for children 1–19 yrs and older (135). The Institute of Medicine recommended that all children (also endorsed by the American Academy of Pediatrics) and adults up to the age of 50 years require 200 IU vitamin D/day and adults aged 51–70 and 71 yrs old need 400 and 600 IU vitamin D/day (136). After correction of their vitamin D status with an oral doses of vitamin D, patients should have a repeat test of their 25(OH)D₃ level to confirm that they are in the normal range. If the 25(OH)D₃ concentration remains persistently low despite several attempts at correction with oral vitamin D, a trial of UVB light therapy (i.e. by tanning lamps) may be considered in order to treating rickets, as demonstrated by a marked increase in the mineralisation of the skeleton (137).

1.8 Plasma and serum samples

Blood (plasma or serum) are widely used matrices in clinical and biological studies and are frequently used to assist in the diagnosis of cases of vitamin D deficiency. Both plasma and serum are aqueous solutions (about 95% w/v water) containing a variety of substances. These include: proteins and peptides (such as albumins, globulins, lipoproteins, enzymes and hormones), nutrients (such as carbohydrates, lipids and amino acids), electrolytes, organic
wastes and a variety of other small organic molecules either suspended or dissolved in them. Compositions of plasma and serum in terms of molecules appear to be relatively similar (based on current analytical techniques) (138).

Both plasma and serum are derived from full blood that has undergone different biochemical processes after blood collection. Plasma is obtained from a blood sample after anti-coagulants are introduced. After centrifuging the sample, three layers are observed and the least dense (non-cellular) portion is plasma. If the blood is allowed to clot and no anticoagulant is added, the upper layer of the fluid is called serum; serum is less viscous than plasma and lacks fibrinogen, prothrombin and other clotting protein (139). Plasma is a straw-coloured liquid in which blood cells are suspended, comprising approximately 50–55% of blood volume, with blood cells (erythrocytes, leukocytes and platelets) accounting for the remaining portion (140).

There is limited evidence that different collecting procedures and the coagulation cascade can influence concentrations of both proteins and metabolites in these matrices. One concern is the effect of anticoagulants on LC–MS/MS assay performance. One study demonstrated that different sample matrices, serum, heparin or K2-EDTA coagulated plasma, did not cause significant variation in 25(OH)D2 and 25(OH)D3 quantification (141). A recent study using a SPE-LC/MS method, confirmed that vitamin D3, 24,25-dihydroxyvitamin D3 and 25-hydroxyvitamin D3 levels in plasma and serum were similar, while levels of 1,25-dihydroxyvitamin D3 were significantly different, being higher in plasma than in serum (142).

1.9 Analytical methods for quantification of vitamin D compounds

A wide variety of methodologies have been developed for the quantification of vitamin D metabolites in serum/plasma samples. A literature review identified approximately 90 publications on the measurement of vitamin D within the period 2008 – 2012 (143). The analysis of vitamin D compounds is further complicated by their compound structural similarity, the predominantly hydrophobic/lipophilic nature of vitamin D compounds and the hydrophilic nature of some of the metabolites, and their instability in the presence of heat or UV light. Accordingly, analysis requires selective and rapid analytical methods. Among these, there are two main types of methods which are used routinely, namely: competitive immunoassays and methods based on chromatographic separation followed by non-
immunological direct detection (HPLC-UV and LC–MS/MS). Immunoassays are frequently commercial kits and include radioimmunoassay (RIA), enzyme-linked immunoassay (ELISA), chemiluminescent linked immunoassay (CLIA), and electro-chemiluminescent (ECL) methods. Immunoassays are readily automated, suitable for high throughput and do not require high level technical skills to obtain satisfactory results. However, the major disadvantage is that of specificity as immunoassays are not able to separate 25(OH)D$_2$ and 25(OH)D$_3$ metabolites on an equimolar basis. In addition, these methods are unable to detect 25(OH)D$_2$, thus making them unsuitable for monitoring any patient on 25(OH)D$_2$ supplementation (144-147). From the results studied by Cavalier et al., in method such as Diasorin RIA, Diasorin Liaison, Roche Elecsys and HPLC (35), the uncertainty used to determine 25(OH)D$_3$ was found to be up to 20%. The authors concluded that a physician should ensure that patients’ results are ≥ 100 nmol/L if the normal status level is considered ≥ 80 nmol/L (35).

Previously, liquid chromatography (LC) with ultraviolet detection (UV) has been a common technique for measuring vitamin D. Unfortunately, lack of specificity in UV detection and high limits of detection (LOD) required high sample volumes of assessment of circulating vitamin D metabolites present at low concentrations (148). Liquid chromatography- tandem mass spectrometry (LC- MS/MS) is currently the most powerful tool for the analysis of vitamin D compounds in biological fluids. In recent years, LC-MS/MS has been established as the technique of choice for vitamin D analysis because it conveys both high specificity and sensitivity (149). LC-MS methods allow the separation of compounds based on their polarities, ionisation behaviours, and mass to- charge (m/z) ratios and can offer very low limits of quantitation. LC-MS methods are also excellent for targeting the identification of low abundance metabolites over a wide polarity range. The low chemical reactivity of these compounds together with their low concentration in human systematic circulation, particularly for 1,25(OH)$_2$D$_3$ (150) are the limiting factors for the development of new analytical methods for their determination. In order to study the patho-biological role of vitamin compounds including the potentially important low-abundance species, it is important that the concentrations are determined. Moreover, LC-MS/MS is able to measure 25(OH)D$_2$ and 25(OH)D$_3$ independently with good recoveries (151). The ability to measure 25(OH)D$_2$ and 25(OH)D$_3$ separately has been the main criterion distinguishing LC- MS/MS method from immunoassays and the HPLC- UV method. However, measurements of vitamin D species from biological fluids such as plasma or serum using mass spectrometry are not
without challenges. These challenges include: abundant isobaric and isomeric interferences in serum or plasma (152), low ionisation efficiencies for mass spectrometric analyses (153) and matrix effects in MS detection (154). Moreover, the use of LC-MS/MS equipment is costly and requires significant technical expertise (155). However, LC–MS/MS has more strengths and fewer weaknesses as identified by users compared with other methods.

A very sensitive analytical method is required to quantify the different vitamin D metabolites in relevant human biological fluids and, simultaneously, be selective enough to differentiate the numerous metabolic species (34). Therefore, increasing the selectivity and sensitivity for determination of vitamin D metabolites has been the aim of various methods and has focused on sample clean-up/extraction and analyte concentration prior to determination.

1.10 Sample preparation/extraction
Analyzing lipid soluble compounds such as vitamin D metabolites in biological fluid samples provides a great challenge to ensure an efficient sample clean-up and to remove other interfering substances prior to analyte separation and/or detection (155). Establishing an appropriate extraction method for vitamin D is crucial as it cannot be assessed by the validation process (143). Therefore, the release of the vitamin from the sample matrix is a crucial step that affects the sensitivity and reproducibility of the analytical process.

Ideally, the extraction method must be capable of dislodging the entire vitamin content from the matrix before analysis. One way of assessing the efficiency of extraction is to compare the results from a variety of extraction methods, as was accomplished in a recent study: six different vitamin D assay methods were compared and the results showed high variability illustrating inconsistency in the extent of vitamin D release from the matrix (156). Most methods performed poorly with samples from pregnant mothers having higher amounts of vitamin D binding proteins. When vitamin D release from such proteins is incomplete, falsely low estimates for in vivo vitamin D status are obtained. The above study included both chromatographic and immunoassay methods as both use some form of extraction to release the vitamin, although in kit-based immunoassays this extraction step is less obvious. Most immunoassays use protein precipitation either by using common precipitant solvents or through reagents provided by suppliers of the kit.
Traditionally, saponification has been used for the extraction of vitamin D from foodstuffs including milk, and protein precipitation is used for the same in plasma and serum. There have been only a limited number of instances where saponification has been used for serum and plasma (143).

Liquid-liquid extraction (LLE) and solid phase extraction (SPE) also have been used as extraction/sample clean-up procedures in vitamin D assays analyses after the release from matrix components such as protein and fat.

1.10.1 Protein Precipitation

Protein precipitation has been frequently used as a fast sample clean-up extraction procedure due to its simplicity by which to release proteins from biological fluids (143, 157). Precipitation is useful as it can be employed to denature the protein, destroying its drug-binding ability depending on the binding mechanism (158). In order to quantify a drug in a serum or plasma sample, it is often necessary to disrupt the protein–drug binding so that the total amount of the drug can be extracted for analysis. Research on protein precipitation is growing and many researchers have used protein precipitation in their analysis of drugs in serum and plasma (157).

A study to determine the most competent protein precipitants for protein removal was undertaken and zinc sulphate (10%, w/v), acetonitrile and trichloroacetic acid (10%, w/v), were consistently found to remove plasma proteins effectively in all species at all precipitant to plasma volume ratios of 2:1 and greater (157). The chosen sample pre-treatment involved manual protein precipitation using acetonitrile with a ratio of sample: acetonitrile of 1:1.5, described by Polson et al. (157) followed by centrifugation to remove the precipitated proteins. Subsequently, evaporation is used to remove the supernatant solvents and the residue is then reconstructed in an appropriate solvent, in accordance with the analytical method used. Despite the fact that protein precipitation is neither as complex nor as time-consuming as saponification, the disadvantage of protein precipitation is that the extract is not very clean and contains a considerable amount of impurities from the matrix. This finding may lead to poor selectivity and ion suppression in LC–MS (143).
1.10.2 Saponification

Traditionally, the process of alkaline saponification for the extraction of vitamins and step-wise HPLC analyses has been widely used for analysis of lipophilic vitamins in animal feeds and the most common procedure by which to extract vitamin D compounds from foodstuff (159). The hydrolysis reaction attacks ester bonds and releases the fatty acids from the glycerol of glycerides and phospholipids, as well as from esterified sterols and carotenoids (160). This reaction also frees vitamin D from any binding matrix that may exist.

Given the lack of stability of vitamin D, it is common to use antioxidants such as pyrogallol (benzene-1,2,3-triol) (161), butylatedhydroxytoluene (2,6-di-tert-butyl-4-methylphenol) and ascorbic acid in the saponification process (162-166), combined with potassium hydroxide in ethanol or water solutions. The importance of potassium concentration in ethanol or methanol in saponification to obtain vitamin D in milk matrix has been reported in various studies. There was no significant difference when either methanol or ethanol were used (167). Ethanolic KOH prevents the formation of emulsions and mixes well with fat, but it requires daily preparation (168). In contrast, aqueous KOH does not mix so well with fat, but is more stable – this probably being the reason why it is more often used (168). With 1.9N KOH, D₂ and D₃ recoveries are less than 40%; recovery doubles when increasing the KOH concentration to 3.8 N (167).

Hot saponification consists of treating the sample with ethanolic or aqueous KOH at temperatures between 60–100 °C and times range of 20–45 min while cold saponification consists of treating the sample overnight with ethanolic or aqueous KOH at room temperature, under slow constant stirring (168). Thermal isomerisation of vitamin D to pre-vitamin D may be avoided in a cold saponification procedure (isomerisation losses of less than 5% under cold conditions versus about 10–20% under hot conditions). Furthermore, this method provides satisfactory extraction and recovery and is simpler to operate with less operator attention (160, 169).

Once saponification has been completed, the non-saponifiable fraction is extracted with organic solvents that are not miscible in water such as heptane, hexane, petroleum ether, di-ethyl ether, pentane or mixtures of these substances. It is usually preferable to use hexane instead of di-ethyl ether since di-ethyl ether is more inflammable and unstable than hexane.
and the latter can be simply removed at low pressure at a temperature below 50°C (160). However, Renken et al. preferred di-ethyl ether because it is less prone to form emulsions (170). In addition, there are various other conditional factors such as sample particle size (mesh), ratio of sample to reagent, extraction time, extraction equipment and pre-purification that can affect extraction efficiency (171). Subsequently, evaporation is used to remove the organic solvents and an appropriate solvent, according to the analytical method used, is used to reconstitute the residue. Nevertheless, the saponification procedure is time-consuming and, in addition, there is a considerable amount of complexity involved in the process.

1.10.3 Liquid-liquid extraction (LLE)

In vitamin D analysis liquid-liquid extraction (LLE), the sample is agitated in the presence of an extracting solvent that is not miscible with the sample (21, 172). Solvents such as hexane, heptane, ether, ethyl acetate, methyl t-butyl ether, dichloromethane, cyclohexane, iso-octane, chloroform, pentane or mixtures of non-polar solvents have been frequently used (143). When the sample/solvent mixture has settled after agitation, and two layers of liquids are visible, the organic layer will contain most of the vitamin D compound. The shaking action ensures that all parts of the sample come into contact with the extraction solvent. The organic layer is then removed, evaporated and the sample residue containing vitamin D compounds is reconstituted in a suitable solvent depending on the subsequent analytical technique to be used. LLE generally provides cleaner extracts than solid phase extraction (SPE) (173) and it is considered inexpensive and simple. However, it is not free of complications, for instance, the use of hazardous solvents, emulsion formation and low efficiency due to matrix effects in the sample, when compared with SPE (143).

1.10.4 Solid phase extraction (SPE)

Solid-phase extraction (SPE) has also been used for the extraction of vitamins from biological fluids (174-176). The SPE procedure consists of an initial conditioning of a reversed phase cartridge, application of the sample, subsequent washing followed by elution with a suitable solvent/solvent mixture. Several different SPE cartridges have been tested and different washing or elution solvents assessed in previous studies (175, 177, 178). After a sample application, metabolites are retained on the sorbent and subsequently washed to remove polar compounds. The sorbent is then eluted using a predominantly organic solvent to isolate the hydrophobic vitamin D compounds. Subsequently, the eluent is evaporated to
dryness and the extract concentrated by reconstituting the residue in a small volume of a suitable solvent, depending on the subsequent analytical method used. Some LC–MS methods using MS/MS detection for the quantification of vitamin D compounds in serum or plasma have employed on-line solid phase extraction after protein precipitation (78, 179). SPE systems have been developed for vitamin D metabolites including 25(OH)D₃, 25(OH)D₂, 24,25(OH)₂D₃, 1,25(OH)₂D₃ and 1,25(OH)₂D₂ (177, 180, 181).

Polar sorbents have been used in SPE clean-up processes of vitamin D compounds. For this class of sorbents, interactions between compounds and stationary phases may take place by hydrogen bonds, dipole-dipole, or π-π interactions (163, 165, 182-185). Some methods (186) have used immunosorbents but since vitamin D metabolites have similar chemical characteristics with other substances in biological fluids, cross reactivity may easily occur with immunosorbent SPE materials (143).

SPE offers benefits such as lower sample volumes needed and less hazardous solvents used when compared to LLE extraction. In addition, there is no emulsion formation in SPE. However, SPE has some drawback; the most significant of which that, SPE is expensive because the SPE cartridges are intended for single use. Furthermore, the SPE clean-up procedure necessitates additional time and effort in method development.

1.11 Derivatisation procedures
Vitamin D metabolites are lipophilic in nature and have low abundance in human biological fluids; this makes the development of analytical assays very challenging. In addition, vitamin D metabolites have low ionisation efficiencies in electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) sources because they lack easily charged groups, which would enhance ionisation efficiencies (187, 188). A common strategy is derivatisation to enhance the assay sensitivity of vitamin D metabolites in various human biological samples; derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), a representative Cookson-type reagent, has often been employed (187). In this reaction, the dienophile moiety of PTAD reacts with the s- cis- diene structure of 25(OH)D₃ substances at carbon positions 19, 10, 6, and 5, resulting in the formation of a stable PTAD adduct (74, 153, 188, 189). Figure 1.7 has illustrated the formation of two epimers (6S and 6R) during derivatisation with PTAD, corresponding to the position of the dienophile relative to the plane of the A ring (153).
However, other derivatisation agents used such as Ampliflex® (23), custom-synthesized DAPTAD (4-(4’-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione) (190) and 2-nitrosopyridine (PyrNO) demonstrate that improved ionisation and higher sensitivity can be achieved compared to PTAD (191).

Improvements in signal response have been achieved by the introduction of this reagent and some studies have reported previously that this process increases in sensitivity over non-derivatised compounds (192-194). One study found that the optimum sensitivity increase for vitamin D-PTAD compounds was achieved when 100-fold molar excess of PTAD over vitamin D compounds was used with the reaction time of one hour (193, 195). Other advantages of derivatisation include the reduction of noise from low molecular impurities in the mobile phase thereby improving signal/noise ratio in the mass spectra by shifting to higher m/z range by derivatisation (195). Unfortunately, derivatisation procedures require time-consuming sample preparation steps thus less suitable for routine clinical application.

![Derivatisation of 25(OH)D₃ using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD).](image)

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**Figure 1.7:** Derivatisation of 25(OH)D₃ using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD). PTAD reacts with the *s-cis*-diene structure of 25(OH)D₃ substances at carbon positions 19, 10, 6, and 5, resulting in the formation of two epimers (6S and 6R). Adapted from (153)
1.12 Quantification

It is common knowledge in analytical chemistry that the instrumental response can be affected by the sample matrix. With mass spectrometric detection in liquid chromatography, co-eluting impurities affect the analyte response due to ion suppression/enhancement. Compounds that are co-eluted with the analyte interfere with the ionization process in the MS detector, thereby causing ionisation suppression or enhancement (196). In mass spectrometry (MS), the ion suppression/enhancement effects due to the matrix can significantly reduce or enhance the analyte response thereby affecting sensitivity (197). Moreover, matrix effects encountered in LC-MS/MS techniques can affect accuracy, precision and the limit of detection (198). The matrix effect is one of the most relevant drawbacks when analysing human blood samples; the properties of vitamin D (such as its lipophilic nature and tight binding to vitamin D-binding protein (DBP)) have the potential to cause substantial matrix effects during sample extraction.

An option can be suggested to minimise or to correct ion suppression/enhancement included: modifications of the MS conditions and carrying out an extensive sample clean-up to reduce matrix effects (199). If matrix suppression/enhancement phenomena cannot be eliminated or made negligible through the application of the strategies described, the only way to obtain accurate data is to remove the contribution from the interferences at the quantification step. The addition of stable isotope labelled internal standard (SIL-IS) to the sample and subsequent quantification using internal standard (IS) calibration has become the most commonly used method to correct matrix effects in MS detection. Since the chemical properties and ionisation process of SIL-IS should be almost identical to those of the analyte, it will elute at the same retention time as the analyte and will experience the same extent of matrix effects (200). Although the magnitudes of the individual responses for the analyte and the internal standard will differ in the presence and absence of ion suppression/enhancement, the ratio of responses will be unaffected. Therefore, the use of stable isotope labelled internal standard (SIL-IS) for each vitamin D compound is essential to correct analytical errors caused by ion suppression effects and to ensure the accuracy and reliability of data (198, 201, 202). Unfortunately, their use may be expensive, especially in a multicomponent analysis or may be impractical due to the lack of available commercial standards.
1.13 Conclusion
This chapter has summarised the different forms of vitamin D and the possible risk factors of vitamin D deficiency in humans. Particular emphasis has been made on the analytical methods used for quantification and sample preparation procedures in a variety of matrices. Our knowledge regarding the consequences of vitamin D deficiency in humans is still incomplete and therefore requires further investigation. There is some evidence that the frequency of measurements of vitamin D in the human population has significantly increased over the last decade since vitamin D deficiency has now been linked to many diseases. This is due to each form of vitamin D having a different biological function, in addition to its established role in bone health. Serum 25(OH)D$_3$ concentration is used to serve as a marker for vitamin D in plasma and serum. However, measuring 25(OH)D$_3$ only provides an incomplete picture that may or may not correlate with function or disease. Vitamin D methods have been compared and critically evaluated at each stage of the quantification including extraction. This has enabled the evaluation of each stage of the analytical process and the selection or development of an appropriate method for a specific application.

Liquid chromatography-mass spectrometry (LC-MS/MS) has emerged as the preferred analytical technique used for vitamin D assays, but immunoassays are widely employed in clinical settings. These approaches are challenging, however, due to instrumental limitations with the detection of vitamin D compounds and the low concentrations of the metabolites in biological fluids. The development of new LC-MS/MS sources as well as improvements on derivatisation procedures are expected to address issues related to the poor ionisation efficiency in ESI or atmospheric pressure chemical ionisation of vitamin D compounds. Ideally, a desirable analytical method for vitamin D metabolites would cover all relevant high-and low-abundance species, using a universal and standardised analytical method (34).

1.14 Research aim
The aim of this research is to develop methods by which to measure quantitatively a wide range of vitamin D compounds, thereby creating a comprehensive picture of vitamin D status. A robust, efficient, reliable and inexpensive analytical method is to be developed to simultaneously measure 12 vitamin D compounds in human blood. Specific research objectives are as follows :-

2. To compare the efficiencies of two different sample preparation methods (protein precipitation and saponification).

3. To determine the appropriateness of the biological sample (serum and plasma) as the sample for vitamin D analysis and the sample size required to measure simultaneously 12 vitamin D compounds in serum and plasma.

4. To develop a method that is amenable for routine analysis.

5. To apply the method to quantify the total vitamin D in other biological fluids.

1.15 Research hypotheses

1. Protein precipitation is a better sample preparation method to extract vitamin D from blood samples when compared to saponification followed by liquid-liquid extraction.

2. There is no significant difference between plasma and serum as the optimum sample matrix for vitamin D assay analysis.

3. LC-MS/MS approaches can simultaneously quantify the concentrations of 12 vitamin D compounds over wide concentration ranges.
CHAPTER 2: SIMULTANEOUS QUANTITATIVE ANALYSIS OF NINE VITAMIN D COMPOUNDS IN HUMAN BLOOD USING LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

2.1 Foreword
This chapter describes the attempts to develop and validate an LC-MS/MS method for the sensitive and accurate quantification of 12 vitamin D compounds in human blood. A comparative evaluation of sample type and extraction method were carried out. Based on this comparison, a sensitive LC-MS/MS method was developed and validated. Much work of this chapter has been published in the journal Bioanalysis, 2016; 8:397–411; doi: 10.4155/bio.15.260. However, to be compatible with the style and the flow of the thesis, some modifications have been introduced. The references have been revised to follow the format used in the thesis, as well as the numbering for Tables and Sections. Sections have been added at the end of the chapter to include additional work that has been undertaken for this chapter but has not been published.

2.2 Introduction
Historically, numerous studies have been undertaken concerning the role of vitamin D in bone health. Recent studies have, however, concentrated more on elucidating the ways in which the vitamin and its metabolites may influence other pathologies (11, 35). In addition to the well-known effect of vitamin D’s role in bone formation, there is now growing evidence that vitamin D deficiency can increase the risk of other major diseases such as cancer, cardiovascular diseases, schizophrenia and depression (5). These findings have led to a significant increase in studies investigating links between vitamin D levels and such conditions.

Although vitamin D has more than 50 metabolites, only two metabolites, namely, 25-hydroxyvitamin D [25(OH)D] and 1,25-dihydroxyvitamin D [1,25(OH)₂D], have been afforded the greatest clinical attention in relation to their quantification (35, 203). Controversy, however, remains regarding the forms of vitamin D and their function; a comprehensive, sensitive and robust assay methodology for vitamin D metabolites is therefore required (48). Vitamin D₂ (D₂ or ergocalciferol) and vitamin D₃ (D₃ or
cholecalciferol), the major forms in ingested food, are considered to be biologically inactive (11) and they are metabolised in the liver to 25(OH)D₂ (25-hydroxyvitamin D₂) and 25(OH)D₃ (25-hydroxyvitamin D₃), collectively referred to as 25(OH)D. Subsequently, they are hydroxylated to 1,25(OH)₂D (collectively referred to as 1,25-dihydroxyvitamin D₂ and 1,25-dihydroxyvitamin D₃) or 24,25(OH)₂D (collectively referred to as 24R,25-dihydroxyvitamin D₂ and 24R,25-dihydroxyvitamin D₃).

Of the four known sulfated conjugates, vitamin D-sulfate (D₃-S and D₂-S) and 25-hydroxyvitamin D-sulfate (25(OH)D₃-S and 25(OH)D₂-S), 25(OH)D₃-S has been the only form found in human and animal blood to date (77). The circulating levels of this compound were found to be similar to or higher than those of 25(OH)D₃ in adults and infants, respectively (19, 77, 80). Since the level of 25(OH)D₃-S is similar to or exceeds that of the major circulating form of vitamin D in adults, it was viewed as being important in researching the significance of the sulfate metabolite in pregnant women and newborns (80). Despite being a major circulating form of vitamin D in man the biological role of 25(OH)D₃-S is not yet known, and has been therefore, considered to be a “storage” form of the major circulating form 25(OH)D₃ (77). The lack of research into the biological function of 25(OH)D₃-S may be due to the scarcity of the methods available for its accurate quantification, until recently, for its accurate quantification (78). In studies where up to six analogues and two epimers of vitamin D were determined simultaneously, sulfate metabolites were not included (204).

Considering the above information, and, given the differences in biological activities of each vitamin D form, it is important to selectively and accurately quantify each form of vitamin D separately. It is also timely to develop an assay that can simultaneously determine as many forms of vitamin D as possible, including the most active forms. An ideal analytical method would cover all ranges of concentration for all vitamin D metabolites with one standardised analytical method (34). We present here the development of a method that is capable of accurately and selectively quantifying nine compounds of vitamin D as well as capable of separating the epimeric compounds of the major circulating form 25(OH)D₃, in human blood. This method will facilitate clinical research into the study of the biological function/s of each form and their potential roles in a variety of clinical conditions.
The majority of the studies on vitamin D have used serum as it is recognised that serum 25(OH)D₃ concentration measurements provide the single best assessment of vitamin D status (203). In contrast, some studies have reported that the levels of 25(OH)D₃ in plasma represent the best indicator of physiological vitamin status. A recent study using a solid phase extraction (SPE)-LC/MS method, confirmed that vitamin D₃, 24,25-dihydroxyvitamin D₃ and 25-hydroxyvitamin D₂ levels in plasma and serum were similar, while levels of 1,25-dihydroxyvitamin D₃ were significantly different, being higher in plasma than in serum (142).

As the potency of 25(OH)D₂ is considered to be less than one third of the potency of 25(OH)D₃ (43), the most common method used to measure vitamin D, immunoassay-based methods, due to their inability to differentiate between these compounds, may overestimate the in vivo vitamin D status. Furthermore, some immunoassay methods are known to not detect 25(OH)D₂ with adequate sensitivity therefore adding more potential inaccuracy to the measured vitamin D status (48). The application of liquid chromatography-tandem mass spectrometry, with its associated high selectivity and sensitivity, has been used increasingly in vitamin D assay methods in recent years (143) and it has greatly facilitated the determination of individual compounds of vitamin D. In terms of quantifying a variety of forms, the most significant study so far has been one that was developed for the quantification of six forms of vitamin D, with two epimers separated (204).

Mass spectrometry (MS) detection is susceptible to matrix effects, in the form of ionisation suppression or enhancement that can detrimentally affect both accuracy as well as sensitivity. Complete chromatographic separation of all interfering matrix compounds from all analytes of interest in every sample is likely unachievable. Therefore, correction for matrix effects using co-eluting (stable isotope labelled) analogues as internal standards for each analyte is the optimal way to ensure the accuracy and reliability of data (201). In this study, we employed an internal standard calibration method using stable isotope labelled analogue for each of the 12 compounds of vitamin D.

Vitamin D metabolites have low ionisation efficiencies in electrospray (ESI) or atmospheric pressure chemical ionisation (APCI) sources due to the lack of charged groups that would help to enhance ionisation efficiencies. The conjugated diene group of vitamin D metabolites, however, renders them a specific target for Diels–Alder derivatisation. The derivatisation reagents present polar groups and accordingly normally bring about a 100–1000-fold increase
in sensitivity over non-derivatised compounds, depending on the vitamin D compound (188). In this study, we applied the commonly used 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (205) to derivatise the vitamin D compounds prior to LC-MS/MS analysis.

Traditionally, a saponification method with subsequent extraction has been used for the determination of vitamin D from foodstuffs including milk, and protein precipitation has been used in plasma and serum analysis. There have been only a limited number of instances where saponification has been used for serum and plasma (143) and, to date, there have been no studies that have compared these two major methods of sample preparation. Therefore, in the following report, saponification and protein precipitation were compared for their effectiveness in extracting vitamin D from human blood samples from volunteer adults.

2.3 Materials and methods

2.3.1 Serum and plasma samples

Blood samples were obtained from volunteer staff and research students of the School of Pharmacy, University of Queensland. Ethical approval was obtained from the Institutional Human Research Department, The University of Queensland. Participants’ blood samples were taken by the pathology laboratory in the UQ Health Care Centre. The sample size was 10 mL. Blood was collected in Vacutainer tubes containing either EDTA or without anticoagulant, for plasma and serum samples, respectively.

2.3.1.1 Serum preparation

Blood samples were collected in yellow-topped evacuated tubes from Becton Dickinson (BD) with no anticoagulant. BD’s trade name for the blood handling tubes is Vacutainer. The sample was incubated in an upright position at room temperature for 30-45 min (not longer than 60 min). The clot was removed by centrifugation at 1000-2000 x g for 15 min at room temperature. The resulting supernatant was designated serum. The samples were maintained at 2-8 °C while handling. The supernatant (serum) was apportioned into 500 µL aliquots in Eppendorf tubes and stored at -80 °C. It is important to avoid freeze-thaw cycles because this can be detrimental to many serum components. Samples were labelled with the relevant information.
2.3.1.2 Plasma Preparation

Blood samples were collected into anticoagulant with EDTA treated tubes (lavender tops). Cells were separated from plasma by centrifugation for 15 min at 1000 - 2000 x g using a centrifuge at room temperature. The resulting supernatant is designated plasma. The samples were maintained at 2-8 °C while handling. Plasma was apportioned into 500 µL aliquots in Eppendorf tubes and stored at -80 °C. It is important to avoid freeze-thaw cycles because this can be detrimental to many plasma components. Samples were labelled with the relevant information.

2.3.2 Chemicals and reagents

All reagents and solvents were of analytical grade or LC-MS grade. PTAD (≥97%) was purchased from Sigma Aldrich (St. Louis, USA). Acetonitrile, ascorbic acid, dichloromethane, ethanol, hexane, methanol and potassium hydroxide were purchased from Merck (Darmstadt, Germany). Formic acid (>99%) was purchased from Fisher Chemical (Geel, Belgium). High-purity water was prepared in-house using a Millipore Milli-Q system (Milford, USA). Water and solvents used for the mobile phases were filtered with a 0.2-µm Supelco Nylon 66 membrane (Supelco, Bellefonte, PA, USA).

Standard vitamin D compounds and several of their stable isotope labelled analogues [D2, D3, D2-S, D3-S, 25(OH)D2, 25(OH)D3, 25(OH)D2-S, 25(OH)D3-S, 24,25(OH)2D2, 24,25(OH)2D3, 1,25(OH)2D2, 1,25(OH)2D3, D2-d3, D3-d3, D2-S-d3, D3-S-d3, 25(OH)D2-S-d3, 25(OH)D3-S-d3, 24,25(OH)2D2-d6, 24,25(OH)2D1-d3, and 1,25(OH)2D3-d6] were purchased from IsoScience (King of Prussia, USA). Other labelled analogues (25(OH)D2-d6 and 25(OH)D3-d6) were purchased from Chemaphor (Ottawa, Canada), (1,25(OH)2D2-d6)Medical Isotopes Inc. (Pelham, USA), and (24,25(OH)2D2-d6) Toronto Research Chemicals Inc. (Ontario, Canada). All compounds had a purity of 97% or better.

2.3.3 Preparation of standards and sample solutions

All preparations were performed under subdued light. Stock solutions in ethanol of all standard compounds were prepared at a concentration of 1µM, were sub-divided into small volumes, and stored in amber Eppendorf tubes at -20°C. All working solutions were prepared
by serial dilution of stock solutions. Several aliquots (500 µL) of plasma and serum were accurately measured and frozen at -80°C until analysis.

2.3.4 Optimisation of saponification followed by liquid-liquid extraction method

Plasma (250 µL) was mixed with 250 µL of two different concentrations (4M or 8M) of potassium hydroxide in ethanol containing 20% ascorbic acid. The saponification reaction was carried out for 20 min at 80°C, 3 h at room temperature or overnight (12 h) at room temperature. This 2x3 variable factorial design was carried out with triplicate samples for each treatment combination, totalling 18 samples. After completion of saponification, the mixture was centrifuged at 4000 x g for 10 min. The supernatant was transferred to a clean Eppendorf tube, and 675µL hexane: dichloromethane mixture (4:1, v/v) was added. The solution was then vortex mixed for 1 min. The upper clear layer was transferred to a new tube and dried under a gentle stream of nitrogen at room temperature. The dry residue was subjected to derivatisation with PTAD, as described below. The derivatised mixture was evaporated to complete dryness under a gentle stream of nitrogen and the residue reconstituted with 50 µL mobile phase (77 %B – see below) prior to LC-MS/MS analysis. Peak areas were used to assess the efficiency of each set of conditions.

2.4 Optimisation of sample preparation

2.4.1 Protein Precipitation

Plasma/serum (500 µL) was mixed with 1000µL of acetonitrile and 25 µL of the combined internal standard solution (to give the same concentrations as those in calibration standards in the final solution injected onto LC-MS/MS), then vortex mixed for 1 min. The mixture was incubated for 15 min at room temperature to allow protein precipitation to be completed and was then centrifuged at 4000 x g for 10 min. The supernatant was transferred to a clean Eppendorf tube and evaporated under a gentle stream of nitrogen gas at room temperature to evaporate most of the acetonitrile. The remaining solution in each tube was then frozen at -80°C for one hour, then evaporated using a freeze drier operated at -80°C (12 h). The dry residue was subjected to a derivatisation reaction with PTAD, as described below.
2.4.2 Saponification

The procedure used was similar to that in 2.3.4 using the optimised conditions: 8M potassium hydroxide in ethanol (500 µL) containing 20% (w/v) ascorbic acid added to plasma/serum (500 µL) and overnight (12 h) saponification at room temperature. The combined internal standard solution (25 µL; to provide the same concentrations as those in calibration standards in the final solution injected to LC-MS/MS) was added to plasma/serum before adding the saponification mixture.

2.5 Derivatisation reaction

The dried residue obtained from protein precipitation or saponification was vortex mixed with 350 µL of derivatising agent, PTAD (10 mM in ACN), for 1 min. The mixture was incubated at the room temperature for 1 hr, then, upon completion of the reaction, the excess PTAD was reacted with ethanol (50 µL). The solvent was evaporated to complete dryness under a gentle stream of nitrogen at room temperature. The dry residue was reconstituted with mobile phase (50 µL; 77%B –section 2.6), and centrifuged before transferring the supernatant into HPLC vials. An aliquot (20µL) was injected into the LC-MS/MS system for analysis. For standards, the mixed standard solution at appropriate concentrations was evaporated under a gentle stream of nitrogen gas to dryness, then derivatised with PTAD and reconstituted with mobile phase as described above.

2.6 LC-MS/MS procedure

An Agilent binary LC system consisting of an Agilent 1290 infinity LC pump, Agilent 1290 well plate auto-sampler and a Poroshell 120 EC-C18 (150 x 2.1 mm, 2.7 µm) column (Agilent Technologies, Santa Clara, CA, USA) was used for separations. A binary solvent consisting of 0.1% formic acid in water (A) and 0.1% of formic acid in methanol (B) was used for separation. Isocratic elution was maintained for 12.5 min at 77%B. The composition of the mobile phase was then changed from 77% B to 100% B over the next 1.5 min. Thereafter, it remained at 100% B over the next 9 min. The mobile phase was then returned to the starting composition of 77% B over the next 1 min, and the column was subsequently re-equilibrated with 77%B for 10 min before injecting the next sample. The total run time including the equilibration of the column was 34 min. The mobile phase flow rate was 0.2 mL/min and the injection volume was 20 µL.
An Agilent 6460 triple quadrupole tandem mass spectrometer equipped with a Jet Stream source and supported by *Mass Hunter Workstation* software (Agilent Technologies, Santa Clara, CA, USA) was used as the detector, in ESI positive mode. For all compounds, the MS parameters were optimised to obtain the highest signal in MRM mode. *Table 2.1* shows the optimised parameters and the MRM and qualifier ions used. Source parameters including gas flow, sheath gas flow, gas temperature and sheath gas temperature were optimised and maintained at 5 and 12 L/min (gas and sheath gas flows, respectively) and at 300°C and 250°C (gas and sheath gas temperatures, respectively). The capillary and nozzle voltages were, respectively, maintained at 5000V and 2000V.
**Table 2.1:** Optimised mass spectrometry parameters used in MS detection

<table>
<thead>
<tr>
<th>Vitamin D compounds with PTAD</th>
<th>MRM pair</th>
<th>Qualifier ion</th>
<th>Fragmentor</th>
<th>Collision energy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Precursor ion - Product ion</td>
<td>(m/z)</td>
<td>(m/z)</td>
<td>(V)</td>
</tr>
<tr>
<td>D₃-PTAD</td>
<td>560-298</td>
<td>161</td>
<td>96</td>
<td>12</td>
</tr>
<tr>
<td>D₃-d₃-PTAD</td>
<td>563-301</td>
<td>-</td>
<td>96</td>
<td>12</td>
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<tr>
<td>D₂-PTAD</td>
<td>572-298</td>
<td>161</td>
<td>96</td>
<td>16</td>
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<tr>
<td>D₂-d₃-PTAD</td>
<td>575-301</td>
<td>-</td>
<td>96</td>
<td>16</td>
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<tr>
<td>25(OH)D₃-PTAD</td>
<td>558-298</td>
<td>161</td>
<td>168</td>
<td>12</td>
</tr>
<tr>
<td>25(OH)D₃-d₆-PTAD</td>
<td>564-304</td>
<td>-</td>
<td>168</td>
<td>12</td>
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<tr>
<td>25(OH)D₂-PTAD</td>
<td>570-298</td>
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<tr>
<td>25(OH)D₂-d₆-PTAD</td>
<td>576-304</td>
<td>-</td>
<td>144</td>
<td>12</td>
</tr>
<tr>
<td>25(OH)D₃-S-PTAD</td>
<td>639-378</td>
<td>161</td>
<td>144</td>
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<tr>
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<td>-</td>
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<tr>
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<td>650-378</td>
<td>161</td>
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<tr>
<td>25(OH)D₂-S-d₃-PTAD</td>
<td>653-381</td>
<td>-</td>
<td>144</td>
<td>12</td>
</tr>
<tr>
<td>1,25(OH)₂D₃-PTAD</td>
<td>574-314</td>
<td>177</td>
<td>139</td>
<td>8</td>
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<tr>
<td>1,25(OH)₂D₃-d₆-PTAD</td>
<td>580-320</td>
<td>-</td>
<td>139</td>
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<tr>
<td>D₃-S-PTAD</td>
<td>640-378</td>
<td>161</td>
<td>124</td>
<td>12</td>
</tr>
<tr>
<td>D₃-S-d₃-PTAD</td>
<td>643-381</td>
<td>-</td>
<td>124</td>
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<td>1,25(OH)₂D₂-PTAD</td>
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<td>1,25(OH)₂D₂-d₆-PTAD</td>
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<tr>
<td>D₂-S-d₃-PTAD</td>
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<td>-</td>
<td>134</td>
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</tr>
<tr>
<td>24,25(OH)₂D₃-PTAD</td>
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<td>161</td>
<td>198</td>
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<tr>
<td>24,25(OH)₂D₃-d₆-PTAD</td>
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<td>-</td>
<td>198</td>
<td>20</td>
</tr>
<tr>
<td>24,25(OH)₂D₂-PTAD</td>
<td>586-298</td>
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<td>178</td>
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</tr>
<tr>
<td>24,25(OH)₂D₂-d₆-PTAD</td>
<td>592-304</td>
<td>-</td>
<td>178</td>
<td>12</td>
</tr>
</tbody>
</table>
2.7 Method Validation

The calibration standards (six levels) were prepared in mobile phase (77% B) to cover the following concentration ranges of the derivatised compounds: 50-1250 nM (25(OH)D₃, 25(OH)D₂, D₂, D₃, and 25(OH)D₃-S); 5-100 nM (25(OH)D₂-S); 0.1-2 nM (1,25(OH)₂D₃ and D₃-S); 0.05-0.4 nM (1,25(OH)₂D₂ and D₂-S); 10-100 nM (24,25(OH)₂D₃) and 1-10 nM (24,25(OH)₂D₂). The (derivatised) internal standard concentrations in all calibration standards were: 500 nM (25(OH)D₃-d₆, 25(OH)D₂-d₆, D₂-d₃, D₃-d₃, and 25(OH)D₃-S-d₃); 50 nM (25(OH)D₂-S-d₃ and 24,25(OH)₂D₃-d₆); 1nM (1,25(OH)₂D₃-d₃, and D₃-S-d₃); 0.1 nM (1,25(OH)₂D₂-d₆, and D₂-S-d₃) and 5 nM (24,25(OH)₂D₂-d₃). Linearity was assessed by plotting the peak area ratios of standard: internal standard against the concentration of the standards using linear regression analysis.

The on-column limit of detection (LOD) was estimated as 3 x noise and limit of quantitation (LOQ) as 10 x noise for each compound. Repeatability was determined by analysing 10 separate aliquots of pooled serum sample followed by the calculation of the percentage relative standard deviation for each vitamin compound. Recovery was assessed by analysing the concentration of each vitamin D analogue in six separate aliquots of spiked and un-spiked serum samples. Each aliquot of serum was spiked to give an added concentration similar to the second level of the calibration range used in this study.

2.8 Results and discussion

2.8.1 LC-MS/MS method development for vitamin D compounds and epi-25(OH)D₃

The quantification of different vitamin D forms has been a recent trend (143) driven partly by the necessity to study the biological function of each form (48) and partly due to the availability of LC-MS/MS technology that possesses the necessary selectivity and sensitivity. The best method available to-date, in terms of the number of forms analysed, was able to quantify six vitamin D compounds (204). The two principal problems with vitamin D assays using LC-MS/MS have been the lack of sensitivity in MS detection (due to the lack of ionisable groups in vitamin D molecules) (153) and the accuracy/reliability concerns due to ion-suppression effects caused by endogenous interfering compounds in complex matrices such as plasma and serum. In this study, we overcame the sensitivity problem by derivatisation with PTAD to enhance ionisation. The inaccuracies introduced due to ion
suppression effects were corrected by using co-eluting labelled analogues of each vitamin D compound in internal standard calibration.

Each derivatised vitamin D compound was individually subjected to MS parameter optimisation. For this experiment, only a small excess (5X) of PTAD was used because the suppression resulting from a large excess of PTAD (as used in the developed LC-MS/MS method) produced very small or non-existent MS signals, when directly infused into MS. The optimized MS parameters are shown in Table 2.1. The internal standard of each form was detected using the same parameters, so that both compounds experience the same conditions. To further ensure that the internal standard and the corresponding unlabelled compound were analysed using the same conditions thus enabling correction of ion suppression effects, chromatograms were checked for compound co-elution.

Chromatography was optimised so that the first peak of interest was satisfactorily remote from early eluting interfering compounds, including the excess PTAD reagent. This minimised the potential ion suppression effects on vitamin D compounds of such early eluting compounds. Another aim of chromatographic optimisation was the separation of the epimeric form from 25(OH)D₃. The use of methanol, when compared to acetonitrile, provided improved selectivity, enabling the separation of epimeric forms. Chromatograms obtained for a standard mixture, using the optimum LC and MS conditions are shown in Figure 2.1. Seven of the vitamin D-PTAD compounds (including the epimeric form of 25(OH)D₃) were chromatographically resolved, and D₂ and D₃ pairs of other forms co-elute. This co-elution poses no problems on method accuracy or selectivity because these forms are mass spectrometrically well separated, based on their MRM transition pairs, and because the use of co-eluting internal standards for each form correct the ion suppression effects from each other. PTAD derivatisation produces two isomers (R and S) for each vitamin compound, but they are not always separated, as has been observed in previous methods (188, 194, 206). The quantification reported here is based on the total peak area of the R and S forms, therefore the resolution of these two isomeric forms is unimportant.
Figure 2.1: LC-MS/MS profiles of 13 vitamin D compounds in the standard mixture
2.8.2 Optimisation of the saponification method

Two approaches (protein precipitation and saponification with extraction) have been used previously to extract vitamin D from blood. However, the use of saponification has been limited (143). In our study, one of the aims was to extract the maximum amount of vitamin D from the matrix. To achieve this, we compared the two methods for their effectiveness in terms of amounts of each vitamin D compound extracted. Although protein precipitation is well established for use with blood samples, saponification is not. We considered that it was important to optimise the saponification method for blood samples prior to its application to a comparison study.

Saponification uses alkaline hydrolysis to break the ester linkages of triglycerides, phospholipids and esterified sterols, thereby releasing the vitamin compounds (207). Saponification procedures have been reported with the use of potassium hydroxide in ethanol or water solutions, containing ascorbic acid as the antioxidant as an alternative to reduce the instability of vitamin D compounds (168). The amount of vitamin D released may be dependent on the concentration of potassium hydroxide used in the saponification reaction and on the time/temperature of the reaction. The concentrations used for extraction of vitamin D compounds from milk (a food matrix somewhat similar to blood) varied from 4-8 M (167) and the temperature and time varied from room temperature to 80°C and 3 h to overnight (12 h) (155, 208). As described in the experimental section, we used a 2x3 factorial design to optimise the plasma saponification methodology. The results for the two major forms of the vitamin are shown in Figure 2.2. The saponification mixture containing 8M ethanolic potassium hydroxide and reacted for 12 h at room temperature extracted the greatest amounts of all major vitamin D forms. Higher concentrations of KOH and extended reaction times release more vitamin. Although a higher temperature could have generally reduced the reaction time, in this case it did not; vitamin D is known to undergo thermal isomerisation to pre-vitamin D at high temperatures. It was reported that less than 5% isomerisation occurs under cold conditions compared to 10 - 20% at 60°C (168).
Figure 2.2: Signal responses for major vitamin D compounds using a range of saponification conditions (error bars represent ±1SD, n = 3)
2.8.3 Comparison of sample types used for the vitamin D assay

The major difference between plasma and serum is the removal of fibrinogen and associated proteins by the coagulation process (138). The absence of the anticoagulant in the serum preparation permits an increase in the concentration of organic solvent used and these conditions then enhance the protein precipitation process (142). There is limited evidence that different collecting procedures and the presence or absence of the coagulation cascade influence the concentrations of both proteins and vitamin D compounds in these matrices. One concern, however, was the effect of anticoagulants on LC-MS/MS assay performance (141). This study demonstrated that different sample matrices, serum, heparin or K$_2$-EDTA coagulated plasma, did not cause significant variation in 25(OH)D$_2$ and 25(OH)D$_3$ quantification (141). In order to do a systematic comparison study, we used pooled samples of human serum and plasma from several healthy adults. As the comparison of the two methods (protein precipitation and saponification with extraction) was not done at this stage, both methods were used for each sample type. The saponification method used was the one reported above. Concentrations of each vitamin D compound were determined using the internal standard calibration method. Results shown in Figure 2.3 are expressed in vitamin D concentrations in serum or plasma; protein precipitation released higher concentrations for nine forms of vitamin D. For all vitamin D compounds, serum produced higher concentration levels of vitamin D. Based on these results, serum was selected as the sample type for the remainder of this study.
Figure 2.3: Comparison between serum and plasma using protein precipitation and saponification procedures for the extraction of nine vitamin D compounds. Error bars represent ±2SD (n=3). PP, Protein precipitation; SN, Saponification
2.8.4 Comparison of methods used for vitamin D assay

To our knowledge, the two sample preparation methods (protein precipitation and saponification) have not been compared for their performance in vitamin D assay methods in serum. Serum samples from 10 individuals were used for this comparison. In our preliminary study, protein precipitation extracted significantly higher amounts of vitamin D when compared to saponification, as shown in Figure 2.4; significant losses of vitamins during saponification have been previously reported (143). Based on the results obtained, further work was undertaken using the protein precipitation method to extract vitamin D from serum.
Figure 2.4: Comparison between protein precipitation and saponification procedures using serum. Mean concentrations of forms in 10 individuals shown. Error bars represent ± 1SD.
2.9 Method validation and application to samples

The LC-MS/MS method developed in 2.6 was combined with the optimal sample type and extraction method, as established in 2.8.3 and 2.8.4, to obtain the optimum assay method for all the forms of vitamin D chosen in this study. This method was then validated, as described in the experimental section. The calibration ranges used for each form of vitamin D were based, where available, on levels reported previously (78, 204, 206, 209, 210). In our study, the calibration standards were prepared in the mobile phase solvent at the same composition as the initial gradient conditions as described in LC-MS/MS section 2.7. Previous work has established that, when isotopically labelled standards are used as internal standards, there is no significant difference in the results when the calibration standard are prepared either in mobile phase solvent or in matrix matched medium (211, 212). Therefore, matrix matching of samples and those of calibration standards was not required since a co-eluting stable isotope labelled internal standard was used for each analyte quantified. This approach represents a significant time advantage and is additional to the role of the internal standards in improving precision and accuracy, as described earlier. For those compounds where the levels were not available, the lowest concentration ranges used for other vitamin D forms were used. Table 2.2 shows the results of regression analysis of the internal standard calibration (peak area ratio of standard/internal standard plotted against concentration of standard). The results show good linearity ($R^2 > 0.99$) for all 12 vitamin D compounds within the concentration ranges of interest. Table 2.2 also shows the on-column LOD and LOQ values in fmol, estimated as described in the experimental section, and the same in terms of concentrations in serum in pM (in parenthesis), calculated based on volume of injection of 20 µL, and taking into account dilution effects.
Table 2.2: Linearity, limit of detection (LOD), limit of quantification (LOQ), repeatability and recovery of all vitamin D compounds with PTAD

<table>
<thead>
<tr>
<th>Vitamin D compounds with PTAD</th>
<th>Linearity equation</th>
<th>R²</th>
<th>Mean ± SD (nmol/L)</th>
<th>RSD (%)</th>
<th>Mean (%) ± SD (nmol/L)</th>
<th>LOD (LOQ) *</th>
<th>LOD (LOQ) **</th>
<th>LOD (LOQ) ***</th>
<th>femtomols</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₃</td>
<td>y = 0.0008x + 0.4025</td>
<td>0.995</td>
<td>12.657 ± 0.0112</td>
<td>0.09</td>
<td>98.5 ± 5.02</td>
<td>14 (47)</td>
<td>1.4 (4.7)</td>
<td>0.28 (0.94)</td>
<td></td>
</tr>
<tr>
<td>D₂</td>
<td>y = 0.0005x + 0.0283</td>
<td>0.996</td>
<td>5.879 ± 0.0546</td>
<td>0.93</td>
<td>100.6 ± 4.13</td>
<td>14 (47)</td>
<td>1.4 (4.7)</td>
<td>0.28 (0.94)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D₃</td>
<td>y = 0.0042x + 0.0824</td>
<td>0.997</td>
<td>30.733 ± 0.7068</td>
<td>2.3</td>
<td>98.56 ± 2.37</td>
<td>19 (50)</td>
<td>1.8 (5.0)</td>
<td>0.38 (1.00)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D₂</td>
<td>y = 0.0023x - 0.0547</td>
<td>0.995</td>
<td>15.016 ± 0.3555</td>
<td>2.37</td>
<td>100.6 ± 7.62</td>
<td>11 (38)</td>
<td>1.1 (3.8)</td>
<td>0.20 (0.76)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D₃-S</td>
<td>y = 0.001x - 0.0256</td>
<td>0.997</td>
<td>4.168 ± 0.0902</td>
<td>2.2</td>
<td>96.2 ± 0.01</td>
<td>30 (100)</td>
<td>3.0 (10)</td>
<td>0.60 (2.00)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D₂-S</td>
<td>y = 0.0115x + 0.0214</td>
<td>0.998</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>43 (143)</td>
<td>4.3 (41.3)</td>
<td>0.86 (2.86)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>y = 0.4489x + 0.1198</td>
<td>0.995</td>
<td>0.245 ± 0.0030</td>
<td>1.23</td>
<td>98.9 ± 0.09</td>
<td>10 (33)</td>
<td>1.0 (3.3)</td>
<td>0.20 (0.66)</td>
<td></td>
</tr>
<tr>
<td>D₃-S</td>
<td>y = 0.5533x - 0.0377</td>
<td>0.991</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28 (130)</td>
<td>2.8 (13.0)</td>
<td>0.56 (2.60)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)₂D₂</td>
<td>y = 1.9038x - 0.0061</td>
<td>0.992</td>
<td>0.051 ± 0.0020</td>
<td>3.71</td>
<td>98.7 ± 7.46</td>
<td>10 (36)</td>
<td>1.0 (3.6)</td>
<td>0.20 (0.72)</td>
<td></td>
</tr>
<tr>
<td>D₂-S</td>
<td>y = 2.3457x + 0.1205</td>
<td>0.992</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>37 (126)</td>
<td>3.7 (12.6)</td>
<td>0.74 (2.50)</td>
<td></td>
</tr>
<tr>
<td>24,25(OH)₂D₃</td>
<td>y = 1.7725x - 0.7883</td>
<td>0.995</td>
<td>16.680 ± 0.1409</td>
<td>0.84</td>
<td>96.4 ± 0.38</td>
<td>18 (45)</td>
<td>1.8 (4.5)</td>
<td>0.36 (0.90)</td>
<td></td>
</tr>
<tr>
<td>24,25(OH)₂D₂</td>
<td>y = 0.0718x - 0.0331</td>
<td>0.992</td>
<td>1.477 ± 0.0023</td>
<td>0.16</td>
<td>95.1 ± 2.74</td>
<td>20 (43)</td>
<td>2.0 (4.3)</td>
<td>0.40 (0.86)</td>
<td></td>
</tr>
</tbody>
</table>

* Concentration in extract
** Concentration in serum
*** On column amounts
The repeatability values \((n = 10)\), determined by analysing multiple aliquots of a pooled human serum sample, and expressed as percentage standard deviations, are shown in Table 2.2. The method is precise with percentage standard deviations within the range 0.09\% to 3.71\%. The accuracy of the method, determined as the mean recoveries ranged from 95\% to 101\% as also shown in Table 2.2. Three of the compounds were not recovered therefore failed the validation. Table 2.3 shows the ranges of vitamin D (nine compounds for which the method was validated) detected in 10 healthy adult serum samples using the optimised validated method. It is worth emphasising that although the number of samples is small, a trend was observed of higher levels of \(\text{D}_2\) and its metabolites being detected in the serum from two volunteers who had been taking vitamin \(\text{D}_2\) supplements (2400IU/day). The lowest levels of vitamin D were detected in those volunteers who were not receiving vitamin D supplements.

Table 2.3: Concentrations ranges of vitamin D compounds in serum from 10 volunteers

<table>
<thead>
<tr>
<th>Vitamin D compounds with PTAD</th>
<th>Concentration ranges (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\text{D}_3)</td>
<td>3.04-36.2</td>
</tr>
<tr>
<td>(\text{D}_2)</td>
<td>0.863-6.46</td>
</tr>
<tr>
<td>(25(\text{OH})\text{D}_3)</td>
<td>19.2-104</td>
</tr>
<tr>
<td>(25(\text{OH})\text{D}_2)</td>
<td>16.4-52.2</td>
</tr>
<tr>
<td>(25(\text{OH})\text{D}_3)-S</td>
<td>6.81-20.1</td>
</tr>
<tr>
<td>(1,25(\text{OH})_2\text{D}_3)</td>
<td>0.148-0.347</td>
</tr>
<tr>
<td>(1,25(\text{OH})_2\text{D}_2)</td>
<td>*0.000 -0.020</td>
</tr>
<tr>
<td>(24,25(\text{OH})_2\text{D}_3)</td>
<td>1.03-2.71</td>
</tr>
<tr>
<td>(24,25(\text{OH})_2\text{D}_2)</td>
<td>0.508-2.29</td>
</tr>
</tbody>
</table>

*Concentration below limit of detection for two samples

Chromatographic profiles of a typical serum sample showing all detected vitamin D forms are shown in Figure 2.5. As evident from Table 2.2 and Figure 2.5, only nine out of twelve compounds were detected in serum. This was not only due to the presence of very low levels in serum but also because the extraction method and/or LC-MS/MS method were not ideal for the three vitamin D forms \(\text{D}_2\)-S, \(\text{D}_3\)-S and \(25(\text{OH})\text{D}_2\)-S. This conclusion was based on the observations that the spiked levels for these three were not recovered, and that the added internal standard compounds of these analytes were also not detected. Further work is necessary to explain these observations.
Figure 2.5: Chromatographic profiles showing all detectable vitamin D compounds in human serum samples
2.10 Conclusion

Although it has been suggested that each member of the family of vitamin D compounds may have different function/s, the specific functions and their relation to human health have not been well explored. The lack of selective quantification methods for each vitamin D compounds may have been a significant contributing factor for the scarcity of clinical research on individual vitamin D compounds. With the expansion of the scope of vitamin D assay methods, such as the method described here, more clinical research will be focused on the role of each vitamin D compound on human health. In time, the roles of D group vitamins will be well established, similar to those of the B group vitamins.
2.11 Optimisation of sample volume and number of liquid-liquid extractions (LLE)

2.11.1 Introduction

In this study, a determination of the minimum sample size required to measure simultaneously all major vitamin D compounds in blood was performed. The number of extractions necessary for liquid-liquid extraction was adjusted to obtain the optimal recovery of vitamin D compounds prior to analysing serum samples using LC-MS/MS. We used a 2 x 2 factorial design to optimise the sample volume and the number of extractions using the protein precipitation method.

2.11.2 Preparation of samples

Serum (250 µL or 500 µL) was mixed with acetonitrile (500 µL or 1000 µL) respectively, then vortex mixed for 1 min. The mixture was incubated for 15 min at room temperature to allow protein precipitation to proceed to completion. The mixture was then centrifuged at 4000 x g for 10 min and the supernatant was transferred to a clean Eppendorf tube. The remaining solution was extracted with hexane: dichloromethane (4:1, v/v) and the solution was then vortex mixed for 1 min. Samples (250 µL or 500 µL) were either one time or three times extracted with 500 µL or 1500 µL of hexane: dichloromethane (4:1, v/v), respectively. Each of the upper clear layers of supernatant was collected and transferred to a clean set of tubes and dried under a gentle stream of nitrogen gas at room temperature and subjected to further derivatisation reaction with PTAD, and analysed as described in sections 2.5 and 2.6 as above. Peak areas were used to assess the efficiency of each set of conditions.

2.11.3 Results and discussion

Initially, different ratios of hexane-dichloromethane for liquid-liquid extraction (LLE) were evaluated in our laboratory (195). Optimum results were obtained with hexane-dichloromethane (4:1, v/v) ratio especially for the metabolites that are relatively more polar (195). Thus, further extractions were conducted using the optimised LLE ratio in this study. Pooled samples of human serum from volunteers were used. The recoveries with 250 µL and 500 µL sample size were determined by using the similar numbers of extraction (three times) compared to one time extraction. The use of a higher volume of extraction solvent by increasing the number of extractions was shown to release higher concentration for seven
vitamin D compounds. While using the similar numbers of extraction, the recoveries with 500 μL and 250 μL sample size were determined. The greater the volume of the sample used in analysis, two-fold vitamin D compounds were identified. For 1,25(OH)2D3 and 1,25(OH)2D2 metabolites, since the level of these compounds in blood is extremely low (picomolar) level, a larger sample is required for quantification: both compounds were not detectable when using a 250 μL sample size. **Figure 2.6** demonstrates that the combination of these two parameter, when optimised provides a better recovery of vitamin D compounds. The extraction conditions thus chosen were based on three-fold LLE extractions and the serum sample volume was chosen to be 500 μL in order to achieve the maximum potential recovery.
**Figure 2.6:** Signal responses for major vitamin D compounds during sample volume and extractions study
2.12 Optimisation of protein precipitation procedure

2.12.1 Introduction

Protein precipitation using acetonitrile was selected based on its high efficiency in precipitating proteins (213). Liquid-liquid extraction (LLE) is a relatively simple and the most common clean-up procedure used for the analysis of vitamin D compounds following protein precipitation. In this study, the sample preparation procedure was compared; protein precipitation with LLE and without LLE in combination with PTAD derivatisation followed by analysis using LC-MS/MS. The sample volume (500 µL) and number of LLE extractions (three times with total volume 1500 µL) were used as per the optimised method in section 2.11.

2.12.2 Sample preparation

2.12.2.1. Protein Precipitation with liquid-liquid extraction (LLE)

Serum (500 µL) was mixed with 1000 µL of acetonitrile, and 25 µL of combined internal standard solution (to give the same concentrations as those in calibration standards in the final solution injected onto LC-MS/MS), then vortex mixed for 1 min. The solution was incubated for 15 min at room temperature to allow protein precipitation to proceed to completion. The mixture was centrifuged at 4000 x g for 10 min, the supernatant transferred to a clean Eppendorf tube and evaporated under a gentle stream of nitrogen gas at room temperature to evaporate the majority of the acetonitrile.

The remaining solution (approximate 500 µL) after the above procedure was extracted three times (using three equal aliquots of 500 µL) with a total volume of 1500 µL hexane: dichloromethane (4:1, v/v) and the solution was then vortex mixed for 1 min. Each of the upper, clear layer supernatants was collected and transferred to a new set of clean tubes and dried under a gentle stream of nitrogen gas at room temperature. The dry residue was then subjected to a derivatisation reaction with PTAD, as described in section 2.5 above. The derivatised mixture was evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted in 50 µL mobile phase (77% B – section 2.6) before LC-MS/MS analysis. All preparations were done under subdued light.
2.13.2.2. Protein Precipitation without liquid-liquid extraction (LLE)

Serum (500 µL) was mixed with 1000 µL of acetonitrile, and 25 µL of combined internal standard solution (to give the same concentrations as those in calibration standards in the final solution injected onto LC-MS/MS), then vortex mixed for 1 min. The solution was incubated for 15 min at room temperature to allow protein precipitation to be completed. The solution was centrifuged at 4000 x g for 10 min, the supernatant transferred to a clean Eppendorf tube and evaporated under a gentle stream of nitrogen gas at room temperature to evaporate most of the acetonitrile. The remaining solution in each tube was then frozen at -80°C for one hour, then evaporated to dryness using a freeze drier operated at -80°C (12 h). The dry residue was subjected to a derivatisation reaction with PTAD, as described in section 2.5 above. The derivatised mixture was evaporated to dryness under a gentle stream of nitrogen and the residue reconstituted with 50 µL mobile phase (77% B – section 2.6) before LC-MS/MS analysis. All preparations were done under subdued light.

2.12.3 Results and discussion

Data were generated by extracting 10 individual human serum samples and Figure 2.7 shows the comparison between the protein precipitation method with LLE and without LLE. Protein precipitation with a subsequent liquid–liquid extraction (LLE) step shows evidence of vitamin D loss and no signals for any vitamin D sulfate compound (Figure 2.7). It was hypothesise that not all vitamin D compounds from the matrix were extracted during the liquid–liquid extraction step and that the extraction and/or LC-MS/MS method proved to be unsuitable for the sulfate compounds. However, using the original method of protein precipitation and by removing the LLE step, the vitamin yields for such lipophilic compounds improved and one sulphate analogue, 25(OH)D₃ was recovered.

The comparison study demonstrates that despite the lengthy drying process, protein precipitation without LLE resulted in high recoveries of vitamin D compounds. Taking into consideration the necessity of exploring assays for multiple compounds, all studies in this chapter then were carried out using the original protein precipitation method without LLE.
Figure 2.7: Comparison between protein precipitation with LLE and protein precipitation without LLE procedures using human serum samples. Mean concentrations of compounds in 10 individuals shown. Error bars represent ±1SD.

LLE, liquid-liquid extraction
CHAPTER 3: SIMULTANEOUS DETERMINATION OF 12 VITAMIN D COMPOUNDS IN HUMAN SERUM USING ONLINE SAMPLE PREPARATION AND LIQUID CHROMATOGRAPHY-TANDEM MASS SPECTROMETRY (LC-MS/MS)

3.1 Foreword
The LC-MS/MS method described in Chapter 2 is capable of determining nine lipophilic forms and a single hydrophilic form [25(OH)D3-S] of vitamin D. It failed to properly accommodate three other sulfate compounds [25(OH)D2-S, D3-S and D2-S]. This chapter continues improvements to the method and aims to develop a method for the quantification of 12 vitamin D compounds simultaneously in human serum using liquid chromatography-tandem mass spectrometry (LC-MS/MS). The main challenge encountered was that of extracting and chromatographing vitamin D compounds with a range of polarities, encompassing both lipophilic and hydrophilic, in a single analytical procedure. The chapter explores in detail the strategies devised to improve the efficiency of extraction and used careful pH control in order to produce adequate chromatography for both lipophilic and hydrophilic forms of the vitamin D compounds. The simplicity of the method was enhanced by removing the lengthy derivatisation procedure and incorporating an online solid phase extraction (SPE). Much work of this chapter has been published in the Journal Chromatography A, 2017; https://doi.org/10.1016/j.chroma.2017.12.012. However, to be compatible with the style and the flow of the thesis, some text and content modifications have been introduced.

3.2 Introduction
Recent literature has described contemporary mass spectrometry assays for the quantitative measurement of multiple vitamin D compounds and their application in clinical research (34, 153, 214). It has been suggested that each member of the family of vitamin D compounds may have different functions beyond the traditionally referenced maintenance of musculoskeletal health towards conditions such as diabetes, cancer, multiple sclerosis, depression, and cardiovascular disease (52, 215). Consequently, the demand for measurements for vitamin D compounds in pathology laboratories has significantly
increased. Unfortunately, many studies investigating links between vitamin D and diseases investigate a single metabolic oxidative pathway, that yielding 25-hydroxyvitamin D [25(OH)D₃ and 25(OH)D₂], to assess the vitamin D status of individuals (216). It is plausible that measuring only 25-hydroxyvitamin D concentrations will provide an incomplete picture of vitamin D status that may or may not correlate with function or disease. An expansion of the range of vitamin D compounds that can be selectively quantified is hence potentially important for future vitamin D research.

Ideally, a benchmark method for all vitamin D metabolites would cover the relevant high and low abundance compounds, and would use universal, standardised analytical techniques (34). Methods for the determination of lipophilic vitamin D compounds, especially 25(OH)D₃, have been reported for a diverse range of biological fluids (23, 143). However, the lack of analytical methods for the determination of the more hydrophilic vitamin D compounds, such as the vitamin D-sulfate conjugate metabolites, has precluded their investigation in clinical studies examining their role and biological functions. While controversy exists regarding their biological roles, it has been reported that 25-hydroxy D₃-sulfate [25(OH)D₃-S] may be a storage form of vitamin D₃ and knowledge of its concentration may be expected to be helpful in the assessment of the vitamin D status, especially that of infants (78).

A previous method has been developed and is discussed in Chapter 2 above. The method uses derivatisation methodologies, to determine the major vitamin D compounds in human serum but was found to be inappropriate for the determination of three of the sulfated (D₂-sulfate, D₃-sulfate and 25-hydroxy D₂-sulfate) vitamin D compounds (217). A separate, non-derivatisation, method has also been developed to quantify these sulfated forms alone, taking into account their hydrophilic nature (84); both of these methods employed C18 columns. It was therefore resolved to undertake analytical method development to quantify simultaneously 12 vitamin D compounds including the sulfated forms [vitamin D₂ and D₃ (D₂ and D₃), 25-hydroxy D₂ and D₃ (25(OH)D₂ and 25(OH)D₃), 24,25-dihydroxy D₂ and D₃ (24,25(OH)₂D₂ and 24,25(OH)₂D₃), 1,25-dihydroxy D₂ and D₃ (1,25(OH)₂D₂ and 1,25(OH)₂D₃), D₂-sulfate and D₃-sulfate (D₂-S and D₃-S) and 25-hydroxy D₂-sulfate and D₃-sulfate (25(OH)D₂-S and 25(OH)D₃-S)] using a simple method with no derivatisation, incorporating some automation, and using a single column. The sensitivity was increased by reducing matrix effects in MS detector (using an additional sample clean up step) instead of
using pre-column derivatisation to improve ionisation in MS. The aim was to develop a method that was sufficiently simple and robust to accurately quantify 12 vitamin D compounds, both lipophilic and hydrophilic forms, using a single extraction and chromatographic run. The principal analytical challenge was that of quantifying compounds with very different lipophilicities in a single analysis procedure.

Vitamin D levels are often analysed in plasma or serum samples. A recent study, reporting on the determination of nine vitamin D compounds, revealed that serum levels of vitamin D compounds were higher than those in plasma samples taken at the same time (217). Protein precipitation is a common approach used to release vitamin D compounds from serum or plasma binding proteins by using acetonitrile (ACN) or trichloroacetic acid (TCA) for protein denaturation (157). However, establishing which protein precipitation method would release more vitamin D compounds and thereby yield a chromatographically cleaner extract has not been studied to date. Protein precipitation does not remove phospholipids which can cause ion suppression in MS (thereby reducing sensitivity), can reduce HPLC column lifetime and increase the need for mass spectrometry maintenance due to contaminant build up on the ion source (218). A sample clean-up technique such as solid phase extraction (SPE) is required to remove phospholipids from the extract before it is subjected to chromatographic separation. However, such a SPE clean-up procedure necessitates additional time and effort in method development. In this chapter, two protein precipitation techniques have been compared, to be used as the initial sample preparation method to remove biological proteinaceous matrices. An on-line SPE method using a guard column was also introduced further to clean-up the extract prior to LC-MS/MS separation. Stable isotope labelled analogues of each targeted compound (twelve in total) were used as internal standards to correct for matrix effects in the MS detector as well as to correct for the procedural errors during sample extraction and clean up. No LC–MS/MS method has been reported which is able to quantify simultaneously the lipophilic and hydrophilic vitamin D compounds in human serum; as stated above, currently available LC–MS/MS methods for the determination of sulfated vitamin D compounds are limited only to the measurement of 25(OH)D3-S in human plasma (217) or separate quantification procedures are used for lipophilic and hydrophilic vitamin D compounds, respectively (84, 195). Hence, the aim of this study was to develop and validate a simple, effective and reliable method that can be used in a routine analytical laboratory to quantify all major vitamin D compounds (both lipophilic and hydrophilic forms) in human serum, providing good precision, accuracy and sensitivity.
3.3 Materials and methods

3.3.1 Serum and plasma samples

Blood samples were obtained from volunteer staff and research students (20 participants) of the School of Pharmacy, University of Queensland. Ethical approval was obtained from the Institutional Human Research Department, The University of Queensland. All volunteers provided written informed consent and relevant information such as the hours of exposure to sunlight during the week of interest, consumption of vitamin D supplements and the type. Serum samples were collected in vacutainer tubes without anticoagulant. Aliquots (500 µL) of the serum samples were accurately measured and frozen at -80°C until analysis.

3.3.2 Chemicals and reagents

All reagents, solvents, unlabelled standard and standard vitamin D compounds were purchased as described in Chapter 2. Trichloroacetic acid (TCA) and 4-methylmorpholine were purchased from Sigma Aldrich (St. Louis, MO, USA); 3-epi-25(OH)D₃ and 3-epi-25(OH)D₃-d₃ were purchased from IsoScience (King of Prussia, PA, USA). Figure 3.1 shows the chemical structures for all stable isotope labelled vitamin D compounds (the analyte structures can be obtained by respectively replacing the deuterium atoms in each structural representation by hydrogen/protium atoms).
Figure 3.1: Chemical structures of the deuterated analogues of vitamin D compounds used in the analysis
3.4 Preparation of standards solutions
All preparations and manipulations of vitamin D containing solutions were done under subdued light. Stock solutions in ethanol of all standard compounds were prepared at a concentration of 1 µM, were sub-divided into small volumes, and stored in amber Eppendorf tubes at -20 °C. All working solutions were prepared by serial dilution of stock solutions. The combination standards contained the concentrations described in section 3.10 below.

3.5 Preparation of sample solution and standard solutions prior to on-line SPE
A frozen serum sample (500 µL) was thawed and spiked with 25 µL of the combined internal standard solution (to give the same concentrations as those in calibration standards in the final solution injected onto LC- MS/MS – as listed in section 3.10), 975 µL of acetonitrile was added, and then vortex mixed for 1 min. The mixture was incubated for 15 min at room temperature to allow protein precipitation to be completed, then centrifuged at 4000 x g for 10 min. The supernatant (1100 µL) was transferred to a clean Eppendorf tube and mobile phase (75%B – section 3.9) added to a final volume of 1600 µL, and vortex mixed. The sample was injected using full loop mode with a 100 µL loop and overfill factor of 15.

With standards, 500 µL of each of the six combination standards containing internal standards was mixed with mobile phase (75% B) to a final volume of 1600 µL and vortex mixed for 1 min before injection.

3.6 Comparison of two solvents to release vitamin D from serum by protein precipitation
The protein precipitation procedure used in this section was similar to our previous work (217). Briefly, an aliquot of serum (250 µL) was mixed with 500 µL of acetonitrile or 2% (w/v) trichloroacetic acid. The mixtures were vortex mixed for 30 s and then incubated for 15 min at room temperature. Following protein precipitation, the mixtures were centrifuged at 4000 x g for 10 min. The supernatants were transferred to clean Eppendorf tubes and evaporated to dryness using a freeze drier (12 h). The residues were reconstituted with 50 µL of mobile phase (75% B) prior to injection of 20 µL in LC-MS analysis. In this experiment the online sample clean-up was not used, and the extracts from two protein precipitation methods were directly injected to the analytical column. Triplicate measurements were performed and peak areas were used to assess the efficacy of each set of conditions.
3.7 Optimisation of mobile phase pH

Mobile phases were prepared by varying the buffer in both the aqueous (solvent A) and organic (solvent B) component as described in Table 3.1. The pH values of the mobile phase solutions were measured using a Mettler Toledo Seven Compact™ S210 pH meter (Mettler Toledo, Switzerland); the pH meter was calibrated with buffer solutions of pH 4.00, 7.00 and 10.00. All experimental settings were as per the optimised SPE method shown in section 3.8.

Table 3.1: Mobile phases used in scouting experiments

<table>
<thead>
<tr>
<th>Mobile phase/pH</th>
<th>2.56</th>
<th>6.42</th>
<th>6.42</th>
<th>7.50</th>
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<tbody>
<tr>
<td><strong>Aqueous</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(A)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water with</td>
<td>Water with 5 mM</td>
<td>Water with 5 mM 4-</td>
<td>Water with 10 mM</td>
<td></td>
</tr>
<tr>
<td>0.01M formic acid</td>
<td>ammonium formate</td>
<td>methylmorpholine adjusted with formic acid</td>
<td>ammonium formate</td>
<td></td>
</tr>
<tr>
<td><strong>Organic</strong></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td>component</td>
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</tr>
<tr>
<td>(B)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methanol with 0.01M formic acid</td>
<td>Methanol with 5 mM ammonium formate</td>
<td>Methanol with 5 mM 4-methylmorpholine adjusted with formic acid</td>
<td>Methanol with 10 mM ammonium formate</td>
<td></td>
</tr>
</tbody>
</table>

3.8 Optimisation of on-line solid phase extraction (SPE) and separation

The optimisation of operating procedure was carried out using a combination standard prepared as in section 3.10. Guard column (SPE) and analytical column conditions were optimised separately, based on the highest peak areas and best peak resolution respectively. Parameters optimised were: stationary phase (PFP and C18), flow rate (0.2-1.2 mL/min), buffer (ammonium formate, 4-methylmorpholine) and mobile phase composition (25:75-60:40 (water: methanol)) summarised in Table 3.2. The on-line clean-up and separation procedure used in this study is shown in Figure 3.2.
Table 3.2: Optimisation of solid phase extraction (SPE)

**SPE Column**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tested variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>PFP, C18</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.2-1.2 mL/min</td>
</tr>
<tr>
<td>Buffer</td>
<td>Ammonium formate, 4-methylmorpholine</td>
</tr>
<tr>
<td>Mobile phase composition (A)</td>
<td>25-40 %</td>
</tr>
<tr>
<td>Mobile phase composition (B)</td>
<td>60-75 %</td>
</tr>
</tbody>
</table>

**Analytical column**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Tested variable</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flow rate</td>
<td>0.2-1.2 mL/min</td>
</tr>
<tr>
<td>Buffer</td>
<td>Ammonium formate, 4-methylmorpholine</td>
</tr>
<tr>
<td>pH of mobile phase</td>
<td>2.56-7.50</td>
</tr>
<tr>
<td>Mobile phase composition (A)</td>
<td>25-40 %</td>
</tr>
<tr>
<td>Mobile phase composition (B)</td>
<td>60-75 %</td>
</tr>
<tr>
<td>Temperature</td>
<td>30-40˚C</td>
</tr>
</tbody>
</table>
Figure 3.2: Scheme of the two valve positions (219); Flow shown in green
3.9 LC-MS/MS procedure

An Agilent binary LC system consisting of an Agilent 1290 infinity LC pump and an Agilent 1290 well plate auto-sampler was used. A pentafluorophenyl (PFP) guard column/ SPE column (2.1 x 5 mm, 2.7 μm; Agilent Technologies, Santa Clara, CA, USA) and PFP chromatographic column (150 x 2.1 mm, 2.7 μm (Agilent Technologies, Santa Clara, CA, USA) (column temperature 40 °C), were installed on the switching valve system as described in Figure 3.2.

For sample clean up and for chromatographic separation (using pumps A and B) the same mobile phases were used: mobile phase A (aqueous 3 mM ammonium formate) and mobile phase B (methanol containing 3 mM ammonium formate). While operating in position A, the solid phase extraction (SPE) column (PFP guard column) was equilibrated with a mixture of the two mobile phases in a ratio A: B (60:40), at a flow rate of 0.8 mL/min (using pump A) for 10 min. Then the sample (1500 μL), obtained following protein precipitation, was injected onto SPE and washed for 2.5 min with the same conditions.

During the period of SPE clean up, in position A, the analytical column was equilibrated using pump B with the two mobile phases in a ratio A:B (75:25) at 0.2 mL/min. After 2.5 min wash, the guard column was connected to the analytical column using the switching valve system to start the chromatography process (position B). The analytical mobile phase was maintained for 11.5 min at 75% B and the composition changed from 75% B to 100% B over the next 1 min, thereafter, it remained at 100% B for the next 9 min. The mobile phase was then returned to the starting composition of 75% B over the next 1 min, and the column was subsequently re-equilibrated with 75% B for 10 min before injecting the next sample. The total run time including the equilibration of the column was 35 min. During the chromatographic run, the SPE guard column was washed using 100% mobile phase B using pump A.

An Agilent 6460 triple quadrupole tandem mass spectrometer equipped with a Jet Stream source and supported by Mass Hunter Workstation software (Agilent Technologies, Santa Clara, CA, USA) was used as the detector. For all compounds, the MS parameters were optimised to obtain the highest signal in MRM mode. The MS was used in dual polarity mode to detect all compounds in a single run: for all lipophilic vitamin D compounds (D2, D3, 25(OH)D2, 25(OH)D3, 24,25(OH)2D2, 24,25(OH)2D3, 1,25(OH)2D2 and 1,25(OH)2D3), ESI
positive ion mode was used and ESI negative mode was used for the four sulfate compounds (D$_2$-S, D$_3$-S, 25(OH)D$_3$-S and 25(OH)D$_2$-S). Deprotonated species ([M–H]$^-$) were selected as precursor ions for all vitamin D-sulfate compounds, whilst for lipophilic vitamin D compounds the protonated precursor ions ([M+H]$^+$) or (M+H-H$_2$O)$^+$ were used. Table 3.3 shows the optimised MRM transition parameters, the fragmentor and collision energy (CE) voltages and electrospray ionisation (ESI) mode used. Source parameters including gas flow, sheath gas flow, gas temperature and sheath gas temperature were optimised and maintained at 5 and 12 L/min (gas and sheath gas flows, respectively) and at 300 °C and 250 °C (gas and sheath gas temperatures, respectively). The capillary and nozzle voltages were maintained, respectively, at 5000 V and 2000 V.
### Table 3.3: Optimised mass spectrometry parameters used in MS detection

<table>
<thead>
<tr>
<th>Vitamin D compounds</th>
<th>MRM transition Precursor ion - Product ion (m/z)</th>
<th>Qualifier ion (m/z)</th>
<th>Fragmentor (V)</th>
<th>Collision energy (V)</th>
<th>ESI mode</th>
</tr>
</thead>
<tbody>
<tr>
<td>D₃</td>
<td>385-159</td>
<td>162</td>
<td>104</td>
<td>24</td>
<td>Positive</td>
</tr>
<tr>
<td>D₃-d₃</td>
<td>388-162</td>
<td>-</td>
<td>104</td>
<td>24</td>
<td>Positive</td>
</tr>
<tr>
<td>D₂</td>
<td>397-159</td>
<td>-</td>
<td>100</td>
<td>43</td>
<td>Positive</td>
</tr>
<tr>
<td>D₂-d₃</td>
<td>400-162</td>
<td>-</td>
<td>100</td>
<td>43</td>
<td>Positive</td>
</tr>
<tr>
<td>Epi-25(OH)D₃</td>
<td>401-159</td>
<td>257</td>
<td>100</td>
<td>28</td>
<td>Positive</td>
</tr>
<tr>
<td>Epi-25(OH)D₃-d₆</td>
<td>407-65</td>
<td>-</td>
<td>100</td>
<td>28</td>
<td>Positive</td>
</tr>
<tr>
<td>25(OH)D₃</td>
<td>401-159</td>
<td>257</td>
<td>100</td>
<td>28</td>
<td>Positive</td>
</tr>
<tr>
<td>25(OH)D₃-d₆</td>
<td>407-165</td>
<td>-</td>
<td>100</td>
<td>28</td>
<td>Positive</td>
</tr>
<tr>
<td>25(OH)D₂</td>
<td>413-159</td>
<td>-</td>
<td>90</td>
<td>24</td>
<td>Positive</td>
</tr>
<tr>
<td>25(OH)D₂-d₆</td>
<td>419-165</td>
<td>-</td>
<td>90</td>
<td>24</td>
<td>Positive</td>
</tr>
<tr>
<td>25(OH)D₃-S</td>
<td>479-96</td>
<td>-</td>
<td>222</td>
<td>36</td>
<td>Negative</td>
</tr>
<tr>
<td>25(OH)D₃-S-d₃</td>
<td>482-96</td>
<td>-</td>
<td>222</td>
<td>36</td>
<td>Negative</td>
</tr>
<tr>
<td>25(OH)D₂-S</td>
<td>491-96</td>
<td>-</td>
<td>222</td>
<td>40</td>
<td>Negative</td>
</tr>
<tr>
<td>25(OH)D₂-S-d₃</td>
<td>494-96</td>
<td>-</td>
<td>222</td>
<td>40</td>
<td>Negative</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>417-399</td>
<td>381</td>
<td>124</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>1,25(OH)₂D₃-d₆</td>
<td>423-405</td>
<td>-</td>
<td>124</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>D₃-S</td>
<td>463-96</td>
<td>-</td>
<td>232</td>
<td>36</td>
<td>Negative</td>
</tr>
<tr>
<td>D₃-S-d₃</td>
<td>466-96</td>
<td>-</td>
<td>232</td>
<td>36</td>
<td>Negative</td>
</tr>
<tr>
<td>1,25(OH)₂D₂ –H₂O</td>
<td>411-151</td>
<td>133</td>
<td>144</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>1,25(OH)₂D₂-d₆</td>
<td>417-157</td>
<td>-</td>
<td>144</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>D₂-S</td>
<td>475-96</td>
<td>-</td>
<td>242</td>
<td>36</td>
<td>Negative</td>
</tr>
<tr>
<td>D₂-S-d₃</td>
<td>478-96</td>
<td>-</td>
<td>242</td>
<td>36</td>
<td>Negative</td>
</tr>
<tr>
<td>24,25(OH)₂D₃</td>
<td>417-381</td>
<td>159</td>
<td>90</td>
<td>12</td>
<td>Positive</td>
</tr>
<tr>
<td>24,25(OH)₂D₃-d₆</td>
<td>420-384</td>
<td>-</td>
<td>90</td>
<td>12</td>
<td>Positive</td>
</tr>
<tr>
<td>24,25(OH)₂D₂</td>
<td>429-393</td>
<td>111</td>
<td>60</td>
<td>4</td>
<td>Positive</td>
</tr>
<tr>
<td>24,25(OH)₂D₂-d₆</td>
<td>435-399</td>
<td>-</td>
<td>60</td>
<td>4</td>
<td>Positive</td>
</tr>
</tbody>
</table>
3.10 Method Validation

The calibration range for each compound was determined based on previously reported concentrations in human serum. The calibration ranges (six levels) were as follows: 0.5-100 nM [25(OH)D₃, 25(OH)D₂, D₃, D₂, 25(OH)D₃-S, 25(OH)D₂-S, D₂-S, D₃-S]; 0.05-2 nM [1,25(OH)₂D₃, and 1,25(OH)₂D₂]; 0.10-15 nM [24,25(OH)₂D₃ and 24,25(OH)₂D₂]. The internal standard concentrations in all calibration standards were: 50 nM [25(OH)D₃, 25(OH)D₂, D₃, D₂, 25(OH)D₃-S, 25(OH)D₂-S, D₂-S, D₃-S]; 0.5 nM [1,25(OH)₂D₃, and 1,25(OH)₂D₂]; 5 nM [24,25(OH)₂D₃ and 24,25(OH)₂D₂]. All calibration standards were prepared in mobile phase of the composition at the beginning of the gradient elution (75%B-section 3.9). Linearity was assessed by plotting the peak area ratios of standard/internal standard against the concentration of the standards using linear regression analysis.

The limit of detection (LOD) was calculated as the concentration corresponding to three times the noise, and the limit of quantification (LOQ) as the concentration corresponding to 10 times the noise, for each compound. Repeatability was determined by analysing 10 separate aliquots of a pooled serum sample followed by the calculation of the percentage relative standard deviation for each vitamin D compound. Recovery was assessed by analysing the concentration of each vitamin D compound in six separate aliquots of spiked and non-spiked serum samples. Each aliquot of serum was spiked to give a final concentration similar to the level four of the calibration range used in this study.

3.11 Results and discussion

3.11.1 Optimisation of protein precipitation method

The most efficient protein precipitants for protein removal have previously been reported to be acetonitrile and trichloroacetic acid (TCA) (157). These precipitants consistently removed plasma proteins effectively in samples from all species examined and at precipitant to plasma volume ratios of 2:1 (157). Figure 3.3 shows the peak areas obtained for each of the 12 vitamin D compounds using the two protein precipitation solvents. Triplicate measurements were performed and peak areas were used to assess the efficacy of each set of conditions. In this comparison study, we were not able to detect three compounds, 25(OH)D₂, 1,25(OH)₂D₂ and 24,25(OH)₂D₂, using either extraction method due to their very low levels in the samples. For all other vitamin D compounds, the peak areas observed with TCA precipitation were only 1-6% of the areas observed with acetonitrile precipitation Protein precipitation with
acetonitrile was, therefore, seen to be more efficient in releasing vitamin D metabolites from the matrix and/or the acetonitrile extract is cleaner and therefore fewer compounds co-elute with the vitamin D peaks resulting in reduced ion suppression in MS detection (220). In general, it has been shown that improving the sample preparation and the chromatographic separation are the two most effective ways of circumventing ion suppression (196) in mass spectrometry.
Figure 3.3: Peak areas for 12 vitamin D compounds obtained after using the two protein precipitation solvents, acetonitrile or trichloroacetic acid (TCA)
3.11.2 Effect of mobile phase pH: implications in liquid chromatography/tandem mass spectrometric bioanalysis for vitamin D–sulfate compounds.

Mobile phase composition and pH in LC-MS/MS analysis are known to impact analyte ionisation, thus affecting the amounts and types of ions formed (221). Vitamin D-sulfates are acidic in nature due to the presence of the readily ionisable sulfate moiety, and are capable, by deprotonation, of producing negatively charged ions. A systematic investigation of the effects of mobile phase pH and organic modifier on ESI-MS response and chromatographic performance was assessed.

The responses at pH (2.56–7.50) for vitamin D-sulfates in LC–ESI negative mode were compared, as noted in section 3.7. The use of a high-pH stable, highly efficient reversed-phase HPLC column enabled the retention of the polar conjugate bases (sulfates). The principal mas ions detected were those of the protonated forms for the sulfate compounds. However, if ammonium adducts were to be formed between sulfates and ammonium ions in the mobile phase, the population of protonated form would thereby be reduced. When both 4-methylmorpholine and ammonium formate were used in this experiment, a higher peak area for the protonated form with 4-methylmorpholine was observed if the compounds formed ammonium adducts. According to the data shown in Figure 3.4, the peak areas in panels C and D are similar, suggesting that no adduct formation is occurring.

These results also show that pH affects the retention time and the peak response. Retention times increased at lower pH values. Based on the poor peak shape obtained and the long retention time at low pH (2.56), the low pH mobile phase with formic acid as the buffer was eliminated. Both the retention time and the peak response at pH 6.42 were favourable and therefore the mobile phase at pH 6.42, with ammonium formate buffer, was chosen for further studies.
Figure 3.4: Chromatograms illustrating the separation of D$_2$-S, D$_3$-S, 25(OH)D$_3$-S and 25(OH)D$_2$-S, using varying pH and buffer conditions: (A) pH 7.50; water/methanol with 10mM ammonium formate (B) pH 2.56; water/methanol with 0.01M formic acid (C) pH 6.42; water/methanol with 5mM ammonium formate (D) pH 6.42; water/methanol with 5mM 4-methylmorpholine adjusted with formic acid
3.11.3 LC-MS/MS method development for vitamin D compounds

It is evident from the literature (157) that serum samples are not necessarily subjected to protein precipitation prior to using SPE. We have, however, reported here a simple manual protein precipitation step, to enhance the SPE recovery. The original methodology used in previous analytical method development involved a protein precipitation step similar to the method used in the chapter, the removal of acetonitrile using nitrogen, a subsequent 12 h freeze drying step followed by the derivatisation of the residual extract before injection onto a column (217). This lengthy, very labour intensive, sample preparation has now been replaced by a simplified off-line preparation involving manual protein precipitation which is then combined with an on-line SPE pre-treatment, equivalent to a trapping column technique, by using a re-usable guard column as the SPE column. The use of a relatively inexpensive guard column as a re-usable SPE cartridge has resulted in diminished operational and consumable costs and has significantly enhanced the lifespan of the analytical column. On-line two-dimensional chromatography, using a column-switching device, has been previously shown to reduce matrix effects (202).

The optimisation of the on-line clean up and separation was undertaken in two steps: the first stage focused on the extraction of the target compounds using the SPE guard column by injecting the combination standard of vitamin D compounds into the guard column and eluting directly to MS detector; the second stage focused on achieving best resolution of compounds by injecting the combination standard onto the complete assembly using a 1.5 mL sample loop. The MS parameters were optimised by directly infusing the vitamin D standard solutions into the MS detector. The predominant product ion was used as the quantifier ion and another ion as the qualifier ion.

There have been previous reports using a PFP column for underivatised vitamin D compounds (222-224). As derivatisation was omitted in this chapter for the reasons outlined above, a PFP analytical column was therefore used. It was also noted that the sensitivities for D-sulfate compounds were considerably enhanced at higher mobile phase pH values. Therefore, mobile phases were prepared using ammonium formate or 4-methylmorpholine. The reason for using 4-methylmorpholine was to prevent any possible adduct formation of some vitamin D compounds with ammonium ions. However, both mobile phases produced similar peak areas for all compounds, confirming that ammonium ions do not appear to form
adducts with vitamin D compounds at these conditions. Therefore, ammonium formate was used as the buffer in the optimised method. The chromatographic separation of 25(OH)D$_3$ from its 3-epi-25(OH)D$_3$ isomer was necessary to enable quantification of these compounds since they have the same mass transitions. Optimisation of the chromatographic separation using different chromatographic gradients and column temperatures enabled the satisfactory resolution of these epimers. This agrees with previous studies that have achieved isomeric separation using PFP columns (26, 224-227). The PFP column uses a variety of separation mechanism; the interaction of the hydroxyl group of 3-epi-25(OH)D$_3$ with the sorbent, allowed epimeric separation. The relative rigidity of the fluorinated bonded phase provides enhanced shape selectivity (288).

Considering the ion-suppression effects of the buffer ions in mobile phase, it is desirable to use the lowest possible concentration of buffer in mobile phase. Therefore, the possibility of using a lower buffer concentration was examined by studying its effect on the chromatography of all the vitamin D compounds, using buffer concentrations 3mM and 5mM at pH 6. As there was no significant change in chromatography between the two concentrations, and there was an improvement in peak response, 3mM ammonium formate was selected as the optimum.

The optimised flow rate, mobile phase composition and column temperature obtained are shown in Table 3.4. Chromatograms obtained for a standard mixture, using the optimum LC and MS conditions are shown in Figure 3.5. The aim of chromatographic optimisation was the separation of the epimeric form from 25(OH)D$_3$ and to ensure that the chromatogram for the first peak of interest was satisfactorily remote from early eluting interfering compounds while achieving the separation of all compounds of interest within the shortest possible time.

**Table 3.4: Optimum condition of the automated SPE step**

<table>
<thead>
<tr>
<th>Variable</th>
<th>SPE Column</th>
<th>Analytical column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type</td>
<td>Pentafluorophenyl (PFP)</td>
<td>Pentafluorophenyl (PFP)</td>
</tr>
<tr>
<td>Flow rate</td>
<td>0.8 mL/min</td>
<td>0.2 mL/min</td>
</tr>
<tr>
<td>Buffer</td>
<td>Ammonium formate</td>
<td>Ammonium formate</td>
</tr>
<tr>
<td>MP composition</td>
<td>40:60 (water: methanol)</td>
<td>25: 75 (water: methanol)</td>
</tr>
<tr>
<td>Temperature</td>
<td>40˚C</td>
<td>40˚C</td>
</tr>
<tr>
<td>pH</td>
<td>6.17 (3 mM ammonium formate)</td>
<td>6.17 (3 mM ammonium formate)</td>
</tr>
</tbody>
</table>
Figure 3.5: LC-MS/MS profiles of 12 vitamin D compounds (lipophilic and hydrophilic) in the standard mixture.
3.11.4 Method validation and application to samples

As shown in Table 3.5, the detection and quantification limits were found to be slightly higher, when compared to the previous method (217). These results may have arisen from the decision to remove the lengthy derivatisation procedure; the derivatisation enhanced the ionisation to a certain extent and additionally increased the signal/noise ratio since the analytes were detected at higher molecular masses where the noise from low molecular weight impurities in the mobile phase are reduced. As one of the main objectives of this chapter was to simplify the method by partially automating the sample preparation thereby making it suitable for routine analysis, the derivatisation step was removed. The lengthy multi-step derivatisation procedure used in vitamin D analysis (217) is not amenable to automation and therefore has not been fully or partially automated to date. The sample is required to be completely dry before derivatisation (188), and this is challenging to achieve in an on-line procedure.

It has been clearly established that, when co-eluting, isotopically labelled analogues are used as internal standards, there is no significant difference in the results when the calibration standards are prepared either in mobile phase solvent or in matrix matched medium (211, 212). Therefore, all samples and the calibration standards were prepared in mobile phase solvent at the same composition as the initial gradient conditions as described in LC-MS/MS section. The assay calibration demonstrated good linearities ($R^2 > 0.99$) for all 12 vitamin D compounds over the concentration ranges used. The concentrations of three of the vitamin D$_2$ compounds, 1,25(OH)$_2$D$_2$, 25(OH)D$_2$-S and D$_2$-S could not be reported, due to levels lower than the limit of quantification in the pooled serum sample used. The method was found to be precise with percentage standard deviations within the range 2.82-4.86%. The accuracy of the method, determined as the recovery values, ranged from 92% to 99%. The SD values reported in the recovery column of Table 3.5 gives an approximation of the precision for the three vitamin D$_2$ compounds absent in the repeatability column.
Table 3.5: Linearity, repeatability, recovery, limit of detection and limit of quantification of all vitamin D compounds including epi-25(OH)D$_3$

<table>
<thead>
<tr>
<th>Vitamin D compounds</th>
<th>Linearity equation</th>
<th>R$^2$</th>
<th>Mean ± SD (nM)</th>
<th>RSD (%)</th>
<th>Mean ± SD (%)</th>
<th>nM</th>
<th>**LOD (LOQ)</th>
<th>***LOD (LOQ)</th>
<th>pmols</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D$_3$</td>
<td>y = 13.551x + 0.0314</td>
<td>0.9974</td>
<td>51.50 ± 1.48</td>
<td>2.87</td>
<td>97.6 ± 1.61</td>
<td>0.0367 (0.122)</td>
<td>0.117 (0.392)</td>
<td>0.0551 (0.184)</td>
<td></td>
</tr>
<tr>
<td>epi-25(OH)D$_3$</td>
<td>y = 4.8937x - 0.1135</td>
<td>0.9957</td>
<td>2.1200 ± 0.0686</td>
<td>3.23</td>
<td>97.9 ± 0.827</td>
<td>0.0367 (0.122)</td>
<td>0.117 (0.392)</td>
<td>0.0551 (0.184)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D$_2$</td>
<td>y = 0.6559x + 0.2549</td>
<td>0.999</td>
<td>10.800 ± 0.341</td>
<td>3.17</td>
<td>97.8 ± 4.18</td>
<td>0.0121 (0.040)</td>
<td>0.0386 (0.129)</td>
<td>0.0181 (0.0603)</td>
<td></td>
</tr>
<tr>
<td>D$_3$</td>
<td>y = 0.0668x + 0.3179</td>
<td>0.9939</td>
<td>14.500 ± 0.627</td>
<td>4.33</td>
<td>92.5 ± 0.291</td>
<td>0.0188 (0.0628)</td>
<td>0.0603 (0.200)</td>
<td>0.0283 (0.0942)</td>
<td></td>
</tr>
<tr>
<td>D$_2$</td>
<td>y = 1.7506x - 2.1213</td>
<td>0.9968</td>
<td>2.230 ± 0.109</td>
<td>4.86</td>
<td>92.2 ± 1.26</td>
<td>0.0475 (0.158)</td>
<td>0.152 (0.507)</td>
<td>0.0713 (0.237)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>y = 0.151x + 0.018</td>
<td>0.9902</td>
<td>0.6240 ± 0.0233</td>
<td>3.57</td>
<td>93.3 ± 0.0230</td>
<td>0.0239 (0.0796)</td>
<td>0.0764 (0.255)</td>
<td>0.0358 (0.119)</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_2$</td>
<td>y = 2.1163x + 0.932</td>
<td>0.9905</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>94.9 ± 0.0490</td>
<td>0.0680 (0.227)</td>
<td>0.217 (0.725)</td>
<td>0.102 (0.340)</td>
</tr>
<tr>
<td>24,25(OH)$_2$D$_3$</td>
<td>y = 1.9274x - 0.966</td>
<td>0.9976</td>
<td>1.1200 ± 0.0357</td>
<td>3.18</td>
<td>93.2 ± 2.88</td>
<td>0.0179 (0.0598)</td>
<td>0.0574 (0.191)</td>
<td>0.0259 (0.191)</td>
<td></td>
</tr>
<tr>
<td>24,25(OH)$_2$D$_2$</td>
<td>y = 5.6412x + 0.9608</td>
<td>0.9978</td>
<td>3.370 ± 0.120</td>
<td>3.6</td>
<td>96.1 ± 0.0430</td>
<td>0.0249 (0.0831)</td>
<td>0.0798 (0.267)</td>
<td>0.0374 (0.125)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D$_3$-S</td>
<td>y = 0.0076x + 0.1845</td>
<td>0.9904</td>
<td>12.000 ± 0.529</td>
<td>4.41</td>
<td>97.8 ± 0.667</td>
<td>0.0585 (0.1950)</td>
<td>0.187 (0.624)</td>
<td>0.0877 (0.292)</td>
<td></td>
</tr>
<tr>
<td>25(OH)D$_2$-S</td>
<td>y = 0.1641x + 0.9365</td>
<td>0.9934</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>95.7 ± 1.35</td>
<td>0.182 (0.606)</td>
<td>0.582 (1.939)</td>
<td>0.273 (0.909)</td>
</tr>
<tr>
<td>D$_3$-S</td>
<td>y = 1.524x - 0.0496</td>
<td>0.9997</td>
<td>1.2000 ± 0.0338</td>
<td>2.82</td>
<td>99.7 ± 1.32</td>
<td>0.0255 (0.0849)</td>
<td>0.0814 (0.272)</td>
<td>0.0382 (0.127)</td>
<td></td>
</tr>
<tr>
<td>D$_2$-S</td>
<td>y = 63.194x + 3.4594</td>
<td>0.9999</td>
<td>N.D</td>
<td>N.D</td>
<td>N.D</td>
<td>97.3 ± 0.0690</td>
<td>0.0329 (0.109)</td>
<td>0.105 (0.351)</td>
<td>0.0493 (0.164)</td>
</tr>
</tbody>
</table>

N.D, not detected; SD, standard deviation

* Concentration in extract

** Concentration in serum

*** On column amounts
Table 3.6 shows the ranges of vitamin D compounds in donated human serum samples (n = 10) collected from volunteers, using the optimised, validated method reported above. The lowest concentrations of vitamin D compounds were found in volunteers who: had not taken vitamin D supplements; did not use fortified vitamin D milk in their daily intake; and who were rarely exposed to sunlight in outdoor activities in the past two weeks. The above information was based on surveys provided by the volunteers prior to sample collection. A number of reports suggest that a 25(OH)D$_3$ concentration below 20 ng/mL (50 nmol/L) (31, 47, 58, 228) is an indication of vitamin D deficiency, whereas concentrations of 21–29 ng/mL (51–74 nmol/L) are considered to indicate insufficiency and concentrations of 30 ng/mL (75 nmol/L) suggested by some to maximise the effect of vitamin D on calcium, bone, and muscle metabolism (228-230). Severe deficiency leads to rickets in children and osteomalacia in adults with concentrations of 25(OH)D$_3$ below 10 ng/mL (25 nmol/L). The chromatographic profiles obtained for human serum are shown in Figure 3.6. The broadness of some peaks may likely to be due to incomplete separation of epimeric forms. The only epimeric form (separated) identified in this study, using a standard compound, is epi-25(OH)D$_3$.

Table 3.6: Concentration ranges of vitamin D compounds in serum from 10 volunteers

<table>
<thead>
<tr>
<th>Vitamin D compounds</th>
<th>Concentration ranges (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D$_3$</td>
<td>18.7-83.2</td>
</tr>
<tr>
<td>epi-25(OH)D$_3$</td>
<td>6.43-47.0</td>
</tr>
<tr>
<td>25(OH)D$_2$</td>
<td>0.0654-14.0</td>
</tr>
<tr>
<td>D$_3$</td>
<td>2.63-18.3</td>
</tr>
<tr>
<td>D$_2$</td>
<td>0.00-3.65</td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_3$</td>
<td>0.263-1.08</td>
</tr>
<tr>
<td>1,25(OH)$_2$D$_2$</td>
<td>0.00-2.62</td>
</tr>
<tr>
<td>24,25(OH)$_2$D$_3$</td>
<td>0.264-3.04</td>
</tr>
<tr>
<td>24,25(OH)$_2$D$_2$</td>
<td>0.00-0.159</td>
</tr>
<tr>
<td>25(OH)D$_3$-S</td>
<td>9.52-43.8</td>
</tr>
<tr>
<td>25(OH)D$_2$-S</td>
<td>0.00-40.0</td>
</tr>
<tr>
<td>D$_3$-S</td>
<td>0.0976-2.75</td>
</tr>
<tr>
<td>D$_2$-S</td>
<td>0.00-0.363</td>
</tr>
</tbody>
</table>
Figure 3.6: Chromatographic profiles showing 12 detectable vitamin D compounds (lipophilic and hydrophilic) in human serum samples
3.12 Conclusion

This study is the first to report the development and validation of an analytical method for the simultaneous detection and quantification of 12 vitamin D compounds (both lipophilic and hydrophilic) in human serum. The high accuracy, selectivity and sensitivity obtained demonstrate the suitability of the proposed method for the determination of vitamin D compounds and their metabolites in man, that are potentially useful in disease diagnosis. The method described above has minimal sample handling; making it amenable for routine analysis. The method can be very likely applied to any biological fluid following modification of the sample preparation step.
CHAPTER 4: STANDARD ADDITION WITH INTERNAL STANDARDISATION TO ELIMINATE MATRIX EFFECTS IN LC- MS/MS - APPLICATION TO VITAMIN D ASSAY

4.1 Foreword
This chapter proposes an alternative approach for method calibration using standard addition with an internal standard. Traditionally, the standard addition method has been used to overcome matrix effects in atomic spectroscopy and has been a well-established method. This chapter proposes a similar application for mass spectroscopic detection following liquid chromatography and demonstrates that the results are comparable to those of the internal standard method using labelled analogues, for a vitamin D assay method. As the conventional standard addition procedure does not address procedural errors, the inclusion of an additional internal standard (not co-eluting) was proposed. Experiments were carried out in human serum and plasma samples. The internal standardisation approach using stable isotope labelled analogues (SIL-IS) results were compared with the proposed method of standard addition. Much of the work reported in this chapter has been submitted to the Analytical Chemistry journal. However, to be compatible with the style and the flow of the thesis, some text and content modifications have been introduced.

4.2 Introduction
According to the Union of Pure and Applied Chemistry (IUPAC) (231), a matrix effect is defined as the combined effect of all components of the sample other than the analyte on the measurement of the quantity while interference refers to the effect of a specific component that is identified as causing an effect on the measurement. When measurements are carried out in complex matrices such as blood, in which the composition varies from person to person, the effects on analytical measurement largely arise from matrix effects rather than interferences. Unlike with interferences, it is challenging to overcome matrix effects because of this variation in matrix composition. Ion suppression/enhancement is the most common form of matrix effect encountered in liquid chromatography–mass spectrometry (LC- MS), regardless of the sensitivity or selectivity of the mass analyser used (232, 233). A study by King et al.(234) indicates that the electrospray ionisation interface (ESI) is more likely to be
impacted in the manner when compared to atmospheric pressure chemical ionisation (APCI) (235).

In general, matrix effects are due to inadequate sample clean-up, especially with complex sample matrices. However, with LC-MS, ion suppression is encountered even when there is no matrix by the trace impurities in mobile phase (236). Furthermore, sample clean-up procedures are designed to remove compounds that are dissimilar to the analyte therefore they cannot remove the compounds that are similar to the analyte that co-elute and cause ion suppression/enhancement in MS. Hence, the only option available is to correct the data for these matrix effects rather than trying to eliminate the matrix effects, by using appropriate calibration methods (154). The use of a stable isotope labelled analogue of analyte as internal standard (SIL-IS) is the generally accepted way of data correction for matrix effects, as it is most likely to completely co-elute with the analyte and to encounter the same amount of ion suppression/enhancement because it has almost identical chemical and physical properties to the target analyte (200). Furthermore, if added at the beginning of sample preparation, it will behave identically during all procedures as well as chromatography. However, it is important to recognise that a certain amount of ion suppression of the analyte will occur due to the co-eluting isotope-labelled internal standard itself (233, 237). As the same amount of internal standard is added to all samples, ion suppression by the internal standard will affect samples containing smaller amounts of analyte to a greater extent. Furthermore, complete co-elution may not occur due to the slight change in lipophilicity of the molecule acquired during the replacement of the carbon bound hydrogen with deuterium (238), therefore the ion suppression effects may not be appropriately corrected. In some studies (239), it was found that there was significant difference in extraction behaviours (recovery) between the analyte and its deuterated analogue, that can also affect the accuracy of the data.

In spite of the above-mentioned drawbacks, at present, the use of internal standard calibration using SIL-IS is the best option available to ensure the removal of matrix effects from LC-MS data. In practice, SIL-IS are generally very expensive and they are often not commercially available. Developing an LC-MS/MS assay for 12 vitamin D compounds (217) was an expensive exercise in consumable as some SIL-IS compounds were purchased and some were synthesised at a high cost. This and the knowledge of drawbacks described in the previous paragraph, prompted the exploration of alternative methods of overcoming matrix effects in LC-MS.
The standard addition method, taught through analytical chemistry textbooks as a calibration method to be used when matrix effects cannot be eliminated by physical means, has been widely used in spectrophotometric analysis, especially in atomic spectroscopy (240-243). Some atomic spectroscopic instruments such as graphite furnace AAS, are equipped with hardware and software to carry out the standard addition on-line. However, the method of standard addition has not been considered as a scheme for overcoming matrix effects in LC-MS except for a few recent reports on its feasibility (154, 244, 245).

The standard addition method of calibration employs exactly the same matrix in all calibration standards and therefore does not require matrix matched calibration standards. Matrix matching is an unachievable task in clinical analysis as the matrix will vary from sample to sample and thus differs from the matrix used for the preparation of calibration standards. In standard addition, each sample has its own calibration curve. Also, it is well suited for endogenous metabolite assays in biological fluids as there is no need for blanks and hence blank matrix in which to prepare calibration standards. The problems with SIL-IS methods as mentioned above, such as ion suppression by co-eluting internal standards or non-co-elution due to the effects of dueteration are not present in the standard addition method. Most attractive practically is the absence of requirement to purchase or synthesise the expensive labelled compounds. The drawback is having to use at least three times the sample volume for analysis. With some prior validation work, the volume of sample used can be reduced to two-fold rather than three, by using a single addition calibration as described in the following discussion section.

In this study, the possibility of operating the method of standard addition in routine LC-MS analysis to compensate for matrix effects was investigated by using a vitamin D assay as an example. The assay for vitamin D compounds used in this study is a good candidate as it simultaneously quantifies many compounds and is validated and well established method (246); vitamin D is also one of the most requested clinical assays in recent times (143). The classical standard addition method is not suitable for assays requiring multi-step sample preparation, as it does not compensate for procedural errors. Therefore, an internal standard was incorporated into the classical standard addition method and was then compared to the classical method. Coordination swapping (247) was also incorporated for ease and practicality. The accuracy of this improved, simplified standard addition method was
validated through the determination of recovery, and by comparison with existing method (using SIL-IS).

4.3 Materials and Methods

4.3.1 Chemical

All reagents, solvents, unlabelled standard and standard vitamin D compounds were purchased as described in Chapter 2.

4.3.2 Sample collection

Blood samples were obtained as described in Chapter 3.

4.4 Sample and standard preparation

For all experiments, samples with known vitamin D compositions (based on prior analysis (246)) were used. For the comparison of methods, plasma samples previously demonstrated to be devoid of 25(OH)D₂ were used; this enabled the use of 25(OH)D₃ as an internal standard. The comparison studies used established methods using SIL internal standards (246) and those using standard addition (as described below in 4.4.2 but using all vitamin D compounds except 25(OH)D₂ listed in 4.3.1 above in the combination standard added). The sample size used in all experiments was 250 µL for each extraction/run.

For validation studies, a pooled sample of serum devoid of the target vitamin D compounds (D₂ and 1,25(OH)₂D₂) and the internal standard (25(OH)D₂) was used. In addition, a blank run was performed (using serum with no added vitamin D compounds) in 4.4.1 and 4.4.2 to ensure the absence of these compounds. From about 3.5 mL of the pooled serum sample, 250 µL aliquots were pipetted for analysis as described in 4.4.1 and 4.4.2 below. To each aliquot, D₂ and 1,25(OH)₂D₂ were added to mimic an original sample concentration of 5 nM and 0.5 nM of each compound, respectively. These analytes were added at the first stage of extraction instead of adding to the pooled serum in order to avoid sample dilution.

4.4.1 External standard calibration method (Method I) and stable isotope-labelled internal standard calibration method (Method II)

A slightly modified version of the published method (246), using half the amount of sample, was used. To a 250 µL volume of serum sample, D₂ and 1,25(OH)₂D₂ were added to give
concentrations of 5 nM and 0.5 nM of each, respectively, and the sample was then spiked with stable isotope-labelled internal standards (D\textsubscript{2}-d\textsubscript{3} and 1,25(OH\textsubscript{2})D\textsubscript{2}-d\textsubscript{6}) to provide final concentrations the same as those in the calibration standards. The mixture was then vortex mixed for 1 min, and 500 µL of acetonitrile was added. The mixture was incubated for 15 min at room temperature to allow protein precipitation to be completed and was then centrifuged at 4000 x g for 5 min. Supernatant (700 µL) was transferred to a clean Eppendorf tube and then mobile phase was added to obtain a final volume of 1600 µL. An aliquot of this extract (1500 µL) was injected to on-line solid phase extraction (SPE) followed by analysis using LC-MS/MS. The standards were prepared and processed as in the published method (246) but with only two vitamin compounds (D\textsubscript{2} and 1,25(OH\textsubscript{2})D\textsubscript{2}) and their corresponding SIL internal standards. There was a total of seven runs (sample run in triplicate and four-point calibration). The concentrations in each of the triplicate samples were calculated using: external standard calibration without using the internal standard responses (method I); and internal standard calibration (method II) (246). The recoveries were calculated using these concentrations and the added amounts (0.5 and 5 nM).

4.4.2 Standard addition calibration method without (Method III) and with (Method IV) internal standard

To a 250 µL volume of serum sample, D\textsubscript{2} and 1,25(OH\textsubscript{2})D\textsubscript{2} were added to give concentration of 5 nM and 0.5 nM of each compound, respectively, and the sample was spiked with the internal standard 25(OH)D\textsubscript{2} to give a final concentration of 0.5 nM. A combination standard (D\textsubscript{2} and 1,25(OH\textsubscript{2})D\textsubscript{2}) was added to the sample to give two different levels of final concentrations (10 nM and 15 nM for D\textsubscript{2}, 1 nM and 1.5 nM for 1,25(OH\textsubscript{2})D\textsubscript{2}). Each of these levels were prepared in triplicate. The mixture was then vortex mixed for 1 min and then mixed with 500 µL of acetonitrile. The mixture was incubated for 15 min at room temperature to allow protein precipitation to proceed to completion and was then centrifuged at 4000 x g for 5 min. An aliquot of supernatant (700 µL) was transferred to a clean Eppendorf tube then mobile phase was added to obtain a final volume of 1600 µL. An aliquot (1500 µL) of this extract was injected to on-line solid phase extraction (SPE) followed by LC-MS/MS. There was a total of nine runs (three sample runs with no spiking, and six sample runs with spiking at two levels). With each of the triplicate samples, the concentration of the sample was calculated using the classical standard addition method by plotting the added concentration (0 nM, 5 nM and 10 nM) vs. peak area for each compound (method II),
with the concentration being the Y-axis intercept. The concentrations for the same samples were also calculated using the internal standard (25(OH)D$_2$) – in this case instead of plotting the peak area as the X-axis, the ratio of peak area of analyte/internal standard peak area was plotted. Concentration was determined using the same approach as method III as negative Y-axis intercept (method IV). The recoveries were calculated using these concentrations and the added amounts (0.5 and 5 nM).

4.5 Results and discussion

The chromatograms obtained for each of the compounds used in validation study are shown in Figure 4.1. A well-established method was used (vitamin D and metabolites) which has been previously validated and published (246), for this study. It was intended to validate the proposed standard addition method through recovery, hence, known amounts of two forms of vitamin D that were not present in the samples were added: D$_2$ and 1,25(OH)$_2$D$_2$. The D$_2$ forms are present only in those individuals who consume plant-based vitamin D (143). As the dietary patterns of the volunteers had been previously collected, the serum from individuals who had no D$_2$ forms in their blood could be pooled. In addition, blanks were run with each method performed to ensure the absence of these D$_2$ forms.
The function of the internal standard used in the proposed method of standard addition is solely to correct for procedural errors and therefore there is no requirement for it to be structurally similar to the analytes, or to co-elute with the analytes. For convenience, another vitamin D₂ form (25(OH)D₂) was used as the internal standard since its chromatographic behavior was established and its absence in the samples used for this study had been previously demonstrated. However, any compound that elutes within the retention times of the analytes can be used for this purpose, as long as it will not be present in any sample. To use the vitamin D method (246) in standard addition mode, the most convenient approach is to use an isotopically labelled analogue of one of the vitamin D forms. With LC-MS/MS this can be separated from all analytes, and it is guaranteed to be absent in samples because it is a labelled compound. Because the labelled internal standard can co-elute with at least one of
the analytes of interest, and cause ion suppression it is best to use the labelled analogue of a compound that is less likely to be present and/or not of significant interest for quantification. Furthermore, it is advisable to check whether any other analyte of interest co-elutes with this internal standard and cause ion suppression. This is applicable for any assay for which sourcing of an internal standard that is absent in all samples is difficult to achieve. The internal standard will also be affected by the matrix effects in MS detection. However, the extent of this effect will be the same for all additions including the zero addition (zero in Y-axis in Figure 4.3 where the added standard concentration is zero) because the co-eluting impurities in the matrix is the same for all three additions. Therefore, the variation in peak areas of the internal standard will be due only to procedural errors but not to matrix effects. When the area of the analyte is divided by that of the internal standard in each chromatogram, this ratio is devoid of the procedural errors. Therefore, in this proposed method, this ratio is used in calibration rather than using area alone as is in the classical standard addition method. As with any internal standard calibration method, it is advisable to check the linear range of the internal standard and confirm that the response is linear within the variation expected (248).

As described in 4.4.1 and 4.4.2, the same sample (containing known amounts of the two analytes of interest) was analysed (in triplicate each) using the external calibration (I), and internal standard calibration using SIL-IS (II). It was also analysed (in triplicate each) using the classical standard addition method (III), and the proposed standard addition method (IV) that incorporated an internal standard.

With both methods III and IV, instead of plotting peak area (or peak area ratio of analyte/internal standard in proposed method) vs. concentration of the added standard (Figure 4.2), the reverse was plotted (Figure 4.3). This co-ordinate swapping method of standard addition calibration (247) is simpler than the classical method, as the concentration value is simply read as the negative value of the Y-intercept (rather than the ratio of the intercept and the slope with the classical method).

Using the conventional linear equation below, where the independent variable standard added, X-axis (usually a concentration) and dependent variable, Y-axis (usually response or signal) (247).

\[ y = a_0 + a_1 x \] (Equation 1)
Ratio of the intercept ($a_0$) and slope ($a_1$) is the result of added amount for analyte; $x_0 = a_0/a_1$.

**Figure 4.2** shows the direct regression calibration plot of the standard addition calibration method for both $D_2$ and $1,25(OH)_2D_2$ compounds.

![Graph showing the direct regression calibration plot for $D_2$ and $1,25(OH)_2D_2$.](image)

**Figure 4.2**: Direct regression of classical standard addition with internal standard calibration method. Concentration observed obtained from the equation, $y = a_0 + a_1x$

where $x_0 = a_0/a_1$

However, in this chapter for the standard addition method, the concentration observed of each vitamin D compound in serum samples was determined directly using a calibration plot of concentration versus the peak area of each and analyte by using the Juris *et al.* coordinate swapping procedure (247) in inverse linear regression, $x = b_0 + b_1y$ (247) (**Figure 4.3**). The
negative Y-intercept from the equation was hence the concentration of the respective vitamin D compound in human serum, \( x_{0,\text{inv}} = -b_0 \). Table 4.1 shows this method as applied to method IV, the proposed standard addition method in this study.

![Graph 1](image1)

**Figure 4.3:** The added concentration vs peak area ratio (analyte/internal standard) for the proposed standard addition method.

The concentrations were determined as the negative intercepts: 4.77 nM for D\(_2\) and 0.479 nM for 1,25(OH)\(_2\)D\(_2\).

Table 4.1 shows a comparison of all four methods based on their recoveries. The recovery calculation was based on the known amounts added, and therefore a recovery of 100 indicates the highest accuracy. The extent of deviation from 100 in this study corresponds to the magnitude of matrix effects that were not removed from the data. The percentage recovery was determined using the following formula:
% recovery = \( \frac{\text{Concentration of sample observed}}{\text{Concentration of sample expected}} \times 100\% \)

As expected, the external calibration (I) has the lowest accuracy as there was no correction of matrix effects. SIL-IS method (II) has high accuracy but the drawbacks discussed above in the introduction may be responsible for the slight loss of accuracy. The classical method of standard addition (III) has lower precision as well as accuracy when compared to the standard addition with internal standard (IV), mainly due to procedural errors rather than matrix effects. The proposed method of standard addition gives the highest accuracy.

**Table 4.1:** Comparison based on recovery for four methods of calibrations (*n = 3*)

<table>
<thead>
<tr>
<th>No.</th>
<th>Methods</th>
<th>Compounds</th>
<th>Concentration (nM)</th>
<th>Recovery ± RSD (%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Expected</td>
<td>Observed</td>
</tr>
<tr>
<td>I</td>
<td>External standard calibration</td>
<td>D2</td>
<td>5</td>
<td>4.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,25(OH)2D2</td>
<td>0.5</td>
<td>0.4</td>
</tr>
<tr>
<td>II</td>
<td>Stable isotope labelled – internal standard (SIL-IS)</td>
<td>D2</td>
<td>5</td>
<td>4.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,25(OH)2D2</td>
<td>0.5</td>
<td>0.45</td>
</tr>
<tr>
<td>III</td>
<td>Standard addition without IS</td>
<td>D2</td>
<td>5</td>
<td>4.27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,25(OH)2D2</td>
<td>0.5</td>
<td>0.47</td>
</tr>
<tr>
<td>IV</td>
<td>Standard addition with IS (SA-IS)</td>
<td>D2</td>
<td>5</td>
<td>4.77</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,25(OH)2D2</td>
<td>0.5</td>
<td>0.48</td>
</tr>
</tbody>
</table>

Quantitative analytical results for each vitamin D compound obtained by standard addition with the internal standard (SA-IS) calibration method were compared with the stable isotope labelled- internal standard (SIL-IS) calibration method. **Table 4.2** shows the data for plasma vitamin D levels from three individual volunteers (1-3). Results were comparable proving that the proposed standard addition method (SA-IS) is an acceptable substitute to the SIL-IS method which is the gold standard in LC-MS for correcting matrix effects.
Table 4.2. Concentrations of 11 vitamin D compounds in plasma samples from three volunteers determined using standard addition with internal standard calibration method (SA-IS) and SIL-IS calibration method. *25(OH)D2 was used as internal standard for SA – IS calibration method.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>SIL-IS</th>
<th>Concentration (nM)</th>
<th>SA-IS (% difference from SIL - IS method)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>25(OH)D3</td>
<td>14.5</td>
<td>17.2</td>
<td>16.01</td>
</tr>
<tr>
<td>25(OH)D2 (IS)</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D3</td>
<td>0.047</td>
<td>1.29</td>
<td>3.20</td>
</tr>
<tr>
<td>D2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>1,25(OH)2D3</td>
<td>5.91</td>
<td>7.25</td>
<td>3.21</td>
</tr>
<tr>
<td>1,25(OH)2D2</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>24,25(OH)2D3</td>
<td>5.66</td>
<td>1.22</td>
<td>3.59</td>
</tr>
<tr>
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<td>0.00</td>
</tr>
<tr>
<td>25(OH)D3-S</td>
<td>2.33</td>
<td>2.22</td>
<td>4.21</td>
</tr>
<tr>
<td>25(OH)D2-S</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D3-S</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>D2-S</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

The major drawback with the standard addition procedure is the necessity of using three times the sample volume as compared to SIL-IS method. As Ellison and Thompson (249) highlighted, standard addition can only be precise and feasible when the analytical calibration curve is linear throughout the targeted concentration range. Therefore, this condition must be established prior to embarking on 2-point or 1-point standard addition procedure.

4.6 Conclusion

At present, internal standardisation using stable isotope labelled analogues of the analytes is the best available method to overcome matrix effects in LC-MS/MS. In this chapter, the problems associated with this method have been discussed and a standard addition method proposed as an alternative. The classical standard addition procedure is only suited to “dilute and shoot” type methods where the procedural errors are minimal. For others such as the vitamin D assay, as used in this study, we introduced an additional internal standardisation step to the classical standard addition method. Comparison with classical standard addition
and internal standardisation using SIL-IS showed that the proposed standard addition method in this paper to be a viable method to overcome matrix effects in LC-MS/MS. The accuracy of the proposed standard addition method was demonstrated to be superior to that of the SIL-IS method.
CHAPTER 5: SAMPLE APPLICATION IN MOUSE BRAIN

5.1 Foreword
This chapter will use the LC-MS/MS method developed in Chapter 3 above in the following application; the quantification of vitamin D metabolites in mouse brain tissue. The tissues samples were collected by Dr Virginie Lam from the School of Public Health, Faculty of Health Sciences, Curtin University, Perth, Australia and she was also involved in the experimental process and data analysis. The specific objective of this collaboration was to provide quantitative information on the concentrations of 12 vitamin D compounds in mouse brain samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS).

5.2 Introduction
Some studies suggest that an inadequate dietary, essential vitamin, mineral or micronutrient supply of any number of such compounds can adversely affect brain function (250-252). One specific nutritional factor that has been linked to the epidemiology of Alzheimer’s disease is vitamin D. Alzheimer’s disease is a group of disorders characterised by neurodegeneration and progressive loss of memory and cognitive function (253). Moreover, epidemiological studies have shown significant associations between vitamin D deficiency and an increased risk of other various neuropsychiatric and neurodegenerative disorders in central nervous system function (254) and brain development in adults (255).

There is also accumulating evidence that severely low serum 25-hydroxyvitamin D [25(OH)D] in adults is linked with a number of non-skeletal diseases. The report by Garcion et al. is of particular interest; accruing evidence relating vitamin D₃ concentrations and the brain function (256) and there are also suggestions that early developmental vitamin D₃ deficiency may be a risk factor for number of disorders associated with cognitive functions. Vitamin D₃ has many effects on adult brain tissue, in vivo and in vitro (256), and there are suggestions (257) that early vitamin D₃ deficiency may be a risk factor for the number of disorders relating to cognitive functions. The activating enzyme of vitamin D, 25-hydroxyvitamin D₃1-alpha-hydroxylase, also known as cytochrome P450 27B1 (CYP27B1), is found in a wide variety of tissues throughout the body, including the brain (258, 259), along with 25-hydroxylase (CYP 2R1) and the enzyme required for the degradation of the biologically active form of vitamin D, 24-hydroxylase (CYP24A1) (260) (Figure 5.1).
Animal studies have shown that the vitamin D receptor (VDR) is also found within specific brain regions, including the hippocampus, amygdala, hypothalamus, thalamus, cortex, and cerebellum (261, 262), which are key areas for cognition (263). It is noteworthy that brain capillaries have significant expression of the VDR and of the enzyme 1-α-hydroxylase (CYP27B1), which converts 25(OH)D₃ to the active metabolite, 1,25(OH)₂D₃.

**Figure 5.1:** Representative diagram of synthesis and metabolism of vitamin D. Figure modified from Kalueff *et al.* (264)

There was a growing evidence that vitamin D is involved significantly in mammalian brain functioning (265). A study based on brain tissue sample, found that vitamin D₃ metabolites in brain tissue are significantly higher in rats fed with a vitamin D diet compared to rats fed a
deficient diet and serum and brain concentrations of vitamin D\textsubscript{3} are strongly correlated (266). Therefore vitamin D\textsubscript{3} status in serum directly affects bioavailability of vitamin D metabolites in brain. The majority of evidence concerning the biology and mechanisms of action of vitamin D in the brain, and most of the direct evidence for effects of vitamin D inadequacy on cognitive or behavioural function, rely on experiments conducted in laboratory rats or mice. A study by Feron et al. (267) demonstrated that vitamin D\textsubscript{3} deficiency has profound effects on the developing brain including changes in volume, shape, cell proliferation and growth factor expression in rats. Moreover, the nuclei of neurons in the certain brain regions of adult rats and mice have been found to contain 1,25(OH)\textsubscript{2}D\textsubscript{3} (268). There is now also an accumulating evidence that 1,25-dihydroxyvitamin D\textsubscript{3} (1,25(OH)\textsubscript{2}D\textsubscript{3}) is involved in brain development (267).

A number of studies have summarised that vitamin D is synthesised in the skin of rats and humans (269, 270), and the distribution of the vitamin D receptor (VDR) in human and rat brains is very similar (271). The apoptotic effects of calcitriol on brain glial cells appears to be similar whether the cells are from humans or rats (272) and serum concentrations of vitamin D - binding protein are similar in laboratory rats and humans (273). A previous study showed that 25(OH)D\textsubscript{3} serum levels decreased in parallel to a similar decrease in 25(OH)D\textsubscript{3} in brain tissue, whilst, 25(OH)D\textsubscript{3} in brain tissue increased with its level in serum in the supplement group (266). Furthermore, decreases in vitamin D metabolites in rat plasma from animals of advancing age were showed by Horst et al.(274). This, therefore, emphasises the potential importance of ensuring appropriate levels of vitamin D supplementation amongst older people both for maintaining bone health and also for improving cognitive performance.

The aim of this study was to investigate the concentrations of 12 vitamin D compounds in mouse brain samples. The quantitative determination of vitamin D and its metabolites in mouse brain samples is a challenging task due to our present level of knowledge of such concentrations in the sample and the presence of interfering endogenous matrix materials. It is likely that the findings from this study may be relevant to improve our understanding of the relationship between vitamin D concentrations and cognition in normal and pathological ageing, using cognitive tasks to assess specific cognitive processes. A developed and validated liquid chromatography mass spectrometry (LC-MS/MS) method for the analysis of a wide range of vitamin D analogues in serum was used to evaluate the level of D\textsubscript{2}, D\textsubscript{3},

5.3 Materials and methods

5.3.1 Brain samples

Mouse brain samples were obtained from the Animal Resources Centre (Murdoch, Western Australia). Ethical approval and all experimental procedures were conducted in accordance with the requirements of the National Health & Medical Research Council accredited Curtin Animal Ethics Committee. Senescence- Accelerated- Mouse- Prone 8 (SAMP 8) and their age-matched controls, Senescence-Accelerated- Mouse- Resistant 1 (SAMR1) mice, were weaned at three weeks of age. Standard rodent chow (Specialty Feeds, Western Australia) and water were provided ad libitum to individually housed mice on a 12 h light/dark cycle (Curtin University Animal Facility). Both six-week and 52-weeks old male and female samples (n = 44) were analysed.

5.3.2 Chemicals and reagents

All reagents, solvents, unlabelled standard and standard vitamin D compounds used in this chapter were purchased as described in Chapter 2.

5.3.3 Preparation of standard curves and linearity range.

Stock solutions in ethanol of all standard compounds were prepared at a concentration of 1 µM, were sub-divided into small volumes, and stored in amber Eppendorf tubes at -20 °C. All working solutions were prepared by serial dilution of stock solutions. The combination standards were further diluted in mobile phase to the appropriate concentrations for the preparation of calibration curves. To determine the linear range of the method, six levels (concentration level) of calibration were prepared and analysed as per samples. The concentrations of brain tissue standard curves used for all compounds were 0.5, 1, 4, 10, 20 and 30 nM.

5.3.4 Preparation of brain sample solution

A sample (approximately 40 - 150 mg of snap frozen mouse brain tissue accurately weighed) was perfused prior to homogenisation in 1100 µL acetonitrile, vortexed for 30 s; the mixture
was then centrifuged at 4000 x g for 10 min. The supernatant was transferred to a clean Eppendorf tube and the mixture was vortex mixed for 30 s and mobile phase was added in order to obtain a final sample volume of 1600 µL. Samples of male (34) and female (10) mouse brain tissue were included in this analysis.

5.4 LC-MS/MS method

The samples were analysed according to the LC-MS/MS method used for the analysis of a range of vitamin D compounds as described in Chapter 3. Briefly, lipophilic and hydrophilic vitamin D compounds were measured using an online SPE method with simple protein preparation using acetonitrile prior to LC-MS/MS analysis. D2, D3, 25(OH)D2, 25(OH)D3, 1,25(OH)2D2, 1,25(OH)2D3, 24,25(OH)2D2 and 24,25(OH)2D3 were assayed using positive ion MS detection while D2-S, D3-S, 25(OH)D2-S, 25(OH)D3-S were quantitated using negative ion MS detection.

5.5 Results and discussions

In this study, calibration plots were derived by using the peak area of the target analyte and the concentration of each compound standard. Regression coefficients (R²) were above 0.99 for all linear calibration models. Table 5.1 shows the average of vitamin D compounds in the overall mouse brain sample (n = 44) collected from male and female mice, using the developed and validated method reported in Chapter 3, while a representative chromatographic profile obtained for detectable vitamin D compounds in the mouse brain samples is shown in Figure 5.2. To our knowledge, this study was the first to attempt to detect and quantitate vitamin D sulphate metabolites in mouse brain; we observed, however, that sulphate forms were not present in any of the samples analysed. We consider that the absence of D2-S, D3-S, 25(OH)2D3-S and 25(OH)2D2-S is due to the potential impact of the blood-brain barrier (BBB) on the transportation of sulfate metabolites into the cerebrospinal fluid. The BBB is a physical and biochemical barrier between the blood and the brain, which prevents entry into the brain of most drugs and endogenous compounds from the blood. Only small lipophilic compounds can diffuse passively through the BBB, while other compounds are usually able to cross the BBB only with the help of carrier proteins (275).

Our results showed that membrane vitamin D3 receptors have also been identified in the brain and the concentrations of 1,25(OH)2D3 in the mouse brain tissue samples is markedly higher than that of other metabolites. This metabolite is able to cross the blood-brain barrier and
bind to nuclear vitamin D₃ receptors in the brain (276, 277). In comparison to human serum concentrations, 1,25(OH)₂D₃ levels in mouse brain were observed to be greater. It has been shown that a physiological concentration of the concentration in brain tissue of 25(OH)D₃ was five percent or less than that of 1,25(OH)₂D₃ due to their polarity differences (278). Owing to the added sulfate group, the polarities of D₂-S, D₃-S, 25(OH)D₃-S and 25(OH)D₂-S are likely greater than the polarity of lipophilic compounds – this is seen with other sulphate metabolites (279). Thus, it might be expected that the concentrations of vitamin D-sulfate compounds would be much reduced or indeed undetectable. This is possibly one of the main reasons that vitamin D-sulfate levels in brain tissue are largely unexplored and almost never measured in clinical practice.

Table 5.1: Average concentration of vitamin D compounds in mouse brain tissue

<table>
<thead>
<tr>
<th>Compound</th>
<th>Average concentration (nmol/kg) (n = 44)</th>
<th>Male (n = 34)</th>
<th>Female (n = 10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>25(OH)D₃</td>
<td></td>
<td>3.78</td>
<td>2.87</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td></td>
<td>23.3</td>
<td>23.1</td>
</tr>
<tr>
<td>24,25(OH)₂D₃</td>
<td></td>
<td>12.2</td>
<td>9.37</td>
</tr>
<tr>
<td>D₃</td>
<td></td>
<td>19.3</td>
<td>19.5</td>
</tr>
<tr>
<td>D₂</td>
<td></td>
<td>11.7</td>
<td>8.02</td>
</tr>
<tr>
<td>25(OH)D₂</td>
<td></td>
<td>2.52</td>
<td>2.67</td>
</tr>
<tr>
<td>1,25(OH)D₂</td>
<td></td>
<td>9.81</td>
<td>11.4</td>
</tr>
<tr>
<td>24,25(OH)₂D₂</td>
<td></td>
<td>5.63</td>
<td>4.16</td>
</tr>
</tbody>
</table>
Figure 5.2: Chromatographic profiles showing eight detectable vitamin D compounds (lipophilic and hydrophilic) in mouse brain samples
The concentrations of vitamin D compounds in brain tissues from mice at six weeks of age and 52 weeks of age were compared and the results shown in Figure 5.3; these samples, although revealing much inter-individual variability suggest an aged-associated decrease in brain concentrations of these compounds. Results in Figure 5.4 shows that the sex of the animal was not linked to statistically significant differences in vitamin D compound concentrations. Although the lack of correlation is clear it is possible that this can be explained by the relatively small sample number, male (n = 34) and female (n = 10), coupled with observed large inter-individual variations, in this study. Therefore, sex differences in the susceptibility to vitamin D deficiency should be explored further.

**Figure 5.3:** Level of eight vitamin D compounds in mouse brain of 44 samples for both sexes aged six weeks and 52 weeks. Bar graph represents mean and error bars indicate standard deviation
Figure 5.4: Sex difference did not lead to statistically significant differences in vitamin D compound concentrations. Results are presented as mean ± SD. There was no statistical difference found in the concentration ranges between male and female samples.

In summary, the present data clearly demonstrate that the developed method to determine vitamin D metabolites in human serum is appropriate to use for mouse brain tissue samples, with a simple modification of the sample preparation step. We have shown sex differences in the susceptibility to vitamin D deficiency that should be explored further. Furthermore, research in animals that combines vitamin D deficiency with relevant animal models of neuropsychiatric and neurodegenerative disorders is also required. In addition, this collaborative study was able to provide the accurate detection and subsequent quantification of 12 vitamin D compounds, previously been determined by the ELISA kits-based method (280), which is unable to separate measure D$_2$ and D$_3$ compounds. Furthermore, with the extensive links between each level of vitamin D compound deficiency and other brain disorders now evident, there is a need for relevant animal models of neuropsychiatric and neurodegenerative diseases.

5.6 Conclusion
Mounting evidence suggests that maintaining optimal vitamin D levels may lower the risk of developing a wide range of brain disorders, this indicates that research focusing on the elucidation of the mechanisms of vitamin D’s actions within the brain will be of significant utility. The developed LC-MS/MS method has proved to be highly sensitive, specific, and
accurate in the quantitation of vitamin D compounds in animal brain tissue. If these observations in mice are relevant to humans, they may have important implications for public health. This study has provided preliminary data that can be used to predict the levels of vitamin D compounds in brain tissue in the developing and ageing adult human brain. Further research work is required to establish the biological implications related to these findings.
CHAPTER 6: GENERAL DISCUSSION AND CONCLUSION

6.1 Foreword

This chapter provides a summary and discussion of the main findings of the studies reported in this thesis. The thesis comprises two papers that have been published (Chapter 2 and Chapter 3) and one chapter (Chapter 4) submitted for publication. The limitations of the thesis are addressed and the implications for future research are present.

6.2 Summary

The primary goal of the studies presented in this thesis was to bring about advances in the field of vitamin D assays for biological sample especially for human blood samples, by developing a highly specific and sensitive LC-MS/MS method. This research started with the following specific aims:

- To develop a sensitive, accurate and robust LC-MS/MS method for the determination of 12 vitamin D compounds (D$_2$, D$_3$, 25(OH)D$_2$, 25(OH)D$_3$, 1,25(OH)$_2$D$_2$, 1,25(OH)$_2$D$_3$, 24,25(OH)$_2$D$_2$, 24,25(OH)$_2$D$_3$, D$_2$-S, D$_3$-S, 25(OH)D$_2$-S and 25(OH)D$_3$-S).
- To compare the efficiencies of two different sample preparation methods (protein precipitation and saponification).
- To determine the appropriateness of the biological sample (serum and plasma) as the sample to be used for vitamin D analysis and the sample size necessitated in order to measure simultaneously 12 vitamin D compounds in serum and plasma.
- To develop a method that is amenable for routine analysis.
- To apply the method to quantify the total vitamin D compounds in other biological fluids/matrices.

Vitamin D now has been reported have many biological functions in humans beyond that relating the skeletal system (5). While there are more than 50 vitamin D metabolites, vitamin D status is usually evaluated by determining the plasma or serum concentrations of the metabolite 25-hydroxyvitamin D (25(OH)D$_3$ and 25(OH)D$_2$), the major circulating compound(s), considered to be a marker for vitamin D (281). However, it is still controversial as to whether the measurement of 25(OH)D alone provides sufficient information to link vitamin D with many other skeletal and non-skeletal disorders (34, 48). The ability to
quantify individual vitamin D form is essential in both nutritional and clinical samples in order to diagnose affected individuals who can receive appropriate treatment (143). In addition, early detection of vitamin D deficiency for new born babies is important; putting them at risk of long-term physical deficits.

Each vitamin D compound has a different function in the body (11, 78, 225). Most biological actions of vitamin D are attributed to the metabolite 1,25-dihydroxyvitamin D (1,25(OH)2D3 and 1,25(OH)2D2) that is, they are primarily responsible for preventing skeletal and non-skeletal disorders (11, 282). The metabolites 24,25-dihydroxyvitamin D (24,25(OH)2D3 and 24,25(OH)2D2) have also been found to play a crucial role in intramembranous and endochondral bone formation and in bone fracture repair (11). 3-Epi-25(OH)D3 is considered as inactive vitamin D compounds and, therefore, the collective measurement of the vitamin D forms and their epimers may contribute to an overestimation of the 25(OH)D level (22). Vitamin D can be also found as hydrophilic forms (vitamin D2-sulfate (D2-S), vitamin D3-sulfate (D3-S), 25(OH) D2-sulfate (25(OH)D2-S) and 25(OH)D3-sulfate (25(OH)D3-sulfate) (84). Hence, the capability to specifically measure a range of different vitamin D forms is highly valuable to investigate clinical disorders and their potential linkages to vitamin D status (34).

Traditionally, immunoassay methods have been applied for the determination of vitamin D in biological fluid samples (283, 284). The main disadvantage is their incapability to differentiate between a number of the different forms of vitamin D (149). Liquid chromatography (LC) methods have also been used for the analysis of vitamin D compounds in biological fluids. The LC methods have an advantage over immunoassays because of their ability to distinguish a variety of vitamin D compounds in a single run with electrochemical (EC) (285) and ultraviolet (UV) (22, 155) detectors. However, recently tandem mass spectrometry (MS/MS) detection has been the method of choice not only due to its ability to accurately quantify multiple analytes in a single but its superior sensitivity, specificity and reproducibility. Nevertheless, poor ionisation efficiency of vitamin D compounds in the atmospheric pressure ionisation (API) source in LC-MS/MS analysis, and vitamin D being present in low levels in biological fluids have been drawbacks (143).

In order to overcome the sensitivity issue, a derivatisation process based on the Diels-Alder reaction has been used to improve in signal response and increase in sensitivity of vitamin D.
over the non-derivatisation assay method (187). Derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) has proven to enhance the ionisation efficiency for small sample volumes such as neonatal biological fluids which the concentrations are too low to be detected by standard LC-MS/MS methods (23, 286, 287).

Another challenge in vitamin D assays has been the extraction of vitamin D compounds, particularly in biological samples. Vitamin D compounds in biological fluid samples are tightly bound to proteins and lipids and it is necessary to disrupt this binding to release the vitamin prior to LC-MS/MS analysis in order to generate free compound for analysis and to avoid matrix effects (143, 153). Therefore, sample preparation includes all processes that are involved prior to separation and detection including extraction and clean-up, and is important in initial stage method development as it frequently improves on the accuracy of the analytical method. Furthermore, sample clean-up directly effects the sensitivity and selectivity of the assay and in addition, adequate sample clean-up protects the chromatographic columns and also reduces matrix effects from co-eluting substances during the ionisation process. Protein precipitation is often used to remove proteins from biological fluids while saponification is commonly used with foodstuffs (143). After protein precipitation or saponification processes, the sample requires further extraction in order to remove other interfering substances prior to separation and/or detection. Liquid-liquid extraction (LLE) and/or solid phase extraction (SPE) are widely used for the extraction procedure in vitamin D analysis. This combination of sample clean up and extraction has resulted in high throughput methods.

Ion suppression/enhancement is the most common form of matrix effect occurred in liquid chromatography–mass spectrometry (LC-MS), when compounds that co-elute with the analyte interfere with the ionisation process, regardless of the sensitivity or selectivity of the mass analyser used. The only option to obtain accurate data is by complete elimination of the interfering compounds that co-elute with the analyte. The inclusion of stable isotope labelled-internal standard (SIL-IS) in the sample is able to remove the contribution from the interferences at the quantification step. SIL-IS calibration methods has become the most commonly used method to correct for matrix effects in MS detection (211) as SIL-IS are almost identical to those of the analyte, and they elutes at the same retention time as the analyte and experiences the similar extents of matrix effects.
The main objective of the research presented in this thesis was to develop an analytical method to measure quantitatively a wide range of vitamin D compounds, thereby creating a comprehensive picture of vitamin D status. Of particular focus is the simultaneous detection and quantification of 12 vitamin D compounds (both lipophilic and hydrophilic) in biological samples. The selected compounds comprised D_2, D_3, 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, 1,25(OH)_2D_2, 1,25(OH)_2D_3, 24,25(OH)_2D_2, 24,25(OH)_2D_3 (the lipophilic forms), vitamin D_2-sulfate (D_2-S), vitamin D_3-sulfate (D_3-S), 25(OH) D_2-sulfate (25(OH)D_2-S) and 25(OH)D_3-sulfate (25(OH)D_3-S) (the hydrophilic forms).

Chapter 2 described initial method development and validation attempts of a simultaneous determination method of 12 vitamin D compounds in human blood using pre-column derivatisation and the separation by using C18 column. Serum (compared to plasma) was found to be a more suitable sample type, and protein precipitation (compared to saponification) a more effective extraction method. Only nine out of twelve compounds were detected in serum; the methods were only able to determine one hydrophilic compound, 25(OH)D_3-S, in human serum. This was not only because the presence of very low levels in serum but also arose due to the derivatisation process, the procedure proving unsuitable for sulfated compounds. The main challenge was that of extracting and chromatographing vitamin D compounds with different polarities, both lipophilic and hydrophilic, in a single analytical procedure.

Additional assay method approaches were studied in Chapter 3; ammonium formate (at pH 6.17) was used as the optimised mobile phase to facilitate the protonation (positive mode) and de-protonation (negative mode). The extraction of all 12 vitamin D compounds was achieved by an optimised protein precipitation method using acetonitrile as the precipitant. The sensitivity was improved by minimising matrix effects in the MS detector rather than by using a lengthy derivatisation procedure; an online solid phase extraction (SPE) using a pentafluorophenyl (PFP) guard column was used for clean-up and the separation was accomplished by using a PFP analytical column. A combination with the SPE method resulted in a significant simplification of the sample preparation procedure, the on-column detection and quantification limits were found to be somewhat slightly higher, when compared to the previous method of Chapter 2. LC-MS/MS resolution of all 12 vitamin D compounds, including the chromatographic separation of 25(OH)D_3 from the isomer 3-epi-25(OH)D_3 was achieved. Stable isotope labelled vitamin D compounds were used as internal
standards for the quantification of all 12 vitamin D compounds in both methods outlined in Chapters 2 and 3. The method can be very likely applied to other biological fluids; mouse brain samples were also similarly analysed following modification of the sample preparation step, as described in Chapter 5.

Developing the LC-MS/MS method for 12 vitamin D compounds can be a financially expensive exercise, in laboratory consumable terms, as SIL-IS compounds are required for each of the 12 vitamin D compounds. In order to develop a method combining assay utility with cost-effectiveness, the developed online method was then applied to study alternatives to the stable isotope labelled – internal standard (SIL-IS) calibration method, in which a standard addition method was combined with an additional internal standard (not co-eluting) to correct for procedural errors. Experiments were carried out on biological human plasma/serum samples. The results obtained show that the proposed method of standard addition yields more accurate results than internal standardisation using stable isotope labelled analogues, based on the recovery results shown in Chapter 4.

In Chapter 5, the method was demonstrated to be easily adapted for other complex matrices such as exist in mouse brain tissue samples. Evidence continues to accumulate, indicating that adequate levels of vitamin D are required for normal brain development and function. In this study, it was confirmed that the vitamin D compound presence in mouse brain samples is significantly not extant in the sulphated forms, but concentrations of 1,25(OH)\(_2\)D\(_3\) in mouse brain sample were markedly higher than the major metabolites, 25(OH)D\(_3\). While mindful of the limitations of analysis of sulfate forms, such findings may also prove useful for directing the association between a patient’s vitamin D levels with diverse clinical neurological outcomes. If these observations in mice are relevant to humans, they could have important implications for public health.

Overall, the studies reported in this thesis have resulted in the development of LC-MS/MS methods for the successful determination of 12 vitamin D compounds (both lipophilic and hydrophilic in nature) in biological samples. The high specificities and sensitivities obtained demonstrate the suitability of the developed methods for the determination of vitamin D compounds and their metabolites in human samples, thus providing results that are potentially useful in disease diagnosis. These methods will therefore enable researchers to study more precisely the activities and functions of each vitamin D compound and the total
amount (and activity) of vitamin D in blood and other biological samples, which is of key importance in the investigation of links between vitamin D and clinical disorders.

### 6.3 Study limitations

The limitations of the studies were discussed in detail in each chapter. In summary, the initial developed method in Chapter 2 was time-consuming and was not best suited for routine analysis application. The studies reported in Chapter 3, permitted the elimination of derivatisation reactions by careful adjustment of pH and then the simplification of sample preparation procedures by partially automating the sample preparation method. The detection and quantification limits subsequently obtained were found to be somewhat higher when compared to the first developed method using a derivatisation step. The lengthy multi-step derivatisation procedure used in vitamin D analysis is not easily amenable to automation and therefore has not been fully or partially automated so far. The sample is required to be completely dry before derivatisation, and this is challenging to achieve in an on-line procedure.

### 6.4 Future research directions

The analytical methods presented in this thesis represent significant improvements upon existing methodologies. Multi-compound determination is one of the most important trends to provide a better diagnostic picture of vitamin D status. Further studies are also necessary to develop a method in order to fulfil the multi-compound determination of vitamin D metabolites with simple solid-phase separation procedures coupled to on-line derivatisation reactions. These represent assay necessities on the pathway to sample analysis automation - to decrease the complexity of the clean-up and increase the sensitivity with the required smaller sample size. Further research work is additionally required to advance the automation of sample preparation procedures, in order to fulfil the demand for high-throughput analysis techniques and making it suitable for routine analysis.

The analytical method developed and implemented in this thesis can be applied to future research examining vitamin D in other biological fluids. With further development, an extended range of vitamin D compounds could also be studied including vitamin D-glucuronide metabolites. It has been demonstrated that conjugation with glucuronic acid plays a quantitatively important role in the enterohepatic circulation of vitamin D. Future studies may focus on linking vitamin D-glucuronide status in brain development and function. Similarly, steroidal sulfates are known to undergo a variety of important
physiological roles, but the roles remain controversial. Further research work is required to better understand this class of vitamin D compounds. It would therefore be of clinical interest to investigate whether 25(OH)D$_3$-S could be epimerised, as it could be a potential vitamin D marker. Also, it would be of greatest interest to investigate whether there are dihydroxy-vitamin D compounds present as the respective sulfate conjugate.

The development of vitamin D Standard Reference Material (SRM) is undertaken by the National Institute of Standards and Technology (NIST). Currently SRM 972a is only available for the following vitamin D compounds: 25(OH)D$_3$, 25(OH)D$_2$, 24,25(OH)$_2$D$_3$ and 3-epi-25(OH)D$_3$ with different levels of endogenous vitamin D metabolites in human serum. In the future, it may be appropriate, and indeed valuable, to compare a comprehensive SRM (ideally with all 12 compounds) against the method reported here.
6.5 Conclusion

There are a number of strengths identified from the studies reported in this thesis. The thesis provided original contributions to the current knowledge in several ways. Firstly, a simple and effective method to simultaneously determine 12 vitamin D compounds in biological fluid was established. The concentration profiles of D$_2$, D$_3$, 25(OH)D$_2$, 25(OH)D$_3$, 1,25(OH)$_2$D$_2$, 1,25(OH)$_2$D$_3$, 24,25(OH)$_2$D$_2$, 24,25(OH)$_2$D$_3$, D$_2$-S, D$_3$-S, 25(OH)D$_2$-S, 25(OH)D$_3$-S, both lipophilic and hydrophilic, in a single analytical procedure were then documented. Following this, the concentrations of these metabolites in human serum and mouse brain were examined. This thesis found that serum was a more suitable sample type when compared to plasma and protein precipitation was the more effective extraction method for vitamin D assay; protein precipitation with acetonitrile proved more efficient in releasing vitamin D metabolites from the matrix when compared to trichloroacetic acid. This thesis has provided new evidence that an additional internal standard (not co-eluting), when combined with a standard addition method can be effectively used to correct for procedural errors. Recoveries determined on human serum samples show that the proposed method of standard addition yields more accurate results than the internal standardisation using stable isotope labelled analogues. In mouse brain samples, concentrations of 1,25(OH)$_2$D$_3$ were higher, when compared to other metabolites (e.g. 25(OH)D$_3$), whereas sulfate compounds were not detected. In summary, this thesis reported an original analytical method to analyse simultaneously 12 vitamin D compounds in biological fluids by using the most advanced and sensitive analytical technique available to date, for both lipophilic and hydrophilic compounds.
CHAPTER 7: REFERENCES


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Appendix 1: Approval letter of ethical clearance of Institutional Human Research Ethics Committee for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2014)

**The University of Queensland**

**Institutional Human Research Ethics Approval**

<table>
<thead>
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<th>Project Title:</th>
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</thead>
<tbody>
<tr>
<td>Chief Investigator:</td>
<td>Dr Amitha Hewativithara</td>
</tr>
<tr>
<td>Supervisor:</td>
<td>None</td>
</tr>
<tr>
<td>Co-Investigator(s):</td>
<td>Fabio Pereira Gomes, Dr Karen Whitfield, Prof Nick Shaw</td>
</tr>
<tr>
<td>School(s):</td>
<td>Pharmacy</td>
</tr>
<tr>
<td>Approval Number:</td>
<td>2014001235</td>
</tr>
<tr>
<td>Granting Agency/Degree</td>
<td>None</td>
</tr>
<tr>
<td>Duration:</td>
<td>31st October 2015</td>
</tr>
</tbody>
</table>

**Comments/Conditions:**

Note: If this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Assurance Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

**Name of responsible Committee:**

Medical Research Ethics Committee

This project complies with the provisions contained in the [National Statement on Ethical Conduct in Human Research](https://www.ahrq.gov/) and complies with the regulations governing experimentation on humans.

**Name of Ethics Committee representative:**

Professor Bill Vicenzino

Chairperson

Medical Research Ethics Committee

Signature [Signature]

Date 15.10.2014
Additional Notes to Ethics Approval

1. The clearance number should be quoted on the protocol coversheet when applying to a granting agency and in any correspondence relating to ethical clearance.

2. Clearance will normally be for the duration of the project unless otherwise stated in the institutional clearance form.

3. Adverse reaction to treatment by subjects, injury, or any other incidents affecting the welfare and/or health of subjects attributable to the research should be promptly reported to the Head of School, the Occupational Health & Safety Unit, and the Ethics Committee.

4. Amendments to any part of the approved protocol (including change of Investigator/s), documents, or questionnaires attached to the clearance must be submitted to the Ethics Committee for approval.

5. Unforeseen events that might affect continued ethical acceptability of the project must be immediately reported to the Ethics Committee.

6. Discontinuation of the project before the expected date of completion must be reported to the Ethics Committee, giving reasons.

7. The Chief/Principal Investigator/s are responsible and accountable for full compliance of the protocol by all investigators.

8. The Committee reserves the right to visit the research site and view materials at any time, and to conduct a full audit of the project.

9. It is the Committee’s expectation, whenever possible, that work should result in publication. The Committee would require details to be submitted for our records.

10. Staff and students are encouraged to contact either the Ethics Officer (3365 3924), or Chairperson on other issues concerning the conduct of experimentation/research (e.g., involvement of children, informed consent) prior to commencement of the project and throughout the course of the study.
Appendix 2: Consent form for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2014)

SCHOOL OF PHARMACY

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Pharmacy Australia Centre of Excellence
20 Cornwall Street
Woolloongabba, QLD 4102
Telephone (07) 3346 1999
Facsimile (07) 3346 1999

CONSENT FORM

Development of an LC-MS-MS method to measure all major vitamin D analogues in blood.

Principal investigator: Dr. Amita Hewawitharana School of Pharmacy, UQ

Co-investigators: Fabio Pereira Gomes School of Pharmacy, UQ (Ph.D. student)
Dr. Karon Whitfield School of Pharmacy, UQ
Professor Nick Shaw School of Pharmacy, UQ

I agree to participate in the above University of Queensland research project. I have had the project explained to me, and I have read the Information Sheet, which I will keep for my records. I understand that signing this consent form means that I am willing to:
- Participate in providing a blood sample
- Allow an investigator to take a medication history
- Allow the information to be collated (anonymously), analysed and written up in a research paper

I understand that the following steps will be undertaken to protect my identity from being made public:
- All participants will be requested not to disclose names or unique identifiers.
- No family names or unique identifiers will be recorded
- All recordings and documents will be kept securely within the School of Pharmacy, University of Queensland, and only the researchers will have access to these records.
- No data will be published that may identify individuals participating in this research.
- I will derive no personal benefit by participating in this research

I also understand that my participation is voluntary, that I can choose not to participate in part or all of the project, and that I can withdraw at any stage of the project without being penalised or disadvantaged in any way.

Signature: ..........................................................

Name (please print) ..................................................

Date: ..../..../2014 Telephone number: (........)..........................
Appendix 3: Participant information sheet for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2014)

PARTICIPANT INFORMATION SHEET

Project title:
Development of an LC-MS-MS method to measure all major vitamin D analogues in blood

Principal investigator: Dr. Amitha Hewawitharana, School of Pharmacy, UQ

Co-investigators: Fabio Pereira Gomes, School of Pharmacy, UQ (Ph.D. student)
Dr. Karen Whitfield, School of Pharmacy, UQ
Professor Nick Shaw, School of Pharmacy, UQ

Vitamin D deficiency has been associated with inflammatory conditions and chronic diseases such as multiple sclerosis, rheumatoid arthritis, type I and II diabetes, certain cancers and some mental disorders including schizophrenia. Early identification of individuals who are vitamin D deficient is essential in order to institute adequate nutritional supplementation. A blood sample is required to assess the vitamin D status in the body.

Liquid chromatography-tandem mass spectrometry (LC-MS-MS) is currently the most powerful tool for analysis of vitamin D compounds in biological fluids. Blood (plasma and/or serum) vitamin D assay is widely used to diagnose vitamin D deficiency. However, the commonly used vitamin D assays measure one or two analogues of vitamin D. We plan to develop a method to determine about 10 analogues therefore to provide a comprehensive picture of the vitamin D status. Sample size will be also optimized, to allow assessment of the vitamin D status in individuals who can only provide limited volumes of blood.

To achieve the accurate results from analysis, the blood samples need to be prepared appropriately. Sample preparation still remains as one of the most time-consuming and error-prone aspects in analysis of vitamin D. Inappropriate sample preparation procedures may lead to an erroneous estimation of the vitamin D status. Most studies have been focused on protein precipitation method for plasma/serum. Saponification method is widely used for vitamin D assay of food but not of serum/plasma. There are no studies showing direct comparison between protein precipitation and saponification to extract vitamin D compounds from blood and to establish the best extraction method for vitamin D analysis of plasma/serum. By establishing the best extraction method and using LC-MS-MS, the accurate level of vitamin D can be measured with high sensitivity and this will positively affect the clinical outcome of the patients. Early identification of individuals who are vitamin D deficient is of key importance so that early supplementation can be instituted.

Therefore, the aims of the study are:

- To investigate the appropriateness of protein precipitation or saponification as the sample preparation method for vitamin D analysis in blood, by comparing the two methods.
- To determine the minimum sample size required for analysis of vitamin D in blood.
• To investigate LC-MS-MS as a suitable method to measure all major vitamin D analogues in blood.

We would like to invite you to participate in our project, which will take no more than 20 minutes.

What will you be asked to do?

You will be asked to provide a blood sample taken by the pathology lab at UQ Health Care Centre, Annerley which is located in PACE. You do NOT need to fast and you can take your regular medication. The amount of blood taken will be 10mL, which is a usual volume for a regular blood test. You may experience mild discomfort when the blood sample is taken. This will take approximately 10 minutes.

The researcher will also take a medication history by asking if you are taking any medications. Medications may include those prescribed by your doctor, any medications that you buy over-the-counter from your pharmacy or any herbal or vitamin products. The researcher will write down all your medications as well as the dosage that you take. The researcher will also ask questions about your diet, whether it is of animal or plant origin or both. This interview will take approximately 10 minutes.

Participation in this project is voluntary and there will be no consequences if you choose not to participate. You are free to withdraw from the project at any time.

The information that we collect from you will be de-identified. The data collected from this study, both paper and electronic forms will be stored in a locked filing cabinet under the supervision of the investigators for a period of 7 years. After this period of time, the data will be destroyed appropriately (paper shredded, CD-ROM erased). Published data will not identify any individuals participating in this project.

If you wish to participate in this project, please sign the consent form.

A summary of the results from this study will be available on request. If interested, please leave your contact details on a separate sheet provided by the researcher or contact one of the investigators.

If you have any queries, please contact Dr. Amita Hewavitharana (3346 1898, a.hewavitharana@pharmacy.uq.edu.au) or Dr. Karen Whitfield (0407063799 or karen.whitfield@uq.edu.au).

This study has been cleared by one of the human ethics committees of the University of Queensland in accordance with the National Health and Medical Research Council’s guidelines. You are of course, free to discuss your participation in this study with project staff (contactable on 3365 2868 or email karen.whitfield@uq.edu.au or amitha.hewavitharana@uq.edu.au)

If you would like to speak to an officer of the University not involved in the study, you may contact the Ethics Officer on 3365 3924 (quote project number ###8).

Thank you for participating in this project.

Fabio Pereira Gomes
Dr Amita Hewavitharana
Dr Karen Whitfield
Professor Nick Shaw
Appendix 4: Questionnaire form for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2014)

**SUPPLEMENT SOURCES OF VITAMIN D QUESTIONNAIRE**

Patient #: ___________________  Gender: M / F  Date of Birth: ___________________

During the past 7 days how often did you take the following supplement over the counter?

<table>
<thead>
<tr>
<th>Supplement Type</th>
<th>What is the name (brand or generic) of the supplement?</th>
<th>How often do you take this supplement? Eg: times/day, times/week, times/month</th>
<th>What is the strength/amount of vitamin D only?</th>
<th>What time did you take your last dose?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod Liver Oil (oil or capsules)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D tablets</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multivitamins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Are you a vegetarian?

☐ No  ☐ Yes

Are you a vegan?

☐ No  ☐ Yes

Are you currently taking any medication prescribed by doctor to treat your vitamin D deficiency?

☐ No  ☐ Yes

If so can you tell us the name and dose of the medication? __________________________________________

Thank you for your time in completing this questionnaire!
Appendix 5: Approval letter of ethical clearance of Institutional Human Research Ethics Committee for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2015)

THE UNIVERSITY OF QUEENSLAND
Institutional Human Research Ethics Approval

Project Title: Development of an LC-MS-MS Method to Measure All Major Vitamin D Analogues in Blood
Chief Investigator: Dr Amita Hewavitharana
Supervisor: None
Co-Investigator(s): Nur Sofiah Abu Kassim, Dr Karen Whitfield, Prof Nick Shaw
School(s): Pharmacy
Approval Number: 2015000690
Granting Agency/Degree: None
Duration: 31st August 2016

Comments/Conditions:

Note: If this approval is for amendments to an already approved protocol for which a UQ Clinical Trials Protection/Innovation Form was originally submitted, then the researchers must directly notify the UQ Insurance Office of any changes to that Form and Participant Information Sheets & Consent Forms as a result of the amendments, before action.

Name of responsible Committee: Medical Research Ethics Committee
This project complies with the provisions contained in the National Statement on Ethical Conduct in Human Research and complies with the regulations governing experimentation on humans.

Name of Ethics Committee representative: Professor Bill Vicenzino
Chairperson
Medical Research Ethics Committee

Signature ___________________________ Date 5/4/2015
Additional Notes to Ethics Approval

1. The clearance number should be quoted on the protocol coversheet when applying to a granting agency and in any correspondence relating to ethical clearance.

2. Clearance will normally be for the duration of the project unless otherwise stated in the institutional clearance form.

3. Adverse reaction to treatment by subjects, injury, or any other incidents affecting the welfare and/or health of subjects attributable to the research should be promptly reported to the Head of School, the Occupational Health & Safety Unit, and the Ethics Committee.

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6. Discontinuation of the project before the expected date of completion must be reported to the Ethics Committee, giving reasons.

7. The Chief/Principal Investigator/s are responsible and accountable for full compliance of the protocol by all investigators.

8. The Committee reserves the right to visit the research site and view materials at any time, and to conduct a full audit of the project.

9. It is the Committee's expectation, whenever possible, that work should result in publication. The Committee would require details to be submitted for our records.

10. Staff and students are encouraged to contact either the Ethics Officer (3365 3924), or Chairperson on other issues concerning the conduct of experimentation/research (e.g., involvement of children, informed consent) prior to commencement of the project and throughout the course of the study.
Appendix 6: Consent form for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2015)

CONSENT FORM

Development of an LC-MS-MS method to measure all major vitamin D analogues in blood.

Principal investigator: Dr. Amitha Hewavitharana School of Pharmacy, UQ
Co-investigators: Nur Sofiah Abu Kassim, UQ (Ph.D. student) 
Professor Nick Shaw School of Pharmacy, UQ
Dr. Karen Whitfield School of Pharmacy, UQ

I agree to participate in the above University of Queensland research project. I have had the project explained to me, and I have read the Information Sheet, which I will keep for my records. I understand that signing this consent form means that I am willing to:

- Participate in providing a blood sample
- Allow an investigator to take a medication history
- Allow the information to be collated (anonymously), analysed and written up in a research paper

I understand that the following steps will be undertaken to protect my identity from being made public:

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- No family names or unique identifiers will be recorded
- All recordings and documents will be kept securely within the School of Pharmacy, University of Queensland, and only the researchers will have access to these records.
- No data will be published that may identify individuals participating in this research.
- I will derive no personal benefit by participating in this research

I also understand that my participation is voluntary, that I can choose not to participate in part or all of the project, and that I can withdraw at any stage of the project without being penalised or disadvantaged in any way.

Signature: .................................................................

Name (please print) ...........................................................

Date: ...... / ...... / 2015  Telephone number: (......).................................
Appendix 7: Participant information sheet for development of an LC- MS/MS method to measure all major vitamin D analogues in blood (2015)

SCHOOL OF PHARMACY

THE UNIVERSITY OF QUEENSLAND

PARTICIPANT INFORMATION SHEET

Project title:
Development of an LC-MS-MS method to measure all major vitamin D analogues in blood

Principal investigator: Dr. Amita Hewavitharana School of Pharmacy, UQ

Co-investigators : Nur Sofiah Abu Kassim, UQ (Ph.D. student)
Professor Nick Shaw School of Pharmacy
Dr. Karen Whitfield School of Pharmacy, UQ

Vitamin D deficiency has been associated with inflammatory conditions and chronic diseases such as multiple sclerosis, rheumatoid arthritis, type I and II diabetes, certain cancers and some mental disorders including schizophrenia. Early identification of individuals who are vitamin D deficient is essential in order to institute adequate nutritional supplementation. A blood sample is required to assess the vitamin D status in the body.

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Therefore, the aims of the study are:

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- To determine the minimum sample size required for analysis of vitamin D in blood.
- To investigate LC-MS-MS as a suitable method to measure all major vitamin D analogues in blood.
We would like to invite you to participate in our project, which will take no more than 20 minutes.

**What will you be asked to do?**

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The researcher will also take a medication history by asking if you are taking any medications. Medications may include those prescribed by your doctor, any medications that you buy over-the-counter from your pharmacy or any herbal or vitamin products. The researcher will write down all your medications as well as the dosage that you take. The researcher will also ask questions about your diet, whether it is of animal or plant origin or both. This interview will take approximately 10 minutes.

Participation in this project is voluntary and there will be no consequences if you choose not to participate. You are free to withdraw from the project at any time.

The information that we collect from you will be de-identified. The data collected from this study, both paper and electronic forms will be stored in a locked filing cabinet under the supervision of the investigators for a period of 7 years. After this period of time, the data will be destroyed appropriately (paper shredded, CD-ROM erased). Published data will not identify any individuals participating in this project.

If you wish to participate in this project, please sign the consent form.

A summary of the results from this study will be available on request. If interested, please leave your contact details on a separate sheet provided by the researcher or contact one of the investigators.

If you have any queries, please contact Dr. Amita Hewavitharana (3346 1898, a.hewavitharana@pharmacy.uq.edu.au) or Prof Nick Shaw (nshaw@pharmacy.uq.edu.au).

This study has been cleared by one of the human ethics committees of the University of Queenslands in accordance with the National Health and Medical Research Council's guidelines. You are of course, free to discuss your participation in this study with project staff (contactable on 3365 2868 or email nshaw@pharmacy.uq.edu.au or amitha.hewavitharana@uq.edu.au)

If you would like to speak to an officer of the University not involved in the study, you may contact the Ethics Officer on 3365 3924 (quote project number ####).

Thank you for participating in this project.

Nur Sofiah Abu Kassim
Professor Nick Shaw
Dr Amita Hewavitharana
Dr Karen Whitfield
Appendix 8: Questionnaire form for development of an LC-MS/MS method to measure all major vitamin D analogues in blood (2015)

**SCHOOL OF PHARMACY**

THE UNIVERSITY OF QUEENSLAND

Pharmacy Australia Centre of Excellence
20 Cornwall Street
Woolloongabba, QLD 4102
Telephone (07) 3346 1900
Facsimile (07) 3346 1999

**VITAMIN D QUESTIONNAIRE**

<table>
<thead>
<tr>
<th>Gender</th>
<th>M / F</th>
<th>Height: cm</th>
<th>Weight: kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Date of Birth:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

1) During the past 7 days how often did you take the following supplement over the counter:

<table>
<thead>
<tr>
<th>Supplement Type</th>
<th>What is the name (brand or generic) of the supplement?</th>
<th>How often do you take this supplement? (Eg: times/day, times/week, times/month)</th>
<th>What is the strength/amount of vitamin D only?</th>
<th>What time did you take your last dose?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cod Liver Oil (oil or capsules)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vitamin D supplements or calcium with vitamin D</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Multivitamins</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2) Are you a vegetarian or vegan? __Yes / No__

3) How many servings of milk do you get daily? _______

4) On average, how much sun exposure have you had in the past week?

- [ ] Less than 5 minutes per day
- [ ] 5–15 minutes per day
- [ ] 15–30 minutes per day
- [ ] More than 30 minutes per day

5) Are you currently taking any medication prescribed by a doctor? __Yes / No__

If so can you tell us the name of the medication? __________________________

Thank you for your time in completing this questionnaire!
18 June 2015

To whom it may concern,

RE: Letter of Authority and Approval for Collection of Serum Samples

I am writing in support of this application and am aware that there is a change of investigator on the project.

I also, as Head of School, give permission for staff and RHD students to be contacted to volunteer for this project.

Sincerely

[Signature]

Professor Peter J. Little, AM
Head of School
Appendix 10: Approval letter of ethical clearance of National Health & Medical Research Council accredited Animal Research Ethics Committee, Curtin University (2017)

28-Sep-2017

Name: John Mano
Department/School: School of Public Health
Email: J_Manoo@curtin.edu.au

Dear John Mano,

RE: Annual report acknowledgement
Approval number: AEC_2014_77

Thank you for submitting an annual progress report to the Animal Research Ethics Office for the project The effect of selected nutraceuticals on age-associated cerebral capillary dysfunction and cognitive deficits.

Your annual progress report was reviewed by the Animal Ethics Committee at its meeting on 21-Sep-2017 and has been approved. Approval is granted for a period of one year. Approval will remain current until 01-Aug-2018. Continuation of approval will be granted on an annual basis following submission of an annual report. Any special conditions noted in the original approval letter still apply.

Standard conditions of approval:

- An Annual Progress Report must be submitted to the Ethics Office annually, on the anniversary of approval.
- An Annual Animal Use Report that captures the relevant details regarding the number of animals used in the preceding year i.e. 1 January to 31 December must be submitted before 31 January of the following year.
- Any amendments to the approved protocol must be submitted to the Ethics Office.
- A Completion Report must be submitted to the Ethics Office on completion of the project.
- Should any animal(s) experience an adverse or unexpected outcome resulting from the experimentation, the AEC is to be notified in writing immediately.
- Please ensure that you quote the Animal Ethics Committee approval number whenever you order animals for this project. Note also that an AEC approval number must be displayed on the cage(s)/aquaria etc used to house/maintain animals during an approved activity.
- If the results of this research will be published, citations should state: “All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes”.

Should you have any queries regarding consideration of your project, please contact the Ethics Officer at aec@curtin.edu.au or on 9266 2784.

Yours sincerely,

[Signature]

De: Beng Chua
Deputy Chair, Animal Ethics Committee