

Implementing an LC-QQQ method for the quantification of vitamin D analogues from serum accounting for epimers and isobars

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Success isn't a result of spontaneous combustion. You must set yourself on fire.

~ Arnold H. Glasow

ABSTRACT

In the early 19th century a ground-breaking discovery was made that linked a dietary deficiency of a fat-soluble vitamin with the childhood disease known as rickets. The vitamin was named vitamin D and extensive research regarding the physiological importance of this vitamin followed ever since. It is currently known that vitamin D plays an important role in maintaining the calcium and phosphate homeostasis in the human body. Less clear evidence states the medical importance of vitamin D in the prevention and cancer, autoimmune disease and diabetes. Current literature shows that vitamin D has five distinct forms, vitamin D₁ to D₅, of which vitamin D₂ and D₃ are the most studied forms. The term “vitamin D” is often wrongfully used to include the vitamin D mother molecule, the vitamin D status indicator (25(OH)D), the biologically active form (1,25(OH)₂D) and biologically inactive form (24,25(OH)₂D). The interest for measurement of these vitamin D analogues is a continuously growing field both on individual and epidemiological level. For decades laboratories have struggled to produce a robust method capable of quantifying these different vitamin D analogues and uncovered a new form of complexity regarding the analysis of these analogues. The identification of the C3-epimeric forms of vitamin D metabolites has forced laboratories to rethink their analytical methods and several concerns were raised regarding the overestimation of the true vitamin D status by current analytical methods. The quantification of the biologically active and inactive forms of vitamin D is reported to be difficult and to date very few LC-MS/MS methods reported in the literature are able to quantify various vitamin D analogues. However, to our knowledge none of these methods are able to include the precursor vitamin D, the 25-hydroxylated metabolites, the biologically active and inactive metabolites, C3-epimers and isobaric compounds in a single run.

Therefore the aim of this study was to develop, optimise and validate a LC-MS/MS method for the quantification of twelve vitamin D analogues in a single run. This was done by optimising the underlying LC-MS/MS parameters to ensure optimal analytical sensitivity in positive ESI mode and sufficient chromatographic separation between analytes with similar chemical properties. Furthermore, the optimised method was validated to ensure the accuracy and precision of the method before implementation into a clinical environment. The vitamin D analogues included in this study were vitamin D₂, vitamin D₃, 25(OH)D₂, 25(OH)D₃, 1,25(OH)₂D₂, 1,25(OH)₂D₃, 24,25(OH)₂D₂, 24,25(OH)₂D₃, 3-epi-25(OH)D₂, 3-epi-25(OH)D₃, 7(OH)4C₃ and 1 α (OH)D₃.

A double liquid-liquid extraction with hexane and ethyl acetate were found to be the most efficient at extracting the vitamin D analogues from a serum matrix after matrix modification

with sodium hydroxide. Recoveries of > 95 % (CV <10 %) were achieved for all the analytes. It was noted that a precursor adduct other than the molecular mass ion for a specific vitamin D analogue can produce a more abundant MS1 signal and that the ESI source parameters vary between analytes with different chemical properties and should therefore be optimised individually for each analyte. Various columns were assessed and sufficient chromatographic separation between the relevant analytes was achieved with an Agilent Technologies Pentafluorophenyl column. Baseline separation was achieved between 25(OH)D₃ and 3-epi-25(OH)D₃ as well as 25(OH)D₂ and 3-epi-25(OH)D₂, which is a requirement for this method to be viable. The method was subjected to a series of validation steps to ensure the accuracy and precision of the method. These included the assessment of the analytical range, LOD, LOQ, inaccuracy, imprecision, stability, interference and recovery. It was found that the optimised method had good linearity ($r > 0.995$), acceptable repeatability (CV < 10 %) and within-lab precision (CV < 15%) and excellent method accuracy (systematic error < 6.60 %). Furthermore, all the analytes proved to be stable for 48 hours after sample preparation with no interferences found for co-eluting analytes. Finally, based on the sigma metric scale specifications, it was calculated that this method proved to be “world class” and very little QC is needed to ensure the quality of the data derived from this method.

Based on the findings in this study, it was concluded that a novel LC-MS/MS method for the quantification of twelve vitamin D analogues in a single run was successfully developed. All the LC-MS/MS parameters were optimised to ensure optimal analytical sensitivity for each analyte and the method was validated based on a series of method validation steps required for implementation into a clinical laboratory. This validation proved this method to be ready for implementation into a clinical environment.

Keywords: Vitamin D, LC-MS/MS optimisation, LC-MS/MS method validation

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ABBREVIATIONS

SYMBOLS

α	-	alpha
$^{\circ}\text{C}$	-	degree Celsius
%	-	percentage
b	-	slope

MEASURING UNIT

g/mol	-	gram per mole
h	-	hour(s)
IU	-	international units
L/min	-	litre per minute
M	-	molar
m/z	-	mass-to-charge ratio
mg	-	milligram
mg/L	-	milligram per litre
min	-	minute(s)
mm	-	millimetre
mM	-	millmolar
mmol/L	-	millimole per litre
ng/ml	-	nanogram per millilitre
pg/ml	-	picogram per millilitre
psi	-	pounds per square inch
rpm	-	revolutions per minute
S/N	-	signal-to-noise ratio
μg	-	microgram
μl	-	microlitre
μm	-	micrometre
V	-	volt

ABBREVIATIONS

25(OH)D ₂	-	25-hydroxyvitamin D ₂
25(OH)D ₃	-	25-hydroxyvitamin D ₃
25(OH)D	-	collectively 25(OH)D ₂ and 25(OH)D ₃
3-epi-25(OH)D ₂	-	25-hydroxyvitamin D ₂ C-3 epimer
3-epi-25(OH)D ₃	-	25-hydroxyvitamin D ₃ C-3 epimer
3-epi-25(OH)D	-	collectively 3-epi-25(OH)D ₂ and 3-epi-25(OH)D ₃
1,25(OH) ₂ D ₂	-	1,25-dihydroxyvitamin D ₂
1,25(OH) ₂ D ₃	-	1,25-dihydroxyvitamin D ₃
1,25(OH) ₂ D	-	collectively 1,25(OH) ₂ D ₂ and 1,25(OH) ₂ D ₃
24,25(OH) ₂ D ₂	-	24,25-dihydroxyvitamin D ₂
24,25(OH) ₂ D ₃	-	24,25-dihydroxyvitamin D ₃
24,24(OH) ₂ D	-	collectively 24,25(OH) ₂ D ₂ and 24,25(OH) ₂ D ₃
7(OH)4C3	-	7 α -hydroxy-4-cholesten-3-one
1 α (OH)D ₃	-	1 α -hydroxyvitamin D ₃

AJS	-	Agilent Jet Stream
ALTM	-	All laboratory trimmed mean
APCI	-	Atmospheric pressure chemical ionisation
APPI	-	Atmospheric pressure photoionisation
CI	-	Confidence interval
CLIA	-	Chemiluminescent immunoassay
CN	-	Cyano
CV	-	Coefficient of variance
CVD	-	Cardiovascular disease
DBP	-	Vitamin D binding protein
DEQAS	-	Vitamin D External Quality Assessment Scheme
EIA	-	Enzyme immunoassay
EQA	-	External quality assessment
ESI	-	Electrospray ionisation
FIA	-	Flow injection analysis
HPLC	-	High-performance liquid chromatography
IOM	-	Institute of Medicine
IS	-	Internal standard

LC-ESI-MS/MS	-	Liquid chromatography - electrospray ionisation - tandem mass spectrometry
LC-MS/MS	-	Liquid chromatography-tandem mass spectrometry
LC-QQQ	-	Liquid chromatography – triple quadrupole
LLE	-	Liquid-liquid extraction
LOD	-	Limit of detection
LOQ	-	Limit of quantification
MDC	-	Medical decision concentration
MMI	-	Multimode ionisation
MRM	-	Multiple reaction monitoring
MS	-	Mass spectrometer
NaCl	-	Sodium chloride
NaOH	-	Sodium hydroxide
NHANES	-	National Health and Nutrition Survey
NIST	-	National Institute of Standards and Technology
PFP	-	Pentafluorophenyl
PPE	-	Protein precipitation extraction
PTH	-	Parathyroid hormone
RAS	-	Renin-angiotensin system
RIA	-	Radio immunoassay
RMP	-	Reference measurement procedure
SD	-	Standard deviation
SIM	-	Selective ion monitoring
SOP	-	Standard operating procedure
SPE	-	Solid phase extraction
TE _a	-	Total error allowable
TIC	-	Total ion chromatogram
UV	-	Ultra violet light
VDR	-	Vitamin D receptor

CHAPTER 1

INTRODUCTION

1.1 BACKGROUND AND MOTIVATION

According to the Third National Health and Nutrition Survey (NHANES 3) it was estimated that more than 1 billion people worldwide are vitamin D deficient (Makariou *et al.*, 2011; Looker *et al.*, 2002). Current evidence shows that vitamin D has a supporting role in maintaining the calcium homeostasis thus preventing rickets or osteomalacia. Furthermore, the beneficial effect of vitamin D on the inhibition of autoimmune diseases, regulation of immune function, the maintenance of anti-proliferative effect on the skin and the inhibition of cancer progression has also been investigated but is less clear (Bailey *et al.*, 2013). Van den Ouweland (2013) stated that the interest for characterisation of the vitamin D status is continuously growing both on individual and epidemiological level.

For decades laboratories have struggled to produce a robust method capable of quantifying different vitamin D metabolites, especially 25-hydroxyvitamin D₃ (25(OH)D₃), which is an indication of an individual's vitamin D status. Further progress regarding the quantification of this metabolite uncovered a new form of complexity regarding the analysis (Bailey *et al.*, 2013). The identification of the C3-epimeric forms of vitamin D metabolites has forced laboratories to rethink their analytical methods. These C3-epimers have identical molecular structures as the relevant analytes, with a stereochemical difference at one of the hydroxyl sites as the only exception (Lensmeyer *et al.*, 2012). Bailey and co-workers (2013) reported the findings of a survey done on participants of the Vitamin D External Quality Assessment Scheme (DEQAS) in 2011. The survey included 1066 international laboratories and 14 different commercial methods for the quantification of vitamin D metabolites. Of the 14 methods, only some of the chromatographic ligand binding assays and liquid chromatography - tandem mass spectrometry (LC-MS/MS) methods were able to distinguish between 25(OH)D₂, 25(OH)D₃ (collectively 25(OH)D) and relevant C3-epimers. The authors also stated that many epidemiological studies have been based on radioimmunoassays (RIA), which were at the time not able to distinguish between the epimers and the 25(OH)D analogues. This contributed to a positive bias. Furthermore, Shah and co-workers (2011)

raised concerns regarding the co-elution of the 25(OH)D analogues and C3-epimers during the analysis with LC-MS/MS. This was supported by Couchman and co-workers (2012), the authors stated that in 2010 only 5 % of all the DEQAS participating laboratories that implemented LC-MS/MS claimed distinguishability between 25(OH)D analogues and relevant epimers. Furthermore, van den Ouweland & Vogeser (2013) and El-khoury *et al.* (2011) stated that isobaric compounds, 7 α -hydroxy-4-cholesten-3-one (7(OH)4C3) and 1 α -hydroxyvitamin D₃ (1 α (OH)D₃), can also cause interferences regarding the analysis of 25(OH)D with LC-MS/MS. Isobaric compounds have similar nominal mass but different molecular formulas. 1 α ,25-Dihydroxyvitamin D₂ (1 α ,25(OH)₂D₂) and 1 α ,25(OH)₂D₃ (collectively 1,25(OH)₂D) are the biologically active metabolites of vitamin D and are produced from the 1 α -hydroxylation of 25(OH)D. Due to the short half life time of 1,25(OH)₂D, 4 to 8 hours, compared to approximately 2 to 3 weeks of 25(OH)D in serum, it is clinically not useful to measure 1,25(OH)₂D as the vitamin D status indicator (Holick *et al.*, 2011). But the quantification of 1,25(OH)₂D can provide useful information regarding acquired and inherited disorders in the metabolism of 25(OH)D and phosphate. This includes disorders like chronic kidney disorders, sarcoidosis, rickets etc. (Holick *et al.*, 2011). Furthermore, in a research environment, the quantification of 1,25(OH)₂D can provide critical information regarding therapy with vitamin D active metabolites (van den Ouweland & Vogeser, 2013) and therefore measurability. The biologically inactive metabolites of vitamin D, 24R,25(OH)₂D₂ and 24R,25(OH)₂D₃ (collectively 24,25(OH)₂D) are the major circulating dihydroxyvitamin metabolites and is formed from the precursor 25(OH)D via C24-hydroxylation. Van den Ouweland & Vogeser (2013) also stated that the measurement of 24,25(OH)₂D in conjunction with 25(OH)D can be used as a novel marker regarding 25(OH)D catabolism as well as a predictor of serum 25(OH)D response to vitamin supplementation. Furthermore, it was found that 24,25(OH)₂D can cross react with immunoassays measuring 25(OH)D and the co-elution of 1,25(OH)₂D and 24,25(OH)₂D analogues when measured with LC-MS/MS, can result in misclassification of the true value of these analytes. These are some of the main reasons for the measurement of the biologically inactive metabolites of 25(OH)D.

Now, the following two questions arise: 1. The first question is: Why implement an LC-MS/MS method when immunoassays can achieve a higher throughput less expensively?

To date the LC-MS/MS assay is considered the “gold standard” for the quantification of vitamin D analogues and within the last decade clinical laboratories’ opinion of this assay changed from it being a labour intensive, expensive and complicated assay to a simple,

robust and highly automated assay achieving greater sensitivity and greater specificity with high throughput (El-Khoury *et al.*, 2011). Furthermore, as evidence showed, commercial immunoassays cannot distinguish between the D₂ and D₃ forms of the vitamin D analogues, the C3-epimers and 25(OH)D analogues and the dihydroxylated vitamin D analogues. Several concerns were raised by Bailey and co-workers (2013) regarding the importance of distinguishability between vitamin D analogues when quantified by means of LC-MS/MS. According to Shah and co-workers (2012) the LC-MS/MS assay is able to distinguish between 25(OH)D₂, 25(OH)D₃, 24,25(OH)₂D₂, 24,25(OH)₂D₃, 1,25(OH)₂D₂, 1,25(OH)₂D₃, isobaric forms as well as epimers of 25(OH)D. Although this has not been achieved in a single run, the authors came very close to achieving this as the ultimate goal.

The second question is 2: Why measure the precursor vitamin D, the 25(OH)D analogues, the biologically active metabolites (1,25(OH)₂D), the biologically inactive metabolites (24,25(OH)₂D), the 25(OH)D epimers and 25(OH)D isobars, when it is known that the 25(OH)D analogues are the best indicator of the vitamin D status ?

Previous evidence shows that the measurement of the 1,25(OH)₂D and 24,25(OH)₂D analogues can provide critical clinical information in a research environment as well as the diagnosis and monitoring of acquired and inherited disorders of vitamin D and phosphate metabolism. Furthermore, based on the concerns raised in respect of the analytical methods, it was shown that it is necessary to include the C3-epimeric and isobaric analogues of 25(OH)D in the analytical run. Sufficient chromatographic separation is also required to ensure that no misclassification of the true vitamin D status can occur due to interferences from these compounds. Lastly, although the D₂ form of vitamin D can only be acquired from the diet and supplementation, the D₂ and D₃ forms undergo identical metabolic transformations in the human body and the quantification of the D₂ form would thus be required to provide the true total value of all the analogues. It is also necessary to include the D₂ form of all the analogues to provide clinical information regarding therapy of specific metabolic disorders when treated with D₂ supplementation.

With the above motivation in mind, the following research questions will be addressed in this study:

1. Can an in-house liquid chromatography-electrospray ionisation-tandem mass spectrometry (LC-ESI-MS/MS) method be developed for the quantification of twelve vitamin D analogues in a single run?
2. Can this method be optimised to ensure the best achievable analytical sensitivity and validated to ensure claimed accuracy and precision?

1.2 RESEARCH AIM AND OBJECTIVES

The aim of this study was the implementation of an LC-QQQ (LC-MS/MS) method for the quantification of vitamin D analogues accounting for interferences from epimers and isobars.

The specific objectives were:

- The optimisation of the underlying LC-MS/MS parameters for optimum analytical sensitivity and sufficient chromatographic separation of relevant analytes;
- Validation of the optimised method through a series of method validation steps to determine the amount of error present within results derived from this assay;
- The calculation of the assay quality control specifications to prevent misdiagnosis in a clinical environment.

1.3 STRUCTURE OF DISSERTATION

This dissertation is a compilation of chapters written specifically to comply with the requirements of the North-West University, Potchefstroom Campus, for the completion of the Masters study (Biochemistry) in dissertation format.

The current chapter, Chapter 1, gives a brief overview of the background and problem statement which includes the motivation for this study. The aim of the study and the objectives for the achievement of the aim are summarised. A description of the layout of the dissertation is included.

Chapter 2 summarises the biochemistry and physiology of vitamin D analogues as well as the clinical significance of these analogues. The procedures and performance of different methods currently used for the quantification of vitamin D analogues are summarised. Furthermore, the analytical function of the underlying LC-ESI-MS/MS parameters are

explained, followed by an overview of the method validation steps and the experimental outline.

Chapter 3 contains the materials and methods used for this study.

In Chapter 4, the results obtained from the optimisation of the LC-MS/MS conditions are shown and discussed. This chapter concludes with a summary of the optimised method.

Chapter 5 shows the results obtained from a series of validation assessments. These results are discussed and the chapter concludes with a method decision chart and quality control specification for the method.

Chapter 6 is a comprehensive conclusion of the results obtained from Chapters 4 and 5. Furthermore, recommendations and future prospects for follow-up studies are discussed.

A detailed standard operating procedure (SOP) and work instruction of the optimised and validated method are attached as an addendum.

CHAPTER 2

LITERATURE REVIEW

BIOCHEMISTRY AND PHYSIOLOGY OF VITAMIN D

2.1 BACKGROUND

In the early 1920's a discovery was made that linked a dietary deficiency of a fat-soluble vitamin with the childhood disease known as rickets (McCollum *et al.*, 1922). McCollum and co-workers further discovered that the vitamin in question was distinct from vitamin A, as initially believed, and declared it as vitamin D (calciferol). However, it was not until 1936 that the true chemical structures of vitamin D₂ and vitamin D₃ were established by Windaus and Thiele (Wolf, 2004). The term 'vitamin D' refers to a subgroup of secosteroid compounds of which the 9,10 carbon-carbon bond of ring B is broken and these secosteroids are a group of tetracyclic steroid derivatives. Although the term 'vitamin D' is internationally used, it is often interpreted wrongly to incorporate vitamin D metabolites like 25-hydroxyvitamin D (25(OH)D) and the biologically active- and inactive forms of vitamin D (Couchman *et al.*, 2012). Figure 2.1 illustrates the chemical backbone of vitamin D compounds. The chemical structure is based on four steroid rings, one of which is open (Battersby *et al.*, 2012).

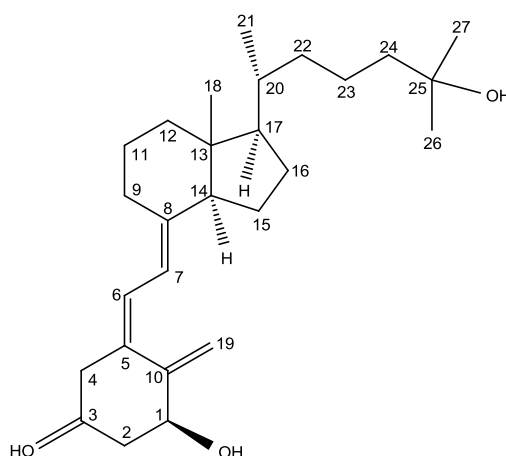


Figure 2.1 Vitamin D chemical backbone. (Adapted from Jones *et al.*, 1998) This illustration shows the chemical backbone of vitamin D₃.

2.2 MAJOR FORMS AND SOURCES OF VITAMIN D

Vitamin D has five distinct forms, D₁ to D₅, which differs in chemical composition. Their chemical compositions are described in the following way: vitamin D₁: combination of ergocalciferol and lumisterol; vitamin D₂: ergocalciferol; vitamin D₃: cholecalciferol; vitamin D₄: dihydroergocalciferol, which is vitamin D₂ without a double bond between carbon 22 and 23; and lastly vitamin D₅: sitocalciferol, which is made from 7-dehydrositosterol (Vanga *et al.*, 2010). The two major and most studied forms are known as ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). As illustrated in Figure 2.2, ergocalciferol differs from cholecalciferol only in the side chain of the chemical structure. Ergocalciferol has a double bond between carbons 22 and 23 as well as a methyl group on carbon 24 (Endries & Rude, 2006:). Ergocalciferol is naturally found mainly in yeast and fungi (Calvo *et al.*, 2013). It is produced from the plant steroid ergosterol, by the exposure to ultraviolet B (UVB) light that activates the reaction of UV irradiation of the ergosterol to ergocalciferol (Jasinghe & Perera, 2005) and consumed by humans through daily diet or vitamin D supplements. As mentioned before, the chemical structure of ergocalciferol differs from the chemical structure of cholecalciferol, but it is still believed that they have similar biological activity in the human body (Jasinghe & Perera, 2005). It has also been reported that ergocalciferol and cholecalciferol has identical responses in the body concerning vitamin D deficiency related diseases (Jurutka *et al.*, 2001).

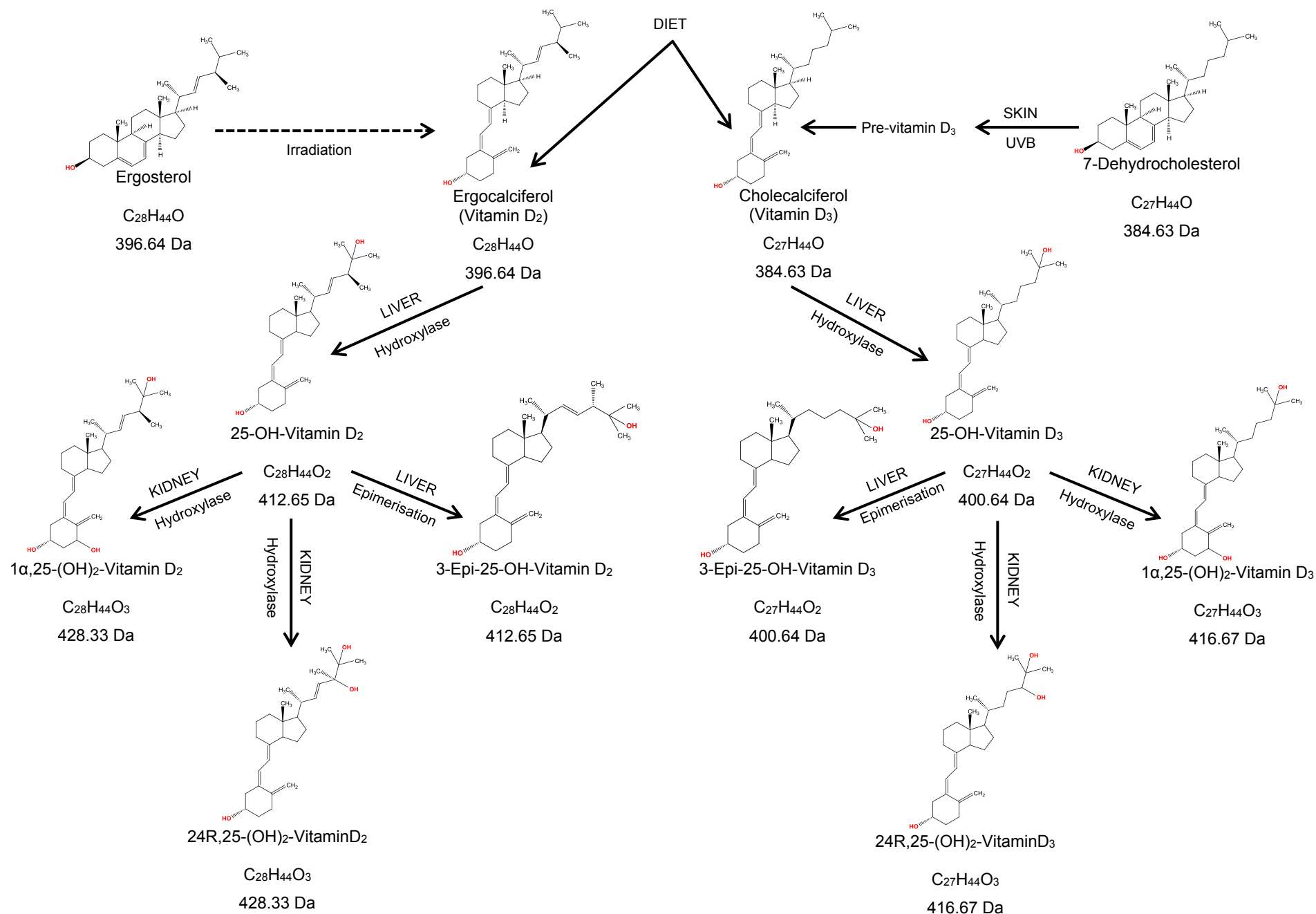


Figure 2.2 Summary of the major metabolic transformations of vitamin D₂ and vitamin D₃ (Adapted from Horst *et al.*, 2005; Endries & Rude, 2006). Both ergocalciferol and cholecalciferol is subject to the same metabolic transformations in human metabolism. 1α,25-dihydroxyvitamin D is considered the biologically active form of vitamin D while 24R,25-dihydroxyvitamin D is considered the inactive form. Solid lines indicate human metabolic transformations and dashed lines indicate plant, yeast and fungi metabolic transformations.

Cholecalciferol is considered the parent compound of the naturally present form of vitamin D in humans and is also regarded the more prominent source between the two major forms contributing up to 80 % of the total vitamin D in humans (Vanga *et al.*, 2010). The immediate precursor of cholecalciferol and cholesterol is 7-dehydrocholesterol present in the skin epidermis of mammals and humans (Horst *et al.*, 2005). When UVB light (290 nm - 320 nm) strikes the skin of humans and mammals the 5-7-diene in ring B of 7-dehydrocholesterol is rearranged, which leads to ring breakage to form previtamin D₃. The previtamin D₃ is a thermodynamically unstable compound and is rearranged to cholecalciferol (vitamin D₃) (Maunsell *et al.*, 2005). Cholecalciferol is thus considered a prosteroid hormone rather than a vitamin. Although only certain foods like fish liver oils, egg yolks, fatty fish and liver naturally contain adequate amounts of vitamin D₃ it is believed that a fair-skinned human can produce up to 20 000 IU or 500 µg vitamin D₃ when sunbathing for 30 minutes (Cannell & Hollis, 2008). Excessive or toxic amounts of cholecalciferol are not the product of prolonged sun exposure; previtamin D₃ can give rise to other non-vitamin D forms like lumisterol and tachysterol through thermal activation which limits the formation of cholecalciferol (Webb *et al.*, 1989: cited by Ross *et al.*, 2011). Several factors have an influence on the production of vitamin D₃ in the skin; these factors include latitude, seasonal change, aging, sunscreen use, skin pigmentation, etc. (Hollis, 2005).

2.3 VITAMIN D METABOLISM

A summary of the metabolic transformations of cholecalciferol and ergocalciferol is illustrated in Figure 2.2. Although the chemical structures of vitamin D₂ and D₃ differ, they follow the same metabolic transformations in humans. Vitamin D₃ is primarily produced from the precursor 7-dehydrocholesterol in the human skin but can also be, like vitamin D₂, consumed through the daily diet and supplements.

Dietary vitamin D (both ergocalciferol and cholecalciferol) has a fat soluble nature and can be absorbed in the small intestine with other dietary fats (Haddad *et al.*, 1993). For the absorption to be most efficient, fat in the intestinal lumen is required to trigger the release of bile acids and pancreatic lipase. At this stage the bile acids are initiating the emulsification of the lipids and the pancreatic lipase hydrolyses the triglycerides into monoglycerides and free fatty acids (Weber, 1983: cited by Ross *et al.*, 2011). Vitamin D, cholesterol and other lipids are packed together into chylomicrons within the intestinal wall. These chylomicrons reach circulation via the lymphatic system and are metabolised in the peripheral tissues that express lipoprotein lipase; especially fat tissue and skeletal muscle which express vast

amounts of this enzyme. The main pathway of vitamin D uptake is incorporation into chylomicrons that reach the systematic circulation via the lymphatics (Ross *et al.*, 2011). Furthermore, vitamin D analogues are bound to the vitamin D-binding protein (DBP) and circulates in the blood. This binding protein is a circulating glycoprotein to which vitamin D, 25(OH)D and 1 α ,25(OH)₂D are bound, allowing their translocation into general circulation (Vanga *et al.*, 2010). The DBP is a high-affinity transport protein that contains 458 amino acid residues. Synthesised by the liver, DBP circulates in excessiveness around 400 mg/L with less than 5% of the DBP binding sites normally occupied. Vitamin D and its metabolites are bound with the following preference; 25-hydroxyvitamin D > 1 α ,25-hydroxyvitamin D >> vitamin D (Endries & Rude, 2006).

Vitamin D undergoes two separate hydroxylation reactions after being released from the DBP into the liver; the first hydroxylation reaction is a hepatic 25-hydroxylation reaction by which vitamin D (both cholecalciferol and ergocalciferol) is hydroxylated at the carbon molecule in the 25 position to 25(OH)D. This reaction is catalysed by the cytochrome P450 enzyme, vitamin D-25-hydroxylase (CYP2R1) (Muszkat *et al.*, 2010). Although serum 25(OH)D is the major circulating metabolite of vitamin D it is considered biologically inactive. Circulating 25(OH)D has a half-life time of 2 to 3 weeks (Endries & Rude, 2006: 1891-1965).

The second hydroxylation reaction is a renal 1- α -hydroxylation reaction by which the cytochrome P450 enzyme, 25-hydroxyvitamin D-1- α -hydroxylase (CYP27B1), hydroxylates 25(OH)D to 1 α ,25(OH)₂D at the first carbon molecule position. The 1 α ,25(OH)₂D metabolite is considered to be the biologically active metabolite of vitamin D and has a half-life time of 4 to 6 hours (Endries & Rude, 2006). With the formation of the biologically active metabolite completed, it now enters general blood circulation to act in distant organs and cells in a hormone-like manner. This active metabolite binds to the vitamin D receptor (VDR) which is then activated and finally has a wide range of physiological implications on the target cells via the nuclear receptors in the cells (Battault *et al.*, 2013). The 1 α ,25(OH)₂D also has a significant role in back regulation by inducing the enzyme 25-hydroxyvitamin D-24-hydroxylase (CYP24A1). This enzyme hydroxylates 25(OH)D to 24R,25-dihydroxyvitamin D (24,25(OH)₂D) at the 24 carbon molecule position. This is the most prevailing dihydroxylated vitamin D metabolite in the serum and is considered to be biologically inactive (Jones *et al.*, 2012). The prevalence of this metabolite reduces the formation of the biologically active form, 1 α ,25(OH)₂D, because less 1 α ,25(OH)₂D precursor, 25(OH)D, is available for 1- α -hydroxylation. The 25-hydroxyvitamin D-24-hydroxylase enzyme also has the ability to metabolise 1 α ,25(OH)₂D and 24,25(OH)₂D to 1 α ,24,25(OH)₃D which is excreted through the

bile into the faeces and very small amounts into the urine (Jones *et al.*, 1998). Jones and his co-workers (2012) also state that the CYP24A1 enzyme is able to catalyse multiple hydroxylation reactions of the carbon molecule on position 24 of both 25(OH)D and 1 α ,25(OH)₂D.

In recent studies, Kamao and his co-workers (2004) discovered and identified a new metabolite involved in vitamin D metabolism. It has been established that vitamin D can alternatively be metabolised through a C-3 epimerisation pathway that yield the metabolite known as 3-epi-25-hydroxyvitamin D (3-epi-25(OH)D). The epimerisation of 25(OH)D is through the conversion of the hydroxyl group, at the C-3 carbon position of the A-ring, from the alpha orientation to the beta orientation (Bailey *et al.*, 2013). The epimerisation pathway occurs in the liver and has a low affinity for the vitamin D receptor but has a high affinity for the DBP. This suggests that the 3-epi-25(OH)D has a longer half-life time than most of the other vitamin D metabolites. Epimerisation seems to be common for all the major metabolites of vitamin D and the exact physiological role of these C-3 epimerised metabolites are largely unknown. However, the 3-epi-25(OH)D metabolite is thought to be the most prevailing of all the 3-epi metabolites (Bailey *et al.*, 2013).

2.4 CLINICAL SIGNIFICANCE OF VITAMIN D

2.4.1 VITAMIN D STATUS

The vitamin D status of an individual is determined through the quantification of total serum 25(OH)D, including 25(OH)D₂ and 25(OH)D₃. As mentioned in the previous section, 25(OH)D is the most abundant circulating serum vitamin D metabolite and thus a good indicator of an individual's vitamin D status.

To date there are no definite reference values to determine an individual's vitamin D status due to the controversy between experts regarding the adequate amounts of vitamin D required per individual (Ong *et al.*, 2012). The most commonly referenced values are those of the Institute of Medicine (IOM) (Ross *et al.*, 2011: 75-124). They state that an individual can be classed as either vitamin D sufficient, insufficient, deficient or toxic based on the total serum 25(OH)D of the individual. The following table summarises the most commonly used reference values to which an individuals' vitamin D status is classified.

Table 2.1 Reference values for total serum 25(OH)D.

Vitamin D status	Total 25(OH)D	
	(nmol/L)	(ng/ml)
Deficiency	< 30	< 12
Insufficiency	30 - 50	12 - 20
Sufficiency	50 - 125	20 - 50
Toxic	> 125	> 50

This table indicates the classification of an individual's vitamin D status according to the reference values stated by the Institute of Medicine (2011) (Ross *et al.*, 2011). Reference values indicated as nano-mole per litre (nmol/L) and nano-gram per millilitre (ng/ml).

As indicated in Table 2.1, an individual is referred to as vitamin D deficient if the total circulating serum 25(OH)D is below 30 nmol/L. It is estimated that 1 billion people worldwide have a vitamin D deficiency or insufficiency (Makariou *et al.*, 2011) and the NHANES 3 concluded 25 – 57 % of adults in the United States adults to be vitamin D deficient (Looker *et al.*, 2002). It is also believed that 40 – 100 % of elderly people in the United States and Europe are vitamin D deficient; this is believed to be due to the decreased concentration of 7-dehydrocholesterol in the skin and in part to alterations in skin morphology associated with ageing. Obesity is another high risk factor associated with vitamin D deficiency; the fat cells of obese people isolate vitamin D and obese people are usually more inactive and have less outdoor activity (Michos *et al.*, 2010). Ethnicity resulting in light or dark skin pigmentation also has an influence on the vitamin D status; individuals with darker skin are more frequently vitamin D deficient since the melanin skin pigmentation absorbs the UVB light resulting in reduced vitamin D synthesis (Martins *et al.*, 2007). Pregnant and lactating women as well as their infants are potentially at risk for vitamin D deficiency. Women who exclusively breast feed their infants are at higher risk of vitamin D deficiency due to the fact that vitamin D sources of the mother are exhausted by the infant through the breast milk; this also puts the breast-fed infant at risk of a vitamin D deficiency (Horst *et al.*, 2005).

Contrary to a vitamin D deficiency, an individual can also become vitamin D toxic. An individual is classified as vitamin D intoxicated if the total serum 25(OH)D exceeds 125 nmol/L. The state of vitamin D intoxication is also known as hypervitaminosis D; this is extremely rare and is the result of excessive intake of vitamin D (Vanga *et al.*, 2010). This state is not due to prolonged sun exposure because previtamin D can give rise to other non-vitamin D forms like lumisterol and tachysterol through thermal activation which limits the formation of vitamin D (Webb *et al.*, 1989: cited by Ross *et al.*, 2011: 75-124). Makariou and

co-workers (2011) reported that an individual can become vitamin D intoxicated if a daily intake of 40 000 IU is exceeded. It has been reported that hypervitaminosis D can lead to hypercalcemia which eventually leads to the calcification of soft tissue resulting in renal and cardiovascular damage (DeLuca, 1974: cited by Ross *et al.*, 2011: 75-124).

2.4.2 CALCIUM AND PHOSPHATE HOMEOSTASIS

It is known that the interactions between dietary calcium intake and renal metabolism of vitamin D plays a key role in maintaining plasma calcium homeostasis (Anderson *et al.*, 2012). Strong evidence prove that a vitamin D deficiency can give rise to bone diseases like rickets, in children, and osteomalacia and osteoporosis, in adults due to a delay in bone mineralisation (Anderson *et al.*, 2012). Further evidence suggests that osteoporosis and the risk of bone fractures can be decreased by treating patients with low 25(OH)D serum levels with vitamin D supplementation (Holick, 2004). In a case of vitamin D deficiency, PTH levels rise and causes further bone demineralisation, leading to osteoporosis and greater susceptibility to fractures (Anderson *et al.*, 2012).

The interest for the measurement of the active vitamin D metabolite, 1,25(OH)₂D, has rapidly increased over the past decade. A review by Lips (2007) states that although the measurement of 25(OH)D is considered more important to indicate a patients vitamin D status, the measurement of 1,25(OH)₂D can reveal critical information regarding errors in the vitamin D metabolic pathway. Lips (2007) states that 1,25(OH)₂D should be measured in case of possible disorders in the 1-OH-hydroxylation pathway in the kidney which is common for chronic renal failure, vitamin D-dependent rickets type 1 and hypophosphatemic rickets (Jacobs & Smith, 1979). Furthermore, in a case of vitamin D-dependent rickets type 2, 1,25(OH)₂D levels will be highly elevated due to an inborn error in the vitamin D receptor pathway.

The most abundant circulating 25(OH)D metabolite, 24,25(OH)₂D, is known as the biologically inactive metabolite of vitamin D. Currently not many applications measure this metabolite but its rapidly starting to become a metabolite of interest. To date, no clear evidence states why the biologically inactive form should be measured but, Bosworth and co-workers (2012) provide evidence for the measurement and correlation between 24,25(OH)₂D and chronic kidney disease. The authors also states that 24,25(OH)₂D is formed from 25(OH)D catabolism by CYP24A1 enzyme, while in the kidney CYP24A1 transcription is induced by fibroblast growth factor-23 (FGF-23) and suppressed by PTH.

Therefore, a possibility for the correlation of 25(OH), 24,25(OH)₂D, FGF-23 and PTH in chronic kidney disease patients still exists and the measurement of 24,25(OH)₂D can provide valuable information regarding the diagnosis, disease monitoring and therapy monitoring (Bosworth *et al.*, 2012).

2.4.3 VITAMIN D DEFICIENCY AND DISEASE ASSOCIATION

CANCER

It has been reported that vitamin D can be related to cancers like breast cancer, colon cancer and prostate cancer. An inverse relationship between the incidence of breast cancer and the amount of sun exposure has been shown through epidemiologic studies (Nagpal *et al.*, 2005). Chen and co-workers (2008) found in a meta-analysis of vitamin D and breast cancer that a higher concentration of circulating 25(OH)D lead to a decrease of 45 % in the risk of breast cancer. It is believed that VDR proteins are expressed in the mammary tissue and breast cancer cells; this enables them to be potential target sites for the biologically active form of vitamin D (Nagpal *et al.*, 2005). With regards to colon cancer; increased VDR protein is found in colon tumours. This supports the epidemiological studies that have suggested an inverse association between vitamin D circulating levels and the incidence of colon cancer (Nagpal *et al.*, 2005). It has also been reported that an intake of 2000 IU vitamin D per day reduced the incidence of colorectal cancer in North America by 27 % (Muszkat *et al.*, 2010). Further studies by Nagpal and co-workers (2005) indicated that 1 α ,25(OH)₂D₃ is able to inhibit the proliferation of prostate cancer cell lines. Evidence shows 25(OH)D to play a significant role in the regulation of prostate cell proliferation, mainly through the VDR and 1 α -hydroxylation (Lou *et al.*, 2004). To conclude, vitamin D poses the potential to decrease the risk of certain cancers, thus a vitamin D deficiency is thought to have the opposite effect regarding cancers.

DIABETES

Diabetes mellitus type 1 is also known as insulin-dependent diabetes and is the result of the body's failure to produce insulin. This is due to autoimmune destruction of the beta cells in the islets of the pancreas that produce insulin, thus leading to an insulin deficiency (Harris, 2005). Harris (2005) reported experiments that indicate the prevention of the development of type 1 diabetes in nonobese diabetic mice with the administration of pharmacologic doses of

the active form of vitamin D; $1\alpha,25(\text{OH})_2\text{D}$. It is believed that vitamin D acts as an immunosuppressive agent regarding type 1 diabetes which is thought to be an autoimmune disease. It is also believed that vitamin D thus inhibits the autoimmune reaction targeted at the beta cells of the pancreas (Hyppönen *et al.*, 2001). Hyppönen and co-workers (2001) also found in an epidemiological study that the risk of type 1 diabetes was significantly reduced through supplementation with vitamin D in infants.

Diabetes mellitus type 2 is characterised through insufficient insulin production of the body as well as resistance of body tissues to insulin. Type 2 diabetes is the most common form of diabetes worldwide and covers approximately 90 % of the total diabetes cases (Smushkin & Vella, 2010). Muscogiuri and co-workers (2012) suggested that an association between type 2 diabetes and vitamin D deficiency is easily explained by their common link to obesity. This is also supported by epidemiological evidence that suggest a direct relationship between these entities. Although fat cells isolate vitamin D, it has been proven that a direct association between decreased $25(\text{OH})\text{D}$ and type 2 diabetes still remains even when confounding factors like body mass index and physical activity are taken into account (Makariou *et al.*, 2011). A cross-sectional survey that included 5677 New Zealand individuals concluded that newly diagnosed type 2 diabetes patients had decreased $25(\text{OH})\text{D}$ serum concentrations (Scragg *et al.*, 1995; Cavalier *et al.*, 2011). This is in correlation with the NHANES 3 survey that concluded an inverse association between the vitamin D status and diabetes in non-Hispanic whites and Mexican-Americans (Scragg *et al.*, 2004; Cavalier *et al.*, 2011).

CARDIOVASCULAR DISEASE

It is reported that the risk of cardiovascular disease (CVD) is increased during winter months, which is characterised by decreased sunshine per day, and at higher latitudes (Zittermann *et al.*, 2005). This correlates directly with the prevalence of vitamin D deficiency which is higher during winter months and at higher latitudes. Evidence concerning CVD in correlation with vitamin D deficiency suggests that adequate levels of vitamin D are necessary for optimal cardiovascular health. Different underlying units of CVD like heart failure, stroke, heart attack, coronary artery disease, hypertension, cardiac arrhythmias and myocardial infarction are associated with a vitamin D deficiency and evidence is presented onwards. (Agarwal & Agarwal, 2012). A study reported by Giovannucci (2009) indicated that a vitamin D deficiency is associated with an increased risk of myocardial infarction. During this study the $25(\text{OH})\text{D}$ levels in 128 patients, admitted with ischemic heart disease, and 409

controls were measured. As mentioned before, vitamin D has a diverse effect on the cardiovascular health and it is believed that it has a great influence on blood pressure through the renin-angiotensin system (RAS) and PTH levels (Zittermann *et al.*, 2005). The renin-angiotensin system regulates electrolyte and volume homeostasis, thus increased RAS stimulation is related to higher blood pressure (Giovannucci, 2009). Animal studies indicated that the active metabolite of vitamin D, $1\alpha,25(\text{OH})_2\text{D}$, suppresses renin gene expression (Li *et al.*, 2004; Giovannucci, 2009) thus indicating that a vitamin D deficiency will lead to elevated renin production and finally increased blood pressure. Further evidence indicated that a vitamin D deficiency resulting in increased PTH levels are associated with many cardiovascular abnormalities. Hyperparathyroidism is associated with disturbances in the RAS, cardiac arrhythmias and functional abnormalities in the vascular wall (Bischoff *et al.*, 2006; Giovannucci, 2009).

ANALYTICAL PROCEDURES FOR THE MEASUREMENT OF VITAMIN D AND ITS METABOLITES

2.5 BACKGROUND

The vitamin D status of an individual is best determined through the quantification of the total circulating serum 25(OH)D. It is widely agreed that 25(OH)D is considered a “difficult” analyte due to method-related variability that is mostly because of the hydrophobic nature of the molecule and its high affinity for the DBP (Carter, 2012; Chen *et al.*, 2012). In the early 1970s, the first methods for measuring 25(OH)D that were based on competitive protein binding after solvent extraction, were described. Later that decade new methods for the measurement of 25(OH)D, which were based on high performance liquid chromatography (HPLC), became available. Furthermore, radioimmunoassays (RIA) were developed in 1985 and were based on incorporating a specific 25(OH)D antibody. Today, the radioactive labels of RIA are mostly replaced by labels employing chemiluminescent substances (CLIA) or enzymes immunoassays (EIA). Since the year 2000, liquid chromatography- tandem mass spectrometry (LC-MS/MS) has become a preferred method for the quantification of 25(OH)D (Wallace *et al.*, 2010). To date, it is still reported that significant variability between assays and laboratories for the quantification of 25(OH)D exist, thus these unanswered questions regarding the preferred assay of quantification needs to be addressed (Couchman *et al.*, 2012).

2.6 IMMUNOASSAY

Immunoassays are commercially available as kits and their main attraction is their high throughput and speed at relatively low cost which is ideal for large sample sizes in clinical laboratories (Schöttker *et al.*, 2012). Since the first use of a competitive binding protein assay for the measurement of 25(OH)D, assay improvement led to a consumer's choice of either a manual immunoassay or an automated immunoassay. Traditional manual immunoassays involve the extraction of 25(OH)D and possibly other hydroxylated vitamin D metabolites from serum or plasma with organic solvents, followed by the reconstitution into a suitable matrix and the quantification by means of an antibody and tracer (Heijboer *et al.*, 2012; Wallace *et al.*, 2010). Improved automated immunoassays are competitive CLIAs that

release the 25(OH)D from its binding proteins during an incubation period and quantify the 25(OH)D by means of a specific antibody, labelled tracer and measurement of relative light units (RLU).

2.6.1 ASSAY PROCEDURE

A recent review by Wallace and co-workers (2010) indicated that the three most widely used manual immunoassays at the time were the Diasorin RIA, Immunodiagnosics Systems (IDS) EIA and the IDS RIA. The laboratory procedure of both the before mentioned RIAs are very similar; both include an extraction step with organic solvents, incubation period and quantification using a 25(OH)D specific antibody and ¹²⁵I-labelled 25(OH)D tracer. The manual EIA incorporates a buffer reagent for the dissociation of 25(OH)D from its binding proteins and 25(OH)D labelled with biotin as diluent of the serum or plasma samples. The microtitre wells are coated with a 25(OH)D specific antibody. After a two hour incubation period an enzyme (horseradish peroxidase) labelled with avidin is added to the samples and binds specifically to biotin complexes. A colour reaction is induced by a chromogenic substrate and the quantification is through the measurement of the colour intensity being inversely proportional to the concentration of 25(OH)D (Wallace *et al.*, 2010). Apart from the concerns that will be discussed later on about the performance of these manual assays, the market indicated them to be too labour intensive and automated methods became desirable.

Wallace and co-workers (2010) reported the first automated assay for the measurement of 25(OH)D to be a chemiluminescent competitive protein binding assay. In 2004, Diasorin introduced their Liaison automated immunoassay but replaced it in 2007/2008 with a reformulation of the Liaison namely Liaison Total automated immunoassay. In 2008, Roche Diagnostics followed by introducing their Elecsys automated immunoassay and in early 2009, the IDS iSYS automated immunoassay became available (Wallace *et al.*, 2010). The before mentioned automated assays are the most widely used of their kind and all share the similarity of incorporating a competitive CLIA basis. The Diasorin Liaison and Liaison Total both incorporate the same 25(OH)D specific antibody coated onto the solid phase and vitamin D linked to an isoluminol derivative as the tracer. The procedure of the Liaison includes an incubation period during which the 25(OH)D is released from its binding proteins and competes with the labelled vitamin D for antibody binding. After a wash step, the flash chemiluminescent reaction is initiated and the light signal is measured which is inversely proportional to the concentration of the 25(OH)D present. The procedure of the Liaison Total is very similar to that of the Liaison assay and the main difference between these two CLIAs

is that the Liaison Total incorporates a two-step incubation period. During the first incubation period 25(OH)D is released from its binding proteins, followed by the addition of the tracer and a further shorter second incubation period. After a wash step, the flash chemiluminescent reaction is initiated and the light signal is measured which is inversely proportional to the concentration of the 25(OH)D present. The basis of the procedures of the Roche Elecsys CLIA and the IDS iSYS CLIA are outlined by Wallace and co-workers (2010) and will not be discussed here, but their performance will be assessed further on. In several papers concerns were raised about the performance of not only the automated immunoassays but also the manual immunoassays (Schöttker *et al.*, 2012; Carter *et al.*, 2004; Becker *et al.*, 2012) and will be discussed further on.

2.6.2 ASSAY PERFORMANCE

Since 1989, the international Vitamin D External Quality Assessment Scheme (DEQAS) have been monitoring the performance of 25(OH)D assays of more than 700 laboratories worldwide (Carter *et al.*, 2010). The DEQAS survey provides an indication of an assay performance based on a comparison of the assay precision to the all laboratory trimmed mean (ALTM). DEQAS data in 2004 reported the Diasorin RIA to have less than 1% bias from the ALTM. At the time, 60 % of the DEQAS returns were made up from Diasorin RIA users. The bias for the same assay increased to -5.4 % from 2004 to 2008 with an average between laboratory imprecision (coefficient of variance, CV %) of up to 20.5 % (Wallace *et al.*, 2010). According to DEQAS reports, the use of the Diasorin RIA decreased from 60 % of all users in 2001 to 7 % in 2009. In a recent study, the Diasorin RIA was compared to the automated Diasorin Liaison assay (Sarafin *et al.*, 2011). The inter- and intra-assay variation (CV %) for the RIA ranged from 1 % to 13.7 % and 8.1 % to 12.0 % respectively. Glendenning and co-workers (2006) reported that the Diasorin RIA underestimates the 25(OH)D₂ compared to HPLC. However, in a study reported by Hollis (2000) the Diasorin RIA recovered 91 - 100 % of both 25(OH)D₂ and 25(OH)D₃. Chen and co-workers (2008) compared the Diasorin to an LC-MS/MS method using a set of 554 plasma samples from the NHANES. The conclusion was made that the RIA gave lower values than the LC-MS/MS at low concentrations and higher values at high concentrations.

Wallace and co-workers (2010) reported the mean DEQAS bias recorded from 2004 to 2008 of the Diasorin Liaison automated immunoassay to range between -16.9 % and -7.9 % with an average between laboratory imprecision (CV %) of up to 21.6 %. Good recovery has been reported for the Liaison; 81 % for 25(OH)D₃ and 89 % for 25(OH)D₂ (Carter *et al.*,

2007). A study by de Koning and co-workers (2013) concluded that the Liaison assay had decreased specificity when the sample had a high concentration of 25(OH)D₂. The Diasorin Liaison Total automated immunoassay replaced the previous Liasison from 2007. It became a very popular method and was used in 2009 by approximately 36 % of all DEQAS participants. The mean DEQAS bias in 2008 for the Liaison Total was -9 % with an average between laboratory imprecision (CV %) of 15.5 %. In a comparative study by Sarafin and co-workers (2011) the inter- and intra-assay variation (CV %) for the Liaison Total ranged from 3.2 % to 8.01 % and 6.9 % to 12.7 % respectively.

In 2009, the IDS EIA accounted for 19 % of the DEQAS returns. The mean DEQAS bias for this assay recorded from 2004 to 2006 ranged between 5.7 % and 23 % with a CV % of up to 18.4 %. After re-calibrating the assay, in 2006 the mean bias were reduced significantly to 5 % (Wallace *et al.*, 2010). Carter and co-workers (2007) indicated very poor recovery of 25(OH)D₂ (56 %) and 25(OH)D₃ (79 %).

In 2008, Roche introduced their Elecsys automated competitive immunoassay that is based on streptavidin-biotin technology. In 2010 only 6 % of the DEQAS participants employed this method. The mean DEQAS bias for this assay recorded in 2008 was 7.6 nmol/L with a CV % of 16.7 % (Wallace *et al.*, 2010). Ong and co-workers (2012) reported that the Elecsys assay has 81 % cross reactivity for 25(OH)D₂ and 98 % for 25(OH)D₃ with a CV % ranging between 3.5 % and 11.5 % when patient samples are tested. The final remark on the Elecsys, based on a decision chart using total errors allowable (TEa) of 25 %, is that this assay shows unacceptable poor performance (Ong *et al.*, 2012).

In conclusion, immunoassays are unable to distinguish between 25(OH)D₂ and 25(OH)D₃ and only measures the total 25(OH)D. Although immunoassays are currently the most popular commercial assays (according to the DEQAS survey) for the measurement of total 25(OH)D the results acquired can vary by up to 20 % between assays and laboratories (Schöttker *et al.*, 2012). Another great concern with these commercial immunoassays are that some are unable to detect the C3-epimer (3-epi-25(OH)D₃) (Bailey *et al.*, 2013) and other hydroxylated vitamin D metabolites like the inactive form (24,25(OH)₂D) (Carter, 2012). Clive and co-workers (2002) indicated that specific immunoassays have 100 % cross-reactivity with the active forms of vitamin D, 1,25(OH)₂D₂ and 1,25(OH)₂D₃. The high cross-reactivity of the immunoassays for other hydroxylated metabolites and their inability to detect epimeric forms of vitamin D can lead to a misdiagnosis of the true vitamin D status of patients.

2.7 LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY

Currently, two non-immunological direct detection methods are available for the measurement of vitamin D and its metabolites. High performance liquid chromatography incorporates chromatographic separation coupled to a detector, e.g. UV detection or mass spectrometer (MS). Since the first LC-MS/MS assay for the measurement of 25(OH)D reported by Watson and co-workers in 1991 (Couchman *et al.*, 2012), improvements in the automation of sample preparation, the speed of the chromatographic steps and the sensitivity of the MS led to a highly sensitive and selective assay for the measurement of 25(OH)D (Couchman *et al.*, 2012; Wallace *et al.*, 2010). The LC-MS/MS assay incorporates chromatographic separation followed by an MS, specifically a tandem MS. To date the LC-MS/MS assay is considered the “gold standard” for the measurement of 25(OH)D and within the last decade clinical laboratories’ opinion of this assay changed from it being a labour intensive, expensive and complicated assay to a simple, robust and highly automated assay achieving greater sensitivity and greater specificity with high throughput (El-Khoury *et al.*, 2011). To date there is no international reference measurement procedure (RMP) for the quantification of 25(OH)D (Schöttker *et al.*, 2012) and several concerns were raised regarding the current LC-MS/MS assay and possible interfering compounds that may affect the outcome of the measurement of 25(OH)D (Maunsell *et al.*, 2005; Carter 2012; Bailey *et al.*, 2013; El-Khoury *et al.*, 2011).

2.7.1 ASSAY PROCEDURE

Each separate phase or step of the LC-MS/MS assay for the quantification of 25(OH)D is varied based on the in-house development of the assay. Each in-house LC-MS/MS assay is developed according to the specific setup and specialised equipment available to the laboratory. Between laboratories, assays vary mainly in the sample preparation step, the mobile- and stationary phase for chromatographic separation and the source conditions that includes the ionisation mode, temperature and gas flow (Wallace *et al.*, 2010). The different sample preparation steps include either one or more of the following: solid phase extraction (SPE) which incorporates a C8 or C18 solid phase, liquid-liquid extraction (LLE) which usually incorporates n-heptane, hexane or dichloromethane and/or protein precipitation extraction (PPE) usually attained with acetonitrile, methanol and/or isopropanol. The mobile phases for chromatographic separation are usually based on an organic phase like acetonitrile or methanol and an aqueous phase. The ratio of organic- to aqueous phase are one of the key elements of retaining sufficient chromatographic separation of compounds

and these ratios are usually assay- and laboratory specific. The stationary phase can vary in terms of chemical properties and dimensions of the packing particles. In most laboratories, an Agilent Technologies Zorbax SB-C18 rapid resolution column, 2.1 x 100 mm, with particle size of 1.8 μm is used for the measurement of 25(OH)D but can vary based on the laboratory's needs. The ionisation mode, as part of the source conditions, are usually electron spray ionisation (ESI) mode, either positive or negative, or atmospheric pressure chemical ionisation (APCI) mode, either positive or negative, for the quantification of 25(OH)D (El-Khoury *et al.*, 2011; Wallace *et al.*, 2010).

Although no RMPs have been defined to date, two RMPs have been developed. The first LC-MS/MS candidate RMP for the measurement of 25(OH)D, by Tai and co-workers (2010), incorporates LLE with hexane and ethyl acetate, an isocratic mobile phase consisting of 66 % methanol and 34 % water and an Agilent Technologies Zorbax SB cyano (CN) column as the stationary phase in positive APCI mode. During the sample preparation step of the second candidate RMP (Stepman *et al.*, 2011), the vitamin D metabolites are extracted with n-hexane and fractionated with Sephadex LH-20 chromatography, thus SPE. The organic mobile phase consists of methanol and the aqueous phase of water, these mobile phases are set to run at a gradient. This assay also incorporates a two dimensional stationary phase for chromatographic separation. The Acquity BEH 300 C4 column (2.1 x 50 mm, 1.7 μm) is employed as the first dimension and an Acquity BEH C18 column (2.1 x 50 mm, 1.7 μm) as the second dimension with the MS source operating in positive ESI mode.

In 2012, Shah and co-workers reported a novel LC-MS/MS assay for the simultaneous quantification of six vitamin D metabolites along with two epimers. This assay incorporates PPE with isopropanol and methanol followed by LLE with dichloromethane and hexane. A gradient program is followed with methanol and water being the mobile phases. This assay also incorporates a two dimensional stationary phase for chromatographic separation, with the first dimension being an Ultrason ES-OVM chiral column (2.0 x 150 mm, 5 μm) and the second dimension an Agilent Technologies Zorbax SB-C18 rapid resolution column (2.1 x 100 mm, 1.8 μm). The MS source is also operated in positive ESI mode.

All LC-MS/MS procedures usually incorporate an internal standard (IS) for the quantification of the metabolites. These ISs can vary between laboratories and the most commonly reported ISs are Standozolol-d₃, hexadeuterated 25(OH)D₃ and one deuterated ¹³C 25(OH)D₃ (Wallace *et al.*, 2010).

2.7.2 ASSAY PERFORMANCE

Couchman and co-workers (2012) reported that the LC-MS/MS assay account for 10 % of all DEQAS returns at the time. The mean DEQAS bias for this assay ranged between -1.3 % and 9.5 % from 2005 to 2008. The average between laboratory imprecision (CV %) for the same time period ranged between 9 % and 21.3 % (Wallace *et al.*, 2010). Experts agreed that this assay is preferred for the measurement of 25(OH)D and other vitamin D metabolites based on the excellent sensitivity and specificity of the assay (Kleerekoper *et al.*, 2011). Although interferences in LC-MS/MS assays are less common, several concerns were raised about possible interfering compounds that could have an effect on the outcome of the measurement of 25(OH)D with this assay. Hydroxylated 25(OH)D metabolites like the inactive form, 24R,25(OH)₂D, and the active form 1 α 25(OH)₂D are indicated as possible interfering compounds. Baecher and co-workers (2012) indicated no significant chromatographical interference of 24R,25(OH)₂D while Shah and co-workers (2012) indicated similar for 1 α 25(OH)₂D. Several reports indicated co-elution of other vitamin D metabolites with 25(OH)D. Bailey and co-workers (2013) reported the clinical importance of the distinguishability between 25(OH)D and its C3-epimers, 3-epi-25(OH)D. Other co-eluting metabolites have been reported; the isobaric compound 7- α -hydroxy-4-cholesten-3-one (7(OH)4C3) which is an endogenous precursor of bile acids and derived from cholesterol (Carter, 2012; Maunsell *et al.*, 2005) as well as another isobaric compound 1 α -hydroxyvitamin D₃ (1 α (OH)D₃) (Alphacalcidol) which is acquired through vitamin D supplementation (El-Khoury *et al.*, 2011; Maunsell *et al.*, 2005). The before mentioned epimers and isobars have identical molecular masses to 25(OH)D₃ and 25(OH)D₂ and a study by Shah and co-workers (2011) indicated that these epimers and isobars can account for as much as 55.3 % of the total 25(OH)D measured in a patient. In the novel LC-MS/MS assay reported by Shah and co-workers (2012) they addressed the issue of these co-eluting compounds and indicated sufficient chromatographic separation between 25(OH)D₂, 25(OH)D₃, their epimers and the isobar 7(OH)4C3. The accuracy of this assay ranged between 85 % and 99.9 % for intra- and inter- day variation.

In conclusion, the LC-MS/MS assay can distinguish between 25(OH)D₂, 25(OH)D₃, 24R,25(OH)₂D₂, 24R,25(OH)₂D₃, 1 α 25(OH)₂D₂, 1 α 25(OH)₂D₃, isobaric forms as well as epimers of 25(OH)D. Although this has not been achieved in a single run, Shah and co-workers (2012) came very close to achieving this as the ultimate goal. Experts are in agreement that this is a highly sensitive and selective method for the measurement of 25(OH)D and other vitamin D metabolites, with high throughput and the possibility of being

fully automated. It is also recognised by clinical laboratories and experts as the “gold standard” for vitamin D quantification.

2.7.3 ASSAY OPTIMISING PARAMETERS

Although the LC-MS/MS assay is considered the “gold standard” for the quantification of vitamin D and its metabolites, this assays’ performance can be improved by the sufficient optimisation of each of the underlying limiting parameters for the specific desired analytes. In this section, the LC-MS/MS is assessed as different parts contributing to the total performance of the assay.

2.7.3.1 LIQUID CHROMATOGRAPHY

A number of fundamentals affect the performance of the chromatography for the selected analytes; these include the efficiency of the stationary phase, the retention of the analytes, the selectivity of the stationary phase, the analyte peak chromatographic separation and finally the van Deemter curve and equation to evaluate the total efficiency of the liquid chromatography.

The efficiency (N) of the stationary phase is used to compare the performance of different columns based on the number of theoretical plates, N, of each column. Thus, a stationary phase with a higher plate number is theoretically more efficient and will have a narrower analyte peak at the specific retention time. The efficiency of a stationary phase is affected by column parameters like the diameter, length, particle size of the stationary phase, the type and viscosity of the eluent, the flow rate as well as the specifics of the analyte affecting its retention (Agilent Technologies: The LC handbook, 2011).

The retention factor (k) measures the period of time that the specific analyte resides in the stationary phase relative to the period of time it resides in the mobile phase. This is thus an indication of the total time by which the analyte is retained in the stationary phase with regards to the total time that it would take the analyte to travel through the column at the specific velocity of the mobile phase (Agilent Technologies: 2011).

The selectivity (α) of the stationary phase for each analyte is defined as the ratio between the retention factors of two analytes. Thus, it is a measure between the maxima of two

analyte peaks and if the selectivity of two analyte peaks are equal to 1 the peaks have the same retention time and will thus co-elute. The greater the calculated value, the greater the peak selectivity. Selectivity is also a factor of change and can be adjusted by altering the mobile phase selection and/or its composition ratio or the column temperature (Agilent Technologies: 2011).

Peak resolution (R_s) indicates the ability of the column to separate analyte peaks of interest. Baseline separation is usually required when measuring analytes that have almost identical chemical structures, thus resulting in identical precursor- and product ion masses, like isobaric, isomeric or epimeric analytes. Baseline separation is achieved with greater resolution, thus analytes that may co-elute can be separated. When calculating the resolution between two peaks, a value of 1 is considered to be the minimum for a measurable separation to occur, a value of 1.6 is considered baseline separation between analyte peaks and ensures the most accurate quantitative results, while a value of 1.7 or greater is required for rugged methods. The resolution equation considers the efficiency, selectivity and retention of the analyte peaks and can be improved by altering any of these parameters (Agilent Technologies: 2011).

Finally, the van Deemter equation is used to evaluate the total efficiency of the column as a function of flow rate. This determines the optimum linear velocity (flow rate) for a method. The van Deemter equation is based on the linear velocity (flow rate), height of the theoretical plate, column length, the calculated number of theoretical plates and the Eddy-diffusion parameter. The goal is to get a small plate height by decreasing particle size and using low viscosity mobile phases (Agilent Technologies: 2011).

By assessing each of the variables discussed above as a whole, the most effective analytical stationary phase accompanied by the best mobile phase can be selected. Underlying factors like flow rate and gradient ratio of the mobile phase, column pressure and column temperature can thus ensure the optimisation of the limiting factors of the liquid chromatography resulting in sufficient separation between analyte peaks.

2.7.3.2 MASS SPECTROMETRY

Mass spectrometry is an analytical technique that can provide both structural and concentration based information of a specific analyte after its conversion to ions. The MS consists of three components namely an ion source, a mass analyser and a detector.

Furthermore each of these components have several underlying parts that can be optimised to improve the desolvation, ionisation and detection of the specific analytes. In this section all the limiting components of the MS will be discussed based on the Agilent Technologies triple quadrupole mass spectrometer (QQQ) with Jet Stream Technology.

Agilent Technologies provides a choice of four ion sources to pair with their QQQ: ESI, APCI, atmospheric pressure photoionisation source (APPI) and multi-mode ionisation source (MMI). Only the optimisation parameters for the ESI source is covered in this section.

The ESI source has been designed to reduce the introduction of unwanted sample components that may interfere with the analysis. The specific design incorporates an advanced nebuliser design that produces a uniform droplet size thus ensuring maximum sensitivity by reducing the solvent cluster background (Cech & Enke, 2001). The nebuliser ensures the formation of a fine mist spray bearing charged droplets from the liquid mobile phase. Furthermore, a high voltage, either positive or negative, is applied to the capillary. This applied voltage results in the liquid eluting from the capillary tip to form a Taylor cone. The drying gas furthermore dries the excess eluting sample and single charged ions left. The charged ions travel through the atmosphere and is introduced into the MS. Based on a tandem MS, the mass-to-charge ratio of a specific precursor ion is analysed (Cech & Enke, 2001).

Through the optimisation of the underlying parameters like the drying gas temperature, drying gas flow rate, capillary voltage and nebuliser pressure, the MS and ion source can be tweaked for optimal analyte sensitivity. These optimal conditions will differ between analytes with different chemical properties as well as the solvent used to introduce the sample into the ion source.

2.7.3.3 SAMPLE PREPARATION

Sample preparation, also known as sample clean-up, refers to the extraction of specific analytes from a biological matrix. The sample clean-up takes place before the physical detection and quantification of the analytes, thus ensuring lowered matrix interferences resulting in improved sensitivity, accuracy and precision. The most commonly used sample clean-up methods with regard to LC-MS/MS analysis is LLE, SPE and PPE (Musteata & Musteata, 2011). Liquid-liquid extraction is based on the principle that one or more solutes are transferred between two immiscible liquids based on the solutes' relative solubility. The

two liquids involved for the extraction procedure are usually a water phase and an organic solvent. Currently LLE is used for the extraction of vitamin D metabolites from a wide variety of sample matrices. Most of these applications also apply a PPE step before the LLE (van den Ouweland *et al.*, 2013). This is due to the high affinity of the vitamin D metabolites for the DBPs. The addition of a common organic solvent, like acetonitrile, causes the binding between the vitamin D metabolites and the DBP to disrupt, resulting in a protein crash. The most common organic solvents used for LLE are heptane, hexane, isopropyl alcohol, ethanol, methanol, and acetone (El-Khoury *et al.*, 2011; Adamec *et al.*, 2011). Solid phase extraction is based on the principle that the solutes are separated from other compounds within the biological matrix based on their affinity for the stationary phase. Musteata and Musteata (2011) describes several vitamin D applications that uses SPE as the sample clean-up method. Most of these applications make use of either a C18 or a C8 stationary phase for the extraction of these metabolites. A review by Musteata and Musteata (2011) compared LLE, SPE and PPE and concluded that most of the sample preparation methods based on PPE prior to LLE obtained a recovery close to 100 % with a CV of less than 15 %. Although recoveries of close to 100 % was reported, most of these applications only included vitamin D₂, vitamin D₃, 25(OH)D₂ and 25(OH)D₃, while very few included the dihydroxylated metabolites, 1,25(OH)₂D₂, 1,25(OH)₂D₃, 24,25(OH)₂D₂ and 24,25(OH)₂D₃. This review article also concluded that LLE resulted in a cleaner sample with higher specificity and recovery.

2.8 ASSAY QUALITY ASSESSMENT (METHOD VALIDATION)

According to Westgard (2008), error assessment is the inner, hidden, deeper, secret meaning of method validation. The principle of method validation is to estimate the total error present within the results derived from an analytical method. Based on the total error allowable for the specific application a conformation can be made regarding the overall performance of the analytical method; if the amount of error is too high, false interpretation of the test results may occur which is unacceptable for clinical laboratories. To ensure the acceptability of the performance of an analytical method, the fundamental parameters of method validation are assessed. These parameters include: accuracy, precision, selectivity, sensitivity and stability (Westgard, 2008). Through the assessment of each of these parameters the quality of the pre-analytical, analytical and post-analytical procedures are assessed.

2.8.1 ACCURACY

The accuracy of the analytical method is defined as the closeness of the agreement between the measured value of a specific analyte and its true value within the sample (Food and Drug Administration: Guidance for industry, 2001). Thus, the accuracy of an analytical method is based on the assessment of the inaccuracy of the method, expressed as a concept of error. This error is defined as systematic error and can be divided into two categories, namely constant systematic error or proportional systematic error (Westgard, 2008). To assess the systematic error of a test method, a method comparison study is conducted. This is done by comparing the test method to a traceable reference method through the measurement of specific analytes within a sample and comparing the values obtained by the test method to the true value of the analyte as determined by the reference method (Westgard, 2008). The National Institute of Standards and Technology (NIST) and DEQAS are examples of proficiency testing schemes that can provide samples of which the true values of analytes, based on the measurement by the reference method, are known. The differences between the measurements of the test method and the reference method are illustrated as a comparison plot. Regression statistics from this comparison plot provides critical information regarding the inaccuracy of the method. Conclusions are also made from this plot regarding the category of the systematic error; either constant or proportional. The total systematic error is then expressed as a percentage and is known as the method bias (Westgard, 2008). Stockl and co-workers (2009) used four models to determine the specifications of trueness and precision for methods measuring vitamin D metabolites. The authors concluded that the total error allowable (TE_a) for a method is 20 % and the method bias should not exceed 10 %.

2.8.2 PRECISION

The precision of an analytical method describes the agreement between the results obtained from repeated analysis of a single biological sample (Food and Drug Administration: Guidance for industry, 2001). The random error or imprecision of the method is divided into two categories, namely the within-day error and the within-laboratory error (Westgard, 2008). The amount of error present for each category is estimated through a replication experiment. The estimation of the within-day random error requires two or more biological samples to be analysed repeatedly on a single day. The within-laboratory random error are estimated when these samples are then analysed over a series of consecutive days. The assessment of the data obtained from these replication experiments thus indicate the amount of random error

caused by any variation of the analysis (Westgard, 2008). These variations may include the pipetting, reagents, temperatures and the analyst himself. The random error is described as the standard deviation (SD) and the CV calculated from the relevant groups of data. Ideally a CV value of less than 15 % is acceptable and must not exceed this percentage for any concentration (Stockl *et al.*, 2009).

2.8.3 SELECTIVITY

The selectivity of an analytical method is its ability to differentiate and correctly quantify the analytes of interest from other compounds present in a complex biological matrix. The biological matrix is complex and the presence of other compounds may cause interference, this is also known as a matrix effect (Whitmire *et al.*, 2011). With regards to LC-ESI-MS/MS, the presence of these interfering compounds may result in ion suppression or enhancement. Ion suppression is more common and is the result of competition for ionisation between the interfering compounds and the analyte of interest (Annesley, 2003). This may result in reduced sensitivity and incorrect quantification. The effect of interfering compounds can be overcome in several ways. Sufficient sample preparation ensures a sample clean-up, thus less interfering compounds. The use of stable isotopes to absolutely quantify the analytes of interest ensures that interferences resulting in ion suppression are compensated for. By performing an interference experiment, the amount of constant systematic error present, due to interferences, can be estimated. The co-elution of analytes of interest can also cause an interference and incorrect quantification due to similarities of these analytes. This is overcome by chromatographically separating these analytes.

2.8.4 SENSITIVITY

Analytical sensitivity can be defined as the increased analytical response linear to the increased analyte concentration. This is the simplest model that adequately describes the relationship between concentration and response (Food and Drug Administration: Guidance for industry, 2001). The analytical sensitivity is best displayed as a calibration curve and the slope of this calibration curve. A calibration curve is obtained by analysing a series of samples with known concentrations of each analyte. These samples should be prepared in the same biological matrix as the intended samples to be analysed by this method (Whitmire *et al.*, 2011). Through the use of linear regression statistics, the $y=mx+c$ model can be used to determine the limit of detection (LOD) and limit of quantification (LOQ) for the method.

Several methods are used to described LOD and LOQ. Shrivastava and Gupta (2011) defined LOD as the lowest concentration of an analyte that can be detected in a sample but not necessarily quantified under the specific method conditions. The LOQ is the lowest concentration of an analyte in a sample that can be quantified with acceptable precision and accuracy. A signal-to-noise ratio method can be applied to analytical methods that exhibits baseline noise to determine LOD and LOQ (Armbruster & Pry, 2008). Through the use of the $y=mx+c$ model, LOD can be expressed as $3S_a/b$ and LOQ as $10S_a/b$; where S_a is the SD of the response and b is the slope of the calibration curve. The SD of the response can be estimated as either the SD of the y -residuals or the SD of the y -intercepts. The linear range or reportable range of the method can also be determined by the calibration curve. The linear range can be defined by the highest and lowest points that fall within 1 SD of the calibration straight line. By assessing the LOD, LOQ and the linear range of the method the analytical sensitivity of the method can be determined and described.

2.8.5 STABILITY

Analyte stability can be defined as the ability of the analyte contained in the biological matrix to retain its initial chemical properties for a period of time stored under specific conditions (Food and Drug Administration: Guidance for industry, 2001). Analyte instability can contribute to the amount of random error seen for the method. Several analyte stability tests should be evaluated for the assessment of the analyte stability. These include freeze and thaw stability, short-term storage stability, long-term storage stability, internal standard stock solution storage stability and post-preparative stability (Food and Drug Administration: Guidance for industry, 2001). For more than 30 years researchers have been familiar with the fact that 25(OH)D and 1,25(OH)₂D are extremely stable in serum (Lisser *et al.*, 1981). Lisser and co-workers (1981) reported that vitamin D metabolites will remain intact when the blood is stored at 24 °C for up to 72 hours. More recent studies provided evidence that vitamin D metabolites are stable when serum is stored at -20 °C for up to 3 years and that multiple freeze and thaw cycles have no effect on these analytes (Hollis, 2008; Agborsangaya *et al.*, 2010). Hollis (2008) also explained an experiment conducted to test the stability of vitamin D metabolites when exposed to UV light. The author concluded that after 2 days of UV light exposure no significant changes were seen in the 25(OH)D and 1,25(OH)₂D serum concentrations. He explained the finding to be due to UV light that poorly penetrates the serum and the main reason to be due to vitamin D metabolites that are bound to the vitamin D binding protein in serum. Hollis (2008) concluded that vitamin D metabolites are very stable in serum and require minimal attention to storage. Agborsangaya and co-

workers (2010) described an experiment that tested the long-term stability of vitamin D metabolites. They found that serum samples stored in biobanks at -25 °C had no effect on the concentration of the vitamin D metabolites. However, the authors also found that the sampling season had an effect on the concentration of these metabolites. They described that the concentration of the vitamin D metabolites, especially 25(OH)D, were significantly lower in samples acquired in winter than in summer. This correlates to studies regarding the synthesis of vitamin D in human skin due to the exposure to sunlight. A letter to the editor by Wielders and Wijnberg (2009) concluded 25(OH)D concentration to be “solid as a rock” at room temperature and under common pre-analytical conditions experienced in medical laboratories.

EXPERIMENTAL OUTLINE

Based on the motivation for this study and the study objectives described in Chapter 1, the following experimental plan was constructed. The aim of the study will be achieved by optimising the underlying parameters of the LC-MS/MS system. These include the optimisation of the mass spectrometry parameters, optimisation of the liquid chromatography conditions for sufficient chromatographic separation of the analytes as well as the optimisation of the sample preparation procedure. Each of these parameters will be optimised for the fifteen analytes included in this study. The optimised method will then be subject to several validation steps to ensure the accuracy and precision of the method. Finally, based on the method validation, the quality assurance of the method will be determined for clinical laboratory implementation followed by a detailed SOP.

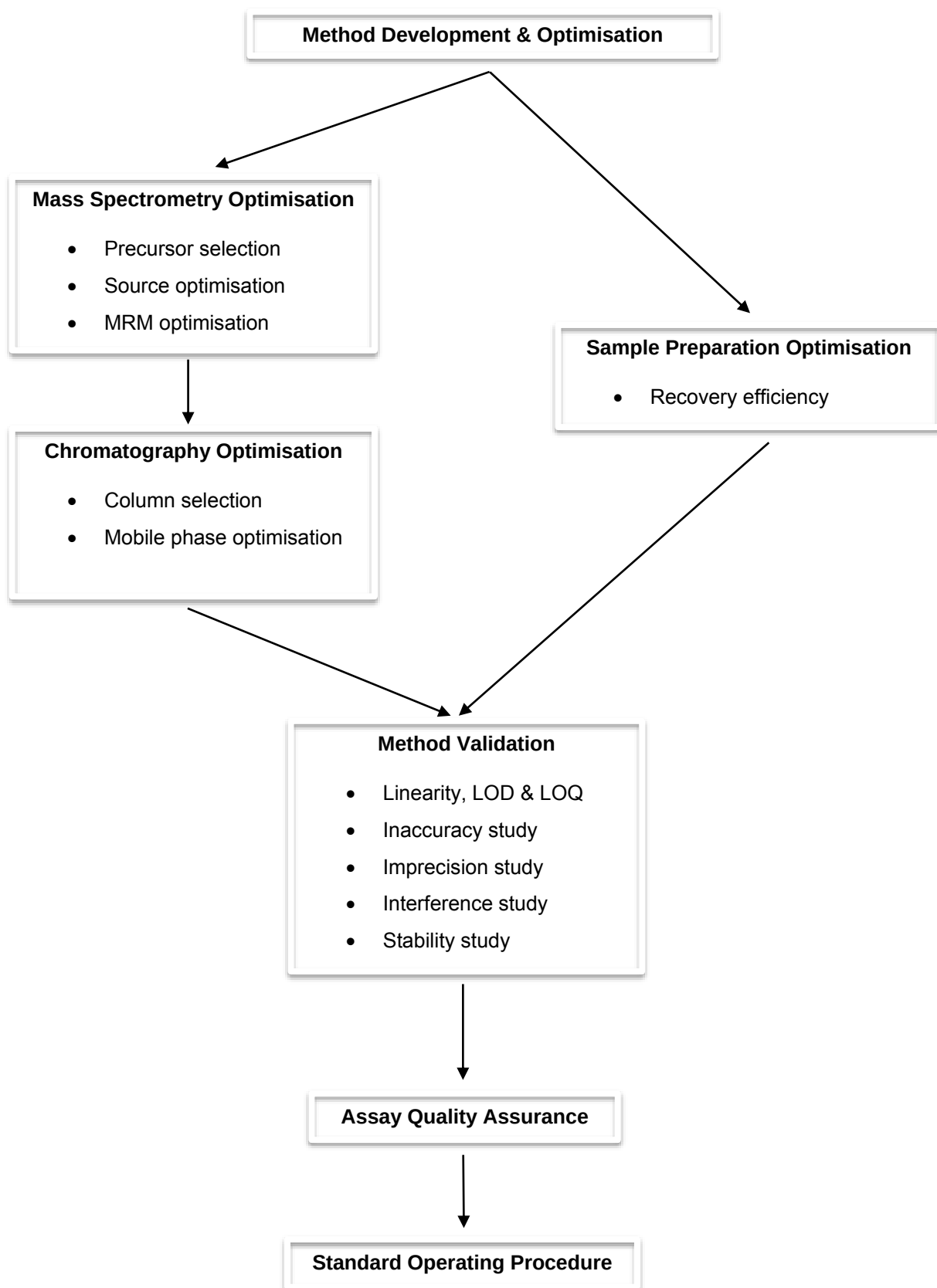


Figure 2.4 Experimental outline for this study. This illustration shows the experimental outline for this study. Each step throughout the experimental procedure correlates to the specific study objective.

CHAPTER 3

MATERIALS & METHODS

3.1 BACKGROUND

The previous chapter entailed the importance of the measurement of vitamin D and its metabolites. Currently, immunoassays are more widely used for the quantification of 25(OH)D and its metabolites. Although the LC-MS/MS assay is considered the “gold standard” for the quantification of vitamin D and its metabolites several concerns were raised regarding the performance of the assay. The biggest concern raised by Bailey and co-workers (2013) is the possible overestimation of the 25(OH)D due to the interference and co-elution of known C3-epimers, 3-epi-25(OH)D₂ and 3-epi-25(OH)D₃, and isobaric compounds, 7(OH)4C3 and 1 α (OH)D₃ (Alphacalcidol). These epimers and isobars have identical molecular masses to 25(OH)D₂ and 25(OH)D₃. A study by Shah and co-workers (2011) indicated that these epimers and isobars may account for as much as 55.3 % of the total 25(OH)D measured in a patient. Therefore sufficient chromatographic separation is required to prevent the co-elution of the epimers and isobars with regard to the 25(OH)D. This will prevent the overestimation and false-high quantification of 25(OH)D. The second concern raised is based on the dihydroxylated metabolites of 25(OH)D, 24R,25(OH)₂D and 1 α ,25(OH)₂D. These metabolites also have similar molecular masses and thus sufficient chromatographic separation is also required for accurate quantification. The need for a quantitative assay that can distinguish between the vitamin D₂ and vitamin D₃, 25(OH)D₂, 25(OH)D₃, the C3-epimers and isobars, 1 α ,25(OH)₂D₂, 1 α ,25(OH)₂D₃, 24R,25(OH)₂D₂ and 24R,25(OH)₂D₃ in a single run still exists. Through the optimisation of each LC-MS/MS parameter, a sensitive method that addresses all of these concerns can be developed. Through the validation of this optimised method the accuracy and precision can be evaluated to ensure the quality of the method for the implementation into clinical laboratories.

3.2 GENERAL MATERIALS

3.2.1 REAGENTS, STANDARDS AND SOLUTIONS

Twelve analytes and three stable isotopes were included in this study. The following analytical standards were purchased from the CertiMass Solution range of IsoScience, USA. Each standard contained 100 mg/L of the specific analyte dissolved in ethanol, with a purity of greater than 99.9 %. These included the following, with catalogue numbers indicated in brackets: vitamin D₂ (50-14-6), vitamin D₃ (67-97-0), 25(OH)D₂ (21343-40-8), 25(OH)D₃ (19356-17-3), 1,25(OH)₂D₂ (60133-18-8), 1,25(OH)₂D₃ (32222-06-3), 24,25(OH)₂D₂ (58050-55-8), 24,25(OH)₂D₃ (55721-11-4), 3-epi-25(OH)D₂ (908126-48-7), 3-epi-25(OH)D₃ (73809-05-9), vitamin D₃-[²H₃] (8066-48-4), 25(OH)D₃-[²H₃] (140710-94-7) and 1,25(OH)₂D₃-[²H₃] (128723-16-0). 1 mg high purity 7(OH)4C₃ (sc-210660) was purchased from Santa Cruz Biotechnology, USA and 1 mg high purity 1α(OH)D₃ (17946) from Sigma Aldrich, South-Africa.

A stock solution was prepared by combining the fifteen analytical standards. The stock solution was serially diluted to a concentration of 100 ng/ml (250 nmol/L) for each analyte. Each analyte was also diluted individually to a concentration of 100 ng/ml in methanol. The analytical standards of each individual analyte as well as the combined stock solution were stored in the dark at -20 °C.

Honeywell Burdick & Jackson, spectrometry grade, methanol (10071675), acetonitrile (10071618), water (10071715), acetone (10071616), isopropyl alcohol (10071758) and heptane (10071791) were purchased from Anatech, South Africa. High purity hexane (34859), formic acid (100987129), ammonium acetate (1001444789), sodium hydroxide (S8045) and sodium chloride (13423) were purchased from Sigma Aldrich, South Africa. Ethyl acetate (1.09623.2500) was purchased from Merck, South Africa and ammonium formate (G1946-85021) from Agilent Technologies, USA.

3.2.2 LIQUID CHROMATOGRAPHY – TANDEM MASS SPECTROMETRY SPECIFICATIONS

The LC system included a 1290 Infinity Binary Pump with Agilent Jet Weaver mixer technology (G4220A), a 1290 Infinity Thermostated Column Compartment (G1316C) and a 1290 Infinity Autosampler (G4226A), Agilent Technologies, USA. The desolvation and

ionisation of sample material was done by positive ESI enhanced with Agilent Jet Stream (AJS) technology. The 6460 triple quadrupole mass spectrometer (G6460A), Agilent Technologies, USA, was used as the mass analyser.

The analytical columns included for the optimisation of the chromatography were the Pursuit 3 Pentafluorophenyl (PFP) (3 x 100 mm, 3 μ m; P.N.: A3061100X030), Pursuit 3 PFP (4.6 x 100 mm, 3 μ m; P.N.: A3051100X046), Zorbax SB-CN (2.1 x 100 mm, 1.8 μ m, P.N.: 828700-905), Zorbax SB-Phenyl (2.1 x 100 mm, 1.8 μ m; P.N.: 828700-912) and Zorbax Eclipse Plus C18 (2.1 x 50 mm, 1.8 μ m; P.N.: 827700-902), purchased from Agilent Technologies, USA.

3.2.3 BIOLOGICAL SAMPLES

Five sets of pooled human serum samples that form part of an external quality assessment (EQA) scheme were purchased from DEQAS, UK. These five sets included twenty samples with NIST RMP assigned 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃ concentration values and twenty samples with LC-MS/MS assigned 1,25(OH)₂D₃ concentration values. These DEQAS samples are pooled serum samples with unmodified matrix (DEQAS, 2014). The concentration value of each specific analyte within each of the samples is shown Table 3.1 and Table 3.2 respectively.

Table 3.1 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃ National Institute of Standards and Technology reference measurement procedure assigned values for Vitamin D External Quality Assessment Scheme samples.

Sample No.	NIST 25(OH)D ₂	NIST 25(OH)D ₃	NIST 3-epi-25(OH)D ₃	NIST Total 25(OH)D	DEQAS ALTM	% Difference
	nmol/L	nmol/L	nmol/L	nmol/L	nmol/L	
421	0.95	57.50	2.33	58.50	55.40	-5.3
422	1.65	36.58	1.70	38.20	36.20	-5.2
423	0.98	84.63	5.68	85.60	81.20	-5.1
424	0.98	46.20	2.58	47.20	47.00	-0.4
425	0.95	46.18	2.50	47.10	46.90	-0.4
426	1.05	33.40	0.40	34.50	32.00	-7.2
427	0.88	75.20	4.50	76.08	72.50	-4.7
428	2.45	52.30	2.80	54.75	51.80	-5.5
429	0.55	58.90	3.00	59.45	58.50	-1.7
430*	22.30	17.60	0.80	39.90	35.00	-12.3
431	1.25	22.60	1.20	23.85	24.90	4.2
432	2.70	48.60	2.90	51.30	50.00	-2.5
433	1.18	90.40	11.70	91.58	88.20	-3.7
434**	4.43	74.20	2.40	78.63	67.20	-14.5
435	0.53	46.10	2.40	46.63	45.80	-1.7
436	1.25	76.70	4.00	77.95	83.20	6.7
437	1.38	33.30	1.40	34.68	35.20	1.4
438	1.80	54.80	2.70	56.60	57.30	1.2
439	1.18	39.70	2.30	40.88	42.60	4.2
440	1.30	47.10	2.30	48.40	51.30	6.0

NIST Total 25(OH)D is the sum of 25(OH)D₂ and 25(OH)D₃. ALTM: All Laboratory Trimmed Mean. % Difference calculated as the difference of the ALTM from the NIST RMP assigned values for the total 25(OH)D. * Sample contained a mixture of endogenous 25(OH)D₂ and 25(OH)D₃. ** Lipaemic sample.

Table 3.2 1,25(OH)₂D LC-MS/MS assigned values for Vitamin D External Quality Assessment Scheme samples.

Sample No.	LC-MS/MS 1,25(OH) ₂ D	DEQAS ALT	% Difference*
	pmol/L	pmol/L	
311	129.3	129.8	0.4
312	95.3	90.5	-5.0
313	101.5	107.8	6.2
314	104.3	110.4	5.8
315	108.0	107.1	-0.8
316	123.2	125.0	1.5
317	109.4	112.7	3.0
318	152.0	161.3	6.1
319	77.3	59.9	-22.5
320	124.7	121.9	-2.2
321	103.4	119.6	15.7
322	89.7	103.7	15.6
323	88.6	103.0	16.3
324	67.1	78.0	16.2
325	55.1	51.6	-6.4
326	115.4	122.9	6.5
327	71.5	68.5	-4.2
328	113.8	111.9	-1.7
329	90.0	86.5	-3.9
330	80.7	81.6	1.1

ALT: All Laboratory Trimmed Mean. % Difference calculated as the difference of the ALT from the LC-MS/MS assigned values for the total 1,25(OH)₂D.

3.3 METHOD DEVELOPMENT & OPTIMISATION

3.3.1 MASS SPECTROMETRY OPTIMISATION

3.3.1.1 PRECURSOR ION SELECTION

Depending on the analytical system conditions, different precursor ions can be selected for each analyte. The intensity of the dihydroxylated analytes can be intensified by choosing the specific adduct as the precursor ion rather than the molecular mass ion $(M+H)^+$ (Adamec *et al.*, 2011; El-Khoury *et al.*, 2011; Shah *et al.*, 2012). These different precursor ions included the ammonium adduct $[M+H]^+ + [NH_3]^+$ and the water loss molecules $[M+H] - [H_2O]^+$, $[M+H] - [2H_2O]^+$ or $[M+H] - [3H_2O]^+$.

On this basis, 0.5 μ l of each analyte standard (100 ng/ml) was infused with MS2 set to scan mode to observe any other abundant precursor adducts. The mobile phase ratio was kept isocratic at 70 % methanol with a flow rate of 0.2 ml/min. From the total ion chromatogram (TIC) for each analyte, the most abundant possible precursor adducts were selected. Another infusion of the same analyte standard was made, with MS2 set to selective ion monitoring (SIM) mode, monitoring only these selected possible precursor adducts. The abundances of the possible precursor adducts were compared and the most abundant precursor was selected for each analyte.

3.3.1.2 SOURCE OPTIMISATION

As explained in 2.7.3.2, several underlying parameters regarding the ESI source can be optimised for each analyte to improve the analytical sensitivity. These parameters are specific to the analytical instrumentation used and included drying gas flow (L/min), drying gas temperature ($^{\circ}$ C), nebuliser pressure (psi), capillary voltage (V), nozzle voltage (V), sheath gas flow (L/min) and sheath gas temperature ($^{\circ}$ C). The optimisation of these parameters was performed by altering each of the parameters individually by means of a flow injection analysis (FIA) program. The FIA program consisted of 20 segments with each parameter being varied four times. The drying gas temperature and the sheath gas temperature were kept constant throughout this optimisation process. The FIA program was set to repeat 20 times, draw sample, inject, wait 0.4 minutes and end repeat. For each of the 20 segments, 0.5 μ L of the analytical standard (100 ng/ml) was infused with MS2 set to SIM mode, monitoring the selected precursor ion. The mobile phase ratio was kept isocratic at 70

% methanol and a flow rate of 0.2 ml/min. The FIA program with the varied parameter settings are shown in Table 3.3.

Table 3.3 Flow injection analysis program for the optimisation of source parameters.

Segment:	Injection Time (min) :	Parameter Change Time (min) :	Drying Gas Temp (°C)	Drying Gas Flow (L/min)	Nebuliser Pressure (psi)	Capillary Voltage (V)	Nozzle Voltage (V)	Sheath Gas Flow (L/min)	Sheath Gas Temp (°C)
1	0.0	0.00	300	(D) 6	15	4000	1000	7	350
2	0.4	0.55	300	4	15	4000	1000	7	350
3	0.8	1.20	300	9	15	4000	1000	7	350
4	1.2	1.80	300	12	15	4000	1000	7	350
5	1.6	2.40	300	6	(D) 15	4000	1000	7	350
6	2.0	3.00	300	6	10	4000	1000	7	350
7	2.4	3.70	300	6	30	4000	1000	7	350
8	2.8	4.30	300	6	60	4000	1000	7	350
9	3.2	4.90	300	6	15	(D) 4000	1000	7	350
10	3.6	5.70	300	6	15	2000	1000	7	350
11	4.0	6.30	300	6	15	5000	1000	7	350
12	4.4	7.10	300	6	15	6000	1000	7	350
13	4.8	7.65	300	6	15	4000	(D) 1000	7	350
14	5.2	8.30	300	6	15	4000	0	7	350
15	5.6	8.95	300	6	15	4000	1500	7	350
16	6.0	9.50	300	6	15	4000	2000	7	350
17	6.4	10.20	300	6	15	4000	1000	(D) 7	350
18	6.8	10.80	300	6	15	4000	1000	9	350
19	7.2	11.40	300	6	15	4000	1000	10	350
20	7.6	12.00	300	6	15	4000	1000	11	350

Green blocks indicate the specific varying parameter. (D): Default as per Agilent Technologies MassHunter Software, (min): minutes, (°C): degree Celsius, (L/min): litre per minute, (psi): pound per square inch.

The drying and sheath gas temperatures were optimised individually for each analyte due to the time needed for the temperature to stabilise. A 0.5 µl infusion of the analyte standard (100 ng/ml) was made with the temperature of one gas being altered. The selected gas temperatures were; 250 °C, 275 °C, 300 °C and 350 °C for the drying gas and 300 °C, 325 °C and 350 °C for the sheath gas. All the other source parameters were kept constant at the default point and MS2 was set to monitor the intensities of each infusion in SIM mode.

3.3.1.3 MULTIPLE REACTION MONITORING OPTIMISATION

The optimisation for the best multiple reaction monitoring (MRM) transition of each analyte was done by means of the MassHunter Optimiser Software (B.04.01), Agilent Technologies, Germany. A single acquisition method was established containing the optimised source

parameters for each analyte precursor ion. The standard solution containing 100 ng/ml of each analyte was infused while the mobile phase was isocratic at 70 % methanol with a flow rate of 0.2 ml/min.

3.3.2 CHROMATOGRAPHIC OPTIMISATION

3.3.2.1 COLUMN SELECTION

A set of five analytical columns were selected to find the one that can achieve baseline separation between 25(OH)D₂ and 3-epi-25(OH)D₂; 25(OH)D₃, 3-epi-25(OH)D₃, 7OH4C3 and 1 α (OH)D₃; 1,25(OH)₂D₂ and 24,25(OH)₂D₂; 1,25(OH)₂D₃ and 24,25(OH)₂D₃. These analytical columns included a Pursuit 3 PFP (3 x 100 mm, 3 μ m), Pursuit 3 PFP (4.6 x 100 mm, 3 μ m), Zorbax SB-CN (2.1 x 100 mm, 1.8 μ m), Zorbax SB-Phenyl (2.1 x 100 mm, 1.8 μ m) and Zorbax Eclipse Plus C18 (2.1 x 50 mm, 1.8 μ m). These columns were assessed based on the retention of the analytes and the chromatographic separation between the relevant analytes.

Each of the columns tested was also subjected to temperatures of 25 °C, 35 °C and 45 °C to note the effect of temperature on the chromatographic separation.

3.3.2.2 MOBILE PHASE OPTIMISATION

Different mobile phases as well as the mobile phase ratio and mobile phase modifiers were assessed based on the chromatographic separation of the analytical peaks. The mobile phases included methanol and acetonitrile as the organic phases and water as the aqueous phase. The mobile phase modifiers included formic acid, ammonium acetate and ammonium formate. These mobile phase modifiers were selected based on their current use in similar vitamin D metabolite quantification applications (Baecher *et al.*, 2012; Stepman *et al.*, 2011; Shah *et al.*, 2012; van den Ouweland *et al.*, 2010).

The effect of the methanol compared to the acetonitrile, as organic phase, was assessed based on the retention and chromatographic separation of the analytes. An injection was made from a stock solution containing 100 ng/ml of the fifteen analytes. The mobile phase was kept isocratic at 70 % organic phase for both methanol and acetonitrile to evaluate the effect of the two different organic phases on the chromatographic parameters. The mobile

phase flow rate, sample injection volume and column temperature were kept constant while the MS parameters were set according to the optimisation as described in 4.5.3.

Mobile phase modifiers were also assessed to evaluate the effect, if any, on the retention and chromatographic separation of the analytes as well as the intensity of the analytical peaks. El-Khoury and co-workers (2011) reported the general use of formic acid, ammonium acetate and ammonium formate, at different final concentrations, from several similar literature applications. Three experiments were performed to note the effect of the formic acid. A single injection was made from a stock sample containing 100 ng/ml of the fifteen analytes of interest. The formic acid was added to the aqueous phase only, thereafter to the organic phase only and finally to both phases, with a final concentration of 0.1 % (26.51 mM). The same experiment was also conducted for the ammonium acetate and ammonium formate but with variation of the final concentration of these modifiers to 0.5 mM, 1.0 mM and 5.0 mM in each solvent. Throughout these experiments, the mobile phase ratio was kept isocratic at 70 % methanol with a constant flow rate, sample injection volume, column temperature and MS parameters set according to the optimisation as described in 4.5.

The chromatographic separation of the analytes was optimised by adjusting the gradient of the mobile phase ratio. The flow rate was set to 0.8 ml/min, according to the column dimensions and was also optimised for optimum peak shape. The optimisation of the mobile phase gradient was done by injecting 5 µl of a stock sample containing 100 ng/ml of each analyte onto the selected column. The optimisation initiated with a 70 % methanol isocratic ratio and was adjusted based on the chromatographic separation of the analytes.

3.3.3 OPTIMISATION OF SAMPLE PREPARATION

From the literature-based evidence, protein precipitation prior to LLE was selected as the extraction method for this application. Hexane, heptane, acetone and ethyl acetate were assessed as extraction solvents based on the solvents' extraction efficiency. The effect of matrix modifiers on the total analyte recovery and sample clean-up was also assessed. These modifiers included sodium hydroxide (NaOH) and sodium chloride (NaCl).

The sample preparation steps described by Shah and co-workers (2011) were used as a guideline for this application. These steps included the following: for the sample pretreatment or protein precipitation, a serum sample was thawed and vortex mixed followed by the addition of the internal standard and an incubation period. Furthermore, an organic solvent

was added and vortex mixed to promote protein precipitation during an incubation period. After a centrifugation step, the supernatant was transferred to a clean tube and subjected to LLE. During LLE, the extraction solvent was added to the supernatant, vortex mixed and centrifuged. The upper organic layer was transferred to a clean tube and the residual lower layer was subject to a further two extractions. All these organic phases obtained were pooled and dried under a gentle stream of nitrogen at room temperature. The dried sample was then reconstituted in a set amount of methanol and water (1:1, v/v) and analysed by LC-MS/MS.

These sample preparation steps were used as a guideline to evaluate the extraction efficiency of the different extraction solvents as well as the influence of the matrix modifiers. For the assessment of the solvents' extraction efficiency or recovery, aliquots of a pooled serum sample were used. Two aliquot serum samples (500 µl) were spiked with 50 µl stock solution containing 100 ng/ml of each analyte, one serum sample before extraction and the other after extraction. Both samples also had the addition of 50 µl (100 ng/ml) stable isotopes as the internal standards before extraction. This was repeated for each extraction solvent. The concentration of each analyte from the serum sample spiked before and after extraction was then determined and the percentage recovery was calculated. The assessment of the matrix modifiers was based on the same principle; two aliquot serum samples were spiked before and after extraction and the first was modified by adding the NaOH or NaCl while the second serum sample matrix was not modified. For this experiment hexane was used as the extraction solvent. The percentage recovery was again calculated to report the effect of the matrix modifiers on the extraction efficiency.

3.4 METHOD VALIDATION

3.4.1 LINEARITY, LIMIT OF DETECTION & QUANTIFICATION

A linearity study reveals the relationship between the concentration of the analyte and the analytical response and is displayed as a calibration curve. Because of the sample preparation procedure that concentrate all the analytes within the serum with a factor 10, the calibration ranges were prepared to account for this concentrating factor.

The linearity of the method for each analyte was investigated. This was done by preparing a calibration range for each analyte. A five point calibration range, ranging between 0.1 – 10 ng/ml (0.25 – 25 nmol/L), for vitamin D₂, vitamin D₃, 1,25(OH)₂D₂, 1,25(OH)₂D₃,

24,25(OH)₂D₂ and 24,25(OH)₂D₃ was prepared by means of serial dilution. Furthermore, a seven point calibration range, ranging between 50 – 1000 ng/ml (125 – 2500 nmol/L), for 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₂, 3-epi-25(OH)D₃, 7(OH)4C3 and 1 α (OH)D₃ was also prepared by means of serial dilution. All the calibrators were made up in methanol containing 100 ng/ml stable isotope. The LOD for each analyte was estimated based on a signal-to-noise (S/N) ratio method from the calibration curve (Shrivastava & Gupta, 2011; Sanagi *et al.*, 2009). Although this method has limitations, the LOD is in this application less important than the LOQ. Therefore, a method proposed by Westgard (2008) was used for the estimation of the LOQ. This method is based on the principle that the LOQ can be estimated as the analyte concentration at which the CV = 20 %.

3.4.2 INACCURACY STUDY

According to Westgard (2008), the inaccuracy or systematic error of a method can be estimated through a comparison of methods experiment. This is done by analysing a series of biological samples by the test method and comparing the quantified results to the true values obtained by the traceable reference method.

The method comparison study was conducted by analysing 40 DEQAS samples in duplicate. Twenty DEQAS samples contained known concentrations of 25(OH)D₂, 25(OH)D₃ and 3-epi-25(OH)D₃ determined by the NIST RMP and the other 20 DEQAS samples contained known concentrations of total 1,25(OH)₂D determined by a traceable LC-MS/MS method. Only these analytes were subjected to the method comparison study due to the unavailability of the other analytes. The sample preparation steps and LC-MS/MS specifications were done and set, respectively, according to the optimised method described in 4.5.

The differences between the measurements of the test method and the reference method were illustrated as a comparison plot. Regression statistics, performed in Microsoft Office Excel 2010, provided information regarding the systematic error of the test method. Further assessment of the systematic error was done to indicate constant or proportional systematic error.

3.4.3 IMPRECISION STUDY

Method imprecision, also known as random error or repeatability, can be determined through an analysis of variance experiment (Westgard, 2008). The calculated variance components can then be used to estimate the within-run and within-laboratory precision.

The imprecision study was conducted by analysing three samples per day over five consecutive days, in triplicate. Serum samples were obtained from healthy individuals and pooled. This pooled serum sample was then divided to have three levels namely high, medium and low. Each level was then spiked with vitamin D₃, 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃, 1,25(OH)₂D₃ and 24,25(OH)₂D₃ to obtain values that reflect close to the MDC for each analyte. These values are shown in Table 5.3. The spiked serum sample for each level was further aliquoted to have 15 samples per level. Per day, each level was analysed in triplicate from three individual samples. The sample preparation steps and LC-MS/MS specifications were done and set, respectively, according to the optimised method described in 4.5.

The values obtained reflect only vitamin D₃, 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃, 1,25(OH)₂D₃ and 24,25(OH)₂D₃ due to the fact that the D₂ form of all the analytes was found to be scarcely present and in low concentration in the pooled sample. But, 25(OH)D₂ was also included in the imprecision study because 25(OH)D₂ can be present in high concentration within a population with that consume vitamin D₂ fortified foods.

A Cochran C test and Dixon Q test were performed on the daily variances and daily averages respectively to assess the data for outliers. The within run and within-laboratory precision was subsequently calculated from the variance components.

3.4.4 INTERFERENCE STUDY

The amount of constant systematic error caused by interfering material within the sample can be estimated by means of an interference experiment (Westgard, 2008). This interference experiment involves two steps. The first step is to indicate possible ion suppression due to interfering material within the sample matrix. This is done by post column infusion of the sample matrix. The second step is to note the inaccurate quantification due to known interfering compounds with identical molecular masses as the analyte of interest. This is done by spiking sample material with known interfering compounds to note the effect on

the quantification of the analytes of interest (Annesley, 2003). Shah and co-workers (2011) indicated that the C3 epimer and isobars can account for as much as 55.3 % of the total 25(OH)D measured in a patient sample and inaccurate quantification due to these interfering analytes should be investigated.

As described in 3.3.3, protein precipitation followed by LLE was the sample preparation method of choice for this application. This sample preparation method results in a clean sample with very little interfering biological matrix (Musteata & Musteata, 2011). Salts found in biological matrices are known to cause ion suppression (Annesley, 2003). These salts have very little retention on analytical columns. As seen in the chromatogram from Figure 4.9, the first analyte of interest eluted at 3.75 minutes. Therefore, the MS diverter valve was configured to divert the eluting sample to waste from 0 to 3 minutes. This diversion effectively diverts the biological salts to waste, thus restricting the possibility of ion suppression due to interfering biological salts.

As seen in Figure 4.10 B, sufficient chromatographic separation was established between 24,25(OH)₂D₂ and 1,25(OH)₂D₂, 24,25(OH)₂D₃ and 1,25(OH)₂D₃, 25(OH)D₃ and isobars 1 α OH D₃ and 7(OH)4C3. Seen in Figure 4.11 A and 4.11 B, baseline separation was also achieved between 25(OH)D₂, 25(OH)D₃ and the relevant C3 epimers. Based on this evidence, no interference studies regarding the co-elution of these analytes were investigated. Due to the co-elution of 25(OH)D₂ and 3-epi-25(OH)D₃ an interference study was conducted to note any ion suppression of either analyte due to interference of the other.

This interference study was conducted by spiking 2 aliquots of pooled serum samples with high concentrations (100 ng/ml final concentration) of 25(OH)D₂ and 3-epi-25(OH)D₃, respectively analysed in triplicate. One aliquoted sample was not spiked and the possibility of ion suppression or enhancement was investigated. These samples were prepared based on the sample preparation steps and the LC-MS/MS conditions were set according to the optimised method described in 4.5.

3.4.5 STABILITY STUDY

Analyte instability can contribute to the total amount of random error seen for the method. Several analyte stability tests should be evaluated for the assessment of the analyte instability. These include freeze and thaw stability, short-term storage stability, long-term storage stability and post-preparative stability (Food and Drug Administration: Guidance for

industry, 2001). Literature is discussed in 2.8.5 and describes that vitamin D analogues are very stable under common pre-analytical conditions experienced in medical laboratories (Wielders and Wijnberg, 2009). The freeze and thaw stability, short-term storage stability and long-term storage stability of vitamin D analogues were also evaluated and all the analogues were found to be very stable (Lisser *et al.*, 1981; Hollis, 2008; Agborsangaya *et al.*, 2010).

Based on this evidence, the pre-analytical stability of the analytes included in this study was not evaluated. Post-preparative stability was assessed by preparing a sample (high, medium and low concentration) based on the sample preparation steps described in 4.5.1. These samples were spiked with the D₃ form of the vitamin D analogues due to the biological scarcity of the D₂. Each sample was analysed three times; 2, 6, 12, 24 and 48 hours after preparation and stored at -4 °C between analyses.

3.5 DATA ANALYSIS

Stable isotopes were used for the quantification of the vitamin D analogues to compensate for any losses during the sample preparation steps as well as possible ion suppression due to complex biological matrix. Only three deuterium marked stable isotopes were available for this study. These stable isotope included vitamin D₃-[²H₃], 25(OH)D₃-[²H₃] and 1,25(OH)₂D₃-[²H₃]. Thus, vitamin D₃, 25(OH)D₃ and 1,25(OH)₂D₃ were absolutely quantified with these isotopes while all the other analytes were relatively quantified according to the isotope with similar chemical properties. Vitamin D₂ were quantified relative to vitamin D₃-[²H₃]. 25(OH)D₂, 3-epi-25(OH)D₂, 3-epi-25(OH)D₃, 7(OH)4C3 and 1α(OH)D₃ were quantified relative to 25(OH)D₃-[²H₃]. 1,25(OH)₂D₂, 24,25(OH)₂D₂ and 24,25(OH)₂D₃ were quantified relative to 1,25(OH)₂D₃-[²H₃]. All the results were quantified using a multi-point calibration curve with the following mathematical approach:

$$x = ((y_{ANA} / y_{ISO}) - c) / m * [ISO]$$

where x: calculated analyte concentration

y_{ANA}: analyte abundance

y_{ISO}: selected stable isotope abundance

c: calibration curve y intercept

m: calibration curve slope

[ISO]: selected stable isotope concentration

CHAPTER 4

RESULTS & DISCUSSION

METHOD DEVELOPMENT & OPTIMISATION

4.1 BACKGROUND

The aim of this study was to implement an LC-MS/MS method for the quantification of vitamin D analogues from a human serum matrix. The D₂ and D₃ forms of vitamin D, 25(OH)D, 1,25(OH)₂D and 24,25(OH)₂D were included in this study. The C3 epimers and known isobars of 25(OH)D were also included to prevent the possibility of interferences and incorrect quantification of the true 25(OH)D concentration. The first part of this study was conducted to optimise the sample preparation steps for maximum recovery of each analyte, the tandem mass spectrometry parameters for analytical sensitivity and the optimisation of liquid chromatographic conditions for sufficient chromatographic separation between the analogues with similar chemical properties.

4.2 MASS SPECTROMETRY OPTIMISATION

4.2.1 PRECURSOR ION SELECTION

As described in 3.3.1.1, Adamec *et al.* (2011), El-Khoury *et al.* (2011) and Shah *et al.* (2012), reported the use of adducts as alternative precursor ions for the quantification of vitamin D analogues. The adduct precursor ion is dependent on the mobile phase and mobile phase modifiers. Thus, for the optimisation of a new assay, different precursor adduct ions for each of the analytes of interest should first be assessed. The most applicable precursor ion should be selected.

Each of the twelve analytes of interest as well as the three isotopes were individually injected. Mass spectrometer 2 was set to scan mode and the TIC of the infusion peak was assessed to find the most abundant precursor ions for each analyte. Figure 4.1 (A – O) illustrates the TIC of all the analytes with the x-axis displaying the mass-to-charge (m/z) ratio and the y-axis the counts of each m/z. Table 4.1 summarises the most abundant possible precursor adducts as seen in Figure 4.1.

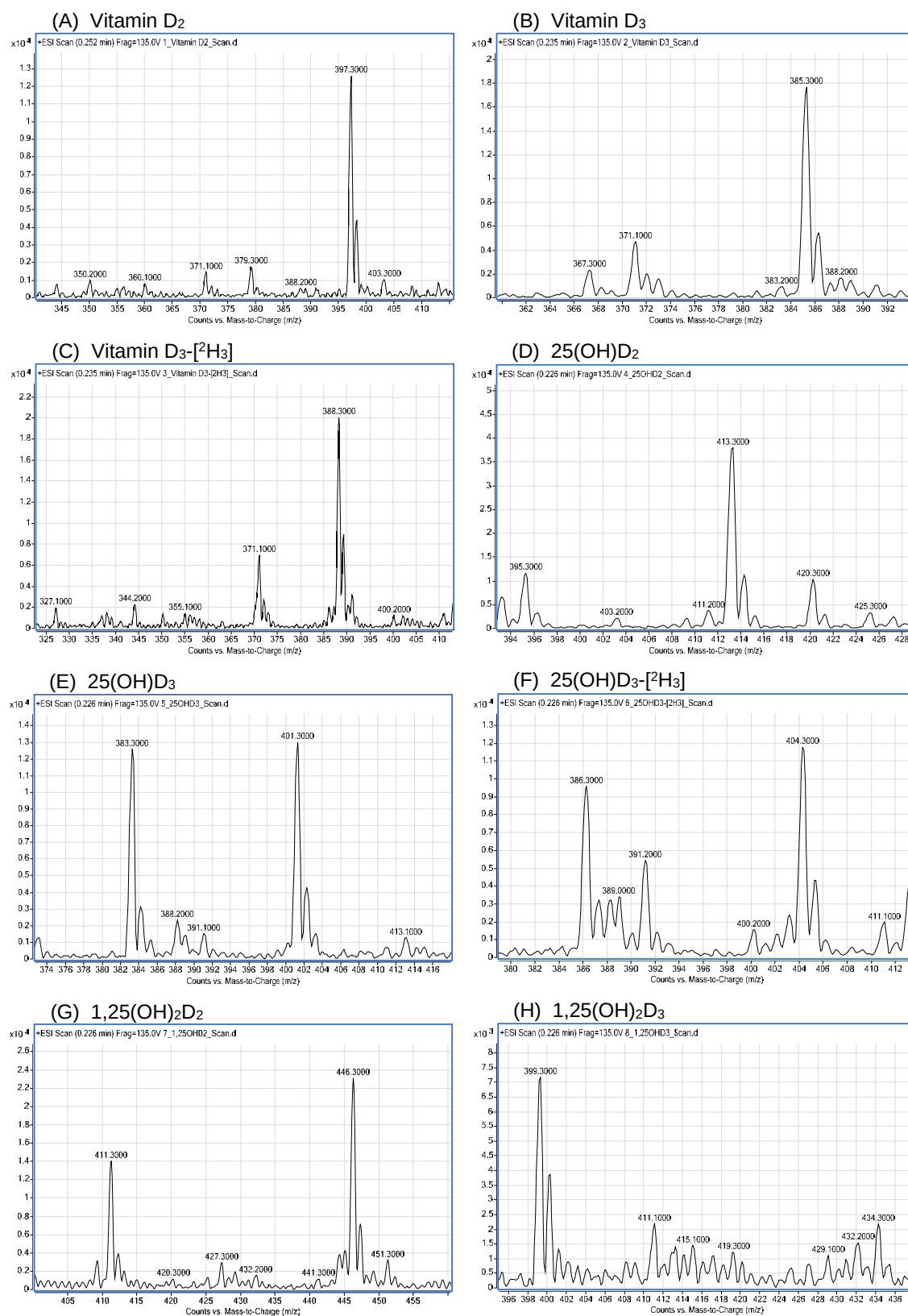


Figure 4.1 (A – H) Total ion chromatogram of vitamin D analogues for the assessment of possible precursor adducts.

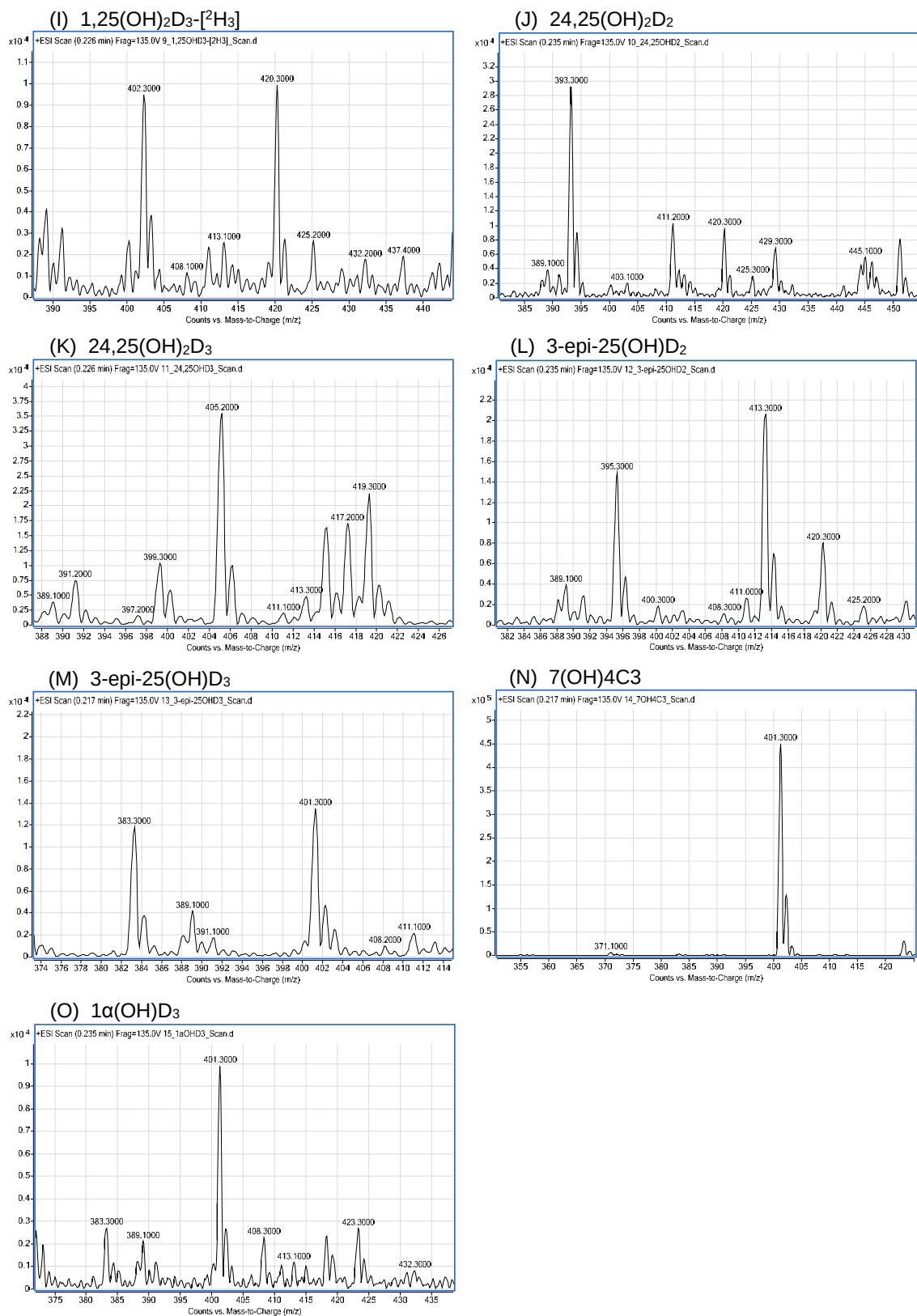


Figure 4.1 (I – O) Total ion chromatogram of vitamin D analogues for the assessment of possible precursor adducts.

Table 4.1 Summary of possible vitamin D analogue precursor adducts.

Analyte	Mass-to-charge (m/z) ratio	Adduct
Vitamin D ₂	397.3	[M+H] ⁺
	379.2	[M+H] ⁺ -[H ₂ O]
Vitamin D ₃	385.3	[M+H] ⁺
	367.3	[M+H] ⁺ -[H ₂ O]
Vitamin D ₃ -[² H ₃]	388.3	[M+H] ⁺
	370.3	[M+H] ⁺ -[H ₂ O]
25(OH)D ₂	413.3	[M+H] ⁺
	395.3	[M+H] ⁺ -[H ₂ O]
25(OH)D ₃	401.3	[M+H] ⁺
	383.3	[M+H] ⁺ -[H ₂ O]
25(OH)D ₃ -[² H ₃]	404.3	[M+H] ⁺
	386.3	[M+H] ⁺ -[H ₂ O]
1,25(OH) ₂ D ₂	429.3	[M+H] ⁺
	411.3	[M+H] ⁺ -[H ₂ O]
	446.4	[M+H] ⁺ + [NH ₃]
1,25(OH) ₂ D ₃	417.3	[M+H] ⁺
	399.3	[M+H] ⁺ -[H ₂ O]
	434.4	[M+H] ⁺ + [NH ₃]
1,25(OH) ₂ D ₃ -[² H ₃]	420.3	[M+H] ⁺
	402.3	[M+H] ⁺ -[H ₂ O]
	437.4	[M+H] ⁺ + [NH ₃]
24,25(OH) ₂ D ₂	429.3	[M+H] ⁺
	411.3	[M+H] ⁺ -[H ₂ O]
	393.3	[M+H] ⁺ -2[H ₂ O]
24,25(OH) ₂ D ₃	417.3	[M+H] ⁺
	399.3	[M+H] ⁺ -[H ₂ O]
3-epi-25(OH)D ₂	413.3	[M+H] ⁺
	385.3	[M+H] ⁺ -[H ₂ O]
3-epi-25(OH)D ₃	401.3	[M+H] ⁺
	383.3	[M+H] ⁺ -[H ₂ O]
7(OH)4C3	401.3	[M+H] ⁺
	383.3	[M+H] ⁺ -[H ₂ O]
1α(OH)D ₃	401.3	[M+H] ⁺
	383.3	[M+H] ⁺ -[H ₂ O]
	418.3	[M+H] ⁺ + [NH ₃]

From Table 4.1 it is notable that alternative precursor adducts may be abundant relative to the molecular mass precursor ion $[M+H]^+$. It is evident that the molecular mass ion $[M+H]^+$ and the water loss adduct $[M+H]^+-[H_2O]$ were the most abundant precursor adducts for all the analytes. The ammonium adduct $[M+H]^++[NH_3]$ was abundant for $1\alpha(OH)D_3$ and the $1,25(OH)_2D$ analytes while $24,25(OH)_2D_2$ was the only analyte that had an abundant double water loss precursor adduct $[M+H]^+-2[H_2O]$.

To compare these possible precursor adducts, another infusion of all the analytes was done. For this infusion, MS2 was set to SIM mode to compare the abundances of these selected precursor adducts for each analyte. The SIM chromatogram for each analyte is shown in Figure 4.2 with the x-axis displaying the m/z and the y-axis the counts of each m/z.

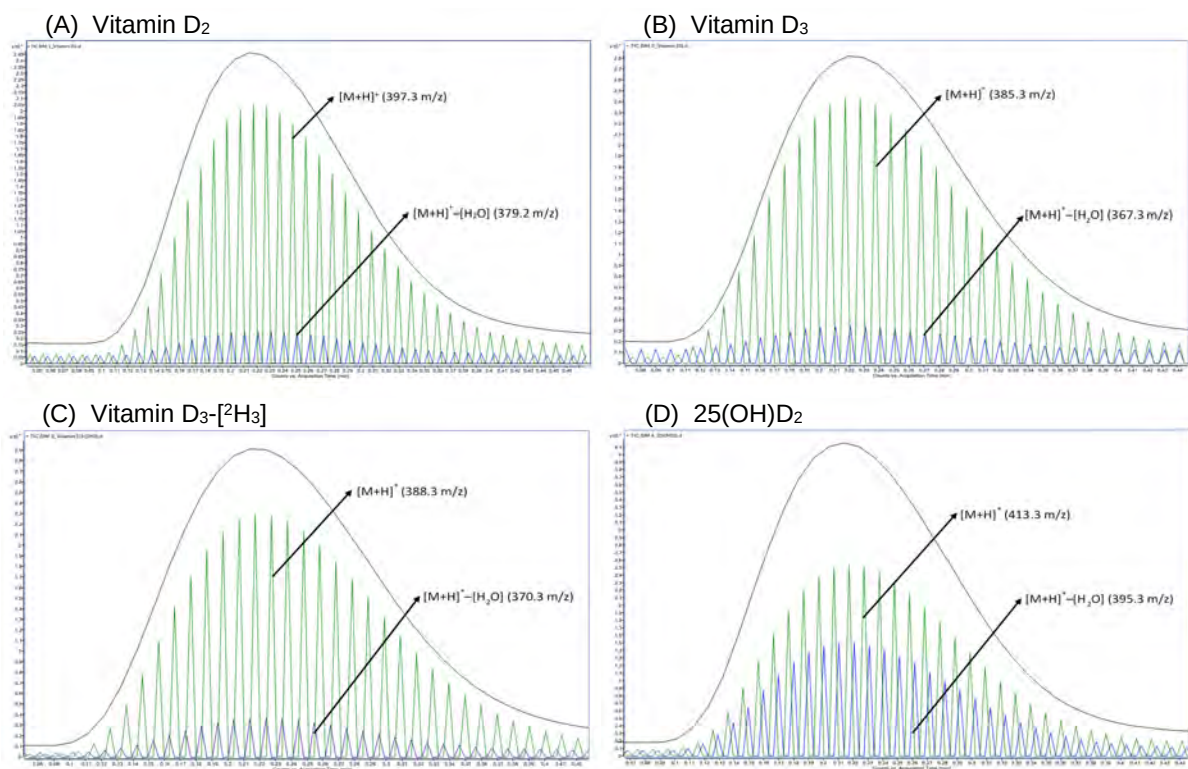
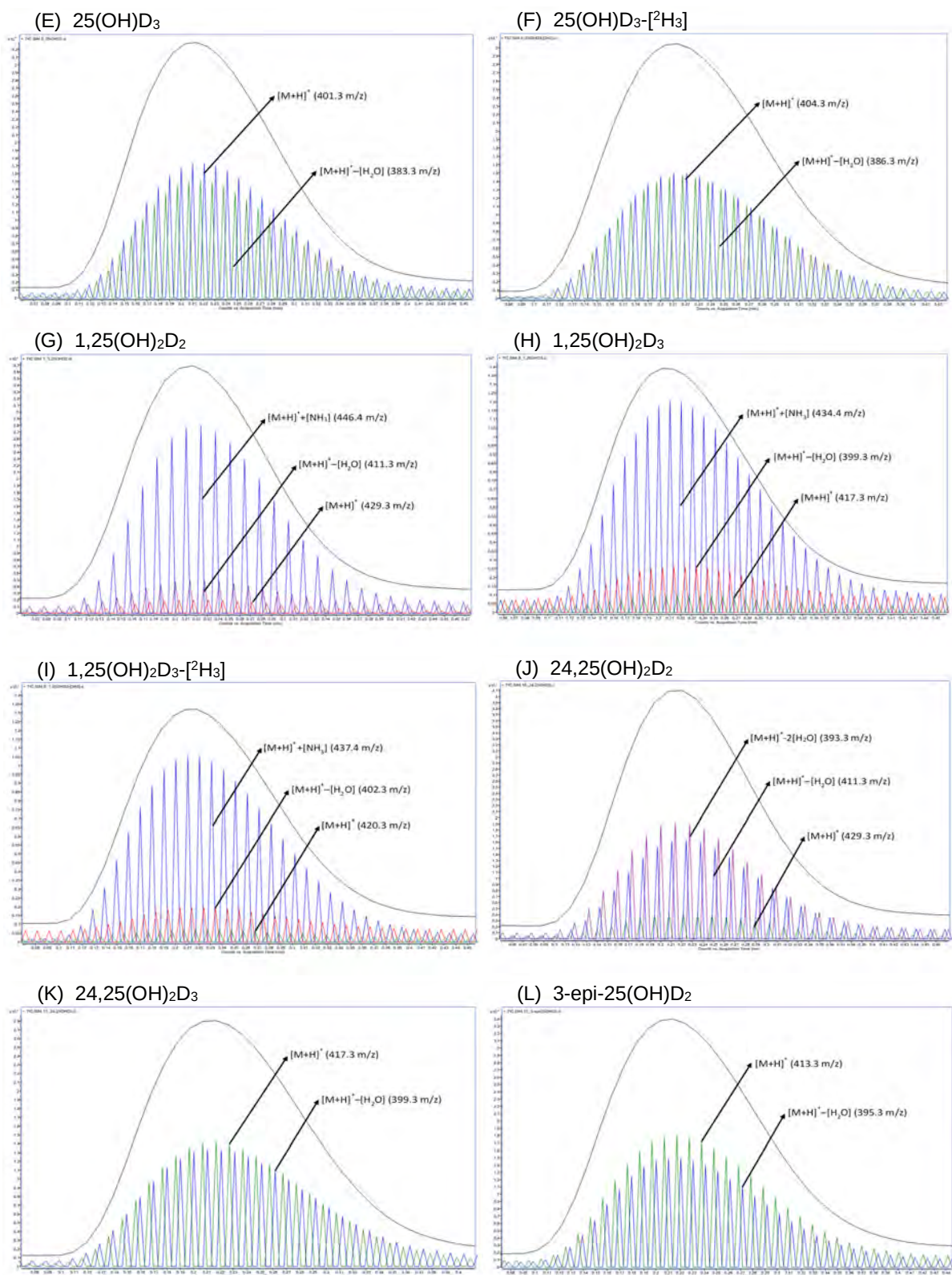


Figure 4.2 (A – D) Selective ion monitoring chromatograms for the comparison of vitamin D analogue precursor adducts.



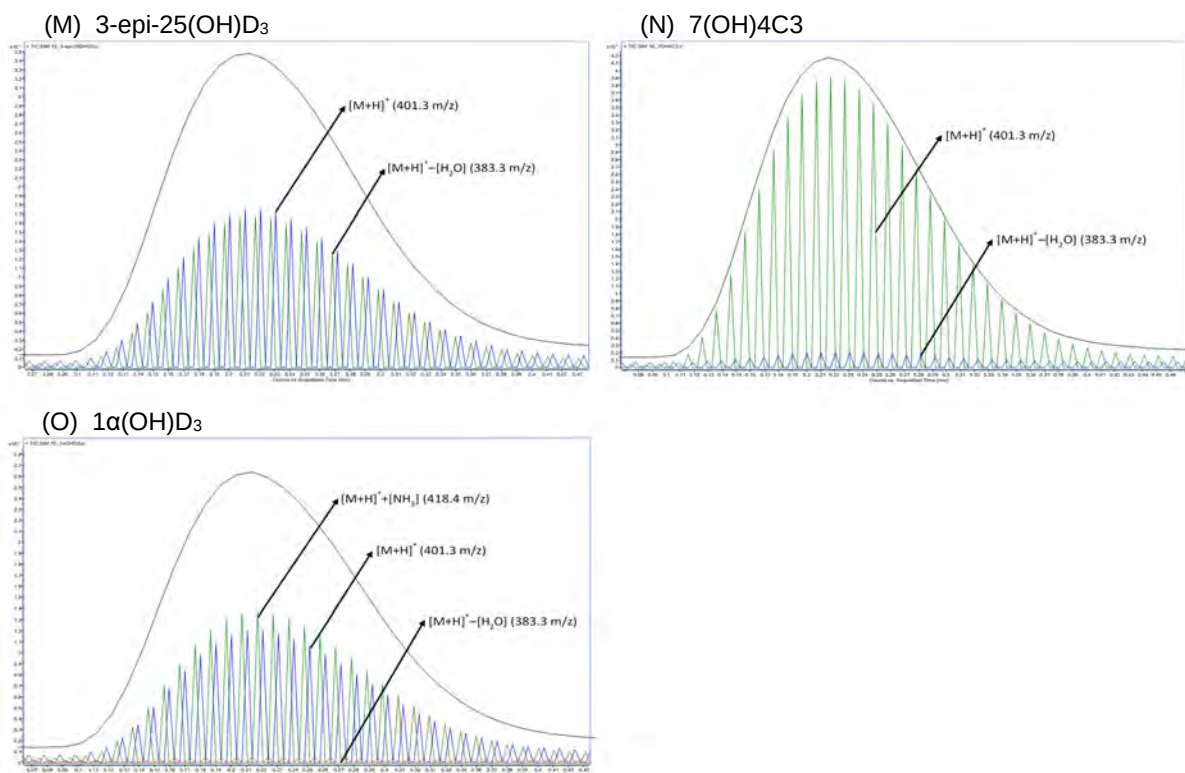


Figure 4.2 (M – O) Selective ion monitoring chromatograms for the comparison of vitamin D analogue precursor adducts.

The final selection of a single precursor adduct was performed by comparing the most abundant precursor adducts as previously described. The SIM chromatograms seen in Figure 4.2 (A - O) illustrate these comparisons. From the SIM chromatogram of each analyte the single most abundant precursor adduct was selected. These selected precursor adducts are shown in the following table.

Table 4.2 Selected precursor adduct for each vitamin D analogue.

Analyte	Mass-to-charge (m/z) ratio	Selected precursor adduct
Vitamin D ₂	397.3	[M+H] ⁺
Vitamin D ₃	385.3	[M+H] ⁺
Vitamin D ₃ -[² H ₃]	388.3	[M+H] ⁺
25(OH)D ₂	413.3	[M+H] ⁺
25(OH)D ₃	401.3	[M+H] ⁺
25(OH)D ₃ -[² H ₃]	404.3	[M+H] ⁺
1,25(OH) ₂ D ₂	446.4	[M+H] ⁺ + [NH ₃]
1,25(OH) ₂ D ₃	434.4	[M+H] ⁺ + [NH ₃]
1,25(OH) ₂ D ₃ -[² H ₃]	437.4	[M+H] ⁺ + [NH ₃]
24,25(OH) ₂ D ₂	393.3	[M+H] ⁺ - 2[H ₂ O]
24,25(OH) ₂ D ₃	417.3	[M+H] ⁺
3-epi-25(OH)D ₂	413.3	[M+H] ⁺
3-epi-25(OH)D ₃	401.3	[M+H] ⁺
7(OH)4C3	401.3	[M+H] ⁺
1α(OH)D ₃	418.4	[M+H] ⁺ + [NH ₃]

The ammonium adduct was selected as the precursor adduct for 1,25(OH)₂D₂, 1,25(OH)₂D₃, 1,25(OH)₂D₃-[²H₃] and 1αOHD₃. Furthermore the molecular mass ion was selected for the rest of the analytes except for 24,25(OH)₂D₂ for which the double water loss adduct was chosen. A review by El-Khoury and co-workers (2011) summarises the use of different precursor adducts for similar applications measuring vitamin D analogues. Similar to the results found with the precursor selection in this study, all the applications measuring underivatised vitamin D analogues used the molecular mass precursor ion [M+H]⁺ for vitamin D₂, vitamin D₃, 25(OH)D₂ and 25(OH)D₃ quantification. Kissmeyer and Sonne (2001) describes the quantification of 1,25(OH)₂D₃ by means of LC-MS/MS with the ammonium adduct [M+H]⁺+ [NH₃] as the selected precursor adduct, while Beacher and co-workers (2012) reported the use of the molecular mass ion [M+H]⁺ as selected precursor ion for the quantification of 24,25(OH)₂D₃ and 3-epi-25(OH)D₃. This is similar to the results found in this study regarding the selection of the precursor adducts. The selected precursor adducts for each analyte, as shown in Table 4.2, were used onwards for the optimisation of the source parameters and the MRM transition parameters.

4.2.2 SOURCE OPTIMISATION

As described in 3.3.1.2, the aim of optimising the source parameters is to intensify the MS1 signal for each analyte precursor adduct. This is achieved by optimising each of the underlying source parameters, both compound and mobile phase dependent parameters, to ensure the most effective desolvation and ionisation possible. Section 3.3.1.2 describes the different source parameters included in the optimisation process as well as the FIA program used to automate the optimisation of each parameter. A summary of the FIA program is shown in the following table.

Table 4.3 Summary of flow injection analysis program for the optimisation of source parameters.

Segment	Drying Gas Flow (L/min)	Nebuliser Pressure (psi)	Capillary Voltage (V)	Nozzle Voltage (V)	Sheath Gas Flow (L/min)
1	(D) 6	15	4000	1000	7
2	4	15	4000	1000	7
3	9	15	4000	1000	7
4	12	15	4000	1000	7
5	6	(D) 15	4000	1000	7
6	6	10	4000	1000	7
7	6	30	4000	1000	7
8	6	60	4000	1000	7
9	6	15	(D) 4000	1000	7
10	6	15	2000	1000	7
11	6	15	5000	1000	7
12	6	15	6000	1000	7
13	6	15	4000	(D) 1000	7
14	6	15	4000	0	7
15	6	15	4000	1500	7
16	6	15	4000	2000	7
17	6	15	4000	1000	(D) 7
18	6	15	4000	1000	9
19	6	15	4000	1000	10
20	6	15	4000	1000	11

Through multiple infusions of a single analyte, with MS2 set to SIM mode, monitoring the specific precursor adduct selected, the following SIM chromatograms were obtained and shown in Figure 4.3 (A – O). By comparing the abundances of the four analyte peaks from four relevant segments, the specific parameter setting resulting in the most abundant peak area was chosen. These figures illustrate the difference in ion abundances as the result of the different ESI source parameter settings, while Table 4.4 shows the integrated data for each analyte peak.

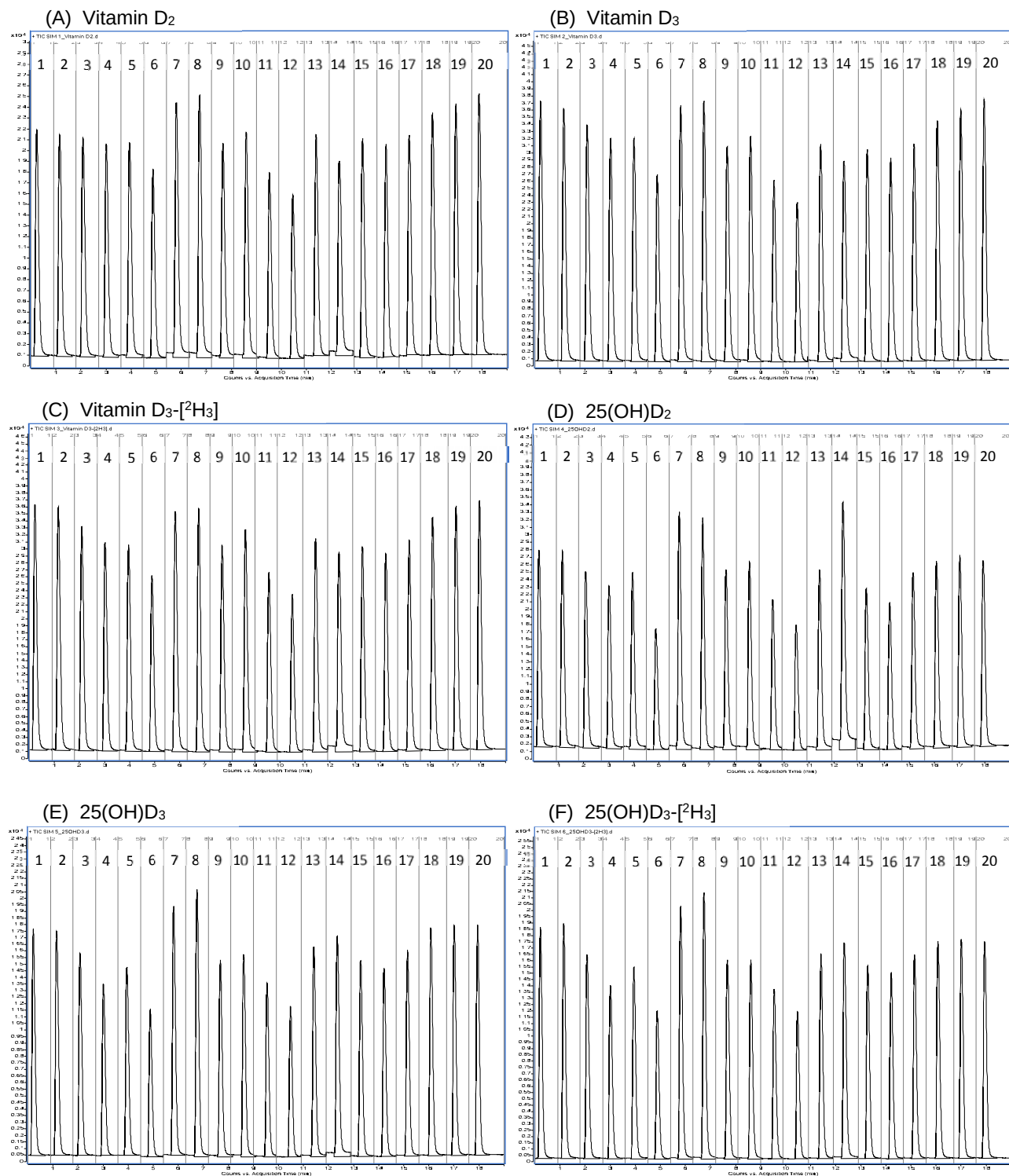


Figure 4.3 (A – F) Selective ion monitoring chromatograms for the optimisation of electrospray ionisation parameters by means of a flow injection analysis program.

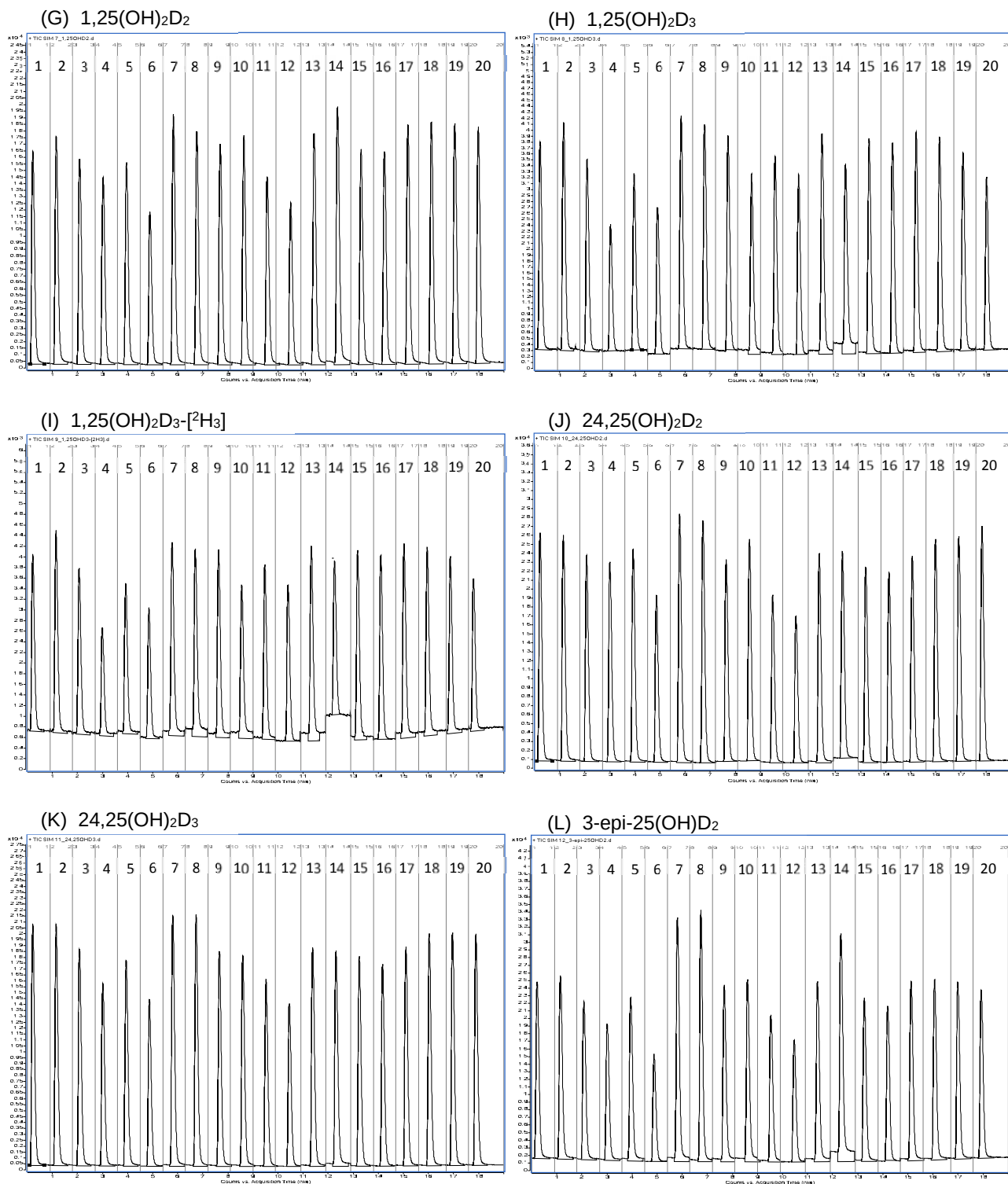


Figure 4.3 (G – L) Selective ion monitoring chromatograms for the optimisation of electrospray ionisation parameters by means of a flow injection analysis program.

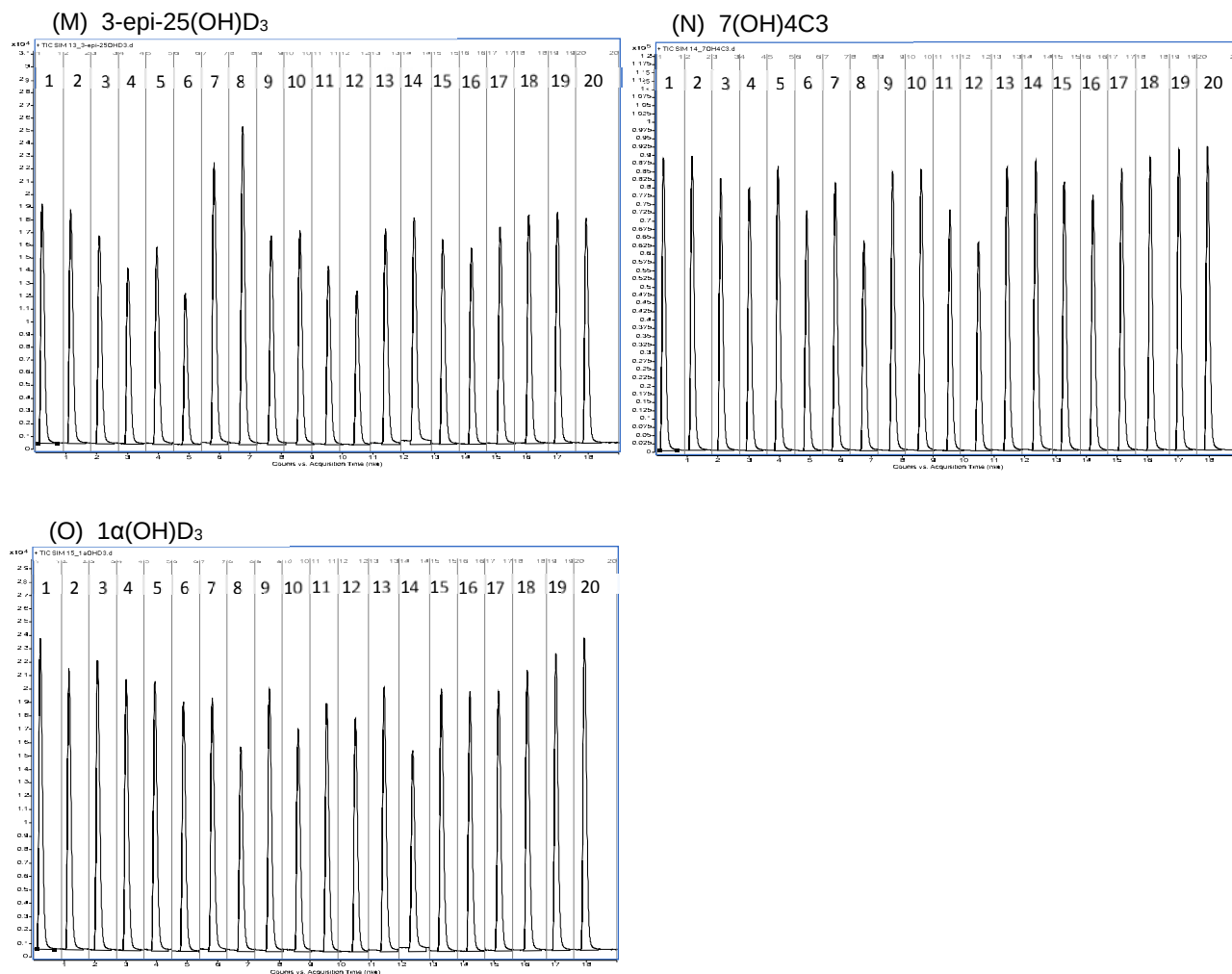


Figure 4.3 (M – O) Selective ion monitoring chromatograms for the optimisation of electrospray ionisation parameters by means of a flow injection analysis program.

Table 4.4 Selective ion monitoring abundance data for the optimisation of electrospray ionisation parameters by means of a flow injection analysis program.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Analyte:	Gas Flow (L/min)				Nebulizer Pressure (psi)				Capillary Voltage (V)				Nozzle Voltage (V)				Sheath Gas Flow (L/min)			
	(D) 6	4	9	12	(D) 15	10	30	60	(D) 4000	2000	5000	6000	(D) 1000	0	1500	2000	(D) 7	9	10	11
Vitamin D ₂	188503	181859	179354	172598	175051	150254	205367	207620	173343	179469	148535	132052	179610	163973	174995	169427	179267	198265	204182	212929
Vitamin D ₃	314874	308146	286644	272674	271649	225618	308957	309391	260875	273148	223879	194809	266746	257047	257086	247923	265973	293552	306837	316865
Vitamin D ₃ -[₂ H ³]	302247	295576	276588	259012	254921	214674	294253	296365	252371	268412	219994	193779	261040	253901	251649	241017	259818	289221	301409	308593
25(OH)D ₂	222085	221179	201914	183921	197648	138611	255427	244596	199193	206774	169003	143382	202745	276211	182680	167811	201204	215170	219814	216130
25(OH)D ₃	141054	139461	127737	107569	119373	92283	155469	162288	123183	124906	106175	92416	128932	139055	122069	116869	128683	139585	141247	140296
25(OH)D ₃ -[₂ H ³]	146401	146542	134173	112953	125320	97077	163088	169728	130373	130893	111564	97133	134886	145337	126856	121495	134013	143829	145132	143478
1,25(OH) ₂ D ₂	132184	143104	128022	116266	125189	96940	152985	140019	136959	141493	116411	102639	144386	164334	134491	133673	150650	151471	152301	149475
1,25(OH) ₂ D ₃	27558	30378	25511	16626	23428	19574	31245	30262	28723	23645	26988	24018	29210	24495	28877	28132	29496	28563	26497	22864
1,25(OH) ₂ D ₃ -[₂ H ³]	25878	29486	24094	15043	21580	18475	28191	26882	27100	21672	25297	23094	28133	22891	26723	26994	28104	27355	25058	21728
24,25(OH) ₂ D ₂	206666	205219	188845	182782	192940	149934	223785	217263	183757	201779	152747	132782	189248	194006	177275	171518	186954	201830	205320	213218
24,25(OH) ₂ D ₃	165458	165367	148357	124495	142482	113678	172077	171046	147791	144608	127961	112656	150866	151292	143769	138314	151986	158990	161593	159865
3-epi-25(OH)D ₂	196163	202085	176325	150155	177459	118426	255837	261290	191501	194417	161256	136267	195950	246420	176945	169434	195452	197404	195013	185743
3-epi-25(OH)D ₃	153673	151911	135051	113802	128809	97070	180006	199863	136303	136965	117127	100813	141069	151231	132891	126660	141529	149245	151290	146237
7OH4C3	684816	682324	639270	612856	666587	564920	630110	494729	651843	661590	561137	488999	667274	683851	632472	601785	665883	692874	711026	717127
1αOHD ₃	181634	164519	170203	159541	159368	147576	149599	121603	155351	131098	145392	137239	156361	116460	155074	154418	154206	165142	176941	185322

(°C): degree Celsius, (L/min): litre per minute, (psi): pound per square inch, (V): voltage.

Figure 4.3 illustrates the effect of specific changes in ESI source parameters on the signal of the fifteen analytes. By comparing the abundances of the parameter specific segments the effect of changing these specific parameters can be seen.

Segments 1 – 4 show the effect of varying drying gas flow; 6 (default), 4, 9 and 12 L/min respectively. The purpose of the drying gas is to ensure efficient desolvation of the sample material and solvent. The data provided in Table 4.4 shows that the flow of the drying gas have a great effect on the abundance of the analytes recorded by MS1. It is notable that the analyte abundance decreases with a higher gas flow rate. The flow rate of the drying gas is a function of the mobile phase flow rate and the composition of the mobile phase being used and should be considered when a final method is set. The drying gas parameters should also not be assessed alone due to the uniqueness of this ESI source that is enhanced with AJS technology. The sheath gas, which is unique to the AJS ESI source, collimates the nebuliser spray, resulting in a brighter source with more ions and fewer solvent droplets. When observing the results of both the drying gas flow and the sheath gas flow (segment 17 – 20; 7 (default), 9, 10 and 11 L/min respectively) it can be seen that sufficient desolvation was achieved with lower drying gas flow and higher sheath gas flow. Again this is a function of the composition and flow rate of the mobile phase and should be brought into consideration. The nebuliser pressure ensures that the mobile phase and sample are sprayed in a fine mist with even droplet size. Segments 5 – 8 (15 (default), 10, 30 and 60 psi respectively) show that for most of the analytes a higher abundance was achieved with higher nebuliser pressure. The dihydroxylated analytes, 1,25(OH)₂D₂, 1,25(OH)₂D₃, 24,25(OH)₂D₂ and 24,25(OH)₂D₃ as well as the two isobars are exceptions, with a slightly higher abundance achieved with lower nebuliser pressure. The nebuliser pressure affects the nebulisation efficiency and is thus also influenced by the flow rate of the mobile phase. During positive ESI the nozzle and capillary voltage ensures the positive charging of the desolvated droplets. As seen in Table 4.4 a nozzle voltage (segments 13 – 16; 1000 (default), 0, 1500 and 200 V, respectively) of 1000 V resulted in higher abundances for the majority of the samples. 25(OH)D₂, 25(OH)D₃ and their C3 epimers were exceptions, with higher abundances seen with a nozzle voltage of 0 V. Higher abundances for the dihydroxylated analytes and the 1 α (OH)D₃ were seen when a capillary voltage of 4000 V was applied. For the rest of the analytes higher abundances were achieved with a lowered capillary voltage of 2000 V.

The drying gas and sheath gas temperatures were optimised individually due to the time needed for the temperature fluctuations to stabilise. By overlaying the SIM chromatograms obtained for each temperature, specific to each gas, the results shown in Figures 4.4 (A – O)

and 4.5 (A –O) were acquired. The x-axis of the SIM chromatograms display the acquisition time in minutes and the y-axis the counts or abundances of the analyte peaks. For accurate comparisons regarding the effect of the varying temperatures on the analyte MS1 signal the SIM chromatograms were integrated and this data is shown in Table 4.5.

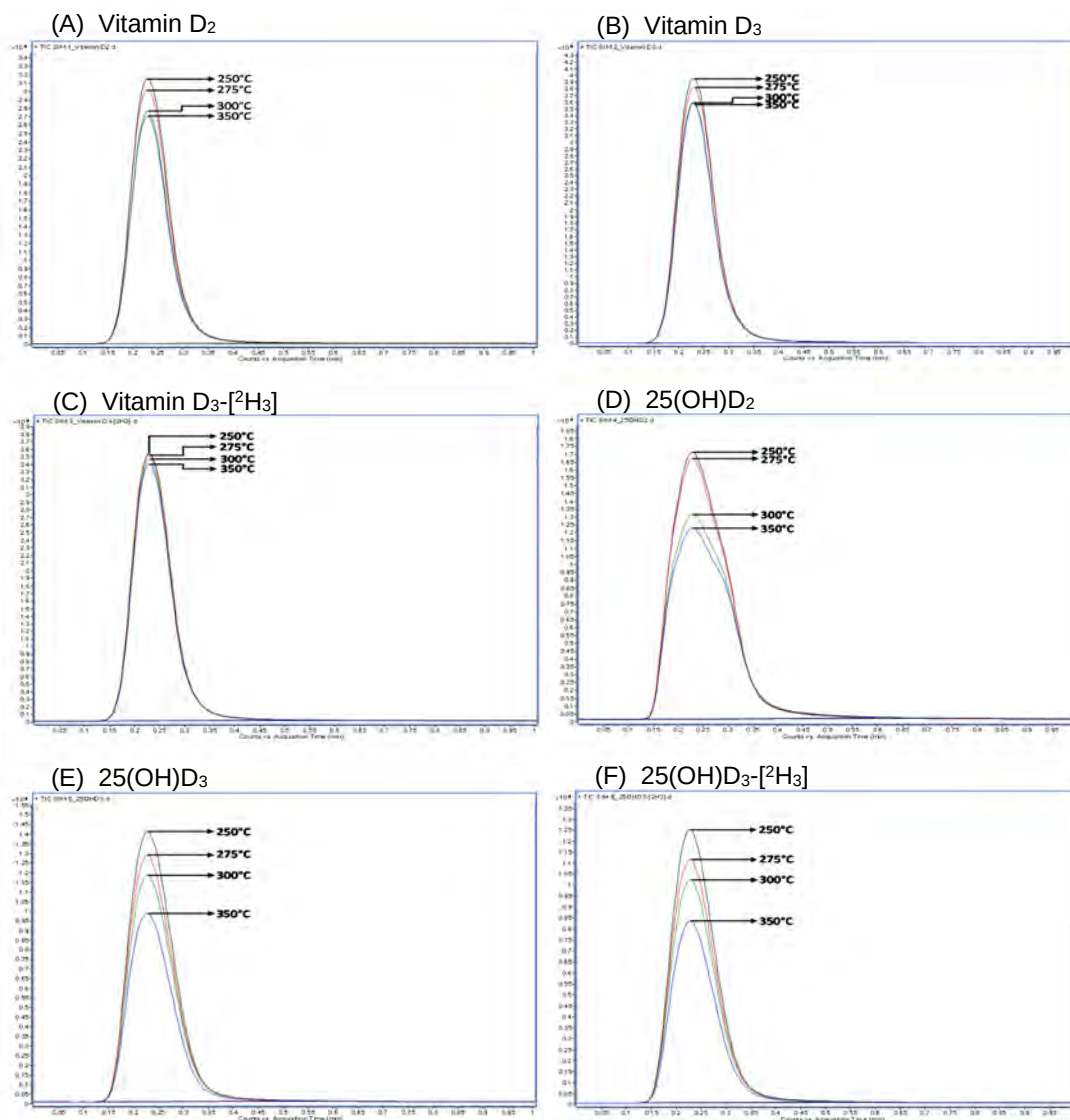


Figure 4.4 (A – F) Selective ion monitoring chromatograms for the optimisation of drying gas temperatures.

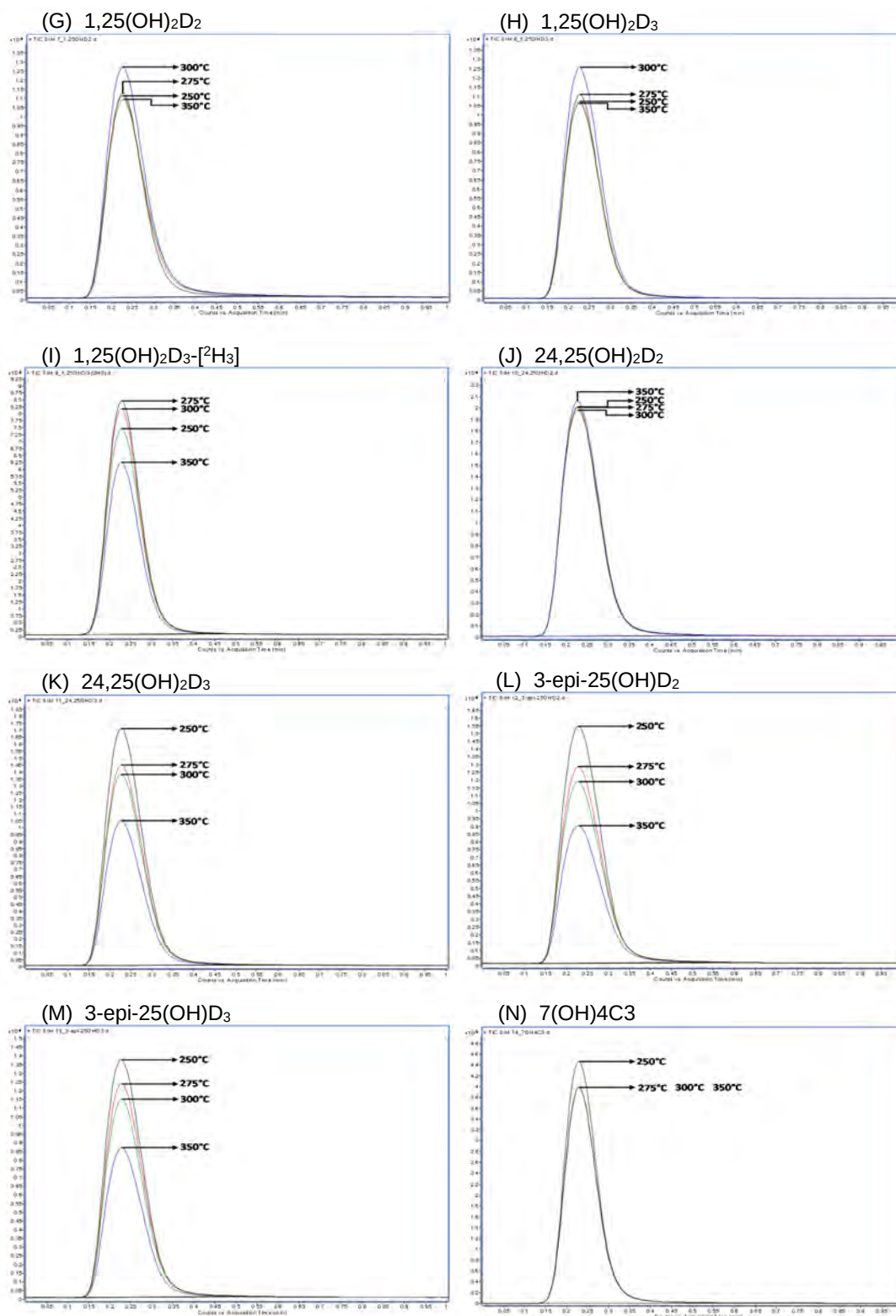


Figure 4.4 (G – N) Selective ion monitoring chromatograms for the optimisation of drying gas temperatures.

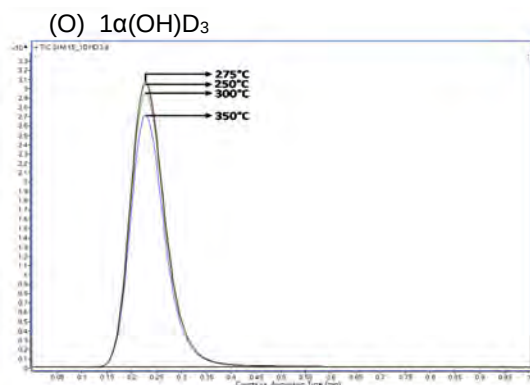


Figure 4.4 (O) Selective ion monitoring chromatograms for the optimisation of drying gas temperatures.

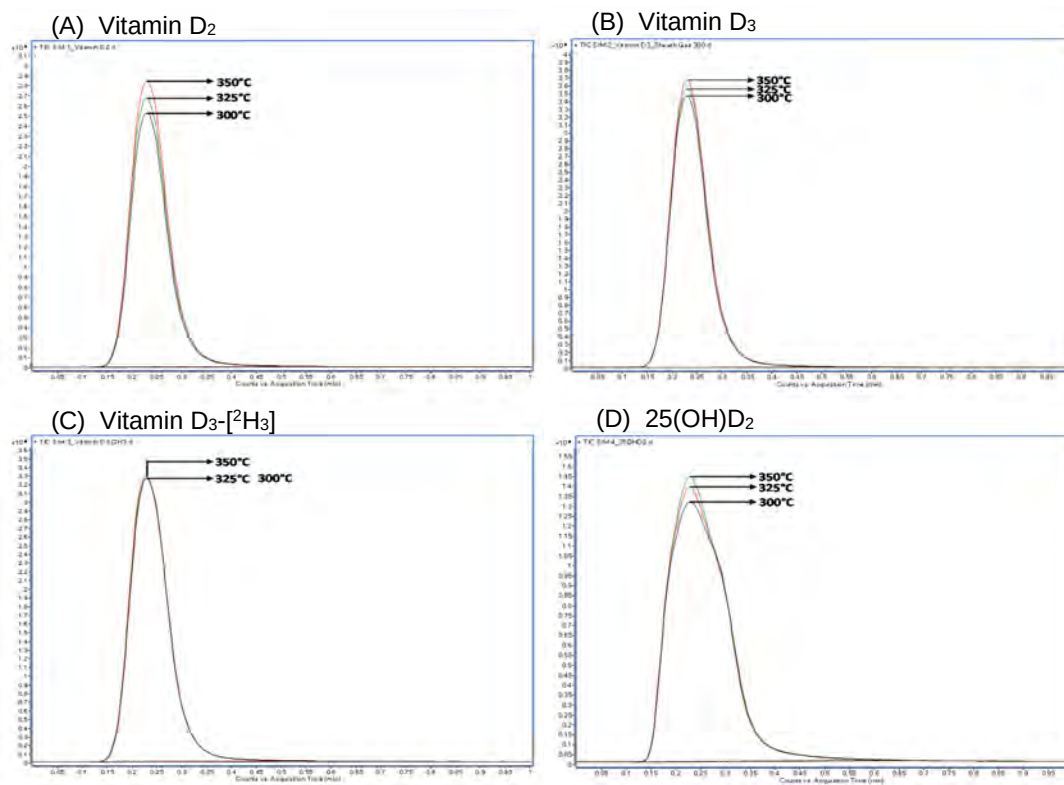


Figure 4.5 (A – D) Selective ion monitoring chromatograms for the optimisation of sheath gas temperatures.

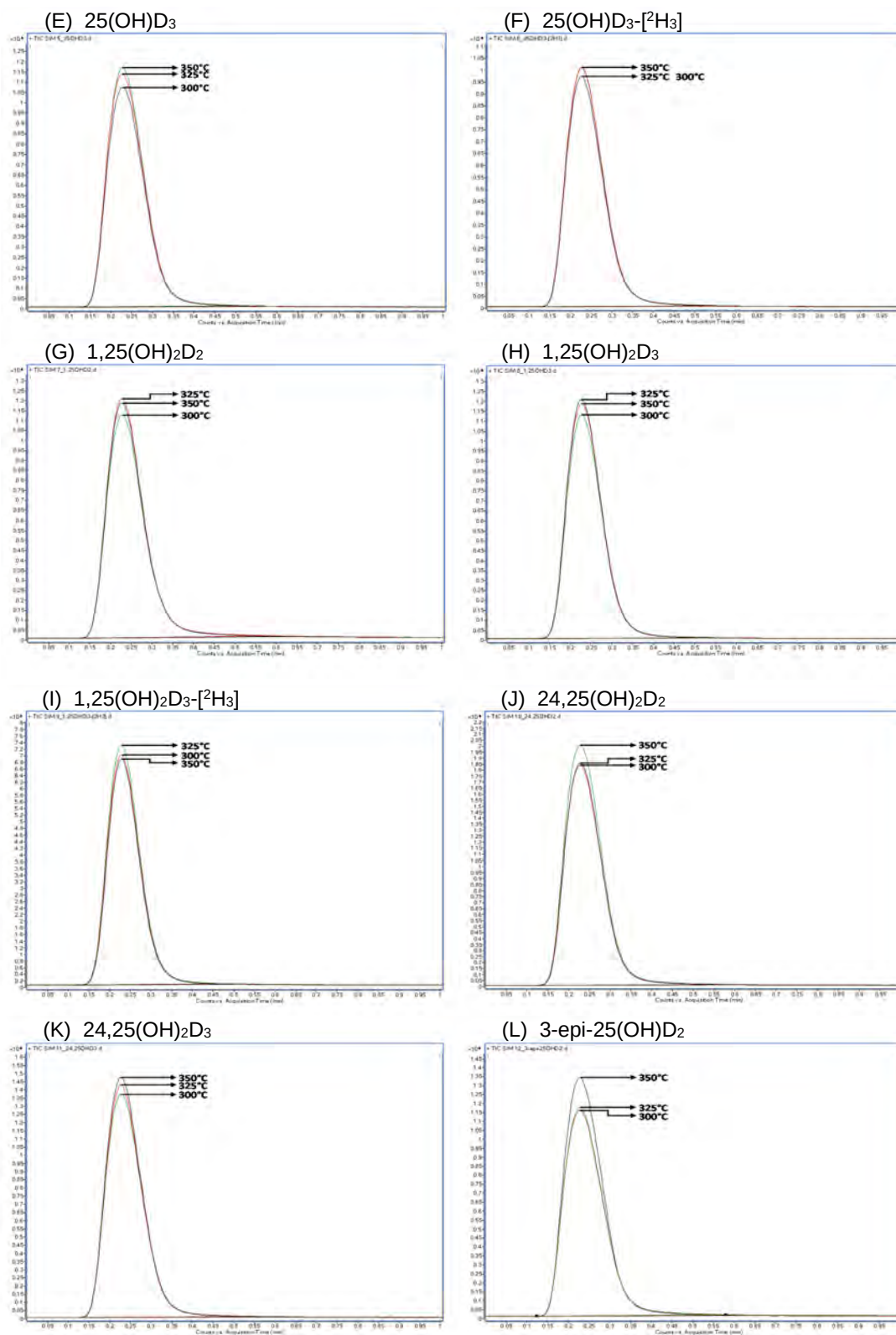


Figure 4.5 (E – L) Selective ion monitoring chromatograms for the optimisation of sheath gas temperatures.

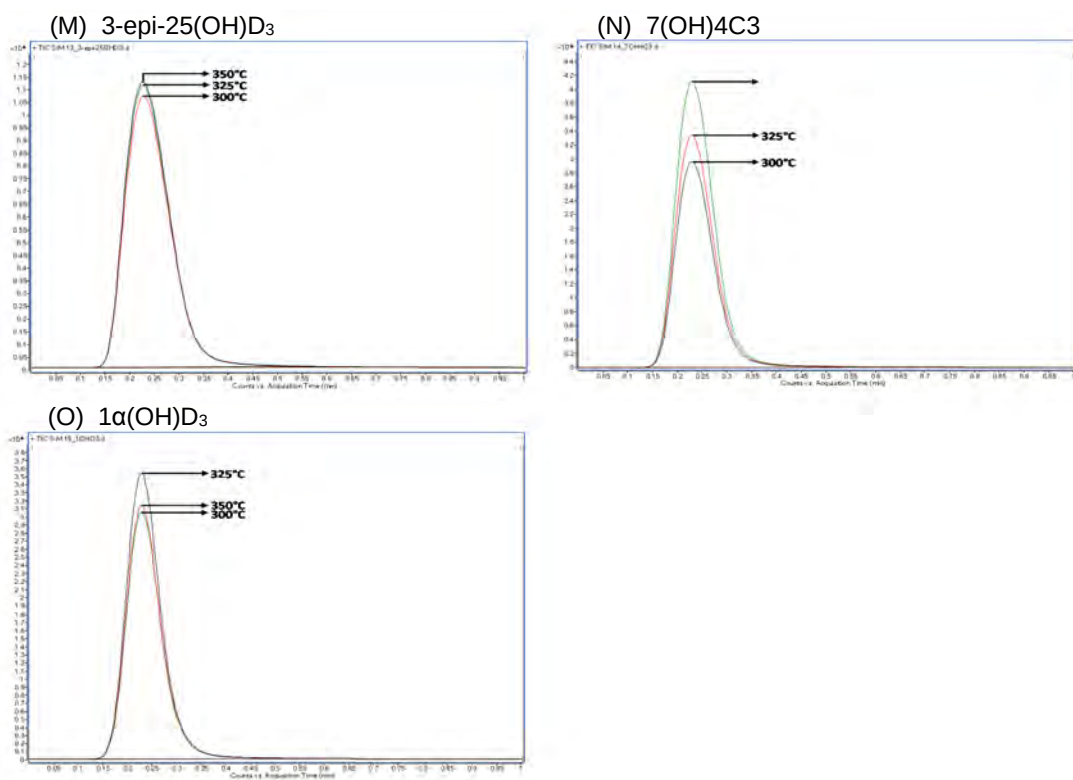


Figure 4.5 (M – O) Selective ion monitoring chromatograms for the optimisation of sheath gas temperatures.

Table 4.5 Selective ion monitoring abundance data for the optimisation of gas temperatures.

Analyte	Drying Gas Temperature (°C)				Sheath Gas Temperature (°C)		
	250	275	300	350	300	325	350
Vitamin D ₂	166990	161905	145165	141492	132322	142292	150539
Vitamin D ₃	209930	203479	188476	186009	181855	188959	193043
Vitamin D ₃ -[² H ₃]	193061	193691	187945	183110	175693	177893	179459
25(OH)D ₂	131984	130011	110785	104522	110205	113168	115458
25(OH)D ₃	88050	81044	74578	64509	67947	71881	73056
25(OH)D ₃ -[² H ₃]	77699	70219	64366	52421	60896	63389	63458
1,25(OH) ₂ D ₂	71345	74126	82126	69330	70381	75388	74387
1,25(OH) ₂ D ₃	65688	63968	76186	64973	68166	71445	71270
1,25(OH) ₂ D ₃ -[² H ₃]	41935	46976	45114	34523	38990	40665	37689
24,25(OH) ₂ D ₂	124664	125098	125476	129382	115529	116086	125755
24,25(OH) ₂ D ₃	105102	90603	86514	65037	85957	87967	90181
3-epi-25(OH)D ₂	99537	85125	79457	60187	77373	78525	87230
3-epi-25(OH)D ₃	86652	78664	72504	54610	68119	70379	71442
7(OH)4C3	2400583	2182296	2187756	2183254	1603229	1831102	2238748
1α(OH)D ₃	152825	151119	146779	133736	152532	175690	157529

Figures 4.4 (A – O) and 4.5 (A – O) illustrate the effect of changes in the drying gas and sheath gas temperatures on the abundances of the analyte precursor adducts recorded by MS1. As seen in the data shown in Table 4.5, the majority of the analytes had a higher abundance at a lowered drying gas temperature of 250 °C. 1,25(OH)₂D₂, 1,25(OH)₂D₃ and 24,25(OH)₂D₂ were exceptions and showed higher abundances with a drying gas temperature of 300 °C. The data shown in Table 4.5 and the illustrations in Figure 4.5, show that the opposite was seen with regard to the effect of changes in sheath gas temperature. All the analytes were more abundant at a higher sheath gas temperature of 325 °C or more. It is also notable that in general, the drying gas temperature had a larger effect on the analyte abundance than the sheath gas temperature. Both the drying gas and sheath gas flow and temperatures, are functions of mobile phase composition and flow rate and should be brought into consideration with the final selection of the MS parameters.

A summary of the optimised ESI parameter settings are shown in the Table 4.6.

Table 4.6 Summary of the optimised electrospray ionisation source conditions for each analyte.

Analyte:	Gas Temp (°C)	Gas Flow (L/min)	Nebuliser Pressure (psi)	Capillary Voltage (V)	Nozzle Voltage (V)	Sheath Gas Temp (°C)	Sheath Gas Flow (L/min)
Vitamin D ₂	250	6	60	2000	1000	350	11
Vitamin D ₃	250	6	60	2000	1000	350	11
Vitamin D ₃ -[² H ₃]	250	6	60	2000	1000	350	11
25(OH)D ₂	250	6	60	2000	0	350	10
25(OH)D ₃	250	6	60	2000	0	350	10
25(OH)D ₃ -[² H ₃]	250	6	60	2000	0	350	10
1,25(OH) ₂ D ₂	300	4	30	4000	1000	325	7
1,25(OH) ₂ D ₃	300	4	30	4000	1000	325	7
1,25(OH) ₂ D ₃ -[² H ₃]	275	4	30	4000	1000	325	7
24,25(OH) ₂ D ₂	350	4	30	4000	1000	350	7
24,25(OH) ₂ D ₃	250	4	30	4000	1000	350	7
3-epi-25(OH)D ₂	250	6	60	2000	0	350	10
3-epi-25(OH)D ₃	250	6	60	2000	0	350	10
7(OH)4C3	250	6	15	2000	1000	350	11
1αOHD ₃	275	6	15	4000	1000	325	11

(°C): degree Celsius, (L/min): litre per minute, (psi): pound per square inch, (V): voltage.

The optimum drying gas temperature and drying gas flow rate for the majority of the analytes were 250 °C and 6 L/min respectively. The following were exceptions: 1,25(OH)₂D₂ (300 °C, 4 L/min), 1,25(OH)₂D₃ (300 °C, 4 L/min), 1,25(OH)₂D₃-[²H₃] (275 °C, 4 L/min), 24,25(OH)₂D₂ (350 °C, 4 L/min) and 1α(OH)D₃ (275 °C, 6 L/min). Although the optimum drying gas temperature for these five analytes was different than those of the other analytes, the data suggest that the analyte abundance will not be greatly affected when applying 250 °C. The five dihydroxylated analytes (1,25(OH)₂D₂, 1,25(OH)₂D₃, 1,25(OH)₂D₃-[²H₃], 24,25(OH)₂D₂ and 24,25(OH)₂D₃) showed optimum analyte abundance with a nebuliser pressure of 30 psi, the two isobars (7(OH)4C3 and 1α(OH)D₃) with a nebuliser pressure of 15 psi and the rest of the analytes with a nebuliser pressure of 60 psi. The dihydroxylated analytes also differed from the rest of the analytes regarding the capillary voltage. The optimum capillary voltage for all the dihydroxylated analytes and 1α(OH)D₃ were 4000 V, while 2000 V was shown to result in the most abundant signal for the rest of the analytes. A nozzle voltage of 0 V was shown to be the optimum for 25(OH)D₂, 25(OH)D₃, 25(OH)D₃-[²H₃], 3-epi-25(OH)D₂ and 3-epi-25(OH)D₃ compared to the 1000 V seen for all the other analytes. The optimum sheath gas temperature seen for the majority of the analytes were 350 °C, with 1,25(OH)₂D₂ (325 °C), 1,25(OH)₂D₃ (325 °C), 1,25(OH)₂D₃-[²H₃] (325 °C) and 1α(OH)D₃ (325 °C) being the

exceptions. The optimum sheath gas flow rate was different for each group of analytes. Vitamin D₂, vitamin D₃, vitamin D₃-[²H₃] and the two 25(OH)D isobars showed a higher abundance with a sheath gas flow rate of 11 L/min, while 25(OH)D₂, 25(OH)D₃, 25(OH)D₃-[²H₃] and the two C3 epimers had a higher abundance with a sheath gas flow rate of 10 L/min. The dihydroxylated analytes (1,25(OH)₂D₂, 1,25(OH)₂D₃, 1,25(OH)₂D₃-[²H₃], 24,25(OH)₂D₂ and 24,25(OH)₂D₃) showed a higher abundance with a lowered sheath gas flow rate of 7 L/min. By referring to the data shown in Table 4.5, it is also concluded that the sheath gas temperature does not greatly affect the abundance of the analytes that initially indicated other optimum temperatures than 350 °C.

4.2.3 MULTIPLE REACTION MONITORING OPTIMISATION

The optimisation of the multiple reaction monitoring (MRM) transition of each analyte was done by infusing the analytes and changing the fragmentor voltage and collision energy settings to find the most abundant characteristic product ion for each analyte. An acquisition method was established for the MRM optimisation process. The optimised precursor ion for each analyte, as shown in Table 4.2, as well as the optimum source conditions, as indicated in Table 4.6, were incorporated in this method. The following table shows the most abundant MRM transition for each analyte, as determined by the Agilent MassHunter Optimiser software, and these selected MRM conditions were used onwards in this study.

Table 4.7 Optimised MRM transition for each analyte.

Analyte	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)
Vitamin D ₂	397.3	69.1	111	24
Vitamin D ₃	385.3	105.0	121	48
Vitamin D ₃ -[₂ H ³]	388.3	370.3	111	4
25(OH)D ₂	413.3	395.3	94	4
25(OH)D ₃	401.3	383.3	99	4
25(OH)D ₃ -[₂ H ³]	404.4	386.3	94	4
1,25(OH) ₂ D ₂	446.4	411.3	94	4
1,25(OH) ₂ D ₃	434.4	399.3	84	4
1,25(OH) ₂ D ₃ -[₂ H ³]	437.4	402.3	89	4
24,25(OH) ₂ D ₂	393.3	243.2	133	8
24,25(OH) ₂ D ₃	417.3	381.3	104	4
3-epi-25(OH)D ₂	413.3	395.3	104	4
3-epi-25(OH)D ₃	401.3	383.3	99	4
7(OH)4C3	401.3	383.3	133	12
1 α (OH)D ₃	418.4	401.3	84	4

The precursor adduct, ESI source parameter settings and MRM transition conditions for each of the fifteen analytes were successfully optimised. The optimisation of the chromatographic conditions for sufficient chromatographic separation of the analytes was then done, with the results shown in the following section.

4.3 CHROMATOGRAPHIC OPTIMISATION

4.3.1 COLUMN SELECTION

Five analytical columns were selected based on their prior use in literature for similar applications. The stationary phases of these columns are different and were assessed based on the retention of the analytes and the chromatographic separation between relevant analytes. Based on the initial performance of these columns, one was selected to be used for this application.

The following results were obtained through the injection of a standard solution, containing 100 ng/ml of each analyte as listed in 3.2. The analytical conditions were kept constant for all the columns, except for the mobile phase flow rate that was adjusted based on the specific column dimensions. The best results were obtained using the Agilent Technologies Pursuit 3

PFP (3x 100 mm, 3 μ m) and Pursuit 3 PFP (4.6 x 100 mm, 3 μ m) columns and only the relevant data is shown. The Agilent Technologies Zorbax SB-CN and SB-Phenyl were rejected from the study based on the insufficient chromatographic separation of the relevant analytes while the Eclipse Plus C18 was rejected based on the poor retention of the analytes with this particular C18 stationary phase. Figures 4.6 and 4.7 show the MRM chromatograms obtained using the two Pursuit 3 PFP columns. The x-axis display the retention times of the analytes, in minutes, and the y-axis the abundances of the analytes.

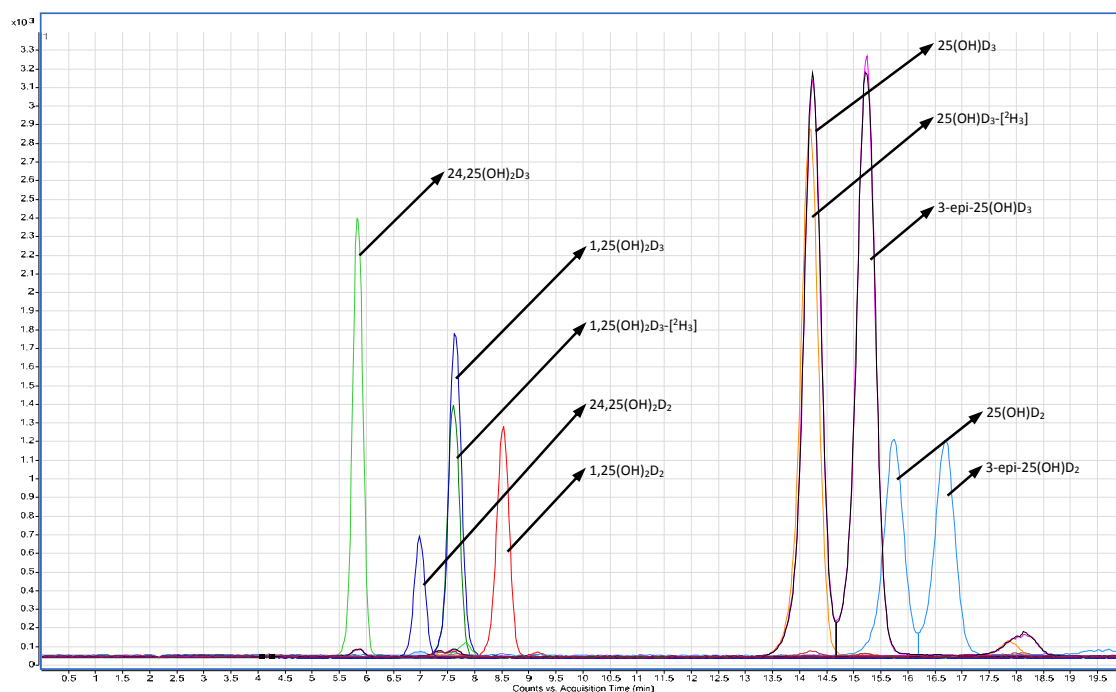


Figure 4.6 Chromatogram illustrating chromatographic separation on the Pursuit 3 Pentafluorophenyl (3 x 100 mm, 3 μ m) as part of column selection.

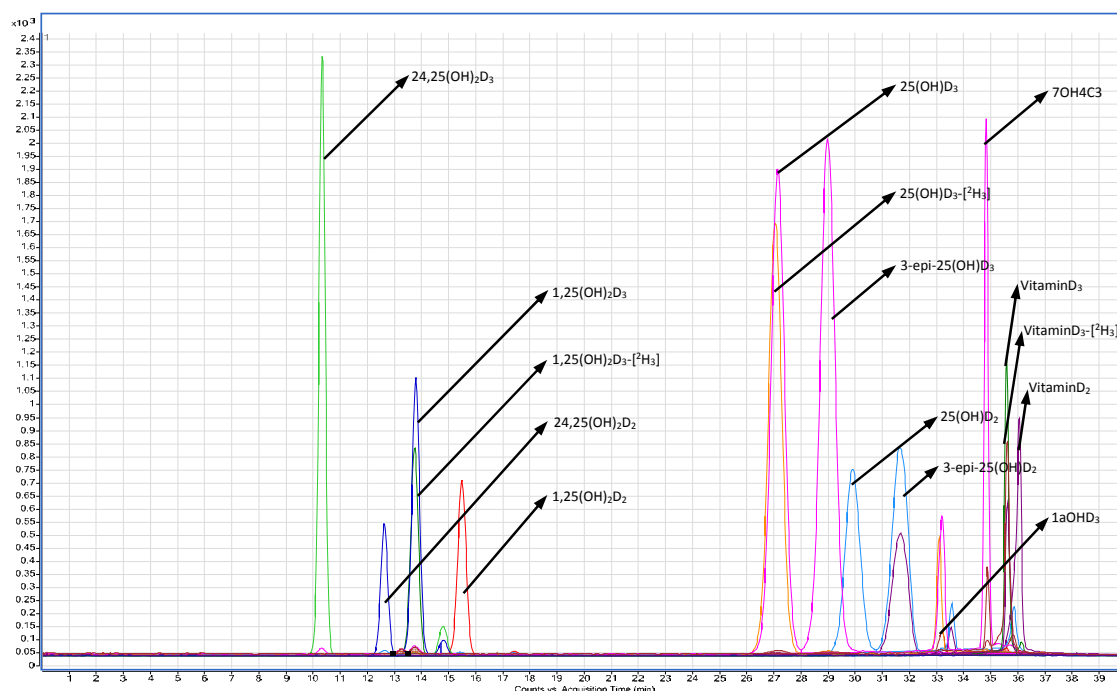


Figure 4.7 Chromatogram illustrating chromatographic separation on the Pursuit 3 Pentafluorophenyl (4.6 x 100 mm, 3 μ m) as part of column selection.

As seen in Figure 4.6, 7(OH)4C3, 1 α (OH)D₃, vitamin D₂, vitamin D₃ and vitamin D₃-[²H₃] did not elute from the PFP (3 mm) within the specific runtime. The rest of the analytes that did elute, had broad peaks with poor chromatographic separation between 25(OH)D₂, 25(OH)D₃ and the relevant C3 epimers. Although these flaws can possibly be resolved by altering the mobile phase gradient, the PFP (4.6 mm) showed better results with similar analytical conditions. As shown in Figure 4.7, very good chromatographic separation between 25(OH)D₂, 25(OH)D₃ and the relevant epimers were obtained with this column. Better retention of the analytes and narrower analyte peaks resulting in better sensitivity were also seen.

No alterations in the mobile phase gradient were tested because the aim was strictly to apply similar analytical conditions to each column. Based on the retention and the chromatographic separation of the analytes, the column that showed enhanced qualities was selected to be used for this application. As shown in Figure 4.7, the PFP (4.6 mm) column showed better retention of the analytes resulting in better chromatographic separation. Therefore the Pursuit 3 PFP (4.6 x 100 mm, 3 μ m) was selected to be used for the chromatographic separation of the analytes further on. An Agilent Technologies LC-MS/MS

application note regarding the analysis of 25(OH)D₂, 25(OH)D₃ and the relevant C3 epimers described the use of a similar Pursuit 3 PFP (4.6 x 100 mm, 3 µm) column for the chromatographic separation of these analytes (Agilent Technologies: 2013). Although this application did not include all the analytes included in this study, it was shown in Figure 4.7 that this particular column would be best suited for the analysis of the other analytes as well.

4.3.2 MOBILE PHASE OPTIMISATION

The most commonly used mobile phases for similar LC-MS/MS applications include water as the aqueous phase and methanol or acetonitrile as the organic phase (El-Khoury *et al.*, 2011). These two organic phases were tested to determine which one should be used together with water as the mobile phase. This was done by injecting a standard solution, containing 100 ng/ml of each analyte, onto the Pursuit 3 PFP (4.6 x 100 mm, 3 µm) column. All the analytical variables that may influence the retention and chromatographic separation of the analytes, like the mobile phase flow rate, mobile phase gradient, sample injection volume and column temperature, were kept constant. Therefore the only variable was set to be acetonitrile compared to methanol, as the organic phase. The mobile phase was set isocratic at 70 % organic phase, acetonitrile or methanol, with a flow rate of 0.8 ml/min. Figures 4.8 and 4.9 illustrate the effect of the use of acetonitrile and methanol, on the retention and chromatographic separation of the analytes. These illustrations are shown as MRM chromatograms with the retention time displayed on the x-axis, in minutes, and the abundances of the analytes on the y-axis.

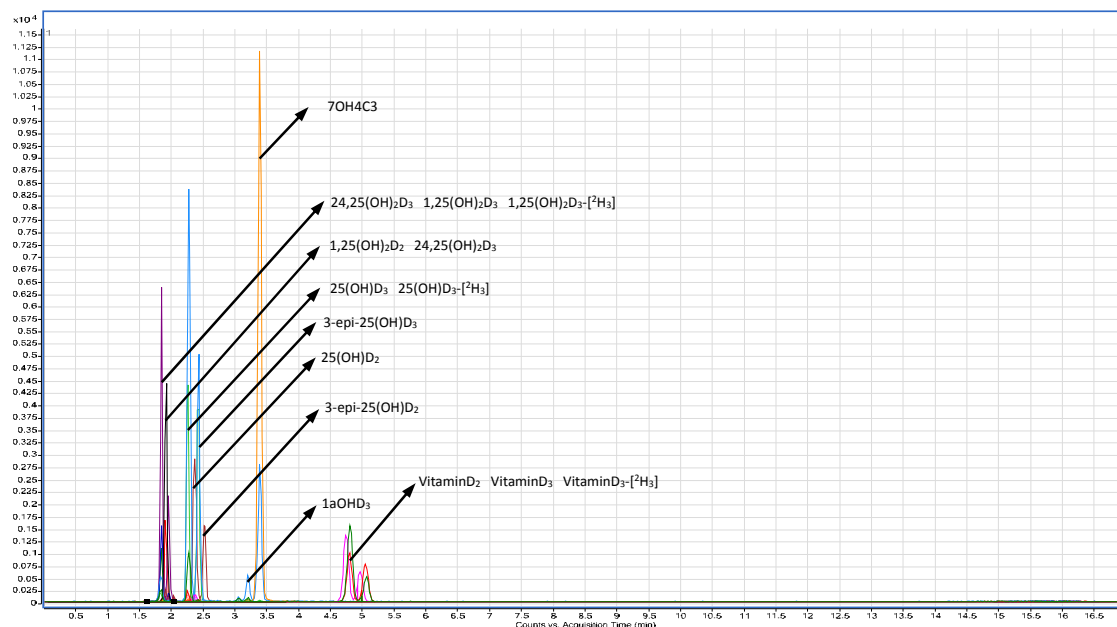


Figure 4.8 The effect of acetonitrile on the retention and chromatographic separation of the analytes.

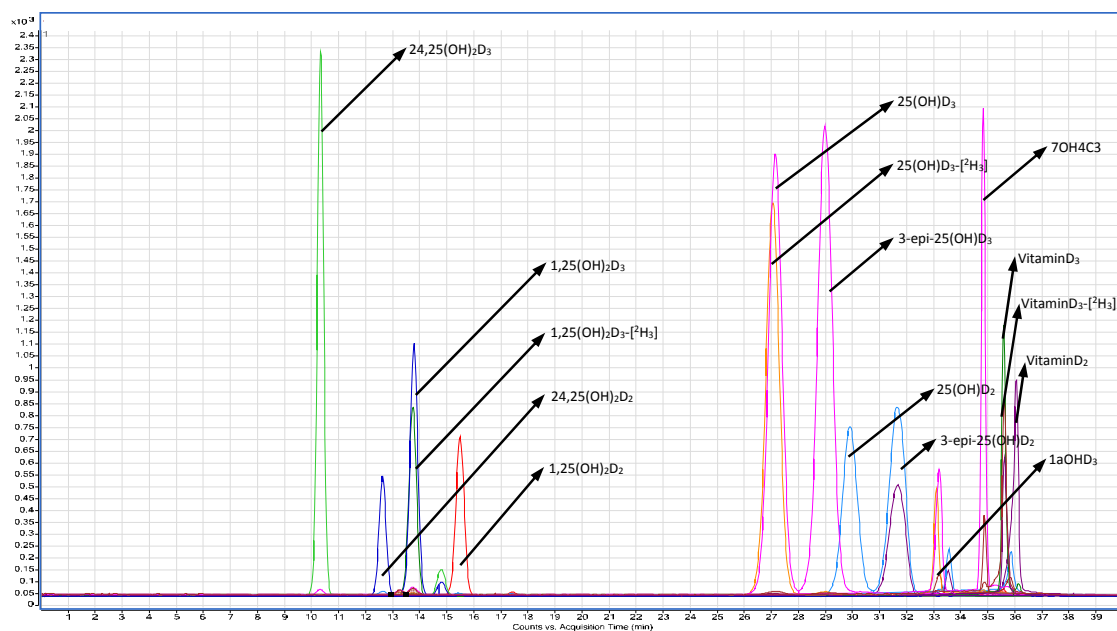


Figure 4.9 The effect of methanol on the retention and chromatographic separation of the analytes.

As seen in Figure 4.8, with the use of acetonitrile as the organic phase, the analytes had poor retention on the column and eluted within 5.5 minutes. Figure 4.9 illustrates the elution of all the analytes within 37 minutes at similar analytical conditions, but with methanol as the organic phase. With regards to the chromatographic separation of the analytes; as seen from Figure 4.8, very poor separation was achieved with the use of acetonitrile and this resulted in the co-elution of relevant analogue groups. Contrary to this, almost baseline chromatographic separation was achieved between 25(OH)D₂, 25(OH)D₃ and the relevant epimers with the use of methanol. Also seen in Figure 4.9, the use of methanol resulted in chromatographic separation between all the dihydroxylated analytes. Further optimisation of the mobile phase gradient was done to ensure baseline separation of all the relevant analytes and is discussed later on. An Agilent Technologies application note regarding the analysis of 25(OH)D₂, 25(OH)D₃ and the relevant C3 epimers also described the use of methanol and water as the mobile phase of choice (Agilent Technologies, 2013). Several literature applications, including a candidate reference measurement procedure, also described the use of methanol as the organic phase for the quantification of a variety of vitamin D analogues (Vogeser *et al.*, 2004; Kissmeyer & Sonne, 2001; Baecher *et al.*, 2012). Based on the results in Figure 4.9, methanol resulted in better retention and superior chromatographic separation of the analytes when compared to acetonitrile and therefore methanol was selected to be used together with water as the mobile phase.

Formic acid, ammonium formate and ammonium acetate were tested as mobile phase modifiers. Briefly, it was found that the addition of 0.1 % (26.51 mM, final concentration) formic acid added to both the water and methanol resulted in improved analytical sensitivity. The addition of formic acid to the mobile phases resulted in a higher solvent pH. Cech and Enke (2001) explained that the analysis of acidic compounds, like vitamin D analogues, with positive ESI-MS was improved using solvents with a higher pH for improved protonation of the analytes. Furthermore, it was found that the addition of 0.5 mM (final concentration) ammonium formate to the water resulted in improved analytical sensitivity. The ammonium formate acts as a buffer (Cech & Enke, 2001) and resulted in the formation of ammonium precursor adducts which led to improved analytical sensitivity for 1,25(OH)₂D₂, 1,25(OH)₂D₃, 1,25(OH)₂D₃-[²H₃] and 1 α (OH)D₃.

The mobile phase gradient was optimised to achieve baseline chromatographic separation between the vitamin D analogues that had similar chemical properties. An Agilent application note achieved baseline separation between 25(OH)D₂, 25(OH)D₃ and the relevant C3 epimers through a 70 % methanol isocratic gradient for 10 minutes (Agilent Technologies, 2013). Based on this application note a 5 μ l injection of a stock solution containing 100 ng/ml

of each analyte was done. The mobile phase gradient was set isocratic at 70 % methanol and the flow rate was kept constant at 0.8 ml/min for a total runtime of 40 minutes. This was done to get a starting point regarding the mobile phase gradient. Based on the retention and chromatographic separation achieved with this run, the mobile phase gradient was adjusted to ensure sufficient chromatographic separation in a more realistic runtime. This gradient proceeded as follows (B indicating the organic phase); time (T): 0, % B: 65 %; T: 10 min, % B: 100 %; T: 12 min, % B: 100 %; T: 14 min, % B: 65 %; with 3 minutes between injections for column equilibration. Figure 4.10 A illustrates the MRM chromatogram obtained with this gradient with the x-axis displaying the retention time, in minutes, and the y-axis the abundances of the analytes.

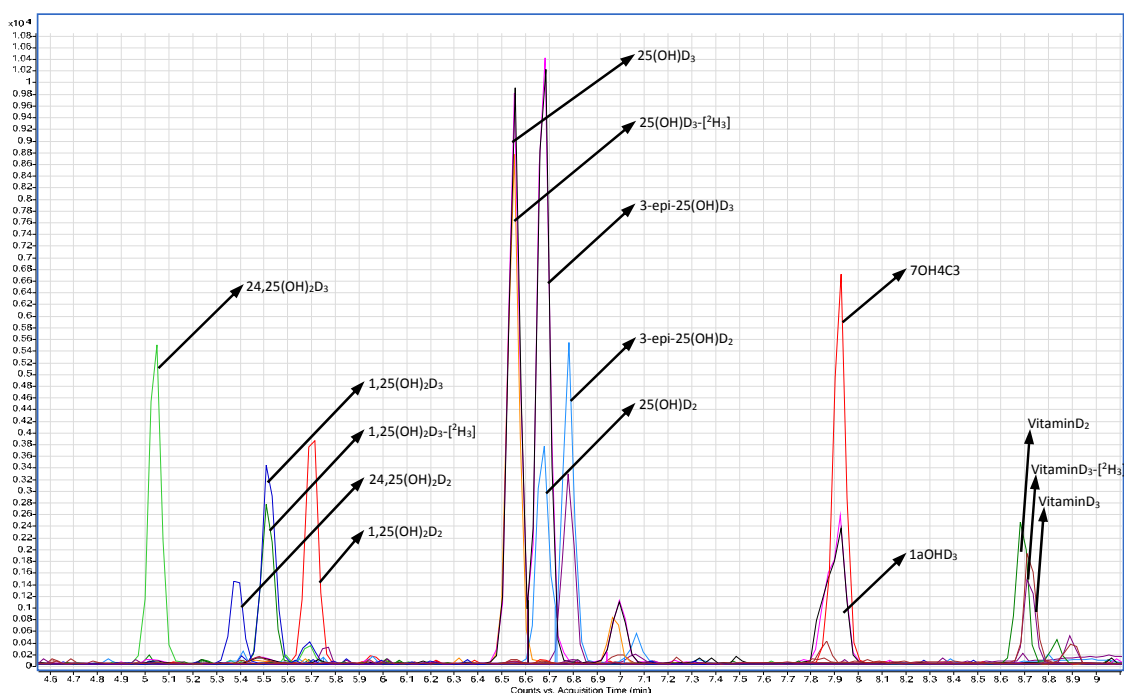


Figure 4.10 A Mobile phase gradient optimisation.

Seen in Figure 4.10 A, all the analytes eluted within 9 minutes. The first analyte, 24,25(OH)₂D₃, had a retention time of 5.061 minutes and good retention of all the analytes were seen. All the dihydroxylated analytes, 1,25(OH)₂D₂, 1,25(OH)₂D₃, 24,25(OH)₂D₂ and 24,25(OH)₂D₃, were chromatographically separated. On the other hand, baseline separation between 25(OH)D₂, 25(OH)D₃ and the C3 epimers were not achieved with this gradient and was therefore adjusted. The adjusted gradient proceeded as follows; time (T): 0, % B: 75 %; T: 10 min, % B: 75 %; T: 19 min, % B: 100 %; T: 20 min, % B: 75 %; with 3 minutes between injections for column equilibration. Figure 4.10 B illustrates the MRM chromatogram obtained

with this gradient with the x-axis displaying the retention time, in minutes, and the y-axis the abundances of the analytes. Table 4.8 shows the retention times of each analyte as seen in Figure 4.10 B.

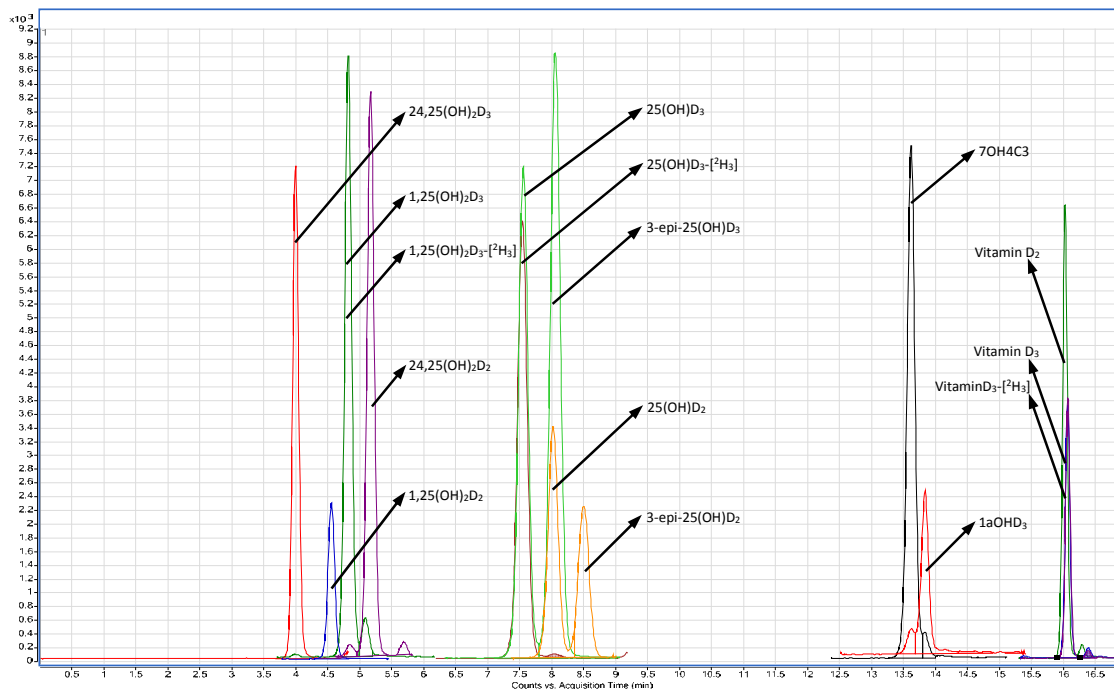


Figure 4.10 B Mobile phase gradient optimisation.

Table 4.8 Analyte retention times with optimised mobile phase gradient.

Analyte	Retention time (min)
24,25(OH) ₂ D ₃	4.01
1,25(OH) ₂ D ₂	4.57
1,25(OH) ₂ D ₃	4.79
1,25(OH) ₂ D ₃ -[² H ₃]	4.80
24,25(OH) ₂ D ₂	5.32
25(OH)D ₃ -[² H ₃]	7.55
25(OH)D ₃	7.56
25(OH)D ₂	8.05
3-epi-25(OH)D ₃	8.09
3-epi-25(OH)D ₂	8.50
7(OH)4C3	13.68
1α(OH)D ₃	13.91
Vitamin D ₂	16.02
Vitamin D ₃	16.13
Vitamin D ₃ -[² H ₃]	16.15

As seen in Figure 4.10 B, all the analytes eluted within 16.5 minutes. With regards to the retention times of the analytes shown in Table 4.8, 1,25(OH)₂D₂ (4.57 min) and 24,25(OH)₂D₂ (5.32 min), 1,25(OH)₂D₃ (4.79 min) and 24,25(OH)₂D₃ (4.01 min) were chromatographically separated. 25(OH)D₃ (7.56 min) were also chromatographically separated from possible interfering isobars, 7(OH)4C3 (13.68 min) and 1α(OH)D₃ (13.91 min). With this particular mobile phase gradient, baseline separation was also achieved between 25(OH)D₃ (7.56 min), 25(OH)D₂ (8.05 min) and their C3 epimers, 3-epi-25(OH)D₃ (8.09 min) and 3-epi-25(OH)D₂ (8.50 min), respectively. The resolution between 25(OH)D₃, 25(OH)D₂ and their C3 epimers was calculated and is shown in Figures 4.11 A and B, respectively. As seen in Figure 4.10 B, the first analyte, 24,25(OH)₂D₃, eluted at 4.01 minutes. This allows time for the MS diverter valve to be switched to waste for the time it takes salts found in the serum matrix to elute, thus preventing ion suppression of the analytes. This mobile phase gradient resulted in sufficient chromatographic separation between relevant vitamin D analogues, sharp analyte peaks with no peak broadening and peak tailing within a 20 minute runtime per sample. Therefore, this mobile phase gradient was selected for the analysis of biological samples.

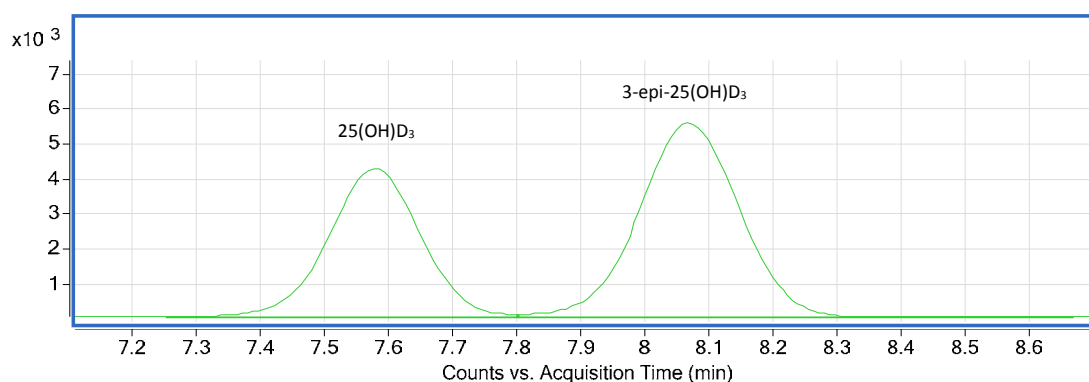


Figure 4.11 A Chromatographic separation between 25(OH)D₃ and 3-epi-25(OH)D₃.

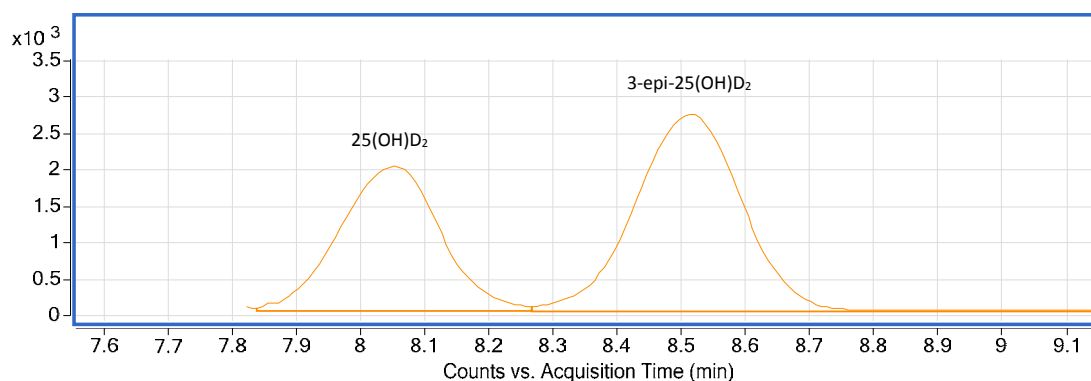


Figure 4.11 B Chromatographic separation between 25(OH)D₂ and 3-epi-25(OH)D₂.

In a recent review regarding the analytical measurement of vitamin D C3-epimers, Bailey and co-workers (2013) described that C3 epimers can cause a positive method bias regarding 25(OH)D₃ and 25(OH)D₂ concentrations. It was also stated that, for an LC-MS/MS to correctly quantify 25(OH)D₃ and 25(OH)D₂ concentrations, the method should be able to distinguish between these analytes and their C3 epimers through chromatographic separation. Figures 4.11 A and B illustrate the chromatographic separation between 25(OH)D₃ and 3-epi-25(OH)D₃, 25(OH)D₂ and 3-epi-25(OH)D₂, respectively. The resolution (R_s) for the relevant analytes were calculated according to the following equation; $R_s = \Delta T_r / \text{Width}_{\text{AVE}}$, where ΔT_r = the retention time differences between the two peaks and $\text{Width}_{\text{AVE}}$ = the average width of the analytical peaks (Agilent Technologies, 2011). The calculated peak resolution between 25(OH)D₂ and 3-epi-25(OH)D₂, and 25(OH)D₃ and 3-epi-25(OH)D₃ was greater than 1.5, thus indicating baseline separation between these analytes.

Sufficient chromatographic separation between relevant vitamin D analogues was achieved through the optimisation of the column, the mobile phase, mobile phase modifiers and mobile phase gradient. Furthermore the optimisation of the sample clean up steps prior to LC-MS/MS analysis was performed and the results shown in Section 4.4.

4.4 OPTIMISATION OF SAMPLE PREPARATION

Protein precipitation prior to LLE was selected for the extraction of the vitamin D analogues from a human serum matrix (Musteata & Musteata, 2011). Hexane, heptane, acetone and ethyl acetate were assessed as possible extraction solvents. This was done by spiking two aliquots of pooled serum, one before extraction and the other after extraction, with a standard solution containing 100 ng/ml of each analyte. This was done for each extraction solvent and the percentage analyte recovery was calculated by comparing the concentration values of the sample spiked after extraction with the one spiked before. The coefficient of variation (CV %) for the recovery efficiency of each analyte with each extraction solvent, was also calculated from 3 replicates. The sample preparation steps listed in 3.3.3 were used to evaluate the different extraction solvents. Table 4.9 shows the percentage mean recovery and CV of each analyte with the different extraction solvents. Thereafter, Figure 4.12 illustrates the recovery efficiency of each extraction solvent for the fifteen analytes.

Table 4.9 Analyte recovery efficiency and coefficient of variance with different extractants.

Analyte	Hexane		Heptane		Acetone		Ethyl acetate	
	Mean recovery %	% CV	Mean recovery %	% CV	Mean recovery %	% CV	Mean recovery %	% CV
Vitamin D ₂	67	8	73	12	97	10	98	8
Vitamin D ₃	58	5	61	9	89	6	95	5
Vitamin D ₃ -[² H ₃]	62	11	58	11	93	8	106	12
25(OH)D ₂	97	6	88	7	94	4	73	8
25(OH)D ₃	102	6	88	4	89	3	81	11
25(OH)D ₃ -[² H ₃]	96	5	91	6	92	6	77	7
1,25(OH) ₂ D ₂	11	28	24	19	56	9	93	13
1,25(OH) ₂ D ₃	44	13	31	14	48	15	98	8
1,25(OH) ₂ D ₃ -[² H ₃]	49	4	40	18	69	12	96	5
24,25(OH) ₂ D ₂	32	8	19	9	78	9	101	6
24,25(OH) ₂ D ₃	29	16	62	4	79	11	91	6
3-epi-25(OH)D ₂	104	6	87	8	93	5	71	9
3-epi-25(OH)D ₃	98	10	96	6	98	4	70	11
7(OH)4C3	91	9	98	8	102	8	94	2
1α(OH)D ₃	87	15	31	18	56	17	14	29

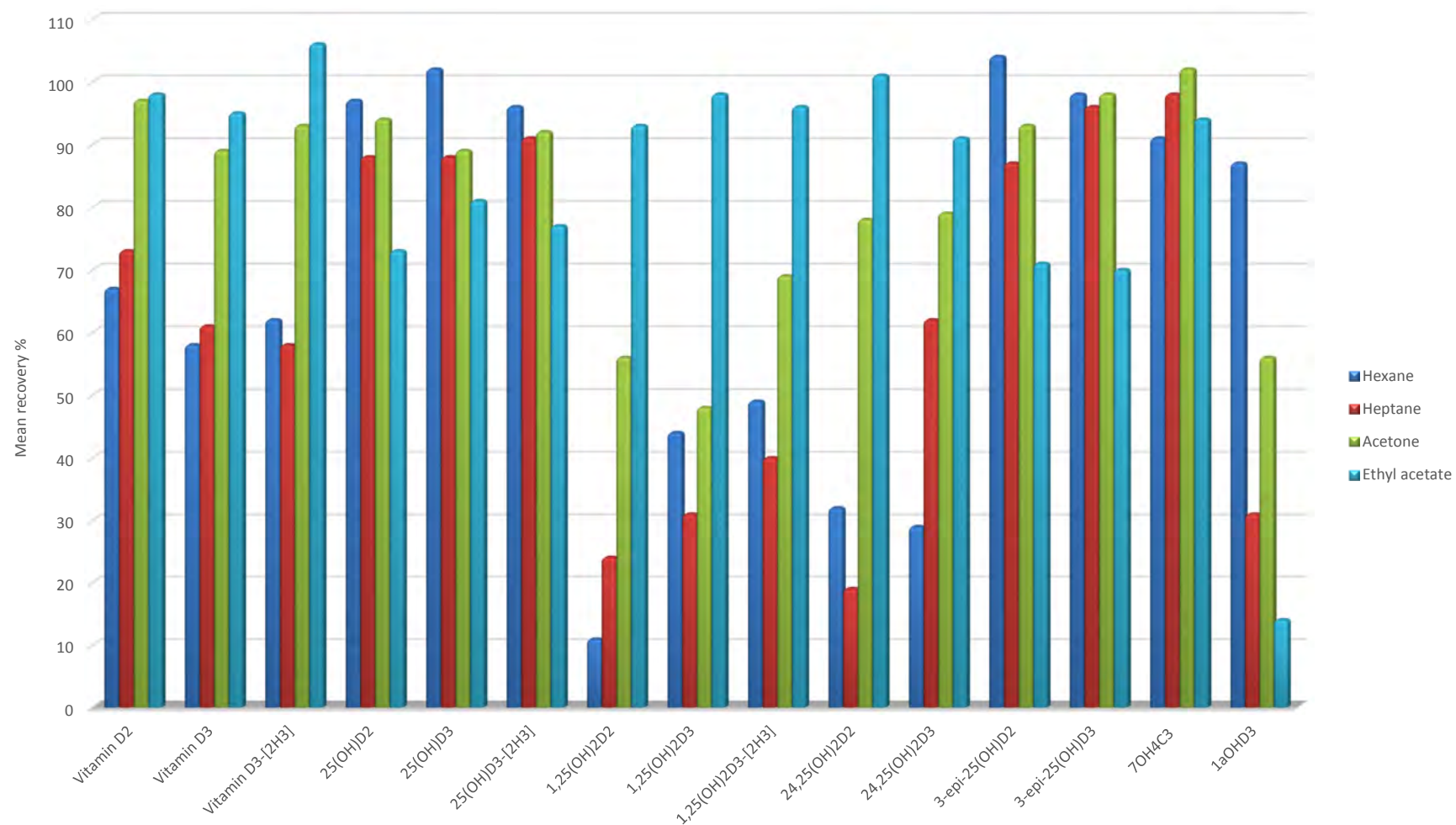


Figure 4.12 Illustration of analyte recovery efficiency with different extractants.

The extraction efficiency of the different extraction solvents was compared. As seen in Table 4.9, the different groups of vitamin D analogues were more efficiently extracted with different solvents. Vitamin D₂, vitamin D₃ and vitamin D₃-[²H₃] were most effectively extracted with acetone or ethyl acetate, showing a mean recovery of >90 % and a CV of <15 % for both extractants. Illustrated in Figure 4.12, 25(OH)D₂, 25(OH)D₃, 25(OH)D₃-[²H₃], 3-epi-25(OH)D₂ and 3-epi-25(OH)D₃ were best extracted with hexane or heptane. A mean recovery of >85 % (CV <10 %) was achieved with heptane, while hexane ensured a mean recovery of >95 % with a CV <10% for these analytes. Hexane, heptane and acetone were not very efficient at extracting the dihydroxylated analytes from the matrix. A mean recovery of <60 % with a CV ranging between 4 – 28 % for 1,25(OH)₂D₂, 1,25(OH)₂D₃, 1,25(OH)₂D₃-[²H₃], 24,25(OH)₂D₂ and 24,25(OH)₂D₃ was achieved with hexane, heptane and acetone. Contrary to this, ethyl acetate extracted these dihydroxylated analytes with an efficiency of >90 % and a CV of <10 %. 1,25(OH)₂D₂ was an exception to this, with a CV of 13 %. As seen in Figure 4.12, 7(OH)4C₃ was effectively extracted with all the solvents, with a mean recovery of >90 % and a CV of <10 %. The other isobar, 1 α (OH)D₃, showed overall poor extraction efficiency. With heptane, acetone and ethyl acetate a recovery ranging between 14 – 56 % (CV of up to 29 %) was achieved. Hexane proved to recover on average 87 % of the 1 α (OH)D₃ with a CV of 15 %.

Based on the evidence regarding the extraction of the analytes with different extraction solvents, both hexane and ethyl acetate was selected as extractants. The first extraction was done with hexane, extracting 25(OH)D₂, 25(OH)D₃, 25(OH)D₃-[²H₃], 3-epi-25(OH)D₂, 3-epi-25(OH)D₃, 7(OH)4C₃ and 1 α (OH)D₃. Thereafter, vitamin D₂, vitamin D₃ and vitamin D₃-[²H₃], 1,25(OH)₂D₂, 1,25(OH)₂D₃, 1,25(OH)₂D₃-[²H₃], 24,25(OH)₂D₂ and 24,25(OH)₂D₃ were extracted with the ethyl acetate. This double extraction proved to be efficient and repeatable for all the analytes. Furthermore, the effect of NaOH and NaCl on the sample clean-up and total analyte recovery was assessed. It was found that neither one of these matrix modifiers had an enormous effect on the recovery efficiency of all the analytes. Vogeser *et al.* (2004) and van den Ouweland *et al.* (2011) described the addition of NaOH to their sample preparation steps to release vitamin D analogues from the vitamin D binding proteins before protein precipitation. When tested, it was found that with the addition of 100 μ l 4 M NaOH to 500 μ l serum resulted in a cleaner sample with less background during analysis. It was also found that NaCl did not have any effect on the sample clean-up and recovery efficiency of the analytes and was therefore not included in the sample preparation steps.

4.5 SUMMARY OF OPTIMISED METHOD

4.5.1 SAMPLE PREPARATION

The sample pretreatment or protein precipitation proceeded with a 500 µl serum sample, thawed and vortex mixed, followed by the addition of 50 µl isotope solution (100 ng/ml of each isotope) and an incubation period of 10 minutes for equilibration of the stable isotopes within the matrix. Furthermore, 100 µl 4 M NaOH was added to release the vitamin D analogues from the vitamin D binding proteins during a 5 minute incubation period. 500 µl methanol was then added and the sample was vortex mixed (30 seconds) to promote protein precipitation during a further incubation period (2 minutes). After a centrifugation step (10 minutes, 10 000 rpm), the supernatant was transferred to a clean tube and subjected to LLE. During the first extraction step, 500 µl hexane was added to the supernatant, vortex mixed (1 minute) and centrifuged (5 minutes, 10 000 rpm). The upper organic layer was transferred to a clean tube and the residual lower layer was subjected to the second extraction step. The second extraction step proceeded with 500 µl ethyl acetate added to the residual lower layer, vortex mixed (1 minute) and centrifuged (5 minutes, 10 000 rpm). The two organic phases were pooled and dried under a gentle stream of nitrogen at room temperature. The dried sample was then reconstituted in 50 µl of methanol and analysed by means of LC-MS/MS.

The initial volume of serum sample was 500 µl, reconstituted in 50 µl after the extraction. These volumes were set in this manner to concentrate the vitamin D analogues with a factor 10 before LC-MS/MS analyses. This was done due to the low (pg/ml) circulating serum concentrations of certain vitamin D analogues. With 100 % extraction of the stable isotopes, concentrations of 100 ng/ml were found with analyses, because no concentrating factor was applied for them.

4.5.2 LIQUID CHROMATOGRAPHY

For the chromatographic separation of the analytes, the Agilent Technologies Pursuit 3 PFP (4.6 mm x 100 mm, 3 µm) was chosen as the stationary phase. Water and methanol was selected as the mobile phases with mobile phase modifiers including 0.1 % (26.51 mM) (final concentration) formic acid added to both the water and methanol, as well as 5 mM (final concentration) ammonium formate added to the water. The mobile phase flow rate was set at 0.8 ml/min with the following gradient; T: 0, % B: 75 %; T:10 min, % B: 75 %; T: 19 min, %

B: 100 %; T: 20 min, % B: 75 %; with a 3 minute post-runtime for column equilibration between 5 µl sample injections. The MS diverter valve was set to divert the eluent to waste between 0 – 3 minutes and back to the MS from 3.1 minutes.

4.5.3 MASS SPECTROMETRY

The MS was set to monitor dynamic MRM for each analyte in positive ESI mode. Table 4.10 shows these dynamic MRM transitions with the retention times based on the chromatogram illustrated in Figure 4.10 B.

Table 4.10 Dynamic multiple reaction monitoring transition for vitamin D analogues.

Analyte	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)	Retention Time (min)
Vitamin D ₂	397.3	69.1	111	24	16.02
Vitamin D ₃	385.3	105.0	121	48	16.13
Vitamin D ₃ -[² H ₃]	388.3	370.3	111	4	16.15
25(OH)D ₂	413.3	395.3	94	4	8.05
25(OH)D ₃	401.3	383.3	99	4	7.56
25(OH)D ₃ -[² H ₃]	404.4	386.3	94	4	7.55
1,25(OH) ₂ D ₂	446.4	411.3	94	4	4.57
1,25(OH) ₂ D ₃	434.4	399.3	84	4	4.79
1,25(OH) ₂ D ₃ -[² H ₃]	437.4	402.3	89	4	4.80
24,25(OH) ₂ D ₂	393.3	243.2	133	8	5.32
24,25(OH) ₂ D ₃	417.3	381.3	104	4	4.01
3-epi-25(OH)D ₂	413.3	395.3	104	4	8.50
3-epi-25(OH)D ₃	401.3	383.3	99	4	8.09
7(OH)4C3	401.3	383.3	133	12	13.68
1α(OH)D ₃	418.4	401.3	84	4	13.91

Due to the use of dynamic MRMs no time segments were included to vary the source parameters for each analyte. Therefore, the source conditions shown in Table 4.11 were selected as the optimum source conditions. Each parameter was chosen based on the order of effect on sensitivity and relevance to each analyte.

Table 4.11 Selected optimum source conditions for positive electrospray ionisation.

Parameter	Value (+)
Gas Temp (°C)	250
Gas Flow (L/min)	6
Nebuliser Pressure (psi)	45
Capillary Voltage (V)	2000
Nozzle Voltage (V)	1000
Sheath Gas Temp (°C)	350
Sheath Gas Flow (L/min)	11

For this method to be viable for implementation into a clinical laboratory, a series of validation experiments were conducted to evaluate the accuracy and precision of this optimised method. The results of the validation assessment are described in Chapter 5. Furthermore, a detailed description of the optimised method is provided in the SOP, Appendix B.

CHAPTER 5

RESULTS & DISCUSSION

METHOD VALIDATION

5.1 BACKGROUND

Westgard (2008) described the principle of method validation as the estimation of the total error present within the results derived from an analytical method. For an analytical method to be viable for implementation into a clinical laboratory, the total amount of analytical error should be acceptable with regards to the total error allowable for the specific application. The optimised method, described in Chapter 4, were subjected to a series of method validation assessments to estimate the total error present in this method. These validation assessments included the method linearity, LOD and LOQ, inaccuracy, imprecision, recovery, interference and analyte stability.

5.2 ANALYTICAL RANGE, LIMITS OF DETECTION & QUANTIFICATION

The analytical range is defined by the College of American Pathologists (CAP) as the “range of numeric results a method can produce without any special specimen pre-treatment, such as dilution, that is not part of the usual analytic process” (Westgard QC, 2014). The analytical range of the method for the quantification of each analyte was investigated. For this study, the quantification of the vitamin D analogues was not done relative to the stable isotope with similar chemical properties. The analytical range was rather investigated to estimate the analytical signal obtained for different concentrations of each analogue. This was done in order to investigate a direct correlation between the analytical signal and the analyte concentration. Whitmire and co-workers (2011) stated that the analytical range should be investigated with calibrators in the same biological matrix as the intended samples, in this case serum. For this study, no blank serum was available and the analytical range was therefore investigated using analytical standards dissolved in methanol. Although this was not ideal, it should be considered that the sample preparation steps of all the biological samples were extensive and included a protein precipitation step followed by a

double LLE. Thus, the complex serum matrix were simplified during the sample preparation steps and the sample was reconstituted in methanol which was closer to the matrix of the calibrators. The sample preparation steps, listed in 4.5.1, commenced with 500 µl serum which was then dried after the extraction process and reconstituted in 50 µl methanol before LC-MS/MS analysis. This resulted in a 10 times higher concentration of all the analytes and therefore the calibration curves for all the analytes were set up to accommodate this concentrating factor. The analytical range of the method was investigated at the concentration ranges shown in Table 5.1. The calibration curves for all the analytes are illustrated in Figure 5.1 (A – L). Furthermore, the linear or analytical range and correlation coefficient (r) for each analyte are summarised in Table 5.1.

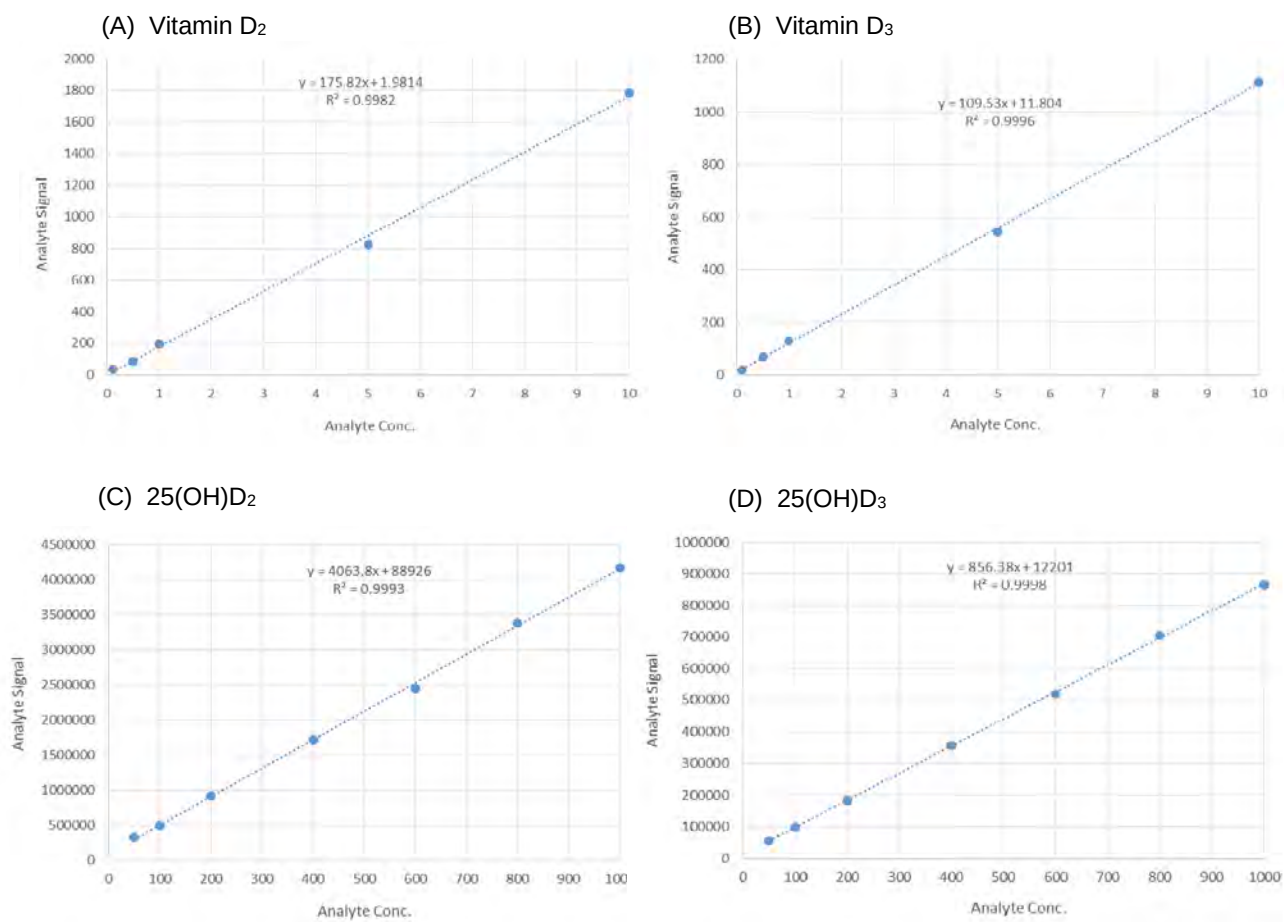
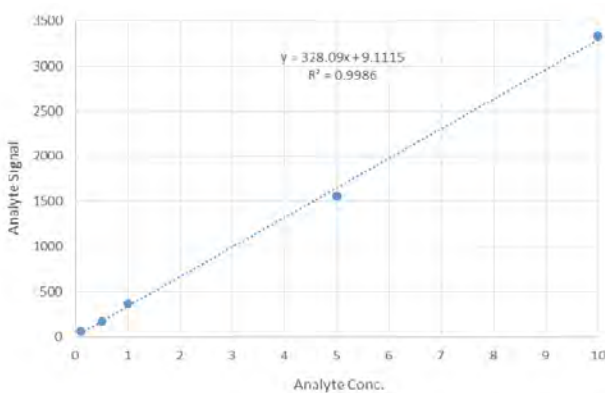
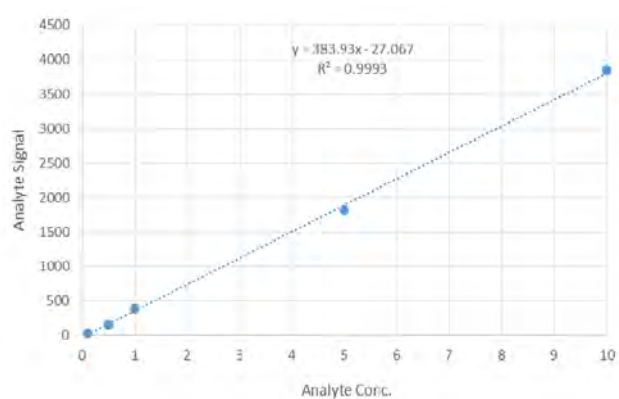


Figure 5.1 (A – D) Vitamin D analogue calibration curves.

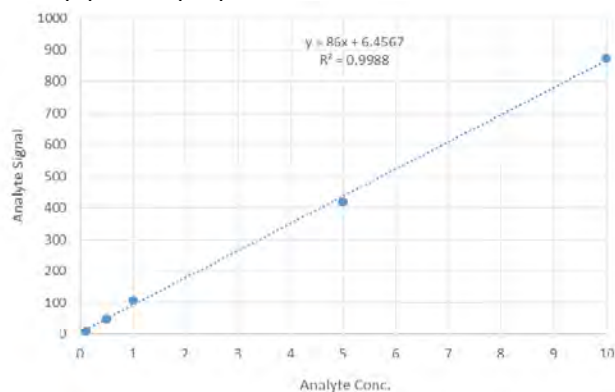
(E) 1,25(OH)₂D₂



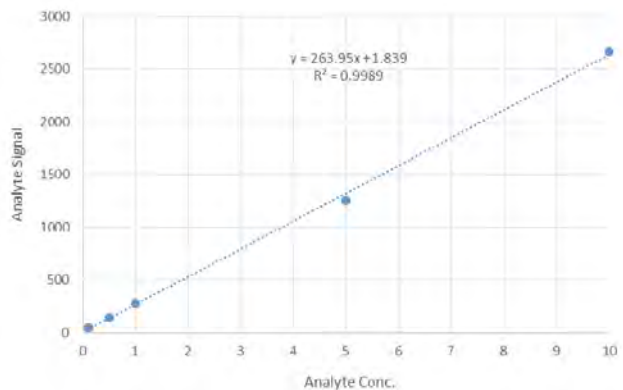
(F) 1,25(OH)₂D₃



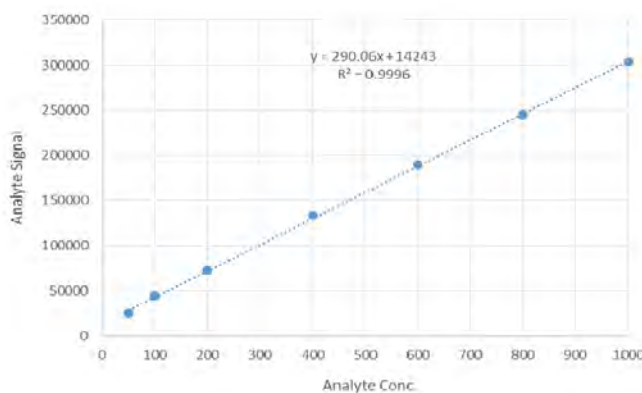
(G) 24,25(OH)₂D₂



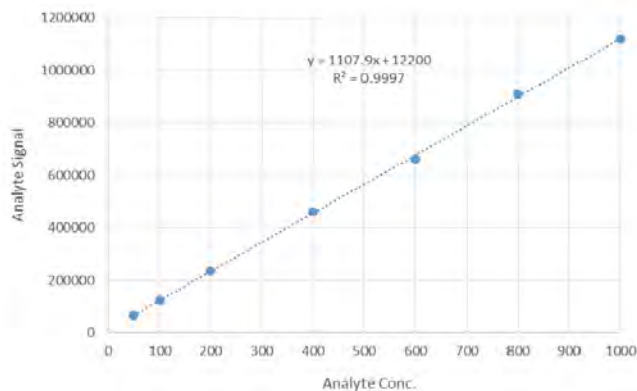
(H) 24,25(OH)₂D₃



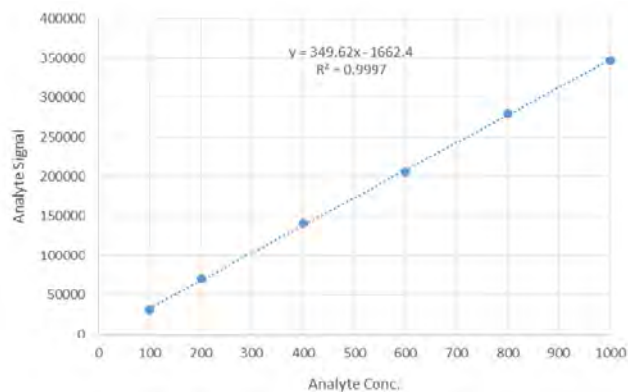
(I) 3-epi-25(OH)D₂



(J) 3-epi-25(OH)D₃



(K) 7OH4C3



(L) 1 α OH D₃

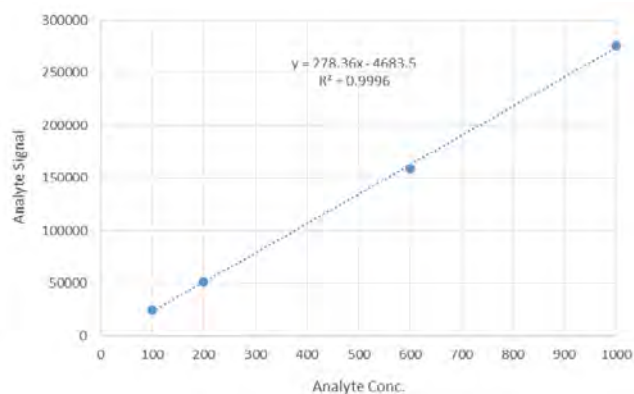


Figure 5.1 (E – L) Vitamin D analogue calibration curves.

Table 5.1 Summary of correlation coefficients and linear ranges for vitamin D analogues.

Analyte	Investigated concentration range		Correlation coefficient (r)	Linear analytical range	
	ng/ml	nmol/L		ng/ml	nmol/L
Vitamin D ₂	0.1 - 10	0.25 - 25	0.996	0.1 - 10	0.25 - 25
Vitamin D ₃	0.1 - 10	0.25 - 25	0.998	0.1 - 10	0.25 - 25
25(OH)D ₂	50 - 1000	125 - 2500	0.999	50 - 1000	125 - 2500
25(OH)D ₃	50 - 1000	125 - 2500	0.999	50 - 1000	125 - 2500
1,25(OH) ₂ D ₂	0.1 - 10	0.25 - 25	0.995	0.1 - 10	0.25 - 25
1,25(OH) ₂ D ₃	0.1 - 10	0.25 - 25	0.998	0.1 - 10	0.25 - 25
24,25(OH) ₂ D ₂	0.1 - 10	0.25 - 25	0.999	0.1 - 10	0.25 - 25
24,25(OH) ₂ D ₃	0.1 - 10	0.25 - 25	0.998	0.1 - 10	0.25 - 25
3-epi-25(OH)D ₂	50 - 1000	125 - 2500	0.998	50 - 1000	125 - 2500
3-epi-25(OH)D ₃	50 - 1000	125 - 2500	0.999	50 - 1000	125 - 2500
7(OH)4C3	50 - 1000	125 - 2500	0.998	50 - 1000	125 - 2500
1 α (OH)D ₃	50 - 1000	125 - 2500	0.998	50 - 1000	125 - 2500

Furthermore, LOD and LOQ for each vitamin D analogue were determined. The LOD was of less importance and therefore a “quick and dirty” signal-to-noise (S/N) ratio method was used for the estimation of the LOD for each analogue. (Sanagi *et al.*, 2009; Shrivastava & Gupta, 2011). These authors showed that the LOD can be estimated as three times the noise seen for the specific analyte; thus an S/N ratio of 3:1. This was calculated from 5 blank injections. These authors also claimed that the LOQ can be estimated on the same principle; LOQ = S/N ratio of 10:1. Westgard (2008) described several limitations of the S/N method for the estimation of LOD and LOQ. Because LOQ was more important for this application, it was decided that a method proposed by Westgard (2008) was to be used for the estimation of LOQ. This method states that the LOQ can be estimated as functional sensitivity. The functional sensitivity is the analyte concentration at which the method CV = 20 %. Table 5.2 shows the LOD and LOQ estimated for each analogue.

Table 5.2 Limits of detection and quantification for vitamin D analogues.

Analyte	LOD		LOQ	
	ng/ml	nmol/L	ng/ml	nmol/L
Vitamin D ₂	0.02	0.05	0.15	0.38
Vitamin D ₃	0.02	0.05	0.15	0.38
25(OH)D ₂	0.30	0.75	0.90	2.25
25(OH)D ₃	0.30	0.75	0.85	2.13
1,25(OH) ₂ D ₂	0.01	0.03	0.15	0.38
1,25(OH) ₂ D ₃	0.01	0.03	0.15	0.38
24,25(OH) ₂ D ₂	0.02	0.05	0.21	0.53
24,25(OH) ₂ D ₃	0.01	0.03	0.18	0.45
3-epi-25(OH)D ₂	0.25	0.63	0.95	2.38
3-epi-25(OH)D ₃	0.30	0.75	0.85	2.13
7(OH)4C3	0.35	0.88	0.96	2.40
1 α (OH)D ₃	0.35	0.88	0.95	2.38

5.3 INACCURACY STUDY

The method inaccuracy, also known as the systematic error (bias) of the method, was estimated through a comparison of methods experiment (Westgard, 2008). This was done by analysing 40 DEQAS samples with known concentrations of 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃ and 1,25(OH)₂D. Twenty DEQAS samples contained known concentrations of 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃ determined by the NIST RPM and the other 20 DEQAS samples contained known concentrations of total 1,25(OH)₂D determined by a traceable LC-MS/MS method. These DEQAS samples are pooled serum without any matrix modifications and are therefore ideal to be used for external quality assessment (DEQAS, 2014). Though linear regression statistics of method comparison data, the slope, slope confidence intervals (95 %), y-intercept and y-intercept confidence intervals (95 %) were calculated. This was done to calculate the amount of proportional systematic error, calculated from the slope, and the amount of constant systematic error, calculated from the y-intercept, that was present for the quantification of the vitamin D analogues. Figure 5.2 (A – D) illustrates the method comparison plot for these analytes with the comparative method shown on the x-axis and the test method on the y-axis. The green linear line shows the y = x theoretical equivalence performance while the blue line shows the results of the test method compared to the comparative method. Table 5.3 shows the results from the linear regression statistics indicating the amount of systematic error seen for each analyte. The raw data showing the mean calculated concentrations of the DEQAS samples are shown in Appendix

A. The total method bias for each analyte was estimated at the MDC. According to Wu (2006) the medical decision concentrations (MDCs) for 25(OH)D₃ and 1,25(OH)₂D are 25 ng/ml serum and 16 pg/ml serum, respectively. MDC values for 3-epi-25(OH)D₃ and 25(OH)D₂ are not yet defined in the literature, but Shah and co-workers (2011) showed that 3-epi-25(OH)D₃ can account for up to 50 % of the total 25(OH)D measured in patients. Cases of such high 3-epi-25(OH)D₃ levels are very rare and therefore, the 3-epi-25(OH)D₃ MDC used for this study was decided as 10 % of the 25(OH)D₃ MDC, thus 2.5 ng/ml. Furthermore, the 25(OH)D₂ can significantly contribute to the total 25(OH)D in a population where vitamin D₂ fortified foods are consumed regularly. Therefore, the MDC of 25(OH)D₂ was decided as 50 % of the 25(OH)D₃ MDC, thus 12.5 ng/ml.

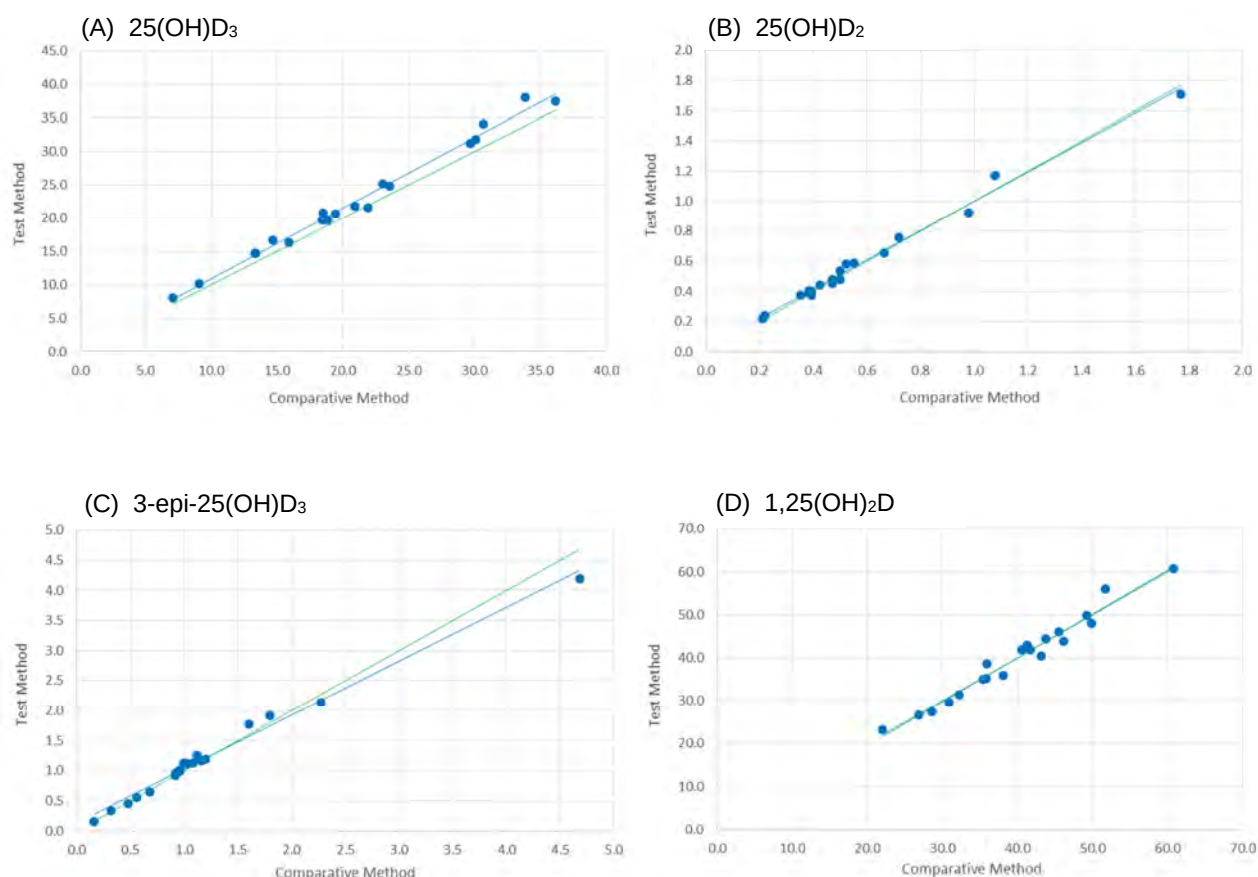


Figure 5.2 (A – D) Method comparison plots for vitamin D analogues. Analyte concentration shown on x-axis and y-axis in ng/ml for 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃ and in pg/ml for 1,25(OH)₂D. The green linear line show the x=y line and the blue the trend line for results derived from the test method.

Table 5.3 Linear regression statistics calculated from the method comparison plots for vitamin D analogues.

	25(OH)D₃	25(OH)D₂	3-epi-25(OH)D₃	1,25(OH)₂D
r	0.994	0.995	0.994	0.984
Slope (95 % CI)	1.060 (1.005 - 1.116)	0.974 (0.928 - 1.019)	0.897 (0.848 - 0.945)	1.02 (0.928 - 1.111)
Proportional Systematic Error	0.06%	-	-0.10%	-
Intercept (95 % CI)	0.231 (-1.004 - 1.466)	0.025 (-0.006 - 0.056)	0.132 (0.058 - 0.206)	-0.819 (-4.567 - 2.928)
Constant Systematic Error	-	-	0.132 ng/ml	-
MDC	25 ng/ml	N/D (12.5 ng/ml)	N/D (2.5 ng/ml)	16 pg/ml
Extrapolated Proportional Systematic Error at MDC	6.00%	-	-0.61%	-
Extrapolated Constant Systematic Error at MDC	-	-	6.60%	-
Method Bias at MDC	6.00%	-	5.99%	-

N/D: Not defined

As seen in Table 5.3, the correlation coefficient (r) for all the analytes were greater than 0.99, except for 1,25(OH)₂D which was 0.98 and the linear regression plot was therefore a good model fit. The r value for all the analytes was also calculated without any comparison outliers and it was found that this did not have any significant effect on the r value. The 95 % confidence estimates of the slope, seen from the method comparison plot for 25(OH)D₂ and 1,25(OH)₂D, included slope = 1 and were therefore not statistically different from the theoretically expected slope = 1, in case of method equivalence. A statistically different slope were seen for 25(OH)D₃ and 3-epi-25(OH)D₃. The slope of 25(OH)D₃ indicated a positive proportional systematic error of 0.06 % while a negative proportional systematic error of 0.10 % was seen for 3-epi-25(OH)D₃. The 95 % confidence estimates of the y-intercepts included y-intercept = 0, in case of method equivalence, for all the analytes except for 3-epi-25(OH)D₃ whose y-intercept indicated a positive constant systematic error of 0.132 ng/ml. The total method bias was calculated as the absolute difference between the theoretical equivalence line and the regression line at the MDC for 25(OH)D₃ and 3-epi-25(OH)D₃ only. The regression statistics of the other analytes were not statistically significant and therefore no bias estimate was calculated. Wallace and co-workers (2010) described the performance of LC-MS/MS assays and reported a method bias ranging between -1.3 % and 9.5 % for the DEQAS samples measured with LC-MS/MS from 2005 to 2008. Based on the evidence it

was concluded that no statistically significant difference between the test method and similar LC-MS/MS assays were found.

5.4 IMPRECISION STUDY

Method imprecision or random error was estimated by means of a replication experiment (Westgard, 2008). This was done by analysing three serum samples per day over five consecutive days, in triplicate. These serum samples were obtained from pooled serum and spiked with the D₃ form of the vitamin D analogues to reflect analyte concentrations close to the MDC. Furthermore, a Cochran C test and Dixon Q test were performed on the data to check for any outlier amongst repeats and outliers amongst days, respectively. Analysis of variance (ANOVA) was used to estimate the repeatability and within-laboratory precision (Westgard QC, 2014). For both the repeatability and within-laboratory precision the SD and CV were calculated. CV values were calculated with the following formula; $\% CV = (SD / \text{Mean}) \times 100$. Table 5.4 shows the random error as repeatability and within-laboratory CV's at different concentration levels in ng/ml. 25(OH)D₂ was also included in the imprecision study because 25(OH)D₂ can be present in high concentration within a population with that consume vitamin D₂ fortified foods.

Table 5.4 Method imprecision calculated as repeatability and within-laboratory precision.

Analyte	Concentration Level	Calculated mean	Repeatability (SD)	Repeatability (CV)	Within-laboratory precision (SD)	Within-laboratory precision (CV)
Vitamin D₃	High (ng/ml)	9.14	0.81	8.89%	0.82	8.94%
	Medium (ng/ml)	15.41	0.72	4.69%	1.26	8.17%
	Low (ng/ml)	40.26	0.84	2.09%	1.23	3.05%
25(OH)D₂	High (ng/ml)	9.87	0.19	1.93%	0.43	4.36%
	Medium (ng/ml)	13.12	0.12	0.91%	0.37	2.82%
	Low (ng/ml)	35.76	0.48	1.34%	1.21	3.38%
25(OH)D₃	High (ng/ml)	20.68	0.23	1.11%	0.77	3.74%
	Medium (ng/ml)	28.67	0.16	0.54%	0.84	2.94%
	Low (ng/ml)	68.59	0.55	0.81%	2.31	3.37%
1,25(OH)₂D₃	High (pg/ml)	55.58	2.57	4.62%	2.68	4.83%
	Medium (pg/ml)	84.31	2.12	2.52%	2.30	2.73%
	Low (pg/ml)	152.34	8.77	5.75%	7.96	5.22%
24,25(OH)₂D₃	High (ng/ml)	0.36	0.01	3.64%	0.04	11.34%
	Medium (ng/ml)	0.73	0.04	5.40%	0.07	10.15%
	Low (ng/ml)	1.19	0.06	4.73%	0.09	7.76%
3-epi-25(OH)D₃	High (ng/ml)	2.24	0.04	1.58%	0.08	3.39%
	Medium (ng/ml)	2.72	0.06	2.11%	0.15	5.66%
	Low (ng/ml)	5.97	0.01	0.25%	0.09	1.47%
7OH4C3	High (ng/ml)	42.61	1.35	3.16%	5.23	12.28%
	Medium (ng/ml)	38.11	1.12	2.93%	5.47	14.36%
	Low (ng/ml)	33.96	0.82	2.40%	4.18	12.31%
1αOHD₃	High (ng/ml)	23.10	1.13	4.87%	2.96	12.83%
	Medium (ng/ml)	24.17	0.58	2.42%	2.13	8.81%
	Low (ng/ml)	20.71	0.79	3.81%	2.04	9.84%

The Cochran C test and Dixon Q test did not detect any outliers in the data and all the results were included in the repeatability and within laboratory precision estimates. Seen in Table 5.4, the repeatability CV of all the analytes at all the concentrations were $\leq 5\%$ with a few exceptions. These exceptions included the low concentration level of vitamin D₃ (8.89 %), the high concentration level of 1,25(OH)₂D₃ (5.75 %) and the medium concentration of 24,25(OH)₂D₃ (5.40 %). According to Stockl *et al.* (2009) and the U.S. Food and Drug Administration Guideline for bioanalytical method validation (2001) a within-laboratory CV value of less than 15 % is acceptable and must not exceed this percentage for any concentration. The within-laboratory CV for all the analytes at all the concentration level were within this cut off value of 15 %. The highest within-laboratory CV (14.36 %) were seen for the medium concentration of 7(OH)4C3 while the lowest CV % of 2.73 % were seen for

the medium concentration level of 1,25(OH)₂D₃. As shown in Table 5.5, Adamec and co-workers (2011) optimised an LC-MS/MS method for the quantification of vitamin D₂, vitamin D₃, 25(OH)D₂ and 25(OH)D₃ and found the repeatability CVs ranging between 2.3 - 16.7 % and within-laboratory CVs ranging between 4.2 - 15.4 % at concentrations ranging between 2 – 200 ng/ml. Shah and co-workers (2012) described an LC-MS/MS method for simultaneous quantification of eight vitamin D analogues. The authors reported repeatability precision CVs ranging between 1.3 – 11.3 % and within-laboratory CVs ranging between 2.2 – 13.3 % at concentrations ranging between 0.08 – 32 ng/ml. Furthermore, a candidate RMP for the quantification of 25(OH)D₃ by LC-MS/MS reported a within-laboratory precision CV ranging between 7.8 % - 12.0%. The repeatability and within-laboratory CVs of these methods are shown in Table 5.5. Based on this evidence it was concluded that the optimised method for the quantification of vitamin D analogues compared well in terms of the total assay precision and the within-laboratory precision were within the cut-off limit of 15 % (U.S. Food and Drug Administration Guideline for bioanalytical method validation, 2001). Any other sources of analytical error was also investigated by means of an inaccuracy study, interference study and analyte stability study. According to Westgard (2008) a recovery experiment should also be included in the method validation steps. A recovery assessment was included in this study but as part of the optimisation of sample preparation steps. The results shown in 4.4, concluded that a recovery of >95 %, with a CV of <10 % was achieved for all the analytes with the optimised sample preparation steps.

Table 5.5 Repeatability and within-laboratory precision of similar LC-MS/MS methods.

Analyte	Adamec <i>et al.</i> , 2011		Shah <i>et al.</i> , 2012		Candidate RMP	
	Repeatability CV (%)	Within-laboratory CV (%)	Repeatability CV (%)	Within-laboratory CV (%)	Repeatability CV (%)	Within-laboratory CV (%)
vitamin D ₂	6.4 - 12.8	6.1 - 13.7	6.3 - 11.3	9.6 - 13.3	-	-
vitamin D ₃	2.5 - 5.2	10.3 - 12.7	1.3 - 4.1	2.3 - 5.3	-	-
25(OH)D ₂	2.7 - 16.7	5.1 - 14.0	2.7 - 3.3	3.4 - 3.9	-	-
25(OH)D ₃	2.3 - 3.7	4.2 - 15.4	2.7 - 6.1	2.9 - 7.4	-	7.8 - 12.0
1,25(OH) ₂ D ₂	-	-	3.2 - 4.4	4.3 - 6.3	-	-
1,25(OH) ₂ D ₃	-	-	3.6 - 4.4	2.6 - 5.6	-	-
24,25(OH) ₂ D ₂	-	-	-	-	-	-
24,25(OH) ₂ D ₃	-	-	-	-	-	-
3-epi-25(OH)D ₂	-	-	3.2 - 7.1	2.2 - 3.5	-	-
3-epi-25(OH)D ₃	-	-	3.1 - 3.5	4.3 - 10.9	-	-
7(OH)4C3	-	-	2.3 - 4.1	3.1 - 5.4	-	-

5.5 INTERFERENCE STUDY

Biological matrices are known to contain large amounts of salts that are known to cause ion suppression with regards to LC-ESI-MS/MS, this is also known as the ion suppression effect (Whitmire *et al.*, 2011). Furthermore, the inaccurate quantification of the analytes of interest can occur due to interfering compounds within the biological matrix with similar chemical properties and identical molecular masses and is known as a matrix effect (Shah *et al.*, 2011). According to van den Ouweland (2013), for an LC-MS/MS assay to correctly quantify vitamin D analogues, the assay must be able to chromatographically distinguish between 1,25(OH)₂D₂ and 24,25(OH)₂D₂, 1,25(OH)₂D₃ and 24,25(OH)₂D₃, 25(OH)D₂ and 3-epi-25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃, 7(OH)4C3 and 1 α (OH)D₃. As described in 3.4.4, protein precipitation prior to LLE was selected as the sample preparation and clean-up steps. It was described by Musteata & Musteata (2011) that this sample preparation method results in a clean sample with very little interfering biological matrix. The possibility of ion suppression due to the presence of biological salts was not investigated because these salts have very little retention on analytical columns. As seen in the chromatogram from Figure 4.9, the first analyte of interest elutes at 3.75 minutes and therefore, the MS diverter valve was configured to divert the eluting sample to waste from 0 to 3 minutes. This diversion effectively diverts the biological salts to waste, thus restricting the possibility of ion suppression due to interfering biological salts. Furthermore, it was shown in Figure 4.10 B that sufficient chromatographic separation was established between 24,25(OH)₂D₂ and 1,25(OH)₂D₂, 24,25(OH)₂D₃ and 1,25(OH)₂D₃, 25(OH)D₃ and isobars 1 α (OH)D₃ and 7(OH)4C3. Also seen in Figure 4.11 A and 4.11 B, baseline separation was achieved between 25(OH)D₂, 25(OH)D₃ and the relevant C3 epimers and therefore no interference study regarding the co-elution of these analytes was investigated. Seen in Figure 4.10 B, 25(OH)D₂ and 3-epi-25(OH)D₃ co-eluted and ion suppression of both were investigated. This was done by spiking aliquoted serum sample with high concentrations of these analytes. Table 5.6 shows the results found to indicate whether a high concentration of either analyte had an effect on the quantification of the other.

Table 5.6 Interference study of co-eluting 25(OH)D₂ and 3-epi-25(OH)D₃

Sample	Mean 25(OH)D ₂		Mean 3-epi-25(OH)D ₃	
	concentration (ng/ml)	CV %	concentration (ng/ml)	CV%
Unspiked	12.46	2.81%	22.27	4.59%
Spiked 25(OH)D ₂	111.4	1.28%	21.98	3.77%
Spiked 3-epi-25(OH)D ₃	12.91	2.72%	122.6	2.98%

Seen in Table 5.6, a sample containing a high concentration of 25(OH)D₂ had no statistically significant ion suppression or enhancement effect on the measured concentration of 3-epi-25(OH)D₃. A mean concentration of 22.27 ng/ml was measured for 3-epi-25(OH)D₃ within the unspiked sample, while a mean concentration of 21.98 ng/ml was measured in the sample containing a high concentration of 25(OH)D₂. The same was also seen for 25(OH)D₂; a mean concentration of 12.46 ng/ml and 12.91 ng/ml was measured in the unspiked sample and sample spiked with 3-epi-25(OH)D₃, respectively. It was therefore concluded that although these analytes co-elute, a high concentration of either analyte did not have any statistically significant ion suppression effect on the other, thus not contributing to the amount of constant systematic error seen for either analyte.

5.6 STABILITY STUDY

Analyte instability can contribute to the amount of random error seen for the method and should therefore be assessed for each analyte. These instability assessments include freeze and thaw stability, short-term storage stability, long-term storage stability, internal standard stock solution storage stability and post-preparative stability (Food and Drug Administration: Guidance for industry, 2001). Literature evidence described in 2.8.5 showed that vitamin D analogues are very stable under common pre-analytical conditions experienced in medical laboratories (Wielders and Wijnberg, 2009). It was also described that vitamin D analogues were found to be very stable when their freeze and thaw stability, short-term storage stability and long-term storage stability were assessed (Lisser *et al.*, 1981; Hollis, 2008; Agborsangaya *et al.*, 2010). Based on literature evidence, the pre-analytical stability of the analytes included in this study was not evaluated. The post-preparative stability of the analytes was assessed to determine if the analytes were stable over a period of time when reconstituted in methanol after the sample preparation. This was done by analysing three

samples, in triplicate, 0, 2, 6, 12, 24 and 48 hours after sample preparation. Table 5.7 shows the amount of error seen between analyses.

Shown in Table 5.7, a CV of $\leq 5\%$ was seen for all the analytes when measured over a period of 48 hours. No trends regarding the CVs were seen and the highest CV percentage, 4.78 %, was seen for 24,25(OH)₂D₃ at medium concentration level, while the lowest, 0.54 %, was seen for 1 α (OH)D₃. The stability CV values shown in Table 5.7 are lower than the repeatability precision CVs shown in Table 5.4 and therefore it was concluded that all the analytes are stable for a period of 48 hours after extraction, when reconstituted in methanol

Table 5.7 Vitamin D analogue stability measured over 48 hours.

	Concentration Level	0 hours (mean concentration)	2 hours (mean concentration)	6 hours (mean concentration)	12 hours (mean concentration)	24 hours (mean concentration)	48 hours (mean concentration)	Mean	SD	CV %
Vitamin D₃	Low (ng/ml)	9.52	9.22	8.75	8.91	9.67	9.21	9.21	0.35	3.79
	Medium (ng/ml)	14.97	14.73	15.27	15.78	14.83	14.98	15.09	0.38	2.54
	High (ng/ml)	41.11	41.13	41.78	40.30	39.79	41.08	40.87	0.71	1.73
25(OH)D₃	High (ng/ml)	19.82	20.21	19.77	19.63	21.46	19.82	20.12	0.69	3.40
	Medium (ng/ml)	28.40	28.78	28.24	27.66	28.59	28.39	28.34	0.38	1.35
	High (ng/ml)	67.13	67.17	67.78	66.98	67.15	67.94	67.36	0.40	0.59
1,25(OH)₂D₃	Low (pg/ml)	54.56	55.91	53.19	54.84	54.00	55.72	54.70	1.03	1.88
	Medium (pg/ml)	84.32	83.17	83.82	84.09	84.98	83.68	84.01	0.62	0.73
	High (pg/ml)	150.97	151.99	148.17	152.98	154.75	149.54	151.40	2.37	1.57
24,25(OH)₂D₃	Low (ng/ml)	0.32	0.35	0.33	0.35	0.33	0.32	0.33	0.01	4.10
	Medium (ng/ml)	0.74	0.74	0.69	0.77	0.79	0.72	0.74	0.04	4.78
	High (ng/ml)	1.22	1.11	1.16	1.26	1.23	1.22	1.20	0.05	4.56
3-epi-25(OH)D₃	Low (ng/ml)	2.12	2.24	2.15	2.33	2.12	2.16	2.19	0.08	3.79
	Medium (ng/ml)	2.68	2.67	2.81	2.59	2.69	2.71	2.69	0.07	2.64
	High (ng/ml)	5.94	5.97	5.85	5.69	5.79	5.88	5.85	0.10	1.75
7OH4C3	Low (ng/ml)	42.66	40.69	41.88	41.72	42.56	42.93	42.07	0.82	1.96
	Medium (ng/ml)	38.97	38.79	38.93	39.58	39.18	37.10	38.76	0.86	2.21
	High (ng/ml)	34.76	35.52	35.11	34.62	34.36	34.09	34.74	0.52	1.48
1αOHD₃	Low (ng/ml)	23.17	25.39	23.85	22.14	23.17	23.90	23.60	1.08	4.59
	Medium (ng/ml)	26.97	27.00	26.75	26.85	26.92	27.18	26.95	0.15	0.54
	High (ng/ml)	23.26	23.37	24.09	23.73	23.01	23.55	23.50	0.38	1.61

5.7 METHOD DECISION SIGMA METRIC

Westgard (2008) explained that the meaning of method validation is error assessment. Based on a total allowable error (TE_a), set for the specific application, the quality control specifications for the validated method can be determined to ensure the accuracy and precision of the method when implemented in a clinical environment (Westgard 2008). The TE_a for the specific application can be determined based on four different models. Models 1 – 4 are respectively based on misclassifications in diagnosis, biological variation data, expert recommendations and state-of-the-art performances (Stockl *et al.*, 2009). These models describe the TE_a which includes specifications for the allowable systematic error (method bias) and the allowable random error (method imprecision). Based on the total allowable specifications set by one of these four models the method sigma metric can be calculated to determine a quality rule set to include maximum tolerable misclassification. According to a biological variation model described by Stockl *et al.* (2009), the TE_a for 25(OH) D_3 was determined as 22.2 %. Current literature doesn't provide quality specifications of the measurement of 25(OH) D_2 and 3-epi-25(OH) D_3 and because the measurement of both these analytes can contribute to the total 25(OH) D , a similar TE_a was used as for 25(OH) D_3 to calculate the sigma metric values. DEQAS stated that for the measurement of 1,25(OH) $_2D$, 80 % of the results should fall within ± 30 % of the ALTM. This was calculated as a TE_a of 46 % based on the calculations described by Stockl *et al.* (2009). Table 5.8 shows the method sigma metric values calculated with the following formula: Sigma metric = (TE_a – systematic error) / random error.

Table 5.8 Method sigma metric calculations.

	25(OH) D_3	25(OH) D_2	1,25(OH) $_2D$	3-epi-25(OH) D_3
MDC	25	12.5	16	2.2
TE_a	22.2%	22.2%	46.0%	22.2%
Bias	6.0%	-	-	5.9%
CV	2.9%	2.8%	4.8%	3.4%
Sigma	5.6	7.8	9.5	4.8

According to Westgard (2008), based on the TE_a for the specific application, the sigma metric of the method can be calculated. Then based on the sigma value, the specific quality control (QC) requirements can be calculated to ensure the quality of the analytical method when implemented. The sigma metric scale ranges from 1 – 6 with each level on the scale

implying the following regarding the method; 1: “unacceptable”, 2: “poor”, 3: “marginal”, 4: “good”, 5: “excellent” and 6: “world class” (Westgard 2008). As seen in the Table 5.8, a sigma of higher than 6 was found for 25(OH)D₂ and 1,25(OH)₂D, thus implying a world class method requiring little quality control procedures. A sigma of 5.6 and 4.8 was found for 25(OH)D₃ and 3-epi-25(OH)D₃, respectively, still implying an excellent method. Based on these findings an in-house QC procedure must be implemented to accommodate the specific quality specifications and to ensure that any method error can be detected before the results derived from this method are compromised.

5.8 CONCLUSION

Based on the results derived from a series of method validation steps it was concluded that the optimised method showed good linearity ($r > 0.995$), acceptable repeatability ($CV < 10\%$) and within-laboratory precision ($CV < 15\%$) and excellent method accuracy (systematic error $< 6.60\%$). Furthermore, all the analytes proved to be stable for 48 hours after sample preparation with no interferences found for co-eluting analytes. Finally, based on the sigma metric scale, it was calculated that this method proved to be both “excellent” and “world class” and very little QC is needed to ensure the quality of the data derived from this method.

Overall, it was concluded that the optimised method for the quantification of twelve vitamin D analogues were validated based on requirements for implementation into a clinical environment.

CHAPTER 6

EVALUATION & CONCLUDING REMARKS

6.1 BACKGROUND

The interest for measurement of various vitamin D analogues is a continuously growing field both on individual and epidemiological level. It is currently known that vitamin D plays an important role in maintaining the calcium and phosphate homeostasis in the human body. Less clear evidence stated the medical importance of vitamin D metabolites regarding cancer, autoimmune disease and diabetes. Currently, the majority pathology laboratories can give an accurate estimate of an individual's vitamin D status by measuring the 25-hydroxylated metabolites of vitamin D, but does not report on a full vitamin D profile. The measurement of vitamin D analogues are known to be a challenge and several concerns were raised regarding the faults in currently used LC-MS/MS methods. The overestimation of an individual's true vitamin D status can occur due to the interference of C3-epimers and isobaric compounds. The quantification of the biologically active and inactive forms of vitamin D was reported to be difficult and to date very few LC-MS/MS methods reported in the literature were able to quantify various vitamin D analogues. None of these methods were able to include the precursor vitamin D, the 25-hydroxylated metabolites, the biologically active and inactive metabolites, C3-epimers and isobaric compounds in a single run. The concerns raised regarding the quantification of vitamin D analogues with LC-MS/MS were addressed in this study. This was done with the main focus set on developing an in-house LC-ESI-MS/MS method for the quantification of twelve vitamin D analogues. Furthermore, this method was optimised for optimal analytical sensitivity and validated to ensure its precision and accuracy before implementation into a clinical environment.

6.2 GENERAL CONCLUSION

The first part of this study focused on optimising an LC-ESI-MS/MS method for the quantification of twelve vitamin D analogues accounting for interferences from epimers and isobars. The underlying parameters of the mass spectrometry conditions were optimised to find the best precursor ion for each analyte, the optimum ESI source conditions and the

MRM conditions that produced the highest analyte signal. These optimum conditions are summarised in 4.5.3. Furthermore, different LC mobile phases and mobile phase modifiers were tested and it was found that methanol and water modified with formic acid and ammonium formate were suited for the intended chromatographic conditions. After the assessment of various columns, it was found that with the Agilent Technologies PFP (4.6 x 100 mm, 3 μ m) column, sufficient chromatographic separation between relevant analytes was achieved. One of the analytical difficulties described in the literature is achieving baseline separation between the 25(OH)D analogues and the relevant C3-epimers. After various attempts, baseline separation was achieved between these analytes in a total run time of 20 minutes. A variety of sample preparation methods were described in the literature and it was decided to implement protein precipitation prior to LLE as the sample preparation method. The principle of different literature described LLE methods was used to optimise the sample preparation steps suited for this application. After a recovery study of all the analytes with various extractants, it was found that a recovery of >95 % (CV <10 %) for all the analytes was achieved with a double extraction using hexane and ethyl acetate. Matrix modifiers were also assessed and it was found that the addition of NaOH, prior to protein precipitation, resulted in higher recoveries, thus confirming literature based evidence that NaOH release vitamin D analogues from the vitamin D binding proteins.

The optimised method was subjected to a series of method validation steps described by Westgard (2008) to ensure the accuracy and precision of the method before implementation into a clinical laboratory. These validation steps focused on error assessment and included the assessment of the analytical range, LOD, LOQ, imprecision, inaccuracy, interference and instability. The analytical range of this method for the quantification of the vitamin D analogues was adjusted to accommodate the sample preparation steps that concentrated all the analytes 10 times higher before LC-MS/MS analysis. A correlation coefficient of >0.995 was found for linear analytical ranges between 1 and 1000 ng/ml, for all the analytes. The LOD and LOQ were different for all the analytes and are summarised in 5.2. The method imprecision or method random error was assessed for each of the twelve analytes at three different concentration. Repeatability CVs of <10 % and within-laboratory CVs of <15 % were found for all the analytes. A Cochran C test and Dixon Q test found no outliers amongst repeats and days, respectively. It was concluded that the optimised method compared well to literature specifications in terms of the total assay precision. The method inaccuracy or method systematic error was assessed for only 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃ and 1,25(OH)₂D due to unavailability of EQA material for the rest of the analytes. DEQAS samples with known concentrations of these four analytes were quantified and a proportional

systematic error of 0.06 % and -0.10 % were found for 25(OH)D₃ and 3-epi-25(OH)D₃, respectively. No proportional systematic error were found for 25(OH)D₂ and 1,25(OH)₂D. A constant systematic error of 6.60 % was found for 3-epi-25(OH)D₃, while none was found for the rest of the analytes. It was concluded that no statistically significant difference between the accuracy of the test method and similar literature LC-MS/MS methods was found. Because of the sample preparation steps and the chromatographic separation between analytes, it was not necessary to investigate interferences due to ion suppression and biological matrix. Interferences due the co-elution of 25(OH)D₂ and 3-epi-25(OH)D₃ were investigated and it was found that a high concentration of either analyte did not have any statistically significant ion suppression effect on the other. Based on literature evidence the only analyte stability study conducted, evaluated the stability of all the analytes for 48 hours after sample preparation steps. It was found that all the analytes are stable for 48 hours (CV <5 %) when reconstituted in MeOH after sample preparation steps. A recovery study was included in the optimisation of the sample preparation steps and was therefore not included as part of the validation steps. The method decision sigma metric for 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃ and 1,25(OH)₂D was calculated based on the TEa specifications from a literature described biological variation study and DEQAS specifications. Sigma metric values of 7.8, 7.2, 4.8 and 9.5 were found for 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₃ and 1,25(OH)₂D, respectively. Based on sigma metric specifications described by Westgard (2008) it was concluded that the optimised method is “world class” and require very little quality control.

It was concluded that the method was optimised for the quantification of twelve vitamin D analogues and validated for the implementation into a clinical laboratory. Thus, the aim and objectives, stated in Chapter 1, was achieved.

6.3 FUTURE RECOMMENDATIONS

The following recommendations should be considered for implementation:

- Although sufficient chromatographic separation between all the analytes was achieved with the PFP (4.6 x 100 mm, 3 µm) column, the 4.6 mm diameter require high flow rates of 0.8 ml/min and a relative long run time of 20 minutes. PFP columns with smaller diameter and particle size should thus be considered.
- Due low concentrations (pg/ml) of 1,25(OH)₂D, the sample preparation used 500 µl serum for extraction to improve LOQ especially for 1,25(OH)₂D. Optimisation of

sample preparation and chemical derivatisation may be considered. This would result in less serum needed for the extraction.

- Due to unavailability of a certified blank serum matrix, the analytical range of the method, for all the analytes, was assessed with calibrators in MeOH. It should therefore be considered to evaluate the use of vitamin D free serum matrix.
- The inaccuracy study was only performed for four of the twelve analytes included in this study. This was due to the unavailability of EQA material for the other eight analytes. Further work regarding the method systematic error for these eight analytes should be considered.

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APPENDIX

APPENDIX A

Table A1 shows the mean calculated concentrations of the DEQAS samples, used for the inaccuracy study, compared to the assigned concentrations as stated by DEQAS.

Table A1 DEQAS samples mean calculated concentrations vs. assigned concentrations.

DEQAS Sample No.	25(OH)D ₃		25(OH)D ₂		3-epi-25(OH)D ₃		DEQAS Sample No.	1,25(OH) ₂ D	
	Assigned Concentration	Mean Calculated Concentration	Assigned Concentration	Mean Calculated Concentration	Assigned Concentration	Mean Calculated Concentration		Assigned Concentration	Mean Calculated Concentration
	nmol/L	nmol/L	nmol/L	nmol/L	nmol/L	nmol/L		pmol/L	pmol/L
421	57.50	62.90	0.96	1.02	2.33	2.45	311	129.30	140.05
422	36.58	41.57	1.66	1.63	1.70	1.63	312	95.30	89.75
423	84.62	95.22	0.98	0.94	5.68	5.35	313	101.50	104.68
424	46.20	49.11	0.98	1.01	2.58	2.79	314	104.30	104.65
425	46.18	51.89	0.96	0.96	2.50	2.82	315	108.00	101.00
426	33.40	36.58	1.06	1.10	0.40	0.39	316	123.20	124.73
427	75.20	79.43	0.88	0.93	4.50	4.79	317	109.40	111.17
428	52.30	54.29	2.45	2.31	2.80	3.15	318	152.00	151.71
429	58.90	61.95	0.55	0.60	3.00	2.97	319	77.30	73.96
430	17.60	19.95	22.30	21.38	0.80	0.85	320	124.70	120.20
431	22.60	25.31	1.25	1.20	1.20	1.15	321	103.40	107.47
432	48.60	51.47	2.69	2.93	2.90	2.91	322	89.70	88.17
433	90.40	93.67	1.18	1.19	11.70	10.48	323	88.60	87.43
434	74.20	78.13	4.42	4.28	2.40	2.52	324	67.10	66.63
435	46.10	49.32	0.53	0.55	2.40	2.49	325	55.10	58.04
436	76.70	85.39	1.25	1.33	4.00	4.43	326	115.40	109.90
437	33.30	36.54	1.38	1.46	1.40	1.40	327	71.50	68.73
438	54.80	53.78	1.80	1.90	2.70	2.80	328	113.80	115.34
439	39.70	40.67	1.18	1.13	2.30	2.29	329	90.00	96.67
440	47.10	49.17	1.31	1.45	2.30	2.38	330	80.70	78.67

APPENDIX B

STANDARD OPERATING PROCEDURE

BACKGROUND

The following standard operating procedure (SOP) contains the purpose, method principle, specimen specifications, equipment and reagents needed for the quantification of twelve vitamin D analogues by means of LC-MS/MS, from human serum. Furthermore, the work instructions specify the assay procedure. These include the worklist creation, sample preparation, LC-MS/MS specifications, specimen analysis, data acquisition and interpretation.

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**PROCEDURE FOR THE QUANTIFICATION OF VITAMIN D ANALOGUES WITH
AN AGILENT 6460 QQQ LIQUID CHROMATOGRAPHY – TANDEM MASS
SPECTROMETER**

Reference number	WI-VITD-001		
Edition	1		
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1 INTRODUCTION AND OVERVIEW

1.1 INTRODUCTION

The term 'vitamin D' refers to a subgroup of secosteroid compounds of which the 9,10 carbon-carbon bond of ring B is broken and these secosteroids are a group of tetracyclic steroid derivatives. Although the term 'vitamin D' is internationally used, it is often interpreted wrongly to incorporate vitamin D metabolites like 25-hydroxyvitamin D and the biologically active- and inactive forms of vitamin D (Couchman *et al.*, 2012). Vitamin D has five distinct forms, D₁ to D₅, which differs in chemical composition. The two major and most studied forms are known as ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Ergocalciferol is naturally found mainly in yeast and fungi (Calvo *et al.*, 2013). It is produced from the plant steroid ergosterol, by the exposure to ultraviolet B (UVB) light that activates the reaction of UV irradiation of the ergosterol to ergocalciferol (Jasinghe & Perera, 2004) and consumed by humans through daily diet or vitamin D supplements. Cholecalciferol is considered the parent compound of the naturally present form of vitamin D in human skin and is also regarded the more prominent source between the two major forms contributing up to 80 % of the total vitamin D in humans (Vanga *et al.*, 2010).

Although the chemical structures of vitamin D₂ and D₃ differ, they follow the same metabolic transformations in humans. Vitamin D undergoes two hydroxylation reactions in humans. The first hydroxylation reaction is a hepatic 25-hydroxylation reaction by which vitamin D (both vitamin D₂ and D₃) is hydroxylated at the carbon molecule in the 25 position to 25-hydroxyvitamin D₂ and D₃. These are used to measure the vitamin D status of patients. The second hydroxylation reaction is a renal 1- α -hydroxylation reaction by which 25(OH)D is hydroxylated to 1 α ,25-dihydroxyvitamin D at the first carbon molecule position. The 1 α ,25-dihydroxyvitamin D is considered the biologically active metabolites of vitamin D. Furthermore, 25(OH)D is hydroxylated to 24R,25-dihydroxyvitamin D at the 24 carbon molecule position and these are considered the biologically inactive metabolites. Epimerisation of 25(OH)D can also occur and is the conversion of the hydroxyl group, at the C-3 carbon position of the A-ring, from the alpha orientation to the beta orientation (Bailey *et al.*, 2013). These C3 epimers of 25(OH)D₂ and D₃ as well as isobaric compounds, 7- α -hydroxy-4-cholesten-3-one (7(OH)4C3) and 1 α (OH)D₃ can cause interferences resulting in false-high interpretation of the vitamin D status (El-Khoury *et al.*, 2011; Maunsell *et al.*, 2005). These possible interferences are compensated for by chromatographically separating 25(OH)D₂ and D₃ from the relevant epimers and isobars.

1.2 PURPOSE

This document contains the procedure for the quantification of twelve vitamin D analogues and three deuterium labelled stable isotopes by means of an optimised and validated liquid chromatography – tandem mass spectrometry (LC-MS/MS) assay. The method is optimised and validated on the Agilent Technologies 6460 triple quadrupole LC-MS with Agilent JetStream (AJS) Technology. This method was optimised and validated at the Centre for Human Metabonomics, North-West University and is intended for the implementation into a clinical laboratory for routine analysis of a full spectrum vitamin D status.

1.3 PRINCIPLE

The basis of this assay is that, different vitamin D analogues can be quantified from human serum. This is achieved through sufficient chromatographic separation between the analytes that have similar chemical properties and the quantification of these analytes by means of multiple reaction monitoring (MRM) in positive electrospray ionisation (ESI) mode. The vitamin D analogues included are; vitamin D₂, vitamin D₃, 25(OH)D₂, 25(OH)D₃, 1 α ,25(OH)₂D₂, 1 α ,25(OH)₂D₃, 24R,25(OH)₂D₂, 24R,25(OH)₂D₃, 3-epi-25(OH)D₂, 3-epi-25(OH)D₃, 7- α -hydroxy-4-cholesten-3-one (7(OH)4C3) and 1 α (OH)D₃.

1.4 DEFINITIONS & ABBREVIATIONS

h	-	hour(s)
L/min	-	litre per minute
M	-	molar
m/z	-	mass-to-charge ratio
min	-	minute(s)
mM	-	millimolar
ng/ml	-	nanogram per millilitre
pg/ml	-	picogram per millilitre
psi	-	pounds per square inch
rcf	-	relative centrifugal force
μ g	-	microgram
μ l	-	microlitre
μ m	-	micrometre
V	-	volt

25(OH)D ₂	-	25-hydroxyvitamin D ₂
25(OH)D ₃	-	25-hydroxyvitamin D ₃
3-epi-25(OH)D ₂	-	25-hydroxyvitamin D ₂ C-3 epimer
3-epi-25(OH)D ₃	-	25-hydroxyvitamin D ₃ C-3 epimer
1,25(OH) ₂ D ₂	-	1,25-dihydroxyvitamin D ₂
1,25(OH) ₂ D ₃	-	1,25-dihydroxyvitamin D ₃
24,25(OH) ₂ D ₂	-	24,25-dihydroxyvitamin D ₂
24,25(OH) ₂ D ₃	-	24,25-dihydroxyvitamin D ₃
7(OH)4C ₃	-	7 α -hydroxy-4-cholesten-3-one
1 α (OH)D ₃	-	1 α -hydroxyvitamin D ₃
AJS	-	Agilent JetStream
ESI	-	Electrospray ionisation
LC-MS/MS	-	Liquid chromatography-tandem mass spectrometry
LLE	-	Liquid-liquid extraction
MeOH	-	Methanol
MRM	-	Multiple reaction monitoring
PFP	-	Pentafluorophenyl

1.5 REAGENT & EQUIPMENT INFORMATION

1.5.1 REAGENT INFORMATION

Reagent	Company	Catalogue number
Vitamin D ₂	IsoScience, USA	50-14-6
Vitamin D ₃	IsoScience, USA	67-97-0
25(OH)D ₂	IsoScience, USA	21343-40-8
25(OH)D ₃	IsoScience, USA	19356-17-3
1,25(OH) ₂ D ₂	IsoScience, USA	60133-18-8
1,25(OH) ₂ D ₃	IsoScience, USA	32222-06-3
24,25(OH) ₂ D ₂	IsoScience, USA	58050-55-8
24,25(OH) ₂ D ₃	IsoScience, USA	55721-11-4
3-epi-25(OH)D ₂	IsoScience, USA	908126-48-7
3-epi-25(OH)D ₃	IsoScience, USA	73809-05-9
Vitamin D ₃ -[² H ₃]	IsoScience, USA	8066-48-4
25(OH)D ₃ -[² H ₃]	IsoScience, USA	140710-94-7
1,25(OH) ₂ D ₃ -[² H ₃]	IsoScience, USA	128723-16-0
Methanol	Anatech, SA	10071675
Acetonitrile	Anatech, SA	10071618
Water	Anatech, SA	10071715
Hexane	Sigma Aldrich, SA	34859
Ethyl acetate	Merck, SA	1.09623.2500
Formic acid	Sigma Aldrich, SA	100987129
Sodium hydroxide	Sigma Aldrich, SA	S8045
Ammonium formate	Agilent Technologies, USA	G1946-85021

1.5.2 EQUIPMENT AND MATERIAL INFORMATION

Equipment	Company	Type / Model
1290 Infinity Binary Pump	Agilent Technologies, USA	G4220A
1290 Infinity Thermostated Column Compartment	Agilent Technologies, USA	G1316C
1290 Infinity Autosampler	Agilent Technologies, USA	G4226A
6460 JetStream triple quadrupole mass spectrometer	Agilent Technologies, USA	G6460A
Pursuit 3 PFP analytical column	Agilent Technologies, USA	4.6mm x 100mm, 3µm (A3051100X046)
Centrifuge	Merck, SA	5417R
-20°C Freezer	Forma Scientific, SA	
Glass ware	Merck, SA	Schott Duran (Different volumes)
Plastic containers	Merck, SA	1.5 ml
Sample vials & pulled point inserts	Agilent Technologies, USA	2 ml screw cap & pulled point vial inserts
Pipettes	Merck, SA	100 µl, 1000 µl
Pasteur pipettes	Sigma Aldrich, SA	2 ml
Vortex	Merck, SA	F202A0230

1.6 SPECIMENS

Patient blood is collected in a safe and correct manner by trained staff at any pathology laboratory. Blood is collected in a 3.5 ml BD Vacutainer® SST II Advance REF 367957 tube. All blood samples must be marked with the patients' credentials and surname for reference purposes and sent via courier services, at ambient temperature, to the Centre for Human Metbonomics, North West University.

2 ASSAY PROCEDURE

2.1 REAGENT PREPARATION

Clearly mark all reagent solutions. Indicate the compound, solution, concentration and date on all prepared solutions. Record all lot numbers, version numbers, production dates and expiry dates of all reagents used.

- **STANDARD SOLUTIONS FOR CALIBRATION CURVE**
 - Prepare a stock standard solution in a volumetric flask containing 1000 ng/ml (final concentration) of each analytical standard, dissolve in methanol, and store at -20 °C.
 - Prepare a stock stable isotope solution in a volumetric flask containing 1000 ng/ml (final concentration) of each stable isotope, dissolve in methanol, and store at -20 °C.
 - Prepare calibration curve solutions containing a final concentration of 1, 5, 10, 20, 40, 60, 80, 100, 200, 400, 600, 800 and 1000 ng/ml of the twelve analytical standards, by serial diluting the prepared stock solution.
 - From the prepared stock isotope solution, add the stable isotopes to the calibration curve solutions to have a final concentration of 100 ng/ml.
 - Store these calibration curve solutions, in the dark, at -20 °C for future use.
- **SODIUM HYDROXIDE SOLUTION**
 - The sodium hydroxide solution is used during the sample preparation steps to release the vitamin D analogues from the vitamin D binding proteins.
 - Prepare a 4 M sodium hydroxide solution by weighing 8.143 g NaOH into a 100 ml volumetric flask and filling it with distilled water. Store at room temperature for future use.
- **LC-MS/MS MOBILE PHASE**
 - Prepare the aqueous phase (mobile phase A) by adding 1 ml formic acid (final concentration of 26.51 mM) and 1 ml 5 M ammonium formate (final concentration of 5 mM) to a 1 L Schott bottle. Fill with spectrometry grade water to 1 L. Sonicate for 20 min to de-gas.

- Prepare the organic phase (mobile phase B) by adding 1 ml formic acid (final concentration of 26.51 mM) to a 1 L Schott bottle. Fill with spectrometry grade methanol to 1 L. Sonicate for 20 min to de-gas.

2.2 GENERATING WORKLIST

A worklist for the batch is generated in Microsoft Excel 2010 using the following template and is given a consecutive worklist number; WL-VITD-0001.

Number:	Sample:	LC-MS/MS position:	Type:	Level:	Comments:
1	Blank 1	No injection	Blank		
2	CAL 1	P1-A1	Calibrator	1	
3	CAL 2	P1-A2	Calibrator	2	
4	CAL 3	P1-A3	Calibrator	3	
5	CAL 4	P1-A4	Calibrator	4	
6	CAL 5	P1-A5	Calibrator	5	
7	CAL 6	P1-A6	Calibrator	6	
8	CAL 7	P1-A7	Calibrator	7	
9	CAL 8	P1-A8	Calibrator	8	
10	CAL 9	P1-A9	Calibrator	9	
11	CAL 10	P1-B1	Calibrator	10	
12	CAL 11	P1-B2	Calibrator	11	
13	CAL 12	P1-B3	Calibrator	12	
14	CAL 13	P1-B4	Calibrator	13	
15	Blank 2	No injection	Blank		
16	01/02/03/2014_1	P1-B5	Sample		
17	01/02/03/2014_2	P1-B5	Sample		
18	01/02/03/2014_3	P1-B5	Sample		
19	02/02/03/2014_1	P1-B6	Sample		
20	02/02/03/2014_2	P1-B6	Sample		
21	02/02/03/2014_3	P1-B6	Sample		
22	03/02/03/2014_1	P1-B7	Sample		
23	03/02/03/2014_2	P1-B7	Sample		
24	03/02/03/2014_3	P1-B7	Sample		
25	04/02/03/2014_1	P1-B8	Sample		
26	04/02/03/2014_2	P1-B8	Sample		
27	04/02/03/2014_3	P1-B8	Sample		
28	05/02/03/2014_1	P1-B9	Sample		
29	05/02/03/2014_2	P1-B9	Sample		
30	05/02/03/2014_3	P1-B9	Sample		
31	QC 1	P1-C1	QC		
32	QC 2	P1-C2	QC		
33	QC 3	P1-C3	QC		
34	QC 4	P1-C4	QC		

As seen in the worklist template, no injection blanks are added before and after the set of calibrators. Thereafter, the samples are added in triplicate followed by the set of four quality control (QC) samples.

2.3 SAMPLE PREPARATION

All samples must be labelled with the appropriate given laboratory number.

2.3.1 RECEIVING / HANDLING OF SAMPLES

Each sample is given a specific laboratory number upon arrival. The laboratory number contains the date and number of sample for that specific day. An example of a laboratory number is: 01/02/03/2014. This number indicates that this is the first sample "01", received on the second day "02", of March "03", year 2014. All samples must be handled with care and as if infectious. Upon arrival samples (SST tubes) are centrifuged for 10 minutes at 2600 rfc (17 °C) and the serum layer is aliquoted (500 µl) in Eppendorf tubes and stored at -20 °C. The remaining blood and tubes are discarded in a biological waste container.

2.3.2 SAMPLE PRETREATMENT

- 1) Thaw a 500 µl serum sample (in 1.5 ml Eppendorf tube) at room temperature for 15 minutes and vortex mix for 30 seconds.
- 2) Add 50 µl of 100 ng/ml stable isotope solution to serum.
- 3) Incubate at room temperature for 10 minutes.
- 4) Add 100 µl 4 M NaOH.
- 5) Incubate at room temperature for 5 minutes.
- 6) Add 500 µl MeOH and vortex mix for 30 seconds.
- 7) Incubate at room temperature for 2 minutes.
- 8) Centrifuge for 10 minutes at 10 600 rcf (17 °C).
- 9) Transfer supernatant to a new Eppendorf tube using a Pasteur pipette.

2.3.3 LIQUID - LIQUID EXTRACTION (LLE)

- 10) Use supernatant obtained from sample pretreatment steps.
- 11) Add 500 µl hexane.
- 12) Vortex mix for 1 minute and centrifuge for 5 minutes at 10 600 rcf (17 °C).
- 13) Transfer the upper organic layer to a new Eppendorf tube using a Pasteur pipette.
- 14) Add 500 µl ethyl acetate to the residual lower layer.
- 15) Vortex mix for 1 minute and centrifuge for 5 minutes at 10 600 rcf (17 °C).

- 16) Transfer the upper organic layer to the Eppendorf tube containing the organic layer from step 13 using a Pasteur pipette.
- 17) Discard the residual lower layer.
- 18) Dry the sample under a gentle stream of nitrogen for $\pm$ 30 minutes at 37 °C).
- 19) Reconstitute the dried sample in 50 μ l MeOH.
- 20) Transfer reconstituted sample to LC-MS/MS screw cap vial with pulled vial insert using a Pasteur pipette.
- 21) Perform LC-MS/MS analysis on an Agilent 6460 JetStream ESI QQQ.

IMPORTANT NOTE: Sample preparation commences with 500 μ l serum and is reconstituted in 50 μ l MeOH after extraction procedure. Thus, the analytes within the sample is concentrated 10 times higher. However, this does not have an effect on the concentration of the stable isotopes, because 50 μ l of 100 ng/ml stable isotope solution is added and after extraction procedure reconstituted in 50 μ l. Thus, the stable isotope concentration in the extracted sample remains 100 ng/ml.

2.4 LC-MS/MS ANALYSIS

2.4.1 LC-MS/MS SPECIFICATIONS

- Liquid chromatography specifications:
 - Analytical column: Pursuit 3 PFP (4.6 mm x 100 mm, 3 μ m)
 - Column compartment temperature: 45 °C
 - Sample injection volume: 5 μ l
 - Mobile phase A: Water + 0.1 % formic acid and 5 mM ammonium formate
 - Mobile phase B: Methanol + 0.1 % formic acid
 - Mobile phase flow rate: 0.8 ml/min
 - Mobile phase gradient:

Time:	%B:
0	75
10	75
19	100
20	75

- Post run time: 3 minutes
- MS diverter valve: diverted LC flow to waste: 0 – 3 minutes
diverted LC flow to MS: 3 – 20 minutes
- Mass spectrometry specifications:
 - AJS ESI: Positive ESI
 - Gas temperature: 250 °C
 - Gas flow: 6 L/min
 - Nebuliser pressure: 45 psi
 - Capillary voltage: 2000 V
 - Sheath gas temperature: 350 °C
 - Sheath gas flow: 11 L/min
 - Delta EMV: 300 V
 - MRM Transitions:

	Precursor Ion	Product Ion	Fragmentor (V)	Collision Energy (V)	Retention Time (min)
Vitamin D ₂	397.3	69.1	111	24	16.02
Vitamin D ₃	385.3	105.0	121	48	16.13
Vitamin D ₃ -[² H ₃]	388.3	370.3	111	4	16.15
25(OH)D ₂	413.3	395.3	94	4	8.05
25(OH)D ₃	401.3	383.3	99	4	7.56
25(OH)D ₃ -[² H ₃]	404.4	386.3	94	4	7.55
1,25(OH) ₂ D ₂	446.4	411.3	94	4	4.57
1,25(OH) ₂ D ₃	434.4	399.3	84	4	4.79
1,25(OH) ₂ D ₃ -[² H ₃]	437.4	402.3	89	4	4.80
24,25(OH) ₂ D ₂	393.3	243.2	133	8	5.32
24,25(OH) ₂ D ₃	417.3	381.3	104	4	4.01
3-epi-25(OH)D ₂	413.3	395.3	104	4	8.50
3-epi-25(OH)D ₃	401.3	383.3	99	4	8.09
7OH4C3	401.3	383.3	133	12	13.68
1αOHD ₃	418.4	401.3	84	4	13.91

2.4.2 DATA ACQUISITION

- Data acquisition can only proceed if the data acquisition method is set up with the LC-MS/MS specifications as stated in 2.4.1. The method should be named according to the laboratory specifications and any revisions of the method should be recorded for reference purposes. An appropriate method name would be VITAMIN D_REV 1.m

- With the data acquisition method setup, the following steps should be performed before analyses:
 - Place mobile phase A (water) and mobile phase B (MeOH) on appropriate LC channels.
 - Flush solvent channels for 5 minutes at 5 ml/min flow rate, with MS diverter valve set to waste.
 - Install analytical column and check for leaks.
 - Set mobile phase flow rate to 0.2 ml/min, 75% B to equilibrate column (± 10 minutes).
 - Perform a check tune and in necessary an auto tune.
 - The analytical system is now ready for sample analysis.

- Compiling LC-MS/MS Worklist:
 - In the “Agilent MassHunter Workstation Data Acquisition” software.
 - Click File, Worklist, New.
 - Copy & paste sample name and position from worklist generated in Excel (from SOP step 2.1).
 - Right-click top-left corner of worklist, click Add Script.
 - Add “Standby script” to the end of the worklist.
 - Again right-click top-left corner of worklist, click Worklist Run Parameters.
 - In Data Path box, set a destination for data files to be saved.
 - Use the date and worklist number as destination folder name. Example: “D:\MassHunter\Data\BCH_Vitamin D\20140302_WL 0001”.
 - Click File, Save as, Worklist.
 - Give appropriate worklist name (identical worklist name as given in SOP step 2.1).
 - Click File, Print, Worklist Report and file for future reference.
 - Worklist now setup.

- Analysing samples:
 - Click File, Method, Open.
 - Choose saved method name. Example: VITAMIN D_REV 1.m.
 - Power on the LC-QQQ by clicking “Power On” tab.
 - All modules statuses will turn green indicating that the instrument is in a ready state (do this 15 minutes before sample analysis).

- If the module status is red, indicating an instrument error, consults the Log book.
- Insert samples in autosampler trays. Starting at position A1 in the top-left corner of plate 1 (plate 1 is to the front and plate 2 at the back).
- Click “Start Worklist Run” tab to start sample analysis.

2.4.3 QUANTITATIVE DATA ANALYSIS

- The quantification of the data is performed relative to the stable isotope with similar chemical properties. Vitamin D₂ and vitamin D₃ are quantified using vitamin D₃-[²H₃], 25(OH)D₂, 25(OH)D₃, 3-epi-25(OH)D₂, 3-epi-25(OH)D₃, 7(OH)4C3 and 1α(OH)D₃ with 25(OH)D₃-[²H₃], 1,25(OH)₂D₂, 1,25(OH)₂D₃, 24,25(OH)₂D₂ and 24,25(OH)₂D₃ with 1,25(OH)₂D₃-[²H₃].
- The analyte concentration of each calibrator level as well as the concentration of the stable isotope in the calibrators are used for this section.
 - Open “MassHunter Quantitative Analysis” software (Quant: Blue Icon).
 - Click File, New Batch.
 - Type an appropriate batch name that contains the date and worklist number in the folder that contains the data. Example: 20140302_WL 0001.
 - Click OK.
 - Click, File, Add Samples.
 - Select and add all the samples, blanks and calibrators from that worklist.
 - Click Method tab, New, New Method from Acquired MRM Data.
 - Select an appropriate data file containing the MRM acquisition data.
 - Click OK.
 - Click “Concentration Setup” tab.
 - Create calibration levels for each analyte by clicking the “Create Levels” tab.
 - Insert the calibration concentration of each level as specified in 1.5.2.
 - Click “ISTD Setup” tab.
 - Flag the stable isotopes as internal standards by clicking in the “ISTD Flag” checkboxes.
 - Enter the stable isotope concentrations in the “ISTD Conc.” boxes.
 - Click the “Validate” tab.
 - If a method error appears consult the log book.

- Click OK.
 - Click “Save” tab.
 - Click “Exit” tab.
 - Click OK when prompt if method should be applied to batch.
 - Add a concentration factor of 0.1 by right-clicking and clicking Add Column.
 - Choose to add column “Dil.”.
 - The concentration factor of x10 is corrected in all the calculated sample concentrations.
 - Click “Analyse Batch” tab.
 - Click File, Save Batch.
 - Click File, Print and print the data and the calibration curves.
 - Alternatively, the data can be exported to Excel for report generation.
- Reference values for serum vitamin D:

Vitamin D status:	Total 25(OH)D:	
	(nmol/L)	(ng/ml)
Deficiency	< 30	< 12
Insufficiency	30 - 50	12 - 20
Sufficiency	50 - 125	20 - 50
Toxic	> 125	> 50

3 HEALTH AND SAFETY

- All the biological sample are to be treated as potentially infectious and should be handled accordingly.
- All personnel must read and sign the relevant Material Safety Data Sheets (MSDS) before handling of any chemicals.
- Protective laboratory-coats, eyewear and gloves are required with the handling and preparation of any biological samples or chemicals.

4 REFERENCES

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