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Development of an assay for total vitamin D content in milk and application to study the effect of pasteurisation in breastmilk

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Abstract

Vitamin D deficiency has been traditionally associated with bone disorders; recent studies, however, show a relationship to several non-skeletal diseases such as autoimmune diseases, rheumatoid arthritis, hypertension, diabetes mellitus, cardiovascular diseases and common cancers. As a result, there has been an increasing global concern on the role of vitamin D in human health.

Vitamin D occurs as a number of metabolites/analogues that vary in their biological functions. Vitamin D_3 (D_3) and 25-hydroxyvitamin D_3 ($25(OH)D_3$) are considered more potent than vitamin D_2 (D_2) and 25-hydroxyvitamin D_2 ($25(OH)D_2$). The 1,25-dihydroxyvitamin D metabolites ($1,25(OH)_2D_3$ and $1,25(OH)_2D_2$) are the most active forms that are responsible for bone health. The 24,25-dihydroxyvitamin D metabolites ($24,25(OH)_2D_3$ and $24,25(OH)_2D_2$) are responsible for intramembranous and endochondral bone formation, and bone fracture repair. The biological roles of sulfated analogues/metabolites, vitamin D-Sulfate (D_3-S and D_2-S) and 25-hydroxyvitamin D-Sulfate ($25(OH)D_3$-S and $25(OH)D_2$-S), are unclear to date, probably due to the lack of sufficiently sensitive assay methods. It has been suggested, however, that sulfated forms are storage forms of the non-sulfated forms, and may have similar potencies. The major circulating form is $25(OH)D_3$, with a long half-life, and is therefore considered as the principal biomarker of vitamin D status. However, considering the varied biological roles of vitamin D analogues/metabolites listed above, it is desirable to assay as many as possible using a sensitive and specific method.

Milk is an important source of vitamin D for various risk populations, including infants. When mothers are unable to breastfeed their infants, pasteurised donor human milk is the most effective alternative source of nutrition. Vitamin D is potentially sensitive to high temperatures and pasteurisation is a thermal procedure potentially responsible for vitamin D degradation. No studies on the impact of pasteurisation on vitamin D content in breastmilk have yet been reported.

There are only few vitamin D assay methods available for unfortified milk. Among them, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been demonstrated to be the best technique in terms of sensitivity and specificity compared with other methods. However, using LC-MS/MS for vitamin D is a challenging task because vitamin D analogues/metabolites have poor MS/MS detectability due to their low ionisation in atmospheric ionisation sources such as electrospray and chemical ionisation. Although there

are eight major analogues/metabolites of vitamin D in biological fluids, currently available LC-MS/MS methods are capable of quantifying only four of these in milk; the dihydroxylated and sulfated forms are not quantified. Furthermore, current methods do not distinguish the major circulating form, 25(OH)D_3, from its inactive epimeric form, resulting in an over-estimation of 25(OH)D_3.

This thesis describes the development and validation of an LC-MS/MS method for the quantitative analysis of eight major forms of vitamin D in milk: D_2; D_3; 25(OH)D_2; 25(OH)D_3; 24,25$(OH)_2D_2$; 24,25$(OH)_2D_2D_3$; 1,25$(OH)_2D_2$ and 1,25$(OH)_2D_3$. Protein precipitation was compared with the commonly used saponification method for extraction of vitamin D from milk and found to be simpler and more effective. A systematic optimisation of the pre-column derivatisation procedure using 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) was performed to enhance the ionisation efficiencies thereby increasing the sensitivities of all vitamin D compounds. Chromatography was also optimised to reduce matrix effects in the MS/MS detector, and co-eluting stable isotope labelled internal standards were used for the calibration of each analogue/metabolite to eliminate matrix effects. The metabolite 25(OH)D_3 and its epimer were chromatographically separated to prevent over-estimation of vitamin D status. The method was validated: detection limits ranged from 0.2 to 0.4 femtomols, repeatability standard deviations ranged from 6.30 to 13.5%, and the recoveries ranged from 88.2 to 105%.

The validated method was then applied to assay for vitamin D compounds in human, cow, mare, goat and sheep milk samples and was also used to evaluate the effect of pasteurisation on the content of vitamin D in breastmilk. Pasteurisation resulted in a significant reduction ($P < 0.05$) in the detected vitamin D compounds and the losses ranged from 10 to 20%.

During the initial method development, an attempt was made to include sulfated vitamin D compounds, D_2-S; D_3-S; 25(OH)D_2-S and 25(OH)D_3-S. However, the method failed to detect sulfated forms in milk, except for traces of 25(OH)D_3-S. Therefore, an LC-MS/MS method, specific for sulfated forms, was developed. A comparative evaluation showed that, for sulfated forms, the ionisation efficiencies of underivatised forms in negative ion mode electrospray ionisation (ESI) were superior to those of derivatised forms with PTAD in positive ion mode ESI. Therefore, underivatised D-S compounds were used with negative MS mode, in an LC-MS/MS method similar to the method for non-sulfated forms. The method was then fully validated: detection limits ranged from 0.20 to 0.28 femtomol, repeatability standard deviations ranged from 2.8 to 10.2%, and recoveries ranged from 81.1 to 102%. The validation and application of this sulfated method was undertaken for both human milk and

serum. All four sulfated vitamin D analogues/metabolites were quantifiable in serum, and three in milk.

Declaration by author

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

I have clearly stated the contribution of others to my thesis as a whole, including statistical assistance, survey design, data analysis, significant technical procedures, professional editorial advice, and any other original research work used or reported in my thesis. The content of my thesis is the result of work I have carried out since the commencement of my research higher degree candidature and does not include a substantial part of work that has been submitted to qualify for the award of any other degree or diploma in any university or other tertiary institution. I have clearly stated which parts of my thesis, if any, have been submitted to qualify for another award.

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Publications during candidature

Review

Gomes FP, Shaw PN, Whitfield K, Koorts P, Hewavitharana AK. Recent trends in the determination of vitamin D. *Bioanalysis*. 2013; 5(24):3063-78.

Book chapter

Hewavitharana AK, and **Gomes FP** (2016). Cholecalciferol: Properties and Determination. In: Caballero, B., Finglas, P., and Toldrá, F. (eds.). The Encyclopedia of Food and Health vol. 2, pp. 42-46. Oxford: Academic Press.

Research papers

Gomes FP, Shaw PN, Whitfield K, Hewavitharana AK. Simultaneous quantitative analysis of eight vitamin D analogues in milk using liquid chromatography-tandem mass spectrometry. *Analytica Chimica Acta*, 2015; 891:211-20.

Gomes FP, Shaw PN, Whitfield K, Koorts P, McConachy H. and Hewavitharana A.K. Effect of pasteurisation on the concentrations of vitamin D compounds in donor breast milk, *International Journal of Food Sciences and Nutrition*, 2016, 67: 16–19.

Gomes FP, Shaw PN and Hewavitharana A.K. Determination of four water-soluble vitamin D analogues in human biological fluids by liquid chromatography-tandem mass spectrometry, *Journal of Chromatography B*, 2016; 1009: 80-86.

Kassim NSA, **Gomes FP**, Shaw PN, and Hewavitharana AK. Simultaneous quantitative analysis of nine vitamin D compounds in human blood using LC-MS/MS, *Bionalysis*, 2016; 8: 397 - 411.

Conference abstracts

Fabio P. Gomes, Paul N. Shaw, Karen Whitfield and Amitha K. Hewavitharana. Comparison of Saponification and Protein Precipitation Methods for the Extraction of Vitamin D in Breast Milk, In: *Mass Spectrometry: Applications to the Clinical Lab (MSACL)*, Salzburg (Austria), 2015. (Poster presentation)

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Publications included in this thesis

Gomes FP, Shaw PN, Whitfield K, Koorts P, Hewavitharana AK. Recent trends in the determination of vitamin D. *Bioanalysis*. 2013; 5(24):3063-78.

Contributor	Statement of contribution
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Paul N. Shaw	Supervision and manuscript revision (40%)
Karen Whitfield	Supervision and manuscript revision (5%)
Pieter Koorts	Supervision and manuscript revision (5%)
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Gomes FP, Shaw PN, Whitfield K, Koorts P, McConachy H and Hewavitharana AK. Effect of pasteurisation on the concentrations of vitamin D compounds in donor breast milk, *International Journal of Food Sciences and Nutrition*, 2016, 67: 16–19.

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Contributions by others to the thesis

Dr. Amitha K. Hewavitharana, Dr. Karen Whitfield, Dr. Pieter Koorts and Prof. Nick Shaw contributed to the conception, design and progress of this research project. They also contributed to the preparation of the manuscripts.

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

None.

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Dedication

This thesis is dedicated to my mother (Maria Carolina Marques Gomes) who I express my eternal heartfelt gratitude. I love her very much and with all force of my heart, without her this work would never be possible. She is all my reasons and inspiration. I thank her very much for the continuous and strong encouragement and support since I was born. I do not have words to express my feelings for her. Although I am far away from her, she is always in my heart and thoughts. We are only one.

Trust in the LORD forever, for the LORD GOD is an everlasting rock.
Isaiah 26:4

Keywords

Vitamin D, LC-MS/MS, derivatisation, saponification, protein-precipitation, pasteurisation

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List of abbreviations

1α(OH)D_3: 1α-hydroxyvitamin D_3

1,25(OH)$_2$D: 1,25-dihydroxyvitamin D

3-epi-25(OH)D_3: 3-epi-25-hydroxyvitamin D

24,25(OH)$_2$D: 24,25-dihydroxyvitamin D

25(OH)D: 25-hydroxyvitamin D

25(OH)D-S: 25-hydroxyvitamin D-Sulfate

AAP: American Academy of Pediatrics

ACE: Acetone

ACN: Acetonitrile

AmAc: Ammonium acetate

AMM: Ammonium formate

amu: Atomic mass unit

APCI: Atmospheric pressure chemical ionisation

APPI: Atmospheric pressure photoionisation

Asc: Ascorbic acid

Borbuff: Borate buffer

BHT: Butylated hydroxytoluene

BuOH: Butanol

C: Chloroform

CAD: Collision gas

CE: Collision energy

CLIA: Chemiluminescence

CN: Cyanopropyl column

CPS*:* Counts per second

CUR: Curtain gas flow

CXP: Collision exit potential

CYHA: Cyclohexane

D_3: Vitamin D_3

D_2: Vitamin D_2

D_3-S: Vitamin D_3-Sulfate

D_2-S: Vitamin D_2-Sulfate

DAD: Diode array detector

DAPTAD: 4-(4-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione

DBS: Dried blood spots

DBP: Vitamin D-binding protein

DCM: Dichloromethane

DEAPTAD: 4-(4-diethylaminophenyl)-1,2,4-triazoline-3,5-dione

DIOX: Dioxane

DP: Declustering potential

DMEQ-TAD: 4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl-)ethyl]-1,2,4-triazoline-3,5-dione

E: Ether

ECLIA: Electrochemiluminescence

EP: Entrance potential

EtOAc: Ethyl acetate

EtOH: Ethanol

ESI: Electrospray ionisation

FA: Formic acid

FAB: Fast atom bombardment

FDA: Food and Drug Administration

FP: Focusing potential

GC: Gas chromatography

H_2O: Water

H: Heptane

HAc: Acetic acid

HEX: Hexane

HIV: Human immune deficiency virus

HMBANA: Human Milk Banking Association of North America

HP: Holder pasteurisation

HPLC: High performance liquid chromatography

HPTLC: High performance thin layer chromatography

HTLV: Human T-lymphoma virus

HTST: High-temperature short-time

IPA: Isopropanol

IS: Ion spray voltage

ISO-OCT: Isooctane

IU: International unit

KOH: Potassium hydroxide

K_2PO_4: Potassium phosphate dibasic

LLE: Liquid liquid extraction

LC-MS: Liquid chromatography-mass spectrometry

LC-MS/MS: Liquid chromatography tandem mass spectrometry

LiAc: Lithium acetate

LOD: Limit of detection

LOQ: Limit of quantitation

M: Methylamine

m/z: Mass/charge ratio

MeCl: Methylene chloride

MeOH: Methanol

MRM: Multiple reaction monitoring

MS: Mass spectrometry

MTBE: Methyl *t*-butyl ether

NaAS: Sodium ascorbate

NaOH: Sodium hydroxide

NB: Nebulizer gas

NEC: Necrotising enterocolitis

NH_2: Amino column

NMR: Nuclear magnetic resonance

P: Pentane

PA: Pyrogallol acid

PB: Phosphate buffer

PBS: Phosphate buffer saline

PC: Preparative column

PE: Petroleum ether

PFP: Pentafluorophenyl

PP: Protein precipitation

PrOH: Propanol

PTAD: 4-phenyl-1,2,4-triazoline-3,5-dione

PY: Pyrogallol

QTAD: 4-(6-quinolyl)-1,2,4-triazoline-3,5-dione

RBWH: Royal Brisbane and Women's Hospital

RIA: Radioimmunoassay

RP: Reversed-phase

RRF: Relative response factor

SDS: Sodium dodecyl sulphate

SERS: Surface-enhanced Raman scattering

SIL: Stable isotope labelled

SN: Saponification

S/N: Ratio of Signal/Noise

SPE: Solid phase extraction

SPME: Solid phase micro extraction

Tol: toluene

TIC: Total ion current

TSP: thermospray

UQ: University of Queensland

UV-B: Ultraviolet B irradiation

V: Volts

WHO: World Health Organization

ZS: Zinc sulphate

1 Chapter 1. Literature review

1.1 Foreword

Chapter 1 reviews the analytical methods used to quantify vitamin D, and within this context introduces vitamin D, its many forms and its metabolism. This review employed a comprehensive search of the most used vitamin D methods for all applications. Particular emphasis was given to sample preparation methods and the different forms of vitamin D measured across different fields of applications such as biological fluids, food and pharmaceutical preparations. This revision compared and critically evaluated a wide range of approaches and methods, and then used this comparative critical evaluation to formulate the best possible vitamin D assay method that is the subject of the subsequent chapters of this thesis. The content of this comprehensive review has been published as a review article in the journal *Bioanalysis* 5 (24): 3063-78 (2013). This article is presented in this chapter, in a slightly altered format to fit with the style of this thesis. Permission was granted from the publisher to reproduce the work in the thesis. Some additional information such as the introduction of sulfated forms of vitamin D is added. Tables 1 and 2 have been updated, to include new analytical methods from 2013 to 2016. The research hypothesis and aims of this thesis are also included.

1.2 Introduction

Vitamin D insufficiency and its deficiency in human populations has been demonstrated to be of significant concern and has become a very important health issue (1). Vitamin D deficiency is not only related to muscle weakness and osteomalacia, but has also been associated with cardiovascular disease, cancer, autoimmune diseases, diabetes mellitus and hypertension (2-5). Furthermore, newborn babies may be affected in their normal growth and development, putting them at risk of long-term physical deficits (6). Vitamin D status is usually evaluated by determining the levels of the metabolite 25-hydroxyvitamin D (25[OH]D), the major circulating form, which is considered the most important biomarker among the many forms of this vitamin in plasma or serum (7). Early detection of vitamin D deficiency is of key importance in order to identify affected individuals who can then receive adequate and appropriate treatment. As a result, many analytical methods based on a variety of techniques have been reported for the assay of vitamin D and its metabolites, or analogues, in complex matrices, especially in biological fluids. Subsequently, many reviews have been published on the topic of vitamin D assay methods. However, most of the reviews published in recent

years have focused on the measurements in biological fluids such as serum or plasma using LC–MS (8-12). While the majority of vitamin methods published have focused on LC–MS, this chapter discusses all methods, including immunoassays, HPTLC, NMR and Raman scattering, since LC–MS still remains an inaccessible instrument for many analytical laboratories. In addition, we have included measurements in all matrices including food, plants, biological fluids and pharmaceutical preparations. Emphasis was given to various forms of vitamin D that were measured, and the various extraction and sample preparation methods employed for this purpose. In general, the development of vitamin D methodologies, using a variety of techniques and in various fields (e.g., biological fluids, foods and pharmaceutical products), has been undertaken in isolation. We believe that a chapter with all vitamin D methods that compares and critically evaluates a wide range of approaches will provide a valuable addition to the literature and will enable the reader to access developments across a number of applications and to select or develop the optimal method for their particular application. For this reason, we have divided all methods into common sections: sample preparation, separation and detection, and quantification, in order that the reader can select the best option from each section and tailor a new method of analysis. The comparisons and evaluations were undertaken for each of the above sections to render this task easier. Our search included comprehensive databases: ISI Web of Science®, Scifinder®, Science Direct, Scopus and PubMed, for all vitamin D methods published recently. The search word used was ‘vitamin D’ and then refined using key words such as ‘quantitation’, ‘determination’ and ‘methods’. Approximately 120 articles were found in total, but only some 90 are included here because others comprise studies where the methods used were not described in detail, thus not enabling us to review them fully in this chapter.

1.3 Forms of vitamin D and its metabolites

Vitamin D has two major forms, cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) **(Figure 1)**.

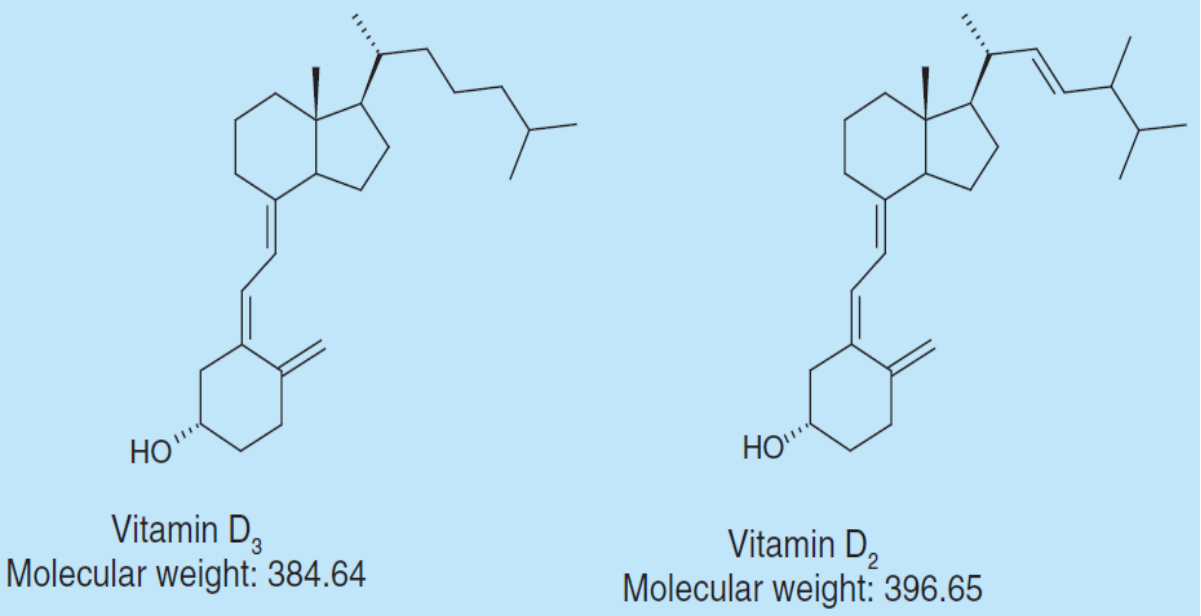

Figure 1. Different forms of vitamin D.

In animals, vitamin D_3 is obtained from food or by photosynthesis in the skin, formed from UV irradiation (UV-B: 290– 315 nm) of 7-dehydrocholesterol in the skin. In plants, vitamin D_2 is produced by the irradiation of ergosterol. Both vitamins D_2 and D_3 are biologically inactive. In humans, they are metabolized in the liver to calciferol (25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3, collectively known as 25(OH)D). Vitamin D that is not metabolized in the liver is stored in the adipose tissue and skeletal muscle, then released during vitamin D deprivation. The major circulating form of vitamin D is 25(OH)D, which is then transported to the kidney where it is hydroxylated to its hormonal and biologically active form, calcitriol (1,25-dihydroxyvitamin D, also called 1a,25-dihydroxyvitamin D, [1,25$(OH)_2$D]) or the form considered inactive, 24,25-dihydroxyvitamin D [24,25$(OH)_2$D] **(Figure 2)**, depending on the calcium and phosphorus requirements of the animal (13).

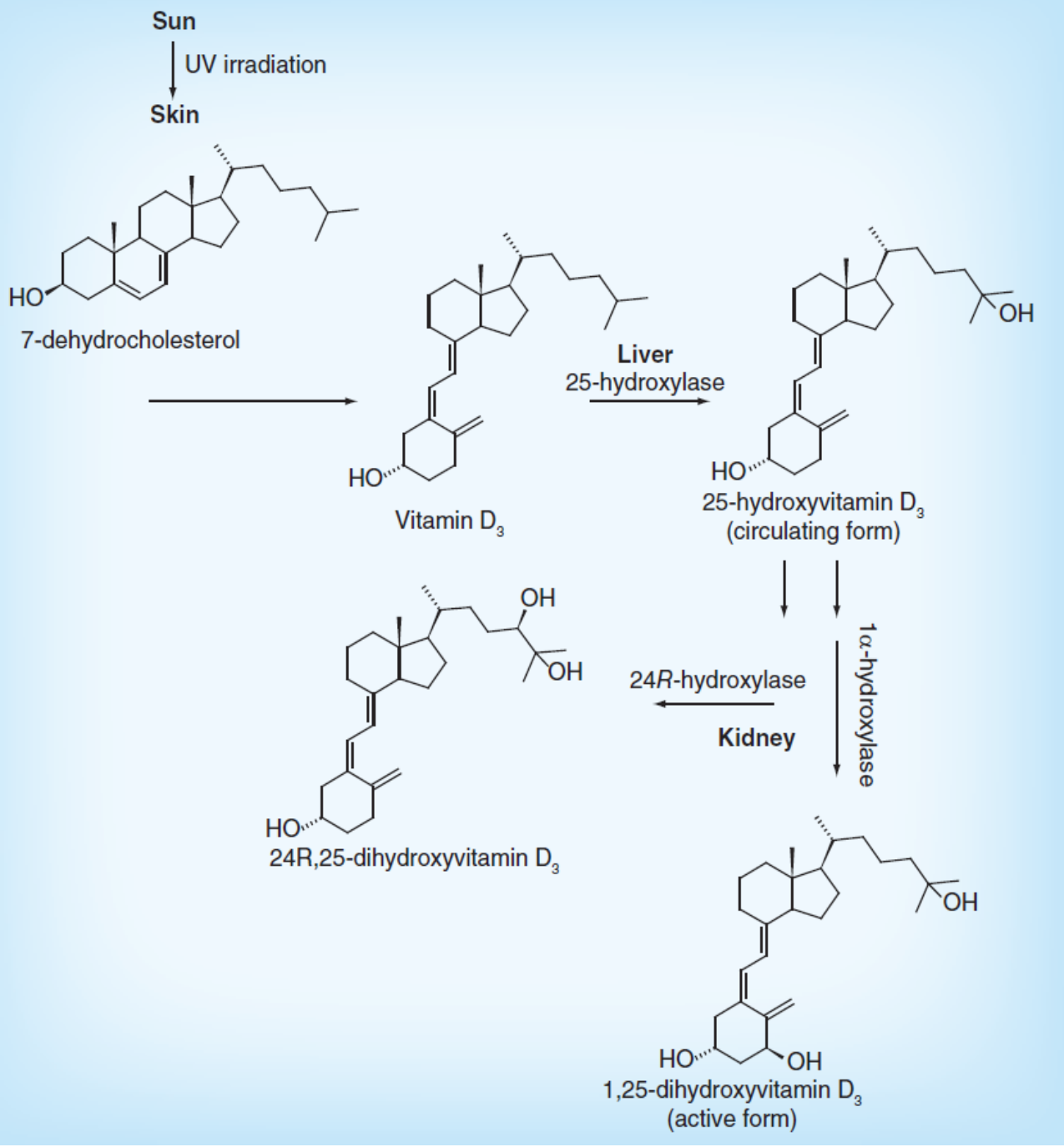

Figure 2. Biosynthesis and metabolism of vitamin D_3 in humans.

Although 24,25$(OH)_2$D has been originally considered to possess little or no activity, other than serving to divert the metabolism of 25(OH)D away from 1,25$(OH)_2$D, some studies indicate that it is a functionally independent hormone that plays a crucial role in intramembranous and endochondral bone formation and in the repair of bone fractures (13).

The process of the conversion to the biologically active forms of vitamin D_2 and D_3 was evident by the lag time between the administration and the appearance of a physiological

response. In animals, the potency of vitamin D_2 was found to be higher than that of vitamin D_3 when the animal is nocturnal and/or when its diet is primarily plant-based (13). In humans, most research has been carried out with vitamin D_3 rather than vitamin D_2, with the former found to be more efficacious than the latter at raising serum vitamin D concentrations (14). In plasma, all forms of vitamin D are bound to vitamin D-binding protein (DBP), however, the binding affinity of $1,25(OH)_2D$ is tenfold less than that of 25(OH)D (with both vitamin D_2 and D_3). Although the dietary forms are mainly the non-hydrolyzed forms, there may be small amounts of 25(OH)D in some foods that can be absorbed far more easily by the body. In some disease states 25(OH)D is the more effective form of supplementation due to its ease of absorption. The normal concentration range of plasma 25(OH)D is 10 to 40 ng/mL. Unlike with photosynthesized vitamin, with dietary intake levels can rise to over 400 ng/mL, leading to vitamin D toxicity. The normal plasma concentration of $24,25(OH)_2D$ in humans ranges from 1 to 4 ng/mL, while that of the potent form, $1,25(OH)_2D$, is maintained within the range 25 to 70 pg/mL by reciprocal changes in the rates of synthesis and degradation at the cellular level (13).

Currently, 25(OH)D ($25(OH)D_2$ and $25(OH)D_3$ collectively) is considered to be the best indicator of vitamin D status in either serum or plasma because this metabolite has been demonstrated to have a half-life much longer and a circulation concentration approximately 1000- times larger than $1,25(OH)_2D$, which allows a precise estimation of vitamin D status from both dietary intake and UV irradiation (15). In addition, 25(OH)D is produced based on its substrate concentration, which is different from $1,25(OH)_2D$ that is limited by the calcium requirements of each individual (13, 16).

Vitamin D can be also found as water-soluble (sulfated) forms. Little clinical and nutritional attention have been given to these conjugated forms. While a few proponents claim their vital roles in humans, mainly on semi-scientific websites (17, 18), a clear evidence is still out. Studies suggest that non- and sulfated forms possess equivalent potencies and also that sulfated forms could be storage forms of non-sulfated forms (19-21). The biosynthesis of sulfated forms still remains to be elucidated (19, 22); however, it has been found that vitamin D_3-Sulfate (D_3-S) is formed in the skin in a similar photochemical process of its non-conjugated form. It was confirmed by the presence of 7-dehydrocholesterol-sulfate (7-DHC-S) in human skin (19). While there is no conclusion about the specific role of each sulfated form, D_3-S has been reported to increase calcium transportation in young rats (23). The metabolite 25-hydroxyvitamin D_3-sulfate ($25(OH)D_3$-S) could be an important vitamin D biomarker, as its levels in human circulation seems to exceed those of its non-conjugated

form (19, 22). Vitamin D_2-Sulfate (D_2-S) has been shown to have an efficient antirachitic activity in rats (24). There are no reports of the metabolite 25-hydroxyvitamin D_2-Sulfate (25(OH)D_2-S) in human or animal biological fluids. The lack of research in to water soluble (sulphated) forms of vitamin D could be due to the lack of analytical methods available to quantify them.

While there are more than 50 vitamin D metabolites, clinical studies has primarily focused on the assessment of 25(OH)D status (25). However, it is still controversial whether the measurement of only 25(OH)D provides sufficient information to link vitamin D with many other non-skeletal disorders such as the aforementioned diseases in the introduction section (25, 26). Hence, the capability to specifically measure different vitamin D forms is highly valuable to investigate clinical disorders that are associated with vitamin D deficiency and/or vitamin D metabolic dysfunction (25).

Recently, the detection of epimeric forms of 25(OH)D and 1,25$(OH)_2$D in infant, paediatric and adult populations has raised the question as to whether these C3-epimers can/should be measured (27). Studies have found that all vitamin D metabolic products can be epimerized (27) and 3-epi-1,25$(OH)_2D_3$ is also capable of binding to the vitamin D receptor (28). The epimerisation routes for 25(OH)D and 1,25$(OH)_2$D are shown in **Figure 3** (29).

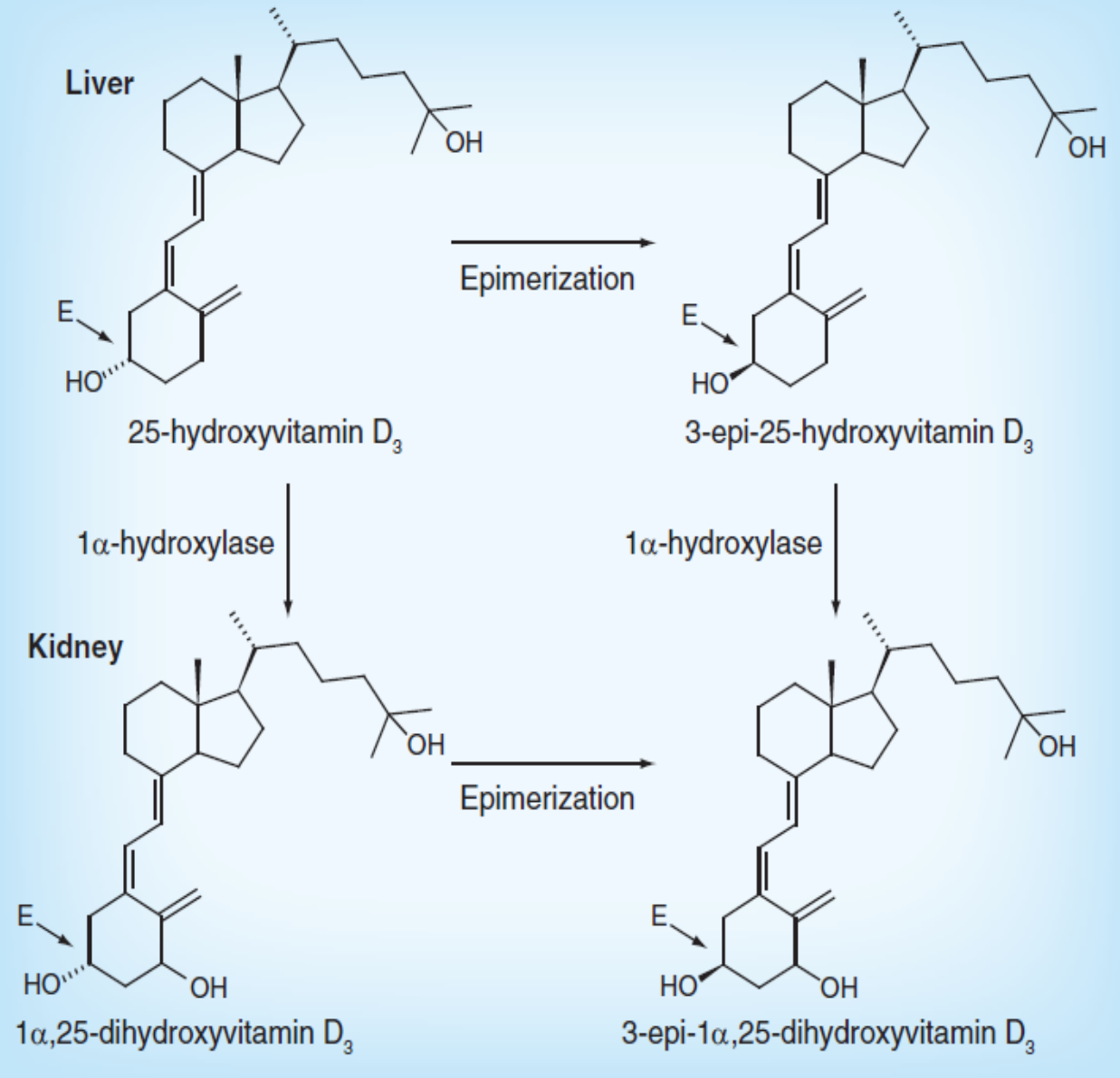

Figure 3. Epimerization routes for 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D_3. E: The epimeric centre. Adapted from (29).

3-Epi-25(OH)D_3 and 3-epi-1,25$(OH)_2D_3$ are considered inactive or relatively less active in terms of their physiological effects, therefore, collective measurement of the vitamin D forms and their epimers may contribute to an overestimation of the vitamin D activity and detrimentally affect the outcome of clinical studies (28). Recent analytical methods have separated the epimers using special columns with LC–MS/MS separation and detection, rather than by measuring the total using techniques such as immunoassay and the more usual LC methods (27).

The main form of vitamin D present in plant-based foods dried in sun or under UV light is vitamin D_2 formed by the irradiation of ergosterol. However, a recent study found that cholesterol containing plant foods such as the Solanaceae family are able to produce vitamin D_3 (30). The major form in animal based foods such as cod liver oil, oily fish, eggs and cow's milk products, is vitamin D_3 (31). However, human and cow's milk both contain small amounts of other metabolites such as $24,25(OH)_2D$ and $1,25(OH)_2D_3$ (32). It was found that $25(OH)D_3$ is absent in cow's milk products (33). In pharmaceutical preparations, in addition to vitamin D_2 and D_3, $1,25(OH)_2D_3$ and its analogues, 1α-hydroxyvitamin D_3 ($1[OH]D_3$) have also been used for the treatment of diseases such as osteoporosis (34). In general, vitamin D analysis in foods has been undertaken on fortified foodstuffs, therefore mainly concentrating on vitamin D_3 and vitamin D_2.

1.4 Analytical methods

1.4.1 Sample preparation

Sample preparation is the first and the critical stage of method development as it directly impacts on the accuracy of the analytical method. The extraction (from the matrix) of the vitamin is a crucial step, as it cannot be assessed by the validation process. While the recovery parameter estimates the accuracy in terms of how much of the added vitamin is recovered, it does not indicate whether the vitamin is completely extracted from the matrix. A recent study that compared six different vitamin D assay methods concluded that inadequate release of the vitamin from the DBP, in some of the methods, was the main reason for the high between-method variabilities they observed (35). In this comparison, most methods performed poorly when such protein levels are high in conditions such as pregnancy.

The sample clean-up step has special significance in chromatographic methods coupled with MS: adequate sample clean-up not only protects the chromatographic columns, but also reduces matrix effects from co-eluting substances during the ionisation process. Particularly for vitamin D compounds in biological fluids or foodstuffs, their very low concentrations demand adequate sample-preparation methods in order to achieve the best sensitivity.

Sample preparation, in this chapter, is divided into extraction, clean-up and derivatisation steps, all processes that are involved prior to separation and detection. Table 1 summarizes each of these steps, along with the forms of vitamin D measured in different matrices, in analytical methods.

Table 1. Summary of sample preparation methods used in the analysis of vitamin D.

Vitamin D analogues	Sample	Extraction	Clean up	Derivatisation	Refs.
$25(OH)D_3$, $25(OH)D_2$ $1\alpha25(OH)_2D_3$, $1\alpha25(OH)_2D_2$ $24,25(OH)_2D_3$	Serum	PP – ACN	LLE - K_2HPO_4:MTBE SPE - RP, ACN	PTAD	(36)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25OHD_3$, 24*R*,$25(OH)_2D_3$	Serum	PP – ACN	Online SPE - RP, MeOH:H_2O	-------	(37)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25OHD_3$	Serum	PP – ZS, MeOH	LLE – HEX:EtOAc	-------	(38)
$1\alpha25(OH)_2D_3$	Serum	PP – ACN	Online SPE - RP, MeOH:ACN:H_2O	-------	(39)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN	Online SPE - RP, MeOH:H_2O	-------	(40)
$1,25(OH)_2D_2$	Serum	PP – ACN	SPE - RP, TBME (96-well plate)	-------	(41)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN	Online SPE (96-well plate)	-------	(42)
$25(OH)D_3$, $25(OH)D_2$ $1\alpha25(OH)_2D_3$, $24,25(OH)_2D_3$	Serum	PP - MeOH:ACN	SPE - RP, ACN:IPA:FA	PTAD	(43)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN	-------	-------	(44)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN	-------	-------	(45)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN	-------		(46)
$1\alpha25(OH)_2D_3$, $1\alpha25(OH)_2D_2$	Serum	PP – ACN IDS antibody	Alcohol SPE - RP, Alcohol	-------	(47)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN (96-well-plates)	-------	-------	(48)

Table 1 (continued).

Vitamin D analogues	Sample	Extraction	Clean up	Derivatisation	Refs.
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN (96-well-plates)	LLE – H (96-well-plates)	-------	(49)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – ACN (96-well-plates)	-------	-------	(50)
25(OH)D	Serum	PP - ACN (RIA) PP - Surfactant:EtOH (CLIA)	-------	-------	(51)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – EtOH	SPE - RP, EtOAc:H PC – CN, IPA:H	-------	(52)
25(OH)D	Serum	PP - EtOH	LLE - HEX:DCM	-------	(53)
$25(OH)D_3$	Serum	PP – EtOH (ELISA)	-------	-------	(54)
D_3	Serum	PP - MeOH	-------	-------	(55)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – MeOH	SPE - RP, MeOH:H_2O (Automated)	-------	(56)
D_2, D_3, $25(OH)D_2$, $25(OH)D_3$	Serum	PP – ACE	-------	-------	(57)
D_3, D_2, $25(OH)D_3$, $25(OH)D_2$, $1\alpha25(OH)_2D_2$ $1\alpha25(OH)_2D_3$, 3-epi-$25(OH)D_3$, 3-epi-$25(OH)D_2$	Serum	PP - MeOH:IPA:FA	LLE - DCM:H	-------	(58)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP - MeOH:ACN	SPE - RP, MeOH	-------	(59)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25(OH)D_3$	Serum	PP - MeOH:IPA	LLE - HEX	-------	(60)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25(OH)D_3$, $1\alpha(OH)D_3$	Serum	PP - MeOH:IPA	LLE - HEX:DCM SPE - silica, E:HEX	-------	(29)

Table 1 (continued).

Vitamin D analogues	Sample	Extraction	Clean up	Derivatisation	Refs.
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25OHD_3$	Serum	PP - ACN:H_2O:ZS	SPE - RP, ACN:ACE (96-well plate)	-------	(28)
$25(OH)D_3$, $25(OH)D_2$	Serum	-------	LLE - HEX:EtOAc	-------	(61)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25OHD_3$	Serum	PP - ACN:MeOH	SPE – MeOH	-------	(62)
D_3	Serum	-------	LLE - MTBE LLE – MTBE SPE - RP – MeOH (96-well plate)	PTAD	(63)
$1,25(OH)_2D_2$, $1,25(OH)_2D_3$	Serum	--------	SPE – Chromabond XTR, PrOH:HEX SPE – silica, PrOH:HEX	Amplifex PTAD	(64)
D_2, D_3, $25(OH)D_2$, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$, $1,25(OH)_2D_3$	Serum	-------	SPE – ACN:H_2O	-------	(65)
$25(OH)D_3$, $24,25(OH)_2D_3$	Serum	PP – ZS:MeOH	LLE – HEX: MTBE	DMEQ-TAD	(66)
$25(OH)D_3$, $25(OH)D_2$	Serum	PP – MeOH	LLE - HEX	-------	(67)
$25(OH)D_3$, 3-epi-$25(OH)D_3$	Serum	PP – EtOH:MeOH:H_2O	LLE - HE (96-well plate)	-------	(68)
$1\alpha25(OH)_2D_3$, $1\alpha25(OH)_2D_2$	Serum	PP - MeOH:ACN	SPE - monoclonal anti-$1\alpha25(OH)_2D$, EtOH	PTAD	(69)

Table 1 (continued).

Vitamin D analogues	Samples	Extraction	Clean up	Derivatisation	Refs.
$25(OH)D_3$, 3-epi-$25(OH)D_3$	Serum or murine skin	--------	SPE – ACN:MeOH	PTAD	(70)
$24,25(OH)_2D_3$, $25(OH)D_3$	Serum or brain	PP - ACN	-------	PTAD	(71)
D_2, D_3, $25(OH)D_2$, $25(OH)D_3$, $24,25(OH)_2D_3$, $1,25(OH)_2D_2$, $1,25(OH)_2D_3$	Serum or plasma	PP – MeOH:FA	SPE - Online	-------	(72)
$25(OH)D_3$, $25(OH)D_2$	Serum or plasma	PP – MeOH	LLE - H	-------	(73)
$25(OH)D_3$, $25(OH)D_2$	Serum or plasma	SN – MeOH:NaOH (96-well plate)	LLE - H (96-well plate)	-------	(74)
$25(OH)D_3$, $25(OH)D_2$ $1,25(OH)_2D_3$, $1,25(OH)_2D_2$, $24,25(OH)_2D_3$	Plasma	PP - ACN	SPE - RP, ACN:EtOAc LLE - EtOAc-H_2O	PTAD	(75)
$25(OH)D_3$, $25(OH)D_2$	Plasma	PP - MeOH	SPE - Online	-------	(73)
$25(OH)D_3$-S	Plasma	PP - ACN	SPE – Oasis-HLB, MeOH	-------	(21)
$25(OH)D_3$	Plasma	PP – EtOH	SPE - RP, MeOH LLE - HEX	-------	(76)
$25(OH)D_2$, $25(OH)D_3$	Plasma	PP – MeOH:H_2O:DIOX	LLE – HEX	-------	(77)
25(OH)D	Plasma	PP - ACN (RIA)	-------	-------	(78)

Table 1 (continued).

Vitamin D analogues	Sample	Extraction	Clean up	Derivatisation	Refs.
$25(OH)D_2$, $25(OH)D_3$	Plasma	PP – MeOH:ACN:ZS	-------	-------	(79)
$25(OH)D_3$, $1\alpha25(OH)_2D_3$ $24,25(OH)_2D_3$, $4\beta25(OH)D_2$	Plasma	PP - ACN	LLE - EtOAc	PTAD	(80)
$25(OH)D_3$, $25(OH)D_2$	Plasma	PP - EtOH:BHT	LLE - ISO-OCT:C	-------	(81)
D_3, D_2, $25(OH)D_3$, $25(OH)D_2$	Plasma	SN – MeOH:AS:KOH	LLE – H	-------	(82)
$25(OH)D_3$, 3-epi-$25(OH)_2D_3$	Dried blood spots	PP - MeOH	SPE – RP, EtOAc	PTAD	(83)
$25(OH)D_3$, $25(OH)D_2$	Dried blood spots	PP – MeOH	LLE - HEX	-------	(84)
$25(OH)D_3$, 3-epi-$25(OH)D_3$	Dried blood spots	-------	-------	PTAD DAPTAD DEAPTAD QTAD	(85)
$25(OH)D_3$	Dried blood spots	PP - MeOH	-------	DAPTAD	(86)
$25(OH)D_3$, $24,25(OH)_2D_3$	Urine	--------	SPE - Oasis® HLB, MeOH:H_2O	DAPTAD	(87)
$25(OH)D_3$	Saliva	PP – ACN	SPE - RP, EtOAc	PTAD	(88)
$25(OH)D_3$	Swine tissue	PP - MeOH	SPE - RP, MeOH	-------	(89)
D_2, D_3, $25(OH)D_3$, $1,25(OH)_2D_3$	Mouse brain	-------	LLE – DCM:MeOH	-------	(90)
D_3, $25(OH)D_3$	Porcine tissues	SN – NaAS:KOH:EtOH	LLE – PrOH:H SPE – silica, PrOH:H	PTAD	(91)
D_3	Spinach leaves	SN - KOH:EtOH:AS	SPE - silica, IPA:H	-------	(30)

Table 1 (continued).

Vitamin D analogues	Sample	Extraction	Clean up	Derivatisation	Refs.
D_2-S, D_3-S, 25(OH)D_2-S, 25(OH)D_3-S	Milk/Serum	PP - ACN	-------	-------	(92)
D_2, D_3, 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$	Milk	PP - ACN	LLE – HEX:DCM	PTAD	(93)
D_3	Beverage	SN – KOH:EtOH:AS	LLE – E:PE PC - silica, MeOH:ACN	-------	(94)
D_3, D_2	Food	SN - EtOH:KOH	LLE - H SPE - silica, MeCl:IPA	-------	(95)
D_3, 25(OH)D_3	Food	SN – PY:EtOH:KOH	SPE - silica, IPA:DCM PC - silica-NH_2, IPA:HEX	-------	(96)
D_3, D_2	Food	SN - Alcohol:PA:KOH	LLE - HEX:BHT	-------	(97)
D_3	Food	SN - KOH:AS	LLE - E:PE	-------	(98)
D_3, D_2	Food	SN - NaAS:MeOH:KOH	LLE - E:P	-------	(99)
D_3	Food	PP - MeOH:ISO-OCT:H_2O	-------	PTAD	(100)
D_3	Food	SN – EtOH:KOH:AS	LLE – HEX:BHT SPE – silica, HEX:EtOAc	-------	(33)

Table 1 (continued).

Vitamin D analogues	Sample	Extraction	Clean up	Derivatisation	Refs.
D_3	Supplement	-------	SPE - NH_2, EtOAc	-------	(101)
D_3	Supplement	-------	-------	-------	(102)
D_3	Pharmaceuticals	PP - ACN:MeOH	-------	-------	(103)

Abbreviations: ACE, acetone; ACN, acetonitrile; AS, ascorbic acid; BHT, butylated hydroxytoluene; C, chloroform; CYHA, cyclohexane; DAPTAD, 4-(4-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione; DCM, dichloromethane; DEAPTAD, 4-(4-diethylaminophenyl)-1,2,4-triazoline-3,5-dione; DIOX, dioxane; DMEQ-TAD, 4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl-)ethyl]-1,2,4-triazoline-3,5-dione; E, ether; EtOAc, ethyl acetate; EtOH, ethanol; FA, formic acid; H, heptane; HEX, hexane; H_2O, water; IPA, isopropanol; ISO-OCT, isooctane; LLE, liquid-liquid extraction; KOH, potassium hydroxide; K_2PO_4, potassium phosphate dibasic; MeCl, methylene chloride; MeOH, methanol; MTBE, methyl *t*-butyl ether; NH_2, amino column; P, pentane; PA, pyrogallol acid; PC, preparative column; PE, petroleum ether; PTAD, 4-phenyl-1,2,4-triazoline-3,5-dione; PrOH, propanol; PY, pyrogallol; NaOH, sodium hydroxide; NaAS, sodium ascorbate; NH_2, amino column; QTAD, 4-(6-quinolyl)-1,2,4-triazoline-3,5-dione; SPE, solid phase extraction; ZS, zinc sulphate.

A step prior to sample preparation (sample collection) that deserves special mention here is the use of DBS. In recent years DBS has been used as a sample collection method, instead of the usual heparinised tubes, especially in neonatal monitoring where the sample size available is very low (104, 105). However, Kvaskoff *et al.* have recently investigated the implications of DBS sampling on quantitation and reported that factors such as gradient distribution of the vitamin on paper, and thus the hole punch position, paper type and blood spot volume, can add variability to the analysis of 25(OH)D (106).

1.4.1.1 Extraction

The protein and lipid portions of biological fluids or foodstuffs containing vitamin D compounds comprise a large group of compounds such as triglycerides, phospholipids and DBPs. The physicochemical properties of these molecules make them a promising potential source of interference. As vitamin D compounds in biological fluids or foodstuffs are bound to proteins and/or lipids, these chemical bonds should be broken to liberate the analyte (32, 107). Hence, it is essential to use rigorous procedures to release vitamin D compounds from these macromolecules and concentrate them. Two approaches have been extensively applied to remove proteins and lipids. One is deproteinisation or protein precipitation (PP), used mainly for biological fluids where the vitamin is released from the proteins by denaturing the protein. The other one is saponification (SN), used mainly for foodstuffs where the vitamin is released by hydrolysis of the ester group formed between the hydroxyl groups of the vitamin and carboxylic acid groups of the proteins.

1.4.1.2 Protein precipitation

PP is frequently used to remove proteins from biological fluids. The methodology is based on mixing the sample with aqueous or organic solvents to precipitate out the proteins by denaturation. Subsequently, the precipitated proteins are removed by centrifugation, the supernatant removed by evaporation and the residue reconstituted in a suitable solvent depending on the analytical method used. As shown in table 1, acetonitrile is the most commonly used precipitant in PP (21, 36, 37, 39-42, 44-51, 75, 78, 92, 93, 108, 109). Other precipitants such as acetone, dioxane, ethanol and methanol have also been used with biological fluids (52-57, 73, 76, 83, 84, 89). Some precipitant solutions based on mixing solvents such as acetonitrile, methanol, ethanol, isopropanol and aqueous zinc sulfate have also been used for the extraction of vitamin D compounds (28, 29, 43, 58-60, 67, 69, 77, 79, 81, 103, 110). In some cases, the precipitant solvent is mixed with a nonpolar extracting

solvent (111). As shown in table 1, some methods have not used either PP or SN extractions (61, 63). Some of these were supplementary preparations, which did not need extraction (101, 102). With radioimmunoassay, acetonitrile is usually used to extract the 25(OH)D (51, 78) while a recent ELISA method used ethanol for the same procedure (54). With chemiluminescence enzyme immunoassay, 25(OH)D is dissociated from protein by a buffer containing 10% ethanol and surfactant (51). Commercially available immunoassay kits use proprietary techniques to displace the vitamin from the binding protein. An important innovation in routine analysis has been the introduction of 96-well plates for PP whereby many samples can be prepared simultaneously (48-50). Although simple and convenient, a major drawback of PP is that the extract is not very clean, containing a considerable amount of impurities from the matrix. Consequently, this phenomenon may lead to ion suppression in LC–MS as well as poor selectivity.

1.4.1.3 Saponification (SN)

SN is commonly used with foodstuffs. Based on well-established principle of alkaline hydrolysis, this procedure consists of mixing the sample with potassium hydroxide in ethanol or methanol solution, and heating to approximately 80°C for approximately 1 h. SN can also be performed cold, overnight at room temperature with magnetic stirring (32). As shown in table 1, the non-saponifiable portion containing the vitamin is then extracted using a nonpolar solvent such as ethyl ether, pentane, petroleum ether, heptane or hexane. Subsequently, the organic phase is removed by evaporation and the residue reconstituted in a suitable solvent depending on the analytical method used.

Potassium hydroxide in ethanol or water solution, containing ascorbic acid as the antioxidant to reduce the instability of vitamin D compounds during the SN procedure, has been the most common option of SN for foodstuffs (30, 98, 112, 113); some methods did not use an antioxidant (95, 114). When methanol was used instead of ethanol, no significant differences were observed (82, 99). Some methods have employed pyrogollol as the antioxidant (96, 97).

Although SN is normally used with foodstuffs, Hymoller *et al.* and Hoofnagle *et al.* have unusually applied this approach for plasma/serum samples; in this procedure, alkalinisation was performed with sodium hydroxide and was found to be efficient for their purpose (74, 82). Ethanolic KOH is preferred to aqueous KOH in the saponification of dairy products because it mixes well with fat and prevents formation of emulsions (32). Disadvantages of the SN procedure are the loss of vitamin during SN and use of carcinogenic organic solvents. Also, interfering substances are not completely removed.

1.4.1.4 Sample clean-up

After the release from matrix components such as protein and fat the sample requires further clean-up in order to remove other interfering substances prior to the separation and/or detection. Sample clean-up often improves the sensitivity and selectivity of the assay.

1.4.1.5 Liquid-liquid extraction (LLE)

LLE is the most common clean-up procedure used for the analysis of vitamin D compounds. In vitamin D analysis LLE consists of adding and mixing of a relatively less polar, water immiscible solvent to extract the hydrophobic vitamin D compounds. The organic layer is then separated, the solvent removed and the sample reconstituted in a suitable solvent depending on the subsequent analytical technique used.

As shown in table 1, LLE in vitamin D assays has commonly employed solvents such as hexane, heptane, ether, ethyl acetate, methyl t-butyl ether, dichloromethane, cyclohexane, isooctane, chloroform, pentane or mixtures of nonpolar solvents. Usually, LLE has been performed manually where several stages of solvent transfers are necessary. Automated multi-well plate methods have recently been introduced for simultaneous LLE to improve the throughput of the assay (49, 63, 74, 110). Although LLE is a relatively simple and inexpensive sample clean-up procedure, practical difficulties remain, such as the handling of emulsion formation and the use of hazardous solvents.

1.4.1.6 Solid phase extraction (SPE)

SPE has also been widely used for analysis of vitamin D, as shown in table 1. In vitamin D analysis, the SPE procedure is usually conducted using reversed phase cartridges. However, polar sorbents where interactions between compounds and stationary phases may occur by hydrogen bonds, dipole–dipole or π-π interactions have also been used in SPE clean-up of vitamin D compounds (29, 30, 95, 101, 113). Some methods used immunosorbents (69) and some others used solid-phase microextraction (63). Preparative columns with polar packing have also been used as a SPE alternative (52, 96, 97).

Despite its drawbacks, such as the existence of silanols and lack of stability in a wide range of pH, silica-based sorbents have proved to be a very effective strategy for clean-up in vitamin D assays. Since many other substances with similar chemical characteristics to vitamin D metabolites are present in biological fluids, cross reactivity may easily occur with immunosorbent SPEs. Although attractive at the first glance, due to its simplicity, solid-phase microextraction lacks robustness owing to the fragile needle. Preparative columns are a good

robust alternative to SPE, especially for routine analysis as it is very effective in isolating the vitamin and it is reusable therefore qualifies as a green alternative.

In comparison to LLE, lower volumes and less hazardous solvents are used with SPE and there are no emulsion formation problems. Despite its attractive characteristics, SPE is not without problems: often the cartridges cannot be used more than once and the procedure is time consuming and costly. Moreover, there may be batch-dependent reproducibility issues related to SPE cartridges. Although the new trend of automation (online-SPE) increases the throughput, it does not necessarily improve the assay in terms of sensitivity.

1.4.1.7 Derivatisation procedures

Due to the poor ionisation of vitamin D compounds in mass spectrometers, a derivatisation procedure based on a Diels–Alder or cycloaddition reaction has been introduced to enhance sensitivity in the determination of vitamin D by LC–MS. For the derivatisation of vitamin D compounds, a Cookson-type reagent, 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD), has been extensively used as dienophile. It directly reacts with the *cis*-diene structure of vitamin D compounds at carbon positions 19, 10, 6 and 5 to produce stable Diels–Alder adducts (115). As shown in Table 1, other Cookson-type reagents such as, 4-(4-dimethylaminophenyl)-1,2,4-triazoline-3,5-dione (DAPTAD) (85-87), 4-(4-diethylaminophenyl)-1,2,4-triazoline-3,5-dione (DEAPTAD) (85), 4-[2-(6,7-dimethoxy-4-methyl-3,4-dihydroquinoxalinyl-)ethyl]-1,2,4-triazoline-3,5-dione (DMEQ-TAD) (66), 4-(6-quinolyl)-1,2,4-triazoline-3,5-dione (QTAD) (85) and Amplifex (64) have been also used as derivatising reagents; however, they are either not commercially available or are significantly more expensive than PTAD. As also noted in Table 1, derivatisation is not very common in the LC–MS analysis of vitamin D, probably due to the time-consuming nature of the procedure, including several steps. However, it is an attractive option for the analysis of minor vitamin D analogues, the concentrations of which are too small to be detected by MS of underivatised compounds, and for small sized samples such as neonatal biological fluids.

1.4.2 Separation and detection

Chromatographic techniques are used to separate vitamin D compounds in the extracts from other interfering compounds, before detection and quantification. Although GC has also been used previously for vitamin D analysis, only LC has performed the separations reported in the last years. Most of these LC methods used MS detection and some used UV detection.

1.4.2.1 Chromatographic separation

As shown in table 2, the column type used for chromatographic separation depends largely on the form or forms of vitamin D assayed.

Table 2. Summary of separation, detection and quantification methods used for the analysis of vitamin D.

Vitamin D analogues	Internal standard	Separation conditions	Detection	Sensitivity (pmol/L)	Refs.
$25(OH)D_3$, 3-epi-$25(OH)D_3$	$25(OH)D_4$	RP - MeOH:AMM:M (Isocratic)	MS/MS - ESI	LOD: 3744 LOQ: 7488	(83)
Vitamin D_3, Vitamin D_2 $25(OH)D_3$, $25(OH)D_2$ $1\alpha25(OH)_2D_2$, $1\alpha25(OH)_2D_3$ 3-epi-$25(OH)D_3$, 3-epi-$25(OH)D_2$	Stanozolol-D_3	Chiral-RP - ACN:FA:AmAc (Gradient)	MS/MS - ESI	LOD: 10 - 50	(58)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_3	RP – MeOH:H_2O:FA:AmAc (Gradient)	MS/MS - ESI	LOQ: 5750	(77)
$25(OH)D_3$, $25(OH)D_2$, 3-epi-$25(OH)D_3$	$25(OH)D_3$-d_6, $25(OH)D_2$-d_3, 3-epi-$25(OH)D_3$-d_3	RP – MeOH:H_2O:FA:AmAc (Gradient)	MS/MS - ESI	--------	(62)
$25(OH)D_3$, $25(OH)D_2$, 3-epi-$25(OH)D_3$	$25(OH)D_3$-d_3, 3-epi-$25(OH)D_3$-d_3	PFP – MeOH:H_2O:FA (Isocratic)	MS/MS - ESI	LOD: 500 LOQ: 2000	(38)
D_3-S, D_2-S, $25(OH)D_3$-S, $25(OH)D_2$-S	D_3-S-d_3, D_2-S-d_3, $25(OH)D_3$-S-d_3, $25(OH)D_2$-S-d_3	RP – MeOH:H_2O:AMM (Gradient)	MS/MS - ESI	LOD: 1.2 – 2.4 LOQ: 4.2 – 9.2	(92)
D_3, $25(OH)D_3$	$25(OH)D_3$-d_6, $25(OH)D_3$-d_6	RP – MeOH:H_2O:FA:M (Gradient)	MS/MS	LOQ: 250*	(91)
$25(OH)D_3$, $24,25(OH)_2D_3$	DAPTAD- d	RP – MeOH:H_2O:AMM (Gradient)	MS/MS - ESI	--------	(87)
D_2, D_3, $25(OH)D_3$, $1,25(OH)_2D_3$	D_3-d_3, 27-hydroxycholesterol-d_6	RP – MeOH:H_2O (Gradient)	MS/MS – APPI	LOD: 1250 - 6250 LOQ: 2500 – 12500	(90)
$25(OH)D_3$, 3-epi-$25(OH)D_3$	$25(OH)D_3$-d_6	PFP – MeOH:H_2O:FA	MS/MS	--------	(68)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6	RP - MeOH:AmAc (Isocratic)	MS/MS	LOD: 10000	(49)

Table 2 (continued).

Vitamin D analogues	Internal standard	Separation conditions	Detection	Sensitivity (pmol/L)	Refs.
$25(OH)D_3$, 3-epi-$25(OH)D_3$	$25(OH)D_2$-d_3	RP – MeOH:H_2O:AMM (Isocratic)	MS/MS - ESI	--------	(85)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25(OH)D_3$, $1\alpha(OH)D_3$	Stanozolol-D_3	RP- Chiral - ACN:H_2O:FA (Gradient)	MS/MS - ESI	LOD: 624	(29)
$25(OH)D_3$, $25(OH)D_2$ $24,25(OH)_2D_3$, 3-epi-$25OHD_3$	$25(OH)D_3$-d_6	PFP - MeOH:AmAc (gradient)	MS/MS - APCI	LOQ: 2000 - 4000	(37)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_3, $25(OH)D_2$-d_3	RP - MeOH:H_2O:FA:AmAc (Gradient)	MS/MS	LOQ: 6250	(73)
$25(OH)D_3$, $25(OH)D_2$,	$25(OH)D_3$-d_6	PFP - MeOH:H_2O:FA (Isocratic)	MS/MS – APCI	LOQ: 17500 - 20000	(73)
$1,25(OH)_2D_3$, $1,25(OH)_2D_2$	$1,25(OH)_2D_3$-d_6 $1,25(OH)_2D_2$-d_6	RP - MeOH:H_2O:FA (Gradient)	MS/MS	LOD: 4.8 - 72	(64)
$24,25(OH)_2D_3$, $25(OH)D_3$	$25(OH)D_3$-d_6	RP - MeOH:H_2O:FA (Gradient)	MS/MS - ESI	LOQ: 250 - 625	(71)
$25(OH)D_3$, $25(OH)D_2$, 3-epi-$25(OH)D_3$	$25(OH)D_3$-d_6 $25(OH)D_2$-d_3	PFP - MeOH:H_2O (Isocratic)	MS/MS - APCI	LOD: 1880 - 4880	(110)
$25(OH)D_3$	$25(OH)D_3$-d_3	RP - MeOH:AMM	MS/MS - ESI	--------	(86)
$25OHD_3$, $25OHD_2$ $1,25(OH)_2D_3$, $1,25(OH)_2D_2$ $24,25(OH)_2D_3$	$25(OH)D_3$-d_6 $1,25(OH)_2D_3$-d_6	RP - MeOH:ACN:H_2O:FA (Gradient)	MS/MS - ESI	LOQ: 62.4	(36)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6 $25(OH)D_2$-d_6	RP - MeOH:H_2O:AmAc:FA (Isocratic)	MS/MS	LOQ: 240 - 780	(74)

Table 2 (continued).

Vitamin D analogues	Internal standard	Analytical conditions	Detection method	Sensitivity (pmol/L)	Refs.
D_2, D_3, 25(OH)D_2, 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$	D_2-d_3, D_3-d_6, 25(OH)D_2-d_3, 25(OH)D_3-d_6, 24,25$(OH)_2D_3$-d_6, 1,25$(OH)_2D_3$-d_6	RP – MeOH:H_2O:AMM (Isocratic)	MS/MS	LOQ: 37.5 - 375	(72)
D_2, D_3, 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$	D_2-d_3, D_3-d_3, 25(OH)D_2-d_6, 25(OH)D_3-d_6, 24,25$(OH)_2D_2$-d_3, 24,25$(OH)_2D_3$-d_6, 1,25$(OH)_2D_2$-d_6, 1,25$(OH)_2D_3$-d_3	RP – MeOH:H_2O:FA (Isocratic/Gradient)	MS/MS - ESI	LOD: 0.25 – 0.5 LOQ: 0.82 – 1.25	(93)
25(OH)D_3, 3-epi-25(OH)D_3	25(OH)D_3-d_3	RP – ACN:H_2O:FA (Gradient)	MS/MS - APCI	----------	(70)
D_2, D_3, 25(OH)D_2, 25(OH)D_3, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$	D_2-d_3, D_3-d_6, 25(OH)D_2-d_3, 25(OH)D_3-d_6, 24,25$(OH)_2D_3$-d_6, 1,25$(OH)_2D_2$-d_6, 1,25$(OH)_2D_3$-d_6	RP – MeOH:H_2O:AMM (Gradient)	MS/MS - ESI	LOD: 0.75 - 190 LOQ: 2.5 - 625	(65)
D_2, D_3, 25(OH)D_2, 25(OH)D_3,	D_3-d_6, 25(OH)D_2-d_6, 25(OH)D_3-d_6,	RP – ACN:H_2O:M:FA:EtOH (gradient)	MS/MS - ESI	LOD: 260	(116)
25(OH)D_3, 24,25$(OH)_2D_3$,	25(OH)D_3-d_3, 24,25$(OH)_2D_3$-d_6	Phenyl – MeOH:H_2O (Gradient)	MS/MS	--------	(66)
25(OH)D_2, 25(OH)D_3,	25(OH)D_3-d_6	RP – MeOH:H_2O:FA (Gradient)	MS/MS - APCI	LOQ: 12000 - 12500	(67)
25(OH)D_3, 25(OH)D_2	25(OH)D_3-d_6	RP - RP - MeOH:ACN:IPA:ACE:H_2O:FA (Gradient)	MS/MS - APCI	LOD: 773.76-2132 LOQ: 2146-3416	(46)
25(OH)D_3, 25(OH)D_2	25(OH)D_3-d_6	RP - MeOH:H_2O:AMM (Gradient)	MS/MS - ESI	LOD: 3300	(81)

Table 2 (continued).

Vitamin D analogues	Internal standard	Analytical conditions	Detection method	Sensitivity (pmol/L)	Refs.
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6	RP - RP - MeOH:ACN:IPA:ACE:H_2O:FA (Gradient)	MS/MS - APCI	LOD: 773.76-2132 LOQ: 2146-3416	(46)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_2$-d_6 $25(OH)D_3$-d_6	RP - MeOH: AMM (Gradient)	MS/MS – APCI MS/MS - ESI	LOD: 500 – 1000 (APCI) LOQ: 1500 - 3000 LOD: 500 – 1200(ESI) LOQ: 1500 – 3600	(79)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6	RP - MeOH:H_2O: AmAc:FA (Gradient)	MS/MS - ESI	LOD: 2500 LOQ: 4000 - 7500	(56)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6 $25(OH)D_2$-d_6	RP - MeOH:H_2O: AmAc:FA (Isocratic)	MS/MS - ESI	LOQ: 3000 - 4000	(42)
$25(OH)D_3$, $25(OH)D_2$	$25OHD_3$-d_6	RP - MeOH:H_2O:FA	MS/MS - APCI	LOQ: 2496	(44)
Vitamin D_3, Vitamin D_2	Vitamin D_3-d_3 Vitamin D_2- d_3	RP - MeOH:H_2O:AMM (Gradient)	MS/MS - APCI	LOD: 1559* LOQ: 4367*	(99)
Vitamin D_3, Vitamin D_2	Vitamin D_3-d_3	RP - MeOH:H_2O:FA (Gradient)	MS/MS - APCI	LOD: 1689-2092* LOQ: 5719-7059*	(97)
1α $25(OH)_2D_3$	1α $25(OH)_2D_3$-d_6	RP - MeOH:LiAc (Gradient)	MS/MS - ESI	LOD: 7.2 LOQ: 36	(39)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6, $25(OH)D_2$-d_6	RP - MeOH:H_2O (Isocratic)	MS/MS - APCI	LOD: 1700-1200 LOQ: 4600-3000	(50)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_6	PFP - MeOH: AmAc:FA	MS/MS - ESI	-------	(84)
$25(OH)D_3$, $25(OH)D_2$	$25(OH)D_3$-d_3 $25(OH)D_2$-d_3	CN - MeOH:H_2O (Isocratic)	MS/MS - APCI	LOD: 374*	(61)
$25(OH)D_3$	$25(OH)D_4$	RP - RP - MeOH:AMM:M (Isocratic)	MS/MS - ESI	LOD: 4.99	(88)

Table 2 (continued).

Vitamin D analogues	Internal standard	Analytical conditions	Detection method	Sensitivity (pmol/L)	Refs.
Vitamin D_3	Vitamin D_3-d_6	RP - MeOH:AmAc (Isocratic)	MS/MS - APCI	LOQ: 1299 - 12999	(63)
Vitamin D_3, Vitamin D_2	Vitamin D_3-d_3 Vitamin D_2-d_3	RP - MeOH:AMM (Gradient)	MS/MS - APCI	LOD: 5042-12219* LOQ: 15378-37437*	(95)
25(OH)D3-S	25(OH)D_3S-d_3	RP – MeOH:H_2O:AMM (Gradient)	MS/MS - ESI	LOQ: 5210	(21)
25(OH)D_3, 25(OH)D_2 24,25$(OH)_2D_3$, 1,25$(OH)_2D_3$ 1,25$(OH)_2D_2$	25OHD_3-d_6 25(OH)D_2-d_6 1,25$(OH)_2D_3$-d_6 1,25$(OH)_2D_2$-d_6	RP - MeOH:H_2O:FA:M (Gradient)	MS/MS - ESI	LOQ: 24.96 - 48	(75)
25(OH)D_3, 25(OH)D_2 1α 25$(OH)_2D_3$ 24,25$(OH)_2D_3$	25OHD_2-d_6, 25(OH)D_3-d_6 1α25(OH)D_3-d_6	RP - MeOH:AMM (Gradient)	MS/MS - ESI	LOD: 1.24 – 2.4 LOQ: 12 – 249	(43)
1α25$(OH)_2D_3$, 1α25$(OH)_2D_2$	1α25$(OH)_2D_3$-d_6 1α25$(OH)_2D_2$-d_6	RP – (No details)	MS/MS - ESI	LOD: 1.5 LOQ: 3.0	(69)
1α25$(OH)_2D_3$, 1α25$(OH)_2D_2$	1α25$(OH)_2D_3$-d_6 1α25$(OH)_2D_2$-d_6	RP - MeOH:H_2O:LiAc (Gradient)	MS/MS - ESI	LOQ: 8.16 - 9.09 (immunotube) LOQ: 37.92 – 32.42 (IDS antibody)	(47)
Vitamin D_3	Vitamin D_3-d_6	RP - MeOH:H_2O:AMM (Gradient)	MS/MS - ESI	LOD: 208 LOQ: 363	(100)
25(OH)D_3, 25(OH)D_2	25(OH)D_3-d_6, 25(OH)D_3-d_6	RP - MeOH:H_2O (Gradient)	MS/MS - APPI	LOQ: 1300	(45)
25(OH)D_3, 1α25$(OH)_2D_3$ 24,25$(OH)_2D_3$, 4β25(OH)D_3	25(OH)D_3-d_6 1α25$(OH)_2D_3$-d_6	RP - ACN:H_2O:FA (Gradient)	MS/MS - ESI	LOD: 2.4 - 9.98 LOQ: 60 - 124	(80)

Table 2 (continued).

Vitamin D analogues	Internal standard	Analytical conditions	Detection method	Sensitivity (pmol/L)	Refs.
Vitamin D_3	Vitamin D_3-d_6	RP-RP - MeOH:ACN:FA (Gradient)	MS/MS - ESI	LOD: 1299 – 2079* (food) 33.79** (Pharmaceuticals) LOQ: 2599 – 4159* (food) 5199** (Pharmaceuticals)	(114)
25(OH)D_3, 25(OH)D_2	25(OH)D_3-d_6, 25(OH)D_2-d_6	CN-RP – MeOH:H_2O:FA (Gradient)	MS/MS - APCI	LOD: 1260 LOQ: 2496	(48)
25(OH)D_3, 25(OH)D_2	25(OH)D_3-d_6	RP - EtOH:H_2O (isocratic)	MS/MS - APCI	LOD: 1223-4507	(40)
Vitamin D_3	Vitamin D_3-d_3	PFP - MeOH:ACN:H_2O:FA (Gradient)	MS/MS - APCI	LOD: 5199-20798*	(30)
Vitamin D_2, Vitamin D_3 25(OH)D_3, 25(OH)D_2	Vitamin D_2-d_6 Vitamin D_3-d_6 25(OH)D_3-d_6 25(OH)D_2-d_3	RP - MEOH:H_2O:T (Gradient)	MS/MS - APPI	LOQ: 2521-4846	(57)
1,25(OH)$_2D_2$	1α,24(OH)D_2-d_8	RP - ACN:H_2O (Gradient)	MS/MS - APCI	LOQ: 58.32	(41)
25(OH)D_3, 25(OH)D_2	25(OH)D_3-d_6	RP – MeOH:AmAc:FA (Gradient)	MS/MS - ESI	LOD: 1200-1500 LOQ: 3500-2000	(59)
25(OH)D_3	25(OH)D_3-d_6	RP - MeOH:H_2O:FA (Gradient)	MS - APCI	LOD: 4992 – 12480*	(89)
25(OH)D_3, 25(OH)D_2 3-epi-25(OH)D_3	25(OH)D_3-d_6 25(OH)D_2-d_3	RP - MeOH:H_2O (Gradient) CN - MeOH:H_2O (Isocratic) PFP - MeOH:H_2O (Isocratic)	MS - APCI	LOD: 2496 – 4992* LOQ: 7488 – 14976*	(60)
Vitamin D_3	Vitamin D_3-d_3	RP - MeOH:ACN:H_2O (Gradient)	MS/M - APCI MS - APCI	-------	(101)
Vitamin D_3	Vitamin D_3-d_3	RP - MeOH:H_2O:AMM (Isocratic) RP - MeOH:H_2O (Isocratic)	MS/MS – ESI MS^n - APCI	LOQ: 260 - 520	(33)

Table 2 (continued).

Vitamin D analogues	Internal standard	Analytical conditions	Detection method	Sensitivity (pmol/L)	Refs.
$25(OH)D_3$, $25(OH)D_2$	-------	RP - MeOH:H_2O (Isocratic)	DAD – 265 nm	LOD: 6000 - 12000 LOQ: 4000	(52)
Vitamin D_3	Vitamin D_2	RP - MeOH:ACN (Isocratic)	DAD – 265nm	LOD: 64995	(103)
Vitamin D_3	-------	RP - SDS:BuOH:PB (Isocratic)	DAD – 230, 280, 300 nm	LOD: 2365848 LOQ: 7097545	(112)
Vitamin D_3, Vitamin D_2 $25(OH)D_3$, $25(OH)D_2$	$1\alpha(OH)D_3$	RP - MeOH:EtOH (Gradient)	UV – 265 nm	-------	(82)
25(OH)D	-------	RP - ACN:MeOH:IPA (Gradient)	UV – 268 nm	LOD: 8000	(53)
Vitamin D_3	-------	RP - MeOH:ACN (Isocratic)	UV – 285 nm	LOD: 12999 LOQ: 117187	(55)
$25(OH)D_3$	Retinyl acetate	RP - MeOH:H_2O (Isocratic)	UV – 265 nm	LOD: 10000	(76)
$25(OH)D_3$, $25(OH)D_2$ 3-epi-$25(OH)D_3$	Laurophenone	CN - MeOH:H_2O (Isocratic) CN - MeOH:H_2O:FA (Isocratic)	MS/MS - APCI UV – 275 nm	LOD: 249.60	(28)
Vitamin D_3, $25(OH)D_3$	Vitamin D_2 $25(OH)D_2$	RP - MeOH-H_2O-HAc (Isocratic) RP - MeOH:H_2O (Isocratic)	MS/MS – APCI UV-DAD – 265, 280 nm	LOD: 998-1039*	(96)
Vitamin D_3	Vitamin D_2	RP - MeOH:MeOH (Isocratic)	MS - APCI UV – 265 nm	-------	(82)
Vitamin D_3	Vitamin D_2	RP-RP - MeOH:ACN:DCM (Gradient)	MS – APCI UV – 265 nm	LOD: 17938 - 188747 LOQ: 59536 - 629159	(102)
$25(OH)D_3$	-------	Anti-$25(OH)D_3$	ELISA -Visible-450nm	LOD: 5500	(54)

Table 2 (continued).

Vitamin D analogues	Internal standard	Analytical conditions	Detection method	Sensitivity (pmol/L)	Refs.
25(OH)D	-------	Anti-25(OH)D	RIA - Radioimmunoassay CLIA - Chemiluminescence ECLIA – Electrochemiluminescence	-------	(51)
25(OH)D	-------	Anti-25(OH)D	RIA - Radioimmunoassay CLIA – Chemiluminescence	-------	(78)
$1\alpha25(OH)_2D_3$	-------	Anti-$1,25(OH)_2D_3$	SERS	LOD: 4.32	(117)
Vitamin D_3	-------	Silica gel plates, C:E	HPTLC	-------	(98)

***Expressed in pmol/kg , **Expressed in pmol/tablet**

Abbreviations:ACE, acetone; ACN, acetonitrile; AmAc, ammonium acetate; AMM, ammonium formate; APPI, atmospheric pressure photoionisation; APCI, atmospheric pressure chemical ionisation; BB, borate buffer; BuOH, butanol; C, chloroform; CN, cyanopropyl column; DAD, diode array detector; DCM, dichloromethane; E, ether; ESI, electrospray ionisation; FA, formic acid; HAc, acetic acid; H_2O, water; LiAc, lithium acetate; LOD, limit of detection; LOQ, limit of quantitation; M, methylamine; MeOH, methanol; PB, phosphate buffer; PBS, phosphate buffer saline; RP, reverse phase; PFP, pentafluorophenyl column; SDS, sodium dodecyl sulphate; SERS, surface-enhanced Raman scattering; T, toluene.

As shown in table 2, D_2 and/or D_3 were separated and detected in foodstuffs (30, 94-99, 111-114). The majority of the methods for serum/plasma separated and detected 25(OH)D_2 and 25(OH)D_3, while others assayed only 25(OH)D_3. Some methods separated epimers (28, 29, 37, 58, 60, 83, 110). A few others have separated and detected many forms of vitamin D compounds (36, 43, 57, 75, 80, 82). Due to the hydrophobic characteristics of vitamin D compounds and the derivatives, reversed-phase (RP) was the most commonly used LC column type. A relatively uncommonly used method, HPTLC, has also been developed to determine D_3 in fish oil (98).

Even though LC–MS/MS provides outstanding specificity, a challenge encountered in vitamin D assay is the separation of epimers. The epimeric form and the corresponding vitamin analogues have the same mass and same fragmentation patterns hence, they cannot be differentiated by LC–MS/MS. This imposes a high demand on the separation step to prevent co-elution of the epimers. PTAD derivatisation has been employed to enhance the MS response of 25(OH)D_3; thus, 25(OH)D_3 has been separated from its epimer by using a RP column after PTAD-derivatisation (70, 83, 93). Other methods have not used any derivatisation procedure and the separation of 25(OH)D_3 from its epimer has been achieved using either a cyano column (28, 60); a RP column in tandem with a chiral column (29, 58); or a pentafluorophenyl column (37, 38, 60, 68, 110). Four analogues excluding epimers were separated using a RP column (57, 82). Four (43, 80) or five (36, 75) analogues excluding epimers were separated using PTDA-derivatisation and a RP column. Four analogues including epimers were separated using a pentafluorophenyl column or RP in tandem with a chiral column (29, 37). Eight analogues were separated using RP in tandem with a chiral column (58).

1.4.2.2 Detection

As shown in table 2, MS has been an increasingly popular detection method for the analysis of vitamin D compounds due to its high specificity and sensitivity. Although few studies (45, 57, 90) have used ionisation sources such as atmospheric pressure photoionisation, ESI and atmospheric pressure chemical ionisation modes remain the most frequently used and the majority of methods used LC–MS/MS rather than LC–MS. The major weakness of MS in vitamin D analysis is the low ionisation ability of vitamin D compounds, and in some cases this problem has been overcome by derivatisation, as discussed in the section titled ‘Derivatisation procedures’. Although UV detection has been largely replaced by MS detection due to its poor specificity and/or sensitivity, it is still used as a less expensive

detection option, as shown in table 2. Based on the chemical structure/chromophore of vitamin D analogues, these compounds absorb at approximately 265 nm. NMR spectrometry has also been assessed to detect four vitamin D analogues (118).

As shown in table 2, there are many different immunoassay kits commercially available for vitamin D assay. With immunoassays, after the initial sample preparation/clean-up step the extract is subjected to an antibody specific vitamin D assay. The detection step of immunoassays is based on different spectroscopic techniques such as UV-visible (54), chemiluminescence (51, 78), Raman scattering (117) or radioactivity measurements (51, 78). Two major limitations of immunoassays for vitamin D analysis are matrix effects and the inability to distinguish between $25(OH)D_3$ and $25(OH)D_2$ (8). Raman (117) spectroscopy for immunoassay has shown high sensitivity, enabling the detection of vitamin D metabolites at very low concentrations; however, this method still cannot distinguish between the different vitamin D metabolites.

1.4.2.3 Quantification

Despite its strong advantages over other analytical techniques, one of the major drawbacks of MS detection is its susceptibility to matrix effects. Matrix effects in MS occur when the compounds that co-elute with the analyte interfere with the ionisation process causing ionisation suppression or enhancement. As there is no way to ensure complete elimination of the interfering compounds that co-elute with the analyte, the only way to obtain accurate data is to remove the contribution from the interferences at the quantification step. The inclusion of stable isotope labelled (SIL) internal standard (IS) in the sample and subsequent quantification using IS calibration has become the most commonly used method to correct for matrix effects in MS detection (119). As the chemical properties and ionisation process of SIL IS are almost identical to those of the analyte, and as it elutes at the same retention time as the analyte and experiences the same extent of matrix effects, it provides the best option to use in IS calibration methods with LC–MS.

As shown in table 2, some MS methods did not employ SIL IS and, therefore, matrix effects were not specifically removed as a result (28, 29, 58, 83, 88). Most methods used SIL IS such as d_6-$25OHD_3$ (36, 37, 40, 42-46, 48-50, 56, 59, 65, 67, 68, 71, 72, 74, 75, 79-81, 84, 89, 91, 110), d_6-$25OHD_2$ (42, 43, 45, 48, 50, 57, 75, 79), d_6-$1,25(OH)_2D_3$ (36, 39, 43, 47, 65, 69, 72, 75, 80) and d_6-$1,25(OH)_2D_2$ (47, 65, 69, 75) for the correction of matrix effects at the quantification step. Some methods used a single SIL IS for all vitamin forms (37, 40, 44, 46, 49, 56, 59, 81, 97) although this does not remove matrix effects from all vitamin D analytes

except the form that co-elutes with the IS (119). Quantification in LC-UV methods has used either IS calibration using non co-eluting compounds such as vitamin D_2 (94, 96, 102, 103), 25(OH)D_2 (96), 1α(OH)D_3 (82), laurophenone (28), retinyl acetate (76) or external standard calibration.

All LOD and LOQ values in Table 2 were presented in the same units (pmol/L or pmol/Kg) so that a comparison of sensitivities was possible. According to Tables 1 and 2, in general, PTAD derivatisation increased the sensitivity although in some cases it was not high relative to other methods (53, 59, 61). Interestingly, some LC–MS/MS methods with no derivatisation (39, 41, 47, 58) and an immunoassay reported very high sensitivities (117).

1.5 Conclusion

This chapter summarises the most used vitamin D methods for all applications. Particular emphasis has been placed on the different forms of vitamin D measured and the sample preparation procedures used in a variety of matrices. This chapter was divided into sections based on the typical steps of an analytical method, and the vitamin D methods were compared and critically evaluated at each step. This enabled the evaluation of each stage of the analytical process and selection or development of an appropriate method for a specific application. In general, saponification was the method of choice to extract the vitamin from food stuff while PP was used with plasma and serum. DBS has been a popular sample collection method for small sample volumes, although a recent study (106) found that this method introduces high variability to the assay. Overall, LC–MS/MS is the most common technique used and it is the best option for the unambiguous identification and accurate quantification of the many different forms of vitamin D.

Vitamins D_2 and D_3 are the forms commonly measured in food. Other native forms were disregarded due to the relatively low levels compared with the often fortified vitamin levels. Although controversy exists regarding the inadequacy of the choice, the form 25(OH)D continues to serve as the marker for vitamin D in plasma and serum (120). However, widespread variation in measurement results for 25(OH)D warranted the establishment of reference measurement procedures and standardisation efforts (121, 122). As evident from the content of this review, assays for forms other than 25(OH)D in plasma and serum are not common, therefore standardisation of methods for these forms is not likely to occur in the near future. The development of new LC–MS/MS sources as well as improvements on derivatisation procedures are expected to address issues related to the poor ionisation efficiency in ESI or atmospheric pressure chemical ionisation of vitamin D compounds.

Advances in automation of sample preparation techniques are in progress and will continue due to the demand for high throughput and greener technologies.

1.6 Research hypothesis

1. The quantification of all individual major forms (twelve rather than four commonly measured) of vitamin D in biological fluids will provide an accurate estimation of the total vitamin D status.
2. The pasteurisation process will affect the concentration of vitamin D in breastmilk.

1.7 Research aims

1. To develop an accurate and sensitive LC-MS/MS method for the quantification of twelve major forms of vitamin D in milk by optimisation of each step of the analytical method: extraction, sample preparation, separation and detection.
2. To validate the developed method.
3. To apply the method to quantify the total vitamin D in milk from several species.
4. To apply the method to evaluate the effect of pasteurisation on the vitamin D content in breastmilk.

2 Chapter 2: Development and validation of a method for the quantitative analysis of eight vitamin D analogues in milk using liquid chromatography-tandem mass spectrometry

2.1 Foreword

This chapter describes the attempts to develop and validate an LC-MS/MS method for the sensitive and accurate quantification of vitamin D compounds in milk. It explores in detail the strategies used to improve the ionisation efficiency of vitamin D compounds, to achieve an efficient extraction method and to produce adequate chromatography. The majority of the content of this chapter has been published in the journal *Analytica Chimica Acta*, 3; 891:211-20 (2015) and it is presented in a slightly modified format to fit with the style of this thesis. Permission has been granted from the publisher to reproduce the work in the thesis.

2.2 Introduction

Vitamin D is essential in the regulation of the intestinal absorption of calcium and phosphate, both of which are vital for healthy homeostasis of bones. Osteomalacia in adults and rickets in infants are the most common adverse effects related to an insufficiency of vitamin D (13). Vitamin D deficiency has also been associated with many non-skeletal disorders such as common cancers, hypertension, cardiovascular disease, diabetes mellitus and autoimmune diseases (4). There has been an increase in the interest in vitamin D status because of its implications on these major diseases (123).

Each vitamin D form has a different function in the body (13, 21, 27). Most biological actions of vitamin D are attributed to the metabolite 1,25-dihydroxyvitamin D ($1,25(OH)_2D_3$ and $1,25(OH)_2D_2$) that is primarily responsible for preventing skeletal and non-skeletal disorders mentioned earlier. The metabolite 24,25-dihydroxyvitamin D ($24,25(OH)_2D_3$ and $24,25(OH)_2D_2$) was originally considered to be inactive but was later found to play a crucial role in intramembranous and endochondral bone formation and in bone fracture repair (13). It has also been suggested that vitamin D_3 (D_3) is more efficiently absorbed by the intestine than vitamin D_2 (D_2) (124, 125). The metabolite 25-hydroxyvitamin D_3 ($25(OH)D_3$) has three times the potency (126) and higher affinity than 25-hydroxyvitamin D_2 ($25(OH)D_2$) to vitamin D-binding protein (DBP), which results in a higher biological half-life (124). $25(OH)D_3$ can be epimerised to an inactive form (3-epi-$25(OH)D_3$) and collective measurement of these two isomers can lead to an overestimation of the vitamin D content (27, 123). Given the diverse and varying activities and biological functions of different

analogues of vitamin D, it is desirable to simultaneously measure all key vitamin D analogues using an analytical method with high specificity and sensitivity (120). The attempts to develop and validate an LC-MS/MS method for the sensitive and accurate quantification of twelve vitamin D forms in milk are described in this chapter.

Numerous vitamin D assays in biological fluids have been reported based on different techniques (123); however, few have been developed for application to milk samples and even fewer for unfortified milk. Immunoassay methods such as competitive protein binding assays (CPBA) have been applied for the determination of vitamin D in unfortified milk (127, 128). The main drawback of immunoassay methods is their inability to distinguish between different forms of vitamin D (123). Liquid chromatography (LC) methods have also been used for the analysis of vitamin D compounds in milk, and they have the advantage over immunoassays because of their ability to differentiate a variety of vitamin D compounds in a single run. Electrochemical (EC) (129) and ultraviolet (UV) (130, 131) detectors have been the detectors of choice until recently. Recently, tandem mass spectrometry (MS/MS) detection has been gaining popularity due to its superior sensitivity and specificity. Nevertheless, poor ionisation efficiency in the atmospheric pressure ionisation (API) of vitamin D compounds has been a drawback (123). In order to address this critical factor, a derivatisation procedure based on the Diels-Alder reaction has been introduced to enhance the ionisation efficiency and thereby the sensitivity of vitamin D assay (132). Derivatisation with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) has proved to be an effective strategy for the analysis of minor vitamin D compounds, the concentrations of which are too small to be detected by standard MS methods and also for small volume samples such as neonatal biological fluids (83, 104). The PTAD derivatisation method was initially optimised for the analogues D_3, lα(OH)D_3, 25(OH)D_3, l,25$(OH)_2D_3$ and 24,25$(OH)_2D_3$, as a post-column derivatisation method to use prior to thermospray mass spectrometry detection. It was found to increase the sensitivity between 7 and 70-fold, depending on the vitamin D compound (133). This method has subsequently been used as a pre-column derivatisation method for many vitamin D analogues, but without rigorous optimisation. In this study, we have optimised the pre-column derivatisation step and compared the sensitivities of derivatised and underivatised forms of four major forms (and one minor form) of vitamin D in biological fluids.

Vitamin D compounds in b iological fluids or foods are bound to proteins and lipids and therefore are required to be released before analysis. Two approaches have been commonly used for this process: protein precipitation and saponification. Traditionally, saponification

was used for foodstuffs while protein precipitation has largely been used for plasma/serum, with the exception of two studies that used saponification for plasma/serum (123). In this study, we compared the performances of the two approaches, saponification and protein precipitation, for their effectiveness in releasing the vitamin from milk. Also, the method reported here can resolve 25(OH)D_3 and its inactive epimeric form and thereby prevent an overestimation of vitamin D concentration in milk samples. To correct for the ionisation suppression or enhancement effects and thereby ensure the highest accuracy and reliability of our LC-MS/MS method (134) we included a co-eluting stable isotope labelled internal standard for each analogue of vitamin D quantified. Since the more widespread availability of MS detection, quantitative methods have been developed for the determination of D_2, D_3, 25(OH)D_2 and 25(OH)D_3 analogues in milk (135). The developments and optimisation procedures outlined in this study have been used to expand the scope to include eight additional vitamin D analogues, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$, vitamin D_2-Sulfate (D_2-S), vitamin D_2-Sulfate (D_3-S), 25-hydroxyvitamin D_2-Sulfate (25(OH)D_2-S) and 25-hydroxyvitamin D_3-Sulfate (25(OH)D_3-S). In addition, the pre-vitamin 7-dehydrocholesterol (7-DHC) was also included in a standard mixture and ensured chromatographic separation from isobaric D_3. The method developed has been validated and subsequently used to quantify and compare the total vitamin D profiles of several different types of milk: human, cow, mare, goat and sheep.

2.3 Material and methods

2.3.1 Chemicals and samples

The following unlabelled and deuterium-labelled (d_3 or d_6) standards: D_2, D_3, 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, D_2-S, D_3-S, 25(OH)D_2-S, 25(OH)D_3-S, D_2-d_3, D_3-d_3, 24,25$(OH)_2D_3$-d_6, 1,25$(OH)_2D_3$-d_3, D_2-S-d_3, D_3-S-d_3, 25(OH)D_2-S-d_3 and 25(OH)D_3-S-d_3 were purchased from IsoSciences (King of Prussia, USA). Other labelled standards were purchased from Chemaphor (Ottawa, Canada) (25(OH)D_2-d_6 and 25(OH)D_3-d_6), Medical Isotopes Inc. (Pelham, USA) (1,25$(OH)_2D_2$-d_6), and Toronto Research Chemicals Inc. (Ontario, Canada) (24,25$(OH)_2D_2$-d_3). All compounds had a purity of 97% or better. PTAD (≥97%) was purchased from Sigma Aldrich (St. Louis, USA). Organic solvents of LC-grade (dichloromethane, hexane, acetonitrile and methanol), and chemicals of analytical grade (potassium hydroxide (KOH), ascorbic acid (Asc) and ethanol (EtOH) were purchased from Merck (Darmstadt, Germany)). LC-MS grade formic acid (>99%) was purchased from Fisher Chemical (Geel, Belgium). High-purity water was

prepared by using a Millipore Milli-Q system (Milford, USA). Human milk samples were kindly donated by the breast milk bank of the Royal Brisbane and Women's Hospital (RBWH), Brisbane, Australia. Cow, mare, goat and sheep milks were kindly donated by Dr John Wright, School of Veterinary Science, The University of Queensland (UQ).

2.3.2 LC-MS/MS analysis

Chromatographic separations were carried out using an Agilent binary LC system consisting of an Agilent 1290 infinity LC pump, an Agilent 1290 well plate auto-sampler and a Poroshell 120 EC-C18 (150 x 2.1 mm, 2.7 µm) column (Agilent Technologies, Santa Clara, CA, USA). For experiments using non-derivatised vitamin D, the separations were carried out using a Pursuit PFP (150 x 3.0 mm, 3 µm) column (Agilent Technologies, Santa Clara, CA, USA). An Agilent 6460 Triple Quadrupole tandem mass spectrometer equipped with a Jet Stream and supported by Mass Hunter Workstation software (Agilent Technologies, Santa Clara, CA, USA) was used as the detector. A binary solvent system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) was used for separation.

Isocratic elution was used for the first 12.5 min (77% B), the composition changed to 100% B over the next 1.5 min, and remained at 100% B for the next 9 min. The composition returned to 77% B during the next 1 min, and the column was then re-equilibrated with 77% B for 10 min before injecting the next sample. The flow rate was 0.2 mL/min^{-1} and the injection volume was 20 µL. Mass spectrometry parameters such as fragmentor and collision energies were optimised to obtain the highest possible sensitivity for each compound, as shown in Table 3.

Table 3. Optimised mass spectrometry parameters used in MRM mode for all vitamin D.

Compound	Precursor ion (m/z)	Product ion (m/z)	Qualifier ion (m/z)	Fragmentor (V)	Collision energy (V)
D_2	572	298	161	96	16
D_2-d_3	575	301	----	96	16
D_3	560	298	161	96	12
D_3-d_3	563	301	----	96	12
25(OH)D_2	570	298	161	144	12
25(OH)D_2-d_6	576	298	----	144	12
25(OH)D_3	558	298	161	168	12
25(OH)D_3-d_6	564	298	----	168	12
1,25$(OH)_2D_2$	586	314	135	144	12
1,25$(OH)_2D_2$-d_6	592	314	----	144	12
1,25$(OH)_2D_3$	574	314	177	139	8
1,25$(OH)_2D_3$-d_3	577	317	----	139	8
24,25$(OH)_2D_2$	586	298	161	178	12
24,25$(OH)_2D_2$-d_3	589	298	----	178	12
24,25$(OH)_2D_3$	574	298	161	198	20
24,25$(OH)_2D_3$-d_6	580	298	----	198	20
D_2-S-PTAD	652	378	161	134	16
D_2-S-d_3-PTAD	655	381	-----	134	16
D_3-S-PTAD	640	378	161	124	12
D_3-S-d_3-PTAD	643	381	-----	124	12
25(OH)D_2-S-PTAD	650	378	161	144	12
25(OH)D_2-S-d_3-PTAD	653	381	-----	144	12
25(OH)D_3-S-PTAD	639	378	161	144	12
25(OH)D_3-S-d_3-PTAD	642	381	-----	144	12
7-DHC	560	365	157	134	24

Table 3 also shows the *m/z* of precursor, product and qualifier ions used in MRM mode for each vitamin D compound. Source parameters such as gas flow, sheath gas flow, gas temperature and sheath gas temperature were optimised and maintained at 5 L/min, 12 L/min, 300 ^{o}C and 250 ^{o}C respectively. The capillary and nozzle voltages were maintained at 5000 and 2000 V respectively. The non-derivatised forms of 25(OH)D_2, 25(OH)D_3 and 3-epi-25(OH)D_3 analogues were analysed according to a published study (38). This method was further adapted to accommodate the compounds D_2 and D_3. Briefly, a binary solvent system consisting of 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B) was used for separation. An isocratic elution was used for the first 12 min (80%, B), the composition changed to 100% B over the next 10 min, and maintained at 100% B for the next 2 min. The composition returned to 80% B during the next 1 min, and the column was then re-equilibrated with 80% B for 10 min before injecting the next sample. The flow rate was

0.2 mL min^{-1} and the injection volume was 20 uL. The mass spectrometer was operated in ESI positive mode. The detection was performed in MRM mode as follows: D_2, *m/z* 397-83; D_3, *m/z* 385-159; 25(OH)D_2, *m/z* 413-83 and 25(OH)D_3 and 3-epi-25(OH)D_3, *m/z* 401-159. The fragmentor and collision energy voltages were maintained at 96 and 24 V, respectively. Source parameters such as gas flow, sheath gas flow, gas temperature and sheath gas temperature were maintained at 5 L/min, 12 L/min, 300^{o}C and 250^{o}C respectively. The capillary and nozzle voltages were maintained at 5000 V and 2000 V, respectively.

2.3.3 Preparation of standard and sample solutions

All sample and standard preparations were carried out under subdued light. All standard compounds were dissolved in ethanol to obtain 10 nM stock solutions that were stored at -20^{o} C. Working solutions were prepared by serial dilutions from the stock solutions. Each milk sample was vortex mixed and 4 mL aliquots were accurately measured and frozen until extraction and analysis.

2.3.4 Optimization of derivatisation reaction with PTAD

The derivatisation reactions between PTAD and five vitamin D analogues, D_2, D_3, 25(OH)D_2, 25(OH)D_3 and 3-epi-25(OH)D_3, were optimised: the molar ratios of PTAD:vitamin were varied from 5 to 30,000; the time of reaction was varied from 1 to 16 h. All procedures were carried out under subdued light. Briefly, a stock solution of PTAD was prepared in acetonitrile at a concentration of 800 µM and working solutions were prepared by serial acetonitrile dilutions from the stock solution. The mixed standard solution (10 µL) was dried down under a gentle stream of nitrogen and then 100 µL of PTAD solution (0.025, 0.5, 5, 50 or 150 µM) was added to give a molar excess of 5, 100, 1000, 10,000 or 30,000-fold respectively, and the solutions vortex mixed for 1 min. These solutions were allowed to react at room temperature for 1, 5 or 16 h, after which time 50 µL of ethanol was added to stop the reaction. The solutions were dried down under a gentle stream of nitrogen and the residues reconstituted with 100 µL mobile phase (77% B composition). These solutions were centrifuged for 5 min at 4000 g before using the supernatant for LC-MS/MS analysis. The underivatised forms of the same analogues were prepared in mobile phase at the same final concentration (1 nM) and both derivatised and underivatised forms were analysed using separate LC-MS/MS procedures, as described above. Peak height ratios (signal/noise) were used to compare the effect of derivatisation.

2.3.5 Optimisation of sample preparation

2.3.5.1 Protein precipitation (PP)

Eight millilitres of acetonitrile was added to 4 mL milk and vortex mixed for 2 min. The samples were incubated at room temperature for 15 min to complete the protein precipitation, and then centrifuged for 10 min at 4000 g. The supernatant was transferred to a fresh centrifuge tube and subjected to further liquid-liquid extraction (LLE), as described below.

2.3.5.2 Saponification (SN)

Four millilitres of 8 M ethanolic KOH solution and 2.8 mL of 1 M of ascorbic acid (Asc) were added to 4 mL milk. The mixture was vortex mixed for 2 min and incubated at room temperature for 12 h, followed by centrifugation for 10 min at 4000 g. The supernatant was transferred to a fresh centrifuge tube and subjected to further liquid-liquid extraction (LLE), as described below.

2.3.5.3 Liquid-liquid extraction (LLE)

Twelve millilitres of hexane:dichloromethane (4:1, v/v) mixture were added to the solution prepared in PP or SN above, and vortex mixed for 2 min. The upper organic layer was transferred to a centrifuge tube and the process repeated for a second time. The two batches of supernatants were pooled and evaporated to complete dryness under a gentle stream of nitrogen at room temperature.

2.3.5.4 Derivatisation procedure

The residue obtained from LLE (for standards and samples) was derivatised with PTAD using the optimum conditions (10,000-fold excess of PTAD with one-hour reaction time) and reconstituted with mobile phase as described above.

2.3.5.5 Method validation

Linearity was estimated by using six mixed standard solutions, derivatised and then dissolved in mobile phase, containing 0.05-5.0 nM (D_2, D_3, 25(OH)D_3 and 24,25$(OH)_2D_3$), 0.05-0.5 nM (D_2-S, D_3-S, 25(OH)D_2-S, 1,25$(OH)_2D_2$, and 1,25$(OH)_2D_3$), 0.05-1 nM (25(OH)D_3-S, 25(OH)D_2, and 24,25$(OH)_2D_2$), 1 nM (D_2-d_3, D_3-d_3, 25(OH)D_3-d_6 and 24,25$(OH)_2D_3$-d_6), 0.2 nM (D_2-S-d_3, D_3-S-d_3, 25(OH)D_2-S-d_3, 25(OH)D_3-S-d_3, 1,25$(OH)_2D_2$-d_6, and 1,25$(OH)_2D_3$-d_3) and 0.2 nM (25(OH)D_2-d_6 and 24,25$(OH)_2D_2$-d_3). The linearity was assessed by plotting the peak area ratios of each unlabelled form and its respective labelled form versus the

concentration of unlabelled standard. The limits of detection (LOD) and limits of quantitation (LOQ) for each compound were determined based on the concentrations (based on peak heights) corresponding to 3x noise and 10x noise respectively. Repeatability was estimated by analysing 10 individual aliquots of a human milk sample followed by the calculation of relative standard deviation of the concentrations determined for each vitamin compound. Recovery was determined by using the concentrations of each vitamin D analogue determined for six aliquots each of spiked and unspiked human milk samples. Each aliquot of milk was spiked to give an added concentration similar to the mid-point of the calibration range.

2.4 Results and Discussion

The quantitative determination of vitamin D and its metabolites in milk is a challenging task. Low abundance of vitamin D in milk, its instability in the presence of heat and light, and the presence of interfering endogenous components in milk were key challenges. Initially, we embarked in analysing the same four major analogues as in a published method available for milk, D_2, D_3, 25(OH)D_2 and 25(OH)D_3 (135) and 3-epi-25(OH)D_3 and optimised the derivatisation step for these compounds. Following the initial development we used targeted multiple reaction monitoring (MRM) using predicted molecular ions and predicted fragment ions for number of other known vitamin D analogue-PTAD derivatives: 652-378 (D_2-S), 640-378 (D_3-S), 650-378 (25(OH)D_2-S), 639-378 (25(OH)D_3-S), 586-314 (1,25(OH)$_2D_2$), 574-314 (1,25(OH)$_2D_3$), 586-298 (24,25(OH)$_2D_2$) and 574-298 (24,25(OH)$_2D_3$). From this study, we screened 1,25(OH)$_2D_2$, 1,25(OH)$_2D_3$, 24,25(OH)$_2D_2$ and 24,25(OH)$_2D_3$ therefore purchased standards for the confirmation of identities and subsequently the stable isotope labelled analogues to use as internal standards for quantification purposes. Subsequent optimisation studies included all eight analogues. Although sulfated forms of Vitamin D were not screened in this study, unlabelled and deuterium-labelled sulfated standards were also acquired for further investigations.

2.4.1 MS optimisation

The MS parameters were optimised by directly infusing the vitamin D-PTAD derivatives into MS detector. Initially, the derivatisation was carried out using 11,000-fold molar excess of PTAD relative to vitamin D, as commonly noted in previous studies (36). We were unable to detect signals for any of the vitamin D compounds. We considered that ionisation suppression by the excess PTAD reagent may have been responsible for this observation and we

subsequently used a 5-fold molar excess of PTAD, thereby obtaining good signals and validating our hypothesis. ESI in positive mode was used to identify the precursor and product ions to be used for quantification in order to develop an MRM method. Two product ions were obtained for each vitamin D-PTAD derivative, the predominant product ion was used as the quantifier ion and the other as the qualifier ion. These ions were detected with high sensitivity and selectivity and resulted from the moiety where PTAD is attached to the cis-diene moiety of the A-ring and the double bond of the opened B ring of vitamin D compounds. Compound dependent parameters such as fragmentor and collision energies were tuned to produce the most intense mass spectral signals for each analyte, as shown in Table 3. Dehydrated precursors $[M\text{-}H_2O+H]^+$ were selected for most of the vitamin D-PTAD compounds (producing the *m/z* 298, 314 and 378 fragments) as their abundances were higher than those of the protonated molecules ($[M+H]^+$). These compounds commonly lose a molecule of water at position 25, which is attributed to the formation of a stable tertiary carbocation (36, 43). For all other vitamin D-PTAD derivatives, the highest signal response was dominated by $[M+H]^+$ (producing the *m/z* 298, 365 and 378 fragments). As shown in Figure 4, the source parameters were also optimised in order to maximise the ESI response of each targeted analyte.

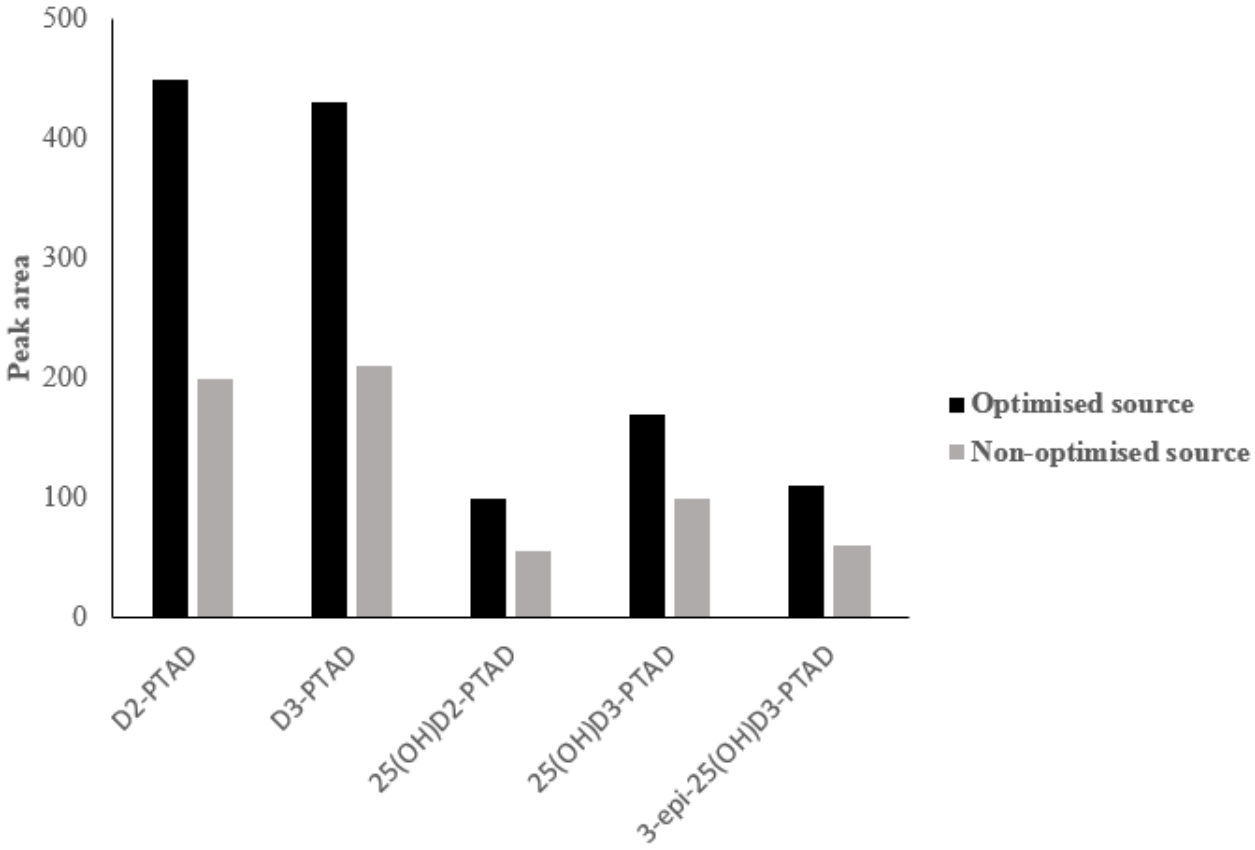

Figure 4. Optimisation of source parameters.

2.4.2 LC optimisation

Chromatographic separation for all vitamin D compounds was optimised with emphasis on baseline separation of the critical isomeric pair: (25(OH)D_3 and 3-epi-25(OH)D_3). Another concern was the potential interference of 7-DHC in the quantification of D_3 because these compounds also possess the same molecular mass. In order to conduct these separations, a range of chromatographic columns were tested including, PFP (as listed in experimental section), and C18 columns with a range of particle sizes. Organic modifiers such as, methanol, acetonitrile and isopropanol were also tested in different ratios and combinations. Unreacted, excess PTAD was observed (using Total Ion Current, TIC mode of MS) eluting early in the chromatogram; we therefore ensured that all vitamin D compounds eluted clear of the excess PTAD. Using lengthier retention times also ensured elimination of the matrix effects from the endogenous interferences, eluting early in the chromatographic separation. The optimal separation was achieved with isocratic-gradient conditions using methanol/water (with 0.1% formic acid) as mobile phase on a C_{18} column with a particle size of 2.7 µm. This mobile phase demonstrated the superior selectivity of the C_{18} column, with a small particle size, toward vitamin D compounds and particularly for the critical pair (25(OH)D_3 and 3-epi-25(OH)D_3), as shown in Figure 5.

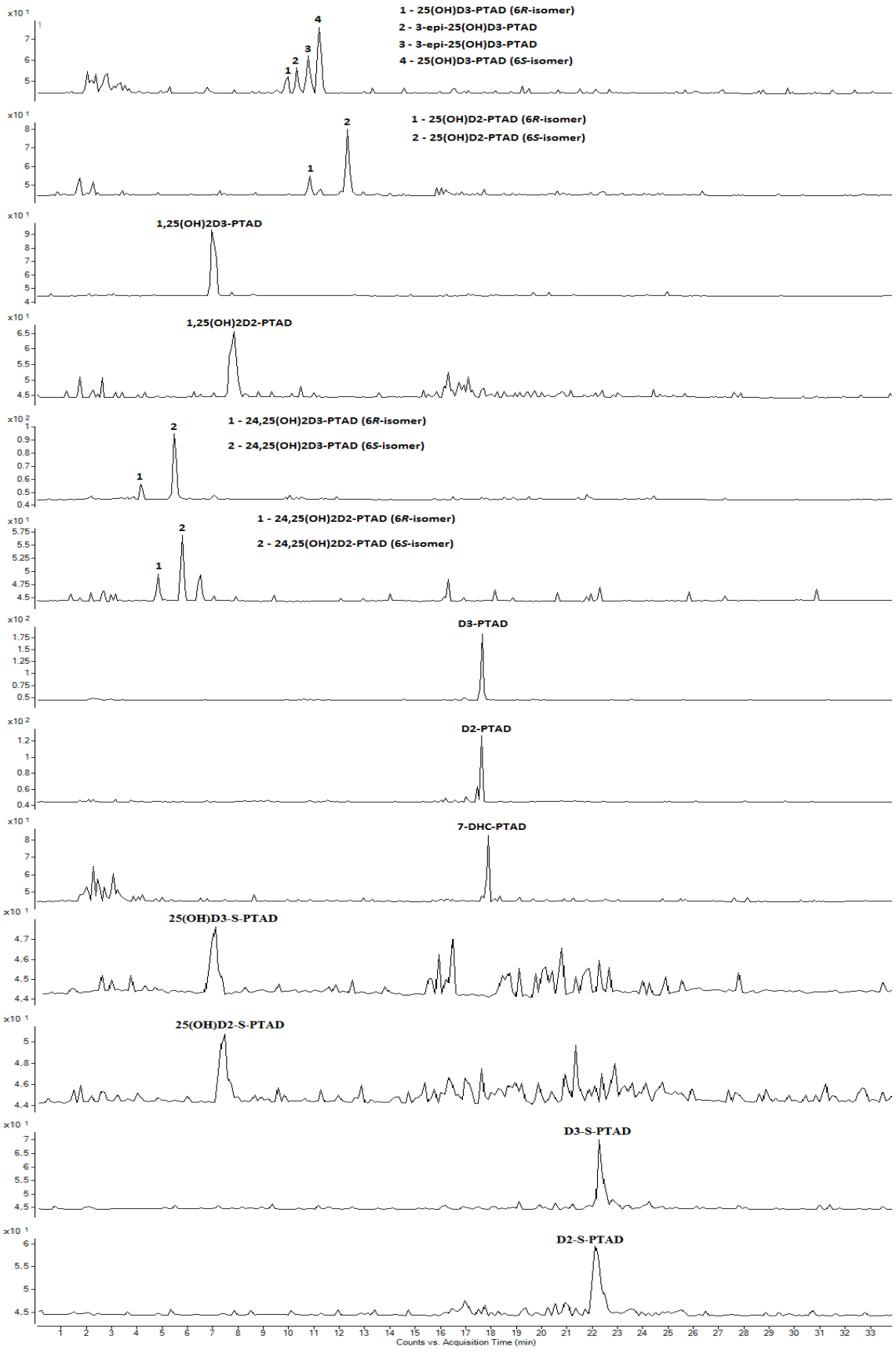

Figure 5. Chromatographic separation of the major (derivatised) vitamin D forms, including 7-dehydrocholesterol, as described in the experimental section.

The use of tandem mass detection and satisfactory chromatographic separation ensured the high specificity of the method. Although methanol as an eluent created higher column backpressures due to its greater viscosity compared to acetonitrile, it also produced superior selectivity. The optimal column temperature was found to be 40 ^{o}C. All vitamin D-PTAD derivatives in the mixture were chromatographically resolved from each other; except for D_2 and D_3. However, these were easily distinguished from each other based on their MRM transition pairs, and the use of co-eluting internal standards for each corrected the effects of ion suppression/enhancement from each other. The isomers (*R* and *S*) of some compounds were not separated under our chromatographic conditions, which also agree with previous methods (36, 43, 100). Because we used the total peak area of both forms for quantification, the separation or non-separation of *R* and *S* isomers does not affect the quantification outcome.

2.4.3 Optimisation of derivatisation reaction with PTAD

Some studies have reported previously that the detectability of vitamin D compounds was improved about 100-fold after the derivatisation (36, 88), whereas others saw improvements of about 20-fold (100). The molar ratios of PTAD:vitamin D used in reported methods varied from 11,000:1 to 571,000:1, and the reaction time varied from 1 to 4 h (36). The results of our optimisation are shown in Figure 6.

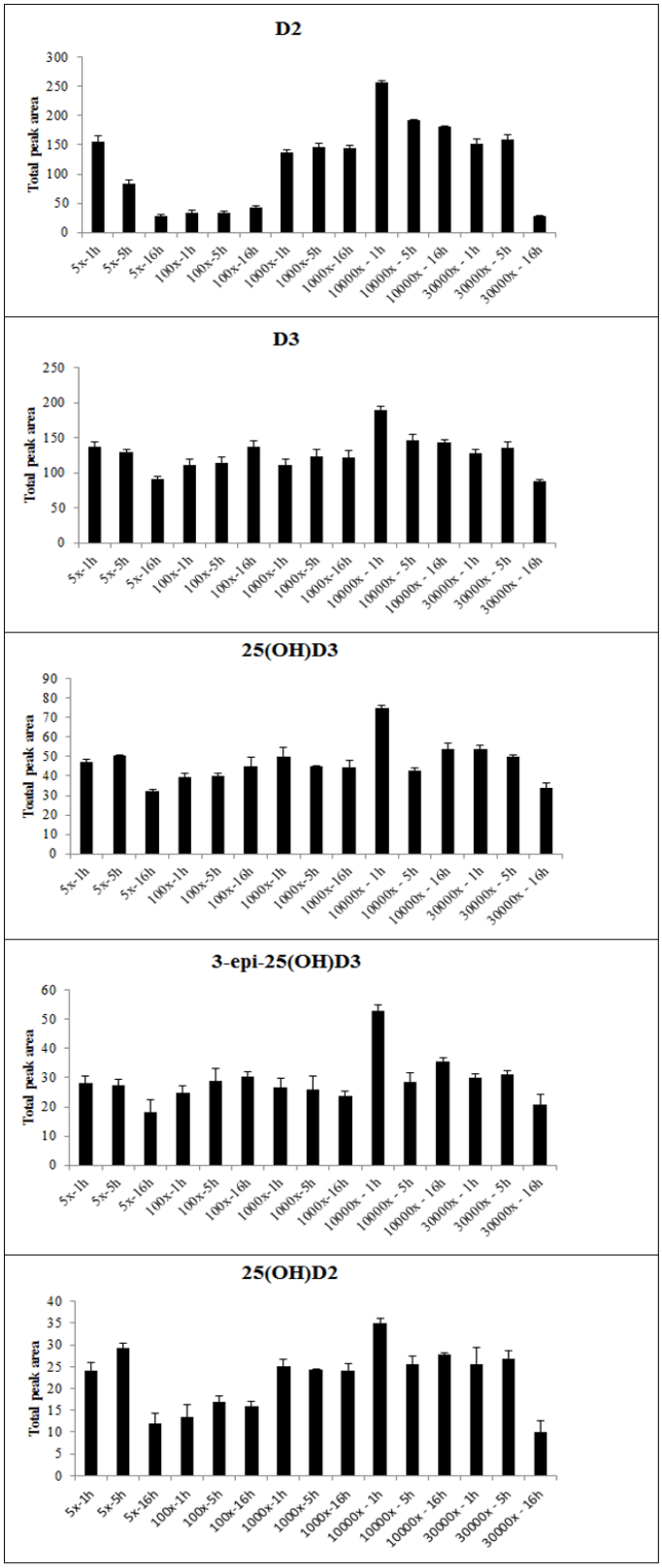

Figure 6. Signal responses for vitamin D analogues with different derivatisation conditions, as described in the experimental section.

Clearly, a 10,000-fold excess of PTAD with one-hour reaction time proved to be the optimum derivatisation conditions, giving rise to the best sensitivity, for all five analogues. One hour was found sufficient to complete the reaction between PTAD and all vitamin D compounds, which agrees with literature reports (88). Table 4 shows the relative response factors, RRF, obtained using peak height ratio of the signal and noise for each analogue calculated relative to the highest peak (D_2-PTAD in this instance), for derivatised and underivatised forms. It also shows the ratio of the RRF values of derivatised/underivatised forms.

Table 4. Relative response factor (RRF) values obtained for derivatised and non-derivatised forms, and the ratios of the two, as described in text.

Compound	**Sensitivity (S/N) PTAD**	**Sensitivity (S/N) Non-PTAD**	**Gain (x-fold)**
D_2	100	1.67	60
D_3	78.3	0.97	80
25(OH)D_2	41.4	0.82	50
25(OH)D_3	58.7	1.04	56
3-epi-25(OH)D_3	45.4	0.90	50

These results indicate that the ionisation efficiencies have been improved some 50-80-fold, following derivatisation with PTAD, using the optimised method. In addition to the detectability, the chromatography (on C_{18}) and the stability of the vitamin D analytes were also improved significantly over the unreacted forms. As the derivatisation shifted the *m/z* range of the reacted forms to higher *m/z* values, it reduced the significant chemical background that is usually present in low *m/z* regions. The background noise was therefore reduced substantially over the unreacted forms giving rise to a higher signal/noise ratio, as shown in the Figure 7.

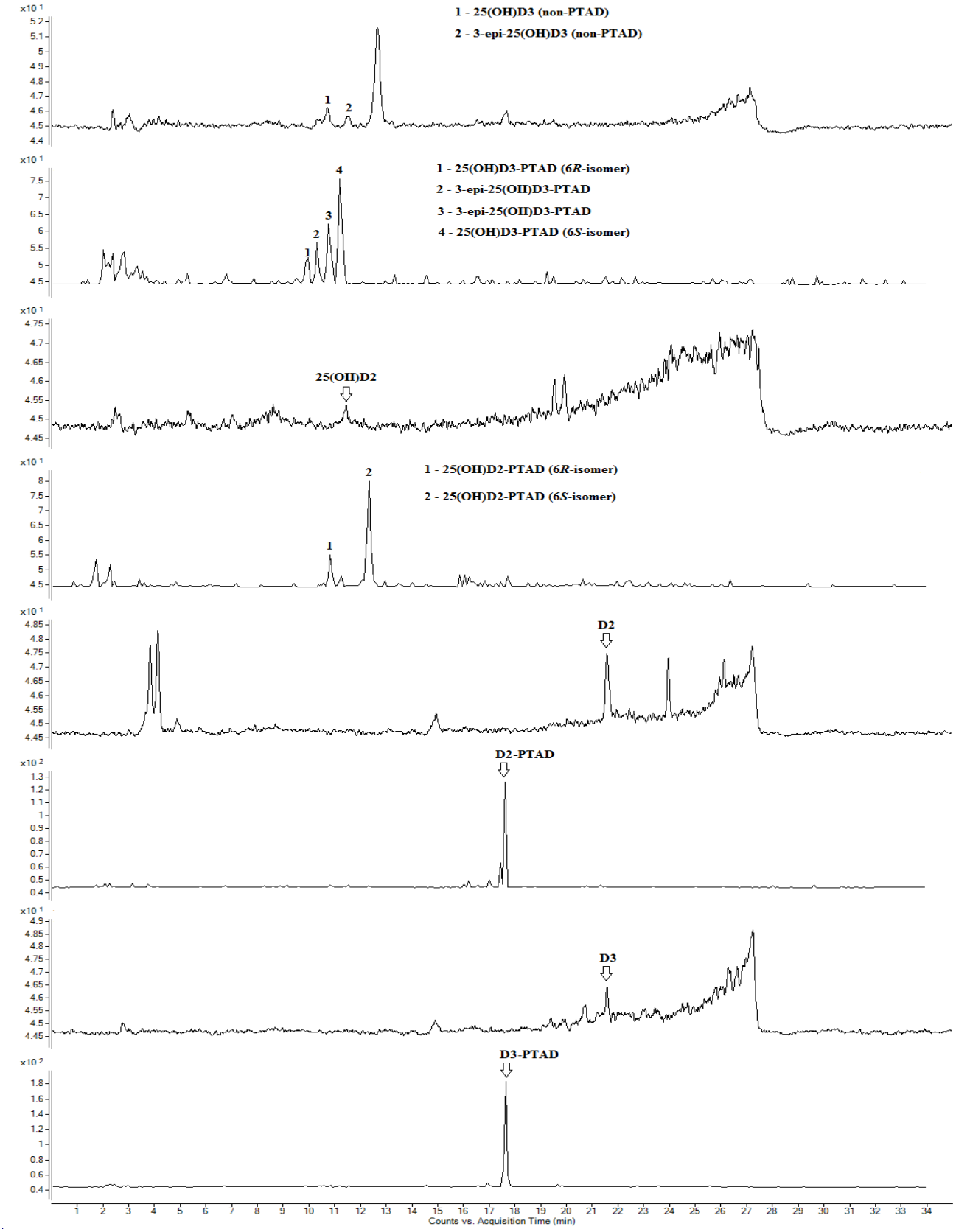

Figure 7. Chromatographic profile of each targeted compound before and after PTAD derivatisation.

The peak areas of derivatised forms that were exposed to light at room temperature for 8 h were not significantly different from those of unexposed to light, indicating the light stability of the derivatised forms (data not shown). PTAD has been reported to generate ketones from secondary alcohols of vitamin D metabolites (36); however, this phenomenon was not observed. Since PTAD is unstable in protic solvents such as water (36), a range of different types of ACN were evaluated for the lowest possible quantity of water: anhydrous, dried (using molecular sieves) and HPLC grade. As shown in the Figure 8, no significant differences were observed when using different types ACN. Hence, further studies were carried out using ACN (HPLC grade); this solvent is cheaper and the preparation less laborious compared with the other types of ACN tested.

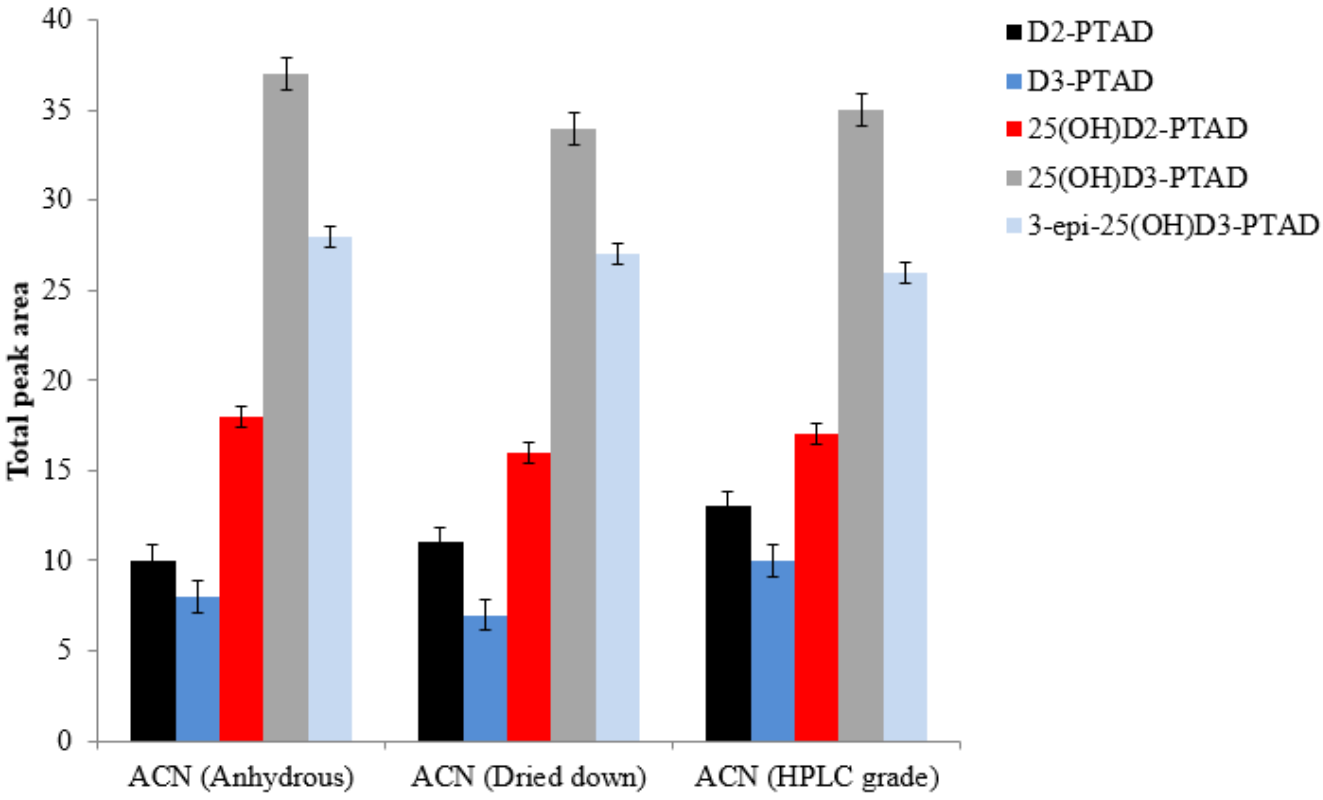

Figure 8. Comparison of different ACN types for PTAD derivatisation. Error bars represent ±1sd.

2.4.4 Optimisation of sample preparation

The efficiencies of two methods (PP and SN) were evaluated by comparing the concentration of each form of vitamin D detected in human milk. The SN procedure was adapted from previous analytical methods for analysis of vitamin D in milk (123). A PP with acetonitrile was selected, based on its high deproteinisation efficiency (136). In our preliminary studies,

we found that the evaporation of solvent to dryness in PP (before the derivatisation) was tedious and time consuming leading to vitamin loss. To overcome this problem, an LLE procedure similar to that used in SN (29, 112) was incorporated, generating an evaporation step that was easier and faster. Furthermore, different ratios of hexane-dichloromethane were evaluated with both SN and PP and the hexane-dichloromethane (4:1, v/v) solution was found optimal; the extraction yield was increased for each compound, especially for the metabolites that are relatively more polar, as shown in the Figure 9.

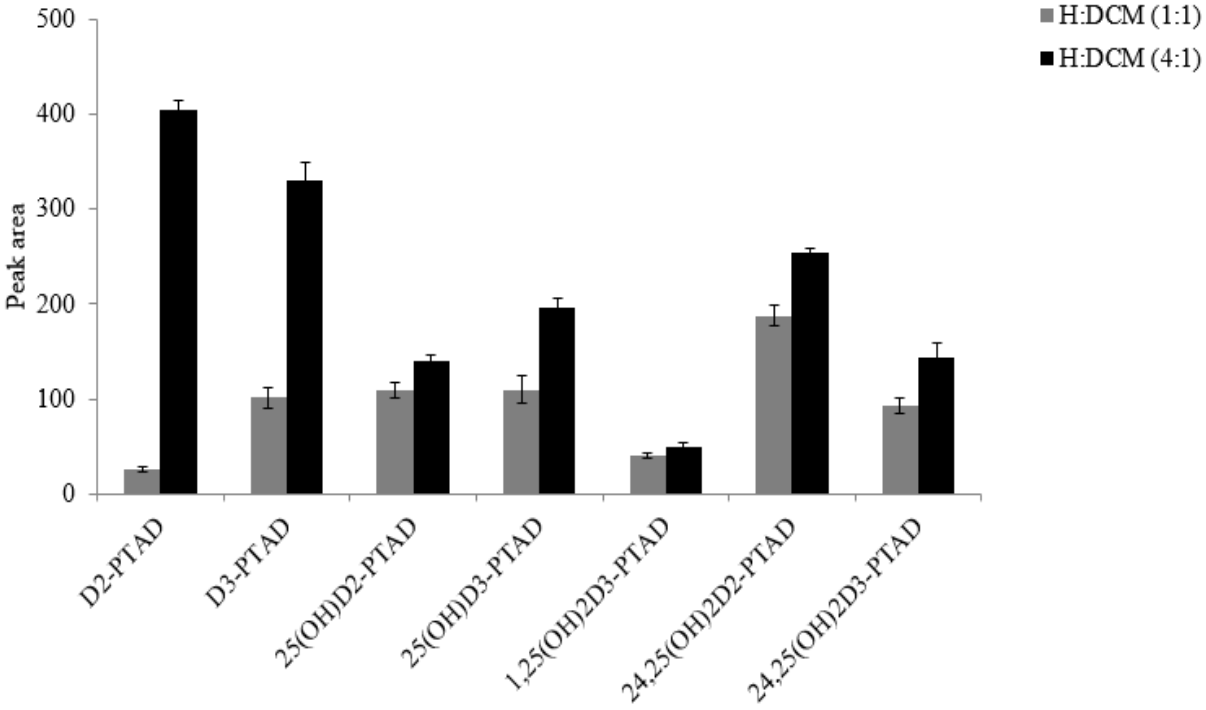

Figure 9. Comparison between different ratios of hexane and dichloromethane. Error bars represent ±1SD.

The use of higher volumes of dichloromethane (4:2 or 4:4) or the omission of dichloromethane decreased the extraction yield. Both PP and SN were conducted using this optimised LLE on human milk (each in triplicate) and the results are shown in Figure 10.

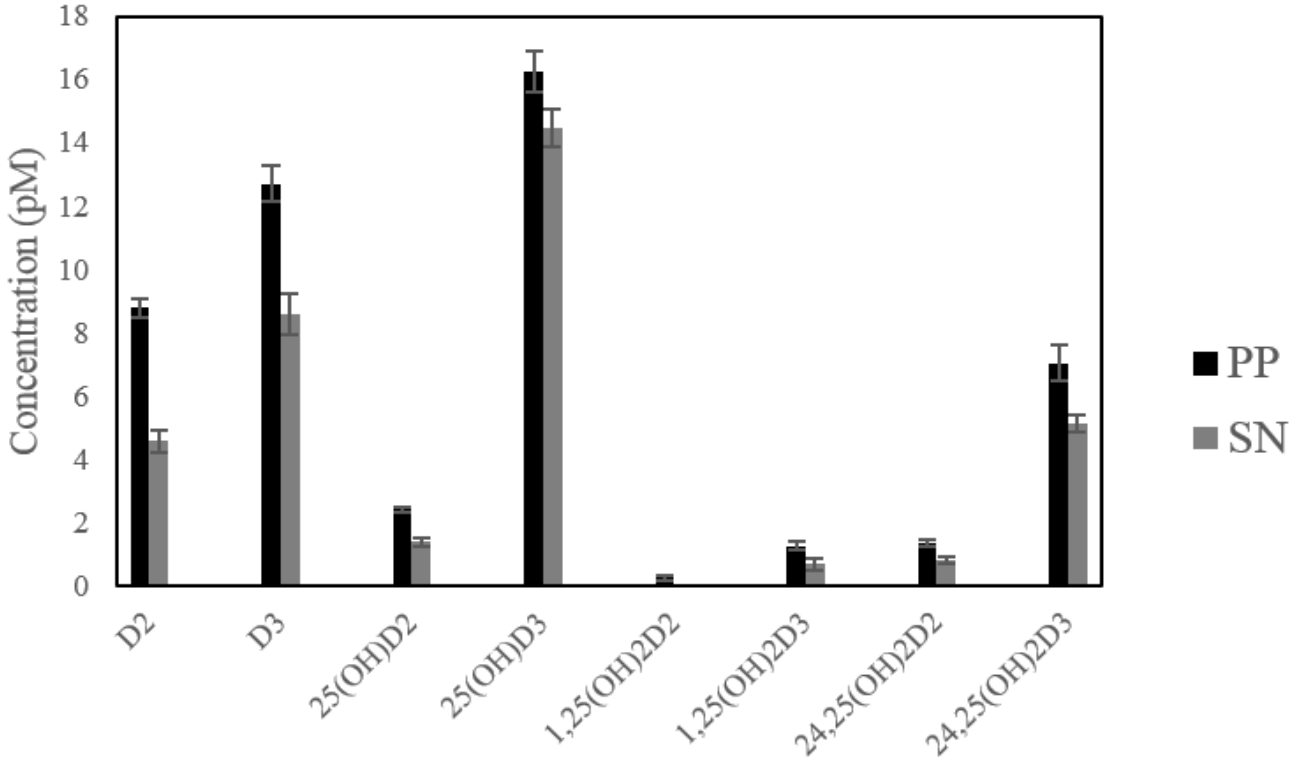

Figure 10. Comparison between PP and SN procedures for the extraction of vitamin D from milk. Error bars represent ±1SD.

Significantly higher amounts of vitamin D were obtained with PP compared to SN which has been the method of choice for milk until this study. SN is well known for its effects on vitamin loss (123) and compensatory approaches such as the use of cold SN and the addition of antioxidants (both employed in our study) were incorporated to minimise these effects. However, the comparison study demonstrates that despite the improvements, vitamin yields are lower with saponification. All further studies were carried out using protein precipitation; a method that was simpler, faster and more efficient in extracting the vitamin.

2.4.5 Method validation

The LOD and LOQ, estimated based on 3x and 10x noise respectively, ranged from 0.20 to 2.86 fmol, as shown in Table 5.

Table 5. Linearity, and detection and quantitation limits for the vitamin D compounds.

Compound	Linearity (r^2) & Upper limit (nM)	LOD* (fmols)	LOD** (pM)	LOD*** (pM)	LOQ* (femtomols)	LOQ** (pM)	LOQ*** (pM)
D_2	0.998 (5)	0.28	14	0.35	0.94	47	1.17
D_3	0.999 (5)	0.28	14	0.35	0.94	47	1.17
25(OH)D_2	0.998 (1)	0.20	11	0.27	0.76	38	0.95
25(OH)D_3	0.999 (5)	0.38	19	0.47	1.00	50	1.25
1,25$(OH)_2D_2$	0.999 (0.5)	0.20	10	0.25	0.72	36	0.90
1,25$(OH)_2D_3$	0.996 (0.5)	0.20	10	0.25	0.66	33	0.82
24,25$(OH)_2D_2$	0.999 (1)	0.40	20	0.50	0.86	43	1.07
24,25$(OH)_2D_3$	0.998 (5)	0.36	18	0.45	0.90	45	1.12
D_2-S-PTAD	0.999 (0.5)	0.74	37	0.92	2.52	126	3.15
D_3-S-PTAD	0.996 (0.5)	0.56	28	0.70	2.60	130	3.25
25(OH)D_2-S-PTAD	0.996 (0.5)	0.86	43	1.10	2.86	143	3.57
25(OH)D_3-S-PTAD	0.996 (1)	0.60	30	0.75	2.00	100	2.50

*On column amounts
** Concentration in extract
*** Concentration in milk

Table 5 also shows LOD and LOQ values calculated based on the concentrations of the extract that was injected to the column (with the volume of injection of 20 µL), and the concentrations in milk (based on 40-fold concentration in the extract). Since isotopically labelled analogues (as internal standards) possess chemical behaviours similar to the corresponding analyte, they are expected to compensate for any sample losses and/or suppression effects that may take place during sample processing and analysis (119, 137, 138). Another advantage of using such internal standards is that it has been previously shown that there is no significant difference in the results when calibration standards are prepared either in mobile phase solvent or in matrix matched medium, when isotopically labelled standards are used as the internal standards (119, 139). Therefore, in our study, the calibration standards were prepared in mobile phase solvent (the starting composition of the run), as described in the experimental section. Calibration standards (six levels) were prepared to include the typical levels of each form reported in milk, as described in the experimental section. Table 6 shows the linearity, along with the upper limits of concentrations used in this study. Good linearities (r^2 of >0.99) were obtained for all analogues for the required concentration ranges. The repeatability values (n = 10), determined by analysing multiple aliquots of the same human milk sample on the same day and expressed as percentage standard deviations, are shown in Table 6.

Table 6. Repeatability and recovery for the targeted vitamin D compounds.

Compound	Repeatability (n=10)		Recovery (n=6)
	Mean ± SD (pM)*	RSD (%)	Mean ± SD (%)
D_2	8.14 ± 0.001	6.30	93.9 ± 3.23
D_3	12.2 ± 0.002	13.2	98.9 ± 6.83
$25(OH)D_2$	2.38 ± 0.001	9.36	95.8 ± 8.02
$25(OH)D_3$	16.7 ± 0.001	4.77	98.2 ± 2.27
$1,25(OH)_2D_2$ **	0.305 ± 0.004	13.0	88.2 ± 3.99
$1,25(OH)_2D_3$	1.22 ± 0.001	9.75	94.5 ± 10.5
$24,25(OH)_2D_2$	1.38 ± 0.004	13.5	96.5 ± 8.25
$24,25(OH)_2D_3$	7.74 ± 0.005	7.68	105 ± 8.29
D_2-S-PTAD	-----	-----	-----
D_3-S-PTAD	-----	-----	-----
$25(OH)D_2$-S-PTAD	-----	-----	-----
$25(OH)D_2$-S-PTAD	-----	-----	-----

* Concentration in milk
** Approximate only (value between LOD and LOQ)

The method is precise with percentage standard deviations within the range 4.76-13.5. The mean recoveries, calculated using the percent ratio of observed/spiked concentrations, were in the range of 88.2-105%, as shown in Table 8. The method was applied to measure eight analogues of vitamin D in human, cow, mare, goat and sheep milk. The sulfate forms were not detected in the milk samples. The chromatographic profiles obtained for human milk are shown in Figure 11.

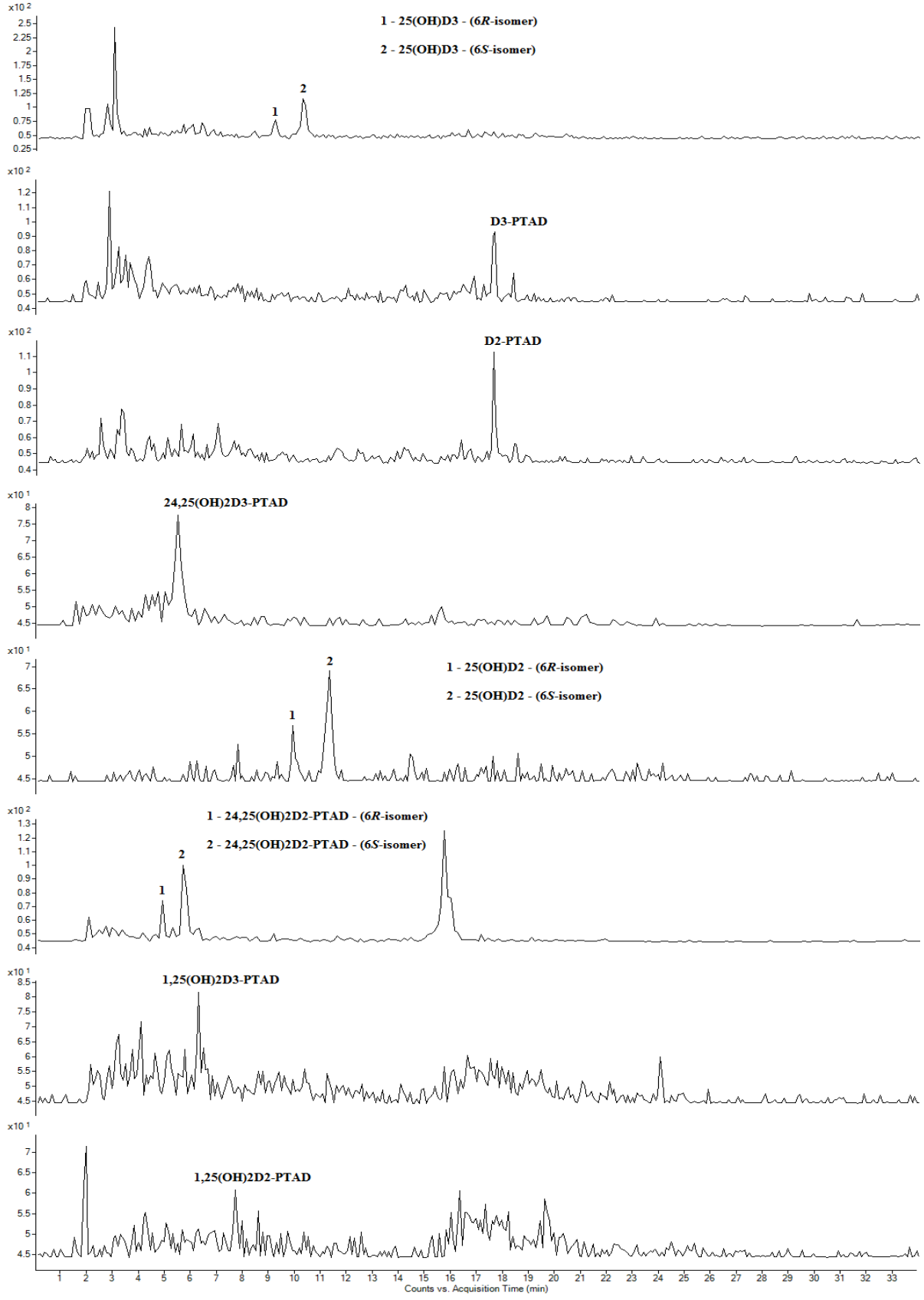

Figure 11. Chromatographic profile of the major vitamin D forms (derivatised) in human milk.

The concentrations of each vitamin D compound quantified are listed in Table 7.

Table 7. Concentration of vitamin D compounds in milk samples from human, cow, goat, mare and sheep.

Compound (pM)	Human	Cow	Mare	Goat	Sheep
D_2	8.63	5.70	----	3.36	----
D_3	13.0	14.1	14.5	12.6	13.9
$25(OH)D_2$	2.34	1.90	----	1.68	----
$25(OH)D_3$	16.0	19.7	13.1	10.9	11.9
$1,25(OH)_2D_2$	0.31	----	----	----	----
$1,25(OH)_2D_3$	1.14	0.41	----	----	----
$24,25(OH)_2D_2$	1.27	0.94	----	0.86	----
$24,25(OH)_2D_3$	7.68	10.7	7.09	7.94	4.69

Vitamin D_3 and $25(OH)D_3$ are the major forms in all species. There may be similarities in the relative levels of each analogue in different species, however, more work is needed to study this trend. It is also important to consider that the content of vitamin D may vary significantly due to factors such as vitamin D supplementation, exposure to sunlight, and storage conditions.

2.5 Conclusions

Despite the lack of accuracy, reproducibility and selectivity of immunoassays, they have been the only methods readily available for the quantification of the dihydroxyl vitamin D forms in milk. The scope of the LC-MS/MS methods available for milk has been limited to the quantification of D_2, D_3, $25(OH)D_2$ and $25(OH)D_3$. The method reported here has expanded the scope to include four additional analogues. The contribution of the inactive form 3-epi-$25(OH)D_3$ has been (chromatographically) removed to prevent overestimation of vitamin D. Adequate retention was achieved for all analytes of interest to minimise the ion suppression effects from early eluting endogenous compounds and excess derivatising agent. The maximum possible sensitivity and specificity for each of the eight (derivatised) analogues has been obtained by optimisation of the ESI source and MRM conditions. The PTAD derivatisation was systematically optimised for pre-column derivatisation. It should be noted that the optimisation of the MS conditions, chromatography, and the derivatisation method are applicable to matrices other than milk, permitting femtomole levels of vitamin D compounds to be quantifiable. The protein precipitation method, with some modifications,

has been applied to milk for the first time and found superior to the commonly used saponification in terms of efficiency of extraction, simplicity and speed. By using an isotope labelled internal standard for each vitamin D compound we have developed an accurate as well as a specific and sensitive quantification method for these compounds. The method presented here was unable to capture sulfated forms in milk samples. This finding may be attributable to the strongly acid characteristics of the sulfate moiety that would facilitate deprotonation rather than protonation in the mass spectrometer. In this context, PTAD derivatisation would not be required to improve their ionisation efficiencies, as these compounds could be readily negatively ionised in solution at a typical pH range. Thus, the ionisation efficiency of the sulfated forms (unreacted in negative mode ESI) will be evaluated and further compared with those from PTAD derivatives in positive mode ESI.

3 Chapter 3: Effect of pasteurisation on the concentrations of vitamin D compounds in donor breastmilk

3.1 Foreword

This chapter describes the effect of pasteurisation on the vitamin D content in donor breastmilk. The analytical method developed and validated in chapter 2 was used for this study. The content of this chapter has been published in the *International Journal of Food Sciences and Nutrition*, 67: 16–19 (2016) and it is presented in a slightly altered format to fit with the style of this thesis. Permission was granted from the publisher to reproduce the work in this thesis.

3.2 Introduction

Breastmilk is the most important nutrient and source of supplementation for both term and preterm infants (140). It is composed of many important nutrients, including vitamin D (4). The content of this vitamin in breastmilk is usually low, even for lactating mothers with adequate vitamin D status (4, 141). Preterm infants are at a significant risk of vitamin D deficiency due to decreased transplacental transfer (142, 143). Such infants also possess smaller vitamin D storage sites such as fat and muscle tissue (144). Vitamin D is essential for the regulation of the intestinal absorption of calcium and phosphate, both of which are vital for healthy homeostasis (13, 123). When mothers are unable to breastfeed their infants, pasteurised donor human milk (PDHM) is considered to be the most effective alternative source of nutrition (145) and it is widely accepted that donor human milk has considerable advantages over formula milk (146). Donor human milk is collected, processed and stored by specialised centres, such as human milk banks (147). Donor breastmilk is obtained from healthy lactating mothers who consent to donate their surplus breastmilk (147, 148) and this milk is pasteurised to reduce microbial growth, ensuring its safety for infant consumption (149). The most common pasteurisation procedure is known as Holder pasteurisation (HP), in which milk is exposed to a temperature of approximately 62.5^{o}C for at least 30 min (145). While this thermal procedure is required to minimise potential infectious diseases, it may be responsible for the destruction or degradation of vitamin D, since this vitamin is potentially sensitive to high temperatures (148). Vitamins D_2 and D_3 (D_2 and D_3) and the metabolites 25-hydroxyvitamins D_2 and D_3 (25(OH)D_2 and 25(OH)D_3) are the four major forms of vitamin D compounds in biological fluids (150). It has been suggested that D_3 is more efficiently intestinally absorbed than D_2 (124, 125). The metabolite 25(OH)D_3 has three times the

potency (126) and higher affinity than 25(OH)D_2 to vitamin D-binding protein (DBP), which results in higher half-life (124). Some studies on the effect of pasteurisation have been reported, including, for example the significant effects of pasteurisation on the content of vitamin C in milk (148, 151, 152). To our knowledge, however, no investigations on the impact of pasteurisation upon the vitamin D content in breastmilk have been reported. The aim of this study was to evaluate the effect of pasteurisation on the content of D_2, D_3, 25(OH)D_2 and 25(OH)D_3 in donor breastmilk. The quantitative determination of vitamin D and its metabolites in milk is a challenging task due to their low levels and the presence of interfering endogenous components. A published liquid chromatography mass spectrometry (LC-MS/MS) method previously developed and validated for the analysis of a wide range of vitamin D analogues in milk was used to evaluate the effect of pasteurisation on the above compounds in donor breastmilk (150).

3.3 Methods

3.3.1 Collection

Breastmilk samples were donated by 16 healthy lactating women. Participants were women (over 18 years old) who wished to donate milk to the Milk Bank at the Royal Brisbane & Women's Hospital (RBWH) and who had met the criteria to donate. The milk bank staff asked the volunteers during the routine interview whether they would be interested in participating in this study. Information sheets were provided to all potential participants and all questions were addressed. Informed consent was provided by all donors to use their breastmilk in this research work; all information linking the samples with specific donors was removed from the samples. The collection of the milk samples from RBWH was approved by RBWH ethics committee and The University of Queensland (UQ) ethics committee. A RBWH Milk Bank has been established for two years; its processes are based on the Good Manufacturing Practice guidelines and it is accredited with ISO 22000 (153). A four millilitre aliquot was used to analyse vitamin D levels (pre-pasteurisation) and another four millilitre aliquot was used to analyse vitamin D levels (post-pasteurisation). Each aliquot was placed in a clean Eppendorf tube and given a unique identifier. The milk samples were stored at -80^{o}C until analysis.

3.3.2 Holder-pasteurisation

The breastmilk samples were processed in a Sterifeed Pasteuriser. An aliquot of each breastmilk sample was transferred into a bottle and submitted to a pre-heated water bath (62.5^0C) for 30min (153).

3.3.3 Chemicals

The unlabelled and labelled standards (D_2, D_3, 25(OH)D_2 ,25(OH)D_3, D_2-d_3, D_3-d_3), were purchased from IsoSciences (King of Prussia, USA), and (25(OH)D_2-d_6 and 25(OH)D_3-d_6) from Chemaphor (Ottawa, Canada). All compounds had a purity of 97% or better. PTAD (≥97%) was purchased from Sigma Aldrich (St. Louis, USA). The organic solvents of LC-grade (dichloromethane, ethanol, hexane, acetonitrile and methanol) were purchased from Merck (Darmstadt, Germany). LC-MS grade formic acid (>99%) was purchased from Fisher Chemical (Geel, Belgium). High-purity water was prepared by using a Millipore Milli-Q system (Milford, USA).

3.3.4 Sample preparation and LC-MS/MS analysis

The milk samples were processed and analysed according to an established LC-MS/MS method for the analysis of a range of vitamin D analogues in milk (150).

3.3.5 Statistical analysis

The results are presented as a percentage increase or decrease of each analyte post-pasteurisation, compared with pre-pasteurisation. Statistically significant differences were assessed by comparing pre- and post-pasteurisation values using the Wilcoxon matched-pairs signed rank test, with $P<0.05$ considered significant, calculated using Prism 6 (GraphPad.com).

3.4 Results

Pasteurisation resulted in a significant reduction ($P< 0.05$) in the content of D_2, D_3, 25(OH)D_2 and 25(OH)D_3 with P values of 0.01 for all targeted analytes. The individual pre- and post-pasteurised sample values are illustrated in the Figure 12.

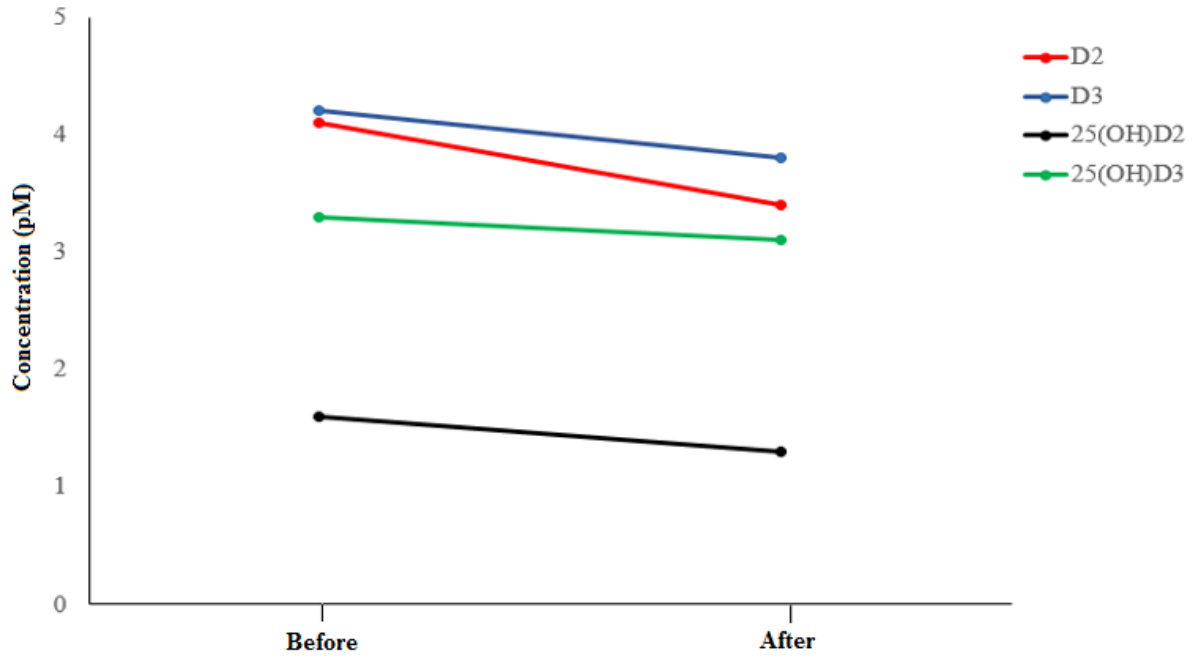

Figure 12. The individual pre- and post-pasteurised sample values.

The mean concentrations of the vitamin D analogues in non-pasteurised milk were 4.1 pM (D_2), 4.2 pM (D_3), 1.6 pM ($25(OH)D_2$) and 3.6 pM ($25(OH)D_3$), as shown in Table 8.

Table 8. Mean/median concentrations (pM) of vitamin D compounds (pre- and post-pasteurisation). Samples of donor breast milk (n=16) were analysed by LC-MS/MS.

Compounds	Pre-pasteurised Mean ± SD	Post-pasteurised Mean ± SD	Pre-pasteurised Median (95% CI)	Post-pasteurised Median (95% CI)
D_2	4.1 ± 0.45	3.4 ± 0.36	4.0 (3.7 - 4.5)	3.3 (3.2 - 3.9)
D_3	4.2 ± 2.41	3.8 ± 2.36	3.4 (2.3 - 6.4)	3.0 (2.3 - 6.0)
$25(OH)D_2$	1.6 ± 0.23	1.3 ± 0.14	1.6 (1.4 - 1.7)	1.3 (1.2 - 1.4)
$25(OH)D_3$	3.3 ± 2.20	3.1 ± 2.16	3.1 (1.8 - 5.0)	2.4 (1.4- 4.7)

The mean concentrations of the vitamin D analogues in post-pasteurised milk were 3.5 pM (D_2), 3.8 pM (D_3), 1.3 pM ($25(OH)D_2$) and 3.1 pM ($25(OH)D_3$), as shown in Table 10. Losses of vitamin D compounds caused by pasteurisation ranged from 10% to 20%.

3.5 Discussion

This study assessed the effect of pasteurisation on key vitamin D compounds in donor breastmilk. Isotopically labelled analogues of each standard compound were used as internal standards to ensure the reliability of the results, provided by the LC-MS/MS method (119). As expected, the concentrations of D_2, D_3, $25(OH)D_2$ and $25(OH)D_3$ in breastmilk were found to be at very low (picomolar) levels, and within the levels reported previously (135, 150).

It is important to note that vitamin D supplementation and seasonal variation may impact significantly on the vitamin D content in breastmilk (141). Other metabolites such as 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and 24,25-dihydroxyvitamin D_3 ($24,25(OH)_2D_3$) were also detected in some samples. A statistical evaluation was not possible with these metabolites due to the very low levels detected in three samples. The epimeric form of $25(OH)D_3$ was detected in two samples. Since these two isomers were chromatographically resolved, there was no over-estimation of the $25(OH)D_3$ status. The method used for this study was capable of only removing the inactive epimeric form from $25(OH)D_3$ but not of quantifying it. Therefore, the effect of pasteurisation on the inactive epimer was not studied.

In this study, we have observed that D_2, D_3, $25(OH)D_2$ and $25(OH)D_3$ concentrations were decreased after pasteurisation, indicating that donor breastmilk has potentially a reduced antirachitic activity when compared to breastmilk provided directly from a lactating mother.

As these compounds cannot be protected from temperature changes during the pasteurisation process, their partial destruction or isomerisation to inactive forms may be unavoidable when using such thermal methods. Vitamin D compounds are potentially thermally sensitive, as observed by their decomposition upon exposure to heat in the presence of air (154) and these substances can be converted irreversibly to cyclised compounds such as, pyro- and isopyro-vitamin D at elevated temperatures (155-158). Cooking temperatures in absence of either air or light have been found to promote the isomerisation of vitamin D to pyro-vitamin D (158). This vitamin can be also thermally converted to its precursor form (159).

Our results demonstrate that vitamin D_2 compounds were more affected by pasteurisation than were the D_3 vitamin compounds, indicating their higher sensitivities to thermal treatments. Hence, the measurement of either D_2 or $25(OH)D_2$ may be potentially regarded as a suitable indicator of the degree of thermal treatment, although D_2 was less affected than $25(OH)D_2$. Another potential source of vitamin loss could have been the adsorption of the vitamin D and/or fat to the pasteurisation vessel; the significance of this effect is not yet known and is a focus of future studies. In general, the activities of vitamins are expressed in

international units (IU). With vitamin D, both cholecalciferol and ergocalciferol are considered to have a similar potency: 1IU = 25 ng. The content of vitamin D in breastmilk is usually very low (about 20 IU/L) (4). As a result, it is often unable to satisfy the current vitamin D recommendations and hence meet the specific needs of premature infants. It has therefore been suggested that preterm infants should be supplemented with vitamin D (160). There appears to be little consensus for the optimal daily intake of vitamin D for preterm infants (161) and vitamin D intake has been suggested to be about 400 IU a day (162). In the case of full term infants who receive donor milk, the adequacy of vitamin levels of the donor milk and hence intake are provided with less attention than those of premature infants. The results of our study, therefore, may have more impact on those full term infants dependent on pasteurised donor milk.

3.6 Conclusions

We have demonstrated that the content of vitamin D compounds in donor breastmilk is consistently very low when compared to some recommendations of dietary requirements and these concentrations are also affected by pasteurisation. Further research work is required to establish the biological implications related to these findings. Since many milk banks have now been established across the world, it is timely and also essential to evaluate the content of vitamin D in pasteurised donor breastmilk with reliable analytical tools such as LC-MS/MS. This approach will then ensure that health care can be provided based on reliable values to establish adequate vitamin D supplementation or/and milk fortification. This study has provided preliminary data that can be used for the improvement of current approaches for milk pasteurisation treatments, permitting minimal effects on the biological activity of vitamin D in breastmilk.

4 Chapter 4: Development and validation of a method for the quantification of four sulfated vitamin D compounds in breastmilk and human serum by liquid chromatography-tandem mass spectrometry

4.1 Foreword

The aforementioned LC-MS/MS method (chapter 2) failed to accommodate both fat- and water soluble forms of vitamin D. It was only capable of determining fat-soluble forms. This chapter describes a new approach taken to analyse the water-soluble forms. A comparative evaluation of the ionisation efficiencies of the underivatised forms of sulfates in negative ion mode ESI and those of the derivatised (using PTAD) forms in positive ion mode ESI was carried out. Based on this comparison, a sensitive LC-MS/MS method was developed, and validated. The content of this chapter has been published in the *Journal of Chromatography B,* 1009: 80-86 (2016) and it is presented in a slightly altered format to fit with the style of this thesis. Permission was granted from the publisher to reproduce the work in this thesis.

4.2 Introduction

It has been commonly accepted that vitamin D has a much wider role to play in the human organism rather than just its role in the homeostasis of healthy bone tissues. A growing body of evidence has been provided on the importance of this vitamin, in reducing the risk of hypertension, common cancers, diabetes mellitus, autoimmune and cardiovascular disorders (4, 13). Despite these findings, vitamin D deficiency still remains a commonly encountered health issue, affecting many individuals across the world (123).

While both fat- and water-soluble forms of vitamin D have been reported in diverse biological fluids, clinical and nutritional attention has been primarily given to the fat-soluble forms (123, 163). Consequently, clinical studies on water-soluble Vitamin D compounds are lacking. It has been suggested that the water-soluble forms (sulfate conjugates) of vitamin D have potencies similar to those of the fat-soluble compounds (20). Although the biosynthesis of the sulfates is unclear (19), a study has shown that vitamin D is not readily sulfated in man, indicating its formation from a conjugated precursor is possible (22). The presence of 7-dehydrocholesterol-sulfate (7-DHC-S) has been reported in human and rat skin tissue, confirming the existence of a precursor for vitamin D_3-sulfate (D_3-S) (19). While controversy exists regarding the specific actions and biological roles (164), it has been reported that vitamin D_3-sulfate (D_3-S), when orally administered in high doses, increases calcium transportation in young rats (23).

25-Hydroxyvitamin D_3-sulfate ($25(OH)D_3$-S) is a major circulating form of vitamin D and its levels in human blood may exceed those of the non-sulfated form, $25(OH)D_3$ (19, 22), the most commonly measured form of vitamin D used to determine vitamin D status (123). Clearly, the measurement of $25(OH)D_3$-S in human blood is thus likely to be important in the assessment of vitamin D status. $25(OH)D_3$-S could be considered a storage form of non-sulfated D_3, as hydrolysis of the conjugate may potentially take place *in vivo* (19, 21).

Vitamin D_2-sulfate (D_2-S) has been detected in chicken tissues and in rabbit urine (23, 165) and the biosynthesis of D_2-S has been achieved *in vitro* (165). It has also been demonstrated that D_2-S possesses a potent antirachitic activity when administered in rats (24).

To our knowledge, the vitamin D metabolite, 25-hydroxyvitamin D_2-sulfate ($25(OH)D_2$-S), has not yet been reported in biological fluids, and its physiological functions are unknown. In fact, the vital roles of D-S forms are still debatable and clear evidence for their biological function in humans has not yet been reported. A potential reason for this is the absence of a sufficiently sensitive analytical method to detect endogenous levels of these compounds.

Methods based on low sensitivity and low specificity, such as colorimetry, have been reported for the analysis of D-S analogues in milk (166, 167). Contradictory results were obtained in different studies for D-S in breastmilk (168), which may be due to a lack of specificity in the analytical methods used and that saponification has been used as an extraction procedure for D-S in milk (169). Although widely used to extract vitamin D analogues from food, saponification may be especially destructive for the water-soluble forms of this vitamin (167, 168).

Hyphenated methods such as liquid chromatography-UV spectrophotometry (LC-UV) have been used for the measurement of D_3-S and $25(OH)D_3$-S (22, 164, 170). They have been largely replaced by liquid chromatography-tandem mass spectrometry (LC-MS/MS), currently considered the technique of choice for vitamin D assays (171). Although LC-MS/MS offers the resolving power of LC systems combined with the high sensitivity and specificity of MS/MS detection, two major problems are associated with this technique in vitamin D assays. Firstly, vitamin D compounds have poor ionisation efficiencies when using atmospheric pressure ionisation (API), which has been solved by derivatising them with 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) or other Cookson type reagents (described in the chapter 1) (123, 171). Secondly, the susceptibility of MS detection to matrix effects such as ionisation suppression can negatively compromise the quality of assay, in terms of sensitivity and accuracy. This has been resolved by the use of a high efficiency separation method to

minimise co-elution and by the use of co-eluting stable isotope labelled analogues as internal standards (123).

The scope of currently available LC-MS/MS methods for the determination of sulfated vitamin D analogues is limited only to 25(OH)D_3-S in human plasma (163). Therefore, we embarked on the development and validation of an LC-MS/MS method for the determination of four sulfated forms, D_2-S, D_3-S, 25(OH)D_2-S and 25(OH)D_3-S in breastmilk and human serum. A simple protein precipitation technique was used as the sample preparation method to remove the targeted analytes from the biological matrices and stable isotope labelled analogues of each targeted form were used as internal standards for accurate quantification. The ionisation efficiencies of the intact forms in negative ion mode electrospray (ESI) were compared to those of the derivatised forms with PTAD in positive ion mode ESI. The aim of this study was to develop a simple, effective and reliable method to quantify the water-soluble forms of vitamin D in biological fluids.

4.3 Material and methods

4.3.1 Chemical and reagents

The unlabelled and deuterium-labelled standards: D_2-S, D_3-S, 25(OH)D_2-S, 25(OH)D_3-S, D_2-S-d_3, D_3-S-d_3, 25(OH)D_2-S-d_3, and 25(OH)D_3-S-d_3 were purchased from IsoSciences (King of Prussia, USA). All compounds had a purity of 97% or better. PTAD (≥97%) was purchased from Sigma Aldrich (St. Louis, USA). Organic solvents of LC-grade (acetonitrile and methanol) and analytical grade (ethanol) were purchased from Merck (Darmstadt, Germany). LC-MS grade formic acid (>99%) was purchased from Fisher Chemical (Geel, Belgium). LC-MS grade ammonium formate (>99%) was purchased from Sigma-Aldrich (St. Louis, USA). High-purity water was prepared by using a Millipore Milli-Q system (Milford, USA). Human milk samples were kindly donated by the breastmilk bank of the Royal Brisbane and Women's Hospital (RBWH), Brisbane, Australia. Serum samples for the study were obtained from volunteer donors of the School of Pharmacy, The University of Queensland. Ethical approvals were obtained from the Institutional Human Research Department, The University of Queensland and from the Royal Brisbane and Women's Hospital.

4.3.2 LC-MS/MS analysis

Chromatographic separations were carried out using an Agilent binary LC system consisting of an Agilent 1290 infinity LC pump, an Agilent 1290 well plate auto-sampler and a

Poroshell 120 EC-C_{18} (150 x 2.1 mm, 2.7 μm) column (Agilent Technologies, Santa Clara, CA, USA). An Agilent 6460 Triple Quadrupole tandem mass spectrometer equipped with a Jet Stream source and supported by Mass Hunter Workstation software (Agilent Technologies, Santa Clara, CA, USA) was used as the detector.

A binary solvent system consisting of 10mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B) was used for separation. Gradient elution was used for the first 7 min (85 to 100% B), and remained at 100% B for the next 5 min. The composition returned to 85% B during the next 1 min, and the column was then re-equilibrated with 85% B for 10 min before injecting the next sample. The total run time including re-equilibration step was 23 min. The flow rate was 0.2 $mLmin^{-1}$ and the injection volume was 20 μL.

Mass spectrometry parameters such as fragmentor and collision energy were optimised to obtain the highest possible sensitivity for each compound, as shown in Table 9.

Table 9. Optimised mass spectrometry parameters used in MRM mode for all vitamin D analogues.

Compound	Precursor ion (m/z)	Product ion (m/z)	Fragmentor (V)	Collision energy (V)
D_2-S	475	96	242	32
D_2-S-d_3	477	96	242	32
D_3-S	463	96	232	36
D_3-S-d_3	465	96	232	36
25(OH)D_2-S	491	96	222	40
25(OH)D_2-S-d_3	493	96	222	40
25(OH)D_3-S	480	96	222	36
25(OH)D_3-S-d_3	482	96	222	36
D_2-S-PTAD	652	378	134	16
D_3-S-PTAD	640	378	124	12
25(OH)D_2-S-PTAD	650	378	154	12
25(OH)D_3-S-PTAD	639	378	144	12

Table 9 also shows the *m/z* of precursor and product ions used in multiple reaction monitoring (MRM) mode for each D-S compound. Source parameters such as gas flow, sheath gas flow, gas temperature and sheath gas temperature were optimised and maintained at 5 L/min, 12 L/min, 300^{o}C and 250^{o}C respectively. The capillary and nozzle voltages were maintained at 5000 and 2000 V respectively.

The derivatised forms were analysed using a previously developed method in our laboratory (chapter 2) (150). The detection parameters used in MRM mode are: D_2-S-PTAD, *m/z* 652-

378; D_3-S-PTAD, *m/z* 640-378; 25(OH)D_2-S-PTAD, *m/z* 650-378 and 25(OH)D_3-S-PTAD, *m/z* 639-378.

4.3.3 Preparation of standard and sample solutions

All sample and standard preparations were carried out under subdued light and standard compounds were dissolved in ethanol to obtain stock solutions (1 µM) that were stored at -20 ^{o}C. Working solutions were prepared by serial dilution from the stock solutions. Each breastmilk sample was vortex mixed and 4 mL aliquots were accurately measured and frozen until extraction and analysis. Each serum sample was vortex mixed and 250 µL aliquots were accurately measured and frozen until extraction and analysis.

For milk samples, eight millilitres of acetonitrile were added to 4 mL milk and vortex mixed for 2 min. The samples were incubated at room temperature for 15 min to complete the protein precipitation, and then centrifuged for 10 min at 4000 g. The supernatant was transferred to a fresh centrifuge tube and evaporated under a gentle stream of nitrogen. After evaporating most of the acetonitrile, the remaining solution in the tube was frozen at -80°C. The frozen sample was then evaporated to dryness using a freeze drier operated at -80°C. The residue was reconstituted in 500 µL of mobile phase (85% B).

For serum samples, a 500 µL aliquot of acetonitrile was added to 250 µL serum and vortex mixed for 2 min. The procedure was performed as described above for milk samples; however, the residue was reconstituted in 50 µL of mobile phase (85% B).

For the analysis of derivatised forms of the standards and samples, a previously developed method in our laboratory was used (150). The derivatised and underivatised forms were prepared to obtain the same final concentrations.

4.3.4 Comparison of positive and negative ESI ionisation methods using derivatised and underivatised forms

The derivatised and underivatised forms of standard compounds (10 nM each in final solution) were analysed using separate LC-MS/MS procedures, as described above. Peak areas were used to compare the ionisation efficiencies in ESI positive ion mode (derivatised forms) and ESI negative ion mode (underivatised forms). Triplicate measurements were performed for each method.

4.3.5 Method validation

Linearity was estimated using six mixed standard solutions, dissolved in mobile phase, with concentrations ranging from 0.05 to 200 nM (D_2-S, D_3-S, 25(OH)D_2-S and 25(OH)D_3-S). Each mixed standard solution contained 25 nM of internal standards (D_2-S-d_3, D_3-S-d_3, 25(OH)D_2-S-d_3 and 25(OH)D_3-S-d_3). The linearity was assessed by plotting the peak area ratios of each unlabelled form and its respective labelled form versus the concentration of unlabelled standard. The limits of detection (LOD) and limits of quantitation (LOQ) for each compound were determined based on the concentrations (as peak heights) corresponding to 3x noise and 10x noise respectively. Repeatability was estimated by analysing 10 individual aliquots of both breastmilk and human serum samples followed by the calculation of relative standard deviation of the concentrations. Recovery was determined by using the concentrations of each targeted analogue determined for six aliquots each of spiked and non-spiked breastmilk and human serum samples. Each aliquot of milk and serum was spiked to give an added concentration similar to the mid-point of the calibration range.

4.4 Results and discussion

4.4.1 Method development

Direct infusion in ESI negative ion mode was used for the identification of the precursor and product ions to be used for the development of MRM method. Deprotonated species ($[M-H]^-$) were selected as precursor ions for all D-S compounds. The predominant product ion observed was the sulfate group, *m/z* 96.9, for each D-S analogue, in agreement with a previous report (21). In addition, the collision-induced dissociation (CID) spectra of the water-soluble forms did not exhibit any other product ion, as the sulfate was the most stable dissociation product. As shown in table 11, compound dependent parameters such as collision energy and fragmentor voltage were optimised to yield the highest mass spectral signal for each compound.

Chromatography was developed to elute all four underivatised sulfates within 10 minutes. Figure 12 shows the overlapped chromatograms of the derivatised and underivatised analogues on the same scale.

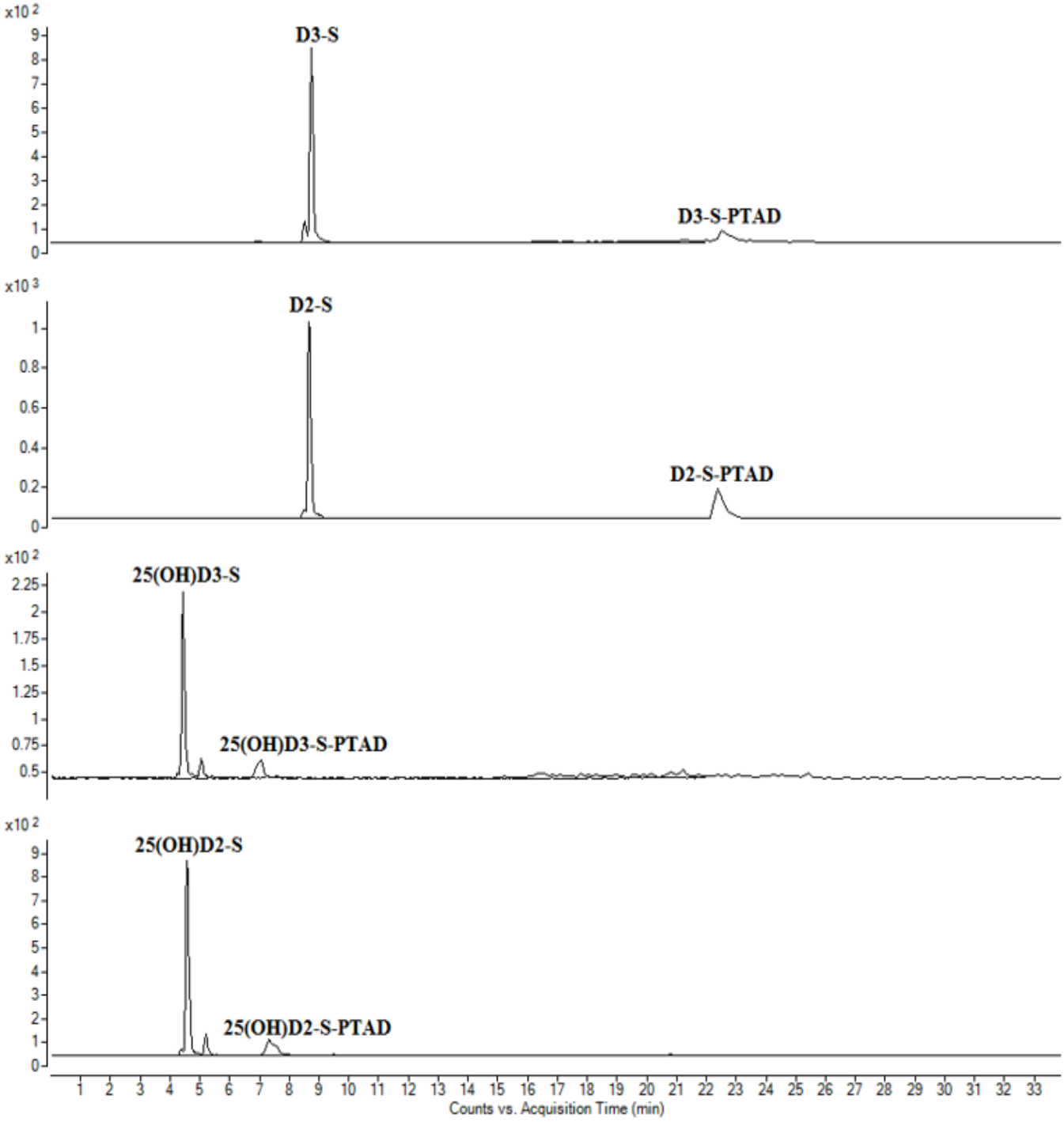

Figure 13. Overlapped chromatographic profiles of the vitamin D sulfates in negative (underivatised compounds) and positive (derivatised with PTAD) ESI modes, as described in the above Material and methods section.

Figure 14 shows the average peak areas obtained for the triplicate analysis with error bars.

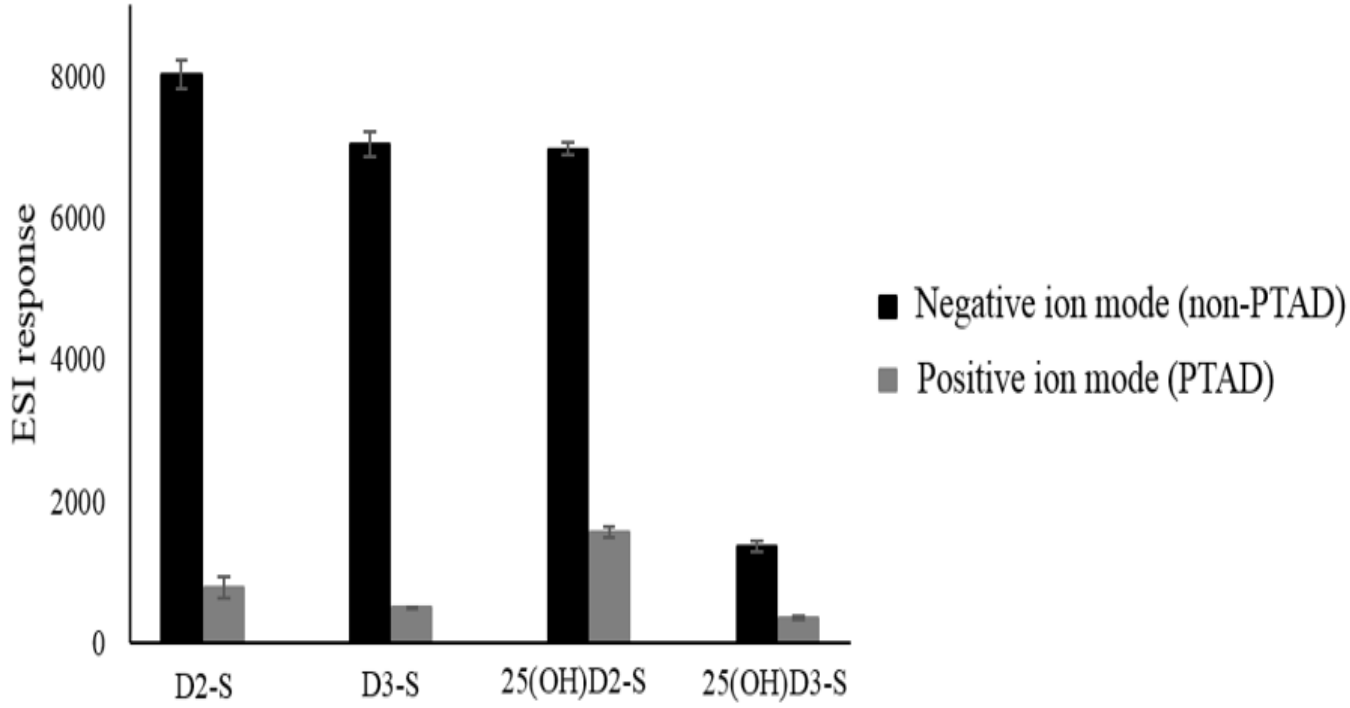

Figure 14. ESI responses (average peak areas) for D-S analogues in negative ion mode (underivatised) and positive ion mode (derivatised with PTAD), as described in the experimental section. Error bars represent ±1SD (n=3).

Table 10 shows the average peak area ratios of underivatised/ derivatised forms. Clearly, a significantly higher (>10 times) ESI response was consistently observed for all underivatised forms, in negative ESI.

Table 10. Peak area ratios obtained for underivatised (negative ion mode) and derivatised (positive ion mode) forms.

Compound	**Ratio**
D_2-S	11
D_3-S	14
25(OH)D_2-S	12
25(OH)D_3-S	10

As mentioned previously, Diels-Alder cycloaddition reaction with PTAD has been widely used to enhance the detection characteristics of fat-soluble forms of vitamin D in API sources (36, 75, 83, 88, 104). Their ionisation efficiencies are increased due to the polar

characteristics of PTAD; thus, positive ion mode is the most effective manner to maximize the ESI response of these compounds (36). Although the introduction of this readily ionisable moiety (PTAD) has rendered improvements in the detectability for the lipid-soluble forms of Vitamin D of about 100-fold, this approach includes time-consuming sample processing steps not necessarily suited for routine clinical and nutritional analysis (36, 88, 171). Despite these limitations, PTAD derivatisation is widely used because the levels of vitamin D in biological fluids are very low and without derivatisation it is not possible to detect many analogues (123).

We initially attempted to develop a method in positive ion mode (derivatised forms) for the simultaneous determination of both fat- and water-soluble forms of vitamin D (150): only 25(OH)D_3-S was detected at very low levels. We then examined the method applicability of both negative (underivatised forms) and positive (derivatised forms) ion modes in human serum analysis (same sample). Whilst it was possible to observe the presence of all four endogenous D-S analogues in negative ion mode, only 25(OH)D_3-S was detected in positive mode (data not shown). The ESI response in positive ion mode for 25(OH)D_3-S was approximately 90% lower than that obtained in negative ion mode. Also, high background noise was observed in the chromatograms obtained in positive ion mode.

Unlike the non-sulfated forms of vitamin D, the sulfated forms are capable of producing a negatively charged ion, by de-protonation, in ESI due to the presence of the ionisable sulfate moiety (21). Since the conjugated sulfates are strong acids, deprotonated molecules would be formed in solution for all D-S forms that were fully ionised in the sample and in mobile phase. As a result, the signal responses of the targeted compounds for underivatised forms were significantly higher than those of the derivatised forms, as illustrated in the Figure 9, indicating that the ionisation efficiency is lower for compounds that still require ionisation by protonation.

Following the above comparison, all further experiments were carried out only in negative mode; the method was simpler (not requiring the lengthy derivatisation step) and faster, yet more sensitive, and therefore well suited for high throughput analysis.

4.4.2 Method validation and application

In this study, co-eluting stable isotope labelled analogues of each compound were used as internal standards to correct for ion suppression/enhancement effects due to co-eluting impurities and to compensate for procedural errors (119). It has been clearly demonstrated that when isotopically labelled standards are used as internal standards, there is no significant

difference in the results when calibration standards are prepared either in mobile phase solvent or in matrix matched medium (119, 139, 150). The samples as well as calibration solutions were therefore prepared in mobile phase (85% B, being the mobile phase composition at the beginning of the gradient). Calibration solutions at six levels were prepared to cover simultaneously the expected concentrations of each form reported in breastmilk and human serum. For the analogues that were not reported, the same ranges as for those that are reported were used. As shown in table 11, the calibration curves were linear (r^2 of >0.99) for each analyte over the concentration ranges used in this study. The on-column LOD and LOQ estimated based on 3x and 10x noise ranged from 0.20 to 0.28 fmol (LOD) and from 0.68 to 0.92 fmol (LOQ), respectively as shown in Table 11.

Table 11. Linearity, and detection and quantitation limits for D-S compounds. LOD and LOQ for milk and serum samples were estimated from on-column values.

Compound	Linearity (r^2)	LOD * (fmol)	LOD ** (pM)	LOD *** (pM)	LOD **** (pM)	LOQ * (fmol)	LOQ ** (pM)	LOQ *** (pM)	LOQ **** (pM)
D_2-S	0.999	0.2	10	1.2	2.0	0.78	34	4.2	6.8
D_3-S	0.999	0.28	14	1.7	2.8	0.92	46	5.8	9.2
25(OH)D_2-S	0.996	0.24	12	1.5	2.4	0.78	39	4.9	7.8
25(OH)D_3-S	0.998	0.24	12	1.5	2.4	0.80	40	5.0	8.0

*On column amounts
** Concentration in extract
*** Concentration in milk
**** Concentration in serum

The LOD and LOQ values were also estimated based on the concentrations of the extracts that were injected to the column (with the volume of injection of 20 µL), and the concentrations in breastmilk and human serum based on 8- and 5-fold concentration in the extracts, respectively, as shown in the same table. Table 12 shows the repeatability values (n= 10) determined by analysing multiple aliquots of the same breastmilk and human serum samples on the same day and expressed as percentage standard deviations.

Table 12. Repeatability and recovery for the targeted compounds in breastmilk and human serum.

Compound	* Repeatability (n=10)		*Recovery (n=6)	** Repeatability (n=10)		**Recovery (n=6)
	Mean ± SD (pM)*	RSD (%)	Mean ± SD (%)	Mean ± SD (pM)**	RSD (%)	Mean ± SD (%)
D_2-S	131 ± 5.1	3.9	81.1 ± 3.21	261 ± 7.3	2.8	84.2 ± 1.64
D_3-S	261 ± 12.8	4.9	86.1 ± 2.81	692 ± 36.2	5.2	85.6 ± 4.01
25(OH)D_2-S	----	----	84.5 ± 4.34	1407 ± 56.4	4.0	95.1 ± 4.57
25(OH)D_3-S	172 ± 13.5	7.9	102 ± 6.25	9493 ± 971	10.2	102 ± 6.78

* Breastmilk
** Human serum

The method was found to be precise for both milk and serum samples with percentage standard deviations, ranging from 2.8 to 10.2%. As shown in table 14, the mean recoveries calculated using the percent ratio of observed/spiked concentrations, ranged from 81.1 to 102% (for both milk and serum samples).

The method was then successfully applied for the determination of four D-S analogues in breastmilk and human serum. Figure 14 illustrates the chromatographic profiles of breastmilk and human serum samples.

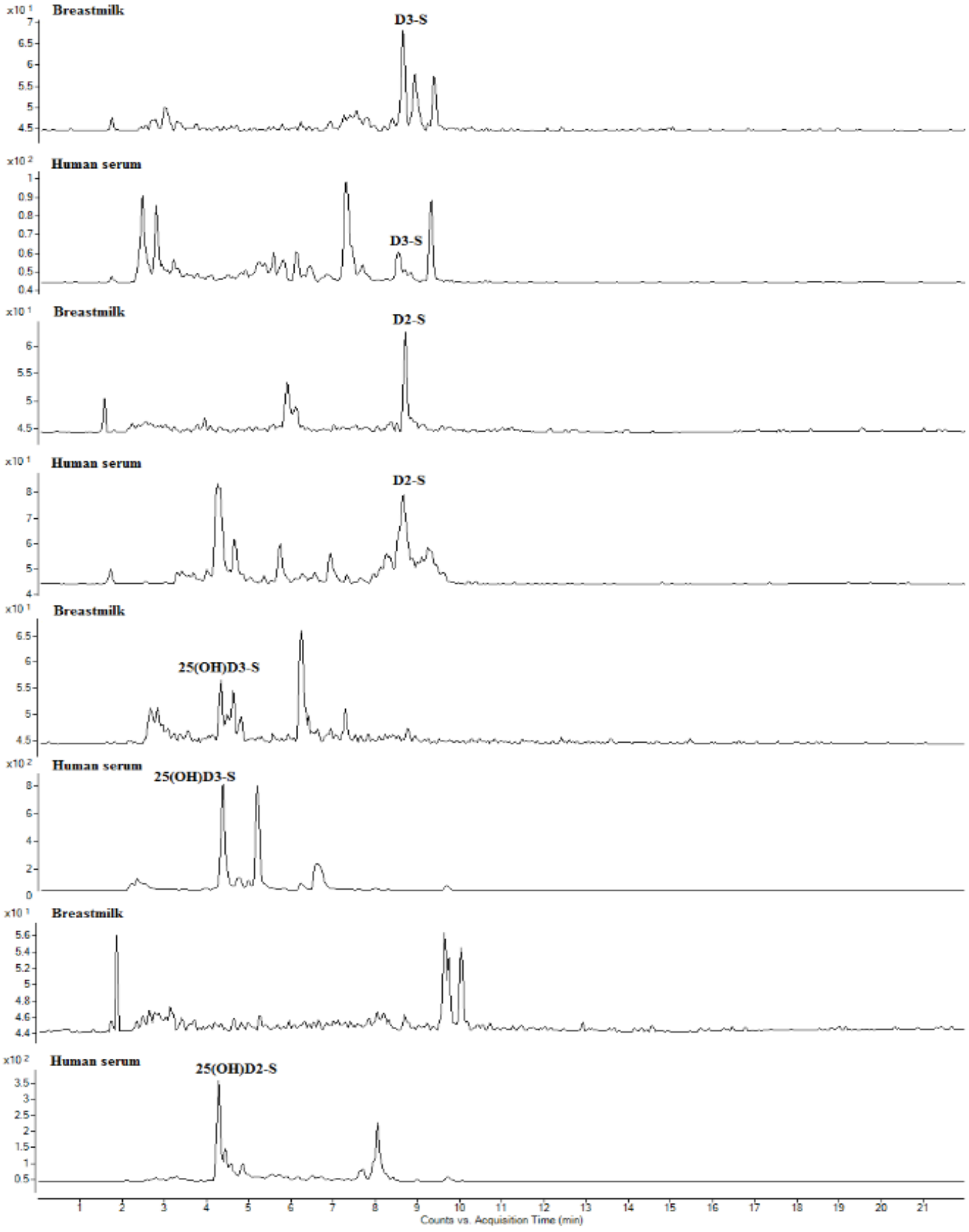

Figure 15. Chromatographic profiles of the D-S analogues (underivatised forms) in breastmilk and in human serum.

As shown in Table 13, the concentrations of all D-S analogues quantified were above their respective LOQ values.

Table 13. Concentrations (± SD) of targeted compounds in milk and serum samples.

Compound (pM)	Breastmilk	Human serum
D_2-S	139 ± 5.42	495 ± 13.9
D_3-S	261 ± 12.8	704 ± 36.6
25(OH)D_2-S	----	1475 ± 59.0
25(OH)D_3-S	87 ± 6.9	10395 ± 106.0

4.5 Conclusions

Despite their poor sensitivities and specificities, colorimetric and LC-UV methods were previously the sole techniques available for the determination of the water-soluble forms of vitamin D in milk. The currently available LC-MS/MS assay methodology is limited to the measurement of only 25(OH)D_3-S in human plasma. The method presented here is a significant improvement from previous analytical methods, and one that will facilitate clinical studies of the four sulfated forms of vitamin D. In negative ion ESI, the sulfated vitamin D analogues showed superior responses when compared to those obtained in positive ion mode (following derivatisation with PTAD), thereby enabling faster sample preparation. Co-eluting, stable isotope labelled internal standards were used for each D-S compound, ensuring high accuracy and precision. The method was validated for both human serum and breastmilk samples, with four major sulfated vitamin D compounds being accurately quantified in serum and three in milk.

5 Chapter 5: Conclusion

The discovery that vitamin D has many biological functions in humans beyond the skeletal systems has reinvigorated interest in the biological roles of this vitamin (4). Vitamin D_3 (D_3) is mainly obtained *in vivo* via the action of ultraviolet radiation on skin while vitamin D_2 (D_2) can be normally only obtained from food (4, 13). Vitamin D and its metabolites differ from each other, in terms of their biological functions; D_2 compounds are less potent in man than D_3 compounds (93, 124). The metabolite 25-hydroxyvitamin D ($25(OH)D_2$ and/or $25(OH)D_3$) is the marker for vitamin D (123). However, $25(OH)D_3$ is the best indicator of the vitamin D status due to its higher half-life and abundance (123, 171). This major circulating form, $25(OH)D_3$, can be epimerised and its measurement, along with 3-epi-25-hydroxyvitamin D_3 (3-epi-$25(OH)D_3$) lead to an erroneous estimation of the vitamin D status (27). Dihydroxy metabolites (1,25-dihydroxyvitamin D ($1,25(OH)_2D_2$ and/or $1,25(OH)_2D_3$)) and 24,25-dihydroxyvitamin D ($24,25(OH)_2D_2$ and/or $24,25(OH)_2D_3$) are biologically active although $1,25(OH)_2D$ is primarily responsible for most of the vitamin D actions (13, 93). Vitamin D can also occur as water-soluble sulfate forms (vitamin D-Sulfate and 25-hydroxyvitamin D-Sulfate) (92). Little is known about this class of vitamin D, as the clinical and nutritional studies have mainly focused previously on the fat-soluble forms (92, 123). Their mechanisms and biological functions still remain to be elucidated although some have suggested that their potencies are equivalent to those of the non-conjugated forms (20). The ability to quantify individual key vitamin D forms in both nutritional and clinical samples is essential: to provide effective information regarding supplementation for at-risk populations; to advance therapeutic strategies for several clinical disorders and, to investigate their physiological roles (43, 93, 171).

Immunoassays have been traditionally used for the quantification of vitamin D compounds in biological fluids; however, these techniques cannot differentiate vitamin D forms from each other and these techniques are well-known for their lack of reproducibility (123, 171). Despite its lack of specificity and sensitivity, liquid chromatography in conjunction with ultraviolet detection (LC-UV) methods have been widely used, especially for vitamin D analysis in fortified milk (93). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is currently the most powerful analytical tool to measure vitamin D compounds in biological fluids, as a result of its high throughput efficiency, high specificity, sensitivity and reproducibility (171). Nevertheless, its applicability to the analysis of vitamin D compounds is limited due to their poor ionisation efficiency in atmospheric pressure ionisation (API) sources and their low levels in biological fluids such as milk (123, 171). Chemical

derivatisation has been used to address this problem and it has been shown to be a valuable strategy to enhance vitamin D compound ionisation efficiencies in API sources; Cookson-type reagents are the most common derivatives for this class of compounds (93, 123, 171). Although there is a wide range of derivatising reagents, 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) is the most popular due to its ease of availability (93). While it has been successfully applied to enhance the detectability of vitamin D compounds in MS/MS detections, the derivatisation reaction with PTAD has not been systematically optimised (93).

Another challenge in vitamin D assays has been the extraction of these compounds from complex matrix. Milk samples are particularly problematic, as a result of many potential interfering compounds, such as sterols, triglycerides, phospholipids and other chemicals that have properties similar to those of vitamin D and its metabolites (32, 93, 123). Vitamin D compounds are tightly bound to proteins and lipids and it is necessary to disrupt this binding to release the vitamin prior to LC-MS/MS analysis in order generate free compound for analysis and to avoid matrix effects (93, 123, 171). Therefore, sample preparation for the analysis of vitamin D in milk has been reported to involve a quite laborious procedure and, even so, it does not necessarily eliminate all interfering compounds (32, 93). Protein precipitation (PP) has been widely used for the extraction (liberation) of vitamin D compounds from plasma/serum while saponification (SN) has been primarily used for their extractions from foodstuffs (123). These extraction procedures have often been used in conjunction with either solid phase extraction (SPE) or liquid-liquid extraction (LLE). This combination has resulted in high throughput methods. SPE is a quite laborious procedure, although capable of automation, and its advantages over LLE in terms of matrix effects are sometimes limited. It is important to note that both adequate sample preparation and chromatography cannot fully eliminate all interfering substances (32, 123). LC-MS/MS methods are particularly affected by such interfering substances. When co-eluting, these interfering substances can either suppress or enhance the mass spectral signal (matrix effects). Isotopically labelled analogues of each analyte are used as internal standards to correct sample losses during sample preparation and to correct matrix effects in MS detection (123, 171).

The isomers, 25(OH)D_3 and 3-epi-25(OH)D_3 cannot be distinguished from each other solely by using MS/MS detection, as a result of their similar mass and fragmentation patterns. Thus, chromatographic separation of this critical pair is required prior to MS/MS detection (93, 123, 171); this is a challenging task, particularly for derivatised species, as result of the presence of an additional peak representing each isomer following the chemical derivatisation

reaction (93). Available LC-MS/MS vitamin D assays in milk are unable to resolve isomers chromatographically and are also not able to quantify the biologically active forms that are found at low levels and/or the sulfate conjugated forms in this matrix.

The primary goal of the studies presented in this thesis was to bring about advances in the field of vitamin D assays for milk samples by developing a highly specific and sensitive LC-MS/MS method. Particular emphasis was placed on sample preparation procedures, LC separation and quantification using MS/MS detection. The selected compounds comprised: D_2, D_3, 25(OH)D_2, 25(OH)D_3, 3-epi-25(OH)D_3, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$ (generally regarded as being the "fat-soluble" forms), vitamin D_2-Sulfate (D_2-S), vitamin D_3-Sulfate (D_3-S), 25-hydroxyvitamin D_2-Sulfate (25(OH)D_2-S) and 25-hydroxyvitamin D_3-Sulfate (25(OH)D_3-S) (the "water-soluble" forms).

The methods presented here represent significant improvements upon existing methodologies. The compounds D_2, D_3, 25(OH)D_2, 25(OH)D_3, 1,25$(OH)_2D_2$, 1,25$(OH)_2D_3$, 24,25$(OH)_2D_2$, 24,25$(OH)_2D_3$ were simultaneously measured in human and animal milk samples, using the method as described in Chapter 2. The metabolites 25(OH)D_3 and 3-epi-25(OH)D_3 were chromatographically resolved; a necessary step to avoid potential overestimation of 25(OH)D_3 status. The ion suppression effects from excess PTAD reagent were minimised, as a result of permitting longer retention times for all of the targeted compounds. The ESI source and MRM conditions were also adjusted for each compound in order to achieve the highest possible sensitivity and specificity and the Diels-Alder cycloaddition reaction with PTAD was thoroughly optimised. PP and SN methods were compared in milk samples and PP was found to be faster and simpler; it was also found superior in terms of extraction efficiency. PP in conjunction with LLE was further optimised. The accuracy of the method was ensured by using isotopically labelled internal standards for each targeted compound. A combination of the above mentioned optimisations, enhancement of the MS parameters and the use of appropriate chromatography resulted in a very sensitive, selective, and accurate method for the determination of the targeted compounds at low levels. This method was successfully applied for the quantification of vitamin D compounds in milk and it is anticipated that this method can easily be adapted for other complex matrices such as human serum. This method was then applied to investigate the effect of pasteurisation on the content of the major vitamin D forms (D_2, D_3, 25(OH)D_2 and 25(OH)D_3) in breastmilk, as described in the chapter 3. In this study, it was confirmed that the vitamin D content in milk is significantly low and it does not satisfy the vitamin D's requirements for either term or preterm infants. Pasteurisation was demonstrated to be a potential source of vitamin D

degradation since losses were observed after this thermal procedure. Pasteurisation is a common procedure adopted by breastmilk banks to ensure its safety for infant consumption. Therefore, the findings reported here will assist health care professionals in providing appropriate vitamin D supplementation for infants where necessary. Such findings may also prove useful for directing improvements in milk pasteurisation procedures, which may reduce losses of vitamin D content in donor breastmilk samples.

This LC-MS/MS method was initially developed for the analysis of both non-conjugated and the sulfated forms. However, the method as described in chapter 2 proved inadequate to determine the sulfated forms, hence, it was necessary to develop a new strategy to determine D_2-S, D_3-S, 25(OH)D_2-S and 25(OH)D_3-S in biological samples. These sulfate conjugate compounds (without derivatisation), when quantified in negative ion ESI, gave a superior MS signal response when compared to the PTAD derivatised forms of the same compounds using positive ion mode. This observation resulted in a significant simplification of the sample preparation procedure, as the derivatisation step proved to be unnecessary. Since the difference between conjugated and non-conjugated forms is the ionisation efficiency, the same stationary phase (C_{18}) was used for all targeted compounds. However, formic acid (at pH 3) was used to facilitate the protonation for derivatised forms in positive mode and ammonium formate (at pH 7) was used to facilitate the de-protonation for non-derivatised forms in negative mode. The method was developed and validated to quantify the four major sulfated forms and is described in Chapter 4. The applicability of the method was demonstrated in samples of both human serum and breastmilk.

Overall, the studies reported in this thesis have resulted in the development of highly specific and sensitive LC-MS/MS methods for the successful determination of twelve key vitamin D compounds in milk. These methods will therefore enable researchers to study more precisely the activities and functions of each vitamin D analogue and the total amount (and activity) of vitamin D in milk and other biological fluids which is of key importance to investigate links between vitamin D and clinical disorders.

As mentioned previously, LC-MS/MS has become increasingly important for the analysis vitamin D and its metabolites in biological fluids. While there is a continuing trend towards the development of new derivatising reagents, a parallel future study is required to improve API sources. It will allow the measurement of dihydroxy-vitamin D metabolites at low levels in biological fluids; eliminating the use of derivatisation reactions and then simplifying sample preparation procedures. Further research work is additionally required to advance the automation of sample preparation procedures, in conjunction with the development of more

specific LC methods, in order to fulfil the demand for high-throughput analysis techniques, and to reduce manual handling errors and biological hazards as well as avoiding the co-elution of vitamin D isomers. The trend towards more rigorous clean-up procedures is necessary in order to achieve high selectivity and a precise quantification of the dihydroxy-vitamin D metabolites at picomolar levels. Further studies are also necessary to reduce sample and extraction solvent volumes. Such an approach will certainly assist in minimising the impact of chemical residues in the environment and reduce associated costs.

Human milk banks routinely use pasteurisation and it is a necessary step to ensure its safety for consumption. Further studies are necessary to evaluate whether vitamin D losses could be also associated with compound adsorption and/or fat adsorption to the pasteurisation container. Thus, the findings obtained in this thesis will be useful to assist such future work.

There are many studies regarding the physiological roles of sulfated forms of vitamin D; but their roles remain controversial. Further research work is required to better understand this class of vitamin D compounds. It would therefore be of clinical interest to investigate whether 25(OH)D_3-S could be epimerised, as it could be a potential vitamin D marker. Also, it would be of greatest interest to investigate whether there are dihydroxy-vitamin D compounds present as the respective sulfate conjugate. The method as presented in this thesis is specific for sulfated forms and hence it will be useful for such investigations on the biological function of these conjugates.

6 Bibliography

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