Paediatric food allergy and the role of vitamin D

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

I acknowledge the support I have received for my research through the provision of an Australian Government Research Training Program Scholarship.

Rosita Zakariaee Abkoo

25/02/2019
Dedication

My humble effort I dedicate to

my beloved mother, father and husband

whose affection, love, encouragement and support

make me able to achieve such success and honour.
Acknowledgements

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<td>1α,25-(OH)2D</td>
<td>1α,25-Dihydroxyvitamin D</td>
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<td>Amplifex</td>
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<td>APTDCI</td>
<td>atmospheric pressure thermal desorption chemical ionisation</td>
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<td>CI</td>
<td>confidence intervals</td>
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<td>Certified Reference Materials</td>
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<td>CV</td>
<td>Coefficient of variation</td>
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<td>Day</td>
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<td>DART</td>
<td>Direct Analysis in Real Time</td>
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<td>Desorption Electrospray Ionisation</td>
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<td>EBF</td>
<td>European Bio-analysis Forum</td>
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<td>Epi-25-OHD3</td>
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<td>EQA</td>
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<td>ERNDIM</td>
<td>European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism</td>
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<td>ETDA</td>
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<td>FT-ICR</td>
<td>Fourier Transform Ion Cyclotron Resonance</td>
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<td>HILIC</td>
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<td>ID</td>
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<td>IDES</td>
<td>Isotope-Dilution Electrospray</td>
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<td>IQC</td>
<td>Internal Quality Control</td>
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<td>ISTD</td>
<td>Internal Standard</td>
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<tr>
<td>LC</td>
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<td>LLE</td>
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<td>LLOD</td>
<td>Low Limit of Detection</td>
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<td>LLOQ</td>
<td>Lower Limit of Quantitation</td>
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<tr>
<td>LMJ-SSP</td>
<td>Liquid Micro-junction Surface Sampling Probes</td>
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<td>MCRI</td>
<td>Murdoch Children's Research Institute</td>
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<td>MetV</td>
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<td>MI</td>
<td>Metabolic Intermediate</td>
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<td>MP</td>
<td>Mobile Phase</td>
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<td>Description</td>
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<tr>
<td>MRM</td>
<td>Multiple Reactions Monitoring</td>
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<td>NBS</td>
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<td>NIST</td>
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<tr>
<td>nm</td>
<td>Nano Meter</td>
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<td>NMIA</td>
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<tr>
<td>NR</td>
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<td>NSQAP</td>
<td>Newborn Screening Quality Assurance Program</td>
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<td>New South Wales</td>
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<tr>
<td>OFC</td>
<td>Oral Food Challenges</td>
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<td>PD</td>
<td>Pharmaceutical Drugs</td>
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<tr>
<td>PFA</td>
<td>Paediatric food allergy</td>
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<tr>
<td>PFP</td>
<td>Pentafluorophenyl</td>
</tr>
<tr>
<td>PK</td>
<td>Pharmacokinetics</td>
</tr>
<tr>
<td>PKU</td>
<td>Phenylketonuria</td>
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<td>PPT</td>
<td>Protein Precipitation</td>
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<tr>
<td>PSI</td>
<td>Pound per Square Inch</td>
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<td>PS-MS</td>
<td>Paper Spray Mass Spectrometry</td>
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<td>PTAD</td>
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<td>r</td>
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<td>Ref</td>
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<td>RepU</td>
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<td>School of Health and Biomedical Sciences</td>
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<td>SRM / MRM</td>
<td>Selective / Multiple Reaction Monitoring</td>
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<td>St</td>
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<td>TDM</td>
<td>Therapeutic Drug Monitoring</td>
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<td>TEa</td>
<td>Total Allowable Error</td>
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<td>TK</td>
<td>Toxicokinetic</td>
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<td>The United Kingdom National External Quality Assessment Service</td>
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<td>UNICEF</td>
<td>United Nations International Children's Emergency Fund</td>
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<tr>
<td>USA</td>
<td>United States of America</td>
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<tr>
<td>UVR</td>
<td>Ultra Violet Radiation</td>
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<td>VAD</td>
<td>Vitamin A Deficiency</td>
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<td>VCGS</td>
<td>Victorian Clinical Genetics Service</td>
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<tr>
<td>VDBP</td>
<td>Vitamin D Binding Protein</td>
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Publications from this work


Conference abstracts


3. Chen Lim, Rosita Zakaria, Nick Crinis, Yvonne Reidley, Que Lam. 1,25 Dihydroxyvitamin D Status of Patients with 25 Dihydroxyvitamin D Deficiency. Poster presented in AACB-AIMS


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Research project summary

Investigation of vitamin D metabolism, its biological role and the relevant quantitative methods of assessment has exponentially increased over the past two decades. Vitamin D plays a significant role in multiple physiological functions in humans. Also, there is some evidence linking vitamin D inadequacy with numerous pathologies in both children and adults. However, research into the role of vitamin D is hampered by lack of large-scale, prospective studies.

Emerging evidence of the association between early life vitamin D deficiency and increased risk of childhood pathologies such as food allergy, eczema and infections, suggests that vitamin D likely plays a role in early development of infants’ immune system and health. In Victoria, the prevalence of both vitamin D insufficiency and challenge-proven food allergy in infants is high and the two could be linked. However, it remains unclear whether low vitamin D levels play a causal role in the development of food allergy, and if so, whether there is a “window of opportunity” during which adequate vitamin D levels are most important for immune development and immune responses to food. Potentially, vitamin D status at birth has implications on the prevalence of food allergy and other pathologies. We have therefore aimed to determine the frequency of vitamin D inadequacy in newborns from Melbourne, Victoria and investigate the relationship between vitamin D level at birth and food allergy in infants.

Vitamin D conventionally is measured as 25-hydroxy vitamin D3 in serum / plasma and the interpretation cut-offs for adults are well-defined. However, for large-scale population studies sample handling procedures including; collection, transport and storage are cumbersome for serum/plasma specimens. Accordingly, the interest in the use of dried blood spots (DBS) as an alternative matrix for vitamin D assessment has increased. Furthermore, there is a particular interest in newborn blood spot screening (NBS) samples as they collect information on various biomarkers including vitamin D at birth. This timing is important as it is prior to the onset of disease, and also because some of the immune deviations that lead to diseases including food allergy are already
thought to be present at birth or shortly afterwards. However, this is limited by the lack of an accurate method for measuring vitamin D levels from DBS/NBS samples.

Analysis of DBS vitamin D has been previously demonstrated. However, there are some analytical and post analytical challenges to be considered such as; traceability, sensitivity and assay robustness. Therefore, to advance the literature regarding measurement of vitamin D we aimed to develop a sensitive and robust local DBS vitamin D method, that is traceable to serum for use in population-based studies.

Following the aims of the project, we collected 2700 archived NBS Guthrie cards from the Victorian Clinical Genetics Services at the Murdoch Children's Research Institute for measurement of 25-hydroxy vitamin D3 (25-OHD3). We examined the association between season of birth, parents’ ethnicity and supplementation intake and vitamin D levels at birth, as well as the association between vitamin D levels and parent-reported eczema and challenge proven food allergy at one year of age, using data from the HealthNuts study. HealthNuts is a long-term population-based study designed to assess different aspects of paediatric food allergy in Victoria, Managed by Murdoch Childrens Research Institute.

To alleviate the potential errors associated with the dried matrix of DBS sample which could span the total testing process, we developed a simple method to estimate haematocrit in archived DBS samples. A formic acid solution was used to elute the haemoglobin content of 3.2 mm spotted blood disk from its dry matrix. A direct spectrometry method was utilised to obtain the spectrum of the extracted haemoglobin in the visible range and the linear relationship between individuals’ haematocrit percentage and haemoglobin concentration applied to estimate the haematocrit level of the blood spot. This method potentially can be incorporated into various DBS analytical sample preparation process for correction of measured analytes of interest.

To evaluate the vitamin D status in NBS samples we then developed a method to quantitate vitamin D from DBS. 25-hydroxy vitamin D3 was eluted and extracted from one 3.2 mm DBS disk using a water-methanol mixture (3:5) followed by a supported liquid extraction and derivatisation
procedures prior to LC-MS/MS analysis. The extracted and derivatised DBS samples were injected onto a LC-MS/MS system (reversed phase Kinetex F5 analytical column, methanol/water gradient) which was operated in positive ESI multiple reactions monitoring mode. The assay has a linear range of 0.2 to 353 nmol/L, with a between run imprecision, assessed from the in-house DBS control samples of 12.9% at 45 nmol/L. NIST-972a traceable commercial standard material was used to calibrate vitamin D measures.

The developed method has been utilised to analyse archived NBS samples to determine the vitamin D status in a Melbourne neonatal population. This was the largest longitudinal population study determining the distribution of vitamin D at birth in Melbournian infants, and has examined whether vitamin D status at birth predicts the risk of food allergy and eczema in early childhood. We found that the prevalence of vitamin D inadequacy is very high among the studied cohort (born between September 2007 and August 2011), comparing the DBS measures with the Institute of Medicine adult values and our defined quintiles for 25-OHD3 measures. However, there was no evidence of an association between the NBS vitamin D levels and challenge proven food allergy (to sesame, peanuts and egg) or eczema in this large cohort of 2615 participants. As would be predicted, season of birth and maternal vitamin D supplementation were associated with DBS vitamin D levels. While no other significant predictor (such as; gender, parents’ region of birth and gestational age) of vitamin D level in DBS samples was observed.

This study confirmed a significant rate of vitamin D inadequacy among infants born in Victoria. Further prospective studies are required to determine if age-adjusted decision limits for infants (different from suggested cut-offs for adults) are required. Current evidence supports the feasibility of safe sun exposure and season-focused supplementation during pregnancy and early infancy, but further studies are required to define recommendations for optimal dosage and timing of supplementation. In conclusion, the outcomes of this PhD project including development of method and analysis of newborn vitamin D levels, did not provide any evidence of association between vitamin D levels at birth and prevalence of food allergy in early childhood.
Chapter 1

Literature review
1. Literature review

1.1. Introduction

Investigation of vitamin D metabolism, its biological role and the relevant quantitative methods of assessment has exponentially increased over the past two decades. Vitamin D plays a significant role in multiple physiological functions in human including; general well-being, immune system development, bone health as well as normal cell growth and maturation (1). However, vitamin D deficiency for children and pregnant women is still a critical global health challenge. Reported rates of vitamin D insufficiency (VDI) (25-hydroxy vitamin D level below 50 nmol/L) in some populations of Victorian children is up to 54.9% (2). This population group might be at substantial risk of bone related diseases and other chronic illnesses or disabilities (3). Also, relatively high prevalence (80%) of vitamin D deficiency (25-hydroxy vitamin D below 22.5 nmol/L) is reported in the metropolitan Melbourne population study among the dark skinned or veil-wearing pregnant women (4).

Emerging evidence of the association between early life vitamin D deficiency and increased risk of childhood pathologies such as food allergy, eczema and infections, suggests that vitamin D likely plays a role in early development of infants’ immune system and health (5). Furthermore, evidence-based studies show that, the rate of food allergy in developing countries is increasing (6-8). Hypothetically, the significant increased incidence of vitamin D inadequacy in pregnant women and infants could be considered as an underlying cause of allergic disorders in children including paediatric food allergy and eczema (9).

Measurement of vitamin D metabolites remains challenging due to their low circulatory concentration, binding proteins and the structural similarity of their analogues. This chapter aims to provide an insight into the complexity of vitamin D measurement and investigates alternative analytical methods which could be applicable to paediatric studies. In addition, this review summarises the general biochemical specification, metabolism and pathophysiology of vitamin D, with the special attention to its role in paediatric food allergy. This chapter also incorporates the findings of two published review papers, which discuss the details of clinical and technical aspects of the project background (9, 10).
1.1.1 Chemical structure

Vitamin D is chemically a group of seco-steroids which possess an open cyclo-pentano-perhydrophenanthrene ring (ring B on carbon 9, 10). These seco-steroids are structurally comparable to the steroid hormone group, and the biologically active vitamin D metabolite; 1,25-dihydroxy vitamin D (1α,25-(OH)2D) (which appears to be the only active form of vitamin D) that exhibits hormone-like behaviour (11). Besides its seco-steroid form, the presence of a side chain provides a more flexible molecular structure compared to the classic steroids (12) (Figure 1.1). 25-hydroxy vitamin D (25-OHD) is the main circulatory analogue of vitamin D with the longest half-life in adults; while its epimer (3-epi-25-OHD) (which appears to be metabolised into a physiologically inactive metabolite) is present in significant levels in paediatric group (13).

![Figure 1.1. Molecular structure of vitamin D3 metabolites; vitamin D metabolites are the group of seco-steroids with the similar molecular structure. 1,25-(OH)2D3 is the most active of the analogues which presents with hormone like biological activity.](Image)

1.1.2 Biochemical pathways and metabolism

There are two distinct metabolic pathways for production and metabolism of vitamin D; 1) vitamin D2 (Ergocalciferol), which is sourced from plants and 2) vitamin D3 (Cholecalciferol), that forms naturally in the skin in response to sun exposure (ultra-violet radiation) from its primary precursor cholesterol (11). Ultra-
violet rays initiate a series of photochemical reactions to convert this seco-steroid to pre-vitamin D3 in the malpighian layer of skin. Pre-vitamin D3 can isomerise to vitamin D3 within 2-3 days. To become biologically active, vitamin D must be double-hydroxylated at position 1 and 25 (Figure 1.2).

Vitamin D metabolites possess a high affinity to the plasma vitamin D binding protein (VDBP). VDBP carries cholecalciferol to the liver; where the 25-hydroxylase enzyme accelerates formation of 25-OHD3. VDBP circulatory level is in a high molar excess compared to the vitamin D metabolites concentration. As such, only very limited portion of metabolites circulate freely (14). The complex of VDBP-25-OHD3 then travels to the kidney for a further hydroxylation step. 1α-hydroxylase enzyme catalyses 25-OHD secondary hydroxylation to produce; 1) the active hormone-like 1α,25-(OH)2D3 (Calcitriol) and 2) the presumably inactive 24,25-(OH)2D3 metabolites (15) (Figure 1.2).

The enzymes responsible for biological activation of vitamin D3 are cytochrome P-450 family members. Hepatic vitamin D 25-hydroxylases (CYP2R1, CYP2D11 and CYP2D25) induce the formation of 25-OHD. In the kidney cytochrome P-450 mono-oxygenase 25(OH)-D 1-alpha hydroxylase (CYP27B1; 1α-(OH) ase) enzymes facilitate the production of the active form of vitamin D; 1α,25-(OH)2D. This CYP27B1 is also expressed in other organs and cells including; placenta during pregnancy, monocytes-macrophages, endothelial, colon, breast, prostate and pancreatic cells. These are consequently known as extra-renal production sites for 1α,25(OH)2D (16).

24,25(OH)2D could be synthesised in kidney by the 24-hydroxylase enzyme (CYP24A1), which compared to 1α,25-(OH)2D is considered a relatively inactive metabolite (17). Hence, vitamin D metabolic degradation and deactivation is recognised as the central function of CYP24A1. Also, CYP24A1 largely is found in all vitamin D target tissues such as; stomach isthmus cells, entero-endocrine cells, pyloric muscle cells, teeth pulp cells, thymus reticular cells, epithelium, neuron cells, adrenal medullary cells, kidney macula densa cells, podocytes, liver Ito cells, female and male reproductive organs, and atrial cardio-myocytes (15, 18). Finally, CYP24A1 mediates a set of reactions resulting in production of excretory forms; calcitroic acid and 1-desoxycalcitroic acid; from the different metabolites of vitamin D (Figure 1.2). These final products of vitamin D metabolic
pathway are predominantly excreted through the bile into the faeces, whereas are only rarely expelled through the urine (19, 20) (Figure 1.2).

Plants and invertebrates synthesize vitamin D2 from ergosterol (21). Chemically, vitamin D2 and D3 are quite similar, and the side chain is the only notable difference between them (Figure 1). This difference is alleged to alter the vitamins’ corresponding manner of binding to VDBP and the metabolic pathway (22). Furthermore, it is understood that the efficacy of vitamin D3 in raising plasma concentration of 25-OHD is significantly higher than vitamin D2. As such, specifically vitamin D3 supplementation is recommended for treatment of vitamin D inadequacy (23).

1.1.3 Regulation

Regulation of 1α,25-(OH)2D is controlled by the complex positive and negative feedback systems, which affect the expression of the hydroxylase enzymes (24). Low circulatory calcium concentration increases 1-α-hydroxylase gene activation and transcription. In turn, elevated parathyroid hormone (PTH) suppresses the gene transcription (25, 26). Besides, the regulation of 24-hydroxylase is controlled through 1α,25-(OH)2D status, plasma calcium concentration and PTH level (25).

Due to highly lipophilic molecular structure of vitamin D metabolites, their circulatory forms are mainly bound to VDBP (27). This binding protein is known to facilitate the dispersal of deactivated and excretory forms of vitamin D to cells which can further deactivate and excrete them (28). Additionally, VDBP-bound sterols are important reservoirs for normal physiological activities and also during limited production / intake circumstances. However, it has been argued that, whereas binding protein accessibility in plasma is an important factor to maintain total circulating levels of 1,25(OH)2D3, VDBP concentration does not affect calcitriol’s biologically active pool (29).

Other factors which can affect metabolism of vitamin D include fibroblast growth factor-23 (FGF-23) which is a phosphaturic factor for renal phosphate excretion, and a known physiological regulator for vitamin D biosynthesis and metabolism. Primarily, production of FGF-23 in bone is stimulated by 1α,25-(OH)2D. In turn, expression of 1α-hydroxylase is suppressed by elevated levels of FGF-23. Furthermore, increased FGF-
23 induces the 24-hydroxylase activity in renal cells (30). Also, it is reported that, production of 1α,25-(OH)2D is stimulated by oestrogen in combination with progesterone or androgen. Also, it is suggested that, oestrogen alone may suppress the synthesis of 24,25-(OH)2D (31).

Within 15-30 minutes of full body skin sun exposure, 10,000 to 20,000 IU of vitamin D3 will be produced. After two hours sun exposure the body will have reached the maximum capacity of vitamin D3 IU intake. The excess amount of pre-vitamin D converts to other inert metabolites such as; Lumisterol and Tachysterol, which are degraded into other inactive metabolites (e.g. Suprasterol). Thus, it is believed that prolonged skin sun-exposure cannot induce vitamin D toxicity (32).
Figure 1.2. Vitamin D metabolism; 7-Dihydroxycholesterol is the precursor for the endogenous synthesis of vitamin D in the skin tissue. The activation and metabolism of vitamin D is mediated by a group of cytochromes P-450 enzymes in the liver, kidney and other target tissues. It appears likely that this group of enzymes play a significant regulatory role for vitamin D function in various organs. Calcitriol (1,25(OH)2-vitamin D3) is known as the most active form of vitamin D with the hormone-like behaviour.
1.2. Are vitamins A and D important in the development of food allergy?

This section has been peer reviewed and published with contribution of other co-authors as review paper (9). My contribution to this study and subsequent publication was included; developing the concept for the study in conjunction with my senior supervisor (RG), performing the literature search / study, collecting data, analysis and interpretation of data, writing the first draft of the publication and reviewing and incorporating all suggestions from the co-authors.

1.2.1 Introduction

Food allergy has a dramatic impact on a child and family’s quality of life (33) and places a major financial burden on the community (34). Of further concern, hospitalizations for food allergy-related anaphylaxis, the most severe expression of a food allergic reaction and a life-threatening condition, have dramatically increased since the early 1990s. Recent studies demonstrate that 8-10% of one-year old infants living in Melbourne Australia have challenge-proven IgE-mediated food allergy (6). This is the highest reported prevalence of challenge-proven food allergy in the world (Figure 1.3) (35). The cause of this epidemic is unknown and identifying contemporary environmental factors that may underlie the increase in food allergy is actively being investigated.

The increase in food allergy may relate to the concordant rise in prevalence of vitamin D insufficiency (VDI) (8). Previous studies have shown that greater than one third (39%) of Australian women of child bearing age has VDI (36). The high rate of VDI may relate to a number of factors including reduced sun exposure, low dietary intake of foods that are naturally rich in vitamin D, as well that lack of routine vitamin D supplementation in this country – unlike in the US and Europe. A number of recent findings suggest an association between early life VDI and food allergy. This is an important hypothesis with clear public health implications. The initial findings of a latitude gradient in the prescription of adrenaline auto-pens for the treatment of food allergy anaphylaxis (higher rates further from the equator) have recently been extended by a large cross-sectional study which found a clear link between low vitamin D status (which was directly measured) and proven food allergy in young infants of Australian born parents (37).
Vitamin A has an important role in immune function (38). The health impact of vitamin A deficiency in developing countries has been recognised for some time; this includes vitamin A’s association with increased risk of morbidity in malnourished infants whom die due to impaired immune system associated by vitamin A deficiency (39). Hence, vitamin A supplementation in association with vaccination programs have been established to combat deficiency in preschool children in these countries (40). Recent evidence suggests that neonatal supplementation of vitamin A is associated with an increase in atopic sensitization and atopic disease (41). In addition, it has also been proposed that retinoic acid (one of the three forms of vitamin A) may influence the acquisition of immune intolerance (42-44). Indeed vitamin A appears to play a key role in enabling humans to consume a wide range foods without stimulating an adverse immune reaction (38).

Figure 1.3. Variation in the documented global prevalence of food allergy in children ≤5 years of age. This map represents information from 14* countries globally and is derived from a mixture of sources which includes parent reports, symptoms and sensitization and oral food challenges (OFC). This data is adapted from information provided through the World Allergy Organization and information provided in reference no. 4. The map was developed using online software from http://www.aneki.com/map.php.

The prevalence, based on percentage reports in the population:

- **OFC High prevalence (>8%)** – i.e. Australia
- **OFC Medium prevalence (4 – 8%)** – i.e. China, Norway, United Kingdom
- **OFC Low prevalence (< 4%)** – i.e. Denmark, Iceland, Thailand
- **Non OFC proven food allergy**, Korea, Sweden, Taiwan* have data based on sensitisation studies. The documented incidence is from 3.2 to 11%.
- Non OFC proven food allergy, Canada, Finland, France, Hong Kong*, Japan and the USA have data based on parent reports. The documented incidence range is from 4.1 to 9.2%.
- **Information not available or not provided**

*Note: the software used to generate this map does not distinguish Taiwan and Hong Kong from mainland China.
Together, these hypotheses have prompted further investigation into the circulating levels of vitamins A and D in relation to food allergy prevalence. Whilst there is no clear evidence examining an interaction between vitamin A and D in association with food allergies, it is expedient to be able to measure these vitamins together. Ideally, the analytical methods utilised for the measurement of these vitamins are both precise and accurate. We also desire methods that are sensitive and specific. The higher order methods which are reported to achieve these desired method characteristics are based on chromatographic techniques for both vitamins.

The chromatographic method group, whilst of a higher order, is technically more challenging and has been shown in recent years to not always be as robust as hoped (45, 46). An alternative method group, immunoassay, is also widely utilised for vitamin D analysis and is considered by many to be an acceptable alternative (47). However, often a limitation of both groups (chromatography and immunoassay) remains their inability to distinguish the various forms or precursors of vitamin D and A respectively (48).

This review aims to examine the relationship between vitamin A and vitamin D and food allergy, and includes a discussion of the measurement techniques, to provide the precise and accurate data required to assess vitamin status in paediatric populations.

1.2.2 What is the link between vitamin A & D and food allergy?

Generally, food allergy is defined as a maladaptive immune response to an inoffensive food related antigen (49). Available epidemiological data propose a dramatic increase in food allergy and related anaphylaxis during the last two decades (50). There are several studies available which provide indirect and direct evidence for vitamin D and suggestive evidence for vitamin A levels in relation to food allergy prevalence.

Vitamin D: The Indirect Evidence

There is some evidence that variation in food allergy prevalence within countries might be linked with ambient ultra violet radiation (UVR) exposure and thus potentially with vitamin D levels. Although few studies internationally have measured food allergy prevalence in population-based samples using the gold standard of oral food challenges, several have assessed the correlation between proxy markers of food allergy status and
latitude. Four studies have been published which report that areas further from the equator with lower ambient UVR levels may have more food allergy (49, 51-53). In Australia, parent-reported egg and peanut allergy are more common in southern States i.e. further from the equator, which have less year round sunlight (54). Also in Australia, prescriptions of hypoallergenic formulas which are used for the treatment of food allergy are more common in southern States, as are prescriptions for epinephrine auto-injectors for the treatment of anaphylaxis (52). Similar trends in prescription of auto-injectors have been described in the US, with these being more common in areas further from the equator (55).

Comparisons between countries with varying ambient UVR levels are theoretically possible. In practice, such studies are hampered by the lack of standardized protocols for measuring food allergy. This limits the ability to make accurate comparisons between studies. The EuroPrevall studies which does use standardized protocols to measure food allergy across multiple countries may provide further insight into potential links between UVR and food allergy prevalence (56). However, even these comparisons are complicated by differences between countries in sun exposure behaviour, such as the use of sunscreen and time spent outside. Such comparisons are further complicated by the varying recommendations for maternal and infant vitamin D supplementation and food chain fortification with vitamin D. In addition to a potential link between UVR exposure on a population level and food allergy, there is some indirect evidence to support a link between individual UVR exposure and food allergy.

Food allergy is widely believed to have increased over recent decades and the available evidence, although scarce and mixed, seems to overall support an increase. Examination of changing vitamin D exposure and food allergy prevalence at the population level over time may support a causal link between vitamin D and food allergy, although the evidence is not clear. The indirect evidence for and against is:

1) **Supporting evidence.** Two studies have shown that patients with food allergy were more likely to have been born in autumn/winter when ambient UVR levels are lower and vitamin D stores may therefore be lower (51, 52). In addition, there has been a contemporaneous decrease in vitamin D levels in many parts of the world due to changes in behaviour leading to less sun exposure over time (57).
2) **Contrary evidence.** On the other hand, time trends over a longer period may not support a link between vitamin D and food allergy (58). To this effect, Wjst argue that in the 1900s when low vitamin D levels were common in England, seen in high rates of rickets, allergic disease in general and presumably food allergy also appears to have been uncommon (59). This observation, whilst important, is of course complicated by a wide range of other environmental factors that have changed over the last century. It has been argued, for instance, that low vitamin D status may only be a risk factor for food allergy in the presence of a ‘Westernised’ gut microbiota (60).

**Vitamin D: The Direct Evidence**

Only a few studies to date have examined the relationship between measured serum vitamin D levels and either food sensitization or allergy. These studies are:

1) In the US-based National Health and Nutrition Examination Survey (NHANES 2003-2006), children with 25-hydroxy vitamin D (25-OHD) <37.5 nmol/L of blood were more likely (odds ratio 2.39; 95% CI, 1.29–4.45) to have allergic sensitization to peanuts than children with 25OHD levels >75 nmol/L (61).

2) A separate study found that in children with four specific genotypes, low vitamin D at birth (cord blood 25-OHD level <27.5 nmol/L i.e. <11 ng/mL) was associated with food sensitization, while this association was not present in children with other genotypes (62). This finding of a gene-vitamin D interaction provides some evidence to support a causal relationship between vitamin D levels and food sensitization.

3) In a recent Australian population-based cohort study of one year old infants, those with vitamin D insufficiency were three times more likely to be egg allergic and eleven times more likely to be peanut allergic compared to those with sufficient serum vitamin D levels (37). Among sensitized infants, those with VDI were six times more likely to be food allergic. This association was only present among infants of Australian-born parents, suggesting a possible gene-environment interaction.
4) Some studies have also suggested that high vitamin D levels or oral supplementation with vitamin D may be associated with an increased risk of allergic disease in general, although these have generally not examined food allergy specifically (57).

5) One recent small study found that higher maternal and cord blood vitamin D levels were associated with a higher risk for food sensitization and allergy in the first two years of life (63). This study, however, was limited by significant loss to follow-up.

These apparently conflicting findings might be explained by a differential immune effect of oral vitamin D supplementation compared with vitamin D derived from UVR exposure. Alternatively, there is some evidence to support a ‘u-shaped association’ whereby both high and low serum vitamin D levels may increase the risk of allergic disease. The timing of adequate vitamin D exposure may also be important, and longitudinal studies investigating the effect of serum vitamin D levels at different time periods from birth through the first years of life are still required to investigate this.

**Vitamin A: Is it worth studying as a potential risk factor?**

To date no study has looked at the role of vitamin A in the development of food allergy, although there is reasonable evidence that it plays a role in immune development. Therefore, the investigation of the role of vitamin A alongside vitamin D in the risk of food allergy development is a worthwhile line of investigation – especially since many foods such as dairy, fish and eggs contain reasonable quantities of both of these fat-soluble vitamins. The role of vitamin A in immune health is reviewed below.

1) **Role in the gut.** Vitamin A plays a vital role in epithelial cell differentiation and immune system development (64, 65) and has a well-established role in gut mucosal immune system development (38). There is emerging evidence that vitamin A may also play a role in immune tolerance and tolerance to food proteins through imprinting of gut-homing specificity on T cells (66). Since the body mucosal surface is the front-line barrier exposed to food allergens, the development of mucosal immune tolerance is essential to normal development.
2) **Role on immune response.** Vitamin A appears to play a key role in maintaining an adequate number of peripheral naïve T cells. To achieve oral tolerance, vitamin A is likely to be essential in enhancing conversion of T cells to regulatory T cells by a TGF mediated mechanism (67-69). There is also some evidence regarding an association of elevated circulatory vitamin A levels and increased risk of IgE mediated sensitization (70). This is through action on T helper-2 cells response resulting in skewing of the immune system (71).

3) **Vitamin A supplementation.** There is a direct association between vitamin supplementation in early childhood and the prevalence of food allergy related asthma (70). Vitamin A deficiency is common in developing countries and to prevent clinical consequences, vitamin A is supplemented in early infancy (72). Although water-based preparations of vitamin A are available, fat soluble based preparations such as peanut oil is usually advocated because of improved preparation and storage. Moreover, peanut oil contains omega-3 and omega-6 fatty acids (72) which are recommended to reduce autoimmune disease and prevent allergy (73, 74). Moreover, it is reported in young children that, the water-soluble form of supplementation increases the odds of developing allergic diseases compared with fat-soluble forms possibly because it is so poorly absorbed (72).

4) **Potential interplay with vitamin D.** Vitamins A and D compete for the same receptor sites. The main relevance of this is that preparation containing both vitamins (such as cod liver oil) may behave differently to pure forms of either. Indeed, this is a factor that should be considered when evaluating the observation that increasing cod liver oil supplementation over the 20th century was associated with an increase in allergic disease (28).

Further research needs to be conducted to establish evidence for or against the role of vitamin A in food allergies.

### 1.2.3 What is the biological plausibility of vitamin D in relation to food allergy?

The role of vitamin D in immune system development and innate epithelial defence and maintaining of intestinal mucosal barrier integrity has been recently demonstrated (60, 75, 76). Vitamin D potentially can promote the pro-sensitization immune imbalance which can compromise immunologic tolerance (77). Hence,
it is suggested that the rapid rise in food allergy may be associated with the increase of vitamin D insufficiency. It is proposed that there is a robust link between vitamin D and food allergy, due to the significant role of vitamin D in maintaining mucosal immunity, healthy gastro-intestinal microbiota and allergen tolerance (49). Additionally, the expression of IL-10-secreting regulatory T cells can be induced by vitamin D (78). On the other hand, there is a correlation between allergen specific T-regulatory cells and food intolerance reactions (63). Thus, vitamin D’s potential role in food tolerance promotion among food sensitized individuals is now thought to be substantial.

1.2.4 How are these vitamins measured?

Vitamins A and D are classified as fat soluble vitamins and both are recognised to have hormonal like actions on nuclear receptors in target tissues. As both compounds are hydrophobic they are transported in the circulation by their respective carrier proteins. To exert their effect both vitamins require conversion to their active forms. To assess the levels of vitamins A and D in blood, the main circulating forms (i.e. retinol and 25-OHD3 respectively) are routinely measured on serum or plasma samples. There has been extensive discussion in the literature relating to the lack of agreement between methods for these vitamins. The following section briefly outlines the method characteristics for the measurement of the main circulating form of these vitamins.

Vitamin A methods

In paediatric laboratories in developed countries\(^1\), serum vitamin A levels are most commonly measured to monitor cystic fibrosis patients and to screen newly arrived immigrants from developing countries. Whereas, in developing countries, vitamin A is almost exclusively measured to assess clinical deficiency; which is supported by World Health Organization (WHO) programs to combat the vitamin A deficiency epidemic.

\(^1\) According to the International Monetary Fund, advanced economies comprise 65.8% of global nominal GDP and 52.1% of global GDP (PPP) in 2010/ IMF GDP data September 2011.
observed in low gross domestic product (GDP) countries. The lower reference limit for serum vitamin A is clearly defined by the WHO as 0.7 µmol/L (with clinical signs of deficiency at 0.35 µmol/L) in children (79). The upper reference limit is less clearly defined with variations seen between laboratories (80). Analysis of this vitamin is almost exclusively by chromatographic separation techniques in laboratories worldwide (80, 81).

Measurement of vitamin A, due to its low circulatory concentration and structural similarity of related analogues remains a challenge. Most laboratories use high performance liquid chromatography with spectrophotometric detection to measure retinol (80, 82). More recently mass spectrometry based detection methods have been reported (83-85). Prior to sample analysis, the serum is prepared by liquid-liquid extraction techniques and includes a protein precipitation step to remove vitamin A from its binding protein. In all assays an internal standard is added to correct for losses in the preparation technique. Commercial calibrators available for vitamin A are usually based on the National Institute for Standards and Technology (NIST) reference material; currently NIST SRM 968e.

Whilst the NIST reference material is available and utilised by commercial calibrator manufacturers, agreement between laboratories for vitamin A results remains a problem (45). This hinders harmonisation efforts in laboratory medicine and presents problems with the transportability of results. In addition, currently there is no reference method available for retinol measurement (86). However, liquid chromatography coupled with tandem mass spectrometry can be considered as a prospective nominee for a retinol measurement reference method which is able to meet precision and accuracy requirements (87).

Further confounding the interpretation of vitamin A results is the problem that serum retinol levels do not always accurately reflect liver retinyl ester levels (i.e. the storage form of vitamin A) (88). However, once vitamin A liver supply is diminished, circulatory levels will diminish too. Hence, serum retinol concentration is still of value to dichotomise deficient from replete patients. Retinol levels are also relevant for acute toxicity but have been reported to be of less value in isolation for chronic toxicity (89). In addition, blood collection is the least invasive collection method to assess vitamin A status.

Rosita Zakariaee Abkoo
**Vitamin D methods**

In general vitamin D refers to two major groups of metabolites: ergocalciferol (vitamin D2) and cholecalciferol (vitamin D3). Ergocalciferol is basically driven from plant sources which are typically ingested in supplementation form. Predominantly cholecalciferol is the endogenous form of vitamin D, that is formed from conversion of 7-dehydrocholesterol to pre-vitamin D3 due to skin tissue UV exposure. Liver hydroxylation results in the 25-hydroxyvitamin form of both D2 and D3. The bio-active form is generated by the kidney as; 1-alpha,25-dihydroxy for either D2 or D3 (24,25-(OH)2D) in combination with the insignificant 24,25-(OH)2D3 analogue (90-92).

Isomerisation of the vitamins D at position 3 of the structure results in a shift in the hydrogen atom at position three from the beta to alpha. Whilst this isomerisation occurs with a number of the compounds in the vitamin D pathway, this process is significant for 25-OHD3, when the epi-25-OHD3 is produced. Significant amounts of 3-epi have been observed in paediatric serum samples (93). Whilst 3epi-25-OHD3 is an inactive isomer of 25-OHD3 it actively cross-reacts and causes overestimation in vitamin D determination (94). 3epi-1,25(OH)2D3 may be formed from 3epi-25-OHD3 hydroxylation. It is suggested that some of the biological activity of cholecalciferol may relate to 3epi-1,25(OH)2D3 (95).

25-OH-Vitamin D3 has a higher circulatory concentration and longer half-life compare with 1,25-(OH)2D3. 25-OHD3 is considered the appropriate analyte to measure in commercial immunoassays (96). The early measurement techniques of competitive binding protein and radio immunoassay techniques were developed to evaluate the circulating levels of vitamin D (97-101). The advantage of these assays was the ability of providing equal recognition of 25-OHD2 and 25-OHD3. Due to molecular structure similarity of the vitamin D analogues (Figure 1.4), as well as, non-specific interference of lipids and other circulatory metabolites; even under further sample solvent extraction pre-treatment, the specificity and sensitivity of immunoassay methods always has been critically questioned (68).
Figure 1.4. LC-MS/MS-MRM chromatogram of 25-OHD analogues and retinol separation in serum sample; 25-OHD3-d3 is the deuterated analogue added to the sample for use as the internal standard. The x-axis represents the acquisition time (minute) and the y-axis is the ion counts. Each of the vitamin D analogues are secosteroids i.e. steroid like chemical structure with the second ring opened. Vitamin A, measured as retinol, is a cyclohexene ring attached to a tetra-isoprene chain. (This figure is modified from reference (102)).

Current immunoassay based technology for the measurement of 25-OHD is the most broadly used (47). Many of these assays are now fully automated and do not require any sample pre-treatment e.g. extraction. The antibodies employed between manufacturers target 25-OHD3 with varying cross reactivity with 25-OHD2 and other forms of vitamin D. Overestimation of vitamin D status is the result of other vitamin D metabolites (such as; 24,25-(OH)2D, 25,26-(OH)2D, and 25,26,(OH)2D-26, 23-lactone) interference (103, 104). Thus, measured vitamin D may be overestimated by 10-20% using either competitive binding or immunoassay techniques (105). This problem of standardisation with established trueness for vitamin D is considered a major disadvantage of immunoassays.

Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) system, potentially provides exceptional specificity in terms of different analytes separation and measurement. LC-MS/MS based methods are superior to immunoassays for result interpretation (106, 107). Utilising multiple reactions monitoring mode (MRM) delivers the greatest specificity, along with liquid chromatography separation of structurally related isomers separation. However, in regard to vitamin D measurement, separation of the 25-OHD3 from
epi-25-OHD3 is still challenging (108). With clear separation of the vitamin D epimers, LC-MSMS is generally considered the working “gold standard” method, for separation and quantitation of 25-OHD2 and 25-OHD3 and epi- 25-OHD3 (109).

3epi-25-OHD3 and 3epi-1,25-(OH)2D3 are product of 25-OHD3 and 1,25-(OH)2D3 epimerization (110). 3-epi-25-OHD3 is biologically considered as an inactive cholecalciferol isomer, which is expressed more significantly in younger children rather than adults (13). Since 3epi-25-OHD3 can cross-react and impede the routine measurement methods, still cannot be neglected with regard to vitamin D determination in both adults and children (93).

Recently a more sensitive method utilising LC-MS/MS, has been described for 3epi-25-OHD3 measurement. Using this method enables us to separate and quantify 25-OHD2 and 25-OHD3, together with 25-OHD3 epimer (3epi-25-OHD3) and its isobars (1-α-OHD3) as well as 7-α-OH-4-cholesten-3-1. The assay sensitivity is equal to 0.62 nmol/L (0.25 ng/mL). Thus, this developed assay can be considered as the very first, specific, reliable, reproducible and robust LC-MS/MS method for the accurate evaluation of Vitamin D (108). However, most of the chromatographic-mass spectrometry methods required a large sample volume. Recently a two-dimensional ultra-performance liquid chromatography separation technique, coupled with tandem mass spectrometry (UPLC-MS/MS) is developed to quantitate 25-OHD3, epi-25-OHD3, and 25-OHD2, requiring a small sample volume (50µl) with 2 nmol/L (0.8 ng/ml) analytical sensitivity.

Assessment of analytical methods due to low circulatory concentration and molecular similarities of the analyte, as well as the samples matrix effect caused by lipophilic nature of retinol and cholecalciferol are critical factors to consider in fat soluble vitamins measurement (54). Routine measurement methods, which are able to maintain clinically desirable and fit for purpose results in terms of sensitivity and specificity, are typically based on chromatography and mass spectrometry techniques. Generally, the requirement of large amounts of blood sample and deep freeze storage condition are critical disadvantages of the methods currently available for population-based assessment of fat soluble vitamins (111). However, few groups around the world have successfully derived vitamin D from Guthrie cards (111, 112) and none to our knowledge have optimized measurement of vitamin A from these sources.

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**Combined Vitamin A and D analysis**

Given the potential for vitamin A, in addition to vitamin D, to have a role in relation to the prevalence of food allergies, it would be ideal to measure these analytes simultaneously. This would be advantageous in terms of laboratory efficiency plus also for the determination the pattern of vitamin A alongside vitamin D. Any such method would still require clear separation of the carbon-3-epimer. Recently, such a method has been presented (102) indicating that simultaneous measurement is possible.

**1.2.5 Alternative sample collection – Guthrie cards**

Classically, analytical methods for vitamin A and vitamin D utilise either serum or plasma separated from whole blood. Mostly, in large population-based studies, especially involving children and infant participants, we face some uncertainties in terms of sample collection and storage. Blood spot card (Guthrie) are suggested as the alternative sampling method (dried capillary blood spot) to store and undertake further blood analyses for large population-based studies. These studies already utilise chromatography / mass spectrometry measurement methods (113-115). It has been demonstrated that retinol, 25-OHD2 and 25-OHD3 are stable in dried blood spot card and measurable, utilising such LC-MS/MS method (112, 116).

Several studies report a high level of agreement for the cholecalciferol and retinol concentrations between venous blood samples and Guthrie. Venous blood samples have a higher haematocrit than capillary samples (117). The implication of this is that dried blood sample may have a slightly lower serum fraction than samples derived from venepuncture. Accordingly, it is expected that lower values for either retinol or cholecalciferol will be obtained from Guthrie compare to serum/plasma, unless adjusted for sample used for analysis. Therefore, in the estimation of vitamin D in dried blood samples such as Guthrie cards, assessment of blood spot size and haemoglobin concentration, needs to be taken into account (118).

**1.2.6 Where to from here?**

In summary, the increase of food allergy may be related to a concordant rise in prevalence of vitamin D insufficiency. In addition, vitamin A is linked to immunity and it is plausible that it may also have a role in
the risk of developing food allergies. However, further studies need to be conducted to investigate, the impact of vitamin A and D insufficiency or excess at birth on food allergy in early childhood. Furthermore, understanding the adverse effects of a high prevalence of VDI in the general population is an important public health initiative, since VDI may be corrected with the simple and inexpensive intervention of dietary Vitamin D supplementation.

Chromatographic separation coupled with mass spectrometric detection is considered the higher order methods for both vitamins. These analytical methods should be fully validated for use in paediatric populations to ensure they are fit for their clinical purpose, including chromatographic separation of the 25-OHD3 from its epimer. To utilise blood spots for the investigation of these vitamins at birth, the LC-MS/MS methods will require further optimisation in order to evaluate the small volume samples driven from dried blood spot cards.

Finally, it is essential to provide the integral population data regarding vitamin D and other fat-soluble vitamins such as vitamin A levels to determine if the hypothesized relationships do exist in early life.”
1.3 Advantages and challenges of dried blood spot analysis by mass spectrometry across the total testing process

This section has been peer reviewed and published with contribution of other co-authors as a review paper (10). My contribution to this study and subsequent publication was included; developing the concept for the study in conjunction with my senior supervisor (RG), performing the literature search / study, collecting data, analysis and interpretation of data, writing the first draft of the publication and reviewing and incorporating all suggestions from the co-authors.

1.3.1 Introduction

“A century ago, for the very first time, Ivar Bang described a dried blood matrix as an unconventional sampling method (119). Later, Robert Guthrie in 1963 introduced the dried blood spot (DBS) technique for screening. Guthrie application of the dried blood spot, and his personal crusade to utilise this approach to screen intellectually disabled children, heralded the introduction of newborn blood spot screening (NBS). Although the particular assay is now defunct, the term “Guthrie card” remains to colloquially describe the DBS collection technique which still underpins today’s newborn screening programs worldwide (120).

The original semi-quantitative bacterial inhibition test developed by Guthrie to screen for phenylketonuria (PKU) was highly sensitive but had a low analytical throughput (121). Through the introduction of advanced analytical techniques, that expanded testing options and improved throughput, the scope of newborn screening blood spot sample applications were extended, this included screening applications for congenital hypothyroidism and cystic fibrosis in many centres (122). Further to this expansion was the introduction of tandem mass spectrometry for newborn screening DBS analysis in 1990s (123).

Mass spectrometry is now the most common technique reported in the literature for dried blood spot analysis (124-127). DBS analysis offers the advantage of collecting a small sample volume, which is easily transported. However, this also means that the concentration of the target analyte is potentially quite low (e.g. less than 1 ng/L) which requires a sensitive and specific assay for detection and quantification. These considerations,
coupled with the expansion of mass spectrometry into clinical laboratories, have led to a surge in the utilisation of this sampling method outside of the scope of newborn screening in the published literature.

Clinicians and researchers have become optimistic about the potential applications of dried blood spot-based mass spectrometry applications (DBS-MS) and it has been used for a range of clinical utilities including drug toxicology and sports doping screening. Scientists and technical analysts on the other hand face challenges regarding how to ensure optimal sensitivity, reproducibility and overall accuracy of DBS quantification. In this review, we aim to bring together clinical and analytical facets to discuss the advantages and current challenges of non-newborn screening applications of DBS quantification by MS.

To address these aims, we performed a key word search of PubMed and MEDLINE online databases in conjunction with individual manual searches to gather information. Keywords for the initial search included; “blood spot” and “mass spectrometry”; while excluding “newborn”; and “neonate”. In addition, databases were restricted to English language and human specific. No time period limit was applied. As a result of this selection criteria, 191 references were identified for review. For presentation, this information is divided into clinical applications and analytical considerations across the total testing process. It is not our intention in this review to highlight all analytical aspects related to quality MS application (as this has been covered extensively elsewhere) but rather to address the analytical aspects pertinent to the DBS matrix.

1.3.2 Clinical Applications

The first application of MS to DBS analysis was reported 40 years ago (in 1976) for fatty acid determination by direct chemical ionisation (128). By the mid-1980s, when gas chromatography (GC) was the technique of choice for separation and analysis of volatile small molecules, derivatised fatty acids were measured from DBS samples using GC-MS (129). In 1990s, when electrospray ionisation became commercially available, liquid chromatography - tandem mass spectrometry (LC-MS/MS) began to be incorporated into the analytical tools for newborn screening laboratories, leading to the significant expansion of screening applications with phenylalanine and tyrosine being two of the early markers (123, 130). Today DBS-MS applications are the workhorse for many NBS laboratories worldwide; and additional tests are continually being added to the

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repertoire. Outside of NBS, an epidemiological study analysing benzoylecgonine was the first reported DBS-MS application (131).

Now, many potentially reliable and compatible MS detection methods are available across a wide range of disciplines (132). Our literature search identified 97 references encompassing 121 distinct biomarkers determined from DBS samples utilising MS technology beyond its application in NBS. Notably, the role of DBS analysis by MS now encompasses translational research and clinical diagnostic analytes in the areas of therapeutic drug monitoring (TDM); pharmakinetics (PK); toxicokinetics (TK); forensic; endocrinology and metabolism; and other areas of bio-analysis. Table 1 provides a list of these biomarkers.

Therapeutic and toxicological drug analysis are the most extensively reported DBS-MS applications in the literature (Table 1). These DBS-MS applications, (encompassing both LC-MS/MS and GC-MS techniques) are particularly fit for population-based studies of multiple biomarkers (133, 134). Similarly, DBS-MS applications are now applied in sport related doping tests for the detection of anabolic, ergogenic and masking agents (135-137).

<table>
<thead>
<tr>
<th>Table 1.1.a) The list of abbreviations</th>
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<tr>
<td>2D: two dimensional</td>
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<tr>
<td>AnalTech: analytical technique</td>
</tr>
<tr>
<td>APTDCI-MS: atmospheric pressure thermal desorption chemical ionisation Mass spectrometry</td>
</tr>
<tr>
<td>D: day</td>
</tr>
<tr>
<td>DAA: drug abuse athletics</td>
</tr>
<tr>
<td>Dab: drug abuse</td>
</tr>
<tr>
<td>Dad: drug adherence</td>
</tr>
<tr>
<td>ESI: electrospray ionisation</td>
</tr>
<tr>
<td>FIA-ESI-MS/MS: flow Injection Analysis-electrospray ionisation tandem mass spectrometry</td>
</tr>
<tr>
<td>FT-ICR-MS: fourier transform ion cyclotron resonance mass spectrometry</td>
</tr>
<tr>
<td>GC: gas chromatography</td>
</tr>
<tr>
<td>GC-HRMS: gas chromatography–high resolution mass spectrometry</td>
</tr>
<tr>
<td>HILIC-MS/MS: hydrophilic Interaction chromatography tandem mass spectrometry</td>
</tr>
<tr>
<td>ID: illicit drug</td>
</tr>
<tr>
<td>IDES-MS/MS: isotope-dilution electrospray tandem mass spectrometry</td>
</tr>
<tr>
<td>LC: liquid chromatography</td>
</tr>
<tr>
<td>LC-HRMS: Liquid chromatography–high resolution mass spectrometry</td>
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<tr>
<td>LLOQ: lower limit of quantitation</td>
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Table 1.1.a) The list of abbreviations cont.

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<td>M</td>
<td>month</td>
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<tr>
<td>MetV</td>
<td>method validation</td>
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<tr>
<td>MI</td>
<td>metabolic intermediate</td>
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<tr>
<td>MS</td>
<td>mass spectrometry</td>
</tr>
<tr>
<td>NR</td>
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<td>PD</td>
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<tr>
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<td>paper spray mass spectrometry</td>
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<td>pharmakinetics</td>
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<td>Ref</td>
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</tr>
<tr>
<td>RepU</td>
<td>reporting unit</td>
</tr>
<tr>
<td>Stab</td>
<td>stability</td>
</tr>
<tr>
<td>TDM</td>
<td>therapeutic drug monitoring</td>
</tr>
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<td>TK</td>
<td>toxicokinetic</td>
</tr>
<tr>
<td>TLC-MS</td>
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</tr>
<tr>
<td>W</td>
<td>week</td>
</tr>
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Table 1.1.b) List of biomarkers

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<tr>
<td></td>
<td>Metabolic Intermediate</td>
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<td></td>
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<tr>
<td></td>
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<td>NR</td>
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<td></td>
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<td>µmol/L</td>
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<td>µg/ml</td>
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<td>(164)</td>
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<td>27</td>
<td>Stroles</td>
<td>APTDCI-MS</td>
<td>NR</td>
<td>Ratio</td>
<td>NR</td>
<td>NR</td>
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**Pharmaceutical Drugs**

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Rosita Zakariaee Abkoo
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### Table 1.1.b) List of biomarkers cont.

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<td>97</td>
<td>Simvastatin</td>
<td>LC-HRMS</td>
<td>0.5-5.0 ng/ml</td>
<td>12w</td>
<td>Y</td>
<td>(180)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLC-MS</td>
<td>5.0-50 ng/ml</td>
<td>NR</td>
<td>NR</td>
<td>(173)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PS-MS</td>
<td>0.25-0.75 ng/ml</td>
<td>NR</td>
<td>NR</td>
<td>(125)</td>
<td></td>
</tr>
<tr>
<td>98</td>
<td>Sirolimus</td>
<td>LC-MS/MS</td>
<td>116 μmol/L</td>
<td>7d</td>
<td>Y</td>
<td>(146, 148, 184, 186)</td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>Analyte</td>
<td>AnalTech</td>
<td>LLOQ</td>
<td>RepU</td>
<td>Stab</td>
<td>MetV</td>
<td>Ref</td>
</tr>
<tr>
<td>----</td>
<td>--------------------------</td>
<td>-------------------</td>
<td>-----------</td>
<td>------</td>
<td>------</td>
<td>------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>99</td>
<td>Sitamaquine</td>
<td>TLC-MS</td>
<td>5.0-50 ng/ml</td>
<td>NR</td>
<td>NR</td>
<td>(173)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>PS-MS</td>
<td>0.25-0.75 ng/ml</td>
<td>NR</td>
<td>NR</td>
<td>(125)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>Sunitinib</td>
<td>PS-MS</td>
<td>0.25-0.75 ng/ml</td>
<td>NR</td>
<td>NR</td>
<td>(125)</td>
<td></td>
</tr>
<tr>
<td>101</td>
<td>Tacrolimus</td>
<td>LC-MS/MS</td>
<td>1 µg/L 20d</td>
<td>Y</td>
<td></td>
<td>(126, 146-148, 184, 186, 217, 218)</td>
<td></td>
</tr>
<tr>
<td>102</td>
<td>Tamoxifen</td>
<td>LC-MS/MS</td>
<td>0.5 ng/ml 20d</td>
<td>Y</td>
<td></td>
<td>(127)</td>
<td></td>
</tr>
<tr>
<td>103</td>
<td>Tenofovir</td>
<td>LC-MS/MS</td>
<td>2.5 ng/ml 6d</td>
<td>Y</td>
<td></td>
<td>(190)</td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>Topiramate</td>
<td>LC-MS/MS</td>
<td>10 µg/ml 194d</td>
<td>Y</td>
<td></td>
<td>(219)</td>
<td></td>
</tr>
<tr>
<td>105</td>
<td>Valproic acid</td>
<td>LC-MS/MS</td>
<td>10 µmol/L 42d</td>
<td>Y</td>
<td></td>
<td>(220)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GC-MS</td>
<td>5 µmol/L 21d</td>
<td></td>
<td></td>
<td>(221)</td>
<td></td>
</tr>
<tr>
<td>106</td>
<td>Vemurafenib</td>
<td>LC-MS/MS</td>
<td>1 µg/ml NR</td>
<td>Y</td>
<td></td>
<td>(222)</td>
<td></td>
</tr>
<tr>
<td>107</td>
<td>Venlafaxine</td>
<td>LC-MS/MS</td>
<td>20 µg/L NR</td>
<td>Y</td>
<td></td>
<td>(206)</td>
<td></td>
</tr>
<tr>
<td>108</td>
<td>4-nitrophthalic acid</td>
<td>TLC-MS</td>
<td>5.0-50 ng/ml</td>
<td>NR</td>
<td>NR</td>
<td>(173)</td>
<td></td>
</tr>
<tr>
<td>109</td>
<td>4-hydroxytamoxifen</td>
<td>LC-MS/MS</td>
<td>0.5 ng/ml 20d</td>
<td>Y</td>
<td></td>
<td>(127)</td>
<td></td>
</tr>
<tr>
<td>110</td>
<td>Illicit Drug</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amphetamines</td>
<td>ESI-MS/MS &amp; GC-MS</td>
<td>2.3-11 ng/ml</td>
<td>NR</td>
<td>Y</td>
<td>(223)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2D-LC-MS/MS</td>
<td>5 ng/ml 6 m</td>
<td>Y</td>
<td></td>
<td>(224)</td>
<td></td>
</tr>
<tr>
<td>111</td>
<td>Caffeine</td>
<td>LC-MS/MS</td>
<td>0.35-250 ng/ml</td>
<td>5d</td>
<td>Y</td>
<td>(194)</td>
<td></td>
</tr>
<tr>
<td>112</td>
<td>Cocainics</td>
<td>ESI-MS/MS &amp; GC-MS</td>
<td>2.3-11 ng/ml</td>
<td>NR</td>
<td>Y</td>
<td>(223)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>2D-LC-MS/MS</td>
<td>5 ng/ml 6 m</td>
<td>Y</td>
<td></td>
<td>(224)</td>
<td></td>
</tr>
<tr>
<td>113</td>
<td>Novel psychoactive substances</td>
<td>LC-ESI-MS/MS</td>
<td>1.0-10 ng/ml</td>
<td>1w</td>
<td>Y</td>
<td>(225)</td>
<td></td>
</tr>
<tr>
<td>114</td>
<td>Opiates</td>
<td>2D-LC-MS/MS</td>
<td>5 ng/ml 6m</td>
<td>Y</td>
<td></td>
<td>(224)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ESI-MS/MS &amp; GC-MS</td>
<td>2.3-11 ng/ml</td>
<td>NR</td>
<td>Y</td>
<td>(223)</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>Δ9-tetrahydrocannabinol</td>
<td>LC-MS/MS</td>
<td>100 ng/ml 3m</td>
<td>Y</td>
<td></td>
<td>(226)</td>
<td></td>
</tr>
</tbody>
</table>
Irrespective of the clinical application, there are specific analytical considerations. Several parameters can impact on the accuracy of DBS measurement (128). The following sections of this review will focus on important considerations for DBS-MS quantification in the pre-analytical, analytical and post-analytical phases.

1.3.3 Pre-analytical

The pre-analytical phase of testing incorporates the processes of 1) blood collection from the patient, 2) its application onto the filter paper, 3) drying and 4) transport and storage of the DBS sample. In addition to the pre-analytical variables identified for routine blood collection, DBS faces additional challenges, including the quality of DBS sample (which is subject to sample collection and spotting variations), choice of collection card, collection (bleeding and blotting), transport and storage. Moreover, biological factors such as sample viscosity, haematocrit level and the nature of the target analyte, may lead to variation in sample quality. To sustain the quality management of the pre-analytical phase, there are defined recommendations for positive patient identification (231), sample collection of capillary blood (232), choice of filter paper (233), application of the sample onto the filter paper (234) and shipment of the DBS sample (235). An example of a standardised protocol for DBS pre-analytical processes is provided in Table 1.2.
Sample Collection

The sample collection technique is important for accurate analysis of the DBS. Capillary blood collection is a common approach, as it usually requires less sample volume and is more patient friendly, compared to venepuncture. Both the World Health Organization (WHO) and the United Nations International Children's Emergency Fund (UNICEF) certify the quality of DBS samples and maintain healthcare workers safety through the provision of guidance manuals and standard operating procedures for DBS sample collection (236, 237). Due to the likelihood of significant sources of artefact formation, specific DBS collection training points are highlighted, including the choice of prick point, lancet type/size, prick depth, pressure rate during blood drop collection, dropping size/speed consistency, uniformity of sample diffusion on the collection card and DBS contamination due to extended air or light exposure during the drying process. Importantly, WHO highlight that “working with DBS, whole blood or plasma requires the same biohazard safety precautions as whole blood” (237).
Table 1. 2. Example dried blood spot sample collection and transport protocol.

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Positive patient identification</td>
</tr>
<tr>
<td>2.</td>
<td>From a capillary collection, allow one drop of blood to penetrate each position of the filter paper. This should be one large drop of blood approx. 20µl</td>
</tr>
<tr>
<td>3.</td>
<td>Repeat for all positions</td>
</tr>
<tr>
<td>4.</td>
<td>Record at least two (prefer 3) identifier on the card (e.g. Name, date of birth, study number, Health record number)</td>
</tr>
<tr>
<td>5.</td>
<td>Record sample collection date and time</td>
</tr>
<tr>
<td>6.</td>
<td>Record the following information in the logbook; a) Participants first name and surname, b) Participants date of birth, c) Study number, d) Card bar code number, e) Date of collection, f) Any other relevant information</td>
</tr>
<tr>
<td>7.</td>
<td>Allow samples to air dry for a minimum of 2 hours (longer may needed in humid or cold environment)</td>
</tr>
<tr>
<td>8.</td>
<td>Store in Biohazard / Specimen Transport Bag in dry place at room temperature until shipment to the destination lab</td>
</tr>
</tbody>
</table>

Rosita Zakariae Abkoo
9. Send dried blood spot sample cards following the protocol to the destination lab with a copy of the logbook entries.

10. Attach a copy of the import (shipping) permit to the outside of the package.

11. Attach a copy of a “manufacturer declaration” signed on your letterhead to the outside of the package. Use the following wording for the manufacturer’s declaration:

   “Manufacturer’s declaration
   To Whom It May Concern:
   The shipment xxx (shipping number) xxx from the Department xxx (your location) xxx contains Human blood. This item is covered in Permit xxx (permit number) xxx under condition number xxx (condition number) xxx. This material is non-toxic, non-infectious, and is intended for clinical laboratory studies.
   Yours Sincerely, Add your signature line here”

12. Ensure that the samples are packaged according to the required standards of shipping bloods (secondary container / absorbent material etc).

13. Label the package with specific destination address. The following details should be used in association with the Permit xxx (permit number) xxx;

   “Attention: Contact person’s name
   Address: Destination lab postal address
   Phone: Destination lab / contact person’s contact number including area code
   Email: Contact person’s email address”

14. Send an email to Destination lab contact person to advice of shipment.

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**Selection of filter paper**

Collection of DBS samples is onto one of two types of untreated solid support: pure cotton filter paper and glass microfiber paper. The choice of paper, including its thickness and density, influences the rate of adsorption and dispersion. As an example, the rate of membrane non-specific analyte adsorption is reduced in glass micro-fibre paper (63). Accordingly, dissimilarities in these solid supports may induce variations in the DBS sample attributes leading to potential differences in analyte stability, commutability, volume per area, and analytical effects (covered later in this review) (238). These DBS specific pre-analytical variables require standardisation and the Clinical and Laboratory Standards Institute (CLSI) offer a guideline NBS01-6 to support DBS collection (233).

The CLSI guideline recommends the use of two specific collection cards: the Whatman 903 and Ahlstrom 226. Both filter papers are approved by the Food and Drug Administration (FDA), Newborn Screening Quality Assurance Program (NSQAP) and also the Centers for Disease Control and Prevention (CDC) (234).

**Sample application to filter paper**

Both the Whatman 903 and Ahlstrom 226 cards have a target collection area printed on the paper to indicate and ensure the 50-75 µl sample volume limits (234). The whole blood drop should be spotted onto the mid-point of the collection area to allow for radial dispersion to the designated edge of the spot. This lateral distribution is uneven; as clearly evaluated by autoradiography (239). Usually, the concentration of biomarkers is decreased along the edge of the DBS, while the middle is affected by the volcano effect (paper chromatography effect) and occasionally a speckled pattern (240). The physical characteristics of the DBS sample is also potentially affected by the patient’s haemoglobin and haematocrit level; which also influences the spreading area of the blotted blood.

Variations in haematocrit (Hct)/haemoglobin (Hb) will affect the relative plasma percentage of the spot. This is important for analytes predominately found in serum / plasma, as the relative amount of plasma in a disk punched from different spots can vary; and particularly exaggerated when the Hct is extremely low.
high or low (241). Whole blood samples with a higher Hct tend to distribute to a lesser extent across the filter paper (i.e. smaller blood spot diameter), and consequently the target analyte diffusion distance is shorter. The inverse applies for lower Hcts. As a result, the determined concentration compared with the “normal” Hct sample would be over or under estimated due to the change in the analyte’s distribution and infusion pattern across the blood spot (242, 243). Hence, for accurate quantitation, ideally the patient’s Hct needs to be determined, by either a separate capillary drop / sample collected at the time of the DBS collection or directly from the DBS card. Figure 1.5 provides a visual demonstration of the effect of Hct on diffusion.

**Analytes stability (storage and transport condition)**

DBS samples should be allowed to completely dry before transport and/or storage. It has been demonstrated that rapid drying and storage in low humidity conditions improves the stability of DBS samples (134). The length of time required for air-drying will depend on the local environmental conditions such as air conditioning, room temperature and humidity. DBS drying usually takes from 90 minutes to approximately 4 hours and ideally, the DBS samples should not be left exposed to direct strong sunlight during this period.

The appropriately dried DBS sample can then be placed in an envelope or similar container for the logistically simple and cost effective transport process (134). As the low volume of the dried specimen significantly reduces the risk of infection transmission compared to the other biological samples (244, 245), it can be transported in small lightweight packages that do not require temperature regulation.
This negates the often cumbersome and expensive processes associated with transport of liquid biological samples (134). Once the DBS samples are received at the destination, the size and properties of the DBS samples make storage relatively easy as minimal space is required; and they can often be stored at room temperature.

The stability of the DBS sample does require consideration, as the relevant stability for different analytes on DBS is quite variable. The stability of numerous blood biomarkers on blotting paper at room temperature has been confirmed for a minimum of 7 days (63), several months (pharmaceutical drugs, opiates and nucleic acids) (246, 247), and even greater than 20 years (vitamin D) (167). Freezing at -20 °C or -80 °C has been shown to further increase the stability of DBS samples; often extending the stability from days / months to years (248-250). In addition, some reports show that some metabolites have better stability in the form of card-blotted whole blood compared to plasma (251).

Despite these exciting reports of long-term stability, there are many recognized potential influences, in addition to storage time, which could affect stability; including the type of filter paper, light exposure, temperature, humidity as well as the nature of the target analyte. Examples of changes to the collection process to improve stability include: 1) rapidly lowering the pH of the spotted blood sample to extend the stability time of some structurally unstable drugs which degrade during the drying process; and 2) use of EDTA as an anticoagulant to preserve and stabilise DBS samples for enzyme activity determination (252, 253). As a result of variations in stability, analyte specific protocols need to be administered for the collection and storage of DBS samples. Table1.1 provides a guide to the stability of DBS samples for different analytes found in the literature.

### 1.3.4 Analytical considerations

The solid form of the DBS sample is not compatible with the most analytical techniques and requires elution of the sample from the filter paper. Accordingly, the testing process commonly includes three main stages: primary sample preparation, sample pre-treatment and sample analysis. Analysis can be further divided into chromatographic and MS separation and detection. In addition, the overall quality
of the analytical process needs consideration. In this section of the review, we will discuss each of these aspects in turn. Figure 1.8 provides a general summary of the DBS-MS process.

**The punch**

Sample preparation usually starts with deportation of a segment of the DBS from the blotter using a manual or automated puncher. Commonly, to minimise the assay bias due to punch location, it is
recommended to take the DBS punch either from the centre or close to the outer edge, consistently (254). The punch size may vary from 3mm punch size to 6mm or whole spot, depending on the method. Techniques have been developed to overcome the haematocrit impact issue and minimize the labour associated with the sample preparation process. These are a number of strategies to overcome the haematocrit effect: 1) Pre-cut DBS and perforated DBS techniques are introduced in DBS handling procedures to recover the haematocrit effect and eliminate the chance of carry-over between the punches. 2) To disregard the haematocrit effect and improve the assay bias, blotting of less whole blood volume (e.g. 10 µl) on the smaller pre-cut disk (3 or 6mm) and analysis of the whole disk is suggested (255-258). 3) Similarly, a two-layered polymeric membrane has been utilised to form a separated secondary dried plasma spot from the whole blood sample to be analysed following solid phase extraction (259). Recently, a novel collection card has been developed for DBS sampling, which generates a volumetric plasma sample (2.5 or 5.0 µL) from a non-volumetric application of whole blood sample. The purported advantages of this collection matrix includes enhanced assay reproducibility and selectivity, with a simplified sample extraction procedure and elimination of the haematocrit effect (260).

**Elution**

For analysis, the analyte of interest firstly requires elution out of the filter paper along with the whole blood matrix by using the appropriate elution and extractor buffers. The choice of extractor materials may vary from one compound to the other. As an example pure methanol is considered as a generic solvent for drugs of abuse extraction of the blood spot sample (261). Water on the other hand impairs the interaction between cellulose and the target analyte’s hydroxyl groups. Hence, the partial addition of water prior to the organic extraction advances the efficiency in certain cases (203). The efficient elution of analytes from the DBS sample is challenging and there is always a chance of analyte loss due to ineffective extraction; poor sample elution is due to either incomplete extraction or analyte degradation. To achieve effective analyte recovery with maximum extraction efficiency, the extraction
parameters, including extractor solution mix, duration, temperature and application of additional solvation energy (sonication), need to be optimised for each individual target metabolite (191, 241).

**Sample pre-treatment**

Depending on the molecular characterisation of the target compound, various sample preparation approaches have been suggested. These sample pre-treatment methods may be incorporated as a combination with each other or may be used in isolation depending on the analytical process developed. These techniques include the classic sample preparation process of 1) protein precipitation (PPT); 2) liquid-liquid extraction (LLE); 3) solid phase extraction (SPE); 4) supported liquid extraction (SLE); and / or 5) derivatisation.

Extraction and derivatisation procedures applied manually (or offline) are considerably time consuming and laborious. Whilst derivatisation is not required for many plasma-based analytes using LC-MS/MS, it is required for many DBS analysis to improve the sensitivity; offsetting the small sample volume. However, as the derivatisation process prolongs the overall analysis time, it is considered to be a limiting factor. It has been a driver for the development of on-line extraction techniques to facilitate the DBS sample pre-analytical treatment.

Automation of sample preparation directly coupled with the LC-MS/MS system has been introduced to improve turn-around time and run cost. PPT is a simple and popular method for automation that has been utilised for therapeutic drug monitoring (262). However, following a single PPT procedure, salts and other endogenous analytes are still present which may cause ion alteration in the MS process. SPE-LC-MS/MS set-up is designed to facilitate online sample desorption. This technique has turned to a time and cost effective method for DBS analysis (247, 263-266). Compared to PPT, SPE presents an improved sample clean-up (218). There are specific challenges with on-line extraction approaches, in comparison with the off-line extraction methods. The significant source of assay bias includes; non-homogenous mixture of internal standard with the analyte in the extract, sample dilution then band broadening and in chromatography separation; inadequate focusing of the extract onto the analytical
column (128). Accordingly, as part of the method development process, certain strategies are required to eliminate these issues.

Technology has been developed that allows for the direct sampling of the DBS, without the need for a change to liquid or elution. As it is described by the manufacturer, “Liquid Micro-junction Surface Sampling Probes (LMJ-SSP) are self-aspirating devices where liquid is pumped to and aspirated away from a surface of interest to a mass spectrometer for integrated extraction and ionisation” (267). By utilising the LMJ-SSP technology, the analyte of interest could be directly extracted from the different surfaces and detected by a mass spectrometer in a short time frame with minimum sample handling (268). The LMJ-SSP device coupled with the MS has been utilised for the determination of proteins in the DBS sample (269), direct tandem mass spectrometer for detection of haemoglobin (154), as well as therapeutic drugs (270, 271). Likewise, novel “on spot” direct derivatisation approaches provide a time and cost effective alternative sample preparation procedure; a technique introduced to determine thiorphan drug (196, 272).

**Sample analysis**

The choice of GC or LC instruments depends on the required sensitivity and target analyte characteristics. Despite the fact that the GC-MS provides selectivity, sensitivity and robustness for many DBS analytes, the literature demonstrates that, it is not as popular as LC-MS/MS (Table1.1). This is likely to be due to the improved specificity and sensitivity afforded by LC-MS/MS for blood spot analysis; associated with a significantly faster and usually more cost effective process compared to GC-MS (273, 274).

By introduction of the two-dimensional chromatography (2D-C) tools (applicable on both GC and LC), the separation efficiency, analytical sensitivity, quantitation accuracy and precision have been improved. This 2D-C process has reduced the DBS matrix and carry-over effects, with reports suggesting improved imprecision and bias (170, 275). With the further addition of on-line extraction joined to the 2D-C system, sensitivity and specificity is maximised when coupled with either a triple-
quadrupole tandem mass spectrometer or a high-resolution quadrupole time of flight mass spectrometry (QTOF-MS) (218, 276).

The advances in ion source technology have enhanced sensitivity for both polar and non-polar analytes from DBS samples (277-279). Selective / multiple reaction monitoring (SRM / MRM) modes in MS/MS detection, focusing on specific transitions, have advanced the assay specificity remarkably along with improving linearity and limits of detection (128, 280). GC-MS analytical techniques often offer a higher separating power and efficient reproducibility compared with LC-MS. Accordingly, GC-MS is still utilised for the determination of volatile biomarkers with low molecular weight and heat resistance fragments. Applications include, steroids, metabolomics and therapeutic drug monitoring studies using DBS samples (135, 176, 196, 221, 281) (Table 1.1).

Negating the pre-analytical clean-up and chromatographic front end separation, direct MS methods and surface sampling techniques coupled with MS have been used for DBS samples (282). Desorption electrospray ionisation (DESI), direct analysis in real time (DART) and direct electro spray ionisation mass spectrometry (ESI-MS) methods have been utilised in order to generate ions from the surface, thus avoiding purification or derivatisation processes (282-289). However, elimination of this primary sample purification and separation may result in loss of sensitivity and precision due to the disintegrated metabolite interferences (288). Thus, the application of non-paper blotting matrices and online SPE in conjunction with the direct MS methods is recommended to enhance the sensitivity and measurement precision (274, 290). The gains of time efficiency and throughput need to be balanced with achieving the desired method performance.

**Quality considerations**

There are some important considerations in relation to the method validation and acceptance criteria for DBS analysis. Accordingly, the European bio-analysis forum (EBF) has described the details of the DBS sample analysis methodology to provide specific recommendations for validation of DBS methods (291). The EBF recommendations document includes specific concepts of; collection card variability, sample-to-sample variability, DBS homogeneity, punch point effect, sample stability, blood physical
parameters effect, matrix effect, extraction recovery, internal standard (ISTD) application and quality control (QC) and calibrator (Cal) preparation. In addition to the detail provided in this document, traceability to the liquid matrix sample, fitness for clinical purpose and reference intervals / decision limits for interpretation require consideration.

Collection cards

To avoid issues of inter-card variability, calibrator and control material should be prepared using identical collection card type/manufacture as the patient samples. If multiple type/manufacturer of cards are used, then a method comparison is required to determine the comparative card stability, extraction recovery and matrix effect (292).

Haematocrit effect

As mentioned earlier, the physical behaviour of blotted whole blood is influenced by different parameters such as; haematocrit level, haemolysis degree and anticoagulant type (if it is applied). Currently the haematocrit is recognised as the most significant parameter affecting blood spot characteristics (drying time, dimension and homogeneity) and assay reproducibility. Haematocrit effect is more substantial when a sub-sample disk punch is analysed, rather than the whole DBS sample. Hence, method validation studies for DBS sample applications also need to include investigations of the impact of haematocrit variation on measurement and assay performance (293).

Application of Internal Standard

The incorporation of the ISs to the DBS sample processing is an important step and ideally should occur early in the process. 1) Collection cards pre-treated with the IS can be prepared prior to the spotting of the blood. This ensures both the ISTD and nominated compounds have undergone the same matrix and extraction effect. However, this approach logistically might not be practicable when dealing with multiple studies. 2) Commonly manual extraction methods utilise the approach of integrating the IS into the DBS elution reagent / extraction solvent. In this method, the ISTD is co-extracted along with
the target analyte. 3) Addition of the IS into the sample along with extraction / preparation process is another simple alternative. However, as the ISTD is not fully incorporated with the paper matrix, variations in elution recovery are not accounted for. 4) Using on-line DBS sample preparation technology, the ISTD is sprayed on the blood spot before the extraction using the Touch-spray technique (294-296).

**Carry-over**

Carry-over is a significant issue for DBS-MS analysis. Carry-over may have different sources including: physical card to card contact during storage; spot to spot originated from the puncher head and post-preparation initiated from the instrument (e.g. auto-sampler and analytical column) (291). As the puncher head is re-used, contamination and sample carry-over are notable concerns. To overcome this issue, either a clean-up step or a blank-card punch in between the samples is recommended (297). To investigate the instrumentation carry-over, two injections of sequential blank DBS extracts should be performed after an injection of a sample with the upper limit of quantitation concentration. The response for the first and second blank matrix should not exceed 20% and 5% respectively of the mean response of the lower limit of detection of the analyte of interest (298).

**Internal Quality control**

Internal quality control (IQC) spiked samples preparation for DBS analysis requires special considerations compared to liquid phase biologic samples. The main challenge of IQC is keeping the matrix consistent with that of patient’s blood spot sample. Both sample dilution and saturation may occur in spiked sample preparations (292). Ideally fresh non-haemolysed whole blood sample with the closely matching haematocrit level of the study group, should be chosen for spiking (298). These IQCs should be spotted onto the filter paper and eluted along with the patient samples.
External Quality Assurance

EQA programs are considered essential tools in evaluating the reliability and traceability of the analytical assay as well as monitoring the quality of the laboratory performance. The United Kingdom National External Quality Assessment Service (UK-NEQAS), the European Research Network for evaluation and improvement of screening, Diagnosis and treatment of Inherited disorders of Metabolism (ERNDIM) and the CDC (Newborn Screening Quality Assurance Program (NSQAP)) provide a variety of schemes for DBS newborn screening testing. However, there is no further EQA program available to assure the accuracy of DBS analysis outside of NBS. Hence, for most DBS analytes discussed in this review we do not have a peer review process to fully gauge laboratory performance. This represents a gap in harmonisation of analytes measured in this matrix.

Calibration

Standards preparation for DBS quantitative analysis includes whole blood fortification (replacement of certain amounts of plasma with the artificial plasma containing a known concentration of target analyte) with a set of commercial or in-house calibrator materials before spotting. The percentage of non-aqueous components replaced with plasma needs to be minimised to prevent solvent effects creating inconsistency between spiked samples (calibrators) and patient samples in terms of spot formation (291). Most DBS analytes are small molecular weight well defined compounds. In principle, full standardisation with traceability should be achievable. However, in practice the DBS matrix adds an extra level of complexity for commutability.

1.3.5 Post-analytical

Post-analytical concerns of the testing process phase mainly include; result reporting and interpretation, assay total error management and turnaround time (299). The key for the introduction of DBS analysis as a diagnostic tool is the cross validation of the method to a reference plasma/serum base assay. However, the fact that the concentration of the biomarkers in whole blood may vary from serum/plasma must be taken into account.
To turn the numerical result generated from the DBS analysis into clinical meaningful results a reference interval (RI) or decision point needs to be established. DBS specific RI have been developed for many analytes (300-304). For analytes routinely measured in liquid whole blood comparative RI can often be transferred to DBS samples. However, it is often more challenging for DBS analytes that require a comparison to serum for their clinical interpretation.

Some DBS analytes, such as vitamin D, require a conversion to their serum equivalent concentration for interpretation. This requires the development of a robust relationship between the measured analyte in the blotted whole blood and the equivalent serum sample. For this, we need to be able to estimate the equivalent blood volume in the blood spot punch. Two main approaches have been described to evaluate the corresponding serum volume; the application of chemical tracers and geometric calculation (128).

Chemical tracers, such as $^{125}$I-albumin, $^{51}$Cr-haemoglobin and $^{125}$I-L-thyroxin, have been used to estimate the equivalent serum volume of the blotted whole blood punch. In this process, the serum volume is determined by comparing radiochemical counts of the blood spot punch with the known volume of whole blood samples in the liquid phase (239). Geometric calculation has been applied to evaluate the ratio of the punched disk to the known volume of the entire spotted whole blood with the pre-measured haematocrit (305, 306). However, application of this approach does not fully take into account the chromatographic effect of the blood distribution on the filter paper, and still raises issues of analytical bias for the final measurement (307). Which is why it is recommended that the punch is taken from the centre of the spot. Considering the fact that these common methods are not applicable to archived DBS samples, direct estimation of Hb concentration on the DBS punch is an alternative but is currently not described in the literature.

1.3.6 Conclusions

The initial widespread application of DBS was utilised for newborn screening. More recently, it has been applied more broadly. Mass spectrometry-based applications are the dominant techniques for
DBS analysis, and liquid chromatography separation is more popular than gas chromatography. Drug monitoring (therapeutic and toxicology) and pharma-toxico-kinetics studies are the major application groups outside of newborn screening. For many analytes, method validation and further bridge experiments are required to develop adjustment rules to convert the results obtained from DBS analysis to the equivalent serum/plasma values. Likewise, establishment of robust reference intervals or decision limits is essential for DBS analytes. It is envisaged, with the inherent advantages of the alternative DBS sampling technique compared to the classic plasma-based strategies, in future micro-sampling-based assays will certainly play a substantial role for analysis of biomarkers.”
1.4 Research Project hypotheses, aims and questions

This research project hypothesises that; vitamin D insufficiency at birth, predicts the higher risk of allergic diseases in early childhood. To address this hypothesis, this project aims to; 1) Develop an accurate method for measurement of vitamin D metabolites from archived dried blood spot samples, 2) Investigate the association between early life vitamin D insufficiency and paediatric food allergy.

Research Questions

1. Can the current dried blood spot sample derivatisation and preparation technique be optimised for LC-MS/MS measurement method?

2. What is the sample specific distribution of vitamin D at birth in a population-based cohort of metropolitan Melbourne?

3. Are vitamin D levels at birth associated with subsequent IgE-mediated food allergy at age 12 months?
Chapter 2

General materials and methods
2 General materials and methods

This chapter defines the design and requirement of the samples, general equipment / tools, chemicals and data analysis procedures utilised in this project. This chapter also includes the details of method validation and population study sample resources, collection, storage and preparation processes. An additional methodology section is included in each chapter to detail specific material and methods used for the study.

2.1 Sample collection and storage

Dried blood spot (DBS) samples were retrieved from a biobank of archived Newborn blood spot Screening (NBS) Guthrie card samples from the Victorian Clinical Genetics Service (VCGS) biobank. These 2700 samples were collected as part of the NBS program and consent for this research was obtained from the guardians. These DBS specimens have been collected from two internationally unique NHMRC funded population cohorts (HealthNuts ref. 1006215 - RMIT ethics approval number: 0000019315) from participants at birth (≤ 3 days old). The NBS sample collection dates varied between 15/03/2006 and 14/08/2010. The participant population includes 1315 female and 1385 male newborns.

DBS samples were collected on the Whatman 903 filter paper collection cards (one drop on each collection area, air dried) and stored in archive boxes at room temperature. In September 2014 Guthrie cards were retrieved from archive boxes, and two 3.2 mm punches were collected in two sets of 96-well plates (one with flat the other with conical bottom). Each DBS punch-plate was covered plates with the web-sealer and stored in archive boxes at room temperature until examination time. DBS samples were ideally punched from the centre of the blood spot (one spot per each plate-well), and in case of unavailability of two intact blood spots, were consistently punched from the edge for both punches. Observations and discrepancies related to the blood spot quality and punch point were recorded. The blank filter paper punch was collected if there was no suitable blood spot available for collection (Figure 2.1).
Whole blood samples (n=80, de-identified human sourced) for method validation studies were collected from the Royal Children Hospital (RCH) Laboratory Services archive (patient’s median age = 10 years) and Austin Pathology (patient’s median age = 63 years). Fresh EDTA specimens (less than 48 hs old) were retrieved from and stored at 4°C before blotting on collection cards (Whatman 903) or separation of plasma. One drop (50 µl) of whole blood was spotted on each spotting area (four per card), then air dried for 2 hours and stored at room temperature cabinet (in plastic specimen storage zipper-bag) until analysis. Plasma samples were prepared by centrifugation of whole blood at 3000 rpm for 5 minutes and stored in -20°C until examination time.

### 2.2 Equipment and tools

Three main sets of equipment were utilised for this project: 1) sample collection and preparation materials, 2) sample analysis equipment and 3) data processing tools.
2.2.1 Sample collection

Study subject DBS samples, in-house quality control and method development/validation samples were collected on FDA proven collection cards; Whatman 903. Semi-automated and manual punchers were used to collect the DBS disk of the spotted samples; 3.2 and 6 mm manual punchers and 3.2 mm semi-automated (Perkin Elmer Precisely 1296-071 Delfia dried blood spot punch, Wallac DBS pincher). VCGS-NBS lab standard operation procedure was followed to punch Guthrie cards. DBS punches were collected, stored and analysed in 96-well plates; Thermo Scientific flat-bottom clear 96-well plate, Cat no. 9502227 for spectrometric scanning and Greiner Bio-one v-bottom 96-well microplates (part no. 65/201). Plates containing DBS punches were covered by a closing-mat for 96-well plate sourced from Agilent Technologies (Santa Clara, California, United States, part no. 5042-1389) for storage and analysis.

Whole blood samples were collected in EDTA contained collection tube: (Becton Dickinson, VIC, Australia, part no. 369651 and 367873). Whole blood and plasma samples were transported and stored in microcentrifuge propylene tubes (1.5mL, Cat no. 72.690.001) supplied by Sarstedt (Adelaide, Australia).

2.2.2 Sample analysis

A range of different equipment and tools were utilised for different studies including; sample preparation, haemoglobin derivatives estimation and vitamin D measurement tools.

Sample preparation

Heraeus centrifuge multifuage 1 S-R and temperature adjustable laboratory oven were sourced from Thermo Scientific (VIC, Australia). Dry block heater, 3x4 nozzle gas manifold, roller mixer and vortex mixer were obtained from Ratek (VIC, Australia). A set of pipettes including 1-25, 20-200, 200-1000 and 10-300 µl were obtained from Thermo Scientific Finnpipette (VIC, Australia) and Eppendorf (NSW, Australia). A water purification system (Milli-Q Direct 8) was sourced from Merck Millipore.
(VIC, Australia) and an electronic balance (Mettler AT261 Deltarange®) used for weight measurement was sourced from Mettler (VIC, Australia). A UCT 69-well plate positive pressure manifold (UCT inc. Bristol, USA, part no. VMFPPMIK) provided by PM Separations (NSW, Australia) was used for the sample extraction process and supported liquid extraction (SLE) plates (Biotage, Uppsala, Sweden, Cat no. 820-0200-P01) were obtained from Shimadzu Oceania (NSW, Australia) for sample purification. Extracted samples were collected, derivatised and loaded into the LC-MS/MS in the Agilent 96-deep well plate (1 ml) (part no. 5042-1389). A rotational vacuum concentrator (RVC 2-33 CP Plus, CHRIST, Osterode, Germany, part no. 101233) supplied from John Morris Scientific (VIC Australia) was used for extracted sample drying. An ultrasonic bath (40kHz, Unisonics, Part No; FXP) obtained from Unisonics Australia (NSW Australia) was utilised for blood spot sample elution and extraction.

General laboratory glassware items were sourced from Schott-Duran (NSW, Australia) and the Pacific laboratory products (Melbourne, Australia) supplied the KIMAX glass tubes (13 × 100 mm, Cat no. 45066A-13100). Glass Pasteur pipettes (Cat no. NAAU92501.01) were acquired from the Merck (VIC, Australia). The screw cap Amber vials (Cat no. 5183-2081) and flat bottom glass inserts (400 μl, Cat no. 5181-3377), were procured from the Agilent Technologies.

**Haemoglobin derivatives estimation**

A PerkinElmer - EnSpire Multimode Plate Reader (2300) using wavelengths of 520-600 nm with band pass width of 8nm was utilised for the spectrophotometric scanning at 540 nm wavelength.

**Vitamin D measurement**

A Liquid chromatography tandem mass spectrometry (LC-MS/MS) system including; Agilent 1290 infinity LC and Agilent 6490 triple quadrupole mass spectrometer supplied from Agilent Technologies (Santa Clara, CA, United States) was used for vitamin D (native and derivatised) metabolites quantification. The columns used for liquid chromatography procedures are: Pursuit Pentafluorophenyl (PFP) column (100 mm × 2 mm× 3 μm, Cat no. A3051100X020), MetaGuard column 2.0 mm Pursuit 3 μm PFP (Cat no. A3051MG2) from Agilent Technologies. The core-shell column (2.6μm, 2.1 mm
x100mm, Kinetex F5, 100A, Cat no. 00D-4723-AN), Security Guard ULTRA Cartridges (UHPLC F5 for 2.1 mm ID columns, Cat no. AJ0-9322) were purchase from PHENOMENEX (Torrance, CA, USA).

Performance specifications of the liquid chromatography and the tandem mass spectrometry systems used in this project for measurement of vitamin D are summarised in table 2.1 and 2.2. Please note that the general concept and structure of these tables has been adopted from Ali Al-Bahrani’s PhD thesis (308).

Table 2.1. Performance specifications of Agilent LC-1290.

<table>
<thead>
<tr>
<th>Specification*</th>
<th>Agilent LC-1290 (309-311)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pump</strong></td>
<td></td>
</tr>
<tr>
<td>Hydraulic system</td>
<td>Two dual pistons in series, pumps with proprietary servo-controlled variable stroke design and smooth motion control</td>
</tr>
<tr>
<td>Settable flow range</td>
<td>0.001 – 5 mL/min, in 0.001 mL/min increments</td>
</tr>
<tr>
<td>Flow precision</td>
<td>≤0.07 % RSD</td>
</tr>
<tr>
<td>Flow accuracy</td>
<td>±1%</td>
</tr>
<tr>
<td>Pressure operating range</td>
<td>Binary Pump: up to 120 MPa (1200 bar) up to 2 mL/min, 80 MPa (800 bar) at 5 mL/min Binary Pump VL: up to 105 MPa (1050 bar) up to 2 mL/min</td>
</tr>
<tr>
<td>Pressure pulsation</td>
<td>&lt; 1 % amplitude</td>
</tr>
<tr>
<td>Gradient formation</td>
<td>High pressure binary mixing</td>
</tr>
<tr>
<td>Delay volume</td>
<td>JetWeaver V35: &lt; 45μL, JetWeaver V100: &lt; 75μL</td>
</tr>
<tr>
<td>Composition range</td>
<td>Settable range: 0 - 100%</td>
</tr>
<tr>
<td>Composition precision</td>
<td>&lt; 0.15 % RSD or 0.01 min SD</td>
</tr>
<tr>
<td><strong>Autosampler</strong></td>
<td></td>
</tr>
<tr>
<td>Injection range</td>
<td>0.1–20 μL in 0.1 μL increments</td>
</tr>
<tr>
<td>Injection precision</td>
<td>&lt;0.3% from 5–40μL, &lt;0.5% from 2–5μL, &lt;0.7% from 0.5–2μL</td>
</tr>
<tr>
<td>Sample viscosity range</td>
<td>0.2–5 centipoise</td>
</tr>
<tr>
<td>Injection cycle time</td>
<td>Typically &lt; 21 s depending on draw speed and injection vol.</td>
</tr>
<tr>
<td>Carryover</td>
<td>Typically &lt; 0.004%</td>
</tr>
<tr>
<td><strong>Column Oven</strong></td>
<td></td>
</tr>
<tr>
<td>Temperature range</td>
<td>10 °C below ambient to 100°C</td>
</tr>
<tr>
<td>Temperature stability</td>
<td>± 0.05°C</td>
</tr>
<tr>
<td>Temperature accuracy</td>
<td>± 0.8°C</td>
</tr>
<tr>
<td>Column capacity</td>
<td>2 columns of 300 mm with individual solvent heating</td>
</tr>
<tr>
<td>Internal volume</td>
<td>3 μL left heat exchanger, 6 μL right heat exchanger</td>
</tr>
</tbody>
</table>

*These performance specifications are based on data analysed by Agilent technologies.

Table 2.2. Performance specifications of Agilent MS/MS-6490.

<table>
<thead>
<tr>
<th>Specification*</th>
<th>Agilent MS/MS-6490 (312)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitivity: 1pg reserpine* quantifying on m/z 609→195 using ESI positive mode</td>
<td>S/N &gt; 50,000:1</td>
</tr>
<tr>
<td>Sensitivity: 1pg chloramphenicol* quantifying on m/z 321→152 using ESI negative mode</td>
<td>S/N &gt; 50,000:1</td>
</tr>
<tr>
<td>Linear dynamic range</td>
<td>&gt; 6.0 x 10⁶</td>
</tr>
<tr>
<td>Mass range</td>
<td>5–1,400 Da</td>
</tr>
<tr>
<td>Maximum scan rate</td>
<td>12,500 Da/sec</td>
</tr>
<tr>
<td>Minimum MRM dwell time</td>
<td>1 milliseconds</td>
</tr>
<tr>
<td>MRM transitions</td>
<td>500 per TS, &gt;40,000 ion transitions per method</td>
</tr>
<tr>
<td>Polarity switching (from positive to negative)</td>
<td>30 milliseconds</td>
</tr>
<tr>
<td>Collision cell ion clearance</td>
<td>&lt; 1 milliseconds</td>
</tr>
<tr>
<td>Agilent Jet stream technology⁵</td>
<td>Yes</td>
</tr>
<tr>
<td>Agilent iFunnel technology⁵</td>
<td>Yes</td>
</tr>
</tbody>
</table>
# Agilent technologies use reserpine and chloramphenicol compounds to compare their MS/MS systems.

“Jet Stream sample introduction, providing high-efficiency ESI ion generation and focusing; a hexabore capillary; and a unique dual-stage ion funnel assembly. Together these technologies reduce neutrals and increase ion sampling to dramatically improve overall signal within the system, delivering significant increases in sensitivity compared with conventional instruments.” (313).

## 2.2.3 Operation of the LC-MS/MS system

The LC-MS/MS system was operated using Agilent MassHunter data acquisition software (version B.4.01) following the standard operation procedure document generated by RMIT MS lab research team and general operation manual published by LC-MS/MS research group, for the Agilent LC-MS/MS 1200/6400 that was available as a hardcopy in the LC-MS/MS laboratory also saved on a shared Google drive.

### 2.3 Reagents and chemicals

The reagents and chemicals used in this project were procured from different manufacturers and suppliers (Table 2.3). The following were obtained from Merck: ethanol gradient grade for HPLC, hexane for liquid chromatography, 2-Propanol for liquid chromatography, methanol for liquid chromatography and nitric acid 65% suprapur. Thermo Scientific also provided Optima methanol for liquid chromatography. Formic acid (purity ≈98%) was supplied by Sigma-Aldrich. Seracon human plasma matrix match diluent (Material no.1800-0027) was purchased from SeraCare. The high purity grade/ extra high pressure/ compressed nitrogen (Purity ≥ 99.99%) was supplied by BOC. Derivatising reagents including Amplifex™ Diene and 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) were purchased from Sciex and Sigma-Aldrich respectively.

Pure compounds and internal standard materials were sourced from different manufacturers. Retinol (purity ≥95%) and lyophilised human haemoglobin were obtained from Sigma-Aldrich. 25-hydroxyvitamin D3 (purity ≥98%), 3-epi-25-hydroxyvitamin D3 (purity ≥98%), 25-hydroxyvitamin D3-[1H3] (purity ≥98%), 25-Hydroxyvitamin D3-[13C5], 25-Hydroxyvitamin D3-[2H6]-Monohydrate and 3-Epi-25-Hydroxyvitamin D3-[13C5] were purchased from IsoSciences.
### Table 2.3. List of reagents and chemicals used in the project.

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Cat*/order no.</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-hydroxyvitamin D2/D3 Calibrator set</td>
<td>62039</td>
<td>ChromSystems (Munich, Germany)</td>
</tr>
<tr>
<td>25-hydroxyvitamin D3 (CAS** No 19356-17-3)</td>
<td>S4163UNL</td>
<td>IsoSciences (Ambler, USA)</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D3-[13C5]</td>
<td>S13125-0.1</td>
<td>IsoSciences (Ambler, USA)</td>
</tr>
<tr>
<td>25-hydroxyvitamin D3-[2H3] (CAS No 140710-94-7)</td>
<td>S4163</td>
<td>IsoSciences (Ambler, USA)</td>
</tr>
<tr>
<td>25-Hydroxyvitamin D3-[2H6]-Monohydrate</td>
<td>S13085</td>
<td>IsoSciences (Ambler, USA)</td>
</tr>
<tr>
<td>2-Propanol (CAS No 67-63-0)</td>
<td>1.01040.2500</td>
<td>Merk (Darmstadt, Germany)</td>
</tr>
<tr>
<td>3-epi-25-hydroxyvitamin D2/D3 Calibrator set</td>
<td>62029</td>
<td>ChromSystems (Munich, Germany)</td>
</tr>
<tr>
<td>3-epi-25-hydroxyvitamin D3 (CAS No 73809-05-9)</td>
<td>S7004</td>
<td>IsoSciences (Ambler, USA)</td>
</tr>
<tr>
<td>3-Epi-25-Hydroxyvitamin D3-[13C5]</td>
<td>14041</td>
<td>IsoSciences (Ambler, USA)</td>
</tr>
<tr>
<td>4-Phenyl-1,2,4-triazoline-3,5-dione (CAS No 4233-33-4)</td>
<td>42579</td>
<td>Sigma-Aldrich (VIC, Australia)</td>
</tr>
<tr>
<td>Amplifex™ Diene</td>
<td>5037804</td>
<td>Sciex (Framingham, USA)</td>
</tr>
<tr>
<td>Compressed nitrogen (N5.0)</td>
<td>Gas Code: 035</td>
<td>BOC (VIC, Australia)</td>
</tr>
<tr>
<td>Ethanol (CAS No 64-17-5)</td>
<td>1.11727.1000</td>
<td>Merk (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Formic acid (CAS No 64-18-6)</td>
<td>94318</td>
<td>Sigma-Aldrich (VIC, Australia)</td>
</tr>
<tr>
<td>Hexane (CAS No 110-54-3)</td>
<td>1.04391.2500</td>
<td>Merk (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Liquid Meter Trax control</td>
<td>970-211870</td>
<td>BioRad Laboratories (California, USA)</td>
</tr>
<tr>
<td>Lyophilised human haemoglobin</td>
<td>H7379</td>
<td>Sigma-Aldrich (VIC, Australia)</td>
</tr>
<tr>
<td>Methanol (CAS No 67-56-1)</td>
<td>1.06018.4000</td>
<td>Merk (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Nitric acid 65% suprapur (CAS No 7697-37-2)</td>
<td>1.00441.1000</td>
<td>Merk (Darmstadt, Germany)</td>
</tr>
<tr>
<td>Optima methanol (CAS No 67-56)</td>
<td>A456-4</td>
<td>Thermo Fisher Scientific (Waltham, USA)</td>
</tr>
<tr>
<td>Quality Assurance Program materials</td>
<td></td>
<td>Royal College of Pathologists of Australasia (NSW, Australia)</td>
</tr>
<tr>
<td>Retinol (CAS No 68-26-8)</td>
<td>R7632</td>
<td>Sigma-Aldrich (VIC, Australia)</td>
</tr>
<tr>
<td>Seracon human plasma matrix match diluent</td>
<td>1800-0027</td>
<td>SeraCare (Milford, USA)</td>
</tr>
<tr>
<td>Serum quality control for vitamin A</td>
<td>10081-10082</td>
<td>UTAK Laboratories Inc. (Valencia, USA)</td>
</tr>
<tr>
<td>Vitamin D quality control</td>
<td>10060-10061-10062</td>
<td>UTAK Laboratories Inc. (Valencia, USA)</td>
</tr>
<tr>
<td>Vitamins A and E calibrator set</td>
<td>3.004</td>
<td>ChromSystems (Munich, Germany)</td>
</tr>
</tbody>
</table>

* Cat no; Catalogue number ** CAS No; Chemical Abstracts Service number

### 2.4 Chemical preparation

#### 2.4.1 Preparation of solutions for liquid chromatography

**Needle wash solution**

To minimise the potential carry-over contamination and clean the auto-sampler needle, needle wash solution was used prior to each sample injection into the LC-MS/MS. The needle wash solution
contained 50% methanol, 10% 2-propanol and 40% water, which was prepared fortnightly and stored at ambient temperature.

**Mobile phases**

Two mobile phases were utilised for the reversed-phase chromatography. The hydrophilic mobile phase consisted of 0.1% formic acid and 2% methanol in Milli-Q water. The organic mobile phase consisted of 0.1% formic acid in methanol.

**2.4.2 Reagent preparation**

All pure compounds and reagents were prepared in amber or aluminium-foil-wrapped glass vials/bottles under the fume hood.

**25-hydroxyvitamin D3 and 3-epi-25-hydroxyvitamin D3 stock solutions**

Stock 1: 25-hydroxyvitamin D3 (12,480 nmol/L) and 3-epi-25-hydroxyvitamin D3 (253,338 nmol/L) were provided in sealed glass ampoules (in 1 mL of ethanol). Stock 2: was prepared by diluting 25-hydroxyvitamin D3 and 3-Epi-25-hydroxyvitamin D3 stock 1 with HPLC grade methanol (10,000 nmol/L). Stock 1 ampoule content was transferred and diluted into an Agilent amber glass vial, which the cap was tightly wrapped with the parafilm and stored at -20 °C.

**Internal standard (25-Hydroxyvitamin D3-[2H3], [3H6], [13C5] and 3-epi-25-Hydroxyvitamin D3- [13C5]) stock solutions**

Stock 1; the internal standard solutions were supplied in sealed glass ampoule (1mL) with 1 g/mL in ethanol. Stock 2; stock 1 was transferred into an aluminium-foil-wrapped glass bottle containing 49 mL of HPLC grade methanol to prepare 50 mL of 1/50 diluted material. Stock 3 (working solutions); was prepared by diluting stock 2 with the HPLC grade methanol (1/20 dilution) at concentrations of 247.75, 246.3 and 246.91 nmol/L of [2H3], [3H6] and [13C5] internal standards respectively.
**Retinol stock solution**

Stock 1: retinol crystal was dissolved into HPLC grade methanol at a 1,000 μmol/L concentration. Stocks 2 and 3 were prepared by diluting stock 1 with HPLC grade methanol at 100 μmol/L and 10 μmol/L concentrations respectively.

**Vitamin D, A and IS mixture in methanol**

Spiked methanol (with vitamin D, A and IS) was used for LC-MS/MS method development and system performance check prior to each analytical batch. Spiked methanol was prepared by diluting each stock solution with HPLC grade methanol to prepare a mixture with concentrations of; 100 nmol/L, 60 nmol/L, 150 nmol/L, 10 μmol/L of 25-OHD3, epi-25-OHD3, 25-OHD3-d6 and retinol respectively. This mixture was stored at -20 °C.

**Derivatising reagents**

As part of the method development process for measurement of vitamin metabolites from DBS samples we trialled PTAD to derivatise and enhance the assay sensitivity. Its working solution was prepared with the concentration of 0.1 g/mL in acetonitrile. The working solution was stored at 4 °C with the stability time of 8 weeks.

Amplifex™ Diene working solution reagent was prepared by the addition of 3 ml of diluent (acetonitrile) into the reagent bottle (contains 1 gr dry Amplifex™ Diene) and was vortexed for 20 seconds followed by 20 seconds sonication. Working solution was stored at -20 °C while was stable for 4 weeks.
2.4.3 Calibrators

Commercial calibrator sets for vitamin D metabolites and vitamins A were sourced from Chromsystems, while dried calibrator spot standards were prepared in-house using Sigma human haemoglobin material.

In-house DBS haemoglobin calibrators

In-house calibrators were prepared from Sigma lyophilised human haemoglobin in milli-Q water. A five-point calibration curve was generated using in-house Hb standard materials (50, 100, 150, 200, 250 g/L). 50µl of each level of standards were spotted on the collection cards (Whatman 903) and air-dried overnight at room temperature (RT).

Commercial calibrators

Lyophilised commercial calibrators were used in this project. Lyophilised serum calibrator sets for 25-hydroxyvitamin D2/D3 (6 Plus1 Multilevel Serum Calibrator set, Masschrom), 3-epi-25-hydroxyvitamin D2/D3 (3 Plus1 Multilevel Serum Calibrator set, Masschrom) and vitamins A and E were purchased from ChromSystems (distributed by PM separations - QLD, Australia). These calibrator sets are traceable to the National Institute of Standards and Technology-standard reference material 972 (NIST972a) (314). Lyophilised calibrators were reconstituted in milli-Q water following the manufacturer’s instructions and the labelled aliquots (in polypropylene microcentrifuge tubes) stored at -20 °C.

2.4.4 Controls

Separate commercial sets of quality control (QC) material for vitamin D metabolites and vitamins A were sourced from UTA, while dried quality control spots were prepared using BioRad human blood control materials and in-house DBS controls.
In-house DBS quality controls

In-house QC sets were prepared for DBS vitamin D and haemoglobin. These control materials were concurrently prepared with the unknown human blood samples at the beginning of the study. Results from the controls were used to monitor the assay performance. The In-house QC was prepared from 50 µl spotted unknown human whole blood sample on the filter paper and air-dried overnight at RT.

Commercial quality controls

Liquid and lyophilised controls were used in this project. Lyophilised serum quality control sets for vitamin A (low and high) and for vitamin D (low, medium and high) were procured from UTAK Laboratories Inc. Liquid three-level human blood quality control material (Meter Trax control, low, mid, high) were purchased from BioRad Laboratories.

External quality control materials were supplied by the Royal College of Pathologists of Australasia (RCPA). RCPA quality assurance program (QAP) for endocrine and vitamins materials were utilised for the method general performance testing. RCPAQAP lyophilised materials were reconstituted in milli-Q water following the manufacturer’s instructions. Samples were analysed after preparation and the excess were stored at 4 °C and -20 °C for retesting (if it was required).

2.5 Glassware cleaning

Glassware was well-maintained and cleaned regularly meeting the requirements of the mass spectrometry analysis and standard laboratory practice. All cleaned reusable glassware was kept in capped containers and allocated cabinets.

Initially bottles and reusable glassware including KIMAX screw cap culture glass tubes were washed with Milli-Q water and rinsed with methanol prior drying in the 50°C oven (to avoid diluting the nitric acid in the following step). Nitric acid solution (65%) and Milli-Q water were used to clean and rinse the glassware respectively prior to the final step of hot oven drying (Appendix 8.1).
2.6 Safety

The biological samples, medical waste and chemicals were handled and disposed following the laboratory safety procedures. The biological samples used included human samples, calibrators, quality controls and external quality assurance materials. These biological specimens need to be considered as potentially infectious agents and were handled and disposed based on the RMIT University, School of Health and Biomedical Sciences (SHBS) laboratory safety procedures (available online and hardcopies accessible in the LC-MS/MS laboratory). Safety Data Sheet (SDS) and risk assessments procedures (provided by the manufacturers and/or created at the commencement of the project) were followed to safely handle the chemicals. A hard copy and electronic copy of safety procedures and documents were available in the LC-MS/MS laboratory and a shared Google drive. Biohazardous waste and disposable labware were also disposed according to the SHBS laboratory waste disposal management strategy procedure (315).

2.7 Data analysis

Agilent MassHunter data acquisition, quantitative analysis (version B.05.00/B5.0.291.0) and qualitative analysis (version B.04.00) software were used for LC-MS/MS data analysis. Agilent LC-MS/MS 1200/6400 general operation manual was available in the LC-MS/MS laboratory. Collected data was stored and backed-up in the main computer of the LC-MS/MS laboratory and an external memory drive; both of which are password protected.

Microsoft Excel software (scatter chart, trend-line equation) was used for calibration curve generation and Hb-drv concentration calculation. “Method Validator” software (Philippe Marquis 1999, version; 1.19) was used for accuracy testing and method correlation studies (Linear regression, Passing-Bablok and Difference Plot). Assay linearity was assessed with LinChecker software (Philippe Marquis 2001, version; 1.1.2.0).
Chapter 3

Determination of Haemoglobin Derivatives in Aged Dried Blood Spots to Estimate Haematocrit
3 Determination of Haemoglobin Derivatives in Aged Dried Blood Spot to Estimate Haematocrit

The levels of the haematocrit and haemoglobin at extreme high or low ends may influence the accuracy of the measurements quantification of the DBS sample. To alleviate the potential errors associated with the dried matrix of DBS sample which could span the total testing process, we aimed to predict the haematocrit of the punched dried blood spot through primary spectrophotometric estimation of its haemoglobin content. This correction could be incorporated into DBS standard operation procedures for measurement of different types of biomarkers.

This chapter has been peer reviewed and published with contribution of other co-authors as an analytical full paper (316). My contribution to this study and subsequent publication was included; developing the concept for the study in conjunction with my senior supervisor (RG), performing the experiments and method development, analysis and interpretation of data, writing the first draft of the publication and reviewing and incorporating all suggestions from the co-authors.

3.1 Introduction

“The micro-sampling technique associated with dried blood spot (DBS) samples is considered to be less invasive, requiring only a small sample volume (usually capillary) applied to filter paper, that is easy to transport and store with a lower risk of infection transmission (119, 121, 317). These advantages have prompted the interest in utilising DBS for clinical assessment outside of newborn blood spot screening (NBS). As such, DBS applications now encompass analytes related to clinical diagnosis, epidemiological studies, therapeutic drug monitoring, pharmacokinetics and toxicokinetic studies (10). However, the accuracy of DBS quantitation requires additional considerations over the conventional “liquid” blood sample. This is due to the physical characteristics of the blotted whole blood sample affecting the quality of the measurement. In particular, potential irregular errors associated with haematocrit (Hct) span the DBS total testing process:
Pre-analytically, the levels of a patient’s haemoglobin (Hb), and therefore Hct, at extreme high or low ends may influence the viscosity and fluidity of the spreading sample (241). Hence, there would be sample volume dissimilarity for a fixed size punch from the DBS originating from whole blood samples with different levels of Hct (Figure 1) (10, 240, 241, 254).

Analytically, Hct can influence the extraction of a measurand from the filter paper. This has been demonstrated in the quantitative bio-analysis of drugs where Hct has been identified as a causal factor of assay bias due to variations in the efficiency of the extraction process (305).

Post-analytically, the reference intervals and decision points for biomarkers and clinical assessments are predominantly described for samples with a liquid matrix (serum/plasma). The accurate conversion of dry matrix results (i.e. from the DBS) requires a consistent relationship with the liquid form (serum/plasma). Hence it is useful to know the Hct value (306).

DBS laboratories are often keen to know the Hct in order to account for, and ideally correct for, associated sample variation across the total testing procedure. The additional collection of a liquid sample (venous or capillary) for Hct measurement at the time of the DBS collection is recommended (318). However, there are many archived DBS samples that have limited biological information (including Hct), where prediction of the spotted blood Hct level is warranted. As such, the indirect (e.g. potassium measurement) or direct determination of Hct from the DBS cards is proposed (319-323).

To alleviate the DBS-Hct problem, scientists have employed a variety of correction techniques. The strategies used can be broadly divided into the following groups: 1) measuring the Hb and/or Hct
independently at the time of collection from a whole blood liquid sample; 2) determining the Hb and/or Hct from the DBS sample; or 3) not determining Hb and/or Hct at all. Where a Hct correction option is sought, practical solutions to minimise bias have been devised. These include: analysis of the entire blood spot; determination of the spotted blood volume by weighing the whole spot; using dried plasma collection; pre-cutting the filter paper prior to application of the blood; assessment or prediction of Hct from the spotted whole blood; and normalising the DBS measurement using the average value of Hct for males/females (10, 167, 318, 324). Whilst each of these correction approaches has merit, none have been widely adopted due to inherent limitations within the individual approach.

Over time, Hb converts to various Hb derivatives (Hb-drv) (Figure 3.2). Estimation of Hb-drv from the actual DBS punch could potentially be used as a surrogate for Hct to account for physiologically low or high Hct levels. We propose this might be possible spectrophotometrically using the eluted (solubilised) punched DBS (hence taking into account both diffusion and extraction differences). In this study, we aimed to predict the Hct of the punched archived DBS through primary spectrophotometric estimation of its Hb-drv content. Successful confirmation of this hypothesis would enable this technique to be incorporated into DBS standard operation procedures, allowing the laboratory to account for Hct in association with their DBS analyte of interest.

**Figure 3.2.** Haemoglobin is an oxygen transport protein in the form of oxyhaemoglobin (Hb-O₂). As soon as fresh whole blood is air exposed outside the body, Hb is completely oxygen saturated and converts into Hb-O₂. Over time, Hb-O₂ is auto-oxidised to met-haemoglobin (met-Hb). Met-Hb eventually denatures into heme-chromes (HC) (known as the most stable Hb derivative). HC is a common name for group of low spin forms of the ferri-Hb (met-Hb) constituting ferri-alpha / beta subunits. The distinct feature of the met-Hb is its six liganded state of the haem iron that formed through the separate alterations of the globin conformation so that atoms endogenous to protein became bound as a sixth ligand of the haem iron. The abundant majority of HC have a proximal (F8) and a distal (E7) His as fifth and sixth ligands respectively (325, 326). Following our observations using direct spectrometry method after week 8 HC is measured.
3.2 Materials and methods

3.2.1 Subjects

Study samples

One hundred and twenty de-identified venous whole blood ethylenediaminetetraacetic acid (EDTA) samples were sourced from two laboratories; Laboratory A - the Royal Children’s Hospital Melbourne (RCH) Laboratory Services (Approval no. S73) (n=60, age range: 9 month – 90 years, median age: 10 years). Laboratory B - Austin Pathology (Approval no. 16.3162) (n=60, age range: 26-81 years, median age: 49 years). Eight of the 60 samples from Laboratory B were specifically used for the initial proof of concept studies and 32 samples from Laboratory B were used to assess punch location; leaving 80 samples (Lab A: n=60 and Lab B: n=20) used for the remaining method development and evaluation studies. All specimens were previously collected by the two hospitals from the in-patients (signed the collection form including informed consent for research purposes) for routine analysis. Information related to age, gender and the laboratories Hb and Hct results were provided. The Hb and Hct levels were determined on the laboratories’ (Siemens ADVIA® 2120i -Lab A and Sysmex XE-5000 –Lab B) instruments. Both laboratories routinely participate in the Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) for Haematology and are accredited to ISO15189-2012. The whole blood samples, prior to initial retrieval, were stored post analysis by the pathology laboratories for up to 48 hours or, 24 hours at 4°C by Lab (A) and (B) respectively.

The retrieved EDTA sample tubes were mixed on a roller mixer for 30 minutes followed by 20 times using “figure 8” hand mixing, prior to blotting on the collection cards. 50 µL of whole blood was spotted on the filter paper (Whatman 903) (eight spots for each sample), then air-dried at room temperature (RT) overnight. To minimize and prevent pre-analytical errors affecting the quality of DBS analysis, we followed (and advise) the recommended standards for blood spot preparation including DBS quality, collection card choice, blood collection technique, transport and storage (10). For note, whilst capillary blood is the standard approach for DBS collection, studies have demonstrated that
variation of measured total Hb from capillary and venous samples is within the acceptable range for screening purposes (327-329). We spotted the whole blood samples onto Whatman 903 paper. This filter paper follows the CLSI recommendation and is approved by the Food and Drug Administration (FDA), Newborn Screening Quality Assurance Program (NSQAP) and also the Centers for Disease Control and Prevention (CDC) (234, 239).

**Calibrators**

Lyophilised human haemoglobin (Sigma-Aldrich, Australia, Cat no. H7379) reconstituted in milli-Q water was used as in-house calibrator. A four-point calibration curve was generated using the in-house Hb standard materials (100, 150, 200, 250 g/L). 50 µL of each level of standard was spotted on the Whatman 903 collection cards and air-dried overnight at RT (Figure 1). Calibrators were prepared and spotted at the same time as the first set of study samples were collected and spotted. In addition, the same calibrator batch was used across the entire study.

**Internal quality control (IQC)**

Commercial IQC (BioRad liquid tri-level human blood quality control material - Cat no. 970X) were spotted on the collection card and air-dried overnight at RT. The IQC material was prepared, and 50µl spotted at the same time and under the same storage conditions as the calibrators and other study samples. The In-house DBS control was prepared 12 months prior to the study commencement date.

**3.2.2 Measurement**

**Pilot study**

The calibrator set along with the commercial and In-house IQC materials and eight pilot whole blood samples were used for the primary method development steps. These were run weekly for 14 consecutive weeks. The aim was to observe the specimen behaviour over time in order to predict the appropriate time of analysis after spotting. Based on our observations and outcomes of this pilot study, a long-term stability testing procedure and other method validation studies were developed.
Sample preparation

100µl of extraction solution of deionised water containing 0.1% formic acid (Sigma-Aldrich, Australia, Cat no. 5.33002) was added to one 3.2 mm DBS. All DBS samples were punched peripherally except for the “effect of punch point on measurement” study. The samples were subjected to 30 minutes shaking at ambient temperature followed by 10 minutes sonication. 85 µL of the sample eluate was then transferred into the standard flat-bottom clear 96-well plate (Thermo Scientific, Australia, Cat no. 9502227) for spectrometric scanning.

Spectral scan and photometric measurement

A PerkinElmer - EnSpire Multimode Plate Reader (2300) using wavelengths of 520-600 nanometre (nm) with band pass width of 8 nm was utilised for the spectrophotometric scanning. Sixty patient samples in conjunction with calibrators and IQC material were prepared as described above and scanned between 520 and 600 nm. Optical density values for all subjects were plotted against the wavelength to investigate the corresponding optimum absorbance. The wavelength of 540 nm was selected for Hb-drv quantitation.

Method evaluation studies

Method evaluation studies were performed on DBS samples between 8 to 12 weeks post spotting time, unless otherwise specified in the relevant section.

Imprecision

The Intra-assay imprecision study was performed across three measured Hb-drv concentrations (average 123, 191 and 227 g/L). The Hb-drv concentration of each sample was estimated in twenty replicates within the same run and the coefficient of variation (CV) calculated as a percentage (CV%). The IQC samples’ results, across the 26 distinct analyses, were used to estimate the inter-assay imprecision.
**Linearity**

One human whole blood sample was used to generate two aliquots of extremely low and high levels of Hb (20 and 270 g/L). Whole blood was spun, and the red cell fraction and separated plasma were mixed in different proportions to generate the highest and lowest levels. Then the two samples were serially mixed at different ratios to generate a final nine-level set (20.00, 51.25, 82.50, 113.75, 145.00, 176.25, 207.50, and 270.00 g/L) to study linearity on human blood matrix-match DBS sample. DBS samples were processed in duplicate as described above. LinChecker software was used for linearity testing.

**Stability**

A total of 60 samples were measured to determine the Hb-driv concentration. Analysis occurred in weeks 2, 8, 12 and 48 weeks of spotting. Measures were plotted against time to monitor the change over the 48 weeks period.

**Punch location**

To determine the effect of punch location on DBS Hb-driv results; each sample was double punched with punch 1) central-punch and 2) peripheral-punch, for the calibrators, controls (n=2) and samples (n=32). All were analysed 40 weeks post collection. The central-punch was used as the “true” value and the peripheral-punch results were compared to the central-punch results with both the central-punch as the calibrator and also the calibrator set from the peripheral-punch. Passing-Bablok regression and Bland-Altman difference plots were assessed to determine if the 95% confidence intervals (CI) demonstrated equivalence based on slope (where the CI should include 1.0) and intercept and mean difference (where the CIs should include zero). A Student Paired T-test was used to determine statistical significance where p<0.05 was deemed to be a significant difference.

**Method comparison**

The correlation study was performed on 80 DBS samples 48 weeks post spotting. Hb and Hct levels of the human whole blood samples tested by two laboratories were considered as the reference values.
Measured Hb-driv values were plotted against the fresh whole blood Hct values measured by the reference method to predict the linear equation and the 95% confidence intervals. DBS Hb-driv measures converted to their equivalent Hct using regression equation.

**Recovery study**

To determine the recovery a human whole blood sample collected on EDTA was used to generate a pair of diluted and spiked samples. Each sample included 450 µL of whole blood while sample A was spiked with 50 µL H₂O and sample B was spiked with 50 µL Hb standard material (250 g/L) (i.e. adding 25 g/L of Hb into the spiked sample). These samples were spotted onto filter paper and analysed 12 weeks post spotting. The calculation used to estimate recovery was:

\[
\text{Recovery} \% = \frac{(\text{Spiked DBS Hb-driv} - \text{Diluted DBS Hb-driv}) \times 100}{25} = \frac{(\text{Sample B Hb-driv} - \text{Sample A Hb-driv}) \times 100}{25}
\]

**3.2.3 Calculations and statistics**

The Ricos C, et al data-base and rules in desirable analytical quality specifications for imprecision, bias and total error for whole blood samples were utilised to evaluate the within-subject and between-subject CV values (330). Microsoft Excel software (scatter chart, trend-line equation) was used to generate the calibration curve and Hb-driv concentration calculation. The “Imathas Boxplot Grapher” software was used to create Whisker and box plot was utilised for the stability study. “Method Validator” software (Philippe Marquis 1999, version; 1.19) was used for accuracy testing and method evaluation studies (linear regression, Passing-Bablok and Bland-Altman difference plots). Assay linearity was assessed with LinChecker software (Philippe Marquis 2001, version; 1.1.2.0). The two-tailed t-test (Microsoft Excel software) was utilised to study the significance of the punch point of DBS samples.
3.3 Results

3.3.1 Spectral scan and measurement method

Our initial method development studies involved performing a spectral scan of all study samples over time. The spectral scan of the 60 study samples (collected from Lab A) on the second week of spotting showed the two maximum absorption peaks at 540 and 576 nm, corresponding to oxy-haemoglobin (Hb-O₂) as the major type of Hb present in fresh DBS samples (Figure 3.3.a). However, following the transition of Hb-O₂ into met-haemoglobin (met-Hb) and then hemi-chrome (HC) and other haemoglobin derivatives, the optimum optical absorption observed was at 540 nm for the aged DBS samples, IQCs and standard materials (Figure 3.3.b).

\[ \text{Absorbance} \]
\[ \text{Wavelength nm} \]

Figure 3.3. Spectral scan of study samples (n=60; from Laboratory A) between 520 and 600 nm at:
a) week 2 of spotting – Corresponding to the absorption spectra of Hb-O₂, b) week 8 of spotting – Hb is denatured and its absorption spectra follows HC pattern

3.3.2 Method evaluation studies

The calibration curves were plotted using In-house calibrators with the concentrations ranging from 100 to 250 g/L of haemoglobin (coefficient of determination \( r^2 = 0.99 \)) (Figure 3.4.a). The linearity experiment determined the reportable range for the Hb-driv assay. The assay was linear for whole blood samples from 82.5 to 207.5 g/L of haemoglobin (Figure 3.4.b). Biological range for Hb concentration for healthy individuals across the different ages and genders is between 90 – 175 g/L (331). Considering the range of calibration points and assay linearity testing data, the concentration within the linear range were deemed fit for purpose using the current method. The calculated intra-run coefficient of variation
(CV %) for different levels was; 3.6, 2.4 and 4.3 %. While the inter-run imprecision was calculated as a CV % of 7.9 and 7.5 for mean concentrations of 117 and 197 g/L respectively. The recovery was 84%.

Figure 3.4. a) Calibration curve; calibrators were prepared and spotted at the same time as other study samples were collected and spotted. The calibrator batch was consistent across the entire study. b) Linearity experiment using human whole blood demonstrating the method is linear between 82.5 and 207.5 g/L Hb-drv.

Figure 3.5. Stability testing results of the 60 DBS samples from Laboratory A analysed over 48 weeks of spotting. Analysis occurred in weeks 2, 8, 12 and 48. This report demonstrates that the samples are stable after week 8. a) Visual plot (vertical dash-lines represent the measurement time points), b) Whisker and Box plot with the box showing the 25th to 75th percentile and whiskers demonstration the lowest and highest results obtained at the time points.

The measured Hb-drv from DBS samples over the 48 weeks, demonstrated that the samples are stable after week 8 (Figure 3.5). Using the Passing-Bablok regression procedure, correlation coefficient (r) value was 0.88 for week 48 of post spotting (slope: 4.3, intercept: 26.6) (Figure 3.6). The second set of
samples were analysed at week 40 of the study using the central and peripheral punches of DBS samples. Comparison of the central and peripheral-punch location demonstrated agreement based on the slope 0.93, intercept 6.1 and difference -6.4 using the central-punch calibrator for both (and difference 2.6 using the central-punch calibrator for both). Using the calibrator punched from the respective samples’ punch locations, the agreement was slightly improved with the slope 1.046, intercept -1.2. The t-Test was also not significant with p>0.05.

Figure 3.6. Comparison of estimated Hb-drv (g/L) and equivalent Hct (calculated based on whole blood Hct and DBS Hb-drv relationship equation) (%) of the 48 weeks old DBS samples (n=80; from both Laboratories A and B). a) Passing-Bablok regression plot for comparison of measured Hb-drv from DBS versus whole blood Hct measure by the reference method (indicating regression line). The equation demonstrating the relationship is y = 4.3x + 26.6; r = 0.88. b) Bland-Altman plot demonstrating a mean difference of 2.4 % (95% CI: 1.8-3.0 %) between calculated DBS Hct (from the Passing-Bablok relationship equation) and measured Hct of original whole blood samples, demonstrated by the black solid line (---), compared to the expected difference of zero demonstrated by the red solid line (-----) and 95% range of difference as demonstrated by the two red dotted lines (- - -).

3.4 Discussion

We have successfully developed and validated a simple assay to estimate the Hct in aged DBS samples, such as those available from newborn screening programs. This assay can be used for bio-bank DBS samples that are at least two months of age. Prior to 8 weeks, the samples are still undergoing change so the level of hemi-chrome (HC) is unstable. The current DBS Hb-drv method can be directly incorporated into the measurement method, or alternatively the analysis can be done on a reserved DBS punch. This will depend on the extraction procedure of the analyte to be measured. We therefore suggest this approach for screening of low and high Hct and subsequent adjustment of the analyte results to improve their quantitation accuracy.
Previously other groups have attempted to correct for Hct effect on DBS analysis (10, 167, 318-324). The obvious approach is to measure Hct on the fresh whole blood at the time of collection. Recently, three studies have looked at Hb fractions, the first two used a spectrophotometric method for the determination of Hb in fresh DBS samples (one to 10 days post collection) from human and lamb blood however, as the Hb molecule on the DBS changes over time (due to instability of Hb), the Hb analysis of “fresh” DBS samples would be different to “archived” DBS samples (332, 333). A third study used non-contact diffuse reflectance spectroscopy technology with a quasi-isosbestic point of 589 nm for samples from 2 days to 5 months post spotting (323). Our method extends further providing a simple and robust assay for archived DBS analysis of Hb-drv that takes into account extraction efficiency and uses a spectrophotometer plate reader; an instrument readily available in most laboratories. In addition, this method is validated for analysis of DBS samples that extends to 48 weeks post spotting.

The starting rationale for our method development was based on the measurement of Hb in the plasma of haemolysed blood samples, as part of the serum/plasma indices measurement on a general biochemical analyser. DBS size for sample analysis varies from as small as one 3.2 mm punch (containing approximately 3 µL of blood) to the analysis of the whole blood spot (containing approximately 50 µL of blood). In either extreme, the DBS sample is highly diluted in the extraction solution as part of the sample preparation process (which includes matrix conversion from solid into the liquid) and the overall concentration of Hb in the extract is low. Likewise, the concentration of Hb in plasma (due to red blood cells haemolysis) is also low (compared to whole blood Hb); which is measured routinely using a direct spectrometric principle (334, 335).

The basic “Beer-Lambert” equation (A=εbc) principal has been previously employed for assessment of Hb concentration in tissue diagnosis (336). We therefore investigated the use of the Hb molar extinction coefficient and a direct spectrometric technique with calibrators for Hb-drv estimation. However, as the extinction coefficient is defined for fresh and intact whole blood samples, and our samples are air exposed (causing the conversion of Hb into its variants; Hb-O₂, met-Hb and HC), this approach was not possible; to achieve this we would need to know the ratio of the various derivatives and apply their
specific extinction $\varepsilon$ coefficients. Hence, we chose to develop aged DBS calibrators for incorporation into our method to generate a standard curve.

We have confirmed that the total Hb-drv concentration is directly measurable by using visible absorption spectroscopy. As the Hb-O$_2$ spectrophotometric band is known to be proportional to the concentration of Hb (337), we scanned for the expected absorption peaks in the visible region between 520-580 nm (338). Both foetal and adult haemoglobin variants (HbF and HbA) are known to follow similar optical absorption spectra patterns with the maximum absorbance at around 540 and 575 nm (339). Over time, Hb-O$_2$ under the influence of oxidants converts to met-Hb, and then transforms to HC resulting in a change in the spectral scan.

The transition time for Hb conversion to Hb-drv is unpredictable. The transition of Hb-O$_2$ into met-Hb and HC is rapid over the first 10 hours, after that the transition rate slows down over the period of 7-10 days (34). Also, the rate of Hb degradation (Hb-O$_2$ into met-Hb then HC) in dried samples become stable after 8 weeks (1500 hrs) of drying or spotting on the filter paper become stable and the change is then close to zero (35). Our stability data supports and extends these studies as we also demonstrate the stability of Hb-drv from 8 weeks and the validation of the Hb-drv for at least 48 weeks (323, 338-340).

Our observations from processing 60 human DBS samples shows the optimum optical density (quasi-isosbestic) to be at 540 nm, which represents a high positive correlation for measured Hb-drv versus the reference Hct. For comparison, in parallel over the three-month period we used two wavelengths for the direct spectrometric method calculation to estimate the total Hb fraction content of the DBS and convert it to its corresponding Hct level. Comparing the correlation coefficient of the measured Hb-drv using both wavelengths against the reference Hct, reading at 576 nm demonstrated inferior correlation at 576 nm compare to 540 nm. Therefore, the wavelength of 540 nm was chosen for Hb-drv estimation of the archived DBS samples. In addition, this Hb-drv spectra pattern is not significantly affected by pH variations (340) and we observed enhanced extraction efficiency with slight acidification of the extraction solution.
Our method validation included assessment of imprecision and bias compared to biological variation (330, 341). The correlation between the measured Hb-drv and the reference Hct were consistently good over the study time (week 1 to 48) (342). However, as it was expected from the Hb degradation process, the slope and intercept continued to change for the first 8 weeks but then remained consistent for the remainder of the study period (48 weeks). The total error was also assessed by comparison to biological variation and Clinical Laboratory Improvement Amendments guidelines for Total Allowable Error (CLIA 88 TEa) (343). We found that our imprecision compared to the biological variation criteria was less than the minimum criteria. Bias, and therefore, TEa was assessed based on the recovery data and also did not meet these specifications. Hence this assay is not fit for the retrospective diagnosis and monitoring of blood pathologies such as anaemia and polycythaemia. However, as biological variation data is generated for Hb in whole blood and our assay is not intended for this purpose we deemed the other method validation components to be acceptable, we propose this method is fit for purpose.

3.5 Conclusions

We have successfully developed a simple Hb-drv method to estimate Hct in aged DBS samples. This method can be incorporated into DBS analytical work-flow for the in-situ estimation of Hct and subsequent correction of the analyte of interest.”
Chapter 4

Determination of Vitamin D from Dried Blood Spot Samples
4 Determination of Vitamin D from Dried Blood Spot Samples

Vitamin D conventionally is measured as 25-hydroxy vitamin D3 in serum / plasma and the interpretation cut-offs are well-defined. However, for large-scale population studies sample handling procedures including; collection, transport and storage are cumbersome for serum/plasma specimens. Accordingly, the interest in the use of dried blood spots (DBS) as an alternative matrix for vitamin D assessment has increased. Following the project aim of investigation of the association between early life vitamin D insufficiency and paediatric food allergy, we developed an accurate method for measurement of 25-hydroxy vitamin D3 (25-OHD3) from archived DBS samples.

This chapter with contribution of other co-authors has been submitted to the (The Journal of Steroid Biochemistry and Molecular Biology) for peer revision and to be published as an analytical full paper.

4.1 Introduction

A century has now passed since the flurry of research leading to the discovery of vitamin D and its role in bone health (344). More recently it is the activities of the current millennium that have expanded our understanding of the vitamin D pathway and the potential implications of vitamin D deficiency on a range of pathologies. As the increasing involvement of vitamin D in physiological functions has been elucidated, requests for evaluation of patient vitamin D status have increased exponentially. The overarching aim of these requests is to clinically stratify patients as replete or deficient in vitamin D (9).

Despite awareness of the importance of vitamin D, deficiency is still considered a global health challenge (345), and the application of vitamin D testing is extended further from monitoring into broader areas such as epidemiological and population studies. The use of the traditional measurement of vitamin D (i.e. serum/plasma 25-OHD3) by automated immunoassay (referenced to LC-MS/MS methods) has limitations when applied to population studies, especially those involving children. These limitations range from the volume of blood required to be collected, how it is transported and associated
cost, through to the amount of storage space required (either refrigerated or frozen). It is these cumbersome steps that have resulted in the push for alternative micro-sampling techniques (i.e. collection and analysis of ≤ 50 µL of biological samples) utilising a dried blood matrix for the assessment of vitamin D.

DBS matrices have been applied to many applications both within and outside of newborn blood spot screening (NBS) (10). The various hydroxylated forms of vitamin D are all small well defined hydrophobic seco-steroids, with 25-OHD3 the most commonly measured. The long-term stability of 25-OHD3 on DBS is detailed in the literature (118, 167, 346, 347) and its analysis by LC-MS/MS has also been previously demonstrated (118, 167, 169-171, 347, 348). Hence this micro-sampling application can be readily applied for the measurement of 25-OHD3. This also means that archived DBS samples e.g. those collected from NBS programmes can be retrospectively analysed for vitamin D population studies.

Whilst, the pre-analytic components are simplified through application of the DBS matrix, there are new analytical and post-analytical challenges to consider using this approach. These considerations include:

1) How to ensure traceability is maintained - Serum/plasma vitamin D is standardised and traceable to Certified Reference Materials (CRM), Reference Measurement Procedures (RMP) and for Reference DBS Measurement Laboratories (RML).

2) The lack of peer review programs to support ongoing standardisation and there are no evidence-based agreed decision limits that are broadly applied (349).

3) How best to achieve the required sensitivity?

4) Interpretation of data by comparison to serum / plasma equivalent and the cut-offs for interpretation are clearly defined (350-352).

To advance the literature regarding measurement of vitamin D the following study aimed to develop a sensitive and robust local DBS vitamin D method, that is traceable to serum for use in population-based studies.
4.2 Materials and Methods

4.2.1 Chemicals, calibrators and quality control materials

**Collection card:** To prepare DBS samples whole blood specimens were blotted on Whatman 903 filter paper, following the Clinical and Laboratory Standards Institute (CLSI) guidelines (234, 239). This filter paper is approved by the Food and Drug Administration (FDA), Newborn Screening Quality Assurance Program (NSQAP) and also the Centers for Disease Control and Prevention (CDC) (239).

**Chemicals:** Hexane for liquid chromatography was purchased from Merck (VIC, Australia): hexane (CAS No. 110-54-3, Cat no. 1.04391.2500), also Thermo Scientific (VIC, Australia) provided Optima methanol for liquid chromatography (CAS No. 67-56-1, Cat no. A456-4). Amplifex™ Diene Reagent was purchased from Sciex (MA, USA) (product no. 5037804) and was prepared following the manufacturer’s instructions and diluted (1:1) with acetonitrile prior to use. Formic acid (purity ≈98%, CAS No. 64-18-6, Cat no. 94318) was obtained from Sigma-Aldrich (VIC, Australia). 25-hydroxyvitamin D3 (purity ≥98%, CAS No. 19356-17-3, Cat no. S4163UNL), 3-epi-25-hydroxyvitamin D3, (purity ≥98%, CAS No. 73809-05-9, Cat no. S7004), 25-hydroxyvitamin D3-[2H6], (purity ≥98%, CAS No. 140710-94-7, Cat no. S4163 were purchased from IsoSciences (PA, USA) via their Australian distributor (PM Separations, QLD, Australia). The nitrogen gas cylinder (N5.0 [Purity ≥ 99.999%], CAS No. 7727-37-9) was supplied by Coregas (VIC, Australia). Seracon human plasma matrix matched diluent (Material Number1800-0027) was purchase from SeraCare (Milford, MA, USA).

**Calibrators:** The multi-level NIST-972a traceable ChromSystems calibrator set (seven levels, Cat no. 62039) was used for 25-OHD3 quantitation. It was prepared according to the manufacturer’s instructions and stored at -20°C. The calibration curve covered blank to 376 nmol/L 25-OHD3 concentrations. Based on our theoretical calculation and other practical studies, an average 3.2 mm DBS punch contains approximately 3.1 µL of whole blood (353, 354), which is 1/16 of the 50 µL of the liquid sample. Different amounts of internal standard (ISTD) material (proportional with the ratio of
liquid samples volume to DBS punch) was added into calibrators, quality control (QC) materials and DBS samples to correlate the 3.2 mm DBS punch results to equivalent liquid matrix concentration.

**Internal quality controls (IQC) and External quality assurance (EQA) materials:** Commercial UTAK lyophilised IQC set (tri-levels: low, mid, high – Cat no: 1060-2) was used for vitamin D. An In-house DBS IQC was prepared using 50 µL of a human whole blood sample, spotted on the filter paper (Whatman 903) and air-dried overnight in ambient condition. The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) samples were used in conjunction with IQC materials to certify the traceability of the measurement, which has target values for vitamin D assigned by National Measurement Institute Australian Government (NMIA). Our laboratory routinely participates in the RCPAQAP for Endocrines (including vitamin D).

### 4.2.2 Subjects

De-identified venous whole blood samples (n=80, collected in ethylenediaminetetraacetic acid (EDTA)) were sourced from the Royal Childrens Hospital Melbourne Laboratory Services (Approval no. S73) (Lab A) and Austin Pathology (Approval no. 16.3162) (Lab B) and used for the method development and evaluation. Information related to age and gender were provided. The plasma 25-OHD3 level for these samples was determined in our laboratory using an adaptation of our previously validated LC-MS/MS method (355). Prior to initial retrieval, the whole blood samples were stored post analysis by the pathology laboratories for up to 48 hours at 4 °C (Lab A) or, 24 hours at 4 °C (Lab B). Samples sourced from Lab A generally included paediatric patients (median age:10 years, gender ratio:1) while whole blood specimens obtained from Lab B compromised the adult population (median age:63 years, gender ratio:1).

A whole blood aliquot (1 mL) of each sample was spun at 2000 revolution per minute for 5 minutes and separated plasma was stored in -20 °C. The EDTA sample tubes were mixed on a roller mixer for 30 minutes followed by 20 times using “figure 8” hand mixing, prior to blotting on to the collection cards. 50 µL of whole blood was spotted on the filter paper (Whatman 903) (eight spots for each
sample), then air-dried in ambient condition overnight. Prepared DBS cards were stored at room temperature in a biohazard specimen zipper plastic bag and light protected.

### 4.2.3 Measurement

**Sample Preparation**

Liquid calibrators, IQC materials and any other liquid matrix samples were prepared in conjunction with the DBS samples (solid matrix);

**DBS, calibrators and control sample preparation,** involved a 3.2 mm DBS disk punched from the whole blood sample spotted on the filter paper and placed into a 96-well micro plate. 125 µl of de-ionised water was added to the DBS disk. Concurrently, 50 µL de-ionised water was added to 50 µL of calibrators and liquid QC materials, followed by 60 minutes shake; the first 30 minutes at room temperature and the second 30 minutes at 60 °C. 75 µl water-methanol mixture containing 10 nmol/L and 100 µL of water-methanol mixture containing 123 nmol/L deuterated ISTD (25-hydroxy vitamin D3-d6) were added into DBS and calibrators/IQCs samples respectively. Samples were subjected to further 10 mins sonication (40kHz, Unisonsics, Part No; FXP, Unisonsics Australia Pty Limited, Australia).

**Supported liquid extraction (SLE) procedure,** was adapted from the manufacturer’s published application note (356). DBS eluate and pre-treated calibrators and IQCs, were purified by using Biotage SLE cartridges (Cat no. 820-0200-P01) and application of 350 µL hexane elution (twice). The sample eluates were collected and dried using a rotational vacuum concentrator (Martin Christ, Part No: RVC2-33PACK, John Morris Scientific Pty Ltd, Australia) at 40°C temperature and 500 rounds-per-minutes speed.

**Derivatisation,** 50 µL ½ diluted (in acetonitrile) Amplifex diene reagent solution added to the dried sample, then vortexed for 20 seconds, followed by 30 mins incubation at 4 °C. The derivatisation
reaction was neutralised by the addition of 75 µL methanol/de-ionised water mixture (1:1). Figure 4.1 demonstrates the derivatisation reaction of 25OH-D3 with Amplifex diene.

![Figure 4.1. Derivatisation reaction of 25OH-D3 using Amplifex Diene label (adapted from Müller et.al (357)). In MS method result of derivatisation, the molecular weight of the derivatised product would be greater than the initial precursor, where mass to back ground noise ratio is relatively decreased.](image)

**Liquid Chromatography – Tandem Mass Spectrometry detection**

The water-methanolic extract was injected onto a core-shell column (2.6 µm, 100 mm, Kinetex F5, Phenomenex Inc., Australia). An Agilent HPLC Model 1260 infinity coupled with the Agilent Model 6490 triple-quadrupole mass spectrometer was used for the sample analysis. Derivatised 25-OHD3 was eluted using a solvent program. The initial solvent was 60% methanol in water (containing 0.1% formic acid) with a flow rate of 0.25 ml/min. The maximum organic solvent gradient was 100% methanol. Multiple reaction monitoring (MRM) and electrospray ionisation (ESI) positive mode was applied for mass spectrometry measurement with the following mass to charge ratio (m/z) for precursor and product ions; 25-OHD3 quantitation using: 732.5 → 673.4 (Collision energy: 30 eV), and qualifier 732 → 217 (Collision energy: 50 eV), with the ISTD 738.4 → 679.4 (Collision energy: 30 eV). Ion source specifications were as follows; gas temperature: 290 °C, gas flow: 11 L/min, nebulizer pressure: 40 psi, sheath gas temperature: 275 °C, sheath gas flow: 7 L/min with the nozzle voltage of 2000 V.

**Assay performance studies**

The method was validated following the Clinical Laboratory Improvement Amendments (CLIA) final rules and recommendations (358). The intra-run imprecision study was performed across three
measured vitamin D concentrations from DBS samples (low: 7, medium: 22 and high: 50 nmol/L). Derivatised 25-OHD3 concentration of each sample was estimated in twenty replicates within the same run and the coefficient of variation calculated. The IQC samples were used to estimate the inter-run imprecision. The lower limit of quantitation was determined for the lowest concentration with coefficient of variation (CV) of <20% for twenty replicates with signal to noise ratio (S/N) >10. In addition to track the assay accuracy and traceability, a pair of RCPAQAP samples were analysed and reported monthly. Furthermore, assay linearity was checked for DBS by testing a set of spotted whole blood samples serially mixed in five different concentrations from low and high levels of 5 and 40 nmol/L.

De-identified whole blood samples (n=80) collected from Lab A and Lab B were used for the method comparison study and to determine the relationship to the serum calibrator. The matching plasma 25-OHD3 level was determined in our laboratory using our previously validated LC-MS/MS method (355). Measured derivatised 25-OHD3 levels from spotted samples were plotted against the plasma 25-OHD3 to predict the linear equation and confidence intervals of 95%. To assess the effect of punch location a set of DBS samples (n=11) with different levels vitamin D (low, medium and high) were tested in duplicate (punched from the centre and edge of the blood spot).

The recovery study was performed by using a human whole blood sample collected in EDTA to generate a set of diluted and spiked samples at low and high concentrations. Each sample included 450 µL of whole blood, where 50 µL H2O was added to the whole blood to generate diluted sample and the other aliquots were spiked with 50 µL 25-OHD3 standard materials (84, 376, 538 nmol/L). DBS samples were analysed after 3 months of spotting. Blank samples including 3.2 mm filter paper punches and 50 µL deionised water were analysed for the carry-over study in triplicate following the highest level of IQC samples for both dried and liquid matrices.

To investigate the matrix effect and ion suppression, a blank sample including washed red blood cells diluted in Seracon (matrix matched vitamin D free serum) (45:55) was spiked with pure 25-OHD3 (25 µg) and spotted on the filter paper. Vitamin D was measured from the dried spiked sample in
conjunction with matrix-free matching derivatised 25-OHD3 (359, 360). Additionally, phospholipids (which could induce ion suppression) were consistently monitored using their common ion transitions; (104→104 and 184→184).

**Quantitation and statistics**

Stöckl D, et al and Fraser C.G recommendations for the desirable analytical quality specifications for imprecision, bias and total error for measurement of vitamin D were utilised to evaluate the within-subject, between-subject CV values and assay fitness for purpose (330, 361, 362). Agilent MassHunter Workstation Data Analysis software (version: B.4.04/B.4.0/B.5.0) was used for qualitative and quantitative analysis. LinChecker software was used for linearity testing (Philippe Marquis 2001, version; 1.1.2.0). “Method Validator” software (Philippe Marquis 1999, version; 1.19) was used for accuracy testing and method evaluation studies (Linear regression, Passing-Bablok and Bland-Altman difference Plot).

**4.3 Results**

25-OHD3 was eluted and extracted from a 3.2 mm DBS disk in conjunction with the liquid matrix calibrators and quality controls using SLE. Derivatised 25-OHD3 and its relative ISTD were separated and detected at 7 minutes retention time on 100% methanol gradient of LC-MS/MS analysis (Figure 4.2). The 7-point calibration constantly demonstrated a coefficient of determination of, at least 0.99 (Figure 4.3). The assay has the lower limit of quantitation of 0.5 nmol/L (S/N = 10) and the upper limit of linearity of 376 nmol/L. Besides the linear range of the calibration curve, the linearity experiment confirmed the linear norm between 5 to 40 nmol/L 25-OHD3 of the raw results for DBS samples (Figure 4.4).
Figure 4.2. Example 25-OHD3 chromatography-mass spectrometric peaks. (A) Chromatogram of 25-OHD3 and 3-epi-25-OHD3 from a 50 μL spiked plasma sample: MRM 401.0 > 383.0 m/z and retention time of approximately 18.4 to 19.2 min [23]. (B) Chromatogram of derivatised 25-OHD3 and 3-epi-25-OHD3 from a 3.2 mm spiked DBS sample: MRM 732.5 > 217.0 m/z and retention time of approximately 7 to 7.5 min.

Figure 4.3. A typical calibration curve generated by MassHunter Quantitative Analysis software; blank to 376 nmol/L 25-OHD3 concentrations using liquid matrix calibrators.

Figure 4.4. Linearity experiment using spotted human whole blood demonstrating the method is linear between 5 to 40 nmol/L 25-OHD3 directly measured of DBS samples.
Calculated within-run CV for low (15 nmol/L), medium (22 nmol/L) and high (42 nmol/L) level of 25-OHD3 was; 10.5, 8.1 and 9.3 % respectively. Therefore, calculated uncertainty of measurement is equal to +/- 21.0 %. This is in addition of bias of calibrator which is unknown. While between-run imprecision was calculated as a CV of 12.9 % for mean concentrations of 7.8 nmol/L for DBS and 8.9, 6.7 and 5.6 % for respectively for low, medium and high level UTAK liquid quality controls.

The comparative accuracy of 25-OHD3 measures of DBS samples compared to their matching plasma samples demonstrated the coefficient of correlation of R = 0.94 for internal quality controls (n = 7), R = 0.91 for Lab A samples (n = 40) and R = 0.96 for Lab B samples (n=40) and R = 0.86 (n=80) for the combined analysis (mixed results of lab A and B) (Figure 4.5).

**Figure 4.5.** Comparison of 25-OHD3 (nmol/L) measures of DBS samples and their matching plasma samples (n=80, combined data analysis from both laboratories A and B). a) Passing-Bablok regression plot for comparison of 25-OHD3 (nmol/L) measures of DBS samples and their matching plasma samples (indicating regression line). The equation demonstrating the relationship is; R=0.86, y = 0.41x -0.2. B) Bland-Altman difference plot demonstrating a mean difference of -2.14% (95% limits of agreement: -5.69 to 1.41 %) between plasma-equivalent DBS vitamin D (from Passing-Bablok relationship equation) and plasma measures (black solid line).

Comparison of the central and peripheral punch location demonstrated agreement based on the slope 1.01 (0.95 confidence interval 0.82 to 1.24), intercept 0.78 (0.95 confidence interval -2.58 to 3.02), difference 0.79 (0.95 confidence interval -0.30 to 1.88) and the paired t-test (two-tailed) P value equals 0.13 (Figure 4.6). The recovery rates were calculated as 94, 84 and 89% respectively for 8.4, 37.6 and 53.8 nmol/L concentrations of 25-OHD3 from DBS samples. Carry-over was determined as <0.1 and <0.5 % respectively for liquid and DBS matrixes. The total ion relative response difference between spiked DBS sample and pure matrix material, was calculated as 11.5% and phospholipid peak retention time was on average 3 minutes distinct from the derivatised vitamin D (Figure 4.7).
Figure 4.6. Comparison of 25-OHD3 (nmol/L) measures of central DBS punch and their matching peripheral DBS punched disk (n=11); a) Passing-Bablok regression plot for comparison of 25-OHD3 (nmol/L) measures of central DBS punch and their matching peripheral punched disk (indicating regression line). The equation demonstrating the relationship is: $R=0.98$, $y = 1.01x -0.8$. B) Bland-Altman difference plot demonstrating a mean difference of 0.79% (0.95 CI: -0.30 to 1.88 %) between central punched DBS and peripheral punched DBS 25-OHD3 (nmol/L) measures (black solid line).

Figure 4.7. Monitored phospholipids along with MRM detection of derivatised 25-OHD3. Phospholipids are known as the major source of interference and potential cause of ion suppression for blood analysis using mass spectrometry. Progressive monitoring of phospholipids demonstrated that there was no significant chromatographic effect on derivatised 25-OHD3 peaks (phospholipids’ structure adapted from Little et.al (363)).

4.4 Discussion

We have successfully developed and validated a LC-MS/MS assay to estimate 25-OHD3 from DBS samples, using one 3.2 mm punch. This assay has improved correlation with liquid matrix, sensitivity, imprecision and/or robustness over other previously published procedures described for vitamin D analysis of DBS sample. In addition, our method extends further to assess vitamin D status using archived DBS samples, which is relevant for epidemiological studied by application of semi-automated
sample preparation and analysis process (increased throughput). Furthermore, measured 25-OH-D3 by the current method is traceable to NIST SRM972 standard reference material as the DBS samples were linked to the 7-point commercial serum calibrators which is reported by the manufacturer to be traceable to NIST, in addition certified external quality control materials (Figure 4.8).

Previously other groups have attempted to measure vitamin D metabolites from DBS samples, however, inadequate assay sensitivity, analytical robustness and/or low throughput has limited their application to large population base studies (118, 169-171, 347, 348, 364, 365). Furthermore, a traceability chain that links the measured vitamin D from the archived DBS sample to the liquid matrix previously has not been clearly defined.

Pre-analytical variables including storage, punch location and haematocrit effect on DBS analysis are considered as a source of bias and inaccuracy of measurement. Additionally, the stability for different biomarkers in dried matrix is quite variable. Thus as part of the method evaluation process, analyte specific stability study from collection to storage and analysis of the DBS sample is highly recommended (10). However, multiple studies have demonstrated that vitamin D metabolites are stable on dried matrix for 8-20 years (118, 167, 346, 347). Furthermore, to improve the accuracy, it has been recommended to consistently punch the blood spot either from the centre or close to the outer edge (254, 366). Nevertheless, more recent reports, similar to our observation from haemoglobin-derivative assay method development/evaluation process, and this current study dispute the punch location and haematocrit effect as the source of bias for DBS analysis (316, 353, 365).

Figure 4.8. The RCPAQAP Endocrine program vitamin D end of cycle report. This report demonstrating the method accuracy and performance. To track the assay accuracy and traceability, a pair of RCPAQAP samples were analysed and reported monthly to control LC-MS/MS method calibration and accuracy to detect 25-OH-D3 in liquid samples including the DBS-extracts. All the values of RCPAQAP analyses, at different concentrations of 25-OH-D3, are within the acceptable range and close to the target values (compared to the different method users including other MS method users. The advantages of our DBS-vitamin D method are; high accuracy, precision and the tractability of the DBS measures to the liquid sample.
The incorporation of SLE and Amplifex derivation reagent for sample preparation have improved sample quality and ionisation efficiency, in conjunction with decreased ion suppression and reduced phospholipid interference. Together these have a significant impact on assay sensitivity and lower limit of quantitation (0.5 nmol/L) compare to the previously developed methods. Vitamin D metabolites are non-polar molecules. Due to lack of naturally charged group, vitamin D metabolites have low ionisation efficiency under ESI or atmospheric pressure chemical ionisation (APCI) conditions. This has resulted in tagging substances being utilised to increase their ionisation capacity. It is reported that, the derivatisation process may improve the sensitivity of MS methods for vitamin D detection by 100-1000 fold (367), which potentially influences the required sample volume for analysis. In MS based methods, as a result of derivatisation process, the molecular weight of the derivatised product is greater than the precursor, thus mass to back ground noise ratio is relatively decreased.

Several highly reactive triazolinedione compounds (TADs) have been described for vitamin D mass spectrometer detection methods. These compounds generally are group members of nitro-aromatic complexes such as phenyl-triazoline-dione analogues and Cookson-type substances (21-24). TADs in general increase the vitamin D metabolites ionisation aptitude with the most stable positive charge. In addition, the rapid derivatising reaction of vitamin D analogues with TADs is stable and rationally irreversible under LC-MS/MS condition (25). 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) has been previously used, and is commercially available, to enhance the sensitivity of LC-MS/MS methods developed for determination of either low concentration metabolites (e.g. 1,25(OH)-D3) or small sample volume analysis (e.g. DBS) (170, 348, 364, 368). Lately Amplifex diene derivatising reagent became available, which has been specifically designed for LC-MS/MS measurements due to an activated dienophile moiety in its molecular structure as well as the positively charged terminal group (Figure 4.1). Our observation and other studies have demonstrated the advanced assay selectivity and sensitivity using Amplifex over PTAD (369). The advantage of Amplifex over other Cookson-type derivatising reagent for vitamin D metabolites analysis is separation of 3-epimer (357, 369) (Figure 2).

Our method validation included assessment of imprecision and bias compared to biological variation (330, 361). Following CLSI guidelines, accuracy studies included the comparison of the experimental
method with the reference method (including regression and Bland-Altman difference plots), and estimation of the recovery rate of target analyte from its matrix as well as interference detection and ion suppression (370). The correlations between the measured vitamin D (25hydroxy-vitamin D3 plus epi-25-OHD3; no 25-OHD2 was identified) and the reference plasma 25-OHD3 were consistently high positive across the mixture of adults and paediatric samples (371). DBS vitamin D measurements are comparable to equivalent plasma values by application of a correction factor derived from the regression slope of and intercept (Figure 4.5).

Assay imprecision experiments, including inter/intra-run reproducibility testing, demonstrated a desirable rate of precision for all studied levels (in agreement with FDA guideline; < 15% coefficient of variation (372)). Accuracy or systematic error, as well as sensitivity of an analytic method is directly influenced by recovery rate and matrix effect (driven by ion suppression and presence of interfering substances) (359, 360). Considering recommendations and available data presenting recovery rate for different MS methods for measurement of 25-OHD3 (rate range of 86-110 %) (361), our assay represents similar acceptable levels of recovery across the pathological range. The ion suppression study demonstrated an insignificant difference (<12%) between the total ion relative response of spiked DBS sample and pure matrix material. In addition, progressive monitoring of phospholipids in conjunction with total ion respond comparison between spiked DBS and matrix free sample, demonstrated that there was no significant chromatographic effect on derivatised 25-OHD3 peaks.

It is important to note the potential limitations from the DBS sample itself. Apart from the commonly raised issues such as Hct effect etc. the age of the DBS may be a significant variable. Whilst others have found vitamin D to be stable long term, during the last 4 years of our studies we have potentially observed a visual colour change in extracts as the control samples grow older. A confounder to this observation is that we were developing and changing the analytic process over this period to generate our final method. It is unclear if this visual change has also an influence in vitamin D extraction efficiency or not. As the answer is not clear, we strongly recommend DBS vitamin D methods to be used for population studies only and not individual patient measurement. Furthermore, for these population studies usually samples should all be of a similar age to negate any potential difference.
Finally, we note that this is an observation and this speculation is not substantiated, therefore does not necessarily counteract the reported long term stability of vitamin D (5, 6).

Conventionally vitamin D is measured from the liquid matrix (serum/plasma) and the cut-off for interpretation and measures of traceability chain is clear. Certainly, traceability along with cross validation of the method to a reference plasma/serum-based method is a vital requirement for offering an analytical method as a diagnostic tool (10, 373). The theoretical ratio between the average amount of blood sample on a DBS punch and the volume of liquid sample that was applied defines the relative quantity of internal standard for both DBS and liquid samples (including commercial standard and control materials) within the same analytical method to maintain the traceability chain link between the dry and liquid matrices. However, to turn the numerical result from DBS analysis into a clinically meaningful result, a reference interval (RI) or decision point needs to be established by further large population studies. In addition, this assay potentially can be a candidate reference measurement procedure for the determination of 25-OHD3 in DBS sample, since our method validation technique meets the ISO 15193:2009 licence criteria including: application of traceable standard material to generate the calibration curve, measurement uncertainty assessment (imprecision and accuracy testing) and systematic assessment of factors influencing results (point of punch and matrix effect studies) (374).

4.5 Conclusions

We have successfully developed an analytical method for quantitation of vitamin D from DBS samples. The traceability of the DBS measures is improved by this approach and potentially current method can be used broadly for other dried matrix measurands which comparison to serum/plasma is required for their interpretation. However, to standardise DBS analysis dedicated EQA programs are crucial. Despite the evidence of vitamin D long-time stability in dried matrices, further prospective longitudinal studies are required to investigate the influence of aging on DBS vitamin D measures. Also, to turn the numerical result from DBS analysis into a clinical meaningful result, age and sample specific RI or decision points need to be established.
Chapter 5

Is vitamin D level at birth associated with paediatric food allergy development in early childhood?
5 Is vitamin D level at birth associated with paediatric food allergy development in early childhood?

Throughout the past decade, there has been a serious concern about the link between Vitamin D status and allergic diseases. Earlier studies have provided some direct and indirect evidence suggesting the inverse correlation between vitamin D levels and prevalence of food allergy (3, 375). In Victoria, the prevalence of both vitamin D insufficiency and challenge-proven food allergy in infants is high and the two have been linked (376). However, it remains unclear whether low vitamin D levels play a causal role in the development of food allergy, and if so, whether there is a “window of opportunity” during which adequate vitamin D levels are most important for immune development and immune responses to food. Potentially, vitamin D status at birth has implications on the prevalence of food allergy and other pathologies. We have therefore aimed to determine the frequency of vitamin D inadequacy in newborns from Melbourne, Victoria and investigate the relationship between vitamin D level at birth and food allergy in infants.

5.1 Introduction

There is evidence that vitamin D supplementation reduces the risk of asthma exacerbations, but no strong evidence for a role of vitamin D in other allergic disorders (9). Vitamin D plays a part in immune system development and function which may be important in evolution and maintenance of food tolerance (60, 75, 377). This includes its involvement in maintaining intestinal mucosal barrier integrity and promoting the expression of IL-10-secreting T-regulatory cells, which is involved in regulation of food antigen intake and maintenance of immune tolerance, as well as prevention of sensitization (77, 378). Hence, it is biologically plausible for a true link to exist between vitamin D insufficiency (VDI) and food allergy (9, 49).

The prevalence of food allergy has a tentative inverse relationship with ambient ultraviolet radiation (UVR) exposure and thus potentially with vitamin D levels (which are known to vary based on variables of ethnicity and latitude) (6). Although few studies internationally have measured food allergy
prevalence in population-based samples using the gold standard of oral food challenges, several have assessed the correlation between proxy markers of food allergy status and latitude (49, 51, 53, 379). According to these studies, the areas further from the equator with lower ambient UVR levels have higher incidence of food allergy. In Australia, parent-reported egg and peanut allergy and prescriptions of hypoallergenic formulas which are used for the treatment of food allergy are more common in southern states with less year-round sunlight, as are prescriptions for epinephrine auto-injectors for the treatment of anaphylaxis (379). Similar trends in prescription of auto-injectors have been described in the United States (US), with these being more common in areas further from the equator (9, 55).

Only a few studies to date have examined the relationship between measured serum vitamin D levels and either food sensitisation or allergy. In the US-based National Health and Nutrition Examination Survey (NHANES 2003-2006), children with 25-hydroxy vitamin D (25-OHD) less than 37.5 nmol/L of blood were more likely (odds ratio 2.39; 95% CI, 1.29–4.45) to have allergic sensitisation to peanut than children with 25-OHD levels greater than 75 nmol/L. A separate study found that in children with specific genotypes, low vitamin D at birth (cord blood 25-OHD Level < 25 nm/L) was associated with food sensitisation, while this association was not present in children with other genotypes (380). In earlier Australian studies of infants of six months and one year old, those with VDI were more likely to develop food or other allergic diseases, and suggests that improving vitamin D status in pregnancy and early infancy may decrease the risk (376, 380).

Some studies have also suggested that high vitamin D levels or oral supplementation with vitamin D may be associated with an increased risk of allergic disease in general, although these have not examined food allergy specifically (50, 72, 381). Conversely, the outcome of another small study on two-year-old children, suggests that higher maternal and cord blood vitamin D levels associates with a higher risk for food sensitisation and allergy (63). These apparently conflicting findings might be explained by a differential immune effect of oral vitamin D supplementation compared with vitamin D derived from UVR exposure. Alternatively, there is some evidence to support a ‘u-shaped association’ whereby both high and low serum vitamin D levels may increase the risk of allergic disease (9).
Dried blood spot (DBS) cards collected for newborn blood spot screening (NBS) for genetic disorders at two to three days of age could be utilised to assess vitamin D levels at birth. Whilst the analysis of vitamin D is usually performed on serum or plasma samples, successful measurement using blood spots has been demonstrated (118, 167, 169-171, 347, 348). There is published data available from relatively small study-group studies (using serum sample) reporting mean maternal vitamin D in neonates (380, 382, 383). More recently Australian (n=259) and European (n=1451) studies attempted to evaluate the neonatal vitamin D status using NBS samples (383, 384). However, to date there is limited information available to define adequate infant levels of vitamin D, which might be different from established thresholds described for the adults. Therefore, larger population studies are required to establish the mean concentration of vitamin D in DBS samples in local cohort. Accordingly, we aimed to utilise our in-house DBS vitamin D method (chapter 4) to, a) provide a description of vitamin D levels in the Melbournian infant population and b) investigate the association between vitamin D status at birth and prevalence of food challenge proven- IgE mediated clinical food allergy and eczema at 12 months of age.

5.2 Subjects and Methods

5.2.1 Study subjects

The current study was conducted as part of the HealthNuts study; a large-scale population-based cohort study (n=5,300). Briefly, the HealthNuts study has aimed to assess the risk-factors and prevalence of the paediatric food allergy (385). For this study, infants were recruited between September 2007 and August 2011, with the age range of 11-15 months. Informed consent was obtained from the parents who then completed a questionnaire containing information on parents’ ethnicity, children’s skin type, season of birth, environmental exposure, supplementation and infant feeding practices in the first year of life. Infants were skin prick tested to sesame, peanut and hen’s egg. All participants were examined for the presence of eczema. Infants with a detectable reaction on skin prick test to any foods were followed up with diagnostic oral food challenges at the Royal Childrens Hospital Melbourne. Those infants with both a positive food challenge and a positive skin prick test or elevated specific IgE level
 (> 0.35 kUA/L) were considered food allergic. We chose not to look at different food-specific IgE cut-offs as the clinical relevance of food-specific IgE in the absence of a history of reaction to foods or a positive oral food challenge is unclear. 2700 participants of the original 5300 provided consent for us to access their NBS samples and are therefore involved in the current analysis including 341 with food allergy and 2148 controls (Table 5.1). Ethical approval was obtained from the Office for Children Human Research Ethics Committee (HREC) (ref. CDF/07/492), Department of Human Services HREC (ref. 10/07), and the Royal Childrens Hospital HREC (ref. 27047).

Table 5.1. HealthNuts participants characteristics (number and percentage) by gender, parents’ country of birth, gestational age at birth, multivitamin / vitamin D supplementation in pregnancy, season of birth, challenge-proven food allergy and eczema.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Guthrie Card Available (%)</th>
<th>Total (%)</th>
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<td>One east Asia</td>
<td>4.11</td>
<td>4.30</td>
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<tr>
<td>Both east Asia</td>
<td>3.56</td>
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</tr>
<tr>
<td>Other</td>
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<td>30.20</td>
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</tr>
<tr>
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<tr>
<td>&gt;36</td>
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<tr>
<td>&lt;=36</td>
<td>6.08</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Not ingested</td>
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<tr>
<td>Ingested</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Summer</td>
<td>23.43</td>
<td>23.56</td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>23.70</td>
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</tr>
<tr>
<td>Spring</td>
<td>27.26</td>
<td>26.65</td>
<td></td>
</tr>
<tr>
<td>Winter</td>
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</tr>
<tr>
<td>Challenge-confirmed food allergy</td>
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<td></td>
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</tr>
<tr>
<td>No</td>
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<tr>
<td>Yes</td>
<td>13.70</td>
<td>10.88</td>
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<tr>
<td>Parent reported eczema</td>
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<td></td>
</tr>
<tr>
<td>No</td>
<td>71.84</td>
<td>73.40</td>
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<tr>
<td>Yes</td>
<td>28.16</td>
<td>26.60</td>
<td></td>
</tr>
</tbody>
</table>

A total of 2700 DBS samples (archived NBS Guthrie cards) from the recruited infants, were retrieved from the Victorian Clinical Genetics Services (VCGS) at the Murdoch Children's Research Institute (MCRI) (BIS ref. 607370 and HealthNuts ethics ref. 1006215) (Appendix 8.3). DBS disks (3.2 mm)
were punched from NBS cards (in duplicate or triplicate, depending on the viability of the blood spots) into 96-well plates and stored at ambient condition prior to analysis.

5.2.2 Vitamin D measurement method

We used our previously developed DBS vitamin D method (chapter 4) for analysis of NBS Guthrie cards. Water-methanol mixture including deuterated internal standard (25-Dihydroxyvitamin D3-[d6]) was used to elute vitamin D content of 3.2 mm spotted blood. The eluate was subjected to a supported liquid extraction (Biotage - ISOLUTE® SLE plate) and derivatisation (Ampliflex Diene Reagent) process prior to liquid chromatography-tandem mass spectrometry (LC-MS/MS) (Agilent 1260 HPLC, 6490 MS/MS) analysis. Derivatised 25-OHD3 was detected using: core-shell Kinetex F5 column, methanol/water gradient, positive ESI mass spectrometry with ion transitions of 732.5 > 673.4 and 738.4 > 679.4 m/z. NIST972a traceable commercial material from Chromsystems was used to calibrate vitamin D. The assay has the lower limit of quantitation of 0.5 nmol/L and the upper linearity limit of 376 nmol/L, with the average coefficient of variation of 12.9% (inter-assay) and 9.3% (intra-assay).

5.2.3 Statistical analysis

Statistical analyses were performed using Stata software (version 14.1, College Station, TX, USA). De-identified data was analysed to generate histogram graphs and whisker box plots and demonstrate the distribution of vitamin D levels within the study group. As the data was not normally distributed, we generated the quintiles of vitamin D measures, and Chi-square test was used to compare the quintiles versus different variables.

5.3 Results

Of the total 2700 collected archived NBS Guthrie cards (aged 8-12 years), 25-OHD3 was measured for 2615 participants (Figure 5.1). From this study group (49.18% female), 93.9% were born at term (> 36 weeks) with 59.0% from Australian born parents, and 13.7 % having challenge-proven food allergy in early childhood (12 months age) (Table 5.2). Compared to the entire HealthNuts study cohort, those
who provided consent for NBS Guthrie card access were more likely to have both parents born in Australia and to have a child with food allergy or eczema. Other characteristics were similar between the two groups (Table 5.2).

The NBS 25-OHD3 measurements were converted to equivalent plasma using the regression equation obtained from correlation study in chapter 4. Predicted 25-OHD3 levels from DBS samples were ≤ 25 nmol/L in 51%, 25-50 nmol/L in 33.7% and >50 nmol/L in 15.3% of the samples (Figure 5.2). For population comparison studies quintiles (<16.15, 16.15-21, 21.3-28.4, 28.4-42.9 and >42.9 nmol/L of 25-OHD3) were generated for NBS vitamin D measures.

**Figure 5.1.** Flow diagram of inclusion criteria in NBS sample analysis. 13.7% (n=341 of total n=2489) of HealthNuts participants with available blood spot vitamin D data have challenge-proven food allergy.

**Figure 5.2.** a) Whisker-Box plot and b) Histogram graph demonstrating the distribution of vitamin D levels in NBS study samples.
Comparison of estimated NBS vitamin D quintiles versus different variables is demonstrated in table 5.2. Season of birth and maternal history of vitamin D supplementation were both associated with vitamin D levels at birth, with higher levels among those born in autumn season and among those with maternal vitamin D supplementation during pregnancy (Pearson chi$^2$(12) = 76.8880, Pr < 0.001 and Pearson chi$^2$(4) = 20.4353, Pr< 0.001 respectively). However, there was no association between vitamin D levels at birth and challenge-proven food allergy or parent-reported eczema. (Figure 5.2). Similarly, the levels of vitamin D were not affected by parents’ country of birth, gestational age or gender of infant (Table 5.3).

![Figure 5.3. Whisker-Box plots demonstrating the comparison between distribution of vitamin D quintiles in infants; a) with/without proven food allergy, b) with/without parents’ reported eczema, c) born in different seasons and d) with/without maternal supplementation history.](image-url)
Table 5.2. The relationship between infant-related variables (parents' birth country, birth gestational age, pregnancy vitamin D supplement, season of birth, challenge-proven food allergy and parent reported eczema) and vitamin D levels (nmol/L) predicted from NBS samples.

<table>
<thead>
<tr>
<th>Variables</th>
<th>NBS estimated vitamin D Quintiles (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>&lt;16.15</td>
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<tr>
<td>Male (n=1316) %</td>
<td>20.14</td>
</tr>
<tr>
<td>Female (n=1259) %</td>
<td>19.78</td>
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<tr>
<td>Total (n=2575) %</td>
<td>19.96</td>
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<td>Pearson chi²(4) = 6.1799, Pr = 0.186</td>
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<table>
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<tr>
<th>Parents' country of birth</th>
<th>Australia (n=1634) %</th>
<th>One east Asia (n=100) %</th>
<th>Both east Asia (n=89) %</th>
<th>Other (n=685) %</th>
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</thead>
<tbody>
<tr>
<td>Male (n=1316) %</td>
<td>21.36</td>
<td>20.69</td>
<td>19.65</td>
<td>19.77</td>
</tr>
<tr>
<td>Female (n=1259) %</td>
<td>25.00</td>
<td>15.00</td>
<td>17.00</td>
<td>19.00</td>
</tr>
<tr>
<td>Total (n=2508) %</td>
<td>20.53</td>
<td>20.06</td>
<td>19.10</td>
<td>19.94</td>
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<td>Pearson chi²(12) = 15.9393, Pr = 0.194</td>
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</table>

<table>
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<th>Gestational age at birth (weeks)</th>
<th>&gt;36 (n=2329) %</th>
<th>&lt;=36 (n=151) %</th>
</tr>
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<tbody>
<tr>
<td>Male (n=1316) %</td>
<td>19.92</td>
<td>19.92</td>
</tr>
<tr>
<td>Female (n=1259) %</td>
<td>21.85</td>
<td>17.22</td>
</tr>
<tr>
<td>Total (n=2480) %</td>
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<td>19.76</td>
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<td>Pearson chi²(4) = 7.6348, Pr = 0.106</td>
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<table>
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<tr>
<th>Vitamin D supplement in pregnancy</th>
<th>Not ingested (n=2158) %</th>
<th>Ingested (n=127) %</th>
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</thead>
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<tr>
<td>Male (n=1316) %</td>
<td>21.08</td>
<td>15.75</td>
</tr>
<tr>
<td>Female (n=1259) %</td>
<td>15.75</td>
<td>18.90</td>
</tr>
<tr>
<td>Total (2285) %</td>
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<td>19.04</td>
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<td>Pearson chi²(4) = 20.4353, Pr &lt; 0.001</td>
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<table>
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<tr>
<th>Season of birth</th>
<th>Summer (n=609) %</th>
<th>Autumn (n=596) %</th>
<th>Winter (n=668) %</th>
<th>Spring (n=711) %</th>
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<tbody>
<tr>
<td>Male (n=1316) %</td>
<td>20.03</td>
<td>20.97</td>
<td>18.86</td>
<td>20.39</td>
</tr>
<tr>
<td>Female (n=1259) %</td>
<td>18.88</td>
<td>16.11</td>
<td>20.36</td>
<td>23.91</td>
</tr>
<tr>
<td>Total (2584) %</td>
<td>20.05</td>
<td>20.01</td>
<td>19.93</td>
<td>20.05</td>
</tr>
<tr>
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<td></td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>NBS estimated vitamin D Quintiles</th>
<th>Variables</th>
</tr>
</thead>
<tbody>
<tr>
<td>mmol/L</td>
<td>Challenge-confirmed food allergy</td>
</tr>
<tr>
<td>No %</td>
<td>Yes %</td>
</tr>
<tr>
<td>&lt;16.15</td>
<td>(n=496)</td>
</tr>
<tr>
<td>16.15-21</td>
<td>(n=491)</td>
</tr>
<tr>
<td>21.3-28.4</td>
<td>(n=494)</td>
</tr>
<tr>
<td>28.4-42.9</td>
<td>(n=492)</td>
</tr>
<tr>
<td>&gt;42.9</td>
<td>(n=494)</td>
</tr>
<tr>
<td>Total</td>
<td>(n=2467)</td>
</tr>
<tr>
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</tr>
<tr>
<td>Pearson chi²(4) = 4.2844, Pr = 0.369</td>
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5.4 Discussion

This is the largest longitudinal population study determining the distribution of vitamin D at birth in Melbournian infants, and it has examined whether vitamin D status at birth predicts the risk of food allergy and eczema in early childhood. We found that the prevalence of vitamin D inadequacy is very high among the studied cohort (born between September 2007 and August 2011), comparing the DBS measures with the Institute of Medicine adult values and our defined quintiles for 25-OHD3 measures. However, there was no evidence of an association between the NBS vitamin D levels and challenge proven food allergy (to sesame, peanuts and egg) or eczema in this large cohort of 2615 participants. As would be predicted, season of birth and maternal vitamin D supplementation were associated with DBS vitamin D levels. While no other significant predictor (such as; gender, parents’ region of birth and gestational age) of vitamin D level in DBS samples was observed.

The large size of study cohort, including a large number of individuals with challenge-proven food allergy and eczema, in a multi-ethnic population with available information of both environmental and individual variables of infants, added to the strength of the study. To estimate vitamin D levels in archived NBS samples we utilised our novel validated analytic method, with enhanced sensitivity and accuracy compare to the previously introduced procedures, as is described in chapter 4. However, absence of availability of maternal vitamin D status or alternatively mothers’ serum / cord blood sample, was a limiting factor for this study, since we were unable to examine the correlation between the predicted vitamin D concentration from DBS with cord-blood and/or maternal levels. Thus, we are not able to compare our results with other studies which have measured cord blood or maternal levels. Some studies have found that the average ratio of birth vitamin D (cord-blood 25-OHD3) to maternal vitamin D is less than 0.50-0.6 (386, 387). Whereas, other study has argued that, within their fifty study subjects, all cord blood vitamin D measures were higher (approximately 40%) than their corresponding maternal levels (388). Hence, the relationship between maternal, cord blood and archived NBS samples remains unclear.
Our study confirmed that, the levels of 25-OHD3 in Victorian infants’ dried capillary blood were very low. Comparing DBS vitamin D measures with defined thresholds for adults, 51% of study population were vitamin D deficient and 85% were insufficient. This high prevalence of infants’ vitamin D inadequacy is in keeping with other Australian and international studies. Recently other two studies also have reported the high prevalence of VDI (45% and 48% ) among healthy Victorian neonates looking at 25-OHD3 measures from NBS samples (n=259, DBS age < 4 years old) and cord bloods (n=233) with the median of 29.2 nmol/L (383, 386). Similarly, a high rate of newborns’ VDI (57%) is reported in New Zealand (n=929) by measuring cord blood 25-OHD3 (382). Consistently, international studies have indicated the low mean values of vitamin D at birth such as; 34 nmol/L in European (> 5 years old) NBS (n=1451), 27.2 nmol/L in an American cord blood cohort (n=241), 28.5 nmol/L (with 56% 25-OHD < 25 nmol/L) in Turkish newborns’ serum (n=750), as well as 80% rate of 25-OHD < 25 nmol/l in Tanzanian neonates (n=170 serum) (supplementation history was not available) (384, 387, 389, 390). Considering the plausible disparity between measures of serum and archived dried blood samples, due to 25-OHD3 degradation or impaired extraction efficiency influenced by aging of the DBS, and lack of age-adjusted 25-OHD3 recommended ranges, we have generated quintiles for NBS 25-OHD3 measures when examining associations with clinical outcomes.

Consistent with the Barwon Infant Study (BIS) findings (n=233) (386), the data from this current study also indicates no association between low levels of vitamin D at birth and the development of paediatric food allergy (at 12 months of age). Furthermore, other studies in US, Germany and Poland have demonstrated that infants’ cord blood vitamin D concentrations were not associated with risk of asthma, wheeze, food allergy or atopic outcomes in children whom were followed up by the age of 5 to 6 years or during the first 2 years of life (63, 391, 392). By contrast, in our cohort (the HealthNuts cohort), low vitamin D levels at 12 months of age were associated with an increased risk of food allergy (393), suggesting that if vitamin D does play a role in the development of food allergy, vitamin D levels in the first year of life might be more important than vitamin D levels at birth. Randomised controlled trials of vitamin D supplementation in the first year of life are required to determine whether vitamin D levels in infancy do in fact play a causal role in the development of food allergy (5). It has been hypothesized...
that vitamin D status during the solid food introduction period could be a more powerful predictor for infants’ food allergy (394) but the outcome of BIS did not support the relationship between 25-OHD3 levels at six months and the prevalence of paediatric food allergy (386).

Findings from our study are in keeping with earlier data associating the levels of infants’ vitamin D with the season of birth. It is well known that solar radiation is the trigger for natural endogenous vitamin D synthesis. The higher UVR during the summer season (compared to the other seasons) and the increased chance of skin exposure lead to the higher levels of maternal vitamin D synthesis as predicted, which may reflect the 25-OHD3 concentrations in autumn born infants (388, 395). For note, the association between season of birth and allergy has been examined previously in this cohort. There was no convincing evidence of an association between season of birth and allergy (37).

Within our study samples, the median of NBS vitamin D was higher in autumn born neonates. Considering the half-life of circulatory 25-OHD3, shifted seasons in Melbourne (climate summer season is delayed compared to the calendar summer) and cultural behaviour, vitamin D levels projects the higher endogenous vitamin D production over late summer and early autumn. In contrast the lowest vitamin D median was observed in infants born in winter and spring time, where the concentrations of 25-OHD3 represent the low rate of vitamin D synthesis during winter with low UVR and the least chance of skin exposure. However, it has been shown that the ambient UVR effects on immune system modulation, are independent from vitamin D pathways, and may protect against allergic disorders development (396, 397).

Vitamin D supplementation in pregnancy was also linked with increased levels of 25-OHD3 at birth. By contrast, in the same cohort, infants whose mothers used vitamin D supplements during pregnancy had lower levels of 25-OHD3 at one year of age (398). This might be due to the fact that, mothers with a history of low vitamin D levels (whose babies are therefore also at risk of low vitamin D) are more likely to be supplemented, and the beneficial effect of maternal supplementation, although present at birth, does not persist to one year of age. Our current study also found low levels of vitamin D in NBS samples even in the presence of maternal vitamin D supplementation, indicating the inefficiency of
supplementation dosage to maintain infants’ adequate vitamin D levels at birth and throughout the first year of life.

5.5 Conclusion

Adding to previous studies which have examined smaller cohorts, our large population study confirmed that the prevalence of vitamin D inadequacy among the infants born in Victoria is high. Nonetheless, further studies are required to develop a robust adjustment factor corresponding to the age of specimens to standardise the measurements of archived dried samples. Further studies are also needed to define age-adjusted reference intervals and recommended ranges in infants. Our study did not provide any evidence of association between challenge proven food allergy and eczema at 12 months of age, suggesting that if vitamin D does play a role in the development of food allergy, maintaining sufficient levels in infancy might be more important than vitamin D levels at birth. Current evidence supports the feasibility of widespread education of safe sun exposure and perhaps season-focused supplementation during pregnancy and early infancy. Also, further studies are required to define recommendations for optimal dosage and timing of supplementation.
Chapter 6

General Discussions and Conclusions
6 General discussion and conclusions

6.1 Discussion

Epidemiological evidence has shown a concordant increase in the prevalence of allergic diseases including food allergy and eczema, and decreased vitamin D levels in both developed and developing countries. Also, it has been revealed that the incidence of allergic reactions is more significant in regions further from the equator with lower ultra violet radiation (UVR). This suggests that vitamin D inadequacy may play a role in allergic disorder development. The current literature describes the biological mechanism by which vitamin D affects the incidence of allergic diseases through its influence on intestinal epithelial barrier integrity and the immune system. In addition, earlier research has shown evidence of a relationship between vitamin D inadequacy and the increased risk of food allergy in early childhood in Victorian population (376).

Validation of this putative association between early life vitamin D insufficiency (VDI) and food allergy has clear public health implications. However, further studies were required to investigate whether levels of vitamin D at birth predict the risk of paediatric food allergy. In addition, we noted that there was no population data available regarding vitamin D levels in Australian infants and children. Accordingly, we aimed to demonstrate the relationship between vitamin D levels at birth and the development of food allergy in early childhood, as well as predicting the sample specific distribution of vitamin D in the Victorian infant population.

In our review of the literature we also proposed a link between vitamin A and the process of immune system development. Increased levels of vitamin A may be associated with an increased risk of development of early childhood food allergies. As vitamin A can potentially be measured in the same mass spectrometry analysis as vitamin D, it was proposed to also investigate the level of vitamin A metabolites in relation to the development of food intolerance / allergy concurrently with vitamin D. DBS samples would contain protein bound retinol, which if the DBS is stored in the dark and moisture protected, would be stable. However, our observational study demonstrated that whereas retinol was
detectable in fresh dried blood spot (DBS) samples, it was undetectable from the older (>4 weeks) DBS samples. Hence, it was not possible to determine the relationship between vitamin A levels and childhood food allergies with the samples available.

Our project used newborn blood spot screening (NBS) Guthrie cards, which were collected from two to three-day old infants and stored in Victorian Clinical Genetics Services (VCGS) bio-bank, to assess vitamin D levels at birth. Whilst the analysis of vitamin D is usually performed on serum or plasma samples, successful measurement in dried matrices has been demonstrated. However, the measurement of vitamin D in children was known to be complicated by the presence of the inactive epi-vitamin D3. As such, mass spectrometry analysis coupled with chromatographic separation was the only accepted method for the quantitation of this isomer from the predominant form of active vitamin D3. A method for the separation of these forms of vitamin D simultaneously with other fat-soluble vitamins including vitamin A had been developed at RMIT University (355). This was used as the foundation for the DBS vitamin D method which constitutes the first part of this thesis.

In preparation for analysis of vitamin D we punched 2700 DBS disks from NBS Guthrie cards from the VCGS bio-bank with the very limited available sample resource (maximum 3 punches per sample), to investigate the association between the rate of VDI at birth and challenge proven IgE mediated food allergy (FA) at 12 months of age. Blood spot samples belonged to the infants recruited for a National Health and Medical Research Council (NHMRC) granted study; HealthNuts. The HealthNuts study is the world's first longitudinal (birth to 10 years age) population (5,300 children) study with unbiassed measurement of challenge-proven paediatric food allergy (PFA) in a Melbourne cohort. This study aims for a better understanding of the natural history of allergic diseases including FA and eczema, and the risk factors for developing these conditions in children. Individual information and allergic disorders related data was already collected (or under collection) from the HealthNuts cohort.

To predict the vitamin D metabolites (including its epimer) levels in the DBS punches, we aimed to optimize and improve sensitivity and specificity of the available in-house liquid chromatography tandem mass spectrometry (LC-MS/MS) method (355). The method was adapted for DBS sample
preparation including extraction and derivatisation, with specific challenges including the limited sample volume and extraction from the solid matrix. Moreover, interpretation is challenging as the recommended ranges for vitamin D is predominantly described for the samples with a liquid matrix (serum/plasma). Hence, for the effective interpretation of the DBS measurements, accurate conversion of dry matrix results to the equivalent liquid form was required. To determine this relationship, we analysed 80 paired serum/plasma and DBS samples.

Accuracy of DBS quantitation has been reported to be Haematocrit (Hct) dependent and requires additional considerations over the conventional “liquid” blood sample. This is due to the physical characteristics of blotted whole blood affecting the quality of the measurement. We discussed these aspects in detailed as part of our review and DBS Hct estimation paper. Potential errors associated with Hct variation span the DBS total testing process. Where a Hct correction option is sought, practical solutions to minimise bias have been devised including assessment or prediction of Hct from the spotted whole blood. Hence, we aimed to develop a method for prediction of Hct of the DBS punch (described in chapter 3).

We successfully developed a simple method to estimate Hct in archived DBS samples. In this method the haemoglobin (Hb) derivative (drv) content of spotted blood sample was eluted from the dried matrix, using a formic acid solution. Then, we utilised a direct spectrometry method to scan the extracts for measurement of Hb-drv. We then applied the linear relationship between an individuals’ Hct percentage and measured Hb-drv to estimate the dried sample’s Hct. Potentially our method can be incorporated into various DBS analytical sample preparation process for correction of measured analytes of interest. However, following our observations throughout method development for measurement of 25-OHD3 concentrations in DBS samples, due to the relative diffusion rate of whole blood samples on the collection card, as well as different degrees of extraction efficiency of samples with different levels of Hb/Hct, the Hct effect might not be as significant in practise for samples with a Hb/Hct level within the normal range. Hence, we consider that the levels of a patient’s Hb (and therefore Hct) may influence the measurement from the DBS sample only at extreme high or low ends of the pathological spectrum.
Therefore, we see our DBS Hb-drv best utilised as a screening tool to predict Hct levels at extremes which may then affect the DBS distribution of an analyte.

For DBS analysis, the change of dry matrix into the liquid and elution of the analyte of interest from the filter paper is the first step. Depending on the chemical characteristics of the target analyte, the choice of an appropriate elution and extractor buffer is critical. Considering the hydrophobic molecular structure of 25-OHD we initially tried a water-methanol mixture (3:5) for elution of vitamin D contents from DBS, in conjunction with 30 minutes shaking at ambient temperatures followed by a brief sonication process. This approach was satisfactory for the trial DBS samples with <3 years spotting age, whereas it was not efficient enough for our archived NBS study samples (older than 8 years). The efficiency of elution could be challenging, as there is always a chance of analyte loss, incomplete extraction or analyte degradation during the extraction process. Hence, to achieve effective analyte recovery, we applied a high-temperature, high-speed shaking procedure with the additional solvation energy (sonication).

A significant improvement on our original vitamin D method was the change from liquid-liquid extraction (LLE) to supported liquid extraction (SLE). This offset the small sample volume and laborious manual procedure, by the process with a 96-well plate. This adapted extraction process was still consistent with the original in-house method by using a water-methanol mixture for protein precipitation followed by hexane for extraction, when method comparison was performed by analysis of multi levels of QAP samples. This extraction technique improved not only the turn-around time, but also the extraction efficiency, sensitivity and overall imprecision of our analytical method.

The limited sample volume of dried blood samples is not compatible with most measurement methods and a distinctive analytical technique is required to support the desirable sensitivity. Whereas, currently, derivatisation is not utilised for most LC-MS/MS analyses for measurement of serum/plasma 25-OHD3, it has been used for many DBS testing studies to maintain the required assay sensitivity. However, the derivatisation process prolongs the overall turnaround time. Moreover, a review of the literature has indicated that the common derivatising reagent used for mass spectrometric measurement of vitamin D
metabolites, 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD), does not allow discrimination of 3-epi-25-OHD3. This inactive epimer is seen at high levels in paediatric samples and it is important to be able to discriminate this from the active 25OHD. Several other research groups had unsuccessfully attempted to separate PTAD-derivatised 3-epi-25OHD3 (365, 368, 399) and Higashi et al. demonstrated structurally that this was not possible to separate the epimer from the isomeric forms during the derivatisation process (348). We felt these outcomes were limiting factors for our large sample population study which needed to be overcome.

As enhanced sensitivity and separation of 3-epi-25OHD3 were the required and primary considerations of our study, we had two options; 1) return to the non-derivatised method, or 2) find an alternative derivatising agent. We first explored option (1) by attempting to develop a 2-dimentional chromatography holding set-up, where we would effectively load the first column (multiple injections) with high volume of the extracted sample. Then, adjusting the gradient by increasing the methanol percentage and directing the sample to the second column for the chromatography separation. This approach worked to a limited degree but was cumbersome for our proposed automation and lacked the required assay robustness. We continued to pursue this approach whilst continuing to review the literature.

A new derivatising agent (Amplifex, Sciex) was commercially released in 2013. We therefore adapted our methodology to utilise this reagent in our vitamin D assay. This derivatising reagent proved successful in separating 3-epi-25OHD3 and demonstrated enhanced signal compared to the PTAD. This was in-part because it separated vitamin D chromatographically from phospholipids (ion transitions; 104 > 104 and 184 > 184 m/z). The package inserts for this reagent stated 2-hours stability. However, we found it to be stable for 4 weeks if stored at -20°C and furthermore it could be effectively used at much lower concentrations than the manufacturer’s recommendation, which were important considerations as the cost of Amplifex is substantially more than PTAD. Ultimately, we succeeded to incorporate DBS vitamin D analysis into the serum/plasma vitamin D assay. To achieve this outcome, we needed to adopt our validated in-house method to derivatisate vitamin D, to attain the traceability link.
Chromatographic and mass spectrometric conditions of the in-house serum/plasma vitamin D assay were optimized for the derivatised 25-OHD3. The assay’s overall performance was enhanced through further studies and improvements applied to the previously referenced LC-MS/MS method. The adjustment included: an altered extraction method for sample preparation (LLE changed to SLE), internal standards and utilisation of a different isotope (25-OHD3-[²H₃] replaced with 25-OHD3-[²H₆] for the derivatised compound and a Carbon-labelled internal standard (13C and epi-13C) were utilised for measurement of native 25OHD3 and its epimer). Variations were qualified through assay evaluation analysis and reporting The Royal College of Pathologists of Australasia Quality Assurance Programs (RCPAQAP) samples continuously (Figure 6.1). However, quantitation of epimer was eventually disregarded in the DBS method due to insufficient sensitivity required for measurement of epimer from DBS samples.

Traceability of the DBS measures to the standardized reference plasma/serum base assay, is a crucial requirement for offering a DBS analytical method as a reliable tool. To maintain the traceability link, we used National Institute of Standards and Technology (NIST) traceable commercial materials to calibrate our vitamin D assay. Furthermore, to certify the accuracy of the measurement, external quality control materials were analysed as part of our analytical process, in conjunction with routine analysis of commercial quality control material (Figure 6.1). We applied the theoretical ratio between the average amount of blood sample on a DBS punch and the volume of liquid samples to define the relative quantity of internal standard for both dry and liquid matrices within the same analytical method. Hence, different amounts of internal standard material, which were proportional to the ratio of liquid sample volume to DBS punch, was added into calibrators, quality control materials and dried blood samples to correlate the dried-matrix measures to equivalent liquid sample concentration. The serum / plasma vitamin D decision points could then be applied to the DBS samples.
Figure 6.1. The RCPAQAP report demonstrating the method accuracy and performance. All the values of RCPAQAP analyses, at different concentrations of 25-OHD3, are within the acceptable range and close to the target values (compared to the different method users including other MS method users. One of the uniqueness of our DBS-vitamin D method is; the traceability of the DBS measures to the liquid sample.

The developed method was then utilised to analyse NBS samples to investigate the prevalence of vitamin D adequacy in a Melbourne paediatric population. The information was used to examine the association between vitamin D levels in early life and the prevalence of paediatric food allergy. This was the first, large population-based study of vitamin D status in new-born infants in Victoria. Our data demonstrated the seasonal pattern of vitamin D levels in archived dried bloods and identified that the prevalence of vitamin D inadequacy is considerably higher among the nominated cohort cut-offs.

The current multi-ethnic study is the broadest investigation of vitamin D measures at birth in relation to Ig-E mediated challenge-proven food allergy at 12 months of age. Among the study cohort there was no evidence of a relationship between vitamin D levels and food allergy or eczema. As expected, the prevalence of lower vitamin D levels was decreased in the population with a history of maternal vitamin D supplementation. However, the high prevalence of vitamin D insufficiency suggests that further pharmacokinetic investigations are required to optimize the dosage regime for effective supplementation.
6.2 Need of a dedicated isotopic internal standard for accurate 3-epi-25-OHD3 quantification by LC-MS/MS.

A requirement for a dedicated isotopic internal standard for accurate measurement of 3-epi-25-OHD3 was examined, and the outcome of the study has been communicated as a letter to the editor in Clinical Chemistry and Laboratory Medicine journal. This study was a sub-study of the HealthNuts project and my contribution to this study and subsequent publication was included; developing the concept for the study in conjunction with my senior supervisor (RG), analysis and interpretation of data, writing the first draft of the publication and reviewing and incorporating all suggestions from the co-authors. This section has been peer reviewed and published with the contributions of other co-authors (400).

“We have read with interest a report by Flynn and colleagues which demonstrates significant enhancement of the 3-epi-25-hydroxy vitamin D3 (3-epi-25-OHD3) signal when co-eluted with 25-hydroxy vitamin D3 (25-OHD3) and also when fully resolved (401). Co-elution of 3-epi-25-OHD3 with 25-OHD3 results in a 60% increase in signal enhancement from the epimer and hence the vitamin D result cannot be considered the sum of the two. With resolution and the use of a specific in-house epi calibrator Flynn demonstrated a reduction in bias to +20%. Hence, Flynn’s group has recommended the chromatographic resolution of 3-epi-25-OHD3 from 25-OHD3 and use of a dedicated 3-epi-25-OHD3 calibrator (401). These recommendations are potentially important for clinical stratification as the epimer is added to the vitamin D3 to quantitate the total vitamin D against the clinical decision points, and these positive biases may change the interpretation of a patient’s vitamin D status.

The +20% bias remains a concern and we believe the choice of isotopic internal standard is also important for the accurate quantitation of 3-epi-25-OHD3. Traditionally, deuterated internal standards directly related to the compound of interest have been applied and ideally, these internal standards should co-elute so that they enter the MS at the same time and conditions. However, for vitamin D analysis the same isotopic internal standard is often used for quantitation of both 25-OHD3 and its epimer (401, 402). We hypothesise that this may be the cause of the overestimation of the resolved 3-
epi-25-OHD3 from Flynn's study. Our aim was therefore to determine the need for the inclusion of a dedicated internal standard in our method for the quantitation of 3-epi-25-OHD3.

Some experts have questioned the stability, and therefore accuracy, of using deuterated internal standards (403-405). This has led to increased advocacy for internal standards with closer physio-chemical properties, such as the use of carbon 13 (\(^{13}\)C) labelled internal standards for LC-MS/MS methods when available (406). As there are now dedicated \(^{13}\)C labelled internal standards commercially available for both 25-hydroxy vitamins D3 and also its epimer, we purchased 25-Hydroxy vitamin-D3-[23,24,25,26,27-\(^{13}\)C5] (abbreviated to \(^{13}\)C) and 3-Epi-25-Hydroxyvitamin D3-[\(^{13}\)C5] (abbreviated to epi-\(^{13}\)C) from Isosciences, via PM Separations, Qld, Australia, to analyse 425 paediatric samples for vitamin D using an adaptation of our published method (308). The blood samples were part of the Vitality trial (407); Royal Children’s Hospital Human Research Ethics Committee approval number 34168H.

Briefly, calibrators, controls and patient samples were initially prepared by supported liquid-liquid extraction employing hexane. For the liquid chromatography separation, a core-shell Kinetex F5 column (Phenomenex Inc., Australia) was used as the stationary phase and a gradient of water and methanol with 0.1% formic acid for the mobile phase was applied. The mass selective detection was performed utilising an Agilent 6490 system in positive ESI mode with multiple reaction monitoring (MRM) applied for quantitation of 25-OHD3 and its epimer (m/z 401>383) and the \(^{13}\)C-labelled isotopes (m/z 406.3>107). The performance of the adopted method is: imprecision of <5.0%; average bias of 0.04 μmol/L; and lower limit of quantitation (LLOQ) of 1.0 nmol/L. The method is standardised using the Chromsystems 25-OHD3 and 3-epi Calibrators (Astral Scientific, NSW, Australia). The 3-epi-25-OHD3 was quantitated against both internal standards and compared.

The results from our study demonstrate that 65 of the 425 paediatric patients had detectable concentrations of 3-epi-25-OHD3 above the LLOQ when quantitated against the \(^{13}\)C-epi internal standard (Figure 6.2). These 65 participants represent 15% of the study cohort and had a median age of 7 months (minimum of 2 months and maximum of 25 months). Comparison of the \(^{13}\)C versus the
epi-$^{13}$C internals standards for quantitation of the epimer demonstrated a significant positive bias of +30% when the non-retention time matched internal standard was applied (Figure 6.3). The data also demonstrates the variation of 3-epi-25-OHD3 in the samples, with 85% having no detectable 3-epi-25-OHD3 to a significant proportion having concentrations up to 30 nmol/L. This in itself confounds the interpretation of results, but the use of a non-epi internal standard would artificially inflate these results and potentially change the clinical interpretation.

The study participants’ 25-OHD3 concentrations ranged from 4 to 150 nmol/L with a median of 58 nmol/L. 159 of these participants had vitamin D deficiency, when epi-25-OHD3 was separated (deficiency defined as less than 50 nmol/L (350-352)). A change in the clinical interpretation occurred
in ten of the 65 patients when the dedicated epi-\textsuperscript{13}C internal standard was applied (in preference to the C13-25-OHD3 internal standard): one patient was reclassified from adequate to insufficient based on the 50 nmol/L decision point; nine patients were reclassified from optimal to adequate based on the 75 nmol/L decision point; and no patients were reclassified based on the 25 nmol/L decision point. These irregular errors potentially confound the clinical interpretation of the vitamin D results in cohorts where epi-25-OHD3 is reported to be present in significant amounts (93, 308, 408).

Still to date, the physiological action of 3-epi-1,25-OHD3 is not known. The 3-epimerisation may be an inactivation mechanism, as the epimer has reduced binding affinity to the vitamin D receptor (409). Yet, it does inhibit parathyroid hormone production as much as calcitriol (409). Regardless of its biological activity, it is worthwhile to measure the epimer accurately and precisely, as its presence can significantly affect the total 25-OHD3 test results of those individuals with detectable concentrations of epi-25-OHD3. This is relevant as the current international guidelines for the interpretation of vitamin D add the forms of 25-OHD together for interpretation against the clinical decision points (350-352).

Accurate assessment of vitamin D status requires both the separation and quantification of epi-25-OHD3. Even with the separation and accurate quantitation of 3-epi-25-OHD3, the summation with 25-OHD3 leads to a change in the clinical interpretation for 18 of our 65 patients with detectable concentrations of epi-25-OHD3. This raises the question of whether future clinical guidelines for vitamin D status should be redefined to be specific for 25OHD3 only (i.e. excluding 3-epi-25-OHD3) to ensure consistent interpretation.

In conclusion, in our study we have demonstrated that the use of a dedicated epimer internal standard corrects the positive bias as hypothesised. We support the prior recommendations of chromatographic resolution and use of a dedicated calibrator for the quantitation of 3-epi-25-OHD3. Based on our findings, we strongly recommend where practical, that serum vitamin D measurement by LC-MS/MS includes the chromatographic resolution of the epimer, a specific calibrator and an epimer matched internal standard, preferably \textsuperscript{13}C is applied when quantitation of the epimer is required. LC-MS/MS methods that do not fulfil these requirements risk overestimating an individual’s vitamin D status.
Quantification of epi-25-OHD3 is also recommended for future discoveries regarding the biological function of epi-25-OHD3 which may require all accurate vitamin D assessments to include epimer quantification. This will allow for clear and accurate data analysis of clinical studies.”

6.3 Conclusions

In this project we have successfully developed a robust vitamin D DBS method that has significant advantages including its ability to separate 25-hydroxy vitamin D epimer if required, with its application extend into liquid sample analysis. Furthermore, we developed a method to predict Hct level in archived blood spot samples, which can be incorporated into DBS analytical work-flow to alleviate the Hct effect at extreme levels. Ultimately the value of a method is in its clinical application. The DBS vitamin D method has been successfully used to address the question of whether there is an association between vitamin D levels at birth and paediatric food allergy. The findings from our large data set of 2489 participants’ samples indicate that the vitamin D levels at birth do not predict / influence the onset of challenge proven food allergy in infants (11-12 months) as no association was found. As newborn vitamin D levels were variable in those with maternal vitamin D supplement, further studies are warranted to determine the appropriate regime to ensure vitamin D sufficiency.
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Appendices

App.1 LC-MS lab glass-ware washing standard procedure

😊 Cleaning Tips

✔ Wash lab glass wares as quickly as possible after use. If a thorough cleaning is not possible immediately, put glass wares to soak in distilled water!

✔ Most new glassware is slightly alkaline in reaction. For precision chemical tests, new glassware should be soaked several hours in acid water (1% solution of hydrochloric or nitric acid) before washing.

1. Wash the tubes with distilled water until all specimen residues are well rinsed. Use the glassware brush if required.
2. Oven-dry tubes at 50°C.
3. Fill the tubes with 65% nitric acid under the chemical fume hood, stand for 10 mins.
4. Pour back nitric acid into the nitric acid bottle for further use. (Nitric Acid recycling frequency; to be determined)
5. Rinse tubes with distilled water five times and stand overnight distilled water- filled tubes.
6. Rinse tubes with Milli-Q water two times.
7. Oven-dry tubes at 50°C.

References:


● Ali Al-Bahrani, PhD thesis
App.2 Development of a DBS vitamin D quantitation method, incorporated into the serum/plasma vitamin D assay

Introduction

Vitamin D is chemically a group of seco-steroids, which possesses an open cyclo-pentanoperhydrophenanthrene ring (ring B on carbon 9, 10). These seco-steroids are structurally comparable to the steroid hormone group. The biologically active vitamin D metabolite (1α-25(OH)2-D), exhibits hormone like behaviour. However, 25(OH)-D (Calcifediol) is the analogue of vitamin D with the higher circulatory concentration and longer half-life in adults, while its epimer (epi-25(OH)-D) is significantly expressed in paediatric group. Apart from its seco-steroid form, the presence of a side chain provides a more flexible molecular structure compare to classic steroids (Figure 3.1).

![Figure 8.1: Chemical structure of Calcifediol](image)

Cholesterol, then 7-Dihydroxycholestrol is the launch point of vitamin D3 (cholecalciferol) formation in the human skin due to sunlight exposure. Ultra-violet light initiates a series of photochemical reactions to convert this seco-steroid to pre-vitamin D3 in malpighi-layer of skin. Pre-vitamin D3 can isomerase to vitamin D3 within 2 or 3 days. Vitamin D metabolites mostly expose a high affinity to the plasma vitamin D binding protein (DBP). However, DBP circulatory concentration is in a great molar excess compare to vitamin D metabolites, only very limited portion of metabolites circulate freely. This vitamin D-binding protein (DBP) carries cholecalciferol to the liver; where the 25-hydroxylase enzyme effects the formation of 25(OH)-D3.The complex of DBP-25(OH)-D3 then takes the journey to the
kidney for a further hydroxylation step, mediated by 1α-hydroxylase to produce the active hormone-like 1α,25(OH)2-D3 (Calcitriol) and 24,25(OH)2-D3 metabolites.

Regulation of 1α,25(OH)2-D is under the control of a complex positive and negative feedback system which effects the expression of the hydroxylase enzymes. Decreased dietary calcium and phosphate intake and elevated PTH, due to low circulatory calcium concentration, induce the activation and gene transcription of 1-α-hydroxylase. In turn, elevated PTH supresses the gene transcription. However, the regulation of 24-hydroxylase is under control of 1α,25(OH)2D status, plasma calcium concentration and PTH level. Along with the regulatory role on mineral homeostasis, a wide range of various physiological functions are assigned to hormonal action of vitamin D. These include; effect on cancer cells growth rate, immune system development and protection against certain immune disorders.

Traditionally vitamin D is measured as serum/plasma 25-hydroxy cholecalciferol and the cut-off for interpretation is clearly defined. However, the collection, transport and storage requirements for serum/plasma are cumbersome for large scale population-based studies. As a result, there has been increased interest in the use of dried blood spots (DBS) as a matrix for the assessment of vitamin D. Following the project aim of investigation of the association between early life vitamin D insufficiency and paediatric food allergy, we developed the accurate method for measurement of vitamin D metabolites from archived DBS samples.

Current SOP explains the procedure of the measurement of 25-hydroxy-vitamin D3 measurement from the serum/plasma and the DBS sample, utilizing liquid chromatography coupled with tandem mass spectrometry technique.

**Principle of the method**

Serum/DBS proteins are precipitated using Water/Methanol mixture. Deuterated 25-hydroxyvitamin D3 (d6) added as internal standards. The analytes are extracted into hexane through solid phase extraction (SLE) procedure. The hexane is recovered and evaporated to dryness under a speed-vacuum condition at 40°C temperature and 500 rpm speed. After evaporation of the hexane eluate residue is
dissolved in methanol-water mixture. Amplifex Diene reagent is used to derivatise vitamin D of the DBS samples.

The methanolic extract is injected onto a core-shell Kinetex F5 column (Phenomenex Inc., Australia). The vitamins are eluted using a solvent program. The initial solvent is 35% methanol in water. The maximum organic solvent gradient is 100% methanol. Vitamin D (non-derivatised) is detected by m/z ratio transitions of 401→383 and 401→365. Vitamin D (derivatised) is detected by m/z ratio transitions of 732.4→673.4 and 732→217.

The calibration curve (7 points) is linear (1/X) and the origin is ignored in the regression analysis. DBS Hct is estimated to correct variations and convert the measures into the equivalent serum.

Formic acid solution (0.1%) is used to elute haemoglobin content of DBS.

**Specimen and sample storage**

**Specimen type**

Serum or EDTA/ heparinised plasma and, also air dried whole blood spot are suitable for vitamin D assay. A minimum volume of 150 µL of light protected serum/plasma and one drop (~ 50 µL) whole blood spotted on the filter paper (Whatman 903) is required.

**Effect of diet / sun exposure**

Non-fasting samples are routinely analysed at RMIT LC-MS/MS lab. Perhaps the seasonal effect may need to be considered for vitamin D results interpretation.

**Specimen storage**

Blood should be centrifuged, and the serum/plasma separated within 30 minutes of venesection. The separated serum or plasma is transferred to a storage tube and stored at -20°C until analysis. Spotted
blood should air dry for minimum 2 hours. Dried blood samples store in darken container, kept in biohazard specimen zipper bag at room temperature.

**Reagents**

**Safety warnings**

Inflammable solvents (hexane and methanol) had to be kept away from sources of ignition including electrical switches that may cause sparking. And, also must be used in a fume hood. Storage bottle were tightly capped when were not in use. The chemical spill kit (located in room under the washing basin) and the safety shower/eye wash stations were located in the room. Skin contact and inhalation of solvent vapour were avoided to prevent solvent toxicity.

Nitrogen gas cylinders were stored in cylinders under high pressure. Ensured the cylinders were secured so that they could not topple and fracture the high-pressure fittings. Standard safe operation procedure of the high pressure shut-off valve was followed before changing the regulator to a fresh cylinder.

**Chemicals**

**Milli-Q water**

Supplies by Millipore water filter-Element A10, located in Biochemistry Laboratory-Module H (223.01.07)

**Nitrogen, high purity**

Nitrogen, high purity, Coregas Cat. No. 221150 or equivalent

**Methanol**

Methanol, gradient grade for liquid chromatography-mass spectrometry, cat no. 1.06018.4000 (Merck). Do not substitute with any other grade of methanol.
N-Hexane

N-Hexane, for liquid chromatography-mass spectrometry, cat no. 1.04391.2500 (Merck).

Formic acid

LC-MS Ultra, eluent additive for UHPLC-MS, cat no. 94318 (Sigma-Aldrich)

Derivatising agent

Amplifex™ Diene reagent, product no. 5037804 (Sciex)-the material prepared following manufacturer instruction as the stock solution. Stock solution was diluted with the diluent (acetonitrile) 1:1 (50%) to prepare the working-solution (prepared fresh per batch).

Stock internal standard solution

Deuterated 25-hydroxyvitamin D3-[2H6], in ethanol, 100 µg/mL, cat no. S4163 (Iso-Science)

Stock internal standard solution preparation (4926 nmol/L)

Deuterated 25-hydroxyvitamin D3 ampule has been removed from storage (-20°C) and allowed it to warm to room temperature. The entire ampule contain was added into the HPLC grade methanol to make up the final volume of 50 mL (including methanol wash step of the ampule to ensure whole content has transferred into the glass Schott storage bottle) and was stored at -20°C (stable for at least one year).

Working internal standard solution; deuterated 25-hydroxyvitamin D3 (d6) in methanol 246 nmol/L

Deuterated 25-hydroxyvitamin D3 stock solution bottle was removed from the storage (-20°C) and allowed the standard material to warm to room temperature. 5 ml of stock internal standard solution was diluted in the HPLC grade methanol to make up the final volume of 100 mL (including methanol
wash step of the ampule to ensure whole content has transferred into the glass Schott storage bottle. Working IS solution was stored -20°C (stable for at least six months).

**Diluted internal standard solution; deuterated 25-hydroxyvitamin D3 (d6) in methanol for liquid matrix and DBS samples (123 and 10 nmol/L) – to be prepared fresh for each batch of analysis**

Working internal standard (IS) solution; deuterated 25-hydroxyvitamin D3 (d6) in methanol (246 nmol/L) was removed from the storage (-20°C) and allowed the standard material to warm to room temperature. Estimate the required volume of internal standard for the batch (100 µL per each liquid sample) and dilute working internal standard solution in milli-Q water-methanol mixture (2:1:1) to make up the total required volume. (e.g. for one 96-well plate batch including 7+3 calibrators and 86 DBS samples mix 1500 µL working IS with 750 µL milli-Q water and 750 µL methanol for the total volume of 3 mL).

Use diluted-IS (123 nmol/L) as the stock material for preparation of DBS-match IS solution; mix 1 part of diluted IS with 11 parts of milli-Q and methanol mixture to obtain the required volume with the concentration of 10 nmol/L. (e.g. for one 96-well plate batch including 7+3 calibrators and 86 DBS samples; mix 675 µL diluted-IS with 1240 µL milli-Q water and 6185 µL methanol).

**Mobile phase A**

10 mL methanol and 500 µl of LC-MS ultra-grade formic acid were added into a LC-MS/MS lab standard washed 1L measuring cylinder to make up to the 500 mL mark by adding milli-Q water. It was refreshed weekly to minimize the possible bug growth incidence.

**Mobile phase B**

500 µl of LC-MS ultra-grade formic acid was added into a LC-MS/MS lab standard washed 500mL measuring cylinder and then HPLC grade methanol was added to make up 500 mL total volume. It was refreshed per run batch to maintain the chromatogram integrity.
Calibrator material

Vitamin D calibrator set

Chromsystems 6PLUS1® Multilevel Serum Calibrator Set, 25-OH-Vitamin D3/D2, cat no. 62039 was used as calibrator for quantification. Stock materials received as 7x1 mL lyophilised materials. Working solutions were prepared according the manufacturer instruction by adding 1mL milli-Q water into each bottle. The bottles were left on the working bench for 10mins, following 5mins on the low speed rolling mixer. Aliquots of 130 µl into 2 mL – properly labelled (ID, lot no, expiry date, reconstitution date, initial) plastic Eppendorf tube were stored at -20°C.

External / internal quality control material

Vitamin D control set

UTAK Vitamin D Plus; 3 levels control, cat no.10060/10061/10062 were used to control the assay performance. Stock materials received as 5 mL lyophilized bottles. Working solution were prepared following the manufacturer instruction by adding 5mL milli-Q water using 5mL glass volumetric pipette into each bottle. The bottles were left on the working bench for 10mins, following 5mins on the low speed rolling mixer. Aliquots of 130 µl into 2 mL – properly labelled (ID, lot no, expiry date, reconstitution date, initial) plastic Eppendorf tube were stored at -20°C.

External quality control

RMIT LC-MS/MS laboratory participates in Vitamins and Endocrine RCPA-QAP program for vitamin D. Materials are received annually to be prepared (following the package insert instruction), analysed and reported bases on the report schedule calendar.

Analytical procedure

Work-list generation
The excel work-list template is generated and used to prepare the work-list, incorporating calibrators, controls and unknown samples (DBS and serum). The patient name and ID were recorded in columns B and C. The sample type was recorded in column D – either serum. For DBS samples, the disc size was entered in column E. Samples were initially prepared in 96 well micro plates.

- Record the patient name and ID in column A.
- Record the sample type in column B – Liquid / DBS
- Print the worksheet and use for the sample preparation
- Let frozen and refrigerated material defrost and reach the room temperature
- Label a 96 micro-well plate matching the work-list numbers

*Note: maximum batch size is 96 for plate format set up.

**Sample Preparation**

Liquid calibrators, QC materials and any other liquid matrix samples were prepared in conjunction with DBS samples (solid matrix);

DBS, calibrators and controls sample preparation, 125µl of 0.1% formic acid solution is added on 3.2 mm punch DBS and 50µL de-ionised water is added to 50µL of calibrators and QC materials samples, followed by 60mins shake; the first 30mins at RT and the second 30mins at 60°C. 75 µl water-methanol mixture containing 10 nmol/L and 100 µL of water-methanol mixture containing 123 nmol/L deuterated internal standard (ISTD) (25-hydroxy vitamin D3-d6) were added into DBS and calibrators/QCs samples respectively. Samples subjected to further 10mins sonication.

DBS eluate and pre-treated calibrators and QCs, purified by using Biotage SLE cartridges (Cat no820-0200-P01) and application of 350 µL hexane elution (twice). Sample eluate is collected and dried using rotational vacuum concentrator (Martin Christ, Part No: RVC2-33PACK, John Morris Scientific Pty Ltd, Australia) at 45°C.
Derivatisation, 50 µL Amplifex diene reagent solution was added to the dried sample, then was vortexed for 20sec, followed by 30mins incubation at 4°C. The derivatisation reaction was neutralised by addition of 75 µL methanol/de-ionised water mixture (1:1).

- If not already punched, punch out one 3mm disk from the DBS directly into the matching well of the plate.
- Note any spot that is not as expected.
- Pipette 50µL of the serum calibrators, control and unknown liquid samples in their assigned micro-plate well numbers matching the work-list order.
- Add 125µl of 0.01% formic acid solution to the DBS punches
- Add 50µl 0.01% formic acid solution to liquid samples
- Cover the plate with the sealing mat
- Shake the plate for 30mins at 700 rpm / RT
- Prepare diluted IS solutions (whilst plate shaking)
  a) Make 1/2 dilution from the 246 nmol/L stock IS (50:25:25 – IS:H₂O:MeOH)
  b) Make 1/12 dilution from liquid matrix match IS working solution (1:11(1:6) - IS: H₂O/MeOH(1:6)) to be added to 3mm DBS
- Take the plate off the shaker and remove the sealing mat
- Add
  a) 100µL of 1/2 diluted IS (123 nmol/L) to the liquid matrix samples
  b) 75µL of 1/12 IS (10 nmol/L) to DBS samples
- Cover the plate with the sealing mat (ensuring same orientation) and shake for 30secs
- Sonicate the plate for 10mins
- Remove the Biotage SLE plate (Part no. 820-0200-P01) from the box and locate it on the collection plate
- Transfer the spiked samples from the extraction plate using multichannel pipette into the matching well of SLE plate
- Let samples absorb for 1 minute
- Apply positive pressure using ‘UCT positive pressure manifold’ briefly onto the SLE plate; 10 bar, for < 1sec
- Wait for 5mins
- Add 350 µl Hexane to SLE wells in two episodes and collect all total app. 700 µl hexane-eluate into the Collection plate (250, 250, 200µl if using 300µl multichannel pipette)
- Leave to elute with gravidity for 5mins
- Apply positive pressure for 1-2sec
- Use the SpeedVac (RCV 2-33 CD Plus) to dry the hexane-eluate (evaporation program no. 22-Rosy)
- Take collection plate out of speed-vac and add 50 µL ½ diluted Amplifex solution (50:50 – Amplifex:AcN) to the dried samples
- Re-seal the collection plate and shuck the plate for 30secs
- Incubate plate at 4°C for 30mins
- Prepare Stop-solution (50:50 – H₂O: MeOH)
- At the end of 30<sup>th</sup> min incubation, take the plate out of the fridge, then take the sealing mat off and add 75 µL Methanol-water mix (50:50) stop-solution, to stop the derivatisation reaction
- Re-seal the plate properly and shake it for 30<sup>sec</sup>
- Place the plate on the LC auto-sampler and set up LC-MS/MS to run

**Chromatographic-Tandem mass spectrometric analysis**

**Chromatographer and Mass spectrometer**

The routine HPLC was an Agilent Model 1260 infinity, it consists of the following modules: Degasser; Quaternary Solvent pump; Columns Oven which is coupled with the Agilent Model 6490 triple-quadrupole mass spectrometer. LC-MS/MS module control, data acquisition and data processing were provided by MassHunter software (Version B.4.04/B.4.0/B.5.0) (Referred to “General Operation for the Agilent 1290/6490” manual for full general operational details).

**Data acquisition work-list example**

Referred to “General Operation for the Agilent 1290/6490” manual for full general operational details.

**LC-MS/MS analytic method**

Referred to “General Operation for the Agilent 1290/6490” manual for full general operational details.

“20180124-29-F5100-binpump” analytical method was used.

**Chromatographic column**

Analytic column is core-shell column (2.6µm, 100mm, Kinetex F5, Phenomenex Inc., Australia)

Guard column is Pursuit 3 PFP-10x2mm (Agilent Technologies cat no. A3051MG2).

**Quantitative data processing**

“Agilent MassHunter Workstation Software Quantitative Analysis Quant method” was used for LC-MS/MS results quantification (Refer to “General Operation for the Agilent 1290/6490” manual for full general operational details).
Quality control reports

Internal QC reports were archived on “MedLab QC” software for each analysis batch, and external QA results were reported to RCPA-QAP website monthly.

Reporting of patients results and reference intervals

Results for Vitamin D are reported in nmol/L. The values >75 nmol/L for 25OH-D3 indicate the vitamin D sufficiency, while vitamin D deficiency is defined as different levels of; Mild (serum 25OHD3: 25-50 nmol/L), Moderate (serum 25OH-D3: 12.5-25 nmol/L), Severe (serum 25OH-D3 < 12.5 nmol/L)

Figure 8.2. Vitamin D external quality control (RCPAQAP) end of cycle report

Figure 8.3. 7 point-calibration curve for derivatised 25-OHvitamin D3
Figure 8.4. Chromatogram of detection of 25OH-vitamin D3 and 3epi-25OH-vitamin D3 from DBS sample.
App.3 3000 NBS Guthrie cards sample retrieval and collection and storage process