Curtin School of Population Health

Investigating dietary vitamin D in Australia

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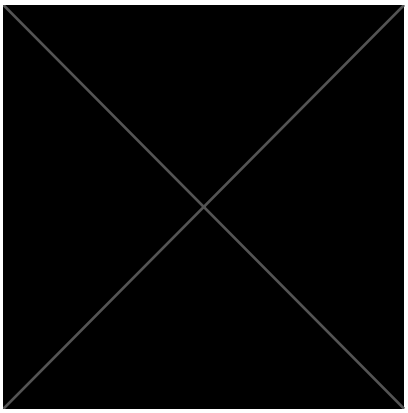
Declaration

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.



Acknowledgment of Country

We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

Acknowledgments

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This has been an incredible adventure. Thank you all!

Publications, presentations, awards and prizes

Work in this thesis contributed to the following publications, conference presentations, awards and prizes:

Publications

1. **Dunlop E**, AP James, J Cunningham, N Strobel, RM Lucas, M Kiely, CA Nowson, A Rangan, P Adorno, P Atyeo, LJ Black. 2021. Vitamin D composition of Australian foods. *Food Chemistry*: 358:129836. doi: 10.1016/j.foodchem.2021.129836.

2. **Dunlop E,** CCJ Shepherd, J Cunningham, N Strobel, RM Lucas, LJ Black. 2022. Vitamin D composition of Australian game products. *Food Chemistry*: 387:132965 2022. doi: 10.1016/j.foodchem.132965

3. **Dunlop E**, JL Boorman, TL Hambridge, J McNeill, AP James, M Kiely, CA Nowson, A Rangan, J Cunningham, P Adorno, P Atyeo, LJ Black. 2022. Evidence of low vitamin D intakes in the Australian population points to a need for data-driven nutrition policy for improving population vitamin D status. *Journal of Human Nutrition and Dietetics*: in press. doi: 10.1111/jhn.13002.

4. **Dunlop E**, M Kiely, AP James, T Singh, LJ Black. 2020. The efficacy of vitamin D food fortification and biofortification in children and adults: a systematic review protocol. *JBI Evidence Synthesis*: 18(12): 2694-2703. doi: 10.11124/JBISRIR-D-19-00373.

5. **Dunlop E**, ME Kiely, AP James, T Singh, NM Pham, LJ Black. 2021. Vitamin D food fortification and biofortification increases serum 25-hydroxyvitamin D concentrations in adults and children: an updated and extended systematic review and meta-analysis of randomized controlled trials. *Journal of Nutrition*: 151(9): 2622-2635. doi: 10.1093/jn/nxab180.

The above publications have been peer-reviewed. Permission has been obtained from the copyright owners to use my published work in which the copyright is held by another party (Appendix I).

Conference abstracts (oral communication selected from abstract)

1. **Dunlop E**, JL Boorman, TL Hambridge, J MacNeill, AP James, M Kiely, CA Nowson, A Rangan, J Cunningham, P Adorno, P Atyeo, LJ Black. 2021. Vitamin D intakes in the Australian population. Dietitians Australia (Western Australia) State Symposium, 5th November 2021, Perth, **Australia** (virtual presentation)

2. **Dunlop E**, AP James, J Cunningham, N Strobel, RM Lucas, M Kiely, CA Nowson, A Rangan, P Adorno, P Atyeo, LJ Black. 2021. A new comprehensive analytical vitamin D food composition database of Australian foods. Dietitians Australia 2021 Conference, 11-13th July 2021, Melbourne, **Australia** (virtual presentation)

3. **Dunlop E**, J Cunningham, N Strobel, RM Lucas, M Kiely, CA Nowson, A Rangan, H Greenfield, AP James, LJ Black. New data for the vitamin D content of Australian foods. 13th International Food Data Conference (IFDC), 14-18th October 2019, Lisbon, **Portugal**

4. **Dunlop E**, CCJ Shepherd, J Cunningham, N Strobel, RM Lucas, LJ Black. New data for vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_2 and 25-hydroxyvitamin D_2 content in Australian camel, crocodile, emu and kangaroo. 13th Asian Congress of Nutrition, 4-7th August 2019, Bali, **Indonesia**

5. **Dunlop E**, J Cunningham, H Greenfield, N Strobel, AP James, RM Lucas, M Kiely, CA Nowson, A Rangan, LJ Black. Developing an Australian food composition database for vitamin D content in foods. 2nd International Conference on Food Analysis (ICFA), 19-22nd November 2018, Melbourne, **Australia**

Conference abstracts (poster communication selected from abstract)

1. **Dunlop E**, CCJ Shepherd, J Cunningham, N Strobel, RM Lucas, LJ Black. Vitamin D in Australian game products, 3rd International Conference on Food Analysis (ICFA), 16-18th November 2021, Melbourne, **Australia** (virtual presentation)

2. **Dunlop E**, JL Boorman, TL Hambridge, J McNeill, AP James, M Kiely, CA Nowson, A Rangan, J Cunningham, P Adorno, P Atyeo, LJ Black. Vitamin D intakes are low in the Australian population. 6th International Vitamin Conference, 22-24th September 2021, Copenhagen, **Denmark** (virtual presentation)

3. **Dunlop E**, J Cunningham, N Strobel, CCJ Shepherd, RM Lucas, LJ Black. Exploring the vitamin D content of Australian bush meats. 10th OCEANIAFOODS Conference, 3-4th September 2019, Auckland, **New Zealand**

Awards and prizes

2021: Premier's Science Awards: Student Scientist of the Year Finalist

Awarded for research achievements to date

https://www.wa.gov.au/organisation/department-of-jobs-tourism-science-and-innovation/premiers-science-awards

2021: Nutrition Society of Australia, Early-Career Development Award

Awarded for the publication "Vitamin D composition of Australian foods"

2021: Dietitians Australia 2021 Conference, New Researcher Award

Awarded for the presentation "A new comprehensive analytical vitamin D food composition database of Australian foods"

2020: Three Minute Thesis (3MT) Curtin University Finalist

"Improving knowledge of vitamin D in Australian Foods"

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Abstract

Vitamin D deficiency (serum 25-hydroxyvitamin D (25(OH)D) concentrations < 50 nmol/L) affects nearly one in four Australian adults, including around one third of young adults and almost 40% of Aboriginal and Torres Strait Islander adults living in remote areas. This is concerning as vitamin D is essential for bone health. Vitamin D deficiency may also be associated with adverse health outcomes, such as diabetes, chronic kidney disease, cardiometabolic risk factors, gastroenteritis and respiratory infection.

The opening chapter provides introductory background information on vitamin D and places the contribution of the thesis amongst the knowledge gaps in the field. The aim of this thesis was to fill the knowledge gaps around dietary vitamin D in Australia in order that food-based strategies may be explored to improve vitamin D status in the population. This aim was fulfilled through four objectives: 1) To develop Australia's first comprehensive vitamin D food composition database for vitamin D_3, 25(OH)D_3, vitamin D_2, and 25(OH)D_2 in commonly consumed foods; 2) To determine the vitamin D content of Australian game products, which may be a useful source of vitamin D for people living in remote areas.; 3) To identify the major food sources of vitamin D and generate the first estimates of usual vitamin D intakes in the Australian population; and, 4) To conduct a systematic review and meta-analysis of randomised controlled trials (RCTs) to evaluate the efficacy of cholecalciferol (vitamin D_3) and ergocalciferol (vitamin D_2) food fortification on serum 25-hydroxyvitamin D concentrations. These objectives were addressed through a series of publications and manuscripts.

Chapter 2 details the development of the first comprehensive analytical vitamin D food composition database for commonly consumed foods in Australia. Food samples (n = 896) representing 98 food products were purchased across three cities and combined into 149 analytical samples. Cholecalciferol (vitamin D_3), 25(OH)D_3, ergocalciferol (vitamin D_2) and 25(OH)D_2 concentrations were measured using a sensitive and specific liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ) method developed at the National Measurement Institute of Australia for this project. The greatest mean (standard deviation) concentrations of vitamin D_3

were found in canned salmon (19.2 (4.8) μg/100 g) and fortified malted chocolate drink powders (17.8 (1.9) μg/100 g). Chicken eggs and chicken leg meat provided the greatest concentrations of 25(OH)D_3. Fortified vegan margarine products and dark chocolate were, respectively, the greatest fortified and naturally-occurring sources of vitamin D_2. 25(OH)D_2 was quantitated in lamb liver and detected in various other meats and animal products.

In Chapter 3, the vitamin D content of selected Australian game products was investigated. Various cuts of camel, crocodile, emu and kangaroo meat were purchased from a central supplier and emu eggs and emu oil were purchased from emu farms. At least one D vitamer was detected in each game product with respective concentrations for vitamin D_3, 25(OH)D_3 and vitamin D_2 of up to 14.5 μg/100 g (emu oil), 5.1 μg/100 g (camel hump) and 1.3 μg/100 g (emu oil). Trace amounts of 25(OH)D_2 were detected in camel and kangaroo meats. Camel meat had a unique 25(OH)D_3-dominated D vitamer profile, while kangaroo meat proved interesting for its relative lack of all D vitamers.

In Chapter 4, our new vitamin D food composition data were used to produce the first estimates of usual vitamin D intakes in the Australian population based on comprehensive vitamin D food composition data. The analytical data were mapped to the 5,740 foods in the 2011-2013 Australian Health Survey food composition database, AUSNUT. The National Cancer Institute Method was used to estimate usual vitamin D intakes by sex and age group. More than 95% of Australians aged ≥2 years were estimated to have inadequate intakes (mean usual intake range: 1.8 to 3.2 μg/day) compared to the Estimated Average Requirement of 10 μg/day recommended by the US Institute of Medicine.

Chapter 5 details the systematic review, meta-analysis and dose-response analyses that were conducted to evaluate the efficacy of vitamin D_3 and vitamin D_2 food fortification on circulating 25(OH)D concentrations. Pooled data from 34 studies conducted in 18 countries supported vitamin D food fortification as an effective strategy to improve circulating concentrations of 25(OH)D. A non-linear dose-response rate was determined (Wald test for nonlinearity $p < 0.001$) with a threshold of ~26 nmol/L at a daily vitamin D dose of ~21 μg.

This body of work advances our knowledge of dietary vitamin D in Australia. Australia now has world-class, comprehensive vitamin D food composition data that demonstrates the importance of measuring the four main D vitamers relevant to food. The first nationally representative estimates of usual vitamin D intakes made using comprehensive food composition data point are concerningly low when coupled with the prevalence of vitamin D deficiency in Australia. The publications included in this thesis provide the data needed to explore food fortification options, which have been demonstrated as effective and could be considered to improve dietary intakes of vitamin D and vitamin D status in the Australian population.

Statement of contribution of others

My contribution to each of the included publications has been detailed and endorsed by co-authors (Appendix II).

Publication	**My contribution**
Vitamin D composition of Australian Foods	Managed the project Purchased samples (Perth sampling round) Curated and quality-checked the data Wrote the paper, wrote the original response to reviewer comments, and compiled the final manuscript and reviewer responses to include Co-Author's suggestions
Vitamin D composition of Australian game products	Managed the project Purchased and arranged transportation of samples Curated and quality-checked the data Wrote the paper
Evidence of low vitamin D intakes in the Australian population points to a need for data-driven nutrition policy for improving population vitamin D status	Calculated total vitamin D concentrations for analysed foods Provided indications for mapping of analytical data to the survey food dataset Analysed usual intakes output data Produced estimates of absolute intakes from food and supplements Wrote the paper, wrote the original response to reviewer comments, and compiled the final manuscript and reviewer responses to include Co-Author's suggestions
The efficacy of vitamin D food fortification and biofortification in children and adults: a systematic review protocol	Co-developed the search strategy Conducted initial database searches Wrote the paper, wrote the original response to reviewer comments, and compiled the final manuscript and reviewer responses to include Co-Author's suggestions
Vitamin D food fortification and biofortification increases serum 25-hydroxyvitamin D concentrations in adults and children: an updated and extended systematic review and meta-analysis of randomized controlled trials	Co-developed the search strategy Conducted the initial search and selected studies Extracted and curated data Conducted statistical analyses and analysed output data Designed figures Wrote the paper, wrote the original response to reviewer comments, and compiled the final manuscript and reviewer responses to include Co-Author's suggestions

Table of contents

List of abbreviations

1,25(OH)D:	1,25-dihydroxyvitamin D_2
25(OH)D:	25-hydroxyvitamin D
ABS:	Australian Bureau of Statistics
AFCD	Australian Food Composition Database
AHS:	2011-2013 Australian Health Survey
AI:	Adequate Intake
AOAC:	Association of Official Agricultural Chemists
BMI:	body mass index
CDC:	US Centers for Disease Control and Prevention
CLIA:	chemiluminescence immunoassay
CMIA:	chemiluminescent microparticle immunoassay
CPBA:	competitive protein-binding assay
CYP:	cytochrome P
EAR:	Estimated Average Requirement
EFSA:	European Food Safety Authority
ELISA:	enzyme-linked immunosorbent assay
FDA:	U.S. Food and Drug Administration
FSANZ:	Food Standards Australia New Zealand
HPLC:	high performance liquid chromatography
IOM:	Institute of Medicine
JBI:	Joanna Briggs Institute
LC-MS/MS:	liquid chromatography with tandem mass spectrometry
LC-QQQ:	liquid chromatography with triple quadrupole mass spectrometry
LOD:	limit of detection
LOR:	limit of reporting
LOQ:	limit of quantitation
NCI:	National Cancer Institute
NHANES:	National Health and Nutrition Examination Survey
NHMRC:	National Health and Medical Research Council of Australia
NIST:	National Institute of Standards and Technology
NMI:	National Measurement Institute of Australia

NNPAS:	2011-2012 National Nutrition and Physical Activity Survey
NRV:	Nutrient Reference Values
NUTTAB:	predecessor to the Australian Food Composition Database
PTAD:	4-Phenyl-1,2,4-triazoline-3,5-dione
RCT:	randomised controlled trial
RDA:	Recommended Dietary Allowance
RDI:	Recommended Dietary Intake
RIA:	radio-immunoassay
RMP:	reference measurement procedures
RPD:	relative percent difference
SD:	standard deviation
SRM:	Standard Reference Material
SUMARI:	Joanna Briggs Institute's System for the Unified Management, Assessment and Review of Information
Tr:	Trace values (>LOD<LOR)
UL:	Upper Level of Intake/Tolerable Upper Intake Level
UVB:	ultraviolet-B
VDE:	vitamin D equivalents
VDSP:	Vitamin D Standardization Program

Thesis outline

This thesis comprises an introduction (Chapter 1), four original research chapters (Chapters 2-5) and a discussion (Chapter 6).

Chapter 1: Background

The opening chapter provides introductory background information on vitamin D. The metabolism of vitamin D and its importance to our health status are explained. We know that vitamin D status is a public health problem globally, including in Australia, where we have shown the prevalence of vitamin D deficiency (serum 25-hydroxyvitamin D concentrations (25(OH)D) <50 nmol/L) to range between 15 to 39% across various population groups. This suggests that many people either do not synthesise enough vitamin D through sun exposure or do not consume enough via food or supplements to compensate. Although supplements are effective, and useful for individuals at greater risk of vitamin D deficiency, they are not considered appropriate as a population-level solution to low vitamin D status. Food-based strategies, such as the addition of vitamin D to staple foods, may safely and effectively improve vitamin D status in the population. However, Australia has not had the necessary data on the vitamin D content of commonly consumed foods or accurate estimates of usual intakes of vitamin D that are needed to model potential fortification scenarios for safety and efficacy. Furthermore, as research has advanced, there has been a need for improved understanding of how the fortification of foods with vitamin D actually impacts vitamin D status. The background highlights, and places the contribution of the thesis amongst, the knowledge gaps in the field.

Chapter 2: Vitamin D composition of Australian foods

Australia has never had a comprehensive food composition dataset for vitamin D due to limitations in the analytical methods available in Australia until recently. In 2017, the National Measurement Institute of Australia developed a new sensitive and specific liquid chromatography with triple quadrupole mass spectrometry (LC-QQQ) method capable of quantitating multiple forms (vitamers) of vitamin D concurrently. We used this method to measure the vitamin D_3, 25(OH)D_3, vitamin D_2 and

25(OH)D_2 content of 98 different food products across a variety of food groups. This was a large-scale project involving the purchase of approximately 900 food samples from across the country and analysis of 149 composite samples.

Australia is not the only country to lack comprehensive vitamin food composition data; in all other food composition databases globally, there are missing data for D vitamers or for D vitamers in certain food groups. Hence, at the time of publishing the data, this vitamin D food composition database was globally significant in that it was the first time that data for all four of the main dietary D vitamers had been published for such a wide range of foods. The data also demonstrated the importance of capturing the contribution of all four dietary D vitamers to accurately represent the vitamin D content of foods.

Chapter 3: Vitamin D composition of Australian game meats

While the prevalence of vitamin D deficiency is of some concern in the general Australian population, it is even more so in remote-dwelling Aboriginal and Torres Strait Islander peoples. Remote community shops often have limited and unreliable supply of fresh produce; even when available, vitamin D-containing foods such as meat and fish can be relatively expensive. There are limited data for the vitamin D content of native and traditional foods globally; however, some studies have shown that some of these foods are important sources of vitamin D. A single composite kangaroo steak sample was included in our larger project described in Chapter 2, revealing a unique D vitamer profile that warranted follow-up analyses. We found no other studies that had investigated the content of all four dietary D vitamers in camel, crocodile or emu products. In this chapter, the vitamin D content of some game products that are freely accessible by people hunting and gathering bush tucker in remote areas of Australia is explored. The LC-QQQ method was used to measure concentrations of vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 in commercial samples of camel, crocodile, emu and kangaroo meat cuts as well as emu eggs and emu oil. These analyses revealed interesting D vitamer profiles in some of the included species and showed that some products provided nutritionally important concentrations of vitamin D. Knowledge of the vitamin D composition of these foods could contribute to promotion of traditional eating habits, which may be both nutritionally and culturally beneficial.

Chapter 4: Estimating usual vitamin D intakes in Australia

The Australian Bureau of Statistics published usual intakes of various nutrients using food consumption data from the 2011-2013 Australian Health Survey (AHS). However, due to the aforementioned lack of comprehensive vitamin D food composition data, this was not the case for vitamin D. Indeed, estimates of usual dietary intakes of vitamin D based on comprehensive food composition data have never been made for the Australian population. The studies detailed in Chapter 2 generated the necessary data to produce the first nationally representative estimates of vitamin D for Australia. Chapter 4 documents the mapping of our new analytical vitamin D food composition data to create a full survey food dataset, and the use of modern statistical methods to combine this dataset with AHS food consumption data to derive usual intake estimates for Australians aged ≥2 years. The findings of this chapter raise the question of whether it might be beneficial to have a more nutritionally-diverse range of routinely fortified foods beyond margarine in Australia? Chapter 5 provides the final set of data required for the modelling that is needed to answer this question.

Chapter 5: Vitamin D food fortification efficacy

In order to determine whether it would be safe and effective to fortify more foods with vitamin D in Australia, we require knowledge of the effect on circulating concentrations of 25(OH)D, that are used as a marker of vitamin D status, by the consumption of foods with added vitamin D. To update globally-relevant data on the efficacy of vitamin D-fortified foods in increasing circulating 25(OH)D concentrations, we conducted a systematic review with meta-analysis and dose-response analyses. We expanded the scope of earlier studies to include children and adults, to conduct separate analyses for vitamin D_3 and vitamin D_2 and to investigate both linear and non-linear dose-response relationships. The greater overall sample size increased the power of this study's findings compared to earlier studies. The findings of this study lend further support to the use of vitamin D food fortification to improve vitamin D status at population level, while the dose-response data can be used to translate a projected increase in vitamin D intake into a predicted increase in circulating 25(OH)D concentration.

Chapter 6: Discussion

The final chapter discusses the thesis' research outcomes, their impact and implications for food policy. Research into vitamin D food fortification modelling that has already been conducted using data from the thesis is briefly described. Some key areas of dietary vitamin D research that warrant attention are detailed, highlighting the need for ongoing work on this topic. The chapter concludes with a summary of the thesis.

Chapter 1: Background

1.1. Vitamin D metabolism

There are two main forms of vitamin D: vitamin D_3 (cholecalciferol) and vitamin D_2 (ergocalciferol). Exposure of skin to sunlight offers the greatest potential source of vitamin D. Ultraviolet B (UVB) light-irradiation causes conversion of the cutaneous provitamin, 7-dehydrocholesterol, to previtamin D_3, which in turn isomerises under the influence of body heat to form vitamin D_3 [1]. Alternatively, we may consume vitamin D, as vitamin D_3 and D_2 exist in certain foods, or may be taken as supplements. Vitamin D_3 and D_2 are then hydroxylated in the liver through the 25-hydroxylase action of cytochrome P450 (CYP) enzymes to form 25-hydroxyvitamin D_3 ($25(OH)D_3$) and $25(OH)D_2$ [1]. These are the main circulating forms of vitamin D, and also may be directly obtained from some foods [2, 3]. The 25(OH)D metabolites are hydroxylated once more in the kidneys by a CYP enzyme with 1 α-hydroxylase activity to form 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and $1,25(OH)_2D_2$ [1]. Although $1,25(OH)_2D$ is commonly considered the active form of vitamin D, 25(OH)D has also been shown to play its own role in calcium homeostasis [4-7], and there is evidence for the localised cellular conversion of 25(OH)D to $1,25(OH)_2D$ [8]. Figure 1.1 depicts the process of vitamin D metabolism.

1.2. Functions

The functions of vitamin D may be categorised by the genomic or non-genomic mechanism of action, or by the skeletal or non-skeletal outcome. Vitamin D plays a crucial role in calcium homeostasis and, therefore, skeletal health via its nutrigenomic action. $1,25(OH)_2D$ upregulates production of proteins required for the movement of ingested calcium from the intestine into circulation [9]. In the event that there is inadequate dietary calcium available for absorption, $1,25(OH)_2D$ will induce calcium resorption from bone to protect calcium homeostasis [10]. If vitamin D is deficient, calcium absorption is affected regardless of calcium intake, and the lack of vitamin D and calcium in combination leads to decreased mineralisation of bone and resorption of calcium from bone [11]. Brittle, osteoporotic bones are a result of reduced bone density in cases of vitamin D deficiency [11]. Over time, vitamin D deficiency

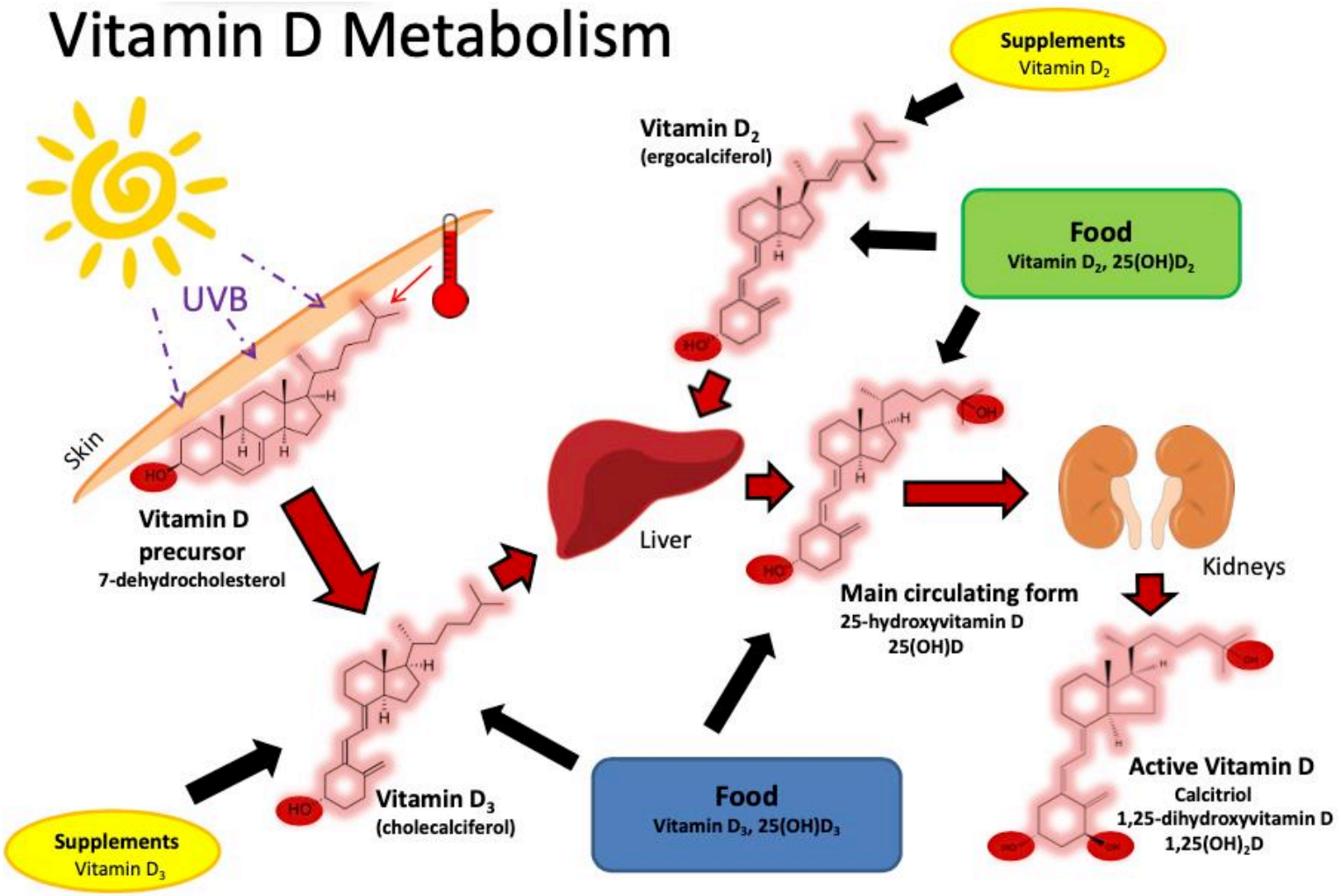

Figure 1.1 Sources and metabolism of vitamin D in humans

can result in softening and weakening of bone due to reduced bone mineralisation [11], manifesting as rickets in children and osteomalacia in adults. Vitamin D also acts through genomic and non-genomic pathways to support muscle function. This, together with vitamin D's effect on bone health, improves mobility and reduces risk of falls and related fractures [11, 12].

Almost all human cells have vitamin D receptors, indicating that the functionality of vitamin D may extend well beyond musculoskeletal health [13]. While *in vitro* and animal studies have supported a protective role of vitamin D in a range of health outcomes, successful translation of these findings to the human setting has proved challenging [14]. This may be because the window of effect, i.e., in earlier life or at a time point prior to disease exposure or onset, is not captured, or because the dosing and feeding regimens used in animal studies are not ethically viable in human studies [15].

An umbrella review published in 2014 found no definite association between low vitamin D status and various non-skeletal health outcomes [16]. A European individual participant data meta-analysis, published in 2017, using standardised serum 25(OH)D concentration data from 26,916 individuals found that those with concentrations < 40 nmol/L were at significantly greater risk of all-cause mortality and cardiovascular mortality in comparison to the reference group with 25(OH)D concentrations 75-99.99 nmol/L [17]. A more recent umbrella review (2021) indicated that low circulating 25(OH)D concentrations were associated with greater risk of all-cause mortality, Alzheimer's disease, hypertension, schizophrenia and type 2 diabetes, while supplementation with vitamin D was associated with all-cause mortality [18].

A 2011 Cochrane Review that included trials (participants n = 94,148) where any dose level of vitamin D was administered, found that supplementation specifically with vitamin D_3 was associated with a slightly reduced risk of all-cause mortality [19]. Findings from that Cochrane Review and another published in 2014 were suggestive of possible benefits of vitamin D supplementation on cancer mortality, but were deemed inconclusive due to the quality of evidence [19, 20]. Similarly, a recent Cochrane Review [21] found that there was insufficient evidence to determine whether vitamin D supplementation was of use in the treatment of coronavirus disease 2019

(COVID-19), which is prevalent and topical at present. Since vitamin D has been suggested as being protective against acute respiratory tract infection [22-24], there has been much speculation about the role that vitamin D may play in the prevention and treatment of COVID-19. While studies [21, 25-31] to date have had mixed results, numerous trials are planned or in progress to investigate this topic further [21, 32]. A great deal more carefully considered and well-designed research is needed to elucidate the role of vitamin D in many health outcomes.

While causal associations are lacking, nationally representative data from the 2011-2013 Australian Health Survey (AHS) have shown that the prevalence of abnormal HDL cholesterol levels (28% compared to 22%) and diabetes (8% compared to 5%) was significantly greater in Australians adults with serum 25(OH)D concentrations < 50 nmol/L than in those with adequate vitamin D status [33]. Similarly, diabetes and chronic kidney disease were more prevalent in vitamin D-deficient participants of the 2012-2013 Australian Aboriginal and Torres Strait Islander Health Survey (AATSIHS) compared to those with adequate vitamin D status. Cross-sectional studies have also found that health conditions, including diabetes and other cardio-metabolic risk factors [34], gastroenteritis [35] and respiratory infection [36], that are prevalent in the Aboriginal and Torres Strait Islander population are more common in people with low vitamin D status. Therefore, while the exact contribution of vitamin D to optimum health is yet to be determined, it would seem prudent to aim for vitamin D sufficiency at the population level [14, 37-39].

1.3. Status

Serum or plasma 25(OH)D concentration is used as a marker of vitamin D status as it is considered to most accurately represent both endogenous and exogenous sources of vitamin D [40]. Although $1,25(OH)_2D$ is the active version of vitamin D, it is not a preferred indicator of status due to its brief circulating half-life (4-6 hours), and its lower circulating levels, such that it is measured in pmol/L rather than the nmol/L concentrations of 25(OH)D. Furthermore, in states of vitamin D insufficiency and deficiency, compensatory measures to protect calcium homeostasis cause increased $1,25(OH)_2D$ production, so that $1,25(OH)_2D$ levels appear normal to high, despite overall low vitamin D status [2].

Numerous assays are used to measure circulating concentrations of 25(OH)D [41]. Liquid chromatography with tandem mass spectrometry (LC-MS/MS) is often considered the preferred method for serum 25(OH)D concentration analysis and directly measures $25(OH)D_2$ and $25(OH)D_3$ independently; however, as it is costly and time consuming, its use is generally limited to research. The true gold standard is a method conducted by a laboratory whose method has been certified to the reference measurement procedures (RMPs) developed under the Vitamin D Standardization Program (VDSP) by the National Institute of Standards and Technology (NIST), Ghent University and the US Centers for Disease Control and Prevention (CDC) [42-44]. Routine, everyday tests are generally carried out using immunoassays, which use 25(OH)D-targeted antibodies and are more efficient, cost effective and simple to perform [45]. At serum 25(OH)D concentrations below 25 nmol/L, immunoassays have been shown to provide relatively accurate measurement, compared to the benchmark for accuracy in evaluation of serum 25(OH)D assays: over all concentrations, mean bias for immunoassays conducted at four different laboratories ranged from -0.7 to 1.7 ng/mL (-1.75 – 4.25 nmol/L, $p < 0.0001$) [45, 46]. At concentrations above the 50 nmol/L cut point value, however, immunoassays have delivered readings up to 25 nmol/L below the true value [45, 46], a considerable proportion of the cut point value.

As many past surveys used immunoassay-derived 25(OH)D data, related prevalence estimates are questionable. The VDSP system and protocols include a mathematical model that allows harmonisation of non-standardised assay data with those from assays that are certified to the RMPs. Harmonisation has been applied to several national datasets with varied effects on prevalence estimates. Initially, it was thought that mean serum 25(OH)D concentrations had declined in the US over the period between the 1988-1994 National Health and Nutrition Examination Survey (NHANES) and the 2000-2006 NHANES [47], both of which used an immunoassay [48]. Following harmonisation of these data, it was determined that there had in fact been no decline in 25(OH)D concentrations in the population of the US during that timeframe [49]. In Europe, harmonisation of serum 25(OH)D data from the Irish National Adult Nutrition Survey revealed a true prevalence estimate of serum 25(OH)D < 30 nmol/L of 11.4% in comparison to the pre-harmonisation estimate of 6.5% [50]; conversely, harmonisation of data from the German Health Interview and

Examination Survey for Adults resulted in a decrease in the estimate of vitamin D deficiency prevalence from 28.0 to 12.9% [39].

Definitions of vitamin D deficiency and sufficiency vary [45]. Deficiency is described by the Institute of Medicine (IOM) as combined $25(OH)D_2$ and $25(OH)D_3$ serum concentrations < 30 nmol/L [51]. This is accompanied by the acknowledgment that some people may be at risk of inadequacy at < 50 nmol/L, but that raising cut-points without sufficient evidence may cause inappropriate overestimations of the prevalence of deficiency [51]. The European Food Safety Authority (EFSA) used a target serum 25(OH)D concentration of 50 nmol/L when determining a recommended adequate intake [52]. Others have argued for higher cut-points [53-55]. The Endocrinology Society recommend cut-points of 50 and 75 nmol/L for vitamin D deficiency and insufficiency, respectively [56], with concerns that those whose status is categorised as insufficient are at risk of negative health outcomes – both of a skeletal and non-skeletal nature [57]. In Australia, vitamin D deficiency is most commonly marked by a cut-point of serum 25(OH)D 50 nmol/L, below which there are further categorisations of mild (30-49 nmol/L), moderate (12.5-29 nmol/L) and severe (< 12.5 nmol/L) deficiency [58, 59].

By the < 50 nmol/L definition, vitamin D deficiency is prevalent globally [37]. Using a standardised analytical method or standardised data, it has been estimated that 18% and 27% of people had 25(OH)D concentrations < 50 nmol/L in the US [60] and in Canada [61], respectively. In Europe, estimates of vitamin D deficiency vary widely between countries and population groups [38, 39]. Although standardised data are not available for all European countries, estimates suggest that high prevalence of vitamin D deficiency is widespread [38, 39].

In Australia, serum 25(OH)D concentrations for the 2011-2013 AHS were analysed at the Douglas Hanly Moir laboratory, which has RMP certification for its LC-MS/MS method [62]. As earlier Australian surveys did not use RMP-certified assays, the data are not suitable for comparison with current data, preventing assessment of trends over time. The AusD Study, which collected data during 2009-2010, provided a prevalence estimate of 29% in Australian adults aged 18-75 years (n = 1002), based on serum 25(OH)D analyses using a non-standardised immunoassay [63]. In contrast,

the Australian Bureau of Statistics estimated that 23% of Australian adults were vitamin D deficient in 2011-2013, based on standardised AHS analyses [58].

Our team has investigated the prevalence of vitamin D deficiency in a number of Australian population groups, based on nationally representative AHS 2011-2013 data. We found that the prevalence of vitamin D deficiency was 17, 32 and 20% in Australian adolescents (12-17 years) [64], young adults (18-24 years) [64] and adults aged ≥25 years, respectively [65]. Based on Census population data, we estimated that this equated to approximately 0.3, 0.6 and 3.3 million Australians with serum 25(OH)D concentrations <50 nmol/L across the same respective age groups in 2011 [64, 65]. Compared to the general population, the prevalence of vitamin D deficiency was higher (27%) in Australian Aboriginal and Torres Strait Islander adults aged ≥18 years, rising considerably to 39% in those living in remote areas [66]. Similarly, African immigrants living in Australia were more likely to be vitamin D deficient (36%), than the general Australian population [67]. This high prevalence of vitamin D deficiency may have health consequences as outlined in the previous section, and there may also be economic burden associated with poor vitamin D status in populations [68-70]. Hence, the promotion of strategies to improve vitamin D status could have wide-ranging benefits in Australia.

1.4. Predictors of deficiency

A number of factors may influence a person's risk of vitamin D deficiency. People with more deeply pigmented skin require a greater dose of UVB irradiation to support sufficient endogenous production of vitamin D compared to lighter-skinned people. Those whose exposure to sunlight is reduced through institutionalisation, full-coverage clothing or indoor work are also at greater risk [59, 71]. As vitamin D is a fat-soluble compound, its sequestration into adipose tissue may reduce circulating stores in obese people [72]. The elderly are at increased risk of vitamin D deficiency, due to the effects of aging on the efficiency of cutaneous synthesis, intestinal absorption and enzymatic hydroxylation of vitamin D [73, 74]. Additionally, appetite often decreases with older age, reducing the potential for dietary vitamin D intake, and impaired mobility may reduce time spent outdoors. However, an increased likelihood of vitamin D deficiency in elderly Australians was not apparent in our

recent investigation [65], possibly due to more prevalent use of vitamin D-containing supplements in older age groups [75].

In the AusD Study, serum 25(OH)D concentration was described as positively associated with age and negatively associated with BMI; other predictors of deficiency included low physical activity level, body mass index ≥ 30.0, greater melanin density (hand), greater percentage of body surface area covered by clothing, state of residence, blood draw in winter compared to summer or autumn and non-use of vitamin D supplements [63]. Our recent work on prevalence and predictors of vitamin D deficiency in Australia revealed predictors of: younger age for African immigrants and men in the general population; region of birth outside Australia for adolescents; region of birth outside Australia and other main English speaking countries for adults; blood draw in winter or spring compared to summer; state of residence; remote residence compared to non-remote residence for Aboriginal and Torres Strait Islander women; greater body mass index for Aboriginal and Torres Strait Islander people and adolescents and adults in the general population; low physical activity for adults (including African immigrants); current smoker compared to ex/non-smoker for women and Aboriginal and Torres Strait Islander men; and non-use of vitamin D or calcium supplements for adults aged ≥ 25 years [64-67].

1.5. Nutritional requirement

Ideally, intake recommendations (or nutrient reference values) should include a Recommended Dietary Allowance/Recommended Dietary Intake (RDA/RDI) and an Estimated Average Requirement (EAR). The RDA is a value of intake that exceeds that required by 97.5% of a population group (by age/sex) [51, 76] and is most suited to assessment of intake in individuals [77]. The EAR is a value of intake that exceeds that required by 50% of a population group [51, 76] and is an appropriate cut point for assessment of intake at the population level [77]. Where there is insufficient evidence to determine an RDA/RDI or an EAR, an Adequate Intake (AI) value may be recommended instead. The AI is a value estimated through observation or experimental determination of the level of intake of people that appear healthy [51, 76] and is not appropriate for use in assessment of nutrient adequacy in populations [77].

Recommendations for optimal intake of vitamin D vary by region. For the US and Canada, the IOM set an EAR of 10 μg/day for people of all ages [51]. More

specifically, an RDA of 15 μg/day was recommended by the IOM for those aged 1-70 years, increasing to 20 μg/day for people aged ≥ 70 years [51]. In Europe, the EFSA set an AI of 15 μg/day for people aged ≥ 1 year [52].

The AI recommended for the majority of the Australian population (those aged 1-50 years) is much lower at 5 μg/day [78]. This increases to 10 and 15 μg/day for people aged 51-70 and > 70 years, respectively [78]. The basis of these AIs is maintenance of serum 25(OH)D concentrations ≥ 27.5 nmol/L with limited sun exposure [78]. However, the National Health and Medical Research Council (NHMRC), that publishes Nutrient Reference Values for Australia and New Zealand (NRVs), have acknowledged that the intake recommendations for vitamin D are in need of update [59, 78]. There has been insufficient evidence to consider an EAR or RDI in Australia; indeed, the research upon which the recommendations for adults are largely based was a cross-sectional study conducted in 2001 that included only women (*n*=861) from the Barwon region of Victoria [79]. The evidence upon which current Australian recommendations are based is outdated and not representative of the population. Hence, Australia is in need of nutrient reference values for vitamin D that are based on nationally representative data.

1.6. Toxicity

As a fat-soluble compound, vitamin D is not readily excreted like water-soluble vitamins. Due to its calcaemic action, persistently excessive intake can result in hypercalcaemia. As feedback mechanisms control the conversion of endogenously synthesised vitamin D [80] and the natural content of foods is generally so low, neither sun exposure nor usual dietary vitamin D intake is likely to cause vitamin D toxicity. Supplements of doses up to 25 μg (1000 IU) per capsule are readily available for purchase in bulk quantities with no requirement of health professional advice; however, excessive consumption would contravene the daily dose stated on packaging.

The precise point of 25(OH)D serum concentration at which the risk of acute toxicity becomes apparent is unclear. Some research has suggested that prolonged elevation of serum 25(OH)D concentrations in the region of 375-500 nmol/L may be dangerous, while other research has indicated that higher concentrations may be tolerated in terms of hypercalcaemia [81]. In a meta-analysis of 26,916 individual

participant serum 25(OH)D concentration data, the risk of mortality did not significantly worsen at concentrations up to 125 nmol/L [17].

The IOM recommends a maximum vitamin D intake of 100 μg/day with the aim that serum 25(OH)D concentrations remain below 125 nmol/L, the threshold at which negative health effects may arise [51]. The NHMRC's upper levels of intake (UL) of 25 and 80 μg/day for Australians aged 0-12 months and 1+ years, respectively [78] are perhaps reasonably conservative given that the points at which detrimental effects may occur with respect to other health conditions are unknown [45, 59, 82].

1.7. Sources

It is possible for humans to synthesise an adequate amount of vitamin D with sufficient sunlight exposure. However, Australia has one of the highest incidence rates of melanoma of the skin globally [83]. Warranted concerns over skin damage and cancer can lead to sun-protective behaviours that prevent or minimise the beneficial vitamin D-producing effects of UVB irradiation. Pending outcomes of the current Sun Exposure and Vitamin D Supplementation Study (SEDS)[84], Australia does not yet have specific guidelines on optimal sun exposure for all people. Even upon completion of the SEDS study, further research will be needed to develop guidelines for people with more deeply pigmented type V-VI skin, as the SEDS study only includes participants with Fitzpatrick skin types of II-IV [85].

Many environmental factors influence the potential for endogenous vitamin D synthesis. Fabric and glass block UVB rays; hence, clothing and indoor lifestyle choices impact on the potential for endogenous vitamin D production, which is furthermore affected by numerous geographical, environmental and climatic conditions. UVB irradiance is greater at more central areas of latitude, while cloud cover may have both limiting and augmentative effects on UVB levels [86]. During winter and at more northerly and southerly points of latitude, solar rays are projected at a more oblique angle than during warmer months or at more central areas of latitude, meaning that fewer UVB photons reach the earth's surface [86]. Furthermore, tendencies towards more indoors lifestyles and sun-protective behaviours have increased in recent times [87]. The aforementioned prevalence of vitamin D deficiency indicates that a considerable proportion of Australia's population do not synthesise a sufficient amount of vitamin D, despite a relatively sunny climate.

Supplements are effective if taken at an adequate dose (e.g., 1000 IU/25 μg/day); however, only 5 per cent of Australian adults take the single vitamin D supplements that deliver this [75]. Prevalence of single vitamin D supplement use is even lower in younger adults (1.5% and 3% in 18-30 and 31-50 year-olds, respectively) [75], who are at greater risk of vitamin D deficiency than older adults [65]. Supplement use in general is associated with health literacy and wealth [88]. Hence, while supplements are necessary and effective for some individuals, they are not considered appropriate as a population-wide solution to deficiency [38].

Vitamin D occurs naturally in low concentrations in only a few foods, such as fish, eggs, meat, some dairy foods and some mushrooms. Traditionally, vitamin D_3 has been thought of as an animal-based source, while vitamin D_2 is derived from fungi and yeast. More recently, vitamin D_2 has been found in animal products [3, 89]. It is thought that the presence of vitamin D_2 in some animal products is due to fungal contamination of animal feed, such as grasses, and that UVB exposure of the ergosterol contained in the grass leads to production of vitamin D_2 [90]. It has also been established that some plants can synthesise vitamin D [91, 92]. Vitamin D_3 and 25(OH)D_3 have been found in the leaves of plants, particularly in those from the Solanaceae family [93-96] as well as in alfalfa/lucerne, which in one study was found also to contain vitamin D_2 [97]. As yet, there does not appear to be any evidence to indicate that vitamin D is found in the edible fruit of plants from the Solanaceae family. We found vitamin D_2 in the dried leaves and berries of the Tasmanian mountain pepper (*Tasmannia lanceolata*) plant and in both the fresh and dried leaves of the lemon myrtle (*Backhousia citridora*) tree [98]. Both vitamin D_2 and D_3 have been found in microalgae [99-101] and macroalgae [100], while UVB exposure has been shown to stimulate vitamin D_3 production in some microalgae [102]. Our exploratory study into the vitamin D content of Australian-grown edible seaweed did not reveal quantifiable amounts in fresh or dried wakame (*Undaria pinnatifida*) or kombu (*Lessonia corrugata*) [98].

Fortified and biofortified foods may also be important sources of vitamin D, where available. These sources are discussed further in section 1.12.

1.8. Measurement in food

In terms of food analysis, vitamin D is structurally complex, lipophilic [103] and its various forms differ in polarity and oxidation potential [104]. Therefore, the extraction and quantitation of vitamin D in foods is challenging compared to many other nutrients.

1.8.1. Early methods and high performance liquid chromatography

In the 1980s, early food-based vitamin D analyses used biological, colorimetric and gas-liquid chromatography assays that, although pioneering for the day, were unable to separate fractions of vitamins D_2 and D_3 and were inaccurate and labour- and time-intensive [105]. Thereafter, high performance liquid chromatography (HPLC) methods, with successive modifications, paved the way forwards [106, 107]. Lipids were saponified and vitamin D fractions were separated from the food matrix by liquid-liquid HPLC [107]. The D_2 and D_3 vitamers were then differentiated through normal phase chromatography with UV and/or diode array detection (DAD) [107, 108]. Internal standards served to correct for limitations in extraction efficiency and to optimize precision [105], while the purification process was improved by addition of solid-phase extraction (SPE) before normal phase extraction [108, 109]. Methods were further enhanced to allow measurement of vitamin D_3 and 25(OH)D_3 in a single analytical run [110] and then further again to allow analysis of four vitamers, vitamin D_3 and vitamin D_2 as well as their respective hydroxylated metabolites, concurrently [111]. These HPLC methods, however, relied heavily on the skill of the analyst to ensure accuracy and remained lengthy, costly and laborious processes [103], thus limiting their commercial application. In 2004, Jakobsen et al. published an improved method that used two HPLC systems to achieve a low limit of quantification (LOQ; 0.03 μg/100 g) [106].

1.8.2. Liquid chromatography mass spectrometry

Also in 2004, an existing liquid chromatography with mass spectrometry (LC-MS) method was improved through addition of atmospheric pressure chemical ionization (APCI) and used to achieve the simultaneous analysis of vitamin D_3 alongside vitamins A and E in the complex matrix of fortified infant formula [112]. The speed of the method was optimised by the use of diatomaceous earth in the SPE step [104, 112].

Use of LC-MS to measure 25(OH)D_3 and 25(OH)D_2 in serum suggested potential for use of the method in the measurement of D vitamers in food, and methods and equipment were optimised further for this purpose [104]. The addition of tandem mass spectrometry (LC-MS/MS) allowed further accuracy [113].

In 2011, a liquid chromatography with ion trap mass spectrometry (LC-IT-MS) method was used in Australia to measure vitamin D_3 in bovine milk, with comparison to, and validation against, the established sensitivity of LC-MS/MS [113]. The new LC-IT-MS method achieved an LOQ of 0.01 µg/100 mL in this food matrix. Following on from this, the measurement of vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 was achieved at in a single analytical run at the National Measurement Institute of Australia (NMI) using LC-IT-MS, achieving respective limits of detection and quantitation of 0.03 and 0.05 µg/100 g in meats [104]. The method was commercially viable and suitable for generation of data on the scale needed for food composition data, allowing up to 112 extractions per week [104]. Oxidation of the hydroxylated vitamers was prevented by expediated transition between extraction and analysis, while variability in the extraction capabilities of SPE or MS was controlled through use of deuterated internal standards [104]. As hardware availability issues forced discontinuation of this method at NMI, a liquid-chromatography with triple quadrupole mass spectrometry (LC-QQQ) method was developed, based on the LC-IT-MS method (ISO7025:2017) [3, 98]. The LC-QQQ method is a highly sensitive and specific method that allows measurement of multiple D vitamers in a single analytical run with limits of detection as low as 0.01 µg/100 g, depending on the food matrix. The full saponification, extraction and derivatisation process remains a relatively lengthy procedure that is conducted over two days and requires specialist skill. Hence, measurement of D vitamers is costly at several times the expense of many other nutrient analyses, and access to comparable quality analytical methods is severely limited globally by the small number of laboratories offering these services.

1.9. Bioactivity

Vitamin D_3 and vitamin D_2 are widely considered as having equal bioactivity, although the findings of some studies have contradicted this. The results of one supplemental study (delivering a dose of 10 µg/day of vitamin D_3, vitamin D_2 or

25(OH)D_3 in a crossover design) published in 2018 suggested that it may take more than twice as much vitamin D_2 to elicit the same increase in serum 25(OH)D concentrations effected by 10 μg/day of vitamin D_3 [114]. Prior to that, a 2012 systematic review and meta-analysis found that vitamin D_3 supplementation was more effective than vitamin D_2 supplementation when delivered as a bolus dose; however, the effect was similar when delivered as a daily dose [115]. One food fortification trial has also suggested smaller increases in circulating 25(OH)D concentrations in intervention groups provided vitamin D_2, compared to intervention groups provided the same dose of vitamin D_3 [116], while others have suggested equal bioactivity of the two vitamins [117, 118]. It should also be noted that there is potential for 25(OH)D assays to underrepresent consumed vitamin D_2 through inaccuracy in measurement of 25(OH)D_2 concentrations [119]. At present, all national food composition databases that distinguish between vitamin D_3 and vitamin D_2 and present a vitamin D equivalents value do so assuming that the two have equal bioactivity.

In the case of 25(OH)D_3, the general consensus is that it is more bioactive than vitamin D_3. Combination of results from five trials [120-124] that administered supplemental 25(OH)D_3 resulted in a mean change in circulating 25(OH)D concentrations of 3.9 nmol/L, with a range of 1.8-5.0 nmol/L [125]. Subsequently, in a trial published in 2018, the increase in circulating 25(OH)D concentrations effected by 25(OH)D_3 supplementation was found to be 1.5 times that of vitamin D_3 supplementation [114], while another study used a Caco-2 model of human enterocytes to determine that 25(OH)D_3 may be more bioavailable than vitamin D_3 as it is more readily incorporated into mixed micelles and taken up by Caco-2 cells [126]. Therefore, it appears that 25(OH)D_3 is at least as bioactive and likely more so than vitamin D_3; however, the degree by which they might differ has not yet been conclusively established. It has been suggested that the bioactivity of the hydroxylated and non-hydroxylated D_2 and D_3 vitamers should be assumed as equal until further evidence is available [125].

The majority of food composition databases treat the hydroxylated and non-hydroxylated D vitamers as equal pending further evidence to the contrary. Currently, a 25(OH)D bioactivity factor of five is used to calculate total vitamin D concentrations in the Armenian [127], Australian [128], Danish [129, 130] , Indian [131] and UK

[132, 133] national food composition databases; however, it was recently announced at the 2021 6th International Vitamin Conference that the vitamers would be considered equal in the forthcoming release of the Danish food composition database.

1.10. Vitamin D food composition data

As at July 2020, there were 64 national food composition databases with data available online representing countries in Asia, Africa, the Americas, Europe, the Middle East and Oceania (Appendix III; Table 1). Of these, 41 included vitamin D as a component. Seven included vitamin D_3 only, eight included vitamin D_3 and vitamin D_2, three included vitamin D_3 and 25(OH)D_3, six included vitamin D_3, vitamin D_2 and 25(OH)D_3, while the Danish food composition database was the only database to include some data for all four vitamers (vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2); it was unclear which vitamers were included in the remaining 16 databases. In many cases, details of the analytical methodology used were not obviously stated, or data were borrowed from multiple sources. Analytical methods that were specified were variations on liquid chromatography or radioimmunoassay. In many databases, it was unclear as to which vitamers had been measured, as data were presented as a total vitamin D value without definition of the vitamers included. A bioactivity factor of five was applied to 25(OH)D in five databases, while documentation for the US and Dutch databases stated that a bioactivity factor was not used due to lack of evidence for a specific factor [134, 135].

Overall, the presence and quality of vitamin D data in national food composition databases varied greatly, and no country had a database that included data for the four D vitamers measured across a wide range of foods. This is likely due to the limited access to the recently developed high-quality assays, and their high cost that is often prohibitive to large-scale analytical food programs. Much of the available vitamin D data globally were derived using older analytical methods that were unable to provide concurrent analysis of multiple vitamers, increasing the cost of multi-vitamer analysis and limiting the number of vitamers able to be measured in each sample. Generally, this has led to vitamin D_3 being measured only in animal products and vitamin D_2 only in non-animal products. Although recent studies have reported the presence of vitamin D_2 and 25(OH)D_2 in some animal products [136, 137],

quantitated values for these vitamers in meat products were not included in any databases.

Similar to other countries, **vitamin D food composition data represented a fundamental knowledge gap for dietary vitamin D in Australia prior to this PhD project**. Data for vitamin D were not included in NUTTAB, the predecessor to the current Australian Food Composition Database. Instead, a supplementary table of vitamin D data for a limited number of foods was provided on the NUTTAB webpage with the caveat that the data were not included within the NUTTAB database due to concerns over the sensitivity and accuracy of the analytical methods available at the time [138, 139]. The initial Australian Food Composition Database release contained some vitamin D data collected through small and mid-scale analytical programs. A Food Standards Australia New Zealand (FSANZ)-commissioned program analysed vitamin D in 22 meat (beef, lamb, pork, chicken), fish (salmon, snapper, hoki, shark and tuna), dairy (milk, cream, and cheese) and egg (chicken) products sampled across the country in 2015 using the aforementioned LC-IT-MS method [138]. The aim of that program was to provide the best possible provisional data for vitamin D in Australian foods within resource constraints [138]. Some seafood species were analysed for vitamin D content using LC-IT-MS in 2010 and 2011 as part of the South Australian Research and Development Institute's (SARDI) 2008/905 Project; however, very few samples (between one and three each of seven species, including finfish, crustacea, mollusc and echinoderm) [140] were able to be analysed for D vitamers due to funding constraints [141]. Due to the limited species and samples analysed, data from both the FSANZ and SARDI projects were pooled to provide values for fatty and non-fatty fish and seafood species for the Australian Food Composition Database [128]. Therefore, the vitamin D contributions to intakes by these species, some of which may be relatively rich sources, may not be differentiated. Other data were collected as circumstances allowed [138] for products such as margarine [142]; however, a large-scale program was still required to provide up-to-date and comprehensive vitamin D food composition data for a wide range of commonly-consumed foods in Australia.

1.11. Usual intakes of vitamin D

Low intakes of vitamin D have been reported in many countries [143]. In Europe, typical intakes were reported as < 5 μg/day [144] and considerably lower than the AI of 15 μg/day [52]. Similarly low mean intakes of vitamin D from food of approximately 4-5 μg/day were reported in the US [60] and Canada [145], although it is likely that these are underestimations as the food composition datasets used did not account for 25(OH)D and data for vitamin D_2 were limited [146-151]. It has been suggested that exclusion of 25(OH)D in food composition data used to estimate intakes may partly explain, in conjunction with the contribution of sun exposure, the gap between higher actual serum 25(OH)D concentrations and the circulating concentrations that would be expected based on intake estimates from food [152].

As discussed in Section 1.8, vitamin D is difficult to measure in food, and to date, Australia has lacked access to a suitably sensitive and specific method capable of producing data on the scale required for food composition. The resulting lack of vitamin D food composition data, together with uncertainty around the 25(OH)D bioactivity factor has precluded the estimation of vitamin D intakes in Australia [153]. **Hence, vitamin D intake was a key knowledge gap in Australia prior to this PhD project, as usual intakes of vitamin D had never been estimated using a comprehensive vitamin D food composition dataset**. In 2002, it was estimated that Australians were consuming 2-2.5 μg/day of vitamin D [154]; however, the vitamin D composition data at the time were extremely limited and were based on analytical methods that are now considered outdated [139, 155, 156]. A subsequent estimate of ~4.3 μg/day was made based on food consumption data from the 2011-2013 Australian Health Survey, but included only a limited selection of meat-based foods, some of which were analysed using the modern LC-IT-MS method [139]. That estimate was based on an assumption that the bioactivity of 25(OH)D_3 is five times that of vitamin D [139].

1.12. Fortification and biofortification

Food fortification has been recommended by the World Health Organization and Food and Agricultural Organization of the United Nations as a safe, effective and relatively economically viable solution to nutrient deficiency at the population level [157]. As concentrations of naturally-occurring vitamin D in most foods are low,

fortification of foods with vitamin D (direct addition of vitamin D to foods) has been suggested as a strategy to safely and effectively improve low vitamin D intakes and status across populations [38, 39, 88, 143, 144]. In some countries, commonly consumed products such as milk, orange juice, bread, breakfast cereals and enriched rice, pasta and noodle products are already fortified in order to increase the dietary supply of vitamin D [143, 144, 158-163]. Where available, vitamin D-fortified staple foods like milk can become primary contributors to vitamin D intakes [60, 145]. In Finland, vitamin D-fortification has been demonstrated as effective in improving vitamin D status at the population level: systematic fortification of fluid milk products and edible oil spreads (e.g., margarine) in 2003 reduced the prevalence of vitamin D deficiency from 56 to 9% by 2011 [162]. Other foods and beverages, such as bread, juice and mineral water, are also permitted for fortification in Finland [162, 164], whereas they are not in Australia [165].

In Australia, edible oil spreads are the only foods to which vitamin D must be added, at a minimum concentration of 5.5 µg/100 g [165]. Vitamin D may be voluntarily added to certain foods, namely dairy foods and their alternatives, formulated beverages, butter and certain breakfast cereals [165]; however, this does not occur routinely. The Australia New Zealand Food Standards Code allows for voluntary food fortification only where there is a demonstrable need in terms of low nutrient status or intakes in the population and where the fortified food is not high in salt, sugar or fat, or of low/no nutritional value [166]. Hence, consumers are protected against fortification and promotion of foods that have limited nutritional value. For example, breakfast cereals may only be fortified with vitamin D if they meet FSANZ' Nutrient Profiling Scoring Criterion (NPSC), which is based on the energy, saturated fat, sugar, sodium, protein, dietary fibre and fruit and vegetable content of foods [167]. To be eligible for vitamin D-fortification, breakfast cereals must score < 4 NPSC points [168] by limiting energy (< 1,340 kJ), saturated fatty acids (< 4.0 g), average sugars (< 18.0 g) and sodium (< 360 mg) per 100 g, or have sufficient protein, fibre, fruit or vegetable content to counteract any excess points accrued due to energy, saturated fat, sugar or sodium content [169]. Australia's Food Standards Code would currently not allow for a fortification system such as that adopted in Finland, where fluid milks are routinely fortified with 1.0 µg/100 g vitamin D and edible oil spreads with 20 µg/100 g [162, 164]. In Australia, the maximum permitted amounts of vitamin D that may

be added to fluid milks/their alternatives and edible oil spreads are 0.8 μg/100 mL and 16 μg/100 g, respectively [165].

Biofortification/bioaddition (for example, addition of vitamin D to animal feed or UVB-irradiation of mushrooms [170, 171], yeast [172], algae [101, 102] or livestock [173-178] in order to increase the natural vitamin D content of their products) is also emerging as a potential strategy to increase vitamin D intakes from food [179]. In Denmark, modelling of biofortification of dairy, chicken (including eggs) and pork products projected a considerable increase in intakes from food compared to intakes that were estimated from current food composition data that is representative of current livestock production in Denmark [180]. That study used results from animal feeding and UVB-exposure trials to project intakes for the biofortification scenario, and assumed equal bioactivity of the D vitamers, including 25(OH)D_3 [180].

Globally, the availability of foods that have been biofortified with the purpose of increasing vitamin D intakes in populations is not yet widespread. In Australia, livestock, such as chickens, are routinely given vitamin D-supplemented feed; however, this tends to be on the basis of maximising animal health for productivity and preventing product loss (e.g., by strengthening egg shells) [181, 182], rather than for the potential benefit to people who consume the food products. UVB-irradiated or 'vitamin D mushrooms' are available in some countries, and although they have been produced in Australia [170, 183], availability is limited.

It is important that any fortification or biofortification program is carefully considered to ensure that it is effective and also safe in terms of not causing persistently excessive intakes that may result in toxicity. This requires modelling of scenarios based on various foods and various fortification concentrations to see how population intakes would be affected [184-190]. Beyond the effect on vitamin D intakes, it is also important to determine the benefit of potential fortification and biofortification scenarios in terms of vitamin D status [191-193]. Earlier systematic reviews, meta-analyses and dose-response studies have used data from randomised controlled trials (RCTs) conducted around the world to estimate the effect of vitamin D from fortified foods on circulating 25(OH)D concentrations [194-198]. Fortification of foods with vitamin D has been determined as effective in increasing circulating 25(OH)D concentrations [194-198]. However, **prior to this PhD project, no previous**

meta-analysis or dose-response study had investigated effects by vitamer, included biofortification trials, investigated a nonlinear dose-response relation in adults, or included both adults and children. It is important to understand these effects so that food-based strategies to improve vitamin D status may be accurately modelled.

Aims and objectives

The overall aim of this thesis was to advance knowledge of dietary vitamin D in Australia and produce the data needed to explore dietary strategies to improve vitamin D status at the population level. In order that potential strategies, such as vitamin D food-fortification, can be explored in Australia the following questions must be answered:

1. How much vitamin D is already in foods that are commonly consumed in Australia?

2. How much vitamin D do people in Australia usually consume from food?

3. How does addition of vitamin D to food affect circulating 25(OH)D concentrations?

The thesis answers these questions through completion of four objectives that directly address the knowledge gaps highlighted in Chapter 1:

1. To develop Australia's first comprehensive vitamin D food composition database for vitamin D_3, 25(OH)D_3, vitamin D_2, and 25(OH)D_2 in commonly consumed foods

2. To determine the vitamin D content of Australian game products, which may be a useful source of vitamin D for people living in remote areas.

3. To identify the major food sources of vitamin D and generate the first estimates of usual vitamin D intakes in the Australian population

4. To conduct a systematic review and meta-analysis of randomised controlled trials to evaluate the efficacy of vitamin D_3 and vitamin D_2 food fortification on serum 25-hydroxyvitamin D concentrations

The studies that were conducted to fulfil this aim and objectives are detailed in Chapters 2 to 5.

Chapter 2: Vitamin D composition of Australian foods

Thesis objective addressed in this chapter:

Objective 1: To develop Australia's first comprehensive vitamin D food composition database for vitamin D_3, 25(OH)D_3, vitamin D_2, and 25(OH)D_2 in commonly consumed foods.

The content of this chapter is covered by Publication 1:

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The vitamin D composition database (Appendix IV) has been adopted by Food Standards Australia New Zealand for inclusion in the Australian Food Composition Database (https://www.foodstandards.gov.au/science/monitoringnutrients/afcd/Pages/Data-provided-by-food-companies-and-organisations.aspx).

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Abstract

Australia needs accurate vitamin D food composition data to support public health initiatives. Previously, limitations in analytical methodology have precluded development of a comprehensive database. We used liquid chromatography with triple quadrupole mass spectrometry (LC-QQQ) to analyse 149 composite samples representing 98 foods (primary samples *n* = 896) in duplicate for vitamin D_3, 25-hydroxyvitamin D_3 (25(OH)D_3), vitamin D_2, 25(OH)D_2. The greatest concentrations of vitamin D_3 were found in canned salmon and a malted chocolate drink powder (fortified); chicken eggs and chicken leg meat contained the most 25(OH)D_3. Margarine (fortified) and chocolate contained the greatest concentrations of vitamin D_2, with smaller amounts found in various meat products. 25(OH)D_2 was detected in various foods, including meats, and was quantitated in lamb liver. These data advance knowledge of dietary vitamin D in Australia and highlight the importance of analysis of these four forms of vitamin D to accurately represent the vitamin D content of food.

Introduction

Australia lacks comprehensive and up-to-date vitamin D food composition data, yet almost one quarter of Australian adults have low vitamin D status (25-hydroxyvitamin D (25(OH)D) concentration < 50 nmol/L) [58, 65]. Lack of high-quality Australia-specific vitamin D food composition data precludes the accurate estimation of vitamin D intakes in the Australian population, which further impedes the estimation of existing and optimal dietary supply of this important bone health promoting nutrient. Sun-exposure, the major potential source of vitamin D, is often limited [199] and Australians are encouraged to protect their skin from prolonged sun exposure to reduce the risk of skin damage and skin cancer [200]. Accurate vitamin D food composition data are needed to investigate potential dietary strategies that may improve vitamin D status at the population level.

In order to accurately represent the vitamin D content of foods, it is necessary to account for the four main forms (D vitamers): vitamin D_3, vitamin D_2, and their respective hydroxylated forms, 25(OH)D_3 and 25(OH)D_2, which may be more bioactive than vitamin D_3 and D_2 [121, 125, 201]. However, inclusion of all four D

vitamers in food composition databases is uncommon. Of the 64 food composition databases listed in the International Network of Food Data Systems (INFOODS) international food composition table/database directory [202], 41 include freely available online-accessible vitamin D food composition data. Of these, only the Danish food composition database provides data for all four D vitamers [129]. The four D vitamers are present in low concentrations in food, are structurally complex and their chemical properties differ. This complicates their extraction from food matrices and their accurate quantitation. Although sensitive, specific and efficient analytical methods have been developed and improved over time [103-108, 111-113, 203], the expense and limited availability of high-quality vitamin D analytical services are limiting factors in acquiring accurate vitamin D composition data.

Due to potential geographical variations in the vitamin D content of foods and differing common foods, it is inappropriate for Australia to borrow vitamin D food composition data from other countries. The Australian Food Composition Database (AFCD) contains some vitamin D data collected through small-scale analytical programs [128]; however, a comprehensive program using modern analytical methods is needed. Liquid chromatography with triple quadrupole mass spectrometry (LC-QQQ) has been used previously to measure D vitamers and other compounds [108, 204, 205]. Recently, a new sensitive and specific LC-QQQ method was developed at the National Measurement Institute of Australia (NMI), allowing accurate quantitation of vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 at low concentrations in a single analytical run, with a throughput speed capable of generating data on a scale suitable for food composition analysis. This study aimed to develop a comprehensive analytical vitamin D food composition database for Australian foods using LC-QQQ.

Materials and methods

Sampling and sample preparation

In order to capture data that most accurately represents foods commonly consumed in Australia, a detailed sampling plan with handling and preparation instructions was developed for ninety-eight food products. These foods were reported as commonly consumed by 2011-2013 Australian Health Survey (AHS) participants and were likely to contain vitamin D (Supplementary Table 1). Foods that were expected to

contain high concentrations of vitamin D, are frequently consumed, and/or tend to be produced and used locally or in surrounding regions were sampled in three cities (Sydney, Melbourne and Perth), which together represent approximately half of Australia's population and are located on both sides of the continent. Otherwise, samples were purchased in a single city. The majority of samples were purchased from supermarkets, while for some foods (e.g., fresh fish, meat) the sampling plan stipulated that a number of samples be purchased from independent speciality shops (e.g., fish monger, butcher). Purchasing occurred in three phases (total primary samples n = 896: Sydney phase 1, August 2018, n = 186; Melbourne phase 2, October-December 2018, n = 516; Perth phase 3, April-June 2019, n = 194) to capture any seasonal variation in vitamin D content.

Samples were chilled from time of purchase and packaged to prevent exposure to heat and light, and loss of any liquid content during transportation. Product names, weights, and place and date of purchase were recorded, as well as country of origin and production method (e.g., wild or aquaculture for fish) where relevant and available. Samples were photographed upon arrival at NMI to confirm that the correct food was purchased. Foods that are usually consumed cooked were prepared (e.g., trimmed, grilled, baked) using common household methods without the addition of other ingredients, except for small amounts of water to prevent sample contents sticking to cooking vessels, if required. Primary samples of the same food type were homogenised to create a composite sample (Sydney n = 31; Melbourne n = 86; Perth n = 32) for each food type for each city in which it was purchased. All composite samples comprised equal amounts of six primary samples, except for dark chocolate, for which there were eight primary samples.

Analysis

All composite samples were analysed at NMI in duplicate for moisture, fat, vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2. The D vitamers were analysed using an LC-QQQ method (ISO17025:2017) that was based on methodology for an earlier liquid chromatography with ion-trap mass spectrometry method [104]. Saponification, extraction and derivatisation procedures were carried out under non-ultraviolet light [206], to minimise vitamin D deterioration, using methods described previously [98]. In brief, the weights of sample aliquots (solid samples: 1-2.5 g, liquid samples: 5-15 g)

were limited by fat content, ensuring that the fat saponified did not exceed 1 g. This was to ensure that the sample fat did not exceed the capacity of the saponification liquor, which was limited to the 50 mL (Falcon® tube) saponification vessel. The saponification mixture consisted of sample, a known amount of chemically labelled internal standard, 1 g sodium ascorbate, 10 mL deionised water, 30 mL ethanol, 2 g potassium hydroxide, and deionised water to make up to 50 mL. The chemically labelled internal standard solution included the following IsoSciences (Ambler, USA) isotopically-labelled metabolites: vitamin D_3 [$^{13}C_5$] carbon-13 labelled standard, 25(OH) D_3 [$^{13}C_5$] carbon-13 labelled standard, vitamin D_2 [$^{2}H_3$] deuterated standard and 25(OH)D_2 [$^{2}H_3$] deuterated standard. Vessels were placed in a shaker bath for approximately 16 hours at 25°C. Vitamin D analytes were then hydrolysed in the ethanolic potassium hydroxide solution (saponification), absorbed onto diatomaceous earth (Agilent Technologies Chem Elut™ 10 mL unbuffered SPE cartridges [part # 12198007]), extracted into petroleum ether, and concentrated by evaporation under nitrogen gas to dryness. The residue was resolvated into heptane, transferred to a liquid chromatography vial and evaporated to dryness under nitrogen. This residue was resolvated into a solution of 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) in anhydrous acetonitrile to form vitamin D-PTAD derivatives. After 10 minutes, the derivatisation reaction was stopped by the addition of water. Extracts with precipitate or a cloudy appearance were centrifuged at 10,000 rpm for one minute. Extracts were transferred to microvials for analysis by LC-QQQ.

Vitamin D_2 and D_3 and their respective 25-hydroxy analytes were separated by reverse phase chromatography on a C18 column (Supelco Ascentis® Express C18, 15 cm x 3 mm, 2.7 μm [Cat#53816-U]). Mobile phase A was prepared using 1 L Milli-Q® water, 1 mL 0.1% formic acid and 0.5 mL 6.4 nM methylamine. Mobile phase B consisted of 1 L methanol, 1 mL 0.1% formic acid and 0.5 mL 6.4 nM methylamine. The isolated vitamin D derivatives entered the triple quadrupole as a methylamine adduct, while the remaining liquid chromatography run was diverted to waste: 0 min to waste; 3.6 min to MS, 5.8 min to waste; 11.2 min to MS, 13.4 min to waste. The flow rate was 0.6 mL/min across the gradient profile (Table 1). Example chromatograms are provided as Supplementary Figure 1. Retention times for internal standards were equal to the non-labelled compounds. The LC-QQQ (1290 Infinity Series LC System and 6460 Triple Quad LC-MS, Agilent Technologies, Santa Clara,

USA) was set up in electrospray ionisation mode with positive polarity. The internal standard(s) corrected for extraction inefficiencies, compensated for instrument injection variation, and negated potential matrix enhancement or suppression effects.

Table 1 Gradient profile

#	Time (min:sec)	Mobile phase* (%)		Flow (mL/min)
		A	B	
1	0.00	20	80	0.6
2	1.00	20	80	0.6
3	13.00	3	97	0.6
4	13.01	0	100	0.6
5	17.00	0	100	0.6
6	17.01	20	80	0.6
7	20.00	20	80	0.6

*Mobile phase A: Milli-Q® water, 0.1% formic acid and 6.4 nM methylamine
Mobile phase B: methanol, 0.1% formic acid and 6.4 nM methylamine.

For each vitamin D analogue there was a precursor ion and three product ions. The product ion with the highest abundance was used for quantitation while the two remaining product ions were termed qualifier ions (Table 2)

Table 2 Quantifier and qualifier ions for liquid chromatography with triple quadrupole mass spectrometry analysis of D vitamers in Australian foods

Compound	Product ions (Da)		
	Quantifier	Qualifiers	
Vitamin D_3 [$^{13}C_5$] PTAD MA	596.4-298.2	596.4-280.2	596.4-161.1
Vitamin D_3 PTAD MA	591.5-298.3	591.5-280.3	591.5-161.2
25(OH)D_3 [$^{13}C_5$] PTAD MA	612.4-298.2	612.4-280.2	612.4-160.9
25(OH)D_3 PTAD MA	607.5-298.2	607.5-280.3	607.5-161.2
Vitamin D_2 [$^{2}H_3$] PTAD MA	606.6-301.2	606.6-283.3	606.6-164.1
Vitamin D_2 PTAD MA	603.5-298.3	603.5-280.2	603.5-161.1
25(OH)D_2 [$^{2}H_3$] PTAD MA	622.6-301.3	622.6-283.3	622.6-164.2
25(OH)D_2 PTAD MA	619.5-298.2	619.5-280.2	619.5-161.2

PTAD MA, 4-Phenyl-1,2,4-triazoline-3,5-dione methylamine
25(OH)D, 25-hydroxyvitamin D

An appropriate calibration standard was selected and, for each analogue, the ion abundance percentage ratios for each qualifier, with respect to its quantitation ion, was set to 100%. For each sample, the relative abundance ratios of the qualifier ions with respect to the quantitation ion were used to verify the identification of each vitamin D analogue. Acceptance criteria were as follows: i) positive result: each qualifier ion ≥ 75% and ≤ 150% of the quantitation ion, and integration of that ion was easily achievable from the background; ii) questionable result (reported as < LOD): a qualifier ion was ≥ 50% and < 75% or > 150% and ≤ 200% of the

quantitation ion, or if the integration of that ion was subjective and operator dependent; iii) unconfirmed result: a qualifier ion was < 50% or > 200% of the quantitation ion. The same process, using the same isotopically-labelled metabolites, was followed for calibration. Quantitation was against a calibration curve of analyte response relative to chemically labelled internal standard versus analyte concentration (example provided as Supplementary Figure 2).

Accuracy of the LC-QQQ method was tested using National Institute of Standards and Technology (NIST) Standard Reference Material® (SRM) 1546a (meat homogenate). Ten 1 g samples of the SRM were saponified to a final volume of 25 mL. From each of these 10 samples, 2 x 10 mL aliquots were extracted to give a total of 20 aliquots for quantitation of vitamin D_3 and 25(OH)D_3.

Moisture was measured using NMI's in-house method, which is based on a previously published AOAC method [207]. Total fat was determined either by Soxhlet [208] or Mojonnier extraction [209].

Quality assurance

Relative percent differences (RPDs) ((differences between replicate values/average of replicates) x 100) between duplicate analyses and percent recoveries were recorded. A number of samples in each analytical run were selected at random for recovery analyses, where the selected sample was spiked with a known concentration (μg/100 g of sample matrix) of each vitamin D analogue analysed. Additionally, samples of an in-house control sample were analysed with each batch. This control was a prepared mixture of infant formula and freeze-dried irradiated mushroom powder. Quality assurance data were verified as meeting the following criteria: RPD ≤ 25% and percent recoveries for spiked and control samples largely within the range of 80-120%. Data outside these acceptable ranges were queried and re-tests arranged if deemed necessary.

The limit of quantitation (LOQ) was defined as the lowest concentration detectable using the specified method, allowing for day-to-day variations in bias and precision. The LOQ was selected as 0.1 μg/100 g for all foods, except those with a high fat content (i.e. butter, margarine, mayonnaise and oil), for which the LOQ was estimated as 0.25 μg/100 g. The limit of detection (LOD) was defined as the lowest

concentration detected during a specific analysis and was matrix dependent. LODs were determined as: (SD of seven replicate analyses carried out on low-level spiked sample matrix) x (t-test value at 99% CI), where the spike concentration was related to the least amount of analyte discernible in comparison to multiple readings of a blank reagent. Trace values were defined as > LOD and < LOQ.

Data handling and quality checks

Data for proximates and D vitamers were checked against the following criteria: combined proximates (moisture and fat) ≤ 100 g edible portion and proximate concentrations similar to published Australian food composition data for the same foods [128]. Concentrations of D vitamers were questioned if differences to data published in Australia and overseas were unlikely to be explainable by expected variations (e.g., due to production method or geographical location) [128, 129, 210]. Data that did not comply with these criteria were queried and re-tests arranged if deemed necessary.

Replicate values were averaged to produce average proximate and D vitamer values for each food. Where foods were sampled in more than one city, values were averaged to produce a national average for each food.

The full analytical dataset will be made available for future releases of the AFCD. Meanwhile, we aggregated the data to demonstrate the wide variety of foods in which we detected and quantitated the D vitamers. Analytical concentrations for individual foods were grouped together according to food type and D vitamer profile. Mean concentrations for each proximate and D vitamer were calculated for each food type group and reported where the mean ≥ LOQ. Ranges were reported for food types where a quantifiable concentration was present. Standard deviations (SD) were reported for food types that included more than one composite sample.

Results

Quality assurance results

The range of LODs was 0.01-0.2 µg/100 g. The mean RPD for vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 was 5, 3, 6 and 2%, respectively. Of 58 recovery percentage results for control and spiked samples across all D vitamers, all

were within the range of 80-120%, except for three samples from Melbourne in which the food matrix complicated analysis (vitamin D3 in basa fish [76%] and Frankfurt sausages [78%], and 25(OH)D2 in ice cream [79%]). The mean (standard deviation [SD]) concentration determined for vitamin D_3 in SRM 1546a was 0.211 (0.010) µg/100 g (NIST reference value = 0.256 (range 0.203-0.309) µg/100 g). The mean (SD) concentration determined for 25(OH)D_3 in SRM 1546a was 0.116 (0.007) µg/100 g (NIST certified value = 0.090 (range 0.078-0.102) µg/100 g).

Analytical results

Vitamin D_3 was found in coffee beverages, vitamin D-fortified breakfast cereals, eggs and egg products, fortified fats, and some unfortified fats, fish, meats, milk products and alternatives, mixed savoury dishes and sweet snack foods (Table 3). We found 25(OH)D_3 in eggs, all meats except kangaroo, and some egg products, unfortified fats, fish, milk products and alternatives, mixed savoury dishes and sweet snack foods. Vitamin D_2 was detected in vitamin D-fortified breakfast cereals, a variety of meats and some snack foods (sweet and savoury), fats and oils, fish and milk products and alternatives. Although only lamb liver contained a concentration that could be quantitated, trace amounts of 25(OH)D_2 were detected in some animal products, mixed savoury dishes and sweet snack foods.

The greatest concentrations, reported as mean (SD), of vitamin D_3 were found in canned salmon (19.25 (4.81) µg/100 g), fortified malted chocolate drink powder (17.85 (1.91) µg/100 g) and fortified breakfast cereals (11.90 µg/100 g). In contrast to canned salmon, fresh Atlantic salmon contained 4.99 (0.31) µg/100 g vitamin D_3, while the concentration of this D vitamer in white fish varieties ranged up to 3.94 µg/100 g. Standard/cage (0.82 (0.28) µg/100 g) and free range/organic (0.78 µg/100 g) chicken eggs and chicken leg meat (0.33 (0.04) µg/100 g) contained the highest concentrations of 25(OH)D_3. Vitamin D_2 was quantitated in a range of both fortified (including margarine, dairy substitutes and dairy products) and unfortified foods (including dark and milk chocolate and various meat products). We were able to quantitate 25(OH)D2 in lamb liver (0.13 µg/100 g).

Table 3 Moisture, fat, vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 concentrations in Australian foods, aggregated by food type

Food type	Food samples included	Primary samples *n*	Composite analytical samples *n*	Moisture (g/100 g ± SD (range))	Fat (g/100 g ± SD (range))	Vitamin D_3 (µg/100 g ± SD (range))	25(OH)D_3 (µg/100 g ± SD (range))	Vit D_2 (µg/100 g ± SD (range))	25(OH)D_2 (µg/100 g ± SD (range))
Beverages	Coffee (cappuccino/ latte/flat white)	6	1	89.2	2.6	Tr	ND	ND	ND
Breakfast cereals – fortified	Breakfast cereals	6	1	2.8	4.1	11.90	ND	0.13	ND
Cereals and cereal products	Bread (white/grain/ wholemeal), rice (white), pastry (puff and shortcrust)	18	3	44.9 ± 24.5 (23.9 - 71.8)	7.7 ± 11.2 (ND - 20.6)	ND	ND	ND	ND
Eggs and egg products	Chicken eggs – cage/standard and free-range/organic, egg mayonnaise	24	4	62.9 ± 25.0 (25.4 - 75.5)	25.2 ± 31.9 (9.2 – 73.0)	1.11 ± 0.67 (0.19 - 1.78)	0.60 ± 0.43 (ND - 1.01)	ND	ND
Fats and oils – unfortified	Butter, lard and dripping, olive oil	30	5	8.4 ± 7.5 (ND - 15.1)	89.7 ± 9.1 (82.4 - 100.0)	0.76 ± 1.27 (ND - 3.02)	ND - 0.21	0.16 ± 0.17 (ND - 0.40)	ND - Tr
Fats and oils – fortified	Margarine, dairy blend spread	24	4	37.5 ± 6.2 (29.7 - 42.7)	60.0 ± 5.0 (55.0 - 66.3)	9.26 ± 2.85 (5.83 - 11.70)	ND	2.73 ± 3.19 (ND - 6.08)	ND
Finfish <5% fat, seafood, coated white fish and seafood products	Barramundi, basa, coated calamari, coated fish, canned tuna, oysters prawns sardines	90	15	65.7 ± 11.6 (49.2 - 83.4)	7.5 ± 6.3 (0.9 - 18.2)	1.05 ± 1.53 (ND - 4.9)	ND	ND-0.11*	ND
Finfish >5% fat	Atlantic salmon, canned salmon, fish fillets (white), mullet, trout	48	8	66.0 ± 4.7 (59.3 - 71.5)	11.1 ± 4.9 (5.1 - 17.6)	8.73 ± 7.1 (1.24 - 22.7)	0.18 ± 0.13 (ND - 0.34)	ND	ND
Fruits and vegetables	Apple, eggplant, potato, tomato	24	4	87.2 ± 7.8 (78.0 - 94.0)	ND	ND	ND	ND	ND
Marsupial animal meat	Kangaroo	6	1	67.9	2.4	ND	ND	Tr	Tr
Milk and milk products – unfortified	Cheese (brie, camembert, cheddar, cream cheese, feta, mozzarella, ricotta), cream, flavoured milk (reduced fat, regular fat), milk (reduced fat, regular fat), yoghurt (regular fat, reduced fat)	156	26	68.5 ± 18.9 (34.3 - 89.8)	13.9 ± 13.8 (1.1 - 38.3)	ND - 0.20	ND - 0.12*	ND-0.11	ND-Tr
Milk, milk products and milk alternatives – fortified	Dairy dessert/yoghurt, infant formula, dairy milk substitute, dairy milk, dairy milk powder, malted chocolate drink powder, toddler formula	54	9	29.5 ± 41.8 (0.8 - 89.2)	11.1 ± 8.9 (1.1 - 25.4)	7.41 ± 8.26 (ND – 19.45)	0.03 ± 0.07 (ND - 0.17)	0.37 ± 0.55 (ND - 1.74)	ND

Mixed savoury dishes	Chicken burger, fried rice with egg/meat/ fish, beef burger with cheese, egg and bacon, beef burger with salad and sauce, meat pie, pizza (tomato/cheese or vegetable/cheese), pizza (meat lovers style), ravioli or tortellini with meat and/or cheese filling), sausage roll, soup with meat or seafood, spring rolls or dumplings with meat or fish, takeaway style mixed dish with beef/lamb/chicken	102	17	56.3 ± 12.0 (40.2 - 85.8)	9.6 ± 3.3 (1.1 - 17.3)	ND - 0.18	ND - 0.17	ND - 0.31	ND-Tr
Monogastric animal meats	Chicken breast (skinless), chicken leg with skin, chicken nuggets, crumbed chicken, pork chops, pork mince	84	14	62.0 ± 6.7 (49.4 - 69.0)	8.9 ± 4.8 (2.2 ± 17.1)	0.42 ± 0.39 (Tr - 1.24)	0.23 ± 0.08 (0.12 - 0.38)	ND	ND
Ruminant animal meats	Beef mince, beef steak, lamb chops, lamb liver	60	10	60.7 ± 5.8 (50.5 - 68.4)	12.2 ± 5.3 (5.0 - 18.8)	0.12 ± 0.09 (ND - 0.33)	0.17 ± 0.06 (Tr - 0.27)	0.17 ± 0.20 (ND - 0.67)	ND - 0.13
Processed meats	Bacon, ham, salami, canned meats, beef sausage, Frankfurts, packaged sliced chicken or turkey	72	12	58.5 ± 11.3 (40.8 - 74.1)	15.4 ± 8.0 (3.3 - 29.2)	0.29 ± 0.38 (0.10 - 1.49)	0.14 ± 0.05 (Tr - 0.25)	ND - 0.18	ND-Tr
Snack foods – sweet	Banana bread, cheesecake, chocolate (dark and milk), chocolate cake, doughnuts, fruit muffin, ice-cream (tub-style, premium chocolate-coated stick-type, stick-type), sweet biscuits	68	11	28.8 ± 22.1 (0.9 – 66.2)	17.2 ± 8.4 (1.7 - 31.0)	ND - 0.24	ND - 0.14	0.52 ± 1.01 (ND - 3.20)	ND-Tr
Snack foods – savoury	Corn chips/extruded snacks (cheese flavour), savoury biscuits (cheese flavour)	12	2	2.3 ± 1.1 (1.5 – 3.1)	24.9 ± 3.5 (22.4 - 27.3)	ND	ND	ND	ND
Spreads	Peanut butter, yeast extract spread	12	2	21.1 ± 27.8 (1.5 - 40.7)	23.9 ± 31.9 (1.3 - 46.4)	ND	ND	ND	ND

Composite samples comprising 6 or 8 primary samples of the same food sample were analysed.
Concentrations ≥LOQ are presented as mean ± SD (range). Exceptions are: food types containing only one composite sample (no SD) and food types that included quantifiable concentrations but had mean concentrations <LOQ (a range is provided). Data for individual food samples will be made available for future releases of the Australian Food Composition Database.
ND, not detected (<LOD); Tr, >LOD<LOQ
LOQ = 0.1 µg/100 g for all foods except high-fat foods. LOQ = 0.25 µg/100 g for high-fat foods (mayonnaise, fats and oils). LODs range 0.01 (dairy milk alternative) to 0.2 (butter and margarine spread) µg/100 g.

Discussion

We have developed Australia's first comprehensive analytical vitamin D food composition database, the complete version of which will be made freely available for future releases of the AFCD. Method validation results are presented for the new LC-QQQ method used, demonstrating that it provides a mean value within the NIST reference range for vitamin D_3 in SRM 1546a. The LC-QQQ mean value for 25(OH)D_3 was 0.01 µg/100 g outside the NIST reference range for 25(OH)D_3; however, due to the challenges of vitamin D measurement in food, such differences may be expected. Fortified foods and oily fish were found to be good sources of vitamin D. Similar to the findings from our pilot study [211], white fish – not typically promoted as a source of vitamin D – was also a source of vitamin D.

It is generally thought that the bioactivity of 25(OH)D_3 may be greater than that of vitamin D_3; however, a lack of evidence for, and consensus on, a defined bioactivity factor has meant that handling of 25(OH)D_3 values varies between food composition databases. To allow for the uncertainty surrounding the 25(OH)D_3 bioactivity factor [125, 201], the AFCD presents values for individual D vitamers unadjusted for bioactivity as well as a 'vitamin D_3 equivalents' value that incorporates a bioactivity factor of five for 25(OH)D_3 [128].

It is important to measure the hydroxylated vitamers of vitamin D (25(OH)D_3 and 25(OH)D_2) since, although the measured concentrations may be low, cumulatively across the diet - even without a bioactivity factor applied - their contribution to dietary requirements may be considerable. In our study, this was particularly evident in chicken eggs, where 25(OH)D_3 (unadjusted for bioactivity) concentrations were more than half the concentrations of vitamin D_3. If the bioactivity of 25(OH)D_3 is greater than vitamin D_3 [121, 125, 201], the contribution of 25(OH)D_3 in eggs may outweigh that of vitamin D_3. Based on our analytical results, in Australia, a standard 120 g serve of eggs [212], would contain approximately 1 µg of 25(OH)D_3 per serve, before adjustment for bioactivity. Independently of vitamin D_3 content, the unadjusted 25(OH)D_3 content alone translates to one fifth of the Australian Adequate Intake (AI) (5 µg/day), one tenth of the Institute of Medicine's (IOM) Estimated Average Requirement (EAR) (10 µg/day), and one fifteenth of the IOM's Recommended Dietary Allowance (RDA) (15 µg/day). In various other beef,

chicken and lamb products, unadjusted 25(OH)D_3 concentrations were either equal to or greater than vitamin D_3. As such, omission of 25(OH)D_3 may cause considerable underestimation of the vitamin D contributions of these foods, and also of vitamin D intakes, which may be further augmented if a bioactivity factor greater than one is determined for 25(OH)D in the future.

Although the human body appears to favour vitamin D_3 over vitamin D_2, the latter is still of use in improving circulating 25(OH)D concentrations in humans [115], and should be included in food composition analyses in order to accurately reflect vitamin D content. Since vitamin D_2 is synthesised from ergosterol in fungi and yeast, but not in animals, the D_2 vitamers are rarely measured in animal products. Vitamin D_2 and 25(OH)D_2, however, have previously been found in cow's milk [137], and concentrations of vitamin D_2 are listed for milk and milk products in some food composition databases (e.g., India has two entries for cheese [131], and Denmark has one entry each for butter and whole milk [129]); however, when surveying other food composition databases, we found no quantitated values for vitamin D_2 in meat products. Barnkob et al. [136] conducted an ultraviolet-B (UVB) exposure study in slaughter pigs raised indoors. They found vitamin D_2 in the rind, subcutaenous fat, lean meat, liver and serum of the control group, as well as 25(OH)D_2 in the rind and serum. Vitamin D_2 content increased in the groups exposed to UVB light. The detection of vitamin D_2 and 25(OH)D_2 in animal products has been attributed to the ingestion of vitamin D_2 in feed, particularly from grasses contaminated with fungi [90]. Indeed, in the study by Barnkob et al. [136], straw and feed were exposed along with the pigs in UVB-irradiated pens.

We found notable amounts of vitamin D_2, which in several cases were naturally-occurring, in various foods, including meat products. Of unfortified foods analysed, the greatest vitamin D_2 concentrations were found in chocolate. Vitamin D_2 is thought to be present in chocolate due to fungal contamination of cocoa beans [213]. This may explain in part, and likely in conjunction with milk solids, the presence of vitamin D_2 in cocoa-containing foods such as chocolate-coated ice-cream products, and malted chocolate drink powder. Indeed, the vitamin D content of analysed chocolate-coated premium stick-type ice-cream products was solely contributed by vitamin D_2, highlighting the need to measure this vitamer in a wide range of foods.

We also found reasonable amounts of vitamin D_2 in several meat products, further reinforcing the importance of measuring all four D vitamers in foods.

While 25(OH)D_2 contributed 20% of the total vitamin D content of lamb livers analysed in our study, the majority of detected amounts of 25(OH)D_2 in other foods were too small to quantitate. When estimating intakes, it is usual practice to allocate a value of LOQ/2 for metabolites detected at concentrations below the LOQ [214] in order to find a middle ground between concentrations that may be closer to either zero or the LOQ. Hence, foods with very low, but detectable, D vitamer concentrations may contribute small, but cumulatively important amounts to overall vitamin D intake estimations.

The major strength of this project was the use of novel, sensitive and specific analytical methodology that allows detection of all four D vitamers at low concentrations. We did not limit the analysis of D vitamers based on preconceptions of the D vitamers that may be found in foods of plant or animal origin, but instead analysed all four D vitamers in every food sampled. Additionally, we developed an extensive sampling plan covering three major cities on both the east and west coasts of Australia, representing where the majority of Australians purchase food. Resource constraints prevented the sampling and analysis of all available food products and of analysis at the primary sample level. Despite careful attention to sampling, averaged, estimated and imputed values taken from these analytical data will not necessarily reflect the nutrient content of a specific food item that is consumed. These are inherent limitations in the production and use of food composition data in general.

Conclusion

We have developed Australia's first comprehensive analytical vitamin D food composition database using extensive sampling and novel, sensitive and specific analytical methodology. Vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 were all detected and/or quantitated in a wide range of foods. All four vitamers are rarely included in food composition databases. These new vitamin D food composition data represent the first step in a major advancement of the understanding of dietary vitamin D in the Australian population and highlight the importance of measurement of all four vitamers in order to accurately represent the vitamin D content of food.

Author contributions:

Eleanor Dunlop: Formal analysis, Data curation, Writing – Original draft, Project administration. **Anthony P James**: Supervision, Writing – Review and editing. **Judy Cunningham:** Methodology, Funding acquisition, Writing – Review and editing. **Norbert Strobel:** Methodology, Validation, Investigation, Writing – Original draft. **Robyn M Lucas:** Conceptualization, Funding acquisition, Writing – Review and editing. **Mairead Kiely:** Funding acquisition, Writing – Review and editing. **Caryl A Nowson:** Funding acquisition, Writing – Review and editing. **Anna Rangan:** Funding acquisition, Writing – Review and editing. **Paul Adorno:** Resources, Funding acquisition, Writing – Review and editing. **Paul Atyeo:** Resources, Funding acquisition, Writing – Review and editing **Lucinda J Black:** Conceptualization, Funding acquisition, Methodology, Supervision, Project administration, Writing – Review and editing.

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Supplementary Table 1 Purchase location and preparation of Australian retail foods analysed for vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 content

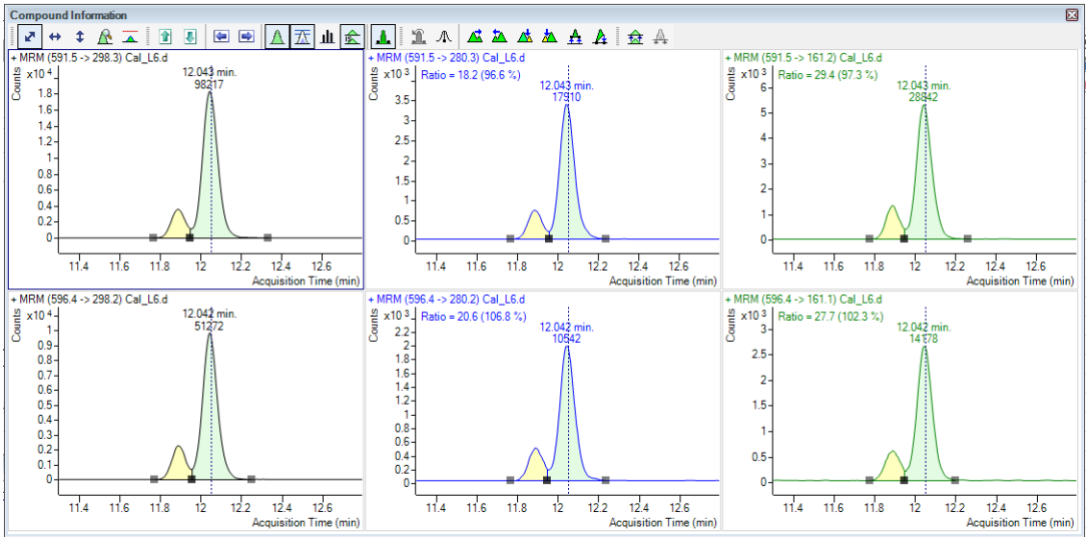
Sample description	Purchase location(s)	Preparation
Apple, unpeeled, raw	Melbourne	Cored
Bacon, partly trimmed	Sydney, Melbourne, Perth	Pan fried without oil
Beef mince, regular fat	Sydney, Melbourne, Perth	Pan fried without oil
Beef, steak, semi-trimmed	Sydney, Melbourne, Perth	External fat removed, grilled
Bread, white, grain and wholemeal, commercial	Melbourne	None
Breakfast cereals, ready to eat, vitamin D -fortified	Melbourne	None
Butter, regular fat	Sydney, Melbourne, Perth	None
Calamari or squid, battered, crumbed or salt & pepper, takeaway or frozen	Melbourne	If frozen, cook according to label instructions
Canned meats, red meat (beef, lamb, ham or pork)	Melbourne	None
Canned salmon, plain or flavoured, drained	Sydney, Melbourne	None
Canned tuna, plain or flavoured, drained	Melbourne, Perth	None
Cheese, brie or camembert	Melbourne	None
Cheese, cheddar	Sydney, Melbourne, Perth	None
Cheese, cream cheese, regular fat	Melbourne	None
Cheese, feta	Sydney, Melbourne	None
Cheese, mozzarella	Sydney, Perth	None
Cheese, ricotta	Melbourne	None
Cheesecake, plain or flavoured	Melbourne	None
Chicken burger, with salad and sauce	Melbourne, Perth	None
Chicken nuggets, frozen or takeaway	Melbourne	If frozen, baked as per label instructions
Chicken, crumbed schnitzels or similar	Melbourne	Baked as per label instructions
Chicken, leg meat with skin	Sydney, Melbourne, Perth	Baked
Chicken, skinless breast fillets	Sydney, Melbourne, Perth	Pan fried without oil
Chocolate, dark, plain	Perth	None
Chocolate, milk, plain	Melbourne	None
Coffee, takeaway, cappuccino, latte & flat white	Melbourne	None
Commercial banana bread	Melbourne	None

Commercial chocolate cakes, iced or un-iced	Melbourne	None
Corn chips or extruded snacks, cheese flavoured	Melbourne	None
Cream, regular fat	Melbourne, Perth	None
Dairy blend, e.g. dairy soft	Melbourne	None
Dairy dessert or yoghurt, children's style, vitamin D fortified	Melbourne	None
Dairy milk substitute (soy, rice, oat, almond), vitamin D-fortified	Melbourne	None
Doughnuts, plain or iced	Melbourne	None
Eggplant	Melbourne	Grilled
Eggs, chicken, free range or organic	Melbourne	Boiled
Eggs, chicken, standard or cage	Sydney, Perth	Boiled
Fish fillets, battered or crumbed, takeaway	Sydney, Melbourne, Perth	None
Fish fingers	Melbourne	Baked as per label instructions
Fish, fillets, basa, skinless	Melbourne	Pan fried + ~40 mL water without oil
Fish, fillets, white type, barramundi, battered, takeaway	Perth	None
Fish, fillets, white type, other	Sydney	Pan fried + ~40 mL water without oil
Fish, whole or fillets, mullet	Sydney	Baked in foil, no oil
Fish, whole or fillets, sardines	Perth	Baked in foil, no oil
Fish, whole or fillets, trout	Melbourne	Baked in foil, no oil
Flavoured dairy milk, reduced fat (~1.5% fat)	Perth	None
Flavoured dairy milk, regular fat (~3.5% fat)	Melbourne	None
Frankfurts, regular	Melbourne	Boiled gently
Fried rice containing egg/meat/fish	Melbourne	None
Frozen crumbed or battered fish	Melbourne	Baked as per label instructions
Frozen puff and shortcrust pastry	Melbourne	Baked as per label instructions
Ham, sliced	Sydney, Perth	None
Hamburger, beef, with cheese, egg and bacon	Sydney, Melbourne	None
Hamburger, beef, with salad and sauce	Sydney, Melbourne	None
Ice cream, tub-style, vanilla flavour, regular fat	Melbourne	None
Ice-cream, premium, stick-type, chocolate-coated	Melbourne	None
Ice-cream, stick-type	Melbourne	None
Infant formula, dairy-based formulas, powder only	Melbourne	None
Kangaroo, steak	Melbourne	Pan fried without oil

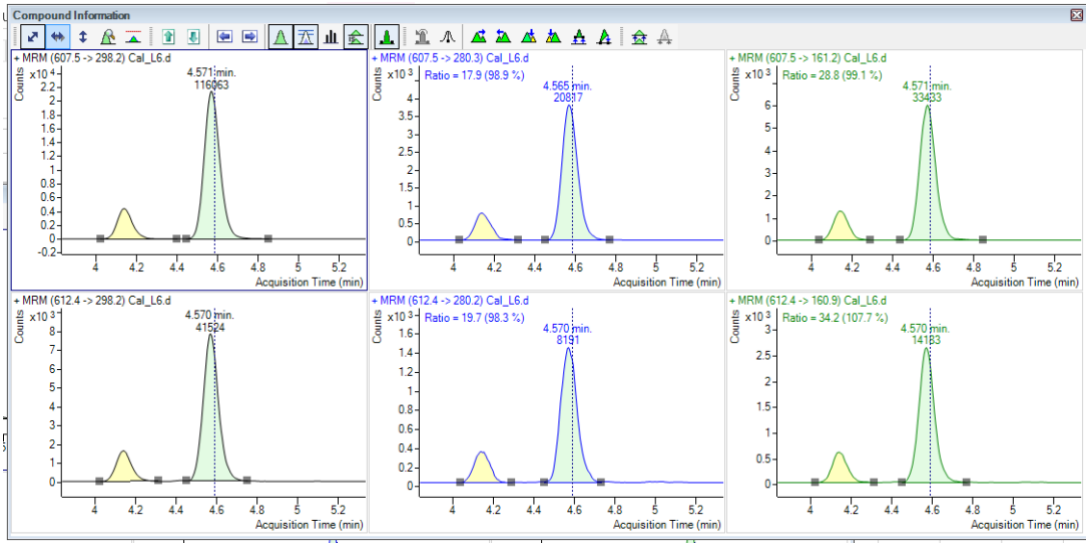
Lamb, chops, semi-trimmed,	Sydney, Melbourne, Perth	Grilled
Lard and dripping	Melbourne	None
Liver, lamb	Melbourne	Pan fried without oil
Margarine spread, regular fat	Sydney, Melbourne, Perth	None
Mayonnaise, regular fat, made with egg	Melbourne	None
Meat pie, standard, fresh or frozen	Melbourne	Baked as per label instructions
Milk powder, regular fat, unfortified (~4% fat when reconstituted)	Melbourne	None
Milk (dairy), plain, reduced fat (~1.5% fat)	Sydney, Melbourne, Perth	None
Milk (dairy), plain, regular fat (~3.5% fat)	Sydney, Melbourne, Perth	None
Milk (dairy), vitamin D fortified (~2% fat)	Melbourne	None
Malted chocolate drink powder, regular flavour	Sydney, Melbourne, Perth	None
Muffin, sweet, fruit	Melbourne	None
Olive oil	Melbourne	None
Oysters, raw	Melbourne	Shelled
Packaged sliced chicken or turkey	Melbourne	None
Peanut butter	Melbourne	None
Pizza, frozen, with tomato & cheese, or with vegetable and cheese topping	Melbourne	Baked as per label instructions
Pizza, takeaway, meat lovers-style	Sydney, Perth	None
Pork Chops, semi-trimmed	Sydney, Melbourne, Perth	Grilled/BBQ
Pork, minced	Sydney, Melbourne, Perth	Pan fried without oil
Potato	Melbourne	Boiled
Prawns (purchased cooked)	Sydney, Melbourne, Perth	None
Ravioli or tortellini, meat and/or cheese filling, no sauce	Melbourne	Boiled as per label instructions
Rice, white	Melbourne	Boiled
Salami, regular fat	Melbourne	None
Salmon, Atlantic, fillets or steaks, fresh	Sydney, Melbourne, Perth	Baked in foil, no oil
Sausage roll, standard	Melbourne	Baked as per label instructions
Sausage, beef	Sydney, Melbourne, Perth	Grilled/BBQ/pan fried
Savoury biscuits, 'Shape' style flavoured biscuits containing cheese powder	Melbourne	None
Soup, prepared, commercial, with meat or seafood	Melbourne	Heated as per label instructions
Spring rolls or dumplings containing meat or fish	Melbourne	Baked or pan fried as per label instructions
Sweet biscuits, plain, including shortbread and Anzacs	Melbourne	None

Takeaway style mixed dish, beef- or lamb-based, with vegetables and sauce	Sydney	None
Takeaway style mixed dish, chicken-based, with vegetables and sauce	Perth	None
Toddler formula	Melbourne	None
Tomato, raw	Melbourne	Stem removed
Yeast extract spread	Melbourne	None
Yoghurt, flavoured or added fruit, full fat (3-5% fat)	Sydney, Melbourne, Perth	None
Yoghurt, flavoured or added fruit, reduced fat (1-2% fat)	Sydney, Melbourne, Perth	None

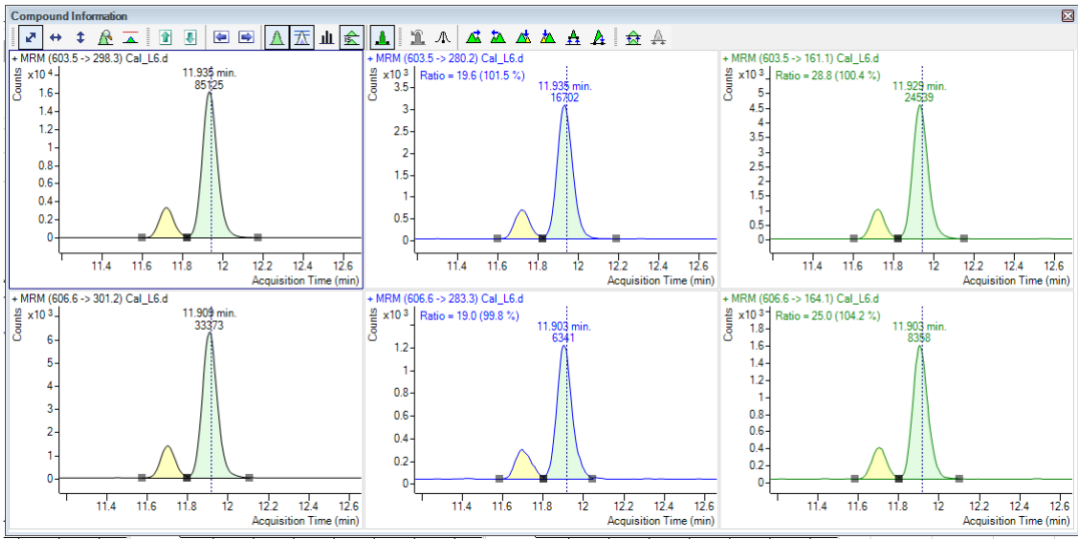
A


Compound Information
+ MRM (591.5 -> 298.3) Cal_L6.d
12.043 min.
98217
+ MRM (591.5 -> 280.3) Cal_L6.d
Ratio = 18.2 (96.6 %)
12.043 min.
17910
+ MRM (591.5 -> 161.2) Cal_L6.d
Ratio = 29.4 (97.3 %)
12.043 min.
28842
+ MRM (596.4 -> 298.2) Cal_L6.d
12.042 min.
51272
+ MRM (596.4 -> 280.2) Cal_L6.d
Ratio = 20.6 (106.8 %)
12.042 min.
10542
+ MRM (596.4 -> 161.1) Cal_L6.d
Ratio = 27.7 (102.3 %)
12.042 min.
14178
Counts
Acquisition Time (min)

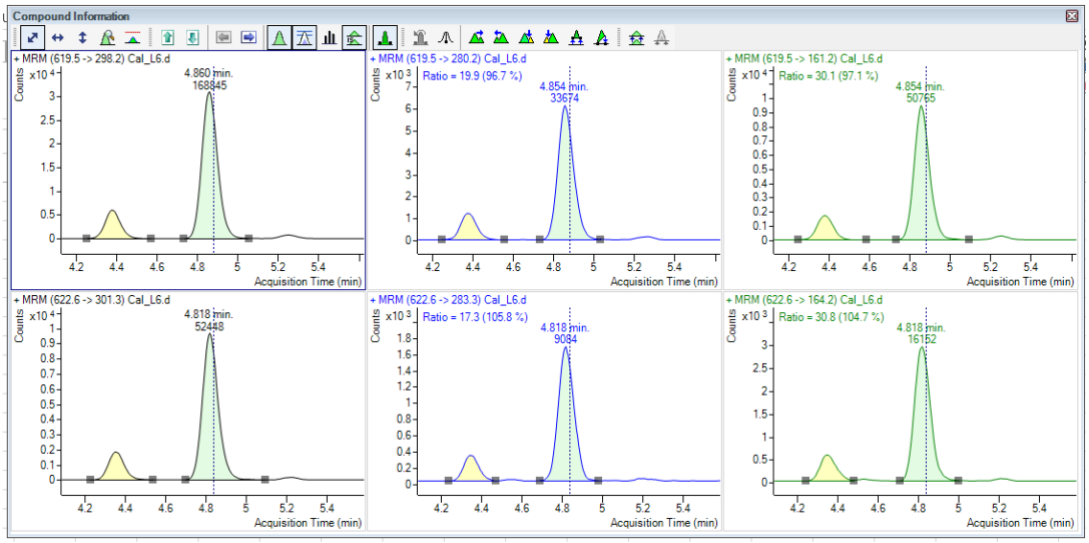
B


Compound Information
+ MRM (607.5 -> 298.2) Cal_L6.d
4.571 min.
116063
+ MRM (607.5 -> 280.3) Cal_L6.d
Ratio = 17.9 (98.9 %)
4.565 min.
20837
+ MRM (607.5 -> 161.2) Cal_L6.d
Ratio = 28.8 (99.1 %)
4.571 min.
33433
+ MRM (612.4 -> 298.2) Cal_L6.d
4.570 min.
41524
+ MRM (612.4 -> 280.2) Cal_L6.d
Ratio = 19.7 (98.3 %)
4.570 min.
8191
+ MRM (612.4 -> 160.9) Cal_L6.d
Ratio = 34.2 (107.7 %)
4.570 min.
14183
Counts
Acquisition Time (min)

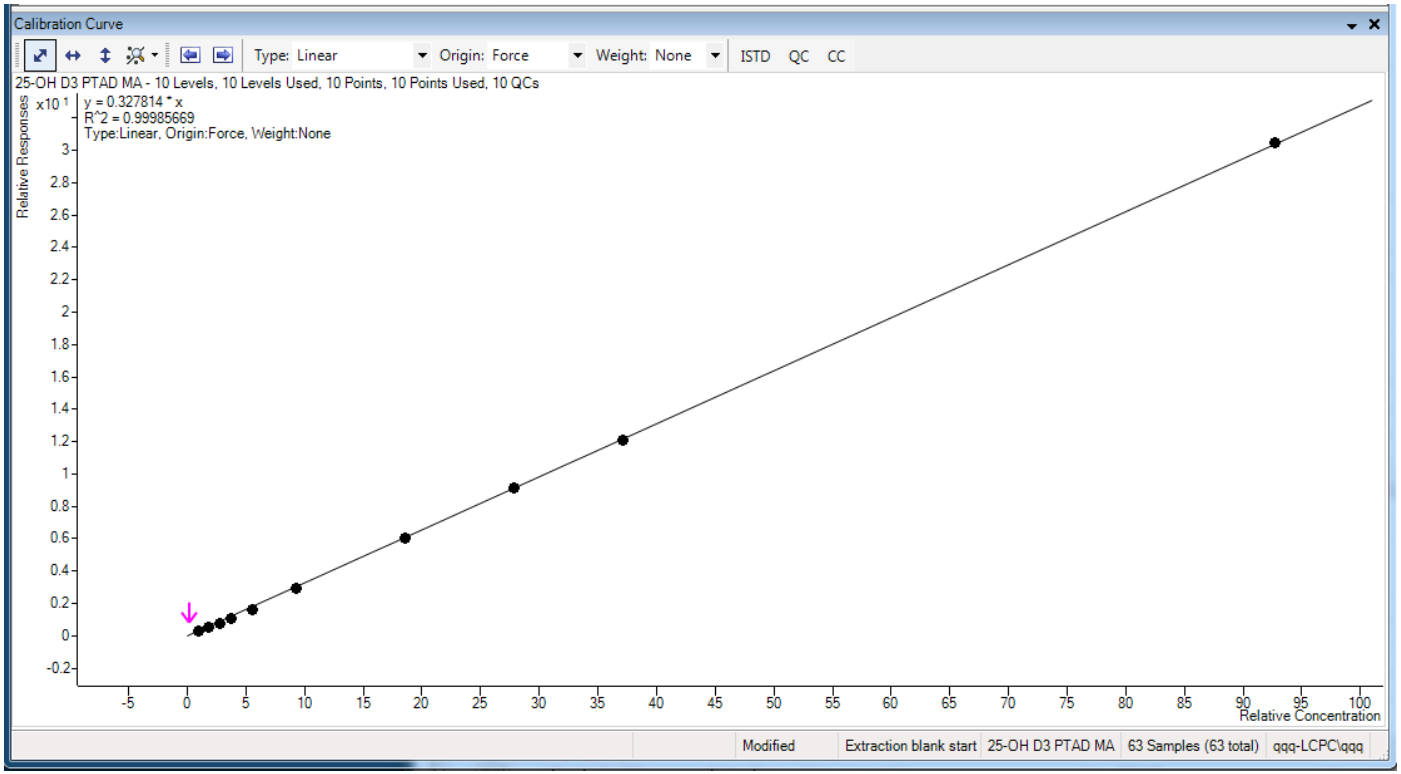
C



D



Supplementary Figure 1 Example chromatograms for (A) vitamin D_3, (B) 25-hydroxyvitamin D_3, (C) vitamin D_2 and (D) 25-hydroxyvitamin D_2



Supplementary Figure 2: Example calibration curve

Chapter 3: Vitamin D composition of Australian game products

Thesis objective addressed in this chapter:

Objective 2: To determine the vitamin D content of Australian game products, which may be a useful source of vitamin D for people living in remote areas.

The content of this chapter is covered by Publication 2:

This is a non-final version of an article published in final form in: **Dunlop E**, CCJ Shepherd, J Cunningham, N Strobel, RM Lucas, LJ Black. 2021. Vitamin D composition of Australian game products. *Food Chemistry*: 387:132965. doi: 10.1016/j.foodchem.2022.132965

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Abstract:

The vitamin D content of many Australian game products is unknown. These foods are potential sources of vitamin D for remote-dwelling Aboriginal and Torres Strait Islander people, of whom 39% are vitamin D deficient (serum 25-hydroxyvitamin D_3 ($25(OH)D_3$) concentrations < 50 nmol/L). Vitamin D_3, $25(OH)D_3$, vitamin D_2 and $25(OH)D_2$ were measured by liquid chromatography-triple quadrupole mass spectrometry (LC-QQQ) in raw meat (camel, crocodile, emu, kangaroo), emu eggs and emu oil. Vitamin D_3 (range, 0.5-14.5 μg/100 g) was found in all products except camel and kangaroo. All samples except kangaroo contained $25(OH)D_3$; some camel samples contained relatively high concentrations (range, 0.4-5.2 μg/100 g). Vitamin D_2 was found in emu products and some kangaroo samples. We detected trace amounts of $25(OH)D_2$ in some camel and kangaroo samples. This study provides valuable insight into foods with a paucity of data on vitamin D content, showing that some are potentially useful sources of vitamin D.

Introduction

Over a quarter (27%) of the adult Australian Aboriginal and Torres Strait Islander (hereafter referred to as Aboriginal) population are vitamin D deficient (serum 25-hydroxyvitamin D (25(OH)D) concentration < 50 nmol/L) [215]. While this is comparable to the general Australian population (23%) [58], there is a distinct difference in the prevalence of vitamin D deficiency within the adult Aboriginal population across the geographic landscape – from 23% amongst those living in non-remote areas to 39% in remote areas [216]. A number of adverse health outcomes with possible links to vitamin D status are prevalent in the Aboriginal population. Although causal associations have not been established, nationally-representative data from the 2012-2013 Aboriginal and Torres Strait Islander Health Survey have shown that diabetes and chronic kidney disease were more common in Aboriginal people with vitamin D deficiency than those who were vitamin D sufficient [216]. Cross-sectional surveys have also shown greater prevalence of diabetes and other cardio-metabolic risk factors [34], gastroenteritis [35] and respiratory infection [36], in Aboriginal people with low vitamin D status. Due to a lack of vitamin D food composition data for foods that are traditionally hunted and foraged by Aboriginal

people, it is not possible to explore traditional and culturally beneficial food-based strategies to improve intakes and status.

Wild game products form part of the traditional and contemporary hunting activities of Aboriginal people [217, 218] and some animals, such as kangaroo, increasingly feature in modern Australian diets [219, 220]. Globally, there has been limited investigation of the vitamin D content of traditional foods [221]; however, studies involving Canadian [222] and Russian [223] Indigenous peoples and their traditional foods have indicated that traditional diets may include important sources of vitamin D.

Accurate measurement of vitamin D in food is challenging and costly. Hence, data presented in national food composition databases are frequently limited by resources and by access to the analytical methods required to measure all four of the main D vitamers found in food (vitamin D_3, vitamin D_2, and their hydroxylated forms, $25(OH)D_3$ and $25(OH)D_2$). The National Measurement Institute of Australia (NMI) recently developed a liquid chromatography with triple quadrupole mass spectrometry (LC-QQQ) method [3, 98] with the capacity to quantitate low concentrations of vitamin D_3, vitamin D_2, $25(OH)D_3$ and $25(OH)D_2$ in food. We used this method to develop an analytical vitamin D food composition database for Australian retail foods [3]; however, the vitamin D content of game animal products in Australia remains largely unexplored. There are numerous logistical barriers to direct acquisition of game products from Australia's vast remote regions. Hence, the aim was to measure vitamin D_3, $25(OH)D_3$, vitamin D_2 and $25(OH)D_2$ in Australian farmed and wild game products (camel, crocodile, emu and kangaroo) sourced from commercial suppliers.

Methods

Sample purchase and preparation

Various cuts (Table 1), weighing between 0.3 and 3.9 kg, of wild camel (*Camelus dromedarius)*, farmed crocodile (*Crocodylus porosus)*, farmed emu (*Dromaius novaehollandiae*), wild Eastern Grey Kangaroo (*Macropus giganteus*) and wild Red Kangaroo (*Macropus rufus)* meat were purchased from Yarra Valley Game Meats (Victoria, Australia) in October 2018 and July 2021. Samples were purchased frozen and transported to NMI in chilled containers, protected from heat and light. Samples

Table 1: Characteristics of game products analysed for vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_2 and 25-hydroxyvitamin D_2

Product type	*n*	Meat cuts included	Production method	Origin
Camel (*Camelus dromedarius)*			Wild	Central Australia
Meat	6	Fillet, mince[a], diced[a], eye fillet, rump, knuckle		
Hump fat/meat	4	Hump		
Crocodile meat (*Crocodylus porosus)*	8	Tail fillet, mince[a], striploin, sweet cut (cheek), body trim[b]	Farmed	Queensland
Emu (*Dromaius novaehollandiae)*			Farmed	
Meat	6	Fan fillet, flat fillet, mince[a]		Victoria
Heart	4	Heart		Victoria
Eggs	8	-		New South Wales, Victoria, Western Australia
Oil	6	-		New South Wales, Victoria, Western Australia
Kangaroo meat (*Macropus giganteus* and *Macropus rufus*)	8	Fillet, rump, mince[a], striploin, tail butt, tail	Wild	Queensland, New South Wales

[a]Mince/diced are a mixture of various cuts
[b]Body trim is the subcutaneous outer layer of flesh

were thawed, removed from packaging and homogenised. Kangaroo tail fur was stripped prior to removing meat and fat from the bone; fur and bone were discarded. Following preparation, individual samples were stored at -20 ^{o}C and protected from light and oxygen until analysis.

Samples of emu eggs (n = 8) and emu oil (total n = 6; 50 mL bottle n = 4, 100 mL bottle n = 2) were sourced from three emu farms located in Western Australia (Free Range Emu Farm), Victoria (Longview Emu Farm) and New South Wales (Emu Logic) in July 2021 (Table 1). The weight of each emu egg, in shell, was recorded (mean egg weight = 636 g, range 590-691 g). The entire contents of all eggs were cracked into a blending container and homogenized to create a composite sample. Following preparation, the composite sample was frozen to < -70 ^{o}C, freeze dried to < 3% moisture by weight and stored at -20 ^{o}C, protected from light and oxygen, until analysis. The entire contents of emu oil bottles were emptied into a blending container and homogenized to create a composite sample. Following preparation, the composite sample was stored at room temperature, protected from light and oxygen, until analysis.

Sample analysis

Details of the LC-QQQ method (ISO17025:2017) used to measure vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 and its validation have been published previously [3]. In addition, we sent four samples (canned salmon, trout, feta cheese and whole chicken eggs) from our earlier study [3] to the Technical University of Denmark for confirmatory testing using LC-MS/MS methods detailed elsewhere [106, 136, 224]. Results returned were within method uncertainties. Briefly, sample aliquot weights were determined in order that the amount of saponified fat was ≤ 1 g, an amount that was able to be saponified within a 50 mL vessel. The sample, a known quantity of chemically labeled internal standard, 1 g sodium ascorbate, 10 mL deionised water, 30 mL ethanol, 2 g potassium hydroxide, and additional deionized water to make 50mL were placed into a capped 50 mL Falcon® tube for saponification in a shaker bath overnight. Following hydrolysis in an ethanolic potassium hydroxide solution, vitamin D analytes were extracted to diatomaceous earth solid phase extraction tubes (Chem Elut™ 10 mL unbuffered SPE cartridges, Agilent Technologies, Santa Clara, USA) and washed through with petroleum ether. After the washes were evaporated

to dryness under nitrogen gas, residues were resolvated into heptane and again evaporated to dryness under nitrogen gas. The resulting residue was resolvated into 4-Phenyl-1,2,4-triazoline-3,5-dione (PTAD) in anhydrous acetonitrile for derivatisation, which was concluded after 10 minutes through addition of water.

Vitamin D analytes were isolated on a reverse phase C18 column (Supelco Ascentis® Express C18, 15 cm x 3 mm, 2.7 μm [Cat#53816-U], Sigma-Aldrich, St. Louis, USA) and were analysed, along with a range of calibration samples by LC-QQQ (1290 Infinity Series LC System and 6460 Triple Quad LC-MS, Agilent Technologies, Santa Clara, USA), which was configured in electrospray ionization mode with positive polarity. Analytes were quantitated against the calibration curve generated from calibration sample analysis.

Moisture was measured using a method developed at NMI, which was based on a previously published AOAC method [207]. The Soxhlet extraction method was used to measure total fat [208].

Quality assurance

Analyses of proximates and all D-vitamers were duplicated, from saponification to quantitation, in all samples. The relative percent difference (RPD) between duplicate analyses was calculated as (difference between replicate values/average of replicates) x 100. Five samples were randomly allocated for recovery analyses, and spiked with a known concentration (µg/100 g of sample matrix) of each D-vitamer. In addition, three samples of an in-house control sample (infant formula and freeze-dried irradiated mushroom powder) were analysed. Measured concentrations in spiked and control samples were recorded and reported as a recovery percentage of the known concentration.

The limit of quantitation (LOQ) represents the lowest concentration that can be quantitated by the specified method. It remains constant and accounts for variations in bias and precision that may occur between analytical runs. The LOQ for all vitamers was estimated as 0.10 µg/100 g for all samples except camel hump and emu oil. A LOQ of 0.25 µg/100 g was estimated for camel hump and emu oil, due to their high fat content and consequential requirement of a reduced analytical sample. The limit of detection (LOD) represents the lowest concentration that can be detected on

a specific day during a specific analytical run and may vary due to a number of factors, including food matrix. LODs were calculated as: (standard deviation (SD) of seven replicate analyses carried out on low-level spiked sample matrix) x (t-test value at 99% confidence interval (CI)), where the spike concentration was related to the least amount of analyte discernible in comparison to multiple readings of a blank reagent. Detected values > LOD and < LOQ, that were subject to greater uncertainty, were reported as 'trace'.

Data handling

Analytical values for duplicate analyses were averaged to give mean values for each sample. For samples that were analysed individually, mean concentrations are reported: camel meat, crocodile (lower-fat cuts and body trim meat were separated due to fat content differences), emu meat, kangaroo meat, emu heart and camel hump. For samples that were composited for analysis (emu oil and eggs), the mean of duplicate analyses is reported. Concentrations of D vitamers per 100 g fat were calculated as follows: (D vitamer per 100 g sample/fat per 100 g sample) x 100. All values, other than those per 100 g fat, are reported per wet weight.

Total vitamin D was calculated as the sum of vitamers for samples containing vitamin D_3 and/or vitamin D_2 only. As 25(OH)D may be between one and five times more bioactive than vitamin D [121, 125, 201], we have presented a range for samples containing quantifiable concentrations of 25(OH)D. The lower bound was calculated based on equal bioactivity of all four vitamers; the upper bound was calculated using a bioactivity factor of five for 25(OH)D_3 and 25(OH)D_2.

Results

In all samples except camel hump and emu oil, for which the LOD was 0.1 µg/100 g, LODs ranged between 0.01-0.06 µg/100 g for all vitamers. The mean RPD was 11, 9, 17 and 7% for vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2, respectively. Recovery from spiked samples ranged from 74 to 126%. Recovery of known amounts from three control samples was 94, 101 and 102%.

All camel cuts contained 25(OH)D_3; however, hump was the only camel cut with detectable vitamin D_3 (Table 2, Supplementary Table 1). The mean concentration of

Table 2. Moisture, fat, vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_2, and 25-hydroxyvitamin D_2 in raw Australian game products[a]

Product	Primary samples *n*	Moisture (g/100 g)	Fat (g/100 g)	Vitamin D_3[b] (µg/100 g)	25(OH)D_3[b] (µg/100 g)	Vitamin D_2[b] (µg/100 g)	25(OH)D_2[b] (µg/100 g)
Camel (*Camelus dromedarius)*							
Meat (fillet, mince[c], diced[c], eye fillet, rump, knuckle)	6	76.0 ± 1.1 (74.8 - 77.3)	1.5 ± 0.7 (0.6 - 2.6)	ND	1.07 ± 0.73 (0.40 - 2.46)	ND	ND-Tr
Hump	4	11.3 ± 5.5 (5.7-17.8)	79.8 ± 7.3 (73.4 - 90.2)	Tr	2.13 ± 2.04 (0.95 - 5.17)	ND	Tr
Crocodile (*Crocodylus porosus)*							
Lower-fat cuts (tail fillet, mince[c], striploin, sweet cut (cheek))	4	77.2 ± 0.5 (76.8 - 77.8)	2.3 ± 1.6 (0.5 - 4.2)	0.48 ± 0.07 (0.39 - 0.65)	Tr	ND	ND
Body trim[d]	4	63.3 ± 5.7 (59.8 - 71.9)	17.2 ± 4.8 (10.3 - 21.1)	1.62 ± 0.63 (0.83 - 2.20)	0.11 ± 0.01 (0.10 - 0.13)	ND	ND
Emu (*Dromaius novaehollandiae)*							
Meat (fan fillet, flat fillet, mince[‡])	6	74.1 ± 1.2 (72.2 - 75.7)	1.7 ± 1.0 (0.5 - 3.2)	0.88 ± 0.18 (0.70 – 1.11)	Tr	0.13 ± 0.03 (Tr – 0.16)	ND
Heart	4	69.7 ± 1.2 (68.7 - 71.3)	13.0 ± 2.8 (9.5 - 16.4)	4.05 ± 0.71 (3.35 - 5.00)	0.14 ± 0.05 (0.10 - 0.20)	0.16 ± 0.07 (0.10 - 0.24)	ND
Eggs	8	69.2	16.0	2.95	0.20	0.20	ND
Oil	6	0.3	100.0	14.50	0.15	1.30	ND
Kangaroo (*Macropus giganteus* and *Macropus rufus*)							
Meat (fillet, rump, mince[c], striploin, tail butt, tail)	8	75.5 ± 1.6 (73.1 - 78.1)	0.4 ± 0.6 (0.0 - 1.9)	ND	ND	Tr	Tr

25(OH)D_3, 25-hydroxyvitamin D_3; 25(OH)D_2, 25-hydroxyvitamin D_2; ND, not detected (<LOD); Tr, trace, >LOD<LOQ

[a]Concentrations ≥ LOQ are presented as mean ± SD (range). Exceptions are emu eggs and emu oil for which composite samples were analysed and the values presented are the mean of duplicate analyses.

[b]Limit of quantitation (LOQ) = 0.10 µg/100g, except camel hump and emu oil for which the LOQ = 0.25 µg/100g; Limit of detection (LOD) = 0.01-0.06 µg/100g

[c]Mince/diced are a mixture of various cuts

[d]Body trim is the subcutaneous outer layer of flesh

25(OH)D_3 was greater for camel hump compared to other camel meat cuts; however, when calculated per 100 g fat, the concentration of this vitamer was more than 25 times greater in meat cuts compared to hump (Table 3). Vitamin D_2 was not detected in any camel samples, but some did contain trace amounts of 25(OH)D_2.

The mean concentration of vitamin D_3 was three times greater in crocodile body trim than in lower-fat crocodile cuts, but was not greatly different when calculated by 100 g fat. 25(OH)D_3 was detected in trace amounts in lower-fat crocodile cuts and found in concentrations close to the LOQ in body trim samples. Neither vitamin D_2 nor 25(OH)D_2 was detected in crocodile samples.

All emu samples contained vitamin D_3, 25(OH)D_3 and vitamin D_2. For all three vitamers, concentrations were greatest in emu oil and lowest in emu meat cuts; for vitamin D_3 and vitamin D_2, this was reversed when calculated by 100 g fat.

Neither vitamin D_3 nor 25(OH)D_3 was detected in kangaroo samples. Vitamin D_2 and 25(OH)D_2 were detected in trace amounts in some kangaroo samples.

Discussion

At least one of the four main D vitamers found in food was detected in each game animal product. Our results provide valuable insight into foods with little to no previously published data on vitamin D content. Some of these foods are potentially useful dietary sources of vitamin D, particularly for Aboriginal people living in remote areas.

The vitamin D profile of camel products was unique among products included in this study and our previous studies of Australian foods. Meat and hump samples contained the greatest concentrations of 25(OH)D_3 of all foods included in our vitamin D studies to date [3, 98]; however, vitamin D_3 was not detected in camel meat and was found in only one camel hump sample. Where 25(OH)D_3 and 25(OH)D_2 are detected in meat, they are often accompanied by their respective non-hydroxylated forms, vitamin D_3 and vitamin D_2 [3, 136, 225, 226]. The absence of vitamin D_3 in camel meat samples may reflect the adaptation of this animal for survival under harsh and arid conditions [227], whereby storing 25(OH)D_3 rather than vitamin D_3 provides a quicker or localised metabolic path to the active form, 1,25-dihydroxyvitamin D_3 [8].

Table 3. Vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_2, and 25-hydroxyvitamin D_2 per 100 g fat in raw Australian game meats[a]

Sample	Fat (g/100 g ± SD)	Vitamin D_3 (µg/100 g fat)	25(OH)D_3 (µg/100 g fat)	Vitamin D_2 (µg/100 g fat)	25(OH)D_2 (µg/100 g fat)
Camel (*Camelus dromedarius)*					
Meat (fillet, mince[b], diced[b], eye fillet, rump, knuckle)	1.2	NA	73.5	NA	2.1
Hump	79.8	NA	2.7	NA	NA
Crocodile (*Crocodylus porosus)*					
Lower-fat cuts (tail fillet, mince[†], striploin, sweet cut (cheek))	2.3	21.0	NA	NA	NA
Body trim[c]	8.9	18.2	1.2	NA	NA
Emu (*Dromaius novaehollandiae)*					
Meat (fan fillet, flat fillet, mince[b])	1.7	52.9	NA	7.8	NA
Heart	13.0	31.1	1.1	1.2	NA
Eggs	16.0	18.4	1.2	1.2	NA
Oil	100.0	14.5	0.2	1.3	NA
Kangaroo (*Macropus giganteus* and *Macropus rufus*) Meat (fillet, rump, mince[b], striploin, tail butt, tail)	0.4	NA	NA	NA	NA

25(OH)D_3, 25-hydroxyvitamin D_3; 25(OH)D_2, 25-hydroxyvitamin D_2; ND, not detected; NA, not applicable: the vitamer was not detected in the sample or the amount detected was too low to be quantitated (limit of detection = 0.01-0.06 µg/100g)

[a]Values of D-vitamer per 100 g fat calculated as ((D vitamer per 100 g sample /fat per 100 g sample) x 100)

[b]mince/diced are a mixture of various cuts

[c]Body trim is the subcutaneous outer layer of flesh

We found the greatest concentration of 25(OH)D_3 in hump flesh; however, when expressed per 100 g fat, the concentration of 25(OH)D_3 was much lower in hump flesh compared to camel meat, which has a low mean fat content compared to other commonly-consumed meats. Jakobsen and Saxholt [137] have proposed the greater polarity of 25(OH)D_3 as an explanation for its presence in fat-free food matrices, while muscle has been demonstrated to act as a storage site for 25(OH)D [228], and may have greater uptake potential of 25(OH)D than fat cells [229]. The 25(OH)D_3 content of camel meat has previously been measured by radioimmunoassay in Moroccan dromedary camel (*Camelus dromedarius*), with 0.42 μg/100 g found in muscle meat [230]. The vitamin D_3 content was not reported in that study and, to our knowledge, there are no other data on the vitamin D profile of camel meat with which to compare our findings. Further research into meat from *Camelus dromedaries* and other camel species from other geographical locations is needed to confirm whether this 25(OH)D_3-dominated vitamin D profile is usual in these species.

Crocodile meat contained vitamin D_3 and 25(OH)D_3, similarly to Australian chicken and pork [3]. We found both vitamers in greater concentrations in the higher-fat body trim than in other lower-fat cuts. The vitamin D_3 (0.6-10.9 μg/100 g) and 25(OH)D_3 (0.1-0.3 μg/100 g) content of two samples of crocodile egg yolk has been measured previously; however, we found no other studies on the vitamin D content of crocodile meat products. Crocodiles are not expected to purposefully ingest fungi or other matter that might provide vitamin D_2; chicken heads and kangaroo meat are commonly used as feed for farmed crocodiles in Australia [231]. Therefore, the absence of vitamin D_2 and 25(OH)D_2 in the meat of crocodiles may be due to their diet.

We found vitamin D_3, 25(OH)D_3 and vitamin D_2 in all emu product samples included in this study. Animals do not synthesise vitamin D_2; rather it is produced in fungi. It is thought that UVB-exposure of fungal-contaminated plant-based animal feed may explain the presence of vitamin D_2 and 25(OH)D_2 in animal products [90, 136]. Emus are farmed outdoors and are opportunistic omnivores. In addition to feed provided, they may consume a wide range of plant matter, insects and small animals. Vitamin D_2 and 25(OH)D_2 have been found in ruminant animal meats (beef and lamb) commonly consumed in Australia [3]. It is unknown whether these vitamers are

obtained from fungal-contaminated feed provided to farmed animals or from other food sources accessible in the local environment.

Our findings for emu meat contradict those of a previous Canadian study that found no detectable vitamin D_3 in emu thigh and leg meat [232]. However, that study used an older high performance liquid chromatography method that lacked the sensitivity (LOD 2.1 μg/100 g) to detect the concentrations of vitamin D_3 found in our emu meat samples (up to 1.1 μg/100 g) and did not examine other D vitamers. To our knowledge, no other studies have investigated the vitamin D content of emu products.

We found that emu eggs contained more than twice the mean concentration of vitamin D_3 found in chicken eggs [3, 211], and the vitamer profiles differ due to the presence of vitamin D_2 in emu eggs. Emu oil had the greatest concentrations of vitamin D_3 and vitamin D_2 of all products included in this study. Emu oil is applied topically for cosmetic purposes due to its moisturising and purported anti-inflammatory properties and to promote hair and skin growth [233]. It may also be ingested, and is thought to lower cholesterol and assist in treatment of internal inflammatory conditions such as inflammatory bowel syndrome and mucositis [233]. Despite being a richer source of vitamin D by concentration than other emu products, it is likely to be consumed in much lower amounts (commonly available capsules contain 750-1000 mg emu oil), and may, therefore provide a similar amount of vitamin D per usual dose/serving.

In contrast to other animal products, kangaroo meat is interesting due to its relative lack of vitamin D. Amongst a variety of meat from mammalian, avian, aquatic vertebrate and reptilian species previously sourced in Australia and analysed for vitamin D content [3, 211], this marsupial animal's meat is unusual for the absence or scarcity of all four D vitamers measured. Neither vitamin D_3 nor 25(OH)D_3 were detected in any kangaroo meat samples despite low LODs ranging from 0.01-0.05 μg/100 g. These results are consistent with data from our previous study [3] that included a composite of six kangaroo steak samples purchased from supermarkets in Melbourne. Kangaroos are herbivores and are not farmed for meat in Australia; meat available for purchase is from wild-caught animals. Serum 25(OH)D concentrations in healthy, free-ranging koalas with no evidence of metabolic bone disease have

previously been reported as being much lower than non-marsupials, prompting speculation that the koala's vitamin D requirements may be relatively low [234]. Similarly, low serum 25(OH)D concentrations have been recorded in other marsupials, specifically wombats and brushtail possums [235]. It does not yet appear to be known why the D vitamer profile of kangaroo meat differs to other animal types, particularly to those that are also herbivores, or whether the D vitamer profile observed in kangaroo meat is specific only to macropods or to marsupials in general.

This study has shown that some Australian game products, that may be hunted and foraged for in remote areas, offer nutritionally important amounts of vitamin D. Availability of foods in remote communities has considerable influence on nutritional intake [236]. Nutritious foods are often in limited and unreliable supply, relatively expensive [237-239] and are frequently passed over in favour of more energy-dense foods with a lower cost per unit of energy Brimblecombe & O'Dea, [240]. Prior to European settlement, traditional diets based on bush tucker would likely have provided a reasonable supply of vitamin D as they included offal and fish [218], both of which are good sources of vitamin D. There is generally less reliance on bush tucker now than in the past, with colonisation marking the most pronounced decline. Despite this, bush tucker contributes up to 50% of food intake for some remote-dwelling people [217]. Our findings have identified some Australian game products as useful sources of vitamin D. One serving (120 g) of emu egg would provide approximately half of the Estimated Average Requirement (EAR) of 10 μg/day [51]. Assuming a bioactivity factor of five for 25(OH)D_3, one serving of camel meat (100 g raw weight) would provide approximately 5 μg total vitamin D. Kangaroo, while being nutritious in other ways and commonly hunted [217], does not contain nutritionally useful amounts of vitamin D. As well as contributing to vitamin D intake, game products are a good source of protein and are often lower in total fat compared to cheaper commercial meats [241]. Encouraging the consumption of bush tucker may be nutritionally and culturally beneficial [242].

A major strength of our study was the use of a highly sensitive and specific LC-QQQ method to measure the four main dietary D vitamers in camel, crocodile, emu and kangaroo products. Given the complexity of measuring D vitamers in food, few international food composition databases include all four vitamers. Due to logistical barriers to collection of samples from remote areas, samples were sourced from

commercial suppliers, and included products from both farmed and wild animals. It was not possible to investigate the effect of the animals' diets on the vitamin D content and profile of their products. Future studies could include wider sampling of a greater range of products, and could examine factors that may affect vitamin D content (e.g., seasonal and geographical variation [225], animals' diets and the effect of cooking).

Conclusions

We investigated the four major D vitamers in Australian camel, crocodile, kangaroo and emu products, revealing interesting variations in profiles and concentrations of D between species. Identifying good sources of vitamin D provides important evidence for developing dietary strategies to promote vitamin D sufficiency in Aboriginal and Torres Strait Islander people living in remote areas.

Authors' contributions*:*

Eleanor Dunlop: Project administration, Data curation, Writing – original draft. **Carrington CJ Shepherd:** Writing – review & editing. **Judy Cunningham:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Norbert Strobel:** Formal analysis, Methodology, Writing – review & editing. **Robyn M Lucas:** Writing – review & editing. **Lucinda J Black:** Conceptualization, Funding acquisition, Data curation, Methodology, Supervision, Writing – review & editing

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Supplementary Table 1. Moisture, fat, vitamin D_3, 25-hydroxyvitamin D_3, vitamin D_2, and 25-hydroxyvitamin D_2 in individually analysed samples of raw Australian game meats

	Moisture (g/100 g)	Fat (g/100 g)	Vitamin D_3[a] (µg/100 g)	25(OH)D_3[a] (µg/100 g)	Vitamin D_2[a] (µg/100 g)	25(OH)D_2[a] (µg/100 g)
Camel diced[b]	77.2	0.8	ND	2.46	ND	Tr
Camel fillet	77.3	1.2	ND	0.68	ND	Tr
Camel mince[b]	75.3	1.8	ND	1.21	ND	Tr
Camel eye fillet	75.0	1.7	ND	0.85	ND	ND
Camel rump	74.8	2.6	ND	0.40	ND	ND
Camel knuckle	76.6	0.6	ND	0.80	ND	ND
Camel hump 1	5.7	90.2	ND	1.00	ND	ND
Camel hump 2	13.6	79.0	ND	1.40	ND	ND
Camel hump 3	8.1	76.6	ND	0.95	ND	ND
Camel hump 4	17.8	73.4	Tr	5.17	ND	Tr
Crocodile mince[b]	76.8	2.9	0.48	Tr	ND	ND
Crocodile strip loin	77.6	0.5	0.41	Tr	ND	ND
Crocodile sweet cut	76.8	4.2	0.65	Tr	ND	ND
Crocodile tail fillet	77.8	1.7	0.39	Tr	ND	ND
Crocodile body trim 1[c]	71.9	10.3	0.83	0.13	ND	ND
Crocodile body trim 2[c]	59.8	21.1	2.20	0.10	ND	ND
Crocodile body trim 3[c]	61.7	17.5	1.40	0.10	ND	ND
Crocodile body trim 4[c]	60.1	19.8	2.05	0.10	ND	ND
Emu fan fillet 1	75.7	0.9	0.80	Tr	0.15	ND
Emu fan fillet 2	73.7	1.2	1.10	ND	0.10	ND
Emu fan fillet 3	74.0	0.5	0.70	ND	0.15	ND
Emu flat fillet 1	72.2	3.2	0.80	ND	0.15	ND
Emu flat fillet 2	75.0	1.8	1.11	Tr	0.16	ND
Emu mince[b]	74.1	2.5	0.78	Tr	Tr	ND
Emu heart 1	69.9	13.4	3.73	0.16	0.24	ND
Emu heart 2	71.3	9.5	4.10	0.10	0.10	ND
Emu heart 3	69.0	12.9	3.35	0.10	0.10	ND
Emu heart 4	68.7	16.4	5.00	0.20	0.20	ND
Kangaroo fillet 1	75.2	0.0	ND	ND	ND	Tr
Kangaroo fillet 2	75.1	0.1	ND	ND	ND	ND
Kangaroo mince[b]	75.4	1.9	ND	ND	Tr	Tr
Kangaroo rump	77.3	0.0	ND	ND	ND	Tr
Kangaroo strip loin	74.7	0.1	ND	ND	ND	ND
Kangaroo tail butt	75.1	0.2	ND	ND	ND	ND
Kangaroo tail 1	78.1	0.9	ND	ND	0.16	0.11
Kangaroo tail 2	73.1	0.2	ND	ND	ND	ND

25(OH)D_3, 25-hydroxyvitamin D_3; 25(OH)D_2, 25-hydroxyvitamin D_2; ND, not detected (<LOD);
Tr, trace, >LOD<LOQ
[a]Limit of quantitation (LOQ) = 0.10 µg/100g, except camel hump and emu oil for which the LOQ = 0.25 µg/100g; Limit of detection (LOD) = 0.01-0.06 µg/100g
[b]Mince/diced are a mixture of various cuts
[c]Body trim is the subcutaneous outer layer of flesh

Chapter 4: Estimating usual vitamin D intakes in Australia

Thesis objectives addressed in this chapter:

Objective 3: To identify the major food sources of vitamin D and generate the first estimates of usual vitamin D intakes in Australian population.

The content of this chapter is covered by Publication 3:

Publication 3: Evidence of low vitamin D intakes in the Australian population points to a need for data-driven nutrition policy for improving population vitamin D status.

This is a pre-copyedited, author-produced version of an article accepted for publication in the Journal of Human Nutrition and Dietetics following peer review. The copyediting process may lead to differences between the version presented in this thesis and the Version of Record. The Version of Record Dunlop E, JL Boorman, TL Hambridge, J McNeill, AP James, M Kiely, CA Nowson, A Rangan, J Cunningham, P Adorno, P Atyeo, LJ Black. 2022. Evidence of low vitamin D intakes in the Australian population points to a need for data-driven nutrition policy for improving population vitamin D status. *Journal of Human Nutrition and Dietetics*: in press. doi: 10.1111/jhn.13002 will be made available online at: https://onlinelibrary.wiley.com/doi/abs/10.1111/jhn.13002.

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Abstract

Nearly one in four Australian adults are vitamin D deficient (serum 25-hydroxyvitamin D concentrations (25(OH)D) < 50 nmol/L) and current vitamin D intakes in the Australian population are unknown. Internationally, vitamin D intakes are commonly below recommendations, although estimates generally rely on food composition data that do not include 25(OH)D. We aimed to estimate usual vitamin D intakes in the Australian population. Nationally-representative food consumption data were collected for Australians aged ≥ 2 years (*n* = 12,153) as part of the cross-sectional 2011-2013 Australian Health Survey (AHS). New analytical vitamin D food composition data for vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 were mapped to foods and beverages that were commonly consumed by AHS participants. Usual vitamin D intakes (μg/day) by sex and age group were estimated using the National Cancer Institute method. Assuming a 25(OH)D bioactivity factor of one, mean daily intakes of vitamin D ranged between 1.84 and 3.25 μg/day. Compared to the Estimated Average Requirement (EAR) of 10 μg/day recommended by the Institute of Medicine, more than 95% of people had inadequate vitamin D intakes. We estimated that no participant exceeded the Institute of Medicine's Upper Level of Intake (63-100 μg/day, depending on age group). Usual vitamin D intakes in Australia are low. This evidence, paired with the high prevalence of vitamin D deficiency in Australia, suggests that data-driven nutrition policy is required to safely increase dietary intakes of vitamin D and improve vitamin D status at the population level.

Introduction

Vitamin D deficiency (serum 25-hydroxyvitamin D (25(OH)D) concentrations < 50 nmol/L [59]) affects many Australians (20% of adults aged ≥ 25 years [65], 32% of young adults aged 18-24 years, and 17% adolescents aged 12-17 years [64]). To date, there has not been an assessment of usual vitamin D dietary intakes in the Australian population using comprehensive vitamin D food composition data and nationally-representative food consumption data. Elsewhere, vitamin D intakes fall short of recommendations. In the US [60], Canada [145] and many European countries [38, 144, 243], estimated mean intakes of vitamin D are ≤ 5 μg/day, which is considerably lower than the Estimated Average Requirement (EAR) of 10 μg/day recommended by the

Institute of Medicine [51]. Those estimates, however, do not appear to have accounted for the contribution of all D vitamers that may be present in food [146-148, 185, 244-250], particularly 25-hydroxyvitamin D (25(OH)D) which is present in some foods, and may be more biologically active than vitamin D itself [125].

Previous estimates of Australian vitamin D intakes were low (2-3 μg/day) [139, 154], but were based on very limited vitamin D food composition data and/or used data produced using outdated analytical methods. The high prevalence of vitamin D deficiency reported recently [59, 64, 65] suggests that intakes are too low to compensate for inadequate safe sun exposure. This is because naturally rich food sources of vitamin D are uncommon and few food products are fortified with vitamin D in Australia. Fortification has been suggested as a potential solution to low vitamin D status [38, 39, 88, 144]. In the Finnish population, vitamin D intakes from food alone were approximately doubled following addition of vitamin D to fluid milk products and fat spreads, and the prevalence of people with serum 25(OH)D concentrations < 50 nmol/L decreased from 56% in 2000 to 9% in 2011 [162]. However, dietary strategies to improve vitamin D status in the Australian population cannot be modelled without an accurate estimate of usual baseline intakes.

The 2011-12 National Nutrition and Physical Activity Survey (NNPAS) provides the most comprehensive and nationally-representative food and dietary supplement consumption data in Australia to date. These food consumption data and the serum 25(OH)D concentrations used to estimate the prevalence of vitamin D deficiency were collected during the same period; therefore, it is relevant to consider them together. However, vitamin D intakes were not estimated due to a lack of locally-relevant vitamin D food composition data [251]. Recently, Australia's first comprehensive analytical vitamin D food composition database was produced [3] using liquid chromatography with triple quadrupole mass spectrometry, a highly sensitive and specific method for measurement of D vitamers. Hence, we aimed to provide the first estimates of usual vitamin D intakes in a nationally-representative sample of the Australian population, and to identify the major food sources of vitamin D, based on new comprehensive vitamin D food composition data.

Methods

We used nationally-representative food (including beverages) and dietary supplement consumption data and new analytical vitamin D food composition data to estimate vitamin D intakes in the Australian population using either the National Cancer Institute (NCI) method (for usual intakes of food; Figure 1) or a determinisitic method (including dietary supplements).

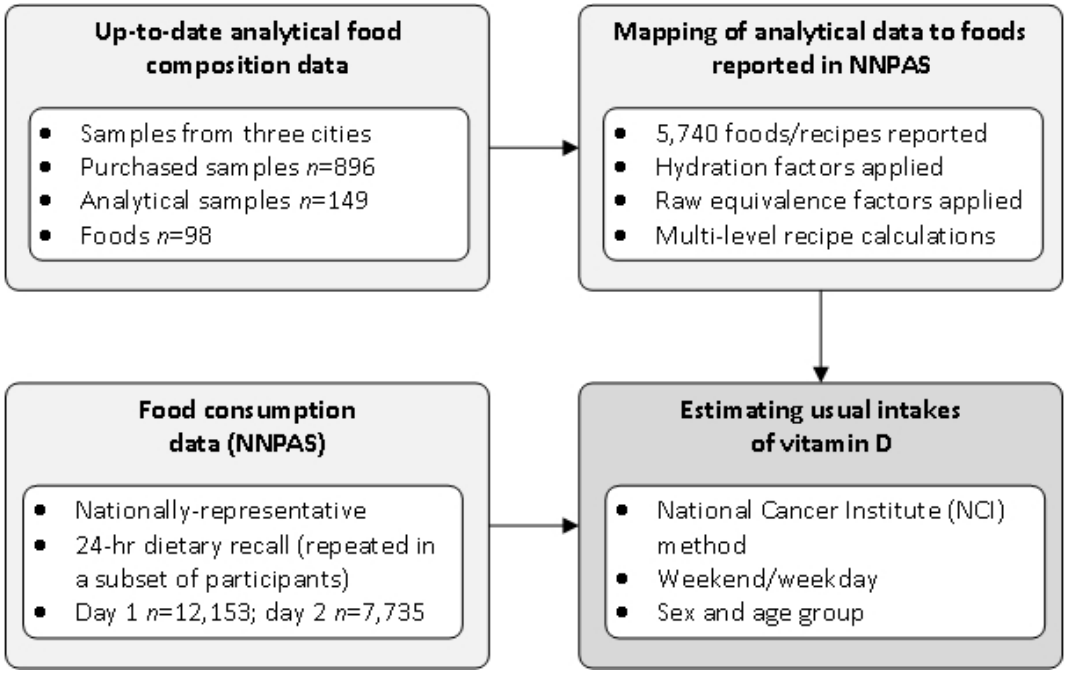

Figure 1. Methods for estimating usual vitamin D intakes in the Australian population
NNPAS, 2011-2012 National Nutrition and Physical Activity Survey

Study population

The Australian Health Survey 2011-2013 (AHS) provided the most recent nationally representative health-related data for the Australian population [62]. Usual residents of metropolitan and rural private dwellings were eligible. An area-based sampling approach was adopted to ensure geographically representative sampling. Approximately 35,000 households were selected with the aim of achieving ~26,000 fully-responding households, allowing for non-response and sample attrition (Supplementary Figure 1). Core demographic, household and other general information (published previously [252]) were collected for one adult or one adult plus one child from 25,080 households. Participants were then allocated to either the

National Health Survey (*n* = 20,426), which focused on health status and conditions, or the NNPAS (*n* = 12,153), which included a food consumption component.

Food consumption data

Food and dietary supplement consumption data were collected for Australians aged ≥ 2 years by trained Australian Bureau of Statistics (ABS) interviewers. Food consumption collection methods have been described in detail by the ABS [62]. In summary, the USDA Dietary Intake Data System [253] was used to collect and code food consumption data. This digital system comprises the Automated Multiple-Pass Method [254] for 24-hour dietary recall, the Post Interview Processing System (PIPS) and Survey Net. The Automated Multiple-Pass Method was modified by the ABS in collaboration with Food Standards Australia New Zealand (FSANZ) to represent foods consumed in Australia. Participants were invited to complete two 24-hour dietary recalls: the first was conducted during an in-person interview (*n* = 12,153) and the second by telephone call (completed by 64% of participants, *n* = 7,735). Where possible, the second interview was scheduled at least eight days after the first and on a different day of the week. These interviews were conducted under the *Census and Statistics Act 1905*. A responsible adult responded for all children aged < 15 years and also for children aged 15-17 years where permission for self-response was denied by a parent or guardian. All data were recorded electronically during interviews. As respondents identified foods and dietary supplements that were consumed, questions specific to the type of these were prompted by the adapted Automated Multiple-Pass Method program in order to determine details about the food and its preparation. The AHS Food Model Booklet [255] aided estimation of the amounts of foods consumed. Interview data were prepared and partially coded in PIPS. Final coding and calculation of the gram weight of consumed items were carried out in Survey Net, which incorporated a food measures database compiled by FSANZ for the AHS [251]. The coded data were imported into Harvest, FSANZ's custom-built dietary modelling software [256].

Vitamin D food composition data

Analytical vitamin D composition data were obtained as previously described [3]. In brief, a sampling plan was developed to include food products that were reported in

the NNPAS as being commonly consumed (as per past nutrition surveys and knowledge of current market availability) or that were expected to contain vitamin D. Between August 2018 and June 2019, 896 primary food samples of 98 different food products were purchased in three cities representing both sides of the continent and where approximately half of Australia's population resides: Sydney (August 2018, 186 samples), Melbourne (October-December 2018, 516 samples) and Perth (April-June 2019, 194 samples). Products were purchased in one, two or three cities depending on the likelihood of high vitamin D concentration in the product, frequency of consumption and whether they are produced and used regionally or distributed nationally from one source. Primary samples were composited into 149 analytical samples each comprising six primary samples per food type per city, with the exception of dark chocolate, for which eight primary samples were combined. Vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2 were analysed in duplicate using a liquid chromatography with triple quadrupole mass spectrometry method at the National Measurement Institute of Australia, a National Association of Testing Authorities-accredited laboratory for measurement of vitamin D in food. A detailed description of the analytical method has been published previously [3].

Calculating vitamin D equivalents from analytical food composition data

Vitamin D equivalents (VDE) were calculated by summing concentrations of the four D vitamers measured, assuming equal bioactivity. Currently, there is no consensus on a bioactivity factor for 25(OH)D [125]. Where it is included in national food composition databases, a bioactivity factor of either one or five is used. Hence, we estimated intakes using both bioactivity factors in order to allow comparison with other studies. Trace values, where the concentration of a nutrient is detected below the limit of reporting (LOR) and cannot be quantitated with certainty, present a risk of under-estimation (if trace values are assigned a zero value) or over-estimation (if trace values are assigned the LOR value) of nutrient intakes. Trace values were, therefore, assigned a value of LOR/2 (LOR = 0.1 μg/100 g for all foods except those with a high fat content, for which the LOR was 0.25 μg/100 g).

Mapping analytical data to consumed foods

Australia's nutrition survey food composition database, AUSNUT [251], is used to estimate usual nutrient intakes based on food consumption data from the NNPAS. As vitamin D was not included in AUSNUT 2011-2013, we mapped our new analytical vitamin D data to AUSNUT food entries.

The process of mapping analytical concentrations to consumed foods was conducted using the same method as for Australian total diet studies [257, 258]. Mapping can consist of direct mapping of concentrations to foods, direct mapping with factors applied, or assigning a recipe. The 5,740 food entries in AUSNUT can be divided into two types: non-recipe foods (individual, staple-type foods, such as rice) and recipe foods (foods that contain more than one non-recipe food, such as fried rice). We manually assigned analytical D vitamer concentrations to all non-recipe foods, with the exception of oral nutritional supplements and meal-replacement products designed for weight loss (n=21), for which label data were used. Methods of data derivation were recorded together with details, such as label data source, where appropriate. The direct mapping with factors method allows the mapping of a food's analytical concentration to other relevant foods with an additional adjustment factor applied to account for food manufacturing or preparation practices such as dehydration or cooking in water [257]. For example, a conversion factor would be applied to the analytical value for powdered infant formula to derive a value for ready-to-drink infant formula [257]. Similarly, a conversion factor would be applied to the analytical value for apple to derive a value for apple juice [257].

Concentration values were derived for recipe foods using Harvest [256]. Harvest allows for multiple levels of nested recipes, i.e. a recipe within a recipe within a recipe. For example, a recipe for 'filled pasta with cheese sauce' contains 'filled pasta' and 'cheese sauce' as separate ingredients. These ingredients are in turn made from recipes. Harvest determines a nutrient concentration for the mixed food based on the nutrient concentrations for ingredient foods and the proportion of an ingredient within a recipe.

Estimating vitamin D concentrations for raw versions of analysed cooked foods

In order to sample as diverse a range of foods as possible with the available funds, we prioritised analysis of foods in the form that they are consumed, i.e. cooked meats, fish and seafood rather than raw; however, some recipe foods include raw versions of these foods as ingredients and, therefore, values for the raw food were estimated. Conversion factors, as explained in the previous section, were applied to cooked meat and seafood concentrations to derive values for raw versions to use in these recipes; however, this did not include retention factors as retention factors were not listed for vitamin D in the AUSNUT 2011-2013 data files [251]. Retention factors for different foods and cooking methods were published in 2002 [259]; however, there is limited up-to-date data on the retention of vitamin D in foods. A recent study examined retention factors for vitamin D in farmed Danish rainbow trout using eight different cooking methods and temperatures, finding that true retention of vitamin D ranged between 85 ± 6% and 114 ± 13% [260]. Hence, it is possible that the use of retention factors may introduce error rather than reduce it. In our study, omitting retention factors should have no major effect on intake estimates as the vitamin D concentration values used were derived from levels of vitamin D in foods as consumed.

Estimating intakes

Usual intakes of vitamin D were estimated using the NCI Method [261]. Implementation of the method was consistent with the approach taken by the ABS and FSANZ in estimating usual nutrient intakes for the NNPAS. Further information about this approach is available elsewhere [62].

In order to apply the NCI method, at least two dietary intakes for a subset of survey respondents are required. Using the NNPAS 24-hour dietary recall data and our new vitamin D food composition data, vitamin D intakes for each respondent for either day one only, or for the two survey days (64% of respondents), were calculated using Harvest, which is the custom-built dietary modelling program used by FSANZ [256]. These Harvest-generated intake data were then used as the input for the NCI method. Rather than using NCI macros for SAS® software, the NCI model was run in the statistical programming software R (version 3.0.3) [262]. FSANZ previously translated the SAS® macros into R code. At the time of translation, FSANZ undertook testing

to validate the R code. Outputs from R were compared and found to be consistent with those from SAS software (unpublished report).

A summary of the specific NCI model set-up is as follows. The amount-only model type was used to estimate usual intakes, as nearly all respondents had a non-zero intake for vitamin D on day one of the NNPAS. The covariates used in the model were sex, age, weekend vs weekday and sequence effect (which considers the potential reporting differences between day one and day two of the nutrition survey). The default of 100 simulations for each respondent was used in the Monte Carlo simulation component of the model. The model was run separately for three population groups: children ≤ 8 years, males ≥ 9 years and females 9 years and over. This ensured that the model fitting was done more specifically using respondents with similar food consumption patterns. Usual vitamin D intakes were then extracted and reported in µg/day by the age/sex groups used in the Nutrient Reference Values (NRVs) for Australia and New Zealand [76].

Estimating adequacy of intakes

Nutrient Reference Values for Australia and New Zealand consist of a recommended Adequate Intake (AI) and Upper Level of Intake (UL) for vitamin D [76]. As the AI is unsuitable for assessment of adequacy of intakes in the population [77], intakes were compared to the US/Canadian EAR of 10 µg/day recommended by the Institute of Medicine [51]. The Australian UL is 80 µg/day for all people aged ≥ 1 years [76], while the Institute of Medicine recommends a UL of 63, 75 and 100 µg/day for those aged 1-3, 4-8 and ≥ 9 years, respectively [51].

Determining percentage contribution of foods to vitamin D intakes

Percentage contributions of foods to vitamin D intakes were derived using Harvest [256] and day one food consumption data. In AUSNUT, foods are organised under food group codes that become more specific as code digits increase. For example, the 2-digit code '13' represents the broad group of cereal based products and dishes. The 3-digit level of this broad group includes subgroups such as code 131: cakes, muffins, scones, cake-type desserts, which in turn expands to a 5-digit level (e.g., code 13301: cakes and cake mixes, chocolate). Percentage contributions of 2- 3- and 5-digit code level food groups were estimated for both 25(OH)D bioactivity factor

scenarios and by NRV age/sex groups [76] as follows: (total vitamin D intake from a food group for all participants/total vitamin D intake from all foods) x 100 [263, 264].

Rounding

The dietary intakes and food contributor estimates are intended to represent habitual vitamin D intakes, which may vary with food and ingredient choice [265]. Preliminary rounding would have rendered some small values to zero, which may not reflect actual intakes over time. For example, some recipes (e.g., a mixed dish such as curry) include an 'undefined fat' ingredient. This 'undefined fat' value is an average of concentrations assigned to the various fats that may be used, e.g., oil, butter, ghee or margarine. As a minor ingredient in a mixed dish, the 'undefined fat' concentration may be close to zero, but cannot be assumed as always zero. Therefore, all values remained unrounded until all data generation steps were complete in order that small concentrations, which may cumulatively contribute to intakes, were accounted for.

Exploring the contribution of dietary supplements to vitamin D intakes

The intake of vitamin D from dietary supplements was not included in our estimates of usual intakes as a limitation of the NCI method is that it cannot make estimations from multimodal distributions [62, 266]. However, we used the NNPAS day one food and vitamin D-containing supplement consumption data to estimate absolute intakes of vitamin D from food and dietary supplements on a single survey day. This was done deterministically using the individual respondent data from the survey unit record file data, via Stata Statistical Software version 15 [267] rather than FSANZ's Harvest program. As previously described [75], the vitamin D composition of dietary supplements reported as consumed was determined using the Australian Register of Therapeutic Goods [268] where possible; otherwise, composition data were obtained directly from manufacturers via website, telephone or email. The vitamin D contents of all dietary supplements reported as consumed were added to absolute daily intakes from food. Dietary supplements that contained vitamin D included single vitamin D supplements, vitamin D-containing multi-nutrient preparations, fish liver oils with naturally-occurring and/or added vitamin D, and fish oils with added vitamin D. These absolute intakes estimates were not compared to an EAR or UL as estimates of

intake from a single day are not suitable for assessment of nutrient adequacy at the population level [77], and may result in overestimation of the prevalence of intakes below the EAR and above the UL [185].

2. Results

Usual intakes of vitamin D

In the population aged 2 years and above, the mean daily usual intake of vitamin D ranged between 1.84 and 3.25 µg/day across the age/sex groups when assuming a 25(OH)D bioactivity factor of one (Table 1). This increased to between 3.48 and 6.09 µg/day when assuming a 25(OH)D bioactivity factor of five. Children aged 2-3 years had the lowest usual vitamin D intakes and mean intakes were lower in females than males across the age groups assessed. We estimated that, across all sex and age groups, more than 90% of people had vitamin D intakes that were below their respective Australian AI (5-15 µg/day, depending on age group) when using a bioactivity factor of 1, and over 30% were under their respective AIs when using a bioactivity factor or 5. More than 95% of the Australian population had inadequate intakes compared to the Institute of Medicine's EAR of 10 µg/day [51] for both scenarios. It was estimated that none of the population had usual intakes above the Australian Upper Level of Intake of 80 µg/day [76] or the Institute of Medicine's UL of 63-100 µg/day [51] for people aged ≥ 1 years for either scenario.

Major contributors to vitamin D dietary intakes

Including all participants aged ≥ 2 years and assuming a 25(OH)D bioactivity factor of one, the greatest contributors to vitamin D intakes were 'Fish and seafood products and dishes' (18.3% (range, 4.6 – 29.4%)). Of these foods, 'Packed fin fish' was the main contributor (7.3% (range, < 1 – 14.6%)). 'Packed fin fish' largely represents canned products and includes popular products such as canned tuna as well as canned salmon, which had the highest analysed concentration of vitamin D [3]. 'Margarine and table spreads' also contributed more than 10% of intake (11.5% (range, 6.3 – 19.7%)) (Table 2). When a 25(OH)D bioactivity of five was used, the greatest contributors in the same respective order of code levels were 'Meat, poultry and game products and dishes' (26.3% (17.8 – 30.6%)), 'Eggs' (8.4% (4.4 – 11.2%)) and 'Chicken eggs' (8.3% (4.4 – 11.2%)). Greatest contributors varied by sex and

Table 1. Usual vitamin D intakes in the Australian population based on food consumption data from the 2011-2012 National Nutrition and Physical Activity Survey for ages ≥2 years, stratified by sex[1]

			25(OH)D bioactivity factor = 1						25(OH)D bioactivity factor = 5					
				Percentile						Percentile				
Age group, y	Sex	*n* [2]	Mean	5th	25th	50th	75th	95th	Mean	5th	25th	50th	75th	95th
			(μg/day)											
2-3	Males	165	2.08	0.92	1.42	1.91	2.55	3.82	3.79	2.08	2.91	3.64	4.51	6.08
2-3	Females	152	1.84	0.81	1.25	1.68	2.26	3.43	3.48	1.89	2.64	3.31	4.14	5.63
4-8	Males	401	2.42	1.07	1.65	2.23	2.96	4.45	4.07	2.23	3.11	3.90	4.83	6.52
4-8	Females	374	2.18	0.94	1.48	1.99	2.68	4.01	3.77	2.03	2.88	3.60	4.50	6.06
9-13	Males	435	3.11	1.39	2.18	2.92	3.83	5.50	5.46	2.68	4.03	5.23	6.63	9.06
9-13	Females	426	2.86	1.20	1.92	2.63	3.54	5.32	4.89	2.37	3.55	4.64	5.93	8.29
14-18	Males	373	3.25	1.50	2.31	3.05	3.99	5.72	6.09	3.12	4.58	5.83	7.34	9.95
14-18	Females	367	2.44	0.99	1.63	2.24	3.03	4.57	4.35	2.05	3.14	4.12	5.30	7.44
19-30	Males	1,116	3.09	1.39	2.18	2.88	3.80	5.47	5.93	2.98	4.44	5.66	7.16	9.74
19-30	Females	1,072	2.70	1.11	1.81	2.48	3.35	5.04	4.69	2.24	3.40	4.45	5.71	7.98
31-50	Males	1,757	3.22	1.46	2.27	3.02	3.94	5.67	5.91	2.97	4.40	5.66	7.13	9.69
31-50	Females	1,778	2.71	1.12	1.83	2.50	3.36	5.03	4.74	2.27	3.45	4.50	5.76	8.02
51-70	Males	1,335	3.20	1.46	2.26	3.00	3.93	5.65	5.74	2.88	4.28	5.49	6.94	9.47
51-70	Females	1,379	2.84	1.18	1.91	2.61	3.51	5.26	4.85	2.34	3.53	4.59	5.89	8.21
≥71	Males	462	3.25	1.48	2.29	3.05	3.98	5.74	5.65	2.82	4.18	5.41	6.82	9.35
≥71	Females	560	2.90	1.21	1.96	2.68	3.60	5.37	4.91	2.37	3.57	4.66	5.96	8.29

[1]Data are presented as mean values. 25(OH)D, 25-hydroxyvitamin D
[2]Weighted to the Australian population in 2011-2012

Table 2. Contribution of foods and beverage types to vitamin D intakes based on food consumption data from the 2011-12 National Nutrition and Physical Activity Survey for ages ≥2 years (n=12,153)[1]

25(OH)D bioactivity factor = 1		25(OH)D bioactivity factor = 5	
Food type	Contribution[2], %	Food type	Contribution[2], %
Food group			
Fish and seafood products and dishes	18.3	Meat, poultry and game products and dishes	26.3
Meat, poultry and game products and dishes	16.1	Cereal based products and dishes	14.7
Cereal based products and dishes	14.9	Egg products and dishes	13.3
Fats and oils	13.7	Milk products and dishes	11.6
Egg products and dishes	9.7	Fish and seafood products and dishes	11.1
Milk products and dishes	6.1	Fats and oils	8.1
Non-alcoholic beverages	5.8		
Cereals and cereal products	5.2		
Food sub-group			
Margarine and table spreads	11.5	Eggs	8.4
Packed (commercially sterile) fish and seafood	7.3	Mixed dishes where cereal is the major ingredient	7.7
Mixed dishes where cereal is the major ingredient	6.7	Beef, sheep and pork, unprocessed	7.4
Eggs	6.1	Dairy milk (cow, sheep and goat)	7.3
Fin fish (excluding commercially sterile)	5.5	Poultry and feathered game	7.3
Food			
Packed fin fish	7.3	Eggs, chicken	8.3
Eggs, chicken	6.0	Chicken	6.8
Monounsaturated margarine spreads, fat content ≥ 65 g/100g	5.0		

[1]Values are frequencies (%) for food and beverage types contributing ≥5% of total vitamin D intakes were included. 25(OH)D, 25-hydroxyvitamin D
[2]Calculated as (total vitamin D intake from a food group for all participants/total vitamin D intake from all foods) x 100

age group, and according to the bioactivity factor assigned to 25(OH)D (Table 3). When assuming equal bioactivity of vitamers, fortified foods (dry beverage flavourings, breakfast cereal and margarine) were major contributors to vitamin D intakes in Australian children aged 2-18 years. When a 25(OH)D bioactivity factor of five was applied, non-fortified foods were the major contributors across all sex and age groups.

Absolute intake of vitamin D from food and dietary supplements

Of 12,153 respondents with day one food consumption data, 2,039 reported taking a supplement that contained vitamin D. The mean (95% confidence interval) absolute intake of vitamin D from food on day one was 2.95 (2.86, 3.04) µg, increasing to 5.27 (5.05, 5.48) µg with vitamin D from dietary supplements added (Supplemental Table 1). The lowest mean absolute intake of vitamin D from food and dietary supplements combined was seen in females aged 2-3 years (2.19 [1.83, 2.55] µg/day), and was greatest for females aged ≥ 71 years (9.50 [8.26, 10.74] µg/day). With dietary supplements included, mean absolute intakes remained below 5 µg/day for all age groups ≤ 18 years, and for males aged 19-70 years and remained below 10 µg/day for all sex and age groups assessed (Supplemental Table 1). Among supplement users only, the mean absolute intake from food and dietary supplements was 17.72 (16.72, 18.72) µg/day, ranging from 4.82 (3.75, 5.91) µg/day in females aged 2-3 y to 24.00 (21.37, 26.64) µg/day in females aged ≥ 71 y (Supplemental Table 2).

Discussion

Usual mean intakes of vitamin D from food were low in the Australian population, at < 3.5 µg/day across all sex and age groups, assuming a bioactivity factor of one for the D vitamers (vitamin D_3, 25(OH)D_3, vitamin D_2 and 25(OH)D_2). Usual vitamin D intakes were lowest in younger age groups and lower in females than males. The overall amount of food consumed may play a role in these differences; however, the EAR of 10 µg/day [51] remains the same for all people aged ≥ 1 years and the mean usual vitamin D intake is estimated as substantially below this recommendation across the age and sex distribution in the Australian population. Our research to date shows that those particularly at risk of vitamin D deficiency in Australia include

Table 3. Highest food and beverage contributors to vitamin D intakes for Australian age/sex groups based on food consumption data from the 2011-12 National Nutrition and Physical Activity Survey (*n*=12,153)

Age, y	25(OH)D bioactivity factor = 1	25(OH)D bioactivity factor = 5
Male		
2-3	Fortified dry beverage flavourings	Milk, cow, fluid, regular whole, full fat
4-8	Fortified dry beverage flavourings	Milk, cow, fluid, regular whole, full fat
9-13	Fortified dry beverage flavourings	Milk, cow, fluid, regular whole, full fat
14-18	Breakfast cereal, mixed grain, fortified, sugars >20 g/100 g	Eggs, chicken
19-30	Packed fin fish	Eggs, chicken
31-50	Eggs, chicken	Eggs, chicken
51-70	Eggs, chicken	Eggs, chicken
>70	Monounsaturated margarine spreads (fortified)	Eggs, chicken
Female		
2-3	Fortified dry beverage flavourings	Milk, cow, fluid, regular whole, full fat
4-8	Monounsaturated margarine spread (fortified); Packed fin fish	Milk, cow, fluid, regular whole, full fat
9-13	Breakfast cereal, mixed grain, fortified, sugars >20 g/100 g	Chicken
14-18	Breakfast cereal, mixed grain, fortified, sugars >20 g/100 g	Eggs, chicken
19-30	Fortified dry beverage flavourings	Egg dishes, savoury
31-50	Packed fin fish	Eggs, chicken
51-70	Packed fin fish	Eggs, chicken
>70	Packed fin fish	Packed fin fish

25(OH)D, 25-hydroxyvitamin D

young adults [64], Aboriginal and Torres Strait Islander people living in remote areas [215] and people born outside Australia or the main English-speaking countries [65]. It is not possible to determine whether the population groups with the lowest usual vitamin D intakes correspond with those with lower vitamin D status, as there has not been a national survey of circulating 25(OH)D concentrations in Australian children aged < 12 years.

Our estimate of usual vitamin D intakes in the Australian population remained relatively low even when a 25(OH)D bioactivity factor of five was applied. The 25(OH)D bioactivity factor of five was used in our secondary model as it is used in a small number of national food composition databases. It is generally accepted that 25(OH)D is more bioactive than vitamin D; however, the extent to which it is has not yet been confirmed, and it has been suggested that the vitamers should be considered equal until definitive data are available [125]. Here, we have shown that vitamin D intakes from food in Australia remain low even under the likely best-case scenario of 25(OH)D being up to five times more bioactive that vitamin D.

Dietary supplements also contribute to intakes of vitamin D and are important to consider when estimating baseline intakes. Our earlier study showed that ~17% of Australians aged ≥ 2 years had consumed a vitamin D-containing supplement in the 24 hours preceding the first 24-hour dietary recall interview [75]. Only 4% of participants had taken a single vitamin D supplement (typical daily dose = 25 µg). Approximately 3% of participants had taken a vitamin D-containing calcium supplement, 11% had taken a vitamin D-containing multivitamin-multimineral supplement and 1% had taken a vitamin D-containing fish oil preparation. The median (range) doses for these preparations, in the same respective order, were 5 (0.1-25), 5 (1-25) and 5 (0.1-25) µg/day. Less than 0.5% of participants had taken fish liver oil with a median (range) dose of 2 (0.2-6) µg/day [75]. We found that, for the majority of the sex and age groups assessed, mean intakes of vitamin D from food and dietary supplements were not substantially greater than intakes from food only. The greatest difference between absolute intakes from food only and with dietary supplements was seen in females aged > 50 years (increase of 5-6 µg/day), who have greater risk of osteoporosis with increasing age. Among supplement users only, there was a greater difference between absolute intakes from food only and from food and dietary supplements. These nationally-representative data suggest that, in 2011-2012,

the majority of Australians either did not use vitamin D-containing dietary supplements, did not report it on the day surveyed due to it being infrequently consumed, or did not take a daily dose sufficient to increase their dietary intake to recommended levels.

Our results indicate that the majority of Australians consume less vitamin D from food than people in the US, Canada and some European countries. This was despite all four D vitamers being measured in all sampled foods, irrespective of animal or plant origin, and accounted for in our estimates. Conversely, food composition data used for US [60, 146, 147], Canadian [145, 148] and some European [185, 244-250, 269] intakes estimates included fewer vitamers and/or not all vitamers were measured in all foods. Caution is needed when comparing intake estimates across countries; however, the gap between intakes in these regions and intakes in Australia could be conceivably greater if the compositional datasets used were of similar scope.

This gap may be due to differing fortification practices. Vitamin D is found naturally in relatively low concentrations in a narrow range of foods [38]. As it can, therefore, be difficult for many people to meet dietary vitamin D requirements through naturally-occuring food sources [38], fortified foods are important sources of vitamin D in countries where they are available [60, 145]. In Australia, only margarine is mandatorily fortified. Although vitamin D is permitted to be added via voluntary fortification to low fat milk, dairy alternatives and breakfast cereals, vitamin D fortification of these products is not routine. In contrast, foods such as dairy products, dairy alternatives and juice are commonly fortified with vitamin D in the US and Canada [51], while fortification practices vary across European countries [38]. In Finland, the proportion of the population with serum 25(OH)D concentrations > 50 nmol/L increased from 44 to 91% following fortification of fluid milk products and fat spreads in 2003 [162]. Moreover, greater improvements in circulating 25(OH)D concentrations were seen in those with concentrations < 30 nmol/L than those with concentrations ≥ 50 nmol/L [162]. Nutrition policy informed by modelling food and nutrient intakes could assist in determining potential fortification strategies to optimise dietary intakes and reduce the prevalence of vitamin D deficiency in Australia.

We estimated that more than 95% of Australians aged ≥ 2 years had vitamin D intakes below the EAR of 10 µg/day recommended by the Institute of Medicine. In

light of this, population-level strategies may be needed to address the low population vitamin D intakes and concomitant low vitamin D status in Australia. However, it should be noted that the aforementioned EAR recommended for the US and Canada is based on minimal sunlight exposure [51], and most Australians have more opportunity for sun exposure than people living in North America. Despite year-round opportunity for sun exposure in many regions of the country, the high prevalence of vitamin D deficiency [64, 65] implies that most Australians do not produce sufficient vitamin D via this source. Even higher prevalence of low vitamin D status, together with low vitamin D intakes, have been reported for some Northern African and Middle-Eastern countries with ample opportunity for sun exposure [38, 270, 271]. Skin pigmentation, cultural clothing practices, sun/heat avoidance and protective measures against skin damage and skin cancer may play a role in the relatively high prevalence of vitamin D deficiency in sunny countries. In Australia, vitamin D dietary supplements may be needed on an individual basis by people with increased dietary vitamin D requirements [51, 76], such as the elderly [38], and others at high risk of vitamin D deficiency. However, relatively few Australians, particularly younger people, use dietary supplements [75], and they may not be effective as a population-wide solution to vitamin D deficiency. Increasing the dietary supply of vitamin D through fortification, on the other hand, is an alternative strategy that could potentially safely improve mean serum 25(OH)D concentrations across the whole population.

Globally, the methods outlined here may be useful to other countries that have, like Australia, lacked comprehensive vitamin D food composition data and are building a new system for estimating usual vitamin D intakes from food. Nationally, our new data on usual vitamin D intakes in the Australian population will allow investigation of potential associations between vitamin D intakes and various health conditions, as well as how health conditions affect intakes, which may be used to inform public health nutrition campaigns. In combination with our new vitamin D food composition data, the data will also allow researchers to predict the effect of adding various concentrations of vitamin D to various foods on circulating 25(OH)D concentrations, and to develop a potential option to improve vitamin D status at the Australian population level.

Major strengths of this study were the use of nationally-representative food consumption data and comprehensive food composition data that included four D vitamers measured using a sensitive and specific LC-QQQ method. Food composition data were based on analytical values for major foods in the form that they would usually be consumed, i.e. cooked meat and seafood. These intakes estimates are, however, subject to the usual limitations of self-reported food consumption data, such as recall bias and measurement error [272], and of food composition data, such as sampling and measurement uncertainty [3]. Although we did not include vitamin D from dietary supplements in the estimation of usual intakes due to limitations of the NCI method, we produced estimations of absolute vitamin D intakes from food and dietary supplements from day one consumption data only. Our findings suggest that vitamin D supplement use in Australia did not sufficiently compensate for low vitamin D intakes from food for the majority of Australians. Due to the age of NNPAS data, food consumption and supplementation practices may have changed over time; however, there are no more recent nationally-representative data available to confirm this.

We have presented estimates of usual vitamin D intakes for the Australian population using nationally-representative food consumption data and comprehensive food composition data. Our new data show that vitamin D intakes from food in Australia are lower than recommendations and lower than in the US, Canada and many European countries. Given the prevalence of low vitamin D status in the population, despite relatively good opportunity for sun exposure, strategies to address low vitamin D intakes from food are needed in Australia. This could include measures such as food-fortification or -biofortification to increase the dietary supply of vitamin D. Our estimate of vitamin D intakes will allow modelling of various food fortification scenarios to inform nutrition policy for improving vitamin D status in the Australian population.

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Author contributions:

Eleanor Dunlop: Conducted research and analyzed data, Wrote the paper. **Julie L Boorman:** Conducted research and analyzed data, Wrote the paper. **Tracy L Hambridge:** Conducted research and analyzed data, Wrote the paper. **Jessica McNeill:** Conducted research and analyzed data, Wrote the paper. **Anthony P James:** Supervised research, Reviewed and edited the paper. **Mairead Kiely:** Designed research, Reviewed and edited the paper. **Caryl A Nowson:** Designed research, Reviewed and edited the paper. **Anna Rangan:** Designed research, Supervised research, Reviewed and edited the paper. **Judy Cunningham:** Designed research, Supervised research, Reviewed and edited the paper. **Paul Adorno:** Provided essential materials, Reviewed and edited the paper. **Paul Atyeo:** Provided essential materials, Reviewed and edited the paper. **Lucinda J Black:** Designed research, Supervised research, Reviewed and edited the paper.

Supplemental Table 1 Absolute vitamin D intakes[1] from food (including beverages), and with dietary supplements in a nationally-representative sample of Australians (*n* = 12,153)

Age group, y	Sex	*n*	Vitamin D intakes from food	Vitamin D intakes from food,and supplements
			μg/day, mean (95% confidence interval)	
≥2	All	12,153	2.95 (2.86, 3.04)	5.27 (5.05, 5.48)
≥2	Males	5,702	3.16 (3.04, 3.27)	4.58 (4.34, 4.82)
≥2	Females	6,451	2.74 (2.62, 2.87)	5.95 (5.59, 6.31)
2-3	Males	228	1.97 (1.62, 2.32)	2.43 (2.01, 2.84)
2-3	Females	236	1.83 (1.51, 2.15)	2.19 (1.83, 2.55)
4-8	Males	397	2.23 (2.04, 2.42)	2.68 (2.41, 2.96)
4-8	Females	392	2.20 (1.81, 2.58)	2.73 (2.86, 3.18)
9-13	Males	392	2.86 (2.54, 3.17)	3.28 (2.83, 3.72)
9-13	Females	395	2.88 (2.48, 3.28)	3.25 (2.80, 3.70)
14-18	Males	403	3.37 (2.95, 3.78)	3.88 (3.34, 4.41)
14-18	Females	369	2.28 (2.02, 2.54)	3.36 (2.42, 4.29)
19-30	Males	739	3.54 (3.13, 3.96)	4.89 (4.10, 5.68)
19-30	Females	853	2.75 (2.45, 3.05)	5.12 (4.22, 6.02)
31-50	Males	1,669	3.12 (2.94, 3.30)	4.80 (4.34, 5.25)
31-50	Females	1,896	2.78 (2.51, 3.06)	5.86 (5.25, 6.47)
51-70	Males	1,341	3.30 (3.07, 3.53)	4.93 (4.50, 5.36)
51-70	Females	1,565	2.90 (2.63, 3.17)	8.07 (7.08, 9.06)
≥71	Males	533	3.26 (2.87, 3.65)	6.15 (5.14, 7.17)
≥71	Females	745	3.04 (2.69, 3.41)	9.50 (8.26, 10.74)

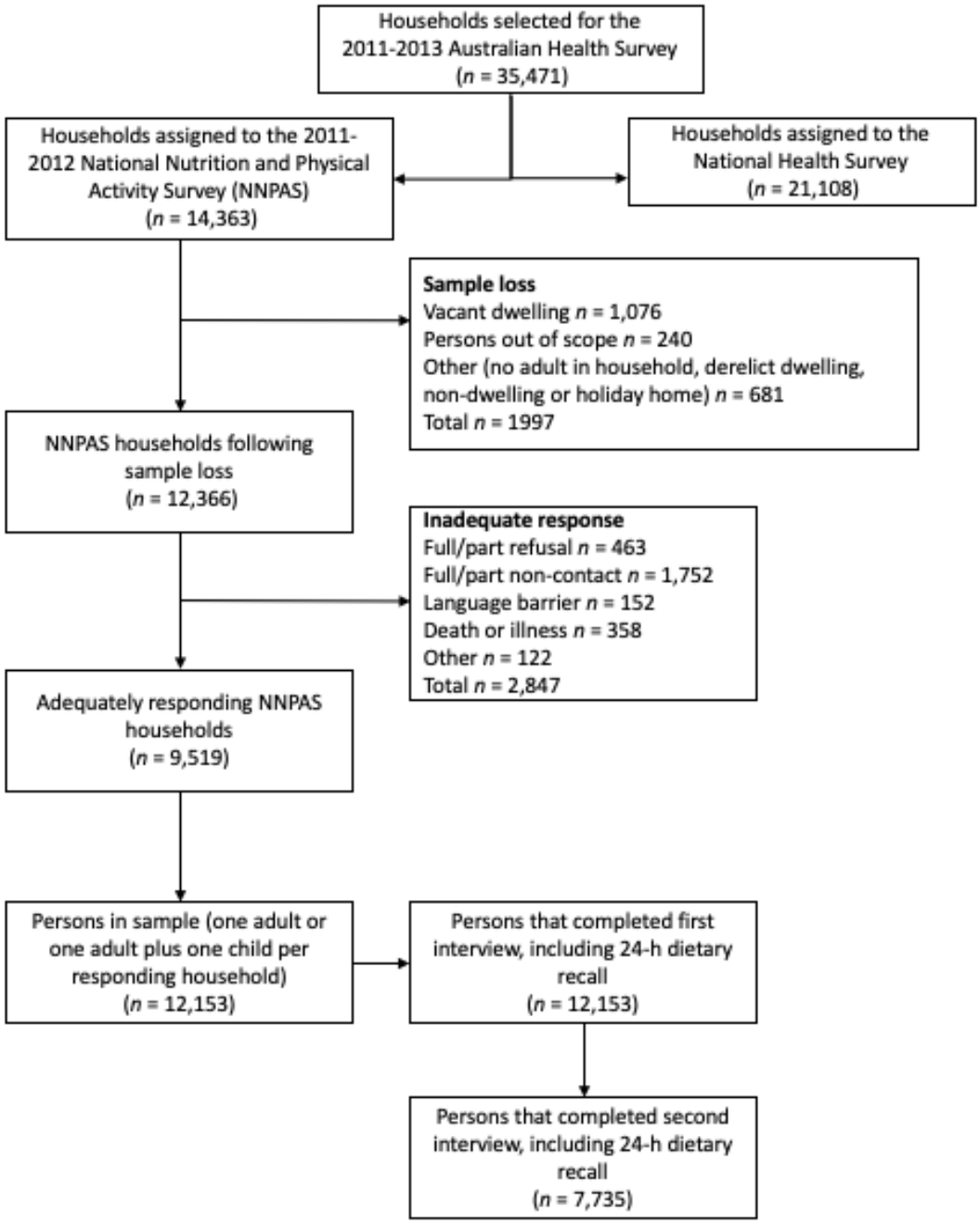
[1]Weighted to the Australian population in 2011-2012.
Day one food and vitamin D-containing supplement consumption data from the 2011-2012 National Nutrition and Physical Activity Survey were used with vitamin D food composition data for vitamin D_3, 25-hydroxyvitaminD_3, (25(OH)D_3) vitamin D_2 and 25(OH)D_2

Supplemental Table 2 Absolute vitamin D intakes[1] from food (including beverages), and with dietary supplements in Australian vitamin D supplement users (*n* = 2,039) which is a subset of the larger nationally representative sample of Australians

Age group, y	Sex	*n*	Vitamin D intakes from food	Vitamin D intakes from food and supplements
			μg/day	
≥2	All	2,039	3.08 (2.88, 3.29)	17.72 (16.72, 18.72)
≥2	Males	695	3.66 (3.28, 4.05)	15.69 (14.30, 17.07)
≥2	Females	1,344	2.74 (2.53, 2.97)	18.93 (17.57, 20.29)
2-3	Males	31	2.12 (1.26, 2.30)	5.14 (4.09, 6.20)
2-3	Females	22	1.56 (0.95, 2.16)	4.82 (3.75, 5.91)
4-8	Males	45	2.27 (1.68, 2.86)	6.82 (5.56, 8.08)
4-8	Females	47	1.88 (1.42, 2.34)	6.36 (4.99, 7.75)
9-13	Males	22	4.23 (2.92, 5.53)	10.06 (7.15, 12.97)
9-13	Females	35	2.98 (1.98, 3.99)	7.11 (5.23, 9.00)
14-18	Males	27	4.34 (1.52, 7.16)	11.14 (6.68, 15.60)
14-18	Females	21	2.00 (1.07, 2.92)	15.03 (8.82, 21.24)
19-30	Males	81	4.56 (3.26, 5.86)	17.37 (12.12, 22.62)
19-30	Females	132	2.34 (1.94, 2.74)	18.15 (13.48, 22.83)
31-50	Males	216	3.60 (2.99, 4.21)	16.38 (14.03, 18.72)
31-50	Females	413	2.94 (2.42, 3.46)	17.50 (15.50, 19.50)
51-70	Males	179	3.68 (2.87, 4.50)	16.63 (14.62, 18.64)
51-70	Females	445	2.82 (2.43, 3.21)	22.04 (19.10, 25.43)
≥71	Males	94	3.19 (2.31, 4.07)	21.22 (17.00, 25.43)
≥71	Females	229	2.98 (2.52, 3.45)	24.00 (21.37, 26.64)

[1]Values are means (95% confidence interval) and are weighted to the Australian population in 2011-2012.

Day one food and vitamin D-containing supplement consumption data from the 2011-2012 National Nutrition and Physical Activity Survey were used with vitamin D food composition data for vitamin D_3, 25-hydroxyvitaminD_3, (25(OH)D_3) vitamin D_2 and 25(OH)D_2



Supplementary Figure 1. 2011-2012 Nutrition and Physical Activity Survey participant flowchart
Persons included in the present study *n*=12,153

Chapter 5: Efficacy of vitamin D food fortification

Thesis objective addressed in this chapter:

Objective 4: To conduct a systematic review and meta-analysis of randomised controlled trials to evaluate the efficacy of vitamin D_3 and vitamin D_2 food fortification on serum 25-hydroxyvitamin D concentrations.

The content of this chapter is covered by Publications 4 and 5:

Publication 4: The efficacy of vitamin D food fortification and biofortification in children and adults: a systematic review protocol.

This is a non-final version of an article published in final form in: Dunlop E, M Kiely, AP James, T Singh, LJ Black. 2020. The efficacy of vitamin D food fortification and biofortification in children and adults: a systematic review protocol. *JBI Evidence Synthesis*: 18(12): 2694-2703. doi: 10.11124/JBISRIR-D-19-00373.

Publication 5: Vitamin D food fortification and biofortification increases serum 25-hydroxyvitamin D concentrations in adults and children: an updated and extended systematic review and meta-analysis of randomized controlled trials.

This is a pre-copyedited, author-produced version of an article accepted for publication in the Journal of Nutrition following peer review. The Version of Record Dunlop E, ME Kiely, AP James, T Singh, NM Pham, LJ Black. 2021. Vitamin D food fortification and biofortification increases serum 25-hydroxyvitamin D concentrations in adults and children: an updated and extended systematic review and meta-analysis of randomized controlled trials. *Journal of Nutrition*: 151(9): 2622-2635. doi:10.1093/jn/nxab180 is available online at: https://academic.oup.com/jn/advance-article/doi/10.1093/jn/nxab180/6296114

The efficacy of vitamin D food fortification and biofortification in children and adults: a systematic review protocol

Abstract

To assess the effect of vitamin D food fortification and biofortification on serum 25-hydroxyvitamin D (25(OH)D) concentrations. Vitamin D deficiency is a global issue with considerable implications for public health. Many people do not receive sufficient sun exposure for adequate endogenous vitamin D synthesis, nor sufficient dietary vitamin D to meet their requirements. Increasing sun exposure may increase risk of skin cancer, and vitamin D supplementation is not favoured as a population-wide solution. Previous systematic reviews have shown a dose-dependent effect of vitamin D-fortified foods on circulating 25(OH)D concentrations. However, previous reviews did not investigate the efficacy of vitamin D fortification and biofortification in children, and several new fortification and biofortification trials in adults have been published since these earlier systematic reviews were conducted. This review will consider randomized controlled trials that investigate the effect of vitamin D food fortification and biofortification on serum 25(OH)D concentrations in children and adults. CINAHL, MEDLINE, Embase, Cochrane Central Register of Controlled Trials and PubMed will be searched for relevant studies published in English. Following a screen of titles and abstracts by a single author, relevant full-text articles will be independently reviewed by two authors. Two authors will independently assess methodological quality using the Joanna Briggs Institute's System for the Unified Management, Assessment and Review of Information (SUMARI) randomized controlled trials critical appraisal tool. Data will be independently extracted by two authors. A meta-analysis will be conducted in SUMARI, and heterogeneity will be statistically tested.

Introduction

Low vitamin D status (25(OH)D concentration < 50 nmol/L) is a global health concern [273], as many people do not receive sufficient sun (ultraviolet-B light [UVB]) exposure or have access to sufficient vitamin D from dietary sources to meet their requirement. Due to the essential role of vitamin D in bone health throughout life,

and potential links between low vitamin D status and various chronic diseases, strategies to increase vitamin D status in the population are under investigation.

Circulating concentrations of 25(OH)D are measured as a biomarker of vitamin D status, rather than the active form, 1,25(OH)D, which has a brief circulating half life (4-6 hours) and 1000-fold lower circulating concentrations than 25(OH)D [2]. Measurement of 25(OH)D is challenging as it is largely protein-bound, and there are two circulating forms, $25(OH)D_2$ and $25(OH)D_3$. However, 25(OH)D has been shown to reliably reflect vitamin D status [40].

A variety of assays may be used to measure serum 25(OH)D; however, accuracy and precision vary [43, 45]. Liquid chromatography-mass spectrometry methods, that are standardised to the standard reference method (RMP) developed by the National Institutes of Standards and Technology, Ghent University and the US Centers for Disease Control and Prevention, are considered the gold standard [42-44]. Where other assays are used, certification of a laboratory's method to the RMPs provides the greatest confidence in an accurate measurement of serum 25(OH)D concentrations [42-44].

The prevalence of 25(OH)D concentrations < 50 nmol/L ranges from 37-48% in the US [274], Canada [61], and Europe [39]. Even in Australia, where the potential for UVB irradiance is greater, almost one quarter of adults have low vitamin D status [58]. The Institute of Medicine (IOM) suggests a vitamin D intake of 10 μg/day as an Estimated Average Requirement for all ages, and a Recommended Dietary Allowance of 15 and 20 μg/day for people aged ≤ 70 and > 70 years, respectively [51]. Usual vitamin D intakes commonly fall short of these recommendations, with estimates of mean dietary vitamin D intakes of < 5 μg/day in Europe [144] and < 6 μg/day in Canada [275]. In the US, even taking into account intake from fortified foods and supplements, the average daily intake of vitamin D was below the Estimated Average Requirement in 70% of individuals aged ≥ 2 years who participated in the 2003-2006 National Health and Nutrition Examination Survey [276].

Potential strategies to improve vitamin D status at the population level are constrained. Increased sun exposure carries increased risk of skin cancer. As a fat-soluble compound, vitamin D is not readily excreted - with persistent

overconsumption, its accumulation in the body may cause toxicity. Therefore, supplements, although effective in increasing 25(OH)D among individuals, are not consistently used across all population sectors, and may be risky as a population-wide intervention due to the potential for toxicity [45, 88].

Diet offers a possible solution; however, vitamin D is found naturally in very few foods and in relatively low concentrations. Consumers may limit or avoid vitamin D-containing foods for various reasons, including cost, religious or cultural practices, and personal preference. Fortification (direct addition of vitamin D to foods) may provide cost-effective population-wide access to modest and safe, but useful, amounts of vitamin D. In the US, a variety of foods, including milk and milk alternatives, cheese, yoghurt and juice, are routinely, and voluntarily, fortified with vitamin D [51]. Although legislation varies between countries, these fortified foods may also be found in some European countries [144]. In Canada, milk, milk substitutes and margarine must be vitamin D-fortified; few other foods are voluntarily fortified [51, 275]. In Australia, it is mandatory for margarine to be fortified and, although certain other foods may be voluntarily fortified, few are fortified in practice. Biofortification (e.g., addition of vitamin D to animal feed [173, 174], or exposure of yeast, mushrooms or livestock to UVB light [136, 170, 175, 277], in order to increase the vitamin D content of produce) is also emerging as a potential strategy.

Strategic fortification and biofortification of staple foods may reduce the prevalence of vitamin D deficiency; however, modelling of fortification/biofortification strategies is required to ensure that they do not place consumers at risk of excessive intake of vitamin D. As such, it is important to understand the treatment effect of vitamin D food fortification and biofortification across various population groups in order to develop safe and effective strategies that increase the dietary supply of vitamin D. An earlier meta-analysis of 16 randomized controlled trials (RCTs) published prior to 2012, with a mean dose of vitamin D from fortified foods of 11 µg/d, showed a combined treatment effect of 19.4 nmol/L (95% CI: 13.90, 24.90), and a serum 25(OH)D increase of 1.2 nmol/L (95% CI: 0.72, 1.68) per 1 µg vitamin D in adults [195]. Due to the limited number of relevant RCTs at that time, that review did not examine the different in effect of the different forms of vitamin D, and included studies with no placebo product provided for control groups. In 2015, a systematic review involving 18 studies of either fortified foods or dietary

supplements, suggested a mean (standard deviation (SD)) serum 25(OH)D increase of 2.19 (0.97) nmol/L per 1 μg of ingested vitamin D in adults; however, only one fortified food study was included [278]. A recent meta-analysis that focused on children found that, of fortified foods, supplements and bolus injections, fortified foods produced the greatest mean change in serum 25(OH)D concentrations [196]. To our knowledge, there are no previous systematic reviews examining the efficacy of vitamin D biofortification (e.g., addition of vitamin D to animal feed or exposure of produce to UVB light) in improving serum 25(OH)D concentrations in humans. In 2016, a Cochrane Library protocol [279] was published for a general investigation of crops biofortified with micronutrients; however, serum 25(OH)D concentration was not listed as an outcome measure.

Several new RCTs have been published since the earlier systematic reviews in adults, providing an increased sample size for review. Hence, this updated review aims to capture recent RCTs and expand the scope of previous reviews by including RCTs of both fortification and biofortification in children and adults. Searches of PROSPERO, the Cochrane Database of Systematic Reviews and the Joanna Briggs Institute (JBI) Database of Systematic Reviews and Implementation Reports revealed no recent or registered related reviews.

Review question

The question of this review is: what is the treatment effect of vitamin D food fortification or biofortification on serum 25-hydroxyvitamin D concentrations in adults and children?

Inclusion Criteria

Participants

The review will consider studies that include children and/or adults with the exception of those with a health condition that may compromise gut integrity and vitamin D absorption.

Intervention(s)

Studies that evaluated foods or beverages fortified or biofortified with vitamin D (vitamin D_2, vitamin D_3, or their hydroxylated forms, 25(OH)D_2 and 25(OH)D_3) will be considered. A daily fortification or biofortification dose of ≥ 5 µg vitamin D must have been provided for at least four weeks (an additional 5 µg would allow the average person with an intake of ~5 µg/day [144, 275, 276] to achieve the IOM's [51] EAR of 10 µg/day).

Comparator(s)

Treatments must be compared to a placebo food or beverage intervention. Studies that use existing/usual diet as a control will be excluded, due to the impact on participant blinding to the intervention.

Outcomes

We will consider studies that include baseline and endpoint serum 25(OH)D concentrations. This outcome may be measured by one of several biochemical analysis methods commonly used to measure serum 25(OH)D concentrations, namely chemiluminescence immunoassay (CLIA), chemiluminescentmicroparticle immunoassay (CMIA), competitive protein-binding assay (CPBA), enzyme-linked immunosorbent assay (ELISA), high performance liquid chromatography (HPLC), liquid chromatography-tandem mass spectrometry (LC-MS/MS) or radio-immunoassay (RIA) [280].

Types of studies

This review will consider only RCTs published in English. Trials must be, at minimum, single-blinded. Studies published from database inception will be included in order to capture all relevant data available to date.

Methods

The proposed systematic review will be conducted according to the Joanna Briggs Institute's methodology for systematic reviews of effectiveness [281]. An application

has been submitted for PROSPERO registration (registration number pending editorial review).

Search strategy

In collaboration with a Health Sciences Faculty Librarian, initial searches were conducted in CINAHL and MEDLINE to identify keywords and terms for RCTs that compared the effect of vitamin D-fortified or -biofortified foods or beverages with an unfortified control food or beverage on serum 25(OH)D concentrations in humans. Database-specific search strategies (Supplementary Figure 1) were developed for CINAHL, MEDLINE, Embase, Cochrane Central Register of Controlled Trials and PubMed in December 2018. With the aim of capturing data for unpublished studies with null or negative results, searches will be conducted in the Cochrane Handbook of grey literature databases, ProQuest Dissertation Publishing, U.S. National Library of Medicine database (ClinicalTrials.gov), World Health Organization International Clinical Trials Registry Platform, All Trials (alltrials.net) and Restoring Invisible and Abandoned Trials. Further main keyword searches will be used to explore any remaining grey literature through the Google and Google Scholar search engines. Bibliographies of included studies will also be screened for relevant articles.

Study selection

Retrieved articles will be exported to EndNote X7 (Clarivate Analytics, PA, USA) where duplicates will be removed and titles and abstracts screened. The remaining articles will be imported to the JBI's System for the Unified Management, Assessment and Review of Information (SUMARI) [282] for independent full-text review by two authors. Any difference in inclusion/exclusion decisions will be resolved through discussion by the two authors. If a consensus is not reached, a third author will be consulted. Reasons for exclusion of articles will be recorded in SUMARI [282].

Assessment of methodological quality

Two authors will independently critically appraise eligible studies for design and methodological quality using the JBI SUMARI [282] RCT critical appraisal tool. The tool comprises 13 questions related to randomisation, blinding, baseline

characteristics of participants, follow-up, measurement of outcomes, statistical analysis and overall design quality. Studies that meet the minimum blinding requirement (single-blinded) and use an appropriate assay, as listed in the inclusion criteria, for serum 25(OH)D concentration analysis will be included: studies that do not meet these will be excluded. Studies will be included regardless of critical appraisal outcomes; however, quality scores (out of 13, relating to whether each of the JBI SUMARI RCT critical appraisal tool questions are satisfied) will be recorded. Authors will discuss any differing opinions on study quality, consulting a third author in order to reach consensus if necessary.

Data extraction

Two authors will independently extract data; if recorded data differ, the authors will collaborate to refer back to articles, involving a third author if required. Data will be extracted to Microsoft Excel, in order to capture the breadth of data required, and to carry out data conversions where necessary. Information on geographical setting (including latitude), study population, age range, study duration, season(s) of intervention, serum 25(OH)D concentration assay, and study quality score will be tabulated (Supplementary Table 1). In a second table (Supplementary Table 2), we will collate data on food/beverage intervention type, measure of compliance, fortified/biofortified, added daily dose of vitamin D (μg), added daily dose calcium (mg) if data are available, mean baseline and endpoint of serum 25(OH)D concentration (nmol/L) and number of participants per group. Multiple study arms of the same trial will be included where appropriate. Where multiple publications exist for the same study population, the main study, or the study with the most comprehensive baseline and endpoint data for the greatest number of participants, will be included; all other duplications will be excluded. Where information is missing, or additional data are required, they will be requested from authors.

Added vitamin D will initially be separated by vitamin D_3, 25(OH)D_3, vitamin D_2, 25(OH)D_2 in case there are a sufficient number of studies to compare the effect of different forms of vitamin D, which may differ in terms of molecular structure and bioactivity. Mean endpoint data for 25(OH)D will be expressed in nmol/L as mean (SD). When required, the following conversions will be used:

Vitamin D (μg) = (vitamin D (IU))/40

[25(OH)D] nmol/L = [25(OH)D] ng/mL x 2.5

SD = standard error of the mean x √n

SD = [(top confidence interval – bottom confidence interval) x √n]/3.92

Two authors will carry out all calculations independently and collaborate to resolve any errors, with the assistance of a third author if necessary.

Data synthesis

Studies will, where possible, be pooled in statistical meta-analysis using JBI SUMARI. Effect sizes will be expressed as weighted final post-intervention mean differences and their 95% confidence intervals will be calculated for analysis. Heterogeneity will be assessed using chi-square tests ($P < 0.10$ = significant heterogeneity) and the I^2 statistic (I^2 of 0% = no heterogeneity, I^2 of 25, 50 and 75% = low, moderate and high heterogeneity, respectively). Statistical analyses will be performed using the random effects model [283]. Where statistical pooling is not possible (e.g., due to high unexplained heterogeneity), the findings will be presented in narrative form including tables and figures to aid in data presentation where appropriate. A funnel plot will be generated in Stata (StataCorp, College Station, TX, USA) to assess publication bias if there are 10 or more studies included in a meta-analysis. Statistical tests for funnel plot asymmetry (Egger test, Begg test, Harbord test) will be performed where appropriate. Reasons for heterogeneity will be explored through subgroup and sensitivity analyses with stratification based on mean baseline 25(OH)D concentrations (< or ≥ 50 nmol/L), latitude (< or ≥ 40 °), dose (< or ≥ 10 μg/d), and, if sufficient data are available, by age (e.g., adults 18-70 and 70+ y, pregnant women; children 0-2 and 2-17 y), form of vitamin D, assay, fortification/biofortification, study quality (JBI SUMARI RCT critical appraisal tool score < or ≥ 10), study duration (< or ≥ 6 months) and by men and women in adults.

Assessing certainty in the findings

We will use the Grading of Recommendations, Assessment, Development and Evaluation (GRADE) approach to grade the certainty of evidence and a Summary of

Findings (SoF) will be created using GRADEPro GDT 2015 (McMaster University, ON, Canada). The SoF will present the following information where appropriate: absolute risks for the treatment and control, estimates of relative risk, and a ranking of the quality of the evidence based on the risk of bias, directness, heterogeneity and inconsistency, precision and risk of publication bias of the review results. We will report the outcome of change in serum 25(OH)D concentration in the SoF.

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Supplementary Figure 1: Search strategy

Initial search was conducted on 11 Dec 2018.

CINAHL (EBSCOhost)

#1	(MH "Biofortification") OR "biofortification"
#2	(MH "Food, Fortified") OR (MH "Function Food") OR "fortified foods"
#3	fortif*
#4	biofortif*
#5	(MH "Vitamin D+") OR "vitamin D" OR (MH "Vitamin D deficiency+") OR (MH "Ergocalciferols") OR (MH "Cholecalciferol")
#6	(#1 OR #2 OR #3 OR #4) AND #5

Limits: humans
Dates of publication: since database inception
Results returned: 223

MEDLINE (Ovid)

#1	exp Vitamin D/
#2	Vitamin deficiency/
#3	("vitamin d" or cholecalciferol or ergocalciferol).ti,ab.
#4	1 or 2 or 3
#5	Food, Fortified/
#6	fortif*.ti,ab.
#7	Biofortification/
#8	biofortif*.ti,ab
#9	5 or 6 or 7 or 8
#10	4 and 9

Limits: humans
Dates of publication: since database inception
Results returned: 821

Embase (Ovid)

#1	exp Vitamin D/
#2	Vitamin deficiency/
#3	("vitamin d" or cholecalciferol or ergocalciferol).ti,ab.
#4	1 or 2 or 3

#5	Food, Fortified/
#6	fortif*.ti,ab.
#7	Biofortification/
#8	biofortif*.ti,ab
#9	5 or 6 or 7 or 8
#10	4 and 9

Limits: humans
Dates of publication: since database inception
Results returned: 1141

Cochrane Central Register of Controlled Trials

#1	vitamin d
#2	MeSH descriptor: [Vitamin D] explode all trees
#3	"vitamin d" or cholecalciferol or ergocalciferol or "vitamin D deficiency"
#4	# 2 or #3
#5	MeSH descriptor: [Food, Fortified] explode all trees
#6	fortif*
#7	MeSH descriptor: [Biofortification] explode all trees
#8	biofortif*
#9	#5 or #6 or #7 or #8
#10	#4 and #9

Limits: humans
Dates of publication: since database inception
Results returned: 65

PubMed

(("vitamin d" OR vitamin D or cholecalciferol OR ergocalciferol OR "vitamin D deficiency" AND ("fortified food" OR "biofortified" OR biofortif* OR fortif*))

Limits: humans
Dates of publication: since database inception
Results returned: 831

Supplementary Table 1: Data extraction table 1

Reference	Location	Population	Age (y)	Study dura-tion	Season of intervention	25-hydroxy-vitamin D assay	Quality score (out of 13)

Supplementary Table 2: Data extraction table 1

			Intervention group							Control group				
Reference	Compliance measured?	Fortified/ biofortified	Food	Added vitamin D_2 dose (μg/d)	Added vitamin D_3 dose (μg/d)	Added Ca dose (mg/d)	Mean baseline 25(OH)D (nmol/L)	Mean end point 25(OH)D (nmol/L)	*n*	Food	Added Ca dose (mg/d)	Mean baseline 25(OH)D (nmol/L)	Mean end point 25(OH)D (nmol/L)	*n*

25(OH)D, 25-hydroxyvitamin D; Ca, calcium

Vitamin D food fortification and biofortification increases serum 25-hydroxyvitamin D concentrations in adults and children: an updated and extended systematic review and meta-analysis of randomized controlled trials

Abstract

Low vitamin D status is a global public health issue that vitamin D food fortification and biofortification may help to alleviate. We investigated the effect of vitamin D food fortification and biofortification in increasing circulating 25-hydroxyvitamin D (25(OH)D) concentrations. We expanded the scope of earlier reviews to include adults and children, to evaluate effects by vitamin D vitamer, and investigate linear and non-linear dose-response relationships. We conducted a systematic review and meta-analysis. We searched CINAHL, MEDLINE, PubMed, Embase, the Cochrane Library and grey and unpublished literature sites for randomized controlled trials, including people of all ages, with the criteria: absence of vitamin D-absorption affecting illness, duration ≥ 4 weeks, equivalent placebo food control, dose quantification, dose ≥ 5 μg/d, baseline and endpoint or absolute change in 25(OH)D concentrations reported, random allocation and participant blinding. Quality was assessed using the Jadad Scale. Data from 34 publications (2398 adults: 1345 intervention, 1053 controls; 1532 children: 970 intervention, 562 controls) were included. Random effects meta-analysis of all studies combined (mean dose 16.2 μg/d) indicated a pooled treatment effect of 21.2 nmol/L (95% confidence interval (CI) 16.2, 26.2), with a greater effect for studies using cholecalciferol than ergocalciferol. Heterogeneity was high (I^2 > 75%). Meta-regression analyses for all studies combined suggested positive effect differences for baseline circulating 25(OH)D concentrations < 50 nmol/L, dose ≥ 10 μg/d and a negative effect difference when the intervention arm included a calcium dose ≥ 500 mg/d greater than the control arm. Dose-response rates were found to be non-linear (Wald test for non-linearity P < 0.001). For all studies combined, a threshold occurred at ~26 nmol/L for a dose of ~21 μg/d). These results support use of vitamin D food fortification to improve circulating 25(OH)D circulations in populations. PROSPERO registration number: CRD42020145497

Introduction

Low vitamin D status (25-hydroxyvitamin D (25(OH)D) concentration < 50 nmol/L) is globally prevalent [273, 284]. Ultraviolet-B light (UVB) irradiance through sun exposure offers the greatest potential source of vitamin D; however, sun exposure is often limited [199]. Low vitamin D status has been estimated to affect 23-40% of people in the US [60], Canada [61], and Europe [39], and despite greater potential for UVB irradiance, 23% of Australian adults [58]. Vitamin D is implicated in metabolic bone diseases, such as nutritional rickets and osteomalacia [51], and recent evidence indicates a role in prevention of acute respiratory tract infections [22, 24], prompting speculation on its potential to mitigate severity of COVID-19 infection [26]. Low serum 25(OH)D concentrations and vitamin D deficiency have also been inversely associated with healthcare expenditure [69, 70].

Food-based solutions to low vitamin D status are safer than increased sun exposure due to skin cancer concerns, and in areas with long winters, food is more reliable than vitamin D supplements, which have limited reach and carry a risk of toxicity [45, 88]. Nonetheless, there are few naturally vitamin D-rich foods and estimates of dietary vitamin D intakes indicate that intake recommendations [51, 52] are not met in the US [60], Canada [275] or Europe [144] and that an inadequate dietary supply of vitamin D is widespread. Addition of vitamin D to the food supply by fortification (direct addition to food) or biofortification (e.g., addition to animal feed or UVB irradiance of animals or produce) may be appropriate strategies to improve vitamin D intakes and status at the population level [88, 144, 162, 163, 285].

Vitamin D food fortification has been demonstrated as safe and effective in improving circulating 25(OH)D concentrations [194-198], notably in Finland through fortification of fluid milk products and fat spreads, where the prevalence of 25(OH)D < 30 nmol/L has been reduced to almost zero through mandatory fortification [162]. Margarine and some fluid milk products are mandatorily vitamin D-fortified in Sweden [286] and Canada [287], and voluntarily, yet routinely, vitamin D-fortified in the US [163]. In Australia, margarine must be vitamin D-fortified; however, few other foods are voluntarily vitamin D-fortified. Recently, an individual participant data-level (IPD) meta-analysis used linear and non-linear regression analyses to determine a dose-response rate of 1.7 nmol/L change in serum 25(OH)D per μg of vitamin D,

based on trials using vitamin D_3-fortified foods with winter endpoints at latitudes ≥40° [194]. Other meta-analyses have estimated treatment effects (ES) and/or dose-response rates for vitamin D fortification in adults (ES 19.4 nmol/L, dose-response rate 1.2 nmol/L/μg [195]); ES 16.9 nmol/L [197]) and children (ES 15.5 nmol/L, dose-response rate 1.2 nmol/L/μg [198]; dose response rate 2.8 nmol/L/μg, with non-linear relation shown in a quadratic-plateau model [196]). However, they either included supplements and bolus injections such that sub-group analyses were not solely fortification-focused, or included non-blinded studies, leading to questionable quality of pooled analyses. Vitamin D food fortification has been shown to result in a greater increase in serum 25(OH)D concentrations than supplements and bolus injections; therefore, it is not appropriate to use data from supplementation trials to assess the efficacy of fortification trials [196]. No earlier food intervention meta-analyses compared different vitamin D vitamers, which may differ in bioactivity [115, 125, 201, 288].

To our knowledge, a review without limits on season or latitude has not yet been conducted to: include children and adults; investigate potential non-linear responses in adults; include biofortification trials. Hence, the objective of our review was to examine the effect of vitamin D-fortification and -biofortification on circulating 25(OH)D concentrations in children and adults, including stratification by vitamer and linear and non-linear dose-response analyses.

Methods

This systematic review and meta-analysis aimed to investigate the effect of vitamin D-fortified or -biofortified foods or beverages with an equivalent non-vitamin D-fortified control treatment, on serum 25(OH)D concentrations in humans. The systematic review protocol [289] was registered with PROSPERO (registration number CRD42020145497).

Data searches and study selection

The search strategy was designed by a Health Sciences librarian to capture all relevant studies. Database and website searches (Supplemental Figure 1) were conducted (EBSCOhost CINAHL, Ovid MEDLINE, Ovid Embase, Cochrane Central Register of Controlled Trials, PubMed, World Health Organization, Open Grey, MedNar and NTIS) to identify relevant articles available initially in December

2018 and then updated on 25 September 2020. Following screening and removal of duplicates, bibliographies were screened for relevant articles. Randomized controlled trials that investigated the effect of vitamin D food fortification or biofortification on serum 25(OH)D concentrations were included. Exclusion criteria were: clinical illness that may affect gut integrity/vitamin D absorption, duration < 4 weeks, no equivalent placebo for control group, no quantification of daily dose or total intake, fortification dose < 5 μg/d, no baseline or endpoint 25(OH)D measurement, non-random allocation, non-blinding of participants. Two reviewers (ED and LB) reviewed full-text articles against inclusion and exclusion criteria and reasons for exclusion were discussed and logged (Supplemental Figure 1).

Data extraction

Data were extracted independently by ED and APJ; inconsistencies were resolved through discussion. The Jadad Scale, which scores studies on a scale of zero to five based on randomization, blinding and description of withdrawals and dropouts, was used to assess study quality [290]. Where absolute change (mean ± SD) was not reported, it was calculated using baseline and endpoint values. The following conversions were used, where necessary, to convert vitamin D doses to μg and 25(OH)D concentrations to mean ± SD in nmol/L: vitamin D (μg) = (vitamin D (IU))/40; SD = standard error of the mean x √n; SD = [(upper CI – lower CI) x √n]/3.92; [25(OH)D] nmol/L = [25(OH)D] ng/mL x 2.5.

Data analysis

Publication bias analyses, pooled pair-wise analyses, subgroup analyses and dose-response meta-analyses were conducted using Stata software [267]. Publication bias was assessed at the outcome level using Egger's test [291] for asymmetry. Duval and Tweedie's "trim and fill" method [292] was used with Begg's test [293] for asymmetry to adjust for publication bias.

For meta-analysis of effect with all studies combined, we pooled results for multiple study arms within the same publication in fixed effects models to give one intervention outcome per publication. We did the same for effect of fortification by D vitamer to give one intervention outcome per vitamer, per publication. We pooled vitamin D dose, mean 25(OH)D change and SD of mean 25(OH)D change values

using weighting values generated by the fixed effects models (vitamin D $dose_{arm1}$ x ($weighting_{arm1}$/100)) + (vitamin D $dose_{arm2}$ x ($weighting_{arm2}$/100)). Random-effects meta-analyses were undertaken to estimate the summary treatment effects of overall vitamin D fortification and its specific forms on circulating 25(OH)D concentrations [294]. Treatment effects (summarized as effect size (ES) or weighted mean difference (WMD) with 95% CI) were derived from the difference in absolute mean change between intervention and control groups. Forest plots, the I^2 statistic (25, 50 and 75% = low, moderate and high heterogeneity, respectively [295]) and the chi-squared statistic ($P < 0.1$ = significant heterogeneity [296]) were used to test heterogeneity.

We conducted sub-group analyses by baseline serum 25(OH)D concentration, vitamin D dose, end season of intervention, sex, age, continent, latitude, study duration (< or ≥ 3 months and < or ≥ 6 months), assay, study quality, health condition and added calcium. Using sub-group analysis results, variables with a statistically significant subgroup effect ($P < 0.1$) were selected for univariate meta-regression [297]. Variables showing $P < 0.2$ in univariate meta-regression were included in multivariate meta-regression models.

Dose-response analyses were performed for overall effect and by vitamer using a one-stage random-effects approach [298]. Studies with multiple intervention arms with the same vitamin D dose and one control group were pooled by fixed-effects meta-analysis to give one data point per dose level per publication. For dose-response by vitamer, studies with multiple intervention arms with the same dose of the same vitamer and one control group were pooled using the fixed-effects meta-analysis model to give one data point per vitamer per dose level per publication. We used Stata packages "*drmeta*" and "*drmeta_graph*" to estimate and plot non-linear dose-response effects using restricted cubic splines with 3 knots (10^{th}, 50^{th}, and 90^{th} percentile) [299]. We reported non-linear dose-response effects if the Wald test for departure from linearity was significant at $P < 0.1$.

Results

Literature search

Initial searches retrieved 3081 publications, of which 1658 were duplications: following title and abstract screening, 128 studies remained (Supplemental Figure 1).

No relevant articles were identified through unpublished and grey literature searches. After full text screening for eligibility, 101 publications were excluded. A further seven publications were identified through bibliography searches and the September 2020 search, leaving 34 publications [116-118, 277, 300-329] for meta-analysis.

Study quality, influence and bias

Of 34 publications, 32 were assigned a Jadad Scale score of ≥3 [116-118, 277, 300-310, 312-328]; of these, 24 were assigned the highest possible score of five [116-118, 277, 300, 301, 303, 305-307, 312, 314-316, 318-324, 326-328] (Table 1). Two publications were assigned a Jadad Scale score of two [311]. Compliance rates were reported in 24 publications [116, 117, 277, 300, 301, 303-306, 308-311, 313-316, 319, 322-325, 327, 329], all with average rates >80%.

Influence analysis showed that point estimates of meta-analyses conducted with each study omitted in turn lay within the confidence interval of the treatment effect for all studies combined. Asymmetry in an Egger's test funnel plot suggested the possibility of publication bias ($P = 0.02$): asymmetry was also evident in a Begg's funnel plot using the "trim and fill" method [292] (Supplemental Figure 2 and Supplemental Table 1), while both fixed and random effects meta-analyses filled with the estimated 12 unpublished studies showed significant outcomes for potential publication bias.

Study characteristics

Data from 2398 adults (*n* intervention 1345, *n* controls 1053) and 1532 children (*n* intervention 970, *n* controls 562) who completed vitamin D fortification and biofortification trials were included. Studies were conducted in 18 different countries across four continents (Asia ([306, 308-312, 314-316, 318, 319, 325, 328, 329]), Europe ([116, 118, 277, 300-302, 305, 307, 313, 321, 327]), North America ([117, 303, 317, 320, 322-324, 326]) and Oceania ([304]), spanning latitudes of 3-60°. Twenty-seven publications included adults only [116-118, 277, 300-322], and seven included only children [323-329] (Table 1). Trials were mixed sex [117, 118, 302, 303, 307, 314-320, 322-324, 326-329] or females only [116, 277, 300, 301, 304-306, 308-313, 321, 325]: none included only males. The majority of studies (*n* = 19) recruited healthy, free-living participants [116-118, 277, 303-305, 307, 309, 316, 320, 322-329]. Four studies recruited postmenopausal women [308, 310, 311, 313], and three recruited people with type 2 diabetes [315, 318, 319]. Various conditions and circumstances (frail elderly [302], institutionalized elderly [301], women in sheltered accommodation [300], postmenopausal women with

Table 1 Summary of selected randomized controlled trials investigating the effect of vitamin D food fortification or biofortification in adults and children

Study	Location	Population	Age, y	Duration, wk	Season	25(OH)D analysis	Jadad Scale score
Adults							
Biancuzzo 2010 (45)	USA (Boston, MA, 42° 21' N)	Healthy adults	18-84	11	Winter 2007	LC-MS/MS	5
Bonjour 2013 (47)	France (location/s not specified)	Institutionalised elderly women	≥60	8	Winter 2011	ELISA	5
Bonjour 2015 (46)	England (Hull, 53° 44' N)	Women in sheltered accommodation	>60	12	Summer 2012	ELISA	5
de Jong 1999 (48)	Netherlands (Wageningen, 51° 58' N)	Frail elderly	≥70	17	NR	CPBA	3
Fisk 2012 (49)	England (London, 51° 30' N)	Healthy adults	18-65	4	Winter 2011	LC-MS/MS	5
Gaffney-Stomberg 2014 (50)	USA (Fort Sill, OK, 34° 42' N)	Recent army recruits	18-42	9	Winter-spring 2013	RIA	5
Green 2010 (51)	New Zealand (Dunedin, 45° 52' S)	Non-pregnant young women	18-45	12	Summer-autumn	RIA	4
Grønborg 2019 (52)	Denmark (Copenhagen, 55° 44' N)	Women of Danish and Pakstani origin	18-50	12	Winter 2016	LC-MS/MS	5
Itkonen 2016 (53)	Finland (Helsinki, 60° 11' N)	Healthy young women	20-37	8	Winter 2014	LC-MS/MS	5
Jafari 2016 (54)	Iran (Isfahan, 32° 39' N)	Diabetic post-menopausal women	NR	12	Autumn 2013-winter 2014	ELISA	5
Keane 1998 (55)	Ireland (Dublin, 53° 21' N)	Community-based elderly	66-91	52	Spring 1993-autumn 1994	CPBA	5
Kruger 2010 (59)	Indonesia (Jakarta, 6° 12' S) and Philippines (Manila, 14° 35' N)	Postmenopausal South East Asian women	>55	16	NR	CLIA	2
Kruger 2012 (58)	China (Beijing, 39° 54' N)	Healthy Chinese postmenopausal women	>55	12	Winter-spring	CLIA	3
Kruger 2018 (56)	Malaysia (location not reported)	Chinese postmenopausal women	>55	52	NR	LC-MS/MS	3
Kruger 2019 (57)	Malaysia (location not reported)	Chinese premenopausal women	30-50	52	N	LC-MS/MS	3
Li 2016 (60)	China (Cangzhou, 38° 19' N)	Women with gestational diabetes mellitus	24-32	16	Winter-spring 2010-2014	NR	5
Manios 2017 (61)	Greece (Athens, 37° 59' N)	Postmenopausal women	55-75	8	Winter-spring 2015	LC-MS/MS	4
Mohammadi-Sartang 2018 (62)	Iran (Shiraz, 29° 36' N)	Adults with metabolic syndrome	20-65	10	Winter-spring 2017	ELISA	5
Nikooyeh 2011 (63)	Iran (location not specified)	Adults with type 2 diabetes	30-60	12	Autumn 2009-winter 2010	HPLC	5
Nikooyeh 2016 (64)	Iran (location not specified)	Healthy adults	20-60	8	Winter 2015	HPLC	5
Rosenblum 2012 (65)	USA (Boston, MA, 42° 21' N)	Healthy overweight and obese adults	18-65	16	Summer 2005-spring 2006 and spring 2006-winter 2006	RIA	3
Salehi 2018 (66)	Iran (Shiraz, 29° 36' N)	Adults with type 2 diabetes	≤75	9	Winter 2015	ELISA	5
Shab-Bidar 2011 (67)	Iran (Tehran, 35° 41' N)	Adults with type 2 diabetes	25-70	12	Autumn 2010-winter 2011	HPLC	5
Tangpricha 2003 (68)	USA (Boston, MA, 42° 21' N)	Healthy adults	22-60	12	Spring	CPBA	5
Toxqui 2013 (69)	Spain (Madrid, 40° 24' N)	Iron-deficient women	18-35	16	NR	ELISA	5
Tripkovic 2017 (70)	England (Surrey, 51° 14' N)	South Asian or white European women	20-64	12	Winter 2011/2012 and winter 2012/2013	LC-MS/MS	5
Wagner 2008 (71)	Canada (Toronto, 43° 39' N)	Healthy adults	18-60	8	Winter 2006	RIA	5
Children							
Brett 2016 (72)	Canada (Montreal, 45° 30' N)	Healthy children	2-8	12	Winter 2014	CLIA	5
Brett 2018 (73)	Canada (Montreal, 45° 30' N)	Healthy children	2-8	24	Autumn 2014-winter 2015	LC-MS/MS	5
Du 2004 (74)	China (Beijing, 39° 54' N)	Healthy girls	10	104	Spring 1999-winter 2001	CPBA	4
Economos 2014 (75)	USA (Boston, 42° 21' N)	Healthy children	6-10	12	Winter-summer 2005 and 2006	CLIA	5
Hower 2013 (76)	Germany (Mulheim 51° 25' N and Dusseldorf 51° 13' N)	Healthy children	2-6	32	Autumn 2010-summer 2011	CLIA	5
Khadgawat 2013 (77)	India (Delhi, 28° 39' N)	Healthy school children	10-14	12	NR	CLIA	5
Rich-Edwards 2011 (78)	Mongolia (Ulaanbaatar 47° 55' N)	School children	9-11	7	Winter 2009	LC-MS/MS	2

25(OH)D, 25-hydroxyvitamin D; CLIA, chemiluminescence immunoassay; CPBA, competitive protein-binding assay; ELISA, enzyme-linked immunosorbent assay; HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography with tandem mass spectrometry; NR, not reported; RIA, radio-immunoassay

type 2 diabetes [306], iron deficiency [321], metabolic syndrome [314], overweight and obesity [317] and gestational diabetes mellitus [312]) were represented in the remaining eight studies. Study participants were aged <18 [323-329], ≥ 18 [116-118, 277, 303-306, 309, 312, 314-317, 319-322] and ≥ 55 ([300-302, 307, 308, 310, 311, 313, 318]) years in 7, 17 and 9 publications, respectively. Study duration ranged from 4 to 104 weeks. Twenty-two publications concluded their intervention in winter/spring [116-118, 277, 301, 303, 305, 310, 312-320, 322-325, 329] while six concluded in summer/autumn [300, 304, 306, 307, 326, 327]: seasons of intervention were not specified in six publications [302, 308, 309, 311, 321, 328]. Various 25(OH)D assays were used (chemiluminescence immunoassay [310, 311, 323, 326-328], competitive protein-binding assay [302, 307, 320, 325], enzyme-linked immunosorbent assay [300, 301, 306, 314, 318, 321], high performance liquid chromatography [315, 316, 319], liquid chromatography-tandem mass spectrometry [LC-MS/MS] [116-118, 277, 305, 308, 309, 313, 324, 329] and radio-immunoassay [303, 304, 317, 322] and unspecified [312]).

Interventions

Various food fortification vehicles were used (Table 2). Most studies used a single food per study arm: milk [307, 318, 321, 325, 327-329], milk powder [304, 308-311], milk-based drinks [118], yogurt [300, 301, 306, 312, 314, 315, 319], cheese [313, 322], fruit juice [116, 117, 317, 320, 326], biscuits [116], snack bars [303], crisp breads and lavash bread [316]. Three studies administered multiple foods within the same study arm every day (egg, yogurt, cheese and crisp breads [305], and yogurt and cheese [323, 324]). One study provided a range of fortified foods (fruit juice, compote, custard, two fruit yogurt options, custard or cheese curd with fruit) from which participants selected two daily [302]. Only one study used biofortified food (bread containing UVB-irradiated yeast) [277]. Three publications studying adults included ergocalciferol (vitamin D_2) and cholecalciferol (vitamin D_3) treatments as separate study arms [116-118], while another publication including adults examined only vitamin D_2 [277]. Twenty-two (17 adults, 5 children) examined only vitamin D_3 [300-306, 312-320, 322-325, 328, 329]. Eight publications (6 adults, 2 children) did not specify vitamer [307-311, 321, 326, 327], and none used 25(OH)D_2 or 25(OH)D_3. Vitamin D doses ranged from 3.3-100 μg/d (overall mean dose weighted by *n* participants = 16.2 μg/d: adults 18.9 μg/d; children 11.8 μg/d). Only one biofortification trial met inclusion criteria [277].

Table 2 Foods, added vitamin D and circulating 25(OH)D concentrations from randomized controlled trials investigating the effect of vitamin D food fortification and biofortification in adults and children[1]

		Intervention group								Control group				
Study	Food	Added ergocalciferol, µg/d	Added cholecalciferol, µg/d	Added vitamin D (form not specified), µg/d	Added Ca, mg/d	Baseline 25(OH)D, nmol/L	Endpoint 25(OH)D, nmol/L	Absolute mean change in 25(OH)D, nmol/L	*n*	Added Ca, mg/d	Baseline 25(OH)D, nmol/L	Endpoint 25(OH)D, nmol/L	Absolute mean change in 25(OH)D, nmol/L	*n*
Adults														
Biancuzzo 2010 (45)	Orange juice		25		350	44.8 ± 27.8	76.8 ± 21.2	32.0 ± 25.2	18	350	49.5 ± 24.0	45.2 ± 16.0	-4.2 ± 14.5	15
	Orange juice	25			350	39.5 ± 25.0	66.0 ± 18.5	26.5 ± 18.0	17					
Bonjour 2013 (47)	Yogurt		10		800	19.2 ± 6.5	44.6 ± 13.5	25.3 ± 9.7	29	280	16.2 ± 3.1	21.4 ± 14.0	5.2 ± 13.0	27
Bonjour 2015 (46)	Yogurt		10		800	34.1 ± 11.8	56.2 ± 11.9	22.0 ± 12.4	24	280	35.1 ± 12.0	41.3 ± 14.3	6.2 ± 7.7	24
de Jong 1999 (48)	Fruit and dairy products		7.5[2]			37.0 ± 20.0	NR	35.0 ± 18.0	37		36.0 ± 20.0	NR	5.0 ± 9.0	34
Fisk 2012 (49)	Malted milk drink	4.8				48.0 ± 26.6	NR	4.9 ± 10.8	8		33.5 ± 13.3	NR	-3.4 ± 7.0	8
	Malted milk drink		5.2			31.3 ± 22.1	NR	11.9 ± 12.5	7					
	Malted milk drink	7.5				41.9 ± 14.1	NR	13.6 ± 13.6	8					
	Malted milk drink		10			30.9 ± 29.1	NR	19.7 ± 14.9	8					
Gaffney-Stomberg 2014 (50)	Snack bar		27.3		1032	58.0 ± 25.8	70.0 ± 15.0	12.0 ± 19.1	83		51.5 ± 20.2	61.5 ± 13.8	10.0 ± 15.5	85
Green 2010 (51)	Milk powder		5			76.0 ± 32.6	65.0 ± 23.1	-11.0 ± 27.4	32		74.0 ± 30.6	53.0 ± 23.8	-21.0 ± 27.3	34
Grønborg 2019 Danish (52)	Egg, yogurt, cheese, crisp breads		20			53.3 ± 17.0	77.8 ± 14.0	26.4 ± 16.0	31		46.2 ± 19.0	44.0 ± 17.0	-2.8 ± 9.0	35
Grønborg 2019 Pakistani (52)	Egg, yogurt, cheese, crisp breads		20			44.5 ± 21.0	54.7 ± 18.0	10.5 ± 18.0	33		49.0 ± 23.0	36.5 ± 16.0	-11.2 ± 12.0	37
Itkonen 2016 (53)	Biofortified bread	26.3				64.6 ± 15.1	66.4 ± 16.5	1.7 ± 3.1	9		66.2 ± 18.6	NR	0	7
Jafari 2016 (54)	Low-fat yogurt		50			62.2 ± 24.6	86.8 ± 26.7	24.6 ± 25.4	30		62.7 ± 23.0	56.1 ± 15.6	-6.6 ± 19.5	29
Keane 1998 (55)	Milk			5		24.0 (<13.8-31.8)	46.2 (24-66.8)	22.3 ± 10.9	24		25.0 (<13.8-32.2)	31.8 (<10-60.2)	6.8 ± 10.9	18
Kruger 2010 Philippines (59)	High-calcium milk powder			9.6	1200	62.0 ± 15.7	86.1 ± 17.0	24.1 ± 16.4	30	54	59.2 ± 15.7	71.0 ± 17.0	11.8 ± 16.4	30
Kruger 2010 Indonesia (59)	High-calcium milk powder			9.6	1200	45.1 ± 11.0	57.8 ± 11.4	12.7 ± 11.0	27	54	43.3 ± 11.0	37.2 ± 11.6	-6.2 ± 11.2	29
Kruger 2012 (58)	High-calcium milk powder			6.4	900	33.1 ± 15.5	39.5 ± 10.4	6.4 ± 12.8	27		29.3 ± 12.03	28.2 ± 11.1	-1.1 ± 11.6	31
Kruger 2018 (56)	Milk powder			15	1200	62.3 ± 17.0	74.8 ± 19.0	12.5 ± 17.4	48	500	64.8 ± 17.3	63.1 ± 19.8	-1.7 ± 18.0	47
Kruger 2019 (57)	Milk powder			15	1000	53.2 ± 16.1	60.8 ± 16.0	7.6 ± 15.4	58	500	48.6 ± 16.1	55.0 ± 15.8	6.4 ± 15.2	55
Li 2016 (60)	Yogurt		25			42.0 ± 11.5	73.8 ± 14.3	31.8 ± 10.8	48		40.5 ± 8.5	39.8 ± 11.2	-0.8 ± 10.2	49
Manios 2017 (61)	Gouda-type cheese		5.7			47.3 ± 15.2	52.5 ± 12.0	5.1 ± 5.6	40		42.9 ± 17.7	38.3 ± 18.9	-4.6 ± 5.7	39
Mohammadi-Sartang 2018 (62)	Yogurt		25		1000	65.1 ± 34.9	98.6 ± 28.8	33.5 ± 18.8	44	600	65.2 ± 32.6	70.2 ± 31.9	2.9 ± 9.6	43
Nikooyeh 2011 (63)	Yogurt drink		25			44.4 ± 28.7	77.7 ± 28.6	33.3 ± 28.6	30		41.6 ± 44.5	37.2 ± 44.0	-4.4 ± 44.2	30
	Yogurt drink + calcium	25			500	44.5 ± 43.7	74.6 ± 39.5	30.1 ± 41.8	30					
Nikooyeh 2016 (64)	Lavash bread		25			33.9 ± 21.9	72.9 ± 23.1	39.0 ± 22.5	30		34.7 ± 30.5	25.4 ± 21.8	-9.3 ± 27.1	30
Rosenblum 2012 (65)	Regular orange juice		7.5		1050	65.0 ± 25.0	NR	12.3 ± 37.5	31	60	67.5 ± 32.5	NR	0.8 ± 25.2	34
	Lite orange juice		7.5		1050	77.5 ± 30.0	NR	5.3 ± 29.3	35	60	82.5 ± 32.5	NR	-17.5 ± 36.0	31

Salehi 2018 (66)	Milk		25			42.5 ± 27.5	77.5 ± 47.5	35.0 ± 50.0	51		40.0 ± 30.0	50.0 ± 42.5	10.0 ± 42.5	50
Shab-Bidar 2011 (67)	Yogurt drink		25		340	38.5 ± 20.2	72.0 ± 23.5	32.6 ± 18.3	50	340	38.0 ± 22.8	33.4 ± 22.8	-2.7 ± 16.6	50
Tangpricha 2003 (68)	Orange juice		25		350	37.0 ± 29.9	94.0 ± 74.8	57.0 ± 26.2	14	350	50.0 ± 34.6	73.0 ± 27.7	22.5 ± 17.3	12
Toxqui 2013 (69)	Fe + D fortified skim milk			5		62.3 ± 20.8	71.1 ± 21.1	8.6 ± 11.2	54		62.9 ± 20.8	63.2 ± 18.3	0.5 ± 11.9	55
Tripkovic 2017 (70)	Juice	15				44.9 ± 29.6	59.7 ± 24.0	14.8 ± 27.2	67		44.8 ± 30.0	33.5 ± 23.6	-11.3 ± 27.4	65
	Biscuit	15				46.1 ± 30.1	61.9 ± 24.2	15.8 ± 27.7	66					
	Juice		15			42.3 ± 29.4	74.0 ± 25.2	31.7 ± 27.5	70					
	Biscuit		15			41.9 ± 29.2	73.0 ± 24.6	31.1 ± 27.2	67					
Wagner 2008 (71)	Cheese		100[3]			50.7 ± 18.9	NR	65.3 ± 24.1	20		55.0 ± 25.3	50.7 ± 24.2	-4.3 ± 9.0	20
	Low-fat cheese		100[3]			57.5 ± 18.4	NR	69.4 ± 21.7	10					
Children														
Brett 2016 (72)	Yogurt drink and cheddar cheese		6.25			59.2 ± 13.2	64.2 ± 10.0	3.7 ± 15.3	25		58.3 ± 14.5	55.8 ± 14.5	-2.5 ± 6.2	24
	Yogurt drink and cheddar cheese		11.25			60.4 ± 10.5	63.7 ± 12.4	3.2 ± 7.3	25					
Brett 2018 (73)	Yogurt drink and cheddar cheese		11.25			65.3 ± 12.2	58.4 ± 8.7	-6.9 ± 1.9	26		67.5 ± 15.1	56.6 ± 13.9	-10.9 ± 14.2	23
Du 2004 (74)	Milk		3.33[4]		245	20.6 ± 8.8	47.6 ± 23.4	27.0 ± 20.5	113		17.7 ± 8.7	17.9 ± 9.0	0.2 ± 8.9	111
Economos 2014 (75)	Orange juice CaD			5	700	64.2 ± 92.8	92.8 ± 36.0	28.6 ± 24.6	53	700	64.3 ± 20.9	80.8 ± 30.5	16.5 ± 26.5	48
	Orange juice CaDEA			5	700	75.6 ± 27.2	95.9 ± 27.7	20.4 ± 25.2	40					
Hower 2013 (76)	Growing up milk			10		54.5 ± 19.2	72.8 ± 15.0	16.5 ± 20.7	35		53.0 ± 19.7	68.0 ± 12.7	10.5 ± 26.2	18
Khadgawat 2013 (77)	Milk		15			28.6 ± 13.1	57.2 ± 16.9	28.6 ± 15.2	243		29.4 ± 13.1	27.1 ± 13.1	-2.3 ± 12.9	237
	Milk		25			29.8 ± 14.0	69.2 ± 21.2	39.4 ± 18.6	233					
Rich-Edwards 2011 (78)	US UHT milk		7.5			25.0 ± 12.5	72.5 ± 25.0	47.5 ± 21.6	37		20.0 ± 10.0	20.0 ± 10.0	0.0 ± 10.0	101
	Mongolian pasteurised milk		7.5			20.0 ± 10.0	50.0 ± 15.0	30.0 ± 13.2	140					

[1]Circulating 25(OH)D concentrations (baseline, endpoint and absolute mean change) are presented as mean ± SD or mean (range). 25(OH)D, 25-hydroxyvitamin D; NR, not reported
[2]Dose data taken from de Jong et al. 2000 (88)
[3]Provided as a weekly dose of 700 µg
[4]Dose per daily portion was 5-8 µg/d; however, milk was not provided during weekends or school holidays

Meta-analyses

Overall effect of interventions

Combining data from the 34 publications indicated a treatment effect (WMD) of 21.2 nmol/L (95% CI 16.2, 26.2); however, heterogeneity was high (I^2 = 96%, chi-squared $P < 0.001$) (Figure 1). Cumulative meta-analysis with random effects indicated that the ES of the earliest included study (1998: daily vitamin D dose 5 µg) [307] was ~6 nmol/L lower than the cumulative ES following addition of the two most recent studies (2019: daily doses 15 and 20 µg) [305, 309] (Supplemental Figure 3).

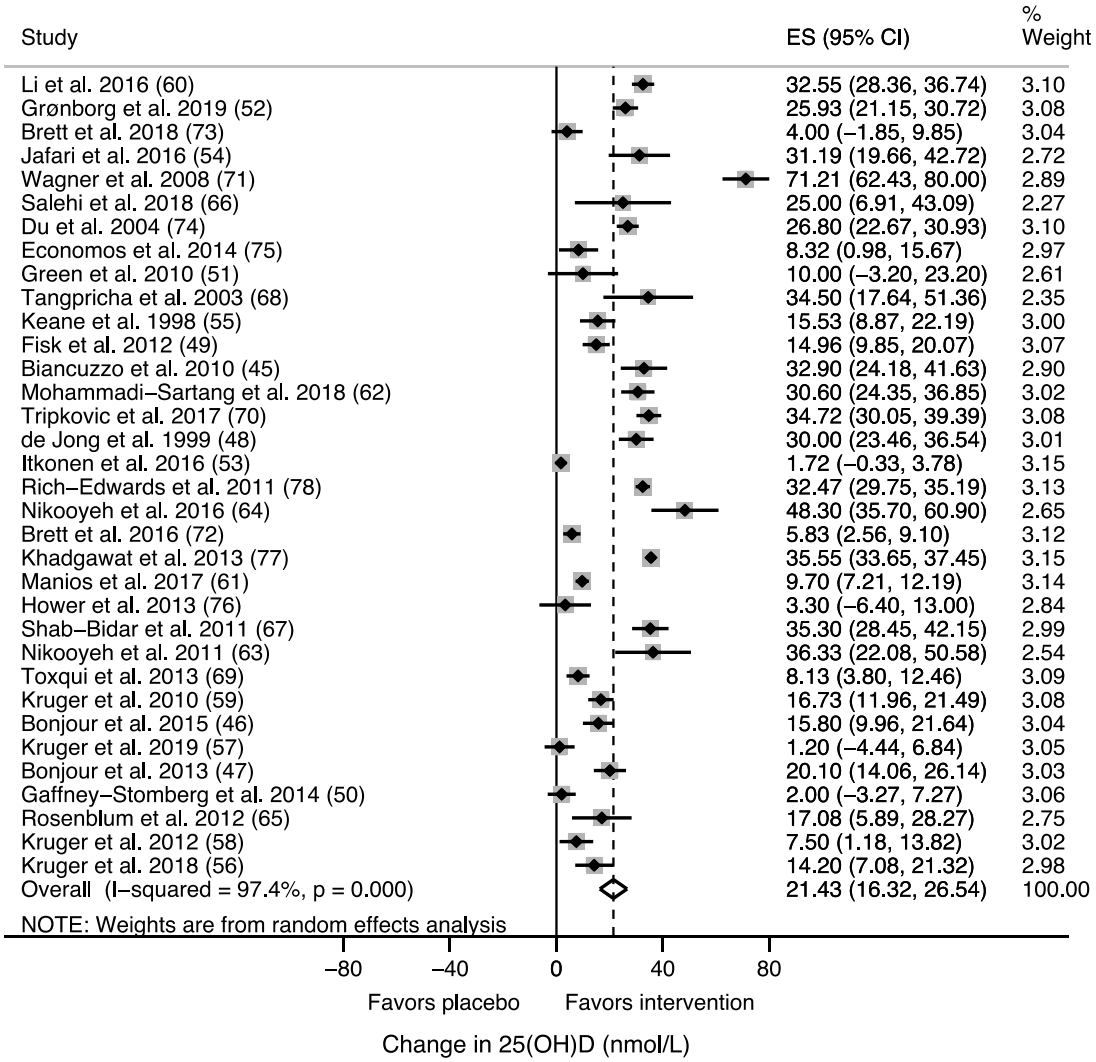

Figure 1 Forest plot for change in circulating 25(OH)D concentrations associated with vitamin D food fortification and biofortification in 34 randomized controlled trials. Treatment effect (ES) values are weighted mean difference (95% CI). 25(OH)D, 25-hydroxyvitamin D

Sub-group analyses for overall effect

Sub-group analyses revealed variations in treatment effects; however, heterogeneity remained high and, in most instances where the ES varied within sub-groups, the daily vitamin D dose varied similarly (Table 3). The ES was considerably greater for baseline circulating 25(OH)D concentrations < 50 nmol/L than ≥ 50 nmol/L and vitamin D treatment dose ≥ 10 μg/d than < 10 μg/d.

Sub-group analyses for overall effect: health and diabetes

When studies were pooled by health condition and circumstance (n = 19), the overall ES for healthy participants without any other circumstance (mean vitamin D dose 18 μg/d) was similar to the overall ES. In contrast, the ES for the three pooled studies including participants with type 2 diabetes (mean vitamin D dose 25 μg/d) was 34.2 nmol/L (95% CI 28.1, 40.3) with low heterogeneity (I^2 = 0%; chi-squared P = 0.566). One study involving participants with gestational diabetes mellitus (vitamin D dose 25 μg/d) had a similarly high ES to the type 2 diabetes trials. When all five studies including participants with any form of diabetes were pooled, the ES remained high with low heterogeneity compared to the pooled ES for all other studies; however, the mean daily dose of vitamin D was correspondingly high (Table 3).

Sub-group analyses for overall effect: effect of addition of calcium to treatment foods

In order to investigate whether the addition of calcium affected the vitamin D treatment effect, we compared the ES between studies in publications that used calcium-enriched treatment foods [117, 300, 301, 303, 308-311, 314, 317, 319, 320, 325, 326] with those that did not [116, 118, 277, 302, 304-307, 312, 313, 315, 316, 318, 321-324, 327-329]. In some studies, calcium concentrations were greater in treatment foods than control foods. With similar mean daily vitamin D doses, studies where the calcium dose was ≥ 500 mg/d greater in the treatment group than the control group had a considerably lower overall treatment than those where the calcium dose was < 500 mg/d greater in the treatment group than the control group (Table 3).

Table 3 Sub-group analysis of overall effect of vitamin D food fortification/biofortification on circulating 25-hydroxyvitamin D concentrations in adults and children for 34 studies included in meta-analysis with corresponding mean daily vitamin D dose[1]

Variable	Publications, *n*	Pooled WMD, nmol/L	Weight, %	*P*-value	I^2, %[2]	Vitamin D dose, µg/d[3]
Low vitamin D status[4]						
Yes	18	26.5 (20.8, 32.2)	52.7	<0.001	95	15
No	16	14.9 (8.62, 21.2)	47.3	<0.001	94	18
Overall				<0.001	96	
Daily vitamin D dose ≥10 µg/d						
Yes	20	26.2 (18.3, 34.2)	58.3	<0.001	97	22
No	14	14.6 (8.75, 20.5)	41.7	<0.001	93	6
Overall				<0.001	96	
End season of intervention						
Winter/spring	22	24.19 (17.8, 30.6)	64.2	<0.001	97	18
Summer/autumn	6	14.9 (9.09, 20.8)	17.1	0.04	56	14
Unspecified	6	17.0 (3.72, 30.2)	18.7	<0.001	98	15
Overall				<0.001	96	
Sex						
Mixed	19	24.9 (17.6, 32.2)	54.0	<0.001	95	19
Female	15	16.8 (10.8, 22.8)	46.0	<0.001	96	13
Overall				<0.001	96	
Adult/child						
Adult	27	22.1 (16.8, 27.4)	78.6	<0.001	95	19
Child	7	17.5 (6.93, 28.2)	21.4	<0.001	97	12
Overall				<0.001	96	
Age, y						
<18	7	17.5 (6.93, 28.2)	21.4	<0.001	97	12
≥18	17	24.1 (16.5, 33.6)	48.8	<0.001	97	21
≥55	9	16.0 (11.2, 20.8)	27.1	<0.001	82	12
Unspecified	1	31.2 (19.7, 42.7)	2.8	-	-	50
Intervention duration						
≥3 months	23	19.2 (13.6, 24.8)	67.6	<0.001	94	14
<3 months	11	25.4 (16.0, 34.9)	32.4	<0.001	98	21
Overall				<0.001	96	
Intervention duration						
≥6 months	6	11.6 (1.95, 21.2)	18.2	<0.001	93	9
<6 months	28	23.4 (17.6, 29.2)	81.8	<0.001	97	18
Overall					96	
Assay						
LC-MS/MS	10	17.1 (8.79, 25.4)	30.6	<0.001	97	13
Other	23	22.5 (16.4, 28.6)	66.2	<0.001	95	17
Unspecified	1	32.6 (28.4, 36.7)	3.18	-	-	25
Overall				<0.001	96	
Jadad quality score						
5	24	23.63 (16.9, 30.4)	69.9	<0.001	97	20
4	3	15.9 (2.41, 29.4)	9.08	<0.001	96	4
3	5	12.9 (1.96, 23.9)	14.8	<0.001	91	11
2	2	22.8 (2.99, 42.5)	6.19	<0.001	95	8
Overall				<0.001	96	
Jadad quality score						
High (≥3)	32	21.1 (15.9, 26.3)	93.8	<0.001	96	17
Low (<3)	2	22.8 (2.99, 42.5)	6.19	<0.001	95	8
Overall				<0.001	96	
Continent						
Asia	14	25.9 (19.6, 32.2)	41.2	<0.001	94	18
North America	8	20.5 (8.1, 32.9)	22.6	<0.001	95	22
Europe	11	16.8 (10.6, 22.9)	33.6	<0.001	94	10
Oceania	1	10.0 (-3.29, 23.2)	2.66	<0.001	-	5
Overall				<0.001	96	
Latitude						
<40°C	15	22.6 (15.2, 30.1)	44.3	<0.001	97	19
≥40°C	19	20.0 (13.2, 26.8)	55.7	<0.001	96	12
Overall				<0.001	96	
Latitude, °C						
60	1	1.72 (-0.33, 3.78)	3.24	-	-	26
55	1	29.2 (22.8, 35.6)	3.09	-	-	20
53	2	15.7 (11.3, 20.1)	6.20	0.95	0	8
51	4	22.3 (10.9, 33.7)	11.5	0.001	83	11
47	1	32.5 (28.9, 36.1)	3.20	-	-	8
46	1	20.1 (14.1, 26.1)	3.11	-	-	10
45	3	5.49 (2.08, 8.89)	8.95	0.70	0	8
43	1	71.2 (58.8, 83.6)	2.72	-	-	100
42	4	21.4 (7.33, 35.5)	10.5	0.004	77	12
40	1	8.13 (3.80, 12.5)	3.18	-	-	5
39	2	17.3 (-1.61, 36.2)	6.28	<0.001	96	4

38	1	32.6 (28.4, 36.7)	3.18	-	-	25
37	1	9.70 (7.21, 12.2)	3.23	-	-	6
35	1	35.3 (28.4, 42.2)	3.07	-	-	25
34	1	2.00 (-3.27, 7.27)	3.14	-	-	27
32	3	38.7 (27.3, 50.2)	7.63	0.14	49	33
29	3	33.4 (29.0, 37.8)	8.62	0.21	36	21
14	1	12.3 (4.00, 20.6)	2.99	-	-	10
3	2	7.5 (-5.22, 20.2)	6.18	0.005	87	15
Not specified	1	31.2 (19.7, 42.7)	2.78	-	-	
Overall				<0.001	96	
Health and situation						
Healthy/no condition	19	21.5 (13.6, 29.4)	56.1	<0.001	98	18
Type 2 diabetes	3	34.2 (28.1, 40.3)	7.51	0.57	0	25
Gestational diabetes	1	32.6 (28.4, 38.7)	3.18	-	-	25
Postmenopausal and type 2 diabetes	1	31.2 (19.7, 42.7)	2.78	-	-	50
Postmenopausal	4	10.0 (7.89, 12.2)	12.4	0.52	0	10
Frail	1	30.0 (23.5, 36.5)	3.09	-	-	7.5
Obese/overweight	1	11.6 (-4.14, 27.2)	2.47	-	-	8
Institutionalised	1	20.1 (14.1, 26.1)	3.11	-	-	10
Iron deficient	1	8.13 (3.80, 12.5)	3.18	-	-	5
Sheltered accommodation	1	15.8 (9.96, 21.6)	3.12	-	-	10
Metabolic syndrome	1	30.6 (24.4, 36.8)	3.10	-	-	25
Overall				<0.001	96	
Healthy						
Yes	19	21.5 (13.6, 29.4)	56.1	<0.001	98	16
No/other situation	15	20.8 (15.0, 26.7)	43.9	<0.001	92	17
Overall				<0.001	96	
Diabetes (any type)						
Yes	29	32.9 (29.6, 36.2)	13.5	0.84	0	29
No	5	19.5 (14.1, 24.8)	86.5	<0.001	97	14
Overall				<0.001	96	
Added calcium						
Yes	14	17.7 (11.3, 24.1)	41.5	<0.001	92	14
No	20	23.6 (16.5, 30.8)	58.5	<0.001	98	18
Overall				<0.001	96	
Calcium dose difference[5]						
≥500 mg/d	8	10.4 (5.04, 15.7)	24.1	<0.001	80	15
<500 mg/d	26	24.6 (18.4, 30.7)	75.9	<0.001	97	16
Overall				<0.001	96	

[1]WMD values are presented as mean (95% CI). LC-MS/MS, liquid chromatography with tandem mass spectrometry; WMD, weighted mean difference
[2]I^2, the variation in WMD attributable to heterogeneity (25, 50 and 75% = low, moderate and high heterogeneity)
[3]Mean daily dose weighted to *n* participants
[4]25-hydroxyvitamin D concentrations <50 nmol/L at baseline
[5]Calcium dose for intervention arm <500 mg/d greater or ≥500 mg/d greater than control arm

Meta-analysis: effect of interventions by vitamer

When combining studies by vitamer in the meta-analysis, the overall treatment effect was greater for the 25 studies investigating vitamin D_3 (ES 26.8 nmol/L; 95% CI 21.1, 32.5; $I^2 = 97\%$; chi-squared $P < 0.001$) than for the four studies investigating vitamin D_2 (ES 17.2 nmol/L; 95% CI 2.78, 31.7; $I^2 = 96\%$; chi-squared $P < 0.001$). However, heterogeneity was high (Supplemental Figures 4 and 5).

Sub-group analyses for effect by vitamer

Results of sub-group analyses for vitamin D_3 followed the same trends as for overall effect (Supplemental Table 2). Due to the few vitamin D_2 trials, available, sub-group analyses confirmed that 7 of 15 variables had only one stratum for vitamin D_2 and 7 of 8 remaining variables had strata with only one study (Supplemental Table 3); therefore, we did not conduct meta-regression analyses for vitamin D_2.

Meta-regressions

Univariate meta-regression: overall effect of interventions

Studies were not included in meta-regression where data were missing for age [306], season [302, 308, 309, 311, 321, 328] or assay [312]. We conducted univariate meta-regression analysis for low vitamin D status at baseline (25(OH)D < 50 nmol/L), vitamin D dose, season at end of intervention, sex, adult/child, age, study duration ≥ 3 or < 3 months and ≥ 6 or < 6 months, assay, study quality, continent, latitude, healthy, presence of diabetes, added calcium, and difference in calcium dose between the intervention and control group. Significant predictors (P < 0.2) selected for multivariate meta-regression were: low baseline vitamin D status, daily vitamin D dose, end season, sex, study duration ≥ 6 or < 6 months, diabetes, and difference in calcium dose.

Multivariate meta-regression: overall effect of interventions

Low vitamin D status (effect difference 9.38 nmol/L; 95% CI 0.17, 18.6), daily vitamin D dose ≥ 10 μg (effect difference 12.0 nmol/L; 95% CI 2.40, 21.6), and difference in daily calcium dose ≥ 500 mg (effect difference -11.9 nmol/L; 95 CI -23.6, -0.30) between intervention and control group were significantly associated with the effect of the interventions (Table 4). The joint test for the five covariates gave a P-value of 0.01, indicating that one of the covariates may be associated with the treatment effect size; however, residual I^2 (92%) and adjusted R^2 (37%) statistics suggest that any variance may be due to differences between studies.

Meta-regression for effect of vitamin D_3

Studies were not included in meta-regression where data were missing for age [306], season [302, 328] or assay [312]. We conducted univariate meta regression analysis for the same variables mentioned for overall effect. Vitamin D daily dose, age group and difference in calcium daily dose were the only variables included in multivariate meta-regression. Vitamin D dose and difference in calcium dose showed similar effects as the overall analysis (Table 4). Again, although the joint test P-value indicated the possibility of an association between one of the covariates and the

Table 4 Multivariate meta-regression models for predictors of the effect of vitamin D fortification and biofortification on circulating 25-hydroxyvitamin D concentrations in adults and children[1]

Predictor	Coefficient, nmol/L	*P*-value
All studies combined		
Low vitamin D status[2]	9.38 (0.17, 18.6)	0.046
Fortification dose ≥10 µg/d	12.0 (2.40, 21.6)	0.016
End season: winter/spring versus summer/autumn	0.00 (-0.01, 0.02)	0.46
Sex: female vs mixed	-2.13 (-6.88, 2.61)	0.36
Study duration ≥ 6 months	-7.61 (-19.4, 4.12)	0.19
Any form of diabetes	0.26 (-14.5, 15.0)	0.97
Intervention arm calcium dose ≥500 mg/d greater than control arm calcium dose	-11.33 (-23.5, -0.30)	0.002
Vitamin D_3 studies only		
Fortification dose ≥10 µg/d	13.2 (4.18, 22.2)	0.005
Age group	0.00 (-0.03, 0.03)	0.94
Intervention arm calcium dose ≥500 mg/d greater than control arm calcium dose	-15.1 (-25.7, -4.48)	0.007

[1]Values are coefficient (95% CI)
[2]Serum 25-hydroxyvitamin D concentrations <50 nmol/L

treatment effect size, a high residual I^2 statistic (96%) and R^2 of 29% indicate that any variance could be due to differences between studies.

Dose-response analyses

Wald test for non-linearity was $P < 0.001$ for dose-response analyses conducted using restricted cubic splines for all studies combined, adults, children and for vitamin D_3 only (Figure 2). Thresholds occurred at ~25.6 nmol/L at a dose of ~21 µg/d for all studies combined and at ~29.9 nmol/L at a dose of ~20 µg/d for vitamin D_3. When studies were separated by adults and children, the rate began to slow at a mean difference of ~18 nmol/L 25(OH)D, but at a higher dose (~10 µg/d) in adults than children (~5 µg/d). There were insufficient data to perform cubic regression analysis for vitamin D_2.

Discussion

This systematic review and meta-analysis included data from 3930 adults and children to provide updated information on the effect of vitamin D food fortification interventions and new treatment effect estimates by vitamer. For all studies combined, the mean increase of 25(OH)D concentrations was 21.2 nmol/L, which was greater for vitamin D_3 than vitamin D_2 interventions. Heterogeneity was high, due to variations in population characteristics, and was not fully explained by stratified or meta-regression analyses. Therefore, estimated treatment effects should

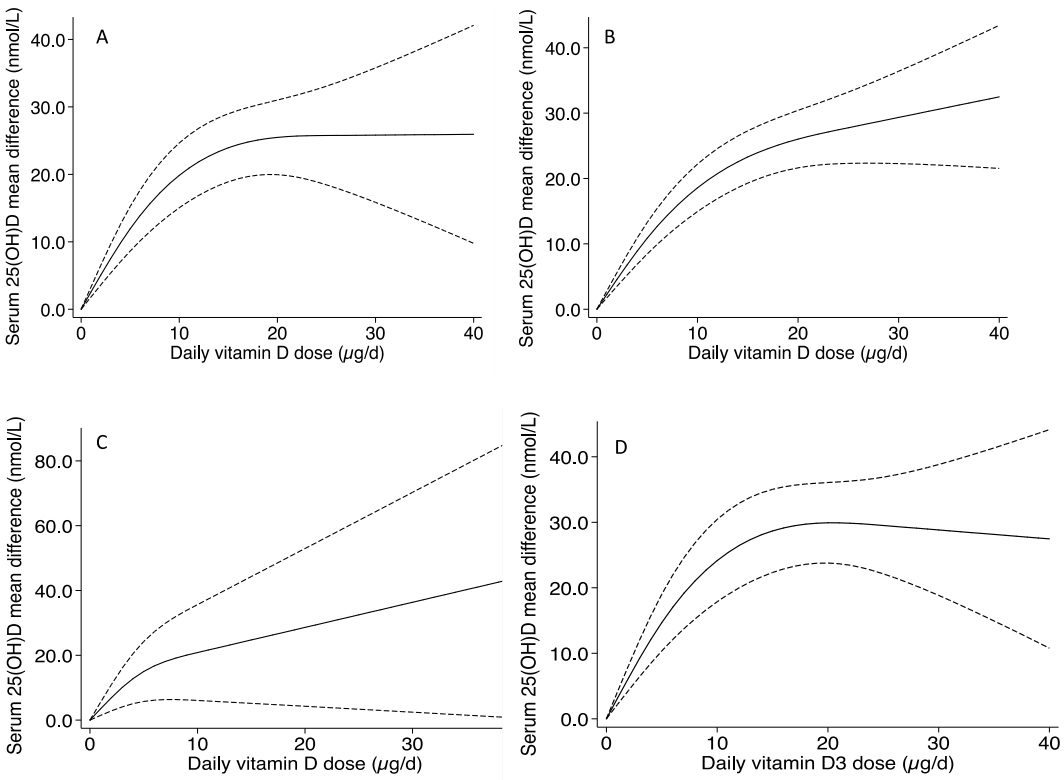

Figure 2 Dose-response results for change in circulating 25(OH)D concentrations associated with vitamin D food fortification and biofortification in randomized controlled trials using restricted cubic spline (with 3 knots at vitamin D doses of 0, 5 and 20 µg/d): (A) Overall (34 publications, 40 data points); (B) Adults (27 publications, 31 data points); (C) Children (7 publications, 9 data points); (D) Vitamin D_3 only (25 publications, 30 data points). *P*-value for non-linearity <0.001 in all four cases. Solid and dotted lines represent the restricted maximum likelihood estimates and 95% confidence intervals, respectively.

be interpreted and used with caution. There was significant non-linearity for all dose-response analyses conducted. Thresholds for mean 25(OH)D values appeared to vary depending on vitamer used and were different between adults and children; however, wide and overlapping 95% confidence intervals meant that it was not possible to draw conclusions on potential variation.

The treatment effect (22.1 nmol/L) and was slightly higher in our present review than the mean increase of 19.4 nmol/L reported in our 2012 review [195]. This may reflect the higher mean dose for adults in the more recent papers available to the current review (~19 µg/d versus ~11 µg/d), which have also added power as well as higher

quality and diversity, lending more confidence to the current estimates. Tangestani and colleagues' meta-analysis reported a lower treatment effect estimate of 16.9 nmol/L in adults [197]. That review did not appear to restrict inclusions based on vitamin D dose or study duration, and it was unclear whether an equivalent placebo food, randomized allocation or double-blinding were requirements for inclusion (several included studies had low Jadad Scale scores of one or two). In children, Al Khalifah and colleagues' meta-analysis produced a lower treatment effect (15.5 nmol/L) compared to our review (17.5 nmol/L); however, Al Khalifah et al. included studies that were either cross-sectional, had daily vitamin D doses < 5 µg/d or undefined daily dose (e.g., ad libitum intake), or used usual diet as a control [198].

Our dose-response analyses, indicating that the effect of vitamin D fortification is non-linear, corroborate the findings of Brett and colleagues [196] for children. We further confirmed that the same appears to be true for adults; however, more evidence is needed to determine whether the shape of dose-response curves differ between vitamers and between adults and children. Consistent with earlier reviews [195, 197], we found that vitamin D food fortification was more effective in those with low baseline 25(OH)D concentrations. Also as expected [195, 196], higher daily vitamin D doses produced greater treatment effects: the effect of using ≥ 10 µg/d on serum 25(OH)D concentrations was consistently higher than using < 10 µg/d.

A unique observation from our analysis was that providing a daily calcium dose ≥ 500 mg/d greater in the intervention than control group generated a markedly lower treatment effect, regardless of whether the vitamin D dose provided was greater or less than 10 µg/d. Regulation of vitamin D conversion to 25(OH)D in humans in the context of calcium availability seems unclear [330, 331]. There was considerable variation in the calcium supplement doses provided (54-1200 mg/d), and in the baseline 25(OH)D concentrations among participants in those studies (approximately 16-82 nmol/L). Therefore, it is possible that the calcium interaction seen in our analysis is an artefact that should be investigated prospectively.

In our analyses, food fortification with vitamin D_3 appeared more effective than with vitamin D_2. In a meta-analysis of supplemental studies, Tripkovic and colleagues found that, when delivered as a bolus dose, vitamin D_3 was more effective than vitamin D_2 in raising circulating 25(OH)D concentrations; however, no significant

difference in efficacy was apparent between the two vitamers with daily supplementation [115]. It may be that some assays used in our included studies underestimated 25(OH)D_2 concentrations, thus underestimating the contribution of vitamin D_2 to overall 25(OH)D concentrations [119] and suggesting a greater gap between the efficacy of vitamin D_2 and vitamin D_3 food fortification. Despite few studies investigating vitamin D_2, our effect estimate for vitamin D_2 was still considerable (17.2 nmol/L). It remains important to assess vitamin D_2-fortified foods (e.g., dairy alternatives and vegan spreads) for people who avoid vitamin D_3-containing foods for cultural, religious or lifestyle reasons.

A major strength of this study and a significant advance since our previous systematic review in 2012, was the number of eligible high-quality publications, which allowed evaluation of effect and dose-response rates by vitamer in adults and children while representing a variety of food fortification vehicles, geographical locations and populations. High heterogeneity remained a limiting factor. There was also evidence of publication bias. Potentially confounding factors (e.g., usual vitamin D and calcium intake, sun exposure, ethnicity), for which sufficient data were unavailable, may have influenced outcomes. An important feature, compliance, was missing from almost one-third of included studies. Variation in the precision and accuracy of 25(OH)D assays is widely acknowledged [43, 45, 46, 332], and may affect outcomes that involve 25(OH)D measurement [333]. As the gold standard is considered to be LC-MS/MS conducted by certified laboratories [42-44], we dichotomized the assay variable as LC-MS/MS or 'other'; however, it should be noted that LC-MS/MS methods conducted by uncertified laboratories may not achieve accurate results while certified laboratories using other assays may. Use of certified laboratories or standardized data [42] in future trials would improve data accuracy. There remains a lack of quality evidence to investigate effects in males, and in African and South American populations. There were insufficient studies to provide meaningful results when stratified by food type and it remains to be clarified whether incorporation of vitamin D into the food matrix through addition or biofortification influences bioavailability. Vitamin D_2 evaluation was limited, while no studies specified use of 25(OH)D_2 or 25(OH)D_3.

In summary, this review provides updated information for use in modeling fortification scenarios that may be helpful in improving vitamin D status at the

population level. It is clear that the addition of vitamin D to food is effective in raising circulating 25(OH)D concentrations in a dose-dependent manner among adults and children, using many food vehicles and in many locations around the globe, regardless of season. The effect on circulating 25(OH)D concentrations was greater when baseline 25(OH)D concentrations were < 50 nmol/L and when the fortification dose was ≥ 10 μg/d. There is much scope for further research to clarify the effects of food matrix, vitamer, and co-supplementation with calcium.

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Author contributions

Eleanor Dunlop: Conducted research, Analyzed data and performed statistical analysis, Wrote the paper. **Mairead Kiely:** Designed the research, Reviewed and edited the paper. **Anthony P James:** Conducted research, Reviewed and edited the paper. **Ngoc Minh Pham:** Analyzed data and performed statistical analysis, Reviewed and edited the paper. **Lucinda J Black:** Designed the research, Conducted research, Reviewed and edited the paper.

Supplemental Table 1 Publication bias results for 34 randomized controlled vitamin D fortification and biofortification trials using Duval and Tweedie's (37) "trim and fill" method[1]

Model	Pooled estimate, nmol/L	Asymptotic		Studies, *n*
		z_value	p_value	
Fixed	9.49 (8.71, 10.3)	23.6	<0.001	46
Random	11.0 (5.38, 16.7)	3.83	<0.001	

[1]Pooled estimates are presented as mean (95% CI)

Supplemental Table 2 Sub-group analysis of overall effect of cholecalciferol food fortification on circulating 25-hydroxyvitamin D concentrations for 25 studies included in meta-analysis with mean daily vitamin D dose[1]

Variable	Publications, *n*	Pooled WMD, nmol/L	Weight, %	*P*-value	I^2, %[2]	Vitamin D dose, µg/d[3]
Low vitamin D status[4]						
Yes	16	29.3 (23.1, 35.4)	63.8	<0.001	95	16
No	9	21.4 (9.8, 33.0)	36.2	<0.001	96	25
Overall				<0.001	96	
Daily vitamin D dose ≥10 µg/d						
Yes	17	30.6 (23.5, 37.6)	67.1	<0.001	95	24
No	8	18.2 (9.37, 26.9)	33.0	<0.001	96	6
Overall				<0.001	96	
End season of intervention						
Winter/spring	20	26.9 (20.4, 33.4)	79.5	<0.001	96	18
Summer/autumn	3	18.9 (8.20, 29.7)	11.8	0.03	71	22
Unspecified	2	33.6 (29.4, 38.8)	8.76	0.12	58	18
Overall				<0.001	96	
Sex						
Mixed	16	27.9 (19.8, 27.0)	62.3	<0.001	96	20
Female	9	24.2 (16.5, 31.9)	37.7	<0.001	95	14
Overall				<0.001	96	
Adult/child						
Adult	20	28.0 (21.4, 34.6)	77.9	<0.001	94	22
Child	5	21.1 (8.92, 33.2)	22.1	<0.001	98	13
Overall				<0.001	96	
Age, y						
<18	5	21.1 (8.92, 33.2)	22.1	<0.001	98	13
≥18	14	30.9 (22.2, 39.6)	53.6	<0.001	93	23
≥55	5	19.3 (11.1, 27.6)	20.5	<0.001	90	13
Unspecified	1	31.2 (19.7, 42.7)	3.81	<0.001	96	50
Overall				<0.001	96	
Intervention duration						
≥3 months	15	25.2 (18.7, 31.8)	60.1	<0.001	94	16
<3 months	10	28.4 (18.2, 38.6)	39.9	<0.001	96	21
Overall				<0.001	96	
Intervention duration						
6+ months	2	15.5 (-6.84, 37.8)	8.75	<0.001	97	5
<6 months	23	27.5 (21.5, 33.5)	91.3	<0.001	96	20
Overall				<0.001	96	
Assay						
LC-MS/MS	7	23.8 (12.8, 34.9)	29.1	<0.001	96	12
Other	18	27.5 (20.9, 34.1)	70.9	<0.001	95	20
Overall				<0.001	96	
Jadad quality score						
5	19	28.4 (21.4, 35.4)	75.4	<0.001	95	23
4	3	15.9 (2.4, 29.4)	12.6	<0.001	96	4
3	2	22.2 (4.35, 40.1)	7.62	0.03	78	8
2	1	32.5 (28.9, 36.0)	4.45	<0.001	-	8
Overall				<0.001	96	
Jadad quality score						
High (≥3)	24	26.2 (20.3, 32.1)	95.6	<0.001	96	19
Low (<3)	1	32.5 (28.9, 36.0)	4.45	-		8
Overall				<0.001	96	
Continent						
Asia	10	32.8 (30.0, 35.6)	39.8	0.019	55	19
North America	7	22.7 (8.63, 36.8)	26.9	<0.001	96	25
Europe	7	23.0 (14.4, 31.6)	29.6	<0.001	94	12
Oceania	1	10.0 (-3.20, 23.2)	3.63	-	-	5
Overall				<0.001	96	
Latitude						
<40°C	11	27.6 (19.3, 36.6)	44.2	<0.001	97	20
≥40°C	14	25.3 (17.3, 33.3)	55.8	<0.001	94	14
Overall				<0.001	96	
Latitude, °C						
55	1	29.2 (22.8, 35.6)	4.28	-	-	20
53	1	15.8 (9.96, 21.6)	4.32	-	-	10
51	3	29.5 (15.9, 43.1)	12.2	0.001	86	12
47	1	32.5 (28.9, 36.0)	4.45	-	-	8
46	1	20.1 (14.1, 26.1)	4.31	-	-	10
45	3	5.49 (2.08, 8.89)	12.4	0.70	0	8

43	1	71.2 (58.8, 83.6)	3.72	-	-	100
42	3	27.5 (11.9, 43.1)	10.1	0.046	68	16
39	1	26.8 (22.7, 30.9)	4.43	-	-	3
38	1	32.6 (28.4, 36.7)	4.42	-	-	25
37	1	9.70 (7.21, 12.2)	4.49	-	-	6
35	1	35.3 (28.4, 42.2)	4.25	-	-	25
34	1	2.00 (-3.27, 7.27)	4.36	-	-	27
32	3	38.7 (27.3, 50.2)	10.4	0.142	49	33
29	3	33.4 (29.0, 37.8)	11.9	0.21	36	21
Overall				<0.001	96	
Health and situation						
Healthy/no condition	14	27.6 (19.2, 36.0)	56.5	<0.001	96.4	17
Type 2 diabetes	3	34.2 (28.1, 40.3)	10.2	0.57	0	25
Gestational diabetes	1	32.6 (28.4, 36.7)	4.42	-	-	25
Postmenopausal and type 2 diabetes	1	31.2 (19.7, 42.7)	3.81	-	-	50
Postmenopausal	1	9.70 (7.21, 12.2)	4.49	-	-	6
Frail	1	30.0 (23.5, 36.5)	4.27	-	-	8
Obese/overweight	1	11.6 (-4.14, 27.2)	3.35	-	-	8
Institutionalised	1	20.1 (14.1, 26.1)	4.31	-	-	10
Sheltered accommodation	1	15.8 (9.96, 21.6)	4.32	-	-	10
Metabolic syndrome	1	30.6 (24.4, 36.8)	4.29	-	-	25
Overall				<0.001	96	
Healthy						
Yes	14	27.6 (19.2, 36.0)	56.5	<0.001	96	17
No/other situation	11	25.0 (17.6, 32.5)	43.5	<0.001	93	20
Overall				<0.001	96	
Diabetes (any type)						
Yes	5	32.9 (29.6, 36.2)	18.4	0.84	0	29
No	20	25.1 (18.7, 31.6)	81.6	<0.001	96	16
Overall				<0.001	96	
Added calcium						
Yes	9	23.2 (15.2, 31.3)	36.1	<0.001	92	16
No	16	28.2 (20.6, 35.9)	63.9	<0.001	97	19
Overall				<0.001	96	
Calcium dose difference[5]						
≥500 mg/d	4	12.4 (2.91, 21.8)	16.3	<0.001	87	18
<500 mg/d	21	29.2 (23.0, 35.3)	83.7	<0.001	96	18
Overall				<0.001	96	

[1]WMD values are presented as mean (95% CI). LC-MS/MS, liquid chromatography with tandem mass spectrometry; WMD, weighted mean difference

[2]I^2, the variation in WMD attributable to heterogeneity (25, 50 and 75% = low, moderate and high heterogeneity)

[3]Mean daily dose weighted to *n* participants

[4]25-hydroxyvitamin D concentrations <50 nmol/L

[5]Calcium dose for intervention arm <500 mg/d greater or ≥500 mg/d greater than control arm

Supplemental Table 3 Sub-group analysis of overall effect of ergocalciferol food fortification/biofortification on circulating 25-hydroxyvitamin D concentrations for 4 studies included in meta-analysis with mean daily vitamin D dose[1]

Variable	Publications, *n*	Pooled WMD, nmol/L	Weight, %	*P*-value	I^2, %[2]	Vitamin D dose, µg/d[3]
Low vitamin D status[4]						
Yes	3	21.6 (7.78, 35.4)	72.9	0.02	83	16
No	1	1.72 (-0.33, 3.78)	27.1	-	-	26
Overall				<0.001	94	
Daily vitamin D dose ≥10 µg/d						
Yes	3	19.2 (-2.03, 40.4)	75.2	<0.001	96	18
No	1	8.30 (-0.62, 17.2)	24.8	-		6
Overall				<0.001	94	
End season of intervention						
Winter/spring	4	16.3 (1.55, 31.0)	100	<0.001	94	17
Overall				<0.001	94	
Sex						
Mixed	2	19.2 (-2.72, 41.2)	48.3	0.002	89	19
Female	2	13.7 (-10.6, 38.1)	51.7	<0.001	96	16
Overall				<0.001		
Adult/child						
Adult	4	16.3 (1.55, 31.0)	100	<0.001	94	17
Overall				<0.001	94	
Intervention duration						
≥3 months	1	26.6 (17.3, 35.9)	24.6	-	-	15
<3 months	3	12.8 (-2.43, 28.0)	75.4	<0.001	92	21
Overall				<0.001	94	
Intervention duration						
<6 months	4	16.3 (1.55, 31.0)	100	<0.001	94	17
Overall				<0.001	94	
Assay						
LC-MS/MS	3	11.7 (-2.70, 26.1)	79.4	<0.001	93	15
Other	1	30.7 (19.4, 42.0)	23.5	-	-	25
Overall				<0.001	94	
Jadad quality score						
5	4	16.3 (1.55, 31.0)	100	<0.001	94	17
Overall				<0.001	94	
Continent						
North America	1	30.7 (19.4, 42.0)	23.5	-		25
Europe	3	11.7 (-2.70, 26.1)	76.5	<0.001		15
Overall						
Latitude						
≥40°C	4	16.3 (1.55, 31.0)	100	<0.001	94	17
Overall				<0.001	94	
Latitude, °C						
60	1	1.72 (-0.33, 3.78)	27.1	<0.001	-	26
51	2	17.4 (-0.53, 35.3)	49.4	0.005	87	14
42	1			-	-	25
Overall				<0.001	94	
Health and situation						
Healthy/no condition	4	16.3 (1.55, 31.0)	100			17
Overall						
Added calcium						
Yes	1	30.7 (19.4, 42.0)	23.5	-	-	25
No	4	11.7 (-2.70, 26.1)	76.5	<0.001	93	15
Overall				<0.001	94	
Calcium dose difference[5]						
<500 mg/d	4	16.3 (1.55, 31.0)	100	<0.001	94	17
Overall				<0.001	94	

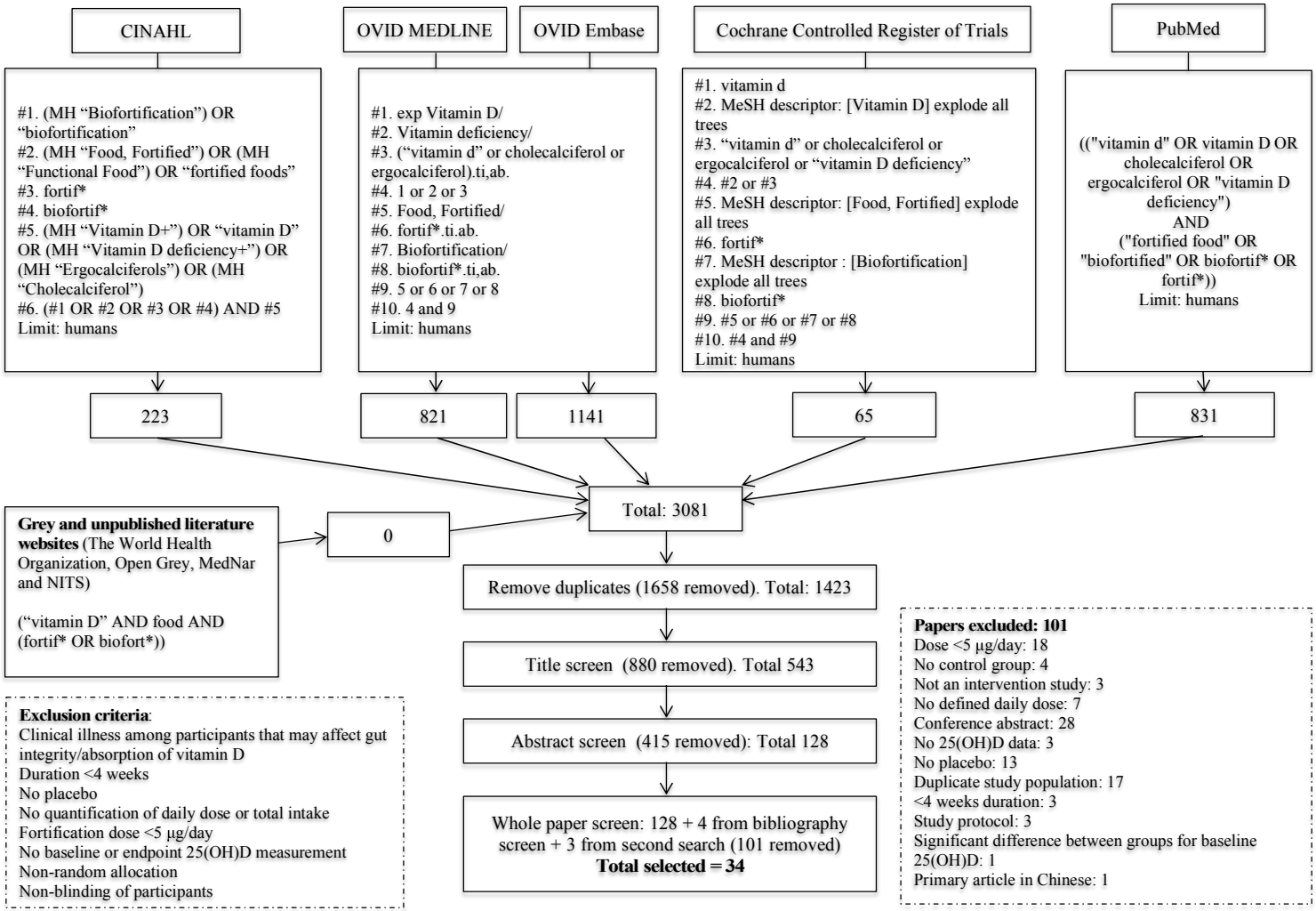
[1]WMD values are presented as mean (95% CI). LC-MS/MS, liquid chromatography with tandem mass spectrometry; WMD, weighted mean difference

[2]I^2, the variation in WMD attributable to heterogeneity (25, 50 and 75% = low, moderate and high heterogeneity)

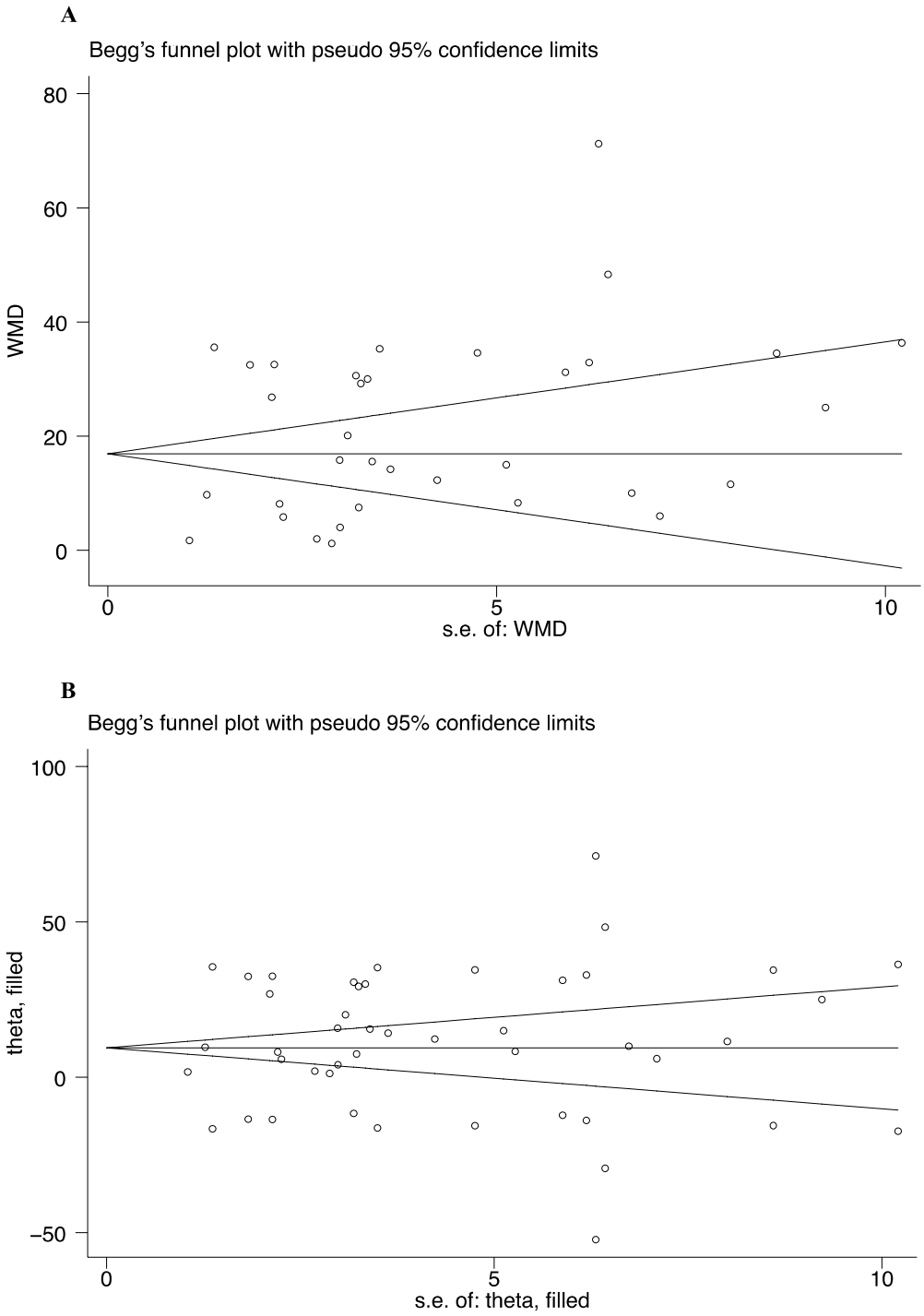
[3]Mean daily dose weighted to *n* participants

[4]25-hydroxyvitamin D concentrations <50 nmol/L

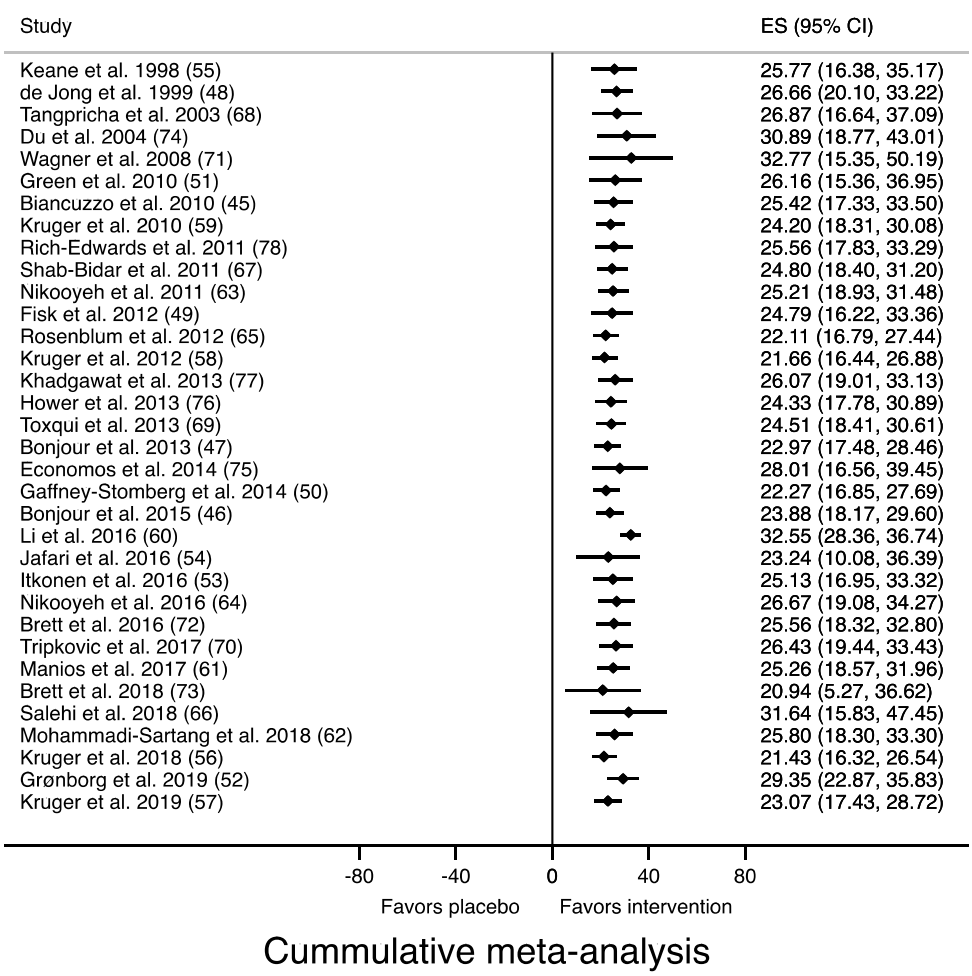
[5]Calcium dose for intervention arm <500 mg/d greater or ≥500 mg/d greater than control arm



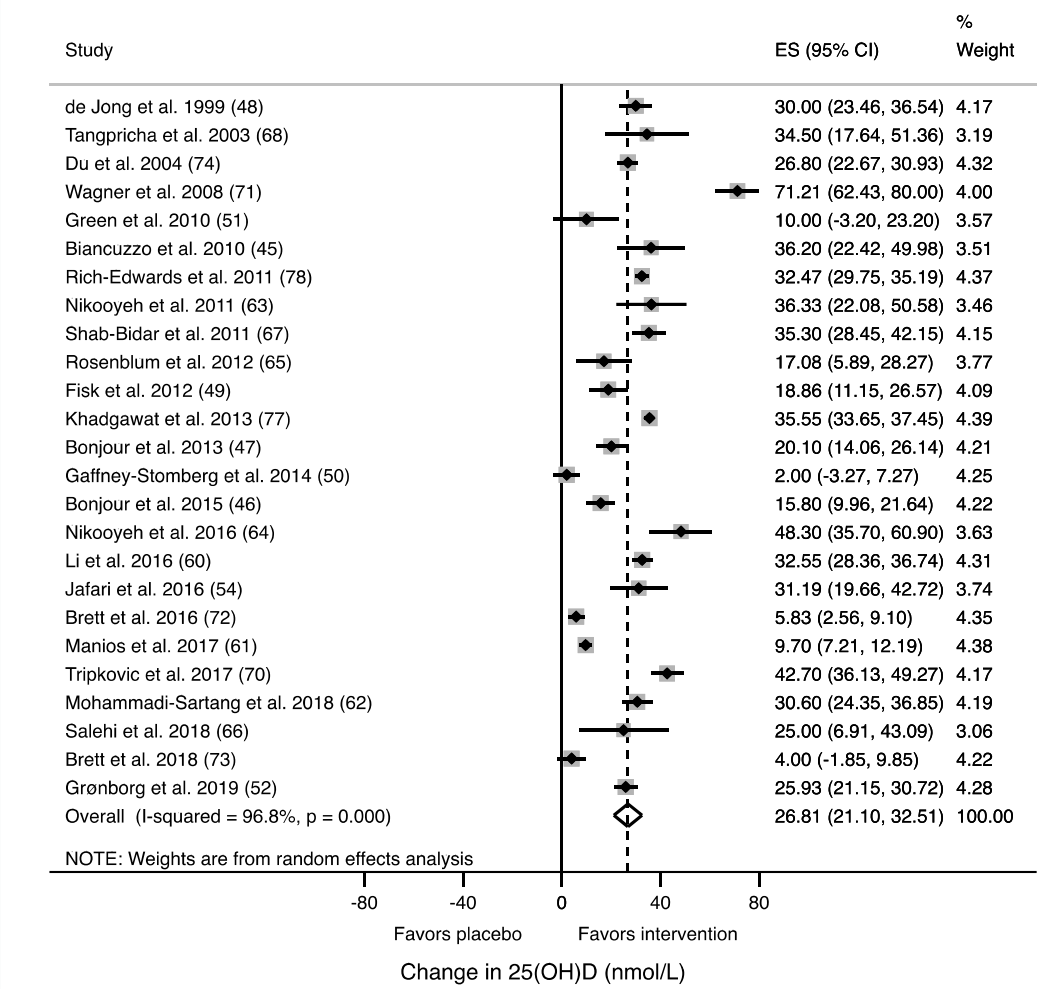
Supplemental Figure 1. Vitamin D food fortification/biofortification efficacy search strategy and study selection procedure



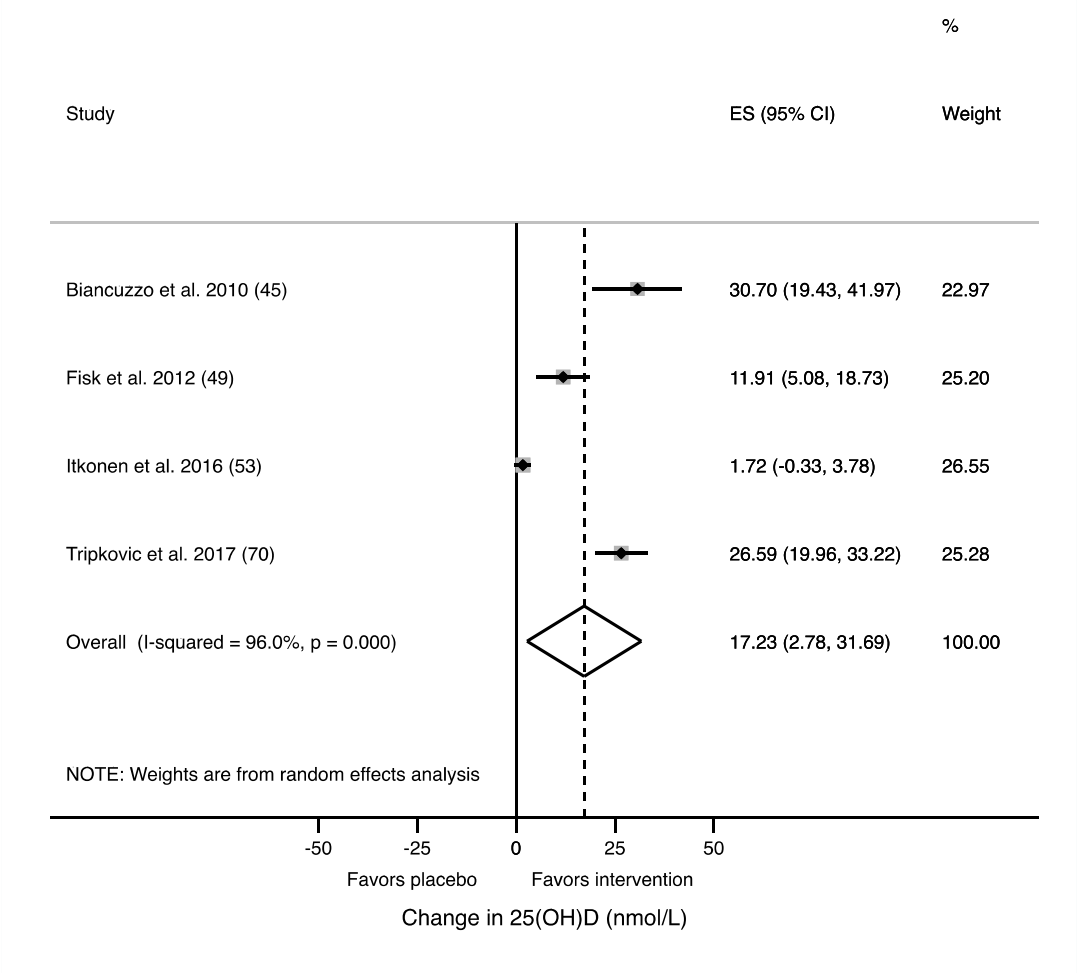
Supplemental Figure 2 Begg's (38) Funnel plots for tests of potential publication bias in a group of 34 publications selected for meta-analysis of randomized controlled trials examining the effect of vitamin D food fortification and biofortification on circulating 25-hydroxyvitamin D concentrations: without trim and fill (A) and with trim and fill (37) (B).



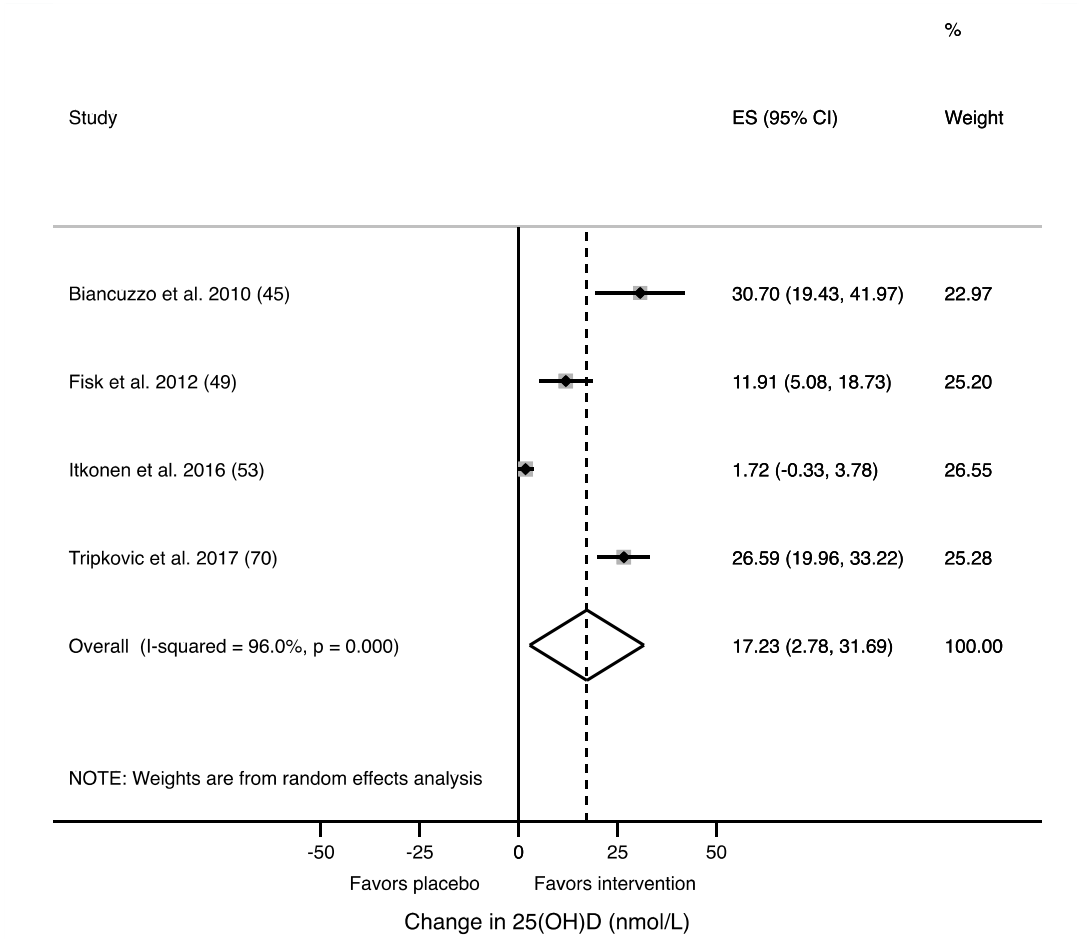
Supplemental Figure 3 Cumulative meta-analysis of 34 randomized controlled trial publications that examined the effect of vitamin D food fortification and biofortification on circulating 25-hydroxyvitamin D concentrations. Treatment effect (ES) values are weighted mean difference (95% CI) of circulating serum 25-hydroxyvitamin D (nmol/L).



Supplemental Figure 4 Forest plot for change in circulating 25(OH)D (nmol/L) associated with cholecalciferol food fortification and biofortification in 25 randomized controlled trials. Treatment effect (ES) values are weighted mean difference (95% CI). 25(OH)D, 25-hydroxyvitamin D



Supplemental Figure 5 Forest plot for change in circulating 25(OH)D (nmol/L) associated with ergocalciferol food fortification and biofortification in 4 randomized controlled trials. Treatment effect (ES) values are weighted mean difference (95% CI). 25(OH)D, 25-hydroxyvitamin D



Supplemental Figure 5 Forest plot for change in circulating 25(OH)D (nmol/L) associated with ergocalciferol food fortification and biofortification in 4 randomized controlled trials. Treatment effect (ES) values are weighted mean difference (95% CI). 25(OH)D, 25-hydroxyvitamin D

Chapter 6: Discussion

This thesis project has fulfilled its objectives and has satisfied the main aim of providing the evidence needed to model vitamin D food fortification strategies for potentially improving vitamin D intakes and status in the Australian population.

6.1. Vitamin D composition of Australian foods

Chapter 2 provides the fundamental vitamin D food composition data that is needed to underpin knowledge around dietary vitamin D in Australia. To our knowledge, this was the first time globally that four D vitamers had been measured in such a broad range of foods, and the publication highlights the importance of measurement of multiple D vitamers across a wide range of food types. The international relevance of the study was evidenced by its publication in *Food Chemistry*, which has an impact factor of 7.5 and is ranked in the top 3% of Food Science journals in Scimago Journal Rankings (2022).

The output of this study has vastly improved the quality and depth of analytical vitamin D food composition data for foods available in Australia. This is especially true for important sources of vitamin D, such as fish, where our new analytical data are more representative of individual fish species. An example of this can be demonstrated in trout and mullet species, for which data are available in the Australian Food Composition Database and in our new analytical dataset, based on the same cooking method. In the Australian Food Composition Database, the same vitamin D_3 (2.0 µg/100 g) and 25(OH)D_3 (0.1 µg/100 g) data are provided for baked mullet and trout. This is because values for both species are derived using a recipe approach based on the same pooled data for raw, non-fatty fish. In our study, samples of these fish species were analysed separately. They were also analysed in the baked form, as they would be consumed, rather than raw, which eliminated the need for conversion factors that may introduce error. We determined vitamin D_3 concentrations of 5.62 and 9.49 µg/100 g, respectively, which are both markedly greater than the 2.0 µg/100 g reported in the Australian Food Composition Database. In mullet, we detected 25(OH)D_3 in trace amounts, whereas a concentration of 0.11 µg/100 g was determined in trout.

The new vitamin D food composition data is reported on the FSANZ website (https://www.foodstandards.gov.au/science/monitoringnutrients/afcd/Pages/Data-provided-by-food-companies-and-organisations.aspx) and will be included in future releases of the Australian Food Composition Database and AUSNUT. The freely available data will allow:

- The general public to look up the vitamin D content of foods;
- Health professionals to assess and the optimise the diets of individuals for vitamin D and to develop public health nutrition resources to promote useful dietary sources of vitamin D;
- Use of the data in a variety of research settings, including estimating vitamin D intakes over time in order to monitor trends;
- Development of food policy, particularly around food fortification and/or biofortification;
- Evaluation of production practices on the vitamin D content of produce;
- Inclusion of vitamin D on more nutrition information panels – currently, only products that are fortified with a known amount of vitamin D include this important nutrient on nutrition information panels;
- Identification and promotion of food sources of vitamin D;
- Development of novel food products targeted at population groups most at risk of vitamin D deficiency.

6.2. Vitamin D composition of Australian game products

The new vitamin D composition data for Australia game products have practical importance in terms of indicating which of the included products may be useful sources of vitamin D, particularly for remote-dwelling Aboriginal and Torres Strait Islander people.

Interestingly, kangaroo meat, which is likely the most widely consumed of the products included in the study, does not appear to offer nutritionally relevant

amounts of vitamin D, whereas camel, crocodile and emu products may be useful sources. This new knowledge is crucial for those who are seeking to increase their vitamin D intake from food and rely on these game products as accessible and nutrient-rich foods. Further, this study revealed some fascinating vitamin D profiles that are unique amongst animal products that have been analysed for vitamin D content in Australia and abroad. These findings challenge assumptions that have been made about the vitamin D content of animal meats.

Prior to this study, it would have been reasonable to assume that animal meats generally contain some vitamin D_3 and some 25(OH)D_3, based on data available internationally for various meat products. Indeed, this assumption was made to estimate the D vitamer concentrations for kangaroo, crocodile and camel meat that are currently provided in the Australian Food Composition Database. Analytical data for various commonly-consumed animal meats (including those from a 2015 analytical program which measured vitamin D in beef, lamb, pork and chicken products [138]) were pooled to estimate values for vitamin D_3 and 25(OH)D_3 in kangaroo, crocodile and camel in the absence of species-specific data. Based on those pooled data, concentrations of 0.2 µg/100 g vitamin D_3 and 0.4 µg/100 g 25(OH)D_3 were assigned for raw kangaroo loin fillet, rump and tail [128]. As the Australian Food Composition Database uses a bioactivity factor of five in calculations of vitamin D equivalents (VDE), the VDE concentration for raw kangaroo meats is shown as 2.4 µg/100 g [128], suggesting that they are reasonable sources of vitamin D. The findings of the two vitamin D composition studies conducted for this thesis project contradict this, suggesting that kangaroo meat is not a useful source of vitamin D and contains trace amounts only of vitamin D_2 and 25(OH)D_2.

Similar to kangaroo, the Australian Food Composition Database reports concentrations of 0.2 µg/100 g vitamin D_3 and 0.5 µg/100 g 25(OH)D_3 for crocodile back leg and tail fillet, and raw camel rump and cube roll, by pooling values for meat from various other animals [128]. These calculated data underestimate the vitamin D_3 content and overestimate the 25(OH)D_3 content of crocodile meat in comparison to our new analytical data and, they do not at all reflect the 25(OH)D_3-dominated profile seen in camel meat in our study.

Data shown in the Australian Food Composition Database for vitamin D in raw emu fan fillet and steak (0.2 µg/100 g vitamin D_3 and 0.7 µg/100 g 25(OH)D_3) were imputed from chicken[128]. This was based on the assumption that the meat of avian species would have a similar vitamin D profile and content. However, based on our new analyses, chicken appears to contain less vitamin D_3, but more 25(OH)D_3 than emu meat. Some emu meat may also contain vitamin D_2; however, we do not yet know whether this is dependent on available food sources, which may differ by geographical location or by wild-caught and farmed emu. Therefore, the D vitamer profiles of emu and chicken may not be as similar as previously assumed.

As with the vitamin D composition study detailed in Chapter 2, this study provides data that are more representative of products from individual species than the pooled data that has been relied upon until now. The new data will allow consumers to make more informed decisions on which foods to eat in order to improve their vitamin D intake, which may be of particular importance to Aboriginal and Torres Strait Islander people living in remote areas. The data will also allow more accurate estimation of vitamin D intakes from these game products.

6.3. Estimation of usual vitamin D intakes in the Australian population

Our new estimates of usual vitamin D intakes are the first to have been made for the Australian population using comprehensive vitamin D food composition data and nationally representative food consumption data. Together with the new vitamin D composition data, these usual intakes estimates will form the basis of future dietary vitamin D research in Australia.

The study's findings indicate that usual intakes of vitamin D in the Australian population are considerably lower than recommendations, regardless of the bioactivity factor applied to 25(OH)D. Assuming a 25(OH)D bioactivity factor of one, there is a ~7-8 µg/day deficit in mean usual intakes in comparison to the Institute of Medicine's EAR of 10 µg/day, and the gap remains at a considerable 4-6.5 µg/day assuming a bioactivity factor of five. A standard 100 g cooked serve [212] of certain fish types (e.g., salmon, trout, mullet, sardine [3]) may deliver enough vitamin D to bridge the gap for some Australians; however, fish is not consumed regularly by a large proportion of the population. In the absence of rich fish sources, a

combination of foods with lower concentrations (e.g., other fish, meats and eggs) would be required daily in quantities unlikely to be realistic for most people. This points to a need for the addition of vitamin D to commonly consumed food/s in order to allow Australians to easily achieve adequacy of vitamin D intake.

Usual vitamin D intakes in Australia also appear to be lower than in the US, Canada and some European countries [38, 60, 144, 145, 243]. The findings further suggest that, due to omission of 25(OH)D, estimates made for populations elsewhere (e.g., the US and Canada) are likely to be underestimated. It has been estimated that, assuming equal bioactivity of the D vitamers, $25(OH)D_3$ contributes 24 and 18% of the total intake of vitamin D from food in Danish children and adults, respectively [180]. The relative contribution of 25(OH)D could be greater still if it has greater bioactivity than vitamin D. Mean intakes estimates of ~5 µg/day made for the US [60] and Canada [145] assuming a 25(OH)D bioactivity factor of one are already greater by 2-3 µg/day than those estimated for the Australian population. If the estimates for the US and Canada were underestimated by ~20%, this would increase the gap between vitamin D intakes in Australia and those countries by a further ~1 µg/day.

It is likely that this gap exists due to differing fortification policies. Fortification of foods with vitamin D is tightly regulated in Australia. Until now, the data required to determine whether vitamin D fortification of a wider range of foods is necessary have not been available. These intakes estimates fill the crucial data gap that has precluded progress being made on dietary strategies to improve vitamin D intakes in the Australian population.

6.4. The effect of food fortification on circulating 25(OH)D

In Chapter 5, new data are presented that further our understanding of how circulating 25(OH)D concentrations are affected through consumption of foods fortified with vitamin D. The vitamin D fortification treatment effect and dose-response data are needed to translate a projected increase in vitamin D intake into an estimate of the effect on vitamin D status. They are based on studies conducted in 18 different countries across four continents, making the data internationally relevant. This is reflected in publication of the study's findings in the highly-regarded *Journal of Nutrition*, which has an impact factor of 4.8 and ranks 11 of 124 journals in the Nutrition and Dietetics category of Scimago Journal Rankings (2022).

Thirty-four publications were included in the systematic review and meta-analysis, providing an overall sample of nearly 4,000 adults and children [334]. This was more than 2.5 times the sample size of 1,513 adults from 15 publications included in an earlier study that was published by our team in 2012. Due to the greater number of available studies, we were able to restrict eligibility to studies that used random allocation, participant blinding and a placebo food that was equivalent to the treatment food. The study confirmed the findings of earlier studies that showed that fortification of foods with vitamin D is effective and, furthermore, that it is most effective in those that need it most, i.e., people with lower vitamin D status. The study described the evidence currently available on this topic, generated treatment effect estimates and, importantly, generated updated dose-response data. The greater number and diversity of the available studies meant that we were able to conduct analyses by adult and child and, for the first time, by D vitamer. We were able to also, for the first time, determine a non-linear dose-response relation in adults.

In the Australian setting we have already used the dose-response data generated by this thesis project to model a fortification scenario (described in Section 6.6) and estimate its effect on vitamin D intakes. The dose-response data generated for the study described in Chapter 5 allowed us to take this modelling an important step further to predict the associated increase in serum 25(OH)D concentrations in adults and children. Modelling studies such as this are required to simulate the effect of any potential vitamin D food fortification strategies, in order to determine whether they are safe and effective across the population.

A change would need to be made to the Australia New Zealand Food Standards Code to accommodate any expansion of the list of foods that are permitted for fortification with vitamin D in Australia. Anyone may submit an application to change the Food Standards Code; however, applications must be carefully assessed by Food Standards Australia New Zealand, with protection of public health and safety of the greatest priority [335]. Any variation to food regulatory measures must also be based on "risk analysis using the best available scientific evidence" [335]. It is data such as that derived through our dose-response studies that can allow such evidence to be established. The modelling studies that may now be conducted may be used to support changes to food regulations and to inform future food policy in Australia. Similarly, the dose-response data generated through this project may be used to

model fortification scenarios in other countries and to develop vitamin D food fortification policy.

6.5. Strengths and limitations

We used a highly sensitive and specific analytical method and national sampling to create new vitamin D food composition data that are representative of foods that are commonly consumed in Australia. We used nationally representative food consumption data together with sophisticated statistical methods that are ideal for estimation of usual intakes from episodically consumed foods to produce the most accurate estimates of usual vitamin D intakes in the Australian population to date. The major strength of our systematic review, meta-analysis and dose-response study was the increased sample size, which permitted expansion of the scope to include analysis by adult and child and by vitamer. A diverse range of populations and food fortification vehicles were represented in the included studies.

The food composition data are subject to the usual limitations of such data, including measurement uncertainties and the inability to produce data that is exactly representative of a specific food item that may be consumed. These limitations may have a flow-on effect on the estimates of vitamin D intake that are based upon them, in addition to the well-acknowledged limitations of self-reported food consumption data captured through recall, such as recall bias and measurement error [272]. Further, although we estimated absolute intakes of vitamin D from food and supplements, we were unable to make estimates of usual intakes from both sources due to the limitation that the NCI method cannot make estimations based on multimodal distributions [62, 266]. Limitations of the systematic review, meta-analysis and dose-response study included high heterogeneity and the indication of publication bias. Only two-thirds of included studies reported on compliance, while important factors in vitamin D status, such as usual vitamin D intake, sun exposure and skin type could not be included in the analyses as data were unavailable. There were not enough eligible studies to allow thorough evaluation of effects by vitamer, food matrix and population group. Although exclusive use of standardised data is a goal for future vitamin D research [332], the historical vitamin D-enriched food trial data currently available did not allow for this to occur in this study.

6.6. Future directions for vitamin D research and policy development

The overall aim of this project was to enable the research of dietary strategies to improve vitamin D status at the population level. The outputs of this project have already been used to model the effect of a fortification scenario that assumed addition of vitamin D to all fluid milks and their alternatives (i.e., soy, nut and other plant-based fluid milk alternatives) at a concentration of 0.8 µg/100 mL. We projected that, under this simulated fortification model, absolute intakes of vitamin D would increase by ~2 µg/day [336]. This would not adequately increase vitamin D intakes in the population and offers only a small potential improvement in circulating 25(OH)D concentration. In this instance, we aimed to simulate a fortification model that would be achievable within the current regulations of the Australia New Zealand Food Standards Code [337]. In the future, expanded fortification models should be simulated, in order to inform potential updates to food fortification regulations, if and when regulations evolve.

The outputs of this project will also enable the potential for biofortification to be assessed in the Australian population. Biofortification of foods with vitamin D has only recently gained momentum in some other countries as a viable strategy for improving the vitamin D content of foods. However, industry support of research into such initiatives in Australia may be impacted, since the incentive to value-add to products and promote for increased marketability is absent in the case of 25(OH)D, the content of which is likely to be augmented by UVB-exposure and 25(OH)D food enrichment biofortification strategies. Currently, only vitamin D_3 and vitamin D_2 are recognised under the Australia New Zealand Food Standards Code as nutritive substances that may be used in foods [338], and for which nutrient and health claims may be made [165, 168]. Therefore, foods may not be fortified with 25(OH)D, and biofortification measures taken to increase the 25(OH)D content of foods cannot be recognised through nutrient or health claims. The data provided by this PhD project could be useful as support for, or arguments against, future changes to sections of the Australia New Zealand Food Standards Code that regulate the use and promotion of the D vitamers in food.

Further to the main aim of enabling research into dietary strategies for improved vitamin D intakes and status, the data provided by this PhD project will allow a wealth of other previously unfeasible research to be conducted.

Our new vitamin D food composition data will be used with food consumption data that will be generated through future Australian national nutrition surveys to provide updated estimates of usual vitamin D intakes, and to assess trends over time. Current and future estimates of usual vitamin D intakes can now be used to explore potential associations (in both directions) between vitamin D intakes and a range of health outcomes.

As well as the aforementioned research areas that are specifically supported by the data generated through this project, there are other vitamin D research areas that have not been fully explored. Broader issues include the absence of definitive data to determine the relative bioactivity of the D vitamers and lack of consensus on cut-points for definitions of vitamin D status. There is also considerable variation and limitations in nutrient reference values. Similar to many other countries, Australia needs population-specific Estimated Average Requirements and Recommended Dietary Intakes for vitamin D. Currently, in order to assess adequacy of vitamin D intakes in Australia [77], we must look to nutrient reference values developed for other populations, since there is no suitable Australia-specific option available. Globally, there remains a lack of comprehensive vitamin D food composition data, and limited laboratories that offer rapid, sensitive and specific methods. In Australia, there is a gap in nationally representative data for vitamin D status in children, and updated data are needed across the rest of the population, since the most recent data from the 2011-2013 Australian Health Survey are now approximately one decade old.

The systematic review and meta-analysis conducted for this project demonstrated that there is a dearth of Australia-specific fortification trials and that there is an international lack of vitamin D food fortification trials in men and in African and South American populations. A global paucity of biofortified food trials means that it is not yet possible to determine whether incorporation of vitamin D into the food matrix through biofortification provides a more bioavailable vitamin D source than fortification [334]. There is a need for animal feeding and UVB-exposure trials, and human biofortified food trials. Concerns over the long-term sustainability of current

animal farming practices suggest that further investigation of the biofortification potential of sustainable protein sources is also warranted. Generally, more well-designed trials are needed across all populations and for numerous food vehicles in order to improve homogeneity when pooling results from multiple studies.

An important consideration for all future studies that involve measurement of circulating 25(OH)D concentrations is use of standardised data, either through use of RMP-certified assays or retrospective standardisation methods [42-44]. The movement to publish only standardised 25(OH)D data has begun [332, 339, 340] and it is hoped that, in the future, it will be possible to conduct studies such as systematic reviews, meta-analyses and dose-response studies using only standardised data.

Collectively, there is great scope for vitamin D research in Australia and overseas.

6.7. Conclusion

This PhD project has produced fundamental data to underpin knowledge around dietary vitamin D in Australia and expands the global knowledge base on this topic. There are some useful food sources of vitamin D available in Australia; however, they are not necessarily widely or commonly consumed. Hence, the majority of Australians do not consume adequate amounts of vitamin D. As poor vitamin D status is prevalent in Australia, strategies to safely improve vitamin D intakes and status are needed. Fortification of food with vitamin D is effective in increasing the 25(OH)D concentrations used as a marker of vitamin D status. Vitamin D food fortification is a strategy that could be further explored in Australia. However, initial modelling of a scenario that fits within Australia's current regulations around food fortification suggests that the limits on concentrations of vitamin D permitted in fortified foods may need to be increased, and the list of foods permitted for fortification in Australia may need to be expanded, to accommodate a model that would adequately improve vitamin D intakes across the population.

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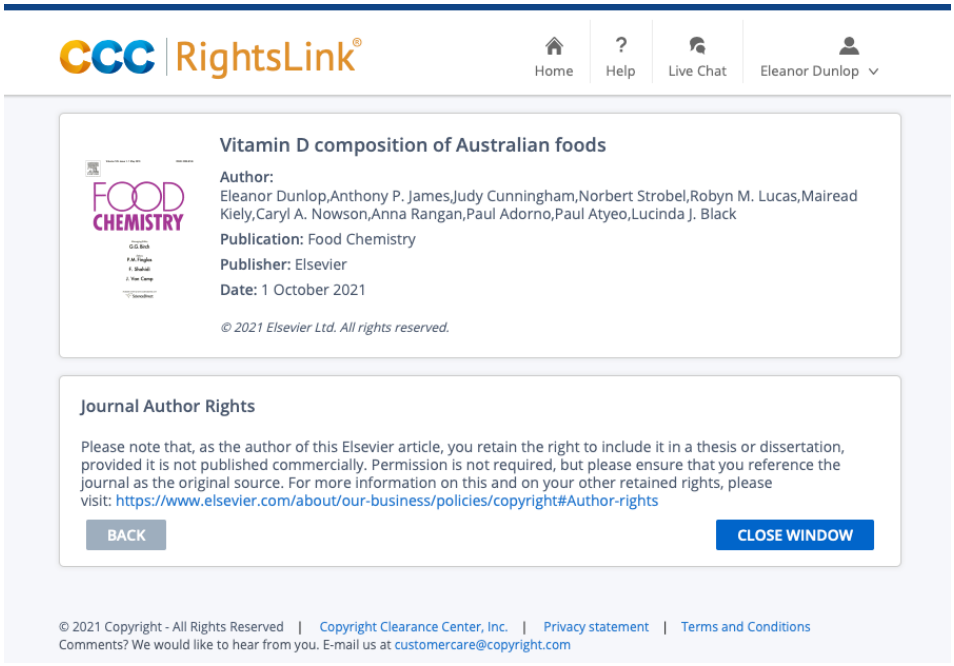
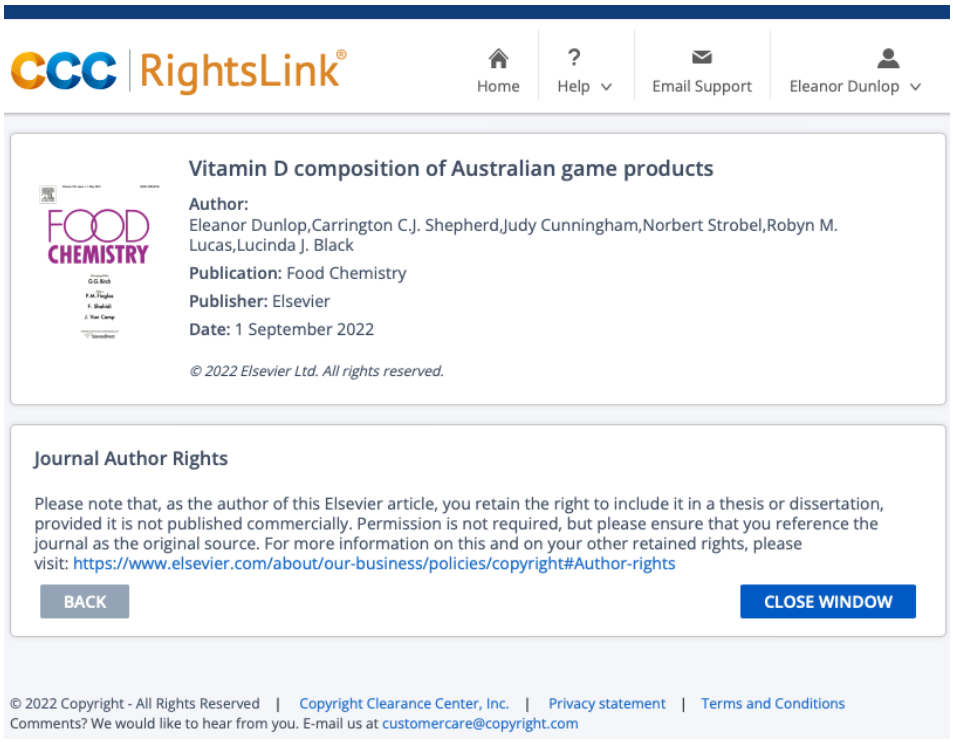
Vitamin D composition of Australian foods



Vitamin D composition of Australian game products



Evidence of low vitamin D intakes in the Australian population points to a need for data-driven nutrition policy for improving population vitamin D status

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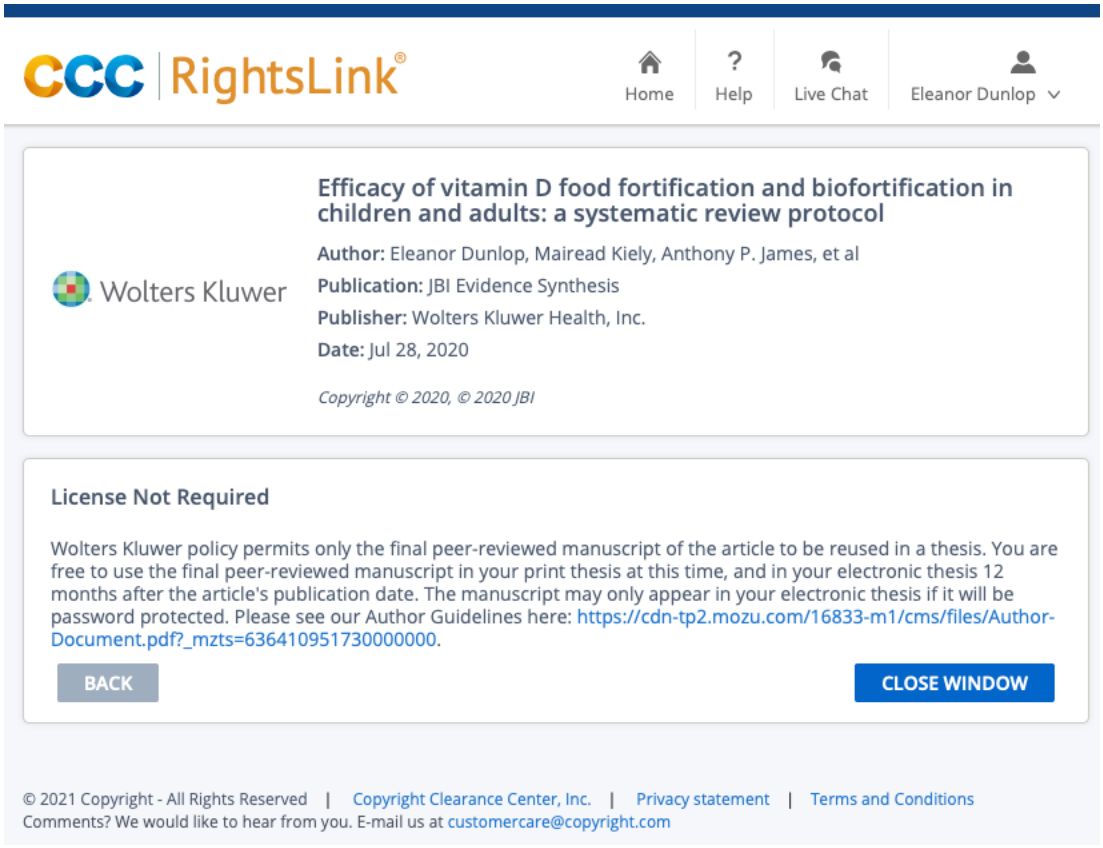

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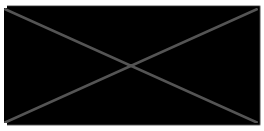
Appendix II: Statements of contribution

To Whom It May Concern

I, Eleanor Shu-ying Dunlop, contributed (managed the project, purchased samples for the Perth sampling round, curated and quality-checked the data, wrote the paper, wrote the original response to reviewer comments and compiled the final manuscript and reviewer response to include Co-Authors' suggestions) to the publication entitled "Vitamin D composition of Australian foods" by E Dunlop, AP James, J Cunningham, N Strobel, RM Lucas, M Kiely, CA Nowson, A Rangan, P Adorno, P Atyeo and LJ Black. 2021. (*Food Chemistry* 358:129836. Doi: 10.1016/j.foodchem.2021.129836).



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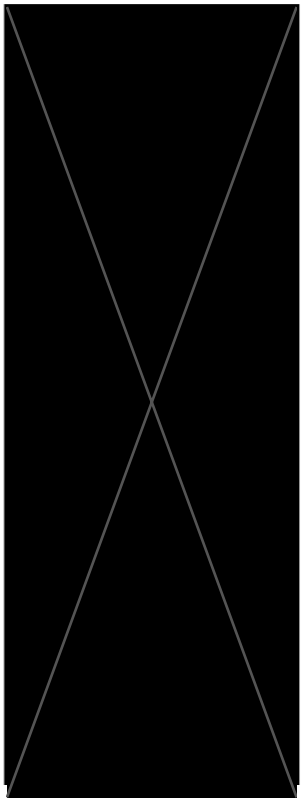
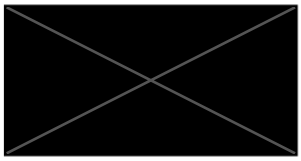
I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Anthony P James		08 / 02 / 2022
Judy Cunningham		08 / 02 / 2022
Norbert Strobel		08 / 02 / 2022
Robyn M Lucas		07 / 02 / 2022
Mairead Kiely		08 / 02 / 2022
Caryl A Nowson		08 / 02 / 2022
Anna Rangan		08 / 02 / 2022
Paul Adorno		09 / 02 / 2022
Paul Atyeo		10 / 02 / 2022
Lucinda J Black		04 / 02 / 2022

To Whom It May Concern

I, Eleanor Shu-ying Dunlop, contributed (managed the project, purchased and arranged transportation of samples, curated and quality-checked the data and wrote the paper) to the manuscript entitled "Vitamin D composition of Australian game products" that is being developed for a publication with Co-Authors.



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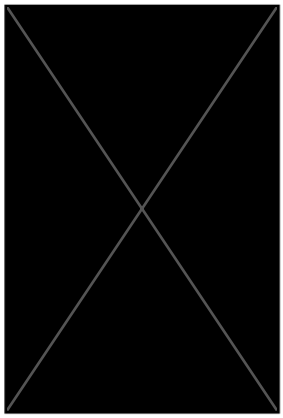
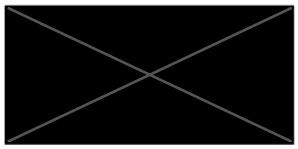
Carrington C J Shepherd		04 / 02 / 2022
Judy Cunningham		04 / 02 / 2022
Norbert Strobel		04 / 02 / 2022
Robyn M Lucas		07 / 02 / 2022
Lucinda J Black		04 / 02 / 2022

To Whom It May Concern

I, Eleanor Shu-ying Dunlop, contributed (calculated total vitamin D concentrations for analysed foods for two bioactivity scenarios, provided indications for mapping of analytical data to the survey food dataset, analysed usual intakes output data, produced estimates of absolute intakes from food and supplements, wrote the paper, wrote the original response to reviewer comments and updated the original manuscript and reviewer response to include Co-Authors' suggestions) to the manuscript entitled "Evidence of low vitamin D intakes in the Australian population points to a need for data-driven nutrition policy for improving population vitamin D status" that is being developed for a publication with Co-Authors.



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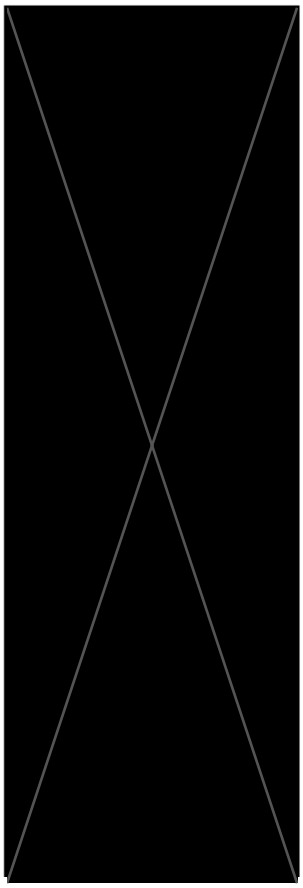
Julie L Boorman		17 / 02 / 2022
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Anna Rangan		07 / 02 / 2022
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Paul Adorno		09 / 02 / 2022
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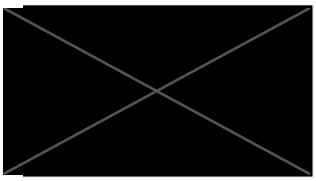
I, Eleanor Shu-ying Dunlop, contributed (co-developed the search strategy, conducted initial database searches, wrote the paper, wrote the original response to reviewer comments and compiled the final manuscript and reviewer response to include Co-Authors' suggestions) to the publication entitled "The efficacy of vitamin D food fortification and biofortification in children and adults: a systematic review protocol" by E Dunlop, M Kiely, AP James, T Singh, LJ Black. 2020. *JBI Evidence Synthesis*: 18(12): 2694-2703. Doi: 10.11124/JBISRIR-D-19-00373.



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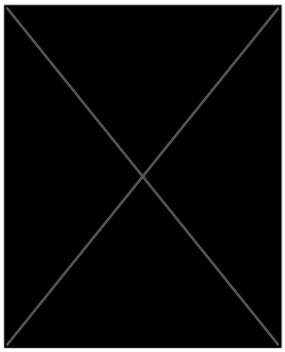
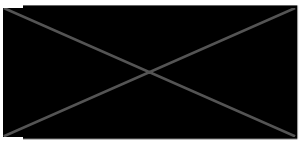


Mairead Kiely	04 / 02 / 2022
Anthony P James	08 / 02 / 2022
Tanya Singh	03 / 02 / 2022
Lucinda J Black	04 / 02 / 2022

To Whom It May Concern

I, Eleanor Shu-ying Dunlop, contributed (co-developed the search strategy, conducted the initial search, selected studies, extracted data, curated data, conducted statistical analyses, analysed output data, designed figures, wrote the paper, wrote the original response to reviewer comments and compiled the final manuscript and reviewer response to include Co-Authors' suggestions) to the publication entitled "Vitamin D food fortification and biofortification increases serum 25-hydroxyvitamin D concentrations in adults and children: an updated and extended systematic review and meta-analysis of randomized controlled trials." by E Dunlop, M E Kiely, A P James, T Singh, N M Pham, L J Black. 2021. *Journal of Nutrition*: 151(9): 2622-2635. Doi: 10.1093/jn/nxab180.



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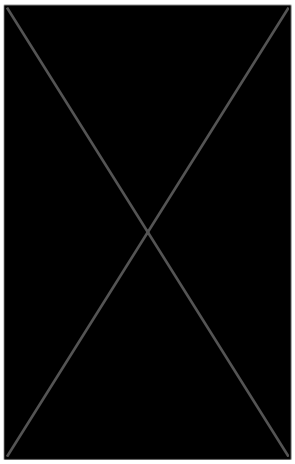
I, as a Co-Author, endorse that this level of contribution by the candidate indicated above is appropriate.

Mairead Kiely		04 / 02 / 2022
Anthony P James		07 / 02 / 2022
Tanya Singh		03 / 02 / 2022
Ngoc Minh Pham		04 / 02 / 2022
Lucinda J Black		04 / 02 / 2022

Appendix III: Survey of vitamin D food composition data

In order to determine the availability of vitamin D food composition data, I surveyed national food composition databases available globally (Table 1). The purpose and timing of this activity was to place the scope and breadth of the new analytical vitamin D food composition database - that was compiled as part of this thesis project - amongst data available elsewhere, at the time that the new database was compiled (2020). I surveyed databases that were included in the International Network of Food Data Systems (INFOODS) international food composition table/database directory [202] and were accessible online 23-31 July 2020. If web links listed in the INFOODS directory failed, Google searches were conducted for the relevant country name and "food composition". I recorded whether or not vitamin D data were included in each publication. Where vitamin D data and documentation were available, I noted the analytical methods used, the forms of vitamin D included, the presence of vitamin D_2 data for meat products and whether a bioactivity factor was applied to data that included 25(OH)D. When necessary, Google Translate was used to navigate and locate relevant information on websites with no English language option.

Table 1. National food composition databases available online surveyed for vitamin D data, method of analysis and vitamers measured, July 2020

Database name or country/area of origin	Reference	Vitamin D data	Method for analytical values (citation of method or user guide if different to main database/tables, where available)	Vitamers measured	Vitamin D_2 in meat products?	25(OH)D bioactivity factor
Africa	U.S. Department of Health and FAO (1968) [341]	No	-	-	-	-
Africa, West	Vincent et al. (2019) [342]	Yes	HPLC - the majority of data are borrowed from the U.S., India, the UK and Denmark [343]	Vitamin D_3 Vitamin D_2	Unclear (presented as "vitamin D")	-
Argentina	Universidad Nacional de Luján (2010) [344]	No	-	-	-	-
Armenia	Ministry of Agriculture of the Republic of Armenia et al. (2010) [127]	Yes	Liquid chromatography (AOAC 995.05 or 982.29) [127]*	Vitamin D_3 Vitamin D_2 25(OH)D_3	Unclear (presented as "vit D")	Yes: five
ASEAN	Institute of Nutrition and Mahidol University (2014) [345]	No	-	-	-	-
Australia	Food Standards Australia New Zealand (2019) [128]	Yes	Various analytical programs over time, including HPLC and LC-IT-MS [104]	Vitamin D_3 Vitamin D_2 25(OH)D_3	No	Yes: five (for total vitamin D – individual 25(OH)D values are unadjusted for bioactivity)
Bahrain	Musaiger (2011) [346]	Yes	Unable to locate	Unclear (presented as "Vit. D" for a limited number of composite dishes)	Unclear	Unclear
Bangladesh	Shaheen et al. (2013) [347]	Yes	Unable to locate	Unclear (presented as "vitamin D")	Unclear	-
Belgium	Nubel (2017) [348]	Yes	HPLC	Vitamin D_3 Vitamin D_2	Unclear (presented as "VIT. D")	-
Brazil	Universidade de São Paulo (USP) and Food Research Center (FoRC) (2020) [349]	Yes	Borrowed data – mainly from USDA [349, 350]	Vitamin D_3 Vitamin D_2	Unclear (presented as "vitamina D")	-
Brazil, regional	Ministéro da Saúde et al. (2015) [351]	No	-	-	-	-
Cambodia	Ministry of Agriculture Forestry and Fisheries of Cambodia et al. (2013) [352]	Yes	Unable to locate	Vitamin D_3 Vitamin D_2	Unclear (presented as "VITD" and "VITDEQ")	-
Canada	Health Canada (2015) [149]	Yes	HPLC or LC/MS [108, 148]	Vitamin D_3 Vitamin D_2	Listed for some meat products (no values >0)	-
Columbia	Colombian Institute of Family Welfare (2018) [353]	No	-	-	-	-

Czech Republic	Centre for Food Composition Database (2020) [354]	Yes	N/A: values are calculated, not analytical	Unclear (presented as "vitamin D")	No	-
Denmark	National Food Institute (2019) [129]	Yes	Various analyses [129], including HPLC [106]	Vitamin D_3 Vitamin D_2 $25(OH)D_3$ $25(OH)D_2$	No	Yes: five
East Asia	Wu Leung et al. (1972) [355]	No	-	-	-	-
Ecuador	LATINFOODS (2018) [356]	No	-	-	-	-
Estonia	National Institute for Health Development (2020) [357]	Yes	Unable to locate	Unclear (presented as "vitamin D" and vitamin D_3")	Unclear	-
FAO/INFOODS Analytical food composition database	FAO (2017) [358]	Yes	Not stated – this database is a compilation of analytical data from various countries [359]	Vitamin D_3 Vitamin D_2 (no entries found) $25(OH)D_3$ (no entries found)	No	Not stated
FAO/INFOODS global database for fish & shellfish	FAO (2016) [358]	Yes	HPLC [360]	Vitamin D_3 (no entries found)	-	-
Finland	National Institute for Health and Welfare (2019) [269]	Yes	Unable to locate	Unclear (presented as "vitamin D")	Unclear	Unclear
France	French Agency for Food (2020) [361]	Yes	Unable to locate	Unclear (presented as "vitamin D")	Unclear	Unclear
Gambia	Prynne and Paul (2011) [362]	No	-	-	-	-
Germany	Federal Ministry of Food and Agriculture (2017) [363]	Yes	Unable to locate	Unclear (presented as "vitamin D"	Unclear	Unclear
Greece	Helenic Health Foundation (2007) [364]	No	-	-	-	-
Iceland	Matís (no date) [365]	Yes	Unable to locate	Unclear (presented as "D-vítamín")	Unclear	Unclear
India	Longvah and Ananthan (2017) [131]	Yes	Vitamin D_3 and $25(OH)D_3$: LC-MS/MS Vitamin D_2: LC-MS	Vitamin D_3 (animal foods) Vitamin D_2 (plant foods and dairy products) $25(OH)D_3$ (animal foods)	No	Yes: five
Indonesia	SouthEast Asian Minsters of Health Organization et al. (2013) [366]	Yes	Borrowed from Vietnam – unable to locate method for Vietnamese source	Vitamin D_3 Vitamin D_2	Unclear (presented as VITD)	-
Italy	European Institute of Oncology (2015) [367]	Yes	Various – borrowed values from several sources	Unclear (presented as "vitamin D")	Unclear	Unclear
Japan	Ministry of Education Culture Sports Science and Technology - Japan (2015) [368]	Yes	HPLC-UV-HPLC	Vitamin D_3	-	-

Kenya	FAO and Government of Kenya (2018) [369]	No	-	-	-	-
Korea	National Institute of Agricultural Sciences (2014) [370]	Yes	Unable to locate	Vitamin D_3 Vitamin D_2	Unclear (it is listed; however, spot checks did not reveal any values – unable to generate a report of values via the website and the PDF versions do not open)	-
Laos	The National Institute of Public Health and Wageningen University (2013) [371]	No	-	-	-	-
Lesotho	Lephole et al. (2006) [372]	Yes	Unable to locate	Unclear (presented as "vitamin D")	Unclear	Unclear
Malawi	MAFOODS (2019) [373]	Yes	Unable to locate	Unclear (presented as "vit D")	Unclear	Unclear
Malaysia	National Technical Working Group of Malaysian Food Composition Database (2011) [374]	Yes	HPLC AOAC method 995.05 – reference not provided (infant formula and enteral products) HPLC [375] (all other samples) [376]	Unclear (no vitamin D values located)	Unclear	Unclear
Mozambique	Korkalo et al. (2011) [377]	No	-	-	-	-
Near East	FAO and USDA (1982) [378]	No	-	-	-	-
Nepal	Nepal Government (2012) [379]	No	-	-	-	-
Netherlands	RIVM (2019) [134, 380]	Yes	Unable to locate	Vitamin D_3 $25(OH)D_3$	-	No
New Zealand	New Zealand Institute for Plant Food Research and Ministry of Health (New Zealand) (2019) [381]	Yes	Vitamin D_3 and vitamin D_2: HPLC-UV $25(OH)D_3$: acetonitrile extraction followed by radioimmunoassay [382]	Vitamin D_3 Vitamin D_2 $25(OH)D_3$	Listed (no values >0)	Unclear
Nigeria	Sanusi (2019) [383]	Yes	Unable to locate	Unclear (presented as "VITDEQ")	Unclear	Unclear
Norway	Norwegian Food Safety Authority (2019) [384]	Yes	HPLC (in fish [385])	Vitamin D_3 $25(OH)D_3$ in fish [385]	Unclear (presented as "vitamin D")	Unclear
Pacific Islands	Dignan et al. (2004) [386]	No	-	-	-	-
Pakistan	Department of Agricultural Chemistry et al. (2001) [387]	No	-	-	-	-
Peru	Bejarano et al. (2002) [388]	No	-	-	-	-
Philippines	Food and Nutrition Research Institute (1997) [389]	No	-	-	-	-
Portugal	National Health Institute Dr. Ricardo Jorge (INSA) (2019) [390]	Yes	Unable to locate	Vitamin D_3	-	-

Serbia	Institute of Medical Research – Centre of Research Excellence (2007) [391]	Yes	Unable to locate	Unclear (presented as "vitamin D")	Unclear	Unclear
Singapore	Health Promotion Board (2011) [392]	Yes	Unable to locate	Unclear (presented as "vitamin D")	Unclear	-
Slovak Republic	Ministry of Agriculture and Rural Development of the Slovak Republic (2016) [393]	Yes	Not available in English – data appear to be borrowed from various sources	Vitamin D_3	-	-
South Africa	SAFOODS (2018) [394]	No	-	-	-	-
Spain	BEDCA Network of the Ministry of Science and Innovation (2017) [395]	Yes	Various - appears to be borrowed and analytical data from several sources	Unclear (presented as "vitamin D")	Unclear	Unclear
Sri Lanka	Perera et al. (1979) [396]	No	-	-	-	-
Sweden	Swedish Food Agency (2020) [397]	Yes	Unable to locate	Unclear (some entries specify sum of vitamin D_3 + vitamin D_2, while others do not)	Unclear	Unclear
Switzerland	Federal Food Safety and Veterinary Office FSVO (2019) [398]	Yes	Various – includes borrowed values from several sources	Vitamin D_3	-	-
Tanzania	Lukmanji et al. (2008) [399]	Yes	Unable to locate	Vitamin D_3	-	-
Thailand	Judprasong et al. (2018) [400]	No	-	-	-	-
Turkey	TürKomp (2014) [401]	Yes	HPLC-UV	Vitamin D_3	-	-
Uganda	Hotz et al.(2015) [402]	No	-	-	-	-
United Kingdom	Public Health England (2019) [132]	Yes	Results of various analytical programs over time, including HPLC [403, 404]	Vitamin D_3 25(OH)D_3	-	Yes: five
United States	USDA (2018) [210]	Yes	HPLC/MS/MS [203]	Vitamin D_3 Vitamin D_2 25(OH)D_3	Unclear: no values located through SR Legacy Nutrient Search** USDA, [405]	No
Vietnam	National Institute of Nutrition and Wageningen University (2013) [406]	Yes	Unable to locate	Vitamin D_3 Vitamin D_2	Unclear (presented as "VITD")	-
Zimbabwe	Chitsiku (1989) [407]	No	-	-	-	-

Searches were conducted 23-31 July 2020

*Written in Armenian – Google Translate used for relevant passages

**As the option to conduct a nutrient search had not yet been added to FoodData Central, the SR Legacy Nutrient Search was used instead.

25(OH)D, 25-hydroxyvitamin D; FAO, Food and Agriculture Organization of the United Nations; HPLC, high performance liquid chromatography; HPLC/MS/MS, high performance liquid chromatography with tandem mass spectrometry; HPLC-UV, high performance liquid chromatography-ultraviolet; LC-IT-MS, liquid chromatography with ion trap mass spectrometry; LC/MS, liquid chromatography with mass spectrometry; LC-QQQ, liquid chromatography with triple quadrupole mass spectrometry; USDA, U.S. Department of Agriculture; VITDEQ, vitamin D equivalents

Appendix IV: A new analytical vitamin D composition database for Australian foods

Table 2: Fat, moisture and vitamin D composition of 98 commonly consumed Australian foods*, per edible portion of food, on fresh weight basis

Sample	Fat	Moisture	Vitamin D_3	25(OH)D_3	Vitamin D_2	25(OH)D_2
	g/100 g		µg/100 g			
Apple, unpeeled, raw	0.0	83.4	ND	ND	ND	ND
Bacon, partly trimmed (pan fried without oil)	18.3	47.8	0.18	0.11	ND	ND
Beef mince, regular fat (pan fried without oil)	18.4	54.5	0.21	0.20	0.22	Trace
Beef, steak, semi-trimmed (grilled)	5.9	66.3	Trace	0.18	Trace	Trace
Bread, white, grain and wholemeal, commercial	2.7	39.0	ND	ND	ND	ND
Breakfast cereals, ready to eat (vitamin D-fortified)	4.1	2.8	11.90	ND	0.13	ND
Butter	83.0	13.9	0.26	Trace	Trace	Trace
Calamari or squid, battered, crumbed or salt & pepper, takeaway or frozen (frozen: cooked as per label instructions)	14.0	49.2	Trace	ND	ND	ND
Canned meats, red meat (beef, lamb, ham or pork)	17.8	58.8	0.34	0.16	0.18	Trace
Canned salmon, plain or flavoured, drained	5.9	70.9	19.25	0.28	ND	ND
Canned tuna, plain or flavoured, drained	3.8	71.0	1.73	ND	Trace	ND
Cheese, brie or camembert	32.0	45.6	ND	ND	ND	ND
Cheese, cheddar	32.9	35.6	0.16	Trace	Trace	Trace
Cheese, cream cheese, regular fat	30.0	57.4	Trace	ND	ND	ND
Cheese, feta	19.7	58.3	ND	ND	ND	ND
Cheese, mozzarella	22.1	47.5	Trace	ND	ND	ND
Cheese, ricotta	10.2	74.2	ND	ND	ND	ND
Cheesecake, plain or flavoured	15.1	39.8	0.15	0.11	ND	ND
Chicken burger, with salad and sauce	10.4	52.6	0.10	Trace	ND	ND
Chicken nuggets, frozen or takeaway (frozen: cooked as per label instructions)	15.2	49.4	0.13	0.15	ND	ND
Chicken, crumbed schnitzels or similar (baked)	12.2	52.7	0.14	0.14	ND	ND
Chicken, drumsticks with skin (baked)	8.4	67.1	0.23	0.33	ND	ND
Chicken, skinless breast fillets (pan fried without oil)	2.7	68.2	0.15	0.22	ND	ND
Chocolate, dark, plain	31.0	0.9	ND	ND	3.20	ND
Chocolate, milk, plain	30.6	1.0	ND	ND	1.56	ND
Coffee, takeaway, cappuccino, latte & flat white	2.6	89.2	Trace	ND	ND	ND
Commercial banana bread	11.6	31.2	0.16	0.14	ND	ND
Commercial chocolate cakes, iced or un-iced	19.1	22.7	0.24	ND	0.15	ND
Corn chips or extruded snacks, cheese dairy flavoured	27.3	1.5	ND	ND	ND	ND
Cream, regular fat	36.6	56.2	0.18	Trace	Trace	ND
Dairy blend, dairy soft etc	66.3	29.7	11.70	ND	ND	ND
Dairy dessert or yoghurt, children's style, vitamin D -fortified	3.3	78.5	0.93	ND	0.18	ND
Dairy milk substitute (soy, rice, oat, almond), vitamin D-fortified	1.1	89.2	ND	ND	1.74	ND
Doughnuts, plain or iced	18.5	23.1	ND	ND	ND	ND
Eggplant (grilled)	0.0	93.5	ND	ND	ND	ND
Eggs, chicken, free range or organic	9.4	75.5	1.36	0.78	ND	ND
Eggs, chicken, standard or cage (boiled)	9.2	75.4	1.44	0.82	ND	ND
Fish fillets, battered or crumbed, takeaway	14.4	54.6	0.73	ND	ND	ND
Fish fingers (baked)	9.4	60.0	0.37	ND	ND	ND
Fish, fillets, basa, skinless (pan fried + ~40mL water. No oil)	1.4	83.4	Trace	ND	ND	ND
Fish, fillets, white type, barramundi, battered, deep-fried	18.2	52.5	3.94	ND	ND	ND
Fish, fillets, white type, other (pan fried + ~40mL water. No oil)	5.1	71.5	1.24	ND	ND	ND
Fish, whole or fillets, mullet (baked in foil, no oil)	10.1	66.1	5.62	Trace	ND	ND

Fish, whole or fillets, sardines (baked in foil, no oil)	4.6	71.8	4.90	ND	Trace	ND
Fish, whole or fillets, trout (baked in foil, no oil)	14.3	64.6	9.49	0.11	ND	ND
Flavoured dairy milk, reduced fat	1.5	84.6	ND	ND	ND	ND
Flavoured dairy milk, regular fat	3.5	81.5	Trace	Trace	Trace	ND
Frankfurts, regular (boiled gently)	14.0	64.9	0.29	0.21	ND	ND
Fried rice containing egg/meat/fish	5.8	59.6	0.13	0.13	ND	ND
Frozen crumbed or battered fish (baked)	9.6	56.3	0.35	ND	ND	ND
Frozen puff and shortcrust pastry (baked)	20.6	23.9	ND	ND	ND	ND
Ham, sliced	3.7	73.9	0.15	Trace	ND	ND
Hamburger, beef, with cheese, egg and bacon	11.2	56.7	0.17	0.16	Trace	ND
Hamburger, beef, with salad and sauce	9.2	56.6	0.11	Trace	Trace	Trace
Ice cream, tub-style, vanilla flavour, regular fat	11.6	59.6	Trace	Trace	ND	ND
Ice-cream, premium, stick-type	15.8	39.3	ND	ND	0.61	ND
Ice-cream, stick-type	1.7	66.2	ND	ND	Trace	ND
Infant formula, dairy-based formulas, powder only	25.4	2.1	7.80	ND	ND	ND
Kangaroo, steak (pan fried without oil)	2.4	67.9	ND	ND	Trace	Trace
Lamb, chops, semi-trimmed (grilled)	13.3	59.6	Trace	0.10	0.24	Trace
Lard and dripping	99.4	0.7	3.02	ND	0.40	ND
Liver, lamb (pan fried without oil)	9.3	65.8	0.18	0.23	0.12	0.13
Margarine spread, regular fat	58.0	40.1	8.45	ND	3.64	ND
Mayonnaise, regular fat, made with egg	73.0	25.4	Trace	ND	ND	ND
Meat pie, standard, fresh or frozen (baked)	13.1	53.5	Trace	Trace	Trace	ND
Milk powder, regular fat, unfortified	23.0	2.1	0.10	0.13	0.11	ND
Milk, plain, reduced fat	1.2	89.7	ND	Trace	ND	ND
Milk, plain, regular fat	3.1	88.3	ND	Trace	ND	ND
Milk, vitamin D fortified	1.7	87.8	0.45	ND	ND	ND
Malted chocolate drink powder, regular flavour	9.7	1.5	17.85	Trace	0.43	ND
Muffin, sweet, fruit	14.0	30.1	0.12	ND	0.00	ND
Olive oil	100.0	0.0	ND	ND	ND	ND
Oysters, raw	2.1	83.4	0.25	ND	0.10	ND
Packaged sliced chicken or turkey	3.8	72.3	0.10	0.11	ND	ND
Peanut butter	46.4	1.5	ND	ND	ND	ND
Pizza, frozen, with tomato & cheese, or with vegetable and cheese topping (baked)	9.7	46.6	ND	ND	ND	ND
Pizza, takeaway, meat lovers' style	10.6	40.7	0.16	0.03	ND	ND
Pork Chops, semi-trimmed, (grilled/BBQ)	6.5	63.1	0.43	0.17	ND	ND
Pork, minced (pan fried without oil)	14.6	57.0	1.05	0.28	ND	ND
Potato (boiled)	0.0	78.0	ND	ND	ND	ND
Prawns, (purchased cooked)	1.0	74.5	Trace	ND	ND	ND
Ravioli or tortellini, meat and/or cheese filling, no sauce (boiled)	7.5	49.8	0.13	Trace	Trace	Trace
Rice, white, boiled	0.0	71.8	ND	ND	ND	ND
Salami, regular fat	29.2	42.3	1.49	0.25	ND	ND
Salmon, Atlantic, fillets or steaks, fresh (baked)	15.7	61.3	4.99	0.25	ND	ND
Sausage roll, standard (baked)	17.3	43.5	Trace	0.11	0.10	ND
Sausage, beef (pan fried without oil)	19.2	57.4	0.15	0.15	0.13	Trace
Savoury biscuits, 'Shape' style flavoured biscuits containing cheese powder	22.4	3.1	ND	ND	ND	ND
Soup, prepared, commercial, with meat or seafood (heated)	1.1	85.8	ND	Trace	ND	ND
Spring rolls or dumplings containing meat or fish (baked)	9.7	59.8	ND	ND	0.31	ND
Sweet biscuits, plain, including shortbread and Anzacs	20.3	2.6	0.15	ND	0.12	Trace
Takeaway style mixed dish, beef- or lamb-based, with vegetables and sauce	9.5	70.9	ND	Trace	Trace	Trace
Takeaway style mixed dish, chicken-based, with vegetables and sauce	7.3	74.8	ND	ND	ND	ND
Toddler formula	16.7	1.9	3.89	ND	ND	ND
Tomato, raw (stem removed)	0.0	94.0	ND	ND	ND	ND
Yeast extract	1.3	40.7	ND	ND	ND	ND
Yoghurt, flavoured or added fruit, full fat	4.4	76.1	Trace	ND	ND	ND
Yoghurt, flavoured or added fruit, reduced fat	1.4	81.4	ND	ND	ND	ND

*Data for individual vitamers are presented as analysed, without adjustment for bioactivity.
ND, not detected; LOR, limit of reporting; Trace, < LOR (0.1 µg/100 g, except for butter, margarine, mayonnaise and oil, for which the LOR = 0.25 µg/100 g)