Title: Vitamin D composition of Australian foods

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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.

Keywords: 25-hydroxyvitamin D; Australia; diet; food composition data; LC-QQQ; vitamin D

1. 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) (Australian Bureau of Statistics, 2014; Malacova et al., 2019). 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 (Alfredsson et al., 2020) and Australians are encouraged to protect their skin from prolonged sun exposure to reduce the risk of skin damage and skin cancer (Cancer Council Australia, 2019). 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 (Cashman et al., 2012; Jakobsen, Melse-Boonstra, & Rychlik, 2019; Ovesen, Brot, & Jakobsen, 2003). 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 (Food and Agriculture Organization of the United Nations (FAO), 2020), 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 (National Food Institute, 2019). 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 (Bilodeau et al., 2011; Byrdwell et

al., 2008; Heudi, Trisconi, & Blake, 2004; Huang, LaLuzerne, winters, & Sullivan, 2009; Jackson, Shelton, & Frier, 1982; Jakobsen, Clausen, Leth, & Ovesen, 2004; Mattila, Piironen, Uusi-Rauva, & Koivistoinen, 1995; Phillips et al., 2008; Strobel, Buddhadasa, Adorno, Stockham, & Greenfield, 2013; Trenerry, Plozza, Caridi, & Murphy, 2011), 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 (FSANZ, 2019); 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 (Adamec et al., 2011; Bilodeau et al., 2011; Bussche et al., 2014). 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.

2. Materials and methods

2.1 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.

2.2 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 (Strobel et al., 2013). Saponification, extraction and derivatisation procedures were carried out under non-ultraviolet light (Gill & Indyk, 2018), to minimise vitamin D deterioration, using methods described previously (Hughes et al., 2018). 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.

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). 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 (AOAC International, 2005a). Total fat was determined either by Soxhlet (Food Science Australia, 1998) or Mojonnier extraction (AOAC International, 2005b).

2.3 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,

200 mayonnaise and oil), for which the LOQ was estimated as 0.25 μg/100 g. The limit of detection
201 (LOD) was defined as the lowest concentration detected during a specific analysis and was matrix
202 dependent. LODs were determined as: (SD of seven replicate analyses carried out on low-level
203 spiked sample matrix) x (t-test value at 99% CI), where the spike concentration was related to the
204 least amount of analyte discernible in comparison to multiple readings of a blank reagent. Trace
205 values were defined as >LOD and <LOQ.
206

207 *2.4 Data handling and quality checks*
208 Data for proximates and D vitamers were checked against the following criteria: combined
209 proximates (moisture and fat) ≤100 g edible portion and proximate concentrations similar to
210 published Australian food composition data for the same foods (FSANZ, 2019). Concentrations of
211 D vitamers were questioned if differences to data published in Australia and overseas were unlikely
212 to be explainable by expected variations (e.g. due to production method or geographical location)
213 (FSANZ, 2019; National Food Institute, 2019; USDA, 2019). Data that did not comply with these
214 criteria were queried and re-tests arranged if deemed necessary.
215

216 Replicate values were averaged to produce average proximate and D vitamer values for each food.
217 Where foods were sampled in more than one city, values were averaged to produce a national
218 average for each food.
219

220 The full analytical dataset will be made available for future releases of the AFCD. Meanwhile, we
221 aggregated the data to demonstrate the wide variety of foods in which we detected and quantitated
222 the D vitamers. Analytical concentrations for individual foods were grouped together according to
223 food type and D vitamer profile. Mean concentrations for each proximate and D vitamer were
224 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.

3. Results

3.1 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 D_3 in basa fish [76%] and Frankfurt sausages [78%], and 25(OH)D_2 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).

3.2 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)D_2 in lamb liver (0.13 µg/100 g).

4. 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 (Dunlop et al., 2017), 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 (Jakobsen et al., 2019; Ovesen et al.,

277 2003), the AFCD presents values for individual D vitamers unadjusted for bioactivity as well as a
278 'vitamin D_3 equivalents' value that incorporates a bioactivity factor of five for $25(OH)D_3$ (FSANZ,
279 2019).
280
281 It is important to measure the hydroxylated vitamers of vitamin D ($25(OH)D_3$ and $25(OH)D_2$) since,
282 although the measured concentrations may be low, cumulatively across the diet - even without a
283 bioactivity factor applied - their contribution to dietary requirements may be considerable. In our
284 study, this was particularly evident in chicken eggs, where $25(OH)D_3$ (unadjusted for bioactivity)
285 concentrations were more than half the concentrations of vitamin D_3. If the bioactivity of $25(OH)D_3$
286 is greater than vitamin D_3 (Cashman et al., 2012; Jakobsen et al., 2019; Ovesen et al., 2003), the
287 contribution of $25(OH)D_3$ in eggs may outweigh that of vitamin D_3. Based on our analytical results,
288 in Australia, a standard 120 g serve of eggs (National Health and Medical Research Council, 2015),
289 would contain approximately 1 μg of $25(OH)D_3$ per serve, before adjustment for bioactivity.
290 Independently of vitamin D_3 content, the unadjusted $25(OH)D_3$ content alone translates to one fifth
291 of the Australian Adequate Intake (AI) (5 μg/day), one tenth of the Institute of Medicine's (IOM)
292 Estimated Average Requirement (EAR) (10 μg/day), and one fifteenth of the IOM's Recommended
293 Dietary Allowance (RDA) (15 μg/day). In various other beef, chicken and lamb products,
294 unadjusted $25(OH)D_3$ concentrations were either equal to or greater than vitamin D_3. As such,
295 omission of $25(OH)D_3$ may cause considerable underestimation of the vitamin D contributions of
296 these foods, and also of vitamin D intakes, which may be further augmented if a bioactivity factor
297 greater than one is determined for 25(OH)D in the future.
298
299 We detected $25(OH)D_3$ in all monogastric and ruminant animal meats; however, $25(OH)D_3$ was not
300 detected in the marsupial kangaroo meat. Conversely, $25(OH)D_2$ was detected in kangaroo, but not
301 in any monogastric meat samples, and only in some ruminant animal meats. Our subsequent
302 analyses (unpublished data) of four individual samples of kangaroo (one of each cut: fillet, rump,

303 mince, tail) suggest that this is not a random sampling event. To our knowledge, it is not yet known
304 why the D vitamer profile of kangaroo meat differs to other animal types, particularly to those that
305 are also herbivores. Kangaroo meat has a lower fat content than most monogastric and ruminant
306 meats; however, muscle has been demonstrated to act as a storage site for 25(OH)D (Abboud et al.,
307 2013), and may have greater uptake potential of 25(OH)D than fat cells (Abboud et al., 2014).
308 Serum 25(OH)D concentrations in healthy, free-ranging koalas with no evidence of metabolic bone
309 disease have previously been reported as being much lower than non-marsupials, prompting
310 speculation that the koala's vitamin D requirements may be relatively low (Pye, Ellis, FitzGibbon,
311 Opitz, Keener, & Hollis, 2013). Similarly, low serum 25(OH)D concentrations have been recorded
312 in other marsupials, specifically wombats and brushtail possums (Fowler & Fraser, 1993). We hope
313 to explore the D vitamer profile of other marsupial meats in another project (National Health and
314 Medical Research Council GNT1184788) that aims to investigate the vitamin D content of foods
315 that are hunted and foraged in Australia. This may provide further indication as to whether the D
316 vitamer profile observed in kangaroo meat is specific only to macropods or to marsupials in
317 general.
318
319 Although the human body appears to favour vitamin D_3 over vitamin D_2, the latter is still of use in
320 improving circulating 25(OH)D concentrations in humans (Tripkovic et al., 2012), and should be
321 included in food composition analyses in order to accurately reflect vitamin D content. Since
322 vitamin D_2 is synthesised from ergosterol in fungi and yeast, but not in animals, the D_2 vitamers are
323 rarely measured in animal products. Vitamin D_2 and 25(OH)D_2, however, have previously been
324 found in cow's milk (Jakobsen & Saxholt, 2009), and concentrations of vitamin D_2 are listed for
325 milk and milk products in some food composition databases (e.g. India has two entries for cheese
326 (Longvah & Ananthan, 2017), and Denmark has one entry each for butter and whole milk (National
327 Food Institute, 2019)); however, when surveying other food composition databases, we found no
328 quantitated values for vitamin D_2 in meat products. Barnkob et al. (Barnkob, Petersen, Nielsen, &

329 Jakobsen, 2018) conducted an ultraviolet-B (UVB) exposure study in slaughter pigs raised indoors.
330 They found vitamin D_2 in the rind, subcutaenous fat, lean meat, liver and serum of the control
331 group, as well as 25(OH)D_2 in the rind and serum. Vitamin D_2 content increased in the groups
332 exposed to UVB light. The detection of vitamin D_2 and 25(OH)D_2 in animal products has been
333 attributed to the ingestion of vitamin D_2 in feed, particularly from grasses contaminated with fungi
334 (Jäpelt, Didion, Smedsgaard, & Jakobsen, 2011). Indeed, in the study by Barnkob et al. (Barnkob et
335 al., 2018), straw and feed were exposed along with the pigs in UVB-irradiated pens.
336
337 We found notable amounts of vitamin D_2, which in several cases were naturally-occurring, in
338 various foods, including meat products. Of unfortified foods analysed, the greatest vitamin D_2
339 concentrations were found in chocolate. Vitamin D_2 is thought to be present in chocolate due to
340 fungal contamination of cocoa beans (Kühn, Schröter, Hartmann, & Stangl, 2018). This may
341 explain in part, and likely in conjunction with milk solids, the presence of vitamin D_2 in cocoa-
342 containing foods such as chocolate-coated ice-cream products, and malted chocolate drink powder.
343 Indeed, the vitamin D content of analysed chocolate-coated premium stick-type ice-cream products
344 was solely contributed by vitamin D_2, highlighting the need to measure this vitamer in a wide range
345 of foods. We also found reasonable amounts of vitamin D_2 in several meat products, further
346 reinforcing the importance of measuring all four D vitamers in foods.
347
348 While 25(OH)D_2 contributed 20% of the total vitamin D content of lamb livers analysed in our
349 study, the majority of detected amounts of 25(OH)D_2 in other foods were too small to quantitate.
350 When estimating intakes, it is usual practice to allocate a value of LOQ/2 for metabolites detected at
351 concentrations below the LOQ (FAO and INFOODS, 2013) in order to find a middle ground
352 between concentrations that may be closer to either zero or the LOQ. Hence, foods with very low,
353 but detectable, D vitamer concentrations may contribute small, but cumulatively important amounts
354 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.

5. Conclusions

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.

Abbreviations:

25(OH)D	25-hydroxyvitamin D
AFCD	Australian Food Composition Database – Release 1
AHS	Australian Health Survey 2011-2013

FSANZ	Food Standards Australia New Zealand
INFOODS	International Network of Food Data Systems
LC-QQQ	liquid chromatography with triple quadrupole mass spectrometry
LOD	limit of detection
LOQ	limit of quantitation
NIST	National Institute of Standards and Technology
NMI	National Measurement Institute of Australia
RPD	relative percent difference
SRM	Standard Reference Material®

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Chemical compounds studied in this article:

Vitamin D_3/cholecalciferol (PubChem CID: 5280795); Vitamin D_2/ergocalciferol (PubChem CID: 5280793); 25-hydroxyvitamin D_3/25-hydroxycholecalciferol (PubChem CID: 5283731); 25-hydroxyvitamin D_2/25-hydroxyergocalciferol (PubChem CID: 5710148).

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6. References

Abboud, M., Gordon-Thomson, C., Hoy, A. J., Balaban, S., Rybchyn, M. S., Cole, L., . . . Mason, R. S. (2014). Uptake of 25-hydroxyvitamin D by muscle and fat cells. *Journal of Steroid Biochemistry and Molecular Biology, 144*, 232-236. https://doi.org/10.1016/j.jsbmb.2013.10.020.

Abboud, M., Puglisi, D. A., Davies, B. N., Rybchyn, M., Whitehead, N. P., Brock, K. E., . . . Mason, R. S. (2013). Evidence for a specific uptake and retention mechanism for 25-hydroxyvitamin D (25OHD) in skeletal muscle cells. *Endocrinology, 154*, 3022-3030. https://doi.org/10.1210/en.2012-2245.

Adamec, J., Jannasch, A., Huang, J., Hohman, E., Fleet, J. C., Peacock, M., . . . Weaver, C. M. (2011). Development and optimization of an LC-MS/MS‐based method for simultaneous quantification of vitamin D_2, vitamin D_3, 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3. *Journal of Separation Science, 34*, 11-20. https://doi.org/10.1002/jssc.201000410.

Alfredsson, L., Armstrong, B. K., Butterfield, D. A., Chowdhury, R., de Gruijl, F. R., Feelisch, M., . . . Young, A. R. (2020). Insufficient Sun Exposure Has Become a Real Public Health Problem. *International Journal of Environmental Research and Public Health, 17*, 5014. https://doi.org/10.3390/ijerph17145014.

AOAC International. (2005a). Dairy samples-determination of total solids and moisture. In W. Horwitz (Ed.), *Official methods of analysis of AOAC International*. Atlanta, GA, USA: AOAC International.

AOAC International. (2005b). Determination of fat-Gravimetric method. In W. Horwitz (Ed.), *Official methods of analysis of AOAC International*. Atlanta, GA: AOAC International.

443 Australian Bureau of Statistics. (2014). Australian Health Survey: biomedical results for nutrients,
444 2011-2012. Retrieved from: https://www.abs.gov.au/statistics/health/health-conditions-and-
445 risks/australian-health-survey-biomedical-results-nutrients/latest-release. Accessed July 17,
446 2020.

447 Barnkob, L., Petersen, P., Nielsen, J., & Jakobsen, J. (2018). Vitamin D enhanced pork from pigs
448 exposed to artificial UVB light in indoor facilities. *European Food Research and*
449 *Technology, 245*, 1-8. https://doi.org/10.1007/s00217-018-3173-6.

450 Bilodeau, L., Dufresne, G., Clément, J., Bertrand, G., Turcotte, J., Robichaud, S., . . . Deeks, A.
451 (2011). Determination of vitamin D3 and 25-hydroxyvitamin D3 in foodstuffs by HPLC
452 UV-DAD and LC-MS/MS. *Journal of Food Composition and Analysis, 24*, 441-448.
453 https://doi.org/10.1016/j.jfca.2010.08.002.

454 Bussche, J. V., Decloedt, A., Van Meulebroek, L., De Clercq, N., Lock, S., Stahl-Zeng, J., &
455 Vanhaecke, L. (2014). A novel approach to the quantitative detection of anabolic steroids in
456 bovine muscle tissue by means of a hybrid quadrupole time-of-flight-mass spectrometry
457 instrument. *Journal of Chromatography A, 1360*, 229-239.
458 https://doi.org/10.1016/j.chroma.2014.07.087.

459 Byrdwell, W. C., Devries, J., Exler, J., Harnly, J. M., Holden, J. M., Holick, M. F., . . . Wolf, W. R.
460 (2008). Analyzing vitamin D in foods and supplements: methodologic challenges. *American*
461 *Journal of Clinical Nutrition, 88*, 554S-557S. https://doi.org/10.1093/ajcn/88.2.554S.

462 Cancer Council Australia. (2019). Preventing skin cancer. Retrieved from:
463 https://www.cancer.org.au/preventing-cancer/sun-protection/preventing-skin-cancer/.
464 Accessed July 14, 2020.

Cashman, K. D., Seamans, K. M., Lucey, A. J., Stöcklin, E., Weber, P., Kiely, M., & Hill, T. R. (2012). Relative effectiveness of oral 25- hydroxyvitamin D3 and vitamin D3 in raising wintertime serum 25- hydroxyvitamin D in older adults. *American Journal of Clinical Nutrition, 95*, 1350-1356. https://doi.org/10.3945/ajcn.111.031427.

Dunlop, E., Cunningham, J., Sherriff, J. L., Lucas, R. M., Greenfield, H., Arcot, J., . . . Black, L. J. (2017). Vitamin D3 and 25-hydroxyvitamin D3 content of retail white fish and eggs in Australia. *Nutrients, 9*, 1-15. https://doi.org/10.3390/nu9070647.

Food and Agriculture Organization of the United Nations. (2020). International Network of Food Data Systems (INFOODS): International food composition table/database directory. Retrieved from: http://www.fao.org/infoods/infoods/tables-and-databases/en/. Accessed July 22, 2020.

Food and Agriculture Organization of the United Nations, & International Network of Food Data Systems (INFOODS). (2013). Food Composition Data online course. Retrieved from: https://elearning.fao.org/course/view.php?id=191. Accessed January 30, 2019.

Food Science Australia. (1998). Crude fat determination - Soxhlet method. Retrieved from: https://meatupdate.csiro.au/infosheets/Crude%20Fat%20Determination%20-%20Soxhlet%20Method%20-%201998.pdf. Accessed April 20, 2020.

Food Standards Australia New Zealand. (2019). Australian Food Composition Database - Release 1. Retrieved from: http://www.foodstandards.gov.au/science/monitoringnutrients/afcd/Pages/default.aspx. Accessed October 6, 2019.

Fowler, A. M., & Fraser, D. R. (1993). Vitamin D metabolism in nocturnal marsupials. *Proceedings of the Wildlife Disease Association (Australasian Section) Annual Conference*, 21-22.

Gill, B. D., & Indyk, H. E. (2018). Analysis of vitamin D_2 and vitamin D_3 in infant and adult nutritional formulas by liquid chromatography-tandem mass spectrometry: A multilaboratory testing study. *Journal of AOAC International, 101*, 256-263. https://doi.org/10.5740/jaoacint.17-0149.

Heudi, O., Trisconi, M.-J., & Blake, C.-J. (2004). Simultaneous quantification of vitamins A, D3 and E in fortified infant formulae by liquid chromatography–mass spectrometry. *Journal of Chromatography A, 1022*, 115-123. https://doi.org/10.1016/j.chroma.2003.09.062.

Huang, M., LaLuzerne, P., winters, D., & Sullivan, D. (2009). Measurement of vitamin D in foods and nutritional supplements by liquid chromatography/tandem mass spectrometry. *Journal of AOAC International, 92*, 1327-1335.

Hughes, L. J., Black, L. J., Sherriff, J. L., Dunlop, E., Strobel, N., Lucas, R. M., & Bornman, J. F. (2018). Vitamin D content of Australian native food plants and Australian-grown edible seaweed. *Nutrients, 10*, Article E876. https://doi.org/10.3390/nu10070876.

Jackson, P. A., Shelton, C. J., & Frier, P. J. (1982). High-performance liquid chromatographic determination of vitamin D3 in foods with particular reference to eggs. *The Analyst, 107*, 1363-1369. https://doi.org/10.1039/AN9820701363.

Jakobsen, J., Clausen, I., Leth, T., & Ovesen, L. (2004). A new method for the determination of vitamin D3 and 25-hydroxyvitamin D3 in meat. *Journal of Food Composition and Analysis, 17*, 777-787. https://doi.org/10.1016/j.jfca.2003.10.012.

507 Jakobsen, J., Melse-Boonstra, A., & Rychlik, M. (2019). Challenges to quantify total vitamin
508 activity: How to combine the contribution of diverse vitamers? *Current Developments in*
509 *Nutrition, 3*, Article nzz086. https://doi.org/10.1093/cdn/nzz086.

510 Jakobsen, J., & Saxholt, E. (2009). Vitamin D metabolites in bovine milk and butter. *Journal of*
511 *Food Composition and Analysis, 22*, 472-478. https://doi.org/10.1016/j.jfca.2009.01.010.

512 Jäpelt, R. B., Didion, T., Smedsgaard, J., & Jakobsen, J. (2011). Seasonal variation of provitamin
513 D2 and vitamin D2 in perennial ryegrass (Lolium perenne L.). *Journal of Agricultural and*
514 *Food Chemistry, 59*, 10907-10912. https://doi.org/10.1021/jf202503c.

515 Kühn, J., Schröter, A., Hartmann, B. M., & Stangl, G. I. (2018). Cocoa and chocolate are sources of
516 vitamin D2. *Food Chemistry, 269*, 318-320.
517 https://doi.org/10.1016/j.foodchem.2018.06.098.

518 Longvah, T., & Ananthan, R. (2017). Indian food composition tables. Retrieved from:
519 https://drive.google.com/file/d/1eqQ578gHiPoIaHaVYjQa_3sFe_LzGhm1/view. Accessed
520 July 23, 2020.

521 Malacova, E., Cheang, P., Dunlop, E., Sherriff, J., Lucas, R. M., Daly, R. M., . . . Black, L. J.
522 (2019). Prevalence and predictors of vitamin D deficiency in a nationally-representative
523 sample of adults participating in the 2011-2013 Australian Health Survey. *British Journal of*
524 *Nutrition, 121*, 894-904. https://doi.org/10.1017/S0007114519000151.

525 Mattila, P. H., Piironen, V. I., Uusi-Rauva, E. J., & Koivistoinen, P. E. (1995). Contents of
526 cholecalciferol, ergocalciferol, and their 25-hydroxylated metabolites in milk products and

raw meat and liver as determined by HPLC. *Journal of Agricultural and Food Chemistry, 43*, 2394-2399. https://doi.org/10.1021/jf00057a015.

National Food Institute, Technical University of Denmark. (2019). Food data, version 4. Retrieved from: https://frida.fooddata.dk. Accessed November 30, 2020.

National Health and Medical Research Council. (2015). Australian Dietary Guidelines. Retrieved from: https://www.eatforhealth.gov.au. Accessed August 10, 2020.

Ovesen, L., Brot, C., & Jakobsen, J. (2003). Food contents and biological activity of 25-hydroxyvitamin D: A vitamin D metabolite to be reckoned with? *Annals of Nutrition & Metabolism, 47*, 107-113. https://doi.org/10.1159/000070031.

Phillips, K. M., Byrdwell, W. C., Exler, J., Harnly, J. M., Holden, J. M., Holick, M. F., . . . Patterson, K. Y. (2008). Development and validation of control materials for the measurement of vitamin D3 in selected US foods. *Journal of Food Composition and Analysis, 21*, 527-534. https://doi.org/10.1016/j.jfca.2008.05.003.

Pye, G. W., Ellis, W., FitzGibbon, S., Opitz, B., Keener, L., & Hollis, B. W. (2013). Serum vitamin D levels in free-ranging koalas (Phascolarctos cinereus). *Journal of Zoo and Wildlife Medicine, 44*, 480-483. https://doi.org/10.1638/2012-0032R.1.

Strobel, N., Buddhadasa, S., Adorno, P., Stockham, K., & Greenfield, H. (2013). Vitamin D and 25-hydroxyvitamin D determination in meats by LC–IT-MS. *Food Chemistry, 138*, 1042-1047. https://doi.org/10.1016/j.foodchem.2012.08.041.

546 Trenerry, V. C., Plozza, T., Caridi, D., & Murphy, S. (2011). The determination of vitamin D3 in
547 bovine milk by liquid chromatography mass spectrometry. *Food Chemistry, 125*, 1314-
548 1319. https://doi.org/10.1016/j.foodchem.2010.09.097.

549 Tripkovic, L., Lambert, H., Hart, K., Smith, C. P., Bucca, G., Penson, S., . . . Lanham-New, S.
550 (2012). Comparison of vitamin D2 and vitamin D3 supplementation in raising serum 25-
551 hydroxyvitamin D status: a systematic review and meta-analysis. *American Journal of*
552 *Clinical Nutrition, 95*, 1357-1364. https://doi.org/10.3945/ajcn.111.031070.

553 U.S. Department of Agriculture. (2019). FoodData Central. Retrieved from:
554 https://fdc.nal.usda.gov. Accessed July 30, 2020.
555