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Article

Vitamin D content of Australian native food plants and Australian-grown edible seaweed

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Abstract: Vitamin D has previously been quantified in some plants and algae, particularly in leaves of the Solanaceae family. We measured the vitamin D content of Australian native food plants and Australian-grown edible seaweed. Using liquid chromatography with triple quadrupole mass spectrometry, 13 samples (including leaf, fruit and seed) were analysed in duplicate for vitamin D_2, vitamin D_3, 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3. Five samples contained vitamin D_2: raw wattleseed (*Acacia victoriae*) (0.03 µg/100 g dry weight (DW)); fresh and dried lemon myrtle (*Backhousia citriodora*) leaves (0.03 and 0.24 µg/100 g DW, respectively); dried leaves and berries of Tasmanian mountain pepper (*Tasmannia lanceolata*) (0.67 and 0.05 µg/100 g DW, respectively). Fresh kombu (*Lessonia corrugata*) contained vitamin D_3 (0.01 µg/100 g DW). Detected amounts were low; however, it is possible that exposure to ultraviolet radiation may increase the vitamin D content of plants and algae if vitamin D precursors are present.

Keywords: LC-QQQ; liquid chromatography; triple quadrupole; vitamin D; 25(OH)D; plants; algae

1. Introduction

Approximately a quarter of Australian adults are deficient in vitamin D (serum 25-hydroxyvitamin D (25(OH)D) <50 nmol/L) [1]. There is seasonal variation in the prevalence of vitamin D deficiency, with 14% of the adult population deficient in summer and 36% in winter [1]. Certain population groups, such as people with dark skin, those wearing covering for religious or cultural reasons, and people living largely indoors, are at greater risk of deficiency due to inadequate sun exposure, particularly in winter months [2]. Although the major source of vitamin D for humans is cutaneous synthesis from sun exposure [3], when sun exposure is inadequate to maintain vitamin D status, dietary sources make a small but useful contribution [4]. In the Australian food supply, fish, meat, eggs, dairy and fortified margarine are known sources of vitamin D_3, while mushrooms are a source of vitamin D_2 and small amounts of vitamin D_3 and D_4 [5,6]. Many animal products also contain the hydroxylated metabolite of vitamin D_3,

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25-hydroxyvitamin D_3 (25(OH)D_3), which likely has a greater biological activity than vitamin D_3 itself [7,8].

The anti-rachitic properties of plants were originally discovered by animal feeding studies [9-11], although the active compound in these early studies was later identified as vitamin D_2 produced from fungal contamination, rather than endogenous synthesis in the plants. More recently, high performance liquid chromatography-ultraviolet (HPLC-UV) with mass spectrometry, or liquid chromatography tandem mass spectrometry (LC-MS/MS), have been used to measure vitamin D metabolites directly in the plant matrix [12-14]. Vitamin D_3 and 25(OH)D_3 have been found in leaves of various plants, especially those of the Solanaceae family; algae are also known to contain vitamin D_2 and/or vitamin D_3, but studies are limited (Table 1).

Table 1. Content of vitamin D_2, vitamin D_3 and 25-hydroxyvitamin D_3 in plants, microalgae and macroalgae derived from previously published literature.

Common Name (*Botanical Name*)	**Plant part**	**Vitamin D_2 (µg/100 g)**	**Vitamin D_3 (µg/100 g)**	**25(OH)D_3 (µg/100 g)**
Plants				
Tomato	Leaf	Not tested	78 (DW)[a]	2 (DW)[a]
(*Lycopersicon esculentum*)	Leaf	Not tested	110 (FW)[b]	1.5 (FW)[b]
	Leaf	Not tested	0.17 (DW)[c]	n/d[c]
Waxy leaf nightshade	Leaf	Not tested	0.32 (DW)[c]	0.08 (DW)[c]
(*Solanum glaucophyllum*)	Cell culture derived from leaf material	Not tested	220.00 (FW)[d]	100.00 (FW)[d]
Potato (*Solanum tuberosum*)	Leaf	Not tested	15 (FW)[b]	n/d[b]
Bell pepper (*Capsicum annuum*)	Leaf	Not tested	n/d[c]	n/d[c]
Day blooming jasmine (*Cestrum Diurnum*)	Leaf	Not tested	10 (DW)[e]	10 (DW)[e]
Zucchini (*Cucurbita pepo*)	Leaf	Not tested	23 (FW)[b]	Not tested
Alfalfa/Lucerne (*Medicago sativa*)	Leaf	4.8 DW)[f]	0.06 (DW)[f]	Not tested
Rimu (*Dacrydium cupressinum*)	Fruit	70 (DW)[g]	11.5 (DW)[g]	Not tested
Algae				
Microalgae				
Phytoplankton	Whole algae	1.9-4.3 (DW)[h]	2.2-14.7 (DW)[h]	Not tested
		5.3 (DW)[i]	80.4 (DW)[i]	Not tested
Zooplankton	Whole algae	n/d[h]	3.0-10.9 (DW)[h]	Not tested
		72.4 (DW)[i]	271.7 (DW)[i]	Not tested
(*Pavlova lutheri*)	Whole algae	3900 (DW)[j]	Not tested	Not tested
(*Tetraselmis suecica*)	Whole algae	1400 (DW)[j]	Not tested	Not tested
Marine centric diatom (*Skeletonema costatum*)	Whole algae	1100 (DW)[j]	Not tested	Not tested
(*Isochrysis galbana*)	Whole algae	500 (DW)[j]	Not tested	Not tested
(*Chaetoceros calcitrans*)	Whole algae	n/d[j]	Not tested	Not tested
Macroalgae				
Japanese Wireweed (*Sargassum muticum*)	Not specified	90 (DW)[j]	Not tested	Not tested

Decimal places are reported as per the original reference. n/d, not detected; DW, dry weight; FW: fresh weight
[a] [15] [b] [13] [c] [14] [d] [12] [e] [16] [f] [17] [g] [18] [h] [19] [i] [20] [j] [21]

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Given the emerging interest in Australian native food plants for local consumption and export [22], we measured vitamin D_2, vitamin D_3, 25-hydroxyvitamin D_2 ($25(OH)D_2$) and $25(OH)D_3$ in a selection of Australian native food plants and Australian-grown edible seaweed. Since the metabolism of calcium and vitamin D are closely linked in animals, and there is a potential link between calcium and vitamin D metabolism in plants [23], we focused on plants and seaweed with high calcium content (Table 2). We used liquid chromatography with triple quadrupole mass spectrometry (LC-QQQ), which has been validated for the detection of low levels of vitamin D metabolites in biological samples [24], modified to suit the complex matrices of plants and algae.

Table 2. Calcium content of Australian native food plants and edible seaweed derived from published literature.

Common Name ***(Botanical Name)***	**Plant part**	**Calcium (mg/100 g)**
Plants		
Wattleseed	Leaf	No data
(*Acacia victoriae*)	Raw seed	No data
	Roasted, milled seed	434[a]
Tasmanian mountain pepper	Fresh leaf	No data
(*Tasmannia lanceolata*)	Dried leaf	495[a]
	Fresh berries	No data
	Dried berries	148[a]
Lemon myrtle	Fresh leaf	No data
(*Backhousia citriodora*)	Dried Leaf	1583[a]
Wakame	Fresh	150[b]
(*Undaria pinnatifida*)	Dried	1100-3000[c]
Algae		
Kombu	Fresh	No data
(*Lessonia corrugata*)	Dried	706[b]

[a] **[25]** [b] **[26]** [c] **[27]**

2. Materials and Methods

2.1 Sample acquisition

Samples of Australian native food plants (wattleseed (*Acacia victoriae*), lemon myrtle (*Backhousia citriodora*) and Tasmanian mountain pepper (*Tasmannia lanceolata*)), and Australian-grown edible seaweeds (wakame (*Undaria pinnatifida*) and kombu (*Lessonia corrugata*)) were sourced from commercial growers or wild harvesters. The selected plants were identified as commonly consumed and commercially available in the Australian food supply [22,25].

Samples were shipped directly from the growers and harvesters to the National Measurement Institute of Australia (NMI), Port Melbourne, Victoria, for preparation and analysis. To maintain their integrity, fresh samples were shipped in an insulated box containing cooler bricks. Details of quantity and source of samples, along with any processing by the growers and harvesters, are outlined in Table 3. Ethics approval was not required for this study.

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96 **Table 3**. Description of plant and algae samples used in the current study.
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Common Name (*Botanical Name*)	Plant part	Quantity	Location	Processing	Edible/ Toxic
			Plants		
Wattleseed	Leaf	1 kg	South Australia	Picked fresh morning of shipping August 2017	Toxic [a]
(*Acacia victoriae*)	Raw seed	200 g	South Australia	Harvested January 2017	Edible [a]
	Roasted, milled seed	188 g	South Australia	Processed as needed, seeds roasted in a rotating drum roaster for 20 min at 400°C, seeds ground in a grain mill	Edible [a]
Tasmanian mountain pepper	Leaf	1 kg	Tasmania	Picked fresh morning of shipping August 2017	Edible [a]
	Dried leaf	200 g	Tasmania	Harvested March-May 2017, warm air-dried	Edible [a]
(*Tasmannia lanceolata*)	Fresh berries	1 kg	Tasmania	Frozen to <4°C	Edible [a]
	Dried berries	200 g	Tasmania	Harvested March-May 2017, warm air-dried	Edible [a]
Lemon myrtle	Leaf	1 kg	New South Wales	Picked fresh morning of shipping August 2017	Edible [a]
(*Backhousia citriodora*)	Dried Leaf	200 g	New South Wales	Machine harvested morning of processing, leaf and sticks separated, leaves dried in an air dryer for 12-24 h	Edible [a]
Algae					
Wakame	Fresh upper leaf and central stem	1 kg	Tasmania	Chilled to 4°C after harvest August 2017	Edible [b]
(*Undaria pinnatifida*)	Dried upper leaf and central stem	100 g	Tasmania	Chilled to 4°C after harvest, processed <48 h after harvest, blanched 45 s at 70°C, plunged into ice water, drained, air dried	Edible [b]
Kombu	Fresh leaf	1 kg	Tasmania	Chilled to 4°C after harvest August 2017	Edible [b]
(*Lessonia corrugata*)	Dried leaf	100 g	Tasmania	Chilled to 4°C after harvest, processed <48 h after harvest, chopped, air-dried	Edible [b]

98 [a] [28] [b] [29]
99

2.2 Sample preparation

Upon arrival at NMI, dried samples were stored at room temperature and fresh samples were stored at <5 °C. Dried and freeze-dried samples were homogenised. Fresh samples were prepared as follows: leaves were cut to 1 cm squares and fresh fruit was blended; weight was recorded; samples were frozen overnight at -70 °C and then freeze dried for 48 hours to -50 °C and <10 mTorr; weight was recorded again; freeze-dried factor was determined. Each dried and fresh sample yielded one analytical sample. Prepared samples were stored between -16 °C and -20 °C until extraction and analysis. Extraction methodology was derived from published [14,30] and NMI methodology (VL294, VL392).

2.3 Sample analysis

Samples were homogenised with 1 g ascorbic acid, 10 mL deionised water, 30 mL absolute ethanol, 2 g potassium hydroxide pellets and 100 µL of 100 ng/mL deuterated internal standard mix, and made to 50 mL with deionised water. The headspace was flushed with nitrogen gas, capped and placed in a shaker for saponification overnight. The samples underwent centrifugation and 10 mL of the ethanol layer was extracted onto diatomaceous earth SPE cartridges (ChemElute Agilent). The organic soluble compounds were washed off with two 30 mL aliquots of petroleum spirits. The washes were collected into 80 mL glass EPA vials and then evaporated to dryness under high purity nitrogen gas. The residue was reconstituted into 400 µL heptane and transferred to a LC vial containing a 400 µL glass insert. Prepared extracts were stored at -20 °C.

Where samples were determined to have high diene content, extract clean-up via normal phase chromatography fraction collection was performed. Extracts were inspected for cold precipitate: if present, the liquid extract was transferred to a fresh 400 µL glass insert. Of the remaining liquid extract, 200 µL were injected onto a normal phase chromatographic system with a silica column, 1 mL/min 2% isopropyl alcohol in heptane mobile phase and a photodiode array detector set to 265 nm. Vitamin D and 25(OH)D fractions were collected.

Fractions of vitamin D and 25(OH)D were combined and evaporated under high purity nitrogen gas. The dry material was reconstituted in 200 µL of dry acetonitrile containing 1 mg/mL of 4-phenyl-1,2,4-triazole-3,5-dione (PTAD) and transferred to a fresh LC vial. Two hours were allowed to complete derivatisation. The sample was evaporated under high purity nitrogen gas. The dry material was reconstituted in 100 µL of methanol and water (70:30) and transferred to a fresh 400 µL glass insert and placed into an LC vial.

Samples were analysed for vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 using LC-QQQ. Calibration samples of vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 were prepared. The calibration concentrations (in ng/mL) were 0, 2.5, 5, 7.5, 10, 15, 25, 50, 75, and 100. Each calibration sample also contained 10 ng/mL of deuterated internal standard for each vitamer (vitamin D analogue) tested. The calibrations and samples were analysed using 1290 Infinity Series LC System/6460 Triple Quad LC-MS fitted with a Jet Stream ESI source (Agilent Technologies, US) in positive ion mode using a Supelco Ascentis Express C18 10cm x 2.1mm, 2.7µm LC chromatographic column.

For each vitamer analysed, each sample was tested in duplicate, and duplicate values were averaged to obtain one mean value for each sample. A third sample, spiked with the same vitamer, was analysed for each sample tested to provide quality control data. The mean percentage recovery and mean relative percentage difference were calculated for each vitamer. At the time of writing, the expected limit of detection, post validation study, is expected to be 0.05 µg/100 g (N.Strobel, email communication, 10 October, 2017).

3. Results

The mean recovery percentage across all samples for vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 was 96%, 98%, 101% and 94%, respectively. Across all samples, the mean relative percentage difference between duplicate samples for vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3 was 71%, 15%, 50% and 56%, respectively.

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Table 4. New data on the content (dry weight) of vitamin D_2, vitamin D_3, 25-hydroxyvitamin D_2 and 25-hydroxyvitamin D_3 in Australian native food plants and edible seaweed.

Common Name (*Botanical Name*)	Plant part	Vitamin D_2 (μg/100 g)	Vitamin D_3 (μg/100 g)	25(OH)D_2 (μg/100 g)	25(OH)D_3 (μg/100 g)
Plants					
Wattleseed (*Acacia victoriae*)	Leaf	n/d	n/d	n/d	n/d
	Raw seed	0.03	n/d	n/d	n/d
	Roasted, milled seed	n/d	n/d	n/d	n/d
Tasmanian mountain pepper (*Tasmannia lanceolata*)	Fresh leaf	n/d	n/d	n/d	n/d
	Dried leaf	0.67	n/d	n/d	n/d
	Fresh berries	n/d	n/d	n/d	n/d
	Dried berries	0.05	n/d	n/d	n/d
Lemon myrtle (*Backhousia citriodora*)	Fresh leaf	0.03	n/d	n/d	n/d
	Dried Leaf	0.24	n/d	n/d	n/d
Algae					
Wakame (*Undaria pinnatifida*)	Fresh upper leaf and central stem	n/d	n/d	n/d	n/d
	Dried upper leaf and central stem	n/d	n/d	n/d	n/d
Kombu (*Lessonia corrugata*)	Fresh leaf	n/d	0.01	n/d	n/d
	Dried leaf	n/d	n/d	n/d	n/d

n/d, not detected

Of the 13 samples tested, six contained quantifiable vitamin D metabolites (Table 4). Five samples contained vitamin D_2: raw wattleseed (0.03 μg/100 g); fresh and dried lemon myrtle leaves (0.03 and 0.24 μg/100 g, respectively); and dried leaves and berries of Tasmanian mountain pepper (0.67 and 0.05 μg/100 g, respectively). Fresh kombu contained 0.01 μg/100 g vitamin D_3. The concentrations of vitamin D metabolites in other samples were below the limit of detection.

4. Discussion

We detected low levels of vitamin D_2 in raw wattleseed, dried leaves and fruit of Tasmanian mountain pepper, and fresh and dried lemon myrtle leaves. Although fungal infection was not tested for in our study, the vitamin D_2 content found in the plants may have been due to fungal contamination [31]. In fact, vitamin D_2 is considered a marker for fungal contamination in some crops, such as ryegrass (*Lolium perenne L.*) and hops (*Humulus lupulus L.*) [32,33]. Vitamin D_3 and, in some cases, 25(OH)D_3 have previously been detected in the leaves of tomato [13-15], waxy leaf nightshade [12,14], potato [13], day blooming jasmine [16], zucchini [13] and alfafa [17]; however, we did not detect these metabolites in our samples of native Australian plants, and only very low levels in seaweed.

Recently, the fruit of the rimu tree (*Dacrydium cupressinum*), a podocarp native to New Zealand, was found to contain substantial amounts of both vitamin D_2 and D_3 [18]. Measured by isocratic reversed-phase HPLC, the average vitamin D_2 and D_3 contents of rimu berries were reported as 70 μg/100g and 11.5 μg/100g, respectively, although no quality control data were provided. In another study, the precursors of vitamin D_2 and D_3 (ergosterol and 7-dehydrocholesterol, respectively) were detected in plant oils [34]. Other studies have found that the vitamin D_3 and 25-hydroxyvitamin D_3 content of leaves and cell cultures of certain plants increases after UV irradiation [14,16,35]. For example, exposure to UV radiation increased the vitamin D_3 content of tomato (*Solanum lycopersicum* L.) leaves by almost 60 times to 100 ng/g, compared to 1.7 ng/g in non-UV-exposed leaves [14]. Future investigations into other potential plant sources of vitamin D, and the effect of exposure to UV radiation, appear warranted by the finding that consumption of plant oils, particularly UV B-irradiated wheat germ oil, increased 25(OH)D plasma concentration in mice [34].

Sargassum, an edible macroalgae [29], was first discovered to have anti-rachitic properties when the lipid fractions of the algae were fed to rats with induced rickets [36]. Since then, vitamin D_2 and vitamin D_3 have been found in microalgae and macroalgae using HPLC, in much larger quantities than found in our study [19-21]. Vitamin D metabolites were largely undetected in macroalgae in our study, with the exception of vitamin D_3 in kombu (*Lessonia corrugata)* measured at 0.01 µg/100g. In other studies, Japanese wireweed (*Sargassum muticum)* was found to contain 90 µg/100g, while vitamin D_2 and D_3 contents in microalgae ranged from not detected to 3900 µg/100g and 2.2 to 271.7 µg/100g, respectively [19-21]. Ergosterol and 7-dehydrocholesterol have also been found in microalgae [20]. As with plants, it has been suggested that significant vitamin D content of algae is dependent on exposure to UV radiation [19,37]. The role of UV radiation has been implicated by the finding that microalgae harvested in summer have a higher vitamin D_2 and D_3 content than those harvested in autumn and winter [19]. Although we detected only low levels of vitamin D_3 in kombu, and no vitamin D_2 or vitamin D_3 in wakame, the algae tested in our study were harvested in the winter months and were not sundried or exposed to UV radiation after harvest.

Plant and algal matrices present challenges for the quantification of vitamin D_2, vitamin D_3, 25(OH)D_2 and 25(OH)D_3, due in part to the presence of interfering compounds such as chlorophyll and lipophilic pigments [37]. Therefore, any method used must be highly sensitive and selective [37]. When compared to single MS, LC-QQQ has higher sensitivity and selectivity when applied to the detection of pesticides in water and soil samples [38]. To our knowledge, this method has not been used previously to detect vitamin D metabolites in complex plant and algal matrices, and is a major strength of our study due to the low detection limits of the instrumentation. The mean recovery from all spiked samples in our study was high, indicating that LC-QQQ is highly accurate in detecting low levels of vitamin D in plant and algal matrices. All samples were measured in duplicate and, where possible, we tested the edible portion in addition to the leaf material. However, we tested only a few species of Australian native food plants and Australian-grown edible seaweed. Although regional and seasonal variation have been shown to influence the vitamin D content of plants [32,37], we analysed only single samples sourced from single locations and during months of relatively low UV radiation levels.

The vitamin D content of the Australian native food plants and Australian-grown edible seaweed tested in our study is not nutritionally relevant to the Australian food supply. However, given that the vitamin D precursors, ergosterol and 7-dehydrocholesterol, have previously been found in both plants and algae, testing the effect of exposure to UV radiation on the vitamin D content of Australian native food plants and Australian-grown edible seaweed may be justified.

Author Contributions: JFB, RML, JLS and LJB conceived and designed the study; LJH sourced the samples and wrote the paper; NS conducted laboratory analyses; LJB, JLS, JFB, RML, ED and NS provided critical revision of the manuscript for important intellectual content. All authors are in agreement and this material has not been published elsewhere.

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