Vitamin D_4 in Mushrooms

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Abstract

An unknown vitamin D compound was observed in the HPLC-UV chromatogram of edible mushrooms in the course of analyzing vitamin D_2 as part of a food composition study and confirmed by liquid chromatography-mass spectrometry to be vitamin D_4 (22-dihydroergocalciferol). Vitamin D_4 was quantified by HPLC with UV detection, with vitamin [3H] itamin D_3 as an internal standard. White button, crimini, portabella, enoki, shiitake, maitake, oyster, morel, chanterelle, and UV-treated portabella mushrooms were analyzed, as four composites each of a total of 71 samples from U.S. retail suppliers and producers. Vitamin D_4 was present (>0.1 µg/100 g) in a total of 18 composites and in at least one composite of each mushroom type except white button. The level was highest in samples with known UV exposure: vitamin D enhanced portabella, and maitake mushrooms from one supplier (0.2–7.0 and 22.5–35.4 µg/100 g, respectively). Other mushrooms had detectable vitamin D_4 in some but not all samples. In one composite of oyster mushrooms the vitamin D_4 content was more than twice that of D_2 (6.29 vs. 2.59 µg/100 g). Vitamin D_4 exceeded 2 µg/100 g in the morel and chanterelle mushroom samples that contained D_4, but was undetectable in two morel samples. The vitamin D_4 precursor 22,23-dihydroergosterol was found in all composites (4.49–16.5 mg/100 g). Vitamin D_4 should be expected to occur in mushrooms exposed to UV light, such as commercially produced vitamin D enhanced products, wild grown mushrooms or other mushrooms receiving incidental exposure. Because vitamin D_4 coeluted with D_3 in the routine HPLC analysis of vitamin D_2 and an alternate mobile phase was necessary for resolution, researchers analyzing vitamin D_2 in mushrooms and using D_3 as an internal standard should verify that the system will resolve vitamins D_3 and D_4.

Introduction

Vitamin D is a 9,10-secosteroid and 6 forms have been identified [1]. Vitamin D_2 (9,10-seco(5Z,7E)-5,7,10(19),22-ergostatetraene-3β-ol; ergocalciferol) and vitamin D_3 (9,10-seco(5Z,7E)-5,7,10(19)cholestatriene-3β-ol; cholecalciferol) are the predominant forms of vitamin D relevant to human nutrition. Vitamin D_3 originates from animal sources, and vitamin D_2 is derived predominantly from fungi, such as yeast [2,3]. The importance of vitamin D in bone (calcium homeostasis) is well established, and vitamin D has been the subject of increased attention in recent years for its role in muscle function, immunology, heart and cardiovascular disease, cancer, and insulin secretion [4,5,6,7,8]. A primary source of vitamin D_3 in humans and many animals occurs from the conversion of 7-dehydrocholesterol in the epidermis to vitamin D_3 during exposure to ultraviolet (UV) radiation present in sunlight [2]. Oily fish and fish liver oils are naturally rich dietary sources of vitamin D_3. Other foods in the U.S. marketplace are fortified (typically with vitamin D_3), including milk, cheeses, yogurts, cereals, margarines, and orange juice.

Mushrooms are a natural source of vitamin D_2. The vitamin D_2 content of mushrooms can be increased dramatically by UV irradiation, whereby ergocalciferol is formed from ergosterol [9,10,11,12,13]. Recent analyses conducted on ten types of mushrooms sampled from the U.S. marketplace showed vitamin D_2 concentrations between 0.03–63.2 µg/100 g (1.2–2528 IU/100 g) fresh weight, with the highest levels in mushrooms exposed to UV during production [14]. Ergosterol is also found in yeast and other fungi [15], and vitamin D_2 is produced industrially by UV irradiation of yeast [3]. Vitamin D_2 is included in some dietary supplements and fortified foods, particularly vegetarian products.

The occurrence of vitamers other than D_3 and D_2 in the food supply has not been widely reported in the literature, nor have their nutritional value and biological effects. In the available studies evaluating the vitamin D content in different mushroom species (including Mattila et al. [16,17,18], Rangel-Castro et al. [19], Teichmann et al. [13]), no vitamers other than D_2 have been

reported. In our recent analysis of the vitamin D$_2$ and sterol content of ten types of mushrooms [14] a second peak having a UV spectrum consistent with vitamin D was present in the HPLC chromatogram of many samples and occurred at a relatively high level in mushrooms that had been exposed to UV light. The vitamin D$_4$ precursor ergosta-5,7-dienol (22,23-dihydroergosterol) was present in all samples. The purpose of this communication is to report on findings that support the identification of vitamin D$_4$

in mushrooms, and the vitamin D$_4$ content of ten types of mushrooms.

Materials and Methods

General experimental procedures

Reagents and standards for extraction and analysis of vitamin D and sterols were as described previously [14]. Authentic vitamin
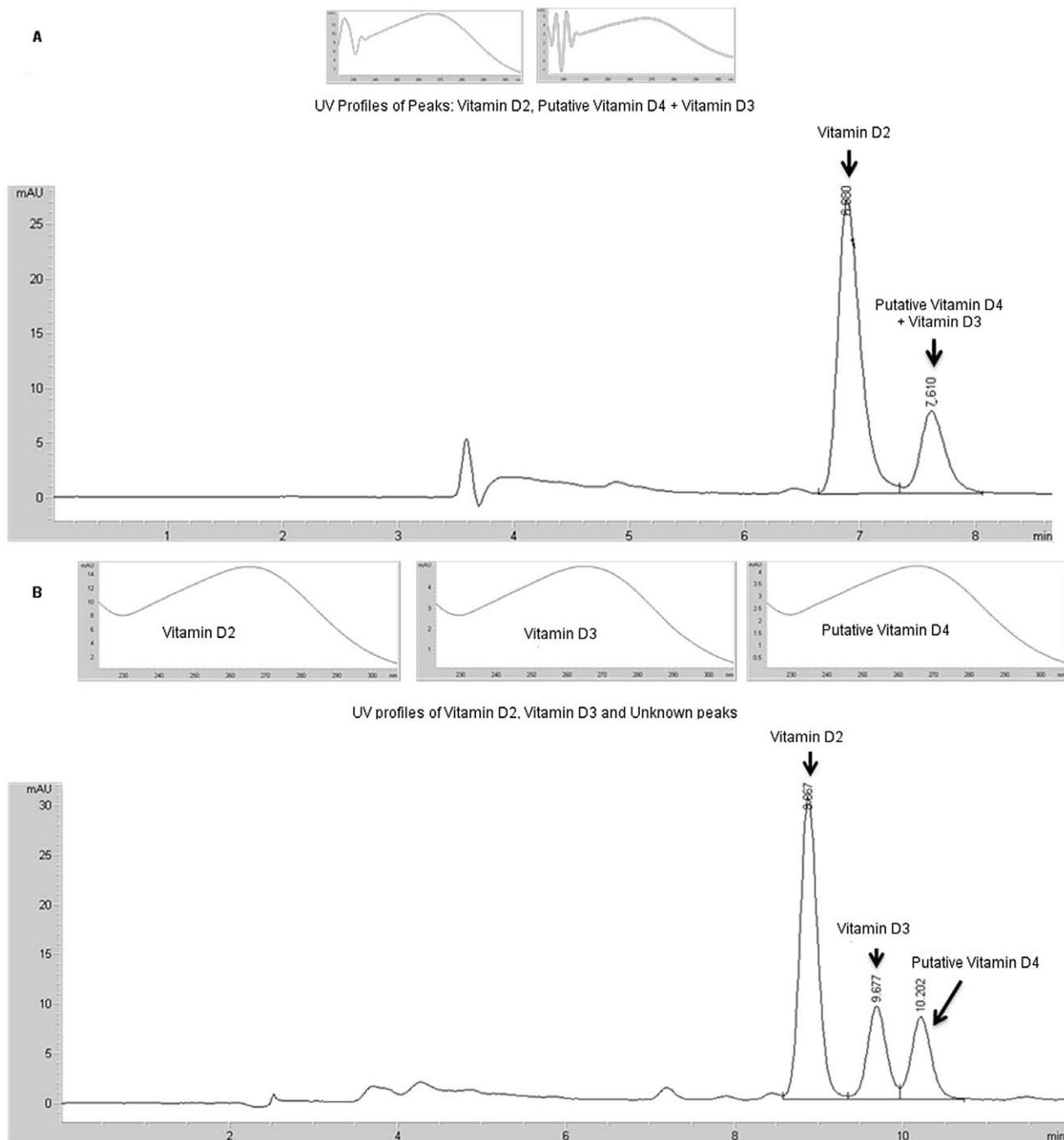
Figure 1. HPLC chromatograms and UV spectra of vitamin D components in a mixed mushroom extract. Chromatography on a Vydac® ODS column developed using (A) acetonitrile:methylene chloride (70:30) (the solvent system used previously for quantitation of vitamin D$_2$ [14]), showing co-migration of the putative vitamin D$_4$ with vitamin D$_3$ in this system; (B) developed with acetonitrile:methanol (1:1) mobile phase, showing separation of the peak containing putative vitamin D$_4$ and vitamin D$_3$ into two components.
doi:10.1371/journal.pone.0040702.g001

Figure 2. High resolution mass spectral comparison of putative vitamin D$_4$ isolated from mushroom. (**A**) Spectrum of HPLC-purified mushroom isolate corresponding to vitamin D$_4$ with structure and breakdown products highlighted. (**B**) Spectrum of vitamin D$_4$ standard.
doi:10.1371/journal.pone.0040702.g002
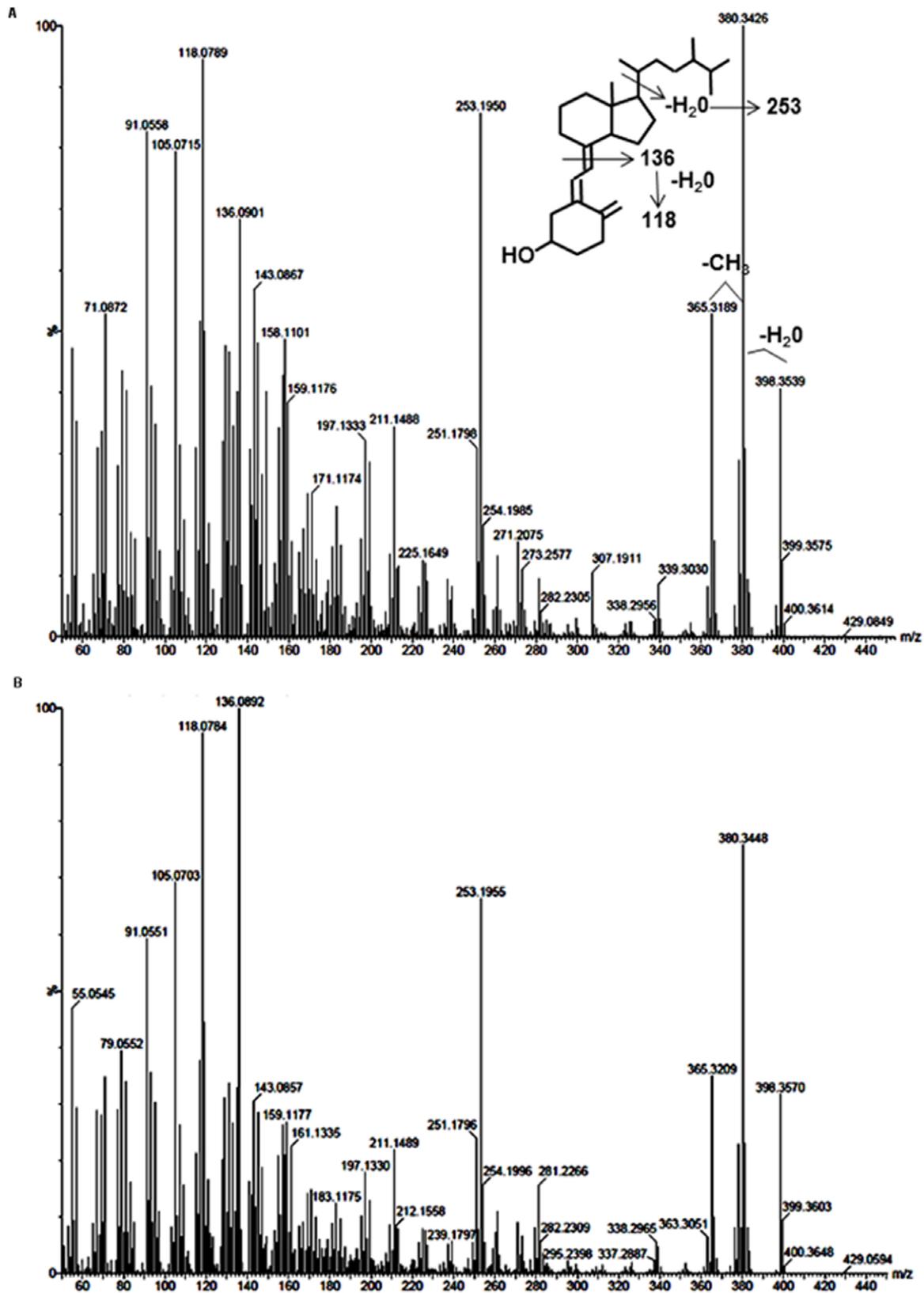
D_4 (manufacturer's specified purity, 98.9% by TLC9) was procured from Lanospharma Laboratories Co., Ltd. (Chongqing, China). Ergosterol and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma-Aldrich Corp. (St. Louis, MO).

Samples

Samples of white button, crimini, portabella, enoki, shiitake, maitake, oyster, morel, UV-treated portabella, and chanterelle mushrooms were the same as described in detail in the previous report on vitamin D_2 and sterols [14] and comprised a total 71 original samples analyzed as four composites of each type of mushroom (two for chanterelle).

Extraction and analysis of sterols and vitamin D

Sterols and vitamin D_2 were quantified as described previously [14]. Sterols were determined as the trimethylsilyl ether (TMS) derivatives, by gas chromatography with flame ionization detection after alkaline saponification of total lipid extracts, with gas chromatography-mass spectrometry (GC-MS) to confirm component identities. Vitamin D_4 was quantified using high-performance liquid chromatography (HPLC) with UV detection and [³H]vita-

min D_3 as the internal standard as described previously for vitamin D_2 [14], except using the HPLC conditions described below.

Identification of the unknown

Mass spectrometry was performed at the High Resolution Mass Spectrometry Facility at the University of Iowa (Iowa City, IA) using a Waters GCT Premier (Waters Corp. Milford, MA). For solid probe high resolution mass spectrometry the ramp temperature used was 100°C/min. For GC-MS the column was a 30m DB-5ms and the ramp started at 170°C, then increased by 10°C per minute with a final temperature of 300°C that was held for 15 minutes. BSTFA derivatization for GC-MS was performed by re-suspending dry samples or standards in a 1:1 mixture of BSTFA:methylene chloride, warming at 40°C for 60 minutes followed by direct injection of an aliquot of a given mixture onto the GC-MS column.

Quality control

A sample of a mushroom control composite previously described [14], that comprised approximately 50% portabella mushrooms and 50% vitamin D enhanced (UV-treated) portabella mushrooms, was analyzed with each batch of samples and used to
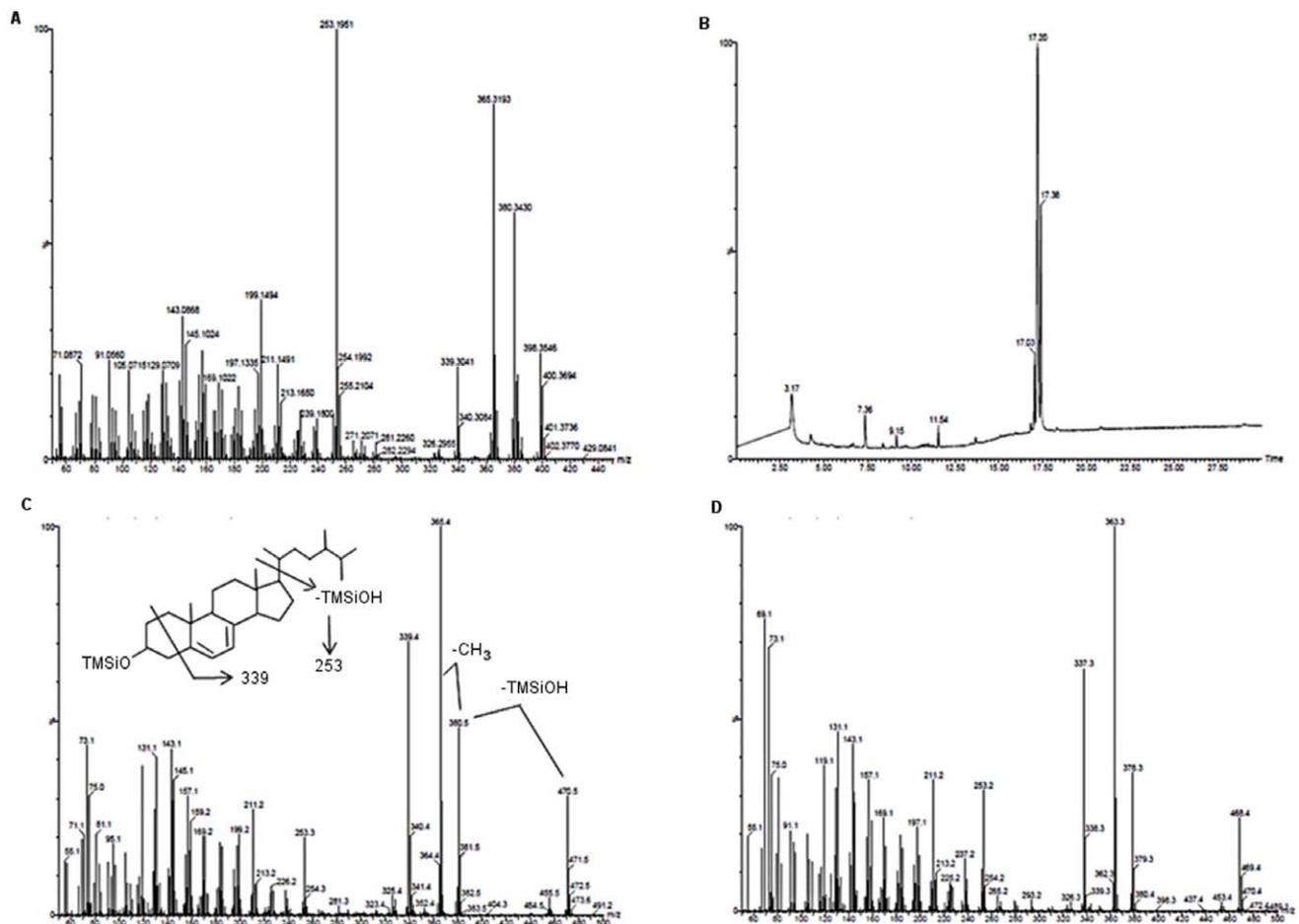
Figure 3. Spectral analysis of putative dihydroergosterol in a mushroom isolate. (**A**) High resolution mass spectrum of purified mushroom isolate corresponding to dihydroergosterol. (**B**) Gas chromatogram of products obtained following derivatization of the purified mushroom isolate with N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA). (**C**) Low resolution GC-MS of derivatized mushroom product at t = 17.20 min corresponding to dihydroergosterol with structure and breakdown products highlighted. (**D**) Low resolution GC-MS of commercially available ergosterol standard following derivatization with BSTFA.
doi:10.1371/journal.pone.0040702.g003

Table 1. Vitamin D_4 and pre-vitamin D_4 (22,23-dihydroergosterol; ergosta-5,7-dienol) content of ten types of mushrooms.

Mushroom	Scientific name	NDB no.[a]	Composite[b]	Moisture (g/100g)	Vitamin D_4 μg/100g fresh weight[c]	Mean	SD	Std Err	22,23-Dihydroergosterol mg/100g fresh weight	Mean
White button	*Agaricus bisporus*	11260	1	92.85	–	–[B]	–	–	5.97	6.03 [B,C]
			2	92.81	–				5.79	
			3	92.35	–				5.86	
			4	92.47	–				6.49	
Enoki	*Flammulina veluptipes*	11950	A1	87.68	–	0.10 [B]	0.21	0.10	17.0	16.5 [A]
			A2	88.47	–				18.0	
			G1	88.28	0.41				17.0	
			1	89.30	–				13.8	
Shiitake	*Lentinus edodes*	11238	1	86.90	0.27	0.51 [B]	0.48	0.24	7.31	6.51 [B,C]
			2	91.41	0.67				7.25	
			3	90.53	1.11				6.15	
			A1	90.11	–				5.34	
Maitake	*Grifola frondosa*	11993	A1	88.37	–	14.5 [A]	17.5	8.76	8.90	6.34 [B,C]
			A2	88.59	–				9.00	
			C1	92.30	35.4				3.53	
			C2	91.92	22.5				3.92	
Oyster	*Pleurotus ostreatus*	11987	A1	89.70	0.81	1.77 [AB]	3.00	1.52	8.55	8.89 [B]
			1	88.77	–				11.7	
			2	90.38	6.29				8.16	
			3	90.54	–				7.13	
Crimini	*Agaricus bisporus*	11266	1	91.92	–	0.31 [B]	0.61	0.31	5.25	5.92 [B,C]
			2	91.22	1.22				6.11	
			A1	93.08	–				5.42	
			B1	92.07	–				6.92	
Portabella	*Agaricus bisporus*	11265	1	90.96	–	0.14[B]	0.27	0.14	6.75	6.18 [B,C]
			2	92.22	–				5.45	
			3	91.29	0.55				6.53	
			4	91.25	–				5.97	
Portabella, uv treated	*Agaricus bisporus*	11998	A1	94.86	0.20	3.62 [AB]	3.22	1.61	4.57	4.70 [C]
			A2	95.12	1.66				3.94	
			B1	94.76	7.05				5.10	
			B2	93.68	5.56				5.20	
Chanterelle	*Cantharellus californicus* or *C. cibarius*	11239	D1	91.09	0.82	1.62 [AB]	1.13	0.80	5.23	4.49 [C]
			D2	88.61	2.42				3.75	
Morel	*Morchella spp.*	11240	E1	89.46	2.36	1.13 [B]	1.31	0.65	7.13	5.79 [B,C]
			E2	90.38	2.15				5.75	
			F1	89.44	–				5.31	
			F2	89.18	–				4.98	

[a]Database entry number from United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference [53]; [b] Composites are combinations of samples from statisitical sampling locations in the U.S., or retail suppliers, as described in Phillips et al. [14]. Composites designated with the same capital letter were from the same supplier. [c] – indicates less than the limit of detection (0.1 μg/100 g fresh weight).
doi:10.1371/journal.pone.0040702.t001

monitor run-to-run precision. Validation of recovery of vitamin D_2 as described in a previous communication [14] was assumed to apply to the extraction of vitamin D_4. The GC-MS analyses described above verified the identity of the analyte peaks.

Data analysis

Means and standard deviations were calculated using Microsoft® Office Excel (Professional Plus edition, 2010; Microsoft Corporation, Redmond, WA), and analysis of variance ($\alpha = 0.05$)
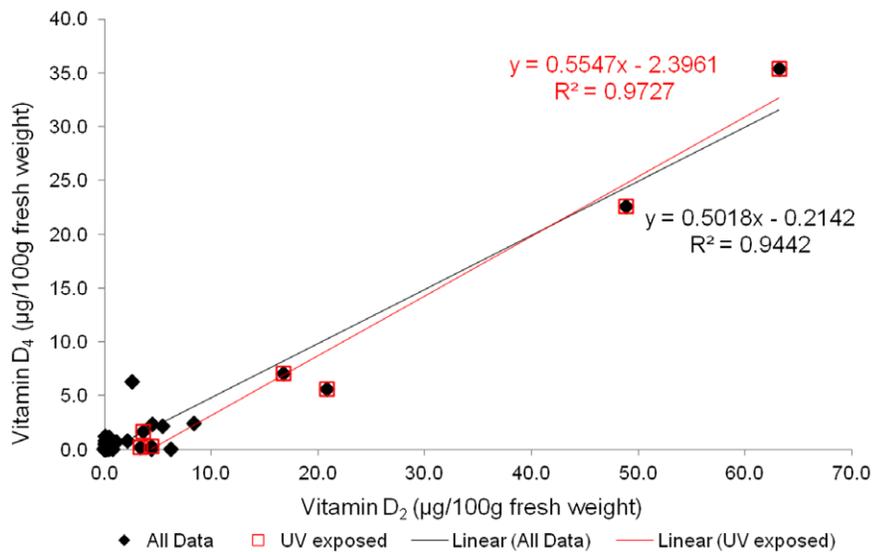
Figure 4. Relationship between the vitamin D$_4$ and vitamin D$_2$ concentrations in ten types of mushrooms (Table 1). Data for vitamin D$_2$ were previously reported [14].
doi:10.1371/journal.pone.0040702.g004

and pairwise comparison of means using the Student-Newman-Keuls test with a 95% confidence interval were performed with XLSTAT (version 2011.2.06; Addinsoft, New York, NY).

Results

Identification of vitamin D unknown in mushrooms

Initially the unknown vitamin D form observed in a variety of mushrooms in previous work [14] was thought to be vitamin D$_3$, because it eluted at the same retention time as a vitamin D$_3$ standard chromatographed under the conditions that were being used for analysis of vitamin D$_2$ and displayed the characteristic UV chromophore. Some literature reports were found on the presence of vitamin D$_3$ in alfalfa, tomato, eggplant and zucchini leaves and some other plants have been reported [20,21,22,23],

Table 2. Comparison of assayed concentrations of ergosterol (vitamin D$_2$ precursor) and 22,23-dihydroergosterol (vitamin D$_4$ precursor) in white and brown button mushrooms.

Component		Range (µg/100g dry weight)	
		This study	Shao et al. [31][a]
Ergosterol	White button	740–795	563–681
	Brown button[c]	725–821	475–938
22,23-Dihydroergosterol	White button	77–86	71–95 [b]
	Brown button[c]	65–87	42–65 [b]
22,23-Dihydroergosterol	White button	10.0–11.2	11.2–14.0
(as percent of ergosterol)	Brown button[c]	7.9–12.0	6.9–10.5

In this study for four samples of each type, and as reported by Shao et al. [31] for one sample at each of three stages of maturity for each mushroom type.
[a]values show the sum of the concentrations in the separately assayed stems and caps.
[b]reported as "ergosterol analogue".
[c]crimini.
doi:10.1371/journal.pone.0040702.t002

but none on nutritional quantities of vitamin D$_3$ or other forms besides D$_2$ in mushrooms.

Figure 1 shows the high-performance liquid chromatography (HPLC) chromatogram of a mushroom extract containing the putative vitamin D$_4$ and spiked with vitamin D$_3$. Figure 1A shows the chromatogram from the solvent system routinely used for vitamin D analysis (acetonitrile/methylene chloride (70/30) as described by Phillips et al. [14]; Figure 1B shows the separation of the vitamin D$_3$ and putative vitamin D$_4$ into two components using an alternate solvent system (acetonitrile:methanol, 1:1), confirming the component was not D$_3$. The unknown was hypothesized to be vitamin D$_4$ (22-dihydroergocalciferol) because it co-eluted with an authentic vitamin D$_4$ using the alternative solvent system and because its precursor is present in mushrooms. Although there have been no previous literature reports of vitamin D$_4$ in mushrooms, vitamin D$_4$ (22,23-dihydroergocalciferol;9,10-seco(5Z,7E)-5,7,10(19)-ergostatriene-3β-ol) is the product of UV irradiation of 22,23-dihydroergosterol, analogous to the formation of vitamin D$_2$ from ergosterol. 22,23-dihydroergosterol (ergosta-5,7-dienol) was present in ten types of mushrooms, as previously reported [14]. Therefore it seemed reasonable to presume conversion of some portion of the 22,23-dihydroergosterol to vitamin D$_4$, and mass spectral studies were conducted to confirm the identity.

Mass spectral confirmation

Material was collected from the putative vitamin D$_4$ peak of a mixture of mushroom types and analyzed by high resolution mass spectrometry and compared with an authentic vitamin D$_4$ standard run under identical conditions. As seen in Figure 2A, the mushroom compound produced a parent molecular ion at m/z 398.3539, in good agreement with the calculated mass value of 398.3549 for vitamin D$_4$. Losses of water and a methyl group are readily apparent (m/z 380.3426 and 365.3189). The prominent peak at 253.1950 corresponds to loss of the vitamin D$_4$ side chain in combination with a water molecule, while peaks at 136.0901 and 118.0789 are characteristic for cleavage of the secosteroid structure and subsequent water loss. All of these fragments were also observed with the authentic vitamin D$_4$ compound when
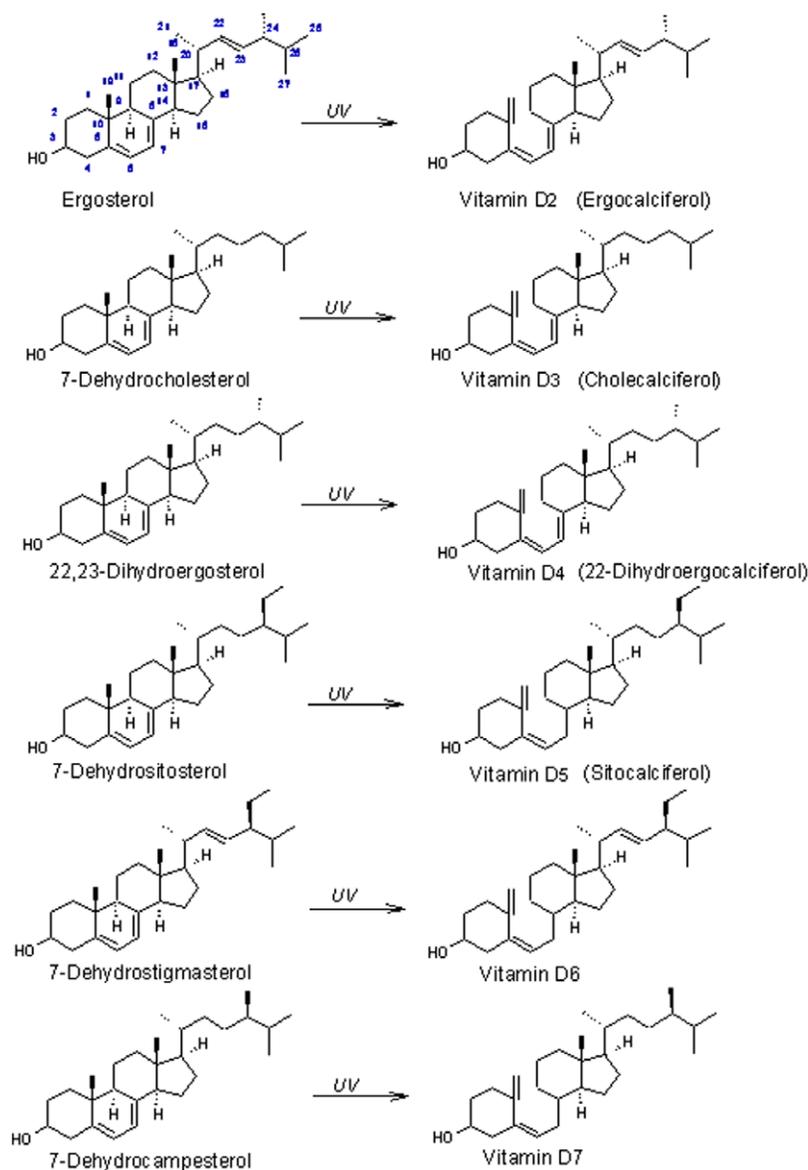
Figure 5. Structure of six forms of vitamin D their sterol precursors.
doi:10.1371/journal.pone.0040702.g005

subjected to the same high resolution analysis (Figure 2B). In addition, low resolution mass spectrometry of TMS-derivatized samples of both the mushroom isolate and vitamin D_4 standard produced analogous mass spectral fragmentations, with parent ions of m/z 470.5 (data not shown), thus verifying the presence of a single hydroxyl moiety and further corroborating the identity of this compound from the mushroom isolate as vitamin D_4.

In a similar manner, high resolution mass spectrometry was also performed on the purported 22,23-dihydroergosterol collected from the mixed mushroom sample; however, in contrast the spectra revealed the presence of at least 2 compounds, with molecular ions evident at m/z 398.3546 and 400.3694 (Figure 3A). The lower mass was in agreement with the prediction for 22,23-dihydroergosterol ($C_{28}H_{46}O$; calculated value 398.3549), while the higher mass suggested an additional saturation of a diene bond, presumably of a 22,23-dihydroergosterol-like molecule ($C_{28}H_{48}O$; calculated value of 400.3705). Because of the apparent complexity of the sample, the mixture was derivatized with BSTFA and

subjected to gas chromatography-mass spectrometry (GC-MS). As seen in Figure 3B, the gas chromatogram of the TMS-derivatized mushroom isolate revealed the presence of 3 peaks. The major peak (17.2 min) produced a parent ion of m/z 470.5, in keeping with the derivatization of a single hydroxyl moiety and consistent with the expected ion mass for the TMS derivative of 22,23-dihydroergosterol (Figure 3C). Ions corresponding to loss of trimethylsilanol (m/z 380.5) followed by a methyl group (m/z 365.4) were readily apparent. The decrease of 131 mass units to produce the ion at m/z 339 is proposed to arise from fragmentation of the A-ring, most likely involving loss of C-2, C-3, C-4 and their substituents [24,25]. Importantly, the presence of the m/z 253.3 ion, representing the core ring structure resulting from loss of the side chain and trimethylsilanol fragments indicates the additional saturation with hydrogen molecules occurred in the side chain. By way of comparison, an authentic ergosterol standard was similarly derivatized with BSTFA and subjected to GC-MS, which produced a single peak at 17.4 minutes (data not

shown). As seen in Figure 3D the fragmentation pattern for derivatized ergosterol standard essentially paralleled that of the mushroom isolate, including the presence of the m/z 253.2 ion; except for the observed decrease in the molecular ion due to unsaturation of the side chain in the standard material. Thus, the data are consistent with the isolation of 22,23-dihydroergosterol from the mushroom extract. Finally, the other 2 peaks observed in the GC trace from the derivatized mushroom isolate (17.03 and 17.38 min) both produced parent ions at m/z 472 and fragments at m/z 255 (data not shown). As noted above, we suspect these may be isomers corresponding to additional saturation of one or the other of the diene bonds in the B-ring of 22,23-dihydroergosterol to produce, for instance, 22,23-dihydrobrassicasterol. Additional experiments will need to be performed to confirm these suspicions; however, the loss of the diene entity would explain the extent to which these compounds could co-migrate with the 22,23-dihydroergosterol and escape detection by HPLC utilizing an ultraviolet light detector to track the purification of the mushroom compounds.

The quantitative values for 22,23-dihydroergosterol that are reported were obtained in the previously reported GC and GC-MS analysis [14], which provided better resolution and eliminated the interference of the other components that were shown to coelute with 22,23-dihydroergosterol in the HPLC system.

Vitamin D$_4$ content of mushrooms

Table 1 summarizes the assayed concentration (fresh weight basis) of vitamin D$_4$ and its precursor, 22,23-dihydroergosterol in ten types of mushrooms (white button, crimini, portabella, enoki, shiitake, maitake, oyster, morel, and UV-treated portabella, and chanterelle) sampled from retail outlets in the U.S. Overall, vitamin D$_4$ was detected (>0.1 µg/100 g) in 18 of the total of 38 composites analyzed and was present at an average concentration of 5.2 µg/100 g. However there was wide variability between and within samples different types of mushrooms. There were 7 samples known to contain mushrooms that had been exposed to UV light during production: the Mushroom CC, the vitamin D enhanced portabella, and the two maitake composites from supplier G (Table 1). All of these samples contained vitamin D$_4$, and in some the concentration was similar to or greater than that of vitamin D$_2$ (previously reported in Phillips et al. [14]). The two maitake mushroom samples that were high in vitamin D$_2$ (63.2 and 48.9 µg/100 g) were also high in vitamin D$_4$ (35.4 and 22.5 µg/100 g, respectively). These mushrooms were presumed to have been exposed to UV light under the growing conditions reportedly used by this producer [26]. Of the mushrooms not known to have received UV exposure, vitamin D$_4$ occurred in at least one composite of each type except white button. In oyster mushrooms the composite highest in vitamin D$_2$ (2.59 µg/100 g) had a vitamin D$_4$ content more than two-fold higher (6.29 µg/100 g). Vitamin D$_4$ exceeded 2 µg/100 g in the morel and chanterelle mushroom samples that contained D$_4$ (all but two morel composites).

Results for a total of 26 analyses of a control composite (Mushroom CC) across multiple assays provided an estimate of the analytical uncertainty in the vitamin D$_4$ concentrations assayed in individual composites. The mean vitamin D$_4$ concentration in the Mushroom CC was 0.14 µg/100g with a standard deviation of 0.042 µg/100 g (standard error, 0.008 µg/100 g). Greater precision at higher concentrations would be expected [27].

The presence of vitamin D$_4$ in all mushrooms with known UV exposure but with no consistency in other samples suggests that vitamin D$_4$ in mushrooms results from incidental or intentional UV exposure. Interestingly, Wang et al. [28] reported variability in the vitamin D level in lichens (*Cladina* spp.) as related to UV exposure at different latitudes. Figure 4 illustrates vitamin D$_4$ concentration as a function of vitamin D$_2$ concentration (previously reported [14]) in the 38 composites of ten types of mushrooms that were analyzed. Overall there was a positive correlation between vitamins D$_4$ and D$_2$. In a separate study of white button mushrooms subjected to controlled UV exposure [29], all of the UV-treated samples contained vitamin D$_4$, with an average of 2.43 µg/100 g fresh weight (range 1.95–2.74), whereas the concentration was <0.1 µg/100 g in the unexposed mushrooms.

Vitamin D$_4$ precursor in mushrooms

The vitamin D$_4$ precursor 22,23-dihydroergosterol was present in all mushroom composites (Table 1). The levels were not correlated with vitamin D$_4$, but differed among species. Enoki mushrooms had a notably higher 22,23-dihydroergosterol content, with an average of 16.5 mg/100g compared to 4.49–8.89 mg/100 g in other types of mushrooms.

There have been other, limited reports on 22,23-dihydroergosterol in mushrooms, although the diversity in common nomenclature for sterols often makes the synonymous identity or close structural similarity among various sterols not readily apparent (see Moss [30] for detailed information on steroid nomenclature). 22-23-Dihydroergosterol [(24R)-24-methylcholesta-5,7-dien-3β-ol] is ergosta-5,7-dienol, and ergosta-5,7-dienol in wild and cultivated mushrooms [*Cantharellus cibarius* and *C. tubaeformis* (chanterelle), *Boletus edulis* (king bolete), *Lentinus edodes* (shiitake), *Pleurotus ostreatus* (oyster), and *Agaricus bisporus* (white button, brown button, crimini), portabella] was reported by Teichmann et al. [13]. Vitamin D$_2$ levels were also analyzed in that study but no chromatograms from the vitamin D analysis were published, so it is not possible to determine if vitamin D$_4$ may have been present. Shao et al. [31] recently reported the ergosterol content of stems and caps of white and brown button mushrooms at different stages of development and identified an "ergosterol analogue" in their HPLC analysis. This component is likely 22,23-dihydroergosterol based on comparison of the concentrations reported to those in the present study, and the fact that this component was identified in all samples of white and brown mushrooms in the present investigation. In the Shao et al. study [31] the sum of the concentration of the "ergosterol analogue" in the saponified extracts of the stems and caps was 0.71–0.95 mg/g dry wt and 0.42–0.65 mg/g dry wt in brown mushrooms (11.2–14.0% and 6.9–10.5% of the ergosterol concentration, respectively). These concentrations were similar to the averages of 0.82 mg/g dry wt and 0.75 mg/g dry wt for 22,23-dihydroergosterol (10.7% and 9.8% of the ergosterol concentration, respectively) in this study (Table 2).

Discussion

The conjugated unsaturation at C-5 and C-7 in the B-ring is the key structural feature of sterols that are converted to vitamin D by UV irradiation. Figure 5 shows the sterol precursors of vitamin D compounds, which differ in the side chain at C-24 and the C22–23 bond. Excellent reviews are available on the metabolism and physiology of vitamin D [5,32,33]. Overall there is very little published on the physiological significance of vitamers other than D$_3$ or their occurrence in foods and other natural products aside from vitamin D$_2$ in mushrooms. Vitamin D$_3$ and D$_2$ are metabolized *in vivo* to the biologically active forms, 1α,25-dihydroxyvitamin D$_3$ and D$_2$ [22,34]. The bioavailability of vitamin D$_3$ is well established, and the bioavailability of vitamin D$_2$ from mushrooms in humans has been shown to be comparable

to that of a vitamin D$_2$ supplement [35,36]. Forms other than D$_3$ have shown lower biological activity in vitamin D dependent cellular functions in some studies. DeLuca et al. [37] synthesized 22,23-[^{3}H]vitamin D$_4$ and compared its metabolism to 22,23-[^{3}H]vitamin D$_3$ in the rat. Vitamin D$_4$ metabolites had a tissue distribution similar to vitamin D$_3$ but were excreted more quickly but also appear to have lower toxicity in high doses compared to D$_3$ [38].

The lower potential toxicity of vitamin D compounds other than D$_3$ has spurred interest in their development as vitamin D analogs for use as potential pharmaceutical agents. The synthetic derivative of vitamin D$_5$, 1α-hydroxyvitamin D$_5$, has shown anti-tumor activity and been studied as an anti-cancer treatment [39,40,41]. Tachibana and Tsuji [42] found the metabolism of 1α,25-dihydroxyvitamin D$_4$ to be similar to that of 1α,25-dihydroxyvitamin D$_2$ in a study involving rats. Jones [43] has written an excellent review on vitamin D analogs, their pharmaceutical applications, and potential mechanisms of action.

Knowledge of the occurrence of lesser known forms of vitamin D and their sterol precursors, particularly in foods, herbal medicines, and materials that may be sources of these compounds is therefore valuable, given the potential value of vitamin D compounds. Some other organisms in which 22,23-dihydroergosterol (ergosta-5,7-dienol; 22-dihydroergosterol) has been reported include *Chlorella* species [44] and various yeasts and fungi [15,45]. It has been found in *Mucor pusillus* [46], a source of a milk curdling protease used in cheese production. Interestingly, anobiid beetles have been shown to synthesize cholesterol from 22-dihydroergosterol supplied by symbiotic yeast, with 7-dehydrocholesterol (the precursor of vitamin D$_3$) as the intermediate [47]. 22,23-dihydroergosterol and also 7-dehydrostigmasterol (another $\Delta 5,7$-sterol) and the precursor of vitamin D$_6$ (Fig. 1) have been reported in *Trypanosoma cruzi*, the organism responsible for Chagas disease [48]. Vitamin D$_5$ is the product of UV irradiation of 7-dehydrositosterol (Fig. 5). 7-dehydrositosterol has been reported in *Rauwolfia serpentina* (snakeroot), a plant commonly used in Chinese herbal medicine [49] and also in algae [50]. 7-dehydrocampesterol, the C-24 epimer of 22,23-dihydroergosterol [51] and the precursor to vitamin D$_7$, has been found in *Crithidia fasciculate* [48] and in *Helianthus annuus* (sunflower) seed oil [52].

Because the vitamin D$_4$ precursor 22,23-dihydroergosterol occurred in all mushrooms analyzed and vitamin D$_4$ was found in approximately half of the samples overall and in all mushrooms with know UV exposure, its presence should be expected in mushrooms exposed to UV light in the commercial production of vitamin D enhanced products, or in wild grown or other mushrooms receiving incidental UV exposure.

Wide variability in the occurrence and vitamin D$_4$ concentration in this relatively large sampling of mushrooms also suggests that the common practice of using vitamin D$_3$ as an internal standard in the HPLC analysis of vitamin D$_2$ in mushrooms will result in errors unless the separation of vitamins D$_3$ and D$_4$ by the chromatographic system is assured.

Further study of the biological activity of vitamin D$_4$ is warranted, given its presence in many commonly consumed mushrooms.

Acknowledgments

Author Contributions

Conceived and designed the experiments: KMP RLH. Performed the experiments: RLH NJK. Analyzed the data: KMP RLH RRS. Contributed reagents/materials/analysis tools: KMP RLH NJK. Wrote the paper: KMP RRS RLH.

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