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Photobiology of vitamin D in mushrooms and its bioavailability in humans

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Abbreviations: 25(OH)D, 25-hydroxyvitamin D; 7-DHC, 7-dehydrocholesterol; cZc, 5,6-s-cis-s-cis previtamin D; *E. huxleyi*, *Emiliania huxleyi*; HPLC, high performance liquid chromatograph; IU, international units; LCMS/MS, liquid chromatography tandem mass spectroscopy; *S. cerevisiae*, *Saccharomyces cerevisiae*; tZc, 5,6-s-trans-s-cis previtamin D; UV, ultraviolet; UVB, ultraviolet B

Mushrooms exposed to sunlight or UV radiation are an excellent source of dietary vitamin D_2 because they contain high concentrations of the vitamin D precursor, provitamin D_2. When mushrooms are exposed to UV radiation, provitamin D_2 is converted to previtamin D_2. Once formed, previtamin D_2 rapidly isomerizes to vitamin D_2 in a similar manner that previtamin D_3 isomerizes to vitamin D_3 in human skin. Continued exposure of mushrooms to UV radiation results in the production of lumisterol$_2$ and tachysterol$_2$. It was observed that the concentration of lumisterol$_2$ remained constant in white button mushrooms for up to 24 h after being produced. However, in the same mushroom tachysterol$_2$ concentrations rapidly declined and were undetectable after 24 h. Shiitake mushrooms not only produce vitamin D_2 but also can produce vitamin D_3 and vitamin D_4. A study of the bioavailability of vitamin D_2 in mushrooms compared with the bioavailability of vitamin D_2 or vitamin D_3 in a supplement revealed that ingestion of 2000 IUs of vitamin D_2 in mushrooms was as effective as ingesting 2000 IUs of vitamin D_2 or vitamin D_3 in a supplement in raising and maintaining blood levels of 25-hydroxyvitamin D which is a marker for a person's vitamin D status. Therefore, mushrooms are a rich source of vitamin D_2 that when consumed can increase and maintain blood levels of 25-hydroxyvitamin D in a healthy range. Ingestion of mushrooms may also provide the consumer with a source of vitamin D_3 and vitamin D_4.

Prehistoric Overview

It is theorized that the origins of vitamin D came from Earth's earliest life forms over 900 million years ago. Primitive life forms were bombarded by the sun's UV (UV) radiation; this caused damage to their UV-sensitive DNA, RNA and proteins.[1] Provitamin D absorbs UV radiation between 240 and 320 nm and thus could act as a sunscreen. Phytoplankton and zooplankton that produced provitamin D_2 (ergosterol) were likely to have their DNA and RNA protected from UV radiation and were able to pass along their genes to their progeny.[1,2] Five hundred million years ago phytoplankton such as *Emiliania huxleyi (E. huxleyi*) were producing provitamin D_2.[1,2] Upon exposure to UVB radiation, provitamin D_2 was converted to previtamin D_2, which then thermally isomerized to vitamin D_2 (**Fig. 1**). It is believed that animals higher on the food chain acquired their vitamin D from phytoplankton and zooplankton.[1]

Land vertebrates require vitamin D to maintain adequate serum calcium levels and bone metabolism. Poikilothermic animals have been studied and were found to contain several provitamin D's in their skin, with the major provitamin D identified as 7-dehydrocholesterol (7-DHC, provitamin D_3).[1] Northern grass frogs and anolis lizards were found to have 1 to 2 orders of magnitude greater concentrations of provitamin D_3 in their skin compared with humans. Therefore, some poikilotherms have a tremendous capability to produce vitamin D_3 in their skin.[1] This finding suggests that the cutaneous production of vitamin D may have played a major evolutionary role in poikilothermic land vertebrates.

Historic Overview

Vitamin D deficiency has been implicated with several bone pathologies, one of the earliest being rickets. In the early 19th century, rickets was considered an epidemic affecting Europe and the United States, particularly in the northern industrialized cities. Autopsy studies conducted in Boston and Leiden, The Netherlands in the late 19th century found 80–90% of children had rickets. Rickets can be caused by vitamin D deficiency, calcium deficiency, acquired and inherited disorders metabolism of vitamin D, calcium and phosphorus. Specific signs and symptoms include, growth retardation, muscle weakness, skeletal deformities, stunted growth and bowed legs.[3,4]

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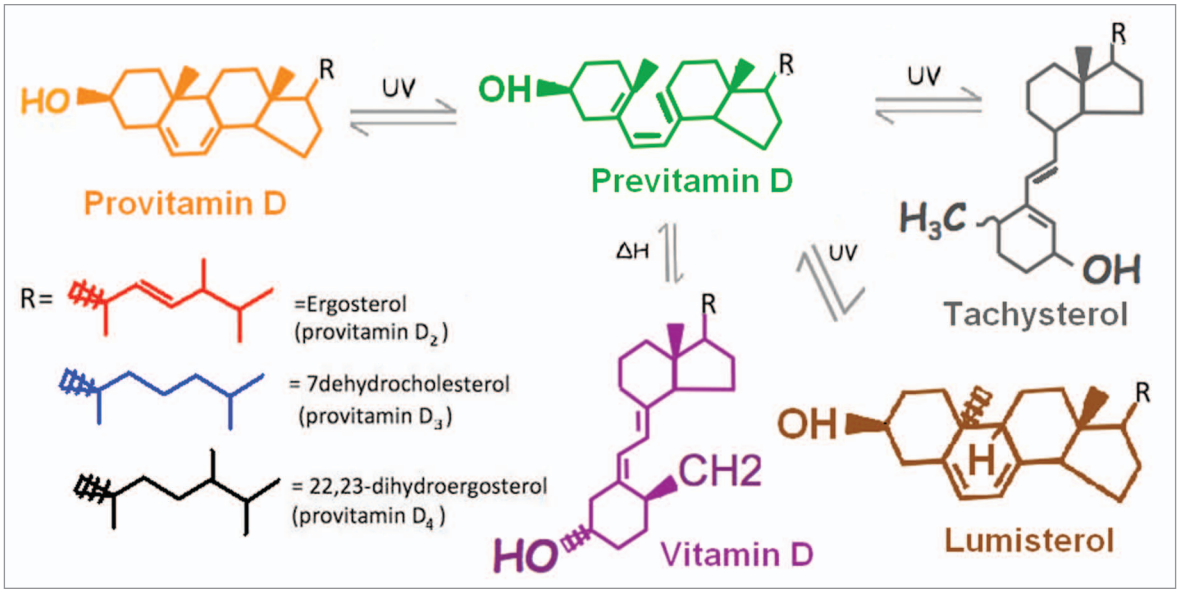

Figure 1. Schematic showing the structures of the provitamin D (side chains labeled R) ergosterol (red), 7-dehydrocholesterol (7-DHC) (blue), 22,23-dihydroergosterol (provitamin D_4) (black). Upon UV irradiation, the provitamin D (orange) is converted to previtamin D (green), which can thermally convert to vitamin D (purple). Previtamin D is converted to photobyproducts tachysterol (dark gray) and lumisterol (brown) (Holick, copyright 2012, reproduced with permission).

By the late 18th and early 19th century, rickets was a common condition for children. In 1822, the Polish physician Sniadecki noticed that children living in Warsaw, Poland, a densely populated city, had a higher incidence of rickets compared with children living in the countryside where sun exposure was more common. Sniadecki was the first to hypothesize the importance of sunlight to bone health.[3] In 1861, Dr. Trousseau of France postulated the etiology of rickets was a lack of sun exposure and diet, and successfully treated patients with cod-liver oil.[4]

In the early 20th century, the German physician Huldschinsky treated patients suffering from rickets with exposure to a mercury-vapor quartz lamp that emitted UVB radiation.[3] After six weeks of treatment, the children had radiologic improvement in their condition, demonstrated by an increase in mineralization in the children's X-ray. In 1921, Hess and Unger observed that exposing children to direct sunlight three to four times a week improved their clinical and radiological manifestations of rickets.[5] Hess and Unger also observed that cod liver oil acted as a preventative measure and treatment for rickets in adolescents.[3]

Vitamin D in Food

In 1918, Mellanby first showed that feeding puppies cod liver oil could prevent rickets from occurring.[6] In 1924, Steenbock and Black observed that UV irradiated foods had an antirachitic effect when consumed by animals.[7] Later, in 1925, Cowell demonstrated that bovine milk exposed for 20 min under a mercury vapor lamp could be used to treat rickets when consumed by adolescents.[8] Within two decades, a wide variety of foods and beverages were fortified with vitamin D.[3] These discoveries lead to the identification of vitamin D as having an antirachitic effect in humans and animals. It was originally assumed that endogenously produced vitamin D was the same as vitamin D produced by irradiated yeast. However, chickens fed vitamin D from irradiated yeast had minimal antirachitic affects, while cod liver oil reversed the effects of rickets.[1-3]

It was then concluded that vitamin D produced from the skin was different than vitamin D produced by irradiated yeast. To distinguish the two forms, they were named vitamin D_2 (ergocalciferol) from yeast and vitamin D_3 (cholecalciferol) from human and animal skin.[3] Vitamin D_2 and vitamin D_3 are structurally very similar. The only exceptions are that vitamin D_2 has a double bond between carbons 22 and 23 and a methyl group on carbon 24 (**Fig. 1**).

Photobiology of Vitamin D_2 in Mushrooms

Mushrooms are fungi and belong to the division Basidiomycota. The vitamin D benefits of edible mushrooms has been known since 1994 when Mattilla et al. extracted provitamin D_2 from wild mushrooms.[9] White button mushrooms (*Agaricus bisporus*) are grown in the dark and therefore contain negligible concentrations of vitamin D_2. White button mushrooms were examined and found to contain 56.3 μg/100 g fresh weight of provitamin D_2, and 0.11 μg/100 g fresh weight of vitamin D_2.[10]

When exposed to UV radiation, mushrooms become an abundant source of vitamin D_2.[11] Mushroom producers have recently begun exposing mushrooms to UV radiation in order to have their product contain vitamin D_2. The photobiology of vitamin D_3 has been well studied in poikilothermic animals and human skin; however, little is known about how vitamin D_2 is produced in irradiated mushrooms.

Photoproduction of Previtamin D_2 in Mushrooms

White button mushrooms were irradiated and studied in order to elucidate the mechanism of vitamin D_2 production. Provitamin D_2 dissolved in methanol (50 μg/mL) in borosilicate ampoules were irradiated and used as a positive control as previously described.[12] Mushrooms and ampoules were irradiated for 5 min, 3.5 inches from a RC-500B Pulsed UV Curing System (Xenon Corporation). After irradiation mushroom samples were obtained with a brass 0.5 cm^2 cork borer to a depth of 0.1 cm at various times for up to 96 h. The samples were homogenized in 6.0 mL of 100% methanol. The samples were centrifuged and the liquid layer was pipetted off and dried with N_2. The dried samples were dissolved in either 0.3% or 0.8% isopropanol in hexane and chromatographed on a high performance liquid chromatograph stacked Agilent 1100 (HPLC) attached to a photodiode detector. Two different columns were used as a stationary phase (5 μm spherical silica gel) to separate provitamin D_2 from its photoproducts and vitamin D_2. A Zorbax RX-SIL column with 0.8% isopropanol in hexane was used to separate lumisterol$_2$ from previtamin D_2 and the Zorbax CN column with 0.3% isopropanol in hexane was used to separate tachysterol$_2$ from vitamin D_2 (**Fig. 2A and B**). The concentrations of photobyproducts were calculated using a conversion factor obtained from a standard curve.

As can be seen in **Figure 2A**, immediately after exposure to 5 min of UV radiation provitamin D_2 was converted to a product that migrated at 6.7 min and had a UV absorption spectrum λ_{max} 260 nm, consistent with previtamin D_2. The peak with retention time 10.6 min had a UV absorption λ_{max} at 265 nm and was consistent with vitamin D_2 (**Fig. 2B**). These findings in irradiated mushrooms are similar to what was observed in irradiated ampoules containing provitamin D_2 and confirms the previous observations by Kalaras et al.[13] (**Fig. 2C and D**).

A 96 h time course was conducted to examine the conversion of previtamin D_2 to vitamin D_2 in UV irradiated white button mushrooms and ampoules. Immediately after irradiation and at various times, the samples were chromatographed to determine the percent conversion of previtamin D_2 and vitamin D_2 (**Fig. 3**). There was a time dependent increase in the amount of vitamin D_2. At 6 h after irradiation, 24% of the previtamin D_2 in white button mushroom had converted to vitamin D_2 whereas only 10% of the previtamin D_2 in the irradiated provitamin D_2 ampoules converted to vitamin D_2.

After 11.5 h, 50% of the previtamin D_2 present in white button mushrooms had converted to vitamin D_2 compared with only 19% of the previtamin D_2 converted to vitamin D_2 in the ampoules (**Fig. 3**). These results mimic what was observed in ampoules containing 7-DHC in methanol and lizard skin after exposure to UV radiation and kept at 25°C (**Fig. 3**). The results demonstrate that the kinetics for previtamin D_2 conversion to vitamin D_2 in mushrooms was enhanced compared with the conversion in an organic solvent, similar to lizard skin.[14]

Photoproduction of Lumisterol$_2$ and Tachysterol$_2$

White button mushrooms were exposed to UV radiation for 1, 2, 3, 4, 5 and 10 min at 25°C (**Fig. 4**). After 5 min of exposure to UV radiation a peak that migrated at 7.3 min and had a UV absorption spectrum λ_{max} 272 nm, consistent with lumisterol$_2$ (**Fig. 2A**) and a peak with retention time 11.7 min that had a UV absorption spectrum λ_{max} 281 nm consistent with tachysterol$_2$ was detected in the irradiated white button mushrooms (**Fig. 2B**). A similar result was observed in irradiated ampoules; previtamin D_2, lumisterol$_2$ and tachysterol$_2$ were detected (**Fig. 2C and D**) and confirms a previous report.[13]

Previtamin D_2 and lumisterol$_2$ were only observed after exposure to 1 min of UV radiation. After 3 min of UV exposure, previtamin D_2, vitamin D_2, lumisterol$_2$ and tachysterol$_2$ were detected (**Fig. 4**). Photoequilibrium of the previtamin D_2 photobyproducts was reached between 4 and 5 min of UV exposure. Although the ratio of previtamin D_2 and photoproducts did not change after 5 min of exposure, continued exposure increased the total amount of previtamin D_2 and its photoproducts. Mushrooms that were not exposed to UV radiation did not contain detectable amounts of previtamin D_2, its photobyproducts or vitamin D_2.

Stability of Lumisterol$_2$ and Tachysterol$_2$ in Mushrooms

A 24 h time course was conducted to study the stability of the photobyproducts of previtamin D_2 in white button mushrooms. White button mushrooms and provitamin D_2 ampoules were irradiated for 5 min; mushroom samples were collected every 2 h for 24 h. As expected, previtamin D_2 and vitamin D_2 decreased and increased, respectively, in a time dependent manner (**Fig. 3**). An extended time course of 336 h was conducted to study the stability of lumisterol$_2$ and tachysterol$_2$ in methanol that were recovered from the irradiation of provitamin D_2. Lumisterol$_2$ and tachysterol$_2$ remained stable in methanol for more than 300 h (**Fig. 5A**). The amount of lumisterol$_2$ remained constant over time in irradiated mushrooms. Though, the amount of tachysterol$_2$ remained constant in irradiated ampoules, in irradiated white button mushrooms, tachysterol$_2$ rapidly declined to undetectable levels within 24 h (**Fig. 5B**). The same rapid disappearance of tachysterol$_2$ was also observed in oyster (*Pleurotus ostreatus*) and shiitake ((*Lentinula edodes*) mushrooms (data not shown).

Vitamin D_4 in Mushrooms

Vitamin D_4 is a form of vitamin D that is structurally similar to vitamin D_3 and was first produced by Windaus and Trautmann in 1937.[15] Vitamin D_4 has a methyl group on carbon 24 of the vitamin D_3 side chain (**Fig. 1**). Vitamin D_4 is produced from the UV irradiation of its precursor, 22,23-dihydroergosterol (provitamin D_4) (**Fig. 1**). Vitamin D_4 was reported to be about 60% as active as vitamin D_3 in healing rickets in rats.[16]

Philips et al. reported the presence of provitamin D_4 in several mushroom species including crimini (*Agaricus bisporus*), portabella (*Agaricus bisporus*), enoki (*Flammulina velutipes*), shiitake,

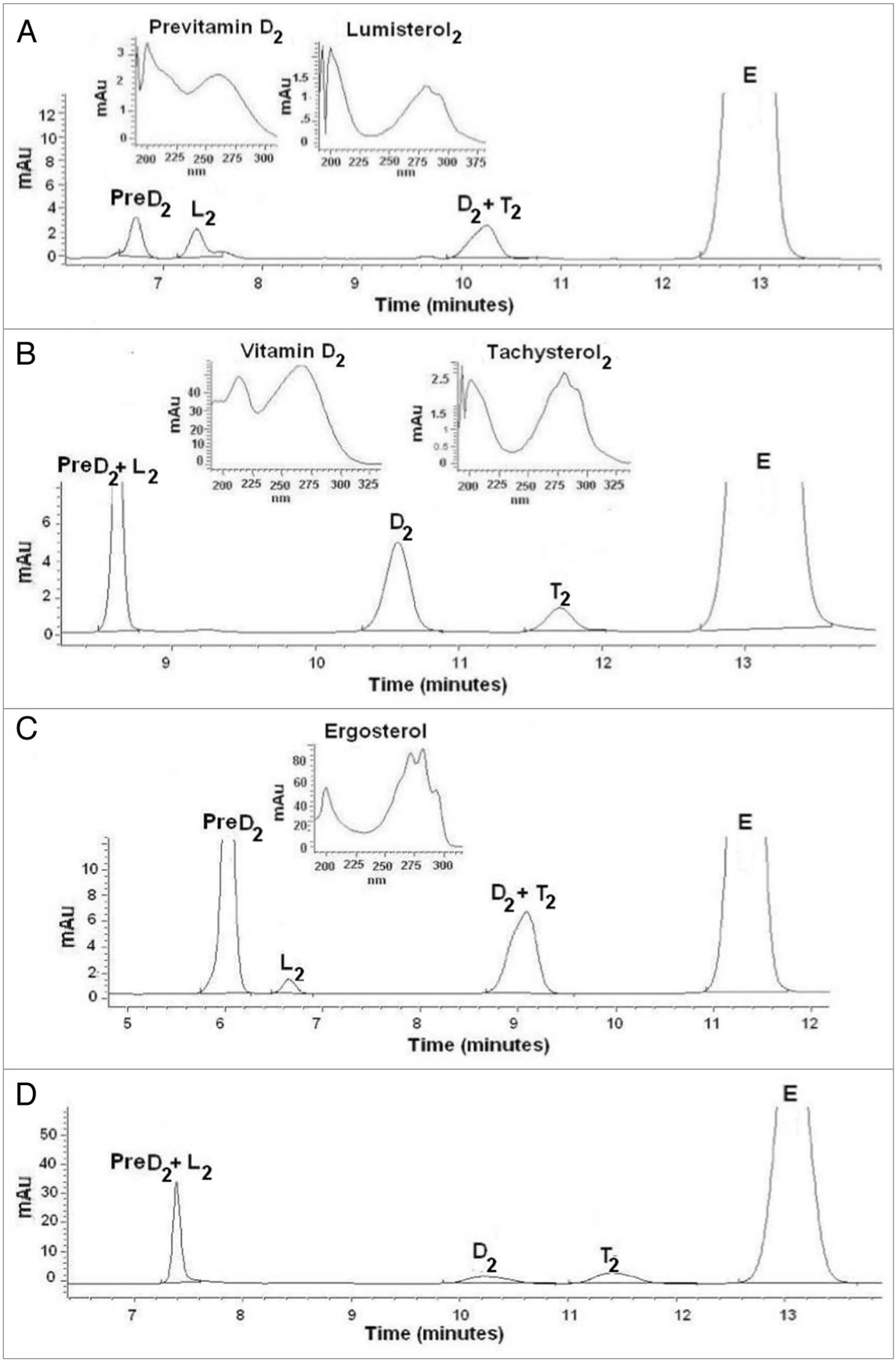

Figure 2. For figure legend, see page 5.

Figure 2. HPLC chromatographs of extracts from white button mushrooms and ampoules irradiated for 5 min under the Xenon RC-500 pulsed UV lamp. The X-axis is retention time in minutes; Y-axis is miliAbsorbance Units (mAU) at 265 nm. A Zorbax RX-SIL column was used in 0.8% isopropanol (IPA) in hexane to separate lumisterol$_2$ (L_2) from previtamin D_2 ($PreD_2$) (**A and C**). A Zorbax CN column in 0.3% IPA in hexane was used to separate tachysterol$_2$ (T2) from vitamin D_2 (D_2) (**C and D**). Irradiated white button mushroom (**A and B**). Irradiated ampoule containing 50 μg/mL of ergosterol (**C-E**).

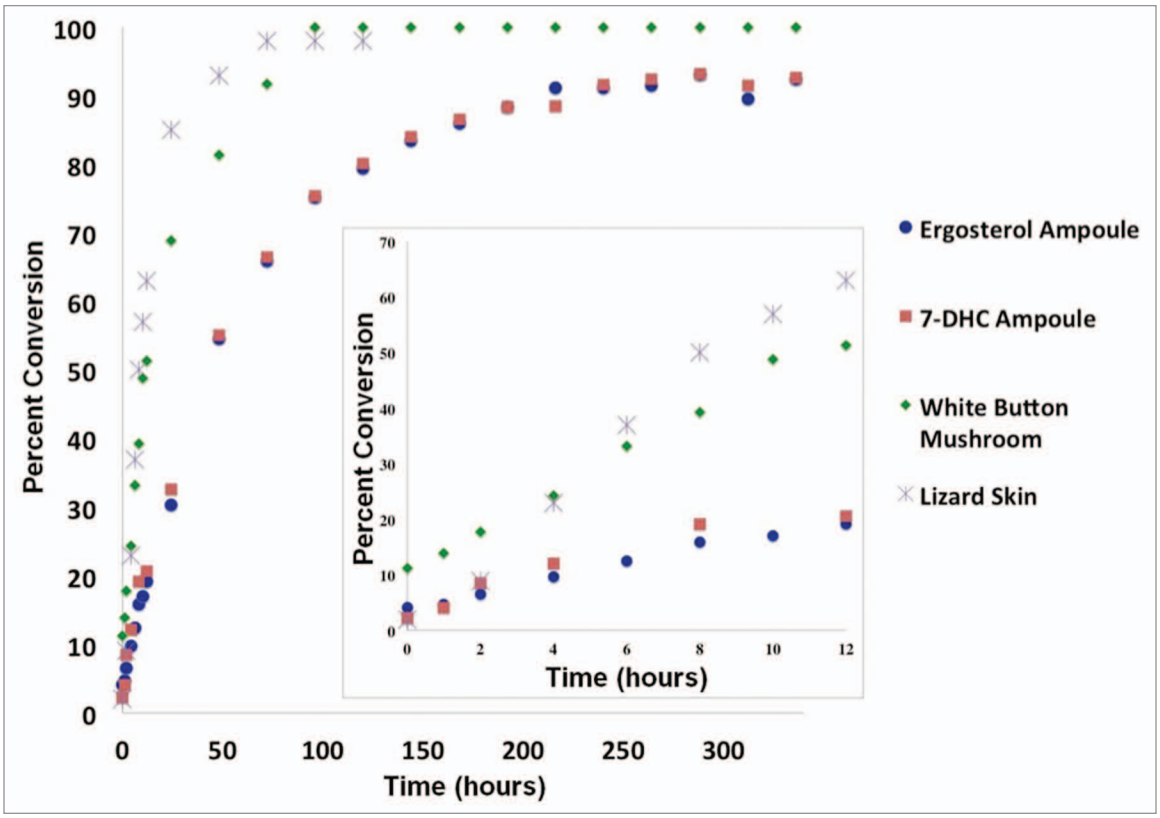

Figure 3. Conversion of previtamin D_2 to vitamin D_2 at 25°C in irradiated white button mushrooms (◆), irradiated ampoules containing 50 μg/mL of ergosterol in methanol (●), 7-dehydrocholesterol (7-DHC) in ampoules (■) and lizard skin (*) at 25°C. Inset is percent conversion of previtamin D_2 to vitamin D_2 and previtamin D_3 to vitamin D_3 between 0 and 12 h.

maitake (*Grifola frondose*), oyster, morel (*Morchella spp)* and chanterelle (*Cantharellus cibarius)*.[17]

We examined crimini, oyster, portabella, shiitake, white button mushrooms, white button mushroom power (Monterey Mushrooms, Inc.) and *Saccharomyces cerevisiae* (*S. cerevisiae)* yeast (Lallemand Inc.) for the presence of provitamin D_2, vitamin D_2, provitamin D_4, vitamin D_4 as well as other potential provitamin Ds and vitamin Ds. Mushroom samples were obtained in a similar manner that was described for white button mushrooms. One gram of *S. cerevisiae* was extracted with 5 mL of methanol for 1 min and sonicated with a sonic disrupter (Teledyne Tekmar). After being centrifuged for 5 min, the liquid layer was removed, and the extraction was repeated 5 additional times. The extracts were combined and taken to dryness under N_2 gas. The dried samples were dissolved in methanol, ethanol and dH_2O (32:8:1 ratio) and chromatographed on a reverse phase HPLC using a Zorbax ODS column.

Analysis of crimini, oyster, portabella, shiitake, white button mushrooms, white button mushroom powder and *S. cerevisiae* yeast using the reverse phase HPLC system with a Zorbax ODS column to separate the various provitamin Ds revealed all samples contained provitamin D_2 and vitamin D_2. In addition the peak with retention time at 9.6 min and a UV absorbance spectrum of a 5,7-diene consistent with provitamin D_4 (**Fig. 6A**).[15] Provitamin D_4 obtained from white button mushroom powder was dissolved in methanol (4 μg/mL) and placed in a borosilicate ampoule and irradiated for 10 min using a RC-500B Pulsed UV Curing System. After irradiation the provitamin D_4 ampoule was dried with N_2 gas and prepared for straight phase HPLC. HPLC analysis revealed peaks with retention times that had UV absorption spectra consistent with provitamin D_4, previtamin D_4, lumisterol$_4$ and tachysterol$_4$, and were used as our standards (**Fig. 6B and C**). HPLC analysis of *S. cerevisiae* yeast revealed peaks that also co-eluted with standard provitamin D_4 and vitamin D_4. Oyster mushrooms were irradiated for 5 min and examined for the presence of vitamin D_4. The dried samples were dissolved in 20% methanol in acetonitrile and prepared for reverse phase HPLC, using a Vydac C18 column to separate the various

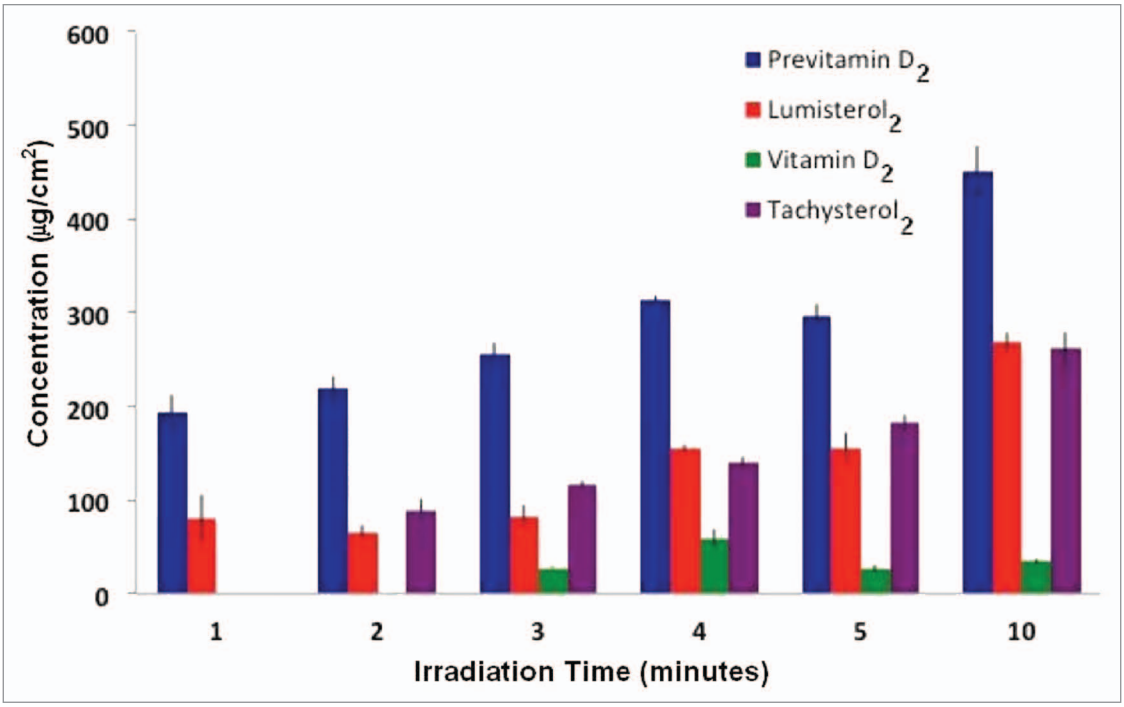

Figure 4. Photobyproducts in irradiated white button mushrooms with various exposure times to UV radiation. Samples dissolved in either 0.3% or 0.8% isopropanol in hexane were chromatographed on a high performance liquid chromatograph, and were analyzed for previtamin D_2 (blue), lumisterol$_2$ (red), vitamin D_2 (green) and tachysterol$_2$ (purple). Mean ± SEM.

vitamin Ds. UV irradiated mushrooms revealed the presence of previtamin D_4 with a retention time of 6.2 min in straight phase; its presence was confirmed by chromatography after its conversion to vitamin D_4. The analysis revealed peaks with retention times of 10.8 and 13 min that had a UV absorbance spectra with λ_{max} 265 nm for the 5,6-cis-triene system consistent with vitamin D_2 and vitamin D_4, respectively (**Fig. 6D**). Provitamin D_2 and provitamin D_4 were detected in the unexposed mushrooms (data not shown). For comparison white button mushroom powder contained ~88% vitamin D_2 and ~12% vitamin D_4, whereas *S. cerevisiae* yeast sample contained ~99% vitamin D_2 and ~1% vitamin D_4.

Vitamin D_3 in Mushrooms

It has been assumed that mushrooms only produce vitamin D_2 when irradiated. However, since some mushrooms can produce vitamin D_4 we reevaluated, by HPLC, mushroom extracts for the possibility of identifying other provitamin Ds. Evaluation of extracts from shiitake mushrooms revealed by reverse phase HPLC a peak with a retention time of 11.5 min that was identified as provitamin D_4, and a peak with a retention time of 10.8 min with an identical UV absorption spectrum consistent with a 5,7-diene (**Fig. 7A**). Co-chromatography studies revealed that this peak was 7-DHC. 7-DHC was found to migrate between provitamin D_2 and provitamin D_4 that had retention times of 10.8, 9.6 and 11.5 min, respectively. Concentrations of the 7-DHC and provitamin D_4 were 5.8 and 2.6 greater in the gills of the mushroom compared with the surface of the mushroom cap. UV irradiated shiitake mushrooms were examined for the presence of vitamin D_3. Shiitake mushrooms were irradiated with the gills facing up for 5 min using a RC-500B Pulsed UV Curing System. Mushroom samples were obtained in a similar manner that was described previously. The samples were chromatographed on reverse phase HPLC using a C18 column. UV irradiated mushrooms revealed the presence of previtamin D_3 with a retention time of 6.3 min in straight phase; its presence was confirmed by chromatography after its conversion to vitamin D_3. Peaks with retention times of 5.1 and 6.1 min had UV absorbance spectra with λ_{max} 265 nm consistent with vitamin D_2 and vitamin D_3, respectively (**Fig. 7B**).

Bioavailability of Vitamin D_2 in Mushrooms

Various foods and beverages have been fortified with vitamin D in the United States for more than 70 y since there are few that contain vitamin D naturally. Milk, orange juice, bread, other dairy products and some cereals have been fortified with this essential vitamin.[18] Mushrooms exposed to sunlight or UV radiation are a good source of vitamin D_2. Several clinical studies have been conducted to determine the bioavailability of vitamin D_2 and vitamin D_3 in fortified foods and beverages and the efficacy of fortification in increasing 25-hydroxyvitamin D [25(OH)D] levels; the measure for determining a person's vitamin D status.[18] Fortification of foods with vitamin D_2 or vitamin D_3 has been shown to be a safe and effective way to increase 25(OH)D levels in children and adults.[18]

Biancuzzo et al. found that there was no difference in total 25(OH)D levels in healthy adults who consumed orange juice that was fortified with 1000 IU of vitamin D_2 or 1000 IUs of vitamin D_3 compared with consuming a supplement containing either 1000 IU vitamin D_2 or 1000 IU vitamin D_3.[19] It was also found that there was no difference in the increase of 25(OH)D_3 levels between subjects who consumed vitamin D_3-fortified orange juice or vitamin D_3 supplements or in 25(OH)D_2 levels of subjects who consumed vitamin D_2-fortified orange juice or vitamin D_2 supplements. Additionally, Natri et al. found that bread fortified with vitamin D_3 increased total serum 25(OH)D levels in women as effectively as a vitamin D_3 supplement.[20]

To date, two groups have reported on the bioavailability of vitamin D_2 in UV irradiated mushrooms. Urbain et al. conducted a five-week single-blinded, randomized, placebo-controlled trial in 26 healthy Caucasian adults with 25(OH)D levels below 20 ng/mL.[21] These subjects were randomized to three groups and assigned to receive either 28,000 IU vitamin D_2 from UV irradiated mushrooms in a soup and placebo, 60 IU vitamin D_2 in soup that contained non-UV-irradiated mushrooms and 28,000 IU vitamin D_2 in a liquid supplement, or 60 IU vitamin D_2 in a non-UV-irradiated mushroom soup and placebo supplement four times a week for four weeks. After four weeks, serum 25(OH)D levels increased significantly and consuming vitamin D_2 from UV-irradiated mushrooms was equally as effective at raising 25(OH)D levels as ingesting the same amount of vitamin D_2 as a supplement.

Stephenson et al. conducted a similar study where subjects were randomized to consume one serving of mushrooms with a standard meal each day for six weeks.[22] Four groups received either one serving of non-UV-irradiated mushrooms plus meal (control), UV irradiated mushrooms containing 352 IU vitamin D_2 with a meal, UV irradiated mushrooms containing 684 IU vitamin D_2 with a meal or a supplement containing 1,128 IU vitamin D_2 with non-UV-irradiated mushrooms. At the end of six weeks, 25(OH)D_2 levels were higher in all groups except the control group. They observed a significant decrease in serum 25(OH)D_3 in the group receiving 684 IU vitamin D_2 in UV irradiated mushrooms and in the group receiving the vitamin D_2 supplement. There was a mean decrease of 25(OH)D_3 of 0.32 ng/mL that was offset by an increase of 0.40 ng/mL in 25(OH)D_2. Overall, however, there was a small decrease in total 25(OH)D levels in the groups consuming UV irradiated mushrooms that contained vitamin D_2.

This observation contributed to the controversy surrounding the efficacy of maintaining total serum 25(OH)D levels after taking supplemental or dietary vitamin D_2 vs. vitamin D_3. Some reports have suggested that vitamin D_3 was more effective than vitamin D_2 at maintaining total serum 25(OH)D levels.[23,24] In contrast, Holick et al., similar to Biancuzzo et al., found that the daily ingestion of a 1000 IU vitamin D_2 supplement was as effective as the daily ingestion of a 1000 IU vitamin D_3 supplement at raising and maintaining total serum 25(OH)D levels.[19,25] Furthermore, taking 50,000 IU vitamin D_2 once a week for eight weeks and every other week thereafter for up to six years increased serum total 25(OH)D levels and is considered to be effective for the treatment and prevention of vitamin D deficiency.[26] Similarly, Demetriou reported that 50,000 IU vitamin D_2 repletion and maintenance therapy in vitamin D deficient patients significantly increased serum 25(OH)D_2 and total 25(OH)D despite a decrease in serum 25(OH)D_3 levels.[27]

We conducted a clinical study to determine if ingestion of vitamin D_2 in a dried white button mushroom extract (Monterey Mushrooms, Inc.) was as effective at increasing and maintaining vitamin D status as supplemental vitamin D_3 and vitamin D_2. Thirty healthy adults were enrolled in the study (6 male, 19 female, mean age 35.2 y) and were randomized to ingest capsules containing 2000 IU vitamin D_2, 2000 IU vitamin D_3 or 2000 IU mushroom vitamin D_2 once a day for three months during the winter. Vitamin D concentrations were verified to be within 10% by HPLC. Twenty-five subjects completed the study. Fourteen subjects were randomized to the mushroom vitamin D_2 group, eight subjects to the supplemental vitamin D_2 group and 3

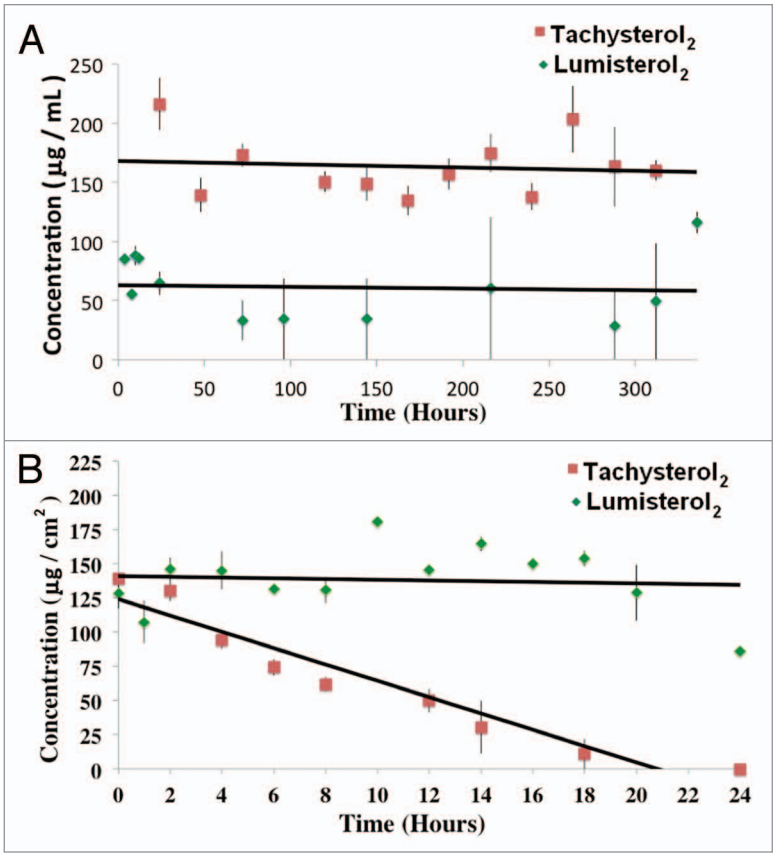

Figure 5. Stability of lumisterol$_2$ and tachysterol$_2$ in irradiated ampoules and mushrooms. Ampoules and mushroom samples were exposed to 5 min of UV radiation. Samples were taken over time. Samples were dissolved in either 0.3% or 0.8% isopropanol in hexane and chromatographed on a high performance liquid chromatograph at 25°C. Mean ± SEM. (**A**) Lumisterol$_2$ (◆) and tachysterol$_2$ (■), in irradiated ampoules. (**B**) Lumisterol$_2$ (◆) and tachysterol$_2$ (■), in white button mushroom samples.

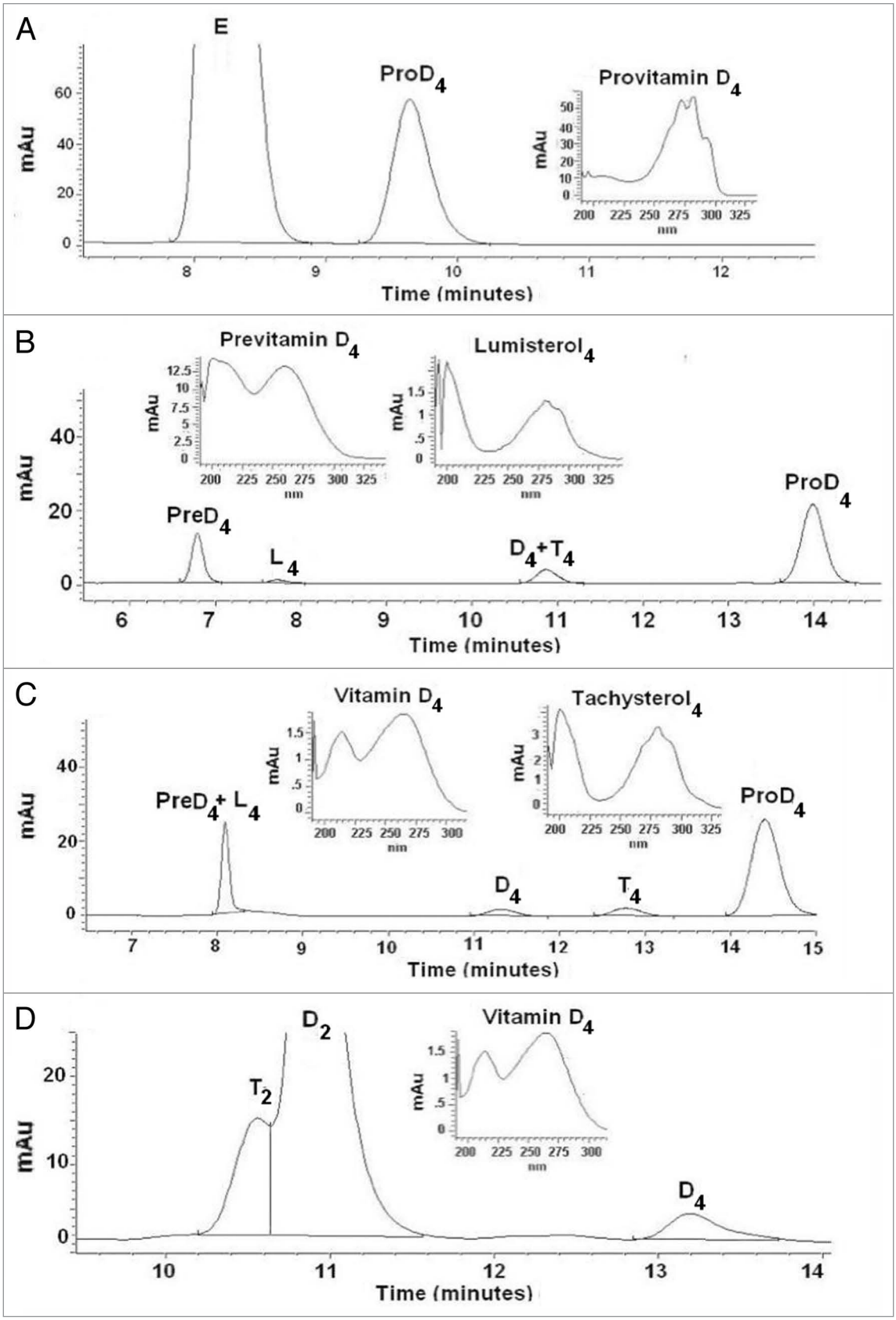

Figure 6. For figure legend, see page 9.

Figure 6. HPLC chromatograms of extracts from white button mushrooms. (**A**) An extract of white button mushroom chromatographed on reverse phase on a Zorbax ODS column. Methanol, ethanol and dH_2O (32:8:1 ratio) to separate the provitamin Ds. Y-axis is miliAbsorbance Units (mAU) at 280 nm. Ergosterol (E), provitamin D_4 ($ProD_4$). (**B**) An ampoule containing provitamin D_4 ($ProD_4$) irradiated for 10 min and chromatographed on a Zorbax RX-SIL column 0.8% isopropanol (IPA) in hexane to separate lumisterol$_4$ (L_4) from previtamin D_4 ($PreD_4$). Y-axis is mAU at 265 nm. Tachysterol$_4$ (T_4), vitamin D_4 (D_4). (**C**) An ampoule containing provitamin D_4 ($ProD_4$) irradiated for 10 min chromatographed on a Zorbax CN column in 0.3% IPA in hexane to separate tachysterol$_4$ (T_4), from vitamin D_4 (D_4). Y-axis is mAU at 265 nm. Previtamin D_4 ($PreD_4$), lumisterol$_4$ (L_4). (**D**) An extract from an oyster mushroom irradiated for 5 min and chromatographed on a C18 column in 20% methanol in acetonitrile to separate vitamin Ds. Y-axis is mAU at 265 nm. Tachysterol$_2$ (T2), vitamin D_2 (D_2), vitamin D_4 (D_4).

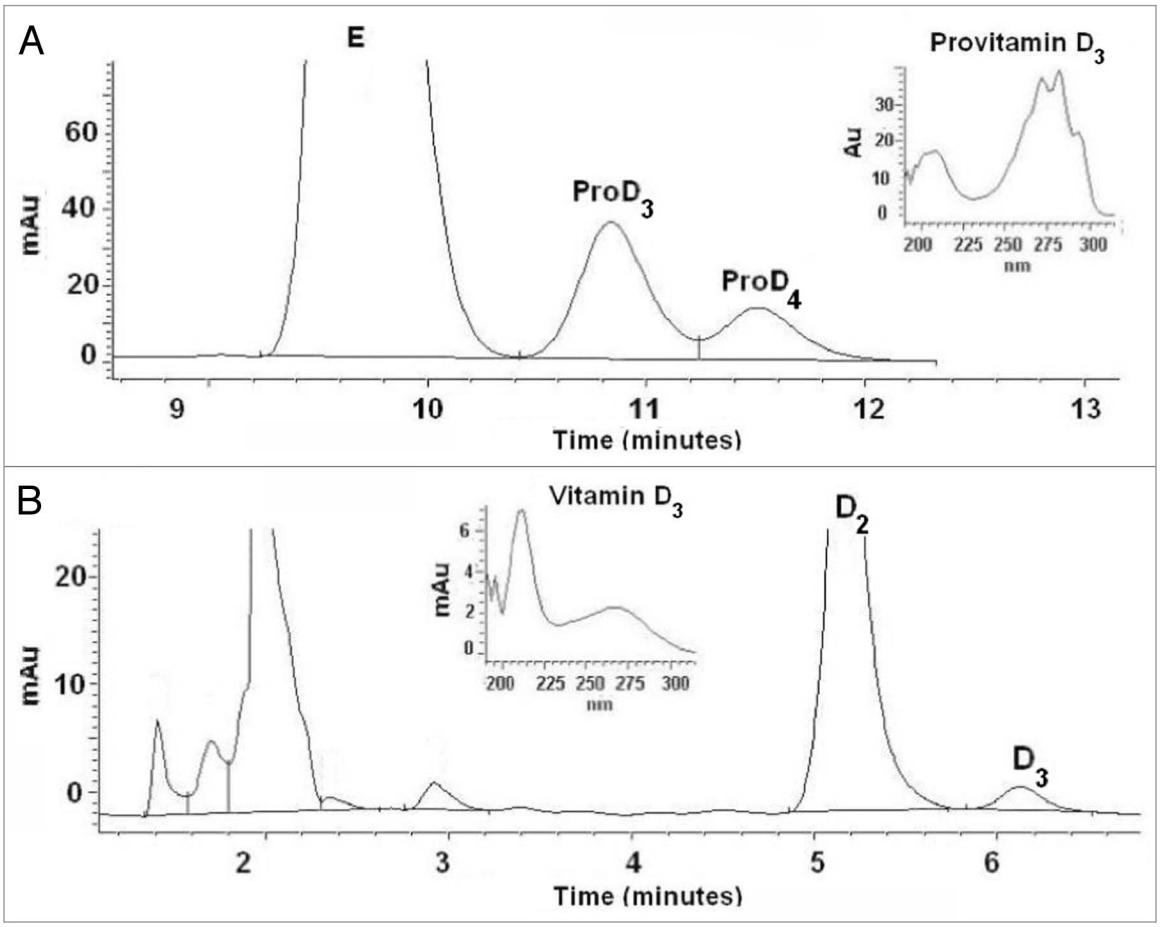

Figure 7. Reverse phase HPLC chromatograms of extracts from shiitake mushrooms. (**A**) An extract from a shiitake mushroom chromatographed on a Zorbax ODS column in methanol, ethanol, and dH_20 (32:8:1 ratio) to separate the provitamin Ds. Y axis is miliAbsorbance Units (mAU) at 280 nm. Ergosterol (E), provitamin D_3 ($ProD_3$), provitamin D_4 ($ProD_4$). (**B**) An extract from a shiitake mushroom irradiated for 5 min and chromatographed on a C18 column in 20% methanol in acetonitrile to separate vitamin Ds. Y axis is miliAbsorbance Units (mAU) at 265 nm. Vitamin D_2 (D_2), vitamin D_3 (D_3).

subjects to the supplemental vitamin D_3 group. Serum concentrations of 25(OH)D_2, 25(OH)D_3 and 25(OH)D were measured once a week for 12 weeks by liquid chromatography tandem mass spectroscopy (LCMS/MS) as previously described.[28]

Subjects in the mushroom vitamin D_2 group had a mean baseline serum 25(OH)D_2 of 0.6 ± 0.3 ng/mL that increased significantly to 18.6 ± 1.4 ng/mL at the end of 12 weeks ($p < 0.0001$). Total serum 25(OH)D levels increased from 20.6 ± 2.4 ng/mL to 30.1 ± 2.6 ng/mL ($p < 0.001$) (**Fig. 8A**).

Subjects in the supplemental vitamin D_2 group had a mean baseline serum 25(OH)D_2 of 1.5 ± 1.2 ng/mL that increased significantly to 13.3 ± 2.0 ng/mL at the end of 12 weeks ($p < 0.001$). Total serum 25(OH)D levels significantly increased from 19.4 ± 2.3 ng/mL to 29.2 ± 2.0 ng/mL ($p < 0.01$) (**Fig. 8B**).

Subjects in the supplemental vitamin D_3 group had a mean baseline serum of 25(OH)D_3 of 16.3 ± 0.6 ng/mL with a final baseline serum level of 34.4 ± 1.3 ng/mL (**Fig. 8C**). Total 25(OH)D increased from 17.1 ± 1.4 ng/mL to 34.4 ± 1.3 ng/mL ($p < 0.05$). The discrepancy in mean baseline serum levels is due to some detectable 25(OH)D_2 of 0.8 ng/mL (**Fig. 8C**).

Baseline serum total 25(OH)D levels were not significantly different between the groups; 17.1 ± 1.2, 19.4 ± 2.3 and 20.6 ± 2.4 ng/mL for the supplemental D_3 and vitamin D_2, and mushroom vitamin D_2 groups respectively. Serum 25(OH)D levels

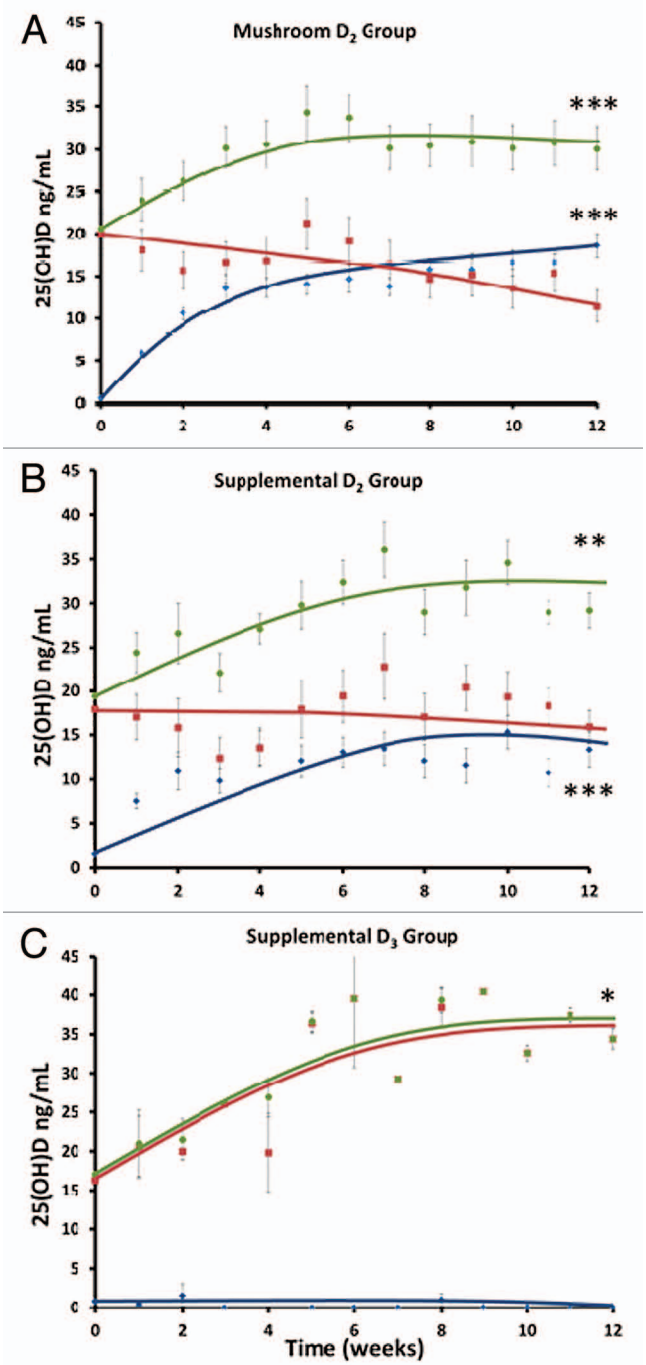

Figure 8. Mean (± SEM) 25-hydroxyvitamin D_2 (◆), 25-hydroxyvitamin D_3 (■) and total 25-hydroxyvitamin D (●) concentrations over time after oral administration of (A) 2000 IU of mushroom vitamin D_2 in capsules (n = 14), (**B**) 2000 IU of supplemental vitamin D_2 in capsules (n = 8) and (**C**) 2000 IU of supplemental vitamin D_3 in capsules (n = 3). The change in total serum 25-hydroxyvitamin D concentrations from baseline to final visits in each group was statistically significant as was the change in serum 25-hydroxyvitamin D_2 concentrations from baseline to final visit in the mushroom vitamin D_2 group and the supplemental vitamin D_2 group. (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). No statistically significant difference was observed between final total 25-hydroxyvitamin D concentrations between all three groups.

gradually increased and plateaued at seven weeks and were maintained for the following five weeks. At the end of 12 weeks, serum total 25(OH)D levels were not statistically significantly different in all three groups: 34.4 ± 1.1, 29.2 ± 2.0 and 30.1 ± 2.6 ng/mL for supplemental vitamin D_3, D_2 and mushroom D_2 respectively.

These results demonstrate ingestion of mushrooms containing D_2 was as effective at increasing and maintaining total serum 25(OH)D levels as supplemental vitamin D_2 and vitamin D_3.

Conclusion

Vitamin D deficiency is a pandemic. It has increased the risk of skeletal and chronic diseases associated with vitamin D deficiency worldwide. Therefore, obtaining vitamin D from sensible sun exposure, foods that naturally contain vitamin D, and from supplementation with vitamin D is imperative to maintain a healthy lifestyle.

Phytoplankton have been producing vitamin D_2 from provitamin D_2 for over 500 million years. The phytoplankton *E. huxlei* contained 1.0 μg/g wet weight of provitamin D_2 and has likely been a source of vitamin D_2 for the oceanic food chain for millions of years.[2] Due to their large quantities of provitamin D_2, fungi like phytoplankton have a huge capacity to produce vitamin D_2 when exposed to UV irradiation.

It is now known that in mushrooms, provitamin D_2 is converted to previtamin D_2 upon UV irradiation. Previtamin D_2 can absorb UVB radiation resulting in the production of the photobyproducts, lumisterol$_2$ and tachysterol$_2$.[13] An evaluation of the thermal isomerization of previtamin D_2 to vitamin D_2 in mushrooms revealed that it was rapidly converted to vitamin D_2 most likely by a non-enzymatic membrane-enhanced catalytic mechanism similar to what was observed in lizard and human skin.[14,29] The planar structure of 7-DHC and provitamin D_2 can fit in between the triglyceride side chains and polar head groups. Upon exposure to UVB radiation the 5,7-diene absorbs the radiation, converting to its respective previtamin D. Previtamin D exists as two conformers cis-cis (cZc) and cis-trans (cZt). In an organic solvent, ~90% exists in the thermodynamically favored cZt while only ~10% exists in the cZc state. Although more thermodynamically stable, the cZt conformer of previtamin D is unable to isomerize to vitamin D; only its less stable cZc can. Thus it takes several days at 25°C for the cZt conformer to convert to cZc, which in turn converts to the thermodynamically stable vitamin D_3. Our observation that previtamin D_2 more rapidly converts to vitamin D_2 in mushrooms than in methanol suggests that this mechanism of a membrane-enhanced conversion of previtamin D_2 to vitamin D_2 has existed for hundreds of millions of years.

Tachysterol$_3$ and lumisterol$_3$ produced in human skin have no biological function on calcium metabolism.[30] Therefore, the physiologic significance of mushrooms producing lumisterol$_2$ and tachysterol$_2$ is unknown. During our investigation on the time dependent conversion of previtamin D_2 to vitamin D_2 in white button mushrooms we observed that the concentration of tachysterol$_2$ began to decline and was undetectable after 24 h. To be certain that this decline was not due to tachysterol$_2$ being unstable,

we conducted a stability evaluation of tachysterol$_2$ in methanol at room temperature for more than one week and found it to be stable. We also observed that this phenomenon also occurred in oyster and shiitake mushrooms suggesting that mushrooms are utilizing in some manner the tachysterol$_2$. This observation may provide insight as to a possible biologic function of tachysterol not only in mushrooms but also in human skin.

It is known that plants and poikilothermic animals contain several different forms of provitamin D.[1,31] Similarly mushrooms are capable of producing more than one provitamin D. It had always been assumed that UV irradiated mushrooms were only capable of producing vitamin D$_2$. Our study confirms that some mushrooms do contain provitamin D$_4$ and we now also report that shiitake mushrooms contain 7-DHC. Therefore during UV irradiation some mushrooms are capable of producing vitamin D$_2$, vitamin D$_3$ and vitamin D$_4$.

Mushrooms exposed to UVB radiation contain a significant amount of vitamin D$_2$ and therefore is an excellent alternative food source for vitamin D, especially for vegans. However, there continues to be concern that vitamin D$_2$ not only is less effective than vitamin D$_3$ in maintaining total serum 25(OH)D concentrations but that the ingestion of vitamin D$_2$ can ultimately result in a decrease in total 25(OH)D concentrations.[23,24] Stephenson et al.[22] reported that ingesting UVB irradiated mushrooms containing vitamin D$_2$ resulted in a small decrease in total 25(OH)D levels. In our study, healthy adults who ingested daily for 3 mo 2000 IUs of vitamin D$_2$ from mushrooms were able to raise and maintain their total 25(OH)D concentrations similar to healthy adults who ingested either 2000 IU supplement containing vitamin D$_2$ or vitamin D$_3$. These results confirm other studies that have demonstrated that ingesting vitamin D$_2$ either from fortified orange juice,[19] a supplement[20] or a pharmaceutical formulation[12,26] were all capable of increasing total circulating 25(OH)D concentrations for at least 3 mo and up to 6 y. Therefore ingesting mushrooms containing vitamin D$_2$ can be an effective strategy to enhance the vitamin D status of the consumer. The observation that some mushrooms when exposed to UVB radiation also produce vitamin D$_3$ and vitamin D$_4$ can also provide the consumer with at least two additional vitamin Ds.

Disclosure of Potential Conflicts of Interest

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References

1. Holick MF. Phylogenetic and evolutionary aspects of vitamin D from phytoplankton to humans. In: Vertebrate Endocrinology: Fundamentals and Biomedical Implications, Vol. 3. Orlando, FL: Academic Press, 1989:7-43.
2. Holick MF. Vitamin D: A millenium perspective. J Cell Biochem 2003; 88:296-307; PMID:12520530; http://dx.doi.org/10.1002/jcb.10338.
3. Holick MF. Resurrection of vitamin D deficiency and rickets. J Clin Invest 2006; 116:2062-72; PMID:16886050; http://dx.doi.org/10.1172/JCI29449.
4. Rajakumar K. Vitamin D, cod-liver oil, sunlight, and rickets: a historical perspective. Pediatrics 2003; 112:e132-5; PMID:12897318; http://dx.doi.org/10.1542/peds.112.2.e132.
5. Hess AF. Rickets including osteomalacia and tetany. Philadelphia, PA: Lea J. Febiger, 1929:401-429.
6. Mellanby T. The part played by an 'accessory factor' in the production of experimental rickets. J Physiol 1918; 52:11-4.
7. Steenbock H, Black A. The reduction of growth-promoting and calcifying properties in a ration by exposure to ultraviolet light. J Biol Chem 1924; 61:408-22.
8. Cowell SJ. Irradiation of milk and the healing of rickets. Br Med J 1925; 1:594-5; PMID:20771981; http://dx.doi.org/10.1136/bmj.1.3352.594.
9. Mattila PH, Piironen VI, Uusi-Rauva EJ, Koivistoinen PE. Vitamin D contents in edible mushrooms. J Agric Food Chem 1994; 42:2449-53; http://dx.doi.org/10.1021/jf00047a016.
10. Phillips KM, Ruggio DM, Horst RL, Minor B, Simon RR, Feeney MJ, et al. Vitamin D and sterol composition of 10 types of mushrooms from retail suppliers in the United States. J Agric Food Chem 2011; 59:7841-53; PMID:21663327; http://dx.doi.org/10.1021/jf104246z.
11. Jasinghe VJ, Perera CO, Barlow PJ. Bioavailability of vitamin D$_2$ from irradiated mushrooms: an in vivo study. Br J Nutr 2005; 93:951-5; PMID:16022766; http://dx.doi.org/10.1079/BJN20051416.
12. Webb AR, Kline L, Holick MF. Influence of season and latitude on the cutaneous synthesis of vitamin D$_3$: exposure to winter sunlight in Boston and Edmonton will not promote vitamin D$_3$ synthesis in human skin. J Clin Endocrinol Metab 1988; 67:373-8; PMID:2839537; http://dx.doi.org/10.1210/jcem-67-2-373.
13. Kalaras MD, Beelman RB, Holick MF, Elias RJ. Generation of potentially bioactive ergosterol-derived products following pulsed ultraviolet light exposure of mushrooms (*Agaricus bisporus*). Food Chem 2012; 135:396-401; PMID:22868105; http://dx.doi.org/10.1016/j.foodchem.2012.04.132.
14. Holick MF, Tian XQ, Allen M. Evolutionary importance for the membrane enhancement of the production of vitamin D$_3$ in the skin of poikilothermic animals. Proc Natl Acad Sci U S A 1995; 92:3124-6; PMID:7724526; http://dx.doi.org/10.1073/pnas.92.8.3124.
15. Windaus A, Trautmann G. Über das krystallisierte Vitamin D4. Z Phys Chem 1937; 247:185; http://dx.doi.org/10.1515/bchm2.1937.247.4-5.185.
16. De Luca HF, Weller M, Blunt JW, Neville PF. Synthesis, biological activity., and metabolism of 22,23-^{3}H vitamin D$_4$. Arch Biochem Biophys 1968; 124:122-8; PMID:4298487; http://dx.doi.org/10.1016/0003-9861(68)90310-X.
17. Phillips KM, Horst RL, Koszewski NJ, Simon RR. Vitamin D$_4$ in mushrooms. PLoS One 2012; 7:e40702; PMID:22870201; http://dx.doi.org/10.1371/journal.pone.0040702.
18. Holick MF. Vitamin D deficiency. N Engl J Med 2007; 357:266-81; PMID:17634462; http://dx.doi.org/10.1056/NEJMra070553.
19. Biancuzzo RM, Young A, Bibuld D, Cai MH, Winter MR, Klein EK, et al. Fortification of orange juice with vitamin D(2) or vitamin D(3) is as effective as an oral supplement in maintaining vitamin D status in adults. Am J Clin Nutr 2010; 91:1621-6; PMID:20427729; http://dx.doi.org/10.3945/ajcn.2009.27972.
20. Natri AM, Salo P, Vikstedt T, Palssa A, Huttunen M, Kärkkäinen MU, et al. Bread fortified with cholecalciferol increases the serum 25-hydroxyvitamin D concentration in women as effectively as a cholecalciferol supplement. J Nutr 2006; 136:123-7; PMID:16365070.
21. Urbain P, Singler F, Ihorst G, Biesalski HK, Bertz H. Bioavailability of vitamin D$_2$ from UV-B-irradiated button mushrooms in healthy adults deficient in serum 25-hydroxyvitamin D: a randomized controlled trial. Eur J Clin Nutr 2011; 65:965-71; PMID:21540874; http://dx.doi.org/10.1038/ejcn.2011.53.
22. Stephensen CB, Zerofsky M, Burnett DJ, Lin YP, Hammock BD, Hall LM, et al. Ergocalciferol from mushrooms or supplements consumed with a standard meal increases 25-hydroxyergocalciferol but decreases 25-hydroxycholecalciferol in the serum of healthy adults. J Nutr 2012; 142:1246-52; PMID:22623385; http://dx.doi.org/10.3945/jn.112.159764.
23. Trang HM, Cole DEC, Rubin LA, Pierratos A, Siu S, Vieth R. Evidence that vitamin D$_3$ increases serum 25-hydroxyvitamin D more efficiently than does vitamin D$_2$. Am J Clin Nutr 1998; 68:854-8; PMID:9771862.
24. Heaney RP, Recker RR, Grote J, Horst RL, Armas LA. Vitamin D(3) is more potent than vitamin D(2) in humans. J Clin Endocrinol Metab 2011; 96:E447-52; PMID:21177785; http://dx.doi.org/10.1210/jc.2010-2230.

25. Holick MF, Biancuzzo RM, Chen TC, Klein EK, Young A, Bibuld D, et al. Vitamin D_2 is as effective as vitamin D_3 in maintaining circulating concentrations of 25-hydroxyvitamin D. J Clin Endocrinol Metab 2008; 93:677-81; PMID:18089691; http://dx.doi.org/10.1210/jc.2007-2308.
26. Pietras SM, Obayan BK, Cai MH, Holick MF. Vitamin D_2 treatment for vitamin D deficiency and insufficiency for up to 6 years. Arch Intern Med 2009; 169:1806-8; PMID:19858440; http://dx.doi.org/10.1001/archinternmed.2009.361.
27. Demetriou ET, Travison TG, Holick MF. Treatment with 50,000 IU vitamin D_2 every other week and effect on serum 25-hydroxyvitamin D_2, 25-hydroxyvitamin D_3, and total 25-hydroxyvitamin D in a clinical setting. Endocr Pract 2012; 18:399-402; PMID:22440989; http://dx.doi.org/10.4158/EP11268.OR.
28. Holick MF, Siris ES, Binkley N, Beard MK, Khan A, Katzer JT, et al. Prevalence of Vitamin D inadequacy among postmenopausal North American women receiving osteoporosis therapy. J Clin Endocrinol Metab 2005; 90:3215-24; PMID:15797954; http://dx.doi.org/10.1210/jc.2004-2364.
29. Tian XQ, Chen TC, Matsuoka LY, Wortsman J, Holick MF. Kinetic and thermodynamic studies of the conversion of previtamin D3 to vitamin D3 in human skin. J Biol Chem 1993; 268:14888-92; PMID:8392061.
30. Holick MF, MacLaughlin JA, Doppelt SH. Regulation of cutaneous previtamin D_3 photosynthesis in man: skin pigment is not an essential regulator. Science 1981; 211:590-3; PMID:6256855; http://dx.doi.org/10.1126/science.6256855.
31. Horst RL, Reinhardt TA, Russell JR, Napoli JL. The isolation and identification of vitamin D_2 and vitamin D_3 from *Medicago sativa* (alfalfa plant). Arch Biochem Biophys 1984; 231:67-71; PMID:6326678; http://dx.doi.org/10.1016/0003-9861(84)90363-1.