

The Pennsylvania State University
The Graduate School
Department of Food Science

**PRODUCTION OF ERGOCALCIFEROL (VITAMIN D₂) AND RELATED
STEROLS IN MUSHROOMS WITH EXPOSURE TO PULSED ULTRAVIOLET
LIGHT**

A Dissertation in
Food Science
by
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ABSTRACT

Vitamin D status in humans worldwide presents itself as one the most important factors in human health. The classic bone diseases associated with vitamin D deficiency, rickets and osteomalacia, are still prevalent among certain subpopulations. In addition, modern research has found a putative link between vitamin D status and the risk of a host of diseases including various cancers, heart disease, stroke, diabetes, multiple sclerosis and rheumatoid arthritis, among others. Compounding the issue is the fact that in modern society, individuals spend less time exposed to sunlight due to various factors. Sunlight exposure is the main source of vitamin D, which is produced from the conversion of 7-dehydrocholesterol in the epidermis through UV irradiation. As such, the use of vitamin supplements and foods containing vitamin D are an important source. However, the number of foods that are sources of vitamin D is limited and more sources are needed.

Mushrooms are an abundant source of ergosterol, which is the precursor to vitamin D₂. Mushrooms have been shown to contain small amounts of vitamin D₂ when grown in the wild. Furthermore, the vitamin D₂ content of cultivated mushrooms has been shown to increase with exposure to artificial UV light. The use of conventional continuous UV systems has been shown to take on the order of minutes to hours of exposure to increase the vitamin D₂ levels to a significant level. The main objective of this study was to investigate the use of pulsed UV (PUV) light as a rapid and predictable method for increasing the vitamin D₂ content of mushrooms and mushroom products.

A study was conducted to assess the effectiveness of a PUV system to increase the vitamin D₂ content of a variety of mushrooms species (*Agaricus bisporus*, white and brown button mushrooms; *Pleurotus ostreatus*, oyster, and *Lentinula edodes*, shiitake) as well as several mushroom powders (*Pleurotus eryngii*, king oyster and *Agaricus bisporus*) and spent growing substrates containing mycelial biomass. Sliced white button mushrooms showed an increase from an initial level of 0 µg/g dw to 5.11 µg/g dw (325% DV/84g serving) after just one pulse. The PUV system generated 3 pulses per second, achieving an exposure time of less than one second with one pulse. Similar results were observed with brown button mushrooms, wherein vitamin D₂ levels increased to 4.43 µg/g dw (362% DV/serving) with one pulse. Oyster mushrooms and shiitake contained

higher initial levels of vitamin D₂, 0.16 µg/g dw and 0.037 µg/g dw respectively, and the vitamin D₂ content increased to 7.95 µg/g dw and 5.71 µg/g dw respectively after one pulse. All mushrooms tested had increasing levels of vitamin D₂ with each additional pulse. The study also found that PUV was effective in increasing the vitamin D₂ content of dried mushroom powders. With 4 pulses the vitamin D₂ content of white button mushroom powder increased from near 0 to ca. 12 µg/g dw and increased with additional exposure of 16 total pulses to ca. 26 µg/g dw. Similar results were seen in king oyster mushroom powder with 10.4 µg/g dw and 23.0 µg/g dw vitamin D₂ with 4 and 16 pulses, respectively. Spent mushroom substrate containing mycelial biomass contained lower levels of vitamin D₂ (under 2 µg/g dw, with a maximum 16 pulses) when exposed to PUV. This study suggests that PUV is an effective and rapid means of increasing the vitamin D₂ content of mushrooms and mushroom products with exposure times on the order of seconds.

A dose-response study of the effect of PUV treatment on vitamin D₂ content of *Agaricus bisporus* was conducted. In addition, factors influencing the conversion of ergosterol to vitamin D₂ and effects of PUV on shelf life were also investigated. Fresh white button mushrooms were treated with PUV wherein vitamin D₂ was found to rapidly increase to over 100% of the recommended dietary allowance (RDA) per serving following 3 pulses. Following 12 pulses, vitamin D₂ levels began to approach a maximum concentration of 27 µg/g dw. A shelf life study of PUV-treated mushrooms was carried out, where it was observed that the vitamin D₂ produced with 3 pulses dropped from 11.9 µg/g dw to 9.05 µg/g dw after 3 days of storage; however, vitamin D₂ levels remained nearly constant after this point throughout the 11-day study. The effect of PUV treatment on sliced and whole mushrooms was also evaluated. Significantly more vitamin D₂ was produced in sliced mushrooms than in whole mushrooms: 113% and 81% less for brown and white button mushrooms, respectively. Vitamin D₂ production in brown compared to white button mushrooms were also examined, and it was observed that whole and sliced brown mushrooms exposed to PUV generated significantly less vitamin D₂ than their white counterparts (33% and 21% less for whole and sliced, respectively). Finally, several quality attributes were assessed in PUV-treated mushrooms. No significant differences between control and PUV treated mushrooms were observed. These

findings suggest that PUV treatment is a viable method for increasing the vitamin D₂ content of fresh mushrooms without adversely affecting quality.

The effect of PUV treatment of mushrooms on the production of photoproducts of ergosterol was investigated. In addition to vitamin D₂, it is known that with UV exposure of ergosterol other products including previtamin D₂, tachysterol₂ and lumisterol₂ are produced. White button mushrooms were sliced and treated in a single layer system with PUV irradiation increments from 0 to 60 pulses to determine the dose-response relationship between irradiation and photoproduct content. All photoproducts increased in a non-linear but dose-dependent manner as a function of increasing PUV irradiation. Vitamin D₂ content approached a maximum value of ca. 124 µg/g dw with 60 pulses, and was observed to be the most abundant photoproduct with respect to yield. With 60 pulses, previtamin D₂ content approached a maximum value of 44.6 µg/g dw, while lumisterol₂ and tachysterol₂ reached maximum levels of 30.5 µg/g dw and 24.7 µg/g dw, respectively.

PUV has been shown to be an effective and rapid means of increasing the vitamin D₂ content of mushrooms. Exposure times of approximately one second are more than sufficient to increase vitamin D₂ to levels that are biologically significant. The shortest exposure time of any previous study using continuous UV regimes to achieve a level equivalent to 100% DV/serving was ca. 8 minutes. PUV treatment of mushrooms was also shown to have little to no effect on quality parameters as they relate to appearance and product quality. The presence of additional photoproducts of ergosterol in mushrooms was also demonstrated for the first time. These products may have some biological significance, which should be investigated in future studies. With the use of PUV, mushrooms can be a viable source of vitamin D₂, which can aid in alleviating the persistent problem of vitamin D deficiency in humans.

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1. Introduction and Review of Relevant Literature

1.1 Introduction

The role of vitamin D in human health has been well studied in the past century. Since the discovery of its role in bone health and prevention of rickets, vitamin D status has been shown to have a putative link to a number of disease states. Although the science is not fully conclusive on the precise role vitamin D has on these diseases, there are still several populations who are at risk for vitamin D deficiency in the United States and around the world.

Vitamin D₃ derived through animal sources from 7-dehydrocholesterol and vitamin D₂ derived from ergosterol through fungal sources are the two predominant forms of vitamin D. Humans produce vitamin D₃ with sunlight exposure of the skin resulting from the UV induced conversion of 7-dehydrocholesterol. However, given the population's increased use of sunscreen and less time spent outdoors, additional sources of vitamin D are often needed. Currently this can be accomplished through oral vitamin supplements or through the diet from foods that are natural sources as well as foods that are fortified. The number and variety of these foods is limited. Sources include fatty fish, fortified dairy products and some fortified orange juices. As such, additional sources are needed.

Mushrooms naturally contain high levels of ergosterol, the precursor to vitamin D₂. Through sunlight or commercial UV irradiation this ergosterol can be converted to vitamin D₂. Because most mushrooms are grown indoors, commercial irradiation is needed to increase the levels of vitamin D₂ to biologically significant levels. The research using conventional UV light to this point has been shown to take on the order of minutes to hours to achieve these levels. These long processing times may prove to be impractical in a commercial setting. Pulsed UV light, on the other hand, is a high intensity broadband system that has the potential for faster throughput in a commercial setting.

1.2 Mushrooms

Mushroom is the common name for the edible portion of fungi, which can grow wild or be obtained through commercial cultivation. Among the edible mushrooms, *Agaricus bisporus*, or the common button mushroom, is the most cultivated in the United States. Several other species of mushrooms including *Lentinula edodes* (shiitake), *Pleurotus ostreatus* (oyster), and *Pleurotus eryngii* (king oyster) are commercially cultivated. Several wild species of mushrooms are not able to be cultivated but are harvested from nature and sold commercially such as *Grifola frondosa* (maitake) and *Cantharellus cibarius* (chanterelle).

Pennsylvania is the largest producer of mushrooms in the United States followed by California and Washington (1). The top three states produced respectively produced 65, 14 and 1.5% of the total 2010-2011 U.S. *Agaricus* mushroom crop, which totaled 845 million pounds valued at \$960 million. The total mushroom crop for 2010-2011 was valued at \$1.2 billion, with specialty mushrooms only accounting for \$50 million for 18 million pounds. Of these specialty mushrooms 9.7 million pounds of shiitake mushrooms, 5.4 million pounds of oyster mushrooms and 1.6 million pounds of other species were sold valued at 30, 12.5 and 5.6 million dollars respectively, The overwhelming majority of mushrooms produced in the United States in 2010-2011 were sold as fresh mushrooms with 86% of the *Agaricus* crop being sold as fresh and 14% sold for processing.

1.2.1 Commercial Mushroom Production

The commercial production of mushrooms in the United States is largely based around the widespread large-scale production of button mushrooms. The growing of button mushrooms can be divided into six steps; Phase I composting, Phase II composting, spawning, casing, pinning and cropping. These procedures are outlined in the Penn State Agricultural extension document *Six Steps to Mushroom Farming* (2). The complete commercial process mushrooms production occurs over the span of 14 weeks.

Production of button mushrooms begins with the preparation of the growing medium called compost. During Phase I composting, the ingredients consisting of mainly wheat straw-bedded horse manure are wetted and turned with the addition of nitrogen supplements and gypsum and formed into piles wherein aerobic microbial fermentation begins to break down the components. During this process, heat, ammonia and carbon dioxide are formed as byproducts and the chemical nature of the compost is changed to a composition best suited for the growth of mushrooms but not to the growth of other fungi or bacteria. Phase I is complete within 6 to 14 days when certain endpoints are reached which include; the compost will have a chocolate brown color, have soft pliable straws, have a moisture content between 68 and 74% and have a strong smell of ammonia.

The role of Phase II composting is to pasteurize the compost and to remove ammonia formed during Phase I. Phase II composting can occur in one of three systems: a zone system, wherein compost is placed in wooden trays; a bed system, wherein the compost is placed directly into the growing beds; and the newer bulk system, wherein the compost is placed in a tunnel designed to allow for computer controlled aeration. During this process, air is incorporated into the compost to allow for microorganisms to grow and reproduce, producing nutrients that are used by the mushrooms during growth. The endpoint is reached when the temperature is between 75 and 80°F, the nitrogen content is between 2.0 to 2.4%, and the moisture content is between 68 and 72%.

The third step in mushroom growing is called spawning. Spawn is the name for cultured mushroom mycelium (thin threadlike cells) propagated from mushroom spores and grown on various grains. Spawn is produced by specialized companies who sell the material to mushroom growers. Spawn acts as the “seed” of mushroom growing. This material is thoroughly mixed into the compost.

Casing involves placing a layer of a mixture of peat moss and ground limestone on top of the spawned compost. The function of this layer is act as a water reservoir to supply water to the mycelium for growth, prevent the compost from drying and providing structure and support for the growing mushroom. Inside the casing, rhizomorphs are

formed from the mycelium fusing together, which leads to the development of mushroom pins.

Pinning takes place through the artful control of air and moisture. Carbon dioxide content of the air must be below 0.08% for pins to form. These pins enlarge as they grow into mushrooms that are harvestable approximately 18 to 21 days after casing is completed.

The final step of mushroom production is cropping. This occurs in what is termed as breaks, flushes or blooms which refers to the 3 to 5 day harvest periods during which mushrooms are harvestable. This cycle repeats for 35 to 42 days and included several days in between each break in which no mushrooms are harvestable. Harvesting is done by hand in order to protect the mushrooms from physical damage.

1.2.2 Chemical Composition of Mushrooms

The proximate analysis of selected mushrooms species are given in **Table 1.1**.

Mushrooms are very high in moisture content, at approximately 90%. Mushrooms are low in fat with the majority of the dry weight consisting of protein and carbohydrate.

Table 1.1. Proximate Composition (% dry weight, except moisture) of Selected Mushroom Species (3)

Component	<i>Agaricus bisporus</i>	<i>Lentinula edodes</i>	<i>Pleurotus ostreatus</i>
moisture	78.3-90.5	90.0-91.8	73.7-90.8
ash	7.7-12.0	3.7-7.0	6.1-9.8
protein	23.9-34.8	13.4-17.5	10.5-30.4
carbohydrate	51.3-62.5	67.5-78.0	57.6-81.8
fat	1.7-8.0	4.9-8.0	1.6-2.2

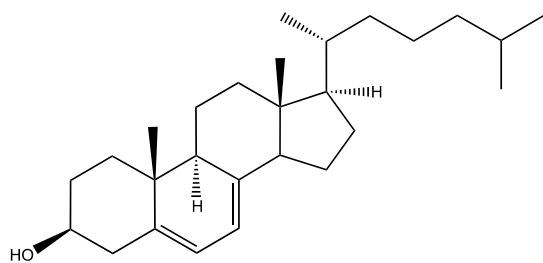
The majority of the fatty acids present in *Agaricus bisporus* are linolenic and palmitic acid, while glutamic acid is the most abundant amino acid (4). Button mushrooms also contain approximately 0.76 mg vitamin B₆, 3.37 mg riboflavin and 37.3 mg of niacin per 100 g of dry weight (dw) (4). Mushrooms are also rich in sterols, with ergosterol being the most abundant, ranging anywhere from 2.73 mg/g dw (5) to 6.54 mg/g dw (6). Button mushrooms have also been found to contain other sterols including ergosta-7,22-dienol, ergosta-7,5-dienol and fungisterol (15.2, 94.0 and 25.8 mg/100 dw, respectively) (6).

These sterols are major constituents of the cell membranes of mushrooms and other fungi including yeasts, which aid in protecting the cytoplasm from the environment as well as modulating bulk physical state and integrity (6,7). In fungal cell membranes this influences membrane rigidity, fluidity and permeability (8,9)

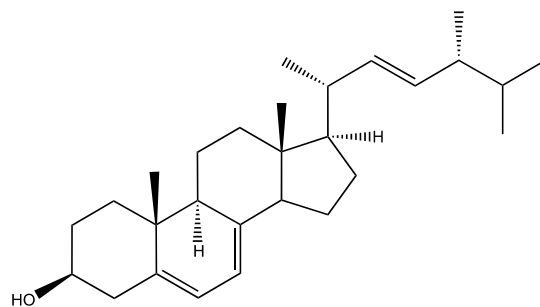
1.3 Vitamin D

1.3.1 Vitamin D Structure

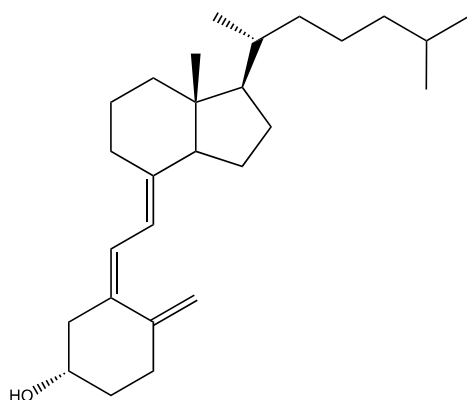
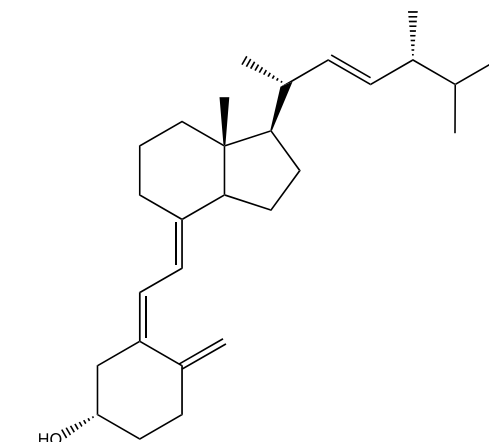
Vitamin D is a fat-soluble secosteroid that exists in two primary forms in nature, ergocalciferol (vitamin D₂) and cholecalciferol (vitamin D₃). Vitamin D₃ is the form found in animal life and is derived from cholesterol. Vitamin D₂ is formed from ergosterol, a sterol that is found abundantly in fungi and in some plants. The structures are given below in **Figure 1.1**. The provitamins (7-dehydrocholesterol and ergosterol) consist of identical sterol backbones (one hydroxyl group on the A ring). The side chain attached to the 5-carbon ring is the distinguishing feature between the two provitamins. Ergosterol contains a double bond between carbon 22 and 23 and contains an additional methyl group (C28) on this side chain in comparison to 7-dehydrocholesterol. The same differences are seen in the vitamin forms in which the B ring is opened.



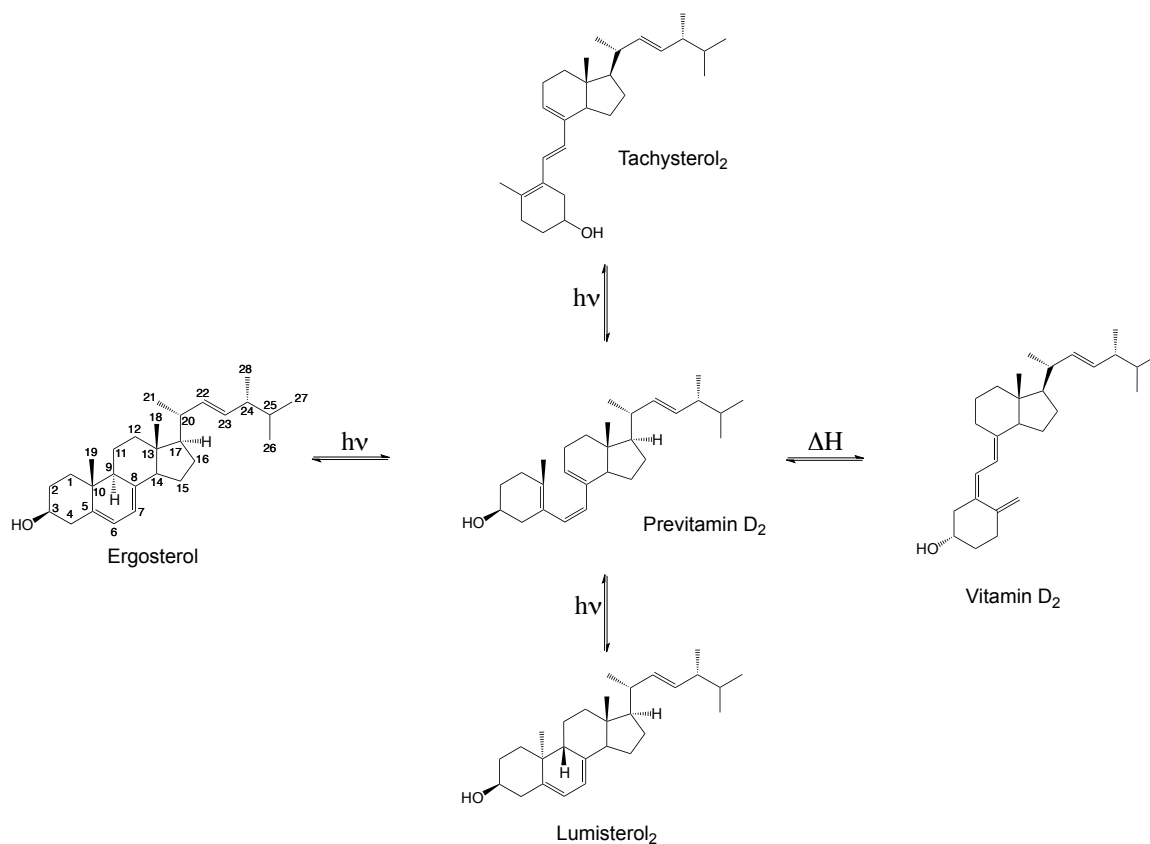
7-Dehydrocholesterol



Ergosterol

Vitamin D₃Vitamin D₂**Figure 1.1.** Structure of 7-dehydrocholesterol, vitamin D₃, ergosterol and vitamin D₂ (10,11)

1.3.2 Vitamin D Synthesis

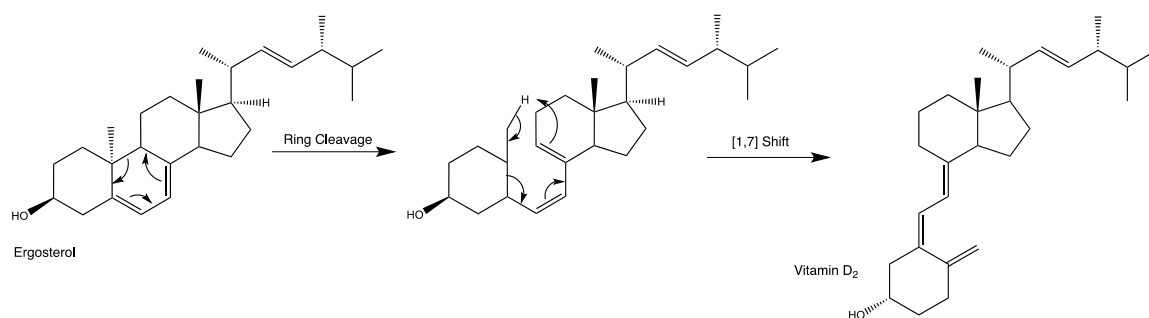


Scheme 1.1. Reaction scheme of photochemical conversion of ergosterol to vitamin D₂ and isomers (10,12,13)

The conversion of ergosterol to vitamin D₂ (D₂) (**Scheme 1.1**) is initiated with the exposure of the precursor ergosterol (also known as provitamin D₂) to UV light which cleaves and opens ring B thus forming previtamin D₂ (PD₂). Through extended exposure to UV light, PD₂ can undergo ring closure to form lumisterol₂ (L), which is a (9β,10α) stereoisomer of ergosterol (9α,10β). PD₂ is thermally labile and is converted to D₂ by a thermally induced [1,7] sigmatropic shift (**Scheme 1.2**) (14). A sigmatropic shift is a pericyclic reaction which involves the rearrangement of a σ-bond to another position in a π-bonded system (15). In the case of the rearrangement of previtamin D to vitamin D, a hydrogen shift occurs from the 1 position of the cleaved B ring to the 7 position, hence termed [1,7].

Tachysterol₂ (T₂) is another photoproduct of this photochemical reaction and is structurally similar to vitamin D₂ except in T₂ the A ring is rotated 180° and the C ring

contains a double bond. Other products such as suprasterols (**Figure 1.2**) and toxisterols (**Figure 1.3**) are known to form as a result of extended irradiation of ergosterol (12) and have been shown to be toxic (16). The ratio of each product formed varies depending on wavelength of the UV irradiation (17).



Scheme 1.2. Ergosterol sigmatropic shift to vitamin D₂ (14,15)

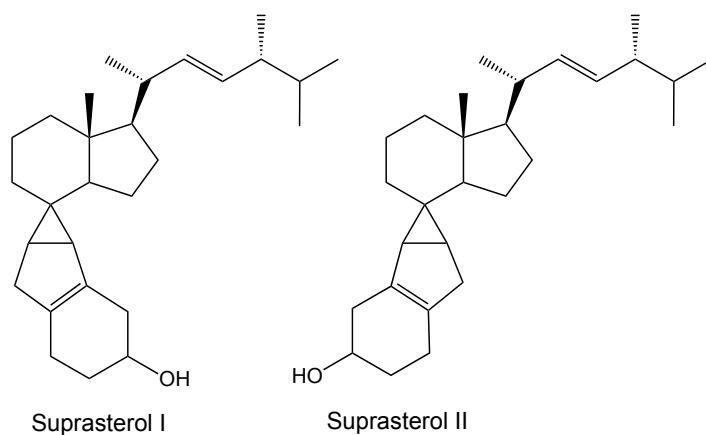


Figure 1.2. Structure of suprasterols (10)

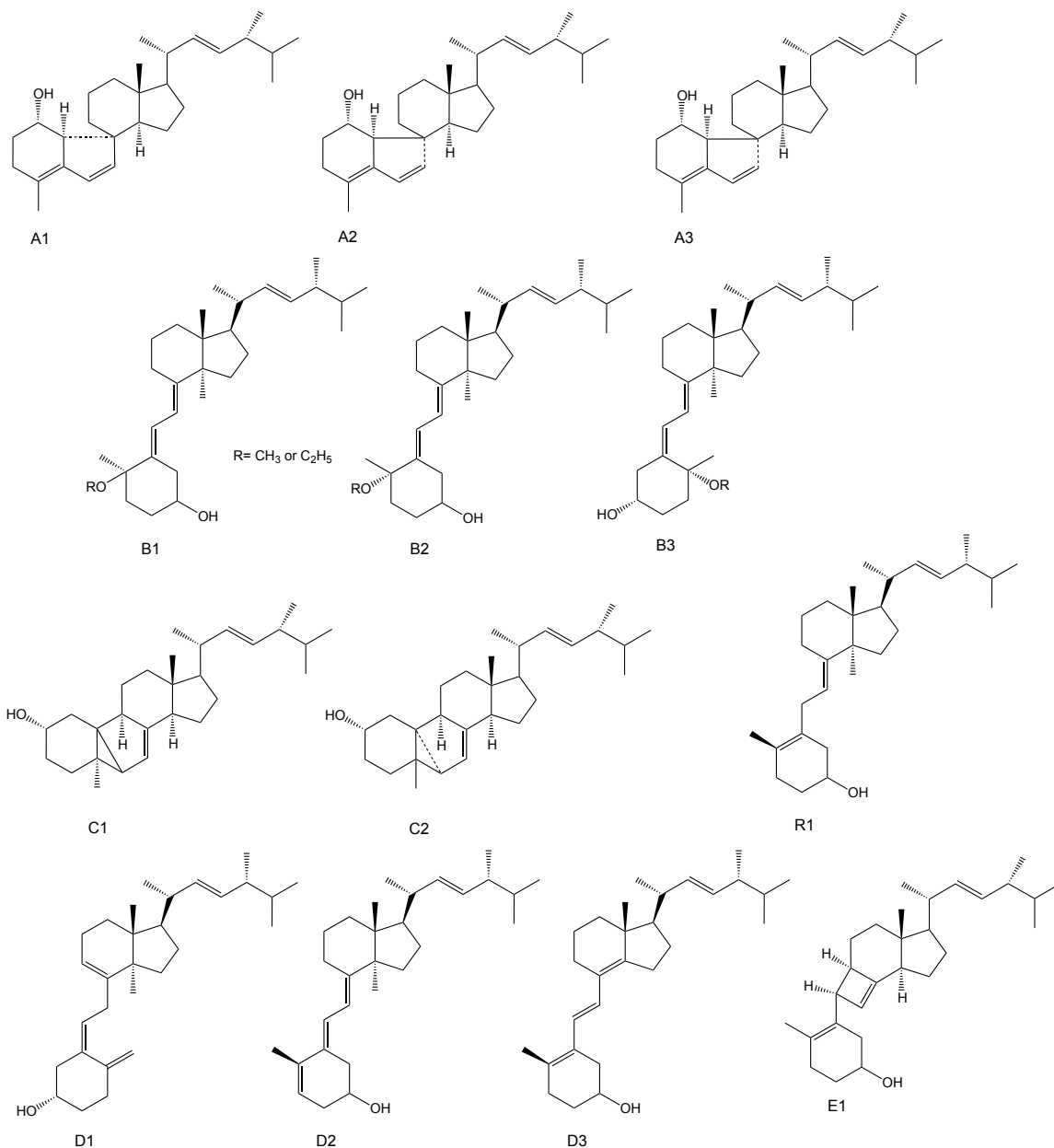


Figure 1.3. Structure of toxisterols (12)

1.3.3 Human Metabolism Of Vitamin D

Vitamin D is converted in the liver and kidneys to the active form 1,25 dihydroxyvitamin D. This active form then plays a role in maintaining blood levels of phosphorus and calcium and also promotes bone mineralization and calcium absorption. The metabolism of vitamin D (18) in humans is presented in **Figure 1.4**.

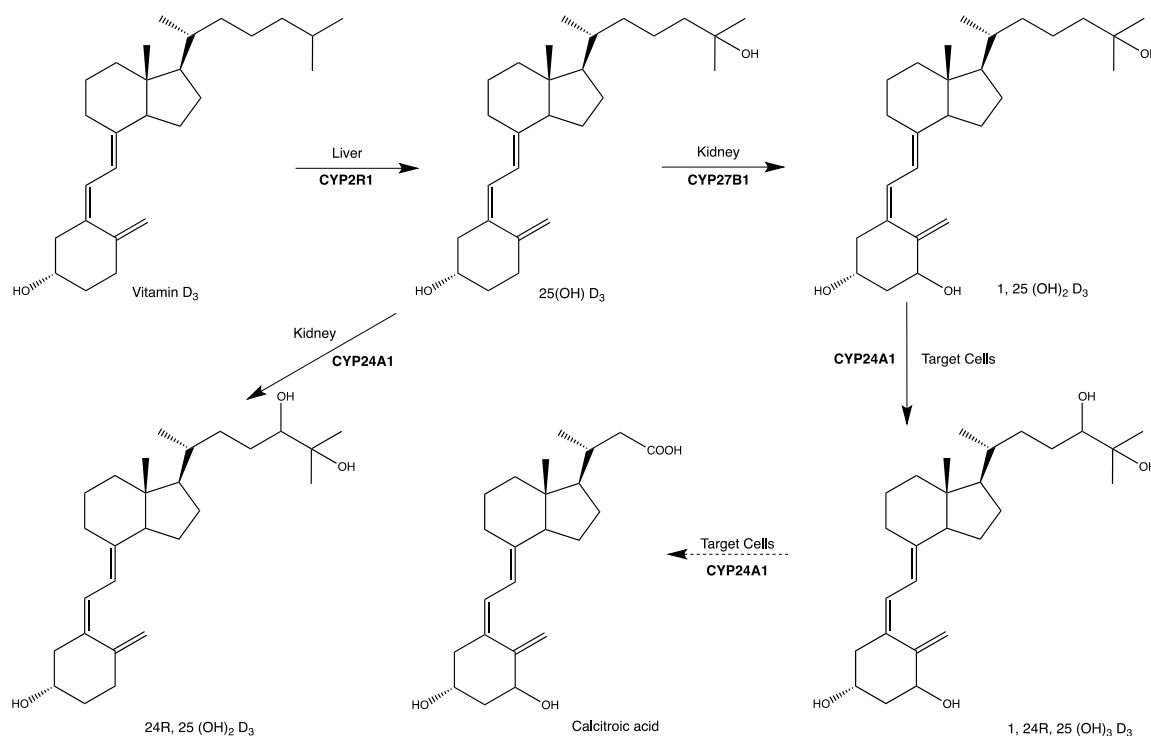


Figure 1.4. Metabolic and catabolic pathway of vitamin D₃ in the body, (18).

The cytochrome p450 (CYP) family of enzymes plays a major role in vitamin D metabolism. The first step in activation of vitamin D occurs in the liver. The enzyme CYP2R1 is a 25-hydroxylase, which converts vitamin D to 25-hydroxyvitamin D (25[OH]D) which is the main circulating form of vitamin D in the blood. The second step involves the enzyme CYP27B1 and the cofactors ferredoxin and ferredoxin reductase in the kidney. The enzyme is a 1 α -hydroxylase, which converts 25[OH]D to 1,25-dihydroxyvitamin D (1,25[OH]₂D). Normal levels of 25[OH]D can vary from less than 10 ng/mL serum in deficient adults to around 75 ng/mL in individuals who work or live in the sun (19–21).

There are several known polymorphisms that affect vitamin D metabolism and thus vitamin D status of an individual. The disease cerebrotendinous xanthomatosis (CTX) results from a mutation of CYP27A1 and affects bile acid and lipid metabolism which can indirectly lead to low levels of 25[OH]D (22). In a study of 15 patients with CTX, all patients were found to have low levels of 25[OH]D which resulted in significant osteoporosis and it was recommended that vitamin D supplements could aid in reducing

symptoms (23). Also CYP2R1 L99P results in vitamin D dependent rickets type 1a (VDDR 1a) from null enzyme activity (18). VDDR type 1 is a polymorphism of CYP27B1 (18), which causes a mutation of the 1 α -hydroxylase and thus 25[OH]D cannot be hydroxylated to the active 1,25[OH]₂D, leading to rickets.

Vitamin D inactivation signaling is a result of the enzyme CYP24A1, which breaks down 1,25[OH]₂D into inactive forms acting as a feedback loop when a certain threshold level of 1,25[OH]₂D is reached thereby neutralizing excess vitamin D levels. CYP24A1 is present in the kidney as well as target cells and acts as a 23- and 24-hydroxylase, but can form 25[OH]D_{26,23}-lactone when the A326G mutation changes it from a 24 to 23-hydroxylating enzyme (18). The role of this metabolite is unknown but a synthetic form was shown to be a vitamin D receptor antagonist (24).

The effectiveness of vitamin D₂ and vitamin D₃ has been a contested subject for many years. Vitamin D₂ has been shown by some to not to be as biologically active as Vitamin D₃ (25,26). Trang et al. found that when both forms were given daily in 4000 IU doses, that vitamin D₃ was 1.7 times as active in increasing 25[OH]D levels than vitamin D₂ (25). Armas et al. observed that with a single 50,000 IU dose of each, that 25[OH]D levels increased for three days afterwards for both forms but levels fell in subjects given vitamin D₂ following three days while continuing to rise in subjects given vitamin D₃ through 14 days after dose administration (26). However, recent research suggests that both forms are converted to the active 1,25 dihydroxyvitamin D form in the body and result in no significant changes in blood levels of the active form when consumed (27). The differences in effectiveness in these studies can be attributed to be a result of the high doses used by Armas et al. and Trang et al. The structural differences do not affect metabolism, as both forms undergo the same activation to form 25[OH]D and 1,25[OH]₂D, eliciting virtually identical biological responses in the body while both forms have binding affinity to the vitamin D receptor and vitamin D binding protein (28). The enzymes involved in side chain metabolism also do not discriminate between vitamin D₂ or vitamin D₃ side chains (18). Ross et al. state that the differences seen in these studies (25–27) does indicate a difference in pharmacokinetics (28).

1.3.4 Photoproducts of Ergosterol and Related Sterols

Recent studies suggest that the photoproducts of ergosterol could be of biological importance. Chen et al. (29) investigated the D₃ series of these compounds and their role in antiproliferative activity in human keratinocytes and affinity to the nuclear vitamin D receptor (VDR). The authors found that while the photoproducts had low binding affinity for VDR there was some antiproliferative activity, as measured by ³H-thymidine incorporation of cultured human keratinocytes, that may have been due to the hydroxylation of tachysterol₃ (T₃) to its 25(OH) and 1α,25(OH) forms. The authors showed that that 25(OH)T₃ has binding affinity for VDR although it is lower than that of 25[OH]D₃.

Also of note is that there are hundreds of vitamin D analogs used for various clinical treatments including rickets, osteomalacia, osteoporosis, renal failure, leukemia, psoriasis, hypo- and hyperparathyroidism, and immune diseases (18). Vitamin D analogs are built upon the steroid backbones of vitamin D with alterations to the side chain being the primary means of differentiation. Many of the vitamin D analogs are based on the first analog developed, dihydrotachsterol₂ (DHT) (30). DHT is a reduction product of vitamin D₂ and is structurally similar to T₂ with the only differences being the lack of double bonds in the A and C rings in DHT leading to a stereochemical difference. Due to the structural similarity of these analogs, it is conceivable that these photoisomers may be of biological relevance.

This is supported by two studies by Holick et al., wherein the authors demonstrated that 5,6-*trans*-25-hydroxycholecalciferol was effective in stimulating calcium transport in the duodenum of vitamin D-deficient rats (31). The authors also demonstrated that isotachysterol₃ and 25-hydroxyisotachysterol₃ both stimulated the transport of calcium in the intestines and bone calcium mobilization in anephric rats (32).

One of the reasons the biological impact of these compounds has not been studied is that the event these compounds would be consumed and circulated throughout the body is rare. Currently there is no data on the circulation of these compounds. However, Holick et al. demonstrated that the affinity of the vitamin D Binding Protein (DBP) is low

for 7-DHC and L_3 and T_3 , which indicates these compounds are less likely to leave the skin once formed (33). The use of the D_2 series of compounds is limited to some food fortification and supplementation and, as such, in most cases authentic vitamin D_2 (i.e., no photoisomers) is the sole compound added. Furthermore, the commercial production of vitamin D_2 can be optimized to maximize the yield of vitamin D_2 and to eliminate the photoisomers (34,35).

Biological study of these compounds is still justifiable on structural grounds because of the similarity of T and D and, also, of E (or 7-DHC) and L. In addition, other factors beyond the binding to DBP could also be involved in health effects, such as the binding to vitamin D receptors located in various tissues in the body.

1.4 Causes and Consequences of Vitamin D Deficiency

The generation of vitamin D_3 in humans principally occurs in the epidermal layer of the skin by exposure to ultraviolet radiation from sunlight (36). 7-dehydrocholesterol absorbs ultraviolet radiation in the B range (290-315nm) and is converted to previtamin D_3 . Once formed, previtamin D_3 rapidly converts to vitamin D_3 . Thus, individuals with limited sun exposure are the most at risk for vitamin D deficiency.

Deficiency of vitamin D has been linked to rickets in children and osteomalacia in adults, which is caused by inadequate calcium levels leading to inadequate bone mineralization. Rickets occurs in infants and children and is the end stage of severe calcium deficiency in which bone has failed to properly grow resulting in irreversible skeletal changes such as bowing of the limbs. Osteomalacia follows a similar pathology in adults and can lead to softening of the bone (28).

Individuals at risk for vitamin D deficiency include: infants who are exclusively breastfed (breast milk is not a significant source of vitamin D) (28); older individuals (elderly often have lower vitamin D intake, less sun exposure and lower dermal conversion of 7-DHC to vitamin D_3 due to lower levels of 7-DHC (37)); people with limited sun exposure (28); people with greater skin melanin content (melanin blocks UV absorption) (28); and people with fat malabsorption (vitamin D is a fat soluble vitamin and therefore fat

absorption is required for absorption of vitamin D) (38). Another problem is the use of sunscreen. Matsuoka et al. (39) showed that with the use of a sunscreen with a SPF of 8, the ability to produce vitamin D is reduced by 95%.

1.4.1 Diseases Linked To Vitamin D Status

Vitamin D is most often associated with its contribution to maintaining circulating levels of calcium and phosphorus. Classically it is thought that a deficiency in vitamin D leads to inadequate bone mineralization. This condition is observed as rickets in children (40) and osteomalacia in adults. Emerging research suggests vitamin D status is linked to many more human disease states.

A recent report has cited links to several diseases including cancer, coronary heart disease, hypertension and stroke, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, mental illness and chronic back pain (19). Vitamin D has also been linked to helping to maintain a healthy immune system and regulation of cell differentiation and growth (28).

1.4.1.1 Cardiovascular Disease

The role of vitamin D deficiency in increased risk of cardiovascular disease has been shown in several epidemiological studies. Wang et al. conducted an analysis of data from the Framingham Offspring Study of participants without prior cardiovascular disease. They found that participants with hypertension and vitamin D deficiency had a higher 5-year rate of cardiovascular disease and twice as high in those with and without hypertension in vitamin D deficient participants (25[OH]D levels ≤ 15 ng/mL) than non deficient participants (25[OH]D > 15 ng/mL). Calculated hazard ratios for cardiovascular events were 1.62 for vitamin D deficient participants and 2.13 in vitamin D deficient hypertensive participants. Vacek et al. conducted an observational retrospective study of academic medical center patients and found that vitamin D deficiency was associated with hypertension (OR= 1.62), coronary artery disease (OR= 2.45), cardiomyopathy (OR= 3.09) and diabetes (OR= 1.67).

In a review by Artaza, it was demonstrated that African Americans who are at the highest risk of cardiovascular disease among ethnic groups also have the highest incidence of vitamin D deficiency and that there may be a putative link. The authors also documented the role 1,25[OH]₂D has on signaling various responses in the body that could lead to an increased risk of cardiovascular disease such as inflammatory response (which can lead to plaque formation in the arteries) as well as reducing platelet aggregation, thrombogenesis (blood clot formation), vascular smooth muscle cell proliferation and vascular calcification.

1.4.1.2 Cancer

There have been numerous papers published on the links between vitamin D status and various cancers. The following is a review of a selection of these works.

1.4.1.2.1 Breast Cancer

The relationship of vitamin D status and breast cancer incidence has been widely studied (41). Garland et al. (42) utilized data on solar irradiance and breast cancer mortality in 87 regions of the United States. The authors observed an inverse relationship of solar irradiance and breast cancer mortality (R value of 0.80, $p = 0.0001$). This indicates that women living in areas farther south or areas with high levels of solar irradiation had lower mortality rate than women living in areas with less sunlight. When the data was adjusted for stratospheric ozone, which blocks sunlight (particularly the UV wavelengths responsible for vitamin D synthesis), the R value increased to 0.82.

The authors also addressed potential confounding variables such as dietary intake of vitamin D. Vitamin D is not abundant in the diet and, as such, most people do not consume significant levels of vitamin D. The potential for differences in consumption of these foods from region to region was stressed as a potential factor.

Mohr et al. (43) also utilized an epidemiological approach using data from 107 countries, but the authors focused on risk of breast cancer rather than mortality. This study used 25[OH]D levels in addition to the data on incidence and latitude to attempt to show a

more direct correlation. The authors found that there was an inverse relationship between serum 25[OH]D levels and breast cancer risk. It was shown that serum levels of 22-34 ng/mL 25[OH]D were associated with the lowest incidence of breast cancer with the highest risk for those individuals with serum levels below 20 ng/mL.

Millen et al. (44) built upon this work but tried to stratify the data based on additional factors associated with geographic location, such as solar irradiance measured in Watts and Langleys, since geographic location is an approximate measure of how much sun exposure an individual would get in terms of total irradiance. However, time spent outside is a major factor. For example, if a person lives in the southern US but spends most of the day indoors, then the amount of sun exposure and thus vitamin D synthesis could be equivalent to an individual who lives in the North but spends more time in the sun. The paper by Millen et al. (44) does mention this and the authors assess this phenomenon by utilizing questionnaires about time spent outdoors. The time was reported as: under 30 minutes; between 30 minutes and two hours; and more than two hours. Women who reported spending less than 30 minutes outside year round had a 20% increased risk compared to women who spent more than 2 hours outside year round. However, the lowest choice could include anywhere from zero sunlight exposure to adequate amounts during the summer in southern locations. The authors supplemented that data with another question about how the individual reacted to sunlight (tanning or burning). One of the conclusions mentioned that time spent outside is a factor in relation to breast cancer risk. That is, spending more time outside would result in more vitamin D synthesis and thus would better illustrate the supposed inverse correlation between vitamin D status and risk of developing breast cancer.

Garland et al (45) conducted a pooled analysis of two earlier studies by Bertone-Johnson et al. (46) and Lowe et al. (47) which utilized 25[OH]D levels in patients with cancer and controls who did not have cancer. Both studies were set up in a very similar fashion. In these matched case-control studies, breast cancer patients were chosen and paired with a control individual that did not have cancer but was similar in terms of age and menopausal status. Time of year blood samples were taken because levels would be lower in winter than in the summer. Bertone-Johnson et al. (46) adjusted their data to more parameters than Lowe et al. (47). This included factors that could have a

relationship with risk of breast cancer such as dietary habits and smoking status.

Bertone-Johnson et al. (46) did not include vitamin D supplement users in the study, because they stated these individuals could have been on treatment for bone loss and thus have a low risk of breast cancer. Bertone-Johnson et al. observed that while cases with cancer had lower 25[OH]D levels than control cases, higher levels were associated with a nonsignificant lower risk of breast cancer (46). Lowe et al. (47) showed more of a difference in relative risk between their quartiles than Bertone-Johnson et al. (46).

The pooled data analysis suggests that a 50% lower risk of breast cancer is associated with 25[OH]D levels greater than 50 ng/ml when compared to a baseline group with less than 10 ng/mL (45). Garland et al. then use this data to develop a suggested intake of vitamin D that would be adequate to reduce the risk of breast cancer. A dose of 2000 IU is suggested which would, according to Garland et al. (45), prevent 66,000 cases of breast cancer per year. The authors utilized the conclusion of Heaney et al. (48) that with each 1000 IU dose of vitamin D, 25[OH]D increases by 10 ng/ml and the number of new cases of breast cancer in 2006 (n=214,000) as the basis for these calculations.

Gissel et al. (49) conducted a meta-analysis of six studies on vitamin D intake and the risk of breast cancer. The authors found no significant association between amount of vitamin D and risk of breast cancer but they observed a trend of lower risk of breast cancer with more than 400 IU/day. This study lacked data regarding sun exposure which is especially important because of the low levels of vitamin D consumption shown in the study (most well below 200 IU/day) and the lack of 25[OH]D levels. These are confounding variables. If the intake in the diet of vitamin D is low, the main way an individual gets vitamin D is through sun exposure. The relative risks shown in this study may have affected by this, as the authors did not observe a definitive association.

These studies have shown that there may be a relationship between vitamin D and the risk of breast cancer. However, more clinical data is needed, as the data presented is purely epidemiological. In addition, an epidemiological study on this topic that included where people live, more precise measures of how much sun exposure each individual gets in an average day, how much vitamin D is taken in through the diet, and what the vitamin D status (25[OH]D levels) of the individuals are coupled with breast cancer

incidence data would present the best case for confirming that there is correlation. These factors are not separate, as many of the studies presented seem to suggest. Instead, they all contribute to the vitamin D status of the individual.

1.4.1.2.2 Colorectal Cancer

Wu et al. (50) completed a similar study to those of Bertone-Johnson et al. (46) and Lowe et al. (47) involving vitamin D status and risk of colorectal cancer. The authors conducted a nested case-control study from the Health Professionals Follow-up Study and then later pooled data from the Nurse's Health Study to strengthen their results. They also stratified by confounding variables including BMI, physical activity, calcium, retinol intake and time of year that the blood samples were taken. They observed a significant trend for reduction of colon cancer but not for colorectal cancer. When the authors pooled the data with the Nurse's Health Study they observed a significant inverse association for risks of both types of cancer and vitamin D status. The OR for colon cancer was 0.54 (highest verses lowest quintile of 25[OH]D) and 0.66 for colorectal cancer.

Wu et al. (50) showed that their pooled data did not differ in the inverse associations of 25[OH]D levels and risk of colorectal cancer by calcium intake. This is important because calcium could be a confounding variable.

Gorham et al. (51) conducted a meta-analysis of five studies that examined 25[OH]D levels and risk of colorectal cancer. Only three of the five studies had a significant inverse trend with regards to 25[OH]D levels and risk of colorectal cancer. The authors concluded that there was 50% lower risk of colorectal cancer with a 25[OH]D level of 34 ng/ml compared to 12 ng/ml. This conclusion is very similar to the conclusion of Garland et al. (45) and their assessment that breast cancer risk is reduced 50% by a 25[OH]D level of 52 ng/ml. The authors address confounding variables by concluding that other studies have shown that there are three lines of epidemiological evidence of an inverse relationship including geographic gradient of UVB exposure, observational studies showing that low levels of 25[OH]D increase risk, and studies showing that low oral intake and increased risk as well Vitamin D Receptor (VDR) polymorphisms increasing

risk. They state that a single confounder could not account for all these associations. However, multiple confounders such as diet and other risk factors could play a role. This study utilized in vivo and in vitro studies in mice to back up these observations. While not perfect models of the effects of vitamin D on decreasing the risk of colorectal cancer in humans, this data is a little more credible than epidemiological studies alone.

Bischoff-Ferrari et al. (52) examined 25[OH]D levels and related them to risk of colorectal cancer and also bone mineral density, dental health, fractures, etc. This study was conducted to examine the preventative relationship between adequate level of intake of vitamin D and multiple diseases.

1.4.1.2.3 Prostate Cancer

Mikhak et al. (53) conducted a nested case-control study on prostate cancer risk and Vitamin D Receptor (VDR) gene polymorphisms within the Health Professionals Follow-up Study. VDR gene polymorphisms could alter the way vitamin D is utilized in gene expression and thus cancer proliferation. VDR polymorphisms may not be directly related to vitamin D deficiency in terms of intake and synthesis from the sun. A detailed literature review of VDR polymorphisms and risk of all types of cancer was completed by Kostner et al. (54). They demonstrated how more than 60 VDR polymorphisms contribute to risk of these cancers.

Schwartz (55) examined the intervention studies that had previously been completed, of which there are more than 20, regarding vitamin D treatment of prostate cancer. The author's main conclusion was that vitamin D intake or synthesis may prevent cancer but that treatment of existing cancer required very high doses of 1,25(OH)₂D.

1.4.1.2.4 Calcium, Vitamin D and Cancer

The role of calcium and vitamin D in reducing the risk of cancer has been studied. As mentioned by Wu et al. (50), there was no difference in risk of colorectal cancer by calcium intake with vitamin D status. Garland et al. (56) suggested that calcium and vitamin D both had roles in preventing colon and breast cancer. The authors show that

there is an inverse dose response relationship with incidence of cancer and calcium intake. Alternatively, the partnership of vitamin D and calcium in the body suggests that both may be required to have an effect.

A clinical study (57) was undertaken to address the roles of vitamin D and calcium in reducing the incidence of cancer. The study took place over four years and consisted of 3 treatment groups, a placebo group, a calcium only group (either 1400 mg calcium citrate or 1500 mg calcium carbonate), and a vitamin D (1000 IU cholecalciferol) plus calcium group (same dose as calcium only). The authors showed in that there was a RR of .402 for the calcium plus vitamin D group compared to the placebo and a 0.532 RR for the calcium only group compared the placebo. The authors then removed the cases of cancer from the first year of the study, stating that they may have been preexisting before the start of the study. With this adjustment the calcium plus vitamin D group had a RR of 0.232 compared to the placebo group while the calcium only group had an RR of 0.587 compared to the placebo. The authors also showed that the vitamin D plus calcium group had increased levels of 25[OH]D after 12 months while the placebo and calcium only groups did not.

There is a lack of clinical data available on vitamin D and cancer. However, Lappe et al. (57) observed that there was some clinical effect with regards to vitamin D and its role in preventing cancer.

1.4.1.2.5 Cancer and Vitamin D: Mechanisms of Action

Although the exact mechanisms of vitamin D and cancer reduction are not fully understood, it is believed that inhibition of cell proliferation and promotion of cell differentiation contribute to this phenomenon. Holt et al. observed that proliferation decreased in colonic epithelial cells with increasing levels of serum 25[OH]D (58). Antiproliferative effects on prostate cancer cells have also been reported in several studies (55,59).

1.4.1.2.6 Vitamin D and Cancer Conclusions

There are some evident links within all the research presented in the sections above. The results across all types of cancers are very similar. For example, the results of the pooled analyses completed by Garland et al. (45) and Gorham et al. (51) were very similar, but were focused on two different types of cancer (breast cancer and colorectal cancer respectively). The trend of increased levels of 25[OH]D and decreased risk of cancer in both studies is the same.

It is also clear from these studies that while each looked at different factors that could lead to deficiency of vitamin D there were no papers that looked at all of them together. Location of residence, time spent outside, skin pigmentation and use of sunscreen, and intake of vitamin D in the diet all contribute to the serum levels of 25[OH]D in the body. Some of these studies also did not include 25[OH]D levels and instead utilized other factors to hypothesize the cause of increased risk of cancer.

Another important point is how prevalent vitamin D deficiency is around the world. Malabanan et al. (60) estimated that 40% of adults are vitamin D deficient. However, Garland et al. (56) puts this into context by observing that vitamin D levels required to eliminate the risk of rickets is many times lower than that for preventing colon cancer. This is a very important point. Even if an individual is at what would be considered sufficient levels of 25[OH]D they could still be at increased risk of cancer. This observation emphasizes the potential role vitamin D may actually have in prevention of cancer as well as the unknown roles vitamin D may have in the prevention of other diseases that have not yet been studied in this context.

The data published thus far shows that there may be an inverse association with vitamin D status (particularly 25[OH]D serum levels) and risk of cancer. Even though most of this data is from epidemiological studies, the supporting data from several studies showing the same general trend from study to study, and for that matter from cancer to cancer, provides for a solid basis for a link between vitamin D status and cancer risk. However, additional properly controlled clinical studies are needed to fully examine the connection.

1.4.1.3 Other Diseases with Putative Links to Vitamin D Status

In several review articles, numerous other disease states have been linked to low vitamin D status. These include psoriasis, tuberculosis, inflammatory bowel disease, multiple sclerosis, rheumatoid arthritis, diabetes and metabolic syndrome (61,62). However, the studies on many of these links are still in the very early stages of gaining an understanding of whether these effects are valid. Recent reports on recommendations for vitamin D intake acknowledge this lack of data and only make recommendations based on bone health (20,28). However, these reports also indicate that more work is needed in this area. Once links to other disease states have been firmly established, the need for higher levels of vitamin D intake will most likely need to be filled through the diet.

1.5 Vitamin D In The Diet

1.5.1 Recommended Intake of Vitamin D

The Dietary Reference Intakes for calcium and vitamin D were revised in December 2010. The previous recommended daily intake of vitamin D was 200 IU for people up to age 50, 400 IU for adults over 51 to 70, and 600 IU for adults over 71 (63). The new Recommended Dietary Allowance (RDA) for vitamin D for most individuals was increased to 600 IU/day (28). The levels are based on the literature regarding only bone health as the authors cite that the literature linking vitamin D status to other diseases is inconclusive. According to the report, the majority of Americans are receiving adequate levels of vitamin D and calcium. However, the report also states that the dietary intake is below the current recommendation, but average blood levels are above their set value of 20 ng/mL. This is below what others have suggested as being adequate. This disparity is attributed to sunlight exposure fulfilling the additional vitamin D. Some subgroups, such as those who do not get enough sun as a result of various factors and those with dark skin pigmentation are still at risk of having low levels of vitamin D. In study by Nesby-O'Dell et al., for example, it was found that 42% of African American women had levels considered to be deficient (≤ 37.5 nmol/L in this study) compared to only 4% of white women (64).

The report also states that any dose over 4,000 IU/day the risk of harm begins and that at over 10,000 IU/day kidney and liver damage set in. The report, however, does not mention how long these levels need to be sustained to cause damage (28). High doses of vitamin D can result in hypercalcemia, which leads to very high levels of calcium in the blood. Toxicity occurs through calcification of soft tissues as well as anorexia, weight loss and fatigue among other symptoms (65).

Vitamin D toxicity is often not the main cause of hypercalcemia, which can be caused by a variety of disorders such as primary hyperparathyroidism or as a result of taking other drugs. Vitamin D toxicity is rare and often occurs from inadvertent overdosing (66).

Doses in the range of 10^4 IU/day for several months can cause toxicity, which is diagnosed by symptoms including anorexia, nausea, and vomiting, often followed by polyuria, polydipsia, weakness, nervousness, pruritus, and eventually renal failure (66).

Vitamin D toxicity resulting from hypercalcemia would most likely be limited to cases where subjects were taking too high of a prescribed dose. Thus, accidental overdose is unlikely.

Vitamin D deficiency is defined as serum level of circulating 25[OH]D under 20 ng/mL, while insufficiency is defined as 25[OH]D levels between 21 and 29 ng/mL (20).

Many studies relating to vitamin D and health outcomes have recommended intake much higher than the current recommendations in order to reach sufficient levels. These values typically fall between 1000 and 2000 IU/day.

1.5.2 Sources of Vitamin D

There are few methods for humans to intake vitamin D into the body beyond sun exposure. These include oral intake via vitamin supplements and dietary intake. As a result of the incidence of deficiency and insufficiency, dietary sources of vitamin D are needed. The number of foods that contain vitamin D is very limited (**Table 1.1**). Foods that are good sources of naturally occurring vitamin D include fatty fish (such as salmon

and mackerel), eggs, and beef liver. Milk and some milk products are another good dietary source of vitamin D, due to intentional fortification. Cereals and calcium-fortified juices (such as orange juice) are also often fortified with vitamin D. However, there are few common vegetarian/vegan sources of vitamin D on the market for consumption.

Table 1.2. Vitamin D content of selected foods (adapted from Vitamin D fact sheet (67) and USDA Nutrient Database (68))

Food	IUs per serving	% DV
Cod liver oil, 1 tablespoon	1,360	340
Salmon (sockeye), cooked, 3 ounces	447	112
Mackerel, cooked, 3 ounces	388	97
Tuna fish, canned in water, drained, 3 ounces	154	39
Orange juice fortified with vitamin D, 1 cup	137	34
Milk, nonfat, reduced fat, and whole, vitamin D-fortified, 1 cup	115–124	29–31
Yogurt, fortified with 20% of the DV for vitamin D, 6 ounces	88	22
Margarine, fortified, 1 tablespoon	60	15
Liver, beef, cooked, 3.5 ounces	49	12
Sardines, canned in oil, drained, 2 sardines	46	12
Egg, 1 large (vitamin D is found in yolk)	41	10
Ready-to-eat cereal, fortified with 10% of the DV for vitamin D, 0.75–1 cup	40	10
Cheese, Swiss, 1 ounce	6	2

O'Donnell et al. (69), in a review, showed that fortification of foods is effective in raising the serum 25(OH) D levels and thus overall vitamin D status in adults. However, it is also evident that for some subpopulations, such as dark-skinned individuals and the elderly, food fortification is not sufficient to maintain a healthy vitamin D status (70). Also, due to an overall decline in milk consumption (70), the main vitamin D fortified food product, the current offering of foods rich in vitamin D is not sufficient for the bulk of the U.S. population. Recently, vitamin D added to orange juice has been shown to be just as effective at maintaining vitamin D status as oral supplements (71). The introduction of calcium and vitamin D fortified orange juice may help to alleviate some of this decline, but the relatively few food sources of vitamin D does not provide for a varied offering in

the diet. The current recommendation for adults is to obtain 600 IU/day to maintain adequate levels of vitamin D (28).

The relative lack of diversity in dietary sources of vitamin D is one of the major shortcomings in availability of vitamin D. Individuals who are lactose intolerant, vegetarian or vegan, or those who do not wish to introduce additional sources of fat into the diet are left with even fewer choices of foods to maintain vitamin D intake. Therefore, additional low-fat, vegetarian sources of vitamin D are needed.

1.6 UV Light

1.6.1 Types of UV Light

Ultraviolet light encompasses the 100-400 nm range of the electromagnetic spectrum and can be subdivided into three main regions. These include UV-A (315-400 nm), UV-B (280-315 nm) and UV-C (200-280 nm) (72). The vacuum UV range encompasses light from 100-200 nm and can only be transmitted in a vacuum.

While sunlight is a natural source of UV irradiation, there are several lamp technologies that allow for artificial generation of UV light. The most common and conventional are mercury vapor lamps. These lamps consist of a silica glass tube in which mercury and an inert filler gas (typically argon) is sealed inside with both ends containing an electrode. The electrodes act to apply a voltage across the gas, which excites the atoms, thus producing UV light. A schematic of a conventional low-pressure mercury gas discharge lamp is given in **Figure 1.5**. These glass lamps can be coated in order to achieve various wavelength emissions.

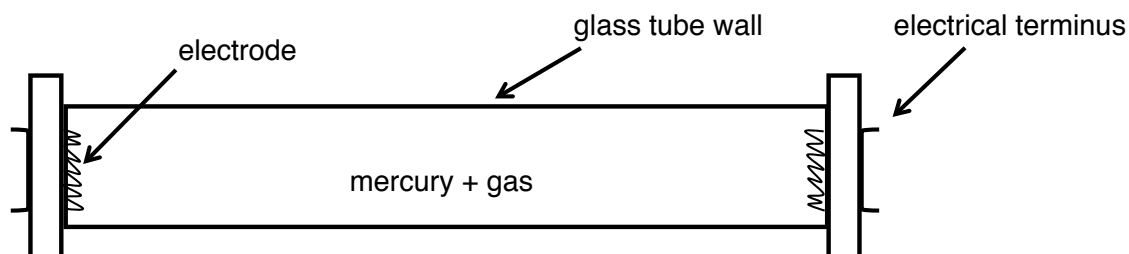


Figure 1.5. Schematic of conventional mercury gas discharge UV lamp (72).

Pulsed UV light is a technology that uses a broad spectrum (100-1100 nm, which encompasses the UV and visible wavelengths) xenon lamp, along with high intensity pulses, delivering energy at a high peak power in a short amount of time (72). Compared to continuous UV light systems, the time for the same amount of UV light irradiation is much shorter. These lamps are also filled with xenon gas as opposed to argon. The relative irradiance across the 100-800 nm spectrum of a typical pulsed UV lamp is shown in **Figure 1.6**. A representative batch pulsed UV system is shown in **Figure 1.7**, which consists of a stainless steel chamber with an access door for sample placement upon adjustable shelves. The broadband, pulsed UV lamp is horizontally situated above the chamber separated by a quartz window.

1.6.2 Applications of UV Light

In food systems and in laboratory settings the main use of UV light is for antimicrobial or germicidal purposes. UV light acts upon the DNA of the microorganism to prevent reproduction. The main mechanism of action is the creation of pyrimidine dimers in the DNA structure, in which adjacent thymine molecules bond, preventing DNA replication and thus microbial reproduction. Studies comparing the different regions of pulsed UV light have found that the UV-C region (200-280nm) is the most effective region. The specific wavelength of 254 nm is generally considered the most effective for microbial inactivation. UV-C for that reason is the most commonly used sub region for UV inactivation of microorganisms. Other factors including heat generation have been linked to the effectiveness of these methods in microbial inactivation, however these are confounding variables and are not part of the mechanism for the UV mediated effects.

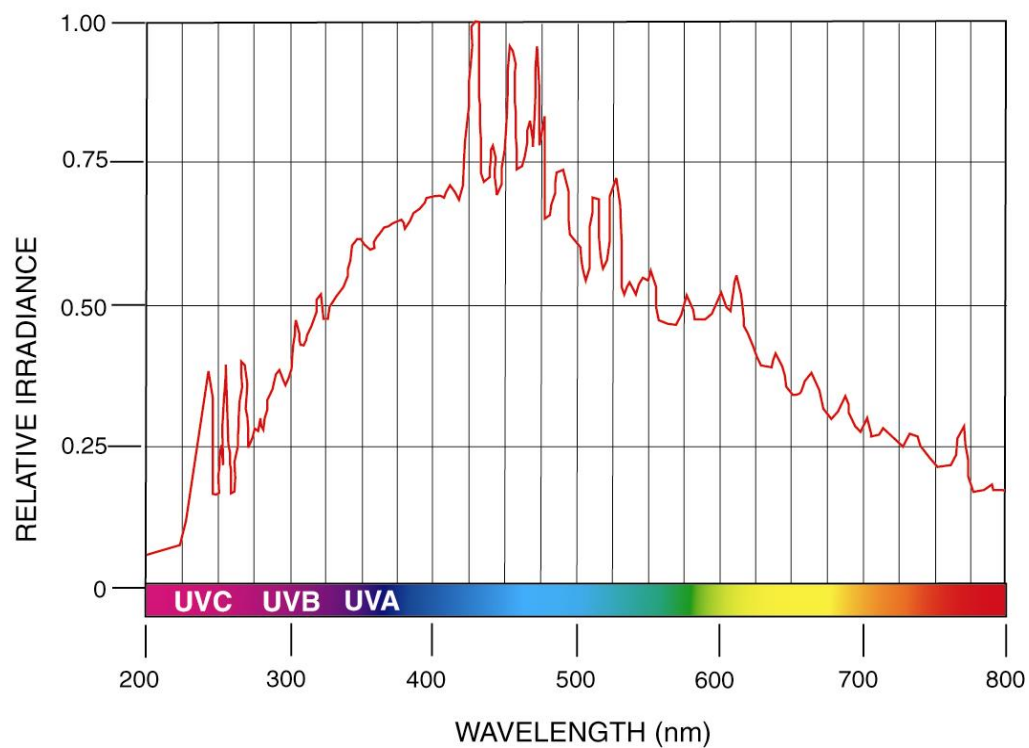


Figure 1.6. UV spectrum of a representative pulsed UV lamp (courtesy: Xenon Corporation)



Figure 1.7. Pulsed UV light batch treatment system

Ultraviolet light in solid food surface decontamination has been shown to be effective in a wide range of foods including shell eggs (73–75), fruits (76,77), and chicken (78). UV light treatment of liquid foods has also been shown to be an effective means to decrease microbial populations, such as in apple juice and cider (79–81).

In pulsed UV antimicrobial processes, it has been demonstrated that three mechanisms play a role in microbial inactivation (82). First are the photochemical effects or the formation of thymine dimers, second are photothermal effects or localized superheating at long exposures, and third are the photophysical effects or structural damages to the microbial cell. Farrell et al. determined that pulsed UV irradiation is effective in reducing levels of both gram-positive and gram-negative microorganisms. Sharma and Demirci demonstrated that *E. coli* O157:H7 inoculated on alfalfa seeds can be reduced by up to 8 log CFU/g, with effects of distance from the lamp, thickness of the sample and treatment time being contributing factors towards total reduction. Krishnamurthy et al. demonstrated that after 5 seconds of pulsed UV irradiation populations of *Staphylococcus aureus* could be reduced by 8 log CFU/mL. Chikthimmah and Beelman demonstrated that overall yeast and mold as well as aerobic bacterial counts on fresh mushrooms could be reduced by over 1 log CFU/g with 30 seconds of exposure to pulsed UV at a distance of 8 cm.

In addition to antimicrobial effects, UV irradiation can also have effects on components of nutritional value. It is known that UV light can have the potential to degrade other vitamins including vitamins A, B₂, B₆, B₁₂, C, E, K, carotene and folic acid which are all light sensitive (72). The most beneficial application of UV irradiation for human health is the ability to convert 7-DHC to vitamin D₃ in human skin or ergosterol to vitamin D₂ in fungi (yeast and mushrooms).

1.7 Vitamin D and Mushrooms

1.7.1 Vitamin D₂ In Mushrooms- Naturally Occurring

Vitamin D₂ has been observed to occur naturally in some wild and cultivated edible mushroom species. A level of approximately 11.9 µg/100g fresh weight (fw) vitamin D₂ would be equivalent to 100% of the Daily Value (DV) in one serving of fresh mushrooms (84 g) and a level of about 1.49 µg/g dry weight (dw) would be 100% of the DV. (These levels were calculated based on 10 µg being equivalent to 400 IU, which is the 100% DV and an average 92% moisture for mushrooms. Every 84 g serving of mushrooms would contain approximately 6.72 g of dry matter.)

Vitamin D levels in cultivated *Agaricus bisporus* (common white button mushrooms) have been shown to be less than 0.02 µg/100g fw (83), less than 1 µg/100g fw (84), 0.21 µg/100g fw (85), 2.2 µg/g dw (5), and 0.06 µg/g dw (86).

Several edible wild species have been discovered to have natural levels of vitamin D₂, including *Cantharellus tubaeformis* (29.82 µg/100g fw) and *Boletus edulis* (58.7 µg/100g fw) (85). Shiitake mushrooms have been shown to have virtually no naturally occurring vitamin D (87).

A retail compositional study of various mushroom species found that of the nine mushroom varieties tested, only maitake (28.1 µg/100g fw), chanterelle (5.30 µg/100g fw) and morel (5.15 µg/100g fw) contained vitamin D levels greater than 1 µg/100g fw (88).

1.7.2 Vitamin D₂ In Mushrooms- UV Light Treatment

Several studies have examined the effects of ultraviolet irradiation on the generation of vitamin D₂ in cultivated *A. bisporus*. The substrate ergosterol is present in all mushroom tissues in shiitake mushrooms, albeit in differing concentrations, being most concentrated in gill tissue followed by cap outer layer and stalk in shiitakes (87). However, this relationship has not been fully studied in button mushrooms.

Studies have been conducted measuring the effects of all three subtypes of UV (UV-A, UV-B and UV-C) on vitamin D₂ generation in *A. bisporus*. Using a UV-A lamp, Jasinghe and Perera reported that after 2 hours of exposure (25.2 KJ/m²), mushrooms had a level of 12.48 µg/g dw vitamin D₂ (87), while Teichmann et al. only showed a level of 0.15 µg/g dw after the same length of exposure (379.1 J/cm²) (84).

Using a UV-B lamp, Mau et al. showed that after 2 hours of exposure (0.986 J/cm²), vitamin D₂ content was 12.48 µg/g dw (a 567% increase compared to the control) (5) while Jasinghe et al. reported 32.24 µg/g dw vitamin D₂ after 1 hour of exposure on each side of the mushroom (35.3 KJ/m² total) (89). A pre-harvest exposure study by Feeney showed a level of 8.8 µg/g dw vitamin D₂ after 6 hours exposure on the mushroom beds (irradiation dose not given, but lamps were 97-90 cm away from the mushrooms, indicating a possible lower dose of irradiation than other studies) (86).

Using a UV-C lamp, Mau et al. showed a level of 7.30 µg/g dw vitamin D₂ after two hours exposure (1.471 J/cm²) (5) while Teichmann et al. found a level of 10.14 µg/g dw after the same length of exposure (379.1 J/cm²) (84). Feeney showed a level of 12.9 µg/g dw after 15 minutes of exposure (dose not given) (86).

A comparative study between UV-B and sunlight treatment of white button mushrooms was conducted by Simon et al. (4). The authors determined that there was no significant difference in vitamin D₂ content using either treatment when with the UV-B treated mushrooms receiving a dose of 1.06 J/cm². The authors were not able to measure dose for the sunlight treated mushrooms in the UV-B range but were able to record of dose of 24.0 J/cm² in the UV-A range. Sunlight, however, consists of all regions of the UV spectrum so wavelengths were not controlled.

A comparative study was completed by Jasinghe et al. (90) following 1 hour exposure to each subtype of UV light the levels of vitamin D₂ in *A. bisporus* were 22.9, 56.5 and 34.4 µg/g dw for UV-A, B and C, respectively. The final delivered dose for each treatment was not normalized, however. The UV-B treated mushrooms received the most total amount of ultraviolet energy (35.3 KJ/m²) followed by UV-A (25.2 KJ/m²) and UV-C (23.0 KJ/m²).

This should also be a closely considered factor when comparing any of the aforementioned studies.

1.7.3 UV Treatment Parameters

The manner in which mushrooms have been treated with UV light differs amongst studies not only by UV type (UV-A, UV-B, UV-C) and duration (dose) but also by several other factors. These include origin and cultivar of the mushroom, time after harvest, orientation of the mushrooms to the light source, sliced or whole preparations, temperature, moisture content, form treated (fresh or dried) and others.

1.7.3.1 Mushroom Origin and Time After Harvest

The timing of post-harvest UV treatment of mushrooms is of interest due to the rapid nature of mushroom spoilage. The literature does not present a complete picture of the effects mushroom origin or time after harvest has on ergosterol content or on the conversion of ergosterol to vitamin D₂. Shao et al. measured ergosterol content in different stages of maturity based on cap size and saw no difference in white button mushrooms but a significant decrease in ergosterol levels as maturity increased in brown button mushrooms (91).

From a commercial standpoint, mushrooms would most likely be exposed to UV on the day of harvest so time after harvest would likely not be an issue. However, most studies on vitamin D₂ enrichment of mushrooms relied on obtaining mushrooms from local vendors instead of the producers on the day of harvest so it is unknown if vitamin D₂ content would be any different on mushrooms treated directly following harvest. Differences in distribution channels across nations present additional ambiguity on how long after harvest the mushrooms in these studies were irradiated. Studies have reported the source of their mushrooms as coming from markets (92), supermarkets (93), and grocery stores (86). Some studies obtained mushrooms through their own cultivation (91) or directly from producers (5) but did not mention if the mushrooms were obtained on the day of harvest or of the amount of time that elapsed before the mushrooms were treated.

Only one study has irradiated mushrooms on the growing beds pre-harvest using UV-B lamps (5). Although other mushrooms in the same study were treated post-harvest, no comparison between the vitamin D content can be made because the post-harvest mushrooms were treated with UV-C under different conditions. Roberts et al. have completed the only work that looks at the effects of treating mushrooms under the same conditions at different days after harvest (94). They obtained mushrooms from a producer and treated on 1 and 4 days post-harvest and found no significant difference between the two days in terms of vitamin D₂ produced. It would still be of benefit to evaluate the difference between mushrooms treated directly following harvest and consecutive days after harvest to fully investigate the effects of senescence on vitamin D₂ production. Additional study is also needed to evaluate the ergosterol content from harvest throughout the entire shelf-life.

1.7.3.2 Temperature and Moisture Content

The conversion of ergosterol to vitamin D₂ is, like any chemical reaction, influenced by various factors. Two important factors that have been studied in detail are the effects of moisture content and temperature. Jasinghe et al. have hypothesized that moisture content would effect the conversion of ergosterol to vitamin D₂ by having a dilution effect on the concentration of ergosterol at higher moisture content and thus decreasing the rate of conversion and that drier mushrooms will have an increased surface area (13). The data thus far seems to suggest a more complicated relationship.

Perera et al. (93) first studied the effect of moisture content on the conversion by adjusting mushrooms to varying levels of moisture in desiccators and subjecting them to a standard treatment of UV-B at 20 cm for 2 hours (dose not reported). One confounding variable in this study is that the mushrooms at lower moisture content would also be older (and thus have senesced more than the higher moisture content mushrooms) since they were left in the desiccator for a number of days until they reached the desired moisture content. The vitamin D content was observed to increase with moisture content from about 30% moisture to 80% and then decline above 80% moisture. Jasinghe and

Perera (87) observed similar results with an optimum moisture content of between 75% and 80% leading to the highest vitamin D₂ content.

Jasinghe and Perera (87) also studied the effect of irradiation temperature on the production of vitamin D₂ and found that 35°C was the optimum temperature leading to the highest amount of D₂. In a follow up study on the kinetics of the conversion, Jasinghe et al. (13) completed a 2X2 factorial design looking at temperature and moisture content and the mushrooms treated at a higher moisture content (80%) resulted in higher vitamin D content than in the lower moisture content mushrooms (60%) . This study had some further limitations that will be discussed in the section on kinetics.

1.7.3.3 Fresh vs. Dried Mushrooms

The work discussed in the previous section established that moisture content plays a role in the conversion of ergosterol to vitamin D₂. Based on this, the form in which mushrooms are treated is also important. Mushrooms are often consumed fresh, but a considerable market exists for dried and powdered mushrooms, which would inherently contain low moisture levels. It is therefore of interest to understand the rate of conversion in both forms.

Choi et al. demonstrated that mushroom powders could be treated in a petri dish and the vitamin D₂ levels could be increased to about 1500 µg/100 g dw after 30 minutes of UV treatment at 254nm (dose not reported) (92). Teichmann et al. treated dried *Cantharellus tubaeformis* with UV-C and UV-A and found a considerable increase in vitamin D₂ using UV-C irradiation but not with UV-A (84). However, no report has compared the vitamin D₂ production from the same lot of mushrooms treated both fresh and dried. Therefore, more work is needed to understand which would be the most efficient form in which to treat mushrooms to obtain maximum or desired levels of vitamin D₂. The form in which a mushroom is treated also depends on the application in which the mushroom would be used.

1.7.3.4 Ergosterol Content

The distribution of ergosterol in different tissues has been suggested as a factor influencing the degree of conversion to vitamin D₂. It is hypothesized that if ergosterol is more concentrated in the gill tissue of a certain mushroom species then slicing of the mushroom and subsequent UV irradiation would yield higher concentrations of vitamin D₂ than if the same mushroom was treated in its whole form. However, this could also be due to the increased surface area available for UV exposure as a result of slicing. The tissues of a button mushroom are shown in **Figure 1.8**.

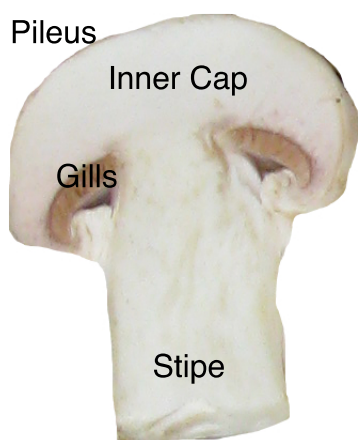


Figure 1.8. Cross sectional view of a white button mushroom

Few studies have investigated the distribution of ergosterol in mushrooms on a quantitative basis but there is a clear picture of the distribution of ergosterol in shiitakes. Choi et al. (92) showed that the ergosterol content in the pileus of shiitakes was higher (139.3 mg/100g dw) than in the stipe (17.3 mg/100g dw) although it is unclear if by pileus they meant the entire cap including the gills as there was no data for the ergosterol content of the gills alone. Jasinghe et al. (95) is one of the only studies that separately measured the ergosterol content of the gills, pileus and stipe. They found that the gills contained the most ergosterol at 10.6 mg/g dw followed by the pileus and stalk with 5.34 and 2.97 mg/g dw respectively.

The ergosterol levels in other mushroom species have not been studied as well. Jasinghe and Perera (87) illustrated the most complete picture of total ergosterol levels

in different mushroom species and showed that the content varies to a great degree and does not correlate to the total amount vitamin D₂ produced given the same amount of irradiation. This is most likely due to morphological differences of the different mushrooms species and whether the tissues containing the highest amounts of ergosterol were orientated towards the UV source.

Among the other mushroom species *Agaricus bisporus* is of most interest in the United States because it is by far the most widely consumed type of cultivated mushroom. The ergosterol content of button mushrooms has not been studied as greatly as shiitakes. Choi et al. (92) found that the ergosterol level in brown button mushrooms was almost twice as high compared to white buttons; however, the vitamin D₂ content after the same UV treatment was lower in brown buttons. In a recent study, the distribution ergosterol in button mushrooms was measured, however the authors did not segregate gill and pileus (cap) tissues (91) and the data gave no clear relationship of which tissue contained more ergosterol.

The level of ergosterol after UV treatment is of interest to aid in understanding the potential vitamin D levels that could be achieved. It would also help to clarify the possibility of the production of excess vitamin D because of the abundance of ergosterol present in mushrooms. Mau et al. quantified the loss of ergosterol with UVC and UVB treatment in several mushroom species (5). Overall, the authors observed greater losses in ergosterol than D₂ produced but large amounts of ergosterol remained unconverted. This phenomenon could be attributed to the creation of other products such as pre-vitamin D, tachysterol and lumisterol.

The total amount of ergosterol present in a mushroom is most likely not the key issue in understanding the conversion of ergosterol to vitamin D₂. The total amount of ergosterol that is exposed to UV light is likely of greater importance. Because of the low penetration of UV in mushroom tissues, the majority of ergosterol present in a mushroom is not available to take part in the reaction. This may be supported by the research that has previously been completed by examining the interplay between mushroom orientation and the effects of slicing when compared to whole mushroom treatment.

1.7.3.5 Orientation of Mushroom Tissues to UV Source

The orientation with respect to gill tissue of a mushroom to a UV light source has shown to be an important factor in determining the ultimate vitamin D₂ content achieved. This is also highly dependent on the species of mushroom being evaluated as morphology varies greatly from species to species. The slicing of mushrooms before UV treatment is of consequence in certain mushroom species such as immature *Agaricus spp.* due to the morphological nature of the gill tissue being internalized within the cap and not exposed. As seen in some mushroom species, such as shiitakes, the ergosterol content of the gill tissues is often greater than in the cap or stipe tissues although data for all mushroom species is not as clear.

The orientation of mushrooms to the UV lamp has varied greatly between studies. Mau et al. treated whole button mushrooms with the veil intact and shiitake mushrooms with 80% of the gills exposed (5), while several studies have reported irradiating each side of the shiitake, either individually or both sides of the same mushroom. As a result, observed vitamin D₂ content in UV-treated mushrooms varies greatly across studies.

Of all the studies on vitamin D₂ in mushrooms, the relationship between the orientation to the lamp, ergosterol content and resulting vitamin D₂ content has been most well studied in shiitake mushrooms. Lee et al. (96) irradiated shiitakes with the cap facing the UV source and also with the gills facing the UV source and found that there was significantly more D₂ produced in the shiitakes irradiated with their gills facing the lamp (doses ranged from 2.5 to 10 J/cm²). Jasinghe et al. (95) observed that when irradiated for 2 hours (25.2 kJ/m²) with the gills facing the UV-A source shiitakes produced 22.8 µg/g dw vitamin D₂ but only 5.16 µg/g dw when irradiated with the caps facing the lamp after 2 hours.

Jasinghe et al. (90) went a step further and irradiated shiitakes in the following manner: 1 hour each side facing, 2 hours each side facing the lamp and 2 hours gills facing the lamp (25.2 kJ/m² dose for all treatments). The authors also conducted a dose response study, irradiating each side individually as well as both sides. In the dose response study, the lowest amount of vitamin D₂ produced were the mushrooms irradiated on only

the cap side and the highest were the mushrooms irradiated on both sides. Shiitakes irradiated for 2 hours on only the gill side contained 24.0 µg/g dw vitamin D₂ while the shiitakes that were irradiated for 2 hours on each side only contained marginally higher vitamin D₂ (38.5 µg/g dw) than shiitakes irradiated for 1 hour on each side (36.1 µg/g dw). It is not clear whether the vitamin D content when treated on only the gill side and only the cap side would add to 38.5 µg/g dw, as the authors did not irradiate only the cap side. This is consistent with the results of the dose response experiment, which saw the vitamin D₂ content level off after 1 hour of treatment because after 1 hour of irradiation vitamin D₂ content reaches a peak level.

The relationship between orientation to the lamp, ergosterol content and vitamin D₂ content is not as well understood in button mushrooms. Jasinghe et al. (87) did not expose the gill tissue of button mushrooms the UV source and saw the lowest overall vitamin D₂ content of all the mushroom species they tested. However, in the same study the authors also showed that button mushrooms had by far the highest ergosterol content. On the other hand, it was found that oyster mushrooms yielded the highest vitamin D₂ content, even though they had one of the lowest ergosterol levels. These effects are likely the cause of the morphological differences between species and the internalization of the tissues containing the highest ergosterol content in a closed cap button mushroom. Jasinghe et al. (13,90) later irradiated both sides of button mushrooms but did not expose the gill tissue in the process and, again, button mushrooms were found to produce the lowest vitamin D₂ content of all species tested.

Roberts et al. (94) exposed button mushrooms to UV light with the cap side up. They indicated that this would be a worst-case scenario, i.e. producing the lowest vitamin D₂ content. However, the idea of worst-case scenario depends on what outcome would be less desirable. In regards to consumer safety, it could also be said that the worst-case scenario would be the highest amount of vitamin D₂ formed, which may result in a product with levels that would be considered too high for consumption. Koyyalamudi et al. (97) also treated button mushrooms button side up (facing the lamp).

Ko et al. (98) investigated the interplay between tissues exposed and vitamin D₂ content. The authors dissected each tissue component (pileus, middle cap and gills) of white

button mushrooms and exposed each to the same level of UV irradiation (10 and 20 kJ/m²). The authors found that the vitamin D₂ content of the tissues increased from the pileus, followed by the middle cap and finally the gills (36.7, 68.6 and 106.4 µg/g dw respectively). This supports the theory that ergosterol is highest in the gill tissue in button mushrooms, although no study has directly measured the ergosterol content of the gills. The authors also treated sliced mushrooms, but there was no direct comparison made to whole buttons.

1.7.4 Ergosterol Levels as Result of UV Irradiation

There have been few studies that have determined the ergosterol content of mushrooms in addition to the vitamin D₂ content following UV treatment. It would be expected that ergosterol content would decrease by approximately the same magnitude that vitamin D₂ content would increase as well as any other additional products that are formed. Several studies have shown a decrease in ergosterol content from control levels following exposure to UV. Mau et al. (5) showed a decrease of ergosterol to ca. 12% of initial levels after 2 hours (0.986 J/cm²) of UV-B treatment in *Agaricus bisporus*. Choi et al. (92) reported a decrease in ergosterol content of shiitake mushrooms using UV at 254 nm and solar irradiation (dose not reported). Teichmann et al. observed a decrease in ergosterol in *Chantarellus tubaeformis* from an initial level of 0.83 mg/g dw to 0.57 mg/g dw with 2 hours of UV-C irradiation and 0.54 mg/g dw with 2 hours of UV-A irradiation (84). The authors also reported a slight decrease of ergosterol in *A. bisporus* from 4.7 to 4.5 mg/g dw after 2 hours irradiation with both UV-C and UV-A (379.1 J/cm² for both treatments).

Two studies have also shown an increase in ergosterol levels with UV treatment. Mau et al. observed an overall increase of ergosterol from an initial level 50.78 µg/g dw in *Agaricus bitorquis* (5). Levels initially decreased to 43.53 µg/g dw after 0.5 hours of UV-C irradiation but then increased to 78.57 µg/g dw after 1 hour (0.606 J/cm²) and subsequently to 254.88 µg/g dw after 2 hours (1.471 J/cm²). A similar effect was seen after UV-B irradiation of *Volvariella volvacea* with an initial ergosterol level of 185.89 µg/g dw which fell to 96.92 µg/g dw after 0.5 hours (0.247 J/cm²), but rose to 233.56 and 215.81 µg/g dw with 1 and 2 hours irradiation respectively (0.493 and 0.986 J/cm²

respectively). They also showed a slight increase after 0.5 hours UV-B irradiation in shiitake mushrooms but after further treatment ergosterol fell below control levels. Perera et al. (93) found in shiitakes irradiated for 2 hours with UV-B irradiation (dose not reported), ergosterol increased with increasing moisture content from 60 to 80% moisture but saw a decrease in ergosterol in mushrooms with moisture levels above 90%. The reason behind any observed increases in ergosterol has been purely speculative to this point and could either be due to metabolic response in the living tissue or simply an error in methodology. Further research is needed in these cases to clarify the exact effect.

1.7.5 Kinetics of the Conversion of Ergosterol To Vitamin D₂ in Mushrooms

Several studies have shown a linear increase in vitamin D₂ content in mushrooms as a function of increasing exposure to UV irradiation to a certain point, and subsequent leveling off to an apparent steady state (90,92). Studies on ergosterol in a model system also have shown this phenomenon as well as with vitamin D₃. Jasinghe et al. (13) also studied the kinetics of the conversion of ergosterol in fresh mushrooms. This study examined the effects of irradiation time, temperature and moisture content of the mushrooms. All of the figures indicate a linear relationship between treatment time and vitamin D₂ content; as treatment time increases so does vitamin D₂ content. The authors then state that this is evidence of zero order kinetics. However, based on the available literature, including previous work by the authors, this was not shown with extended UV treatment of mushrooms. The equations generated in this study (13) are based on the assumption of zero order kinetics. The authors did not generate an equation that takes irradiation time and moisture and temperature all into account. There are separate equations for vitamin D₂ production in different mushrooms at 30°C and 80% moisture and then one equation based on 2X2 factorial design for temperature and moisture at a single irradiation time of 2 hours, there are several problems with this. First, a 2X2 factorial may not be appropriate because it would imply a linear relationship. Previous studies by the authors indicate the effect of temperature and moisture content is in not linear. Secondly, by using only one dose of irradiation, the equation is only true for that one dose. The use of additional treatment levels in the experiments may be of benefit to determine interactions between all factors. Then, all factors could be combined into one

equation. The authors also used UV-A light, which had been shown in previous studies (including their own) to be the least effective wavelength band in the generation of vitamin D₂. The conversion of ergosterol to vitamin D₂ in mushrooms is complex and based on many variables and this study provides a good starting point for future research.

The irradiation of ergosterol does not only produce vitamin D₂. The other main products include pre-vitamin D₂, tachysterol and lumisterol, which are reversible. Extended UV irradiation is also known to generate irreversible products such as the class of compounds known as toxisterols and also suprasterols. Therefore, a more thoughtful, thorough examination of the kinetics of the conversion of ergosterol to vitamin D₂ in mushrooms is needed that includes all of these parameters.

1.8 Stability of Vitamin D

There is limited literature on the heat stability of vitamin D and even less when it is in mushrooms. Mushrooms are cooked in a variety of ways by consumers (e.g., sautéing, frying) and can be thermally processed at a commercial level (e.g., canning). These processes involve thermal treatment, which is a function of time and temperature, and the vitamin D₂ content may also be impacted by the matrix the mushrooms are processed in. For this reason research would have to be done on an individual process basis.

Mattila et al. (99) investigated the stability of vitamin D in mushrooms when pan fried without fat at an undisclosed temperature for 5 min. The retention of vitamin D was 87% for *C. cibarius* mushrooms and 99% for *C. tubaeformis* mushrooms. The other foods tested in the paper all showed vitamin D retention above 90%. Another paper (100) examined the stability of vitamins during heat treatment. The few sources cited in the literature showed that vitamin D losses were generally not more than 20%.

Havinga (10), examined the conversion products of vitamin D at various temperatures. At a temperature range of 100-180°C it was observed that conversion of ergosterol to vitamin D still takes place, but ring closures occur forming additional products. This could

be the same mechanism for vitamin D degradation under cooking conditions.

Based on the literature on both cooking and chemical structure, it appears that vitamin D is relatively stable during cooking. However, more research is needed to analyze practical situations and to determine what products are being formed as a result. It is unclear what products are formed as a result of thermal degradation of vitamin D₂ but the products outlined by Havinga at elevated temperatures in solution may give a starting point for identification of products formed at high temperatures in cooked mushrooms.

The shelf life stability of vitamin D₂ present in irradiated mushrooms is also of importance. Roberts et al. (94) stored white button mushrooms at 2.2°C for 4 days after UV-B treatment and determined that while there was an initial reduction in vitamin D₂ from the initial levels after 2 days, but there was no difference in vitamin D₂ from 2 days to 4 days post treatment. Levels remained above 150% DV through 4 days. Hu (101) showed similar results in UV-B treated white and brown button mushrooms, although some increases occurred after the initial decrease from day 0. These studies show that there may be some degradation of vitamin D₂ while in storage but it is unclear if the levels are consistent over an extended shelf life study. Further research is necessary to determine the retention of vitamin D₂ in mushrooms throughout the entire shelf life.

1.9 Bioavailability of Vitamin D from Mushrooms

The bioavailability of vitamin D₂ from UV irradiated and wild mushrooms has been studied in both animal models and in humans. Jasinghe et al. conducted a study in rats given a vitamin D deficient diet for one week followed by the same diet plus either control mushroom powder or mushroom powder enriched with 1 µg per day for 4 weeks (95). They found that in the control rats serum 25[OH]D levels were 6.06 nmol/L while the rats receiving the vitamin D₂ enriched mushrooms had levels of 129.42 nmol/L. The authors also found that femur bone density increased in the rats fed vitamin D₂ enriched mushrooms. Koyyalamudi et al. found that in rats fed 0, 50, 100, or 200 mg/kg body weight of vitamin D₂ enriched mushroom powder (17.6 µg/g dw) for three weeks, serum 25[OH]D levels increased from zero in the control rats to 3.13 nmol/L in the 50 mg/kg

group while also increasing in the two higher groups (though not significantly) (97). Lee et al. conducted a study of vitamin D₂ enriched mushroom powder with and without calcium fortification in mice (102). The authors found that mice fed vitamin D₂ and calcium enriched diets had significantly higher femur density and tibia thickness. Calvo et al. also found that in rats fed diets containing no vitamin D, 400 IU and 600 IU from irradiated mushrooms, rats had significantly higher 25[OH]D levels when on the vitamin D₂ enriched diets (118 and 159 ng/mL for 400 IU and 600 IU diets, respectively) than the control diet (4 ng/mL) (103).

In humans, vitamin D₂ enriched mushrooms have also been found to illicit a similar response as found in animal models. Outila et al. conducted a study on the bioavailability of vitamin D₂ from wild mushrooms (104). In that study, three groups (n=9 per group) receiving either 14 µg vitamin D₂ per day from wild mushrooms, a vitamin D₂ supplement or a placebo, serum 25[OH]D increased in both vitamin D₂ groups, but not in the placebo group over the course of the study. In a one man study, Ozzard et al. observed an increase in serum 25[OH]D of 129% (17 to 39 nmol/L) after 3 months of daily ingestion of mushrooms irradiated in the subject's home (105). Urbain et al. conducted a 5 week, prospective study on three groups; placebo (n=9), mushroom plus vitamin D₂ (n=8), and vitamin D₂ supplement (n=9) (106). The authors observed no difference in mushroom or supplement groups over the course of the study but did see an increase in serum 25[OH]D in both groups (3.9 and 4.7 nmol/L per week in the mushroom and supplement groups, respectively) and a decrease in the placebo group. The data generated from these studies indicates that vitamin D₂ produced in mushrooms is biologically active.

1.10 Potential Toxicity and Safety of Vitamin D in Mushrooms

As described earlier there is a risk of vitamin D toxicity with repeated ingestion of high doses of vitamin D. Therefore, it is unlikely that a one-time overdose of mushrooms with an excessive amount of vitamin D would cause serious health concerns. However, it is important to assure that vitamin D-enriched mushrooms do not contain excessive amounts to protect against accidental overdose.

To ensure a safe product is being released to the market, stringent processing parameters would have to be set to ensure a uniform product with a consistent reasonable level of vitamin D per serving. A reasonable level would be the new RDA of 600 IU in one serving or 84g of mushrooms. This level would be well below the daily levels listed previously that would be considered toxic. This can be achieved through processing validation and training as well as process monitoring and product testing. The level of vitamin D₂ has been shown to be controllable through the manipulation of irradiation dose based on distance from the lamp and time of exposure. If these two variables are controlled these mushrooms should be a consistent and safe product.

1.11 Browning and Quality Concerns In Mushrooms

Several of the studies on the generation of vitamin D₂ in fresh cultivated mushrooms have shown that after treatment, appearance of the mushrooms was significantly altered. Mushroom browning after UV treatment was seen by Mau et al. (5) and Jasinghe and Perera (87). Feeney looked at the browning effects following treatment in more depth (86). The study noted that after 15 minutes of UV treatment the L-value decreased from 93 in the control to 85 in the treated mushrooms (a L-value of 100 corresponds to white while a value of 0 corresponds to black). The color of mushrooms, especially white button mushrooms, is known to be an important quality factor to consumers, and thus brown color in white mushrooms may be unacceptable to consumers and negatively impact sales.

Tyrosinase in the mushroom tissue is responsible for the browning of white button mushrooms. The substrates are L-tyrosine and γ -L-glutaminyhydroxybenzene (GHB) (107), which form into catechols and then their corresponding quinones, which then spontaneously react to form melanins (108). Melanins are also the same type of pigment that colors human skin and are responsible for blocking sunlight and causing vitamin D deficiency in dark skinned individuals (109). Browning in mushrooms is typically mediated through cell disruption and subsequent mixing of substrates that results in the chemical reaction that forms polymers of melanin, giving the mushrooms a brown color. Tissue disruption usually occurs through bruising that occurs during handling of the mushroom or bacterial spoilage (*Pseudomonas spp.*).

Although the exact cause of browning of mushrooms as a result of conventional UV light treatment has never been investigated, it can be hypothesized it results from a similar tissue disruption mechanism. This could take place through several mechanisms. The UV irradiation itself is of high energy and thus could cause cell disruption through any number of possible photo-induced reactions. Another possible mechanism of tissue disruption could be from conformational change of the membrane components. Ergosterol is a membrane component. If the ergosterol conformation changes as a result of vitamin D formation, cell disruption may occur through membrane deformation thus allowing polyphenol oxidase to initiate browning.

1.12 Limitations of Previous Research on Vitamin D₂ Enriched Mushrooms

The previous research that has been completed using continuous UV systems has shown that increases in vitamin D₂ levels in mushrooms are possible but several limitations have also been documented. Factors such as band of UV (UV-A, UV-B, UV-C), origin of the mushrooms, time after harvest before treatment, temperature, moisture content, ergosterol levels, orientation of various tissues to the UV source have been shown to effect the final vitamin D₂ levels in UV irradiated mushrooms. Proper control of these parameters, for not only research purposes but also for processing concerns, is of major importance. In addition, the kinetics of this process, the stability of vitamin D₂ produced and effects on mushroom quality (such as browning) have not been adequately investigated.

Perhaps most importantly from a commercial standpoint, continuous UV systems have also been shown to take hours of treatment to obtain nutritionally significant levels of vitamin D₂. The long treatment times required by this technology, and the deleterious effect on quality attributes (i.e. browning) are obvious barriers to the mushroom industry adopting vitamin D enrichment by continuous UV. Pulsed UV systems on the other hand, could potentially ameliorate these issues.

2. Significance and Hypotheses

2.1 Significance

Vitamin D deficiency and insufficiency is a serious health problem that affects many people around the world. Deficiency and insufficiency can affect bone health and also may be linked to a variety of diseases. While the links to these diseases are not yet fully understood, as research progresses the need for increased levels of vitamin D will most likely be an important issue. Currently the recommended level for daily intake is 600 IU/day for most individuals (28). Others suggest that intake of up to 2000 IU/day may also be beneficial to increase or maintain levels of vitamin D in the blood (20). Several populations are at risk for vitamin D deficiency including infants who are exclusively breastfed, older individuals, individuals with limited sun exposure, persons with greater skin melanin content and people with fat malabsorption (28). For people in these populations, greater care must be taken to ensure proper intake of vitamin D.

The number of sources of vitamin D is limited to sun exposure, vitamin supplements and some fortified foods. Therefore, the creation of additional sources of vitamin D is justified. Mushrooms hold great potential as a source of vitamin D as seen in previous work, but a more rapid means of enrichment is needed. This process has been documented to take anywhere from minutes to hours to achieve mushrooms that have biologically significant levels of vitamin D₂. Vitamin D₂ from mushrooms has been shown to be bioavailable in humans (104–106).

The main goal of these studies is to investigate the effects of pulsed UV light treatment of mushrooms and mushroom products. This is important because a reliable and rapid methodology to increase vitamin D₂ content of mushrooms is needed in order to be commercially viable. The short exposure time of PUV is the main advantage of the use of this system over conventional continuous UV applications. Any further effects that would be of benefit such as improvements in product quality, shelf life, microbial load, etc. would be of interest and add to overall value.

2.2 Thesis objectives and Hypotheses

- I. Determine if PUV is an effective means to increase the vitamin D₂ content of fresh mushrooms and mushroom products.

Hypothesis: PUV is in essence the same technology as continuous UV with the major difference being PUV utilizes a high intensity broadband lamp that delivers UV irradiation in short pulses. Therefore, PUV irradiation will be an effective and rapid means of increasing the vitamin D₂ content of mushrooms and mushroom products.

- II. Determine the Dose/Response relationship between PUV irradiation and vitamin D₂ content of fresh mushrooms.

Hypothesis: PUV irradiation of mushrooms will result in an increasing vitamin D₂ content with increasing exposure time.

- III. Determine if the vitamin D₂ produced is stable under storage conditions typical of consumer refrigeration for the duration of the shelf life.

Hypothesis: Vitamin D₂ produced as a result of PUV irradiation in mushrooms will remain relatively stable throughout storage at refrigeration temperatures.

- IV. Determine the effect of a PUV treatment that would be used to increase vitamin D₂ levels of nutritional significance on the microbial load of fresh mushrooms.

Hypothesis: PUV treatment will have no effect on microbial load when used at a level necessary to increase vitamin D₂ content to a nutritionally significant level.

- V. Determine the effects on mushroom quality attributes after a PUV that would be used to increase vitamin D₂ levels of nutritional significance.

Hypothesis: Mushroom quality will not be changed with the use of PUV irradiation.

- VI. Determine if pre-vitamin D₂, tachysterol₂, and lumisterol₂ are also generated in addition to vitamin D₂ in fresh mushrooms irradiated with PUV and determine the relationship between the rate these compounds are formed if present.

Hypothesis: Following PUV treatment the photoproducts of ergosterol including previtamin D₂, vitamin D₂, tachysterol₂, and lumisterol₂ will be formed.

3. Pulsed UV Light Treatment as a Rapid Method for Increasing Vitamin D₂ Content of Mushroom Products

3.1 Abstract

A study was conducted to assess the effectiveness of a PUV system to increase the vitamin D₂ content of a variety of mushrooms species (*Agaricus bisporus*, white and brown button mushrooms; *Pleurotus ostreatus*, oyster, and *Lentinula edodes*, shiitake) as well as several mushroom powders (*Pleurotus eryngii*, king oyster and *Agaricus bisporus*) and spent growing substrates containing mycelial biomass. Sliced white button mushrooms showed an increase from an initial level of 0 µg/g dw to 5.11 µg/g dw (325% DV/84g serving) after just one pulse. The PUV system generated 3 pulses per second, achieving an exposure time of less than one second. Similar results were observed with brown button mushrooms, wherein vitamin D₂ levels increased to 4.43 µg/g dw (362% DV/serving) with one pulse. Oyster mushrooms and shiitake contained higher initial levels of vitamin D₂, 0.16 µg/g dw and 0.037 µg/g dw respectively, and the vitamin D₂ content increased to 7.95 µg/g dw and 5.71 µg/g dw respectively after one pulse. All mushrooms tested had increasing levels of vitamin D₂ with each additional pulse. The study also found that PUV was effective in increasing the vitamin D₂ content of dried mushroom powders. With 4 pulses the vitamin D₂ content of white button mushroom powder increased from near 0 to ca. 12 µg/g dw and increased with additional exposure of 16 total pulses to ca. 26 µg/g dw. Similar results were seen in king oyster mushroom powder with 10.4 µg/g dw and 23.0 µg/g dw vitamin D₂ with 4 and 16 pulses, respectively. Spent mushroom substrate containing mycelial biomass contained lower levels of vitamin D₂ (under 2 µg/g dw, with a maximum 16 pulses) when exposed to PUV. This study suggests that PUV is an effective and rapid means of increasing the vitamin D₂ content of mushrooms and mushroom products with exposure times on the order of seconds.

3.2 Introduction

Mushrooms present one of the few natural sources of vitamin D₂ available in the diet. Mushrooms are abundant in ergosterol (5,92,93), the substrate that is converted to

vitamin D₂. This process occurs through ring cleavage of ergosterol by exposure to UV light and subsequent thermal rearrangement to vitamin D₂ (10). This conversion can be mediated naturally in wild mushrooms by exposure to sunlight (4,6), or through exposure to commercial UV light (4,5,13,84,88,90,94,95,97,98). The commercial implications of this process are that a product, which has little to no vitamin D₂ content, can be treated so that the vitamin D₂ content is increased to a desired level. As previous research has shown, however, the use of conventional continuous UV light can take between 15 minutes (86) to 2 hours (5,13,84,87,90) to achieve an increase in vitamin D₂ content to an acceptable level (e.g. 100% of the DV in one serving of mushrooms).

Pulsed UV light is a technology that delivers high intensity pulses of broadband light in a short period of time (72). The result of this is that an equivalent dose of irradiation can be delivered with pulsed UV in seconds that would otherwise take minutes to hours to achieve with conventional continuous UV applications.

This study aims to determine the effectiveness of a pulsed UV-B type lamp treatment on increasing the vitamin D₂ levels in four commonly consumed mushroom varieties, *Agaricus bisporus* (White and Brown Button mushrooms), *Pleurotus ostreatus* (Oyster), and *Lentinula edodes* (Shiitake). In addition, air-dried mushroom powders of *Pleurotus eryngii* (King Oyster) and *Agaricus bisporus* were examined for the ability to produce vitamin D₂ in the same manner. Mycelial biomass grown on sterile grain substrate and commercial spent mushroom substrate containing some mycelial biomass were also treated and evaluated. The effect of pulsed UV treatment on ergothioneine and selenium content of mushrooms was also examined to determine the efficacy of producing a product that is a good source of all three nutrients. The results of this chapter were the basis for a U.S. provisional patent application (110).

3.3 Materials and Methods

3.3.1 Materials

Fresh mushrooms were obtained from Modern Mushroom Farm (Avondale, PA) and the Pennsylvania State University Mushroom Test and Demonstration Facility (MTDF). All mushrooms were grown using typical commercial practices. All mushrooms were protected from extraneous light exposure throughout the experiments and stored under refrigeration temperatures until use.

White button mushroom powders were obtained from air-dried *Agaricus bisporus* with and without selenium enrichment grown at the Penn State MTDF using the methods of Werner and Beelman (111). King oyster mushroom powder was obtained from Golden Gourmet Mushrooms (Oceanside, CA).

Spent mushroom substrates (Maitake and King Oyster) and King Oyster mycelial biomass grown on sterile oats were obtained from Golden Gourmet Mushrooms.

3.3.2 PUV Irradiation

A Steripulse®- XL 3000 (Xenon Corporation, Wilmington, MA) was used for pulsed UV light exposure. The system consisted of closed stainless steel chamber with a moveable shelf to adjust sample distance from the lamp. Above the chamber, a quartz window was situated 5.8 cm from the lamp, which was suspended horizontally along the length of the chamber. A B-type lamp was used which had UV-C wavelengths filtered out to protect against ozone formation. The system generated 505 Joules per pulse at 3 pulses per second. At 3.2 cm from the window the broadband energy was 0.873 J/cm^2 per pulse. Energy was measured using an Ophir Nova radiometer (Ophir-Spiricon, LLC, North Logan, UT) equipped with a pyroelectric head. The detector head was placed 3.2 cm from the quartz window and energy was averaged over three pulses.

3.3.2.1 PUV Irradiation of Fresh Mushrooms

Brown and white button mushrooms were sliced to expose gill tissue. They were randomly placed in 150g lots into polystyrene containers. Oyster and Shiitake mushrooms were divided into 150g lots and were arranged in the system so that there was a single layer of mushrooms. All samples were placed in the pulsed UV chamber at a distance of 3.2 cm from the quartz window.

Brown and white button mushrooms were exposed for 0, 1, 2, 3, and 4 pulses. All treatments were replicated three times. Oyster and Shiitake mushrooms were exposed for 0, 1, 2, and 3 pulses. All treatments were replicated twice. Differences in treatments were due to limited availability of the oyster and shiitake mushrooms.

3.3.2.2 PUV Irradiation of Mushroom Powders and Spent Mushroom Substrates

Mushroom powders from air-dried *Agaricus bisporus* with and without selenium enrichment were treated in 5g lots in uncovered Petri plates at a distance of 3.2 cm from the quartz window. King Oyster mushrooms were air-dried and treated at the same distance. The powders were treated with 0, 4, 8, and 16 pulses.

Spent mushroom substrates (Maitake and King Oyster) were treated either before or after air-drying. Dry samples were treated in 5g lots and wet samples (approximately 62% moisture) were treated in 20g lots. The Maitake substrate was treated at 0, 4, and 8 pulses. King Oyster substrates were treated at 0, 8 and 16 pulses.

Commercially dried King Oyster mycelial biomass grown on sterile organic oats were treated at 0, 4, 8, and 16 pulses before and after being ground into powder form.

The King Oyster mushroom powder was also evaluated for ergothioneine content. Ergothioneine content was determined by the method of Dubost et al. (112). In brief, one gram of freeze-dried mushroom powder was extracted using 20 mL of cold ethanolic extraction solution (10 mM diethiothreitol, 100 μ M betaine and 100 μ M 2-mercapto-1-methyl imidazole in 70% ethanol) by mixing in a small blender. A 1 % ethanolic solution

of SDS was added (4mL) and the sample was mixed and centrifuged to remove insoluble material. The supernatant (1 mL) was then evaporated to dryness under nitrogen and resuspended in 0.5 mL water. The filtered extract was then injected onto two C-18 columns and ergothioneine was analyzed by HPLC (isocratic mobile phase of 50 mM sodium phosphate in water, 3% acetonitrile, 0.1% triethylamine, flow rate of 1 mL/minute). Ergothioneine levels are reported as mg/g dry weight.

3.3.3 Vitamin D₂ Analysis

Mushroom samples were snap frozen (-80°C) directly following treatment and lyophilized. All other samples were air-dried. Following drying, freeze-dried mushrooms and mushroom products were homogenized into powders. The powders were sent to Medallion Labs (Minneapolis, MN) for analysis of vitamin D₂ content as described by Roberts et al. (94). Briefly, each mushroom sample was saponified with dihydrotachysterol (internal standard), ascorbic acid, and pyrogallol acid in an ethanol/KOH solution, as described previously (94). The samples were then extracted using heptane, dried, and reconstituted in cyclohexane and heptane (1:1). The solution underwent preparative HPLC cleanup and the fraction containing the internal standard and vitamin D₂ was collected, dried under nitrogen, reconstituted in 1:1 cyclohexane/heptane and quantitated by reverse-phase HPLC with mass spectrometry detection. Vitamin D₂ values are reported as µg/g dry weight (dw).

Vitamin D₂ values for powders and substrates are presented as µg/g dry weight (dw). Vitamin D₂ values of fresh mushrooms are also presented based on the % DV (400 IU) in a serving (84g) of fresh mushrooms.

3.3.4 Statistical Analysis

One-way ANOVA was performed to compare means of vitamin D₂ content across all treatments for a given mushroom product. Tukey's Test was utilized to determine significant difference between means. Statistical analysis was performed using SPSS software (IBM). ($\alpha = 0.05$)

3.4 Results and Discussion

3.4.1 PUV Irradiated Fresh Mushrooms

After exposure to increasing amounts of PUV light there was an increase in vitamin D₂ content of every mushroom product tested. With each additional pulse, the mushrooms were exposed to an additional dose of irradiation and thus more energy was available for vitamin D₂ synthesis from ergosterol.

Fresh, sliced white button mushrooms showed an increase from an initial vitamin D₂ level of 0 µg/g dw to 5.11 µg/g dw (325% DV/serving) after just one pulse (**Figure 3.1**) of PUV. After 4 pulses the level of vitamin D₂ increased to 13.4 µg/g dw. Similar results were seen in fresh sliced brown button mushrooms (**Figure 3.2**) where vitamin D₂ increased from an initial level of 0.06 µg/g dw at 0 pulses to 4.43 µg/g dw (362% DV/serving) after one pulse. The level increased to 11.1 µg/g dw after 4 pulses. Several studies have shown a similar increase of vitamin D₂ in whole button mushrooms with increasing UV dose, although levels varied among studies (5,13,84,94,97).

Using UV-B, Mau et al. saw an increase from an initial level of 2.2 µg/g dw to 5.74, 8.51 and 12.48 µg/g dw with 0.5, 1 and 2 hours of exposure (0.247, 0.493, 0.986 J/cm² respectively) (5). Roberts et al. utilized differing intensities of lamps (0.46, 0.75 and 1.0 mW/cm²) but normalized to total dose (0.5, 1.0 and 1.5 J/cm², respectively) to achieve decreasing treatment times [(18.1, 36.2 and 54.4 minutes), (11.1, 22.2 and 33.3 minutes), and (8.3, 16.7, and 25 minutes) with respect to intensity] and found no significant difference between intensities (94). However, the authors did observe a similar increase in vitamin D₂ levels with increasing dose [(3.75, 4.53 and 7.28 µg/g dw), (3.48, 5.58 and 7.20 µg/g dw), and (3.83, 6.2 and 7.43 µg/g dw) with respect to intensity]. Teichmann et al. observed similar results using UV-C irradiation with much higher doses (84.7, 189.5 and 379.1 J/cm²) at 0.5, 1 and 2 hours where they observed an increase in vitamin D₂ levels from an initial level of 0.07 to 5.32, 7.24 and 10.14 µg/g dw with respect to dose (84). The results of these studies indicate that PUV is much more rapid means of achieving similar vitamin D₂ content in button mushrooms with treatment times on the order of seconds, compared to minutes (94) or hours (5,84).

Only one study (98) used sliced buttons mushrooms. The authors found that when the mushrooms tissues were treated with the gill tissue facing the lamp, more vitamin D was produced (14.4 $\mu\text{g/g dw}$) than when treated with the cap facing the lamp (8.48 $\mu\text{g/g dw}$) with a 10 kJ/m^2 dose. This is evidence that more ergosterol is available to take part in the reaction when the gill tissue is exposed.

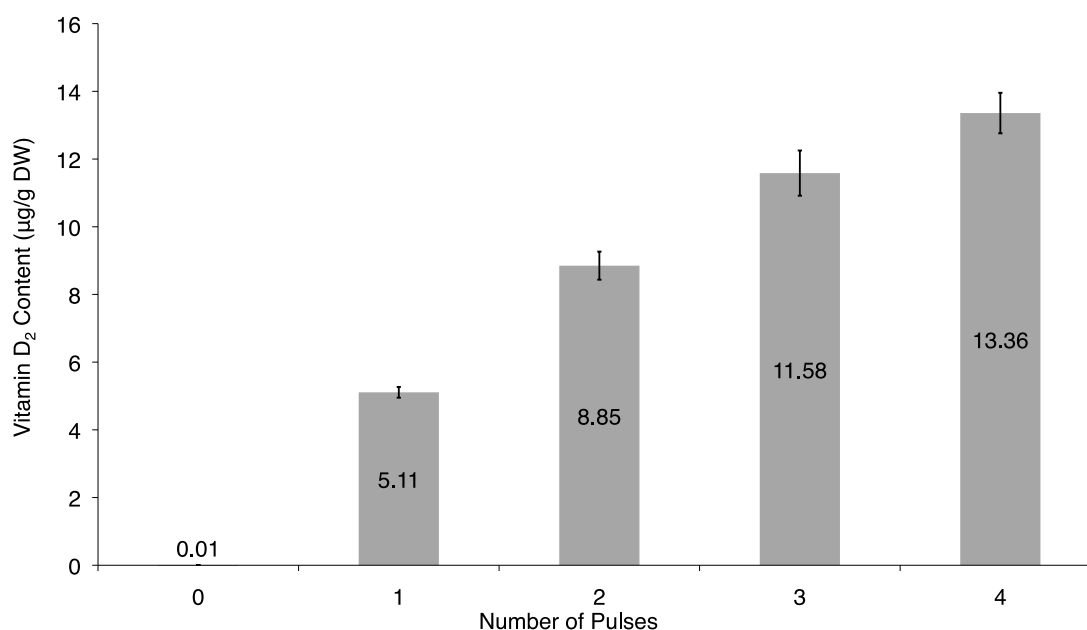


Figure 3.1. Vitamin D₂ content of white button mushrooms (*Agaricus bisporus*) after pulsed UV light exposure (B-type lamp). Error bars represent standard deviation of the three replications.

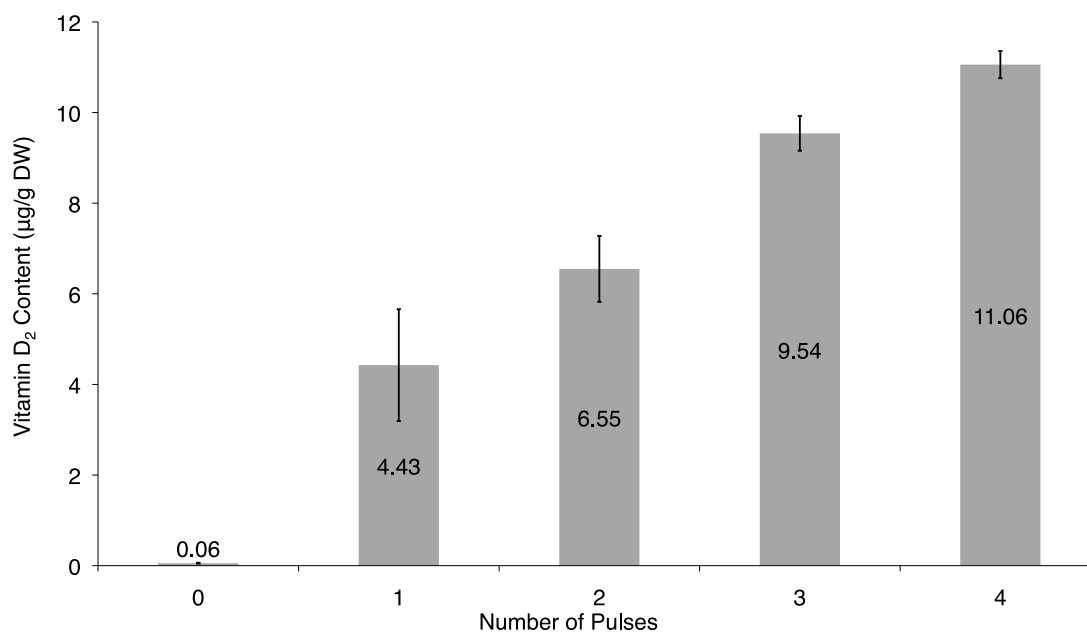


Figure 3.2. Vitamin D₂ content of brown button mushrooms (*Agaricus bisporus*) after pulsed UV light exposure (B-type lamp). Error bars represent standard deviation of the three replications.

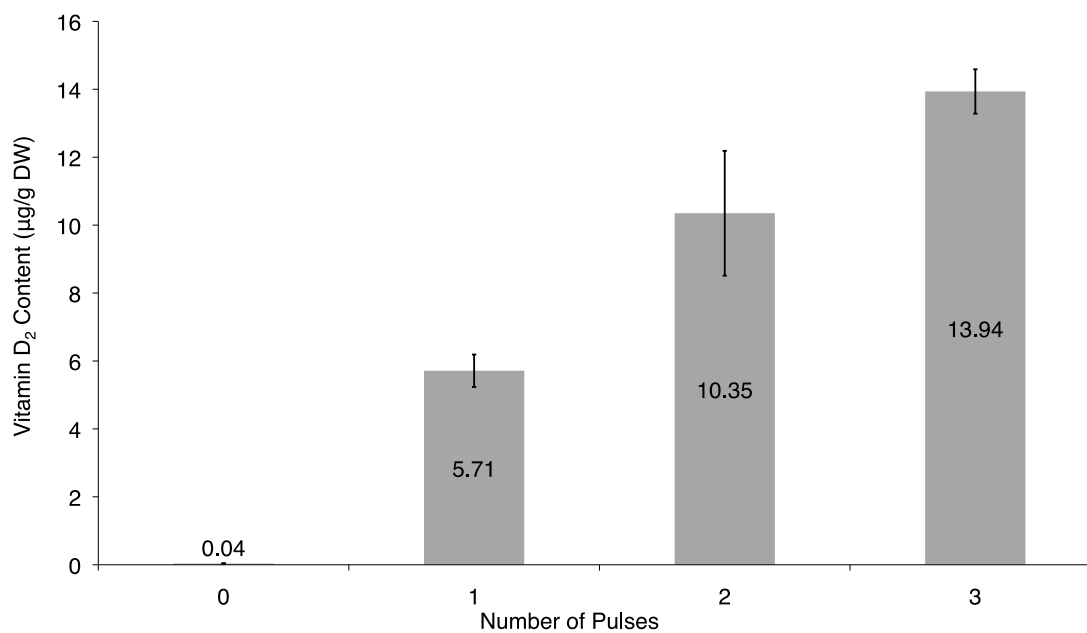


Figure 3.3. Vitamin D₂ content of shiitake mushrooms (*Lentinula edodes*) after pulsed UV light exposure (B-type lamp). Error bars represent standard deviation of the two replications.

After PUV treatment, fresh Shiitake mushrooms (**Figure 3.3**) showed an increase in vitamin D₂ content from an initial level of 0.037 µg/g dw at 0 pulses to 5.71 µg/g dw (490% DV/serving) after one pulse. The vitamin D₂ content after 3 pulses was 13.9 µg/g dw. A few studies have also previously demonstrated this increase using continuous UV sources in shiitake mushrooms (5,13,98). Mau et al. observed that with 0.5, 1 and 1.5 hours (0.247, 0.493, 0.986 J/cm² respectively) of UV-B irradiation vitamin D₂ increased from an initial level of 2.16 µg/g dw to 3.71, 4.69 and 6.58 µg/g dw, respectively (5). Ko et al. observed very high levels of vitamin D₂ when shitakes were treated with the gills facing the source. At doses of 25, 50 and 75 kJ/m², vitamin D₂ content increased to 13.8, 40.7 and 61.9 µg/g dw respectively (98). Jasinghe et al. observed a linear increase from 10 to 40 minutes of UV-A exposure (0.21 kJ/m²/min) from approximately 20 to 35 µg/g dw, yielding the equation $[D_2 = 11.97 + 0.643(t)]$, where t = time] (13).

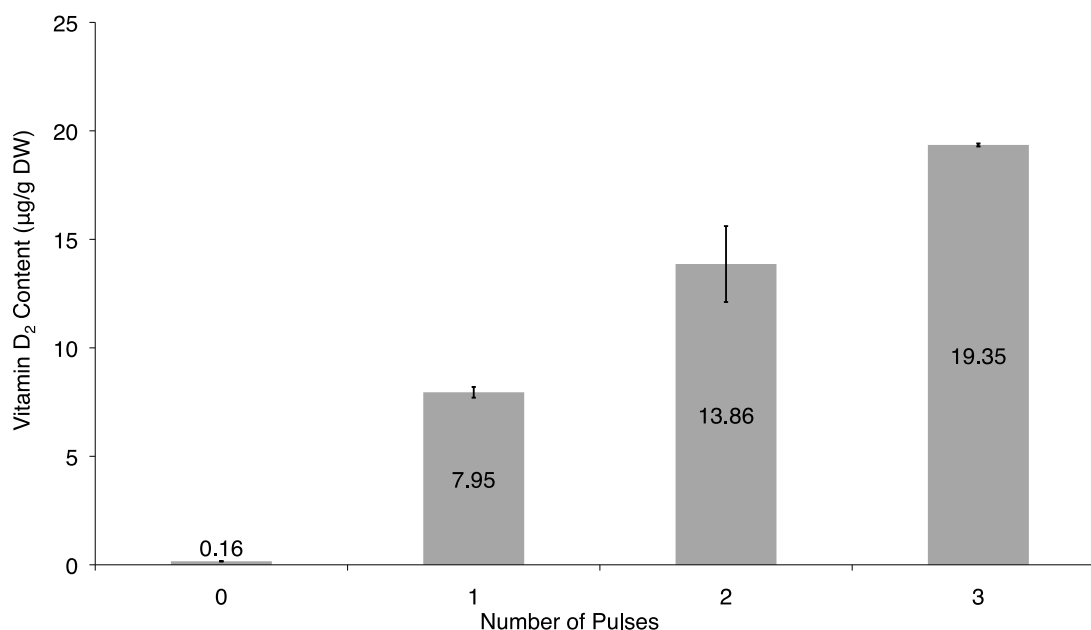


Figure 3.4. Vitamin D₂ content of oyster mushrooms (*Pleurotus ostreatus*) after pulsed UV light exposure (B-type lamp). Error bars represent standard deviation of the two replications.

Fresh Oyster mushrooms contained an initial level of vitamin D₂ of 0.16 µg/g dw at 0 pulses and increased to a level of 19.4 µg/g dw after 3 pulses (**Figure 3.4**). A few studies have previously shown an increase in vitamin D₂ content of UV treated oyster mushrooms (13,87,90). Compared to several other species of mushrooms in two

studies, oyster mushrooms were found to have the highest vitamin D₂ content when treated for 2 hours at different total doses. With a dose of 25.2 kJ/m², oyster mushrooms irradiated contained 45.1 µg/g dw (87). However, when treated on each side for 1 hour (total 2 hours) with UV-B with a total dose of 35.3 kJ/m², a vitamin D₂ content of 184 µg/g dw was achieved (90). These levels are much higher than those achieved in this study, however total dose is much lower in this study. The only other study in which a dose-response of UV irradiation and vitamin D₂ content in oyster mushrooms was conducted by Jasinghe et al (13). The authors observed and increase from approximately 15 to 45 µg/g dw with 10 to 40 minutes of UV-A exposure (0.21 kJ/m²/min) respectively and gave an equation $[D_2 = 6.78 + 0.890(t)]$, where t = time].

The oyster and shiitake showed higher amounts of vitamin D₂ content after pulsed UV light exposure than the brown and white mushrooms. Jasinghe and Perera (90) showed that oyster mushrooms produced the most vitamin D₂ compared to other mushroom types using the same treatment. The higher vitamin D₂ content in this study is most likely due to the thickness of the layer of mushrooms in the system. The brown and white button mushrooms were placed in polystyrene containers to simulate a package of sliced mushrooms being treated. The Oyster and Shiitake mushrooms were treated as whole mushrooms since their morphology did not permit for even distribution when packaged together. The single layer of the Oyster and Shiitake mushrooms was similar to how these mushrooms would be treated if the pulsed UV system were placed over a line where the mushrooms were being transported on a conveyor belt in a single layer. An additional study would be needed to directly compare the vitamin D₂ content of the *Agaricus* mushrooms to the Oyster and Shiitake mushrooms.

This study demonstrates that after a very short exposure time of about 1 sec (system generates 3 pulses per second) the vitamin D₂ content of these mushroom varieties can be increased from undetectable or very low levels to upwards of 800% DV/serving. Previous studies using continuous UV light has been shown to take at least 5 minutes (86) or up to 2 hours (5,84) of exposure to obtain similar values (13.8, 12.48 and 10.14 respectively).

3.4.2 PUV Irradiated Mushroom Powders and Spent Mushroom Substrates

This study also showed that increasing the vitamin D₂ contents of several mushroom products such as powders and substrates is possible. The vitamin D₂ content of selenium enriched (200 ppm) and control (10 ppm) mushroom powder (*Agaricus bisporus*) was increased in a similar manner (**Figure 3.5**) from 0.013 and 0.025 µg/g dw at 0 pulses to 27.8 and 25.3 µg/g dw, respectively, with a treatment of 16 pulses. Mushroom powders enriched in both selenium and vitamin D₂ are important because this product has more intrinsic nutritional value than powders not enriched in one or both of these nutrients. As seen in **Figure 3.6**, mushrooms are also a rich source of the antioxidant ergothioneine.

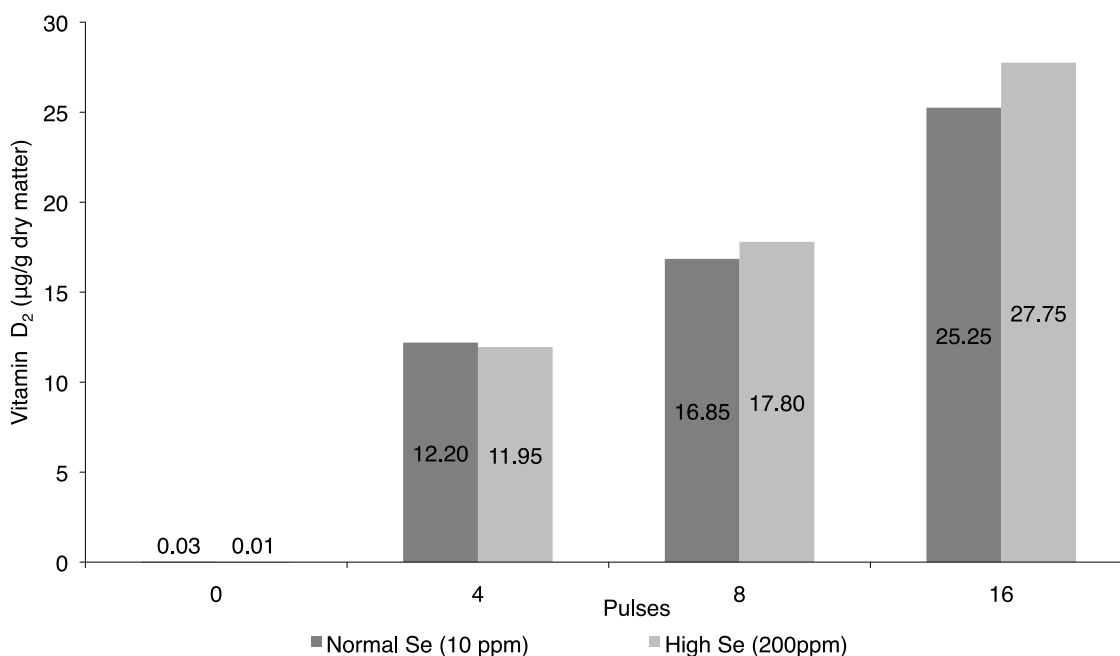


Figure 3.5. Vitamin D₂ content of pulsed UV treated selenium enriched and normal air-dried *Agaricus bisporus* mushroom powder.

The vitamin D₂ content of air-dried King Oyster powder was increased from 0.092 µg/g dw at 0 pulses to 23.0 µg/g dw after 16 pulses. The ergothioneine content of the dried products remained constant ca. 1.3 mg/g for all treatments (**Figure 3.6**) indicating that pulsed UV treatment had no effect on ergothioneine levels. The results of these experiments illustrate that mushrooms and their products enriched in selenium and

vitamin D₂ and naturally containing high levels of ergothioneine could be marketed as an excellent source of all these nutrients.

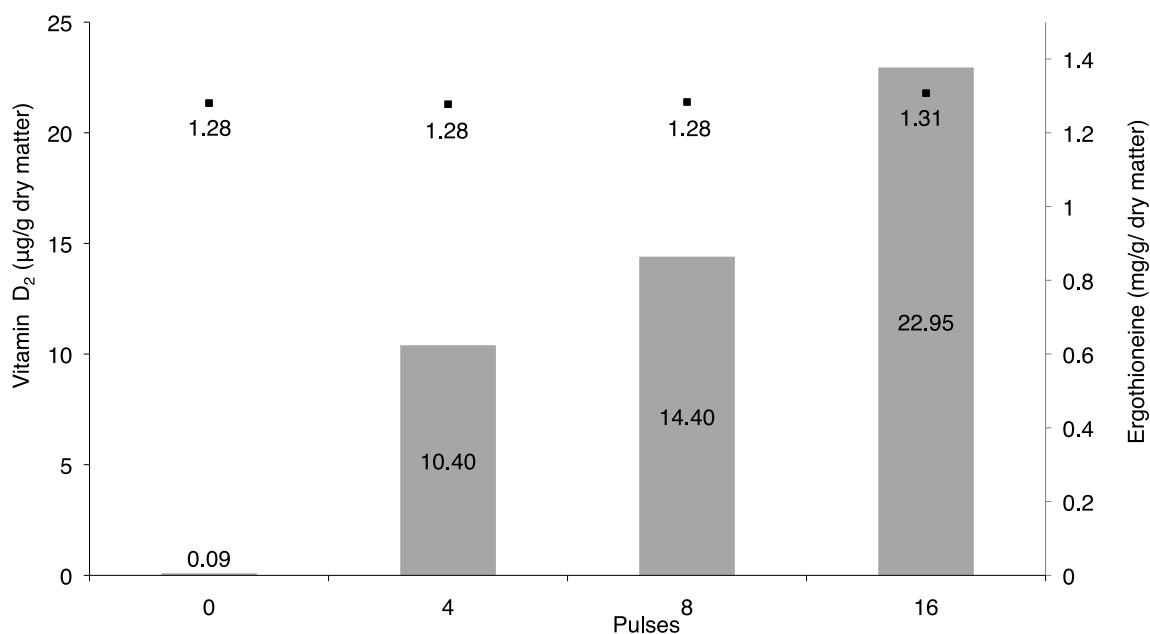


Figure 3.6. Vitamin D₂ (bars) and ergothioneine (black squares) contents of pulsed UV treated king oyster mushroom powder.

Mushroom mycelial biomass grown on sterile organic oats showed similar increases in vitamin D₂ with increasing exposure although levels were not as high as with pure fruiting body material. Vitamin D₂ dried King Oyster mycelial biomass increased significantly when ground from 0 to 1.78 µg/g dw however when exposed before grinding the level only rose to 0.072 µg/g dw (**Figure 3.7**). One limiting factor of this research is the ratio of mycelium to substrate is unknown. This ratio most likely varies based upon several factors such as initial level of spawn inoculum and stage of growth before the substrate was treated. This is, however the first instance of mycelium undergoing UV irradiation to increase vitamin D₂ content.

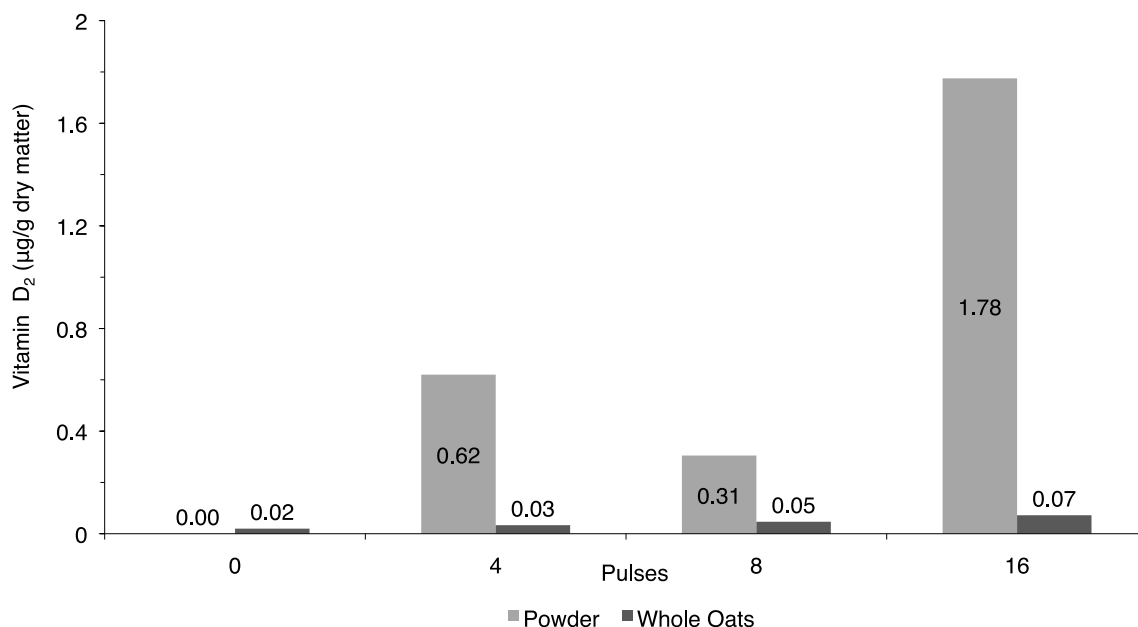


Figure 3.7. Vitamin D₂ content of pulsed UV treated king oyster mycelium grown on oat substrate irradiated in both whole oat and ground powder form.

King Oyster spent substrates pressed of excess water and treated with pulsed UV light before and after air drying (**Figure 3.8**) showed slightly higher vitamin D₂ content when treated wet (2.26 µg/g dw compared to 1.71 µg/g dw at 16 pulses). The opposite effect was seen with Maitake spent substrate (**Figure 3.9**). The undried substrate showed less conversion after 8 pulses (0.45 µg/g dw compared to 0.85 µg/g dw). Perera et al. (93) illustrated the effect of moisture content on the conversion of vitamin D₂ in mushrooms. They found that 85% moisture was the optimum level where the most vitamin D₂ was produced. Any moisture content lower or higher resulted in less vitamin D₂. More research is needed into this phenomenon, as the limitations of this portion of the study did not allow for adequate replication. In addition, a comparison that properly controls for surface area and dose is needed.

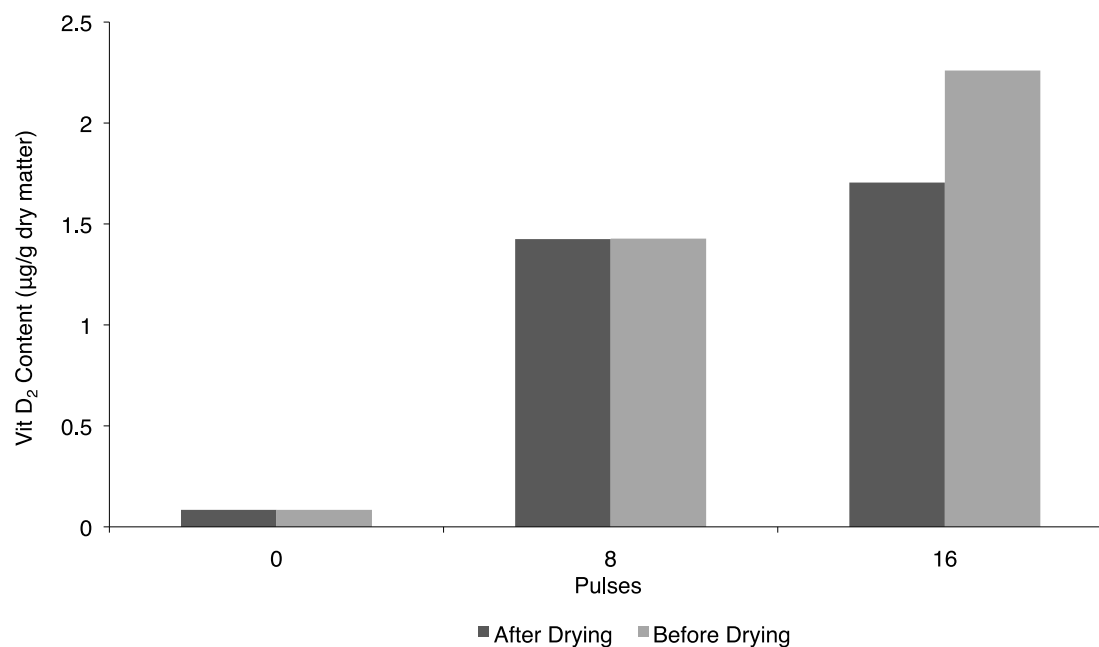


Figure 3.8. Vitamin D₂ content of pulsed UV treated spent king oyster substrate. Samples were treated before and after air-drying.

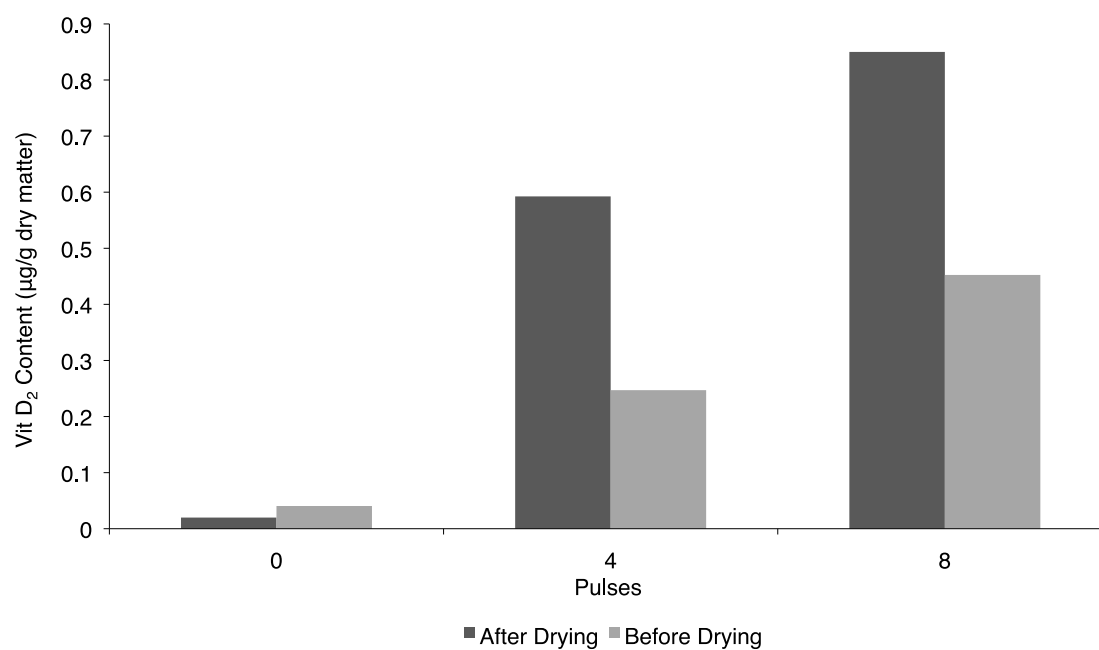


Figure 3.9. Vitamin D₂ content of pulsed UV treated spent maitake substrate. Samples were treated before and after air-drying.

3.5 Conclusions

In this study, we observed that all four mushroom varieties tested contained practically zero vitamin D₂. However, upon PUV irradiation vitamin D₂ content could be increased with one pulse (less than one second) to 5.11, 4.43, 5.71 and 7.95 µg/g dw in white button, brown button, shiitake and oyster mushrooms, respectively. Vitamin D₂ increased with subsequent PUV irradiation, reaching 13.36 and 11.06 µg/g dw in white and brown button mushrooms, respectively, with 4 pulses and 13.94 and 19.35 µg/g dw in shiitake and oyster mushrooms, respectively, with 3 pulses.

It was also observed that mushroom products including powders, spent growing substrates and grain inoculated with mycelium could be irradiated with PUV to increase vitamin D₂ content from practically zero in all cases. In white button mushroom powders with and without selenium enrichment, vitamin D₂ increased to approximately 12 µg/g dw after 4 pulses and increased with subsequent exposure to approximately 27 µg/g dw after 16 pulses. There was no significant difference between control powder and selenium-enriched powder. In king oyster mushroom powder similar levels were achieved after 4 and 16 pulses (10.40 and 22.95 µg/g dw, respectively). In king oyster powder, ergothioneine levels were measured with PUV treatment, no significant change was observed. Mushroom mycelial biomass and spent substrates showed a lower potential for vitamin D₂ enrichment. Grain inoculated with king oyster mycelium reached a vitamin D₂ content of 1.78 µg/g dw after 16 pulses when treated in powder form. Spent king oyster mushroom substrate contained approximately 2 µg/g dw after 16 pulses whereas spent maitake mushroom substrate contained less than 1 µg/g dw after the same treatment.

Pulsed UV technology has been shown to be a more practical method of UV irradiation of mushrooms for the mushroom industry than previous methods due to the shorter amount of time needed for exposure to achieve high amounts of vitamin D₂. The UV-B bulb used in this study was found to be highly effective in converting ergosterol to vitamin D₂ and would appear to be more practical than UV-C bulbs for commercial use since there would be no generation of ozone that could compromise worker safety.

The ergothioneine content of mushrooms in both fresh and powder form did not appear to change significantly with pulsed UV treatment. This shows that fresh mushrooms or mushroom products high in selenium, vitamin D₂ and ergothioneine are a plausible product. This is important from a “whole food” concept, wherein consuming bioactive components as part of a food matrix may be of greater impact to health than supplementation of each component alone. The significance of this phenomenon in mushrooms containing these compounds are yet unknown.

The outcome of this study allows for many practical applications. We have shown that a rapid method of increasing the vitamin D₂ content is achievable using pulsed UV light. The mushrooms produced can be sold fresh and would allow consumers an additional source of vitamin D in the diet. These products are also a non-animal source of vitamin D. The results of the powder and substrate enrichment show that these materials could be used as a food ingredient to increase the vitamin D content of any number of food products or even animal feeds. Overall, this study illustrates the potential health benefits of PUV irradiated mushrooms as an abundant source of vitamin D₂.

4. Effects of Postharvest Pulsed UV Light Treatment of White Button Mushrooms (*Agaricus bisporus*) on Vitamin D₂ Content and Quality Attributes

4.1 Abstract

A dose-response study of the effect of PUV treatment on vitamin D₂ content of *Agaricus bisporus* was conducted. In addition, factors influencing the conversion of ergosterol to vitamin D₂ and effects of PUV on shelf life were also investigated. Fresh white button mushrooms were treated with PUV wherein vitamin D₂ was found to rapidly increase to over 100% of the recommended dietary allowance (RDA) per serving following 3 pulses. Following 12 pulses, vitamin D₂ levels began to approach a maximum concentration of 27 µg/g dw. A shelf life study of PUV-treated mushrooms was carried out, where it was observed that the vitamin D₂ produced with 3 pulses dropped from 11.9 µg/g dw to 9.05 µg/g dw after 3 days of storage; however, vitamin D₂ levels remained nearly constant after this point throughout the 11-day study. The effect of PUV treatment on sliced and whole mushrooms was also evaluated. Significantly more vitamin D₂ was produced in sliced mushrooms than in whole mushrooms: 113% and 81% less for brown and white button mushrooms, respectively. Vitamin D₂ production in brown compared to white button mushrooms were also examined, and it was observed that whole and sliced brown mushrooms exposed to PUV generated significantly less vitamin D₂ than their white counterparts (33% and 21% less for whole and sliced, respectively). Finally, several quality attributes were assessed in PUV-treated mushrooms. No significant differences between control and PUV treated mushrooms were observed. These findings suggest that PUV treatment is a viable method for increasing the vitamin D₂ content of fresh mushrooms without adversely affecting quality.

4.2 Introduction

Vitamin D deficiency has recently become a major public health issue. Current research suggests that inadequate intake of vitamin D results in more than rickets and osteomalacia, a consequence of inadequate bone mineralization (28). Links to vitamin D deficiency and diseases such as cardiovascular disease (113) and cancer (57) have been documented. Other diseases with links to vitamin D deficiency include

hypertension, stroke, diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, periodontal disease, macular degeneration, mental illness, and chronic pain (19).

Humans are capable of converting cholesterol present in the epidermis to vitamin D₃ (cholecalciferol) following sun exposure (36). Individuals with limited sun exposure are most at risk for vitamin D deficiency. Other groups at risk include infants who are exclusively breastfed, the elderly, obese individuals and those with dark skin pigmentation (28). Apart from sun exposure and dietary supplements, vitamin D can be acquired through the diet, although few foods contain sufficient endogenous levels. These include fatty fish (*e.g.*, salmon, mackerel), eggs, and bovine liver. Fortified foods (*e.g.*, milk, orange juice, cereals) are, therefore, an important source of vitamin D in the American diet. Vitamin D₃ used for fortification is typically produced from cholesterol obtained from lanolin, an animal derived product, and is not considered vegan (114). Mushrooms, however, represent a potential source of vitamin D that is not derived from animals and, therefore, can be considered vegan (87).

Mushrooms contain high levels of pro-vitamin D₂ (ergosterol) and studies have shown that some wild mushroom species contain naturally occurring levels of vitamin D₂, ranging from 2.91- 58.7 µg/100g fresh weight (6,84,85). This most likely results from sun exposure of wild mushrooms, which promotes the conversion of endogenous ergosterol to ergocalciferol (vitamin D₂). The vitamin D₂ content of mushrooms can also be enhanced through the use of artificial UV light (4,5,13,84,87,88,90,94,97,98). These studies have shown that exposure to UV light can increase vitamin D₂ content in mushrooms from non-detectable levels to over 100 µg/g dry weight. The vitamin D₂ produced in UV irradiated mushrooms has been shown to be biologically active in humans (106).

The use of continuous UV irradiation to increase vitamin D₂ content, however, is not without its limitations. The exposure time required to generate adequate amounts of vitamin D₂ is on the order of minutes to hours, which may be too long to be practical for mushrooms produced under commercial conditions. Significant browning of white button

mushrooms exposed to continuous UV has also been reported (5,84,97) which is a serious issue with respect to consumer acceptability.

In contrast of continuous UV, pulsed UV light (PUV) is a technology that utilizes a broad spectrum (100-1100 nm) lamp along with high intensity pulses, delivering high amounts of energy in a relatively short amount of time. Compared to continuous UV light systems, the time required to deliver the same dose of UV irradiation is much shorter with PUV (72). In addition, continuous UV systems do not typically employ broadband lamps, and are only capable of encompassing one of the three principal regions of the UV spectrum: UV-A (400-315 nm), UV-B (315-280 nm) and UV-C (280-100 nm). The use of a broadband PUV system for vitamin D₂ enrichment could prove to be more attractive for commercial use due to the decreased exposure times and the potential for higher throughput.

Previous research conducted in our laboratory (110) demonstrated that PUV, using a C-type lamp (encompassing all regions of the UV spectrum amplified in the UV-C region), could be used to rapidly produce vitamin D₂ in fresh mushrooms. In **Chapter 3** the same effects were demonstrated using a B-type lamp.

The present study was conducted to determine methods needed to optimize PUV treatment using the B-type lamp (UV-C wavelengths excluded) to produce significant amounts of vitamin D₂ in fresh mushrooms following very short exposure times, as well as to study the factors influencing the amount of vitamin D₂ produced. A dose-response study was also carried out, in part to determine the maximum yield of vitamin D₂ obtainable by PUV. The effects of our PUV process on the shelf life of fresh mushrooms and their quality attributes were evaluated. The retention of the vitamin D₂ produced by this process during postharvest storage was also determined.

4.3 Materials and Methods

4.3.1 Materials

Mushrooms (*Agaricus bisporus* (J.E. Lange) Imbach) were obtained from the Pennsylvania State University Mushroom Test and Demonstration Facility (MTDF) on the day of harvest and were treated the same day. All mushrooms were protected from light exposure throughout the experiments. Materials for microbial enumeration included buffered peptone water (BPW), plate count agar (Standard Methods Agar) and dichloran-rose Bengal-chloramphenicol agar (DRBC) (BD Difco, Sparks, MD).

4.3.2 Dose-Response Study

To determine the dose-response of PUV treatment to vitamin D₂ production in fresh mushrooms, white button mushrooms (*Agaricus bisporus*) were cut into 7 mm slices and weighed into 150 g lots in polystyrene containers. A Steripulse®- XL 3000 (Xenon Corporation, Woburn, MA) was used with a B-type lamp for PUV experiments. The lamp was situated 5.8 cm from a quartz window. The apparatus generated 3 pulses/s at 505 J/pulse. At 3.18 cm from the quartz window the broadband energy was 0.791 J/cm²/pulse. Energy was measured using an Ophir Nova radiometer (Ophir-Spiricon, LLC, North Logan, UT) equipped with a pyroelectric head. The detector head was placed 3.18 cm from the quartz window and energy was averaged over three pulses. Mushroom samples were treated in increments of 3 pulses from 0 to 18, at a distance of 3.18 cm from the quartz window, in order to simulate treatment in a commercial package.

4.3.3 Mushroom Variety, Slicing and Package Weight

White and brown button (*Agaricus bisporus*) mushrooms were treated as described above with 3 pulses, either as whole mushrooms or 7 mm slices. White button mushrooms were also weighed into 150 and 230 g weight packages to determine the effect of the amount of mushrooms treated in a single package on vitamin D₂ generation.

4.3.4 Shelf Life Study and Vitamin D Retention Analysis

White button mushrooms were sliced and treated in 150 g lots with either 0 or 3 pulses of PUV. Packages were subsequently overwrapped with PVC film, and three holes were punched in the top of each package to replicate commercial packaging. Mushrooms were stored at 3°C to replicate storage conditions under refrigeration. Whole package samples were taken during storage at days 0, 3, 6, and 11 and tested for vitamin D₂ content and quality attributes.

4.3.5 Microbiological Analysis

The microbiological population of PUV treated mushrooms was analyzed on days 0 and 11 of the shelf life study. Samples (150 g) were transferred to a Waring LB10S laboratory blender and diluted 1:1 with buffered peptone water (BPW) and homogenized for 30 s. Following serial dilution with BPW, samples were plated on Standard Methods Agar and DRBC agar for analysis of aerobic plate count and yeasts and molds, respectively. The inoculated plates were incubated under aerobic conditions at 25°C for 48-92 h after which colonies were enumerated.

4.3.6 Quality Assessment

Three markers of quality were assessed to determine differences between PUV treated and untreated mushrooms. Weight loss, whiteness and visual appearance were measured at days 0, 3, 6 and 11 of storage at 3°C. To determine weight loss, weight changes were monitored over the course of storage and percent weight loss was normalized to day 0 values. Whiteness was assessed in terms of L-value using a Minolta Chroma Meter (Model CR-200, Japan) (n=20 measurements per sample).

4.3.7 Vitamin D₂ Analysis

Samples were snap frozen (-80°C) directly following treatment or after a predetermined number of days of storage, depending on the experiment. Following lyophilization, freeze-dried mushrooms were homogenized and analyzed for vitamin D₂ content

according to established methods (94). Briefly, each mushroom sample was saponified with dihydrotachysterol (internal standard), ascorbic acid, and pyrogalllic acid in an ethanol/KOH solution, as described previously (94). The samples were then extracted using heptane, dried, and reconstituted in cyclohexane and heptane (1:1). The solution underwent preparative HPLC cleanup and the fraction containing the internal standard and vitamin D₂ was collected, dried under nitrogen, reconstituted in 1:1 cyclohexane/heptane and quantitated by reverse-phase HPLC with mass spectrometry detection. Vitamin D₂ values are reported as µg/g dry weight (dw).

4.3.8 Statistical Analysis

All experiments were performed in triplicate. One-way ANOVA and Tukey's Test ($\alpha = 0.05$) were performed using Minitab 15 (Minitab Inc., State College, PA, USA) to compare means of each treatment and determine significant difference.

4.4 Results and Discussion

The results of the dose-response study (**Figure 4.1**) suggest a non-linear relationship between PUV irradiation dose and vitamin D₂ content of fresh mushrooms. The initial levels of vitamin D₂ in untreated mushrooms were less than 0.005 µg/g dw, and rapidly increased to 12.6 µg/g dw after 3 pulses. This is equivalent to 518% of the RDA of vitamin D per serving of fresh mushrooms (84 g fresh weight) based on the current RDA of 600 IU for vitamin D (28). Vitamin D₂ levels in mushroom samples then appeared to level reach a maximum concentration of approximately 27 µg/g dw or 1130% RDA/serving between 12 and 18 pulses.

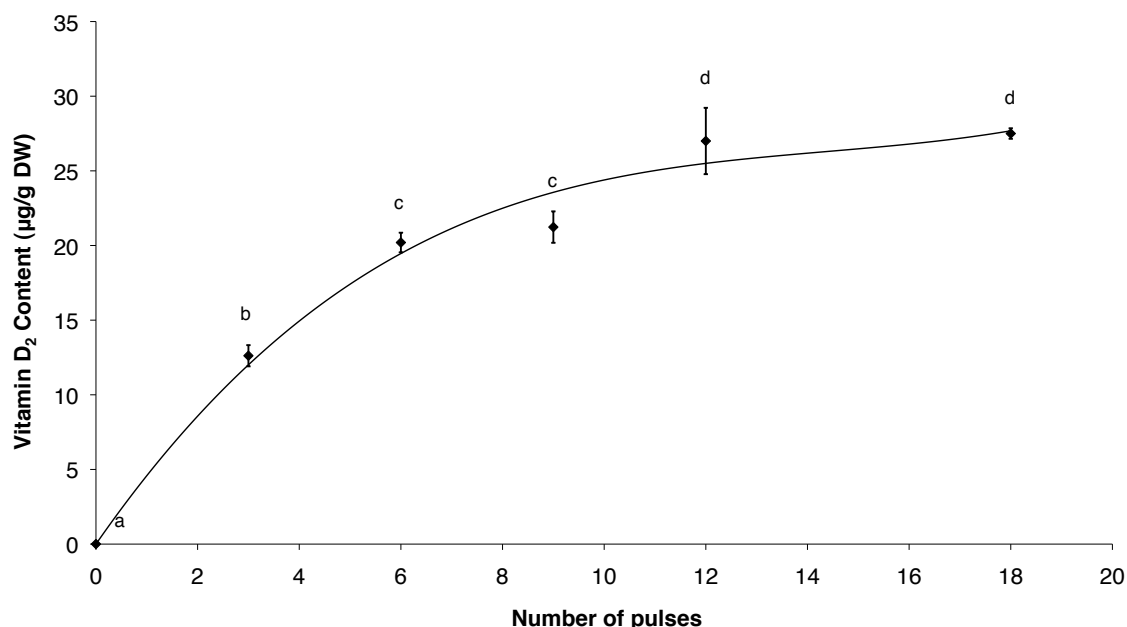


Figure 4.1. Vitamin D₂ content of sliced fresh white button (*Agaricus bisporus*) mushrooms treated with pulsed UV light in increments of 3 pulses (3 pulses = 1 s exposure time). Error bars represent standard deviations. Mean values with lower case letters that are the same are not significantly different ($p < 0.05$).

Jasinghe et al. (13) studied the kinetics of vitamin D₂ conversion from ergosterol in fresh mushrooms. D₂ production was observed to increase linearly as a function of exposure time to continuous UV irradiation, suggesting zero order kinetics. However, our results indicate that the conversion of ergosterol to vitamin D₂ may not follow zero order kinetics after exposure to the relatively higher levels of UV irradiation used in our study. In a separate study, Jasinghe et al. reported similar kinetics in shiitake mushrooms treated up to 120 minutes with UV-A irradiation, wherein the maximum D₂ concentration obtained was ca. 30 µg/g dw with the gill tissue facing the lamp (90). This phenomenon observed in our study could potentially be due to a low depth of penetration of UV irradiation due to the thickness of the batch of mushrooms being treated as opposed to a single layer system. It has also been suggested that photodegradation of D₂ may result with prolonged exposure. In addition, side products such as lumisterol and tachysterol may be formed with prolonged exposure (115).

For all the subsequent studies, a dose of 3 pulses was chosen to represent what would be a reasonable treatment in terms of both D₂ produced and ease of implementation in a commercial setting. The high level of D₂ produced, even with this one-second treatment, allowed for the various other parameters to be evaluated at what would probably be the upper level of commercial treatment. This treatment would therefore be the most rigorous commercial treatment and any effects on browning, microbial inactivation, etc. would be visible. If no effects were seen with this treatment, any lower dose treatment (i.e. to achieve a D₂ content of 400 IU/serving or 100% of the Daily Value) would likely not suffer any benefits or consequences seen in a higher dose. Implementing a full range of doses to investigate the following parameters would have been beyond the scope of this study.

Brown and white button mushrooms were treated with 3 pulses of PUV in sliced and whole form. There was significantly more vitamin D₂ in sliced mushrooms ($p < 0.05$) compared to whole in both brown (113% more) and white (81% more) mushrooms (**Figure 4.2**).

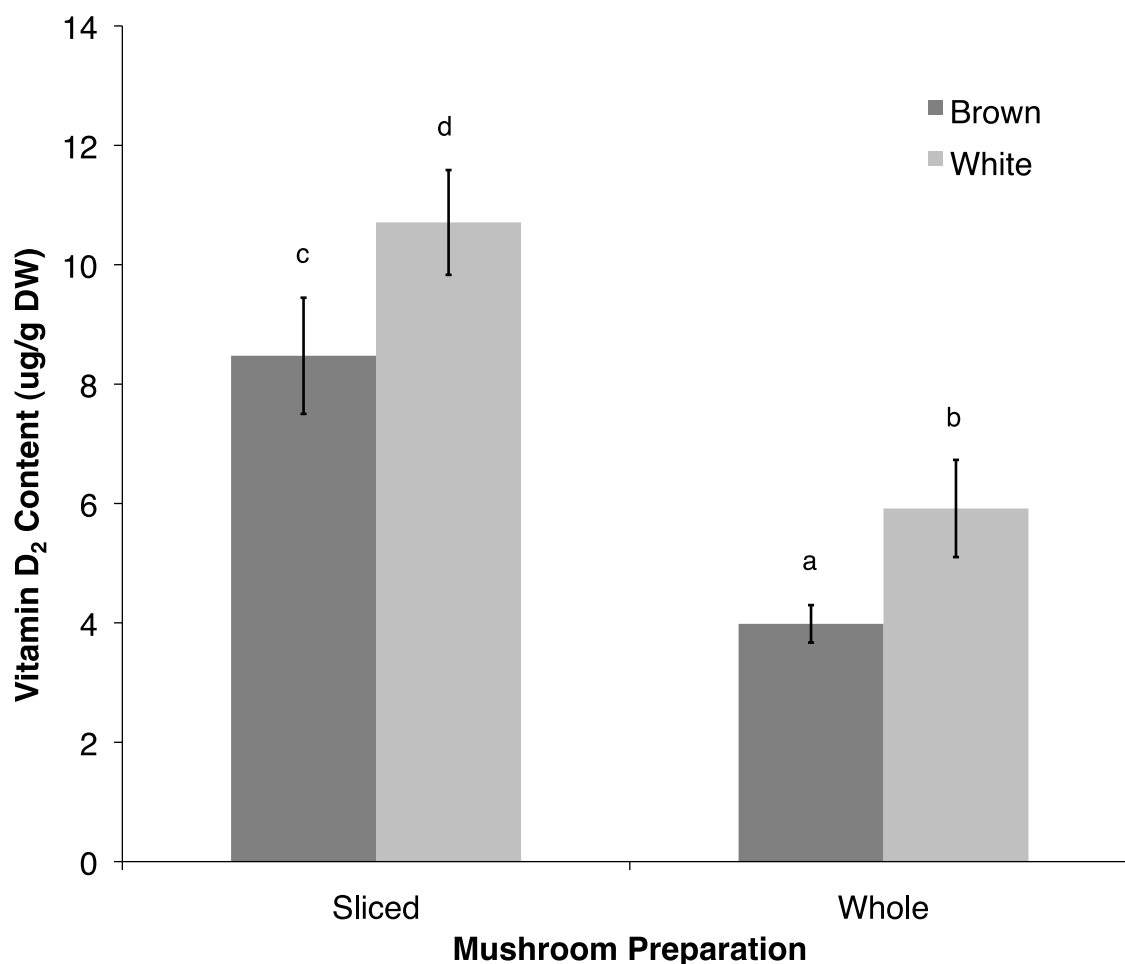


Figure 4.2. Vitamin D₂ content of sliced fresh white and brown button (*Agaricus bisporus*) mushrooms treated with pulsed UV light (3 pulses = 1 s exposure time, control mushrooms received 0 pulses) in either sliced or whole form. Mean values with lower case letters that are the same are not significantly different ($p < 0.05$). (Control mushrooms contained 0.0106 and 0.265 $\mu\text{g/g}$ in brown and white varieties respectively.)

This is most likely due to increased exposure of the gill tissue in the sliced mushrooms. Ergosterol levels have been shown to vary in different mushroom tissues. Jasinghe and Perera (87) showed that shiitake mushrooms contained more ergosterol in the gill tissue than the cap while Ko et al. (98) demonstrated that treating the gill tissue of button mushrooms resulted in more vitamin D than when the pileus was treated. Based on our results, and the previously mentioned ergosterol distribution studies, it is conceivable that ergosterol is concentrated within the gill tissue of the brown and white button mushrooms used in the present study. As slicing increases gill tissue exposure to the

light source, one would expect higher final levels of D₂ in sliced versus whole samples. However, further research is needed to establish the ergosterol content of the various tissues of *Agaricus bisporus*, and to determine if this phenomenon is simply a result of the exposed gill tissue or a surface area effect (*i.e.*, the surface-area-to-volume of gill tissue is higher than that of the smooth cap). There was also significantly less ($p < 0.05$) D₂ in brown button varieties compared with whites in both the sliced (21% less) and whole (33% less) treatment groups. It is likely that the higher levels of pigmentation in the brown mushroom samples shielded endogenous ergosterol from the UV source.

The penetrating effect of PUV on mushroom samples was investigated within the context of D₂ production. When a full commercial package of mushrooms (230 g) was treated in a batch system, the final vitamin D₂ content (7.6 µg/g dw) was 40% lower ($p < 0.05$) than those treated in 150 g batches (11.9 µg/g dw) (**Figure 4.3**). These data suggest that the top layer of mushrooms were exposed to a higher proportion of UV light and, therefore, generated higher levels of D₂. Based on these results, it is suggested that PUV treatment of mushrooms in a single layer would result in a more even distribution of D₂ throughout all mushrooms. From a commercial standpoint, this would be practical if the PUV apparatus is placed on a processing line directly following slicing before packaging. For whole mushrooms, which are often picked directly into the final packaging this may prove to be less practical.



Figure 4.3. Vitamin D₂ content of sliced fresh white button (*Agaricus bisporus*) mushrooms treated in two different weight packages with pulsed UV light (3 pulses = 1 s exposure time). Mean values with lower case letters that are the same are not significantly different ($p < 0.05$).

A shelf life study of PUV treated mushrooms was carried out, where it was observed that 76 % of the D₂ produced was retained in all samples throughout storage (**Figure 4.4**). The mushrooms had an initial level of 11.9 µg/g dw after treatment with 3 pulses of PUV. After 3 days of storage (3°C) the level dropped by 24% to a final concentration of 9.05 µg/g dw; however, this level did not change significantly ($p < 0.05$) for the duration of the storage study. A previous study (94) found a similar initial decrease and subsequent leveling off of vitamin D₂ levels in mushrooms stored at 2.2°C, although the study was only carried out for 4 days.

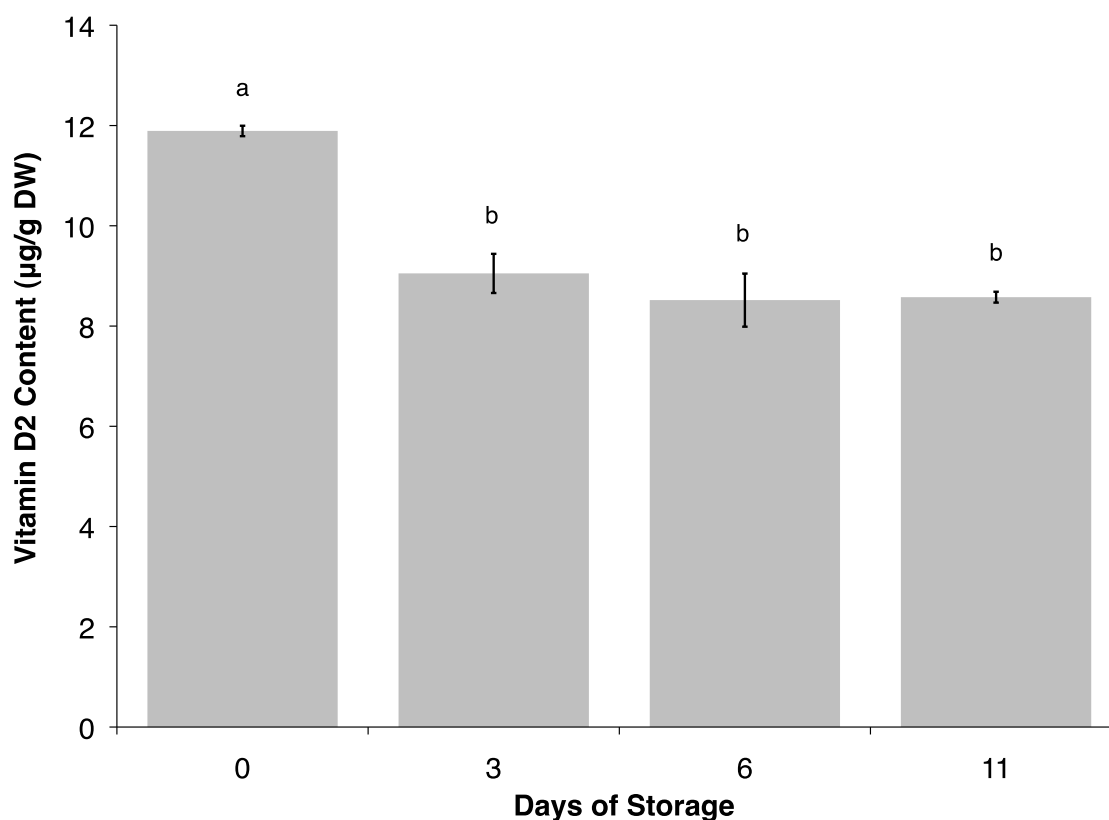


Figure 4.4. Vitamin D₂ content of sliced fresh white button (*Agaricus bisporus*) mushrooms treated with pulsed UV light (3 pulses = 1 s exposure time) and stored for up to 11 days at 3°C. Mean values with lower case letters that are the same are not significantly different ($p < 0.05$).

A visual quality assessment indicated no significant difference ($p < 0.05$) in untreated and PUV treated mushrooms: no discernable difference in whiteness and no overall difference in visual appearance between untreated and PUV treated white button mushrooms at any time point during storage through 11 days (**Figures 4.5 and 4.6**).

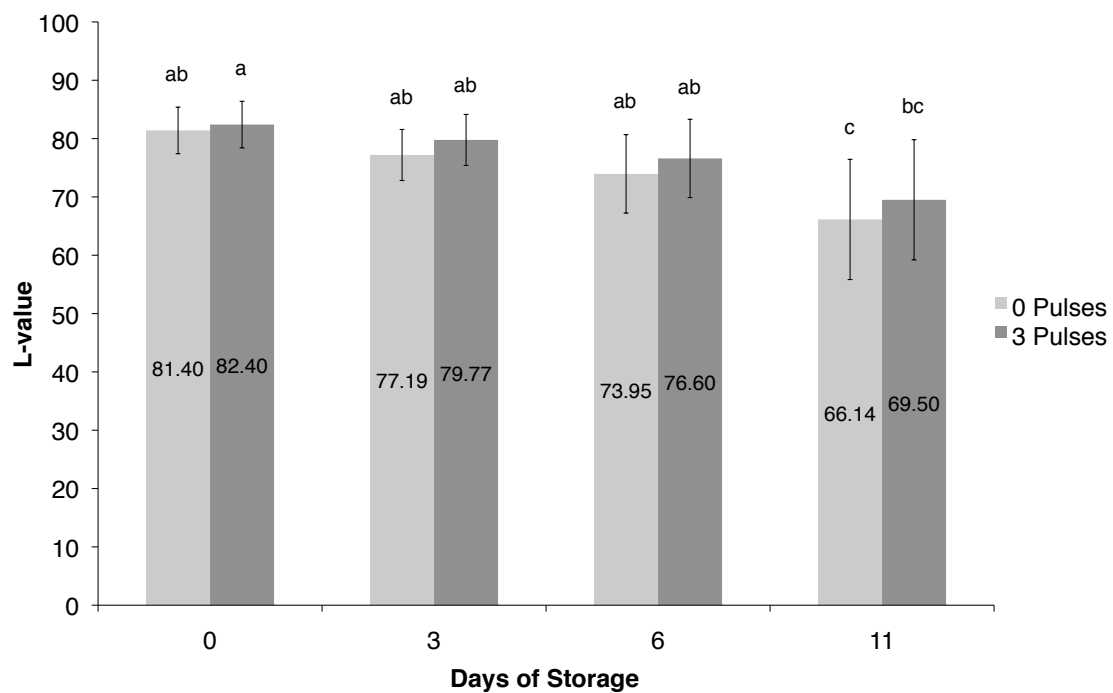


Figure 4.5. Whiteness (L-value) of untreated and pulsed UV light treated sliced fresh white button (*Agaricus bisporus*) mushrooms. L-values of 0 and 100 represent black and white, respectively. Mean values with lower case letters that are the same are not significantly different ($p < 0.05$)



Figure 4.6. Photograph of sliced fresh white button mushrooms after storage at 3°C for 0 days (a), 3 days (b) 6 days (c) and 11 days (d). The top row of each quadrant represents untreated mushroom; the bottom row of each quadrant represents mushroom treated with 3 pulses of pulsed UV light

Previous studies using continuous UV light have shown browning of white button mushrooms (5,84,97). One study also reported browning in addition to a decrease in moisture content with continuous UV treatment (98). PUV does not appear to yield the same deleterious effects, however further research is needed to investigate the basis of this phenomenon. No significant difference ($p < 0.05$) in weight change of PUV treated mushrooms from day 0 through 11 days of storage was observed compared to the control (**Table 4.1**). Weight change is a measure of degradation of the mushroom during storage as moisture and other components are lost through desiccation. These results indicate that PUV treatment of fresh mushrooms does not have any deleterious effects on the quality parameters measured.

Table 4.1. % Weight loss in pulsed UV treated sliced fresh white button (*Agaricus bisporus*) mushrooms from initial weight (150 g) after storage at 3°C for up to 11 days.

Number of Pulses	Days of Storage		
	3	6	11
0	1.69±0.62	3.42±0.33	5.33±0.24
3	1.87±0.76	3.76±1.18	6.19±1.60

The use of PUV irradiation in food processing is mainly implemented as an antimicrobial intervention (72). The treatment regimen used in this study was examined to determine if the level of irradiation necessary to achieve reasonable levels of vitamin D₂ was effective in reducing microbial loads, as well. Neither total aerobic plate count nor yeast and mold levels were reduced with a 3-pulse batch treatment (**Table 4.2**).

Table 4.2. Total aerobic plate counts and yeast and mold counts of pulsed UV treated (3 pulses) and untreated sliced white button (*Agaricus bisporus*) mushrooms directly following treatment and after 11 days of storage at 3°C.

	Day 0		Day 11	
	Control (CFU/g)	Treatment (CFU/g)	Control (CFU/g)	Treatment (CFU/g)
Total Aerobic Plate Count	6.21 ± 0.16	6.27 ± 0.19	8.74 ± 0.19	8.55 ± 0.22
Yeasts and Molds	5.88 ± 0.09	6.02 ± 0.34	8.21 ± 0.79	8.11 ± 0.18

Chikthimmah and Beelman reported that with treatment times varying from 4-30 seconds of PUV, the microbial loads of fresh mushrooms could be reduced by 0.9 to 1.6 log CFU/g (116). In this present study, a lower dose of PUV irradiation was employed, which could explain why no reduction in microbial load was observed. This could also be due to the use of the B-type lamp, which does not include the most germicidal region of the UV spectrum (*i.e.*, UV-C). Another possible explanation for the lack of a microbicidal effect is that all mushrooms treated in their commercial packages were not fully exposed to PUV radiation: the top layer was likely the only surface that was exposed to PUV radiation and, thus, the microbial population of the lower layer of mushrooms were not affected. Therefore, use of PUV to reduce microbial levels in fresh mushrooms may not be a practical method because the levels of irradiation needed to reduce microbial levels may

result in levels of vitamin D₂ that exceed the tolerable upper intake level (UL) of 4000 IU/day (28).

4.5 Conclusions

In this study, it was observed that vitamin D₂ content of sliced white button mushrooms treated in a batch system increased from practically zero to 12.6 µg/g dw after 4 pulses and increased non-linearly to approach a maximum value of approximately 27 µg/g dw between 12 and 18 pulses. It was also observed that sliced mushroom produced more vitamin D₂ than whole mushrooms (113 and 81% more in brown and white buttons, respectively) and brown button mushrooms produced less vitamin D₂ than their white counterparts (21 and 33% less in sliced and whole, respectively). When treated in a batch system it was observed that mushrooms treated in a full commercial package (230 g) produced 40% less vitamin D₂ than when treated in a 150 g package. In the shelf life portion of this study a 24% decrease was observed in vitamin D₂ levels after 3 days of storage at 3°C but levels remained constant afterward through 11 days. No significant differences in quality attributes measured were observed (color, appearance, microbial load, and weight change).

The observations of this study demonstrate the use of PUV treatment of fresh mushrooms for the purpose of increasing vitamin D₂ content is an effective and rapid method with no known deleterious effects on product quality. The shortest exposure time of any previous study using continuous UV regimes to achieve a level equivalent to 100% DV/serving was ca. 8 minutes (94). In contrast, we have demonstrated in this present study that PUV systems are capable of achieving similar levels of vitamin D₂ with one second or less of total exposure time and without negative effects on appearance. The PUV treatment described in this paper does not have an effect on microbial load, which suggests all mushrooms in a commercial package are not exposed to PUV irradiation. Therefore, this study suggests that sliced mushrooms treated in a single layer of small batch system may be the optimal method for enrichment. Furthermore, it was shown that the vitamin D₂ produced under the parameters of this study was stable under commercially relevant storage conditions. Future study into other quality attributes such as flavor and the effect on other bioactive compounds would be of interest.

5. The Effect of Pulsed UV Light Treatment on Vitamin D₂, Tachysterol₂, Lumisterol₂ and Previtamin D₂ Content of White Button Mushrooms (*Agaricus bisporus*)

5.1 Abstract

The effect of PUV treatment of mushrooms on the production of photoproducts of ergosterol was investigated. In addition to vitamin D₂, it is known that with UV exposure of ergosterol other products including previtamin D₂, tachysterol₂ and lumisterol₂ are produced. White button mushrooms were sliced and treated in a single layer system with PUV irradiation increments from 0 to 60 pulses to determine the dose-response relationship between irradiation and photoproduct content. All photoproducts increased in a non-linear but dose-dependent manner as a function of increasing PUV irradiation. Vitamin D₂ content approached a maximum value of ca. 124 µg/g dw with 60 pulses, and was observed to be the most abundant photoproduct with respect to yield. With 60 pulses, previtamin D₂ content approached a maximum value of 44.6 µg/g dw, while lumisterol₂ and tachysterol₂ reached maximum levels of 30.5 µg/g dw and 24.7 µg/g dw, respectively.

5.2 Introduction

The important role that vitamin D has on human health has been well established (28,117), with research suggesting that inadequate intake or production results in more than rickets and osteomalacia, a consequence of inadequate bone mineralization (20). Links to vitamin D deficiency and diseases such as cardiovascular disease (113) and cancer (57) have been documented. Other diseases with links to vitamin D deficiency include hypertension, stroke, diabetes, multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, periodontal disease, macular degeneration, mental illness, and chronic pain (19).

The generation of vitamin D₃ in humans occurs principally in the epidermal layer of the skin (36) as a result of exposure to UV radiation from sunlight. The cholesterol precursor, 7-dehydrocholesterol, absorbs ultraviolet radiation in the B range (290-315 nm) and is

converted to previtamin D₃. Once formed, previtamin D₃ rapidly converts to vitamin D₃. This reaction can also be initiated in fungi through natural or artificial UV irradiation of ergosterol, a structural analog of cholesterol, to form vitamin D₂ (D₂).

The conversion of ergosterol to D₂ (**Scheme 1.1, Chapter 1**) is initiated upon exposure of the precursor ergosterol (also known as provitamin D₂) to UV irradiation, which results in cleavage and opening of the B-ring to yield previtamin D₂ (PD₂). Through extended exposure to UV irradiation, PD₂ can undergo ring closure to form lumisterol₂ (L), a (9β,10α) stereoisomer of ergosterol (9α,10β). PD₂ is thermally labile and is converted to D₂ by a thermally induced [1,7] sigmatropic shift (10). Tachysterol₂ (T₂) is another photoproduct of this photochemical reaction and is structurally similar to vitamin D₂; however, the A-ring of T₂ is rotated 180° and the A and C rings contain a double bond. Other products such as toxisterols and suprasterols are known to form as a result of extended irradiation of ergosterol (12).

Recent studies suggest that the photoproducts of cholesterol could be of biological importance, which may have applicability to ergosterol-derived products in mushrooms. Chen et al. investigated the D₃ series of these compounds and their role in antiproliferative activity and affinity to the nuclear vitamin D receptor (VDR) (29). The authors found that while the photoproducts had low binding affinity for VDR, some antiproliferative activity was observed that may have been due to the hydroxylation of T₃ to its 25(OH) and 1α,25(OH) forms. The authors showed that 25(OH)T₃ has binding affinity for VDR although 1,000 fold lower than 1,25[OH]₂D₃.

Also of note is that many of the of vitamin D analogs used for various clinical treatments are based on the first analog developed, dihydrotachsterol₂ (DHT) (30). DHT is a reduction product of D₂ and is structurally similar to T₂ with the only difference being the lack of double bonds in the A and C rings in DHT. Due to the structural similarity of these analogs, it is conceivable that these photoisomers may be of biological relevance. This is supported by two studies by Holick et al., wherein the authors demonstrated that 5,6-*trans*-25-hydroxycholecalciferol (the 5,6-*trans* isomer of 25[OH]D₃) was effective in stimulating calcium transport in the duodenum of vitamin D-deficient rats (31). The authors also demonstrated that isotachysterol₃ and 25-hydroxyisotachysterol₃ both

stimulated the transport of calcium in the intestines and bone calcium mobilization in anephric rats (32). The structures of these compounds are vary in structure to tachysterol₃ in that isotachysterol₃ has a double bond between C-8 and C-14 while in tachysterol₃ the double bond is C-8 to C-19. 25-hydroxyisotachysterol₃ is the 25-hydroxylated form of isotachysterol₃.

Holick et al. demonstrated that the affinity of the vitamin D binding protein (DBP) is low for 7-DHC and L₃ and T₃ formed *in vivo*, which indicates that these compounds are less likely to leave the skin (33). Thus, circulating concentrations of the photoisomers of 7-DHC or ergosterol in the blood would be minimized. However, irradiated foods containing the precursors could contain these photoproducts. D₂ is used for some food fortification and supplementation. The commercial production of D₂ can be optimized to maximize the yield of D₂ and to eliminate the photoisomers (34,35).

The consumption of D₂ enriched mushrooms is perhaps a unique case because irradiated mushrooms are one of the only products a human would consume that would potentially contain not only D₂ but also its photoisomers. The only other irradiated natural product would be irradiated yeast added as an ingredient or foods that contain D₂ from irradiated yeast. While the effect of UV on D₂ production in mushrooms has been well established (5,13,84,87,90,94,97,98), the effect on the generation of other photoproducts in mushrooms has not been studied. We have previously demonstrated that the use of a PUV system with a B-type lamp is a rapid method for increasing the D₂ content of fresh mushrooms (118). The present study focuses on the relationship between D₂ production and the production of ergosterol-derived products in fresh mushrooms when subjected to PUV treatment.

5.3 Materials and Methods

5.3.1 Materials

White button mushrooms (*Agaricus bisporus*) were obtained from the Pennsylvania State University Mushroom Test and Demonstration Facility on the day of harvest and were PUV treated on the same day. All mushrooms were protected from extraneous light

exposure throughout the experiments. Crystalline vitamin D₂ (ergocalciferol), ergosterol and vitamin D₃ (cholecalciferol) standards were obtained from Sigma-Aldrich Co (St. Louis, MO, USA). Crystalline previtamin D₂, tachysterol₂ and lumisterol₂ were prepared and purified by UV irradiation of ergosterol based on previously published methods (119). All other chemicals and solvents used were of analytical or HPLC grade.

5.3.2 Pulsed UV Treatment

A Steripulse®- XL 3000 (Xenon Corporation, Woburn, MA) was used with a B-type lamp for PUV experiments. The lamp was situated 5.8 cm from a quartz window. The apparatus generated 3 pulses/s at 505 J/pulse. At 3.18 cm from the quartz window the broadband energy was 0.791 J/cm²/pulse. Energy was measured using an Ophir Nova radiometer (Ophir-Spiricon, LLC, North Logan, UT) equipped with a pyroelectric head. The detector head was placed 3.2 cm from the quartz window and energy was averaged over three pulses.

To determine the dose-response of PUV treatment to vitamin D₂, T₂, L₂ and PD₂ production in fresh mushrooms, white button mushrooms (*Agaricus bisporus*) were cut into 7 mm slices and were treated in increments of 3 pulses from 0 to 60, at a distance of 3.18 cm from the quartz window. Samples were snap frozen (-80°C) directly following treatment and subsequently lyophilized (Virtis Genesis XL). Following lyophilization, freeze-dried mushrooms were homogenized and analyzed for product formation.

To determine heat generated by the UV system, temperature was measured using a thermocouple (Omegaette HH306, Omega Engineering, Inc., Stamford, CT) with a wire probe inserted just below the surface of a mushroom as well as with an infrared (IR) thermometer (Omega TrueRMS Supermeter, Omega Engineering, Inc., Stamford, CT) to monitor surface temperature. Temperature did not rise significantly at lower treatments, however with prolonged irradiation mushroom surface temperature rose significantly.

Table 5.1. Temperature of PUV irradiated mushrooms

Number of Pulses	Thermocouple (°C)	IR (°C)
0	22.9 ± 0.2	20.3 ± 0.6
3	23.6 ± 0.4	21.3 ± 0.6
9	24.5 ± 0.9	25.0 ± 1
15	25.1 ± 1	29.3 ± 2
30	27.7 ± 3	33.7 ± 0.6
60	31.1 ± 5	41.3 ± 3

5.3.3 Extraction of Ergosterol-Derived Products from Mushrooms

The method used for the extraction of the photoproducts of ergosterol was adapted from previously published methods for vitamin D extraction from mushrooms. Approximately 1 g of mushroom powder and 20 µg vitamin D₃ (internal standard) were weighed and added to a centrifuge tube containing 10 mL dimethyl sulfoxide, mixed by vortex and placed in a 45°C sonicating water bath for 30 minutes. A mixture of methanol:water (1:1 v/v, 10 mL) was then added and the sample mixed by vortex before adding 20 mL hexane. The resulting mixture was vortexed, returned to the sonicating water bath for an additional 30 minutes, re-vortexed, and finally centrifuged for 10 minutes. The upper layer was removed and the bottom layer was separated from the pellet and extracted twice in a separatory funnel with 20 mL of hexane. The upper layers were combined and brought to dryness in a rotary evaporator. The residual material was then dissolved in 5 mL of methanol and filtered over a 0.45 µm PTFE syringe filters.

5.3.4 Chromatographic Separation and Quantification of Photoproducts of Ergosterol in Mushrooms

The separation of the photoproducts of ergosterol required the use of three separate HPLC methods to fully resolve and allow for the quantification of all analytes. As seen in **Figure 5.1**, a reverse phase system was able to resolve vitamin D₂, however T₂ and L₂ coeluted under these conditions. PD₂ and L₂ were separated using a silica column; however, vitamin D₂ and T₂ coeluted using this method (**Figure 5.2**). A third HPLC method using a cyano (CN) bonded column was able to fully resolve T₂, yet PD₂ and L₂ were observed to co-elute (**Figure 5.3**).

For vitamin D₂ analysis, filtered mushroom extract (20 µL) was injected onto a reverse phase HPLC system (Shimadzu 10ADvp pump with sample introduction by means of a 20ADvp temperature-controlled autosampler (4 °C)). The column was a Supelcosil LC-18 (4.6 x 150 mm, 5 µm; Supelco Inc., Bellefonte, PA). The mobile phase was methanol/water (95/5 v/v) which was delivered at a flow rate of 1 mL/min. Vitamin D₂ was detected at 265 nm using a Shimadzu SPD-M10Avp photodiode array detector, with quantitation based on an external standard curve prepared from vitamin D₂. Vitamin D₂ values are reported as µg/g dry weight (dw).

Previtamin D₂ and lumisterol₂ were analyzed by taking mushroom extract (1 mL) to dryness using a vacuum centrifuge, followed by extraction in 1 mL of hexane, of which 20 µL was injected onto a normal phase column by means of a HPLC system (Hewlett Packard Series 1050). The column was a Zorbax Rx-Sil (4.6 x 150 mm, 5 µm; Agilent Technologies). The solvent was delivered isocratically at a flow rate of 2 mL/min, and the mobile phase used was hexane/isopropanol (99.7/0.3 v/v). PD₂ was detected at 260 nm and L₂ was detected at 270 nm using a HP Series 1050 photodiode array detector, with quantitation based on an external standard curve prepared from PD₂ and L₂. PD₂ and L₂ values are reported as µg/g dry weight (dw).

Tachysterol₂ was extracted and analyzed in a similar manner. Briefly, hexane extracts of mushroom powder were injected (20 µL) onto a normal phase HPLC system (Hewlett Packard Series 1050). The column was a Zorbax CN (4.6 x 150 mm, 5 µm; Agilent Technologies). The mobile phase was hexane/isopropanol (99.7/0.3 v/v) and was delivered isocratically using a flow rate of 1.5 mL/min. T₂ was detected at 280 nm using a HP Series 1050 photodiode array detector, with quantitation based on an external standard curve prepared from authentic T₂. T₂ values are reported as µg/g dry weight (dw).

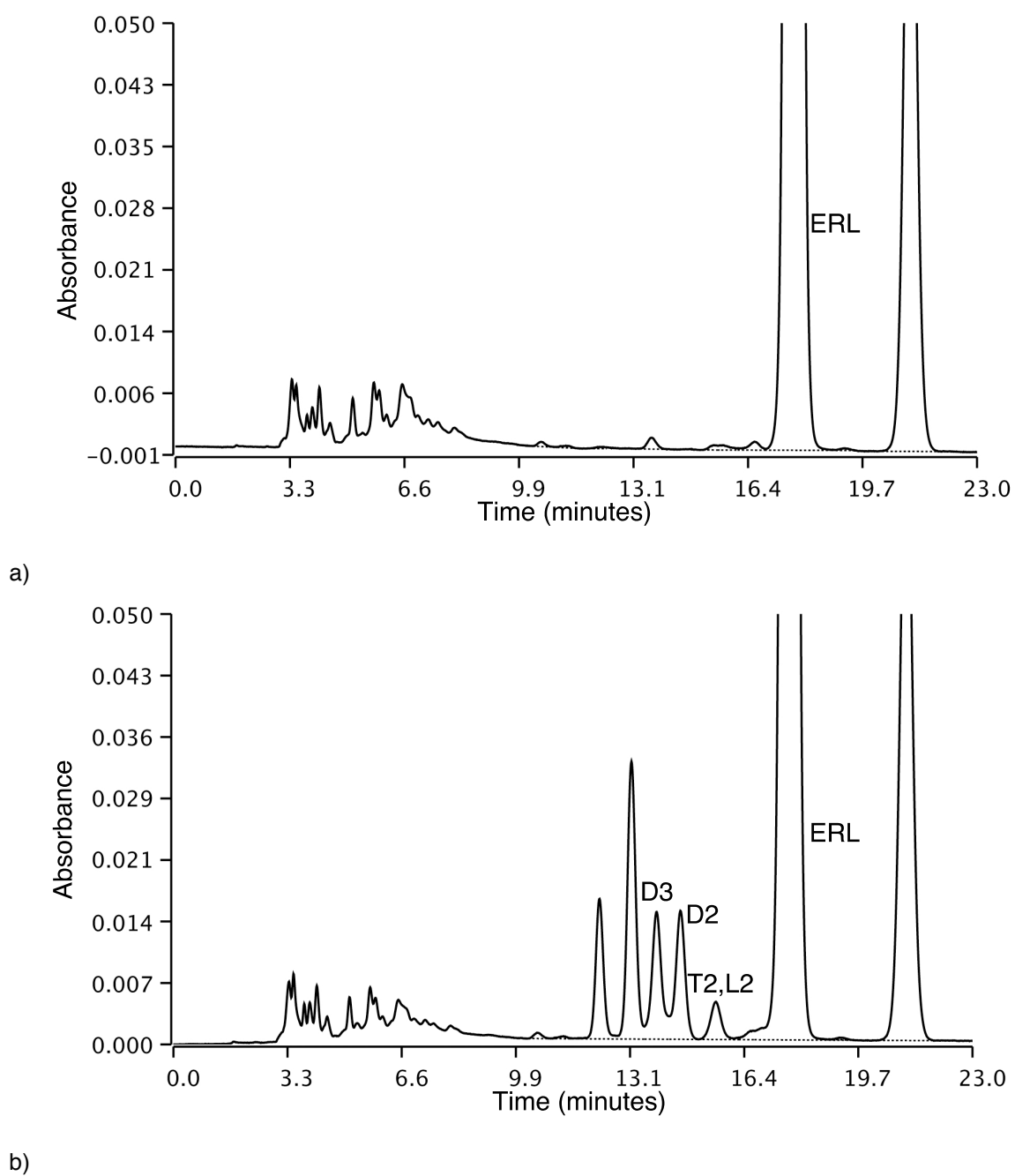


Figure 5.1. Reverse phase HPLC chromatograms of control (a) and PUV exposed (b) white button mushrooms

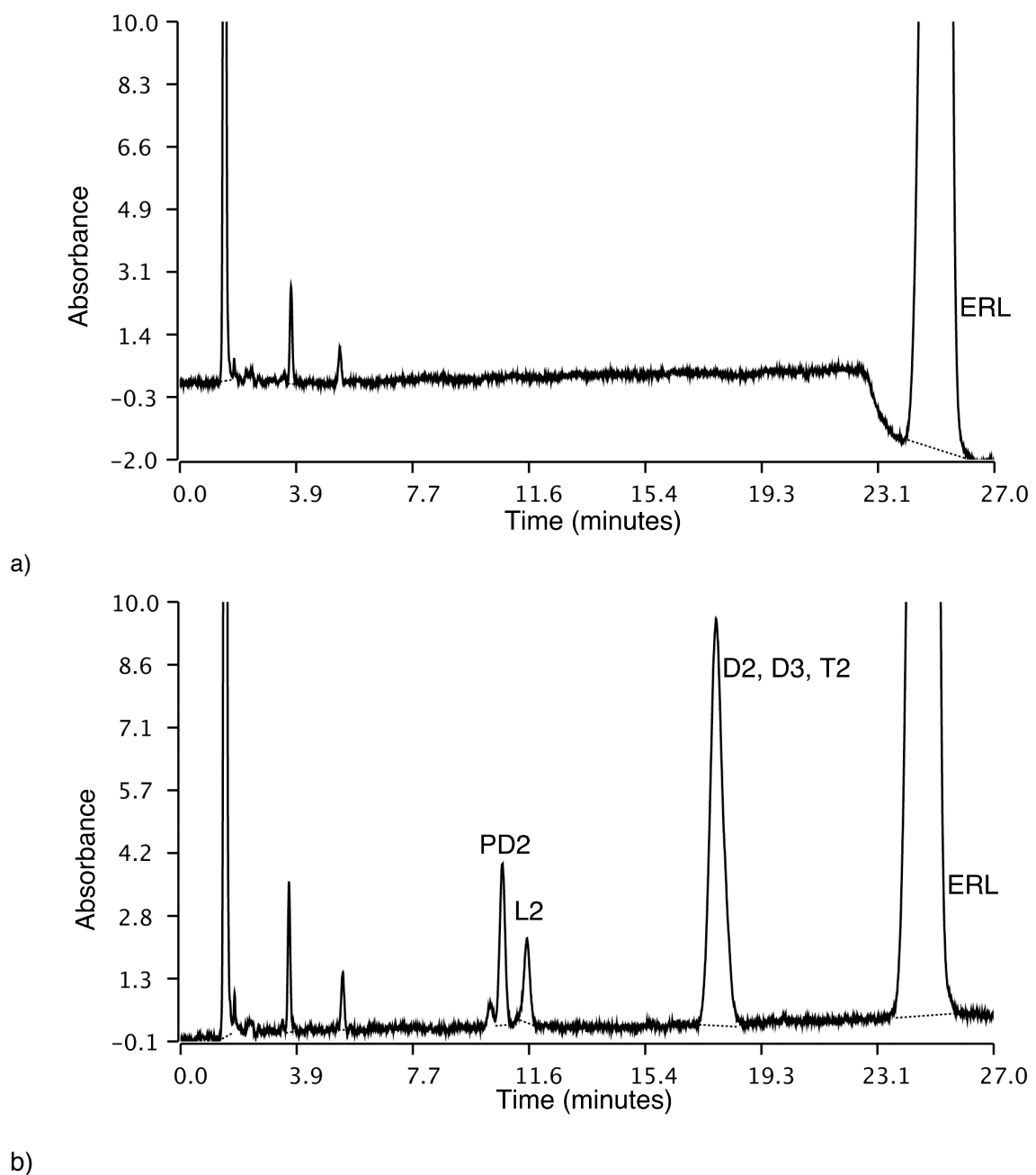
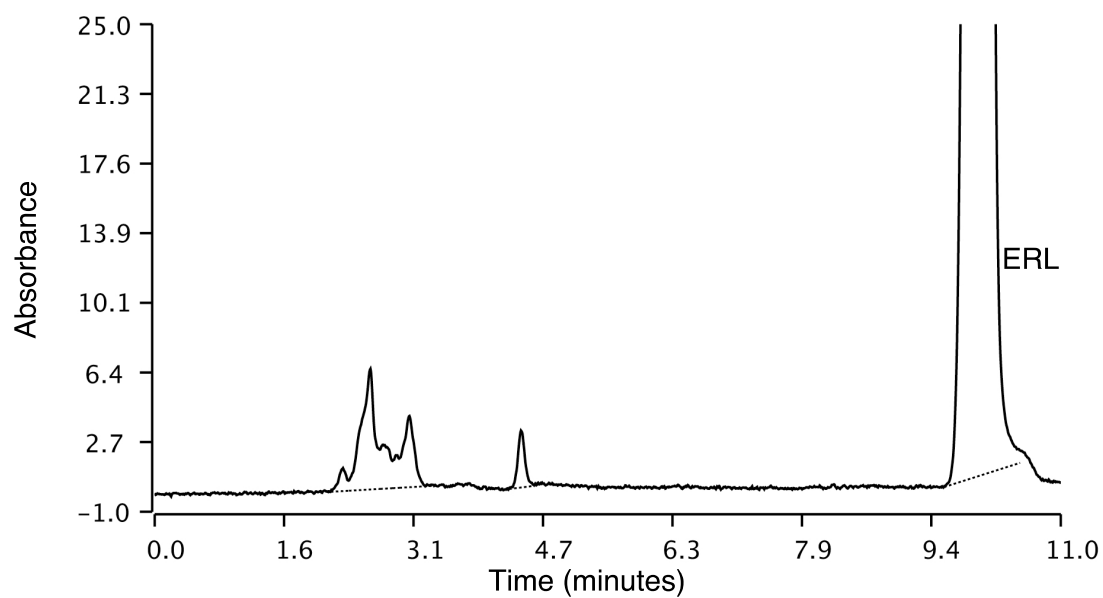
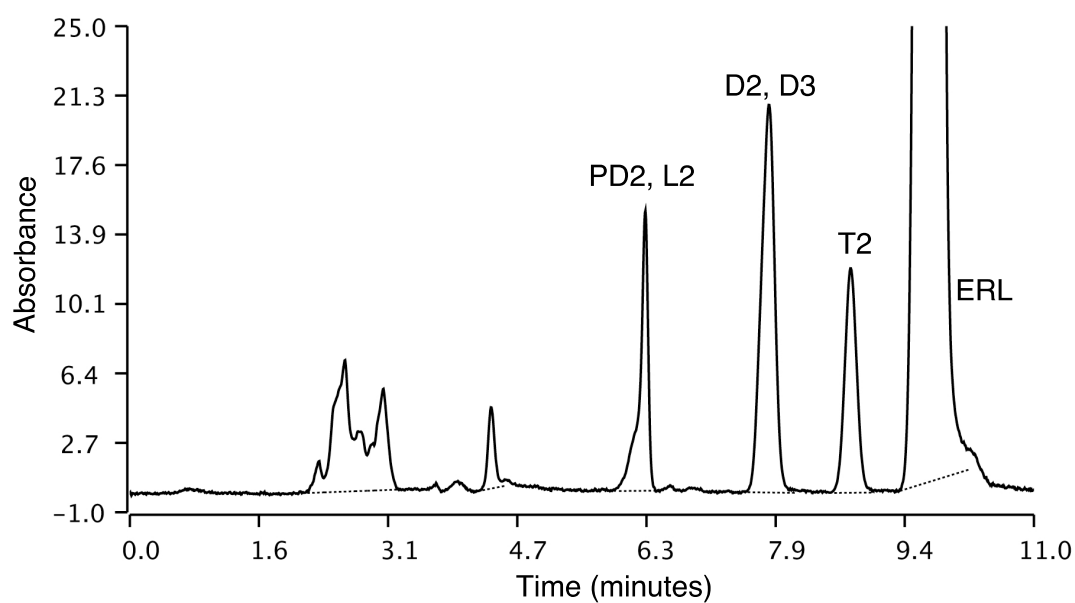


Figure 5.2. Normal phase (silica column) HPLC chromatograms of control (a) and PUV exposed (b) white button mushrooms



a)



b)

Figure 5.3. Normal phase (CN column) HPLC chromatograms of control (a) and PUV exposed (b) white button mushrooms

5.3.5 Statistical Analysis

All experiments were performed in duplicate. One-way ANOVA and Tukey's Test were performed using Minitab 15 (Minitab Inc., State College, PA, USA) and SPSS (IBM) to compare means of photoproducts between treatment levels to determine significant difference between means. ($\alpha = 0.05$)

5.4 Results and Discussion

Vitamin D₂ production was observed to increase in sliced white button mushrooms as a function of PUV dose (**Figure 5.4**), as predicted by our previous studies. The untreated control mushrooms contained no detectable levels of vitamin D₂. Following 3 pulses, the equivalent to 1 s of exposure, vitamin D₂ content increased to 32.2 µg/g dw, which is equivalent to 1300% DV of vitamin D per serving of fresh mushrooms (84 g fresh weight) based on the current RDA of 600 IU for vitamin D. Vitamin D₂ levels approached a maximum concentration of 124 µg/g dw (5100% DV/serving) upon treatment with 60 pulses (20 s total exposure). These levels are above the current tolerable upper level of intake of vitamin D (*i.e.*, 4000 IU/day, or 100 µg/day) (28); however, such conditions were required in order to accurately determine the kinetics of formation of L₂, T₂, and PD₂.

There were no detectable levels of any of the photoisomers in untreated mushroom samples. Following 3 pulses, the L₂ content was 3.05 µg/g dw and by 60 pulses had reached 30.5 µg/g dw (**Figure 5.5**). The T₂ content was 2.79 µg/g dw after 3 pulses and with 60 pulses reached a level of 24.7 µg/g dw (**Figure 5.6**). The PD₂ content was 9.77 µg/g dw after 3 pulses and with 60 pulses reached a level of 44.6 µg/g dw (**Figure 5.7**). The dose-response curves for all photoisomers tested were similar to that of D₂ in that product yields increased as a non-linear function of pulse number.

The photochemical reaction of ergosterol to yield PD₂ and other photoisomers has been previously characterized (10,12,120,121). This reaction, however, has not been fully investigated in mushrooms and instead has, to this point, focused solely on D₂ production. Jasinghe et al. investigated the kinetics of this conversion but focused on treatment parameters (time, temperature and moisture content) and only measured D₂

production (13). The kinetics of this reaction has been studied in model systems (122) in the case of the D₃ series in human skin (33,123,124), lizard skin (125) and liposomes (126). In most cases, the production of the photoproducts is non-linear with respect to dose. However, the relative amount of each photoproduct formed was observed to vary by matrix.

The non-linear increase in the photoproducts of ergosterol (**Figures 5.4-7**) has been demonstrated for both the D₂ and D₃ series (17,33,121,122). A steady state concentration of D₂ and D₃ series photoproducts was observed as the substrate was exposed to increasing irradiation. The isomerization of these products was also shown to be reversible (12). It has been demonstrated that upon extended irradiation by UV, the formation of suprasterol I and II from vitamin D and “over-irradiation products” or toxisterols from T₂ and PD₂ occurs, which is a non-reversible reaction (10,12). It is unlikely that these products would be present in the mushrooms treated in this study, as the main reaction products have not reached the steady state concentration after 60 pulses, although these compounds should be analyzed in future studies. The level of vitamin D₂ present in these samples may be initially too high for human consumption and, as such, these toxisterols may not be of concern since the levels of the photoproducts are low in mushrooms treated with low doses of UV.

The reaction pathway of ergosterol to its photoproducts is also dependent upon the wavelength of the incident UV source (120,127,128). Norval et al. calculated the percentages of PD₃, T₃ and L₃ in the photostationary state based on wavelength used for irradiation (17). The ratio between the three isomers varied with wavelength with virtually no L₃ produced in the UVC range (254 nm) while, at longer UVB wavelengths (310 nm), less T₃ was produced. The main reaction product observed at all dose levels is D₂ followed by PD₂, L₂ and T₂ respectively. The PUV apparatus used in the present study encompasses a broad range of light including all wavelengths of the UV spectrum. Therefore, depending on the spectral characteristics of the light generated by the lamp, the ratio of the photoproducts is expected to vary. The PUV lamp used in this study was a B-type lamp, which excluded wavelengths in the UV-C region while allowing more photons in the UV-B region to pass. Based on this, the results of this study are in agreement with the literature.

The conversion of PD₂ to vitamin D₂ is a thermally initiated [1,7] sigmatropic shift that occurs at a rapid rate at elevated temperatures (e.g., human body temperature); therefore, isomerization could occur in the body (129,130). As such, the PD₂ content of PUV treated mushrooms is of importance. The effect of thermally processing mushrooms on the vitamin D₂ content has been studied (99) and was shown to result in a small decrease in D₂. PD₂ could be converted in the body when ingested and, thus, the actual dose of vitamin D₂ would be higher than the measured content in the mushrooms under this scenario.

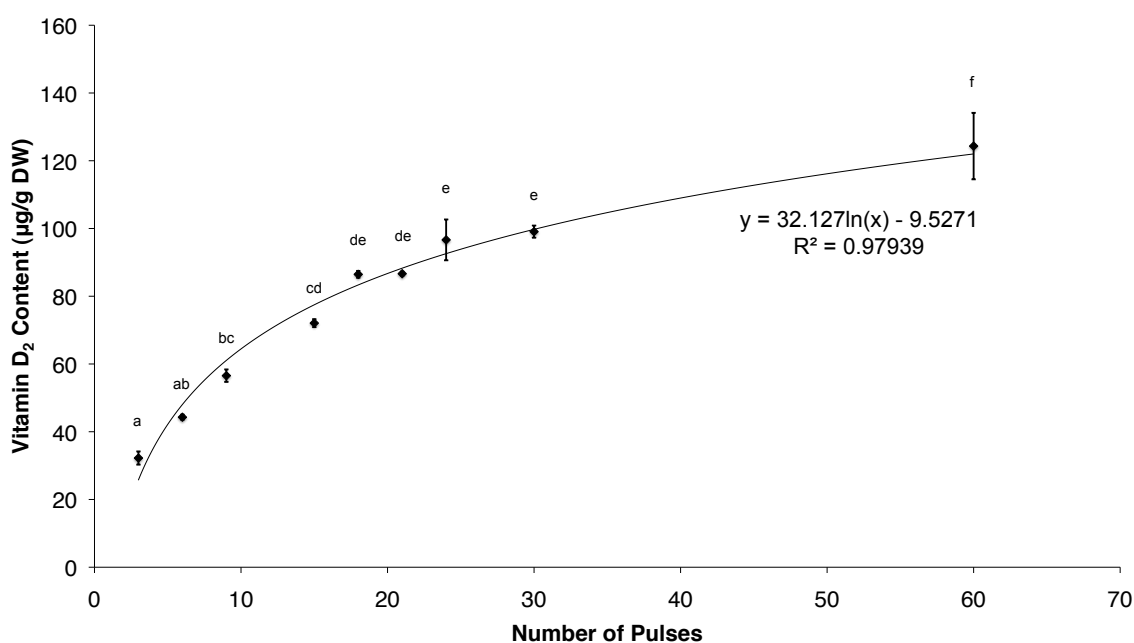


Figure 5.4. Dose response of vitamin D₂ content of PUV treated white button mushrooms

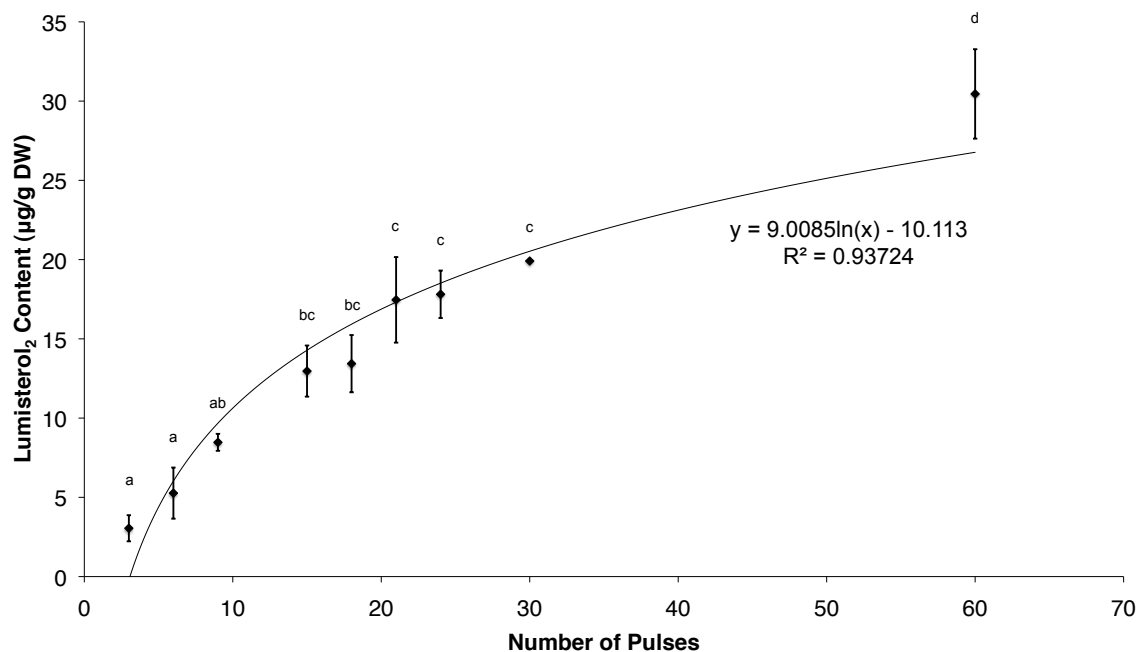


Figure 5.5. Dose response of lumisterol₂ content of PUV treated white button mushrooms

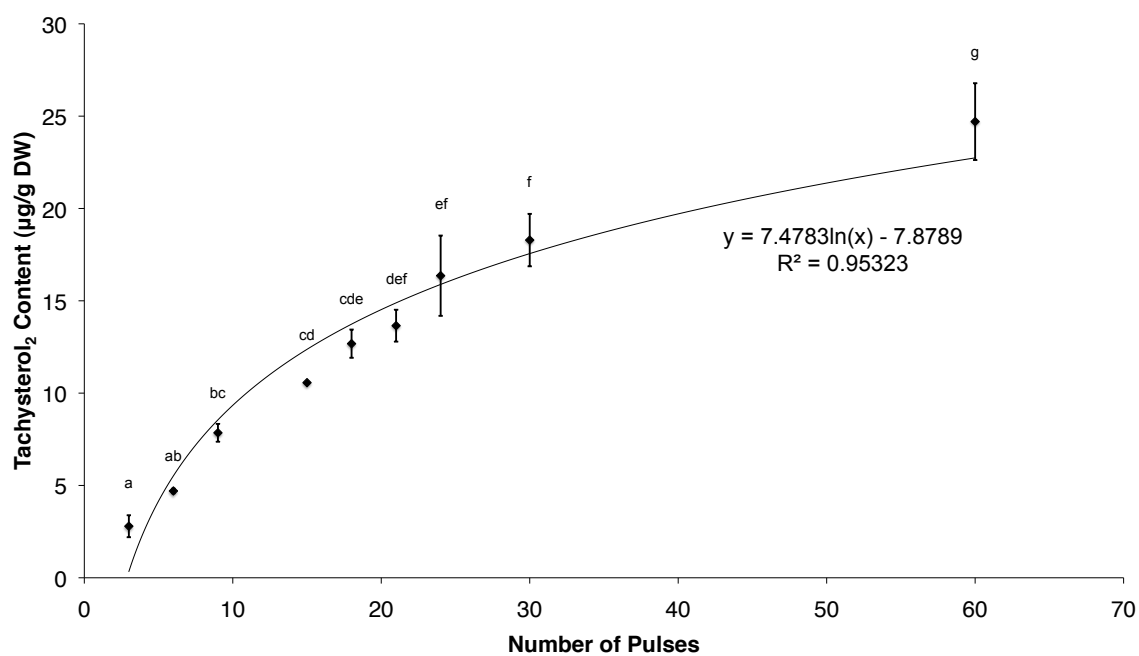


Figure 5.6. Dose response of tachysterol₂ content of PUV treated white button mushrooms

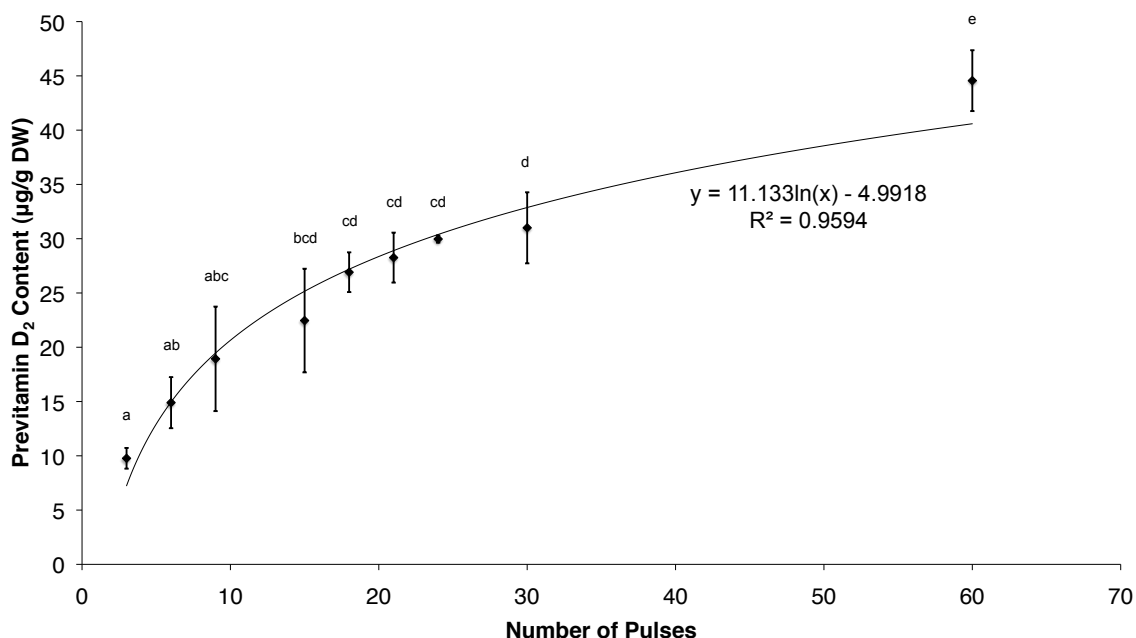


Figure 5.7. Dose response of previtamin D₂ content of PUV treated white button mushrooms

5.5 Conclusions

It was observed that when sliced button mushrooms are irradiated with PUV in a single layer the increase in vitamin D₂ trends similarly to mushrooms treated in a batch system (demonstrated in **Chapter 4**). In this single layer treatment regimen, mushrooms were treated with an extended dose of PUV irradiation and vitamin D₂ reached much higher levels overall at each dose. This is most likely due to the dilution effect of a batch system. Mushrooms contained practically zero vitamin D₂ with no irradiation and increased to 32.2 µg/g dw with 3 pulses and increased to a maximum value of 124 µg/g dw with 60 pulses. Each of the photoproducts increased in a similar manner with increasing PUV irradiation. While vitamin D₂ was the most plentiful product, PD₂, L₂ and T₂ were formed in decreasing order of abundance. PD₂ increased from 9.77 µg/g dw with 3 pulses to 44.6 µg/g dw after 60 pulses, while L₂ increased to 3.05 and 30.5 µg/g dw with 3 and 60 pulses, respectively, and T₂ content reached 2.79 and 24.7 µg/g dw with 3 and 60 pulses respectively.

The results of this study show, for the first time, the production of the additional photoproducts previtamin D₂, lumisterol₂ and tachysterol₂ in addition to vitamin D₂ from

ergosterol in mushrooms resulting from UV irradiation. This phenomenon shows that care must be taken when irradiating mushrooms for the purposes of increasing D₂ content. The presence of these photoproducts is an example of the “whole food” concept and suggests that PUV irradiated mushrooms may have implications on health beyond the benefits of vitamin D₂ alone. Future studies are needed to investigate the effects the photoproducts identified in the present study have on human health.

6. Conclusions and Suggestions For Future Research

6.1 Conclusions

In **Chapter 3**, we observed that all four mushroom varieties tested contained ca. zero vitamin D₂ with no irradiation; however, upon PUV irradiation, vitamin D₂ content increased with as little as one pulse (less than one second) to 5.11, 4.43, 5.71 and 7.95 µg/g dw in white button, brown button, shiitake and oyster mushrooms, respectively. Thereafter, vitamin D₂ content increased with subsequent PUV irradiation, reaching 13.36 and 11.06 µg/g dw in white and brown button mushrooms, respectively, with 4 pulses and 13.94 and 19.35 µg/g dw in shiitake and oyster mushrooms, respectively, with 3 pulses.

Mushroom products including powders, spent growing substrates and grain inoculated with mycelium were also irradiated with PUV to increase vitamin D₂ content from ca. zero in all cases. White button mushroom powders with and without selenium enrichment, treated with 4 pulses had vitamin D₂ increase to approximately 12 µg/g dw, which increased with subsequent exposure to approximately 27 µg/g dw after 16 pulses. There was no significant difference in vitamin D₂ production between control powder and selenium-enriched powder. In king oyster mushroom powder similar levels were achieved after 4 and 16 pulses (10.40 and 22.95 µg/g dw, respectively). In king oyster powder, ergothioneine levels were measured with PUV treatment, no significant change was observed. Mushroom mycelial biomass and spent substrates showed a lower potential for vitamin D₂ enrichment. Grain inoculated with king oyster mycelium reached a vitamin D₂ content of 1.78 µg/g dw after 16 pulses when treated in powder form. Spent king oyster mushroom substrate contained approximately 2 µg/g dw after 16 pulses

whereas spent maitake mushroom substrate contained less than 1 $\mu\text{g/g dw}$ after the same treatment.

I have shown that pulsed UV technology could be a more practical method of UV irradiation of mushrooms for the mushroom industry than previous methods due to the shorter amount of time needed for exposure to achieve high amounts of vitamin D₂. The UV-B bulb used in this study, which filtered out UV-C wavelengths, was found to be highly effective in converting ergosterol to vitamin D₂ and would appear to more practical for commercial use than bulbs that did not filter out UV-C wavelengths, since there would be no generation of ozone that could compromise worker safety.

The ergothioneine content of mushrooms in both fresh and powder form did not appear to change significantly with pulsed UV treatment. This shows that fresh mushrooms or mushroom products high in selenium, vitamin D₂ and ergothioneine can be practically achieved. Thus, these mushrooms could be marketed as a good or excellent source of all of these nutrients.

The outcome of this study allows for many practical applications. We have shown that a rapid method of increasing the vitamin D₂ content is achievable using pulsed UV light. The mushrooms produced can be sold fresh and would allow consumers an additional source of vitamin D in the diet. These products are also a non-animal source of vitamin D. The results of the powder and substrate enrichment show that these materials could be used as a food ingredient to increase the vitamin D content of any number of food products or even animal feeds. Overall, this study illustrates the potential health benefits of PUV irradiated mushrooms as an abundant source of vitamin D₂.

In **Chapter 4**, it was observed that the vitamin D₂ content of sliced white button mushrooms treated in a batch system increased from practically zero to 12.6 $\mu\text{g/g dw}$ after 4 pulses and increased non-linearly to approach a maximum value of approximately 27 $\mu\text{g/g dw}$ between 12 and 18 pulses. It was also observed that sliced mushroom produced more vitamin D₂ than whole mushrooms (113 and 81% more in brown and white buttons, respectively) and brown button mushrooms produced less vitamin D₂ than their white counterparts (21 and 33% less in sliced and whole, respectively). When

treated in a batch system it was observed that mushrooms treated in a full commercial package (230 g) produced 40% less vitamin D₂ than when treated in a 150 g package. In the shelf life portion of this study a 24% decrease was observed in vitamin D₂ levels after 3 days of storage at 3°C but levels remained constant afterward through 11 days. No significant differences in quality attributes measured were observed (color, appearance, microbial load, and weight change).

The observations in **Chapter 4** demonstrate the use of PUV treatment of fresh mushrooms for the purpose of increasing vitamin D₂ content is an effective and rapid method with no known deleterious effects on product quality. The shortest exposure time of any previous study using continuous UV regimes to achieve a level equivalent to 100% DV/serving was ca. 8 minutes (94). In contrast, we have demonstrated in this present study that PUV systems are capable of achieving similar levels of vitamin D₂ with one second or less of total exposure time and without negative effects on appearance. The PUV treatment described in this paper does not have an effect on microbial load, which suggests all mushrooms in a commercial package are not exposed to PUV irradiation. Therefore, this study suggests that sliced mushrooms treated in a single layer of small batch system may be the optimal method for enrichment. Furthermore, it was shown that the vitamin D₂ produced under the parameters of this study was stable under commercially-relevant storage conditions. Future study into other quality attributes such as flavor and the effect on other bioactive compounds would be of interest.

It was observed in **Chapter 5** that when sliced button mushrooms are irradiated with PUV in a single layer the increase in vitamin D₂ is similar to mushrooms treated in a batch system (demonstrated in **Chapter 4**). In this single layer treatment regimen, mushrooms were treated with an extended dose of PUV irradiation and vitamin D₂ reached much higher levels overall at each dose. This is most likely due to the dilution effect of a batch system. Mushrooms contained practically zero vitamin D₂ with no irradiation and increased to 32.2 µg/g dw with 3 pulses and increased to a maximum value of 124 µg/g dw with 60 pulses. Each of the photoproducts increased in a similar manner with increasing PUV irradiation. While vitamin D₂ was the most plentiful product, PD₂, L₂ and T₂ were formed in decreasing order of abundance. PD₂ increased from 9.77 µg/g dw with 3 pulses to 44.6 µg/g dw after 60 pulses, while L₂ increased to 3.05 and

30.5 $\mu\text{g/g dw}$ with 3 and 60 pulses, respectively, and T_2 content reached 2,79 and 24.7 $\mu\text{g/g dw}$ with 3 and 60 pulses respectively.

The results in **Chapter 5** show, for the first time, the production of the additional photoproducts previtamin D_2 , lumisterol₂ and tachysterol₂ in addition to vitamin D_2 from ergosterol in mushrooms resulting from UV irradiation. These results suggest that care must be taken when irradiating mushrooms for the purposes of increasing D_2 content. The presence of these photoproducts suggests that PUV irradiated mushrooms may have implications on health beyond the benefits of vitamin D_2 alone. Future studies are needed to investigate the effects the photoproducts identified in the present study have on human health.

The work presented here demonstrates that pulsed UV light can be a viable method for rapidly increasing the vitamin D_2 content of fresh mushrooms, mushroom powders and other mushroom products containing ergosterol. Previous work using conventional continuous lamps has also shown to be effective in increasing the vitamin D_2 content but with a much longer treatment time needed. In a commercial setting PUV may prove to be more effective because of the time saved.

It has also been shown that the vitamin D_2 produced is relatively stable during storage. This is important for producers due to regulatory issues related to labeling and the amount of vitamin D_2 present in a commercial mushroom product. It is also of importance overall because of the health benefit associated with obtaining a desired amount of vitamin D.

In addition to the vitamin D_2 content, it was shown that PUV technology has no deleterious effects on quality attributes when compared to non-irradiated mushrooms. These attributes are mainly related to consumer acceptance based on visual appearance and color. However, it was also observed that PUV irradiated mushrooms showed no difference in deterioration compared to non-irradiated mushrooms over an extended shelf life study.

This study, for the first time, has shown the effects of PUV irradiation of mushrooms on the content of the other photoproducts of ergosterol. These include pre-vitamin D₂, tachysterol₂, and lumisterol₂. These products are of interest for two main reasons. First, the relationship between the rate at which these products are formed compared to vitamin D₂ is important to the overall reaction kinetics of the entire process. The production of these compounds follows a pathway in which the first product formed is previtamin D₂ and from there additional compounds are formed. Secondly, because these products are all isomers of each other, they may impart additional health consequences. However, future study is needed. Other examples of this phenomenon in food include lycopene from tomatoes which is found to be more active in its *cis* form than in the *trans* conformation (131) and mixed tocopherols having more of an inhibitory effect on lipid peroxidation than α -tocopherol alone (132).

In summary, the vitamin D₂ content of mushrooms can easily and rapidly be produced using pulsed UV irradiation. These mushrooms provide an ideal source for delivery of vitamin D₂ because of the biological potential the other photoproducts that are produced, as well as other important nutrients such as potassium, selenium and riboflavin and ergothioneine.

6.2 Future Work

6.2.1 Comprehensive Comparison of Conventional UV and PUV Irradiated Mushrooms

In order to better understand the differences between mushrooms treated with conventional UV systems and PUV systems, a direct comparison needs to be made. This can be accomplished when certain parameters are properly controlled. The main factor would be total irradiation dose. The main difference between these technologies is the ability of PUV systems to deliver a high dose of energy in a short period of time. If the dose is equalized between both systems a better comparison can be made. In addition, because PUV is a broadband system, the wavelengths used in each system must be controlled. This can be achieved by utilizing several different lamps with varying wavelengths and the use of filters on both types of lamps.

6.2.2 Ergosterol

The relationship between starting levels of ergosterol and final vitamin D₂ content is not fully understood. The data generated in this study suggest that the ergosterol that is present at the surface of the mushroom is likely the only ergosterol that is physically able to take part in the photochemical conversion. This is most likely due to the low penetrating properties of UV irradiation. In future studies, it would be beneficial not only to collect data on total levels of ergosterol in a mushroom but also to understand how much ergosterol is actually available to take part in the conversion to vitamin D₂. This can be done through subjecting incrementally thinner slices of mushrooms to PUV irradiation and measuring ergosterol and vitamin D₂. This would also serve to generate data to understand the penetration depth of PUV irradiation.

6.2.3 Tissue Dissection and Evaluation of Ergosterol

The distribution of ergosterol in various tissues of mushrooms has been shown to vary from species to species. A future study should be undertaken, wherein mushrooms of various species are dissected so as to separate the outer cap layer, cap, gills and stipe and the ergosterol content measured in all tissues. In addition, each tissue could be subjected to PUV irradiation and vitamin D₂ content measured to examine the effects of ergosterol content and/or morphology on the conversion.

6.2.4 Surface Area Effects on Vitamin D₂ Production in Mushrooms

The relationship between vitamin D₂ content in mushrooms treated with gill tissue exposed and unexposed (such as a whole mushroom), wherein more vitamin D₂ is produced in mushrooms with gill tissue exposed, could be explained by two hypotheses. This phenomenon could be due to a higher ergosterol content in the gill tissues, which by slicing and exposing this tissue to UV irradiation could lead to higher levels of vitamin D₂ than when not directly exposed. Conversely, this effect could also be due to surface area effects, whereby slicing increases the total surface area of the mushroom and thus more ergosterol is available to take part in the conversion to vitamin D₂. A series of experiments should be conducted to determine the surface area and ergosterol

mediated effects on vitamin D₂ content. This could involve irradiating powders of the different mushroom tissues to control for surface area or preparing mushroom samples so that surface area is uniform.

6.2.5 Additional Parameters of Mushroom Quality

In addition to the quality parameters measured in this study (color, appearance, weight change, microbial load) it would be beneficial to examine other quality parameters that could be affected by PUV irradiation. This includes other dietary components of significance, lipid oxidation and flavor.

In this study, PUV irradiation was shown to have an effect on vitamin D₂ content, but not on ergothioneine content. It would be beneficial to ensure that this treatment does not degrade any other nutritional components. In a recent study it was shown that UV-B or sunlight irradiation does not significantly affect riboflavin, vitamin B₆, and niacin content as well as having no significant effect on fatty and amino acids (4). However, this study was only conducted with one dose of UV irradiation. A comprehensive evaluation of all bioactive components contained in mushrooms with increasing doses of UV irradiation should be undertaken. This will ensure that UV irradiation has no negative effects on the health benefits of mushrooms.

UV irradiation is known to have effects on lipid peroxidation. It is unclear at this point if UV irradiation of mushrooms induces oxidation on the fatty acids that are contained therein. If oxidation is occurring, this could negatively impact flavor. A simple way to evaluate these effects would be to employ a blinded sensory analysis on control and UV treated mushrooms to determine if a difference is detectable by consumers. This could be a triangle test, in which three samples are given where 2 are the same and one is different and the odd one out must be identified. Additional sensory evaluation should be undertaken to determine if there are any flavor defects or other sensory issues in UV treated versus control mushrooms.

6.2.6 Expansion of Shelf Life Studies

The shelf life study conducted in **Chapter 4** was only conducted at refrigeration temperature and using one starting UV dose. This was done mainly to get a general idea of overall effect of storage on vitamin D₂ content. Additional time points would have also added too many variables, which would have been financially prohibitive and time consuming. As a basis for future work, however, the work in this thesis provides for a good starting point. There are several factors that should be expanded on in future studies.

First, these experiments should be replicated at additional temperatures. The original study used a temperature of 3°C, which approximates storage conditions by consumers. However, mushrooms are also stored under retail conditions, which are typically in the 12°C range. Therefore, this temperature would make the most sense for additional study of the stability of vitamin D₂ and other shelf life factors. Additionally, abuse conditions such as an elevated temperature of 22°C could be examined.

Secondly, more starting points for PUV irradiation dose/vitamin D₂ initial content would be of interest. By only selecting one initial dose, it is unknown if the degradation kinetics are concentration dependent. Based on the small amount of data available in the literature (28) this may not be the case, but further study is warranted.

6.2.7 Photoproducts of Ergosterol

6.2.7.1 The Role of Photoproducts of Ergosterol in Human Health

The other major photoproducts identified in mushrooms this study in addition to vitamin D₂ include previtamin D₂, tachysterol₂ and lumisterol₂. The role the photoisomers play in human health is not fully understood. However, based on the fact these products are structurally similar and are often similar to vitamin D analogs, which are used for clinical treatment, there may be a chance that these products are of some additional benefit. Alternatively, these compounds could have deleterious effects on human health.

Clinical studies in which these compounds are administered to humans would ultimately give a definitive answer to the role these compounds have on human health. However, initial studies using in vitro assays and mouse models should be utilized to minimize costs and to guard against potential toxicity. If these compounds are found to have a beneficial effect on human health, the case for vitamin D₂ enriched mushrooms, as a healthful will be much stronger.

6.2.7.2 Production of Toxisterol and Additional Photoproducts

In addition to the photoproducts identified and quantified in this study, the photochemical reaction pathway is known to produce many more compounds, such as the toxisterols. Future studies should focus on identifying whether or not these compounds are produced in mushrooms and the quantity produced if present. These compounds may have health benefits or be potentially harmful and thus once these compounds are identified the impact on health can be determined.

6.2.7.3 Photoproducts of Ergosterol Production Using Continuous UV Irradiation

It is unknown if the photoproducts of ergosterol observed in this study using PUV irradiation are also produced when mushrooms are treated using conventional continuous UV irradiation. If these products were shown to be of importance to human health it would be of interest to investigate the formation of these products using all available lamp technologies. Therefore, a simple study using continuous lamps of all three major sub-regions of the UV spectrum (UV-A, UV-B and UV-C) should be conducted and the photo isomers quantified as described in this study.

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Curriculum Vitae

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Publications

Michael Kalaras, Robert Beelman and Ryan Elias. "*Effects of Postharvest Pulsed UV Light Treatment of White Button Mushrooms (Agaricus bisporus) on Vitamin D₂ Content and Quality Attributes*". Journal of Agricultural and Food Chemistry. 2012, 60 (1), pp 220–225

Robert Beelman and Michael Kalaras, Inventors. "*Methods and compositions for improving the nutritional content of mushrooms and fungi*" – United States Patent Application 20090269441.

Presentations

Mushroom Industry Conference. 2011. Mendenhall, PA. "*Vitamin D and Mushrooms*". Presentation.

Mushroom Industry Conference. 2010 University Park, PA. "*Vitamin D and Mushrooms: Enrichment With Pulsed UV Light*". Presentation.

IFT Annual Meeting and Food Expo. 2010. Chicago, IL. "*Post-harvest Production and Retention of Vitamin D₂ in Fresh Mushrooms Treated With Pulsed UV Light and Effects on Quality*". Poster.

IFT Annual Meeting and Food Expo. 2009. Anaheim, CA. "*Vitamin D₂ Enrichment of Fresh Mushrooms Exposed to Pulsed Ultraviolet Light*". Poster.

Mushroom Industry Conference. 2009. Avondale, PA. "*Vitamin D Enrichment of Fresh Mushrooms Using Pulsed UV Light*". Presentation.

Scholarships and Awards

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