# CONVERSION OF ERGOSTEROL IN EDIBLE MUSHROOMS TO VITAMIN $D_2$ BY UV IRRADIATION

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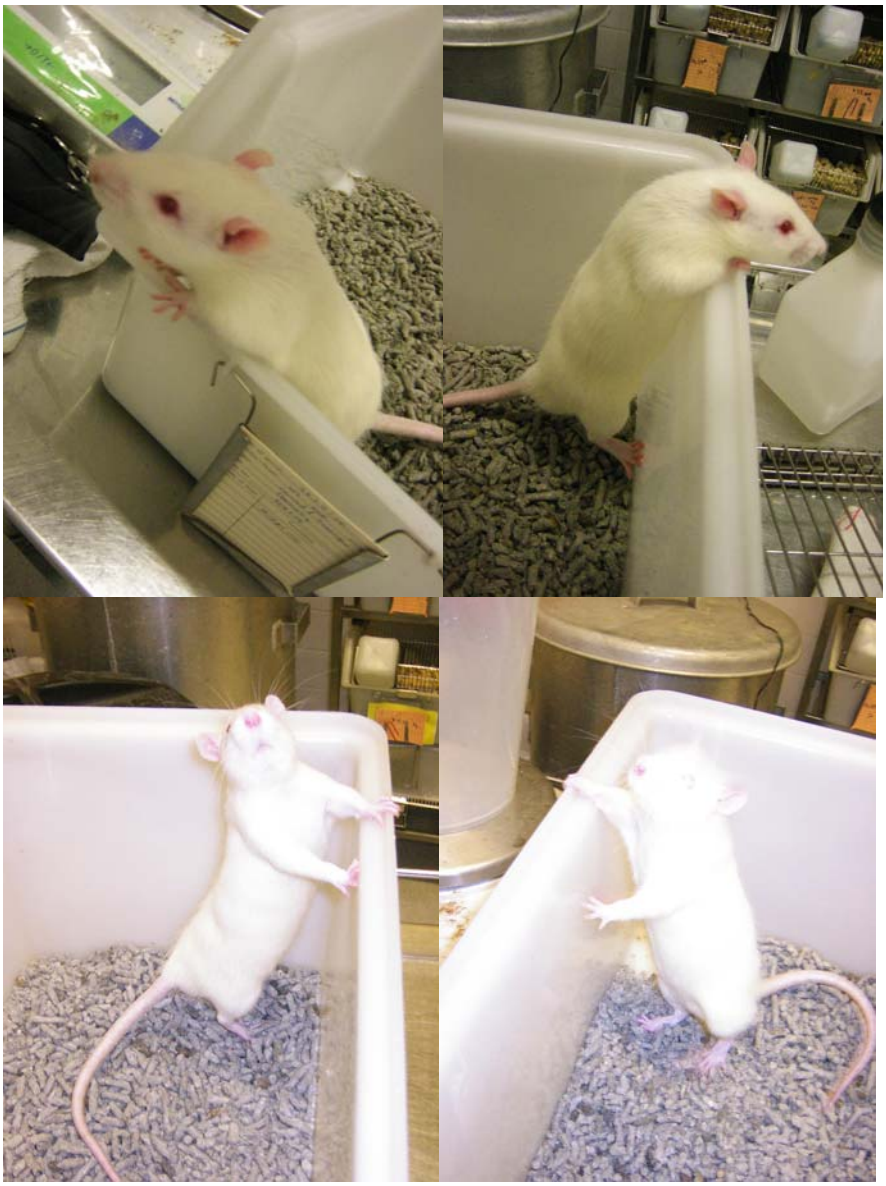
**DEDICATION**

This thesis is dedicated to the rats who sacrificed their lives for the advancement of science……



I can assure the readers that all the rats involved in this study were **treated in a humane fashion** in accordance with the guidelines of the National University of Singapore, **painlessly killed under anesthesia**, and disposed of in a manner prescribed by the National University of Singapore.

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## Summary

This project was planned to be carried out in two phases. In the first phase, the conversion of ergosterol in a variety of mushrooms to vitamin $D_2$ by irradiation was studied under different UV conditions (UV-A, UV-B, and UV-C) including an investigation of the kinetics of conversion of ergosterol to vitamin $D_2$. In the second phase, the bioavailability of vitamin $D_2$ from irradiated mushrooms was investigated in an animal model in order to predict the clinical applications of vitamin $D_2$ from irradiated mushrooms.

Analysis of ergosterol content in different tissues of Shiitake mushrooms showed a significant difference ($p < 0.01$) in its distribution. The conversion of ergosterol in whole mushrooms to vitamin $D_2$, by exposure to UV irradiation was significantly affected ($p < 0.01$) by the orientation of the mushroom tissues to the UV radiation. The highest ergosterol content was found in Button mushrooms (7.80 ± 0.35 mg/g DM) while the lowest was in Enoki mushrooms (0.68 ± 0.14 mg/g DM). The conversion of ergosterol to vitamin $D_2$ was about four times higher when gills were exposed to UV-A radiation compared with when the outer caps were exposed to the same radiation. The lowest conversion to vitamin $D_2$ (12.48 ± 0.28 μg/g DM) was observed for button mushrooms while the highest value (45.10 ± 3.07 μg/g DM) was observed for oyster mushrooms. The optimum moisture and temperature of mushrooms for this conversion was around 80 % (wet weight basis) and a temperature of around 35 $^{o}$C.

Fresh Shiitake mushrooms (*Lentinula edodes*), Oyster mushrooms (*Pleurotus ostreatus*), Button mushrooms (*Agaricus bisporus*), and Abalone mushrooms (*Pleurotus cystidus*) were irradiated with Ultraviolet-A (UV-A; wavelength 315 – 400), Ultraviolet-B (UV-B; wave length 290 – 315 nm), and Ultraviolet-C (UV-C; wave length 190 – 290 nm). Irradiation of each side of the mushrooms for one-hour, was found to be the optimum period of irradiation in this conversion. The conversion of ergosterol to vitamin $D_2$ under UV-A, UV-B, and UV-C was shown to be significantly different ($p < 0.01$). The highest vitamin $D_2$ content (184.22 ± 5.71 μg/g DM) was observed in Oyster mushrooms irradiated with UV-B at 35 $^{o}$C and around 80 % moisture. On the other hand, under the same conditions of irradiation, the lowest vitamin $D_2$ content (22.90 ± 2.68 μg/g DM) was observed in Button mushrooms.

Kinetics of conversion of ergosterol to vitamin $D_2$ has been investigated in cultivated edible mushrooms. It was observed that the rates of conversion of ergosterol to vitamin $D_2$ differed between different types of mushrooms. Both initial moisture content and temperature of irradiation influenced the conversion of ergosterol, and a 2 x 2 factorial design was used to study this influence. It was shown that the conversion of ergosterol to vitamin $D_2$ followed zero-order kinetics, where the rate constant varied with temperature according to the Arrhenius equation ($A_o = 7.32\ s^{-1}$; $E_a = 51.5\ kJ\ mol^{-1}$).

Having previously optimized a method for the conversion of ergosterol to vitamin $D_2$ in mushrooms, the study then examined the vitamin D enriched mushrooms (*Lentinula edodes*) for their bioavailability of the vitamin, using an animal model. Thirty male

Wistar rats were fed for one week with a diet deficient in vitamin D. After this one-week period, six rats were randomly selected and sacrificed for analysis of initial Bone Mineral Density (BMD), and serum level of 25-hydroxyvitamin D [(25(OH)D]. A group of 12 rats of the test animals received 1 μg of vitamin $D_2$/day from irradiated mushrooms for a period of four weeks until sacrificed. The remaining 12 rats were fed un-irradiated mushrooms at the same level to act as controls. At the end of a four week period, mean serum 25(OH)D level of the experimental group was 129.42 ± 22.00 nmol/L whereas it was only 6.06 ± 1.09 nmol/L in the control group. Femur BMD of the experimental group of animals was significantly higher ($p < 0.01$) than the control group. It may be concluded from the results that vitamin $D_2$ from UV-irradiated mushrooms is well absorbed and metabolized in this model animal system. Significant increase in femur bone mineralization ($p < 0.01$) was shown in the presence of vitamin $D_2$ from irradiated mushrooms compared with the controls.

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**ABBREVIATIONS**

| | |
|---|---|
| $1,25(OH)_2D$ | 1,25-dihydroxyvitamin D |
| $1,25(OH)_2D_2$ | 1,25-dihydroxyvitamin $D_2$ |
| 25(OH)D | 25-hydroxyvitamin D |
| $25(OH)D_2$ | 25-hydroxyvitamin $D_2$ |
| ACN | Acetonitrile |
| Alpha-MSH | alpha-Melanocyte Stimulating Hormone |
| AVA | Agri-food and Veterinary Authority |
| BMD | Bone Mineral Density |
| CHF | Congestive Heart Failure |
| DM | Dry Matter |
| IDDM | Insulin Dependant Diabetes Mellitus |
| NACLAR | National Advisory Committee for Laboratory Animal Research |
| PTH | Para Thyroid Hormone |
| RDA | Recommended Dietary Allowances |
| UV-A | Ultraviolet – A (wavelength; 315 – 400 nm) |
| UV-B | Ultraviolet – B (wavelength; 290 – 315 nm) |
| UV-C | Ultraviolet – C (wavelength; 190 – 290 nm) |
| VDD | Vitamin D Deficiency Disorders |

## LIST OF PUBLICATIONS BASED ON THIS STUDY

### Oral paper presentations based on this study

1. Vitamin $D_2$ and ergosterol in Shiitake mushrooms. HSA – NUS joint scientific seminar, April 9 2003, Singapore
2. Conversion of ergosterol to vitamin $D_2$ in shiitake mushrooms during drying. Regional workshop on drying technology, the third seminar and workshop, July 21 – 25 2003, Bogor, Indonesia.
3. Enhancement of vitamin $D_2$ in cultivated edible mushrooms. Regional conference for young chemists 2004 (RCYC 2004), April 13 – 14 2004, Penang, Malaysia.
4. UV-B irradiation enhances vitamin $D_2$ content in edible mushrooms. Institute of food technology annual meeting & food expo 2004 (IFT 2004), July 21 – 26 2004, Las Vegas, Nevada, USA.
5. Irradiated edible mushrooms to address the unrecognised epidemic among elderly; vitamin D deficiency, $5^{th}$ APRU doctoral students conference, August 9 – 13 2004, University of Sidney, Australia.
6. Can irradiated edible mushrooms be used as a vitamin D supplement for the population effected by vitamin D deficiency disorders? World Congress on Clinical Nutrition 2004 (WCCN 2004), November 30 – December 3, 2004, Phuket, Thailand.

**Poster paper presentations based on this study**

1. Simultaneous analysis of ergosterol and vitamin D in Shiitake mushrooms (*Lentinula edodes*) and effect of UV-B irradiation on the conversion of ergosterol to vitamin $D_2$. Singapore International Chemical Conference 3 (SICC 2003), Frontiers in Physical and Analytical Chemistry, December 15 – 17 2003, Singapore.
2. Can irradiated edible mushrooms be used as an alternative dietary source to prevent vitamin D deficiency common in elderly population? 2nd Asia pacific conference & exhibition on anti-ageing medicine 2003, September 8 – 11 2004, Singapore.
3. Can humans obtain vitamin D without their exposure to UV radiation from sunlight? 3rd Asia pacific anti-ageing conference and exhibition 2004, June 24 – 27 2004, Singapore.

**International journal paper publications based on this study**

1. Perera CO, Jasinghe VJ, Ng FL & Mujumdar AS (2003) The effect of moisture content on the conversion of ergosterol to vitamin D in Shiitake mushrooms. Drying technology 21, 1091 – 99.
2. Jasinghe VJ & Perera CO (2004) Distribution of ergosterol in different tissues of mushrooms and its effect on the conversion of ergosterol to vitamin $D_2$ by UV irradiation. Food Chem 92, 541-46.

**3.** Jasinghe VJ, Perera CO & Barlow PJ (2005) Bioavailability of vitamin $D_2$ from Irradiated Mushrooms; an *in-vivo* study. Br J Nut 93, 951-55.

**4.** Jasinghe VJ, Perera CO & Sablani SS (2005) Kinetics of the conversion of ergosterol in edible mushrooms. J Food Eng (under consideration).

**5.** Jasinghe VJ & Perera CO (2005) Ultraviolet irradiation: the generator of Vitamin $D_2$ from edible mushrooms. Food Chem (in press).

# PART I

# INTRODUCTION AND EXPERIMENTAL

# CHAPTER 1

# INTRODUCTION

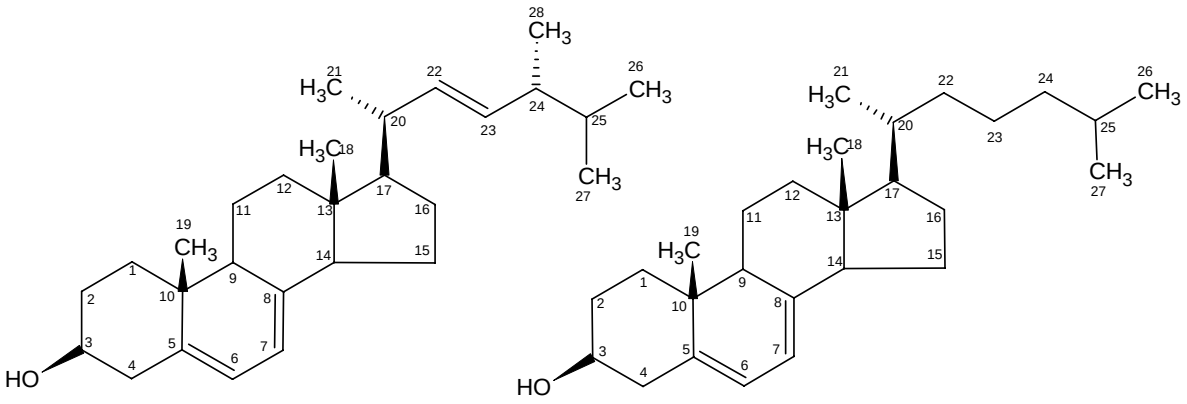
# CHAPTER 1

# INTRODUCTION

## 1.1: Vitamin D

In 1919, vitamin D, sometimes referred to as the "sunshine vitamin", was discovered by Sir Edward Mellanby (Mellanby, 1919) as part of his experiments on rickets. The main role of vitamin D is it's functioning as a hormone in maintaining calcium homeostasis, important in the mobilization, retention, and bone deposition of calcium and phosphorous (Webb, 1990; Morgan, 2001; Holick, 2001;). Even though the role of vitamin D in invertebrates is not clear, phytoplanktons and zooplanktons have been producing vitamin D for more than 500 million years (Holick, 2003). Therefore it might suggest that there are some other hidden functions of vitamin D in the human body, which have yet to be elucidated.

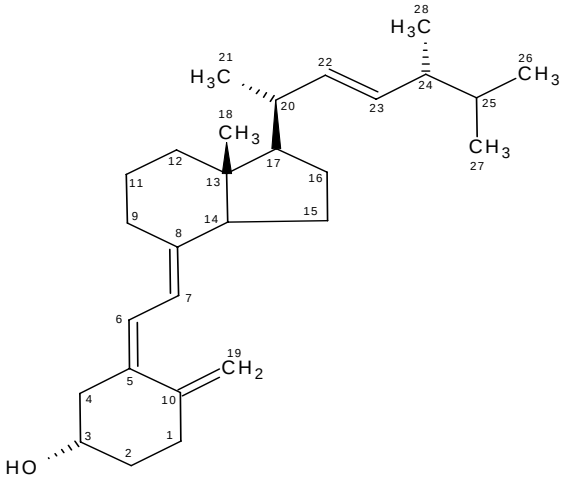
Vitamin D is the generic name of a closely related group of vitamins exhibiting similar biological activity to cholecalciferol (vitamin $D_3$). Ergocalciferol (vitamin $D_2$) is the synthetic form of vitamin D that can be formed from the plant steroid called ergosterol, by UV irradiation. Vitamin $D_2$ and $D_3$ can be further classified into vitamin $D_4$ (22,23 dihydroergocalciferol); vitamin $D_5$ (sitosterol or 24-ethylcholecalciferol); and vitamin $D_6$ (stigmasterol) according to their side chain structures (Napoli *et al*. 1979). Vitamins $D_2$ and $D_3$ have very similar structures except that vitamin $D_2$ has one more double bond and a methyl group compared with vitamin $D_3$. Figure 1.1 illustrates the chemical structures of previtamin $D_3$, vitamin $D_3$, previtamin $D_2$, and vitamin $D_2$.



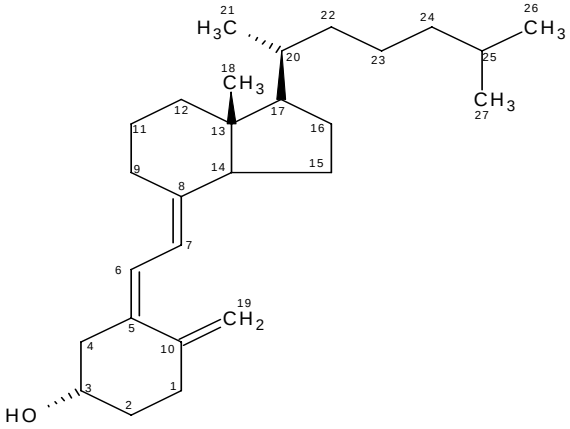
Ergosterol



7-Dehydrocholesterol



Vitamin $D_2$ (ergocalciferol)



Vitamin $D_3$ (cholecalciferol)

**Figure 1.1:** The chemical structures of ergosterol (previtamin $D_2$), 7-dehydrocholesterol (previtamin $D_3$), vitamin $D_2$, and vitamin $D_3$ (source: Horst & Reinhardt, 1997)

Vitamin D, along with the vitamins A, E, and K is categorized into the group of "fat soluble" vitamins. Overdosing of vitamin D is potentially toxic in view of its hypercalcemic effect (Adams & Lee, 1997; Marriott, 1997). However there are no

reported cases of vitamin D overdose (Marriott, 1997), on the contrary, there are concerns about the validity of current recommended dietary allowances (RDA). Some believe that the current RDA is not adequate (Hanly *et al*. 1985; McKenna *et al*. 1985; McKenna *et al*. 1995; Chapuy *et al*. 1997; McKenna & Freaney, 1998; Compston, 1998; Cheetham, 1999; Vieth, 1999; Heaney, 2000; Vieth, 2000) and even up to100 μg vitamin $D_3$ /day is a safe intake (Vieth *et al*. 2001).

### 1.1.1: Recommended daily dietary allowances (RDA)

In Singapore, current recommendations are only around 2.5 – 10 μg/day (Health Promotion Board, 2004). The RDA for vitamin D in the United States is 10 μg/day for children and 5 μg/day for adults (National Research Council, 1989). Table 1.1 illustrates vitamin D intakes by age according to FAO & WHO recommendations.

**Table 1.1:** Recommended dietary allowances for vitamin D by age groups

| Age group | Recommended Dietary Allowances (μg/day) |
|---|---|
| Infants | |
| 0-6 months | 5 |
| 7-12 months | 5 |
| 1-3 years | 5 |
| 4-6 years | 5 |
| 7-9 years | 5 |
| Adolescents, 10-18 years | 5 |
| Adults, | |
| 19-50 years | 5 |
| Older adults, 51-65 years | 10 |
| Elderly adults, 65+ years | 15 |
| Pregnant women | 5 |
| Lactating women | 5 |

Source: FAO/WHO (1998), expert consultation on human vitamin and mineral requirements

## 1.2: Vitamin D metabolism

Vitamin D undergoes a series of metabolic changes, in order to form biologically active analogues as illustrated in Figure 1.2.

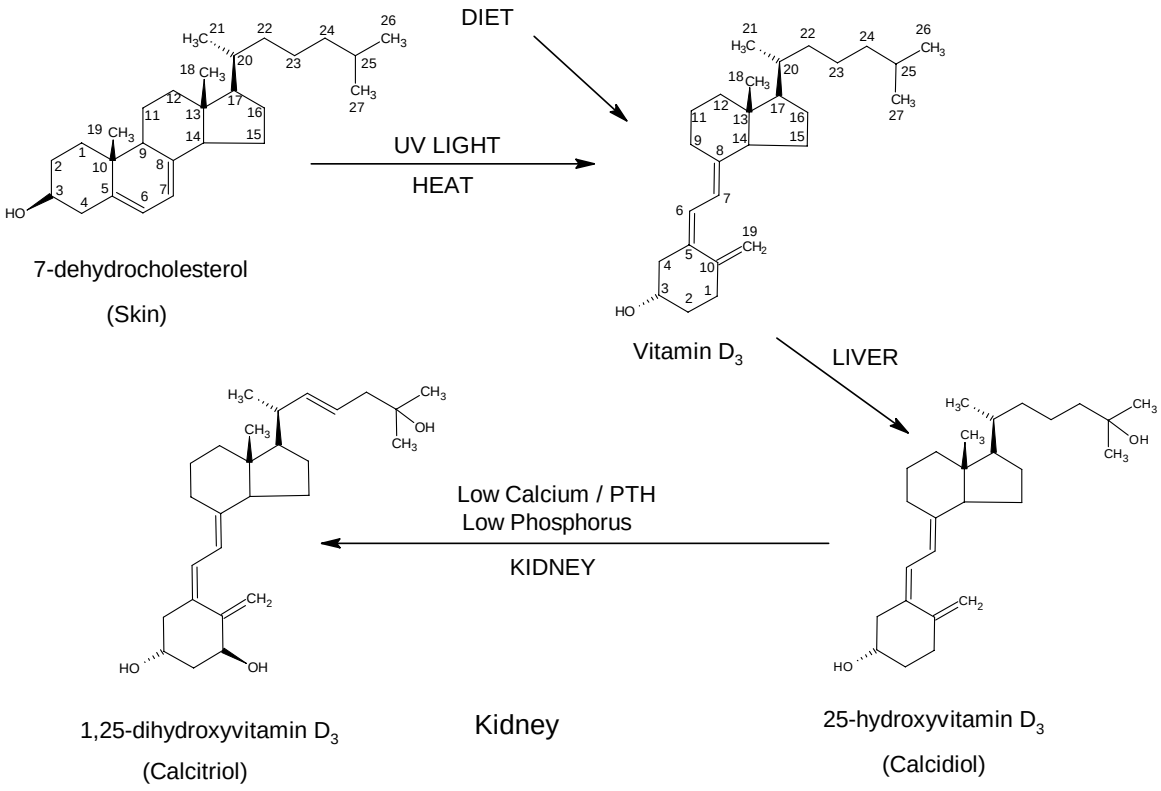



**Figure 1.2:** Pathways of Vitamin $D_3$ metabolism (source: Horst & Reinhardt, 1997)

Vitamin D activation is initialized by 25-hydroxylation in the liver (Horst & Reinhardt, 1997), and its metabolism is controlled by the physiological loop, which starts with calcium sensing by the calcium receptor of the parathyroid gland (Brown *et al*., 1998). In vitamin D deficiency, low serum calcium levels or elevated serum phosphate concentrations, stimulate the parathyroid gland to release Para Thyroid Hormone (PTH)

(Garabedian *et al*. 1972; Fine *et al*. 1993; McKenna & Freaney, 1998; Feldman, 1999). Increase in serum PTH concentration causes increased renal phosphate excretion which, in turn causes decreased intracellular phosphate. The combined effects of increased PTH and decreased phosphate, induce 1α-hydroxylase, which stimulates the production of 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] in the kidney (Feldman *et al*. 1996). This process is auto regulated by inhibiting the production of PTH by increased serum calcium concentrations (Herfarth *et al*. 1992; Feldman *el al*. 1996) and this process is linked with calcium homeostasis. Apart from its unique action on mineral homeostasis, a number of additional benefits of 1,25$(OH)_2$D have been discovered which are discussed below.

### 1.3: Clinical importance of vitamin D

Vitamin D is now known to have many beneficial clinical applications in animals other than those previously reported. There are a number of reviews on the link between vitamin D deficiency and chronic diseases now available (Ponsonby *et al*. 2002; Zitrermann, 2003; Heaney 2003). They are discussed below.

#### 1.3.1: Cancer

There are a number of malignancies associated with insufficient solar UV-B radiation and suggestions that these could be reduced significantly by increased UV-B exposure or supplementary vitamin D consumption (Grant, 2002a). High cancer mortality rates have been reported in the USA due to inadequate doses of solar UV-B (Grant, 2002b).

Furthermore, vitamin D deficiency has been shown to be associated with several types of cancers such as, breast (John *et al*. 1999; Grant, 2002a; O'Kelly & Koeffler 2003; Lowe *et al*. 2003; Berube *et al*. 2004), prostrate (Luscombe *et al*. 2001; Hansen *et al*. 2001; Tuohimaa *et al*. 2001; Polek & Weigle, 2002; Chen & Holick, 2003; Chen *et al*. 2003; Wang *et al*. 2003), skin (Braun & Tucker, 1997; Majewski *et al*. 2000; Kamradt *et al*. 2003), and a number of reports showing evidence of relationship between vitamin D deficiency and colon cancers are now available (Sadava *et al*. 1996; Pritchard *et al*. 1996; Mokady *et al*. 2000; Platz *et al*. 2000; Tangpricha *et al*. 2001; Lamprecht & Lipkin, 2001; Burton, 2001; Peters *et al*. 2001; Ogunkolade *et al*. 2002). It is now well established that apart from having an important role in calcium homeostasis and skeleton maintenance, the active analogs of vitamin D act as growth regulators on hyperproliferative cells including cancer cells.

### 1.3.2: Heart diseases

Congestive Heart Failure (CHF) has been found to correlate with serum vitamin D concentrations (Zittermann *et al*. 2003), and therefore it has been suggested that vitamin D deficiency may be a contributing factor in the pathogenesis of CHF in adults. In addition, vitamin D deficiency has been found to contribute to the heart failure in infants (Carlton-Conway *et al*. 2004). It has been well elucidated how vitamin D is associated with muscle weakness (Zittermann, 2003) and the CHF associated with vitamin D deficiency may also be explained in the same way. Moreover, there are a number of observations of cardiovascular diseases, which are associated with vitamin D

insufficiency that have been reported in the literature (Segall, 1989; Williams & Lioyd, 1989; Mancini *et al*. 1996; Norman *et al*. 2002; Zittermann *et al*. 2003). All these findings suggest that vitamin D plays a favourable role in the prevention of heart diseases.

### 1.3.3: Diabetes

In the past few decades, there has been a rapid increase in the incidence of insulin dependant diabetes mellitus (IDDM) worldwide, especially in Europe (Bingley & Gale, 1989; Diabetes epidemiology research international group, 1990; Karvonen *et al*. 1993; EURODIAB ACE study group, 2000; Levy-Marchal *et al*. 2001). It has been reported in some European countries that more children who later develop IDDM have been found to be born in spring and summer (Rothwell *et al*. 1996; Rothwell *et al*. 1999; Mikulecky *et al*. 2000; Ursic-Bratina *et al*. 2001; Songini & Casu, 2001; McKinney *et al*. 2001). In addition, fewer than expected diabetic children have been born in these countries at the end of summer in October (Samuelsson *et al*. 1999) when serum vitamin D concentrations are high (Vieth *et al*. 2001). Vitamin D deficiency in infancy or pregnancy has been found to be associated with IDDM (Billaudel *et al*. 1998; Bourlon *et al*. 1999; THE EURODIAB Substudy 2 Study Group, 1999; Stene *et al*. 2000; Hypponen *et al*. 2001). Furthermore, Syndrome 'X', is the term used to describe a cluster of disorders linked to insulin resistance with the potential risk of glucose intolerance (Reaven, 1995), and the risk of Syndrome 'X' is associated with vitamin D deficiency (Boucher, 1998).

### 1.3.4: Obesity

Obesity is emerging as a global public health crisis (WHO, 1997), and it is recognized as a major public health problem of global significance (Gill *et al*. 1999). The current estimates of global prevalence exceed 250 million (WHO, 1997). There is evidence reported in the literature that vitamin D deficiency has been linked with obesity (Heldenberg *et al*. 1992; Cantorna, 2000; Shi *et al*. 2001; Speer *et al*. 2001; Kamycheva *et al*. 2002).

Immunoreactive alpha-melanocyte stimulating hormone (alpha-MSH) plays an important role in energy metabolism and the production of this vital hormone is stimulated by UV exposure (Altmeyer *et al*. 1986). Obesity in humans and rodents is associated with high circulating leptin levels (El-Haschimi *et al*. 2000). Alpha-MSH acts on the brain to control the hormone leptin, which is produced by fat cells and insulin, which regulate food intake and body weight (Baskin *et al*. 1999; Schwartz, 2001). Decrease in body weight, serum concentrations of leptin and insulin have been observed in a human study where alpha-MSH has been given to human subjects over a period of six weeks (Fehm *et al*. 2001). However the long-term effect of alphaMSH on the control of body fat has yet to be fully elucidated. In addition, people who are obese are likely to be deficient in vitamin D because of decreased bioavailability of vitamin D due to its deposition in the adipose tissue (Wortsman *et al*. 2000).

In addition to the above described major chronic diseases, vitamin D deficiency has been found to be associated with arthritis (McAlindon & Felson, 1996; Braun & Tucker, 1997), hypertension (Rostand, 1997; Krause *et al*. 1998; Pfeifer *et al*. 2001) psoriasis (Fleischer *et al*. 1997; Kira *et al*. 2003) etc. Moreover, Vitamin D has been suggested for therapeutic applications in the treatment of several diseases including hyperproliferative diseases, secondary hyperparathyroidism, post transplant survival, and various malignancies (Peleg, 1997; Mehta & Mehta, 2002;).

The evidence discussed in this section strongly suggests that vitamin D deficiency is not only associated with skeleton bone disease but also with a number of chronic diseases. Hence, maintenance of healthy vitamin D status could be useful in the prevention of a wide spectrum of chronic diseases throughout the general population.

**1.4: Vitamin D deficiency**

The common results of severe vitamin D deficiency disorders are rickets in children and osteomalacia in adults (Feldman, 1999; Morgan, 2001). There are number of investigations that have been carried out on vitamin D deficiency disorders all over the world. Out of 824 elderly persons from 11 European countries, 36 % of men and 47 % of women had 25(OH)D concentrations below 30 nmol/L (van der Wielen *et al*. 1995). Vieth *et al*. (2001) observed vitamin D deficiency status is common in winter in Canadian women and revealed that their vitamin D intake was not sufficient to prevent it. They have suggested that the RDA for vitamin D is too low to prevent the insufficiency.

On the other hand in Finland, vitamin D intake was low and hypovitaminosis D was common in 9 – 15 year old apparently healthy Finnish girls, (Erkkola *et al*. 1998; Lehtonen-Veromma *et al*. 1999), and suggested that the daily dietary vitamin D supplementation with 10 μg/day was insufficient in preventing hypovitaminosis. Furthermore, it has been reported that British pre-school children are at risk of vitamin D deficiency (Davies *et al*. 1999, Lawson *et al*. 1999), and it was observed that most of the children with low haemoglobin levels show low plasma vitamin D values.

Almost all countries, which have conducted surveys in order to investigate the prevalence of vitamin D deficiency, have reported high prevalence of vitamin D deficiency among their populations. Vitamin D deficiency incidences have been reported in Netherlands (Meulmeester *et al*. 1990), Argentina (Oliveri *et al*. 1994, Oliveri *et al*. 2004), Pakistan (Henriksen *et al*. 1995), Kuwait (El-Sonbaty & Ghaffar, 1996), France (Chapuy *et al*. 1997), United States of America (Semba *et al*, 2000; Nesby-O'del *et al*. 2002, Gordon *et al*. 2004), China (Yan *et al.* 2000; Du *et al*. 2001), Australia (Diamond *et al*. 2000), India, (Goswami *et al*. 2000; Wayse *et al*. 2004), Bangladesh (Islam *et al*. 2002), Switzerland (Ginty *et al*. 2004), Ireland (Hill *et al*. 2004), and Norway (Henriksen *et al*. 1995; Holvik *et al*. 2004). Furthermore, in 2001, Vitamin D deficiency was reported as an unrecognized epidemic among the elderly population, and more than 50 % of elderly persons, living in their own homes and nursing homes in the USA were found to be deficient in vitamin D (Holick, 2001).

## 1.5: Sunlight as a source of vitamin D

Naturally, humans obtain vitamin D through cutaneous synthesis in the presence of ultraviolet B (UV-B) from sunlight and as well as from the diet. UV-B (UV-B; wave length 290 – 315 nm) represents approximately 1.5 % of the total solar spectrum (Hollosy, 2002). The precursor of vitamin $D_3$, 7-dehydrocholesterol found in the adipose tissues of the body can be converted to vitamin $D_3$ in the skin, and this process is supported by sunlight (Feldman *et al*. 1996).

Sunlight is the most important source of vitamin D for most of the people in the UK since the content of vitamin D in the largely unfortified British diet is low (Burns *et al*. 2003). Furthermore, sunlight is the major determinant of vitamin D stores in southern Tasmanian population (Jones *et al*. 1999). The cutaneous production of vitamin D under exposure to sunlight depends on number of factors such as latitude, season, exposure to direct sunlight, skin colour, and age (Holick, 1987; Webb *et al*. 1989; Need *et al*. 1993).

Sunscreens suppress cutaneous vitamin D synthesis (Matsuoka *et al*. 1987). Age-related decline in skin thickness may contribute to the age-related decline in 25(OH)D (MacLughlin & Holick, 1985; Need *et al*. 1993). Environmental factors such as latitude, season, and time of the day influence the cutaneous production of vitamin D (Holick, 1995).

The prevalence of vitamin D deficiency is higher in people with darker skins than people with white skins (Harris & Hughes 1998; Serhan *et al*. 1999; Shanna *et al*. 2002). Moreover, people with dark skins need to spend up to six times longer in the sun to obtain the same amount of vitamin D as a white person since the increased skin pigment can greatly reduce the penetration of ultraviolet radiation into the skin (Clemens *et al*. 1982). However, "Sun bingeing" may cause skin cancers (Wharton & Bishop, 2003). In addition, excessive exposure to ultraviolet radiation, produces undesirable inactive byproducts of previtamin D, such as tachysterol and lumisterol by photoisomerization (Havinga *et al*. 1960; Havinga, 1973). The evidence reviewed in this section suggest that there are a number of factors involved in cutaneous production of vitamin D under exposure to sunlight, and therefore the adequate exposure is not easily defined. On the other hand, still there are pro and counter arguments on the risks and benefits of sunlight among the scientific community, which keeps the question unreciprocated.

### 1.6: Dietary sources of vitamin D

Vitamin $D_3$ may be obtained in limited amounts from animal food products such as butter, margarine, milk & milk products, liver and other meats, and eggs. Oily fish (including mackerel, sardines, salmon and trout) and fish liver oils provide more substantial amounts of vitamin D but are eaten only by a minority of people. Vitamin D rich dietary sources are tabulated in Table 1.2.

**Table 1.2:** Vitamin D rich food sources[a]

| Dietary source | Vitamin $D_3$ (μg/100 g) |
|---|---|
| Cod liver oil | 250 |
| Salmon (raw) | 30.0 |
| Halibut Greenland (raw) | 15.0 |
| Rainbow trout (raw) | 13.0 |
| Salmon (canned) | 13.0 |
| Sardine in tomato source (canned) | 12.0 |
| Mackerel (raw) | 5.5 |
| Egg yolk (chicken)(raw) | 4.0 |
| Tuna (raw) | 2.9 |
| Mackerel in tomato source (canned) | 2.4 |
| Chicken | 1.5 |
| Milk, dry, whole, (powder) | 1.2 |

[a]: Source: Danish food composition databank (2004)

In the United States, vitamin D is added to milk and recently; in 2003, the Food and Drug Administration (FDA) released a regulation allowing the addition of vitamin D to calcium-fortified juices (Linda, 2003). In 2004, additional food fortifications as well as dietary and supplement have been recommended in the USA (Moore *et al*. 2004). However, milk fortified with vitamin D is not permitted in the UK and some other European countries.

### 1.7: Feasibility of use of cultivated edible mushrooms as a vitamin D source

The evidence gathered suggest that vitamin D deficiency among the word population is dramatically increasing. Accumulating clinical evidence suggests that the vitamin D deficiency increases the risk of a large spectrum of chronic diseases including cancers, heart diseases, diabetes, obesity, arthritis, hypertension and psoriasis. Since the feasibility of sunbathing for vitamin D is still complicated and the risk/benefit has yet to be elucidated, the best idea is to look for alternative dietary sources.

Edible mushrooms are very popular among the world population for their unique flavour and medicinal value. Furthermore, mushrooms are considered a delicacy, highly accepted by vegetarians as well as non-vegetarians and could be used to supplement vitamin D in the diets of those populations at risk of vitamin D deficiency. Vitamin $D_2$ is the form of vitamin D that could be provided from mushrooms, and this form has some remarkable advantages over vitamin $D_3$.

Vitamin $D_2$ is more effective for bone mineralization than vitamin $D_3$ (Tjellesen *et al*. 1985), and vitamin $D_2$ is less toxic compared with vitamin $D_3$ (Mehta & Mehta, 2002). In addition, vitamin $D_2$ does not have hypercalcemic effects (Mawer *et al*. 1995).

In nature, a limited amount of vitamin $D_2$ has been reported in some wild edible mushrooms, however, cultivated edible mushrooms have been shown to be devoid of vitamin $D_2$ (Mattila *et al*. 1994; Mattila *et al*. 2002; Perera *et al*. 2003; Jasinghe and Perera, 2004). Naturally, wild mushrooms may be exposed to UV radiation, which

comprises 8 – 9 % of the total solar spectrum (Hollosy, 2002), and this could be the reason for the presence of a limited amount of vitamin $D_2$ in wild mushrooms. The commercially available cultivated mushrooms may not be exposed to the sunlight, which is essential in the natural production of vitamin $D_2$. Nevertheless, ergosterol in mushrooms can be converted to vitamin $D_2$ by UV irradiation (Mau *et al*. 1998; Perera *et al*. 2003; Jasinghe and Perera, 2004).

### 1.7.1: History of mushrooms

The history of use of mushrooms has been estimated to begin more than 6500 years ago by the rock paintings found in Tassili (Algeria), Tadrart Acacus (Libya), Ennedi (Chad), and Djebel Ouenat (Egypt) (Samorini, 2001). Mushrooms have been identified as a food of high medicinal value since very early times in China. The use of medicinal mushrooms in China has been described for at least 2000 years and more than 100 species have been used as traditional Chinese medicines (Hobbs, 2001). A number of different edible mushroom varieties have been used for the prevention and treatment of diseases such as tumors, fungal infections, viral infections, cardiovascular diseases, hypercholesterolemia, hypertension, and diabetes (Breene, 1990; Chihara, 1992; Ooi & Liu, 1999; Wasser & Weis, 1999; Ooi, 2001).

### 1.7.2: Widespread cultivated edible mushrooms and their medicinal properties

#### 1.7.2.1: Shiitake mushrooms (*Lentinula edodes*)

Shiitake mushrooms are also known as 'oak mushrooms' since they are naturally grown on logs of oak. The colour of the mushroom is light brown and it has a strong unique flavor. Synthetic logs mainly made out of sawdust and other agricultural wastes are being used in growing Shiitake in farms and they are largely produced in China, Japan, and South Korea. In 1997, the world production of Shiitake mushrooms was estimated to be 1.5 million metric tons, which accounts for 25.4 % of world production of cultivated mushrooms (Chang, 1999a).

Shiitake mushrooms have been used in traditional medicines and a number of investigations have been reported in the literature on their clinical efficacy. An antitumor active polysaccharide called “Lentinan (β-D-glucans)” has been isolated from Shiitake mushrooms (Chihara *et al*. 1970a; Chihara *et al*. 1970b; Chihara, 1992; Ikekawa, 2001; Kirchhoff, 2001; Yap & Ng, 2001). In addition, Shiitake mushrooms display anti-inflammatory, antiviral, antibacterial, and antiparasitic medicinal properties (Wasser & Weis, 1999; Dighe & Agate, 2000). Furthermore, anti hypertensive properties of Shiitake mushrooms have been observed in rats (Kabir & Kimura, 1989).

### 1.7.2.2: Button mushrooms (*Agaricus bisporus*)

This variety of mushrooms is also known as 'the white cultivated mushroom', since they are white in colour. The major regions of cultivation are Europe, North America, and China. Button mushrooms are the most extensively cultivated mushrooms in the world. It was estimated that the world production of Button mushrooms to be 1.9 million metric tons in 1997, which accounts for 31.8 % of the world production of cultivated mushrooms (Chang, 1999a). Antitumor active polysaccharides have been found in Button mushrooms (Mizuno *et al*. 1995). In addition, *Agaricus bisporus* has positive effects on insulin-depended diabetes mellitus (Swanston-Flatt *et al*. 1989) and *Agaricus* species display antibacterial properties as well (Dighe & Agate, 2000).

### 1.7.2.3: Pleurotus spp.

Among the different edible *Pleurotus* species, Oyster mushrooms (*Pleurotus ostreatus*) and Abalone mushrooms (*Pleurotus cystidus*) are popular in the world. Physically, Oyster and Abalone mushrooms are more or less similar except that Abalone mushrooms are light yellow in colour compared with light ash colour of Oyster mushrooms. However, the texture and flavor of these two types are different. *Pleurotus* mushrooms are the third most important mushrooms in production in the world, and it has been estimated that the production of *Pleurotus* species in 1997 was around 1 million metric tons, which accounts for 14.2 % of total world production of cultivated mushrooms (Chang, 1999a). China is the main producer of *Pleurotus* species however, they are cultivated worldwide.

*Pleurotus* species display antifungal, antitumor, antiviral, antibacterial , and antiparasitic medicinal properties (Wasser & Weis, 1999; Solomko, 2001; Gerasimenya *et al*. 2002). In addition, antibiotic, anti-inflammatory, hypoglycemic, and hypocholesterolemic medicinal properties have been observed in *Pleurotus* species (Bobek *et al*. 1991; Bobek *et al*. 1993; Bobek *et al*. 1995; Gunde-Cimerman, 1999; Wasser & Weis, 1999; Ikekawa, 2001; Gunde-Cimerman & Plemenitas, 2001;).

#### 1.7.2.4: Enoki mushrooms (*Flammulina velutipes*)

This species of mushroom is also called 'winter mushroom'. Although this mushroom is gathered from the wild, it is also now cultivated particularly in Japan. The world production of Enoki mushrooms was estimated to be around 0.3 millions metric tons in 1997 accounting for 4.6 % of the total world production of cultivated mushrooms (Chang, 1999a). Enoki mushrooms display antifungal, anti-inflammatory, antitumor, and antiviral medicinal properties (Wasser & Weis, 1999; Ikekawa, 2001; Badalian *et al*. 2001).

### 1.7.3: The world production of edible mushrooms

The world production of edible mushrooms has been increased significantly from 0.341 million metric tons in 1965 followed by 1.2 million metric tons in 1981, 4.9 million metric tons in 1994, and finally it reached 6.1 million metric tons in 1997, keeping the average annual increase around 12 % (Chang, 1999a; Chang, 1999b). The annual

increase in the world market of cultivated edible mushrooms is getting bigger. This impact is expected to continue and expand through the 21st century (Chang, 1999b).

In 1994, the value of the world mushroom production and mushroom medicinal products was estimated to be worth approximately 14 billion US dollars (USD), which was equal to the value of coffee production in 1997 (15 billion USD) (Chang, 1999b). Furthermore, Shiitake mushrooms are very popular among the Asian countries, and as an example, the import of fresh or chilled Shiitake mushrooms into Japan in 1997 was 26,000 metric tones worth over USD140 million (Anon, 1997a). In the same year the import of dried Shiitake mushroom to Japan was over 9000 metric tonnes worth over USD100 million (Anon, 1997b).

### 1.7.4: Ergosterol in mushrooms and its conversion to vitamin $D_2$

The history of research on ergosterol in fungi starts from the 1970's and a number of studies have been carried out in this field. Ergosterol, the precursor of vitamin $D_2$ is abundant in most of the fungi (Weet 1974; Nes 1977; Szymczak, 1979; Yokokawa, 1980; Yokokawa, & Mitsuhashi, 1981). Ergosterol content in cultivated mushrooms varies according to the composition of the cultivation media (Trigos, 1996; Trigos, 1997). Furthermore, ergosterol content also varied among the different mushroom species (Mau *et al*. 1998) and among the different cultivars as well (Yoshida *et al*. 1979). Mattila *et al*. (2001) showed that ergosterol was the most abundant sterol found in mushrooms, and its content was higher in cultivated mushrooms (6.02 – 6.79 mg/g DM) than in wild mushrooms (2.96 – 4.89 mg/g DM).

The effect of light source of UV has been found to be an important factor on the conversion of ergosterol under UV irradiation (Kobayashi & Yasumura, 1972). Mau *et al*. (1998) observed the conversion of ergosterol in mushrooms to vitamin $D_2$ under UV irradiation, and it was reported that the conversion was higher under UV-B compared with UV-C. It was also reported that vitamin $D_2$ in fresh common mushroom (*Agaricus bisporus*) and high temperature mushrooms (*A. bitorquis*) irradiated with UV-C at 12 $^{o}$C for 2 hours increased from 2.20 and 4.01 μg/g of dry weight to 7.30 and 5.32 μg/g respectively. However, the effect of UV-A, which represents approximately 6.3 % of the incoming solar radiation (Hollosy, 2002), on the conversion of ergosterol in mushrooms to vitamin $D_2$ has not been reported in the literature. Even though Mau *et al*. (1998) has reported the effect of UV-B and UV-C on the conversion of ergosterol in mushrooms to vitamin $D_2$, the maximum value of vitamin $D_2$ that they obtained was only 12.28 μg/g. In their study, there was also no mention of the orientation of mushrooms to the UV source when they were irradiated, which I found to be one of the most important factors in the conversion of ergosterol to vitamin $D_2$. In addition, they did not study the effect of moisture content and the temperature of irradiation of the mushrooms on this conversion in order to maximize the yield of vitamin $D_2$. Ergosterol in mushrooms has been discovered for over three decades; however, there are only a handful of investigations that have been carried out on the conversion of ergosterol in mushrooms to vitamin $D_2$. The mechanism of the conversion of ergosterol to vitamin $D_2$ is shown in Figure 1.3.

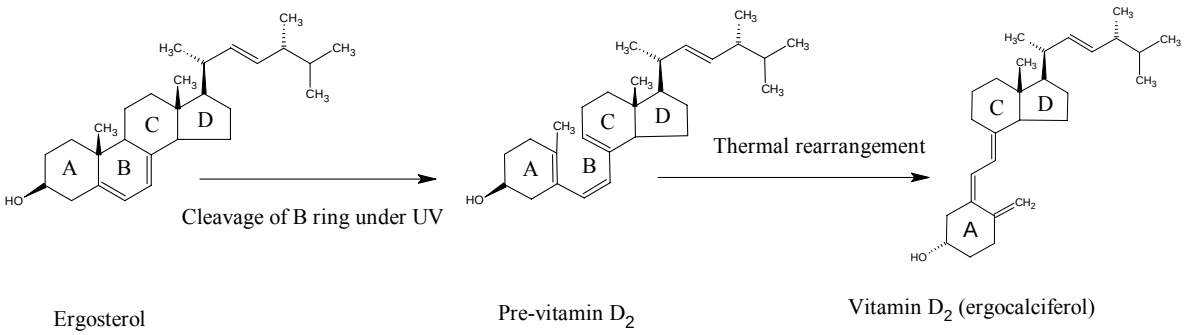



**Figure 1.3:** The mechanism of conversion of ergosterol to vitamin $D_2$ (Sources: Havinga, 1973; Horst & Reinhardt, 1997).

Photochemical cleavage of the B ring of ergosterol takes place under UV radiation, and then the intermediate (pre-vitamin $D_2$) formed, undergoes subsequent thermal rearrangement to form vitamin $D_2$ (ergocalciferol). However, pre-vitamin $D_2$ intermediate also absorbs UV and prolonged irradiation may produce undesirable byproducts such as tachysterol and lumisterol by photoisomerization (Havinga *et al*. 1960; Havinga, 1973; Braun *et al*. 1991).

### 1.8: Bioavailability of vitamin D

Bioavailability has been defined as 'that fraction of an oral dose (parent compound or active metabolite) from a particular preparation that reaches the systemic circulation' (Schumann *et al*. 1997). This same concept of bioavailability is applicable to nutritional studies.

There are a few studies on the bioavailability of vitamin D. However, most of the studies have been carried out using supplements. The absorption of vitamin D from supplements in humans is estimated at around 55 – 99 % and the values from food sources are probably lower (Van-den-Berg, 1997). Theoretical, animal and human models are used in bioavailability studies. It is intended in this study to use an animal model for the study of bioavailability of vitamin $D_2$ from irradiated Shiitake mushrooms.

The biologically active metabolite of vitamin $D_2$ is 25-hydroxyvitamin $D_2$ (Suda *et al*. 1969), and measurement of this compound is considered to be the best indicator of vitamin D status (Holick *et al*. 1986). Furthermore, 25(OH)D, which is the major circulating form of vitamin D, is more suitable as an index of vitamin D status than $1,25(OH)_2D$, since the half life of 25(OH)D is more than 7 days and it is circulated in the body at a concentration some 1000 times higher than $1,25(OH)_2D$ (Holick, 2004). Therefore, serum levels of 25(OH)D may be used as a sensitive indicator in the investigation of bioavailability of vitamin $D_2$ in *in-vivo* studies. In addition, Vitamin D deficiency is also associated with bone loss and incidence of fractures (Parfitt *et al*. 1982; Lips & Obrant 1991; Dawson-Hughes *et al*. 1995) and therefore measurement of BMD could also be used to evaluate vitamin D deficiency status.

Except for one human bioassay study (Outila *et al*. 1999), there appears to be no reported data on bioavailability of vitamin $D_2$ from natural food sources. Hence the focus of this study is to investigate the bioavailability of vitamin $D_2$ from irradiated edible mushrooms

in order to understand its biological activity and the possibility of using this food source to help in eradication of vitamin D deficiency from affected or at risk populations.

### 1.8.1: Widespread animals use in bioavailability studies

Among the animals used in laboratory studies, rat strains are commonly used in pharmaceutical studies. Sprague Dawley (SD) and Wistar (WI) rats are the most common strains of rats used in laboratory research on a worldwide basis. WI rats are good breeders giving approximately 2.5 young per female per week. They have a long life-span of approximately 30 months and are excellent parents. Wistar strain is the second most commonly used strain in laboratory research on a worldwide basis. Figure 1.4 illustrates a normal growth chart of male and female rats of Wistar strain.

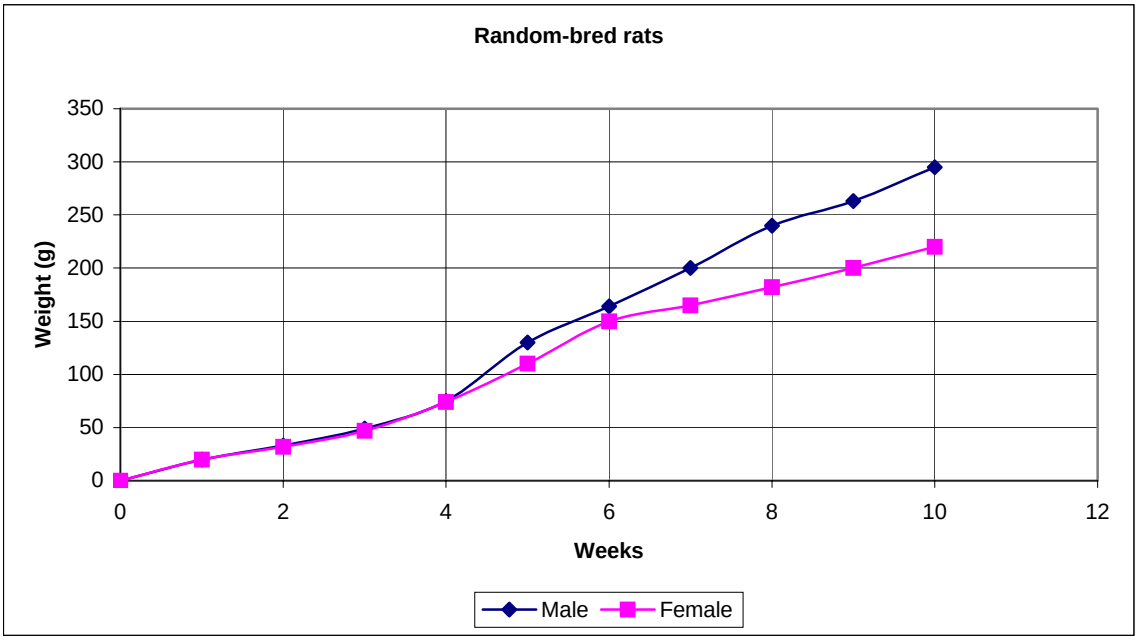



**Figure 1.4:** A normal growth chart of SD and WI rats (Source: Animal Holding Unit, 2004)

The weight gain of male rats was reported to be higher than that of female rats (Animal Holding Unit, 2004). The growth rate of male Wistar, rats during the period of their growth from 4-10 weeks (the period of the experiment) was relatively linear, which was important to minimize the variability during growth of the animals. The growth chart clearly indicates that from three weeks to around 10 weeks is the period of their most rapid growth. In this period, vitamin D may be a critical factor involved in their growth since it is one of the major determinants of the bone mineralization process. In addition, this may be the period that vitamin D metabolites, calcium, and phosphorous are essential when they lay down their skeleton, which requires calcium and phosphorous, and vitamin D metabolites are essential in the metabolism of these two minerals.

## 1.9: The objectives of the research

### 1.9.1: Ergosterol and vitamin $D_2$ content of the different parts of the mushrooms

Mushrooms are all similar in shape and structurally, they can be divided into cap, stalk and gills. These parts differ structurally as well as morphologically, and so the chemical composition is also likely to vary. Hence determination of the distribution of ergosterol and vitamin $D_2$ in different parts of the mushroom could be helpful for the interpretation of results for the conversion studies in the later stages of this project. In this study, ergosterol content of three different parts of mushrooms (caps, gills, and stalk) will be separately investigated.

### 1.9.2: Effect of irradiation on the conversion of ergosterol to vitamin $D_2$

There are two tissues in mushrooms (caps & gills), which could be orientated to the UV-source when they are irradiated. Physically and morphologically, the caps and gills of the mushrooms are different. In addition, the composition of ergosterol in caps and gills could be quite different, which could be an important factor to decide which tissue should be turned towards the source of UV when they are irradiated, in order to maximize the conversion of ergosterol to vitamin $D_2$. Therefore a further aim of this study was to investigate the effect of irradiation on the type of mushroom-tissue being irradiated on the conversion of ergosterol to vitamin $D_2$.

### 1.9.3: Effect of moisture content of mushrooms on the conversion of ergosterol to vitamin $D_2$ by UV irradiation

Water is needed as the reaction medium for most chemical reactions to occur in nature. Even though it provides the medium, dilution effect at high moisture contents may lead to lower reaction rates. On the other hand, low moisture levels make an undesirable environment for the mobilization of reactants leading to lower reaction rates. Moisture content of a food plays an important role in shelf life and the optimum moisture content for the different reactions may be different. Hence, the objective of this experiment was to investigate how the conversion of ergosterol in mushrooms to vitamin $D_2$ by UV irradiation takes place under different moisture levels. This could be useful to determine

the optimum moisture content of mushrooms for the conversion in order to maximize the yield of vitamin $D_2$.

### 1.9.4: Effect of temperature on the conversion of ergosterol in mushrooms to vitamin $D_2$ by UV irradiation

Reaction rates vary with the concentrations of reactants and as well as the temperature at which the reaction takes place. As the temperature increases, molecular motion increases. In the case of enzyme catalyzed reactions, as the speed of enzyme and substrate molecules increases, the chance for collisions and the formation of enzyme-substrate complexes increases. Thus as the temperature rises, the reaction rate increases too. Above the optimal temperature however, this does not apply. As the temperature rises above the optimal level then, an increasing number of enzymes become denatured. Fewer and fewer enzymes are able to fit with their substrates at the active sites. The reaction rate decreases until at some high temperature, all the enzymes are denatured, and reactions cease. However, some reactions prefer low temperatures since they need gentle collisions at slower speeds, to form bonds between molecules in order to cause reaction. Therefore, there is an optimum temperature where the reaction proceeds at its maximum. Above or below that optimal temperature, the reaction rate decreases. Hence the objective of this experiment was to investigate the optimum temperature of the conversion of ergosterol in mushrooms to vitamin $D_2$.

### 1.9.5: Effect of the band of UV applied (UV-A, UV-B, and UV-C) on the conversion of ergosterol in mushrooms to vitamin $D_2$

There are data on the irradiation of mushrooms under UV-B and UV-C in the literature (Mau *et al*. 1998), and the general conclusion is that UV-B is better for this conversion than UV-C. However, no previously reported data are available on the conversion of ergosterol in mushrooms under UV-A. The cleavage of the "B" ring of ergosterol is the initializing point of this conversion, and this happens under UV radiation. Therefore it was intended in this experiment to investigate the best range of wavelengths for this conversion.

### 1.9.6: Kinetics of conversion of ergosterol in mushrooms to vitamin $D_2$

Since the morphological structures of different cultivated edible mushrooms are varied, the rate of the conversion of ergosterol to vitamin $D_2$ could also vary. The kinetics of this conversion in different types of edible mushrooms could be useful as a prediction of yield of vitamin $D_2$ after certain periods of irradiation. In this experiment, vitamin $D_2$ content as affected by the time period of irradiation was studied. The combined effect of moisture & temperature on the conversion of ergosterol to vitamin $D_2$ in a 2 x 2 factorial design was also studied.

### 1.9.7: Bioavailability of vitamin $D_2$ from edible mushrooms.

There are only a few studies on the bioavailability of vitamin D, but most of these studies have been carried out using supplements. Bioavailability of vitamin $D_2$ from edible mushrooms was studied in order to understand the nutritional benefits of this popular delicacy as a vitamin D source. There appear to be no reported data on the bioavailability of vitamin $D_2$ from irradiated edible mushrooms in the past. Theoretical, animal and human models are used commonly in bioavailability studies. However, it is difficult to use human models unless in collaboration with a medical study. Data from animal models are more reliable than those from theoretical models. Therefore an animal model was used for the study of bioavailability of vitamin $D_2$ from UV irradiated Shiitake mushrooms.

# CHAPTER 2

# MATERIALS AND METHODS

# CHAPTER 2

# Materials and methods

## 2.1: Materials

### 2.1.1: Raw materials

Fresh Shiitake mushrooms (*Lentinula edodes*), Oyster mushrooms (*Pleurotus ostreatus*), Button mushrooms (*Agaricus bisporus*), Abalone mushrooms (*Pleurotus cystidus*) and Enoki mushrooms (*Flammulina velutipes*) were purchased from a local supermarket for the preliminary studies, and were used immediately in the experiments. These are the most commonly used mushrooms in South East Asia. All samples were purchased between February, 2003 and August, 2004 during 2003/4.

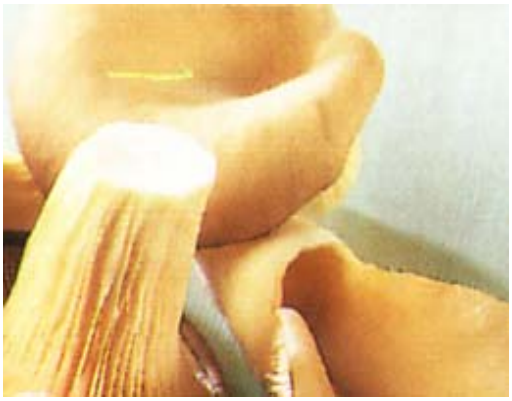
Shiitake mushrooms from local mushrooms producers are also available in the market. Since the uniformity of mushrooms is important, only Shiitake mushrooms, from a local farm were purchased and used immediately in this study unless otherwise stated. The other types of edible mushrooms used in the study (Button, Oyster, Enoki, and Abalone) were cultivated in local mushroom farms in Singapore. Photographs of different types of mushrooms used in this study are shown in Figure 2.1.

**Button mushrooms**

**Shiitake mushrooms**

**Enoki mushrooms**

**Abalone mushrooms**



**Oyster mushrooms**

**Figure 2.1:** Pictures of edible cultivated mushrooms used in this study

### 2.1.2: Chemicals

Ascorbic acid and sodium hydroxide pellets were purchased from BDH laboratory suppliers (99 % pure, BDH laboratory suppliers, Poole, England). Ethanol (99 % pure) was purchased from Riverbank chemicals, Singapore. Potassium hydroxide (85 % pure), Methanol (99.8 % pure), and Acetonitrile (99.8 % pure) were purchased from Merck chemicals, Darmstadt, Germany. The working standards, cholecalciferol (Sigma chemicals, St. Louis, MO, USA), ergocalciferol (98 % pure), and ergosterol (95 % pure) Aldrich chemicals, Milwaukee, WI, USA), were used in this study. Diethyl ether (99.9 % pure) was obtained from J.T.Baker chemicals, Phillipsburg, NJ, USA and n-Pentane was obtained from (98.8 % pure) from Tedia chemicals, Fairfield, OH, USA.

### 2.1.3: Apparatus

#### 2.1.3.1: UV lamps

UV-A Mineralight UVGL – 25, [UVP, Inc, San Gabriel, U.S.A.] with UV-A lamp, UV-B MODEL UVM-57, [UVP, Inc, Upland, CA, U.S.A.], and UV-C Mineralight UVGL – 25, [UVP, Inc, San Gabriel, U.S.A.] with UV-C lamp, were used for the irradiation of the mushrooms.

#### 2.1.3.2: Intensity meter

The light intensity of UV lamps was measured by an optical radiometer model MS-100, equipped with UV-A sensor (MS-136 UV sensor), UV-B sensor (MP-131 UV-B sensor), and UV-C sensor (MS-125 UV Sensor), all from UVP, Inc, Upland, CA, USA.

#### 2.1.3.3: Water bath

MEMMERT water bath with a digital thermal controller (Schutzart DIN 40050-IP 20, Memmert Gmbh+Co.KG, Schwabach, Germany) was used in saponification of mushroom samples at 80 $^{o}$C.

#### 2.1.3.4: Rotavapor

BUCHI rotavapor R-200 equipped with vacuum pump (BUCHI Vac V-500), and heating bath (BUCHI B-490), BUCHI Labortechnik AG, Meierseggstrasse Postfach Flawil, Switzerland) was used for concentrating of the samples by rotary evaporation.

#### 2.1.3.5: Freeze Drier

LABECONCO bench top freeze-drying system (LABCONCO FREEZONE 1 L, Labconco Corporation Kansas City, Missouri) equipped with Edwards vacuum pump

(EDWARDS RV3, Edward vacuum international, Crawley, Sussex, England) was used in freeze-drying the mushroom samples.

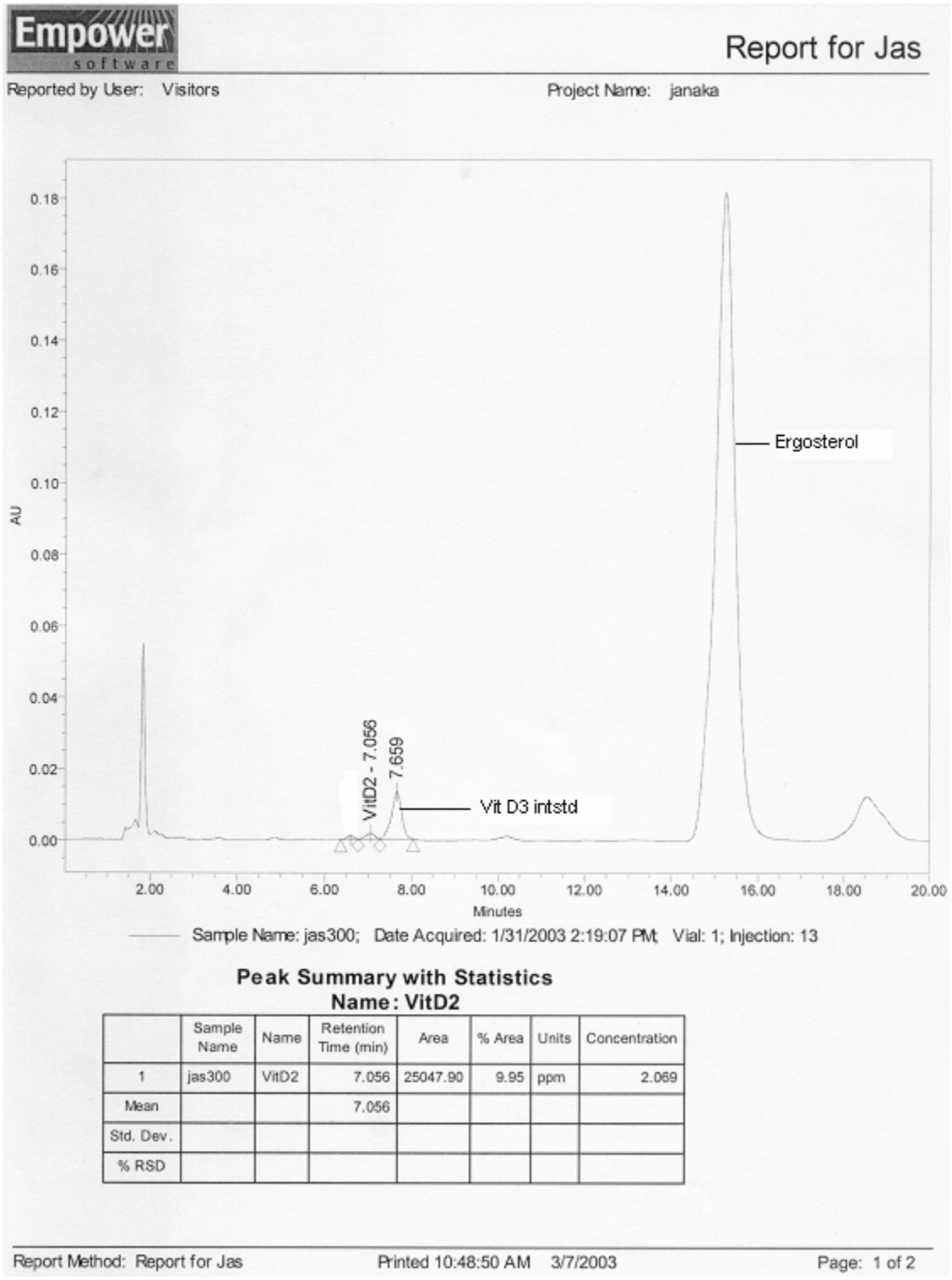
#### 2.1.3.6: HPLC system

Waters 600E HPLC system equipped with Waters 486 tunable absorbance UV detector (Waters, Milford, MA, USA) and a reverse phase C18 column (Maxsil 5 C18, 250 X 4.6 mm, Phenomenex, Torrance, CA, USA), were used for the chromatographic analyses.

## 2.2: Methods

### 2.2.1: Calibration of the instrument

UV scans for ergosterol, vitamins $D_2$, and $D_3$ was carried out and it was determined that all compounds could be detected by using UV absorption at the wavelength 282 nm. Acetonitrile (ACN) / methanol system was used as the solvent system and it was determined that ACN to methanol ratio of 75:25 was the best mobile phase in order to obtain fine peak resolution at a flow rate of 2.3 ml/min. Vitamin $D_3$ was selected as the internal standard, as it has similar properties compared with vitamin $D_2$. Also, it did not interfere with the target compounds in the sample. Other advantages are the ability to detect the selected internal standard using the same wavelength of 282 nm and being well separated from the peaks of the target compounds. A typical HPLC chromatogram of an irradiated mushroom extract is shown in Figure 2.2.




| | Sample Name | Name | Retention Time (min) | Area | % Area | Units | Concentration |
|---|---|---|---|---|---|---|---|
| 1 | jas300 | VitD2 | 7.056 | 25047.90 | 9.95 | ppm | 2.069 |
| Mean | | | 7.056 | | | | |
| Std. Dev. | | | | | | | |
| % RSD | | | | | | | |

**Figure 2.2:** A HPLC chromatogram of an irradiated mushroom extract

The calibration of standard compounds was arranged according to the expected amounts of ergosterol and vitamin $D_2$ in the samples, in order to obtain accurate quantifications of the target compounds. The calibration range for the ergosterol was 100 ppm to 1000 ppm and the calibration range for vitamin $D_2$ and $D_3$ was 1 ppm – 50 ppm. A series of standards of Vitamin $D_2$, $D_3$, and ergosterol were prepared. Then an aliquot of 20 µl of pre-prepared standard samples were injected to the HPLC system. Three different calibration curves were plotted for quantifying ergosterol, vitamin $D_2$ and vitamin $D_3$ (Appendix 1).

#### 2.2.1.1: Linearity range

The linearity range was tested according to the expected concentrations. The checked linearity ranges and their correlation coefficients are given in Table 2.1.

**Table 2.1**: The linearity ranges of vitamin $D_2$, $D_3$, and ergosterol and their correlation coefficients.

| Standard | Checked linearity range | Correlation coefficient ($R^2$) |
|---|---|---|
| Vitamin $D_2$ | 1ppm – 50 ppm | 0.9997 |
| Vitamin $D_3$ | 1ppm – 50 ppm | 0.9998 |
| Ergosterol | 100 ppm – 1000 ppm | 0.9998 |

### 2.2.2: Sample preparation

#### 2.2.2.1: Ergosterol and vitamin $D_2$ content of different parts of Shiitake mushrooms

The aim of this study was to find out whether there was significant variation in the distribution of ergosterol within the different morphological parts of mushrooms. This could be useful in optimization of the irradiation process investigated in the later stages of this project.

Different batches of fresh Shiitake mushrooms were purchased from the market and good quality well developed mushrooms were selected for the study. Then with the help of a sharp blade, the outer layer of the cap (approximately 1mm thickness), gills and the stalk were carefully removed. These three parts were separately freeze-dried and kept in a vacuum desiccator covered by aluminium foil in order to prevent the samples from being exposed to any light source, until ready for the preparation of samples for further analysis.

#### 2.2.2.2: Effect of irradiation on the conversion of ergosterol to vitamin $D_2$

It was found from the previous study that the distribution of ergosterol in different types of mushroom tissues varied. Hence, the specific tissue of mushroom, which is being irradiated, could effect the conversion of ergosterol to vitamin $D_2$. Therefore, it was intended in this study to determine the effect of irradiation on the type of mushroom-

tissue being irradiated on the conversion of ergosterol to vitamin $D_2$. This could be useful in further optimization of the conversion of ergosterol to vitamin D in the latter stages of this project.

Shiitake mushrooms were divided equally into two lots, one lot was placed with their gills facing the UV source and the other lot was placed with their caps facing the UV source in the irradiation chamber. Then the mushrooms were irradiated with UV-A at ambient temperature (27 $^{o}$C) for two hours. The mushroom samples were placed 15 cm away from the UV source when they were irradiated. After the irradiation treatment the mushroom samples were separately freeze-dried and kept in a vacuum desiccator for further analysis.

### 2.2.2.3: Ergosterol and vitamin $D_2$ contents in different types of edible mushrooms and the conversion of ergosterol to vitamin $D_2$ by UV-A irradiation

The first part of this study was carried out in order to investigate the composition of ergosterol and vitamin $D_2$ in cultivated edible mushrooms. Fresh Shiitake, Oyster, Abalone, Button, and Enoki mushrooms were purchased from the market. They were then freeze-dried and kept in a vacuum desiccator for further analysis of vitamin $D_2$ and ergosterol.

It was found from the previous study (2.2.2.2) that the conversion of ergosterol to vitamin $D_2$ was higher when the mushrooms were irradiated with their gills facing the source of

UV than when their gills were facing away from it (caps facing the source of UV). Therefore as a preliminary study, the second part of this study was carried out to investigate the conversion of ergosterol in mushrooms to vitamin $D_2$ by UV-A irradiation. Another batch of fresh Shiitake, Oyster, Abalone, Button, and Enoki mushrooms was irradiated with their gills facing the source of UV-A. The irradiations were performed for two hours at ambient temperature of 27 $^{o}$C. The mushroom samples were placed 15 cm away from the UV source when they were irradiated. The irradiated mushroom samples were separately freeze-dried and kept in a vacuum desiccator for later analysis.

#### 2.2.2.4: Effect of moisture content of the mushrooms on the conversion of ergosterol to vitamin $D_2$

The aim of this part of the study was to observe the optimum moisture content on the conversion of ergosterol to vitamin $D_2$. The findings could be helpful to determine how much moisture should be removed from the mushrooms, before subjecting them to the process of irradiation in order to optimize the conversion.

Shiitake mushrooms were freeze-dried to different moisture levels and samples having different moisture levels were separately irradiated with their gills facing the UV-A source. The irradiations were performed for two hours at ambient temperature of 27 $^{o}$C and mushroom samples were placed 15 cm away from the UV source during irradiation. Irradiated samples were freeze-dried and kept in a vacuum desiccator for further analysis.

The moisture contents of different mushroom samples were measured gravimetrically by drying samples in an air convection drier at 105 $^{o}$C for at least 20 hr.

### 2.2.2.5: Effect of temperature on the conversion of ergosterol in mushrooms to vitamin $D_2$

The aim of this part of the study was to find the optimum temperature for the conversion of ergosterol to vitamin $D_2$ and the findings could be used to fulfill the conditions for maximization of the yield of vitamin $D_2$.

Shiitake mushrooms were irradiated with their gills facing the UV-A source at a distance of 15 cm away from the samples in an irradiation chamber. The irradiation was applied at a given temperature for two hours. Then the study was repeated with fresh mushrooms for different temperatures. The temperature of 12 $^{o}$C was achieved by keeping the irradiation chamber in a refrigerator maintained at 12 $^{o}$C. The other temperatures (25, 35, 45, and 65 $^{o}$C) were achieved by keeping the irradiation chamber in an oven at different temperatures from 25 ± 1 $^{o}$C to 65 ± 1 $^{o}$C. Following the appropriate treatment regime the irradiated mushroom samples were freeze-dried and kept in a vacuum desiccator for further analysis.

### 2.2.2.6: Effect of the orientation of mushroom tissue to the source of UV and the duration of irradiation on the conversion of ergosterol to vitamin $D_2$

Prolonged irradiation of mushrooms leads to photo degradation of vitamin $D_2$ and discoloration of the mushrooms. These changes, particularly physical change in colour may lead irradiated mushrooms to be deemed unacceptable by the consumers. Therefore, the optimum period of irradiation on this conversion process should be investigated in order to prevent the mushrooms from over-irradiation, which promotes adverse chemical reactions. Hence, the objective of this part of the work was to investigate the optimum period of irradiation and the effect of orientation of mushrooms to the source of UV on the conversion of ergosterol to vitamin $D_2$.

In the first experiment, fresh Shiitake mushrooms were subjected to three different irradiations with the same source of UV-A. The first lot of mushrooms was irradiated for two hours with their gills facing the UV source. The second lot of mushrooms was irradiated with their gills facing the UV source for one hour and then they were further irradiated for another hour with their caps facing the UV source, and the third lot was irradiated with their gills facing the UV source for two hours and then they were further irradiated for another two hours with their caps facing the UV source. These samples were separately freeze-dried and kept in a vacuumed desiccator for later analysis. As before, the source of irradiation was placed at a distance of 15 cm away from the samples in an irradiation chamber and the tests carried out at ambient temperature of 27 $^{o}$C.

In the second experiment, the effect of period of irradiation on the conversion of ergosterol to vitamin $D_2$ was investigated. One lot of Shiitake mushrooms was irradiated for different time periods starting from 10 minutes up to two hours with their gills facing the UV source, and another lot of Shiitake mushrooms was irradiated with their caps facing the UV source. This study was repeated by irradiating of each side of mushrooms for different time periods to investigate the combine treatment effect on the conversion of ergosterol to vitamin $D_2$.

### 2.2.2.7: Conversion of ergosterol in mushrooms to vitamin $D_2$ under different bands of UV

The conversion of ergosterol under different bands of UV (UV-A, UV-B, and UV-C) was investigated. The conversion of ergosterol to vitamin $D_2$ is initialized by the cleavage of "ring B" of ergosterol under UV. The energy supplied by different wavelengths on this initialization process could vary and this may effect the overall reaction rate of the conversion. Hence in this experiment, it was proposed to investigate the conversion of ergosterol under UV-A, UV-B, and UV-C at optimum temperature and moisture content for the conversion.

Fresh Shiitake, Oyster, Button, and Abalone mushrooms were irradiated with UV-A, UV-B, and UV-C. In this study, optimum temperature of 35 ± 1 $^{o}$C (Jasinghe and Perera, 2004) was maintained during the irradiation. The moisture content of the mushrooms was pre-adjusted to an optimum of approximately 80 % (Jasinghe and Perera, 2004) by

keeping them in a vacuum dryer at room temperature before they were subjected to the irradiations. First, the mushrooms were irradiated for one hour with their gills facing the UV source. Then they were further irradiated for another hour with their caps facing the UV source. The mushrooms were placed at a distance of 15 cm away from the UV source in an irradiation chamber when they were irradiated. The irradiated mushrooms were freeze-dried and kept in a vacuum desiccator for later analysis.

**2.2.2.8: Kinetics of the conversion of ergosterol in mushrooms to vitamin $D_2$, and combined effect of the temperature of irradiation and the moisture content of mushrooms on the conversion of ergosterol in mushrooms.**

Kinetics of the conversion of ergosterol in mushrooms to vitamin $D_2$ is very important for prediction of the yield of vitamin $D_2$. However, there appears to be no reported data on this aspect. Hence the objective of the study was to investigate the kinetics of the conversion of ergosterol in mushrooms to vitamin $D_2$. This could be useful in predicting the yield of vitamin $D_2$ after the irradiation process.

For this study, fresh Shiitake, Oyster, Abalone, and Button mushrooms were subjected to UV-A irradiation for different time periods. Each side of the mushrooms was irradiated with UV-A source. The mushrooms were irradiated at ambient temperature (27 $^{o}$C). Freeze-dried irradiated mushroom samples were kept in a vacuum desiccator for later analysis. In order to study the conversion kinetics of ergosterol to vitamin $D_2$ in Shiitake

mushrooms, the experiment was repeated at three different temperatures, 25, 30 and 35 $^{o}$C.

The joint effect of factors on a reaction can be investigated in factorial designs. In this part of the study, a 2 x 2 full factorial design was used to investigate the combined effect of the temperature of irradiation and the moisture content of mushrooms on the conversion of ergosterol to vitamin $D_2$. Following the 2 x 2 factorial model by Montgomery (2001), four treatment combinations were used. All the irradiation treatments were carried out with their gills facing the UV source for two hours.

The first lot of mushrooms was irradiated at low temperature (25 $^{o}$C) and low moisture content (approximately 60 %, but the exact moisture content was determined using the oven method), and the second lot of mushrooms was irradiated at high temperature (35 $^{o}$C) and low moisture content (60 %). The third lot of mushrooms was irradiated at low temperature (25 $^{o}$C) and high moisture content (approximately 80 %), and the fourth lot of mushrooms was irradiated at high temperature (35 $^{o}$C) and high moisture content (approximately 80 %). The high and low levels of two factors were selected within the linearity limits of the two factors on the conversion of ergosterol to vitamin $D_2$, which had been previously observed (Jasinghe and Perera, 2004). The each side irradiated mushroom samples were separately freeze-dried and kept in a vacuum desiccator for later analysis.

In order to study the conversion kinetics of ergosterol in vitamin $D_2$, three different temperatures, 25, 30 and 35 $^{o}$C were selected. Each side Shiitake mushrooms were irradiated for different time periods from 10 minutes to 60 minutes at the above

mentioned three different temperatures. Again a 2 x 2 factorial design was used in this study. The moisture content of fresh Shiitake mushrooms was measured gravimetrically by drying samples in an air convection drier at 105 $^{o}$C for at least 20 hr.

### 2.2.3: Bioavailability of vitamin $D_2$ from irradiated edible mushrooms

#### 2.2.3.1: Animal model

For this part of the study using an animal (rat) model, all the procedures were performed according to an approved project protocol, which complied with the Singapore Agri-Food and Veterinary Authority (AVA) regulations, and abided with National Advisory Committee for Laboratory Animal Research (NACLAR), by laboratory animal centre National University of Singapore. All animals involved in this study were treated in a humane fashion in accordance with the guidelines of the National University of Singapore, and disposed of in a manner prescribed by the animal holding unit, National University of Singapore.

Thirty male WI rats, around the age of three weeks and having an approximate weight of 55 g (average weight 54.32 ± 5.12 g) were obtained from the laboratory animal-breeding centre, National University of Singapore. All the animals were subjected to behavioural observation (activeness, alertness, and brightness), physical examination (abnormal discharges from nose and eyes, shininess of the hair coat), and verification that the

animals were in good health when they were received. All the rats were housed in individual plastic cages (Figure 2.3) at 25 $^{o}$C under incandescent lighting.


**Figure 2.3:** Rat cages

### 2.2.3.2: Feeding of the animals

Initially, all the rats were given a diet deficient in vitamin D with 0.47 % calcium and 0.3 % phosphorous (Diet TD89123, Teklad Premier Laboratory Diets, Madison, WI) in order to induce vitamin D deficiency. After one week, six rats were randomly selected (Group 1), and sacrificed to analyze initial BMD and serum levels of 25(OH)D.

A group of 12 rats (Group 2) was administered daily a known amount of lyophilised, powdered, irradiated Shiitake mushrooms, having 1 μg of vitamin $D_2$ while the control group (Group 3) received the same amount of non-irradiated Shiitake mushroom, confirmed to be free of vitamin $D_2$. The test diets (freeze-dried, irradiated and non irradiated mushroom powders) were administrated in liquid form (35.7 mg of mushroom powder suspended in 0.5 ml of deionised water) directly into the rat's stomachs through a gavage tube (Figure 2.4), while both groups were given free access to deionised water and the vitamin D deficient diet.

**Figure 2.4:** Gavage needle with the syringe

The feeding steps of test diets are shown in Figure 2.5

Step 1 Step 2

**Figure 2.5:** Steps of gavage feeding of a rat

The feeding plan is shown in Figure 2.6.

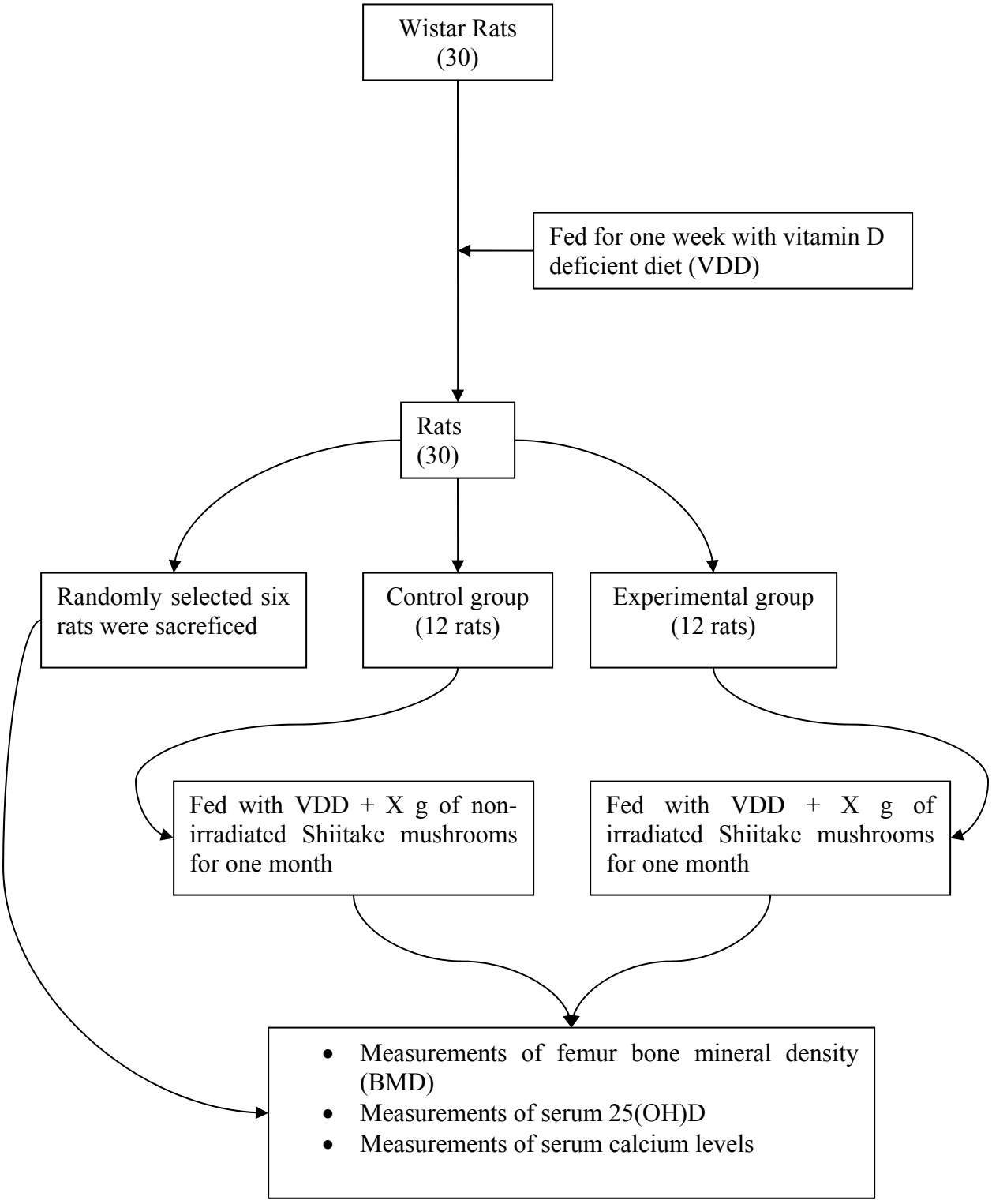



**Figure 2.6:** Animal feeding plan

The daily dietary intake and the weight gain were measured using an electronic analytical balance. The test diets were given to Groups 2 and 3 for a period of four weeks, and at the end of the fourth week, all the rats were sacrificed for the analysis of femur BMD and level of serum 25(OH)D. Blood samples were collected by cardiac puncture (Figure 2.6) before sacrificing the animals at the end of the period of study. Serum plasma of heparine anti-coagulated blood samples was separated by centrifugation of blood samples at 5 $^{o}$C. Separated serum samples were kept in a freezer, maintained below –20 $^{o}$C for later analysis.

**Figure 2.7:** Blood drawing by cardiac puncture

### 2.2.4: Measurements of 25(OH)D, serum calcium and BMD

Commercially available $^{125}$I-based RIA kits are being used in clinical investigations of 25(OH)D (Hollis, 2000). Serum 25(OH)D was analysed using Gamma-B 25(OH)D $^{125}$I RIA kit (DiaSorin and IDS Ltd, Boldon, UK.) as directed by manufacturer's product guidelines. This radioimmunoassay method does not discriminate between 25(OH)$D_2$

and 25(OH)$D_3$. Serum 25(OH)D bound radioactivity was measured by a gamma well-counting system (Berthold DPC Gamma-C12 multi crystal gamma counter, Berthold, Wilberg, Germany). Serum calcium levels were measured by an automated VITROS 950 chemistry system (Ortho-Clinical Diagnostics, Inc, Raritan, NJ, USA). The BMD of femur bones was measured by Lunar DPX-L Dual-Energy X-ray Bone Densitometer (DEXA); software version 1.3, (Lunar DPX-L, Lunar Corp., Madison, WI, USA). DXEA scanning is a non-invasive method of BMD analysis (Figure 2.8). The measurements of femur bone lengths were taken by adjusting the cursor pointer to the exact end points of the scanned images of femur bones. A DXEA image of a rat is shown in Figure 2.9

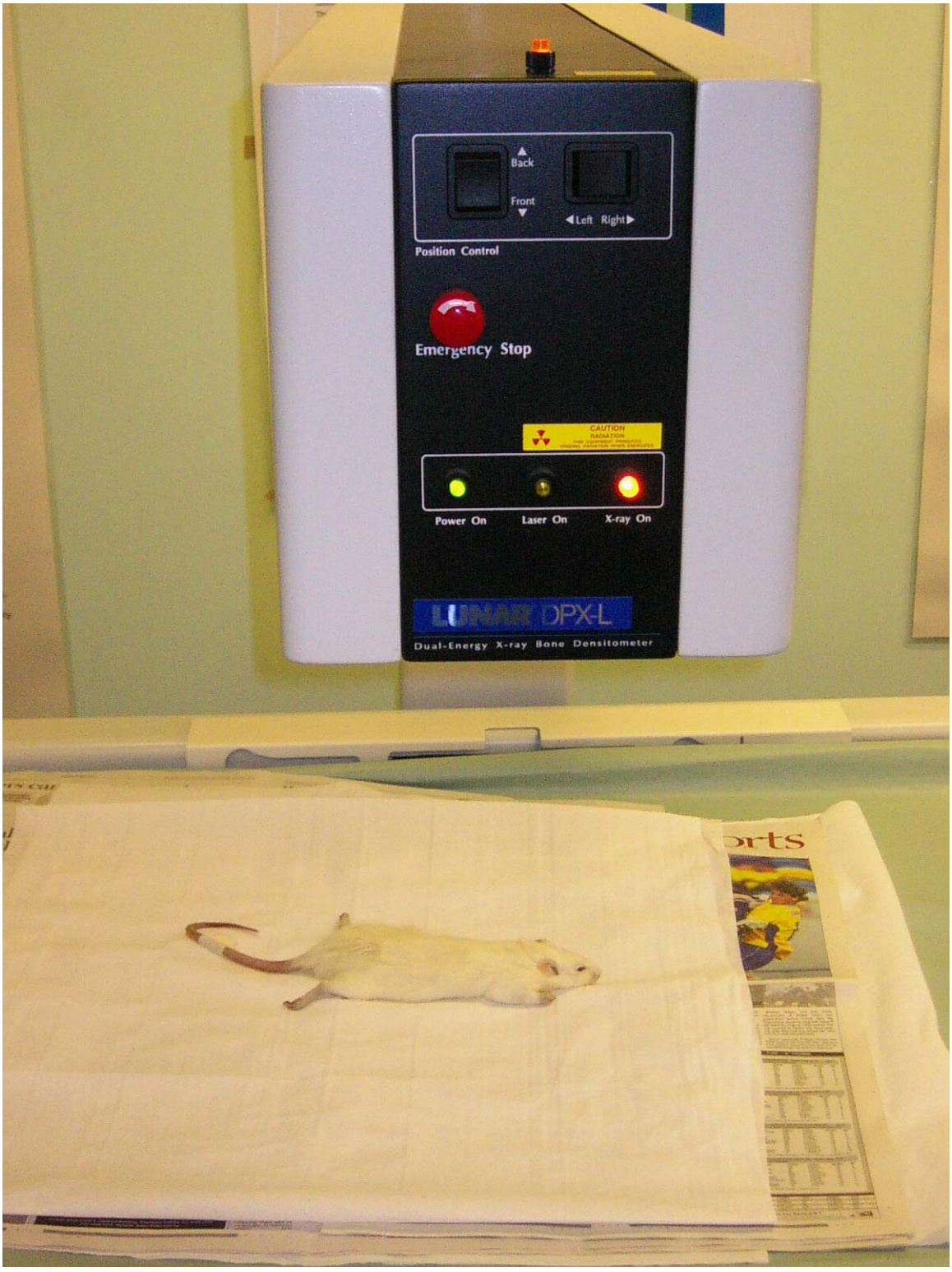



**Figure 2.8:** DXEA scanning of a rat

DXEA scanning of a rat

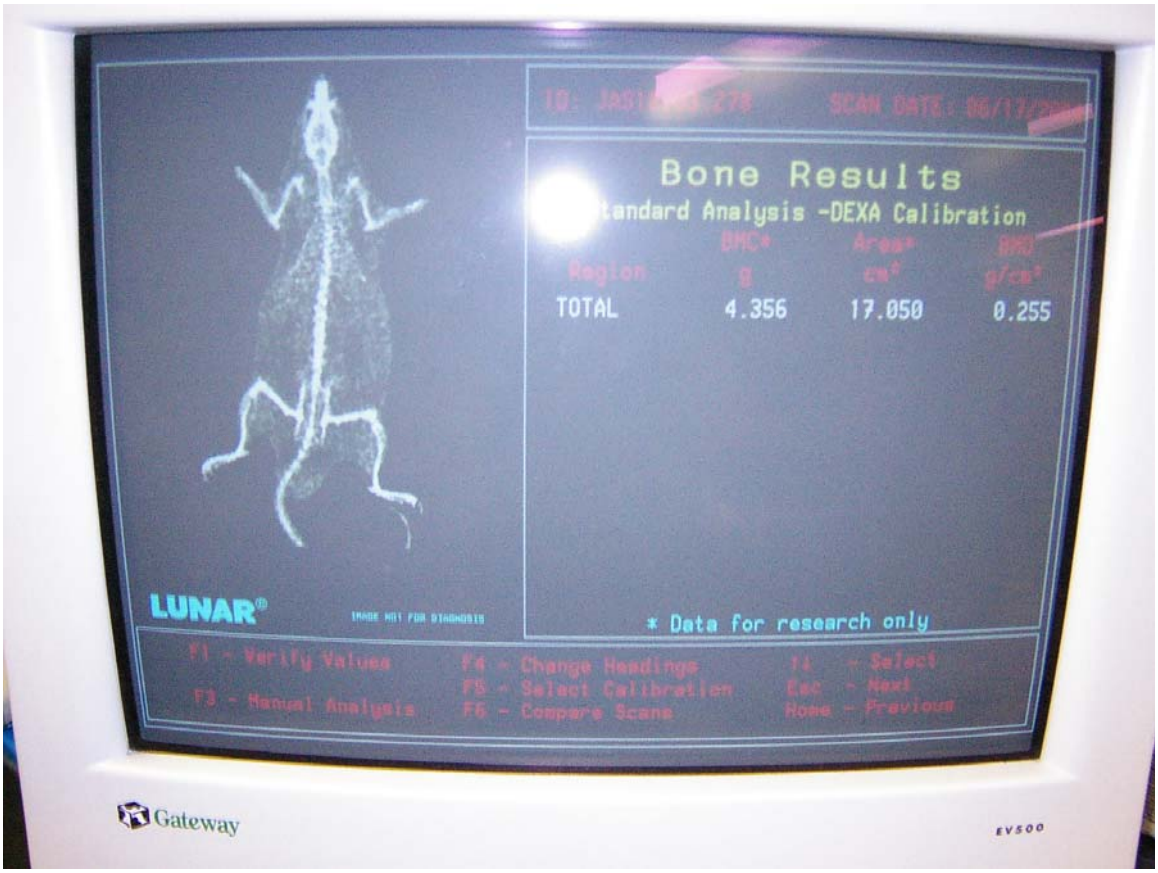



**Figure 2.9:** A DXEA image of a scanned rat

### 2.2.5: Simultaneous analysis of ergosterol and vitamin $D_2$

The analysis and quantification of vitamin $D_2$ were performed according to the method of Mau *et al*. (1998), modified as follows.

Freeze dried mushroom sample powders (0.5 g) were accurately weighed into 250 ml round bottom flasks and mixed with 4 ml of sodium ascorbate solution (17.5 g of sodium ascorbate in 100 ml of 1M NaOH), 50 ml of ethanol (99 %), and 10 ml of 50 % potassium hydroxide. The mixture was saponified under reflux at 80 $^{o}$C for 1 h, then, it was immediately cooled to room temperature and transferred into a separating funnel. The mixture was first extracted with 15 ml de-ionized water, followed by 15 ml ethanol (99 %) and then with a three-stage use of n-pentane of volumes 50, 50 & 20 ml respectively. The pooled organic layers were washed three times with 50 ml of 3 % KOH in 5 % ethanol and then finally with de-ionized water until neutralized. The organic layer was transferred into a round bottom flask, rotary evaporated to dryness at 40 $^{o}$C, and immediately re-dissolved in 5 ml ethanol (99 %).

The samples were passed through 0.45 μm Non-Pyrogenic filters (Schleicher & Schuell, Dassel, Germany). A volume of 20 μl of filtered sample was injected into a Waters 600E HPLC system equipped with Waters 486 tunable absorbance UV detector (all from Waters, Milford, MA, USA), and eluted through a reverse phase C18 column (Maxsil 5 C18, 250 X 4.6 mm, Phenomenex, Torrance, CA, USA) using acetonitrile / methanol (75:25) as the mobile phase at a flow rate of 2.3 ml/min. The UV detection of the eluate

was performed at 282 nm. Vitamin $D_2$ was qualitatively determined by comparing the retention times of standards obtained, and quantification was done by means of a calibration curve.

### 2.2.6: Statistical analysis

The results were statistically analyzed by analysis of variance [ANOVA, Vassar stats statistical computations, (http://vassun.vassar.edu/~lowry/VassarStats.html)]. The evaluation of equality of means was carried out by the one-way analysis of variance using the F distribution to assess significance. The data were expressed as means ± SD (standard deviation). The test results were considered significant only after reaching $p < 0.01$.

# PART II

# RESULTS AND DISCUSSION

# CHAPTER 3

# CONVERSION OF ERGOSTEROL TO VITAMIN $D_2$

# CHAPTER 3

## Conversion of ergosterol in mushrooms to vitamin $D_2$

### 3.1: Ergosterol and vitamin $D_2$ content in different parts of Shiitake mushrooms

The aim of this study was to investigate the concentration of ergosterol in different parts of Shiitake mushrooms. The analysis of ergosterol content of three different parts (cap, gills, and stalk) showed that Shiitake mushrooms contained remarkably high amounts of ergosterol and its distribution differed between the different parts of the mushroom tissues. Table 3.1 shows ergosterol contents in different parts of Shiitake mushrooms.

**Table 3.1:** Ergosterol contents of the different parts of Shiitake mushrooms.

| Part of the mushroom (n = 27) | Mean Ergosterol (mg/g, DM[a] ± S.D) |
|---|---|
| Gills | 10.6 ± 0.99 |
| Outer layer of the Cap | 5.34 ± 0.64 |
| Stalk | 2.97 ± 0.56 |

[a]Dry matter

The results show that the distribution of ergosterol within the mushroom tissues was significantly different ($p < 0.01$). The highest concentration of ergosterol was found in the gills, while the lowest was present in the stalk of mushrooms. The concentration of ergosterol in gills was almost twice that found in the outer layer of the caps, which in turn had almost twice that found in the stalks.

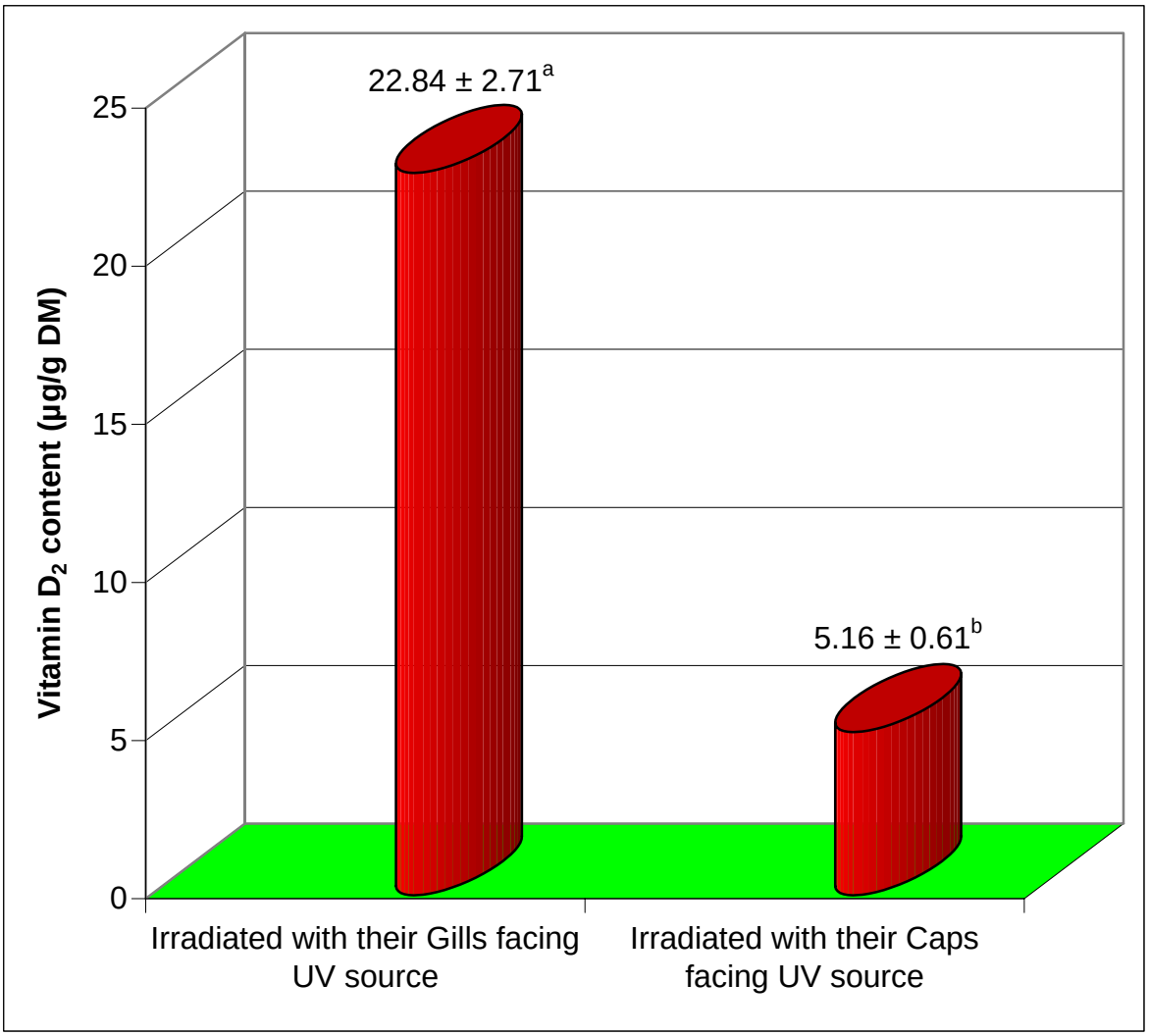
On the other hand, the analysis of non-irradiated mushrooms showed that cultivated Shiitake mushrooms were totally devoid of vitamin $D_2$. Mattila *et al*. (2002) also reported that vitamin $D_2$ was almost totally absent in cultivated mushrooms and the results of this study support this hypothesis. This was probably due to non-exposure of cultivated mushrooms to sunlight. The most of commercial mushrooms are grown in rooms, which are prevented from exposure to direct sunlight. In contrast, a few studies carried out in Japan reported very low amounts (0.0004 μg/g DM) of vitamin $D_2$ (Takeuchi *et al*. 1984), and 0.23 – 1.10 μg/g DM (Takamura *et al*. 1991) in cultivated Shiitake mushrooms. Mattila *et al*. (1994) also reported limited amounts of vitamin $D_2$ in wild edible mushrooms, and this was probably due to the exposure of mushrooms to sunlight.

### 3.2 Effect of irradiation on the conversion of ergosterol to vitamin $D_2$

It was observed from the results in Section 3.1, that ergosterol content in gills was almost double than that of the outer layer of the cap. The aim of this part of study was to investigate the effect of orientation of the different mushroom tissues to the source of irradiation on the conversion of ergosterol to vitamin $D_2$.

A UV-A lamp (Mineralight UVGL – 25, San Gabriel, U.S.A. with UV-A lamp) was used for the irradiation of mushrooms, and the UV light intensity at a distance of 15 cm away from the source was measured by an optical radiometer [MS-100, UVP, Inc, Upland, CA, USA, equipped with UV-A sensor (MS-136 UV sensor, UVP, Inc, Upland CA, USA)]. The calculated irradiation dose after two-hour irradiation period, at a distance of 15 cm

away from the source was 25.2 kJ/m$^2$. The effect of UV irradiation of mushroom tissues, namely, the caps and gills, exposed to the source of irradiation is shown in Figure 3.1.




[a,b]: Values shown are mean values of 12 replicates ± SD. Values with different superscript letters are significantly different ($p < 0.01$). Mushrooms were irradiated with UV-A at ambient temperature (27 $^oC$). Moisture content of mushrooms was found to be around 89 % (w.b.).

**Figure 3.1:** Vitamin $D_2$ contents of Shiitake mushrooms subject to the two different orientations of the tissues to the source of irradiation

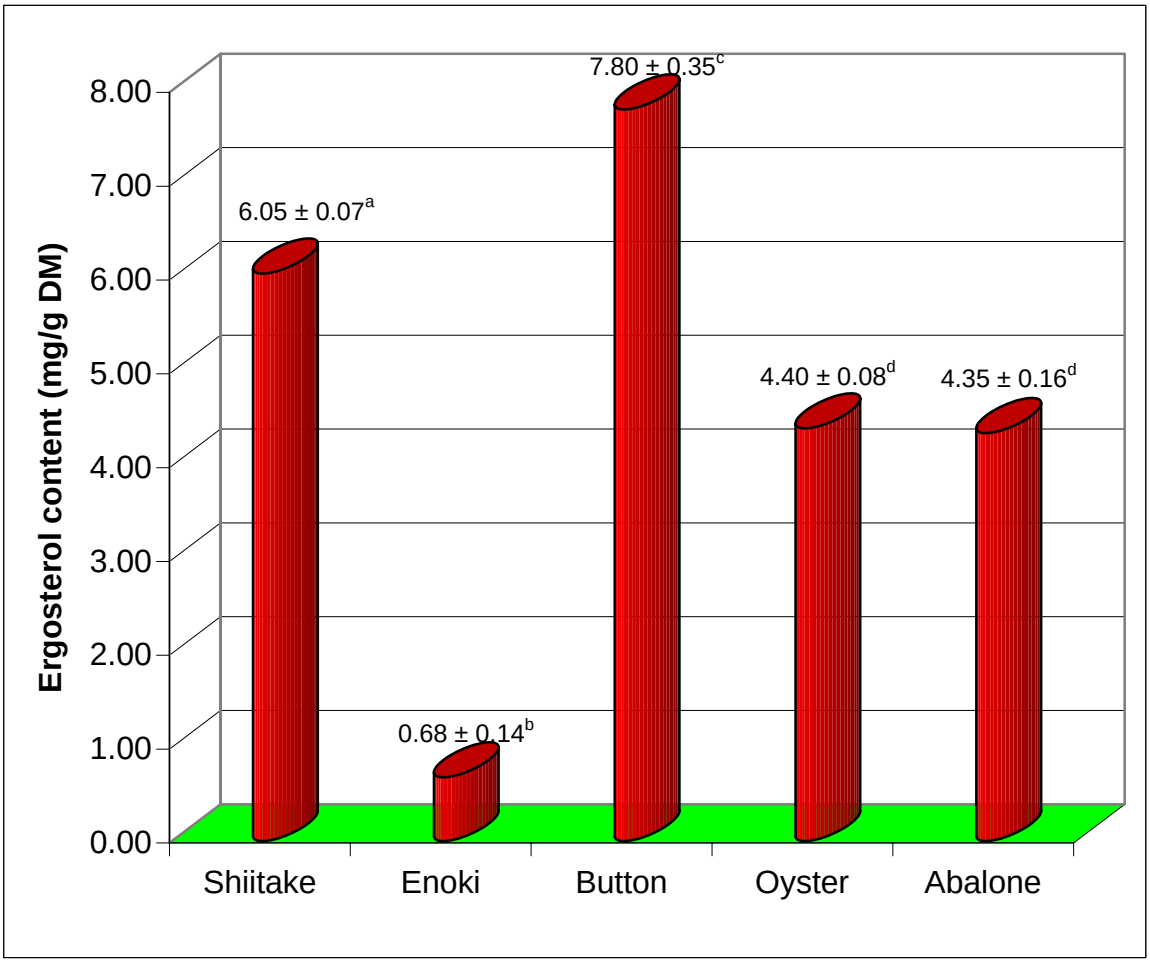
The results showed a high rate of conversion of ergosterol to vitamin $D_2$ when the mushrooms were irradiated with their gills facing the UV source. When the gills were facing the source of irradiation, the yield of vitamin $D_2$ was 22.84 ± 2.71 μg/g DM, whereas, when they were facing away from the source of irradiation (caps facing the source of irradiation), the yield of vitamin $D_2$ was only 5.16 ± 0.61 μg/g DM. Vitamin $D_2$ yield values obtained from these two orientations were significantly different ($p < 0.01$). These values are three to four times higher than those reported by Mau *et al*. (1998) for the conversion of ergosterol to vitamin $D_2$ in Shiitake mushrooms by UV-B and UV-C irradiation.

Even though the concentration of ergosterol in gills of Shiitake mushrooms was only about twice higher than that of the outer layer of the cap (Table 3.1), Figure 3.1 clearly shows a conversion factor of approximately 4. This high level of conversion of ergosterol to vitamin $D_2$ may be due to the fine morphology of the gills, which allows greater exposure of the surfaces to irradiation than in the case of the caps, and in addition, the dark colour of caps could effect the penetration of UV radiation into mushroom tissues.

### 3.3: Ergosterol and vitamin $D_2$ contents in different types of edible mushrooms

A range of different types of mushrooms was investigated for their ergosterol content. The overall ergosterol contents of different types of mushrooms varied. The highest ergosterol content was found in Button mushrooms (7.80 ± 0.35 mg ergosterol/g DM)

while the lowest was in Enoki mushrooms (0.68 ± 0.14 mg/g DM). Oyster mushrooms contained 4.40 ± 0.08 mg/g DM and the value was more or less the same for Abalone mushrooms (4.35 ± 0.16 mg/g DM). Shiitake mushrooms contained 6.05 ± 0.07 mg/g DM of ergosterol. This is in agreement with the values found by Mattila *et al*. (2002) who observed a value of 6.79 mg/g DM of ergosterol in Shiitake mushrooms. Ergosterol contents of different types of non-irradiated mushrooms are shown in Figure 3.2.

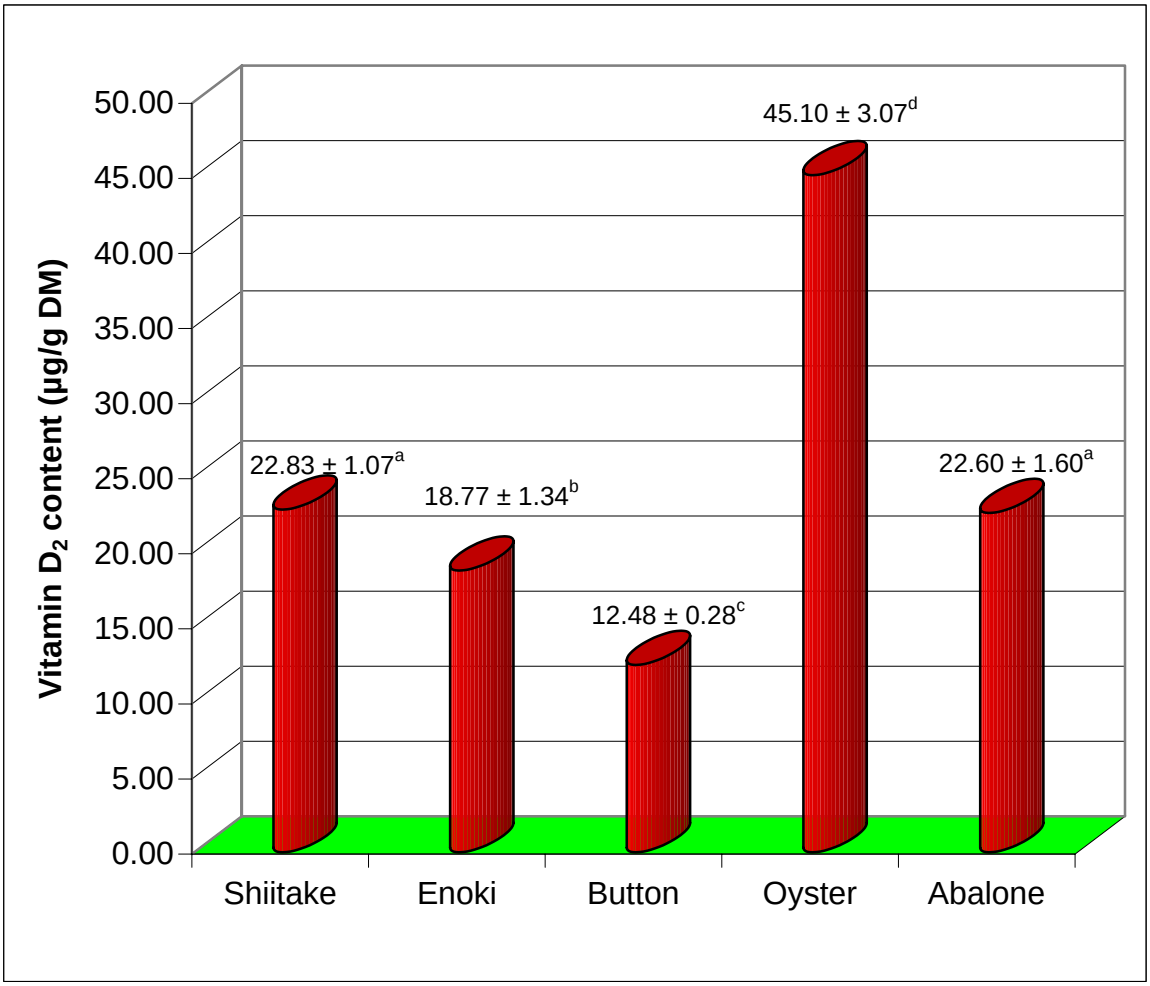



[a-d]: Values shown are mean values of 6 replicates ± SD. Values with different superscript letters are significantly different ($p < 0.01$).

**Figure 3.2:** Ergosterol contents of different types of mushrooms

### 3.4: Conversion of ergosterol to vitamin $D_2$ by UV irradiation

Vitamin $D_2$ content of different types of mushrooms subjected to 2 h of UV-A irradiation with their gills facing the source of irradiation is shown in Figure 3.3.




[a-d]: Values shown are mean values of 6 replicates ± SD. Values with different superscript letters are significantly different [a & b ($p < 0.05$), others ($p < 0.01$)]. Mushrooms were irradiated with their gills facing UV-A source at 27 $^{o}$C. Moisture contents of the mushrooms were found to be around 89 % (w.b.).

**Figure 3.3:** Vitamin $D_2$ contents of the different types of mushrooms subjected to irradiation for two hours; with their gills facing the UV-A source

Button mushrooms showed the lowest vitamin $D_2$ content (12.48 ± 0.28 μg/g DM) despite having the highest levels of ergosterol in them. Since the gills of button mushrooms were not opened, the gills of button mushrooms could not be exposed to UV source when they were irradiated. This could lead to lower conversion rate. On the other hand, the mono-oxygenase activity in button mushrooms was reported to be higher than that in other mushrooms (Espin *et al*. 2000) which contribute to a higher transformation of vitamin $D_2$ to 25(OH)$D_2$ and 25(OH)$_2D_2$. This higher transformation of vitamin $D_2$ to other products, could reduce the overall conversion of ergosterol to Vitamin $D_2$.

Overall the conversion of ergosterol to vitamin $D_2$ is very low on weight basis. Even though ergosterol in mushrooms was found in milligrams, the yield of vitamin $D_2$ from this conversion was only in micrograms. The possible reason for this lower than expected conversion of ergosterol to Vitamin $D_2$ may be due to the low depth of penetration of UV rays. UV-A is known to penetrate to a depth of 60-90 μm (approximately the thickness of epidermis) whereas UV-B and UV-C penetrate only to a depth of less than 10 μm in human skin (Freeman *et al*. 1962; Anderson & Parrish, 1981).

UV penetration in different mushrooms could also vary depending on the presence of pigments on the mushroom tissue. Oyster mushrooms on the other hand showed the highest vitamin $D_2$ content (45.10 ± 3.07 μg/g DM) among the different mushrooms tested. The yield of vitamin $D_2$ obtained from Abalone mushroom, which had more or less similar ergosterol content compared with Oyster, was only 22.60 ± 1.60 μg/g DM. Once again this may be due to their morphological differences and / or to the presence of

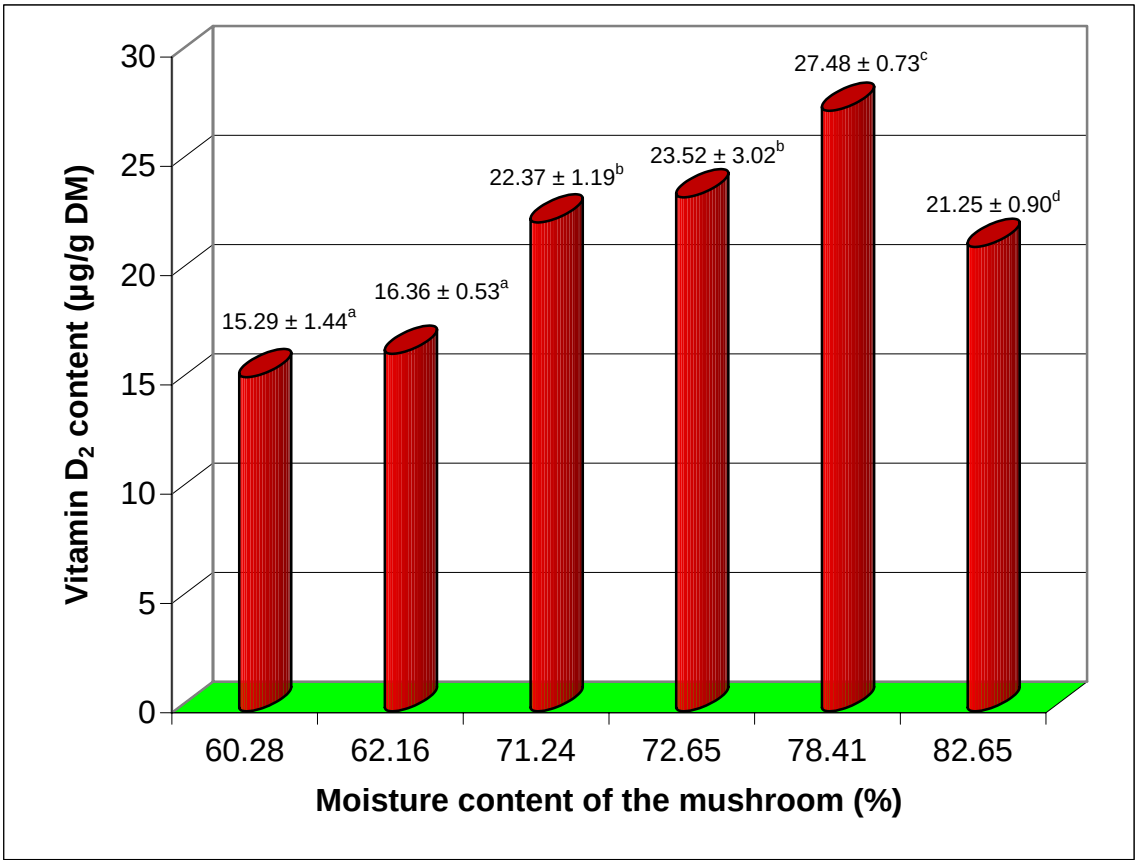
active mono-oxygenases, which convert vitamin $D_2$ to the hydroxy form. Vitamin $D_2$ content in Shiitake mushrooms after two-hour UV-A irradiation was 22.83 ± 1.07 μg/g DM. Mau *et al*. (1998) have reported 6.58 μg/g DM and 12.48 μg/g DM of vitamin $D_2$ from Shiitake mushrooms and Button mushrooms respectively, after two-hour UV-B irradiation at 12 $^{o}$C. However, no indication of the orientation of the mushroom tissues to the source of UV irradiation was indicated in their study. The values obtained for Shiitake mushrooms in this study were significantly higher than those reported by others and this may be due to the specific orientation of the mushroom to the UV source.

### 3.5: Effect of moisture content of mushrooms on the conversion of ergosterol to vitamin $D_2$

In this study, the irradiations were performed for two hours with the mushroom's gills facing the source of UV-A, at ambient temperature of 27 $^{o}$C. The mushroom samples were placed 15 cm away from the UV source during irradiation, and the calculated irradiation dose after a two-hour irradiation period was 25.2 kJ/m$^2$. The results obtained from irradiating Shiitake mushrooms at different moisture contents with their gills facing the UV source, show that the best conversion takes place at a moisture content of around 78 % on a wet basis (Figure 3.4).

At the high moisture content (the moisture content of fresh mushrooms are around 89 %), the conversion was significantly ($p < 0.01$) lower than at 78.4 % moisture content. This may be due to the dilution effect of ergosterol at very high moisture content, which is

likely to bring about a lower conversion rate. At low moisture levels, the specific surface area of the tissue is increased due to evaporation of moisture, and consequently the exposure to oxygen is increased resulting in the oxidation of vitamin $D_2$. Furthermore, irradiation also contributes to an oxidative atmosphere Vayalil *et al*. (2003), and photo-degradation of vitamin $D_2$ may occur. It can be concluded from the results that irradiation of mushrooms at a moisture-content of around 70 % - 80 %, enhances the yield of vitamin $D_2$ significantly ($p < 0.01$).



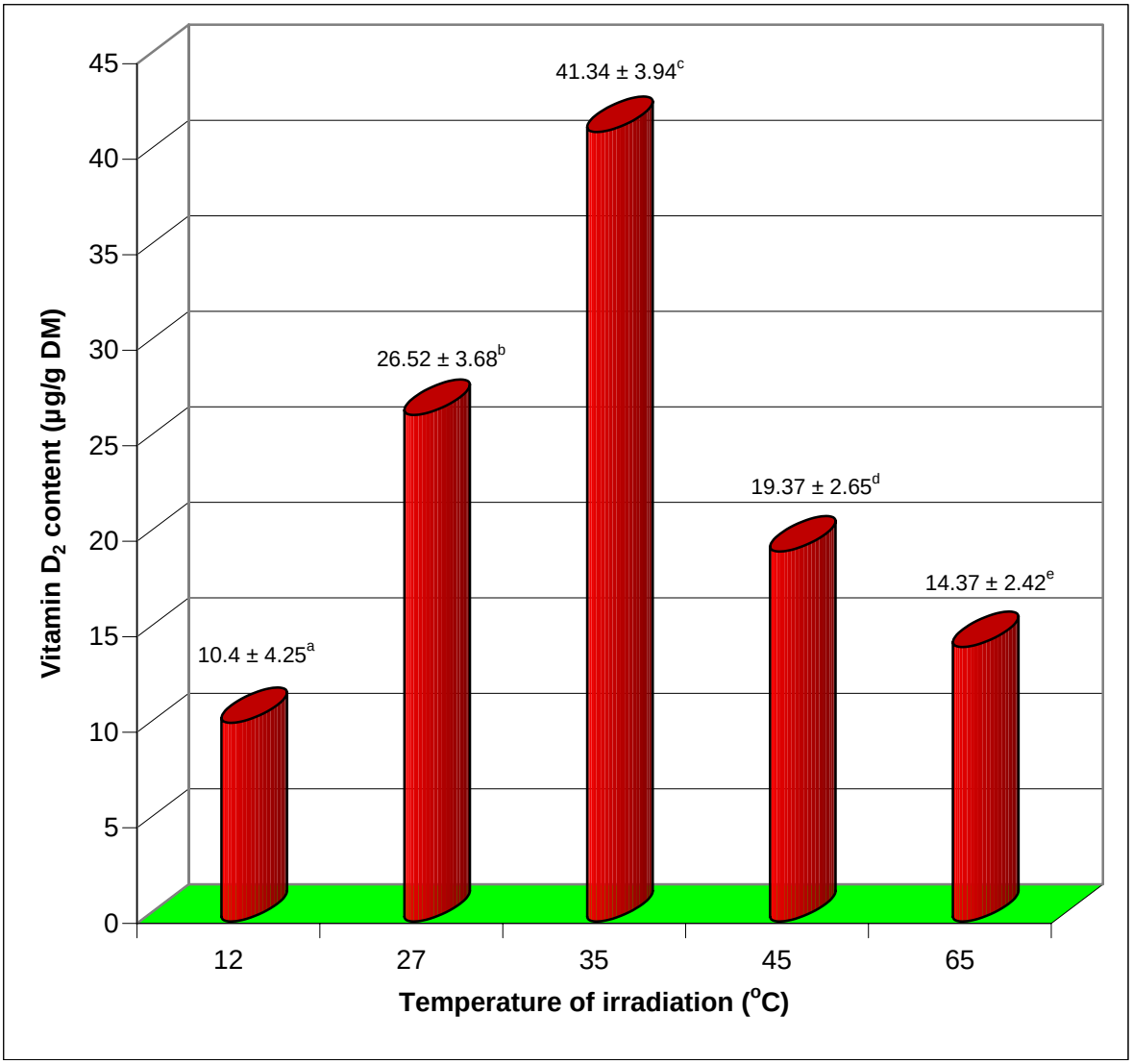

[a-d]: Values shown are mean values of 6 replicates ± SD. Mushrooms were irradiated at 27 [o]C. Values with different superscript letters are significantly different [b & d ($p < 0.05$), others ($p < 0.01$).

**Figure 3.4:** Effect of moisture content of mushrooms on the conversion of ergosterol to vitamin $D_2$

### 3.6: Effect of temperature on the conversion of ergosterol to vitamin $D_2$

In this part of study, Shiitake mushrooms were irradiated for two hours with their gills facing UV-A source, at different temperatures. The mushroom samples were placed 15 cm away from the UV source during irradiation, and the calculated irradiation dose after two-hour irradiation period was 25.2 $kJ/m^2$. The moisture content of mushrooms was 89 % on a wet basis. Figure 3.5 shows the effect of irradiation temperature on the conversion of ergosterol to vitamin $D_2$ in Shiitake mushrooms.

The yields of vitamin $D_2$, after irradiation at 12, 27, 35, 45, and 65 $^oC$ were significantly different ($p < 0.01$). The results clearly suggest that irradiation of mushrooms at about 35 $^oC$, enhance the conversion leading to the highest yield of vitamin $D_2$. The decrease in conversion rate beyond 35 $^oC$ was probably due to many concurrent events that may occur: heat stress (oxidative), cell death, formation of browning pigments, further transformation of vitamin $D_2$ as well as photo-degradation by irradiation.



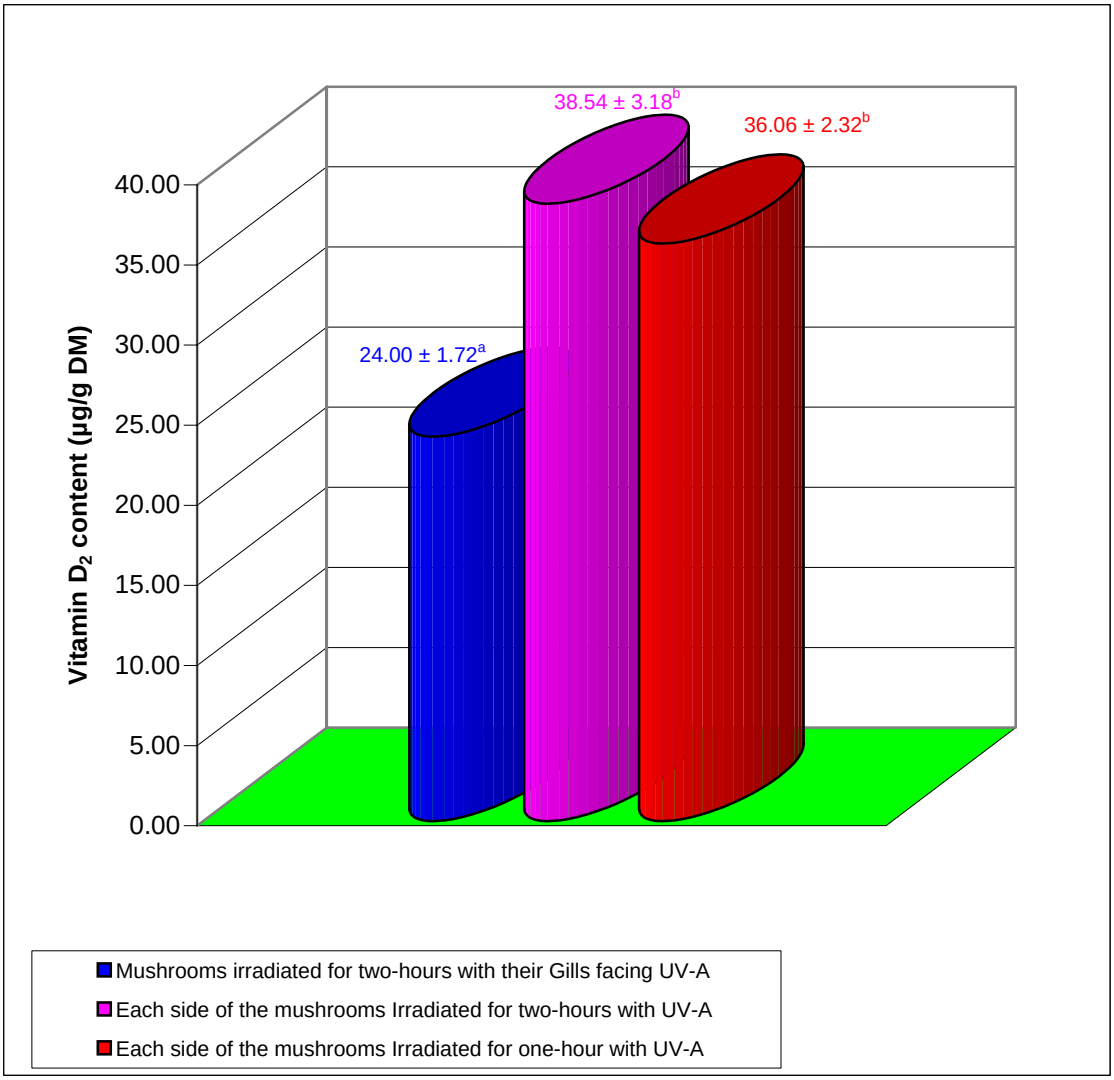

[a-d]: Values shown are mean values of 6 replicates ± SD. Values with different superscript letters are significantly different ($p < 0.01$). Moisture content of mushrooms was found to be around 89 % on a wet basis (w.b.).

**Figure 3.5:** Effect of temperature of irradiation on the conversion of ergosterol to vitamin $D_2$

### 3.7: Effect of different orientations of mushrooms to the UV source and duration of irradiation on the conversion of ergosterol to vitamin $D_2$

In the previous study (Section 3.2), it was found that the conversion of ergosterol was higher in mushrooms when they were irradiated with their gills facing the UV source, than when they were irradiated with their caps facing the UV source. One of the aims of this experiment was to investigate the effect of orientation of mushrooms and the time of exposure to the UV source on the conversion of ergosterol to vitamin $D_2$.

In this study, fresh Shiitake mushrooms were subjected to three different irradiations with UV-A. The first lot of mushrooms was irradiated for two hours with their gills facing the UV source, the second lot of mushrooms was irradiated with their gills facing the UV source for one hour and then they were further irradiated for another hour with their caps facing the UV source, and the third lot was irradiated with their gills facing the UV source for two hours and then they were further irradiated for another two hour with their caps facing the UV source. The calculated irradiation doses were 25.2, 25.2, and 50.4 $kJ/m^2$ accordingly. Vitamin $D_2$ contents of Shiitake mushrooms subjected to different orientations and times of exposure to UV irradiations are shown in Figure 3.6.




[a-c]: Values shown are mean values of 6 replicates ± SD. Values with different superscript letters are significantly different ($p < 0.01$). Mushrooms were irradiated at 27 $^{o}$C and the moisture content of mushrooms was found to be around 89 % (w.b.).

**Figure 3.6:** Effect of orientation of mushrooms and the duration of irradiation on the conversion of ergosterol to vitamin $D_2$.

The yield of vitamin $D_2$ after the irradiation of each side of the mushrooms (cap and gills) for one hour and vitamin $D_2$ yield after the irradiation of each side of the mushrooms for two hours were shown to be significantly higher ($p < 0.01$) than those observed from the

mushrooms irradiated with their gills facing the UV source for two hours. The vitamin $D_2$ yield obtained from irradiation of each side of the mushrooms for two-hours was 38.54 ± 3.18 μg/g DM) whereas it was 36.06 ± 2.32 μg/g DM when each side of the mushrooms were irradiated for one-hour. However, the difference between the two values was shown not to be significant ($p = 0.154$).

In humans, UV penetrates the outer most layers of the skin (epidermis, dermis) and there are some other factors such as skin colour, thickness, and body fat that effect the penetration of UV in to the skin (Holick *et al*. 1980; MacLughlin & Holick, 1985; Need *et al*. 1993). Similar factors (except the effect of fat layer in human skin) may interfere with UV penetration into mushrooms as well and therefore it is not clear to what extent that UV penetrates into the mushrooms tissues.

Figure 3.7 shows the effect of time period of irradiation of each side of the mushrooms on the conversion of ergosterol to vitamin $D_2$. The conversion of ergosterol in mushrooms to vitamin $D_2$ is almost completed within one hour, and this could be the reason that prolonged irradiation of each side after one-hour, does not contribute much in this conversion. The calculated irradiation dose in this study was 0.21 $kJ/m^2/min$ and average moisture content of mushrooms was found to be around 89 % (w.b).

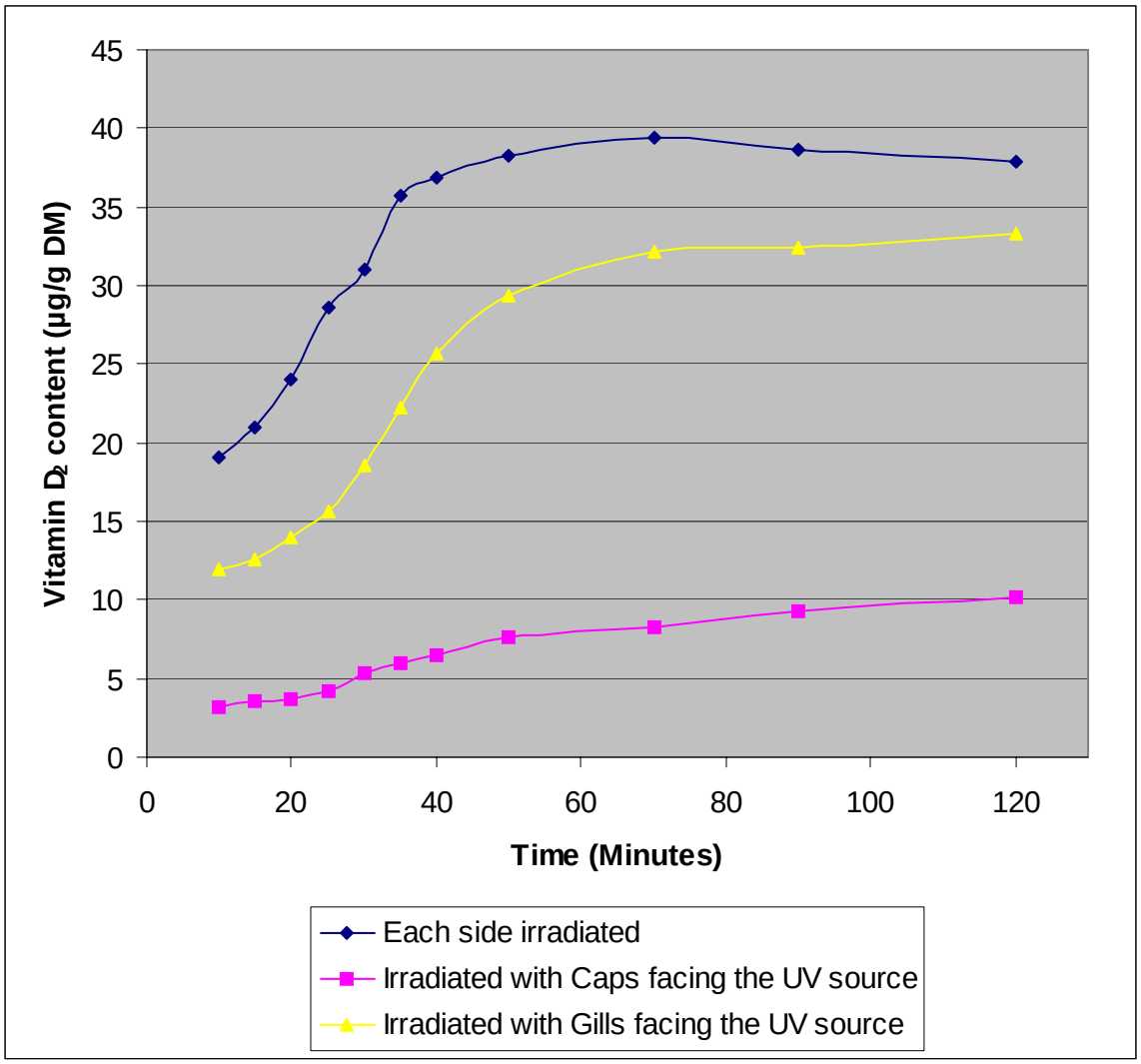



**Figure 3.7:** The effect of time of UV-A irradiation of Shiitake mushrooms on the conversion of ergosterol to vitamin $D_2$.

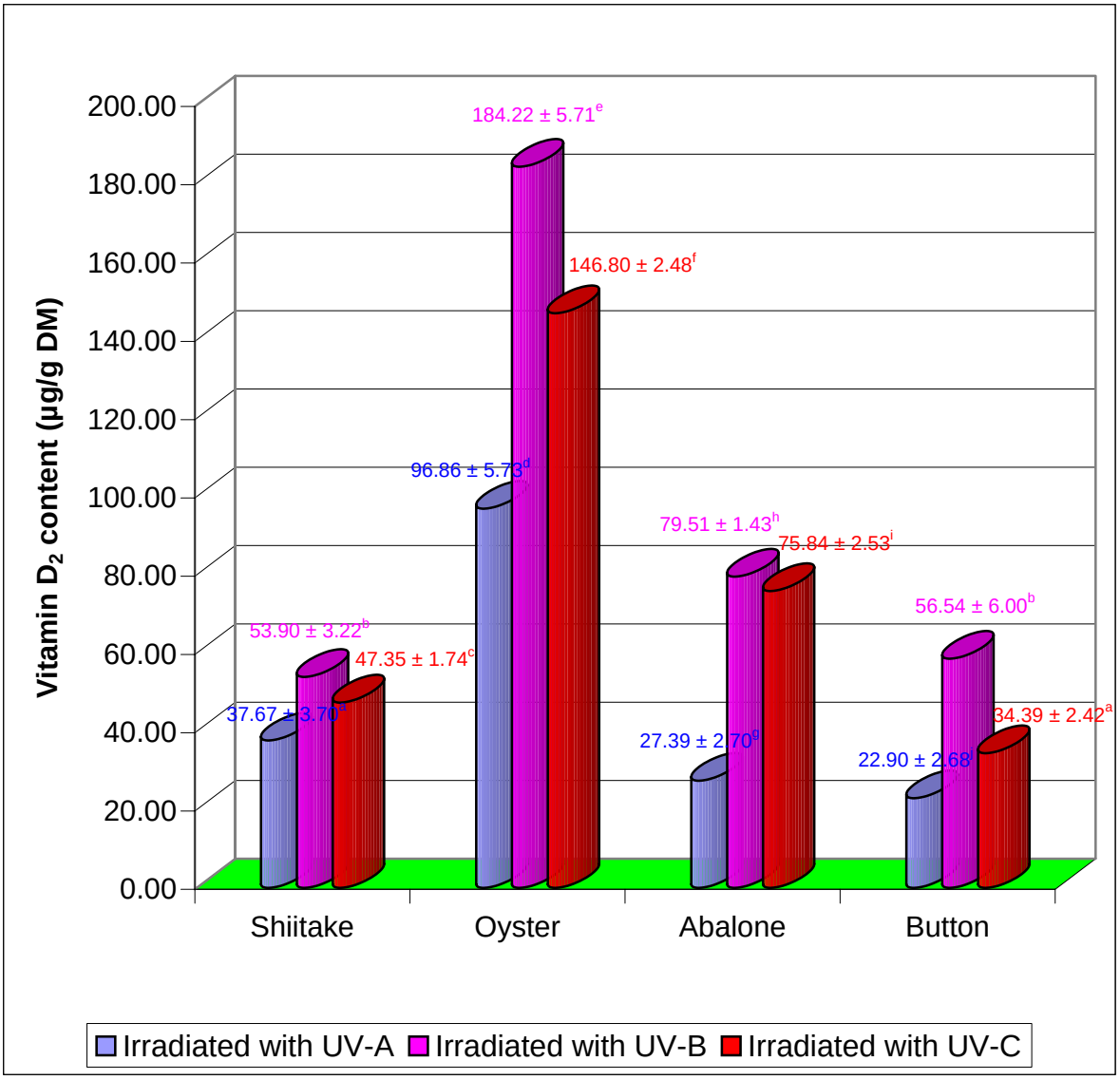
It is reflected in the graph that prolonged irradiation does not increase vitamin $D_2$. Previtamin D intermediates also absorb UV, produce tachysterol and lumisterol by photoisomerization (Havinga *et al*. 1960; Havinga 1973), and prolonged irradiation produces irreversible "over-irradiation products" by dimerization, and ring cleavage of the sterols (Braun *et al*. 1991). These may be the reasons for the slight reduction in

vitamin $D_2$ content close to the two-hour period of irradiation, when each side of the mushrooms were subjected to UV-A irradiation. In addition, irradiation also contributes to an oxidative atmosphere (Vayalil *et al*. 2003), and prolonged exposure of vitamin D to UV may result in photo-degradation of vitamin $D_2$ (Webb *et al*. 1989).

### 3.8: Conversion of ergosterol to vitamin $D_2$ by different bands of UV (UV-A, UV-B, and UV-C).

In this study, the moisture content of mushrooms was adjusted to around 80 % by removing the moisture in a vacuum dryer at ambient temperature, and irradiation was performed at 35 $^{o}$C, since these were found to be the optimum conditions for this conversion (Jasinghe and Perera, 2004). Figure 3.8 illustrates the effect of different bands of UV radiation on the conversion of ergosterol to vitamin $D_2$.

The yields of vitamin $D_2$ under UV-A, UV-B, and UV-C are significantly different ($p < 0.01$). The calculated radiation doses of UV-A, UV-B, and UV-C after two-hour period of irradiation (one-hour each side) were 25.2, 35.3, and 23.0 kJ/m$^2$ respectively. The results clearly indicate that the conversion of ergosterol to vitamin $D_2$ under UV-C was significantly higher ($p < 0.01$) than that under UV-A and the conversion under UV-B was significantly higher ($p < 0.01$) than those under UV-A or UV-C.




[a-j]: Values shown are mean values of 6 replicates ± SD. Values with different superscript letters are significantly different [b &c, h & i ($p < 0.05$), others ($p < 0.01$). The moisture content of mushrooms was around 80 %, and irradiation was performed at 35 $^{o}$C,

**Figure 3.8:** The conversion of ergosterol to vitamin $D_2$ under UV-A, UV-B, and UV-C.

The highest yields of vitamin $D_2$ were obtained under UV-B irradiation. However, under UV-B, mushrooms received 50 % more irradiation dose than under UV-C. Therefore the vitamin $D_2$ yields under UV-B and UV-C cannot be reconciled. Mau *et al.* (1998) have

reported a value of 6.58 μg/g DM of vitamin $D_2$ from Shiitake mushrooms after a two-hour period of UV-B irradiation. However, the orientation of mushrooms to the UV source and the moisture content were not reported. The temperature of irradiation, reported in their study (12 $^{o}$C) was much lower than that maintained in the current study (35 $^{o}$C). Temperature of irradiation plays an important role in this conversion as shown earlier and this may be one of the reasons why they obtained low conversion rates. In addition, the irradiation dose used in their study (9.86 kJ/m$^2$) was much lower than the irradiation dose used in this study (35.3 kJ/m$^2$) and finally the orientation of the mushrooms to UV source is most important, as shown earlier (Jasinghe & Perera, 2004).

# CHAPTER 4

# KINETICS OF THE CONVERSION, COMBINED EFFECT OF MOISTURE CONTENT AND TEMPERATURE ON THE CONVERSION OF ERGOSTEROL IN MUSHROOMS TO VITAMIN $D_2$

# CHAPTER 4

## Kinetics of the conversion, combined effect of moisture content and temperature on the conversion of ergosterol in mushrooms to vitamin $D_2$

### 4.1: Kinetics of the conversion of ergosterol to vitamin $D_2$

Kinetics are useful to investigate the order of a reaction, and can be used in prediction of the products, which resulted from a particular reaction. In this part of the work, Shiitake, Oyster, Abalone, and Button mushrooms were irradiated at a temperature of around 27 $^{o}$C for different time periods in order to investigate the conversion kinetics with regards to time of irradiation. The moisture content of those mushrooms was found to be 89 % (w.b.). The calculated UV-A irradiation dose, received by the mushrooms was 0.21 kJ/m$^2$/min.

Vitamin $D_2$ contents in different types of edible mushrooms, subjected to UV-A irradiation for different time periods, are shown in Figure 4.1 The highest rate of conversion of ergosterol to vitamin $D_2$ was observed in Oyster mushrooms while that of the lowest was observed in Button mushrooms.

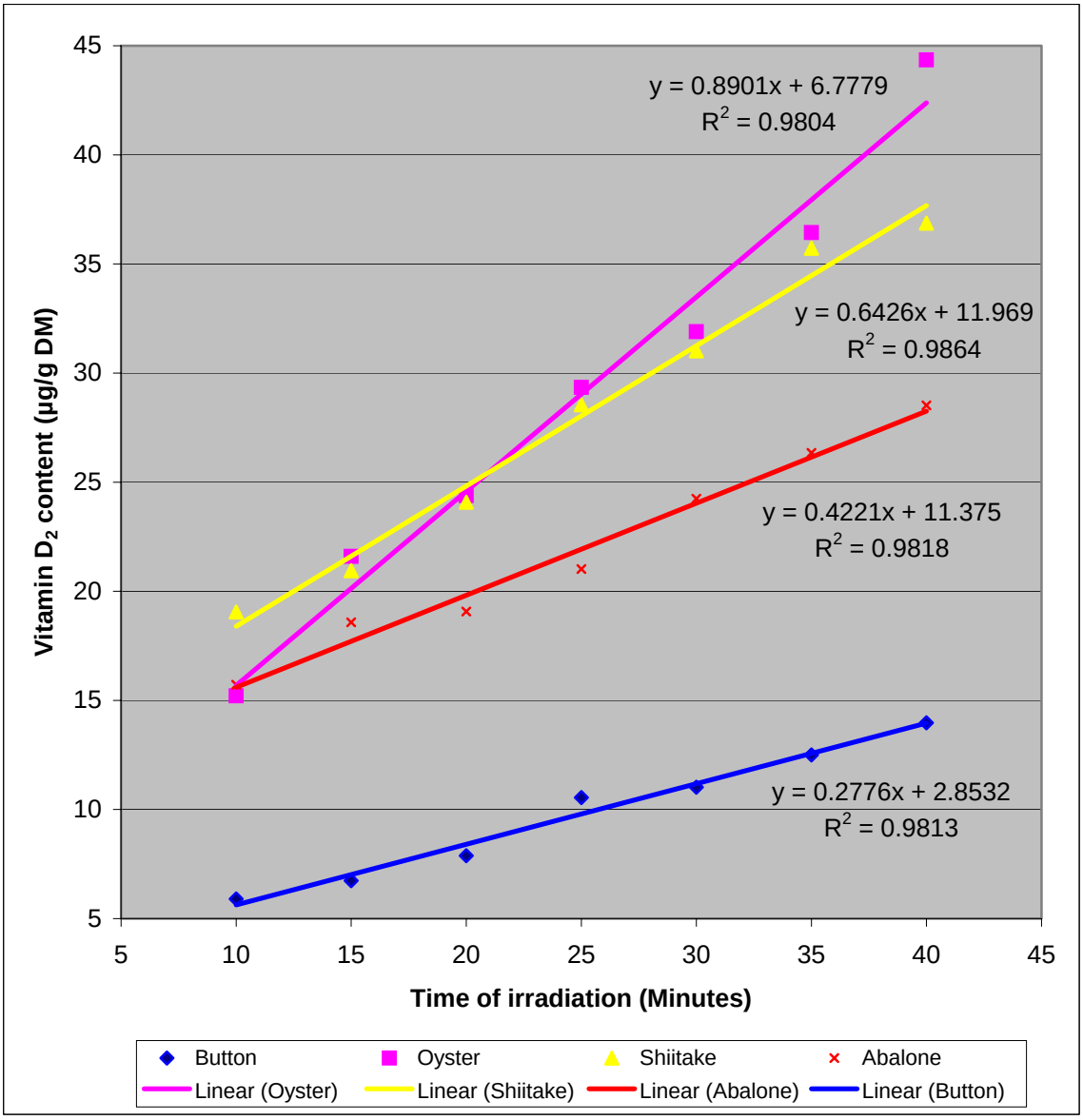



**Figure 4.1:** Effect of irradiation time on the conversion of ergosterol to vitamin $D_2$ in different types of edible mushrooms. Each side of the mushrooms was irradiated at 27 $^{o}$C and 89 % moisture content (w.b.)

The conversion of ergosterol to vitamin $D_2$ in different types of mushrooms under the said conditions can be predicted from the following equations:

*Oyster: amount of vitamin $D_2$* = *6.78 + 0.890*t* (1)

*Shiitake: amount of vitamin $D_2$* = *11.97 + 0.643*t* (2)

*Abalone: amount of vitamin $D_2$* = *11.38 + 0.422*t* (3)

*Button: amount of vitamin $D_2$* = *2.85 + 0.278*t* (4)

where amount of vitamin $D_2$ conveted from ergosterol is in μg/g DM and *t* is the time of irradiation in min.

### 4.1.1: Kinetic model of ergosterol conversion

The results of our experimental studies show that the conversion of ergosterol to vitamin $D_2$ during irradiation of mushroom increased linearly with time (Figure 4.1, Table 4.1). The amounts of vitamin $D_2$ versus time period of irradiation were well correlated ($R^2 \geq 0.98$) This trend was consistent with all three temperatures and time period used in this study. Hence a zero order reaction equation is a reasonable model to use for kinetics of conversion of ergosterol to vitamin $D_2$.

$$\frac{dC}{dt} = KC^0 \quad (5)$$

where *C* is the concentration of vitamin $D_2$ (g/g DM), t is the time of irradiation (s) and *K* is the reaction rate constant ($1s^{-1}$). One of the most common practices to model temperature dependence of reaction rates is to use Arrhenius equation (Banga & Singh, 1994). Yang et al (1998) also used this approach to model influence of temperature on reaction rates of conversion of ergosterol in solution to vitamin $D_2$. Hence in this study

the temperature dependence of the reaction rate constant *K* could also be described by an Arrhenius equation.

$$K = A_0 \exp\left(\frac{-E_a}{RT}\right) \quad (6)$$

Where $A_0$ is the reaction frequency factor, $E_a$ is the activation energy of conversion of ergosterol to vitamin $D_2$ ($Jmol^{-1}$), *R* is the gas constant (8.314 J K $mol^{-1}$) and *T* is the absolute temperature (K).

### 4.1.2: Kinetic model parameters

The kinetic model described in equations 5 and 6 was used as the basis of an effort to derive physical parameters for the experimental data obtained in this study. Thus, equation 5 was used to derive kinetic parameters at different irradiation times for each experimental temperature (Figure 4.2).

The gradient of straight lines in Figure 4.2 constitutes the constant *K* for each temperature, which was used in equation 6 to obtain the reaction frequency factor ($A_0$ = 7.32 $s^{-1}$) and the activation of energy of conversion ($E_a$ = 51.5 kJ $mol^{-1}$; $R^2$ = 0.94) (Figure 4.3). These kinetic parameters obtained are valid only for the temperature range of 25 $^oC$ to 35 $^oC$ tested in the experiment. Yang et al. (1998) reported the value of activation energy of conversion in the range of 4.2 to 28.7 $kJ/mol^{-1}$ depending upon the solvent and wavelength of UV light used. These kinetic parameters can be used to estimate the amount of vitamin $D_2$ yield for different times of irradiation within the temperature range studied.

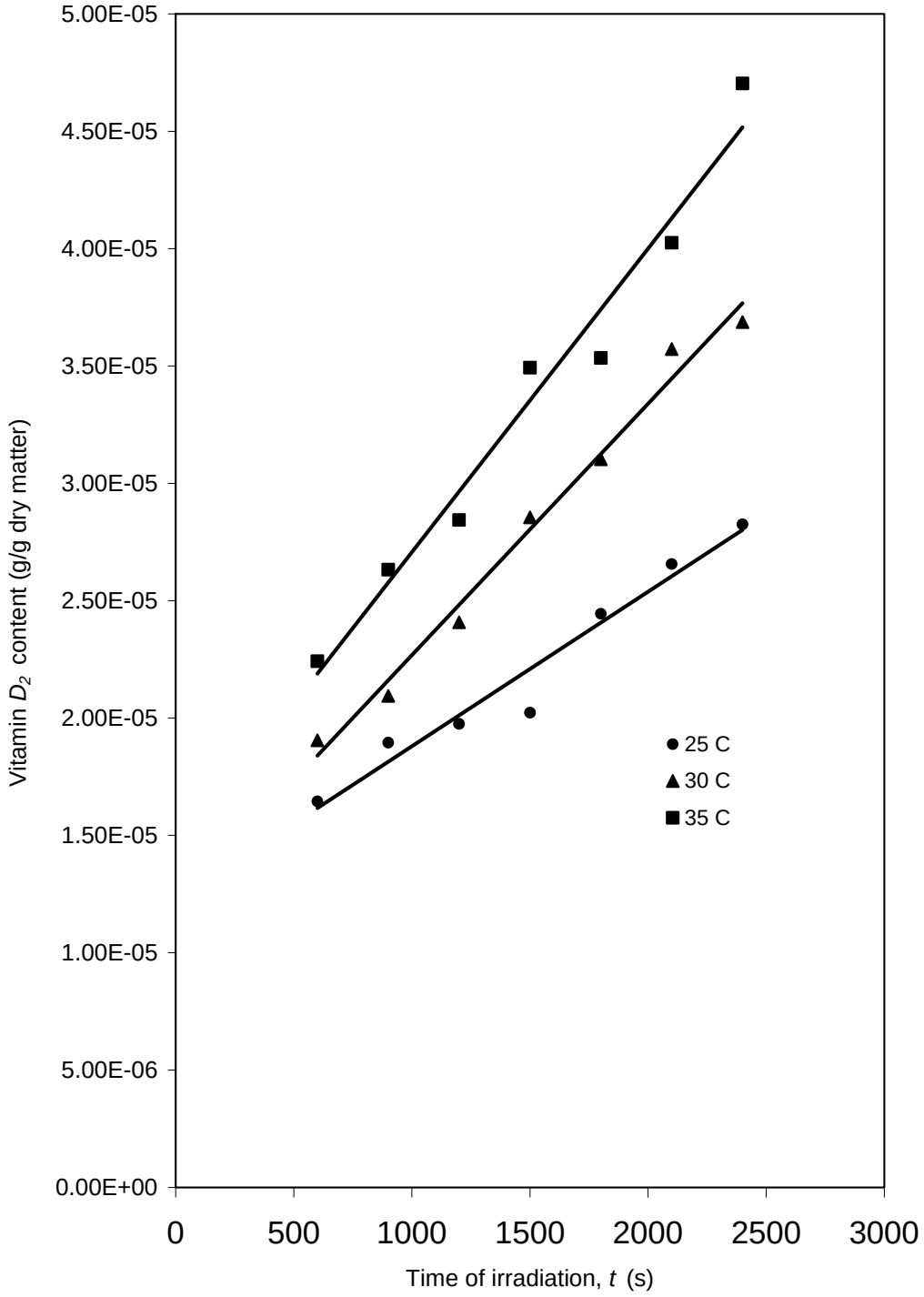



**Figure 4.2:** Modeling of the kinetic parameters for the experimental data in Table 4.1 in terms of reaction rate constant at different temperature

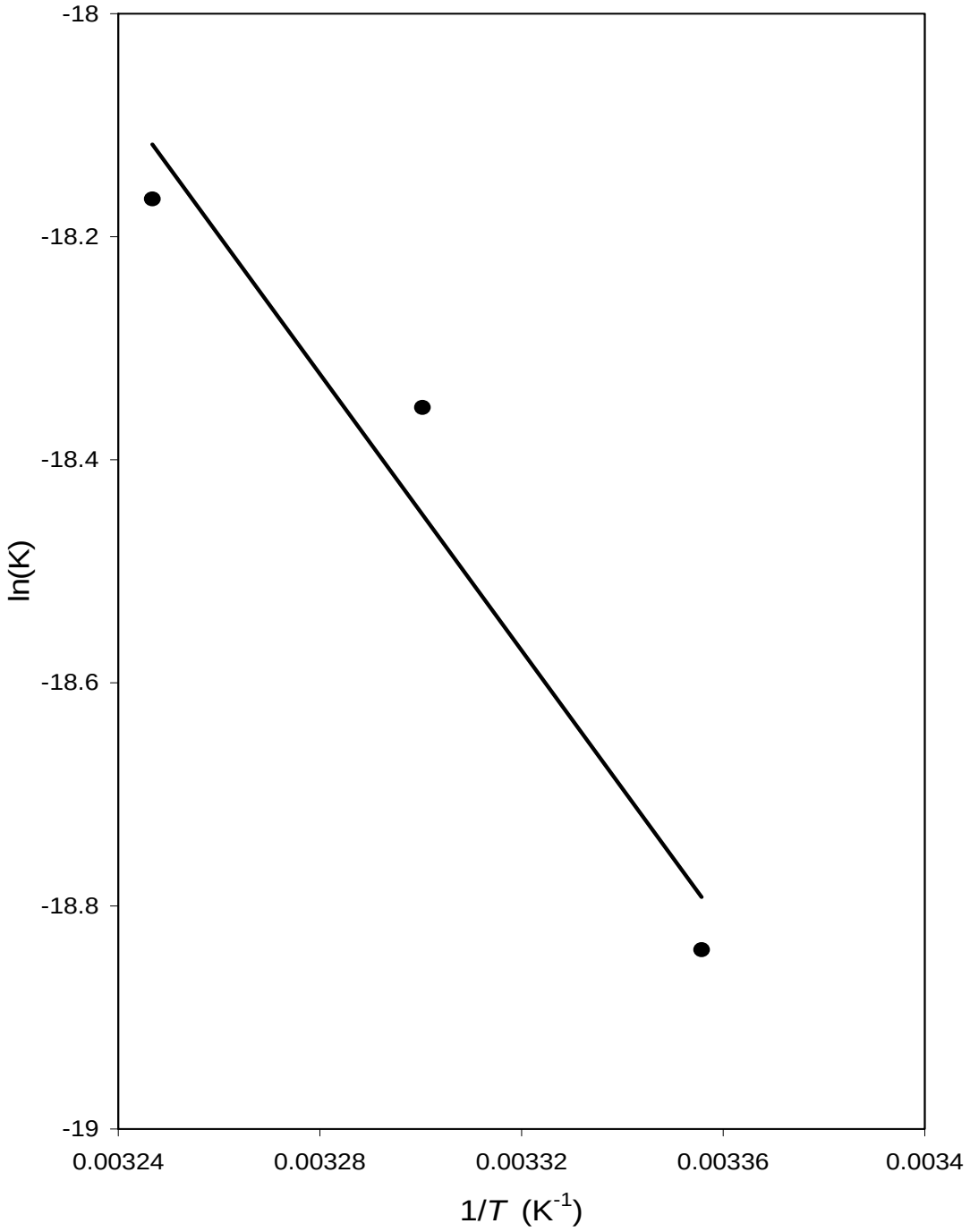



**Figure 4.3:** Modeling of the kinetic parameters for the experimental data in Table 4.1 in terms of temperature dependence of reaction rate constant using Arrhenius equation.

**Table 4.1:** Vitamin $D_2$ content (g/g DM) in Shiitake mushrooms, each side irradiated at different temperatures and times

| Time (s) | (Vitamin $D_2$ content x $10^5$) | | |
|---|---|---|---|
| | Temperature ($^oC$) | | |
| | 25 | 30 | 35 |
| 600 | 1.65 | 1.91 | 2.24 |
| 900 | 1.90 | 2.09 | 2.63 |
| 1200 | 1.98 | 2.41 | 2.84 |
| 1500 | 2.02 | 2.86 | 3.49 |
| 1800 | 2.44 | 3.10 | 3.53 |
| 2100 | 2.66 | 3.57 | 4.03 |
| 2400 | 2.83 | 3.69 | 4.70 |

### 4.2: Combine effect of moisture content and irradiation temperature on the conversion of ergosterol to vitamin $D_2$

Moisture content of mushrooms and the temperature of irradiation, effect the conversion of ergosterol to vitamin $D_2$ (Jasinghe & Perera, 2004). The combined effect of these two factors was studied in a 2 x 2 factorial model. The results are summarized in Table 4.2.

A 2 x 2 factorial design experiment was used, data were collected for four experimental conditions. The highest average vitamin $D_2$ yield (44.8 μg/g DM) was observed in mushrooms, having 80 % moisture and irradiated at a temperature of 35 $^0$C, which was followed by those having 60% moisture and irradiated at 35 $^0$C (29.7 μg/g DM). The

lowest yield of vitamin $D_2$ (8.05 μg/g DM) was observed in mushrooms having 80 % moisture and irradiated at 25 $^0$C. The average values were calculated from six experimental replicates. The tabulated data were analyzed by fitting them to 2 x 2 factorial model.

**Table 4.2:** Vitamin $D_2$ content in Shiitake mushrooms, each side irradiated at different moisture content and temperatures.

| Factor | | Treatment combination | Replicates of vitamin $D_2$ contents (μg/g DM) | | | | | | Average |
|---|---|---|---|---|---|---|---|---|---|
| T[a] (°C) | M[b] (%) | | 1 | 2 | 3 | 4 | 5 | 6 | |
| 25 | 60 | T low, M low | 7.78 | 8.33 | 9.80 | 7.90 | 7.78 | 6.81 | 8.05 |
| 35 | 60 | T high, M low | 31.10 | 29.00 | 29.40 | 30.60 | 27.24 | 30.50 | 29.70 |
| 25 | 80 | T low, M high | 21.00 | 22.30 | 20.50 | 20.20 | 22.90 | 21.00 | 21.30 |
| 35 | 80 | T high, M high | 44.70 | 44.10 | 46.10 | 45.50 | 40.40 | 47.60 | 44.80 |

[a]Temperature of irradiation
[b]Moisture content of mushrooms

The complete analysis of Two-way ANOVA with repeated measures on both factors was carried out using the Vassar statistical analysis software and it is summarized in Table 4.3. Based on p- values, it can be concluded that the main effects [factor *A* (temperature of irradiation, T, °C) and factor B (moisture content of mushroom, M, fraction)] were statistically significant ($p < 0.01$) however; interaction effect between two factors was shown to be not significant ($p > 0.05$).

**Table 4.3:** Analysis of variance for the experiment data obtained in 2 x 2 factorial design

| Source of Variation | Sum of squares | Degrees of freedom | Mean square | F | p |
|---|---|---|---|---|---|
| T | 3044.0 | 1 | 3044.03 | 554.5 | < 0.01 |
| M | 1206.0 | 1 | 1206.01 | 1747.8 | < 0.01 |
| TM | 4.98 | 1 | 4.98 | 2.69 | 0.1619 |
| Total | 4304.9 | 3 | | | |

A multiple regression analysis was performed to correlate vitamin $D_2$ yield in Shiitake mushrooms with regards to temperature of irradiation and moisture content of the mushrooms, which resulted in following equation:

$$D_2 = -91.3 + 2.25 * T + 71 * M \quad (7)$$

The two factors considered, namely, temperature and moisture content were well correlated in regression model equation ($R^2 = 0.98$). The mean relative error and standard deviation of relative error were 5.24 % and 4.90 %, respectively.

The amount of ergosterol converted to vitamin $D_2$ increased linearly with time of irradiation. The combined effect of the temperature of irradiation, and the moisture content of mushrooms, was well correlated in the 2 x 2 factorial model. The expected

vitamin $D_2$ yield in Shiitake mushrooms can be predicted using equation 7. The rate of conversion of ergosterol to vitamin $D_2$ in different types of mushrooms was found to be different. More experimental data are required on other types of mushrooms to develop prediction equation for vitamin $D_2$ yield. The kinetic model parameters presented can be used to optimize the yield of vitamin $D_2$ in a given process.

Compared with high amounts of ergosterol present in cultivated mushrooms (quantitatively in milligrams/g DM), the amounts of vitamin $D_2$, obtained from the irradiation process were lower (only in micrograms/g DM). However, the current RDA of vitamin D for human is from 5 μg to 15 μg. The kinetic parameters, investigated in this study could also be used to control the process of this conversion in order to obtain different amounts of vitamin $D_2$ as required.

# CHAPTER 5

# BIOAVAILABILITY OF VITAMIN $D_2$

# CHAPTER 5

## Bioavailability of vitamin $D_2$

### 5.1: Bioavailability of vitamin $D_2$ from irradiated Shiitake mushrooms

The serum concentration of 25(OH)D is the barometer of vitamin D status (Holick, 2001), and therefore this measurement can be used in bioavailability studies of vitamin D.

Bioavailability of vitamin $D_2$ from irradiated Shiitake mushrooms was studied in a rat model. All animals involved in this study were treated in a humane fashion in accordance with the guidelines of the National University of Singapore, and disposed of in a manner prescribed by the animal holding unit, National University of Singapore.

All the subjects survived until they were sacrificed at the end of the study and neither physiological nor behavioural abnormalities were observed in any group. The ranges of physical measurements for the rat subjects are tabulated in Table 5.1.

The bodyweights at the beginning and end of the study did not differ among groups ($p < 0.01$). Furthermore, the lengths of femur bones did not differ among groups. No significance difference ($p < 0.01$) was shown in daily dietary intakes of Group 3 and Group 2. Group 1 was used to evaluate vitamin D deficiency status of animals before the administration of test diets.

**Table 5.1:** Basic measurements of rat group physical parameters(1)

| Measurement | Group 1[2] | Group 2[3] | Group 3[3] |
|---|---|---|---|
| Average body weight at commencement of the experiment (g) | 54.32 ± 5.12 | 99. 24 ± 4.64 | 93.92 ± 7.68 |
| Body weight when sacrificed (g) | 89.40 ± 4.63 | 311.87 ± 23.36 | 294.55 ± 19.06 |
| Average daily dietary intake (g) | 10.56 ± 2.51 | 22.78 ± 3.42 | 22.35 ± 3.14 |
| Length of right femur (mm) | 18.89 ± 1.15 | 20.68 ± 1.96 | 20.86 ± 2.05 |
| Length of left femur (mm) | 18.70 ± 1.23 | 19.87 ± 3.56 | 20.11 ± 1.33 |

[1]Group 1, on vitamin D deficient diet for one-week; Group 2, on vitamin D deficient diet for one-week and then irradiated mushrooms + vitamin D deficient diet for four weeks; Group 3, on vitamin D deficient diet for one-week, and then non-irradiated mushrooms + vitamin D deficient diet for four weeks.
[2]Measurments after one week; n = 6, mean ± SD.
[3]Measurments after five weeks; n = 12, mean ± SD.

Figure 5.1 shows the growth charts and daily dietary intakes of the test groups. The growth chart shows slight increment of the growth curve of Group 2 over the Group 3 after one week, when the test diets were begun to administrate. However, this difference was shown not to be significant ($p = 0.603$).

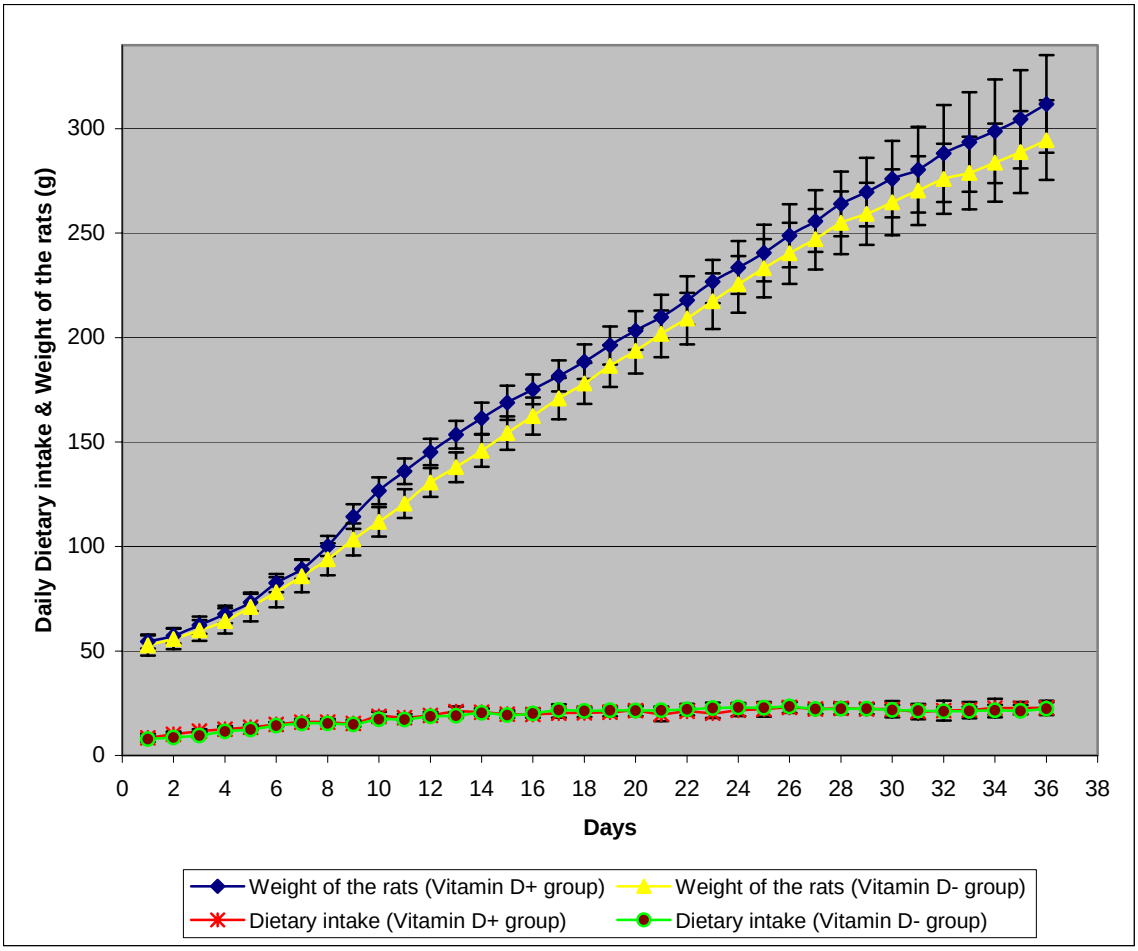
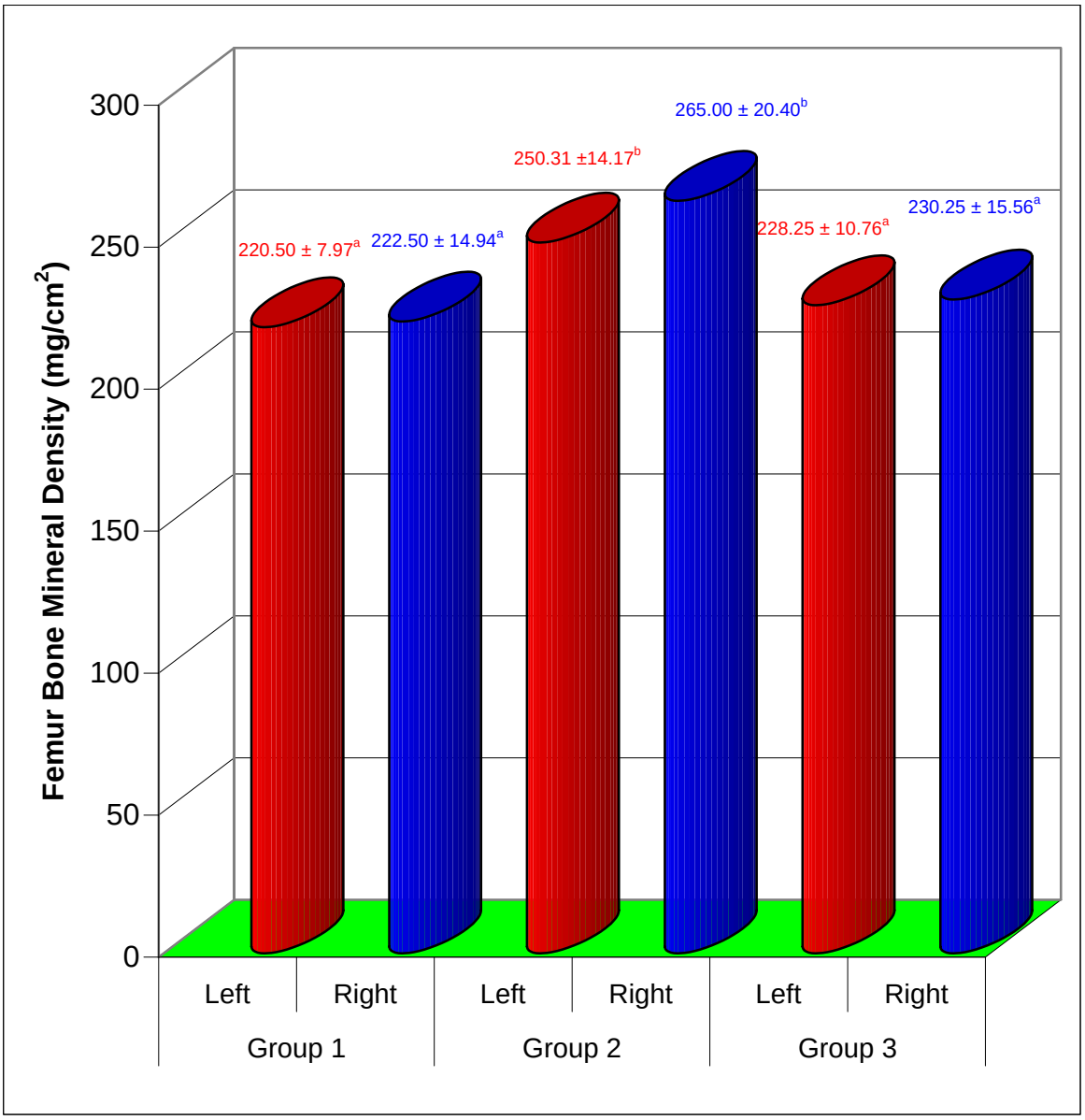



**Figure 5.1:** The growth charts and daily dietary intakes of the experimental group and the control group.

In this study, the difference in daily dietary intake of Groups 2 & 3 was shown to be not significant ($p = 0.853$). Assuming only vitamin $D_2$ was formed in mushrooms under UV radiation; Group 2 received 1 μg of vitamin $D_2$ from irradiated mushrooms while Group 3 received a similar diet but lacking in vitamin $D_2$. Since the animals were housed under incandescent light, cutaneous synthesis of vitamin D was not expected to interfere with the results. The BMD of femur bones was measured by Lunar DPX-L Dual-Energy X-ray

Bone Densitometer (DEXA); software version 1.3, (Lunar DPX-L, Lunar Corp., Madison, WI, USA). Figure 5.2 illustrates femur BMD of the three different groups.




[a]: Values with different superscript letters are significantly different ($p < 0.01$).

**Figure 5.2:** Femur BMD of initial, control, and experimental group

Femur BMDs of Group 2 were significantly higher ($p < 0.01$) than those of the other two groups. Difference in BMD between Group 1 and Group 3, was shown to be not significant ($p = 0.332$). In addition, the BMD values of right and left femurs within the groups were similar and the difference between values was shown not to be significant (Group 1, $p = 1.00$; Group 2, $p = 0.434$; Group 3; $p = 0.487$).

DEXA is a useful tool for measuring intact and excised rat leg bone mineral density (Nagy *et al*. 2001). In this study, it was shown that vitamin $D_2$ from irradiated mushrooms increased femur BMD of laboratory rats. Since vitamin D is directly involved in bone mineralization (Schapira *et al*. 1995; Erben *et al*. 1997a,b; Erben *et al*. 1998; Kaastad *et al*. 2001; Erben *et al*. 2002), the results of the current study show that in laboratory rats, vitamin $D_2$ from irradiated edible mushrooms has an important positive effect on the femur bone mineralization, especially during the period that the rats lay-down their skeleton.

Serum 25(OH)D was analysed using Gamma-B 25(OH)D $^{125}$I RIA kit (DiaSorin and IDS Ltd, Bolton, UK.), serum calcium levels were measured by an automated VITROS 950 chemistry system (Ortho-Clinical Diagnostics, Inc, Raritan, NJ, USA), at the Singapore National University Hospital. Serum 25(OH)D, and calcium concentrations of the groups are shown in Table 5.2.

**Table 5.2:** Serum 25(OH)D and serum calcium concentrations of rat groups[1]

| Variable | Group 1[2] | Group 2[3] | Group 3[3] |
|---|---|---|---|
| Serum 25(OH)D (nmol/L) | $18.06 \pm 5.26^{a}$ | $129.42 \pm 22.0^{b}$ | $6.06 \pm 1.09^{c}$ |
| Serum Calcium (mmol/L) | $2.61 \pm 0.32^{a}$ | $2.28 \pm 0.11^{b}$ | $1.60 \pm 0.24^{c}$ |

[1]Each sample was subjected to triplicate analysis. Group 1, on vitamin D deficient diet for one-week at the beginning; Group 2, on irradiated mushrooms + vitamin D deficient diet for four weeks; Group 3, on non-irradiated mushrooms + vitamin D deficient diet for four weeks. Means with different superscript letters are significantly different, $P < 0.01$.The statistical analyses were based on ANOVA and Turkey's HSD test.
[2]mean value ± SD; n = 6.
[3]mean value ± SD; n = 12.

The results show that serum 25(OH)D concentration of Group 2 clearly differs from that of Group1 and 3. Serum 25(OH)D concentration of Group 2, which received 1 μg of vitamin $D_2$ daily from mushrooms for four weeks, was 129.42 ± 22.00 nmol/L whereas it was only 6.06 ± 1.09 nmol/L in Group 3, which received no vitamin $D_2$. A decrease in 25(OH)D concentration was observed in Group 3 compared with Group 1 but on the other hand, a remarkable increase was observed in Group 2. The serum calcium levels among groups were also significantly different.

In contrast to what might have been expected, the serum calcium level of Group 2 was significantly lower compared with Group 1. This could be due to a higher rate of bone mineralization in Group 2 (which received vitamin $D_2$ from mushrooms) compared with Group 1. This is supported by the observation that there was a significantly higher BMD

and lengths of femur bones in Group 2. In addition, lowered serum levels of PTH, raised serum ionised calcium levels, and an age related decline in duodenal calcium absorption have all been reported and could be contributing factors to this difference (Liang *et al*. 1989; Takamoto *et al*. 1990; Agnusdei *et al*. 1998; Schulz & Morris, 1999).

The current results clearly indicate that vitamin $D_2$ from irradiated mushrooms was well absorbed in the laboratory rats since the serum concentration of 25(OH)D of the experimental group was remarkably higher than the control group. Vieth & Milojevic (1995) reported a value of 58 ± 8 nmol/L of 25(OH)D in a similar rat study using vitamin $D_3$ as a supplement. In this study, the quantities of vitamin D administered were considerably higher than the amount of vitamin D given there, and this may be the reason for the observation of high values of serum 25(OH)D in this study.

Since vitamin D influences several steps in the active calcium transport system, (Bronner, 1987; Bronner, 1992; Gueguen & Pointillart, 2000), measurement of serum calcium concentration is a useful tool to predict vitamin D deficiency. Serum calcium concentration of Group 2 was significantly higher than that of the value for Group 3. Thus, it was clearly indicated that Group 3, fed only on vitamin D deficient diet, was indeed deficient in vitamin D.

The dose of vitamin $D_2$ for rats, which was used in this study, was around 3 μg/Kg body weight. If this dose is converted to an average body weight of a human (70 Kg), it is around 200 μg/day. This is around 20 times higher compared with current RDA of

vitamin D for adults (10 μg/day), which some workers believe to be inadequate (Hanly *et al*. 1985; McKenna *et al*. 1985; McKenna *et al*. 1995; Chapuy *et al*. 1997; McKenna & Freaney, 1998; Compston, 1998; Cheetham, 1999; Vieth, 1999; Heaney, 2000; Vieth, 2000), and even up to100 μg vitamin $D_3$/day is a safe intake (Vieth *et al*. 2001). Irradiated edible mushroom powder could be used in fortification of human food supplements or the fresh form of irradiated mushrooms could be used directly for human consumption.

# PART III

# CONCLUSIONS AND FUTURE WORK

# CHAPTER 6

# CONCLUSIONS AND FUTURE WORK

# CHAPTER 6

## Conclusions and future work

### 6.1 Conclusions

In this study, edible cultivated mushrooms were found to be a very rich source of ergosterol. However, no detectable vitamin $D_2$ was observed in cultivated Shiitake, Oyster, Abalone, Button and Enoki mushrooms. The distribution of ergosterol in different parts of shiitake mushroom varied. Gills of mushrooms contain the highest value of ergosterol followed by the cap and the least amount was found in the stalk of the mushrooms.

The orientation of mushrooms to the source of irradiation was very important in conversion of ergosterol to vitamin $D_2$. The conversion rate of ergosterol to vitamin $D_2$ was approximately 4 times higher when gills were facing the UV source than when they were facing away from the source of irradiation. Mushrooms should be irradiated with their gills facing the UV source in order to maximize the conversion of ergosterol to vitamin $D_2$. However, it can be concluded from the results that irradiation of each side of mushrooms (cap & gills) for one hour is the optimum period of irradiation in conversion of ergosterol in mushrooms to vitamin $D_2$.

Temperature of irradiation and the moisture content of mushrooms play an important role in this conversion. The moisture content of fresh mushrooms was found to be around 89 % (w.b.) while the optimum moisture content of mushrooms for the conversion of

ergosterol to vitamin $D_2$ was found to be around 70 % - 80 % (w.b.). Hence, approximately 10 % – 15 % of moisture should be removed from fresh mushrooms before they are subjected to irradiation treatment. Irradiation of mushrooms should be carried out at a temperature of around 35 $^{o}$C in order to optimize the conversion of ergosterol to vitamin $D_2$.

The conversion of ergosterol in mushrooms to vitamin $D_2$ was studied under UV-A, UV-B, and UV-C. The yields of vitamin $D_2$ under UV-A, UV-B, and UV-C are significantly different. It can be concluded from the results that UV-C is more effective in this conversion than UV-A. UV-B was found to be the best source of irradiation in the conversion of ergosterol in mushrooms to vitamin $D_2$. Nevertheless, mushrooms received considerably higher dose of irradiation under UV-B compared with the irradiation doses under UV-A and UV-C. Under the conditions of UV irradiation, there was no apparent browning of the mushrooms, however, it was found that UV-C irradiation for 2 h caused considerable browning during subsequent storage.

Study of kinetics of conversion of ergosterol to vitamin $D_2$ shows the amount of ergosterol converted to vitamin $D_2$ increased linearly with time of irradiation. The combined effect of the temperature of irradiation, and the moisture content of mushrooms, was well correlated in the 2 x 2 factorial model. The expected vitamin $D_2$ yield in Shiitake mushrooms can be predicted using the equation,

$$\text{Vitamin } D_2 = -91.3 + 2.25 * T + 71 * M.$$

The rate of conversion of ergosterol to vitamin $D_2$ in different types of mushrooms was found to be different. More experimental data are required on other types of mushrooms to develop prediction equation for vitamin $D_2$ yield. The kinetic model parameters presented in this study can be used to optimize yield of vitamin $D_2$ in a given process.

The results of the kinetic study could be used in dried and fresh mushroom industry to improve the nutritional/functional value of mushrooms. Mushrooms can be subjected to an appropriate dose of radiation under known conditions in order to get predicted amount of vitamin $D_2$ before drying. The results also could be applicable to fresh mushroom industry in order to add more nutritional value to fresh mushrooms.

Bioavailability of vitamin $D_2$ from irradiated mushrooms was studied in an animal model in order to investigate the feasibility of use of irradiated mushrooms as a vitamin D supplement as the final stage of this project. It was found that serum 25(OH)D levels of a rat group fed with irradiated mushrooms were remarkably high compared with a control group. Hence, current results strongly suggest that vitamin $D_2$ from irradiated mushrooms is well absorbed in animals. On the other hand, femur BMD's of rats were also significantly higher in the experimental group compared with the control group. Thereby, it can be concluded from the results that not only vitamin $D_2$ from irradiated mushrooms is absorbed by animals but also the absorbed vitamin is metabolized to its active metabolites, and actively participates in the bone mineralization process. Therefore, the current results suggest that irradiated edible mushroom powder could be used in fortification of human food supplements or the fresh form of irradiated

mushrooms could be used for human consumption. Even under normal conditions, 5 g of fresh Shiitake mushrooms irradiated for 15 minutes with UV-A, or UV-B is more than enough to obtain the recommended vitamin D allowances for adults (10 μg/day) of vitamin D.

The term "irradiation" used here is totally different from the process of irradiation, now being used to a limited extent in the food industry. The term "irradiation" usually refers to high energy gamma irradiation using radioactive materials such $^{137}$Ce. Since the consumers might mistake the UV irradiated products as being gamma irradiated, the term "UV exposed" could be used in place of "UV irradiated" in order to differentiate between the two processes. In food processing industry, UV irradiation is used extensively to sterilize freshly squeezed juices, surface sterilization of solid food particles in order to eradicate micro organisms (Wilson *et al*. 1997 and Pan *et al*. 2004). Hence there appears to be no scientifically valid reason to consider the UV exposure of foods to be harmful or to cause health hazards to consumers.

In this study the relatively high levels of vitamin $D_2$ obtained from mushrooms (4-5 times higher than the maximum conversion of ergosterol by Mau et al 1998), irradiated mushroom powder high in vitamin $D_2$ could be used in food fortification. Even though vitamin $D_2$ is a fat soluble vitamin, that from irradiated mushroom powder could be incorporated with food products without a fat base. This is an advantage of using irradiated mushroom powder as an additive of vitamin D since the incorporation of

irradiated mushroom powder would not change the caloric value or fat composition of the product significantly.

Irradiated mushroom powder could be used in pharmaceutical industry to develop vitamin $D_2$ pills or tablets to use in eradicating vitamin D deficiency from the effected population.

However, the optimal therapeutic dosage of vitamin, and the effect of its administration on the other animal organs, especially the liver, heart and kidney, hypercalcemic effect, and the systemic metabolism to its active analogs, have yet to be elucidated.

## 6.2 Future work

### Clinical application of irradiated mushrooms in therapeutic diets

The literature reviewed it this thesis, clearly indicates that many common diseases (heart diseases, obesity, diabetes, cancers, hypertension, and arthritis) are associated with vitamin D deficiency. In addition, vitamin D is now being used in therapeutic applications in the treatment of several diseases including hyperproliferative diseases, secondary hyperparathyroidism, post transplant survival, and various malignancies. Investigation using clinical studies of therapeutic potential of vitamin $D_2$ from irradiated mushrooms and its applications could be useful for dieticians, nutritionists, and other relevant health professionals for formulation of therapeutic diets.

**Analysis of active analogs of vitamin $D_2$ from irradiated mushrooms and their hypercalcemic effect**

Conversion of vitamin $D_2$ from irradiated mushroom to other active analogs in animals is still unknown. Hence a study of the metabolism of vitamin $D_2$ from irradiated mushroom in animals could be useful in future therapeutic applications of this product.

The optimal therapeutic dosage of vitamin D, and the effect of its administration on the other animal organs, especially the liver, heart and kidney, hypercalcemic effect, and the systemic metabolism to its active analogs, need to be elucidated.

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# APPENDICES

## Appendix 1: Calibration curves

### 1.1: Calibration curve for the vitamin $D_2$

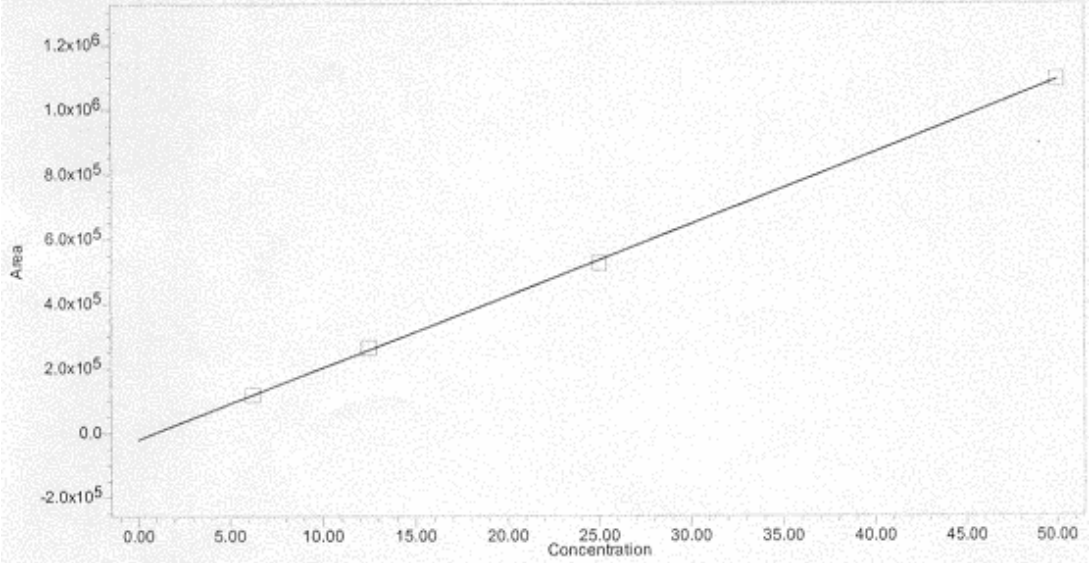
Empower software

LC Calibration Report

Reported by User: Visitors

Project Name: janaka

| | | | |
|---|---|---|---|
| Processing Method: | jas | Project Name: | janaka |
| Processing Method ID | 1317 | System: | HPLC_1 |
| Calibration ID: | 1318 | Channel: | 486 |
| Date Calibrated: | 1/14/2003 11:59:30 AM | Proc. Chnl. Descr. | |




Name: VitD2; RT: 7.252; Fit Type: Linear (1st Order); Cal Curve Id: 1319; R: 0.999879; R^2: 0.999758; Weighting: None; Equation: Y = 2.22e+004 X - 2.09e+004

**Peak: VitD2**

| | Sample Name | Result Id | Name | Level | X Value | Response | Calc. Value | % Deviation | Manual |
|---|---|---|---|---|---|---|---|---|---|
| 1 | janakaVitD2std6.25ppm | 1320 | VitD2 | 1 | 6.250 | 117255.000 | 6.226 | -0.39 | No |
| 2 | janakaVitD2std12.50ppm | 1321 | VitD2 | 2 | 12.500 | 263079.000 | 12.799 | 2.39 | No |
| 3 | janakaVitD2std25.00ppm | 1322 | VitD2 | 3 | 25.000 | 524741.000 | 24.594 | -1.62 | No |
| 4 | janakaVitD2std50.00ppm | 1323 | VitD2 | 4 | 50.000 | 1091246.000 | 50.131 | 0.26 | No |

**Peak: VitD2**

| | Ignore |
|---|---|
| 1 | No |

| | Ignore |
|---|---|
| 2 | No |

| | Ignore |
|---|---|
| 3 | No |

| | Ignore |
|---|---|
| 4 | No |

Report Method: LC Calibration Report Printed 10:52:33 AM 1/15/2003 Page: 1 of 1

## 1.2: Calibration curve for the vitamin $D_3$

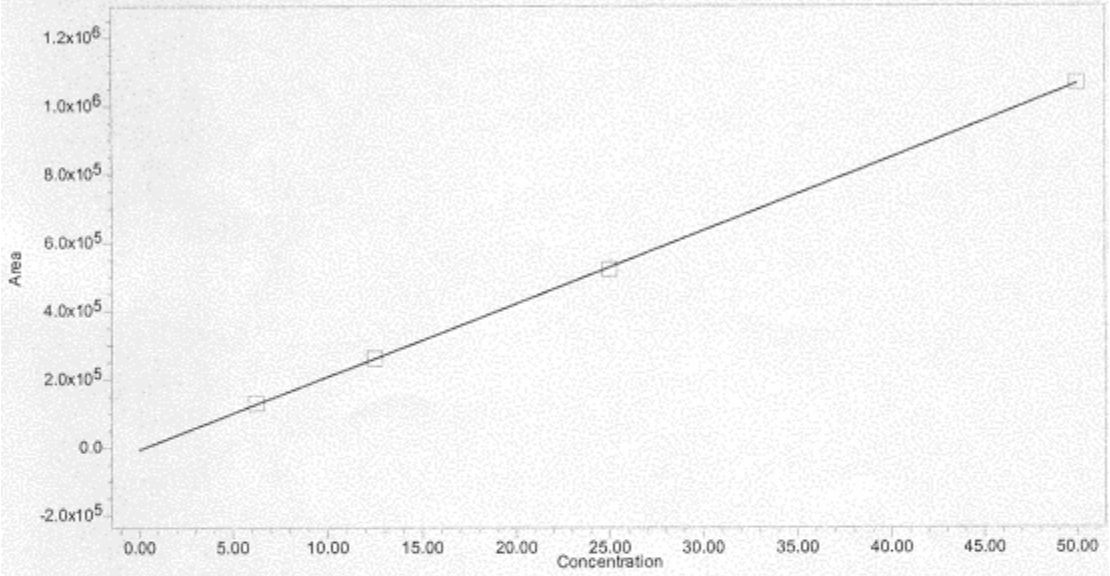
Empower software

# LC Calibration Report

Reported by User: Visitors

Project Name: janaka

| | | | |
|---|---|---|---|
| Processing Method: | JasD3 | Project Name: | janaka |
| Processing Method ID | 1444 | System: | HPLC_1 |
| Calibration ID: | 1446 | Channel: | 486 |
| Date Calibrated: | 1/14/2003 4:02:46 PM | Proc. Chnl. Descr. | |




Name: VitD3; RT: 7.882; Fit Type: Linear (1st Order); Cal Curve Id: 1447; R: 0.999938; R^2: 0.999876; Weighting: None; Equation: Y = 2.14e+004 X - 6.31e+003

**Peak: VitD3**

| | Sample Name | Result Id | Name | Level | X Value | Response | Calc. Value | % Deviation | Manual |
|---|---|---|---|---|---|---|---|---|---|
| 1 | janakaVitD3std6.25ppm | 1445 | VitD3 | 1 | 6.250 | 130506.569 | 6.399 | 2.39 | No |
| 2 | janakaVitD3std12.50ppm | 1448 | VitD3 | 2 | 12.500 | 261697.000 | 12.536 | 0.29 | No |
| 3 | janakaVitD3std25.00ppm | 1449 | VitD3 | 3 | 25.000 | 521441.000 | 24.685 | -1.26 | No |
| 4 | janakaVitD3std50.00ppm | 1450 | VitD3 | 4 | 50.000 | 1065432.000 | 50.130 | 0.26 | No |

**Peak: VitD3**

| | Ignore |
|---|---|
| 1 | No |

| | Ignore |
|---|---|
| 2 | No |

| | Ignore |
|---|---|
| 3 | No |

| | Ignore |
|---|---|
| 4 | No |

Report Method: LC Calibration Report Printed 10:51:46 AM 1/15/2003 Page: 1 of 1

## 1.3: Calibration curve for the ergosterol

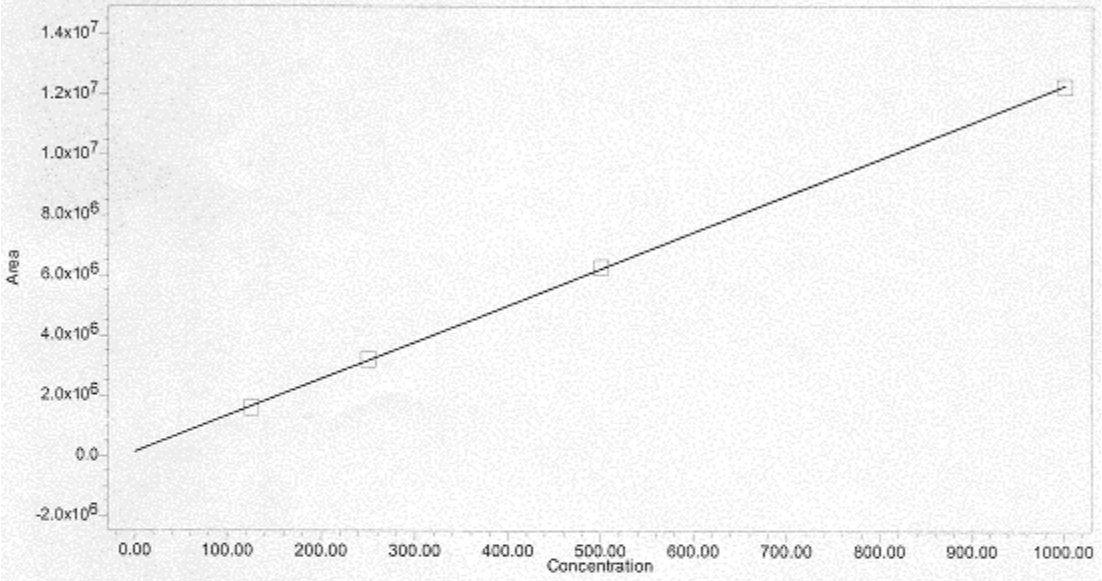
Empower software

LC Calibration Report

Reported by User: Visitors

Project Name: janaka

| | | | |
|---|---|---|---|
| Processing Method: | JasErgo | Project Name: | janaka |
| Processing Method ID | 1460 | System: | HPLC_1 |
| Calibration ID: | 1462 | Channel: | 486 |
| Date Calibrated: | 1/14/2003 4:22:04 PM | Proc. Chnl. Descr. | |




Name: Ergo; RT: 15.713; Fit Type: Linear (1st Order); Cal Curve Id: 1463; R: 0.999945; R^2: 0.999889; Weighting: None; Equation: Y = 1.22e+004 X + 1.52e+005

**Peak: Ergo**

| | Sample Name | Result Id | Name | Level | X Value | Response | Calc. Value | % Deviation |
|---|---|---|---|---|---|---|---|---|
| 1 | janakaErgostd125.00ppm | 1461 | Ergo | 1 | 125.000 | 1615647.000 | 120.312 | -3.75 |
| 2 | janakaErgostd250.00ppm | 1464 | Ergo | 2 | 250.000 | 3228458.000 | 252.863 | 1.15 |
| 3 | janakaErgostd500.00ppm | 1465 | Ergo | 3 | 500.000 | 6283057.000 | 503.909 | 0.78 |
| 4 | janakaErgostd1000.00ppm | 1466 | Ergo | 4 | 1000.000 | 12293871.000 | 997.916 | -0.21 |

**Peak: Ergo**

| | Manual | Ignore |
|---|---|---|
| 1 | No | No |

| | Manual | Ignore |
|---|---|---|
| 2 | No | No |

| | Manual | Ignore |
|---|---|---|
| 3 | No | No |

| | Manual | Ignore |
|---|---|---|
| 4 | No | No |

Report Method: LC Calibration Report Printed 10:49:54 AM 1/15/2003 Page: 1 of 1