VITAMIN D ACCUMULATION IN KING OYSTER MUSHROOM STIMULATED BY UV RADIATION

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Philosophy

September, 2015

CERTIFICATE OF ORIGINALITY

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September 2015

Abstract

Vitamin D plays a critical role in regulating calcium and phosphorus homeostasis in the human body and deficiency of vitamin D can result in rickets, osteomalacia and other diseases such as cancers, diabetes and cardiovascular diseases. Currently, vitamin D deficiency or insufficiency is still common worldwide with an estimated total number of one billion people, and over 50% of the population in Asia. Edible mushrooms are a good source of various vitamins but have a low or negligible content of vitamin D. UV radiation has been found effective to stimulate the accumulation of vitamin D from the conversion of ergosterol in edible mushrooms. King oyster mushroom (*Pleurotus eryngii*) is one of the most popular and tasty edible mushrooms. However, the content and dynamic trend of vitamin D in king oyster mushroom during UV irradiation are still not well characterized. This study was focused on the accumulation of some important vitamin D species (e.g. D2 and D4) and their dynamic relationships in King oyster mushrooms during UV-B irradiation.

Our first study was on the stimulation of vitamin D2 and vitamin D4 accumulation in the oyster mushrooms by UVB irradiation for various periods of time. The contents of vitamin D2 and D4 showed a linear increase with time (D2: $y = 0.998x + 15.75$, $r^2 = 0.973$; D4: $y = 0.081x + 0.389$, $r^2=0.834$), and provitamin D2 a linear decrease ($y = -0.003x+2.09$, $r^2=0.942$) within 30 minute of UVB irradiation. The relationship between vitamin D2 and provitamin D2 can be fitted to a parabolic model ($y=7127x^2-29150x+298311$). Vitamin D2 and vitamin D4 showed a linear correlation ($y=10.91x+13.58$, $r^2=0.935$), indicating a synchronic increase during UVB radiation. Another

major part of our study was to develop effective high performance liquid chromatographic (HPLC) methods for quantitative analysis of the vitamin D contents in the mushroom with simplified sample preparation. These HPLC methods showed high precision and repeatability and recovery for quantification of vitamin D2, provitamin D2 and vitamin D4.

In summary, this study has developed effective HPLC methods for quantification of major vitamin D species in mushrooms, the dynamic trend of vitamin D2, provitamin D2 and vitamin D4 during UVB irradiation. These will provide useful references for enhancement of vitamin D accumulation in mushrooms by UV radiation and for quantitative analysis of the vitamin D contents.

Conference presentations

1. Zhang, H. L. Wu, J.Y. (2014) Stimulation of vitamin D2 accumulation in king oyster mushroom by ultraviolet irradiation. The 21st Symposium on Postgraduate Research of Chemistry, Hong Kong

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3. Zhang, H. L. Wu, J.Y. (2015) Simultaneous quantification of free provitamin D2 and vitamin D2 in mushroom using RP-HPLC-DAD. XV Chemometrics in Analytical Chemistry, Changsha

4. Zhang, H. L. Wu, J.Y. (2015) Effects of UV exposure on vitamin D2 and vitamin D4 contents in mushroom. The 2nd Macau Symposium on Biomedical Sciences 2015, Macau

5. Zhang, H. L. Wu, J.Y. (2015) Effects of UV exposure on vitamin D4 contents in mushroom. The 11th International Postgraduate Symposium on Chinese Medicine, Hong Kong

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List of abbreviations and symbols

DAD	Diode Array Detector
HPLC	High-performance Liquid Chromatography
IU	International Unit
MS	Mass Spectrometer
RDA	Recommended Dietary Allowance
RSD	Relative Standard Deviation
UV	Ultraviolet
UVA	Ultraviolet A
UVB	Ultraviolet B
UVC	Ultraviolet C
VD2	Vitamin D2
VD4	Vitamin D4
Ds	D2, D3…D7
SBG	Shuang Bao Gu
SD	Standard Deviation
XBG	Xing Bao Gu
XB	Xiang Gu
BG	Ben Gu

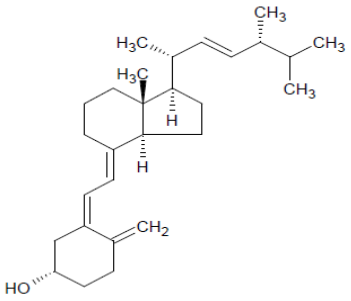
PG	Ping Gu
LZG	Ling Zhi Gu
LC-MS	Liquid Chromatography-Mass Spectrometer
LOD	Limits of Detection
LOQ	Limits of Quantification
WHO	World Health Organization
FAO	Food and Agriculture Organization
IOM	Institute of Medicine

Chapter 1 Introduction

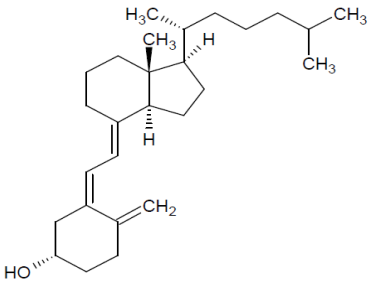
Vitamin D is a steroid prohormone substance with fat soluble property, which was discovered in the early 20th century in cod liver oil (McCullum EV, 1922; Zhang & Naughton, 2010). So far, several different series of the vitamin D, including vitamin D2 to vitamin D7, have been discovered and identified in organism. However, only vitamin D2 and vitamin D3 show the strongest biological activities in the body and are closely related with human nutrition (Kubodera, 2009; Tsugawa et al, 1999). The other forms of vitamin D, such as vitamin D4, also show biological activities and nutrition values like vitamin D2 or D3 (De Luca et al, 1968). Nevertheless, the biological activities of other forms of vitamin D are relatively lower than vitamin D2 or vitamin D3 in human (Hirsch, 2000; Napoli et al, 1979). For instance, the biological activities of vitamin D4 is about 60 % as active as vitamin D2 or D3 in rats (De Luca et al, 1968). Vitamin D5 showed much lower biological activities, the biological activities of vitamin D5 is around 1/80 as active as vitamin D2 or D3 (Napoli et al, 1979).

Vitamin D is a group compound of 9, 10-secosteroids, which is steroid molecules with an opened 9, 10-bond of the B-ring under UV environment (Dixon & Mason, 2009). Vitamin D2 is come from steroid compound called ergosterol under UV environment, and vitamin D4 come from a steroid compound called 22, 23-didydroergosterol UV environment. The chemical structures of ergosterol (provitamin D2), 22, 23-didydroergosterol (provitaminD4) and corresponded vitamin D2, vitamin D4 are showed in Figure 1-3. The chemical structures of other form vitamin D, including vitamin D2 to vitamin D7, are showed in Figure 1-1. The corresponded

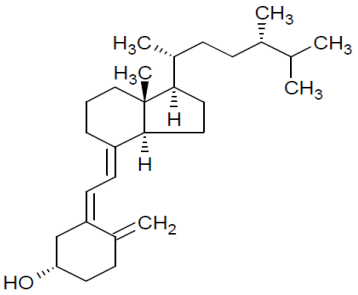
provitamin D2-D7 name and relative information is listed in Table 1-1 (Hirsch, 2000; Phillips et al, 2012).



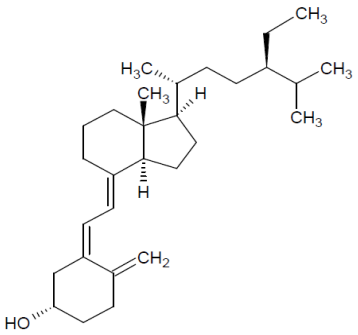
Vitamin D2



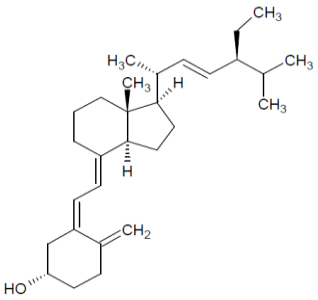
Vitamin D3



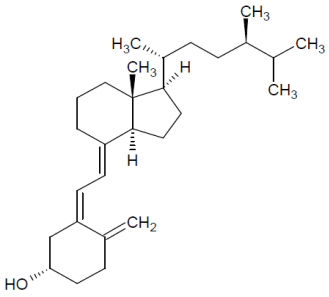
Vitamin D4



Vitamin D5



Vitamin D6



Vitamin D7

Figure 1-1.The chemical structures of vitamin D2, D3…D7

Table 1-1. Vitamin Ds and related provitamin Ds

Vitamin D	Provitamin D
D2	ergosterol
D3	7-dehydrocholesterol
D4	22,23-dihydroergosterol
D5	7-dehydrositosterol
D6	7-dehydrostigmasterol
D7	7-dehydrocampesterol

Vitamin D plays several critical roles in regulating homeostasis of calcium and phosphorus as well as enhancing bone mineral density in the body. Calcium and phosphorus are necessary substance in growth and maintenance of normal health in the body, therefore vitamin D play a role like controller in regulating calcium and phosphorus in body (Hirsch, 2000; Perez-Lopez, 2007). Phosphorus absorption in the body is just around 60% if do not have vitamin D, but if have vitamin D in the environment. Vitamin D can help phosphorus absorption increase to more than 75% in the body. Similarly for phosphorus absorption, intestinal calcium absorption is just around 10% in the body if do not have vitamin D2, but if have existence of vitamin D, intestinal calcium absorption can increase to over 30% in the body (DeLuca, 2004; Heaney et al, 2003; Holick, 2007). The study demonstrated that the intestinal calcium absorption showed significantly decreased in the body, when vitamin D concentration in blood was less than a level of 30 ng per mL (Heaney et al, 2003).

Recently, more and more research evidences indicate that vitamin play a wide and more important role in the body. Vitamin D2 deficiency does not just results in diseases related with rickets or bone health, but also related with incidence of other diseases, including cancers, hypertension, diabetes, infectious diseases, and cardiovascular diseases (Bilinski & Boyages, 2013; Burgaz et al, 2011; Dini & Bianchi, 2012; Ginde et al, 2009; Holick, 2007; Hossein-nezhad & Holick, 2013; Liu et al, 2012; Luczynska et al, 2013; Mohr et al, 2010; Pittas et al, 2010; Yin et al, 2013). The study demonstrated that data from over 900 women showed women with intake the highest vitamin D lowered 50% risk of breast cancer compared women with the lowers intake of vitamin D (Garland et al, 2006). A prospective study showed that there was an inverse relationship between 25-hydroxyvitmain D concentration and type 2 diabetes. The data collected from more than 3000 cases showed that when 25-hydroxyvitmain D level more than 30 ng per ml can reduce risk of type 2 diabetes compared with the level lower than 20 ng per ml level(Forouhi et al, 2012).

Currently, it is widely accepted by people that the blood concentration of 25-hydroxyvitmain D is the most useful marker to assess vitamin D status in the body. Although different societies may have little difference in definition for vitamin D deficiency, the standard of vitamin D deficiency from US Endocrine Society is widely used by most regions or countries. The US Endocrine Society recommended that vitamin D deficiency is defined as concentration of 25-hydroxyvitmain D in bloodlowerthan20 ng per mL; vitamin D insufficiency is defined as range of from 21 ng to 29 ng per mL, and vitamin D sufficiency is defined as concentration level with higher than 30 ng per mL, or higher for children and adults, as showed in Table 1-2 (Holick et al, 2011). Also, the society recommends that it is a better level to maintain

bone health that blood concentration of 25-hydroxyvitmain D is in the range from 40 to 60 ng per mL. Thus, the concentration of 40 to 60 ng per ml was considered as an ideal level to avoid disease occurrence related with vitamin D2 deficiency.

Currently, vitamin D deficiency or insufficiency is still common worldwide, according to the definition or assessing standard of vitamin D deficiency from US Endocrine Society(George et al, 2012; Holick et al, 2011). It was roughly estimated that around one-sixth people worldwide are vitamin D deficiency or insufficiency. All the peoples regardless of race and age showed phenomenon of vitamin D deficiencies or insufficiency (Lips, 2010; van Schoor & Lips, 2011;Wacker & Holick, 2013). In addition, vitamin D deficiency or insufficiency is highly prevalent in China. The studies showed that with more than 40% people in China are vitamin D deficiency or insufficiency (Du et al, 2001; Lu et al, 2012; Yu et al, 2015; Zhang et al, 2013;Zhen et al, 2014).

Additionally, the research evidences demonstrated that around 78% to 98% people are vitamin D deficiency or insufficiency in Asia. Particularly, vitamin D deficiency is quite prevalent in India and Mongolia (Marwaha et al, 2011; Rich-Edwards et al, 2011). In Africa and Middle-east, vitamin D deficiency is very common (Gannage-Yared et al, 2000; Prentice et al, 2009;Siddiqui & Kamfar, 2007). In Southern America, vitamin D insufficiency is quite common (Lips et al, 2006; Oliveri et al, 2004). Vitamin D status is only better in United States and Australia, which is estimated around 30% people are vitamin D deficiency (Looker et al, 2011; van der Mei et al, 2007). In summary, vitamin D deficiency or insufficiency is not rare, but still very common worldwide, particular in group of young children, pregnant women, and elderly people (Lips, 2010). A global status of vitamin D deficiency is showed in Figure 1-2. Thus, it is necessary and

significant to daily supplement vitamin D for most of people in the world by consumption of dietary foods or health care products.

Table 1-2. Definition of vitamin D deficiency

Definition	25-hydroxyvitmain D (ng/mL)	Influence
Deficiency	Less than 20	Weak mineralization
Insufficiency	21-29	Bone loss
Sufficiency	31-100	Ideal range

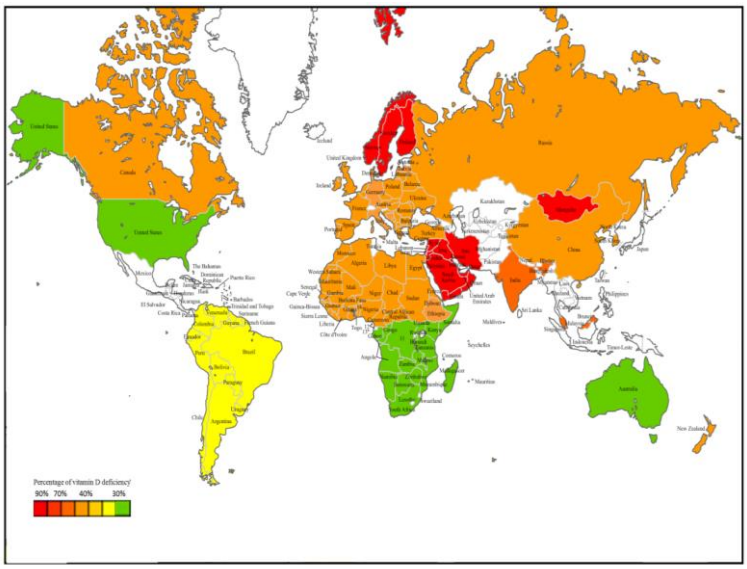


Figure 1-2. Global status of vitamin D deficiency in general population

Several health or medicine organizations, such as Institute of Medicine committee of US, US Endocrine Society, WHO and FAO, have published daily reference intakes of vitamin D to provide guidance dosages of vitamin D2 intakes. The Institute of Medicine (IOM) committee recommend dietary reference intake of vitamin D is 600

IU per day for children or adults. For the people with over 70 year, with 800 IU per day is suggested for intake, according to latest guideline of vitamin D intake of IOM released on year of 2010 (Ross et al, 2011). In addition, The US Endocrine Society recommendation in Practice Guidelines also has a reference intake of vitamin D. vitamin D intake dosage of 600 to 1000 IU per day is suggested for children and adolescents; but for the adults more than 18 year, vitamin D intake dosage of 1500-2000 IU per day is suggested by US Endocrine Society (Wacker & Holick, 2013).

In year of 2004, World Health Organization (WHO) and Food and Agriculture Organization (FAO) of the United Nations had a publication for vitamin D reference intakes. This reference intakes of vitamin D from WHO and FAO is lower than IOM dosage since it considers all people from world, but not for a particular country. Thus, it is suggested a lower reference intake dosage of vitamin D.

The daily reference intakes for vitamin D from organization of WHO and FAO are suggested in range from 200 to 600 IU per day in general dosage. The WHO and FAO recommended that 200 IU per day of vitamin D for infants and children, 200IU per day for adults, and 600IU per day for individuals who are more than 65 years (World Health Organization. & Food and Agriculture Organization of the United Nations., 2004).

In addition, the other organizations from other countries also have reference standard for daily vitamin D intake, such as Swiss Federal Nutrition Council, Belgian Health Council, Dutch Health Council, and DACH (Germany, Austria and Switzerland) Council etc. Although each organization may have a tiny difference in dosage of vitamin D intake, all of them follow a same principle which is the older people with over 70 years need to supplement higher dosage vitamin D compared with young adults. The dietary reference intake of vitamin D from another organization is showed in Table

1-3. (Balvers et al, 2015; Holick et al, 2011; Nutrition, 2012; Rizzoli et al, 2013; Spiro & Buttriss, 2014; World Health Organization. & Food and Agriculture Organization of the United Nations., 2004).

In summary, daily supplement of vitamin D should be in range of 200-800 IU to avoid incidence diseases related with vitamin D deficiency, based on reference intake of vitamin D from all organizations. The people over 70 years should supplement higher dosage of vitamin D, with daily supplement should be more than 600 IU.

European Society for Clinical and Economic Aspects of Osteoporosis and steroarthrisis (ESCEO) thinks it would be a basic level of 25-hydroxyvitmain D concentration of 20 ng per for osteoporosis patients to maintain healthy bone (Rizzoli et al, 2013). ESCEO thinks that the patients of high risk of fracture require a basic level for 25-hydroxyvitmain D is a concentration over30 ng per mL. The evidence and publications of these organizations show vitamin D plays vital role in the body. Those organizations provide a dosage for daily supplement vitamin D for maintain a health bone, and further demonstrate importance for supplement of vitamin D.

Table 1-3. Dietary reference: recommended intake of vitamin D by various organizations

Organization	Recommended intake (IU)			
	Age 1-18	Age 19-60	Age 61-70	Age >70
Institute of Medicine (RDA)	600	600	600	800
US Endocrine Society	600-1000	1500-2000	1500-2000	1500-2000
Swiss Federal Nutrition Council	600	600	800	800
Belgian Health Council	400-600	400-600	400-600	600
Germany, Austria and Switzerland	800	800	800	800
WHO/FAO (2004)	200	200-400	400-600	600

RDA= Recommended Dietary Allowance; IU= International Unit; 10 μg=400 IU.

Currently, most people rely on health care products to supplement vitamin D. Nevertheless, health care products are relatively expensive. For some people, it is hard to keep consistency to supplement vitamin D by consuming health care products, since they are unaffordable. Additionally, health care products are also inconvenient way compared with food sources to supplement vitamin D. Mushrooms are considered as one of the good food sources for supplement vitamin D, since it contains abundant provitamin D2 (ergosterol) and shows other functions (Calvo et al, 2013; Feeney et al, 2014; Jasinghe & Perera, 2005; Mattila et al, 2002; Teichmann et al, 2007).

It has been known that provitamin D2 undergoes photosynthesis to convert into vitamin D2 under exposure of UV environment or sunlight (Boomsma et al, 1977; Havinga, 1973; Havinga, 1960;Kalaras et al, 2012). UV light can cleave the B-ring of provitamin D2 to produce previtamin D2. Then, previtamin D2 is converted to vitamin D2 by thermally induced sigmatropic shift. Based on the theory of conversion between provitamin D2 and vitamin D2, UV light was therefore incorporated to enhance vitamin D2 enrichment in mushrooms. Similarly, vitamin D4 can be converted from provitamin D4 in the mushroom under UV environment or sunlight, showed in Figure 1-3.

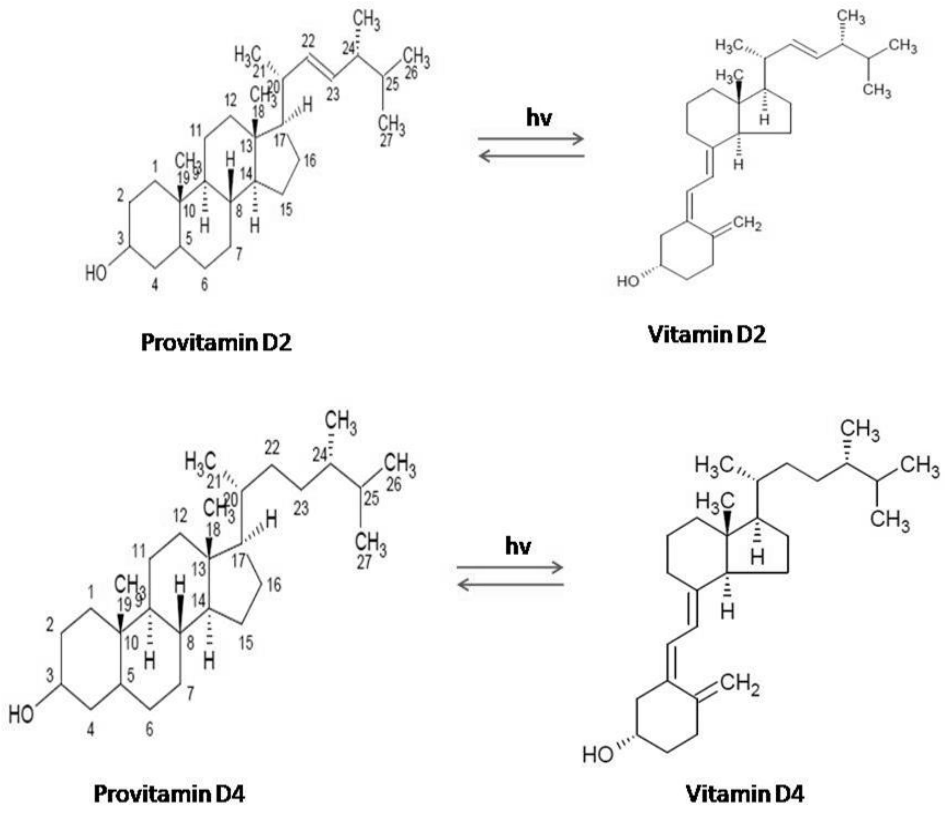


Figure 1-3. Conversional diagrams of vitamin D's and provitamin D's: (A) vitamin D2 and provitamin D2; (B) vitamin D4 and provitamin D4

Mushrooms may provide good source for supplement vitamin D2 for human. There were studies that reported vitamin D2 content and content dynamic trend in some mushrooms. King oyster mushroom (*Pleurotus eryngii*) is one of the most popular and tasty edible mushrooms. However, vitamin D2 content and content dynamic trend in king oyster mushroom during UV irradiation have not yet study so far. Vitamin D4 content and content dynamic trend in king oyster mushroom during UV irradiation was never reported previous study. Provitamin D2 content dynamic trend in king oyster mushroom during UV irradiation is still unknown. Additionally, the relationship between vitamin D2 and vitamin D4 in king oyster mushroom was never reported, and the relationship between vitamin D2 and provitamin D2 was never studied in previous research. Thus, it is necessary to illustrate contents and content dynamic trends for vitamin D2, vitamin D4 and provitamin D2 in king oyster mushroom during UVB irradiation.

Our study is to investigate vitamin D2 and vitamin D4 content in mushrooms and its dynamic trend in mushrooms and to evaluate content of vitamin D2 and vitamin D4 in mushrooms upon exposure of UV irradiation. It will be quite significant for humans if could supplement vitamin D2 by consumption of edible mushrooms. It is not only save huge cost in buying supplement vitamin D2, but also provide convenient approach to supply vitamin D2 for human if this technology were applied for stimulating production of vitamin D2 in edible mushrooms. Finally, it would provide a convenient and low-cost approach to supplement vitamin D2 by consuming mushrooms for human.

In addition, there are some methods to quantify vitamin D2 in mushrooms and some methods to analyse provitamin D2 in mushroom, but these methods are separately used for quantification of vitamin D2 and provitamin D2in mushrooms (Jedlickova et

al, 2008; Phillips et al, 2011; Tong et al, 2014). Hence, these methods suffer from several defects used for quantification of vitamin D2 and provitamin D2 in mushrooms, such as time-consuming, low-efficiency and low-accuracy, if used different methods to separately quantify vitamin D2 and provitamin D2 in mushroom. So far, there has not yet a method for simultaneous quantification of vitamin D2 and provitamin D2 in the mushrooms. Hence, it is necessary to develop a method to simultaneous quantification of vitamin D2 and provitamin D2 in the mushrooms.

In our study, we first reported a new approach to simultaneously quantify vitamin D2 and provitamin D2 in mushroom by HPLC-DAD method. Our approach to simultaneously quantify vitamin D2 and provitamin D2 will provide a simple approach to show the content and content dynamic trend for vitamin D2 and provitamin D2 in the mushroom during UV irradiation. The methods were successfully applied to find the content dynamic trend vitamin D2 and provitamin D2 in mushrooms.

Additionally, there has not yet a HPLC-DAD method for quantification of vitamin D4 in mushroom, and vitamin D4 content in king oyster mushroom is still unknown. Therefore, there is a need to develop a HPLC-DAD method to quantify vitamin D4 in mushrooms before unveiling content dynamic trend of vitamin D4 in the mushroom. Moreover, there was no study to focus on vitamin D4 dynamic trend and content in king oyster mushroom in previous research. Thus, it is significant to unveil vitamin D4 content and content dynamic trend in king oyster mushroom.

In our study, we first developed a HPLC-DAD method to quantify vitamin D4 in king oyster mushroom. The developed method provides an approach to demonstrate content and content dynamic trend for vitamin D4 in king oyster mushroom. Our study

demonstrated the vitamin D4 content and dynamic trend in king oyster mushroom. The developed method will provide a new approach to quantify vitamin D4 in mushrooms.

In the present study, we develop a HPLC-DAD method to quantify vitamin D2 and provitamin D2 in the mushroom, and demonstrate content and content dynamic trend for vitamin D2 and provitamin D2 in king oyster mushroom during UVB irradiation. We develop a new HPLC-DAD method to quantify vitamin D4 in mushroom, and demonstrate content and content dynamic trend for vitamin D4 in king oyster mushroom during UVB irradiation. In addition, we first demonstrate the content relationship between vitamin D2 and vitamin D4 in king oyster mushroom during UVB irradiation. Our study demonstrated content relationship between vitamin D2 and provitamin D2 in king oyster mushroom during UVB irradiation. The content and content relationship will provide a useful information and guidance for supplement vitamin D in king oyster mushroom.

Chapter 2 Objectives and significance

The main aim(s) of the research project is to investigate effects of UV radiation on vitamin D2 and vitamin D4 in mushrooms irradiated by UV light, and to study on content dynamic trend and relationship for vitamin D2 and provitamin D2, as well as vitamin D2 and vitamin D4 in mushrooms. Additionally, our study is to develop a HPLC method to quantify vitamin D2 and provitamin D2, and to develop a method approach to quantify vitamin D4 in mushrooms by using HPLC-DAD method. We will pursue the following specific aims:

(1) To develop a method to quantify vitamin D2 and provitaminD2 in mushroom using HPLC-DAD.

(2) To investigate the effects of UV irradiation for both vitamin D2 and provitamin D2 in mushroom.

(3) To develop a method to quantify vitamin D4 in mushrooms by HPLC-DAD.

(4) To investigate the effects of UV irradiation on vitamin D4 in the mushroom.

(5) To find content relationship between vitamin D2 and provitamin D2 in the mushroom during UV irradiation.

(6) To construct content relationship between vitamin D2 and vitamin D4 in the mushroom during UV irradiation.

Our study will provide basic information for content of vitamin D2 in king oyster mushroom after UV irradiation, and provide a content dynamic trend in king oyster mushroom. In our study, we showed provitamin D2 content dynamic trend in king oyster mushroom. In addition, we will demonstrate vitamin D4 content and content

dynamic trend in king oyster mushroom.

Secondly, we developed a HPLD-DAD method to simultaneously quantify vitamin D2 and provitamin D2 in mushroom. The developed method will provide an approach to simultaneously quantify vitamin D2 and provitamin D2 in mushrooms. Our study developed a HPLC-DAD method to quantify vitamin D4 in mushrooms. The developed method will provide an approach to quantify vitamin D4 in mushrooms.

Thirdly, our study will provide basic reference information for supplement vitamin D2 in mushrooms. We demonstrate that enhancement of vitamin D2 in mushroom by UVB light is feasible for supplement vitamin D2 in mushrooms. Vitamin D2 in the mushroom irradiated by UVB light can be significant enhanced in content. Our study will demonstrate content relationship vitamin D2 and vitamin D4 in mushroom during UV irradiation. Also, we will provide a content relationship between vitamin D2 and provitamin D2 in mushroom during UV irradiation. In this study, we will first provide a systemic and detail study for the relationship between vitamin D2 and vitamin D4, and vitamin D2 and provitamin D2 in the mushroom. Our study for both method development of vitamin D2, vitamin D4 and provitamin D2, as well as their content dynamic trend will provide useful reference for study on vitamin Ds in mushrooms.

In addition, our study will pave a way for further study on biological activities of vitamin D in mushrooms. Vitamin D2 and vitamin D4 are closely related with in bone mineral intensity. Our study for vitamin D2 and vitamin D4 in mushroom provide important content information for further biological activities, such as bone mineral intensity.

Chapter 3 Literature review

3.1 Physiological functions of vitamin D

The nutritional and physiological significance for human health of vitamin D has been well known so far. Deficiency or inadequate level of vitamin D in the body can result in rickets and osteomalacia. Recently, vitamin D deficiency have been associated with incidence of other diseases, such as cancers, cardiovascular diseases and infectious disease (Cantorna et al, 2014; Dong et al, 2010; Forouhi et al, 2012; George et al, 2012; Holick, 2006; Holick, 2007; Holick & Chen, 2008; Hossein-nezhad & Holick, 2013; Judd & Tangpricha, 2009; Kumar et al, 2009; Reinhold et al, 2008; Reis et al, 2009; Skinner et al, 2006; Stolzenberg-Solomon et al, 2010; Welsh et al, 2012).

In the early of last century, rickets was one of most popular diseases and widespread in children in Europe, with estimated that more than eight percent children in Europe had diseases related with bone problem since vitamin D deficiency (Holick, 2005; Holick, 2006). The study demonstrated there was correlation between 25-hydroxyvitmain D level and bone mineral density. The results showed that 25-hydroxyvitmain D, a serum level of vitamin D status, can significantly increase bone mineral density, when level of 25-hydroxyvitmain D concentration is higher than concentration of 40 ng per mL or more higher level concentration (Bischoff-Ferrari et al, 2006).

In recent years, vitamin D deficiency has been correlated with bone loss or turnover, and a wide range of other diseases, such as cardiovascular disease, diabetes, hypertension and cancers etc. (Adewoye et al, 2006; Chien et al, 2015; Deleskog et al, 2012; Forouhi et al, 2012; Garland et al, 2007; Holick, 2008; Hossein-nezhad & Holick,

2013; Judd & Tangpricha, 2009; Park & Lee, 2012; Shui et al, 2012; Spina et al, 2006; Wang et al, 2008; Weng et al, 2013). The study demonstrated that bone turnover showed higher signal, when 25-hydroxyvitmain D level was below than concentration of 10ng per mL. However, there showed a lower signal when 25-hydroxyvitmain D level more than concentration of 20 ng per mL in the body (Sai et al, 2011). A recent study collected more than 13000 cases, including over 6000 men and over 7000 women, with 8-year follow-up to demonstrate that correlation between 25-hydroxyvitmain D level and death of heart failure and cardiovascular diseases. The results found 25-hydroxyvitmain D concentration less than 20 ng per mL showed more than 2-fold higher risk of heart failure death than those who blood 25-hydroxyvitmain D concentration was over 30 ng per mL. The study suggested there was a significant inverse correlation between blood 25-hydroxyvitmain D concentration and heart failure and cardio vascular diseases death (Liu et al, 2012). A study collected more than 450 participants of pancreatic cancer, and control over 1000 participants, showed that 25-hydroxyvitamin D level there was an inverse association between 25-hydroxyvitamin D level and pancreatic cancer. A lower 25-hydroxyvitamin D level indicated a higher risk of pancreatic cancer (Wolpin et al, 2012).

In addition, vitamin D has been related to various infectious diseases, such as influenza, tuberculosis(Dini & Bianchi, 2012; Yamshchikov et al, 2009). In the last century, it had been reported that vitamin D was successfully used for treatment pulmonary tuberculosis(Phelan, 1947).A recent study showed that there was inverse associated between blood level of 25-hydroxyvitmain D and risk of active tuberculosis (Wilkinson et al, 2000). Also, a few studies reported that vitamin D correlated with risk of autoimmune diseases (Antico et al, 2012; Grant, 2008). A study collected more

than 400 cases showed that lower concentration of 25-hydroxyvitmain D in blood correlated with higher risk of multiple sclerosis (Munger et al, 2006).

3.2 Ultraviolet classification

Ultraviolet (UV) radiation was discovered in year of 1801(Barth, 1987). UV light can be divided as three different subclasses according to its wavelength range, which are UVA, UVB and UVC light. UVA is defined as in a range of 400-315 nm; UVB wavelength is defined in range of 315-280 nm; UVC wavelength is defined in range of 280-100 nm (IARC, 1992). Medium wavelength UVB shows the capability to stimulate vitamin D conversion or production from provitmain D in the body or mushrooms. Recently, UVB technology has been used for enhancing vitamin D2 contents in mushrooms since it shows ability in stimulate conversion of vitamin D2 in mushrooms.

3.3 Stimulation of vitamin D2 accumulation by UV radiation

Several studies were conducted to enhance vitamin D2 enrichment in mushrooms by UV irradiation (Jasinghe & Perera, 2005; Jasinghe & Perera, 2006; Kalaras et al, 2012; Ko et al, 2008; Mattila et al, 2002; Mau et al, 1998; Perera et al, 2003; Phillips et al, 2011; Simon et al, 2011; Teichmann et al, 2007). Mattila et al study found that vitamin D2 was almost totally absent in cultivated mushrooms, e.g. *A. bisporus, P. ostreatus,* and *L.edodes,* if did not irradiated by UV light (Mattila et al, 2001). However, they found that some of wild mushrooms, such as *C. tubaeformis* and *C. cibarius*, contained high concentrations of vitamin D2 relatively (Mattila et al, 2001). Jasinghe et al conducted a study which revealed that the highest vitamin D2 content was found in oyster mushrooms irradiated by UVB light, and the lowest vitamin D2 content was found in button mushrooms under the same conditions of irradiation (Jasinghe & Perera,

2006). Another study from Simon et al demonstrated that vitamin D2 content upon exposure to UVB light was basically comparable to content of vitamin D in the mushrooms exposed to sunlight for 2.5 h (Simon et al, 2011). Mau et al demonstrated that UVC light is also possible to stimulate vitamin D2 production in fresh mushrooms of *Agaricusbisporus*, *Lentinula edode* and *Volvariellavolvacea*. Vitamin D2 content in the mushrooms irradiated by UVC light could be enriched (Mau et al, 1998). Subsequently, Phillips et al conducted a detail study to quantify vitamin D2 and sterol contents in ten mushrooms from USA. They demonstrated that vitamin D2 content was in range from 0.1 to 0.3 μg in 100 g in white button, acrimini, portabella mushrooms. For mushroom of enoki, shiitake and oyster, the contents of vitamin D was between 0.4 and 0.7 μg in 100 g. the study showed that mushroom of Chanterelle showed higher contents in vitamin D2. And content of vitamin D2 in portabella mushroom irradiated by UV was in 3.4 to 20.9 μg (Phillips et al, 2011). The some studies about vitamin D2 in mushroom are listed in Table 3-1.

Several studies have been conducted for stimulating vitamin D2 enrichment in mushrooms irradiated by UV light, and demonstrated that UV irradiation is an effective approach to enhance vitamin D2 enrichment. However, most these studies only focused on vitamin D2 enrichment in white button mushroom or oyster mushroom. Up to date, there has not yet a detail study was conducted for research on vitamin D2 enrichment in king oyster mushroom.

In addition, vitamin D2 is come from provitamin D2 in mushrooms under UV environment. Up to date, most of those studies were either research on vitamin D2 content in the mushrooms as showed above, or concentrated on those studies which only researched on provitamin D2 content in mushrooms (Chang et al, 2005; Koyama

et al, 1984; Liu et al, 2011; Tong et al, 2014). Most of those studies did not focus on the provitamin D2 content dynamic trend, but only quantification vitamin D4 content in mushrooms. Those studies did not focus on both vitamin D2 and provitamin D2 dynamic trend in mushrooms during UVB irradiation. Also, there has not yet study to focus on provitamin D2 dynamic trend in king oyster mushroom during UVB irradiation. Thus, provitamin D2 content dynamic trend in the mushroom was never unveiled in previous studies. The relationship between vitamin D2 and provitamin D2 in king oyster mushroom during UV irradiation was also never reported in previous studies focused on vitamin D content. It is therefore significant to unveil the relationship between vitamin D2 and provitamin D2 in the mushroom during UV irradiation.

Also, there has not yet a detail study to focus on dynamic trend for both vitamin D4 and vitamin D2 inking oyster mushroom irradiated by UV light. It is still unclear that the content of vitamin D4 and vitamin D4 dynamic trend in king oyster rmushroom, and the relationship between vitamin D2 and vitamin D4 in the mushroom during UV irradiation. Hence, it is necessary to demonstrate the content and content relationship between vitamin D2 and vitamin D4 in king oyster mushroom during UV irradiation, and to unveil the content relationship between vitamin D2 and provitamin D2 in king oyster mushroom during UV irradiation.

Table 3-1.Vitamin D2 contents in mushrooms

Mushrooms	Scientific name	Content (μg/100g)
Portobello	*Agaricus bisporus*	11.15
White button mushroom	*Agaricus bisporus*	100*(68.6±4.90) (dw)
Shiitake	*Lentinus edodes*	100*(61.9±10.6) (dw)
Maitake	*Grifola frondosa*	0.08–63.2
Wild Chanterelle	*Cantharellus tubaeformis*	29.8
Morel	*Morchella esculenta*	2.18–8.41
Wild Chanterelle	*Cantharellus cibarius*	12.8
Wild button	*Agaricus bisporus*	0.7-2.3
Wild Porcini	*Boletus edulis*	58.7

3.4 Analysis of vitamin D2 and provitamin D2 in mushrooms

There have many methods to analyse vitamin D2 in mushroom and many methods analyse provitamin D2 in mushrooms(Huang et al, 2015; Kalaras et al, 2012; Koyyalamudi et al, 2011; Perera et al, 2003; Phillips et al, 2011; Tong et al, 2014).However, all those methods are separately used for quantification of vitaminD2 and provitamin D2 in mushroom. Those methods either quantify vitamin D2 in mushrooms, or quantify provitamin D2 in mushroom. It would be time-consuming and lower accuracy if used those two methods to monitor content dynamic trend for both vitamin D2 and provitamin D2 in mushrooms during UV irradiation. Up to date, there are not yet a HPLC-DAD method to simultaneously quantify vitamin D2 and

provitamin D2 in mushrooms to monitor content dynamic trend for both vitamin D2 and provitamin D2 in mushroom. Hence, it is necessary and significant to develop a method to quantify vitamin D2 and provitamin D2 in mushrooms.

In addition, most all these studies involve in saponification process in sample preparation, which is an expensive and time-consuming handling method. The saponification handling of vitamin D2 in mushrooms was used in many papers for vitamin D2 analysis (Kalaras et al, 2012; Ko et al, 2008; Mattila et al, 2002; Mau et al, 1998; Perera et al, 2003; Phillips et al, 2011; Simon et al, 2011; Teichmann et al, 2007). The more detail step for saponification process was described in Jasinghe's paper (Jasinghe & Perera, 2006). There are basically four steps in saponification sample preparation of vitamin D2 in mushrooms: the first step is to saponify by potassium hydroxide by heating 80℃ for one hour, and then to be extracted by n-pentane for three times. After that, to wash by 3% potassium hydroxide for three time, and then wash by de-ionized water for several times until neutral. Finally, to evaporate the organic solvent, and re-dissolved the sample by the solvent for further analysis.

The process of saponification preparation is commonly used for the removal of ester forms in analysis of steroid compounds. This process can convert esterifies forms to the free form for steroid compounds (Slavin & Yu, 2012; Zak, 1977). However, these steps for sample preparation not only increase labour time and experimental cost, but also need good experimental skills to keep consistence in the operations for each step involving in the whole process. Also, these complicated preparation steps increase the probability of the error since sample loss and operational deviation during multi-step operations. There has not a simple method for sample handling and analysis of vitamin

D2 in mushrooms was reported so far. Thus, it is significant to quantify vitamin D2 by using simple sample preparation.

Currently, those methods for quantification of vitamin D2 in mushrooms and another methods for quantification of provitamin D2 in mushrooms, but these methods are separately quantified for vitamin D2 and provitamin D2 in mushrooms (Jedlickova et al, 2008; Phillips et al, 2011; Tong et al, 2014). These methods cannot provide a simple and accurate an approach to monitor content dynamic trend for both vitamin D2 and provitamin D2 in mushrooms during UV irradiation. Therefore, there is a need to develop an approach to simultaneously quantification of vitamin D2 and provitamin D2 in the mushrooms.

The developed new approach to simultaneously quantify vitamin D2 and provitamin D2 in the mushroom by HPLC-DAD method, which will provide a simple approach to research on dynamic trend for vitamin D2 and provitamin D2 in king oyster mushroom.

3.5 Vitamin D4 in mushrooms

Although lots of studies were conducted to enhance vitamin D2 enrichment in mushroom, most of those studies did not focus on vitamin D4 content in mushrooms. Vitamin D4 is a form of vitamin D, known as 22, 23-dihydroergocalciferol. Vitamin D4 is converted from 22, 23-dihydroergosterol under UV environment. There have quite limit reports for vitamin D4 content in mushrooms. Vitamin D4 content in king oyster mushroom was not reported in previous study, and vitamin D4 dynamic trend in king oyster mushroom irradiated by UV light is unveiled. So far, there only has one paper which reported the content of vitamin D4 in the mushrooms. (Phillips et al, 2012).

Currently, the research evidences indicate that vitamin D4 show quite similar biological activities as vitamin D2 or D3 in the body. However, vitamin D4 exhibit lower biological activities compared with vitamin D2 or D3 in the body.

For biological activity of increase bone intensity, vitamin D4 has showed biological activities as vitamin D3 or D2, which is around 60% as active as vitamin D3 or D2 in rats or human (De Luca et al, 1968). Mushroom irradiated by UV light can produce vitamin D2, and also can stimulate production of vitamin D4. Thus, biological functions for increasing bone density from mushroom irradiated by UV light, which are not only contributed to vitamin D2, but also have contribution of vitamin D4 in mushroom. Hence, it is therefore significantly to quantify vitamin D4 content in the mushroom.

In addition, quantification of vitamin D4 in mushrooms has been used by LC-MS method. But there has not yet a HPLC-DAD method to quantify vitamin D4 in mushroom so far. Hence, it is quite necessary to develop HPLC-DAD method for quantification of vitamin D4 in mushrooms, to provide a simple approach to quantification of vitamin D4 in mushrooms. Also, content and content dynamic trend of vitamin D4 in king oyster mushroom has never been reported in previous studies. Hence, it is very significant to develop a simple HPLC-DAD method for quantification of vitamin D4 in king oyster mushroom.

Chapter 4 General materials and methods

4.1 Chemicals and reagents

Vitamin D2 standard was purchased from International Laboratory (South San Francisco, USA). Provitamin D2 (ergosterol) standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Vitamin D4 standard was purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol for extraction and methanol for HPLC analysis was purchased from International Laboratory (South San Francisco, USA). Acetonitrile HPLC analysis was purchased from International Laboratory (South San Francisco, USA). De-ionized water was prepared by using a Millipore Milli-Q Plus system (Millipore, Bedford, USA). Sodium ascorbate (International Laboratory, South San Francisco, USA), potassium hydroxide (85% pure, International Laboratory, South San Francisco, USA), ethanol (International Laboratory, South San Francisco, USA), *n*-pentane (International Laboratory, South San Francisco, USA), Formic acid (International Laboratory, South San Francisco, USA).

4.2 Materials

The fresh mushroom listed following: *Pleurotuseryngii* (Product of Korea, Good Friend King Oyster Mushroom™, Shiu Pong TradingCo., limited), *Agaricus bisporu* (Product of Malaysia, Champ Fungi Sdn. BhD) and *Pleurotuso streatus* Product of China, Shenzhen YongJia Fresh Produce CO., LTD), *baby protabello* (Product of Malaysia, The Dairy Farm Company Ltd.) Ben Gu (Product of China, Shanghai Fangyuan Mushroom CO., LTD), *Pleurotus eryngii* (Product of Hong Kong, Tai Tong Organic EcoPark Ltd), *Pleurotus eryngii* (Product of China, Shenzhen YongJia Fresh

Produce CO., LTD), *Lentinusedodes* (Product of Taiwan, Wan Bang Trading Limited), *Hypsizygustessellates* (Tak Ling Trading Co., Limited). All the mushrooms were purchased from local market, and were prepared and used immediately after purchase.

UVB light (Beijing Zhongyiboteng Tech CO., LTD. Wavelength: 285-320nm), or UVA light (Beijing Lighting Research Institute. Wavelength: 320-400nm), UVC light (Jiang Su Juguang Photoelectric CO., LTD. Wavelength: 253.7nm). Nylon membrane syringe filters (Heaion, China).

4.3 Methods

The fresh king oyster mushrooms were cut out cap and bottom, and then were cut into slices of $1cm^2$. The fresh sliced $1cm^2$ mushrooms were placed in Petri dish, and then transferred into UV chamber for irradiation. After UV radiation, the mushroom samples were transferred into vacuum-drier for dryness. The vacuum dried mushroom samples were kept in a vacuum-container until next step use of analysis.

The sliced mushrooms were irradiated by UV light under various conditions. The sliced mushrooms were irradiated by different UV lights, including UVA, UVB, and UVC light. The sliced mushrooms were irradiated with six-time period from 10 minute to 150 minute, including 10, 20, 30, 60, 120, 150 minute. After the UV irradiation of the mushrooms, the HPLC method was used for quantification of vitamin D2, vitamin D4 and provitamin D2 in mushrooms to monitor content and content dynamic trend of vitamin D4, vitamin D2 and provitamin D2 in mushroom.

Firstly, A HPLC method for quantification of vitamin D4 in mushrooms was developed using HPLC-DAD. This developed method will be used for research on content dynamic trend for vitamin D4 in the mushroom during UV irradiation. Secondly,

Another HPLC method for quantification of vitamin D2 and provitamin D2 was developed by HPLC-DAD. This developed method will be used for research on content dynamic trend for vitamin D2 and provitamin D2 in the mushroom during UVB irradiation. Based on those developed methods for quantification vitamin D2 and provitamin D2 as well as vitamin D4 in mushrooms, our study mainly focuses on effects of UV irradiation on content and content dynamic trend of vitamin D4, vitamin D2 and provitamin D2 in mushrooms during UV irradiation. The basic strategy diagram and work flow is showed in Figure 4-1.

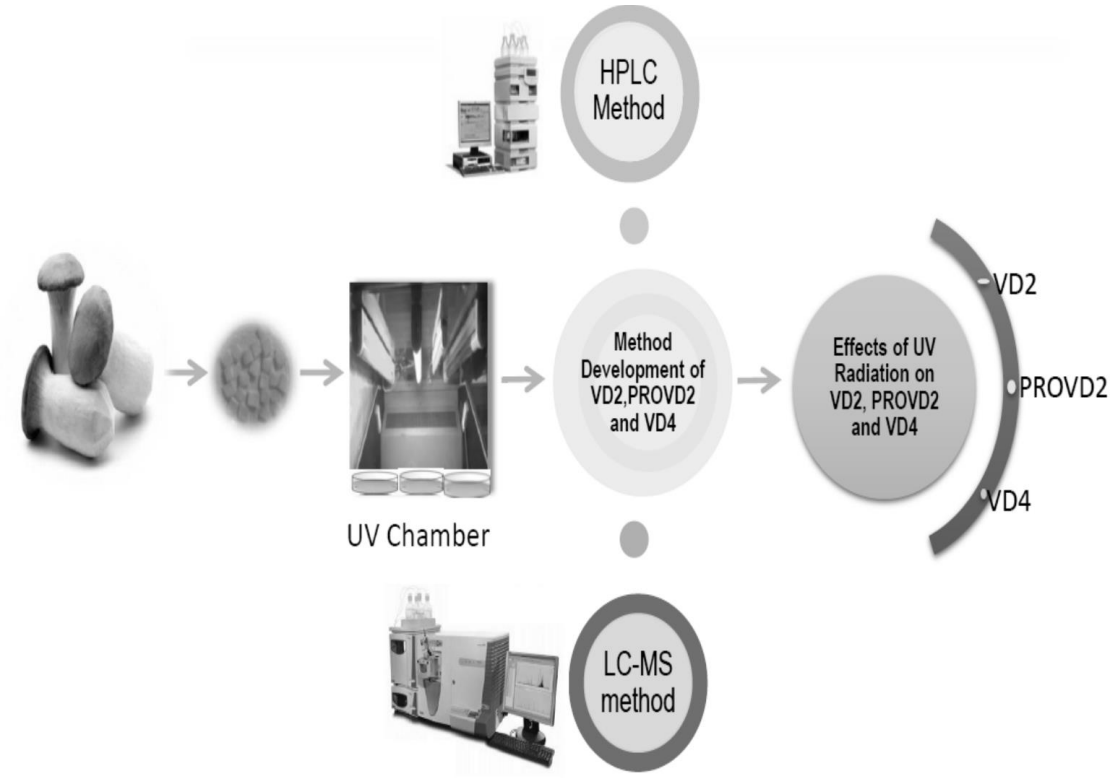


Figure 4-1. The strategy diagram and work flow for study on content and content dynamic trend of vitamin D4, vitamin D2 and provitamin D2 in mushrooms.

Chapter 5 Effects of UV radiation on vitamin D2 in mushroom

5.1 Method development of vitamin D2 quantification

5.1.1 Sample preparation and extraction

The sliced fresh king oyster mushroom (5g) were irradiated by UVB light and were vacuum-dried after UVB irradiation. The vacuum-dried irradiated mushrooms were kept in vacuum-container until next step use of analysis. Vacuum-dried mushroom samples were extracted with 7 mL methanol for one hour in boiling methanol by using reflux extraction, and then cooled down room temperature immediately. The extraction solution was then filtered through 0.45 μm nylon membrane syringe filters (Heaion, China) before the HPLC-DAD analysis. For the saponification handling of vitamin D2 in mushrooms, the saponification process was performed according to the procedure described in the Jasinghe's paper (Jasinghe & Perera, 2006).

5.1.2 HPLC-DAD Analysis

HPLC analyses were performed on an HPLC system of Agilent Series 1100 liquid chromatography system (Agilent Technologies, USA). The HPLC analysis system was equipped with quaternary pump, a vacuum degasser, an auto-sampler and a diode array detector (DAD). The solvents which constituted the mobile phase included aqueous solution (A) and MeOH (B). The separation was achieved on Waters Atlantis dC18 column (5 μ, 4.6× 250mm) at ambient temperature, and was carried out by gradient elution of 0–6 min, 80–90% B; 6-15 min, 90–100% B; 15–27 min, 100% B; and finally, reconditioning the column with 80% B isocratic for 10 min. The flow rate of mobile

phase was set at 1 mL/min. the detection wavelength was set at 265nm and the injection volume was 10μl.

Identification of vitamin D2 in king oyster mushroom in two ways: firstly, we compared the retention time of vitamin D2 of mushroom samples with vitamin D2 standard under the identical chromatographic conditions based on HPLC-DAD (Figure 5-1). Secondly, HPLC-MS was performed to further confirmation (Figure 5-3). HPLC–MS analysis was performed on Thermo Finnegan Surveyor liquid chromatography system, which coupled with an LCQ Advantage ion-trap mass spectrometer and an electrospray ionization (ESI) source (Thermo Fisher, San Jose, CA, USA). The optimized parameters of the mass spectrometer were showed as follows: Sheath gas flow rate (art), 10; Aus/sweep gas flow rate (art), 10; Spray voltage, 4.0 KV; Capillary temperature, 350 ℃; Capillary voltage, 3 V; Tube lens offset, 40 V. The full scan MS analysis was obtained in the range of m/z 200–600, and scan mode was performed in positive ion mode. The Xcalibur 1.3 software was used for control the HPLC-MS system.

5.1.3 Calibration curve, limit of detection and quantification

Methanol stock solution of standard compound was prepared, and then diluted to suitable concentrations. More than six different concentrations of the standard solution were tested in two replicates, and the calibration curve was plotted by the peak areas versus the concentration of vitamin D2. The linear regression equation of calibration curve was shown as following: y=24.61x-0.035 (x, Concentration; y, Area), r^2=0.9999. Test ranges of calibration carve were from 160.00-0.63 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) of vitamin D2 in the chromatographic

conditions were determined at an S/N of around three and ten, respectively. LOD and LOQ of the vitamin D2 were 0.08 μg/mL and 0.22 μg/mL, respectively.

5.1.4 Precision, repeatability, stability and recovery

Intra- and inter-day variations were tested for measuring precision of the developed method. For intra-day variation test, the standard solution was injected for six replicates for each level (n=6), and three levels were tested within one day. While inter-day variations, three levels were tested in two replicates for consecutive three days (n=6). The relative standard deviation (RSD) was used for the assessment of variation. The results showed that the RSD of intra-and inter-day precision were less than 0.20 % and 0.77%, respectively, see Table 5-1.

The repeatability of chromatographic method was determined by testing three levels of extracted mushroom samples. The extracted mushroom samples of 4g, 5g and 6g (fresh weight) were analyzed for three levels tests. Each level of the mushroom extracted samples was analyzed six replicates (n=6), and results were assessed by RSD (%). The results demonstrated that the repeatability of chromatographic method was less than 0.77%. Three test levels of low, middle, and high were less than 0.57%, 0.10%, and 0.77%, respectively, as showed in Table 5-2.

The sample stability was tested by storing the mushroom sample at room temperature within 48 hours. The extracted XBG sample was analysed at 0, 2, 4, 6, 8, 20, 24 and 48 hours, respectively, after the store at room temperature. The results showed that the RSD of stability was less than 0.75%. The results of stability indicated that vitamin D2 was relatively stable within 48 hours. The recovery was tested by adding known amount of standard into amount of sample. The mixture samples were

extracted and analysed as according to in section 5.1.1. The recovery was performed by test three times of mixture samples. The recoveries were calculated by using formula as following: Recovery (%) = 100 % × (amount found – amount original)/amount spiked. The average recovery was 96.04% from three replicates tests.

Table 5-1. Inter- and intra-day precisions

Concentration (μg/mL)	Intra-day (n=6)			Inter-day (n=6)		
	Observed conc (μg/mL)	Accuracy (%)	RSD (%)	Observed conc (μg/mL)	Accuracy (%)	RSD (%)
80.00	79.92	99.90	0.06	80.74	100.93	0.77
20.00	20.28	101.40	0.10	20.27	101.40	0.10
5.00	5.20	104.00	0.20	5.22	104.44	0.43

Accuracy (%) = 100%× mean of measured concentration/nominal concentration

Table 5-2. The repeatability of chromatographic method

Compound	Weight (g)	RSD (%, n=6)
Vitamin D2	4	0.57
	5	0.10
	6	0.77

Table 5-3. Recovery of vitamin D2

Original(μg)	Spiked(μg)	Found(μg)	Recovery (%)	RSD (%)
15.73	13.60	28.79	96.04	0.57

Recovery (%) =100%× (amount found – amount original)/amount spiked

Table 5-4. Comparison of vitamin D2 contents in three mushrooms

Scientific Name	Chinese Name	Saponification Mean (μg/g)	Methanol Mean (μg/g)
Pleurotuseryngii	Xing Bao Gu	59.82	63.23
Agaricusbisporus	Shuang Bao Gu	103.61	109.70
Pleurotusostreatus	Ping Gu	86.89	99.87

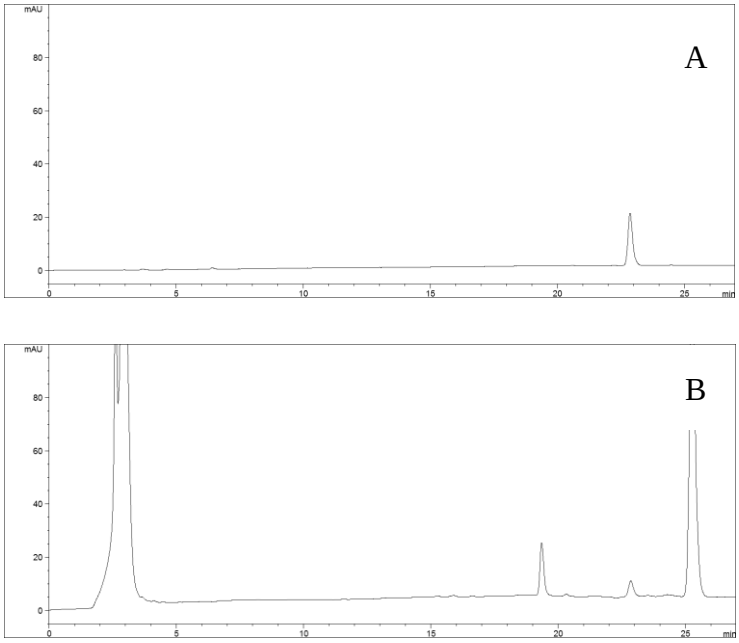


Figure 5-1. HPLC chromatograms of vitamin D2 standard and extraction sample of XBG: (A) Vitamin D2 standard; (B) extraction sample of XBG irradiated by UVB

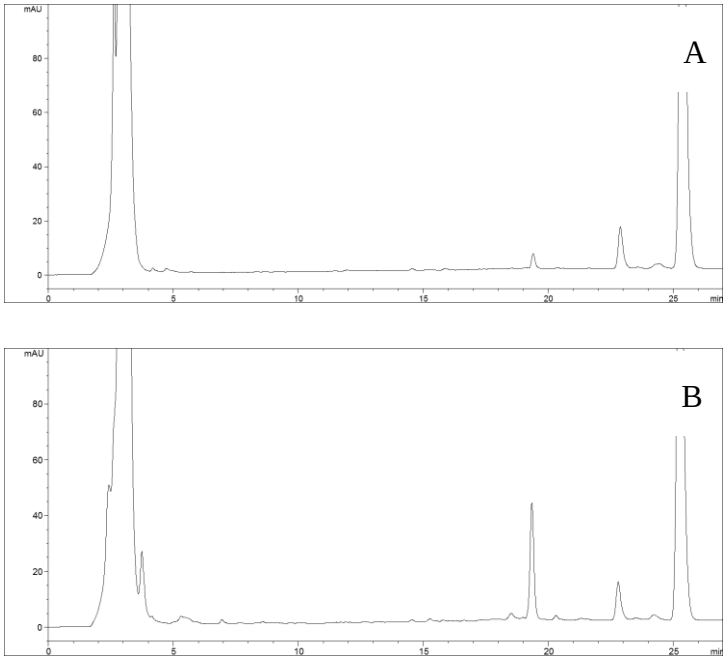
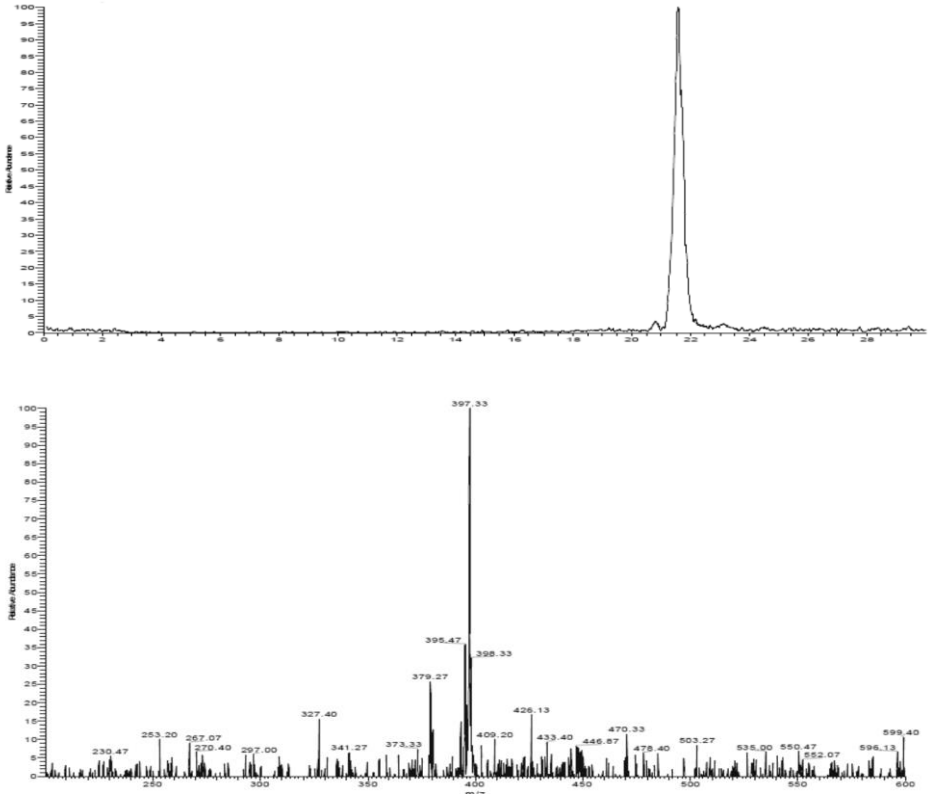
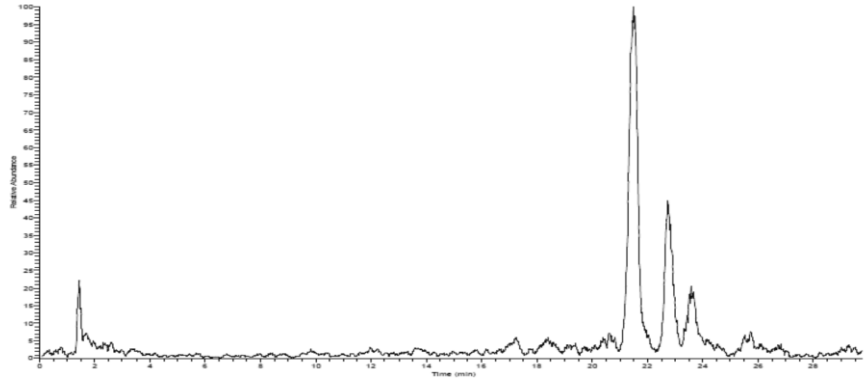
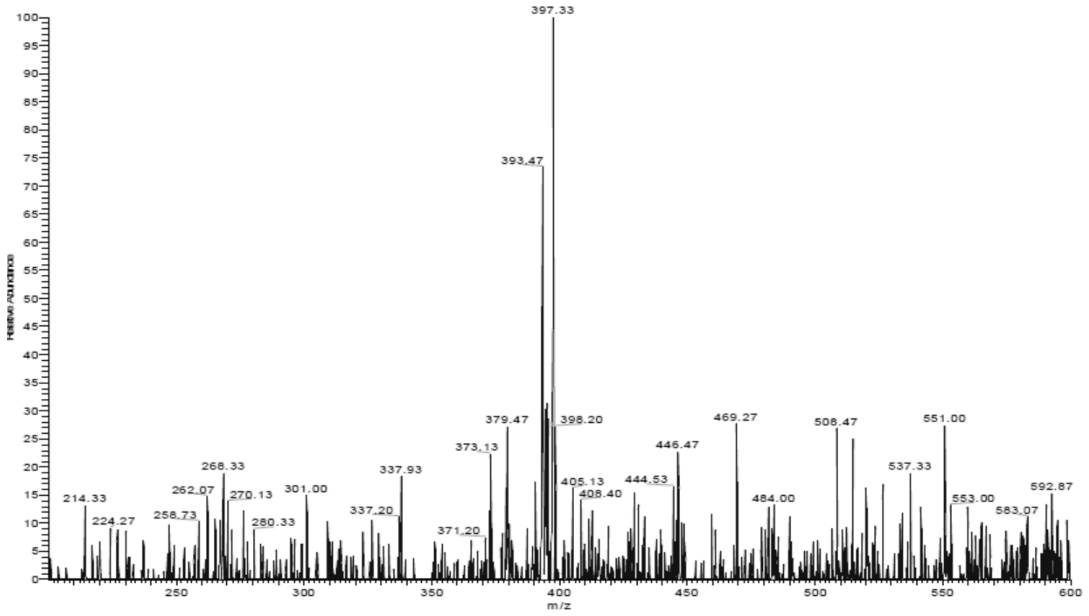


Figure 5-2. Representative HPLC chromatograms of mushroom extraction samples: (A) SBG sample; (B) PG sample

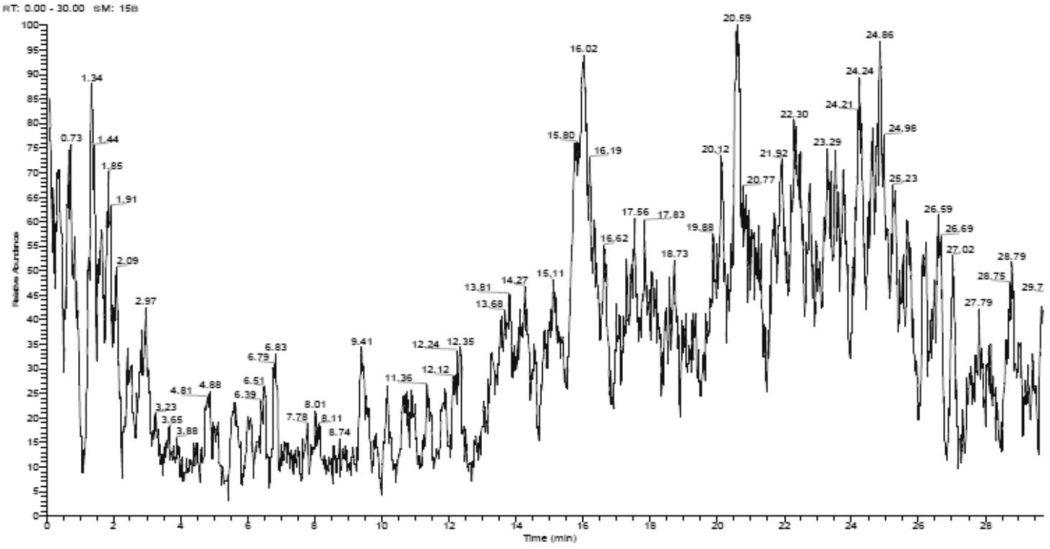

Relative Abundance
397.33
395.47
398.33
379.27
327.40
426.13
253.20
267.07
270.40
230.47
297.00
341.27
373.33
409.20
433.40
446.67
470.33
478.40
503.27
535.00
550.47
552.07
596.13
599.40

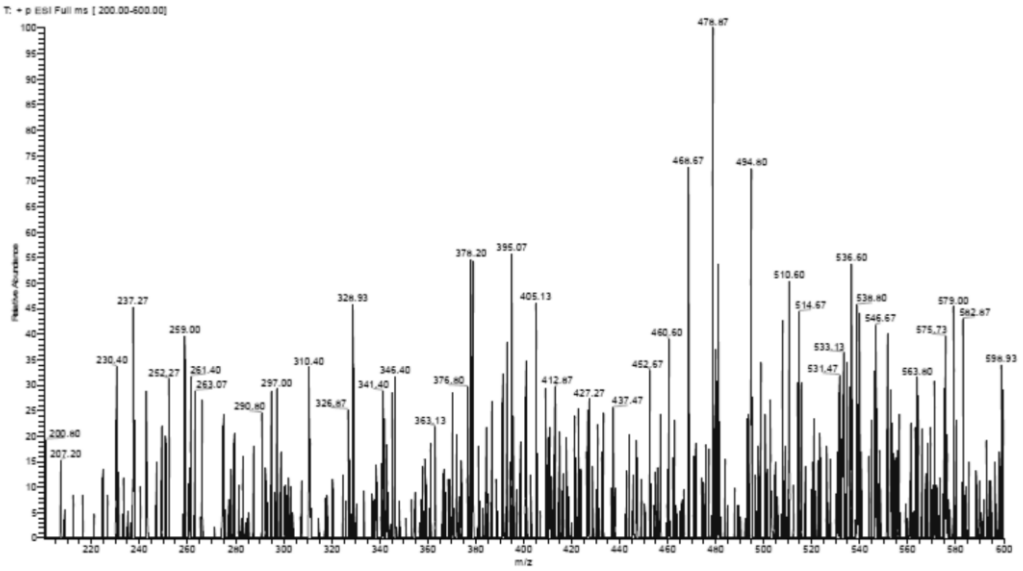
A


Relative Abundance
Time (min)


Relative Abundance
m/z
397.33
393.47
379.47
398.20
373.13
469.27
446.47
508.47
551.00
337.93
268.33
405.13
444.53
537.33
262.07
270.13
301.00
408.40
484.00
592.87
214.33
337.20
553.00
224.27
258.73
280.33
371.20
583.07

B


RT: 0.00 - 30.00 SM: 15B
Relative Abundance
Time (min)
20.59
16.02
24.86
1.34
24.24
24.21
22.30
15.80
24.98
0.73
1.44
16.19
20.12
23.29
1.85
21.92
1.91
20.77
25.23
17.56
17.83
26.59
2.09
16.62
19.88
26.69
18.73
27.02
2.97
15.11
28.79
14.27
13.81
28.75
13.68
29.7
27.79
6.83
6.79
12.24
12.35
9.41
6.51
12.12
4.81
4.88
11.36
3.23
6.39
8.01
3.65
7.78
8.11
3.88
8.74



C

Figure 5-3. LC-MS chromatograms of vitamin D2 standard and extraction sample of XBG. (A) vitamin D2 standard the mass spectrum; (B) vitamin D2 mass spectrum of XBG mushroom sample; (C) methanol solvent control and mass spectrum at vitamin D2 retention time.

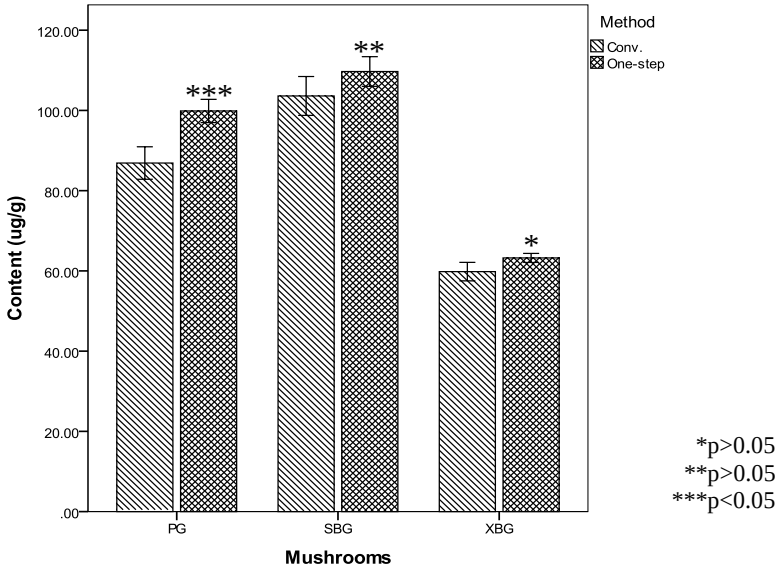


Figure 5-4. Comparison of vitamin D2 contents in three mushrooms

5.2 Vitamin D2 contents in mushrooms

5.2.1 Irradiation procedure

The fresh king oyster mushrooms were removed cap and bottom, and then were cut into slices of 1cm^2. The fresh sliced mushrooms (5 g) were placed in Petri dish, and then transferred into UV chamber for irradiation. The sliced mushrooms were exposed to UVB light (Beijing Zhongyiboteng Tech CO., LTD. Wavelength: 285-320nm), or UVA light (Beijing Lighting Research Institute. Wavelength: 320-400nm), UVC light (Jiang Su Juguang Photoelectric CO., LTD. Wavelength: 253.7nm), respectively. After UV irradiation, the mushroom samples were separately vacuum-dried and then kept in a vacuum-container until next step of analysis.

With the developed method and mushroom samples, several irradiation conditions, such as, different UV light, irradiation temperature, irradiation time and UV intensity, would be changed to try to explore some factors which may affect vitamin D2 contents in mushroom.

5.2.2 Vitamin D2 contents under various UV lights

The sliced fresh king oyster mushrooms were subjected to three different types of UV light. The fresh sliced mushrooms were transferred into UV chamber for irradiation by UVA, UVB and UVC light, respectively. All the irradiations were performed at 35℃ for 0.5 hour.

The results were shown in Table 5-5 and Figure 5-5. The results showed that the UVB light was most effective light for stimulating vitamin D2 accumulation in king oyster mushrooms. The UVC light also showed effect to enhance vitamin D2 enrichment in the mushroom. However, vitamin D2 contents in king oyster mushroom

irradiated by UVC light was lower than UVB light. Vitamin D2 in the mushroom was irradiated by UVA light was undetectable. The result might imply that UVA light, which do not have effect to stimulate vitamin D2 production in the mushroom.

Table 5-5. Vitamin D2 contents in the mushroom under various UV lights.

UV type	Mean(μg/g)	SD (n=5)
UVB	5.79	0.52
UVC	2.78	0.35
UVA	-	-

- Undetectable

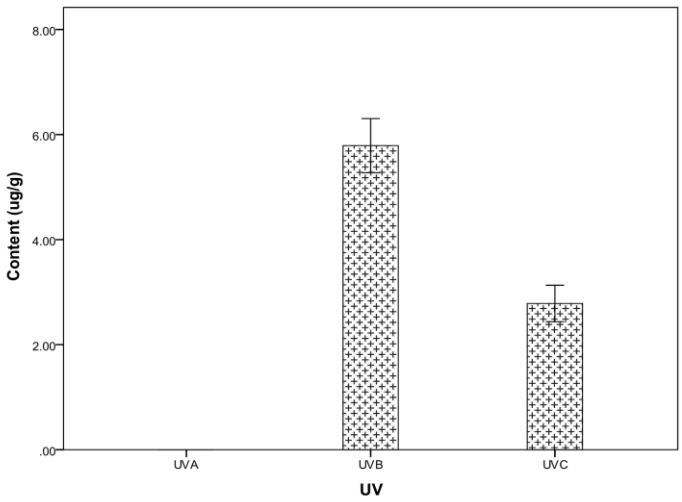


Figure 5-5. Vitamin D2 contents in the mushroom under various UV light.

5.2.3 Vitamin D2 contents in different time

With comparison between different UV light, the UVB light showed maximum production for vitamin D2 in the mushrooms. The different time effects of stimulating vitamin D2 were performed with irradiation of UVB light. The sliced fresh mushrooms were placed into UV chamber (50cm×55cm×35cm, L×W×H)) for UV irradiation. The irradiations were performed under 35℃ at time period of 0.25 hour, 0.5 hour, 1.0 hour, 1.5 hours, 2.0 hours, and 2.5 hours. After the UV irradiation, the mushroom samples were processed as described in section 4.3. Each tested samples comprised five samples. The results were shown in Table 5-6 and Figure 5-6.

The highest content of vitamin D2 in the mushroom was found in irradiation time of 0.5 hour. The lowest content of vitamin D2 was found at irradiation time of 0.25 hour. The content of vitamin D2 showed relatively stable trend when irradiation time more than 0.5 hours. The results showed that the content of vitamin D2 in the mushrooms upon exposure of UVB light from 1.0 hour to 2.5 hours were in relatively dynamic stable range. The results demonstrated that vitamin D2 content linear increase was occurred within 30 minute in the mushroom.

Table 5-6. Vitamin D2 contents in the mushroom with different time period

Time (h)	Mean (μg/g)	SD (n=5)
0.25	4.92	1.45
0.50	11.84	1.58
1.00	10.74	1.41
1.50	9.19	0.47
2.00	10.51	0.92
2.50	10.38	1.05

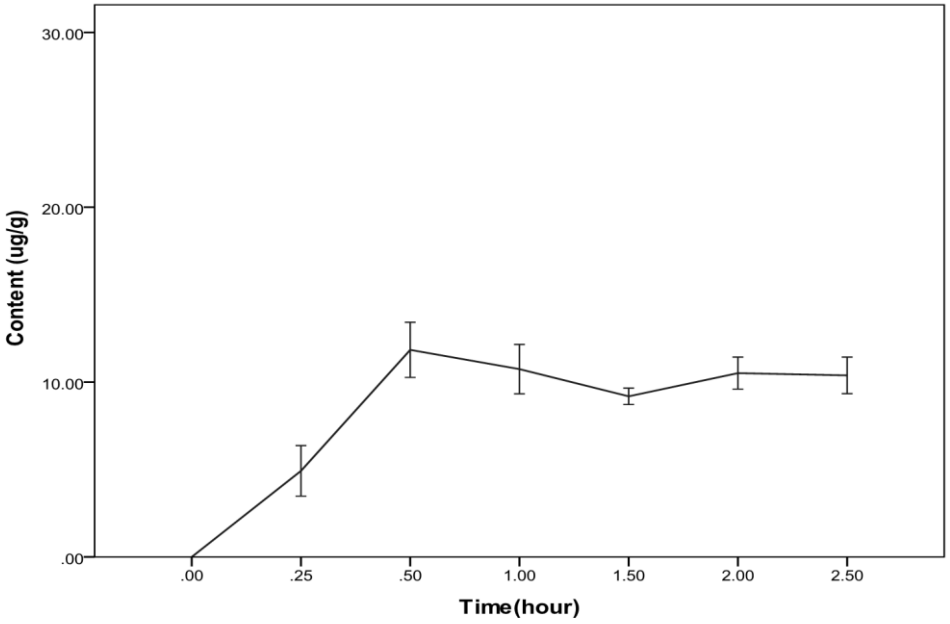


Figure 5-6. Vitamin D2 contents in the mushroom irradiated with different time.

5.2.4 Vitamin D2 contents under different temperatures

The fresh sliced mushrooms were transferred into UV chamber, and then were irradiated in temperature of 35℃, 30℃ and 25℃ for 0.5 hour and 1 hour, respectively.

After the UV irradiation, the mushroom samples were processed as described in section 5.1.1. Each tested samples included five paralleled samples. The results were shown in Table 5-7 and Figure 5-7.

Vitamin D2 contents irradiated at 25 ℃ and 30℃ were lower than irradiated at 35 ℃ under same time period. There were basically no different in vitamin D2 yields between irradiation of 25 ℃ and irradiation of 30℃. Our results demonstrated that the optimum UV irradiation temperature for enhancing vitamin D enrichment in king oyster mushroom was found under irradiation at 35℃.

Table 5-7.Vitamin D2 contents in the mushroom irradiated with different temperatures

Temperature (℃)	Time (h)	Mean (μg/g)	SD (n=5)
35	0.5	11.84	5.04
	1.0	10.74	1.41
30	0.5	8.23	1.39
	1.0	8.02	1.19
25	0.5	9.01	2.20
	1.0	7.35	1.16

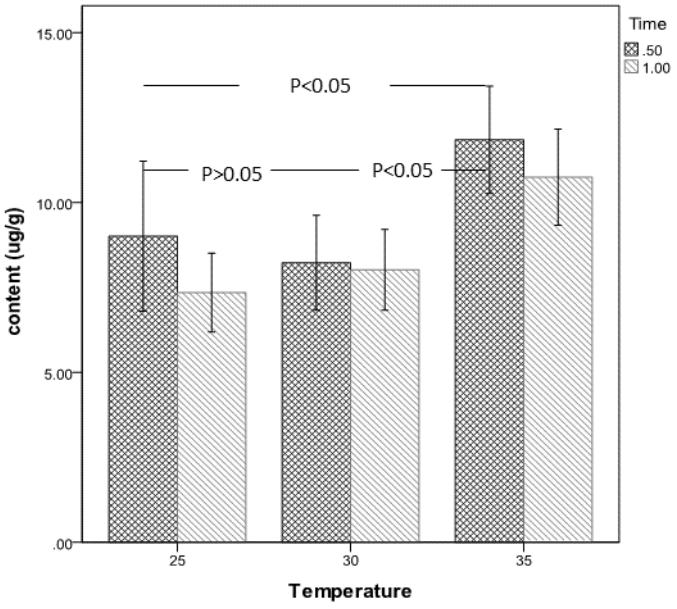


Figure 5-7. Vitamin D2 contents in the mushroom under different temperatures

5.2.5 Vitamin D2 contents on various UV intensity

The fresh sliced king oyster mushrooms were transferred into UV chamber, and then were irradiated under UVB different intensity by 95 μw/cm^2, 148 μw/cm^2. The UV irradiation was performed at 35℃ for 0.5 hour, respectively. After the UV irradiation, the mushroom samples were processed as described in section 5.1.1. Each tested samples included five paralleled samples. The results were shown in Table 5-8 and Figure 5-8.

The results showed that vitamin D2 under irradiation of intensity of 148 μw/cm^2 showed somewhat higher than irradiation of intensity of 95 μw/cm^2. However, there was no significantly different in vitamin D2 content between intensity of 95 μw/cm^2 and intensity of 148 μw/cm^2. The results may indicate that higher UV intensity could improve efficiency of vitamin D2 accumulation.

Table 5-8. Vitamin D2 contents in king oyster mushroom

Intensity (μw/cm^2)	Mean (μg/g)	SD (n=5)
148	12.60	2.17
95	11.84	1.58

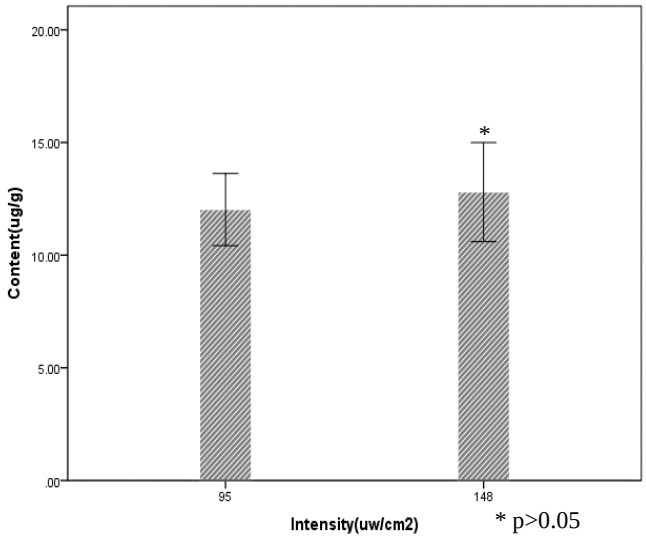


Figure 5-8. Vitamin D2 contents in the mushroom irradiated by different intensity.

5.2.6 Investigation of vitamin D2 contents in mushrooms

With successful finding of optimum condition for vitamin D2 enrichment in king oyster mushroom, several different mushrooms were used for investigation the contents of vitamin D2 enrichment upon exposure of UVB irradiation. Eight mushrooms were investigated based on optimum irradiation condition; the mushrooms were listed in Table 5-9. All mushrooms were irradiated under optimum condition, which was at 35℃

for 0.5 hour. Each tested group sample included five samples. The results were shown in Table 5-9 and Figure 5-9.

The investigation results found that they can be divide three levels for vitamin D2 contents in the mushrooms: the highest level content of vitamin D2 was found in XBG and SBG mushrooms; middle level content was found in JZG, BG and XG; and the lowest level content was found in LZG and PG.

The results showed that there was significant difference in vitamin D2 content between the highest level content and the lowest content. The results of vitamin D2 contents among three XBG, including from China mainland, Hong Kong, South Korea, showed that vitamin D2 contents in XBG from South Korea higher than from China and Hong Kong.

The highest content of vitamin D2 was found in XBG from South Korea among all investigated mushrooms, and lowest content of vitamin D2 was found in LZG. The investigation results found that XBG and SBG have showed higher contents in vitamin D2 after UVB irradiation; the lowest contents of vitamin D2 were found in LZG; and JZG showed middle contents in vitamin D2 compared with other mushrooms

Table 5-9. Vitamin D2 contents in other mushrooms by the present method

Chinese Name	Abbreviation	Scientific Name	Content (μg/g)
Ben Gu	BG	*Lyophyllumshimeji*	6.42
Ping Gu	PG	*Pleurotusostreatus*	5.52
HK Xing Bao Gu	HXB	*Pleurotuseryngii*	9.84
Shuang Bao Gu	SBG	*Agaricusbisporus*	11.01
Xiang Gu	XG	*Lentinusedodes*	8.32
Korea Xing BaoGu	KXB	*Pleurotuseryngii*	12.28
Lin ZhiGu	LZG	*Hypsizygustessellatus*	4.30
Xing BaoGu	XBG	*Pleurotuseryngii*	12.02

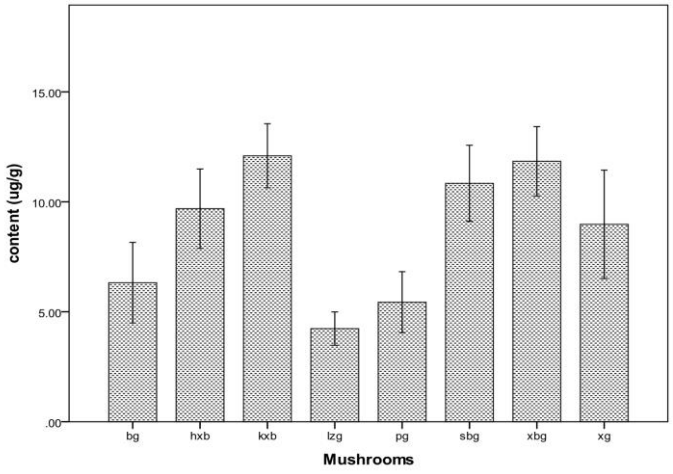


Figure 5-9. Vitamin D2 contents in other mushrooms.

5.3 Results and discussion

5.3.1 Validation of HPLC-DAD method

The calibration curve of vitamin D2 showed good linear regression (r^2=0.9999) within test range. The precision of the HPLC-DAD method was determined by intra- and inter-day precision. Intra-day precision was tested by determination of three levels of the standard solutions for 6 times within one day, while the inter-day precision was tested within consecutive three days. The RSD were used for assessment its intra- and inter-day precision. The RSD of intra- and inter-day precision was less than 0.20% and 0.77%, respectively. The repeatability of chromatographic method was determined by measuring three levels of XBG extraction samples for six replicates. The RSD of repeatability of chromatographic method were 0.82 %, and the three levels at low, middle and high level were less than 0.50%, 0.59% and 0.82%, respectively. The developed method showed that good overall recovery of more than 95%. The stability results indicated that vitamin D2 in the mushroom with methanol extraction was relative stable within 48 hours. Validation tests of the method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D2 in the mushrooms.

Vitamin D is a group of secosteroid compound (Perez-Lopez, 2007), which is possible to form esterifies form in appropriate environment. Saponification handling is quite complicated process with many operation steps, so saponification process in sample preparation of vitamin D2 in mushrooms is a time-consuming, expensive and low-efficient. Moreover, the multi-step saponification process increases the possibility of sample loss in the multi-step sample handling.

Herein, we developed one-step methanol extraction method for fast extraction of vitamin D2 in mushrooms, which can complete extraction within one hour. The new method maintains vitamin D2 yields comparable to conventional saponification method. Three different mushrooms were compared using saponification method and one-step

methanol extraction, our results demonstrated that the yields of vitamin D2 in the mushrooms using one-step methanol extraction was no less than saponification content.

Our results indicated that one-step methanol extraction is feasible for extraction vitamin D2 in the mushrooms and maintain vitamin D2 yields comparable with saponification process. With one-step handling approach, the sample handling efficiency could significant to increase the sample handling efficiency compared with conventional method, which provide a new approach to fast quantification of vitamin D2 in the mushrooms. Additional, we noticed that vitamin D2 was almost undetectable in the mushrooms if they did not irradiate by UVB light.

Three different mushrooms, including *Pleurotuseryngii*, *Agaricusbisporus* and *Pleurotusostreatus,* were compared by using the conventional method and our one-step extraction method, the HPLC chromatograms showed in Figure 5-1. The results showed very similar trend in comparison of two handling methods that vitamin D2 yields from conventional saponification handling was somewhat lower than our one-step extraction for the three mushrooms, see Figure 5-4. It may be due to methanol extraction can reduce sample loss in the sample handling, while saponification method experience more than 10-fold steps which increase the probability of sample loss. Therefore, vitamin D2 yield of one-step extraction showed somewhat higher than conventional handling method. The developed method will provide a simple method to estimate vitamin D2 content in mushrooms.

5.3.2 Vitamin D2 contents under various conditions

The results showed that the UVB light was most effective light for stimulating vitamin D2 accumulation in the mushroom. The UVC light had also showed effect to

enhance vitamin D2 enrichment in the mushroom. However, vitamin D2 content in the mushroom irradiated by UVC light showed lower vitamin D2 in the mushroom compared with UVB light. UVA light almost do not have effect to stimulate vitamin D2 enhancement in the mushroom.

The results indicated there were basically no different for vitamin D2 content under irradiation of between UV intensity of 95μw/cm^2 and intensity of 148 μw/cm^2. However, the higher intensity of UVB irradiation showed somewhat higher vitamin D2 content.

Vitamin D2 contents in other mushrooms were measured under same UV environment. Our results found that vitamin D2 content in the mushrooms can be divided as three levels. The highest level content of vitamin D2 was found in XBG and SBG mushrooms. The middle level content was can be found in JZG, BG and XG. The lowest level content was found in LZG.

5.4 Summary

In conclusion, we developed a HPLC-DAD method to quantify vitamin D2 in mushrooms. The validation test showed that developed method showed good linearity, precise and recovery. The developed method provides a simple method to estimate vitamin D2 content in mushrooms.

In our study, the UVA, UVB, UVC light were applied for comparing effect for vitamin D2 accumulation in mushrooms. The results showed that the UVB light was most effective light for stimulating vitamin D2 accumulation in mushrooms. The UVC light had also showed effect to enhance vitamin D2 enrichment in the mushroom. Vitamin D2 in king oyster mushroom irradiated by UVA light was undetectable. Our

results showed that UVA light do not have effect to stimulate vitamin D2 enhancement in the mushroom

Vitamin D2 contents in other mushrooms were investigation after UV irradiation. We found that vitamin D2 in the mushrooms can be divided as three levels in contents. The highest level content of vitamin D2 was found in XBG and SBG mushrooms. Middle level contents of vitamin D2 was found in JZG, BG and XG. The lowest level content of vitamin D2 was LZG.

With exploration for affecting vitamin D2 accumulation under different conditions, the results suggested that irradiation time is one of most important factors to affect vitamin D2 accumulation in mushrooms. The results pave a way for further study on content dynamic trend for vitamin D2, provitmain D2, and vitamin D4 in the mushroom. Thus, the content dynamic trends of provitamin D2, vitamin D2, vitamin D4 in the mushroom will be symmetrically investigated.

Chapter 6 Effects of UV radiation on provitamin D2 and vitamin D2 in mushroom

6.1 Method development for quantification of provitamin D2 and vitamin D2

6.1.1 Sample preparation and extraction

The sliced 1cm^2 fresh mushrooms were irradiated by UV light and were vacuum-dried after UV irradiation. The vacuum-dried irradiated mushrooms were smashed as around 850 μm particle size. And then the samples were kept in vacuum-container until next step use of analysis. Vacuum-dried mushroom samples of 0.5 g were extracted with 7mL methanol for one hour in boiling methanol by using reflux extraction, and then cooled down room temperature immediately. The methanol extraction solution was then filtered through 0.45 μm nylon membrane syringe filters (Heaion, China) before use of HPLC-DAD analysis.

6.1.2 HPLC-DAD Analysis

HPLC analyses were performed on an HPLC system of Agilent Series 1100 liquid chromatography system (Agilent Technologies, USA). The HPLC analysis system was equipped with a vacuum degasser, a quaternary pump, an auto-sampler and a diode array detector (DAD). The solvents which constituted the mobile phase included aqueous solution (A) and ACN/MeOH (8:2; v/v) (B). Chromatographic separations were performed on Thermo Accucore XL C18 column (4.6× 250mm,4μ) coupled with guard column of Agilent Eclipse XDB-C18 (4.6x12.5mm,5μ) at ambient temperature, and gradient elution as following: 0–19 min, 93–100% B; 20-45 min, 100% B; and finally, reconditioning the column with 93% B isocratic for 5 minute. The flow rate of

mobile phase was set at 1 mL/min of chromatographic condition. The detection wavelength was set at 265nm for vitamin D2, and set at 282 nm for provitmain D2. The injection volume was 10µl.

Identification of provitamin D2 and vitamin D2 from the mushroom by comparing the retention time and UV spectra of provitamin D2 and vitamin D2 of mushroom samples with standard compounds of provitamin D and vitaminD2 under the identical chromatographic conditions based on HPLC-DAD method. The chromatograms were showed in Figure 6-3.

6.1.3 Calibration curves, limits of detection and quantification

Ethanol stock solution of standard compounds was prepared, and then diluted to appropriate concentrations. More than six different concentrations of the standard solution were tested. And the calibration curves of provitamin D2 and vitamin D2 were plotted by the peak areas versus the concentration of the provitamin D2 and vitamin D2. The linear regression equations of calibration curve were shown in Figure 6-1 and Figure 6-2. The calibration curves of provitamin D2 and vitamin D2 were calculated using peak area (y) versus concentration (x). The test range of vitamin D2 was in a range of 117.22 - 0.46 µg/mL, and provitamin D2 was in range of 317.30 -1.24 µg/mL. The linearity of the equation (r^2) for both vitamin D2 and provitamin D2 were over 0.9999, 0.9999, respectively, see Table 6-1.

Ethanol stock solution were diluted a series of concentrations by using ethanol solvent. The limits of detection (LOD) and limits of quantification (LOQ) of provitamin D2 and vitamin D2 of the chromatographic conditions of were determined at signal-to-noise ratio (S/N) around three and ten, respectively. The LODs of provitamin D2 and

vitamin D2 were 0.10 µg/mL and 0.07 µg/mL, respectively. The LOQs of provitamin D2 and vitamin D2 were 0.33µg/mL and 0.23 µg/mL, respectively. The results of LODs and LOQs for provitmain D2 and vitamin D2 were shown in Table 6-1.

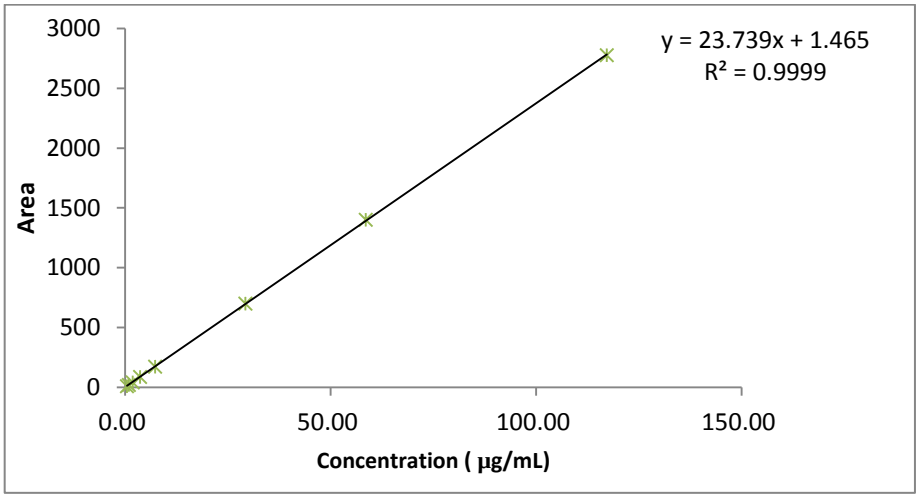


Figure 6-1. The calibration curve of vitamin D2

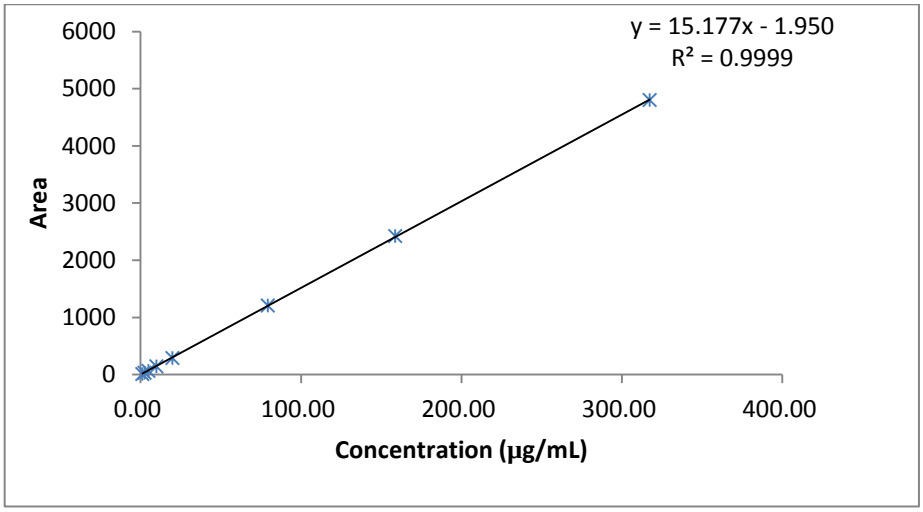


Figure 6-2. The calibration curve of provitamin D2

6.1.4 Precision, repeatability and recovery

The precision of the HPLC-DAD method was determined by intra- and inter-day precision. Intra-day precision was tested by determination of three levels of standard solutions for 6 times within one day (n=6). The inter-day precision was determined within consecutive three days and each day was determined three times (n=9). For precision of vitamin D2, the RSD of intra- and inter-day precision of vitamin D2 were less than 0.36% and 1.03%, respectively. The RSD of inter-day precision at three levels of low, middle and high level were less than 0.55%, 1.03%, 0.71%, respectively, see Table 6-2. For precision provitamin D2, the RSDs of intra- and inter-day precision of provitamin D2 were less than 0.06% and 0.50%, respectively. The RSDs of inter-day precision at three levels of low, middle and high level were less than 0.30%, 0.50%and 0.34%, respectively (see Table 6-2). The intra- and inter-day precision for both vitamin D2 and provitamin D2 at three level tests were less than 1.03% in RSD value.

The XBG extracted samples were analysed to measure repeatability of chromatographic method. The repeatability was determined by three levels of XBG extraction samples and each level was measured for six replicates. The RSD of vitamin D2 repeatability of chromatographic method was less than 0.90%. The levels of low, middle and high tests were less than 0.90%, 0.54% and 0.74%, respectively. The RSD of provitamin D2 repeatability of chromatographic method was less than 0.98%. The low level, middle and high level tests were less than0.15%, 0.47% and 0.98%, respectively, see Table 6-2.

The extracted XBG sample was stored at room temperature for stability test. The stability was performed by analysing the store mushroom sample of different time period. The extracted sample was analysed at 2, 4, 6, 12, 13, 25 hours for test of stability.

The results indicated that the RSD of stability for vitamin D2 and provitamin D2 was less than 0.79% and 1.98%, respectively.

The recovery was tested by adding known amount of standard into amount of mushroom sample. The mixture samples were prepared, extracted and analyzed as described in sample preparation in section 6.1.1 and section 6.1.2. The recovery was performed three times according to the method described as above. The average recovery of vitamin D2 was 92.64% from three replicated tests. The RSD of recovery of vitamin D2 was 1.49%. The average recovery of provitamin D2 was 96.70% from three replicate tests, and RSD value was 3.68%. The overall recovery for both vitamin D2 and provitamin D2 was over 92 % from three replicate tests, see Table 6-3. The recoveries were calculated by using formula as following: Recovery (%) = 100 % × (amount found – amount original)/amount spiked.

Table 6-1. Calibration curves, LOD and LOQ of the standard compounds

Compounds	Regression equation	r^2	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Vitamin D2	y = 23.739x +1.465	0.9999	117.22-0.46	0.07	0.23
Provitamin D2	y = 15.177x -1.950	0.9999	317.30-1.24	0.10	0.33

Table 6-2. Intra- and inter-day precisions

Conc (μg/mL)	Intra-day (n=6)			Inter-day (n=9)		
	Observed Conc (μg/mL)	Accuracy (%)	RSD (%)	Observed Conc (μg/mL)	Accuracy (%)	RSD (%)
Vitamin D2						
61.38	61.44	100.10	0.06	60.87	99.17	0.71
15.35	15.27	99.47	0.08	15.05	98.07	1.03
3.84	3.78	98.38	0.36	3.76	97.92	0.55
Provitamin D2						
148.68	147.99	99.54	0.06	148.50	99.88	0.30
37.17	37.12	99.88	0.06	37.01	99.57	0.50
9.29	9.49	102.16	0.27	9.45	101.68	0.34

Accuracy (%) = 100%×mean of measured concentration/nominal concentration.

Table 6-3. Recovery of vitamin D2 and provitamin D2

Compounds	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)	RSD (%, n=3)
Vitamin D2	9.28	10.34	18.87	92.64	1.49
Provitamin D2	458.90	497.33	939.81	96.70	3.68

Table 6-4. The repeatability of chromatographic method

Compounds	Weight(g)	RSD(%, n=6)
Vitamin D2	0.4	0.90
	0.5	0.54
	0.6	0.74
Provitamin D2	0.4	0.15
	0.5	0.47
	0.6	0.98

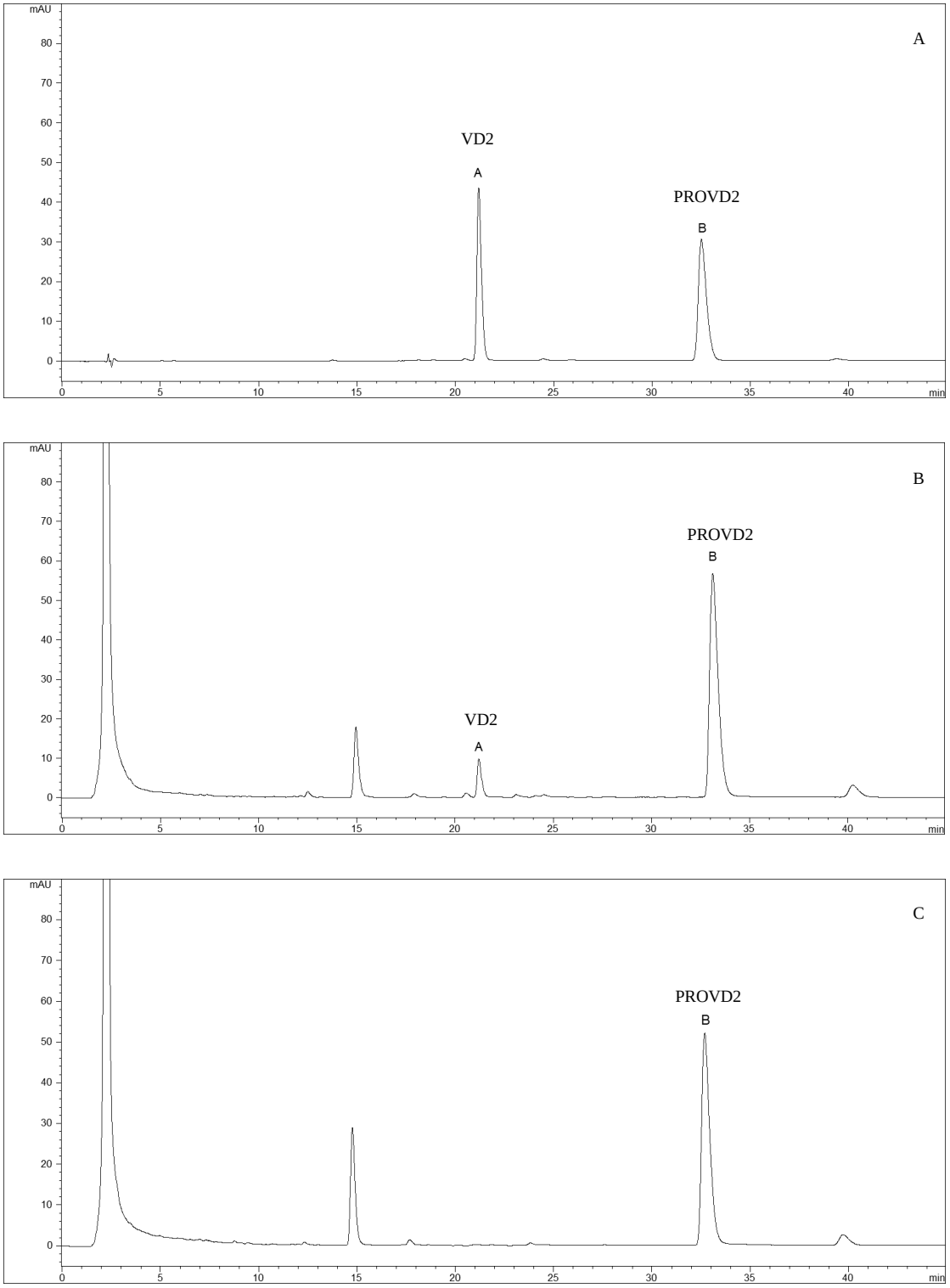


Figure 6-3. Representative HPLC chromatograms of standards and extract mushroom sample: (A) vitamin D2 and provitamin D2 standard; (B) extract irradiated mushroom sample; (C) extract the mushroom sample without UV irradiation.

6.2 Vitamin D2 and provitamin D2 contents in mushroom

6.2.1 Irradiation procedure

The fresh king oyster mushrooms were cut out cap and bottom, and then were cut into slices of 1cm^2. The fresh sliced mushrooms were placed in Petri dish, and then transferred into UV chamber for irradiation. The sliced mushrooms were exposed to UVB light (Beijing Zhongyiboteng Tech CO., LTD. Wavelength: 285-320nm). After UV radiation, the mushroom samples were dried by using vacuum-drier. And then the mushroom samples were kept in a vacuum-container until next step use of analysis.

6.2.2 Dynamic trend of vitamin D2 content in the mushroom

The king oyster mushroom was irradiated by six different time period to evaluate contents dynamic process between vitamin D2 and provitamin D2 based on our method. The results indicated that vitamin D2 content showed a linear increasing trend ($y = 0.998x + 15.75$, $r^2=0.973$) under UVB irradiation within 30 minute as showed in Figure 6-4. The results revealed that vitamin D2 content showed very slowly increase after UV irradiation of one hour. The results indicated that it is linear change in content for both vitamin D2 and provitamin D2 in the mushroom during UVB irradiation within 30 minute.

The result demonstrated that linear change of content occurred within the first 30 minute for both vitamin D2 and provitamin D2. The content of provitamin D2 was relatively dynamic stable in a range with UVB irradiation between 1 hour and 2.5 hour, and vitamin D2 tended to be a steady trend between 1 hour and 2.5 hour, see Figure 6-6. Our study was the first time report to monitor content dynamic process for both vitamin D2 and provitamin D2 in king oyster mushroom during UVB irradiation. Additional, we noticed that vitamin D2 was almost undetectable in the mushrooms if the

mushrooms were not irradiated by UVB light, which indicate that UVB irradiation is an effective way to enhance vitamin D2 content in the mushrooms.

For vitamin D2, the dynamic trend in the mushroom during UVB irradiation from our results showed quite similar figure compared with previous reported results (Jasinghe & Perera, 2006). All the results showed that high efficiency accumulation of vitamin D2 in the mushroom occurred within one hour, and vitamin D2 tended to be relative stable range after one-hour irradiation.

6.2.3 Dynamic trend of provitamin D2 content in the mushroom

The results indicated that provitamin D2 showed a linear decreasing trend ($y=-0.003x+2.09$, $r^2=0.942$) under UVB irradiation within 30 minute, as showed in Figure 6-4.The results indicated that provitamin D2 content maintain in a relative stable dynamic range after UVB irradiation of one hour. Provitamin D2 content showed a relative stable range between 1 and 2.5 hours.

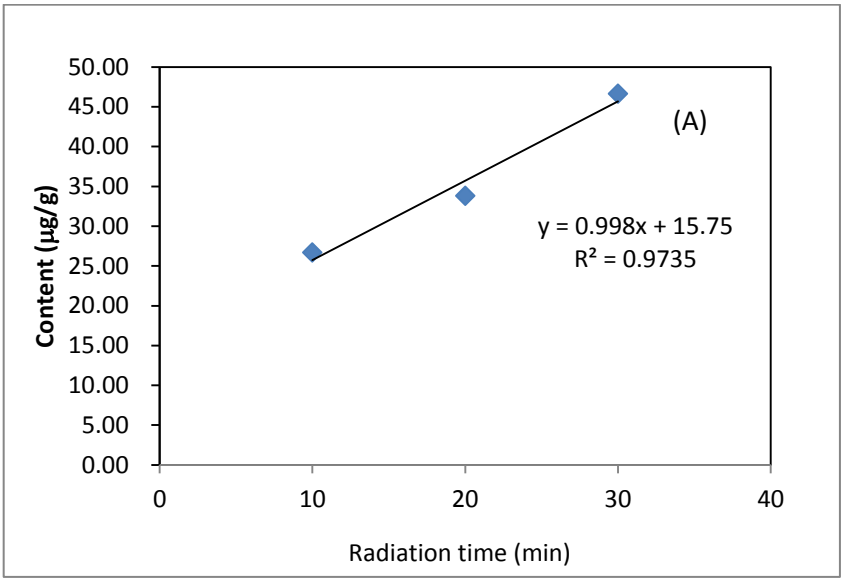
In addition, we constructed a content relationship between vitamin D2 and provitamin D2 in the mushroom under UVB irradiation within 30min, which can be express a parabolic equation showed as: $y=7127x^2-29150x+29831$, (y=vitamin D2, x=provitamin D2), see Figure 6-5. The relation equation of between vitamin D2 and provitminD2 may provide a simple way to estimate vitamin D2 content exposed to UVB light within 30 minute when the provitamin D2 content have been known in king oyster mushroom. For provitamin D2, we were the first time to demonstrate provitamin D2 dynamic trend in the mushroom during UVB irradiation. Our study was the first time to show that provitamin D2 dynamic trend in the mushroom irradiated by UV light within 30 minute.

Table 6-5. Contents of provitamin D2 in the mushroom

Time (min)	Provitamin D2 (mg/g)	SD (n=3)
10	2.06	0.01
20	2.01	0.02
30	1.99	0.01
60	1.91	0.00
90	2.04	0.03
120	1.87	0.04
150	1.99	0.03

Table 6-6. Contents of vitamin D2 in the mushroom

Time (min)	Vitamin D2 (μg/g)	SD (n=3)
10	26.68	0.81
20	33.81	1.37
30	46.64	0.54
60	74.66	4.70
90	67.80	1.79
120	89.59	0.83
150	78.60	1.32



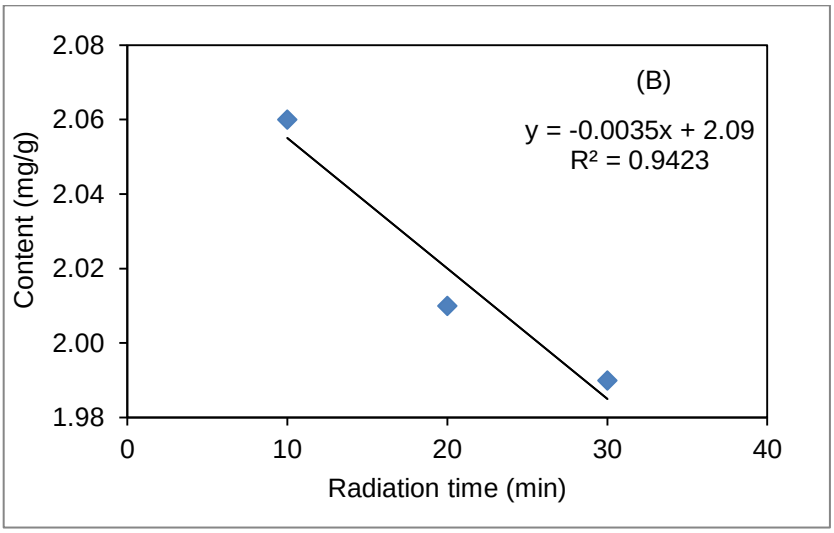


Figure 6-4. (A) Vitamin D2 content dynamic trend within 30 minute; (B) Provitamin D2 content dynamic trend within 30 minute

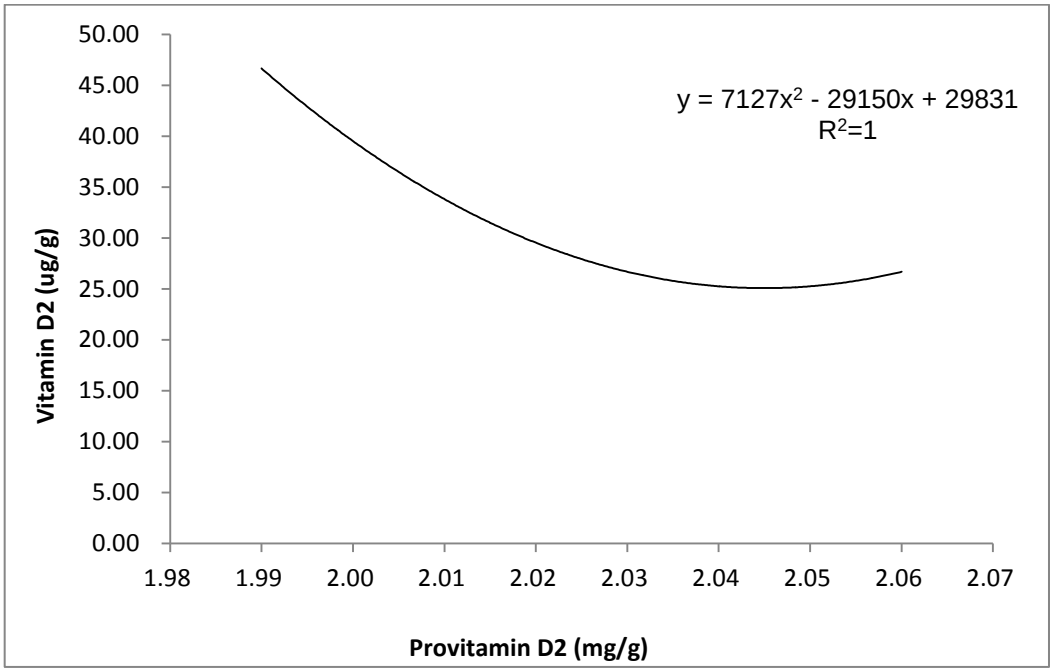
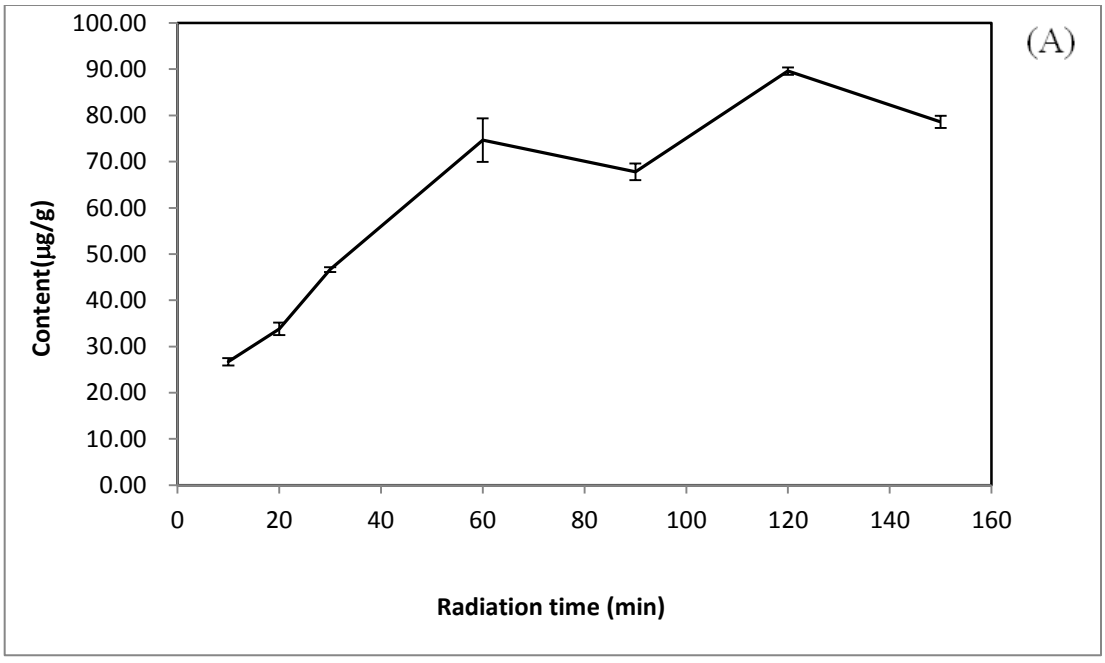


Figure 6-5. The relationship between vitamin D2 and provitamin D2 in the mushroom



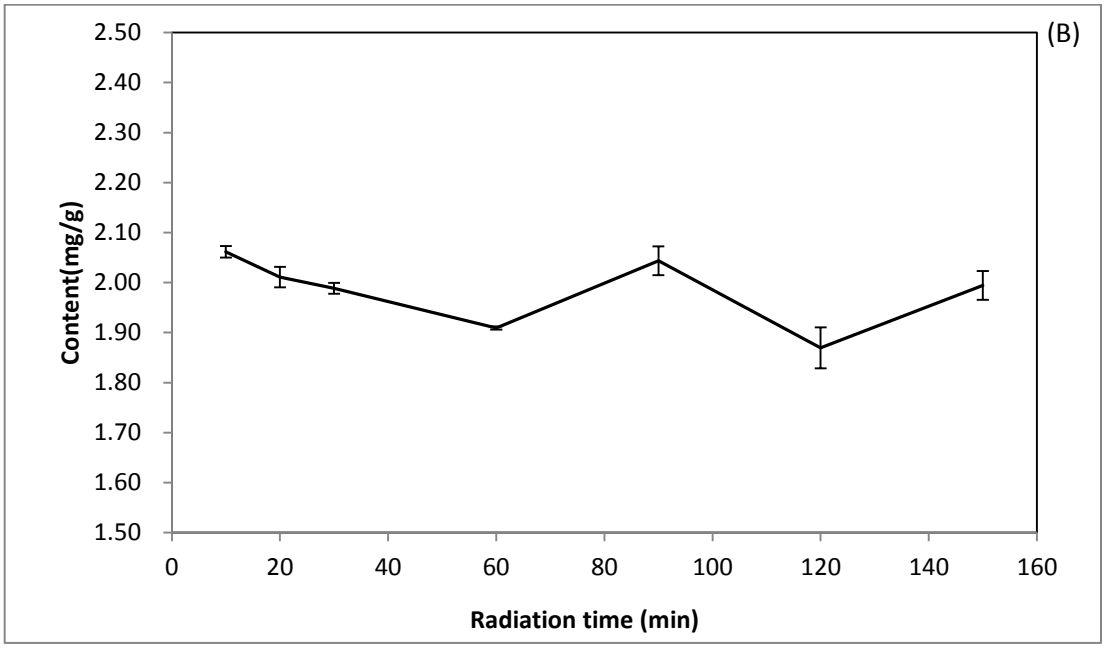


Figure 6-6. (A) Vitamin D2 content dynamic trend with 150 minute; (B) Provitamin D2 content dynamic trend within 150 minute

6.3 Results and discussion

6.3.1 Validation of HPLC-DAD method

The developed method showed good linearity within test range for both vitamin D2 and provitamin D2 with r^2=0.9999 and r^2=0.9999, respectively. The LODs were 0.07μg/mL and 0.10 μg/mL, and LOQs were 0.23 μg/mL and 0.33 μg/mL for vitamin D2 and provitamin D2, respectively, as showed in Table 6-1.

The intra-day precision was tested by determination of three levels of the standard solutions for six times within one day (n=6), while the inter-day precision was tested within consecutive three days (n=9). The RSD of inter-day precision of vitamin D2 and provitamin D2 were less than 1.03% and 0.50%, respectively. The results indicated that the developed method showed good precision for both vitamin D2 and provitamin D2. The repeatability of chromatographic method was determined by three levels of XBG

extraction samples for six replicates. The RSD of repeatability of chromatographic method of vitamin D2 and provitamin D2 were less than 0.90% and 0.98%, respectively. The results indicated that the developed method showed a good repeatability for both vitamin D2 and provitamin D2 in mushroom. The stability of vitamin D2 and provitamin D2 was performed within 24 hours. The RSDs of stability for vitamin D2 and provitamin D2 were 0.79% and 1.98%, respectively. The results indicated that vitamin D2 and provitamin D2 are relatively stable within 24 hours. The recoveries of vitamin D2 and provitamin D2 were performed by measuring three times. The overall recoveries of vitamin D2 and provitamin D2 were between 92%-97%. The developed method showed a reasonable recovery for both vitamin D2 and provitamin D2.

The results demonstrated the developed method showed good intra-and inter day precise, repeatability of chromatographic method, and recovery. Validation tests of the developed method showed that the HPLC-DAD method is precise, accurate for determination of vitamin D2 and provitmain D2 in the mushroom. The method was successfully used for quantification of vitamin D2 and provitamin D2 in the mushroom. The developed method will provide a new approach to quantify vitamin D2 and provitamin D2 in mushrooms.

6.3.2 Dynamic trend of provitamin D2 and vitamin D2

The king oyster mushroom was irradiated by six different time period to evaluate content dynamic process for both vitamin D2 and provitamin D2 based on our method. The results indicated that vitamin D2 content showed a linear increasing trend ($y = 0.998x + 15.75$, $r^2=0.973$) exposed to UVB light within 30 minute. Contents of provitamin D2 in the mushroom showed a linear decreasing trend ($y=-0.003x+2.09$, $r^2=0.942$) exposed to UVB light within 30 minute. The content of provitamin D2 was relatively dynamic stable in a range with UVB irradiation between one hour and 2.5

hour and vitamin D2 tended to be a steady trend between 1 hour and 2.5 hour. Ourt study was the first time to report content dynamic process for both vitamin D2 and provitamin D2 in king oyster mushroom during UVB irradiation. Contents of provitamin D2 and vitamin D2 were showed in Table 6-5 and Table 6-6.

Additional, we noticed that vitamin D2 was almost undetectable in the mushrooms if the mushrooms were not irradiated by UVB light. This result indicated that UVB irradiation is an effective way to enhance vitamin D2 content in the mushrooms. Vitamin D2 contents in mushrooms can be rapidly accumulated in high level after short time period UVB irradiation.

6.3.3 The relationship between provitamin D2 and vitamin D2

Our results indicated that there was a content relationship between vitamin D2 and provitamin D2 in the mushroom during UVB irradiation within 30min. The relationship can be expressed as parabolic equation: $y=7127x^2-29150x+29831$, (y=vitamin D2, x=provitamin D2). Firstly, the parabolic equation between vitamin D2 and provitamin D2 demonstrated content relationship between vitamin D2 and provitamin D2 in the mushroom during UVB irradiation. Secondly, the parabolic equation can be used for calculation of content of vitamin D2 or provitamin D2 as long as known one of parameters. The equation provides a way to calculate the content for both provitamin D2 and vitamin D2 in the mushroom during UVB irradiation with the first 30 minute. Our study was the first time to demonstrate the relationship between vitamin D2 and provitamin D2 in mushroom during UVB irradiation.

6.4 Summary

In conclusion, we developed a method to quantify vitamin D2 and provitamin D2 in the mushrooms. The validation tests of the method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D2 in the mushrooms. The method was successfully used for quantification of vitamin D2 and provitamin D2 in the mushroom. The developed method will provide a new approach to quantify vitamin D2 in mushrooms.

The results indicated that provitamin D2 and vitamin D2 content showed a linear increasing trend ($y = 0.998x + 15.75$, $r^2=0.973$) exposed to UVB irradiation within 30 minute. Contents of provitamin D2in the mushroom showed a linear decreasing trend ($y=-0.003x+2.09$, $r^2=0.942$) exposed to UVB irradiation within 30 minute. The content of provitamin D2 was relatively dynamic stable in a range with UVB irradiation between one hour and 2.5 hour and vitamin D2 tended to be a steady trend between 1 hour and 2.5 hour.

The relationship between vitamin D2 and provitamin D2 in the mushroom during UVB irradiation within 30 min can be express by an equation. The relationship can be expressed as parabolic equation: $y=7127x^2-29150x+29831$, (y=vitamin D2, x=provitamin D2, $r^2=1$). The parabolic equation provides a way to calculate content relationship between vitamin D2 and provitamin D2 in the mushroom during UVB irradiation. The parabolic relation equation provides an approach to calculate the contents for vitamin D2 and provitamin D2 in the mushroom in specific time from 10 to 30 minute.

Chapter 7 Effects of UV radiation on vitamin D4 in mushroom

7.1 Method development for quantification of vitamin D4

7.1.1 Sample preparation and extraction

The sliced fresh mushrooms were irradiated by UVB light and were vacuum-dried after UVB irradiation. The vacuum-dried irradiated mushrooms were smashed by homogeniser as powder around 850 μm particle size, and then were kept in vacuum-container until next step analysis.

Vacuum-dried 0.7g (dry weight) mushroom samples added four ml of ascorbic acid sodium salt, a 10 ml solution of 50% potassium hydroxide, and 50ml ethanol (99%), and then formed a mixed solution. The ascorbic acid sodium salt solution was prepared as two gram of ascorbic acid sodium salt in ten mL of sodium hydroxide (1M). The mixed solution was refluxed at 80 degrees for one hour for saponification. After that, it was cooled down to room temperature, and was transferred into separation funnel for separation. The mixture solution was extracted by fifteen ml Milli-Q water and then fifteen ml ethanol, and then extracted by n-pentane with volumes 50, 50 and 50 ml, respectively. The pooled organic layers were washed by 50 ml of a solution (3% potassium hydroxide in 5% ethanol) for three times, and then washed by Milli-Q water for three times until neutralized. Finally, the organic layer was evaporated by rotary evaporator at 40 ℃. After the evaporation of organic solvent, the sample was dissolved in five ml ethanol.

7.1.2 HPLC-DAD Analysis

HPLC analyses were performed on HPLC system of Agilent Series 1100 (Agilent Technologies, USA). The HPLC system was equipped with a vacuum degasser, a

quaternary pump, an auto-sampler and a diode array detector (DAD). The solvents which constituted the mobile phase included aqueous solution (A) and MeOH (B). The solvents which constituted the mobile phase included 0.05% formic acid aqueous solution (A) and ACN/MeOH (8.2:1.8, v/v) (B). Chromatographic separations were performed on Thermo Accucore XL C18 column (4.6× 250mm,4μ) coupled with guard column of Agilent Eclipse XDB-C18 (4.6×12.5mm,5μ) at ambient temperature, and gradient elution as following: 0–19 min, 93–100% B; 20-27 min, 100% B; and finally, reconditioning the column with 93% B isocratic for 10 min. The flow rate of mobile phase was set at 1 mL/min. the detection wavelength was set at 265nm and the injection volume was 20μl. Identification of vitamin D4 from the mushroom by comparing the retention time of vitamin D4 of mushroom sample with vitamin D4 standard under the identical chromatographic conditions based on HPLC-DAD method.

7.1.3 Calibration curve, limit of detection and quantification

Methanol stock solutions of standard compounds was prepared, and then diluted to suitable concentrations. More than seven different concentrations of the solution were tested in two replicates, and the calibration curve was plotted by the peak areas versus the concentration of vitamin D4. The linear regression equation of calibration curve was shown as following: y = 31.957x - 0.418 (x, Concentration; y, Area, r^2=0.9999). Test ranges of calibration carve were from 30.07-0.12 μg/mL. The limit of detection (LOD) and limit of quantification (LOQ) of vitamin D4 in the chromatographic conditions were determined at an S/N of around 3 and 10, respectively. LOD and LOQ of the vitamin D4 were 0.04 μg/mL and 0.12 μg/mL, respectively, see Table 7-1.

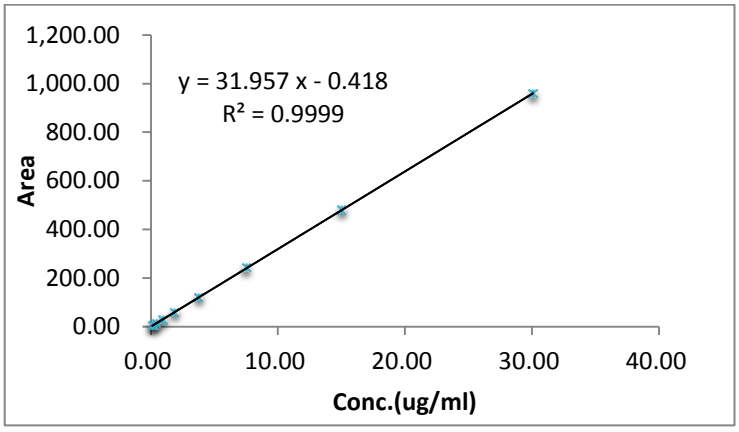


Figure 7-1. Calibration curve of vitamin D4

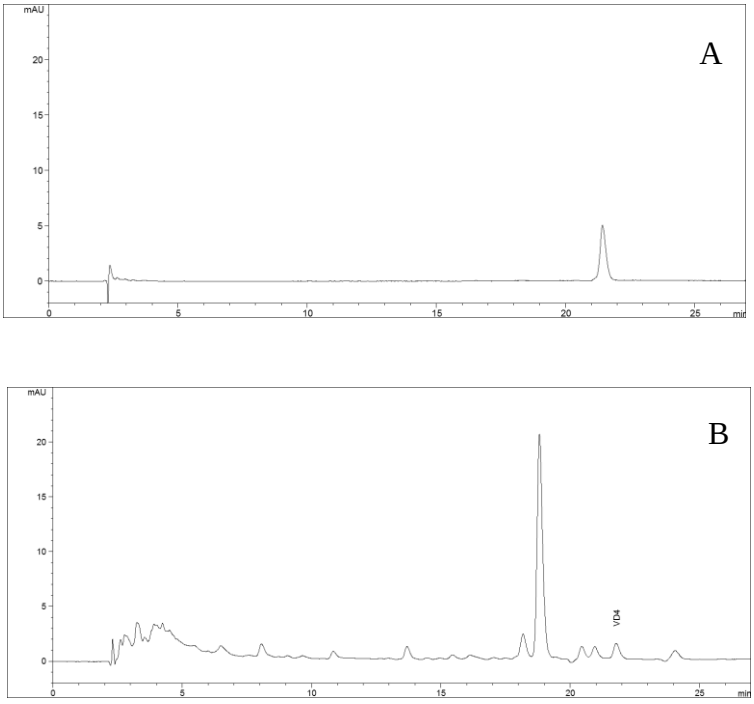


Figure 7-2. Representative HPLC chromatograms of vitamin D4: (A) vitamin D4 standard compound; (B) The mushroom sample

7.1.4 Precision, repeatability and recovery

The precision of the developed method was measured by testing intra- and inter-day variations. For intra-day variation test, the standard solution was injected for six replicates for each level (n=6), and three levels were examined within one day. While inter-day variations, each level of standard solution was injected for three replicates,

and three levels were examined for consecutive three days (n=9). The relative standard deviation (RSD) was used for the assessment of variation. The RSD of intra-and inter-day were less than 0.44 % and 0.63%, respectively. For inter-day precision, three levels of low, middle and high tests were less than 0.63%, 0.62%, and 0.63%, respectively, as showed in Table 7-2.

The different levels of extracted mushroom samples were tested for the repeatability of chromatographic method. Three levels of mushroom sample were test for assessment of repeatability. The mushroom samples of 0.7g, 0.8g and 0.9gwere extracted and prepared according to procedure described in section 7.1.1. Each level of XBG mushroom samples was analyzed six replicates (n=6), and results were assessed by RSD (%). The RSD of repeatability of chromatographic method was less than 1.20%. The tests levels of low, middle and high level were less than1.16%, 0.65% and 1.20%, respectively, see Table 7-3.

The stability was performed by testing extracted mushroom sample with 48 hours. The mushroom extracted sample was stored at 4 ℃, and then analyzed at 1, 3, 29, 32 and 48 hours for stability test. The results showed that the RSD of stability was less than 0.85%.

The recovery was tested by adding known standard into amount of the mushroom sample. The mixture samples were prepared and analysed according to the method as described in section 7.1.1 and section 7.1.2. The recoveries were calculated by using formula as following: Recovery (%) = 100 %× (amount found – amount original)/amount spiked. The average recovery was 90.16% from two replicates tests, see Table 7-4 .

Table 7-1. Calibration curve, LOD and LOQ of vitamin D4

Compound	Regression equation	r^2	Range (μg/mL)	LOD (μg/mL)	LOQ (μg/mL)
Vitamin D4	y = 31.957x - 0.418	0.9999	30.07-0.12	0.04	0.12

Table 7-2. Intra- and inter-day precisions

Conc (μg/mL)	Intra-day (n=6)			Inter-day (n=9)		
	Observed conc (μg/mL)	Accuracy (%)	RSD (%)	Observed conc (μg/mL)	Accuracy (%)	RSD (%)
30.07	30.09	100.07	0.07	30.06	99.97	0.08
3.76	3.81	101.41	0.48	3.80	101.07	0.62
0.94	0.88	93.54	0.44	0.87	92.84	0.63

Table 7-3. The repeatability of chromatographic method

Compound	Weight (g)	RSD (n=6, %)
Vitamin D4	0.7	1.16
	0.8	0.65
	0.9	1.20

Table 7-4. Recovery of vitamin D4

Compound	Original (μg)	Spiked (μg)	Found (μg)	Recovery (%)
Vitamin D4	1.46	1.89	3.16	90.16

Recovery (%) =100%× (amount found – amount original)/amount spiked

7.2 Vitamin D4 contents in the mushroom

7.2.1 Irradiation procedure

The fresh king oyster mushrooms were cut out cap and bottom, and then were cut into slices of 1cm^2. The fresh sliced 1cm^2 mushrooms were placed in Petri dish, and then transferred into UV chamber for irradiation. The sliced mushrooms were irradiated under UVB light at 35℃ for time period of 10, 20 and 30 minute, respectively. After UV radiation, the mushroom samples were transferred into vacuum-drier for dryness, and then the mushroom samples were kept in a vacuum-container until next step use of analysis.

7.2.2 Vitamin D4 contents in mushroom

After the irradiation the mushroom samples were prepared according to the procedure described in section 7.1.1. And then the samples were analysed according to the developed method. The mushroom sample were irradiated by three different time period, including 10, 20, 30 minute. The result showed that contents of vitamin D4 in the mushroom was in a range from 1.42 to 3.06 μg/g in king oyster mushroom within 30 min irradiation, as showed in Table 7-5.

Vitamin D4 contents showed a linear increase trend within the first 30-minute irradiation. The linear equation can be expressed as: $y = 0.081x + 0.389$, $r^2=0.834$, (y=vitamin D2, x= time) as showed in Figure 7-3. The results indicated that vitamin D4 showed a linear increase trend within irradiation of 30 minute. The linearity of vitamin D4 ($r^2=0.834$) was not as good as vitamin D2 ($r^2=0.934$) in mushroom during UVB irradiation.

Table 7-5. Contents of vitamin D4 in the mushroom

Time(min)	Vitamin D4 (μg/g)
10	1.42
20	1.61
30	3.06

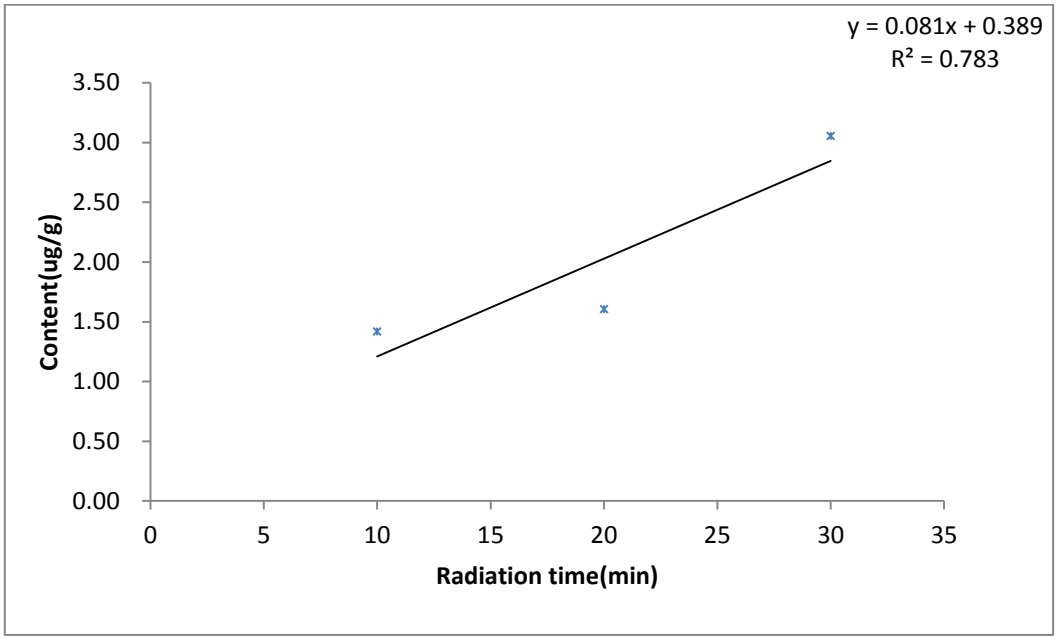


Figure 7-3. Content dynamic trend of vitamin D4 in the mushroom

7.3 Results and discussion

7.3.1 Validation of HPLC-DAD method

The calibration curve of vitamin D4 showed good linear regression (r^2=0.9999) within test range. The precision of the HPLC-DAD method was determined by intra- and inter-day precision. Intra-day precision was tested by determination of three levels of the standard solutions for six times within one day, while the inter-day precision was tested within consecutive three days. The RSD of intra- and inter-day precision were less than 0.48% and 0.63%, respectively. The results indicated that vitamin D4 showed a good precision with the developed method. The repeatability of chromatographic method was performed by determining by three levels of the mushroom extraction samples for six replicates. The RSD of repeatability of chromatographic method was less than 1.16%. The three test levels of low, middle and high were less than1.20%, 0.65% and 1.16 %, respectively. The results demonstrated that the developed method for quantification of vitamin D4 showed a reasonable repeatability with RSD value less

than 1.20%. The recovery was performed by adding known amount of vitamin D4 into the mushroom sample. The two tested was tested for recovery. The overall recovery of vitamin D4 was more than 90%. The developed method for determination of vitamin D4 in mushroom showed that good overall recovery. The validation tests of the developed method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D2 in the mushroom. The developed method for quantification of vitamin D4 in mushroom will provide an approach to determine vitamin D4 in mushrooms.

7.3.2 Vitamin D4 in mushroom

The results showed that contents of vitaminD2 was about 15-20 fold higher than contents of vitamin D4 in the mushroom. The results indicated that vitamin D4 in mushroom during UVB irradiation showed a linear increasing trend within 30 minute ($y = 0.081x + 0.389$, $r^2=0.834$, y= vitamin D4, x= time). The dynamic trend of vitamin D4 in mushroom showed a similar trend compared with vitamin D2 increased trend, which all showed linear increase trend within the first 30 minute. However, increasing linearity showed by vitamin D4 was not as good as increasing linearity showed by vitamin D2 in the mushroom. The reason of vitamin D4 showed a relative weaker linearity may be due to the mushroom contained quite small amount of vitamin D4, which caused that it was not easy to form good linearity.

7.3.3 The relationship between vitamin D4 and vitamin D2

The results indicated that both vitamin D4 and vitamin D2 showed a linear increase with time within 30 minute. Therefore, vitamin D4 and vitamin D2 in the mushroom also have a potential relationship in contents. The results indicated that when vitamin D4 contents were increasing along with irradiation time, vitamin D2 contents were also

increasing along with irradiation time. Thus, both vitamin D4 and vitamin D2 showed a relationship with similar increasing trend in the mushroom. The relationship can be constructed as linear relationship between vitamin D4 and vitamin D2 in the mushroom during UVB irradiation. The relationship between vitamin D4 and vitamin D2 can be expressed as: $y=10.91 x+13.58$, $r^2=0.935$. Firstly, the relation equation between vitamin D4 and vitamin D2 demonstrated content relationship of vitamin D4 and vitamin D2 in the mushroom. Secondly, the relation equation between vitamin D4 and vitamin D2 demonstrated the synchronism in increasing trend between vitamin D4 and vitamin D2 in the mushroom during UV irradiation. The equation indicated that there was a very good synchronism in increasing for both vitamin D2 and vitamin D4 in the mushroom.

The results indicated that mushroom irradiated by UVB light can stimulate production of vitamin D2, but also stimulate vitamin D4 production in king oyster mushroom. Vitamin D2 and vitamin D4 in the mushroom irradiated by UV light showed a linear increase within 30 minute. There was a linear relationship between vitamin D4 and vitamin D2 in the mushroom during UVB irradiation.

Table 7-6. Vitamin D4 and vitamin D2 contents in the mushroom

Time (min)	VD2 contents (μg/g)	VD4 contents (μg/g)
10	26.68	1.42
20	33.81	1.61
30	46.64	3.06

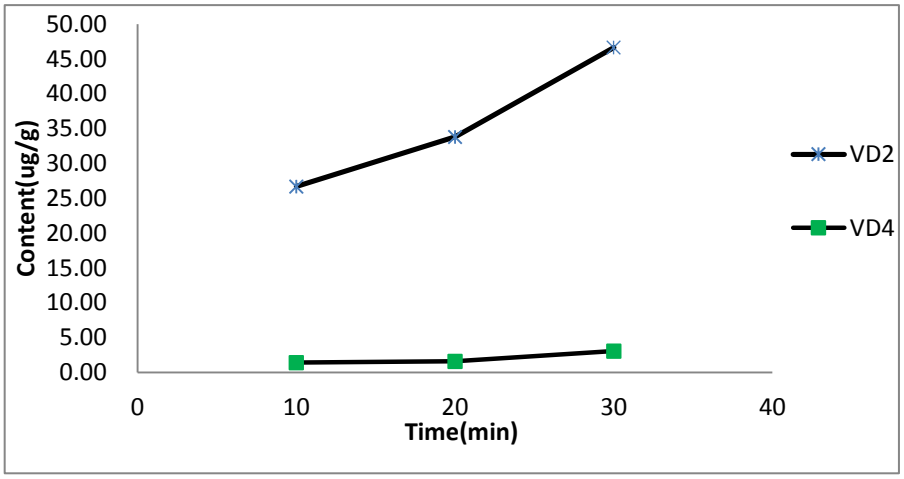


Figure 7-4. Content and dynamic trends of vitamin D4 and vitamin D2 in the mushroom

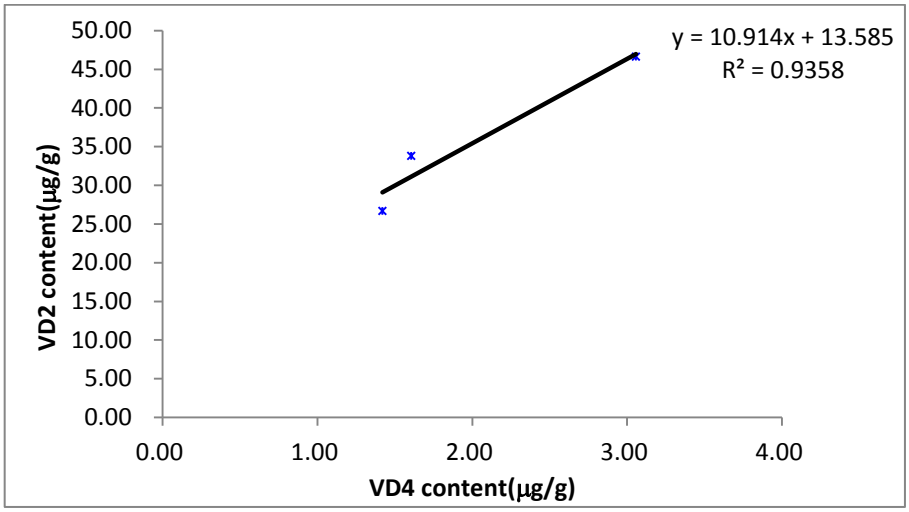


Figure 7-5. The content relationship between vitamin D4 and vitamin D2 in the mushroom

7.4 Summary

In conclusion, we develop a HPLC-DAD method for quantification of vitamin D4 in mushrooms. Validation tests of the method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D2 in the mushroom. The developed method for quantification of vitamin D4 in mushroom will provide an approach to determine vitamin D4 in mushroom.

The contents of vitamin D4 in the mushroom were in a range from 1.42 to 3.06 μg/g in king oyster mushroom within 30 min irradiation. The content dynamic trend of vitamin D4 in mushroom during UVB irradiation showed a linear increasing trend within 30 minute. The linear equation can be expressed as: $y = 0.081x + 0.389$, $r^2=0.834$. Dynamic trend of vitamin D4 in mushroom showed a similar trend compared with vitamin D2 increased trend, which all showed linear increase trend within the first 30 minute. Vitamin D4 and vitamin D2 showed a relationship with similar increasing trend in the mushroom. The relationship can be constructed as linear relationship between vitamin D4 and vitamin D2 in the mushroom during UVB irradiation. The relationship between vitamin D4 and vitamin D2 can be expressed as a linear equation ($y=10.91 x+13.58$, $r^2=0.935$, y=vitamin D2, x= vitamin D).

Chapter 8 General results and discussion

8.1 The HPLC methods for vitamin D2, provitamin D2 and vitamin D4

The developed method of quantification of vitamin D2 and provitamin D2 showed good linearity within test range for both vitamin D2 and provitamin D2 with r^2=0.9999 and r^2=0.9999, respectively, see Table 6-1. The RSD of inter-day precision of vitamin D2 and provitamin D2 were less than 1.03% and 0.50%, respectively. The RSD of repeatability of chromatographic method of vitamin D2 and provitamin D2 were less than 1.27% and 0.20%, respectively. The overall recovery of vitamin D2 and provitamin D2 were between 92%-97%. The developed method showed good intra-and inter day precise, repeatability, and recovery. Validation tests of the method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D2 in the mushrooms. The method was successfully used for quantification of vitamin D2 and provitamin D2 in the mushroom.

The validation testes of quantification for vitamin D4 in mushrooms showed good linear regression (r^2=0.9999) within test range. The precision of the HPLC-DAD method was determined by intra- and inter-day precision. The RSD of intra- and inter-day precision were less than 0.44% and 0.63%, respectively. The repeatability of chromatographic method of chromatographic method was determined by three levels of the mushroom extraction samples for six replicates. The three test levels of low, middle and high were less than1.20%, 0.65% and 1.16 %, respectively. The developed method for determination of vitamin D4 in mushroom showed that good overall recovery. The recovery of vitamin D4 was more than 90%. Validation tests of those methods showed that this HPLC-DAD method is precise, accurate for determination of

vitamin D4, vitamin D2 and provitamin D2 in the mushroom. The developed method will provide a new approach to quantify vitamin D2 and provitamin D2 in mushrooms, and provide a method to quantify vitamin D4 in mushrooms.

8.2 Effects of UV irradiation on vitamin D4

8.2.1 Dynamic trend of vitamin D4 in the mushroom

The results indicated that vitamin D4 in mushroom during UVB irradiation showed a linear increase trend within 30 minute (r^2=0.83). Dynamic trend of vitamin D4 in mushroom showed a similar trend compared with vitamin D2 trend, which was that both of vitamin D4 and vitamin D2 showed linear increasing trend within the first 30 minute. However, increasing linearity showed by vitamin D4 was not as good as increasing linearity showed by vitamin D2 in the mushroom. The reason of vitamin D4 showed a relative weaker linearity may be due to the mushroom contained quite small amount of vitamin D4. Thus it showed weaker linearity compared with vitamin D2 in the mushroom during UVB irradiation.

The results indicated that mushroom irradiated by UVB light can stimulate vitamin D2 production, but also stimulate vitamin D4 production in king oyster mushroom. The results may imply that the biological activities for bone mineralization showed by mushroom, which not only contribute vitamin D2, but also contribute vitamin D4 in the irradiation mushroom.

8.2.2 The relationship between vitamin D4 and vitamin D2

The results showed that content of vitamin D2 was about 15-20 fold higher than content of vitamin D4 in the mushroom. Because both vitamin D4 and vitamin D2 showed a linear increase with time within 30 minute, therefore, vitamin D4 and vitamin D2 in the mushroom also have a potential relationship in content. The results indicated

that when vitamin D4 content increased with time, vitamin D2 content also increased along with time. Thus, there was a relationship between vitamin D4 and vitamin D2 in the mushroom. The relationship can be constructed as linear relation between vitamin D4 and vitamin D2 in the mushroom during UVB irradiation. The relationship between vitamin D4 and vitamin D2 in the mushroom can be expressed as: $y=10.91 x +13.58$, $r^2=0.935$. The linear equation indicated that there was a good linearity between vitamin D4 and vitamin D2 in the mushroom.

Vitamin D2 and vitamin D4 in the mushroom irradiated by UV light showed a linear increase within 30 minute. There was a linear relationship between vitamin D4 and vitaminD2 in the mushroom during UVB irradiation. The results indicated that contents of both vitamin D4 and vitamin D2 in the mushroom irradiated by UVB light was simultaneously increased and showed similar trend within 30 minute.

8.3 Effects of UV radiation on vitamin D2

8.3.1 Vitamin D2 content under different UV light

In our study, we compared three different UV light for stimulate vitamin D2 production. The results showed that the UVB light was most effective light for stimulating vitamin D2 accumulation in king oyster mushrooms. The UVC light had also showed capability to stimulate vitamin D2 production in the mushroom. However, the results demonstrated that vitamin D2 content in king oyster mushroom irradiated by UVC light was less than vitamin D2 content in the mushroom irradiated by UVB light. The results showed that vitamin D2 irradiated by UVA light was undetectable in the mushroom, which indicated that UVA light was almost no effect to stimulate vitamin D2 production in the mushroom.

Our results demonstrated that UVB is an effective light to stimulate vitamin D2 production in the mushroom. UVC light showed less effective in stimulation production of vitamin D2. UVA light was almost no effect to stimulate vitamin D2 production in the mushroom.

8.3.2 Vitamin D2 contents in other mushrooms

In the present study, we investigated vitamin D2 contents in other mushrooms. The results demonstrated that the highest level content of vitamin D2 was found in XBG and SBG mushrooms. Middle content level of vitamin D2 was found in JZG, BG and XG; and the lowest level content of vitamin D2 was found in mushroom of LZG and PG.

The results of vitamin D2 contents among three types of XBG, including from mainland China, Hong Kong, South Korea, showed that similar contents in vitamin D2. However, the XBG from South Korea showed higher content of vitamin D2 than from China mainland and Hong Kong. The results demonstrated that the highest content of vitamin D2 was found in XBG, and lowest content of vitamin D2 was found in LZG. The investigation results found that XBG and SBG have showed higher contents in vitamin D2 after UVB irradiation. The lowest contents of vitamin D2 were found in LZG; and JZG showed middle contents in vitamin D2 compared with other mushrooms.

8.4 Effects of UV radiation on vitamin D2 and provitamin D2

8.4.1 Dynamic trend of provitamin D2 in the mushroom

The king oyster mushroom was irradiated by six different time period to evaluate content dynamic process between vitamin D2 and provitamin D2 based on our method. The results indicated that provitamin D2 showed a linear increasing trend ($y=-0.003x+2.09$, $r^2=0.942$) under UVB irradiation within 30 minute. The results

demonstrated that it is significantly change in content for provitamin D2 in the mushroom during UVB irradiation within 30 minute. The content of provitamin D2 maintain in a relative stable dynamic range after UVB irradiation of one hour.

The result demonstrated that content linear change occurred within first 30 minute for provitamin D2. The contents of provitamin D2 was relatively dynamic stable in a range with UVB irradiation between 1 hour and 2.5 hour. It was the first report to monitor contents dynamic process for both vitamin D2 and provitamin D2 in king oyster mushroom during UVB irradiation.

8.4.2 Dynamic trend of vitamin D2 in the mushroom

The results indicated that vitamin D2 contents showed a linear increasing trend ($y = 0.998x + 15.75$, $r^2=0.973$) exposed to UVB irradiation within 30 minute. The results demonstrated that it is significantly change in contents for vitamin D2 in the mushroom during UVB irradiation within 30 minute. The results revealed that vitamin D2 contents showed very slowly increase after UV irradiation of one hour. The contents of vitamin D2 tended to be a relatively steady trend between 1 hour and 2.5 hour. All the results showed that high efficiency accumulation of vitamin D2 in the mushroom occurred within 1 hour, and vitamin D2 tended to be relatively stable range.

8.4.3 The relationship between provitamin D2 and vitamin D2

In our study, we demonstrated a relationship between provitamin D2 and vitamin D2 in the mushroom during UVB irradiation. Our results indicated that thecontent relationship between vitamin D2 and provitamin D2 in the mushroom under UVB irradiation within 30min could be expressed as $y=7127x^2-29150x+29831$, (y=vitamin D2, x=provitamin D2). The relation equation of between vitamin D2 and provitmin D2

may provide a simple way to estimate vitamin D2 content exposed to UVB light within 30minute when the provitamin D2 content have been known for king oyster mushroom.

In this study, we were the first time to demonstrate that relationship between provitamin D2 and vitamin D2 in the mushroom during UVB irradiation, which can be expressed as parabolic equation. This equation can be used for calculation vitamin D2 and provitamin D2 content in the mushroom within 30 minutes.

Chapter 9 General conclusions and future work

9.1 Method for quantification of vitamin D4 in mushroom

In the present study, we developed a method to quantify vitamin D4 in mushrooms. The developed method for determination of vitamin D4 in mushroom showed that good linearity, precise, repeatability and reasonable recovery. The validation tests of the method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D4 in the mushroom. The developed method for quantification of vitamin D4 in mushroom will provide an approach to determine vitamin D4 in mushroom. Vitamin D4 in mushrooms is relatively new, it is quite significant to determinate vitamin D4 in mushrooms. However, there has not yet a HPLC-DAD method to quantify vitamin D4 in mushroom so far. Herein, we developed HPLC–DAD method to quantify vitamin D4 in mushroom. The developed method will provide an approach to quantification of vitamin D4 in the mushrooms.

9.2 Vitamin D4 in mushroom

In our study, the results indicated that vitamin D4 in mushroom during UVB irradiation showed a linear increasing trend within 30 minute. The dynamic trend of vitamin D4 in mushroom showed a similar trend with vitamin D2. Both of them showed a linear increasing trend within the first 30 minute.

The results showed that content of vitaminD2 was about 15-20 fold higher than content of vitamin D4 in the mushroom. Vitamin D4 and vitamin D2 showed a linear increase with time within 30 minute. The relationship can be constructed as linear relation between vitamin D4 and vitamin D2 in the mushroom during UVB irradiation.

The relationship between vitamin D4 and vitamin D2 in the mushroom can be expressed as: $y=10.91 x + 13.58$, $r^2=0.935$.

There was a good linear relationship between vitamin D4 and vitaminD2 in the mushroom during UVB irradiation, which indicated that vitamin D2 and vitamin D4 in the mushroom irradiated by UV light showed synchronic increase within 30 minute. Our study was first to demonstrate that there has a linear relationship between vitamin D2 and vitamin D4 in the mushroom during UVB irradiation within 30 minute.

9.3 Method for quantification of vitamin D2 and provitamin D2

In our study, we developed a HPLC method to determine vitamin D2 and provitamin D2 in the mushroom. The developed method showed good linearity within test range, good intra-and inter-precise and accuracy for both vitamin D2 and provitamin D2. The validation tests of the method showed that this HPLC-DAD method is precise, accurate for determination of vitamin D2 and provitamin D2 in the mushroom. The developed method has been successfully used for quantification of vitamin D2 and provitamin D2 in mushroom to simultaneously quantify vitamin D2 and provitamin D2 in the mushroom. The developed method for simultaneous quantification vitamin D2 and provitamin D2 will provide a new approach to quantify vitamin D2 and provitamin D2 in mushrooms.

9.4 Vitamin D2 and provitamin D2 in mushroom

In our study, the results indicated that vitamin D2 content showed a linear increasing trend ($y = 0.998x + 15.75$, $r^2=0.973$) exposed to UVB irradiation within 30 minute. Our results indicated that provitamin D2 showed a linear decreasing trend ($y=-0.003x+2.09$, $r^2=0.942$) under UVB irradiation within 30 minute. The results

demonstrated that it is significantly change in content for vitaminD2 and provitamin D2in the mushroom during UVB irradiation within 30 minute. The results revealed that vitamin D2 content showed very slowly increase after UV irradiation of one hour. The content of vitamin D2 tended to be a steady trend between 1 hour and 2.5 hour. The results indicated that high efficiency accumulation of vitamin D2 in the mushroom occurred within 1 hour.

Our study demonstrated a relationship between provitamin D2 and vitamin D2 in the mushroom during UVB irradiation. In the present study, we demonstrated the content relationship between vitamin D2 and provitamin D2in the mushroom under UVB irradiation within 30minute. The relationship can be expressed as a parabolic equation: $y=7127x^2-29150x+29831$, (y=vitamin D2, x=provitamin D2). Our study was the first time to show content dynamic process and relationship for both vitamin D2 and provitamin D2 in king oyster mushroom during UVB irradiation.

9.5 Vitamin D4, vitamin D2 and provitamin D2 in mushroom

In our study, we found a content relation between vitamin D4 and vitamin D2. The content of vitamin D2 was about 15-20 fold higher than content of vitamin D4 in the mushroom. They showed linear increasing trend within 30 minute for both vitamin D4 and vitamin D2 in the mushroom. Vitamin D2 and vitamin D4 showed a linear relationship in the mushroom during UVB irradiation. The relationship between vitamin D4 and vitamin D2 can be expressed as: $y=10.91\ x+13.58$, $r^2=0.935$. The relation equation between vitamin D4 and vitamin D2 demonstrated content relationship of vitamin D4 and vitamin D2 in the mushroom. The equation indicated that the synchronism in increasing trend between vitamin D4 and vitamin D2 in the mushroom during UV irradiation.

Provitamin D2 in the mushroom showed a linear deceasing trend within 30 minute. Vitamin D2 showed a linear increasing trend in the mushroom during within 30 minute. The relationship between vitamin D2 and provitamin D2 can be expressed a parabolic equation, which could be expressed as $y=7127x^2-29150x+29831$, (y=vitamin D2, x=provitamin D2). The parabolic equation between vitamin D2 and provitamin D2 demonstrated content relationship of provitamin D2 and vitamin D2 in the mushroom during UV irradiation.

In our study, it was the first time to demonstrate the relationship between vitamin D2 and vitamin D4 in mushroom, and the relationship between vitamin D2 and provitamin D2 in mushroom during UV irradiation. Our study will provide important content information of vitamin D2 and vitamin D4 in the mushroom, and pave an important way for further study on biological activities.

9.6 Future studies

Our study provides content information for vitamin D2, vitamin D4 and provitamin D2 in the mushroom. Also, we provide the content relationship between vitamin D2 and vitamin D4 in the mushroom, and relationship between vitamin D2 and provitamin D2 in the mushroom. Our study will provide important information for content of vitamin D2 and vitamin D4 in the mushroom, and provide important step for biological activities.

In next step, other mushrooms can be used for test for vitamin D2 and provitamin D2 in mushrooms, and vitamin D4 to compare the contents between different mushrooms.

In addition, it would be significant to conduct biological activities test. Vitamin D biological functions are closed related with bone mineralization and bone health. In the

future work, the mushroom irradiated by UV light can be used for test in vivo to further demonstrate its biological functions to evaluate the effects of mushrooms irradiated by UV light. The mushrooms with UV irradiation testes in vivo test will provide more systematic information for effects of the mushroom to increasing bone density.

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