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OPTIMISATION AND VALIDATION OF AN HPLC-DAD METHOD FOR ANALYSIS OF ERGOSTEROL AND ERGOCALCIFEROL IN EDIBLE MUSHROOMS EXPOSED TO UV-B RADIATION

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ABSTRACT

Vitamin D is essential for maintaining bone health, calcium and phosphorus metabolism, and proper immune system function. Its deficiency is a global concern, and one potential solution for enhancement of vitamin D intake by consumption of biofortified mushrooms. The aim of this study was to optimise the analytical determination of ergosterol and ergocalciferol (vitamin D_2) using the HPLC-DAD method, evaluate the method based on selected validation parameters, and apply it to the analysis of shiitake mushrooms (*Lentinula edodes*). The experimental material was subjected to the influence of a selected stress factor (UV-B radiation) to potentially transform the precursor ergosterol into ergocalciferol. Method validation established detection limits and limits of quantification for ergosterol and ergocalciferol - LODs were 2.69 µg.mL^{-1} and 1.84 µg.mL^{-1}, and LOQs were 8.06 µg.mL^{-1} and 5.53 µg.mL^{-1}, respectively. Fruiting bodies of shiitake mushrooms were exposed to UV-B irradiation for different durations (0–60 min). The pre-treatment of the experimental material included drying, saponification, and subsequent liquid-liquid extraction of the analytes, followed by low-pressure evaporation and reconstruction. Using high-performance liquid chromatography with diode-array detection (HPLC-DAD) analysis, the concentrations of ergosterol and ergocalciferol were determined. The results indicate a positive correlation between UV-B exposure duration and the formation of vitamin D_2. Longer exposure to UV-B radiation led to a higher ergocalciferol concentration, confirming the effectiveness of this method for mushroom biofortification. The findings highlight the importance of mushrooms as a natural source of vitamin D and confirm that UV-B exposure can enhance their nutritional value, making them a promising dietary component for addressing vitamin D deficiency.

Keywords: shiitake mushrooms, ergosterol, ergocalciferol, HPLC-DAD analysis, method validation, conversion

INTRODUCTION

Mushrooms represent a unique biological entity, occupying a position between plants and animals. They contain a wide range of bioactive compounds, including polysaccharides, beta-glucans, and phenolic compounds, which contribute to their potential health benefits, such as antioxidant properties and immune support (**Bains et al., 2021; Yue et al., 2021; Ma et al., 2013**). Additionally, they serve as an excellent source of proteins, with an amino acid profile comparable to that of animal proteins (**Fogarasi et al., 2020; Nagy et al., 2017**). Mushrooms are particularly rich in glutamine, leucine, valine, glutamic acid, and aspartic acid (**Reis et al., 2012**). Besides these compounds, mushrooms are an abundant source of essential vitamins, particularly B vitamins (riboflavin, niacin, and pantothenic acid), as well as minerals such as selenium, potassium, and magnesium (**Rózsa et al., 2019; Yadav & Negi, 2021**). This unique combination of nutrients makes mushrooms a valuable component of a healthy, balanced diet (**Strong et al., 2022; Martinez-Medina et al., 2021**). It is important to note that some mushrooms contain compounds that can be harmful in excessive amounts, such as toxic elements or naturally occurring toxins (**Qin et al., 2024; Ronda et al., 2022; White et al., 2019**). Mushrooms also contain sterols, particularly ergosterol, which is a key component of fungal cell membranes (**Nzekoue et al., 2022**). When exposed to ultraviolet (UV-B) radiation, ergosterol can be converted into biologically active forms of vitamin D, primarily vitamin D_2 (ergocalciferol) (Fig. 1) (**Rodrigues, 2018**), enhancing the overall nutritional profile of mushrooms. This process makes mushrooms one of the few non-animal sources of vitamin D. Research suggests that mushrooms exposed to UV radiation can provide a significant amount of vitamin D, which is particularly beneficial for individuals with lactose intolerance, dairy allergies, or those following vegetarian or vegan diets (**Nölle et al., 2017**). The biofortification of mushrooms with vitamin D can be influenced by several factors, including the mushroom species, duration and intensity of UV exposure, and specific environmental conditions in which they are cultivated. The importance of vitamin D for human health cannot be overlooked, as it plays a crucial role in regulating calcium and phosphorus levels necessary for maintaining bone health (**Capozzi et al., 2020**). Sufficient vitamin D levels also support immune function, muscle performance, mood regulation, and may reduce the risk of chronic diseases (**Charoenngam et al., 2019**). Often referred to as the "sunshine vitamin," vitamin D is primarily synthesised in the skin upon exposure to sunlight. However, some individuals may struggle to maintain adequate vitamin D levels due to factors such as geographical location, seasonal variations, and lifestyle choices (**Cashman, 2020; Holick, 1995**). Vitamin D deficiency represents a significant global health issue, associated with a higher incidence of conditions such as osteoporosis, osteomalacia, rickets, autoimmune disorders, and increased vulnerability to infections (**Dissanayake et al., 2022**). In response to rising public health challenges, researchers are actively investigating alternative vitamin D sources. Enhancing the vitamin D synthesis process in mushrooms could maximise their potential as a sustainable and affordable supply of this vital nutrient, offering a promising strategy for promoting public health and overall well-being.

Vitamin D_2 is formed by the process of photoconversion of ergosterol, which converts it to vitamin D_2. This process involves a series of photochemical and thermal reactions, similar to those that occur in human skin when exposed to sunlight (**Mau et al., 1998**). The actual conversion of ergosterol to ergocalciferol occurs in two stages. The first step is photolysis, in which UV radiation disrupts the bonds between the carbons in the B-ring of ergosterol, resulting in the formation of previtamin D_2. This is followed by thermal isomerization, in which its structure changes to the biologically active vitamin D_2 (**Papoutsis et al., 2020**).

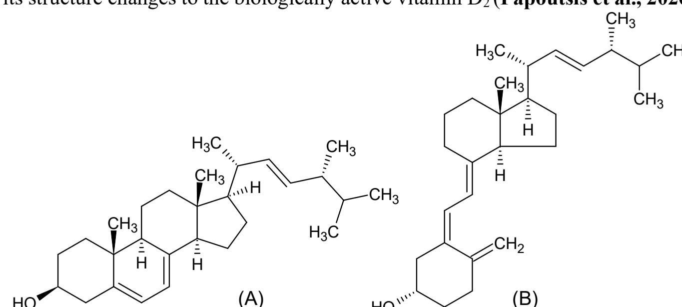

Figure 1 Structure of ergosterol (A) and ergocalciferol (B)

The development of reliable analytical methods for vitamin D detection and quantification is essential due to its important role in human health. Several techniques, including HPLC-UV, HPLC-PDA, GC, LC-MS, GC-MS, HPLC-GC-MS and LC-MS/MS, have been employed for this purpose, each with specific advantages and limitations, such as high instrument costs and sensitivity **(Amithabh et al., 2024; Stokes et al., 2018; Yin, 2018; Nestola & Thellmann, 2015; Momenbeik et al., 2010)**. LC-MS/MS is a particularly powerful technique, offering high sensitivity and selectivity by combining liquid chromatography with mass spectrometry, enabling precise detection of vitamin D compounds even at trace levels. However, its high cost and the need for specialized expertise restrict its widespread use. In contrast, common HPLC-UV or HPLC-DAD is commonly utilized due to its efficiency, selectivity, and affordability, though they lack the sensitivity required for trace analysis.

In this study, we focused on optimising the analytical determination of ergocalciferol and its precursor ergosterol using the HPLC-DAD method, as well as applying of UV-B radiation to evaluate changes of ergocalciferol content. We used fruiting bodies of shiitake mushrooms (*Lentinula edodes*), which were exposed to UV-B irradiation to investigate the effect of exposure on the conversion of ergosterol to ergocalciferol.

MATERIAL AND METHODS

Reagents and Standard Solutions

Ergosterol and ergocalciferol were obtained from Sigma-Aldrich (Darmstadt, Germany), as well as L-ascorbic acid. Centralchem (Bratislava, Slovakia) supplied potassium hydroxide (p.a.). Methanol (p.a.) was sourced from Honeywell Riedel-de Haën GmbH (Niedersachsen, Germany), while hexane was obtained from Supelco Inc. (Pennsylvania, United States). For the HPLC analysis, HPLC-purity solvents methanol (≥99.9%) and acetonitrile (≥99.9%) (Sigma-Aldrich, Darmstadt, Germany) were used as the mobile phases. To prepare the standard solutions, 1.0 mg of both ergosterol and ergocalciferol standards were weighed and dissolved in 1 ml of methanol. The final standard mixtures were prepared by pipetting 0.4 - 100 µL of ergocalciferol standard and 1.6 - 400 µL of ergosterol standard into 2 mL HPLC vials and diluting with the methanol (HPLC quality) to reach 1 mL of standard mixtures for individual concentration levels. These solutions were used for measurements for the calibration curve in HPLC analysis and for determination of selected validation parameters.

Instrumentation

The analytical method for determination of ergosterol and ergocalciferol was optimized and evaluated for the instrument Agilent 1260 Infinity II HPLC (Agilent Technologies, Santa Clara, CA, United States) with a diode-array detection. The separation was performed in isocratic mode with the composition of mobile phase methanol:acetonitrile 5:95 % (v/v) over 10 min. The analysis was performed on a Kinetex C18 column (100 mm × 4.6 mm × 2.6 µm, Phenomenex, California, USA). The flow rate was maintained at 0.8 $ml.min^{-1}$, and the injection volume for both the standard mixture and the sample was 5 µl. Each standard mixture and sample was injected three times to ensure accuracy. The signal was recorded at wavelengths of 265 nm and 285 nm. For the identification and quantification of both analytes, retention time and UV spectrum were used. The concentration of ergosterol and ergocalciferol was expressed in $mg.kg^{-1}$ of dry weight (DW).

Mushroom samples preparation and irradiation by UV-B

To increase the vitamin D_2 content, we used fruiting bodies of the edible shiitake mushrooms (*Lentinula edodes*), which we obtained from the mushroom urban farm Drž hubu (Bratislava, Slovakia). Before starting the experiment, we determined the water content in the fruiting bodies using a KERN DLB 160-3A moisture analyzer (Kern & Sohn GmbH, Balingen, Germany). In the experiment, we exposed the mushrooms to various periods of UV-B radiation. The source of UV-B radiation was a Compact-Fluorescent UVB 10.0 lamp (26W, 230V, Repti Planet, Plaček Pet Product company, Poděbrady, The Czech Republic), providing irradiation in the UV-B and visible spectrum.

Irradiation was carried out on mushrooms growing on the growing substrate at selected time intervals (0-60 min). The characteristics of the experimental variants are summarized in Table 1. After the specified time interval (0 – control, 15, 30 and 60 minutes), approximately 100 g of sample (whole fruiting bodies) was taken from each variant and dried in a laboratory drying oven with forced air circulation Memmert UN 160 (Memmert GmbH + Co. KG, Schwabach, Germany). Sample preparation for analysis was completed by homogenization using a laboratory homogenizer IKA A 10 basic (IKA-Werke GmbH & Co KG, Staufen, Germany).

Data processing

Statistical analysis was conducted for data evaluation, with a rapid method validation performed using Excel (Microsoft, Redmond, WA, USA). An in-house validation of the method for determining ergosterol and ergocalciferol was carried out, focusing selected validation parameters such as intermediate precision, linearity, and the limits of the method by determining of the limit of detection (LOD) and limit of quantification (LOQ). Intermediate precision was assessed by evaluating intraday and interday precision based on two parameters: detector response (as a peak area) and retention time of both analytes. Linearity was determined through regression analysis using repeated measurements of standard mixture within concentration ranges of 1.6–240 $µg.mL^{-1}$ for ergosterol and 0.4–100 $µg.mL^{-1}$ for ergocalciferol, with coefficients of determination (R^2) calculated. LOD and LOQ values were derived from the calibration curve using the upper limit approach **(Hegedűs et al., 2010)**. The results of ergocalciferol determinations in shiitake mushrooms were statistically analysed using Past 4.03 software. The effect of the irradiation method for biofortification was evaluated, and significant differences between samples exposed to different UV-B irradiation durations were tested using the Kruskal-Wallis test followed by Dunn's post hoc test.

RESULTS AND DISCUSSION

Determination of selected validation parameters of the HPLC-DAD method

The HPLC-DAD method for determining ergosterol and ergocalciferol was optimized and validated to establish its reliability. The accuracy and consistency of the method were verified, and key validation parameters were calculated to assess the performance of the HPLC method for analysing ergosterol and ergocalciferol in mushroom samples after appropriate sample preparation. A standard mixture of ergosterol and ergocalciferol was used to evaluate repeatability and intermediate precision, expressed as RSD (%) values from repeated measurements conducted within a single day and over three consecutive days. The results of the selected validation parameters are presented in the Table 1.

Table 1 Selected validation parameters for the ergosterol and ergocalciferol in standard mixture

Compound	Intraday precision					Interday precision			
	Concentration	Retention time		Peak area		Retention time		Peak area	
	$µg.mL^{-1}$	min	RSD %	Response	RSD %	min	RSD %	Response	RSD %
Ergosterol	80.00	5.60	0.09	563.4	0.08	5.60	0.11	563.8	0.15
Ergocalciferol	20.00	4.67	0.08	326.6	0.13	4.66	0.10	326.9	0.18

Linearity	Concentration range	Linear regression equation	Coeficient of determination
Ergosterol	1.6 - 240 $µg.ml^{-1}$	$y = 6.7514x + 5.5164$	$R^2 = 0.9998$
Ergocalciferol	0.4-100 $µg.ml^{-1}$	$y = 17.753x - 9.7828$	$R^2 = 0.9993$

Limits of the method	Unit	Limit of detection (LOD)	Limit of quantification (LOQ)
Ergosterol	$µg.mL^{-1}$	2.69	8.06
Ergocalciferol	$µg.mL^{-1}$	1.84	5.53

Note: The concentrations of ergosterol and ergocalciferol in the standard mixture are presented as mean values. For intraday precision, the values were calculated from five repeated measurements within a single day, while for interday precision, they were derived from ten repeated measurements over three consecutive days.

The table presents validation parameters for the determination of ergosterol and ergocalciferol using HPLC. Intraday and interday precision were assessed by evaluating the retention time and peak area of both compounds. The relative standard deviation (RSD%) values for retention time and peak area indicate high precision, as the variability of retention time did not exceeded 0.2% for both evaluated analytes in interday and intraday precision, as well. Evaluation of intraday precision of peak area showed RSD % up to 0.1% and for interday variability was up to 0.2%. We observed a slight delay of retention times of both analytes in the samples compared to standard solutions. Within one day a mean delay of retention time for samples was 0.03 min. for ergocalciferol and 0.02 min. for ergosterol. The slight shift was probably due to matrix effect of the mushroom extracts.

Linearity was confirmed over a broad concentration range, with demonstrated excellent correlation coefficients ($R^2 > 0.999$). Calibration dependence was used for calculation of limit of detection (LOD) and limit of quantification (LOQ) based on the upper limit approach **(Hegedűs et al., 2010)**. Sensitivity of the method was determined by calculating the limit of detection (LOD) and limit of quantification (LOQ), where ergosterol had an LOD of 2.69 $\mu g.mL^{-1}$ and LOQ of 8.06 $\mu g.mL^{-1}$, while ergocalciferol showed an LOD of 1.84 $\mu g.mL^{-1}$ and LOQ of 5.53 $\mu g.mL^{-1}$. These results confirm that the method provides reliable quantification of both compounds with high precision, accuracy, and sensitivity.

High-performance liquid chromatography is a widely used method for the determination of vitamin D_2 due to its cost-effectiveness compared to liquid chromatography-mass spectrometry. While LC-MS offers superior sensitivity and selectivity, it requires expensive instrumentation, specialized maintenance, and highly trained personnel, making it less accessible for routine analysis in many laboratories. The obtained limits of quantification (LOQ) for ergosterol (8.06 $\mu g.mL^{-1}$) and ergocalciferol (5.53 $\mu g.mL^{-1}$) in this study are higher compared to those reported by **Magalhães et al., (2007)**, who achieved significantly lower LOQs of 0.195 $\mu g.mL^{-1}$ for ergosterol and 0.113 $\mu g.mL^{-1}$ for ergocalciferol using the LC-MS technique. This highlights the superior sensitivity of LC-MS for vitamin D analysis. However, when compared to the HPLC method used by **Momenbeik et al., (2010)**, which reported an LOD of 2.18 $\mu g.mL^{-1}$ for vitamin D, our results for ergosterol and ergocalciferol are comparable, demonstrating the effectiveness of the HPLC method in routine analysis despite its lower sensitivity than LC-MS.

Determination of ergosterol and ergocalciferol in Shiitake mushrooms

Vitamin D_2 is rapidly synthesized in mushrooms when exposed to UV light, leading to increased research on enhancing vitamin D levels in commercially grown mushrooms (**Salemi et al., 2021; Singh & Joshi, 2023**). Some wild and cultivated mushroom species naturally contain varying amounts of vitamin D_2, influenced by ambient UV exposure (**Mattila et al., 2002**). The concentration of vitamin D_2 can vary significantly, with reported values ranging from 0.03 to 63.2 $\mu g.100g^{-1}$ (fresh weight) in retail mushrooms sampled in the U.S. Several factors impact vitamin D_2 production, including exposure duration, UV intensity, wavelength, and the positioning and surface area of the gills. These variables play a crucial role in determining the effectiveness of UV treatment in boosting vitamin D_2 content in mushrooms.

The method for separation and determination of ergosterol and ergocalciferol was applied on real shiitake mushrooms, exposed to the UV-B irradiation in different duration intervals (0-60 min). The Table 2 presents summary statistics of ergosterol and ergocalciferol content in shiitake mushrooms exposed to the UV-B irradiation and in the Figure 2 is shown the change of ergocalciferol content regarding to the irradiation duration. In the ergosterol content, the overall mean across all samples was 3703.7 $mg.kg^{-1}$ DW, while the overall median was 3851.1 $mg.kg^{-1}$ DW. The overall mean across all samples was 2.42 $mg.kg^{-1}$ DW, while the overall median was 2.60 $mg.kg^{-1}$ DW. The highest standard deviation was observed across all samples combined (0.38 $mg.kg^{-1}$ DW).

Table 2 Descriptive summary statistics for determination of ergosterol and ergocalciferol

Parameter	Ergosterol $mg.kg^{-1}$ DW	Ergocalciferol $mg.kg^{-1}$ DW
N	15	15
Min	3063.5	1.90
Max	4127.8	2.90
Mean	3703.7	2.42
Median	3851.1	2.60
SD	405.4	0.38

Note: N – no. of measurements; Min – minimum value; Max– maximum value; Mean – mean value; Median– median value; SD – standard deviation.

The effect of UV-B radiation on ergocalciferol content in shiitake mushrooms was evaluated, as shown in Table 3 and Figure 2. The control sample (SHI_K), which was not exposed to UV-B radiation, exhibited the lowest ergocalciferol content (1.87 $mg.kg^{-1}$ DW). With increasing exposure time, a gradual rise in ergocalciferol concentration was observed, reaching 2.13 $mg.kg^{-1}$ DW after 5 minutes (SHI_5), 2.63 $mg.kg^{-1}$ DW after 15 minutes (SHI_15), and peaking at 2.85 $mg.kg^{-1}$ DW after 30 minutes of irradiation (SHI_30). However, after 60 minutes of exposure (SHI_60), a slight decrease in ergocalciferol content (2.60 $mg.kg^{-1}$ DW) was detected. These results indicate that UV-B irradiation effectively enhances ergocalciferol synthesis in shiitake mushrooms, with an optimal exposure duration of 30 minutes. The observed decline in ergocalciferol content at 60 minutes, highlighting the importance of optimizing UV-B treatment duration for maximal vitamin D_2 enrichment.

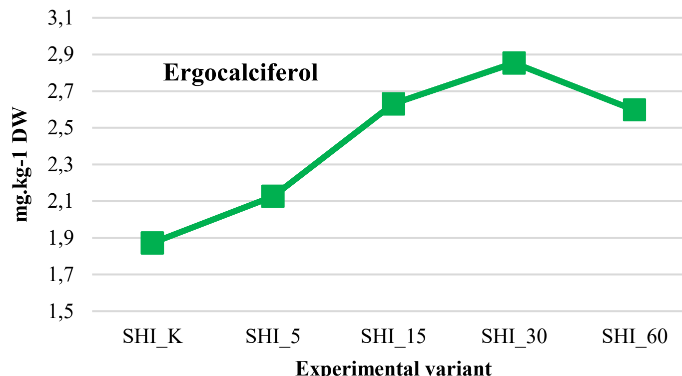

Figure 2 Dependence of ergocalciferol concentration in relation with duration of UV-B irradiation
Note: DW – dry weight; SHI_K – control variant with no irradiation by UV-B light; SHI_5 – variant irradiated for 5 minutes by UV-B; SHI_15 – variant irradiated for 15 minutes by UV-B; SHI_30 – variant irradiated for 30 minutes by UV-B; SHI_60 – variant irradiated for 60 minutes by UV-B

The most significant change in ergocalciferol content was observed in the SHI_30 sample, where it increased by 52%. Changes were also noted in SHI_5 and SHI_15, with a 13% increase after 5 minutes of irradiation and a 40% increase after 15 minutes. In the SHI_60 sample, which was exposed to UV-B radiation for 60 minutes, ergocalciferol content increased by 38% compared to the control, which is 14% less than in SHI_30, suggesting that excessive exposure time may have led to degradation (**Li & Min, 1998**). The differences in final ergocalciferol content may also depend on initial variations in the mushrooms **(Judprasong et al., 2023; Hu et al., 2020**). Statistical tests were used for evaluation of the dataset. The Shapiro-Wilk test was used to assess whether a dataset follows a normal distribution. It calculates a test statistic (W) and a corresponding p-value to determine if the data significantly deviate from normality. In case of ergocalciferol, the dataset does not follow a normal distribution (Shapiro-Wilk $W = 0.8643$; $p(\text{normal}) = 0.02785$, $p < 0.05$), thus non-parametric ANOVA statistical tests were used. The Kruskal-Wallis test was used followed by post-hoc Dunn's test to determine which specific groups of experimental variants differ from each other. The results are summarized in the Table 3.

Table 3 Evaluation of experimental variants by non-parametric ANOVA tests (p-values)

Variants	Ergocalciferol Dunn´s post hoc test SHI_5	SHI_15	SHI_30	SHI_60
SHI_K	0.4058	0.03364	0.0008837	0.04215
SHI_5		0.196	0.01264	0.2298
SHI_15			0.2298	0.9264
SHI_30				0.196

Note: $p<0.05$ There is a significant difference between sample medians in ergocalciferol content

In case of ergosterol content in experimental variants, the Kruskal-Wallis test confirmed a significant difference between some group medians. Dunn's post hoc test identified specific significant differences, particularly involving SHI_K and SHI_30, which differ from multiple groups (green colour p values are highlighted in the Table 3).

CONCLUSION

This study successfully optimized and validated an HPLC-DAD method for the quantification of ergosterol and ergocalciferol in shiitake mushrooms, demonstrating high precision, accuracy, and sensitivity. The method exhibited excellent linearity ($R^2 > 0.999$) across a broad concentration range, with low LOD and LOQ values, confirming its suitability for routine analysis.

The effect of UV-B irradiation on ergocalciferol synthesis in shiitake mushrooms was observed, with the most significant increase occurring after 30 minutes of exposure, where ergocalciferol content increased by 52%. Shorter irradiation times (5 and 15 minutes) led to moderate increases (13% and 40%, respectively), while prolonged exposure (60 minutes) resulted in a decrease. These findings indicate that UV-B treatment is a promising method for enhancing vitamin D_2 content in mushrooms, with an optimal exposure time of 30 minutes. Overall, this research highlights the potential of controlled UV-B irradiation for vitamin D_2 enrichment in shiitake mushrooms while emphasizing the importance of optimizing exposure duration to prevent degradation. Future studies should focus on refining UV treatment conditions to maximize ergocalciferol yield while preserving the nutritional integrity of mushrooms.

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