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Simple HPLC-DAD-based method for determination of ergosterol content in lichens and mushrooms

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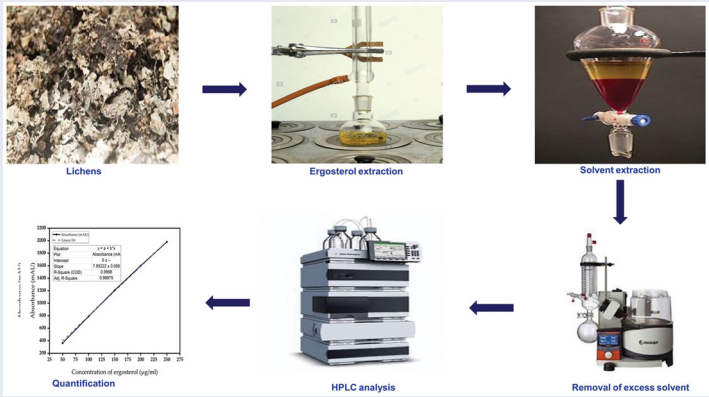
ABSTRACT

A novel yet simple high-performance liquid chromatography (HPLC) method with diode array detector has been developed and validated for the quantification of ergosterol (Pro-vitamin D_2) in commercially available lichens and cultivated edible mushrooms as per International Conference on Harmonization (ICH) guidelines. The extraction of ergosterol in the lichens and mushrooms was carried out with alcoholic KOH via reflux, and the extracts were processed for HPLC analysis. The chromatographic identification of ergosterol from the extracts was achieved using C_{18} column (5 μm, 4.6 × 250 mm) thermostatted at 30 °C using an isocratic elution of 100% methanol at a flow rate of 1.5 mL/min. The elution was monitored at 280 nm using a diode array detector. In this method, ergosterol was separated from the extract within a short interval of time (7.0 (±0.1) min). The developed method exhibited good linearity (r^2>0.9998), limit of detection (0.03 μg/mL), limit of quantification (0.095 μg/mL), interday and intraday precision and accuracy. A total of five lichen samples and three mushroom samples were analyzed using this method to quantify the amount of ergosterol present in them. This work, thus, highlights the possible use of lichens as an excellent source of ergosterol.

GRAPHICAL ABSTRACT



KEYWORDS

Ergosterol; Pro-Vitamin D_2; Lichens; HPLC; Method validation

Introduction

Vitamin D is a fat-soluble vitamin that majorly helps in absorption of calcium by the body for enriching the bones and blocks the release of parathyroid hormone, to make them strong and healthy.[1,2] Vitamin D also assists in treating multiple ailments like diabetes, heart diseases, high blood pressure, neurodegenerative diseases, preventing cancer, and improving the muscle function and immune system.[3] The deficiency of Vitamin D results in several medical issues like muscle weakness, depression, hypertension, cardiovascular problems and the softening of bones in children and adults.[4,5] Vitamin D can be obtained naturally from sunlight, and food sources like oil-rich fish, fish liver oils, egg yolk and red meat.[6] An exposure of at least 30 min to the sun is necessary, in order for the human body to produce, the required amount of vitamin D.[5] However, in the current times, our reliance on sun for vitamin D has reduced largely, owing to the risk of skin cancers and increase in the use of sunscreens.

Vitamin D deficiency is observed primarily in people having vegetarian diet only.[7] There are two main forms of vitamin D, namely vitamin D_2 and vitamin D_3.[8] Vitamin D_2 can be obtained by irradiating UV light on a plant sterol

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called ergosterol. However, vitamin D_3 can be synthesized inside the human body, by the transformation of cholesterol present under the skin, on exposure to sunlight.[9,10]. Studies have reported that both these forms are equally effective in regulating serum 25-hydroxyvitamin D levels in humans.[11] This vitamin D_2 serves as the dietary source of vitamin D for vegetarian population. Therefore, it is important to know the sources of ergosterol which can be further converted into vitamin D_2.

Lichens are a group of organisms, where fungus and algae grow together in a close symbiotic association. These have been traditionally used for various medical purposes like pulmonary diseases, eczema, respiratory diseases and arthritis. More than 800 secondary metabolites have been reported to be produced by lichens like depsidones, xanthones, depsides, usnic acid, cyclic peptides, steroids, diterpenes, triterpenes, etc.[12,13] Because of the sensitivity of the lichens toward certain pollutants, these are also used as bioindicators of the environment.[14] Since lichens are edible, identifying and quantifying of ergosterol in them and converting the ergosterol content present in them into vitamin D_2 makes it a great source of vitamin D for those who prefer a vegetarian diet. It is also found that ergosterol is one of the prominent components of lichens that can be used as a marker for identifying the lichens.[15]

Mushrooms have been classified under the plant based diet and are one of the major sources of ergosterol. Significant work has been carried out in identifying the quantity of ergosterol present in mushrooms.[7,16–19] Most of the methods used for identification of ergosterol are UV spectrometry and HPLC methods. However, the reported HPLC methods are associated with high retention times in identifying the ergosterol.[20–24] HPLC has been employed for various qualitative and quantitative applications in natural products, pharmaceutical formulations, pharmacokinetic studies, water analysis, and food analysis.[25–30] In the present study, we set out to develop a simple, robust and accurate high-performance liquid chromatography (HPLC) method for the determination of ergosterol from commercially available lichens and cultivated edible mushrooms. This developed method was further applied for the quantification and comparison of ergosterol content in lichens and mushrooms.

Materials and methods

Sample

Lichen samples were obtained from commercial sources. All the lichen samples obtained from commercial sources were identified (*Evernia*, *Parmotrema* species1, *Parmotrema* species 2, *Parmelia* species 1, *Parmotrema tinctorum*) by Dr G.N. Hariharan, Advisory Member of Indian Lichenological Society Advisory Committee & Director Biotechnology Department, M.S. Swaminathan Research Foundation, Taramani, Chennai. Mushroom samples (*Hypsizygus ulmaris*, *Pleurotus florida*, and *Calocybe indica*) were obtained from Mushroom Laboratory at the Indian Institute of Horticultural Research (IIHR), Bangalore, India.

Chemicals and standard solutions

Ergosterol hydrate (96%) was purchased from Alfa Aesar. Ascorbic acid was purchased from Sigma Aldrich. Methanol (HPLC grade), n-hexane (HPLC grade) and potassium hydroxide pellets were purchased from Merck. The standard solutions of ergosterol were made using HPLC-grade methanol. Ascorbic acid solution was made using de-ionized water.

Instruments

The analysis of ergosterol in the extracts were carried out by the Agilent 1260 Infinity Quaternary LC VL with Photodiode Array Detector. We used a Rotary evaporator from Heidolph Instruments of the type: Heizbad Hei-VAP for drying the excess solvent in the reaction mixture. The sonication was carried out in an Ultrasonic water bath from BANDELIN SONOREX of Type: RK 103 HPLC. The complete dryness of the sample was carried out in the Sample Concentrator: Stuart Sample Concentrator, Model No: SBH13 OD13.

Method for extraction of ergosterol

The extraction of ergosterol was carried out using a modified method reported earlier by Raina et al.[7] and Barreira et al.[22] Briefly, the extraction of ergosterol was performed using alcoholic potassium hydroxide. The powdered sample (0.5 g) was saponified with 12.5 mL of 25% alcoholic KOH (methanolic KOH) and 0.5 mL of 0.1 M ascorbic acid by refluxing in a water bath for 3 h. During the process of reflux, 5 mL of methanol was added into the reaction mixture along the wall of condenser to wash down for every one hour of the reaction. After the saponification, the reaction mixture was allowed to cool down to room temperature and transferred into a separatory funnel and then 10 mL of methanol was added over the residue to recover the residual ergosterol. The reaction mixture in the separatory funnel was extracted with 20 mL n-hexane for three times and the organic layers were pooled together. These pooled organic layers were evaporated using rotary evaporator and nitrogen purging. The obtained residue was then re-dissolved in 2.5 mL of HPLC grade methanol and filtered through a 0.2-µm PTFE syringe filter for HPLC analysis. All the extractions were done in triplicate.

HPLC analysis

The extracts were analyzed using Agilent 1260 Infinity Quaternary LC coupled with a photodiode array detector HPLC system. The separation of ergosterol in the extracts was achieved on a C_{18} column (Agilent Zorbax Eclipse plus C_{18}, 5 µm, 4.6 × 250 mm) thermostatted at 30 °C. The mobile phase used was an isocratic elution of 100% methanol with a flow rate of 1.5 mL/min. The volume of the sample injected was 10 µL. The identification of ergosterol was done on the basis of retention time of standard ergosterol. The

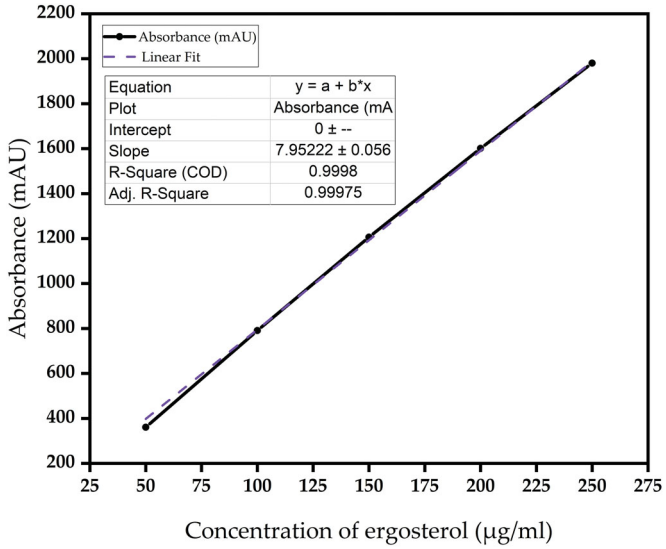

Figure 1. Calibration plot of ergosterol with concentrations ranging 50–250 µg/mL.

confirmation was carried out by matching the UV spectrum and its quantification was done against standard curve obtained by injecting different concentrations of the ergosterol standard.

Method validation

Parameters like linearity, precision, and accuracy of the method were verified for validating the developed novel method.[31]

A linear relationship

A linear relationship for the method is obtained by the calibration plot. The calibration plot is attained by plotting peak areas of standard ergosterol against the concentrations of standard ergosterol.

Precision

Intra-day and inter-day analysis were carried for the samples to validate the precision of the developed method. The intra-day analysis was carried out by analyzing the sample for three times within the same day. The inter-day analysis was carried by analyzing the same sample for three consecutive days.

Accuracy

The accuracy of the method is verified by spiking a known concentration of standard solution to the sample and obtaining the recovery percentage.

Limit of detection (LOD)

Limit of detection is the lowest amount of analyte in any sample which can be detected, but not necessarily quantified as a precise value by an analytical instrument.

Table 1. A summary of the linearity, LOD, LOQ, recovery percentage.

Validation parameters	
Linearity range (µg/mL)	50–250
Regression equation	$y = 7.95222x$
Correlation coefficient (r^2)	0.9998
Limit of detection (µg/mL)	0.03 µg/mL
Limit of quantification (µg/mL)	0.095 µg/mL
Mean recovery	
Parmotrema sp2 + 50 µg/mL ergosterol standard	93.46%
Parmotrema sp2 + 150 µg/mL ergosterol standard	90.20%
Parmotrema sp2 + 250 µg/mL ergosterol standard	91.40%

Limit of quantification (LOQ)

Limit of quantification is the lowest amount of analyte in any sample which can be determined with a definite precision and accuracy.

Both LOD and LOQ were determined by injecting the known concentrations of series of dilutions of ergosterol under the same chromatographic conditions of the method.

Results and discussion

Validation of developed method

The calibration plot was obtained by using standard solutions of ergosterol and plotting the peak areas against the concentration of the standard solutions. The range of standard ergosterol concentrations used for obtaining the calibration plot is 50–250 µg/mL. The correlation coefficient obtained ($r^2 > 0.9998$) indicated good linearity as plotted in the following Figure 1.

LOD and LOQ were determined by taking the signal to noise ratio as 3:1 and 10:1 respectively. The LOD of the ergosterol is 0.03 µg/mL and its LOQ is 0.095 µg/mL. The accuracy of the method was obtained by spiking the known concentrations of standard ergosterol to the *Parmotrema* sp2 sample prior to extraction and calculating the percentage recovered. The known concentrations of ergosterol standards used for spiking are 50 µg/mL, 150 µg/mL and 250 µg/mL. The results of analytical parameters like linearity, LOD, LOQ, and mean recovery are summarized in the Table 1. The precision of the developed method was verified by intra-day analysis and inter-day analysis. The inter-day and intra-day analysis were carried out for *Parmotrema* sp2 sample and its results are reported in Table 2.

Ergosterol content present in lichen

Ergosterol in powdered lichen and mushroom samples was identified and quantified using reverse-phased HPLC. The extraction of ergosterol from the sample was performed in triplicate and the extracts were injected into octadecyl (C_{18}) column and analyzed in reversed phase system with 100% methanol as mobile phase. Ergosterol in the sample was identified by comparing the retention time and UV absorption spectrum of the standard ergosterol. The wavelength for the detection of ergosterol in the sample was set at 280 nm. The retention time of the standard ergosterol was observed to be 7.0 (±0.1) min. The typical chromatograms of ergosterol in the extracts (lichen, mushroom samples and standard) and standard ergosterol (50 − 250 µg/mL) are

Table 2. A summary of inter-day and intra-day analysis.

Intraday analysis	Run 1	Run 2	Run 3	Mean	Stdev	R.S.D (%)
Peak area (mAU)	3128.2453	3151.93	3177.39	3152.522	±24.5782	0.78
Retention time (min)	7.062	7.061	7.058	7.060333	±0.0017	0.024074
Interday analysis	Day 1	Day 2	Day 3			
Peak area (mAU)	3199.6333	3241.71802	3231.8	3224.384	22.00071	0.682323
Retention time (min)	7.078	7.246	7.103	7.142333	0.090644	1.269109

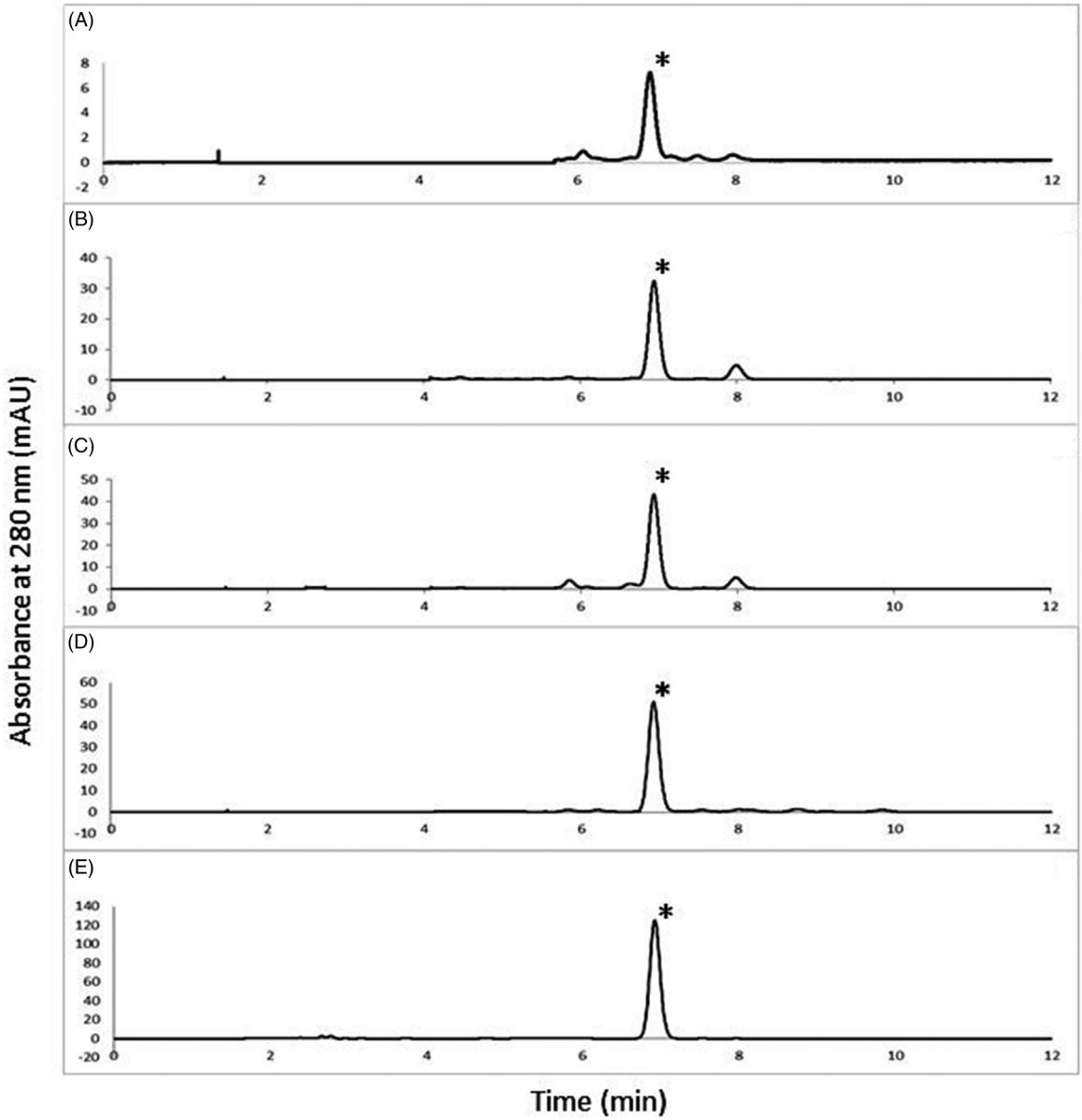

Figure 2. HPLC chromatograms of ergosterol in mushroom and lichen extracts: (A) *Calocybe indica*, (B) *Pleurotus florida*, (C) *Hypsizygus ulmaris* and (D) *parmotrema* sp2 extract, (E) ergosterol standard. *Indicates the ergosterol peak in the chromatogram.

captured in the Figures 2 and 3, respectively. The amount of ergosterol present in one-gram dry weight of lichen and mushroom samples was calculated using the calibration plot. The quantities of ergosterol present in the powdered lichen and mushroom samples are summarized in Table 3.

The average of the triplicate data was used to estimate the quantity of ergosterol present in one-gram dry weight of powdered lichen and mushroom samples. Many researchers have isolated and estimated the quantity of ergosterol in the mushrooms. As per literature, the ergosterol content per gram dry weight of mushroom present in several mushrooms are as follows: *Volvariella volvacea* (159 µg/g),[7] *Cantharellus cibarius* (230 µg/g),[32] *Agaricus bisporus* (273.97 µg/g), *Lentinus edodes* (297.09 µg/g).[18] The mushrooms (*H. ulmaris*, *P. florida*, and *C. indica*) used in our study were found to contain 157.79 µg/g, 189.99 µg/g, and 34.98 µg/g of ergosterol, respectively.

But in the present study, we focus primarily on the estimation of ergosterol in commercially available lichens and compared them with few mushroom samples. The results

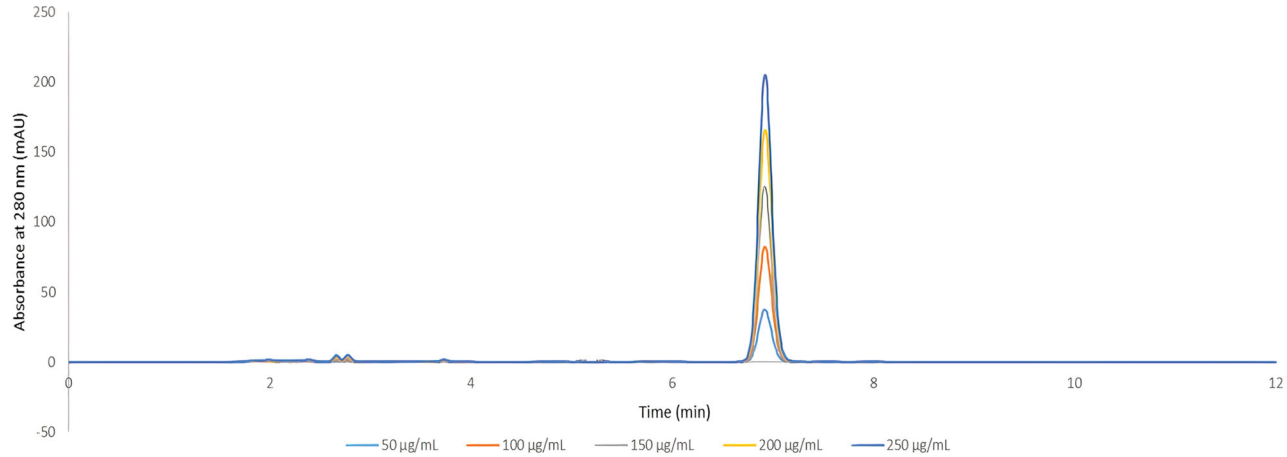

Figure 3. Overlayed chromatogram of standard ergosterol concentrations 50 µg/mL, 100 µg/mL, 150 µg/mL, 200 µg/mL, and 250 µg/mL.

Table 3. Quantity of ergosterol present per gram dry matter in lichen and mushroom samples.

Sample name	Ergosterol (µg/g DW)	Stdev (±µg)	R.S.D (±%)
Hypsizygus ulmaris	157.79	9.54	6.05
Pleurotus florida	189.99	6.65	2.97
Calocybe indica	34.98	3.11	8.91
Evernia	84.90	3.37	3.97
Parmotrema sp1	262.76	11.72	4.46
Parmotrema sp2	309.4	6.25	2.02
Parmelia sp1	435.20	42.08	9.67
Parmotrema tinctorum	499.22	31.24	6.26

DW: Dry weight; Stdev: standard deviation; R.S.D: relative standard deviation.

demonstrated that lichens contain more amount of ergosterol as compared to the mushrooms. Therefore, lichens can be promoted in the daily consumption and formulations as a source of vitamin D_2.

Further, the novel method developed proves to be advantageous in multiple ways. Firstly, it allows the separation of ergosterol from the extract in a short time interval of 7 min using Agilent Zorbax Eclipse Plus C_{18} column (5 µm, 4.6 × 250 mm). Thus, this method can be employed to analyze ergosterol content in other samples in a faster and more efficient manner. The retention time achieved in this method is significantly lesser as compared to previously reported method. Thus, this method reduces the time required for analysis, thereby increasing the number of samples that can be studied. Additionally, this method exhibits a good reproducibility in terms of linearity, precision and accuracy for identifying and estimating the quantity of ergosterol in any sample.

Conclusions

Ergosterol is a pro-vitamin D_2 which can be converted into vitamin D_2 on irradiation of UV light. Therefore, it is important to identify the sources of ergosterol and use them for production of vitamin D_2. In the present study, we have developed a RP-HPLC method for the estimation of ergosterol in commercially available lichens and cultivated edible mushrooms. The developed method is simple, accurate, robust and demonstrates the adequate quantification of ergosterol in lichens and mushrooms in less than 10 min as compared to previously reported methods. In this method, ergosterol in the extracts is eluted at 7(±0.1) min. Validation results of the developed method demonstrated that the method has good linearity ($r^2 > 0.9998$), inter-day precision (Peak area: RSD 0.68%, Retention time: 1.26% RSD), intra-day precision (Peak area: RSD 0.78%, Retention time: 0.02% RSD) and accuracy (recovery range: 90.20–93.46% for three different concentrations) as per the ICH guidelines. From the results of the present study, we report that ergosterol content in lichens and mushrooms is varied. It is very clear from this work that lichens contain more amount of ergosterol as compared to mushrooms. We thus conclude that lichens are a good source of ergosterol. Hence, lichens could be a better alternative for vitamin D as a food additive in commercial products.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

References

[1] Jasinghe, V. J.; Perera, C. O. Ultraviolet Irradiation: The Generator of Vitamin D2 in Edible Mushrooms. *Food Chem.* **2006**, *95*, 638–643. DOI: 10.1016/j.foodchem.2005.01.046.

[2] Khundmiri, S. J.; Murray, R. D.; Lederer, E. PTH and Vitamin D. *Compr. Physiol.* **2016**, *6*, 561–601. DOI: 10.1002/cphy.c140071.

[3] Mullin, G. E.; Dobs, A. Vitamin D and Its Role in Cancer and Immunity: A Prescription for Sunlight. *Nutr. Clin. Pract.* **2007**, *22*, 305–322. DOI: 10.1177/0115426507022003305.

[4] Lee, N. K.; Aan, B. Optimization of Ergosterol to Vitamin D2 synthesis in *Agaricus bisporus* powder using ultraviolet-B radiation. *Food Sci. Biotechnol.* **2016**, *25*, 1627–1631. DOI: 10.1007/s10068-016-0250-0.

[5] Marwaha, R. K. Status of Vitamin D Deficiency in India. *World J. Endocr. Surg.* **2013**, *5*, 0–0. DOI: 10.5005/wjoes-5-3-v.

[6] Koyyalamudi, S. R.; Jeong, S. C.; Pang, G.; Teal, A.; Biggs, T. Concentration of Vitamin D2 in White Button Mushrooms (*Agaricus bisporus*) Exposed to Pulsed UV Light. *J. Food Compos. Anal.* **2011**, *24*, 976–979. DOI: 10.1016/j.jfca.2011.02.007.

[7] Raina, S.; Sodhi, H. S.; Sethi, S. Identification of Ergosterol in Mushrooms. Proceedings of the 8th International Conference on Mushroom Biology and Mushroom Products (ICMBMP8) 2014, 2014, pp 247–251.

[8] Cardwell, G.; Bornman, J. F.; James, A. P.; Black, L. J. A Review of Mushrooms as a Potential Source of Dietary Vitamin D. *Nutrients.* **2018**, *10*, 1498–1411. DOI: 10.3390/nu10101498.

[9] Jasinghe, V. J.; Perera, C. O. Distribution of Ergosterol in Different Tissues of Mushrooms and Its Effect on the Conversion of Ergosterol to Vitamin D2 by UV Irradiation. *Food Chem.* **2005**, *92*, 541–546. DOI: 10.1016/j.foodchem.2004.08.022.

[10] Holick, M. F. Environmental Factors That Influence the Cutaneous Production of Vitamin D. *Am. J. Clin. Nutr.* **1995**, *61*, 638–645.

[11] Holick, M. F. Vitamin D: A D-Lightful Health Perspective. *Nutr. Rev.* **2008**, *66*, S182–S194.

[12] Pratibha, P.; Sharma Mahesh, C. GC-MS Analysis and Biological Activities of Medicinally Important Lichen: *Parmelia perlata*. *Int. J. Pharmacogn. Phytochem. Res.* **2016**, *8*, 1975–1985.

[13] Stojanović, I. Ž.; Radulović, N. S.; Mitrović, T. L.; Stamenković, S. M.; Stojanović, G. S. Volatile Constituents of Selected Parmeliaceae Lichens. *J. Serbian. Chem. Soc.* **2011**, *76*, 987–994.

[14] Jayanthi, S.; Priya, P.; Devi, D. M.; Smily, J. B. Lichens: Origin, Types, Secondary Metabolites and Applications. *J. Acad. Indus. Res.* **2012**, *1*, 45–49.

[15] Dahlman, L.; Zetherström, M.; Sundberg, B.; Näsholm, T.; Palmqvist, K. Measuring Ergosterol and Chitin in Lichens. *Protoc. Lichenol.* **2002**, 348–362. https://doi.org/10.1007/978-3-642-56359-1_21

[16] Roberts, J. S.; Teichert, A.; McHugh, T. H. Vitamin D2 Formation from Post-Harvest UV-B Treatment of Mushrooms (*Agaricus bisporus*) and Retention during Storage. *J. Agric. Food Chem.* **2008**, *56*, 4541–4544. DOI: 10.1021/jf0732511.

[17] Sławińska, A.; Fornal, E.; Radzki, W.; Skrzypczak, K.; Zalewska-Korona, M.; Michalak-Majewska, M.; Parfieniuk, E.; Stachniuk, A. Study on Vitamin D2 Stability in Dried Mushrooms during Drying and Storage. *Food Chem.* **2016**, *199*, 203–209. DOI: 10.1016/j.foodchem.2015.11.131.

[18] Mau, J.-L.; Chen, P.-R.; Yang, J.-H. Ultraviolet Irradiation Increased Vitamin D 2 Content in Edible Mushrooms. *J. Agric. Food Chem.* **1998**, *46*, 5269–5272. DOI: 10.1021/jf980602q.

[19] Teichmann, A.; Dutta, P. C.; Staffas, A.; Jägerstad, M. Sterol and Vitamin D2 Concentrations in Cultivated and Wild Grown Mushrooms: Effects of UV Irradiation. *LWT – Food Sci. Technol.* **2007**, *40*, 815–822. DOI: 10.1016/j.lwt.2006.04.003.

[20] Chiocchio, V.; Matković, L. Determination of Ergosterol in Cellular Fungi by HPLC. A Modified Technique. *J. Argentine. Chem. Soc.* **2011**, *98*, 10–15.

[21] Yuan, J. P.; Wang, J. H.; Liu, X.; Kuang, H. C.; Huang, X. N. Determination of Ergosterol in Ganoderma Spore Lipid from the Germinating Spores of *Ganoderma lucidum* by High-Performance Liquid Chromatography. *J. Agric. Food Chem.* **2006**, *54*, 6172–6176. DOI: 10.1021/jf0617059.

[22] Barreira, J. C. M.; Oliveira, M. B. P. P.; Ferreira, I. C. F. R. Development of a Novel Methodology for the Analysis of Ergosterol in Mushrooms. *Food Anal. Methods.* **2014**, *7*, 217–223. DOI: 10.1007/s12161-013-9621-9.

[23] Raina, S.; Sodhi, H. S.; Sethi, S. Identification of Ergosterol in Mushrooms. Proceedings of the 8th International Conference on Mushroom Biology and Mushroom Products (ICMBMP8) 2014, 2014.

[24] Van Trung, H.; Tuan, N. N.; Thanh, N. T.; Thi, T. Determination of Ergosterol and Ergosterol Peroxide in Higher Fungi Species by High-Performance Liquid Chromatography. *J. Pharmacogn. Phytochem.* **2018**, *7*, 2376–2379.

[25] Ameeduzzafar , El-Bagory, I.; Alruwaili, N. K.; Imam, S. S; Alomar, F. A.; Elkomy, M. H.; Ahmad, N.; Elmowafy, M. Quality by Design (QbD) Based Development and Validation of Bioanalytical RP-HPLC Method for Dapagliflozin: Forced Degradation and Preclinical Pharmacokinetic Study. *J. Liq. Chromatogr. Relat. Technol.* **2020**, *43*, 53–65. DOI: 10.1080/10826076.2019.1667820.

[26] Gilani, S. J.; Imam, S. S.; Ahmed, A.; Chauhan, S.; Mirza, M. A.; Taleuzzaman, M. Formulation and Evaluation of Thymoquinone Niosomes: Application of Developed and Validated RP-HPLC Method in Delivery System. *Drug Dev. Ind. Pharm.* **2019**, *45*, 1799–1806. DOI: 10.1080/03639045.2019.1660366.

[27] Imam, S. S.; Aqil, M.; Akhtar, M.; Sultana, Y.; Ali, A. Optimization of Mobile Phase by 32-Mixture Design for the Validation and Quantification of Risperidone in Bulk and Pharmaceutical Formulations Using RP-HPLC. *Anal. Methods.* **2014**, *6*, 282–288. DOI: 10.1039/C3AY41562G.

[28] Rejczak, T.; Tuzimski, T. Application of High-Performance Liquid Chromatography with Diode Array Detector for Simultaneous Determination of 11 Synthetic Dyes in Selected Beverages and Foodstuffs. *Food Anal. Methods.* **2017**, *10*, 3572–3588. DOI: 10.1007/s12161-017-0905-3.

[29] Tuzimski, T. Application of SPE-HPLC-DAD and SPE-TLC-DAD to the Determination of Pesticides in Real Water Samples. *J. Sep. Sci.* **2008**, *31*, 3537–3542. DOI: 10.1002/jssc.200800368.

[30] Tuzimski, T. Use of Thin-Layer Chromatography in Combination with Diode-Array Scanning Densitometry for Identification of Fenitrothion in Apples. *J. Planar. Chromatogr. - Mod. TLC.* **2005**, *18*, 419–422. DOI: 10.1556/JPC.18.2005.6.3.

[31] de Sá Viana, O.; et al. Development and Validation of a HPLC Analytical Assay Method for Efavirenz Tablets: A Medicine for HIV Infections. *Brazilian. J. Pharm. Sci.* **2011**, *47*, 97–102.

[32] Villares, A.; Mateo-Vivaracho, L.; García-Lafuente, A.; Guillamón, E. Storage Temperature and UV-Irradiation Influence on the Ergosterol Content in Edible Mushrooms. *Food. Chem.* **2014**, *147*, 252–256. DOI: 10.1016/j.foodchem.2013.09.144.