

## Design of experimental approach for maximal extraction of vitamin D<sub>2</sub> from mushrooms

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### Summary

Vitamin D<sub>2</sub> is the standard form of fortification and supplementation of food in most countries because of its good bioavailability and its ability to substitute vitamin D<sub>3</sub>. In this study, tetrabutylammonium bromide/decanoic acid-based hydrophobic deep eutectic solvent was employed for green extraction of ergosterol from mushrooms. Extraction factors, stirring time, sonication time, solvent volume and solvent ratio were optimized by response surface methodology to attain maximum extraction yield of 3900 mg ergosterol per kilogram dry weight mushrooms. The extract was purified using preparative high performance liquid chromatography (HPLC). The extracted ergosterol was subjected to ultraviolet radiation and maximum ergocalciferol yield of 1290.3 mg·kg<sup>-1</sup> dry weight mushroom was obtained. This study also describes a HPLC method for analysing the stability of Vitamin D<sub>2</sub> and its validation using the International Council for Harmonisation guidelines. The method was optimized exposing a standard ergocalciferol solution to various stress conditions. The degradation products formed during initial elucidation by HPLC were further analysed by electrospray ionization mass spectrometry.

### Keywords

ergocalciferol; vitamin D<sub>2</sub>; deep eutectic solvent; mushroom; response surface; stability

Vitamin D is responsible for calcium homeostasis in human body, increasing intestinal absorption of calcium, magnesium and phosphate and having multiple other biological effects. The most important compounds in this group are vitamin D<sub>3</sub>, also known as cholecalciferol, and vitamin D<sub>2</sub>, known as ergocalciferol [1].

Vitamin D<sub>3</sub> is synthesized in the skin by the photochemical conversion of pro-vitamin D<sub>3</sub>, but enough irradiation is available throughout the year only in places that lie below the latitude of 35°. Studies in regions of the world located at low latitudes, such as the Middle East, have shown a high prevalence of vitamin D deficiency, ranging from 50 % to 97 %. As the exposure to natural sunlight is variable in people belonging to different locations, the recommendations about the extent of

exposure to sunlight that may be considered safe are uncertain, considering the cases of skin cancer patients. Thus, intake of additional vitamin D through diet and using food supplements is highly recommended [1].

Vitamin D deficiency is a condition dangerous to health as it may lead to osteoporosis or rickets, skin-pigmentation (increases melanogenesis) and has also been linked to hypertension, autoimmune diseases, diabetes and cancer [2]. Vitamin D has an important role in both progression of healthy pregnancy and long-term health of offsprings. Recent evidence has suggested that vitamin D has an association with multifactorial diseases of pregnant women, such as bacterial vaginosis, pre-term birth, gestational diabetes or preeclampsia [1].

Apart from the primary source of vitamin D

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(solar UV-B irradiation), dietary sources of vitamin D include oily fish such as salmon (approximate contents of  $2.68 \text{ mg}\cdot\text{kg}^{-1}$ ), mackerel and sardines, some fish oils such as cod liver oil (contents of  $0.06 \text{ kg}\cdot\text{kg}^{-1}$ ) or egg yolk (approximate contents of  $13.4 \text{ mg}\cdot\text{kg}^{-1}$ ). Mushrooms also contain vitamin D but in the form of ergocalciferol, vitamin D<sub>2</sub>. Vitamin D<sub>2</sub> is another important compound in the D group of vitamins, which is the standard form of fortification and supplementation of food in most countries, because it shows good bioavailability and substitute for vitamin D<sub>3</sub>. Mushrooms contain vitamin D<sub>2</sub> in much lower quantities as compared to its precursor ergosterol. Ergosterol can be converted to vitamin D<sub>2</sub> by irradiation of whole fruiting bodies or slices of mushrooms, which has been a common procedure used for decades [3]. However, exposure of an ergosterol extract to ultraviolet (UV) radiation for conversion to ergocalciferol is not a commonly used approach. Mushroom extracts for UV exposure can be obtained employing various processes, including the innovative, more effective ones such as supercritical fluid extraction, sonication or microwave assisted extraction [4].

Conventionally, Vitamin D is extracted using organic solvents like ethanol, hexane and chloroform. Most of these organic solvents are environmentally hazardous, toxic and inflammable, contribute to volatile organic compounds in atmosphere and serve as a cause of global warming. Hence, environmentally more acceptable, “greener” solvents for the extraction of vitamin D have become the need of today. One such alternative solvent category that may be employed for extracting vitamin D is the deep eutectic solvents (DESs). DESs are regarded as ‘green solvents’ as they exhibit low volatility, are water-miscible, non-flammable, non-toxic, biocompatible and biodegradable, and hence able to overcome the problems associated with conventional organic solvents. Considering the physico-chemical properties of vitamin D (non-polar, fat-soluble vitamin), hydrophobic deep eutectic solvents (HDESs) were chosen to extract vitamin D from mushrooms. HDES composed of decanoic acid, as the hydrogen bond donor (HBD), and tetrabutyl ammonium bromide, as the hydrogen bond acceptor (HBA), was employed for extracting vitamin D<sub>2</sub> and ergosterol [5]. Further, the study involved comparison of HDES with other DESs with regard to extraction efficiency, and optimization of extraction with respect to different process parameters, using Design of Experiments (DoE) approach, to maximize the yield of vitamin D.

It is often difficult to accurately quantify vita-

min D because of the complexity of matrices it is present in, due to its low content in the natural sources from which it is extracted or due to the interference of other similar compounds during analysis. Over the last decade, high performance liquid chromatography (HPLC) based quantification has become the technique of choice for analysing vitamin D [6, 7] due to its accuracy in quantification and its ability to resolve it from structurally similar impurities [8, 9]. Moreover, stability-indicating HPLC methods may also be developed to understand the degradation of compounds when exposed to diverse conditions such as action of acids, bases, oxidizing agents, temperature and UV light (photolytic degradation) [10]. Stability testing is important to verify the quality of a drug or bioactive compound, especially in presence of other chemically similar compounds. A fast and simple stability-indicating method is thus the need of today for quality assurance of a product and to validate the assigned shelf-life [11, 12]. Till date, majority of the stability-indicating methods related to vitamin D were based on analysis of vitamin D<sub>3</sub> (cholecalciferol), which is structurally similar to vitamin D<sub>2</sub> (ergocalciferol). However, ergocalciferol is an important form of vitamin D that is used in food supplements and fortified food products and has gained wide popularity as the “vegan vitamin D” [13]. Thus, it is important to develop a stability-indicating method for ergocalciferol to quantify and study its stability in various vitamin D food supplements and fortified food products that are prepared using diverse processes. In this work, we report a reverse phase HPLC-UV method for accurate quantification and stability testing of ergocalciferol. The reported method was validated according to the International Council for Harmonisation (ICH) guideline Q2 (R1) and degradation studies were conducted according to the ICH guideline Q1A (R2) [14].

## MATERIALS AND METHODS

### Materials

*Agaricus bisporus* mushrooms were purchased from the local market (Mumbai, Maharashtra, India). The mushrooms were lyophilized, pulverized and the resulting powder was stored in a desiccator. Tetrabutyl ammonium bromide (laboratory reagent grade,  $\geq 98.5\%$ ), decanoic acid (laboratory reagent grade,  $\geq 99\%$ ), choline chloride (pure,  $\geq 99\%$ ), glycerol (analytical reagent grade,  $\geq 99.5\%$ ), 1,4-butanediol (laboratory reagent grade,  $\geq 99\%$ ), propane-1,2-diol (pure,  $\geq 99.5\%$ ), lactic acid (pure,  $\geq 88\%$ ), chloroform

(laboratory reagent grade,  $\geq 99.5\%$ ) and hexane (laboratory reagent grade,  $\geq 95\%$ ) were purchased from SDFine Chemicals (Mumbai, India). D(+)-glucose (HPLC grade,  $\geq 99.5\%$ ) was purchased from Sigma Aldrich (St. Louis, Missouri, USA). Ethylene glycol (pure,  $\geq 98\%$ ) was purchased from Sisco Research Laboratories (Mumbai, India). Ergocalciferol standard (vitamin D<sub>2</sub>; HPLC grade) was purchased from Supelco (Bellefonte, Pennsylvania, USA) and ergosterol standard (HPLC grade,  $\geq 95\%$ ) was purchased from Sigma Aldrich. Acetonitrile (HPLC grade,  $\geq 99.7\%$ ) and methanol (HPLC grade,  $\geq 99.7\%$ ) were purchased from HiMedia Laboratories (Mumbai, India). Isopropanol (HPLC grade,  $\geq 99.8\%$ ) was purchased from SDFine Chemicals. Deionized water was prepared by a Milli-Q Ultrapure Water System (Millipore, Billerica, Massachusetts, USA). Other chemicals and reagents used in the experiments were of analytical reagent grade.

#### Preparation of hydrophobic deep eutectic solvent

HDES was prepared using decanoic acid as HBD and tetrabutyl ammonium bromide as HBA, the molar ratio was 1:2 (other ratios are mentioned in Tab. 1). The weighed constituents were transferred to a reagent bottle and stirred on a magnetic stirrer at 50 °C until a clear liquid was obtained. Thereafter, it was maintained at 30 °C overnight. Other DESs used for comparison were prepared using the same method as used for preparing HDES. The composition and ratio for each DES is listed in Tab. 2.

#### Extraction of ergosterol from mushrooms

For extraction using HDESs, DESs and organic solvents, 1 g of lyophilized mushroom powder was added to 20 ml of the respective solvents. The mixture was stirred for 2 h and then sonicated for 45 min to achieve maximum extraction. The mixtures were centrifuged at 6700  $\times$ g for 10 min,

**Tab. 1.** Design of Experiment runs and response.

Run order	Standard order	Solvent volume [ml]	Solvent ratio	Stirring time [min]	Sonication time [min]	Ergosterol [mg·kg <sup>-1</sup> ]
1	26	12.5	1.75	62.5	22.5	3367.00
2	3	5.0	2.00	5.0	0.0	2246.20
3	2	20.0	1.50	5.0	0.0	1729.20
4	18	27.5	1.75	62.5	22.5	3428.15
5	14	20.0	1.50	120.0	45.0	3898.80
6	23	12.5	1.75	62.5	22.5	2713.00
7	7	5.0	2.00	120.0	0.0	3050.00
8	9	5.0	1.50	5.0	45.0	3396.50
9	27	12.5	1.75	62.5	22.5	3282.50
10	31	12.5	1.75	62.5	22.5	3252.50
11	5	5.0	1.50	120.0	0.0	3095.50
12	29	12.5	1.75	62.5	22.5	2975.25
13	20	12.5	2.25	62.5	22.5	3342.25
14	1	5.0	1.50	5.0	0.0	2091.20
15	8	20.0	2.00	120.0	0.0	3204.00
16	15	5.0	2.00	120.0	45.0	3237.80
17	12	20.0	2.00	5.0	45.0	3369.20
18	6	20.0	1.50	120.0	0.0	3221.60
19	4	20.0	2.00	5.0	0.0	2730.40
20	16	20.0	2.00	120.0	45.0	3903.20
21	21	12.5	1.75	52.5	22.5	2717.50
22	19	12.5	1.25	62.5	22.5	3142.50
23	24	12.5	1.75	62.5	67.5	3507.50
24	11	5.0	2.00	5.0	45.0	3380.70
25	28	12.5	1.75	62.5	22.5	3414.00
26	22	12.5	1.75	177.5	22.5	3532.75
27	30	12.5	1.75	62.5	22.5	3049.00
28	13	5.0	1.50	120.0	45.0	3344.20
29	10	20.0	1.50	5.0	45.0	3900.80
30	17	2.5	1.75	62.5	22.5	0.00
31	25	12.5	1.75	62.5	22.5	3384.75

**Tab. 2.** Statistical evaluation of design of experimental data (Analysis of variance).

Source	Degrees of freedom	Adjusted sums of squares	Adjusted mean squares	F value	p value
<b>Model</b>	14	6108655	436332	6.92	0.000
<b>Linear</b>	4	4851463	1212866	19.23	0.000
Solvent volume	1	329234	329234	5.22	0.037
Solvent ratio	1	29624	29624	0.47	0.504
Stirring time	1	1373486	1373486	21.78	0.000
Sonication time	1	3119118	3119118	49.46	0.000
<b>Square</b>	4	98794	24698	0.39	0.811
Solvent volume × Solvent volume	1	22004	22004	0.35	0.564
Solvent ratio × Solvent ratio	1	1522	1522	0.02	0.879
Stirring time × Stirring time	1	37433	37433	0.59	0.453
Sonication time × Sonication time	1	45400	45400	0.72	0.410
<b>2-Way interaction</b>	6	1135695	189283	3.00	0.039
Solvent volume × Solvent ratio	1	13753	13753	0.22	0.647
Solvent volume × Stirring time	1	48963	48963	0.78	0.392
Solvent volume × Sonication time	1	107338	107338	1.70	0.212
Solvent ratio × Stirring time	1	37433	37433	0.59	0.453
Solvent ratio × Sonication time	1	189769	189769	3.01	0.103
Stirring time × Sonication time	1	738439	738439	11.71	0.004
<b>Error</b>	15	946034	63069		
Lack-of-Fit	9	770430	85603	2.92	0.102
Pure Error	6	175604	29267		
Total	29	7054689			

**Validation**

Variable	Setting
Solvent volume [ml]	15
Solvent ratio	2
Stirring time [min]	60
Sonication time [min]	45

**Experiment**

Result	Ergosterol yield [mg·kg <sup>-1</sup> ]
Fit	3564.69
Standard error fit	124.09
95% confidence interval	(3300.20, 3829.18)
95% prediction interval	(2967.63, 4161.75)

at 25 °C. A volume of 1 ml of the supernatant was withdrawn and diluted two-fold with the HPLC mobile phase. Then it was filtered through a membrane filter (pore size 0.45 µm) and analysed by HPLC.

**HPLC analysis of ergocalciferol and ergosterol**

HPLC system Series 1260 (Agilent Technologies, Santa Clara, California, USA), equipped with a variable wavelength spectrophotometric detector and a ChemStation data acquisition system, was used. The chromatographic separation was performed on a reverse phase column (C18; 250 mm × 4.6 mm, particle size of 5 µm) from Agilent Technologies. Mixture of methanol/acetonitrile/isopropyl alcohol (60:30:10, v/v) was used as the mobile phase. The column temperature was 30 °C and flow rate was 1.2 ml·min<sup>-1</sup>. Sample injection volume was 20 µl. Detection was done at 265 nm. The content of ergocalciferol and ergosterol was calculated by means of calibration curves

established with regression equations Eq. 1 for ergocalciferol and Eq. 2 for ergosterol.

$$y = 23.154x + 39.443 \quad (R^2 = 0.995) \quad (1)$$

$$y = 20.846x + 77.469 \quad (R^2 = 0.998) \quad (2)$$

**Method validation**

The HPLC method was validated for selectivity, linearity, method and system precision, and repeatability. For linearity testing, 1 mg of standard ergocalciferol was dissolved in 10 ml mobile phase to prepare a stock solution containing 100 mg·l<sup>-1</sup> of ergocalciferol. The stock solution was diluted with the mobile phase to prepare standard solutions containing 1, 5, 10, 15, 20, 40, 60 and 80 mg·l<sup>-1</sup> of ergocalciferol, to plot the calibration curve. The method precision and system precision were determined by repeatedly injecting solutions containing 1 mg·l<sup>-1</sup> (low concentration) and 40 mg·l<sup>-1</sup> (intermediate concentration) of ergocalciferol, on the

same day and the following five days, to check the intra- and inter-day precision. Injection repeatability was evaluated by injecting the same samples (1 mg·l<sup>-1</sup> and 40 mg·l<sup>-1</sup>) five times. Due to structural similarities between ergosterol and ergocalciferol, the same method was used for quantification of ergosterol.

#### Forced degradation of ergocalciferol

Forced degradation of vitamin D<sub>2</sub> was performed under five different stress conditions, namely, hydrolytic (acid degradation and base degradation), oxidative, thermal and photolytic conditions. A 100 mg·l<sup>-1</sup> stock solution of ergocalciferol was prepared by dissolving 1 mg of standard ergocalciferol in 10 ml of the mobile phase. Degradation was facilitated by mixing 1 ml of the ergocalciferol stock solution with 1 ml of 0.1 mol·l<sup>-1</sup> hydrochloric acid, for acid degradation, 1 ml of 0.1 mol·l<sup>-1</sup> sodium hydroxide for base degradation and 1 ml of 15% v/v H<sub>2</sub>O<sub>2</sub> for oxidative degradation. The samples for acid and base degradation were maintained at 80 °C for 20 min, while the sample for oxidative degradation was held at 30 °C for 1 h. Thermal degradation was executed by incubating 1 ml of the stock solution at 80 °C for 2 h. In case of photolytic degradation, 1 ml of the stock solution was placed in a UV-B cabinet for 2 h in a glass vial.

After the degradative treatments, the samples were cooled to 30 °C, wherever applicable, neutralized with HCl or NaOH, wherever necessary to stop the reactions, and the final volumes were made up to 5 ml with the mobile phase. In case of oxidative, thermal and photolytic degradation, the samples were diluted without neutralization. The degradation studies were conducted according to the ICH guideline Q1A (R2) [14].

#### LC-MS analysis of the degradation products

Fragmentation of the individual product peaks obtained after degradation of ergocalciferol, under various stress conditions, were studied using a HPLC Series 1260 attached to a single Quad mass spectrometer (Agilent Technologies). Electrospray ionization was employed in positive mode, from *m/z* 100 to 500, to confirm whether the developed HPLC–UV method was stability-indicating one. The operating conditions for the mass spectrometer (MS) were as follows: drying gas temperature of 350 °C, drying gas flow rate of 12 l·min<sup>-1</sup>, nebulizer pressure of 241.32 MPa and capillary entrance voltage of 3000 V. The chromatographic conditions were the same as those described under HPLC analysis of ergocalciferol and ergosterol. A variable wavelength detector

was coupled with the LC–MS system to identify the LC-MS peaks corresponding to the degradation peaks.

#### Experimental design and statistical analysis

The variable range of experimental parameters was determined using one-factor-at-a-time approach and the suitable resulting range was employed during experimental design to obtain the experimental runs for further optimization. The selected factors were further optimized to attain the highest extraction yield of ergosterol (ergocalciferol content was below the detection limit, hence only ergosterol yield was optimized, which was eventually used to yield ergocalciferol upon UV exposure). Central composite design (CCD) was employed to study the effects of extraction volume, stirring time, sonication time and HBD/HBA ratio, at three levels (–1, 0, 1), on the extraction yield of ergosterol, which was represented as the response. Thirty-one experiments, including seven replicates at centre points, were performed in random order. Tab. 1 shows the experimental runs as the actual values of the variables, with order and response data collected after experimentation. The experiments were designed and regression analysis was carried out using Minitab 17.3.1 software (Minitab, Coventry, United Kingdom). The fitness of the proposed model was estimated by assessing *p*-value, based on analysis of variance (ANOVA).

#### Purification of ergosterol from extract using preparative HPLC

A 1260 Infinity Preparative LC System (Agilent Technologies), equipped with a diode-array spectrophotometric detector and a ChemStation data acquisition system, was used for separation of ergosterol. The chromatographic separation was performed on an Eclipse XBD-C18 column (250 mm × 9.4 mm, particle size of 5 µm) from Agilent Technologies. Mixture of methanol/acetonitrile (70:30, v/v) was used as the mobile phase. The column temperature was 30 °C and flow rate was 3 ml·min<sup>-1</sup>. Sample was prepared by diluting the extract with mobile phase (1:1) and subsequent filtering through a membrane filter (pore size 0.45 µm). Ergosterol was collected by fraction collection manually.

## RESULTS AND DISCUSSION

#### Factor screening

Four factors were considered to check their effect on the extraction of ergosterol from mush-



rooms, namely, solvent ratio, solvent volume, stirring time and sonication time [15, 16]. At deciding the ratio of tetrabutylammonium bromide to decanoic acid, during the preparation of HDES, the molar ratio of 1:1 did not yield a stable solvent. Other ratios employed were 1:2 and 1:3, which were stable. However, the solvent obtained at the molar ratio of 1:3 was too oily, thus resulting in extended time requirement to clean the column, even after dilution with mobile phase. Moreover, the extraction yield obtained with this solvent was comparable to the amount of ergosterol that could be extracted with the solvent prepared using the molar ratio of 1:2. Since the possibilities of varying these ratios was enormous, the response surface methodology (RSM) software was utilised to decide the ratio of the components of HDES.

The stirring time range considered for RSM was 5 min to 2 h. It was found that stirring itself resulted in considerable extraction of ergosterol. However, stirring followed by sonication reduced the total time required for extraction and also facilitated cell disruption to allow for maximum extraction of ergosterol. Sonication time was varied over the range of 0 min to 45 min. The solvent volume was evaluated over the range of 5 ml to 20 ml of the solvent per gram of dry mushroom powder and when the ratios of tetrabutylammonium bromide to decanoic acid was varied between 1:1.5 and 1:2. The RSM software suggested combination of reaction variables at the suggested endpoints of the reaction variables, between the ranges and also at values above and below the indicated ranges.

#### Optimization of the extraction factors

DoE gives a prediction model based on interaction between the experimental factors. It replaces the trial-and-error procedure with a well-set model, and optimizes the data reducing the experimental runs, thus reducing the experimental time and cost. In this study, the amount of ergosterol was regarded as the response for the model. Four factors, namely, the solvent volume, the solvent ratio, the stirring and the sonication time were optimized to obtain a regression model. The analysis of variance data is shown in Tab. 2. The model was analysed from  $p$  values, predicted by the software based on  $F$ -test, which should be less than 0.05 ( $p < 0.05$ ) for the model to be significant. The data, which were found to be significant based on the analysis of the  $p$  values ( $p < 0.05$ ), indicated that the model was suitable for the extraction. Also individual factors were found to be significant, except for the solvent ratio ( $p = 0.504$ ; non-significant), as was also suggested

by the experimental results. Lack of fit resulted in  $p = 0.102$ , which was not significant, and hence supported the claim that the model did efficiently fit the obtained data. Sonication time and stirring time were found to be interdependent, as evident from the two-way interaction data ( $p = 0.004$ ) and hence were adjusted to attain the maximum extraction in the shortest possible time. The method was further validated by prediction analysis, the experimental values were found to be within the confidence interval and the standard error predicted was small, which again suggested the high accuracy of the model (Tab. 2).

A second order polynomial equation for the yield of extraction was obtained by multiple regression analysis of the data, as stated in Eq. 3.

$$R = 816 - 11.5A + 1006B + 13.57C + 57.8D - 0.65A^2 - 118B^2 - 0.0111C^2 - 0.0799D^2 + 15.6AB + 0.128AC + 0.485AD - 3.36BC - 19.4BD - 0.1661CD \quad (3)$$

where,  $A$  is the solvent volume,  $B$  the solvent ratio,  $C$  the stirring time,  $D$  the sonication time and  $R$  the response.

Three-dimensional response surface plots representing the model equation were constructed to visualize the effect of factors on the yield of extraction, considering two factors at a time. These plots revealed the two-way interaction curves. Here, the curve for the stirring time and sonication time was relatively steeper, as compared to the other curves, since these two factors were interdependent while the other factors were independent. These plots may be used as a reference to view the trend of the significant factors and also to study their interactions, if any (Fig. 1). These results were consistent with ANOVA.

#### Comparison of extraction efficiency using various solvents

Various organic solvents, e. g. chloroform or hexane, were previously used for extracting vitamin D from various sources. Deep eutectic solvents were explored extensively for the extraction of vitamins. Theoretically, bioactive compounds can be extracted effectively using solvents which have polarity similar to that of the compound being extracted. Vitamin D (pro-vitamin D, ergosterol has similar hydrophobic properties, being a fat-soluble vitamin, may be extracted effectively with solvents which are hydrophobic. Thus, HDES was chosen considering the physico-chemical properties of vitamin D and ergosterol. Other DESs were chosen based on literature reports of

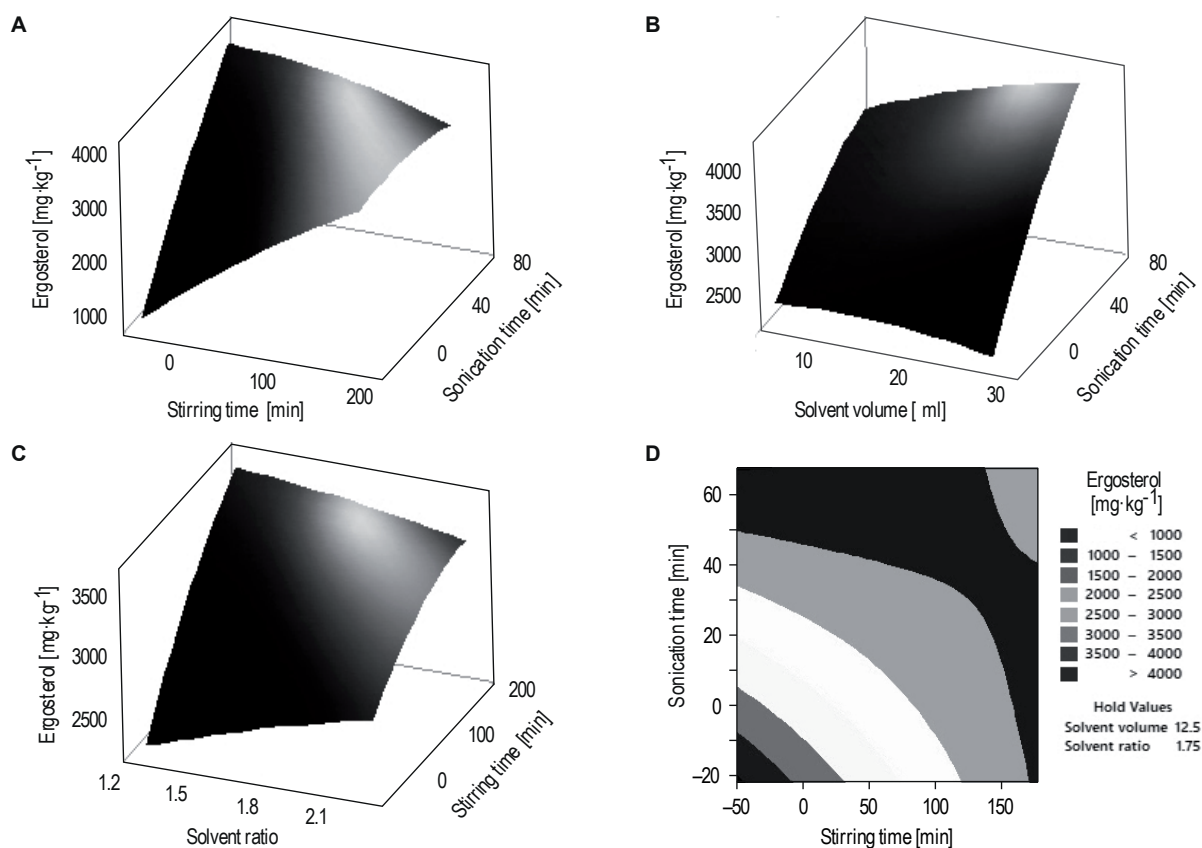


Fig. 1. Response surface methodology plots for two-way interactions.

A – stirring and sonication times, B – sonication time and solvent volume, C – stirring time and solvent ratio, D – contour plot.

alternative DESs, based on butane-1,4-diol, propane-1,2-diol and ethylene glycol, having been successfully used for the extraction of hydrophobic compounds like shinone IIA. Also lactic acid- and glucose-based ternary solvents were reported as effective in extracting water-insoluble compounds like quercetin or cinnamic acid, and hence were chosen for this study. The extraction efficiency of HDES and the other DESs was further compared with those of the commonly used organic solvents that were reported to be suitable for the extraction of vitamin D. All the extractions were carried out using the same conditions.

In Tab. 3, yield of ergosterol is listed as extracted with HDES, DESs of various composition and with organic solvents from mushrooms. These results were compared graphically in Fig. 2. It is clearly shown that HDES was able to extract the highest amounts of ergosterol, as compared to other DESs, whereas the extraction yield was comparable to that obtained using conventional organic solvents. The amounts of ergosterol extracted from the *A. bisporus* mushrooms with the two organic solvents used in this study were comparable to the data reported previously. Thus,

HDES was found to efficiently extract ergosterol from mushrooms, leaving behind minimal residual ergosterol in the mushroom mass after extraction. Tab. 1 shows extraction of  $3900 \text{ mg} \cdot \text{kg}^{-1}$  dry weight, which is almost equivalent to total content present in *A. bisporus* mushrooms [17]. Apart from extraction efficiency, the solvent was also found to be selective, as minimum interfering impurity peaks were observed in the chromatogram upon injection of a diluted extract, which demonstrated no need for saponification. Amongst DESs, the solvent based on choline chloride and butane-1,4-diol (molar ratio of 1:4) resulted in maximum extraction of ergosterol, the solvents based on choline chloride/glucose/water (molar ratio of 5:2:5) and choline chloride/glycerol/urea (molar ratio of 1:1:1) were found to exhibit the least extraction efficiency. Thus, HDES may be used to replace the organic solvents during vitamin D extraction from mushrooms, without compromising the yield and, at the same time, may eliminate the environmental drawbacks associated with the use of organic solvents. Also, based on initial studies and DoE-related data, it may be stated that stirring by itself is enough for extracting considerable

**Tab. 3.** Extraction performance of solvents.

Solvent	Composition	Molar ratio	Aspect at room temperature	Ergosterol yield [mg·kg <sup>-1</sup> ]
<b>Hydrophobic deep eutectic solvent</b>				
HDES	Tetrabutyl ammonium bromide/decanoic acid	1:2	Transparent liquid	3665.50
<b>Deep eutectic solvents</b>				
DES1	Choline chloride/ethylene glycol	1:2	Transparent liquid	57.32
DES2	Choline chloride/1,2-propanediol	1:2	Transparent liquid	190.28
DES3	Choline chloride/1,2-propanediol	1:3	Transparent liquid	438.90
DES4	Choline chloride/1,4-butanediol	1:2	Transparent liquid	160.34
DES5	Choline chloride/1,4-butanediol	1:4	Transparent liquid	938.28
DES6	Choline chloride/glycerol	1:3	Transparent liquid	122.82
<b>Ternary deep eutectic solvents</b>				
TDES1	Lactic acid/glucose/water	5:1:3	Transparent liquid	254.96
TDES2	Choline chloride/glucose/water	5:2:5	Transparent liquid	24.48
TDES3	Choline chloride/glycerol/urea	1:1:1	Transparent liquid	26.15
TDES4	Choline chloride/glycerol/urea	1:2:2	Transparent liquid	54.22
<b>Organic solvents</b>				
ORGS1	Chloroform	–	–	3282.10
ORGS2	Hexane	–	–	3898.20

amount of ergosterol from mushrooms (Tab. 1, run 15 of RSM). The hydrophobic character of HDES may have solubilized the cell membrane, which is a known mechanism of cell disruption by organic solvents, thus leading to good extraction efficiency. Furthermore, sonication was observed to reduce the extraction time, and can be used for large scale process using special sonication equipment.

#### Analytical method optimization and validation

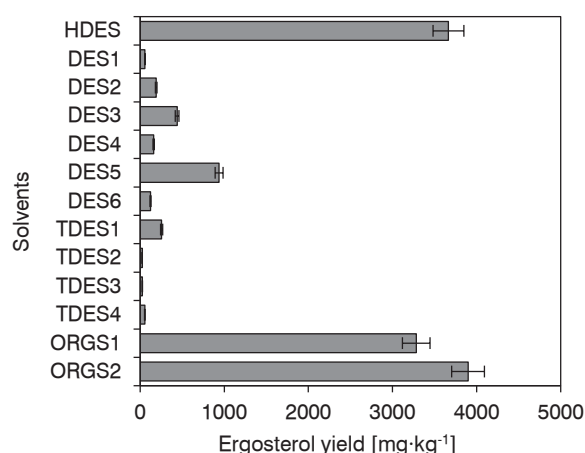
The stability-indicating method developed for ergocalciferol was initially optimized using samples that underwent acid-mediated degrada-

tion. This enabled us to study the resolution of the method by changing the mobile phase composition, temperature and flow rate. The sample after acid degradation was chosen for the optimization studies based on information from the published literature that the peak of the product of acid degradation was the most difficult to resolve from the peak of native ergocalciferol, as compared to the peaks of degradative products due to other stresses [12, 18]. The optimized method also resulted in a good resolution of the peaks of degradation products subjected to other stresses, from the peak of the native compound.

The developed method was validated in accordance with the ICH guideline Q2 (R1) [14]. The method was found to be selective in resolving the peaks of degradative products, arising due to exposure to various degradative conditions, and no interfering peaks were found at the retention time of ergocalciferol. Also, the method distinctly separated ergosterol from ergocalciferol, present in the mushroom extract, as well as from a mixture of individual standards, thus indicating its selectivity for vitamin D<sub>2</sub>, even in presence of closely related compounds like ergosterol (Fig. 3).

Linearity was evaluated by injecting nine calibration standards of ergocalciferol, in the concentration range of 1–100 mg·l<sup>-1</sup>, and plotting a calibration curve, which exhibited a regression coefficient ( $R^2$ ) of 0.995 (Fig. 4). Thus, the developed method demonstrated excellent linearity, which can be used to effectively quantify ergocalciferol in the tested concentration range.

Stability of ergocalciferol in the mobile phase



**Fig. 2.** Ergosterol yield after extraction with different solvents.

Composition of solvents is given in Tab. 4.



was assessed by injecting a standard solution (40 mg·l<sup>-1</sup>) of the compound at 0, 8 and 24 h after preparation. Ergocalciferol was found to be stable in the mobile phase and no peaks of degradation products or significant change in the peak area were observed at these time points.

System precision and method precision were examined in terms of repeatability, intra-day and inter-day precision. The method resulted in a constant retention time ( $R_t$ ) for both, ergocalciferol ( $R_t = 6.7$  min) and ergosterol ( $R_t = 7.6$  min), as shown in Fig. 3.

Method precision, expressed as relative standard deviation ( $RSD$ ), should be below 5 % [18] for a result to be statistically significant.  $RSD$  for repeatability was found to be 0.3 % and 0.4 %, for concentrations of 1 mg·l<sup>-1</sup> and 40 mg·l<sup>-1</sup>, respectively, thus confirming that the results were reproducible.

The inter-day and intra-day precision were within the required range of < 5 % [12, 18], once again indicating that the results were reproducible on the same day and also over a period of time, given that the samples were stored as per the conditions stated in material safety data sheet (MSDS) [18].

The limit of detection ( $LOD$ ) and limit of quantification ( $LOQ$ ) were calculated from the regression lines, using the Eq. 4 and Eq. 5.

$$LOQ = 10S/b \quad (4)$$

$$LOD = 3.3S/b \quad (5)$$

where  $S$  is the standard deviation of the intercepts and  $b$  is the average slope of the calibration curve.

The  $LOD$  and  $LOQ$  values were found to be 0.10 mg·l<sup>-1</sup> and 0.3 mg·l<sup>-1</sup>, respectively.  $LOD$  indicated that the method could precisely detect a concentration as low as 0.1 mg·l<sup>-1</sup>, while the  $LOQ$  value confirmed that concentration of ergocalciferol, as low as 0.3 mg·l<sup>-1</sup>, could be quantified.

#### Forced degradation study of ergocalciferol

Vitamin D is prone to oxidative, hydrolytic, photolytic and thermal degradation [19, 20]. Standard ergocalciferol samples were subjected to various degradative stresses and the post degradation concentrations were analysed using the standard curve (Fig. 4). Degradation by various stresses was studied using HPLC with variable wavelength spectrophotometric detection (Fig. 5). Peaks from HPLC analysis were further studied using LC-MS, the results of which are presented in Tab. 4. The results showed that content of vitamin D<sub>2</sub> decreased when subjected to various stress conditions. Also, additional well resolved peaks

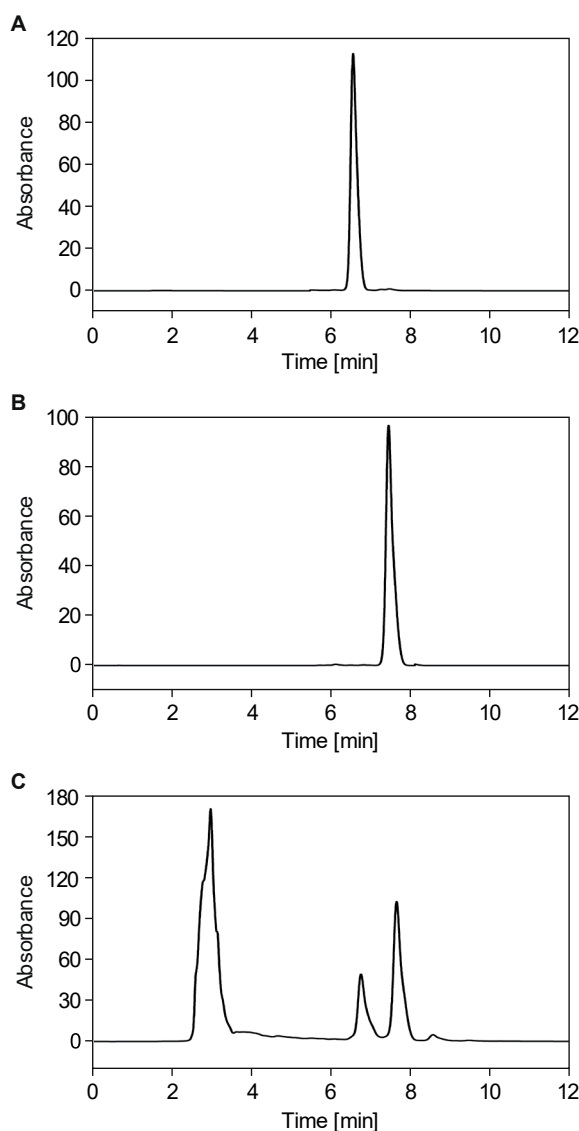


Fig. 3. Chromatograms from method validation.

A – ergocalciferol standard, B – ergosterol standard, C – mushroom extract.

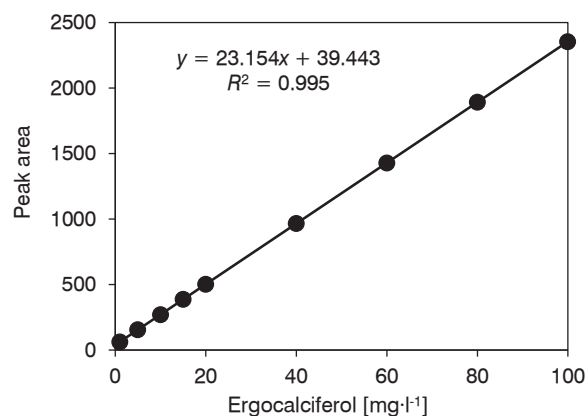
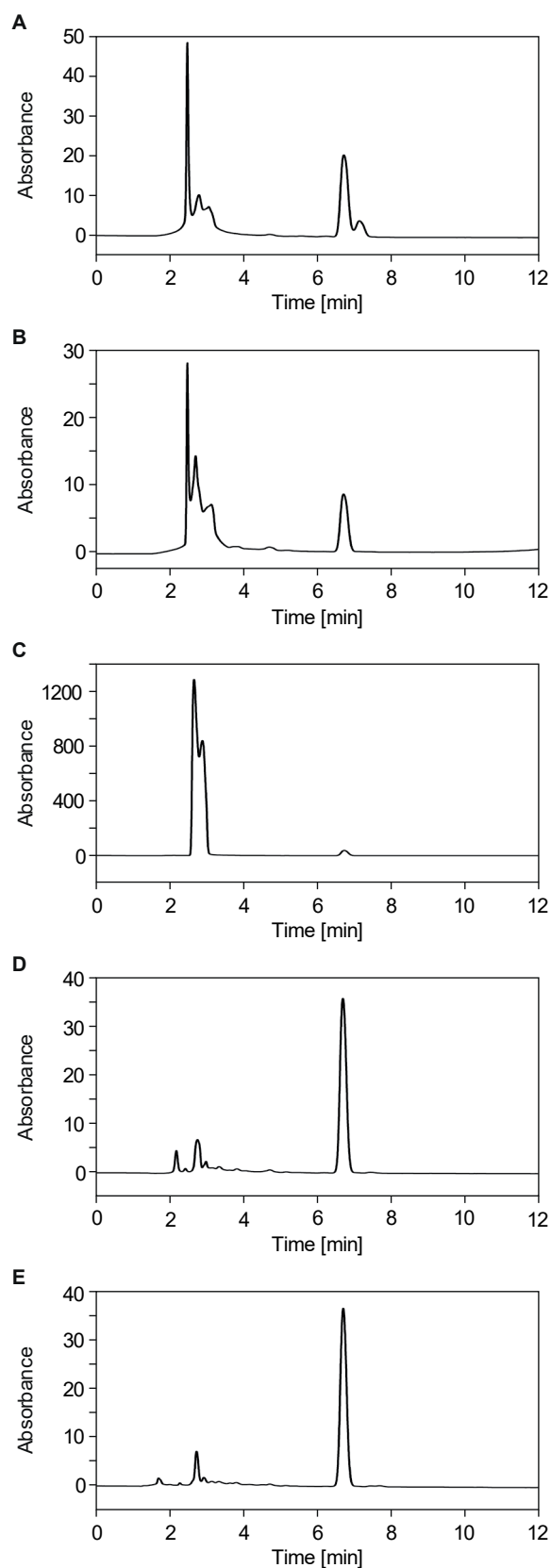


Fig. 4. Calibration curve of ergocalciferol.



**Fig. 5.** Chromatograms of ergocalciferol from stability testing.

A – acid treatment, B – base treatment, C – oxidation, D – thermolysis, E – photolysis.

were observed, which were absent in the chromatogram of the standard vitamin D<sub>2</sub>, at the same content. This proved that vitamin D<sub>2</sub> was indeed vulnerable to oxidative, hydrolytic, photolytic and thermal stresses. The ability of the method to resolve the peaks of degradative products from the peak of standard vitamin D<sub>2</sub> confirmed it to be a stability-indicating method.

The chromatograms of the samples subjected to oxidative and UV degradation, as shown in Fig. 5, revealed varying contents of the common degradation product, having a retention time of approximately 2.7 min. However, the chromatograms of samples exposed to oxidative degradation also showed the presence of an additional peak at the retention time of 2.2 min, as indicated in Tab. 4. The chromatograms of samples subjected to acid and base hydrolysis (Fig. 5A, Fig. 5B) revealed the presence of common degradative products. However, the chromatogram of the acid-hydrolysed sample showed an additional peak, having the retention time of 7.15 min, very close to the peak of vitamin D (6.7 min; Fig. 5A). This peak was interpreted to be of tachysterol, as identified by LC-MS (indicated in Tab. 4). The LC-MS analysis of this peak showed a molecular ion at  $m/z$  384.9, which was indicative of tachysterol. This compound has a very similar structure to that of vitamin D. The result confirmed the efficient resolution capability of the developed HPLC method to separate structurally similar compounds. Base hydrolysis resulted in highest degradation, followed by acid degradation, which was similar to the results reported for cholecalciferol [18]. For ergocalciferol, complete degradation has been reported upon acid and base hydrolysis. This may be due to the unsaturation present in the structure of ergocalciferol [2]. Oxidative stress resulted in the least degradation, as the oxidation of ergocalciferol was temperature-dependent [21], with degradation being negligible at the temperature of 30 °C, at which the test was conducted.

#### Exposure to UV radiation for conversion of ergosterol to ergocalciferol

Ergosterol is a vitamin D<sub>2</sub> precursor. UV radiation cleaves the B ring of ergosterol to give ergocalciferol. Ergosterol is converted to pre-ergocalciferol, which in turn isomerizes to ergocalciferol. Both pre-ergocalciferol and ergocalciferol absorb UV radiation and are converted to a variety of by-products.

The extracted ergosterol was exposed to UV-B radiation to convert it into ergocalciferol. Extraction of ergosterol was optimized to attain maximum possible content of vitamin D from the

**Tab. 4.** Liquid chromatography-mass spectroscopy analysis of degradation products.

Degradation	Stress conditions	Number of integrated peaks	Retention time $R_t$ [min]	Corresponding mass fragments identified ( $m/z$ )
Acid	0.1 mol·l <sup>-1</sup> HCl, 80 °C, 20 min	4	2.495 2.811 3.074 7.150	243.3; 211.2; 102.2 304.2; 181.2; 104.1 181.1; 338.1 384.9 (molecular ion); 248.9; 226.9; 113.0 (tachysterol)
Base	0.1 mol·l <sup>-1</sup> NaOH, 80 °C, 20 min	3	2.495 2.811 3.074	243.3; 211.2; 102.2 304.2; 181.2; 104.1 181.1; 338.1
Oxidative	15% H <sub>2</sub> O <sub>2</sub> , 30 °C, 1 h	2	2.200 2.772	171.2; 307.0; 385.3 353.3; 376.3
Thermal	80 °C, 2 h	2	2.664 2.890	179.1; 315.0; 381.1 135.1; 113.0; 248.9; 271.1
Photolytic	UV light, 2 h	1	2.761	353.3; 376.3

mushroom powder, so that it could be converted to vitamin D<sub>2</sub> by exposing to UV radiation. Batches of extracts with initial ergosterol concentration of 128 mg·l<sup>-1</sup> were subjected to UV-B radiation for various time periods to optimize the time required to obtain the maximum of vitamin D. As the time of exposure increased, the amount of ergocalciferol increased. Maximum conversion was attained after 48 h. Nevertheless, some amount of ergosterol was still present in the solution after 48 h of UV exposure. However, further exposure to UV radiations resulted in higher degradation of ergosterol rather than in its conversion to ergocalciferol. Thus, exposure for 48 h was found to be optimal for obtaining maximum amount of vitamin D. Based on the DoE data, maximum ergosterol was extracted and exposed to UV to get the corresponding high yield of vitamin D<sub>2</sub>. The maximum yield obtained was 1290.3 mg·kg<sup>-1</sup> dry weight of mushrooms.

In a similar kind of study with fruiting body exposure to UV radiation, 12.48, 6.58 and 7.58 mg·kg<sup>-1</sup> dry weight of vitamin D<sub>2</sub> in *A. bisporus* mushroom were reported [22]. In another study with direct exposure of the fruiting body, maximum vitamin D<sub>2</sub> yield was reported to be 23.1 mg·kg<sup>-1</sup> [23], which was less compared to our study. Vitamin D content was enhanced in numerous investigations as discussed above by exposing the mushrooms directly to UV radiation for a short period of time instead of extracting and exposing the extract. However, this was found to result in lower yields and prolonged exposure of mushrooms to UV radiations altered their appearance and rendered them rubbery, which made it difficult to pulverize the mushrooms for extraction. Thus, shorter periods of UV exposure were recommended [24]. Thus, UV exposure after ex-

traction was found to be more suitable and resulted in the maximum yield of ergosterol from the mushroom powder. Our study presents the highest yield of vitamin D from *A. bisporus* mushrooms till today.

## CONCLUSION

Ergosterol was efficiently extracted from *A. bisporus* mushrooms using HDES based on tetrabutyl ammonium bromide and decanoic acid. DoE approach was used to optimize the extraction of ergosterol, wherein the highest yield of 3900 mg·kg<sup>-1</sup> dry weight of mushroom was obtained after 5 min of stirring, 45 min of sonication, and using 20 ml of the extracting solvent per 1 g of lyophilized mushroom powder. Use of HDES resulted in the highest extraction efficiency, when compared with other binary and ternary DESs. The extracted ergosterol was purified by preparative HPLC. The conversion of ergosterol to ergocalciferol by UV-B exposure was studied and the exposure time was optimized to attain a maximum ergocalciferol content of 1290.3 mg·kg<sup>-1</sup> dry weight of mushrooms. The ergocalciferol yield obtained in this study is the highest reported for *A. bisporus* mushrooms.

This study showed that hydrophobic deep eutectic solvents may be employed as „green solvents“ for efficient extraction of ergosterol and related compounds like ergocalciferol or cholecalciferol.

Stability studies were conducted on ergocalciferol to determine the effect of hydrolytic, oxidative, photolytic and thermal stresses and to ensure the stability of ergocalciferol during analysis or production. It was found that the highest degra-

dation was observed due to base-mediated hydrolysis, while oxidative stress resulted in the least degradation of ergocalciferol. Stability-indicating methods, like the one developed, can be used for product qualification and assurance and to validate the assigned shelf-life of a drug or bioactive compound or formulations containing these.

The HPLC and the LC-MS analysis together revealed that the developed HPLC method was a stability-indicating one and is suitable for analysing the stability of vitamin D<sub>2</sub> during various extraction processes from natural matrices like mushrooms. The optimized method was successfully validated and the examined parameters met the defined acceptance criteria.

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