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A Case Study on Surplus Mushrooms Production: Extraction and Recovery of Vitamin D_2

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Abstract: The presented case study illustrates the possibility of adding value to the biological surplus remaining from the mushroom cultivation industry. In essence, the unused mushroom parts were submitted to UV-C irradiation, with the purpose of increasing the vitamin D_2 content and validating its extraction. Vitamin D_2 concentration in three different mushroom species (*Agaricus bisporus*, *A. bisporus* Portobello, and *Pleurotus ostreatus*) was obtained by high-performance liquid chromatography (HPLC), by means of an ultraviolet (UV) detector. The method was validated using an *A. bisporus* Portobello sample, and its reproducibility and accuracy were confirmed. Independently of the UV-C irradiation dose, the effect on vitamin D_2 concentration was significant, allowing it to increase from less than 4 μg/g dry weight (dw) to more than 100 μg/g dw in all mushroom species. These results are good indicators of the feasibility of industrial surplus mushrooms as sustainable vitamin D_2 food sources, besides contributing to strengthen the circularity principals associated to the mushroom production chain.

Keywords: surplus mushroom; natural resources; UV-C irradiation; vitamin D_2; natural-based ingredients; circular economy

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1. Introduction

The global mushroom market share is dominated by *Agaricus bisporus* (J.E.Lange) Imbach (button mushroom), *Lentinula edodes* (Berk.) Pegler (shiitake mushroom) and *Pleurotus ostreatus* (Jacq.: Fr.) P. Kumm. (oyster mushroom) [1]. The production of mushrooms and truffles is dominated by Asia (78.2%), followed by Europe (14.7%), and then the Americas (6.2%) [2]. During mushroom production, a percentage as high as 20% of surplus might be generated [3]. These mushrooms have low industrial application, because they are in an advanced stage of maturation, or they have deformed lids and/or stems that do not meet the specifications established by retailers, so they are considered mushrooms of low economic value. These unused mushrooms are often discarded, even though their high nutritional compounds (e.g., proteins, carbohydrates) and valuable chemical compounds (e.g., amino acids, polysaccharides, sterols) are not compromised [3–6].

Currently, the disposal procedures (such as incineration, burying, and landfilling) employed to eliminate these surplus mushrooms generate some cost and may have an environmental impact; these techniques can cause water source contamination, acidification, eutrophication, air pollution, depletion of natural resources, eco-toxicity, among others [3,6].

In this sense, innovative alternatives to add value to this surplus mushroom production need to be explored. The irradiation of surplus mushrooms to obtain vitamin D_2 is a sustainable strategy to increase vitamin D availability. In Europe, for example, assessments

of vitamin D intake showed that for 77–100% of adults (19–64 years old) and 55–100% of elderly adults (>64 years old), vitamin D intake is inadequate [7,8]. In recent years, with advances in the food industry, in parallel with consumer demand for natural-based options, fortified or enriched foods with natural vitamin D_2 are an innovative alternative, particularly for specific groups such as vegans [9,10]. In addition, surplus mushroom production can be used to prepare vitamin D_2-enriched extracts that could be applied by the pharmaceutical industry as nutritive supplements [3].

In their natural state, mushrooms present very low concentrations of vitamin D_2 [11]. Nonetheless, several researchers have found them to be a rich source of ergosterol (a precursor of vitamin D_2), which can be converted into vitamin D_2 by artificial UV irradiation [11,12]. Studies assessing the effects of radiation on ergosterol conversion into vitamin D_2 using UV light are mostly available for cultivated species namely the ones with high production value [11,13]. There are several examples of mushroom species where some amounts of vitamin D_2 have been developed after irradiation [11,13–16]. To the best of the authors' knowledge, the present study provides the first report of the use of surplus mushrooms as a sustainable source of vitamin D_2.

For natural vitamin D_2 to be a real and promising alternative, it is necessary to find suitable methodologies for its extraction, and to develop effective recovery processes. Several technologies are available to extract and quantify vitamin D_2 [17]. However, the effectiveness of the employed methodologies is affected by factors such as time, temperature, power, and solvent type. It is therefore important to combine the best operational conditions to achieve the best vitamin D_2 recovery indices [3,18].

In view of the growing consumer demand for natural-based ingredients, the objective of this case study was to set the UV-C irradiation and extraction conditions that maximize vitamin D_2 contents in the surplus mushrooms production and, meeting the concept reduce-reuse-recycle to minimize the surplus in the mushrooms production sector. The bioactive effects and potential toxicity of vitamin D_2-enriched extracts were also evaluated.

2. Materials and Methods

2.1. Samples Information, UV-C Irradiation and Reagents

The surplus production from *P. ostreatus* and *A. bisporus* (Portobello and white mushroom) were supplied by Ponto Agrícola, Baião, north of Portugal. Subsequently, the fresh samples were sliced (2 to 3 mm) and divided into the following four groups with twenty specimens in each group: control (non-irradiated, 0.0 mJ/cm^2), sample 1 (200 mJ/cm^2), sample 2 (800 mJ/cm^2) and sample 3 (3200 mJ/cm^2) [12,19].

The irradiation was performed at the Centro de Investigação de Montanha of Instituto Politécnico de Bragança, Portugal and took place in an ultraviolet (UV-C) radiation chamber (JP Selecta, Barcelona, Spain) with the following different exposure times: 0, 2, 6 and 10 min. Before analysis, the samples were lyophilized and reduced to a fine, dried powder, and mixed to obtain homogenized samples.

The standard of pure vitamin D_2 was purchased from Acrōs Organics (Fair Lawn, NJ, USA). HPLC-grade acetonitrile (99.9%) and n-hexane (95%) were purchased from Fisher Scientific (Lisbon, Portugal). Dimethyl sulfoxide was purchased from Fisher Scientific (Loughborough, UK), sulforhodamine B and ellipticine were acquired from Sigma-Aldrich (St. Louis, MO, USA), four human tumor cell lines were acquired from Leibniz-Institut DSMZ and one non-tumoral cell line was obtained from ATCC, LGC Standards (Middlesex, UK).

Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). Methanol and acetonitrile were of HPLC grade, from Lab-Scan (Dublin, Ireland).

2.2. Method Proof Assays

The sensitivity and linearity of the HPLC analysis were determined and the method was validated by the instrumental precision, repeatability, and accuracy, using the best

extract obtained. Precision was accessed after six extractions of the same sample; each one being analyzed twice in the same day. The repeatability was accomplished by analyzing the mushroom sample, six times in the same day. The accuracy of the method was evaluated by the standard addition procedure (percentage of recovery), with three additional levels (25%, 50%, and 100% of the peak/area concentration), each one in triplicate. The standard mixture (vitamin D_2) was added to the sample and the extraction procedure was carried out [20].

2.3. Extraction Methodology and Chromatographic Analysis

Vitamin D_2 was extracted and analyzed according to the method described by Huang et al. [21], with some modifications. Mushroom powder (2.5 g) was mixed with 10 mL of dimethyl sulfoxide and ultrasound-oscillated at 45 °C for 30 min. Then 10 mL of methanol and water (1:1, *v*/*v*) and 20 mL of hexane were added, and the mixture was ultrasound-oscillated at 45 °C for 30 min and centrifuged at 5200× *g* for 5 min (Centurion K24OR, West Sussex, UK). The residue was extracted twice with 20 mL of hexane and centrifuged. The combined filtrate was rotary evaporated (Hei-VAP Advantage, Heidolph, Germany) at 40 °C to dryness, redissolved in 1 mL of methanol (Fisher Scientific, Loughborough, UK), and filtered using a 0.1 µm Whatman nylon filter (Millipore, Billerica, MA, USA) before HPLC injection.

The HPLC system (Knauer system, Smartline 1000, Berlin, Germany) coupled to a UV detector (Knauer Smartline 2500) was used under the same conditions described and optimized by Barreira et al. [22]. Chromatographic separation was performed with an Inertsil 100A ODS-3 reverse phase column (5 µm, 250 × 4.6 mm, BGB Analytik AG, Boeckten, Switzerland) at 35 °C. The mobile phase used was acetonitrile/methanol (70:30, *v*/*v*) at a flow rate of 1 mL/min, with an injection volume of 20 µL and the wavelength was 280 nm. Subsequently, the results were analyzed using the Clarity 2.4 software (DataApex, Pod Ohradska, Czech Republic). Vitamin D_2 was quantified based on a calibration curve obtained from a commercial standard vitamin D_2, and the results were expressed in µg per g of dry weight (dw).

2.4. Bioactivity of the Vitamin D_2-Enriched Extract

According to the extraction results, the most potent extract (*A. bisporus* Portobello irradiated with UV-C, 6 min, 3200 mJ/cm^2) was chosen to test the bioactivity in cell lines. In this assay, four human tumor cell lines (MCF-7—breast adenocarcinoma, NCI-H460—non-small cell lung cancer, AGS—gastric cancer, and CaCo-2—colorectal adenocarcinoma) and one non-tumoral cell line of bone origin (h-FOB 1.19—human osteoblasts) were used.

Cell proliferation in the presence and absence of functional extract and pure vitamin D_2 was assessed using the sulforhodamine B (SRB) assay. For this assay, the extract prepared above was dissolved in water at a concentration of 8 mg/mL, the procedures were performed as described previously by the authors [23,24], and the final concentrations were 400, 100, 25 and 6.25 mg/mL. Ellipticin was used as a positive control. Absorbance was measured at 495 nm and the results were expressed as GI_{50} values (sample concentration that inhibited 50% of cell growth) in µg per mL.

2.5. Statistical Analysis

All analyses (extractions) were performed in triplicate and each replicate was also quantified three times. Data were expressed as mean ± standard deviation, presenting the significant numbers in agreement with the magnitude of the corresponding standard deviation.

All statistical tests were applied considering a 5% significance level (IBM SPSS Statistics for Windows, Version 22.0. Armonk; IBM Corp., Armonk, NY, USA). The results were compared through two-way analysis of variance (ANOVA) with type III sums of squares, performed using the general linear model (GLM) procedure. The analyzed statistical factors were "exposure time" (ET) and "ultraviolet radiation" (UV-C) and their effects were

classified through the HSD Tukey's test. The statistical interaction among these two factors was verified in all cases.

3. Results and Discussion

3.1. Method Validation

For this case study, before the surplus mushroom extract analysis, the correlation coefficient (R^2), linearity range, and limits of detection and quantification (LOD and LOQ, respectively) of the methodology employed to determine vitamin D_2, were fully validated (Table 1). After the linearity check (linearity range: 0.78–50 µg/mL), a seven-level calibration curve (y = 11.909x + 6.9688) was made, using the peak/area ratio versus concentration of the standard concentration (µg/mL), reaching a correlation coefficient of 0.9992. The average of the double determinations for each level was used.

Table 1. Calibration parameters of the method for vitamin D_2 detection and quantification, and method validation parameters using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm^2).

Calibration Curve	Correlation Coefficient (R^2)	Linearity Range (µg/mL)	Limit	
			LOD [1] (µg/mL)	LOQ [2] (µg/mL)
y = 11.909x + 6.9688	0.9992	0.78–50	1.67	5.07
Precision CV, % (n = 6)	Accuracy (recovery, %)		Precision CV, % (n = 6)	
0.82	1.35		94	

[1] LOD: limit of detection of the chromatographic method; [2] LOQ: limit of quantification of the chromatographic method; CV: coefficient of variation.

The LOD, calculated as the concentration corresponding to three times the standard error of the calibration curve, divided by the inclination, was 1.67 µg/mL, while the LOQ, i.d., the concentration corresponding to ten times the calibration error, divided by the inclination, was 5.07 µg/mL.

In order to evaluate the instrumental precision, the mushroom sample (*A. bisporus* Portobello, irradiated for 6 min at 3200 mJ/cm^2) was injected six times, and the chromatographic method proved to be precise, according to the coefficient of variation (CV) of 0.82%. Repeatability was evaluated by applying the whole extraction procedure six times to the same sample, and the CV value obtained was low (1.35%). The method accuracy was evaluated by the standard addition procedure (% of recovery). The standard mixture was added to the samples in three concentration levels (25%, 50% and 100% of the peak/area concentration, each one in duplicate) before the extraction. The method showed good recovery results, with an average of 94% (Table 1).

3.2. Conversion Conditions

The starting point is the use of surplus mushrooms as a sustainable material to obtain vitamin D_2, avoiding the use of mushroom suitable for commercialization.

In this sense, Table 2 presents the vitamin D_2-enriched extracts content in different mushroom species, exposed to different UV-C radiation doses and exposure times.

As it is mandatory in any two-way ANOVA, the possible interaction among the assayed factors was verified (ET × UV-C). Since the interaction proved to be significant ($p < 0.050$) in all the cases, it became obvious that the effect of one factor depends on the level of the second.

Therefore, the variation induced by every single factor could not be classified. Nonetheless, it was possible to observe some evident trends, as confirmed by the individual p-values of each factor. A significant increase (from less than 4 µg/g dw to more than 100 µg/g dw in all the cases) in vitamin D_2 concentration was observed with the application of this irradiation type, most likely due to the conversion of ergosterol naturally present in the assayed mushroom. Furthermore, there were no significant differences in the result of using 200, 800 or 3200 mJ/cm^2, which indicates that the vitamin D_2 increase may be

achieved with the least energetic consumption, making this processing approach more competitive and with minimal environmental impact.

Table 2. Vitamin D_2 content in different mushroom species exposed to different UV-C radiation doses and exposure times (ET). The results are presented as mean ± SD [1].

		Vitamin D_2-Enriched Extracts (μg/g dw)		
		A. bisporus	***A. bisporus* Portobello**	***P. ostreatus***
ET/min	0	3.77 ± 0.02	3.7 ± 0.2	2.38 ± 0.04
	2	84 ± 7	109 ± 6	97 ± 11
	6	125 ± 8	124 ± 11	125 ± 11
	10	122 ± 1	127 ± 5	119 ± 7
ANOVA *p*-value [2]		<0.001	<0.001	<0.001
UV-C (mJ/cm^2)	0	3.77 ± 0.02	3.7 ± 0.2	2.38 ± 0.04
	200	104 ± 22	113 ± 9	107 ± 20
	800	111 ± 21	119 ± 9	114 ± 12
	3200	116 ± 19	128 ± 10	119 ± 14
ANOVA *p*-value [2]		<0.001	<0.001	<0.001
ET × UV-C *p*-value [3]		<0.001	0.035	<0.001

[1] Results are reported as mean values of each parameter (ET or UV-C), combining all exposure times and irradiation doses (from ET or UV-C). [2] If $p < 0.05$, the corresponding parameter presented a significantly different value for at least one ET or UV-C. [3] The interaction among factors was significant in all cases; thereby the statistical classification could not be indicated.

With regard to exposure time, there were no significant differences upon irradiating mushrooms during 6 or 10 min, but the intermediate assayed time was better than the 2 min. Hence, the optimal exposure time, considering the results obtained with the surplus of assayed mushroom species, turned out to be 6 min. The origin of the mushroom, applied dose, time after harvest, positioning of the mushrooms to the light source, fresh or dried samples, whole or sliced samples, the method by which vitamin D_2 has been extracted, among others, influence the results obtained [12].

3.3. Vitamin D_2-Enriched Extracts

As for the mushrooms evaluated in this work (e.g., for 6 min at 3200 mJ/cm^2), vitamin D_2-enriched extract levels in the Portobello *A. bisporus* samples reached a maximum concentration of 124 μg/g dw, and in the white *A. bisporus* and *P. ostreatus* samples they reached values of 125 μg/g dw (Table 2, Figure 1).

In UV-C-irradiated *P. ostreatus* samples, Hu et al. [13] reported a maximum concentration of approximately 24 μg/g dw of vitamin D_2 content. Teichmann et al. [25] reported 10.14 μg/g dw in white *A. bisporus* samples, Guan et al. [26] reported 13.4 and 9.5 μg/g dw in white and Portobello *A. bisporus* samples, respectively, and Jasinghe and Perera [14] reported 34.4 μg/g dw in white button mushrooms. Similarly, for UV-C-irradiated shiitake (*Lentinula edodes*) mushroom, Xu et al. [27] obtained an increase in vitamin D_2 content, until 20.11 μg/g dw.

Concerning UV-B irradiation, Urbain et al. [28] and Urbain et al. [29] obtained 56.8 and 67.1 μg/g dw of vitamin D_2, respectively, in button mushrooms; Nölle et al. [30] reported that fresh whole *A. bisporus*, followed by freeze-drying, obtained 45 μg/g dw of vitamin D_2, and slicing before UV-B irradiation led to a ten-fold increase.

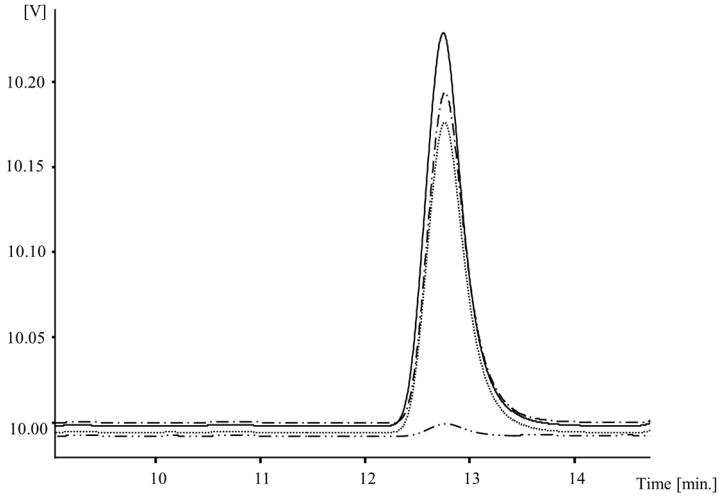

Figure 1. Vitamin D_2-enriched extracts chromatogram profile of *Agaricus bisporus* Portobello (-), white *A. bisporus* (.-.-) and *Pleurotus ostreatus* (...) irradiated with UV-C (6 min at 3200 mJ/cm^2), and *A. bisporus* Portobello control samples (..-..-).

There are dissimilarities in the irradiation process and conditions to maximize the photoconversion of ergosterol into vitamin D_2 in mushrooms, and most of these cited studies were performed with the whole intact mushroom, with a longer irradiation time (20 min) and higher irradiation dose.

In this sense, based on the case study considered in this work, we make the first attempt to establish the irradiation conditions and extraction procedure needed to maximize ergosterol conversion to vitamin D_2 from surplus mushroom production, avoiding the need to use mushroom samples that are suitable to be commercialized.

3.4. Bioactivity of the Vitamin D_2-Enriched Extract

The in vitro cytotoxicity of the vitamin D_2-enriched extract and pure vitamin D_2 was analyzed. The effect of the vitamin D_2-enriched extract and pure vitamin D_2 in human tumoral cell lines (MCF-7, NCI-H460, AGS and CaCo) and non-tumoral bone cell line (h-FOB 1.19) growth are presented in Table 3. The GI_{50} values represent the extract concentrations that cause a 50% inhibition of cell growth.

Table 3. Antiproliferative and cytotoxicity activities of vitamin D_2-enriched extracts using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm^2) and pure vitamin D_2 (mean ± SD, n = 9).

	MCF-7	NCI-H460	AGS	CaCo	h-FOB 1.19
Vitamin D_2-enriched extracts (GI_{50} μg/mL)	>400	293 ± 17 ^{b}	82 ± 9 ^{c}	377 ± 24 ^{a}	>400
Vitamin D_2 pure (GI_{50} μg/mL)	>400	>400	>400	>400	>400

The cytotoxicity results were expressed as GI_{50} values, corresponding to the sample concentration that inhibited 50% of the net cell growth. In row, different letters mean significant differences ($p < 0.05$).

The sample of pure vitamin D_2 tested did not reveal cytotoxicity at the evaluated concentrations (GI_{50} values > 400 μg/mL) for all the cell lines tested (tumoral and non-tumoral). However, the vitamin D_2-enriched extract presented effective activity in the AGS (82 μg/mL) tumoral cell line, and moderate activity in the NCI-H460 (293 μg/mL) and CaCo (377 μg/mL) tumoral cell lines.

The results obtained indicate that these effects may be related to the compounds (including ergosterol, phenolic compounds, organic acids, etc.) present in the mushroom extract, since the mushrooms are a rich source of bioactive compounds [31]. It is noteworthy

that neither vitamin D_2-enriched extracts or pure vitamin D_2 presented cytotoxicity against the non-tumoral bone cell, h-FOB 1.19 (GI_{50} > 400 µg/mL).

4. Conclusions

Based on the case study considered, vitamin D_2-enriched extracts were obtained by HPLC-UV, using a methodology that proved to be reproducible and accurate. Vitamin D_2 was identified and quantified, and *A. bisporus* Portobello was the species with the highest total content. The recovery of vitamin D_2 from surplus mushrooms presents an interesting valorization and sustainable approach.

The use of vitamin D_2-enriched extracts from surplus mushroom production could benefit several bio-based industries, since the applications of vitamin D_2 from this sustainable material are nonexistent. Accordingly, the development of food applications of mushroom vitamin D_2-enriched extract, from surplus mushroom production, can be considered and valued. It could be of added value to promote the agricultural sector or the pharmaceutical industries.

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