



ValorNatural – Valorização de Recursos Naturais através da Extração de Ingredientes de Elevado Valor Acrescentado para Aplicações na Indústria Alimentar.

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Sumário

Este entregável diz respeito ao trabalho desenvolvido e submetido à revista *Agriculture* e refere as condições de otimização da extração e obtenção da vitamina D2 a partir do excedente da produção de cogumelos. Foram avaliados o efeito bioativo e a potencial toxicidade do extrato enriquecido com vitamina D2.

Índice

1. Identificação	5
2. Informação	6

1. Identificação

<i>Deliverable</i>	E.5.1.7. Publicação dos ingredientes com melhor capacidade de aumento da absorção de cálcio e sem toxicidade
<i>Tipo de deliverable</i>	Publicação
<i>Nível de disseminação</i>	Público
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2. Informação

Amostragem e irradiação UV-C

O excedente da produção de *Pleurotus ostreatus* e *Agaricus bisporus* (Portobello e cogumelo branco) foi fornecido pelo Ponto Agrícola, Baião, Norte de Portugal. Posteriormente, as amostras foram divididas em quatro grupos com vinte espécimes em cada grupo: controlo (não irradiado, 0,0 mJ/cm²), amostra 1 (200 mJ/cm²), amostra 2 (800 mJ/cm²) e amostra 3 (3200 mJ/cm²). A irradiação foi realizada no Centro de Investigação de Montanha do Instituto Politécnico de Bragança e decorreu numa câmara de radiação ultravioleta (UV-C) (JP Selecta, Espanha) com diferentes tempos de exposição: 0, 2, 6 e 10 min (**Figura 1**). Antes da análise, as amostras foram liofilizadas, trituradas e homogeneizadas.

Ensaios de validação do método

A sensibilidade e linearidade da análise por Cromatografia Líquida de Alta Eficiência (HPLC) foram determinadas e o método foi validado de acordo com a precisão instrumental, repetibilidade e exatidão, utilizando, para tal, o melhor extrato obtido. A precisão foi determinada após seis extrações da mesma amostra e cada uma analisada em duplicado no mesmo dia. A repetibilidade foi determinada fazendo a análise das amostras seis vezes no mesmo dia. A precisão do método foi determinada pelo procedimento da adição do padrão (percentagem de recuperação), com três níveis de adição (25, 50 e 100%) e analisados em triplicado. A mistura padrão (vitamina D2) foi adicionada à amostra e o procedimento de extração foi realizado.

Metodologia de extração e análise cromatográfica

A vitamina D2 foi extraída e analisada de acordo com o método descrito por Huang et al. [1] com algumas modificações.

Foram adicionados 5 mL de DMSO:H₂O (9:1) a 2,5g de cada amostra liofilizada, procedeu-se à incubação em banho-maria a 60 °C durante 10 min. De seguida, as amostras foram arrefecidas em gelo, adicionou-se 10 mL de isoctano e ficaram sob agitação constante durante a noite à temperatura ambiente. Após esta etapa, adicionou-se 2 mL de água desionizada e deixou-se em repouso (15 min.) para separar o DMSO do isoctano e, em seguida, as amostras foram centrifugadas (2000 × g, 10 min, centrífuga refrigerada,

Centurion K24OR, West Sussex, Reino Unido). Posteriormente, retirou-se o sobrenadante das amostras e procedeu-se à sua evaporação ($\pm 40\text{ }^{\circ}\text{C}$, Hei-VAP Advantage, Heidolph, Germane). Os extratos secos foram redissolvidos em 1 mL de metanol, filtrados (nylon Whatman de $0,1\text{ }\mu\text{m}$) e analisados por cromatografia líquida [2].

A análise cromatográfica foi realizada por cromatografia líquida de alta eficiência (HPLC, sistema Knauer, Smartline 1000, Berlin, Germany) acoplada a um detetor ultravioleta (UV, Knauer Smartline 2500), operando nas condições descritas pelos autores Barreira et al. [3]. A separação cromatográfica foi realizada com uma coluna de fase reversa Inertsil 100A ODS-3 ($5\text{ }\mu\text{m}$, $250 \times 4,6\text{ mm}$, BGB Analytik AG, Boeckten, Suíça) a $35\text{ }^{\circ}\text{C}$. A fase móvel utilizada foi metanol/acetonitrilo (30:70, v/v) com o fluxo de 1 mL/min, volume de injeção de $20\text{ }\mu\text{L}$ e comprimento de onda de 280 nm (**Figura 1**).

Posteriormente, os resultados foram analisados utilizando o software Clarity 2.4 (DataApex, Pod ohradská, República Checa), a vitamina D2 foi quantificada com base na curva de calibração obtida com o padrão comercial da vitamina D2 (Acrós Organics, Nova Jérсия, EUA), e os resultados foram expressos em μg de VitD2 por g de massa seca.

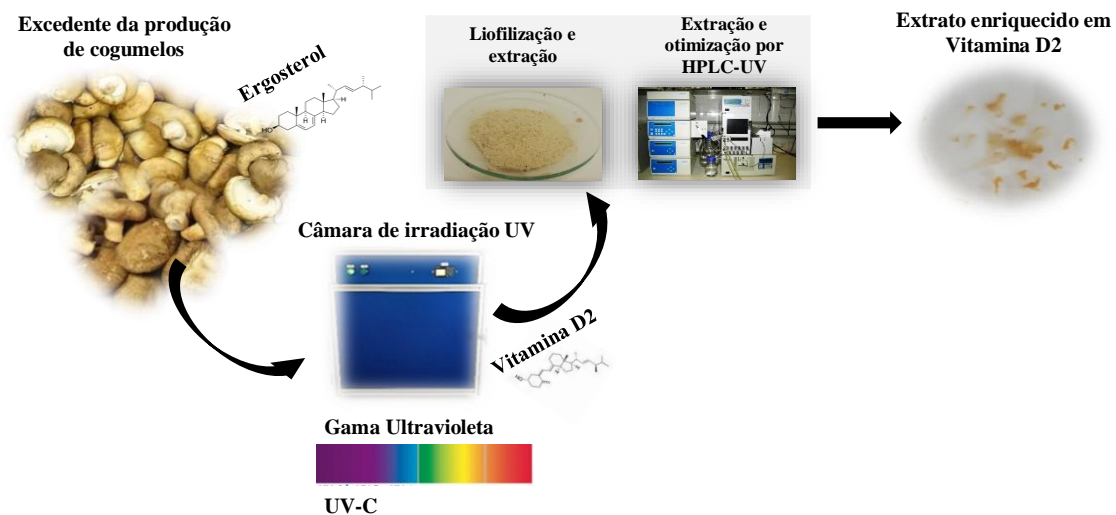


Figura 1. Representação esquemática do processo para a obtenção do extrato enriquecido em vitamina D2.

Bioatividade dos extratos enriquecidos em vitamina D2

De acordo com os resultados da extração, o melhor extrato (*A. bisporus* Portobelo irradiado com UV-C, 6 min, 3200 mJ/cm²) foi escolhido para testar a bioatividade em linhas celulares. Neste ensaio, três linhas de células tumorais (MCF-7 - adenocarcinoma de mama, NCI-H460 – carcinoma de pulmão e AGS - carcinoma gástrico) e uma linha de células não tumorais de origem óssea (h-FOB 1.19 - Osteoblastos humanos) foram utilizadas. A proliferação celular na presença e ausência do extrato enriquecido em vitamina D2 e vitamina D2 pura foi avaliada usando o ensaio sulforodamina B (SRB).

Para este ensaio, os extratos foram dissolvidos em água na concentração de 8 mg/mL, os procedimentos foram realizados conforme descrito pelos autores [4, 5] e, as concentrações finais foram 400, 100, 25 e 6,25 mg/mL. A elipticina foi usada como controlo positivo. A absorvância foi medida a 495 nm e os resultados foram expressos como valores de GI₅₀ (concentração de extrato que inibiu 50% do crescimento celular) em µg por mL.

Método de validação

Antecedente à análise dos extratos, o coeficiente de correlação (R^2), o intervalo de linearidade e os limites de deteção e quantificação (LOD e LOQ) da metodologia utilizada para determinar a vitamina D2 foram validados.

Após a verificação da linearidade (intervalo de linearidade: 0,78-50 µg/mL), a curva de calibração com sete níveis ($y = 11,909x + 6,9688$) foi realizada utilizando a relação pico/área *versus* concentração do padrão (µg/mL), verificando-se um coeficiente de correlação de 0,9992.

O LOD foi de 1,67 µg/mL e o LOQ foi de 5,07 µg/mL. Para avaliar a precisão instrumental, a amostra de cogumelo (*A. bisporus* Portobello, irradiado durante 6 min a 3200 mJ/cm²) foi analisada seis vezes, e o método cromatográfico utilizado evidenciou ser preciso, apresentando o coeficiente de variação (CV) de 0,82%. Para a repetibilidade obteve-se o CV de 1,35%. A precisão do método foi avaliada pelo procedimento da adição do padrão (% de recuperação) e apresentou resultados de recuperação de 94%.

Condições de conversão

A **Tabela 1** apresenta o conteúdo da vitamina D2 nas diferentes espécies de cogumelos expostas a diferentes intensidades de radiação UV-C e tempos de exposição.

Verificou-se uma interação significativa ($p < 0,050$) entre o tempo de exposição (TE) e as intensidades de irradiação UV-C aplicadas. No que diz respeito ao TE, não se verificaram diferenças significativas com os tempos de 6 ou 10 min., sendo o tempo de 6 min. o que apresentou melhores resultados comparativamente com o tempo de 2 min. Assim, o tempo ótimo de exposição, considerando os resultados obtidos é de 6 min.

No que diz respeito ao efeito da irradiação UV-C, verificou-se um aumento significativo do conteúdo de vitamina D2 (de aproximadamente 4 $\mu\text{g/g}$ de massa seca para mais de 100 $\mu\text{g/g}$ de massa seca em todos os casos). Não houve diferenças significativas no resultado da utilização de 200, 800 ou 3200 mJ/cm^2 .

Tabela 1. Conteúdo em vitamina D2 nas espécies de cogumelos expostas a diferentes intensidades de radiação UV-C e tempos de exposição. Os resultados são apresentados como média \pm DP.

		Vitamina D2 ($\mu\text{g/g}$ massa seca)		
		<i>A. bisporus</i>	<i>A. bisporus</i> Portobello	<i>P. ostreatus</i>
Tempo de exposição (TE)/min.	0	3,77 \pm 0,02	3,7 \pm 0,2	2,38 \pm 0,04
	2	84 \pm 7	109 \pm 6	97 \pm 11
	6	125 \pm 8	124 \pm 11	125 \pm 11
	10	122 \pm 1	127 \pm 5	119 \pm 7
ANOVA <i>p</i> -valor		<0,001	<0,001	<0,001
UV-C (mJ/cm^2)	0	3,77 \pm 0,02	3,7 \pm 0,2	2,38 \pm 0,04
	200	104 \pm 22	113 \pm 9	107 \pm 20
	800	111 \pm 21	119 \pm 9	114 \pm 12
	3200	116 \pm 19	128 \pm 10	119 \pm 14
ANOVA <i>p</i> -valor		<0,001	<0,001	<0,001
TE \times UV-C <i>p</i> -valor		<0,001	0,035	<0,001

No que se refere aos cogumelos avaliados neste trabalho, o *A. bisporus* Portobello foi o que apresentou melhores resultados. Comparativamente com os resultados descritos na literatura, neste trabalho obtivemos níveis significativamente superiores de vitamina D2.

A origem do cogumelo, a intensidade de luz aplicada, o tempo após a colheita, o posicionamento dos cogumelos relativamente à fonte de luz, método pelo qual a vitamina D2 foi extraída, entre outros, influenciam os resultados obtidos [6].

Nesse sentido, neste trabalho foi feita uma primeira tentativa de identificação das condições de irradiação e o procedimento de extração necessário para maximizar a conversão do ergosterol em vitamina D2 a partir dos excedentes da produção de cogumelos.

Bioatividade do extrato enriquecido em vitamina D2

Foi analisada a citotoxicidade "*in vitro*" do extrato enriquecido em vitamina D2 e do extrato da vitamina D2 pura. O efeito dos extratos nas linhas celulares tumorais (MCF-7, NCI-H460, AGS e CaCo) e na linha celular óssea não tumoral (h-FOB 1.19) (dado pela concentração necessária para inibir em 50% o crescimento das células (GI₅₀), são apresentados na **Tabela 2**.

Tabela 2. Atividade antiproliferativa e citotóxica dos extratos de *Agaricus bisporus* Portobello irradiado com UV-C (6 min, 3200 mJ / cm²) e da vitamina D2 pura (média ± DP, *n* = 9).

	MCF-7	NCI-H460	AGS	CaCo	h-FOB 1.19
Extrato enriquecido em vitamina D2 (GI ₅₀ µg/mL)	>400	293±17	82±9	377±24	>400
Vitamina D2 pura (GI ₅₀ µg/mL)	>400	>400	>400	>400	>400

O extrato da vitamina D2 pura não apresentou citotoxicidade, com valores de GI₅₀ superiores a 400 µg/mL para todas as linhas celulares testadas. Porém, o extrato enriquecido em vitamina D2 apresentou atividade efetiva contra a linha celular tumoral AGS (82 µg/mL) e atividade moderada contra as linhas tumorais NCI-H460 (293 µg/mL) e CaCo (377 µg/mL). O extrato enriquecido em vitamina D2 foi mais eficaz do que o extrato da vitamina D2 pura para a maioria das linhas avaliadas. Estes resultados provavelmente indicam que esse efeito pode estar relacionado com os compostos (incluindo ergosterol, compostos fenólicos, ácidos orgânicos, etc.) presentes no extrato, uma vez que os cogumelos são uma fonte em compostos bioativos [7]. De referir que, nem o extrato enriquecido em vitamina D2 nem a vitamina D2 pura apresentaram citotoxicidade contra a linha celular óssea não tumoral h-FOB 1.19 (GI₅₀> 400 µg / mL).

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Article

A Case Study on Surplus Mushrooms Production: Extraction and Recovery of Vitamin D₂

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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 D₂ content and validating its extraction. Vitamin D₂ 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 D₂ 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 D₂ food sources, besides contributing to strengthen the circularity principals associated to the mushroom production chain.

Keywords: surplus mushroom; natural resources; UV-C irradiation; vitamin D₂; 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 D₂ 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 D₂ are an innovative alternative, particularly for specific groups such as vegans [9,10]. In addition, surplus mushroom production can be used to prepare vitamin D₂-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 D₂ [11]. Nonetheless, several researchers have found them to be a rich source of ergosterol (a precursor of vitamin D₂), which can be converted into vitamin D₂ by artificial UV irradiation [11,12]. Studies assessing the effects of radiation on ergosterol conversion into vitamin D₂ 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 D₂ 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 D₂.

For natural vitamin D₂ 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 D₂ [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 D₂ 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 D₂ 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 D₂-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/cm²), sample 1 (200 mJ/cm²), sample 2 (800 mJ/cm²) and sample 3 (3200 mJ/cm²) [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 D₂ was purchased from Acrôs 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 D₂) was added to the sample and the extraction procedure was carried out [20].

2.3. Extraction Methodology and Chromatographic Analysis

Vitamin D₂ 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 D₂ was quantified based on a calibration curve obtained from a commercial standard vitamin D₂, and the results were expressed in µg per g of dry weight (dw).

2.4. Bioactivity of the Vitamin D₂-Enriched Extract

According to the extraction results, the most potent extract (*A. bisporus* Portobello irradiated with UV-C, 6 min, 3200 mJ/cm²) 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 D₂ 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 GI₅₀ 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 D₂, 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 D₂ detection and quantification, and method validation parameters using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm²).

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/cm²) 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 D₂, avoiding the use of mushroom suitable for commercialization.

In this sense, Table 2 presents the vitamin D₂-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 D₂ 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/cm², which indicates that the vitamin D₂ increase may be

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

Table 2. Vitamin D₂ 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)		
		<i>A. bisporus</i>	<i>A. bisporus</i> Portobello	<i>P. ostreatus</i>
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 <i>p</i> -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 <i>p</i> -value ²		<0.001	<0.001	<0.001
ET × UV-C <i>p</i> -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 D₂ has been extracted, among others, influence the results obtained [12].

3.3. Vitamin D₂-Enriched Extracts

As for the mushrooms evaluated in this work (e.g., for 6 min at 3200 mJ/cm²), vitamin D₂-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 D₂ 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 D₂ 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 D₂, 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 D₂, and slicing before UV-B irradiation led to a ten-fold increase.

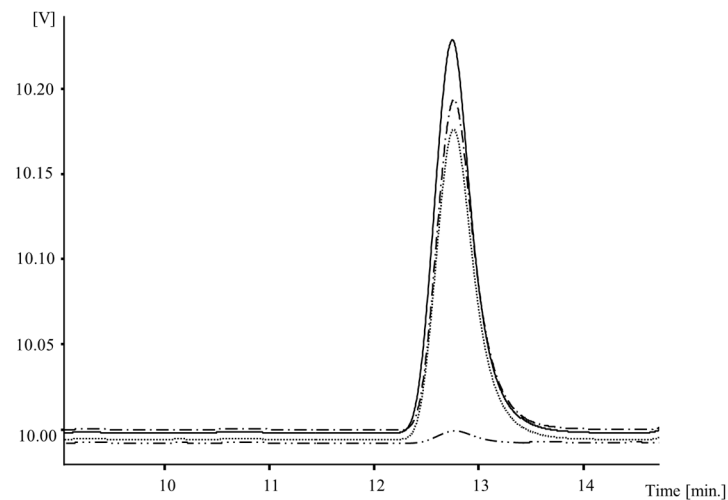


Figure 1. Vitamin D₂-enriched extracts chromatogram profile of *Agaricus bisporus* Portobello (-), white *A. bisporus* (-.-) and *Pleurotus ostreatus* (...) irradiated with UV-C (6 min at 3200 mJ/cm²), and *A. bisporus* Portobello control samples (-.-.-).

There are dissimilarities in the irradiation process and conditions to maximize the photoconversion of ergosterol into vitamin D₂ 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 D₂ from surplus mushroom production, avoiding the need to use mushroom samples that are suitable to be commercialized.

3.4. Bioactivity of the Vitamin D₂-Enriched Extract

The *in vitro* cytotoxicity of the vitamin D₂-enriched extract and pure vitamin D₂ was analyzed. The effect of the vitamin D₂-enriched extract and pure vitamin D₂ 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 GI₅₀ values represent the extract concentrations that cause a 50% inhibition of cell growth.

Table 3. Antiproliferative and cytotoxicity activities of vitamin D₂-enriched extracts using *Agaricus bisporus* Portobello irradiated with UV-C (6 min, 3200 mJ/cm²) and pure vitamin D₂ (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 GI₅₀ 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 D₂ tested did not reveal cytotoxicity at the evaluated concentrations (GI₅₀ values > 400 µg/mL) for all the cell lines tested (tumoral and non-tumoral). However, the vitamin D₂-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 D₂-enriched extracts or pure vitamin D₂ presented cytotoxicity against the non-tumoral bone cell, h-FOB 1.19 (GI₅₀ > 400 µg/mL).

4. Conclusions

Based on the case study considered, vitamin D₂-enriched extracts were obtained by HPLC-UV, using a methodology that proved to be reproducible and accurate. Vitamin D₂ was identified and quantified, and *A. bisporus* Portobello was the species with the highest total content. The recovery of vitamin D₂ from surplus mushrooms presents an interesting valorization and sustainable approach.

The use of vitamin D₂-enriched extracts from surplus mushroom production could benefit several bio-based industries, since the applications of vitamin D₂ from this sustainable material are nonexistent. Accordingly, the development of food applications of mushroom vitamin D₂-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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