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Lentinula edodes. The main process of ergosterol and Vitamin D2 extraction involves methodologies with long extraction times such as Soxhlet extraction, or mechanically agitated extraction with or without saponification

From waste to a nutraceutical quality:
a green and sustainable approach for the recovery of Vitamin D

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Introduction

Food waste is produced in all the phases of food life cycle, i.e. during agricultural production, industrial manufacturing, processing and distribution. Up to 42% of food waste is produced by household activities, 39% losses occurring in the food manufacturing industry and 14% in food service sector (ready to eat food, catering and restaurants), while 5% is lost during distribution (Kumar et al., 2017). Recent reports shows development of high value products (such as cosmetics, foods and medicines) from agro-industrial by-products. There is growing interest of consumers towards food bioactives that provide beneficial effects to humans in terms of health promotion and disease risk reduction. Nowadays, there is growing trend in the food industry toward the development and manufacture of functional and nutraceutical products.
Edible mushroom production has tremendously increased over the last 4 decades (over a 30-fold increase) to meet the continuously growing market demand (Royse et al., 2017; Amin et al., 2021). Beyond their

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Agaricus bisporus. The health benefits of ergosterol and Vitamin D2 stimulated their investigation in the most consumed mushrooms including Agaricus bisporus, Lentinula edodes, and Pleurotus ostreatus

excellent flavor and sensory properties, mushrooms are being increasingly consumed due to their nutritional quality and various health benefits (Gonzalez et al., 2020). Indeed, dried mushrooms are high in proteins (19–37 g/100 g), B vitamins (50–120 mg/100 g), minerals (0.7–1.6 mg/100 g), dietary fibers (7–10 g/100 g), and low in fats (1.6–4.5 g/100 g) (Bekiaris et al., 2020). Moreover, mushrooms are a rich source of various bioactive compounds including potent antioxidants such as L-ergothioneine and glutathione, and hypocholesterolemic compounds such as β-glucans and mycosterols (fungal sterols) (Morales et al., 2017; Nachimuthu et al., 2019). Ergosterol (5,722-ergostatrien-3β-ol), the major product of mycosterol biosynthesis, is an important component of fungal cell membranes that maintain membrane structural integrity, permeability, and fluidity. Moreover, ergosterol has demonstrated various health-promoting effects including immunoactivity, anti-cancer, anti-inflammatory, and hypocholesterolemic effects (Correa et al., 2018). Furthermore, ergosterol shows an additional important interest, being a precursor of vitamin D2, which plays a key role in calcium absorption and bone health (Khare et al., 2021). Recently, Sizar's group showed that the deficiency of Vitamin D was associated with some diseases like cancer, diabetes, heart, and autoimmune diseases (Sizar et al., 2021). Similarly, Jain et al. (2020) concluded that COVID-19 patients with Vitamin D deficiency have more serious inflammation, leading to higher morbidity and mortality. Although the important role of Vitamin D in human life, half of the population in the world has a Vitamin D insufficiency, and the number is still rising, especially among people following vegan and vegetarian diets (Fallon & Dillon, 2020). Therefore, it becomes necessary to find innovative sources of Vitamin D.

It is important to note that, under UV light exposition (270–310 nm), ergosterol can be converted into vitamin D2 (ergocalciferol) (Salemi et al., 2021).

The health benefits of ergosterol and Vitamin D2 stimulated their investigation in the most consumed mushrooms including *Agaricus bisporus*, *Lentinula edodes*, and *Pleurotus ostreatus* (Jiang et al., 2020a). The main process of ergosterol and Vitamin D2 extraction involves methodologies with long extraction times such as Soxhlet extraction, or mechanically agitated extraction with or without saponification (Patnana et al., 2021). More recently, faster non-conventional extraction techniques, such as supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and ultrasound-assisted extraction (U.A.E) have been also considered. The latter (U.A.E), compared to other non-conventional techniques, is more economical, simple, and can be performed under atmospheric conditions.

U.A.E is based on the principle of acoustic cavitation that involves the growth of preexisting microbubbles until they reach an unstable size, which collapses damaging the cell structure of the fungal matrix and thereby facilitating the penetration of solvent and the release of bioactive compounds (Picot-Allain et al., 2021). U.A.E efficiency is influenced by different parameters including the sample pretreatment (drying, size reduction), the type of solvent, the pH, the temperature, the time, the solid/solvent ratio (SSR), the frequency, and the ultrasonic power (Kumar et al., 2020).

The aim of this work is to extract ergosterol and Vitamin D2

from mushrooms waste and to convert ergosterol quantity in Vitamin D2.

Sample preparation

The lyophilized mushrooms powdered samples (100 mg) were extracted using a U.A.E system Labsonic LBS 2 (FALC, Bergamo, Italy). Different U.A.E parameters were assessed to optimize the extraction of ergosterol and vitamin D. Moreover, the presence of conjugated sterols (esterified and glycosides) was studied through the application of acidic and basic hydrolysis treatment. White button samples were used for the optimization of the extraction conditions comparing the levels of ergosterol and vitamin D. Due to the undetectable concentration of vitamin D2 in mushrooms before UV irradiation, the recovery of vitamin D was studied spiking the samples with vitamin D3 (200 Qg/g) (Subramaniam et al., 2021). After extraction, the obtained extract was carefully collected and then filtered using 0.45 Qm filters before HPLC analysis 2.3.1. Optimization of the extraction solvent 6 extraction solvents were compared: ethanol (EtOH), methanol (MeOH), acetonitrile (ACN), hexane (Hex), chloroform (CHCl3), and MeOH/CHCl3 (1:2). 100 mg of lyophilized button mushrooms were extracted with 10 mL of solvent under agitation with a magnetic stirrer at room temperature for 120 min. Extracts from Hex, CHCl3, and MeOH/ CHCl3 were dried under nitrogen and dissolved in 10 mL of EtOH before HPLC analysis. Ergosterol and vitamin D were analyzed using a 1260 Infinity HPLC equipment (Agilent Technologies, Santa Clara, CA, USA), made of a quaternary pump, an autosampler, and a diode array detector (DAD). The chromatographic separation was performed on a Gemini C18 analytical column (250 × 3.0 mm, 5 Qm) preceded by a security guard column C18 (4 × 3 mm, 5 Qm), (Phenomenex, Torrance, CA, USA). The mobile phase consisted of phase A made of water and phase B made of methanol at a flow rate of 0.5 mL/min.

Results and discussions

Mushroom samples were freeze-dried until the complete removal of water. The moisture contents were respectively 91.3 3 0.4 %, 91.2 3 0.8 %, and 90.8 3 0.4 %, for white buttons, portobello buttons, and oyster mushrooms. These levels are closed to those reported in the literature (Lin et al., 2019). Lyophilization was applied as a suitable technique for mushroom drying compared to hot-air drying (80 °C), which is reported to produce a great loss (around 30 %) of vitamin D2 and ergosterol (Bernas & Jaworska, 2017). After freeze-drying, mushrooms were milled and sieved to obtain a standardized particle size and thus, reduce recovery variations from sample to sample.

The HPLC method was validated by evaluating its linearity, sensitivity, and interday/intraday reproducibility. The linearity was assessed by injecting 7 standard solutions of ergosterol (1-200 Qg/ mL) and vitamin D2 (0.1-100 Qg/mL) at different concentrations. The calibration curves obtained showed a coefficient of determination (R^2) of 0.999 for both analytes, demonstrating thus, the linearity of the method. The intraday reproducibility was evaluated by calculating the %RSD between successive analyses (n = 3) performed the same day, while the interday reproducibility was determined on repeated analyses performed on 3 consecutive days. The intraday reproducibility was between 0.04 and 0.6 %, while the interday reproducibility ranged between 0.05 and 0.7 %. These results demonstrate the excellent reproducibility of the analytical method. The sensitivity was determined through the limit of detection (LOD) and the limit of quantification (LOQ). The LODs and LOQs were estimated as the concentrations of analytes giving signal-to-noise ratios (S/N) of 3:1 and 10:1 respectively. The validated method showed a LOD between 0.02 and 0.03 Qg/mL, while the LOQ ranged between 0.05 and 0.09 Qg/mL.

Various solvents have been used for the extraction of ergosterol and vitamin D in the literature. The most used solvents are hexane (Guan et al., 2016), ethanol (Francisco et al., 2018a, 2018b), and chloroform/ methanol (Villares et al., 2014). In this study, 6 solvents were tested (EtOH, MeOH, ACN, Hex, CHCl3, and MeOH/CHCl3) and were compared in terms of recoveries (%) vitamin D and concentrations (mg/g dw) for ergosterol. According to the results reported in Fig. 1, ACN shows the lowest extraction efficiency ($p < 0.05$)

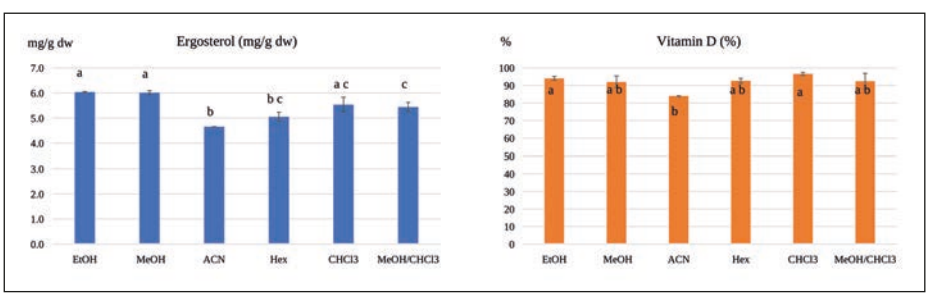

Fig. 1. Effect of solvent on ergosterol and vitamin D extraction in mushrooms. Values with different upper-case letters showed statistically significant differences ($p < 0.05$).

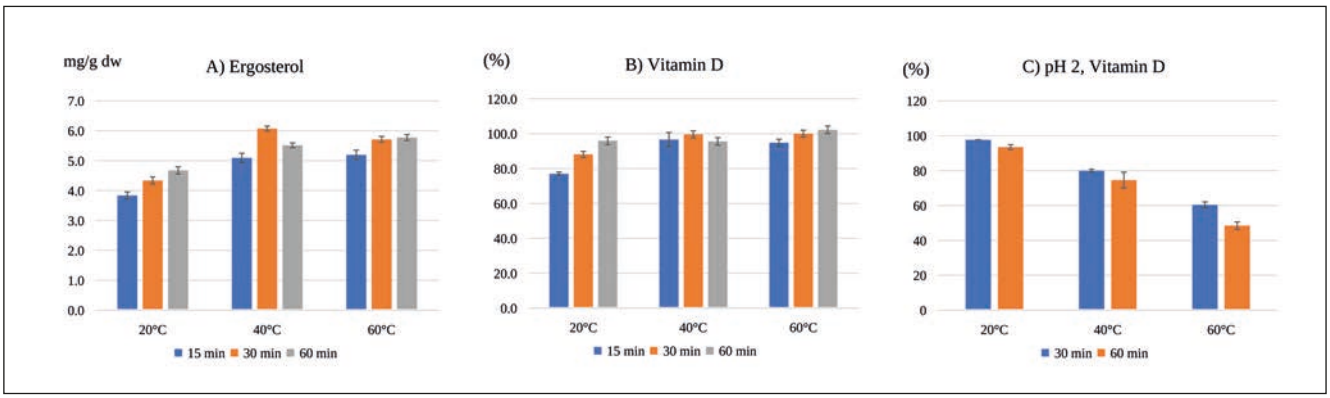

Fig. 2. Effect of time and temperature on the extraction of A) ergosterol (mg/g dw), B) vitamin D (%), and C) vitamin D (%) at pH 2. Values in the same graph with different upper-case letters showed statistically significant differences ($p < 0.05$).

for ergosterol and vitamin D. High extraction was obtained with EtOH, MeOH, and CHCl3 for both analytes. However, the best solvent resulted to be EtOH with extraction levels, which tended to be higher than MeOH, CHCl3, and MeOH/CHCl3 for ergosterol (6.04 3 0.02 mg/g dw) and were close to vitamin D recoveries obtained with CHCl3 (97 3 0.8 %). A similar result was reported by Heleno et al. (2016) but the comparison was limited to 3 solvents (Hex, EtOH, and limonene) for just ergosterol extraction. Hexane shows good recoveries for vitamin D (92.6 3 1.4 %) but a lower extraction efficiency for ergosterol (5.05 3 0.18 mg/g dw)
compared to more polar organic solvent (EtOH, MeOH, and CHCl3). Therefore, extraction methods using hexane or organic solvent with similar polarities can cause an underestimation of the ergosterol content in mushrooms.
Time and temperature are important parameters in UAE. In this study, all the parameters were optimized using EtOH as the extraction solvent. The optimization of time and temperature was assessed maintaining fixed the other UAE parameters. The results showed that the highest extraction yields were obtained at 40 ◦C and 60 ◦C with an extraction time between 30 and 60 min (Fig. 2). At 20 ◦C, the levels of ergosterol and vitamin D increased with time from 15 to 60 min.

However, at higher temperatures, extraction time longer than 30 min did not significantly improve the extraction yield. Therefore, 40 ◦C for 30 min was selected as optimal U.A.E. condition for ergosterol and vitamin D in mushrooms. These results are similar to those reported by Taofiq et al. (2019), in which 30 min resulted as the best extraction time for the U.A.E of ergosterol in mushrooms. However, in Taofiq et al. study, the effect of longer extraction times (> 30 min) and higher temperatures (40-60 ◦C) was not assessed.

The increased request for foods with high contents in provitamin and vitamin D requires the development of a standardized, fast, and costeffective extraction method to compare the results obtained from different research teams. Based on U.A.E optimization, the developed extraction method presents the advantage of being efficient, easy to handle, and reproducible (Canales et al., 2021; Singh et al., 2022). Indeed, although it is not possible to rigorously compare levels obtained from different studies due to the variations linked to crops and environmental conditions, it is important to note that the levels obtained in this study are in the range of the highest levels reported in the literature for buttons (6.8–7.6 mg/g dw) and oyster mushrooms (3.3–4.5 mg/d dw) (Blumfield et al., 2020; Jiang et al., 2020b). These findings confirm thus, the high efficiency of the developed U.A.E method. To our know knowledge, this study is the first to thoroughly investigate the influence of the various U.A.E parameters for optimizing the extraction yields of ergosterol and vitamin D2 from edible mushrooms. EtOH resulted to be the best solvent for the simultaneous extraction of ergosterol and vitamin D2. Besides, being a GRAS solvent, EtOH can be effectively and safely used for the food or nutraceutical applications of mushrooms extracts. U.A.E temperature of 40 ◦C for 30 min at a frequency of 40 kHz and a power of 240 W allows a fast and efficient mushroom extraction. The assessment of the SSR and the impact of the number of extraction shows that U.A.E allows the optimal extraction of the analytes with low solvent in a single extraction step. U. A.E is based on the phenomena of acoustic cavitation, which is influenced by the ultrasonic wave frequency (kHz) and ultrasonic intensity (W/cm2) (Aslam et al., 2021; Geow et al., 2021). In sterol extraction from mushrooms, various U.A.E frequencies between 20 and 65 kHz have been reported (Papoutsis et al.,

2020). However, no study has assessed the effect of this important parameter on ergosterol or vitamin D extraction. Basic U.A.E allowed an improvement of ergosterol levels due to the extraction of ergosteryl esters, which represent about 4% of the total ergosterol in Agaricus bisporus and 7.6 % in Pleurotus ostreatus (Table 3). Hammann and Vetter (2016), reported that esterified ergosterol contributed to only 3% of the total ergosterol content in the button Agaricus bisporus. However, the traditional method used for ergosteryl esters extraction was very long requiring hot saponification, extraction with hexane, and sample concentration (Papoutsis et al., 2020). To our knowledge, this study is the first to report a direct basic U.A.E for the extraction of esterified ergosterol. The obtained results emphasize thus, the advantage of U.A.E for the direct extraction of mycosterol and vitamin D from mushrooms.

The optimized U.A.E method was applied on 3 mushroom samples: white button, portobello button, and oyster. Extractions were performed to quantify the free and esterified ergosterol in mushrooms. Moreover, Vitamin D2 content was evaluated after UV-C irradiation. Fig. 3 shows the chromatogram of irradiated white button mushrooms through U.A. E., in which ergosterol and vitamin D2 can be observed.

Relating to vitamin D2 analysis, UV-C irradiation was performed to increase vitamin D levels and thus, apply the optimized U.A.E. method on mushroom samples with detectable levels of vitamin D2. The developed method, which showed good recoveries for vitamin D (98 3 1.8 %) during optimization allowed to efficiently extract vitamin D from mushroom powder.

Conclusions

Optimized U.A.E methods have been developed for ergosterol and vitamin D extraction in mushrooms. U.A.E is a powerful extraction technique, which significantly reduces the extraction time, showing high efficiency in terms of ergosterol and vitamin D extraction yields. The developed methods could be considered as reference methods for the extraction of ergosterol and photoisomers in mushrooms.

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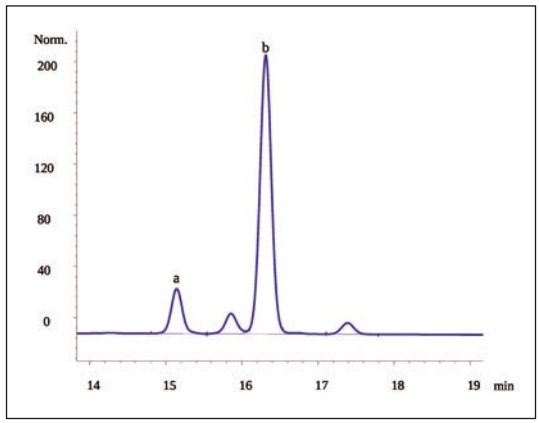

Fig. 3. HPLC-DAD chromatogram of mushrooms (A. bisporus Portobello) after 30 min of UVC irradiation. Extraction was performed through U.A.E: 100 mg in 5 mL of ethanol at 40 ◦C for 30 min, 40 kHz, and 240 W. a Vitamin D2 b Ergosterol

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