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Optimization of ultrasound-assisted extraction to obtain mycosterols from *Agaricus bisporus* L. by response surface methodology and comparison with conventional Soxhlet extraction

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ABSTRACT

Ergosterol, a molecule with high commercial value, is the most abundant mycosterol in *Agaricus bisporus* L. To replace common conventional extraction techniques (*e.g.* Soxhlet), the present study reports the optimal ultrasound-assisted extraction conditions for ergosterol. After preliminary tests, the results showed that solvents, time and ultrasound power altered the extraction efficiency. Using response surface methodology, models were developed to investigate the favourable experimental conditions that maximize the extraction efficiency. All statistical criteria demonstrated the validity of the proposed models. Overall, ultrasound-assisted extraction with ethanol at 375 W during 15 min proved to be as efficient as the Soxhlet extraction, yielding 671.5 ± 0.5 mg ergosterol/100 g dw. However, with *n*-hexane extracts with higher purity (mg ergosterol/g extract) were obtained. Finally, it was proposed for the removal of the saponification step, which simplifies the extraction process and makes it more feasible for its industrial transference.

1. Introduction

Mushrooms are appreciated worldwide, not only for their texture and flavour, but also for their nutritional and medicinal properties (Ferreira, Barros, & Abreu, 2009; Kalac, 2012). These organisms are a rich source of vitamins, fibre, amino acids and proteins (Heleno, Barros, Sousa, Martins, & Ferreira, 2010; Mattila et al., 2001). Free fatty acids, mono-, di- and triglycerides, sterols, and phospholipids can be found in the lipidic fraction (Heleno, Barros, Sousa, Martins, & Ferreira, 2009). Regarding their medicinal properties, there is evidence demonstrating the benefits of mushroom consumption, due to the richness in bioactive compounds, such as phenolics, tocopherols, ascorbic acid, carotenoids (Ferreira et al., 2009), and mycosterols, in particular ergosterol (5,7,22-ergostatrien-3β-ol), that represent ~90% of the sterol fraction of *Agaricus bisporus* L. (Barreira, Oliveira, & Ferreira, 2014).

Mushrooms produce ergosterol as the primary sterol (Barreira et al., 2014; Villares, García-Lafuente, & Ramos, 2012), which have antioxidant, anti-inflammatory and antitumor properties (Barreira & Ferreira, 2015; Villares et al., 2012), and could also exhibit hypocholesterolemic effects, similar to the bioactive phytosterols (Barreira & Ferreira, 2015; Teichmann, Dutta, Staffas, & Jägerstad, 2007).

For decades, traditional methods, such as Soxhlet extraction, maceration and percolation, have been used everywhere for many different purposes. In terms of efficiency, the traditional methods, and in particular the Soxhlet extraction, are described as the universal chemical extraction process. Nonetheless, by itself it is an optimized extraction system and in addition, literature offers a high amount of practical examples that report the favourable conditions. Additionally, its sister in industrial applications, the repeated-maceration-extraction, is often used by the food processing industries and researchers, with the purpose of effortlessly

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extracting major and minor compounds. However, these methodologies require large extraction times and quantities of solvents.

Emerging techniques, such as ultrasound-assisted extraction (UAE), microwave assisted extraction, supercritical fluid and the extraction with pressurized solvent, have been studied to extract sterols, increasing also the extraction yield and improving the extraction conditions (Wang & Weller, 2006; Xiao, Yuan, & Li, 2013). In comparison with conventional procedures, they are less time-consuming and require less amount of polluting solvents. Supercritical fluid extraction and pressurized solvent extraction are the most common showing high ergosterol extraction yields (Gil-Ramírez et al., 2013). However, the UAE also increases the sterols yield (Table 1) making it an interesting technique to be explored in the extraction of mycosterols and, in particular, ergosterol (Villares, Mateo-Vivaracho, Garcia-Lafuente, & Guillamón, 2014; Villares et al., 2012; Yuan, Kuang, Wang, & Liu, 2008).

Nevertheless, the UAE yield of ergosterol also varies widely depending on the applied extraction conditions (type of solvent, time, liquid-to-solid ratio, ultrasound power, among others), which makes it necessary to study its performance. In this research, first, the variables and factors that play a very significant role in the enhancement of the extraction yield were selected, and then a statistical multi-response optimization was performed using a response surface methodology (RSM). The RSM is a mathematical tool statistically designed to describe the relation between independent variables and one or more responses, enabling process optimization with a reduced number of experimental trials (Samarama et al., 2014).

Furthermore, most of the studies available in literature report a saponification step to eliminate interferences of other lipidic molecules, with the objective of purifying the extract and, therefore, leading to an enriched ergosterol extract (Barreira et al., 2014). Nevertheless, this step may eventually be eliminated without significant effect on the ergosterol concentration (Phillips et al., 2011; Shao, Hernandez, Kramer, Rinner, & Tsao, 2010; Gil-Ramírez et al., 2013).

This study aims to improve the extraction of mycosterols from *A. bisporus* (evaluated as the content in ergosterol) by testing different conditions, such as solvent (e.g., *n*-hexane, ethanol and limonene), extraction times (5–15 min) and ultrasound power (250–500 W). By means of RSM, the joint effect of time and ultrasound power on the extraction yield was described for each one of the selected solvents. To the best of our knowledge, the optimization of the ergosterol UAE by RSM was never been reported previously. The experimental values obtained under optimal UAE and Soxhlet extraction conditions were compared. Moreover, in order to reduce the process complexity, the pertinence of the saponification step was evaluated.

2. Material and methods

2.1. Samples

A. bisporus L. bioresidues were purchased from a local mushrooms production enterprise "Mogaricus Cogumelos – Sociedade Unipessoal Lda." The samples were weighed, lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA) and reduced to a fine dried powder (20 mesh) for subsequent assays.

2.2. Standards and reagents

Methanol and acetonitrile were of HPLC grade from Fisher Scientific (Lisbon, Portugal). The standards of sterols (ergosterol, cholecalciferol) were purchased from Sigma (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA). All other chemicals and solvents were of an analytical grade and purchased from common suppliers.

2.3. Ergosterol extraction

2.3.1. Conventional extraction by Soxhlet

The lyophilized powdered samples (4.5 g) were extracted with 150 ml of each solvent (*n*-hexane, ethanol or limonene) during 4 h (12 cycles), refluxing in a Soxhlet apparatus. Before the extraction, an adequate volume of cholecalciferol (internal standard) was added to each sample. The solvent was, thereafter, evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland).

2.3.2. Ultrasound-assisted extraction

The UAE was carried out using an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA), comprising an ultrasound power in the range between 100 and 500 W, at a frequency of 20 kHz, equipped with a digital timer. The lyophilized powdered samples (3 g) were extracted with 100 ml of each selected solvent (*n*-hexane, ethanol and limonene) into the ultrasonic device at different times and ultrasound power ranges, as defined by the RSM design. Before the extraction, an adequate volume of cholecalciferol (internal standard) was added to each sample. After ultrasonic extraction, the extracts were filtered through Whatman n° 4 paper and evaporated under reduced pressure to remove the solvent.

In both extractions, the final residue was dissolved in methanol at 10 mg/ml and filtered through a 0.2 μm nylon filter for ergosterol quantification by HPLC-UV analysis.

2.4. Saponification step

The saponification step was performed according to a procedure described by Barreira et al. (2014). Briefly, approximately 0.05 g of the extract was transferred to a dark bottle. A solution of ascorbic acid 0.1 M (1 ml) and potassium hydroxide solution 2 M (5 ml) were added to the sample. The saponification was carried out by shaking the mixture at 125 rpm in a thermostated (60 °C) bath for 45 min. After cooling at room temperature, the resulting mixture was filtered and treated with 2.5 ml of saturated sodium chloride solution and 5 ml of *n*-hexane. The samples were then stirred for 1 min in the vortex mixer. The *n*-hexane phase containing sterols was collected. The aqueous layer was then re-extracted with a new aliquot of 5 ml *n*-hexane. Both *n*-hexane fractions were combined, and dried by passing through anhydrous sodium sulphate. The *n*-hexane phase was evaporated to dryness under reduced pressure. The resulting residue was dissolved in 1 ml of methanol and filtered through a 0.2 μm filters for HPLC-UV analysis.

2.5. Ergosterol quantification

The analyses were performed according to a procedure descried by Barreira et al. (2014), using an HPLC equipment coupled to an UV detector. The equipment for analysis consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), degasser system (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and a UV detector (Knauer Smartline 2500). Data were analysed using Clarity 2.4 Software (DataApex). Chromatographic separation was achieved with a Inertsil 100A ODS-3 reversed-phase column (4.6 × 150 mm, 5 μm, BGB Analytik AG, Boeckten, Switzerland) operating at 35 °C (7971R Grace oven). The mobile phase was acetonitrile/methanol (70:30, v/v), at a flow rate of 1 ml/min, and the injection volume was 20 μl; the detection was performed at 280 nm. Ergosterol was quantified by comparing the area of its peak with the calibration curve obtained from a