Ultrasound-assisted extraction of mycosterols from *Agaricus bisporus* L. vs conventional Soxhlet extraction

Patrícia do Carmo Claro Diz

Final dissertation report submitted to

Escola Superior de Tecnologia e Gestão

Instituto Politécnico de Bragança

To obtain the Master Degree in

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This dissertation does not include the critiques and suggestions made by the jury

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ABSTRACT

Ergosterol is the most abundant mycosterol in *Agaricus bisporus* L. (cultivated mushroom most consumed worldwide) and has high commercial value since, among other applications; it is used by the pharmaceutical industry in the preparation of several drugs. 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 the type of solvent, extraction time and ultrasound power alter notably the extraction efficiency. Using response surface methodology, models were developed to investigate the favourable experimental conditions that maximize the extraction efficiency. All the 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, *n*-hexane conducted to extracts with higher purity (mg ergosterol/g extract) due to the high affinity for lipophilic compounds.

In this work it was also concluded that, to simplify the extraction process under industrial conditions, the saponification step often done in the purification of sterol extracts, can be eliminated, without significant differences in the ergosterol content.

RESUMO

O ergosterol é o micoesterol mais abundante na espécie *Agaricus bisporus* L. (cogumelo cultivado mais consumido em todo o mundo) e apresenta elevado valor comercial uma vez que, entre outras aplicações, é utlizado pela indústria farmacêutica na preparação de diferentes fármacos. Com o intuito de substituir as técnicas de extração convencionais mais comuns, como por exemplo a técnica de extração por Soxhlet, o presente estudo teve como principal objectivo determinar as condições ótimas de extração assistida por ultrassons para a molécula de ergosterol.

Após a realização de vários testes preliminares, os resultados mostraram que os parâmetros tipo de solvente, tempo de extração e potência dos ultrassons alteram consideravelmente a eficiência de extração. Utilizando a metodologia de superfície de resposta, foram desenvolvidos modelos para investigar as condições experimentais favoráveis à maximização da eficiência de extração. Todos os critérios estatísticos demonstraram a validade dos modelos propostos. No geral, a extração assistida por ultrassons com etanol a 375 W durante 15 minutos mostrou ser tão eficiente quanto a extração por Soxhlet, extraindo 671,5 ± 0,5 mg ergosterol/100 g ms. No entanto, a utilização de *n*-hexano conduziu a extratos com uma pureza superior (mg de ergosterol/g de extrato) uma vez que este solvente apresenta uma maior afinidade para moléculas lipofílicas.

Neste trabalho concluiu-se ainda que, no sentido de simplificar o processo de extração em condições industriais, o passo de saponificação frequentemente realizado na purificação de extratos de esteróis, poderá ser eliminado, não alterando significativamente o conteúdo em ergosterol.

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ABBREVIATIONS LIST

ANOVA	one vary analysis of variance
ASE	accelerated solvent extraction
COX	cyclooxygenase
DAD	diode array detector
DW	durbin-watson coefficient
GC	gas chromatography
HPLC	high resolution liquid chromatography
DAD	diode array detector
UV-Vis	ultraviolet visible detector
MAE	microwave-assisted extraction
MAPE	mean absolute percentage error
MSE	mean squared error
RMSE	root mean square of the errors
RSM	response surface methodology
SFE	supercritical fluid extraction
UAE	ultrasound-assisted extraction

1. Introduction

1.1. Mushrooms as a source of mycosterols

1.1.1. Nutritional and bioactive compounds from mushrooms

Mushrooms are worldwide appreciated not only for their texture and flavour but also for their nutritional and medicinal properties (Ferreira et al., 2009; Kalac, 2012). In nutritional terms, mushrooms are a highly nutritious food and have been compared to meat, eggs, milk, since they have an amino acid composition similar to that of animal protein (Longvah & Deosthale, 1998; Ferreira, 2011). In fresh mass, mushrooms have a content of 80 to 90% in water (Kalac, 2012). They contain vitamins such as thiamine, riboflavin, ascorbic acid, vitamin D_2, a high content of minerals (calcium, iodine, phosphorus) and also appreciable amounts of fibers (Kalac, 2012). In its lipid portion, it can be found free fatty acids, mono-, di- and triglycerides, sterols, and phospholipids (Heleno et al., 2009).

In medicinal terms, there is scientific evidence demonstrating the benefits of consumption of mushrooms because of their therapeutic properties. Some of their studied bioactivities include prebiotic, antioxidant, anti-inflammatory and antimicrobial activities (Ferreira et al., 2009; Azevedo et al., 2012; Alves et al., 2012). Several species have demonstrated immunomodulatory action, acting at the level of the immune system, affecting the immune response, with antitumor or immunosuppressive effect (Ferreira et al., 2010). They are also used for treatment and prevention of cardiovascular diseases such as hypertension, hypercholesterolemia and diabetes (Helm et al., 2009). The exhibited biological properties are due to the fact that the mushrooms possess a great variability of bioactive compounds, namely phenolic acids, tocopherols, ascorbic acid, carotenoids (Ferreira et al., 2009), and mycosterols, in particular ergosterol (5,7,22-ergostatrien-3β-ol) that represents ~90% of the sterol fraction of *Agaricus bisporus* (Barreira et al., 2014).

1.1.2. Ergosterol and other mycosterols

Sterols are special forms of steroids that can be found in animals (zoosteroids), plants (phytosteroids) and fungi (mycosteroids) (Barreira & Ferreira, 2015). They consist in a tetracyclic structure of four rings linked together, of three to six carbons and other ring with five carbons (steroid nucleus) (**Figure 1**) with a hydroxyl group at the C_3 position and an aliphatic chain linked to the steroid nucleus (Fahy et al., 2005).

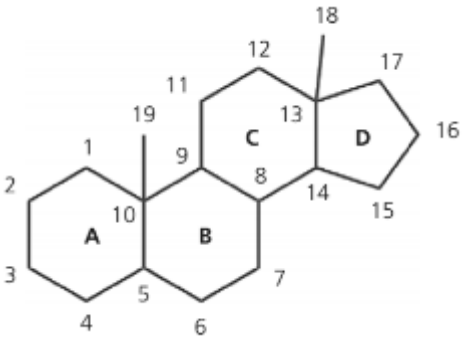

Figure 1. Steroid nucleus (Barreira & Ferreira, 2015).

Sterols play an important physiological role to the extent that they are vital compounds of the plasma membrane, being also responsible for maintaining the structure and viability of cells. Ergosterol (1) is the main sterol in fungi, but Zymosterol (2) fungisterol (3), ergosta-5,7-dienol (4), 24-methyl cholesterol (5), and methylene cholesterol (6) (**Figure 2**) are further examples of important sterols in fungi (Mattila et al. 2002; Barreira & Ferreira, 2015).

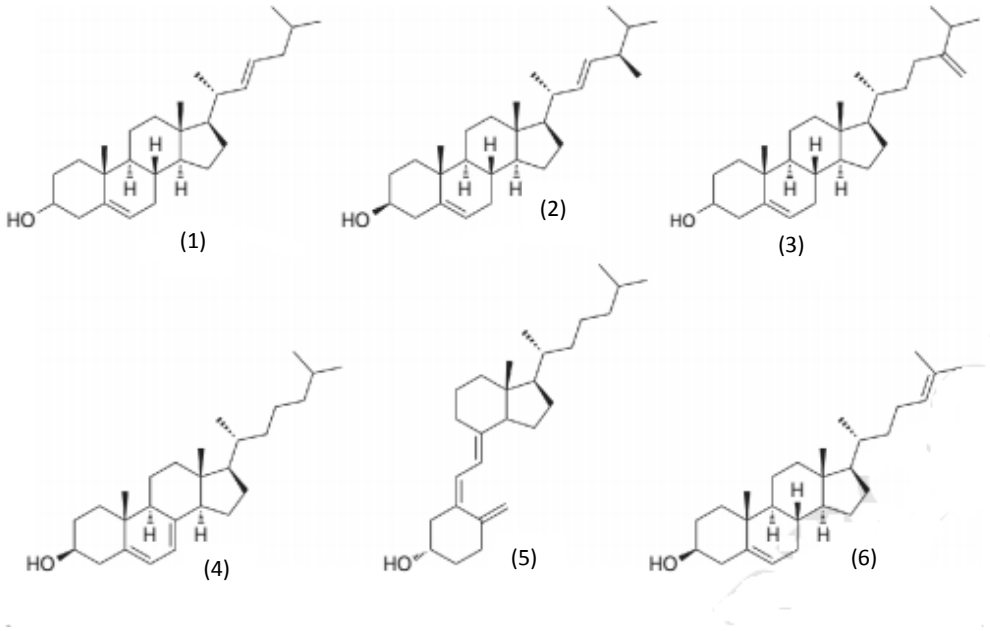

Figure 2. Chemical structures of common mycosteroids: (1) ergosterol; (2) zymosterol; (3) fungisterol; (4) ergosta-5,7-dienol; (5) 24-methyl cholesterol; (6) methylene cholesterol (Barreira & Ferreira, 2015).

Ergosterol with the scientific name 5,7,22 ergostatrien-3β-ol, has the empirical formula $C_{28}H_{44}O$ and molecular weight 396,36 g/mol. It is a solid compound, crystalline, colourless and its melting point is in the order of 161-166ºC. Furthermore, ergosterol supports the temperature of 250 °C without decomposition. It has a side chain with a double bond at C_{22} and two double bonds at the level of positions C_5 and C_7 (ring B), giving a maximum absorption in the UV in a range of 240 to 300 nm (Barreira et al., 2014). This compound is practically insoluble in water, presenting reasonable solubility in organic solvents, such as chloroform. When exposed to ultraviolet light it can be transformed in ergocalciferol (vitamin D_2), the most important pro-vitamin D (Villares et al., 2012).

Agaricus bisporus has a considerable content of mycosterols, being ergosterol the most abundant one, representing 90% of its sterol fraction (Barreira et al., 2014). According to the literature, ergosterol content varies according to the mushroom species, within the same species, different cultivars and the mushroom maturation stage (Jasinghe & Perera, 2005). These authors reported that the concentration of sterols in mushrooms, particularly ergosterol, depends on the part of the mushroom tissue, developmental stage and growth conditions. Savón et al. (2002) also described that in addition to the specific genetic characteristics, ergosterol content in mushrooms also depends on environmental factors such as light, heat, temperature, humidity and type of substrate used in mushrooms growth.

Several studies have demonstrated that ergosterol and its peroxidation products may contribute to potential health benefits and significant physiological functions that include antioxidant, antimicrobial and antitumoral activity (Villares et al., 2012; Barreira & Ferreira, 2015). In addition, these compounds allow a reduction in pain-related inflammation, incidences of cardiovascular disease and inhibition of cyclooxygenase (COX) enzymes (Yasukawa et al. 1994; Zhang et al. 2002; Ravi& Abplanalp, 2003).

Recent studies showed that ergosterol could be metabolized *in vivo* to generate new bioactive products that have been found to be able to inhibit the proliferation of skin cells in culture, as demonstrated in human keratinocytes, melanoma cell lines (Yuan et al. 2007), and other tumour cell lines (Yasukawa et al. 1994; Ravi & Abplanalp, 2003). The antitumor activity of ergosterol may be due to the direct inhibition of angiogenesis (development of new blood vessels from the existing ones) induced by solid tumors

(Ravi & Abplanalp, 2003). Ergosterol is absorbed in the alimentary tract, accumulated in the adrenals and other organs, and can be metabolized *in vivo* to generate newer bioactive products, such as 17,24- dihydroxyergosterol, which has been found to inhibit the proliferation of abnormal skin cells in culture, as demonstrated in human keratinocytes and melanoma cell lines (Slominski et al. 2005). In other studies, ergosterol was isolated from the lipid fraction of the *Agaricus brasiliensis* mushroom, where it was reported the volume reduction and inhibition of tumor growth in mice with sarcoma 180 (Takaku *et al*., 2001; Zaidman *et al*., 2005).

Moreover, ergosterol can present similar properties as phytosterols from vegetables showing hypocholesterolemic properties since it is able to reduce cholesterol levels in serum (Gil-Ramírez et al., 2013).

Several mycosterols, including ergosterol and other mycosterols, in particular ergosta-7,22-dienol, ergosta-5,7-dienol and ergosta-7-enol, existing in several species of mushrooms have been identified and quantified in different mushroom species, being the most studied ones: *Agaricus bisporus* L., *Agrocybe aegerita* (V. Brig.) Singer, *Amanita caesarea* (Scop.) Pers., *Boletus edulis* Bull., *Calocybe gambosa* (Fr.) Donk, *Cantharellus cibarius* Fr., *Cantharellus* spp. Adans. Ex Fr., *Craterellus cornucopioides* (L.) Pers., *Fistulina hepatica* (Schaeff.) With., *Flammulina veluptipes* Singer , *Grifola frondosa* (Dicks.) Gray, *Hygrophorus marzuolus* (Fr.) Bres., *Lactarius deliciosus* (L. ex Fr.) S.F.Gray, *Lentinus edodes* (Berk.) Pegler, *Macrolepiota procera* (Scop.) Singer, *Morchella esculenta* Fr., *Morchella* spp. Dill. Ex Pers., *Pleurotus eryngii* (DC.) Quél., *Pleurotus ostreatus* (Jacq. Ex Fr.) P.Kumm., *Termitomyces albuminosus* (Berk.) R. Heim, *Tuber aestivum* Vittad., *Tuber indicum* Cooke et Massee., *Tuber melanosporum* Vittad (Yuang et al., 2008; Shao et al., 2010; Phillips et al., 2011; Villares et al., 2012; Gil Ramírez et al., 2013; Barreira et al., 2014; Villares et al., 2014). According to these authors, ergosterol values referenced for *A. bisporus* range from 77 mg/100 g to 865 mg/100 g dry weight (dw), and for the other species between 27 and 151 mg/100 g dw.

1.2. Extraction methodologies for mycosterols

1.2.1. Extraction techniques applied to mushrooms

Among the solid-liquid extraction methods most commonly used in mycosterols extraction from macrofungi (mushrooms), stand out the conventional: maceration and Soxhlet extraction; and emerging: ultrasound-assisted extraction, microwave-assisted extraction, supercritical fluid extraction and accelerated solvent extraction. These novel techniques have been developed in order to decrease the extraction time and the solvent consumption, increasing the extraction yield, and enhancing the extracts quality (Wang & Weller, 2006).

Table 1 shows the main advantages and disadvantages of extraction methodologies mentioned, which are discussed in detail in the following sections.

Table 1. Advantages and disadvantages of different techniques applied to the extraction of ergosterol and other mycosterols (Wang & Weller, 2006).

Technique	Advantages	Disadvantages
Maceration	Simple and inexpensive method	Requires high extraction times
Soxhlet	Standard method; uses large amount of sample; no requires filtration; low costs	Slow method; requires large amounts of solvent and has high solvent losses by evaporation
Ultrasound	Rapid technique; uses large amount of sample; low costs	It requires large amounts of solvent, filtration
Supercritical Fluid	Rapid technique; CO_2 is innocuous; selective method when varying pressure and temperature; requires small amounts of solvent and can be fully automated	Use of higher pressures; high costs
Microwave	Rapid technique; requires small amounts of solvent when compared with Soxhlet; allows complete control of extraction parameters;	Requires filtration
Accelerated solvente	Rapid technique; requires a minimal amount of solvent; fully automated and easy to operate	It has very high cost

1.2.2. Conventional methodologies

1.2.2.1. Maceration extraction

In this extraction method, the sample is placed in a closed container for a period of time at room temperature or under heat and/or stirring to accelerate the dissolution of the compounds in the extraction medium. Maceration is the most commonly used conventional technique and several studies exist in the literature describing its use to extract mycosterols. Among the existing studies, it appears that the authors refer often to a saponification step as a way of purifying the extract, obtaining a more concentrated ergosterol extract by removal of interfering molecules as tocopherols and other lipidic compounds (Phillips et al., 2011; Gil-Ramirez et al., 2013).

However, in another study, the authors reviewed the amount of ergosterol extracted by maceration with and without the saponification step, varying the solvent used for extraction, and have verified that the saponification step does not significantly influence the amount of ergosterol extracted (Shao et al., 2010).

Different solvents are used in the extraction by maceration as can be seen in **Table 2**, noting that the solvent type significantly affects the amount of sterols extracted. From among the mushrooms analysed, it can be stated that *A. bisporus* is one of the richest species in ergosterol, compared with the other tested species.

Table 2. Mycosterols obtained by maceration of different species of mushrooms.

Samples	Saponification step	Solvent	Ergosterol (mg/100g dw)	Ergosta-7,22-dienol (mg/100 g dw)	Ergosta-5,7-dienol (mg/100 g dw)	Ergosta-7-enol (mg/100 g dw)	References
A. bisporus (white) (J.E.Lange) Emil J. Imbach	Yes	Ethanol	335±0.06[B] 346±0.08[A]				
	No	Hexane->methanol->water[a]	329±0.08[B] 339±0.08[A]				
		Hexane	332±0.12[B] 343±0.16[A]		Nd		Shao et al., 2010
A. bisporus (brown) (J.E.Lange) Emil J. Imbach	Yes	Ethanol	482±0.10[B] 456±0.05[A]				
	No	Hexane->methanol->water[a]	475±0.10[B] 449±0.09[A]				
		Hexane	477±0.03[B] 451±0.02[A]				
A. bisporus (white) (J.E.Lange) Emil J. Imbach	Yes	Hexane/ ethyl acetate (85:15, v/v)	56.3[b] (563)[c]	1.78[b] (17.8)[c]	6.03[b] (60.3)[c]	1.34[b] (13.4)[c]	Phillips et al., 2011
A. bisporus (crimini) (J.E.Lange) Emil J. Imbach			61.4[b] (614)[c]	2.32[b] (23.2)[c]	5.92[b] (59.2)[c]	1.7[b] (17.0)[c]	
A.bisporus (portobello) (J.E.Lange) Emil J. Imbach			62.1[b] (621)[c]	2.57[b] (25.7)[c]	6.18[b] (61.8)[c]	1.75[b] (17.5)[c]	
A. bisporus (portobello, UV) (J.E.Lange) Emil J. Imbach			51.1[b] (511)[c]	1.73[b] (17.3)[c]	4.70[b] (47.0)[c]	1.28[b] (12.8)[c]	
Cantharellus spp. Fr.			46.3[b] (463)[c]	1.68[b] (16.8)[c]	4.49[b] (44.9)[c]	2.02[b] (20.2)[c]	
Flammulina veluptipes Singer			35.5[b] (355)[c]	1.49[b] (14.9)[c]	16.5[b] (165)[c]	2.32[b] (23.2)[c]	
Grifola frondosa (Dicks.) Gray			79.2[b] (792)[c]	1.79[b] (17.9)[c]	6.34[b] (63.4)[c]	1.94[b] (19.4)[c]	
Lentinus edodes (Berk.) Pegler			84.9[b] (849)[c]	2.26[b] (22.6)[c]	6.51[b] (65.1)[c]	5.03[b](50.3)[c]	
Morchella spp. Dill. Ex Pers.			26.3[b] (263)[c]	-	5.79[b] (57.9)[c]	-	
Pleurotus ostreatus (Jacq. Ex Fr.) P.Kumm.			68.0[b] (680)[c]	1.66[b] (16.6)[c]	8.89[b] (88.9)[c]	1.7[b] (17.0)[c]	
A. bisporus (Fungisem H5) (J.E.Lange) Emil J. Imbach	Yes	Methanol/water (55:45, v/v)	561±0.76	29±0.03	70±0.09	38±0.04	Gil-Ramirez et al., 2013
A. bisporus (Fungisem H15) (J.E.Lange) Emil J. Imbach			865±0.69	42±0.17	151±0.20	64±0.11	
A. bisporus (Gurelan 60) (J.E.Lange) Emil J. Imbach			594±0.14	27±0.01	86±0.03	37±0.03	
A. bisporus (Mispaj 365) (J.E.Lange) Emil J. Imbach			740±0.38	29±0.03	123±0.36	47±0.10	
A. bisporus (Somycel A15) (J.E.Lange) Emil J. Imbach			515±0.12	30±0.00	99±0.02	50±0.01	

[a]a -> represents a sequence of steps [b]results expressed in mg/100 g fw; [c]based on fresh mushrooms that have a content of 90% water; A-Stem; B-Cap. dw- dry weight; fw- fresh weight.

1.2.2.2. Soxhlet extraction

Soxhlet extraction is one of the most common extraction techniques and most widely used for conventional extraction of solid samples. It consists of a sample destillation process repeated a number of times (Luque-García & Luque de Castro, 2004). In conventional Soxhlet, the sample is placed in a thimble-holder and during operation gradually filled with condensed fresh solvent from a distillation flask (**Figure 3**). When the liquid reaches an overflow level, a siphon aspirates the whole content of the thimble holder and unloads it back into the distillation flask, carrying the extracted compounds in the bulk liquid. This operation is repeated until complete extraction is achieved (Wang & Weller, 2006).

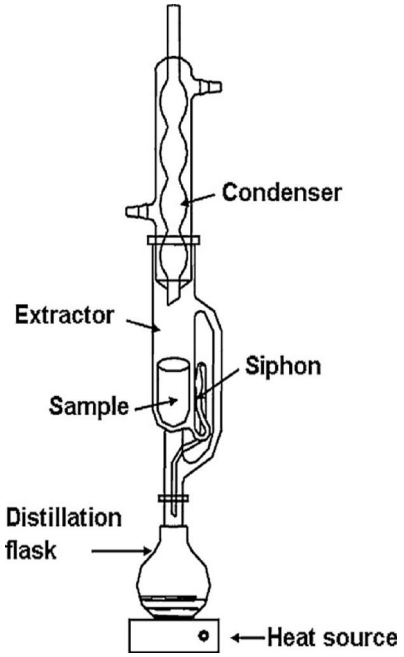

Figure 3. Conventional Soxhlet extractor (Luque de Castro & Priego-Capote, 2010)

The main advantages of this method are to be a very simple methodology that requires little training and can extract more sample mass than most of the latest techniques (such as supercritical-fluid extraction). Besides that, the sample is repeatedly in contact with fresh portions of the extractant, which facilitates the displacement of the transfer equilibrium. Also, the sample throughput can be increased by performing several simultaneous extractions in parallel, which is facilitated by the low cost of the basic equipment (Luque de Castro and Priego-Capote, 2010). Lastly, no filtration is required after the process. However, conventional Soxhlet extraction has also significant

drawbacks as the long time required for the extraction and the large amount of organic solvent wasted, which is not only expensive to dispose off but also can cause environmental pollution itself (Luque de castro & Garcia-Ayuso, 1998; Wang & Weller, 2006).

In the soxhlet technique hexane is the commonly used solvent, described as one of the most efficient for the extraction of lipidic compounds (Barreira et al., 2014). These authors studied various solvents and mixtures (dichloromethane/methanol; chloroform/methanol) and various techniques for extracting sterols (maceration and ultrasonic bath), concluding that the soxhlet extraction using hexane as solvent resulted in higher amounts of ergosterol. In **Table 3**, it is observed that *A. bisporus* has a significantly higher amount of ergosterol compared to the other species analysed.

T**able 3.** Ergosterol content obtained by Soxhlet extraction with hexane of different species of mushroom, after a saponification step (Barreira et al., 2014).

Samples	**Ergosterol (mg/100 g dw)**
Agaricus bisporus (J.E.Lange) Emil J. Imbach	352±1
A. bisporus Portobello (J.E.Lange) Emil J. Imbach	77±1
Amanita caesarea (Scop.) Pers.	231±1
Boletus edulis Bull.	234±2
Cantharellus cibarius Fr.	129±1
Fistulina hepática (Schaeff.) With.	108±1
Flammulina velutipes Singer	189±2
Lactarius deliciosus (L. ex Fr.) S.F.Gray	55±1
Lentinus edodes (Berk.) Pegler	217±2
Macrolepiota procera (Scop.) Singer	118±2
Morchella esculenta Fr.	43±2
Pleurotus eryngii (DC.) Quél.	187±1
Pleurotus ostreatus (Jacq. Ex Fr.) P.Kumm.	104±1

dw – dry matter.

1.2.3. Emerging methodologies

1.2.3.1. Ultrasound-assisted extraction (UAE)

The ultrasound technique is a process that uses the energy of the waves (mechanical) transmitted in frequency higher than 20 kHz. This energy is used in order to convert electrical energy into vibrational impulses (Castro & Capote, 2007; Serradilla et al., 2007). The vibrational impulses are described as cyclic compression and decompression waves whose overall effect is to cause cavitation phenomenon; cycles form, grow and collapse of bubbles formed during propagation of the waves. Expansion pulls molecules apart and compression pushes them together. The expansion can create bubbles in a liquid and produce negative pressure. The collapse of the bubbles within the matrix causes disruption of cell structure, increasing the release of extractable compounds and enhancing the mass transference to the solvent (Wang & Weller, 2006).

There are two commons devices for ultrasounds extraction. The more widely used is the bath system but the one which offers more advantages is the probe system. This provides directly cavitation in the solution, being more efficient (Priego-Capote & Luque de Castro, 2004).

The main benefits of using ultrasound in solid–liquid extraction include the increase of extraction yield and faster kinetics. Ultrasound can also reduce the operating temperature allowing the extraction of thermolabile compounds. It is necessary to take into account sample characteristics such as moisture content, particle size, solvent used for the extraction in order to obtain an efficient and effective ultrasound-assisted extraction. Furthermore, many factors govern the action of ultrasound including frequency, pressure, temperature and sonication time (Wang & Weller, 2006).

The UAE has been used as an alternative to extraction by Soxhlet (Wang & Weller, 2006), being methanol, dichloromethane and chloroform the most used solvents in this technique in a ratio that can increase the extraction efficiency of sterols. Analysing the literature (**Table 4**), it appears that the UAE is efficient in the obtainment of ergosterol.

Table 4. Ergosterol content obtained by ultrasound assisted extraction with different species of mushrooms.

Samples	Equipment	Solvent	Ergosterol (mg/100 g dw)	References
Agrocybe aegerita (V. Brig.) Singer	Probe	Methanol/dichloromethane (75:25, v/v)	351±0.06A 144±0.04B 240±0.05C	Yuan et al., 2008
Lentinus edodes (Berk.) Pegler			170±0.03[a] 402±0.08B 111±0.03C	
Termitomyces albuminosus (Berk.) Pegler			215±0.04[a] 170±0.03B 402±0.08C	
Tuber aestivum Vittad.	Bath	Chloroform/methanol (2:1, v/v)	151±0.20	Villares et al., 2012
Tuber indicum Cooke et Massee.			128±0.11	
Tuber melanosprrum Vittad.			180±0.12	
A. bisporus (J.E.Lange) Emil J. Imbach	Bath	Chloroform/methanol (2:1, v/v)	642±0.15	Villares et al., 2014
Boletus edulis Bull.			400±0.53	
Calocybe gambosa (Fr.) Donk			361±0.18	
Cantharellus cibarius Fr.			23±0.01	
Craterellus cornucopioides (L.) Pers.			44±0.00	
Hygrophorus marzuolus (Fr.) Bres.			681±0.72	
Lactarius deliciosus (L. ex Fr.) S.F.Gray			32±0.02	
Lentinus edodes (Berk.) Pegler			364±0.02	
Pleurotus ostreatus (Jacq. Ex Fr.) P.Kumm.			331±0.17	

A- Pileus; B- Gills; C- Stipe; dw- dry weight.

1.2.3.2. Microwave-assisted extraction (MAE)

Microwave heating is also called dielectric heating, existing two main mechanisms for the transformation of electromagnetic energy into heat. The first mechanism is called dipole rotation and relates to the alignment of the molecules (which has permanent or induced dipoles) and to the applied electric field. When the electric field is removed, the molecules return to a disordered state, and the energy that was absorbed into this guidance in these dipoles is dissipated as heat. The second mechanism is called ionic conduction and consists on the heat generated by friction losses and occurs through the migration of dissolved ions due to the action of an electromagnetic field. These losses

depend on the size, charge, conductivity of dissolved ions and the interaction of the latter with the solvent (Sanseverino, 2002).

With this method, the samples can contain moisture traces that serve as the target for the microwave heating. Due to microwave heating, the moisture is heated up inside the sample cells, evaporating and generating high pressures on the cell. The pressure pushes the cell wall from inside, stretching and ultimately rupturing it (Mandal et al., 2007), thus the exudation of active constituents from the cells occurs, hence increasing the yield of the constituents (Tatke & Jaiswal, 2011).

The capacity of the solvent to absorb microwave energy and transferring it in the form of heat, molecule to molecule, strongly depends on the solvent dissipation factor. The factor of dielectric loss (ε''), measure the efficiency of electromagnetic energy conversion into heat. The dielectric constant of the substance (ε') is a measure indicating its polarity. The reason (ε'' / ε'), is numerically equal to tan δ (tan δ = ε'' / ε') and it is called by dissipation factor, which means the ability of a sample to convert electromagnetic radiation into heat (as much higher is this value, higher is the heating of the substance by microwave energy). Solvents such as ethanol, methanol and water are polar and are good solvents to be heated by microwave energy (Brachet et al., 2002). Less polar solvents (aliphatic or aromatic hydrocarbons) or substances with zero dipole moment (as CCl_4 and CO_2) weakly absorb microwave radiation. MAE has been considered as a potential alternative to traditional solid–liquid extraction methods. It has been used because it reduces the extraction time, reduces solvent usage and improves extraction yield (Wang & Weller, 2006).

This technique has also been applied to the extraction of sterols, namely phytosterols (Xiao et al., 2013). As far as we know, there are no references in the literature to the extraction of mycosterols using microwave assisted extraction, however taking into account its characteristics we consider this technique a promising technique for this purpose, since it presents a number of variables that can be optimized to obtain a high extraction efficiency of such molecules.

1.2.3.3. Supercritical fluid extraction (SFE)

A supercritical fluid is defined as any substance whose temperature and pressure are above their critical values (T_c and P_c, respectively) (**Figure 4**), not co-existing a distinct region of gas or liquid above its critical point.

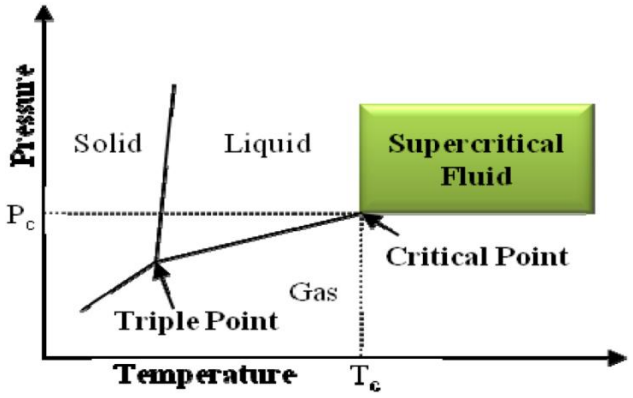

Figure 4. Phase diagram for a pure substance.

In the supercritical state, the fluids exhibit physical and chemical properties such as density, diffusivity and viscosity intermediate between gas and liquid. This allows the supercritical fluids to be able to dissolve compounds which are sparingly dissolved by a gas or liquid. According to Ribeiro et al. (2007), properties related to the solubilisation capacity, such as density, are similar to those typical of a liquid, while properties related to the transport of matter such as the diffusivity and viscosity, can reach values typical of a gas. Supercritical solvents, combining the desirable characteristics of both liquid and gas, are great solvents with high diffusivity and low viscosity. As a result, supercritical fluid extraction becomes a fast and efficient process.

The supercritical fluids existing in the market span a wide range of temperatures and operating pressures. However, among all the supercritical solvents, CO_2 is the most widely used, due not only to a very particular set of physical and chemical characteristics, but also economic (Medina et al., 1988). Carbon dioxide is non-toxic, non-flammable and non-corrosive, being also cheap and available in large quantities in the market with a high degree of purity. Carbon dioxide presents low critical temperature and pressure (73.8 bar and 31.1ºC, respectively), meaning that low temperature values are needed in the extraction process (Nodar, 2002).

The major disadvantage in using CO_2 as the supercritical fluid is related to their nonpolar nature, which is not favourable to the extraction of polar compounds. However, this limitation may be overcome by adding a polar organic solvent. It is therefore usual to add to the supercritical fluid an organic compound - co-solvent (typically a polar compound of average volatility, methanol, ethanol, acetone, among others), in order to change not only the power of the supercritical fluid for solubilisation, but also its selectivity (Wang & Weller, 2006).

Therefore, this technique presents some advantages compared to conventional techniques such as the use of low extraction temperatures, minimizing the loss of flavour, colour and nutritional value of thermolabile products; the possibility of using low toxic solvents minimizing the environmental impact; the easy control of selectivity and modification of the fluids ability to penetrate into the matrix, by setting the temperature and operating pressure; the shorter extraction time when compared conventional solvents (Lang & Wai, 2001; Wang & Weller, 2006; Shi et al., 2007).

The main disadvantage of the extraction with supercritical fluids is the need to operate at high pressures, which require the use of expensive and technologically complex installations. The high degree of complexity of the phase equilibrium at high pressures and the possibility of loss of volatile components may be other obstacles to the development of new applications using supercritical fluids (Wang & Weller, 2006).

This technique is currently the most sophisticated used in the extraction of sterols due to the above mentioned features, being efficient and profitable, and being possible to optimize all the extraction conditions and variables (Wang e Weller, 2006). Several authors have used this technique, optimizing the various extraction conditions as the optimum pressure with or without the addition of ethanol as co-solvent. For the best extraction conditions (9 MPa without ethanol as a co-solvent), the concentration of the various mycosterols obtained for the species *A. bisporus* was: ergosterol- 550 mg/100 g dw, ergosta-7,22-dienol- 420 mg/100 g dw, ergosta-5,7-dienol- 440 mg/100 g dw and fungiesterol- 520 mg/100 g dw) (Gil-Ramírez et al., 2013).

1.2.3.4. Accelerated solvent extraction (ASE)

The use of a pressurized fluid to obtain extracts is known to be a rapid and efficient method for the extraction of compounds from plant sources. Pressurized solvents are of particular interest, since their physico-chemical properties can be adjusted by changing the pressure and temperature, which allows control of the solubilizing power and selectivity of the solvent for the process (Pronyk & Mazza, 2009).

A pressurized fluid is heated to high temperatures and pressures below the critical. The extraction is performed using a cylindrical steel vessel containing the solid matrix and a solvent above its boiling point, which is maintained in the liquid phase due to a pressure increase. Increasing the temperature increases the solubility in the solvent and accelerates the diffusion of soluble compounds, allowing efficient extraction in a short

time. This technique is based on changing the polarity and solubility of the solvent by the combination of high pressure and high temperature (temperatures can range from 50 to 200 ° C and pressures between 1500 and 2000 bar) (Wang & Weller, 2006).

The extraction method with pressurized fluid is similar to Soxhlet extraction, except for the fact that solvents are used near its supercritical region, where they have properties that allow higher extraction. Due to its characteristics, this technique allows high extraction efficiency with smaller volume and extraction time than conventional extractions (Wang & Weller, 2006). As Soxhlet extraction, this technique is not suitable for thermal unstable matrices and their industrial application is limited due to the high pressures required (Marcic et al., 2005).

The pressurized extraction solvent is also one of the most currently used. In this technique it is possible to optimize a set of variables including the extraction ratio sand/sample (the sand is used as an inert filler to improve process efficiency); number and cycle time and pressure. Several authors have used this extraction method for sterols extraction from *A. bisporus*, using ethanol as solvent, and obtained for the best conditions (50ºC, 10.7 MPa, 5 cycles, 5 min, ratio sample/sand: 1:4) high concentrations of sterols: ergosterol (450 mg/100 g dw), ergosta-7,22-dienol (380 mg/100 g dw), ergosta-5,7-dienol (410 mg/100 g dw) and fungisterol (430 mg/100 g dw) (Gil Ramírez et al., 2013). These authors conducted a comparative study between this technique and the extraction by SFE and concluded that the SFE conducted to less content of extract but in the other hand, also conducted to higher contents in ergosterol.

1.3. Comparison of the extraction conditions reported in literature

From the analysis of **Tables 2-4** it appears that in most of the cases, the application of the mentioned emerging techniques increases the extraction of ergosterol, when compared with conventional techniques. Among the emerging techniques applied, the extraction with pressurized solvent is leading to higher levels of ergosterol. However, extraction techniques assisted by ultrasound and microwave are shown as well as promising techniques since they lead to high levels of ergosterol when comparing to the conventional techniques. With regard to solvents, the most used are hexane and ethanol, depending on the extraction method. The use of these solvents with different polarities

is due to the fact that the ergosterol molecule presents an amphipathic structure, being able to have affinity for different polarity solvents (Barreira et al., 2014).

The saponification step is used by most of the authors with the aim of purifying the extract, becoming more concentrated in ergosterol, however, analysing **Tables 2-4** it can be stated that this step can be eliminated, without significantly affecting the concentration of ergosterol present.

From an analysis of **Tables 2-4**, it is also observed that, among the studied mycosterols, ergosterol is the most abundant mycosterol in the studied samples, ranging from 3-9 mg per g of dried mushroom in *A. bisporus* (Mattila et al, 2002; Villares et al, 2012), that is also the species presenting the highest concentration of this molecule.

The optimization of all the parameters and the selection of the most efficient technique to extract ergosterol is an important topic, since it is a bioactive molecule with numerous potential applications in the food, cosmetic and pharmaceutical industries.

The optimization of its extraction without the saponification step is a priority, aiming to reduce the complexity of the process.

1.4. Analytical methods for ergosterol identification and quantification

The quantification of ergosterol in mushrooms, after extraction, has been performed currently by two methods, namely, by high resolution liquid chromatography (HPLC) (Yuan et al., 2006; Yuan et al., 2007; Yuan et al., 2008; Shaos et al., 2010), and gas chromatography (GC) (Schwadorf & Muller, 1989; Phillips et al., 2011). The development of methods for HPLC allowed a considerable reduction of analysis time/response, without prejudice to the recovery and resolution. The HPLC quantification method has advantages over the GC method since it permits the use of lower temperatures during analysis, thereby reducing the risk of isomerisation of the double bonds of the methyl esters (Czauderna & Kowalczyk, 2001; Li et al., 2001) and that it is not necessary to derivatize the sample, allowing to reduce the analysis time (Knothe, 2001).

The determination of ergosterol by HPLC is performed mainly with ultraviolet-visible detector (UV-Vis) (Schwadorf & Muller, 1989) or diode array detector (DAD) (Yuan et al., 2006; Yuan et al., 2007; Yuan et al., 2008; Shaos et al., 2010). Ergosterol is easily

detected in the UV region because it has absorption peaks range 240 to 300 nm (Barreira et al., 2014).

In the quantification of ergosterol and other sterols, different HPLC techniques have been used such as reverse phase (Yuan et al., 2006; Yuan et al., 2007; Yuan et al., 2008; Shaos et al., 2010) and normal phase (Schwadorf & Muller, 1989). Most studies for determination of ergosterol in mushrooms uses the HPLC method with reversed phase C18 column and UV detection at 280 and 282 nm (Yuan et al., 2006; Yuan et al., 2007; Yuan et al., 2008; Shaos et al., 2010).

Most of the methods for ergosterol detection use columns with 5 μm particle size (Schwadorf & Muller, 1989; Yuan et al., 2006; Yuan et al., 2008; Shao et al., 2010), and with 4 μm (Yuan et al., 2007). According to the literature, the column temperature is generally 20 ° C (Yuan et al., 2007) to 30ºC (Yuan et al., 2008).

1.5. Motivation and objectives

Mushrooms are worldwide appreciated not only for their texture and flavor but also for their nutritional and medicinal properties (Ferreira et al., 2009; Kalac, 2012). These organisms are rich sources of vitamins, fibres, amino acids and proteins (Mattila et al., 2001; Heleno et al., 2010). Free fatty acids, mono-, di- and triglycerides, sterols, and phospholipids can be found in the lipidic fraction (Heleno et al., 2009). Regarding their medicinal properties, there is scientific evidence demonstrating the benefits of consumption of mushrooms because of 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 represents ~90% of the sterol fraction of *Agaricus bisporus* L. (Barreira et al., 2014).

Mushrooms produce ergosterol as the primary sterol (Villares et al. 2012; Barreira et al., 2014), which has been demonstrating antioxidant, anti-inflammatory and antitumor properties (Villares et al., 2012; Barreira & Ferreira, 2015), and could also exhibit hypocholesterolemic effects similarly to the bioactive phytosterols (Teichmann et al., 2007; Barreira & Ferreira, 2015).

Thus, conventional methods such as maceration and Soxhlet extraction have been used for ergosterol extraction. Due to the high extraction time and large quantities of solvents required in these methodologies, other emerging alternatives have been studied to

extract sterols, increasing also the extraction yield and improving the extraction conditions (Wang & Weller, 2006; Xiao et al., 2013). Emerging techniques include ultrasound-assisted extraction (UAE), microwave assisted extraction (MAE), supercritical fluid and the extraction with pressurized solvent. Supercritical fluid extraction and pressurized solvent extraction are the most common leading to an increase in ergosterol extraction yields (Gil-Ramírez et al., 2013). However, the UAE also increases the sterols yield (**Tables 3-4**) making it an interesting technique to be explored in the extraction of mycosterols and, in particular, ergosterol (Yuan et al., 2008; Villares et al., 2012; Villares et al., 2014).

Nevertheless, the UAE yield of ergosterol also varies widely depending on the extraction conditions applied (solvent, time, liquid-to-solid ratio, extraction power, among others), which makes 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., 2015).

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 (Shao et al., 2010; Phillips et al., 2011; Gil-Ramirez et al., 2013).

Therefore, this study aimed to improve the extraction of mycosterols from *A. bisporus* (evaluated by the content in ergosterol), testing different conditions such as solvent (e.g., *n*-hexane, ethanol and limonene), time (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 our knowledge, the optimization of the ergosterol UAE by RSM was not previously reported. The experimental values obtained under optimal UAE and Soxhlet were compared. Moreover, in order to diminish the process complexity, the pertinence of the saponification step was evaluated.

2. Material and methods

2.1. Samples

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

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 analytical grade and purchased from common sources.

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 evaporated under reduced pressure (rotary evaporator Büchi R-210, Flawil, Switzerland).

2.3.2. Ultrasound-assisted extraction

The UAE was carried out in an ultrasonic device (QSonica sonicators, model CL-334, Newtown, CT, USA) with an ultrasound power range between 250 to 500 W, equipped with a digital timer and a temperature controller. 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 time 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 nº 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 oil sample 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 thermostatic bath (60 °C) 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

commercial standard. Quantification was performed using the internal standard method and cholecalciferol was used as internal standard.

2.6. Responses format values to present the results

The results were expressed in two response (*Y*) format values: Y_1, in mg of ergosterol in 100 g of mushroom dry weight material (mg/100 g dw), which was specifically used to analyze the ergosterol extraction yields; and Y_2, in mg of ergosterol obtained in the extract (mg/g extract, either by Shoxlet or UAE technique), which was specifically used to evaluate the ergosterol purity in the extracts. Both responses were equally analysed, but more considerations regarding the first one (mg/100 g dw) were provided in the results presentation because, it would be the guiding response in terms of optimization or industrial transference. Note that by dividing those responses Y_1/Y_2, we will obtain g of extract/100 g dw of mushroom (the % of extracted material).

2.7. Response surface methodology

The RSM family designs are used for modelling and analysis of problems in which a response of interest is influenced by several variables. The RSM was used to optimize the UAE with the purpose of finding favourable conditions that would result in similar efficiencies to those obtained by the Soxhlet system.

2.7.1. Preliminary tests to assess the effect of variables and collateral factors on ergosterol extraction.

Initial tests were carried out to screen the appropriate variables to determine their experimental domain for an appropriate RSM design. Independent variables including solvent concentration, extraction time (*t*) and ultrasound power (*P*) were preliminary tested, as well as other collateral factors such as solvents and mushrooms growth conditions. Additionally, other variables such as solvent-to-material ratio were selected based on a literature review (**Table 2-4**).

2.7.2. Experimental design

From the preliminary experiments, the variables *t* and *P* and the solvents factor were the significant ones selected. Therefore, the combined effects of these variables on

ergosterol extraction yield with two of the most relevant solvents (*n*-hexane and ethanol) were studied using full factorial design (three replicates per condition). The structure of a full factorial design implies that all combinations of three values for each factor (minimum, mean and maximum) are studied. The number of experiments *n* for *k* factors is given as $n=3^k$. Experimental runs were randomized, to minimize the effects of unexpected variability in the observed responses. The variables were coded according to the following equation:

$$X = \left(x_a - x_0\right) / \Delta x \qquad [1]$$

where X is the coded value for the variables t and P, x_a is the corresponding actual value, x_0 is the actual value in the centre of the domain, and Δ_x is the increment of x_a corresponding to a variation of 1 unit of X.

2.7.3. Mathematical model

Response surface models were fitted by means of least-squares calculation using the following Box-Behnken design equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \qquad [2]$$

where Y is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and n is the number of variables. As pointed out, two different response formats were used as the dependent variable: Y_1, response format value in mg/100 g dw to analyze the ergosterol extraction yields; and Y_2, the response format value in mg/g extract to analyze the ergosterol purity in the extracts.

2.8. Numerical methods and statistical analysis

All fitting procedures, coefficient estimates and statistical calculations were performed on a Microsoft Excel spreadsheet. Fitting and statistical analysis of the experimental results to the proposed equations were carried out in four phases:

Coefficients determination. Parametric estimates were obtained by minimization of the sum of quadratic differences between observed and model-predicted values, using the nonlinear least-square (quasi-Newton) method provided by the macro *Solver in Microsoft Excel 2003* (Kemmer & Keller, 2010), which allows quick testing of a hypotheses and its consequences (Murado & Prieto, 2013).

2.8.1. Coefficients significance

The determination of the parametric confidence intervals was calculated using the *'SolverAid'* (Prikler, 2009). The model was simplified by dropping terms, which were not statistically significant p-value *(p)* > 0.05.

2.8.2. Model consistency

The Fisher *F* test (α=0.05) test was used to determine whether the constructed models were adequate to describe the observed data (Shi & Tsai, 2002).

2.8.3. Other statistical assessment criteria

To re-check the uniformity of the model the following criteria were applied: a) The 'SolverStat' macro (Comuzzi et. al., 2003), which is used for the assessment of parameter and model prediction uncertainties; b) R^2 is interpreted as the proportion of the variability of the dependent variable explained by the model; c) Adjusted coefficients of multiple determination (R^2_{adj}), which is a correction to R^2 taking into account the number of variables used in the model; d) Bias and accuracy factors of all equations were calculated to evaluate the fittings to experimental data, such as the mean squared error (MSE), the root mean square of the errors (RMSE) and the Mean Absolute Percentage Error (MAPE); e) The Durbin-Watson coefficient (DW) is used to check if the residuals of the model are not autocorrelated; and f) The analysis of variance table (ANOVA) is used to evaluate the explanatory power of the variables.

3. Results and discussion

For decades, traditional methods such as Soxhlet extraction, maceration and percolation, have been used everywhere for many different purposes. They are often time-consuming and require large quantities of polluting solvents in comparison to other emerging technologies, such as UAE. However, in terms of efficiency (extraction yield and purity), the traditional methods and in particular the Soxhlet extraction is 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, has been used in the last decades by the food processing industries and researchers with the purpose of extracting more efficiently major and minor compounds. However, are the conventional system extractions more efficient than emerging technologies? Can emerging technologies aided by mathematical tools find optimal conditions that would be as efficient as the conventional extraction processes? Authors aim to address these questions among others in the following paragraphs using Soxhlet and UAE as the representative techniques of traditional and modern extraction approaches, respectively.

3.1. Efficiency of ergosterol extraction by Soxhlet. Traditional approach

3.1.1. Recommended conditions on the Soxhlet process

Solvents define a major part of the environmental performance of processes in chemical industry and also of the impact on cost, safety and health issues. The choice of an extracting solvent is the first crucial step towards optimization, which has a strong impact on the yield of extraction. Different solvents will yield a different amount and composition of extracts. Therefore, a suitable extracting solvent should be selected for the extraction. In this research, either with Soxhlet or UAE techniques, we have avoided the use of toxic or hazardous organic solvents (such as benzene, cyclohexane, dichloromethane) as part of a more green industrial processes. The solvents selected for analysis were *n*-hexane, ethanol and limonene.

Other extraction conditions in Soxhlet were selected following the convenient ones indicated by other authors (Barreira et al., 2014; Savón et al., 2002; Jasinghe & Perera,

2005), in which they agree to use values around 12 cycles over a period of 4 h at the corresponding boiling point temperature of each solvent.

3.1.2. Soxhlet extraction

As presented in **Table 5**, ethanol proved to be the most efficient solvent to extract the molecule of ergosterol (676 ± 3 mg/100 g dw), followed by limonene (261 ± 11 mg/100 g dw) and *n*-hexane (186.1 ± 0.3 mg/100 g dw). Besides being also a non-polar molecule, limonene is slightly more polar than *n*-hexane, being able to extract higher contents of ergosterol. On the contrary, when expressing the results in mg/g extract, *n*-hexane gave the highest value (108.8 ± 0.2 mg/g extract), followed by ethanol (56.3 ± 0.2 mg/g extract) and limonene (3.39 ± 0.17 mg/g extract). This is related with the purity of the extracts; *n*-hexane is the less polar solvent, thus it presents a higher affinity with non-polar molecules such as lipophilic compounds. Ethanol, the most polar solvent tested, besides ergosterol, can also extract other compounds such as polyphenols, decreasing the purity of the extract. Limonene generated the less pure extract, due to its ability to extract many other molecules besides non-polar ones. Barreira et al. (2014) also used *n*-hexane for ergosterol extraction, obtaining 352 ± 1 mg/100 g dw (**Table 3**), a higher content than the 186.1 ± 0.3 mg/100 g dw obtained in the present study. This can be explained by differences in the mushroom's cultivation conditions (e.g. light, temperature and moisture), and related secondary metabolites production. This was also supported by Savón et al. (2002) and Jasinghe & Perera (2005), who described that the concentration of ergosterol depends on the tissue and developmental stage of the mushroom.

Table 5. Comparative perspective of the ergosterol extraction from *A. bisporus* in terms of extraction yield (Y_1) and extract purity (Y_2); and extract purity improvement after saponification step (last column).

SOLVENT	EXTRACTION CONDITIONS	ERGOSTEROL EXTRACTION		
		Y_1 (mg/100 g dw)	Y_2 (mg/g extract)	mg/g extract after saponification
n-Hexane	Soxhlet (4 h, 12 cycles)	186.1± 0.3	108.8 ± 0.2	144.2 ± 3.1
Ethanol		676± 3	56.3 ± 0.2	184.21± 0.04
Limonene		261 ± 11	3.39 ± 0.17	5.4 ± 1.6
n-Hexane	UAE (5 min, 500 W)	129.2 ± 0.2	110.8 ± 0.1	143 ± 1
Ethanol		577.2 ± 1.0	36.56 ± 0.06	102 ± 7
Limonene		205 ± 13	23 ± 2	38 ± 1
n-Hexane	UAE (15 min, 375 W)	152.2 ± 0.5	113.9 ± 0.4	146.9 ± 7
Ethanol		671.5 ± 0.2	36.72 ± 0.01	77 ± 11
Limonene		372.0 ± 0.1	12 ± 1	22 ± 2

dw- dry weight

3.2. Efficiency of UAE of ergosterol optimized by RSM. Modern technologies

According to literature, the UAE yields of ergosterol varied widely (**Table 2-4**), which makes necessary a study to select and optimize the conditions that favours the process. At one hand, classical methods to optimize the process variables involve changing one variable at a time, keeping the others at fixed levels. This is a laborious and time-consuming method that often does not guarantee the determination of optimal conditions (Box et. al., 2005). On the other hand, carrying out experiments with every possible combination of all possible variables is impractical because of the large number of experiments required (De Lean et. al., 1978; Prieto et. al., 2015). Therefore, a statistical RSM design was applied to generate a second-order polynomial model to

investigate the best possible experimental conditions that maximize the ergosterol yield extraction. However, previous to the design of a multi-response optimization system using a RSM, preliminary tests are needed to simplify the variables and factors that play a very significant role in the enhancement of the extraction yield.

3.2.1. Preliminary tests to assess variables and factors that affect the UAE of ergosterol

A brief combined summary of some of the results obtained is displayed in **Table 5** and its conclusions are detailed described in the following sections.

3.2.1.1. Effect of extracting solvent and amount of solvent

As previously discussed in the Soxhlet extraction section, solvents are the key choice to unlock the sustainability and profitability of processes at industry level. Ethanol, limonene and *n*-hexane were preliminary tested using UAE keeping other extraction conditions constant. The results (**Table 5**) show that ethanol produced the highest extraction yields followed by limonene and *n*-hexane. The suitability of different polarity solvents in the extraction of ergosterol is due to the fact that the ergosterol molecule presents an amphipathic structure, allowing an affinity with solvents with different polarities (Barreira et al., 2014). On one hand, the most common extraction solvent is *n*-hexane and on the other hand, limonene is a very expensive solvent. Therefore, authors have selected *n*-hexane and ethanol solvents for evaluating their ability to extract ergosterol under RSM design.

3.2.1.2. Effect of liquid-to-solid ratio

Commonly, a large liquid-to-solid ratio can dissolve constituents more effectively, leading to an enhancement of the extraction yield. However, at one hand, large ratios will cause too waste solvent. On the other hand, a small liquid-to-solid ratio will result in a lower extraction yield. Therefore, the choice of a proper solvent volume could be significant. The effect of liquid-to-solid ratio on the ergosterol yield has been well investigated in the literature (**Table 2-4**). Authors point out that within a wide range suitable for industrial purposes, no significant differences were noticed when the liquid-to-solid ratio increased or decreased in any of the previous mentioned solvents. Thus, an intermediate liquid-to-solid ratio of 30 g/L was used in the subsequent experiments.

3.2.1.3. Effect of ultrasound power

The effect of ultrasound power on the ergosterol extraction yield was investigated,being an important factor in the extraction of target compounds. In this study, extraction was carried out at different ultrasound powers ranging from 250 to 500 W while other extraction parameters were constant. The results showed that the yield of ergosterol improved from 250 to 500 W, thus these ranges were selected for the subsequent experiments.

3.2.1.4. Effect of extraction time

The effect of extraction time on the ergosterol yield was investigated keeping other extraction parameters constant. The results showed that the yield of ergosterol increased from 5 to 15 min. No evidences were observed that higher time extractions would provided a significant improvement on the extraction yield. Also, the data didn't show decomposition phases, but this fact depends on the other variables that remained constant. Therefore the time range 5 to 15 min was selected. In addition, when higher times than 15 min were considered, authors found that the ultrasound device found difficulties to maintain the temperature constant. Even if higher times could be considered the effect on the variable temperature has to be accounted, which would increase the number of experiments and would make much more complex the determination.

3.2.2. UAE optimization by RSM application

The RSM experiment was designed based on the above preliminary experimental results. The type of solvent, *t* and *P* alter notably the efficiency of the UAE process, meanwhile less relevant effects were observed for solvent-to-material ratio and different mushrooms growth conditions. A full factorial design of three levels was applied and Box-Behnken second-order polynomial model to predict the extraction yield for each solvent was developed. The results obtained are presented in **Figure 5** and **Figure A1** (supplementary material), and in **Tables 6 and 7**.

Table 6. Two connected but different features are presented: a) The experimental domain of the variables *t* (X_1) and *P* (X_2) in natural and coded values. The coded values are presented between brackets and are used to compute the RSM factorial design; and b) The experimental results of ergosterol extraction to the experimental domain of the RSM design (in triplicate, r_1 to r_3) using ethanol and *n*-hexane solvents in two different response formats (Y_1 and Y_2). Y_1, format value in mg/100 g dw to analyse the ergosterol extraction yields and Y_2, in mg/g extract to analyse the ergosterol purity in the extracts.

EXPERIMENTAL DOMAIN		**ERGOSTEROL RESPONSES**					
		n-Hexane			*Ethanol*		
X_1: *t (min)*	X_2: *P (W)*	r_1	r_2	r_3	r_1	r_2	r_3
		Y_1 ; Y_2	Y_1 ; Y_2	Y_1 ; Y_2	Y_1 ; Y_2	Y_1 ; Y_2	Y_1 ; Y_2
5 (-1)	*250 (-1)*	114 ; 85	113 ; 85	113 ; 85	443 ; 55	444 ; 54	441 ; 57
5 (-1)	*375 (0)*	115 ; 103	121 ; 100	112 ; 98	525 ; 47	524 ; 46	525 ; 46
5 (-1)	*500 (+1)*	129 ; 119	129 ; 120	129 ; 110	578 ; 39	576 ; 33	578 ; 35
10 (0)	*250 (-1)*	135 ; 99	140 ; 102	142 ; 103	531 ; 47	512 ; 43	509 ; 45
10 (0)	*375 (0)*	126 ; 127	129 ; 126	127 ; 127	600 ; 38	610 ; 40	591 ; 40
10 (0)	*500 (+1)*	124 ; 125	123 ; 125	124 ; 120	603 ; 36	593 ; 36	603 ; 39
15 (+1)	*250 (-1)*	149 ; 105	149 ; 105	149 ; 105	596 ; 43	594 ; 39	591 ; 40
15 (+1)	*375 (0)*	142 ; 98	142 ; 101	142 ; 97	671 ; 25	671 ; 27	662 ; 29
15 (+1)	*500 (+1)*	126 ; 127	126 ; 124	127 ; 134	630 ; 36	617 ; 36	621 ; 36

Table 7. Results of the full factorial design with 3 levels of the combined effect of time (*t*) and ultrasound power (*P*) on the extraction of ergosterol (mg/100 g dw) according to Eq. [2] and analysis of significance of the proposed model.

		Y_1 (mg/100 g dw)		Y_2 (mg/g extract)	
		n-Hexane	*Ethanol*	*n-Hexane*	*Ethanol*
Fitting coefficients obtained from Eq. [2] and showed on Eqs. [3], [4], [5] and [6]					
Intercept	b_0	129.52±2.44	597.67±8.08	116.29±3.29	37.81±1.40
Linear effect	b_1	9.83±2.99	56.61±5.71	4.97±1.80	-5.61±0.76
	b_2	-3.72±0.99	41.01±3.71	12.82±1.80	-5.34±0.76
Interactive effect	b_{12}	-9.58±0.66	-26.42±3.01	-2.01±0.12	3.72±0.94
Quadratic effect	b_{11}	ns	ns	-11.43±3.12	ns
	b_{22}	ns	-38.78±2.89	ns	4.03±1.32
Statistical information of the fitting analysis					
Observations		27	27	27	27
R^2		0.9276	0.9748	0.9146	0.9533
R^2adj		0.9008	0.9678	0.8902	0.9411
MEC		163.4	5148.9	268.5	378.2
RMSE		12.7	71.7	10.6	13.1
MAPE		2.195	1.599	1.956	2.859
DW		1.907	3.030	1.701	1.010

ns: non-significant coefficient; *R^2*: Correlation coefficient; *R^2adj*: The adjusted determination coefficient for the model; *MSE*: The mean squared error; *RMSE*: The root mean square of the errors; *MAPE*: The Mean Absolute Percentage Error; and *DW*: The Durbin-Watson statistic.

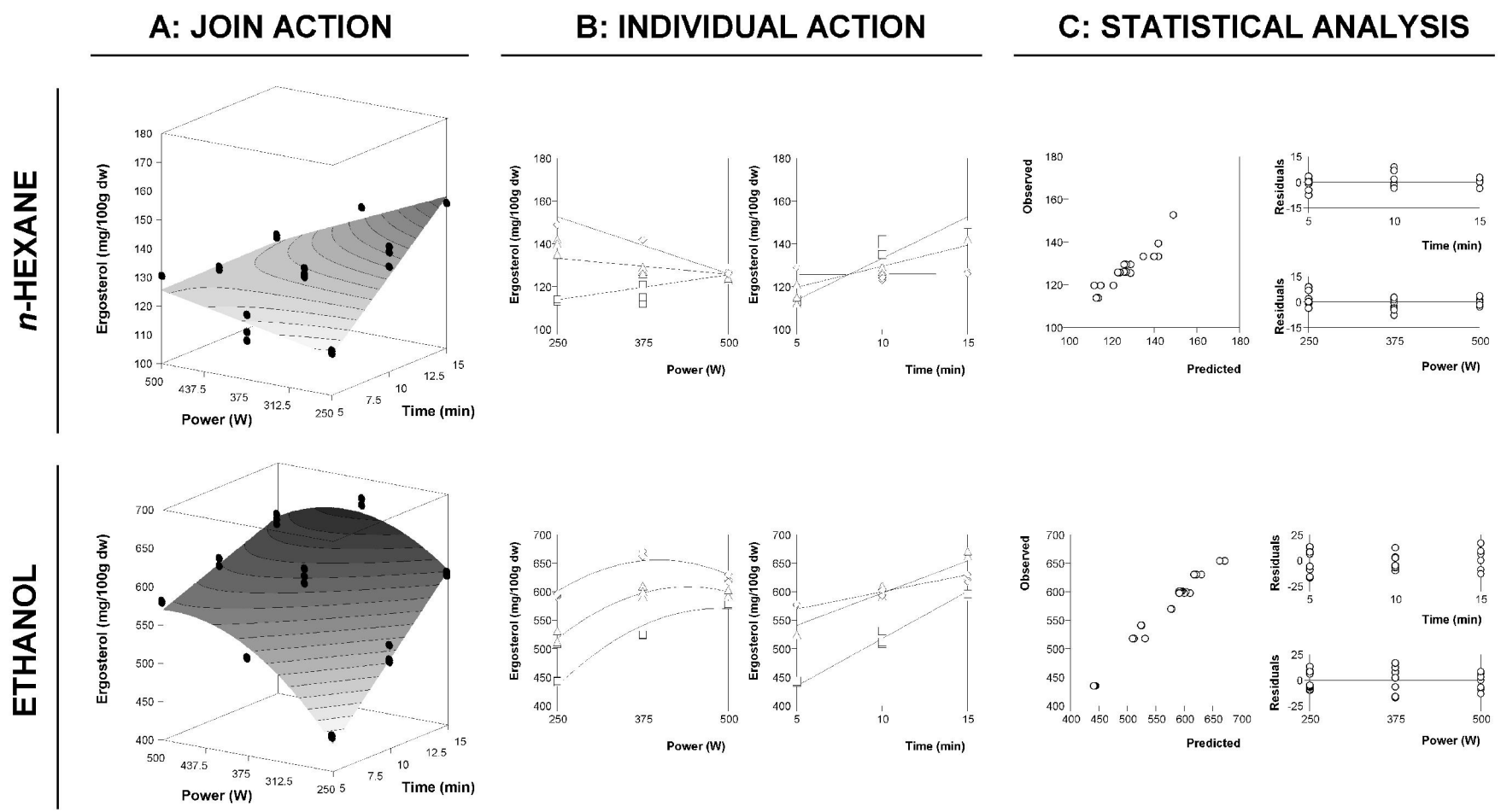

Figure 5. Shows the results in the response value format Y_1 (mg/100 g dw) using *n*-hexane and ethanol as solvent of the extraction. **A:** Ergosterol extraction yield (mg/100 g dw) as a function of extracting time (t) and ultrasound power (P). Points (●) represent the obtained experimental results (**Table 6**) according to the statistical design described**.** The net surface represents the theoretical three-dimensional response surface predicted with the second order polynomial Eqs. [3] and [4]. Estimated parametric values of are shown in **Table 7**. **B:** Two-dimensional representation of the fitting results of Eqs. [3] and [4] (solid line) to the experimental points (□ minimum, △ medium and ◇ maximum variable values) of the combined effect of P and t on ergosterol yield. **C:** To illustrate the statistical description, two basic graphical criteria are used: the ability to simulate the changes of the response and the residual distribution as a function of each of the variables.

3.2.2.1. Theoretical response surface model

Table 6 shows the results in two different response format values (Y_1 and Y_2) obtained after running 27 trials (9 genuine combination conditions and 3 replicates per condition) for each of the solvents used according to the statistical RSM design. Estimated coefficient values of Eq. [2], parametric intervals and numerical statistical criteria are shown in **Table 7**, for each of the responses (Y_1 and Y_2) and for each one of the solvents. These coefficients that showed effects with p-values higher than 0.05 are not significant (ns) at the 95% confidence level and consequently were discarded for model development.

Mathematical models were built through non-linear least-squares estimations based on the coded experimental plan and the response results (**Table 6**) obtaining the following second-order polynomial equations according to Eq. [2]:

when Y_1 response format value (mg / 100 g dw) was considered:

for hexane: $$Y_1^{hex} = 129.52 + 9.83t - 3.72P - 9.58tP \quad [3]$$

and for ethanol: $$Y_1^{eth} = 597.67 + 56.61t + 41.01P - 26.42tP - 38.78\mathrm{P}^2 \quad [4]$$

when Y_2 response format value (mg / g extract) was considered:

for hexane: $$Y_2^{hex} = 116.29 + 4.97t + 12.82P - 2.01tP - 11.43t^2 \quad [5]$$

and for ethanol: $$Y_2^{eth} = 37.81 - 5.61t - 5.34P + 3.72tP + 4.03\mathrm{P}^2 \quad [6]$$

where t is time, P is power, Y is the response, sub-indices 1 and 2 are the response format values and super-indices *eth* and *hex* accounts for ethanol and *n*-hexane solvents.

As explained, not all the parameters present in the second-order polynomial Box-Behnken design model of Eq. [2] were used, since some terms were non-significant (**Table 7**).

Although the model coefficients obtained are empirical and cannot be associated with physical or chemical significance, they are useful to predict the results of untested operation conditions (Rodríguez-Nogales et al., 2007). The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level and when a factor has a negative effect, the response

is lower at high level. The higher the absolute value of a coefficient is, the more important the weight of the corresponding variable is.

Figure 5 and Figure A1 show the results for *n*-hexane and ethanol as solvent of the extraction for each of the response value formats (Y_1 and Y_2), respectively. Each figure is divided into three subsections (A, B and C). The subsection A shows the three-dimensional response surface plots of the ergosterol concentration as a function of *t* and *P* predicted with their respective second order polynomial equation described by Eqs. [3], [4], [5] and [6]. Points (●) represent the obtained experimental results (numerical values in **Table 6**). Estimated parametric values are shown in **Table 7**. The subsection B shows two-dimensional representation of the fitting results to Eqs. [3], [4], [5] and [6] (solid line) to the experimental points (□ minimum, △ medium and ◇ maximum variable values) of the combined effect of *P* and *t*. Finally the subsection C illustrates the capacity to predict the results obtained and the residual distribution as a function of each of the variables *P* and *t*.

The Y_1 response format value (mg/100 g dw) which assess the ergosterol extraction yield shows: (1) The *n*-hexane response with a linear effect between both variables, positive for *t* and negative for *P*, and a negative interactive effect between both variables. In consequence, the extraction yield increases as t increases and decreases as *P* increases, but decreases stronger than increases as *t* and *P* increases due to the stronger negative effect of *P*. The optimum combinations would be found in several parts of the surface described. (2) The ethanol response with a much complex scenery, in which *t* and *P* affects positively to the compound extraction, but the interactive and quadratic *P* terms of the model show a negative effect. In consequence, both *t* and *P* causes the extraction yield to increase until they reach a maximum (or optimum), any further increase on *t* and *P* from the optimum would cause a decrease on the extraction concentration. The optimum combinations would be found at one single point on the surface.

On the other hand, for the Y_2 response format value (mg/g extract) that assess the concentration of ergosterol in the extract (and, therefore, the purity of the extract in ergosterol), totally opposite tendencies as those described for the Y_1 response are shown. A brief summary is described next: (1) The *n*-hexane response shows a positive linear effect between both variables (*t* and *P*), but the interactive and quadratic *t* terms of the model show a negative effect. In consequence, the concentration of the extract increases

as *t* and *P* increases, but decreases in a non-linear way when *t* and *P* increases. Strong decomposition effects are shown as t increases. (2) In the ethanol case, the mathematical response shows a negative effect as *t* and *P* increases, resulting on a decrease of the purity of the extract. The interactive and quadratic terms of the model show a positive effect. In consequence, both *t* and *P* causes a decrease of the ergosterol concentration in the extract.

The behaviour of the extraction can be understood by of the second-order polynomial Box-Behnken models described in Eqs. [3], [4], [5] and [6] or in their graphical representation in **Figure 5 and Figure A1**. However, to make more explicit the appealing combinations of yield (Y_1) and purity (Y_2) response format values depicted in the extraction of ergosterol, **Figure 6** shows the isolines of each response to describe visually the tendencies and guide easily the selection of the most favourable conditions.

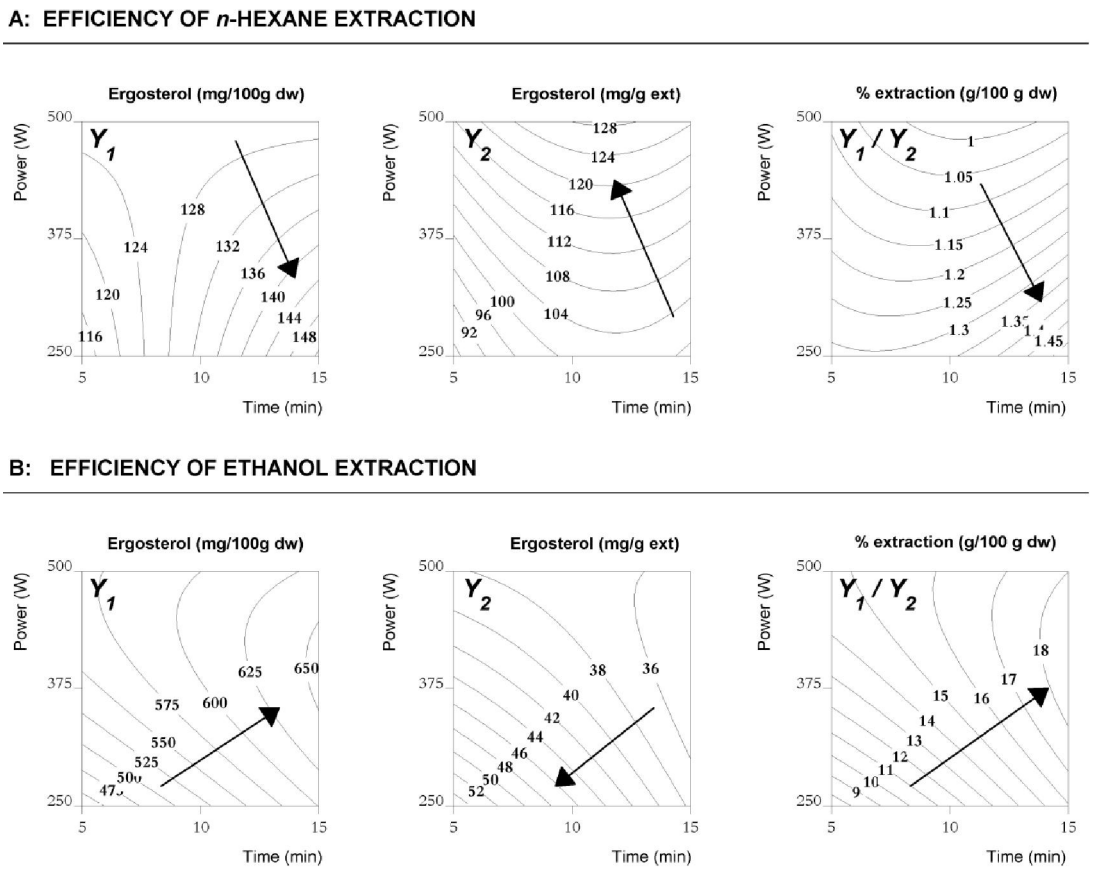

Figure 6. Shows the isolines of both response value formats (Y_1, mg/100 g dw and Y_2, mg/g extract) to describe visually the tendencies of each response and guide the selection of the most favourable conditions, taken into account simultaneously the ergosterol extraction yields and extract purity in ergosterol. Note that the third graphical response, is actually obtained by dividing the responses Y_1/Y_2, which provides g of extract/100 g dw of mushroom, in other words, the % of the extracted material.

3.2.2.2. Statistical and experimental verification of predictive models

This multivariable characterization of the Box-Behnken second-order polynomial model is especially robust, minimizing the effects of random and systematic errors, allowing researchers to squeeze the utmost of the results. As stated by many authors before (De Lean et al., 1978; Murado & Prieto, 2013), optimally and efficient data analysis should involve simultaneous description of all curves, rather than fitting each one individually. The simultaneous curve-fitting reduces the number of parameters needed to analyse the response, it is a more informative approach and provides better estimations of parameters, and finally reduces their interval of confidence (De Lean et al., 1978; Murado & Prieto, 2013). In addition, once all the modes of action are mathematically known, if the experimental curves obtained do not span the full range and some of them fail to provide information about one or more of the parameters of the equation, the multivariable application describes simply and accurately all the areas. Additionally, by standardizing the response, the results obtained are less dependent on the experimental conditions, which, in practice, is one of the common problems when analyzing the efficacy of response factors.

The statistic lack of fit, used to test the adequacy of the models obtained demonstrated that considerable improvement was achieved by the exclusion of the statistically non-significant effects (**Table 7**). This was also verified by the high R^2 and R^2_{adj} values indicating the percentage of variability of each response that is explained by the model (**Table 7**). Additionally, **Figure 5 and Figure A1** (subsections C) show the distribution of residuals always randomly scattered around zero and grouped data and autocorrelations were not observed. This means that these models are workable and can be applied in the subsequent prediction and optimisation stages and also indicates a good agreement between the experimental and predicted values which implies that the variation are explained by the independent variables.

Finally, **Table A1** (supplementary material) shows the analysis of variance (ANOVA) for the regression equation. The linear term and quadratic term were highly significant ($p < 0.01$). The lack of fit was used to verify the adequacy of the model and was not significant ($p > 0.05$), indicating that the model could adequately fit the experimental data.

3.2.2.3. Optimal conditions that maximize the extraction and its applicability for industrial purposes

The optimal values of the selected variables for ethanol extraction can be obtained by solving the regression Eqs. [3], [4], [5] and [6], by equating the partial derivatives to zero and decoding the code value to its natural value.

As well as in the Soxhlet extraction, the ethanol proved to be the most efficient solvent, extracting the highest levels of ergosterol in UAE. Therefore, solving Eq. [4] indicates that the optimal (maximal) time condition results in a linear relation with the *P* variable, meanwhile the optimal power condition resulted to be the centre of the domain (375 W). Since in previous tests we had evaluated that 15 min was an asymptotic value for the variable *t*. It can be affirmed that the conditions that lead to the maximum extraction concentration of ergosterol in ethanol are on the surroundings to 375 W for 15 min (671.5 ± 0.5 mg/100 g dw). To confirm these results, tests were performed in triplicate under optimized conditions.

Using ethanol as solvent, the ergosterol content in terms of mg/100 g dw increased with the increase of *P* (**Figure 6**). Otherwise, with respect to mg/g extract, the content in ergosterol decreased, meaning that the ethanol is extracting other molecules, which increases the yield of the extraction, but with a decrease in the purity of the extract in terms of ergosterol. These results are in agreement with other studies on this subject (**Table 3-4**), where several authors studied various solvents (methanol, dichloromethane and chloroform) in a ratio that increased the extraction efficiency of sterols. Villares et al. (2014), reported an extraction of 642 ± 0.15 mg/100 g dw of ergosterol from *A. bisporus* using chloroform/methanol in an ultrasound bath; this value is very similar to the one obtained in this work (671.5 ± 0.5 mg/100 g dw).

In consequence, from an industrial point of view, the extraction with ethanol on the surroundings of 500 W and 5 min lead to the extraction content of 577.2 ± 1.0 mg/100 g dw with a higher purity content of ergosterol. The value is significant less optimal than the maximum content (671.5 ± 0.5 mg/100 g dw), but in terms of ergosterol purity, time and energy reductions may be considered as more favourable conditions.

3.3. Comparison of the efficiency of ergosterol extraction by UAE and Soxhlet techniques

The advantages of the UAE over other conventional methods, such as the Soxhlet, are related to time and amount of solvent used. As described in **Table 5** and **Table 6**, the Soxhlet extraction takes about 4 h to extract the same content of ergosterol while the UAE optimized by RSM yields to the same quantity in about 15 min, using less amount of solvent. Both methodologies conducted to very similar amounts of ergosterol in terms of concentration, mg/g extract and mg/100 g dw. This aspect might be explained by cavitation phenomenon; cycles form, grow and collapse of bubbles formed during propagation of the waves. The ultrasound sonication is defined as the application of waves with high frequency and their interaction with substances. The collapse of the bubbles within the matrix causes disruption of cell structure, increasing the release of extractable compounds and enhancing the mass transference to the solvent (Wang & Weller, 2006).

Independently of the theoretical explanation that lies behind the faster extraction of mycosterols by UAE in comparison with conventional techniques, the application of both methodologies in an industrial environment requires the removal of the usual saponification step in order to turn the process more practical, profitable and sustainable.

3.4. Pertinence of the saponification step

In order to diminish the process complexity, the pertinence of the saponification step was evaluated. To date most authors use it with the objective of purifying the extract. However, the saponification step may be an unworthy time-consuming operation; indeed, it could be the bottleneck of any possible industrial transference of mycosterol's extraction from *A. bisporus*. Analysing **Table 5** by comparing Y_2 (obtained before the saponification step) and the ergosterol content in the extract after the saponification, it can be observed that in the case of the ethanolic extract, this step increased higher its purity, while for the *n*-hexane and limonene extracts the purity was almost similar. This is explained by the fact that the polarity of ethanol is higher than the one of *n*-hexane and limonene, which leads to a less pure extract. In fact, *n*-hexane and limonene present a higher selectivity for the lipophilic compounds compared to ethanol. The results

suggest that the saponification step can be avoided without significant differences to the final results, in particular in the case of all *n*-hexane and limonene extracts and even for the ethanol extract obtained in the recommended UAE conditions (15 min, 375W).

4. Conclusions and future perspectives

Overall, UAE is a powerful modern extraction technology that proved to be an efficient methodology in terms of ergosterol extraction yield and extract purity. Additionally, UAE significantly decreased the extraction time when compared with Soxhlet extraction (from 4 h to 15 min). The RSM was successfully employed to optimize the extraction and several experimental parameters.

The results showed that the variables extraction solvent, ultrasound power, and extraction time all had significant effects on the concentration of mycosterols. In statistical terms, the high value of the adjusted determination coefficient for each solvent, which was higher than R^2_{adj}=0.9 in all cases, and the no-significant difference between predicted and experimental values demonstrated the validity of the optimization model proposed. Ethanol proved to be a better solvent to extract higher levels of ergosterol when compared with *n*-hexane and limonene.

The optimal extraction conditions are ethanol at t = 15 min and P = 375 W, which yields an ergosterol content of 671.5 ± 0.5 mg/100 g dw of *A. bisporus* mushroom. Furthermore, in the case of the ethanolic extract, the saponification step increased its purity to 21%, while for the *n*-hexane extract (without saponification step) the purity was similar. Other emerging methodologies such as microwave-assisted extraction can be applied with foreseen interesting results.

The results reported in this work were submitted to Food Chemistry journal (Q1, IF= 3.391).

Various studies have been applied to the extraction of sterols, in particular phytosterols (Xiao et al., 2013). The mentioned authors concluded that microwave-assisted extraction (MAE) is an efficient sample preparation technique and has good potential on the extraction of phytosterols from the algae; when compared with traditional solvent extraction it gives much higher extraction yields for shorter extraction times. Until the moment there are no reports in the literature for the extraction of mycosterols, namely ergosterol using this technique. However taking into account the MAE characteristics it can also be a promising technique for this purpose, since it presents a number of variables that can be optimized to obtain a high extraction efficiency of such molecules. With the aim of optimizing the most adequate extraction conditions for ergosterol from

A. bisporus using MAE, an experimental design was elaborated to achieve this goal (**Table A2)**. This study is on-going being the main future perspective of the present work

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Annexes

Annexe 1.

Table A1. ANOVA table for the model of ergosterol extraction with ethanol and *n*-hexane solvents.

Source	*df*	*SS*	*MS*	$F_{statistic}$	*Pr > F*
Response format Y_1 (mg/100 g dw)					
a) Ergosterol extraction with n-hexane					
Model	5	3110	622	40	< 0.0001
Error	23	322	15.3		
Lack of fitting	19	321	16.9	50	0.020
Pure error	2	0.667	0.333		
Total corrected	26	3432			
b) Ergosterol extraction with ethanol					
Model	5	105346	21069	160	< 0.0001
Error	22	2763	131		
Lack of fitting	19	2523	132	1.107	0.578
Pure error	2	239	119		
Total corrected	26	108110			
Response format Y_2 (mg/g extract)					
a) Ergosterol extraction with n-hexane					
Model	5	3562	546	39	< 0.0001
Error	22	298	16.2		
Lack of fitting	19	286	17.6	47	0.033
Pure error	2	0.888	0.342		
Total corrected	26	3258			
b) Ergosterol extraction with ethanol					
Model	5	1053	452	12	< 0.0001
Error	21	266	12		
Lack of fitting	20	244	12	121	0.058
Pure error	2	1.21	0.752		
Total corrected	26	2552			

df: degree of freedom; *SS*: Sum of squares; *MS*: Mean square.

Annexe 2.

Tables A2. Experimental design for extractions in MAE.

						Center		
		Center	Interval	-1,68	-1,00	0,00	1,00	1,68
t	Min	12,00	5,00	4	7	**12**	17	20
T	ºC	135,00	45,00	59	90	**135**	180	211
S/L	g/L	10,00	5,00	1,6	5,0	**10,0**	15,0	18,4

Run	Coded variables			Natural variables			Responses		
	t (min)	*T (ºC)*	*S/L (mg/mL)*	*t (min)*	*T (ºC)*	*S/L (g/L)*	*mg/100g dw*	*mg/g ext*	*% ext*
1	-1.00	-1.00	-1.00	7.0	90	5.0			
2	1.00	-1.00	-1.00	17.0	90	5.0			
3	-1.00	1.00	-1.00	7.0	180	5.0			
4	1.00	1.00	-1.00	17.0	180	5.0			
5	-1.00	-1.00	1.00	7.0	90	15.0			
6	1.00	-1.00	1.00	17.0	90	15.0			
7	-1.00	1.00	1.00	7.0	180	15.0			
8	1.00	1.00	1.00	17.0	180	15.0			
9	-1.68	0.00	0.00	3.6	135	10.0			
10	1.68	0.00	0.00	20.4	135	10.0			
11	0.00	-1.68	0.00	12.0	59	10.0			
12	0.00	1.68	0.00	12.0	211	10.0			
13	0.00	0.00	-1.68	12.0	135	1.6			
14	0.00	0.00	1.68	12.0	135	18.4			
15	0.00	0.00	0.00	12.0	135	10.0			
16	0.00	0.00	0.00	12.0	135	10.0			
17	0.00	0.00	0.00	12.0	135	10.0			
18	0.00	0.00	0.00	12.0	135	10.0			
19	0.00	0.00	0.00	12.0	135	10.0			
20	0.00	0.00	0.00	12.0	135	10.0			

Annexe 3.

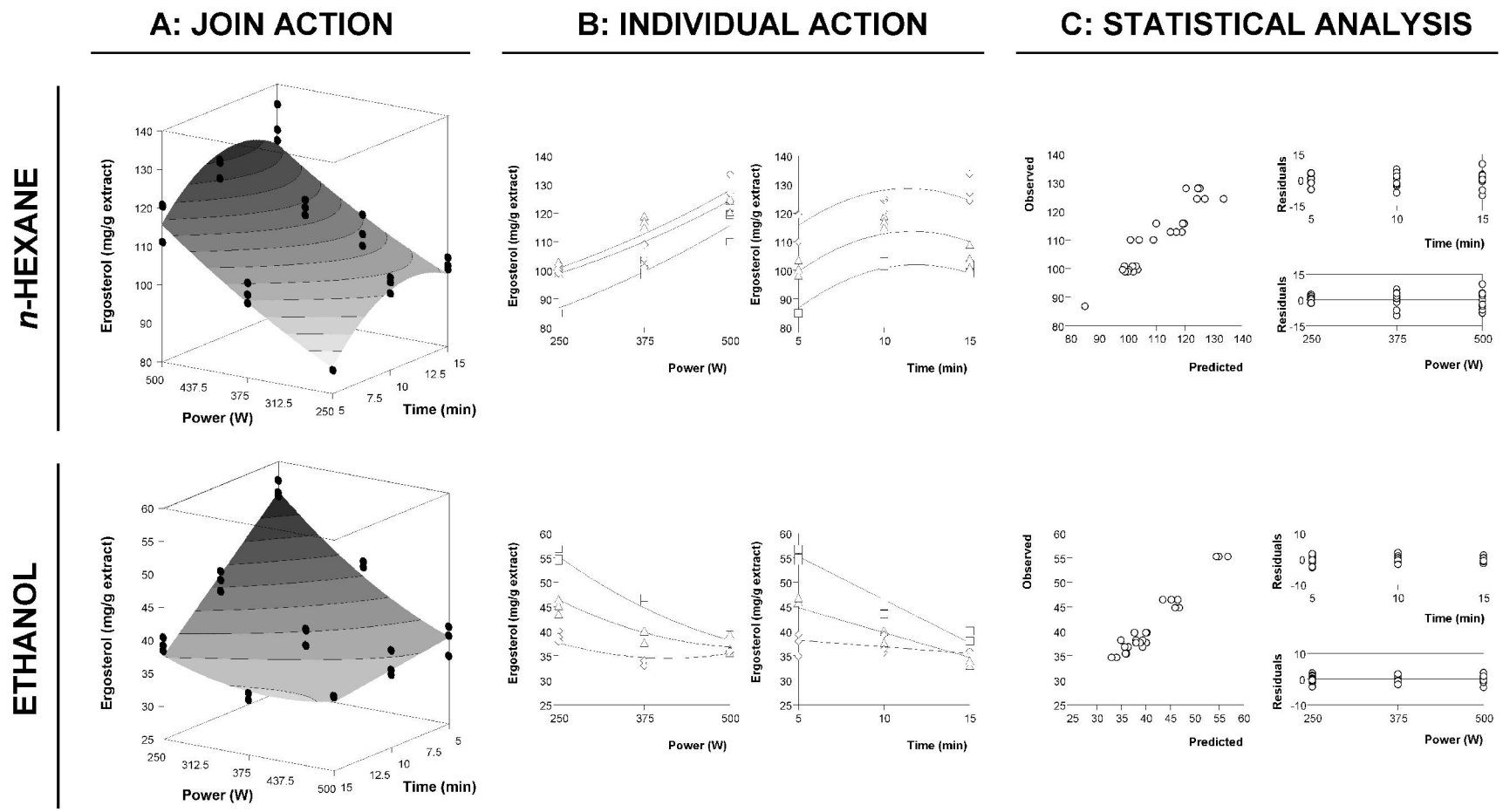

Figure A1. Shows the results in the response value format Y_2 (mg/g extract) using *n*-hexane and ethanol as solvent of the extraction. **A:** Ergosterol purity in the extract (mg/g extract) as a function of extracting time (*t*) and ultrasound power (*P*). Points (●) represent the obtained experimental results (Table 6) according to the statistical design described**.** The net surface represents the theoretical three-dimensional response surface predicted with the second order polynomial Eqs. [5] and [6]. Estimated parametric values of are shown in Table 7. Please note that for the ethanol 3D figure the scales are organized in reverse way to allow readers full view of the surface. **B:** Two-dimensional representation of the fitting results of Eqs. [5] and [6] (solid line) to the experimental points (□ minimum, △ medium and ◇ maximum variable values) of the combined effect of *P* and *t* on ergosterol yield. **C:** To illustrate the statistical description, two basic graphical criteria are used: the ability to simulate the changes of the response and the residual distribution as a function of each of the variables.