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

**Running title:** Optimization of ultrasound-assisted extraction to obtain mycosterols

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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 alter notably 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 375W 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 the removal of the saponification step, which simplifies the extraction process and makes it more feasible for its industrial transference.

**Keywords**: *Agaricus bisporus* L.*;* Ergosterol; Soxhlet extraction; Ultrasound-assisted extraction; Response Surface Methodology; Saponification.

## 1. Introduction

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, fiber, 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 mushrooms consumption 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).

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 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, is often used by the food processing industries and researchers with the purpose of extracting effortlessly 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 et al., 2013). In comparison with conventional procedures, they are less time-consuming and require less amount of polluting solvetns. 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 (Yuan et al., 2008; Villares et al., 2012; Villares et al., 2014).

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 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).

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

*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 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 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 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 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 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 Soxhlet or UAE technique), which was specifically used to evaluate the ergosterol purity in the extracts. Both responses were equally analyzed, 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 proportion, 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 1**).

*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 $\Delta x$ 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 parametric confidence intervals were 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

3.1. Efficiency of ergosterol extraction by Soxhlet. Traditional approach

*3.1.1. Recommended conditions on the Soxhlet process*

The 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) and described in Material & Methods. For the selection of the appropriate solvent, we have avoided the use of toxic or hazardous organic solvents (such as benzene, cyclohexane, dichloromethane) and used, as part of a more green industrial process, the solvents of *n*-hexane, ethanol and limonene.

*3.1.2. Soxhlet extraction*

As presented in **Table 2**, ethanol proved to be the most efficient solvent to extract 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 1**), 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.

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

According to literature, the UAE yields of ergosterol varied widely (**Table 1**), 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 involved 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 2** and its conclusions are detailed described in the following sections.

*Effect of extracting solvent and solvent proportion*. 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 2**) show that ethanol produced the highest extraction yields followed by limonene and *n*-hexane. The suitability of the use 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.

*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 1**). 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.

*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. The increase of extraction process using ultrasound is partially attributed to the cavitation phenomena, but also to the molecular breakdown effects. In this study, extraction was carried out at ultrasound powers ranging from 100 to 500 W while other extraction parameters were constant. The results showed that the ergosterol yield of improved from 250 to 500 W, thus these ranges were selected for the subsequent experiments.

*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 provide a significant improvement on the extraction yield. In addition, 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 times higher 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 1** and **Figure A1** (supplementary material), and in **Tables 3** and **4**.

*Theoretical response surface model.* **Table 3** 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 4**, 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 3**) obtaining the following second-order polynomial equations according to Eq. [2]:

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

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

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

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

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

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

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 4**).

Although the model coefficients obtained are empirical and cannot be related with physical or chemical significance, they are useful to predict the results of untested operation conditions (Ramirez et al., 2000). 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 1** 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 3**). Estimated

parametric values are shown in **Table 4**. 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. Therefore, 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 hypothesis behind the strong decomposition effects of ergosterol may be related with the combination of multiple causes. As time increases, also increases the temperature, variable that has been pointed out as one of the major causes of ergosterol degradation (Kadakal & Artik, 2007). In addition, the cavitation effect produced by the ultrasonic device also increases, a phenomenon that has been pointed out as one of the main reasons for the extraction and destruction of compounds in UAE.

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 1** 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 2** shows the isolines of each response to describe visually the tendencies and guide easily the selection of the most favourable conditions.

*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, Munson, & Rodbard, 1978; Murado & Prieto, 2014), 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 analyze the response, it is a more informative approach and provides better estimations of parameters, and finally reduces their interval of confidence (De Lean, Munson, & Rodbard, 1978; Murado & Prieto, 2014). 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 4**). 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 4**). Additionally, **Figure 1** 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.

*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 stated 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 2**). 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 1**), 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 ultrassound 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 around 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 2** and **Table 3**, 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*. Analyzing **Table 2** 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).

**Conclusions**

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 decreases 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 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 the best 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 in *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 the purity was similar. Other emerging methodologies such as microwave-assisted extraction can be applied with foreseen interesting results.

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## TABLES

**Table 1.** Ergosterol extraction reports available in the literature.

| Samples | Extraction procedure | Saponification step | Solvent | Ergosterol (mg/100 g dw) | References |
|---|---|---|---|---|---|
| *Agaricus bisporus* L. | Soxhlet | Yes | *n*-Hexane | 352±1 | Barreira et al., 2014 |
| *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 hepatica (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 | |
| *Agrocybe aegerita* (V. Brig.) Singer | Ultrasound (Probe) | No | Methanol/ dichloromethane (75:25, v/v) | 351±0.06A<br>144±0.04B<br>240±0.05C | Yuan et al., 2008 |
| *Lentinus edodes* (Berk.) Pegler | | | | 170±0.03A<br>402±0.08B<br>111±0.03C | |
| *Termitomyces albuminosus* (Berk.) R. Heim | | | | 215±0.04A<br>170±0.03B<br>402±0.08C | |
| *Tuber aestivum* Vittad. | Ultrasound (Bath) | No | Chloroform /methanol (2:1, v/v) | 151±0.20 | Villares et al., 2012 |
| *Tuber indicum* Cooke & Massee | | | | 128±0.11 | |
| *Tuber melanosprrum* Vittad. | | | | 180±0.12 | |
| *Agaricus bisporus* L. | Ultrasound (Bath) | No | 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 | |

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

**Table 2.** 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 ± 0.5 | 36.72 ± 0.01 | 77 ± 11 |
| Limonene | | 372.0 ± 0.1 | 12 ± 1 | 22 ± 2 |

**Table 3.** 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 analyze the ergosterol extraction yields and $Y_2$, in mg/g extract to analyze the ergosterol purity in the extracts.

| **EXPERIMENTAL DOMAIN** | | **ERGOSTEROL RESPONSES** | | | | | |
|---|---|---|---|---|---|---|---|
| | | *n-Hexane* | | | *Ethanol* | | |
| $X_1$: *t (min)* | $X_2$: *P (W)* | $r_1$ $Y_1$ / $Y_2$ | $r_2$ $Y_1$ / $Y_2$ | $r_3$ $Y_1$ / $Y_2$ | $r_1$ $Y_1$ / $Y_2$ | $r_2$ $Y_1$ / $Y_2$ | $r_3$ $Y_1$ / $Y_2$ |
| *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 4.** 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 |
| $R^2$adj | | 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.

**FIGURES**

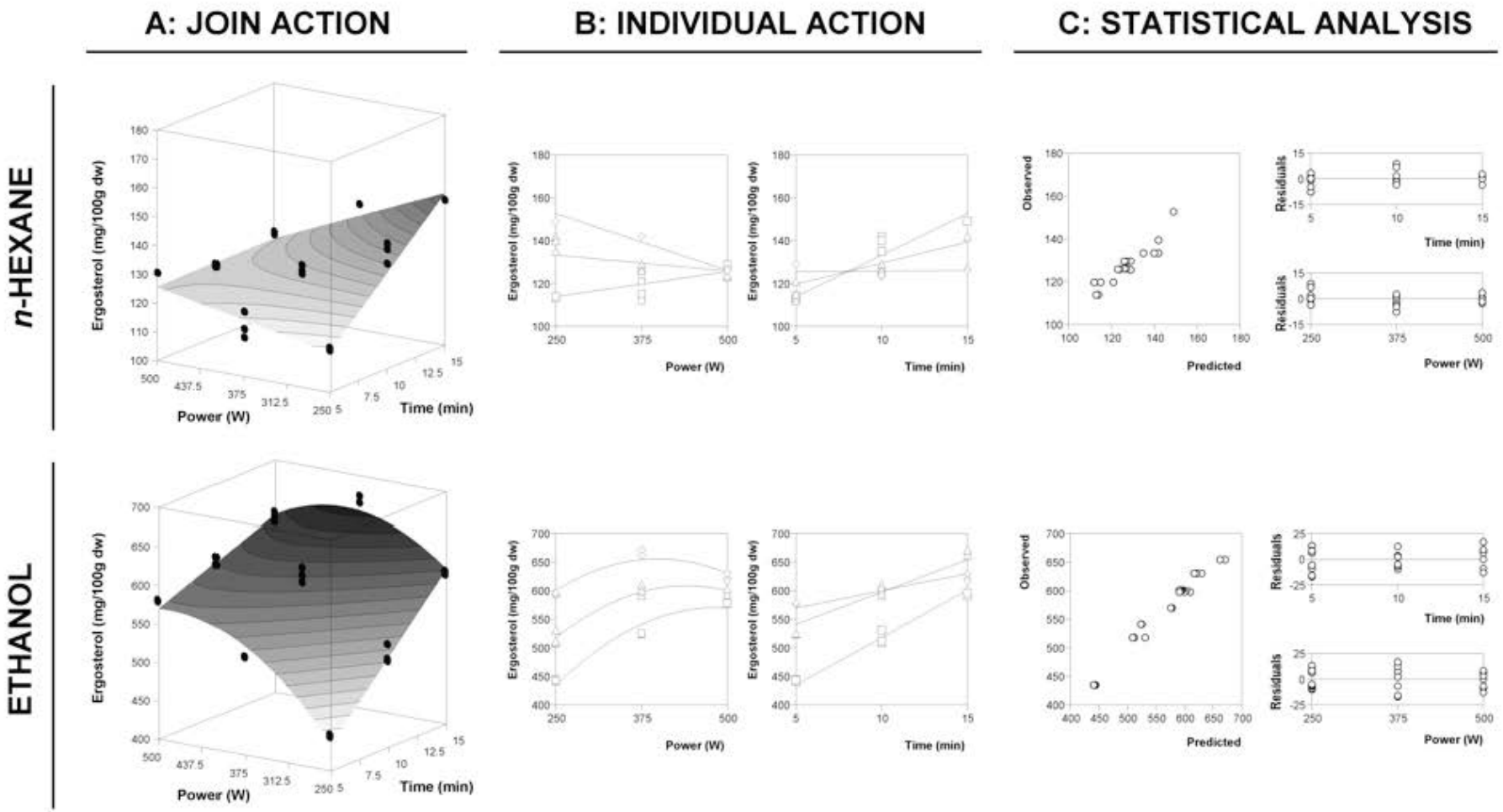


**Figure 1.** Shows the results in the response value format $Y_1$ (mg/100 g dw) using *n*-hexane and ethanol as solvent of the extraction. **A:** Ergosterol extraction yield (mg/100 g dw) as a function of extracting time ($t$) and ultrasound power ($P$). Points (●) represent the obtained experimental results (**Table 3**) 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 4**. **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.

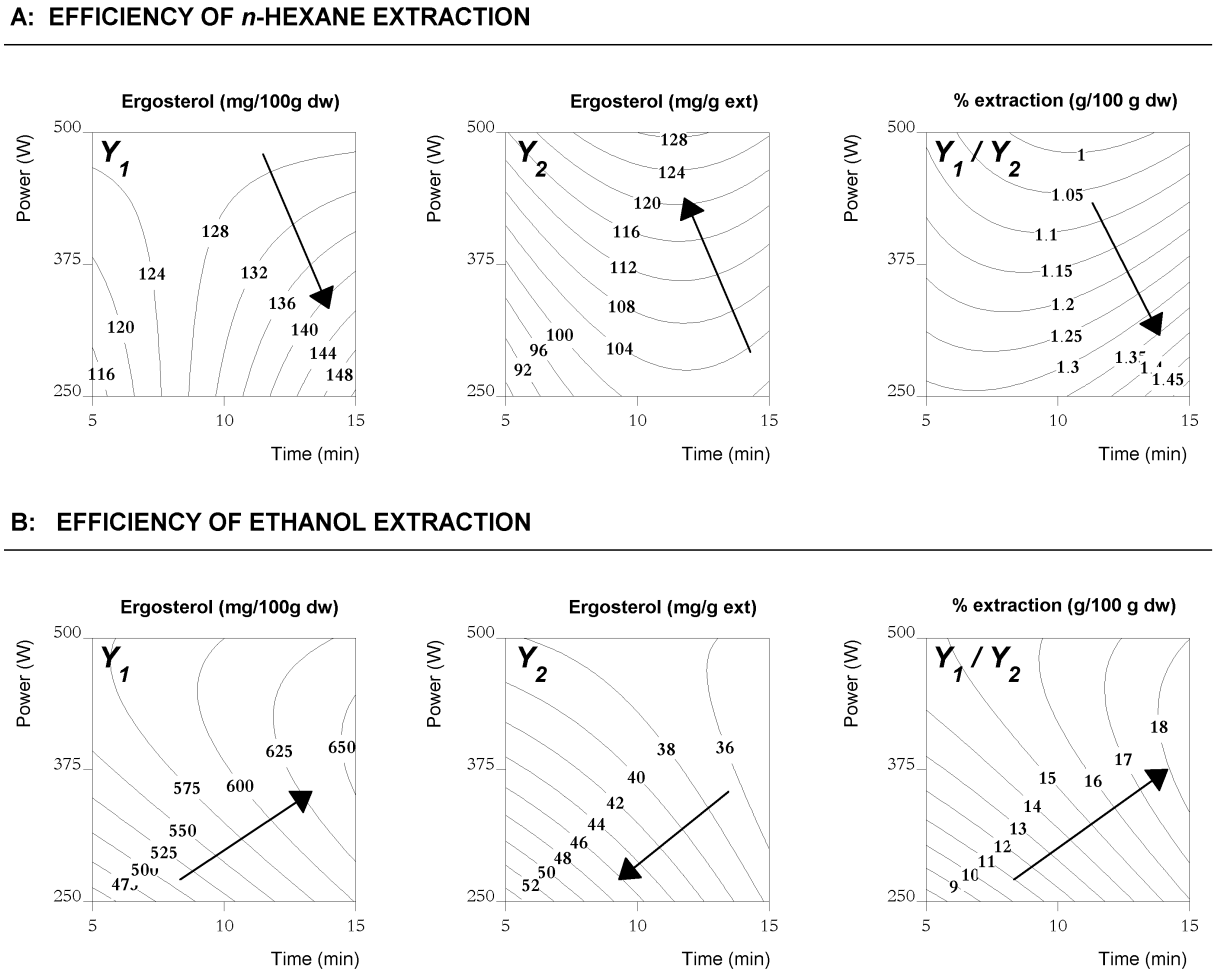


**Figure 2**. Shows the isolines of both response value formats ($Y_1$, mg/100 g dw and $Y_2$, mg/g extract) to describe visually the tendencies of each response and guide the selection of the most favourable conditions, taken into account simultaneously the ergosterol extraction yields and extract purity 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.