Optimization of microwave-assisted extraction of ergosterol from *Agaricus bisporus* L. by-products using response surface methodology

Running title: Optimization of microwave-assisted extraction of ergosterol

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Abstract

This work intends to valorise by-products of the industrial processing of mushrooms to obtain ergosterol as a value-added compound. *Agaricus bisporus* L. is the world's most consumed mushroom and one of the richest sources of ergosterol. Microwave-assisted extraction was used to replace conventional techniques that are time-consuming and need large amounts of solvent. Time (3-20 min), temperature (60-210 ºC) and solid-liquid ratio (1-20 g/L) were found the relevant variables to analyze the extraction process. To maximize the ergosterol extraction yield, response surface methodology was used to optimize the process. The global optimal extraction conditions were determined and comprise: 19.4±2.9 min, 132.8±12.4 ºC and 1.6±0.5 g/L, yielding 556.1±26.2 mg of ergosterol per 100 g of mushroom by-products. In the MAE optimal conditions, it was possible to obtain ergosterol in a similar value to the one obtained in other works when using the Soxhlet extraction method with a significant decrease in the time of extraction. The results show the potential of using the by-products of an agroindustry, mushrooms processing industry, as productive sources of ergosterol.

Keywords: *Agaricus bisporus* by-products; valorisation; ergosterol; microwave-assisted extraction; optimization

1. Introduction

Among two thousand species of edible mushrooms, only a handful is worldwide cultivated and processed at industrial level, in which *Agaricus bisporus* L. is included (Chou et al., 2013; Leiva et al., 2015; Royse, 2014). During the mushroom's manufacturing process a large amount of by-products is generated (Wu and Zivanovic, 2004). Some examples are the volva and bottom part of the stems due to their tough texture, or the organic matter present in the effluent generated in the washing and blanching stages. In addition, during mushrooms cultivation and harvest, the specimens with irregular dimensions and shape are discarded.

Apart from the large amounts of the mentioned solid wastes, there is also a surplus production leading to a glut in the market, distress sale and low profit to the growers. Depending on the size of the mushroom industry, the amount of by-products ranges between 20 to 35% in weight of fresh mushrooms (Gil-Ramírez et al., 2013; Vasylenko et al., 2008). These by-products with a high nutritional value are being wasted, but also demand industries to deal with their environmental impact and associated managing costs (Leiva et al., 2015). Current industrial strategies for mushroom's by-products are based in low-economic income solutions (e.g., animal feed and compost). Since mushrooms production/consumption rates are expected to increase in the next years, the environmental problem is expected to growth proportionally and new alternative and profitable solutions need to be explored (Royse, 2014). Several approaches have been postulated, including the production of biofuel, chitin and chitosan, β-glucan and sterols (Chou et al., 2013; Gil-Ramírez et al., 2013; Vasylenko et al., 2008; Wu and Zivanovic, 2004). However, innovative research is crucial to proper evaluate the most feasible and economically viable solutions for theses raising by-product materials from fungal sources.

Among the possibilities, ergosterol obtainment is quite attractive. It is the most abundant mycosterol, especially in *Agaricus bisporus* L. (90% of its sterols fraction) (Barreira et al.,

2014; Gil-Ramírez et al., 2014), and has been related with different bioactive properties (Barreira and Ferreira, 2015; Villares et al., 2012). Moreover, ergosterol can be converted by irradiation, into vitamin D for sale as a dietary supplement and food additive (Asinghe and Perera, 2005; Teichmann et al., 2007).

A broad spectrum of solid liquid extraction techniques is widely used for the extraction of natural products. Conventional methods used for many decades include Soxhlet extraction, maceration, and percolation, among others. They are often time-consuming and require large quantities of solvents, including hazardous ones (Wang et al., 2013). Emerging technologies, such as supercritical fluid extraction, microwave-assisted extraction (MAE), pressurized solvent extraction (PSE) and ultrasound-assisted extraction (UAE) are fast and efficient extraction technologies that have been used in the last decades by the food processing industries and researchers with the purpose of extracting more efficiently major and minor compounds present in natural matrices, saving energy and reagents, avoiding losses and optimizing the extracting yields (Gil-Ramírez et al., 2013; Heleno et al., 2016; Villares et al., 2014).

According to literature data, the extraction yields of ergosterol can vary widely depending on the type of solvent, extraction time and technologies applied. Due to the low ergosterol concentration present in the cell membranes, it is necessary to study its performance during the extraction. Classical methods to optimize the process variables involve changing one variable at a time, keeping the others at fixed levels (Prieto et al., 2011). Single factor analysis are laborious and time-consuming methods and often does not guarantee the determination of the optimal conditions (Box et al., 2005; Wang et al., 2013). On the other hand, carrying out experiments using every possible combination of the test variables is impractical due to the associated large number of experiments required (Rodríguez-Nogales et al., 2007). One viable strategy consists on selecting, firstly, the variables playing a significant role in the extraction

yield, and then applying the statistical multi-response optimization using a response surface methodology (RSM).

The present study aimed at optimizing the MAE process to extract ergosterol from *A. bisporus* discarded by-products having in view applications in food, pharmaceutical and cosmetic industries. To the author's best knowledge, there are no other reports in the literature describing MAE optimization for ergosterol extraction. This technology has been applied mostly for phenolics and phytosterols extraction (Mustapa et al., 2015; Roselló-Soto et al., 2015). The results obtained in the present study will be compared with the ones obtained by our research group in the optimization of UAE in comparison with the conventional extraction technique, Soxhlet extraction (Heleno et al., 2016).

By means of RSM, the joint effect of the variables time (*t*), temperature (*T*) and solid/liquid ratio (*S/L*) on ergosterol extraction yield was described. With this study, where individual and interactive effects among variables were studied, the authors expect to give a contribution towards the understanding of the real potential of ergosterol extraction and related industrial applications.

2. Materials and methods

2.1. *Samples*

The *Agaricus bisporus* L. discarded by-products were obtained from a local mushrooms production company, Mogaricus Cogumelos - Sociedade Unipessoal Lda. All the samples were weighted, lyophilized (FreeZone 4.5 model 7750031, Labconco, Kansas City, MO, USA), and reduced to a fine dried powder (20 mesh) (Ultra Centrifugal Mill ZM 200, Porto, Portugal).

2.2. *Standards and reagents*

Methanol and acetonitrile of HPLC grade from Fisher Scientific (Lisbon, Portugal) were used. The standards of sterols (ergosterol, cholecalciferol) were purchased from Sigma-Aldrich (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. *Settings and procedure for ergosterol microwave-assisted extraction*

MAE process was performed using a Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) using closed vessels. The lyophilized powdered samples were extracted at different *t*, *T* and *S/L* ratio in the range defined by the RSM design. Before the extraction, an adequate volume of cholecalciferol (internal standard) was added to each sample. The main internal settings of the device during the MAE process were as follows:

1) Because pressure and *T* are correlated; moreover, the power applied is also linked to the *t* needed to reach the selected *T* or pressure. In consequence, the *T* variable was selected as the main controlled one and power variable was set to the maximum one (400 W) in order to ensure that the *t* to reach the selected *T* is the minimum one (less than 20 sec for the highest *T* used). Therefore, to maintain *T* constant, the power would not be applied constantly along the duration of the experiment.
2) The fixed hold-time option was "off" since the t needed to reach the desired *T* when the microwave power was set to a power of 400 W was very short.
3) The cooling option was decided to be "on", which make possible to stop the extraction process by lowering *T* reached in less than 1 min in the worse conditions scenery.

After microwave extraction, samples were filtered through a Whatman paper filter (nº4), the residue discarded and the filtered material evaporated under reduced pressure to remove the

solvent. Finally, the obtained residue was dissolved in methanol at a concentration of 10 mg/mL, filtered through a 0.2 μm nylon filter for ergosterol quantification.

2.4. *Ergosterol quantification*

The identification and quantification of ergosterol were performed according to the procedure descried in Barreira et al. (2014). The HPLC equipment consisted of an integrated system with a pump (Knauer, Smartline system 1000, Berlin, Germany), a degasser module (Smartline manager 5000), auto-sampler (AS-2057 Jasco, Easton, MD) and a UV detector (Knauer Smartline 2500). Data were analyzed using Clarity 2.4 Software (DataApex). The used column was a Inertsil 100A ODS-3 reversed-phase (4.6×150 mm, 5 μm, BGB Analytik AG, Boeckten, Switzerland) operating at 35 °C (7971R Grace oven). Acetonitrile/methanol (70:30, *v/v*) mobile phase at a flow rate of 1 mL/min was used. The injected sample volume was 20 μL and detection was performed at 280 nm. Ergosterol was quantified based on a calibration curve obtained with a commercial standard in the adequate range of concentrations. Quantification was performed using the internal standard method with cholecalciferol as the internal standard.

2.5. *Response format values to express the results*

The results were expressed in two response (Y) format values: Y_1, as the content of ergosterol in the extracted residue, which was specifically used to analyze the extract purity in ergosterol (% E, w/w); and Y_2, as mg of ergosterol per 100 g of mushroom dry weight (dw) material, which was used to analyze the extracted ergosterol yield (mg E /100 g dw). Additionally, by dividing the obtained responses (Y_2/Y_1) is obtained the amount of extracted residue in mg per g of mushroom dry weight, which can be used to express the extracted residue yield (mg R /g dw).

2.6. *Response surface methodology*

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

Initial tests were carried out to screen the right variables and determine their experimental domain for an appropriate RSM design (Box et al., 1987). Variables including type of solvent (*n*-hexane, limonene, chloroform, methanol and ethanol), solvent proportions (50-100%), *t* (1-45 min), *T* (50-220 ºC), and *S/L* ratio (1-60 g/L) were preliminary tested. Other MAE instrumental factors that may alter the efficiency of the extraction were also analysed such as the stirring rate and absorption level.

2.6.2. Experimental design

From the preliminary experiments, the variables *t* (min units, X_1), *T* (ºC units, X_2) and *S/L* ratio (g/L units, X_3) were found significant and thereafter selected. The coded and natural values for the selected variables are presented in **Table 1**. Therefore, the combined effects of these variables on the format value responses, Y_1, Y_2 and Y_2/Y_1 previously described, were studied using the *circumscribed central composite design* as proposed by Box et al. (1957). In this design the experimental points are generated on a sphere around the centre point. The centre point is assumed as an optimum position for the response and is repeated in order to maximize the prediction precision (Box et al., 2005). This design also requires 5 levels of each factor and 3 replicates per coordinate. The number of repetitions n_0 of the centre point is calculated by the following formulas for *k* factors based on the uniform precision:

$$\gamma = \frac{(k+3)+\sqrt{9k^2+14k-7}}{4(k+2)}; \quad \text{where:} \quad n_0 = floor\left(\gamma\left(\sqrt{2^k}+2\right)^2 - 2^k - 2k\right) \qquad [1]$$

where floor designates the highest integer value smaller than the argument. The number of experiments *n* for *k* factors is given as:

$$n = 2^k + 2k + 1 \quad [2]$$

Experimental runs were randomized, to minimize the effects of the unexpected variability in the observed responses. Independent variables coded values and natural ones of the factorial design are coded and decoded by the following expressions:

$$v_c = \left(v_n - v_0\right) \Big/ \Delta v_n \quad \text{and} \quad v_n = v_0 + \Delta v_n \times v_c \quad [3]$$

where v_n and v_c are the natural (n) and the coded (c) values in the centre of the experimental domain, v_0 is the initial value and Δv_n is the increment of v_n per unit of v_c.

2.6.3. Mathematical model

Response surface models were fitted by means of least-squares using the following second order polynomial model:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad [4]$$

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, different response formats were used as dependent variables (Y_1, Y_2 and Y_2/Y_1).

2.7. *Numerical methods, statistical analysis and graphical illustrations*

All fitting procedures, coefficient estimates and statistical calculations were performed using a Microsoft Excel spreadsheet and graphical illustrations presented were developed in the software DeltaGraph V6. Fitting and statistical analysis of the experimental results according 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 and Keller, 2010), which allows a quick testing of a hypotheses and its consequences (Murado and Prieto, 2013).

- *Coefficients significance:* The determination of the parametric confidence intervals done using the '*SolverAid*' (Prikler, 2009). The model was simplified by dropping the terms which were not statistically significant at α=0.05.

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

- *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 that 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 quality of 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) to check if the residuals of the model are not auto-correlated; and f) The Analysis Of Variance table (ANOVA) to evaluate the explanatory power of the variables.

3. Results

3.1. *Preliminary experiments to select the relevant variables and instrumental factors to centre their experimental domain previous to the RSM application*

Although there are previous research examples that optimized the extraction of ergosterol from other natural matrices (Bhuyan et al., 2015; Dahmoune et al., 2015), the results are not

generalizable due to the diversity of phytochemicals present. In order to find the extracting optimal conditions it is necessary to take into account the variables that affect the behaviour of microwave in solid solvent systems. These variables can be divided into non-microwave factors (solvent type and *S/L* ratio) and intrinsic microwave factors (*t* and *T*). Preliminary tests were investigated individually for determining their experimental domain (keeping other ones constant) for obtaining a proper RSM design by analyzing their general pattern responses. The analysis was based only for the Y_2 response format value that translates the ergosterol per 100 g of mushroom dry weight (dw) material (mg E /100 g dw).

The extracting solvent type is the key for the separation of the desired compounds. Due to the ergosterol amphipathic chemical structure, different solvents can be used for obtaining high yield extractions such as *n*-hexane, limonene, chloroform, methanol and ethanol. In a previous work, Heleno et al. (2016) obtained the extract with the highest content of ergosterol using ethanol as the extraction solvent. Binary mixtures of solvents with water were also tested (ranges 50-100%), but in all cases, the extraction yield lead to results inferior to those derived from the ethanolic extraction.

Concerning *S/L* ratio, the tested range was 1 to 60 g/L. Small values lead to a more effective dissolution, causing an extraction yield enhancement, but also a waste of solvent. A large *S/L* ratio will result in a lower extraction yield of ergosterol and a waste of raw materials. Significant differences were found for *S/L* ratios between 1.5 to 18 g/L selecting them as suitable for the RSM analysis.

Regarding the variables from the MAE system (*t* and *T*), The effect of *t* was investigated within the range of 1 to 45 min, meanwhile *T* and *S/L* ratio were held at 135 ºC and 15 g/L, respectively. The effect of extraction *t* showed strong ergosterol decomposition phases at *t* higher than 20 min, but this fact is also dependent on the other variables that remained constant. Therefore, a range comprised from 4 to 20 min was selected. The effect of *T* was

investigated within the range of 50 to 220 ºC, meanwhile *t* and *S/L* ratio were held at 15 min and 15 g/L, respectively. Relevant ergosterol extractions were found within the temperature ranges of 60 to 210 ºC, and therefore this range was selected.

Consequently, the RSM experiment to optimize efficiently the MAE process regarding the extraction of ergosterol, was designed based on the above preliminary experimental results, using five variation levels for *t*, *T*, and *S/L* ratio as independent variables. The coded and natural values for the selected variables are presented in **Table 1**.

3.2. *Response surface methodology output*

Based on the above preliminary experimental results, an optimization study for ergosterol extraction from *A. bisporus* involving RSM methodology was performed to evaluate the optimal extraction conditions of *t*, *T*, and *S/L* ratio.

3.2.1. Theoretical response surface model

The results obtained according to the statistical design described in **Table 1** are shown in **Table 2** for each of the defined responses (Y_1 and Y_2) computed. After fitting Eq. [4] to the three possible responses' format values (Y_1, Y_2 and Y_2/Y_1), the estimated parametric values, parametric intervals and numerical statistical criteria were obtained (**Table 3)**. Those coefficients, which showed effects with p-values higher than 0.05, are not significant at the 95% confidence level and 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 2**) thus obtaining the following second-order polynomial equations according to Eq. [4] for each of the response format values assessed:

for Y_1 response: $$Y_1 = 1.77 - 0.31T - 0.08t^2 - 0.1tT + 0.1tS/L - 0.13T\,S/L \qquad [5]$$

for Y_2 response:
$$Y_2 = 510.5 + 5.1t - 16.3T - 33.5\,S/L - 21.2t^2 - 74.9T^2 - 20.0\,S/L^2 - 19.2tT - 33.9\,tS/L - 22.3T\,S/L \quad [6]$$

for Y_2/Y_1 response:
$$Y_2/Y_1 = 287.2 + 5.1t + 28.3T - 18.4\,S/L - 35.9T^2 - 12.7\,S/L^2 - 32.3\,tS/L \quad [7]$$

where t is time (min), T is temperature (ºC), S/L is solid-liquid ratio (g/L) and Y is the response. Meanwhile the response subindices *1* and *2* describes the purity in ergosterol of the extracted residue (% E, w/w) and the total extracted ergosterol from the mushroom (mg E /100 g dw), respectively. Additionally, the relation (Y_2/Y_1) describes the extracted residue yield (mg R /g dw). Although the statistical consistent model parameters obtained are empirical and cannot be associated with a mechanistic meaning, they are useful to predict the results of untested operation conditions. The sign of the effect marks the response performance. 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 the high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable.

The equations [5] and [6] for Y_1 and Y_2 response format value shows a highly complex scenery. All linear, quadratic and interactive effects are found playing an important and significant role. In consequence, the extraction yield increases as t and T increases due to the strong positive linear effect, but decreases as S/L ratio increases due to the negative effect. The quadratic and interactive effects caused a stronger decrease than increase as t, T and S/L ratio increase. The optimum combinations would be found at one single point on the response. On the other hand, for the Y_2/Y_1 response format value, which assesses the concentration of ergosterol in the extract (and, therefore, the purity of the extract in ergosterol), totally opposite tendencies of those described for Y_1 and Y_2 were found. First, the equation [7] presents much simple scenery. It shows in almost all significant terms (linear, quadratic and interactive

effects) negative parameters. In consequence, the purity of the extract decreases generally as *t*, *T* and *S/L* ratio increases, and in a practically linear form.

Figure 1 shows the extraction results for each of the response value formats (Y_1, Y_2 and Y_2/Y_1). Each figure is divided into two subsections (A and B). The subsection A shows the combination of the three-dimensional response surface plots predicted with their respective second order polynomial equation described by Eqs. [5], [6] and [7] as a function of each the involved variables (*t*, *T* and *S/L*). The binary action between variables is presented when the excluded variable is positioned at the centre of the experimental domain (*t*=12 min; *T*=135 ºC; and *S/L*=10 g/L). The subsection B illustrates the capacity to predict the obtained results and the residual distribution as a function of each of the variables. In almost all combinatory 3D responses, the amount of extracted material (format Y_1) and the ergosterol content (Y_2) increase to an optimum value and then decreases as a function of each one of the assessed variables (*t*, *T* and *S/L*). Conversely, with respect to the yield of the extracted residue (Y_2/Y_1), the patterns show that variables *t* and *S/L* ratio changes the response in a lesser extent than the *T* does, and the patterns are opposite to the ones found in the other responses. From this analysis it can be understood that: 1) ergosterol content is extracted proportionally to the residue in what concerns *t* and *S/L* variables; and 2) extract purity in ergosterol is significantly reduced by the increase of *T*. Therefore, one out of two options is occurring; either other compounds than ergosterol are extracted as the *T* increases, or ergosterol molecule is proportionally destroyed as *T* increases.

The extraction behaviour can be understood by means of the parametric values of the second-order polynomial models described in Eqs. [5], [6] and [7] or represented graphically in Figure 1. However, to make more explicit the appealing combinations, Figure 2 shows the summarized individual 2D responses as a function of the defined variables for all the proposed response criteria (Y_1, Y_2 and Y_2/Y_1) in order to describe visually the tendencies and

guide easily the selection of the most favorable conditions. Each graph shows two lines. The thinner one represents the response of the variable when the other two are positioned at the centre of the experimental domain (t=12 min; T=135 ºC; and S/L=10 g/L). The thicker one represents the variable response when the other variables are located at the optimal conditions found (t=20.4 min; T=131.6 ºC; and S/L=1.6 g/L). The dots (⊙) presented alongside each line highlights the location of the optimal value. The differences between the two dots in each line are the optimization improvements performed with the aid of the RSM statistical tool.

3.2.2. Statistical and experimental verification of predictive models

This multivariable characterization by second-order polynomial model is especially robust, minimizing the effects of random and systematic errors, allowing researchers to squeeze the utmost of the results. 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, reduces their interval of confidence, and overall, reduces the number of the needed experimental trials. When studying 5 levels of 3 independent variables with 2 replicas per condition, the full response analysis would imply 250 possible combinations, but if using the RSM 60 trials are enough (14 genuine independent combinations, 6 replicas at the center of the experimental domain and 3 replicas per coordinate).

The lack-off-fit test used to evaluate the adequacy of the models demonstrated that no considerable worsening was achieved by the exclusion of the statistically non-significant effects (**Table 3**), so that this procedure never produces better results. In fact we have less adjusting non-significant parameters, that, once discarded, should increase deviations but increasing the physical meaning of the remaining. This was also verified by the achieved high R^2 and R^2_{adj} values, indicating the percentage of variability explained by the model (**Table 3**). Additionally, **Figure 1** (subsection B) shows the distribution of residuals always randomly scattered around zero; grouped data and autocorrelations were not observed. This means that

these models are workable and can be applied in the subsequent prediction and optimization stages. It also indicates a good agreement between the experimental and predicted values which implies that the variation is explained by the independent variables. Finally, Table A1 (Supplementary material) shows the analysis of variance (ANOVA) for each of the non-linear regression equations (Eqs. [5], [6] and [7]). All terms were highly significant ($p < 0.01$). The lack-of-fit test was used to verify the model adequacy that was not significant ($p > 0.05$), indicating that the model could adequately fit the experimental data.

3.2.3. Numerical optimal conditions that maximize the extraction

When equating partial derivatives of Eqs. [5], [6] and [7] to zero, solving the system variables and decoding the code value to its natural value, the optimal condition results are found, as well as, the maximal response values. The conditions that maximize the response and the optimal response value are presented in **Table 4** for each of the parametric estimation criteria. For the Y_1 response format value, the optimal conditions found were at 20.4±1.8 min, 59.4±2.5 ºC and 18.4±0.3 g/L producing a maximum response value of 2.99±0.4 % of E. For Y_2, the optimal condition values were at 19.4±2.9 min, 132.8±12.4 ºC and 1.6±0.5 g/L producing a maximum response of 556.1±26.2 mg E/100 g dw mushroom. For Y_2/Y_1, the optimal conditions were found to be at 20.4±1.8 min, 152.7±12.5 ºC and 1.6±0.2 g/L obtaining a maximum response of 387.5±15.3 mg R/g dw of mushroom.

Finally, the intermediate conditions, for the extract yield, ergosterol yield and extract purity in ergosterol, maximizing the response values were depicted using a simplex method tool to solve non-linear problems. Limitations were made to the variable coded values to avoid unnatural conditions (*i.e.*, lower times than 0). The conditions that maximize the response and the optimal response value are presented in **Table 4**. For the Y_1, Y_2 and Y_2/Y_1 the global optimal conditions found were at 20.4±4.2 min, 131.6±3.6 ºC and 1.6±0.4 g/L producing a

maximum response value of 1.32±0.1 % E, 555.3±23.6 mg E/100 g dw mushroom, and 379.6±18.2 mg R/g dw of mushroom.

4. Discussion

MAE has been used and optimized for the extraction of bioactive compounds from natural matrices. This technique is considered as a potential alternative to traditional solid–liquid extraction methods due to its reduced extraction time, solvent usage and improved extraction yield (Wang and 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 describing the optimization of the ergosterol extraction from *A. bisporus* using microwave-assisted extraction. However, taking into account its characteristics it is a promising technique for this purpose, since MAE presents a number of variables that can be optimized to obtain a high extraction efficiency of such molecules. There are several reports in the literature describing the determination of the ergosterol content in *A. bisporus* by means of different extraction techniques. Shao, Hernandez, Kramer, Rinker, & Tsao (2010) characterized several mushrooms in terms of ergosterol content by using maceration with ethanol and finding a value of 346±0.08 mg of E/100 g dw in *A. bisporus*. In another study, Phillips et al. (2010) extracted 563 mg E/100g dw from the same mushroom species but using hexane/ethyl acetate as the extraction solvent mixture. Gil-Ramirez et al. (2013) extracted 561±0.76 mg E/100g dw also using the maceration technique and a mixture methanol/water as solvent. Also with respect to the conventional techniques, Barreira et al. (2014) optimized the Soxhlet extraction with hexane obtaining 352±1 mg E/100g dw in *A. bisporus*. The values obtained by the previous authors refer to ergosterol content after a saponification step.

Regarding the emerging technologies there are also several reports using the ultrasound assisted extraction (UAE). The UAE has been used as an alternative to extraction by Soxhlet

and maceration (Wang and Weller, 2006) being methanol, dichloromethane and chloroform the most used solvents at a ratio solid/liquid capable to increase the extraction efficiency of sterols. Villares et al. (2014) used an UAE bath system and chloroform/methanol as the extraction solvent obtaining 642±0.15 mg E/100 g dw in *A. bisporus*. In a previous study of our research group describing the optimization of UAE parameters for ergosterol extraction, a value of 671.5±0.5 mg E/100 g dw was obtained with ethanol (Heleno et al., 2016). Concerning the supercritical fluid extraction (SFE), 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 the authors achieved 550 mg E/100 g dw from *A. bisporus* being identified also other mycosterols (Gil-Ramírez et al., 2013). Another important technique used for this purpose is the accelerated solvent extraction (ASE); several authors have used this extraction method for sterols extraction from *A. bisporus*, using ethanol as solvent, and obtained 450 mg E/100 g dw (Gil-Ramírez et al., 2013). Comparing the results obtained in the present work with the available literature, MAE can also be a powerful technique to the extraction of this molecule since at the optimized conditions (19.4±2.9 min, 132.8±12.4 ºC and 1.6±0.5 g/L), high levels of ergosterol (556.1±26.2 mg E/100 g dw) were obtained. The major hold back would be related with the low S/L relations needed (1.6±0.4 g/L) to optimize the process for Y_2 and Y_2/Y_1 responses causing the need of a high proportion of solvent. However, by using higher S/L relations upt o 8 g/L, low reductions of Y_2 and Y_2/Y_1 responses would be found. Furthermore, additionally to similar ergosterol yields, this technique is less time consuming and decreases the process complexity since the saponification step is avoided.

5. Conclusions

In general, most of the directives from industrialized countries converge into reach a high level of environmental protection, in which the production of by-products must be restricted either by promoting 'clean' technologies or alternatives to valorise these less valuable side-streams. In this context, this work provides an environmentally friendly, eco-designed and profitable solution that allows the integration of the mushrooms industry into the industrial ecosystem in a more sustainable way. The obtained results indicate the viability of using *A. bisporus* as a productive source of ergosterol and MAE as suitable technique for its extraction. The extraction process was successfully optimized by applying the RSM in order to achieve high ergosterol yield using ethanol as the extraction solvent. The results showed that extraction time, temperature and solid to liquid ratio had significant effects on the ergosterol extraction yield. At the best optimized conditions, it was possible to recover 556.1±26.2 mg of ergosterol/100 g dw mushroom. The results presented are the initial stage to attempt a transfer the methodology to industrial level.

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Tables

Table 1: Experimental domain and codification of independent variables in the *circumscribed central composite design*.

Coded values	**Natural values**		
	t (min)	*T (ºC)*	*S/L ratio (g/L)*
-1.68	3.59	59.32	1.59
-1	7	90	5
0	12	135	10
+1	17	180	15
+1.68	20.41	210.68	18.41

Table 2: Results of the response surface experimental plan for the optimization of the *t*, *T* and *S/L* of residue and ergosterol extraction (Y_1 and Y_2, respectively). Three replicates (r_{1-3}) were performed for each condition and for each response.

VARIABLES			ERGOSTEROL CONTENT EXTRACTED RESIDUE (% E)			ERGOSTEROL CONTENT MUSHROOM (mg E /100 g dw)		
X_1: *t* (min)	X_2: *T* (ºC)	X_3: *S/L* (g/L)	r_1	r_2	r_3	r_1	r_2	r_3
7 (-1)	90 (-1)	5 (-1)	1.72	1.74	1.67	380.2	386.2	369.8
17 (1)	90 (-1)	5 (-1)	1.76	1.77	1.73	440.4	441.6	432.3
7 (-1)	180 (1)	5 (-1)	1.92	1.91	1.92	409.5	406.9	409.3
17 (1)	180 (1)	5 (-1)	1.34	1.33	1.33	486.0	483.9	485.0
7 (-1)	90 (-1)	15 (1)	2.14	2.12	2.09	384.8	381.4	375.1
17 (1)	90 (-1)	15 (1)	1.90	1.88	1.87	401.7	397.1	395.2
7 (-1)	180 (1)	15 (1)	1.54	1.56	1.54	413.3	418.5	413.3
17 (1)	180 (1)	15 (1)	1.68	1.64	1.65	265.2	260.1	260.4
3.6 (-1.68)	135(0)	10 (0)	1.39	1.41	1.37	427.9	433.2	422.8
20.4 (1.68)	135(0)	10 (0)	1.64	1.62	1.60	476.0	470.3	462.8
12 (0)	59.3 (-1.68)	10 (0)	2.97	2.95	2.97	356.7	354.0	356.6
12 (0)	210.7 (1.68)	10 (0)	0.90	0.88	0.90	239.7	234.3	240.6
12 (0)	135 (0)	1.6 (-1.68)	1.86	1.96	1.99	492.3	518.7	526.3
12 (0)	135 (0)	18.4 (1.68)	1.56	1.55	1.54	395.6	392.2	388.8
12 (0)	135 (0)	10 (0)	1.57	1.57	1.57	490.8	491.3	490.9
12 (0)	135 (0)	10 (0)	1.68	1.70	1.71	500.9	507.2	510.8
12 (0)	135 (0)	10 (0)	1.89	1.89	1.86	552.4	551.1	541.6
12 (0)	135 (0)	10 (0)	1.91	1.92	1.92	544.3	546.4	548.3
12 (0)	135 (0)	10 (0)	1.87	1.85	1.85	489.4	485.2	483.6
12 (0)	135 (0)	10 (0)	1.87	1.85	1.85	489.4	485.2	483.6

Table 3: Parametric results of the *circumscribed central composite design* with 5 levels for the combined effect of t (x_1), T (x_2), and S/L (x_3) on the residue and ergosterol extraction (format values Y_1, Y_2 and Y_2/Y_1) according to Eq. [4]. Analysis of significance of the parameters (α=0.05) are presented in natural and percentage (between brackets) values. Additionally the statistical information of the fitting procedure to the model is presented.

		RESPONSES					
		Y_1 *(% E)*		**Y_2** *(mg E/100 g dw)*		**Y_2/Y_1** *(mg R/g dw)*	
Fitting coefficients obtained							
Intercept	b_0	1.779±0.016	*(1%)*	510.58±13.3	*(3%)*	287.17±15.1	*(5%)*
Linear effect	b_1	*ns*		5.16±8.8	*(17%)*	5.15±1.0	*(19%)*
	b_2	-0.315±0.015	*(5%)*	-16.29±8.8	*(54%)*	28.31±10.0	*(35%)*
	b_3	*ns*		-33.47±8.8	*(26%)*	-18.36±10.0	*(54%)*
Quadratic effect	b_{11}	-0.076±0.015	*(20%)*	-21.21±8.6	*(40%)*	*ns*	
	b_{22}	*ns*		-74.89±8.6	*(11%)*	-35.91±9.7	*(27%)*
	b_{33}	*ns*		-19.97±8.6	*(43%)*	-12.69±9.7	*(77%)*
Interactive effect	b_{12}	-0.096±0.020	*(21%)*	-19.20±11.5	*(60%)*	*ns*	
	b_{13}	0.095±0.020	*(21%)*	-33.91±11.5	*(34%)*	-32.27±9.7	*(30%)*
	b_{23}	-0.128±0.020	*(16%)*	-22.27±11.5	*(52%)*	*ns*	
Statistical information of the fitting analysis							
Obs		60		60		60	
df		54		50		53	
R^2		0.9751		0.9151		0.9172	
R^2adj		0.9526		0.8998		0.9095	
MEC		0.12		2760.9		6810.2	
RMSE		0.34		52.5		82.5	
MAPE		2.52		5.10		3.24	
DW		2.33		3.08		2.15	

ns: non significant coefficient; *Obs*: Number of observations; *df*: Number of degrees of freedom; 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.

Table 4: Variable conditions (*t*, *T*, and *S/L*) in natural values that lead to optimal response values in terms of the parametric estimations for each of the individual responses. Additionally, an intermediary variable condition values that optimize the response for all reactions are presented.

CRITERIA	***OPTIMAL VARIABLE CONDITIONS***			***OPTIMUM RESPONSE***	
	X_1: *t (min)*	X_2: *T (ºC)*	X_3: *S/L (g/L)*		
Individual optimal variable conditions					
Y_1	20.4±1.8	59.4±2.5	18.4±0.3	2.99±0.4	*% E*
Y_2	19.4±2.9	132.8±12.4	1.6±0.5	556.1±26.2	*mg E/100 g dw*
Y_2/Y_1	20.4±1.8	152.7±12.5	1.6±0.2	387.5±15.3	*mg R/g dw*
Global optimal variable conditions					
Y_1				1.32±0.1	*% E*
Y_2	20.4±4.2	131.6±3.6	1.6±0.4	555.3±23.6	*mg E/100 g dw*
Y_2/Y_1				379.6±18.2	*mg R/g dw*

Figures

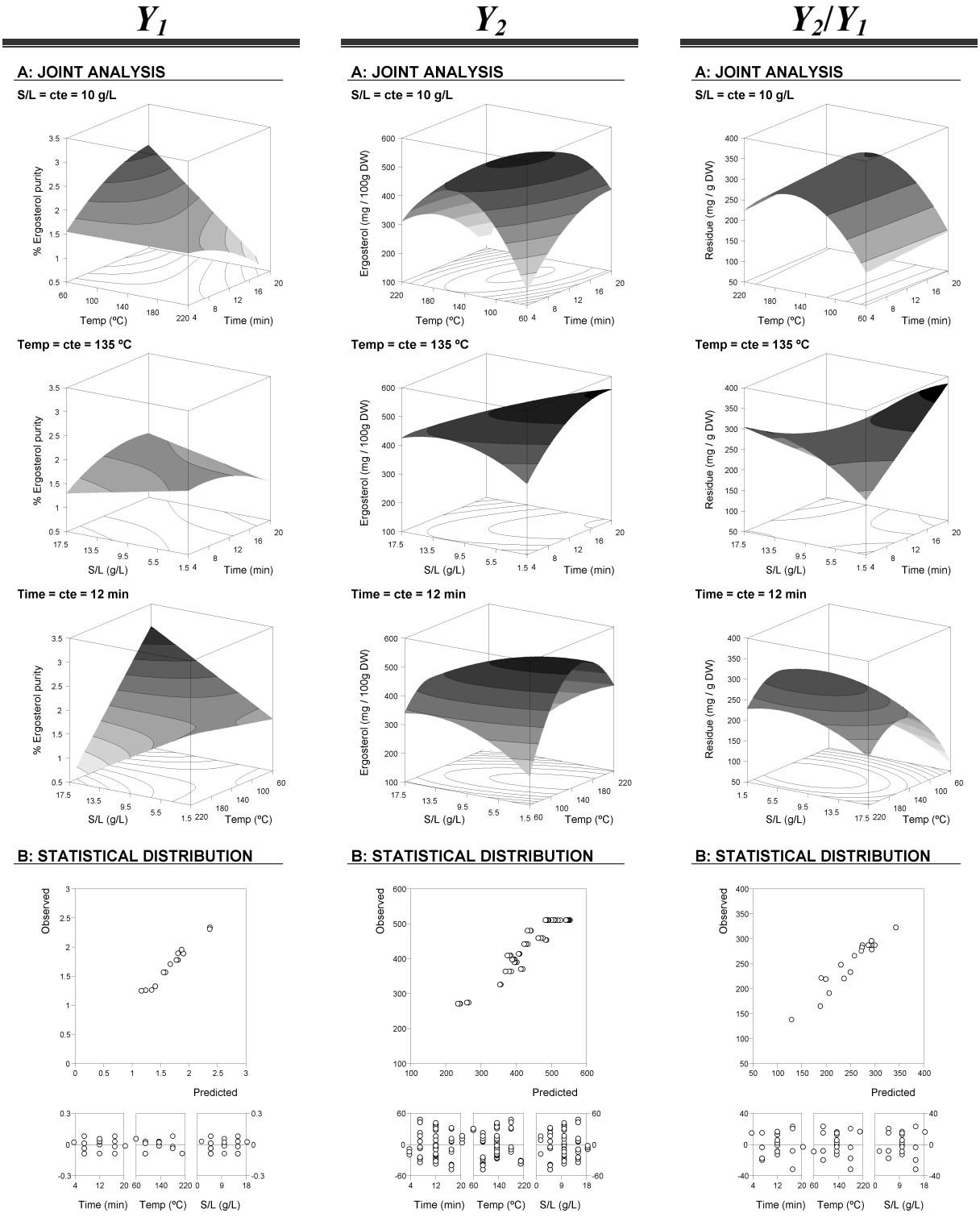

Figure 1: Shows the graphical results in terms of the extraction behavior. *Part A*: Shows the joint graphical 3D analysis as a function of each the variables involve (*t*, *T* and *S/L*). Each of the net surfaces represents the theoretical three-dimensional response surface predicted with the second order polynomial of Eqs. [5], [6] and [7] (format values Y_1, Y_2 and Y_2/Y_1, respectively). The binary action between variables are presented when the excluded variable is positioned at the centre of the experimental domain (*t*=12 min; *T*=135 ºC; and *S/L*=10 g/L). The statistical design and results are described in **Table 2**. Estimated parametric values are shown in **Table 3**. *Part B*: To illustrate the goodness of fit, two basic graphical statistic criteria are used. The first one, the ability to simulate the changes of the response between the predicted and observed data; and the second one, the residual distribution as a function of each of the variables. Note all the differences in the axes scales.

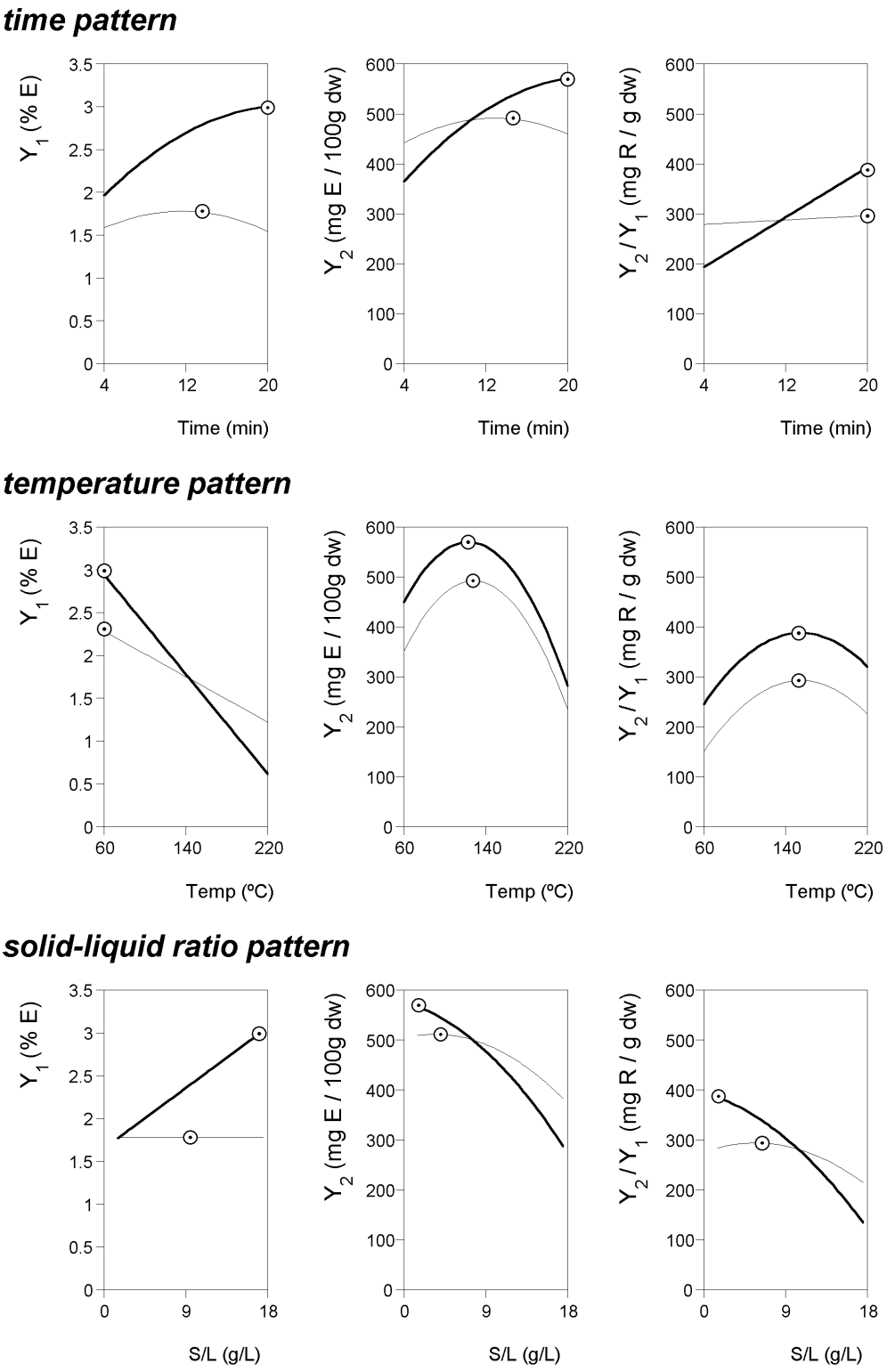

Figure 2: Individual 2D responses of all criteria proposed (format values Y_1, Y_2 and Y_2/Y_1) for each variable. Each graph shows two lines. The thinner one represents the response of the variable when the others are positioned at the centre of the experimental domain (t=12 min; T=135 ºC; and S/L=10 g/L). The thicker one represents the variable response when the other variables are located at the optimal conditions found (**Table 4**). The dots (⊙) presented alongside each line highlights the location of the optimum value. Lines and dots are generated by the theoretical second order polynomial models of Eqs. [5], [6] and [7]. Parametric fitting values obtained are presented in **Table 3**. Optimal conditions and responses in **Table 4**.