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Development of a natural preservative obtained from male chestnut flowers: Optimization of heat- assisted extraction technique

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Abstract

The aim of this work was to optimize the extraction conditions of phenolic compounds (PC) from male chestnut flowers using heat-assisted extraction in developing extracts rich in PC for its potential industrial application as a natural ingredient. The study conditions of time (*t*), temperature (*T*), solvent (*S*, water-ethanol mixtures) and solid-to-liquid ratio (*S/L*) were optimized. The responses used were the quantification of the fourteen major individual PC identified by HPLC-DAD-ESI/MS (seven hydrolysable tannins and seven flavonoids). The recovering of hydrolysable tannins was higher than flavonoids, being trigalloyl-HHDP-glucoside the major one. The conditions that maximized the PC content were at *t*=20.0±37.7 min, *T*=25.0±5.7 ºC, *S*=0.0±8.7% ethanol and *S/L*=82.8 g/L producing an extract with 86.5 mg PC/g of extract. The results highlight the potential of valorising chestnut flowers agro-residues as a productive source of PC for the development of bio-based ingredients for food/pharmaceutical/cosmeceutical industrial applications able to compete with synthetic compounds.

Keywords: Heat-assisted extraction; *Castanea sativa*; Male chestnut flowers; Natural ingredients; Hydrolysable tannins; Flavonoids; Extraction optimization.

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1. Introduction

The chestnut tree (*Castanea sativa* Mill.) fruit represent one of the most economically important agro-food material in the northeastern region of Portugal, in which the fruit represents the most exported plant part to Europe [1]. Despite the importance of the fruit for the region, previous scientific work can be mentioned to illustrate how almost all parts of the chestnut tree have been studied in order to find potential industrial applications [2,3]. Among them, the most relevant are: 1) chestnut wood, which is used in the production of furniture and it is considered of high quality [4]; 2) chestnut leaves and flowers, which have been used since ancient times in the preparation of infusions due to the high concentration of active phenolic compounds (PC) beneficial to the human health, especially in treatments of colds, cough or diarrhoea [5]; and 3) chestnut honey, although it is not a standard by-product from the production of chestnut fruits, it is highly appreciated and its production is totally attached to the chestnut tree agro-industry. Outside of those uses, tones of agro-residues are generated annually (branches, leaves, flowers, etc) and used, in the better cases, as natural fertilizers or, in a less environmental friendly case, incinerated. Reduction of environmental impacts of by-products from industrial processes have been continually highlighted in the last two decades, during which scientists have emphasized the transformation of industries using advanced sustainable process of agro-industrial activities [6]. As a consequence, typically discarded by-products generated, have been valorized [7].

Recent research has shown that male chestnut flowers (CF) or extracts possess high abundance of PC that can be used in the preservation of foods due to their capacity to inhibit lipid peroxidation and microbial proliferation [4,8,9], and used as a natural ingredient while enhancing the health of consumers [10]. These properties as well as the medicinal effects referred above have been related to their PC [4,8,9]. In this regard, recently studies have incorporated CF into different Portuguese products such as cheese and dried cakes [11,12], and

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results have confirmed the potential of these natural matrices in the development of new food products which can meet consumers expectations.

From a different perspective, recent scientific evidences have related the consumption of synthetic compounds in foods with undesirable effects in human health. Such results are pushing the food-industry to look for alternatives that meet consumers' needs towards a more natural market [13]. In this way, the food industry has been searching towards substitution of this type of synthetic additives by natural ingredients obtained from plants, mushrooms or algae, with already proven human health benefits [14].

In order to turn natural additives into a real and efficient alternative to the widely used artificial analogues, it is necessary to find promising sources and develop sustainable and efficient recovery processes for these compounds. However, the efficiency of these processes is affected by considered variables (e.g., time, temperature, ultrasonic power and solvent) [15–17]. The production of natural ingredients is more complex than it seems, since it always requires complex studies on the category of compounds as well as the best conditions and methodologies to be applied in the extraction [18–20]. Therefore, it is necessary to use appropriate experimental designs and optimization tools to determine optimal extraction conditions that should lead to the best response values. Moreover, different extraction parameters such as the solvent used, time and energy, as well as the possible loss of natural compounds, should be also taken into consideration [21]. To guarantee a maximum yield with the minimum of time, solvent and energy used, it is essential to select and optimize the best extraction conditions [22]. Through response surface methodology (RSM) it is possible to optimize the relevant variables simultaneously, obtaining mathematical solutions capable of describing, within the tested experimental interval, the ideal conditions that maximize the used response criteria [23].

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Therefore, this study intends to optimize the conditions for the recovery of PC from CF using one of the most known techniques for the extraction of natural compounds, the heat-assisted extraction (HAE), in order to be used in the food industry as a natural additive. The three most relevant independent variables for each process were combined in a RSM system for the extraction process optimization.

2. Material and Methods

2.1. Sample collection and location

Male chestnut (*Castanea sativa* Mill.) flowers (CF) were collected near Bragança (Samil) in the northeastern region of Portugal in June of 2017 (41º46´52´´N, 6º45´54´´W). The samples were lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) and reduced to a fine powder (~20 mesh). The obtained powder was mixed to guarantee the sample homogeneity and stored in a desiccator at room temperature (~25 ºC), protected from light, until further analysis.

2.2. Extraction technique selected. Heat-assisted extraction (HAE)

The tested variables and appropriate ranges were obtained based on the combination of preliminary single variable experiments and previous HAE extractions studies found in the bibliographic material available [19,20,24,25]. The variables and ranges tested were: time (t or X_1, 20 to 120 min), temperature (T or X_2, 25 to 85 ºC) and ethanol solvent proportion (S or X_3, 0 to 100%). The solid/solvent ratio was kept constant (30 g/L). The applied solvent was a mixture of ethanol/water, characterized in terms of ethanol content (%, w/w). The experimental procedure was performed by adding the dried powdered CF (600 mg) in a glass-reactor with 20 mL of solvent and then inserted in a thermostatic water bath under continuous

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electro-magnetic stirring (CIMAREC i Magnetic Stirrer with a fixed agitation speed 500 rpm, Thermo Scientific, San Jose, CA, USA) at the required conditions of the work plan (*t*, *T* and *S*).

2.3. Analytical responses used for optimization purposes

After the extraction procedure, the extracts were divided in two portions and subjected to the following analytical procedures.

2.3.1. Determination of extraction yield

The residue (R) resulting from each extraction was determined gravimetrically in crucibles, first by partial evaporation of the solvent at 60 ºC and then by a heat treatment at 100 ºC for 24 h. The results were expressed in percentage (%).

2.3.2. Chromatographic PC identification and quantification

Each single experimental point was filtered through a 0.22 µm disposable LC filter disk before chromatographic analysis, which was performed with a HPLC-DAD-ESI/MS (Dionex Ultimate 3000 UPLC, Thermo Scientific, San Jose, CA, USA) system (Pinela et al., 2018). Detection was carried out by DAD, using 280, 330, and 370 nm as the preferred wavelength, coupled to a Linear Ion Trap LTQ XL mass spectrometer (Thermo Finnigan, San Jose, CA, USA) prepared with an ESI source and working in negative mode. Data acquisition was performed using a Xcalibur® data system (Thermo Finnigan, San Jose, CA, USA). The PC were characterized according to their UV, mass spectra, retention times in comparison with authentic standards when available, and with literature. For quantification, calibration curves were generated by injection of known concentration (2.5–100 µg/mL) of standard

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compounds: ellagic acid (y = 26719x - 317255; R^2 = 0.999); gallic acid (y = 131538x + 292163; R^2 = 0.997); quercetin 3-*O*-glucoside (y = 34843x – 160173; R^2 = 0.999).

2.4. Response values

The quantified PC were grouped in two forms: a) by groups, as hydrolysable tannins (*Hta*), flavonoids (*Fla*) and total phenolic compound (*Phe*, including all quantified polyphenols); b) major compounds (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor compounds (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*). Therefore, the response criteria to optimize the extraction conditions of *Hta*, *Fla* and *Phe* from CF using RSM were: extraction *yield* (in %, which provides information regarding the quantity of extracted residue) and the compounds content in the individual and grouped terms (mg/g R, which was specifically used to evaluate the compounds purity in the extracts).

2.5. Experimental design, modelling and optimization

2.5.1. Experimental design

A five-level Central Composite Circumscribed Design (*CCCD*) coupled with RSM was accomplished to optimize the HAE conditions for the extraction of PC from CF. The coded and natural values of the independent variables X_1 (time, *t* in min), X_2 (temperature, *T* in ºC) and X_3 (solvent, *S* in % of ethanol, v/v) are presented in **Table A1** (supplementary material). More details of the experimental design can be found in previous optimization in the bibliographic material [19,20,24,25].

2.5.2. Mathematical modelling

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The data produced under the RSM experimental design presented in **Table A1** were fitted by means of least-squares analysis with the following second order polynomial model that assumes linear interactive solutions:

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

Y denotes all the response values to be assessed (described above), X_i and X_j are the independent variables used as during the extraction (conditions of t, T and S), b_0 is the constant factor, b_i is the factor of linear effect, b_{ij} is the factor of interaction effect, b_{ii} is the factor of quadratic effect, and n is the number of variables.

2.5.3. Procedure to optimize the variables to a maximum response

A *simplex* method developed *ad hoc* and previously described [24,26] was used to optimize nonlinear solutions obtained by Eq. (1) in order to maximize all response values assessed (described above) individually or globally. Restrictions were imposed to the responses and variables to elude awkward solutions.

2.6. Dose-response study of the solid-to-liquid ratio

The study of the solid-to-liquid ratio (*S/L* or X_4, expressed in g/L) was achieved by a dose-response at the optimal conditions of the variables established at the RSM (X_1, X_2, and X_3). The goal was to accomplish the *S/L* conditions that guides to a supplementary productive processes for industrial uses. As described previously [27,28], to depict the response effect as function of the *S/L*, the Weibull (W) equation for increasing (↑) and decreasing (↓) responses was used (with some parametric modifications to fit the searched purposes):

$$\uparrow W(X_4) = K \exp\left[\ln\left(1 - {}^{n}\!/_{100}\right)\left(\frac{X_4}{m_n}\right)^a\right] \quad \text{or} \quad \downarrow W(X_4) = K - K \exp\left[\ln\left(1 - {}^{n}\!/_{100}\right)\left(\frac{X_4}{m_n}\right)^a\right] \quad (2)$$

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where K is the maximum extraction value (the units would be in mg/g R for all the responses except for the extraction yield that would be in %), a is a shape parameter related to the maximum slope of the response, n is any desired level between 0 to 100% of the responses that would be achieved and m_n would be the *S/L* value (X_4) for the selected n response level (m_{10}, m_{25}, m_{75}, m_{95}, etc.). For example, if the n value is selected as 99%, the m_n parameter will display de *S/L* needed to achieve the 99% of the assessed response ($m_{99\,\%}$). When the response shows increasing patterns (↑), the Weibull equation that is used to describe the response will present a m_n parameter of n=99%. When the response shows decreasing patterns (↓), a m_n parameter with n=50% will be used. A more detail information can be found elsewhere [28]. K and m_n are important parameters for evaluating the *S/L* effect since they are responsible for providing information related to the response pattern.

2.7. Numerical and statistical methods

Mathematical analysis, coefficient estimates and statistical determinations were achieved as previously described by other researchers in order to provide the strongest and updated analysis possible [19,20,24,25]. In brief, a) the model parameters were determined by the quasi-Newton algorithm (least-square) with the aid of the macro '*Solver*' in Microsoft Excel minimizing the differences between observed and predicted values; b) the coefficient significance was evaluated using the '*SolverAid*' macro to determine their intervals (α=0.05); and c) the model uniformity was verified by means of numerous statistical principles: i) the Fisher F-test (α=0.05) applied for the evaluation of the competence of the numerical solutions to predict the experimental data; ii) the '*SolverStat*' macro was applied for the evaluation of numerical uncertainties in the developed mathematical models [29]; iii) the R^2 was interpreted as the proportion of variability to assess the parametric results during the fitting procedure.

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197 **3. Results and Discussion**

198 **3.1. Experimental data and response criteria for RSM optimization**

199 The HPLC phenolic profile (for hydrolysable tannins identification recorded at 280 nm and
200 for flavonoid identification recorded at 370 nm) of the CF extract obtained can be seen in
201 **Figure 1**. **Table 1** shows the retention time (Rt), wavelengths of maximum absorption in the
202 visible region (λ_{max}), mass spectral data and identification of PC of the peaks displayed in
203 **Figure 1.** Identification of PC was carried out and cross checked through their
204 chromatographic characteristics, such as retention time, mass spectrum, UV absorption. In
205 total, fourteen different PC were detected of which seven were hydrolysable tannins and the
206 other seven were flavonoids. In the present work the trigaloyl-HHDP-glucoside and
207 quercetin-3-*O*-glucuronide were the major compounds within the two classes found
208 (hydrolysable tannin and flavonoid, respectively). All the detected compounds have been
209 previously identified [8,30]. Of the fourteen compounds identified, seven (*P1*, *P2*, *P3*, *P4*, *P6*,
210 *P10* and *P12*) were considered minor, because they were found in very low amounts of which
211 only two were classified as flavonoids.
212 The content in the final residue produced and the compounds distribution are strongly
213 influenced by the extraction conditions. As already described [24], trying to understand the
214 effects of each of the variables involved in an extraction system individually, while other
215 variables are fixed, it is not as efficient as analysing all the effects in conjunction. Therefore,
216 the first approach to optimize the efficiency of the HAE system for the recovery of PC in CF
217 was to perform a simple independent test of each variable (data not showed) to set the
218 convenient ranges for an optimization study under RSM system. Once it was performed, the
219 application of a RSM was conducted for three variables in a *CCCD* with five levels, being the

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final ranges selected as: *t* (20-120 min), *T* (25-85ºC), and *S* (0-100 %). A full explanation of the coded and natural values of the tree variables designated is presented in the first part of **Table 2**, and in the second part are presented the experimental values of the 28 experimental runs of the *CCCD* design. For optimization purposes, the quantified PC (**Table 2**) were grouped in two forms: a) by group of compounds, as hydrolysable tannins (*Hta*), flavonoids (*Fla*) and total phenolics (*Phe*, including all quantified phenolics); b) major compounds (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor compounds (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*). Therefore, the *yield* of the extraction (in %) and the compounds content in the individual and grouped terms were used as response criteria to optimize the conditions for their extraction from CF using RSM.

The values of the extraction *yield* ranged from 8.02 to 42.83 % (or g R/100 g CF dw) with the experimental runs nº 16 and 3, respectively (**Table 2**). The highest group of compounds detected were *Hta* and ranged from 4.23 to 43.62 mg/g R, corresponding to the experimental runs nº 6 and 15, respectively. The *Fla* group ranged from 4.62 to 17.49 mg/g R (experimental runs nº 6 and 18, respectively). Regarding the individual content of the identified compounds (*P1* to *P14*, more details in **Table 1**), in which compound *P7* (35.41 mg/g R, experimental run 15) showed the highest content followed by *P5* (6.81 mg/g R, run 18), *P8* (6.34 mg/g R, run 19), *P9* (4.51 mg/g R, run 15), *P11* (2.21 mg/g R, run 15), *P13* (1.56 mg/g R, run 18), and *P14* (1.32 mg/g R, run 18). All of them comprised the response criteria used for optimizing the conditions that favours their maximization.

3.2. Mathematical solutions to the RSM experimental data produced

The development of mathematical models to understand and predict the effects of independent variables on certain response variables is essential in a variety of research areas. The validation of the precision of these models becomes essential to fit the experimental data

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[24,28]. The 12 response values (*yield*, *Hta*, *Fla* and *Phe*, *P5*, *P7*, *P8*, *P9*, *P11*, *P13*, *P14* and the total sum of the minor compounds) presented in **Table 2** were fitted by least-squares estimations with Eq. (1), to develop the nonlinear mathematical equations for each response value proposed.

The estimated coefficient values resulted by the polynomial model of Eq. (1) and the coefficient of correlation (R^2) for each parametric response of the extraction method was presented in Part A of **Table 3**. The complexity of the possible interactions between the different variables is presented by the values that translate the response patterns. Nevertheless, some of the coefficients were not significant (*ns*) and as such were not used in Eq. (1) for model construction. On the other hand, the coefficients considered significant obtained a 95% confidence level ($\alpha = 0.05$) were evaluated and presented in Part A of **Table 3**. The final model solutions for each of the 12 assessed responses (Eqs. 3 to 14) are presented in **Table A2** (supplementary material). R^2 coefficients higher than 0.85 were obtained in all cases, which indicates that the model can explain each response in a viable way. This implies that the variation of the experimental results can be explained by the independent processing variables by using the precise parametric values presented in **Table 3**, which validates the models of Eqs. (3) to (14). Therefore, the validated models are numerically applied in the subsequent prediction and optimization steps, which permits the determination of the optimal conditions that will maximize the responses.

Based on the mathematical expressions, no associations were found between the response variables of *Hta*, *Fla* and *Phe*. The variables involved can be ordered by the relevance of the parametric values in descending order: $S > T >> t$. Those results are in accordance with similar studies on the extraction of bioactive compounds, in which authors also revealed that *S* is one of the most relevant variable [31]. All the independent variables analysed presented quadratic or nonlinear effects considered reasonable. The variables T and S presented strong values for the

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quadratic effect. In turn, the variable *t* appears almost always as insignificant. As regards, the interactive effects showed minor relevance while describing the behaviour of almost all responses. The results were illustrated in the response surface plots, to make the combined effects more explicit and to visually describe the extraction trends, discussed next.

3.3. Illustrative description of the effects of the RSM variables on all the response values assessed and optimal values achieved

The parametric coefficients of Eqs. (3) to (14), presented in **Table 3**, are useful to understand the response value behaviour. However, the global comprehension of response patterns could be misunderstood, therefore to simplify the process, in **Figure 2** and **Figure 3**, 2D and 3D graphical illustrations are developed.

Figure 2 illustrates the 3D response surface plots of extraction *yield* and grouped PC as *Hta*, *Fla*, *Phe* and sum of minor compounds (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*). Each of the responses presented in **Figure 2** are described by two main parts (A and B). Part A that shows the 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 3**) predicted with Eq. (1). While, part B of **Figure 2** illustrates the goodness of fit through two graphical representations that can be used as a statistical criteria: 1) the ability to simulate response changes between the observed and predicted values; and 2) the residual distribution as a function of each variable. It is possible to confirm that the optimum value can be found in a single point in almost all combinations, which allows to find easily the extraction conditions that guides to an absolute maximum. Although the responses are altered as a function of the tested variables, the final analytical solutions found were robust and statistically consistent. For all response values, when the predicted and observed data is presented in a graphical illustration, it can be seeming that they show linear solutions, demonstrating accurate correlation between the solutions described by the models

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developed and the experimental data found. Additionally, no group of residual values or autocorrelations were observed.

Figure 3 (part A) illustrates the 2D graphical response of the effects of the independent variables for all the response values assessed of *yield*, *Hta*, *Fla*, *Phe* and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. The lines in all graphs of **Figure 3** (part A) are generated using the theoretical values (**Table 3** part A) predicted with Eq. (1). By applying a *simplex* method to solve nonlinear problems, the optimum individual condition maximizing the recovery of PC were determined and presented in part B of **Table 3**. The dots (⊙) in **Figure 3** (part A) represent the optimal values for an easier interpretation of the effects of the independent variables on the extraction process. In conclusion, it can be summarized the optimal conditions that lead to maximum responses are as follow:

- For *yield*, the optimal conditions were: *t*= 120.0±12.4 min, *T*= 85.0±6.7 ºC and *S*= 44.5%, producing 48.87±2.99% of R.
- For *Hta*, the optimal conditions were: *t*= 20.0±3.3 min, *T*= 25.0±3.7 ºC and *S*= 44.5±9.7% of ethanol (v/v), producing 41.14±0.96 mg/g R.
- For *Fla*, the optimal conditions were: *t*= 20.0±1.7 min, *T*= 85.0±14.7 ºC and *S*= 100.0±17.7% of ethanol (v/v), producing 14.38±0.33 mg/g R.
- For *Phe*, the optimal conditions were: *t*= 20.0±3.7 min, *T*= 25.0±5.7 ºC and *S*= 0.0±8.7% of ethanol (v/v), producing 55.37±2.20 mg/g R.

Similarly, the extraction of the major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds were affected in a different way by the variables tested, with the majority being favoured by lower times as follows:

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- For *P5*, *P14* and sum of the grouped minor compounds (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) the optimal conditions were: *t*= 20.0 min, *T*= 85.0 ºC and *S*= 100.0%, originating 6.00±0.85, 1.31±0.57, and 18.53±1.33 mg/g R, respectively. Compound *P7* showed similar optimum values, but *S*= 0.0% producing 33.14±0.10 mg/g R.
- For *P9* and *P11* the optimal conditions were: *t*= 20.0 min, *T*= 25.0 ºC and *S*= ~0.0%, originating 4.47±0.73 and 2.21±0.56, respectively. Compound *P8* showed similar optimum values, but *t*= 120.0 min producing 6.65±0.83 mg/g R. Compound *P13* showed similar results, but *S*= 100.0±4.7% min producing 1.55±0.60 mg/g of R.

Optimizing extraction systems to recover bioactive compounds from natural matrices has received special attention in the last decades [20,32], mainly because the optimized results obtained are relevant for an eco-friendly alternative to industries. The main benefits are the time, solvent and energy reduction, which also reduces the emitted pollutants to the environment [33]. All those are among the current objectives of a sustainable ‘‘green” chemistry [33]. The results showed that the optimum conditions to maximize PC in the selected experimental domains were *t*= 20.0±3.7 min, *T*= 25.0±5.7 ºC and *S*= 0.0±8.7% of ethanol (v/v) producing 55.37±2.20 mg/g R. Therefore, these conditions were used for the optimization of the *S/L* effect by dose-response and described below.

3.4. Analytical description of the solid-to-liquid effect at the optimum conditions of the variables assessed under RSM

As mentioned in the bibliography [34,35], the idyllic *S/L* should be one that permits the solvent to appropriately enter into the structure of the plant-based material, dissolving the maximum target compounds and using the minimum solid to liquid relation. Consequently, once the optimum conditions of the extraction variables are achieved by the polynomial models

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described above for the PC content maximization, a study aiming to assess the *S/L* pattern was directed in the predicted conditions. Additional trials were conducted to discover the limit value of *S/L* at lab-scale conditions. The results exhibited that over 120 g/L the process could not be standardized correctly, thus the *S/L* dose-response procedure was planned from 5 to 120 g/L.

The dose-response results to *S/L* effects of all the response values assessed was performed by fitting the Eq. (2). All fitting responses showed statically consistent parametric coefficients and robust model solutions. **Table A3** (supplementary material) presented all the obtained parametric values. The effects of all the response values assessed caused by the *S/L* are explicitly shown in **Figure 3** part B, in which the experimental data produced are illustrated by points (○) and the predictions developed by Eq. (2) are showed by the lines. Overall, a non-linear effect is detected for all responses as the *S/L* dose-response increases, causing a saturation-increasing (↑) and decreasing effects (↓). For the *Hta, Phe,* minor compounds and major compounds of *P5* and *P7* a saturation-increasing (↑) effect was found, while a saturation-decreasing effect (↓) was identified for the extraction *yield*, *Fla* and major compounds of *P8*, *P9*, *P11*, *P13* and *P14*. The results are analysed taking into account the parameters K and m_n with response level at 50 or 99 % [28]. The maximum extraction value (obtained as a function of the *S/L* dose-response) is demonstrated by parameter K. At the industrial level it is important to note that it was possible to verify that low m_n values are required to achieve high extraction levels with short dose-response values which consequently translates into limiting the reduction in the amount of solvent required. A brief conclusion of the results achieved can be seem bellow:

- For the dose-responses that caused saturation-increasing (↑) effects (*Phe, Hta,* minor compounds and major compounds of *P5* and *P7*), which means that initially increases as the *S/L* increases, but when a certain *S/L* level is reached (parametric value $m_{99\%}$

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from Eq. (2)), the response remains constant (parametric value *K* from Eq. (2)). Under this pattern, it was possible to find a maximum of *Phe* of 205.4 mg/g of R (value *K*) at 187.8 g/L (value $m_{99\%}$). The *Hta* presented a maximum value of 85.7 mg/g of R (165.7 g/L), the compounds *P5* and *P7* a maximum value of 8.6 mg/g of R (112.8 g/L) and 47.6 mg/g of R (124.3 g/L). Meanwhile, the sum of all minor compounds (*P8*, *P9*, *P11*, *P13* and *P14*) showed a maximum of 116.6 mg/g of R at 182.2 g/L.

- For the dose-responses that caused saturation-decreasing (↓) effects (*extraction yield*, *Fla* and major compounds *P8*, *P9*, *P11*, *P13* and *P14*), which means that the response initially decreases to zero as *S/L* increased. The maximum extraction level is obtained at relatively low *S/L* (parametric value *K* from Eq. (2), as described in Table A3), which may probably reflect the total available response content in the CF. In this scenery, the response of *extraction yield* showed a maximum value of 54.96%, the content in *Fla* showed values of 35.35 mg/g of R and the major compounds *P8* (4.31 mg/g of R), *P9* (4.31 mg/g of R), *P11* (3.12 mg/g of R), *P13* (3.58 mg/g of R) and *P14* (2.58 mg/g of R).

In consequence, by applying a routine to solve all equations, the solution that globalize all responses and maximize the *S/L* dose-response will be 82.8 g/L producing a total PC of 86.5 mg/g of R.

3.5. Comparison with other studies involving the extraction of PC in *C. sativa*

Studies have indicated that the bioactive properties (mainly antioxidant and antimicrobial activity) presented in extracts obtained from plant-based material are related to the major PC composition and exacerbated by potential synergistic interactions between them and other relevant compounds [36]. In recent years the demand for natural additives from plant-based materials has increased exponentially, and PC, specifically *Fla* have been given a great

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interest, probably due to their ability to inhibit the growth of relevant microbial strains [37,38]. Different chestnut products such as leaves, wood, fruits and bark have already been studied and characterized, presenting a great potential as source of bioactive PC, specifically hydrolysable and condensed tannins [39].

The use of CF as infusions and decoctions for medicinal purposes has been recognized since ancient times for the treatment of diverse symptomatology, namely, in the treatment of colds, coughs and diarrhoea [4,40]. Characterization of PC, especially in relation to their bioactivity potential, is indispensable to draw conclusions regarding the possibility of applying them at a food study level as natural ingredients [41]. Previous studies have analysed the nutritional and bioactive properties of CF [2,3,30], and some authors report that the most bioactive molecules are normally found in flowers rather than in the fruits [2,30,42].

The results found in this work are in line with the findings in other studies, in which *Hta* were found the predominated compounds over the *Fla* [30], and trigalloyl-HHDP-glucoside is the major compound [8]. Thus Barros et al. [30], showed the phenolic characterization of a methanolic extract of male flowers of *C. sativa* at soft extracting conditions, finding a total amount of 18.97±0.04 mg PC/g of fresh weight material, in which PC were composed of *Hta*, *Fla*, and phenolic acids. The compounds detected by Barros et al. [30] were different from those found in the present study, but the major compounds present were *Hta* and the trigalloyl-HHDP-glucoside was also the predominate compound, which is in agreement with those presented in **Table 2**. Another study of CF revealed the profile of twenty-seven PC that despite being a much higher number than the one presented in the present study, the trigalloyl-HHDP-glucoside compound was found to be the main molecule [8]. The differences found between the results of the same plant-based material are likely to be related with climatic conditions. As it has been proved, when comparing plant-based material from two different ecosystems [43],

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414 climatic conditions appear to be a determining factor in the production of PC and
415 consequently their bioactive properties.

416

417 **4. Conclusions**

418 Nowadays, it is important for the food industry to find novel sources and efficient extraction
419 methods that can be used for the production of bio-based ingredients. Natural additives have
420 been increasingly added to food products by the food industry in order to replace synthetic
421 compounds to meet the new demands of consumers. CF has been exploited and revealed high
422 antioxidant power and natural high abundance of PC, which could be used as a natural
423 ingredient to preserve food.

424 The simplicity of using conventional extraction methodologies (HAE or maceration) to
425 recover bioactive compounds from natural matrices are evident from an environmental and
426 economical point of view. In this regard, knowing the optimal conditions for maximization
427 purposes is an important step that guides the choice of a suitable and sustainable process. The
428 values predicted by the models are in close agreement with the experimental observations,
429 proving the validity of the model and the utility of the predictions for a future scale up of the
430 studied process. Therefore, the results presented provide significant conclusions that allow the
431 comparison between different extraction conditions, in terms of efficiency and decision
432 making process, which may help to reduce costs at industrial level related to energy, solvent,
433 instrumental, etc.

434 The lack of optimization approaches, specifically in what concerns PC extraction, contributed
435 to detract the use of these natural solutions in the food industry. The study concludes that
436 several conditions of extraction, reduce both economic and ecological impacts of the process,
437 in the extraction of PC from CF at an industrial level. In conclusion, the present study

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438 contributes in the valorisation of CF by the obtainment of rich extracts in PC, that can
439 potentially be applied as a natural ingredient in different industrial fields.

440

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450

Captions

Figure captions

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Figure 1. Example of a HPLC profile of PC of the chestnut flower extract obtained.

Figure 2. Response surface plots of extraction yield and grouped phenolic compounds: T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and minor compounds (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*). Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 3**) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (**Table 3**). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable.

Figure 3. 2D graphical response of the effects of the independent variables for all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. Dots (⊙) represent the optimal values. In each plot, each independent variable was positioned at the optimal value of the other two variables (**Table 3**).

Table captions

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of PC.

Table 2. Variables, natural values, ranges and experimental RSM results of the *CCCD* for the optimization of the three main variables involved (X_1, X_2 and X_3) in the HAE for all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. Three replicates were performed for each condition for each technique.

Table 3. First part of the table shows the fitting coefficients and R^2 determined for the models obtained for of all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. The second part of the table shows the optimal processing conditions of extraction in the HAE and the maximal response values produced.

Supplemental material captions

Table A1. Experimental domain and codification of independent variables in the *CCCD* factorial design with 5 range levels.

Table A2. Mathematical models of the extraction process derived from the second-order polynomial model with interactions of Eq. (1).

Table A3: Parametric results of the dose-response model of Eq. (2) for of all the response values assessed in terms of the variation of the *S/L* ratio: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds.

Figures

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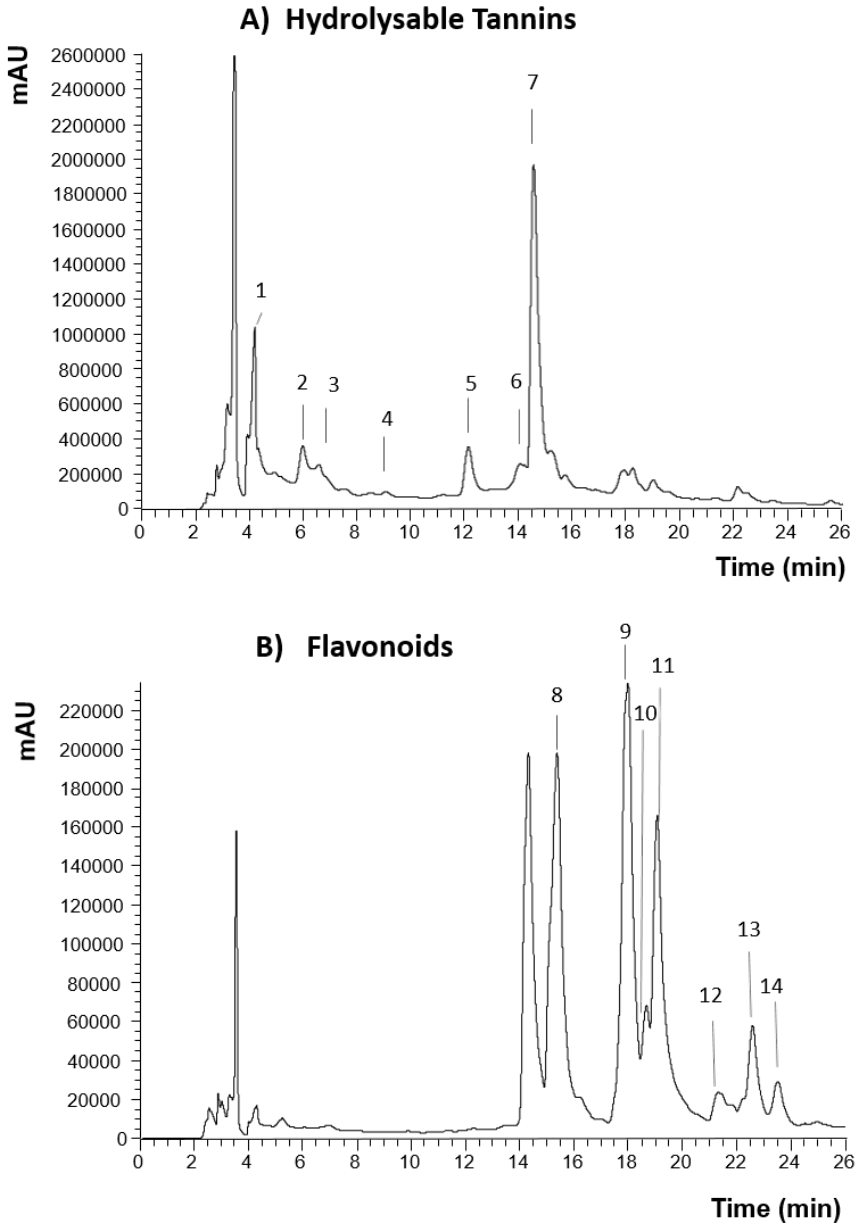

Figure 1. Example of a HPLC profile of phenolic compounds of the chestnut flower extract obtained, at 280 nm (A) and 370 nm (B).

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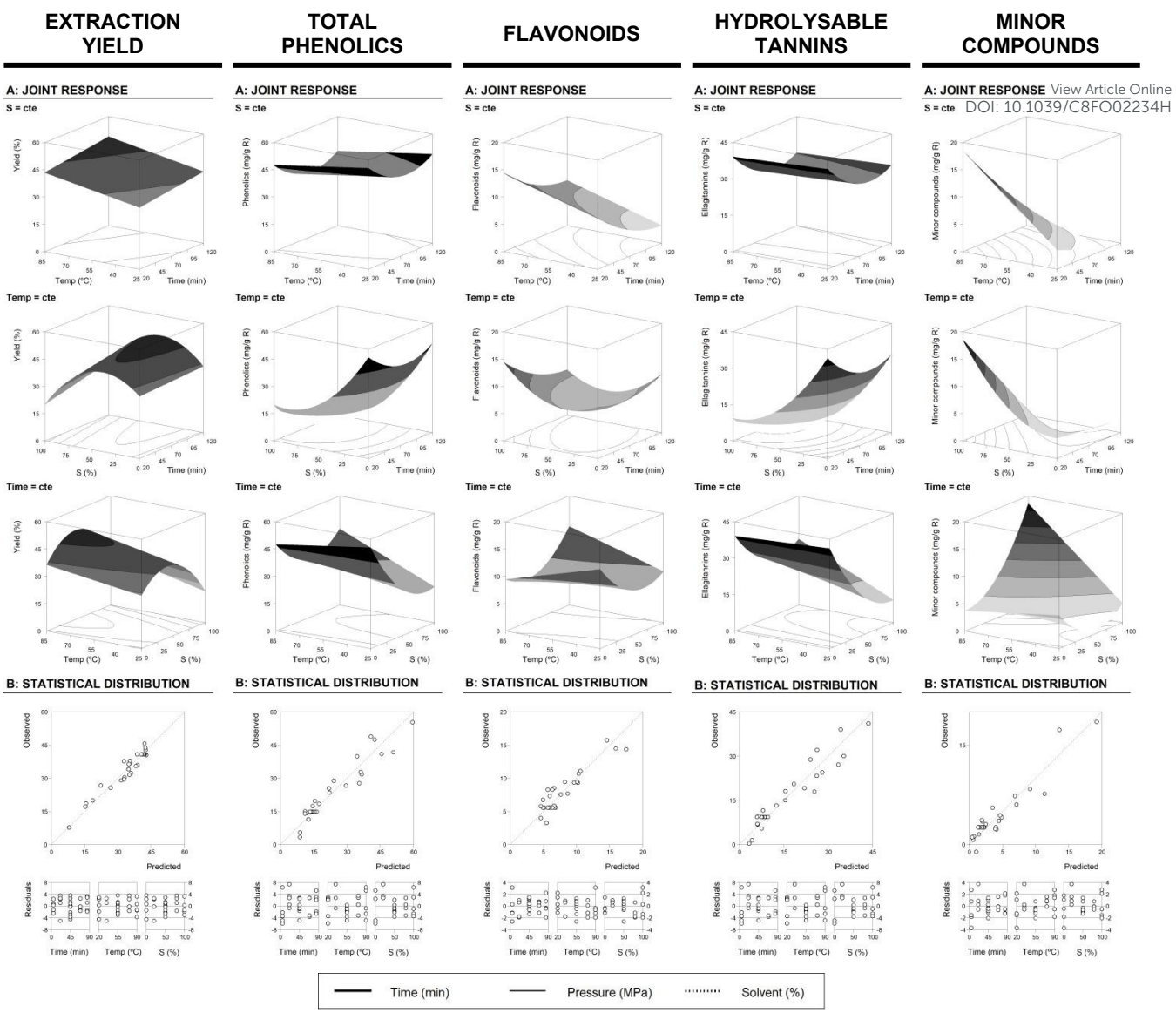

Figure 2. Response surface plots of extraction yield and grouped phenolic compounds: T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and minor compounds (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*). Part A: 3D analysis as a function of each independent variable. The grid surfaces were built using the theoretical values (**Table 3**) predicted with Eq. (1). For representation purposes, the excluded variable was positioned at the optimum of their experimental domain (**Table 3**). Part B: illustration of the goodness of fit through two graphical statistical criteria, namely the ability to simulate response changes between observed and predicted values and the residual distribution as a function of each variable.

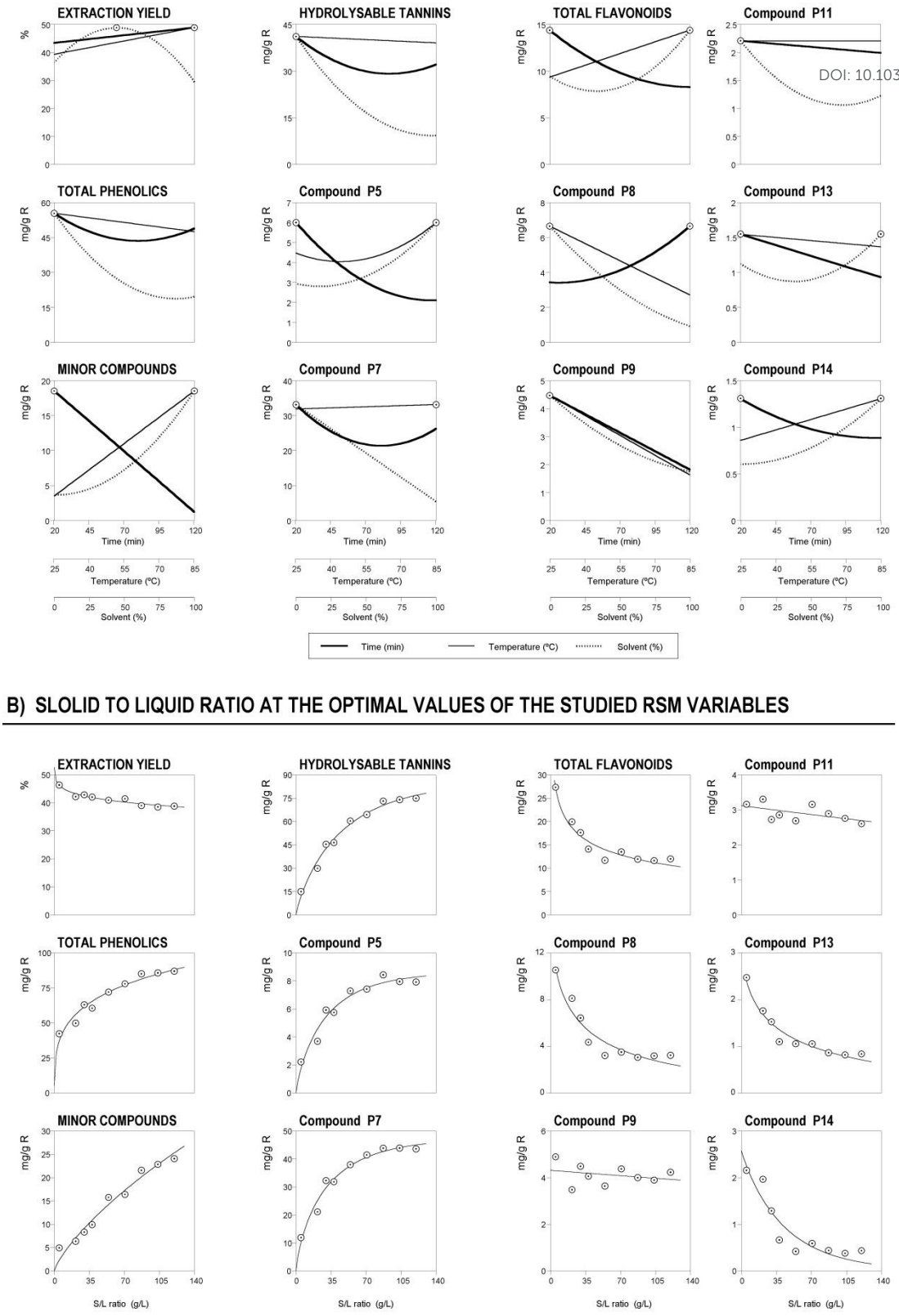

Figure 3. 2D graphical response of the effects of the independent variables for all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. Dots (⊙) represent the optimal values. In each plot, each independent variable was positioned at the optimal value of the other two variables (**Table 3**).

Tables

Table 1. Retention time (Rt), wavelengths of maximum absorption in the visible region (λ_{max}), mass spectral data and identification of phenolic compounds.

Peak ID	Rt (min)	λ_{max} (nm)	Molecular ion [M-H]$^-$ (*m/z*)	MS^2 (*m/z*) (% base peak)	Tentative identification	Phenolic compound classification
1	4.7	280	783	481(30),301(100)	Bis-HHDP-glucoside[A]	hydrolysable tannin
2	6.1	277	633	463(20),301(100)	Galloyl-HHDP-glucoside[A]	hydrolysable tannin
3	6.6	275	937	767(3),637(21),467(100),301(5)	Trigalloyl-HHDP-glucoside[A]	hydrolysable tannin
4	9.0	272	637	593(100),469(19),169(5)	Galloyl derivative[B]	hydrolysable tannin
5	12.1	275	939	631(23),469(100),169(5)	Pentagalloyl-glucoside[B]	hydrolysable tannin
6	13.9	275	937	767(3),637(19),467(100),301(5)	Trigalloyl-HHDP-glucoside[A]	hydrolysable tannin
7	14.5	273	937	767(3),637(20),467(100),301(3)	Trigalloyl-HHDP-glucoside[A]	hydrolysable tannin
8	15.2	355	479	317(100)	Myricetin-3-*O*-glucoside[C]	flavonoid
9	17.7	353	477	301(100)	Quercetin-3-*O*-glucuronide[C]	flavonoid
10	18.5	353	477	301(100)	Quercetin-3-*O*-glucuronide[C]	flavonoid
11	18.8	354	463	301(100)	Quercetin-3-*O*-glucoside[C]	flavonoid
12	21.1	343	593	285(100)	Kaempferol-3-*O*-rutinoside[C]	flavonoid
13	22.4	347	447	285(100)	Kaempferol-3-*O*-glucoside[C]	flavonoid
14	23.3	350	477	315(100)	Isorhamnetin-3-*O*-glucoside[C]	flavonoid

Phenolic compounds used for quantification: compounds [A]- ellagiic acid (y = 26719x - 317255; R^2 = 0.999); compounds [B]- gallic acid (y = 131538x + 292163; R^2 = 0.997); compounds [C]- quercetin 3-*O*-glucoside (y = 34843x – 160173; R^2 = 0.999).

Table 2. Variables, natural values, ranges and experimental RSM results of the *CCCD* for the optimization of the three main variables involved (X_1, X_2 and X_3) in the HAE for all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. Three replicates were performed for each condition for each technique.

CODED VALUES			YIELD	CLASS			COMPOUNDS IDENTIFIED BY HPLC							
							MAJOR							MINOR
X_1	X_2	X_3		*Hta*	*Fla*	*Phe*	P5	P7	P8	P9	P11	P13	P14	P1,2,3,4,6,10,12
t (min)	*T (ºC)*	*S (%)*	*(%)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*	*(mg/g R)*
-1(40,3)	-1(37,2)	-1(20,3)	38.12	26.11	10.07	36.18	1.62	21.64	2.24	3.03	1.40	0.93	0.69	4.64
-1(40,3)	-1(37,2)	1(79,7)	26.73	6.10	4.95	11.05	0.45	3.15	1.15	1.50	0.96	0.90	0.45	2.49
-1(40,3)	1(72,8)	-1(20,3)	42.83	28.00	8.67	36.66	1.46	23.44	2.48	1.78	1.69	0.79	0.57	4.46
-1(40,3)	1(72,8)	1(79,7)	35.94	15.49	6.60	22.09	2.29	4.02	2.88	1.31	0.79	0.77	0.85	9.17
1(99,7)	-1(37,2)	-1(20,3)	32.77	25.34	10.21	35.55	2.59	13.45	3.06	1.79	1.75	0.85	0.69	11.37
1(99,7)	-1(37,2)	1(79,7)	32.99	4.23	4.62	8.85	0.80	3.43	0.98	1.14	0.84	0.68	0.38	0.60
1(99,7)	1(72,8)	-1(20,3)	42.55	21.98	7.62	29.61	2.00	17.71	2.01	1.18	1.54	0.71	0.55	3.91
1(99,7)	1(72,8)	1(79,7)	35.52	6.48	4.62	11.10	0.76	4.25	0.66	1.96	0.74	0.64	0.62	1.47
1.68(120)	0(55)	0(50)	42.41	7.95	6.61	14.56	0.97	6.54	2.95	1.44	1.16	0.58	0.48	0.44
-1.68(20)	0(55)	0(50)	35.45	18.41	5.67	24.08	1.14	12.37	1.23	1.74	1.12	0.81	0.77	4.90
0(70)	-1.68(25)	0(50)	38.82	7.51	5.13	12.64	0.86	5.31	1.30	1.58	1.03	0.73	0.49	1.34
0(70)	1.68(85)	0(50)	42.06	12.48	4.94	17.41	1.27	7.21	1.28	1.35	1.12	0.59	0.61	3.99
0(70)	0(55)	-1.68(0)	35.24	23.99	10.59	34.58	1.86	17.03	2.58	2.58	2.12	0.91	0.61	6.89
0(70)	0(55)	1.68(100)	15.61	6.06	5.96	12.01	0.45	2.10	1.31	1.52	1.22	1.14	0.77	3.51
-1.68(20)	-1.68(25)	-1.68(0)	22.30	43.62	15.94	59.56	3.98	35.41	4.14	4.51	2.21	1.23	0.96	7.13
-1.68(20)	-1.68(25)	1.68(100)	8.02	5.93	9.64	15.57	4.72	1.20	2.02	1.84	1.33	1.55	0.92	1.97
-1.68(20)	1.68(85)	-1.68(0)	34.81	34.23	8.26	42.49	2.73	31.50	1.29	1.41	2.20	0.86	0.60	1.90
-1.68(20)	1.68(85)	1.68(100)	18.71	33.44	17.49	50.93	6.81	11.82	4.82	1.93	1.43	1.56	1.32	21.25
1.68(120)	-1.68(25)	-1.68(0)	31.44	26.23	14.59	40.82	4.13	11.75	6.34	1.50	1.79	0.93	0.76	13.61
1.68(120)	-1.68(25)	1.68(100)	15.33	3.31	5.47	8.79	2.28	1.04	1.14	0.89	1.14	1.00	0.27	1.04
1.68(120)	1.68(85)	-1.68(0)	34.96	35.27	10.33	45.61	3.65	30.64	2.59	1.07	2.02	0.87	0.67	4.10
1.68(120)	1.68(85)	1.68(100)	32.70	15.55	6.35	21.89	1.46	14.09	0.85	2.45	0.77	0.70	0.87	0.71
0(70)	0(55)	0(50)	41.99	7.48	6.38	13.86	0.62	6.86	1.16	1.54	1.16	0.61	0.51	1.40
0(70)	0(55)	0(50)	41.71	8.43	6.84	15.27	0.59	7.26	1.23	1.45	1.33	0.61	0.51	2.29
0(70)	0(55)	0(50)	38.68	9.62	6.68	16.30	0.66	8.30	1.22	1.61	1.19	0.63	0.50	2.20
0(70)	0(55)	0(50)	40.58	8.42	5.95	14.37	0.57	7.22	1.01	1.68	0.98	0.60	0.48	1.84
0(70)	0(55)	0(50)	42.20	7.73	5.67	13.39	0.67	6.56	1.14	1.53	0.82	0.60	0.49	1.57
0(70)	0(55)	0(50)	41.00	8.67	5.88	14.55	0.70	7.30	1.01	1.56	0.95	0.63	0.48	1.93

Table 3. First part of the table shows the fitting coefficients and R^2 determined for the models obtained for of all the response values assessed: *Extraction yield* (%), T. hydrolysable tannins (*Hta*), T. Flavonoids (*Fla*), T. Phenolics (*Phe*) and major (*P5*, *P7*, *P8*, *P9*, *P11*, *P13* and *P14*) and minor (*P1*, *P2*, *P3*, *P4*, *P6*, *P10* and *P12*) compounds. The second part of the table shows the optimal processing conditions of extraction in the HAE and the maximal response values produced.

Response variables	A: Fitting coefficients obtained after applying the RSM equation											B: Optimal conditions and response values			
	Intercept	*Linear effect*			*Quadratic effect*			*Interactive effect*			R^2	*t (min)*	*T (ºC)*	*S (%)*	*Optimum*
	b_0	b_1 (t)	b_2 (T)	b_3 (S)	b_{11} (t^2)	b_{22} (T^2)	b_{33} (S^2)	b_{12} (tT)	b_{13} (tS)	b_{23} (TS)					
Extraction yield	40.83±1.02	1.75±0.21	2.92±0.61	-3.86±0.31	ns	ns	-5.56±0.58	ns	0.63±0.13	0.43±0.03	0.9324	120.0±12.4	85.0±6.7	44.5±9.7	48.87±2.99
Hydrolysable tannins (Hta)	9.34±1.67	-2.68±0.32	2.34±0.22	-6.50±0.92	2.38±0.48	ns	3.04±0.28	ns	ns	1.76±0.18	0.9102	20.0±3.3	25.0±3.7	0.0±6.7	41.14±0.96
Flavonoids (Fla)	5.56±0.59	-0.72±0.13	ns	-1.12±0.13	0.53±0.19	ns	1.28±0.39	ns	-0.65±0.23	0.89±0.23	0.8468	20.0±1.7	85.0±14.7	100.0±1.7	14.38±0.33
Total Phenolics (Phe)	14.90±1.09	-3.40±0.16	2.11±0.16	-7.62±1.06	2.91±1.26	ns	4.32±1.26	ns	-0.87±0.76	2.65±0.76	0.9110	20.0±3.7	25.0±5.7	0.0±8.7	55.37±2.20
Compound P5	0.37±0.26	-0.31±0.14	ns	ns	0.37±0.18	0.37±0.18	0.41±0.18	-0.12±0.10	-0.39±0.10	0.15±0.10	0.8671	20.0±5.2	85.0±6.7	100.0±9.7	6.00±0.85
Compound P7	7.88±1.53	-1.68±0.32	2.09±0.92	-6.15±0.92	2.81±0.86	ns	ns	1.02±0.65	1.25±0.35	ns	0.8921	20.0±3.6	85.0±7.7	0.0±1.7	33.14±0.10
Compound P8	1.22±0.19	ns	-0.17±0.11	-0.43±0.11	0.33±0.12	ns	0.28±0.12	-0.20±0.07	-0.37±0.07	0.39±0.07	0.9009	120.0±2.4	25.0±2.7	0.0±1.7	6.65±0.83
Compound P9	1.55±0.15	-0.23±0.09	-0.13±0.09	-0.17±0.09	ns	ns	0.15±0.09	0.19±0.07	0.14±0.07	0.24±0.07	0.9328	20.0±3.1	25.0±1.7	0.0±2.7	4.47±0.73
Compound P11	1.06±0.08	-0.06±0.01	ns	-0.29±0.05	ns	ns	0.19±0.05	ns	ns	ns	0.8850	20.0±1.6	25.0±1.7	0.0±3.7	2.21±0.56
Compound P13	0.64±0.07	-0.10±0.02	-0.05±0.04	0.05±0.04	ns	ns	0.15±0.04	ns	-0.05±0.03	ns	0.8889	20.0±1.3	25.0±1.7	100.0±4.7	1.55±0.60
Compound P14	0.51±0.02	-0.08±0.01	0.04±0.01	0.02±0.01	0.04±0.02	ns	0.06±0.02	0.02±0.01	-0.04±0.01	0.07±0.01	0.9357	20.0±0.5	85.0±12.7	100.0±5.7	1.31±0.57
Compound P1,2,3,4,6,10,12	2.61±0.25	-0.89±0.29	ns	-0.53±0.19	ns	ns	1.35±0.37	-1.12±0.28	-1.40±0.28	1.53±0.28	0.8855	20.0±3.0	85.0±17.7	100.0±2.7	18.53±1.33

ns: non-significant coefficient; R^2: Correlation coefficient.
Optimum values of the optimized conditions are all presented in mg/g R except for the extraction yield that is expressed in %.

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Graphical abstract

Development of a natural preservative obtained from male chestnut flowers: Optimization of heat- assisted extraction technique

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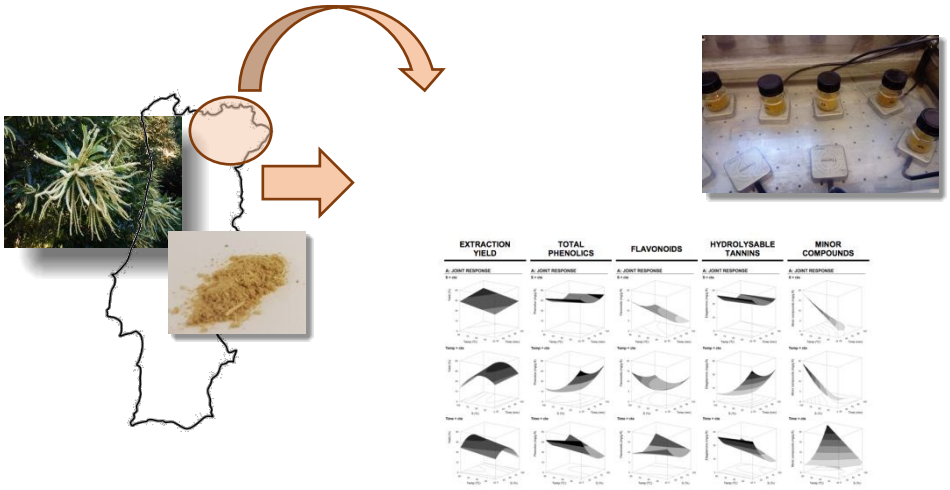

The phenolic compounds extraction optimization from male chestnut flowers allowed the obtainment of a natural ingredient with potential application in the food industry.