# Microwave-assisted extraction of phenolic acids and flavonoids and production of antioxidant ingredients from tomato: a nutraceutical-oriented optimization study

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**Abstract**

The production of natural extracts requires suitable processing conditions to maximize the preservation of the bioactive ingredients. Herein, a microwave-assisted extraction (MAE) process was optimized, by means of response surface methodology (RSM), to maximize the recovery of phenolic acids and flavonoids and obtain antioxidant ingredients from tomato. A 5-level full factorial Box-Behnken design was successfully implemented for MAE optimization, in which the processing time (*t*), temperature (*T*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) were relevant independent variables. The proposed model was validated based on the high values of the adjusted coefficient of determination and on the non-significant differences between experimental and predicted values. The global optimum processing conditions (*t*=20 min; *T*=180 ºC; *Et*=0 %; and *S/L*=45 g/L) provided tomato extracts with high potential as nutraceuticals or as active ingredients in the design of functional foods. Additionally, the round tomato variety was highlighted as a source of added-value phenolic acids and flavonoids.

**Keywords:** Microwave-assisted extraction; Phenolic compounds; Antioxidant activity; Central composite design; *Lycopersicon esculentum*.

## 1. Introduction

Phenolic compounds are a group of secondary metabolites widely spread throughout the plant kingdom. Tomato (*Lycopersicon esculentum* Mill.) fruits, apart from being a functional food rich in carotenoids, vitamins and minerals [1,2], is also an important source of phenolic compounds, including phenolic acids and flavonoids [3]. As antioxidants, these functional molecules play an important role in the prevention of human pathologies [4,5] and found many applications in nutraceutical, pharmaceutical and cosmeceutical industries [6]. Therefore, obtaining added-value functional compounds from natural sources, such as tomatoes, is highly desirable by the food industrial sector. Furthermore, the global nutraceutical market has grown in the last decade and a large percentage of the developed nutraceuticals and functional foods are driven by plant-based products [7].

Tomato is a key element of the Mediterranean diet [8] and the second most important vegetable crop worldwide, being consumed either fresh or in the form of processed products. In Trás-os-Montes, North-eastern Portugal, native population's lifestyle has highlighted the importance of local tomato varieties, which are grown using extensive farming techniques and considered as very tasty and healthy foods [9]. Among them, the common variety of tomato, locally known as "tomate Redondo" (round tomato), was reported as a source of *p*-coumaric acid and quercetin derivatives, as well as of the non-phenolic compound benzyl alcohol dihexose [3]. The *p*-coumaric acid has antioxidant, antilipidemic, antihypertrophic and cardioprotective properties [10,11]. Quercetin shows a wide range of biological and pharmacological effects, including antioxidant, anti-inflammatory, antitumor and antibacterial activities, as well as neuroprotective, hepatoprotective, cardioprotective, anti-atherosclerotic, anti-thrombotic and antihypertensive effects [12–15]. In tomato, quercetin is commonly found in the glycoside, *i.e.*, esterified with rutinose. Rutin, known as vitamin P, also display a remarkable array of health-promoting effects and is widely used in the industry [16]. In turn,

benzyl alcohol, an aromatic alcohol, is used in cosmetic formulations, as local anaesthetic, and as a flavouring substance in foods and beverages [17]. Furthermore, epidemiological studies support the protective effect of tomatoes against certain degenerative diseases associated to oxidative stress, including cardiovascular diseases and various types of cancer [18]. Meanwhile, there has been an increasing concern to develop and include phenolic-rich functional foods in the diet in order to improve the nutritional and health status.

Extraction is an important analytical step in the isolation of compounds from plant materials prior to chromatographic identification, or from a preparative point of view, to produce functional ingredients to use in new formulations [7,19]. Today, microwave-assisted extraction (MAE) is gaining many merits due to the higher extraction rate and superior products quality at lower cost. In fact, this novel green technology is considered as a potential alternative to conventional solid-liquid extraction of bioactive compounds from plant matrices [20]. However, the MAE efficiency depends on several variables which may not be generalised for all plant materials due to the diverse nature of existing bioactive phytochemicals, being necessary to select and optimize the processing conditions as a function of the used matrix and taking into account the desired responses.

Apart from the large amounts of industrial by-products derived from tomato processing, sometimes a surplus production of this fruit occur, which can be sustainably used for functional ingredients recovery. In a previous study conducted by Li et al. [20], optimal extraction conditions were determined based on the ferric reducing antioxidant power (FRAP) and oxygen radical absorption capacity (ORAC) assays. These optimized conditions were then used in the analysis of phenolic compounds. However, non-phenolic compounds can influence antioxidant responses. Therefore, an RSM optimization based on chromatographic analysis is more accurate and desired, once the optimal conditions obtained from antioxidant responses may not match the conditions for the extraction of individual compounds. In

addition, the low range of extraction time (≤ 3.68 min) originated non significant results. Our study aimed at determining the optimal MAE conditions for maximizing the recovery of functional phenolic compounds and the antioxidant capacity of extracts from tomato. Different variables (processing time, temperature, ethanol concentration, microwave power, and solid/liquid ratio) were investigated and the extraction process optimized using a central composite design coupled with response surface methodology (RSM). The content of the major phenolic compounds (two phenolic acids: benzyl alcohol dihexose and a *cis p*-coumaric acid derivative; and two flavonoids: quercetin pentosylrutinoside and quercetin-3-*O*-rutinoside) and the antioxidant activity (DPPH free-radical scavenging activity and reducing power) were evaluated as responses.

## 2. Material and methods

### 2.1. Standards and reagents

HPLC-grade acetonitrile was from Fisher Scientific (Lisbon, Portugal). Formic acid was purchased from Prolabo (VWR International, France). The phenolic compound standards (*p*-coumaric acid, caffeic acid and rutin) were from Extrasynthese (Genay, France). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was from Sigma (St. Louis, MO, USA). All the other chemicals were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (Millipore, model A10, Billerica, MA, USA).

### 2.2. Preparation of tomato extracts

#### *2.2.1. Plant material*

A common farmers' variety of tomato, known as "tomate redondo or batateiro" (round tomato), widely cultivated in rural communities from Miranda do Douro, North-eastern Portugal, was chosen for this study. Fruits at the ripen stage were hand-harvested randomly from the middle of six plants, in selected homegardens of two villages in the studied area. Ripeness was established according to local consumers' criteria based in morphological descriptors such as size, texture, and colour patterns of pericarp. According to local standards, the visual tonality of mature tomatoes was evaluated as corresponding to nº 42 in Red Group, using the colour chart of the Royal Horticultural Society. Six tomato fruits (pericarps without jointed pedicels and seeds) were lyophilized (Free Zone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) and kept at -20 °C until analysis.

*2.2.2. Microwave-assisted extraction*

The MAE process was performed using a Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) in closed vessels of high-precision glass. Ethanol:water mixtures were used since ethanol has low toxicity and efficiency for the extraction of phenolic compounds. The presence of a polar hydroxyl group and a non-polar end was also taken into account. The solvent volume was fixed at 20 mL. The powdered samples were extracted using different time (*t*), temperature (*T*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) conditions that ranged as defined by the RSM design (Table 1). During processing, samples were stirred at 600 rpm using a magnetic stirring bar and irradiated at 200 W (a preliminary study presented in Fig. A.1 of the supplementary material indicated that the microwave power has no effect on the extraction process). After that, the mixture in the extraction vessel was quickly cooled in the processing chamber. The mixture was centrifuged at 6000 rpm for 10 min, the pellet was discarded and the supernatant was carefully collected for further analysis. The dry weight (dw) obtained from each solution was evaluated to determine the extraction yield (g extract/g

sample). A schematic representation of the sequential steps followed in this work is shown in Fig. A.2 provided in the supplementary material.

## 2.3. Chromatographic analysis of the main phenolic compounds

After the MAE process, the extract solutions were purified using Sep-Pak® C-18 3 cc Vac Cartridges (Phenomenex, Torrance, CA, USA), wetted and activated with methanol followed by water; sugars and other polar substances were removed with 10 mL of water, and phenolic compounds were further eluted with 5 mL of methanol. The methanolic extracts were concentrated under vacuum, re-dissolved in 1 mL of water:methanol (80:20, v/v) and filtered through 0.22 μm disposable LC filter disks. The analysis of the main compounds in the tomato extracts was performed by high-performance liquid chromatography (HPLC) (Shimadzu 20A series UFLC, Shimadzu Corporation, Kyoto, Japan) as described previously by the authors [3]. Double online detection was carried with a diode array detector (DAD) operating at 280 and 370 nm as preferred wavelengths. The target phenolic compounds were identified according to their UV spectra and retention time. For the quantitative analysis, a baseline to valley integration with baseline projection mode was used to calculate peak areas. External standards were used for quantification. The results were expressed in mg per g of extract.

## 2.4. Evaluation of the antioxidant activity

Two *in vitro* assays were applied to evaluate the antioxidant activity of the tomato extracts, which were successively diluted to different concentrations using the same extraction solvent.

### *2.4.1. DPPH free-radical scavenging activity*

The solutions with different concentrations (30 µL) were mixed with a methanolic solution (270 µL) containing DPPH free-radicals ($6 \times 10^{-5}$ M) in a 96-well plate. The reaction mixture was left to stand for 60 min in the dark. After that, the reduction of DPPH free-radicals was determined by measuring the absorbance at 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc, Winooski, VT, USA) [1]. The nonlinear dose-response of the asymptotic end-point values of the solutions was calculated by the Weibull model as previously described by Prieto et al. [21] using the Eq. (1).

$$DPPH^{\bullet}(A) = K \exp\left[-(\ln 2)^{1-\alpha}\left(\frac{2v_m A}{K\alpha}\right)^{\alpha}\right] \quad (1)$$

in which A is the dose of antioxidant. The parameter *K* is the starting value of DPPH free-radicals (30 µM). The $\alpha$ shape parameter is related with the maximum slope of the response. The parameter *vm* corresponds to the average number of DPPH molecules reduced per g of extract (µM DPPH/g extract), which is a value of maximal predictability and, therefore, was used as response.

*2.4.2. Reducing power*

The reducing power assay evaluates the capacity of the extracts to convert potassium ferricyanide ($Fe^{3+}$) into potassium ferrocyanide ($Fe^{2+}$), which reacts with ferric chloride to form a ferric-ferrous complex that can be monitored spectrophotometrically. The solutions with different concentrations (0.5 mL) were mixed with sodium phosphate buffer (200 mM, pH 6.6, 0.5 mL) and potassium ferricyanide (1% w/v, 0.5 mL). The mixture was incubated at 50 ºC for 20 min, and then trichloroacetic acid (10% w/v, 0.5 mL) was added. The mixture (0.8 mL) was poured into the 48-well plates, with deionised water (0.8 mL) and ferric chloride (0.1% w/v, 0.16 mL), and the absorbance was measured at 690 nm using the microplate reader described above [1]. The dose-response results showed a linear dependency

and the linear Eq. (2) with zero intercept was used to compute the average number of reduced molecules.

$$RP(A) = mA \tag{2}$$

in which A is the dose of antioxidant. The slope parameter *m* corresponds to the average number of molecules that are reduced per g of extract (μM $Fe^{2+}$/g extract) and was used to compute the potential antioxidant activity of the extracts.

## 2.5. Experimental design

### *2.5.1. Experimental design*

The influence of different independent variables was investigated using a one-factor-at-a-time approach to select the significant ones and to determine a preliminary range for each variable. Based on these experimental results presented in Fig. A.1 of the supplementary material, the variables $X_1$ (time, min), $X_2$ (temperature, ºC), $X_3$ (ethanol concentration, %) and $X_4$ (solid/liquid ratio, g/L) were selected for the RSM design. Then, the combined effects of these four variables on the extraction of phenolic acids and flavonoids and production of functional (antioxidant) ingredients from tomato were studied using a *central composite design* as proposed by Box et al. [22]. The responses were solved using 25 independent combinations and 7 replicates at the centre of the experimental domain, which implies 625 possible combinations. In this design, the points of experiments are generated on a sphere around the centre point. The centre point is supposed to be an optimum position for the response and is repeated to maximize the prediction [23]. This design also requires 5 levels of each factor. The number of repetitions $n_0$ of the centre point was calculated using the formulas presented in Eq. (3) for *k* factors based on uniform precision.

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

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

$$n = 2^k + 2k + 1 \quad (4)$$

Experimental runs were randomized to minimize the effects of unexpected variability in the observed responses. The independent variable coded values and the natural ones of the factorial design were coded and decoded by the expressions in Eq. (5).

$$v_c = \left(v_n - v_0\right) / \Delta v_n \quad \text{and} \quad v_n = v_0 + \Delta v_n \times v_c \quad (5)$$

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

*2.5.2. Box-Behnken mathematical model*

The response surface models were fitted by means of least-squares regressions using the following Box-Behnken design equation:

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (6)$$

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 $n$ the number of variables. The responses of the parametric estimations of the antioxidant activity assays and of the chromatographic quantification of the main phenolic acids and flavonoids were used as dependent variables.

2.6. Fitting procedures and statistical analysis

The fitting procedures of equations to the responses were performed by means of a Microsoft Excel spreadsheet. Coefficients estimation and statistical calculations of the experimental results to the proposed equations were carried out in three phases:

1) Coefficients estimation was obtained by minimization of the sum of quadratic differences between the observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* [24].
2) The significance of the coefficients of the parametric confidence intervals was calculated using the "*SolverAid"* [25]. The model was simplified by dropping terms, which were not statistically significant (*p*-value > 0.05).
3) The uniformity of the model was checked by applying the following statistical assessment criteria: a) The Fisher *F*-test ($\alpha$=0.05) was used to determine whether the constructed models were consistent to describe the observed data; b) The 'SolverStat' macro was used for the assessment of the parameter and model prediction uncertainties [26]; c) $R^2$ and $R^2_{adj}$ were interpreted as the proportion of variability of the dependent variable explained by the model; d) The fitting to experimental data was evaluated by calculating the bias and accuracy factors of all equations, such as the Mean Squared Error (MSE), the Root Mean Square of the Errors (RMSE), the Mean Absolute Percentage Error (MAPE); and the Durbin-Watson coefficient (DW).

## 3. Results & discussion

### 3.1. Response criteria for the RSM analysis

Fig. 1 shows the phenolic profile of the tomato extract obtained under the run nº 9 of the RSM design, whose processing conditions are presented in Table 2. Benzyl alcohol dihexose ($P_1$) and *cis p*-coumaric acid derivative ($P_2$) were the major phenolic acids, while quercetin pentosylrutinoside ($F_1$) and quercetin-3-*O*-rutinoside ($F_2$) were the main flavonoids, in

agreement to Barros et al. [3]. These compounds were identified by comparison of their UV spectra and retention time with those of commercial standards. The quantification results are presented in Table 2 for the different runs of the RSM design. The levels of phenolic acids ranged from 0.94 to 6.80 mg/g extract for $P_1$ and from 2.14 to 17.86 mg/g extract for $P_2$ and were achieved with the runs nº 14 and 21, respectively. For the flavonoids, the amounts of $F_1$ ranged from 0.38 to 2.94 mg/g extract and were achieved with the runs nº 5 and 20, respectively; while the $F_2$ contents ranged from 0.61 to 4.83 mg/g extract and were achieved with the experimental runs nº 16 and 12, respectively. The results of this chromatographic quantification were used as response criteria to optimize the MAE conditions by RSM.

The MAE conditions were also optimized based on two antioxidant activity assays. Fig. 2 illustrates the responses of the DPPH free-radical scavenging activity (on the left-hand side) and reducing power (on the right-hand side) for the extracts obtained under the conditions designed by the RSM. Each half of Fig. 2 shows 25 subfigures that correspond to the dose-response antioxidant activity of the extracts obtained with the 25 genuine combinations of the RSM design. In each subfigure, dots (●) represent the values of standardized substrate (μM of DPPH free-radicals or reduced $Fe^{2+}$) and lines (—) represent the fitted responses to the model of Eq. (1) (for the DPPH free-radical scavenging activity responses) and Eq. (2) (for the reducing power responses). The obtained parametric fitting values, confidence intervals and statistical information are presented in Table A.1 provided in the supplementary material. All coefficients showed significant parametric intervals at the 95% confidence level ($\alpha$=0.05) and the correlation coefficients were always higher than 0.98. Table 2 shows the parametric values of *vm* (μM DPPH/g extract) and *vm* (μM $Fe^{2+}$/g extract) achieved with the Eqs. (1) and (2), respectively, for the 32 runs of the experimental RSM design. The values of *vm* ranged from 0.27 to 5.96 μM DPPH/g extract and were achieved with the runs nº 23 and 16, respectively; while the values of *vm* ranged from 18.5 to 173.9 μM $Fe^{2+}$/g extract and were attributed to the

experimental runs nº 2 and 8, respectively. These parametric values were used as response criteria in the RSM optimization.

3.2. Development of the theoretical response surface models and statistical verification

Fitting the models to the selected responses is crucial to elucidate how precisely the RSM mathematical model can predict ideal variances. The models for each response were built by fitting the Box-Behnken second-order polynomial model of Eq. (6) (independent variables in coded values) to the experimental values (Table 2) through nonlinear least-squares estimations. The resulting models are presented below.

When the peaks of the major phenolic acids were considered:

$$Y_{P1} = 4.7 - 0.7x_3 - 0.9x_4 - 0.7x_1^2 - 0.6x_2^2 + 0.4x_3^2 - 0.6x_1x_3 + 0.5x_2x_3 - 0.4x_3x_4 \quad (7)$$

$$Y_{P2} = 12.8 + 1.3x_3 - 2.4x_4 - 1.7x_1^2 - 1.6x_2^2 + 1.0x_3^2 - 2.4x_1x_3 + 1.3x_2x_3 - 1.1x_3x_4 \quad (8)$$

When the peaks of the major flavonoids were considered:

$$Y_{F1} = 1.6 - 0.3x_1 - 0.5x_2 - 0.3x_4 - 0.3x_1x_2 - 0.3x_1x_4 + 0.3x_2x_3 - 0.2x_3x_4 \quad (9)$$

$$Y_{F2} = 2.4 - 0.4x_1 - 0.7x_2 - 0.5x_4 + 0.4x_1x_2 - 0.5x_1x_4 + 0.6x_2x_3 - 0.3x_2x_4 - 0.4x_3x_4 \quad (10)$$

When the antioxidant activity assays were considered:

$$Y_{DPPH} = 1.1 + 0.5x_2 + 0.5x_3 + 0.6x_4 - 0.3x_1^2 - 0.5x_2^2 - 0.3x_4^2 - 0.4x_1x_2 + 0.4x_2x_3 + 0.6x_2x_4 \quad (11)$$

$$Y_{PR} = 29 + 21x_2 - 10x_3 + 17x_4 + 6x_1^2 + 16x_2^2 + 5x_3^2 + 12x_4^2 + 10x_1x_2 - 9x_2x_3 - 21x_3x_4 \quad (12)$$

where $X_1$ (processing time), $X_2$ (temperature), $X_3$ (ethanol concentration), $X_4$ (solid/liquid ratio), $Y$ is the response, sub-indices indicate the analytical criterion used as response for RSM.

Not all the parameters of Eq. (6) were used for building the model, since some coefficients were statistically non-significant (Table 3). The significant ones were empirical and useful to

predict the results of untested operating conditions [27]. The sign of the effect marks the performance of the response. In this way, when a factor has a positive effect, the response is higher at the high level, and when a factor has a negative effect, the response is lower at high level. The higher the absolute value of a coefficient, the more important the weight of the corresponding variable.

The complexity of the developed response models was very similar. Almost all of them present a combination of linear, quadratic and interactive coefficients and, in all of them, the four variables involved played a significant role. Nonetheless, based in the mathematical expressions, the responses of the antioxidant activity were more complex than those found for the individual phenolic acids and flavonoids. The antioxidant activity depends on the global contribution of different compounds, including interactions among them, and not only on a single molecule determined by HPLC. Indeed, as noted, the complexity of the mathematical equations can be related to the number of factors that affect the response.

The lack of statistical fit, used to test the adequacy of the obtained models, demonstrated that no considerable improvement was achieved by the exclusion of the statistically non-significant effects (Table 3). This was also verified by the high values of $R^2$ and $R^2_{adj}$ indicating the percentage of variability of each response that is explained by the model (Table 3). Additionally, the distribution of residuals was always randomly scattered around zero and grouped data and autocorrelations were not observed (data not shown). This means that these models are workable and can be applied in the subsequent prediction and optimisation stages. It also indicates a good agreement between the experimental and predicted values. Finally, Table A.2 (supplementary material) shows the results of the analysis of variance (ANOVA) for each of the nonlinear regression Eqs. (7) to (12). All coefficients were highly significant ($p < 0.01$). The lack of fit, used to verify the adequacy of the model, was not significant ($p > 0.05$), indicating that the model could adequately fit the experimental data.

The patterns of the extraction can be explained by means of the parametric values of the second-order polynomial models described in Eqs. (7) to (12), but can also be depicted by their graphical representation. The Fig. 3, 4 and Fig. A.3 (supplementary material) show the 3D response surfaces of phenolic acids, flavonoids and antioxidant activity in function of the four studied variables. The individual 2D graphical responses of all the studied independent variables are presented in Fig. A.4 of the supplementary material. The variables excluded in each 3D and 2D graphs were positioned at the centre of their experimental domain, i.e., $t$=10 min, $T$=120 ºC, $Et$=50 % and $S/L$=25 g/L.

### 3.3. Effect of extraction variables on the main phenolic compounds

In the phenolic profile (Fig. 1) of the tomato extracts was possible to notice two main peaks corresponding to benzyl alcohol dihexose ($P_1$) and a *cis p*-coumaric acid derivative ($P_2$). Both compounds were affected in a similar way by the processing conditions, as can be observed comparing each 3D graph showed in the bottom diagonal part of Fig. 3 (for $P_2$) with those presented in the bottom diagonal part of Fig. A.3 (for $P_1$) in the supplementary material. The variables excluded in each 3D graph were positioned at the centre of their experimental domain, *i.e.*, $t$=10 min, $T$=120 ºC, $Et$=50 % and $S/L$=25 g/L. Focussing on the particular case of $P_2$, it was verified an increase in the extraction yield with the increase in $T$ until 144.64 ºC followed by a gradual decrease probably due to degradation phenomena. The $t$ had a similar quadratic effect; the recovery of $P_2$ increased up to 3.15 min of processing and then decreased. The effect of the type of extraction solvent was linear and water ($Et$=0%) was the preferred extraction medium. In fact, phenolic compounds are polar molecules, so the extraction yield increases with increasing water content according to the "like dissolves like" principle [28]. Additionally, water may enhance swelling of cell material, increasing the contact surface area between plant matrix and solvent, resulting then in an increased extraction yield [29]. The $S/L$

had linear and quadratic effects and the higher ratio (45.0 g/L) favoured the extraction yield. At an industrial scale, high ratios are desirable since it is important to maximize the extraction (thus productivity) with a minimal solvent consumption (more sustainable process). It was also found an interactive effect between *S/L* and the other 3 extraction variables (Table 3).

In the HPLC profile (Fig. 1), recorded at 280 nm, it was also possible to assign quercetin pentosylrutinoside ($F_1$) and quercetin-3-*O*-rutinoside ($F_2$) as being the two most abundant flavonoids in the tomato extracts. The interactive effects of the studied independent processing variables on the extraction of these functional compounds are presented in Fig. 3 and Fig. A.3 (supplementary material). The top diagonal part of Fig. A.2 shows the response surfaces of $F_1$ and the top diagonal part of Fig. 3 illustrates the response surfaces of $F_2$. The variables excluded in each 3D graph were positioned at the centre of their experimental domain, *i.e.*, *t*=10 min, *T*=120 ºC, *Et*=50 % and *S/L*=25 g/L. As verified for phenolic acids, the processing conditions also affected the extractability of both flavonoids in a similar way. The increased in *t* and *T* probably led to a decomposition of the analytes, decreasing linearly the recovery of these compounds. Similarly, the higher *Et* revealed a less affinity for the selected flavonoids. The *S/L* had a non-significant effect, but it interacted with *T* and *Et* (Table 3).

### 3.4. Effect of extraction variables on the antioxidant activity

The effects of the studied independent variables on the antioxidant activity of tomato extracts are presented in Fig. 4. The top diagonal part shows the response surfaces of the DPPH free-radical scavenging capacity and the bottom diagonal part represents the response surfaces of the reducing power. The variables excluded in each graph were positioned at the centre of their experimental domain, *i.e.*, *t*=10 min, *T*=120 ºC, *Et*=50 % and *S/L*=25 g/L. The results are expressed in μM of protected substrate (DPPH free-radical or $Fe^{2+}$) per g of extracted

material. The response surfaces of both *in vitro* assays were somewhat similar, except for *S/L*, *i.e.*, high ratios favoured the DPPH free-radical scavenging activity in a positive linear manner, while low ratios led to a higher reducing power in linear and quadratic forms. It was found that higher processing *t* and *T* allowed obtaining tomato extracts with stronger antioxidant properties. Pure ethanol (100 %) was suitable to increase the antioxidant potential of the extracts, as observed for flavonoids, but in a manner contrary to that observed for phenolic acids. In fact, non-phenolic compounds can contribute to the antioxidant activity since increasing the *Et* also increases the solvent affinity towards less polar compounds. An interactive effect of *T* with *t* and *S/L* (Table 3) affected the response surfaces of both *in vitro* assays. Noteworthy consequences on the DPPH free-radical scavenging capacity were also induced by an interactive positive effect between *T* and *Et*. The response surfaces of the reducing power were also influenced by the interaction between *Et* and *S/L*.

### 3.5. Optimal extraction conditions

The operating conditions that maximize the extraction of the major phenolic acids and flavonoids and the antioxidant activity of the tomato extracts are presented in Table 4. The optimal processing conditions for each phenolic acid were as follows: *t*=5.51 min, *T*=146.69 ºC, *Et*=0.0 %, and *S/L*=45 g/L for benzyl alcohol dihexose ($P_1$); and *t*=3.15 min, *T*=144.64 ºC, *Et*=0 %, and *S/L*=45 g/L for the *cis p*-coumaric acid derivative ($P_2$); and allowed obtaining the following maximum recovery: 8.99±0.58 mg/g extract for $P_1$ and 24.8±0.9 mg/g extract for $P_2$. These optimal conditions were very similar, which can facilitate the obtainment of both compounds simultaneously, as reinforced by the following intermediate conditions optimised for both compounds: *t*=4.38 min, *T*=145.6 ºC, *Et*=0 %, and *S/L*=45 g/L. In fact, there were only slight differences in *t* and *T*. Regarding flavonoids, the optimal processing conditions for quercetin pentosylrutinoside ($F_1$) and quercetin-3-*O*-rutinoside ($F_2$) were exactly the same:

*t*=20 min, *T*=60 ºC, *Et*=100 %, and *S/L*=45 g/L; and allowed obtaining a maximum recovery of 6.78±0.45 mg/g extract for $F_1$ and 11.7±0.6 mg/g extract for $F_2$. Compared with the optimal operating conditions for phenolic acids, the extraction of flavonoids demanded a lower *t*, *T* and *S/L* but a higher *Et*.

Curiously, the optimal processing conditions that allowed obtaining tomato extracts with maximal antioxidant activity differed in the *S/L* (45.0 g/L for the DPPH free-radical scavenging activity and 5.0 g/L for the reduction power), but the other processing conditions were exactly the same for both *in vitro* assays. As observed for flavonoids, a higher *Et* (100.0 %) favoured the antioxidant activity of the tomato extracts. Moreover, contrary to that observed for phenolic acids and flavonoids, a longer processing *t* (20.0 min) was required as well as a higher *T* (180.0 ºC). The MAE was conducted in closed vessels; so it was possible to increase the temperature above the boiling point of the solvent. The increase in *T* may improved the extraction efficiency by increasing desorption of antioxidants from active sites in the tomato matrix, and because the decrease in the surface tension and solvent viscosity may improved sample wetting and matrix penetration, respectively [30]. These results diverge from those previously described by Li et al. [20], achieved with the FRAP and ORAC assays. The authors also verified that the independent variables of *T* and *Et* had significant effects on the response of both *in vitro* assays. However, the proposed optimization model was characterized by a shorter *t* (< 2.06 min) and a lower *T* (96.5 ºC) and *Et* (< 66.2 %). These divergent results can be justified by the different mechanisms of action of the performed *in vitro* assays and by variations in the antioxidants profile of the analyzed tomato varieties. The FRAP assay is based on the reducing power of antioxidants, whereas the ORAC assay has been used to evaluate the antioxidant capacity of hydrophilic compounds against the peroxyl radical-induced oxidation initiated by thermal decomposition of AAPH (2,20-azobis-(2-methylpropionamidine) dihydrochloride).

In this study, global processing conditions were also computed in order to promote all the evaluated responses and thus originate tomato extracts with high amounts of phenolic acids and flavonoids and increased antioxidant properties. These MAE conditions were calculated using a simplex method tool to solve linear problems. Restrictions were made to the variable coded values to avoid the variable involved in the equations to consider unnatural conditions (*i.e.*, lower times than 0). As observed in Table 4, the global optimum processing conditions were based on high processing *t* (20 min), *T* (180 ºC) and *S/L* (45.0 g/L) and low *Et* (0.0 %), and allowed obtaining tomato extracts with the highest responses as possible. Thus, based on the different processing conditions shown in Table 4, the tomato samples could be processed differently according to the intended purpose, namely for recovery of phenolic acids, recovery of flavonoids, production of extracts with maximized antioxidant activity, or ingredients with increased levels of functional phenolic compounds and with high antioxidant capacity.

## 4. Conclusions

The combined effects of the independent variables of *t*, *T*, *Et* and *S/L* on the extraction of phenolic compounds and production of antioxidant extracts from tomato were investigated. A 5-level full factorial Box-Behnken design of 25 combinations and 7 replicates at the centre of the experimental domain was successfully implemented for MAE optimization by RSM. The MAE conditions were optimized for each response, as well as for the set of all responses. Under the global optimum conditions (*t*=20 min, *T*=180 ºC, *Et*=0 %, and *S/L*=45 g/L), the values for $P_1$, $P_2$, $F_1$ and $F_2$ were 7.57±0.77 mg/g extract, 18.8±1.9 mg/g extract, 3.67±0.37 mg/g extract and 7.47±0.76 mg/g extract, respectively; for DPPH free-radical scavenging activity and reducing power the values of 4.02±0.41 μM DPPH/g extract and 203.7±20.6 μM $Fe^{2+}$/g extract were obtained, respectively. The proposed optimization model was statistically validated by the high values of the adjusted coefficient of determination and by the observed

non-significant differences between the experimental and predicted results. This study highlighted the analyzed tomato variety as a source of added-value phenolic compounds. Moreover, using the optimal processing conditions, it was possible to produce functional extracts with high potential as nutraceuticals or as active ingredients in the design of functional foods, which can be also extended to other industrial fields such as pharmaceutical and cosmeceutical industries.

**Abbreviations**

*t* : Processing time

*T* : Processing temperature

*Et* : Ethanol concentration

*S/L* : Solid/liquid ratio

$P_1$ : Benzyl alcohol dihexose

$P_2$ : *cis p*-Coumaric acid derivative

$F_1$ : Quercetin pentosylrutinoside

$F_2$ : Quercetin-3-*O*-rutinoside

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**Appendix A - Supplementary material**

**Fig. A.1** Results of the preliminary study carried out to select significant variables and determine optimum ranges for an appropriate RSM design. The independent variables of extraction time (0-15 min), temperature (60-180 ºC), ethanol concentration (0-100 %), solid/liquid ratio (5-150 g/L), and microwave power (100-400 W) were investigated. The extraction yield (% of dry weight) and the amounts of total phenolic (mg of gallic acid equivalents per g of extract) and flavonoids (mg of catechin equivalents per g of extract) were evaluated as responses. The shaded results showed statistically significant differences and the corresponding independent variables were selected for the MAE optimization by RSM.

**Fig. A.2** Schematic representation of the sequential steps carried out in this study.

**Fig. A.3** Matrix combination for the response surfaces of selected phenolic compounds. The top diagonal part shows the response surface of $F_1$ (benzyl alcohol dihexose) and the bottom diagonal part shows the response surface of $P_1$ (quercetin pentosylrutinoside). For representation purposes, the variables excluded in each 3D graph were positioned at the centre of the experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The parametric fitting values are presented in Table 3.

**Fig. A.4** Individual responses of all studied parameters. The variables excluded in each of the 2D graphs were positioned at the centre of their experimental domain (*t*=10 min; *T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The parametric fitting values are presented in Table 3.

**Table A.1** Parametric estimations and statistical information of the mathematical models of the Eq. (1) for the DPPH free-radical scavenging activity and Eq. (2) for the reducing power. All coefficients showed effects with significant parametric intervals at the 95%

confidence level. The estimated numerical values of *vm* (μM DPPH/g extract) and *m* (μM $Fe^{2+}$/g extract) are presented in Table 2.

**Table A.2** ANOVA table for the 5-level Box-Behnken central composite design for the combined effect of *t*, *T*, *Et* and *S/L* on the extraction yield of phenolic acids and flavonoids and on maximizing of the antioxidant activity of the extracts according to Eq. (6) and presented in Eqs. (7)-(12).

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**Figure captions**

**Fig. 1** HPLC profiles of phenolic compounds in the tomato extracts (a representative case of the run nº 9 presented in Table 2). Phenolic acids ($P_1$, benzyl alcohol dihexose and $P_2$, *cis p*-coumaric acid derivative) were recorded at 370 nm and flavonoids ($F_1$, quercetin pentosylrutinoside and $F_2$, quercetin-3-*O*-rutinoside) were recorded at 280 nm.

**Fig. 2** Illustration of the responses obtained for the DPPH free-radical scavenging activity (left-hand side) and reducing power (right-hand side) under the RSM experimental design presented in Table 1. Each graph illustrates one of the 25 independent variable combinations. In all cases, the response shows dots (●) representing the standardized substrate (μM of DPPH radicals or μM of reduced $Fe^{2+}$) values in a dose-response manner; and lines (—) representing the fitted responses to the mathematical models of Eq. (1) and Eq. (2). The parametric fitting values obtained by least-squares estimations are presented in Table A.1.

**Fig. 3** Matrix combination for the response surfaces of selected phenolic compounds ($F_2$ and $P_2$). In the top diagonal part is presented the response surface of $F_2$ (quercetin-3-*O*-rutinoside) and in the bottom diagonal part is presented the response surface of $P_2$ (*cis p*-coumaric acid derivative). For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain ($t$=10 min; $T$=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The parametric fitting values are presented in Table 3.

**Fig. 4** Matrix combination for the response surfaces of the antioxidant activity of the tomato extracts. In the top diagonal part is presented the response surface of the DPPH free-radical scavenging activity and in the bottom diagonal part is presented the response surface of the reducing power. For representation purposes, the variables excluded in each 3D graph were positioned at the centre of their experimental domain ($t$=10 min;

*T*=120 ºC; *Et*=50 %; and *S/L*=25 g/L). The parametric fitting values are presented in Table 3.

## Figures

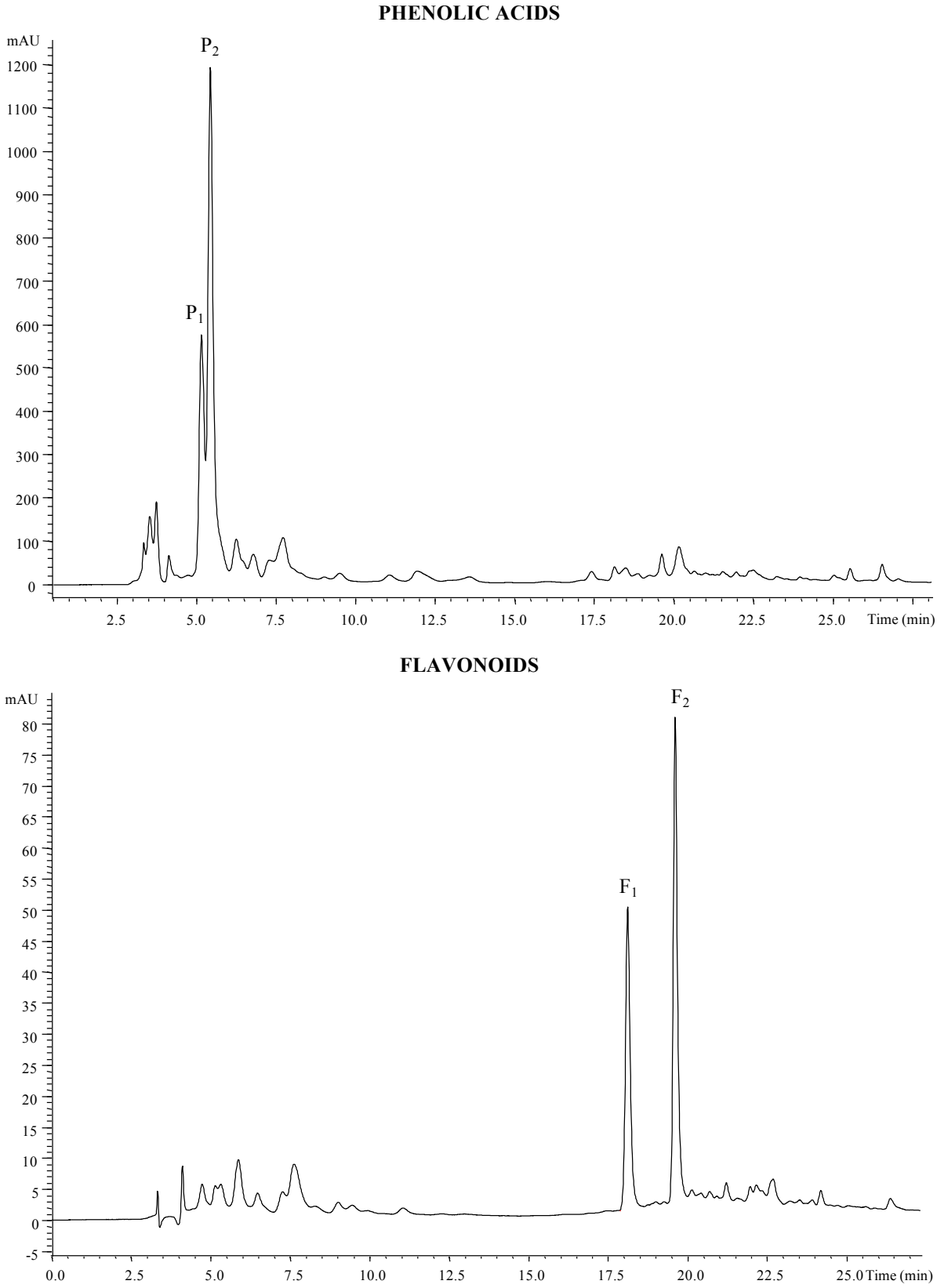
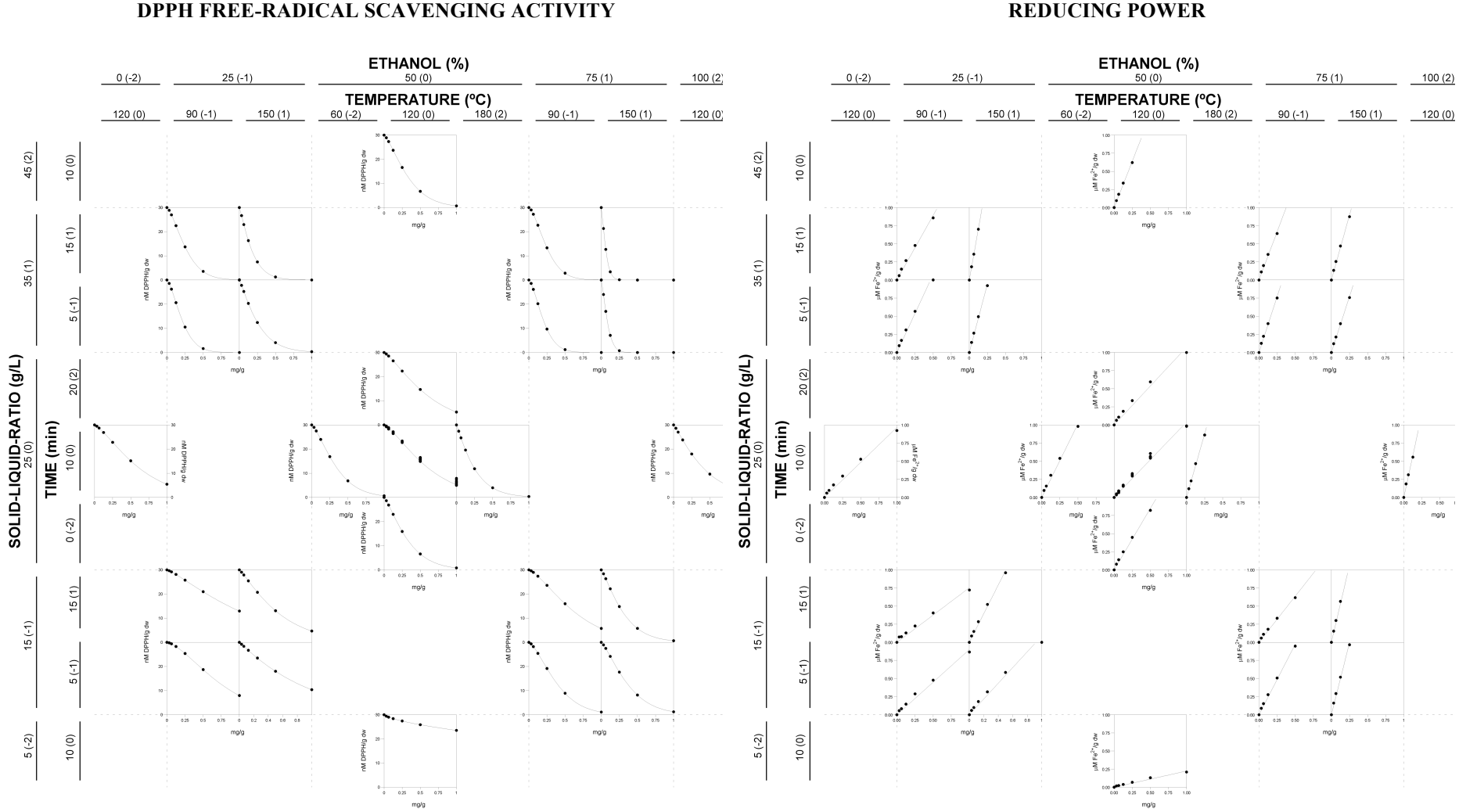



Fig. 1


DPPH FREE-RADICAL SCAVENGING ACTIVITY
REDUCING POWER
ETHANOL (%)
0 (-2)
25 (-1)
50 (0)
75 (1)
100 (2)
TEMPERATURE (ºC)
120 (0)
90 (-1)
150 (1)
60 (-2)
120 (0)
180 (2)
90 (-1)
150 (1)
120 (0)
SOLID-LIQUID-RATIO (g/L)
45 (2)
35 (1)
25 (0)
15 (-1)
5 (-2)
TIME (min)
10 (0)
15 (1)
5 (-1)
20 (2)
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nM DPPH/g dw
µM Fe+2/g dw
mg/g

Fig. 2

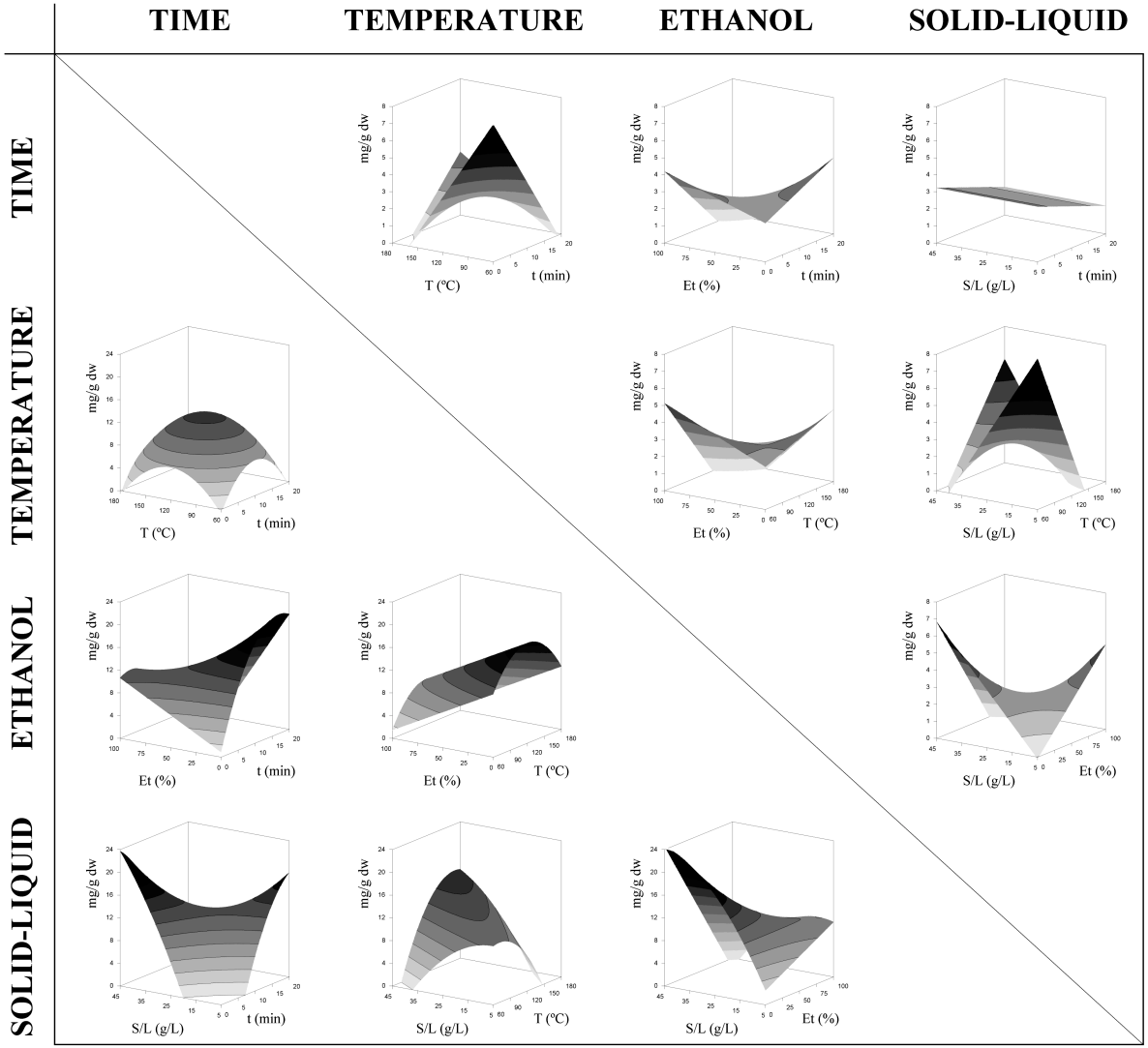
TIME
TEMPERATURE
ETHANOL
SOLID-LIQUID
TIME
TEMPERATURE
ETHANOL
SOLID-LIQUID
mg/g dw
T (ºC)
t (min)
Et (%)
S/L (g/L)

Fig. 3

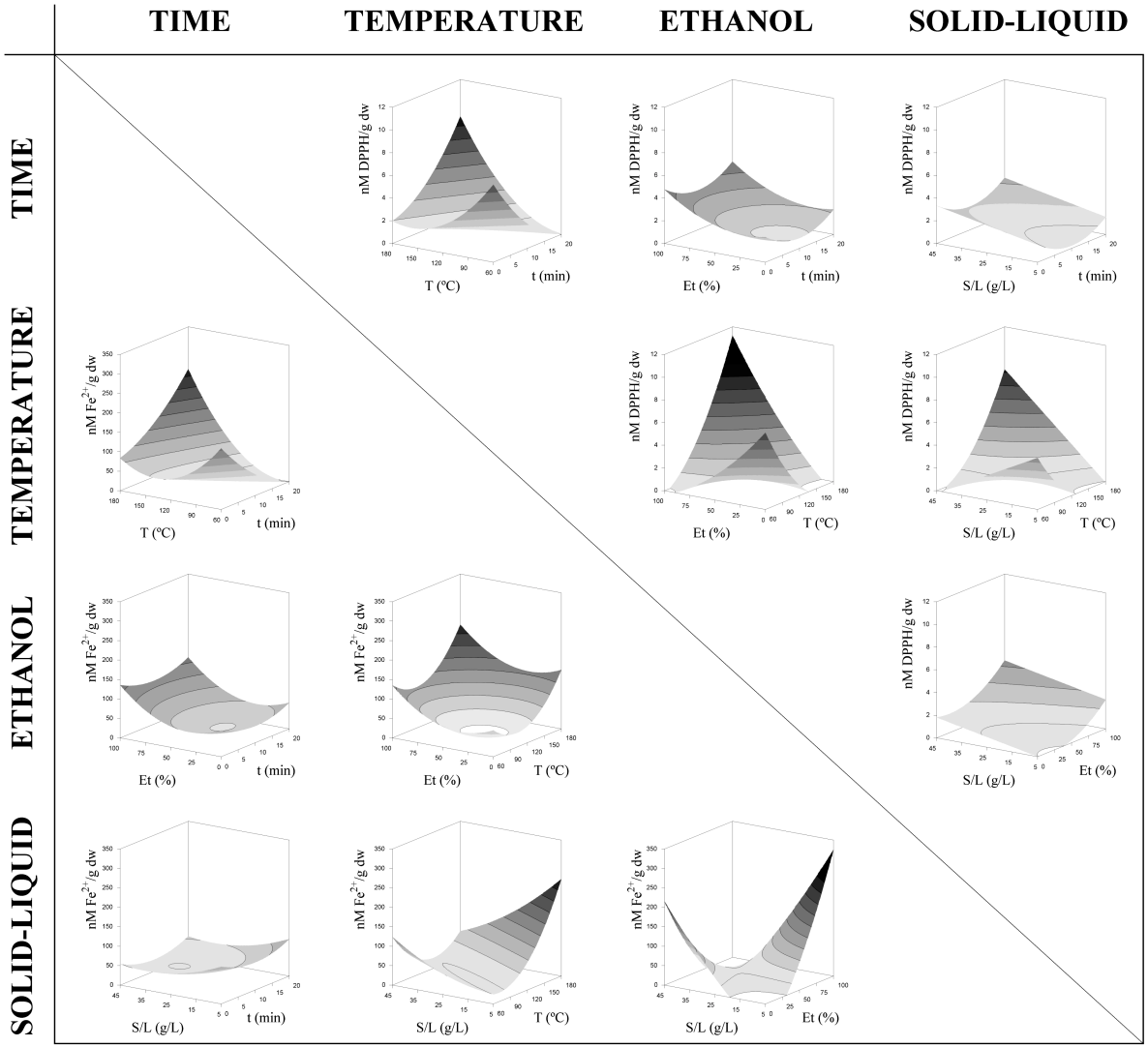
TIME
TEMPERATURE
ETHANOL
SOLID-LIQUID
TIME
TEMPERATURE
ETHANOL
SOLID-LIQUID
nM DPPH/g dw
nM Fe2+/g dw
T (ºC)
t (min)
Et (%)
S/L (g/L)

Fig. 4

## Tables

Table 1 - Coded and natural values of the optimization parameters used in the RSM analysis. The four independent variables $X_1$ (time, min), $X_2$ (temperature, ºC), $X_3$ (ethanol concentration, %) and $X_4$ (solid/liquid ratio, g/L) were combined in a 5-level full factorial design of 25 combinations and 7 replicates at the centre of the experimental domain.

| **CODED VALUES** | **NATURAL VALUES** | | | |
|---|---|---|---|---|
| | *$X_1$: t (min)* | *$X_2$: T (ºC)* | *$X_3$: Et (%)* | *$X_4$: S/L (g/L)* |
| -2 | 0 | 60 | 0 | 5 |
| -1 | 5 | 90 | 25 | 15 |
| 0 | 10 | 120 | 50 | 25 |
| +1 | 15 | 150 | 75 | 35 |
| +2 | 20 | 180 | 100 | 45 |

Table 2 - Numerical values of the responses obtained under the conditions designed in Table 1. The values of phenolic acids and flavonoids were obtained by HPLC quantification. The estimated numerical values of *vm* (μM DPPH/g extract) and *m* (μM $Fe^{2+}$/g extract) were achieved using the Eq. (1) and Eq. (2), respectively.

| **RUN** | **EXPERIMENTAL DOMAIN** | | | | **RSM RESPONSES** | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | ***Phenolic acids*** | | ***Flavonoids*** | | ***Antioxidant activity*** | |
| | $X_1$: *t* | $X_2$: *T* | $X_3$: *Et* | $X_4$: *S/L* | $P_1$ | $P_2$ | $F_1$ | $F_2$ | *DPPH* | *RP* |
| | *min* | *ºC* | *%* | *g/L* | *mg/g extract* | *mg/g extract* | *mg/g extract* | *mg/g extract* | *μM DPPH/g extract* | *μM $Fe^{2+}$/g extract* |
| **1** | -1(5) | -1(90) | -1(25) | -1(15) | 3.30 | 9.81 | 2.58 | 3.67 | 1.92 | 31.8 |
| **2** | 1(15) | -1(90) | -1(25) | -1(15) | 4.19 | 13.27 | 2.28 | 3.29 | 1.16 | 18.5 |
| **3** | -1(5) | 1(150) | -1(25) | -1(15) | 1.31 | 3.85 | 0.40 | 0.67 | 0.48 | 55.4 |
| **4** | 1(15) | 1(150) | -1(25) | -1(15) | 2.49 | 8.25 | 0.87 | 1.49 | 1.30 | 85.9 |
| **5** | -1(5) | -1(90) | 1(75) | -1(15) | 1.61 | 3.96 | 2.94 | 4.51 | 2.42 | 96.6 |
| **6** | 1(15) | -1(90) | 1(75) | -1(15) | 3.08 | 10.04 | 1.34 | 2.75 | 1.47 | 68.7 |
| **7** | -1(5) | 1(150) | 1(75) | -1(15) | 1.14 | 3.75 | 1.02 | 1.22 | 2.74 | 158.5 |
| **8** | 1(15) | 1(150) | 1(75) | -1(15) | 2.01 | 7.32 | 0.59 | 0.68 | 3.47 | 173.9 |
| **9** | -1(5) | -1(90) | -1(25) | 1(35) | 5.00 | 14.13 | 2.42 | 3.35 | 2.21 | 50.8 |
| **10** | 1(15) | -1(90) | -1(25) | 1(35) | 3.57 | 9.23 | 1.72 | 2.46 | 1.50 | 37.8 |
| **11** | -1(5) | 1(150) | -1(25) | 1(35) | 5.76 | 16.29 | 1.82 | 2.88 | 1.83 | 70.6 |
| **12** | 1(15) | 1(150) | -1(25) | 1(35) | 5.45 | 12.88 | 2.73 | 4.83 | 2.66 | 99.1 |
| **13** | -1(5) | -1(90) | 1(75) | 1(35) | 3.35 | 9.81 | 2.35 | 3.50 | 2.32 | 52.6 |
| **14** | 1(15) | -1(90) | 1(75) | 1(35) | 0.94 | 2.14 | 0.47 | 0.84 | 1.60 | 39.2 |
| **15** | -1(5) | 1(150) | 1(75) | 1(35) | 3.21 | 9.43 | 1.20 | 1.70 | 4.75 | 56.8 |
| **16** | 1(15) | 1(150) | 1(75) | 1(35) | 1.73 | 4.30 | 0.42 | 0.61 | 5.96 | 71.3 |
| **17** | -2(0) | 0(120) | 0(50) | 0(25) | 2.20 | 6.14 | 2.48 | 3.69 | 2.26 | 50.3 |
| **18** | 2(20) | 0(120) | 0(50) | 0(25) | 1.47 | 4.37 | 0.72 | 1.24 | 1.98 | 41.6 |
| **19** | 0(10) | -2(60) | 0(50) | 0(25) | 2.44 | 6.06 | 2.63 | 3.90 | 2.09 | 55.8 |
| **20** | 0(10) | 2(180) | 0(50) | 0(25) | 2.00 | 5.48 | 0.38 | 0.79 | 3.51 | 119.7 |
| **21** | 0(10) | 0(120) | -2(0) | 0(25) | 6.80 | 17.86 | 2.14 | 3.47 | 0.96 | 34.0 |
| **22** | 0(10) | 0(120) | 2(100) | 0(25) | 2.60 | 7.50 | 0.80 | 1.32 | 2.80 | 104.0 |
| **23** | 0(10) | 0(120) | 0(50) | -2(5) | 1.21 | 4.79 | 1.63 | 2.53 | 0.27 | 51.5 |
| **24** | 0(10) | 0(120) | 0(50) | 2(45) | 4.50 | 11.72 | 1.68 | 2.62 | 1.72 | 33.1 |
| **25** | 0(10) | 0(120) | 0(50) | 0(25) | 4.60 | 12.64 | 1.65 | 2.54 | 1.13 | 29.0 |
| **26** | 0(10) | 0(120) | 0(50) | 0(25) | 4.73 | 12.87 | 1.77 | 2.71 | 1.14 | 28.6 |
| **27** | 0(10) | 0(120) | 0(50) | 0(25) | 4.53 | 12.35 | 1.59 | 2.45 | 1.13 | 28.8 |
| **28** | 0(10) | 0(120) | 0(50) | 0(25) | 4.71 | 12.75 | 1.61 | 2.47 | 1.06 | 28.8 |
| **29** | 0(10) | 0(120) | 0(50) | 0(25) | 4.72 | 12.79 | 1.62 | 2.48 | 1.11 | 31.8 |
| **30** | 0(10) | 0(120) | 0(50) | 0(25) | 4.70 | 12.74 | 1.60 | 2.47 | 1.09 | 29.0 |
| **31** | 0(10) | 0(120) | 0(50) | 0(25) | 4.74 | 12.84 | 1.62 | 2.49 | 1.04 | 30.7 |
| **32** | 0(10) | 0(120) | 0(50) | 0(25) | 4.73 | 12.80 | 1.62 | 2.48 | 1.08 | 28.8 |

Table 3 - Parametric estimations of the 5-level full factorial design fitted to the second-order polynomial model of Eq. (6), confidence intervals of the estimated parameter values ($\alpha$=0.05) and statistical information of the model proposed for each response.

| | | **PHENOLIC ACIDS** | | **FLAVONOIDS** | | **ANTIOXIDANT ACTIVITY** | |
|---|---|---|---|---|---|---|---|
| | | *$P_1$* *mg/g extract* | *$P_2$* *mg/g extract* | *$F_1$* *mg/g extract* | *$F_2$* *mg/g extract* | *DPPH* *µM DPPH/g extract* | *RP* *µM $Fe^{2+}$/g extract* |
| ***Fitting coefficients obtained by Eq. (6) and showed in Eqs. (7)-(12)*** | | | | | | | |
| Intercept | $b_0$ | 4,70±0.25 | 12,82±0.70 | 1,58±0.07 | 2,44±0.12 | 1,14±0.20 | 29,44±8.29 |
| Linear effect | $b_1$ | ns | ns | -0,33±0.09 | -0,39±0.14 | ns | ns |
| | $b_2$ | ns | ns | -0,48±0.09 | -0,69±0.14 | 0,48±0.13 | 20,97±4.79 |
| | $b_3$ | -0,93±0.16 | -2,40±0.46 | -0,30±0.09 | -0,46±0.14 | 0,64±0.13 | 16,99±4.79 |
| | $b_4$ | 0,69±0.16 | 1,33±0.46 | ns | ns | 0,45±0.13 | -10,33±4.79 |
| Quadratic effect | $b_{11}$ | -0,70±0.15 | -1,76±0.41 | ns | ns | 0,32±0.12 | 6,07±4.32 |
| | $b_{22}$ | -0,60±0.15 | -1,64±0.41 | ns | ns | 0,49±0.12 | 16,53±4.32 |
| | $b_{33}$ | ns | ns | ns | ns | 0,26±0.12 | 11,85±4.32 |
| | $b_{44}$ | -0,44±0.15 | -1,01±0.41 | ns | ns | ns | 5,17±4.32 |
| Interactive effect | $b_{12}$ | ns | ns | 0,29±0.10 | 0,43±0.17 | 0,42±0.16 | 9,79±5.86 |
| | $b_{13}$ | ns | ns | -0,32±0.10 | -0,47±0.17 | ns | ns |
| | $b_{14}$ | -0,63±0.20 | -2,41±0.56 | ns | ns | ns | ns |
| | $b_{23}$ | ns | ns | ns | -0,28±0.17 | 0,60±0.16 | ns |
| | $b_{24}$ | 0,53±0.20 | 1,34±0.56 | 0,34±0.10 | 0,63±0.17 | 0,41±0.16 | -8,79±5.86 |
| | $b_{34}$ | -0,44±0.20 | -1,05±0.56 | -0,25±0.10 | -0,43±0.17 | ns | -21,54±5.86 |
| ***Statistical information of the fitting analysis*** | | | | | | | |
| Obs | | 32 | 32 | 32 | 32 | 32 | 32 |
| DF | | 22 | 22 | 23 | 22 | 21 | 20 |
| $R^2$ | | 0.9545 | 0.9482 | 0.9420 | 0.9348 | 0.9502 | 0.9407 |
| $R^2$adj | | 0.9421 | 0.9284 | 0.9137 | 0.8964 | 0.9165 | 0.8996 |
| MSE | | 4.52 | 30.99 | 1.00 | 2.34 | 2.61 | 2649.6 |
| RMSE | | 2.12 | 5.56 | 1.00 | 1.53 | 1.61 | 51.47 |
| MAPE | | 9.19 | 9.008 | 11.54 | 10.97 | 10.09 | 12.93 |
| DW | | 1.36 | 1.15 | 1.99 | 2.12 | 1.43 | 1.50 |

*ns:* non-significant coefficient; *DF:* Degree of freedom; *$R^2$:* Correlation coefficient; *$R^2$adj:* 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 - Optimal processing conditions in natural values that lead to optimal response values.

| CRITERIA | OPTIMAL PROCESSING CONDITIONS | | | | RESPONSE OPTIMUM | |
|---|---|---|---|---|---|---|
| | $X_1$: t (min) | $X_2$: T (ºC) | $X_3$: Et (%) | $X_4$: S/L (g/L) | | |
| ***For each phenolic acid*** | | | | | | |
| *P1* | 5.51 | 146.69 | 0.0 | 45.0 | 8.99±0.58 | *mg/g extract* |
| *P2* | 3.15 | 144.64 | 0.0 | 45.0 | 24.8±0.9 | *mg/g extract* |
| ***For each flavonoid*** | | | | | | |
| *F1* | 2.0 | 60.0 | 100.0 | 5.00 | 6.78±0.45 | *mg/g extract* |
| *F2* | 2.0 | 60.0 | 100.0 | 5.00 | 11.7±0.6 | *mg/g extract* |
| ***For each antioxidant activity*** | | | | | | |
| *DPPH* | 20.0 | 180.0 | 100.0 | 45.0 | 14.28±0.6 | *μM DPPH/g extract* |
| *RP* | 20.0 | 180.0 | 100.0 | 5.0 | 445.0±3.1 | *μM $Fe^{2+}$/g extract* |
| ***Intermediate processing conditions for phenolic acids, flavonoids and antioxidant activity*** | | | | | | |
| *P1* | 4.38 | 145.6 | 0.0 | 45.0 | 8.96±1.22 | *mg/g extract* |
| *P2* | | | | | 24.7±3.37 | *mg/g extract* |
| *F1* | 2.0 | 60.0 | 100.0 | 5.0 | 6.78±1.11 | *mg/g extract* |
| *F2* | | | | | 11.75±1.9 | *mg/g extract* |
| *DPPH* | 20.0 | 180.0 | 100.0 | 45.0 | 6.91±1.58 | *μM DPPH/g extract* |
| *RP* | | | | | 265.4±60.6 | *μM $Fe^{2+}$/g extract* |
| ***Global processing conditions*** | | | | | | |
| *P1* | 20.0 | 180.0 | 0.0 | 45.0 | 7.57±0.77 | *mg/g extract* |
| *P2* | | | | | 18.8±1.9 | *mg/g extract* |
| *F1* | | | | | 3.67±0.37 | *mg/g extract* |
| *F2* | | | | | 7.47±0.76 | *mg/g extract* |
| *DPPH* | | | | | 4.02±0.41 | *μM DPPH/g extract* |
| *RP* | | | | | 203.7±20.6 | *μM $Fe^{2+}$/g extract* |