Optimization of microwave-assisted extraction of hydrophilic and lipophilic antioxidants from a surplus tomato crop by response surface methodology

José Pinela[a,b], M.A. Prieto[a,c,*], Maria Filomena Barreiro[d], Ana Maria Carvalho[a], M. Beatriz P.P. Oliveira[b], J.A. Vázquez[e], Isabel C.F.R. Ferreira[a,*]

[a] Mountain Research Centre (CIMO), ESA, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 1172, 5301-855 Bragança, Portugal

[b] REQUIMTE/LAQV, Faculty of Pharmacy, University of Porto, Rua Jorge Viterbo Ferreira, nº 228, 4050-313 Porto, Portugal

[c] Nutrition and Bromatology Group, Faculty of Food Science and Technology, University of Vigo, Ourense Campus, E32004 Ourense, Spain.

[d] Laboratory of Separation and Reaction Engineering (LSRE), Associate Laboratory LSRE/LCM, Polytechnic Institute of Bragança, Campus de Santa Apolónia, 1134, 5301-857 Bragança, Portugal

[e] Grupo de Reciclado y Valorización de Materiales Residuales (REVAL), Instituto de Investigacións Mariñas (IIM-CSIC), r/Eduardo Cabello, 6., Vigo 36208, Galicia, Spain.

* Authors to whom correspondence should be addressed: Isabel C.F.R. Ferreira (e-mail: iferreira@ipb.pt; telephone +351-273-303219; fax +351-273-325405) and M.A. Prieto (e-mail: michaelumangelum@gmail.com; telephone +34 654-694-616)

Abstract

Tomato is the second most important vegetable crop worldwide and a rich source of industrially interesting antioxidants. Hence, the microwave-assisted extraction of hydrophilic (*H*) and lipophilic (*L*) antioxidants from a surplus tomato crop was optimized using response surface methodology. The relevant independent variables were temperature (*T*), extraction time (*t*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*). The concentration-time response methods of crocin and β-carotene bleaching were applied, since they are suitable *in vitro* assays to evaluate the antioxidant activity of *H* and *L* matrices, respectively. The optimum operating conditions that maximized the extraction were as follows: *t*, 2.25 min; *T*, 149.2 ºC; *Et*, 99.1 %; and *S/L*, 45.0 g/L for *H* antioxidants; and *t*, 15.4 min; *T*, 60.0 ºC; *Et*, 33.0 %; and *S/L*, 15.0 g/L for *L* antioxidants. This industrial approach indicated that surplus tomatoes possess a high content of antioxidants, offering an alternative source for obtaining natural value-added compounds. Additionally, by testing the relationship between the polarity of the extraction solvent and the antioxidant activity of the extracts in *H* and *L* media (polarity-activity relationship), useful information for the study of complex natural extracts containing components with variable degrees of polarity was obtained.

Keywords: *Lycopersicon esculentum*; microwave-assisted extraction; β-carotene/crocin bleaching assay; concentration-time response modelling; response surface methodology

1. Introduction

Tomato (*Lycopersicon esculentum* Mill.) is the second most important vegetable crop worldwide after potato and is consumed either fresh or in the form of processed products. In 2013, about 164 million of tones were produced in the world, having been registered an increase of 2.6 million of tones over 2012 (FAOSTAT, 2015). Apart from the large amounts of solid wastes produced by the processing industry, sometimes there is also a surplus production that leads to glut in the market, distress sale and low profit to the growers (Oliveira, 2006; Sashimatsung et al., 2011). One solution for the problem of this glut may be its sustainable use for the recovery of value-added antioxidant compounds with applications in food, pharmaceutical and cosmeceutical industries. In fact, tomato is a rich source of hydrophilic and lipophilic antioxidants (Barros et al. 2012; Pinela et al., 2012). The hydrophilic fraction is constituted mainly by ascorbic acid and soluble phenolic compounds, while the lipophilic fraction contains carotenoids (mostly lycopene), tocopherols, sterols and lipophilic phenolics. Each of these compounds has their own function in the human organism, acting at different locations, but also working conjunctly, having the ability to offer protection against oxidative stress and various degenerative diseases (Carocho and Ferreira, 2013a, 2013b; Friedman, 2013). Besides, according to some reports, antioxidants belonging to the hydrophilic fraction have a far more significant impact on total antioxidant activity than does antioxidants of the lipophilic fraction (García-Valverde et al., 2013; Kotíková et al., 2011).

The antioxidant activity can be monitored using a large variety of assays, each one based on a specific mechanism of action, including hydrogen atom transfer, single electron transfer, reducing power, and metal chelation, among others (Carocho and Ferreira, 2013a; Shahidi and Zhong, 2015). For this reason, it is important to understand

the mechanisms behind the selected assay for a suitable evaluation of the antioxidant potential. Crocin and β-carotene bleaching reactions are two *in vitro* assays appropriate for the antioxidant activity evaluation of hydrophilic (*H*) and lipophilic (*L*) matrices, respectively, and can provide useful information in the study of complex natural extracts containing components with variable degrees of polarity (Prieto et al., 2013; Prieto and Vázquez, 2014). Both assays are reproducible, especially accurate, and yields a low experimental error (Prieto et al., 2014).

To recover antioxidants from plant-based products is necessary to follow suitable extraction methods that ensure and preserve its integrity and bioactivity. That's why the industry is looking for more efficient processes based on enhanced innovation capacity. Among them, microwave-assisted extraction (MAE) has gained significance due to its shortened extraction time, higher extraction rate, reduced solvent consumption and superior product's quality at lower cost (Dahmoune et al., 2015; Gallo et al., 2010), being one of the dominant trends of the "green chemistry" movement (Michel et al., 2011). However, the extraction process efficiency depends on some variables and operating conditions (Bhuyan et al., 2015; Dahmoune et al., 2015), which may not be generalized for all plant materials due to the diverse nature of existing bioactive phytochemicals. Therefore, selection and optimization of variables and operating conditions for the MAE of antioxidants from tomato is necessary.

One-factor-at-a-time approaches are commonly used to optimize extraction processes; but it is well-known that optimal operating conditions or interactions between variables cannot be predicted with this methodology. Both problems may be overcome by employing the response surface methodology (RSM), a powerful statistical tool used to predict the optimum experimental conditions to maximize or minimize various independent variables. Indeed, RSM describes the relationship between independent

variables and one or more responses, enabling process optimization such as the extraction of bioactive molecules from natural sources with a reduced number of experimental trials.

This study aimed at determining the optimal extraction conditions for *H* and *L* antioxidants from a tomato surplus. Four independent variables (temperature, extraction time, ethanol concentration and solid/liquid ratio) were studied and the extraction process was optimized by RSM. The concentration-time response methods of β-carotene and crocin bleaching were applied, which are appropriate for the evaluation of antioxidant properties of *L* and *H* fractions, respectively.

2. Material and methods

2.1. Equipment and reagents

Equipments: Biotage Initiator Microwave (Biotage® Initiator+, Uppsala, Sweden) using closed high precision glass vials. Multiskan Spectrum Microplate Photometer using 96-well polypropylene microplates.

Reagents: Linoleic acid (CID 5280450); β-Carotene (CID 5280489); Crocin (CID 5281233); 2,2'-Azobis(2-amidinopropane) (AAPH or ABAP, CID 1969). All other chemicals and solvents 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. Plant material

A common tomato farmers' variety known as "tomate redondo or batateiro" (round tomato), and widely cultivated in rural communities from Miranda do Douro, North-eastern Portugal, was chosen for this study. Surplus tomatoes at the ripe stage were

hand-harvested randomly from the middle of six plants, in selected homegardens of two villages in the studied area. The ripening stage was established according to local consumers' criteria based in morphological descriptors such as size, texture, and pericarp colour. Six tomatoes (pericarps without jointed pedicels and seeds) were frozen and lyophilized (Free Zone 4.5, Labconco, Kansas City, MO, USA), reduced to a fine dried powder (20 mesh) using a grinding machine and kept at -20 °C until analysis.

2.3. Microwave-assisted extraction of *H* and *L* antioxidants

The MAE process was performed using a Biotage Initiator Microwave apparatus in closed vials. The dried powdered samples were extracted at different time (*t*), temperature (*T*), ethanol concentration (*Et*) and solid/liquid ratio (*S/L*) ranging as defined by the RSM design (Fig. 1). The solvent volume was fixed at 20 mL. During extraction, samples were stirred at 600 rpm using a magnetic stirring bar and irradiated at 200 W. After that, the reaction mixture in the closed vial was quickly cooled in the processing chamber and then centrifuged at 6000 rpm for 10 min. The pellet was discarded and the supernatant was carefully collected, evaporated under reduced pressure to remove the solvent and finally re-suspended in distilled water for further analysis. The dry weight (dw) of the suspended solids in the supernatant of each solution was determined to compute the extraction yield (g extract/g samples).

2.4. Determination of the concentration-time dependency of *L* and *H* antioxidants

β-Carotene (Marco, 1968) and crocin (Bors et al., 1984) methods (βCM and CM, respectively) are widely used to evaluate the antioxidant activity of different matrices. Both *in vitro* assays share some analytical similarities as depicted in the next points.

2.4.1. Reaction conditions

βCM conditions (Prieto et al., 2012): 2 mg of β-carotene (βC, 1 μM in the final reaction), 0.25 mL of linoleic acid and 2 g of Tween-40 were dissolved in 20 mL of chloroform, vigorously mixed, followed by chloroform evaporation (45 °C/~15 min). To the resulting oily residue were added 300 mL of buffered Mili-Q water (100 mM Briton, pH=6.5) at 45 °C. The absorbance at 470 nm of the prepared reagent was ~1.40.

CM conditions (Prieto et al., 2015): 4 mg of crocin (Cr, 100 μM in the final reaction) and 75 mg of AAPH (7.68 mM in the final reaction) were dissolved in 30 mL of a 100 mM Briton buffer, pH=5.5, in Mili-Q water. The absorbance at 450 nm of the prepared reagent was ~1.40.

2.4.2. Procedure

The procedure was performed by adding 50 μL of sample extract and 250 μL of reagent into the wells (350 μL) of the microplate. The microplate-reader was programmed at intervals of 3, 5 and 10 min (initiation, propagation and asymptotic phases), during a period of 200 min (total of 30 measures). The sample extracts were analyzed kinetically for eight different concentrations obtained by serial dilution (1/1, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64 and the control) in distilled water.

2.4.3. Quantification

The area under the curve (*AUC*) (Eq. 1), computed by any numerical integration method such as the trapezoidal rule, proved to be a highly robust criterion, able to summarize in a single and direct value the global feature of any kinetic profile.

$$AUC = \frac{R_1 \Delta t_1}{2} + \sum_{i=2}^{i=n-1} R_i \Delta t_i + \frac{R_n \Delta t_n}{2} \quad (1)$$

where i is the number of data measured over time t, R_i are the responses along an arbitrary time series, and Δt is the interval of each measurement.

The AUC of a concentration-response of an antioxidant was normalized against to the AUC obtained with the control, leading to the formulation of the relative area units or protected substrate ($\bar{P}$) in percentage (Eq. 2), as defined similarly by other authors (Dávalos, 2004; Huang et al., 2002; Naguib, 2000) for antioxidant responses.

$$\bar{P}(A) = S_0\left(\frac{AUC_C - AUC_A}{AUC_C}\right)\frac{100}{S_0} \qquad (2)$$

where AUC_C and AUC_A are the area units corresponding to the kinetic profiles found in the absence (control, C) and presence of an antioxidant concentration A, respectively, and S_0 is the initial substrate in the reaction (for the CM, the substrate is equivalent to 100 μM of Cr, and for the βCM it is equivalent to 1 μM of βC).

The relationship in Eq. (2) establishes that AUC_C (control) is also the maximum response achievable; consequently the values obtained were also standardized in percentage. In addition, by normalizing the response, the results obtained are less dependent on the experimental conditions, which, in practice, is one of the common problems when analyzing the efficacy of response factors.

The asymptotic variation of $\bar{P}$ as function of an antioxidant compound suggests that some radical-generating property of the system can be saturated (Gieseg and Esterbauer, 1994). This type of concentration-response patterns should behave in a similarly accumulative way with a number of different antioxidant compounds found in the extract material. Therefore, in general, this patterns can be adjusted by a group of mathematical expressions (mechanistic or not) that translates the pattern of the response into parameters that allow to deduce the meaning and/or quantify the effect of the dependent variable in a simple and global mode. Previous researchers discussed the

applicability of different mathematical expressions (Prieto et al., 2014); therefore, following their views, the Weibull cumulative distribution function was selected (Weibull and Sweden, 1951). Thus, the variation of $\bar{P}$ as function of increasing concentrations of an antioxidant (A) can be described satisfactorily using the Weibull model rearranged for our own purposes as follows in Eq. (3).

$$\bar{P}(t,A) = P_m \left\{ 1 - \exp\left[-\ln(2)^{1-a} \left(\frac{2V_m}{P_m a} A \right)^a \right] \right\} \quad (3)$$

The parameter P_m is the averaged maximum protected substrate, asymptotic value of the response (% μM of βC or Cr), which is specific of each A agent. The parameter V_m corresponds to the average amount of protected molecules per gram of extracted material (% μM of protected substrate/g extract). The parameter a is a shape parameter related to the slope that can produce potential profiles (a<1), first order kinetic ones (a=1) and a variety of sigmoidal profiles (a>1).

In addition, the concentration needed to reach 50% of the maximum protective effect (the so called IC_{50}) can be determined according to Eq. (4).

$$IC_{50} = \frac{Ka \ln 2}{2V_m} \text{ ; therefore } \bar{P}(t,A) = P_m \left\{ 1 - \exp\left[-\ln(2) \left(t/IC_{50} \right)^a \right] \right\} \quad (4)$$

where IC_{50} is the concentration producing the half-maximal response and all other notations remain with the same meaning as above.

2.5. Response surface methodology

The RSM family designs are used for modelling and analyzing problems in which a response of interest is influenced by a set of variables. RSM was applied to optimize the MAE process with the purpose of finding the favourable processing conditions that would result in a higher extraction rate of H or L antioxidants.

2.5.1. Response criteria for evaluating the antioxidant capacity

The responses used in the RSM analysis were based in the numerical values of the parametric coefficients P_m, V_m and IC_{50} of Eqs. (3) and (4). The information provided by the combination of the values of the three response criteria represents a robust tool to compare the activity of different antioxidant agents based on the parametric concentration-time estimations.

2.5.2. Preliminary experiments

Preliminary single-factor experiments were conducted in order to select the significant variables and/or collateral factors in extraction process and to determine the preliminary range of the optimum level of each factor for an appropriate experimental RSM design. In this primary screening trial, the following variables and factors were considered:

- Internal independent variables of the microwave equipment: Pressure (1-30 bar), stirring rate (0-1000 rpm), microwave power (0-400 W), temperature (40-300 ºC) and extraction time (no limits).
- Internal factors of the instrument software: Absorption level (*very low*, *low*, *normal*, *high*, or *very high*), fixed hold time (if *on* the time countdown starts when the target temperature or pressure is reached, *i.e.*, the initial time taken to reach the set conditions is not included in the heating time; if *off* the time countdown starts when the heating starts), cooling (*on* or *off*), pre-stirring (during the fixed hold time, if selected; *on* or *off*) and vial type (2-5 mL or 10-20 mL).
- External independent variables and factors: Solid/liquid ratio and ethanol concentration. The type of solvent used in the extraction.

2.5.3. Experimental design

From the preliminary study, the independent variables X_1 (extraction time, min), X_2 (temperature, ºC), X_3 (ethanol concentration, %) and X_4 (solid/liquid ratio, g/L) were selected. Then, the combined effects of these variables on the extraction yield of *H* and *L* antioxidant were studied using *central composite design* as proposed by Box et al. (1957). 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 in order to maximize the prediction precision (Box and Hunter, 2005). This design also requires five levels for each factor. The number of repetitions n_0 of the centre point is calculated by the formulas present in Eq. (5) for *k* factors based on the uniform precision.

$$\gamma = \frac{(k+3)+\sqrt{9k^2+14k-7}}{4(k+2)}; \quad \text{where:} \quad n_0 = floor\left(\gamma\left(\sqrt{2^k}+2\right)^2 - 2^k - 2k\right) \tag{5}$$

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

$$n = 2^k + 2k + 1 \tag{6}$$

Experimental runs were randomized, to minimize the effects of unexpected variability in the observed responses. Independent variables coded values and natural ones of the factorial design are coded and decoded by the expressions in Eq. (7).

$$v_c = (v_n - v_0)/\Delta v_n \quad \text{and} \quad v_n = v_0 + \Delta v_n \times v_c \tag{7}$$

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

2.5.4. Box-Behnken mathematical model

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

$$Y = b_0 + \sum_{i=1}^{n} b_i X_i + \sum_{\substack{i=1 \\ j>i}}^{n-1} \sum_{j=2}^{n} b_{ij} X_i X_j + \sum_{i=1}^{n} b_{ii} X_i^2 \quad (8)$$

where Y is the dependent variable (response variable) to be modelled, X_i and X_j define the independent variables, b_0 is the constant coefficient, b_i is the coefficient of linear effect, b_{ij} is the coefficient of interaction effect, b_{ii} the coefficients of quadratic effect and n is the number of variables. As pointed out, three different response formats based in the parametric estimations (P_m, V_m and IC_{50}) of Eqs. (3) and (4) were used as the dependent variable for each H and L antioxidant analytical reaction (Y_{Pm}^{H}; $Y_{V\tau}^{H}$; $Y_{IC_{50}}^{H}$; Y_{Pm}^{L}; $Y_{V\tau}^{L}$; and $Y_{IC_{50}}^{L}$).

2.6. Numerical methods and statistical analysis

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

1) Coefficients determination: Parametric estimates were obtained by minimization of the sum of the quadratic differences between observed and model-predicted values, using the nonlinear least-squares (quasi-Newton) method provided by the macro *Solver* in *Microsoft Excel* 2003 (Kemmer and Keller, 2010), which allows a quick testing of a hypotheses and its consequences (Murado and Prieto, 2013).
2) Coefficients significance: The determination of the parametric confidence intervals were calculated using the '*SolverAid*' (Prikler, 2009). The model was simplified by dropping terms, which were not statistically significant p-value > 0.05.

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

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

3. Results and discussion

3.1. Preliminary study

As listed in the material and methods section, the MAE efficiency may be affected by five internal (pressure, stirring rate, microwave power, extraction time, and temperature) and two external (solid/liquid ratio and ethanol concentration) independent variables and five instrument/software factors (absorption level, fixed hold time, vial type, cooling option, and solvent type). Although there are previous research examples (Bhuyan et al., 2015; Dahmoune et al., 2015), the results are not generalizable for all plant materials due to the diverse nature of existing bioactive phytochemicals.

Therefore, this preliminary study allowed screening of appropriate independent variables and determining their optimum experimental domain for an appropriate experimental RSM design. Variables/factors were investigated by testing a broad range, keeping other ones constant and analyzing their antioxidant responses.

- Type of extracting solvent is the key for separating *H* and *L* compounds. Water is the polar solvent with greater interest in biological processes than any other solvent. Ethanol has a polar hydroxyl group and dissolves many ionic compounds, but also has a non-polar end, which will contribute to dissolve non-polar substances. In this study, binary interactions of ethanol-water mixtures were selected due to their straight *H* affinity, but also because ethanol increases the *L* character of the aqueous-ethanolic mixture. All the tested ethanol concentrations give rise to significant differences; thus, the range from 0 to 100 % was selected.
- Pressure and temperature were correlated. The selected irradiation power was applied in the early stage of the extraction process to reach the selected temperature/pressure in a short period of time. After that, it was automatically applied in less intensity (estimated by the microwave system) to keep constant the solution temperature/pressure. In consequence, the microwaves power was set at 200 W and the temperature was selected as the main controlled variable, since relevant differences were found within the range 60 to 180 ºC.
- Lower solid/liquid ratios can lead to a more efficient dissolution of constituents, but also to a waste of solvent. At an industrial scale, higher ratios are desirable since it is important to maximize the extraction yield (thus productivity) with a minimal solvent consumption (more sustainable process). Significant differences were found for all tested ratios in this preliminary study, being the range from 5 to 45 g/L selected for RSM analysis.

- The temperature caused strong decomposition phases of antioxidants for extraction times higher than 20 min, but this effect depended on the other variables that remained constant. Therefore, extraction times ranging from 0 to 20 min were selected.
- The cooling option showed relevant effects. It is used at the end of the MAE process to cool the sample. When *off*, the cooling time of the solution after processing was longer, affecting the extraction of antioxidants. Therefore, it was used *on* to quickly cool the solution and stop faster the extraction process, making the process more accurate.
- When testing the effects of other factors, such as the absorption level, fixed hold time and vial type, no significant changes were found. Therefore, a *normal* absorption level and vials of 10-20 mL were selected for further analysis. The fixed hold time was turned *off*, since the initial time taken to reach the set temperature or pressure was negligible.

Therefore, the RSM experiment was designed based on these preliminary results, using five variation levels of extraction time (0-20 min), temperature (60-180 ºC), ethanol concentration (0-100%) and solid/liquid ratio (5-45 g/L) as independent variables to optimize efficiently the MAE process, regarding the *H* and *L* antioxidant properties of the extracts. The coded values and their natural values are presented in Fig. 2. Note that, for simplification reasons, the RSM design reduces the number of experimental trials. When studying 5 levels of 4 independent variables, the response would imply 625 possible combinations, but using RSM the experiment could be solved in 25 independent combinations and 7 replicates at the centre of the experimental domain.

3.2. Concentration-time antioxidant responses for RSM

Several reviews have discussed the numerous *in vitro* methods developed to evaluate the antioxidant activity of plant extracts and their controversial aspects regarding differences in the generated radicals, variables (mainly pH and temperature), reagents, and quantification procedures (Carocho and Ferreira, 2013a; Frankel and Meyer, 2000; Jiménez-Escrig et al., 2000). However, when determining the bioactivity of a sample, the final activity response also depends on the degree of polarity of the intermediate reaction components of the applied method. For example, when evaluating the bioactivity of extracts obtained with *H* and *L* extraction solvents using the oxidative hemolysis inhibition assay (OxHLIA) (method in which the thermal decomposition of AAPH generates *H* radicals and the lipid peroxidation of the erythrocytes membranes generates *L* radicals (Niki et al., 1988)), no clear conclusions can be made because both *H* or *L* antioxidants delay the hemolysis and, therefore, produce an antioxidant response. To the best of our knowledge only few articles have addressed the *H* and *L* intermediate components activity (Arnao et al., 2001; Prieto et al., 2013; Prior et al., 2003). Therefore, the lack of selective methods to differentiate the activity of *H* and *L* antioxidants is a current issue in the evaluation of antioxidant responses that need to be outlined in the following years.

In order to reduce the variability of experimental conditions, allowing meaningful comparisons, and to quantify the power of the antioxidants in function of the degree of polarity, the response models of CM and βCM were selected because they provide a microsystem for *H* and *L* oxidation processes, respectively (Prieto et al., 2013). The βC is an *L* oxidizable substrate that can join the system of lipid micelles in which the oxidation reaction is accomplished. The method is especially sensitive to antioxidants in a lipidic environment, producing a very low response to *H* antioxidants, even to

powerful ones. In turn, Cr is an *H* oxidizable substrate and *L* antioxidants produce very low responses in the reaction system.

Fig. 3 and Fig. 4 show an illustration of the antioxidant responses obtained for the tomato extracts produced under the experimental RSM design presented in Fig. 2 for each *H* and *L* antioxidant reaction (CM and βCM), respectively. In both figures, two well differentiated sections are presented at the left- and right-hand sides, showing the visual variable distribution of the 25 genuine combinations. The left-hand side shows the combinations of the concentration-time responses of seven serial dilutions (○:1/1, ▲: 1/2, △: 1/4, ■: 1/8, □: 1/16, ◆: 1/32, ◇: 1/64) and the control (●) for the remaining substrates (% μM Cr and βC). Meanwhile, the right-hand side shows the concentration-time transformation into the concentration-response values of the *AUC* computed by the numerical integration method in Eq. (1) and standardized in percentage of protected substrate (Cr or βC) by Eq. (2). The dots (●) are the raw values and lines (—) the fitted responses to the mathematical model of Eq. (3) or (4). The parametric fitting values of Eqs. (3) and (4) are presented in Table 1. The estimated numerical values of P_m, V_m and IC_{50} were the three meaningful ways considered to evaluate the effectiveness of the antioxidant response by RSM.

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

Table 1 shows the results of the parametric fitting coefficients (data presented in Fig. 3 and Fig. 4) for each *H* and *L* reaction obtained after running 32 trials (25 genuine combinations and 7 replicates) following the experimental RSM design. Estimated coefficient values of Eq. (8), parametric intervals and numerical statistical criteria are shown in Table 2, for each coefficient used as response criteria and for *H* and *L*

reactions. The coefficients that showed effects with *p-values* higher than 0.05 are not significant (*ns*) at the 95% confidence level and consequently were discarded for model development.

Mathematical models were built through nonlinear least-squares estimations based on the coded experimental plan and the response results, obtaining the following second-order polynomial Eq. (8):

when the hydrophilic *CM* was considered:

$$Y_{Pm}^{H} = 30.5 + 8.7x_3 + 6.9x_4 + 7.3x_1^2 + 13.1x_4^2 + 13.1x_1x_2 - 4.5x_2x_3 \quad (9)$$

$$Y_{V\tau}^{H} = 26.5 - 8.1x_1 - 17.2x_3 - 6.9x_2^2 + 9.1x_3^2 + 7.0x_4^2 \quad (10)$$

$$Y_{IC_{50}}^{H} = 0.40 + 0.04x_2 + 0.27x_3 + 0.05x_2^2 + 0.05x_3^2 + 0.16x_1x_2 - 0.05x_2x_3 \quad (11)$$

when the lipophilic *βCM* was considered:

$$Y_{Pm}^{L} = 96.1 + 8.1x_1 - 7.1x_2 + 5.1x_3 - 5.6x_4 - 10.7x_1^2 - 9.8x_2^2 - 8.6x_3^2 - 11.8x_4^2 - 7.7x_1x_2 + 13.1x_2x_3 \quad (12)$$

$$Y_{V\tau}^{L} = 13.1 - 2.9x_1 - 3.2x_3 - 2.0x_4 + 5.9x_2^2 + 2.3x_1x_3 + 1.6x_1x_4 - 1.7x_2x_3 + 6.2x_2x_4 \quad (13)$$

$$Y_{IC_{50}}^{L} = 2.97 + 0.76x_3 + 0.31x_4 - 0.24x_1^2 - 0.07x_3^2 - 0.22x_4^2 - 0.24x_1x_4 + 0.43x_2x_3 + 0.27x_3x_4 \quad (14)$$

where X_1 (extraction time), X_2 (temperature), X_3 (ethanol concentration), X_4 (solid/liquid ratio), Y is the response, sub-indices indicates the coefficient criteria (P_m, V_m and IC_{50}) used as responses for RSM and super-indices H and L accounts for the H (CM) and L (βCM) reactions.

The multivariable characterization of the Box-Behnken second-order polynomial model is especially robust, minimizing the experimental errors, allowing explain the utmost of the results. In addition, once a model is designed, if the experimental data obtained do not span the full range and some of them fail to provide information about one or more of the parameters of the equation, the multivariable application describes simply and

accurately all the areas. As explained, not all the parameters of Eq. (8) were used for building the model, since some terms were non-significant (Table 2). Model coefficients obtained are empirical and cannot be associated with physical or chemical significance. However, they are useful to predict the results of untested operation conditions (Ranic et al., 2014). 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. Based in the mathematical expressions, it was found that the responses in the *L* environment were much more complex than those found in the *H* one.

The statistic lack of fit, used to test the adequacy of the obtained models, demonstrated that no considerable improvement was achieved by the inclusion of the statistically non-significant effects (Table 2). This was also verified by the high R^2 and R^2_{adj} values indicating the percentage of variability of each response that is explained by the model (Table 2). The distribution of residuals always randomly scattered around zero and grouped data and autocorrelations were not observed. This means that these models are workable and can be applied in the subsequent prediction and optimization stages. Finally, the analysis of variance (ANOVA) was computed for the regression equations. The lack of fit was used to verify the adequacy of the model and was not significant ($p > 0.05$), indicating that Eqs. (12) to (14) adequately fit the experimental data.

3.4. Effect of *Et* and *T* variables as representative case of the typical *H* and *L* trends

The three response criteria (P_m, V_m and IC_{50}) characterize singular features of the response. Previous to the complete analysis of the *H* and *L* antioxidant extraction trends,

the information provided by each parametric response criteria, which were used in the RSM design, was individually analyzed. As an illustrative case study, it was selected the effect of the variables *Et* and *T*, meanwhile the variables *t* and *S/L* were positioned at the centre of their experimental domain (*t*=10 min and *S/L*=25 g/L). Graphical 3D representations are displayed in Fig. 5 and the parametric fitting values are present in Table 2. In general, it can be observed that the *H* and *L* antioxidant activity of the tomato extracts have opposite trends for *Et* and *T*. In more specific terms, for each criterion it can be concluded that:

a) The parameter P_m of Eq. (3) shows the maximum specific capability of the antioxidant agent to protect the substrate (% μM of Cr or βC) and, the higher the P_m value, the more powerful the protective capability of the antioxidant. In general, we can speculate that the more complex the content in antioxidant molecules in the extract (which act at different *H* or *L* oxidation levels), higher the parameter P_m. These types of extracts are usually obtained with longer extraction times. The conditions that favour the *H* activity of the P_m value were at high ranges (↑) of *Et* and low ranges (↓) of *T* or, in a much less active manner, at ↓ *Et* and ↑ *T*. In contrast, the *L* activity was found at intermediate ranges (↔) of *Et* and *T*, leading to a clear optimum at 50 % *Et* and 120 ºC. The inversion effect of the polarity-activity relationship proposed by the polar paradox theory is visible in these results (Porter, 1993). Actually, the speculated effect of the non-polar end of ethanol on the activity of the tomato extracts was not observed; they even showed an improvement of the *H* antioxidant activity at ↑ *Et*, while the *L* antioxidant activity decreased sharply at both ends of *Et*.

b) The parameter V_m of Eq. (3) corresponds to the average amount of protected molecules of Cr or βC per gram of extracted material (% μM of protected

substrate/g extract), which is a value of maximal predictability. The higher the V_m value, the more powerful the antioxidant. There are a diverse number of compounds that would present a high specific protection, but only few would be present in an enough amount to show its activity. Therefore, the highest values should appear when an extraction peak of an antioxidant with a high specific protection would be found. The conditions that maximize the V_m response of the *H* activity were at ↓ *Et* and ↔ *T*; while for the *L* activity were found at ↓ *Et* and ↑ *T*. The effect of the inversion of the polar activity on the optimal response was not as evident as for the parameter P_m, but the opposite trends remain present as can be seen in each 3D surface.

c) The parameter IC_{50} of Eq. (4) provides directly the classical IC_{50} (g of extract), which will effectively summarize all effects of the other two responses. It provides the amount of extract needed to achieve a very specific response (50%). The lower the IC_{50} value, the more powerful the antioxidant. The lowest values should be found in an intermediate position between those speculated in the previous criteria. For the IC_{50} criteria, the conditions that maximize the response for the *H* antioxidant activity were at ↓ *Et* and ↓ *T*, while for the *L* activity were at ↓ *Et* and ↑ *T*.

The data published in literature often focus on only one response parameter, but each of them describes different intrinsic characteristics of the response. The information provided by the combination of the three values represents a robust tool to compare the activities of different antioxidant agents based on the parametric concentration-time estimations. By analyzing all parametric nonlinear values for the experimental RSM design, a more rigorous evaluation of the extraction efficiency of *H* and *L* antioxidants is accomplished.

3.5. Nonlinear relationship between extraction solvent polarity and antioxidant activity

Matrix combination of the 3D responses for the *H* and *L* environmental reactions obtained for the P_m, V_m and IC_{50} are presented in Fig. 6, Fig. 7 and Fig. 8, respectively. In addition, a simplified way to present the results in a 2D format for all responses is presented in Fig. S1 of the supplementary material. Eqs. (12) to (14) were used to simulate the surfaces. In each graphical illustration, the top diagonal part presents the response surfaces for *L* reactions and the bottom diagonal part presents the response surfaces for *H* reactions. 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).

In general, the inversion effect of the polarity-activity relationship was observed in almost all responses. The effects accounted between the *t*, *T* and *S/L* variables would describe the conditions that optimize the *H* and *L* antioxidant responses of the tomato extracts. However, the variable *Et* did not perform as theoretically expect. Such fuzziness between the polarity of extraction solvent and the antioxidant activity of the extracts in *H* and *L* environments (the so-called polarity-activity relationship) was found interesting enough to be considered. Generally, the extraction ability of solvents can be grouped in three main types: non-polar, polar aprotic and polar protic solvents (Huffman et al., 2012; Kislik, 2012). The choice of the extracting solvent is the first crucial step towards the optimization of any extraction method (Sultana et al., 2009), which has a strong impact on the type of molecules that would be separated. In turn, antioxidants are classified into two broad divisions (Arnao et al., 2001), depending on whether they are soluble in water (*H*, such as ascorbic acid) or in lipids (*L*, such as α-tocopherol). When performing an extraction, it is well known that *L* antioxidant

molecules are mostly extracted in non-polar solvents (*i.e.*, *n*-hexane) and *H* antioxidant molecules in polar ones (*i.e.*, water), according to the "like dissolves like" principle, as confirmed by several authors that separated effectively the molecular *H* and *L* character of molecules by applying different solvent combinations in conjunction with different extraction procedures (Watanabe et al., 2014). However, based on the achieved results, the separation of extracts according to their molecular polarity character does not guarantee that their polar or non-polar target activity can be separated as well. As stated before (Prieto et al., 2013), when testing the activity of *H* and *L* antioxidant extracts (hexane and methanol solvents, respectively), it was confirmed that *H* and *L* antioxidant extracts, normally in a much lesser extent, remain active in the opposite environment. In addition to that complex scenery, there are amphiphilic molecules presenting an affinity with solvents of various polarities (Taresco et al., 2015).

Thus, if their polarity-activity is not totally related with their distribution in the extracting solvents as defined by the polarity index (*i.e.*, dielectric constant), we may be using extracts for *L* environments (*i.e.* oils) with a high content in molecules with a *H* antioxidant activity and vice versa. Nonetheless, it is recognized that antioxidants with a clear *H* and *L* character can cause the opposite effect when applied in the opposite environment (*i.e.*, ascorbic acid can initiate lipid oxidation in conjunction with metal cations) (Zhang and Omaye, 2001). Actually, according to the polar paradox theory (Porter, 1993), polar antioxidants are more effective in less polar media, while non-polar or amphiphilic antioxidants tend to be more effective in a media of relatively higher polarity. The higher efficiency of *L* antioxidants in oil-in-water emulsions would be due to their tendency to concentrate at the interfacial membrane where the oxidation is supposed to occur, while more *H* antioxidants would tend to segregate into the

aqueous phase where they would be much less effective (Frankel et al., 1994). Our results support this phenomenon.

A possible hypothetical foundation behind the mechanisms that caused this effect could be the microwave absorbing properties of the solvent (Dahmoune et al., 2015). Polar molecules strongly absorb microwave energy because of the permanent dipole moment, and the degree of absorption increases with the dielectric constant. A simple comparison between water and ethanol shows that ethanol has a lesser ability to obstruct the microwaves as they pass through, but has a greater ability to dissipate the microwave energy into heat. This strong absorption provides an increase of the temperature inside the sample, leading to the rupture of cells by the *in situ* water. In some cases it can promote the degradation of the target antioxidants and, in other cases, can increase the diffusivity of the target antioxidants in the matrix.

Knowing all that, when describing the antioxidant activity of components of a complex natural extract as a function of the degree of polarity, scientific studies typically involve a first extraction step with solvents with different polarity index, followed by testing their activity by different analytical procedures. However, such a link between polarity-activity cannot be straightforward performed and further analyses are need. In the literature there are few reports addressing the previously mentioned associated issues (Jayasinghe et al., 2013; Li et al., 2015). However, it would be interesting to perform studies considering the following issues: a) a well-defined group of *in vitro* methods that could separate the polar activity of compounds in *H* and *L* antioxidants; b) a representative set of natural materials sources extracted with a set of solvents demonstratives of the different polarity index; c) a complex optimization of variable conditions that affect the extraction of *H* and *L* antioxidants to ensure and preserve its integrity and bioactivity; and d) clear target applications with a marked *H* and *L*

character to prove in an *in vivo* form, whether or not the relation between the aspects stated in a), b) and c) are validated. The combination of all these requirements seems to be a labour-intensive approach, being out the context of this work.

3.6. Optimal extraction conditions for *H* and *L* antioxidants

The fitting results (Table 2) obtained by applying Eq. (8) to all the response criteria (P_m, V_m and IC_{50}) are presented in Eqs. (9) to (11) for the *H* reaction and in Eqs. (12) to (14) for the *L* reaction. By finding the partial derivatives of these regression equations, equating them to zero (Table S1 of the supplementary material) and solving the equations system, the coded values that optimize the response criteria were obtained. Then, the coded variable values were introduced in the original Eqs. (9)-(14) and the optimal response values were found. Finally, by decoding the coded values, the conditions that maximize the response were transformed into natural values.

The operating conditions that maximize the extraction of the tomato antioxidants and the optimal response values are presented in Table 3, for each parametric estimation criteria (P_m, V_m and IC_{50}) and analytical reaction (*H* and *L*). For *H* antioxidants, the optimal conditions for P_m were at 180.0 ºC, with 56.8 % ethanol and 45.0 g/L of sample, during 18.7 min; for V_m were at 120.0 ºC, with 0.0 % ethanol and 5.0 g/L of sample, during 2.5 min; and for IC_{50} were at 90.0 ºC, with 44.0 % ethanol and 17.0 g/L of sample, during 14.5 min; and for *L* antioxidants, the optimal conditions for P_m were at 93.6 ºC, with 44.0 % ethanol and 21.3 g/L of sample, during 13.4 min; for V_m were at 180.0 ºC, with 100 % ethanol and 5.0 g/L of sample, during 2.2 min; and for IC_{50} were at 169.1 ºC, with 91.7 % ethanol and 10.9 g/L of sample, during 2.6 min. Optimal extraction conditions based on all the response criteria (P_m, V_m and IC_{50}) were also determined for *H* and *L* antioxidants. Based on these values, it was found that the

extraction of *L* antioxidants demands a longer *t* (15.4 min) but a lower *T* (60.0 ºC), *Et* (33.0 %) and *S/L* (15.0 g/L), comparing to the operating conditions outlined for *H* antioxidants (*i.e.*, t, 2.25 min; T, 149.2 ºC; Et, 99.1 %; and *S/L*, 45.0 g/L). These intermediate extraction conditions, and others that were optimized for each response criteria (P_m, V_m and IC_{50}) of both *H* and *L* antioxidants, were depicted using a simplex method tool to solve linear problem. Restrictions were made to the variable coded values that did not allowed the set of equations consider unnatural conditions (*i.e.*, lower times than 0). Additionally, optimal extraction conditions for both *H* and *L* antioxidants based on all the response criteria were determined (*i.e.*, *t*, 12.1 min; *T*, 122.3 ºC; *Et*, 100 %; and *S/L*, 27.2 g/L), which allow to obtain the maximum extraction yield of both antioxidants simultaneously.

4. Conclusions

Optimal MAE conditions for *H* and *L* antioxidants from a surplus tomato crop were determined in this study. A five-level full factorial Box-Behnken design was successfully implemented and RSM used for analysis. The independent variables of *t*, *T*, *Et* and *S/L* had significant effects on MAE. To predict the optimal extraction conditions, a second-order polynomial model assuming interactive effects was fitted to each response and the regression coefficients were determined using the least-squares method. Optimal MAE conditions for *H*, *L* and both antioxidants were determined based on the parametric response criteria P_m, V_m and IC_{50}. Overall, MAE proved to be a powerful and efficient innovative methodology to extract the tomato antioxidants. In statistical terms, the high values of the adjusted coefficient of determination (R^2_{adj} > 0.90) and the non-significant difference between predicted and experimental values demonstrated the validity of the proposed optimization model. The results also indicated

that the antioxidant capacity of the *H* fraction was much higher than the *L* one. Additionally, a discussion on the relationship between the extraction capacity of the solvent in function of its polarity and the antioxidant activity of the extracts in *H* and *L* media (the so-called polarity-activity relationship) was initiated, providing useful information in the study of complex natural extracts containing ingredients with opposite degrees of polarity.

Acknowledgments

The authors are grateful to the Foundation for Science and Technology (FCT, Portugal) for financial support to CIMO (PEst-OE/AGR/UI0690/2014), REQUIMTE (UID/QUI/50006/2013) and J. Pinela (SFRH/BD/92994/2013); to FCT/MEC and FEDER under Programme PT2020 for financial support to LSRE (UID/EQU/50020/2013), and to QREN, ON2 and FEDER (Project NORTE-07-0162-FEDER-000050); to the Xunta de Galicia for financial support for the post-doctoral researcher of M.A. Prieto.

References

Arnao, M.B., Cano, A., Acosta, M., 2001. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chem. 73, 239–244.

Barros, L., Dueñas, M., Pinela, J., Carvalho, A.M., Santos Buelga, C., Ferreira, I.C.F.R., 2012. Characterization and quantification of phenolic compounds in four tomato (*Lycopersicon esculentum* L.) farmers' varieties in northeastern Portugal homegardens. Plant Foods Hum. Nutr. 67, 229–234.

Bhuyan, D.J., Van Vuong, Q., Chalmers, A.C., van Altena, I. A., Bowyer, M.C., Scarlett, C.J., 2015. Microwave-assisted extraction of *Eucalyptus robusta* leaf for the optimal yield of total phenolic compounds. Ind. Crops Prod. 69, 290–299.

Bors, W., Michel, C., Saran, M., 1984. Inhibition of the bleaching of the carotenoid crocin a rapid test for quantifying antioxidant activity. Biochim. Biophys. Acta 796, 312–319.

Box, G., Hunter, J., 1957. Multi-factor experimental designs for exploring response surfaces. Ann. Math. Stat. 28, 195–241.

Box, G., Hunter, J., Hunter, W., 2005. Statistics for experimenters: design, innovation, and discovery. Wiley. ISBN: 978-0-471-71813-0

Carocho, M., Ferreira, I.C.F.R., 2013a. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem. Toxicol. 51, 15–25.

Carocho, M., Ferreira, I.C.F.R., 2013b. The role of phenolic compounds in the fight against cancer--a review. Anticancer. Agents Med. Chem. 13, 1236–58.

Comuzzi, C., Polese, P., Melchior, A., Portanova, R., Tolazzi, M., 2003. SOLVERSTAT: a new utility for multipurpose analysis. An application to the

investigation of dioxygenated Co (II) complex formation in dimethylsulfoxide solution. Talanta 59, 67–80.

Dahmoune, F., Nayak, B., Moussi, K., Remini, H., Madani, K., 2015. Optimization of microwave-assisted extraction of polyphenols from Myrtus communis L. leaves. Food Chem. 166, 585–595.

Dávalos, A., 2004. Extending applicability of the oxygen radical absorbance capacity (ORAC-fluorescein) assay. J. Agric. Food Chem. 52, 48–54.

FAOSTAT, 2015. Food and Agriculture Organization of the United Nations. Statistics division. Available at: http://faostat3.fao.org/browse/Q/QC/E. Accessed 5 March 2015.

Frankel, E.N., Meyer, A.S., 2000. The problems of using one-dimensional methods to evaluate multifunctional food and biological antioxidants. J. Sci. Food Agric. 80, 1925–1941.

Frankel, E.N., Huang, S.W., Kanner, J., German, J.B., 1994. Interfacial phenomena in the evaluation of antioxidants: bulk oils vs. emulsions. J. Agric. Food Chem. *42*, 1054-1059.

Friedman, M., 2013. Anticarcinogenic, cardioprotective, and other health benefits of tomato compounds lycopene, α-tomatine, and tomatidine in pure form and in fresh and processed. J. Agric. Food Chem. 61, 9534–9550.

Gallo, M., Ferracane, R., Graziani, G., Ritieni, A., Fogliano, V., 2010. Microwave assisted extraction of phenolic compounds from four different spices. Molecules 15, 6365–74.

García-Valverde, V., Navarro-González, I., García-Alonso, J., Periago, M.J., 2013. Antioxidant bioactive compounds in selected industrial processing and fresh consumption tomato cultivars. Food Bioprocess Technol. 6, 391–402.

Gieseg, S.P., Esterbauer, H., 1994. Low density lipoprotein is saturable by pro-oxidant copper. FEBS Lett. 343, 188–194.

Huang, D., Ou, B., Hampsch-Woodill, M., Flanagan, J., Prior, R., 2002. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. J. Agric. Food Chem. 50, 4437–4444.

Huffman, B.A., Poltash, M.L., Hughey, C.A., 2012. Effect of polar protic and polar aprotic solvents on negative-ion electrospray ionization and chromatographic separation of small acidic molecules. Anal. Chem. 84, 9942–9950.

Jayasinghe, C., Gotoh, N., Wada, S., 2013. Pro-oxidant/antioxidant behaviours of ascorbic acid, tocopherol, and plant extracts in n-3 highly unsaturated fatty acid rich oil-in-water emulsions. Food Chem. *141*, 3077–3084.

Jiménez-Escrig, A., Jiménez-Jiménez, I., Sánchez-Moreno, C., Saura-Calixto, F., 2000. Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-diphenyl-1-picrylhydrazyl. J. Sci. Food Agric. 80, 1686–1690.

Kemmer, G., Keller, S., 2010. Nonlinear least-squares data fitting in Excel spreadsheets. Nat. Protocols 5, 267–281.

Kislik, V.S., 2012. Solvent extraction: classical and novel approaches. Elsevier. ISBN: 978-0-444-53778-2

Kotíková, Z., Lachman, J., Hejtmánková, A., Hejtmánková, K., 2011. Determination of antioxidant activity and antioxidant content in tomato varieties and evaluation of mutual interactions between antioxidants. LWT - Food Sci. Technol. 44, 1703–1710.

Li, Y., Fabiano-Tixier, A. S., Ruiz, K., Castera, A. R., Bauduin, P., Diat, O., Chemat, F. (2015). Comprehension of direct extraction of hydrophilic antioxidants using

vegetable oils by polar paradox theory and small angle X-ray scattering analysis. Food Chem. *173*, 873–880.

Marco, G.J., 1968. A rapid method for evaluation of antioxidants. J. Am. Oil Chem. Soc. 45, 594–598.

Michel, T., Destandau, E., Elfakir, C., 2011. Evaluation of a simple and promising method for extraction of antioxidants from sea buckthorn (*Hippophaë rhamnoides* L.) berries: Pressurised solvent-free microwave assisted extraction. Food Chem. 126, 1380–1386.

Murado, M.A., Prieto, M.A., 2013. NOEC and LOEC as merely concessive expedients: Two unambiguous alternatives and some criteria to maximize the efficiency of dose–response experimental designs. Sci. Total Environ. 461-462, 576–586.

Naguib, Y.M., 2000. A fluorometric method for measurement of oxygen radical-scavenging activity of water-soluble antioxidants. Anal. Biochem. 284, 93–98.

Niki, E., Komuro, E., Takahashi, M., Urano, S., Ito, E., Terao, K., 1988. Oxidative hemolysis of erythrocytes and its inhibition by free radical scavengers. J. Biol. Chem. 263, 19809–14.

Oliveira, M., 2006. The evolution of the portuguese processed tomato sector: situation and prospects on the global market. New Medit, Mediterr. J. Econ. Agric. Environ. 1, 38–46.

Pinela, J., Barros, L., Carvalho, A.M., Ferreira, I.C.F.R., 2012. Nutritional composition and antioxidant activity of four tomato (*Lycopersicon esculentum* L.) farmer' varieties in Northeastern Portugal homegardens. Food Chem. Toxicol. 50, 829–34.

Porter, W.L., 1993. Paradoxical behavior of antioxidants in food and biological systems. Toxicol. Ind. Health, *9*, 93–122.

Prieto, M.A., Vázquez, J.A., 2014. *In vitro* determination of the lipophilic and hydrophilic antioxidant capacity of unroasted coffee bean extracts and their synergistic and antagonistic effects. Food Res. Int. 62, 1183–1196.

Prieto, M.A., Murado, M.A., Vázquez, J.A., Curran, T.P., 2013. A new microplate procedure for simultaneous assessment of lipophilic and hydrophilic antioxidants and pro-oxidants, using crocin and β-carotene bleaching methods in a single combined assay: Tea extracts as a case study. Food Res. Int. 53, 836–846.

Prieto, M.A., Rodríguez-Amado, I., Vázquez, J.A., Murado, M.A., 2012. β-Carotene assay revisited. Application to characterize and quantify antioxidant and prooxidant activities in a microplate. J. Agric. Food Chem. 60, 8983–8993.

Prieto, M.A., Vázquez, J.A., Murado, M.A., 2014. A critical point: The problems associated with the variety of criteria to quantify the antioxidant capacity. J. Agric. Food Chemestry 62, 5472–84.

Prieto, M.A., Vázquez, J.A., Murado, M.A., 2015. Crocin bleaching antioxidant assay revisited: Application to microplate to analyse antioxidant and pro-oxidant activities. Food Chem. 1, 299–310.

Prikler, S., 2009. Robert de Levie: Advanced Excel for scientific data analysis, 2nd ed. Anal. Bioanal. Chem. 395, 1945.

Prior, R. L., Hoang, H., Gu, L., Wu, X., Bacchiocca, M., Howard, L., Hampsch-Woodill, M., Huang, D., Ou, B., & Jacob, R. 2003. Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORACFL)) of plasma and other biological and food samples. J. Agric. Food Chem. 51, 3273–3279.

Ranic, M., Nikolic, M., Pavlovic, M., Buntic, A., Siler-Marinkovic, S., Dimitrijevic-Brankovic, S., 2014. Optimization of microwave-assisted extraction of natural

antioxidants from spent espresso coffee grounds by response surface methodology. J. Clean. Prod. 80, 69–79.

Sashimatsung, Giribabu, M., Lanusunep, 2011. A study on Marketable surplus and Price Spread of Tomato in Mokokchung District of Nagaland. Int. J. Humanit. Soc. Sci. Invent. 2, 37–42.

Shahidi, F., Zhong, Y., 2015. Measurement of antioxidant activity. J. Funct. Foods 1–25.

Shi, P., Tsai, C.-L., 2002. Regression model selection: a residual likelihood approach. J. R. Stat. Soc. B (Statistical Methodol.) 64, 237–252.

Sultana, B., Anwar, F., Ashraf, M., 2009. Effect of extraction solvent/technique on the antioxidant activity of selected medicinal plant extracts. Molecules 14, 2167–2180.

Taresco, V., Crisante, F., Francolini, I., Martinelli, A., D'Ilario, L., Ricci-Vitiani, L., Buccarelli, M., Pietrelli, L., Piozzi, A., 2015. Antimicrobial and antioxidant amphiphilic random copolymers to address medical device-centered infections. Acta Biomater. 22, 131–40.

Watanabe, J., Oki, T., Takebayashi, J., Takano-Ishikawa, Y., 2014. Extraction Efficiency of Hydrophilic and Lipophilic Antioxidants from Lyophilized Foods Using Pressurized Liquid Extraction and Manual Extraction. J. Food Sci. 79, C1665–C1671.

Weibull, W., Sweden, S., 1951. A statistical distribution function of wide applicability. J. Appl. Mech. 18, 293–297.

Zhang, P., Omaye, S., 2001. Antioxidant and prooxidant roles for β-carotene, α-tocopherol and ascorbic acid in human lung cells. Toxicol. Vitr. 15, 13–24.

Figures

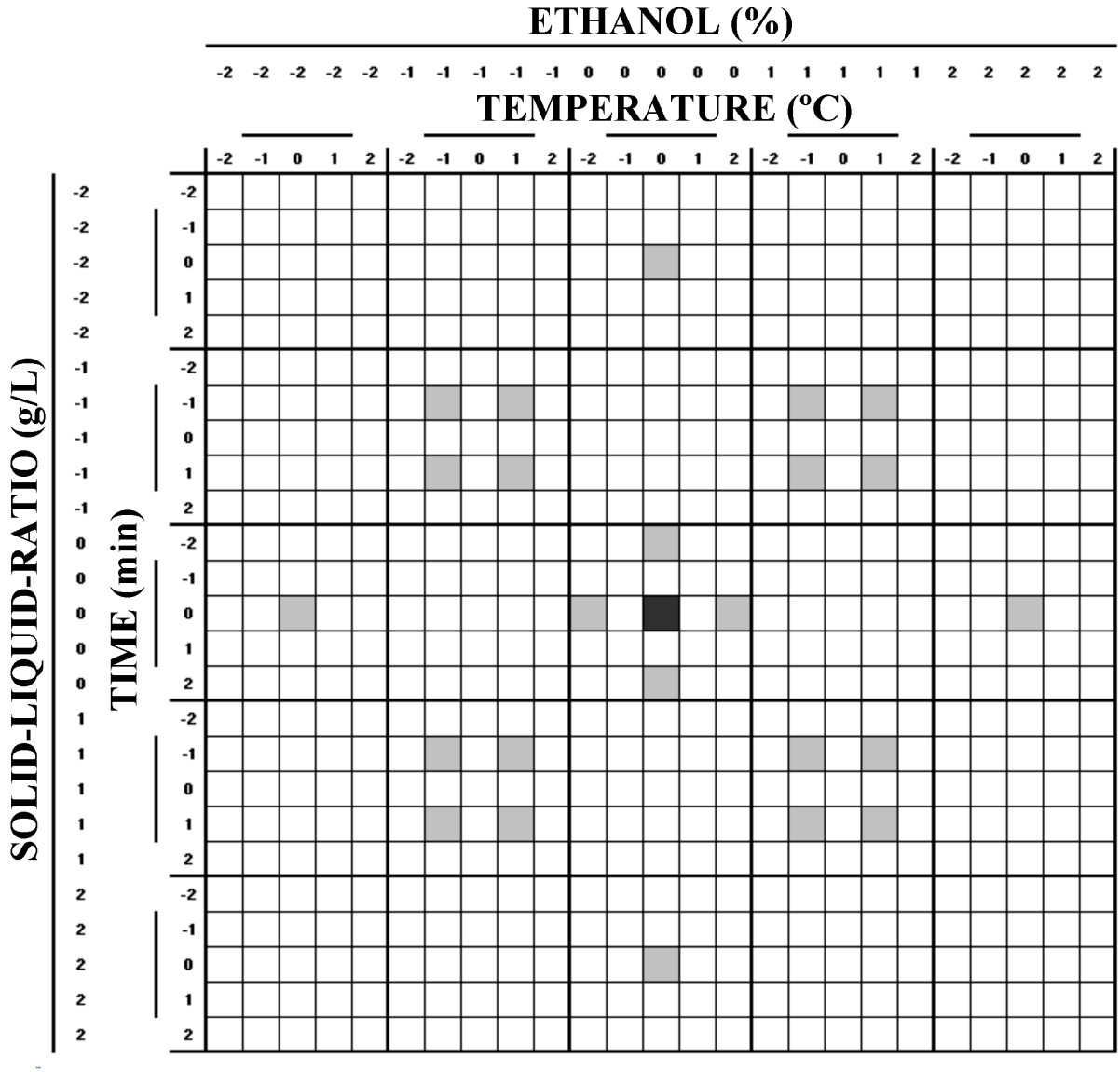

Fig. 2 - Visual representation of the applied experimental RSM design. Four independent variables (extraction time (X_1), temperature (X_2), ethanol concentration (X_3), and solid/liquid ratio (X_4)) were combined in a five-level full factorial design of 25 independent variable combinations (grey grid) and 7 replicates in the centre of the experimental domain (dark grid). Coded values (-2, -1, 0, +1, +2) are in natural values X_1 (*t, min*: 0, 5, 10, 15, 20), X_2 (*T*, ºC: 60, 90, 120, 150, 180), X_3 (*Et*, %: 0, 25, 50, 75, 100) and X_4 (*S/L*, g/L: 5, 15, 25, 35, 45).

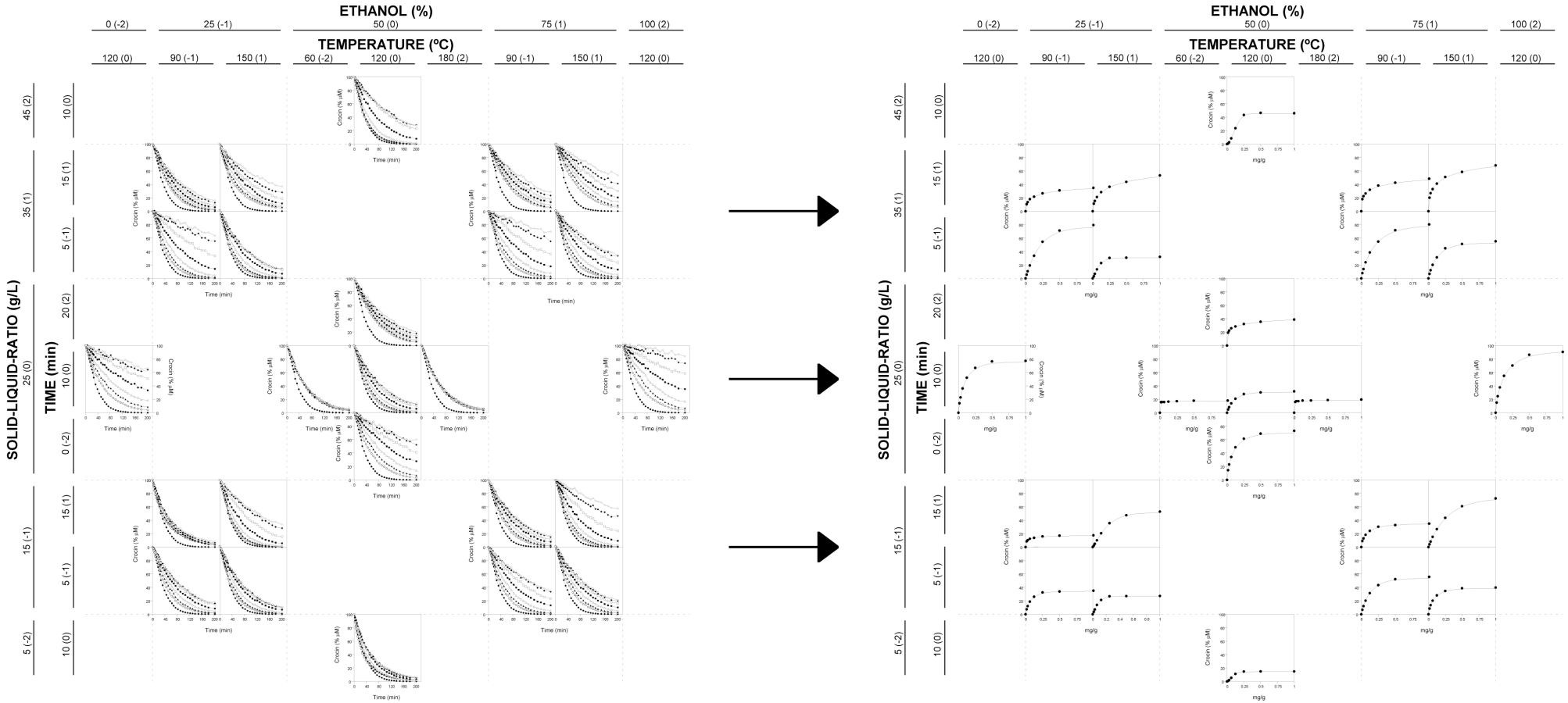

Fig. 3 - Illustration of the *H* responses obtained for the CM under the experimental RSM design presented in Fig. 2. On the left-hand side, each graph illustrates one of the 25 independent variable combinations and inside each graph can be seem the concentration-time responses of seven serial dilutions (○:1/1, ▲: 1/2, △: 1/4, ■: 1/8, □: 1/16, ◆: 1/32, ◇: 1/64) and the control (●) of the extracted material. On the right-hand side, each graph shows: 1) Dots (●), which represents the standardized $\overline{P}$ (protected percentage of Cr) values in a concentration-response format obtained by applying Eq. (2) to the concentration-time responses presented in the left-hand side; and 2) Lines (—), fitted responses to the mathematical model of Eq. (3). The obtained parametric fitting values are presented in Table 1.

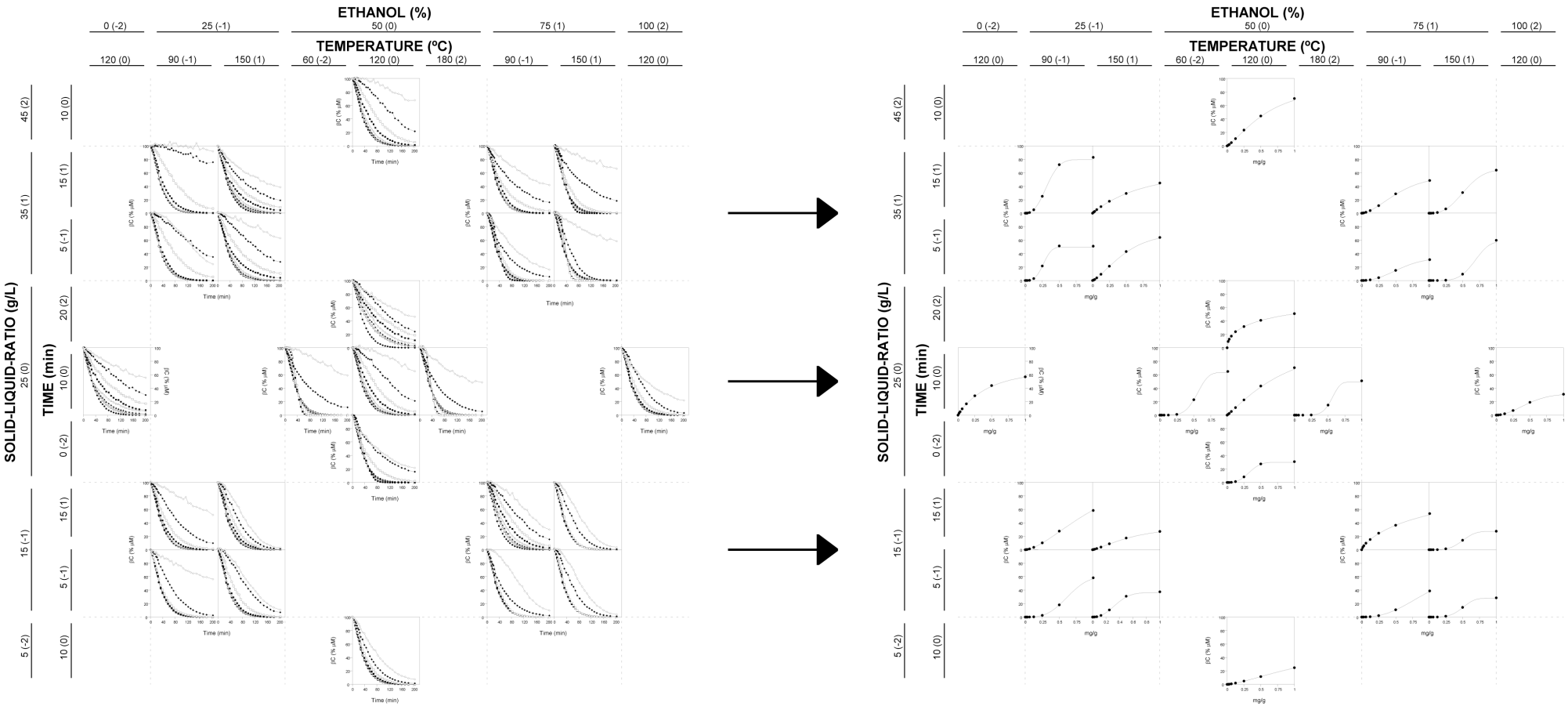

Fig. 4 - Illustration of the *L* responses obtained for the βCM under the experimental RSM design presented in Fig. 2. On the left-hand side, each graph illustrates one of the 25 independent variable combinations and inside each graph can be seem the concentration-time responses of seven serial dilutions (○:1/1, ▲: 1/2, △: 1/4, ■: 1/8, □: 1/16, ◆: 1/32, ◇: 1/64) and the control (●) of the extracted material. On the right-hand side, each graph shows: 1) Dots (●), which represents the standardized $\overline{P}$ (protected percentage of βC) values in a concentration-response format obtained by applying Eq. (2) to the concentration-time responses presented in the left-hand side; and 2) Lines (—), fitted responses to the mathematical model of Eq. (3). The obtained parametric fitting values are presented in Table 1.

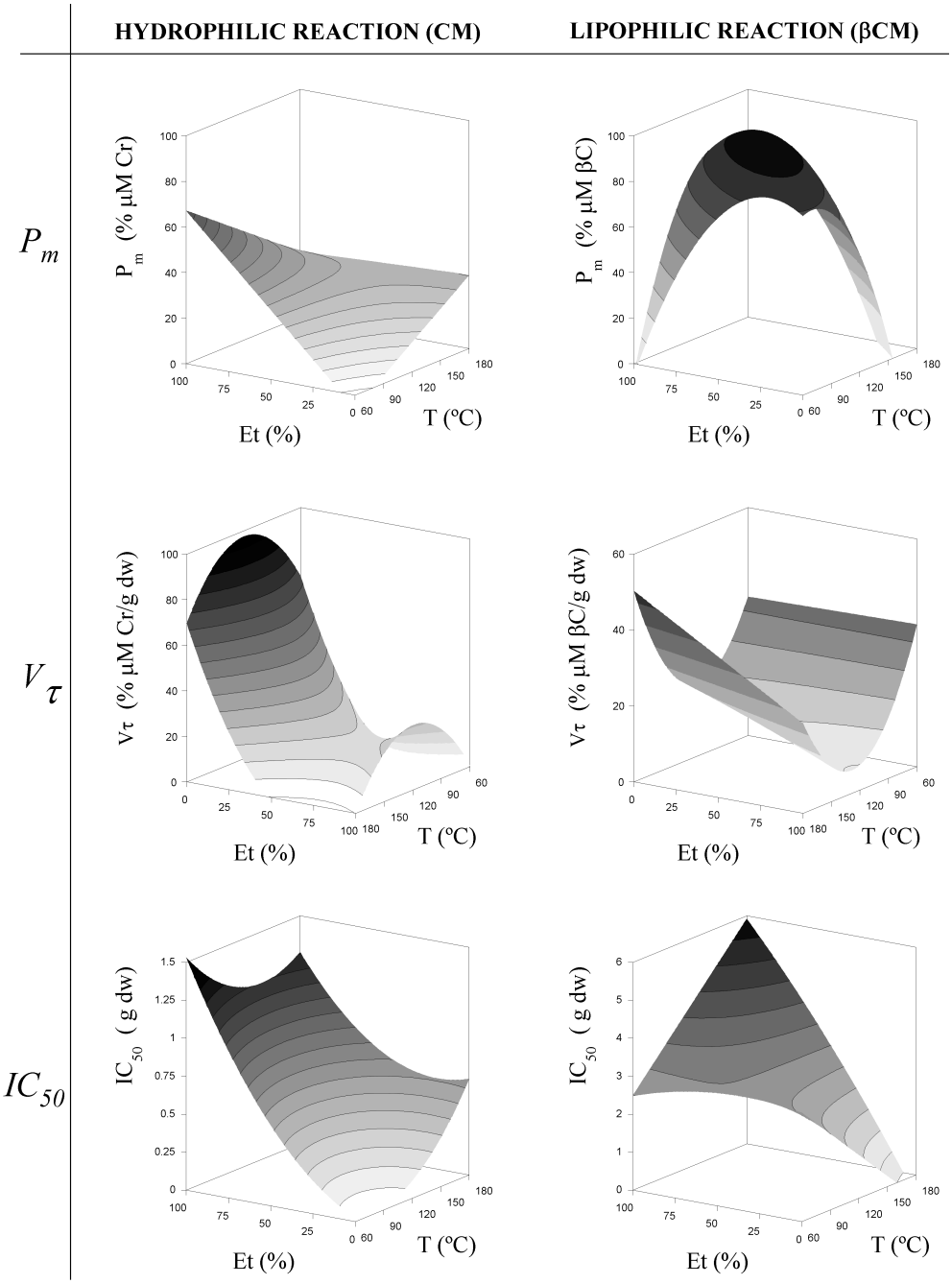

Fig. 5 - Response surfaces of the effects of ethanol concentration (*Et*) *vs* temperature (*T*) on *H* and *L* antioxidant reactions for the parametric response criteria P_m, V_m and IC_{50}. For representation purposes, the variables *t* and *S/L* were positioned at the centre of their experimental domain (*t*=10 min and *S/L*=25 g/L). The obtained parametric fitting values are presented in Table 2.

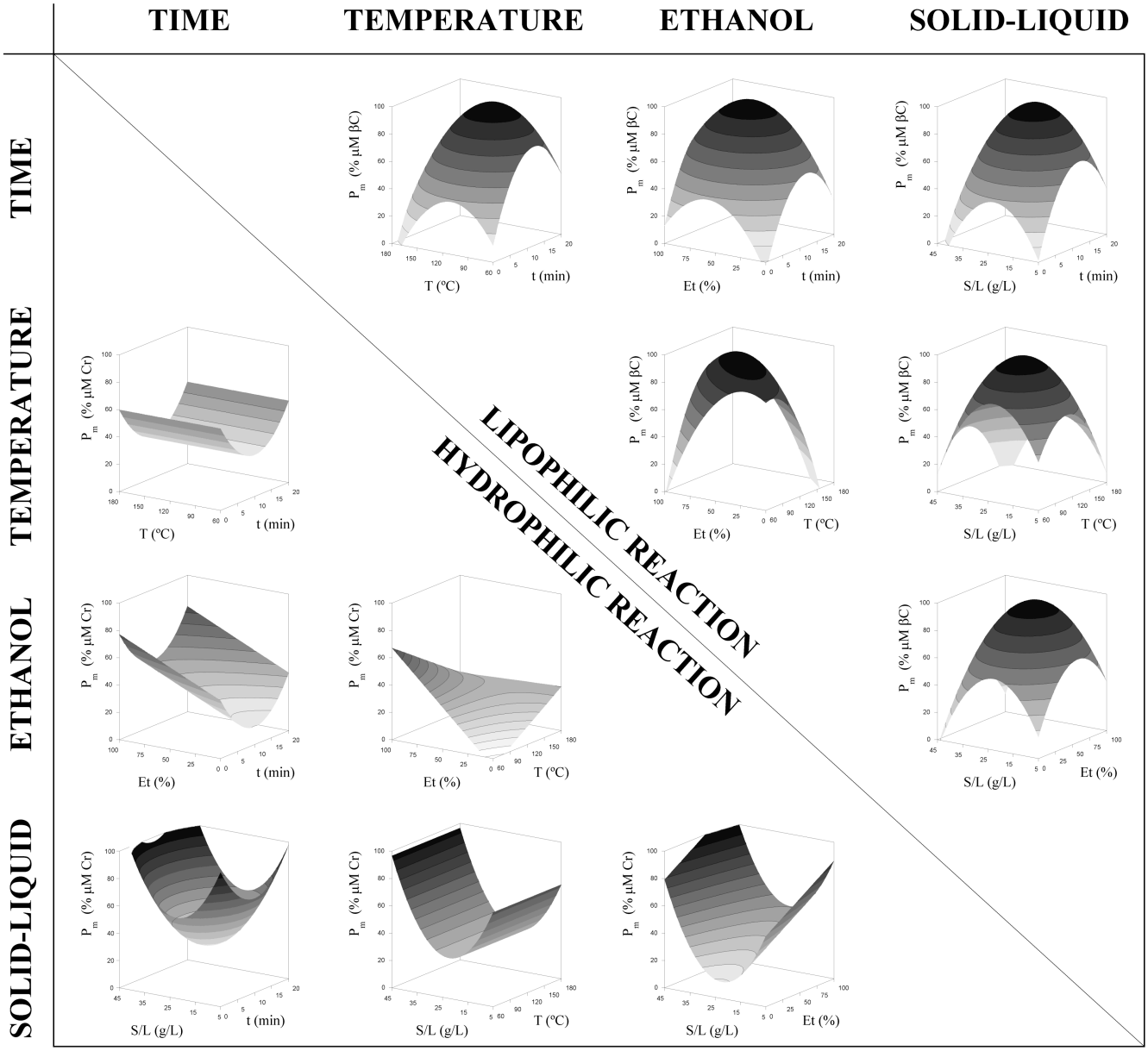

Fig. 6 - Matrix combination of the response surfaces of the *H* and *L* antioxidant reactions obtained for the parametric coefficient P_m (% µM of Cr or βC), which is organized as follows: a) in the top diagonal part is presented the response surface of the *L* reaction; and b) in the bottom diagonal part is presented the response surface of the *H* reaction. 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 obtained parametric fitting values are presented in Table 2.

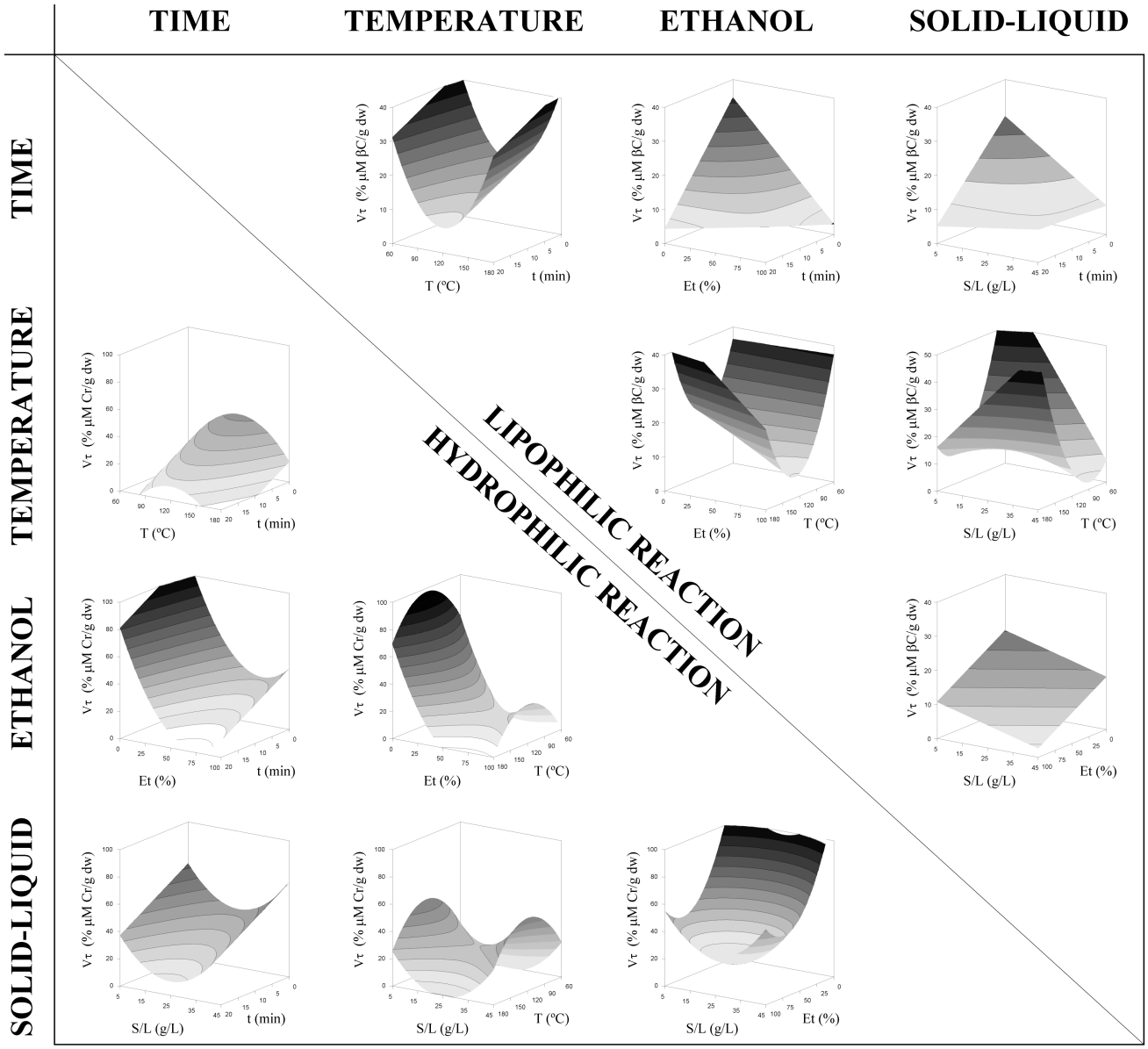

Fig. 7 - Matrix combination of the response surfaces of the *H* and *L* antioxidant reactions obtained for the parametric coefficient V_m (% µM of Cr or βC/g extract), which is organized as follows: a) in the top diagonal part is presented the response surface of the *L* reaction; and b) in the bottom diagonal part is presented the response surface of the *H* reaction. 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 obtained parametric fitting values are presented in Table 2.

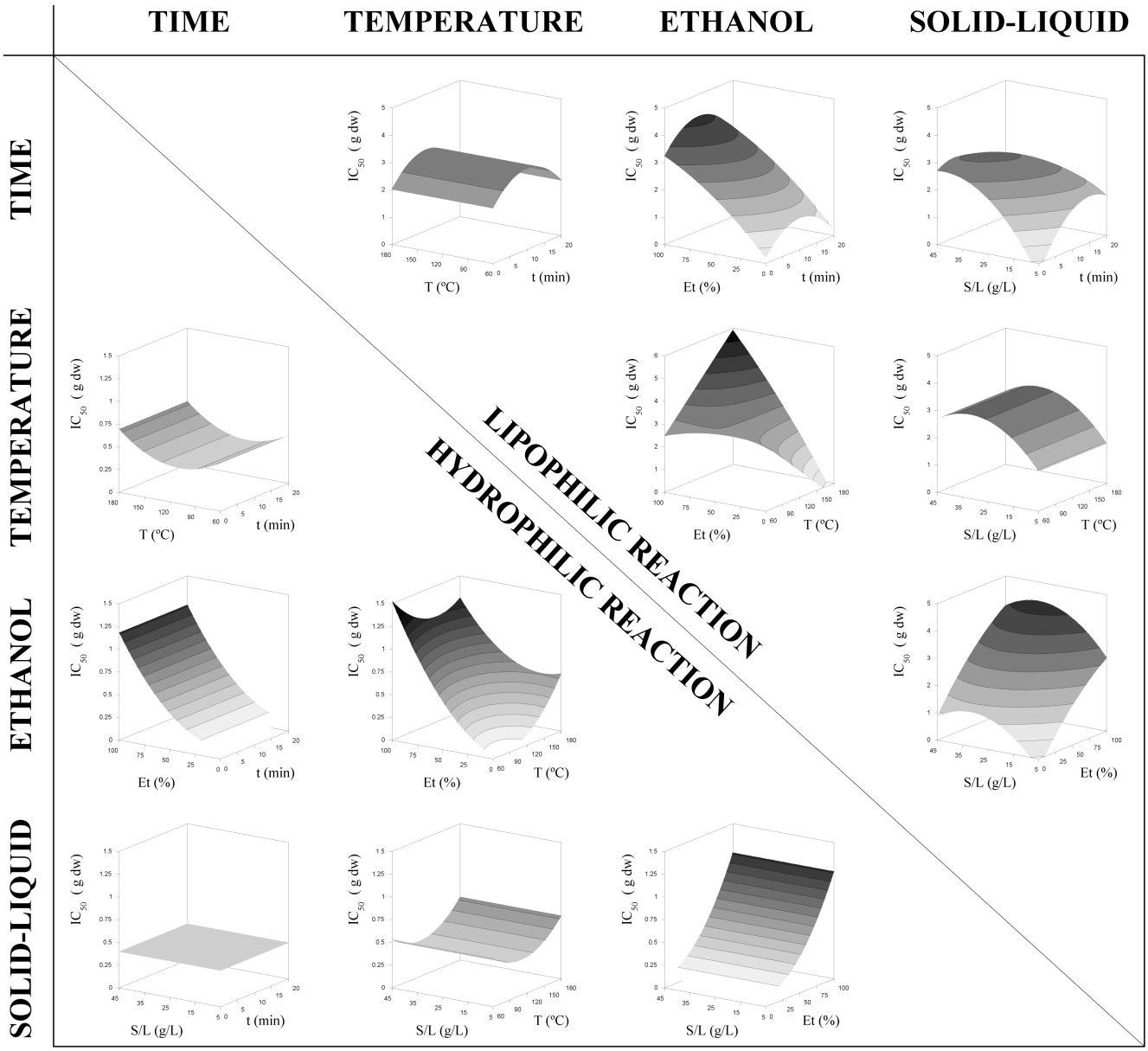

Fig. 8 - Matrix combination of the response surfaces of the *H* and *L* antioxidant reactions obtained for the parametric coefficient IC_{50} (g extract), which is organized as follows: a) in the top diagonal part is presented the response surface of the *L* reaction; and b) in the bottom diagonal part is presented the response surface of the *H* reaction. 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 obtained parametric fitting values are presented in Table 2.

Tables

Table 1 - Estimated numerical values of the parameters (P_m, V_m and IC_{50}) of Eqs. (3) and (4), after fitting the concentration-response values presented in the right-hand side of Fig. 3 and Fig. 4 (for CM and βCM, respectively) of the tomato extracts obtained under the experimental RSM design presented in Fig. 2.

RUN	EXPERIMENTAL DOMAIN				PARAMETRIC ANTIOXIDANT RESPONSES								STATISTICS	
					CM (HYDROPHILIC REACTION)				βCM (LIPOPHILIC REACTION)				R^2_{adj}	
	X_1: *t*	X_2: *T*	X_3: *Et*	X_4: *S/L*	P_m	IC_{50}	*a*	v_m	P_m	IC_{50}	*a*	v_m	*CM*	*βCM*
	min	*°C*	*%*	*g/L*	*% μM Cr*	*g extract*	--	*% μM Cr/g extract*	*% μM βC*	*g extract*	--	*% μM βC/g extract*		
1	-1(5)	-1(90)	-1(25)	-1(15)	34.08±0.47	0.17±0.01	0.84±0.14	58.24±4.75	59.64±1.52	1.88±0.17	3.06±0.32	33.69±1.39	0.9884	0.9569
2	1(15)	-1(90)	-1(25)	-1(15)	17.50±1.42	0.06±0.00	0.42±0.03	44.24±2.52	94.39±2.35	2.43±0.22	1.56±0.13	20.99±3.93	0.9567	0.9906
3	-1(5)	1(150)	-1(25)	-1(15)	26.63±1.81	0.19±0.02	1.16±0.03	56.07±0.36	36.28±1.70	1.02±0.03	2.35±0.00	28.87±4.35	0.9587	0.9727
4	1(15)	1(150)	-1(25)	-1(15)	51.12±3.09	0.51±0.04	1.18±0.16	41.33±4.78	31.79±1.05	1.41±0.04	1.25±0.04	9.79±1.25	0.9760	0.9896
5	-1(5)	-1(90)	1(75)	-1(15)	53.46±0.31	0.29±0.03	0.90±0.01	57.66±6.18	58.77±1.60	2.56±0.05	2.38±0.41	18.96±1.15	0.9744	0.9641
6	1(15)	-1(90)	1(75)	-1(15)	35.08±3.07	0.18±0.02	0.64±0.11	43.38±0.02	86.41±3.34	2.09±0.02	0.75±0.15	10.73±1.64	0.9706	0.9766
7	-1(5)	1(150)	1(75)	-1(15)	38.52±1.12	0.19±0.00	0.80±0.09	57.32±9.27	28.00±1.27	1.50±0.07	4.02±0.09	26.00±1.72	0.9549	0.9827
8	1(15)	1(150)	1(75)	-1(15)	72.90±5.31	0.59±0.01	0.98±0.09	41.77±4.87	27.31±1.19	1.50±0.10	3.81±0.23	24.01±4.50	0.9752	0.9847
9	-1(5)	-1(90)	-1(25)	1(35)	76.55±3.65	1.06±0.04	0.99±0.09	24.92±4.07	49.85±2.10	1.90±0.14	3.22±0.25	29.27±4.71	0.9726	0.9654
10	1(15)	-1(90)	-1(25)	1(35)	37.83±2.73	0.59±0.02	0.47±0.01	10.34±1.78	79.68±2.70	2.24±0.16	2.52±0.03	31.09±4.89	0.9921	0.9787
11	-1(5)	1(150)	-1(25)	1(35)	30.50±2.12	0.53±0.03	1.23±0.18	24.75±1.05	69.58±3.48	2.84±0.09	1.36±0.19	11.52±1.89	0.9828	0.9787
12	1(15)	1(150)	-1(25)	1(35)	61.51±5.82	1.18±0.12	0.53±0.09	9.55±1.57	57.48±3.64	3.44±0.06	0.98±0.07	5.70±0.25	0.9787	0.9531
13	-1(5)	-1(90)	1(75)	1(35)	78.81±2.66	0.97±0.07	0.88±0.13	24.92±4.79	33.10±2.59	3.81±0.27	2.15±0.08	6.48±0.24	0.9886	0.9919
14	1(15)	-1(90)	1(75)	1(35)	58.61±2.29	0.68±0.05	0.36±0.06	10.65±0.27	53.69±1.90	3.42±0.09	1.63±0.20	8.87±0.38	0.9677	0.9802
15	-1(5)	1(150)	1(75)	1(35)	52.40±4.11	0.67±0.04	0.91±0.02	24.62±1.73	57.62±5.40	4.76±0.10	4.54±0.18	19.03±3.35	0.9621	0.9740
16	1(15)	1(150)	1(75)	1(35)	80.03±7.95	0.82±0.01	0.44±0.01	14.79±2.66	64.42±4.82	3.65±0.14	2.65±0.26	16.21±1.22	0.9884	0.9920
17	-2(0)	0(120)	0(50)	0(25)	70.12±4.78	0.35±0.04	0.77±0.15	53.75±2.98	30.00±2.69	1.65±0.03	2.85±0.31	17.97±0.72	0.9742	0.9818
18	2(20)	0(120)	0(50)	0(25)	51.04±1.47	0.37±0.03	0.27±0.03	12.73±1.61	75.06±5.76	2.01±0.15	0.52±0.08	6.74±1.17	0.9840	0.9787
19	0(10)	-2(60)	0(50)	0(25)	32.22±2.89	0.48±0.03	0.05±0.01	1.20±0.10	63.15±4.18	2.77±0.05	4.68±0.57	36.97±5.78	0.9570	0.9858
20	0(10)	2(180)	0(50)	0(25)	34.89±0.90	0.63±0.04	0.06±0.01	1.15±0.15	49.13±3.50	2.78±0.11	6.15±0.14	37.60±1.55	0.9832	0.9728
21	0(10)	0(120)	-2(0)	0(25)	75.52±6.22	0.35±0.03	0.82±0.10	60.82±6.17	64.81±5.05	1.57±0.05	0.94±0.09	13.44±0.53	0.9586	0.9685
22	0(10)	0(120)	2(100)	0(25)	91.54±3.46	0.47±0.02	0.75±0.06	50.61±1.06	31.65±0.18	2.21±0.04	1.90±0.18	9.46±1.02	0.9710	0.9626
23	0(10)	0(120)	0(50)	-2(5)	14.31±0.87	0.08±0.01	1.67±0.29	104.79±7.95	41.18±3.07	0.84±0.08	1.44±0.03	24.35±0.89	0.9569	0.9628
24	0(10)	0(120)	0(50)	2(45)	45.20±0.57	1.11±0.03	1.82±0.33	25.84±0.25	80.94±0.64	4.13±0.51	1.23±0.04	8.37±0.10	0.9551	0.9755
25	0(10)	0(120)	0(50)	0(25)	30.39±1.56	0.38±0.04	0.91±0.02	25.43±0.89	97.33±6.29	3.03±0.31	1.11±0.18	12.35±0.56	0.9728	0.9592
26	0(10)	0(120)	0(50)	0(25)	30.39±2.04	0.38±0.02	0.91±0.00	25.43±4.30	97.33±8.12	3.13±0.11	1.26±0.11	13.63±1.08	0.9585	0.9870
27	0(10)	0(120)	0(50)	0(25)	30.39±0.50	0.38±0.04	0.91±0.01	25.43±2.09	97.33±2.22	3.05±0.16	1.14±0.14	12.58±1.01	0.9700	0.9843
28	0(10)	0(120)	0(50)	0(25)	30.39±1.15	0.38±0.03	0.91±0.03	25.43±0.14	97.33±4.47	3.05±0.16	1.12±0.01	12.34±0.38	0.9637	0.9834
29	0(10)	0(120)	0(50)	0(25)	30.39±1.50	0.38±0.01	0.91±0.08	25.43±0.27	97.33±0.94	3.04±0.28	1.10±0.15	12.21±1.68	0.9725	0.9575
30	0(10)	0(120)	0(50)	0(25)	30.39±2.12	0.38±0.04	0.91±0.11	25.43±5.02	97.33±3.58	2.80±0.28	1.08±0.04	13.03±0.14	0.9888	0.9782
31	0(10)	0(120)	0(50)	0(25)	30.39±1.83	0.38±0.02	0.91±0.00	25.43±2.82	87.46±3.60	2.66±0.30	1.43±0.17	16.30±2.64	0.9886	0.9823
32	0(10)	0(120)	0(50)	0(25)	30.39±0.57	0.38±0.01	0.91±0.13	25.43±1.54	97.33±8.41	3.00±0.18	1.16±0.19	13.02±2.59	0.9827	0.9906

Table 2 - Estimated coefficient values of Eq. (8), parametric intervals and numerical statistical criteria for each parametric response criteria of the *H* and *L* reactions.

		HYDROPHILIC REACTION			**LIPOPHILIC REACTION**		
		P_m	IC_{50}	V_m	P_m	IC_{50}	V_m
Fitting coefficients obtained from Eq. (8) and showed in Eqs. (9)-(14)							
Intercept	b_0	30.50±2.74	0.40±0.05	26.49±3.10	96.10±0.05	2.97±0.37	13.09±0.01
Linear effect	b_1	ns	ns	-8.10±1.79	8.02±0.05	ns	-2.87±0.01
	b_2	ns	0.04±0.04	ns	-7.13±0.03	ns	ns
	b_3	8.70±2.09	0.27±0.04	-17.22±1.79	5.10±0.03	0.76±0.17	-3.20±0.01
	b_4	6.92±2.09	ns	ns	-5.65±0.04	0.31±0.11	-2.02±0.01
Quadratic effect	b_{11}	7.29±1.87	ns	ns	-10.72±0.07	-0.24±0.10	ns
	b_{22}	ns	0.05±0.03	-6.96±1.79	-9.82±0.03	ns	5.94±0.01
	b_{33}	ns	0.06±0.03	9.08±1.79	-8.59±0.06	-0.07±0.03	ns
	b_{44}	13.02±1.87	ns	6.68±1.61	-11.80±0.08	-0.22±0.10	ns
Interactive effect	b_{12}	13.21±2.56	0.16±0.05	ns	-7.70±0.03	ns	ns
	b_{13}	ns	ns	ns	ns	ns	2.35±0.02
	b_{14}	ns	ns	ns	ns	-0.24±0.02	1.57±0.02
	b_{23}	-4.78±2.56	-0.05±0.05	ns	13.04±0.02	0.43±0.12	-1.72±0.02
	b_{24}	ns	ns	ns	ns	ns	6.21±0.02
	b_{34}	ns	ns	ns	ns	0.27±0.07	ns
Statistical information of the fitting analysis							
Observations		32	32	32	32	32	32
R^2		0.9526	0.9236	0.9743	0.9422	0.9437	0.9223
R^2adj		0.9331	0.9136	0.9612	0.9067	0.9058	0.9019
MSE		767.23	0.15	854.45	1127.20	1.50	152.70
RMSE		27.70	0.38	29.23	33.57	1.22	12.35
MAPE		5.99	12.63	22.22	8.14	7.97	9.72
DW		2.39	2.20	1.43	2.32	1.36	2.32

ns: no significant coefficient; **R^2**: Correlation coefficient; **R^2adj**: The adjusted coefficient of determination 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 3 - Operating conditions that maximize the extraction of *H* and *L* antioxidants from tomato and optimal response values for the parametric response criteria (P_m, V_m and IC_{50}) and antioxidant reactions (*H* or *L*). The independent variables *t*, *T*, *Et* and *S/L* are presented in natural values.

	OPTIMAL EXTRACTION CONDITIONS				**RESPONSE OPTIMUM**
	X_1: *t (min)*	X_2: *T (ºC)*	X_3: *Et (%)*	X_4: *S/L (g/L)*	
For H antioxidants					
P_m (*H*)	18.7	180.0	56.8	45.0	100 *% μM Cr*
V_m (*H*)	2.5	120.0	0.0	5.0	136.11 *% μM Cr/g extract*
IC_{50} (*H*)	14.5	90.0	44.0	17.0	0.051 *g extract*
For L antioxidants					
P_m (*L*)	13.4	93.6	44.0	21.3	100 *% μM βC*
V_m (*L*)	2.2	180.0	100.0	5.0	78.70 *% μM βC/g extract*
IC_{50} (*L*)	2.6	169.1	91.7	10.9	0.025 *g extract*
For each response criteria of both H and L antioxidants					
P_m (*H*)	15.4	127.6	93.2	33.8	100.0 *% μM Cr*
P_m (*L*)					43.4 *% μM βC*
V_m (*H*)	3.9	63.3	0.0	5.0	108.93 *% μM Cr/g extract*
V_m (*L*)					74.79 *% μM βC/g extract*
IC_{50} (*H*)	13.9	112.7	89.0	5.3	0.06 *g extract*
IC_{50} (*L*)					0.05 *g extract*
For H and L antioxidants based on all the response criteria					
P_m (*H*)					100 *% μM Cr*
V_m (*H*)	2.25	149.2	99.1	45.0	60.2 *% μM Cr/g extract*
IC_{50} (*H*)					0.09 *g extract*
P_m (*L*)					92.4 *% μM βC*
V_m (*L*)	15.4	60.0	33.0	15.0	42.9 *% μM βC/g extract*
IC_{50} (*L*)					0.38 *g extract*
For both H and L antioxidants based on all the response criteria					
P_m (*H*)					100.0 *% μM Cr*
P_m (*L*)					39.1 *% μM βC*
V_m (*H*)	12.1	122.3	100.0	27.2	46.39 *% μM Cr/g extract*
V_m (*L*)					9.64 *% μM βC/g extract*
IC_{50} (*H*)					0.47 *g extract*
IC_{50} (*L*)					0.47 *g extract*