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Optimization of Phenolics and Flavonoids Extraction Conditions and Antioxidant Activity of Roasted Yerba-Mate Leaves (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) using Response Surface Methodology

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ABSTRACT

This study focused on maximizing the extraction of total phenolics and flavonoids as well as the antioxidant activity measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay from roasted yerba mate (*Ilex paraguariensis*) as a function of time (5, 7.5 and 10 min) and temperature of extraction (60, 75 and 90°C). The data were subjected to Response Surface Methodology and the results showed that polynomial equations were significant, did not present lack of fit, and presented adjusted determination coefficients above 98%, proving their suitability for prediction purposes. Using the desirability function, the optimum operating conditions to obtain a higher extraction of antioxidants was found to be 10 min of extraction at 90°C, and the tea prepared under these experimental conditions presented 427.74 mg of gallic acid equivalents per liter and 80.02% of inhibition of the DPPH radical. The flavonoid content was highly correlated ($r = 0.9046$, $p < 0.001$) to the antioxidant capacity.

Key words: Phenolic composition, correlation analysis, desirability function, phytochemicals, functional foods.

INTRODUCTION

Ilex paraguariensis A. St.-Hil. Var. *paraguariensis* (Aquifoliaceae) tea is widely consumed in South America, especially in Brazil, Argentina and Paraguay. The tea made from the leaves has several phytochemicals such as caffeic acid, chlorogenic acids, 3,4-dicaffeoylquinic acid, 3,5-dicaffeoylquinic acid, and 4,5-dicaffeoylquinic acid, catechins, amino acids, caffeine, quercetin, kaempferol, ascorbic acid, B₁/B₂ vitamins, and rutin (Carini et al. 1998, Dutra

et al. 2010, Berté et al. 2011). All these compounds (solely or their interactions) are responsible for the alleged physiological effects of yerba-mate aqueous extract (tea) (Bracesco et al. 2011).

Several *in vitro*, *ex vivo*, *in vivo* (using animals) and epidemiological protocols have indicated high antioxidant (Schinella et al. 2009, Reta et al. 2012), antitumor (Mejía et al. 2010, Puangraphant et al. 2011), and anti-inflammatory (Arçari et al. 2011a) effects of aqueous extracts of yerba mate, such as the commercial ready-to-drink beverages available in the marketplace in different countries, including

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the inhibition of high-density lipoprotein (HDL) oxidation induced by peroxy radicals (Menini et al. 2007), inhibitory ability against low-density lipoprotein (LDL) oxidation (Bracesco et al. 2003), increase of gene expression of some antioxidant enzymes found in many organs (Matsumoto et al. 2009), decrease in the serum concentration of LDL-cholesterol with a respective increase of the serum antioxidant capacity and glutathione content (Arçari et al. 2011b).

Brazilians consumed more than 3 tons of dried tea leaves between 2009 and 2011, and roasted yerba-mate accounted for 70% of this total. The main reasons for the high consumption of such a tea are its sensory properties and tradition (Euromonitor International 2012). Beverage companies aim at delivering a required and demanded by consumers, and in this regard, consumers have become aware of the direct relationship between the regular consumption of foods rich in phenolic compounds, such as green tea or red wine, and the decreased risk of a range of cardiovascular and neurodegenerative diseases. In order to obtain a yerba-mate tea with optimized chemical composition as extracted by water, the most suitable statistical tool employed to maximize the compounds with the highest biological activity is the Response Surface Methodology (RSM). In accordance with Bas and Boyaci (2007), Granato et al. (2010a), Cruz et al. (2010) and Peña et al. (2014), RSM is a powerful mathematical technique based on regression analysis used to develop and improve (optimize) products and processes that have two or more factors that influence the response. RSM has been widely used by food companies to develop food products as well as to analyze, model and optimize processes (Granato et al. 2010b, Liu et al. 2012, Delgado et al. 2012, Ellendersen et al. 2012).

Food companies employ many technological operations and formulations to prepare the yerba-mate leaves, which create intrinsic differences in

the chemical composition and pharmacological activities of commercial teas and, therefore, there is not a global process optimized by means of statistical techniques employed to extract phenolic compounds that display antioxidant activity from roasted yerba-mate. Consumers do not have access to neither chemical composition nor antioxidant capacity assays but they do have access to kitchen thermometers and chronometers, thus it would be interesting to investigate the effects of temperature and extraction time on the chemical composition and on the *in vitro* antioxidant activity of yerba-mate teas. In fact, no study was found regarding the response surface optimization of phenolic compounds and antioxidant activity of roasted yerba-mate teas. Based on these considerations, this study aimed at optimizing the experimental extraction conditions to obtain a tea made from roasted yerba-mate leaves (*Ilex paraguariensis*) that presents a high content of total phenolic compounds, flavonoids and *in vitro* free-radical scavenging activity.

MATERIALS AND METHODS

CHEMICALS

Folin–Ciocalteu reagent and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and catechin, gallic acid, methanol, sodium nitrite, aluminum chloride, sodium hydroxide, and sodium carbonate were of analytical grade.

EXPERIMENTAL DESIGN AND TEA PREPARATION

In order to assess the effect of temperature (60, 75 or 90°C) and extraction time (5, 7.5 or 10 min) on the extraction of total phenolic compounds, flavonoids and on the antioxidant activity towards DPPH radical, a full factorial design (3^2) was applied and two replicates in the center point were added to the experiment to fit the surface plot for the responses and to estimate the pure error of the multiple regression models (Myers et al. 2009),

totaling 11 tea preparations. The ranges of time and temperature of extraction adopted in this work were based on a preliminary study.

Grounded roasted yerba mate leaves (brand Leão Junior[®], place of production: Paraná, Brazil; species: *Ilex paraguariensis* A.St.-Hil., Aquifoliaceae) were acquired in the commerce of São Paulo, Brazil. A detailed quality control report about the botanical authenticity of the plant material was performed by the company. In order to prepare each tea sample (Table I), a total of 2.0 grams of the plant material

was extracted with 100 mL of distilled water (ratio 1:50 of plant material:solvent) in a 500 mL glass beaker. Firstly, the water was heated up to each temperature value and added to a flask containing the plant material. The temperature inside the flask was regulated with a thermostatic bath with temperature control and the extraction procedure was carried out under magnetic stirring. The yerba mate and the water were thermostabilized at the specific temperatures, and the total extraction time was in accordance with the experimental design (Table I). The initial time

TABLE I
Coded and real values of temperature and extraction time of roasted yerba-mate leaves and results from the chemical and antioxidant assays.

Samples	Coded values		Real values		Response variables		
	Extraction time	Temperature	Extraction time (min)	Temperature (°C)	Total phenolic compounds ¹	Total flavonoids ²	Antioxidant activity ³
1	-1	-1	5	60	349.92d	280.93c	52.56d
2	-1	0	5	75	394.44c	280.93c	59.36c
3	-1	1	5	90	428.31a	421.75a	80.65a
4	0	-1	7.5	60	352.39d	242.00d	52.38d
5	0	1	7.5	90	416.89b	324.54b	63.38b
6	1	-1	10	60	385.50c	268.06c	54.58d
7	1	0	10	75	417.84b	318.10b	62.14bc
8	1	1	10	90	427.74a	419.65a	80.02a
9	0	0	7.5	75	420.13ab	318.10b	53.61d
10	0	0	7.5	75	417.84b	317.38b	55.11d
11	0	0	7.5	75	416.89b	320.96b	54.31d
Pooled standard deviation (PSD)					27.99	63.14	10.18
P-value for homogeneity of variances (Hartley's test)					0.71	0.19	0.83
P-value (one-way ANOVA)					< 0.001	< 0.001	< 0.001

Note: Data presented as mean (n = 3) ± PSD; ¹Expressed as mg GAE/L; ²Expressed as mg CTE/L; ³Expressed as % of inhibition of the DPPH radical. Different letters in the column represent statistical different results (p < 0.05) in accordance with the Fisher LSD *post-hoc* test.

was set when yerba mate was added to the water. The mixture was then filtered and immediately put into Falcon tubes and frozen at -18°C until further analysis.

DPPH FREE-RADICAL SCAVENGING ACTIVITY ASSAY

The free-radical scavenging activity of tea samples towards the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was determined in triplicate using the method proposed by Brand-Williams et al. (1995), with

minor modifications. Briefly, a 100-μL aliquot of yerba mate tea (diluted seven times in distilled water) was mixed with 3.9 mL of a methanolic DPPH solution (125 μmol/L). The mixture was left to react in the dark for 30 min at 25°C, and then the absorbance was read at a wavelength of 517 nm using a spectrophotometer (Model 432, Femto Ltda, São Paulo, Brazil). Methanol was used as the negative control (blank). The DPPH free radical scavenging activity of each tea sample was calculated using Equation 1:

$$\text{Antioxidant activity towards DPPH radical (\%)} = [1 - (A_{\text{sample}}/A_{\text{blank}})] \times 100 \quad (1)$$

DETERMINATION OF TOTAL PHENOLIC CONTENT

The total phenolic compound content of each tea sample (diluted three times in distilled water) was determined in triplicate using the Folin-Ciocalteu method (Singleton and Rossi 1965). The absorbance was measured using a spectrophotometer (Model 432, Femto Ltda, São Paulo, Brazil) at the wavelength of 725 nm. The total phenolic content was determined by a standard curve (total phenolic concentration = 126.85 x absorbance; $r = 0.9869$; $p < 0.001$) of gallic acid (0 to 165 mg/L), and the results were expressed as mg of gallic acid equivalents per liter of tea (mg GAE/L).

DETERMINATION OF TOTAL FLAVONOID CONTENT

The total flavonoid content of the teas (diluted three times in distilled water) was determined using a modified colorimetric method employing aluminum chloride (Jia et al. 1999). The absorbance was measured at a 510 nm wavelength against a prepared blank (water) using a spectrophotometer (Model 432, Femto Ltda, São Paulo, Brazil). The flavonoid content was determined using a standard curve of catechin (0–250 mg/L) and the results, determined from a regression equation (flavonoid concentration = $476.55 \times \text{absorbance}$; $r = 0.9996$; $p < 0.001$), were expressed as milligrams of catechin equivalents per liter of tea (mg CTE/L). Data presented are the averages of three independent measurements.

STATISTICAL ANALYSES

Data were presented as mean \pm pooled standard deviation. RSM is applied when at least a pair of samples differ ($p < 0.05$) from each other, that is, when the p -value from ANOVA is below 0.05. In this sense, differences among samples were checked by using one-way analysis of variance (ANOVA) followed by Fisher Least Significant Difference (LSD) *post-hoc* test (Granato et al. 2014). Prior to this analysis, the homogeneity of variances and

normality of each response variable were confirmed by using the Hartley and the Shapiro-Wilk tests, respectively (Granato et al. 2011a). P -values below 0.05 were regarded as significant. The quality of the mathematical models fitted by RSM was evaluated by ANOVA, based on the F -test and on the percentage of total explained variance (R^2) and also on the adjusted determination coefficient (R^2_{adj}), which provide a measurement of how much of the variability in the observed response values could be explained by the experimental factors and their linear and quadratic interactions (Granato et al. 2010b). A second-order polynomial quadratic equation (Equation 2) was used to fit the results:

$$Y = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{i < j} \beta_{ij} x_i x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \varepsilon \quad (2)$$

where: Y is the predicted response, β_0 is a constant, β_j , β_{ij} and β_{jj} are the regression coefficients and x_j and x_i are the levels of the independent variables (temperature and extraction time). Experimental data were then fitted to the selected regression model to achieve a proper understanding of the correlation between each factor and different responses. This was obtained by estimating the numerical values of the model terms (regression coefficients), whose significance was statistically judged in accordance with the t -statistic at a confidence interval of 95%. Non-significant ($p > 0.05$) terms were deleted from the initial equation and data were refitted to the selected model. The goodness-of-fit of the regression model was evaluated by the correlation coefficient (r), the coefficient of determination (R^2), and also by the probability value (p -value) of the regression and lack-of-fit. To visualize the effects of temperature and extraction time and the relationships between the factors and the response variables, 2D contour plots of the fitted regression equations were generated using the Statistica v. 7 software (Statsoft, USA). A simultaneous optimization using the desirability

function was performed in order to maximize the total phenolic compounds, the flavonoid content and also to maximize the antioxidant capacity of teas towards the DPPH radical (Derringer and Suich 1980).

In order to assess the correlation between response variables, the mean values of each tea preparation were initially checked for normal distribution by using the Shapiro-Wilk test. Secondly, the Pearson's (parametric data) and Spearman's rank (non-parametric data) coefficients were calculated for each pairwise of variables and the p-value for each coefficient was provided.

RESULTS AND DISCUSSION

The total phenolic compounds of yerba mate tea preparations varied from 349.92 to 428.31 mg

GAE/L and the total flavonoid content ranged from 268.06 to 421.75 mg CTE/L of tea, corroborating the results obtained in previous studies for yerba mate and other types of teas (Seeram et al. 2008, Kodama et al. 2010, Reta et al. 2012, Zielinski et al. in press). The DPPH free radical scavenging activity was relatively high and values ranged from 52.38 to 80.65% of inhibition of the radical (Table I).

The experimental data, for all response variables, were homoscedastic ($p \geq 0.19$) and there were significant ($p < 0.001$) differences among samples when the one-factor analysis of variances was employed, which is a basic requirement for RSM application. Response surface plots are presented in Figure 1.

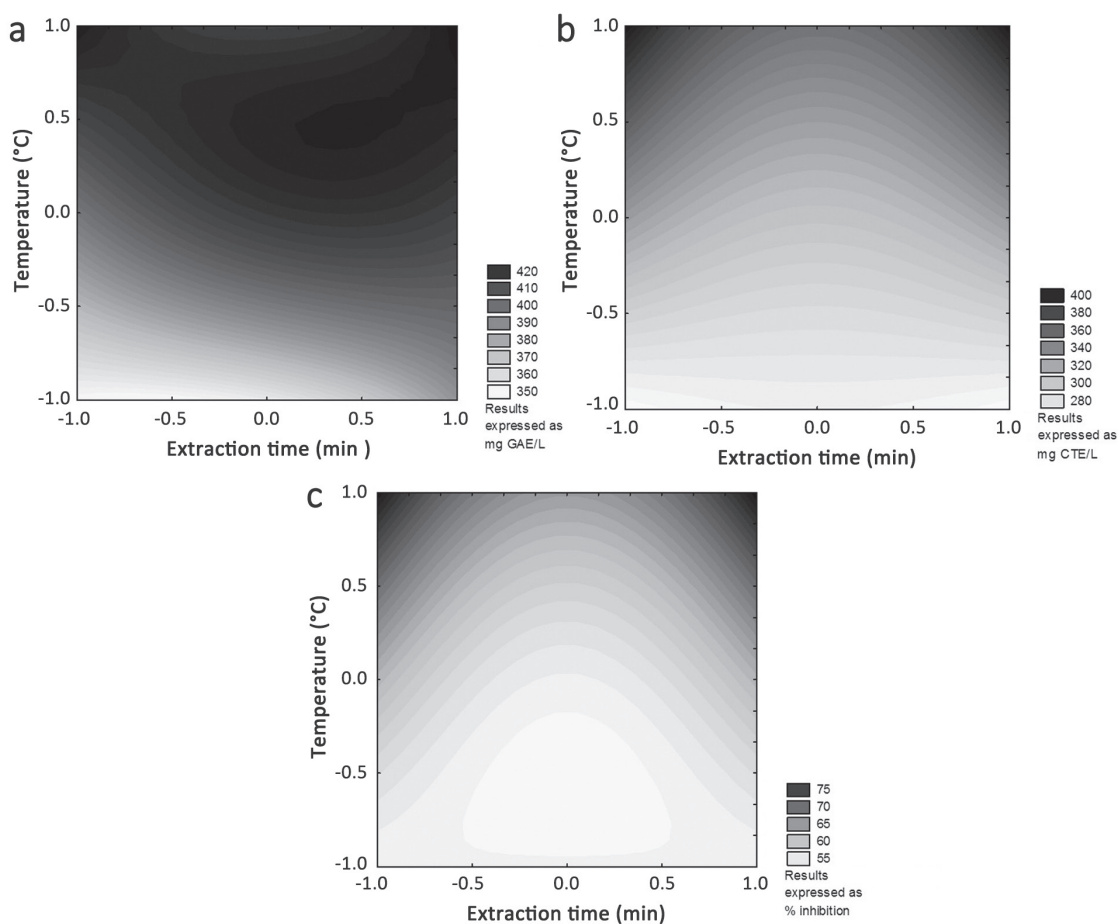


Figure 1 - Response surface plots to show the effect of extraction time (min) and temperature (°C) on the total content of phenolic compounds (A), flavonoids (B), and antioxidant activity towards DPPH (C) of roasted yerba mate leaves.

The linear correlation analysis showed that the total phenolic compounds correlated well with the content of total flavonoids ($r = 0.7596$, $p < 0.01$, $n = 11$), while the antioxidant activity of yerba mate tea infusions was significantly correlated to the content of total flavonoids, according to the linear regression analysis (Figure 2). Likewise, Grujic et al. (2012) verified a correlation between the DPPH free radical scavenging activity and the total

phenolic compounds ($r = 0.85$) and the flavonoid content ($r = 0.74$). The antioxidant activity flavonoids is due to the number and acidity of their phenolic hydroxyl groups and to the resonance between the free electron pair on the phenolic oxygen and the benzene ring, which increases electron delocalization, conferring a nucleophilic character upon the substitution position adjacent to the hydroxyl group (Cheynier 2006).

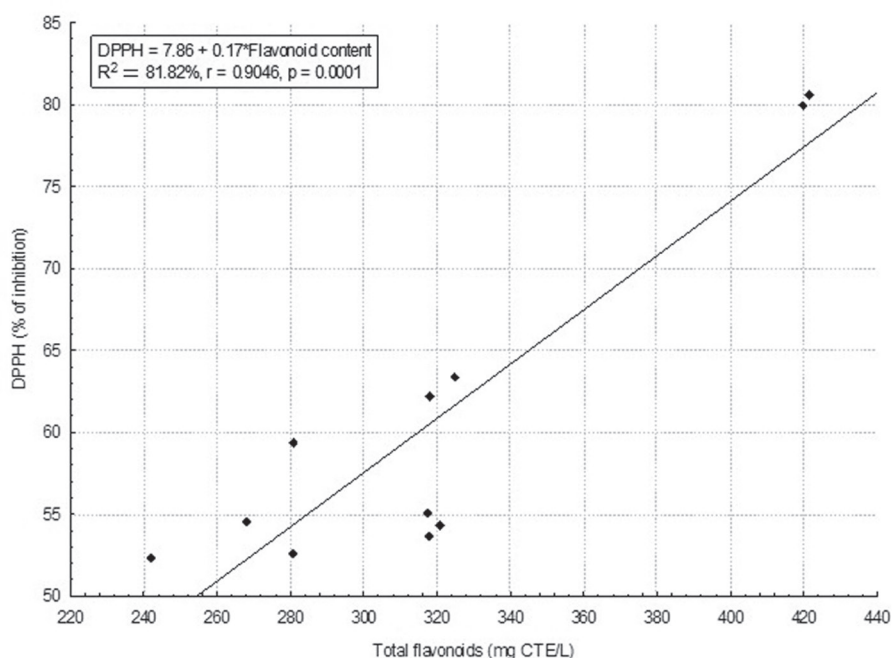


Figure 2 - Linear Regression Analysis between the total flavonoid content and the antioxidant capacity towards the DPPH radical.

The total content of phenolic compounds was significantly correlated with the free-radical scavenging activity towards DPPH radical ($\rho = 0.6895$, $p = 0.02$; $n = 11$). These results are in accordance with those reported elsewhere (Cao et al. 2007, Hartwig et al. 2012). As also outlined by Cheynier (2006), Cao et al. (2007) and Granato et al. (2011b) it is widely accepted that the antioxidant capacity measured by various *in vitro* methods depends on several intrinsic factors and experimental conditions: quantity and interactions among phenolic compounds present in the test material, the concentration and chemical type of the free radical, the time employed in the

assay, the dilution of the sample, pH, solubility, stereochemical structure, among others.

According to the data shown in Table II, it is possible to observe that the regression models proposed for the responses were highly significant ($p < 0.0001$). Indeed, no lack-of-fit ($p \geq 0.105$) was found and both R^2 and R^2_{adj} were higher than 98%, indicating that the empirical models fit the experimental data satisfactorily and may be used for prediction purposes. For the total flavonoid content ($R^2 = 99.89$, $R^2_{adj} = 99.63$, $p < 0.0001$) and phenolic compounds ($R^2 = 99.72$, $R^2_{adj} = 99.29$, $p < 0.0001$), the quadratic and linear effects of

extraction time and temperature as well as the linear and quadratic effects of the interaction between extraction time and temperature were significant ($p < 0.05$). For the DPPH model ($R^2 = 98.91$, $R^2_{adj} = 98.19$, $p < 0.0001$), the linear and quadratic effects

of temperature as well as the quadratic effect of extraction time and the interaction between the linear effect of temperature and the quadratic effect of extraction time were deemed significant to represent the experimental data.

TABLE II
Mathematical equations that describe the response variables (phenolics, flavonoids and DPPH) in response to temperature and extraction time of roasted yerba-mate leaves.

Response variables	Regression equation	R^2 (%)	R^2_{adj} (%)	p-value (regression)	p-value (lack-of-fit)
Total phenolic compounds (mg GAE/L)	$Y = 418.29 + 9.74ET - 12.15ET^2 + 30.86T - 33.65T^2 - 9.04ET*T + 25.37ET^2*T^2$	99.72	99.29	< 0.0001	0.242
Total flavonoids (mg CTE/L)	$Y = 318.81 + 18.58ET + 41.27T - 19.30ET^2 - 35.54T^2 - 22.33ET*T^2 + 31.83ET^2*T + 83.63ET^2*T^2$	99.89	99.63	< 0.0001	0.105
Antioxidant activity (% inhibition of the DPPH radical)	$Y = 53.78 + 5.50T + 7.81ET^2 + 4.94T^2 + 7.88ET^2*T$	98.91	98.19	< 0.0001	0.183

Note: ET = Extraction time (min); T = Temperature (°C).

In practice, high values (> 70%) of determination coefficients (R^2) are reasonable indicators of suitability of regression models to describe the influence of the independent variables (extraction time and temperature) on the dependent variables (flavonoids, phenolic compounds and DPPH) (Bas and Boyaci 2007). It is possible to observe that the modeling of experimental data allowed for the generation of useful mathematical equations for general use, within the experimental range tested in this study, to predict the behavior of the system under different factor combinations. Indeed, food companies that want to develop mate teas from roasted leaves with a functional appeal (high antioxidants content or high concentration of phenolic compounds, for example) may use the approach employed in the study to test new ranges of extraction time and temperature or even other technological conditions to enhance the extraction of antioxidants from yerba mate leaves.

It is noteworthy that the modeling of the *in vitro* antioxidant activity of roasted yerba-mate teas is an important result seeing that food companies could use this statistical approach as the basis for

developing products with a functional claim based on the antioxidant activity measured by *in vitro* protocols. Herein, the development of functional beverages with high content of phenolic compounds and antioxidant activity has been widely studied (Owczarek et al. 2004, Mello et al. 2009, Soccol et al. 2012) and the industry could benefit from these studies, that is, new functional food products, especially bioactive beverages, could be developed.

Another aspect that should also be mentioned here is the fact that many studies are conducted to improve (not optimize) the extraction conditions of flavonoids and phenolic compounds from medicinal herbs that exert antioxidant activity by using the one-at-a-time approach, that is, only one variable (for example: time, temperature, agitation, etc) is tested to render a higher content of phenolic compounds/antioxidant activity (Nishiyama et al. 2010). This method seems to be attractive but does not depict the interaction between/among factors that influence the responses, leading to non-realistic optimization conditions. In this regard, RSM is considered to be the best statistical approach to assess the influence of different experimental

factors and their linear and quadratic interactions on analytical responses and also to model and optimize the extraction conditions of phenolics that display important biological activities (Li et al. 2010, Chen et al. 2012).

Nishiyama et al. (2010) used the 'one-at-a-time' approach to assess the effects of the infusion time, type of packaging of the herb (in bulk or in sachets) and brewing condition on the extraction of bioactive phenolic compounds from Brazilian green tea and authors verified that the use of the herb in bulk and 5 min of infusion was the most favorable condition for the extraction of bioactive compounds. On the other hand, Li et al. (2010) used RSM to model the extraction of kaempferol from tea seed cake as a function of extraction time, temperature, pressure and ethanol content. The authors verified that the best combination of those factors to maximize the

extraction of kaempferol was an extraction time of 150 min, pressure of 20 MPa, temperature of 80°C, and 60% aqueous ethanol solution. Likewise, Chen et al. (2012) used a Box–Behnken design followed by RSM analysis to maximize the extraction of a tea carbohydrate that presents a considerable antitumor activity. For this purpose, authors employed temperature, extraction time and solvent–solid ratio as independent variables and verified that the best experimental conditions to obtain an optimized extraction of carbohydrates were an extraction temperature of 90°C, extraction time 30 min, and solvent–solid ratio 5:1.

The final result for the simultaneous optimization using the desirability function (desirability index of 0.9279) suggested that the tea made from yerba-mate leaves extracted at 90°C for 10 min was the most adequate experimental conditions to

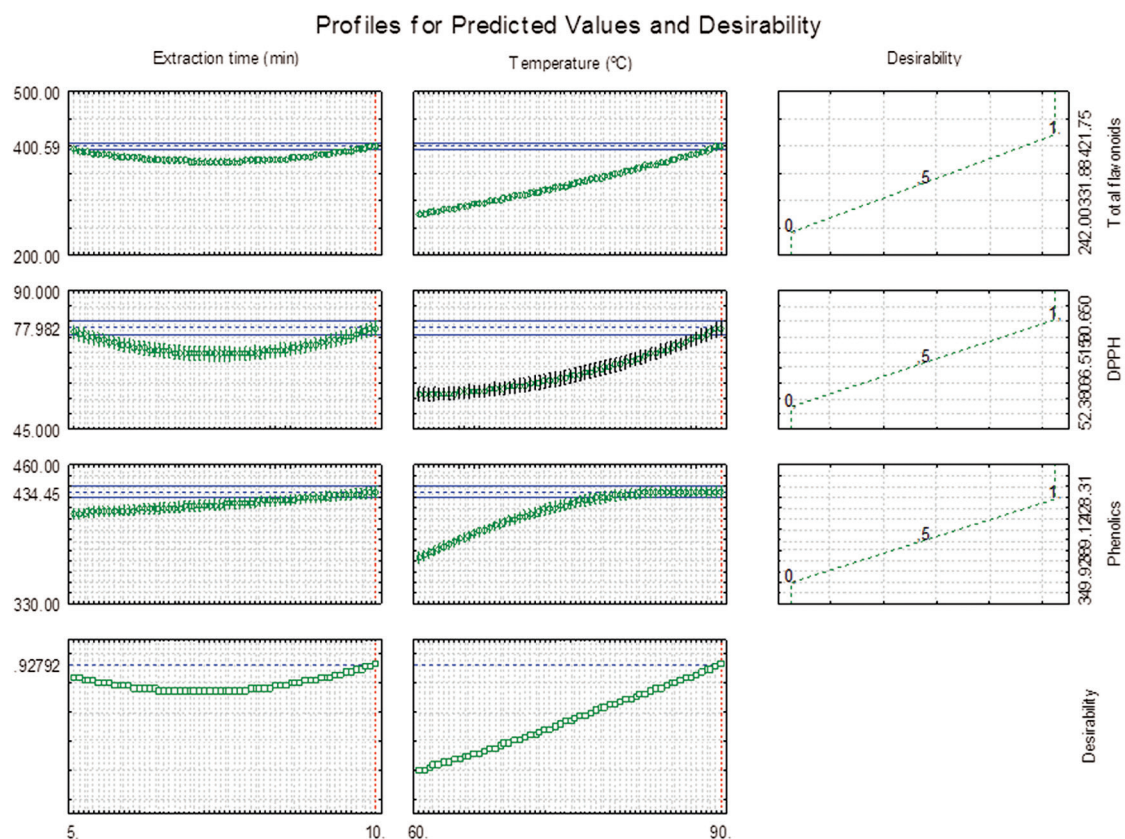


Figure 3 - Optimization of bioactive compounds from roasted yerba mate leaves using the desirability function (Derringer and Suich approach).

achieve the best solution for this combination of variables (Figure 3). Indeed, this result corroborates the findings from the initial experimental data, where tea sample #8 (90°C, 10 min of extraction) presented the highest content of phenolics, flavonoids and also antioxidant capacity.

CONCLUSIONS

Response Surface Methodology was used to model and optimize the experimental conditions to extract phenolic compounds (including flavonoids) from roasted yerba-mate leaves that exert antioxidant activity towards DPPH radical. The best combination of extraction time and temperature were found to be 10 minutes at 90°C, which rendered a mean phenolic content of 427.74 mg GAE/L and 80.02% of inhibition of the DPPH radical. The total flavonoid content correlated closely to the antioxidant capacity, corroborating the fact that this phenolic class is responsible for the beneficial health effects of yerba-mate tea consumption. RSM proved to be effective in optimizing the extraction conditions of bioactive phenolic compounds from roasted *I. paraguariensis* leaves and results may be used to have an idea about the antioxidant activity and content of total phenolic compounds and flavonoids when different combination of extraction time and temperature are used to prepare teas.

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RESUMO

Este trabalho objetivou maximizar a extração de fenólicos e flavonoides totais bem como a atividade antioxidante de erva-mate tostada (*Ilex paraguariensis*) frente ao radical livre 1,1-difenil-2-picrilhidrazila (DPPH) em função do tempo (5, 7,5 e 10 min) e a temperatura de extração (60, 75 e 90°C). Os dados

foram submetidos à metodologia de superfície de resposta e os resultados mostraram que as equações polinomiais foram significativas, não apresentaram falta de ajuste e obtiveram coeficientes de determinação ajustados acima de 98%, comprovando a sua adequação para fins preditivos. Usando a função de desejabilidade, as condições ótimas para maximizar a extração de compostos antioxidantes foram de 10 min de extração a 90°C, e o chá preparado sob estas condições experimentais apresentou 427,74 mg de equivalentes de ácido gálico por litro e 80,02% de inibição da o radical DPPH. O teor de flavonoides foi altamente correlacionado ($r = 0,9046$, $p < 0,001$) com a atividade antioxidante.

Palavras-chave: Composição fenólica, análise de correlação, função de desejabilidade, fitoquímicos, alimentos funcionais.

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