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ORIGINAL ARTICLE

Enzyme-assisted extraction of polyphenols from green yerba mate

Extração de polifenóis de erva-mate verde assistida por enzimas

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Abstract

The enzyme-assisted extraction of bioactive compounds from plants has been studied as an alternative green technology and the carbohydrases have been candidates to improve the extraction process of numerous such compounds from plants. Polyphenols are secondary plant metabolites, generally involved in the defense against different types of stress and yerba mate (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) is a natural source of these antioxidant compounds. The aim of this work was to evaluate the enzyme-assisted extraction of polyphenols from green yerba mate employing response surface methodology (RSM), in order to determine the best extraction conditions. The independent variables were temperature (33.2 to 66.8 °C), enzyme concentration (0 to 336 FGBU/100g), reaction time (19 to 221 minutes) and pH (2.82 to 6.18). The use of carbohydrases increased the extraction of polyphenols from about 38.67% to 52.08%. The present results showed that all the independent variables were significant at the linear level and that temperature and pH were not significant at the quadratic level. The interactions of temperature and pH; enzyme and reaction time; and enzyme and pH were significant. The regression model presented a determination coefficient (R^2) close to 0.85 and a fitted value close to 0.45. Considering the results of this study and their industrial viability, the best conditions for the extraction of polyphenols from green yerba mate are a temperature of 50.0 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH value of 4.50. This study was the first RSM-based report of the optimization of the enzyme-assisted extraction of total phenolic compounds from green yerba mate.

Keywords: Carbohydrases; Beta-glucanase; Polyphenols; Bioactive compounds; *Ilex paraguariensis*; Response surface methodology.

Resumo

A extração de bioativos de plantas assistida por enzimas tem sido estudada como uma tecnologia verde alternativa. As carboidrases foram candidatas para melhorar o processo de extração de numerosos compostos bioativos de plantas. Os polifenóis são metabólitos secundários de plantas geralmente envolvidos em defesa contra diferentes tipos de estresse. A erva-mate (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) é uma fonte natural desses compostos antioxidantes. O objetivo deste trabalho foi avaliar a extração de polifenóis auxiliada por enzimas para determinar as melhores condições para o uso, empregando metodologia de superfície de resposta (RSM). As variáveis independentes foram: temperatura (33,2 to 66,8°C), concentração enzimática (0 to 336 FGB / 100 g), tempo de reação (19 to 221 minutos) e pH (2,82 to 6,18). O uso de carboidrase aumentou a extração de polifenóis de 38,67% para 52,08%. Os resultados mostraram que todas as variáveis independentes foram significativas a nível linear e a temperatura e o pH não se mostraram significativos nos níveis quadráticos. As interações de temperatura e pH, tempo de reação e concentração da enzima e pH e concentração da enzima foram significativas. O modelo de regressão apresentou coeficiente de determinação (R^2) próximo de 0,85 e ajustado perto de 0,45. Considerando-se os resultados deste estudo e a viabilidade industrial, as melhores condições para a obtenção de polifenóis de erva-mate são temperatura de 50,0°C, concentração de enzima 168 FGBU / g de folhas secas, 120 minutos de tempo de reação e pH 4,5. Este estudo é o primeiro relato de otimização da extração de polifenóis de erva-mate verde assistida por enzimas baseada em RSM.

Palavras-chave: Carboidrases; Beta-glucanase; Polifenóis; Compostos bioativos; *Ilex paraguariensis*; Metodologia de superfície de resposta.

1 Introduction

Ilex paraguariensis A. St.-Hil. is a plant that belongs to the family Aquifoliaceae and is widely distributed in Latin American countries such as Brazil, Argentina, Paraguay and Uruguay (Anesini et al., 2012). In these countries, "mate" is a tea made from the infusion of the dry leaves of this plant and is one of the most consumed non-alcoholic beverages. The discovery of various substances with important biological activities in mate has also increased its consumption in other countries (Mejia et al., 2010), and due to this interest, there has been an increase in research related to the properties of yerba mate in the last 20 years (Bracesco et al., 2011). In these studies it was shown that mate had antioxidant, antibacterial, antiviral, antitumor and antimutagenic properties (Bastos et al., 2007; Geetha et al., 2004; Heck et al., 2008). Regarding its composition, the yerba mate extract presents flavonoids and vitamins such as vitamins A, B, C and E, amongst other substances (Menini et al., 2007). Furthermore, the yerba mate extract is very rich in polyphenols, with levels even higher than those in green tea and wines (Gugliucci et al., 2009).

The extraction efficiency of polyphenols from yerba mate is influenced by herb particle size, solvents, pH, time, temperature and agitation (Craft et al., 2012). The conventional extraction of polyphenols uses solvents such as ethyl alcohol or derived aqueous mixtures and has been used by many researchers (Bae et al., 2015). However, another method using enzymes has been developed and used to obtain plant-derived polyphenols, increasing the nutritional value of wines, juice preparations and extracted oils, amongst other substances (Kapasakalidis et al., 2009). Enzymes such as cellulases, pectinases, xylanases and proteases are proteins that act in the degradation of compounds such as plant cell walls (plant cell wall degrading enzymes PCWDEs) (Beg et al., 2001), and can improve the extraction of polyphenols from green yerba mate. For instance, cellulases act on the degradation of cellulosic compounds by catalysing the breakdown of ß (1 → 4) glycosidic bonds that bind the glucose molecules needed for cellulose formation. All the aforementioned enzymes are produced by various microorganisms, including actinomycetes, bacteria and fungi (Sharma et al., 2016). The enzyme-based extraction of bioactive compounds from plants is a potential alternative to conventional solvent-based extraction methods (Puri et al., 2012), for example, the RSM based optimized enzyme-assisted extraction of antioxidant phenolics from underutilized watermelon (*Citrullus lanatus* Thunb.) (Mushtaq et al., 2015). Enzyme-assisted extraction has the advantage of being a green alternative to

conventional methods (Mushtaq et al., 2017). The aim of this research was to evaluate the best reaction time, temperature, enzyme concentration and pH to obtain polyphenols for industrial use from green yerba mate, using enzyme-assisted extraction as an alternative to conventional solvent extraction. For this purpose, a factorial experimental design and response surface methodology (RSM) were used, since these have been used as powerful statistical tools for studying the mutual interactions amongst variables over a range of values, while reducing the number of experimental trials (Moreira et al., 2014).

2 Material and methods

2.1 Substrates

Yerba mate (*Ilex paraguariensis* A. St. Hil.) leaves were collected in July 2016 from the region of São Mateus do Sul in the State of Paraná, Brazil. They were then processed in a traditional manner for enzymatic inactivation in a process called "sapeco". For this, the yerba is submitted to a temperature of 400 °C at the inlet, and 65 °C at the outlet, with a mean time of 8 seconds. It is then dried in a dryer with a temperature ranging from 90 to 110 °C for about 3 hours, after which it is ground to a particle size of 2 to 3 mm. The specimen was identified and deposited (voucher number 394262) at the Municipal Botanical Museum of Curitiba in the state of Paraná, Brazil. The commercial blend of enzymes Viscozyme® L, batch KTNO2241 (Novozymes, Denmark), was used for the enzymatic hydrolysis with 100 FGBU/g (FGBU = Fungal Beta-Glucanase units). This blend contains a range of carbohydrases consisting of arabanase, beta-glucanase, cellulase, hemicellulase and xylanase, and 1 unit of this enzyme (which hydrolyses beta-glucan to reducing sugars) corresponds to the production of 1 µmol glucose per minute at pH 5.0 and 30 °C in 30 minutes. For the analysis of the polyphenols, the Folin Ciocalteau reagent (Sigma-Aldrich, USA) was used with gallic acid (Chromadex, USA) as the standard.

2.2 Experimental design and statistical analysis

The influence of temperature, enzyme (FGBU/g), reaction time and pH on the extraction of the polyphenols was evaluated using a 2^{4-1} CCRD (Central Composite Rotatable Design) with 8 axial points and 4 central points (Table 1). This design was used to determine properties such as randomness, rotatability and orthogonality for the best fit (Myers & Montgomery, 2002). The quality of the polynomial model equation was determined statistically according to the coefficient of determination R^2 and *p*-values below 0.05 were considered statistically significant. A pure error was used for the ANOVA analysis and all statistical analyses were carried out using the Statistica 13.2 software.

Table 1. Levels of the variables of the 2^{4-1} CCDR with 8 axial points and 4 central points used to evaluate the extraction of polyphenols from green yerba mate.

Variable	Level				
	-1.68179	-1	0	+1	+1.68179
(1) Temperature (°C)	33.2	40.0	50.0	60.0	66.8
(2) Enzyme (FGBU/100 g)	0	68	168	268	336
(3) Reaction time (min)	19	60	120	180	221
(4) pH (25 °C)	2.82	3.50	4.50	5.50	6.18

2.3 Assays

In the response surface optimization assays, 20 conical flasks (250 mL) were used to mix 4 g of yerba mate + 100 mL of water and enzyme according to Table 1. The biotechnological experiment was carried out in a Dubnoff-type Metabolic Bath (Novatécnica, Brazil). The pH was evaluated by direct reading using a

Q400A pH meter (Quimis, Brazil) according to the procedure described in the Brazilian Pharmacopoeia (European Directorate for the Quality of Medicines & HealthCare, 2014).

2.4 Assay for the total polyphenolic compounds

The total polyphenolic compound concentrations in the dried leaves of the green yerba mate (*Ilex paraguariensis* A. St. Hil.) and in the enzymatic extracts were determined according to the European Pharmacopoeia (European Directorate for the Quality of Medicines & HealthCare, 2014) using the Folin Ciocalteau (FC) reagent, determining the absorbance in a SP-1105 spectrophotometer (Spectrum, China) at 760 nm, with gallic acid as the standard. This assay is based on the chemical reduction of the reagent containing sodium molybdate and sodium tungstate, which reacts with phenols and other substances such as ascorbic acid, aromatic amines, sugars and xanthines (Singleton et al., 1999).

3 Results and discussion

The experiments were carried out according to a central composite rotatable design (CCRD) considering the follow model: 2^{4-1} CCRD + 8 axial points + 4 central points. The coded values of the experimental factors and their levels for CCRD can be seen in Table 1. The incomplete design was carried out in a random manner in order to minimize the effect of non-controlled variables. A 4-factor design with 5-levels is suitable for exploring a quadratic response surface and constructing a second-order polynomial equation. An analysis of the results obtained from the CCRD allowed for made the following Equation (1) to be obtained:

$$\begin{aligned} TP \;=\; & 7.81077 - 0.110909 * Temp. - 0.000107837 * Temp. * Temp. - 0.270076 \\ & * Enzyme - 0.163313 * Enzyme * Enzyme + 0.012548 * Time - 3.54366e - 005 * Time \\ & * Time - 1.73507 * pH - 0.00316604 * pH * pH + 0.0076881 * Temp. * Enzyme - 6.875e \\ & - 005 * Temp. * Time + 0.0323881 * Temp. * pH + 0.00153333 * Enzyme * Time + 0.108952 * \\ & Enzyme * pH + 0.000425 * Time * pH \end{aligned} \quad (1)$$

Table 2 shows the results obtained for the total polyphenol compounds extracted at the different temperatures (°C), enzyme concentrations (FGBU/100g), reaction times (min) and pH values. According to the Pareto chart (Figure 1) and ANOVA analysis (Table 3), the effects of all the variables were significant ($p \leq 0.05$) for the linear levels, while temperature and pH were not significant at the quadratic levels. The interactions between (1) temperature and (4) pH; (2) enzyme concentration and (3) reaction time; (2) enzyme concentration and (4) pH were also significant ($p \leq 0.05$). The regression model presented a determination coefficient (R^2) close to 0.85 and a fitted coefficient close to 0.45. If the fitted R-squared decreases when a predictor improves the model less than expected by chance, the lower value obtained in this study can be justified due to the lack of difference in the quadratic values for the variables of temperature and pH. The commercial enzyme used, Viscozyme® L, was previously used to optimize the extraction of polyphenol compounds from green tea (*Camellia sinensis*) and was the best choice amongst other commercial enzymes available, such as Celluclast®, Cytolase®, Econase®, Pectinex®, Rapidase® and Ultraflo® (Hong et al., 2013). The same enzyme was also used for the pre-treatment of green tea residues and to improve the extraction of metabolites, where it was applied under mild conditions (temperature below 60 °C), since this preserves the integrity of other by-products such as pigments and polyphenols (Zhang et al., 2016). Many studies have demonstrated that the use of enzymes increases the amount of phenolic compounds extracted as well as the antioxidant activity (Cerda et al., 2013). The enzymatic extraction of polyphenolic compounds occurs via the hydrolytic degradation of polysaccharides from the plant cell wall, which retains the phenolic compounds in a network of polysaccharides and lignin bound by hydrogen or hydrophobic bonds. Another mechanism of enzymatic action is direct catalysis causing disruption of the ether and / or ester bonds between the phenols and polymers of the plant cell wall (Pinelo & Meyer, 2008).

Table 2. Total polyphenolic compounds (mg GAE/g) obtained from green yerba mate under different conditions of temperature (°C), enzyme concentration (FGBU/100 g), reaction time (min) and pH.

Run	Temp. (°C)	Enzyme concentration (FGBU/100 g)	Reaction time (min)	pH	Total polyphenols (mg GAE/ g)
1	60.00	268	180.00	3.50	3.180 ± 0.005
2	60.00	268	60.00	3.50	2.745 ± 0.022
3	60.00	68	180.00	5.50	3.241 ± 0.066
4	40.00	268	60.00	5.50	2.281 ± 0.018
5	60.00	68	60.00	5.50	3.072 ± 0.018
6	40.00	68	180.00	3.50	2.890 ± 0.005
7	40.00	268	180.00	5.50	2.983 ± 0.080
8	40.00	68	60.00	3.50	2.658 ± 0.033
9	33.20	168	120.00	4.50	2.435 ± 0.055
10	66.80	168	120.00	4.50	3.400 ± 0.066
11	50.00	0	120.00	4.50	2.084 ± 0.070
12	50.00	336	120.00	4.50	2.890 ± 0.036
13	50.00	168	19.00	4.50	1.803 ± 0.022
14	50.00	168	221.00	4.50	3.371 ± 0.044
15	50.00	168	120.00	2.82	2.788 ± 0.005
16	50.00	168	120.00	6.18	3.090 ± 0.013
17 (CP)	50.00	168	120.00	4.50	3.168 ± 0.078
18 (CP)	50.00	168	120.00	4.50	3.125 ± 0.013
19 (CP)	50.00	168	120.00	4.50	3.255 ± 0.093
20 (CP)	50.00	168	120.00	4.50	3.130 ± 0.063

mg GAE/g = mg gallic acid equivalent/g; FGBU = Fungal Beta-Glucanase units; CP = central point.

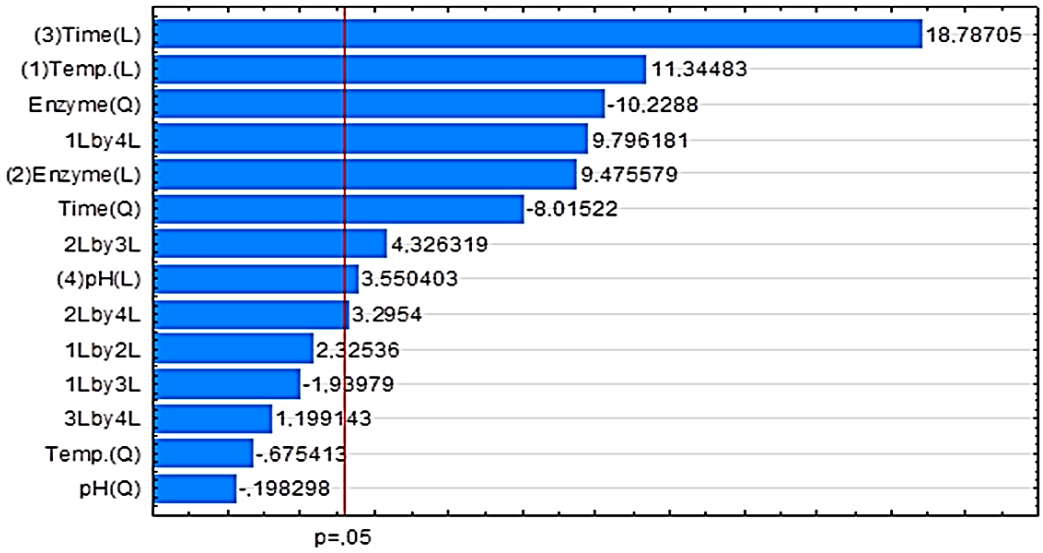

Figure 1. Pareto chart for the effects calculated from the responses of the 2^{4-1} CCRD + 8 axial points + 4 central points used to evaluate the extraction of polyphenolic compounds from green yerba mate considering (1) temperature (°C), (2) enzyme concentration (FGBU/100 g), (3) reaction time (min) and (4) pH ($p < 0.05$).

Table 3. Analysis of variance (ANOVA) for the 2^{4-1} CCRD + 8 axial points + 4 central points used to evaluate the extraction of polyphenolic compounds from green yerba mate.

Factor	SS	Df	MS	F	P
(1) Temperature (L)	0.465612	1	0.465612	128.7052	0.001469
(1) Temperature (Q)	0.001650	1	0.001650	0.4562	0.547813
(2) Enzyme (L)	0.324818	1	0.324818	89.7866	0.002492
(2) Enzyme (Q)	0.378511	1	0.378511	104.6285	0.001992
(3) Reaction time (L)	1.276867	1	1.276867	352.9532	0.000329
(3) Reaction time (Q)	0.232413	1	0.232413	64.2438	0.004054
(4) pH (L)	0.045602	1	0.045602	12.6054	0.038080
(4) pH (Q)	0.000142	1	0.000142	0.0393	0.855489
1L by 2L	0.019562	1	0.019562	5.4073	0.102581
1L by 3L	0.013612	1	0.013612	3.7628	0.147742
1L by 4L	0.347170	1	0.347170	95.9652	0.002261
2L by 3L	0.067712	1	0.067712	18.7170	0.022767
2L by 4L	0.039287	1	0.039287	10.8597	0.045892
3L by 4L	0.005202	1	0.005202	1.4379	0.316550
Lack of fit	0.508290	2	0.254145	70.2511	0.003023
Pure error	0.010853	3	0.003618		
Total SS	3.596007	19			

Variables and interactions in bold express significance ($p < 0.05$). $R^2 = 0.85563$ Adjusted. = 0.45141. SS = sum-of-squares. Df = degrees of freedom. MS = mean square. F = f-ratio. P = p-value.

The presence of an enzyme increased the extraction of polyphenolic compounds from the dry green leaves of yerba mate (Figure 2a). Regarding enzyme concentration, it can be seen that the concentration of 336 (FGBU/100 g) (Table 2, run 12) increased the quantity of polyphenols by 38.67% in relation to extraction without the enzyme under the same experimental conditions (Table 2, run 11). An increase in the extraction of polyphenols was observed at the central points in which an intermediate enzyme concentration of 168 FGBU/100 g of dry green leaves of yerba mate was used, under the same experimental conditions of runs 11 and 12. For this scenario, the extraction of polyphenols increased by an average of 52.08% (four central points) (Table 2, runs 17-20) in relation to run 11 (without enzyme). This effect could be explained by the enzyme/substrate ratio, in which the excess of enzyme was used at quadratic levels and because the quantity of total polyphenols extracted decreased under this condition. Thus the best enzyme concentration for industrial viability was 168 FGBU/100 g of dry green leaves of yerba mate. The best conditions for the extraction of phenolics and flavonoids from roasted yerba mate leaves (*Ilex paraguariensis* A. St.-Hil., Aquifoliaceae) were determined using Response Surface Methodology (Bassani et al., 2014). The extraction of total polyphenolic compounds from yerba mate using ethanol was previously studied and the best parameters were: reaction time of 103 min, extraction temperature of 71 °C and ethanol concentration of 61% (Bae et al., 2015). The enzyme-assisted extraction of secondary metabolites is considered to be a green and clean technology unlike the use of conventional solvents such as alcohols (methanol, ethanol), acetone, diethyl ether and ethyl acetate, which are used for the extraction of polyphenols despite several disadvantages such as possible hazardous effects on human health due to solvent residues in the final products, and the disposal of these solvents can also cause environmental problems (Mojzer et al., 2016). Enzymatic extraction is an alternative for the extraction of secondary metabolites, such as, for example, the enzymatic extraction of pilocarpine from *Pilocarpus jaborandi*, which increased the extraction 3.08 fold as compared to the control treatment (Cho et al., 2013). From green tea, enzymatic extraction showed a significant increase of 4% to 15% in the total polyphenolic compound content of the green tea extract when compared to the non- enzymatic treatment (Hong et al., 2013). Figure 2b shows that the extraction of polyphenolic compounds increased at higher temperatures: at a temperature of 66.80 °C the amount of total polyphenolic compounds extracted increased by 7.27% in relation to the central point that used a temperature of 50 °C. Therefore an increase of 16.8 °C is justified since it improves the extraction of the polyphenols. According to the literature, enzyme

activity can be decreased by protein denaturation, which is an irreversible process involving the loss of the primary structure with the associated cleavage of covalent bonds at temperatures above 80 °C (Daniel et al., 1996). Nonetheless, the higher temperature did not show a significant effect when the pH was below 3.5 (Figure 2c). This result is in agreement with the current optimal conditions in which the ideal pH value is 3.3 to 5.5 (Hong et al., 2013). The reaction time had a significant effect on the extraction of polyphenols from green yerba mate (Figure 2d), although the amount of polyphenols extracted only increased by 6.35%. Enzyme action is pH dependent (Bisswanger, 2014) and this was also observed in the present study where there was a significant interaction between (2) enzyme concentration and (4) pH (Figure 1 and Table 3). The change in pH alone was not significant and did not increase the extraction of polyphenols from yerba mate (Figure 2e), but the interaction between enzyme concentration and pH was observed to produce such an effect, although neither the pH alone for a short reaction time nor the interaction between the pH and reaction time produced a significant difference (Figure 2f). The optimization algorithm allowed for the elaboration of profiles for predicted response values and desirability functions (Figure 3); the red line indicates the best conditions and a desirability score of 1.0: temperature of 50.0 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH of 4.50.

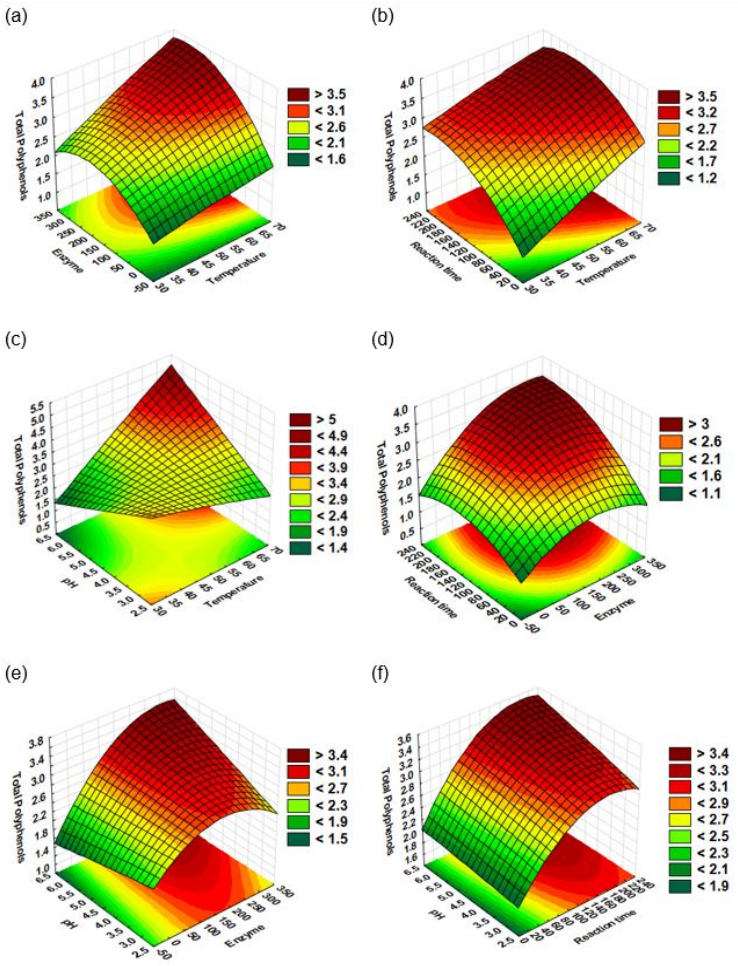

Figure 2. Response surfaces for the 2^{4-1} CCRD + 8 axial points + 4 central points to evaluate the extraction of polyphenolic compounds from green yerba mate. (a) temperature (°C): enzyme concentration (FGBU/100g). Fixed reaction time 120 minutes and fixed pH 4.5; (b) temperature (°C): reaction time (minutes). Fixed enzyme concentration 168 FGBU/100g and pH 4.5; (c) temperature (°C): pH. Fixed enzyme concentration 168 FGBU/100g and reaction time 120 minutes; (d) reaction time (min): enzyme concentration (FGBU/100g). Fixed temperature 50 °C and pH 4.5; (e) pH: enzyme concentration (FGBU/100g). Fixed temperature 50 °C and reaction time 120 minutes; (f) pH: reaction time (min). Fixed temperature 50 °C and enzyme 168 FGBU/100g.

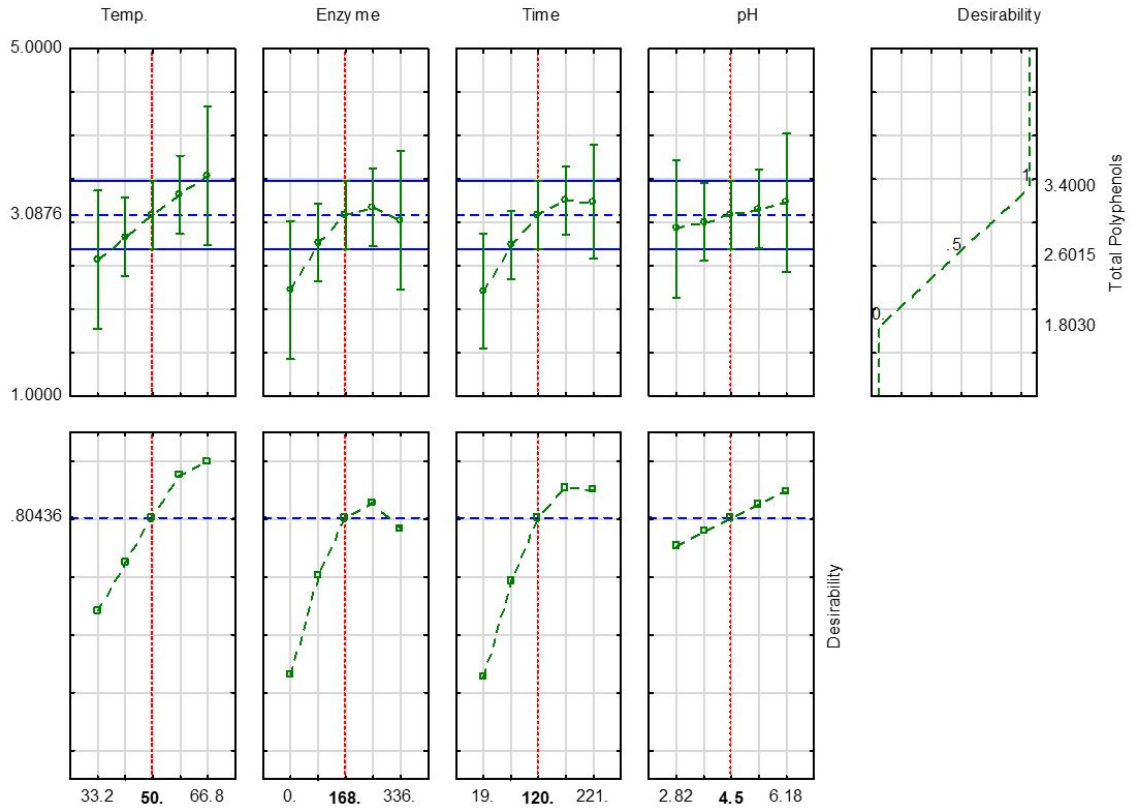

Figure 3. Profiles for the predicted values and desirability functions for the maximum extraction efficiency of total polyphenols from green yerba mate. Dotted red lines indicate the optimal values.

4 Conclusion

In this work a 2^{4-1} CCRD + 8 axial points + 4 central points was used to evaluate the extraction variables (temperature, enzyme concentration, reaction time and pH) and possible interactions for the extraction of total polyphenolic compounds from the dried leaves of green yerba mate. By using response surfaces, contour curves and derivation techniques the optimum application conditions for the enzyme investigated were defined. It was concluded that the effects of all the variables studied (temperature, enzyme concentration, reaction time and pH) were significant at the linear levels although temperature and pH were not significant at the quadratic levels, parameters that can be explained by the optimal enzymatic conditions. The interactions between temperature and pH, enzyme concentration and reaction time and enzyme concentration and pH were significant. Based on the results obtained in this study and taking into account industrial viability, the authors suggest that the optimal conditions for the extraction of total polyphenols from green yerba mate are a temperature of 50.0 °C, enzyme concentration of 168 FGB/100 g, reaction time of 120 minutes and pH of 4.5. The enzyme-assisted extraction of polyphenols from green yerba mate was considered a good alternative to conventional solvent extraction, since enzymes are renewable matter that further contribute to sustainable practices.

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