http://dx.doi.org/10.5935/0103-5053.20150186

J. Braz. Chem. Soc., Vol. 26, No. 10, 2054-2062, 2015.
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0103 - 5053 $6.00+0.00

Article

Chemical Characterization and Optimization of the Extraction Process of Bioactive Compounds from Propolis Produced by Selected Bees *Apis mellifera*

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The extraction process of bioactive compounds from propolis produced by selected bees was optimized using a 2^3 factorial design planning to evaluate the effect of ethanol concentration, time and temperature on the extraction of total phenolic compounds and antioxidant activity. Analysis of total flavonoids, antioxidant activity and chemical characterization performed by high-performance liquid chromatography with photodiode array (HPLC-PDA) detection were carried out in optimal conditions of extraction. Optimizing results suggest that the best condition for extraction of bioactive compounds was obtained in higher levels: time (45 min), temperature (70 °C) and concentration of ethanol (80%). The extract evaluated in optimal conditions presented good antioxidant activity by the radical scavenging 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and ferric reducing antioxidant power (FRAP) methods and chromatographic analysis identified phenolic acids: *para*-coumaric, ferulic and caffeic, indicating that this material is a potential source of bioactive compounds.

Keywords: factorial design**,** chemical characterization, phenolic compounds, antioxidant activity

Introduction

Propolis is a resinous material collected by bees from various parts of plants and is used in protection of the hive and currently is consumed as a functional food. There are several types of propolis and each one has different pharmacological properties, which depends on its chemical composition and varies according to the region where it is produced and collected.[1]

The Brazil is the one of the largest producers of propolis, being the largest part of the production destined for export and another part marketed in the form of alcoholic extract. The Brazilian product is highly valued and has unique characteristics, typical of propolis originated in tropical regions, which give various biological activities to it such as anti-inflammatory,[2] antioxidant,[3,4] antitumoral[5] and antibacterial.[1]

The compounds that have antioxidant activity, such as the phenolic compounds, have been the subject of several studies. These compounds act as protectors of the human body against excessive radicals, naturally produced during aerobic metabolism, which initiates a process of oxidative stress. This process may cause numerous diseases, including cancer, anemia and cardiovascular problems.[6]

Propolis produced by Africanized honey bees (*Apis mellifera* L.) selected to increase honey production, is been produced in Federal Technological University of Paraná, Dois Vizinhos Campus, Paraná, Brazil. The population of these bees is the result of a selection process of the queen's weight to emergency through genetic animal evaluation. The queen's weight is used as the selection criteria due to the fact that this is genetically linked to honey production,

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as well as the production of honey is positively associated to the production of propolis. The choice of the colonies for this experiment was based on phenotypic information on the potential of bees.

Propolis extraction conditions such as temperature, time and concentration of solvent can influence the extraction of phenolic compounds and antioxidant activity.[7,8] Therefore, it is important to apply experimental design, which allow scientists to evaluate the influence of input variables (factors) and output variables (responses) with the main objective of increasing the processes efficiency by reducing costs and the number of experiments.[9]

No study had been conducted with this propolis yet and, for this reason, there were interests that will be evaluated in relation to bioactivity. Within this context, the aims of this work were the optimization of extraction process of bioactive compounds, the evaluation of antioxidant activity and also the chemical characterization of this propolis using the high performance liquid chromatography technique.

Experimental

Materials

The reagents 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) (trolox), 2,4,6-tripyridyl-s-triazine (TPTZ), methanol and Folin-Ciocalteu reagent were obtained from Sigma. The authentic standards (HPLC grade) of ferulic acid, vanillic acid, gallic acid, caffeic acid and *para*-coumaric acid were purchased from Extrasynthese Co. with purity ≥ 99%.

Propolis samples were obtained from selected colonies of Africanized honey bees *Apis mellifera* from March to August of 2013 in an apiary located in the city of Dois Vizinhos, State of Paraná, in southern Brazil. The samples were cleaned, crushed with liquid nitrogen, homogenized, weighed and stored at −6 °C until the moment of the analysis.

Experimental design

Optimization of extraction conditions for total phenolic content (Z_1) and antioxidant activity by the DPPH free radical scavenging method (Z_2) was performed using factorial design and analyzed according to surface response methodology (SRM). Independent variables of the process were ethanol concentration (X_1), temperature (X_2) and extraction time (X_3). The factorial design was selected for each optimization process variable in 3 levels with 11 experiments including 3 replicates at the center point. The levels of the independent variables and also their encoded values are presented in Table 1. Experimental data was analyzed using Environment R (version 3.1.1) as the statistical software.

The dataset presented was adjusted according to the following first-order polynomial equation 1:

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 \quad (1)$$

where Y is the expected response; β_0 represents the intersection; β_1, β_2 and β_3 are the linear coefficients, β_{12}, β_{13} and β_{23} are the coefficients of interaction, and x_1, x_2 and x_3 are independent variables, ethanol concentration, temperature of extraction and extraction time, respectively.

Table 1. Three factors, three levels, factorial design and experimental data of the responses investigated of the propolis extract

Run	Factor 1 (X_1): ethanol concentration[a] / %	Factor 2 (X_2): temperature / ºC	Factor 3 (X_3): time / min	Response1(Z_1): total phenolic content[b] / (mg GAE g^{-1})	Response 2 (Z_2): DPPH[c] / (μg trolox g^{-1})
1	50 (−1)	30 (−1)	15 (−1)	2.79 ± 0.04	31.6 ± 0.12
2	80 (+1)	30 (−1)	15 (−1)	3.95 ± 0.28	47.0 ± 0.12
3	50 (−1)	70 (+1)	15 (−1)	3.33 ± 0.16	49.8 ± 0.13
4	80 (+1)	70 (+1)	15 (−1)	3.56 ± 0.01	58.5 ± 0.04
5	50 (−1)	30 (−1)	45 (+1)	3.37 ± 0.04	39.9 ± 0.14
6	80 (+1)	30 (−1)	45 (+1)	4.68 ± 0.09	58.4 ± 0.01
7	50 (−1)	70 (+1)	45 (+1)	2.42 ± 0.15	78.1 ± 0.06
8	80 (+1)	70 (+1)	45 (+1)	5.75 ± 0.24	87.5 ± 0.13
9	65 (0)	50 (0)	30 (0)	3.61 ± 0.01	57.9 ± 0.06
10	65 (0)	50 (0)	30 (0)	3.56 ± 0.02	56.0 ± 0.06
11	65 (0)	50 (0)	30 (0)	3.96 ± 0.02	58.2 ± 0.07

[a]Ethanol concentration in v/v; [b]total phenolic content in propolis extract as acid gallic equivalents (mg GAE g^{-1}); [c]antioxidant activity expressed as equivalent of μmol trolox g^{-1}. The numbers in parentheses, in the three columns of the factors, represent the levels of each variable used in the factorial design.

Total phenolic content (TPC)

The total phenolic content (TPC) was determined by the colorimetric analysis using the Folin-Ciocalteau reagent as described by Singleton *et al.*[10] In a test tube, 0.5 mL of the ethanolic extract of propolis (EEP) (1:25), 2.5 mL of Folin-Ciocalteau reagent diluted 1:10 and 2.0 mL of Na_2CO_3 4% were added. After incubation in the dark during a period of 2 h at room temperature, the absorbance (740 nm) was measured by spectrophotomer (model UV-Vis lambda 25, Perkin Elmer). The blank was carried out using 0.5 mL of ethanol:water 80:20 (v/v) in the place of ethanolic extract to "zero out" the spectrophotometer. The total phenolic content of the extracts was determined by comparison with a calibration curve of gallic acid as a standard ($r^2 = 0.994$, limit of detection (LOD) = 3.63 μg mL^{-1}, limit of quantification (LOQ) = 12.13 μg mL^{-1} and coefficient of variation (CV) = 3.7% and represented as mg gallic acid equivalents (GAE) g^{-1} of propolis. Analyses were carried out in triplicate.

Total flavonoid content (TFC)

The concentration of the total flavonoid content (TFC) present in EEP was performed using the colorimetric method described by Jurd and Geissman.[11] An aliquot of 0.5 mL of the EEP (1:5) was added in a two series of tubes and identified with and without the addition of nitrate. In the tubes that received nitrate, 4.3 mL of ethanol 80% was added and those that did not receive nitrate, 4.4 mL ethanol 80% was added. In all tubes, 0.1 mL of potassium acetate 1 mol L^{-1} was added. In the series of tubes identified for receiving nitrate, 0.1 mL of aluminium nitrate $Al(NO_3)_3$ 10% was added. A negative control (blank) was also prepared for the test with 4.9 mL ethanol 80% and 0.1 mL potassium acetate. The tubes were shaken and left in incubation in the dark and after 40 min of incubation the absorbance was measured in the spectrophotometer at 415 nm. The total flavonoid contents of the extracts were determined by comparison with the calibration curve of the quercetin standard ($r^2 = 0.997$, LOD = 0.61 μg mL^{-1}, LOQ = 2.05 μg mL^{-1}) and represented as mg quercetin equivalents g^{-1} of propolis. The analyses were done in triplicate.

Antioxidant activity using the ferric reducing antioxidant power (FRAP) method

The ferric reducing antioxidant power (FRAP) method used was proposed by Benzie and Strain.[12] The FRAP reagent was obtained from the mixture of 25 mL of acetate buffer 0.3 mol L^{-1}; 2.5 mL of a solution of 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ), 10 mmol L^{-1} and 2.5 mL of iron chloride in aqueous solution 20 mmol L^{-1}, and used after the preparation. The method consisted of a mixture of 100 μL of the EEP (1:50) with 3 mL of FRAP reagent. The mixture was homogenized and kept in a water bath at 37 °C for 30 min and then the absorbance was measured by spectrophotometer at 595 nm. The FRAP reagent was used as a blank and an aqueous solution of ferrous sulfate was used for calibration curve ($r^2 = 0.998$, LOD = 32.04 μmol L^{-1}, LOQ = 106.81 μmol L^{-1}) and the potential of the antioxidants in the propolis extract to reduce Fe^{III} to Fe^{II} was expressed in μmol Fe^{II} g^{-1} of propolis. All analyses were carried out in triplicate.

Antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) free radical scavenging method

DPPH free radical scavenging activity was measured as described by Brand-Willians *et al.*[13] The reaction medium consisted of 0.5 mL of the EEP (1:50), 3 mL of ethanol and 0.3 mL of DPPH radical solution 0.5 mmol L^{-1} in ethanol. The mixture was incubated in the absence of light at room temperature for 45 min and subsequently, the absorbance was measured using a spectrophotometer (model UV-Vis lambda 25, Perkin Elmer) at 517 nm. Ethanol was used instead of propolis extract solutions as a blank and the quantification was carried out from an analytical curve using trolox as standard ($r^2 = 0.998$, LOD = 22.18 μmol L^{-1}, LOQ = 73.93 μmol L^{-1} and CV = 4.75%) and the results were expressed in μmol of trolox *per* gram of propolis (μmol trolox g^{-1}). All analyses were carried out in triplicate.

Antioxidant activity using the 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid) (ABTS) method

The antioxidant activity by the ABTS method was performed according to Re *et al.*[14] with some modifications. The $ABTS^{\bullet+}$ radical was formed by the reaction of 7 mM ABTS with 140 mM potassium persulfate, incubated at 25 °C in the dark for 12-16 h. The radical was diluted with ethanol to obtain the absorbance value of 0.700 ± 0.200 at 734 nm. Under dark conditions, 3.0 mL of the $ABTS^{\bullet+}$ radical solution was added to 30 μL of EEP (1:5) and the absorbance was read at 734 nm in a spectrophotometer after 6 min. Ethanol was used instead of propolis extract solutions as a blank and trolox was used as reference ($r^2 = 0.998$, LOD = 111.97 μmol L^{-1} and LOQ = 373.23 μmol L^{-1} and the results of the antioxidant activity were expressed as μmol of trolox equivalent antioxidant capacity (TEAC) g^{-1}. All analyses were carried out in triplicate.

High-performance liquid chromatography with photodiode array detection (HPLC-PDA)

The HPLC-PDA method was performed according to Francisco and Resurreccion[15] method with some modifications. For the analysis by HPLC-PAD was injected a volume of 10 µL of EEP at a concentration of 3% in a chromatograph coupled to a photodiode array (PDA) detector (Varian, model 920-LC). The analytical column used was a Varian C-18 RP (250 mm × 4.6 mm, 5 µm) maintained at 30 °C. The mobile phase used was a mixture of water (solvent A) and methanol (solvent B), both acidified with 0.1% acetic acid, with elution in gradient mode. The gradient started with 5% of B up to 7% of B in 7 min, 20% of B in 15 min, 50% of B in 30 min, 90% of B in 50 min, and 95% of B in 55 min, keeping this concentration for 10 min, at a flow rate of 1 mL min^{-1}. This work used authentic standards of ferulic acid, vanillic acid, gallic acid, caffeic acid and coumaric acid. The identification was performed by comparison of retention times and absorption in ultraviolet at wavelengths of 280 nm and 320 nm.

Limit of detection (LOD) and limit of quantification (LOQ)

The values of LOD and LOQ were obtained using the data from the calibration curve equation in accordance with the International Conference on Harmonization (ICH) Guideline.[16] The determination of the respective limits of detection and quantification was carried out from the standard deviation of the intercept and the slope of the calibration curve as shown in equation 2 and equation 3, respectively.

$$LOD = 3 \times \frac{s}{b} \quad (2)$$

$$LOQ = 10 \times \frac{s}{b} \quad (3)$$

where s is the *s*tandard deviation of the intercept of the calibration curve and b is the slope of the calibration curve.

Precision

The precision of the method was expressed by coefficient of variation (CV, %), of the data set as described by ICH.[16] To evaluate precision a total of 6 determinations of the test extracts were evaluated and the CV was obtained by dividing the standard deviation and the mean:

$$CV = \frac{s}{c} \times 100 \quad (4)$$

where s is the standard deviation and c is the mean.

Results and Discussion

Antioxidant activity of EEP determined by DPPH method varied from 31.6 to 87.5 µmol trolox g^{-1} (Table 1). The surface response analysis for antioxidant activity showed that the main effects of ethanol concentration, temperature, time of extraction and the interactions: concentration *versus* temperature and temperature *versus* time were statistically significant ($p \leq 0.001$) (Table 2). All significant effects with the exception of the concentration *versus* temperature interaction were positive, indicating that, it is possible to notice an increase in responses when these are analyzed at the levels −1 and +1.

The concentration of TPC ranged from 2.42 to 5.75 mg GAE g^{-1} (Table 1) and the effects of ethanol concentration, time and the interaction of ethanol concentration and time were statistically significant ($p \leq 0.05$) (Table 2).

The result of the analysis of variance (ANOVA) for the antioxidant activity (Table 2) indicates that the fitted model is considered statistically significant and predictive, with 95% of confidence, since the F calculated value is 150.07 times superior to F table and the lack of adjustment was not significant at the same confidence level. The coefficient of determination (r^2) was of 0.994 demonstrating that the model is suitable for predicting the data obtained (equation 5).

$$DPPH\ (\mu mol\ trolox\ g^{-1}) = 56.5 + 6.5X_1 + 12.1X_2 + 9.6X_3 - 2.0X_1X_2 + 4.7X_2X_3 \quad (5)$$

The analysis of variance (ANOVA) result obtained for TPC (Table 2) indicate that the fitted model is statistically significant and can be used for predictive purposes, with 95% of confidence, since the calculated F value was 7.58 times the F table and the lack of adjustment was not significant at the same confidence level. The coefficient of determination (r^2) was 0.833, indicating that 83.3% of the variability of the data can be explained by the proposed model (equation 6).

$$TPC\ (mg\ GAE\ g^{-1}) = 3.73 + 0.76X_1 + 0.32X_3 + 0.40X_1X_3 \quad (6)$$

The influence of the factors (independent variables) in the variable response was evaluated by using multiple linear regression and response surface methodology (RSM). Figure 1 is a three-dimensional representation of response surface showing the influence of the concentration of ethanol (X_1) and temperature (X_2) on antioxidant activity and total phenolic compounds (TPC) of the EEP. It is

Table 2. Analysis of variance (ANOVA), regression coefficients of the models for the responses antioxidant activity (DPPH) and total phenolic content of extracts of propolis, r^2 values and *F*-value

Term	DPPH coefficient	Phenolic compounds coefficient
β_0	56.5[a]	3.73[a]
β_1	6.5[a]	0.76[a]
β_2	12.1[a]	0.03[b]
β_3	9.6[a]	0.32[c]
β_{12}	–2.0[a]	0.13[b]
β_{13}	0.47[b]	0.40[d]
β_{23}	4.7[a]	–0.002[b]
r^2	0.994	0.833
Main effects		
Ethanol concentration	***[a]	***[a]
Temperature	***[a]	ns[b]
Time	***[a]	*[c]
F value	454.71	24.93
F statistic table	3.03[e]	3.29[e]
F ratio[f]	150.07	7.58

[a]significant at $p < 0.001$(***); [b]ns: not significant at $p > 0.05$; [c]significant at $p < 0.05$ (*); [d]significant at $p < 0.01$ (**); [e]95% of confidence level; [f]F ratio: (*F*-value/*F*-tabular value).

possible to conclude that the concentration of ethanol in higher levels, 80% (v/v), contributed to an increase in antioxidant activity by radical DPPH method (Figure 1a) and extraction of TPC (Figure 1b).

The increase in antioxidant activity by DPPH method was observed with an increase in temperature (up to 70 °C) at higher ethanol concentration (Figure 1). On the other hand, the effect of temperature on the extraction of TPC was not significant (Table 2), indicating that both, lower and superior temperature levels produce the same effects in the contents of phenolic compounds. Cunha *et al.*[17] observed an increase in the efficiency of the extraction of phenolic compounds of propolis when ethanol was used in a concentration equal or greater than 70%. Karacabey and Mazza[7] also observed the influence of ethanol concentration and temperature on the extraction of compounds with antioxidant activity of grape waste.

These results confirm that the rates of diffusion and solubility of analytes are increased by higher temperatures, favoring the extraction of bioactive compounds.[18] However, when choosing the ideal temperature for the extraction it is necessary to consider the fact that excessively high temperatures may cause the degradation and/or volatilization of some compounds, resulting in a decrease in the process efficiency.[19]

The concentration of ethanol also affected significantly the content of phenolic compounds and antioxidant activity of EEP. This effect can be related to a change in solvent polarity due to change of concentration of ethanol. An increase in the concentration of ethanol causes a decrease in polarity, since ethanol has a dipole moment (1.69 D) lower than water (1.85 D), which favors the extraction of less polar compounds, affecting the composition and quantity of phenolic compounds extracted, which affects also the antioxidant activity.[7]

The effects of ethanol concentration (X_1) and time (X_3) on antioxidant activity and total phenolic compounds (TPC) of the EEP are presented in Figures 2a and 2b, respectively. It can be concluded that both factors, ethanol concentration, in high levels (80%) and time at high levels (45 min) are responsible for linear increases in antioxidant activity and TPC.

The total content of phenolic compounds is increased by high ethanol concentrations (80%, v/v) and time (45 min) (Figures 2a and 2b). By assessing the interaction between the ethanol concentration and extraction time it was possible to observe that this interaction was significant

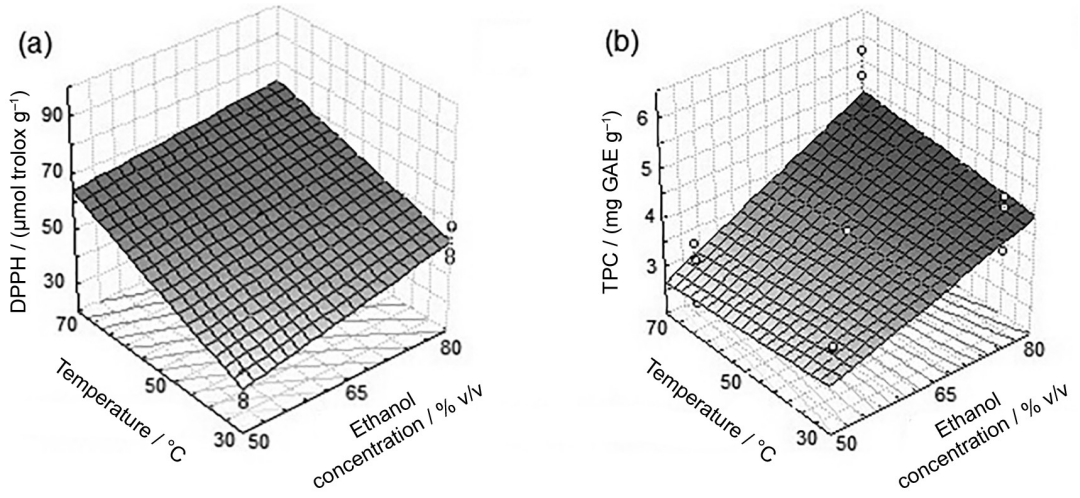

Figure 1. Response surfaces for the effects: ethanol concentration and temperature for the free radical DPPH in (a) and TPC in (b).

only for extraction of phenolic compounds (Figure 2b and Table 2). An increase in extraction time (from 15 to 45 min) at higher concentrations of tested ethanol provided the largest extraction of these compounds.

Although less important than temperature and repetitions in the extraction process, the extraction time significantly affected the extraction of compounds with antioxidant capacity in methanolic extracts produced from the banana residue.[19]

Evaluating Figure 3 it is possible to conclude that both time (X_3) and temperature (X_2) at high levels, as well as the interaction of these variables, influenced the antioxidant activity (Figure 3a), while the content of phenolic compounds was influenced only by time, also at high levels (45 min) (Figure 3b).

According to Bachir *et al.,*[18] mass transfer to the solvent is related to the time and temperature of extraction. The mass transfer increases over time until a maximum of extraction has been achieved. The temperature accelerates the diffusion, which promotes an increase in extraction; however, high temperatures can degrade compounds with antioxidant activity.

Analyzing the response surfaces of Figures 1, 2 and 3 it is possible to verify that the optimum conditions for extraction of compounds with antioxidant activity, as well as the total phenolic content, were obtained using the higher levels of the variables, extraction temperature of 70 °C, time for extraction of 45 min and concentration of 80% ethanol (v/v).

Antioxidant activity, total flavonoids and chemical characterization by HPLC of the EEP in optimum extraction conditions

The antioxidant activity of ethanolic extract of propolis produced in optimum extraction condition was assessed by two distinct *in vitro* methods, ability of scavenger radical ABTS and FRAP. The ABTS method is a more indirect method used to evaluate the antioxidant activity and characterizes for its simplicity and speed, allowing its application in routine analysis in any laboratory.[14] The antioxidant activity of the EEP measured by ABTS method presented a value of 95.88 ± 4.4 μmol of trolox g^{-1} with a CV of 4.61%. Bonvehí and Gutiérrez,[20] when examining the antioxidant activity of propolis samples prepared with different solvents, obtained values between 420 and 1430 μmol trolox g^{-1}.

The FRAP is a indirect method based in a reduction of complex Fe^{III}-TPTZ (2,4,6-tripyridyl-*s*-triazine) to Fe^{II} form by an antioxidant under acidic conditions, and an intense blue color develops with an absorption maximum at 593 nm.[12] The value found for the antioxidant activity measured by FRAP in this study was from 259.30 ± 9.50 μmol of Fe^{II} g^{-1} in equivalent of ferrous sulphate with a CV of 3.67%. Mihai *et al.*,[21] when analyzed 20 propolis samples collected in Transylvania, obtained FRAP values ranging from 0.74 to 2.54 mmol Fe^{II} g^{-1}. Cottica *et al.*[8] analyzed organic propolis collected in the Maringa, State of Parana, Brazil, found values ranging from 528 to 1365 μmol of Fe^{II} g^{-1}. There are few studies available in the literature which evaluated the EEP by FRAP method, therefore, emphasizing the importance of this study.

The content of total flavonoids obtained in this study was $1.86 \pm 5.66 \times 10^{-2}$ mg quercetin g^{-1} with a CV of 3.04%. Cottica *et al.*[8] analyzed organic propolis extracts which were produced by varying the ethanol concentration and the amount of propolis and obtained contents in the range of 2.5 to 176 mg quercetin g^{-1}. They concluded that extracts produced with the mixture of solvents ethanol:water (96:4, v/v) are the ones that presented a higher content of total

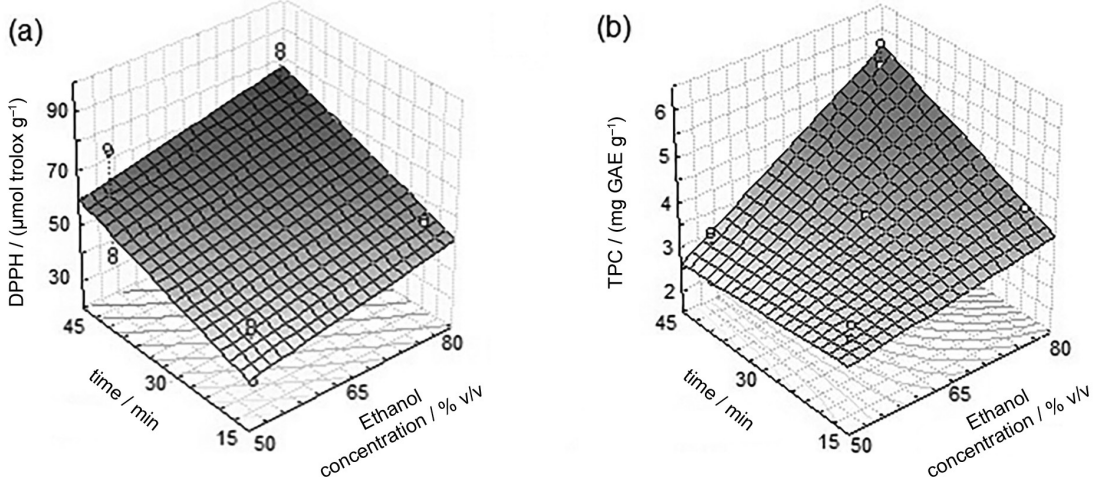

Figure 2. Response surfaces for the effects: ethanol concentration and time for the free radical DPPH in (a) and TPC in (b).

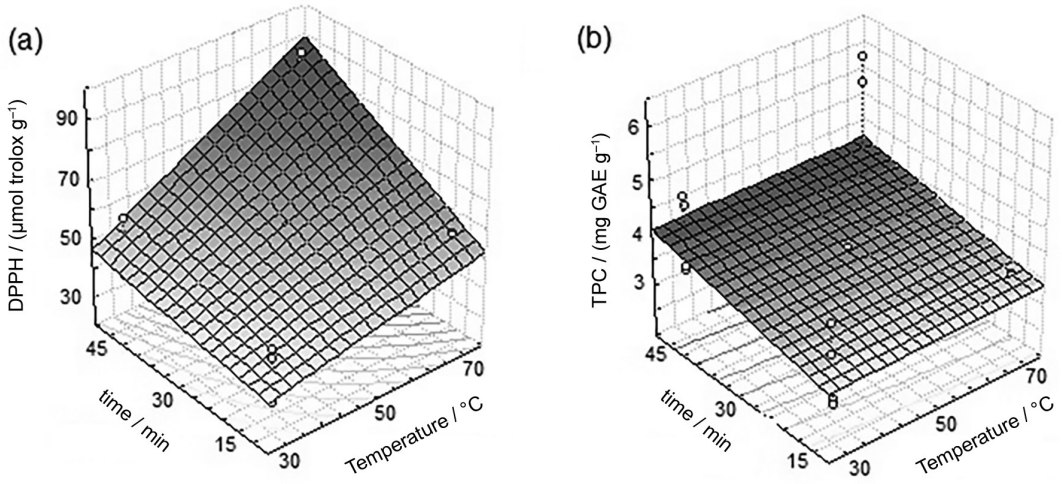

Figure 3. Surface responses for the effects: temperature and time for the free radical DPPH in (a) and TPC in (b).

flavonoids, which corroborates with the results of this study. Cabral *et al*.[22] obtained values from total flavonoids of 3.2 and 61.5 mg quercetin g^{-1} for ethanolic extract of propolis G6 and G12, respectively.

Despite the low concentration of flavonoid content, the EEP showed antioxidant activity which suggests that chemical other compounds than flavonoids in propolis can be responsible by biological activity produced by selected bees.

The phenolic acids present in the EEP were identified by using high performance liquid chromatography (HPLC) (Figure 4). The extract showed a complex chemical composition with multiple peaks in different retention times and a chemical profile with substances of polar and nonpolar nature. The phenolic acid that presented the highest content in EEP was the *para*-coumaric acid (37.83 mg 100 g^{-1}) followed by caffeic and ferulic acids.

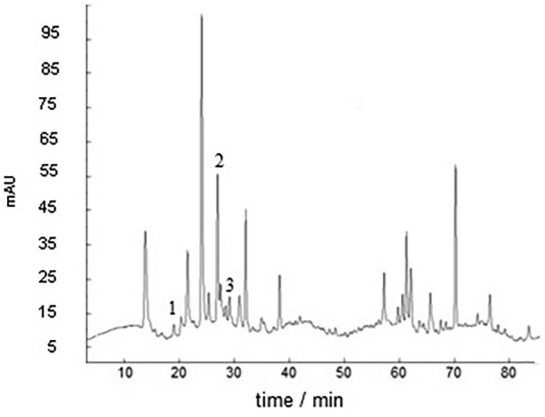

Figure 4. HPLC chromatogram of EEP obtained at 280 nm. Peak identification: (1): caffeic acid; (2): *para*-coumaric acid and (3): ferulic acid.

The chromatographic method used for the identification of phenolic compounds was effective, since it showed high selectivity and resolution for most of the analyzed peaks. Alencar *et al.*[23] identified in EEP of red propolis by HPLC-DAD the ferulic acid, the isoflavone daidzein and the flavonoid quercetin. Castro *et al.,*[24] indicate that cinnamic acid derivatives compounds are the most abundant substances in samples of propolis, which corroborates with the results of this study.

Differences in the phenolic content and antioxidant activity may be due to the different locations where propolis has been harvested. It is reported in the literature that differences in chemical composition of propolis are due to the local flora available to bees at the site of collection.[25] This is a new type of propolis and a chemical and biological characterization can help to define the quality, composition and bioactivity of propolis produced by selected bees *Apis mellifera.*

All the compounds identified and quantified were within their respective limits of detection (LOD) and limits of quantification (LOQ) (Table 3). The standard curves with six points (5.0 μg mL^{-1}; 10.0 μg mL^{-1}; 20.0 μg mL^{-1}; 40.0 μg mL^{-1}; 60.0 μg mL^{-1} and 80.0 μg mL^{-1}) were generated for each phenolic compound studied by plotting area under the peak *versus* phenolic acid concentration and all Pearson correlation coefficients, r, were > 0.994, indicating a positive linear relationship between concentration and peak area a linear in the range studied (Table 3).

The precision together with accuracy, recovery, robustness, etc., determines the analytical measurement errors and it is the main criteria used to judge the quality of an analytical method.[26] The calculated coefficient of variation for the EEP ranged from 2.27-5.14% (n = 3) for the three identified compounds in the samples (Table 3). García-Falcon *et al*.[27] obtained a CV value that ranged from 1 to 9% for the simultaneous determination of phenolic compounds in wine.

Table 3. Chromatographic parameters of phenolic compounds analyzed by HPLC

Phenolic compound	Retention time / min	Band UV / nm[a]	Regression equation	r[b]	LOD[c]	LOQ[d]	Concentration / (mg $100g^{-1}$ ± SD)[e]	Precision / %
Galic acid	8.6	272	y = 0.393x – 0.558	0.999	1.10	3.32	nd[f]	nd[f]
Vanilic acid	23.8	260, 280	y = 0.329x – 0.594	0.994	1.18	3.57	nd[f]	nd[f]
Caffeic acid	24.3	323	y = 1.005x – 3.448	0.998	0.94	2.85	14.22 ± 0.53	3.72
Para-coumaric acid	28.3	309	y = 0.798x – 1.144	0.998	0.12	0.35	37.05 ± 1.90	2.27
Ferulic acid	29.3	322	y = 0.982x – 0.558	0.997	0.31	0.94	15.44 ± 0.35	5.14

[a]λ_{max}: maximum absorption wavelength; [b]r: correlation coefficient = $\sqrt{r^2}$; [c]LOD: limit of detection in μg mL^{-1}; [d]LOQ: limit of quantification in μg mL^{-1}; [e]SD: standard deviation; [f]nd: not detected.

Conclusions

The results of this study confirm that the optimization of extraction conditions is important to obtain extracts rich in phenolic compounds and antioxidant activity from propolis. In addition, using factorial design it was clearly noticed the relationship between the responses, the extraction conditions and the interactions between the different extraction conditions tested.

Furthermore, the results obtained from the 2^3 factorial design indicate that the independent variables: ethanol concentration, extraction time and temperature are considered statistically significant in the extraction process of bioactive compounds in the propolis sample, being that the best response was obtained with the combination of the higher levels of the variables studied. The chromatographic analysis allowed the identification of three phenolic acids derived from hydroxycinnamic acid, common in Brazilian propolis. Regarding bioactivity, it is possible to conclude that both the total phenolic content and antioxidant activity are promising in the propolis produced by selected Aficanized honey bees *Apis mellifera*.

Acknowledgements

The authors are thankful to CNPq and Central de Análises (UTFPR - *campus* Pato Branco).

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Submitted: May 11, 2015
Published online: July 28, 2015

FAPESP has sponsored the publication of this article.