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## ORIGINAL RESEARCH ARTICLE

### Quantification of three phenolic classes and total phenolic content of propolis extracts using a single UV-vis spectrum

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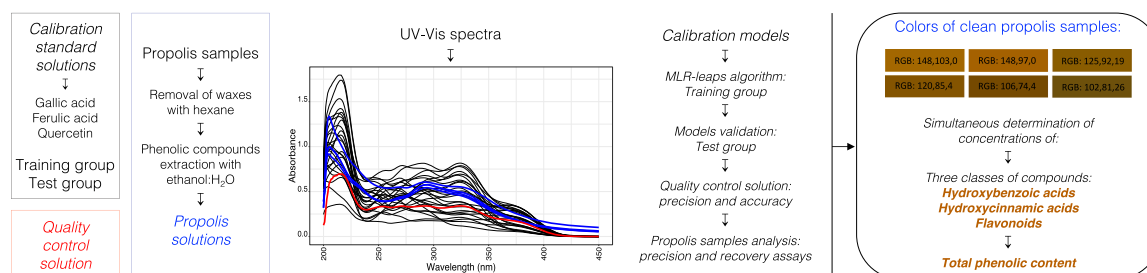
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This work presents a methodology for simultaneously measuring the total content of three classes of phenolic compounds (hydroxybenzoic acids, hydroxycinnamic acids and flavonoids), as well the total phenolic content using a single UV-vis spectrum. To test the methodology, samples of propolis were used. Firstly, an experimental design based in the Response Surface Methodology established that the best hydroethanolic solution for propolis phenolic compounds extraction had 80% of ethanol. Secondly, calibration models were developed with multiple linear regression, coupled with the leaps algorithm for variable selection, using UV-vis spectra of mixed standard solutions (gallic acid, ferulic acid and quercetin mixtures), all with orthogonal concentrations established by a multilevel fractional factorial design. The model's estimation and prediction performance had linearity between predicted and expected concentration values, with slope ranging from 0.996–1.04 and determination coefficients higher than 0.995. A quality control solution presented acceptable repeatability and accuracy (relative standard deviation percentage and percentage relative error were lower than 4.8%). Analysis of propolis phenolic compounds extracts showed acceptable precision (relative standard deviation percentages lower than 1.6%) and acceptable accuracy (recoveries assays ranged between 98 and 112%). Overall, the present multi-parametric analytical technique can be a first approach for chemical characterization of phenolic compounds extracts, such as that obtained from the propolis samples, considering its advantages of simplicity, rapidity, precision and accuracy. Moreover, this methodology can be adapted to other sample matrices, provided that the typical UV-vis absorption spectra of their classes of phenolic compounds shows partial overlapping, allowing multivariate calibration.

### Cuantificación de tres clases fenólicas y contenido fenólico total de extractos de propóleos utilizando un único espectro UV-Vis

Este trabajo presenta una metodología para medir simultáneamente el contenido total de tres clases de compuestos fenólicos (ácidos hidroxibenzoicos, ácidos hidroxicinámicos y flavonoides), así como el contenido fenólico total usando un solo espectro UV-Vis. Para probar la metodología, se utilizaron muestras de propóleos. En primer lugar, un diseño experimental basado en la metodología de la respuesta en superficie estableció que la mejor solución hidroetanólica para la extracción de compuestos fenólicos de propóleos tenía un 80% de etanol. En segundo lugar, se desarrollaron modelos de calibración con regresión lineal múltiple, junto con el algoritmo de saltos para la selección variable, utilizando espectros UV-Vis de soluciones estándar mixtas (ácido gálico, ácido ferúlico y mezclas de quercetina), todas con concentraciones ortogonales establecidas por un diseño factorial fraccional multinivel. La estimación del modelo y el rendimiento de la predicción tenían linealidad entre los valores de concentración previstos y esperados, con una pendiente que variaba de 0,996 a 1,04 y coeficientes de determinación superiores a 0,995. Una solución de control de calidad presentó aceptable repetibilidad y precisión (porcentaje de desviación estándar relativa y porcentaje de error relativo inferiores al 4,8%). El análisis de extractos de compuestos fenólicos de propóleos mostró una precisión aceptable (porcentajes de desviación estándar relativa inferior al 1,6%) y una precisión aceptable (los ensayos de recuperación variaron entre 98 y 112%). En general, la presente técnica analítica multiparamétrica puede ser un primer enfoque para la caracterización química de extractos de compuestos fenólicos, como el obtenido a partir de las muestras de propóleos, considerando sus ventajas de simplicidad, rapidez, precisión y precisión. Además, esta metodología puede adaptarse a otras matrices de muestra, siempre que los espectros de absorción UV-Vis típicos de sus clases de compuestos fenólicos muestren solapamiento parcial, permitiendo la calibración multivariante.



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**Keywords:** Propolis analysis; hydroxybenzoic acids; hydroxycinnamic acids; flavonoids; total phenolic content; single UV-vis spectrum; experimental design; multivariate calibration

## Introduction

Propolis is a resinous substance produced by bees for beehive protection, as a cement product to seal cracks or open spaces, to sterilize the queen-bee posture site and to embalm dead invaders (Almeida & Menezes, 2002; Bankova et al., 2016). Honey bees collect the basic propolis substances from sprouts, flower-buds, trees and other vegetal-tissue resinous exudates. The collected material is processed with enzymatic and salivary secretions and mixed with wax (Castaldo & Capasso, 2002; Sforcin, 2007; Sforcin & Bankova, 2011). The chemical composition of propolis includes phenolic compounds (phenolic acids, their esters, and flavonoids) as major and, as minor compounds, fatty acids, carbohydrates, aldehydes, amino acids, ketones, chalcones, dihydrochalcones, terpenoids, vitamins, and inorganic substances (Bankova et al., 2016; Castaldo & Capasso, 2002; Sahinler & Kaftanoglu, 2005). Phenolic acids and flavonoids have the most research interest (Havsteen, 2002; Lima et al., 2009).

Propolis may exhibit various colors and consistency due to its chemical composition, which depends on the plant sources (Lotfy, 2006) and the edaphoclimatic conditions. Due to this very complex chemical composition, propolis has various biological and pharmacological activities such as: antibacterial, anti-inflammatory, antifungal, antiprotozoan, antiviral activities, hepatoprotective, antioxidant, antitumor (Banskota, Tezuka, & Kadota, 2001; Ghisalberti, 1979; Lotfy, 2006; Sforcin, 2007; Sforcin & Bankova, 2011). It is thus fundamental to develop a simple and fast analytical method that allow the determination of several quality parameters in propolis samples. In general, the most measured parameters in propolis extract are total phenolic and total flavonoid contents (Choi et al., 2006; Lima et al., 2009; Mello, Petrus, & Hubinger, 2010), through two independent spectrophotometric analysis. Usually the analysis is performed with spectrophotometric methods, for example, the determination of propolis total phenolic content can be based on the Folin-Ciocalteu method (Choi et al., 2006; Dias, Pereira, & Estevinho, 2012; Lima et al., 2009; Mello et al., 2010; Moreira, Dias, Pereira, & Estevinho, 2008; Silva, Rodrigues, Feás, & Estevinho, 2012) and total flavonoid content, on the aluminum complexation method (Choi et al., 2006; Dias et al., 2012; Mello et al., 2010; Silva et al., 2012).

To obtain more chemical information, we proposed to verify whether the analytical concept used by Mazza, Fukumoto, Delaquis, Girard, and Ewert (1999) for the simultaneous determination of different phenolic classes in wine sample analysis by analyzing only one solution, and also adopted by Obied, Allen, Bedgood, Prenzler, and Robards (2005) for olive mill waste analysis, could be applied to propolis extracts. They applied an

ultraviolet-visible spectrophotometry method that allowed the simultaneous determination of different phenolic classes using a single spectrum: total phenols at 280 nm using gallic acid as standard, total hydroxycinnamic acid derivatives at 320 nm using caffeic acid as standard, flavonols at 360 nm using quercetin as standard and, at 520 nm, for anthocyanins using cyanidin chloride as standard. Note that, propolis extracts have higher phenolic content and present huge differences in matrix composition in relation to the two above works (Mazza et al., 1999; Obied et al., 2005). All these works used the UV-vis spectrophotometry analysis because of its advantages, such as rapid analysis, low-cost and simple technique.

So, in the present work it was intended to obtain calibration models for the simultaneous concentration determination of three classes of phenolic compounds (hydroxybenzoic acids, hydroxycinnamic acids and flavonoids) and total phenolic compounds using the information present in a single UV-vis spectrum. For this purpose, we used multiple linear regression with wavelength selection, by applying leaps algorithm (a variable feature algorithm) to overcome possible interferences within these three compound classes, in order to obtain linear calibration models and as well, a validation process with standard solutions and propolis samples to show the models predictive power.

## Materials and methods

### Chemicals and reagents

All reagents were of analytical quality and used as purchased. The following compounds were used as standards: gallic acid (1-Hidrate) from Panreac (99%, Spain), ferulic acid from Fluka ( $\geq 99\%$ , USA) and quercetin from Aldrich ( $>95\%$ , Belgium). As solvents, ethanol and hexane were acquired to Panreac (HPLC quality, Spain, 99.9%) and VWR (Analytical quality, France, 97.9%), respectively. Other reagents were concentrated hydrochloric acid from Carlo Erba (France, 37% and  $d = 1.18$ ), sodium carbonate from Merck (Germany) and Folin-Ciocalteu reagent from Panreac (Spain,  $d = 1.234$ ). The deionized water used in all analytical work was of type II.

### Sampling

Six Portuguese *Apis mellifera* propolis samples were collected for this study in several Portuguese regions. They had different colors, which ensured variability in the phenolic composition. Propolis samples were obtained by scraping its material from honey combs or by removal from propolis trap panels. Table 1 indicates the geographical origin, the general characteristics and the method of production of each propolis sample. The

Table 1. Portuguese propolis samples.

Geographical origin	Color of clean propolis	Sampling process	Portugal subregion–region
Braga	RGB: 120,85,4	Scraping	Minho–North
Montesinho	RGB: 148,97,0	Trap panels	Trás-os-Montes–North
Lousã	RGB: 102,81,26	Scraping	Baixo Mondego–Center
Macedo de Cavaleiros	RGB: 148,103,0	Scraping	Trás-os-Montes–North
Pinhel	RGB: 106,74,4	Scraping	Beira Interior–Center
Viana do Castelo	RGB: 125,92,19	Scraping	Minho–Lima–North

color of each sample was defined by photograph (Nikon D7000 Digital SLR Camera with 18–105 mm VR Lens) using ColorChecker Passport (X-Rite) to control the color profile. After adjusting the image colors in the Photoshop software (Adobe Photoshop CS5 Extended, Version 12.0), a rectangular section of the image was used, which included the sample variation in color tones, to set the average color and get the colors in the RGB format.

### Sample preparation

After a first manual separation of extraneous macro-materials, the raw propolis sample was frozen ( $-24^{\circ}\text{C}$ ) and ground to obtain a powder. A wax-free propolis sample was obtained using hexane to remove the wax. The procedure consisted in placing under magnetic stirring for one hour, a mixture in proportion of 5 g of raw propolis to 50 ml of hexane. The mixture was filtered and a second washing to the collected propolis material was carried out, again using 50 ml of hexane. After a new filtration, the solid material was dried for 1 h at  $40^{\circ}\text{C}$  in a drying oven (Memmert Heating Drying Oven UL 60). This final product was in the form of a powder, had a soft odor and the color was similar to the initial sample color. Each wax-free propolis sample was stored at  $-24^{\circ}\text{C}$  (Freezer Gram F 410 LH) until used in analytical assays.

### Phenolic compounds extraction optimization

In order to optimize phenolic compounds extraction conditions from the Portuguese propolis, a Response Surface Methodology was used to evaluate the influence of two factors: pH of the HCl solution, used as the aqueous phase, and ethanol percentage in the extracting hydroethanolic solution. A Box-Wilson central composite design was used with center points (block 1) and expanded with a group of “star” points (block 2) in order to estimate a second-degree polynomial model for the response variable considering these two factorial variables (Lenth, 2009; Myers, Montgomery, & Anderson-Cook, 2009).

### Solutions prepared

The work involved the study of the effect of pH between 0.9 and 5.1 as well as, the percentage of

Table 2. Experimental design for extraction of phenolic compounds.

Assay number	Block	Real values		Coded values	
		pH	Ethanol %	pH	Ethanol %
1	1	3.00	60	0	0
2		3.00	60	0	0
3		4.50	85	+1	+1
4		3.00	60	0	0
5		1.50	35	−1	−1
6		4.50	35	+1	−1
7		1.50	85	−1	+1
8	2	3.00	60	0	0
9		3.00	24.6	0	−1.4
10		3.00	95.4	0	+1.4
11		0.88	60	−1.4	0
12		5.12	60	+1.4	0
13		3.00	60	0	0
14		3.00	60	0	0

ethanol in the hydroethanolic extraction solution used, between 24.6 and 95.4%. The Table 2 shows the assays carried out defined by the experimental design (Lenth, 2009; Myers et al., 2009). In these studies, a mass of 0.1 g of a wax-free propolis of the Lousã sample was mixed with 21 ml of each pH value and hydroethanolic solution defined by the experimental design and, after magnetic agitation was filtered into a flask of 25 ml, and the final volume was adjusted with the respective pH–hydroethanolic solution. The optimal extractor solution will be obtained using the response surface methodology statistical analysis and, as response, the total phenolic content determined in the hydroethanolic extracts, using the Folin-Ciocalteu method (Obied et al., 2005).

### Total phenolic content analysis

The determination of total phenolic content in the propolis filtered solution was performed according to the method of Singleton and Rossi (1965) modified by Obied et al. (2005). In a 10 ml volumetric flask containing 7 ml water was added 0.1 ml of filtered propolis solution extract. Then 0.5 ml of Folin-Ciocalteu reagent was added and after 1 min, 1.5 ml of aqueous sodium carbonate solution (20% w/v). The flask was shaken and the volume adjusted to 10 ml with water. After 1 h at ambient temperature and in the dark, the absorbance was read at 760 nm. The results were expressed in milligrams equivalents of gallic acid per gram of wax-free propolis.

### Multivariate calibration

The three classes of phenolic compounds (hydroxybenzoic acids, hydroxycinnamic acids and flavonoids) to be analyzed were represented by three standard compounds: gallic acid; ferulic acid and quercetin, respectively. For the multivariate calibration, it was applied a multilevel fractional factorial design ( $5^{k-(k-2)}$ , where  $k$  is the number of factors) (Jeff Wu and Hamada 2011). This experimental design establishes the mixing of factors and levels guaranteeing that all compounds concentrations in the mixing solutions are uncorrelated or orthogonal to each other. Considering the case of three factors (compounds) and five levels (concentrations), the Table 3 shows their combination in each solution.

### Calibration standard solutions

So, for the multivariate calibration, 25 mixed solutions of gallic acid, ferulic acid and quercetin were prepared using as solvent the optimal extractor solution. For this, three stock solutions were prepared of each referred pure compound (gallic acid, ferulic acid and quercetin) with concentrations of  $1000 \text{ mg l}^{-1}$ . The mixed solutions of those compounds prepared using five levels of concentrations (10, 20, 40, 60 and  $80 \text{ mg l}^{-1}$  for gallic and ferulic acids; 10, 20, 35, 50 and  $60 \text{ mg l}^{-1}$  for quercetin). With this design was also possible to establish the total

phenolic content by assuming the sum of the masses of those three phenolic compounds,

$$[\text{TPC}, \text{mg l}^{-1}] = [\text{Gallic acid}, \text{mg l}^{-1}] + [\text{Ferulic acid}, \text{mg l}^{-1}] + [\text{Quercetin}, \text{mg l}^{-1}],$$

showing satisfactory variability and amplitude in the dynamic interval of the calibration (calibration dynamic interval between 40 and  $210 \text{ mg l}^{-1}$  of phenolic compounds).

The UV-vis spectrum of each assay was obtained, using the Obied et al. methodology (Obied et al., 2005), by mixing 1 ml of the prepared solution with 1 ml of ethanol at 95% containing 0.1% hydrochloric acid and adding aqueous 2% hydrochloric acid solution until the final volume is adjusted to 10 ml (spectrum solution).

### Sample solutions

Each propolis sample was prepared by dissolving 0.125 g of wax-free propolis sample in 25 ml of the extractor solution. This solution was filtered and dried at  $40^\circ \text{C}$  in a drying oven (Memmert Heating Drying Oven UL 60) until a resin product of constant mass was obtained (clean propolis sample). The propolis solution for analysis was prepared by dissolving 400 mg of clean propolis sample in 10 ml of the extractor solution. A diluted propolis solution was prepared by diluting 0.4 or 0.7 ml of the previous solution (the measured volume depends on the total amount of phenolic compounds) in 10 ml of the extractive solution. It was with this solution that the spectrum solution was prepared. This procedure was repeated until at least three concordant results were obtained in each sample, in order to establish the method precision.

### Samples recovery assays with standard addition solution

For recovery assays, a mixed solution of concentration  $100 \text{ mg l}^{-1}$  of each standard compound (gallic acid, ferulic acid and quercetin) was prepared, using the extractor solution as solvent (standard addition solution). Depending on the levels of phenolic compounds that each sample had, 0.4 or 0.7 ml of the diluted propolis solution and 1 ml of the standard addition solution was added in a 10 ml flask and checked with the extractive solution. This solution was used to prepare the spectrum solution. The standard addition procedure was carried out until at least 3 concordant results were obtained for all samples in order to establish the method accuracy.

### UV-vis spectrophotometry analysis

All spectra were obtained in the UV-vis spectrophotometer (VWR UV-3100PC spectrophotometer) in the wavelength range between 190 and 1100 nm, using a quartz cuvette, using scan step of 1.0 nm, with recording speed of 35 nm/s and reproducibility of absorbance measuring of 0.002.

Table 3. Multilevel fractional factorial design for three factors and five levels.

Assay number	Gallic acid $\text{mg l}^{-1}$	Ferulic acid $\text{mg l}^{-1}$	Quercetin $\text{mg l}^{-1}$
1	40	40	35
2	40	10	20
3	10	20	10
4	20	10	60
5	10	80	60
6	80	80	35
7	80	40	20
8	40	20	60
9	20	80	20
10	80	20	50
11	20	60	50
12	60	60	35
13	60	40	60
14	40	80	50
15	80	60	60
16	60	80	10
17	80	10	10
18	10	10	35
19	10	40	50
20	40	60	10
21	60	10	50
22	10	60	20
23	60	20	20
24	20	20	35
25	20	40	10

Source: Jeff Wu and Hamada (2011).



### Statistical analysis

Data processing was performed with R software (R version 3.3.2, 2016-10-31), a free software environment for statistical computing and graphics. In the optimization study to establish the best procedure conditions for propolis phenolic compounds extraction a Response Surface Methodology was applied. The Response Surface Methodology was implemented by the R package *rsm* (Lenth, 2009) that allowed to generate a standard 2-variable design with 8 corner points and 3 center points (a Box-Wilson central composite design with center points and expanded with a group of "star" points), as well to fit a first order or second-degree polynomial model and also testing the significance of their interaction. After defining the model, its adequacy was evaluated by checking diagnostics and plots of the estimated response surface model (Maroco, 2007), by verifying: randomness and normality of the residuals; cook's distance (values greater than 1 are indicative that these are excessively influential in the model); leverage values (values below 0.2 are acceptable, values between 0.2 and 0.5 are risky and values higher than 0.5 indicate the presence of an influential value or outlier); model's *p*-value (to evaluate the significance of the model obtained using the significance level of 0.05); determination coefficient value ( $R^2$ , to verify the amount of variance explained by the model); and, relative standard error (*rse*, to confirm the magnitude of the model errors).

For the multivariate calibration, in order to analyze simultaneously the three classes of phenolic compounds, a multilevel fractional factorial design was established to obtain mixed solutions of three compounds with five concentration levels to assure orthogonality between solutions (Jeff Wu & Hamada, 2011). These solutions were analyzed to obtain the UV-vis spectra. A multiple linear regression (MLR) coupled with a variable selection algorithm, was used to obtain several best models between concentrations and absorbance at selected wavelengths. The leaps package of R software (Lumley & Miller, 2017) was set to perform exhaustive search for the best subsets of the variables, using the efficient branch-and-bound algorithm. It allowed to program the search for the best models, which have the highest adjusted  $R^2$ , having subset sizes between 1 and 3 variables (results do not depend on a penalty model for model size).

The data split was carried out by applying the *k*-means clustering algorithm, present in the package *prospectr* (Stevens & Ramirez-Lopez, 2013), which selects the assays (using the spectra data) that were closer to the formed cluster centroids for the train group. The mixed solutions were divided into two groups: train group for MLR model construction (80% of mixed solutions); test group to verify the predictive performance of the obtained model (20% that corresponds to 5 of the mixed solutions).

The selected models, for each standard phenolic compound (representing the classes of hydroxybenzoic

acids, hydroxycinnamic acids and flavonoids) and total phenolic content, were evaluated in its quality by checking intercept importance, ANOVA results, diagnostics and plots of the estimated model, as previously described.

Further analysis involved the evaluation of the models' ability to estimate and predict using the train and test group mixed standard solutions, respectively. The linearity between the concentrations calculated by the model and those expected was checked (slope and intercept should be close to the theoretical values, 1 and 0, respectively), as well as, the *p*-value and determination coefficient value.

Finally, the selected models were used to predict the concentrations of the classes of phenolic compounds and total phenolic content in the samples. The precision of analysis was studied by repeatability assays and the accuracy by recovery assays, which is an important quality assessment tool of sample analysis. For this procedure, a standard addition solution was used, adding to sample (method of standard addition) a small known quantity of the compounds under estimation, which was subjected to analysis for the total amount of compounds present. Recovery results were obtained by subtracting the concentrations of the added compounds to those total amounts in sample with addition of mixture standard solution and comparing these results with samples concentrations obtained without standard addition solution. Satisfactory recoveries give confidence in the accuracy of the method of analysis (Harvey, 2000).

Also, the standard addition solution was used as an independent quality control solution (reference solution) to establish precision and accuracy of analysis.

### Results

Since the propolis samples has a complex and resinous matrix, optimization of the phenolic compounds extraction process is important to assure that the extract obtained is suitable to measure its quality.

#### Phenolic compounds extraction optimization

The optimization of the best analytical conditions for phenolic compounds extraction was studied based in the Response Surface Methodology. Two factors were considered: pH of the HCl solution varying between 0.9 and 5.1, used as the aqueous phase, and ethanol percentage in the extracting hydroethanolic solution, between 24.6 and 95.4%.

The extracts obtained were evaluated in its total phenolic content using the Folin-Ciocalteu colorimetric method (Obied et al., 2005).

The minimum and maximum yields for this analysis were of 2.3 and 11.7 mg eq. of gallic acid per ml of propolis hydroethanolic solution, showing that the composition of the hydroethanolic solution clearly affects the amount of phenolic compounds extracted.

In the fit of the response variable (total amount of phenolic compounds) to the two factors studied, it was verified that a second-degree polynomial was the best function to fit the experimental data (Figure 1), being the ethanol percentage the influential variable, with a positive and negative sign for the respective linear and quadratic terms (both with  $p$ -value  $< 0.001$ ), while pH variable (as well, the quadratic term) and interaction terms were not significant ( $p$ -values  $> 0.26$ ). The model obtained had also significant intercept ( $p$ -value  $< 0.001$ ).

The final model was significant ( $p$ -value  $< 0.001$ ) and had an adjusted determination coefficient ( $R^2$ ) of 0.994, meaning that the fitted model explained more than 99% of the data variability. Moreover, the model obtained showed to have random residues that followed a normal distribution confirming the model fitting suitability to the data. The values of Cook's distance according to the assay number were lower than 0.5, indicative that there were no values excessively influential in the model. In the case of the leverage values assessment, it was found results above 0.6 (leverage values higher than 0.5 indicate the possible presence of influential value or outlier) but, since they corresponded to assays with low residues and were related to the lower and higher response values, it was merely an indication that the fitted model will pass close to those particular extreme observations (Maroco, 2007).

The results from the experimental assays indicated that the optimal experimental condition for extracting the phenolic compounds in propolis samples were pH 2.4 and 79.4% of ethanol in the extracting hydroethanolic solution. In the following tests, the pH value was set equal to 3 (although, pH value was not a significant factor in the range studied) and the ethanol percentage in

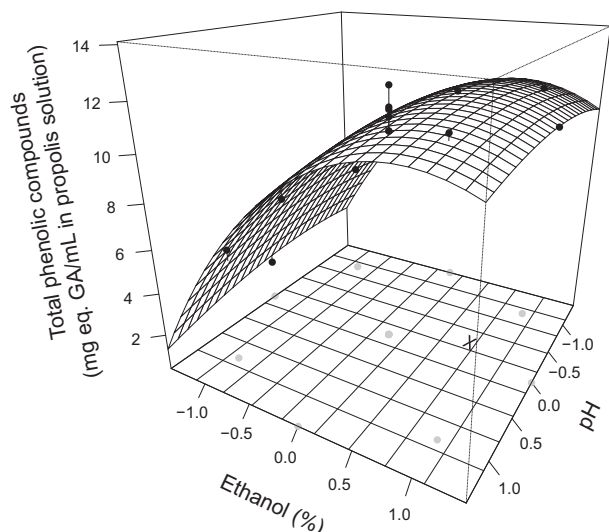


Figure 1. Results (mg eq. gallic acid  $l^{-1}$ ) of Response Surface Methodology design applied to establish optimal propolis phenolic extraction values for the coded values of pH and ethanol percentage in the extracting hydroethanolic solution. The “X” letter marks the optimal point.

the extracting hydroethanolic solution to 80%, being in accordance with the conditions used in other studies (Dias et al., 2012; Falcão et al., 2013; Silva et al., 2012).

### UV-vis spectra of individual standard solutions

The simultaneous quantification of the three classes of phenolic compound (hydroxybenzoic acids, hydroxycinnamic acids and flavonoids) was carried out by using standard mixed solutions of gallic acid, ferulic acid and quercetin, representative compounds of those classes, respectively. The Figure 2(A) shows the spectra of the

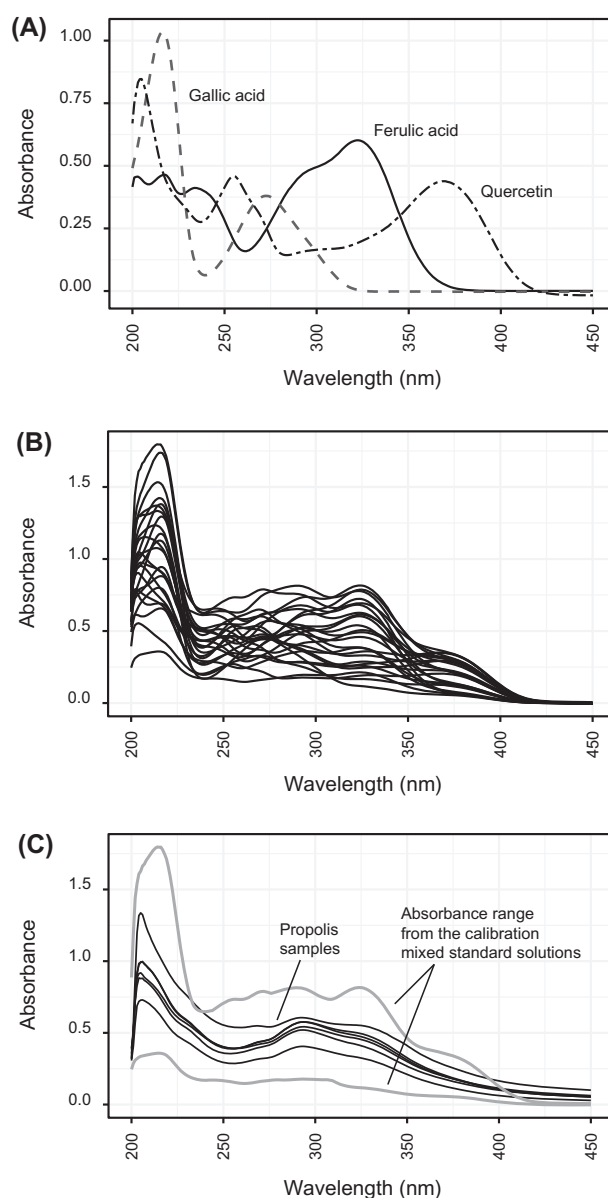


Figure 2. UV-vis Spectra of the: (A) gallic acid, ferulic acid and quercetin standard solutions with concentration of  $1000 \text{ mg } l^{-1}$ ; (B) mixed standard solutions of gallic acid, ferulic acid and quercetin prepared according to experimental design for multivariate calibration; (C) propolis samples and the maximum and minimum absorbance limits of the mixed standard solutions.

three individual standard solutions, with concentrations of  $1000 \text{ mg l}^{-1}$ , in the wavelength range of 200 to 450 nm. The gallic acid spectrum shows absorption peaks at 215 and 275 nm; the ferulic acid at 320 and a shoulder at 280 nm; and, quercetin at 205, 255 and 370 nm. It was possible to visualize that there are distinct zones of UV-vis absorption between the three compounds. The spectrum of gallic acid showed greater overlap by the other two spectra and so, the estimation/prediction model is expected to be more complex. This figure allowed to infer that it would be possible to extract information from the spectra to quantify the total phenolic compounds levels of those classes, because each class of phenolic compounds has a characteristic spectrum (Andrade & Seabra, 2005; Robbins, 2003), although with intensity variations in absorption pattern.

#### UV-vis spectra of mixed standard solutions

Twenty-five mixed standard solutions of gallic acid, ferulic acid and quercetin were prepared with five concentration levels, according to the mixing order of the concentrations defined by a fractional factorial design, so that the concentration levels between these solutions were orthogonal. Due to this fact, the total phenolic content was also analyzed. All solutions were prepared with the 80% of ethanol in the extracting hydroethanolic solution and 20% of HCl aqueous solution with pH value of 3 (optimal extraction solution). The mixed solutions presented concentrations varying between 10 and  $81 \text{ mg l}^{-1}$  for gallic acid, between 11 and  $85 \text{ mg l}^{-1}$  for ferulic acid and 10 and  $61 \text{ mg l}^{-1}$  for quercetin and, for the total phenolic content (sum of the phenolic standard compounds masses used in each mixed solution preparation), between 41 and  $206 \text{ mg l}^{-1}$ .

The spectra of all standard mixed solutions are shown in Figure 2(B) and, generally, they show absorbance less than 1.4, except for the absorbance values obtained in the wavelength range of 200–225 nm. The multivariate calibration was performed using the spectra obtained in the wavelength range between 200 and 450 nm, since at higher wavelengths there was no evidence of absorbance. As referred previously, when using spectra, it is possible to have components overlapped and so, a multivariate statistical method could be required to extract information from these spectra about the amount of each compound. Also, most signals in spectrum are correlated, which allowed to simplify the study by considering the spectra matrix with wavelengths of values with termination zero and five. So, for the quantification of the three standard compounds, which will be representative of total amounts of three classes of phenolic compounds in samples, and the total content in phenolic compounds, the leaps algorithm was used, for the selection of wavelengths to which the absorbance reading would be made, coupled with the MLR technique.

#### Calibration models

In this study, the multivariate calibration was based on a set of 25 spectra with known concentrations of standard compounds representative of the three classes of phenolic compounds. This data was divided into training group (80% of the spectra of the standard mixing solutions) and test group (20% that corresponds to 5 spectra of the standard mixed solutions) using the *k*-means clustering algorithm. The test group presented concentration levels within the limits defined by the solutions of the training group; the gallic acid concentrations in the mixed standard solutions ranged from  $10\text{--}81 \text{ mg l}^{-1}$ ; ferulic acid, between 11 and  $85 \text{ mg l}^{-1}$ ; quercetin, from  $20\text{--}51 \text{ mg l}^{-1}$ ; and, total phenolic content, between 102 and  $201 \text{ mg l}^{-1}$ .

This division made possible to establish the MLR-leaps model using the training data and to verify the ability of the model to predict the concentrations using the test data, as new mixing solutions, by comparing the predicted model results with expected values. The MLR-leaps technique was applied in order to obtain the linear models with measurements of 1 to 3 different wavelengths. All models were tested for the importance of the intercept in the equation, and it was verified that in some of the models it was not significant, but all had large confidence intervals, far from the theoretical value (zero), which originated final models with very high errors. Their removal allowed the calibration models to be significant ( $p < 0.001$ ) and represent more than 99% of the variability of the analytical data. Each final MLR model was selected considering the overall quality results of the:  $R^2$  and rse; linear relation (without the intercept since  $p$ -value was  $> 0.05$ ) between the concentrations estimated by the model and the expected values for the train group, by using the  $R^2$ , rse, slope and its confidence interval; linear relation (without the intercept since  $p$ -value was  $> 0.05$ ) between the concentrations predicted by the model and the expected values for the test group, by using the  $R^2$ , rse, slope and its confidence interval. Table 4 shows all those results for the selected models and Figure 3 presents the plots obtained for the linear relation between the values obtained by each model and the expected results of the train and test groups.

In general, the selected models present measurements at two wavelengths, except for quercetin that with a single wavelength allows to obtain acceptable prediction results. As can be seen, the models explained 99%, or more, of the variability found in the analytical data and with low and similar total errors (rse), except in the quantification of total phenolic content, which has an error greater than twice the error of the quantification models of the individual standard phenolic compounds. Each model obtained was significant ( $p < 0.001$ , Table 4), indicative of a good fit of the analytical data. Also, all independent variables selected for each model were significant ( $p < 0.001$ ), considering the ANOVA results.



Table 4. The selected models and their results of fitting quality, as well as the estimation and prediction performance results using the train and test data, respectively.

Compound	Wavelengths (nm)	Model equation	Model performance		
			$R^2$	rse	p-value
Gallic acid	275 345	$C = 192 (\pm 3, p\text{-value} < 0.001) \times \text{Abs}_{275} - 156 (\pm 5, p\text{-value} < 0.001) \times \text{Abs}_{345}$	0.9977	2.18	<0.001
Ferulic acid	325 380	$C = 129 (\pm 2, p\text{-value} < 0.001) \times \text{Abs}_{325} - 78 (\pm 4, p\text{-value} < 0.001) \times \text{Abs}_{380}$	0.9984	2.15	<0.001
Quercetin	375	$C = 182 (\pm 3, p\text{-value} < 0.001) \times \text{Abs}_{375}$	0.9945	3.02	<0.001
TPC	220 345	$C = 186 (\pm 8, p\text{-value} < 0.001) \times \text{Abs}_{220} + 75 (\pm 9, p\text{-value} < 0.001) \times \text{Abs}_{345}$	0.9975	6.36	<0.001

Compound	Estimation with train data					Prediction with test data				
	$R^2$	rse	p-value	Slope	CI	$R^2$	rse	p-value	Slope	CI
Gallic acid	0.9977	2.12	<0.001	1.00 ( $\pm 0.01$ , $p\text{-value} < 0.001$ )	[0.97; 1.02]	0.9989	1.91	<0.001	1.00 ( $\pm 0.02$ , $p\text{-value} < 0.001$ )	[0.95; 1.05]
Ferulic acid	0.9984	2.10	<0.001	0.998 ( $\pm 0.009$ , $p\text{-value} < 0.001$ )	[0.98; 1.02]	0.9995	1.51	<0.001	1.01 ( $\pm 0.01$ , $p\text{-value} < 0.001$ )	[0.98; 1.04]
Quercetin	0.9945	3.01	<0.001	0.99 ( $\pm 0.02$ , $p\text{-value} < .001$ )	[0.96; 1.03]	0.9988	1.76	<0.001	1.03 ( $\pm 0.02$ , $p\text{-value} < 0.001$ )	[0.98; 1.08]
TPC	0.9975	6.18	<0.001	1.00 ( $\pm 0.01$ , $p\text{-value} < 0.001$ )	[0.97; 1.02]	0.9992	4.01	<0.001	1.01 ( $\pm 0.01$ , $p\text{-value} < 0.001$ )	[0.97; 1.04]

Notes: TPC = Total phenolic content;  $R^2$  = Determination coefficient; rse = Residual standard error; CI = Confidence interval.

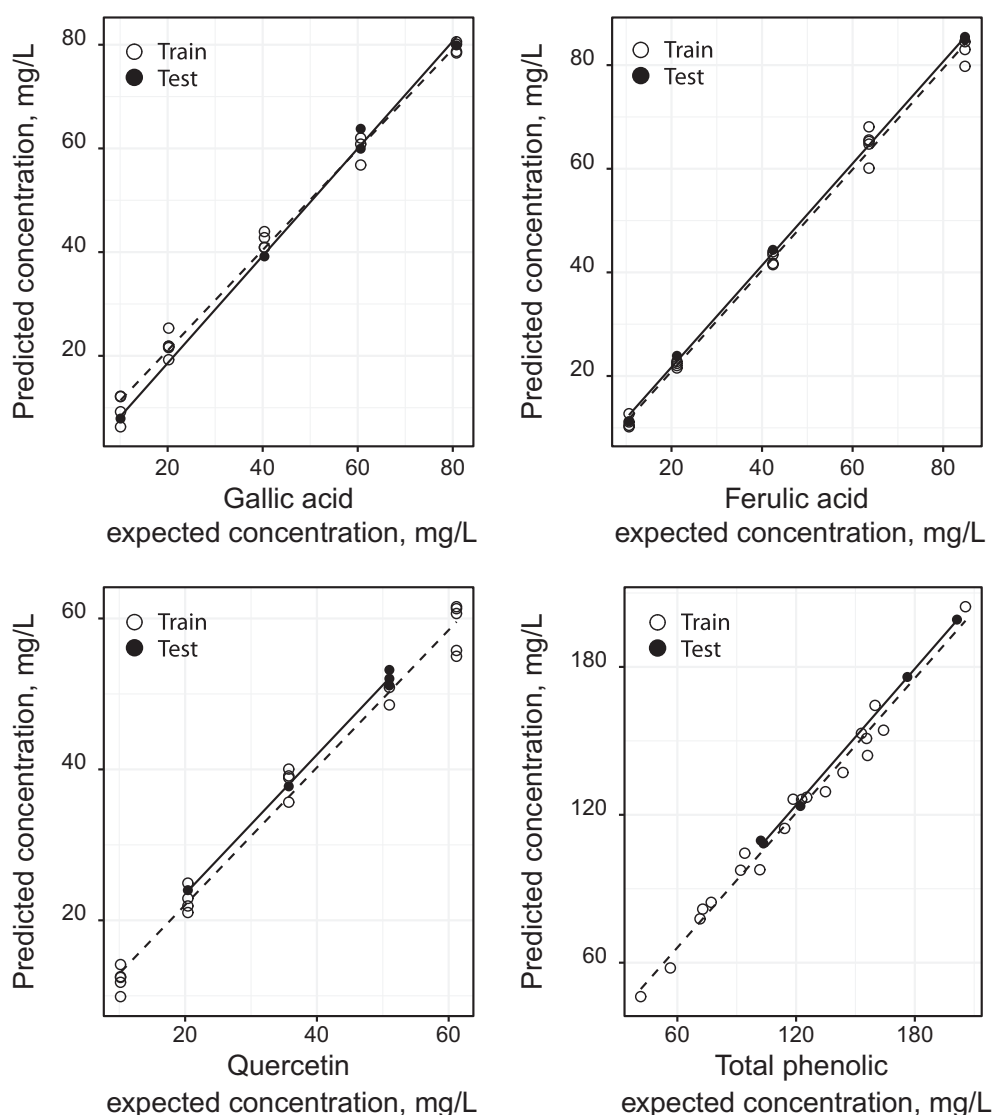


Figure 3. Linear relation between the values obtained by each model and the expected results of the train and test groups for the gallic acid (represents the compounds of the class of hydroxybenzoic acids), ferulic acid (represents the class of hydroxycinnamic acids), quercetin (represents the class of flavonoids) and total phenolic content.

Table 5. Mean and standard deviation for the concentrations of total phenolic compounds and each class of phenolic compounds analyzed in samples of propolis (values presented with two different units).

Sample	Hydroxybenzoic acids class mg GA l <sup>-1</sup>	Hydroxycinnamic acids class mg FA l <sup>-1</sup>	Flavonoids class mg QC l <sup>-1</sup>	Total phenolic content mg TPC l <sup>-1</sup>
Braga	35.8 (±0.5)	49.0 (±0.5)	32.6 (±0.2)	119.2 (±0.9)
Lousã	38.4 (0.6)	54.0 (±0.4)	46.8 (±0.6)	147.4 (±0.6)
Montesinho	34.3 (±0.5)	46.1 (±0.4)	32.9 (±0.5)	114.3 (±0.2)
Macedo de Cavaleiros	32.9 (±0.2)	51.0 (±0.2)	33.7 (±0.4)	121.0 (±0.4)
Pinhel	36.6 (±0.1)	41.3 (±0.3)	29.1 (±0.2)	106.8 (±0.2)
Viana do Castelo	28.2 (±0.5)	33.5 (±0.5)	20.3 (±0.4)	83.2 (±0.8)

Sample	Hydroxybenzoic acids class mg GA g <sup>-1</sup> propolis	Hydroxycinnamic acids class mg FA g <sup>-1</sup> propolis	Flavonoids class mg QC g <sup>-1</sup> propolis	Total phenolic content mg TPC g <sup>-1</sup> propolis
Braga	222 (±3)	303 (±3)	202 (±1)	737 (±8)
Lousã	136 (±2)	191 (±2)	165 (±2)	520.2 (±0.7)
Montesinho	212 (±3)	285 (±3)	204 (±3)	707.1 (±0.9)
Macedo de Cavaleiros	204 (±1)	316 (±1)	209 (±3)	749 (±2)
Pinhel	227.3 (±0.8)	257 (±2)	182 (±1)	664 (±1)
Viana do Castelo	176 (±3)	209 (±3)	127 (±2)	518 (±5)

Notes: GA = Gallic acid; FA = Ferulic acid; QC = Quercetin; TPC = Total phenolic content.

The selected models were also evaluated in its estimation quality by verifying: linearity between the concentrations predicted by the model and those expected ( $R^2$  and slope should be close to the theoretical values, 1, and rse, the least possible); residues random distribution and normality; Cook distances and leverage values according to the standardized residuals. In the linearity study, the intercept was removed since it was not significant in model ( $p$ -value > 0.05). All adjusted models presented normality and randomness in the residues, as well as low Cook distances (values lower than 0.5) and leverage values (values lower than 0.6), indicating that there was no evidence of extreme values or outliers. Table 4 shows acceptable estimative results with  $R^2$  and slopes values greater than 0.99. In the case of slopes, their values can be considered theoretical (1) because this value is within the confidence interval. The overall errors are also low and, as expected, in the same order as those presented in the model. Similar study was carried out to evaluate the predictive capacity of the models, using the test group mixed standard solutions, by comparing the expected results with the concentrations measured with the calibration models. The results were slightly better than those obtained for the model's estimation study (using training group data), showing good predictive capacity of these models with orthogonal mixed standard solutions.

#### Propolis samples analysis

Figure 2(C) shows the spectrum of each propolis sample, where it can be seen that the spectra are in the middle zone of the dynamic range of multivariate calibration, ensuring a correct application of the developed

analytical method. These spectra can be used as examples of a typical spectrum for a Portuguese clean propolis extract (wax-free and purified). As mentioned above, the results of the application of the calibration models obtained to the spectra of the propolis samples are expressed into total concentrations of the respective classes of the standard phenolic compounds used. The mean concentrations and respective errors obtained for each sample are presented in Table 5. The samples from Viana de Castelo and Lousã regions had the lowest levels of phenolic compounds in the clean propolis extract (mg TPC g<sup>-1</sup> propolis), while the sample of Macedo de Cavaleiros, had the highest levels. In the analyzed samples, the class of hydroxycinnamic acids was the most predominant, followed by the class of hydroxybenzoic acids and, at lower levels, the class of flavonoids. Two exceptions were the samples from regions of Lousã and Macedo de Cavaleiros, where the levels of flavonoids compounds were higher than those of hydroxybenzoic acids.

It was verified that, in general, the percentage relative standard deviation of the concentration results was less than 1.6%, indicating that the developed methodology allows acceptable precisions. In order to verify the agreement between the results of the total concentrations obtained for the classes of hydroxybenzoic acids, hydroxycinnamic acids and flavonoids in relation to that obtained for the total content of phenolic compounds for each sample, the percentage of the mean deviation between this value and the calculated value, by adding the measured total concentrations of the three classes of compounds, was computed. These results were, in general, less than 2.8%, except for the Lousã sample, where the value of 5.6% was obtained. These results

are indicative that the established prediction models gave generally acceptable results.

### Quality control solution analysis

A mixture solution of the three phenolic standards (gallic acid, ferulic acid and quercetin) with concentrations close to  $100 \text{ mg l}^{-1}$  for each compound was used as quality control solution, in the study of the precision and accuracy of models prediction. The values of the concentrations measured showed acceptable precision since the relative standard deviation percentages were lower than 0.5%. Accuracy was also generally acceptable since the relative error percentages obtained were 3.8, 4.7 and 4.2%, respectively for the standard phenolic compounds analyzed. For the total phenolic content, the relative error percentage obtained was of 2.1%. It was also verified that the variation between the total concentration of phenolic compounds obtained and the total concentration calculated by the sum of the total concentrations of each standard phenolic compound analyzed in the mixture solution was low (mean deviation percentage of 0.3%), showing that the models applied were robust in predicting concentrations in new synthetic solutions.

### Recovery assays study

In the samples recovery trials study, the previous standard mixture solution was used as the standard addition solution, by adding a volume which established a concentration increase of  $10 \text{ mg l}^{-1}$  of each standard compound, in the samples. Analyzes of samples with standard addition showed, as expected, acceptable precisions, since the relative standard deviations of the concentrations were less than 1.6%. The total concentrations of each class of phenolic compounds and total phenolic content analyzed in the samples with and without addition of standard showed results of recovery rates varying between 98 and 112%. With two exceptions, the determination of total concentration of hydroxybenzoic acids class and total phenolic content in the Lousã sample that presented lower values (84 and 92%, respectively) but, also considered acceptable.

### Results in literature

As discussed earlier, works on propolis commonly involves the determination of total concentration in phenolic compounds and/or classes of phenolic compounds because the medicinal properties of this product are usually attributed to these compounds. The results are frequently presented in units of mg equivalent of gallic acid per g of propolis extract or as percentage values. In this study, Table 5 show the amounts obtained for classes of phenolic compounds (values varied between 109 and  $254 \text{ mg g}^{-1}$  of propolis extract) and total phenolic content (values were higher than

$419 \text{ mg g}^{-1}$  of propolis extract) in samples of propolis. These results were higher than those reported in some works (Dias et al., 2012; Moreira et al., 2008; Silva et al., 2012) in which the object of study were Portuguese propolis samples. Moreira et al. (2008) used two Portuguese propolis samples obtained in the Bornes and Fundão regions, where the levels of total phenolic compounds were of 329 and 151 mg equivalent of gallic acid per g of propolis extract (dry methanolic extract obtained at room temperature), respectively. Dias et al. (2012) studied four propolis samples from four neighboring Portuguese regions of Trás-os-Montes district with different floral origins. The samples extracts were obtained using 80% of ethanol/water (at  $70^\circ\text{C}$  for 1 h) and they showed total phenolic content varying between 11 and 28% and total flavonoids content, between 3 and 12%. Silva et al. (2012) evaluated the efficacy of three extraction procedures (80% of ethanol/water, 80% of methanol/water and aqueous extract) using Portuguese propolis samples, having selected the ethanol/water extract (obtained at  $70^\circ\text{C}$  for 1 h), because it was the most effective for extracting phenolic compounds. For the extract selected, three samples from different Portuguese regions possessed different levels of total phenolic and flavonoids concentrations: Bragança region, 278 mg equivalent of gallic acid per g of propolis extract and 142 mg equivalent of catechin per g of propolis extract, respectively; Coimbra region, 157 mg equivalent of gallic acid per g of propolis extract and 98 mg equivalent of catechin per g of propolis extract, respectively; Beja region, 87 mg equivalent of gallic acid per g of propolis extract and 25 mg equivalent of catechin per g of propolis extract, respectively.

However, the results were in agreement with those reported by Falcão et al. (2013). It was a study of phenolic quantification of forty Portuguese propolis samples collected from six different geographical regions, which phenolic compounds extraction was carried out with 80% of ethanol/water (at  $70^\circ\text{C}$  for 1 h). Due to the high sampling variability, the total phenolic acids ranged between 5 and 96 mg per g of propolis extract and total flavonoids varied between 14 and 535 mg per g of propolis extract.

So, although the above-mentioned studies show differences in the extraction procedure, the overall results obtained in this work can be considered as expected for Portuguese propolis samples.

### Discussion

The present work established a new analytical methodology based on analysis of a single UV-vis spectrum that allowed to obtain the total concentrations of three classes of phenolic compounds and total phenolic content in a propolis extract. All results showed that the analytical methodology developed in this work presented acceptable global quality of precision and accuracy both in the analysis of reference solutions and in

samples. Also, the results from total content of phenolic compounds can be used to establish a state of statistical control when compared with the calculated value by adding the measured total concentrations of the three classes of phenolic compounds. This procedure gives reliable information, which can be used as a first approach to propolis chemical characterization, important to establish the products quality in propolis application studies such as those of biological activities. Moreover, it should be noted that this methodology can be adapted to other sample matrices, provided that an extract of phenolic compounds is obtained and that standards of compounds representative of their classes of phenolic compounds have UV-vis absorption spectra with partial overlapping.

### Conflict of interest

The authors declare that they have no conflict of interest.

### Compliance with Ethics Requirements

This article does not contain any studies with human or animal subjects.

### Disclosure statement

No potential conflict of interest was reported by the authors.

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