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Article

Applicability of an HPLC method for analysis of alcoholic and glycolic Brazilian green-propolis extracts

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Brazilian green propolis has been widely used in food and pharmaceutical products due to its valuable source of phenolic compounds and versatile biological activities. The development and validation of analytical methods are extremely useful for the characterization and quality control of products containing propolis. Therefore, the aim of this study was to optimize, validate and investigate the applicability of a reversed-phase HPLC method for analysis of different types of Brazilian green propolis extracts (glycolic and ethanolic). The method showed to be selective for the propolis phenolic markers. The analysis of variance and residues demonstrated that the method had significant linear regression, without lack of fit. It was also a precise, accurate and robust method, which was of utmost importance to analyze both glycolic and ethanolic extracts and at different concentrations. Moreover, as these products can display most complex matrices to analyze, a valid HPLC method can also prove to be specific and versatile.

Keywords: Propolis. High-performance liquid chromatography (HPLC). Glycolic extract. Ethanolic extract. Phytochemicals.

INTRODUCTION

Propolis (PRP) is a gum resin produced by bees *Apis mellifera* L. from various plant sources around the hive (Burdock, 1998; Bruschi, Franco, Gremião, 2003). In addition to these plant materials, bees also add salivary secretions and enzymes (Greenaway, Scaysbrook, Whatley, 1990; Moreira, 1986). Consequently, PRP is used in the defense of the hive, sealing gaps, and protecting against microorganisms, humidity, and the entrance from intruders and dirt (Bruschi *et al*., 2002; Marcucci, 1995).

The chemical composition of PRP is complex and is related to the vegetation around the hive (Cabral *et al*., 2012). In general, this bee product contains 50-60% resinous compounds and gums, 30-40% waxes, 5-10% volatile oils and aromatic acids, 5% balsams and pollen grains, and 5% of other substances such as polyphenols (flavonoids and phenolic acids), vitamins, mineral salts and impurities (Burdock, 1998; De Francisco *et al*., 2018; Castro *et al*., 2014; Escriche, Juan-Borrás, 2018; Bruschi *et al*., 2003). It has a wide variety of compounds that are important for biological activities, such as terpenoids, steroids, flavonoids, phenolic acids, and esters (Park *et al*., 2002; Longhini *et al*., 2007; Bruschi *et al*., 2006).

During the last decades, PRP has been extensively used for the improvement of human nutrition and health. It is a valuable source of phenolic compounds and displays versatile biological activities such as antibacterial, fungicide, antioxidant, anti-inflammatory, antiviral and immunostimulant (Bruschi *et al*., 2002; Cabral *et al*., 2012; De Francisco *et al*., 2018; Castro *et al*., 2014; Escriche, Juan-Borrás, 2018; Lustosa *et al*., 2008). For this reason, PRP has been widely used in food and pharmaceutical products.

As the PRP chemical composition can vary from different regions and harvest seasons (De Francisco *et al*., 2018; Castro *et al*., 2014), it may present a heterogeneous composition of biologically active substances. Over the last 30 years, Brazilian green PRP has shown great interest, and it is the object of intensive research (Bruschi

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et al., 2002; Marcucci *et al*., 2001; Dota *et al*., 2011; Said dos Santos *et al*., 2020; Said dos Santos *et al*., 2021; Rosseto *et al*., 2017). This PRP type is well known and employed due to its antimicrobial activities (Bruschi *et al*., 2016; Oliveira *et al*., 2006; Ota *et al*., 2001). Therefore, it is essential to seek quality control and standardization of PRP formulations (Pereira, Bruschi, 2013; Coneac *et al*., 2014). Moreover, the *in natura* drug is usually processed, and the extractive solution may be obtained using ethanol or propylene glycol-water as a solvent, producing ethanolic or glycolic extracts, respectively. Ethanol is the most applied liquid of extraction, due to the better dissolution of resins it induces in comparison to the propylene glycol-water mixture. However, glycolic extract is less aggressive towards mucous membranes and skin (Longhini *et al*., 2007). These extracts are utilized as final or intermediate dosage forms in pharmaceutical products and food supplements (Burdock, 1998; Escriche, Juan-Borrás, 2018; Bruschi *et al*., 2006).

The liquid, thin layer and gas chromatography and/or mass spectrometry have been proposed for analysis of PRP extracts (Bankova, Popov, Marekov, 1982; Christov, Bankova, 1992; Pereira *et al*., 1998; Maciejewicz *et al*., 2001; Bruschi *et al*., 2003; Nunes *et al*., 2012; Rosseto *et al*., 2017). Most of these analytical procedures showed to be composed of a long time of analysis, with complex steps, and also dependent of several and expensive analytical standards and reagents. In this context, high-performance liquid chromatography (HPLC) is one of the most utilized technologies for the analysis of PRP extracts, constituting a very useful and smart strategy for the separation and determination of PRP main substances (Bruschi *et al*., 2003; Escriche, Juan-Borrás, 2018). However, during the first decade of this century, HPLC methodologies were proposed for the analysis of Brazilian green PRP extracts, mainly those containing ethanol (Bruschi *et al*., 2003; Rosseto *et al*., 2017).

Therefore, it is very important the development of valid, and more suitable methods, for the analysis of PRP in routine procedures, such as characterization and quality control of PRP extracts prepared using different solvents like ethanol and propylene glycol (Ota *et al*., 2001; Pereira, Bruschi, 2013). Hence, the aim of this work was to optimize and validate a reversed-phase HPLC method for analysis of both ethanolic and glycolic PRP extracts. Two well-known phenolic standards (chrysin and *p*-coumaric acid) were utilized as markers for a faster and simpler HPLC method for analysis of the PRP extracts. The development and validation of the methodology were based on internationally recognized guidelines (European Commission, 2002; ICH, 2005; Magnusson, Örnemark, 2014), and the linearity, specificity, accuracy, limits of detection and quantification, repeatability, reproducibility and robustness were determined. Moreover, the method was evaluated as an application that considered two different types of PRP extracts (ethanolic and glycolic).

MATERIAL AND METHODS

Material

Chrysin (purity≥97%) and *p*-coumaric acid (purity≥98%) were purchased from Sigma-Aldrich (Sao Paulo, SP, Brazil). Methanol (HPLC grade) was purchased from Honeywell (Charlotte, NC, USA). All other materials and solvents were of analytical reagent grade. Ultra-purified water was obtained in-house using a water purification system (Evoqua Water Technologies, Pittsburgh, PA, USA).

Apparatus and analytical conditions

All chromatographic analyses were performed on a HPLC system model Prominence-i LC-2030C 3D (Shimadzu®, Tokyo, Japan), equipped with a micro vacuum degasser, auto-sampler for automatic sample injection, column oven, and photodiode array (PDA) detector. The LabSolutions Lite software (Shimadzu®, Tokyo, Japan) was utilized for data acquisition and elaboration. The PDA detector allowed the collection of absorbance spectrum for each PRP phenolic compound, which was identified by comparison with the standards. It used an analytical C_{18}Hypersil® Gold PFP column (250 x 4.6 mm), packed with 5 µm particle size (Thermo Scientific®, Waltham, MA, USA), and equipped with a drop-in guard cartridge. The mobile phase consisted of acetic acid aqueous solution (1.5%, v/v) as solution A and methanol as solution B. The gradient elution was

performed from 0 to 45% of solution A in 20 min. The flow rate was 1.0 mL/min, the injection volume was 20 µL, and the column temperature was set at 25°C.

Two analytical standards were used as markers for HPLC analysis: chrysin and *p*-coumaric acid. Chrysin was dispersed in methanol at a concentration of 100 µg/mL and from this first solution, seven others were prepared at concentrations of 0.2, 0.3, 0.6, 0.9, 2.0, 3.0 and 4.0 µg/mL. For *p*-coumaric acid, it was prepared a solution in methanol at a concentration 50 µg/mL. From this solution, eight other dilutions were prepared at 0.03, 0.05, 0.07, 0.1, 0.3, 0.4, 0.5 and 0.6 µg/mL. All solutions were properly filtered through 0.45 µm (13 mm) PTFE membrane (Merck Millipore, Cork, Ireland) before HPLC analysis at wavelength $\lambda = 310$ nm.

Validation of analytical methodology

The proposed method was validated according to internationally recognized guidelines (European Commission, 2002; ICH, 2005; Magnusson, Örnemark, 2014), and the following parameters were determined: specificity, linearity, the limit of detection (LOD), the limit of quantification (LOQ), precision, accuracy and robustness. In all cases, a p-value < 0.05 was taken to denote significance, and the software Statistica 12.5® (StatSoft Company, Tulsa, OK, USA) was used throughout.

Specificity

Specificity is defined as the ability of the method to unequivocally analyze the presence of sample components and other interferences (European Commission, 2002; ICH, 2005; Magnusson, Örnemark, 2014; Lopes, Bruschi, Mello, 2009). It must ensure that the method differentiates the related compounds present in the sample, and in addition, must demonstrate that the result is not affected by other compounds. Therefore, the specificity of the methodology was analyzed by using the markers (chrysin and *p*-coumaric acid) standard stock solutions and the stock solutions of PRP extract samples. Moreover, the marker's peak on different samples was also analyzed, considering the complex mixtures and the solvents (ICH, 2005; Junqueira, Borghi-Pangoni, Bruschi, 2018).

Linearity, analysis of the lack of fit and residual analysis

The linearity between the peak's area versus concentration was determined through calibration curves obtained on three different days with the standard solutions of chrysin or *p*-coumaric acid, as previously described. Five replicate curves for each marker were employed. The dependence between the area of peak versus the concentration of standard was treated by linear regression. The residual analysis was performed by calculating the F value from the ratio between the mean square of the regression (MSreg) and the mean square of the residue (MSres), at the selected confidence level. As higher as the ratio of MSreg/MSres and $F_{reg,res}$ was, the more significant was the regression (Pimentel, Neto, 1996). The analysis of lack of fit was determined by calculating the ratio between the mean square of lack of fit (MS_{laf}) and the mean square of pure error (MS_{pe}). If $MS_{laf}/MS_{pe} < F_{laf,pe}$, the value of the model is considered satisfactory and adjusted (Junqueira, Borghi-Pangoni, Bruschi, 2018; Pimentel, Neto, 1996).

Limit of detection and limit of quantification

The limit of detection (LOD) and limit of quantification (LOQ) were determined from the calibration curves considering their standard deviation and slope (Pereira, Bruschi, 2013; ICH, 2005), according to the following equations Eq. (1) and Eq. (2):

$$LOD = \frac{3.\sigma}{S} \quad (1)$$

$$LOQ = \frac{10.\sigma}{S} \quad (2)$$

Where σ is the standard deviation and S is the slope of the calibration curve.

Precision

The precision was estimated at two levels: intermediate precision, between different days and different analysts (two), and repeatability (intra-day). The time interval between the days was carried out on three consecutive days. The intraday was determined

using three samples in a short time. Analysis of variance was used to estimate the total variability of the analytical method. Precision was expressed as relative standard deviation (RSD) of standard concentrations (ICH, 2005; Lopes, Bruschi, Mello, 2009; Junqueira, Borghi-Pangoni, Bruschi, 2018). The analysis was performed in triplicate.

Accuracy

The accuracy was reported as percentage recovery, when comparing the difference between the theoretical concentration and the value found for each concentration. It was calculated by practical concentration of the analyte divided by the theoretical value, and multiplied by 100. The results were expressed as recovery data and the values should be within 80-120% (ICH, 2005; Junqueira, Borghi-Pangoni, Bruschi, 2018). Data were evaluated by ANOVA. The analysis was performed in triplicate.

Robustness

Robustness is the measure of an analytical procedure of not being affected by small variations in parameters that indicate reliability during normal use (ICH, 2005). The robustness of the HPLC analytical methodology was evaluated using three different wavelengths (305, 310 and 315 nm). These values were utilized considering the small wavelength variations of the equipments, in order to get a greater safety interval for the analyses, considering the complexity of the PRP samples to be analyzed (Bruschi *et al*., 2003; ICH, 2005; Rosseto *et al*., 2017). ANOVA was utilized to evaluate the variability of the results.

Applicability of method

Preparation of PRP extracts

Brazilian green PRP was obtained from hives of *Apis mellifera* L. bees, located at the northwest of Parana state, found in a eucalyptus reserve with the predominance of a native shrubby plant *Baccharis dracunculifolia* (Asteraceae). This research was registered in Brazil under SISGEN N° AC7A2F5. All PRP extracts were prepared by turbo extraction technique. The ethanolic extracts were obtained using ethanol 96 ºGL and the comminuted drug in the drug:solvent (w/w) ratios were 30:70 (EE30%) and 10:90 (EE10%). The glycolic extracts were obtained using the comminuted drug and an aqueous solution of propylene glycol 50% (w/w) in the drug:solvent (w/w) ratios of 30% (GE30%) and 10% (GE10%). Afterward, the final dispersions were filtered through grade 3 filter paper (Bruschi *et al*., 2002; Rosseto *et al*., 2017).

Sample preparation from PRP extracts

All PRP extracts (EE10%, EE30%, GE10% and GE30%) were submitted to extraction and separation of polyphenol fraction. A sample of PRP extract (1.0 mL) plus 1.0 mL of acetone and 5.0 mL of water were added in a separation funnel. This mixture was three times extracted with 5.0 mL of ethyl acetate. Ethyl acetate was filtered, rendering S1. The solvent of S1 was evaporated using a water bath at 40°C. The residue was dispersed in methanol up to 2.0mL, rendering S2. This methanolic solution S2 was used for spectrophotometric and HPLC analysis as previously described.

RESULTS AND DISCUSSION

Method validation

Validation means ensuring that routine analyses can reproduce consistent values compared to a reference value (European Commission, 2002; ICH, 2005; Magnusson, Örnemark, 2014). The proposed methodology must be valid, as variation can occur and damage the reliability of the results. The validation of an analytical method, as part of quality control, aims to ensure, through experimental studies, that the method meets the necessary requirements of analytical applications, thus guaranteeing the reliability of results (ICH, 2005). Nunes and collaborators utilized a HPLC methodology for quality control of propolis extract that took a time of 60 min per run and was performed at two main wavelengths: 280 and 340 nm (Nunes *et al*., 2012). In this work, the proposed method shows the advantage of 20 min of run time, and covered a wider range of wavelengths for the final analysis of components. In another work, Coneac and collaborators analyzed PRP

extracts of propolis by HPLC and they observed that the separation of the markers did not occur accordingly, which was not considered as a method of good specificity (Coneac *et al.*, 2014). In the analysis of a complex matrix such as PRP extracts, the method, its characteristics, as well as its usefulness determine the parameters that must be considered (Bruschi *et al.*, 2003).

Specificity

The specificity is defined as the ability of a method to measure the analyte accurately and specifically in the presence of components in the sample matrix (ICH, 2005). The conditions utilized resulted in chromatograms displaying peaks in a run time of 20 min, with retention time of 4.9 min and 16.5 min for *p*-coumaric acid and chrysin, respectively (Figure 1). This relatively short run time was considered as good, in view of other proposed methods that displayed longer time of analysis (Bankova, Popov, Marekov, 1982; Christov, Bankova, 1992; Pereira *et al*., 1998; Maciejewicz *et al*., 2001; Bruschi *et al*., 2003; Nunes *et al*., 2012; Rosseto *et al*., 2017). Moreover, the selectivity of both methods was observed with no interference of solvents. It was not observed absorption of another substance than the marker in the wavelength range, besides that, the peaks were well resolved. The absorption of another material than the markers were not observed in the visible range utilized, in addition peaks were well resolved.

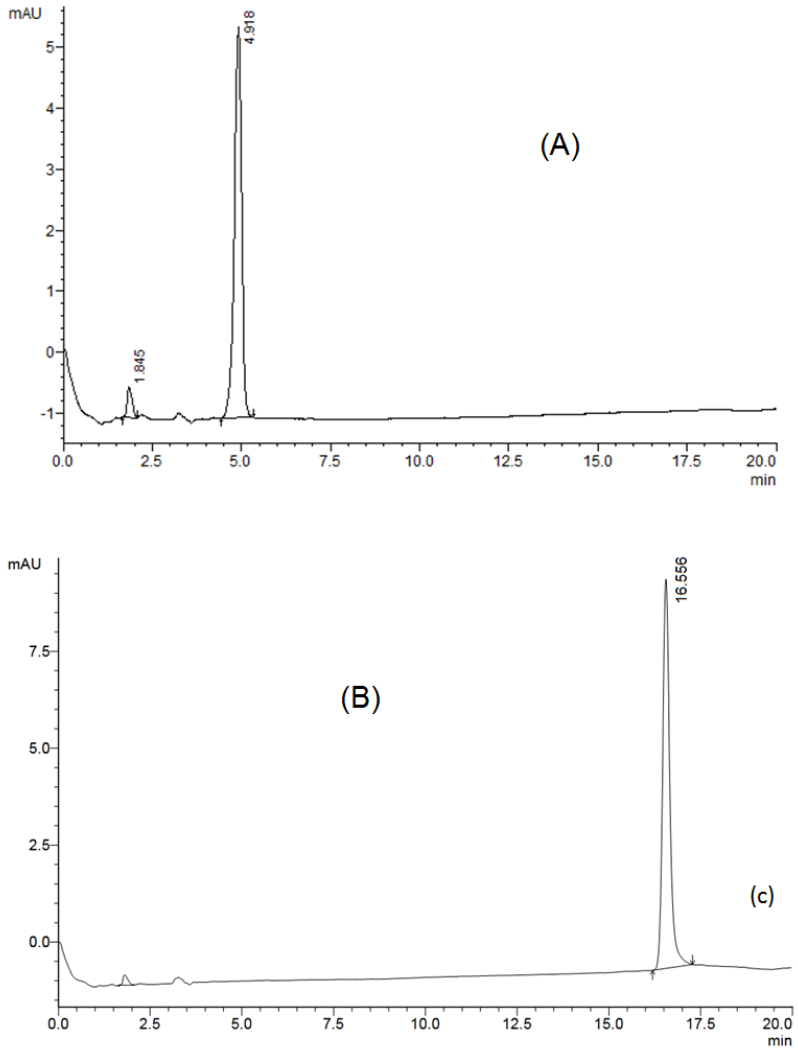

FIGURE 1 - HPLC chromatograms of *p*-coumaric acid (A) and chrysin (B) at wavelength (λ) 310 nm.

Linearity

The linearity of an analytical procedure denotes its ability (within a given range) to obtain test results that are directly proportional to the concentration (quantity) of the substance to be analyzed in the sample (European Commission, 2002; ICH, 2005; Magnusson, Örnemark, 2014). The results for the investigation of linearity of the HPLC analytical method are displayed in Table I.

The linearity of both chromatographic analyses was investigated from dilutions of the *p*-coumaric acid and chrysin solution, as previously described (Figure 1S and Figure 2S). The representative linear equation for *p*-coumaric acid was $y = 144779x - 364.59$ (R = 0.9971) and for chrysin was $y = 50093x - 3550$ (R = 0.9984).

The analysis of regression showed the results for lack of fit (Table I). In addition, the residual analysis was applied to study the difference between observed *y* value and *y* value estimated by regression model, consequently, showed the significance of the regression model.

Both analytical methods displayed a highly significant regression, since the values of MS_{reg}/MS_{res} were higher than the $F_{reg,res}$ value (Table I). Moreover, the methods also displayed MS_{laf}/MS_{pe} values less than the respective $F_{laf,pe}$ value (Table I) and they did not present lack of fit. Therefore, both analytical curves displayed linearity and could be used (ICH, 2005).

TABLE I - Analysis of variance (quadratic model) for validation of HPLC method for determination of *p*-coumaric acid or chrysin

	Sum of squares	Degree of freedom	Mean square	F-value	$F\text{-critical}_{95\%}$
(*p*-Coumaric acid)					
Regression	36994810508	1	36994810508	6597.41	4.10
Residual	213084212.7	38	5607479.282		
Lack-of-fit	49422772	6	8237129	1.61	2.40
Pureerror	163661440	32	5114420		
(Chrysin)					
Regression	$2.28602x10^{11}$	1	$2.28602x10^{11}$	15003.79	4.05
Residual	716105313.8	47	15236283.27		
Lack-of-fit	152099858	6	25349976	1.64	2.30
Pure error	741236241	48	15442422		

Limit of detection and limit of quantification

The limit of detection (LOD) is the smallest amount of analyte in a sample that can be detected, but not necessarily quantified. The limit of quantification (LOQ) is the lowest amount in a sample that can be quantitatively determined with adequate precision and accuracy (ICH, 2005). The values of LOD were 0.0135 µg/mL and 0.0561 µg/mL, and the values of LOQ were 0.0408 µg/mL and 0.1700 µg/mL for the markers *p*-coumaric acid and chrysin, respectively. These values were very low and they can enable the analysis of a low amount of markers in PRP extract samples (ICH, 2005; Bruschi *et al*., 2003; Rosseto *et al*., 2017)

Precision

To evaluate the precision parameter of methodology, the intermediate precision and repeatability were performed (Table II). The results for intraday precision displayed a mean relative standard deviation (RSD) smaller than 5% for the replicates of all levels of *p*-coumaric acid and chrysin within each day, which shows showing the repeatability of both analyses. Moreover, the intermediate precision results (interday and interanalysts precision) also showed a mean RSD lower than 5%. There were no significant differences between the tests ($p<0.05$), either intraday, interday or with different analysts. Thus, it was observed a good precision of both analyzed markers (ICH, 2005).

TABLE II - Precision data of HPLC methodology for analysis of *p*-coumaric acid and chrysin

PRECISION						
	Repeatability			**Intermediate precision**		
p-coumaric acid (µg/mL)	Intraday	RSD (%)	Interday	RSD (%)	Interanalist	RSD (%)
0.03	0.0192 ± 0.0003	1.32	0.0198 ± 0.0010	4.83	0.0329 ± 0.0015	4.69
0.10	0.0867 ± 0.0021	2.41	0.0903 ± 0.0031	3.46	0.0938 ± 0.0045	4.85
0.30	0.3179 ± 0.0051	1.61	0.2842 ± 0.0104	3.66	0.2780 ± 0.0135	4.87
Chrysin (µg/mL)	Intraday	RSD (%)	Interday	RSD (%)	Interanalist	RSD (%)
0.30	0.3653 ± 0.0064	1.75	0.3200 ± 0.0072	2.24	0.3263 ± 0.0126	3.85
2.00	1.9032 ± 0.0619	3.25	1.9603 ± 0.0347	1.77	1.9281 ± 0.0775	4.02
4.00	4.0239 ± 0.1597	3.97	4.0930 ± 0.0642	1.57	4.0708 ± 0.1975	4.85

RSD = Relative standard deviation

Accuracy

The accuracy of an analytical method is expressed as the percentage of recovery, being determined by the difference between the theoretical concentration, and the real value found for each concentration (ICH, 2005). The accuracy data for both methods was expressed in the percentage of recovery (Table III). The mean values obtained were 99.24 ± 0.24% and 98.17 ± 1.39% for *p*-coumaric acid and chrysin, respectively. These results are in accordance with the limits established, being between 80 - 120% (ICH, 2005).

TABLE III - Accuracy of HPLC methodology for analysis of *p*-coumaric acid or chrysin

ACCURACY			
***p*-coumaric acid (µg/mL)**	***p*-coumaric acid concentration found (µg/mL)**	**Recovery (%)**	**Mean recovery (%)**
0.40	0.3924 ± 0.0098	96.41 ± 2.36	
0.50	0.49936 ± 0.0125	98.15 ± 2.83	98.22 ± 0.24
0.60	0.6163 ± 0.0158	100.12 ± 2.58	

TABLE III - Accuracy of HPLC methodology for analysis of *p*-coumaric acid or chrysin

ACCURACY			
Chrysin (µg/mL)	Chrysin concentration found (µg/mL)	Recovery (%)	Mean recovery (%)
0.30	0.3177 ± 0.0152	103.91 ± 5.36	
0.90	0.8982 ± 0.0457	97.93 ± 5.30	98.17 ± 1.39
3.00	2.8332 ± 0.0915	92.65 ± 2.93	

Robustness

The robustness data (Table IV) should demonstrate the reliability of the analysis considering the variations of method parameters, and the interference of minor changes in the experimental conditions for the assay (ICH, 2005). The wavelength (310 nm) changed to 305 and 315 nm. The ANOVA analysis did not show significant differences between the results obtained using different conditions ($p>0.05$). Therefore, considering the possibility of small wavelength variations of the equipments during the analysis and also the complexity of potential PRP samples to be analyzed (Bankova, Popov, Marekov, 1982; Maciejewicz *et al*., 2001; Bruschi *et al*., 2003; ICH, 2005; Nunes *et al*., 2012; Rosseto *et al*., 2017), the analytical methodology is considered as robust.

TABLE IV - Evaluation of robustness of the HPLC methodology for analysis of of *p*-coumaric acid and chrysin, using different wavelengths

***p*-Coumaric acid (µg/mL)**	**305 nm**	**310 nm**	**315 nm**	**RSD (%)**
0.40	0.3834± 0.0028	0.3914± 0.0026	0.37599 ± 0.0021	2.01
0.50	0.4903 ± 0.0109	0.4990 ± 0.0113	0.4797 ± 0.0108	1.97
0.60	0.5980 ± 0.0144	0.6088 ± 0.0144	0.5860 ± 0.0137	1.91
Chrysin (µg/mL)	**305 nm**	**310 nm**	**315 nm**	**RSD (%)**
0.30	0.3172 ± 0.0048	0.3273 ± 0.0056	0.3325 ±0.0059	3.03
0.90	0.8949 ± 0.0362	0.9156 ± 0.0336	0.9383 ± 0.0378	2.56
2.00	1.9030 ± 0.0515	1.9775 ± 0.0560	2.0187 ± 0.0574	3.09

RSD = Relative standard deviation

Method applicability

Regarding the quality of food and pharmaceutical products containing PRP, a common problem is the number of ways in which PRP extracts are produced and marketed without standardization and control. Moreover, PRP extracts are produced using water, ethanol, and also propylene glycol as a solvent, resulting in preparations with different physicochemical, nutritional, biological and pharmacological properties (Bruschi *et al.*, 2002; Castro

et al., 2014; Escriche, Juan-Borrás, 2018; Rosseto *et al*., 2017; Pereira, Bruschi, 2013). The growth in the use of preparations containing Brazilian green PRP demands approaches to the quantification of assets and therefore, a series of analytical procedures have been proposed (Castro *et al*., 2014; Escriche, Juan-Borrás, 2018; Pereira, Bruschi, 2013; Bankova *et al*., 2001; Banskota, Tezuka, Kadota, 2001; De Funari, Ferro, Mathor, 2007).

Food and pharmaceutical products containing PRP can be prepared using ethanolic or glycolic propolis extracts. These extractive solutions are frequently prepared using a PRP ratio from 10 to 30%. Therefore, two types of propolis extracts (ethanolic and glycolic) at two levels of drug concentration (10 and 30%, w/w) were prepared and their contents of *p*-coumaric acid and chrysin were determined using the validated HPLC method (Table V; Figures 2 and 3).

TABLE V - Analysis of propolis extracts by HPLC method

Propolis extract	Content (%, w/v)	
	p-coumaric acid	Chrysin
EE10%	0.0026 ± 0.00001	0.0041 ± 0.00003
EE30%	0.0121 ± 0.0.00002	0.0180 ± 0.0002
GE10%	0.0021 ± 0.00001	0.0009 ± 0.00002
GE30%	0.0096 ± 0.0001	0.0014 ± 0.00002

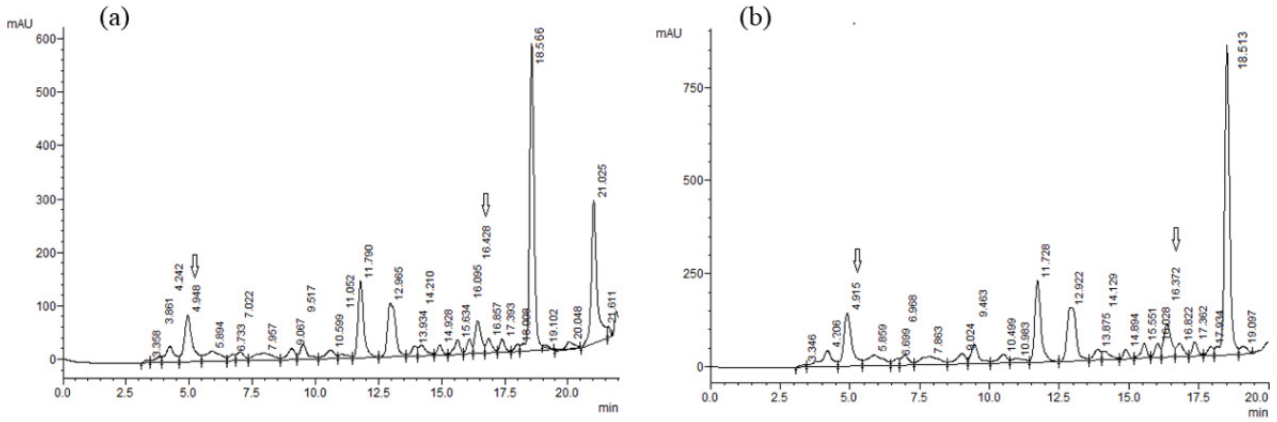

FIGURE 2 - Chromatogram of ethanolic propolis extracts obtained by HPLC at 310 nm: (a) EE10%; (b) EE30%. Arrows show the markers *p*-coumaric acid (4.948 min and 4.915 min) and chrysin (16.428 min and 16.372 min).

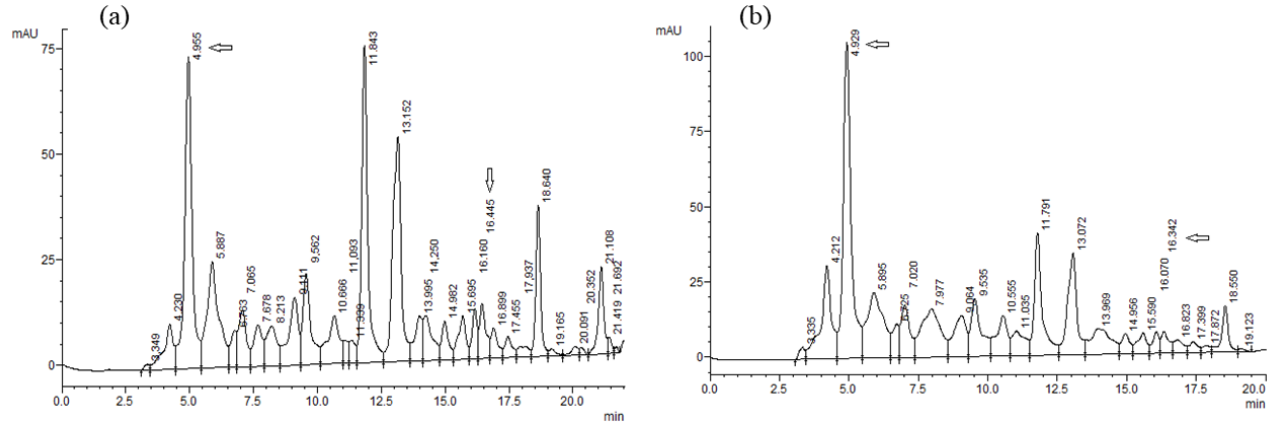

FIGURE 3 - Chromatogram of glycolic propolis extracts obtained by HPLC at 310 nm: (a) GE10%; (b) GE30%. Arrows show the markers *p*-coumaric acid (4.955 min and 4.929 min) and chrysin (16.445 min 16.342 min).

During the analysis of complex biologic matrices like PRP extract samples, the methodology type and its use determine the parameters to be evaluated (Bruschi *et al*., 2003). Moreover, regarding the high number of compounds and chemical complexity of PRP, the peak resolution of markers could be considered (Bankova, Popov, Marekov, 1982; Christov, Bankova, 1992; Pereira *et al*., 1998; Maciejewicz *et al*., 2001; Bruschi *et al*., 2003; Nunes *et al*., 2012; Rosseto *et al*., 2017).

The analytical methodology demonstrated to be robust for the analysis of different extracts. Both of them exhibited suitable content of *p*-coumaric acid and chrysin (Bruschi *et al*., 2002; Rosseto *et al.,* 2017; Pereira, Bruschi, 2013). However, ethanol proved to be the best solvent for extracting PRP in the different concentrations tested. Propylene glycol-water solvent could extract PRP, but the resulted extracts showed a lower content of markers. Compared with ethanolic extracts, the chrysin content in the glycolic extracts (GE10% and GE30%) was relatively more reduced than the *p*-coumaric acid. The solvent propylene glycol-water is more polar than ethanol, and it could extract more *p*-coumaric acid, which also shows more polar characteristics than chrysin. Therefore, the identification and quantification of phenolic compounds of the different extracts from Brazilian green PRP samples demonstrated to be effective (Cavalaro *et al*., 2019). During this work, the obtained and standardized PRP extracts in the laboratory were evaluated for the development of the method in question. Products already commercialized were not used since the proposal was, to obtain an adequate and robust method for the analysis of ethanolic and glycolic propolis extracts, and that the same methodology could be used for both extracts. Thus, considering the results, the proposed HPLC method can be utilized in future studies involving the analysis of market PRP extracts.

CONCLUSION

The HPLC method was valid for analysis of *p*-coumaric acid and chrysin markers. All evaluated parameters were in agreement with the established guidelines. Validation experiments confirmed the good accuracy, precision and recovery of the methodology. Moreover, it demonstrated to be useful for the analysis of propolis extracts containing different solvents and drug ratio, as they can be applied in the future for analysis of food and pharmaceutical products. However, it should be considered that these products can show complex matrices to analyze, and the HPLC method showed also to be specific and versatile.

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CONFLICTS OF INTEREST

No conflict of interest mentioned.

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