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Accuracy Enhancement of the Folin-Ciocalteu Method for Propolis

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Abstract

This study presents an optimization of the Folin-Ciocalteu spectrophotometric method for the determination of total phenol content. Multivariate optimization using factorial planning 2^2 with a central point and central composite planning was constructed to evaluate the influence of variables in the process and maximize radiation absorption with minimal radiation scattering caused by solid formation. X-ray fluorescence and X-ray diffraction spectrometry were used to evaluate the chemical composition of solids formed and nephelometric and spectrophotometric studies were also used to evaluate whether the type, origin, dilution and dry extract contents of commercial propolis extracts would significantly influence the increase in radiation scattering and absorption. The optimized methodology added several advantages, such as reduction of reagents, time analysis, and higher accuracy.

Keywords

Folin-Ciocalteu, Multivariate Optimization, Propolis, Total Phenol

1. Introduction

Phenolic compounds include a wide variety of molecules that have hydroxyl groups bound to aromatic rings. Polyphenols are divided into several classes according to the number of phenolic rings that the molecule can contain and the structural elements that connect to these rings. The main groups of polyphenols are flavonoids, phenolic acids, tannins (hydrolyzable and condensed), stilbenes, and lignans [1].

Many analytical procedures have been developed for the quantification of total phenolic compounds, and although separation methods such as gas chromatography and high-efficiency liquid chromatography are powerful techniques used

for the isolation and identification of phenolic compounds in complex samples, dissolution techniques are time-consuming, expensive, and not suitable for routine analysis. For quantification of total phenolic compounds, many available methods are based on the reaction of phenolic compounds with a colorimetric reagent, thus allowing their measurement in the visible region [2]. Among these methods, Folin-Ciocalteu (FC) has been frequently applied [3] [4], and studies have shown that total phenols, determined using this method, can be correlated with antioxidant activity [5].

The spectrophotometric method using the FC reagent was initially developed in 1927 to determine tyrosine [6]. The FC reagent consists of a mixture of sodium molybdate or phosphomolybidic acid, sodium tungstate, or phosphotungstic acid, and other reagents such as orthophosphoric acid and hydrochloric acid [7]. The method is based on the oxidation of phenolate ions under alkaline conditions, while the FC reagent is reduced [8]. After the reaction with phenols, a blue color is produced, with absorption at 765 nm, attributed to complexed Mo(V) species [7].

Many of the active components present in propolis, such as phenolic acids and flavonoids, have a phenolic group that can be evaluated using this method. The sodium carbonate buffer (pH = 11.9) is added to the medium to obtain an alkaline medium, necessary for the reaction, and when is added to the solution containing the FC reagent (pH around 0.9), the pH of the solution is maintained at around 8.5. The basic medium provides the deprotonation of phenolic groups facilitating the process of complexation of these compounds with the metals present in the FC reagent.

There are several studies in the literature involving the study of substances capable of generating an alkaline medium, such as NaOH, NH_4OH, K_2CO_3, Na_3PO_4, and Na_2CO_3, in several concentrations. In the studies of Box [9], Lowry and collaborators [10], the use of NaOH generated turbidity in the prepared solutions, thus interfering in the values found for absorbances. Folin and Denis [11] compared the performance of three alkalis, showing that NH_4OH and K_2CO_3 caused the formation of precipitate, and for this reason the authors preferred the Na_2CO_3, which provided clearer solutions. Swain & Hillis [12] also found in their study that Na_2CO_3 generates more reproducible results than K_2CO_3, NaOH, or Na_3PO_4. Thus, Na_2CO_3 is one of the most used compounds to obtain and maintain the pH of alkaline solutions in the tests involving the FC reagent.

The FC reagent reacts not only with phenols but also with various other types of compounds, such as aliphatic tertiary amines, tryptophan, hydroxylamine, hydrazine, some purines, and several other organic and inorganic reducers. Among the wide variety of nitrogen compounds that have high reactivity with FC, tertiary aliphatic amines, aromatic amines (primary, secondary and tertiary), N-hydroxyl compounds, N-amino compounds, and compounds containing five heterocyclic nitrogen members can be highlighted [9] [13]. In this sense, nitrogenous substances represent a serious problem in the application of this reagent for the study of phenolic compounds.

In several studies in the literature [14]-[33] involving the FC method for the determination of total phenols in propolis extracts, many experimental conditions are described (Table 1).

Table 1. Examples of experimental conditions for the determination of total phenols in propolis extracts described in the literature.

Folin-Ciocalteu Conc. - Vol.	Incubation Time (min)	Na_2CO_3 Conc. - Vol.	Reaction time (min) - Temperature (˚C)	l (nm)	Ref.
pure - 4 mL	-	10 %m/m - 6 mL	120 - 25	760	14
pure - 6 mL	-	20 %m/m - 6 mL	120 - 25	760	15
pure - 500 mL	1	saturated - 200 mL	120 - 25	765	16
pure - 0.5 mL	3	20 %m/m - 2 mL	30 - 25	760	17
0.2 N - 5 mL	-	saturated - 4 mL	120 - 25	765	18
1:10 - 2.5 mL	-	4 %m/m - 2 mL	5 - 50	760	19
pure - 0.25 mL	3	7.5 %m/m - 2 mL	5 - 25	720	20
0.2 N - 100 mL	5	7.5 %m/m - 80 mL	60 - 25	735	21
pure - 0.5 mL	-	10 %m/m - 2 mL	60 - 25	760	22
2 M - 0.5 mL	-	1 %m/m - 2 mL	120 - 20	765	23
10 %m/m - 125 mL	4	10% - 100 mL	120 - 25	745	24
pure - 100 mL	2	5% - 800 mL	20 - 40	760	25
pure - 1 mL	10	8% - 2 mL	10 - 25	730	26
pure - 15 mL	3	10% - 30 mL	120 - 24	765	27
pure - 1.5 mL	-	7% - 3 mL	60 - 25	765	28
0.2 N - 2.5	5	7.5% - 2 mL	180 - 30	765	29
0.2 M - 2.5	10	7.5% - 48 mL	30 - 25	765	30
pure - 0.2 mL	3	20% - 1 mL	60 - 25	760	31
2 N - 0.5 mL	-	15% - 2 mL	120 - 25	760	32
pure - 5 mL	5	saturated - 1 mL	60 - 25	725	33

Beyond water, different solvents are used to dilute the solutions, such as ethanol and methanol [18] [30], as well as the use of an incubation time, before the addition of the buffer, different proportions of FC reagent and buffer, different times and temperatures of reaction variables and different wavelengths of reading.

Therewith, this study found which variables influence the process of determination of total phenols in commercial propolis extracts using the FC spectrophotometric method and optimize instrumental conditions to obtain a faster and more accurate method.

2. Experimental

2.1. Samples and Reagents

To determine the total phenol content, the FC reagent was commercially acquired (Imbralab). The reagent consists of a mixture of sodium molybdate, sodium tungstate, orthophosphoric acid, hydrochloric acid, and lithium sulfate. Sodium Carbonate was used as a buffer, and 20 %m/v solutions were prepared. The gallic acid (Sigma-Aldrich) was used as the standard for the determination of total phenolic compounds, being solubilized in deionized water for five minutes in an ultrasound bath, at a concentration of 1000 μg mL^{-1}, used on the same day and staying out of the light. Extracts of common and green commercial propolis, produced in the south and southeast regions of Brazil, were used in the optimization of the spectrophotometric methodology.

2.2. Instrumentation and Software

For spectrophotometric studies, a Hewlett-Packard 8451A spectrophotometer, with quartz cells with an optical path of 10 mm was used. The absorption spectra of each sample were obtained by performing a wavelength scan from 190 to 820 nm, with increments of 2 nm.

In the nephelometric studies, a Shimadzu RF-5301 spectrofluorometer was used, with a xenon lamp, using quartz cells with an optical path of 10 mm and a wavelength of 600 nm.

An energy dispersive spectrometer, Shimadzu EDX-800, equipped with a rhodium tube and liquid nitrogen-cooled Si(Li) detector, was used in X-ray fluorescence analyses. The excitation conditions were 50 kV tube voltage, 28 mA tube current, 100-second spectrum acquisition time, and the atmosphere used was atmospheric air. The range of elements evaluated varied from sodium to uranium, without the use of any filter.

A Rigaku diffractometer, Geiger Flex model, operating in conventional geometry θ-2θ, was used in X-ray diffraction analyses. The radiation used was K_α copper (λ = 1.54 Å), and the measurements were performed at room temperature.

2.3. Optimization of the Spectrophotometric Methodology

To determine the significant variables of the method, adequate amounts of a standard solution of gallic acid and FC reagent were added to the 25.00 volumetric flasks, which remained in the dark for eight minutes (incubation time). After this period, adequate amounts of sodium carbonate buffer were added, and the volume was completed with deionized water. Reaction periods in the dark and room temperature, were evaluated in the range of thirty minutes to two hours.

It was studied the significance of some variables for the determination of total phenols in propolis extracts: concentration of gallic acid, the volume of the FC reagent, volume of sodium carbonate buffer at 20 %m/v, and reaction time. After the selection of significant variables, a complete factorial planning with a central

point was used to evaluate the influence of variables on the determination of total phenolic compounds, as well as their possible interactions.

From the evaluation of the influence of variables and their interactions, a central composite design was constructed using as a central point the values that indicated an increase in the response in factorial planning. To find the maximum absorption of radiation and a minimum of scattering of that, due to the possible presence of suspended solids, a new central composite planning was later performed.

2.4. X-Ray Fluorescence and X-Ray Diffraction Analysis

The solutions that presented suspended solids in the optimization process were filtered and the residue obtained was washed with deionized water. The white crystalline solid was dried at around 70˚C and then macerated for fluorescence and X-ray diffraction analysis.

2.5. Merits Parameters

To evaluate the quality of the adjustments made by the least square regression, chosen in the principles of the previous item, was used standardized residues, which consist of the residue divided by standard deviation at each point of calibration curve. For a good fit, the standardized residuals must have values, in module, near the unit [34].

After verifying the absence of discrepant values, for each concentration level, and evaluation of homoscedasticity, the linearity of the analytical curve was evaluated with the determination coefficient (R^2), covering the range from 1.0 to 8.0 μg mL^{-1} for gallic acid. This concentration range was chosen because the range includes most of the absorbances presented by propolis extract solutions. The analytical curves were constructed with standard aqueous solutions, with independent triplicate at each point of the calibration, read in random triplicate.

For the determination of detection and quantification limits, ten independent blank replicates (FC reagent and Na_2CO_3 buffer 20 %m/v), fortified at the lowest concentration of analyte (gallic acid) used in the analytical curve were prepared. These solutions were later analyzed in random triplicates, and the standard deviation of the measurements was used in the calculation of these merit figures (LD = 3 s; LQ = 10 s).

The addition of analyte to blank solutions was performed to find more realistic values, and the presence of the analyte in the samples will provide signals that will involve the influence of possible interfering species and not just the fluctuation of the blank one [35] [36]. The measurements were performed using the appropriate conditions found in the multivariate optimization.

The precision of the FC spectrophotometric method, expressed as repeatability, was obtained from the relative standard deviation of results from seven independent samples, analyzed in random triplicate, fortified at three concentration levels for each analyte (1.0, 4.0, and 8.0 μg mL^{-1} of gallic acid).

3. Results and Discussion

3.1. Multivariate Optimization

Although the literature describes different wavelengths of reading, it was observed that the wavelength of 760 nm provides maximum absorption for gallic acid and the propolis extracts studied, and this value was used (Figure 1). For some samples studied, it is possible to note the occurrence of hypsochromic displacement, that is, displacement of the maximum absorption to shorter wavelengths, due to the presence of different phenolic compounds, but the maximum absorption values did not vary significantly in the 750 - 770 nm interval.

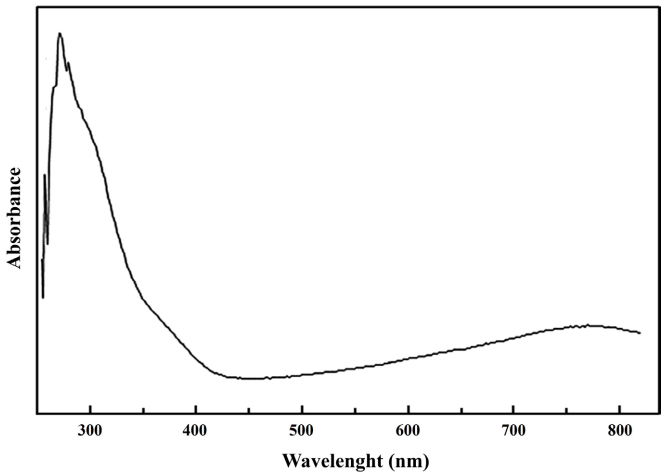

Figure 1. Typical electronic spectra of FC test.

Subsequently, the reaction time in the range of 30 to 120 min was evaluated in three levels of gallic acid concentration (1.0, 5.0, and 10.0 μg mL^{-1}). In this interval, the absorbance in the three levels of concentration of gallic acid remains constant, demonstrating that thirty minutes are suitable for the complete reaction, not requiring long periods such as two hours, which is the most used period described in the literature. Shorter times can be enough to complete the reaction.

A factorial planning 2^2 with central point was performed to verify the influence of FC reagent volume (low level = 0.5 mL and high level = 5.0 mL) and buffer volume (low level = 0.5 mL and high level = 10.0 mL) variables in radiation absorption at 760 nm. The gallic acid concentration was fixed at 10.0 μg mL^{-1} because it is the highest concentration that will be used in later studies, and the reaction time was set at thirty minutes because it did not present a significant influence on the instrumental response, as seen previously.

The variables studied and the interaction between them presented significant positive effects, with buffer volume being the most important effect (Figure 2). It can be observed that when the buffer volume is increased to a fixed volume of FC reagent, there is an increase in the absorption of radiation, but this effect is more pronounced with the volume of 5.0 mL of FC reagent. With the buffer volume fixed at the lower level of 0.5 mL, it is observed that when the FC reagent volume

increases there is a decrease in the absorbance value, while in the volume of fixed buffer at 10 mL, there is an increase in the same. The highest response value was obtained using 5.0 mL of FC reagent and 10 mL of buffer.

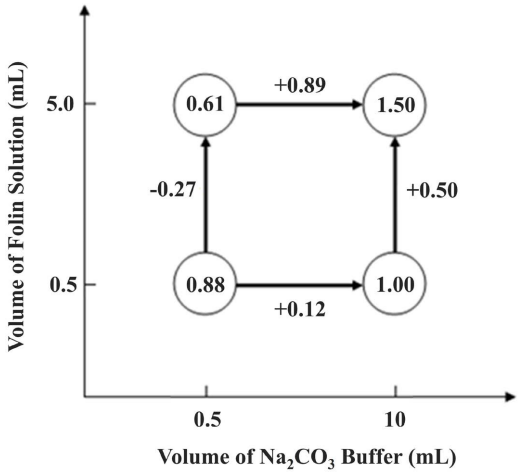

Figure 2. Diagram for interpretation of the effects of Folin and buffer volumes on factorial planning 2^2. The values at the vertices of the square are the mean responses (absorbances).

As the aim of this study was to maximize the absorbance value, studies in the region of 5.0 mL of FC reagent and 10.0-mL buffer were carried out. A central composite design was constructed to optimize the quantities of the FC reagent and buffer, using the volumes of reagents mentioned above as central point of planning, with a total eleven tests.

During the experiments, it was noted that the increase in the absorbance of the solutions was provided by the increase in the turbidity of the solutions, indicating the formation of solids, which were separated for further evaluation of their chemical composition, by spectrometric studies of X-rays (Figure 3).

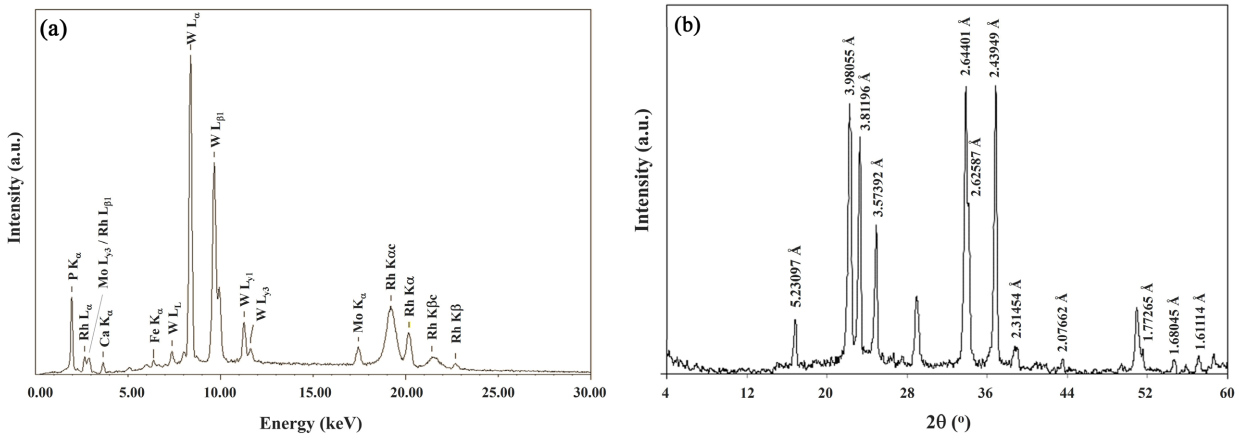

Figure 3. X-ray spectra of the solid responsible for the turbidity of solutions in FC test. (a) Fluorescence; (b) Diffraction.

The X-ray fluorescence spectra (Figure 3(a)) show the presence of phosphorous, molybdenum and tungsten, elements present in FC solution (rhodium is due

to the equipment) and the crystalline solid diffractogram (**Figure 3(b)**), showed an interplanar distance compatible to lithium phosphate [37]. No peaks were observed for compounds containing tungsten and carbonates in the diffractogram because they may be in amorphous form or small concentrations.

This way, it can be inferred that the white crystalline solid is formed by a mixture containing the presence of lithium phosphate and carbonate(s) because bubbles have formed in its mixture with hydrochloric acid. The FC reagent has in its formulation both phosphate and lithium ions, the latter coming from lithium sulfate, one of the FC reagent components. According to Box [9], lithium-ion is added to the formulation of the FC reagent to increase the solubility of the complexes formed with molybdenum and tungsten.

The solutions of the central composite design that presented the formation of solids had the pH evaluated to verify its influence on the precipitation of lithium phosphate. All evaluated solutions presented pH around 8.6, indicating that this does not contribute to the precipitation of lithium phosphate, but due to the increase in the concentration of the FC reagent in the solution, which reaches almost 50% of the volume of the prepared solution, thus exceeding its solubility coefficient.

To avoid the formation of these solids, care was taken to evaluate the maximum absorption of radiation for a minimum scattering of that, resulting in the central composite design with central points in the volume of 1.5 mL of FC reagent and 3.0 mL buffer (20 %m/v), for solutions with a concentration of gallic acid at 10.0 μg mL^{-1}, with a reaction time of thirty minutes. The response variables used were absorbance at 760 nm and scatter intensity at 600 nm.

The solutions prepared under the appropriate conditions presented several colors, ranging from green to grayish-blue. The green color indicates that the relationship between the volumes of FC reagent and buffer were not suitable to produce phenolic complexes, which give the solution an intense blue coloration, despite the low intensities of radiation scattering. For the solutions with grayish-blue coloration, lower absorption values of radiation and higher spreading values were obtained.

The response surfaces obtained (**Figure 4**) show a maximum absorption with critical values of 2.3 mL of FC reagent and 5.3 mL buffer at the maximum point (**Figure 4(a)**) and a minimum of radiation scattering with critical values of 1.6 mL of FC reagent and 0.8 mL buffer (**Figure 4(b)**).

Radiation scatterings at all points of central composite design were compared to the scattering of the deionized water used in the preparation of standard solutions and samples of propolis extracts. At this stage, it can be observed that the use of 0.5 mL of FC reagent and 1.0 mL buffer at 20 %m/v, for 25 mL solutions, generated scatterings similar to that of deionized water. Thus, the above volumes were fixed with the best option for the determination of total phenols through the FC spectrophotometric method, because they present at the same time low radiation scattering and adequate values of radiation absorption.

Comparing the optimized concentration of Na_2CO_3 present in the solutions

(0.008 %m/v) with those used in the literature (0.0014 to 0.14 %m/v) [14]-[33], calculating the concentrations based on the described procedures, can be noted that most of the measurements are subject to solids formation, directly influencing the accuracy of the results obtained. Concentrations lower than 0.008 may not be sufficient to buffer the medium and impair the formation of the complex and higher concentrations may lead to the formation of solids, although unnoticeable to the naked eye.

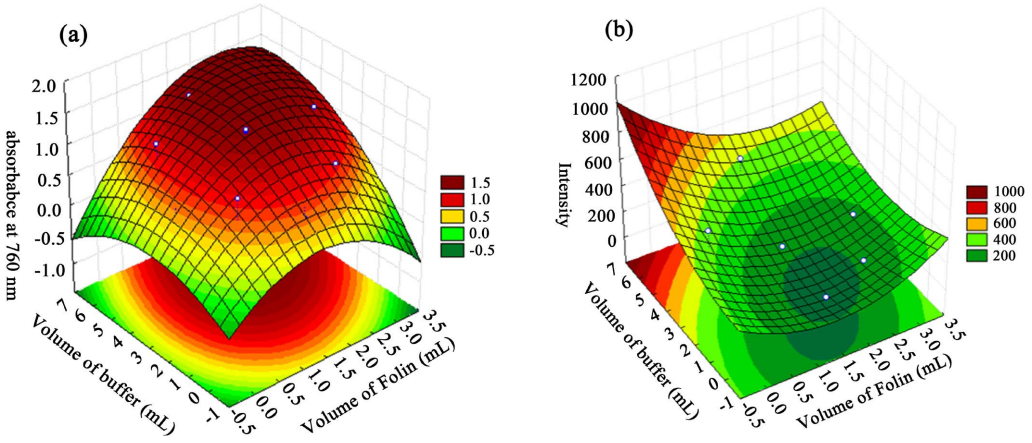

Figure 4. Response surfaces for central composite designs. (a) For maximum absorption of radiation (760 nm); (b) For a minimum scattering (600 nm).

The experimental optimization of the FC spectrophotometric method had as advantages the reduction in the amounts of FC reagent and buffer and the reduction of the analysis time compared to the methods described in the literature. Additionally, it provides greater accuracy, minimizing the influence of suspended solids that can be formed in the reaction. Therefore, FC reagent and Na_2CO_3 buffer volumes should be optimized according to FC reagent composition to avoid that.

3.2. Evaluation of Radiation Scattering in Propolis Extracts

After optimization of the experimental conditions, the method was used to determine the content of total phenols in propolis extracts with different levels of dry extract, previously determined using a gravimetric methodology described by Barbeira *et al.* [38]. A volume of 5 µL of the extract was used together with 0.5 mL of FC reagent and 1.0 mL buffer (20 %m/v), following the optimized conditions, with the respective intensities of radiation scattering in 600 nm and the levels of dry extract (%m/v) and total phenols (%m/m of dry extract), whose results are presented in **Table 2**. All samples were under Brazilian legislation that establishes a minimum total phenol content of 0.5 %m/m, to the dry extract content [39].

The dry extract content had no connection with the small turbidity observed in some samples, being characteristic of the chemical composition of each extract. To evaluate whether this turbidity causes significant changes in the content of phenolic compounds determined using the method, the magnitude of radiation scattering caused by these samples was analyzed. **Figure 4(b)** shows that the

region of minimum radiation scattering encompasses the range that goes up to the intensity value of 200. Among the samples evaluated, the highest spreading value was for sample C3 (intensity equal to 196.7), a value close to the region of minimum radiation scattering. Eventually, larger scatterings can be suppressed with a greater dilution of the sample.

Table 2. Dry extract and total phenol contents, and respective radiation scattering for different propolis samples.

Region/Type	Sample	Dry Extract (%m/v)	Total Fenols (%m/m)	Scattering (600 nm)
-	deionised water	-	-	4.5
-	blank	-	-	48.4
Southeast common	C1	5.43 ± 0.11	52.5 ± 1.1	87.7
	C2	3.51 ± 0.12	24.93 ± 0.61	75.5
	C3	2.74 ± 0.13	52.1 ± 1.2	193.7
	C4	15.05 ± 0.19	20.72 ± 0.47	31.8
	C5	15.48 ± 0.45	28.39 ± 0.40	122.9
	C6	16.69 ± 0.39	9.25 ± 0.21	185.4
South common	C7	5.57 ± 0.21	22.77 ± 0.49	98.1
	C8	5.40 ± 0.12	48.44 ± 1.3	104.7
	C9	5.56 ± 0.11	16.80 ± 0.38	155.2
	C10	15.58 ± 0.19	29.73 ± 0.77	111.6
	C11	15.82 ± 0.26	31.71 ± 0.79	122.1
	C12	16.32 ± 0.13	16.38 ± 0.37	146.6
Southeast green	V1	5.83 ± 0.11	17.63 ± 0.27	95.8
	V2	6.10 ± 0.12	17.71 ± 0.29	101.9
	V3	7.24 ± 0.17	30.91 ± 0.67	51.3
	V4	17.88 ± 0.61	26.48 ± 0.58	31.8
	V5	16.50 ± 0.21	23.28 ± 0.52	63.5
	V6	15.93 ± 0.31	31.69 ± 0.76	24.2

3.3. Figures of Merit

In the evaluation of the least-squares method is best suited to the data of this study, the variance at each point of the calibration was used to verify the behavior of the dataset. A total of eight points, in the concentration range of 1.0 to 8.0 μg mL^{-1}, were studied in the analytical curve for gallic acid.

The data obtained for gallic acid, showed variances with random values, indicating that the data have a homoscedastic behavior, as well as its residues that

indicated random behavior. Thus, linear regression was constructed based on the ordinary least squares method, which is a simpler method because it does not require any weighting. The analysis of standardized residuals showed values close to the unit, in module, indicating that the regression is well adjusted in the concentration range studied, presenting a correlation coefficient of 0.998.

The limits of detection and quantification were determined by adding gallic acid at the lowest concentration used in the calibration curve (1.0 μg mL^{-1}), to obtain realistic values caused by possible matrix interferences and not only to blank fluctuations. The merit parameters found for the spectrophotometric method are presented in Table 3.

Table 3. Merit figures for Folin-Ciocalteu spectrophotometric optimized method.

Parameter		Values
Limit of Detection		0.08 μg mL^{-1}
Limit of Quantification		0.26 μg/mL
Working Range		0.26 to 8.0 μg mL^{-1}
Repeatability	1.0 μg/mL	2.1%
	4.0 μg/mL	2.2%
	8.0 μg/mL	2.4%

To evaluate the accuracy of the FC spectrophotometric method, three concentration levels (low, medium, and higher levels) used in the calibration curve for gallic acid were examined, each level with seven independent replicates analyzed in random triplicate. The precision was expressed in terms of the coefficient of variation (%), obtaining adequate values, showing that the optimized methodology is adequate for the quantification of total phenols in commercial propolis extracts at different concentration levels.

The accuracy of the methodology using HPLC-DAD in the quantification of phenolic compounds in propolis has already been verified by other authors [14] [40] [41]. Popova *et al.* [14] obtained recoveries of about 84% - 109%, Luo *et al.* [40] 84.2 to 118.6%, while Pellati *et al.* [41], the recovery was 96 to 105%, thus demonstrating the accuracy of the proposed methodology.

4. Conclusions

The optimization of the FC spectrophotometric methodology for the determination of phenols in propolis extracts added several advantages, such as the reduction of reagents, analysis time, and higher accuracy. With the verification of the occurrence of radiation scatterings under certain conditions of analysis, many studies present in the literature involving the determination of phenols not only in propolis but also in other types of matrices may have the accuracy of the method compromised.

In many works, extreme conditions are used, such as a large amount of FC

reagent and buffer used in the determination of total phenols in propolis extracts at a given concentration. The formation of suspended solids, which can often be omitted by the blue coloration presented by the solutions, as verified in this work, can compromise the accuracy of the method.

In this article, it can be verified through fluorescence spectrometry and X-ray diffraction that the chemical composition of the crystalline solid formed under experimental conditions that involved large amounts of FC reagent and buffer is mainly lithium phosphate.

The amounts used of FC reagent and Na_2CO_3 buffer were also adequate for the analyses in propolis extracts, providing low spreading values, which had no connection with the type of propolis used in the extraction, with the production region, or with the dry extract content of commercial propolis extracts.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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