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A Rapid and Eco-Friendly Method for Determination of Hydrolysable Tannins and Its Application to Honey Samples

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Abstract This paper describes the development of a rapid and clean method for the determination of hydrolysable tannins in honey samples, focusing on an easy and green sample pretreatment and low generation of waste. The proposed method is based on the reaction between KIO_3 and hydrolysable tannin compounds in acetate buffer medium (pH 4.75), with formation of a colored product measured at 525 nm. The analytical conditions were optimized using experimental design (central composite design). The linear range obtained was 40.0–740 mg L^{-1} ($r = 0.998$), and the detection (LOD) and quantification (LOQ) limits were 12.2 and 40.8 mg L^{-1}, respectively. Acetate buffer was used to ensure formation of the colored product obtained by reaction between the chromogenic reagent and the analyte. The method showed good results when applied to honey samples, with recoveries in the range of 78.3–96.1 %, and the use of diffuse reflectance spectroscopy coupled with spot test analysis provided low reagent consumption and minimized waste generation. The results obtained with the proposed method were confirmed by a spectrophotometric method.

Keywords Honey · Hydrolysable tannin · Diffuse reflectance spectroscopy · Eco-friendly method

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Introduction

Polyphenols are organic compounds that are widely found in plants and some foods. The production of honey from pollen derived from diverse flora results in the inclusion of polyphenolic compounds, particularly tannins, in the honey matrix.

Honey is a sweet product of animal origin, produced by bees as a food source from the nectar of plants, and its characterization contributes to ensuring a high-quality product and safeguarding the health of consumers (Uthurry et al. 2011; Codex 2001). A growing number of studies provide evidence of the importance of the antioxidant capacity of honey for therapeutic purposes (Gorjanovic et al. 2013; Gheldof et al. 2002; Ciappini and Stoppani 2014; Eterafi-Oskouei and Najafi 2013). Honey is consumed in large quantities worldwide, and in Brazil, imports in January 2015 were around 3.76×10^4 kg of common honey and 9.20×10^5 kg of organic honey (National Honey Report 2015).

Tannins are high-molecular-weight polyphenolic compounds found in two principal classes, hydrolysable and condensed tannins. Particular features of these compounds are their astringency and high antioxidant capacity (Mueller-Harvey 2001). These structures include water-soluble phenolic compounds and have high molecular weights of between 500 and 3000 Da (Chung et al. 1998).

Hydrolysable tannins contain either gallotannins or ellagitannins. The hydrolysis of gallotannins yields glucose and gallic acids, while ellagitannins contain hydroxydiphenoyl residues that produce ellagic acid after hydrolysis (Chung et al. 1998; Romani et al. 2006). Condensed tannins, otherwise known as proanthocyanidins, are compounds consisting of oligomers and polymers of flavan-3-ol units, usually linked via C4-C6 or C4-C8 bonds (Romani et al. 2006).

Tannins are present in the matrix of honey as part of its natural composition, rather than due to external contamination. Bees collect the nectar of plants, and the tannins present in the nectar are transferred to the honey during its production (Uthurry et al. 2011).

The antioxidant capacity of tannin compounds present in foods, including the gallic and ellagic acids of honey, is highly important in terms of human health (Escuredo et al. 2013). Studies of the biological action of these compounds have revealed significant activity against certain microorganisms (Scalbert 1991) as well as carcinogenic and hepatotoxic substances (Chung et al. 1998). Tannins exhibit antiinflammatory and healing action and can inhibit HIV reverse transcriptase (Kilkuskie et al. 1992; Khanbabaee and Ree 2001). The presence of tannins in various natural matrices can show seasonality. Salminen et al. (2001) observed seasonal variation of hydrolysable tannins in leaves of *Betula pubescens*. Many tannins can act as scavengers of the free radicals that are associated with degenerative diseases such as cancer, multiple sclerosis, and atherosclerosis, with the tannins interacting with the active oxygen to form stable radicals.

Few analytical methods for the determination of hydrolysable tannins in honey have been reported in the literature, which highlights the importance of the study of hydrolysable tannins presented in this work, since honey is a product that is very widely consumed (Uthurry et al. 2011). The determination of tannins in complex matrices such as honey usually requires laborious procedures and/or extensive cleanup processes, and recoveries can be low. For matrices such as water, tea, and fruits, techniques used have included high-performance liquid chromatography (HPLC) (Ahmed et al. 2014; Silva et al. 2013; Xie et al. 2012), HPLC and capillary electrophoresis (Juang et al. 2004), and HPLC coupled with mass spectrometry (Campillo et al. 2015; Zywicki et al. 2002). The difficulty in analyzing hydrolysable tannins, compared to condensed tannins, has led to the study of these compounds being ignored. Mixtures of hydrolysable tannins have been analyzed using general tannin assays, such as precipitation with proteins or metals, and by colorimetric determination of total phenols (Mueller-Harvey 2001).

The development of new analytical methods is one of the most important goals of Green Chemistry. These methods aim to reduce or eliminate the use of hazardous substances and organic solvents, among other improvements, and are gaining ground in all aspects of chemistry (Keith et al. 2007; Anastas 1999; de La Guardia and Garrigues 2011). The fact that a new methodology is environmentally friendly is as important a factor as the analytical features of the method (Anastas 1999). Diffuse reflectance spectroscopy coupled with spot tests is considered a green analytical technique because it uses very small quantities of reagent and no organic solvents, reducing costs and producing minimal amounts of waste products. The technique offers speed and simplicity, while protecting human health and the environment. An important feature of the diffuse reflectance technique is that it can be performed using portable equipment, with filter paper providing a good surface for the spot test (Dias et al. 2006). Other methodologies using diffuse reflectance Fourier transform infrared spectroscopy have been reported in the literature (Pappas et al. 2015; Kyraleou et al. 2015).

Hydrolysable tannins react with potassium iodate (KIO_3) to produce a purple-colored product (Willis and Allen 1998). The reaction was first described by Haslam (1965), although its application in analytical determinations was subsequently reported by Bate-Smith (1977). The method proposed here is based on the reaction between hydrolysable tannins and potassium iodate in a buffered medium, with the reaction product being measured at $\lambda_{max} = 525$ nm. Experimental design was used to find the best conditions for determination of the analyte in honey samples.

The aim of the present work was therefore to develop a rapid, simple, and environmentally friendly method for the determination of hydrolysable tannins (Fig. 1) in honey, using a diffuse reflectance spectroscopy technique combined with spot tests, without any need for elaborate pretreatment of the honey samples.

Experimental Section

Apparatus

The diffuse reflectance spectroscopy measurements employed a handheld integrating sphere (ISP-REF, Ocean Optics, Dunedin, USA) connected to a fiber optic mini-spectrometer (USB 2000, Ocean Optics). Spectral acquisition was carried out using SpectraSuite software (Ocean Optics).

Reagents and Solutions

Ultrapure water (18 MΩ cm; Milli-Q System, Millipore) was used to prepare the solutions. Qualitative filter paper (Whatman no. 1) was used as the solid platform for the reaction. Potassium iodate (purchased from Mallinckrodt) was used as the chromogenic reagent, with an aqueous stock solution prepared at a concentration of 3.00×10^4 mg L^{-1} for the development of the central composite design. After optimization of the conditions, a solution of KIO_3 at a concentration of 3.1×10^4 mg L^{-1} was prepared and used for the subsequent experiments, as indicated by the central composite design. Acetate buffer solution was prepared using a mixture of sodium acetate (0.1 mol L^{-1}) and acetic acid (0.1 mol L^{-1}), both purchased from Sigma-Aldrich. Tannic acid (Sigma-Aldrich) was used as the standard, with a stock solution prepared at a concentration of 1000 mg L^{-1} in an aqueous medium containing 3 mL of ethanol. Working standard solutions in the range

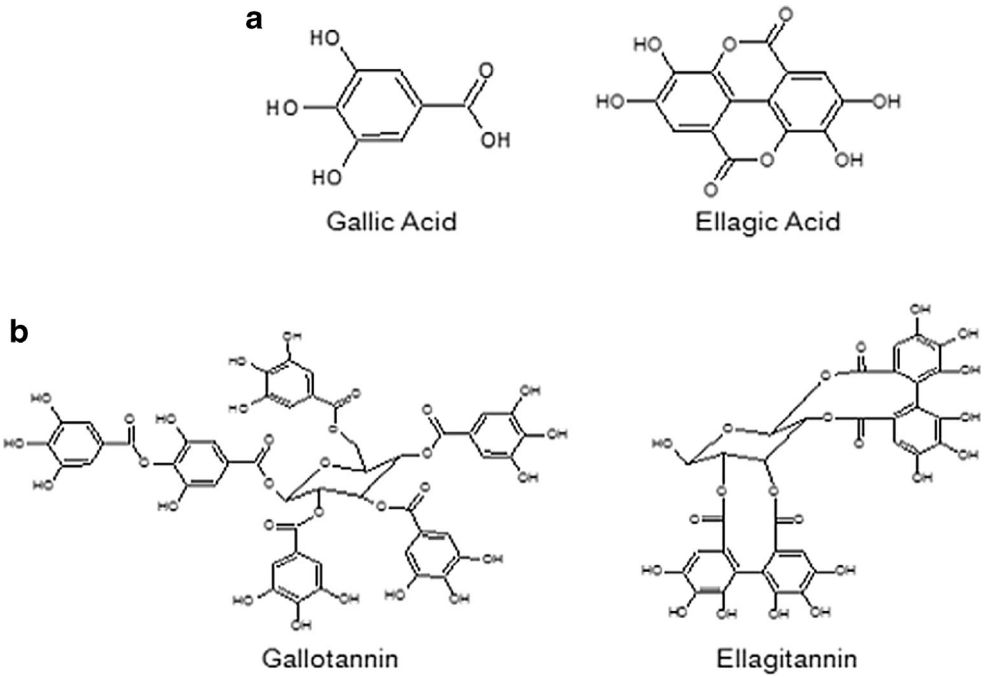

Fig. 1 **a** Chemical structures of precursor molecules of the gallotannins (gallic acid) and ellagitannins (ellagic acid) and **b** chemical structures of gallotannins and ellagitannins

of 40.0–740.0 mg L^{-1} were prepared by suitable dilution of the stock solution.

Honey Samples

Eight samples of orange and eucalyptus flower honey were purchased from different outlets in the municipalities of Araraquara and Matão (São Paulo State) and Cambé (Paraná State) and were used to evaluate the performance of the proposed method.

Sample Preparation

A portion (5.0 g) of the sample was solubilized in deionized water, with the aid of a magnetic stirrer, and transferred to a 25-mL flask. The solution was filtered through a 0.45-μm PVDF membrane (Millex-HV, Millipore), followed by washing of the membrane with two portions (7 mL each) of ethanol:water solution (50 %, *v*/*v*) in order to ensure complete removal of the analyte. Three different ethanol:water ratios were tested for the washing process (30:70, 50:50, and 70:30), and the best results were obtained with the 50:50 mixture. The washing solutions were transferred to the 25-mL volumetric flask, and the volume was completed with the ethanol:water solution.

Procedure for the Spot Test Reaction

A study of the influence of the volumes and the order of addition of the chromogenic reagent, tannic acid standard solution, and acetate buffer solution was performed in order to obtain the best absorbance values. The results obtained are shown in the "Results and Discussion" section.

Experimental Design

Evaluation of all the parameters involved in the method was performed using experimental design methodology. A multivariate analysis was applied in order to optimize the following selected variables: KIO_3 concentration and pH of the buffer solution. The statistical analyses employed the Minitab 16 and Statistica 8.0 software packages.

Analytical Curves

After optimization of the variables, tannic acid standard solutions (40, 100, 200, 300, 400, 500, 600, and 740 mg L^{-1}) were spotted onto filter papers (Whatman no. 1), in triplicate, followed by addition of the buffer solution and the chromogenic reagent solution (in that order). The absorbance of the colored product was measured at 525 nm after 20 min of reaction.

Study of Interferences

Interferences were evaluated by studying the behavior of the reaction in the presence of the major compounds commonly present in honey (glucose, fructose, and hydroxymethylfurfural).

Concentrations 10 times greater than that of tannic acid were evaluated under the same conditions described for the samples.

Standard Additions

The proposed method was validated using standard additions and investigation of possible matrix effects associated with the honey. Samples were spiked with tannic acid at 50, 100, 150, 200, and 250 % of the initial concentration of the spiked sample and were then analyzed by the proposed method.

Comparative Method

The honey samples were also analyzed by a comparative method, as described by Nunes et al. (2009). This was based on reaction of the tannins with iron(III) chloride in a medium containing sodium dodecyl sulfate (SDS), triethanolamine, and isopropanol. The colored product formed by reaction between $FeCl_3$ and the tannins was measured spectrophotometrically at 715 nm.

Results and Discussion

The proposed method is based on the reaction between hydrolysable tannins and KIO_3 as the chromogenic reagent. KIO_3 reacts with hydrolysable tannins to produce a transient purple-colored product that can be measured at 525 nm. Evaluation of the stability of this transient product revealed that the color reached maximum absorbance after 20 min of reaction, with this value being maintained for 15 min, after which the color started to disappear, with formation of a yellow product. The formation of this yellow degradation product has been described previously (Bossu et al. 2006).

In preliminary experiments, the effects of the buffer medium and the order of addition of the solutions to the filter paper were evaluated. Different buffer media were used in order to identify the pH at which the colored product was formed by reaction between the chromogenic reagent and hydrolysable tannins. The influence of the buffer medium was evaluated using the following three different buffers: $H_3PO_4/H_2PO_4^-$ (pH 2.14), $H_2PO_4^-/HPO_4^{2-}$ (pH 7.00), and acetic acid/sodium acetate (pH 4.75). The results showed that at pH 2.14 and pH 7.00, the transient purple-colored product was not obtained, with formation of yellow and brown products, respectively. Formation of the transient colored product only occurred in the presence of the acetate/acetic acid buffer (pH 4.75). It can be seen that the reaction was strongly influenced by the pH and that formation of the transient purple product occurred at pH 4.75.

The influence of the order of addition of the analyte, chromogenic reagent, and buffer solutions to the filter paper was also evaluated. The results of these tests showed that the best conditions for the reaction were provided by addition of the analyte, followed by the buffer solution, and lastly, addition of the chromogenic reagent.

In the sample filtration step, the analyte was completely retained on the 0.45-μm PVDF micropore membrane. Preliminary tests performed with tannic acid standard solution demonstrated the need for two successive steps of washing the membrane with portions of ethanol:water solution (50 %, *v*/*v*) in order to completely remove the analyte. A third wash was performed in order to confirm total elution of the analyte, which indicated that only two washes were required. Figure 2 shows the spectra obtained after reaction of the KIO_3 chromogenic reagent with the first, second, and third washing solutions in pH 4.75 buffer medium (where *A* is the spectrum for a tannic acid standard solution and *B* is the spectrum for a sample solution).

Optimization of Variables

Central Composite Design

Multivariate analysis was used to optimize the variables, employing a central composite design to identify the optimal conditions that provided the best analytical results. A response surface analysis was applied, using the selected variables, KIO_3 concentrations in the range from 1.50×10^4 to 3.00×10^4 mg L^{-1} and buffer pH from pH 2.14 to pH 6.50.

Table 1 presents the central composite design matrix, considering different combination of the factors. From the preliminary experiments, it was possible to select lower (−1) and upper (+1) levels for the variables, resulting in 13 experiments (carried out in triplicate) for analysis of the influence and significance of the factors. In all experiments, the concentration of the tannic acid standard solution was kept constant at 700 mg L^{-1}.

From the data shown in Table 1, a central composite design graph was constructed (Fig. 3) to identify the optimal conditions for the analysis, considering the absorbance values obtained for each combination of variables. A statistically significant quadratic model, accounting for 89.0 % of the variance, was fitted to the data, describing the relation between the maximum absorbance and the optimal experimental conditions. The quadratic regression model is given by Eq. (1).

$$Z = -0.0866 + 0.0053*x - 0.0000835*x^2 + 0.130*y - 0.0171*y^2 + 0.000865xy \quad (1)$$

where Z is the response factor corresponding to the absorbance value and x and y are the KIO_3 concentration and buffer pH, respectively. From the surface obtained, the critical values found for the KIO_3 concentration and the buffer pH were 3.10×10^4 mg L^{-1} and pH 4.75 (acetate buffer), respectively.

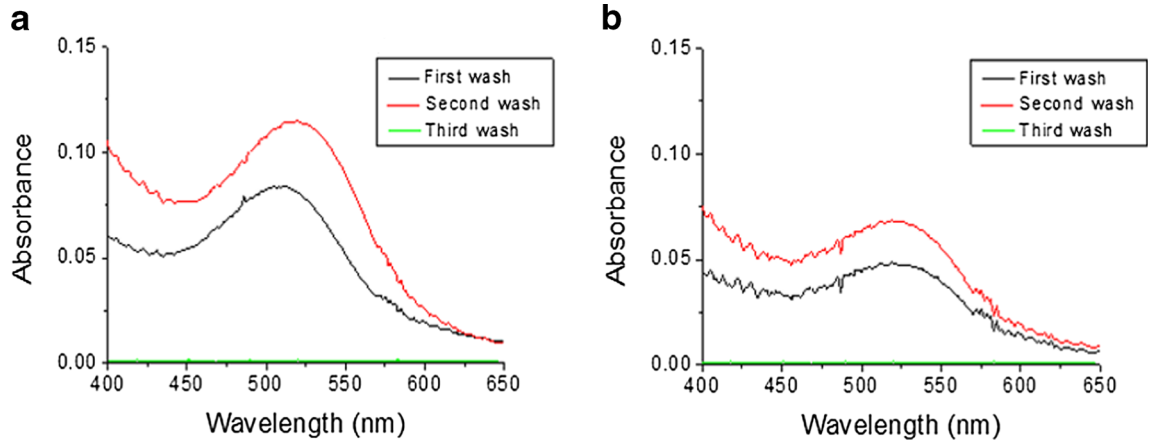

Fig. 2 **a** Spectrum obtained for the reaction between tannic acid standard solution ($C_{\text{tannic acid}} = 700.0$ mg L^{-1}) and KIO_3 after washing of the 0.45-μm membrane and **b** spectrum obtained for the reaction between a honey sample and KIO_3 after washing of the 0.45-μm membrane

Analytical Features

The proposed method was evaluated using the following parameters: linear range, precision, accuracy, limit of detection (LOD), and limit of quantification (LOQ).

After optimization of the variables and obtaining the best analytical conditions, a linear analytical curve was constructed using concentrations of tannic acid in the range of 40–740 mg L^{-1}. The repeatability was evaluated using intra- and inter-day measurements at two concentrations; for a 140 mg L^{-1} standard solution of tannic acid, the %RSD values were 1.94 and 2.34, respectively, while for a concentration of 640 mg L^{-1}, the values were 1.16 and 3.93, respectively. These results were considered acceptable and showed that the proposed method could be used for the analysis of hydrolysable tannins in honey samples. Table 2 presents the analytical parameters of the proposed method.

The LOD and LOQ values were determined according to IUPAC recommendations (Long and Winefordner 1983), using the expressions $3 \times (s/b)$ and $10 \times (s/b)$, respectively, where s is the standard deviation of measurements of the blanks ($n = 10$) and b is the slope of the linear range. The calculated LOD and LOQ values were 12.2 and 40.8 mg L^{-1}, respectively.

Table 1 Central composite design matrix

Experiments	Factors[a]	
	KIO_3 concentration (mg L^{-1})	pH
1	1.50×10^4 (−1)	2.14 (−1)
2	3.00×10^4 (+1)	2.14 (−1)
3	1.50×10^4 (−1)	6.50 (+1)
4	3.00×10^4 (+1)	6.50 (+1)
5	1.20×10^4 (−√2)	2.14 (−1)
6	3.30×10^4 (√2)	2.14 (−1)
7	1.50×10^4 (−1)	1.00 (−√2)
8	1.50×10^4 (−1)	7.00 (√2)
9	2.25×10^4 (0)	4.75 (0)
10	2.25×10^4 (0)	4.75 (0)
11	2.25×10^4 (0)	4.75 (0)
12	2.25×10^4 (0)	4.75 (0)
13	2.25×10^4 (0)	4.75 (0)

[a] The coded values are in parentheses

Study of Interferences

The study of potential interfering compounds was performed considering the typical composition of honey samples, with evaluation of the effects of hydroxymethylfurfural (HMF), glucose, and fructose. No interferences in the proposed method were observed for the interferent concentrations tested.

Analysis and Recovery Using Honey Samples

The hydrolysable tannin contents were quantified for eight samples of honey. Table 3 presents the results obtained for honey samples from different origins and flowers, using the proposed and comparative methods. It can be seen that the eucalyptus honey samples had higher contents of hydrolysable tannins, compared to the orange honey samples. This could have been due to the different floral origins, as well as other factors such as the type of soil, source of the nectar, geographic origin, and seasonal climatic variations (Al-Mamary et al. 2002). It can also be noted that the samples from Paraná State showed higher concentrations of these compounds, compared to samples from São Paulo state, which could be attributed to different seasonal and environmental conditions.

The production of honey is carried out using the nectar of plants. Several components (water, organic acids, carbohydrates, pollen, and wax) are due to maturation of the matrix,

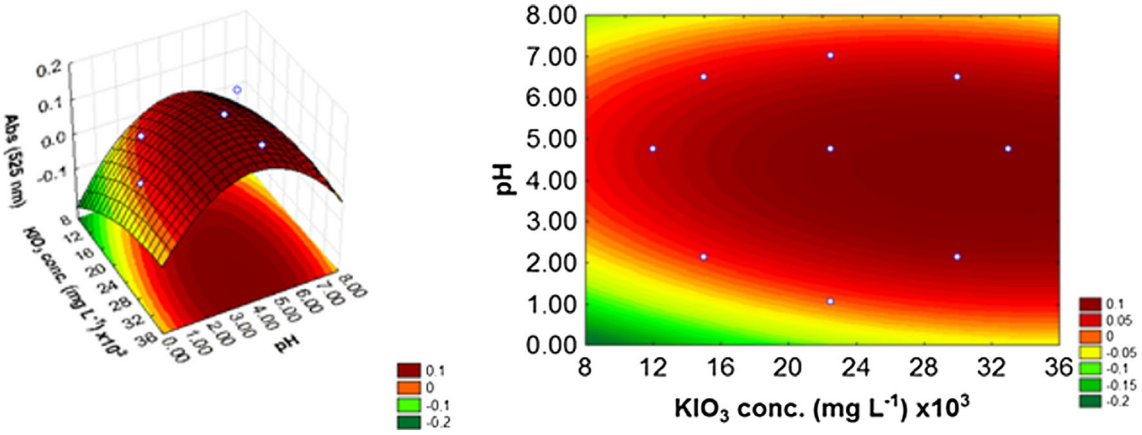

Fig. 3 Central composite design response surface and its projection, obtained for absorbance values as a function of KIO_3 concentration and pH

while others are added by the bees and can be derived from plants of different regions. In the case of honey derived from the same flora, there can be variations due to seasonal climatic fluctuations or different geographic origins. The proportions of the components of honey largely depend on the source of the nectar, and for the same flora, the mineral content can vary from 0.04 % for pale honey to 0.2 % for dark honey, depending on the type of soil in which the plant grows (Anklam 1998).

The data given in Table 3 show that there was consistency between the concentrations found using the proposed and comparative methods. Application of the paired *t* test resulted in value below the tabulated value, indicating that there was no significant difference between the methods.

The accuracy of the proposed method was evaluated by determining the recovery of known concentrations of tannic acid spiked into honey samples, taking into account the initial concentrations naturally present. Two samples (one from orange flora and one from eucalyptus flora) were selected for the study. Spiking was performed at concentrations 50, 100, 150, 200, and 250 % higher than already present in the honey. Table 4 shows the recovery results for the proposed method, from which it could be concluded that the new technique provided good accuracy and an absence of matrix effects.

Table 2 Figures of merit of the proposed method

Parameters	Values
Linear range	40.0–740.0 mg L^{-1}
Coefficient of correlation (*r*)	0.998
Slope	1.70×10^{-4}
Limit of detection[a]	12.2 mg L^{-1}
Limit of quantification	40.8 mg L^{-1}
Wavelength	525 nm
Calibration curve	$A = 0.011 + 1.703 \times 10^{-4}\ C_{\text{tannic acid}}$

[a] Standard deviation of the blank(s) = 6.94×10^{-4}

The use of the proposed method to determine hydrolysable tannins offers several advantages, compared to the analytical methods described in the literature, which require large volumes of organic solvents such as acetonitrile, diethyl ether, formic acid, methanol, and dichloromethane (Ahmed et al. 2014; Xie et al. 2012), generating chemical wastes that contribute to environmental pollution. The earlier methods are also generally more time consuming. The proposed method is simple, fast, low cost, and sufficiently sensitive for determination of the target analytes in honey samples. Furthermore, the sample treatment only requires ethanol, which is a renewable organic solvent. Other important features are that there is no need for any elaborate sample pretreatment or cleanup steps, reagent and sample consumption is low, and waste generation is minimal (about 0.381 mg per analysis) when compared to other methods described in the literature.

Table 3 Quantification of hydrolysable tannins in honey samples by the proposed and comparative methods

Samples	Proposed method (mg kg^{-1})	Comparative method (mg kg^{-1})
1[a, e]	519.3 ± 31.7	516.8 ± 10.7
2[b, c]	290.5 ± 11.7	294.5 ± 10.7
3[b, d]	327.6 ± 10.7	327.7 ± 10.9
4[b, c]	321.4 ± 10.7	322.1 ± 10.7
5[a, d]	383.3 ± 11.5	380.9 ± 10.7
6[a, c]	445.1 ± 0.0	448.4 ± 0.0
7[b, e]	340.4 ± 21.5	337.3 ± 21.5
8[a, d]	383.8 ± 10.7	381.1 ± 11.4

Student's *t* test calculated value = 0.326 and tabulated value = 2.365 (95 % confidence level)

[a] Eucalyptus honey samples

[b] Orange honey samples

[c] Sample from Araraquara/SP Municipality

[d] Sample from Matão/SP Municipality

[e] Sample from Cambé/PR Municipality

Table 4 Recovery values obtained using the proposed method.

Sample	Added value (mg kg^{-1})	Found value (mg kg^{-1})	Recovery (%)
Eucalyptus honey sample no. 8	–	383.8[a]	–
	191.9	519.9	90.3
	383.8	674.0	87.8
	575.7	922.1	96.1
	767.6	1039.7	90.3
	959.5	1268.1	94.4
			$\mu^b = 91.8 \pm 3.4$
Orange honey sample no. 2	–	290.5[a]	–
	145.3	341.2	78.3
	290.5	468.3	80.6
	435.8	617.3	85.0
	581.0	744.3	85.4
	726.3	889.7	87.5
–	–	–	$\mu^b = 83.4 \pm 3.8$

[a] Quantification of hydrolysable tannins in the honey samples

[b] Average ± relative standard deviation (RSD) of five determinations

Conclusions

A new method is presented for determination of hydrolysable tannins in honey samples. The method can be successfully applied for the determination of the analyte in the matrix studied, avoiding possible environmental damage caused by the disposal of solvents, because their use is minimal, compared to other methodologies described in the literature. Advantages of the diffuse reflectance spectroscopy technique include operational simplicity, speed, and low analytical cost. The methodology is environmental friendly and in accordance with the principles of Green Chemistry, with low consumption of reagents and samples, and generates minimal waste during the analyses. Another important feature of the proposed method is that it does not require any elaborate cleanup steps or other sample pretreatments.

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Compliance with Ethical Standards On behalf of all the authors of this original research paper, the author Helena Redigolo Pezza declares that this article does not contain any studies involving human participants or animals performed by any of the authors.

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Conflict of Interest Helena Redigolo Pezza declares that he has no conflict of interest. Tiago Augusto Catelani declares that he has no conflict of interest. Dayana Borges Bittar declares that he has no conflict of interest. Leonardo Pezza declares that he has no conflict of interest.

Informed consent Not applicable.

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