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Rapid Determination of Hydrogen Peroxide Using Peroxidase Immobilized on Amberlite IRA-743 and Minerals in Honey

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Hydrogen peroxide and trace metals (K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, and Li^+) were determined in 14 samples of Brazilian commercial honeys. The method for the determination of H_2O_2 is based on selective oxidation of H_2O_2 using an on-line tubular reactor containing peroxidase immobilized on Amberlite IRA-743 resin. Reactors presented high stability for at least 2 weeks under intense use. The results show a simple, accurate, selective, and readily applied method to the determination of H_2O_2 in honey. The trace metals were determined by capillary zone eletrophoresis. Mean contents of 656, 69.1, 71.8, 36.0, 21.4, and 1.70 mg/kg were found, respectively, for K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, and Li^+ in the analyzed honeys. The cations were identified by comparison of the relative migration times of their peaks with the Ba^{2+} migration time used as reference. The electrophoretic analysis was simple and rapid and did not require any other preparation of sample than dilution and filtration.

KEYWORDS: Capillary zone eletrophoresis; honey; hydrogen peroxide; peroxidase immobilized; trace metals

INTRODUCTION

Honey is a complex matrix of components, which presents a considerable analytical challenge. Honey is produced by bees from the nectar of plants, as well as from honeydew. Bees and plants are the sources of components such as carbohydrates, water, traces of organic acids, enzymes, aminoacids, pigment; and others like pollen and wax arise during honey maturation (*1*). The chemical analysis of honey can be attributed to three main purposes: determination of the geographical and botanical origin, verification of adulteration, and identification of pharmacological active compounds. Both the first and second points assist with certification of quality of the product, which is commonly used as a food, and the third area allows for the examination of content for the use of honey as a medicine.

Hydrogen peroxide is a product of many biological reactions catalyzed by several oxidase enzymes. All honeys contain peroxide, which imbues them with antibacterial properties. It has been shown that the antibacterial activity of honey occurs because of hydrogen peroxide generation (*2*−5). Therefore, the determination of hydrogen peroxide is important in the characterization and selection of honey samples for use as an antimicrobial agent. Hydrogen peroxide is generated by the enzyme glucose oxidase when honey is diluted, and maximum levels of hydrogen peroxide encontered in the diluted honeys are in the range of 1−2 mmol/L (*6*). Dilution is needed for decreasing the acidity of the medium and for adjusting the pH for proper action of glucose oxidase. Weston (*5*) stated that the level of hydrogen peroxide in honey is essentially determined by the amount of catalase, which originates from flower pollen, and glucose oxidase, which originates from the hypopharyngeal glands of bees. Manzoori et al. (*7*) have proposed the spectrofluorometric determination of hydrogen peroxide in several honey samples using the crude extract of kohlrabi (*Brassica oleracea gongylodes*), a rich source of peroxidase.

Strategies have been investigated to adapt quantification methods to the range of sample concentrations with low cost. In the analytical methods using enzymes, the reduction in cost of the determination is generally associated with reduced enzyme consumption. Recently, various ion-exchange resins have gained considerable significance not only for separation purposes but also as carriers of catalytic active substances. Considerable attention has been paid to their application for immobilization of enzymes (*8*−*13*). The resins should meet several requirements. Their porous structures must be strong enough to withstand the enhanced pressure usually applied in forced flow bioreactors. Furthermore, the membrane material must be chemically and physically resistant. These requirements can be met by many aromatic and aliphatic polyamides. Therefore, a resin prepared from these polymers is a suitable substrate for the immobilization of enzymes (*14*). The covalent binding of

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the enzyme to the polymer matrix is one of the most prospective methods for immobilization.

The mineral content in floral honeys ranges from 0.020% to 1.028%, with an average content of 0.169% (*15*). The mineral content of honey is recognized as an environmental indicator at least since 1984. Taking into account cations present in honey, potassium is predominant and calcium, sodium, and magnesium are present in minor quantities. Manganese, copper, iron, nickel, lithium, and cadmium are also present in honey as trace elements. The mineral and trace element content in honey samples could serve as an indicator of environmental pollution and could assist in determining the geographical origin of honey. Different methods for the determination of mineral content in honey have been proposed in previous papers (*1*, *16*−20), some of them with classification purposes, but always according to geographical origin. None has been used to establish a classification of honey according to its botanical origin. The determination of cations in honey has been realized by various procedures, including atomic absorption and emission spectroscopy (*21*), X-ray fluorescence (*22*), flame emission photometry (*23*), inductively coupled plasma atomic emission spectrometry (*24*), and capillary zone electrophoresis (*19*). Fodor and Molnar (*25*) have investigated environmental contamination in honey produced in industrial areas. Recently, Torres et al. (*1*) analyzed 11 elements in 40 honey samples from different locations in Spain and of different botanical origins. Luque et al. (*19*) determined minerals in 25 honey samples and obtained good results as to the classification of the samples according to their geographical origin.

In the present work, we describe a versatile method for the spectrophotometric determination of micromolar hydrogen peroxide in commercial Brazilian honey samples using an on-line tubular reactor containing peroxidase immobilized on resin (Amberlite IRA-743) and the determination of trace metals (K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, and Li^+) using capillary zone electrophoresis without any treatment of the honey samples.

MATERIALS AND METHODS

Preparation of the Enzymatic Reactor. The procedure adopted to immobilize the peroxidase enzyme was fast and very simple (*9*). Amberlite IRA-173 resin (originally manufactured for applications involving boron extraction) was selected as support, which has active amine groups in its chemical structure. The enzyme immobilization process begins with the addition of 100 μL of 0.1 % glutaraldehyde to 250 mg of resin, and this mixture was stirred for 5 min. Subsequently, an amount of 200 units of enzymes was introduced into the mixture and stirred for 10 min more. In the next step, the resin was transferred to a length of Tygon tubing (2.5 mm i.d. and 25 mm long) having one of its extremities closed with a thin layer of glass wool to assemble the reactor. At this point, the other extremity of the tubing was then closed with glass wool. To adapt the enzymatic reactor to a FIA (flow injection analysis) system, the tubing (0.8 mm i.d) was attached at each of its extremities with the aid a small piece of silicone tubing (1.3 mm i.d. and 5 mm long). Finally, the reactor was washed with 10 mmol/L phosphate buffer solution (pH 7.0) to remove the excess of peroxidase.

Reagents and Chemicals. All reagents used were of analytical grade and were used as received. Hydrogen peroxide and mono- and dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Phenol with greater than 99.5% purity and 4-aminoantipyrine were obtained from Aldrich (Milwaukee, WI). Solutions were prepared by dissolving the solids in distilled water that was previously treated with a Nanopure system. Commercial peroxidase (EC 1.11.1.7, 115 U/mg) was obtained from Sigma (St. Louis, MO). The Amberlite IRA-743 ion-exchange resin and glutaraldehyde were obtained from Aldrich (Milwaukee, WI). Aqueous stock solutions of phenol and 4-aminoantipyrine were prepared using a 0.10 mol/L pH 7.0 mono- and dihydrogen phosphate buffer. Diluted solutions of hydrogen peroxide were prepared daily using deionized water.

Analytical standards of K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, Li^+, and imidazole were obtained from Sigma. Stock standard solutions were obtained by dissolution of the cations in deionized water. They were stored at 4 °C when not in use. All solutions were freshly prepared and filtered through a 0.45 μm membrane.

Instrumentation and Procedure. In this work, a flow system was employed for determination of the hydrogen peroxide. The solutions were propelled by pressurization, utilizing an aquarium air pump to avoid the undesirable pulsation observed when peristaltic pumps are employed (*9*, *26*, 27). Control of the flow rate was done by adaptation of the aquarium valve outlet with a pinched Tygon tube inserted in the line. Teflon tubing of 0.5 mm i.d. was used throughout the flow system. The flow system used during the development of this work consisted of two lines. In the first, reagents were added to the system, and in the second, the sample was introduced prior to mixing in the reaction coil with the reagents for color generation. A Shimadzu U.V.1601 PC spectrophotometer operated from a microcomputer was used for colorimetric assays. Glass cuvettes with 1 cm optical path length and 1.5 mL volume were obtained from Hellma Ltda (Concord, ON). Temperature control was achieved with a THERMOMIX 18 BU B thermostatic bath (Braun Biotech International). The temperature control for all assay measurements to within ±0.1 °C was maintained by performing all assay reactions at constant room temperature with thermally equilibrated reagents. The system consists of an aquarium air pump, a pinch valve, sampling loop, a tubular reactor ($\varnothing = 0.25$ and 2.5 cm of length) with peroxidase chemically immobilized on Amberlite IRA-743 resin, cuvettes, and a spectrophotometer.

With phenol and 4-aminoantipyrine concentrations present in sufficient quantity, the rate of color generation (antipyrilquinoneimine dye) at 505 nm is proportional to the rate of hydrogen peroxide consumption. The generation of color by reaction of phenol (PhOH) + 4-aminoantipyrine ($Am-NH_2$) + H_2O_2 in the presence of the peroxidase may be represented by the following overall reaction (*28*):

$$\mathrm{PhOH} + \mathrm{Am{-}NH_2} + 2\mathrm{H_2O_2} \xrightarrow{\text{peroxidase}} \text{antipyrilquinoneimine dye} + 4\mathrm{H_2O}$$

The concentrations of the principal ions (K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, and Li^+) were measured using a capillary electrophoresis system with an automatic sampler and a UV−vis detector (CE Hewlett-Packard) with indirect detection at 214 nm. Running buffer contained 10 mmol/L imidazole of background electrolyte with the pH adjusted to 3.60 by adding 1 mol/L acetic acid, which also serves as a complexing agent. Prior to use, new capillaries were treated with 1.0 mol/L NaOH (10 min), purified water (30 min), and background electrolyte (30 min). Fused-silica capillaries with 75 μm i.d. and 60 cm in length were employed. The distance from the point of injection to the window of on-column detection was 42.5 cm. Sample injection was carried out in hydrodynamic mode (50 mbar) during 5 s. The separation run was at a constant voltage of +25 kV at 25 °C and is achieved in 4.5 min. Indirect UV detection was performed at 214 nm.

Samples. This work was carried out on 14 samples from Brazil. The samples were stored in the dark at room temperature prior to analysis. For determination of hydrogen peroxide, 1 g of honey was dissolved in 10 mL of purified water and injected into the flow-injection system. For the electrophoretic analysis, 2.5 g of honey was dissolved in 25 mL of purified water, filtered through a 0.45 μm membrane, and injected directly without any other sample pretreatment. Each sample was injected in triplicate.

RESULTS AND DISCUSSION

Immobilized Peroxidase and Chemical Variables for Determination of H_2O_2. The specific activities of free and immobilized peroxidase were determined spectrophotometrically (at 505 nm) under static conditions (*8*). The method was based on the change of the solution color resulting from the oxidative coupling of 4-aminoantipyrine, phenol by hydrogen peroxide, in the presence of the enzyme peroxidase. The properties of peroxidase immobilized on Amberlite IRA-743 modified with 0.1 wt % glutaraldehyde were studied to determine the optimum pH and temperature, reuse, and efficiency for the complete oxidation of the hydrogen peroxide, a fundamental condition for applications in the determination of hydrogen peroxide in honey. The activity of hydrogen peroxide at different pH values

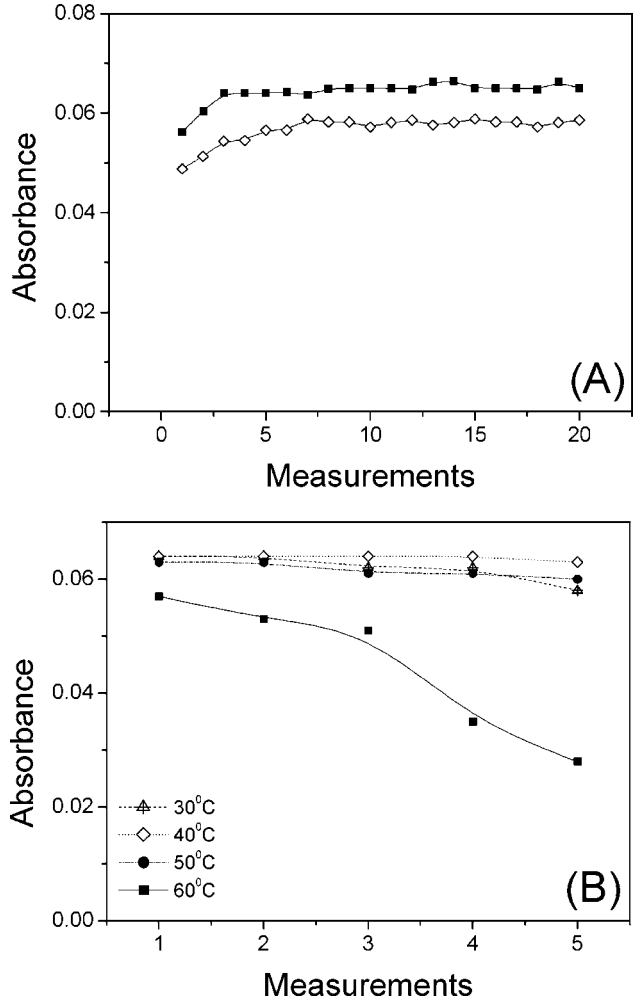

Figure 1. Activity of peroxidase (**A**) with used (◇) and new (■) Amberlite IRA-743 resin and (**B**) stabilities of the immobilized peroxidase at different temperature values between 20 and 60 °C for repetitive injections of 1×10^{-5} mol/L hydrogen peroxide. Conditions are as follows: 0.10 mol/L phosphate buffer (pH 7.0); sample volume, 200 μL; flow rate, 1.5 mL/min; λ, 505 nm.

varying from 4.0 and 9.0, with injection of 200 μL of 1.5×10^{-5} mol/L hydrogen peroxide, was studied. The optimum pH for peroxidase immobilized in the tubular reactor was 7.0. The activity of immobilized peroxidase using 200 μL of 1.0×10^{-5} mol/L hydrogen peroxide in used and new resin is shown in the **Figure 1**A, where similar results were obtained. The activity of peroxidase at different temperatures using 200 μL of 1.0×10^{-5} mol/L hydrogen peroxide was also studied. The temperature of the system (enzymatic reactor and bobbin of reaction coil) was controlled using a thermostatic bath. The optimum temperature for peroxidase immobilized onto Amberlite IRA-743 was determined to be 40 °C (**Figure 1B**).

To examine the efficiency of the tubular reactor containing immobilized peroxidase on the resin, experiments involving consecutive injections of hydrogen peroxide solutions were performed. The response of the spectrophotometric sensor for injection of 200 μL of 1.5×10^{-5} mol/L hydrogen peroxide with immobilized peroxidase was determined. **Figure 2** shows the stabilities of peroxidase immobilized in Amberlite IRA-743 resin. An important characteristic observed for the immobilized enzyme was a storage stability of at least 2 weeks under intense use with hydrogen peroxide standard. After this period, a decrease of about 30−45% of the enzyme activity was observed. When applied in the determination of hydrogen peroxide in

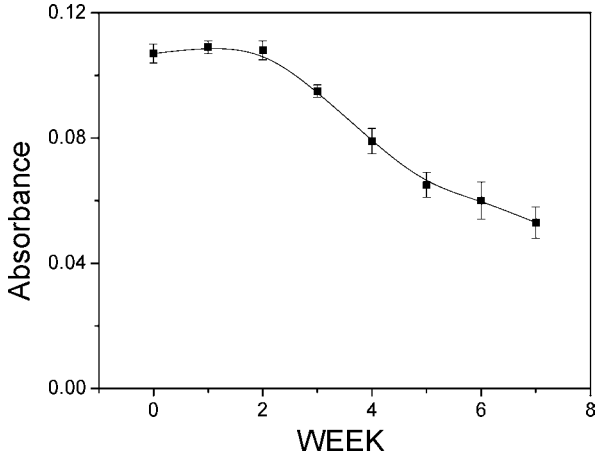

Figure 2. Repetitive injections of 1.5×10^{-5} mol/L hydrogen peroxide in different weeks. Conditions are as follows: 0.10 mol/L phosphate buffer (pH 7.0); sample volume, 200 μL; flow rate, 1.5 mL/min; λ, 505 nm.

honey, the enzymatic reactor showed a loss in the enzyme activity after 50 injections, requiring construction of a new reactor. When not in use, the reactors were stored in a freezer at −20 °C.

Those reagents that directly influence formation of the absorbing species were evaluated to determine optimum concentrations that provide high absorbance without utilization of a large excess of reagents. Various concentrations of phenol, peroxidase, and 4-aminoantipyrine were evaluated. The peroxidase and 4-aminoantipyrine gave no increase above 150 U/mL and 0.5 mmol/L, respectively. Phenol over the range 1.5−4.0 mmol/L showed an increase in absorbance of 14%. The optimum phenol, 4-aminoantipyrine, and peroxidase concentrations have been determined to be 1.87 mmol/L, 0.5 mmol/L, and 120 U/mL, respectively. A premixed reagent that includes phenol, 4-aminoantipyrine, peroxidase, and phosphate buffer yields results comparable to those obtained with the multiple reagent line FIA system, when prepared daily.

The influence of parameters such as flow rate and sample volume was also studied. A spectrophotometric response for injections of 200 μL of 1.0×10^{-5} mol/L hydrogen peroxide as a function of the flow rate, varying from 0.5 to 5.0 mL/min, was evaluated. At high flow rates, the peroxidase immobilized in the tubular reactor was unable to transform hydrogen peroxide completely to antipyrilquinoneimine dye. A flow rate of 1.5 mL/min was chosen as the most favorable, since it combines good reproducibility, high throughput, and lower consumption of carrier solution.

The influence of the sample volume on the analytical signal was also evaluated. Loops with internal volumes varying from 50 to 250 μL were tested. When the volume of the sample was increased, the spectrophotometric signal increased, but the time required for each analysis also increased. A volume of 200 μL was chosen as the working volume in subsequent experiments. For all the volumes studied, the peroxidase immobilized in the tubular reactor was sufficient to transform the hydrogen peroxide completely to antipyrilquinoneimine dye.

Calibration Plot. The calibration plot showed the proportionality between the absorbance and hydrogen peroxide concentrations for successive injections of 200 μL of hydrogen peroxide from 1 to 100 μmol/L. The hydrogen peroxide concentration was calculated using

$$\text{Abs} = 7665.1[\text{hydrogen peroxide}]\ (\text{mol/L}) - 0.00333 \quad (1)$$

The correlation coefficient obtained by linear regression was 0.9995. The detection limit for the conditions adopted in the

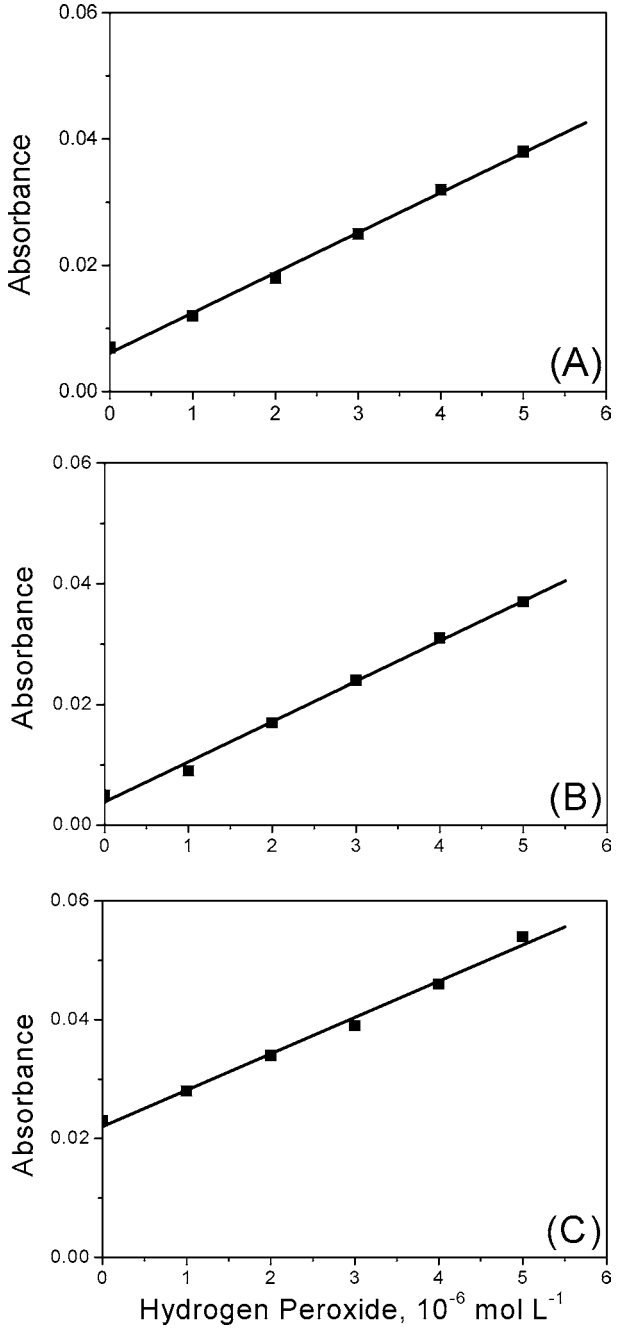

Figure 3. Standard additions curves for three honey samples: (**A**) sample 3; (**B**) sample 5; (C) sample 7.

present study was determined to be 0.7 μmol/L (3 times the standard deviation of the blank) (*29*).

Determination of H_2O_2 in Honey. To quantify hydrogen peroxide in honey, the samples to be analyzed, after dilution with phosphate buffer, were mixed on-line with phosphate buffer solution used as the carrier solution. Manzoori et al. (*7*) showed with when honey was diluted with phosphate buffer of pH 7.4, the maximum concentration was reached sooner (after about 20 min). After about 30 min, the concentration decreased rapidly. The results clearly showed that dilution of honey led to the continuous release of H_2O_2, which can act as a source of antibacterial agent (*7*).

The assay uses phenol, 4-aminoantipyrine, and peroxidase chemically immobilized on Amberlite IRA-743 resin as color-generating substrates. The spectrophotometeric method developed in this work that uses enzyme immobilized in the resin ion-exchange compared with the amperometric method did not present any significant difference in the results. The H_2O_2 concentration values determined by the two FIA method with standards and in six samples were compared using the linear regression procedure and the paired *t*-test (*29*). The confidence interval for the slope and intercept were 0.97 ± 0.03) and 0.04 ± 0.02 μmol^{-1} L^{-1}, respectively, for a 95% confidence level. Taking into account these results, no significant differences between the methods were observed, which strongly indicates the absence of systematic errors. **Figure 3** shows standard addition calibration for three different samples of honey. **Table 1** shows results of the analyses performed by spectrophotometry developed in this work using the enzyme immobilized on an ion-exchange resin for 14 different samples (in triplicate). Recovery experiments on honey solutions spiked with different amounts of H_2O_2 were also carried out.

Table 1. Hydrogen Peroxide and Mineral Content (mg/kg) of Honey Samples

sample no.	geographical origin	H_2O_2 (mg/kg)	cation[a] (mg/kg)					
			K^+	Ca^{2+}	Na^+	Mg^{2+}	Mn^{2+}	Li^+
1	Viçosa	155 ± 2	650	19.2	237	54.5	128	1.51
2	Teresópolis	135 ± 4	144	156	6.06	41.5	ND	ND
3	Teresópolis	71 ± 6	384	34.3	27.0	72.0	132	1.78
4	Teresópolis	131 ± 6	291	34.0	3.77	16.0	ND	1.36
5	Teresópolis	28 ± 1	758	50.9	79.6	25.2	ND	2.44
6	Teresópolis	9 ± 0	476	19.2	60.3	22.8	1.46	1.60
7	Teresópolis	214 ± 4	412	18.4	21.7	20.0	0.981	2.43
8	Espírito Santo	158 ± 5	435	56.0	302	24.8	ND	ND
9	Belo Horizonte	139 ± 3	178	117	50.0	13.9	ND	2.13
10	Tabuleiro	142 ± 4	758	140	46.1	49.9	10.0	ND
11	Coronel Pacheco	62 ± 3	1.40×10^3	166	99.7	80.3	15.4	2.39
12	Coronel Pacheco	91 ± 3	915	87.4	41.6	42.2	5.93	2.47
13	Volta Redonda	147 ± 6	1.01×10^3	10.2	6.51	11.9	ND	2.68
14	Juiz de Fora	47 ± 1	1.37×10^3	59.1	23.8	28.4	5.40	2.95
mean		109	656	69.1	71.8	36.0	21.4	1.70
V_{min}		28	144	10.2	3.77	11.9	0.981	1.51
V_{max}		214	1.40×10^3	166	302	80.3	132	2.95

[a] ND: not detectable..

The method recoveries obtained for the hydrogen peroxide ranged from 85% to 102%. Such values confirm the accuracy of the proposed method. The results obtained in this work were equivalent to that of Manzoori (*7*). This determination demonstrated the potential of the method for the detection of hydrogen peroxide in honey using peroxidase immobilized in a tubular reactor. The very high sensitivity combined with the low volume of the flow cell allows us to work with small sample volumes and at low concentrations. These advantages offer a very favorable way for the rapid analysis of hydrogen peroxide in honey samples. The main disadvantage of the present method was the fact of the honey inactivates the peroxidase after 50 injections, requiring construction of a new reactor.

Mineral Content. Mineral content was determined by identifying and quantifying six cations (K^+, Ca^{2+}, Na^+, Mg^{2+}, Mn^{2+}, and Li^+), which represent more than 99% of the total cations (**Figure 4**). These cations were identified by comparison of the relative migration times of their peaks with the Ba^{2+} migration time used as a reference. These cations were quantified by using an external standard calibration.

Calibration curves were determined for several different concentrations of a mixture of cation standard solutions. Each calibration solution was injected in triplicate. Plotting concentration against peak area and applying the least-squares method produced calibration curves for each compound. A regression analysis for each cation was made.

The detection limit was calculated as $s_b + 3s$, where s_b is the average signal of 10 blank injections and s the standard deviation. The quantification limit was calculated as $s_b + 10s$, where s_b is the average signal of 10 blank injections and s the

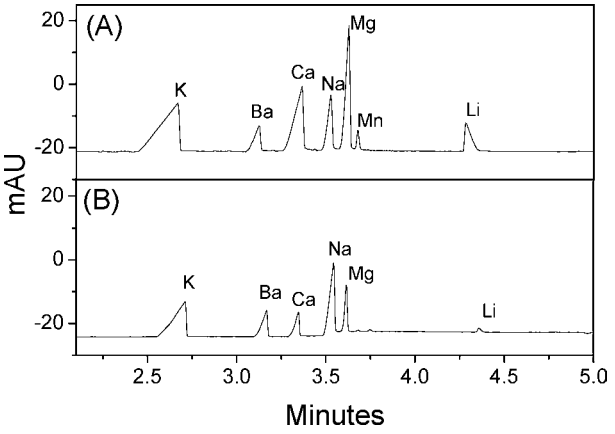

Figure 4. Electropherograms of analyzed cations by proposed method in (**A**) standard and (**B**) sample 4 (geographical origin, Teresópolis). Ba^{2+} was added as a reference compound for the calculation of relative migration times.

Table 2. Detection and Quantification Limit Obtained with the Method of Standard Additions for Analyzed Cations in Honey

cation	detection limit (μmol L^{-1})	quantification limit (μmol L^{-1})
K^+	0.117	0.347
Ca^{2+}	0.683	1.39
Na^+	0.795	1.22
Mg^{2+}	0.463	1.86
Mn^{2+}	0.048	0.130
Li^+	0.098	0.224

Table 3. Mineral Content (mg/kg) Obtained by Other Authors

	cation (mg/kg)					
ref	K^+ ($\times 10^3$)	Ca^{2+}	Na^+	Mg^{2+}	Mn^{2+}	Li^+
15	0.94	50	47	27	2.2	
19	1.22	93	85	54	11	2.3
20	0.68	181	389	77		
21	1.34		115	77	5	0.05
22		66			0.8	
24	0.66			76	0.7	
30	0.47	48	96	37	3	
31		192		71	11	0.27
32	1.03			130	30	

standard deviation. **Table 2** shows the detection and quantification limit obtained for each cation.

The precision study comprised repeatability and reproducibility assays. Ba^{2+} was used as a reference compound for the identification of cations in honey samples because it was not present in honey samples at the detection limit of this method. In the study of the precision of migration times, better results were obtained for relative migration times. The relative standard deviations (RSD) of the repeatability and the reproducibility were $\leq$0.36% and $\leq$0.58% for relative migration times and $\leq$1.78% and 2.54% for absolute migration times.

We have established the accuracy of the cation analysis by using the method of standard additions. Different amounts of each cation standard were added to equal volumes of the sample and then diluted to the same volume. Furthermore, to test whether there was a matrix effect, the recovery assay must be analyzed with different concentrations of sample. If regression lines obtained from the comparison of recoveries are parallel, we can conclude that there was no matrix effect. There was no matrix effect for the determination of cations in honey samples by the method used. **Table 1** shows the results of the analyses of 14 different samples (in triplicate) obtained for each cation. **Table 3** shows results obtained by other authors (*15*, *19*−*22*, *24*, *30*−*32*). The results are close to those obtained in this work in all cases.

The advantage of this method is that the separation and quantification of the cations for electrophoretic analysis were simple and rapid and did not require any other preparation of sample other than dilution and filtration. It represents a great improvement for the determination of cations in honey samples because calcination of the samples is not necessary and thus could be useful for routine analysis. The cation contents of honey could be used to establish the geographical origin of samples.

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