

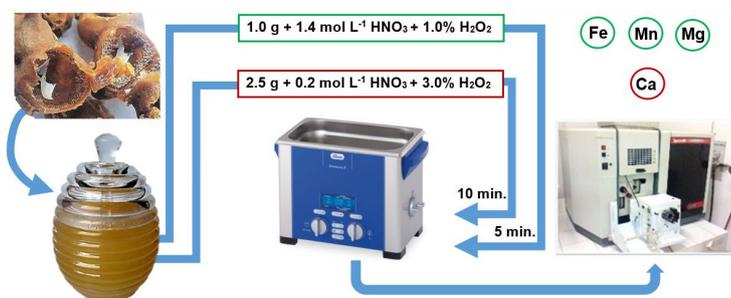
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Development of an Analytical Method for Rapid Metal Determination in Stingless Bee Honey

Gislaine Pucholobek *, Camila Kulek de Andrade , and Sueli Pércio Quináia 

Stingless bee honey is a product used for different purposes due to its composition, in which the presence of metallic species is highlighted. Depending on their amount, they may have either a nourishing or a toxic effect. Flame atomic absorption spectrometry (FAAS) is a commonly used technique for quantifying metals in samples such as honey. However, because of the complexity of the matrix, the sample must go through a preparation step for the analytes to be available for quantification. In the present work, a fast and efficient sample preparation method for the quantification of metallic ions in stingless bee honey was optimized, from the direct analysis of slurries using FAAS. The samples were suspended using honey (1.0 g and 2.5 g), nitric acid (1.4 mol L⁻¹ and 0.2 mol L⁻¹), hydrogen peroxide (1% and 3%) and ultrasonic agitation (5 and 10 minutes). Then, the proposed analytical method was validated by evaluating parameters such as linearity, limits of detection and quantification, precision and accuracy, all with adequate values compared to those established in the literature.

Graphical abstract



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1. Introduction

Stingless bee honey has been standing out in the traditional market of this type of product and has aroused interest due to its intrinsic characteristics, which, when compared to the commonly consumed honey of the species *Apis mellifera*, differs in acidity, sugars, moisture content, metals and other characteristics [1].

Stingless bees are widespread and reported in different tropical and subtropical regions, with more than 500 species identified in 32 genera [2]. Although the presence of stingless bees is significant in different biomes and the values added to

the honey of this species are relatively higher than the honey of the *Apis mellifera* [3], the fact that there are no legislations that meet their specificities is limited to supervision and, consequently, the marketing of this product [4].

In addition to stingless bee honey being a product with distinct sensory characteristics, it is important to highlight the benefits that these bees add to the pollination of native plants [5-6], in the use of honey for therapeutic purposes [7] and as an indicator of environmental contamination [8].

Currently, the interest of the scientific community in the study of stingless bee honey is increasing [9], due to the fact that although it is a product with great characteristics and uses, the official methods available for the quality control of this product are established with basis only on the honey characteristics of the species *Apis mellifera* [10].

One of the parameters used by international organizations to assess honey quality (*Apis mellifera*) is metal content, which can be related to its botanical source, geographical area, environmental conditions, bee species and storage conditions [11].

Techniques based on absorption or atomic emission have been used to quantify metals in honey. Flame atomic absorption spectrometry (FAAS) is often used because it is characterized as a technique with good analytical performance and low operating costs [12-14]. However, because of the complexity of the matrix, it is necessary for the sample to go through a preparation step so that analytes are available for quantification [15].

According to Pohl et al. [15], in a review carried out on the most commonly used procedures for honey sample digestion, one of the most traditional is dry digestion. However, due to the high preparation time and the high temperatures employed, analyte losses may occur as a result of volatilization. Microwave-assisted wet digestion is often used for the digestion of samples such as honey, with the addition of reagents such as HNO₃ (65%) and H₂O₂ (30%) in large and distinct proportions from 3: 4 [16] to 9: 1 [17], respectively.

Specifically for stingless bee honey, among the methods used for sample preparation and further determination of metals, as reported in the literature, one can cite the incineration of samples in a muffle furnace and the subsequent solubilization of the ashes obtained in an acid medium [1, 18], nitro-perchloric digestion [8], solubilization of honey samples in HNO₃ [19] and the gravimetric method (described by the Brazilian legislation for the *Apis mellifera* honey based on the Codex Alimentarius (2001) [11, 20]. Considering the aforementioned methods, all take a long preparation time and/or use large volumes of reagents.

In this sense, slurry sampling presents itself as a potential alternative for simplifying this step, as it articulates advantages of liquid and solid sampling, minimizing interferences that may attribute errors during this step, such as long exposure time of the sample and the use of high volumes of reagents as noted above [21-23].

Therefore, the present work aims at developing an analytical method for the rapid determination of metals in Brazilian stingless bee honey samples and complex matrix of commercial and environmental interest, using flame atomic absorption spectrometry (FAAS).

2. Material and Methods

Reagents and Clean Techniques

To minimize and eliminate possible sources of sample contamination, a set of procedures of cleaning techniques was adopted during sample handling and analysis [25]. Briefly, the glassware was washed with detergent and running water, then left in a HNO₃ bath (5%) for a minimum of 24 h, and then rinsed with ultrapure water. They were later placed in decontaminated drying trays and stored in plastic bags until they were used. The solutions used in the procedures were prepared with ultrapure water obtained by the Milli-Q system supplied by Millipore Corporation®. Fe, Mn, Mg and Ca

standard solutions were made by diluting 1000 mg L⁻¹ standard solutions (Biotec®) until the desired concentration. An ultrasonic bath (frequency 37 kHz; 100% power; Elma®) was employed to prepare the honey slurries.

For the determination of Fe, Mn, Mg and Ca, a flame atomization atomic absorption spectrometer - FAAS (Varian model AA 220) was used. For Fe, Mn and Mg, an oxidizing flame was employed using an air/acetylene mixture in a flow rate of 13.5 L min⁻¹ and 2.00 L min⁻¹, respectively. For Ca, the flame used was reductive, with the mixture of N₂O/acetylene, with a flow rate of 10.0 L min⁻¹ and 6.35 L min⁻¹, respectively. The measurements were performed in triplicate, considering the integration of the absorbance signal, with a total time of 6 seconds of analysis. Hollow cathode lamps (Photron, Agilent and Luxan) were used for the elements studied, using the operating conditions recommended by the equipment manufacturer.

Samples

For the optimization and validation studies, we used a sample of native bee honey of the species *Tetragonisca angustula* (jataí), collected in 2018 in the region of Nova Tebas, Paraná, Brazil. After the sample collection, the same was stored in a polypropylene bottle and kept under refrigeration from 6 to 8 °C.

Analytical Method Optimization

The performance of the proposed analytical method was evaluated by comparing three slurry sampling procedures with microwave assisted acid digestion. Method A [24] was performed using 1.0 g of honey with the addition of HNO₃ (0.1 mol L⁻¹); in method B [25], the suspension was prepared with 1.0 g of honey, HNO₃ (0.2 mol L⁻¹) and H₂O₂ (3%), whereas in method C [26] the suspension was prepared using only ultrapure water. Ultrasonic agitation was used for 10 minutes in all the methods. Considering this study, for Ca and Mg it was not necessary to enrich the samples. For Fe and Mn, however, it was necessary to enrich the samples with 1.0 mg L⁻¹ of the metallic ions, since these elements are naturally found in honey in low concentrations.

For microwave digestion, 1.0 g honey was used with the addition of 4.0 mL of HNO₃, 1.0 mL of H₂O₂ and 1.0 mL of H₂O to the samples. The honey solutions were then transferred to Teflon flasks and taken to digestion using a microwave system (Anton Paar) and a heating ramp (from 0 to 150 °C) for 50 minutes. The operating conditions used in the microwave were established as recommended by the equipment manufacturer.

After choosing the appropriate method, an univariate optimization was performed by evaluating the honey mass (0.5 to 2.0 g), HNO₃ concentration (0.1 to 0.9 mol L⁻¹), H₂O₂ concentration (1 to 9%) and ultrasonic agitation time (5 to 20 min).

Validation of the Analytical Method

The validation of the optimized condition was performed by assessing the limits of detection and quantification, linearity, precision and accuracy of the method.

The limits of detection and quantification were obtained through 10 consecutive measurements of the analytical blank and calculated according to the AOAC validation guide [27].

To evaluate the linearity of the method, different analytical curves were constructed for the analytes, in order to verify if

the extraction medium or the honey matrix could interfere in the quantification of the metals of interest. The procedure

used to construct each curve is shown in Table 1.

Table 1. Conditions of preparation of the analytical curves for Fe, Mn, Mg and Ca.

Metals	External standard – ES	Extrator medium – EM	Standard addition – SA
Fe	Metallic standard in aqueous medium in the range from 0.1 to 1.2 mg L ⁻¹	Metallic standard in the range from 0.1 to 1.2 mg L ⁻¹ + HNO ₃ (0.2 mol L ⁻¹) + H ₂ O ₂ (1%) + 5 min of ultrasonic agitation	Metallic standard in the range from 0.1 to 1.2 mg L ⁻¹ + 1.0 g of honey + HNO ₃ (0.2 mol L ⁻¹) + H ₂ O ₂ (1%) + 5 min of ultrasonic agitation
Mn			
Mg	Metallic standard in aqueous medium in the range from 0.2 to 0.8 mg L ⁻¹	Metallic standard in the range from 0.2 to 0.8 mg L ⁻¹ + HNO ₃ (0.01 mol L ⁻¹) + H ₂ O ₂ (0.09%) + 5 min of ultrasonic agitation	Metallic standard in the range from 0.2 to 0.8 mg L ⁻¹ + 0.01 g of honey + HNO ₃ (0.01 mol L ⁻¹) + H ₂ O ₂ (0.09%) + 5 min of ultrasonic agitation
Ca	Metallic standard in aqueous medium in the range from 0.4 to 2.0 mg L ⁻¹	Metallic standard in the range from 0.4 to 2.0 mg L ⁻¹ + HNO ₃ (0.01 mol L ⁻¹) + H ₂ O ₂ (0.09%) + 5 min of ultrasonic agitation	Metallic standard in the range from 0.4 to 2.0 mg L ⁻¹ + 0.01 g of honey + HNO ₃ (0.01 mol L ⁻¹) + H ₂ O ₂ (0.09%) + 5 min of ultrasonic agitation

Source: The authors (2019).

The data obtained from the analytical curves in an aqueous medium, medium similar to the extraction medium and standard addition, were used in the linear regression calculation using the Minitab 16 software. The linearity data for the analytical curves were evaluated by the parameters: *F* regression, *F* lack of fit and analysis of the determination coefficient (*R*²).

The precision of the method was assessed by the relative standard deviation values obtained on the same day and on different days, and by the Horrat value, through the equations established by the AOAC guide [27].

The accuracy of the analytical method was evaluated by comparing dry digestion, the method traditionally used for the digestion of samples such as honey, and the validated procedure of suspension preparation. Dry digestion was carried out using the methodology described by the IAL (2008), which charred 1.0 g of stingless bee honey in porcelain capsules with the aid of a Bunsen Nozzle, which was later calcined in a muffle furnace at 450 °C for 4 hours. The resulting ashes were dissolved in 1.0 mL of HNO₃ (65% w/v) and enlarged to 10 mL [28].

3. Results and Discussion

Analytical Method Optimization

When comparing the different sample preparation procedures for honey [24-26], it was observed that in samples with or without metal enrichment, the extraction percentages ranged from 70% (Fe) to 100% (Mn). The highest extraction rates were for methods A and B, with percentages higher than 80% for all the metals evaluated, while method C showed adequate extraction only for Mg, demonstrating how easy it was to extract this metal in complex matrices such as honey. Method B was chosen due to the best results for all the metals, in which HNO₃ (0.2 mol L⁻¹) and H₂O₂ (3%) were used for the preparation of the slurries.

Then, the univariate optimization of method B was performed, varying factors such as honey mass, HNO₃ and H₂O₂ concentrations, and ultrasonic agitation time. The univariate optimization study was more adequate when compared to the multivariate study, because of the characteristics of the matrix used, since the variations between the effects were within the deviations, and no significant differences were observed between the conditions evaluated in the planning.

When evaluating the factors individually, there were no significant variations for the quantification of Fe and Mn, since

they were the metals found in smaller amounts in honey, while Mg and Ca were determinants to establish the condition of each factor.

When analyzing the first factor, honey mass, it was observed that larger masses (2.5 g) were more adequate for the quantification of Ca, while smaller masses (1.0 g) were more suitable for Mg. After evaluating the HNO₃ concentration, it was observed that for Fe, higher concentrations of HNO₃ (1.4 mol L⁻¹) are required, while for the other metals the extraction is already effective using acid in lower concentrations (0.2 mol L⁻¹).

In the study of H₂O₂ concentration, it was concluded that percentages of 1% (for Fe, Mn and Mg) and 3% (for Ca) were efficient for the preparation of suspensions and the quantification of metals. Regarding the ultrasonic agitation time, times between 5 (for Fe, Mn and Ca) and 10 (for Ca) minutes were necessary.

The optimum sample preparation conditions for the quantification of metals in stingless bee honey were 1.0 g honey, 1.4 mol L⁻¹ HNO₃, 1.0% H₂O₂ and 5 minutes of ultrasonic agitation for metals Fe, Mn and Mg, while for Ca the conditions established were 2.5 g honey, 0.2 mol L⁻¹ HNO₃, 3.0% H₂O₂ and 10 minutes of ultrasonic agitation. After the optimization of the analytical method, a validation study was performed.

Validation of the Analytical Method

The linearity of the method was evaluated by comparing different analytical curves as shown in Figure 1. As it can be observed, there were no significant differences between the inclines of the evaluated curves.

From the data obtained in the analytical curves with ES, EM and SA, the coefficients of linear regression and of determination (*R*²) were calculated, as well as the evaluation of the models of linear regression and lack of adjustment, at 95% confidence level, represented in Tables 2 and 3.

As it can be seen in Table 2, where the regression parameters are presented, the inclinations between the ES, EM and SA curves are similar and, statistically, do not differ from each other.

For *R*², the values ranged from 95.5 to 99.9, that is, the correlation coefficients (*r*) ranged from 0.98 to 1.00, corroborating the linear relationship between the absorbance signal and the metal concentrations.

By analyzing the confidence intervals (CI) for each curve, it was possible to observe that, for all the metals, the CI for SA were similar to the CI for EM and ES. This indicates that both

the EM and the ES curves could be used to quantify Fe, Mn, Mg and Ca in honey slurry, which is advantageous, as it

reduces the analysis costs and increases the analytical frequency.

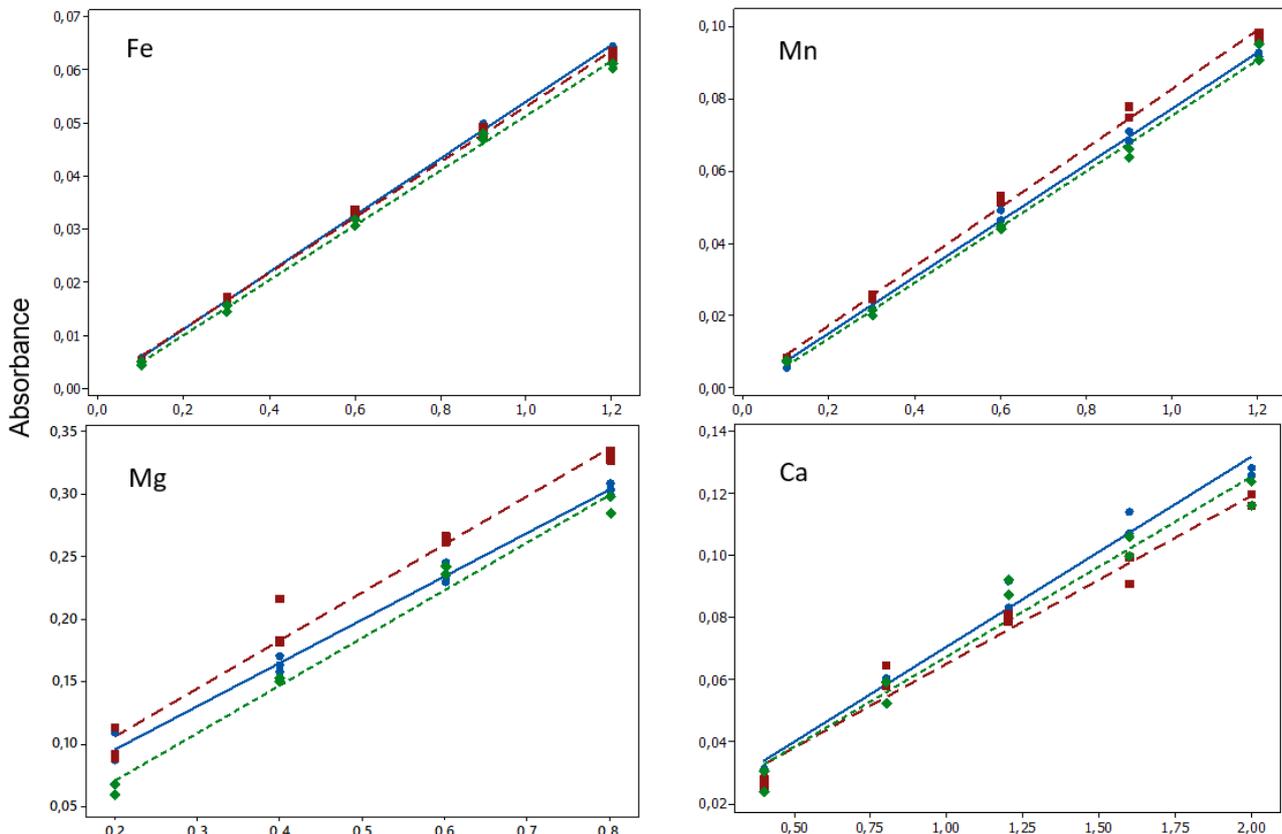


Fig. 1. Analytical curves obtained in the determination of Fe, Mn, Mg and Ca by FAAS. Legend: ES (●) – External Standard; EM (■) – Extraction Medium; SA (◆) – Standard Addition. Source: The authors (2019).

Table 2. Parameters of the analytical curves for Fe, Mn, Mg and Ca.

Calibration		Regression Parameters		
		Equation of the line ¹	Confidence Interval (CI)	R ²
Fe (0.1 – 1.2)	ES	$A = 0.0774 (\pm 0.001) \times [\text{Fe}]$	(0.07458 – 0.08022)	99.8
	EM	$A = 0.0818 (\pm 0.002) \times [\text{Fe}]$	(0.07809 – 0.08550)	99.7
	SA	$A = 0.0769 (\pm 0.002) \times [\text{Fe}]$	(0.07254 – 0.08126)	99.5
Mn (0.1 – 1.2)	ES	$A = 0.0538 (\pm 0.0005) \times [\text{Mn}]$	(0.05263 – 0.05497)	99.9
	EM	$A = 0.0523 (\pm 0.0008) \times [\text{Mn}]$	(0.05043 – 0.05417)	99.8
	SA	$A = 0.0516 (\pm 0.0008) \times [\text{Mn}]$	(0.04972 – 0.05348)	99.8
Mg (0.2 – 0.8)	ES	$A = 0.0252 (\pm 0.005) + 0.348 (\pm 0.009) \times [\text{Mg}]$	(0.32697 – 0.36902)	99.2
	EM	$A = 0.0282 (\pm 0.010) + 0.385 (\pm 0.017) \times [\text{Mg}]$	(0.34547 – 0.42452)	97.7
	SA	$A = 0.387 (\pm 0.019) \times [\text{Mg}]$	(0.34004 – 0.43396)	98.3
Ca (0.4 – 2.0)	ES	$A = 0.00901 (\pm 0.004) + 0.0613 (\pm 0.003) \times [\text{Ca}]$	(0.05476 – 0.06783)	98.1
	EM	$A = 0.0107 (\pm 0.004) + 0.0542 (\pm 0.003) \times [\text{Ca}]$	(0.04648 – 0.06192)	96.7
	SA	$A = 0.0582 (\pm 0.004) \times [\text{Ca}]$	(0.04853 – 0.06787)	95.5

Source: The authors (2019). Notes: The concentration units of the calibration curves are given in mg L⁻¹. (ES: External Standard; EM: Simulation of the Extractor Medium; SA: Standard Addition).¹ The values in parentheses correspond to the confidence intervals of the linear and angular coefficients.

In addition to the evaluation of regression parameters, for the ES curves adopted in the study, we also analyzed F regression and F lack of fit values at a 95% confidence level, data shown in Table 3.

The F_{obs} values ranged from 192.55 (Ca) to 11252.40 (Mn), and all of them were higher than the $F_{critical}$ values, indicating that linear regression is significant for all the analytical curves. To assess the lack of fit of the linear models, F_{iof} values were analyzed, ranging from 0.76 (Fe) to 9.98 (Mg), where all were below the $F_{critical}$ values and with p values above 0.05. In this sense, the ES curves present acceptable linearity, without lack of adjustment, to quantify metals Fe, Mn, Mg and Ca in

stingless bee honey, using FAAS.

Taking into account the concentration of metals in samples such as honey and the complexity of the matrix, it is necessary to know which concentration of the analyte can be detected or quantified by the proposed method. Thus, limits of detection (LOD) and quantification (LOQ) of the optimized method for the quantification of Fe, Mn, Mg and Ca in stingless bee honey were determined using the FAAS technique. The data are shown in Table 4. The limits of detection ranged from 0.2 to 4.3 $\mu\text{g g}^{-1}$, and of quantification ranged from 0.6 to 13 $\mu\text{g g}^{-1}$. Regarding the technique used and the matrix analyzed, the values of LOD and LOQ can be considered acceptable.

Table 3. Data of linearity for Fe, Mn, Mg and Ca.

Calibration	Evaluation of Linearity			
	Regression		Lack of fit	
	F_{obs}	P	F_{lof}	P
Fe (0.1 – 1.2)	4011.52	0.00	0.76	0.565
Mn (0.1 – 1.2)	11252.40	0.00	1.94	0.241
Mg (0.2 – 0.8)	1357.81	0.00	0.14	0.869
Ca (0.4 – 2.0)	467.76	0.00	3.38	0.112

Source: The authors (2019). Notes: The concentration units of calibration curves are given in mg L^{-1} . (ES: External Standard; EM: Extractor Medium; SA: Standard Addition). Fe, Mn and Ca: $F_{critical}$ (1.8; 0.05) for Regression = 5.32 and $F_{critical}$ (3.5; 0.05) for Lack of fit = 5.41. Mg: $F_{critical}$ (1, 10, 0.05) for Regression = 4.96 and $F_{critical}$ (2, 8, 0.05) for Lack of fit = 4.46.

Table 4. Limits of detection and quantification obtained for Fe, Mn, Mg and Ca, employing Slurry Sampling and FAAS.

Element	LOD ($\mu\text{g g}^{-1}$)	LOQ ($\mu\text{g g}^{-1}$)
Fe	0.2	0.7
Mn	0.2	0.6
Mg	0.2	0.6
Ca	4.3	13

Source: The authors (2019).

Table 5. Precision study values (repeatability and intermediate precision), Horrat value, and accuracy study using slurry sampling for the honey preparation and determination of metals by FAAS.

Element	(%RSD)		Horrat	Extraction (%)
	Repeatability	Intermediate Precision		
Fe	1.4	6.9	0.5	95.6
Mn	4.8	9.8	0.6	90.5
Mg	3.4	9.6	1.2	96.8
Ca	0.1	9.0	1.2	86.5

Source: The authors (2019).

4. Conclusions

Slurry sampling proved to be an efficient resource when compared to traditional methods of sample digestion for metal quantification using the flame atomic absorption spectrometry (FAAS) technique, with advantages such as reagent reduction and time optimization during the analysis step of preparing complex samples such as honey, thereby minimizing analysis costs. The analytical method developed for the determination of Fe, Mn, Mg and Ca in stingless bee honey proved to be adequate, since the results obtained in the validation of this method are within the acceptability criteria of national and international regulatory agencies. Given this, the work developed presented significant contributions for the determination of metals in honey from native bees, which may be relevant to help in the regulation, commercialization, supervision and characterization of this product, taking into consideration its characteristics and specificities, distinct from honeys commonly marketed.

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In order to verify the reliability of the developed method, the precision of the method was also evaluated through the tests of repeatability, intermediate precision and Horrat value. The accuracy of the proposed method using the slurry sampling procedure was also evaluated by comparing it with the dry digestion method [28], which is traditionally used for the digestion of samples such as honey. The values obtained are shown in Table 5.

For repeatability, it was observed that the relative standard deviation (% RSD) values were lower than 5%, while for intermediate precision, the RSD values were lower than 10%. In this sense, the RSD values can be considered acceptable, since the validation guides used are limited to 11% for this criterion, considering the concentration of analytes obtained in this type of matrix. The same can be observed for the Horrat value, and in this study, for all the metals, it was below 1.2 and within the acceptability range for this parameter (0.3 - 1.3).[27]

Table 5 shows also that the extraction rates were above 86% for all the metals evaluated in stingless bee honey. Considering the acceptability range for this parameter (80 - 100%), the method obtained adequate accuracy since the values obtained are within this range [29].

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