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Efficiency of two digestion methods in determining the presence of metals (Cd, Cu, Cr, Pb and Zn) in geopropolis produced by *Melipona scutellaris*

Abstract

This study evaluates the efficiency of acid extraction and total digestion to determine the presence of metals in geopropolis produced by the stingless bee *Melipona scutellaris*. Geopropolis samples were collected at five meliponaries in the city and in the metropolitan region of Salvador, Bahia State, Brazil. The sample treatment methods comprised acid extraction and total digestion. The Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES) technique was used to quantify Cd, Cu, Cr, Pb, and Zn. Geopropolis samples submitted to both digestion methods showed statistical differences. For both methods, Cr and Zn showed the highest concentrations, while those of Cd were the lowest. The Cr concentration for determination by total digestion was 37.53 mg/kg, while for acid extraction it was 32.90 mg/kg. For Zn, the concentration was 17.65 mg/kg and 8.85 mg/kg for total digestion and acid extraction, respectively. The total digestion method showed the highest concentrations of the metals evaluated; however, acid extraction (USEPA 3050b) is a more straightforward procedure for metal evaluation in geopropolis samples and presented values that support the use of geopropolis as a bioindicator. The acid extraction method USEPA 3050b, in combination with detection using ICP OES, showed efficiency in analyses carried out to determine metals in geopropolis.

Keywords: *Melipona scutellaris*; stingless bee; acid extraction; total digestion; ICP OES.

Eficacia de dos métodos de digestión para determinar metales (Cd, Cu, Cr, Pb y Zn) en geopropóleos producidos por *Melipona scutellaris*

Resumen

El objetivo de este artículo fue evaluar la eficacia da extracción ácida y de la digestión total para determinación de metales absorbidos en geopropóleos producidos por *Melipona scutellaris*. Se colectaron muestras de geopropóleos en cinco meliponarios localizadas en la región metropolitana del Salvador, estado de Bahia, Brasil. Se utilizaron como métodos de tratamiento para muestras, la extracción ácida y la digestión total. Para determinar los metales Cd, Cu, Cr, Pb y Zn se utilizó Espectroscopia de Emisión Óptica con Plasma Acoplado Inductivamente (ICP OES). Se encontraron diferencias estadísticas en la concentración de metales hallados en las muestras de geopropóleos sometidas a los diferentes métodos de digestión. Para ambos métodos de digestión, las mayores concentraciones de metal fueron observadas para Cr y Zn. Entre los metales evaluados Cd presentó la menor concentración. La concentración de Cr para la determinación por digestión total fue de 37,53 mg/kg y para la extracción ácida fue de 32,90 mg/kg. Para el Zn, la concentración fue de 17,65 mg/kg y 8,85 mg/kg para la digestión total y la extracción ácida, respectivamente. El método de digestión total mostró los mayores valores para concentraciones de los metales evaluados. Entretanto, la extracción ácida, USEPA 3050b, es un procedimiento más simple para la evaluación de metales en muestras de geopropóleos y también mostró valores que pueden satisfacer la necesidad de su uso en evaluaciones de colmenas como bio-indicador. El método de extracción ácida USEPA 3050b en combinación con la detección a través de ICP OES se mostró eficiente para el análisis de metales en geopropóleos.

Palabras clave: *Melipona scutellaris*; abeja sin aguijón; extracción ácida; digestión total; ICP OES.

Eficiência de dois métodos de digestão para determinar metais (Cd, Cu, Cr, Pb e Zn) na geoprópolis produzida por *Melipona scutellaris*

Resumo

O objetivo deste estudo foi avaliar a eficiência da extração ácida e da digestão total para determinação de metais adsorvidos na geoprópolis produzida por *Melipona scutellaris*. Foram coletadas amostras de geoprópolis em cinco meliponários, situados em Salvador, Estado da Bahia e região metropolitana. Foram utilizados como métodos de tratamento das amostras a extração ácida e a digestão total. Para determinação dos metais Cd, Cu, Cr, Pb e Zn utilizou-se a Espectrometria de Emissão Óptica com Plasma Indutivamente Acoplado (ICP OES). Houve diferença estatística na concentração de metais encontrados nas amostras de geoprópolis submetidas aos diferentes métodos de digestão. Para ambos os métodos de digestão as maiores concentrações de metais foi observada para o Cr e Zn. Dentre os metais avaliados o Cd apresentou a menor concentração. A concentração de Cr para determinação por digestão total foi de 37,53 mg/kg e para extração ácida de 32,90 mg/kg. Para o Zn, a concentração foi de 17,65 mg/kg e 8,85 mg/kg para digestão total e extração ácida, respectivamente. O método de digestão total apresentou os maiores valores para concentrações dos metais avaliados. No entanto, a extração ácida, USEPA 3050b, é um procedimento mais simples para a avaliação dos metais em amostras de geoprópolis e também apresentou valores que podem satisfazer a necessidade de utilização na avaliação deste produto da colmeia como bioindicador. O método de extração ácida USEPA 3050b em combinação com a detecção por ICP OES mostrou eficiência para análise de metais em geoprópolis.

Palavras-chave: *Melipona scutellaris*; abelha sem ferrão; extração ácida; digestão total; ICP OES.

Introduction

Sample digestion is usually necessary to determine total metal concentrations in a material, since the application of different acid digestion methods can release different amounts of analytes in the digested solution [1-3]. An appropriate choice of the digestion method to be used in the analytical procedure is essential if element concentrations are to be determined, because complementing the digestion method with the quantification technique ensures the accuracy of the procedure [4-5].

The total digestion of samples containing silicates generally includes mixtures of nitric, hydrochloric and hydrofluoric acids (HNO_3, HCl, and HF respectively). This procedure is used to release analytes from the adsorbed, exchangeable, oxidizable, reduced and residual fractions of the sample matrix [6-7]. Despite HF efficiency in digesting silicates, its use becomes dangerous and requires great care in handling; thus, it is not recommended for use in an analytical routine [2, 7-8].

Acid extraction using HNO_3 and HCl, or HNO_3 and hydrogen peroxide (H_2O_2), are an alternative to the use of nitric-fluoric acid mixtures [6]. Positive results have been achieved by Sastre et al. [7] using acid extraction in soil samples for Cd, Pb, and Cu, when compared to the results using nitric-fluidic solutions. Sample pretreatment is lengthy [10-11], subject to errors, and can be costly [9]. Therefore, understanding the best procedure for sample digestion can avoid these obstacles, making the process faster.

Several techniques can be used to detect metals in hive products, such as Inductively Coupled Plasma Atomic Emission Spectrometry (ICP AES), Inductively Coupled Plasma Optical Emission Spectrometry (ICP OES) and Differential pulse anodic stripping voltammetry (DPASV) [13-14]. Determination by ICP OES is the most commonly used technique in studies of geopropolis, presenting satisfactory results [15-17]. The principal advantage of this technique is the possibility of selecting the optimum λ (wavelength) for each element in any type of sample. In addition, there are no restrictions on the number of elements that can be studied [18].

Geopropolis is a product of stingless beehives, which has aroused the interest of researchers because of its use in environmental quality monitoring, as well as for its chemical characterization [15, 19-20]. Geopropolis is a particular type of propolis, because it contains a mixture of resin and exudates from various plant sources, mixed with wax, silt, and soil particles, which gives it specific characteristics [21-22]. Thus, evaluation of digestion procedures is crucial to determining inorganic constituents, such as Cd, Cu, Cr, Pb, and Zn in geopropolis produced by Meliponini. This study assesses the efficiency of acid extraction and total digestion to determine metals adsorbed in geopropolis produced by *Melipona scutellaris*.

Material and methods

Study site and sampling

Samples of geopropolis were collected from meliponaries in the city of Salvador and in the metropolitan region, Bahia State, Brazil. Samples were collected between June 2015 and July 2016. The sites of the meliponaries are highly urban-industrial, characterized by the dispersion of pollutants into the atmosphere, water, and soil. Pollutants come from various sources, including automotive vehicles, chemical factories and landfill.

Samples were collected in the Camaçari Industrial Pole (Polo Industrial de Camaçari - PIC) located in a densely populated area, close to large water springs, a forest belt and preservation areas containing forest and rural communities. The Aratu Industrial Center (Centro Industrial de Aratu - CIA) is located near Salvador, Bahia State; it has heavy traffic and is densely populated but also contains environmental protection areas, such as the São Bartolomeu Park. Salvador city's landfill site borders both the CIA and the PIC, and is also located in a densely populated area, with heavy traffic and many surrounding communities (Table 1).

Table 1. Origin of the geopropolis samples of *Melipona scutellaris* (Hymenoptera: Apidae, Meliponini), from five meliponaries located in Salvador, Bahia, Brazil, and the metropolitan region.

Meliponaries	Code	Geographic coordinate	Description
1	G1	S 12º51'28.3" W 38º21'54.3"	Adjacent to landfill - 1 km away from the CIA-Airport highway.
2	G2	S 12º32'28.0" W 38º21'42.3"	Distanced from the urban perimeter, access by unmetalled road and away from the highway.
3	G3	S 12º49'58.7" W 38º22'27.4"	Urban perimeter and close to the CIA-Airport highway.
4	G4	S 12º43'55.5" W 38º23'51.6"	Near the Camaçari Industrial Pole (PIC) and approximately 100 meters from the highway.
5	G5	S 12º50'38.1" W 38º21'12.1"	Urban perimeter and close to the CIA-Airport highway.

Collection and storage of geopropolis samples

Geopropolis samples were available inside the hive of *M. scutellaris* and nitrile gloves were used at collection to avoid sample contamination. The samples were packed in sterile plastic bags and properly identified. A pistil and ceramic mortar were used to macerate the samples, which were then sieved through a 500-nm nylon mesh. Subsequently, the samples were submitted to the digestion processes.

Acid digestion of samples

Acid extraction

The geopropolis samples (n = 15) were submitted to acid extraction using the USEPA 3050b method [23]. A mass of 0.5 g of each sample was used. For the analytical procedure, a 3 mL aliquot of HNO_3 was added, followed by 2 mL of H_2O_2. The analytical grade reagents were 65% HNO_3 and H_2O_2 30%. The recovery analysis was performed with the reference material (sample San Joaquin Soil, 2709), subjected to the same evaluation method used for the geopropolis samples.

Procedures for sample digestion

A 0.5 g sample of geopropolis was placed in a digester tube, followed by 10 mL of HNO_3 solution and the content homogenized. The tube was closed with a reflux funnel and the mixture was rested for 5 min. The samples were placed in a digester block at 95 ± 5 °C (with a reflux funnel) for 10-15 min, without boiling. The tubes were cooled and 5 mL of 65% HNO_3 added. The mixture was then heated at 95 ± 5 °C in a digestion block for 30 min, under reflux.

The solution was allowed to evaporate in a digestion block until the volume was reduced for 2 h under reflux to about 5 mL at 95 ± 5 °C. After cooling, 2 mL of ultrapure water (18.2 Mohm.cm) and 4 mL of H_2O_2 30% were added to the samples. Then, the digestion tube was closed using the reflux funnel to complete the reaction. Aliquots of H_2O_2 (1 mL) were carefully added until effervescence became minimal or the overall sample appearance did not change with the addition of H_2O_2.

The tubes closed using the reflux funnel were heated to 95 ± 5 °C in a digestion block, without boiling for 2 h, until the volume had reduced to about 5 mL. The samples were cooled at room temperature (25 ºC).

Subsequently, 10 mL of concentrated HCl were added to the samples and the mixture was heated to 95 ± 5 °C for a further 15 min. After cooling, the digest was diluted with ultrapure water (18.2 Mohm.cm) in a 50 mL volumetric flask. The dilute was transferred (all contents) to 50 mL Falcon tubes, which were centrifuged at 3,000 rpm for 10 min to determine the metals.

Total Digestion

The geopropolis samples (n = 15) were digested following the method described by Malavolta et al. [24] and Krug [25]. A mass of 0.5 g of each sample was used. The analytical grade reagents were 65% HNO_3, perchloric acid ($HClO_4$), and HF. At the end of the process, the digested sample was transferred to a 25 mL volumetric flask, and ultrapure water (18.2 Mohm. cm) was added to complete the flask volume. The digested samples were placed in Falcon tubes (50 mL).

Determination of metals in the geopropolis sample

An Inductive Coupled Plasma Optical Emission Spectrometer (ICP OES - Spectrometer Thermo Scientific – iCAP™ 6300 Duo, Thermo, Germany) was used to quantify the metals in the geopropolis samples and in the reference soil (certified reference sample - San Joaquin Soil, 2709). The conditions for analysis and the detection limits of the metals are described in Table 2. For this study, Cd, Cu, Pb, Cr, and Zn were selected. To validate the analytical method, accuracy was evaluated by the repeatability of experimental results using real samples, and expressed as mean and standard deviation. The accuracy was verified by the calibration curve, using standard solutions of each metal studied, the procedure being performed in triplicate.

Table 2. Conditions of the ICP OES analysis to quantify metals and limits of detection (LoD) in samples of geopropolis and reference soil (San Joaquin Soil, 2709).

Parameters - ICP OES	Operation conditions
Power RF	1150 W
Nebulization flow	0.70 L/min
Auxiliary gas flow	0.50 L/min
Internal standard	Yttrium (Y)
Integration time and reading	15 s
Gas Purity (Argon)	99.999%

Metals	Wavelength (nm)	LoD - Geopropolis/Soil (mg/kg)
Cd	226.5	0.025
Cr	267.7	0.010
Cu	324.7	0.025
Pb	220.3	0.050
Zn	213.8	0.010

Statistical analysis

The experimental design was completely randomized, with three replications. All analyses were performed in triplicate and the descriptive statistics were calculated for each variable, such as minimum, maximum, average, and standard deviation values. The T-test was employed to compare means at 5% probability. Statistical analyses were performed using the R program (version 3.3.2) [26].

Results and Discussion

The comparison of digestion methods used in the preparation of geopropolis samples showed a statistically significant difference according to the T-test, at 5% probability. The coefficient of variation for this experiment ranged from 8.52 to 13.49%, respectively, for Pb and Zn (Figure 1).

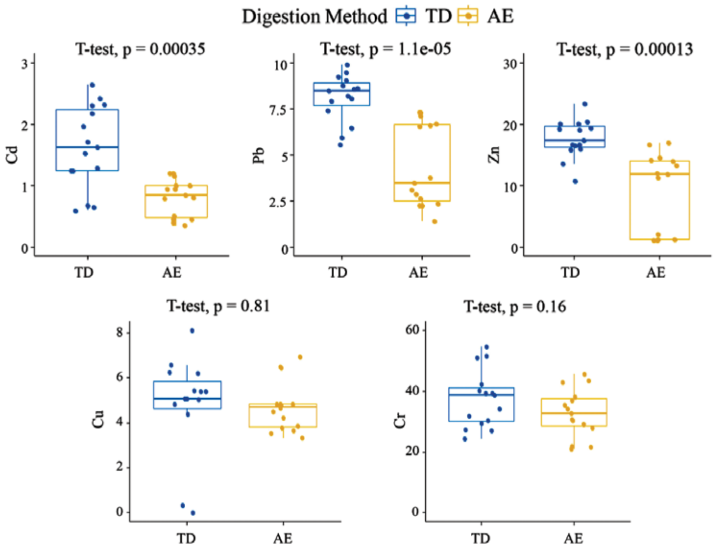

Figure 1. Boxplot graphics for the metals (mg/kg) evaluated in different geopropolis samples for acid extraction (AE) and total digestion (TD) (means compared by T-test at 5% probability).

The geopropolis samples submitted to different digestion methods showed statistical differences (Table 3). The results revealed that, in general, the samples that underwent total digestion presented the highest concentrations of metals. This was expected, as all material is digested in the process, including silicates to which some metals remain adhered. The highest concentrations of metals were observed for Cr and Zn while, for both digestion methods, Cd returned the lowest levels. However, in a study by da Cruz Ferreira et al. [17] Cd was below the detection limit. The authors used a microwave-assisted acid digestion, indicating the precision of the acid extraction and total digestion methods also used in the present study, stressing that the advantage in using acid extraction in the preparation of the sample is greater simplicity in relation to total digestion.

Concentrations of Cr and Cu did not differ statistically for the different sample processing methods. However, other metals showed a difference (Table 3). The samples submitted to total digestion had higher concentrations of metals than those submitted to acid extraction.

Table 3. Comparison of the mean concentration (mg/kg) of metals in geopropolis samples produced by *Melipona scutellaris* in relation to total digestion (TD) and acid extraction (AE).

Metals	Digestion Method		p-value *T-test*	Reference concentration*	
	TD	AE		QuR	Prev
Cd	1.62±0.67 a	0.80±0.30 b	0.0003	<0.50	1.30
Cr	37.53±9.49 a	32.9±7.86 a	0.1561	40.00	75.00
Cu	4.56±2.46 a	4.73±1.12 a	0.8079	35.00	60.00
Pb	8.12±1.28 a	4.38±2.23 b	0.0000	17.00	72.00
Zn	17.65±3.08 a	8.85±6.54 b	0.0001	60.00	300.00

Means followed by the same letter on the line do not differ according to the T-test at 5% probability.
*QuR (Quality Reference - natural soil content), Prevention (Prev) defined by CETESB [27].
The values presented for TD and AE are mean and standard deviation.

The efficiency of a digestion protocol for solid waste depends on the sample, the chemical composition of metals in the waste matrix and the acids used in the digestion process [28-29]. Our results reveal that the total digestion method in geopropolis, which contains significant amounts of soil [21-22], has greater capacity to recover metals.

In a study conducted by Uddin et al. [30], digestion using a mixture of HCl and HNO_3 at a ratio of 1:3 was the most efficient method for recovery of As, Cd, Pb, Ni, Zn, and Fe in herbal samples, compared to a mixture of HNO_3 and $HClO_4$ at a ratio of 2:1. For the geopropolis matrix in our study, the HNO_3, $HClO_4$ and HF mixture was more efficient at determining Cd, Cu, Cr, Pb and Zn in samples. However, the use of HF implies a greater risk for the handler when performing chemical analyses [29].

Metal recovery using the certified reference sample (San Joaquin Soil, 2709) and the USEPA 3050b method was accurate at indicating the digestion method of geopropolis (Table 4). In addition, it is in accordance with the limits of detection (LoD) for certified reference samples and geopropolis (Table 2). All the metals studied showed concentrations within the certified range, with 100% recovery. Nevertheless, Zn was below the certified value, a result that is similar to the findings of Navarro et al. [5]. The range in our study refers to the metals adsorbed.

The study of metal recovery, using the certified reference sample (San Joaquin Soil, 2709) and the USEPA 3050b method showed accuracy when indicating the digestion method in geopropolis (Table 4), where all metals under study showed concentrations within the certified sample range, obtaining 100% recovery. Only Zn was below the range of certified value. A similar result was obtained by Navarro et al. [5], emphasizing that the range presented in the actual study refers to metals adsorbed.

Table 4. Determination of metals in a certified reference sample (San Joaquim Soil, 2709) to validate the method.

Metals	Metal concentration (mg/kg)				
	Cd	Cr	Cu	Pb	Zn
LoD	0.005	0.002	0.005	0.01	0.002
RS**	2.1050	71.9500	28.3500	12.4800	67.6000
IVRS-RS	-	60-115	26-40	12-18	87-120

LoD = limit of detection; **RS = reference sample (San Joaquin Soil, 2709); IVRS-RE = Range of certified recovery values in the reference sample.

The results of our study are important, because acid extraction by the USEPA 3050b to determine bioavailable metals in the environment is a technique of acid digestion of samples and is, therefore, simpler than other methods. According to Silva et al. [31], metals trapped in the silicate mineral structure are not mobile in the environment and therefore are excluded from many transport and pollution processes. Similarly to many studies of hive products [15, 32-33], our study aims to highlight the potential of these bee products in biomonitoring environmental pollution, as acid extraction may be applied in the treatment of the sample matrix.

Toxic metals such as Cd, Pb, Cu, Cr and Zn are important in environmental samples, due to their effects on ecosystems [34-35]. With the development of cities, large quantities of waste associated with industrial activities, modern agriculture, mineral extraction, and pesticides are released into the environment [36].

The determination of metal concentrations in the analyzed geopropolis samples revealed a wide spectrum of values, considering the concentrations in decreasing order, Cr> Zn> Pb> Cu> Cd (Table 3; Figure 1).There is no specific legislation covering the concentration of metals in geopropolis. However, as the characteristics of this material mean that 90% of its composition is represented by the soil fraction [15], the criteria established for soil by CETESB [27] and CONAMA [37] were used. Thus, an exploration of the data presented in Table 3 showed that the geopropolis samples of the area studied present the average values of the metals according to the prevention limits (Prev) established by CETESB [27], namely: Cd (1.30 mg/kg), Cr (75.00 mg/kg) Cu (60.00 mg/kg), Pb (72.00 mg/kg), Zn (300.00 mg/kg) (Table 3).

According to Bonsucesso et al. [15] geopropolis can be an indicator of environmental pollution, as these authors found higher concentrations of toxic metals in the geopropolis obtained in urban areas than in rural or semi-rural locations. Thus, this study can contribute to the choice of sample preparation methods for metal determination, considering the interest of researchers in the use of beehive products for environmental monitoring [15-16, 32-33].

The USEPA 3050b method can be an efficient alternative for metal extraction in geopropolis, because it is simpler and less risky than the digestion method using mixtures of HNO_3, $HClO_4$, and HF. The use of HF and HCl in sample treatment can increase research costs. Additionally, it is more dangerous because of the reagents used in the analysis [38].

The choice for the best procedure of sample digestion should take into account simplicity, speed, reduced use of reagents, as well as methodological procedures that allow the dissolution of a large number of samples capable of generating accurate results [39]. Therefore, sample digestion has potential as an effective technique for subsequent analytical determinations [40]. The results obtained for the geopropolis sample matrix revealed acid extraction to be a promising alternative.

Conclusions

The total digestion method presented the highest values of metal concentrations in geopropolis. However, acid extraction using the USEPA 3050b method is a simpler procedure for evaluating the metal content in geopropolis samples. Acid extraction using the USEPA 3050b method in combination with detection by ICP OES is efficient in determining metals in geopropolis. This is therefore the recommended method when the metals adsorbed in the sample are the ones intended to identify.

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