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Detection of metals by differential pulse anodic stripping voltammetry (DPASV) in pollen collected from a fragment of the atlantic forest in Piracicaba/SP

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Abstract

Detection techniques of trace elements have high costs for routine analysis while differential pulse anodic stripping voltammetry (DPASV) has low operational costs and few structural requirements. This technique was considered a good alternative for pollen analysis. In the analyses, Zinc, Copper and Lead showed concentrations higher than the threshold established for human daily intake of food; however, Cadmium concentrations did not exceed the limits. The high concentrations found may be explained by the geographical locality of the apiary, which is located near the main road to access the city and a water and sewage treatment plant. In addition, other anthropogenic processes, namely agricultural, industrial and residential activities may have influenced. The technique of voltammetry can be considered a good alternative for pollen routine analysis, providing accuracy at lower costs. The study provides information about the contamination of the matrix of bee pollen.

Keywords: bee, biomonitoring, detection limits, trace elements.

INTRODUCTION

Interest in consumption of safe products has been increasing in the past years worldwide. People have become more concerned about the health conditions and well-being, seeking food with no or minimal concentrations of chemical additives (preservatives, acidulants, among others).

In the group of natural foods, safety plays a key role in their marketing and acceptance in the market, receiving a great deal of attention from inspecting agencies. Over the years, bee products (pollen, royal jelly, bee venom) as well as honey have gained a significant market share in the sector of natural products.

According to Ordinance No. 3 of 2001 of the Brazilian Ministry of Agriculture and Supply (Brasil 2001), pollen is defined as the result of agglutination of flower pollen, made by

worker bees through nectar and saliva substances, collected at the hive entrance. Although pollen has well-defined rules as to their physical-chemical compositions, the presence of trace metals (heavy metals) in pollen is another factor has greatly concerned inspecting agencies. Produced in areas near urban environments to facilitate the activity, pollen may be subject to contamination sources (roads, farms, buildings, etc.) that go unnoticed by beekeepers (Pohl *et al.*, 2009).

Bees move around an approximately 10 km radius to search for food source quality, and during those trips, they come into contact with many contamination sources. Heavy metals in the atmosphere can be deposited on flowers, water, air, soil and hairs of bee body, and thus, be transported the hives along with pollen grains (Porrini *et al.*, 2003).

It is known that trace metals such as sodium, potassium, calcium, iron, zinc and copper can be considered essential for

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the biological metabolism of living organisms. Other metals such as lead, cadmium, mercury, aluminum are classified as micro-contaminants of the environment, toxic or non-essential to living organisms and at high concentrations, can be even lethal (Oga *et al.*, 2008)

Detection techniques of trace metals generally have high costs to be used in routine analysis (Tonietto *et al.*, 2012). However, differential pulse anodic stripping voltammetry (DPASV), has low operating costs and few structural requirements, besides having a scanning of “ppt” (parts per trillion) (Rodrigues 1998).

Therefore, this study aimed to apply the voltammetry technique as an alternative to measure trace metals in pollen for in routine analysis, providing accuracy at lower costs over other methods. The present study aimed to also provide information about the contamination of the matrix of pollen.

MATERIALS AND METHODS

The experiment was conducted in a fragment of the Atlantic forest located at the Department of Entomology and Acarology at College of Agriculture “Luiz de Queiroz”, campus ESALQ/USP, in the municipality of Piracicaba, in São Paulo state, Brazil. The site was chosen for having a 7-km radius and its proximity to agricultural, industrial and urban areas, a sewage and water treatment plant of the Piracicamirim River and main road to access the city. Thus, the bee products generated could be exposed to contamination sources.

In the beehive, we kept five colonies of Africanized honey bees (*Apis mellifera* L) Langstroth-type boxes. The pollen was sampled during one year in each of the four seasons (summer, autumn, winter and spring) with two collections, one at the beginning and another at the end of the season (A and B winter; C and D spring; E and F summer and G and H autumn). Were installed a front-type trap to obtain at least 50 g of pollen per beehive per season. The beehives were kept under conditions to favor pollen collection, standardization of the bee population and percentage of pollen retained in the traps.

Samples

Pollen loads in the traps were removed every collection day, cleaned by grooming, weighed and stored in 250 mL plastic bottles, sealed and stored in a freezer at - 26°C until analysis. In all analyses, we used samples in a fresh base.

Analysis of minerals and trace metals: Zn, Cu, Cd and Pb

Sample digestion

We weighed approximately 0.5 g of the pollen sample in a 250 mL flat-bottomed flask and 5 mL of concentrated nitric acid (HNO₃, Sigma-Aldrich®) was added, with a reflux condenser connected to the flask mouth. The flasks were kept on a plate heated at 120°C for the mixture remain in reflux

30 minutes. Afterwards, the material was cooled to room temperature, then 2 mL of nitric acid (HNO₃) and 3 mL of concentrated perchloric acid (HClO₄, Sigma-Aldrich®) were added to submit to reflux for 60 minutes once again. Thereafter, the condenser was removed for the elimination of acids and concentration of the sample. The concentrate was diluted in ultrapure water (Mohm 18.2 cm) in a 25 mL volumetric flask. The blank solution containing only acid used in the digestion underwent a similar procedure (Pereira, 2010).

Voltammetry

The trace metals (Cu, Pb, Cd and Zn) were quantified in a voltammetric analyzer 797 VA ComputraceMetrohm® using electrochemical measures. The differential pulse voltammetry is an electroanalytical technique based on phenomena occurring at the interface between the electrode surface and the thin layer of solution adjacent to the surface. The method is considered dynamic, because the electrochemical cell is operated in the presence of electrical current ($i > 0$), which, in turn, is measured by the controlled application of potential (Skoog *et al.*, 1992) Therefore, information on the analyte (aqueous solution containing ions) is obtained by measuring the magnitude of the electrical current that appears in the working electrode by applying a voltage between a working and an auxiliary electrode. The registration of the current potential may be related to the amount of analyte found in the electrode interface (Montenegro *et al.*, 1991).

The equipment is coupled to a computer with the 797 VA Computrace software version 1.3.1 Metrohm® to record and read the measures. To determine zinc (mg Kg⁻¹), cadmium (µg Kg⁻¹), lead (mg Kg⁻¹) and copper (mg Kg⁻¹), we used the technique of differential pulse anodic stripping voltammetry (DPASV) (Taddia *et al.*, 2004).

The standard addition method was used for the determination of the metals. The reagents and standard solutions used were from Sigma-Aldrich® and QuimLabSpecSol®. The solutions were prepared with ultrapure water (Mohm 18.2 cm) and all flasks were kept in HNO₃ at 10% for 24 hours for decontamination. Prior to use, all material was rinsed with ultrapure water. The parameters and conditions of the analyses are shown in Table 1.

Statistical Analyses

The data for the Zn, Cu, Pb and Cd were analyzed by analysis of variance (ANOVA). We checked the ANOVA assumptions using the optimal transformation family of Box-Cox (Box *et al.*, 1964) and the Hartley (1950) test was used to verify the homogeneity variances. The experimental design was completely randomized and the treatments comprised different pollen types. Once the ANOVA assumptions were verified, we applied the F test ($p < 0.05$) to investigate possible differences between the levels of the Pollen factor. We proceeded with the analysis by performing test of means comparisons, two by two, in the Tukey test ($p < 0.05$). In order

Table 1 – Parameters and conditions used in the polarographic cell for the determination of Zn²⁺, Cd²⁺, Pb²⁺ and Cu²⁺ by DPASV

Voltametric Parameters	Unity	Zn ²⁺ , Cd ²⁺ , Pb ²⁺ and Cu ²⁺
Dilluted sample volume	mL	1
Ultrapure water volume	mL	10
Electrolytes		
KCl 1.5 mol L ⁻¹ and Acetate of Sodium 0.5 mol L ⁻¹	mL	2
Working electrode		HMDE
No. of drops		4
No. of additions		2
No. of replications		3
Reference electrode		Ag/AgCl (KCl 3 mol L ⁻¹)
Auxiliar electrode		Platinum
Shake speed	2000	RPM
Purge time (N ₂ ultrapure)	s	300
Additional purge time	s	10
Deposition potential	V	-1.15
Deposition time	s	90
Equilibrium time	s	10
Pulse amplitude	mV	0.05
Initial potential	V	-1.15
Zn ²⁺	V	-0.98
Cd ²⁺	V	-0.61
Pb ²⁺	V	- 0.38
Cu ²⁺	V	-0.16

to verify if the seasons can be classified using the concentrations of heavy metals (Zn, Cu, Pb and Cd), we used the canonical discriminant analysis. Data were analyzed using the computer program (SAS/Stat 9.1.3 Service Pack 2, 2003).

RESULTS AND DISCUSSION

The analysis of Zn, Cu, Pb and Cd showed different profiles in each season. Thus, a number of variables such as climate, season and botanical origin must be considered when using bees or bee products to monitor trace metals in the environment (Bogdanov 2006; Embrapa Clima Temperado 2008). The mineral composition depends not only on the botanical origin, but on the growth conditions such as soil type and geographical origin (Serra-Bonvehi *et al.*, 1997).

The average zinc concentrations found in pollen collected in the four seasons showed no significant differences in the variance analysis. In this study, the maximum Zn content was 259.52 mg Kg⁻¹ and minimum 124.58 mg Kg⁻¹, which are very

high compared to the recommended daily intake for adults of about 15 mg Kg⁻¹ (Franco 1999). Serra-Bonvehi *et al.* (1997) found different results for pollens collected in Spain where the Zn contents did not exceed a maximum of 81.1 mg Kg⁻¹ and minimum of 18.8 mg Kg⁻¹, which are similar to results for other honey products in that country. In Australia, Somerville & Nikol (2002) found Zn contents similar those in this study, which may be linked to the dominant *Eucalyptus* species in Brazil that has great Zn contents. Morgano *et al.*, (2012) found Zn contents that ranged from 41.4-65.3 mg Kg⁻¹, and only one sample presented a lower content (5.1 mg Kg⁻¹).

Intoxication cases may occur either through ingestion of contaminated food and beverages, by exposure to dust and fumes with high zinc concentrations or skin contact with zinc salts. Zinc becomes dangerous when combined with other metals such as cadmium and lead (Rocha *et al.*, 1985) (Table 2).

The results show that only Cadmium levels in spring exceeded the limit recommended for daily intake (Table 2). FAO (2005) established a provisionally tolerable weekly intake of 7µg Kg⁻¹ of body mass index. Considering the threshold for a 70 Kg person, the cadmium concentration found did not exceed 490 µg Kg⁻¹. In the other seasons, cadmium showed concentrations below tolerable limits. Morgano *et al.*, (2010) apud Leita, reported high Cd contents (near 50 m) in urban centers with high traffic intensity that surpassed the concentration of 2.1 mg Kg⁻¹ in the pollen collected by bees in this region. Morgado *et al.*, (2010) found Cd in 79% of the samples, which was greater than in this study (<0.001-0.233 mg Kg⁻¹) compared to samples collected in Italy (<0.015-0.090 mg Kg⁻¹, wet weight), in the United Kingdom (<0.07-0.13 mg Kg⁻¹) and Spain (0.07-0.10 mg Kg⁻¹) (Maff 1995).

Cadmium is listed in 8th place as hazardous substances (Salgado 2008) Cadmium in the environment is absorbed and retained by aquatic and terrestrial plants, by animals that feed on these plants and ultimately by humans, as the last link of the food chain (Ramos 2005).

Lead concentrations exceeded the tolerable limits in all samples (Table 2). The weekly tolerable intake is 25µg Kg⁻¹ of body mass (WHO 2005), which is equivalent to 3.57 µg Kg⁻¹ of body weight per day. It is worth noting that decree No. 685 of August 27, 1998, of the National Agency of Health Vigilance

Table 2 - Mean values ⁽¹⁾ of trace elements zinc (mg Kg⁻¹), copper (mg Kg⁻¹), lead (mg Kg⁻¹) and cadmium (µg Kg⁻¹) in pollen loads collected in hives of *Apis mellifera* in the municipality of Piracicaba, São Paulo state, Brazil, in four seasons between 2010-2011.

Season	Metals			
	Zn ²⁺	Cd ²⁺	Pb ²⁺	Cu ²⁺
Winter	193.63±21.42 A	115.80±50.29BC	29.620±2.38 A	85.66 ±21.95A
Winter	259.52±57.93A	231.04±55.54 B	27.320±3.85A	65.16±7.58 AB
Spring	231.04±50.81 A	565.54±85.34 A	32.020±6.04 A	53.36±6.87 AB
Spring	212.22±34.39 A	245.08±17.18 B	25.340±1.89 A	44.98±4.50 B
Summer	223.30±34.86 A	195.00±21.45 B	25.440±3.90 A	37.32 ±4.72B
Summer	229.38±50.29 A	159.22±41.20BC	26.200±3.82 A	55.52±7.05 AB
Autumn	187.72±14.46 A	199.90±35.74 B	26.400 ±4.60A	38.66±6.38 B
Autumn	124.58±14.58AB	112.60±9.56 BC	23.320 ±3.84A	36.70 ±5.67B

Means followed by the same letters do not differ statistically (p< 0.05) in the Tukey test. ⁽¹⁾

(Brazil), established lead threshold for several foodstuffs, however, there is no reference to levels in bee products. Thus, lead concentration in soils varies from region to region, so in regions close to roads with heavy traffic and industries, lead levels are much higher than in isolated areas (Larini 1993). The high level of lead in spring may be explained by a higher occurrence of wind in this season, which would transport the metal over long distances.

Lead showed a high concentration in the first sampling of spring when cadmium concentrations were also high. Morgano *et al.*, (2010) conducted studies in São Paulo State, Brazil, and found pollen with higher Cd and Pb contents in the spring in the month of October, and Pb was present in 63% of the samples. Similarly to results found for Cd contents, São Paulo State showed the highest Pb contents ($<0.01-0.44$ mg Kg⁻¹). The same condition was reported in studies carried out on pollens in Europe ($<0.02-0.329$ mg Kg⁻¹ in Italy; $0.3-1.5$ mg Kg⁻¹, in the United Kingdom and $0.1-0.14$ mg Kg⁻¹ in Spain) (Maff 1995).

The study of metal content of Swiss honeys and honeydew in contaminated areas (large cities, roads, incineration areas) showed a positive relationship between Pb and Cd in honey and their concentrations in the environment. The authors also noted that while Pb concentration increases in double, Cd concentrations did not change. This is because cadmium reaches the soil and contamination occurs in the soil, while Pb contamination occurs in the air. The cited authors noted that metal concentrations in honey are directly related to botanical resources, considering, therefore, honey as a good indicator of Pb concentrations in various regions of a contaminated area within a radius of 2 Km (Bogdanov 1986).

The daily intake threshold for copper established by the Brazilian legislation for adults is 900 µg (Brasil 2005). In healthy adults and not occupationally exposed to copper, the main pathway for the copper ingestion is oral. Chronic intoxication in humans is uncommon and is usually associated with cases of accidental ingestion (Lima *et al.*, 2001).

Like other metals, copper is dispersed in the environment by wind, rain and water streams. Water is an important source of copper exposure, especially in households where pipes are made of copper. Copper concentration in food generally varies from 0.2 to 44 µg g⁻¹ of dry weight (Lima *et al.*, 2001).

Copper showed an average of 85.6 mg Kg⁻¹ in the first sampling in winter and 36.7 mg Kg⁻¹ in the second sampling in fall. Morgano *et al.*, (2012) found concentrations between 10-18 mg Kg⁻¹ for the São Paulo State. This high contamination level can be associated to the proximity of the area with the water and sewage treatment plant, since copper sulphate is used to inhibit algae growth in tanks of the treatment plant (Ramos 2005).

Kalbande *et al.*, (2008) evaluated pollen grains from different species under different conditions of exposition to the environment and observed that there is a significant difference between the fresh pollen and pollen from urban centers in terms of metal contents in the pollen. The concentrations of

Pb, Zn and Cd reached 200% greater in urban pollen compared to fresh pollen, depending on the species.

Will hardly reported cases of intoxication with ingestion of pollen once the maximum daily consumption of this product in humans is 25 g that hardly reach the maximum limit of daily ingestion of metals studied (Zn, Cd, Pb and Cu).

Canonical Discriminant Analysis

In the set of samples evaluated, including heavy metals concentration (Zn, Cd, Pb and Cu) and the seasons when the samplings were made, we attempted to determine whether the seasons would discriminate among one another.

Evaluating the sample dispersion and the ellipses formation in some groups (Fig. 1), there is a separation on one of the canonical axes which represents the first sampling in spring that was the only sampling to distant from the others. In order to verify if the seasons can be classified by concentrations of heavy metals (Zn, Cu, Pb and Cd), we used a canonical discriminant analysis based on Mahalanobis distance, taking into account the correlations between the variables with which different patterns can be identified and analyzed. Therefore, it was possible to determine similarity between the samples of winter, summer, autumn and spring 2, considering correlations in the data set.

These results for the metals analyzed in pollen corroborate data found by (Bromenshenk *et al.*, 1984; Kovacheva *et al.*, 2000; Lozak *et al.*, 2002; Atrouse *et al.*, 2004; Rissato *et al.*, 2006 & Sobukola *et al.*, 2008), where accidental contamination may occur due to environmental pollution caused by pesticides and fertilizers in water and soil, or air caused by heavy traffic of vehicles emitting pollutants.

Thus, concentrations of trace metals in honey bee products reflect the profile of metal of a given region, which may be a useful tool to assess contamination levels of the environment. Therefore, determining trace metals concentrations is of great importance especially for quality control of honey bee products, since higher levels of metals are undesirable because of their toxicity (Bogdanov *et al.* 2003).

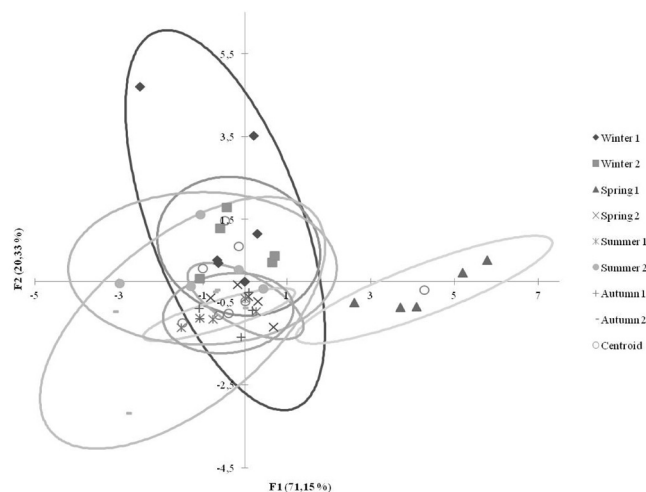


Figure 1 – Discriminant functions of seasons and metal concentrations.

The technique of voltammetry to measure trace elements in pollen can be considered a good alternative for routine analysis, providing accuracy at lower costs. The results of analyses show that the metals zinc, copper and lead have concentrations higher than thresholds established for daily intake of food. The discussions were based on daily consumption of food once again that there is no data in the literature with respect to pollen as environmental indicator. Cadmium concentrations did not exceed thresholds for human consumption, however, a high concentration was found in pollen. The concentrations of trace metals found could be associated with the geographic locality of the apiary and influenced by the proximity to various sources of pollution such as vehicle traffic, water and sewage treatment plant, agricultural, industrial and residential areas.

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