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Short communication

The effect of different propolis harvest methods on its lead contents determined by ET AAS and UV–visS

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Abstract

Argentinean propolis is exported to different countries, specially Japan. The market demands propolis quality control according to international standards. The analytical determination of some metals, as lead in food, is very important for their high toxicity even in low concentrations and because of their harmful effects on health. Flavonoids, the main bioactive compounds of propolis, tend to chelate metals as lead, which becomes one of the main polluting agents of propolis. The lead found in propolis may come from the atmosphere or it may be incorporated in the harvest, extraction and processing methods. The aim of this work is to evaluate lead level on Argentinean propolis determined by electrothermal atomic absorption spectrometry (ET AAS) and UV–vis spectrophotometry (UV–visS) methods, as well as the effect of harvest methods on those contents. A randomized test with three different treatments of collection was made to evaluate the effect of harvest methods. These procedures were: separating wedges (traditional), netting plastic meshes and stamping out plastic meshes. By means of the analysis of variance technique for multiple comparisons (ANOVA) it was possible to conclude that there are significant differences between scraped and mesh methods (stamped out and mosquito netting meshes). The results obtained in the present test would allow us to conclude that mesh methods are more advisable than scraped ones in order to obtain innocuous and safe propolis with minor lead contents. A statistical comparison of lead determination by both, ET AAS and UV–visS methods, demonstrated that there is not a significant difference in the results achieved with the two analytical techniques employed.

Keywords: Lead determination; ET AAS; UV–visS; Propolis samples; Harvest method effects

1. Introduction

Much attention has been given to the determination of heavy metals, especially some toxic metals as lead, for their high toxicity even in low concentrations and because of their harmful effects on health [1]. Lead is a toxic and pervasive chemical which causes neurological, physiological and behavioural problems in children ranging from raised hearing threshold and a decrease in IQ at low lead levels in blood to acute encephalopathy, memory loss and death at higher lead levels in blood [2]. The scientific literature on lead is extensive and numerous reviews have been published. A very complete monograph was prepared by the fifty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [3], in has been given which the critical effects on humans are explained and the relationship between the health effects of current levels of exposure to lead and the impact on health that might be anticipated from reducing exposure, are defined.

The main sources of lead intake in humans are inhaled air, diet and drinking water. A provisional tolerable weekly intake (PTWI) of lead from drinking water and diets has been established by WHO to be 25 $\mu g\,kg^{-1}$ body weight for people in all age groups [4].

The estimated weekly intake of lead in each of the regional diets derived from the proposed Codex limits is 17 $\mu g\,kg^{-1}$ bw in the Middle Eastern diet, 15 $\mu g\,kg^{-1}$ bw in the Far Eastern diet,

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13 μg kg^{-1} bw in the African diet, 13 μg kg^{-1} bw in the Latin American diet, and 20 μg kg^{-1} bw in the European diet. The contribution of each food category to the total intake varies from 1 to 90 μg day^{-1} [3]. Considering the low levels of lead concentration in foods, sensitive analytical techniques are required to obtain adequate detection limits.

Propolis is a mixture of various amounts of beeswax and resins collected by the honeybee from plants, particularly from exudated and leaf buds. It can be assumed that in the process of collecting and modelling the resins, they are mixed with some saliva and other secretions of bees as well as with wax. The composition of propolis depends on the type of plants accessible to the bees. The major compounds are resins composed of flavonoids and phenolic acids or their esters, which often form up to 50% of all the ingredients. The rest of the components are formed by 25–35% of waxes and fatty acids, 10% of volatiles essential oils, 5% of pollen and 5% of other organics and minerals [5].

Propolis is another medicinal marvel from the beehive. One of the most widely known and extensively tested properties of propolis is its antibacterial activity. Many scientific tests have been conducted with a variety of bacteria, fungi, viruses and other microorganism [6]. Many of these tests have shown a positive control of the organisms by various extracts and concentrations of propolis. General medicinal uses of propolis include treatment of the cardiovascular and blood systems, respiratory apparatus (for various infections), dental care, dermatology (tissue regeneration, ulcers, excema, mycosis, mucous membrane infections and lesions), digestive tracts, liver protection and support and many others. Some references to these applications can be found in Apimondia [7]. Propolis is generally used to make dietary supplements.

The main bioactive compounds of propolis are phenols and specially flavonoids [8]. These compounds have important antioxidant [9] and antimicrobial [10,11] properties. They reduce the lipidic peroxidation and the effect of free radicals [12] thus contributing to reduce the risk of heart diseases [13].

Flavonoids tend to chelate metals as iron and copper, which are catalytic compounds of chemical reactions that form free radicals [14]. But the same property allows flavonoids to form chelates with heavy metals [15] such as lead, which becomes one of the main polluting agents of propolis. The lead found in propolis may come from the atmosphere or it may be incorporated in the harvest, extraction and processing [16].

In Brazilian Propolis, Alcici et al. [16, op.cit] determined concentrations from 2.7 to 3.1 mg kg^{-1} of lead in propolis collected by netting, whereas in propolis of scraped, with painting rests, the values found ranged from 19 to 48 mg kg^{-1}. In the United Kingdom in 1995, Food Standards Agency [17] reported problems of elevated values of lead, from 2.3 to 461 mg kg^{-1}, for 20 samples of propolis provided by members of the British Beekeepers Association and analysed by using inductively coupled plasma mass spectrometry (ICP-MS). Food and Drug Administrations, from the USA, reported some cases of contamination with lead of dietary supplements made from propolis in 1994 [18]. Lead concentration ranged from just above the statutory general limit of 1 mg kg^{-1} for lead in food up to 1570 mg kg^{-1} in the worst case. Estimated exposures to lead by consumers of the worst-affected products exceeded the PTWI for lead and on advice of the Department of Health (DH) and the Ministry of Agriculture, Fisheries and Food (MAFF), the supplier, immediately withdrew the affected products. The source of lead in these products was traced to the use of lead-based paint in the beehives of one supplier of raw propolis.

The Committee in Food Additivies (JECFA) considered the results of a quantitative risk assessment and concluded that the concentrations of lead found currently in food would have negligible effects on the neurobehavioral development of infants and children and stated the importance of reducing exposure to lead.

The maximum limit fixed by the Japan Propolis Conference is 20 mg kg^{-1} [19] and the limit fixed by the Codex Alimentarius for foods in general is 2 mg kg^{-1} [3].

For all these reasons the present study is very important since one of the possible sources of lead in propolis could come from its harvest method.

In the present work, an evaluation of the lead level in propolis was made by two analytical methods, electrothermal atomic absorption spectrometry (ET AAS) [20] and UV–vis spectrophotometry by means of chelating methods [21], for several treatments of collection. We intend to demonstrate how the harvest method affects lead contents in propolis. Besides, a statistical comparison for both analytical methods was made.

2. Experimental

2.1. Instrumentation

Lead determination was realized by a Graphite Furnace Atomic Absorption Spectrometer Perkin-Elmer (Norwalk, CT, USA), Aanalyst 100, equipped with an auto-sampler AS 72, an HGA 800 furnace and deuterium background correction. A hollow cathode lamp was used as radiation source, lamp current of 10 mA and with 0.70 nm slit. Pyrolytically coated tubes with integrated platforms were used (Part. No. B3000407, Perkin-Elmer). The graphite furnace program followed was the default program provided by the software manufacturer. The pyrolysis temperature was optimized at 850 °C and the atomization temperature used was 1800 °C. Argon (high purity 99.9%) was used as purge gas. Magnesium nitrate was used as a matrix modifier.

To carry out lead determination by UV–vis molecular absorption spectrophotometry, the general dithizone method according to AOAC with a Hewlett-Packard Diode Array Spectrophotometer 8452A was employed.

2.2. Reagents

All reagents used were of the highest available purity with analytical grade at least.

(a) Water: deionised, distilled, 18 MΩ cm, obtained from a NANOpure (Barnstedt, IA, USA).

(b) nitric acid: Merck (Darmsatdt, Germany), p.a., 65%, sub-boiling distilled.
(c) Modifier stock solution: solution of $Mg(NO_3)_2$ (Part. No. BO190634, Perkin-Elmer) of 10,000 mg L^{-1} (20 °C). A volume of 5 μL will provide 0.06 mg of magnesium nitrate.
(d) Standard solutions of $Pb(NO_3)_2$: suitable standard is available from Merck, 1000 μg mL^{-1}. An intermediate 10 μg mL^{-1} standard diluting with 5% (v/v) nitric acid was prepared.
(e) Calibration Blanck: Nitric acid, approximately 5% sub-boiling, prepared by dilution of the 65% acid with deionized distilled water.
(f) Dithizone: Merck (Darmsatdt, Germany), p.a.
(g) $CHCl_3$: Merck (Darmsatdt, Germany), p.a.
(h) Ammoniacal citrate-cyanide: Merck (Darmsatdt, Germany), p.a.

2.3. Harvest methods and sample treatment

The samples of propolis were taken from one assay of the Project "Physico-chemical characterization of Argentinean Propolis", implemented in the experimental field of INTA LEALES (National Institute of Agricultural Technology), located in the department of Leales (at longitude 65° west and at latitude 27° south), in the province of Tucuman, Argentina. A randomized test was made with three different methods of harvest repeating 10 times each of them:

1- *Separating wedges* (traditional): wood pieces, of approximately 3 cm long × 1 cm wide × 0.3 cm thick, were placed on top of a hive, below the covers, and deposited propolis was scraped with a spatula.
2- *Plastic netting meshes*: A plastic thread weave of 55 cm long and 45 cm wide, was placed in the beehive in the same way as the separating wedges. It was observed that the bees tried to seal the holes in the mesh filling them with propolis. Then the propolized mesh was refrigerated at −18 °C so that propolis became rigid and brittle, and the mesh was twisted to separate the propolis from the mesh.
3- *Stamped out plastic meshes*: two plates with stamped out grooves, of 41 cm long and 25 cm wide and 0.4 cm thick, were located in the same way as the other methods described. The bees also stimulated themselves to cover the slots in the mesh with propolis. After that, the propolized mesh was cooled at −18 °C and then it was twisted to separate the rigid propolis from it.

Eight samples were taken in each treatment in different periods of time during 1 year. The sample treatment consisted in weighing 5 g of propolis and placing it in a furnace set at 400 °C during 4 h. Higher temperatures produced an important mass loss of lead (20% or more). The ashes was dissolved in 10 mL of HNO_3 20%, v/v on a hot plate, filtered with Watman 42 paper to eliminate the carbonaceous residue and diluted to 50 mL with distilled and deionised water. Recovery studies were also made by adding an adequate mass of lead to the propolis samples weighted (Section 2.5).

2.4. Analytical techniques

2.4.1. ET AAS

The present technique is based in the standard method for the determination of lead in sugars and syrups by ET AAS [20]. The quantification by direct calibration against aqueous standards is accomplished using peak area measurements determined at 283.3 nm. The working calibration standards of 20, 15, 10 and 5 μg L^{-1} were prepared from the 1 μg mL^{-1} lead stock automatically with the autosampler. The volume injected was 20 μL of 5% (v/v) nitric acid blank, calibration standards and sample solutions and 5 μL of modifier working solution. The samples were diluted 1 + 99 to carry out the lead determinations by ETAAS.

The detection limit (DL) of the methodology was determined in base on 10 replicates of the sample preparation blank (1 + 99 dilution), then the standard deviation was calculated and multiplied for 3, divide for the analytical curves slopes found along the method development.

2.4.2. UV–vis spectrophotometry

Ten millilitre of a digested sample was introduced into a 125-mL separatory funnel, 50 mL ammoniacal citrate-cyanide solution and 10 mL of dithizone solution in chloroform ($CHCl_3$) were added. The funnel was shaked vigorously for 1 min, and the layers were separated. Two millilitre of chloroform were discarded and then the absorption cell was filled. The absorbance of the cherry-lead dithizonate extract was meassured at 510 nm using a blank to adjust the zero of the spectrophotometer. The calibration curve with five standards of 20, 15, 10, 5 and 2 μg Pb solution was constructed. Lead standards were prepared from the 1 μg mL^{-1} lead stock solution and the same procedure, as in the samples, was followed.

2.5. Recovery and validation studies

Since the method of standard addition is considered as a validation method [22], we used it in order to demonstrate the validity of ETAAS method for lead determinations in propolis and to control the loss of analyte in the process. Ten portions of one propolis sample were taken. Four of them were spiked with an appropriate volume of lead stock standard solution. All the samples were prepared following the treatment proposed (Section 2.3). The average quantity of Pb found was 2.21 (μg L^{-1}) in the six portions without addition and with a 1 + 99 dilution. This value was taken as the base value. The quantity of lead added was: 5, 10, 15 and 20 μg L^{-1}. The average recovery was 95.75%.

3. Results and discussion

The data of average lead contents obtained on the whole samples of propolis for each method of harvest and each analytical technique were shown in Table 1. The analysis carried out with propolis collected in separating wedges presented average lead level ranged from 7.0 to 8.9 mg kg^{-1}, while the propolis collected by mesh methods presented results that varied from

Table 1
Average lead contents obtained for each method of harvest and by two analytical techniques with standard errors (n = eight samples in each treatment)

Methods of harvest	UV–visS Pb (mg kg^{-1}) ±S.E.	ET AAS Pb (mg kg^{-1}) ±S.E.
Separating wedges	7.0 ± 1.5	8.9 ± 2.5
Netting mesh	1.6 ± 0.1	1.8 ± 0.4
Stamped out mesh	1.2 ± 0.1	1.5 ± 0.1

1.2 to 1.8 mg kg^{-1}. These lead levels obtained were inferior to the maximum limit fixed by the Japan Propolis Conference (20 mg kg^{-1}) and in the case of the mesh method they were inferior to the limit fixed by the Codex Alimentarius for foods in general (2 mg kg^{-1}).

The effect of the harvest procedure on the lead contents was evaluated by means of the statistical analysis of variance (ANOVA) shown in Table 2. The P level was obtained by adequate statistical software. Considering that the P value was <0.01, it can be concluded that there are significant differences between the scraped method and the mesh methods (stamped out and netting meshes). The best harvest method would be stamped out plastic netting meshes, because it provides a lower lead content in propolis.

On the other hand, comparing both analytical techniques, ET AAS provides greater lead values than UV–visS for the same samples with more dispersion. However, no statistical differences between both analytical techniques were demonstrated considering the analysis of variance (ANOVA) shown in Table 2, as it can be seen, the P level obtained was 0.261 ($p > 0.05$).

An important difference between both analytical methods is the detection limit. For UV–visS the detection limit is 0.1 mg kg^{-1}, whereas for ET AAS the detection limit is 0.05 mg kg^{-1}. ET AAS is more reliable than colorimetric methods at lower concentrations. Anyway, both techniques are adequate for the lead level required by the Codex Alimentarius for foods.

Fig. 1 shows the dispersion of data for each treatment of harvest. The lead values obtained in the propolis samples collected with the method of separating wedges presents greater dispersion than mesh methods. No significant difference was observed in the lead contents by samples of different periods of year, for each harvest method ($p < 0.05$). The contribution of instrumental methods to this dispersion is not significative, because the data for each analytical method by ANOVA were analyzed, and the dispersion was similar. This would indicate that scraped methods are less reproducible than mesh methods, due to the characteristics of the procedure itself.

Table 2
Analysis of variance by two-way layout

Factor	SS[a]	DF[b]	MS[c]	F-ratio	P level[d]
Treatment	439.9	2	220.0	18.00	0.000
Techniques	15.9	1	15.9	1.30	0.261
Interaction	19.9	2	9.9	0.81	0.451
Error	440.0	36	12.2		
Total	915.7	41			

[a] SS, sum of squares.
[b] DF, degrees of freedom.
[c] MS, mean of squares.
[d] P level, probability level.

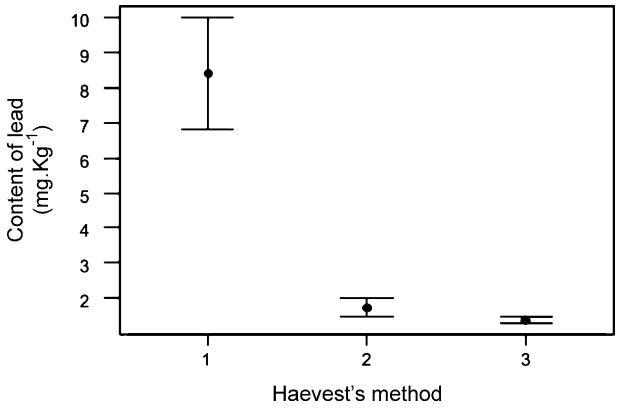

Fig. 1. Dispersion of data for each method of harvest

Treatment	Mean
1 Separating wedges	8.42
2 Netting mesh	1.75
3 Stamped out mesh	1.37

Beekeepers paint their hives and frames with several products like paints and varnish that usually contain lead to protect them from climate. Thus, in the scraped method (separating wedges), the mayor part of lead could come from paint rests. When propolis from beehive is scraped with a spatula, it probably carries away rests of it. Lead could also come from metallic objects present in the beehives (nails and clams, wires like the ones used in frames, metallic spacers and metallic queen excluder). Most of the lead present in propolis collected in mesh could come from the environment. Its origin could be the ant detonator used in gasoline, such as lead tetraethyl and the level in propolis depends on the distance of the apiary from cities and routes [16]. Significant differences are not detected between both mesh methods.

4. Conclusions

The results obtained in this work would allow us to conclude that the harvest methods of meshes are more advisable than scraped methods to obtain innocuous and safe propolis, with minor lead content. Hence, to obtain a better quality product we suggest that beekeepers use the mesh method of harvest. The propolis obtained by these cited methods presented lead level under 2 mg kg^{-1} while the other methods provided average values of 8 mg kg^{-1}.

Furthermore, no statistical difference between results obtained with ET AAS and UV–visS methods was observed. Thus, in laboratories of quality control without Atomic Absorption Spectrometer, the UV–vis molecular absorption spectrometry could be employed. This last technique allows getting low concentrations of lead present in propolis in the levels fixed by the Codex Alimentarius.

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References

[1] J. Christensen, J. Kristiansen, in: Seiler (Ed.), Lead Handbook on Metals in Clinical and Analytical Chemistry, Sigel and Sigel, New York, 1994, p. 425.

[2] WHO-CEHA, Applied Research, Lead Levels and Sources of Lead among Children Living in and near Karachi, Pakistan, December, 2000 http://www.emro.who.int/ceha/applied_research2.asp (last access December 2005).

[3] FAO/WHO, The 53rd Meeting of the Joint Expert Committee on Food Additives, 2000 http://inchem.org/documents/jecfa/jecmono/v44jec12.htm (last access December 2005).

[4] Total Diet Studies: A Recipe for Safer Food. GEMS/food. Food Safety Department, World Health Organization, 2005 http://www.who.int/foodsafety/publications/chem/en/gems_brochure.pdf (last access December 2005).

[5] W. Greenaway, J. May, T. Scaysbrook, F.R. Whatley, Identification by gas chromatography–mass spectrometry of 150 compounds in propolis, Zeitschrift fur Naturforschung C 46 (1990) 111–121.

[6] R. Krell, Value-added products from beekeeping. FAO Agricultural services bulletin No. 124, 1996 http://www.fao.org/docrep/w0076e00.htm (last access December 2005).

[7] N. Atiasov, M. Guseva, V. Kuprianov, Apimondia Publishing House, A remarkable hive product: propolis, Bucharest, 1978, 250 pp.

[8] E. Bedascarrasbure, L. Maldonado, A. Alvarez, A. van der Horst, E. Rodriguez, Content of flavonoids in the Argentinean propolis, in: Honeybee Science Journal, Tamagawa University, Tokio, Japan, 2001.

[9] W. Fierro, El propóleos, un producto con destacable capacidad antioxidante, Meeting Antioxidantes y Calidad de Vida 2001, vol. 7, 33 (2001) pp. 5–11.

[10] A. Tabera, E. Bedascarrasbure, L. Maldonado, A. Alvarez, A.van der Horst, Actividad antibacteriana de propóleos argentino enfrentado a Stafilococcus Aureus. Anales del Congreso Internacional de Propóleos, Buenos Aires, set. 2000, pp. 97, http://www.apinetla.com.ar/congreso/trabajos.htm.

[11] A.G. Hegazi, F. El Hady, Influence of egyptian propolis as antifungical agent, Anales del Congreso Internacional de Propóleos, Buenos Aires, set. 2000, pp. 114, http://www.apinetla.com.ar/congreso/trabajos.htm.

[12] M. Moreno, M.I. Isla, A. Sampietro, M. Vattuone, Comparison of the free radical-scavenging activity of propolis from several regions of Argentina, J. Ethnopharmacol. 71 (1–2) (2000) 109–114.

[13] M. Hertog, E. Feskens, P. Hollman, M. Katan, D. Kromhout, Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study, Lancet 342 (1993) 1007–1011.

[14] E. Cadenas, Sustancias flavonoides, Meeting Antioxidantes y Calidad de Vida 6–28 (1999) 7–12.

[15] B. Havsteen, The biochemistry and medical significance of the flavonoids, Pharmacol. Ther. 96 (2002) 67–202.

[16] N. Alcici, M. Freire, Heavy metals in propolis: practical and simple procedures to reduce the lead level in the Brazilian propolis, First German Bee Products and Apitherapy Congress with International Participation, 2002.

[17] UK Food Standards Agency, maff uk—analysis of bee products for heavy metals Food Survaillance, Information Sheet, Number 53 February 1995 http://archive.food.gov.uk/maff/archive/food/infsheet/1995/no53/53bee.htm (last access December 2005).

[18] FDA Enforcement Report, October 19th 1994 http://www.fda.gov/bbs/topics/ENFORCE/ENF00343.htmL (last access December 2005).

[19] T. Yamamoto, Present State of Basic Studies on Propolis in Japan Speech of the International Conference on Bee Product Properties Application and Apitherapy, Tel-Aviv, Israel, May, 1996.

[20] Method GS2/3-24, The Determination of Lead in Sugars and Syrups by a GFAAS Method-Official, ICUMSA, 1998.

[21] AOAC Official method 934.07, Lead in food, General Dithizone Method. 15th ed., 1993.

[22] E. Prichard, G.M. Mackay, J. Points, Trace Analysis: A Structures Approach to Obtaining Reliable Results, The Royal Society of Chemistry, UK, 1996, p. 38.