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COLD VAPOR ATOMIC ABSORPTION AND MICROWAVE DIGESTION FOR THE DETERMINATION OF MERCURY IN HONEY, POLLEN, PROPOLIS AND BEES OF GREEK ORIGIN

MARAGOU N.C.[1,*]
PAVLIDIS G.[1]
KARASALI H.[1,*]
HATJINA F.[2]

[1]*Laboratory of Chemical Control of Pesticides*
Department of Pesticides Control and Phytopharmacy
Benaki Phytopathological Institute
8 St. Delta Street, Kifissia, 14561, Athens, Greece
[2]*Division of Apiculture, Institute of Animal Science*
Hellenic Agricultural Organization 'DEMETER', Nea Moudania, Greece

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*to whom all correspondence should be addressed:
e-mail: e.karassali@bpi.gr; n.maragou@bpi.gr

ABSTRACT

In the present study a fast and reliable cold vapor atomic absorbance method was developed and validated for the determination of mercury in four beehive matrices (honey, pollen, propolis and bees) from Greece. For the sample preparation microwave digestion with H_2O_2 and HNO_3 in closed vessels under pressure was applied. The analysis of the apiary products showed that the mercury content is < 0.05 μg g^{-1} for honey, < 0.06 μg g^{-1} for pollen, < 0.3 μg g^{-1} for propolis and < 0.8 μg g^{-1} for honey bees d.w. The findings of the present study, in comparison with the literature data, do not trigger a special concern regarding mercury contamination of honey, pollen, propolis and bees from any of the sampled areas in Greece.

Keywords: Bio-indicators; Apicultural products; CVAAS; Toxic elements

1. Introduction

Mercury (Hg) is a metal that is released into the environment from both natural and anthropogenic sources. Its main global uses concern production of batteries, gold mining, and the chlor-alkali industry. Mercury compounds have also been extensively used in fungicide and pesticide products, antiseptics and disinfectants, as well as in the production of dental amalgam (EFSA 2008).

Mercury has been associated with several adverse human health effects. In particular, mercury exposure is related to toxic effects on the nervous, digestive and immune systems, and on lungs, kidneys, skin and eyes (WHO 2016). Because of its toxic properties, monitoring of mercury in environmental and food samples is essential in order to perform reliable risk assessments and take appropriate actions for the protection of the environment and human health (EFSA 2012).

For this scope honey bees and their products, such as honey and pollen, have been proposed as bioindicators of environmental contamination related to Hg among other xenobiotics (Balayiannis and Balayiannis, 2008; Bargańska *et al.*, 2016). Monitoring of mercury levels in honey is important not only for bio-indication of environmental contamination purposes, but also because of the potential human dietary exposure, taking into consideration that honey is regarded as one of the most natural and healthier foods. In addition to this, determination of Hg in honey and propolis is significant as another potential route of human exposure to this element is via the use of pharmaceutical and cosmetic products containing honey or propolis. These

apicultural products are used in medicine and cosmetics because of their antimicrobial, antioxidant, anti-inflammatory and antitumour properties (Burdock, 1998; Kalogeropoulos *et al.*, 2009; Melliou and Chinou, 2011; Burlando and Cornara, 2013; Tsiapara *et al.*, 2009).

Mercury has been determined in honey samples from several countries (Bilandžić *et al.*, 2014; Pisani *et al.*, 2008; Vieira *et al.*, 2014; Meli *et al.*, 2015; Ru *et al.*, 2013; Domínguez *et al.*, 2012; Santos Depoi *et al.*, 2010; Toporcák *et al.*, 1992), whereas limited literature data are available regarding the determination of mercury in propolis (Bonvehí and Bermejo, 2013; Cvek *et al.*, 2008; Matin *et al.*, 2016), pollen (Roman, 2009; Morgano *et al.*, 2010) and honey bees (Perugini *et al.*, 2011; Matin *et al.*, 2016; Toporcák *et al.*, 1992). For the sample preparation of these matrices the most often applied technique is microwave assisted digestion (Pisani *et al.*, 2008; Ru *et al.*, 2013; Roman, 2009; Morgano *et al.*, 2010). Microwave assisted digestion in closed vessels under pressure has gained popularity as a simple and fast dissolution technique that minimizes acid consumption, the risk of sample contamination, and the loss of volatile elements (Korn *et al.*, 2008).

Cold vapor atomic absorption spectroscopy is a simple, sensitive and of low cost technique that has been applied for the determination of Hg in food samples (Ferreira *et al.*, 2015). During this technique, mercury is first converted to Hg^{2+} after oxidation of the sample. Afterwards Hg^{2+} ions are reduced, usually with sodium tetrahydroborate in acidic medium, in order to produce volatile elemental Hg. The elemental Hg is transferred with the aid of carrier gas to a long-pass absorption tube and the determination is completed by measuring the absorbance at 253.7 nm (Skoog *et al.*, 1998). Cold vapor atomic absorption spectroscopy has rarely been applied for the determination of mercury in honey (Vieira *et al.*, 2014; Domínguez *et al.*, 2012), while to the best of our knowledge this technique has not been applied in samples of pollen, bees and propolis.

Apiculture is a traditional activity in Greece and apicultural products are produced and consumed at a large national scale. Nevertheless, although the status of the metal pollution of the environment of Greece has been presented in the past (Farmaki and Thomaidis, 2008), no information is available on the quality of the Greek apicultural products as regards the contamination of these products by Hg.

The aim of this study was the development of a reliable analytical method in order to investigate the possible contamination of bees and apicultural products of Greek origin by the toxic element of Hg in rural and industrialized areas. In particular a fast and reliable cold vapor atomic absorption method in combination with the efficient microwave-assisted digestion technique with closed vessels under pressure was developed and validated for the determination of Hg in honey, pollen, propolis and honeybees. The validated analytical method was applied to thirty two samples collected from the Northern and Western part of Greece.

2. Materials and Methods

2.1. Sampling

Four beehive matrices (honey, pollen, propolis and bees) from thirteen apiaries from Northern and Western part of Greece were collected between spring 2013 and August 2014, from rural, industrialized areas and agricultural areas near mines. Samples were stored at -15 °C in their original plastic container until analysis. In total, 32 samples were analysed for Hg. The location of each sample along with the industrialized activities of the area is illustrated in Figure 1. The samples were classified in four groups (Group 1: Kozani, Group 2: Sindos, Group 3: Chalkidiki and Group 4: Arta) based on their geographical origin as presented in Table 1.

2.2. Materials and reagents

Certified mercury (Hg) standard solution of 1000 μg ml^{-1} in nitric acid, metal-free water, metal-free HCl (30%) and metal-free HNO_3 (65%) were purchased from Fisher Scientific (UK). H_2O_2 was obtained from Carlo Erba (France), $NaBH_4$ (99%) from Acros Organics (USA) and NaOH (98%) from Panreac Quimica (Spain). Stock solution of 5 μg ml^{-1} Hg was prepared in 0.1N HNO_3 and was used for further dilutions in order to prepare calibration standard solutions of 5 – 10 – 20 ng ml^{-1}.

Figure 1. Sampling areas and industrialized activities.

Table 1. Origin of honey, propolis, pollen and bee samples

No of sample	Matrix	Geographical region	Sample name
1	Honey	Group 1 - Kozani	Kozani No 1
2	Honey	Group 1 - Kozani	Kozani No 2
3	Honey	Group 2 - Sindos	Sindos No 1
4	Honey	Group 2 - Sindos	Sindos No 2
5	Honey	Group 3 - Chalkidiki	Apicult. Institute
6	Honey	Group 3 - Chalkidiki	Sani
7	Honey	Group 3 - Chalkidiki	Ag. Mamas
8	Honey	Group 3 - Chalkidiki	M. Panagia
9	Honey	Group 4 - Arta	Arta No 1
10	Propolis	Group 1 - Kozani	Kozani No 1
11	Propolis	Group 2 - Sindos	Sindos No 1
12	Propolis	Group 2 - Sindos	Sindos No 2
13	Propolis	Group 3 - Chalkidiki	Apicult. Institute
14	Propolis	Group 3 - Chalkidiki	Ag. Mamas
15	Propolis	Group 3 - Chalkidiki	M. Panagia
16	Pollen	Group 1 - Kozani	Siatista
17	Pollen	Group 1 - Kozani	Chromio
18	Pollen	Group 1 - Kozani	Kozani No 1
19	Pollen	Group 1 - Kozani	Kozani No 2
20	Pollen	Group 2 - Sindos	Sindos No 1
21	Pollen	Group 2 - Sindos	Sindos No 2
22	Pollen	Group 3 - Chalkidiki	Ag. Mamas
23	Pollen	Group 3 - Chalkidiki	Apicult. Institute
24	Pollen	Group 3 - Chalkidiki	Sani
25	Pollen	Group 3 - Chalkidiki	M. Panagia
26	Pollen	Group 4 - Arta	Arta No 1
27	Pollen	Group 4 - Arta	Arta No 2
28	Bees	Group 1 - Kozani	Kozani No 1
29	Bees	Group 2 - Sindos	Sindos No 1
30	Bees	Group 2 - Sindos	Sindos No 2
31	Bees	Group 3 - Chalkidiki	Apicult. Institute
32	Bees	Group 3 - Chalkidiki	M. Panagia

2.3. Sample preparation

Preliminary experiments were conducted in order to optimize the conditions of microwave digestion. The optimised parameters were the sample weight and the temperature. Figure 2 describes the flow chart of the analytical procedure with the optimised parameters. For the bee samples drying at 100 °C for 48 hours preceded microwave digestion. Depending on the matrix, an aliquot of the samples (0.1 – 1g) was treated with 5 ml ultrapure HNO_3 (65%) and 2.5 ml H_2O_2 (30%) of analytical grade. Special care was taken in order not to have the sample stuck on the walls of the vessel but all of it on the bottom. The samples were digested in the microwave oven (CEM MARS 5, model MD 9132, USA) in Omni XP-1500 tubes. The temperature program was as follows: 0 – 15 min ramp to 210 °C and hold another 15 minutes at 210°C. After digestion, tubes were let to cool down, the pressure was carefully released and the yellow/brown gases were let to escape under sonication, until complete decolorization. After the removal of the gases, samples were quantitatively transferred to 25 ml volumetric flasks and were diluted with water before measurement with CVAAS. Every microwave digestion cycle consisted of 8 samples, two blanks (5 ml HNO_3 (65%) and 2.5 ml H_2O_2 (30%)) and two spiked samples.

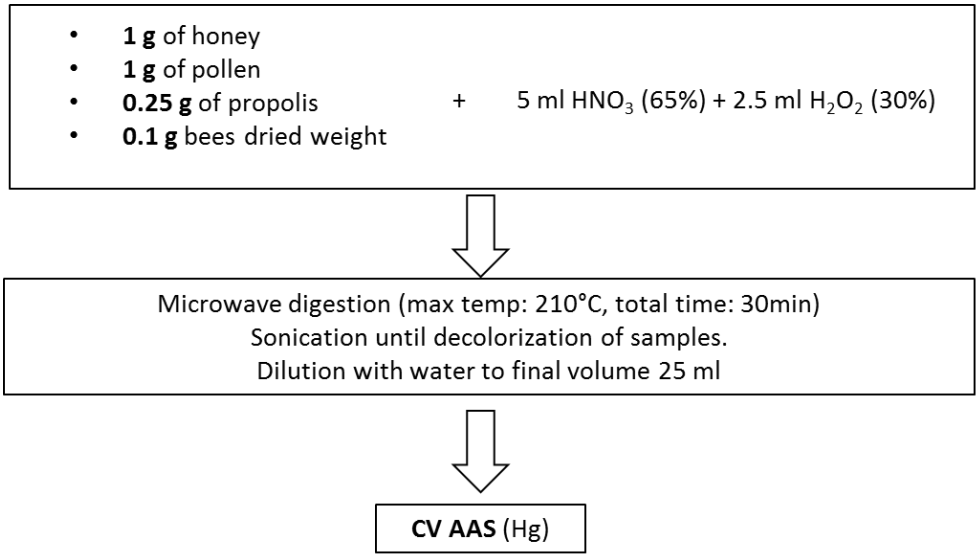

Figure 2. Flow chart of the analytical procedure

2.4. Analytical determination

An atomic absorption spectrometer in combination with a Vapor Generator (Shimadzu model AA-6500F) was used for the determination of Hg. For the cold vapor generation of mercury the carrier liquid was an aqueous solution of 5M HCl and the reducing agent was 0.4% w/v $NaBH_4$ in 0.5% w/v NaOH. The volatile mercury was transferred with the aid of argon gas to the quartz tube, which was positioned at a height of 16 mm. The absorbance was measured at 253.7 nm wavelength using a normal lamp of Hg (Hamamatsu Photonics, Japan) and a deuterium lamp for the background correction.

2.5. Method validation

Standard calibration curves were prepared by measuring standard solutions of Hg in 0.1 N HNO_3 at three calibration levels (5 – 10 – 20 ng ml^{-1}). Linear regression analysis was performed using the absorbance against analyte concentration. The instrumental limit of detection (LOD_{instr}) was defined as $3 \times S_{y/x}$, where $S_{y/x}$ stands for the standard error of the predicted y-value for each x in a regression. For each of the four matrices the method limit of detection (LOD_{meth}) was calculated based on the LOD_{instr}, the amount of the sample and the dilution factor. For the assessment of the accuracy and the precision, the method was applied to honey, propolis and bee samples that were spiked with mercury at appropriate fortification levels (Table 2). Analysis of three replicates of the spiked samples was conducted for the repeatability test. The recovery was calculated by subtracting the concentration measured in the non-spiked sample from that measured in the

spiked sample and then dividing with the spiked concentration. The matrix of pollen was regarded of similar complexity as propolis, so further recovery experiments were not considered necessary.

3. Results and Discussion

3.1. Method performance

Satisfactory linearity was obtained for a concentration range of 5 –20 ng ml^{-1}, with linear regression line y = 0.0124x - 0.0295, squared correlation coefficient (r^2) equal to 0.996, and standard error $S_{y/x}$ 0.0088. The instrumental LOD_{instr} was calculated to be 2.1 ng ml^{-1}. The method limits of detection (LOD_{meth}) varied depending on the matrix analysed. In particular the LOD_{meth} for honey was 0.05 μg g^{-1}, for pollen 0.06 μg g^{-1}, for propolis 0.3 μg g^{-1} and for bees 0.8 μg g^{-1} dw. It is noted that the obtained method limits of detection are relatively high due to the available instrumental technique. Lower LODs could be obtained only by ICP-MS. In addition to this, the amount of honey bee samples was limited, therefore low weighted mass for this matrix had to be applied. Nevertheless it is noted that since there are no regulated limits for the content of mercury in honey, there is not a specific requirement for the LOD from a regulatory point of view. Still, according to the Commission Regulation (EC) No 1881/2006 as regards setting maximum levels for certain contaminants in foodstuffs, the maximum levels for mercury in fishery products and in muscle meat of fish range between 0.5 – 1 mg kg^{-1} wet weight (Commission EC, 2006). The LOD_{meth} of the proposed method is comparable to these maximum limits. The accuracy of the method expressed as % recovery and the precision of the method expressed as % RSD, are presented in Table 2. The reported fortification levels refer to the amount of the element (μg) per amount of sample (g). The recoveries of Hg ranged between 64 and 129% with % RSDs between 0.8 and 12.8.

Table 2. Accuracy and precision data for Hg in honey, propolis and bees (n = 3)

Honey			Propolis			Bees		
Fortification level (μg g^{-1})	**Recovery (%)**	**%RSD**	**Fortification level (μg g^{-1})**	**Recovery (%)**	**%RSD**	**Fortification level (μg g^{-1})**	**Recovery (%)**	**%RSD**
0.12	129	13	0.5	64	6.1	5	86	0.8
0.5	95	5.9	2	66	4.9	20	96	1.4

3.2. Hg concentrations in samples

For honey, mercury content determined in the present study was in all cases < 0.05 μg g^{-1}. These data are in agreement with the concentrations of Hg reported in literature from a number of countries which were in the low ng per g levels. In particular the concentrations reported from recent studies ranged between < 0.001 up to 0.034 μg g^{-1} (Bilandžić *et al.*, 2014; Pisani *et al.*, 2008; Vieira *et al.*, 2014, Meli *et al.*, 2015; Ru *et al.*, 2013; Domínguez *et al.*, 2012; Santos Depoi *et al.*, 2010).There is only one reference reporting increased mercury levels in honey from industrially contaminated areas ranging between 0.050 – 0.212 μg g^{-1} (Toporcák *et al.*, 1992).

For pollen, Hg content determined in the present study was < 0.06 μg g^{-1} (LOD_{meth}). Corresponding studies in pollen from agricultural and former military airfield in Poland reported Hg concentrations of 0.0038 and 0.0066 μg g^{-1} d.w, respectively (Roman, 2009). Similar levels were reported for Brazilian pollen ranging between 0.0004 – 0.0068 μg g^{-1} (Morgano *et al.*, 2010).

For propolis, Hg content determined in the present study was < 0.3 μg g^{-1} (LOD_{meth}). Corresponding studies in propolis from Spain reported mercury content of 0.008 ± 0.0025 μg g^{-1} (Bonvehí and Bermejo, 2013) and in propolis from Croatia mercury was found to be between 0.003 – 0.053 μg g^{-1} (Cvek *et al.*, 2008).

For honey bees, Hg content determined in the present study was below the LOD of the method (0.8 μg g^{-1} dw). The LOD_{meth} in honey bees is relatively high because of the limited amount of this matrix, however it is considered that the generated data are indicative and comparable with the few available data in the literature. The Hg content reported in honeybees from Italy is below 0.01 μg g^{-1} (Perugini *et al.*, 2011).

Another study reported mercury levels of 0.004 – 0.024 μg g^{-1} in several parts of honeybee bodies from uncontaminated areas, whereas very increased concentrations between 0.028 – 2.255 μg g^{-1}, were determined in the same matrices from industrially contaminated areas (Toporcák *et al.*, 1992).

4. Conclusion

The present work describes a successful application of microwave digestion and CVAAS for the determination of Hg in the complex matrices of honey, pollen, propolis and bees. The method was applied in thirty two samples of honey, pollen, propolis and bees and it was found that mercury levels were < 0.05 μg g^{-1} in honey, < 0.06 μg g^{-1} in pollen, < 0.3 μg g^{-1} in propolis and < 0.8 μg g^{-1} dw in honey bee samples. The findings of the present study, in comparison to the data presented in literature, do not trigger a special concern regarding mercury contamination of honey from any of the sampled areas in Northern and Western part of Greece, nor indicate measurable contamination of honeybees, pollen and propolis. However it is noted that further research is required in order to draw safe conclusions. Furthermore, as very limited data are available worldwide regarding the mercury content in bees, pollen and propolis, further research and generation of data from several countries would be enlightening and could support more realistic environmental and human health risk assessment in the future.

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