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Analysis of Cadmium and Lead in honey: direct-determination by Graphite Furnace Atomic Absorption Spectrometry

Andrea Colabucci, Anna Chiara Turco, Maria Ciprotti, Marco Di Gregorio, Angela Sorbo, Laura Ciaralli

European Union Reference Laboratory for Chemical Elements in Food of Animal Origin (EURL-CEFAO), Department of Food Safety and Veterinary Public Health, Italian National Institute of Health, V.le Regina Elena, 299- 00161 Rome, Italy

ABSTRACT
One of the emerging issues regarding the analysis of inorganic contaminants in food is the determination of Cadmium (Cd) and Lead (Pb) in honey. In fact, this kind of analysis is foreseen in most of the National Residue Control Plans (NRCPs) that European Union Member States (EU MSs) have to perform according to Directive 96/23.

With the aim to provide technical support to the EU National Reference Laboratories (NRLs), an easy-to-use method on direct determination (DD) of Cd and Pb in honey by graphite furnace atomic absorption spectrometry (GF-AAS) after dissolution in an aqueous mixture of 2.5 % HNO_3 (v/v) and 12.5 % H_2O_2 (v/v) was in-house validated and distributed to the NRLs network. Analyses were carried out on a batch prepared for the feasibility study of the EURL-CEFAO 19th PT on honey. The validation levels (20 and 100 µg/kg for Cd and Pb, respectively) were chosen similar to those that would have been proposed for the PT.

The method proved its efficacy in terms of analytical performance; it appears to be low cost and time saving compared to a microwave sample preparation. In addition, it also decreases the environmental impact, being the amount of acid and reagents considerably reduced.

Section: RESEARCH PAPER

Keywords: method validation; chemical elements; honey; atomic absorption

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Corresponding author: Andrea Colabucci, e-mail: andrea.colabucci@iss.it

1. INTRODUCTION

According to the task listed in article 32 of Reg. 882/2004 [1] the EURL-CEFAO is responsible of the annual evaluation for group B3c (chemical elements) of the National Residue Control Plans, that the European Union Member States (EU MSs) have to perform in accordance with the requirements of Directive 23/96/EC [2]. In general terms, these plans foresee the analyses of different food products in order to monitor the presence of unauthorised substances, residues of veterinary medicinal products and chemical contaminants that may represent a risk for public health.

As for chemical elements, the EU MSs usually choose to investigate those for which maximum levels (MLs) are set in the relevant EU legislation (CR 1881/2006) [3] but other element/matrix combinations can be selected according to each MS control strategy. Following this last approach the number of EU MSs that include the analysis of chemical elements in honey had increased in 2012. In particular, according to the relevant EURL-CEFAO evaluation, cadmium and lead in honey were considered of interest by 26 EU MSs although no ML for chemical elements in this matrix was set in EU legislation at that time. However, values at which samples are rejected being non-compliant, namely action levels (ALs), were set for Cd and Pb in 14 EU countries. The other eleven MSs that analyzed honey did not set or gave any indication on the levels considered. The ALs were spread all over the European Union ranging from presence (few μg/kg) up to hundreds of μg/kg.

According to the EFSA report 2012 [4], the presence of lead represented the most important cause for sample rejection. Furthermore, as the differences in rules adopted by the Member States may hinder the functioning of the common market, the possibility of setting an harmonised maximum level for lead in honey was discussed at EU level and a ML was set in CR 1005/2015 [5], amending CR 1881/2006 [3], to be adopted as of 1 January 2016.

As a consequence of this picture, the availability of analytical methods suitable to quantify chemical elements in honey becomes a key point for both the EU National Reference Laboratories and laboratories dealing with official controls. The lack of Certified Reference Materials (CRMs) [6] as well as the scarcity of specific proficiency tests [7] make it difficult to assess the trueness and the performance of these methods. According to its tasks [1], the EURL-CEFAO organized in 2013 an interlaboratory comparison for its network on the determination of cadmium and lead mass fraction in honey making especially efforts to prepare an adequate material for this proficiency test [8]. In fact, from a physical point of view, honey is a high-viscosity liquid foodstuff containing a range of important nutritional complementary elements including a complex mixture of carbohydrates [9]. The difficulties in obtaining an homogeneous and stable material have never led to the effective production of a reference material certified for chemical elements [10], [11]. Therefore, this proficiency test was particularly valuable because of the production of a material suitable to check the NRLs capability in dealing with this matrix. As the basal content of cadmium and lead was found negligible in the honey used as start material, it was spiked with these chemical elements so as to obtain a mass fractions of ~ 0.02 mg/kg and ~ 0.10 mg/kg for Cd and Pb, respectively. These values were selected on the basis of a rough estimate of the ALs mean value but were also considered adequate to make the exercise as profitable as possible. Furthermore, the EURL-CEFAO developed, validated and distributed among the NRLs "ad-hoc" in-house methods to quantify Cd and Pb in honey so as to fulfil another's key task, namely providing national reference laboratories with details of analytical methods.

The validation was carried out with the two analytical techniques used in the network (ICP-MS and GF-AAS) around the values of mass fraction foreseen for the PT. This activity was of particular interest due to the intrinsic difficulty in the analysis of a high sugary matrix such as honey is. In particular for GF-AAS two different sample preparation approaches were investigated: the first one was based on the microwave sample digestion (MW GF-AAS) while the second one, consisting in a direct determination (DD GF-AAS), was chosen on the basis of information gathered from a survey of the literature [9], [12].

The method validation was planned and conducted according to the EURL-CEFAO internal procedure based on the Eurachem Guide [13].

This paper describes the full "in house" method validation of the direct determination of Pb and Cd in honey by GF-AAS and its efficacy and capabilities especially considering its low-cost and time saving peculiarities.

2. MATERIALS AND INSTRUMENTATIONS

All the steps of the material preparation were performed in the EURL-CEFAO facilities.

The different types of honey were purchased at the retail store. In particular ~ 15 kg of the wildflower one were used for the screening, the production of the preliminary batch and the final PT items. Moreover, other types of honey (chestnut, flowers, honeymoon, eucalyptus and melon) having different fluidity where bought to test the method and for screening purposes.

All chemical reagents were at least of suprapure grade: HNO_3 67-69 % (v/v) (Romil, Cambridge, UK) and H_2O_2 30 % (v/v) (Romil) to dissolve or digest the samples.

The spiking solution and the calibrants were prepared from Cd and Pb elemental stock solutions of high purity grade at 1000 μg/mL in 2 % HNO_3 (volume fraction) (High-Purity standards, Charleston, USA directly traceable to SRM 3100 Series Spectroscopic Standard Solutions) and diluted in high purity deionised water (resistivity 18 MΩ cm) (Zeneer UP 900 Water Purification System, Human Corporation, Seoul, Korea) up to the final concentrations.

TFM^{TM} polytetrafluoroethylene vessels and microwave ovens (Ethos 1 and Ethos-900, Milestone, Sorisole, Italy) were used for the microwave assisted acid digestions.

The mass fractions of the elements were determined by graphite furnace atomic absorption spectrometry with Zeeman effect background correction (Z-GF-AAS AAnalyst 800, PerkinElmer, Waltham, USA) using THGA graphite tubes and electrodeless discharge lamps (Cd and Pb, PerkinElmer). Matrix modifiers were prepared starting from magnesium nitrate ($Mg(NO_3)_2 \cdot (6H_2O)$, Suprapur Merck, Darmstadt, Germany), ammonium dihydrogen phosphate ($NH_4(H_2PO_4)$ Suprapur Merck) and palladium nitrate ($Pd(NO_3)_2$, PerkinElmer sol. 1 % in 15 % HNO_3).

An inductively Coupled Plasma Mass Spectrometer (ICP-MS, Elan DRC II, Perkin Elmer, Waltham, USA) with cyclonic spray chamber and Meinhard quartz concentric nebulizer (Meinhard, Golden, CO, USA) was employed for screening and comparison purposes.

Minitab 15 Statistical Software was used for statistical scopes.

The following equipments were used to prepare the preliminary batch and the final sample for the 19th EURL-CEFAO PT:

- an OLS200 Shaking Water Bath (Grant Instruments, Cambridge, UK) to warm up the jars in order to enhance the fluidity of honeys and to pasteurize the final samples;
- polypropylene beakers for food use (5 L) to collect the sample before homogenization;
- two technical balances (Mettler-Toledo, Greifensee, Switzerland 4200S and 16001L) and an analytical balance (Mettler-Toledo AT 261 DR);
- a T 25 Ultra-Turrax high-speed homogenizer (Ika-Werke GmbH & Co. KG, Germany) equipped with a stainless steel anchor stirrer rod for liquids characterized by medium/high viscosity;
- a granite machine (FBM L (Bras International spa, Italy) for higher quantity of highly viscous liquids equipped with a dispenser.

3. METHOD VALIDATION

3.1. General scheme

A preliminary study on the possible interferences on the determination of Cd and Pb was carried out especially evaluating the occurrence of a matrix effect. Linearity of the calibration curve used for quantification purposes, limit of detection (LoD) quantification (LoQ), repeatability [14], intermediate precision and trueness were the parameters assessed during the validation process. In particular, the repeatability, LoD and LoQ were calculated according to what stated CR 333/2007 [15], even if it is mandatory only for element/matrix combinations for which MLs are set in CR 1881/2006 [3] and following amendments.

3.2. Instrumental set-up and preliminary studies

Ashing and atomization curves were performed to allow the EURL to set the best temperatures in the furnace programmes: Cd 700 °C–1550 °C and for Pb 600 °C–1700 °C. The instrumental conditions are reported in Table 1.

These investigations, as well as the analysis foreseen for the validation, were performed using spare parts (e.g. graphite tubes) at about their half-life to better reproduce the typical working conditions of the laboratory. Moreover, it is to be underlined, that even if a carbonaceous deposit may remain in the graphite tube, its working life is not sensibly reduced.

The high matrix effect, observed for both elements, lead to analyze samples using the matrix matched calibration approach. This choice was also due to the necessity to overcome the problems raised from the different density between standard aqueous solutions and the sample resulting in an underestimation of both element mass fractions evidenced in screening experiments (ca. 20 %).

Moreover, matrix modifier concentrations were more diluted compared to those used when analyzing microwave digested samples and prepared in Triton X 0.05 % to enhance the fluidity of the whole injected solutions.

The calibration curves were constructed using 5 point (0 and base excluded) to cover all the concentrations of interest (0.25 – 2 and 2 – 20 μg/kg for Cd and Pb, respectively), diluting one part of the aqueous standard solutions in one part of basal honey (1:1). The curves were found linear (minimum correlation coefficient 0.9995) in the investigated range.

Table 1: Instrumental conditions for Cd and Pb.

Parameter	Cd	Pb
Ashing T (°C)	700	600
Atomization T (°C)	1550	1700
Volume injected (µL)	20	20
Matrix modifier	$NH_4H_2PO_4$ 0.06 g/L in 0.01% Triton X	$NH_4H_2PO_4$ 0.06 g/L + $Mg(NO_3)_2$ 0,05 g/L in Triton X 0.01%
Calibration points (µg/L)	0.25, 0.5, 1.0, 1.5, 2.0	2.5, 5, 10, 15, 20
Calibration curve	Method of Addition Calibrate	Method of Addition Calibrate
Integration mode	Peak Area	Peak Area
Number of replicates	2	2
Minimum sample dilution	1:1	1:1
Wavelength (nm)	228.8	283.3
Background correction	Zeeman effect	Zeeman effect
Lamp	Electrodeless discharge lamp	Electrodeless discharge lamp
Lamp current (mA)	230	440

These investigations, as well as the analysis foreseen for the validation, were performed using spare parts (e.g. graphite tubes) at about their half-life to better reproduce the typical working conditions of the laboratory. Moreover, it is to be underlined, that even if a carbonaceous deposit may remain in the graphite tube, its working life is not sensibly reduced.

3.3. Limits of detections and limits of quantification

LoDs and LoQs were assessed according to the indications set in CR 333/2007 [15]. Twenty-one samples from five different types of honey were analyzed. In particular, four samples of honeymoon, chestnut, eucalyptus, flowers each, and five of the wildflowers honey were spiked with a small amount of the analytes (0.1 μg/kg and 2 μg/kg for Cd and Pb respectively) so as to have a quantifiable analytical signal. The samples were then analysed for Cd and Pb content and the means and the standard deviations of each group were determined. The sample means were statistically compared by using the ANOVA test and no significant differences were found considering an alpha of 0.05 (p-value = 0.173 and 0.582 for Cd and Pb, respectively) so the pooled standard deviation was used to calculate LoD and LoQ for both Cd and Pb (Figure 1).

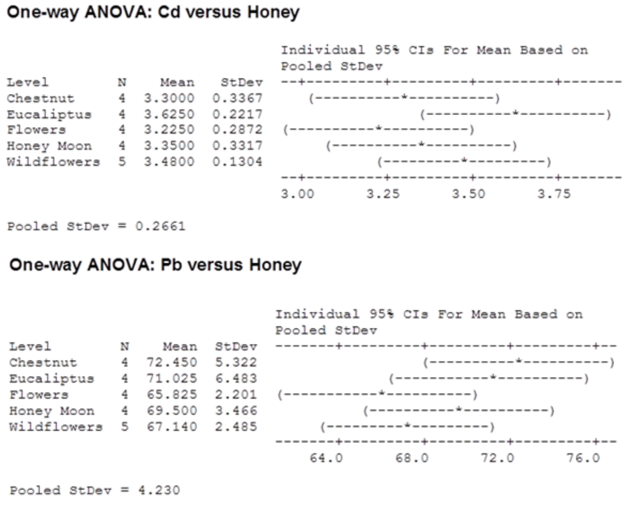
In particular, the pooled standard deviation was multiplied by 3 to derive the LoDs (0.8 μg/kg and 13 μg/kg for Cd and Pb, respectively) and by 10 to derive the LoQs (2.7 μg/kg and 42 μg/kg for Cd and Pb respectively). The obtained values for these parameters fulfilled the internal requirements established by the EURL.

3.4. Assessment of repeatability and intermediate precision

The method performance under repeatability condition [14] was evaluated analyzing a set of 10 samples (from the preliminary batch) versus two different calibration curves in the same day.

As no suitable reference material (RM) with certified values of the analytes of interest was available, the mean values of the results (n=20) was compared with those obtained analysing the material both with GF-AAS and ICP-MS after microwave assisted sample digestion (MW).

The use of the “surrogate” recovery (spiking of the sample with a known amount of the analytes before the digestion) was



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One-way ANOVA: Cd versus Honey

                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level        N    Mean   StDev  --+---------+---------+---------+-------
Chestnut     4  3.3000  0.3367     (----------*----------)
Eucaliptus   4  3.6250  0.2217                  (----------*----------)
Flowers      4  3.2250  0.2872  (----------*----------)
Honey Moon   4  3.3500  0.3317       (----------*----------)
Wildflowers  5  3.4800  0.1304             (---------*---------)
                                --+---------+---------+---------+-------
                                3.00      3.25      3.50      3.75

Pooled StDev = 0.2661

One-way ANOVA: Pb versus Honey

                               Individual 95% CIs For Mean Based on
                               Pooled StDev
Level        N    Mean  StDev  -------+---------+---------+---------+--
Chestnut     4  72.450  5.322                  (----------*----------)
Eucaliptus   4  71.025  6.483              (-----------*----------)
Flowers      4  65.825  2.201  (-----------*----------)
Honey Moon   4  69.500  3.466            (----------*----------)
Wildflowers  5  67.140  2.485       (---------*---------)
                               -------+---------+---------+---------+--
                                   64.0      68.0      72.0      76.0

Pooled StDev = 4.230
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Figure 1. Test one-way ANOVA for pooled standard deviations.

discarded as considered of lesser significance. In fact, the "surrogate" recovery is effective when samples are quantified versus an external calibration (e.g. to verify if matrix effect occurs). In this method, samples were quantified versus a calibration curve prepared on spiked honey so that any interference found in the calibration step would probably similarly affect the quantification of the samples.

The mean values of mass fraction calculated from the 20 results showed a good agreement among the different procedures and analytical techniques applied for both Cd and Pb as summarised in Table 2.

This accordance obtained in the different sample preparation/analytical technique combinations as well as the foreseen mass fractions, given by the sum of the incurred and the spiked mass fractions, were considered good evidences to judge the methods as "fit for the purpose".

A further proof that can also be considered is the good recoveries obtained (96 % for Cd and 102 % for Pb) on the material used for the EURL-CEFAO 17th PT on Infant Formula analyzed by ICP-MS during the repeatability assessment. This matrix was chosen considering its similarity honey in nutritional components, as reported in the NIST triangle for food equivalence.

The values of the relative standard deviations were compared with the Horrat$_r$ value [16]. The criterion to be < 1, prescribed in the EURL-CEFAO procedure, was met.

Moreover it was found that, using DD GF-AAS, lower standard deviations were observed compared to those obtained when analysing the samples after microwave assisted acid digestion; this effect was probably due to the minimum handling during sample preparation for analysis.

The intermediate precision of the method [14] was evaluated as described: six independent aliquots of the material used to assess the repeatability underwent the afore-mentioned sample treatment in two different periods of time (April 2014 and July 2014) and then were analysed for the content of Cd and Pb.

The results coming from each set of analysis (n=12) were combined with the first six results obtained during the repeatability assessment to calculate the standard deviation used to derive the parameters obtained in intermediate precision. As prescribed in the internal procedure, they were collected in a period of time longer than 5 months. In particular, the intermediate precision conditions were simulated by changing the operator in the second run, while another atomic absorption spectrometer (same model and brand) was employed by the first user in the third analytical run.

3.5. certainty estimate

The estimate of bias, that is the systematic measurement error, is a crucial parameter to assess the uncertainty of a measurement method. The analytical result given by a laboratory needs a "true" value to be compared with and this can be done using a certified reference material. As previously reported, no CRM for chemical elements in honey was available on the market. For this reason accuracy was evaluated versus the assigned value of the EURL-CEFAO 19th PT on honey, obtained by consensus from the participants (National Reference Laboratories of the European Union) according to ISO 13528 [17]. This approach was chosen bearing in mind that the low value of uncertainty of the material, that does not take into account all the parameters considered when dealing with a CRM or a RM [18], [19], could lead to an underestimation of the method uncertainty. For this reason the contributions of the non-significant bias and instrumental drift were included in the uncertainty budget.

Table 2: Results obtained under repeatability conditions using GF-AAS DD, GF-AAS MW and ICP-MS MW .

Cd	GF-AAS DD	GF-AAS MW	ICP-MS MW
Mean$_r$ (µg/kg)	20.9	21.1	21.5
s$_r$ (µg/kg)	0.3	0.5	0.6
RSD$_r$ (%)	1.4	2.4	2.6
r (µg/kg)	0.8	1.4	1.7

Pb	GF-AAS DD	GF-AAS MW	ICP-MS MW
Mean$_r$ (µg/kg)	115	112	113
s$_r$ (µg/kg)	2	4	3
RSD$_r$ (%)	1.7	3.6	2.7
r (µg/kg)	6	11	8

It is also to be underlined that the consensus values were, anyway, obtained from the results of outstanding laboratories (EU NRLs). Moreover the assigned values (0.0199 mg/kg and 0.102 mg/kg for Cd and Pb, respectively) were in full agreement with the grand mean of the results obtained by the EURL-CEFAO (0.0201 mg/kg and 0.106 mg/kg for Cd and Pb, respectively) when assessing the sufficient homogeneity of the material [8] according to the International Harmonized Protocol [20].

According to the internal procedure based on the Eurachem guide for quantifying uncertainty [21], two sets of 4 samples each were analysed together with the sets for the intermediate precision assessment and a third set in September 2014 to cover a time interval longer than 5 months. The mean of the values obtained for Cd and Pb for the twelve samples was considered. A test for the significance of the recovery was performed using the student test. The t_{calc} from (1) was compared with 2-tailed critical values for n-1 (i.e. 11) degrees of freedom at 95 % confidence (t_{crit} = 2.20) using the average recovery ($\overline{\mathrm{R}}$ec) and its standard uncertainty ($u_{\overline{\mathrm{R}}\mathrm{ec}}$):

$$t_{calc} = \frac{|1-\overline{\mathrm{R}}\mathrm{ec}|}{u_{\overline{\mathrm{R}}\mathrm{ec}}} \tag{1}$$

The t_{calc} both for Cd (1.71) and for Pb (0.97) were lower than the t_{crit} = 2.2. Even if the comparison between t_{calc} and t_{crit} showed that the bias was not significant, the contribution of the bias was however included in the uncertainty budget [22].

The combined uncertainty was estimated taking into account the figures obtained in intermediate precision, bias and instrumental drift according to the following equation:

$$u_C = C \mathrm{x} \sqrt{\left(\frac{s_R/\sqrt{m}}{C_R}\right)^2 + \left(\frac{u_{bias}}{C_{bias}}\right)^2 + \left(\frac{\mathrm{drift}_{max}}{\sqrt{m}}\right)^2} \tag{2}$$

s_R and C_R are the standard deviation and the mean value of the measurements in intermediate precision conditions, and m is the number of replicates analyzed in routine analysis (three in the EURL-CEFAO procedure).

The drift (continuous change in an indication, related neither to a change in the quantity being measured nor to a change of any recognized influence quantity of the instrument [14] was also included.

The uncertainty of the bias was estimated taking into account the figures obtained in intermediate precision condition (mean value, bias, standard deviation):

$$\frac{u_{bias}}{C_{bias}} = \sqrt{\left(\frac{\mathrm{bias}}{M_{RM}}\right)^2 + \left(\frac{s_{RM}}{M_{RM}\sqrt{m}}\right)^2 + \left(\frac{u_{bias}}{C_{RM}}\right)^2} \tag{3}$$

The maximum uncertainties associated, using 2 as a coverage factor [15], were 10 % and 12 % for Cd and Pb respectively, complying with the maximum allowable value set in CR 333/2007 [15]. The figures of merit are reported in Table 3.

Table 3: figures of merit of the method.

Parameter	Cd	Pb
Validation Level (µg/kg)	20	100
s_r (µg/kg)	0.3	0.002
RSD_r (%)	1.4	1.7
r (µg/kg)	0.8	0.006
s_R (µg/kg)	0.2	0.004
RSD_R (%)	1.0	3.5
R (µg/kg)	0.6	0.011
LoD (µg/kg)	0.8	13
LoQ (µg/kg)	2.7	42
Recovery (%)	103.5	102.9
u (µg/kg)	1	6
u (%)	5	6
U (µg/kg)	2	12
U (%)	10	12

4. CONCLUSIONS

Method validation was carried out on an in-house prepared honey sample with ad-hoc mass fractions of Cd and Pb. The long standing experience as producer of PT test items permitted the EURL-CEFAO to obtain a suitable material, whose homogeneity and stability for the time necessary for the validation, were verified according to internal procedures based on ISO/IEC 13528.

Validation levels were chosen taking into account a rough mean of the action levels set by the EU MSs in their 2012 National Residue Control Plans for these element/matrix combinations. This choice has proved to be of particular relevance considering the recent introduction of a maximum level for lead (CR 1005/2015 amending CR 1881/2006) in this matrix at a value consistent with the mass fraction used in the EURL method validation.

The preliminary results produced applying the direct determination method were in good agreement with those obtained by ICP-MS and GF-AAS after MW sample digestion. The minimum sample handling also allowed the DD GF-AAS method to provide high quality data either under repeatability or intermediate precision conditions (Table 1).

Moreover, notwithstanding the lack of a proper certified reference material, it was possible to verify the capability of this method, in terms of accuracy, versus leftover test items material employed for the EURL-CEFAO 19th PT on honey.

As reported in Table 3, the good recovery for both the elements (103.5 % and 102.9 % for Cd and Pb, respectively) versus the consensus values produced by outstanding laboratories (NRLs of the EU MSs) is a further proof of the appropriateness of the method performance. This outcome demonstrates the suitability of the method for the direct determination of Cd and Pb in honey by graphite furnace atomic absorption spectrometry in terms of capability and precision. Its main strong point is the minimum sample handling resulting in a very low cost, time saving and more "eco-friendly" process.

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