

## Variations in lead, cadmium, arsenic, and mercury concentrations during honeybee wax processing using casting technology

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Beeswax foundations are a necessary material in intensive modern beekeeping. Heavy metals can accumulate in these foundations for decades, as it is a common beekeeping practice to recycle wax. Beeswax samples were analysed using GFAAS for As, Cd, Pb, and Hg concentrations during the production of beeswax foundations using casting technology with a prolonged cooling and sedimentation phase. Significant differences were determined in the concentrations of As, Cd, Pb ( $p < 0.01$ , all), and Hg ( $p < 0.05$ ) between the three levels of wax in a double-walled steel casting container and comb foundations (CF) during the processing stage. Concentrations ( $\text{mg kg}^{-1}$ ) of the examined metals in comb foundations as the final product ranged as follows: As 0.01–0.88; Cd 1.26–3.55; Pb 82.5–171, and Hg 0.29–1.46. All examined element concentrations demonstrated similar distribution and ratio in different layers, ranging from the lowest concentrations in layers from which wax material is used for comb foundations as the final product, to the highest concentrations in sedimented layer, which represents waste. The obtained results suggest that the described method could effectively eliminate a significant amount of heavy metals from the initial material used for the production of new beeswax foundations.

**KEY WORDS:** *arsenic; beeswax; cadmium; comb foundations; lead; mercury*

Beeswax is a natural honeybee product. It is an extremely complex mixture consisting mainly of esters of higher fatty acids, alcohols, hydrocarbons, proteins, and other minor substances (1-2). Beeswax is primarily used in beekeeping to produce comb foundations but also in the chemical, cosmetic, pharmaceutical, and food industries. In hives, it is vital for the honeybee colony, as it is a construction material for comb cells where nectar and honey are stored (3). Successful comb management is crucial for beekeeping practice and quality, and the sensory properties of beeswax depend greatly on production methods (4). The production or processing of beeswax comb foundations has been described elsewhere (5).

Wax, along with other apian products, can be a useful tool for collecting information about environmental contamination with toxic metals (6-10). Honey could be considered a primary bio-indicator of pollution and therefore used to assess the presence of metals and pesticides in soil, water, and plants (11). Beeswax combs represent a sink or an outlet for many environmental contaminants and, when in the hive for toxins as well, because of a specific lipid-based chemical composition (3).

As, Cd, Pb, and Hg are the main toxic heavy metals in environmental pollution that can contaminate honeybee products, primarily nectar and honeydew. Due to the order and social activity of foragers and house bees, combs and the inside of the hives can also be contaminated (12). The presence of these metals depends on the environmental pollution of air, water, and soil (13, 14) and may cause a wide range of toxic effects, including gastrointestinal, muscular, reproductive, neurological or behavioural effects. Such adverse health effects have been previously documented in birds and mammals, especially for Pb (15-17). Discharge of this highly toxic effluent can seriously impact the environment, as these metals cannot be destroyed or degraded, and their accumulation through the food chain leads to ecological issues with dire consequences (18, 19).

Pursuant to the European legislation, beeswax specification values were set to  $5 \text{ mg kg}^{-1}$  for Pb,  $3 \text{ mg kg}^{-1}$  for As and  $1 \text{ mg kg}^{-1}$  for Hg (20). The FAO/WHO Expert Committee on food additives also set a concentration of  $2 \text{ mg kg}^{-1}$  for Pb in wax, and proposed a maximum residue limit (MRL) for the EU of  $1 \text{ mg kg}^{-1}$  (21-22). The committee of the European Food Safety Authority (EFSA) (23) recommended that the specification for Pb should be set as low as possible, due to its possible toxic effects.

There are very few studies on the heavy metal contamination of beeswax (24), and data on its influence on comb foundation

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quality, as well as the correlations with the rearing and biological characteristics of honeybee brood in contaminated combs are insufficient. It is not known how effective the production of casting comb foundations with a long sedimentation and cooling period is in reducing heavy metal concentrations. In everyday beekeeping conditions, beekeepers collect old, dark, and destroyed combs during the active beekeeping season and then deliver the total amount of wax to the craft unit. Also, because most beekeepers do not have enough collected wax for one round of its processing, commonly few beekeepers join raw material together in the form of wax sheaves for annual comb foundation remount. In the processing stage of beeswax, after melting and homogenisation, several layers of wax are deposited within heated water containers made of high-grade steel during a 48-hour sedimentation period. This step in processing is necessary for an additional cleansing of melted wax. Impurities sink to the lowest part of the wax and must be removed from the container after the sedimentation period, and only the pure upper layers of wax should be used for production of new comb foundations.

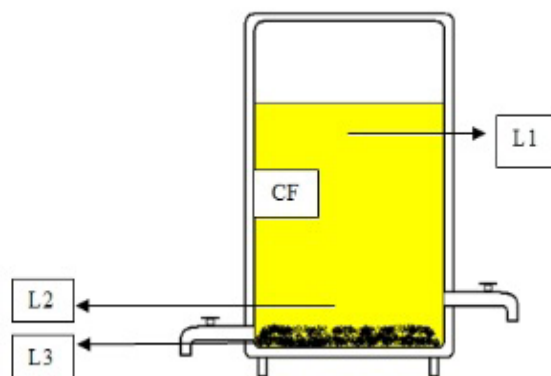
The objective of this study was to determine and compare the concentrations of As, Cd, Pb, and Hg from different layers of melted wax in containers during the processing and production of wax comb foundations, including comb foundations as the final product.

## MATERIALS AND METHODS

### Beeswax samples

A total of 24 samples of melted wax were collected from three double-wall heatable steel water containers, 200 L volume each, during the comb foundation production. Samples were taken 48 hours after melting, homogenisation, sterilisation of beeswax at 125 °C for one hour, and the sedimentation process. During sedimentation, melted wax was kept at an automatically set temperature of 75 °C.

The levels of wax in the container where samples were taken are shown in Figure 1. A long handled grab was used to sample the surface layer (L1). The container had two pipes at different levels with a 7 % elevation difference. The lowest level faucet served first to sample dark sediment (L3) and then lighter wax from the second layer (L2). Beekeepers usually deliver raw wax material in the form of beeswax sheets to a wax production craft unit. Therefore, when using containers such as this, from the higher level faucet, the final material for the comb foundation is slid directly onto a cooled roller that prints the comb foundations. In our case, prior to the contact with the roller, the last sample of the final wax material was taken (CF – material for the comb foundations). Upon collection, all wax samples (100 g) were placed into clean plastic containers, labelled, and transferred to the laboratory and kept at 4–8 °C until analysis.



**Figure 1** Wax sampling levels in a double-walled steel container 48 hours after the processing of raw material, using a casting method with a prolonged cooling and sedimentation period

### Reagents

All reagents were of analytical reagent grade, HNO<sub>3</sub>, H<sub>2</sub>O<sub>2</sub>, and HCl (Analytical Grade, Kemika, Croatia). Ultra-pure water (Milli-Q Millipore, 18.2 M MΩ/cm resistivity) was used for all dilutions. Plastic and glassware were cleaned by soaking in diluted HNO<sub>3</sub> (1/9, v/v) and rinsed with distilled water prior to use. Calibrations were prepared with element standard solutions of 1000 mg L<sup>-1</sup> of each element supplied by Perkin Elmer. Stock solution was diluted in HNO<sub>3</sub> (0.2 %). Matrix modifiers (all Perkin Elmer, USA) in each atomisation were: for Cd 0.005 mg Pd(NO<sub>3</sub>)<sub>2</sub> and 0.003 mg Mg(NO<sub>3</sub>)<sub>2</sub>, for Pb 0.050 mg NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> and 0.003 g Mg(NO<sub>3</sub>)<sub>2</sub>, and for As 0.01 mg Pd(NO<sub>3</sub>)<sub>2</sub> and 0.005 mg Mg(NO<sub>3</sub>)<sub>2</sub>.

### Microwave digestion

Samples (2 g) were digested with 5 mL HNO<sub>3</sub> (65 % v/v), 1 mL H<sub>2</sub>O<sub>2</sub> (30 % v/v) with a microwave oven. A blank digest was carried out in the same way. The Multiwave 3000 microwave closed system (Anton Paar, Germany) was used for sample digestion. The digestion program first began at a potency of 1200 W and was then ramped for 10 min, after which the samples were held at 1200 W for 10 min. The second step began at a potency of 0 W and was held as such for 15 min. Digested samples were diluted to a final volume of 50 mL with double deionised water.

Samples were run in batches that included two replicates of an individual sample, blanks, a standard for the calibration curve, and two spiked specimens. All metal concentrations were determined on a wet weight basis as mg kg<sup>-1</sup>. Detection limits (LOD) were determined as the concentration corresponding to three times the standard deviation of ten blanks and were found to be (mg kg<sup>-1</sup>): As 0.01, Cd 0.0004, Pb 0.005, Hg 0.0005.

To calculate the recovery percentages, five wax samples spiked with known amounts of element analytical standards were processed as follows (mg kg<sup>-1</sup>): for As 0.02; for Cd 0.02, for Hg 0.02, for Pb 0.05. The quality of data showed good

**Table 1 a, b** Instrumental conditions for the determination of Pb, Cd, and As by graphite furnace atomic absorption spectrometry (GFAAS) and Hg, using a mercury analyser, in beeswax samples

Table 1a

Conditions for graphite furnace atomic absorption spectrometry (GFAAS) and heating program			
	As	Cd	Pb
Wavelength (nm)	193.7	228.8	283.3
Argon flow (mL min <sup>-1</sup> )	250	250	250
Sample volume (µL)	20	20	20
Modifier volume (µL)	5	5	5
Drying 1	110 (1, 30)	110 (1, 30)	110 (1, 30)
Drying 2	130 (15, 30)	130 (15, 30)	130 (15, 30)
Ashing	1600 (10, 20)	700 (10, 20)	900 (10, 20)
Atomisation	2000 (0, 5)	1550 (0, 5)	1850 (0, 5)
Cleaning	2450 (1, 3)	2450 (1, 3)	2450 (1, 3)

Table 1 b

Conditions for mercury analyser	
	Hg
Wavelength (nm)	253.65
Drying time (s)	60
Decomposition time (s)	150
Wait time (s)	45
Weight / volume of sample	100 mg 100 mL <sup>-1</sup>
Working range	0.05–600 ng

accuracy, with calculated recoveries for elements (%): As 93.5, Cd 95.7, Hg 98.2, Pb 97.8.

#### Analysis of metals

The analyses of As, Cd, and Pb were conducted at 193.7, 228.8, and 283.3 nm by graphite furnace-atomic absorption spectroscopy using an AAnalyst 600 (Perkin Elmer, USA). Mercury levels in wax samples were quantified without acid digestion using AMA-254 (Advanced Mercury Analyser, Leco, Poland), which employs direct combustion of the sample in an oxygen-rich atmosphere. The instrumental settings and optimising temperature programs of the graphite spectrometer and mercury analyser are summarised in Tables 1a and 1b.

#### Statistical analysis

Statistical analyses were performed using STATA® 13.1 (StataCorp LP®, USA). Concentrations of Pb, As, Cd, and Hg in wax samples were expressed as the minimum and maximum concentrations, median, mean ± standard deviation (SD). The Shapiro-Wilk test was used for the determination of data distribution and the Kruskal-Wallis test for the determination of differences between the concentrations of elements in wax samples. Statistically significant differences were expressed as the level of probability ≤0.05 and 0.01.

## RESULTS AND DISCUSSION

The results for heavy metal concentrations in wax samples are given in Table 2. Significant differences in the concentrations

of As, Cd, Pb ( $p < 0.01$ , all), and Hg ( $p < 0.05$ ) were determined between the four observation levels for wax samples. The highest concentrations of all four examined metals were determined in the wax samples taken at the sediment level (L3), which can be considered the concentrations in raw material, and the lowest were detected in the surface (L1) and middle layers (L2), which is where most of the material for the final product, the comb foundation (CF), is located. The concentrations in L3 were three times higher than in L2, and a hundred times higher than in L1. The concentrations of As decreased in the following order: L3 > L2 > L1 > CF. L1 and L2 concentrations of Cd were approximately fifty times lower than the concentration in L3. The concentrations of Cd increased in the following order: L2 < L1 < CF < L3, and they were about fifty times higher in L3, than in L1, L2, or CF. Cadmium concentrations found in L1, L2, and CF observation points were lower or similar to published data (25). Mercury concentrations from L1, CF, and L2 were about fifteen times lower than in L3. The content of harmful metals (As, Hg) was within the maximum tolerable levels according to the regulations for nutritional products or additives (21-23), with the exception of Pb. The obtained Pb concentrations appear very high, and although the concentrations from L1 and L2 were approximately a hundred times lower than in L3, high amounts were detected in CF. This fact is difficult to discuss due to a lack of detailed data on wax manipulation and storage prior to its delivery to the wax production craft unit. Also, the measured Pb concentrations were higher than the previously reported results on the mineral content in honeybee products within the frame of environmental pollution monitoring (13,

**Table 2** Concentrations of heavy metals in beeswax samples determined at four observation levels, during the production of wax foundations using a casting method with a prolonged cooling and sedimentation period

Element (mg kg <sup>-1</sup> )	Statistics	L1	L2	L3	CF
		(surface level) n=6	(middle level) n=6	(sediment level) n=6	(comb foundation) n=6
As**	Mean±SD	0.69±1.06	2.69±3.12	70.2±49.0	0.15±0.36
	Median	0.005	1.56	67.1	0.01
	Min-Max	0.005–2.14	0.005–6.96	19.3–144	0.01–0.88
Cd**	Mean±SD	1.61±0.47	1.47±0.46	84.0±69.1	2.24±0.83
	Median	1.68	1.35	75.4	2.42
	Min-Max	0.92–2.26	1.02–2.16	16.8–202	1.26–3.45
Hg*	Mean±SD	1.20±1.36	0.67±0.23	9.50±15.1	0.83±0.39
	Median	0.52	0.73	1.49	0.78
	Min-Max	0.33–3.79	0.29–0.87	0.99–38.9	0.29–1.46
Pb**	Mean±SD	58.1±21.5	48.0±19.1	6312±4043	115±35.1
	Median	65.3	54.0	6438	105
	Min-Max	28.1–82.2	17.3–66.3	1338–10802	82.5–171

Statistically significant differences between the elements: \*p<0.05, \*\* p<0.01

25). These authors stated that dust fallout could contain significant amounts of Pb and Cd, which could be linked to the manner of storage of raw wax. There is a lack of knowledge about the possible negative effects of the examined metals on honey brood and also the need for beeswax quality control regarding contaminants and adulteration (26).

Studies on heavy metal contamination of honeybees, honey or pollen are well documented (27, 10, 28, 29). However, beeswax is analysed very rarely, likely due to the fact that it is not consumed. From another aspect, honey ripens in wax combs, and may later be consumed or used in medicine or cosmetics. Also, comb contamination with heavy metals could likely affect honeybee brood development, after-emergence vitality, queen productivity or longevity of adult bees. For all these physiological parameters, pesticides have been proven to have a negative effect (30-33). Heavy metal concentrations are not routinely examined in beeswax foundation production. There is a probability they will be washed during the process of comb melting but without sufficient knowledge of the ratio. Moreover, heavy metal contaminants resist the wax melting temperature. Therefore, they can accumulate for decades, as it is a common beekeeping practice to recycle wax almost continuously in the form of comb foundations, upon which honeybees construct a complete comb. Contaminants from the environment can reach the raw materials of bee products and can be transported to the hive as a consequence of bees' physiological activities (34-35). It is also known that pesticides and acaricides used in regular beekeeping practices, or other fat soluble and persistent substances, can easily accumulate within (24). Recently, Reddy et al. (36) concluded that honeycomb biomass is an environmentally friendly and attractive option for removing heavy metal ions from water or wastewater.

Stratification of melted wax during a prolonged cooling and sedimentation period could have an effect on metal precipitation in the lower wax layers. Although the wax in the upper layers is not completely free of heavy metal traces, it

could be efficiently washed using the described technology. In this study, all examined element concentrations demonstrated a similar distribution and ratio in different layers, ranging from the lowest concentrations in L1 and L2, from which wax material is used for CF as the final product, to the highest concentrations in L3, which represents waste. Although a more detailed study is necessary, it can be stated that in the light of these preliminary results, the concentrations of the examined elements in CF are significantly lower than in the initial wax material, with the exception of Pb. The obtained results suggest that the described method could effectively eliminate a significant amount of heavy metals from the initial material used for the production of new beeswax foundations and its use can be recommended. Generally, beeswax samples should be analysed more often during wax processing season, with respect to toxic metals contamination.

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### Koncentracije olova, kadmija, arsena i žive tijekom prerade pčelinjeg voska metodom lijevanja

U intenzivnom modernom načinu pčelarenja satne su osnove nužan materijal. Teški metali mogu se taložiti u satnim osnovama tijekom više desetljeća, a uobičajena je pčelarska praksa kontinuirano preradivati vosak. Uzorci pčelinjeg voska analizirani su GFAAS-om za utvrđivanje koncentracija As, Cd, Pb i Hg tijekom proizvodnje satnih osnova primjenom tehnologije lijevanja s produženom fazom hlađenja i sedimentacije. Utvrđene su značajne razlike u koncentracijama As, Cd, Pb ( $p < 0,01$ ; svi) i Hg ( $p < 0,05$ ) između triju izdvojenih slojeva voska u čeličnim spremnicima dvostrukih stijenki i satnim osnovama tijekom prerade voska. Koncentracije (mg/kg) pretraživanih metala u satnim osnovama kao konačnom proizvodu utvrđene su u rasponu: As 0,01-0,88; Cd 1,26-3,55; Pb 82,5-171 i Hg 0,29-1,46. Najniže koncentracije pretraživanih elemenata utvrđene su u slojevima materijala za izradu satnih osnova kao konačnog proizvoda, a najviše u istaloženom sloju otpada. Dobiveni rezultati upućuju na mogućnost da se primjenom opisane metode mogu učinkovito ukloniti značajne količine teških metala iz sirovine voska korištenog za preradu u nove satne osnove.

KLJUČNE RIJEČI: pčelinji vosak; satne osnove; olovo; arsen; kadmij; živa