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Original Scientific Article

CONTENT OF FIVE TRACE ELEMENTS IN DIFFERENT HONEY TYPES FROM KOPRIVNICA-KRIŽEVCI COUNTY

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Summary: Multifloral and unifloral honeys [black locust (*Robinia pseudoacacia* L.), chestnut (*Castanea sativa Mill.*), lime (*Tilia spp.*), indigobush (*Amorfa fruticoza* L.) and rapeseed (*Brassica napus*)] were collected from Koprivnica-Križevci County in northwestern Croatia during 2010 and 2011. The concentrations of Cd, Pb, Hg, As and Cu were determined and mean levels of elements (μg/kg) in honey samples measured were: in multifloral 1.26 for Cd, 163 for Pb, 135 for As, 1.35 for Hg and 11.7 for Cu; in black locust 1.52 for Cd, 182 for Pb, 23.2 for As, 0.46 for Hg and 7,697 for Cu; in lime 2.92 for Cd, 340 for Pb, 116 for As, 0.74 for Hg and 7,798 for Cu. Significant differences in Hg and Cu levels were observed between honey types. Average Cu levels found in lime and black locust honey types were much higher than those reported in other European countries. The highest element contents measured in different honey types were: Cd 4.0 μg/kg and As 502 μg/kg in rapeseed, Hg 6.11 μg/kg in chestnut, Pb 2,159 μg/kg in black locust and Cu 79,167 μg/kg in indigobush. Lead concentrations measured in all honey types were much higher than levels obtained in Italy, Slovenia, Poland, Romania and Turkey. These indicate that special attention should be paid to ensuring positions of hive in zones of bee forage that are more distant from highways and railways. The results presented indicate a differentiation of the trace element content of honeys of different botanical origin obtained from the same area.

Key words: different honey types; metals; As; Cd; Cu; Hg; Pb

Introduction

Honey is a sweet natural product, produced by honeybees (*Apis mellifera*) that collect nectar from flowers and turn it into a product considered to be a delicious food and known to be a healthier nutritional choice than sugar (1). It is mainly composed of fructose and glucose (65 %) and water (18 %), while protein contents are low (2). The mineral content in honey is low and ranges from about 0.04 % in pale honeys to 0.2 % in dark honeys (3). Honeybees are estimated to forage on plants growing over a relatively large area (more than 7 km^2) and when going from flower to flower, are also in contact with air, water and soil, branches and leaves. Therefore, honey is the result of a bio-accumulative process useful for collecting information about the environment and may be considered a bioindicator of environmental pollution (4).

The great variability of the trace element content in different honey types is closely related to botanical and geographical origin with regards to soil and climate characteristics (5). Environmental pollution factors that may contribute to the presence of metals in honey may be caused by non-ferrous metallurgy, industrial smelter and factories, leaded petrol, and agrochemicals such as cadmium-containing fertilizers, and organic mercury and arsenic-based pesticides still in use in some countries (6, 7).

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In addition to the environmental importance, the determination of trace metals is important regarding to quality control of honey and because the fact that today's total production of honey in the world is increasing. The European Union is the world's largest consumer of honey (1, 8).

In the past decade, different trace metals content have been determined in different honey types in European countries: France (9), Italy (10-12), Slovenia (13, 14), Poland (15), Czech Republic (16), Romania (6), Spain (17-19) and Turkey (20-23). In most of these studies, the botanical influence on honey composition has been neglected and only the differences among regions and the geographical origin of honey have been tested.

In Croatia, the most common unifloral honey types are black locust (*Robinia pseudoacacia* L.), chestnut (*Castanea sativa Mill.*) and lime (*Tilia spp.*), while the unifloral honeys indigobush (*Amorfa fruticoza* L.) and rapeseed *(Brassica napus)* are rarer. Unifloral honeys originating from *Lavandula stoechas* and *Salvia officinalis* are also rare and are indicative of the southern geographical origin in the country.

The aim of the present study is to examine the concentrations of the toxic trace metals Cd, Pb, Hg, As and Cu in different honey types collected in the same geographical region, in Koprivnica-Križevci County in northwestern Croatia.

Materials and methods

Sample collection

A total of 14 different honey type samples were collected: 3 multifloral, 5 black locust (*Robinia pseudoacacia* L.), 3 lime (*Tilia spp.*), 1 chestnut (*Castanea sativa Mill.*), 1 indigobush (*Amorfa fruticoza* L.) and 1 rapeseed *(Brassica napus)*. Honey samples were produced and collected during 2010 and 2011 from individual beekeepers near the towns Križevci, Đurđevac, Koprivnica and Virovitica in Koprivnica-Križevci County (Table 1).

Koprivnica-Križevci County covers an area of about 1,746 km^2 and the urban centres Križevci, Đurđevac, Koprivnica and Virovitica each have populations of 20,000 to 55,000. The region is relatively clean, as economic activities are primarily agricultural with cultivated fields and woods, and a high representation of vineyards. The region has no pronounced industrial activities and vehicular traffic is rather low in comparison with European standards and the more populated, urbanized and industrialized Centre region around the capital city, Zagreb.

Following collection, honey samples (500 g) were placed into clean glass bottles, labelled and brought to the laboratory and stored at 4–8°C until analysis.

Table 1: Geografical and botanical origin of honey samples

Sample no.	Sample identification	Honey type (common name)	Geographical origin	Year
1	BL 1	Black Locust	Đurđevac	2010
2	BL 2	Black Locust	Đurđevac	2011
3	BL 3	Black Locust	Đurđevac	2011
4	BL 4	Black Locust	Koprivnica	2011
5	BL 5	Black Locust	Križevci	2011
6	L 1	Lime	Đurđevac	2011
7	L 2	Lime	Đurđevac	2010
8	L 3	Lime	Koprivnica	2011
9	MF 1	Multifloral	Koprivnica	2011
10	MF 2	Multifloral	Đurđevac	2011
11	MF 3	Multifloral	Križevci	2011
12	C	Chestnut	Đurđevac	2010
13	I	Indigobuch	Virovitica	2010
14	R	Rapeseed	Koprivnica	2010

Standard preparation

Acid used, HNO_3 and HCl were of analytical reagent grade (Kemika, Croatia). Ultra high purity water processed through a purification system NIRO VV UV UF 20 (Nirosta d.o.o. Water Technologies, Osijek, Croatia) was used for all dilutions. Calibrations were prepared with element standard solutions of 1 g/l of each element (Perkin Elmer, USA). Stock solution was diluted in HNO_3 (0.2 %). In the preparation of Hg standards, 1 ml of HNO_3 conc., 0.1 ml 10 % $K_2Cr_2O_7$, and 0.1 ml HCl conc. were added to all working standards and prepared in brown glass volumetric flasks. As matrix modifiers in each atomization for As, Cd, Cu and Pb, 0.005 mg $Pd(NO_3)_2$ and 0.003 mg $Mg(NO_3)_2$ (Perkin Elmer, USA) were used.

Plastic and glassware were cleaned by soaking in diluted HNO_3 (1/9; v/v) and by subsequent rinsing with high purity water and drying prior to use.

Sample preparation

Honey samples (0.5 g) were digested with 4 ml HNO_3 (65 % v/v) and 2 ml H_2O_2 (30 % v/v) with a Multiwave 3000 microwave closed system (Anton Paar, Germany). A blank digest was carried out in the same way. The digestion programme began at a power of 500 W, ramped for 1 min and hold for 4 min. The second step began at a power of 1000 W ramped for 5 min and hold for 5 min. The third step began at a power of 1400 W, ramped for 5 min and hold for 10 min. Digested samples were diluted to a final volume (50 ml) with double deionised water.

Determination of metals

Atomic absorption spectrometry by AAnalyst 800 and AAnalyst 600 (Perkin Elmer, USA) equipped with an AS 800 and AS 600 autosamplers (Perkin Elmer, USA) was used for measurement of As, Cd, Cu and Pb concentrations. Argon was used for graphite furnace measurements. Pyrolytic-coated graphite tubes with a platform were used. The atomic absorption signal was measured in peak area mode against a calibration curve.

Mercury in honey samples were quantified using the AMA-254 (Advanced Mercury Analyzer, Leco, Poland) without acid digestion by direct combustion of the sample in an oxygen-rich atmosphere. The operating parameters for working elements are presented in Table 2.

Detection limits for the five metals were determined as the concentration corresponding to three times the standard deviation of twenty blanks. All samples were run in batches that included blanks, a standard calibration curve, two spiked honey samples, and one duplicate. In order to determine the method accuracy and to calculate the recovery percentage, ten honey samples were spiked with known amounts of Cd, Pb, As, Hg and Cu analytical standards.

Data analysis

Statistical analysis was performed using the software package Statistica 6.1 (StatSoft® Inc., USA). One-way analysis of variance was used to test for differences in honey metal concentrations. Data were log-transformed to improve normality prior to analysis to meet the underlying assumptions of the analysis of variance; the values given are therefore geometric means. The differences between the metal concentrations in different honey types were analyzed using the *t*-test. A probability level of $p \leq 0.05$ was considered statistically significant.

Results and Discussion

In this study, concentrations of heavy metals and Cu were determined in six different honey types from the same geographical origin under the same climatic conditions.

The quality of data was checked by analysis of the recovery rate with spiked honey samples for Cd, Pb, Hg As and Cu and showed good accuracy, with recovery rates for metals of 95.9 % to 99.2 % (Table 3). The limits of detection (LODs, μg/kg) were: Cd 1.0, Pb 4.7, Hg 0.1, As 5.0 and Cu 1.2. The concentrations of the five elements in multifloral and unifloral honey samples are reported in Table 4. Statistical analyses by one-way ANOVA showed a significant difference in Cu levels ($p<0.01$). However, no significant differences were observed in Cd, As, Hg and Pb levels. It has to be point out that element levels results presented for honey types chestnut, rapeseed and indigobush is for one sample only.

In the present study, Cd content in all honey samples range from 1 to 5 µg kg^{-1}. No significant differences were observed between different honey types. Literature values for different honey type originating from European countries are given in Table 5. Cadmium multifloral levels observed were similar to contents obtained in different geographical regions of Turkey (23), Macedonia (3.63 µg/kg; 24) and to previously reported levels in multifloral honey samples from different regions in Croatia (1.51 µg/kg; 25). In general, Cd levels in all honey types were 2 to 10 times lower than those reported in Italy (12) and Turkey (21, 22) and more than 100 times lower than the high Cd levels found in mixed flower honey from Bologna, Italy (11).

Lead is one of the most widespread metal pollutants and, like Cd, can reach humans through air, water and food. This metal had no beneficial role in human metabolism and produces a progressive toxicity and can cause health disorders such as fatigue, sleeplessness, hearing and weight loss. A provisional tolerable weekly intake (PTWI), as acceptable levels of major toxic elements that can be ingested on a weekly basis and may accumulate in the body established by WHO (The World Health Organization) and FAO (Food and Agriculture

Table 2: Instrumental conditions for atomic absorption spectrometry and mercury analyzer and graphite furnace program (temperature and time) for Pb, Cd, Cu, As and Hg determination in honey samples

Conditions for graphite furnace atomic absorption spectrometry				
	Lead	Cadmium	Copper	Arsenic
Wavelength (nm)	283.3	228.8	324.8	193.7
Argon flow (ml/min)	250	250	250	250
Sample volume (µl)	20	20	20	20
Modifier volume (µl)	5	5	5	5
Heating pogram tempetature °C (ramp time (s), hold time (s))				
Drying 1	110 (1, 30)	110 (1, 30)	110 (1, 30)	110 (1, 30)
Drying 2	130 (15, 30)	130 (15, 30)	130 (15, 30)	130 (15, 30)
Ashing	900 (10, 20)	700 (10, 20)	1200 (10, 20)	1600 (10, 20)
Atomisation	1850 (0, 5)	1550 (0, 5)	2000 (0, 5)	2000 (0, 5)
Cleaning	2450 (1, 3)	2450 (1, 3)	2450 (1, 3)	2450 (1, 3)

Conditions for determination on mercury analyzer	
Wavelength (nm)	253.65
Drying time (s)	60
Decomposition time (s)	150
Wait time (s)	45
Weight / volume of sample	100 mg / 100 ml
Working range	0.05 – 600 ng

Table 3: Trace metal concentrations and recoveries in spike honey samples

Element	Spiked value (µg/kg)	Measured value (µg/kg)	Recovery (%)
Cd	10	9.67 ± 0.33	96.7
Pb	50	48.9 ± 6.45	97.8
Hg	10	9.92 ± 0.14	99.2
As	10	9.59 ± 0.27	95.9
Cu	50	49.4 ± 5.23	98.8

Table 4: Concentrations of trace elements Cd, Pb, As, Hg and Cu (mg/kg) in honey samples of different botanical origin from Koprivnica-Križevci County

Sample identification	Cd (mg/kg)	Pb (mg/kg)	As (mg/kg)	Hg (mg/kg)	Cu (mg/kg)
BL 1	4.0	270	11.0	0.90	318
BL 2	1.0	134	10.1	0.62	1091
BL 3	1.0	62.1	12.2	0.13	1272
BL 4	2.0	2159	499	0.53	124
BL 5	1.0	41.3	10.0	0.51	665
Geometric mean	1.52	182	23.2	0.46	515 [ab]
L 1	5.0	303	512	0.75	47.5
L 2	2.0	364	11.1	1.21	195
L 3	2.5	358	279	0.45	86.1
Geometric mean	2.92	340	116	0.74	92.7 [ac]
MF 1	2.0	438	185	2.41	10.1
MF 2	1.0	134	118	0.83	12.1
MF 3	1.0	73.2	112	1.22	13.2
Geometric mean	1.26	163	135	1.35	11.7 [bc]
C	1.0	1637	30.1	6.11	53.4
I	1.0	159	11.1	1.62	154
R	4.0	180	502	1.11	29.2

Significant differences between honeys: [a] BL:L $p < 0.05$; [b] BL:MF $p < 0.01$; [c] L:MF $p < 0.01$;

Table 5: Overview of the element contents in different honey types from different countries

	Country Honey type (reference)			
Element (µg/kg)	Italy MF1-mixed floral (11) C-chestnut (11) MF2- multifloral (12) MF3- multifloral (10)	Slovenia MM-monofloral (13)	Spain MM-multifloral + monofloral (18)	Turkey MF1 -multifloral (21) MF2 -multifloral (22) MF3-multifloral (23)
Cd	MF1 305 C < 50 MF2 4.25	-	-	MF1 10.9 – 21.2 MF2 1.1 – 17.9 MF3 0.38 – 2.03
Pb	MF1 620 C < 50 MF2 76.4	MM 1.86 - 4.3	-	MF1 17.6 – 32.1 MF1 8.4 – 106 MF3 1.54 – 36.7
Hg	MF2 < 2	-	-	-
As	MF2 6.59	MM 1.24 to 1.49	-	-
Cu * mg/kg	MF1 890 C < 50 MF2 647 MF2 0.31*	MM 1.4 to 2.7 *	MM 0.1-1.73*	MF1 0.25 – 1.1* MF2 0.23 – 2.41* MF3 9.97 – 29.5

Organization): Cd (7 μg/kg b.w.), Pb (25 μg/kg b.w.), As (15 μg/kg b.w.) and Hg (5 μg/kg b.w.) (26-28). In 2000 the maximum residue limit (MRL) values of 0.1 mg/kg for Cd and 1 mg/kg for Pb were proposed for the European Union. However, until today there are no established MRL values for heavy metals in honey. In Croatia there is not established maximum permitted levels for the Cd, Hg, Pb, As and Cu. According to the published data, only in Macedonia maximum permitted value for Cd and Cu in honey is set to be 0.03 mg/kg for Cd and 1 mg/kg for Cu (24, 29). In the present study, obtained mean concentrations of Cd and Pb were below MRL values proposed by EU.

Lead contents decreased in the following order: chestnut > black locust > lime > multifloral > rapeseed > indigobush. The lowest and highest Pb concentrations obtained were 159 μg/kg in indigobush honey and 2,159 μg/kg in black locust honey. Mean Pb concentrations measured in black locust, lime and multifloral honeys were much higher (in some cases for more that 4,000 times) than levels obtained in other countries: Italy (12), Poland (70 μg/kg; 15), Turkey (21, 22) and Romania (0.07 μg/kg; 6). However, only in multifloral honey were Pb levels for 3.8-time lower than those found in mixed flower honey from Italy (11). With regards to fact that studied region has no pronounced industrial activities and vehicular traffic is rather low it is not clear why higher concentrations of Pb were determined. It is only be assume that may be due to the position of hives in zones near highways and railways during, which is often the case. Other factor that may contribute is that in soils, and suspended air particulates, concentrations of Pb were influenced by distance from highway and direction of prevailing winds. Also it is demonstrated that Pb accumulation in and on plants next to highways were caused principally by aerial deposition and not by, at least to any great extent, absorption by the plant from contaminated soil (30). On the other hand, Pb is relatively unavailable to plants when the soil pH level is above 6.5. In case of pH values less than 6.5 there is actual increase of Pb uptake by the plant itself from soil. Recent study of soil metal content in Croatia shown that Pb is present in much higher amounts in soil (25.3–27.0 μg/g d.w.) than for example Cd (0.2 μg/g d.w.) (31).

In this study, As levels ranged from the lowest level of 11.1 μg/kg in indigobush honey to 502 μg/kg in rapeseed honey. In comparison to the very few literature data, As levels obtained were higher than those found in Siena County, Italy (12), but more than 9 time lower than levels in Slovenia (13).

Environment pollution by mercury may be caused by industrial activities, mining and combustion and pollution from agricultural sources (32). Several reports were available for Hg concentrations in honey samples. Mercury levels ranged from 50 to 212 μg/kg in contaminated and from 1 to 3 μg/kg in uncontaminated areas in Slovakia (33). In multifloral and other honey types examined in Siena County in Italy, Hg levels were lower than the quantification level of 2 μg/kg (12). In the present study, Hg ranged from a minimal of 0.13 μg/kg in black locust honey to a maximum of 6.11 μg/kg in chestnut honey.

As an essential element, Cu may influence growth, skin pigmentation, bone mineralisation, gastrointestinal and heart function. However, Cu may also generate toxic effects such as dermatitis, liver cirrhosis and neurological disorders, while acute Cu poisoning causes symptoms of nausea, vomiting and abdominal and muscle pain (34). The provisional permitted daily intake for Cu determined in an average adult with 60 kg body weight is 3 mg (35). Copper can enter the food chain and also honey through the mineralisation of crops and due to its application on a large number of crop pests as a fungicide, bactericide and herbicide in the vicinity of the hives locations (36).

In the present study, Cu concentrations ranged from the lowest concentration of 10.1 μg/kg measured in multifloral honey to the highest of 1,272 μg/kg in black locust honey. Mean Cu levels determined in multifloral honey were significantly lower than those of lime and black locust honey types ($p < 0.01$, both). Also, significantly higher Cu concentration was determined in black locust than in lime honey ($p < 0.05$). Differences in the element content between different honey types have also been reported in previous studies (12, 16, 23, 37).

In previous studies, upper Cu limit determined in other parts of the world was around 2 μg/g (38). Average Cu levels found in multifloral honey (11.7 μg/kg) were more than 25 lower than those reported in previous studies from Italy (10, 11, 12), and more than 110 and 230 times lower than the highest level measured in Turkey (21, 22), Spain (18) and Slovenia (13). Also, the mean Cu level determined were more than 90 times lower than previously obtained results for multifloral honey samples from different regions in Croatia (1074 μg/kg; 25). On the other hand, the results obtained were similar to levels found in the Black Sea region in Turkey (23).

Copper levels determined in chestnut honey in this study are higher than those from Bologna, Italy (11) but for 8 times lower than those found in chestnut honey from Anatolia, Turkey (0.42 mg/kg; 39). On the other hand, Cu levels determined in monofloral honeys black locust and lime was lower that those found in Slovenia (13) and Spain (18). A soil metal study in Croatia showed low Cu levels raging form 10 to 25.4 μg/g d.w. in region studied (31).

The quantities of heavy metals in honey are usually so small that even 100 g eaten daily would not contribute appreciably to dietary requirements. However, if in the environment is increased one or more elements these increased content of it in honey which then has a significant impact. As a result of present study, trace element levels of Pb and Cu determined in multifloral honey were lower than in unifloral honey black locust, lime and chestnut honeys obtained in the same geographical region. Accordingly, these results represent a differentiation of the trace element content in honeys of different botanical origin obtained from the same geographical region.

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VSEBNOST PETIH ELEMENTOV V SLEDOVIH V RAZLIČNIH VRSTAH MEDU IZ OKROŽJA KOPRIVNICA-KRIŽEVCI

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Povzetek: V okrožju Koprivnica-Križevci na severozahodnem delu Hrvaške so bili med leti 2010 in 2011 zbrani vzorci medu, nabrani na več rastlinah, ali sortnega medu robinje (*Robinia pseudoacacia* L.), kostanja (*Castanea sativa Mill.*), lipe (*Tilia spp.*), grmaste amorfe (*Amorfa fruticoza* L.) in kapusnice (*Brassica napus*). Koncentracije vsebnosti Cd, Pb, Hg, As in Cu ter srednje vrednosti elementov (µg/kg) v vzorcih medu so bile: v medu, nabranem na več rastlinah: 1,26 za Cd, 163 za Pb, 135 za As, 1,35 za Hg in 11,7 za Cu, v medu z robinje: 1,52 za Cd, 182 za Pb, 23,2 za As, 0,46 za Hg in 7,697 za Cu ter v medu z lipe 2,92 za Cd, 340 za Pb, 116 za As, 0,74 za Hg in 7,798 za Cu. Opažene so bile značilne razlike v nivoju Hg in Cu med vrstami medu. Povprečna raven Cu v medu lipe in robinje je bil bistveno višji kot v ostalih evropskih državah. Najvišja izmerjena vrednost elementov v različnih vrstah medu je bila: Cd 4,0 µg/kg in As 502µg/kg v medu kapusnice, Hg 6,11 µg/kg v medu kostanja, Pb 2,159 µg/kg v medu robinje in Cu 79,167 µg/kg v medu grmaste amorfe. Vsebnosti svinca, izmerjenega v vseh vrstah medu, so bile bistveno višje kot izmerjene vsebnosti v Italiji, Sloveniji, Poljski, Romuniji in Turčiji. Podatki kažejo na to, da je potrebno pozorneje izbrati lokacije čebelnjakov v področjih, kjer čebele nabirajo med. Pomembno je tudi, da so oddaljena od avtocest in železnic. Rezultati kažejo na razlike v vsebnosti elementov v sledovih v vzorcih medu, ki imajo različno botanično poreklo, pridobljeno na istem področju.

Ključne besede: različni tipi medu; kovine; As; Cd; Cu; Hg; Pb