Macedonian Journal of Chemistry and Chemical Engineering, Vol. **36**, No. 1, pp. 23–33 (2017)

MJCCA9 – 724

ISSN 1857-5552
e-ISSN 1857-5625
DOI:10.20450/mjcce.2017.1004

Received: July 25, 2016
Accepted: January 27, 2017

Original scientific paper

ASSESSMENT OF HEAVY METALS IN PROPOLIS AND SOIL FROM THE PELAGONIA REGION, REPUBLIC OF MACEDONIA

Biljana Bogdanova Popov[1*], Vesna Karapetkovska Hristova[2], Stefce Presilski[2], Mohammad Ali Shariati[3], Stevo Najman[4]

[1]*Veterinary Faculty, University Ss. Kliment Ohridski, Prilepska n. n., Bitola, Republic of Macedonia*
[2]*Faculty of Biotechnical Sciences, University Ss. Kliment Ohridski, Partizanska n. n., Bitola, Republic of Macedonia*
[3]*All-Russian Research Institute of Phytopatology of Federal Agency of Scientific Organizations of Russia, Leninsky Prospekt 32a, Moscow, Russia*
[4]*Faculty of Medicine, University of Niš, Bulevar Zorana Djindjica 81, Niš, Serbia*
[*]biljana.bogdanova@uklo.edu.mk

In this study, the contents of five representative heavy metals (Cr, Pb, Zn, Cd and Cu) were determined in soil and propolis samples from four locations in southwestern Macedonia using atomic absorption spectrometry. The aim was to pinpoint the key factors that influence the content of heavy metals in propolis and to establish whether there is a connection between the contents of heavy metals in soil and in propolis from the same location. Generally, at all of the locations, the relative concentrations of heavy metals in soil were found to decrease in the following order: Zn>Cr>Cu>Pb>Cd. The highest mean values for concentrations of heavy metals in these soils were found to be: 72.03, 38.28, 26.64, 17.15 and 0.60 mg kg^{-1} for Zn, Cr, Cu, Pb and Cd, respectively, and they are all below the target values from the new Dutch list. The general trend of the heavy metal contents in propolis from the same four locations, in decreasing order, is: Pb>Cr>Zn>Cu≈Cd. Generally, the propolis samples from the highland locations (Orle and Rapeš) had lower overall contents of heavy metals than the lowland locations (Novaci and Makovo). All of the analyzed propolis samples meet the requirements of the Macedonian legislation and the international organizations for the maximum allowed levels for heavy metals. Attempts were made to find a correlation between the heavy metal contents in soil and propolis. According to our aim, the investigation presented herein offers one step towards a complete picture of ecological safety of the specific areas in the Republic of Macedonia. To do so, it is necessary to perform additional studies and to find appropriate biomonitoring methods. Further studies are needed to complete the picture and to determine the major pathways of incorporation of heavy metals in beehive products.

Key words: heavy metals; soil; propolis; atomic absorption spectrometry; honeybee; biomarker

ПРОЦЕНА НА ТЕШКИ МЕТАЛИ ВО ПРОПОЛИС И ВО ПОЧВА ОД ПЕЛАГОНИСКИОТ РЕГИОН, РЕПУБЛИКА МАКЕДОНИЈА

Во оваа студија, со користење на атомска апсорпциона спектрометрија, беше одредена содржината на пет тешки метали (Cr, Pb, Zn, Cd и Cu) во примероци почва и прополис, од четири локации во југозападниот дел на Македонија. Целта беше да се посочат клучните фактори кои влијаат на содржината на тешките метали во почвата и во прополис од иста локација. Општо земено, на сите локации релативните концентрации на тешки метали во почва беа со следниот опаѓачки редослед: Zn>Cr>Cu>Pb>Cd. Највисоките средни вредности од концентрациите на тешки метали во овие почви изнесуваат: 72,03; 38,28; 26,64; 17,15;0,60 mg kg^{-1} за Zn, Cr, Cu, Pb и Cd, соодветно, и сите тие се под целните вредности претставени во Холандската листа. Општиот тренд на содржината на тешки метали во прополисот од истите четири локации, е со следниот опаѓачки редослед: Pb>Cr>Zn>Cu≈Cd. Генерално, примероците од прополис од планинските локации (Орле и Рапеш) имаат пониска севкупна содржина на тешки метали од прополисот од низинските локации (Новаци и Маково). Сите анализирани примероци на прополис ги исполнуваат барањата на македонските стандарди, како и на меѓународните организации за максимални дозволени концентрации на тешки метали. Обидите беа направени за да се најде корелација помеѓу содржината на тешки метали во почва и во прополис. Според нашата цел, презентираните истражувања претставуваат еден чекор кон добивање целосна слика за еколошка безбедност на специфични области во Република Македонија. За да се направи тоа, потребни се дополнителни студии и наоѓање соодветен метод за биомониторинг. Потребни се дополнителни истражувања за да се комплетира сликата, како и откривање на главните патишта преку кои тешките метали се инкорпорираат во пчелините производи.

Клучни зборови: тешки метали; почва; прополис; атомска апсорпциона спектрометрија; пчела; биомаркер

1. INTRODUCTION

The emergency created by environmental contamination brought on by human activities (both industrial and domestic) implies that tools are needed to determine the quality and quantity of pollutants in the environment. Pollution with heavy metals is a global problem caused by rapid technological advances and human exploitation of natural resources [1]. Determination of the concentrations of heavy metals in the environment (air, soil and water) is an important issue in order to understand biogeochemical processes and to monitor ecosystem health. In order to make a valid assessment of these complex processes, appropriate bioindicator-based techniques must be developed and assessed. One of the approaches is to use a suitable organism as a bioindicator. From previous studies it has been established that the honeybee (*Apismellifera* L.) and its products (honey, pollen, wax and propolis) can potentially be highly useful as heavy metal biomonitors[1–3]. Honeybees are continuously exposed to contaminants present in the widespread area (~ 5 km^2) around the apiary for the duration of their active foraging period. Air and soil contain heavy metals and can be potential sources of contamination of a bee colony and its products[4]. Additionally, honeybees are highly sensitive to organic pollutants and pesticides and the effects can be measured by the mortality of the species and the concentration of the pollutants in the corresponding products.

In this context a special place belongs to propolis, a honeybee product, as a crucial bioindicator for the determined specific group of heavy metals [4–6]. The chemical composition of propolis is very complex and more than 300 constituents have been identified. It contains mainly resin (50–55%), wax (30%), essential oils (8–10%), organic material, minerals, pollen, and mechanical impurities [7–9]. In general, the composition of propolis is directly related to that of the bud exudates collected by the honeybees from various plants, and these bud exudates depend on many ecological factors [7–9]. Propolis exhibits some functional properties such as antibacterial, anti-inflammatory, antiviral, antioxidative, and analgesic activity and many others[10–12].

Our study had several aims. The main aim was to pinpoint the key factors that influence the contents of heavy metals in propolis and to establish whether there is a connection between the contents of heavy metals in soil and in propolis from the same location or locations. For that purpose it was decided to focus on a small selected region near to the city of Bitola (10–17 km from it), with similar vegetation and with a difference in the elevation (altitude) between locations. There are very few published studies that take into consideration both the contents of the heavy metals in soil and the contents of the same heavy metals in propolis (and other bee products). Additionally, extra caution should be taken in terms of the techniques and type and quality of equipment used to gather and store the propolis in order to avoid its contamination with heavy metals, which has been addressed by several authors [13–16].

Our second goal was to determine whether the gathered samples of propolis are safe for consumption based on the contents of the selected heavy metals (chromium, lead, zinc, cadmium, and copper). It is important to know whether the soil from the selected area of southeastern Pelagonia (Republic of Macedonia) is safe for agricultural and related produce and whether the propolis from the same area meets the stringent criteria for heavy metals set by the Food and Agriculture Organization (FAO), World Health Organization (WHO), EU [17], and Macedonian legislation. In the present work, the contents of five elements (Cr, Pb, Zn, Cd and Cu) were measured in soil and propolis using atomic absorption spectrometry (AAS). A biomonitoring survey involving soil and propolis samples was carried out in four locations in southwestern Macedonia, in the vicinity of the city of Bitola.

2. EXPERIMENTAL

The propolis samples used as research material concerning the *ApismelliferaMacedonica*subspecies were collected monthly from 2012 to 2013 from four different locations in R. Macedonia, situated in the central part of the Balkan Peninsula. The selected region is between the geographic latitudes of 40°50″ and 42°20″ N and longitudes of 20°27′ and 23°05″ E, and further, we subdivided it into two groups: two lowland areas near to river areas around the village of Novaci (41°2′34.49″ N, 21°27′35.81″ E, 600 m a.s.l.), the village of Makovo (41°7′4.91″ N, 21°36′31.6″ E, 620 m a.s.l.) and two mountain areas or highland areas named the village of Rapeš (41°6′9.73″ N, 21°38′50.18″ E, 802 m a.s.l.) and the village of Orle (41°8′53.82″ N, 21°36′48.28″ E,780 m a.s.l.). All of the above mentioned areas were situated in rural areas for the duration of this work, and a geographical map (taken from Google Maps) of R. Macedonia is shown in Figure 1.

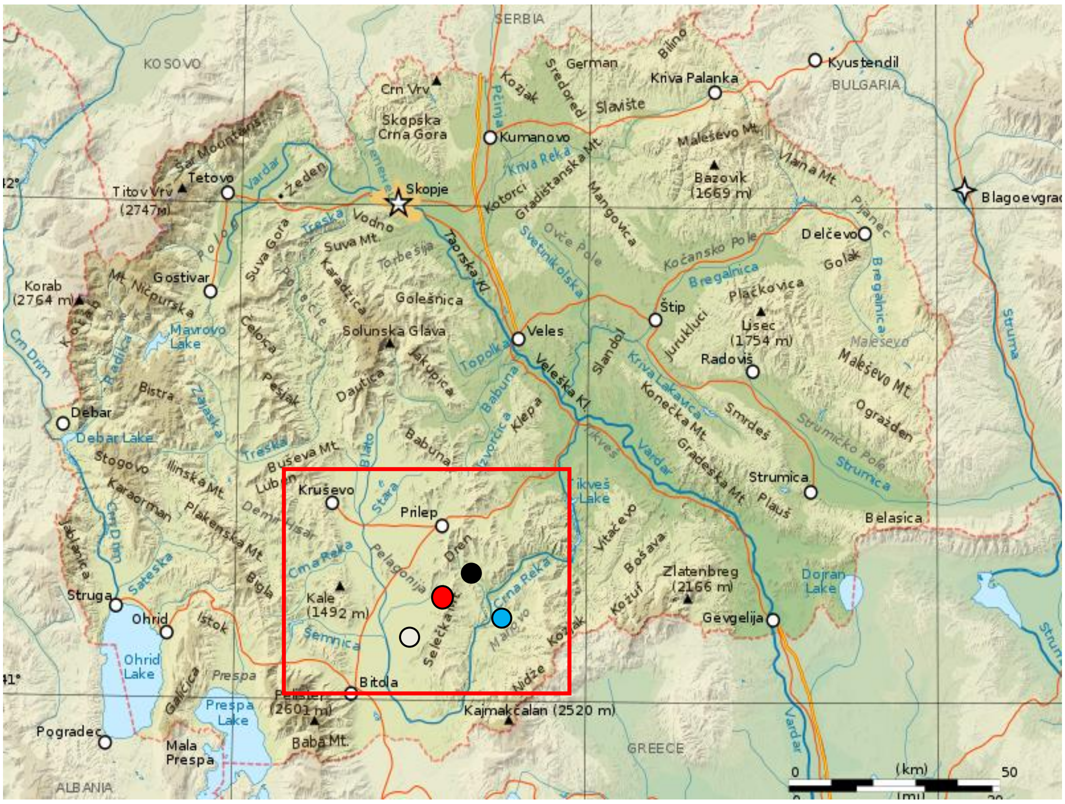

Fig.1. Geographical map of the Republic of Macedonia (taken from Google Maps). The red spot indicates the village of Novaci, and the white spot indicates the village of Makovo (lowland areas). The black spot indicates the village of Orle, and the blue spot indicates the village of Rapeš (highland areas).

2.1. *Sample preparation for atomic absorption analyses*

The quantitative analysis of the selected propolis samples was carried out using AAS in the Laboratory for Sanitary Chemistry at the Centre for Public Health – Bitola, and the Scientific Tobacco Institute at Prilep, Macedonia. The heavy metal content in the samples was determined using Varian, Spectra AA 220 atomic absorbtion spectrophotometer according to an official method that is recommended by the European Commission Joint Research Centre [17] specifically for the determination of heavy metals in feed and food. In general, the samples (0.10 g) were dissolved/digested in a mixture of 25.0 ml of concentrated nitric acid and 10.0 ml of concentrated sulfuric acid in a beaker and analyzed using AAS according to EN 14084:2003.

2.2. *Preparation of soil samples for analysis*

All of the soil samples were collected from the four specified areas at least 5 km from the observed bee hives. Approximately 3.0 kg of soil was collected at a depth of 30 cm. Later on these samples were sealed in two layers of plastic bags and transported to the laboratory for further analysis [18–20]. Laboratory tests were performed in an accredited laboratory for soil control, water, fertilizers, and plants. Sample pre-treatment was done in accordance with ISO 11464:2006. Samples were first air-dried and then crushed (ground) and sieved through a 2 mm sieve. The soil samples were prepared by the *aqua regia* extraction method (ISO 11466:1995) using a 3:1 mixture of hydrochloric and nitric acid. The extraction was carried out for two hours on a heated water bath using a reflux condenser. The soil sample (3.0 g) was dissolved in the reflux digestion vessels by adding 21 ml of concentrated hydrochloric acid and 7 ml of concentrated nitric acid. Once the solution started to boil, the temperature was maintained at 180 $^{\circ}$C for two hours. The solution in each vessel was quantitatively transferred to 100 ml volumetric flasks and subsequently analyzed by AAS.

2.3. *Preparation of propolis samples for analysis*

All of the propolis samples were collected during the summer/fall season. The samples were obtained from five Langstroth-type beehives at each apiary from four different areas. They were disposed according to the recommendations given in the "Health certificate of bee colonies and apiary products". Later on, these samples were representative of the particular apiary directly from the hives. They were placed in dark flacon-type containers and kept in a dry clean place until analysis. The collection of propolis was performed using the scraping method. The propolis was obtained from the inner surface of the hive discarding placed in the background, which is usually heavily polluted. The wooden walls and frames were scraped down with a special sharp instrument [21]. The grouping of propolis was closely related to the honeybee pasture in the local area where the propolis was produced and was performed according to previously published work [22]. The samples were pooled, combined in identical quantities (10.0 g), and classified into four different groups, and for the final statistical task the number of samples (N) was 40. One gram of each sample of propolis was weighed with a precision of 0.1 mg in a quartz pot and placed on a heating plate for about 30 minutes. Afterwards, the samples were subjected to another stage of heating in a muffle oven at 500 $^{\circ}$C for three hours. To the obtained ash distilled water (5.0 ml) was added followed by 5.0 ml of 5% HNO_3 (Trace Pure). The obtained material was filtered and diluted with distilled water to 25.0 ml in volumetric flasks.

2.4. *AAS analysis of propolis samples*

The abovementioned prepared samples were placed in a specialized assay cuvette (1.0 ml) to remove the moisturizing effect and were then measured using AAS. Individual standards were used for measuring each element of interest: Trace CERT – lead standard for AAS, 1000 mg l^{-1} ± 4 ml Fluka; cadmium standard for AAS, 1000 mg l^{-1} ± Fluka; zinc standard for AAS, 1000 ml l^{-1} ± Fluka; chromium standard for AAS, 1000 mg l^{-1} ± Fluka; and copper standard for AAS, 1000 mg l^{-1} ± Fluka. The contents of all elements were expressed in milligrams per kilogram.

2.5. *Chemical method for soil analysis*

The physical clay content and mechanical composition of the soil were determined with sodium pyrophosphate by an international method called the "B method" and described by various authors [23, 24]. For this purpose, the analysis was carried out with a 0.4% solution of sodium pyrophosphate; fractionation of mechanical elements was performed according to international classifications [25] and has been performed by others [26]. The humus content was determined by the so-called Tyurin method. This is a modified method where the oxidation of carbon from the humus is performed by using 0.1% potassium dichromate and has been successfully used by other researchers [23, 24]. The total nitrogen content in the soil was determined by a modified micro-Kjeldahl method according to ISO 11261:1995. The soil solution, which was a combination of water and potassium chloride with soil, was subjected to colorimetric treatment and later the pH values were determined using a pH meter (ISO 10390:2005). The carbonate content in the soil was determined volumetrically with a Šajblercalcimeter, according to ISO 10693:1995 (Soil Quality – Determination of Carbonate Content – Volumetric Method). The contents of readily available phosphorus (expressed as P_2O_5) and potassium (expressed as K_2O) were determined and were evaluated with the help of a validated Egner-Riehm-Domingo (A-L) method [27]. More details about the above mentioned parameters are given in Table 1.

2.6. *Statistical analysis*

In order to provide a detailed description, we used computations in which continuous variables are presented as mean values, standard deviations, and medians. The statistical significance of the differences in continuous variables between areas was determined by ANOVA in the case of a normal distribution and the Kruskal-Wallis test if the distribution deviated from the normal. After the ANOVA, the statistically significant difference between the variables of individual locations was subsequently determined with post hoc tests. The level of statistical significance was $p < 0.05$. Statistical analysis was conducted using the software packages SPSS 15.0 and STATISTICA 8.0. Details of the complete statistical analysis were provided in earlier publications by the same author [28, 29].

3. RESULTS AND DISCUSSION

As mentioned before, the first part of the study aimed to determine the contents of the selected heavy metals (Cu, Pb, Cd, Cr and Zn) in the soil in a region at least 5 km from the four selected locations (Novaci, Makovo, Orle and Rapeš) where the bee hives were positioned. As a starting point, the soil samples were subjected to standard chemical tests, and the mechanical composition, texture and chemical properties of the soil were determined (Table 1).

T a b l e 1

Determined soil properties from the four selected locations in the Pelagonia region

Parameters	Area			
	Novaci	Makovo	Orle	Rapeš
pH (H_2O)	neutral soil	neutral soil	neutral soil	neutral soil
pH (KCl)	neutral soil	neutral soil	neutral soil	neutral soil
K_2O	extremely high secure	very high secure	extremely high secure	extremely high secure
P_2O_5	very high secure	very high secure	extremely high secure	extremely high secure
$CaCO_3$	medium carbonate	medium carbonate	medium carbonate	medium carbonate
Mechanical structure	light loam	light loam	light loam	medium loam
Humus	low secure	medium secure	medium secure	very high secure

The mechanical composition, texture, and chemical properties of the soil are presented in Table 1. According to the classification, the soil was mainly light to medium loam. From Table 1, it is evident that the pH of the soil was neutral in all of the examined locations in R. Macedonia. With respect to the behavior of K_2O and P_2O_5, their contents in the examined soil samples varied from ones that are highly secure in lowland areas to extremely highly secure in the highland areas. The analysis of soil composition showed that all soil samples had medium levels of carbonate. The contents of the major elements are most frequently a result of the dominant geological formations in the area. As far as the Pelagonia region is concerned, the dominant geological formations in the area are Quaternary sediments, Precambrian and Paleozoic schists and gneisses, volcanic rocks, and Paleozoic and Mezozoic carbonates [30, 31].

The values of the contents of the selected heavy metals in soils in the investigated areas and the levels of the statistically significant differences between individual regions are given in Table 2. Generally, for all of the locations, the relative concentrations of heavy metals in soil were found to decrease in the following order: Zn > Cr > Cu > Pb > Cd. The highest contents of Cu and Cd were determined in Makovo, and the highest contents of Pb and Cr in the soil were determined for the location of Novaci, while the highest content of Zn was measured in the location of Rapeš.

The determined mean Cu content in soils from the different locations was in the following order: 8.95 mg kg^{-1} (Rapeš), 12.8 mg kg^{-1} (Orle), 15.41 mg kg^{-1} (Novaci), and 26.64 mg kg^{-1} (Makovo). Stafilov and co-workers determined [30, 32] that the mean content of Cu in the Pelagonia region of R. Macedonia was 17 mg kg^{-1}. The determined content of Pb in soil in our work ranged from 8.26 mg kg^{-1} for Orle to 17.15 mg kg^{-1} for Novaci, which is in agreement with the average value of 14 mg kg^{-1} given by Dimovska *et al*. for the Bitola region [32].

T a b l e 2

Concentration of elements Cu, Pb, Cd, Cr and Zn (mg kg^{-1}) in the soil samples from different areas in the Republic of Macedonia

Element	Area			
	Novaci	Makovo	Orle	Rapeš
Cu***	15.41 ± 2.48 c*d*** (16.40)	**26.64** ± 1.52 acd*** (26.85)	12.08 ± 1.85 d** (12.10)	8.95 ± 0.74 (9.05)
Pb***	**17.15** ± 1.17 cd*** (17.55)	14.01 ± 0.84 cd*** (14.15)	11.62 ± 1.02 d*** (11.45)	8.26 ± 0.71 (8.20)
Cd***	0.25 ± 0.05 (0.27)	**0.60** ± 0.09 cd*** (0.58)	0.36 ±0.09 a* (0.36)	0.42 ± 0.05 a*** (0.41)
Cr***	**38.28** ± 0.83 bd***c* (38.45)	34.47 ± 2.14 d*** (33.80)	36.02 ± 1.78 d*** (36.80)	27.67 ± 1.46 (27.80)
Zn***	29.57 ± 2.01 (29.15)	53.26 ± 2.02 ac*** (53.55)	43.52± 1.62 a*** (43.40)	**72.03** ± 1.55 abc** (72.20)

Note. Data are given as means ± SD (medians); * – $p< 0.05$, ** – $p < 0.01$, *** – $p < 0.001$; c – Novaci, d – Makovo, c – Orle, d – Rapeš.

Similarly, it was established that the lowest content of Cr (27.67 mg kg^{-1}) occurred in the village with the highest elevation (Rapeš). The highest content of chromium, 38.28 mg kg^{-1}, was found in Novaci. Both of these findings are within the range of published data [33], where the Cr content varies from 16 to 540 mg kg^{-1} with a median value of 74 mg kg^{-1}. The lowest cadmium content, 0.25 mg kg^{-1}, was found in Novaci, and is very similar to the content for the Pelagonian region reported by Stafilov and Šajn[31], which varied between 0.1 and 21 mg kg^{-1} with a median value of 0.2 mg kg^{-1}.

The highest content of Zn was determined in Rapeš, (72.03 mg kg^{-1}), while the lowest content of Zn was determined in Novaci (29.57 mg kg^{-1}). According to Jordanoska *et al*. [34] and Stafilov *et al*. [33], large differences in Zn content (1.4–780 mg kg^{-1}, median 55 mg kg^{-1}) were found in soils from the Pelagonia region. A detailed study was carried out by Stafilov and co-workers regarding the distribution of zinc in the soil over the Bitola region [32]. They determined that the content of Zn varied between 3.4 and 220 mg kg^{-1} with a median value of 26 mg kg^{-1}. It is important to note that Zn is constantly being transported by nature in a process called natural cycling, where rain, snow, ice, sun and wind erode zinc-containing rocks and soil.

T a b l e 3

Concentrations of Cu, Pb, Cd, Cr and Zn (mg kg^{-1}) in the soil samples from lowland and highland sampling areas in the Bitola region and their corresponding values from the Dutch list

Element	Lowland areas (Novaci and Makovo)	Highland areas (Orle and Rapeš)	Dutch list target value	Dutch list intervention value
Cu***	**21.03** ± 6.10*** (20.65)	10.52 ± 2.11 (9.75)	36	190
Pb**	**15.58** ± 1.89*** (15.05)	9.94 ± 1.92 (9.65)	85	530
Cd***	**0.42** ± 0.19 (0.40)	0.39 ± 0.08 (0.40)	0.8	12
Cr***	**36.38** ± 2.51** (37.05)	31.85 ± 4.57 (31.25)	100	380
Zn***	41.42 ± 12.31 (40.70)	**57.78** ± 14.71** (58.25)	140	720

Note. Data are given as means ± SD (medians); ** – $p < 0.01$, *** – $p < 0.001$.

The values of the concentrations of elements in the soil in lowland and highland areas are given in Table 3. With the exception of Zn, concentrations of all investigated elements in the soil are higher in lowland areas. The concentration of zinc is statistically higher in the mountain areas (58.25 mg kg^{-1}). The conclusion of this portion of the study is that, in general, the concentrations of the studied heavy metals are in agreement with the previous studies and indicate that the soil is not contaminated with heavy metals (Cu, Pb, Cd, Cr, Zn) and is suitable for agricultural and related uses. This is evident from Table 3, where the highest values of concentrations of heavy metals in soil are below the target values from the new Dutch list (http://www.contaminatedland.co.uk/std-guid/dutch-l.htm).

In this work, it was assumed that raw propolis is an ecologically pure product that can be utilized as a bioindicator of environmental pollution by determining the levels of toxic elements accumulated in it [27, 34]. Both soil and propolis were taken from the same four locations in R. Macedonia. The obtained values of the concentrations of the selected heavy metals in propolis from the sampling locations are given in Table 4. All of the samples have relatively low levels of metals that are below the maximum residue limits in accordance with scientific opinions and SCOOP report on heavy metals in food. The highest concentrations were obtained for the area of Makovo (Cd is found in the same concentration and for area Novaci). Except for the concentrations of Cr, the lowest concentrations were found in Orle. According to the results obtained for the copper content, it can be seen that the concentrations determined in samples from lowland and highland areas are 0.027 mg kg^{-1} and 0.019 mg kg^{-1} respectively (the highest concentration of Cu of 0.029 mg kg^{-1}was found in the village of Makovo). In the literature [22, 35], the published concentrations of Cu in raw propolis are higher than those found in this investigation (Cu_{avg} = 8.94 mg kg^{-1} [22]; Cu_{avg} = 7.12 mg kg^{-1}) [35].

T a b l e 4

Concentrations of Cu, Pb, Cd, Cr and Zn (mg kg^{-1}) in propolis samples from different locations in the Republic of Macedonia (N = 40)

Element	Area			
	Novaci	Makovo	Orle	Rapeš
Cu***	0.024 ± 0.006 (0.026)	**0.029** ± 0.005 c***d** (0.028)	0.018 ± 0.005 (0.017)	0.020 ± 0.006 (0.020)
Pb**	0.040 ± 0.006 c* (0.039)	**0.042** ± 0.006 c**d* (0.044)	0.031 ± 0.006 (0.034)	0.034 ± 0.007 (0.036)
Cd***	**0.030** ± 0.003 cd*** (0.029)	**0.030** ± 0.003 cd*** (0.029)	0.013 ± 0.002 (0.013)	0.014 ± 0.002 (0.013)
Cr***	0.037 ± 0.003 cd*** (0.038)	**0.038** ± 0.005 cd*** (0.038)	0.013 ± 0.002 (0.013)	0.012 ± 0.002 (0.012)
Zn***	0.030 ± 0.06 c*d** (0.032)	**0.031** ± 0.06 c**d*** (0.030)	0.021 ± 0.06 (0.021)	0.020 ± 0.05 (0.018)

Note. Data are given as means ± SD (medians); * – $p < 0.05$, ** – $p < 0.01$, *** – $p < 0.001$; c – Orle, d – Rapeš

Furthermore, it is evident from Table 4 that low concentrations of lead were found in propolis samples in all studied areas. The relative concentrations of Pb were found to increase in the following order: 0.031 mg kg^{-1} (Orle) > 0.034 mg kg^{-1} (Rapeš) > 0.040 mg kg^{-1} (Novaci) > 0.042 mg kg^{-1} (Makovo). The results obtained here are in good agreement with the data published by Formicki and Bogdanov [6, 36], who point out that low level of lead (Pb_{min} = 0.06 mg kg^{-1}) was found in the propolis collected in Poland taken from the Raciechowice location. The content of lead in propolis collected in 2013 was determined by Serra–Bonvehi and Bermejo Oranteson [37], and their reported value of 3.80 mg kg^{-1} is higher compared to the value determined in our study.

We have collected some important data about cadmium and its content and reported them in Table 4. The relative concentration of Cd was found to increase in the following order: 0.013 mg kg^{-1} (Orle) > 0.014 mg kg^{-1} (Rapeš) > 0.030 mg kg^{-1} (Makovo) = 0.030 mg kg^{-1} (Novaci). The present values for the concentrations of Cd were significantly lower than the value for Cd content (Cd = 12.5 ± 4.7 mg kg^{-1}) in propolis samples from a different location in Malopolska, Poland, given by some authors [6]. However, in a more recent study carried out by Roman et al., values for Cd concentration in propolis of 0.069–0.802 mg kg^{-1}were reported [38]. This is very similar to the content of Cd in propolis (0.04–0.82 mg kg^{-1}) reported by Stafilov and Kulevanova [39]. In their study, the highest concentration of Cd in propolis (0.82 mg kg^{-1}) was found in a sample from Veles, where a metallurgical facility for lead and zinc was active at the time.

The lowest concentration of Cr (0.012 mg kg^{-1}) in propolis samples was detected in the village of Rapeš, whereas the highest content of Cr was found in Makovo (0.038 mg kg^{-1}). Chromium was determined in Macedonian propolis (except in the Bitola region) and the values were in the range of 1.50–17.3 mg kg^{-1} (Table 6). The closest values for Cr concentration in propolis in the literature were reported by Conti and Botre [4] and ranged from 0.0183 to 0.0703 mg kg^{-1} (Table 7). They determined the content of three heavy metals in honey, wax, pollen and propolis gathered in Rome, Italy. This is an especially relevant publication because they had taken extra measures to avoid metal contamination of the samples.

The values of the concentrations of all heavy metals in propolis in lowland (Novaci and Makovo) and highland (Orle and Rapeš) areas are given in Table 5. It is evident that the concentrations of all of the investigated heavy metals in propolis are higher in lowland areas, especially for Pb (0.041 mg kg^{-1}).

Overall, our results for heavy metal contents in propolis correspond with lower-range concentrations reported in the literature as summarized in Table 6. This outcome may be due to the fact that the sampled and assayed beehive material (i.e. freshly produced propolis) contained a higher percentage of water than more seasoned propolis, thus leading to generally lower concentrations of heavy metals. Additionally, the collection and analysis of samples referred to freshly produced material, which limited the study to a period of maximal honeybee activity and therefore did not provide an opportunity to investigate the long-term accumulation of heavy metals in the different matrices.

T a b l e 5

Concentrations of Cu, Pb, Cd, Cr and Zn (mg kg^{-1}) in propolis samples from lowland and highland areas of the Republic of Macedonia (N = 40)

Element	Lowland areas (Novaci and Makovo)	Highland areas (Orle and Rapeš)
Cu	**0.027** ± 0.006 *** (0.027)	0.019 ± 0.005 (0.018)
Pb	**0.041** ± 0.006 *** (0.043)	0.033 ± 0.006 (0.035)
Cd	**0.030** ± 0.003 *** (0.029)	0.013 ± 0.002 (0.013)
Cr	**0.038** ± 0.004 *** (0.038)	0.012 ± 0.002 (0.013)
Zn	**0.030** ± 0.06 *** (0.031)	0.020 ± 0.06 (0.019)

Note. Data are given as means ± SD (medians);
*** – $p < 0.001$.

T a b l e 6

Concentrations of Cu, Pb, Cd, Cr and Zn (mg kg^{-1}) in propolis samples from different locations in Europe

Element	Area			
	Macedonia [39]	Poland [38]	Spain [37]	Croatia [40]
Cu	0.08 – 67.9	1.09 – 18.32	2.1 – 4	0.3 – 6
Pb	1.30 – 19.5	0.39 – 18.29	0.07 – 4	0.3 – 64
Cd	0.04 – 0.82	0.006 – 0.811	n.r.	n.r.
Cr	1.50 – 17.3	n.r.	0.3 – 3	0 – 1
Zn	n.r.	10.91 – 115.22	163 – 1236	8 – 933

n.r.–not reported

T a b l e 7

Concentrations of Cu, Pb, Cd, Cr and Zn (mg kg^{-1}) in honey, pollen propolis and wax samples from Rome, Italy [4]

Element	Area			
	(Conti, 2001) Honey	(Conti, 2001) Pollen	(Conti, 2001) Propolis	(Conti, 2001) Wax
Cu	n.r.	n.r.	n.r.	n.r.
Pb	0.033 – 0.045	0.020 – 0.335	0.0106 – 0.0432	0.0566 – 0.206
Cd	0.020 – 0.063	0.015 – 0.0901	0.0062 – 0.00659	0.015 – 0.052
Cr	0.084 – 0.102	0.030 – 0.112	0.0183 – 0.0703	0.005 – 0.012
Zn	n.r.	n.r	n.r.	n.r.

n.r.–not reported

Our relatively low values for the selected heavy metals are comparable to the values for Pb, Cd and Cr obtained by Conti and Botre [4]. Apart from their study, there are not many details in the available literature about the detailed procedures of selection of geographic area, duration and time-frame of sampling, construction of beehives (preferably from wood), or proper sample collection in an effort to avoid any kind of metal contamination. It is well known that the acidic nature of bee products (honey, propolis, wax, etc.) may enhance the corrosion of beekeeping tools made of galvanized steel, aluminum, and brass, as well as containers used for storage and shipment. Storing bee products in galvanized containers can be source of Zn contamination, iron/steel based parts can be a source of Cr, and brass components can be a source of Zn and Cu [14, 15].

If one takes the maximum permitted levels set by the Macedonian legislation for some heavy metals in honey (0.03 mg $kg^{-1}$for Cd, 1 mg $kg^{-1}$for Cu, 20 mg kg^{-1} for Fe, and 10 mg $kg^{-1}$for Zn), then it can be concluded that all of the samples meet the criteria. It should be noted that the samples from the lowland locations (Novaci and Makovo) have a borderline concentration of Cd of 0.030 mg kg^{-1}.

As a next step, in order to find out whether there is a connection between the content of (certain) heavy metals present in soil and in propolis from the same location, statistical treatment was carried out using the gathered data for the concentrations of the selected heavy metals in soil and propolis. The obtained results are given in Table 8along with correlation coefficients, standard errors of estimates, mean absolute errors and Durbin-Watson statistics. The correlation coefficient of 0.924 for the mean concentration of copper in soil and propolis indicates that there is a relatively strong relationship between the variables. In the case of Pb, the correlation coefficient is equal to 0.688, indicating a moderately strong relationship between the variables. Based on the same statistical parameters, there is also a moderately strong relationship between the variables for Cr and Zn. In the case of Cd, the correlation coefficient is 0.145, indicating a relatively weak relationship between the variables. The finding for Cd is quite peculiar because its content compared to the other investigated metals in the soil is the lowest (about 20–100 times lower than the other heavy metals); however in the propolis samples its concentration is almost the same as the Cu, Pb and Cr concentrations. Compared to these three metals there seems to be a concentration of Cd from soil to propolis. Since there is no metallurgical plant in the vicinity of the locations, the most logical pathway seems to be from the soil to plants. It is essential to analyze Cd in propolis and other bee products because of its toxicity as well as the fact that Cd can be transported through the root system into the nectar of the plants [13].

Table 8

Correlation between the mean concentrations of Cu, Pb, Cd, Cr and Zn in soil samples And propolis samples determined in this work

Element	Cu	Pb	Cd	Cr	Zn
Correlation coefficient	0.924	0.688	0.145	0.583	–0.535
Standard error of est.	3.6	3.3	0. 2	4.6	18.5
Mean absolute error	2.5	2.3	0.1	3.0	13.1
Durbin-Watson statistic	2.1	2.3	2.7	2.9	3.0
	(P = 0.77)	(P = 0.87)	(P = 0.70)	(P = 0.88)	(P = 0.92)

These results must be analyzed and used with caution because the accumulation of heavy metals in propolis is a complex process and this is just the first step towards pinpointing the relevant factors. Many factors influence the heavy metal contents of propolis, such as the geographical and botanical origin, soil, atmosphere, beekeeping equipment and practices, elemental composition of nectar, season of the year, rainfall, and anthropogenic activities, among others. Our present results indicate that the concentrations of the selected heavy metals in the samples of propolis and soil samples from the same areas generally do not indicate contamination and are well below the limits specified by standard regulations.

4. CONCLUSIONS

In this study, the contents of five representative heavy metals (Cr, Pb, Zn, Cd and Cu) were determined in soil and propolis samples from four locations in southwestern Macedonia in the vicinity of the city of Bitola. Generally, for all of the locations, the relative concentrations of heavy metals in soil were found to decrease in the following order: Zn>Cr>Cu>Pb>Cd, and the highest values of the concentrations of heavy metals in these soils are below the target values from the new Dutch list. The general trend of the heavy metal contents in propolis from the same four locations, in decreasing order, is Pb>Cr>Zn>Cu≈Cd. Generally, the propolis samples from the highland locations (Orle and Rapeš) had lower overall contents of heavy metals than those from the lowland locations (Novaci and Makovo). All of the analyzed propolis samples meet the requirements of the Macedonian legislation and the international organization for the maximum allowed levels of heavy metals. According to our aim, the investigation presented herein provides one step towards a complete picture of the safety of the specific areas in R. Macedonia, which is necessary for further ecomonitoring. Further studies are needed to complete the picture and to determine the major pathways of incorporation of heavy metals in beehive products.

Acknowledgments. The study was supported by the Ministry of Science and Technological Development of the Republic of Serbia (project no. III41017). The authors would like to express their gratitude to Vesna Prodanovska Poposka for her help in the preparation of the manuscript.

REFERENCES

[1] J. H. Duffus, Heavy metals a meaningless term, *Pure Appl. Chem.*, **74**, 793–807 (2002).

[2] J. O. Duruibe, M. O. C. Ogwuegbu, J. N. Egwurugwu, Heavy metal pollution and human bio toxic effects, *Int. J. Physical Sci*, **2**(5), 112–118 (2007).

[3] L. Järup, Hazards of heavy metal contamination,Department of Epidemiology and Public Health, *Br. Med. Bull.*, **68**, 167–182 (2003).

[4] M. E. Conti, F. Botre, Honeybees and their products as potential bioindicators of heavy metals contamination, *Environ. Monit. Assess.*, **69**(3), 267–282 (2001).

[5] A. Roman, Concentration of chosen trace elements of toxic properties in bee pollen loads, *Pol. J. Environ. Stud.*, **18**(2), 265–272 (2009).

[6] G. Formicki, A. Gren, R. Stawaez, B. Zysk, A. Gal, Metal content in honey, propolis, wax, and bee pollen and implications for metal pollution monitoring, *Pol. J. Environ. Stud.*, **22**(1), 99–106 (2013).

[7] K. Salomao, A. P. Dantas, C. M. Borba, L. C. Campos, D. G. Machado, N. F. R. Aquino, S. L. de Castro, Chemical composition and microbicidal activity of extracts from Brazilian and Bulgarian propolis, *Lett. Appl. Microbiol.*, **38**, 87–92 (2004).

[8] E. L. Ghisalberti, Propolis: A Review, *Bee World*, **60**(2), 59–84 (1979).

[9] V. S. Bankova, S. L. de Castro, M. C. Marcucci, Propolis: recent advances in chemistry and plant origin, *Apidologie*, **31**(1), 3–15 (2000).

[10] A. G. Hegazi, F. K. A. Hady, Egyptian propolis. 3:Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands, *Zeitschrift für Naturforschung C*, **57**, 395–402 (2002).

[11] A. F. N. Ramos, J. L. Miranda, Propolis: A review of its anti-inflammatory and healing actions, *J. Venom. Anim. Tox. Incl. Trop. Dis.*, **13**(4), 697–710 (2007).

[12] B. Truseva, I. Todorov, M. Ninova, H. Najdenski, A. Daneshmand, V. Bankova, Antibacterial mono- and sesquiterpene esters of benzoic acids from Iranian propolis, *Chem. Central J.*, **4**, 8–16 (2010).

[13] E. Stankovska, T. Stafilov, R. Šajn, Monitoring of trace elements in honey from the Republic of Macedonia by atomic absorption spectrometry, *Environ. Monit. Assess.*, **142**(1–3), 117–126 (2008).

[14] S. Silici, O. D. Uluozlu, M. Tuzen, M. Soylak, Assessment of trace element levels in Rhododendron honeys of Black Sea Region, Turkey, *J. Hazard. Mater.*, **156**, 612–618 (2008).

[15] P. Pohl, I. Sergiel, Direct analysis of dark honeys for total content of copper and its fractionation forms, *Food Anal. Methods*, **5**(1), 113–118 (2012).

[16] T. Pehlivan, G. Aziz, Determination of Heavy Metals Contents of Some Monofloral Honey Produced in Turkey, *J. Appl. Pharmac. Sci.*, **5**(8), 042–045 (2015).

[17] EN 14084:2003: Foodstuffs – Determination of trace elements - Determination of lead, cadmium, zinc, copper and iron by atomic absorption spectrometry (AAS) after microwave digestion; (AAiCE-Methods-Manual-V5 2008).

[18] B. Balabanova, T. Stafilov, K. Baceva, Biomonitoring of atmospheric pollution with heavy metals in the copper mine vicinity located near Radoviš, Republic of Macedonia, *J. Environ. Sci. Health*, Part A, **45**(12), 1504–1518 (2010).

[19] A. Mbiri, A. Onditi, N. Oyaro, E. Murago, Determination of essential and heavy metals in Kenyan honey by atomic absorption and emission spectroscopy, *J. Agric. Sci. Techn*, **13**(1), 107–115 (2011).

[20] L. Barandovski, M. V. Frontasyeva, T. Stafilov, R. Šajn, S. Pavlov, V. Enimiteva, Trends of atmospheric deposition of trace elements in Macedonia studied by the moss biomonitoring technique, *J. Environ. Sci. Health, Pt. A: Environ. Sci. Eng. Toxic Hazard. Subst. Control*, **47**(13), 2000–2015 (2012).

[21] A. F. Raed, K. N. Ibrahim, M. D. Rula, A. Z. Mosa, Honey bee hive modification for propolis collection, *Jordan J. Agric. Sci.*, **4**(2), 138–147 (2008).

[22] A. Roman, E. Popiela, Z. T. Dobrzański: The propolis as a bioindicator of environmental heavy metals pollution. In: *Proceedings of the XV International Congress of the International Society for Animal Hygiene*. Vienna, Austria, 2011.

[23] S. D. Orlov, A. L. Grishina: *Practicum on Chemistry on Humus*. Moscow University, Moscow, USSR, 1981.

[24] D. Dilek, A. Aksoy, Heavy metals in vegetables in Turkey are within safe limits for Cu, Zn, Ni and exceeded for Cd and Pb, *J. Food Qual.*, **29**, 252–265 (2006).

[25] G. Filipovski, S. Pradhan, *Moisture retention characteristics in the soils of the Socialist Republic of Macedonia*, Macedonian Academy of Sciences and Arts, Skopje, 87, 1980.

[26] H. Resulović: *Methods of investigation of physical properties of soils*. The Yugoslav Society of Soil Science, Belgrade, Yugoslavia, 1971.

[27] V. Pelivanoska, B. Jordanoska, T. Mitkova, M. Markovski: Heavy metal contents in soil and oriental tobacco manufactured in Republic of Macedonia. In: *Proceedings of the International Conference 100 years Bulgarian Soil Science*, 2011.

[28] P. B. Bogdanova, K. V. Hristova, M. A. Ayaz, S. Najman, Monitoring of heavy metals and trace elements contamination in the soil and vegetables and air pollution in Republic of Macedonia, *Int. J. Enhan. Res. Sci. Techn. Eng. (IJERSTE)*, **3**(1), 205–214 (2014).

[29] B. P. Bogdanova, S. Najman, K. V. Hristova, M. A. Ayaz, Inductively coupled plasma–optical emission spectroscopy (ICP-OES) approach for determination of heavy metals, metalloid and trace element in soil and vegetables, *Indian Hortic. J.*, **4**(2), 98–104 (2014).

[30] A. Puteska, B. Dimovska, T. Stafilov, R. Šajn, Distribution of chemical elements in soil samples from the Pelagonia Region, Republic of Macedonia, *Geol. Croat.*, **68**(3), 261–272 (2015).

[31] T. Stafilov, R. Šajn, *Geochemical Atlas of Macedonia*, Faculty of Natural Sciences and Mathematics, Skopje, Macedonia, 2016.

[32] B. Dimovska, T. Stafilov, R. Šajn, K. Bačeva, Distribution of lead and zinc in soil over the Bitola Region, Republic of Macedonia, *Geol. Maced.*, **28**(1), 87–91 (2014).

[33] T. Stafilov, R. Šajn, A. Puteska, B. Dimovska, *Geochemical Atlas of Pelagonia*, Faculty of Natural Sciences and Mathematics, Skopje, Macedonia, 2015.

[34] B. Jordanoska, T. Stafilov, V. Pelivanovska, K. Bacheva, Assessment of the content of chemical elements in soil and its properties used for tobacco cultivation in the Republic of Macedonia, *Bulg. J. Agric. Sci.*, **20**(2), 255–266 (2014).

[35] C. Porini, G. S. Anna, G. Stefano, S. Ghini, P. Medrzycki, F. Grillenzoni, L. Bortolotti, E. Gattavecchia, G. Celli, Honey bees and bee products as monitors of the environmental contamination, *Apiacta*, **38**, 63–70 (2003).

[36] S. Bogdanov, Contaminants of bee products, *Apidologie*, **37**, 1-18 (2006).

[37] J. S. Bonvehi, F. J. O. Bermejo, Element content of propolis collected from different areas of South Spain, *Environ. Monit. Assess.*, **185**(7), 6035–6047 (2013).

[38] A. Roman, B. Madras-Majewska, E. Popiela-Pleban, Comparative study of selected toxic elements in propolis and honey, *J. Apic. Sci.*, **55**(2), 97–106 (2011).

[39] T. Stafilov, S. Kulevanova, Determination of some trace elements in propolis by atomic absorption spectrometry, *Anal. Lab.*, **3**, 270–274 (1994).

[40] J. Cvek, M. Medić-Šarić, D. Vitali, I. Vedrina-Dragojević, Z. Smit, S. Tomić, The content of essential and toxic elements in Croatian propolis samples and their tinctures, *J. Apic. Res.*, **47**(1), 35–45 (2008).