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Original Article

QUANTIFICATION OF SELECTED TRACE AND MINERAL ELEMENTS IN ROYAL JELLY FROM BULGARIA BY ICP-OES AND ETAAS

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Abstract

The objective of the present study was to investigate selected trace and mineral elements in Royal Jelly (RJ) from Bulgaria. A total of 30 RJ samples were included in the study. The analytical procedure consisted of the microwave digestion of the RJ samples with nitric acid followed by instrumental measurement. Concentrations of Al, Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P, Sr and Zn were determined using inductively coupled plasma optical emission spectroscopy (ICP-OES), while As, Cd, Co, Ni and Pb were determined by electrothermal atomic absorption spectrometry (ETAAS). Our results showed that elements K, Mg, Ca represented 96% from the total mineral content of the RJ samples from Bulgaria, while the most abundant trace element was Na, followed by Zn. The elements Ba, Cr, Cu, Fe, Mn and Sr were found in trace concentration levels and elements As, Pb, Cd, Co and Ni in microconcentration levels. Selected mineral and trace elements were found in relatively constant concentration levels in all of the analyzed RJ samples. It was concluded that chemical element content did not depend on geographical origin and was under homeostatic adjustment in RJs.

Keywords: Bulgaria, ETAAS, ICP-OES, mineral elements, royal jelly, trace elements

INTRODUCTION

Royal jelly (RJ) is a product secreted from the hypopharyngeal and mandibular glands of young worker honeybees (*Apis mellifera*) and serves as food for the bee queen and the larvae up to three days old. The composition of RJ has been studied by several authors, but data available in the literature are inadequate and unstructured with considerable variety. This is probably due to the variability of the product itself and to the different methods used for its analysis. RJ is mainly composed of water (60–70%), proteins (9–18%), carbohydrates (7–18%), lipids (3–8%), amino acids, vitamins and minerals. In fresh RJ, the mineral content varies between 0.8% and 3% (Sabatini et al., 2009). Royal jelly typically is contaminated by pollen grains (Ricciardelli d'Albore & Battaglini Bernardini, 1978; Piana et al., 2006).
Royal Jelly is a natural food product widely used in diets and cosmetics. This elemental analysis of

RJ is of great interest from a nutritional and toxicological point of view and requires food quality control (Chen & Chen, 1995). The biological activities of RJs are variable and have been correlated to the content of trace elements. Researchers believe that the health benefits are attributed to such various ingredients present in RJs as B-complex vitamins, fatty acids and trace elements (Kim & Lee, 2010). While being a natural source of essential elements for consumers, RJ could also contain As, Cd, Cr, Ni and Pb which are harmful to human health. These elements may exist in RJ as free metal ions, as small labile complexes or as complexes with RJ proteins and other macromolecules. Previous studies by Serra Bonhevi (1991) and Nation & Robinson (1971) on the content of trace and mineral elements in RJ were very limited. In a study on the mineral content by Stocker et al. (2005) was determined the concentrations of 28 trace elements in acid digested RJ samples from various botanical, geographical and geological origins. At present there

is an international standard which specifies the production and sanitary requirements for royal jelly and establishes a series of organoleptic and chemical test methods to control its quality (ISO/DIS 12824:2016), there is no information about trace and mineral element content.

The elemental analysis of RJs from different geographical regions is important both to define the content of essential elements, which ensures health benefits as well as to confirm the potential absence of toxic elements, which could adversely affect humans. Results from systematic investigations on mineral content of royal jellies from Bulgaria have not been presented and published until now.

The purpose of the present study was the quantification of major and trace chemical elements in samples of RJ with a geographical indication from Bulgaria. The analyses were focused on the determination of such essential elements as Ca, Co, Fe, K, Mg, Na, P and Zn and such toxic elements as As, Cd, Ni and Pb. Results obtained will be discussed in based on the suggestion that RJ is a form of insect lactation level and shows homeostatic control of chemical element content.

MATERIAL AND METHODS

Samples

The present study was conducted using thirty representative RJ samples with guaranteed origin. Most of the samples were collected from private beekeepers from different regions of Bulgaria: ten samples from the western region around the city of Sofia, seven samples from the eastern Black sea region around the city of Varna and seven samples from the mountain region around city of Lovech. Six other samples came from the market but their geographical origin was known to be from northern Bulgaria: three RJ samples from the region of the city of Veliko Tarnovo and three RJ samples from the region of city of Razgrad (see Fig. 1). The amount of each RJ sample was around 20 g. All samples were collected using the artificial cups method according to Grout (1992). The samples were stored in dark glass containers and kept at −20 °C until analysis. All measurements were performed in 2014.

Fig. 1. Map of RJ sampling localities in Bulgaria.

Reagents

All reagents were of analytical-reagent grade and allaqueous solutions were prepared with MilliQ water (MilliporeCorp., Milford, MA, USA). The stock standard solutions were Certipur® ICP Multi-element standard solution IV (23 elements in diluted nitric acid), Merck Millipore, 1000 mg/L and Certipur® Reference material GF AAS Multi Element Standard solution XVIII, Merck Millipore, 100 mg/L. The working standard solutions were prepared weekly through appropriate dilutions. Concentrated HNO_3 (65%, suprapure, Merck) was used for RJ wet digestion. A stock standard solution of Pd 250 µg/mL in 5% v/v HCl was injected (10 µL) into the graphite furnace to obtain permanent modifier coatings.

Procedures for sample preparation

An accurately weighed RJ sample (0.5 g) was placed in PTFE vessels of a microwave digestion system and 5 mL concentrated HNO_3 was added. The vessels were capped closed, tightened and placed in the rotor of a microwave oven. The digestion was carried out following the program – 200W/5min; 0W/5min; 500W/5min. The vessels were cooled down to the room temperature. Samples were quantitatively transferred into calibrated volumetric flasks and made up to 25 mL with MilliQ water. Blank samples were passed through the whole procedure.

Instruments

Inductively coupled plasma optical emission spectrometry (ICP-OES) was used for the determination of Al, Ba, Ca, Cr, Cu, Fe, K, Mg, Mn, Na, P, Sr and Zn in acid digested RJ samples. The ICP-OES measurements were carried out with inductively coupled plasma optical emission spectrometer (ICP-OES) (Jobin Yvon, Ultima 2). The optimized instrumental parameters are presented in Tab. S1 (Supplementary materials). Calibration against standard calibration curve prepared with aqueous standard solution is recommended.

Electrothermal atomic absorption spectrometry (ETAAS) was used for the determination of toxic element (As, Cd, Co, Ni and Pb) content in RJ samples without preliminary sample digestion. Electrothermal atomic absorption measurements were carried out on a Perkin-Elmer (Norwalk, CT, USA) Zeeman 3030 spectrometer with an HGA-600 graphite furnace. The light sources were hollow cathode lamps for Co, Ni and Pb and an electrodeless discharge lamp for As and Cd. The spectral bandpass for all analytes was 0.7 nm. Pyrolytic graphite-coated tubes with pyrocoated platforms were used as atomizers. Sample aliquots of 20 µL were injected into the graphite furnace using autosampler AS-70. All measurements were carried out with at least three replicates and values measured were based on integrated absorbance. Pretreatment and atomization curves, prepared for Cd and Pb, showed that ETAAS measurement of Pb was possible without a modifier with the use of loss-free pretreatment at 450°C. ETAAS measurement of As and Cd were performed in the presence of 10 µL 250 µg/mL Pd solution with the use of 900°C (As) and 600°C (Cd) as loss-free pretreatment temperatures. Optimal instrumental parameters for direct ETAAS measurements of As, Cd, Ni and Pb are summarized in Tab. S2 and Tab.S3 (see Supplementary material). Matrix interferences evaluated through the ratio of the slopes of calibration curves obtained in the presence of 2% (w/v) royal jelly solution in 0.1 mol/L HNO_3 and for aqueous standards calls for standard addition method for calibration.

Statistical analysis

Each sample was analyzed in triplicates and a calculated mean was used for statistical treatment. Univariate analysis, which involves description of the distribution of each element in sample set as central tendency (including the mean, median, and mode) and results dispersion as the range and quartiles of the data-set as well as measures of spread such as the variance and standard deviation) was performed. The shape of the distribution was defined via such indices as skewness and kurtosis. Characteristics of a variable's distribution were depicted in graphical form through box plotes. Data was performed using the SPSS Statistical Package, version 21 for Windows (IBM Corp.).

Table 1

Concentrations of mineral elements (mg/kg) in RJ samples (three parallel analyses of each sample) from Bulgaria: calculated values for central tendencies represented as mean and median; dispersion of results represented as standard deviation; median relative standard deviation; minimal and maximal values; kurtosis as characteristic of extreme deviations from the mean value; and skewness as characteristic of population asymmetry

Element,	Mean	Median	Standard deviation, SD	RSD*, %	Min value	Max value	Kurtosis	Skewness
Al	3.0	2.75	1.37	46	1.67	7.9	1.16	0.2
As	0.025	0.023	0.014	56	0.011	0.074	1.52	1.64
Ba	0.36	0.33	0.13	56	0.17	0.60	-1.38	0.12
Ca	153	156	9	6	139	163	-1.15	-0.49
Cd	0.003	0.003	0.002	67	0.001	0.006	0.56	1.28
Co	0.007	0.007	0.002	29	0.003	0.010	0.204	0.65
Cr	0.68	0.42	0.62	91	0.20	2.3	-1.59	0.63
Cu	4.4	4.4	0.3	6	4.0	4.9	-1.54	0.12
Fe	17	17	2.5	15	12	21	-0.84	0.069
K	2031	2015	48	2	1978	2102	-1.6	0.41
Mg	259	262	11	4	241	273	-0.82	-0.43
Mn	1.12	1.26	0.44	39	0.34	1.69	-1.25	-0.18
Mo	0.14	0.14	0.02	14	0.10	0.16	-1.65	-0.89
Na	95	95	2	2	91	98	-0.38	-0.35
Ni	0.068	0.069	0.017	25	0.035	0.094	-0.77	0.46
P	2009	2007	39	2	1927	2096	-1.63	-0.06
Pb	0.15	0.07	0.28	187	0.02	0.98	3.53	1.87
Se	0.41	0.48	0.16	39	0.04	0.53	-1.89	-0.76
Sr	0.36	0.35	0.14	39	0.19	0.62	-1.19	0.13
V	0.013	0.013	0.005	38	0.007	0.029	-1.79	-0.56
Zn	21	21	2	10	19	29	1.07	0.98

*Relative standard deviation, represented as Mean/SD*100

Results

The results obtained for all studied elements from the analysis of 30 RJs are summarized in Tab. 1 with the use of descriptive statistics.

The most abundant elements in all the RJ samples were K, P, Mg, Ca and Na with average concentrations of 2031 mg/kg, 2009 mg/kg, 259 mg/kg, 153 mg/kg, and 95 mg/kg, respectively. Moreover, their concentrations in all studied RJ samples were quite similar and very close to the mean value (Relative Standard Deviation, RSD is between 1 and 5 %). The skewness values were fairly symmetrically distributed from -0.35 (Na) to 0.41 (K). The percentage distribution of major elements as well as box plots diagrams for Ca, Ma and Na are depicted at Fig. 2 and Fig. 3 respectively.

The other elements were presented at lower concentration levels as trace elements. The RSD values for essential elements Cu, Fe, Mo and Zn were below 15% with the skewness values were moderately skewed below 1. The concentration levels of toxic elements As, Cd, Ni and Pb broadly varied, although for all RJs they were below permissible limits for honey. The data are quite asymmetrically distributed and
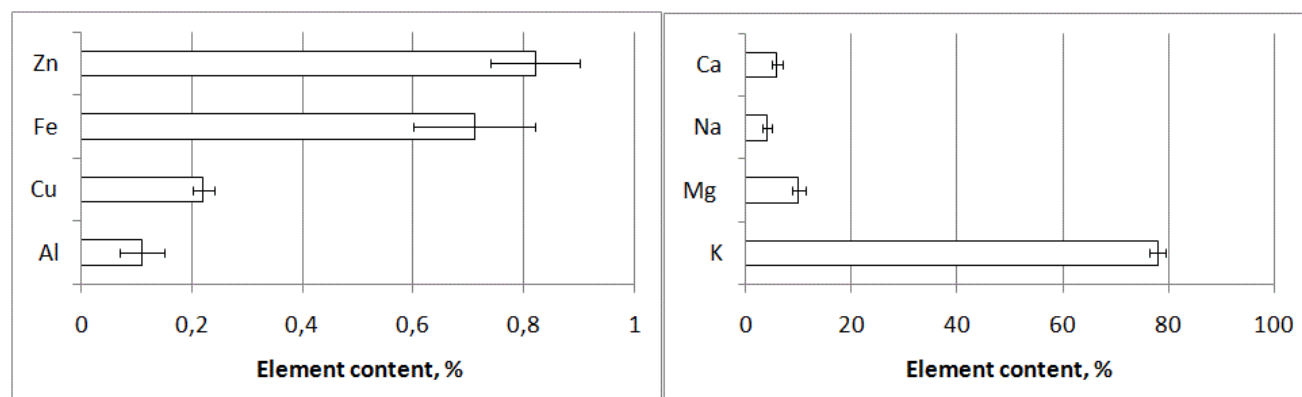
Fig. 2. Major element content (%) in RJ samples from Bulgaria.

heavily tailored, with extreme deviation from the mean, which means the serious influence of external factors. Evidently the content of these elements is not under homeostatic control.

DISCUSSION

Regardless of origin, potassium (K) occurred at the highest concentration level and represented 78% of the total mineral content determined in the samples (Fig. 2). Magnesium (Mg) was the second largest with respect to content level (10%) followed by Ca (6%) and Na (3.7%). The elements Zn and Fe were determined as less than 1%. Potassium is known to be the most abundant element in honey and bee pollen which are precursors for RJ (Stocker et al.,

2005; Terrab et al., 2005; Szczęsna, 2007) and was also found at very high concentrations of 11 180 mg/kg in bee tissue (Kump, Necemer, & Šnajder, 1996). However, K levels in all these matrices widely varied depending on the geographical region. In addition, the levels in honey and pollens depended on their botanical origin but the ones in all studied RJs from the present study were almost constant and only slightly varied, independently from geographical region or botanical origin which is consistent with conclusions by Stocker et al. (2005).

The same conclusion was valid for P content in studied RJ samples, whose values ranged from 1927 mg/kg to 2096 mg/kg (Tab. 1) independently from geographical origin or botanical origin. It was confirmed in the present study that concentrations of essential elements K, Na, P, Ca, Mg which are important for bee-larvae growth were almost constant and evidently under homeostatic control. This conclusion is visualized in Fig. 3 representing box plots diagrams for Ca, Mg and Na.

Considering the almost constant concentration levels of elements K, Na, Ca and Mg, the average ratios K/Na and Mg/Ca, characterizing Bulgarian RJs, are calculated. The average K/Na ratios varied between 20.6 and 22.8 and were very close to the ratios reported by Stocker et al. (2005). The Mg/Ca ratios had a relatively small range – from 1.5 to 1.9 and are a bit lower than these declared by Stocker et al. (2005). This decrease in Mg/Ca ratios could be explained with how soil properties
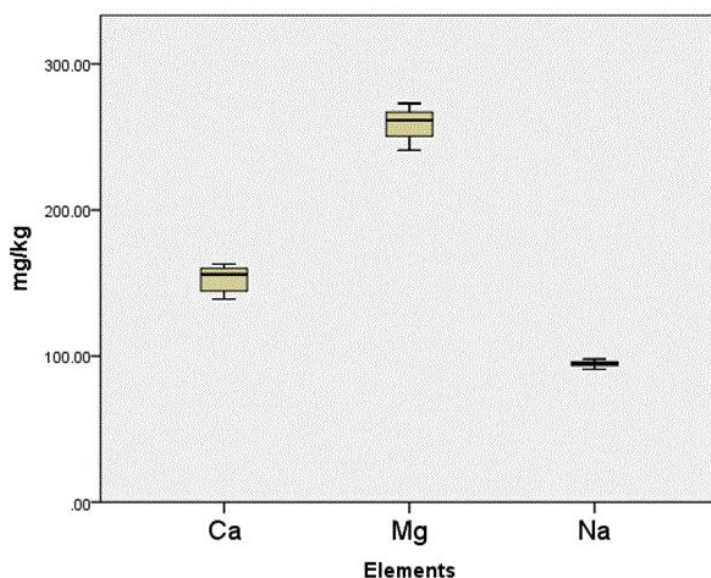

Fig. 3. Box plot diagram of the elements Ca, Mg and Na in RJ samples from Bulgaria. For each element minimum, maximum and median values are shown.
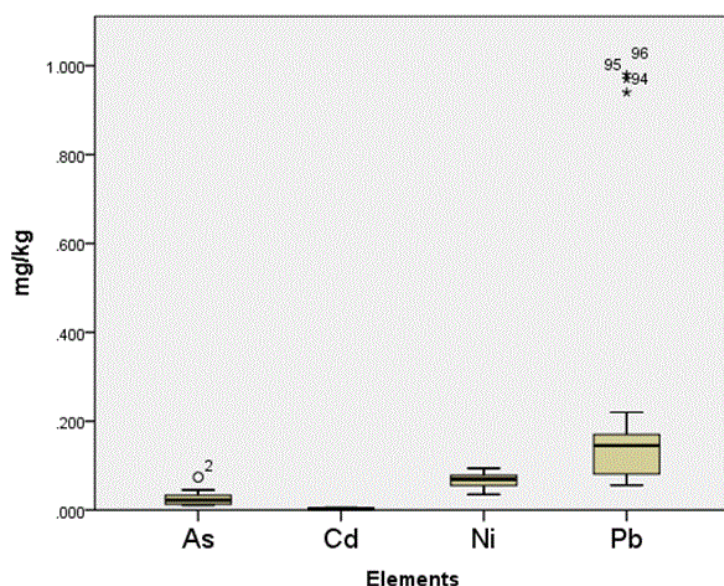
Fig. 4. Box plot diagram of the elements As, Cd, Ni and Pb in RJ samples from Bulgaria. For each element minimum, maximum and median values are shown.

influence the bioavailability of both elements to plants in Bulgaria. Stocker et al. (2005) stated that K/Na ratios were more dependent on seasonal and geographical variation than the Mg/Ca ratios.

The elemental analysis of different RJs from Bulgaria did not show a strong correlation between mineral elements' concentration and respective geographical region. Ivanov & Chervenakova (1985) from Bulgaria found higher average values for K and Zn and lower values for Ca, Fe and Cu in comparison to values reported by Serra Bonhevi, Spain (1991). The differences between these elements were not more than 5.5 µg/g with the exception of K. Mureşan et al. (2016) reported similar results to ours for Ca, Fe and Cu concentrations in RJ, and again K, Mg and Ca were the predominant mineral elements.

The results obtained for other trace elements Al, Ba, Co, Cr, Cu, Fe, Mn, Sr and Zn found in RJ samples showed a wider range of variations (see Tab. S1, Fig. S1 in Supplementary material). The wide range in the content of essential elements Fe, Cu and Zn suggested stronger correlation with the geographical origin of RJ. The variations in the concentrations of elements Al, Mn, Cr, Sr was due the result of exogenous contamination with dust aerosol as reported by Stocker et al. (2005). Evidently the content of trace elements

was affected by the seasonal or geographical origin of the samples from one side and from plants physiology from other side. However, the variations in the concentrations of these elements in royal jelly were much lower than those observed for the associated honey. Results obtained in the frame of this study did not show strong a correlation between essential elements' concentrations and respective geographical region.

The potential toxic elements As, Cd, Ni and Pb could reach honey, pollen, propolis and wax through the air, water, plants and soil and be transported into the bee hive by the bees themselves. Kump et al. (1996) reported that bee tissue was more sensitive to such pollutants as Pb than bee honey. The elements Pb, Cd, Co and Ni have been widely studied as concentration levels in honey samples from different botanical and geographical origin (Chudzinska & Baralkiewicz, 2010; Tuzen et al., 2007; Madejczyk & Baralkiewicz, 2008; Vanhanen, Emmertz, & Savage, 2011), and honey has been confirmed to be an indicator for the degree of anthropogenic impact on the environment. Because honey and pollen are the precursor substances for RJ production, toxic element content in RJ should also be relevant to their environmental concentrations. Results obtained in the present study undoubtedly showed almost equal and quite low content of toxic elements for all studied RJs. Fig. 4 presents the box plot diagram of the variations of these elements. Since all RJ samples were from unpolluted regions, the wide concentration ranges could be considered as a result of external contamination although at very low levels.

Up to now, international Maximum Residue Limit (MRL) levels have never been set for toxic elements content in RJ, but values of 0.1 mg/kg for Cd and 1 mg/kg for Pb in honey have been suggested for the EU (Bogdanov et al., 2007). The MRL levels for Pb and Cd in bee pollen are 0.5 mg/kg and 0.03 mg/kg, respectively. (Campos et al., 2008). The average values for Pb and Cd in all studied Bulgarian RJs are lower than the

above mentioned MRL.

The low toxic element content in RJs obtained in the present study might be explained with the "filtering" properties of the hypopharyngeal and mandibular glands of young worker honeybees. For this reason, RJ is the bee product which is least influenced by such contaminants as toxic elements.

Though the number of samples analyzed in this preliminary investigation was small, the results for the elemental composition of RJs were promising. The combination of ETAAS and ICP-OES measurements is suitable as a useful tool for the accurate quantification of trace and mineral elements in RJ samples. Insignificant differences were found for chemical element content in RJs independent of their geographical and botanical origin. These results confirmed once more the idea that trace and mineral element concentrations in RJ are under homeostatic control and RJ might be accepted as a form of lactation at the insect level.

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Quantification of Selected Trace and Mineral Elements in Royal Jelly from Bulgaria by ICP-OES and ETAAS

Elemental Composition of Bulgarian Royal Jelly

Supplementary material

Table S1

Optimized instrumental parameters for ICP-OES measurements

Parameter	
Generator power	1100 W
Plasma gas flow rate	13 L min^{-1}
Sheath gas flow rate	0.2 L min^{-1}
Nebulizer	0.8 L min^{-1} at 3 bars
Sample uptake	0.8 mL min^{-1}
Type of nebulizer	Glass concentric
Type of spray chamber	Glass cyclonic
Argon humidifier	No
Wavelengths, nm	Al 396.153; Ba 455.403; Ca 422.673; Cr 284.325; Cu 324.754; Fe 259.940, K 766.490; Mg 279.806; Mn 257.610; Na 589.59; P 177.495; Sr 421. 552; Zn 213.856

Table S2

The optimized HGA-600 temperature program for ETAAS measurements

Parameter	Drying	Pyrolysis	Atomization	Cleaning
Temperature (°C)	130	Varies*	Varies*	Varies*
Ramp time (s)	10	20	0	1
Hold time (s)	10	60	5	2
Reading			On	
Ar flow mL min^{-1}	300	300	0	300

*See Table 3S

Table S3

The optimized HGA-600 temperatures for As, Cd, Co, Ni and Pb meassurement

Element	Pyrolysis(°C)	Atomization(°C)	Cleaning(°C)
As	900	2200	2300
Cd	600*	1400	1600
Co	1200	2300	2400
Ni	1000	2200	2300
Pb	500	1500	1600

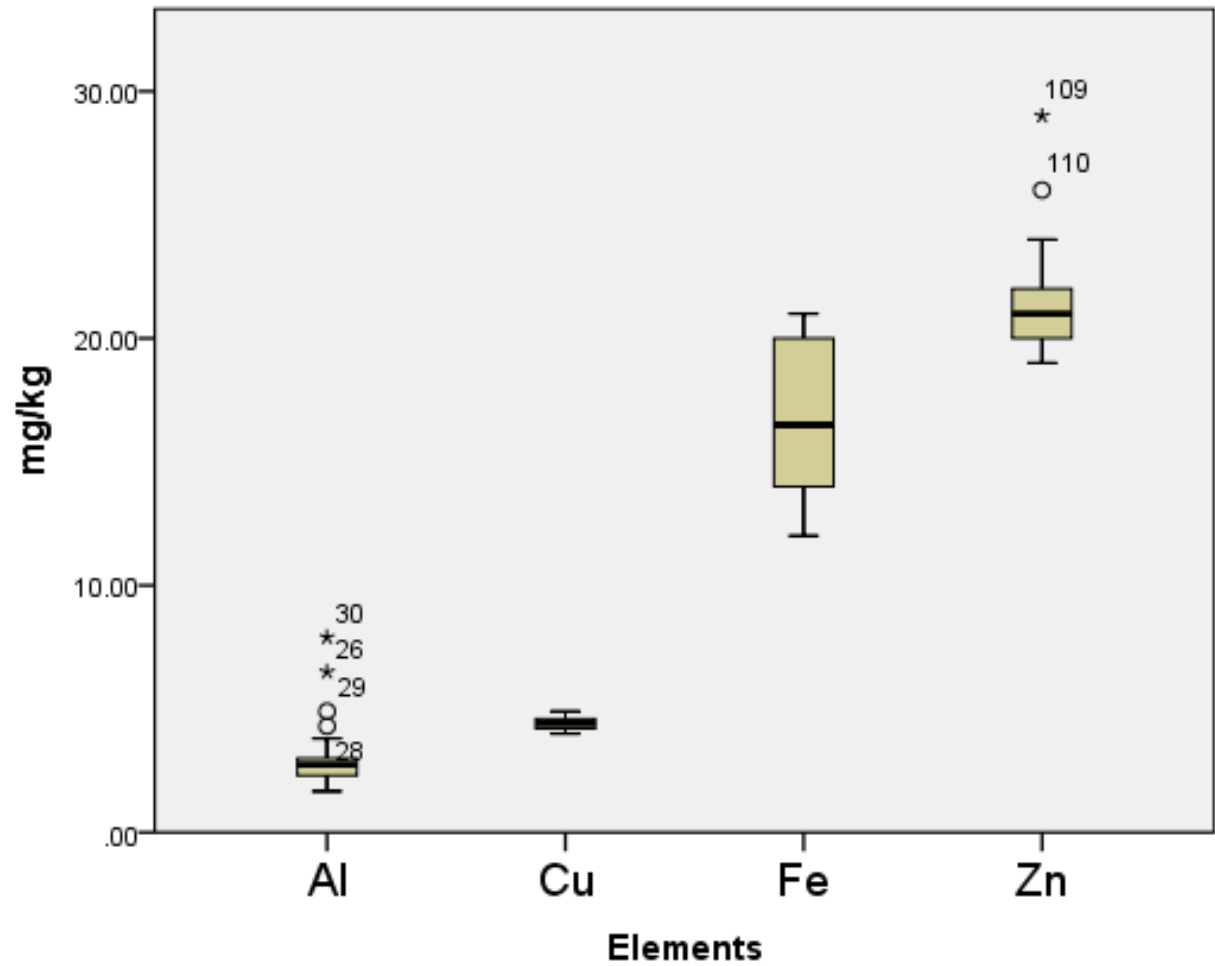*In the presence of Pd as modifier

Fig. S1. Box plot diagram of the elements Al, Cu, Fe and Zn in RJ samples from Bulgaria. For each element minimum, maximum and median values are shown.