Global NEST Journal, Vol 19, No 3, pp 423-429

Major and minor element levels in Greek apicultural products

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Received: 21/12/2016, Accepted: 07/03/2017, Available online: 18/10/2017

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Abstract

In the present study, four beehive matrices (honey, propolis, pollen and bees), sampled from thirteen apiaries from Northern and Western Greece, were analyzed for the presence of Cr, Cu, Mn, Fe, Zn, Mg, and Ca. The proposed method involved microwave digestion with H_2O_2 and HNO_3, dilution with water and direct determination by flame atomic absorption spectrometry. The results showed that the levels of elements in all apicultural products present the following order: Ca > Mg > Zn ≈ Fe > Mn > Cu. Cr was detected at LOD level (0.3 μg g^{-1}) only in some pollen samples from industrial areas. The most abundant elements determined were Ca (3.42 mg g^{-1}) and Mg (1.5 mg g^{-1}) in pollen and propolis. It was also demonstrated that all detected elements are accumulated at higher concentrations in pollen, propolis and bees and to a much lesser extent in honey. Dependence of the content of Mg in honey and of the content of Zn and Cu in pollen, on the geographical origin of samples was identified.

Keywords: micro-elements, macro-elements, honey, pollen, propolis, bees.

1. Introduction

Honey is a natural sweet commodity produced by honeybees which contains mainly simple sugars, water, proteins, flavonoids, phenolic compounds, free amino acids, organics acids, vitamins and all of the minerals that are essential to human health (Gonzalez-Miret *et al.* 2005; Formicki *et al.* 2013). It is used both as food and as medicine due to its high nutritional, antimicrobial, antibacterial and anti-inflammatory properties (Meliou and Chinou 2004, 2011; Kalogeropoulos *et al.,* 2009; Tsiapara *et al.,* 2009; Mandal and Mandal, 2011).

Bee pollen and propolis are bee products that also possess anti-inflammatory and antioxidant properties due to high concentrations of flavonoids and other phenolic compounds (Cowan 1999; Çelemli *et al.,* 2013; Formicki *et al.,* 2013). Pollen is an apicultural product collected from the male plant reproductive cells and used by the bees as the main protein and vitamin food source for the larvae (Yang *et al.,* 2013). Each pollen pellet has a characteristic color, size, morphology, flavor and composition specific to the floral species and cultivar (Di Paola-Naranjo *et al.,* 2004). Pollen contains all nutrients that are necessary for plant growth and development, and it is rich in sugars, proteins, amino acids, lipids, vitamins, minerals, nucleic acids, enzymes, and phenolics (Yang *et al.,* 2013). Pollen is also important for the health and development of the different bee life stages (Avni *et al.,* 2014).

Propolis is produced by honeybees after mixing plant resins with their own secretions. This apicultural product has been proposed as a germicide for food packaging or as a chemical preservative in some food products (Tosi *et al.,* 2007), and even as a natural agent against bee's pathogenic bacteria (Özkirim *et al.,* 2015).

The main natural source of micro and macro elements in apicultural products is the consumption of plants by bees, as well as the soil composition on which plants grow (Eremia, Dabija and Dodon 2010). Certain elements are transported through the root system to plants and their composition in nectar reflects the soil characteristics. Although the amount of micro- and macro-elements depends on apicultural products' botanical origin (Gonzalez-Miret *et al.,* 2005; Lachman *et al.,* 2007), the environmental pollution should also be considered as an additional source of some micro and toxic elements in apicultural products such as Cd, Ni, Cr, Cu, Fe, Pb and Zn. These elements may jeopardize the quality and the safety of apicultural products, and exhibit a potential hazard to human health (Crane 1984; Pohl *et al.*, 2009).

Several aspects of the quality of honey and other apicultural products have been studied during the last years. Numerous studies deal with the determination of macro, micro and toxic elements' content in apicultural products (Tuzen *et al.,* 2007; Pohl *et al.,* 2011; Van der Steen *et al.*, 2011; 2015; Meli *et al.* 2015), often in order to use them as bioindicators (Jones 1987; Saunier *et al.* 2013; Krakowskaa 2015), to certify their geographical origin (Bilandzic *et al.* 2011; Batista *et al.,* 2012; Bonvehí and Bermejo, 2013; Pellerano 2012; Conti *et al.,* 2014), or to assess the quality of honey (Devillers *et al.,* 2002; Lachman *et al.,* 2007; Pisani *et al.,* 2008). Other studies focus on the use of apiary products for biomonitoring of other environmental contaminants (Balayiannis and Balayiannis, 2008; Bargańska *et al.,* 2016).

Although apiculture is very popular in Greece and apicultural products are produced and consumed at a large national scale, very limited information is available on the quality of Greek apicultural products regarding the content of macro and microelements, as well as the contamination of these products by toxic elements (Farmaki and Thomaidis, 2008; Ioannidou *et al.,* 2005). The aim of this study was to address this lack of information by generating data for the elemental composition of Greek honey, propolis, pollen, and honeybees. In particular, a reliable Flame Atomic Absorption Spectroscopy method in combination with microwave-assisted digestion was developed and validated for the determination of Cr, Cu, Mn, Fe, Zn, Mg, and Ca in honey, propolis, pollen, and honeybees, collected from several areas of Northern and Western Greece. The validated analytical method was applied to thirty-two samples and the content of the elements was determined and compared to corresponding data from other countries. Finally, statistical analysis was performed in order to examine the effect of the geographical origin on the elemental composition of the apicultural products.

2. Materials and methods

2.1. Sampling

Four beehive matrices (honey, propolis, pollen and bees) from thirteen apiaries from Northern and Western part of Greece were collected and provided by the local beekeepers, between spring 2013 and August 2014, from rural areas, industrialized areas and some agricultural areas near mines. Samples were stored at -15 °C in their original plastic container until analysis. In total, 32 samples were analysed for macro-elements (Mg, Ca) and micro-elements (Zn, Cu, Fe, Cr, and Mn). The location of the sampling points along with the industrialized activities of the area is illustrated in the map of Figure 1. The samples were classified in three groups based on the geographical origin, as presented in Table 1 (Group 1: Kozani/Sindos, Group 2: Chalkidiki and Group 3: Arta).

2.2. Standards and materials

Analytical grade standard solutions of 1 g L^{-1} of the following elements: chromium (Cr), zinc (Zn), copper (Cu), manganese (Mn), iron (Fe), calcium (Ca) and magnesium (Mg), metal-free water and HNO_3 (65%) were purchased from Fisher Scientific (UK). H_2O_2 was obtained from Carlo Erba (France). Stock solutions of each element were prepared in 0.1 mol L^{-1} HNO_3. The stock solutions were used for further dilutions.

2.3. Sample preparation, analytical determination and data analysis

Figure 2 describes the flow chart of the analytical procedure followed for all the elements. An aliquot of the samples (0.1 – 1g) was treated with 5 mL metal-free HNO_3 (65%) and 2.5 mL H_2O_2 (30%). The samples were digested in a microwave oven (MARS, model MD 9132, CEM Corporation, USA) in Omni XP-1500 tubes. The temperature program was as follows: 0 – 15 min ramp to 210°C and hold another 15 min at 210 °C. Every microwave digestion cycle consisted of 8 samples, two blanks (5 mL HNO_3 (65%) and 2.5 mL H_2O_2 (30%)) and two spiked samples. After digestion, tubes were let to cool down, the pressure was carefully released and the yellow/brown gases were let to escape under sonication, until complete decolorization. After the removal of the gases the samples were quantitatively transferred to 25 mL volumetric flasks and were diluted with water.

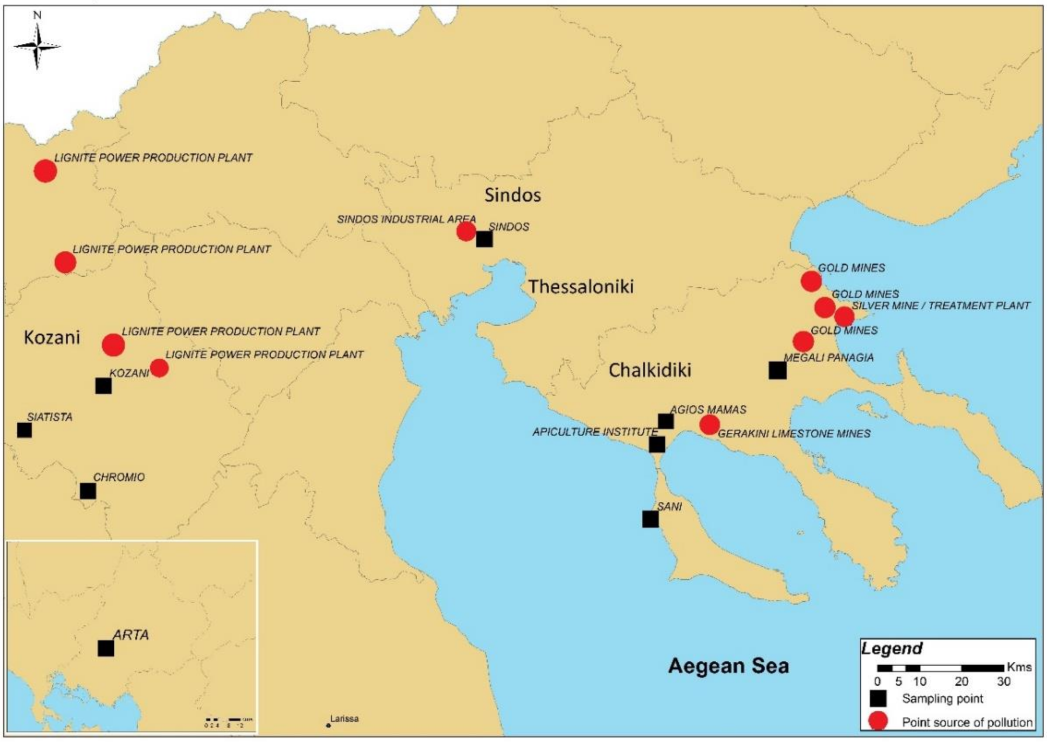

Figure 1. Sampling areas and industrialized activities

Table 1. Origin of honey, propolis, pollen and bee samples

Nº	Matrix	Sample name	Geographical region
1	Honey	Kozani No 1	Group 1
2	Honey	Kozani No 2	Group 1
3	Honey	Sindos No 1	Group 1
4	Honey	Sindos No 2	Group 1
5	Honey	Apicult. Institute	Group 2
6	Honey	Sani	Group 2
7	Honey	Ag. Mamas	Group 2
8	Honey	M. Panagia	Group 2
9	Honey	Arta No 1	Group 3
10	Propolis	Kozani No 1	Group 1
11	Propolis	Sindos No 1	Group 1
12	Propolis	Sindos No 2	Group 1
13	Propolis	Apicult. Institute	Group 2
14	Propolis	Ag. Mamas	Group 2
15	Propolis	M. Panagia	Group 2
16	Pollen	Siatista	Group 1
17	Pollen	Chromio	Group 1
18	Pollen	Kozani No 1	Group 1
19	Pollen	Kozani No 2	Group 1
20	Pollen	Sindos No 1	Group 1
21	Pollen	Sindos No 2	Group 1
22	Pollen	Ag. Mamas	Group 2
23	Pollen	Apicult. Institute	Group 2
24	Pollen	Sani	Group 2
25	Pollen	M. Panagia	Group 2
26	Pollen	Arta No 1	Group 3
27	Pollen	Arta No 2	Group 3
28	Bees	Kozani No 1	Group 1
29	Bees	Sindos No 1	Group 1
30	Bees	Sindos No 2	Group 1
31	Bees	Apicult. Institute	Group 2
32	Bees	M. Panagia	Group 2

Flame atomic absorption spectrometer (Model AA-6500F, Shimadzu Corporation, Kyoto, Japan) with air-acetylene flame was used for the determination of Cr, Zn, Cu, Mn, Fe, Ca and Mg. For the determination of the macro-elements of Ca and Mg, samples of pollen, propolis and bees were further diluted because of their high concentrations. The FAAS conditions applied were those proposed by the manufacturer.

The range of measured concentrations of each element for each matrix and the corresponding mean value were calculated. In cases where the element concentration was below the LOD, a value of LOD/2 was used for the statistical analysis and the calculation of the mean. The effect of the geographical region on the levels of the detected elements in honey, pollen and propolis was examined by performing t-test between Groups 1 and 2 which are described in Table 1. Group 3 was not used in the statistical analysis because of the limited number of samples. The statistical analysis was performed with the Excel program and a probability level of $p > 0.05$ was considered statistically non-significant.

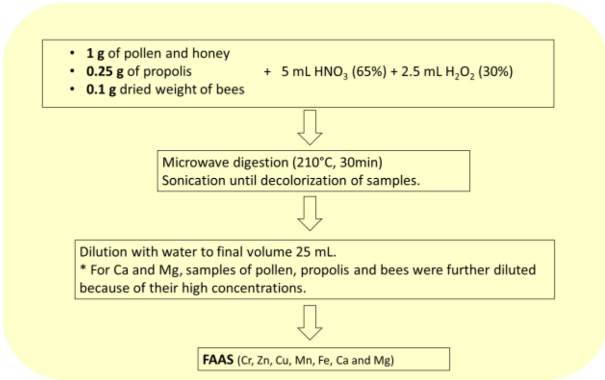

Figure 2. Flow chart of the analytical procedure

2.4. Method validation

Standard calibration curves were prepared daily by measuring standard solutions of the elements in 0.1 mol L^{-1} HNO_3 at four calibration levels (Table 2). Linear regression analysis was performed using the absorbance against analyte concentration.

For the assessment of the accuracy and the precision, the method was applied to honey, pollen, propolis and bee samples that were spiked with the measured elements at appropriate fortification levels (Table 3). Analysis of three replicates of the spiked samples was conducted for the repeatability test. The recovery was calculated by subtracting the concentration measured in the non-spiked sample from that measured in the spiked sample and then dividing with the spiked concentration.

For each of the four matrices the method limit of detection (LOD) was defined as 3 × $S_{unsp.sample}$, where $S_{unsp.sample}$ stands for the standard deviation of the response of 6 independent replicate analyses of the corresponding unspiked sample. In cases where no signal was obtained from the unspiked sample, the standard deviation of the response of a low level spiked sample was used for the determination of the LOD.

3. Results and discussion

3.1. Method performance

Table 2 presents the range of the calibration concentrations of the standard solutions, the linear regression lines and the squared correlation coefficients (r^2) which all exceeded 0.99. The accuracy of the method expressed as % recovery and the precision of the method expressed as % RSD, are presented in Table 3. The reported fortification levels refer to the amount of the element (μg) per amount of sample (g). The recoveries of the elements ranged between 70% and 115%, except for Cr which was 56% and 65% in propolis and bees, respectively. The corresponding % RSDs ranged between 1.4 and 20.

Table 2. Calibration lines of standard solutions

	Calibration range (μg mL^{-1})	Linear regression lines	r^2
Cr	0.1 – 0.4	y = 0.0227x+0.0023	0.9999
Cu	0.05 – 0.4	y = 0.0437x+0.0031	0.9939
Mn	0.05 – 0.4	y = 0.0403x + 0.0031	0.9978
Zn	1 – 4	y = 0.1274x+0.0545	0.9998
Fe	0.1 – 0.8	y = 0.0177x+0.0041	1.0000
Ca	0.5 – 4	y = 0.0251x + 0.0066	0.9994
Mg	0.2 – 1.6	y = 0.2296x-0.0012	0.9949

Table 3. Accuracy and precision data for all elements in all matrices (n = 3)

	Honey			Propolis			Pollen			Bees		
	Fortification level (μg g^{-1})	Rec. (%)	%RSD	Fortification level (μg g^{-1})	Rec. (%)	%RSD	Fortification level (μg g^{-1})	Rec. (%)	%RSD	Fortification level (μg g^{-1})	Rec. (%)	%RSD
Cr	2.5	80	6.4	10	56	9.2	1	74	8.4	100	65	*n=2*
Cu	2.5	103	3.3	2.5	85	1.4	5	83	4.0	125	109	3.4
Mn	2.5	115	3.5	5	108	9.1	25	80	4.9	125	107	20
Zn	25	98	5.9	100	79	3.1	25	70	8.1	125	81	12
Fe	5	91	12	50	103	*(n=1)*	25	100	6.3	125	96	14
Ca	50	93	14	*n.d*	*n.d*	*n.d*	500	82	10	1000	90	15
Mg	20	93	7.6	200	96	3.3	250	100	14	500	86	2.5

n.d: not determined

3.2.Element concentrations in samples

The concentration ranges of the elements in the analysed samples are summarized in Table 4 and the mean concentration of each element for each matrix is illustrated in Figure 3. The method limits of detection varied depending on the metal and the matrix analysed (Table 4).

Cr was detected only in pollen samples, in particular in the four samples of Kozani and in one sample of Sindos at the LOD level (0.3 μg g^{-1}). Similar results for the content of Cr in pollen samples have been obtained from Poland (0.2 - 0.3 μg g^{-1}) (Grembecka and Szefer, 2013), and a wider range of concentrations has been determined in pollen samples from Canada, 0.54 - 22 μg g^{-1} (Cloutier-Hurteau *et al.,* 2014). Concentration of Cr in honey has been found up to 0.5 μg g^{-1}, higher content of Cr has been determined in propolis, 5.6 – 7.8 μg g^{-1} (Grembecka and Szefer, 2013) and concentration in worker honeybees from the Netherlands ranged between 0.15 - 0.28 μg g^{-1} dry matter (Van der Steen *et al.*, 2011).

In the present study the most abundant element was Ca and the highest concentrations were found in samples of propolis with an average value of 2476 μg g^{-1}. The mean content of Ca in pollen was 1345 μg g^{-1}, in bees 1411 μg g^{-1} dry weight and in honey 43 μg g^{-1}. The high content of Ca in pollen and in propolis is also verified with results obtained from corresponding matrices which range between 707 and 2020 μg g^{-1} (Stanciu *et al.,* 2011; Grembecka and Szefer, 2013). Mg comes second in the order of concentration level for all examined matrices with mean values ranging between 33 μg g^{-1} for honey and 928 μg g^{-1} for pollen. The results are in accordance with corresponding published results in honey from Poland (Ca: 20 – 70 μg g^{-1}; Mg: 6.5 – 60 μg g^{-1}) (Grembecka and Szefer, 2013) and honey from the Czech Republic (Ca: 64.9 μg g^{-1}; Mg: 50.1 μg g^{-1}) (Lachman *et al.,* 2007), while higher values for Ca were determined in Croatian honey samples with mean value at 345.3 μg g^{-1} but similar for Mg, 23.9 μg g^{-1} (Bilandžić *et al.,* 2014).

The mean concentration of Zn in honey, determined in the present study, was 6 μg g^{-1}, while the highest levels of this element was determined in propolis, 143 μg g^{-1}; in dried bee samples, 104 μg g^{-1} and in pollen 71 μg g^{-1} (Figure 3). Corresponding published data on the concentration of Zn in Czech (Lachman *et al.,* 2007), Polish (Grembecka and Szefer, 2013) and Croatian honey (Bilandžić *et al.,* 2014) report values between 0.2 – 8 μg g^{-1}. Data on pollen and propolis from Poland report values between 27 and 44.8 μg g^{-1} (Grembecka and Szefer, 2013), data on pollen from Canada report values between 59.5 and 205 μg g^{-1} (Cloutier-Hurteau *et al.,* 2014). Additionally, Zn values in worker honeybees was found to be in the range between 59.18 and 100.46 μg g^{-1} dry weight (Van der Steen, Kraker and Grotenhuis, 2011), which is in agreement with the results of the present study.

Fe was detected in three of the nine honey samples and the statistical mean concentration was 1 μg g^{-1}. The highest levels of this element was determined in pollen, 169 μg g^{-1}, in propolis, 88 μg g^{-1} and in dried bee samples, 83 μg g^{-1} (Figure 3). Corresponding published data on the concentration of Fe in Croatian and Polish honey report values between 0.6 and 6.7 μg g^{-1} (Grembecka and Szefer 2013; Bilandžić *et al.,* 2014). Data on pollen and propolis from Poland report values between 32 and 48.9 μg g^{-1} (Grembecka and Szefer, 2013).

Mn was detected in only one honey sample, M. Panagia from Chalkidiki at a concentration of 0.7 μg g^{-1} (Table 4), while it was detected in all samples of pollen (mean: 29 μg g^{-1}) and in most of the propolis samples (mean: 10 μg g^{-1}) and dried bees (mean: 17 μg g^{-1}) (Figure 3). Corresponding published data on the concentration of Mn in Czech and Polish honey report values between 0.1 – 4.7 μg g^{-1} (Lachman *et al.,* 2007; Grembecka and Szefer, 2013). Data on pollen and propolis from Poland report values between 14 and 24.7 μg g^{-1} (Grembecka and Szefer, 2013) and data on pollen from Canada report values between 19.7 and 117 μg g^{-1} (Cloutier-Hurteau *et al.,* 2014). It should be noted that similar values were determined for Mn in honeybees from the Netherlands (Van der Steen, *et al.,* 2011)

Cu was detected in all honeybee samples at mean concentration 16 μg g^{-1} dried weight and in all pollen samples at mean concentration 9 μg g^{-1}. Regarding propolis only half of the samples were found to contain Cu at concentrations up to 6.1 μg g^{-1}, while it was not determined in none of the honey samples. Regarding the content of Cu in honey bees, similar results were presented by other studies (Roman 2010; Van der Steen *et al.,* 2011). According to Roman, (2010), Cu was an element present in bodies of worker honeybees with a mean content of 22.6 μg g^{-1} d.m, whereas Cu accumulation in honey was at a concentration many fold lower: Cu - 0.82 μg g^{-1} d.m. Additionally, other studies determined Cu in Czech honey (Lachman *et al.,* 2007) and Polish honey (Grembecka and Szefer, 2013) between 0.1 and 2.2 μg g^{-1}, while higher values are reported for Croatian honey, mean of 14.4 μg g^{-1} (Bilandžić *et al.,* 2014). Data on pollen and propolis from Poland report values between 3.6 and 11.2 μg g^{-1} (Grembecka and Szefer, 2013) and data on pollen from Canada report values between 0.54 and 27.7 μg g^{-1} (Cloutier-Hurteau *et al.,* 2014).

The statistical analysis showed that there is a significant difference in the content of Mg in honey (N_1=4, N_2=4, p=0.040), and in the content of Zn (N_1=6, N_2=4, p=0.027) and Cu (N_1=6, N_2=4, p=0.037) in pollen, from the two tested geographical areas. Correlation between the micro-, macro- and trace elements' content of the apicultural products and their geographical origin has been reported in other studies as well (Gonzalez-Miret *et al.,* 2005; Lachman *et al.,* 2007; Van der Steen *et al.,* 2011).

Chromium was detected at the LOD level (0.3 μg g^{-1}) in five out of six pollen samples of Group 1 (Kozani/Sindos), whereas it was not detected in samples of the other geographical origin. These findings could possibly be attributed to the specific industrialized zone areas; however, no safe conclusion can be drawn as the non-quantifiable data did not permit a statistical analysis.

It is noted that all the samples of the present study were also measured for Cd and Pb. However, no detection was observed as the LODs for these toxic elements were ≥ 0.5 μg g^{-1}.

Table 4. Major and minor element concentration ranges (min – max) expressed as μg g^{-1} in the four different matrices (n: number of samples)

	Honey (n=9)	Propolis (n=6)	Pollen (n=12)	Bees **Dry weight* (n=5)
Ca	< LOD up to 73.6 (LOD = 40)	1462 – 3418	544 – 2472	1106 – 1877
Mg	13.6 – 74.5	284 – 401	411 – 1496	655 – 1147
Zn	< LOD up to 33.9 (LOD = 2.8)	30.7 – 383.8	41.1 – 93.9	86.1 – 149.5
Fe	< LOD up to 2.0 (LOD = 1.8)	78.2 – 433	49.3 – 199.1	49.4 – 113.9
Mn	< LOD up to 0.7 (LOD = 0.3)	< LOD up to 24.6 (LOD = 1.3)	14.4 - 63.6	< LOD up to 69.7 (LOD = 3.2)
Cu	< LOD (LOD =0.6)	< LOD up to 6.1 (LOD = 2.5)	5.7 - 15.1	12.7 – 23.4
Cr	< LOD (LOD = 0.7)	< LOD (LOD =3)	≤ LOD (LOD = 0.3)	< LOD (LOD = 7.5)

LOD: method limit of detection

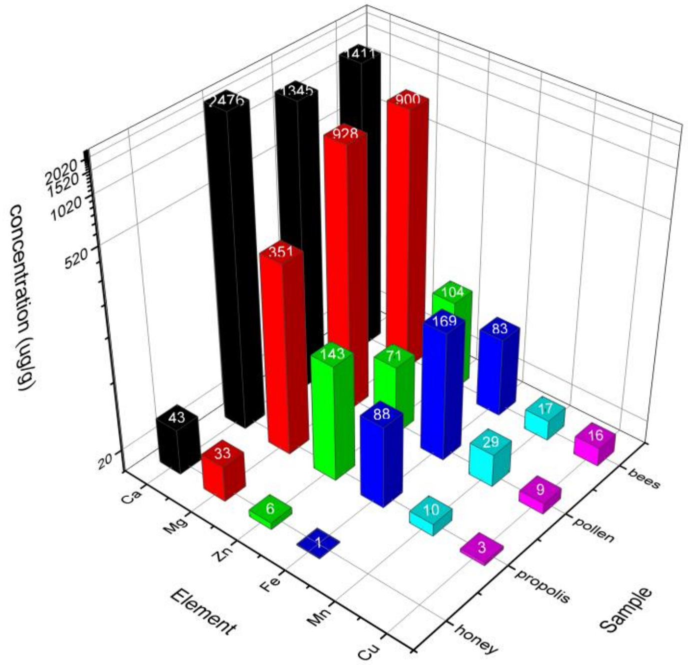

Figure 3. Graph of the mean concentrations (μg g^{-1}) of measured elements in honey, propolis, pollen and bee samples

4. Conclusions

In the present study it is demonstrated that the levels of elements in honey, propolis, pollen and bees present the following order: Ca > Mg > Zn ≈ Fe > Mn > Cu. It is noted that Cu was not detected in honey and Mn was detected in only one honey sample. Cr was detected only in pollen samples, in particular in the five out of six samples of Kozani and Sindos at LOD level (0.3 μg g^{-1}), possibly because of the industrialized zone area. It is also shown that all the detected elements are accumulated at higher concentrations in pollen, propolis and bees and to a much lesser extent in honey. Finally, statistical analysis showed that there is dependence of the content of Mg in honey and of the content of Zn and Cu in pollen, on the selected sampling areas of Northern and Western Greece. These data may act as the basis for further research.

Acknowledgements

This work was partly supported by an EC & Greek Government project, awarded to Division of Apiculture- Hell. Agr. Org. 'DEMETER' under 797/2007 Directive of EC. We would also like to thank Dr. A. Papachristoforou for his assistance, the beekeepers who provided all the samples and the two Greek companies which also partly supported this project: Anel Standard Pantelakis (Beekeeping equipment) and APIVITA SA (Bee Products & Herbs).

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