Global NEST Journal, Vol 20, No 3, pp 541-550
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# Antioxidant activities and heavy metal contents of *Castanea sativa* honey

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Received: 15/02/2018, Accepted: 26/06/2018, Available online: 26/09/2018

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https://doi.org/10.30955/gnj.002628

**Abstract**

Honey is one of the most valuable foods in terms of its antioxidant nature and antioxidant activity. In this study, their botanic origins, total flavonoid content, total phenol content, the hydrogen peroxide scavenging activity (HPSA) (in terms of $SC_{50}$ (µg $mL^{-1}$)), ferric reducing antioxidant power capacity (FRAP), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity (in terms of $SC_{50}$), metal-chelating activity (MCA) (%) and heavy metal amounts were examined to determine the qualities of honey samples that gathered from Giresun city of Black Sea region in Turkey. According to the melissopalynological analysis, the botanic origins of the samples are *Castanea sativa* (Chestnut) Miller and uni-floral. The HPSA, FRAP, DPPH, MCA (%), total phenol content (TPC), and total flavonoid content (TFC) were found between 251.99±0.48-258.64±1.22 µg $mL^{-1}$, 71.34±0.09-73.71±0.20%, 584.86±0.06-595.04±0.29 µg $mL^{-1}$, 36.73±0.00-36.86±0.09%, 93.82±1.05-173.15±2.46 mg GAE 100 $g^{-1}$ and 5.51±0.19-8.29±0.05 mg CAE/100 g, respectively. For comparison of these results, Butylated Hydroxy Anisole (BHA), Butylated Hydroxy Toluene (BHT) and α-Tocopherol (TOC) were used as standard antioxidant compounds. In addition, it was observed that honey samples were been contaminated with most of the heavy metals (Al, Ca, Cr, Cu, Fe, Li, Mg, Mn, Ni, Rb, and Zn) to a lesser or greater extent, whereas others (Te, Tl, and U) were never detected in these samples. However, Cd and Cs were detected only in one sample and Pb in another sample. Finally, the results indicate that although honey is an important source of nutrients, nevertheless it could be affected by environmental pollution.

**Keywords:** Antioxidant activity, bio-monitoring, contamination, honey, metal.

## 1. Introduction

*Castanea sativa* Mill is one of the most important pollen and nectar resource for honey bee (*Apis mellifera*) colonies (Coffey and Breen, 1997; Giovanetti and Aronne, 2011). In Turkey, it grows in native and culture forms mainly at the Black Sea, Marmara, and Aegean regions, and also is a significant agricultural production. Furthermore, chestnut is usually blossoming in June.

Honey is an important food and a natural mixture containing mainly carbohydrates, proteins and a large portion of minor components (trace elements, vitamins, aroma compounds, polyphenols etc.) (Bogdanov *et al.,* 2008). Chemical composition and flavor of the honey are dependent on its botanic origin and process. Chestnut honey is dark colored, slightly bitter, and high in quality as well as having a strong flavor, heavy aroma, a slow crystallization rate, and mostly *Castanea sativa* pollen (>87% in Greek Food Code) (Alissandrakis *et al.,* 2011; Yang *et al.,* 2012). Chestnut honey is an important source of nutrients for human health, because of its bioactive compounds, such as phenolic compounds. Having said that, heavy metals, metallic and toxic chemical elements with high-density in honey may be a threat for health.

Recently, the chemical pollutants, due to the usage of several chemical compounds, increase in environmental matrices such as water, soil, and air as a result of urbanization, agricultural activities, and industrialization (Matin *et al.,* 2016). Environmental pollution by pesticides, heavy metals, organic pollutants, pathogens, and radioactivity can be passed on to living organisms (human, animal, and plants). Heavy metals among these contaminants are the natural constituents of rocks. Therefore, they present in the soils in different proportions and forms (Kocaer and Başkaya, 2003).

Air pollution and agricultural events affect the soil media because heavy metals are adsorbed via organic matters, minerals, and carbonates that are in the soil (Alahabadi *et al.,* 2017). The plants can be considered as an indicator for environmental conditions due to the use of water, soil, and air. The heavy metal accumulation in plants shows variability depending on the metal type, plant species, and plant tissues. Some heavy metals such as Co, Cu, Fe, Mn, Ni, and Zn are important for normal growth of plant at a

certain level, but in very high levels are toxic and they cause the plant to die.

Some of them such as Cd, Pb, Hg, and Cr etc., have extremely toxic effects on plants (Matin *et al.,* 2016). Also, the scientific literature indicates that Ag, Cd, Cr, Cu, Hg, Mn, Ni, Pb and Zn are the heavy metals that are mostly found in food (Nielsen, 1984). Honey bees can be considered as a contaminant multi sample collector (Matin *et al.,* 2016). Bees can carry contaminants to their hives. Because of the contact of bees with water, soil, air, and plant, heavy metals found in honey reflect their amount in the region (Dinkov and Stratev, 2016; Matin *et al.,* 2016). Therefore, honey has been recognized as an environmental marker (Przybylowski and Wilczynska, 2001). Recently, the heavy metal contents of honey samples have been investigated in several countries such as Poland (Przybylowski and Wilczynska, 2001), France (Devillers *et al.,* 2002), Slovenia (Golob *et al.,* 2005), Italy (Pisani *et al.,* 2008), Turkey (Tuzen *et al.,* 2007; Silici *et al.,* 2008; Citak *et al.,* 2012), Croatia (Bilandzic *et al.,* 2011) and China (Ru *et al.,* 2013).

The aims of the present study are to evaluate (i) pollen spectrums, (ii) antioxidant activities and, (iii) the existence of metals in honey samples. Honey is considered valuable food for the human, but its content or the effect of environmental pollution on it is not evaluated by the consumer. However, there is limited study about this situation in the literature. The main goal of this study is to draw attention to the heavy metal pollution in chestnut honey. Additionally, it is also to produce useful information for beekeepers and the honey industry, by characteristics of high-quality honey.

## 2. Experimental methods

### *2.1. Sample solution*

The honey samples were prepared by slightly modified of the sample preparation method given Louveaux *et al.* (1978). 10 grams of homogenous honey samples from each three honey samples were completely dissolved in 50 mL of distilled water. For that, 45°C hot water bath was used. The prepared sample solution was used in the further analyses.

### *2.2. pH and Brix measurement*

The pH value of samples was measured by pH meter (Rodríguez-Flores *et al.,* 2016). The calibration of the digital pH meter was made at the room temperature by using buffer solutions of pH 4 and 7. The refractive index and Brix value of the samples were measured by a refractometer calibrated with the distilled water regularly. Each honey sample was analyzed three times and the mean values were evaluated to determine the honey quality. The refractive index was converted to moisture by reference to the standard table (Annex 2).

### *2.3. Determination of DPPH radical scavenging activity*

The DPPH free radical scavenging activities of samples were assayed according to the method described by Blois *et al.* (Blois *et al.,* 1958). In brief, 3 mL of sample solutions and 1 mL of DPPH$^{\bullet}$ (0.1 mM) dissolved in ethanol were mixed and blended properly. The mixtures were kept at room temperature in a dark environment. After 30 min, the absorbance of mixtures was analyzed at 517 nm. The free radical scavenging activities of mixtures were calculated by absorbance estimation graphics with UV/VIS spectrophotometer (Optizen, Korea). The decrease in absorbance estimation was accepted as a result of high level of free radical scavenging activity. The results were expressed as $SC_{50}$ values (the concentration μg $mL^{-1}$ required for 50% scavenging of DPPH radical).

### *2.4. Determination of hydrogen peroxide scavenging activity*

The hydrogen peroxide scavenging activities of samples were assayed according to the method described by Ruch *et al.* (Ruch *et al.,* 1989). Briefly, the samples were dissolved in 0.04 M phosphate buffer (pH = 7.4) and 3.4 mL of the samples were mixed with 0.6 mL of 40 mM $H_2O_2$ (prepared in the same buffer). The absorbance of the mixture was measured at 230 nm with UV/VIS spectrophotometer versus blind sample after 10 min (Optizen, Korea). Phosphate buffer without hydrogen peroxide was used as a blank. The decrease in absorbance value showed a high level of hydrogen peroxide scavenging activity. The results were expressed as $SC_{50}$ values (μg $mL^{-1}$).

### *2.5. Metal-chelate activity analysis*

The metal-chelate activities and standard antioxidant materials of honey samples were estimated according to the method described by Dinis *et al.* (Dinis *et al.,* 1994). Briefly, 0.05 mL of 2 mM $FeCl_2$ and 0.4 mL of extract solution were mixed. 0.2 mL of 5 mM ferrozine was added and the reaction was initiated. This mixture was vigorously agitated and kept at room temperature for 10 minutes. The absorbance of the mixtures was measured at 562 nm with UV/VIS spectrophotometer (Optizen, Korea). The decrease of absorbance estimation demonstrated a high level of metal-chelate activities of the extracts and the standard antioxidant materials. The metal-chelate activity of extract and the standard antioxidant material were computed according to the equation (1) below:

$$\text{Ferrous Ions Chelating Activity}(\%) = [1 - (A_S/A_C)] \times 100 \quad (1)$$

where $A_C$ is the absorbance value of control, $A_S$ is absorbance value of the extract or standard material.

### *2.6. Total phenolic compound estimation*

The total phenolic compound of extracts was estimated according to the Folin-Ciocalteu method defined by Slinkard and Singleton (Slinkard and Singleton, 1977). The samples (10-250 mg $mL^{-1}$) were added to ethyl alcohol (0.5 mL) and then mixed with deionized water (7.0 mL) and 1 mL of Folin-Ciocalteu reagent (Sigma-Aldrich). After 3 minutes $Na_2CO_3$ (% 2.0, 2.0 M) was added and the mixtures kept remaining at room temperature. In this period, the mixture was shaken occasionally for 2 h. The absorbance of compounds was measured at 760 nm with UV/VIS spectrophotometer (Optizen, Korea). Total phenol compounds of the samples were calculated with the aid of Gallic acid calibration curve ($R^2$ = 0.9827).

*2.7. Total flavonoid content estimation*

Total flavonoid component of extracts was analyzed according to the aluminum chloride colorimetric method (Chung *et al.,* 2002). Deionized water (1.5 mL) mixed with the samples (0.5 mL). Subsequently, aluminum chloride (0.1 mL, 10%), potassium acetate (0.1 mL, 1 M) and deionized water (2.8 mL) were added and then it was left at the room temperature for 30 min. The absorbance of the mixtures was determined at 415 nm with UV/VIS spectrophotometer (Optizen, Korea). The total flavonoid compounds of samples were found with the aid of the catechin's calibration curve that was used as a standard ($R^2$ = 0.9720).

*2.8. Heavy metal analyses*

In the present study, bioaccumulation and biosorption of heavy metals in honey samples were examined and 17 heavy metals were determined by using ICP-MS Spectrometer (Model Bruker 820-MS). Microwave digestion method was used to prepare the honey samples. 0.5 grams of each honey samples were digested with 5 mL of $HNO_3$ (ultrapure) and 2 mL of HCl at 210°C at 1600 Watt for 35 min in CEM MARS-5 Closed Vessel Microwave Digestion System. These solutions were colorless and clear after cooling were placed in a capped falcon tube and were completed to 50 mL with deionized water. And these samples were analyzed by Bruker 820-MS ICP-MS after filtered by a membrane filter of 0.45 µm. The calibration curve was made by using the certified multi-element standard. An intermediate stock of 10 mg $L^{-1}$ was prepared from the main stock solution and a calibration curve was plotted from standard stocks of 5, 10, 20, 50, 100 and 250 µg $L^{-1}$. The samples were prepared in triplicate and each of them was measured 10 times in ICP-MS device. The blind sample was prepared with 1% $HNO_3$. The standard slope, LOD (detection limit), and LOQ (determination limit) were determined (Sengul, 2016).

*2.9. Statistical analysis*

Experimental results were given as mean ± standard deviation (SD) of the three parallel measurements. Analysis of variance was performed by ANOVA procedures. Significant differences between means were determined by Duncan's Multiple Range tests. $p$ values < 0.05 were regarded as significant. Both operations were done with SPSS (version 15.0.0, SPSS Inc., Chicago, IL, USA) for windows.

## 3. Results and discussions

*3.1. Pollen profile*

The determined taxa in the samples and the amounts of pollen from these taxa are shown in Table 1. The pollen profiles of the honey samples consist of 92-97% of *C. Sativa* pollen. Rodríguez-Flores *et al.* determined that *C. Sativa* pollen was presented with a range between 70.4% and 90.2% of the pollen spectra (Rodríguez-Flores *et al.,* 2016). Alissandrakis *et al.* declared that relative pollen frequencies of chestnut honey were 89-95% of *C. Sativa* pollen (Alissandrakis *et al.,* 2011). Yang *et al.* claimed that samples contain 82.47-98.04% of chestnut pollen. Chestnut honey is an "over-represented" type of honey and according to the Greek Food Code, uni-floral chestnut honey must include at least 87% of *C. Sativa* pollen (Yang *et al.,* 2012). Our samples also show similarity with the Greek Food Code and other studies in terms of pollen percentage of *C. Sativa*.

**Table 1.** Pollen spectra (%) of the honey samples from Black sea region

| Samples | Apiaceae | Castanea sativa | Trifolium | Medicago | Xanthium |
|---|---|---|---|---|---|
| Sample 1 | 2 | 92 | 6 | | |
| Sample 2 | - | 97 | 4 | 0.5 | 0.5 |
| Sample 3 | 2 | 95 | 3 | | |

*3.2. pH, refractive index, Brix and moisture*

The honey samples were acidic in a pH range of 4.98-5.24 (Table 2). Giorgi *et al.* reported that the pH values of chestnut honey taken from Veneto and Lombardy Region of Italy were ranged from 4.60 to 5.21 (Giorgi *et al.,* 2011). Kropf *et al.* are determined 5.28-5.61 pH for chestnut honey (Kropf *et al.,* 2010). Bentabol Manzanares *et al.* are indicated that pH values in chestnut honey are observed as 4.20-6.59 (Bentabol *et al.,* 2017). The values obtained from the present study are compatible with the results reported by these studies for chestnut honey.

High Brix value (soluble sugars) is an important criterion for the quality of honey. In our investigation, the sugar content is found between 75.50 and 76.17 °Bx and that is lower than literature. Kasperová *et al.* are determined that sugar contents of samples ranged from 78 to 83 °Bx (Kasperová *et al.,* 2012). Perna *et al.* are determined that sugar content of the chestnut honey was in the range of 80.93-81.68 °Bx (Perna *et al.,* 2012). Early harvesting of chestnut honey and climatic conditions may be the reason for the lower Brix values. According to the instructions of Commission of Food, the moisture content of honey should be 21% for honey; however, our results are slightly over 21% (Table 2). A higher than the humidity of 20% in honey will often cause fermentation.

It is necessary to use a combination of antioxidant tests, comparative analyses, and statistical evaluation to detect the antioxidant behavior of honey. Furthermore, a positive correlation between heavy metal and total phenol contents is reported (Perna *et al.,* 2012).

Hydrogen peroxide, not very reactive molecule as well as other reactive oxygen species, is generated in the biological systems, but it can be toxic for the cells due to hydroxyl radical formation within the cells. For this reason, hydrogen peroxide scavenging activity is very significant for protection of pharmaceuticals and food systems (Güder *et al.,* 2014). According to the results of

antioxidant test parameter, samples had effective hydrogen peroxide scavenging activity as much as standard compounds ($p$<0.05). These results of activity of the samples and standards were ranged from 251.99±0.48 to 258.64±1.22 and 147.49±1.95 to 216.26±0.50 µg $mL^{-1}$, respectively (Table 2).

**Table 2.** Some physicochemical and biochemical properties of Chestnut honey samples

| Samples | pH | Brix | Refractive index (20°C) | Moisture | HPSA (µg $mL^{-1}$ $SC_{50}$) | FRAP (250%) | DPPH (µg $mL^{-1}$ $SC_{50}$) | MCA (%) | TPC (mg GAE/ 100 g) | TFC (mg CAE /100 g) |
|---|---|---|---|---|---|---|---|---|---|---|
| Sample 1 | 5.1 | 75.6 | 1.4835 | 21.2 | 253.9±0.6$^{D}$ | 71.3±0.1$^{C}$ | 584.9±0.1$^{D}$ | 36.7±0.0$^{A}$ | 173.2±2.5$^{C}$ | 8.3±0.1$^{C}$ |
| Sample 2 | 5.0 | 75.5 | 1.4819 | 21.8 | 252.0±0.5$^{D}$ | 73.7±0.2$^{D}$ | 585.1±0.1$^{D}$ | 36.9±0.1$^{A}$ | 93.8±1.1$^{A}$ | 5.5±0.2$^{A}$ |
| Sample 3 | 5.2 | 76.2 | 1.4818 | 21.9 | 258.6±1.2$^{E}$ | 73.7±0.2$^{D}$ | 595.0±0.3$^{E}$ | 36.7±0.0$^{A}$ | 113.8±0.5$^{B}$ | 7.5±0.0$^{B}$ |
| BHA | | | | | 184.1±1.5$^{B}$ | 92.0±0.8$^{E}$ | 8.5±0.1$^{A}$ | 90.0±0.3$^{C}$ | | |
| BHT | | | | | 147.5±2.0$^{A}$ | 54.2±0.7$^{B}$ | 9.0±0.1$^{B}$ | 86.3±0.4$^{B}$ | | |
| TOC | | | | | 216.3±0.5$^{C}$ | 33.0±0.7$^{A}$ | 12.0±0.1$^{C}$ | 93.4±0.6$^{D}$ | | |

**In same column with different letters indicate significant difference (p<0.05)*

The reducing power assay can be accepted as an important parameter for antioxidant potential of samples. In this assay, the existence of resultants like the antioxidant substances in the antioxidant samples causes the formation of the $[Fe(CN)_6]^{3-}$ to the $[Fe(CN)_6]^{4-}$. For this reason, $Fe^{2+}$ can be monitored by measuring the formation of Perl's Prussian Blue ($Fe_4[Fe(CN)_6]_3$) at 700 nm. Consequently, the yellow color of the test solution turns into various shades of green and blue depending on the reducing power of antioxidant samples due to the complex formation (Temel *et al.,* 2015). The activities of standards (BHA, BHT, and TOC) were determined as 92.02±0.84, 54.16±0.71 and 32.98±0.72% while the ferric reducing antioxidant powers (FRAP) of samples were found as 71.34±0.09, 73.71 ± 0.20 and 73.68 ± 0.18% (Table 2). The samples show higher FRAP than standards (BHT and TOC) ($p$<0.05).

As DPPH radical accepts an electron or a hydrogen radical, it becomes a stable diamagnetic molecule. DPPH radical has the maximum absorbance peak at 517 nm but this absorbance value decreases in the existence of an antioxidant or a radical species due to the reaction between the antioxidant molecule and radical. When DPPH radical transforms non-radical form, the color of solutions turns from purple to yellow (Gür *et al.,* 2017). DPPH radical scavenging activity of antioxidants can be an important parameter owing to their hydrogen or electron donating properties. DPPH free radical scavenging and metal chelating activities of samples demonstrate very lower activity than standards ($p$<0.05). DPPH radical scavenging activities of samples were ranged from 584.86 ± 0.06 to 595.04 ± 0.29 µg $mL^{-1}$ (Table 2). Can *et al.* studied Turkish honey. In their study, DPPH radical scavenging activity of chestnut was found as 20.05 mg $mL^{-1}$ in terms of $SC_{50}$ (Can *et al.,* 2015). On the other hand, different parameters were investigated from the honey samples of Lombardia region (Italy). DPPH scavenging activities of chestnut samples were ranged from 12.03 to 52.28 mg $mL^{-1}$ (Giorgi *et al.,* 2011). Yet, being observed of Turkish honey in terms of its antioxidant and antimicrobial activity, DPPH activity was found as 66.17 mg $mL^{-1}$ (Kolaylı *et al.,* 2008). When these results are compared with our data, it can be concluded that our samples have effective DPPH scavenging activity than others.

In this assay, $Fe^{+2}$ ions constitute complex with ferrozine and this complex density can be monitored via spectrophotometry at 562 nm. In the existence of chelating agent, $Fe^{+2}$/ferrozine complex degenerate. However, the degradation of absorbance value can be detected easily (Kizilpinar *et al.,* 2017). As it is seen in Table 2, the standards show a very efficient metal-chelating activity than samples but all samples have similar activity ($p$<0.05).

Phenolic compounds show antioxidant properties as a result of their ability to scavenge free radicals, and active oxygen species such as free radicals, singlet oxygen, and hydroxyl radicals. Flavonoids are very valuable plant components due to their active hydroxyl groups for antioxidant activity (Güder and Korkmaz, 2012). The mean value for total phenolic content (TPC) was 126.92 mg GAE/100 g, with a range between 93.82±1.05 mg GAE/100 g and 173.15±2.46 mg GAE/100 g, while the mean value for the total flavonoid content (TFC) was 7.11 mg CAE/100 g, with a range between 5.51±0.19 mg CAE/100 g and 8.29±0.05 mg CAE/100 g ($p$<0.05) (Table 2). When the chestnut honey from Southern Italy was examined, the TPC and TFC were found as 14.67 mg GAE/100 g and 14.05 mg QE/100 g (Perna *et al.,* 2012). Can *et al.* found the TPC and TFC of chestnut honey as 98.26 mg GAE/100 g and 8.10 mg QE/100 g, respectively (Can *et al.,* 2015). TPC of chestnut honey samples from Italy ranged from 43.12 to 82.49 mg GAE/100 g (Giorgi *et al.,* 2011). Additionally, the TPC of different types of honey samples from Anatolia was determined as 239 mg CA/100 g (Küçük *et al.,* 2007). Moreover, the TPC of Turkish honey from different regions was investigated. Phenolic content of chestnut honey was found as 1074 mg GAE/100 g (Cavrar *et al.,* 2013). The studied indicate that chestnut honey's phenolic content as 430 mg GAE/100 g (Kolaylı *et al.,* 2008). When compared the results with the literature, our samples have higher total phenolic contents than overseas' honey, but not honey of Turkey.

### *3.3. Heavy metals*

In the present study, bioaccumulation and biosorption of heavy metals, and 17 heavy metal concentrations (Al, Ca, Cd, Cr, Cs, Cu, Fe, Li, Mg, Mn, Ni, Pb, Rb, Te, Tl, U, Zn) were observed in three honey samples. As it is being seen in Table 3, honey samples were investigated of being

contaminated to a low or great extent by the heavy metals (Al, Ca, Cr, Cu, Fe, Li, Mg, Mn, Ni, Rb, Zn) while some heavy metals (Te, Tl, U) were never encountered. However, Cd and Cs were only detected in Sample 1, and Pb in Sample 3.

The low levels of Cu could be associated with a naturally occurring metallic element in soil. Also, Cu that is found in all animals and plants is an important element to the health of all living organisms and humans in small amounts. The provisional maximum tolerable daily intake (PMTDI) is 0.5 mg $kg^{-1}$ BW (body weight) for copper (WHO, 1982). Therefore, it is required to think the intake of Cu from varied sources like honey etc. But too much Cu can lead to damaging effects in the body. In this study, Cu concentrations were at higher levels than 0.5 mg $kg^{-1}$ in all honey samples (Table 3) which are 0.868 mg $kg^{-1}$ for Sample 1, 0.743 mg $kg^{-1}$ for Sample 2, and 1.080 mg $kg^{-1}$ for Sample 3. These findings were higher than the other studies reported in previous studies in New Zealand (0.25 mg $kg^{-1}$; 163-182 µg $kg^{-1}$) (Buldini *et al.,* 2001; Vanhanen *et al.,* 2011), Italy (647; 310 µg $kg^{-1}$) (Caroli *et al.,* 1999; Pisani *et al.,* 2008), and Ireland (2 mg $kg^{-1}$) (Downey *et al.,* 2005). The findings are diverse from region to region. The geological structure of the regions, the diversity in growing the plants, the use of different fertilizers, and various industrial activities may be the reason of this variety.

Cd is commonly found in industrial areas, is an extremely toxic metal. The leaves of plants directly absorb Cd (Alloway, 1990). WHO declared that the tolerable daily intake (TDI) is 50 µg for a 60 kg adult. And the provisional tolerable weekly intake (PTWI) limit is 25 µg $kg^{-1}$ BW for Cd (WHO, 2013). According to the results, Sample 1 contains 0.642 mg $kg^{-1}$ of Cd, while others have not. Cd content of honey samples were higher than those reported in China (1.34 µg $kg^{-1}$) (Ru *et al.,* 2013), Italy (305 µg $kg^{-1}$) (Buldini *et al.,* 2001), and Macedonia (3.63 µg $kg^{-1}$) (Stankovska *et al.,* 2006). As a result of its use in several industrial processes, Cd releases into the environment and enters the products by plant uptake from contaminated water and soil. Cd contents in the honey samples show variety according to the industrialization activities of the region (Aghamirlou *et al.,* 2015).

Pb is largely found in the environment due to exhaust emissions of motor vehicles. Lead pollution cannot be ignored out since the most of the beehives were situated along the main roads. According to the results, Sample 3 contains 0.510 mg $kg^{-1}$ of Pb, others have not. FAO and WHO (2002) reported that the acceptable limit for Pb was 2 mg $kg^{-1}$. It has been reported that there was a positive correlation between Cd and Pb (WHO, 2006). They attributed to common polluting sources (sewage, fertilizers, fossil fuels and atmospheric deposition from the burning of fuels, the industrial discharges) (Finger *et al.,* 2014; Formicki *et al.,* 2013). Furthermore, Pb can be found in several products and regions. Pb can reach into plants through the air, water, and soil. So, some foods such as honey can contain this heavy metal. Pb has not a beneficial role in human metabolism and can cause health problems (Aghamirlou *et al.,* 2015). The determined Pb content in Sample 3 was lower than the acceptable limits and the following findings in Slovenia (5.94 mg $kg^{-1}$) (Golob *et al.,* 2005), Italy (2.37 mg $kg^{-1}$) (D'Ambrosio and Marchesini, 1982) and was higher than the results from Poland (0.048 mg $kg^{-1}$) (Przybylowski and Wilczynska, 2001), Taiwan and mainland China (0.007-0.029 mg $kg^{-1}$) (Wang *et al.,* 2012).

Zn is one of the main elements required for the body to keep the building proteins and immune system. High level of Zn can cause anemia and decreasing absorption of Cu and Fe (WHO, 1982). The sources of anthropogenic Zn in the soil are smelter slags and discharged wastes, mine tailings, fertilizers and wood preservatives including Zn. It can be toxic at high levels. The mean suggested Zn as TDI in foods is predicted to be 12-15 mg/day or the PMTDI is 0.3-1.0 mg $kg^{-1}$ BW (WHO, 1982; ATSDR, 2005). In Table 3, when the results compared with other studies, Zn concentrations of all honey samples were higher than from Siena county of Italy (1.820 mg $kg^{-1}$) (Pisani *et al.,* 2008), China (1.330 mg $kg^{-1}$) (Ru *et al.,* 2013), and New Zealand (1.18 mg $kg^{-1}$) (Downey *et al.,* 2005), Zn contents of Sample 1 and Sample 2 were lower than those results in Slovenia (3.61 mg $kg^{-1}$) (Golob *et al.,* 2005), Iran (6.638 mg $kg^{-1}$) (Aghamirlou *et al.,* 2015), Poland (7.76 mg $kg^{-1}$) (Przybylowski and Wilczynska (2001). Some researchers consider that the source of Zn can be the galvanized containers keeping honey (González Paramás *et al.,* 2000).

Ni is a compound that occurs in the environment only at very low levels. The most important factors of nickel contamination are mine spoilage, liquidation furnaces and refinery arresters (Vural, 1993). Bees may collect pollen and nectar of plant that accumulates nickel in polluted soils. From Table 3, Ni concentrations of the honey samples were in a wide range (0.097-0.423 mg $kg^{-1}$). Nickel concentration of Sample 3 was found significantly higher than the others that can be related to more industrial activities used Ni. Ni is found in the water, soil, air and it shows uniform distribution through the soil. The maximum allowable limit of Ni was determined as 5 mg $kg^{-1}$ body (weight $day^{-1}$) by WHO (WHO, 1983). Our results for three honey samples were lower than this limit value (Table 3). We compared the results of other studies with previous data, Ni concentrations of all honey samples were higher than three studies from Spain and Turkey (<0.002, not determined) (Yılmaz and Yavuz, 1999; Latore *et al.,* 1999), and lower from Iran (0.652 mg $kg^{-1}$) (Aghamirlou *et al.,* 2015).

The $7^{th}$ most abundant element on earth is Cr (Mohanty and Kumar Patra, 2013). It is one of the most noxious heavy metals and one of the 129 priority contaminants that are listed by EPA (Sharma *et al.,* 2012). Trivalent Cr is a necessary nutrient. For adults, the daily intake of 30-100 µg $kg^{-1}$ is recommended by Department for Environment, Food and Rural Affairs and the Environment Agency (Duarte-Davidson, 2002). According to the WHO, the supplementation of Cr should not exceed 250 µg $kg^{-1}$ and the Expert Group on Vitamins and Minerals (EVM)

reported that TDI of about 0.15 mg Cr(II) $kg^{-1}$ BW $day^{-1}$ (or 10 mg/person/day) would be without adverse health effects (EFSA, 2010). Cr is used in various industries like chemical production, electroplating, tanning, metallurgy, production of paints and pigments, wood preservation, and pulp and paper production. These industries play a major role in chromium pollution with an adverse effect on biological and ecological species (Ghani, 2011). According to our findings, Cr concentrations were found as 1.054 mg $kg^{-1}$ in Sample 1, 0.818 mg $kg^{-1}$ in Sample 2, 0.966 mg $kg^{-1}$ in Sample 3. From these regulations and data, Cr contents of honey samples were higher than the acceptable levels. When honey contacts with stainless steel surfaces, a rise in Cr content of honey can occur due to the corrosive influence of acidity in honey in the harvesting, processing, preparation for the market (Tuzen *et al.,* 2007).

Ca, Mg, and Fe are the main mineral elements of honey. The mineral property of a honey is a significant parameter of the environmental pollution when heavy metals are determined. Besides, the main mineral content can also reflect the nutritional value of honey whereas it can be taken into consideration as a bio-indicator of the geographical origin of honey (Silva *et al.,* 2009). Ca was the prominent element in the honey samples ranging from 269.687 mg $kg^{-1}$ to 404.840 mg $kg^{-1}$. According to the data, the Ca contents of honey samples in the present study were higher than the other works in Hungary (47.9 mg $kg^{-1}$) (Czipa *et al.,* 2015), Portugal (40.3 mg $kg^{-1}$) (Alves *et al.,* 2013), Serbia (20.1 mg $kg^{-1}$) (Jevtić *et al.,* 2012), and the Ca concentrations of Sample 1 and 2 were found lower than in Italy (356 mg $kg^{-1}$) (Pisani *et al.,* 2008). The RDA (Recommended Dietary Allowance) in Hungary that was detected from WHO technical reports for Ca was 800 mg $day^{-1}$ for adults (Czipa *et al.,* 2015). Mg was 2nd most abundant element in the honey samples (Table 3). When we compared our findings with previous data, Mg concentrations of all honey samples were lower than from Italy (138 mg $kg^{-1}$) (Pisani *et al.,* 2008), New Zealand (86.3 mg $kg^{-1}$) (Vanhanen *et al.,* 2011), and were higher than in Hungary (1.91-35.1 mg $kg^{-1}$) (Czipa *et al.,* 2015), Brazil (72.9 mg $kg^{-1}$) (Batista *et al.,* 2012), and Macedonia (30 mg $kg^{-1}$) (Stankovska *et al.,* 2008). The RDA (Recommended Dietary Allowance) in Hungary that was determined from WHO technical reports for Ca was 375 mg $day^{-1}$ for adults (Czipa *et al.,* 2015). Fe is the 2nd most abundant metal in the earth's crust. Fe is a crucial element for growth and survival of almost all living organisms (Valko *et al.,* 2005). The highest level (10.866-39.240 g $kg^{-1}$) of iron is possibly due to its natural abundance in the environment, the use of mineral crop boost and ground water. Fe contents in honey samples were found as 20.515 mg $kg^{-1}$ for Sample 1, 6.280 mg $kg^{-1}$ for Sample 2, and 15.800 mg $kg^{-1}$ for Sample 3. Fe contents showed similarity to the other study in Turkey (Yücel and Sultanoğlu, 2013), and higher than performed in Portugal (0.70-7.06 mg $kg^{-1}$) (Alves *et al.,* 2013), in Serbia (0.58-4.21 mg $kg^{-1}$) (Jevtić *et al.,* 2012), and in New Zealand (0.67-3.39 mg $kg^{-1}$) (Vanhanen *et al.,* 2011). The PMTDI of Fe is determined as 0.8 mg $kg^{-1}$ BW (body weight) (WHO, 1983). It is known that these metals in honey content are related to soil characteristics and botanical origin (Pohl, 2009). However, the mineral properties do not show the quality of honey according to the EU Directive (EU, 2001). So, the determination of these metals is beneficial to evaluate the nutritional value of honey and to designate the differentiation of a botanical origin (Alves *et al.,* 2013).

Mn has been proved to be essential for plant growth. USDA database (2005) of the nutrients reports the characteristic levels of some trace elements in honey such as Mn (0.8 mg $kg^{-1}$). In this study, the amounts of Mn were between approximately 21.584 and 26.521 mg $kg^{-1}$ in chestnut honey. The data obtained from the study for Mn were higher than the other studies performed in Brazil (0.08-18.8 mg $kg^{-1}$) (Batista *et al.,* 2012), Hungary (0.029-4.23 mg $kg^{-1}$) (Czipa *et al.,* 2015), New Zealand (0.18-4.75 mg $kg^{-1}$) (Vanhanen *et al.,* 2011).

Al content of honey samples were found as 10.407 mg $kg^{-1}$ for Sample 1, 6.511 mg $kg^{-1}$ for Sample 2, and 18.821 mg $kg^{-1}$ for Sample 3. TDI is 8.57 mg for a 60 kg adult. PTWI value of Al is 1 mg $kg^{-1}$ BW (WHO, 2006). The findings were higher than the other studies performed in Hungary (0.103-4.39 mg $kg^{-1}$) (Czipa *et al.,* 2015), in Brazil (0.23-7.40 mg $kg^{-1}$) (Batista *et al.,* 2012), and were in the similar range with a study performed in New Zealand (0.21-21.3 mg $kg^{-1}$) (Vanhanen *et al.,* 2011). Al content of honey samples may be caused by beekeeping equipment and containers during the storage. The galvanized and aluminum containers can be the source of Al and Zn pollutions.

**Table 3.** The concentrations of the determined heavy metals in the honey samples (mg $kg^{-1}$)

| Metals | Sample 1 | Sample 2 | Sample 3 |
|---|---|---|---|
| Li | 0.53±0.02 | 0.40±0.00 | 0.42±0.00 |
| Mg | 78.20±0.08 | 73.52±0.06 | 85.23±0.06 |
| Al | 10.41±0.00 | 6.51±0.01 | 18.82±0.00 |
| Ca | 352.48±0.05 | 269.69±0.08 | 404.84±0.09 |
| Cr | 1.05±0.00 | 0.82±0.00 | 0.97±0.00 |
| Mn | 21.58±0.07 | 21.95±0.01 | 26.52±0.01 |
| Fe | 20.52±0.09 | 6.28±0.01 | 15.80±0.03 |
| Ni | 0.10±0.01 | 0.13±0.00 | 0.42±0.00 |
| Cu | 0.87±0.01 | 0.74±0.00 | 1.08±0.00 |
| Zn | 2.59±0.00 | 3.45±0.00 | 5.10±0.00 |
| Rb | 29.27±0.05 | 9.94±0.00 | 13.29±0.01 |
| Cd | 0.64±0.08 | 0 | 0 |
| Te | 0 | 0 | 0 |
| Cs | 0.06±0.00 | 0 | 0 |
| Tl | 0 | 0 | 0 |
| Pb | 0 | 0 | 0.51±0.01 |
| U | 0 | 0 | 0 |

Rb is associated with the floral origin, soil characteristics and agricultural activities (Pohl, 2009). Rb contents in honey samples were determined as 29.274 mg $kg^{-1}$ for Sample 1, 9.943 mg $kg^{-1}$ for Sample 2, and 13.293 mg $kg^{-1}$ for Sample 3. These results were higher than the other

studies performed in Spain (1.5 µg $kg^{-1}$) (Latorre *et al.,* 1999), Brazil (1.65-3.67 µg $kg^{-1}$) (Batista *et al.,* 2012), Malaysia (1.944-6.511 mg $kg^{-1}$) (Chua *et al.,* 2012).

The 25$^{th}$ most abundant element is Li in the earth's crust. Li is used in the battery, glass and ceramics, greases, metallurgy, chemical and pharmaceutical and rubber industries. The Li demand is rising in the future due to its application in strategic areas. Li is found in igneous rocks. Li can reach into plants through water and soil as a result of the wild storage of lithium-containing products. And these products, especially batteries, also contain the other toxic metals like Cu, Pb, Ni and organic chemicals (Meshram *et al.,* 2014). The Li concentrations of the honey samples are given in Table 3. There are no enough studies related to the Li content of the honey. In a study performed in Spain, Li concentration of the honey sample was reported as 9.2 µg $kg^{-1}$ (Latorre *et al.,* 1999) and this value was lower than our results.

## 4. Conclusion

In this study, the honey samples were classified as a chestnut honey. However, the detected parameters differed as a result of the pollen constituents from different taxa and frequencies. The qualities of the studied honey comply with the standards established by Codex Alimentarius (2001) and European Honey Directive (2001) and they can be classified as a good quality honey.

In the present study, seventeen metals were analyzed in three honey samples. The findings indicated that honey samples were effective in assessing the level of heavy metal pollution in the environment. Therefore, complex studies performed in all bee products can be useful for monitoring of environmental pollution with heavy metals, especially for the comparison of heavy metal contents with standard limits depending on body weight and honey consumption. However, the toxic element and mineral content of a honey can present any evidence of geographical, industrial and agricultural structures. Some suggestions can be given to decrease the metal contents of honey such as limiting the dangerous fertilizers consumption, monitoring of the agricultural soils and waters used in agricultural areas, controlling the quality of foods, considering the distance between industries and agricultural areas/flower gardens/etc., supplying some standards limits for hazardous components in the foods, air pollution monitoring, producing of food packing taking corrosive substances into account. The determination of metal levels in the honey can be useful for both the monitoring of the current situation and the avoiding of further problems.

**Acknowledgement**

The authors are grateful to Özlem Çapanoğlu Cox, Elif Esra Temiz, and Levent Gürel for their kind help with the English language. We wish to extend our thanks to the English-editing-service of Turkey.

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