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Original article

Metal content of southern Italy honey of different botanical origins and its correlation with polyphenol content and antioxidant activity

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Summary Seventy-eight samples of southern Italy honey from five different floral origins (chestnut, eucalyptus, citrus, multifloral and sulla) were screened to quantify the polyphenol and metal contents, evaluate the antioxidant activity and determine the correlations between the parameters analysed. The average polyphenol content was 12.06 mg gallic acid equivalent per 100 g honey and 7.92 mg quercetin equivalent per 100 g honey, for total phenolic and flavonoid contents, respectively. The antioxidant activity ranged from 58.40% (eucalyptus honey) to 60.42% (chestnut honey) in the ABTS assay, from 152.65 μM Fe (II) (citrus honey) to 881.34 μM Fe (II) (chestnut honey) in the FRAP assay, and from 54.29% (citrus honey) to 78.73% (chestnut honey) in the DPPH assay. Fe and Zn were the most abundant among the tested metals, while Cd, Co and Mo were those less present. Chestnut honey presented the highest polyphenol content, antioxidant activity and metal content. The correlations between the analysed parameters were statistically significant ($P < 0.05$). The correlations between metal content and both total phenolic and antioxidant activities were particularly interesting, suggesting a relationship between metal and polyphenol contents in honey.

Keywords ABTS, DPPH, FRAP, honey, metal content, total flavonoid, total phenolic.

Introduction

Honey is produced by honeybees from the nectar of different plants and honeydew. It contains many different substances, mainly sugars such as fructose, glucose and sucrose (65–75% of total soluble solids) in addition to various organic and inorganic acids, enzymes, vitamins, hormones, flavonoids, proteins, amino acids and elements. Honey composition depends on several factors, such as floral source used to collect the nectar, geographical origin, climatic conditions and seasonality (Frankel *et al*., 1998; Al-Mamary *et al*., 2002; Ghedolf *et al*., 2002; Gheldof and Engeseth, 2002). Southern Italy honeys represent a wide and diversified typology, consisting in more than 30 unifloral honeys with plenty of multifloral ones. This is attributable to the production areas having different climatic characteristics and a high diversity of the botanical species collected by bees. The high variability of the products therefore highlights the importance of honey authenticity, a fundamental requirement for all food products that can easily be adulterated. The authenticity of food products must be understood both as production authenticity and as origin authenticity. Numerous methods, both old and new generations (HPLC-MS, GC-MS, NMR, NIR, ES-MS), are used for the authentication of food products. However, given the high variability found in honey, conditioned by a wide range of factors, the application of multivariate analysis (Cluster analysis, Principal Component analysis, Causal Layered Analysis) is very frequent. This is useful for the authentication of products according to their botanical and geographical origin (Tzouros & Arvanitoyannis, 2001; Arvanitoyannis *et al*., 2005; Arvanitoyannis & Vlachos, 2007). The consumption of honey is partly related to the high sugary power, but the presence of substance, such as flavonoids and phenolic acids, highlights its role as a nutritional source of natural antioxidants responsible for protecting human health (Ghedolf *et al*., 2002; Gheldof and Engeseth, 2002). During the past decade, the use of honey as a therapeutic substance has been revalorised in a more scientific setting, as its antibacterial, antioxidant, anti-inflammatory and antitumoural properties were demonstrated (Tonks *et al*., 2001; Orsolic *et al*., 2005). Many authors highlighted a close

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correlation between antioxidant activity and polyphenol content (Gheldof and Engeseth, 2002; Beretta *et al*., 2005; Meda *et al*., 2005; Blasa *et al*., 2006). Among the components responsible for the antioxidant effect, there are flavonoids (chrysin, pinocembrin, pinobanksin, quercitin, kaempferol, luteolin, galangin, apigenin, hesperetin, myricetin) and phenolic acids (caffeic, p-coumaric, ferulic, ellagic, chlorogenic) (Gheldof and Engeseth, 2002). Those antioxidant compounds are synthesised by plants as secondary products serving in plant defence mechanisms to counteract reactive oxygen species (ROS) (Peterson & Dwyer, 1998; Robards *et al*., 1999; Wollgast & Anklam, 2000; Havsteen, 2002). A direct relationship has been found between phenolic content and antioxidant activity of the plants (Robards *et al*., 1999; Al-Mamary *et al*., 2002). The antioxidant polyphenols ability is linked to several mechanisms, such as free radical-scavenging, hydrogen-donation, single oxygen quenching, metal ion chelation, and action as substrate for superoxide and hydroxyl radicals (Bogdanov *et al*., 2008). Honey is also the result of a bioaccumulation process of many metals that can play an important role in a number of biochemical processes (Garcia *et al*., 2005). Co, Fe, Mo, Cr and Zn are oligoelements essential for a broad range of life functions, even if the body needs small amounts of them. Cd and Pb are considered among the most toxic metals. Indeed, international organisations such as the Food and Agriculture Organisation and the World Health Organisation have established the maximum daily intake: 7 μg Cd per kg body weight (FAO WHO, 2003) and 25 μg Pb per kg body weight (FAO WHO, 1993). These transition metals are present in honey at a low concentration. However, they are easily absorbed by the body as they are highly bio-available. Metal concentrations in different honey types largely depend on the mineral composition of nectar or honeydew, with regard to their botanical and geographical origin (Rashed & Soltan, 2004; Pisani *et al*., 2008). Many authors consider honey a biomarker of the bees' forage area and it may be useful as an environmental indicator of heavy metal pollution (As, Cd, Pb, Hg) (Przybylowski & Wilczynska, 2001; Tuzen *et al*., 2007; Rashed *et al*., 2009). The presence of metals may be caused by external sources such as industrial smelter pollution, emissions from factories, non-ferrous metallurgy, leaded petrol from busy highways, by incorrect procedures during the honey processing and conservation phases, and agrochemicals, such as Cd-containing fertilisers, organic Hg, and As-based pesticides that are still in use in some countries (Bratu & Beorgescu, 2005; Pisani *et al*., 2008; Rashed *et al*., 2009). Several authors (Dietz *et al*., 1999; Sahw *et al*., 2004) reported that high concentrations of Fe, Cu, Co, Zn, Cr, Mo induce oxidative stress in cells and tissues of the plant in the following ways: (i) transfer of electrons directly in single-electron reactions, which generate free radicals, (ii) influence on metabolic pathways, especially in the thylakoid membrane, and (iii) inactivation of the antioxidant enzymes responsible for ROS detoxification. Parry *et al*. (1994) and Diáz *et al*. (2001) found that plants subjected to heavy metals show an increase in the activity of the enzymes involved in the metabolism of phenolic compounds, suggesting their ex novo synthesis. The aim of our study was to quantify the total phenolic, flavonoid and metal contents, and to evaluate the antioxidant activity and the correlations between the analysed parameters in honeys from different floral origins of southern Italy. Because of the lack of a specific o cial analytical approach for determination of antioxidant property of honey, the comparison of various methods based on different principles and experimental conditions is necessary to determine the antioxidant behaviour of honey (Bertoncelj *et al*., 2007). The antioxidant assays here used were as follows: the ABTS assay, which highlights the activity of both hydrophilic and lipophilic antioxidants; the DPPH assay, used to detected the ability of some compounds to act as ROS scavengers; and the FRAP assay, which uses reductants in a redox-linked colorimetric method employing an easily reduced oxidant in stoichiometric excess.

Materials and methods

Chemicals

All used chemicals and solvents were of analytical grade. DPPH (1,1-diphenyl-2-picrylhydrazyl), TPTZ (2,4,6-tripyridyl-s-triazine), ABTS 2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt, gallic acid, quercetin, HCl, $FeSO_4$ $7H_2O$, $FeCl_3$, NaOH, $AlCl_3$, $K_2S_2O_8$, HNO_3, H_2O_2 were purchased from Sigma-Aldrich (Milan, Italy). Folin–Ciocalteu's reagent was purchased from Carlo Erba (Milan, Italy).

Samples

Seventy-eight honey samples of southern Italy were collected directly from beekeepers during the 2009 harvest. The floral origin of honey samples was directly characterised by the beekeeper in relation to the location where the beehives were situated and to the accessibility of plant food sources. The honey purity was carefully checked by pollen analysis. Pollen analysis was carried out according to DIN 10760 (DIN, 2002; Von der Ohe *et al*., 2004). On the basis of this analysis, the honey samples were classified into five categories: sixteen samples of chestnut honey (predominant pollen (PP): *Castanea sativa*; frequency (F): 75–90%), eighteen samples of sulla honey (PP: *Hedysarum* spp.; F: >50%), twelve samples of citrus honey (PP: *Citrus* spp.; F:65–76%), fourteen samples of eucalyptus honey

(PP: *Eucalyptus* spp.; F:82–93%) and eighteen samples of multifloral honey. Multifloral honey resulted being composed of a mixture of pollen belonging to different plant species with a synchronised flowering. Honey samples were stored at 4 C in the dark until analysed. The experiments were performed using freshly prepared 10% honey solutions in distilled water. The artificial honey (80% sugar, w v), serving as a blank, was prepared according to White (1979) by dissolving 4 g of fructose, 3 g of glucose, 0.8 g of maltose and 0.2 g of sucrose in distilled water to make a solution of 10 mL final volume. The solution was mixed for 1 h at 80 C. The antioxidant activity was calculated by subtracting the obtained values for the blank from that of each sample. All tests were performed in triplicate.

Determination of total phenolic and flavonoid contents

The Folin–Ciocalteu method as modified by Beretta *et al.* (2005) was used to determine the total phenolic content. This content was expressed as mg of gallic acid equivalent (GAE) per 100 g honey. Total flavonoid content was determined using the Dowd method, as adapted by Arvouet-Grand *et al.* (1994), and the results expressed as mg of quercetin equivalent (QE) per 100 g honey.

Determination of antioxidant activity in the reaction with ABTS cation radicals

A modification of the original method of Re *et al.* (1999) was applied to assess the scavenging capacity of honey samples in the reaction with the ABTS cation radical. ABTS $^{+}$ was produced by reacting 2,2′-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) diamonium salt (ABTS) with potassium persulphate ($K_2S_2O_8$). A stock solution (50 mL) of ABTS (2 mM) was prepared in phosphate-buffered saline (PBS), pH 7.4. ABTS $^{+}$ was produced by reacting 50 mL of stock solution with 200 μL of 70 mM $K_2S_2O_8$. The mixture was left in the dark at room temperature for 12–16 h before use. For the evaluation of antioxidant capacity, the ABTS $^{+}$ solution was diluted with PBS to obtain an absorbance of 0.700 ± 0.020 at 734 nm. Two millilitres of ABTS $^{+}$ solution were mixed with 100 μL of honey solution in a cuvette, and the decrease in the absorbance was measured after 10 min. The blank reagent was prepared by adding artificial honey instead of the sample. The percentage decrease in the absorbance at 734 nm was calculated by the formula:

$$I = [(A_B - A_A)/A_B] \times 100$$

where I = ABTS $^{+}$ inhibition, %; A_B = absorbancy of a blank sample (t = 0 min); A_A = absorbancy of a tested honey solution at the end of the reaction (t = 10 min).

Determination of antioxidant activity in the reaction with DPPH radicals

When the DPPH radical (DPPH) reacts with an antioxidant, it is reduced. The changes in colour (from deep violet to light yellow) were measured at 517 nm using a UV Vis spectrophotometer. The DPPH radical-scavenging activity of honey samples was determined according to the procedure described by Beretta *et al.* (2005), with some modifications. Honey samples were dissolved in distilled water at a concentration of 30–600 mg mL^{-1}. The assay mixture contained 1.9 mL of 130 μM DPPH dissolved in methanol, 1 mL of 0.1 M acetate buffer (pH 5.5) and 100 μL of honey solution. The mixture was shaken on a vortex mixer and then incubated for 60 min at 37 C in a water bath in the dark. The absorbance of the remaining DPPH was determined at 517 nm against a blank. The blank was honey at the same concentration as described earlier containing all reagents except DPPH . Antioxidant activity was expressed as a per cent of inhibition of DPPH radical and calculated from the same equation as for ABTS.

Determination of antioxidant activity by ferric reducing antioxidant power (FRAP) assay

The FRAP assay measures the change in absorbance at 593 nm owing to the formation of blue-coloured Fe^{2+}-TPTZ from colourless oxidised Fe^{3+}-TPTZ by the action of electron-donating antioxidants (Katalinic *et al.*, 2004). The assay was performed according to procedure described by Bertoncelj *et al.* (2007), with some modifications. The FRAP reagent was prepared by mixing 10 volumes of 300 mM acetate buffer (pH 3.6), 1 volume of 10 mM TPTZ in 40 mM HCl and 1 volume of 20 mM $FeCl_3$. It was daily prepared and warmed to 37 C before use. Honey samples (5 g) were diluted with 50 mL of 0.25 M phosphate buffer (pH 7.2). Aliquots of 200 μL of honey samples solutions were mixed with 1.8 mL of FRAP reagent, and the absorbance of each mixture was measured at 593 nm against a blank (artificial honey) after incubation at 37 C for 15 min. Aqueous standard solutions of $FeSO_4$ $7H_2O$ (100–1000 μM) was used for the calibration curve, and the results were expressed as the FRAP value (μM Fe(II)) of the honey solution.

Metals analysis

Five millilitres of nitric acid (0.1 M) plus 1 mL of hydrogen peroxide (30%, v v) were added to a beaker containing the ash of 5 g of honey sample; the mixture was stirred and then heated on a hotplate to almost complete dryness. Two millilitres of the HNO_3 (0.1 M) was added and the mixture was made up to a 10 mL with distilled water. The metal concentration was

determined in triplicate by inductively coupled plasma-atomic emission spectrometry (ICP-OES; model iCAP 6000, Thermo-Scientific, Cambridge, UK). The reference wavelengths for each metal were chosen avoiding interferences with the other elements analysed. Blanks (only HNO_3 and H_2O_2) and a standard stock solution of 50 ppm for each element was analysed for reference purposes. Results were expressed as mg metal per kg honey (ppm).

Statistical analysis

Statistical analysis was performed using the general linear model procedure of statistical analysis system (SAS, 1996), using a monofactorial model:

$$y_{ik} = \mu + \alpha_i + \epsilon_{ik}$$

where μ = average mean; α_i = effect of botanical origin (1, ,5); and ϵ_{ik} = experimental error. Before setting the values, expressed in percentage terms, they were subjected to angular transformation. The Student's *t*-test was used for all variables comparisons. A Pearson's correlation test was conducted to determine the linear correlation among the variables. Differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

Results and discussion

Total content of phenolic compounds and flavonoids

Polyphenols are important components of the honey present in small amounts and derived from the pollen of the plants visited by bees. The content in phenolic compounds and flavonoids of the studied 78 honeys is reported in Table 1. Total phenolic (mg GAE per 100 g honey) and total flavonoid (mg QE 100 g honey) contents showed a high and significant ($P < 0.05$) variability among the different types of honey. The values ranged between 10.82 and 14.67 mg GAE per 100 g honey for total phenolic content, and between 5.09 and 14.05 mg QE per 100 g honey for total flavonoid content. The average polyphenol contents (12.06 mg GAE per 100 g honey and 7.92 mg QE per 100 g honey, for total phenolic and flavonoid contents, respectively) was similar to those found in Italian honeys by Pichichero *et al.* (2009), while it resulted significantly higher than the values reported by Ferreres *et al.* (1992) in rosemary honey, by Martos *et al.* (1997) in Tunisian honey and by Socha *et al.* (2011) in Polish honey. The polyphenol content was highest in chestnut honey ($P < 0.05$): around 80% more than the total average value of flavonoids (14.05 vs. 7.92 mg QE per 100 g honey), and around 22% more than the total average value of phenolic compounds (14.67 vs. 12.06 mg GAE per 100 g honey). The lowest content in total phenolic was found in sulla honey (10.82 mg GAE per 100 g honey). These results are in agreement with those reported by other authors (Beretta *et al.*, 2005; Bertoncelj *et al.*, 2007; Pichichero *et al.*, 2009). The differences in the values of total phenolic and flavonoid contents among the analysed honey samples were likely due to the variation of their floral source (Aljadi & Kamaruddin, 2004; Beretta *et al.*, 2005; Bertoncelj *et al.*, 2007).

Table 1 Total phenolic and flavonoid contents measured in honey samples of different botanical origins

	Parameter	
Honey	Phenolic content (mg GAE per 100 g honey)	Flavonoid content (mg QE per 100 g honey)
Chestnut	14.67 ± 4.64[a]	14.05 ± 8.03[a]
Eucalyptus	11.29 ± 2.50[b]	5.73 ± 1.88[b,c]
Multifloral	11.59 ± 3.22[b]	8.80 ± 4.49[b]
Citrus	11.92 ± 3.82[a,b]	5.09 ± 2.51[c]
Sulla	10.82 ± 6.89[b]	5.96 ± 5.84[b,c]
Total	12.06 ± 4.58	7.92 ± 5.97

Mean values from three repetition ± standard deviations.
[a,b,c]Means in the same column with different letters are significantly different according to the Student's t-test ($P < 0.05$).

Antioxidant activity

Among the main factors responsible for the biological and nutraceutical activities, phenolic substances of honey have a key role (Al-Mamary *et al.*, 2002; Aljadi & Kamaruddin, 2004). Most plants contain an extensive number of polyphenols, and each plant tends to have a distinctive profile. Furthermore, the concentration and type of polyphenols depend on the floral origin of honey. The antioxidant activity of phenolic compounds lies mainly in their chemical structure and is manifested by different mechanisms (Michalak, 2006). Honeys from different floral sources possess strong antioxidative activities and are strong ROS scavengers (Nagai *et al.*, 2006). The antioxidant activity of honey samples was assessed by three different tests: the ABTS, FRAP and DPPH assays. In general, the results showed that all the tested samples exhibited antioxidant activity that varied in a wide range (Table 2). Average values varied from 58.40% (eucalyptus honey) to 60.42% (chestnut honey) in the ABTS assay, from 152.65 μM Fe (II) (citrus honey) to 881.34 μM Fe (II) (chestnut honey) in the FRAP assay and from 54.29% (citrus honey) to 78.73% (chestnut honey) in the DPPH assay. These values are higher than those reported by other authors (Bertoncelj *et al.*, 2007; Al *et al.*, 2009), suggesting a higher comparative nutraceutical potential. In all the assays,

Table 2 ABTS, FRAP and DPPH values of honey samples from different botanical origins

	Parameter		
Honey	**ABTS (I %)**	**DPPH (I %)**	**FRAP (μM Fe(II))**
Chestnut	60.42 ± 2.12[a]	78.73 ± 9.35[a]	881.34 ± 327.21[a]
Eucalyptus	58.40 ± 1.65[b]	72.12 ± 7.86[a]	252.02 ± 121.07[b]
Multifloral	58.99 ± 1.28[b]	62.26 ± 8.94[b]	208.97 ± 114.27[b]
Citrus	58.88 ± 1.26[b]	54.29 ± 8.70[c]	152.65 ± 114.27[b]
Sulla	58.42 ± 2.18[b]	64.55 ± 16.95[b]	240.07 ± 240.29[b]
Total	59.02 ± 1.86	66.39 ± 13.57	347.01 ± 333.91

Mean values from three repetition ± standard deviations.
[a,b,c] Means in the same column with different letters are significantly different according to the Student's t-test ($P < 0.05$).

chestnut honey presented the greater antioxidant activity. In particular, the FRAP value of chestnut honey was very high (881.34 μM Fe(II); $P < 0.05$) if compared with the other studied honeys, exceeding by more than 2.5 times the average (347.01 μM Fe(II)). The antioxidant activity of honey samples as measured by the FRAP assay decreased in the order: chestnut >eucalyptus (252.02 μM Fe(II)) >sulla (240.07 μM Fe(II)) >multifloral (208.97 μM Fe(II)) >citrus. Citrus honey showed a mean activity of 152.65 μM Fe(II), about six times lower than that of the chestnut honey. The FRAP value in chestnut honey was considerably higher than that reported by Beretta *et al.* (2005) and Bertoncelj *et al.* (2007). The antioxidant activity of honey samples measured by DPPH assay decreased in the same order of the FRAP assay. By contrary, the trend of the antioxidant activity evaluated by ABTS assay partly confirmed the results obtained by DPPH and FRAP assays. Indeed, except for chestnut honey, the differences between honey samples were not significant. DPPH and ABTS values showed a low variability around the mean value, indicating the close link with the botanical species. The differences found among the honey types strengthen the widely accepted theory that the antioxidant activity of honey varies greatly depending on the floral source and on external factors, such as season and environment, as well as the processing method used (Gómez-Caravaca *et al.*, 2006).

Metal content

In this study, eight metals were identified and quantified: Cd, Co, Cr, Zn, Fe, Pb, Mo and As (Table 3). As multifloral honey is not representative of a specific areal, the comparisons between the metal contents of unifloral honey and multifloral ones are not very important. On the other hand, these values are interesting to get an indication of the parameter in the grazing areas that are characterised by heterogeneous floristic associations. No As above the detection limit was observed in the samples. Fe and Zn were the most abundant among the tested metals, with an average concentration of 15.043 and 11.846 ppm, respectively. The concentrations of Cd, Co and Mo showed values below 0.1 ppm, while those of Cr and Pb ranged between 0.521 and 0.889 ppm and between 0.169 and 0.498 ppm, respectively. Generally, Cd content in our honeys (0.013 ppm) was similar to the mean values found by Przybylowski & Wilczynska (2001)in floral Polish honeys (0.015 ppm), while the levels of Pb and Zn were higher, approximately 3.5-fold and 2-fold higher, respectively. The heavy metals content was higher than that found by other authors (Buldini *et al.*, 2001; Osman *et al.*, 2007; Pisani *et al.*, 2008). The high level of Zn and Fe found in our samples may be related to a source of contamination, such as beekeepers, equipment and tools used, and environment (Gajek *et al.*, 1987; Caroli *et al.*, 1999). The contamination level of Pb and Cd, very toxic elements, resulted below the levels of risk defined by WHO (1982). Therefore, the studied honeys can be

Table 3 Mineral content measured in honey samples of different botanical origins.

	Type of honey					
Parameter (ppm)	**Chestnut Mean ± SD**	**Eucalyptus Mean ± SD**	**Multifloral Mean ± SD**	**Citrus Mean ± SD**	**Sulla Mean ± SD**	**Total Mean ± SD**
Cd	0.017 ± 0.007[a]	0.006 ± 0.004[b]	0.013 ± 0.004	0.014 ± 0.015[c]	0.017 ± 0.009[a]	0.013 ± 0.009
Co	0.046 ± 0.012[a]	0.010 ± 0.010[b]	0.014 ± 0.011	0.026 ± 0.026[c]	0.033 ± 0.029[d]	0.026 ± 0.022
Cr	0.830 ± 0.632[a,c]	0.532 ± 0.439[b]	0.521 ± 0.338	0.762 ± 0.364[a]	0.889 ± 0.704[c]	0.707 ± 0.528
Fe	27.294 ± 8.858[a]	9.624 ± 5.543[b]	11.610 ± 3.899	12.477 ± 6.278[c,d]	14.213 ± 12.715[d]	15.043 ± 10.115
Mo	0.056 ± 0.029[a]	0.064 ± 0.041[a,c]	0.093 ± 0.090	0.103 ± 0.055[b]	0.077 ± 0.037[c]	0.078 ± 0.056
Pb	0.498 ± 0.340[a]	0.190 ± 0.243[b,d]	0.169 ± 0.161	0.367 ± 0.318[c]	0.222 ± 0.272[d]	0.289 ± 0.276
Zn	17.869 ± 21.247[a]	7.247 ± 2.971[b]	9.389 ± 5.471	9.672 ± 4.254[c]	15.056 ± 12.381[d]	11.846 ± 11.236
As	nd	nd	nd	nd	nd	nd

nd, below detection limit (not detected).
[a,b,c,d]Means in the same row with different letters are significantly different according to the Student's t-test ($P < 0.05$).

considered safe for consumption as far as these parameters were concerned. There are no specific maximum residue limit values for honeys, but thresholds of 0.1 mg kg^{-1} for Cd and 1 mg kg^{-1} for Pb have been suggested for European Union (Byrne, 2000). Fernández-Torres *et al.* (2005) have found a significant relationship between botanical origin and composition of metals of honey. Afik *et al.* (2008) detected a similar mineral content in the nectar and honey of citrus and avocado. Our results (Table 3) showed a high variability around the mean metal value. This is likely influenced by other factors not considered in the present study such as the geographical origin. Generally, the metal content of chestnut honey was significantly higher than that other honeys ($P < 0.05$). This honey was especially rich in Fe, Zn and Pb (27.294, 17.869 and 0.498 ppm, respectively), whose levels resulted higher than those reported by González-Miret *et al.* (2005). The Cr content varied from 0.521 to 0.889 ppm, showing the highest levels in sulla and chestnut honey. The Pb content presented the highest level in chestnut honey ($P < 0.05$). We hypothesised that high level of Pb (in chestnut honey) and Cr (in chestnut and sulla honeys), transported from plants to honey, could be the symptom of a diffuse-type pollution because of the fuel combustion by cars, as observed by Aspetti *et al.* (2002). Our honeys showed high variability in Fe content, certainly because of the different botanical sources, and these values decreased in the following order: 27.294 ppm (chestnut) < 14.213 ppm (sulla) < 12.477 ppm (citrus) < 11.610 ppm (multifloral) < 9.624 ppm (eucalyptus). This can be explained taking into account that, among trace minerals, Fe is the most important for the classification of monofloral honeys, because it is a micronutrient that plays an important role in plants (Bogdanov *et al.*, 2007). The honeys here studied revealed an antioxidant activity significantly correlated with the content in heavy metal, as well as to the content in flavonoids and phenolic compounds.

Correlations

Another objective of this study was to evaluate the correlation between the analysed parameters of the different types of honey (Table 4). The positive and statistically significant correlation was found between total phenolic and flavonoid contents ($r = 0.30$; $P < 0.01$). This result is similar to that reported by Meda *et al.* (2005) in honey from Burkina Faso, while Alvarez-Suarez *et al.* (2010) reported a higher correlation coe cient ($r = 0.83$). The low correlation coe - cient was likely caused by the methods used to determine total phenolic and flavonoids: the Folin–Ciocalteu method determines an overestimation of the content in total phenolic, because the reactive compounds can react with the chemical groups of amino acids and proteins, while the aluminium chloride method, used to determine total flavonoids, tends to underestimate its content, because it is specific only for flavones and flavonols as repoted by Chang *et al.* (2002). Kenjerić *et al.* (2007) found that the amount of polyphenols, especially flavonoids, was higher in the honey produced under high temperatures and humidity conditions. Comparing our data with those reported for different types of fruit and vegetables (Marinova *et al.*, 2005), we found that polyphenol contents of the honey samples was similar to that found in leek and beans. These results showed that honey is comparable to fruits and vegetables (Cao *et al.*, 1996; Wang *et al.*, 1996), indicating that it may have a role as a dietary sources of antioxidants in highly palatable form. A positive correlation was found between total flavonoid contents and FRAP value ($r = 0.66$; $P < 0.001$), while a lower correlation was found between total phenol contents and FRAP value ($r = 0.36$; $P < 0.001$), suggesting that the reducing power of honey may be due to flavonoid contents that can reduce Fe^{+3} to Fe^{+2}. The antioxidant ability depends on the number and position of OH- groups present in the flavonoid structure. The values of the correlation coe cient between total phenolic content and DPPH value ($r = 0.48$; $P < 0.001$) and between total flavonoid content and DPPH value ($r = 0.43$; $P < 0.001$) were positive, further suggesting the relationship between polyphenol concentrations and relative antioxidant potential of the honeys. A positive correlation ($r = 0.61$; $P < 0.001$) was found between flavonoid content and ABTS value. Beretta *et al.* (2005) showed values of r much higher than those found in this study: 0.885 for phenolic content measured by FRAP and 0.918 for phenolic content measured by DPPH. In this study, the correlations between the antioxidant assays, measured as ABTS, FRAP and DPPH assays, were analysed (Table 4). The r value obtained from

Table 4 Correlation matrix (Pearson correlation coefficients) among the considered parameters

	Phenolic content	Flavonoid content	ABTS	DPPH	FRAP
Flavonoid content	0.30**				
ABTS	0.48***	0.61***			
DPPH	0.48***	0.43***	0.62***		
FRAP value	0.36***	0.66***	0.56***	0.61***	
Cd	0.29**	0.004^{ns}	0.31**	0.41***	0.15^{ns}
Co	0.34***	0.22*	0.49***	0.55***	0.49***
Cr	0.48***	-0.16^{ns}	0.23*	0.31**	0.22*
Fe	0.58***	0.42***	0.56***	0.67***	0.73***
Pb	0.37***	0.05^{ns}	0.13^{ns}	0.30**	0.38***
Zn	0.38***	0.14^{ns}	0.40***	0.32**	0.48***

***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; ns not significant.

ABTS and DPPH assays were slightly lower ($r = 0.62$; $P < 0.001$) than that reported by Baltrusaityte *et al.* (2007) ($r = 0.716$). This suggests that the reaction kinetics of radical scavenging in the two systems are different. The correlation between FRAP and DPPH values was statistically significant ($r = 0.61$; $P < 0.001$), but this correlation coe cient was statistically lower if compared with that reported by Bertoncelj *et al.* (2007) ($r = 0.894$; $P < 0.05$). The low values of correlations confirm that phenolic compounds contribute to the antioxidant activity, but are not solely responsible for it. The type and quantity of the phenolic compounds and the presence of non-phenolic antioxidant (catalase, ascorbic acid, proteins) could have contributed to the antioxidant activity. The correlations between metal content and both polyphenol content and antioxidant activity were analysed (Table 4). No correlation was observed between the Mo content and both polyphenol content and antioxidant activity. The data obtained showed a positive and significant correlation ($P < 0.01$) between total phenolic and metal contents, with $r > 0.30$. In particular, a statistically significant ($P < 0.001$) correlation was observed between total phenolic and Co, Cr, Fe, Pb, Zn contents ($r = 0.34$, 0.48, 0.58, 0.37 and 0.38, respectively). The correlation coe cients between total flavonoid and metal content were low and not significant, except for the correlations between total flavonoid and both Co and Fe content, with $r = 0.22$ ($P < 0.05$) and 0.42 ($P < 0.001$), respectively. Despite a wealth of literature information on mineral content of honey and on the induced accumulation of phenolic compounds in plants treated with metals, to our knowledge, there are no available reports on the correlation between metal content and both total phenolic and antioxidant activities. The induction of phenolic compound biosynthesis was observed in wheat in response to nickel toxicity (Diáz *et al.*, 2001), and in maize to response to aluminium (Winkel-Shirley, 2002). *Phaseolus vulgaris* exposed to Cd^{2+} accumulates soluble and insoluble phenolics (Smeets *et al.*, 2005). Our results support the thesis reported by many authors (Bishnoi *et al.*,1993; Diáz *et al.*, 2001; Sivaci *et al.*, 2007), according to which higher levels of heavy metals in the plant induce polyphenols synthesis. In addition, some researchers suggested that an imbalance of minerals would change the content of polyphenols: P deficiency may lead to an increase in flavonoids level (Lillo *et al.*, 2007); Zn-deficient or Zn-excess conditions, in bean plants, cause changes in the antioxidant enzyme activities (Prabhu Inbaraj & Muthuchelian, 2011). Tewari *et al.* (2006) recorded an increase in activities of antioxidative enzymes such as superoxide dismutase in mulberry Mg-deficient plants. Our results (Table 4) also showed a positive and statistically significant ($P < 0.05$) correlation between metal content and antioxidant activity. The interaction of flavonoids with metal ions may also change the antioxidant properties and biological effects of the flavonoids (Kostyuk *et al.*, 2001). Only the correlations between ABTS value and Pb content, and between FRAP value and Cd content were not significant. Noteworthy, high values of r and a high statistical significance ($P < 0.001$) in the correlations between Fe, Co and Zn content and ABTS, FRAP and DPPH values were observed. Pohl and Sergiel (2010) demonstrated that Cd, Fe and Mn can form stable complexes with a variety of organic substances present in the honey matrix, including organic acids, proteins, amino acids, polyphenols, vitamins and aroma compounds. Other authors (Bors *et al.*, 1990; Moridani *et al.*, 2003) hypothesise that the biological activity of an organic ligand could increase when co-coordinated or mixed with suitable metal ions, because of their ability to act as free radical acceptor. Bukhari *et al.* (2008) showed that the Co-quercetin complex is a much more effective free radical scavenger than the free flavonoid. Chaoui *et al.* (1997) found that in *Phaseolus vulgaris,* some antioxidant enzymes can be activated, notably in the upper plant parts, in response to the oxidative stress induced by Cd and Zn. Sulaiman *et al.* (2011), in Malaysian bananas, have found no correlation between mineral content and antioxidant activity, except for a positive correlation between Mn content and DPPH value that might be due to the role of Mn in activating enzymes that enhance the biosynthesis of flavonoids (Gordon, 2007).

Conclusions

The studied honeys showed high levels of phenolics and flavonoids, which function as effective natural antioxidants. The polyphenol content in honey resulted to be a product peculiarity conferred by the botanical origin, which markedly influences the antioxidant activity measured by ABTS, FRAP and DPPH assays. The significant effect of the botanical origin on the antioxidant activity was confirmed by the low variability around the mean value of the studied parameters. Among the studied honeys, chestnut honey presented the highest polyphenol content, antioxidant activity and metal content. The high variability of mean metal content highlighted the influence of other factors not related to the botanical species, such as the geographical origin. Positive and statistically significant correlations were observed between the parameters analysed. The correlations between the metal content and both total phenolic and antioxidant activities were particularly interesting, suggesting the influence of minerals on the polyphenol contents in plant and consequently in honey. Overall, it can be concluded that the different types of the studied honey can be considered particularly useful for human nutrition because of the presence of bioavailable antioxidant compounds.

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