International Journal of
Food Science & Technology
International Journal of Food Science and Technology 2013

Original article

A comparative study on phenolic profile, vitamin C content and antioxidant activity of Italian honeys of different botanical origin

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(Received 27 November 2012; Accepted in revised form 24 March 2013)

Summary The aim of our study was to identify and quantify the phenolic acids, flavonoids and vitamin C and to evaluate the antioxidant activity in ninety Italian honeys of different botanical origins (chestnut, sulla, eucalyptus, citrus and multifloral). The results showed that total phenolic and flavonoid contents varied from 11.08 to 14.26 mg GAE per 100 g honey and from 5.82 to 12.52 mg QE per 100 g honey, respectively. HPLC–UV analysis showed a similar but quantitatively different phenolic profile of the studied honeys. Vitamin C is present in all samples. Multifloral honey showed the highest amount of the detected total phenolic compounds and the highest vitamin C content. The DPPH value varied from 55.06 to 75.37%. Among the unifloral honeys, chestnut honey presented the highest levels of phenolic acids, flavonoids and vitamin C, which are closely associated with its high antioxidant activity. The results show that honey contains high amount of biologically active compounds, which play an important role in defining the nutraceutical quality of the product, and that the distribution of these compounds is influenced by the botanical origin.

Keywords Antioxidants, botanical source, DPPH, flavonoids, honey, HPLC analysis, phenolic acids, total polyphenol, vitamin C.

Introduction

Honey has a complex composition consisting of a high concentration of sugars, water, minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids and enzymes. These components define both the physical properties and the nutritional and nutraceutical characteristics of the product itself (Yaoa *et al*., 2005). Italy has the highest number of honey varieties in Europe: thirty two unifloral and different varieties of multifloral. In 2009, honey production reached about 2600 tons per year in southern Italy, which means about 22.8% of the total national production (Rete Rurale Nazionale, 2007–2013). Southern Italy honeys represent a wide and diversified typology, consisting in more than thirty 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 consumption of honey is partly related to the high sugary power, but during the past decade, the use of honey as a therapeutic substance has been revalorised in a more scientific setting (Tonks *et al*., 2001; Orsolic *et al*., 2005). Many authors highlighted a close correlation between antioxidant activity and polyphenol content (Gheldof & Engeseth, 2002; Beretta *et al*., 2005; Meda *et al*., 2005; Blasa *et al*., 2006). The honey phenolic compounds are products of secondary plant metabolism, and their content varies according to the species, variety, organ considered, physiological stage, pedoclimatic characteristics (Robards *et al*., 1999). The antioxidant capacity of the phenolic compounds is attributed to their chemical structure. They are characterised by an aromatic ring with one or more hydroxyl substituents and, in many cases, serves in plant defence mechanisms to counteract reactive oxygen species (Peterson & Dwyer, 1998; Havsteen, 2002). These compounds are classified into two groups: phenolic acids, including phenolic esters (Amiot *et al*., 1989; Sabatier *et al*., 1992; Andrade *et al*., 1997a,b), and flavonoids (Ferreres *et al*., 1991, 1992, 1993, 1994a,b; Martos *et al*., 2000a,b). Polyphenols may act in various processes, such as free radical scavenging, hydrogen donation, single oxygen quenching, metal ion chelation, substrate for superoxide and hydroxyl radicals (Rice-Evans *et al*., 1995; Kähkönen *et al*., 1999; Michalak, 2006; Bogdanov *et al*., 2008).

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doi:10.1111/ijfs.12169

In recent years, many researchers have suggested the use of phenolic compounds as floral markers for honeys from different botanical and geographical origin (Martos *et al*., 2000a). Among the components with antioxidant activity are also included the water-soluble vitamins, such as vitamin C (L-ascorbic acid). Vitamin C acts as a reducing agent capable of rapidly scavenging a number of reactive oxygen (ROS) and nitrogen (RSN) species, providing an important antioxidant protection: in the eye, against photolytically generated free radical damage; in neutrophils, against ROS produced during phagocytosis; and in semen, against oxidative damage to sperm deoxyribonucleic acid (DNA) (Frei *et al*., 1988; Fraga *et al*., 1991; Levine *et al*., 1994; Delamere, 1996). Leon-Ruiz *et al*. (2011) found that L-ascorbic acid can be considered as marker for different honey types. The aim of our study was to identify and quantify the phenolic acids, flavonoids and vitamin C and to evaluate the antioxidant activity in Italian honeys of different botanical origins (chestnut, sulla, eucalyptus, citrus and multifloral).

Figure 1 Map of Italy showing the sampling sites of the honey samples.

Materials and methods

Chemicals

All used chemicals and solvents were of analytical grade. L-ascorbic acid, gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, ferulic acid, benzoic acid, (−)-epicatechin, (+)-catechin, (−)-gallocatechin, rutin, quercitin, myricetin, hesperetin, HPLC-grade methanol, potassium phosphate monobasic for HPLC, DPPH (1,1-diphenyl-2-picrylhydrazyl), $AlCl_3$ were purchased from Sigma-Aldrich (Milan, Italy). Folin–Ciocalteus reagent was purchased from Carlo Erba (Milan, Italy).

Samples

Ninety honey samples of southern Italy (Fig. 1) were collected from individuals apiarists during the 2009 harvest in the corresponding flowering season: chestnut honey (*Castanea sativa*) was collected between April and October; eucalyptus honey (*Eucalyptus* spp.) between June and August; citrus honey (*Citrus* spp.) between April and May, sulla honey (*Hedysarum* spp.) between April and June and multifloral honey between April and October. The honey purity was carefully checked by pollen analysis carried out according to DIN 10760 (DIN, 2002; Von der Ohe *et al*., 2004). 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. A sugar analogue (80% sugar, w/v), serving as a blank, was prepared by dissolving 0.2 g of sucrose, 0.8 g of maltose, 4 g of fructose and 3 g of glucose in distilled water to make a solution of 10 mL final volume (White, 1979). All tests were performed in triplicate.

Determination of total phenolic and flavonoid contents

The total phenolic content of honeys was estimated according to the Folin–Ciocalteu method as modified by Beretta *et al*. (2005). Gallic acid (0–200 mg L^{-1}) was used as standard to derive the calibration curve, and the results were 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). Quercetin (0–200 mg L^{-1}) was used as standard to derive the calibration curve, and the results were expressed as mg of quercetin equivalent (QE) per 100 g honey.

HPLC–UV analysis of phenolic compounds and vitamin C

Preparation of honey samples

Sample preparation for HPLC was carried out as described by Liang *et al*. (2009). Briefly, three grams of honey was put into a flask of 10 mL capacity and dissolved with 5 mL distilled water. The flask was placed in an ultrasound (US) water bath apparatus (Elma Transsonic 460/H, Singen, Germany) for 10 min at 25 °C. The honey sample was homogenised and filtered through a 0.45-μm cellulose acetate membrane filter (Sigma-Aldrich), and it was kept in the freezer until the chromatographic analysis.

Identification and quantification of phenolic compounds
The honeys' phenolic compounds analysis was performed in liquid chromatography equipped with Varian ProStar Pump model 210, Rheodyne injector with a 20-μL loop, UV–VIS detector Varian ProStar model 325 and using Galaxie™ chromatography software (Varian, Inc., Walnut Creek, CA, USA). The analyses were carried out with the internal standard (ISTD) calibration method. The samples were injected in the HPLC system using a Hypersil gold C18 column (250 × 4.6 mm, 5 μm) connected with a Hypersil gold guard column (10 × 4.0 mm, 5 μm) (Thermo Fischer Scientific, Milan, Italy). The mobile phase consisted of potassium dihydrogen phosphate buffer at pH 2.92 (eluent A) and methanol (eluent B). The injection volume for all samples was 20 μL. The elution was with flow rate 0.4 mL min^{-1}, and the gradient programme was as follows: 100–95% A (5 min), 95–85% A (40 min) and which then becomes isocratic until the end of analysis in the 60 min. The phenolic compounds were detected at 320 and 280 nm, and identification was carried out by comparing retention time and spectral characteristics of unknown analytes with those from commercial standards (Yao *et al*., 2003).

Identification and quantification of vitamin C
The vitamin C analysis was performed by isocratic RP-HPLC method with UV detection at 254 nm. Isocratic elution with potassium dihydrogen phosphate buffer at pH 2.92: methanol (95:5, v/v) was used at a flow rate of 0.5 mL min^{-1}. Vitamin C was identified by comparing its UV spectrum and retention time with that of standard. The content of L-ascorbic acid was calculated on the basis of the calibration curve of L-ascorbic acid (0.05–1.00 mg mL^{-1}), and the results were expressed as mg of L-ascorbic acid per kg honey. The validation parameters for HPLC method for both polyphenols and vitamin C content were showed in Table 1. These parameters were calculated according to ICH guidelines based on the standard deviation of the regression lines of specific calibration curves (ICH, 1995). Accuracy of phenolic compounds and vitamin C was found by studying level of recovery adding the standards at three different concentrations to the honey samples prior to applying the extraction procedure. Intraday and interday precisions were analysed six times in the same day and for three consecutive days by injecting the same sample solution. The limits of detection (LOD) and quantification (LOQ) were separately determined at a signal-to-noise ratio (S/N) of 3 and 10, respectively. The LOD and LOQ were experimentally verified by diluting known concentrations of compounds until the average responses were approximately three or ten times the standard deviation of the responses for six replicate determinations.

DPPH radical scavenging activity

DPPH radical scavenging activity of honeys 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 $DPPH^{\bullet}$ 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 DPPH solution in the absence of sample was used as control, and the methanol was used as blank. The absorbance of the remaining $DPPH^{\bullet}$ was determined at 517 nm using a UV–Vis spectrophotometer 1204 (Schimadzu, Tokyo, Japan) against a blank. The radical scavenging activity was expressed as a per cent of inhibition of DPPH radical and calculated by the following equation:

$$\text{Percentage of DPPH inhibition } (I) = [(A_B - A_A)/A_B] \times 100$$

where, I = DPPH inhibition,%; A_B = absorbance of the control; A_A = absorbance in the presence of the honey solution.

Statistical analysis

Statistical analysis was performed using the general linear model (GLM) 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); 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. Differences between means at the 95% ($P < 0.05$) confidence level were considered statistically significant.

Results and discussion

Melissopalynological analysis

The results of the honey pollen analysis are showed in Table 2. This analysis confirmed the botanical origin of honey, and the samples were classified into five categories, namely chestnut, sulla, citrus, eucalyptus and multifloral honeys. Multifloral honey was composed of a mixture of pollen belonging to different plant species with a synchronised flowering. Pollen analysis of multifloral honey showed the presence of more than 200 types of pollen and among these none were dominant. The pollen data reflected the patterns in the

Table 1 Validation parameters for HPLC method

Compounds	Concentration range (mg L^{-1})	Regression equation	R^2	Recovery (R %) $n = 6$	RSD (%)	Precision (% RSD) Intraday $n = 6$	Precision (% RSD) Interday $n = 6$	LOD (mg L^{-1})	LOQ (mg L^{-1})
Vitamin C	0.05–1	$Y = 0.0084x^2 + 0.2762x$	1	101.38	1.23	0.63	1.13	0.02	0.12
Gallic acid	1–20	$Y = 0.0001x^2 + 0.2377x + 0.1257$	0.9999	99.32	3.00	0.60	1.42	0.04	1.78
Chlorogenic acid	1–20	$Y = 2E{-}05x^2 + 0.2035x$	0.9997	99.55	1.11	0.51	1.96	0.13	1.25
Caffeic acid	1–20	$Y = 0.0003x^2 + 0.2035x$	0.9994	99.79	1.12	0.80	2.80	0.03	0.60
p-Coumaric acid	1–20	$Y = 2E{-}05x^2 + 0.2037x$	0.9998	98.50	1.16	1.40	2.60	0.06	0.24
Ferulic acid	1–20	$Y = 7E{-}05x^2 + 0.2055x$	1	100.00	2.23	1.80	2.37	0.08	0.13
Benzoic acid	1–20	$Y = -6E{-}05x^2 + 0.3626x$	0.9997	99.01	1.54	0.71	1.76	0.18	1.21
Gallocatechin	1–20	$Y = 0.0013x^2 + 0.4516x$	0.9999	98.76	1.24	0.30	1.32	0.06	0.28
Catechin	1–20	$Y = -4E{-}05x^2 + 0.2627x$	0.9997	99.80	1.36	0.72	0.96	0.05	0.12
Epicatechin	1–20	$Y = 0.0005x^2 + 0.3438x$	0.9998	99.97	1.44	0.66	0.80	0.04	0.14
Myricetin	1–20	$Y = 0.001x^2 + 0.4876x$	0.9993	99.90	1.09	1.37	1.65	0.06	0.50
Quercetin	1–20	$Y = -0.0003x^2 + 0.2803x$	0.9992	100.80	2.37	0.67	0.88	0.03	0.15
Rutin	1–20	$Y = 0.001x^2 + 0.421x$	0.9995	100.4	1.57	0.48	1.60	0.07	1.23
Hesperetin	1–20	$Y = -1E{-}0.6x^2 + 0.2021x$	0.9998	98.70	1.30	1.10	1.30	0.08	0.40

LOD, limit of detection; LOQ, limit of quantification; RSD, relative standard deviation, expressed as %.

Table 2 Pollen analysis of honey samples

Honey sample	No. of sample	Characterising Pollen	Frequency (%)	Others pollens
Chestnut	18	*Castanea sativa*	75–90	*Eucalyptus* sp., *Rhamnaceae, Rubus* sp. *Trifolium repens, Trifolium pratense, Cruciferae, Echium vulgare, Umbelliferae, Hedysarum coronarium*
Eucalyptus	18	*Eucalyptus* spp.	82–93	*Castanea sativa, Cruciferae, Asparagus acutiflolius, Trifolium repens*
Citrus	18	*Citrus* spp	65–76	*Cruciferae, Eucalyptus, Castanea sativa, Hedysarum coronarium, Rhamnaceae, Rubus* sp., *Echium* sp.
Sulla	18	*Hedysarum* spp.	>50	*Trifolium pratense, Lotus corniculatus, Rubus* sp., *Vicia* sp., *Trifolium repens, Onobrychis viciifolia*

vegetation characterising the bees pasture area, significantly influenced by the geographical origin.

Total phenolic and flavonoid contents

Polyphenols are important components of the honey present in small amounts and derived from the pollen of the plants visited by bees. Total phenolic and flavonoid contents of the studied honeys are reported in Table 3. The values ranged from 11.08 to 14.26 mg GAE per 100 g honey for total phenolic content and from 5.82 to 12.52 mg QE per 100 g honey for total flavonoid content. The average polyphenol content (12.01 mg GAE per 100 g honey and 8.05 mg QE per 100 g honey for total phenolic and flavonoid contents, respectively) was in close agreement with the results found in Italian honeys by other authors (Pichichero *et al*., 2009; Perna *et al*., 2012), while Socha *et al*. (2011), in Polish lime and multifloral honeys, and Martos *et al*. (1997), in Tunisian eucalyptus, orange and multifloral honeys, found lower values. The polyphenol content of honey samples decreased in the order: chestnut > multifloral (9.02 mg QE per 100 g honey) > sulla (6.76 mg QE per 100 g honey) > eucalyptus (6.16 mg QE per 100 g honey) > citrus for total flavonoid content, and in the order: chestnut > citrus (12.08 mg GAE per 100 g honey) > multifloral (11.38 mg GAE per 100 g honey) > sulla (11.26 mg GAE per 100 g honey) > eucalyptus for total phenolic content. The values found in studied samples match the results reported by other authors (Beretta *et al*., 2005; Bertoncelj *et al*., 2007; Pichichero *et al*., 2009; Perna *et al*., 2012). Total phenolic and flavonoid contents were the highest in chestnut honey ($P < 0.05$). This result was in close agreement with that found by Perna *et al*. (2012), who also found a positive and statistically significant correlation between total phenolic and flavonoid contents. Conversely, Andrade *et al*.

Table 3 Total phenolic and flavonoid contents measured in honeys of different botanical origins

	Parameter	
Honey	Phenolic content (mg GAE per 100 g honey)	Flavonoid content (mg QE per 100 g honey)
Chestnut	14.26 ± 4.14[a]	12.52 ± 6.33[a]
Eucalyptus	11.08 ± 2.80[b]	6.16 ± 1.34[b,c]
Multifloral	11.38 ± 3.38[b]	9.02 ± 3.15[b]
Citrus	12.08 ± 3.25[a,b]	5.82 ± 2.17[c]
Sulla	11.26 ± 5.26[b]	6.76 ± 4.44[b,c]
Total	12.01 ± 3.58	8.05 ± 3.64

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 ($P < 0.05$).

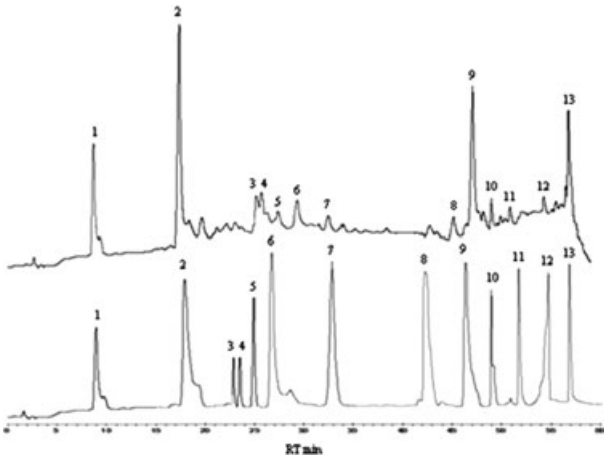

Figure 2 HPLC chromatograms (detected at 280 nm) of chestnut honey and standard mixture of polyphenols. Peaks: 1, gallic acid; 2, gallocatechin; 3, epicatechin; 4, catechin; 5, chlorogenic acid; 6, caffeic acid; 7, benzoic acid; 8, p-coumaric acid; 9, ferulic acid; 10, rutin; 11, myricetin; 12, quercetin; 13, hesperetin (internal standard).

(1997b) found that chestnut honey is rich in phenolic acids and poor in flavonoids. The differences in the total phenolic and flavonoid contents among the analysed honeys were likely due to the variation of their botanical sources (Aljadi & Kamaruddin, 2004; Beretta *et al*., 2005; Bertoncelj *et al*., 2007). The variability around the mean value highlighted the influence of other factor not related to the botanical species, such as the geographical origin.

HPLC analysis of honey phenolic compounds

In this study, thirteen phenolic compounds were identified and quantified by HPLC–UV analysis: seven flavonoids and six phenolic acids (Fig. 2). The results showed that most honeys have similar but quantitatively different phenolic profile (Tables 4 and 5). Our results showed a high variability around the mean value for both phenolic acids and flavonoids. This is related at very low concentrations of the studied compounds, found in some honeys, and due to different geographical origins. In general, phenolic acids such as gallic acid, chlorogenic acid, caffeic acid, p-coumaric acid, benzoic acid and ferulic acid and flavonoids such as gallocatechin, rutin, myricetin and quercetin were detected in all the analysed honey samples. In studied honeys, gallic acid and gallocatechin were the most abundant compounds. Multifloral honey showed the highest amount of the detected total phenolic compounds (Tables 4 and 5). This could be due to the fact that this honey is the product of a grazing area that is characterised by heterogeneous floristic association with a synchronised flowering. Catechin and epicatechin were detected in chestnut, eucalyptus and sulla honeys (Table 4), and the values ranged between 1.50 and 7.94 mg per kg honey and between 0.98 and 4.36 mg per kg honey, respectively. The lowest content in gallocatechin was found in eucalyptus honey (12.41 mg per kg honey; $P < 0.05$), while it resulted significantly the highest in multifloral honey (105.91 mg per kg honey). Hesperetin was detected only in citrus and multifloral honeys ($P < 0.05$), with a mean content of 4.09 and 8.12 mg per kg honey, respectively. The hesperetin content, detected in honey samples, was higher than that reported by other authors (Ferreres *et al*., 1994a,b,c; Liang *et al*., 2009), while it resulted lower than the content reported by Hamdy *et al*. (2009). The higher hesperetin content detected in multifloral honey is attributable to the simultaneous presence of pollen and nectar from different botanical species, in particular officinal herbs, in which the concentration of this flavonoid is high (Socha *et al*., 2009). Moreover, in citrus honey, the hesperetin is considered a marker of the botanical origin (Ferreres *et al*., 1993), while in multifloral honey, the presence of this flavonoid is fluctuating and is influenced by many factors, such as botanical sources, seasonal and environmental conditions. The values found in studied samples match the results reported by Socha *et al*. (2009), who found in Polish multifloral honey a mean hesperetin content higher than that found in lime honey. Quercetin is a flavonoid ubiquitous in unifloral honeys arising from herbaceous and arboreal plants (Tomás-Barberán *et al*., 2001; Yao *et al*., 2003; Truchado *et al*., 2008; Socha *et al*., 2009). Yao *et al*. (2004), in Australian eucalyptus honeys, found a mean quercetin content of 0.33 ± 0.03 mg per 100 g honey. In our study, chestnut and multifloral honeys showed the highest amount of quercetin (1.50 and 1.42 mg per kg honey, respectively), followed by eucalyptus honey (1.05 mg per kg honey), while citrus honey showed the lowest amount

Table 4 The amounts of flavonoids in the analysed honey samples

	Amount of flavonoids (mg per kg honey)							
Honey	**Epicatechin**	**Catechin**	**Gallocatechin**	**Rutin**	**Myricetin**	**Quercetin**	**Hesperetin**	**Sum of flavonoids**
Chestnut	4.36 ± 9.11[a]	7.94 ± 16.81[a]	66.08 ± 63.89[a]	3.49 ± 3.97[a]	1.32 ± 1.11[a,c]	1.50 ± 1.28[a]	nd	84.69
Eucalyptus	0.98 ± 1.92[b]	1.92 ± 3.50[b]	12.42 ± 21.10[b]	1.69 ± 1.45[b,c]	0.78 ± 0.39[b]	1.05 ± 0.77[b]	nd	115.86
Multifloral	nd	nd	105.91 ± 130.9[c]	2.28 ± 2.18[b]	1.67 ± 1.71[a]	1.42 ± 1.17[a]	8.12 ± 10.81[a]	119.4
Citrus	nd	nd	41.55 ± 33.51[a,d]	1.07 ± 0.95[c]	0.70 ± 0.51[b]	0.67 ± 0.61[c]	4.09 ± 4.59[b]	48.08
Sulla	1.12 ± 2.15[b]	1.50 ± 3.72[b]	38.96 ± 59.13[d]	2.14 ± 2.05[b]	1.05 ± 0.75[c,b]	0.98 ± 0.67[b,c]	nd	45.75

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

Table 5 The amounts of phenolic acids in the analysed honey samples

	Amount of phenolic acids (mg per kg honey)						
Honey	**Gallic acid**	**Chlorogenic acid**	**Caffeic acid**	**p-Coumaric acid**	**Benzoic acid**	**Ferulic acid**	**Sum of phenolic acids**
Chestnut	24.81 ± 22.55[a]	4.38 ± 6.0[a,b]	9.97 ± 9.22[a]	5.00 ± 4.65[a]	1.50 ± 1.73[a]	16.66 ± 33.71[a]	62.32
Eucalyptus	53.01 ± 72.22[b]	2.84 ± 3.01[a,c]	6.51 ± 7.46[b]	3.40 ± 2.65[b]	0.89 ± 0.56[b]	3.73 ± 7.24[b]	70.38
Multifloral	39.44 ± 48.07[c]	4.87 ± 7.73[b]	13.83 ± 15.03[c]	3.61 ± 2.73[b]	0.56 ± 0.43[b,c]	3.55 ± 4.96[b]	65.86
Citrus	11.92 ± 7.83[d]	2.25 ± 2.71[c]	5.31 ± 5.87[b]	1.52 ± 1.63[c]	0.46 ± 0.44[c]	2.66 ± 6.92[b]	24.12
Sulla	40.07 ± 32.36[c]	3.25 ± 3.75[a,b,c]	6.81 ± 7.03[b]	2.87 ± 2.14[b]	0.78 ± 0.71[b,c]	3.94 ± 8.28[b]	57.72

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

(0.67 mg per kg honey; $P < 0.05$). The quercetin content was similar to the mean value found in Egyptian citrus honey (0.60 ± 0.01 mg per 100 g honey) by Hamdy *et al*. (2009), while it resulted higher than the value reported by Pichichero *et al*. (2009) in Italian chestnut, sulla and multifloral honeys. These last authors, however, have not detected this flavonoid in citrus honey. Rutin, also called rutoside, is the glycoside between the flavonol quercetin and the disaccharide rutinose. The mean rutin content was the highest in chestnut honey (3.49 mg per kg honey; $P < 0.05$), followed by multifloral honey (2.28 mg per kg honey), while citrus honey showed the lowest content (1.07 mg per kg honey). The highest content in myricetin was found in multifloral and chestnut honeys (1.67 and 1.32 mg per kg honey, respectively; $P < 0.05$), followed by sulla honey (1.05 mg per kg honey). The myricetin content was similar to (for chestnut honey) or higher than (for sulla honey) the content found by Pichichero *et al*. (2009), who have not detected this flavonoid in multifloral honey. Yao *et al*. (2004), in Australian eucalyptus honeys, found a higher content of myricetin when compared with our results. The content of identified phenolic acids is reported in Table 5. The main phenolic acid found was gallic acid, which showed values between 11.92 and 53.01 mg per kg honey, in agreement with that found by Yao *et al*. (2004). The gallic acid content of honey samples decreased in the order: eucalyptus > sulla (40.07 mg per kg honey) > multifloral (39.44 mg per kg honey) > chestnut (24.81 mg per kg honey) > citrus, confirming what reported by other authors (Yao *et al*., 2003, 2004; Gómez-Caravaca *et al*., 2006; Ramanauskiene *et al*., 2009). In chestnut honey, the gallic acid content was considerably lower than that found in Turkey chestnut honeys by Sarikaya *et al*. (2009), while it was higher than the content found in Italian chestnut honeys by Pichichero *et al*. (2009). Several authors (Ghedolf *et al*., 2002; Truchado *et al*., 2008) showed that hydroxycinnamic acids, such as caffeic acid, p-coumaric acid and ferulic acid, derived from propolis that the bee would directly incorporate into honey. In plants, p-coumaric acid is an intermediate metabolite in the synthesis of more complex phenolic compounds (Pryce, 1972; Grace & Logan, 2000). The mean p-coumaric acid content (3.28 mg per kg honey) was significantly higher than the value found by Pichichero *et al*. (2009). The values ranged from 1.52 (citrus honey) to 5.00 mg per kg honey (chestnut honey; $P < 0.05$). The ferulic acid content ranged between 2.66 (citrus honey) and 16.66 mg per kg honey (chestnut honey; $P < 0.05$), showing a mean value of 6.10 mg per kg honey. These values were similar to those reported by other authors (Yao *et al*., 2004; Al

et al., 2009). Caffeic acid was detected in all the analysed honey samples. The values varied from 5.31 (citrus honey) to 13.83 mg per kg honey (multifloral honey). The caffeic acid content was higher than that found in Italian chestnut, sulla and multifloral honeys (Pichichero *et al*., 2009), while it resulted lower than the content found in Turkey chestnut honeys (Sarikaya *et al*., 2009). Chestnut honey showed the highest content in hydroxycinnamic acids ($P < 0.05$), confirming what reported by D'Arcy (2005). The p-coumaric acid and ferulic acid contents in chestnut honey were higher than the values found in Bulgarian honeys (Dimitrova *et al*., 2007). Chlorogenic acid is a product from an esterification of caffeic acid with quinic acid. The ester bond occurs between the carboxyl group of caffeic acid and 3-hydroxyl group of quinic acid. The highest content in chlorogenic acid was found in multifloral honey (4.87 mg per kg honey), followed by chestnut honey (4.38 mg per kg honey). These values were higher than those reported by Pichichero *et al*. (2009). Chestnut honey showed the highest content in benzoic acid (1.5 mg per kg honey; $P < 0.05$), while the other studied honeys showed values below 1 mg per kg honey. These values were lower than those reported by Dimitrova *et al*. (2007). As the plants contain an extensive number of polyphenols, and each plant tends to have a distinctive profile, the variation among the honey samples in their concentration and type of polyphenols is due to variation in their botanical origin (Aljadi & Kamaruddin, 2004; Perna *et al*., 2012). Furthermore, within each plant, large variations may occur, particularly because of environmental conditions and growth or maturation stage of the plant itself (Cheynier, 2005).

Vitamin C content

The vitamin C content of the studied ninety honeys is reported in Table 6. Overall, the results showed that vitamin C is present in all samples. In particular, multifloral honey presented the highest vitamin C content (5.38 mg per kg honey; $P < 0.05$). In unifloral honeys, the content ranged between 2.68 mg per kg honey (citrus honey) and 3.92 mg per kg honey (chestnut honey). These values were similar to those found in citrus and eucalyptus honeys, while they were lower than those found in sulla honeys by Ciulu *et al*. (2011). Ghedolf *et al*. (2002) did not find vitamin C in analysed honeys. Haydak *et al*. (1942) reported that the vitamin C content in honey varies greatly depending on the honey botanical and geographical sources.

DPPH radical scavenging activity

Honey contains many biologically active compounds able to counteract the action of reactive oxygen species (ROS), such as polyphenols, vitamin C, organic acids, catalase, glucose oxidase, amino acids and proteins (Frankel *et al*., 1998; Fahey & Stephenson, 2002; Aljadi & Kamaruddin, 2004; Beretta *et al*., 2005; D'Arcy, 2005; Inoue *et al*., 2005; Blasa *et al*., 2006). The antioxidant activity of honey samples was assessed by DPPH assay. DPPH scavenging is widely used to test the free radical scavenging activity of several natural products (Ahn *et al*., 2007). DPPH is a stable free radical, and any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption (Huang *et al*., 2005). In general, the results showed that all the tested samples exhibited antioxidant activity (Table 6). In particular, chestnut and eucalyptus honeys presented the highest antioxidant activity (75.37 and 73.04%, respectively; $P < 0.05$), while citrus honey showed the lowest activity (55.06%; $P < 0.05$). The results confirm what has been found in previous work (Perna *et al*., 2012). It is difficult to make direct comparisons between our DPPH values and available literature data because the reaction conditions employed by different authors are different. However, we can say that our results are in line with those presented by other authors (Meda *et al*., 2005; Blasa *et al*., 2006; Bertoncelj *et al*., 2007), who reported that dark honeys, as well as chestnut honey, tended to be highly active in the reaction with DPPH and showed a higher polyphenol content. The differences found among the honey types were due to the variation of their content in biologically active compounds, such as polyphenols and vitamin C. Vitamin C is called an antioxidant because, by donating two electrons from a double bond between the second and third carbons of the 6-carbon molecule, it prevents other compounds from being oxidised. However, by the very nature of this reaction, vitamin C itself is oxidised in the process. Khalil *et al*.

Table 6 Vitamin C content (mg L-ascorbic acid per kg honey) and radical scavenging activity in DPPH·reaction system (I %) of different type of honeys

	Parameter	
Honey	Vitamin C content (mg L-ascorbic acid per kg honey)	DPPH (I %)
Chestnut	3.92 ± 0.17^{a}	75.37 ± 7.87^{a}
Eucalyptus	3.83 ± 0.19^{a}	73.04 ± 7.52^{a}
Multifloral	5.38 ± 0.51^{b}	64.03 ± 7.75^{b}
Citrus	2.68 ± 0.14^{c}	55.06 ± 7.04^{c}
Sulla	$3.57 \pm 0.21^{a,c}$	66.60 ± 12.71^{b}
Total	3.89 ± 0.29	66.82 ± 8.20

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 ($P < 0.05$).

(2012), in Algerian honeys, found a statistically significant correlation between vitamin C content and antioxidant activity, measured by DPPH assay, confirming the antioxidant properties of vitamin C. Many authors highlighted a close correlation between antioxidant activity and polyphenol content (Gheldof and Engeseth, 2002; Vela *et al*., 2007; Estevinho *et al*., 2008; Ferreira *et al*., 2009). Important for the antioxidant activity of the phenolic compounds is the ability to inactivate free radicals, by donating a hydrogen atom or an electron. Consequently, they are converted in stable phenolic radicals, because they are able to delocalise the unpaired electron within the aromatic structure, and eventually they may react with other free radicals and inactivate them (Halliwell, 1996). The antioxidant activity of phenolic acids, in the DPPH assay, is connected with: (i) the effect of –CH=CH–COOH group, (ii) the relationship between the number and positions of hydroxyl groups in the aromatic ring, and (iii) the methoxy substituents in the ortho position to the OH (Cuvelier *et al*., 1992; Rice-Evans *et al*., 1996, 1997; Chen & Ho, 1997). In fact, Socha *et al*. (2011) found a high correlation between DPPH assay and gallic acid content, which presents three hydroxyls group in the aromatic ring. The antioxidant activity of the flavonoids is due on the presence of some important structural features: the o-diphenolic group in the B ring (phenyl constituent), the 2–3 double bond conjugate with 4-oxo function in C ring (heterocyclic benzopyran ring), and the hydroxyl groups in positions 3 and 5 in A ring (fused aromatic ring) (Bors *et al*., 1990; Heim *et al*., 2002). Ghedolf *et al*. (2002) reported that the antioxidant activity is the result of the overall action of biologically active components that may act synergistically. Among the studied unifloral honeys, chestnut honey presented the highest levels of phenolic acids and flavonoids, and the highest vitamin C content, which are closely associated with its high antioxidant activity.

Conclusions

This study has allowed us to make the necessary considerations for the best use of the different honeys, also according to the needs of consumers. The results, through the study of the composition of honey, allow us to define the specificities of the botanical species. In fact, the metabolites secreted by the plant, as phenotypic expression of their genome, are collected and processed by honeybees becoming components of the hive products. Among these, the biologically active compounds, such as vitamin C and polyphenols, are the main components responsible for the antioxidant effect of honey and therefore play an important role in defining the nutraceutical quality of the product itself.

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