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Original Article

DETERMINATION OF ANTIOXIDANT CAPACITY, FLAVONOIDS, AND TOTAL PHENOLIC CONTENT IN EUCALYPTUS AND CLOVER HONEYS

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Abstract

Polyphenolic compounds reportedly produce physiological effects that are beneficial to health. Bee products are particularly rich in polyphenolic compounds. We determined the antioxidant capacity and the phenolic and flavonoid compounds content of 81 samples of honey. We used the Folin-Ciocalteu reagent method to evaluate the total phenolic content. The antioxidant activities were evaluated using in vitro scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH) and hydroxyl radicals (OH), Trolox equivalent antioxidant capacity (TEAC), and ferric-reducing antioxidant capacity (FRAC). Total phenolic content ranged from 40.3 to 193.0 mg gallic acid equivalents (GAE)/100 g; the flavonoid content varied from 1.4 to 7.5 mg quercetin equivalents (QE)/100 g. Eucalyptus honeys exhibited significantly higher phenolic content and free radical-scavenging activity than clover honey samples ($p<0.05$ for all). Principal component analysis explained 73% of the differences observed in antiradical activity with respect to floral origin. Total phenolic content may be more useful than the radical-scavenging assay for detecting antioxidant capacity in honey; it also represents the variable that most appropriately discriminated among these honeys.

Keywords: **DPPH, ferric-reducing antioxidant capacity, flavonoids in honey, hydroxyl radicals, polyphenols, Trolox equivalent antioxidant capacity.**

INTRODUCTION

Recently, there has been an increasing interest in the antioxidant capacity of food. Nutritive antioxidant capacity plays an important role as a health-protecting factor. Extracts with antioxidant properties are already being sold as food ingredients, and are also widely used as additives in food processing to prevent or delay spoilage of food (Blasa et al., 2007). In vegetables, this capacity is attributed to the presence of phenolic compounds, mainly flavonoids. It is agreed that the antioxidant activity of flavonoids results from a combination of iron-chelating properties and free-radical capture (Bertonceij et al., 2007), while phenolic acids work as antioxidants by employing free radical-scavenging mechanisms (Hamdy et al., 2009). Others also mention enzymatic oxidation inhibition (enacted by lipoxygenase, ciclooxigenase, myeloperoxidase, and xanthine oxidase), which avoids the in vivo generation of reactive oxygen species (ROS) and organic hydroperoxides (Aljadi and Kamaruddin, 2004; Baltrušaitytė et al., 2007).

Bee products are especially rich in these bioactive compounds. Honeys have a rich phenolic profile consisting of benzoic acid and its esters, cinnamic acid and its esters, and flavonoid aglycones (Hamdy et al., 2009). They can also contain a wide variety of nitrogen compounds (alkaloids, chlorophyll derivates, amino acids and amines), carotenoids, and vitamin C, whose antioxidant activities are widely recognized (Buratti et al., 2007; Pérez et al., 2007). However, the antioxidant activity of honey varies greatly depending on its floral source (Liviu et al., 2011).

Honeys differ not only with respect to chemical composition (volatile compounds, carbohydrates, and phytochemicals), physical properties (color, viscosity, hygroscopic properties, and pH) and taste, but also with respect to biological activity. Some honeys have a stronger biological activity than others. Con-

sequently, it is a reasonable expectation that the composition and properties of honeys from various locations might differ (Kaškonienė et al., 2009).
Many methods have been used to determine honey's antioxidative activity, including: the determination of flavonoids and total phenolic content (Beretta et al., 2005); radical formation and radical scavenging, as in 2,2-diphenyl-1-picrylhydrazyl (DPPH), and the measurement of superoxide radical-scavenging activity (Gheldof et al., 2002; Aljadi and Kamaruddin, 2004; Meda et al., 2005); the ferric-reducing antioxidant power assay (Aljadi and Kamaruddin, 2004); oxygen radical absorbance capacity (ORAC), Trolox equivalent antioxidative activity (TEAC); and enzymatic or non-enzymatic measurements of lipid peroxidation inhibition (McKibben and Engeseth, 2002). In most cases, it is necessary to use several tests to obtain good reliability (Roginsky and Lissi, 2005).
Recently developed biological methods are more relevant than the popular in vitro assays, because they take into account some aspects of the metabolism, intake, and location of antioxidant compounds in cells. However, these methods have been questioned because no correlation has been observed between their results and biological activities (Blasa et al., 2007).
The aim of this work was to measure the antioxidant capacity, total phenolic compound content, and flavonoid content in eucalyptus and clover honeys, in order to estimate the nutritional relevance of antioxidant capacity, which also contributes to the characterization of these honeys.

MATERIAL AND METHODS

Samples

Eighty-one honey samples collected between 2006 and 2010 were analyzed. In every case, samples were collected in accordance with CODEX Stan 12 (2001), in the Argentinean phytogeographical region known as the Pampeana Region. This region is characterized by grass pasture but has been highly altered by the development of many crops (*Triticum aestivum* L., *Zea mays* L., *Glycine max* (L.) Merr., *Oryza sativa*) and animal feed harvests (*Lotus* sp., *Medicago sativa* L., *Trifolium repens* L., *Trifolium pratense* L., *Melilotus albus*), with predominance of non-native species and poor pollen diversity. As the Pampeana Region is mainly committed to agriculture and ranching, crops and their weeds have largely replaced native vegetation, creating an important resource for honey production in the region (Fagúndez and Caccavari, 2006).
According to a palynological analysis (Loveaux et al., 1978), 53 honeys were from clovers (*Trifolium* sp.) and 28 were from eucalyptus (*Eucalyptus* sp.). Upon receipt, honey samples were centrifuged and stored at -18ºC in the dark until analysis. Sample analysis was performed within 4 months of sample harvest.

Quantification of bioactive compounds

Total soluble phenolic compound content

In order to measure the total soluble phenol content of honey samples, we employed phenolic compounds to reduce a phosphowolframate-phosphomolybdate complex to blue products (Singleton et al., 1999). Each honey sample (4.0 ± 0.01 g) was diluted to 25 mL with distilled water and filtered through Whatman Nº 1 paper. Ten milliliters of distilled water were added to 1 mL of this solution and mixed with 1 mL of Folin-Ciocalteu reagent, shaking gently. After 2 min, 2 mL of saturated sodium carbonate solution were added to the mixture and adjusted to 25 mL with distilled water. After incubation in the dark at room temperature for 2 h, the absorbance of the resulting solution was measured at 725 nm in a Varian Cary 50 spectrophotometer (Las Vegas, USA). Gallic acid was used as standard for the calibration curve (0.01 - 2.90 mM; Y = 42.40X + 0.21; R^2 = 0.998). The mean of two readings was used and expressed as milligrams of gallic acid equivalents (GAE) per 100 g honey.

Flavonoid compound content

In order to determine flavonoid content, to each honey sample (2.5 ± 0.01 g) we added 0.5 mL of $AlCl_3$ 5%, and then brought the total volume to 25 mL with distilled water. Diluted samples were incubated for 30 min in the dark at room temperature, after which absorption readings were obtained at 425 nm (Woisky and Salatino, 1998). The total flavonoid content was determined using a standard curve, with quercetin as the standard (0.00 - 28.70 mM; Y = 15.33X - 0.189; R^2 = 0.997). The mean of two readings was used and expressed as milligrams of quercetin equivalents (QE) per 100 g honey.

Antioxidant capacity

The antioxidant capacity of different honey samples was evaluated using the whole sample. Spectrophotometric assays were performed using a Varian Cary 50 spectrophometer (Las Vegas, NV, USA).

DPPH radical-scavenging activity

We used the method suggested by Brand-Williams et al. (1995) to assess DPPH radical-scavenging activity. Each honey sample (0.7 ± 0.01 g) was diluted in 50 mL of water. Two milliliters of this

solution were mixed with 2 mL 0.1 M DPPH ethanol solution (freshly prepared). The mixture was shaken vigorously and left to stand for 60 min in the dark, after which stable absorption values were obtained. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm; absorbance was read after adjustment at 1 with DPPH solution. Results were expressed as milligrams of Trolox equivalents (TE) per 100 g honey, using the standard calibration curve (0.00 - 63.40 mM; Y = 41.91X - 0.13; R^2 = 0.999).

Trolox equivalent antioxidative capacity (TEAC) or ABTS assay

The ABTS assay was performed according to Arnao et al. (2001). The ABTS radical ($ABTS^{\bullet+}$) was obtained by reacting 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) 7 mM solution with 2.45 mM potassium persulfate. The solution was stored in the dark at room temperature between 12 - 16 h, and then diluted in ethanol to read absorbance of 0.70 ± 0.02 at 734 nm. Four milliliters of ABTS solution were mixed with 0.1 mL of 10% honey aqueous solution. This mixture was kept in the dark for 24 h. Absorbance was read at 734 nm against ethanol; absorbance was adjusted at 0.7 with ABTS solution. Results were expressed as milligrams of Trolox equivalents (TE) per 100 g honey, based on the standard calibration curve (0.00 - 0.50 mM; Y = -364.86X + 241.95; R^2 = 0.973).

Ferric-reducing antioxidant capacity (FRAC)

The reducing capacity of a compound can reflect its antioxidant capacity (Oyaizu, 1986). Two milliliters of 0.03 g/mL honey aqueous solution were mixed with 2 mL sodium phosphate buffer solution (0.2 M, pH = 6.6) and 2 mL 1% potassium ferricyanide ($K_3[Fe(CN)]_6$). The homogenized mixture was incubated at 50°C for 20 min. Then, 2 mL of trichloroacetic acid were added. Five milliliters of distilled water and 1 mL ferric chloride 1% ($FeCl_3$) were added to 5 mL of the homogenized mixture. The absorbance was read at 700 nm. Reductive capacity was expressed according to the calibration curve (0.00 - 113.56 mM; Y = 67.06X - 215.90; R^2 = 0.993) using ascorbic acid as the standard. The results were expressed as milligrams of ascorbic acid equivalent (AAE) per 100 g honey.

OH radical-scavenging activity

OH radical-scavenging activity was measured by assessing the inhibitory capacity of the degradation of desoxyribose (Halliwel et al., 1987). In the presence of ascorbic acid, H_2O_2-ferric-EDTA complex produces the Fenton reaction. Free radicals attack desoxyribose, degrading it into fragments that react with thiobarbituric acid to yield a pink chromogen. The color of the solution changes when honey competes for the OH.

This assay was performed by first mixing 0.1 mL desoxirribose solution (28 mM) with 200 μL of 0.02 g/mL honey aqueous solution Next, 0.5 mL phosphate buffer (40 mM, pH = 7.4), 0.1 mL ferric chloride (1 mM), 0.1 mL EDTA (1.04 mM), 0.1 mL H_2O_2 (1 mM), and 0.1 mL L-ascorbic acid (1 mM) were added. After incubation at 37°C for 1 h in a thermostatic bath, 0.5 mL thiobarbituric acid (1% P/V in 0.05 M NaOH) and 0.5 mL 2.8% (v/v) trichloriacetic acid were added. This mixture was left to react for 10 min at 100°C, and absorbance was measured at 530 nm. Quercetine was used as a reference substance (QE), and the corresponding concentration was calculated using a calibration curve (0.00 - 113.56 mM; Y = -0.001X + 0.0005; R^2 = 0.994).

Statistical analysis

The assays were performed in duplicate, and the results were expressed as mean values and standard deviation. Student's t-test was performed on the replicated samples. We considered $p<0.05$ statistically significant.

Principal component analysis (PCA) was conducted to characterize each floral origin with respect to antioxidant activity. PCA is a multivariate statistical tool that indicates the relationship between a given group of variables from a data matrix and the similarity between individuals. The data matrix can be viewed as a multidimensional space with one dimension for each variable, and each sample as a point in that space. The matrix reduces by linear combinations of the original variables; the sample space is reduced, while retaining the highest proportion of the variance present in the original data (Johnson, 2004).

Descriptive and multivariate statistical analyses were performed using SAS® v 9.3 (SAS Institute, 2011).

RESULTS

Quantification of bioactive compounds

Total phenolic content was between 40.30 and 193.03 mg GAE per 100 g honey (mean 93.75 ± 37.06 GAE/100 g, n = 81). The flavonoid content in honey samples ranged from 1.42 to 7.48 mg QE/100 g honey (mean 3.52 ± 1.19 mg QE/100 g, n = 81).

Fifty percent of eucalyptus honey samples exhibited a minimum total phenol level of 106.7 mg GAE/100 g and a minimum flavonoid level of

3.6 mg QE/100 g. In contrast, 75% of clover honey samples exhibited a maximum total phenol level of 100.4 mg GAE/100 g and a maximum flavonoid level of 3.9 mg QE/100 g. Clover honey samples contained significantly lower mean levels of phenolic compounds than eucalyptus honey samples (82.73 ± 28.04 mg GAE/100g honey (n = 53) vs. 109.37 ± 41.44 mg GAE/100 g honey (n = 28); $p \leq 0.05$). Eucalyptus honey samples also contained significantly more flavonoid compounds than clover honeys ($p \leq 0.05$). The average flavonoid compound content was equal to 4.03 ± 1.22 mg QE/100 g for eucalyptus honey and 3.29 ± 1.10 mg QE/100 g for clover honey samples.

Antioxidant capacity

The box plots in Figure 1 depict the differences in antioxidant activity between eucalyptus and clover honey. The TEAC of eucalyptus honey samples is significantly higher than that of clover honey samples ($p \leq 0.05$).

The OH values ranged from 0.52 to 2.03 mg QE/100 g (mean 1.34 ± 0.33 mg QE/100 g, n = 81) and the FRAC values ranged from 2.50 to 98.10 mg AAE/100 g.

After the data matrix was standardized, we performed a PCA regarding several variables: total phenolic compound content, total flavonoid compound content, reducing capacity (FRAC), TEAC, and capture capability from DPPH radicals (DPPH) and OH radicals (OH). Eigenvalues for the three first principal components (PCs) were: $\lambda 1$ = 2.34, $\lambda 2$ = 1.19, and $\lambda 3$ = 0.78. This finding explained 73% of total data variation, an acceptable criterion to represent the original information with only three new latent variables.

Figure 2 shows the correlation circle of the loading of the variables on the components. Each variable is a point whose coordinates are defined by the loading on the PCs (Tab. 1). The plot describes the correlation between the variables. Projecting the

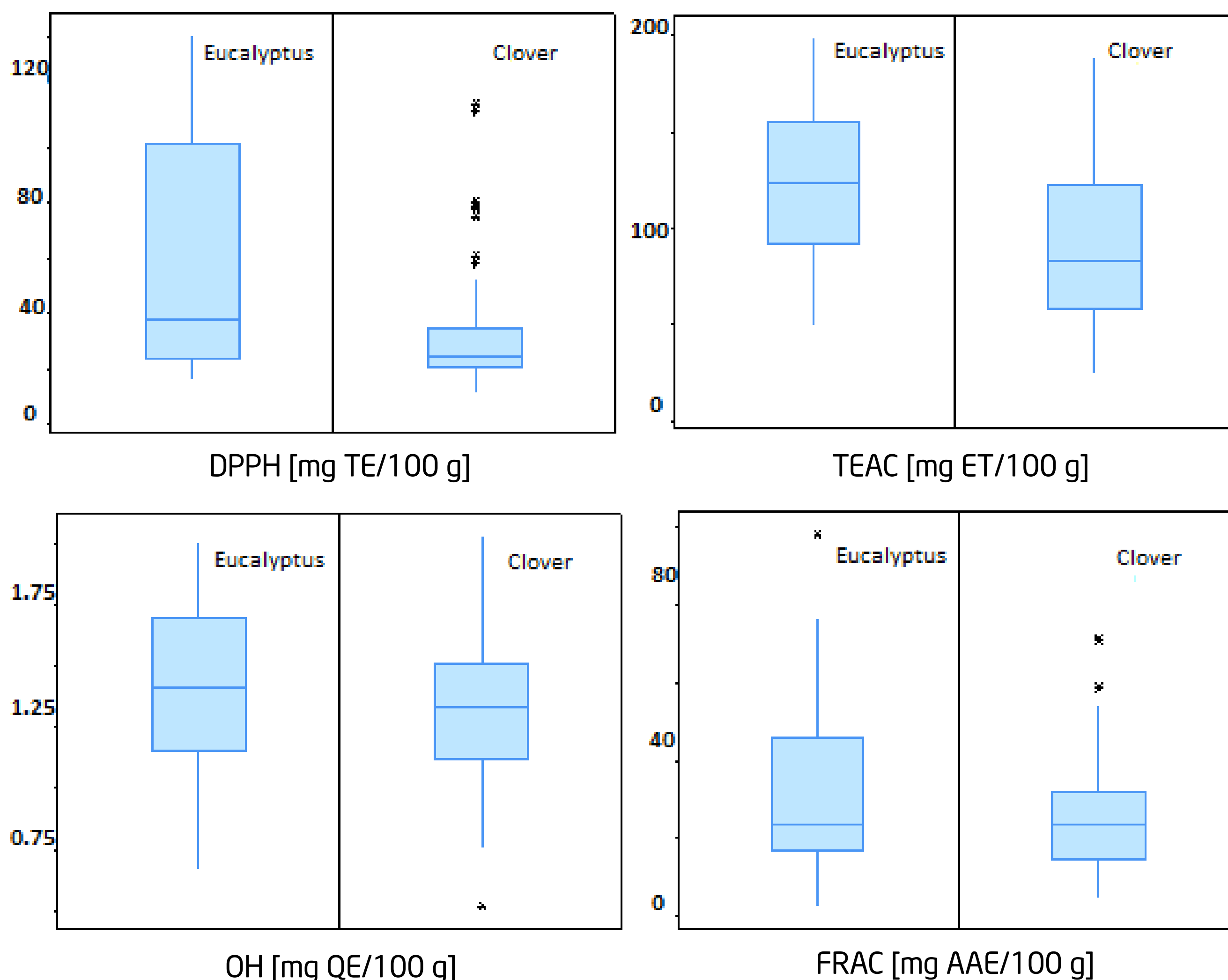

Fig. 1. Box and whisker plots show the minimum and maximum (vertical line), median (center horizontal line), first and third quartiles (box), and outliers (asterisk) of the antioxidant capacity of clover and eucalyptus honeys.

Table 1.
Loading and cumulative variance for the three first principal components (PCs) of the principal content analysis regarding antioxidant capacity

PC	Variables						Cumulative variance %
	Phenol content	Flavonoid content	FRAC	DPPH	TEAC	OH	
1	0.52(*)	0.44	0.46	0.35	0.43	-0.92(*)	41
2	0.08	0.32	-0.35	0.29	-0.11	0.82(*)	19
3	0.05	0.22	-0.00	-0.82(*)	0.45	0.26	73

(*) Loadings involved in the differentiation of honey are highlighted in bold.
FRAC - ferric-reducing antioxidant capacity; DPPH - 2,2-diphenyl-1-picrylhydrazyl radical-scavenging activity; TEAC - Trolox equivalent antioxidative capacity; OH - hydroxyl radical-scavenging activity

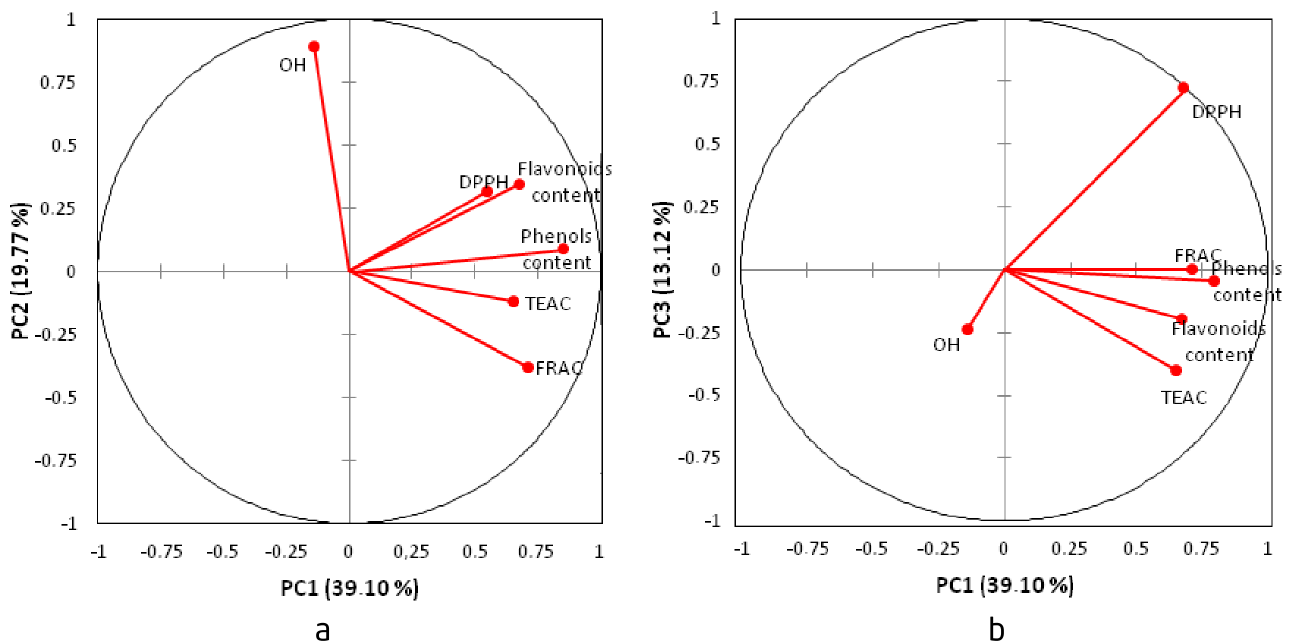

Fig. 2. Circle of correlations and plot of the loading of the variables (a) with principal components (PCs) 1 and 2, and (b) with PCs 1 and 3.

arrows onto the axis, it can be seen that phenol content is the most important variable for the first principal component (PC1), OH is the most important variable for the second principal component (PC2), and DPPH is the most important variable for the third principal component (PC3). The angle between two arrows represents the correlation of the respective variables. Smaller angles between vectors indicate an increased correlation between the respective variables. There is no linear dependence (i.e., no correlation) if the angle is 90 degrees. The OH radical-scavenging activity exhibited no correlation with the other antioxidant parameters assayed.

DISCUSSION

Figure 3 shows comparative results of total soluble phenolic compound content in honey samples from different sources. Meda et al. (2005) reported total phenolic contents between 32.59 and 114.75 mg GAE/100 g in multifloral honeys and honeydews. Vit et al. (2008) reported values between 47.4 and 265.49 mg GAE/100 g in Czech honeys. Others observed narrower ranges (Gheldof et al., 2002; Otilia et al., 2005; Bertonceij et al., 2007; Sosa Martínez et al., 2009; González Lorente et al., 2008; Álvarez Suárez et al., 2009), with the exception of Muñoz et al. (2007), who reported values between 0.0 and 8.82 mg GAE/100 g regarding Chilean honey. Comparative data of flavonoids content are shown in Figure 4. Meda et al. (2005) identified mean flavonoids content values of 2.57 ± 2.09 mg QE/100 g in multifloral honeys and honeydews. Values reported by Vit et al. (2008) ranged from 1.90 to 15.74 mg QE/100 g for Czech honeys. Others authors reported smaller ranges (Baltrušaitytė et al., 2007; Muñoz et al., 2007; Álvarez Suárez et al., 2009; Sosa Martínez et al., 2009).

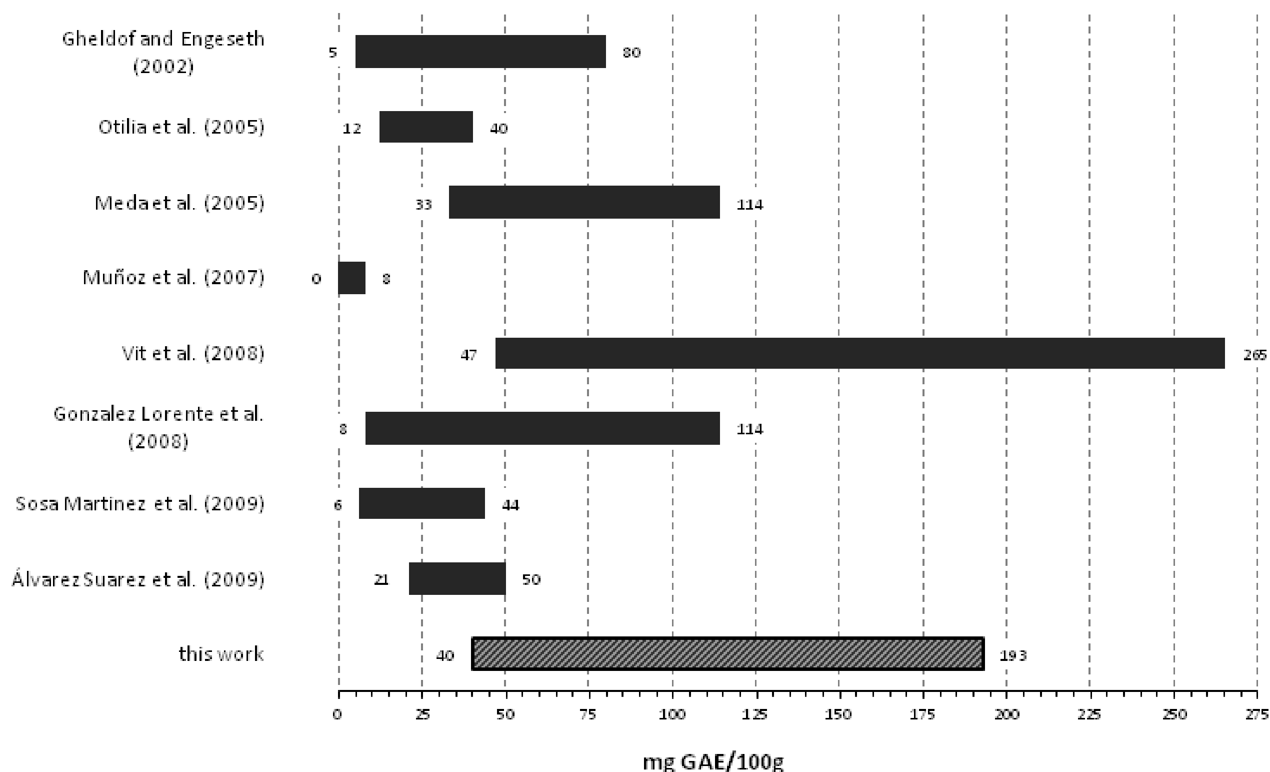

Fig. 3. Total soluble phenolic compound content in honey samples from different sources.

DDPH values were similar to those observed by González Lorente et al. (2008) and higher than those reported by Vela et al. (2007). In agreement with the results obtained in this work, published reports of TEAC showed contents ranging between 43.55 and 294.5 mg TE/100 g (Vit et al., 2008; Álvarez Suárez et al., 2009).

The range of OH values found in this study agrees with that published by Rodríguez et al. (2007). No background FRAC values were observed in honey, to establish a basis for comparison. Montenegro et al. (2013) determined the ferric-reducing ability of plasma (FRAP) in Chilean honey samples, which ranged from 0.1 to 0.59 mmol Trolox equivalent/kg honey.

When free radical-scavenging activity was analyzed, variables with more discrimination power were similar to the ones reported by Iurlina et al. (2011) for honeys of *Trifolium* species, *Melilotus* species, *Medicago sativa*, and *Lotus* species. They were associated with higher phenolic compound content and with OH and DPPH radical capture capacities.

Similar results had been reported in comparative research about fruits (Liu et al., 2008) and regarding pollen antioxidant activity from seven countries (Fuenmayor et al., 2011).

The lack of correlation between OH radical scavenging and the other scavenging assays might suggest that the mechanism of the OH capture reaction differs from the electron-transfer reactions or that this parameter is independent of the content of flavonoids and phenolic compounds. The correlations between total phenolic compounds, total flavonoid compounds, FRAC, and TEAC indicate that it would probably be unnecessary to perform all of these determinations.

Folin–Ciocalteu's method is largely used to evaluate total phenolic compounds. The determination of total soluble phenolic compounds is simple, and has the advantage that Folin–Ciocalteau's solution is stable and can be bought commercially, decreasing analytical errors. But the phosphowolframate-phosphomolybdate complex also reacts with other non-phenolic reducing compounds, such as sugars and amino acids, leading to an overestimation of the phenolic content (Georgé et al., 2005). In fact, honey samples contain some of these compounds, as well as other antioxidants that can lead to an increase in the absorbance values and to positive errors in the determination of phenolic content when the Folin-Ciocalteu method is employed. Because the test does not accurately represent the total phenolic compound content, it would be appropriate to give it a different name (e.g., total antioxidant activity) (Prior et al., 2005).

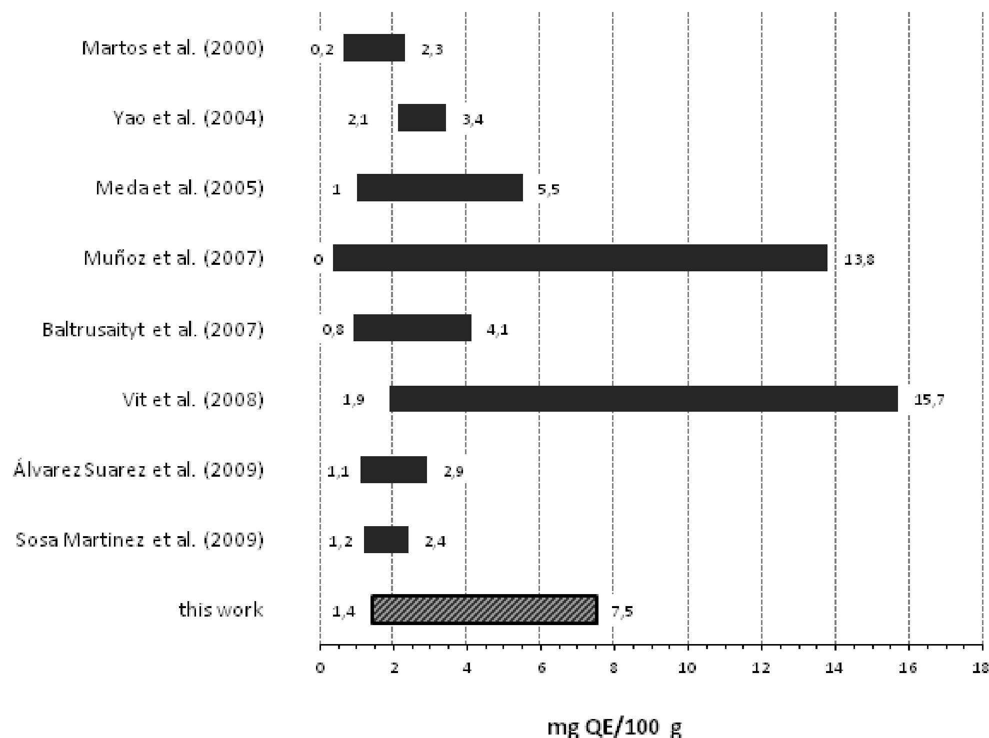

Fig. 4. Total flavonoid compound content in honey samples from different sources.

All honey samples exhibited antioxidant properties different from zero. Tea, red wine, and chocolate are foods with recognized antioxidant capacity. Among them, chocolate contains greater total phenolic compounds per portion than honey. While honey contains 18 mg GAE per portion, chocolate contains up to 611 mg GAE per portion. Black tea contains 124 mg GAE, green tea contains 165 mg GAE, and red wine contains 340 mg GAE (Won Lee et al., 2003). The total phenolic compound content is also reportedly higher in red fruits (Vasco et al., 2008). However, the antioxidant capacity of honey was similar to the reported antioxidant capacity of tomatoes (Wu et al., 2004).

In addition to phenols and flavonoids, honey contains small amounts of proteins, enzymes, amino acids, minerals, trace elements, and vitamins. Thus, it is considered a food of nutritional interest. However, most of the properties of honey require an intake of between 50 and 80 g in order to have an effect (Bogdanov et al., 2008).

CONCLUSION

Results obtained in this study confirm that honey's composition depends to a great extent on its botanical origin. Thus, floral origin is also likely to influence most of honey's health-promoting properties.

It is necessary to reach a consensus regarding the most appropriate analytic method with which to determine the in vitro antioxidant capacity of honey. An interesting alternative would be to evaluate in vitro antioxidant capacity by determining the total phenolic compounds in a sample, in combination with an OH radical-scavenging activity assay.

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