Antioxidant activity of Portuguese honey samples: Different contributions of the entire honey and phenolic extract

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ABSTRACT

The antioxidant activity of Portuguese honeys was evaluated considering the different contribution of entire samples and phenolic extracts. Several chemical and biochemical assays were used to screen the antioxidant properties of entire honeys with different colour intensity and phenolic extracts: reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging capacity, and inhibition of lipid peroxidation using the β-carotene linoleate model system and the thiobarbituric acid reactive substances (TBARS) assay. The amounts of phenols, flavonoids, ascorbic acid, β-carotene, lycopene and sugars present in the samples were also determined. The highest antioxidant contents and the lowest EC_{50} values for antioxidant activity were obtained in the dark honey. An analysis of variance was carried out to evaluate the influence of the colour intensity and extraction method in the antioxidant properties and phenolic contents. A discriminant analysis was also performed, giving satisfactory results once the six samples were clustered in six individual groups obtained through the definition of two discriminant analysis dimensions.

1. Introduction

Honey is nectar collected from many plants and processed by honey bees (*Apis mellifera*). This natural product is widely appreciated as the only concentrated form of sugar available worldwide (FAO, 1996) and is also used as a food preservative (Cherbuliez & Domerego, 2003).

Honey has been reported to contain about 200 substances (complex mixture of sugars, but also small amounts of other constituents such as minerals, proteins, vitamins, organic acids, flavonoids, phenolic acids, enzymes and other phytochemicals) and is considered to be an important part of traditional medicine (White, 1979). It has been used in ethnomedicine since the early humans, and in more recent times its role in the treatment of burns, gastrointestinal disorders, asthma, infected and chronic wounds, skin ulcers, cataracts and other eye ailments has been "rediscovered" (Castaldo & Capasso, 2002; Marcucci, 1995; Molan, 1992; Orhan et al., 2003). This beneficial role is partially attributed to honeys antibacterial activity. However, since some of these diseases are a consequence of oxidative damage, it seems that part of the therapeutic properties of honey is due to its antioxidant capacity. Additionally, the presence of hydrogen peroxide, as well as minerals (particularly copper and iron), in honey, may lead to the generation of highly reactive hydroxyl radicals as part of the antibacterial

system (McCarthy, 1995; Molan, 1992); thus, it is evident that mechanisms must be available in honey to control the formation and removal of these reactive oxygen species. Furthermore, honey, as a source of antioxidants, has been proven to be effective against deteriorative oxidation reactions in food, caused by light, heat and some metals (McKibben & Engeseth, 2002), such as enzymatic browning of fruit and vegetables (Chen, Mehta, Berenbaum, Zangerl, & Engeseth, 2000), lipid oxidation in meat (Gheldof & Engeseth, 2002; McKibben & Engeseth, 2002; Nagai, Inoue, Kanamori, Suzuki, & Nagashima, 2006), and inhibit the growth of foodborne pathogens and food spoilage organisms (Mundo, Padilla-Zakour, & Worobo, 2004; Taomina, Niemira, & Beuchat, 2001). Overall, honey serves as a source of natural antioxidants (Al-Mamary, Al-Meeri, & Al-Habori, 2002; Aljadi & Kamaruddin, 2004; Antony, Han, Rieck, & Dawson, 2000; Beretta, Granata, Ferrero, Orioli, & Facino, 2005; Gheldof, Wang, & Engeseth, 2002; Küçük et al., 2007; Nagai, Sakai, Inoue, Inoue, & Suzuki, 2001; Vit, Soler, & Tomas-Barberan, 1997), which play an important role in food preservation and human health by combating damage caused by oxidising agents e.g., oxygen, namely reducing the risk of heart disease, cancer, immune-system decline, cataracts, different inflammatory processes, etc. (The National Honey Board, 2003).

The antioxidants present in honey include both enzymatic: catalase (Schepartz, 1966), glucose oxidase, peroxidase (Ioyrish, 1974) and non-enzymatic substances: ascorbic acid, α-tocopherol (Crane, 1975), carotenoids, amino acids, proteins, organic acids, Maillard reaction products (Al-Mamary et al., 2002; Aljadi & Kamaruddin,

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2004; Baltrušaityte, Venskutonis, & Čeksteryt, 2007; Bertoncelj, Doberšek, Jamnik, & Golob, 2007; Gheldof, Wang, & Engesetg, 2001; Gheldof et al., 2002; Schramm et al., 2003; The National Honey Board, 2003), and more than 150 polyphenolic compounds, including flavonoids, flavonols, phenolic acids, catechins, and cinnamic acid derivatives. In the literature, several studies for the identification and quantification of antioxidant components of honeybee products have been reported (Buratti, Benedetti, & Cosio, 2007; Ferreres, Tomas-Barberan, Soler, & Garcia-Vigera, 1994; Gheldof et al., 2002).

Many methods for determining the antioxidative activity in honey have been used, e.g., determination of total phenolic content (Beretta et al., 2005), radical formation and following scavenging as in 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radical-scavenging activity measurements (Aljadi & Kamaruddin, 2004; Chen et al., 2000; Gheldof & Engeseth, 2002; Gheldof et al., 2002; Gülçin, Büyükokuroğlu, Oktay, & Küfrevioğlu, 2003; Kefalas, Gotsiou, & Chougoui, 2001; Meda, Lamien, Romito, Millogo, & Nacoulma, 2005; Nagai et al., 2001; Taomina et al., 2001), the ferric-reducing/antioxidant power (FRAP) assay (Aljadi & Kamaruddin, 2004) and enzymatic or non-enzymatic measurements of lipid peroxidation inhibition (Chen et al., 2000; McKibben & Engeseth, 2002; Nagai et al., 2001).

Although it has already been demonstrated that honey has antioxidant activity and different antioxidant compounds, nothing is reported about the different contributions of the entire honeys and their phenolic extracts to those properties. Accordingly, in this work, the antioxidant properties of the entire samples, and phenolics extracts were evaluated through several chemical and biochemical assays: DPPH (2,2-diphenyl-1-picrylhydrazyl) radical-scavenging activity, reducing power, inhibition of β-carotene bleaching and inhibition of lipid peroxidation in pig brain tissue through formation of thiobarbituric acid reactive substances (TBARS). This is also the first study reporting antioxidant activity of Portuguese honey, particularly from a region with high amounts of this natural product (Northeast Portugal).

2. Materials and methods

2.1. Samples

Three unifloral honeys, to the extent of natural limitations, were obtained in Northeast Portugal (Parque Natural de Montesinho) from experienced producers. The type and region of the honey samples, as well as the family, scientific, common and local names of the plants that form the basic flora of the honey samples, are shown in Table 1. The three samples were obtained in June 2007, and the tests were performed within 2 months following collection. Upon receipt, honeys were centrifuged and stored at 21 °C in the dark until used.

2.2. Standards and reagents

All the solvents were of analytical grade purity: methanol and diethyl ether were supplied by Lab-Scan (Lisbon, Portugal). The standards used in the antioxidant activity assays: BHA (2-tert-butyl-4-methoxyphenol), TBHQ (tert-butylhydroquinone), ʟ-ascorbic acid, α-tocopherol, gallic acid and (+)-catechin were purchased

from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). The standard butylated hydroxytoluene (BHT) was purchased from Merck (Darmstadt, Germany). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.3. Colour analysis

Honey samples were heated to 50 °C to dissolve sugar crystals, and the colour was determined by spectrophotometric (AnalytikJena 200-2004) measurement of the absorbance of a 50% honey solution (w/v) at 635 nm. The honeys were classified according to the Pfund scale after conversion of the absorbance values: mm Pfund = $-38.70 + 371.39 \times$ Abs (White, 1984).

2.4. Phenolics extraction

The honey sample (10 g) was mixed with acid water (pH 2 with HCl; 0.02 M), until it was totally fluid, after which it was filtered through cotton to remove any solid particles. The filtrate was poured into a column (35×2 cm) with Amberlite XAD-2 (Fluka Chemie; porosity 9 nm, particle size 0.3–1.2 mm; 150 g). The phenolic compounds were retained in the column whilst sugars and other polar compounds were eluted with the aqueous solvent. The column was washed with the acid solution (pH 2 with HCl, 100 ml) and then with distilled water (~100 ml). The phenolic fraction was eluted with methanol (~100 ml) and dried under reduced pressure (40 °C). The residue was redissolved in methanol to a known final concentration.

2.5. Bioactive compounds quantification

Phenols, flavonoids, ascorbic acid, carotenoids and sugars were determined according to procedures previously described by us (Barros et al., 2007).

For phenolic compounds determination, 1 ml of sample was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm. Gallic acid was used to calculate the standard curve (0.01–0.08 mM; $Y = 2.3725X + 0.0021$; $R^2 = 0.1000$) and the results were expressed as mg of gallic acid equivalents (GAEs) per kg of honey.

For flavonoid contents determination, the sample (250 μL) was mixed with 1.25 ml of distilled water and 75 μL of a 5% NaNO$_2$ solution. After 5 min, 150 μL of a 10% AlCl$_3 \cdot$ H$_2$O solution was added. After 6 min, 500 μL of 1 M NaOH and 275 μL of distilled water were added to the mixture. The solution was mixed well and the intensity of pink colour was measured at 510 nm. (+)-Catechin was used to calculate the standard curve (0.022–0.34 mM; $Y = 0.9990X - 0.0497$; $R^2 = 0.9961$) and the results were expressed as mg of (+)-catechin equivalents (CEs) per kg of honey.

For acid ascorbic determination, the sample (100 mg) was extracted with 10 ml of 1% metaphosphoric acid for 45 min at room temperature and filtered through Whatman No. 4 filter paper.

Table 1
Characterisation of the monofloral honey samples.

Honey sample	Family	Scientific name	Common name	Local name	Region
Light	Laniaceae	Rosmarinus officinalis	Rosemary	Rosmaninho	Gimonde
Ambar	Boraginaceae	Echium vulgare	Viper's bugloss	Erva viperina	Bragança
Dark	Ericaceae	Erica australis	Heather	Urze	Portela

The filtrate (1 ml) was mixed with 9 ml of 2,6-dichlorophenolindophenol and the absorbance was measured within 30 min at 515 nm against a blank. The content of ascorbic acid was calculated on the basis of the calibration curve of authentic L-ascorbic acid (0.020–0.12 mg/ml; $Y = 3.2453X - 0.0703$; $R^2 = 0.9963$) and the results were expressed as mg of ascorbic acid/kg of honey.

For carotenoids determination, the sample (100 mg) was vigorously shaken with 10 ml of acetone–hexane mixture (4:6) for 1 min and filtered through Whatman No. 4 filter paper. The absorbance of the filtrate was measured at 453, 505 and 663 nm. Contents of β-carotene and lycopene were calculated according to the following equations: lycopene (mg/100 ml) = -0.0458 $A_{663} + 0.372 A_{505} - 0.0806 A_{453}$; β-carotene (mg/100 ml) = 0.216 $A_{663} - 0.304 A_{505} + 0.452 A_{453}$. The results were expressed as mg of carotenoid/kg of honey.

For reducing sugars quantification the DNS (dinitrosalicylic acid) method and glucose was used to calculate the standard curve (0.25–1.50 mg/ml; $Y = 0.0009X - 0.1809$; $R^2 = 0.9942$); the results were expressed as g of reducing sugars/kg of honey.

2.6. Antioxidant activity

2.6.1. DPPH radical-scavenging activity

Various concentrations of water honey solutions or phenolic extracts (0.3 ml) were mixed with 2.7 ml of methanolic solution containing DPPH radicals (6×10^{-5} mol/l). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was determined by measuring the absorption at 517 nm (Hatano, Kagawa, Yashura, & Okuda, 1988). The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: % RSA = $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution when the sample extract has been added at a particular level and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radicals scavenging activity (EC_{50}) was calculated by interpolation from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

2.6.2. Reducing power

Various concentrations of water honey solutions or phenolic extracts (2.5 ml) were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) was added, the mixture was centrifuged at 1000 rpm for 8 min (Centorion K24OR-2003 refrigerated centrifuge). The upper layer (5 ml) was mixed with 5 ml of deionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm: higher absorbance indicates higher reducing power (Oyaizu, 1986). The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated by interpolation from the graph of absorbance at 700 nm against extract concentration. BHA and α-tocopherol were used as standards.

2.6.3. Inhibition of β-carotene bleaching

The antioxidant activity of water honey solutions or phenolic extracts was evaluated by the β-carotene linoleate model system. A solution of β-carotene was prepared by dissolving 2 mg of β-carotene in 10 ml of chloroform. Two millilitres of this solution were pipetted into a 100 ml round-bottom flask. After the chloroform was removed at 40 °C under vacuum, 40 mg of linoleic acid, 400 mg of Tween 80 emulsifier, and 100 ml of distilled water were added to the flask with vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into different test tubes containing 0.2 ml of different concentrations of the water honey solutions or phenolic extracts. The tubes were shaken and incubated at 50 °C

in a water bath. As soon as the emulsion was added to each tube, the zero time absorbance was measured at 470 nm using a spectrophotometer. Absorbance readings were then recorded at 20-min intervals until the control sample had changed colour. A blank, devoid of β-carotene, was prepared for background subtraction (Mi-Yae, Tae-Hun, & Nak-Ju, 2003). Lipid peroxidation (LPO) inhibition was calculated using the following equation: LPO inhibition = (β-carotene content after 2 h of assay/initial β-carotene content) × 100. The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. TBHQ was used as standard.

2.6.4. Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from pigs (Sus scrofa) with a body weight of ∼150 kg, dissected and homogenised with a Polytron in ice-cold Tris–HCl buffer (20 mM, pH 7.4) to produce a 1:2 (w/v) brain tissue homogenate which was centrifuged at 3000g for 10 min. An aliquot (0.1 ml) of the supernatant was incubated with the water honey solutions or phenolic extracts (0.2 ml) in the presence of $FeSO_4$ (10 μM; 0.1 ml) and ascorbic acid (0.1 mM; 0.1 ml) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 ml), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 ml), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3000g for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)–TBA complex in the supernatant was measured by its absorbance at 532 nm (Ng, Liu, & Wang, 2000). The inhibition ratio (%) was calculated using the following formula: Inhibition ratio (%) = $[(A - B)/A] \times 100\%$, where A and B were the absorbance of the control and the sample solution, respectively. The extract concentration providing 50% lipid peroxidation inhibition (EC_{50}) was calculated by interpolation from the graph of antioxidant activity percentage against extract concentration. BHA was used as standard.

2.7. Statistical analysis

The assays were carried out in triplicate and the results are expressed as mean values and standard deviation (SD). The statistical differences represented by letters (Tables 2 and 3) were obtained through one-way analysis of variance (ANOVA) followed by Tukey's honestly significant difference post hoc test with $\alpha = 0.05$, coupled with Welch's statistic. Discriminant function analysis was done to determine which variables discriminate between the six naturally occurring groups (ELH, PLH, EAH, PAH, EDH and PDH). These treatments were carried out using SPSS v. 16.0 program.

3. Results and discussion

The antioxidant properties of different Portuguese honey samples were evaluated using the whole sample and the phenolic extract. Numerous tests have been developed for measuring the antioxidant capacity of food and biological samples. However, there is no universal method that can measure the antioxidant capacity of all samples accurately and quantitatively. Clearly, matching radical source and system characteristics to antioxidant reaction mechanisms is critical in assessing antioxidant capacity assay methods (Prior, Wu, & Schaich, 2005). To screen the antioxidant properties of the samples, several chemical and biochemical assays using animal cells were performed: scavenging activity on DPPH radicals (measuring the decrease in DPPH radical absorption after exposure to radical scavengers), reducing power (measuring the conversion of a Fe^{3+}/ferricyanide complex to the ferrous form),

Table 2
Total phenolics (mg/kg) and EC_{50} values (mg/ml) obtained for the antioxidant activity of the honey samples (mean ± SD; n = 3). In each column different letters mean significant differences ($p < 0.05$).

Samples		Phenolics	DPPH scavenging activity	Reducing power	β-Carotene bleaching inhibition	TBARS assay
Light honey	Entire (ELH)	226.16 ± 0.22 c	168.94 ± 19.20 a	48.95 ± 1.61 d	75.51 ± 0.04 a	2.47 ± 0.03 a
	Phenolic extract (PLH)	132.17 ± 0.05 f	90.78 ± 5.10 c	94.11 ± 1.95 a	23.61 ± 2.85 c	0.96 ± 0.02 d
Ambar honey	Entire (EAH)	406.23 ± 17.22 b	130.49 ± 1.38 b	17.95 ± 0.60 e	39.25 ± 0.17 b	1.92 ± 0.05 b
	Phenolic extract (PAH)	168.44 ± 1.99 e	88.53 ± 18.26 c	80.48 ± 0.20 b	12.01 ± 0.96 d	0.71 ± 0.01 e
Dark honey	Entire (EDH)	727.77 ± 0.23 a	106.67 ± 2.48 bc	13.26 ± 0.20 f	37.03 ± 0.01 b	1.75 ± 0.04 c
	Phenolic extract (PDH)	204.24 ± 0.63 d	84.98 ± 1.19 c	73.50 ± 0.03 c	12.13 ± 1.38 d	0.58 ± 0.02 f

Table 3
Correlations established between phenolics and antioxidant activity EC_{50} values.

Antioxidant activity assay	Entire honey				Phenolic extract			
	Equation	R^2	F	p	Equation	R^2	F	p
DPPH scavenging activity	$y = -0.118x + 188.980$	0.915	10.741	0.188	$y = -0.080x + 101.637$	0.982	56.042	0.084
Reducing power	$y = -0.064x + 55.96$	0.714	2.502	0.359	$y = -0.286x + 130.8$	0.967	30.065	0.115
β-Carotene bleaching inhibition	$y = -0.068x + 81.65$	0.649	1.852	0.403	$y = -0.159x + 42.78$	0.746	2.936	0.336
TBARS assay	$y = -0.001x + 2.645$	0.802	4.061	0.293	$y = -0.005x + 1.646$	0.966	29.014	0.117

inhibition of β-carotene bleaching (by neutralising the linoleate-free radical and other free radicals formed in the system which attack the highly unsaturated β-carotene models), and inhibition of lipid peroxidation in brain tissue (measured by the colour intensity of MDA–TBA complex).

From the analysis of Table 2 we can conclude that all the samples revealed good lipid peroxidation inhibition measured in the TBARS assay (EC_{50} values lower than 2.5 mg/ml and lower than the values obtained in the other antioxidant activity assays). Dark honey presented, in all the assays, better antioxidant activity (lower EC_{50} values) than the other honey samples (ambar and light). In fact, the increase of the colour intensity seems to be related to an increase in the antioxidant properties and in the phenolic contents (Table 3). The entire dark honey (EDH, 727.77 mg/kg) and its phenolic extract (PDH, 204.24 mg/kg) revealed the highest phenolic content, followed by the amber sample, and the light honey with the lowest values (226.16 mg/kg for the entire honey, ELH, and 132.17 mg/kg for the phenolic extract, PLH; Table 2).

A significantly higher phenolic content was observed in the entire samples when compared with their phenolic extracts obtained after separation with amberlite and elution with methanol. Phenolic compounds include different subclasses (flavonoids, phenolic acids, stilbenes, lignans, tannins and oxidised polyphenols) displaying a large diversity of structures, some of which may escape the usual methodologies of analysis, commonly carried out by HPLC (high performance liquid chromatography) coupled to distinct detection devices. Various reasons for this exist, such as the existence of isomers, difficulty for chromatographic separation of some compounds, lack of commercial standards, or the structure has not yet been elucidated (Georgé, Brat, Alter, & Amiot, 2005). The method of Folin–Ciocalteu's is, therefore, largely used to evaluate total phenolics despite all the interferences of this assay since the reagent (mixture of phosphotungstic acid and phosphomolibdic acid) also reacts with other non-phenolic reducing compounds

leading to an overvaluation of the phenolic content. For instance, ascorbic acid is a widespread reducing agent that can interfere in the Folin–Ciocalteu reaction. Other reducing substances such as some sugars and amino acids could also interfere. (Georgé et al., 2005). In fact, the honey samples contain some of these compounds as well as other antioxidants (Table 4) that can lead to an increase in the absorbance values and to positive errors in the determination of phenolics by the Folin–Ciocalteu method. From the analysis of Table 4 we can also conclude that dark honey contain the highest concentrations of other antioxidants such as flavonoids (587.42 mg/kg), ascorbic acid (145.80 mg/kg) and β-carotene (9.49 mg/kg), which can also contribute to the better antioxidant properties observed in this sample. No significant statistical difference between the sugar contents in the different honeys was observed. All the samples presented a very high percentage of sugars (~75%) as was already expected because these compounds are the most abundant nutrients in honey (Mendes, Proença, Ferreira, & Ferreira, 1998).

The better scavenging activity and lipid peroxidation inhibition showed by the phenolic extracts in comparison to the entire honeys proved once more, that other substances than phenols might be present in the entire samples. This fact is also supported by the higher correlation coefficients obtained for the phenolic extracts (Table 3). Only in the case of the reducing power assay was there higher EC_{50} values (lower reducing power) for the phenolic extracts in comparison to the entire honeys (Table 2). This is probably due to the presence of sugars in the entire samples which have good reducing capacity (e.g. glucose and fructose) and, therefore, contribute to higher reducing power values than the values obtained in the phenolic extracts.

In order to determine which variables discriminate between the six naturally occurring groups (ELH, PLH, EAH, PAH, EDH and PDH) a discriminant function analysis was carried out, following the stepwise method. In stepwise discriminant function analysis, the

Table 4
Other antioxidant compounds present in the honey samples (mean ± SD; n = 3). In each column different letters mean significant differences ($p < 0.05$).

Samples	Flavonoids (mg/kg)	Ascorbic acid (mg/kg)	β-Carotene (mg/kg)	Lycopene (mg/kg)	Reducing sugars (g/kg)
Light honey	123.62 ± 0.17 c	140.01 ± 0.05 c	9.32 ± 0.01 a	6.55 ± 0.01 a	753.23 ± 22.47 a
Ambar honey	342.70 ± 1.72 b	143.86 ± 1.16 b	8.64 ± 0.06 b	6.19 ± 0.00 b	725.89 ± 5.81 a
Dark honey	587.42 ± 0.46 a	145.80 ± 0.02 a	9.49 ± 0.15 a	6.12 ± 0.03 c	749.90 ± 14.80 a

Table 5
The four most important parameters defined for discrimination between honey samples and extraction method considering antioxidant activity and phenolic content.

	Wilks' Lambda	F-Remove (2.71)	p-Level	Tolerance	1-Tolerance (R^2)
Phenolics	0.0000	686.5187	0.0000	0.5875	0.4125
TBARS assay	0.0000	35.8342	0.0000	0.5712	0.4288
Reducing power	0.0000	183.8875	0.0000	0.8052	0.1948
β-Carotene bleaching inhibition	0.0000	40.9027	0.0000	0.8676	0.1324

model of discrimination is built step by step. At each step, all variables are reconsidered to find which one will contribute most to the discrimination between groups. That variable will then be included in the model, restarting the process. The values of *F* to enter (3.84) and *F* to remove (2.71) are the guidelines of the stepwise procedure. A variable is entered into the model if its *F*-value is greater than the *F*-enter value and its removed if the *F*-value is less than the *F*-remove value. *F*-enter must be greater than *F*-remove, and both values must be positive. The *F*-value for a variable indicates its statistical significance in the discrimination between groups. Discriminant analysis defines an optimal combination of varieties in a way that the first function furnishes the most general discrimination between groups, the second provides the second most, and so on (Benitez, Nogales, Campos, & Ruano, 2006). Table 5 shows the results from applying the algorithm for selecting honey samples according with colour intensity and extraction method. Wilk's Lambda is a variable selection method for stepwise discriminant analysis that chooses variables for entry into the equation on the basis of how much they lower Wilk's Lambda. At each step, the variable that minimises the overall Wilk's Lambda is entered.

Tolerance is the proportion of a variable's variance not accounted by other independent variables in the equation. If it is low, it will contribute little information to the model and might cause problems.

The discriminant analysis (DA) defined four dimensions, but only the first two explained 97.5% of the observed variance, so only they were considered in the canonical analysis (Fig. 1). The first DA dimension separates mainly EDH and EAH from the other samples (means of the canonical variance: EDH = −91.142, EAH = −34.756, ELH = 28.621, PLH = 51.197, PAH = 29.787 and PDH = 16.294), and was more strongly correlated with reducing power and phenolic content. This result confirmed that there is a direct correlation between phenolic content and colour intensity. The second DA dimension reveals the separation of ELH, PLH and PDH (means of

the canonical variance: ELH = 54.925, PLH = −9.032 and PDH = −28.078), and proved to be more accurately correlated with β-carotene bleaching inhibition and TBARS formation inhibition.

Overall, it can be concluded that the samples submitted to phenolic extraction clustered proximately being well separated from the entire honeys (Fig. 1). The proximity of all the extracted samples indicate that a methanolic extraction after separation in Amberlite led to more reliable and accurate antioxidant activity results in a non-honey type dependent manner. The use of the whole extract instead of individual antioxidants allows advantage to be taken of additive and synergistic effects of different phenolic compounds present in the samples. This effect is more easily seen in the phenolic extracts than in the entire honey, once the extracted compounds should be structurally related and therefore, responsible for similar biological properties.

Acknowledgement

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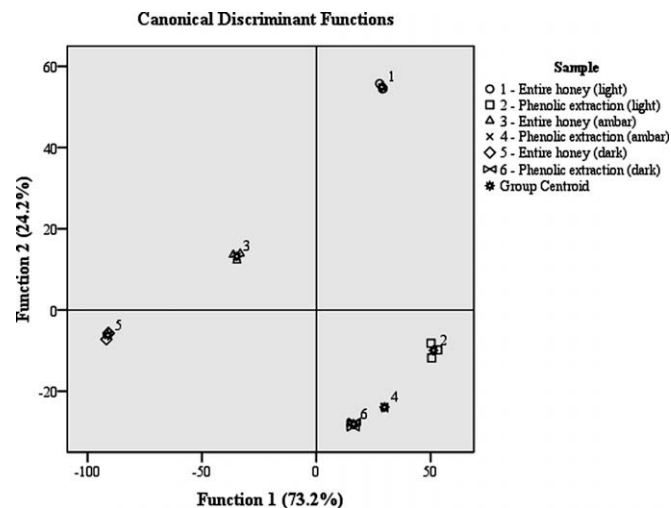

Fig. 1. Canonical analysis of honey samples based on antioxidant activity assays and phenolic contents.

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