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Article

Antioxidant Activity and Phenolic Composition of Brazilian Honeys and their Extracts

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Foram avaliadas as atividades antioxidantes e o conteúdo de polifenóis de cinco méis silvestres e quatro de laranjeira, bem como de seus extratos fenólicos. A identificação e quantificação das substâncias fenólicas foram realizadas por HPLC-DAD (cromatografia líquida de alta eficiência com detecção por arranjo de diodos). A atividade sequestradora de radicais livres, determinada por 2,2-difenil-1-picrilhidrazil (DPPH$^{\bullet}$) e expressa como CE_{50}, variou de 10,81 a 52,64 mg mL^{-1} para mel e de 6,17 a 52,87 µg mL^{-1} para extrato. A atividade antioxidante dos extratos foi também determinada pelos métodos com 2,2'-azinobis-(3-etil-benzotiazolino-6-ácido sulfônico) sal diamônio (ABTS) e poder antioxidante de redução do ferro (FRAP), e os resultados variaram de 46,53 a 383,49 mmol TE 100 g^{-1} e de 34,99 a 408,14 mol Fe(II) 100 g^{-1}, respectivamente. Também foram avaliados os teores de fenóis totais e flavonóides pelos métodos de Folin-Denis e complexação com cloreto de alumínio, respectivamente. A atividade antioxidante e o teor de fenóis totais mostraram-se altamente correlacionáveis.

The antioxidant activities and total polyphenolic content of five multifloral and four orange blossom Brazilian honey samples and their phenolic extracts were investigated. Identification and quantification of phenolic compounds were carried out by HPLC-DAD (high-performance liquid chromatography with diode-array detection). The radical scavenging activities, determined with 2,2-diphenyl-1-picrylhydrazyl (DPPH$^{\bullet}$) and expressed as EC_{50}, ranged from 10.81 to 52.64 mg mL^{-1} for honey and 6.17 to 52.87 µg mL^{-1} for the extracts. The extract antioxidant activities were also determined by 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) and ferric reducing antioxidant power (FRAP) assays, and the results varied from 46.53 to 383.49 mmol TE 100 g^{-1} and 34.99 to 408.14 mol Fe(II) 100 g^{-1}, respectively. It was also evaluated the total phenolic and flavonoid contents by the Folin-Denis and aluminum chloride methods, respectively. Antioxidant activities and total phenolic contents were found to be highly correlated.

Keywords: orange blossom Brazilian honey, multifloral Brazilian honey, honey phenolic compounds

Introduction

Honey is the most important primary product of beekeeping, both from a quantitative and an economic point of view, and honey has been used throughout much of history. Honey from *Apis mellifera* is undoubtedly the most widely collected honey, and this variety has been spread around the world. The quality of honey is judged by the botanical or floral origin of the honey as well as its chemical composition.[1] Sugars and water represent the main chemical constituents of honey (95%), whereas proteins, vitamins, amino acids, phenolic acids and flavonoids constitute the minor components.[2] Honey is rich in phenolic compounds, which act as natural antioxidants, and are becoming increasingly popular because of their potential role in contributing to human health. These compounds can also be used as indicators in studies into the floral and geographical origins of honey.

The use of analytical procedures for determination of phenolic acids or flavonoids, individually or as a group simultaneously, has been related to the floral and geographical origins of honey.[3]

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Honey is known to be rich in both enzymatic and non-enzymatic antioxidants, including glucose oxidase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, amino acids and proteins.[4] According to previous literature, honey composition and antioxidant capacity depend on the nectar floral source. In addition, seasonal and environmental factors as well as processing may also have an effect on honey composition and antioxidant activity.[5]

Previous studies indicate that among minor honey constituents, polyphenols such as phenolic acids and flavonoids may function as natural antioxidants in our diet.[6] These antioxidants can play an important role in food preservation and human health by combating damage caused by oxidizing agents. For this reason, honey has recently received much attention.

Several studies on the phenolic composition have been carried out in European honeys,[7] however, little information is available on the phenolic profiles of Brazilian honeys.

The aim of this study was identify and quantify phenolic compounds in nine Brazilian honeys and to determine their antioxidant activities, as well as on their phenolic extracts.

Experimental

Honey samples

Nine *Apis mellifera* honey samples were collected for this study (Table 1). Four samples of multifloral honeys (RLS1, RLS2, RLS3 and RLS4) and three samples of orange blossom honey (RLL1, RLL2 and RLL3) were obtained from beekeepers. Two samples of orange blossom honey (RLL4 and RLL5) were purchased from the local market. The samples were collected between June 2003 and March 2004 from different geographical regions of Rio de Janeiro and São Paulo States (Table 1). The botanical origin was confirmed by the traditional qualitative microscopic analysis and frequency determination of the classes of pollen grains in the honey samples.[8] The honey was considered as monofloral when more than 45% of typical pollen grains of the botanical specie was present.[8] Samples were stored at 4 °C until they were analyzed.

Phenolic extractions

The procedure for the fractionation of honey was carried out as previously described.[3,6,9] Honey samples (50 g) were thoroughly mixed with 250 mL of distilled water and adjusted to pH 2 with concentrated HCl and filtered through cotton to remove solid particles. The filtrate was mixed with 75 g of Amberlite XAD-2 resin, pore size 9 nm and particle size 0.3-1.2 mm (Supelco, Bellefonte, PA, USA) and magnetically stirred for 10 min. The mixture was then packed into a glass column (25 × 2.0 cm). The column was washed with 250 mL of acidified water (pH 2 with HCl) and then subsequently rinsed with 300 mL of neutral distilled water to remove all sugars and other polar compounds of honey. The phenolic compounds were eluted from the sorbent with 500 mL of methanol. The methanol extracts were concentrated under vacuum at 40 ºC in a rotary evaporator. The residue was dissolved in 10 mL of distilled water and extracted five times with 10 mL of ethyl acetate. The extracts were combined and the solvent was removed under vacuum. The dried residue was then redissolved in 1 mL of methanol (HPLC grade) and filtered through a 0.45 μm pore size membrane filter (Sartorius Stedim Biotech, Germany). Three replicate extractions were performed for each sample, and the standard deviation did not exceed 5%. The yields of extracts were expressed as a mean of three extractions and ranged from 10 to 53 mg *per* 50 g of product.

Determination of total phenolic content

The modified Folin-Dennis method[10] was used to determine the total phenolic content. Each honey sample

Table 1. Honey samples analyzed in this work

Sample code	Kind of honey	Botanical origin (% of pollen)	Harvest date	Localization
RLS1	multifloral	multifloral	March 2004	Paraty-RJ
RLS2	multifloral	multifloral	December 2003	Itararé-SP
RLS3	multifloral	multifloral	January 2004	Itararé-SP
RLS4	multifloral	multifloral	March 2004	Juquitiba-SP
RLL1	orange blossom	*Citrus* sp	March 2004	Botucatu-SP
RLL2	orange blossom	*Citrus* sp	March 2004	Botucatu-SP
RLL3	orange blossom	*Citrus* sp	March 2004	Itararé-SP
RLL4	orange blossom	*Citrus* sp	June 2003	Niterói-RJ[a]
RLL5	orange blossom	*Citrus* sp	March 2004	Botucatu-SP[a]

[a]Obtained from local markets.

(100 mg) and each phenolic extract (0.1 mg) were diluted to 1.0 mL with distilled water. Each solution (0.5 mL) was then mixed with 2.5 mL of Folin-Denis reagent (Sigma-Aldrich Chemie, Steinheim, Germany), and after 5 min, 2 mL of a 14% sodium carbonate (Na_2CO_3) solution was added. After incubation at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm against a Milli-Q water blank using a Shimadzu spectrophotometer (UVmini-1240, Japan). Gallic acid (Sigma-Aldrich Chemie, Steinheim, Germany) was used as the standard to produce the calibration curve (0-0.022 mg mL^{-1}, $y = 4.75098\ x + 23.73359$, $R = 0.99313$) (R: correlation coefficient). The mean of three analyses was used, and the total phenolic content was expressed in gallic acid equivalents (mg GAE 100 g^{-1} of honey or g GAE 100 g^{-1} of extract).

Determination of total flavonoid content

Total flavonoid content was determined using a colorimetric method as previously described.[11] Briefly, 3 mL of a 2% aluminum trichloride ($AlCl_3$; Vetec, Brazil) methanolic solution were added to the same volume of honey (300-600 mg mL^{-1} in methanol:water) or extract (0.2 mg mL^{-1} in methanol) solution. After 30 min of incubation, the absorbance values were measured at 415 nm against a methanol (HPLC grade) blank. Quercetin (Sigma-Aldrich Chemie, Steinheim, Germany) was used as a standard to produce the calibration curve (0-0.020 mg mL^{-1}, $y = 6.95836\ x + 27.0114$, $R = 0.99296$). The total flavonoid content was calculated from the mean of three analyses and expressed as quercetin equivalents (mg QE 100 g^{-1} of honey or g QE 100 g^{-1} of extract).

Determination of free radical scavenging activity with DPPH (2,2-diphenyl-1-picrylhydrazyl)

The scavenging activity of honey samples and extracts for the radical DPPH (Sigma-Aldrich Chemie, Germany), was measured as described by Zhang and Hamauzu[12] with some modifications. The purple color of 2,2-diphenyl-1-picrylhydrazyl (DPPH) decays in the presence of an antioxidant, and the change in the absorbency can be spectrophotometrically monitored at 517 nm.

A volume of 29 µL of a 0.3 mmol L^{-1} DPPH methanolic solution was added to 71 µL of various concentrations of honey (5 to 50 mg mL^{-1} in water:methanol 1:1), honey extract (5 to 100 µg mL^{-1} in methanol) or phenolic standard (5 to 500 µmol L^{-1} in methanol). The mixtures were kept in the dark for 30 min at room temperature, and the absorbance of the remaining DPPH was determined at 517 nm using a microplate reader.[13] A mixture of 29 µL of honey, extract or standard solution was used as a blank, and a mixture of 29 µL of DPPH solution with 71 µL of methanol was used as a negative control. The radical scavenging activity was calculated as percentage of DPPH discoloration using equation 1:

$$AA\% = 100 - \left(\frac{Abs_{sample} - Abs_{blank}}{Abs_{control}} \right) \times 100 \quad (1)$$

where AA% = inhibition percentage, Abs_{blank} = average absorption of a blank sample at the end of the reaction, Abs_{sample} = average absorption of a tested honey, honey extract or phenolic standard at the end of the reaction, and $Abs_{control}$ = average absorption of negative control at the end of the reaction.

The effective concentration providing 50% inhibition (EC_{50}) was calculated from the graph of scavenging effect percentage against honey, honey extract or phenolic standard concentration. All experiments were carried out in triplicate.

Determination of antioxidants using ABTS [2,2'-azino-bis-(3-ethylbenzothiazoline-6- sulfonic acid) diammonium salt]

The $ABTS^{\bullet+}$ radical was prepared by mixing 5 mL of an aqueous 7 mmol L^{-1} ABTS solution with 0.44 mL of an aqueous 140 mmol L^{-1} solution of potassium persulfate. The reaction was kept in the dark for 16 h, and 2 mL of this solution was then dissolved in absolute ethanol until it reached an absorbance of 0.700 ± 0.005 at 734 nm.

The reaction consisted of mixing 50 µL of a methanolic extract solution (0.2 mg mL^{-1}) with 5 mL of the final $ABTS^{\bullet+}$ solution. After 6 min, the absorbance of the reaction was measured at 734 nm using absolute ethanol as a blank.[14] The $ABTS^{\bullet+}$ solution was daily prepared, and the reactions were carried out in the dark. All reactions were done in triplicate. The obtained absorbance values were plotted in a Trolox curve (Sigma-Aldrich Chemie, Steinheim, Germany; 0.000-2.400 mmol L^{-1}; $y = -26.37778\ x + 0.65164$; $R = -0.9997$), and the results were expressed in mmol TE 100 g^{-1}.

Antioxidant activity using FRAP (ferric reducing antioxidant power)

The FRAP assay was done to access the ability of the honey extracts to reduce the ferric 2,4,6-tripyridyl-*s*-triazine complex (Fe^{3+}-TPTZ complex) to its blue ferric form (Fe^{2+}-TPTZ complex). The FRAP reagent was daily prepared by mixing 10 mL of an aqueous 10 mmol L^{-1} TPTZ

(2,4,6-tripyridyl-*s*-triazine) solution in 40 mmol L^{-1} HCl, 10 mL of a 20 mmol L^{-1} $FeCl_3$ solution and 100 mL of a 0.3 mol L^{-1} acetate buffer (pH 3.6). The reaction was then carried out by mixing 0.5 mL of a methanolic extract solution (0.2 mg mL^{-1}) with 4.5 mL of the FRAP reagent, and the absorbance was measured at 593 nm after incubation for 10 min at 37 °C.[15] All reactions were done in triplicate. The blank consisted of 0.5 mL of methanol in 4.5 mL of the FRAP reagent. The absorbance values were plotted on a $FeSO_4.7H_2O$ curve (Vetec, Brazil; 100-1000 μmol L^{-1}; $y = 0.0018\,x + 0.00107$, $R = 0.99961$), and the results were expressed in mmol Fe(II) 100 g^{-1}.

HPLC analysis of the phenolic profile of honey

Polyphenol identification analyses were carried out using a liquid chromatograph (LC-20AT, Shimadzu, Japan) with a diode array detector (DAD, SPD-M20A) coupled to an LCSolution ChemStation data-processing station. The column used was a C_{18} LiChroCART (250 mm × 4 mm; 5 μm; Merck, Darmstadt, Germany), operated at 35 °C. The mobile phase consisted of solvent A (water and acetic acid, 99:1) and solvent B (water, acetonitrile and acetic acid, 59:40:1). The gradient program was as follows: from 25 to 100% B in 30 min, and then isocratic by 10 min. The injection volume was 20 μL, and the flow rate was 1 mL min^{-1}. Chromatograms for the phenolic acids were recorded at 270 nm and for flavonoids at 360 nm. The identification of phenolic compounds was based on a comparison of chromatographic data (retention times and UV spectra) with authentic markers, while quantification was performed through external calibration data with the same compounds. Quantitative results were expressed as mg of compound *per* 100 g of honey.

Authentic markers available at the market were used for chromatographic comparison of data. Gallic acid, protocatechuic acid, syringic acid, *p*-hydroxybenzoic acid, sinapic acid, vanillic acid, *p*-coumaric acid, *p*-methoxybenzoic acid, cinnamic acid, rutin, isoquercetin, morin and quercetin were supplied by Sigma-Aldrich Chemie (Steinheim, Germany).

Statistical analysis

Differences in percentage antioxidants, the total phenolics and the correlations between the phenolic content in honey and its antioxidant activity were analyzed applying the software Unscrambler X version 10.1. Significance level was $p < 0.01$ unless otherwise indicated, and correlation coefficients R varied from 0.99006 to 0.99956.

Results and Discussion

Nine honey samples of different floral origins and their respective extracts were tested to evaluate their antioxidant properties and to find the relationship of total phenolic contents, flavonoid contents and antioxidant activity with floral and geographical origins. The total phenolic (TP) contents varied from 34.0 to 78.2 mg GAE 100 g^{-1} for honey and 12.72 to 76.65 g GAE 100 g^{-1} for the extract (Tables 2 and 3). The multifloral honey (sample RLS1) showed the highest TP content (78.2 mg GAE 100 g^{-1}) and the highest radical scavenging activity (RSA = 10.81 mg mL^{-1}), while the orange blossom honey extract RLL1 showed the highest phenolic content and also the highest radical scavenging activity (TP = 76.65 g GAE 100 g^{-1}; RSA 5.48 μg mL^{-1}). The GAE values for individual honey samples confirmed that the phenolic contents are significantly dependent on the floral sources, which is in agreement with other previous data.[2,16]

Table 2. Total phenolic (TP) and total flavonoid (TF) contents and radical scavenging activity (EC_{50}) of the honey samples[a]

Honey	TP / (mg GAE 100 g^{-1})	TF / (mg QE 100 g^{-1})	Radical scavenging activity (RSA) EC_{50} / (mg mL^{-1})
RLS1	78.2 ± 2.7	–	10.81 ± 0.50
RLS2	42.8 ± 1.9	0.25 ± 0.03	19.74 ± 1.62
RLS3	57.2 ± 2.4	1.60 ± 0.16	18.42 ± 1.47
RLS4	54.0 ± 2.3	4.27 ± 0.43	17.52 ± 1.10
Mean	58.05 ± 14.78	1.53 ± 1.96	16.62 ± 3.98
RLL1	35.7 ± 2.4	0.30 ± 0.03	36.22 ± 3.82
RLL2	38.8 ± 3.6	0.28 ± 0.04	40.80 ± 4.68
RLL3	53.2 ± 2.9	–	29.85 ± 2.67
RLL4	40.1 ± 2.9	–	33.21 ± 2.51
RLL5	34.0 ± 1.8	0.24 ± 0.01	52.64 ± 4.70
Mean	40.36 ± 7.58	0.17 ± 0.15	38.54 ± 8.85

[a]All data expressed on a honey weight basis as means ± SD ($n = 3$).

The total flavonoid (TF) contents of the honey samples and their extracts were also determined. Flavonoid content was lower than phenolic content and ranged from 0.00 to 4.27 mg QE 100 g^{-1} for honey and 0.16 to 5.34 g QE 100 g^{-1} for the extracts (Tables 2 and 3). The greatest TF content was reported for the multifloral honey RLS4 (4.27 mg QE 100 g^{-1}) and the orange blossom honey extract RLL1 (5.34 g QE 100 g^{-1}).

The TP average values found for the analyzed samples are higher than those previously reported in honeys from other origin, such as Cuban honeys (21.39-59.58 mg GAE 100 g^{-1}),[16] and Czech honeys

Table 3. Total phenolic (TP) and total flavonoid (TF) contents, DPPH, ABTS and FRAP tests of the honey extracts[a]

Extract	TP / (g GAE 100 g^{-1})	TF / (g QE 100 g^{-1})	FRAP / (mol Fe(II) 100 g^{-1})	ABTS / (mmol TE 100 g^{-1})	DPPH:EC_{50} / (μg mL^{-1})
RLS1	25.60 ± 0.37	0.50 ± 0.01	95.18 ± 3.21	137.79 ± 4.82	51.45 ± 5.59
RLS2	71.76 ± 1.52	0.95 ± 0.01	408.14 ± 10.02	316.48 ± 9.82	8.17 ± 0.27
RLS3	24.22 ± 0.49	0.30 ± 0.01	109.99 ± 11.23	125.67 ± 9.95	37.62 ± 1.99
RLS4	30.05 ± 0.83	0.38 ± 0.02	78.51 ± 4.24	58.66 ± 15.20	30.60 ± 2.33
Mean	37.91 ± 22.71	0.53 ± 029	172.96 ± 157.32	159.65 ± 110.19	31.96 ± 18.07
RLL1	76.65 ± 0.56	5.34 ± 0.03	438.69 ± 2.78	383.49 ± 6.15	5.48 ± 0.03
RLL2	50.39 ± 1.18	0.23 ± 0.01	376.66 ± 1.60	236.71 ± 3.32	10.50 ± 0.39
RLL3	47.62 ± 0.74	0.16 ± 0.01	375.73 ± 6.99	207.99 ± 3.83	10.73 ± 0.20
RLL4	12.72 ± 0.32	0.19 ± 0.01	34.99 ± 4.24	46.53 ± 1.11	52.87 ± 1.10
RLL5	40.97 ± 0.73	0.34 ± 0.01	303.51 ± 1.60	210.54 ± 4.42	16.16 ± 0.47
Mean	45.67 ± 22.88	1.25 ± 2.89	305.92 ± 158.84	217.05 ± 119.68	15.22 ± 10.75

[a]All data expressed on extract weight basis as means ± SD (n = 3).

(8.99-21.52 mg GAE 100 g^{-1}).[10] However, the results obtained for TF (0.25-4.27 mg QE 100 g^{-1}) are lower than values as previously reported for other honeys, such as Portuguese honey (12.36-58.74 mg QE 100 g^{-1}),[17] Tualang honey (4.74-22.76 mg QE 100 g^{-1})[18] and Indian honey (4.23-0.10 mg QE 100 g^{-1}).[19]

The radical scavenging activities (RSA) of the different honeys and phenolic extracts are also summarized in Tables 2 and 3. The radical scavenging activities of the honey samples, expressed as DPPH-EC_{50}, varied from 10.81 to 52.64 mg mL^{-1}. The antioxidant capacity was high and widely differed among the honey samples. The lowest scavenging activity was found in the orange blossom honey RLL5 (52.64 mg mL^{-1}) while the highest activity was found in the multifloral honey RLS1 (10.81 mg mL^{-1}). The highest average RSA was observed for multifloral honey samples (16.62 mg mL^{-1}), though the multifloral extracts showed lower antioxidant activity than orange blossom honey extracts. Most likely, the differences in antioxidant activity between the tested samples mainly depend on the floral source of honey.

The radical scavenging capacities of the tested honey extracts were also evaluated in FRAP and $ABTS^{\bullet+}$ radical reaction systems. All honey extracts exhibited scavenging potential towards both radicals.

The observed range of FRAP values [438.69 mmol Fe(II) to 34.99 mmol Fe(II)] for honey extracts is comparable to the reducing capacity range of raw Millefiori honey. A range of 61.75-124.5 mmol Fe(II) was found for monofloral honeys[20] and a range of 71.0-478.5 mol Fe(II) was found for heterofloral honey samples.[21]

Based on the correlation matrices (Tables 4 and 5), each coefficient was considered in order to establish the correlations between different couples of assays. The highly significant correlation between total phenol content and DPPH activity ($R = -0.8146$, $p < 0.01$) of honeys suggests that phenolics are the major components responsible for the antioxidant effects. Similarly, the correlations of antioxidant activity with TP content were also apparent in the extracts ($-0.8918 \leq R \leq 0.9580$, $p < 0.01$).

Table 4. Correlation matrix between the results of total phenolic (TP) and total flavonoid (TF) contents and DPPH for the honey samples

	TP	TF	DPPH:EC_{50} (honey)
TP	1	–	–
TF	0.1759	1	–
DPPH:EC_{50} (honey)	–0.8146	–0.3704	1

As shown in Table 5, there were high correlations among the three methods for antioxidant activity measurement ($-0.8257 \leq R \neq 0.9457$, $p < 0.01$), which could be confirmed by the linear correlation graphics shown in Figure 1, indicating a great degree of equivalence among these measurements. There was also a correlation

Table 5. Correlation matrix between the results of total phenolic (TP) and total flavonoid (TF) contents, FRAP, ABTS and DPPH for the extracts

	TP	TF	FRAP	ABTS	DPPH:EC_{50} (extract)
TP	1	–	–	–	–
TF	0.6558	1	–	–	–
FRAP	0.9258	0.4693	1	–	–
ABTS	0.9580	0.6917	0.9259	1	–
DPPH:EC_{50} (extract)	–0.8918	–0.4108	–0.9457	–0.8257	1

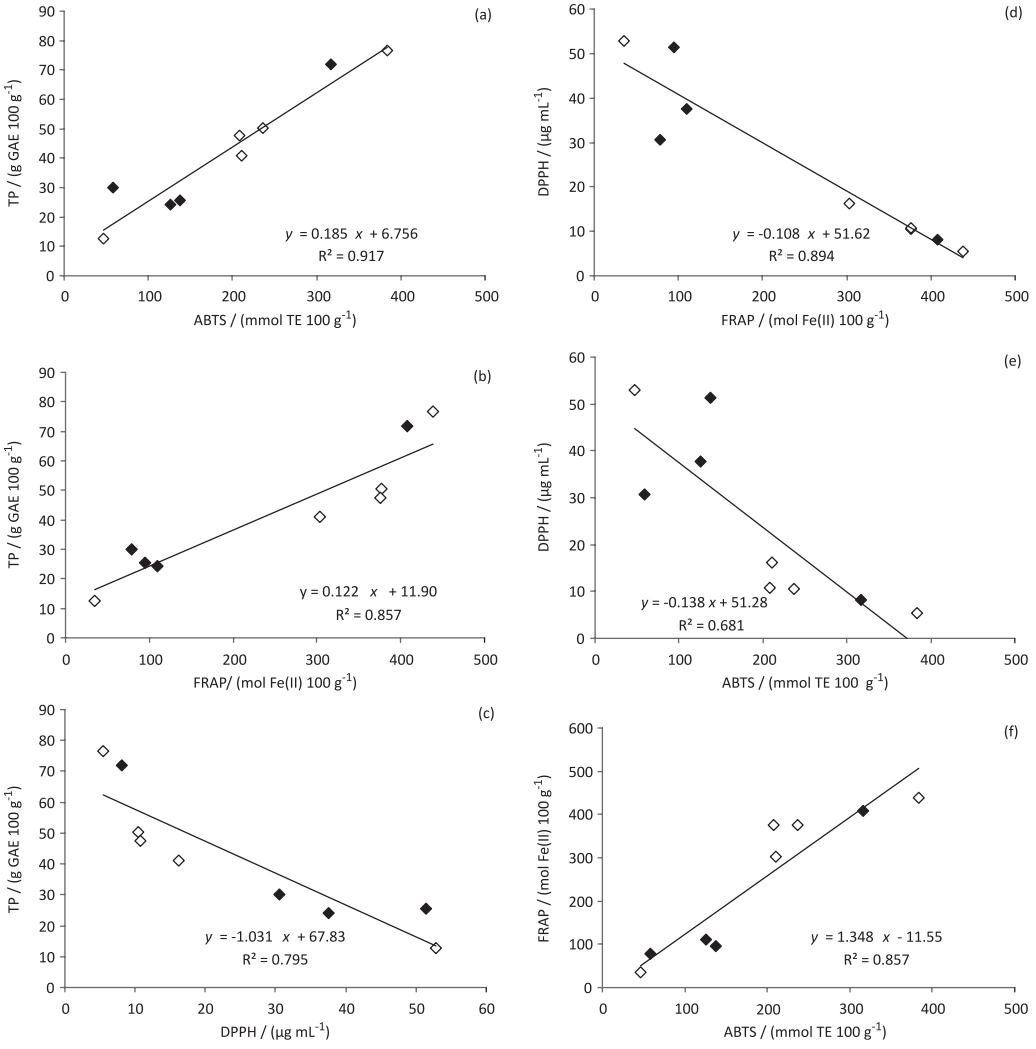

Figure 1. Linear correlations for the extracts between: (a) TP and ABTS, (b) TP and FRAP, (c) TP and DPPH, (d) DPPH and FRAP, (e) DPPH and ABTS, and (f) FRAP and ABTS. White dots: orange blossom honey extracts and black dots: multifloral honey extracts.

with the total phenolic contents. Figures 1a-1c show the correlation of the total phenolic compounds of the extract with ABTS, FRAP and DPPH, respectively, while Figures 1d-1f show the correlation between each of these antioxidant activity methods using the results obtained for the extracts.

The lower correlation and poor statistical significance obtained for total flavonoid content and the honey antioxidant activities ($R = -0.3704$; $p = 0.2$) were assumed to be caused by the very low flavonoid concentration in honey, which eventually reached zero. Although flavonoids are well known to have many important biological properties, including antioxidant activity, their low content in the analyzed honeys made them poor contributors to overall antioxidant capacity of honeys.

The darker multifloral honeys showed higher antioxidant activities and phenolic content than the lighter orange blossom honeys, though the pattern was different for the extracts. The orange blossom honey extracts showed much better results than the multifloral extracts, especially in sample RLL2 (5.48 µg mL^{-1} for DPPH), which may be due to the extraction process. It is known that minerals, especially iron, can complex with phenolic compounds, enhancing their antioxidant activity and color.[22] The water cleansing process during Amberlite XAD-2 extraction can remove most of the honey mineral contents, as their complexes are mainly water soluble. In addition, glycosylated phenolic compounds, which can also contribute to antioxidant capacity of honey, may not be extracted to the methanolic phase or they may be lost in the aqueous phase. Therefore, there might be considerable changes in antioxidant activity and phenolic compounds when comparing the honey and its extract.

It was reported that phenolic compounds are the main components responsible for the antioxidant effects of honey, though some other compounds may also be

involved. Therefore, the evaluation of total phenolic contents and antioxidant activities of honeys may be used as parameters for the assessment of honey quality, as well as the identification of the phenolic constituents.[3,23]

The composition profile of phenolic compounds was analyzed by HPLC. Chromatographic analysis also showed that phenolic acids prevailed in the multifloral honeys. These results coincide with the results obtained by the Folin-Denis method (Table 2): multifloral honey contained a high amount of phenolic compounds, while in orange honey it was the lowest.

The composition and content of individual phenolic compounds in the samples were different and depended on the botanical origin of honeys. Figure 2 shows the typical HPLC chromatograms obtained for some orange blossom and multifloral honey extracts.

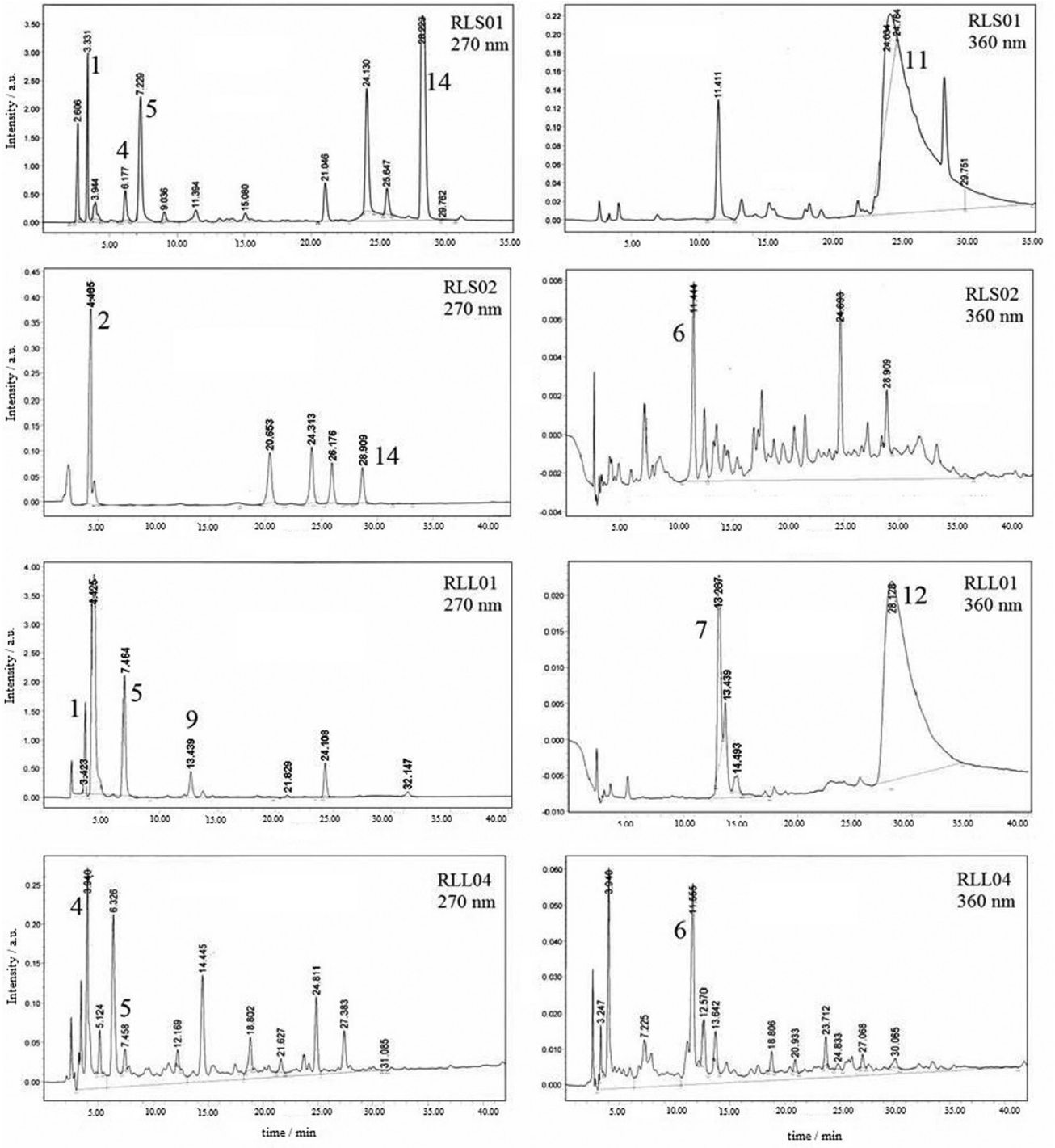

Figure 2. HPLC chromatograms of multifloral and orange blossom honey extracts. For peak identification, see Table 6.

About fourteen phenolic compounds were identified (four flavonoids and ten phenolic acids) and the phenolic pattern of honey contained gallic acid, protocatequic acid, syringic acid, *p*-hydroxibenzoic acid, vanillic acid, *p*-coumaric acid, sinapic acid, *p*-methoxybenzoic acid, cinnamic acid and *p*-methoxycinnamic acid, as well as the flavonoids rutin, isoquercetin, morin and quercetin. Some of these identified phenolic compounds were already reported in other honeys.[2,6] The results of the quantitative determination of phenolic acids and flavonoids in those types of honeys are presented in Table 6. Among these compounds, phenolic acids showed higher concentrations than flavonoids. Some other compounds present in the chromatogram that had similar flavonoid and phenolic acid spectra and chromatographic behavior but they could not be identified due to lack of availability of standard compounds. Comparison with other values found in literature is difficult since previous studies employed different sample preparation and extraction conditions to those used in this work.[24]

The radical scavenging activities of the identified phenolic compounds were also screened in order to evaluate their possible contribution to antioxidant capacity of extracts. Their retention times, λ_{max} (nm) and antioxidant activity (expressed as EC_{50}) are related in Table 7.

According to Table 7, the most hydroxylated compounds, especially in *ortho* positions such as quercetin and gallic acid,

Table 6. Contents of phenolic compounds in the honey samples (mg 100 g^{-1} honey)

entry	Phenolic compound	Multifloral honey				Orange blossom honey				
		RLS1	RLS2	RLS3	RLS4	RLL1	RLL2	RLL3	RLL4	RLL5
1	Gallic acid	7.278	–	–	–	0.005	–	–	–	–
2	Protocatechuic acid	–	0.881	0.967	–	–	1.040	6.673	–	1.307
3	Syringic acid	–	–	–	–	–	–	–	–	1.376
4	*p*-Hydroxybenzoic acid	9.805	–	3.680	5.813	–	–	–	0.823	–
5	Vanillic acid	17.154	–	–	2.895	21.152	–	–	0.142	–
6	*p*-Coumaric acid	–	0.033	7.770	7.788	–	0.515	–	0.671	0.905
7	Rutin	–	–	–	–	0.389	–	–	–	–
8	Isoquercetin	–	–	–	2.960	–	–	–	–	–
9	Sinapic acid	0.699	–	–	–	9.102	3.980	–	–	–
10	*p*-Methoxybenzoic acid	–	–	9.015	7.855	–	–	–	–	–
11	Morin	20.268	–	–	1.773	–	–	–	–	–
12	Quercetin	–	–	2.942	–	2.169	–	–	–	–
13	Cinnamic acid	–	–	–	–	–	0.304	–	–	0.219
14	*p*-Methoxycinnamic acid	22.453	0.254	–	–	–	1.935	–	–	0.754

Table 7. Phenolic standards, retention times, maximum absorption and radical scavenging activity (EC_{50}) of the identified phenolic compounds

entry	Phenolic compound	t_R / min	λ_{max} / nm	EC_{50} [b] / (μmol L^{-1})
1	Gallic acid	3.404	272	6.83 ± 0.04
2	Protocatechuic acid	4.190	259, 295	14.97 ± 0.84
3	Syringic acid	5.936	274	12.24 ± 0.27
4	*p*-Hydroxybenzoic acid	6.325	256	1502.15 ± 25.81
5	Vanillic acid	7.509	260, 292	131.07 ± 6.24
6	*p*-Coumaric acid	11.377	310	239.74 ± 2.78
7	Rutin	13.292	256, 354	7.62 ± 0.11
8	Isoquercetrin	13.409	256, 354	9.33 ± 0.36
9	Sinapic acid	13.572	294 *sh*[a], 323	20.60 ± 0.47
10	*p*-Methoxybenzoic acid	19.969	257	2821.68 ± 118.55
11	Morin	25.114	252, 353	16.34 ± 0,38
12	Quercetin	27.416	256, 368	6.46 ± 0.06
13	Cinnamic acid	27.669	278	3040.07 ± 37.75
14	*p*-Methoxycinnamic acid	28.797	306	1363.71 ± 51.47

[a]*sh* = shoulder; [b]radical scavenging activity was expressed as DPPH-EC_{50}.

showed the highest antioxidant activities (EC_{50} = 6.46 and 6.83 μmol L^{-1}, respectively), while the least hydroxylated compounds showed inferior antioxidant activities.

Accordingly, the honey extract RLL1, with the highest antioxidant activity (EC_{50} = 5.48 μg mL^{-1}) showed the presence of quercetin, rutin and gallic acid (2.169, 0.389 and 0.005 mg 100 g^{-1} of honey, respectively), and also considerable amounts of vanillic and sinapic acids (21.152 and 9.102 mg 100 g^{-1} of honey, respectively). The honey extract RLL4, with the least antioxidant capacity (EC_{50} = 52.87 μg mL^{-1}) showed the presence of only three phenolic compounds in low amounts: *p*-hydroxybenzoic (0.823 mg 100 g^{-1} of honey), vanillic (0.142 mg 100 g^{-1} of honey) and *p*-coumaric (0.671 mg 100 g^{-1} of honey) acids, which have low antioxidant activities (Table 7; entries 4, 5 and 6, respectively). Although it had the highest antioxidant activity (EC_{50} = 8.17 μg mL^{-1}), the honey extract RLS2 showed the presence of only three phenolic compounds: protocatechuic (0.881 mg 100 g^{-1}), *p*-coumaric (0.033 mg 100 g^{-1}) and *p*-methoxycinnamic (0.254 mg 100 g^{-1}) acids. As these compounds exhibit different antioxidant properties, they should influence the RSA of the tested honey samples. These findings show that other components, which were not analyzed in our study, should also play an important role in defining RSA of honey.

Though being more valorized among consumers because of its characteristic flavor, light color and scent, the orange blossom honeys studied in this work generally had poorer phenolic contents and antioxidant capacities when compared to the multifloral honeys. That may be due to characteristic secondary metabolism of citric plant, or even to a wider nectar and pollen source in multifloral areas. Therefore, the less appealing and less expensive multifloral honeys should be more popularized among consumers because of their prominent functional properties.

Conclusions

The present study shows that different honeys contain high levels of phenolics and flavonoids and that the distribution of these compounds is influenced by the floral origin of honey. The antioxidant capacity in multifloral honey was higher than in orange blossom honeys, which could be related to their higher phenolic contents. Orange blossom honeys had lower amounts of phenolic compounds, but they also showed good radical scavenging activity, though less than that seen in multifloral honeys. Phenolic acids prevailed in the all samples. Therefore, the analyzed honeys may be considered easily accessible natural sources of antioxidants and valuable additions to the everyday diet.

HPLC-DAD analysis showed that the extracts are particularly rich in a variety of phenolic acids including *p*-coumaric, protocatechuic, *p*-methoxybenzoic, *p*-hydroxybenzoic and *p*-methoxycinnamic acids, and the flavonoids quercetin and morin. However, the content of individual compounds was different.

The phenolic fractions had much lower EC_{50} values than the corresponding honey. Orange blossom honey extracts showed higher antioxidant capacities and phenolic content than multifloral honey extracts, in contrast to what was observed for honey. Finally, the total antioxidant capacity of honey is likely the result of the combined activities and interactions of a wide range of compounds, including organic acids, enzymes, and possibly other minor components. However, phenolic compounds are well known to be the major contributors of this property, enriching honey quality as a functional food. These results revealed that the Brazilian honeys studied proved to be a good source of antioxidants that might serve to protect human health.

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