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Antioxidant Characterization of Native Monofloral Cuban Honeys

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Five typical Cuban monofloral honeys were analyzed for their in vitro total antioxidant capacity (TAC), phenolic compounds, and ascorbic acid content. Identification and quantification of phenolics were carried out by HPLC-DAD-ESI/MS. Fourteen phenolic compounds could be identified (eight phenolic acids and six flavonoids), including three glycosylated derivatives. Similar contents of total phenolics were found in the different honeys, although they differed in their qualitative profiles. A significant (positive) correlation was found between the results of TAC obtained by parallel FIA-ABTS system and ORAC assay ($r = 0.9565$, $p < 0.001$). Similar correlations were also established between total phenolics and TAC, determined by either the ORAC ($r = 0.9633$; $p \leq 0.001$) or the TEAC assay ($r = 0.9582$; $p \leq 0.001$). Honeys were fractionated by solid-phase extraction into four fractions, and the relative contribution of each fraction to TAC was calculated. Phenolic compounds were significant contributors to the antioxidant capacity of the honeys, but they were not uniquely responsible for it. The antioxidant activity appeared to be a result of the combined activity of a range of compounds including phenolics and other minor components. Ascorbic acid was not detected.

KEYWORDS: Phenolic acids; flavonoids; HPLC-MS; ORAC; TEAC

INTRODUCTION

Honey is produced by bees from plant nectars, plant secretions, and excretions of plant-sucking insects. Its composition is rather variable and primarily depends on the floral source, although certain external factors also play a role, such as seasonal and environmental factors and processing. It consists of a saturated solution of sugars, of which fructose (38%) and glucose (31%) are the main contributors, but it also contains a wide range of minor constituents, among them phenolic compounds (*1*, *2*). Although studies on the basic composition of honeys started a hundred years ago, the interest in honey phenolics is relatively recent. Phenolic compounds in honey are mainly flavonoid aglycones and phenolic acid derivatives (benzoic and cinnamics acids and their respective esters) (*3*−*8*). Recently, the presence of certain amounts of some flavonoid glycosides has also been reported in some floral honeys (*6*).

Phenolic compounds have a plant origin, and thus the phenolic composition in the honey varies depending on the vegetation of the area visited by the bee (*4*). With this in mind, phenolic compounds have been proposed as potential chemical markers for authenticating the geographical and botanical origin of honey. Flavonoids are the most common phenolics in floral honeys, and characteristic profiles could be expected in unifloral honeys depending on the corresponding plant source (*9*, *10*). It has also been shown that a strong correlation exists between the antioxidant activity of honeys and their phenolic composition and especially the total phenolic content (*11*, *12*). Thus, characterization of phenolics and other components in honey that might have antioxidant properties is essential to improve our knowledge about honey as a source of nutraceuticals and would also be an important tool to contribute to their authentication. Several studies on the phenolic composition have been carried out in European honeys, especially by Ferreres and co-workers (*9*, *10*, *13*−*15*). However, little information is available on the phenolic profiles of honeys from Cuban floral sources. The objective of this study was to identify and quantify these compounds in five different typical monofloral Cuban honeys and to determine their total antioxidant capacities. Furthermore, the contribution to the antioxidant activity of different fractions isolated from the honeys was also evaluated.

MATERIALS AND METHODS

Honeys Samples and Chemicals. Five different types of Cuban monofloral honeys were collected. The floral sources and number of samples analyzed were Christmas vine [*Turbina corymbosa* (L.) Raf; 18 samples], morning glory (*Ipomoea triloba* L., 16 samples), black mangrove (*Avicennia germinans* Jacq., 16 samples), linen vine [*Govania polygama* (Jack) Urb, 17 samples], and singing bean [*Lysiloma latisiquum* (L.) Benth, 16 samples]. All honey samples were certified by the National Center of Apiculture Research of Cuba Havana University, Cuba. Samples were collected and designated by the time of the year and the place from which each honey sample was taken; it was noted if those places coincided with

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the floral maps designed in that Center. All samples were tested by their organoleptic characteristics (flavor, scent), the usual available physicochemical tests [ashes (%), electrical conductivity (mS/cm), color (mm Pfund), pH, free acidity (mequiv/kg), humidity (%)], diastases index (U Schade), qualitative tests for authenticity, and HMF test (mg/kg) for quality according to the Official Methods of Analysis of the Association of Official Analytical Chemists (AOAC) (*16*). The botanical origin was confirmed by the traditional qualitative microscopic analysis and frequency determination of the classes of pollen grains in the honey samples (*17*). The different pollen morphologies were compared with reference slides from the Ecology and Systematic Institute (University of Havana, Cuba). Pollen analysis revealed that in all samples the percentage of typical pollen grains of the botanical specie was >51%, sufficient to classify them as monofloral, as reported in the above used methodology and accepted for these floral honeys (*18*).

Fresh honey samples weighing 250 g were packed and sealed in amber glass bottles and stored at 4 °C in the dark until processing. The samples were kept at room temperature (25 ± 2 °C) overnight before analyses were performed. An artificial honey reflecting the main components of honey was prepared by dissolving 1.5 g of sucrose, 7.5 g of maltose, 40.5 g of fructose, and 33.5 g glucose in 17 mL of deionized water (*19*). This solution was included in the study to evaluate the contribution of the predominant sugars to the assayed activities.

2,2′-Azinobis(3-ethylbenzothiazolne-6-sulfonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox), fluorescein, and potassium chloride were purchased from Fluka Chemie (Buchs, Switzerland). 2,2′-Azobis(2-methylpropionamidine) dihydrochloride (AAPH), Amberlite XAD-2 resin, 3,4,5-trihydroxybenzoic acid (gallic acid), (+)-catechin, sodium carbonate anhydrous, Folin−Ciocalteu's phenol reagent, catalase, hydrogen peroxide (H_2O_2), ferrous sulfate ($FeSO_4$), ascorbic acid, sucrose, maltose, fructose, and glucose were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). All chemicals and solvents were of analytical grade.

Determination of Total Phenolic and Flavonoid Content. The Folin−Ciocalteu method (*20*) was used to determine total phenolic content. Each honey sample (1 g) was diluted to 10 mL with distilled water and filtered through Minisart filter of 45 μm (PBI International, Milan, Italy). This solution (0.5 mL) was then mixed with 2.5 mL of 0.2 N Folin−Ciocalteu reagent for 5 min, and 2 mL of 0.7 M sodium carbonate (Na_2CO_3) was then added. After incubation in the dark at room temperature for 2 h, the absorbance of the reaction mixture was measured at 760 nm against sugar analogues using a Beckman Du 640 spectrophotometer (Beckman Instruments Inc., Fullerton, CA). Gallic acid was used as standard to produce the calibration curve (50−300 mg/L). The mean of three analyses was used, and the total phenolic content was expressed in gallic acid equivalents (mg of GAE/kg of honey).

Total flavonoid content was determined using a colorimetric method as previously described (*21*). Briefly, 0.25 mL of honey solution (50%, w/v) in methanol or (+)-catechin standard solution was mixed with 1.25 mL of distilled water in a test tube, followed by the addition of 75 μL of a 5% $NaNO_2$ solution. After 6 min, 150 μL of a 10% $AlCl_3 \cdot 6\,H_2O$ solution was added and allowed to stand for another 5 min before the addition of 0.5 mL of 1 M NaOH. The mixture was brought to 2.5 mL with distilled water and mixed well. The absorbance was immediately measured against the blank (the same mixture without the sample) at 510 nm using a spectrophotometer. The linearity range of a (+)-catechin curve was used for calibration (5−50 mg/L). The total flavonoid content was calculated from the mean of three analyses and expressed as (+)-catechin equivalents (mg of CE/kg of honey).

Vitamin C Analysis. Vitamin C in honeys was analyzed by reversed-phase HPLC, as previously described by our group (*22*) with minor modifications. Triplicate extracts were prepared by diluting 5 g of honey to 10 mL with dithiothreitol solution (4.2 mM in 0.1 M K_2HPO_4, pH 7.0) and mixing thoroughly. One milliliter of extract and 1 mL of 4.5% metaphosphoric acid were mixed, and 20 μL was injected into the HPLC. The HPLC system (Shimadzu Corp., Kyoto, Japan) consisted of a Waters 600 controller, a Waters 996 photodiode array (PDA) detector set at absorbances of 262 and 244 nm, and a column incubator at 30 °C. The HPLC column used was a YMC Pack Pro, 150 × 4.6 mm. A linear gradient was generated using 50 mM KH_2PO_4 (pH 4.5) (solvent A) and methanol (solvent B) starting at 100% A and decreasing to 70% A in 8 min. The flow rate was 0.8 mL/min.

Total Antioxidant Capacity (TAC). The TAC of the honey samples was determined by the Trolox equivalent antioxidant capacity (TEAC) and the oxygen radical absorbance capacity (ORAC) assays.

The TEAC assay was performed according to the method of Re et al. (*23*), partially improved by our group (*24*), which combines a flow injection analysis system (FIA-ABTS assay). This method is based on the ability of antioxidant compounds to quench the ABTS radical cation ($ABTS^{\bullet+}$) and reduce the radical to the colorless neutral form. Honey was diluted in distilled water (50%, w/v) and filtered through Minisart filter of 45 μm (PBI International), and then 10 μL was injected into a serpentine-knotted reaction coil and allowed to react with the $ABTS^{\bullet+}$ working solution pumped into the coil at a flow rate of 1.2 mL/min. The extent of decolorization of the reagent, expressed as percentage of absorbance inhibition, is then plotted as a function of concentrations of the antioxidants in the sample. The linearity range of the Trolox (0.03−2.5 mM) calibration curve was used. TEAC results were expressed as micromoles of Trolox equivalents per gram of honey (μmol of TE/g of honey). The mean of five analyses was used, and the results reported are as mean ± standard deviation (SD).

The ORAC assay was based on the procedure previously described (*25*). Free radicals are produced by the radical generator AAPH, which oxidizes the fluorescent compound fluorescein, leading to a loss in fluorescence. All reagents were prepared in phosphate buffer (pH 7.0, 75 mM), and Trolox (6.25−200 μM) was used as standard. The honey samples were suitably diluted in the phosphate buffer. Each well of a 96-well microplate contained, in a final volume of 200 μL of assay solution, 150 μL of fluorescein (0.08 μM) and 25 μL of honey solution (1 mg/mL final concentration) preincubated for 10 min at 37 °C, and then 25 μL of AAPH (150 mM) was added. After the addition of AAPH, the plate was shaken automatically for 3 s, and the fluorescence was measured every 2 min for 120 min with emission and excitation wavelengths of 485 and 530 nm, respectively, using a microplate fluorescence reader (Synergy Multi-Detection Microplate Reader; Bio-Tek, Instruments, Inc., Winooski, VT) that was maintained at 37 °C. The ORAC values were calculated as area under the curve (AUC) and expressed as micromoles of Trolox equivalents per gram of honey (μmol of TE/g of honey).

Fractionation of Honey on Amberlite XAD-2 Resin. The procedure for the fractionation of honey was adapted from those of Ferreres et al. (*3*) and Andrade et al. (*14*). The different honey samples (50 g each) were mixed with 5 parts of acidified water (pH 2 with HCl) until completely fluid and filtered through cotton to remove solid particles. The filtrate was then passed through a column (25 × 2 cm) of Amberlite XAD-2 resin (pore size = 9 nm, particle size = 0.3−1.2 mm). Sugars and polar compounds were eluted with acidified water (350 mL) (fraction 1); the column was washed with 300 mL of neutral water (fraction 2), and phenolic compounds were further eluted with methanol (600 mL). The methanol phase was concentrated under vacuum at 40 °C (Büchi R-114, Donau, Flawil, Switzerland), suspended in water (5 mL) and extracted three times with diethyl ether (5 mL each). The ether layers were collected, evaporated under vacuum, and redissolved in methanol (fraction 3). Hesperetin was used as internal standard. The fractions were concentrated under vacuum and stored at −20 °C until further analysis. Each honey sample was fractionated and analyzed in triplicate.

All of the fractions, as well as the remaining water layer after ether extraction (fraction 4), were analyzed for antioxidant activity by the ORAC assay to determine their relative contribution to the total ORAC activity of the honey. Prior to the ORAC assay, all fractions were redissolved in 5 mL of the same solvent used for their elution. When methanol or acidified water was used for fractionation, methanol or acidified water was also used in the blank and standard.

Fraction 3, which was expected to contain phenolic compounds, was also analyzed by HPLC-MS. With this aim the concentrated ether layers were redissolved in methanol/H_2O (50:50, v/v) to prevent the elution of the compounds in the elution front.

HPLC-DAD-ESI-MS/MS Analysis of Honey Phenolics. Polyphenol identification analyses were carried out using a Hewlett-Packard 1100 series liquid chromatograph (Agilent Technologies, Waldbronn, Germany) with a quaternary pump and a diode array detector (DAD) coupled to an HP ChemStation (rev. A.05.04) data-processing station. The column used was a C_{18} LiChroCART (Merck, Darmstadt, Germany) (RP-18e, 250 mm × 4 mm; 5 μm), operated at 35 °C. The mobile phase

Table 1. Total Antioxidant Activity of Honey and Content of Different Potential Antioxidant Components[a]

floral source	total antioxidant capacity: ORAC (μmol of TE/g)	total antioxidant capacity: ABTS (μmol of TE/g)	phenol content (mg of GAE/kg of honey)	flavonoid content (mg of CE/kg of honey)	ascorbic acid (μg/100 g of honey)
linen vine (n = 17)	12.89 ± 0.28 a	2.94 ± 0.23 a	595.8 ± 16.82 a	25.2 ± 0.32 a	ud[b]
morning glory (n = 16)	9.26 ± 0.46 b	2.01 ± 0.21 b	347.5 ± 23.85 b	15.8 ± 0.30 b	ud
singing bean (n = 16)	8.12 ± 0.23 c	1.95 ± 0.14 b	298.6 ± 25.01 c	14.2 ± 0.27 b	ud
black mangrove (n = 16)	7.45 ± 0.37 d	1.22 ± 0.24 c	233.6 ± 15.58 d	17.8 ± 0.50 c	ud
Christmas vine (n = 18)	4.59 ± 0.51 e	1.03 ± 0.28 d	213.9 ± 14.50 d	10.9 ± 0.38 d	ud
artificial honey	1.09 ± 0.10 f	0.21 ± 0.01 e	nd[c]	nd	nd

[a] Values are expressed as means ± standard deviation (SD). Mean values within a column sharing the same letter are not significantly different by Tukey's multiple range test ($p < 0.05$). Each sample was analyzed in triplicate. [b] ud, undetectable. [c] nd, nondetermined.

consisted of H_2O/formic acid (99:1, v/v) (eluent A) and methanol/isopropanol (90:10, v/v) (eluent B). The gradient program was as follows: from 10 to 30% B over 20 min, from 30 to 40% over 10 min, from 40 to 60% B over 10 min, from 60 to 80% over 5 min and then isocratic by 5 min. The injection volume for all samples was 100 μL, and the flow rate was 1 mL/min. Identification of honey phenolics was carried out by comparing retention time and spectral characteristics of unknown analytes with standards using the HP ChemStation software (HP Hewlett-Packard ChemStation, rev. A.05.04). Spectroscopic data from all peaks were accumulated in the range of 240−400 nm. Chromatograms for the phenolic acids were recorded at 290 nm and for flavonoids at 360 nm. For calibration appropriate volumes of standard stock solutions (1000 mg/L) were diluted, and different concentration levels were analyzed. Individual phenolic acids were quantified using a calibration curve of the corresponding standard compound, and flavonoids were quantified using a quercetin calibration curve and expressed in terms of quercetin equivalents.

The mass spectrometer was a Finnigan LCQ (San Jose, CA) equipped with an electrospray ionization (ESI) system and an ion trap mass analyzer, which were controlled by LCQ Xcalibur software. Nitrogen was used as both auxiliary and sheath gas at flow rates of 6 and 1.2 L/min, respectively. The capillary voltage was 10 kV and the capillary temperature, 225 °C. MS spectra were acquired in the negative and positive ionization modes between m/z 100 and 800. The MS detector was programmed to perform a series of two consecutive scans: a full scan and an MS/MS scan of the most abundant ion in the first scan, using normalized collision energy of 50%.

Statistical Analysis. Statistical analysis was performed using Statistica software (Statsoft Inc., Tulsa, OK). Data were subjected to a one-way variance analysis for mean comparison, and significant differences between honey type, total ORAC, and the sum of the four fractions after the elution from the Amberlite XAD-2 resin were calculated according to HSD Tukey's multiple-range test. Data were expressed as mean ± standard deviation (SD). Correlations were calculated on a honey mean basis, according to Pearson's test.

RESULTS AND DISCUSSION

Total Phenolic (TP) and Flavonoid (TF) Contents. The mean values and SDs of the TP content are shown in **Table 1**. To compare the values obtained for the five honey groups, a Tukey HSD test for comparison of means was carried out, and it was observed that the evaluated parameters showed a high power to discriminate between the different groups. According to these results, linen vine honey had the highest TP content (595.8 mg of GAE/kg of honey), followed by morning glory honey (347.5 mg of GAE/kg of honey), whereas the lowest contents were measured in black mangrove and Christmas vine (233.6 and 213.9 mg of GAE/kg of honey, respectively). TF contents are also shown in **Table 1**. The values varied from 10.9 mg of CE/kg in Christmas vine honey to 25.2 mg of CE/kg in linen vine honey. A significant correlation ($r = 0.8697$, $p \leq 0.001$) was found between TP and TF contents.

The TP average values found the analyzed samples are similar to those previously reported in honeys from other origins (ranging between 226.16 and 406.23 mg of GAE/100 g) (*5*, *11*, *26*−*29*). Similarly, the results obtained for TF (10−25 mg of CE/kg) are in the same range of values as previously reported for other monofloral honeys, such as eucalyptus honey (20−25 mg of CE/kg), sunflower and rape honeys (15−20 mg of CE/kg), fir, lavender, ivy, and acacia honeys (5−10 mg of CE/kg), and arbutus and chestnut honeys (< 5 mg of CE/kg) (*26*−*29*).

The aluminum chloride spectrophotometric method has been previously used for the quantification of flavonoids in propolis and honey extracts (*21*), but it does not determine equally all flavonoid groups and, thus, it might underestimate the TF content. In this study a detailed analysis of the flavonoids was also performed by HPLC, which provides a more reliable determination of these compounds (see below).

Ascorbic Acid Content. Besides phenolic compounds, honey may contain other compounds known to act as antioxidants, such as ascorbic acid and enzymes (glucose oxidase and catalase) (*1*). Low levels (< 5 mg/100 g) of ascorbic acid have been reported in honey (*2*), although some authors (*26*) determined values between 140 and 145 mg/kg in Portuguese honeys (spectrophotometric quantification). In our study no ascorbic acid could be detected in any of the five monofloral Cuban honeys analyzed, using HPLC coupled to photodiode array detection. A similar result was obtained by Gheldof et al. (*11*) and attributed to a loss during processing and storage of the honeys.

Total Antioxidant Capacity (TAC). TAC was analyzed by the ORAC and ABTS assays, widely used as screening methods for the antioxidant activity in foods and beverages, including honey (*11*, *31*−*33*). The results are shown in **Table 1**. A significant correlation was found between TAC values in both assays ($r = 0.9565$, $p < 0.001$). An artificial honey solution was included in the assay to evaluate the contribution of the predominant sugars to the antioxidant activities (*11*, *19*). As expected, in all cases the values were lower than those determined in the honey samples ($p < 0.0001$) and similar to the ones previously reported for sugar analogues (*11*). The TAC values ranged from 12.89 to 4.59 μmol of TE/g (ORAC) and from 2.94 to 1.03 μmol of TE/g (ABTS), respectively. Linen vine honey showed the highest antioxidant activity in both assays, whereas the lowest values were found in Christmas vine honey. ORAC values ranging between 3 and 9 μmol of TE/g were obtained by Gheldof et al. (*11*, *12*) in commercial monofloral honeys from different floral sources. As for the ABTS assay, TEAC values between 0.62 and 1.14 μmol of TE/g were determined by Zalibera et al. (*33*) in different Slovak honeys, and Baltrušaité et al. (*32*) reported a percent of $ABTS^{\bullet+}$ radical cation decolorization between 76.5 and 81.9%. In all cases, the ranges of TAC values are similar to those found in our samples, suggesting that monofloral Cuban honeys favorably compare with honeys from other geographical areas with regard to antioxidant activity.

It has been reported that the antioxidant capacity of honeys is comparable to that of fruits and vegetables on a fresh weight

Table 2. Fractional Antioxidant Activities (ORACs) of Honeys from Different Floral Origins and the Sum of the Antioxidant Activities of the Four Honey Amberlite XDA-2 Fractions

	ORAC values expressed as μmol of TE/g					
floral source	total ORAC[a]	acidified water fraction[b]	neutral water fraction[b]	ether-extracted methanol phase[b]	water after ether extraction fraction[b]	sum of ORACs of four fractions[c]
linen vine (n = 17)	12.89 ± 0.28 a	4.65 ± 0.12	1.30 ± 0.02	1.17 ± 0.09	2.36 ± 0.24	9.48 ± 0.24
morning glory (n = 16)	9.26 ± 0.46 b	3.24 ± 0.16	0.94 ± 0.06	1.22 ± 0.04	1.58 ± 0.37	6.58 ± 0.17
singing bean (n = 16)	8.12 ± 0.23 c	3.62 ± 0.21	0.72 ± 0. 02	0.86 ± 0.02	1.74 ± 0.12	6.16 ± 0. 26
black mangrove (n = 16)	7.45 ± 0.37 d	2.08 ± 0.14	0.76 ± 0.04	0.83 ± 0.01	0.95 ± 0.25	4.62 ± 0.32
Christmas vine (n = 18)	4.59 ± 0.51 e	1.92 ± 0.11	0.26 ± 0.01	0.43 ± 0.01	0.73 ± 0.13	3.21 ± 0.20

[a] Total ORAC values of honey. [b] Individual ORAC values of the four fractions collected after the elution from the Amberlite XAD-2 resin. [c] Total ORAC is significantly higher than the sum of ORAC of the four fractions by HSD Turkey's multiple-range test. Mean values within a column sharing the same letter are not significantly different by Tukey's multiple-range test ($p < 0.05$). Each sample was analyzed in triplicate.

basis (*5*, *8*, *11*, *12*). Our results also support that assumption. ORAC values in the analyzed honeys (4−13 μmol of TE/g) were in the same range as those found in many fruits and vegetables (0.5−19 μmol of TE/g of fresh weight) (*34*,*35*). Although honey is not consumed in quantities equivalent in mass to those of most fruits and vegetables, it may be used as a healthy alternative to sugar in many products and thereby serve as a supplementary source of dietary antioxidants.

Phenolic Compounds and Antioxidant Activity. It is assumed that phenolic compounds play an important role in the antioxidant capacity of fruits, vegetables, and beverages. In our study significant correlation was found between the content of total phenolics and the TAC values obtained with the ORAC (r = 0.9633; $p \leq 0.001$) and the ABTS assay (r = 0.9582; $p \leq 0.001$). In addition, a significant correlation was found between total flavonoid content and TAC determined by either the ORAC (r = 0.9483, $p \leq 0.001$) or the ABTS assay (r = 0.8315, $p \leq 0.001$).

Samples were fractionated on an Amberlite XAD-2 resin to obtain four fractions that were tested for their antioxidant activity to determine their relative contribution to the TAC of the honeys. The ORAC assay was used for this assay as it determines the capacity of a sample to scavenge a variety of radicals initially triggered by the carbon-centered radicals generated upon decomposition of AAPH, thus giving a broader idea of the antioxidant capacity of the sample than the ABTS assay, which measures the capacity to reduce a single type of radical. The results are shown in **Table 2**. In all honeys, the sum of the ORAC activities of the fractions was significantly lower than the total ORAC activity of the honey ($p \leq 0.001$), similar to the result also reported by Gheldof et al. (*11*). Because of the complex composition of honey, interactions between different antioxidant components are likely important in terms of the overall antioxidant activity of honey. The lower antioxidant capacity of the fractions sum with respect to the total antioxidant capacity of honeys might suggest synergistic interactions among the antioxidant components from the various phases. However, loss of some antioxidants during the fractionation procedure cannot be overlooked as an explanation. More studies are needed to further investigate synergistic interactions between different honey components.

For all honeys, the first acidified water phase (fraction 1) had the highest ORAC activity, followed by the water layer obtained after ether extraction of the evaporated methanol phase (fraction 4), the ether-extracted methanol phase (fraction 3), and the neutral water phase (fraction 2). A similar observation was made by Gheldof et al. (*11*), who obtained ORACs values between 1.87 and 4.78 μmol of TE/g in the acidified water fraction, from 0.11 to 0.90 μmol of TE/g in the methanol fraction, and from 0.29 to 1.77 μmol of TE/g in the water layer after ether extraction.

Interestingly, the acidic water-soluble fraction displayed the greatest antioxidant capacity, even though the methanol fraction was expected to contain most of the phenolics (*36*). The relative contribution of this latter fraction to the total ORAC activity of the honey ranged from 9.35% (singing bean honey) to 13.17% (morning glory honey). It must be taken into account that most carbohydrates are present in the first water-soluble fraction and should contribute to some extent to the antioxidant activity. Nevertheless, in the analysis of the artificial honey solution containing the predominant sugars of honey, a low ORAC value was obtained (1.09 ± 0.10 μmol of TE/g), suggesting that other antioxidant compounds exist in the fraction that are accounting for the antioxidant activity. A possible explanation might be the presence of polar glycosidic flavonoids that would elute in this fraction, as reported by Truchado et al. (*6*). In fact, some compounds of this type have been found in the samples, as below discussed, although it could not be checked whether they are actually concentrated in this fraction. Further studies are, thus, required to ascertain this point.

Another interesting aspect was that the aqueous layer after ether extraction of the methanol fraction showed greater ORAC values than the ether-extracted methanol phase (fraction 3). Ether extraction is an extra step in the purification of this phenolic fraction, which leaves phenolic polymers and some possible residual sugars in the aqueous layer, as proposed by Ferreres et al. (*13*). This suggests that phenolic polymers could be contributing more to the antioxidant activity than other phenolic compounds present in the methanol fraction.

Analysis of Phenolic Compounds by HPLC-DAD-ESI-MS/MS. In **Figure 1** representative HPLC chromatograms of the different extracts of honeys are shown. The compounds were identified on the basis of their UV and mass spectra obtained by HPLC-DAD-ESI/MS in negative mode, as well as their chromatographic behavior compared to external standards when available. UV spectra, mass characteristics, and identity of the peaks are indicated in **Table 3**, and their quantification in the honeys is shown in **Table 4**. Up to 14 peaks could be assigned to phenolic compounds and identified as phloroglucinol (peak 1), phenolic acids (peaks 2−5), and flavonoids (peaks 8−15). All of the identified flavonoids belonged to the group of flavonols. Eight compounds were present in all samples: vanillic, caffeic, and *p*-coumaric acids and the flavonols quercetin, isorhamnetin, kaempferol methoxykaempferol, and kaempferol-7-*O*-rhamnoside (**Table 3**). There was another relevant peak present in all samples that could not be identified (peak 7), although it might not correspond to a phenolic compound (see below).

The identity of phloroglucinol, phenolic acids, and flavonol aglycones could be confirmed positively by comparison with external standards and/or our compound library, whereas the identity of peaks 9, 10, and 12 remains tentative.

Among phenolic acids, the hydroxycinnamic acids, that is, caffeic acid (peak 3), *p*-coumaric acid (peak 5), and ferulic acid (peak 6), were predominant. These compounds are thought to be

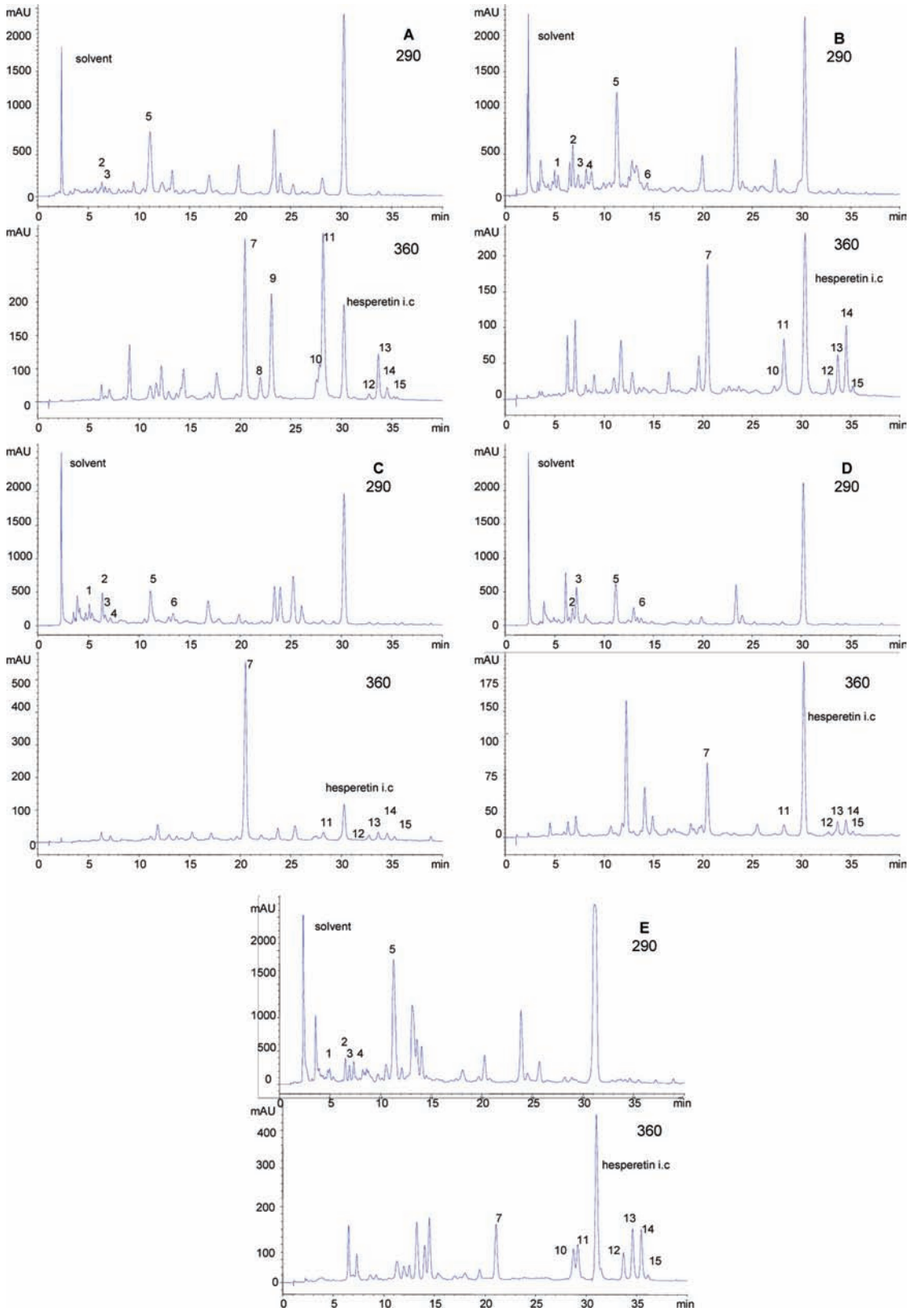

Figure 1. HPLC-DAD chromatograms of honey phenolic fraction at 290 nm for phenolic acids and 360 nm for flavonoids: (**A**) singing bean; (**B**) linen vine; (**C**) morning glory; (**D**) Christmas vine; (**E**) black mangrove. Peaks: 1, phloroglucinol; 2, vanillic acid; 3, caffeic acid; 4, syringic acid; 5, *p*-coumaric acid; 6, ferulic acid; 7, unidentified compound; 8, myricetin; 9, quercetin-diglycoside; 10, quercetin- *O*- rhamnoside; 11, quercetin; 12, kaempferol-7-*O*-rhamnoside; 13, kaempferol; 14, isorhamnetin; 15, 8-methoxykaempferol. Hesperetin was used as internal standard.

derived from propolis (*6*, *11*, *14*, *36*) that the bee would directly incorporate into honey.

Five peaks (8, 11, and 13−15) were assigned to flavonol aglycones, a type of compound widely reported in honey. Quercetin and kaempferol were found in many types of (monofloral) honeys (see, e.g., refs *6*, *8*, *11*, and *37*) and suggested as suitable floral markers for eucalyptus honey (*10*). Kaempferol has also been proposed as a possible botanical marker for rosemary honey (*4*).

Table 3. t_R and UV and MS Spectra (MS, $[M-H]^-$; MS^2 $[M-H]^-$) from the Different Phenolic Compounds Identified in the Various Honeys Analyzed

peak	phenolic compound	t_R (min)	UV (nm)	$[M-H]^-$	MS^2 $[M-H]^-$
1	phloroglucinol	4.80	255	*[a]	*
2	vanillic acid	5.85	260, 294	*	*
3	caffeic acid	7.0	298sh, 324	*	*
4	syringic acid	8.45	274	*	*
5	*p*-coumaric acid	11.10	232, 310	*	*
6	ferulic acid	13.35	296, 322	*	*
7	unknown	20.45	260, 354, 387sh	241	213
8	myricetin	22.00	255, 286sh, 308sh, 374	317	
9	quercetin-diglycoside	23.10	232, 256, 278sh, 350	625	301
10	quercetin-*O*- rhamnoside	27.85	256, 268sh, 296sh, 350	447	301
11	quercetin	28.20	255, 300sh, 370	301	
12	kaempferol-7-*O*-rhamnoside	32.75	263, 320sh, 367	431	285
13	kaempferol	33.65	266, 320sh, 366	285	267
14	isorhamnetin	34.55	255, 267sh, 301sh, 370	315	300
15	8-methoxykaempferol	35.20	268, 292sh, 340	315	285

[a] Compounds were not analyzed by the mass spectrometer.

Table 4. Phenolic and Flavonoid Contents of the Various Honeys Analyzed[a]

		floral source				
peak	phenolic compound	linen vine (n = 17)	morning glory (n = 16)	black mangrove (n = 16)	singing bean (n = 16)	Christmas vine (n = 18)
1	phloroglucinol	22.87 ± 4.08	21.59 ± 5.91	14.84 ± 3.41	nd[b]	nd
2	vanillic acid	35.20 ± 3.17	29.66 ± 4.08	21.74 ± 4.63	24.40 ± 4.25	15.22 ± 4.19
3	caffeic acid	33.94 ± 4.40	25.17 ± 5.59	23.23 ± 3.14	22.06 ± 5.32	23.47 ± 5.23
4	syringic acid	36.14 ± 5.27	26.07 ± 4.13	15.77 ± 5.63	nd	nd
5	*p*-coumaric acid	107.94 ± 3.42	63.85 ± 4.29	155.82 ± 3.64	74.23 ± 4.42	25.82 ± 5.78
6	ferulic acid	50.08 ± 2.58	55.10 ± 5.75	nd	nd	23.06 ± 5.54
8	myricetin	nd	nd	nd	30.27 ± 6.39	nd
9	quercetin-diglycoside	nd	nd	nd	79.25 ± 5.32	nd
10	quercetin-rhamnoside	22.71 ± 2.09	nd	27.96 ± 5.05	36.39 ± 6.16	nd
11	quercetin	39.71 ± 8.99	23.28 ± 1.15	30.21 ± 8.41	95.35 ± 5.72	24.84 ± 2.93
12	kaempferol-7-*O*-rhamnoside	23.43 ± 4.07	17.32 ± 4.53	22.30 ± 2.12	19.27 ± 0.75	19.19 ± 0.68
13	kaempferol	32.46 ± 6.64	19.59 ± 6.32	35.08 ± 8.61	47.78 ± 7.29	24.47 ± 3.30
14	isorhamnetin	32.90 ± 7.01	21.34 ± 5.32	34.57 ± 6.48	26.13 ± 4.00	26.87 ± 3.98
15	8-methoxykaempferol	20.99 ± 2.27	16.76 ± 3.21	19.01 ± 1.11	19.90 ± 1.58	19.27 ± 0.86
total identified phenolic content		458.37 ± 25.71	319.73 ± 18.46	400.53 ± 38.51	475.03 ± 29.69	202.21 ± 11.23
total identified flavonoid content		172.20 ± 14.70	98.29 ± 10.38	169.13 ± 14.16	354.34 ± 28.41	114.64 ± 12.16

[a] Values expressed as means (μg/100 g of honey) ± SD. [b] nd, not detected.

The presence of isorhamnetin was identified in different monofloral Italian honeys by Fiorani et al. (*38*). Another peak that could be assigned to a flavonol aglycone was peak 15. This compound showed a molecular ion $[M-H]^-$ at m/z 315, releasing a major MS^2 fragment at m/z 285 attributed to the possible loss of a methoxy group (−30 amu), which allowed its identification as a methoxy derivative of kaempferol. Its UV−vis spectrum and retention time were consistent with those of 8-methoxykaempferol in our spectra library. The presence of methoxykaempferol in honey has been documented in various studies (*38*−*40*).

In our samples, these four flavonols were detected in all of the analyzed honeys, thus raising doubts that they could be indicative of any given floral origin. By contrast, myricetin (peak 8) was detected in only the singing bean honey, although the widespread nature of this flavonol makes also doubtful that it can be considered as a floral marker for this honey.

In early works on honey flavonoids only aglycones were identified, leading to the suggestion that only these derivatives were present in honey because the nectar flavonoid glycosides were fully hydrolyzed by the bee enzymes (*41*, *42*). That assumption was further rejected following the detection of some glycosides in different honeys (*6*, *37*). In our samples three compounds (peaks 9, 10, and 12) were identified as flavonol glycosides. Peak 9 was detected in only singing bean honey, which was also the richest in flavonoids. This compound showed a UV−vis spectrum characteristic of a flavonol/quercetin derivative and a negative molecular ion at m/z 625 $[M-H]^-$, releasing a MS^2 fragment at m/z 301 (quercetin) $[M-H]^-$. The loss of −324 amu is interpreted as corresponding to two hexose residues. On the other hand, the fact that only one MS^2 fragment is released points out that both sugars constitute a disaccharide and are not attached on different positions of the quercetin structure; in that case more than one fragment would be expected from the alternate cleavage of each sugar residue. The nature of the hexoses could not be established (e.g., glucose, galactose) nor could the position of attachment to quercetin.

Peak 10 was detected in three of the monofloral honeys. This compound showed a molecular ion at m/z 447 $[M-H]^-$ releasing a MS^2 fragment at m/z 301 (quercetin). The loss of a fragment with −146 amu and the fact that its UV−vis spectrum was not modified in the UV region, thus discarding the presence of a *p*-coumaroyl substituent, allow assignment of the compound as a quercetin-rhamnoside.

Peak 12 had a negative molecular ion at m/z 431 $[M-H]^-$ and released an MS^2 fragment at m/z 285 (kaempferol) from the loss of a rhamnose residue (−146 amu). Its UV−vis spectrum showed maximum wavelength in band I at 367 nm, indicating that the hydroxyl in position 3 of the aglycone was free (*43*). A compound with these same characteristics was found by Truchado et al. (*6*) in acacia honey and identified as kaempferol-7-*O*-rhamnoside.

Peak 7 was found in all honey samples. A negative molecular ion $[M - H]^-$ at m/z 241 was observed that released a major fragment at m/z 213 (loss of C=O − 28 amu). The compound found in peak 7 presented mass characteristics similar to those of a compound previously reported by Truchado et al. (*37*) (peak CH5 in the paper of those authors) and suggested by them as a possible floral marker of chestnut honey. That compound was not identified, but it was tentatively assigned as a possible intermediate in the tryptophan metabolism pathway (*37*). No further contribution to the identification of this compound could be made in our study and, thus, the compound remains unidentified. If the compound was the same as the one detected by Truchado et al. (*37*), the possibility of being a marker for European honeys should be ruled out.

In this study relevant amounts of flavonoids in glycosidic form have been detected in different Cuban honeys, suggesting that this might be a characteristic of their origin, either geographical or floral. Further studies are, however, required to confirm this proposal, as well as a more detailed phenolic analysis to confirm the identities of the compounds. Fractionation on Amberlite XAD-2 resin has been indicated as inappropriate for extraction of polar glycosidic flavonoids (*6*) and, therefore, some (minority) compounds of this type might have escaped to the methodological approach used. The use of extraction using reversed-phase SPE cartridges (C-18) combined with HPLC-DAD-MS-MS detection could be a more suitable alternative (*6*).

The total phenolic and flavonoid contents in the studied honeys, determined by a modification of the Folin−Ciocalteu method (*20*) and by the aluminum chloride spectrophotometric method (*21*), respectively, were strikingly higher than the phenolic contents quantified by HPLC analysis (**Table 4**). Similar observations have been reported in estimating phenolics in honey (*11*) and other foodstuffs and beverages (*30*). We were unable to quantify all phenolics separated by HPLC, and some phenolics might have eluted in the first water phase (fraction 1), thus escaping detection. However, the Folin−Ciocalteu method might have overestimated the honey total phenolics, as it is well-known that this reagent determines not only phenolic compounds but rather total reducing substances.

In conclusion, this study reports, for the first time, the potential antioxidant composition of the most important monofloral Cuban honeys and confirms that they contain relevant concentrations of phenolic acids and flavonoids. The results presented here suggest that the antioxidant capacity in honey is likely the result of the combined activity and interactions of a wide range of compounds, including phenolics, as well as possibly enzymes and other minor components.

ABBREVIATIONS USED

TAC, total antioxidant capacity; ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); TE, Trolox equivalents; TP, total phenolic content; GAE, gallic acid equivalents; TF, total flavonoid content; (+)CE, catechin equivalents; TEAC, Trolox equivalent antioxidant capacity assay; FIA-ABTS, flow injection-ABTS; ORAC, oxygen radical absorbance capacity assays.

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