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Identification and Quantification of Antioxidant Components of Honeys from Various Floral Sources

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Little is known about the individual components of honey that are responsible for its antioxidant activity. The present study was carried out to characterize the phenolics and other antioxidants present in honeys from seven floral sources. Chromatograms of the phenolic nonpolar fraction of the honeys indicated that most honeys have similar but quantitatively different phenolic profiles. Many of the flavonoids and phenolic acids identified have been previously described as potent antioxidants. A linear correlation between phenolic content and ORAC activity was demonstrated ($R^2 = 0.963$, $p < 0.0001$). Honeys were separated by solid-phase extraction into four fractions for sugar removal and separation based on solubility to identify the relative contribution of each fraction to the antioxidant activity of honey. Antioxidant analysis of the different honey fractions suggested that the water-soluble fraction contained most of the antioxidant components. Specific water-soluble antioxidant components were quantified, including protein; gluconic acid; ascorbic acid; hydroxymethylfuraldehyde; and the combined activities of the enzymes glucose oxidase, catalase and peroxidase. Of these components, a significant correlation could be established only between protein content and ORAC activity ($R^2 = 0.674$, $p = 0.024$). In general, the antioxidant capacity of honey appeared to be a result of the combined activity of a wide range of compounds including phenolics, peptides, organic acids, enzymes, Maillard reaction products, and possibly other minor components. The phenolic compounds contributed significantly to the antioxidant capacity of honey but were not solely responsible for it.

KEYWORDS: Honey; antioxidants; phenolics; protein; HPLC; ORAC

INTRODUCTION

In the past several years, there has been increasing evidence of the antioxidant capacity of honey. Honey can prevent deteriorative oxidation reactions in foods, such as lipid oxidation in meat (*1*, *2*) and enzymatic browning of fruits and vegetables (*3*−*5*). Honey has therefore great potential to serve as a natural food antioxidant. In a previous study, it was demonstrated that honey is similar in antioxidant capacity to many fruits and vegetables on a fresh weight basis, as measured by the oxygen radical absorbance capacity (ORAC) assay (*6*). The antioxidant activity of honey, however, varies greatly depending on the honey floral source (*6*, *7*). There is a lack of knowledge about the profiles of antioxidant substances in honeys from various floral sources. The variation in these profiles might be responsible for the widely varying abilities of honeys to protect against oxidative reactions.

Honey is a remarkably complex natural liquid that is reported to contain at least 181 substances (*8*). The composition of honey is rather variable and primarily depends on the floral source; however, certain external factors also play a role, such as seasonal and environmental factors and processing. Honey is a supersaturated solution of sugars, of which fructose (38%) and glucose (31%) are the main contributors. A wide range of minor constituents is also present in honey, many of which are known to have antioxidant properties. These include phenolic acids and flavonoids (*9*, *10*), certain enzymes (glucose oxidase, catalase) (*8*), ascorbic acid (*8*), carotenoid-like substances (*11*), organic acids (*12*), Maillard reaction products (*8*), and amino acids and proteins (*13*). The antioxidant activity of phenolic compounds might significantly contribute to the human health benefits of plant foods (*14*, *15*) and beverages such as red wine and tea (*15*−*17*). We have previously demonstrated a strong correlation between the antioxidant activity of honeys and the total phenolic content ($R^2 = 0.963$, $p < 0.0001$) (*6*). In several studies on European honeys, Ferreres and co-workers have shown that honeys have a rich phenolic profile consisting of benzoic acids and their esters, cinnamic acids and their esters, and flavonoid aglycones (*9*, *10*, *18*−*20*). However, little information is available on the phenolic profiles of honeys from floral sources common in North America. Characterization of the phenolics and other components in honey that might be responsible for its antioxidant effects is essential to improve our knowledge about honey as a source of antioxidants. The objective of this study was therefore to identify and quantify the antioxidants of

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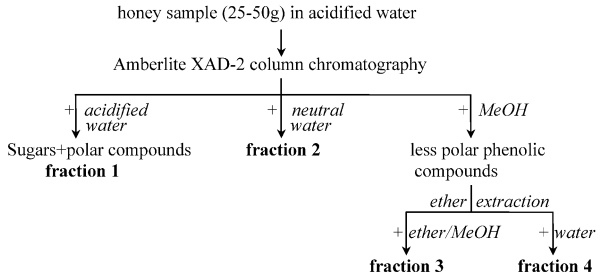

Figure 1. Diagram of the fractionation of honey samples using Amberlite XAD-2 column chromatography.

seven different honeys and to determine the antioxidant capacities of some of the isolated (phenolic) compounds and/or fractions.

MATERIALS AND METHODS

Materials. Commercial honeys from the following floral sources were obtained from Moonshine Trading Co. (Winters, CA): fireweed (*Epilobium angustifolium*), tupelo (*Nyssa aquatica*), and Hawaiian Christmas berry (*Schinus terebinthifolius*). Buckwheat (*Fagopyrum esculentum*) honey was purchased from Wixson Honey Co. (Dundee, NY). The source of clover (*Melilotus* spp.) honey was Sue Bee (Sioux City, Iowa), and the acacia (*Robinia pseudoacacia*) honey source was Langnese Honig KG (Bargteheide, Germany). Soybean (*Glycine max*) honey (extracted in 1996) was locally obtained from the University of Illinois Beekeeping Facility (Urbana, IL). All of these honeys are vended as "monofloral", meaning that the honey must derive at least 51% of the constituent nectar or 45% of contaminant pollen from a single floral source (*21*). Thus, honeys collected can contain nectars from more than one source, but the nominate floral type predominates.

AAPH [2,2′-azobis(2-amidinopropane) dihydrochloride] was purchased from Wako Chemicals, Inc. (Richmond, VA), and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) from Fisher Scientific (Pittsburgh, PA). All other chemicals were obtained from Sigma Chemicals (St. Louis, MO).

Fractionation of Honey on XAD-2 Resin for Phenolic Analysis. The procedures for the fractionation of honey and the high-performance liquid chromatography (HPLC) analysis of honey phenolics were adapted from Ferreres et al. (*18*) and Andrade et al. (*10*) and are diagrammed in **Figure 1**. Honey (25−50 g) was dissolved in 250 mL of acidified water (pH 2.1) and filtered through a column of Amberlite XAD-2 resin. 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 recovered with methanol (600 mL). The methanol extract was concentrated under vacuum at 40 °C and suspended in water (5 mL). This solution was extracted three times with diethyl ether (5 mL) to further purify the flavonoids (*18*). The ether extract was concentrated and dissolved in methanol (0.25 mL) (fraction 3) and stored at −80 °C until further analyzed. Recovery of the internal standard (hesperetin) was 95%. The other three collected fractions [acidified water phase, neutral water phase, and water phase after ether extraction (fraction 4)] were stored at 4 °C until further analysis. Each honey sample was fractionated and analyzed in triplicate.

HPLC and Liquid Chromatography/Mass Spectrometry (LC/MS) Analysis of Honey Phenolics. HPLC was performed using a 150 mm × 3.9 mm i.d., 5-μm XTerra RP18 instrument (Waters, MA) with diode array detection (DAD) at 285 and 340 nm. Gradients were generated using 0.05% formic acid in water (A) and methanol (B), starting at 95% A; decreasing to 85% A in 10 min, to 70% A in 5 min, to 60% A in 5 min, to 55% A in 10 min, to 40% A in 20 min, and to 20% A in 2 min; and holding for 8 min. The flow rate was 0.8 mL/min. Identification of honey phenolics was carried out by comparing retention time and spectral characteristics of unknown analytes and standards using the HP ChemStation 1050A software (Hewlett-Packard, Palo Alto, CA).

Major peaks were further isolated, and their identities were confirmed by LC/MS. Positive-ion-mode electrospray ionization (ESI)-MS spectra were recorded on a Waters Alliance 2690 LC/MS system (Waters, MA) using the following conditions: source temperature 150 °C, desolvation temperature 275 °C, capillary voltage 3.53 eV, and cone voltage 16 eV. Spectra were scanned over a mass range of *m*/*z* 120−650. Chromatographic separation was done on a 150 mm × 2.1 mm i.d, 5 μm Discovery C18 (Supelco, PA) column with UV detection set at 285 and 340 nm. The same solvents and solvent gradient were used as the HPLC analysis but the flow rate was reduced to 0.4 mL/min.

In addition, separation of the honey phenolic components in the methanol extract by HPLC was followed by ORAC analysis of the column fractions. With sampling at 5-min intervals, 12 4.8-mL fractions were collected from the 60-min HPLC run of the honey extracts. The fractions were dried and suitably reconstituted in methanol:phosphate buffer, 75 mmol/L, 1:99, prior to ORAC analysis.

ORAC Assay. The ORAC assay was based on the procedure by Cao et al. (*22*) and modified as previously described (*6*). When methanol or acidified water was used for fractionation, methanol or acidified water was also used in the blank and standard. Each time methanol was utilized, it was diluted with buffer to 1:99 without an effect on the 1:1 relationship between Trolox and the ORAC value. Each honey sample and isolated fraction was analyzed at least three times.

Protein Extraction and Analysis. Honey proteins were extracted as described by Ates et al. (*23*), and the protein precipitate was subjected to the ORAC assay. The protein content of the honeys was determined by the method of White and Rudyj (*13*). Each honey protein precipitation, ORAC assay of protein fraction, and honey protein content determination was performed at least in triplicate.

Analysis of Total Phenolics, 5-(Hydroxymethyl)-2-furaldehyde, and Gluconic Acid. Total phenolics were assayed by a modified version of the Folin−Ciocalteau method as described previously (*6*). The hydroxymethylfuraldehyde content was determined by the UV spectrophotometric method (*24*) as an indicator of the extent of the Maillard reaction. The gluconic acid content was measured by the method described by Mato et al. (*25*), using a Boehringer Mannheim enzymatic kit supplied by Bioform (Marshall, MI). Each honey sample was analyzed for total phenolics, hydroxymethylfuraldehyde, and gluconic acid contents with a minimum of triplicate analyses.

Assay for Peroxide Accumulation in Honey. The combined activities of glucose oxidase and catalase were assessed by measuring peroxide accumulation in honey as described by White and Subers (*26*). Each honey sample was analyzed in triplicate.

Vitamin C Analysis. Determination of ascorbate by HPLC was based on the methods reviewed by Lee and Coates (*27*). 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% *m*-phosphoric acid were mixed, and 20 μL was injected onto the HPLC. The stationary phase of the HPLC was a 150 mm × 3.9 mm i.d., 5-μm XTerra RP18 (Waters, MA) column. 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, and detection was at 263 nm.

Statistical Analysis. All results are presented as means ± SEM. Correlation and multiple regression analyses were performed using SAS Software (SAS Institute, Cary, NC, version 8, 1999). The total ORAC values of the honeys and the ORAC values of the sum of their four fractions were compared by a Wilcoxon signed-rank test.

RESULTS

Analysis of Phenolic Honey Antioxidants. HPLC chromatograms and LC/MS data of the phenolic fractions of the honeys indicated that most honeys tested had similar, but quantitatively different, phenolic profiles (**Figures 2**, **3A**, and **4A** and **Table 1**). The main flavonoids identified were the flavanones pinobanksin and pinocembrin and the flavones chrysin and galangin. Hawaiian Christmas berry (HCB) honey and tupelo honey, however, had different phenolic profiles,

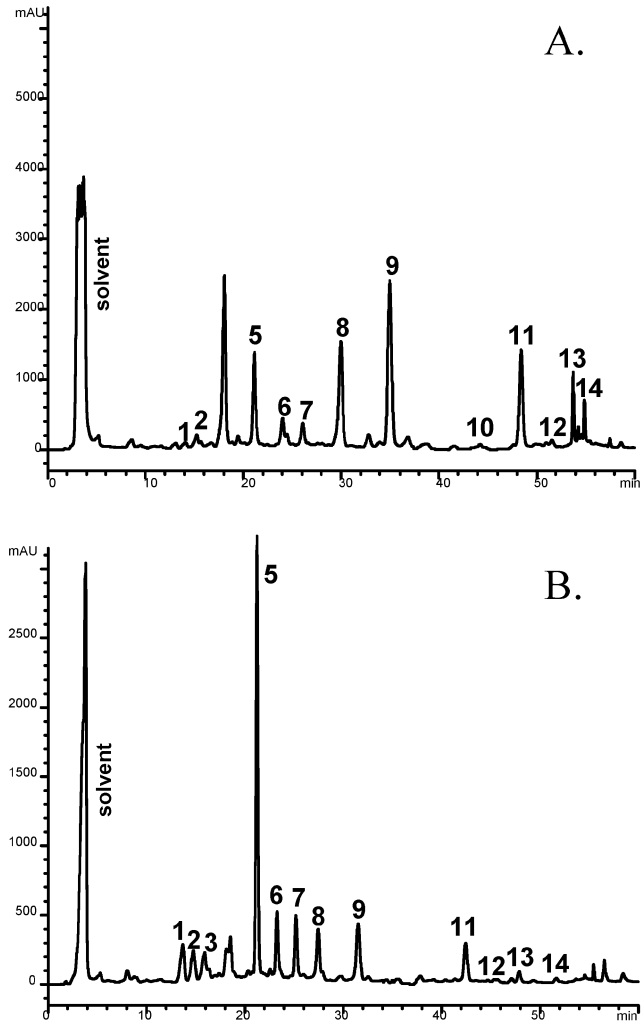

Figure 2. HPLC chromatograms of honey phenolic extracts at 285 nm: (A) soy honey, (B) buckwheat honey. For peak identification, see **Table 1**.

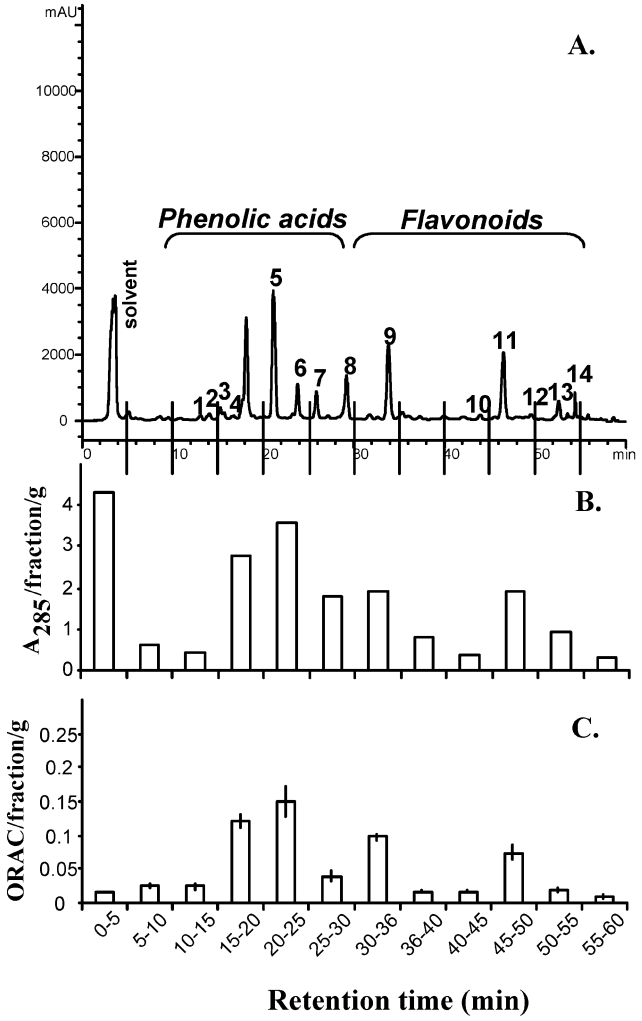

Figure 3. HPLC chromatogram of clover honey phenolics (A) at 285 nm. For peak identification, see **Table 1**. Fractions were collected from the HPLC every 5 min, their absorbance at 285 nm was measured (B), and the ORAC value of each fraction (C) was determined. ORAC assays from each HPLC collection were performed in triplicate.

lacking, or containing less, of the main flavonoids (**Figure 4A** and **Table 1**). Some of the unidentified compounds were quantified as the percentage of the total absorbance of the chromatograms at 285 nm to provide an approximate measure of their presence in the analyzed honey (**Table 1**). A commercial standard of pinobanksin was not available; therefore, quantification of this flavanone was based on the structurally similar flavanone pinocembrin.

ORAC Analysis of Honey Fractions. Separation of the phenolic components in the methanol extract of the honeys by HPLC, followed by ORAC analysis of the column fractions, enabled us to determine the relative abilities of the fractions to scavenge peroxyl radicals. The ORAC values and absorbances at 285 nm of the fractions of the methanol extract of clover honey and HCB honey separated by HPLC are shown in **Figures 3** and **4**. Linear correlation analyses of the absorbance/fraction with the ORAC/fraction of all honeys suggest a dose-dependent ORAC activity ($R^2 = 0.818$, $p < 0.0001$).

To determine the relative contribution of the phenolic (methanol) fraction 3 and other fractions eluting from the Amberlite XAD-2 resin to the total ORAC activity of the honey, the four fractions were tested via the ORAC assay (**Table 2**). For each honey, the first acidified water phase (fraction 1) had the highest ORAC activity, followed by the water phase obtained after ether extraction (fraction 4), the methanol phase (fraction 3), and the neutral water phase (fraction 2). The water-soluble phases of the honeys displayed the greatest antioxidant capacity, in contrast to the methanol phase, which was expected to contain most of the phenolics and thus the highest antioxidant content. The relative contribution of the antioxidant activity of the methanol phase to the total ORAC activity of the honey ranged from 3.6% (fireweed honey) to 10% (Hawaiian Christmas berry honey). For all honeys, the sum of the ORAC activities of the four fractions was significantly lower than the ORAC activity of the full honey ($p \leq 0.001$).

Analysis of Water-Soluble Honey Antioxidants. Although the relationship between the phenolic content and the antioxidant capacity of honey was strong, it was of interest to determine whether there were other significant nonphenolic contributors to the antioxidant capacity of honey. Thus, the relationship between the concentrations of other honey substances and the ORAC activity was determined. The ORAC activities of the various honeys are presented in **Table 3**, together with the concentrations of total phenolics, protein, peroxide, hydroxymethylfuraldehyde, gluconic acid, and ascorbic acid. The honey protein content ranged from 160 to 550 mg/100 g of honey, and a significant correlation with the ORAC activity of honey was obtained ($R^2 = 0.674$, $p = 0.024$). The ORAC activity of the honey protein fraction was also determined (**Table 3**) and

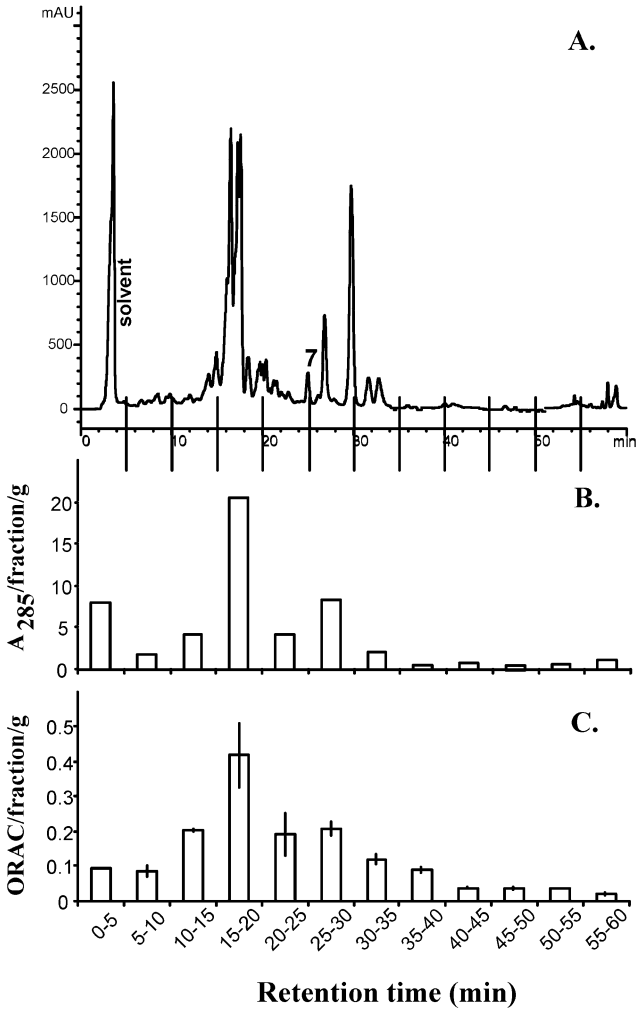

Figure 4. HPLC chromatogram of Hawaiian Christmas berry honey phenolics (A) at 285 nm. For peak identification, see **Table 1**. Fractions were collected from the HPLC every 5 min, their absorbance at 285 nm was measured (B), and the ORAC value of each fraction (C) was determined. ORAC assays from each HPLC collection were performed in triplicate.

found to correlate well with the level of protein ($R^2 = 0.708$, $p = 0.018$), accounting for between 9.7% (acacia honey) and 17.5% (buckwheat honey) of the ORAC activity of the complete honey. Multiple regression analysis revealed that the phenolic content accounted for an overwhelming part (96%, $p < 0.0001$) of the variation in the ORAC activities of the honeys. No significant correlation could be established between the ORAC activity and the inverse level of hydrogen peroxide or the amount of hydroxymethylfuraldehyde, gluconic acid, or ascorbic acid. In general, the levels of hydrogen peroxide accumulation and ascorbic acid were very low, if not undetectable. The level of hydroxymethylfuraldehyde, used as an indicator of the Maillard reaction, varied among the different honeys (from 1.7 to 15 mg/100 g of honey), and the gluconic acid content ranged from 1.8 to 7.4 g/kg of honey.

DISCUSSION

Several components of honey have the potential to serve as antioxidants, including phenolics, peptides, organic acids, enzymes, vitamins, and Maillard reaction products. The purpose of this study was to characterize these antioxidant components in honeys from seven different floral sources. Acacia, fireweed, clover, soy, and buckwheat honey contained phenolic acids and flavonoids similar to those reported in honeys from other floral sources (*9*, *10*, *18*−*20*), including *p*-hydroxybenzoic acid, *p*-coumaric acid, *cis*,*trans*-abscisic acid, cinnamic acid, pinobanksin, pinocembrin, and chrysin. The total phenolic contents of the honeys, determined by a modification of the Folin−Ciocalteu method (*28*), were strikingly higher than the phenolic contents quantified by HPLC analysis. Similar observations have been reported in estimating phenolics of other foodstuffs and beverages (*29*). 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.

Phenolic analyses of soy and buckwheat honey were of particular interest because of the well-studied antioxidant activity of the corresponding plant sources. The antioxidant properties of soy and buckwheat have been attributed to high levels of specific flavonoids, i.e., genistein and daidzein in soybeans (*30*, *31*), and rutin in buckwheat (*32*). However, the phenolic profile differs throughout a plant; thus, the phenolic profile of the plant nectar, and the corresponding honey, might be quite different from that of other plant tissues. Neither genistein, daidzein, nor rutin was found in soy or buckwheat honey.

The major flavonoids in honey (pinocembrin, pinobanksin, and chrysin) have also been found in propolis (*33*). Propolis (bee-glue) is the resinous substance collected by honeybees from the buds of various trees and is used to repair the hives (*34*). Many studies have demonstrated the in vitro and in vivo antioxidant activities of propolis (*35*−*37*), attributed to the large amount of flavonoids present (up to 22%) (*38*). Pinocembrin and pinobanksin, the main flavonoids of propolis and honey, have been demonstrated to be potent antioxidants (*39*, *40*). Small amounts of propolis might be incorporated into honey (*34*); therefore, propolis flavonoids might contribute to the phenolic composition of honey. In temperate areas, the main sources of propolis are poplar bud exudates (*41*). Where *Populus* species are not common, different resin sources are incorporated in the propolis. This might explain the different phenolic pattern found in honey from tropical areas such as Hawaiian Christmas berry honey as compared to honeys from more temperate areas. The phenolic profile of HCB honey was very different from the phenolic pattern of the other honeys tested; likewise, none of the peaks of HCB honey corresponded to any of the phenolics previously reported to be present in honeys from other floral sources. Konig (*42*) demonstrated that the phenolics of propolis from Hawaii were different from those of nontropical propolis samples. However, Konig was unable to identify the Hawaiian propolis phenolics.

To determine the relative contribution of each phenolic fraction to the total antioxidant activity of the methanolic honey extract (fraction 3), individual honey fractions were tested for ORAC activity after HPLC separation. The peroxyl-scavenging capacity of the fractions appeared to be more dependent on the abundance of compounds in the fraction than on specific compounds. Because none of the ORAC-specific activities of the unknown compounds/fractions appeared prominently higher than the ORAC activities of the other fractions, it was beyond the scope of this study to further isolate and identify the unknown compounds and determine the correlation between individual phenolics and overall antioxidant activity.

Although many flavonoids are effective antioxidants, the methanol fraction containing those phenolics (fraction 3) was not the main contributor to the total antioxidant activity of any

Table 1. Phenolic Acid and Flavonoid Content of the Various Honeys Analyzed[a]

peak	MW	phenolic compound	buckwheat	Hawaiian Christmas berry	soy	tupelo	clover	fireweed	acacia
1	154	unknown	(3.01 ± 0.51)[b]	–[c]	(0.61 ± 0.04)	–	(0.77 ± 0.02)	(0.99 ± 0.05)	(0.86 ± 0.12)
2	138	*p*-hydroxybenzoic acid	14.8 ± 0.73	–	3.54 ± 0.12	–	2.86 ± 0.17	1.54 ± 0.24	1.40 ± 0.09
3	168	vanillic acid	1.35 ± 0.51	–	–	5.91 ± 0.67	0.89 ± 0.26	–	tr[d]
4	198	syringic acid	–	–	–	–	0.60 ± 0.16	–	tr
5	164	*p*-coumaric acid	11.8 ± 0.14	–	3.55 ± 0.05	–	4.89 ± 0.30	1.52 ± 0.31	–
6	264	*cis*,*trans*-abscisic acid	5.55 ± 1.21	–	2.82 ± 0.29	12.0 ± 1.38	2.31 ± 0.74	6.31 ± 1.35	2.71 ± 0.36
7	148	cinnamic acid	2.16 ± 0.03	1.96 ± 0.29	1.27 ± 0.03	1.19 ± 0.60	0.61 ± 0.28	2.71 ± 0.43	0.12 ± 0.01
8	286	unknown	(5.28 ± 0.22)[b]	–	(10.0 ± 0.25)	–	(6.88 ± 0.35)	–	(3.13 ± 0.07)
9	272	pinobanksin[e]	5.74 ± 0.84	–	15.6 ± 0.72	0.84 ± 0.04	6.61 ± 0.36	0.93 ± 0.15	2.68 ± 0.10
10	302	quercetin	–	–	2.43 ± 0.15	–	1.57 ± 0.15	–	0.61 ± 0.05
11	256	pinocembrin	3.53 ± 0.38	–	10.6 ± 0.69	0.92 ± 0.13	7.04 ± 0.61	0.73 ± 0.06	1.81 ± 0.12
12	286	kaempferol	tr	–	3.57 ± 0.45	tr	3.92 ± 0.77	–	0.45 ± 0.07
13	254	chrysin	1.37 ± 0.47	–	3.95 ± 0.40	tr	2.05 ± 0.14	0.45 ± 0.07	0.93 ± 0.11
14	270	galangin	0.24	–	3.99 ± 0.38	tr	1.64 ± 0.33	–	0.92 ± 0.18
total identified phenolic content			46.5 ± 0.57	1.96 ± 0.29	50.7 ± 3.00	20.9 ± 1.93	33.1 ± 2.23	14.8 ± 2.55	11.6 ± 0.95

[a] Values expressed as means (mg/kg of honey) ± SEM ($n \geq 3$). [b] Unknown compounds are quantified as the % of the total absorbance of the chromatograms at 285 nm (values in parentheses). [c] – = not detected. [d] tr = trace amounts. [e] Quantification of pinobanksin is expressed in terms of pinocembrin equivalents.

Table 2. Fractional Antioxidant Activities[a] (ORACs) of Honeys from Various Floral Sources and the Sum of the Antioxidant Activities of the Four Honey Fractions[b]

		ORAC (μmol of TE/g)				
floral source	total ORAC (μmol of TE/g)	acidified water fraction	neutral water fraction	methanol fraction	water after ether extraction fraction	sum of ORACs of four fractions[c] (μmol of TE/g)
buckwheat	9.75 ± 0.48	4.78 ± 0.30	0.72 ± 0.09	0.73 ± 0.06	1.77 ± 0.21	7.99 ± 0.27
Hawaiian Christmas berry	8.87 ± 0.33	4.13 ± 0.22	0.72 ± 0.12	0.90 ± 0.03	2.30 ± 0.23	7.97 ± 0.14
soy	8.34 ± 0.51	3.99 ± 0.59	0.61 ± 0.06	0.62 ± 0.03	1.88 ± 0.13	7.53 ± 0.26
tupelo	6.48 ± 0.37	2.46 ± 0.17	0.38 ± 0.01	0.48 ± 0.02	1.37 ± 0.06	4.64 ± 0.10
clover	6.05 ± 1.00	3.10 ± 0.22	0.29 ± 0.04	0.28 ± 0.02	0.72 ± 0.06	4.39 ± 0.22
fireweed	3.09 ± 0.27	1.87 ± 0.08	0.10 ± 0.01	0.11 ± 0.00	0.20 ± 0.01	2.31 ± 0.04
acacia	3.00 ± 0.16	2.10 ± 0.09	0.27 ± 0.03	0.15 ± 0.01	0.29 ± 0.01	2.78 ± 0.08

[a] Individual activities of the four fractions collected upon loading each honey on Amberlite XAD-2 resin. [b] Data expressed as means ± SEM ($n \geq 3$). [c] Total ORAC is significantly higher than the sum of ORAC of the four fractions by one-sided Wilcoxon signed-rank test ($p \leq 0.001$).

Table 3. Antioxidant Activities (ORACs) of Honeys from Various Floral Sources and of the Honey Protein Extract and Contents of Various Potential Antioxidant Components[a]

floral source	ORAC (μmol of TE/g)	ORAC of protein extract (μmol of TE/g)	total phenolics[b] (mg/kg)	protein (mg/100 g)	peroxide (μg/g/h)	hydroxymethyl-furaldehyde (mg/100 g)	gluconic acid (g/kg)	ascorbic acid (mg/100 g)
buckwheat	9.75 ± 0.48	1.71 ± 0.25	456 ± 55.6	552 ± 15.4	nd[c]	5.21 ± 0.15	2.59 ± 0.24	–[d]
Hawaiian Christmas berry	8.87 ± 0.33	1.41 ± 0.16	251 ± 26.3	294 ± 7.18	2.54 ± 0.36	4.70 ± 0.79	7.44 ± 0.31	–
soy	8.34 ± 0.51	1.47 ± 0.22	278 ± 21.8	290 ± 4.71	0.60 ± 0.06	14.9 ± 0.36	3.98 ± 0.08	–
tupelo	6.48 ± 0.37	0.90 ± 0.09	183 ± 8.97	211 ± 2.59	2.60 ± 0.18	8.81 ± 0.69	1.81 ± 0.04	–
clover	6.34 ± 0.21	1.13 ± 0.13	130 ± 10.8	257 ± 3.91	0.18 ± 0.03	3.06 ± 0.14	3.59 ± 0.10	–
fireweed	3.09 ± 0.27	0.54 ± 0.04	61.6 ± 6.15	155 ± 2.80	1.69 ± 0.10	5.73 ± 0.19	2.14 ± 0.04	–
acacia	3.00 ± 0.16	0.29 ± 0.05	46.0 ± 2.03	159 ± 2.36	2.34 ± 0.10	1.73 ± 0.19	2.14 ± 0.06	–
correlation (R^2)[e]			0.963	0.674	0.023[f]	0.144	0.280	
p value			<0.0001	0.024	0.774	0.401	0.220	

[a] All data expressed on a honey weight basis as means ± SEM ($n \geq 3$). [b] Data expressed in units of milligrams of gallic acid equivalent (GAE) per kilogram of honey. [c] nd = not determined. [d] – = undetectable. [e] Correlation of various components with ORAC activity of the respective honey. [f] Inverse correlation.

of the investigated honeys. The aqueous layer after ether extraction (fraction 4) had greater ORAC values than the methanol phase. Ether extraction is an extra step in the purification of honey flavonoids, leaving darker phenolic polymers and contaminant sugars in the aqueous layer (*18*). Hence, the darker phenolic polymers contributed more to the antioxidant activity (measured as ORAC values) than the analyzed phenolic compounds. Most antioxidant compounds, however, eluted in the first water-soluble fraction with the carbohydrates. A sugar analogue had a low ORAC value (*6*), suggesting the presence of antioxidant compounds other than reducing sugars in the water-soluble phase. Interestingly, Weston et al. (*43*) also found that the unidentified compound(s) responsible for the high nonperoxide antimicrobial activity of active manuka honey eluted with the carbohydrates in the water phase. A portion of the water-soluble phenolics might have

possibly eluted in this first fraction. Because of the high sugar content of this fraction, chromatographic isolation of these phenolics is challenging, but studies are currently underway to characterize the phenolics in this fraction.

Some nonphenolic components that contribute to the overall antioxidant capacities of honeys were also quantified, including proteins, gluconic acid, ascorbic acid, peroxide, and hydroxymethylfuraldehyde. ORAC analysis of the honey protein fraction revealed that approximately 16% of the peroxyl-scavenging abilities of honey was contributed by the protein fraction. The protein concentrations of the tested honeys were similar to those reported for other honeys (*13*). A significant correlation between the protein content and the ORAC activity of honey was found ($R^2 = 0.674$, $p = 0.024$). Lee and co-workers (*4*) discovered that a specific honey peptide was responsible for the inhibitory effect of honey on the enzymatic browning of fruits and vegetables by polyphenol oxidase. Honey was demonstrated to inhibit enzymatic oxidation of polyphenols and also to reduce a portion of oxidized phenols to the original polyphenols, similar to the reduction mechanism known to occur with ascorbic acid, leading them to suggest that this honey peptide functions with a reducing capacity similar to that of ascorbic acid. Recently, Ates et al. (*23*) partially characterized the peptide from honey that inhibits polyphenol oxidase, but further characterization of the specific peptide(s) responsible for this antioxidant activity is needed.

Organic acids, such as gluconic, citric, and malic acids, might also contribute to the observed antioxidant capacity of honey. Organic acids chelate metals and hence can synergistically enhance the action of other antioxidants, such as phenolics (*44*). Gluconic acid is the predominant honey organic acid, present at 50-fold higher levels than other acids (*12*). Gluconic acid was thus selected as an indicator of the organic acid concentration in the honeys. No significant correlation was established between the level of gluconic acid and the ORAC activity of the honeys.

Low levels (less than 5 mg/100 g) of ascorbic acid, a water-soluble antioxidant, have been reported in honey (*8*). Ascorbic acid was undetectable in selected commercial honeys as compared to data in the literature based on raw honeys. This is most likely due to processing and storage of commercial honeys used in this study. Similarly, it was expected that the level of enzymes would be greatly reduced due to processing and storage of the honeys. Glucose oxidase and catalase, two enzymes present in honey, are effective antioxidants used in combination in food systems because of their ability to remove oxygen from food systems (*44*). Hydrogen peroxide accumulation was measured to estimate the combined activity of glucose oxidase, catalase, and peroxidase. Hydrogen peroxide has been found to be the major contributor to the antimicrobial activity of honey (*45*, *46*). As peroxide is a pro-oxidant, the inverse correlation between ORAC activity and hydrogen peroxide level was investigated. All honeys contained low levels of hydrogen peroxide as compared to literature values of hydrogen peroxide accumulation in raw honeys (*26*). This might be due to a high level of catalase in the honeys, scavenging the formed peroxide, or, as mentioned above, to processing treatments and storage of the honeys.

Although the levels of certain antioxidant components are lowered upon processing and storage of honey, as discussed above, the formation of other antioxidant compounds might, in fact, be stimulated. Mild heat treatment and/or prolonged storage of honey lead to compositional changes due to caramelization of the carbohydrates, Maillard reaction, and decomposition of fructose in the acid medium of honey (*47*). These reactions might result in the formation of hydroxymethylfuraldehyde, other furfural compounds, and Maillard reaction products. Many of these compounds act as antioxidants (*48*). Therefore, hydroxymethylfuraldehyde was analyzed as a measure of the extent of the Maillard reaction. The hydroxymethylfuraldehyde concentration varied among the different honeys, yet there was no correlation between the ORAC values and the hydroxymethylfuraldehyde content ($R^2 = 0.144$, $p = 0.4$). The hydroxymethylfuraldehyde concentration of soy honey (14.9 mg/100 g) was higher than those of all other honeys. This is probably due to the age of the soy honey, which was extracted in 1996. Fresh soy honey, extracted in 2000, had an ORAC value similar to that of soy honey extracted in 1996 (*6*), even though the hydroxymethylfuraldehyde value was significantly lower (0.6 mg/100 g, data not shown). This indicates that hydroxymethylfuraldehyde was not a major contributor to the antioxidant activity of honey. The Maillard reaction, however, is very complex and leads to a wide range of breakdown products of hydroxymethylfuraldehyde, which might also contribute to the antioxidant activity of honey but are more difficult to characterize.

Because of the complex composition of honey, interactions between different antioxidant components are likely important in terms of the overall antioxidant activity of honey. A comparison was made of the ORAC values of the whole honeys with the sum of the ORAC values of their four fractions. The antioxidant capacity of the sum of the fractions was lower than the antioxidant capacity of the whole honey. This might suggest synergistic interactions among the antioxidant components from the various phases. However, loss of some antioxidants during the extraction procedure cannot be overlooked as an explanation. More experiments are needed to further investigate synergistic interactions between different honey components.

Many other components that have not been investigated in the present study might also contribute to total antioxidant activity. Salicylic acid, for example, has been found in honey (*49*) and is known to neutralize oxygen free radicals (*50*). Different amounts and types of minerals can also influence the antioxidant activity of the honeys. The mineral content varies in honeys from approximately 0.04% in pale honeys to 0.2% in some dark honey samples (*51*).

In conclusion, the results of the current study suggest that the levels of single phenolic or other compounds in honey are too low to have a major individual antioxidant significance. Hence, the total antioxidant capacity of honey is likely the result of the combined activity and interactions of a wide range of compounds, including phenolics, peptides, organic acids, enzymes, Maillard reaction products, and possibly other minor components.

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