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Amino Acid Composition and Antioxidant Capacity of Spanish Honeys

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The amino acid composition of 53 honey samples from Spain, consisting of 39 floral, 5 honeydew, and 9 blend honeys, has been determined. Physicochemical characteristics, polyphenolic content, amino acid composition, and estimation of the radical scavenging capacity against the stable free radical DPPH of the honey samples were analyzed. The resulting data have been statistically evaluated. The results showed that pH, acidity, net absorbance, electrical conductivity, and total polyphenolic contents of the honeys showed a strong correlation with the radical scavenging capacity. The correlation between the radical scavenging capacity of honey and amino acid contents was high with 18 of the 20 amino acids detected, with correlation values higher than those obtained for polyphenolic content. These results suggest that the amino acid composition of honey is an indicator of the sample's scavenging capacity.

KEYWORDS: Honey; antioxidant activity; DPPH; free amino acids

INTRODUCTION

Honey is a natural complex product produced by honeybees from the nectar of blossoms or from exudates of trees and plants to produce nectar honeys or honeydews, respectively. Honey composition depends on the plants visited by the bees and on the climatic and environmental conditions. The strong sweetening capacity of honey is due to the presence of the monosaccharides fructose and glucose as majority components (60−85%) and phenolic compounds, minerals, proteins, free amino acids, enzymes, and vitamins as minor components (*1*). Amino acids in honeys are attributable to the bees or to plant sources. The former amino acids are common to many honeys, while the second ones depend on the botanical and geographical origin of the honey. In this context, amino acid honey composition is described by several authors as a suitable method to determine the botanical differentiation of honeys (*2*−*8*).

The amount of total free amino acids in honey corresponds to between 10 and 200 mg/100 g (*2*, *9*), with proline as their major contributor, corresponding to around 50% of the total free amino acids (*4*). To determine and quantify the free amino acids in honey, gas (*2*, *7*, *8*, *10*) and liquid (*3*−*6*) chromatography methods have been described, although the latter can determine a higher number of these compounds (*3*, *4*).

On other hand, recent scientific evidence supports the effectiveness of some honeys as antioxidant (*11*−*13*) and antibacterial (*14*−*17*) agents, and although these activities have been related to some physicochemical parameters or components (*18*−*21*), the components have not yet been precisely identified. In this context, studies on the relationship between free amino acid content and antioxidant honey capacity have not yet been described. Only recently, a good correlation has been reported between proline contents and the radical scavenging activity of honeys (*22*).

The main tests used in the evaluation of in vitro antioxidant activity of honey are DPPH (*23*−*26*) and ORAC (*13*, *24*) assays. DPPH assay is highly accepted, because it is a simple method to evaluate the radical scavenging activity by means of a spectrophotometer, which involves common laboratory equipment.

The aim of the present work was to assess whether the amino acid composition of a honey can yield information about its radical scavenging capacity, to distinguish floral honeys from honeydew honeys. To carry out this study, 53 samples of artisan honeys from the same geographical area have been analyzed.

MATERIALS AND METHODS

Honey Samples. Fifty-three honey samples, from the 2002 harvest, were provided by local beekeepers settled in Madrid (central Spain). All samples were artisanally produced, obtained by centrifugation, and unpasteurized. Characterization of honeys as floral, blends (floral with honeydew), and honeydew honeys was carried out through melissopalinological and physicochemical analyses. Samples were kept in the

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Table 1. Mean and Deviation Values of Scavenging Activity and Physicochemical Variables in 53 Honey Samples[a]

	floral honeys ($n = 39$)		blend honeys ($n = 9$)		honeydew honeys ($n = 5$)	
	mean	SD	mean	SD	mean	SD
radical scavenging effect (%)	20.7 c	19.1	41.1 b	11.2	70.0 a	12.8
moisture content (%)	16.16 a	0.92	16.07 a	0.75	15.46 a	0.77
pH	4.12 c	0.34	4.41 b	0.12	4.96 a	0.14
free acidity (mequiv/kg)	27.9 b	7.7	37.4 a	5.7	35.9 a	4.0
total acidity (mequiv/kg)	31.3 b	8.6	40.3 a	6.4	37.4 ab	4.8
electrical conductivity (10^{-4} S cm^{-1})	4.44 c	2.19	7.48 b	1.21	10.44 a	0.10
ash content (%)	0.22 c	0.17	0.39 b	0.72	0.63 a	0.11
fructose + glucose content (%)	58.58 a	6.62	57.72 a	5.69	58.66 a	6.35
total phenol content (mg/g of honey)	0.66 b	0.22	0.87 a	0.16	1.03 a	0.13
net absorbance ($A_{560} - A_{720}$)	0.22 c	0.12	0.43 b	0.14	0.64 a	0.15

[a] Means with different letters within a column are significantly different ($p = 0.05$).

Table 2. Mean and Deviation Values of Proline, Protein, and Total Amino Acid Contents in the Analyzed Honey Samples[a]

	floral honeys ($n = 39$)		blend honeys ($n = 9$)		honeydew honeys ($n = 5$)	
	mean	SD	mean	SD	mean	SD
proline	57.55 b	13.95	74.09 a	19.46	72.68 a	11.97
total free amino acids	56.08 c	26.36	80.71 b	24.78	141.65 a	38.60
proteins	85.63 a	28.74	93.39 a	16.56	83.21 a	9.25

[a] Means with different letters within a column are significantly different ($p = 0.05$).

Table 3. Average Free Amino Acid Contents (mg/100 g) in Honeys from Different Floral Sources[a]

	floral honeys ($n = 39$)		blend honeys ($n = 9$)		honeydew honeys ($n = 5$)	
free amino acid	mean	SD	mean	SD	mean	SD
aspartic acid (Asp)	4.18 c	2.90	7.48 b	2.32	13.22 a	2.21
glutamic acid (Glu)	7.01 c	7.10	14.34 b	5.07	27.60 a	5.22
asparagine (Asn)	7.48 b	7.47	12.47 b	3.14	21.63 a	7.72
serine (Ser)	2.61 c	1.23	3.65 b	0.68	5.37 a	1.19
glutamine (Gln)	5.14 c	3.36	9.97 b	4.77	16.22 a	5.31
histidine (His)	1.84 a	1.19	0.75 b	1.13	nd	
glycine (Gly)	1.39 c	0.2	1.61 b	0.27	2.1 a	0.26
threonine (Thr)	2.15 b	0.57	2.45 b	0.35	3.45 a	0.63
arginine (Arg)	3.71 c	1.38	5.03 b	2.11	7.36 a	1.17
β-alanine (b-Ala)	1.91 ab	0.09	1.98 a	2.27	1.80 b	0.11
α-alanine (a-Ala)	2.69 c	1.31	3.89 b	0.69	5.33 a	1.64
γ-aminobutiric acid (Gaba)	2.46 b	2.52	3.56 b	1.63	8.86 a	3.67
tyrosine (Tyr)	11.60 a	3.61	10.13 a	1.89	5.82 b	1.57
valine (Val)	2.13 b	0.32	2.40 a	0.27	2.69 a	0.35
tryptophan (Trp)	3.16 b	0.35	3.17 b	0.20	3.75 a	0.33
phenylalanine (Phe)	22.34 a	10.68	19.42 a	5.10	8.42 b	2.97
isoleucine (Ile)	2.11 b	0.12	2.22 a	0.14	2.25 a	0.16
leucine (Leu)	2.33 a	0.67	2.13 a	0.24	1.99 a	0.06
ornitine (Orn)	2.07 b	0.24	2.39 a	0.48	2.62 a	0.15
lysine (Lys)	3.68 a	0.49	3.36 ab	0.37	3.11 b	0.21

[a] nd = not detected. Means with different letters within a column are significantly different ($p = 0.05$). For proline data see **Table 2**.

dark and at room temperature (<30 °C). Analyses were performed in less than a 6 month period after harvesting.

Melissopalynological Analysis. Microscopic analysis of honey sediment composition was essentially performed according to the method described by Louveaux et al. (*27*) using a non-acetolytic technique to preserve honeydew elements, with the modifications proposed by Terradillos et al. (*28*). A Leica DMR light microscope, fitted with a digital camera and coupled to an Image Analysis system (Leica QWin Std software) for morphometric measurements of pollen grains, was used in these analyses. Identification of pollen grains was carried out using a collection of reference pollens from the Madrid area (*29*).

Physicochemical Analysis. The parameters electrical conductivity, ash content, and water content were determined according to the Harmonized Methods of the International Honey Commission (*30*), whereas pH and free and total acidity were evaluated according to the Spanish Official Methods for Honey Analysis (*31*). Thus, the pH and acidities were determined in solutions of honey (10 g) in deionized water (75 mL). Moisture was determined by refractive index measurement at 20 °C in an Abbe refractometer and correlation with Chataway charts and electrical conductivity by measurement in a Crison GLP31 conductimeter in a solution of 20% honey dry matter in deionized water. Ash content was determined by calcinations at 550 °C until constant weight, with the precaution of including a previous caramelization step on a heating plate to control production of foams and sample losses.

Net absorbance (NA), as a color parameter, was measured by UV−vis spectrophotometry (Perkin-Elmer Lambda 10 spectrophotometer) and defined as the difference between absorbance measurements at 560 and 720 nm (*32*). Glucose and fructose contents were extracted by SPE with Sep-Pak C_{18} filters (Waters) and subsequently analyzed by HPLC using a RECEX RCM-Monosaccharide precolumn and column (Phenomenex), at 90 °C, and a K-2301 refractive index detector. Elution was performed using water (HPLC grade) as the eluent at a flow rate of 1.0 mL min^{-1}. Finally, total phenol content was determined with

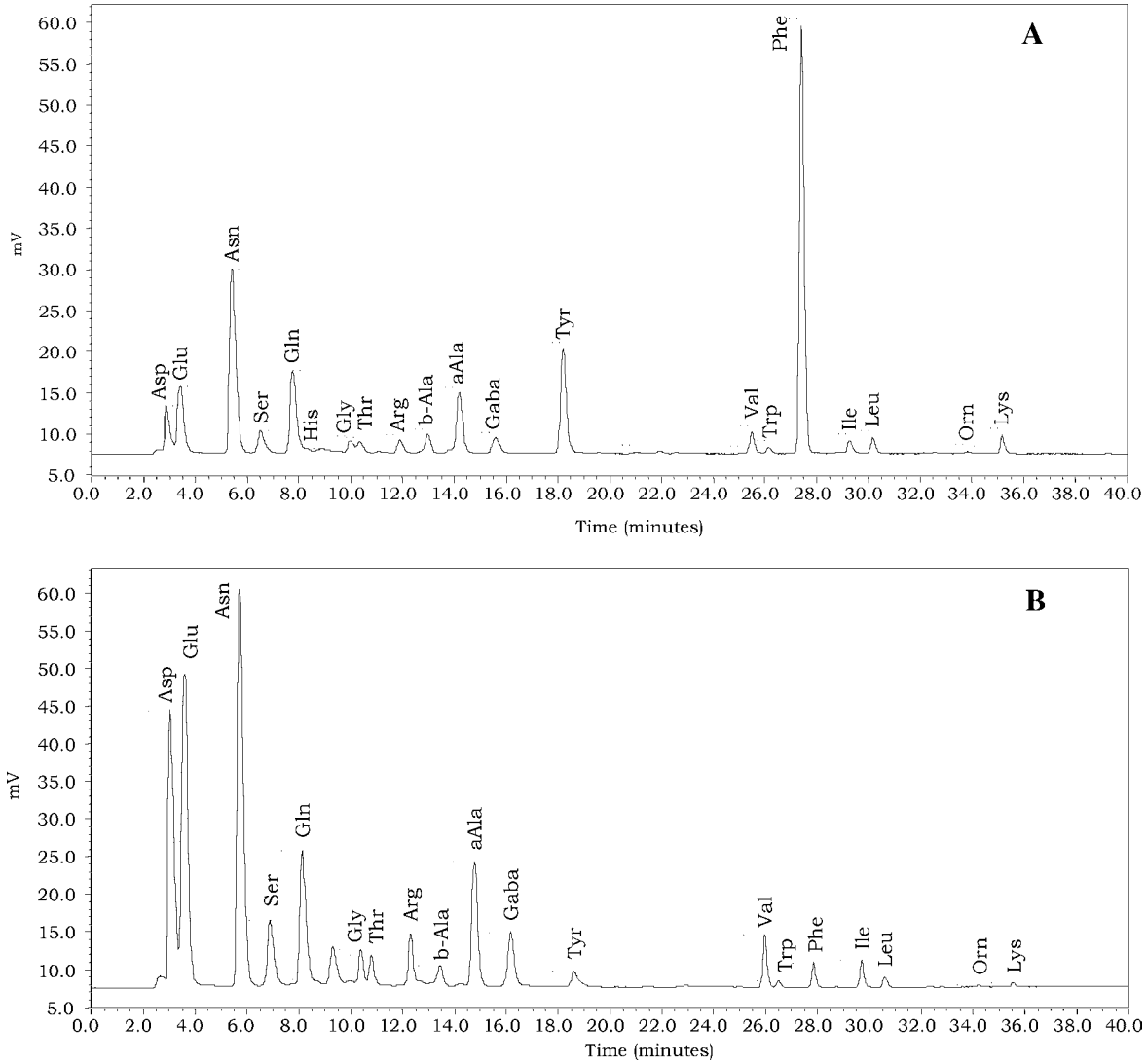

Figure 1. Characteristic chromatograms of amino acids in a floral honey (**A**) and in a honeydew honey (**B**).

the Folin−Ciocalteu reagent by measurement of absorbance at 670 nm, using gallic acid as the standard.

Spectrophotometric Analysis of Protein, Amino Acid, and Proline Contents. The protein content was determined by the method of Bradford (*33*). Total free amino acids were quantified by the Cd−ninhydrin method (*34*). The proline content was determined according to the colorimetric method of Ough (*35*).

HPLC Determination of Amino Acids. Free primary amino acids submitted to a precolumn derivatization with *o*-phthaldialdehyde (OPA; Sigma) were analyzed and quantified using the Iglesias et al. method (*4*).

DPPH Radical Scavenging Activity. Radical scavenging activity of honey, in the presence of the stable free radical DPPH, was determined spectrophotometrically. Briefly, 1.25 mL of a honey solution (0.025 g/mL) was mixed with 1.5 mL of a 90 mg/L solution of DPPH• in methanol, and after 5 min of incubation, the absorbance was read at 517 nm against a water/methanol (1:1) blank. The honey scavenging capacity was estimated using a standard curve of ascorbic acid, and the results are given as percentage equivalents of ascorbic acid, in terms of DPPH• depletion.

Statistical Analysis. Data were processed using SPSS 12.0 software for Windows. The Levene test was used to check equality of variances, and one-way ANOVA (LSD test) was used to estimate statistically significant differences ($p = 0.05$). Canonical discriminant analyses were performed to compare the three types of honeys.

RESULTS AND DISCUSSION

The physicochemical parameters of honey samples were previously considered to be related to their botanical origin (*4*, *26*). These data, together with palynological results were, therefore, previously used to assign honey samples as floral, blends, or honeydew honeys.

Table 1 shows the mean values and standard deviation of the main physicochemical parameters obtained for the three groups, as well as their significance levels. To compare the values obtained for the three honey groups, a Tukey HSD test for comparison of means was carried out, and it was observed that the majority of the parameters evaluated showed a high power to discriminate between floral and honeydew honeys, with the exception of moisture and percentage of glucose plus fructose. According to these results, honeydew honeys are characterized by higher values of pH, electrical conductivity, ash percentage, and net absorbance than floral honeys, whereas blends showed intermediate values. The percentage of glucose plus fructose, which had been previously described as a high discriminatory variable (*4*, *36*), did not show any differentiation capacity to distinguish between both honey groups. These results are in agreement with our previous studies (*26*).

The antioxidant activity of the honey samples was calculated as a percentage of DPPH decoloration, which was established

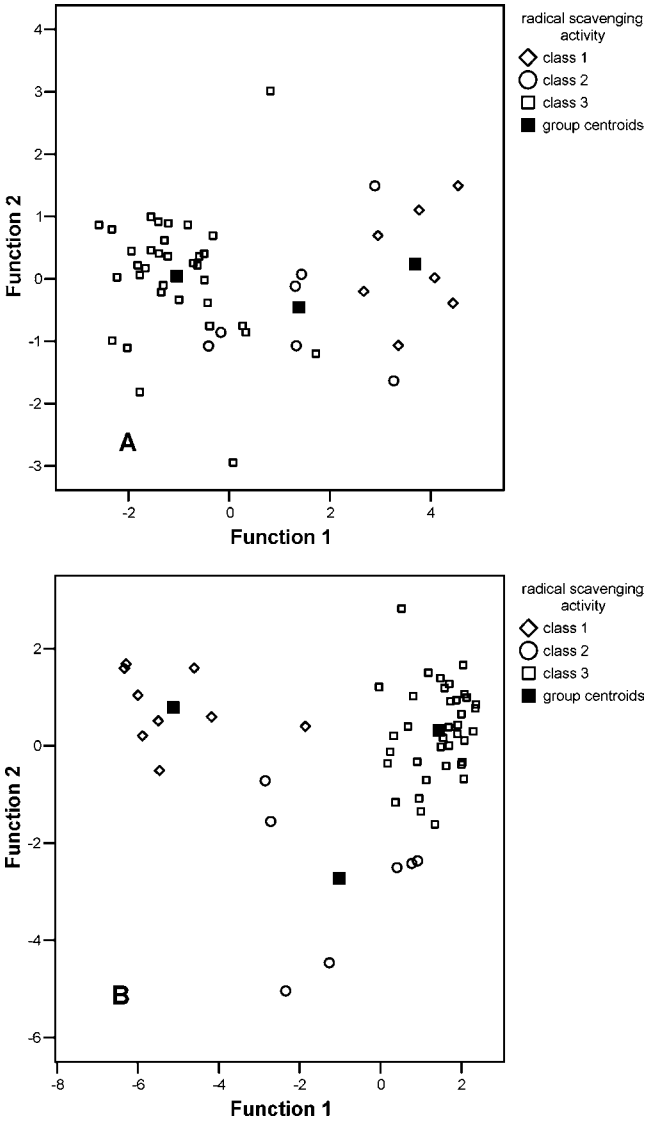

Figure 2. Scatter plots of the first and second canonical variables of the canonical discriminant analysis of the physicochemical honey characteristics (**A**) and amino acid composition (**B**). Classes 1, 2, and 3 represent the different radical scavenging activity of the samples (high, medium, and low, respectively).

by absorbance measurements exactly 5 min after mixing of the honey and DPPH solutions. The results obtained showed a higher percentage of radical scavenging capacity in honeydew honeys (**Table 1**) and are in accordance with recent studies that found the highest antioxidant capacity in the darkest colored honey (*12*, *13*, *26*). As expected (*26*), the results showed a high correlation between the radical scavenging capacity and the parameters electrical conductivity, net absorbance, ash content, total polyphenol content, pH and acidity of honey (with correlation coefficients of 0.876, 0.852, 0.842, 0.809, 0.743, and 0.518, respectively). The correlation obtained between the antioxidant capacity and the total polyphenolic content suggests that the phenolic compounds are, in part, responsible for the antioxidant effects of honey, but there are obviously other factors involved. Moreover, the parameter electrical conductivity accounted for the higher percentage of variance (>76%) in radical scavenging honey capacity, and the latter is closely related to the concentration of mineral contents, organic acids, amino acids, and proteins. It is known that the antioxidant capacity of honey is the result of the combined activity of a wide range of compounds, including phenolic compounds, peptides, organic acids, enzymes, Maillard reaction products, and, possibly, other minor components (*13*). In our study, the relationship between protein and amino acid contents and honey radical scavenging activity was evaluated. Although different studies on the amino acid compositions of honeys have been described, the main objective of these was to use amino acid honey composition as a method to determine the botanical differentiation of honeys (*2*−*8*). However, studies were not found in the available literature that evaluate the correlation between the radical scavenging activity of honeys and their free amino acid compositions. Recently, Meda et al. (*22*) found a higher correlation between this activity and proline content than with total phenolic content and suggest that the amino acid content of a honey should be given more importance when determining its antioxidant activity.

When the contents of proline, total free amino acids, and proteins (**Table 2**) in these honey samples were evaluated, good correlation coefficients were obtained between the first two honey components and the radical scavenging activity (r = 0.571 and 0.825, respectively, $p < 0.001$), and a low one was obtained with protein content ($r = 0.300$, $p < 0.05$). In contrast to the results reported by Meda et al. (*22*), in our studies a better correlation was found between this activity and total phenolic content than with proline content (r = 0.809 and 0.571, respectively, $p < 0.001$), whereas this correlation is higher when total free amino acid content is considered ($r = 0.825$, $p <$ 0.001). These results support the recommendation made by Meda et al. (*22*) to take into account the amino acid content of honey when evaluating its antioxidant activity, and for this purpose, the minor amino acid honey components were evaluated individually (results are described below).

A separate evaluation of floral and honeydew honeys showed that proline and total free amino acid contents (**Table 2**) were parameters with a high differentiation capacity to distinguish between both honey groups, whereas protein content did not show any differentiation capacity. These results are in agreement with the results obtained by Iglesias et al. (*4*).

A total of 24 amino acids were evaluated in these honey samples, for which average values for floral, blend, and honeydew honeys, of the 21 amino acids detected, are reported in **Table 3**. As expected, the majority amino acid in all these groups was proline, and following a decreasing order of concentration with values up to 10 mg/100 g, were glutamic acid, asparagine, glutamine, and aspartic acid in honeydew honeys; phenylalanine and tyrosine in floral honeys; and phenylalanine, glutamic acid, asparagine, and tyrosine in blend honeys. **Figure 1** shows the chromatographic amino acid profile in a floral honey (**A**) and in a honeydew honey (**B**). As in previous studies, the proline concentration is higher in honeydews than in the floral honeys, and proline and phenylalanine are the majority amino acids found in floral honeys (*4*).

Correlation studies and canonical discriminant analyses were carried out to evaluate the relative ability of the physicochemical honey characteristics and amino acid composition to distinguish among three honey groups with different radical scavenging activities (high, medium, low), which are highly related to honeydew, blend, and floral honeys, respectively. In these analyses, significant differences were obtained between radical scavenging activity and the amino acid components of honey, except for β-alanine and leucine. The amino acids with the highest correlation with the antioxidant capacity of the samples were aspartic acid, glutamic acid, glycine, threonine, and glutamine with Pearson's correlation coefficients of 0.844,

0.835, 0.814, 0.831, and 0.817, respectively. It was, therefore, observed that the amino acids which presented the highest correlation coefficients corresponded to polar amino acids, because these amino acids can have a negative charge (such as aspartic acid and glutamic acid) or no charge (such as glycine, threonine, or glutamine). According to this, these amino acids could make an important contribution to the electrical conductivity of the honey, which is a parameter closely related to the radical scavenging activity of a honey.

Three different groups of honeys were established to carry out the corresponding canonical discriminant analyses. Honeys included in these groups showed percentages of radical scavenging activity greater than 55% for high-activity honeys, between 55% and 40% for medium-activity honeys, and less than 40% for low-activity honeys. In this study, two canonical discriminant analyses were carried out separately with a total of six physicochemical variables and nineteen amino acids as variables. These analyses showed that the first two discriminant functions provided a good summary of the original data of the variables considered. Thus, the proportions of the total accumulated dispersion with the two functions were 0.998 for physicochemical variables and 1.000 for amino acid variables, and the canonical correlations were 0.874 and 0.930, respectively. The procedure produced two discriminant functions. **Figure 2** shows the scatter plots of the first and second canonical functions of the canonical discriminant analysis of the physicochemical honey characteristics (**A**) and amino acid composition (**B**), where classes 1, 2, and 3 represent the three groups of honeys on the basis of their radical scavenging activity (high, medium, and low, respectively).

The results obtained in these assays showed a better differentiation, on the basis of honey activity, considering free amino acid contents instead of total polyphenol content or physicochemical honey characteristics. Thus, as also occurred in a previous work (*26*), the honeys evaluated showed a high correlation between the radical scavenging capacity and the parameters conductivity, net absorbance, and total polyphenol content. The higher correlation between the radical scavenging activity and many of the identified amino acids suggested that these compounds are very closely related to this honey activity, even more than their polyphenol components.

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