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Protein Analysis of Honeys by Fast Protein Liquid Chromatography: Application to Differentiate Floral and Honeydew Honeys

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Fast protein liquid chromatography on a Superdex 75 HR column has been applied to analyze the proteins of 29 honeys, 12 of floral origin and 17 from honeydew. The molecular masses were comprised between 13100 and 94000 Da. Seven peaks have been separated; four of them were present in all of the honeys, and three were only present in some honeys. Direct observation of the chromatograms of the floral and honeydew honeys did not reveal any information about their botanical origins. However, both types of honeys can be distinguished with the percentages of the areas of four of the seven chromatographic peaks obtained.

KEYWORDS: Floral honey; honeydew honey; proteins; FPLC; differentiation

INTRODUCTION

Honey is described by the European Union (*1*) as the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature. According to its origin, the main types of honey are blossom honey or nectar honey and honeydew honey. The origin determines to some extent its quality and price. Both consumers and administrations want to know the origin of a honey and, in the case of floral honeys, the identity of the plants from which the pollen comes. Thus, there is a need for methods that allow the authentication of the origin of honeys.

The classical approach to determine the botanical origin of a honey is by palynological analysis. However, highly trained workers, not always available in the control laboratories, must carry out this analysis. For this reason, there are many references in the literature to studies centered on identifying parameters that can establish a honey's origin using simpler techniques, which can be performed in any reasonably equipped laboratory. For example, the discriminatory capacities of phenolic compounds (*2*, *3*), volatile compounds (*4*−*7*), carbohydrates (*8*, *9*), and amino acids (*10*, *11*), among others, have been studied. Recently, physical methods such as infrared and fluorescence spectroscopy have been applied to the authentication of the botanical and geographical origins of honey (*12*−*14*). In spite of the great number of studies realized and because of the variability of the honeys, more research is necessary since valid results for all of the types of honeys have not been obtained with any of the existing methods.

Attempts have also been made to use the information provided by honey proteins to detect their floral origin. Honey proteins are minority compounds, of great importance for the enzymatic character of some of them (*15*−*17*). They proceed from the bee and also from the plants that they feed on and can, therefore, provide information about the floral or honeydew nature of a honey. The proteins from floral honeys and also from bees only fed with sugar have been partially characterized, and the molecular masses and isoelectric points of the majority proteins have been determined. According to different authors, their molecular masses range from 10 to 80 KDa (*18*−*22*) and the isolectric points are between 4.1 and 7.7 (*18*, *22*−*23*). The origin of each protein detected is not certain although Anklam (*24*) and Baroni et al. (*25*) suggest that the concentration of proteins that comes from the bees is much higher than that of plant origin.

The technique with the highest specificity to determine a honey's origin corresponds to immunoassay analysis to determine the nature of the pollens present. Baroni et al. (*21*, *25*) have detected sunflower (*Helianthus annuus*) and eucalyptus pollens (*Eucalyptus* sp.) in honeys by immunoblotting and competitive enzyme-linked immunosorbent assay. One drawback of this very reliable technique for studying the nature of proteins is that the respective immunosera are required. Usually a large number of different pollens are present in a honey, so it

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is impractical, especially in laboratory tests, to have at one's disposal specific immunosera for all of them.

The proteins of honeydew honeys have been less studied than those of floral honeys. We have only found one work in the literature (*26*) focused on the study of honeydew honey proteins. The authors used a Sephadex G-200 column to obtain the chromatography profiles of seven honeys of different origins, finding no differences between the profiles for floral honeys and honeydew honeys. The present research has been carried out, due to the scarce knowledge of the honeys' proteins, with the aim of contributing to the knowledge of this fraction and to explore the possibility of using a simple technique such fast protein liquid chromatography (FPLC) to distinguish floral or blossom honeys and honeydew honeys. For this purpose, 29 artisan honeys from the Madrid community, assigned by palynological and physicochemical analyses into floral or honeydew honeys, were studied. Chromatographic profiles of the protein fraction were obtained, and multivariate statistical techniques were applied to the data obtained.

MATERIALS AND METHODS

Honey Samples. Twenty-nine honey samples from the same harvest were provided by local beekeepers with hives settled in a small geographic area of about 2000 km^2 in central Spain. All samples were artisanally produced, obtained by centrifugation, and unpasteurized.

Pollen Analysis. Melissopalynological analysis of honey samples was essentially performed according to Louveaux et al. (*27*) using a nonacetolytic technique in order to preserve honeydew elements (fungal spores and mycelia, microalgae, and others). The modifications proposed by Terradillos et al. (*28*) for exine cleansing and staining were incorporated. Palynomorphs were identified according to a specifically prepared collection of reference pollens from the Madrid area (*29*).

Physicochemical Parameters. Glucose and fructose contents were determined by high-performance liquid chromatography (HPLC) with a RECEX-Monosaccharide precolumn and column, at 90 °C and using H_2O (HPLC grade) as the eluent at a flow rate of 1.0 mL min^{-1} and a refractive index detector. Free amino acids were quantified by the Cd−ninhydrin method (*30*). The protein content was determined by the Bradford dye-binding assay (*31*). The total nitrogen was determined by the Kjeldahl method with a Tecator Digestion System and a Kjeltec 1030 Auto Analyzer (Tecator AB, Höganäs, Sweden).

Elimination of the Low Molecular Weight Compounds. Twenty-five grams of honey was suspended in 25 mL of distilled water and dialyzed (Cellu Sep T1, Membrane Filtration Products, Inc., Seguin, TX; EEUU, molecular mass cutoff 3500 Da) against tap water for 48 h. The dialysate was lyophilized and then stored at −20 °C until analysis. The lyophilized samples were reconstituted to 2 mL with 0.3 M ammonium acetate buffer.

Chromatographic Separation. Gel filtration chromatography was performed using a FPLC system (Pharmacia-LKB, Uppsala, Sweden) with an UV detector at 280 nm. A Superdex 75 HR 10/30 column (Pharmacia-LKB) was used. A 100 μL amount of the reconstituted sample was injected on the column and eluted with a mobile phase of 0.3 M ammonium acetate buffer at a flow rate of 1 mL/min. The molecular masses of the proteins were determined by the plot of log molecular masses vs retention times using a low molecular mass calibration kit from Amersham Biosciences (Uppsala, Sweden). Standard proteins were as follows: α-lactalbumin (MW 14400), tripsin inhibitor (MW 20100), carbonic anhydrase (MW 30000), ovalbumin (MW 43000), albumin (MW 67000), and phosphorylase b (MW 94000). The R^2 value was 0.98 and indicated that the fit was acceptable.

Statistical Analysis. The statistical methods used for data analysis were a two-sample *t*-test and Mann−Whitney U test to determine if there were significant differences between both types of honey samples and forward stepwise discriminant analysis to select the variables most useful in differentiating the two groups. The STATISTICA program for Windows, version 7.1, was used for data processing (StatSoft, Inc., 2005, www.statsoft.com.). This program was run on a personal computer.

Table 1. Relation of Honey Samples and Assignment of the Samples to the Floral Group or the Honeydew Group[a]

predominant palynomorphs	key label
floral honeys	
Rosmarinus officinalis	F-01
Rosa sp., *Rubus* sp.	F-02
Rosa sp., *Rubus* sp.	F-03
Labiatae	F-04
Labiatae	F-05
Labiatae	F-06
Leguminosae	F-07
Rosa sp., Labiatae, *Erica multiflora*	F-08
Rosa sp., *Eucaliptus* sp., *Echium* sp.	F-09
Rosa sp., *Echium* sp.	F-10
Rosa sp., *Rubus* sp., Labiatae, *E. multiflora*	F-11
Rosa sp., Labiatae, *Echium* sp., others	F-12
honeydew honeys	
Rosa sp., *Rubus* sp.	H-01
Rosa sp., *Rubus* sp.	H-02
Rosa sp., *Rubus* sp.	H-03
Rosa sp., *Rubus* sp.	H-04
Rosa sp., *Rubus* sp.	H-05
Rosa sp., *Rubus* sp., Leguminosae	H-06
Rosa sp., *Rubus* sp., Labiatae	H-07
E. multiflora, Labiatae	H-08
Rosa sp., *Rubus* sp., *E. multiflora*	H-09
Rosa sp., *Rubus* sp., *E. multiflora*, Labiatae	H-10
Rubus sp., Labiatae	H-11
Rosa sp., *Rubus* sp., *Genista* sp., Labiatae	H-12
E. multiflora	H-13
E. multiflora, *Taraxacum vulgare*	H-14
E. multiflora, *Rosa* sp., *Rubus* sp.	H-15
E. multiflora, *Rosa* sp., *Rubus* sp.	H-16
Rosa sp., *Rubus* sp.	H-17

[a] The honey type, predominant palynomorphs, and key used are also shown.

RESULTS AND DISCUSSION

The samples studied and their assignment to the group of floral or honeydew honeys by their physicochemical data and palynological analysis are shown in **Table 1**. This table also shows the pollens predominant in each of the honeys. Of the 12 floral honeys studied, only one, F-01, is a monofloral honey, from rosemary. The other honeys are multifloral, and very few species are represented in their pollens. Six honeys present pollen from labiates, seven from *Rosa* species, three from *Echium*, two from *Erica*, and three from *Rubus*, leguminaceous species and *Eucaliptus*, respectively. Pollens of these same species appear in the honeydew honeys. The explanation for the fact that relatively few species are represented in these honeys is that they all come from the same relatively small geographical area, of around 2000 km^2. These honeys formed part of a wider study (*32*) for which the main objective was to test the capacity of free amino acid content data to discriminate between floral and honeydew honeys. The physicochemical parameters of all of the honeys were within the limits stipulated by the legislation (*33*), except for the glucose plus fructose content in the rosemary honey (F-01), which presents a content of this parameter of 42 g/100 g, below the lower limit established by the legislation, 60 g/100 g. The total nitrogen content was also determined, obtaining a mean value of 79.10 mg/100 g in the floral honeys, significantly lower than the total nitrogen mean value in honeydew honeys of 131.44 mg/100 g. The free amino

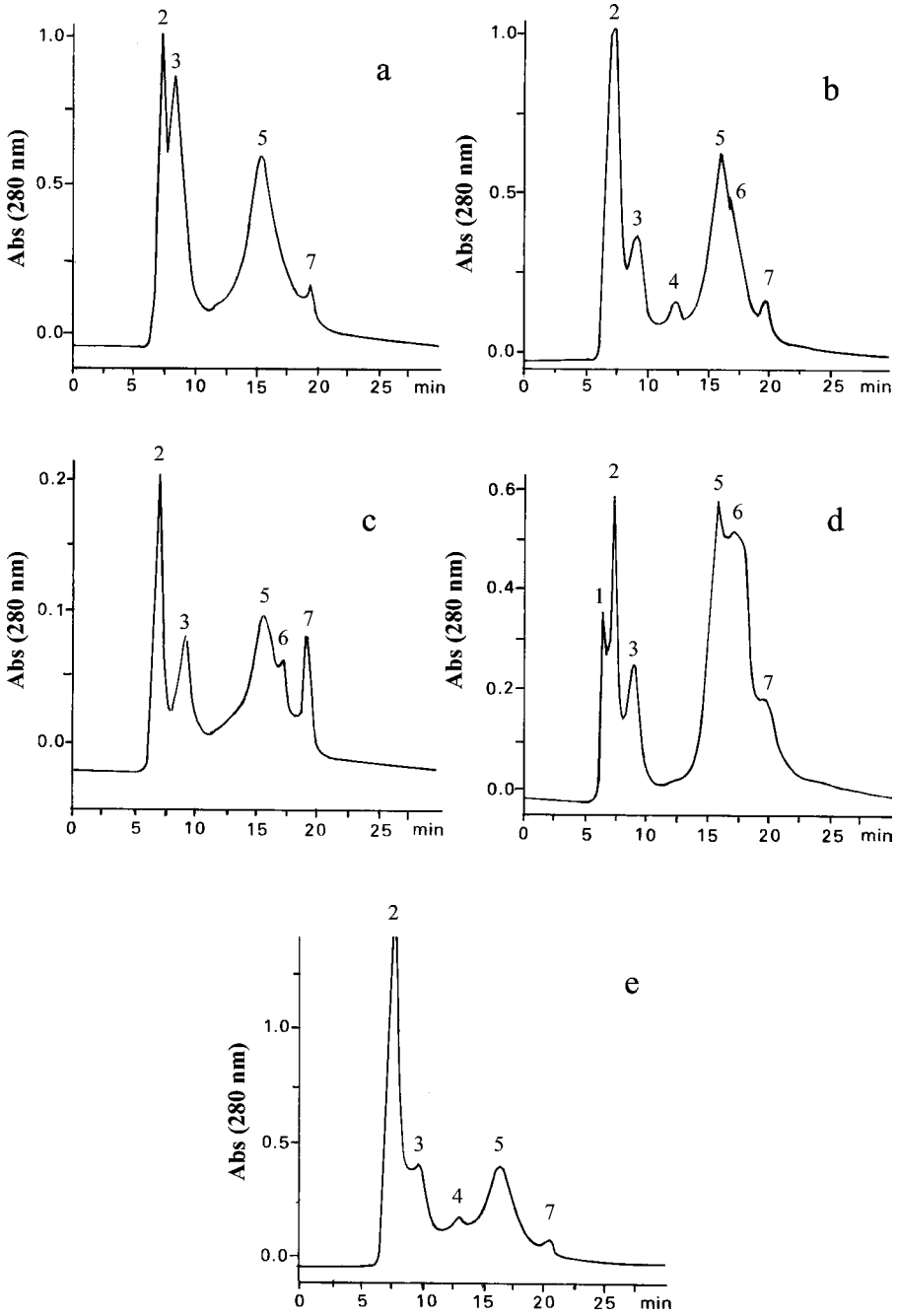

Figure 1. FPLC chromatograms of a honey sample for each of the five (**a**–**e**) chromatographic profiles obtained. Chromatographic conditions: Superdex 75 HR 10/30 column; eluent, 0.3 M ammonium acetate buffer; flow rate, 1 mL/min; detection, at 280 nm. (**a**, **b**) Honeydew honeys and (**c**–**e**) floral honeys.

acid content of floral honeys was 45.72 mg leucine/100 g and was also significantly lower than that recorded in honeydew honeys, 109.78 mg leucine/100 g. There were a great dispersion of the data of protein content in the honeys, from 3.41 to 301.75 mg BSA/100 g, and the mean values of the concentration of proteins of both groups of honey were not significantly different, 111.70 mg BSA/100 g and 135.56 mg BSA/100 g in floral honeys and in honeydew honeys, respectively. Mean values for proteins were within the range detected by Bogdanov (*34*) in a study of 34 honeys, 16 Swiss ones, and 18 from other countries and by da Azeredo et al. (*35*) in a study on 12 Brazilian honeys. The low total nitrogen, free amino acid, and protein contents of 56.1 mg/100 g, 14.1 mg leucine/100 g, and 3.4 mg BSA/100 g, respectively, in the rosemary honey were noteworthy.

In the chromatographic analysis of the dialyzed and lyophilized fractions of the honeys, a total of seven chromatographic peaks were obtained. The chromatograms obtained correspond to five different chromatographic profiles. **Figure 1** shows, as an example, the chromatogram of a honey sample for each of the profiles obtained. The peaks 2, 3, 5 and 7 are present in all of the honeys studied while peaks 1, 4, and 6 are only present in some of the samples. **Figure 1a** shows the chromatogram of a representative honey of the group that only has the peaks 2, 3, 5, and 7. **Figure 1b** shows the chromatogram for a honey with the peaks 4 and 6. The chromatogram of **Figure 1c** represents the group of honeys that present peak 6 as well as peaks 2, 3, 5, and 7. **Figure 1d** shows the chromatogram for a honey that also presents peaks 1 and 6, and **Figure 1e** shows

Table 2. Sum of the Areas of the Seven Peaks Considered (P1–P7) and Their Normalized Area Percentage[a]

		normalized area percentage						
key label	sum of the areas	P1[b] 94000	P2 83500	P3 61200	P4 42000	P5 13100	P6 <13000	P7 <13000
				floral honeys				
F-01	617	7.8	25.8	21.1		27.7	13.9	3.7
F-02	3025	6.1	13.6	11.1		28.8	27.6	12.8
F-03	1327		35.6	12.0	3.2	26.7	17.8	4.8
F-04	1368		23.1	16.5		41.4	12.4	6.6
F-05	1097		23.2	25.9		32.1	13.8	5.0
F-06	3304		18.3	19.1	7.1	36.6	14.9	4.0
F-07	894		22.0	19.7		34.9	10.3	13.1
F-08	2504		51.2	13.3	1.0	31.5		3.1
F-09	4391		47.4	15.9	7.3	27.7		1.6
F-10	835		16.2	15.0	3.0	35.9	24.0	6.0
F-11	1633	7.2	11.6	16.8		24.7	32.0	7.7
F-12	4230		49.4	14.2	3.4	30.8		2.2
				honeydew honeys				
H-01	924		11.3	9.0		79.4		0.3
H-02	4751	7.0	8.2	13.3		41.1	27.4	2.9
H-03	2027		19.3	12.1		40.9	27.3	0.4
H-04	3781		38.7	8.7		52.0		0.6
H-05	1852		18.5	12.4		41.3	27.5	0.4
H-06	1580		20.3	0.9		33.7	44.8	0.2
H-07	4183		32.5	15.1		52.2		0.2
H-08	2297		16.3	22.6		35.4	25.8	0.0
H-09	5830		31.1	17.7		50.0		1.2
H-10	2198		19.7	21.3		32.9	25.7	0.4
H-11	963	4.0	5.9	9.2		44.5	35.8	0.4
H-12	2755		23.0	17.8		58.1		1.1
H-13	4334		18.8	32.4		46.2		2.6
H-14	7621		41.7	1.4		56.5		0.4
H-15	5546		39.5	3.7		55.1		1.6
H-16	2601	8.7	9.8	15.5		61.9	3.2	0.9
H-17	539		8.3	10.9	2.8	74.4		3.5

[a] The approximated molecular mass (Da) is also shown. [b] For the identity of the peaks, see **Figure 1**.

the chromatogram for one of the honeys in which peak 4 also appears without peak 6. The area of the detected peaks for each honey and the sum of the area vary greatly from one honey to the next, since they have very different protein contents, as mentioned previously. Simple observation of the chromatograms does not reveal any relationship between the presence or the absence of each peak and the nature of the pollens present or their assignment to the floral or honeydew honey groups. Bergner and Diemair (*26*) did not observe differences between the chromatographic profile of the proteins of the floral honeys and the honeydew honeys obtained by Sephadex G-200 chromatography.

In order to make the data comparable, the area percentages were calculated, with a sum of 100. **Table 2** shows the sum of the areas of the seven chromatographic peaks, the approximate molecular mass, and the normalized area percentages of the seven peaks in the 29 samples analyzed. Of the seven chromatographic peaks obtained, the four first ones corresponded to proteins in the molecular mass range 42000−94000 Da, while peaks 5−7 are polypeptides with molecular masses equal to or less than 13100 Da. The molecular masses observed in these honeys are similar to those reported by other authors for honeys from Korea (*19*), Argentina (*21*), and the United States (*22*).

Forward stepwise discriminant analysis was applied to data in **Table 2** to determine whether the percentage distribution of the areas of the peaks could differentiate both types of honeys. Values of 4.0 and 3.9 were used for *F* statistics to enter and to remove variables, respectively. Four of the seven peak areas quantified were selected as follows: P7, P4, P5, and P6. A 100% correct assignment of the samples was obtained by either the standard or the leave-one-out cross-validation procedures applied to the protein' peaks selected. The raw canonical discriminant function was

$$\text{canonical score} = 3.3269 + 0.3659 \times \text{P4} - 0.09897 \times \text{P5} - 0.04911 \times \text{P6} + 0.39215 \times \text{P7}$$

that provides negative values for the samples of the honeydew group (between −4.40 and −0.23) and positive values for those of the floral group (between 1.20 and 4.50). From the matrix structure factor, the normalized percentages P7 and P5 are most correlated with the canonical variable, the first one with a positive correlation ($r = 0.44$) and the second one with a negative correlation ($r = -0.40$). Provided that the values of the canonical variable are positive for the samples of the floral group, it is possible to deduce that these samples will have greater values of the percentage of P7 and smaller values of the percentage of P5 than samples from the honeydew group. **Figure 2** shows the categorized scatter plot of the honey samples for P7 and P5 percentages and the straight line ($-0.31008 \times \text{P7} + 0.06832 \times \text{P5} = 1.9699$) of the corresponding canonical discriminant function for these two variables only, which provides positive score values for the samples of the group of honeydew honeys (above the line $-0.31008 \times \text{P7} + 0.06832$

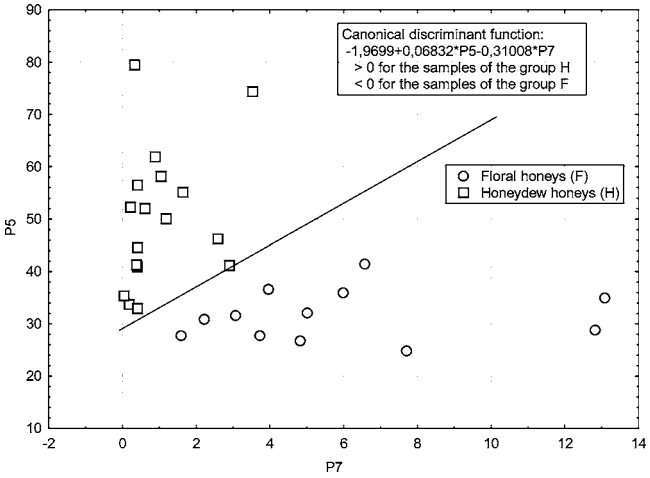

Figure 2. Categorized scatterplot of the honey samples by P7 and P5 normalized area percentages and the straight line of the corresponding canonical discriminant function for these two only variables.

Table 3. Mean and Standard Deviation (SD) Values of the Normalized Area Percentage and the Result of the *t*-Test for Comparison of the Two Means

		floral honeys ($n = 12$)		honeydew honeys ($n = 17$)		
variables	molecular mass (Da)	mean	SD	mean	SD	result of *t*-test
P1	94000	1.8	3.2	1.2	2.7	NS
P2	83500	28.1	14.2	21.3	11.5	NS
P3	61200	16.7	4.2	13.2	8.0	NS
P4	42000	2.1	2.8	0.2	0.7	*
P5	13100	31.6	4.9	50.3	13.2	**
P6	<13000	13.9	10.6	12.8	16.0	NS
P7	<13000	5.8	3.7	1.0	1.1	**

*, significant differences ($P < 0.05$); **, significant differences ($P < 0.01$); NS, not significant differences.

$\times$ P5 $-$ 1.9699 $>$ 0) and negative values for those of the group of floral honeys (under the line, $-0.31008 \times$ P7 $+$ 0.06832 $\times$ P5 $-$ 1.9699 $<$ 0). **Table 3** shows the mean values and standard deviations of the percentages of peak areas in the two groups of honeys and the results of the *t* test for comparison of the two means that coincide with the obtained by means of the Mann−Whitney U test. It can be observed that the samples of floral honeys have greater values in the variables P7 and P4 and smaller values in P5, as compared to the samples of honeydew honeys.

Considering the four variables (P4, P5, P6, and P7), selected by the forward stepwise discriminant analysis, and the matrix with the Euclidean distances among the 29 samples of honeys, the *k* nearest neighbors classification method was applied for $k = 5$, and all 29 samples were correctly classified, except the sample F-10, with this nonparametric procedure.

In summary, the results obtained from the FPLC analysis of honey proteins in a Superdex 75 column indicate that their molecular masses are in the range of <13000−94000 Da. The chromatograms obtained from the 29 studied honeys correspond only to five chromatographic profiles. In spite of the fact that the direct observation of the chromatograms did not reveal any information about their botanical origin, floral honeys and honeydew honeys can be distinguished using the percentages of the areas of four of the seven chromatographic peaks obtained using discriminant analysis.

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