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Assessment of the Floral Origin of Honey by SDS-Page Immunoblot Techniques

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We report on the development of a novel alternative method for the assessment of floral origin in honey samples based on the study of honey proteins using immunoblot assays. The main goal of our work was to evaluate the use of honey proteins as chemical markers of the floral origin of honey. Considering that honeybee proteins should be common to all types of honey, we decided to verify the usefulness of pollen proteins as floral origin markers in honey. We used polyclonal anti-pollen antibodies raised in rabbits by repeated immunization of Sunflower (*Elianthus annuus*) and Eucalyptus (*Eucalyptus sp.)* pollen extracts. The IgG fraction was purified by immunoaffinity. These antibodies were verified with nitrocellulose blotted pollen and unifloral honey protein extracts. The antibodies anti-Sunflower pollen, bound to the 36 and 33 kDa proteins of Sunflower unifloral honey and to honey containing Sunflower pollen; and the antibodies anti-*Eucalyptus sp.* pollen bound to the 38 kDa proteins of *Eucalyptus sp.* unifloral honey in immunoblot assays. Satisfactory results were obtained in differentiating between the types of pollen analyzed and between Sunflower honey and Eucalyptus honey with less cross reactivity with other types of honey from different origin and also with good sensitivity in the detection. This immunoblot method opens an interesting field for the development of new antibodies from different plants, which could serve as an alternative or complementary method to the usual melissopalynological analysis to assess honey floral origin.

KEYWORDS: Honey; protein; floral origin; botanical origin; Western blot; electrophoresis; and melissopalynology

INTRODUCTION

Among different bee products, honey has the major commercial attention because of its consumption without change and its multiple applications as a food constituent. World honey production rose to 1,215,936 Mt during the year 2000, with China (253,691 Mt), the U.S. (101,000 Mt), and Argentina (91,000 Mt) as the main producers (*1*). Though the bulk market price of honey is conditioned by the quantity offered and also by some quality parameters (freshness, color, moisture, etc.), there is an important market segment that pays surplus prices for unifloral honey and/or for origin-certified honey (delicatessen), especially in European countries (*2*). In addition to the market price, the honey sweetness (mainly related to the fructose amount), color, and flavor are strongly associated with its botanical and geographical origin (*2*, *3*).

The standard procedure for assessing honey botanical origin is melissopalynology, which consists of the microscopical analysis of the pollen present in the honey after filtration or centrifugation (*4*). However, melissopalynology requires previous knowledge of pollen morphology and specialized professional personnel to achieve reliable results. Besides, the melissopalynology could be difficult to apply in filtered processed honey, mainly because of pollen scarcity after filtration. Melissopalynology is also limited when the pollen present in the honey is infrarepresented as is the case with citrus honey. Many researchers are looking for an alternative method for melissopalynology based on the physical and chemical attributes of honey, including the analysis of standard physical and chemical parameters (pH, acidity, moisture, HMF, diastase activity, sugar profile, etc.) (2, *5*, *6*) as well as the use of chemometrics to get statistically reliable results (*7*−*9*). Even though physical and chemical analysis associated with chemometrics gives satisfactory results for honey classification, it should be mentioned that many of the parameters used to discriminate between types of honey from different origin change with the time and storage conditions or are unstable (*10*, *11*); thus, chemometrics could be a good approach to assess the origin in fresh honey, but its results should be handled with care when they are from processed or stored honey. Other

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Table 1. Unifloral Honey Melissopalynological Analyses

taxon	2	10	12	52	60	79	95	103	106
Melilotus albus			0.8%		28.2%		60%	4.8%	3%
Prosopis sp.	85.5%	7.4%	2.9%				18%		83.5%
Helianthus annuus					45.6%	90%	11%		
Brassica sp.		1.8%					5%	4%	
Argemone hummenmanni							2%		
Tamarix gallica							2%		
Chenopodium sp.		0.6%					0.9%		
Baccharis sp.	2.4%						0.9%		
Geoffroea decorticans		10.4%			11.7%				7.7%
Argemone subfusiformis								1%	2.6%
Eruca sp.									1.4%
Prosopis caldenia				83%		6.8%			
Heteroteca latifolia						2.3%			
Medicago sativa				6.3%		0.7%			
Salsola cali						0.2%			
Hirsfeldia incana			0.5%	8.3%	2.6%				
Condalia mycrophylla				2%					
Eucalyptus sp.			92%		3.2%			84.5%	
Lycium cestroides		0.6%						2%	
Hyalis argentea					3.2%				
Vicia sp.					3.5%				
Poaceae	6%								
Acacia sp	6%	0.6%							
Tripudantus		0.6%							
Parquinsonia		1.8%							
Larrea divaricata		74.8%	2.9%						
Schinus sp.		1.2%							

authors have looked for honey classification through the use of chemical markers such as flavonoids (*12*−*14*) or through the analysis of natural volatile honey compounds (*15*).

Proteins are minor honey components; however, they are used as internal standard in the evaluation of adulteration by stable carbon isotope ratio (*16*). Honey proteins come from honeybee and also from plants (pollen and nectar) (*16*, *17*). Most of the publications on honey proteins are related to honeybee enzymes (*18*). To the extent of our knowledge, there is only one report on the use of protein electrophoresis to distinguish between commercial and natural Galicia (Spain) honey (*19*).

The main goal of our work was to evaluate the use of honey proteins as chemical markers of the floral origin of honey. Considering that honeybee proteins should be common to all types of honey, we decided to verify the usefulness of pollen proteins as floral origin markers in honey. Because the sensitivity of SDS−PAGE was not enough to detect pollen proteins in honey, we decided to use immunoblot assays employing anti-pollen antibodies raised from Sunflower and Eucalyptus pollen extracts. These immunoblot assays showed satisfactory results discriminating between the different types of pollen analyzed and between Sunflower and Eucalyptus honey.

MATERIALS AND METHODS

Protein Extracts. *Pollen.* The species of pollens studied were *Celtis tala* (Tala), *Eucalyptus sp* (Eucalyptus), *Prosopis sp.* (Algarrobo), *Prosopis caldenia* (Caldén), and *Helianthus annuus* (Sunflower), which were obtained in fields from the Province of Córdoba (Argentina). The protein extraction was carried out by the method of Park et al. (*20*). Briefly, 200 mg of pollen was defatted with diethyl ether and was then extracted in 100 mL of carbonate buffer (0.125 mol·L^{-1} NH_4HCO_3− 0.015 mol·L^{-1} NaN_3, pH 7.5) for 24 h at 4 °C with constant stirring. The extract was centrifuged at 27,000*g* for 30 min at 4 °C, and the supernatant was dialyzed using membranes with cut off of 3.5 kDa against distilled water for 48 h. The dialyzed supernatant was subsequently freeze-dried and stored at −20 °C until use (*20*). Protein concentrations were determined according to the method of Bradford (*21*).

Honey. Six different types of unifloral honey (*Prosopis caldenia*, *Prosopis sp*., *Eucalyptus sp*., *Helianthus annuus*, *Melilotus albus*, and *Larrea divaricata*) were used. Melissopalynological analyses were performed for each studied honey (**Table 1**). The protein extraction was carried out by the method of Bauer et al. (*17*). Briefly, 10 g of honey was suspended in 10 mL of distilled water, and proteins were extracted by overnight shaking at 4 °C. After the mixture was centrifuged at 27,000*g* for 30 min at 4 °C, the supernatant was dialyzed, freeze-dried, and stored at −20 °C, as above. Protein concentrations were determined according to the method of Bradford (*21*).

Preparation of Polyclonal Antibodies. Polyclonal antibodies against total proteins of pollen (anti-pollen antibodies) were raised in rabbits by repeated immunization with 100 μg of pollen protein extract of *Helianthus annuus* or *Eucalyptus sp.* pollen which was emulsified in complete Freund's adjuvant (*22*, *23*). The IgG fraction of rabbit antibodies was obtained from rabbit serum by fractionation with ammonium sulfate 40% and then by anionic exchange chromatography DEAE-Sephacell (*24*). Titers of antibodies were determined by dot blot using the different extracts of pollen dotted in nitrocellulose membranes. Thereinafter, the membranes were incubated with goat anti-rabbit IgG conjugated with peroxidase antibody and revealed with chemoluminiscence reaction (ECL Reagent, NEN Life Science Products).

Electrophoretic Procedures. SDS−PAGE was carried out by the method of Laemmli (*25*) under reducing conditions (Dithiotritol 50 mM), using 12% polyacrylamide gels. Electrophoresis was carried out using 5 μg of protein for pollen extracts and 20 μg of protein for honey extracts in each lane, for 1 h at 140 V using a Mini Protean III electrophoresis cell (Bio-Rad Laboratories, Hercules, CA), and proteins were detected by Coomassie Brilliant Blue R-250 staining. The molecular masses of the proteins were determined from the plot of log Mr versus relative mobility using protein molecular masses standards from Bio-Rad (broad range).

Immunoblot. Electrophoretically separated protein bands were transferred to nitrocellulose membranes using a transfer buffer (25 mmol·L^{-1} Tris, 192 mmol·L^{-1} Glycine, and 20% ethanol, pH 7.5) and blocked with defatted dried milk 5%. Then they were incubated with anti-pollen antibodies diluted 1:7,500 for anti-Sunflower pollen antibodies and 1:1,500 for anti-Eucalyptus pollen antibodies in TBS−T (Tris 0.25 M, NaCl 0.75 M; Tween 20 0.5%; pH 7.3) for 2 h at room temperature. After the membranes were washed with TBS−T, they were incubated with goat anti-rabbit IgG conjugated with peroxidase diluted

Table 2. Classification and Assignment Results of Discriminant Analysis

classification matrix for pollen

forward mode

	predicted group					
actual group	*Prosopis caldenia*	*Prosopis sp.*	*Eucalyptus sp.*	*Helianthus annuus*	*Celtis tala*	% correct
Prosopis caldenia	22	0	0	0	0	100
Prosopis sp.	4	18	0	0	0	82
Eucalyptus sp.	0	0	16	0	0	100
Helianthus annuus	0	1	0	8	0	88
Celtis tala	0	0	3	0	17	85
total	26	19	19	8	17	91

backward mode

	predicted group					
actual group	*Prosopis caldenia*	*Prosopis sp.*	*Eucalyptus sp.*	*Helianthus annuus*	*Celtis tala*	% correct
Prosopis caldenia	16	6	0	0	0	73
Prosopis sp.	4	10	8	0	0	45
Eucalyptus sp.	0	0	16	0	0	100
Helianthus annuus	0	0	1	8	0	89
Celtis tala	0	2	0	0	16	80
total	20	18	27	8	16	74

1:100,000. Then the membranes were washed with TBS−T, and bound antibodies were detected by chemoluminiscence reaction as above.

Statistical Analysis. To get statistical evidence on the differences between several pollen protein profiles from SDS−PAGE, discriminant analysis (DA) was carried out as described previously (*26*). To accomplish DA we assigned eleven different variables to each pollen protein profile. The first variable was an arbitrary number to identify the plant from which the pollen originated. The other 10 variables corresponded to the relative mobility of protein bands (Rf), thus we divided Rf into 10 groups (Rf > 0 and ≤ 0.10; Rf > 0.10 and ≤ 0.20, etc.), with one variable representing each group. Each experimental Rf was loaded into the corresponding group/variable. Null value was assigned to the corresponding group when we did not observe protein bands within these Rf values. Classification matrix as well as discriminant functions were obtained by using both backward and forward stepwise models (*26*).

RESULTS AND DISCUSSION

Our first experiments were directed to determining whether pollens from different plants show characteristic proteins (unique protein profile which would allow differentiation between pollens). We carried out SDS−PAGE using proteins extracted from pollen as described in Material and Methods. **Figure 1** shows the SDS−PAGE profile from pollen protein extracts. In **Figure 1** we can see multiple protein bands ranging from ∼21 to ∼120 kDa, showing bands common to more than one pollen (**Figure 1**, lanes 2 and 3) and bands specific to only one type of pollen (**Figure 1**, lane 1). To discriminate between the different protein profiles obtained for each type of pollen studied, we applied DA to the results as described in Material and Methods. The classification matrix, as well as discriminant functions obtained from DA, are shown in **Table 2** and **Table 3**. From **Table 2** (forward stepwise mode) we observe that the studied pollen can be discriminated up to a 91% certainty by using all of the SDS−PAGE protein bands (**Table 3** forward stepwise mode). It is also remarkable that at least 74% discriminant certainty can be obtained (**Table 2** backward stepwise mode) using only 4 groups of protein bands (**Table 3** backward stepwise mode).

From DA of the protein profile analysis between different pollens, we observed that the entire protein profile points out the difference between pollens rather than a characteristic protein band. This last observation resembles the result obtained by another research group with Galicia (Spain) honey (*19*), where they used DA to differentiate between 82 natural honey samples collected from hives located in Galicia and 24 manufactured honey samples commercially available from Santiago de Compostela (Spain).

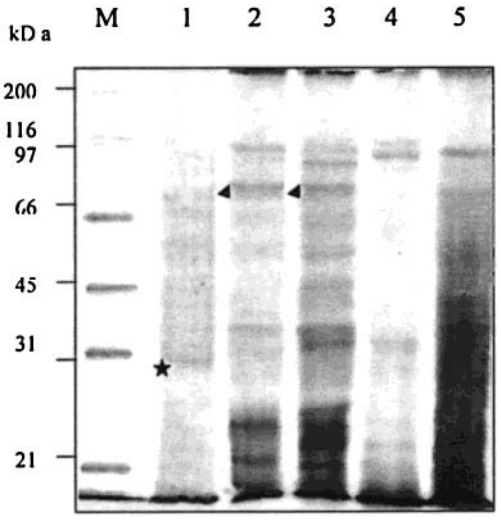

Figure 1. Analysis of five types of pollen by SDS−PAGE under reducing conditions (12% gels). M: Molecular weight markers. Proteins (5 μg) were loaded in each lane: lane 1, *Celtis tala* pollen; lane 2, *Prosopis caldenia*; lane 3, *Prosopis sp.*; lane 4, *Eucalyptus sp.* pollen; and lane 5, *Helianthus annuus* pollen. Gel was stained with Coomassie Brilliant Blue R−250. (Arrows point to a common band of *Prosopis sp*. and *Prosopis caldenia* pollen, and star indicates a characteristic band of *Celtis tala* pollen.)

According to these results, we thought it likely to find these differences between the types of pollen in unifloral honey protein profiles. However, using a similar electrophoretic procedure with honey proteins, the protein profiles obtained were remarkably similar for each of the unifloral honey samples analyzed, revealing two bands at molecular weight of ∼80 and ∼66 kDa in all of them (**Figure 2**). We believe these proteins are from honeybee origin (*17*) and that the amount of these honeybee proteins is much higher than those coming from pollen; thus,

Table 3. Classification Functions for Discriminant Analysis of Rf Values for Pollen

forward mode

Rf values	*Prosopis caldenia*	*Prosopis sp.*	*Eucalyptus sp.*	*Helianthus annuus*	*Celtis tala*
>0–0.1[a]					
>0.1–0.2[a]					
>0.2–0.3	68.3565	54.8985	72.2268	62.2157	15.1083
>0.3–0.4	58.8327	35.9281	66.1996	28.3008	69.4079
>0.4–0.5	–14.4273	–1.3156	5.5466	–20.9808	–2.8824
>0.5–0.6	8.5591	6.2863	11.5969	4.2326	13.8520
>0.6–0.7	–0.0242	0.6224	11.9515	–5.9498	7.2542
>0.7–0.8	–7.2755	–0.1429	–6.7480	–7.3986	–9.5131
>0.8–0.9	8.1337	14.4015	11.0385	–3.7166	–1.8810
>0.9–1	–2.0099	–2.1805	–5.6459	–2.7576	–2.6591
constant	–93.4739	–84.9694	–87.5611	–75.3107	–79.6944

backward mode

Rf values	*Prosopis caldenia*	*Prosopis sp.*	*Eucalyptus sp.*	*Helianthus annuus*	*Celtis tala*
>0.2–0.3	83.4100	76.7675	78.5346	73.8565	27.6850
>0.3–0.4	31.9061	21.6966	35.3109	4.3078	34.7414
>0.4–0.5	1.8369	10.9977	13.3070	–1.3780	9.4968
>0.8–0.9	19.1412	21.9509	21.0500	5.3508	11.0268
Constant	–26.4789	–26.1838	–31.7386	–12.3140	–13.3570

[a] No protein bands were found in these Rf value ranges.

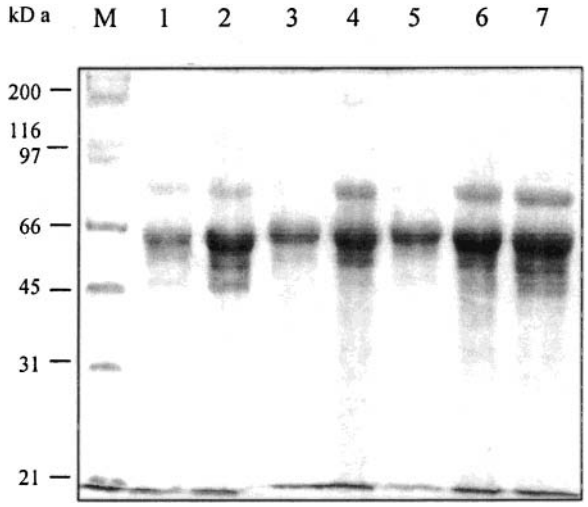

Figure 2. Analysis of the proteins of seven types of unifloral honey by SDS–PAGE under reducing conditions (12% gels). M: Molecular weight markers. Proteins (20 μg) were loaded in each lane: lane 1, sample 60 (*Helianthus annuus* honey); lane 2, sample 103 (*Eucalyptus sp.* honey); lane 3, sample 52 (*Prosopis caldenia* honey); lane 4, sample 79 (*Helianthus annuus* honey); lane 5, sample 106 (*Prosopis sp.* honey); lane 6, sample 10 (*Larrea divaricata* honey); and lane 7, sample 95 (*Melilotus albus* honey). Gel was stained with Coomassie Brilliant Blue R-250.

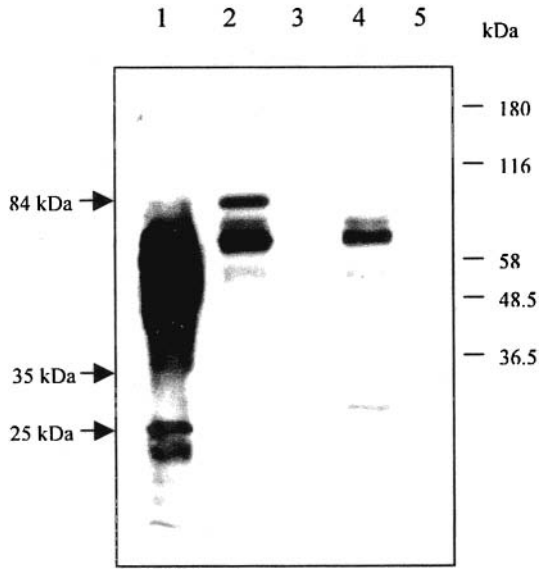

Figure 3. Immunoblot analysis. Protein extracts of five types of pollen were processed by electrophoresis, blotted, and immunodetected with anti-Sunflower pollen antibodies diluted 1:7.500. Lane 1, *Helianthus annuus* pollen; lane 2, *Celtis tala* pollen; lane 3, *Prosopis caldenia* pollen; lane 4, *Prosopis sp.* pollen; and lane 5, *Eucalyptus sp.* pollen.

pollen proteins could be present in amounts less than the detection limit for this technique (even using silver staining; data not shown). These results did not allow us to distinguish among the different unifloral honeys tested as we could not recognize the differences observed between the types of pollen.

Looking for a more sensitive and specific method to study pollen proteins in honey, we decided to use immunoblot assays, using in this case Sunflower and Eucalyptus pollen. We raised polyclonal antibodies against total proteins of pollen in rabbits, to be used in immunoblot assays to identify pollen proteins in honey.

To evaluate the specificity of the anti-pollen antibodies, immunoblot assays with different pollen extracts were carried out. **Figure 3** shows that anti-Sunflower pollen antibodies bound to specific proteins of diverse molecular weight in Sunflower pollen extracts (**Figure 3**, lane 1), ranging from ~ 84 to ~ 35 kDa, and to a characteristic double band at ~25 kDa. On the other hand, anti-Sunflower pollen antibodies also exhibited minimal cross-reaction with *Prosopis caldenia* (**Figure 3**, lane 2) and *Prosopis sp*. (**Figure 3**, lane 4) pollen, and it did not react against *Celtis tala* or *Eucalyptus sp*. pollen (**Figure 3**, lanes 3 and 5, respectively). It is remarkable that the double band observed at ~25 kDa is present only in sunflower pollen. Thus, these results show high specificity of anti-Sunflower pollen antibodies in recognizing these charasteristic protein bands from Sunflower pollen.

In a similar way, we tested the specificity of anti-Eucalyptus pollen antibodies. **Figure 4** shows that antibodies react with Eucalyptus pollen proteins with different molecular weight

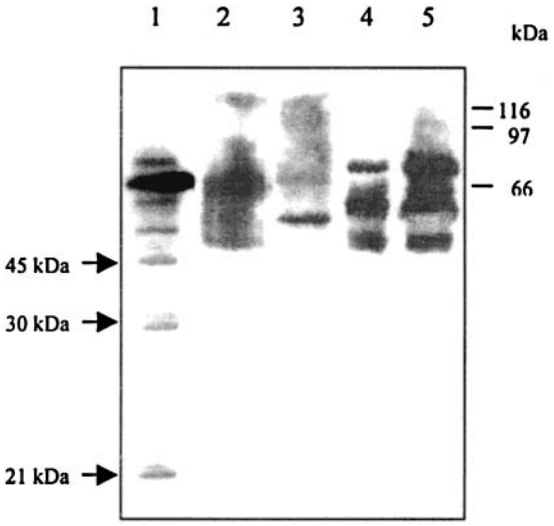

Figure 4. Immunoblot analysis. Pollen extracts were processed by electrophoresis, blotted, and immunodetected with anti-Eucalyptus pollen antibodies diluted 1:1.500. Lane 1, *Eucalyptus sp.* pollen; lane 2, *Prosopis sp.* pollen; lane 3, *Prosopis caldenia* pollen; lane 4, *Celtis tala* pollen; and lane 5, *Helianthus annuus* pollen.

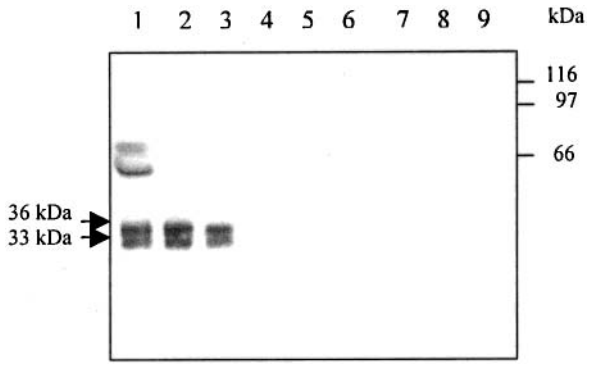

Figure 5. Immunoblot analysis. Unifloral honey protein extracts were processed by electrophoresis, blotted, and immunodetected with anti-Sunflower pollen antibodies diluted 1:7.500. Lane 1, sample 79 (*Helianthus annuus* honey); lane 2, sample 60 (*Helianthus annuus* honey); lane 3, sample 95 (*Melilotus albus* honey); lane 4, sample 103 (*Eucalyptus sp.* honey); lane 5, sample 12 (*Eucalyptus sp.* honey); lane 6, sample 52 (*Prosopis caldenia* honey); lane 7, sample 2 (*Prosopis sp*. honey); lane 8, sample 106 (*Prosopis sp*. honey); and lane 9, sample 10 (*Larrea divaricata* honey).

(ranging from ~120 to ~20 kDa) but shows cross-reaction with the other types of pollen protein extracts analyzed. Despite the cross-reaction, the anti-Eucalyptus antibodies bound to three different Eucalyptus pollen proteins of ~45, 30, and 20 kDa (**Figure 4**, lane 1) which were not recognized in the other types of pollen in this study. So far, these three proteins can be considered as characteristic of Eucalyptus pollen.

To evaluate whether some specific protein of pollen can be found in unifloral honey, we carried out immmunoblot assays with different honey protein extracts. Thus, anti-Sunflower pollen antibodies were probed with Sunflower honey. **Figure 5** shows an immunoblot assay of unifloral honey revealing a double protein band of ~36 and ~33 kDa in Sunflower honey that were not revealed in other unifloral honeys analyzed **(Figure 5**, lanes 1 and 2). In addition to this result, these antibodies bound to the same proteins in Melilotus honey (**Figure 5**, lane 3) which contains 11% of Sunflower pollen in its composition, demonstrating the high sensitivity of the proposed method. On

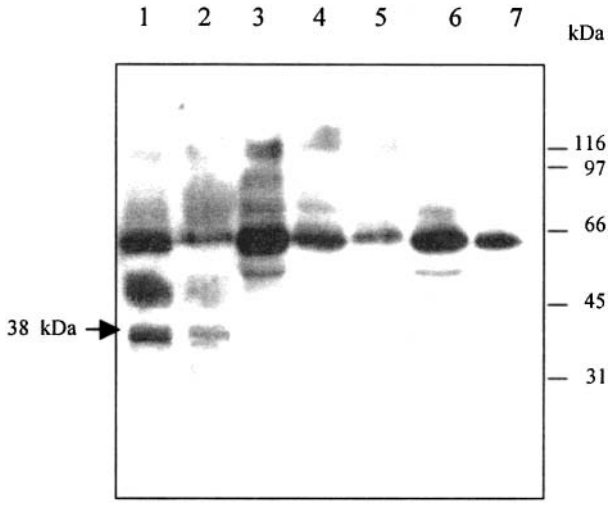

Figure 6. Immunoblot analysis. Unifloral honey protein extracts were processed by electrophoresis, blotted, and immunodetected with anti-Eucalyptus pollen antibodies diluted 1:1.500. Lane 1, sample 12 (*Eucalyptus sp.* honey); lane 2, sample 103 (*Eucalyptus sp.* honey); lane 3, sample 79 (*Helianthus annuus* honey); lane 4, sample 60 (*Helianthus annuus* honey); lane 5, sample 52 (*Prosopis caldenia* honey); lane 6, sample 2 (*Prosopis sp*. honey); and lane 7, sample 95 (*Melilotus albus* honey).

the basis of these results we conclude that these antibodies have high specificity, as they bound only to proteins in honey containing Sunflower pollen.

Likewise we probed anti-Eucalyptus pollen antibodies with Eucalyptus honey. As with pollen extracts, anti-Eucalyptus antibodies showed cross-reaction with honeys from different botanical origins (**Figure 6**). However, we can recognize a single band of ~38 kDa that appears only in honey containing Eucalyptus pollen (**Figure 6**, lanes 1 and 2). Thus, the purified Eucalyptus polyclonal antibodies showed good specificity as they were able to recognize characteristic proteins present only in those honey samples that contained Eucalyptus pollen.

One remarkable fact from this study is the observation that proteins recognized by both polyclonal antibodies in pollen extracts have different molecular weights than those recognized in honey extracts (**Figures 3**−**6**). The discrepancy between the molecular weights observed in pollen and honey extract could be a consequence of the action of honeybee saliva proteolytic enzymes on pollen proteins. This hypothesis is supported by recent work demonstrating that, during the course of bees' work on nectar, the amount of pollen decreases while the enzyme activity increases (*27*, *28*). Thus, it is reasonable to think that protease activity during bee work, and also during honey ripening, could afford protein fragments arising from the original pollen protein. Polyclonal antibodies are able to recognize a number of different epitopes of protein fragments coming from the starting protein (*24*).

Considering all of our results, we remark that the pollen from different plants could be significantly differentiated by means of SDS−PAGE coupled with discriminant analysis. This differentiation is based on the evaluation of the entire protein profile rather than a characteristic protein. In addition to the recognition through the SDS−PAGE profile, it is possible to generate antibodies from pollen proteins which can be used to detect the presence of such proteins, or their pollen proteins fragments, in honey. Therefore, it is reasonable to think of using pollen proteins as markers of the floral origin of honey, especially by using immunoblot assays. Further research is

necessary to expand the scope of the present work to other pollen types, as well as to create a group of antibodies that will let us determine the different percentages of pollen present in honey, to finally obtain in this way, an alternative or complementary method for the botanical characterization of honey.

ABBREVIATIONS USED

SDS−PAGE, Sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Mr, relative mass.

LITERATURE CITED

(1) FAO (Food and Agriculture Organization) FAOSTAT Database 2001 (Accessible through www.fao.org).

(2) Pérez-Arquillué, C.; Conchello, P.; Ariño, A.; Juan, T.; Herrera, A. Physicochemical Attributes and Pollen Spectrum of Some Unifloral Spanish Honey. *Food Chem.* **1995**, *54*, 167−172.

(3) Schmidt, J. O. Bee Products: Chemical Composition and Application. In *Bee Products*; Mizrahi, A., Lensky, Y., Eds.; Plenum Press: New York, 1996; pp 15−26.

(4) Lutier, P. M.; Vaissière, B. E. An Improved Method for Pollen Analysis of Honey. *Rev. Paleobotany Palynology* **1993**, 129−144.

(5) Serra Bonvehi, J.; Ventura Coll, F. Physicochemical Properties, Composition and pollen Spectrum of French Lavender (*Lavandula stoechas* L.) Honey Produced in Spain. *Z. Lebensm.-Unters. Forsch.* **1993**, *196*, 511−517.

(6) Serra Bonvehi, J.; Ventura Coll, F. Characterization of Citrus Honey (*Citrus* Spp.) Produced in Spain. *J. Agric. Food Chem.* **1995**, *43*, 2053−2057.

(7) Sancho, M. T.; Muniategui, S.; Huidobro, J. F.; Simal-Lozano, J. Discriminant Analysis of Pollen Spectra of Basque Country (northern Spain) Honey. *J. Apic. Res.* **1991**, *30*, 162−167.

(8) Krauze, A.; Zalewski, R. I. Classification of Honey by Principal Component Analysis on the Basis of Chemical and Physical Parameters. *Z. Lebensm.-Unters. Forsch.* **1991**, *192*, 19−23.

(9) Peña Crecente, R.; Herrero LaTorre, C. Pattern Recognition Analysis Applied to Classification of Honey from Two Geographic Origins. *J. Agric. Food Chem.* **1993**, *41*, 560−564.

(10) Sancho, M. T.; Muniategui, S.; Huidobro, J. F.; Simal Lozano, J. Aging of Honey. *J. Agric. Food Chem.* **1992**, *40*, 134−138.

(11) Wunderlin, D. A.; Pesce, S. F.; Amé, M. V.; Faye, P. F. The Decomposition of Hydroxymethulfurfural in Solution and the Protective Effect of Fructose. *J. Agric. Food Chem.* **1998**, *46*, 1855−1863.

(12) Ferreres, F.; Andrade, P.; Tomás-Barberán, F. A. Flavonoids from Portuguese Heather Honey. *Z. Lebensm.-Unters. Forsch.* **1994**, *199*, 32−37.

(13) Tomás-Barberán, F. A.; Ferreres, F.; García-Viguera, C.; Tomás-Lorente, F. Flavonoids in Honey of Different Geographical Origin. *Z. Lebensm.-Unters. Forsch.* **1993**, *196*, 38−44.

(14) Ferreres, F.; García-Viguera, C.; Tomás-Lorente, F.; Tomás-Barberán, F. A. Hesperetin: A Marker of the Floral Origin of Citrus Honey. *J. Sci. Food Agric.* **1993**, *61*, 121−123.

(15) D'Arcy, B. R.; Rintoul, G. B.; Rowland, C. Y.; Blackman, A. J. Composition of Australian Honey Extractives. 1. Norisoprenoids, Monoterpenes, and Other Natural Volatiles from Blue Gum (*Eucalyptus leucoxylon*) and Yellow Box (*Eucalyptus melliodora*) Honey. *J. Agric. Food Chem.* **1997**, *45*, 1834−1843.

(16) White, J. W.; Winters, K. Honey Protein as Internal Standard for Stable Carbon Isotope Ratio Detection of Adulteration of Honey. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 907−911.

(17) Bauer, L.; Kohlich, A.; Hirschwehr, R.; Siemann, U.; Ebner, H.; Scheiner, O.; Kraft, D.; Ebner, C. Food Allergy to Honey: Pollen or Bee Products? *J. Allergy Clin. Immunol.* **1996**, *97*, 65−73.

(18) Edelhauser, M.; Bergner, K. G. The Proteins of Honey. Part 9. Honey Sucrase: Isoelectric Focusing and Origin. *Z. Lebensm.-Unters. Forsch.* **1989**, *188*, 237−242.

(19) Rodríguez Otero, J. L.; Paseiro, P.; Simal, J. Intento de Caracterización de las Mieles Naturales de Galicia Mediante las Fracciones Proteicas Separadas por Electroforesis. *Ann. Bromatol.* **1990**, XLII-1, 83−98.

(20) Park, J. W.; Ko, S. H.; Kim, C. W.; Jeoung, B. J.; Hong C. S. Identification and characterization of the major allergen of the *Humulus japonicus* pollen. *Clin. Exp. Allergy* **1999**, *29*, 1080−1086.

(21) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.

(22) Chiabrando, G.; Zalazar, F.; Aldao, M.; Vides, M. A. Rapid and Sensitive Enzyme Immunoassay for low concentration of albumin in human urine. *Clin. Chim. Acta* **1994**, *225*, 155−163.

(23) Yantoro, C.; Vides, M. A.; Vottero de Cima, E. Studies on macromolecular composition of rabbit seminal plasma. *J. Reprod. Fertil.* **1972**, *29*, 229−238.

(24) Tijssen, P. Practice and Theory of Enzyme Immunoassays. In *Laboratory Techniques in Biochemistry and Molecular Biology*; Burdon, R. H., Knippenberg, P. H., Eds.; Elsevier Sciences Publishers: Amsterdam, The Netherlands, 1985; pp 43−121.

(25) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T. *Nature* **1970**, *227*, 680−685.

(26) Wunderlin, D. A.; Díaz, M. P.; Amé, M. V.; Pesce, S. F.; Hued, A. C.; Bistoni, M. A. Pattern Recognition Techniques for the Evaluation of Spatial and Temporal Variations in Water Quality. A Case Study: Suquía River Basin (Córdoba, Argentina). *Water Res.* **2001**, *35*, 2881−2894.

(27) Gonstarki, H.; Hoffman, J. Uber AUFnahme und Verarbentung dez zuckerhaltigen Nahrung bei der Honigbiene. *Z. Bienenforschung* **1962**, *6*, 184−198.

(28) Von der Ohe, W. Unifloral honey: Chemical conversion and pollen reduction. *Grana* **1994**, *33*, 292−294.

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