JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Development of a Competitive ELISA for the Evaluation of Sunflower Pollen in Honey Samples

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We report the development of a rapid, specific, and sensitive enzyme-linked immunoassay (ELISA) for the evaluation of sunflower pollen in honey as a method alternative to melissopalynology, which is considered the standard technique for the evaluation of floral origin of honey. Two 33−36 kDa proteins, identified as characteristic of sunflower pollen, were isolated and used as coating antigens in the competitive ELISA. We verified its analytical performance by evaluating reproducibility, specificity, and exactitude in relation to melissopalynology. The competitive ELISA developed during this work is able to quantify sunflower pollen in honey, with a detection limit of 10%, showing linear response between 10 and 90%. The method afforded low cross reactivity with honey from other floral origin, thus evidencing an adequate selectivity. We also observed a significant correlation ($r = 0.975$; $p < 0.001$) when the proposed ELISA was referenced to melissopalynology. Hence, we conclude that the competitive ELISA constitutes a valuable and feasible alternative for authentication of sunflower honey. This work opens the possibility to develop similar assays for other pollen types.

KEYWORDS: Honey; floral origin; melissopalynology; proteins; ELISA

INTRODUCTION

The botanical origin of honey determines several properties of honey, such as color, flavor, and chemical composition. Thus, the origin of honey establishes its price to a significant proportion. Floral origin is routinely authenticated by melissopalynology (*1*). However, this method shows various limitations depending on the beekeeping techniques, size of pollen grains, and need of a complete pollen library for comparison. Achieving a good qualitative and quantitative result by melissopalynology is time-consuming as well as dependent on the ability and judgment of experts performing the analysis (*2*).

In recent years, alternatives to pollen analyses have been developed to characterize honey more widely and accurately. Some of these alternative methods involve measurements of parameters associated with physical and chemical characteristics of honey (pH, acidity, moisture, HMF, diastase activity, sugar profile, etc.). These parameters also allow the assessment of the geographical or floral origin of honey, when combined with statistical techniques (*3*−*6*). Other authors have looked for honey classification through the use of chemical markers such as flavonoids (*7*−*10*) or through the analysis of natural volatile compounds in honey (*11*−*14*).

Recently, we reported that pollen proteins can be used as chemical markers of the floral origin of honey (*15*). Considering that honeybee proteins should be common to all types of honey (*16*), we demonstrated the usefulness of pollen proteins for honey floral classification. Pollen from different plants was significantly differentiated by means of SDS−PAGE coupled with discriminant analysis. By immunoblot techniques, using antibodies raised in rabbits against pollen protein extracts, we were able to detect sunflower pollen proteins in unifloral sunflower honey. These proteins were characterized as a double band of 33−36 kDa, which were undetected in other unifloral honey (*15*).

In the present work, our starting hypothesis was that the level of 33−36 kDa proteins, contained in honey, may be representative of its floral origin. Therefore, herein we describe the development of a competitive ELISA to quantify 33−36 kDa proteins in honey. To achieve this purpose, these proteins were partially isolated from unifloral sunflower honey by chromatographic procedures. The competitive ELISA was tested and

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10.1021/jf049068e CCC: $27.50
Published on Web 11/02/2004

optimized by using honey having different percentages of sunflower pollen. Results obtained were quantitatively compared with melissopalynology.

MATERIALS AND METHODS

Protein Extracts and Polyclonal Antibodies. Honey protein extracts, and polyclonal antibodies against sunflower pollen proteins, were obtained as previously reported (*15*).

Honey Protein Isolation by Anion Exchange Chromatography. The 33−36 kDa proteins were partially isolated by anion exchange chromatography using a FPLC system (Amersham Bioscience, Sweden). An extract of sunflower honey proteins (500 μg) was loaded on Mono Q HR 5/5 column (1 mL), equilibrated with 30 mL buffer Tris 20 mM, pH 7.5 (buffer A). The column was then washed with 10 mL of buffer A. Thereafter, proteins were eluted with 20 mL using a linear gradient from 0 to 100% NaCl 0.5M in buffer A. Proteins were eluted at a flow rate of 60 mL/h and monitored at 280 nm. Fractions (0.5 mL) were collected and analyzed for the presence of 33−36 kDa proteins by immunoblot using antisunflower pollen antibodies. Positive fractions for 33−36 kDa proteins were pooled, and its protein concentration was determined according to the method of Bradford (*17*). These fractions were used as coating antigen for the ELISA procedure.

ELISA: Development and Evaluation. General Protocol. Microtiter plates were coated with the coating antigen diluted with Tris 20 mM, NaCl 0.5M, pH 7.5 (100 μL/well) and incubated overnight at 4 °C. Afterward, plates were washed three times with phosphate saline buffer containing Tween 20 (PBST buffer: Na_2HPO_4 10 mM, KH_2PO_4 3 mM, NaCl 120 mM, pH 7.5; Tween 20 0.05%). Subsequently, antisunflower pollen antibodies (diluted in PBST buffer) were added. For noncompetitive assays, we used 150 μL/well of the antibodies solution, whereas competitive ELISA requires 100 μL/well antibodies and a 50 μL sample (diluted in PBST) per well. After the addition of antibodies and sample, plates were incubated for 3 h at room temperature (23 ± 2 °C) and washed three times with PBST buffer. Next, 150 μL of antirabbit IgG-HRP (1/10 000 in PBST) was added to each well and incubated for 3 h at room temperature. Then, plates were washed three times with PBST buffer. Color development is achieved by adding an *o*-phenylendiamine solution (200 μL/well of a solution containing 20 g/L in buffer citrate: citric acid 0.4 M, Na_2HPO_4 0.05 M, pH 5). After 15 min in darkness at room temperature, color development was stopped by adding H_2SO_4 1 M (100 μL/well). Color development was measured at 490 nm using a microtiter plate reader (Model 550 Microplate Reader, Bio Rad).

Procedure Optimization. To obtain the optimal coating antigen/antibodies ratio, we evaluate different combinations of coating antigen (25 and 50 ng) and antibodies dilutions (1/100 to 1/3000) by noncompetitive ELISA. Next, we standardize the competitive ELISA using standard competitive antigens (SCA). SCA were honey samples containing 90, 45, and 11% of sunflower pollen as assayed by melissopalynology in according to Loveaux (*1*). On the other hand, as a negative control, we used honey that did not contain sunflower pollen, which was used to determine the unspecific competition of the antigen/antibodies bond. Both SCA and negative control were diluted using PBST buffer. Different amounts of SCA and negative control (100 and 250 ng of honey protein per well) were assayed. We also evaluated different conditions of temperature and preincubation time for antibodies and antigens. Therefore, antibodies and competitive antigens were added to the microtiter plate either immediately or after a pre-incubation step (30 min at 37 °C or 16 h at 4 °C). Results are expressed as inhibition percentage, calculated from eq 1, and plotted versus percentage of sunflower pollen.

$$\left(1 - \frac{\text{absorbance with competitor}}{\text{absorbance of control}}\right)100 \qquad (1)$$

Optimized Competitive ELISA. Microtiter plates were coated with 25 ng of coating antigen (100 μL/well) in Tris 20 mM, NaCl 0.5 M, pH 7.5 and incubated overnight at 4 °C. Thereafter, coated plates were washed three times with PBST (300 μL/well). Then, 100 ng of either SCA or unknown honey sample diluted in PBST (50 μL/well) and polyclonal antibodies (100 μL/well of a solution 1/600 in PBST) were added and incubated 3 h at room temperature (23 ± 2 °C). Finally, the antirabbit IgG-HRP and color development steps were processed as described previously.

Comparison between Competitive ELISA and Melissopalynology. Protein extracts from six different unknown honey samples (100 ng protein/well) were analyzed by the optimized competitive ELISA. A calibration curve was constructed using a SCA (containing 90% of sunflower pollen) diluted with PBST. This curve was used to determine the percentage of sunflower pollen present in each unknown honey. Unknown honey samples were also analyzed by melissopalynology according to Loveaux (*1*). Results obtained by both methods were statistically analyzed by linear regression analysis.

Specificity of Antipollen Antibodies. To assess the cross-reactivity of polyclonal antibodies used, we tested protein extracts, from honey of different floral origin, by competitive ELISA. Honey samples were previously evaluated by melissopalynology and determined as unifloral. IC_{50} (50% inhibition of control) for each honey (20−300 ng of honey protein/well) was determined in competitive experiments, following the optimized protocol described previously. The cross-reactivity (CR) was calculated according to eq 2.

$$CR = \frac{IC_{50}(\text{sunflower honey})}{IC_{50}(\text{honey from different origin})} \times 100 \qquad (2)$$

RESULTS AND DISCUSSION

Honey Protein Isolation by Anion Exchange Chromatography. We have previously demonstrated that a double protein band (33−36 kDa) was characteristic of sunflower honey (*15*). We hypothesized that the quantity of these proteins in honey could be representative of the percentage of sunflower pollen in honey. To demonstrate this hypothesis, we developed a competitive ELISA, looking to quantify sunflower pollen proteins in honey. To use 33−36 kDa proteins as coating antigen, we partially isolated these proteins from sunflower honey by anion exchange chromatography, using a FPLC system. Sunflower honey protein extract afford an elution profile, which is shown in **Figure 1A**. Each fraction was analyzed by immunoblot to test for the presence of 33−36 kDa proteins, characteristic of sunflower pollen (*15*). **Figure 1B** shows that 33−36 kDa proteins were eluted at 19−21% of buffer gradient, which corresponds to a NaCl concentration of 90−100 mM (fraction 30 and 31). These fractions were collected, pooled, and used as coating antigen. From **Figure 1**, it is evident that only a little fraction of the whole honey protein extract contains 33−36 kDa proteins, while the rest correspond to honeybee proteins as well as other unspecific plant proteins. This result is in accordance with our previous report, where we had demonstrated that honeybee proteins are majority within extracts of different honey samples (*15*). However, trace proteins, like the 33−36 kDa isolated here, provide the opportunity and the challenge to develop powerful tools for food authentication (*18*).

Standardization of ELISA Procedure. Previously, we had characterized a polyclonal antibody against sunflower pollen proteins as having high affinity and specificity for 33−36 kDa proteins (*15*). Herein, to obtain the highest antibody affinity, specificity, as well as the lowest unspecific background, different antigen/antibodies ratios were tested by noncompetitive ELISA. **Figure 2** illustrates the reactivity of different dilutions of these antibodies, when tested against two different concentrations (25 and 50 ng) of the coating antigen. We selected the optimal antigen/antibodies ratio considering the combination that gave an absorbance close to 1.0 unit as well as the lowest background. Considering these conditions, we assume that the major

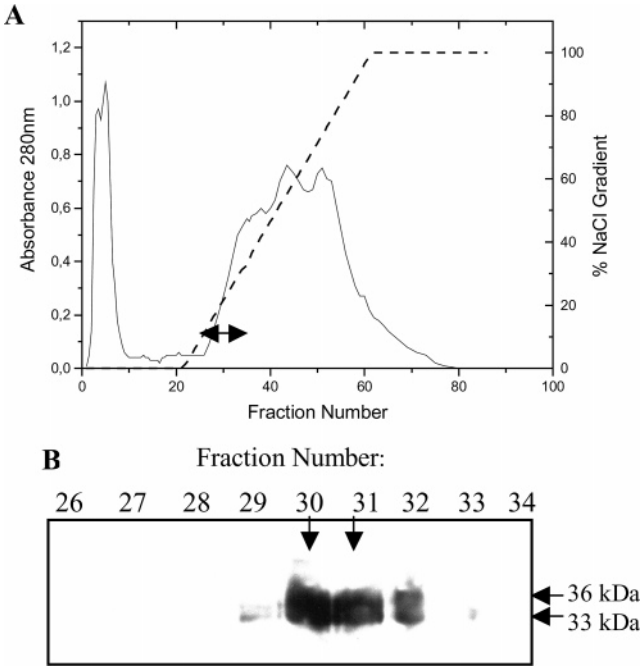

Figure 1. Isolation of 33–36 kDa proteins: (**A**) Anion exchange chromatography of a protein extract of sunflower honey. Protein elution was monitored at 280 nm (solid thick line); horizontal arrows indicate fractions shown in panel **B**. (**B**) Immunoblot analysis. Fractions separated by SDS–PAGE 12%, blotted, and immunodetected with anti-sunflower pollen antibodies diluted 1:7500. Arrows indicate fractions selected due to the presence of 33–36 kDa proteins.

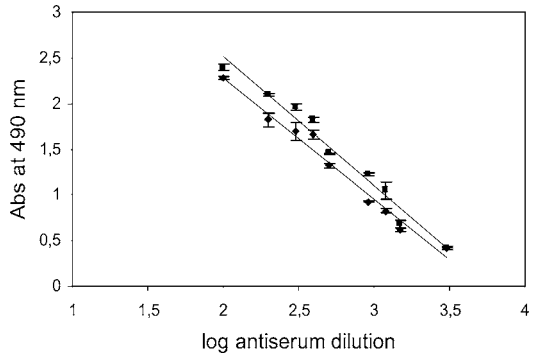

Figure 2. Titration curves to evaluate optimal antigen/antibodies ratio. Microplates were coated with 25 ng (◆) and 50 ng (■) of coating antigen antigen (33–36 kDa proteins). Antibodies dilution ranged from 1:100 to 1:3000 in PBST buffer.

sensitivity of antigen/antibodies couple was obtained when 25 ng of coating antigen and 1:900 dilution of polyclonal antibodies were used.

Development of Competitive ELISA. The standardized ELISA procedure was optimized by assaying 100 ng (**Figure 3A**) or 250 ng (**Figure 3B**) per well of either negative control or SCA containing 90, 45, or 11% of sunflower pollen using different incubation conditions. **Figure 3A,B** shows line plots relating the percentage of inhibition with the percentage of sunflower pollen present in SCA. Each plot presents three results, corresponding to different temperatures and incubation time. From **Figure 3A**,**B**, we conclude that preincubation affords the highest background signal (>30% of inhibition) without significant changes in sensitivity with respect to tests carried out without preincubation. The better analytical condition was observed when using 100 ng competitive antigens (SCA or negative controls) and polyclonal antibodies (diluted 1:900) added immediately to the microtiter plate without preincubation (**Figure 3A**).

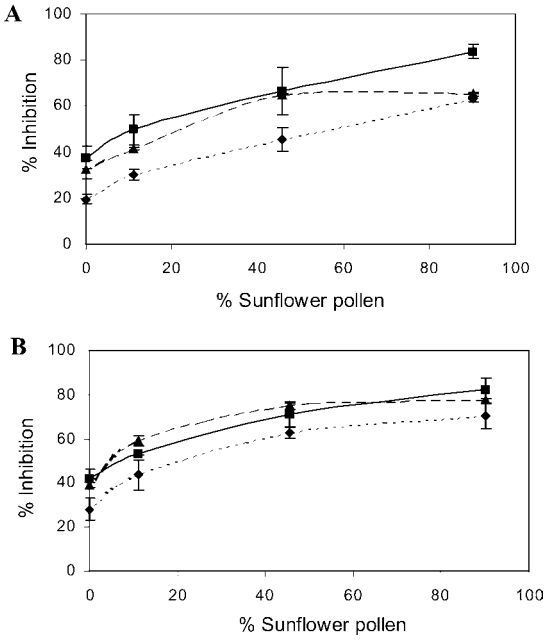

Figure 3. Optimization of competitive ELISA. Inhibition plots were constructed using different amounts of competitive antigens: 100 ng (**A**) and 250 ng (**B**) at three different incubation conditions (■) 16 h at 4 °C; (▲) 30 min at 37 °C; and (◆) without preincubation.

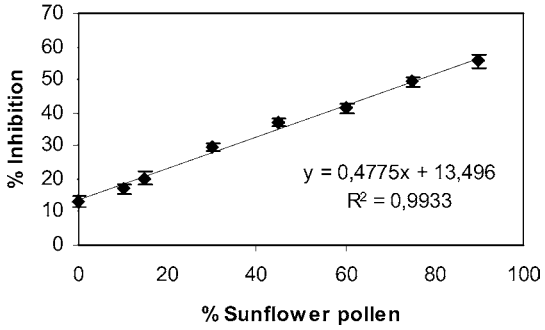

Figure 4. Calibration curve for the evaluation of sunflower pollen by competitive ELISA. The straight line indicates the percentage of inhibition obtained by ELISA, plotted vs the percentage of sunflower pollen present in honey solutions (prepared from honey containing 90% sunflower pollen as evaluated by mellisopalynology). Each point represents the mean ± one standard deviation from five determinations.

To quantify the percentage of sunflower pollen present in honey samples, we constructed a calibration curve, applying the optimized competitive ELISA, using successive dilutions of a SCA containing 90% sunflower pollen (evaluated by melissopalynology). **Figure 4** shows that the optimized ELISA renders a linear inhibitory response from 10 to 90% of sunflower pollen. Negative controls (containing 0% of sunflower pollen) show an unspecific inhibition of ≈13% (**Figure 4**). Nevertheless, the inhibition percentage observed for 10% sunflower pollen is significantly different with respect to negative controls. Thus, we assume that 10% of sunflower pollen in honey is the detection limit for the optimized ELISA.

The reproducibility of the assay was determined by comparing calibration curves on successive days, with further evaluation of the relative imprecision expressed as the coefficient of variation (CV%) at different points of the calibration curve. CV% obtained were ≈14% ($n = 5$) for negative control (unspecific competition), whereas for sunflower honey contain-

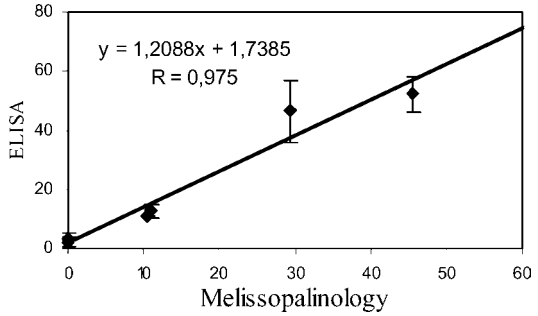

Figure 5. Correlation between percentages of sunflower pollen in honey determined by both melissopalinology and competitive ELISA. Each point represents the mean ± one standard deviation from five determinations.

Table 1. Response of the Competitive ELISA When Tested with Different Unifloral Honey Samples as Competitive Antigens

honey sample	IC_{50} (ng/well)	% cross-rectivity[a]
H. annuus	43	100
L. divaricata	147	29.25
P. caldenia	237	18.14

[a] Percentage of cross-reactivity calculated according to the following formula: %CR = (IC_{50} of sunflower honey/IC_{50} of honey of other floral origin) × 100.

ing 10−15% and 30−90% of sunflower pollen, the CV% was less than 9 and 4%, respectively. This is especially valuable to differentiate between unifloral (≥45% of pollen) and multifloral (<45% of pollen) honey samples.

Comparative Analysis of ELISA versus Melissopalynology. To evaluate the effectiveness of the proposed assay, it was necessary to compare it with the standard melissopalynology. Thus, we analyzed six unknown honey samples by both methods. **Figure 5** shows results obtained from a comparative analysis. **Figure 5** indicates that the developed ELISA has significant correlation ($R > 0.975$, $p < 0.001$) with results obtained by melissopalynology.

Specificity. We evaluate the specificity of the competitive ELISA by testing unifloral honey from different floral origin: *Larrea divaricata*, *Prosopis caldenia*, and *Helianthus annuus* (sunflower). Results are summarized in **Table 1**, which shows IC_{50} and relative cross reactivity (CR). As showed in **Table 1**, *L. divaricata* and *P. caldenia* honey need 3.4- and 5.5-fold the amount of protein than *H. annuus* to yield the same IC_{50}. In terms of cross-reactivity, these values are equivalent to 29 and 18%, respectively.

Immunochemical techniques such as the proposed ELISA have the advantages of being sensitive, specific, and often provide the necessary limit of detection to analyze directly low concentrations of the target protein. In addition, these techniques have advantages over many current classical methods in speed and cost analysis (*19*−*21*).

The developed competitive ELISA allows us both to identify and to quantify sunflower pollen in honey, when it is present over 10% with an adequate linear response between 10 and 90% of pollen. It shows selectivity and specificity to detect sunflower pollen as well as a significant correlation with the standard melissopalynology. Furthermore, competitive ELISA results show good precision in the evaluation of pollen quantity (CV% ranged from 4 to 14%). Alhough the method is less sensitive than melissopalynology, it presents the advantage of analyzing simultaneously several samples in a reduced time. In addition, ELISA results are less dependent on the operator expertise, while melissopalynology is considerably influenced by the judgment ability of the analyst. Moreover, to the extent of our knowledge, this is the only method, alternative to melissopalynology, which allows quantifying the percentage of sunflower pollen present in a honey sample.

These results open an interesting field to develop similar assays for other types of pollen, which could lead to rapid and specific methods alternative to melissopalynology, providing precise results.

ABBREVIATIONS USED

ELISA, enzyme-linked immunoassay; FPLC, fast pressure liquid chromatography; PBST, phosphate saline buffer containing Tween 20; IgG-HRP, goat IgG anti-rabbit horseradish peroxidase conjugate; SCA, standard competitive antigens; CR, cross-reactivity; CV%, coefficient of variation.

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Received for review June 9, 2004. Revised manuscript received September 14, 2004. Accepted September 15, 2004. We thank CONICET (National Research Council−Argentina), as well as Secretaria de Ciencia y Técnica−Universidad Nacional de Córdoba, for fellowship and financial support.

JF049068E