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ARTICLE

# New Criterion for Evaluation of Honey: Quantification of Royal Jelly Protein Apalbumin 1 in Honey by ELISA

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The 55 kDa major protein of royal jelly, named apalbumin 1, is an authentic protein of honey and pollen pellet, and for its quantification an enzyme-linked immunosorbent assay (ELISA) was developed using specific polyclonal anti-apalbumin 1 antibody. The limit of detection for apalbumin 1 was 2 ng $mL^{-1}$. The floral honeys contained apalbumin 1 as follows: acacia, 0.011%; linden, 0.010%; chestnut, 0.029%; rape, 0.010%; and dandelion, 0.014%. The saccharose syrup honey contained only 0.001% of apalbumin 1. The average amount of apalbumin 1 relating to the total protein content of analyzed honey samples was 23.39%, whereas in SCCH apalbumin 1 presented only 4.81% of total proteins of honey. Apalbumin 1 is thermostabile in honey at 80 °C incubated for 40 min. ELISA results show good precision in the evaluation of apalbumin 1 quantity in honey (CV ranged from 0.69 to 4.25%).

**KEYWORDS: Honey; apalbumin1; royal jelly; ELISA; adulteration**

## INTRODUCTION

The authenticity of honey and other honeybee products has long been evaluated on the basis of properties that can be defined by chemical, physical, and instrumental analyses. Nowadays, it is generally understood that the quality of honeybee products should be defined in terms of physiological functions of their authentic components, first of all, proteins.

The proteins are besides sugars and water the third most important honey component. Proteins as high molecular weight compounds of honey originate from nectar, pollen, and secretions of cephalic glands of honeybees. The protein content of honey varies from 0.05 to 0.79% (*1*−*4*). Preliminary screening of honeys by SDS-PAGE (*5*) and protein profiles of floral and honeydew honeys by liquid chromatography (*6*) showed heterogeneous composition, but there are not sufficient data about the identity and quantity of the honey proteins. Recently, to assess a floral origin of honey (*7*), an ELISA was used for quantification of 33−36 kDa proteins of sunflower pollen in honey as an alternative method to the standard melissopalynological analysis. Other authors attempted to classify honeys by chemical markers such as flavonoids (*8*).

The most specific constituents of honey are proteins of honeybee origin. Attention has to be mainly paid to enzymes converting components of nectar to honey. The identified honey proteins until now are enzymes related to carbohydrate metabolism such as α-glucosidase (*9*), glucose oxidase (*10*), β-glucosidase (*11*), and amylase (*12*). The second important group of authentic honeybee proteins in honey is royal jelly (RJ) proteins (*13*−*19*). We have found that the major protein of RJ, named apalbumin 1, is the dominant protein of honey (*20*). It was found that apalbumin 1 (*20*) stimulated secretion of cytokine TNF-α by murine macrophages, whereas the deproteinized honey had no effect on the release of TNF-α (*21*). In addition, apalbumin 1 enhances the proliferation of hepatocytes (*22*), and the C-terminus of apalbumin 1 may be also a precursor form of the antimicrobial peptides jelleines (*23*). Apalbumin 1 is a multifunctional glycoprotein and in RJ is present as a monomer (55 kDa), in oligomeric form (approximately 420 kDa), and in water-insoluble aggregates formed by interaction with fatty acids. Microscopic observations showed that apalbumin 1 forms in aqueous solutions of honey self-assembled membranes similar to those occurring in RJ (*14*) that provide an improved evaporation of water during processing of nectar to honey. Formation of filamentous spider weblike networks upon immobilization of the flower pollen dust and formation of pollen pellet predestine apalbumin 1 as an important factor participating in the processing of honey and honeybee pollen. In light of these data we propose that the quantification of apalbumin 1 could be used as a criterion for the evaluation of authenticity of honeybee products.

The aim of this work was to develop an immunochemical method for the quantification of major RJ protein apalbumin 1 in honeys of different botanical and geographical origin as a tool for preliminary testing of honey authenticity. The presented ELISA has a potentially wide application not only for detection of honey adulteration by low-cost syrups but also for quantification of apalbumin 1 in other honeybee products such as bee pollen and RJ and for determination of RJ content of various preparations used in food products, apitherapy, cosmetics, and pharmacy.

## MATERIALS AND METHODS

**Royal Jelly.** Honeybee (*Apis mellifera carnica*) RJ was collected 48 h after obtaining 1-day-old larvae from A. Kostrian apiary, Slovakia, and stored at −20 °C. The RJ was diluted in water 1:10 (w/v), stirred at room

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temperature for 30 min, and centrifuged at 15000*g* at 4 °C for 20 min. For experiments, the supernatant, as water-soluble RJ proteins, was used.

**Honeybee Pollen Pellet Extraction.** Honeybee rapeseed (*Brassica napus*) pollen pellet in water at a concentration of 100 mg $mL^{-1}$ was homogenizated by vortexing for 5 min at 20 °C. After centrifugation (14000*g* for 30 min), the supernatant was filtered through an 0.8 $\mu$m membrane filter (Millipore Corp.) and concentrated by Microsep centrifugal column of molecular weight cutoff (MWCO) 3 kDa (Pall Life Sciences).

**Nectar Collection.** Nectar collected from acacia (*Robinia pseudoacacia*) and linden (*Tilia* spp.) flowers was concentrated two times by Microsep centrifugal column, MWCO 3 kDa (Pall Life Sciences), and stored at −20 °C prior to use.

**Monofloral Honeys.** Samples of honeys from acacia (*R. pseudoacacia*), linden (*Tilia* spp.), rapeseed (*B. napus*), dandelion (*Taraxacum officinale*), and chestnut (*Castanea sativa*) from CRA, Unità di Ricerca di Apicoltura e Bachicoltura, Bologna, Italy and Slovakia (2008 season) were stored at 18 °C in the dark before use. The samples for analysis were prepared by vortexing of honey with water (1:1, w/v, Milli-Q, Millipore) for 5 min at room temperature. Pollen-free honeys were prepared by filtration of diluted samples through an 0.8 $\mu$m membrane filter (Millipore Corp.). The floral origin of honey was confirmed by standard melissopalynological analysis.

**Saccharose Syrup Honey (SCCH).** A honey chamber of a bee colony was equipped with two new empty combs and fed 1 L of saccharose syrup (saccharose/water 1:1, w/v) for 3 days during floral dearth in October 2007. The SCCH was then harvested from the new combs and stored at 18 °C in the dark.

**Preparation of Artificial Dandelion Honey.** About 250 pieces of dandelion blooms were put into 800 mL of water and boiled for 10 min. After cooling, the mixture was left overnight at room temperature, then filtered and supplemented with 1.5 kg of saccharose and the juice from two lemons. The mixture was boiled for about 35 min under permanent agitation until a viscous consistency was obtained. The dandelion artificial honey was stored at 18 °C in dark.

**Standard and Antibodies.** Apalbumin 1, as a standard protein, was prepared by ultracentrifugation of RJ (*14*), freeze-dried, and stored at −20 °C. Polyclonal antibody against apalbumin 1 (anti-apa1 antibody) was obtained by immunization of rabbits with a recombinant apalbumin 1 (*25*) and stored at −20 °C. Peroxidase-conjugated anti-rabbit IgG (SwAR Px) was obtained from Institute of Sera and Vaccines (Prague, Czech Republic).

**Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blot (WB) Analysis.** Honey proteins were separated by 12% SDS-PAGE (*24*) and then stained by Coomassie Brilliant Blue or (for WB analysis) electroblotted onto a 0.45 $\mu$m NC-membrane (Macherey-Nagel, GmbH & Co. KG, Duren, Germany). Apalbumin 1 was detected using specific polyclonal anti-apa1 antibody as was described previously (*16*, *20*, *25*).

**Determination of Total Protein.** The protein content of the samples was determined by microplate microassay according to the Bradford method (*26*). To 100 $\mu$L of the sample or its dilution was added 100 $\mu$L of Quick Start Bradford reagent (Bio-Rad, Laboratories, Inc.). After incubation of the microplate at room temperature for 15 min, the absorbance was measured at 595 nm on Microplate spectrophotometer, PowerWaveXS (BioTek Instruments, Inc.). Bovine serum albumin (BSA; Sigma) was used as standard.

**Optimization of ELISA.** Conditions for the method were performed initially to determine the optimum dilution of antibodies and antigen, optimum incubation period, washing buffers, and washing steps. Several buffers were tested for washing plates: phosphate buffer saline (PBS; 137 mM NaCl, 2.7 mM KCl, 100 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4), PBS-T (PBS with 0.05% Tween 20), Tris buffer saline (TBS; 100 mM Tris and 150 mM NaCl, pH 7.5), and TBS-T (TBS with 0.05% Tween 20). A calibration curve from 0 to 500 ng $mL^{-1}$ of apalbumin 1 was tested in the 40% glucose and 40% fructose water solution (G/F) at different dilutions (40%, 8%, 0.8%, 0.08%, 0.008%, 0.0008%, 0.00008%) corresponding to total sugar content of assayed honey samples (50, 10, 1, 0.1, 0.01, 0.001, and 0.0001%). Control analysis was performed (1) without antigen (apalbumin1) and without (primary and secondary) antibodies, (2) without antigen (apalbumin 1) and with antibodies, (3) with antigen (standard solution of apalbumin 1, 200 ng $mL^{-1}$) and without antibodies, for each dilution of G/F solution.

**Determination of Apalbumin 1 in Honeys by ELISA.** The 96-well/flat-bottom microtiter plates (Brand, Germany) were coated with 100 $\mu$L/well of antigen-diluted honey samples (0.05 and/or 0.001%) in Milli-Q water and/or standard solution of apalbumin 1 in G/F solution (0.04 and/or 0.008%). The plates were incubated overnight at 4 °C, then washed five times with TBS buffer and blocked with milk buffer (2% nonfat milk in TBS, 400 $\mu$L/well) for 1 h at room temperature. The plates were then incubated with polyclonal rabbit anti-apa1 antibody (*25*) diluted 1:2000 in milk buffer (100 $\mu$L/well) for 2 h, washed five times with TBS, and incubated with peroxidase-conjugated anti-rabbit IgG diluted 1:5000 in milk buffer (100 $\mu$L/well) for 1 h. The plates were finally washed 10 times with TBS. Detection was done by adding 100 $\mu$L/well of 3% 2,2′-azinobis(3-benzthiazoline-6-sulfonic acid (ABTS; Southern Biotech) in 50 mM citrate buffer, pH 4.3, supplemented by hydrogen peroxide (0.03%). After incubation for 15 min in darkness at room temperature, color development was stopped by adding 100 $\mu$L/well of ABTS Peroxidase Stop Solution (1% SDS), and the absorbance at 405 nm was read in a Microplate Spectrophotometer PowerWave XS (BioTek Instruments).

**Thermal Stability of Apalbumin 1.** Acacia honey (0.5 g) was incubated separately at 30, 60, 80, and 100 °C for 40 min at 450 rpm in a Thermomixer comfort 5355 (Eppendorf AG). The 40% glucose and 40% fructose water solution with and without addition of apalbumin 1 at a concentration of 200 $\mu$g $mL^{-1}$ was assayed as control sample. The samples were then diluted 1:1 (w/v) in Milli-Q water and checked for stability of the proteins by 12% SDS-PAGE and by WB analysis using anti-apa1 antibody.

**Data Processing and Statistic.** For the ELISA, fitting of the curves was performed with Gen5 software (BioTek). Statistical data evaluation was carried out with Microsoft Office Professional 2007.

## RESULTS AND DISCUSSION

**Apalbumin 1 as Authentic Protein of Honey and Pollen Pellet.** The common feature of RJ and honey samples analyzed by SDS-PAGE (**Figure 1A**, lanes 2 and 4−9) was the presence of protein

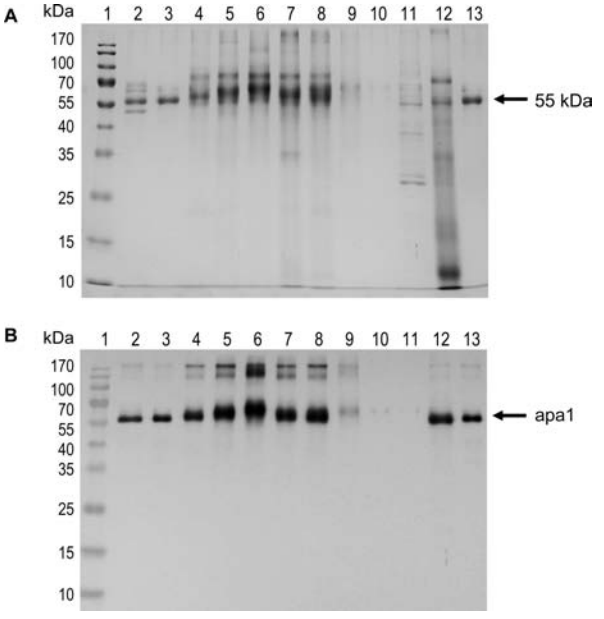


**Figure 1.** Electrophoretic profile of some honeys, nectar, and pollen pellet and immunochemical detection of apalbumin 1 (apa1) in the samples. (**A**) 12% SDS-PAGE, Coomassie Brilliant Blue staining. Lanes: 1, protein MW marker; 2, royal jelly (1 mg $mL^{-1}$); 3, apa1 (0.5 mg $mL^{-1}$); 4−9, 50% honey samples (acacia, linden, chestnut, rapeseed, dandelion, and saccharose syrup honey); 10, 50% artificial dandelion honey; 11, acacia nectar (concentrated 2 times); 12, pollen pellet extract (50 mg $mL^{-1}$); 13, apa1 (0.5 mg $mL^{-1}$). (**B**) Western blot analysis using polyclonal rabbit anti-apa1 antibody. Sample order is the same as in **A**, but diluted 10 times, except acacia nectar and artificial dandelion honey, which were at the same concentration as in **A**.

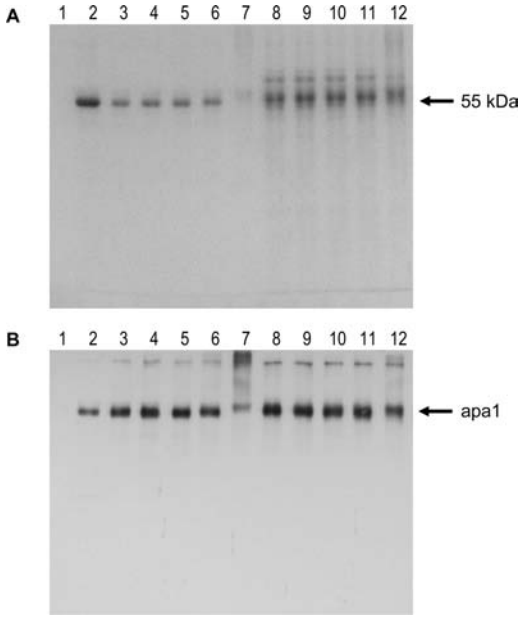


**Figure 2.** Thermal stability of apalbumin 1 (apa1) in honey and in G/F solution. (**A**) 12% SDS-PAGE, Coomassie Brilliant Blue staining. Lanes: 1, 40% G/F solution; 2, apa1 (0.5 mg $mL^{-1}$); 3, apa1 (0.2 mg $mL^{-1}$) in 40% G/F solution without incubation; 4−7, apa1 (0.2 mg $mL^{-1}$) in G/F solution after incubation at 30, 60, 80, and 100 °C, respectively; 8, 50% acacia honey; 9−12, 50% acacia honey after incubation at 30, 60, 80, and 100 °C. (**B**) Western blot analysis using polyclonal rabbit anti-apa1 antibody. The samples order is the same as in **A**, but diluted 10 times.

bands in range from 50 to 70 kDa with dominance of a 55 kDa protein corresponding to the major protein of RJ, apalbumin 1 (**Figures 1A** and **2B**, lanes 3 and 13). The protein patterns of SDS-PAGE of analyzed samples showed significant differences in protein profile between floral honeys (**Figure 1A**, lanes 4−8) and SCCH (**Figure 1A**, lane 9). The protein pattern of concentrated acacia nectar (**Figure 1A**, lane 11) varies widely from that of honey samples, whereas the presence of a 55 kDa protein band is apparent. The artificial dandelion honey showed only indication of protein bands (**Figure 1A**, lane 10).

Western blot analysis showed that the RJ, honey samples, and honeybee pollen pellet contained the 55 kDa protein, immunoactive with polyclonal anti-apa1 antibody (**Figure 1B**). A comparison of the intensity of the bands on WB membrane of the honey samples with one other showed variable contents of apalumin 1 in floral honeys (**Figure 1B**, lanes 4−8) and minute band of apalbumin 1 in SCCH honey (**Figure 1B**, lane 9). The presence of two protein bands in the range from 150 to 170 kDa in honeys immunoactive with anti-apa1 antibody can be explained by the presence of aggregated forms of apalbumin 1. The proteins of concentrated acacia nectar (**Figure 1B**, lane 11) and tilia nectar (not shown), as well as artificial dandelion honey (**Figure 1B**, lane 10), showed no immunochemical activity to polyclonal anti-apa1 antibody on WB analysis, even if the protein bands are clearly visible on Coomassie Brilliant Blue staining of SDS-PAGE (**Figure 1A**, lane 11) at the same concentrations of the samples.

These data confirmed RJ protein apalbumin 1 as an authentic honeybee protein of honey and pollen kit and showed it as a suitable marker for evaluation of honeybee products.

**Thermal Stability of Apalbumin 1.** The determining factor of applicability of a particular protein as a marker incorporated to evaluation of food quality is its thermal stability. Apalbumin 1 as a component of honey during harvesting, storage, and industrial processing is exposed to various temperatures. For investigation of the effect of temperature on the stability of apalbumin 1 in honey, the samples (acacia, chestnut, linden, rape, and dandelion honey, respectively) were heated at 30, 60, 80, and 100 °C, respectively, for 40 min. Apalbumin 1 (200 $\mu$g $mL^{-1}$) in water solution of 40% glucose and 40% fructose was used as a control. The samples afterward were dissolved in water 1:1 (w/v) and used for SDS-PAGE. Apalbumin 1 in G/F solution heated at 30, 60, and 80 °C (**Figure 2A**, lanes 4−6) was observed as a single band like in unheated control sample (**Figure 2A**, lane 3). The substantial part of apalbumin 1 heated to 100 °C in G/F solution for 40 min was degraded (**Figure 2B**, lane 7). The protein patterns of acacia honey after heating at 30, 60, and 80 °C (**Figure 2A**, lanes 9−11) were not changed in comparison with the unheated control sample (**Figure 2A**, lane 9). After 40 min of incubation of the acacia honey at 100 °C, there was observed some partial protein degradation (**Figure 2A**, lane 12).

On the other hand, the WB analysis of apalbumin 1 in G/F and in acacia honey after thermal treatment did not show degradation products of the protein (**Figure 2B**, lanes 4−6 and 9−11). Only after heating of apalbumin 1 in G/F solution at 100 °C for 40 min was the protein aggregated to the high molecular weight oligomeric forms (**Figure 2B**, lane 7). In the case of acacia honey heating at 100 °C for 40 min (**Figure 2B**, lane 12) decreased protein intensity only, but the presence of oligomeric structures was not so significant in comparison to that in G/F solution. These results can be explained by the higher stability of apalbumin 1 in honey, rather than in G/F solution, even after thermal treatment, probably as a consequence of protein−protein interactions present in honey. A similar thermal stability of apalbumin 1, as in acacia honey, was observed also in chestnut, linden, dandelion, and rape honeys (not shown). The thermal stability of apalbumin 1 in honey predestines apalbumin 1 as a marker for the objective evaluation of honey also in food products.

**Standardization of ELISA.** Honey contains 80% of sugars, mostly the monosaccharide glucose, 31.3%, and fructose, 38.2%, and the rest belongs to small amounts of at least 22 other, more complex sugars (10.5%) (*1*−*3*). For direct immunochemical determination of proteins in honey, the presence of sugars of analyzed samples should be taken into account. As uniform conditions for the determination of apalbumin 1 in honey we decided to use a glucose/fructose solution (1:1) as a universal chemically defined medium for the method that also reflected a total sugar content similar to those in honey. The calibration curve of apalbumin 1 was prepared in dilutions of 40% glucose (G) and 40% fructose (F) solution in water (G/F), which corresponds to the total sugar content (80%) in honey.

First, a calibration curve of apalbumin 1 in a broad concentration range from 5 to 500 ng $mL^{-1}$ was prepared in a 96-well plate in different dilutions of G/F, corresponding to the sugar content of diluted honey, as presented in **Figure 3**. For exclusion of unspecific reactions a control analysis was performed: 1, without antigen (apalbumin 1) and without antibodies; 2, without antigen (apalbumin 1) and with antibodies; 3, with antigen and without antibodies, for each dilution of G/F. The preliminary screening of conditions showed the optimal calibration curve of apalbumin 1 in dilutions of G/F from 0.008 to 0.08%, which corresponded to dilution of honey from 0.01 to 0.1%. We have found the higher concentrations of sugars make the binding of antigen more difficult, because of the high viscosity of the sample, which results in lower sensitivity and increasing limit of detection (LOD). Whereas in 0.0008% G/F, the LOD of apalbmin 1 was 2 ng $mL^{-1}$, in 0.8% G/F the LOD was found to be 20 ng $mL^{-1}$.

For routine determination of apalbumin 1 in honey two calibration curves are sufficient to prepare in a concentration range from 2 to 100 ng $mL^{-1}$ of apalbumin 1, one in 0.04% G/F

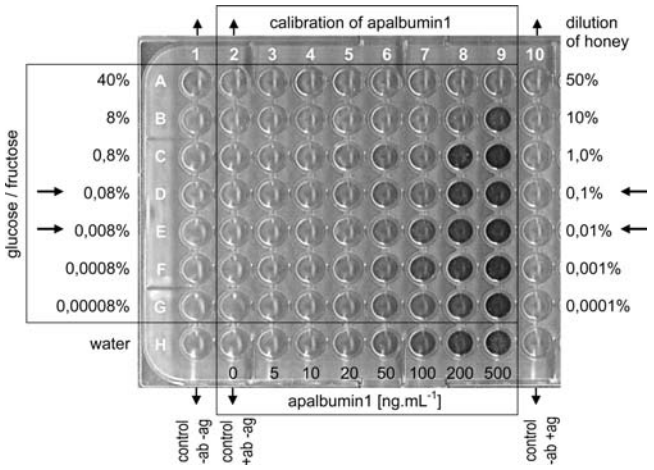


**Figure 3.** Modeling of quantification of apalbumin 1 in G/F solution by ELISA. Calibration curves in the concentration range from 5 to 500 ng $mL^{-1}$ of apalbumin 1 in G/F solution at dilutions from 40 to 0.00008%, corresponding to dilutions of honey from 50 to 0.0001%, in water were tested on the ELISA plate. Controls were done in the absence (−) and/or presence (+) of antibodies (ab) and in the absence (−) and/or presence (+) of antigen (ag). Dilutions of honeys from 0.1 to 0.01% (indicated by arrows) of most samples were optimal for the determination of apalbumin 1, and the corresponding calibration curve of apalbumin 1 in G/F solution is at dilutions from 0.08 to 0.008%.

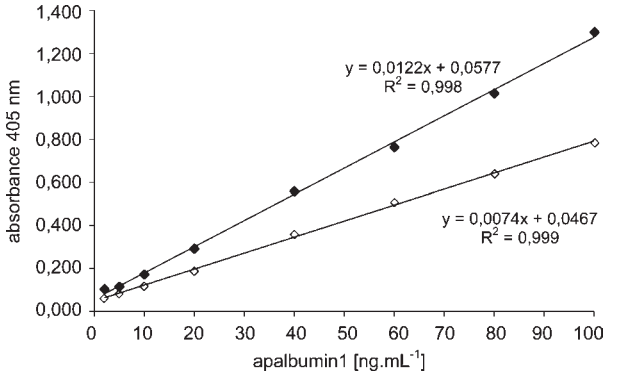


**Figure 4.** Calibration curve of apalbumin 1 in G/F solution. The calibration curve from 2 to 100 ng $mL^{-1}$ of apalbumin 1 in 0.04% G/F solution (○) was used for apalbumin 1 determination in 0.05% dilution of honey samples; the calibration curve of apalbumin 1 in 0.008% G/F (●) was used for 0.01% dilution of the honeys. The curves were prepared using triplicates for each standard concentration.

and the second in 0.008% G/F, for analysis of honeys at dilutions in water to 0.05 and 0.01%, respectively (**Figure 4**). For samples with higher concentrations of apalbumin 1, for which a higher dilution is required, the calibration curve of apalbumin 1 in corresponding G/F dilution is needed. In general, the presented ELISA method is applicable for determination of apalbumin 1 in dilutions of honey from 0.1 to 0.01%. Dilutions of honey lower than 0.1% lead to low sensitivity of the method through interfering high sugar content; dilution of honey higher than 0.001% is not available for concentration of apalbumin 1 below the LOD of the method. The working range of the calibration curve was from 2 to 100 ng $mL^{-1}$ of apalbumin 1 in 0.008 and 0.04% G/F. The curves were generated as a mean of three measurements with coefficient of variance (CV) of each calibration point ±5% and showed good correlation index of $R^2 = 0.999$ for apalbumin 1 in 0.008% G/F and $R^2 = 0.998$ in 0.04% G/F (**Figure 4**). Different incubation times were tested for coating and antibody reactions. The coating was performed overnight at 4 °C under static conditions. The coating, however, could be performed also within 2 h at room temperature under agitation, yielding similar results. TBS buffer was used instead of the convenient PBS in all washing steps, without any detergents, because of its higher sensitivity and lowest interfering reactions.

**Apalbumin 1 Content of Honey Samples.** Despite growing research in proteomics and physiomics of RJ proteins (*27*−*29*), the quantification of RJ proteins in honey and honeybee products is still missing. We indicated the possibility to use apalbumin 1 as a tool for control of authenticity of honey by immunochemical analysis (*20*). In the presented work the simple method for first screening of honey authenticity was developed on the basis of ELISA determination of apalbumin 1. Apalbumin 1 was determined by the presented method in 27 honey samples, 5 samples of each of 5 floral origin (acacia, linden, chestnut, rapeseed, and dandelion) and 2 saccharose syrup honeys. The honeys were obtained from apiaries of different regions in Slovakia and Italy. Averages of apalbumin 1 concentrations of five analyses of honey samples are presented in **Table 1**, as well as total protein content of the honey determined according to the Bradford method. **Table 2** shows values of apalbumin 1 and total protein in different floral honeys as the average of five samples of the same floral origin. The ELISA results show good precision in the evaluation of apalbumin 1 quantity with coefficient of variance ranging from 0.69 to 4.25%. (**Table 1**). Only one Slovakian acacia honey (**Table 1**, sample 1) and an Italian chestnut honey (**Table 1**, sample 15) showed CV values 6.67 and 7.45%, respectively. The highest amount of apalbumin 1 was found in chestnut honey (310.87 $\mu g\ g^{-1}$, sample 15) and the lowest (81.22 $\mu g\ g^{-1}$, sample 18) in rapeseed honey. A minimum apalbumin 1 content of 7.61 $\mu g\ g^{-1}$ was determined in SCCH (**Table 1**, sample 27). The artificial dandelion honey (nonhoneybee honey) prepared under the common recipe was used as negative control for immunochemical determination of apalbumin 1 in real honeybee honeys. In this artificial honey no apalbumin 1 was detected (**Table 1**, sample 28).

The apalbumin 1 content in honey does not depend significantly on geographical origin or honeybee line. This was confirmed, for example, by low variability of apalbumin 1 in acacia honeys (**Table 1**, samples 1−5). Even if the honey samples were produced by *A. mellifera ligustica* (Italy) or *A. mellifera carnica* (Slovakia), no significant differences in apalbumin 1 content in honeys of the same floral origin were found. The general view on the total protein and apalbumin 1 contents in honey summarized in **Table 2** showed the variability of concentrations of apalbumin 1 in different honeys reflected mainly their botanical origin. Distribution of apalbumin 1 content in honeys of plants growing wild such as acacia, chestnut, and meadow flower of dandelion was not so significant in comparison to honeys of cultivated crops such as rapeseed (**Table 2**; **Figure 5**). The broader distribution of apalbumin 1 content in rapeseed honeys can be explained by different selected strains of cultivated rapeseed plant subspecies. In general, the floral honeys contain from 0.01 to 0.03% of apalbumin 1, whereas the apalbumin 1 content in SCCH (0.001%) is > 10 times lower. Even if the apalbumin 1 content is determined by the botanical origin of the honey, the differences between the monofloral honeys are not so significant as to apply them for floral origin determination of the honey. On the basis of the large screening of multifloral and honeydew honeys, mostly from the Czech Republic and Slovakia (more than 150 samples were analyzed, data not shown), apalbumin 1 was detected in concentrations > 53 $\mu g\ g^{-1}$. Overall, it can be supposed that a concentration of apalbumin 1 in honey below 50 $\mu g\ g^{-1}$ indicates the presence of industrial glucose syrups in honey or dilution of

**Table 1.** Total Protein and Apalbumin 1 Contents of Monofloral Honeys from Slovakia and Italy[a]

| | | | total protein | | | apalbumin 1 | | | |
|---|---|---|---|---|---|---|---|---|---|
| sample | honey origin | regional origin | av[b] ($\mu$g g$^{-1}$) | CV[c] (%) | % of honey | av[b] ($\mu$g g$^{-1}$) | CV[c] (%) | % of total protein | % of honey |
| 1 | acacia | Slovakia | 283.77 | 7.45 | 0.028 | 103.28 | 6.67 | 36.40 | 0.010 |
| 2 | acacia | Slovakia | 322.92 | 1.96 | 0.032 | 126.11 | 1.70 | 39.05 | 0.013 |
| 3 | acacia | Slovakia | 323.10 | 2.00 | 0.032 | 106.47 | 2.27 | 32.95 | 0.011 |
| 4 | acacia | Italy | 288.51 | 3.03 | 0.029 | 108.59 | 1.07 | 37.64 | 0.011 |
| 5 | acacia | Italy | 338.81 | 11.84 | 0.034 | 98.74 | 4.52 | 29.14 | 0.010 |
| 6 | linden | Slovakia | 553.85 | 1.73 | 0.055 | 96.29 | 3.93 | 17.39 | 0.010 |
| 7 | linden | Italy | 602.79 | 3.67 | 0.060 | 96.76 | 1.00 | 16.05 | 0.010 |
| 8 | linden | Italy | 558.57 | 1.20 | 0.056 | 109.82 | 0.78 | 19.66 | 0.011 |
| 9 | linden | Italy | 383.13 | 1.12 | 0.038 | 100.30 | 2.29 | 26.18 | 0.010 |
| 10 | linden | Italy | 321.59 | 7.31 | 0.032 | 100.78 | 2.00 | 31.34 | 0.022 |
| 11 | chestnut | Italy | 878.81 | 1.48 | 0.088 | 280.29 | 3.16 | 31.89 | 0.028 |
| 12 | chestnut | Italy | 807.28 | 3.52 | 0.081 | 277.76 | 2.36 | 34.41 | 0.028 |
| 13 | chestnut | Italy | 1025.65 | 2.29 | 0.103 | 265.12 | 1.55 | 25.85 | 0.027 |
| 14 | chestnut | Italy | 1042.27 | 3.75 | 0.104 | 303.05 | 4.47 | 29.08 | 0.030 |
| 15 | chestnut | Italy | 966.97 | 1.35 | 0.097 | 310.87 | 7.45 | 32.15 | 0.030 |
| 16 | rapeseed | Slovakia | 470.64 | 4.78 | 0.047 | 94.61 | 3.56 | 20.10 | 0.010 |
| 17 | rapeseed | Slovakia | 547.34 | 2.58 | 0.055 | 96.38 | 2.31 | 17.61 | 0.010 |
| 18 | rapeseed | Slovakia | 574.17 | 2.82 | 0.057 | 81.22 | 2.30 | 14.15 | 0.008 |
| 19 | rapeseed | Slovakia | 653.59 | 1.24 | 0.065 | 137.68 | 4.25 | 21.07 | 0.014 |
| 20 | rapeseed | Slovakia | 1008.64 | 3.50 | 0.101 | 102.44 | 3.38 | 10.16 | 0.017 |
| 21 | taraxacum | Italy | 1120.33 | 3.88 | 0.112 | 140.86 | 2.64 | 12.57 | 0.014 |
| 22 | taraxacum | Italy | 921.20 | 3.55 | 0.092 | 138.52 | 3.23 | 15.04 | 0.014 |
| 23 | taraxacum | Italy | 1007.46 | 0.74 | 0.101 | 136.77 | 2.88 | 13.58 | 0.014 |
| 24 | taraxacum | Italy | 616.88 | 5.65 | 0.062 | 129.75 | 1.56 | 21.03 | 0.013 |
| 25 | taraxacum | Italy | 862.74 | 7.21 | 0.086 | 136.18 | 0.69 | 15.78 | 0.014 |
| 26 | saccharose syrup | Italy | 187.78 | 5.80 | 0.019 | 7.72 | 7.20 | 4.11 | 0.001 |
| 27 | saccharose syrup | Slovakia | 130.67 | 4.24 | 0.013 | 7.61 | 5.41 | 5.82 | 0.001 |
| 28 | artificial dandelion honey | Slovakia | 164.66 | 3.96 | 0.016 | <LOD[d] | | | |
| 29 | royal jelly | Slovakia | 5062.60 | 3.03 | | 3352.26 | 2.57 | 66.22 | |

[a] The values of protein concentration of the honeys are expressed as a mean of five parallel analyses of the same sample. [b] Average of five analyses of the sample. [c] CV, coefficient of variance. [d] LOD, limit of detection.

**Table 2.** Average of Total Protein and Apalbumin 1 in Monofloral Honeys[a]

| | | apalbumin 1 ($\mu$g g$^{-1}$) | | | apalbumin 1 (%) | |
|---|---|---|---|---|---|---|
| honey origin | total protein[b] ($\mu$g g$^{-1}$) | min | max | av[b] | total protein | honey |
| acacia ($n$ = 5) | 311.42 | 98.74 | 126.11 | 108.64 | 34.88 | 0.011 |
| linden ($n$ = 5) | 483.99 | 96.29 | 109.82 | 100.79 | 20.83 | 0.010 |
| chestnut ($n$ = 5) | 944.20 | 265.12 | 310.87 | 287.42 | 30.44 | 0.029 |
| rapeseed ($n$ = 5) | 650.88 | 81.22 | 137.68 | 102.47 | 15.74 | 0.010 |
| dandelion ($n$ = 5) | 905.72 | 129.75 | 140.86 | 136.42 | 15.06 | 0.014 |
| SCCH ($n$ = 2) | 159.22 | 7.61 | 7.72 | 7.66 | 4.81 | 0.001 |

[a] The average values of protein content were calculated as a mean of five different samples of the same floral origin, presented in **Table 1**. [b] Average of ($n$) honey samples.

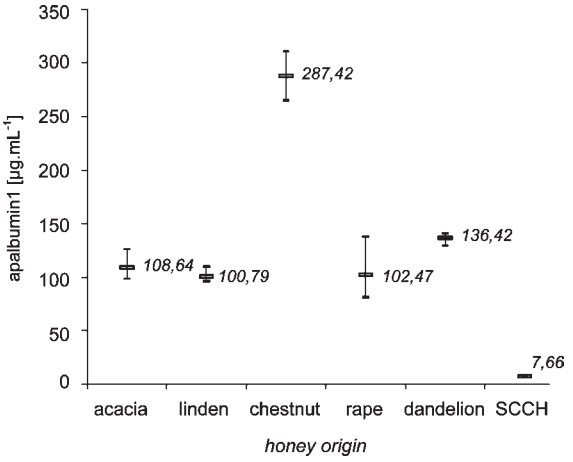


**Figure 5.** Sample distribution of apalbumin 1 content in honey samples of different origin (concentration of apalbumin 1 in acacia, linden chestnut, rapeseed (rape), dandelion, and saccharose syrup honeys (SCCH) according to **Table 2**). Bars indicate minimum, maximum, and average values of apalbumin 1 concentration of five samples of the same floral honey.

floral honey with the honey obtained by feeding of a honeybee colony with saccharose syrup.

The other important factor for honey quality examination is apalbumin 1 proportion to total protein content of honey. The protein content of honey samples determined by microassay according to the Bradford (*26*) method is shown in **Table 1**, and average values of the protein concentration in honeys of different floral origin (presented in **Table 1**) are summarized in **Table 2**. The highest concentration of proteins, 944.20 $\mu$g g$^{-1}$, was determined in chestnut honey, whereas acacia honey contains only 311.42 $\mu$g g$^{-1}$ of the proteins. In SCCH honey the content of total proteins (159.22 $\mu$g g$^{-1}$) was 4 times less than average amount of total proteins of floral honeys (659.24 $\mu$g g$^{-1}$). The average amount of total proteins of analyzed floral honeys (0.066%) corresponded to general data on protein content of honey, which was in range from 0.05 to 0.79% (*1*−*4*). As shown in **Table 1**, protein content depended on the botanical origin of honey rather than the geographical locality. **Figure 6** shows that the ratio of apalbumin 1 to total proteins of honey can be considered as supporting criterion for adulteration of honey. We have found apalbumin 1 in acacia honey at 34.88% of total protein and in dandelion it was 15.06%, whereas in SCCH it was 4.81% of total protein only. Apalbumin 1 content of RJ determined by the presented ELISA method (3.35 mg g$^{-1}$) correspond to 66.22% of total RJ proteins,

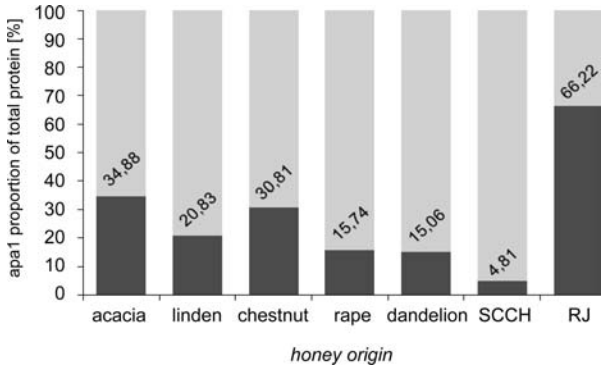


**Figure 6.** Apalbumin 1 proportion of total protein in honey samples and in RJ (proportion of apalbumin 1 (%) of total protein content of acacia, linden chestnut, rapeseed (rape), dandelion, and saccharose syrup honeys (SCCH) according to **Table 2**).

which validates apalbumin 1 as a major protein of RJ and a new factor for the standardization of RJ.

The developed ELISA can be easily performed in certification laboratories for control of honey quality and authenticity. It can be used for first screening of honey adulteration based on addition of industrial sugar syrups (e.g., corn syrup, high-fructose corn syrup) and/or of honeys from feeding bees with saccharose syrup. The method has potentially wide application in food products, apitherapy, cosmetics, and pharmacy.

## ABBREVIATIONS USED

ABTS, 2,2′-azinobis(3-benzthiazoline-6-sulfonic acid); ab, antibody; ag, antigen; apa1, apalbumin 1; BSA, bovine serum albumin; CV, coefficient of variance; ELISA, enzyme-linked immunosorbent assay; G/F, glucose/fructose solution; LOD, limit of detection; MWCO, molecular weight cutoff; PBS, phosphate-buffered saline; PBS-T, phosphate-buffered saline with 0.05% Tween 20; TBS, Tris buffer saline; TBS-T, Tris buffer saline with 0.05% Tween 20; RJ, royal jelly; SCCH, saccharose syrup honey; SDS, sodium dodecyl sulfate; WB, Western blot.

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