doi: 10.17221/422/2015-CJFS

Quantification of Bee-Derived Peptide Defensin-1 in Honey by Competitive Enzyme-Linked Immunosorbent Assay, a New Approach in Honey Quality Control

Ivana VALACHOVÁ[1], Marcela BUČEKOVÁ[2,3] and Juraj MAJTÁN[2,3,4]

[1]Department of Zoology, Faculty of Natural Sciences, Comenius University in Bratislava, Bratislava, Slovak Republic; [2]Institute of Zoology and [3]Laboratory of Molecular Apidology and Apitherapy, Institute of Molecular Biology, Slovak Academy of Sciences, Bratislava, Slovak Republic; [4]Department of Microbiology, Faculty of Medicine, Slovak Medical University, Bratislava, Slovak Republic

Abstract

Valachová I., Bučeková M., Majtán J. (2016): **Quantification of bee-derived peptide defensin-1 in honey by competitive enzyme-linked immunosorbent assay, a new approach in honey quality control**. Czech J. Food Sci., 34: 233–243.

We established and evaluated a polyclonal antibody based competitive enzyme-linked immunosorbent assay for the quantification of defensin-1 in honey. The assay showed an inhibitory concentration (IC_{50}) value of 111.5 ± 15.41 ng/ml with a detection limit of 7.8125 ng/ml. The regaining of defensin-1 in spiked 'artificial honey' was between 87.05 and 112.96% with relative standard deviation less than 9.2%. Sensitivity and specificity of the test were experimentally validated on a sample of 20 different honeys. The antibacterial activity of these honey samples showed a significant concentration-dependent correlation with the production of defensin-1 (n = 20; r = –0.6598; P = 0.0016). The assay provides a specific and sensitive method for the screening of defensin-1 in honey. The method to detect honeybee-derived proteins in honey is a promising approach to verifying the authenticity of honey. The defensin-1 ELISA could also be used for the rapid screening of honeys suitable for medicinal purposes.

Keywords: competitive ELISA; honey adulteration; antibacterial peptides; *Apis mellifera*

Honey is consumed as a healthy food and is also extensively used in folk and clinical medicine to treat a broad spectrum of injuries, in particular burns and chronic wounds (Oskouei & Najafi 2013). Several clinical studies have demonstrated that honey can improve and speed up the healing process (Dunford *et al.* 2000; Eddy & Gideonsen 2005; Molan 2006). High quality of honey is therefore essential for its usage in human consumption and as a potential medical treatment.

Current international honey standards are specified in the European Honey Directive (2002) and in the Codex Alimentarius Standard for Honey (2001). When placed on the market for human consumption, honey must meet precisely defined composition criteria including sugar, moisture, and water-insoluble contents, electrical conductivity, free acids, diastase activity, and hydroxymethylfurfural content. In addition, honey must not (*i*) contain any foreign organic or inorganic matter, flavours or odours, (*ii*) begin to ferment, (*iii*) have an artificially changed acidity, and (*iv*) have been heated in such a way that the natural enzymes are either destroyed or significantly inactivated (EU Honey Regulations 2002). The adulteration of honey, based on the addition of industrial sugar syrups (e.g. corn syrup, high-fructose corn syrup) and/or of honeys from bees feed with saccharose syrup, has increased exponentially (Elflein & Raezke

Supported by the Scientific Grant Agency of the Ministry of Education of the Slovak Republic and the Slovak Academy of Sciences – VEGA, Grant No. 2/0007/14.

doi: 10.17221/422/2015-CJFS

2008; Tosun 2013). Therefore, the need has arisen for more effective quality control methods aiming at detecting adulteration and verifying the authenticity of honey. As new, better and faster analytical methods are available nowadays, the introduction of new norms using these new methods is necessary.

One possible approach in honey quality control is to focus on the detection of specific honey constituents such as enzymes derived from bee salivary secretions. During the processing of nectar into honey, bees enrich nectar with enzymes which are able to cleave complex sugars into simple sugars. In addition to enzymes that digest sugars, bees add several other unique substances [e.g. antibacterial peptide defensin-1; glucose oxidase (GOX), the enzyme responsible for the formation of hydrogen peroxide (H_2O_2); major royal jelly proteins (MRJP) with nutritional value] into nectar during processing, which give honey many of its biological properties (White *et al.* 1963; Fujiwara *et al.* 1990; Seeley 1992; De Grandi-Hoffman & Hagler 2000; Buttstedt *et al.* 2014). Proteins and amino acids in honeys are attributed both to animal and vegetal sources, including fluids and the nectar secretions of the salivary glands and pharynx of honeybees (Sak-Bosnar & Sakac 2012; Escuredo *et al.* 2013). When analysing the proteome content of honey from chestnut, acacia, sunflower, eucalyptus, and orange in order to see if any plant proteins present would allow the proteotyping of these different varieties, it turned out that all major proteins identified in honey were of bee origin (Girolamo *et al.* 2012). Detection and quantification of these proteins of bee origin can serve as a marker to select high quality honeys and also to identify honey adulterations. Preliminary screening of honeys by SDS-PAGE (Marshal & Williams 1987) and protein profiles of floral and honeydew honeys by liquid chromatography (Inglesias *et al.* 2006) have shown heterogeneous compositions, while more evidence is necessary to identify and quantify honey proteins. Recently, two enzyme-linked immunosorbent assays (ELISA) have been established, using the quantification of MRJP1, also described as apalbumin 1 in honey (Bilikova & Simuth 2010) and in royal jelly (Yamaguchi *et al.* 2013). MRJP1 is the most abundant protein in royal jelly and also in honey (Hanes & Simuth 1992). The average amount of MRJP1 relating to the total protein content of analysed honey samples measured by ELISA was 23.39% (Bilikova & Simuth 2010). The disadvantage of using MRJP1 quantification as a criterion for the evaluation of honey quality is its abundance. A preferable approach seems to focus on other bee-derived components, such as the peptide defensin-1. Bee defensin-1 is naturally found in honeys in amounts ranging from 0.04 to 5.17 μg/g of honey (n = 20), while adulterations can decrease such contents.

The antibacterial properties of honey have been extensively studied and several factors have been identified: low pH, high osmolarity, H_2O_2, antibacterial bee defensin-1, methylglyoxal (only in Manuka honey), polyphenolic compounds, and various other phytochemicals (Molan 1992; Adams *et al.* 2008; Mavric *et al*. 2008; Kwakman *et al.* 2010, 2011; Alvarez-Suarez *et al.* 2012; Majtan *et al.* 2012). Bee defensin-1 and H_2O_2 represent the major antibacterial factors in medicinal honey (Kwakman *et al.* 2011). Honeybee defensin-1 was first isolated from royal jelly (described as royalisin) (Fujiwara *et al.* 1990) and subsequently it was found in honey as well (Kwakman *et al*. 2010). Defensin-1 is a peptide belonging to the insect defensin group, is composed of 51 amino acids, which has a molecular weight of 5.52 kDa. Insect defensins are mostly active against Gram-positive bacteria, and less frequently against Gram-negative bacteria (Bulet *et al.* 1999; Bulet & Stocklin 2005). They are either inducibly expressed in the fat body during systemic immune responses or constitutively expressed in tissues which are in continuous contact with potentially infectious sources in the environments [e.g. the salivary glands of *Lucilia sericata* (Valachova *et al.* 2013), the blood storing-stomach of *Triatoma brasiliensis* (Araujo *et al.* 2006), the epidermis of the body wall of *Musca domestica* (Wang *et al.* 2006 and others)]. The defensin-1 expression was determined in nurse honeybees by RT-PCR in hypopharyngeal, mandibular, and thoracic salivary glands (Klaudiny *et al.* 2005). In bees upon infection, the defensin-1 expression is induced in the fat body, from where it is secreted into the haemolymph as a part of the immune response (Casteels-Josson *et al.* 1994; Casteels 1998). Recently, it has been shown that defensin-1 is found in all examined types of larval jelly and honey, including Manuka honey, but its amount varies significantly. The content of defensin-1 in different samples has been semi-quantified by immunoblotting (Klaudiny *et al.* 2012; Majtan *et al.* 2012).

In the present study, we established and evaluated a polyclonal antibody based competitive ELISA for the quantification of defensin-1 in honey. This assay can be used for the purpose of the qualitative analysis of honeys, particularly in order to select honeys with high potential antibacterial activity.

doi: 10.17221/422/2015-CJFS

MATERIAL AND METHODS

Honey samples. Samples of raw honey (n = 20) were received from beekeepers from several regions of Slovakia between May and August 2013 (Table 1). The samples included liquid and crystallised honeys from monofloral or polyfloral sources. After reception, honey samples were transferred to sterile plastic containers and stored at room temperature in the dark. Honey samples were numbered at the beginning of the experiment and were tested blindly to avoid any bias. The identification of a dominant nectar/honeydew source of honey was performed by the beekeepers based on the availability of flora for nectar/honeydew foraging, the location of the apiary and the organoleptic characteristics of the honey.

Microorganisms. The antibacterial activity of honey samples was assessed against a control strain of *Staphylococcus aureus* CCM 4223 obtained from the Department of Medical Microbiology, Slovak Medical University (Bratislava, Slovakia) and originally purchased from the Czech Collection of Microorganisms (Masaryk University, Brno, Czech Republic).

Determination of antibacterial activity. The antibacterial efficacy of various honey samples was evaluated by the minimum inhibitory concentration (MIC) assay according to Bucekova *et al.* (2014). Determination of MIC was conducted according to the recommendations of the Clinical and Laboratory Standards Institute (2003). Briefly, one bacterial colony was suspended in phosphate-buffered saline (PBS) buffer, pH 7.2, and the turbidity of the suspension was adjusted to 10^8 colony forming units (CFU)/ml and diluted with the Mueller-Hinton broth medium to a final concentration of 10^6 CFU/ml. Ten microlitre aliquots of suspension were inoculated into each well of sterile 96-well polystyrene plates (Sarstedt, Nümbrecht, Germany). The final volume in each well was 100 µl, consisting of 90 µl of sterile medium or diluted honey and 10 µl of bacterial suspension. After 18 h of incubation at 37°C, the bacterial growth inhibition was determined spectrophotometrically by monitoring the optical density at 490 nm compared to negative controls. The MIC was defined as the lowest concentration of honey inhibiting bacterial growth. All tests were performed in triplicate and were repeated three times.

A serial twofold dilution series of each honey was prepared from 40% to 50% (w/v) honey solution, resulting in final concentrations of 50, 40, 25, 20, 12.5, 10, 6.25, 5, 3.12, 2.5, and 1.25%.

Anti-honeybee defensin-1 polyclonal antibody. An affinity-purified rabbit polyclonal anti-honeybee defensin-1 antibody was purchased from GenCust Europe (Dudelnag, Luxembourg). Two New Zealand rabbits immunised with a synthetic peptide corresponding to the C terminus of bee defensin-1

Table 1. Honey samples analysed

Honey sample	Dominant nectar/honeydew source	Geographic origin in Slovakia	Harvesting in 2013
1	*Robinia pseudoacacia*	Veľký Krtíš	June
2	*Robinia pseudoacacia*	Lamač	June
3	*Robinia pseudoacacia*	Lamač	June
4	*Robinia pseudoacacia*	Borský Mikuláš	June
5	*Robinia pseudoacacia*	Borský Mikuláš	June
6	*Robinia pseudoacacia*	Borský Mikuláš	June
7	*Tilia platyphyllos*	Jarovce	June
8	*Tilia platyphyllos*	Slovenská Lupča	June
9	*Castanea sativa*	Lamač	June
10	*Helianthus annuus*	Jarovce	June
11	*Brassica napus*	Jarovce	June
12	*Abies alba*	Slovenská Lupča	August
13	*Abies alba*	Čergov	August
14	multifloral	Čergov	July
15	multifloral	Podpolanie	May
16	multifloral	Vysoká nad Uhom	June
17	multifloral	Vysoká nad Uhom	July
18	multifloral	Vysoká nad Uhom	August
19	multifloral	Inovec	May
20	multifloral	Inovec	August

doi: 10.17221/422/2015-CJFS

(CRKTSFKDLWDKRFG) provided antibodies that were then affinity-purified using this peptide. The antibodies were resuspended in molecular grade water (concentration 0.81 mg/ml, 1.04 mg/ml), diluted 1 : 1 with glycerol and stored at – 20°C.

Specificity of anti-honeybee defensin-1 polyclonal antibody. Immunospecificity of the rabbit polyclonal anti-honeybee defensin-1 antibody was evaluated by immunoblot assays. The gland extracts of unspecified hive bees (5 glands/200 µl of PBS pH 7.2) were electrophoresed (15 µl) on a 16.5% Tricine-SDS-PAGE gel using a Mini-Protean II electrophoresis cell (Bio-Rad, Hercules, USA). The proteins were transferred onto a 0.22 µm nitrocellulose membrane (Sigma-Aldrich, Dorset, UK) in 48 mM Tris, 39 mM glycine, and 20% methanol using the semi-dry blotting procedure. The membrane was blocked for 1 h in a TBST buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.05% Tween 20) containing 5% non-fat dried milk and then incubated overnight with the rabbit polyclonal anti-honeybee defensin-1 antibody diluted 1 : 2000 in TBST-blocking buffer. After washing with TBST, the membrane was incubated for 2 h in blocking buffer containing the goat anti-rabbit HRP-linked antibody (Promega, Medison, USA) diluted 1 : 2500. Immunoreactive bands were detected using a solution containing dissolved SigmaFast 3,3-diaminobenzidine tablets (Sigma-Aldrich, UK).The production of defensin-1 in the honeybee glands was studied using whole-mount immunohistochemical staining as described previously (Zitnanova *et al.* 2001). Briefly, glands of female workers of unspecified age of maturity were dissected in PBS and fixed overnight in 4% paraformaldehyde in PBS (pH 7.2). The tissues were washed with PBS [0.3% Triton X-100 (PBST)], pre-absorbed with 5% normal goat serum and incubated in the rabbit polyclonal anti-honeybee defensin-1 antibody diluted 1 : 1000 in PBST for 2 days, washed with PBST and incubated overnight with FITC-labelled goat anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch Lab., West Grove, USA) diluted 1 : 100. FITC-labelled glands were then washed in PBST and mounted in glycerol containing 4',6'-diamino-2-phenylindole (DAPI; 2 mg/ml) (Sigma, St Louis, USA). The fluorescently labelled preparations were observed and imaged by a TCS SPE (Leica Microsystems, Wetzlar, Germany) confocal system using 405 and 488 nm lasers for excitation. Optical sections were merged and processed using the LAS AF software (Leica Microsystems, Germany).

In situ hybridisation. *In situ* hybridisation was used for detection and localisation of the *def* expression in different tissues of worker bees performing different tasks. Primers for the synthesis of a digoxigenin (Dig)-labelled probe were designed based on the honeybee *def* cDNA sequence as follows: sense primer, 5'-AAAATC-TATTTTATTGTCGGCCTTC-3'; antisense primer, 5'-CGAAACGTTTGTCCCAGAGA-3'. The 280-bp amplicon was re-amplified using the PCR Dig Probe synthesis kit (Roche Applied Science, Mannheim, Germany) to synthesise the Dig-labelled antisense DNA probe. The Dig-labelled probe was purified using a PCR Purification kit (Promega, USA) and stored at –20°C. The hypopharyngeal glands, salivary glands, and mandibular glands, fat bodies and guts were dissected from honeybees performing a particular task, fixed in 4% paraformaldehyde (w/v) at 4°C overnight, and subjected to the whole-mount *in situ* hybridisation procedure as described elsewhere (Kim *et al*. 2006).

Competitive enzyme-linked immunosorbent assay. For the quantification of defensin-1 in honey samples, a new ELISA was developed to optimise sensitivity. The principle for the assay lies in competition between sample defensin-1 and a defensin-1-peroxidase conjugate for binding to the rabbit polyclonal anti-honeybee defensin-1 antibody. The defensin-1-peroxidase conjugate was purchased from GenCust Europe (Luxembourg). Horseradish peroxidase (HRP) was crosslinked to a synthetic peptide corresponding to the C terminus of bee defensin-1 (CRKTSFKDLWDKRFG) with glutaraldehyde. The conjugate (1.3 mg/ml) was purified by size-exclusion chromatography, diluted 1 : 1 with glycerol and stored at –20°C.

The ELISA was carried out as described by Kingan *et al.* (1997) with minor variations: firstly, 0.5 µg of the affinity-purified goat anti-rabbit IgG (Abcam, Cambridge, UK) was adsorbed in 100 µl PBS (pH 7.2) to the wells of an ImmunoGrade plate (BrandTech, Essex, USA) for 18 h at 4°C. The contents were discarded and, without rinsing, 200 µl of PBS with 5% BSA and 0.05% Tween-20 (blocking buffer) was added to each well to block excess binding sites. After 2 h, the blocking buffer was discarded and 30 µl of the rabbit polyclonal anti-honeybee defensin-1 antibody, diluted 1 : 8000 in blocking buffer, was added to each well. Samples and standards (a synthetic peptide corresponding to the C terminus of bee defensin-1; CRKTSFKDLWDKRFG) were then added in 100 µl aliquots, followed by 20 µl of defensin-1-peroxidase conjugate diluted 1 : 10000 in blocking buffer. The

doi: 10.17221/422/2015-CJFS

plate contents were then mixed for 5 min on a plate shaker (Mini Shaker PSU-2T; Biosan, Riga, Latvia) and then incubated overnight, without mixing, at 4°C. The plate was then rinsed three times with washing buffer (PBS with 0.05% Tween-20), developed with the 3,3',5,5'-tetramethylbenzidine (TMB) liquid substrate system for ELISA (Sigma-Aldrich, UK) for 30 min, quenched with 2 M H_2SO_4, and read at 450 nm on an ELISA plate reader (Bio-Rad, USA). The absorbances were processed by a four-parameter logistic transformation performed using GraphPad Prism (GraphPad Software Inc., La Jolla, USA). A typical curve for defensin-1 ELISA is shown in Figure 1. The concentration of defensin-1 required to produce 50% inhibition of the conjugate binding antibody was represented as the IC_{50}.

Recovery of defensin-1 spiked in 'artificial honey' measured by competitive ELISA. A spiking experiment was carried out to determine the recovery of defensin-1. A synthetic peptide corresponding to the C terminus of bee defensin-1 (CRKTSFKDLWDKRFG) was added to a 10% solution of 'artificial honey' to a final concentration of 50, 100, and 200 ng/ml. 'Artificial honey', a control solution with a sugar content similar to that of natural honey, was prepared by dissolving 39 g D-fructose, 31 g D-glucose, 8 g maltose, and 3 g sucrose in 19 g of distilled water; this was stored at room temperature in the dark (Majtan & Majtan 2010). The content of

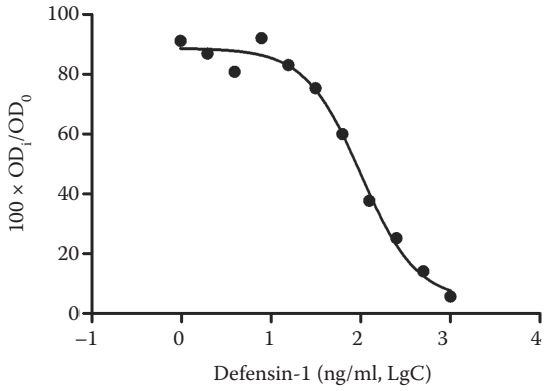

Figure 1. Dose-response curve of enzyme-linked immunosorbent assay used in the quantification of defensin-1 in honey

This assay is based on the competition of defensin-1 in a honey sample with a synthetic fragment of defensin-1 covalently coupled to horseradish peroxidase; the fitted curve for defensin-1 values was plotted using a four-parameter logistic equation; OD_i/OD_0 is the ratio of optical density in the presence of defensin-1 (OD_i), and optical density in the absence of competitor defensin-1 (OD_0)

defensin-1 spiked in 'artificial honey' was analysed by the ELISA described previously. Recovery of defensin-1 was calculated from the concentration values according to the following equation:

R (%) = (measured protein concentration/theoretical protein concentration) × 100

Detection of defensin-1 in honey samples by competitive ELISA. Each honey sample (2.5 g) was dissolved in 2.5 ml of PBS until completely fluid. Insoluble particles were removed from the liquid solution by centrifugation at 14 000 *g* at 4°C for 10 minutes. Serial dilutions of each honey were prepared from 50% (w/w) honey solution, resulting in final concentrations of 10, 5, and 2.5%, i.e. concentrations that fell in the linear portion of the curve. All measurements were performed in triplicate and were repeated three times. The data were expressed as mean values with the standard error of the mean (SEM). The measured amount of defensin-1 in honey sample was expressed as a percentage of total proteins. Total proteins in honey samples were measured using the Quick Start Bradford protein assay (Bio-Rad, USA) as described in the instruction manual.

Statistical analysis. The Pearson correlation test was used for correlation analysis between the antibacterial activity/content of defensin-1 in honeys. Data with *P*-values smaller than 0.05 were considered statistically significant. All statistical analyses were performed using GraphPad Prism (GraphPad Software Inc., La Jolla, USA).

RESULTS

Localisation of def expression in workers performing distinct tasks. Using the specific cDNA probe, we detected strong expression of *def* mRNA in the hypopharyngeal glands of all selected bees (Figure 2). The expression level in hypopharyngeal glands remained strong throughout the behavioural development of the worker bees from cleaners to foragers. No expression of *def* was detected in mandibular, head salivary, and thorax salivary glands of the worker bees. Similarly, no *def* expression was detected in the fat body and gut (data not shown).

Characterisation of anti-honeybee defensin-1 polyclonal antibody. An affinity-purified rabbit polyclonal anti-honeybee defensin-1 antibody was prepared to quantify defensin-1 in honeys derived from different bee colonies. First, the immunospecificity of the an-

doi: 10.17221/422/2015-CJFS

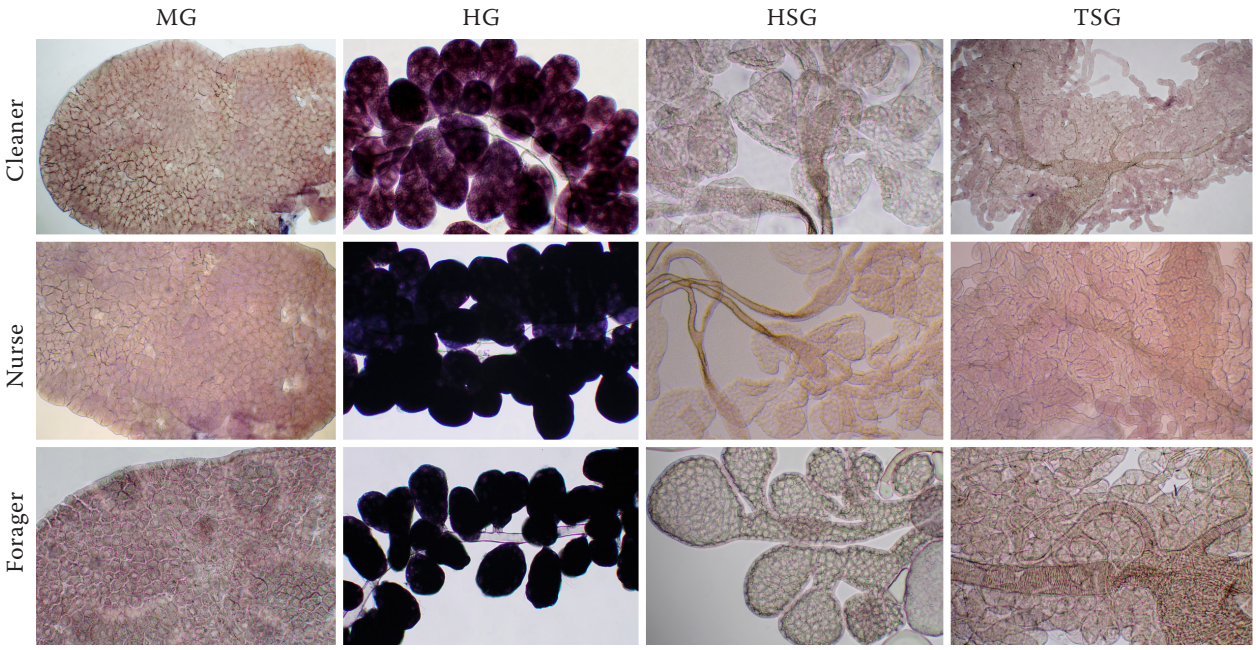

Figure 2. Localisation of *def* expression in different glands of workers specialised in different tasks performed within a colony at different ages

The expression was localised in glands by *in situ* hybridisation; workers performing the following tasks were used in the study: cleaner, nurse, and forager; MG – mandibular glands; HG – hypopharyngeal glands; HSG – head salivary glands; TSG – thorax salivary glands (scale bar 100 μ)

tibody was evaluated by immunoblot assays. Extracts of mandibular, hypopharyngeal, head salivary, and thorax salivary glands were separated on SDS-PAGE gels, blotted onto nitrocellulose membrane and immunostained with the rabbit polyclonal anti-honeybee defensin-1 antibody. A specific immunoreactive band of around 5 kDa in size was detected only in extracts of hypopharyngeal glands suggesting that the antibody has high specificity, and there was no cross-reaction with other proteins in the extracts (Figures 3A, B).

Using whole-mount immunohistochemical staining, secretory granules with defensin-1 were stained specifically only in the hypopharyngeal glands of unspecified hive bees (Figure 3C). The hypopharyngeal glands are composed of clusters of acini that deliver secretions into a collecting duct running to the mouthparts. A single acinus has up to twenty-two cell secretory units, each comprising a secretory cell and a canal cell. Strong defensin-1 immunoreactivity was detected in the secretory granules in the cytoplasm of secretory cells, from where they are transported via canal cells into the collecting duct (Figure 3C).

Development of the competitive ELISA. The rabbit polyclonal anti-honeybee defensin-1 antibody was used to establish a calibration curve for defensin-1 in the competitive ELISA because it had both strong specificity and appropriate affinity. The optimal concentration of defensin-1-HRP and antibody was determined to be 130 ng/ml and 101.25 ng/ml, respectively. The linear portion of the curve was 7.8125–1000 ng/ml. The concentration of defensin-1 which inhibited 50% of the total binding of antibody (IC_{50}) was 111.5 ± 15.41 ng/ml.

A recovery test was performed using a 10% solution of 'artificial honey'. Recoveries of defensin-1 were calculated using the calibration curve established with a synthetic peptide corresponding to the C terminus of bee defensin-1. When spiked with a synthetic peptide corresponding to the C terminus of bee defensin-1 at three different levels, the average recoveries varied from 87.05% to 112.96%. In addition, the relative standard deviation (RSD) ranged from 4.3% to 9.2%, which indicated that the rabbit polyclonal anti-honeybee defensin-1-based competitive ELISA gave sensitive and reliable reproducibility.

Quantification of defensin-1 in honey samples. Sensitivity and specificity of the test were experimentally validated on a sample of 20 different honeys. The amount of defensin-1 in honey samples was examined in their 10, 5, and 2.5% solutions with PBS using the newly developed competitive ELISA (Table 2). Honey samples No. 17, 18, and 19 con-

doi: 10.17221/422/2015-CJFS

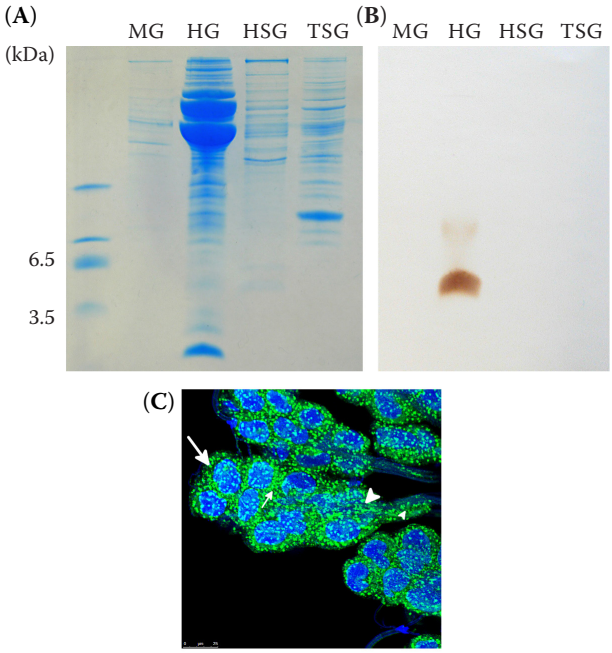

Figure 3. Immunospecificity of an anti-defensin-1 polyclonal antibody evaluated by immunoblot assays and immunohistochemistry: (**A**) Gland extracts of unspecified hive bees (5 glands/200 µl of PBS) were electrophoresed on a 16.5% Tricine-SDS-PAGE gel and stained with Coomassie Blue R; (**B**) After immunoblotting with a purified rabbit polyclonal antibody against honeybee defensin-1, a single band was visible only in the extract of hypopharyngeal glands; (**C**) Using whole-mount immunohistochemical staining with the rabbit polyclonal antibody against honeybee defensin-1, secretory granules in the hypopharyngeal glands were stained green

Nuclei of cells were stained blue using a nuclear dye (4',6'-diamino-2-phenylindole); the hypopharyngeal glands were composed of clusters of acini; a single acinus was composed of up to 22 cell secretory units, each comprising a secretory cell (arrow) and a canal cell (arrowhead); secretory granules with defensin-1 were readily visible in the cytoplasm of secretory cells (small arrow) from where they were transported via canal cells into the collecting duct (small arrowhead); MG – mandibular glands; HG – hypopharyngeal glands; HSG – head salivary glands; TSG – thorax salivary glands

tained the highest concentrations of defensin-1 (3.69, 5.17, and 3.32 µg of defensin-1 per gram of honey, respectively). The measured amount of defensin-1 in honey samples was then expressed as a percentage of total proteins (Figure 4A). The solutions of 20 honey samples analysed, of various botanical and geographical origins, showed differences in their defensin-1 contents. Differences in the defensin-1 content were observed between the honey samples of different and also of similar botanical origin. They were also observed between honey samples of different geographical origins and harvesting time.

Antibacterial activity of honey samples. The MIC values of 20 natural honeys showed different levels of activity against a control strain of *S. aureus* CCM 4223 (Figure 4B). The MICs of honeys against *S. aureus* ranged from 2.5% to 40%. Honey samples No. 17, 18, and 19, of the multifloral origin, and which contained the highest ratio of defensin-1, also possessed the strongest antibacterial activity against the tested bacteria.

The Pearson test correlated the level of bee defensin-1 and the antibacterial activity of honeys and results revealed that there was a significant negative, concentration-dependent correlation between the production of defensin-1 and antibacterial activity against *S. aureus* (n = 20, r = –0.6570, P = 0.0016) (Figure 4C) Therefore, honey samples containing low amounts of bee defensin-1 showed low activity against *S. aureus*.

Table 2. Average concentration of total proteins and defensin-1 in different honey samples

Honey sample	Mean concentration ± SEM (µg/g of honey)	
	total proteins	defensin-1
1	229.51 ± 5.62	0.99 ± 0.24
2	384.16 ± 22.75	1.82 ± 0.20
3	360.74 ± 4.86	1.47 ± 0.09
4	247.92 ± 0.98	1.04 ± 0.20
5	241.64 ± 5.96	0.82 ± 0.31
6	277.87 ± 4.02	1.10 ± 0.14
7	323.46 ± 4.40	0.90 ± 0.03
8	407.03 ± 5.55	1.69 ± 0.05
9	373.66 ± 22.68	0.62 ± 0.20
10	351.24 ± 6.19	1.16 ± 0.24
11	337.39 ± 0.92	0.19 ± 0.27
12	307.43 ± 2.42	1.48 ± 0.65
13	356.90 ± 13.52	1.09 ± 0.26
14	348.88 ± 6.47	0.04 ± 0.05
15	312.56 ± 0.58	0.28 ± 0.01
16	286.43 ± 1.60	0.70 ± 0.34
17	379.88 ± 12.60	3.69 ± 0.13
18	421.92 ± 3.62	5.17 ± 1.39
19	498.47 ± 13.06	3.32 ± 0.49
20	442.30 ± 0.20	0.66 ± 0.15

All measurements were performed in triplicate and were repeated three times; the data are expressed as mean values with the standard error of the mean (SEM)

doi: 10.17221/422/2015-CJFS

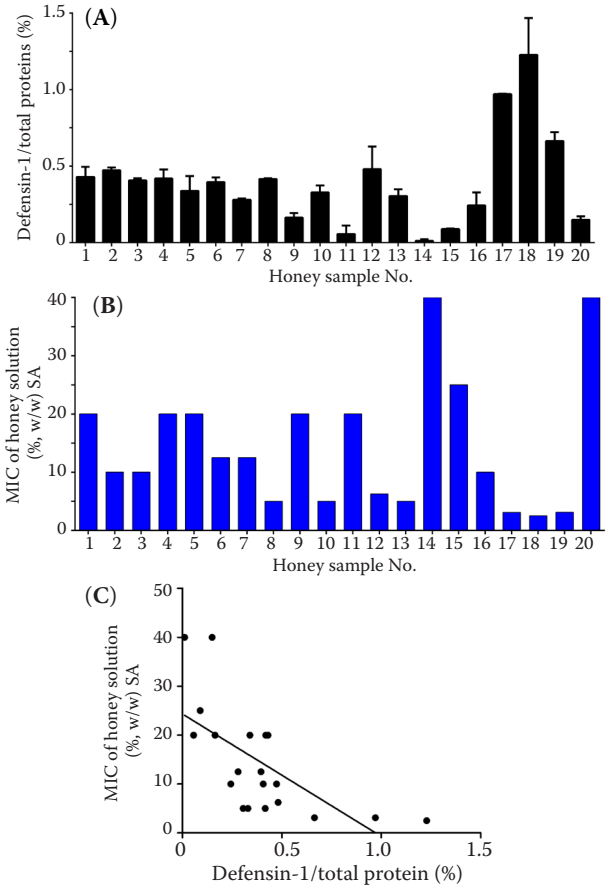

Figure 4. Defensin-1 production in honey samples: (A) The quantified amount of defensin-1 in honey samples expressed as a percentage of total proteins. The levels of defensin-1 in honey samples were quantified using the newly developed enzyme-linked immunosorbent assay. Total proteins in honey samples were measured using the Quick Start Bradford protein assay (data are expressed as mean values ± SEM); (**B**) Antibacterial activity of honeys against a *Staphylococcus aureus* isolate. The activity was determined by the minimum inhibitory concentration (MIC) assay (the MIC was defined as the lowest concentration of honey solution to inhibit bacterial growth); (**C**) A negative correlation was determined using the Pearson test of antibacterial activities (MIC values) against the ratio of defensin-1 over total proteins

DISCUSSION

Most of the methods used in honey quality control are based on biochemical and physical analyses (EU Honey Regulation 2002). Other analyses target the detection of substances foreign to honey such as insecticides (Wang *et al.* 2011), toxins (Oplatowska *et al.* 2014), and antibiotics (Yu *et al.* 2013; Wang *et al.* 2015) in the honey to prevent potential hazards to consumers.

Previous attempts to develop methods for the quantification of bee-derived proteins in honey or royal jelly have been used for authentication of honeys. Most of these assays quantify MRJP1 levels (Bilikova & Simuth 2010; Yamaguchi *et al.* 2013) or chemical markers such as the natural product leptosperin presented solely in Manuka honey (Kato *et al.* 2014) or specific proteins of sunflower pollen to assess the floral origin of honey as an alternative method to the standard melissopalynological analyses (Baroni *et al.* 2004). The key requirement for a successful immunochemical assay is the availability of antibodies with high specificity and desired affinity. We have shown that the rabbit polyclonal anti-honeybee defensin-1 antibody developed in this study has high specificity, and there was no cross-reaction with other proteins in honey. Another important requirement for honey authentication is the identification of a bee-specific marker which is present in each honey. Defensin-1 fulfilled this criterion very well. As we demonstrated by *in situ* hybridisation and immunohistochemistry, defensin-1 is constitutively and solely produced in the hypopharyngeal glands before being secreted into honey by bee workers at different stages of development and performing different tasks.

Using a polyclonal antibody, we developed a competitive ELISA for the detection of defensin-1. The assay showed an IC_{50} value of 111.5 ± 15.41 ng/ml with a detection limit of 7.8125 ng/ml. In order to evaluate the validity and reliability of the rabbit polyclonal anti-honeybee defensin-1 antibody-based competitive ELISA, recovery tests and validation studies were performed for the determination of defensin-1 in 'artificial honey' and also in 20 different honey samples.

Analyses of 20 different honey samples revealed that the amount of defensin-1 varied significantly from honey to honey. Defensin-1 was measurable in all samples, in 10% to 2.5% solutions of honey, where the concentrations of defensin-1 fell in the linear portion of the calibration curve. The content of defensin-1 varied (0.04–5.17 µg/g of honey) among honey samples of different floral sources but also within the similar floral source of different geographical origins. The highest amount of defensin-1 was found in honey samples of multifloral origin. For more explicit conclusions whether the variability is influenced by genetic and/or environmental factors future work is needed on a larger set of samples.

We showed previously that the levels of another bee-derived protein, GOX, varied significantly among samples (Bucekova *et al.* 2014). The variation in

doi: 10.17221/422/2015-CJFS

GOX and defensin-1 contents may be associated with genetic diversity of honeybees (various genotypes). Our previous studies documented that the levels of the bee antimicrobial peptide defensin-1, semi-quantified by immunoblotting, vary in larval jelly and honey samples, and the results indicated that the variations are determined by genetic factors (Klaudiny *et al.* 2012; Majtan *et al.* 2012). Using *in situ* hybridisation, we have shown that the defensin-1 is constitutively expressed only in the hypopharyngeal glands of all types of workers, from where it is secreted into the honey and larval jelly. Previously it has been reported that defensin-1 is expressed also in other bee glands (Klaudiny *et al.* 2005). However it is possible that during dissections the fat body has been unwittingly included into mRNA extracts and *def* mRNA from this tissue was subsequently amplified by RT-PCR, leading to the inaccurate localisation of expression. The expression of defensin-1 could be induced also in the fat body upon infection, as we detected in immune-challenged bees, by injuring with a needle soaked in a lipopolysaccharide solution (data not shown). It has also been shown that defensin-1 is one of the ten most frequently expressed genes in foragers and nurses (Liu *et al.* 2011). However, its expression differs among individuals of a population as evidenced by RT-PCR (Majtan *et al.* 2012). The different rates of expression may be among the factors affecting the final levels of defensin-1 in honeys. Variations in the amount of MRJP1 in natural honeys have also been described (Bilikova & Simuth 2010). A significant correlation exists between the production of defensin-1 (quantified by ELISA in this study) and GOX levels [semi-quantified in the same samples in our previous work (n = 20, r = 0.7757, P < 0.0001; data not shown)]. It is likely that the levels of more bee-derived proteins/peptides in honey, including those contributing to the antibacterial activity of honey (e.g. defensin-1 and GOX), could be influenced by honeybee genetic/epigenetic factors. Higher production of defensin-1 and GOX by honeybees positively affects the total antibacterial activity of honey. Our results are consistent with another study showing that high protein concentrations in honey tend to result in low MIC values (i.e. good antimicrobial activity) while low protein concentrations (due to impurities or adulterations in honeys) result in decreased antimicrobial activity (Khan *et al.* 2014).

It has been mentioned above that GOX-mediated generation of H_2O_2 significantly affects the total antibacterial activity of honey. The maximum levels of accumulated H_2O_2 occurred in honey solutions diluted to concentrations between 30% and 50% (v/v) with at least 50% of the maximum levels occurring at 15% to 67% (v/v). Maximum levels of H_2O_2 reached in the diluted honeys were in the range of 1–2 mmol/l (Bang et al. 2003).

In conclusion, we have established and evaluated a polyclonal antibody based competitive ELISA for the quantification of defensin-1 in honey. The method to detect honeybee-derived proteins in honey can be a promising approach to verify the authenticity of honey. In addition, we showed that antibacterial bee-derived defensin-1 is a regular component of honey and it significantly contributes to the total antibacterial activity of honey against Gram-positive bacteria. Quantification of the defensin-1 level could therefore be useful in order to select honeys with a high potential antibacterial effect.

Acknowledgements. We would like to thank Dr Ladislav Roller for help with the confocal microscopy.

References

Adams C.J., Boult C.H., Deadman B.J., Farr J.M., Grainger M.N., Manley-Harris M., Snow M.J. (2008): Isolation by HPLC and characterisation of the bioactive fraction of New Zealand manuka (*Leptospermum scoparium*) honey. Carbohydrate Research, 343: 651–659.

Alvarez-Suarez J.M., Giampieri F., González-Paramás A.M., Damiani E., Astolfi P., Martinez-Sanchez G., Bompadref S., Quilesg J.L., Santos-Buelgab C., Battinoa M. (2012): Phenolics from monofloral honeys protect human erythrocyte membranes against oxidative damage. Food and Chemical Toxicology, 50: 1508–1516.

Araujo C.A.C., Waniek P.J., Stocka P., Mayer C., Jansen A.M., Schaub G.A. (2006): Sequence characterization and expression patterns of defensin and lysozyme encoding genes from the gut of the reduviid bug *Triatoma brasiliensis*. Insect Biochemistry and Molecular Biology, 36: 547–560.

Bang L.M., Bantting C., Molan P.C. (2003): The effects of dilution rate on hydrogen peroxide production in honey and its implications for wound healing. Journal of Alternative and Complementary Medicine, 9: 267–273.

Baroni M.V., Chiabrando G.A., Costa C., Fagúndez G.A., Wunderlin D.A. (2004): Development of a competitive ELISA for the evaluation of sunflower pollen in honey samples. Journal of Agricultural and Food Chemistry, 52: 7222–7226.

Bilikova K., Simuth J. (2010): New criterion for evaluation of honey: quantification of royal jelly protein apalbumin 1

doi: 10.17221/422/2015-CJFS

in honey by ELISA. Journal of Agricultural and Food Chemistry, 58: 8776–8781.

Bucekova M., Valachova I., Kohutova J., Prochazka E., Klaudiny J., Majtan J. (2014): Honeybee glucose oxidase – its expression in honeybee workers and comparative analyses of its content and H_2O_2-mediated antibacterial activity in natural honeys. Naturwissenschaften, 101: 661–670.

Bulet P., Hetru C., Dimarcq J.L., Hoffmann D. (1999): Antimicrobial peptides in insects; structure and function. Developmental & Comparative Immunology, 23: 329–344.

Bulet P., Stocklin R. (2005): Insect antimicrobial peptides: structures, properties and gene regulation. Protein & Peptide Letters, 12: 3–11.

Buttstedt A., Moritz R.F.A, Erler S. (2014): Origin and function of the major royal jelly proteins of the honeybee (*Apis mellifera*) as members of the yellow gene family. Biological Reviews, 89: 255–269.

Casteels P. (1998): Immune response in Hymenoptera. In: Brey P.T., Hultmark D. (eds): Molecular Mechanisms of Immune Responses in Insect. London, Chapman & Hall: 92–10.

Casteels-Josson K., Zhang W., Capaci T., Casteels P., Tempst P. (1994): Acute transcriptional response of the honeybee peptide-antibiotics gene repertoire and required post-translational conversion of the precursor structures. Journal of Biological Chemistry, 269: 28569–28575.

Clinical and Laboratory Standards Institute (2003): Methods for dilution of antimicrobial susceptibility tests for bacteria that growth aerobically. Approved Standard, 6th Ed. NCCLS document M7-A6. Wayne, NCCLS.

Codex Alimentarius (2001): Codex Standard for Honey, Codex Stan 12-1981, Rev.1 (1987), Rev.2 (2001).

De Grandi-Hoffman G., Hagler J. (2000): The flow of incoming nectar through a honey bee 513 (*Apis mellifera* l.) colony as revealed by a protein marker. Insectes Sociaux, 47: 302–306.

Dunford C., Cooper R., Molan P., White R. (2000): The use of honey in wound management. Nursing Standard,15: 63–68.

Eddy J.J., Gideonsen M.D. (2005): Topical honey for diabetic foot ulcers. Journal of Family Practice, 54: 533–535.

Elflein L., Raezke K.P. (2008): Improved detection of honey adulteration by measuring differences between $^{13}C/^{12}C$ stable carbon isotope ratios of protein and sugar compounds with a combination of elemental analyser-isotope ratio mass spectrometry and liquid chromatography-isotope ratio mass spectrometry (δ ^{13}C-EA/LC–IRMS). Apidologie, 39: 574–587.

Escuredo O., Míguez M., Fernández-González M., Seijo M.C. (2013): Nutritional value and antioxidant activity of honeys produced in a European Atlantic area. Food Chemistry, 138: 851–856.

EU Council (2002): Council Directive 2001/110/EC of 20 December 2001 relating to honey. Official Journal of the European Communities, L 10: 47–52.

Fujiwara S., Imai J., Fujiwara M., Yaeshima T., Kawashima T., Kobayashi K. (1990). A potent antibacterial protein in royal jelly. Purification and determination of the primary structure of royalisin. Journal of Biological Chemistry, 265: 11333–11337.

Di Girolamo F., D'Amato A., Righetti P.G. (2012): Assessment of the floral origin of honey via proteomic tools. Journal of Proteomics, 75: 3688–3693.

Hanes J., Simuth J. (1992): Identification and partial characterization of the major royal jelly protein of the honey bee (*Apis mellifera* L.). Journal of Apicultural Research, 31: 22–26.

Inglesias M.Z., Martin-Alvarez P.J., Polo C., de Lorenzo C., Pueyo E. (2006): Protein analysis of honeys by fast protein liquid chromatography: application to differentiate floral and honeydew honeys. Journal of Agricultural and Food Chemistry, 54: 8322–8327.

Kato Y., Araki Y., Juri M., Fujinaka R., Ishisaka A., Kitamoto N., Nitta Y., Niwa T., Takimoto Y. (2014): Immunochemical authentication of manuka honey using a monoclonal antibody specific to a glycoside of methyl syringate. Journal of Agricultural and Food Chemistry, 62: 10672–10678.

Khan F., Hill J., Kaehler S., Allsopp M., van Vuuren S. (2014): Antimicrobial properties and isotope investigations of South African honey. Journal of Applied Microbiology, 117: 366–379.

Kim Y.J., Zitnan D., Cho K.H., Mizoguchi A., Schooley D., Adams M.E. (2006): Endocrine programming of an innate behavior through central release of peptide cotransmitters. Proceedings of the National Academy of Sciences of the United States of America, 103: 14211–14216.

Kingan T.G., Zitnan D., Jaffe H., Beckage N.E. (1997): Identification of neuropeptides in the midgut of parasitized insects: FLRFamides as candidate paracrines. Molecular and Cellular Endocrinology, 133: 19–32.

Klaudiny J., Albert S., Bachanova K., Kopernick J., Simuth J. (2005): Two structurally different defensin genes, one of them encoding a novel defensin isoform, are expressed in honeybee *Apis mellifera*. Insect Biochemistry and Molecular Biology, 35: 11–22.

Klaudiny J., Bachanová K., Kohútová L., Dzůrova M., Kopernický J., Majtán J. (2012): Expression of larval jelly antimicrobial peptide defensin1 in *Apis mellifera* colonies. Biologia (Bratislava), 67: 200–211.

Kwakman P.H., Te Velde A.A., de Boer L., Vandenbroucke-Grauls C.M., Zaat S.A. (2010): How honey kills bacteria. FASEB Journal, 24: 2576–2582.

doi: 10.17221/422/2015-CJFS

Kwakman P.H., Te Velde A.A., de Boer L., Speijer D., Vandenbroucke-Grauls C.M., Zaat S.A. (2011): Two major medicinal honeys have different mechanisms of bactericidal activity. PLoS ONE, 6(3): e17709. doi:10.1371/journal.pone.0017709

Liu F., Li W., Li Z., Zhang S., Chen S., Su S. (2011): High-abundance mRNAs in *Apis mellifera*: comparison between nurses and foragers. Journal of Insect Physiology, 57: 274–279.

Majtan J., Klaudiny J., Bohova J., Kohutova L., Dzurova M., Sediva M., Bartosova M., Majtan V. (2012): Methylglyoxal-induced modifications of significant honeybee proteinous components in manuka honey: Possible therapeutic implications. Fitoterapia, 83: 671–677.

Majtan J., Majtan V. (2010): Is manuka honey the best type of honey for wound care? Journal of Hospital Infection, 74: 305–306.

Marshall T., Williams K.M. (1987): Electrophoresis of honey: characterization of trace proteins from complex biological matrix by silver staining. Analytical Biochemistry, 167: 301–303.

Mavric E., Wittmann S., Barth G., Henle T. (2008): Identification and quantification of methylglyoxal as the dominant antibacterial constituent of Manuka (*Leptospermum scoparium*) honeys from New Zealand. Molecular Nutrition & Food Research, 52: 483–489.

Molan P.C. (1992): The antibacterial activity of honey: 1. The nature of the antibacterial activity. Bee World, 73: 5–28.

Molan P.C. (2006): The evidence supporting the use of honey as a wound dressing. International Journal of Lower Extremity Wounds, 5: 40–54.

Oplatowska M., Elliott C.T., Huet A.C., McCarthy M., Mulder P.P., von Holst C., Delahaut P., van Egmond H.P., Campbell K. (2014): Development and validation of a rapid multiplex ELISA for pyrrolizidine alkaloids and their N-oxides in honey and feed. Analytical and Bioanalytical Chemistry, 406: 757–770.

Eteraf-Oskouei T., Najafi M. (2013): Traditional and modern uses of natural honey in human diseases: a review. Iranian Journal of Basic Medical Sciences, 16: 731–742.

Sak-Bosnar M., Sakac N. (2012): Direct potentiometric determination of diastase activity in honey. Food Chemistry, 135: 827–831.

Seeley T.D. (1992): The tremble dance of the honey bee: message and meanings. Behavioral Ecology and Sociobiology, 31: 375-383.

Tosun M. (2013): Detection of adulteration in honey samples added various sugar syrups with $^{13}C/^{12}C$ isotope ratio analysis method. Food Chemistry, 138: 1629–1632.

Valachova I., Bohova J., Palosova Z., Takac P., Kozanek M., Majtan J. (2013): Expression of lucifensin in *Lucilia sericata* medicinal maggots in infected environments. Cell and Tissue Research, 353: 165–171.

Wang J.X., Zhao X.F., Liang Y.L., Li L., Zhang W.W., Ren Q., Wang L.C., Wang L.Y. (2006): Molecular characterization and expression of the antimicrobial peptide defensin from the housefly (*Musca domestica*). Cellular and Molecular Life Sciences, 63: 3072–3082.

Wang S., Liu J., Yong W., Chen Q., Zhang L., Dong Y., Su H., Tan T. (2015): A direct competitive assay-based aptasensor for sensitive determination of tetracycline residue in honey. Talanta, 131: 562–569.

Wang R., Wang Z., Yang H., Wang Y., Deng A. (2012): Highly sensitive and specific detection of neonicotinoid insecticide imidacloprid in environmental and food samples by a polyclonal antibody-based enzyme-linked immunosorbent assay. Journal of the Science of Food and Agriculture, 92: 1253–1260.

White J.W., Schepartz A.I., Subers M.H. (1963): The identification of inhibine, the antibacterial factor in honey, as hydrogen peroxide and its origin in a honey glucose-oxidase system. Biochimica et Biophysica Acta, 73: 57–70.

Yamaguchi K., He S., Li Z., Murata K., Hitomi N., Mozumi M., Ariga R., Enomoto T. (2013): Quantification of major royal jelly protein 1 in fresh royal jelly by indirect enzyme-linked immunosorbent assay. Bioscience, Biotechnology, and Biochemistry, 77: 1310–1312.

Yu F., Yu S., Yu L., Li Y., Wu Y., Zhang H., Qu L., Harrington P.B. (2013): Determination of residual enrofloxacin in food samples by a sensitive method of chemiluminescence enzyme immunoassay. Food Chemistry, 149: 71–75.

Zitnanova I., Adams M.E., Zitnan D. (2001): Dual ecdysteroid action on epitracheal glands and the central nervous system preceding ecdysis of *Manduca sexta*. Journal of Experimental Biology, 204: 3483–3495.

Received: 2015–08–27
Accepted after corrections: 2016–05–23
Published online: 2016–06–06

Corresponding author:

RNDr. Ivana Valachová, PhD., Univerzita Komenského v Bratislavě, Přírodovědecká fakulta, Katedra zoologie, Mlynská dolina, Ilkovičova 6, 842 15 Bratislava 4, Slovenská republika; E-mail: ivana.valachova@uniba.sk