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Evaluation of Antioxidant Activity and Flavonoid Composition in Differently Preserved Bee Products

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Abstract

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Antioxidant potential and composition of phenolic compounds were studied in pure bee products (beebread – BB, bee pollen – BP) and in their mixtures with honey and vegetable oils [sea buckthorn (SBO), flax seed (FSO), royal jelly (RJ)], and the alga Spirulina (SA). The values of total phenolic content (TPC) found in the raw weight (RW) of material of BP, BB, SA, and RJ were 23.3, 21.2, 15.4, and 10.7 GAE mg/g, respectively. The methanolic extract of pure BP possessed higher $ABTS^{\cdot+}$scavenging capacity (5.37–6.47 TE mg/g RW) than BB (4.86–5.70 TE mg/g RW). The values of oxygen radical absorbance capacities (ORAC) of methanolic extracts of BB and BP were 626.30 and 894.04 TE mg/g RW, respectively. An analysis of flavonoids in the products by the ultra performance liquid chromatography showed that pure BP possesses a broader spectrum of compounds than pure BB. Three forms of glycosides were identified in BP: quercetin 3-*O*-sophoroside, quercetin dihexoside and isorhamnetin 3-glucoside. Rhamnetin and isorhamnetin as well as their glycosides and kaempferol 3-*O*-α-L-(2"-*E*-*p*-coumaroyl-3"-*Z*-*p*-coumaroyl)-rhamnoside were determined in the samples of pollen mixed with honey (BPH).

Keywords: methanolic extracts; glycosides; pollen; beebread; vegetable oils

Honeybee-collected pollen and beebread are recognised as a well-balanced food (González-Güerca *et al*. 2001; Čeksterytė & Jansen 2012). These beehive products also possess several useful pharmacological properties, such as antibiotic, antineoplastic, and antioxidant activity. Honeybee-collected pollen has been reported as a free radical scavenger and lipid peroxidation inhibitor, the properties associated with the phenolic content (Campos & Cunha 1994; Campos *et al*. 1997). It has also been suggested that the pollen of different botanical origin possesses different antioxidant capacity, which is more related to the specific flavone and phenolic acid profiles than to the total content of flavones (Almaraz-Abarca *et al*. 2004). The highest content of phenolic compounds was found in darker honeys such as buckwheat and heather. Honey phenolics consist mainly of flavonoids and phenolic acids; however, the concentration of the latter group has been reported to be higher (Kaškonienė *et al*. 2009). Food supplements based on bee products, mainly on honey and pollen or beebread mixes, are widely used in the treatment of various health disorders.

Antioxidant properties, phenolic profiles of honey, and beebread from Lithuania were previously studied and it was reported that flavonoid content is higher in beebread and its antioxidant capacity is remarkably stronger than that of honey (Čeksterytė *et al*. 2006; Baltrušaitytė *et al*. 2007). Croatian researchers

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studied the influence of thermal processing on the total phenolic content and antioxidant activity in honey. The authors stated that a short thermal treatment at a relatively low temperature had no negative impact on resultant indicators (Šarić *et al.* 2013). However, the authors did not show the relation between antioxidant activity and humidity as well as total phenolic content and humidity at different duration and temperature of honey heating. The concentration of phenolic compounds in the extracts produced depends on many factors: the use of means and solvents for extraction, its concentration, and length of material maceration (Kasparavičienė *et al*. 2013). Japanese scientists indicated that the antioxidant capacity of beebread extracts of Lithuanian origin isolated with 20°C water was higher as compared to the extracts prepared with boiling water/ethanol or ascorbic acid (Nagai *et al*. 2004). Antioxidants such as ascorbic acid, tocopherols, ubiquinol-10, flavonoids, polyphenols, glutathione, glutathione peroxidases, and reductase, catalases and other peroxidases protect lipids, proteins and DNA against damage by reactive oxygen species in the human body (Sies 1997). The same antioxidant classes occur in many products, such as tea, medicinal plants, spices, fruits, vegetables, honey, beebread, etc. (Wettasinghe & Shahidi 2000; Riemersma *et al*. 2001; Čeksterytė *et al*. 2006). Bioactive properties and content of compounds in beebread are associated with its botanical origin. More than 200 compounds were identified in beebread samples collected in the Baltic region. In this study, it was found that all beebread samples, extracted with organic solvents of different polarity, contained phenolic antioxidants, unsaturated fatty acids, carbohydrates, free amino acids, C_{21}–C_{35} alkanes, unsaturated alcohols, carbohydrate acids (Isidorov *et al*. 2009). Beebread contains a wide variety of compounds; however, their activity also depends on the preparation for use, e.g. drying and/or storage conditions. The addition of plant extracts to honey may increase the radical scavenging capacity of its phenol fraction. Beebread prepared for use with honey and some wax particles possesses higher radical scavenging capacity than pure natural honey. Radical scavenging capacity of different Lithuanian honey varied within a wide range, from 43.0% to 86.4%, while for beebread it was from 81.5% to 93.0% (Baltrušaitytė *et al*. 2007).

It is believed that the lack of endogenous antioxidative defence may be compensated for by exogenous antioxidants, mainly obtained with foods and natural food supplements. *Spirulina platensis (*commonly referred to as spirulina) blends with honey are also produced; however, bioactive properties of such products have not been studied until now. Spirulina is a bluish green alga possessing nutritional and therapeutic value; it is approved as a dietary supplement in the USA (Gilroy *et al*. 2000). Spirulina and its extracts isolated with ethanol, water, and methanol possess antioxidant capacity and may inhibit lipid peroxidation (Kuriakose & Kurup 2011; Seo *et al*. 2013). Antioxidant properties of spirulina are associated with phycocyanin, a pigment-protein complex, and polysaccharides having a direct effect on reactive oxygen species. For instance, the concentration of the antioxidant enzyme superoxide dismutase, which reduces the rate of oxygen radical generating reactions, reaches 1700 units/g in spirulina. Spirulina is also rich in minerals, iodine, zinc, selenium, molybdenum, iron, and therefore microelement-enriched algae are used as functional food (Varga *et al*. 1999; Molnár *et al*. 2013).

The composition of beekeeping products was studied individually while no data on bee products combined with vegetable oils and algae in their composition has been found yet. The composition of vegetable oils shows a variety of fatty acids and the presence of vitamin E in their content (Schwartz *et al*. 2008; Vingering *et al*. 2010).

The broad spectrum of different components that complement the composition of bee products could provide a synergistic effect in the latter products. From this point of view, flax seed, sea buckthorn oils, and the alga spirulina may be considered as promising components, providing antioxidants in the mixes. Oils usually contain strong lipophilic antioxidants, possessing vitamin E activity (tocols). Even though honey-pollen and honey-beebread mixtures are widely used and consumed, there is very little evidence available to support their many medical claims.

The aim of this study was to evaluate the composition of phenolic compounds and the antioxidant properties of different samples of beebread and pollen mixtures with honey from Lithuania using a combination of chromatographic and spectroscopic methods.

MATERIAL AND METHODS

Bee pollen-honey and beebread-honey composition with preparation of additives. Pollen loads were collected from early spring to mid-July at the divisions of the apiary of Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry (LAMMC),

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located at different sites of Kėdainiai district. For collecting bee pollen, a standard pollen trap was mounted on the hive entrance and maintained throughout the collection period. Every day, pollen was taken from the traps, cleaned and kept in air-tight plastic bags in a refrigerator at 5–8°C. Beebread was also collected in the apiaries (LAMMC) situated in different locations of Kėdainiai district. After removal from the combs it was cleaned and only beebread pieces of a desirable length of 0.3–1.0 cm were used for analysis. Samples of beebread (BB) were dried at 35°C or 40°C to the moisture level of 8.0–10.0%. One part of the fresh bee pollen (BP) samples and dried beebread was kept in hermetically sealed dishes in a refrigerator at 5–8°C. The other part of the pollen and beebread was used for conservation under provided target for research: beebread or pollen was mixed with honey at a ratio of 1 : 2 (samples BBH and BPH); flax seed oil (FSO), sea buckthorn oil (SBO), royal jelly (RJ) or the alga spirulina (SA) were added to prepared BBH or BPH. A mixture of honey with SA (0.5%) was additionally prepared. All the specimens used for tests and the concentrations of the additives FSO, SBO, RJ, and SA are shown in Table 1. Botanical composition of pollen and beebread samples was determined according to Louveaux *et al*. (1978).

Preparation of methanol extracts for flavonoid study. Two grams of sample (6 g of fresh royal jelly and pollen and 3 g of the alga spirulina) were dissolved in 2 ml of distilled water using a vortex mixer, then mixed with 8 ml of methanol and extracted for 5 min using a vortex mixer. The resulting solution was centrifuged for 15 min (5000 rpm) and filtered through a vacuum filter. After filtration, the methanol-water solution was evaporated to dryness under vacuum. The resulting dried extracts were dissolved in methanol and stored until analysis (4°C). Each sample of oil (10 g) was dissolved in 10 ml of hexane and extracted three times with 6 ml of methanol-water (60 : 40) at room temperature for 2 min using a shaker. After maceration and filtration the separated methanol-water fraction was stored in a freezer until examination.

Determination of total phenolic content. The total phenolic content (TPC) was measured with Folin-Ciocalteu reagent (Singleton *et al*. 1999). Briefly, 30 µl (0.1%) of sample were mixed with 150 µl of

Table 1. Total phenolic content and antioxidant activity of bee products, and their mixtures with plant oils measured in methanol extracts

Sample	Additives	TPC (mg GAE/g)[a]	Antioxidant activity (mg TE/g)[b]		
			DPPH	ABTS	ORAC
Beebread (BB)	–	21.2 ± 0.08	1.14 ± 0.09	4.86 ± 0.99	626.30 ± 0.35
Royal jelly	–	10.7 ± 0.03	0.82 ± 0.28	1.54 ± 0.80	237.74 ± 0.22
Beebread mixed with honey	–	4.1 ± 0.02	1.24 ± 2.80	5.35 ± 0.72	225.06 ± 0.98
	FSO 2%	4.0 ± 0.04	1.25 ± 2.49	5.70 ± 2.30	166.00 ± 0.78
	FSO 2%, SBO 1%	4.0 ± 0.03	1.46 ± 0.30	5.38 ± 1.18	318.03 ± 1.50
	FSO 2%, RJ 2%	4.0 ± 0.52	1.39 ± 1.07	5.35 ± 3.61	163.04 ± 0.65
	SBO 1%	3.9 ± 0.03	1.37 ± 0.45	5.10 ± 1.62	307.46 ± 1.50
	SBO 2%, RJ 2%	3.9 ± 0.05	1.25 ± 2.79	5.15 ± 3.24	265.64 ± 1.10
Bee pollen (BP)	–	23.3 ± 0.01	1.07 ± 0.14	6.47 ± 0.07	894.04 ± 0.69
Pollen mixed with honey	–	3.5 ± 0.05	1.44 ± 0.48	5.29 ± 2.98	133.95 ± 0.77
	FSO 2%	3.6 ± 0.05	1.32 ± 2.00	5.62 ± 1.65	311.08 ± 0.65
	FSO 2%,SBO 1%	3.6 ± 0.04	1.52 ± 0.05	5.47 ± 1.74	266.77 ± 0.98
	FSO 2%, RJ 2%	3.5 ± 0.03	1.49 ± 1.70	5.82 ± 1.55	182.35 ± 2.52
	SBO 1%	3.6 ± 0.03	1.36 ± 1.09	5.37 ± 2.09	185.79 ± 1.37
	SBO 2%, RJ 2%	3.6 ± 0.05	1.19 ± 2.19	5.69 ± 1.92	257.26 ± 0.65
Mean	beebread	7.0 ± 0.22	1.24 ± 0.20	4.80	288.66 ± 147.9
	bee pollen	6.9 ± 0.25	1.28 ± 0.24	5.16	308.62 ± 243.1
$P \leq 0.05$ probability level (between BB and BP)		0.7	0.08	0.47	107.49

[a]expressed as mg of gallic acid equivalents (GAE) per 1 g of raw material; [b]expressed as mg Trolox equivalents (TE) per 1 g of raw material; values are mean ± SD of mean of triplicate analyses; FSO – flax seed oil; SBO – sea buckthorn oil

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10-fold diluted (v/v) Folin-Ciocalteu reagent and 120 µl of 7.5% Na_2CO_3. After mixing all reagents, the microplate was placed in a microplate reader (Biotek El 808; BioTek, Winooski, USA) and shaken for 30 seconds. After incubation for 30 min at room temperature the absorbance was measured at 765 nm. All measurements were performed in triplicate. A series of gallic acid solutions in the concentration range of 0.025–0.35 mg/ml was used for the calibration curve. The results were expressed relatively to the raw weight (RW) of material, mg of gallic acid equivalents per g of RW (mg GAE/g RW). All chemicals used in the experiments were of analytical grade. The dry weight of solid residues of methanolic extracts was determined from the additionally prepared samples by the thermogravimetric principle (Rubinson 1987).

Radical scavenging capacity (RSC). DPPH$^{\bullet}$ scavenging capacity of extracts was determined by a slightly modified spectrophotometric method (Brand-Williams *et al.* 1995) using a 96-well microplate reader. For each well an aliquot of 7.5 µl of extract was mixed with 300 µl of DPPH$^{\bullet}$ (6×10^{-5} mol/l) and the decrease of absorbance was measured during 40 min at 515 nm by comparing with a blank sample containing the same amount of methanol and DPPH$^{\bullet}$. The final RSC values were calculated using a regression equation ($y = 313.33x - 5.2077$; $R^2 = 0.99$), based on the calibration curve prepared using 0.01–0.04 mmol/l Trolox solutions. The antioxidant capacity of each sample is expressed as mg of Trolox equivalent per gram of sample (mg/TE/g RW).

ABTS$^{\bullet+}$ scavenging activity. ABTS$^{\bullet+}$ decolourisation assay was performed according to Re *et al.* (1999). For reaction 3 µl of methanolic extract were mixed with 300 µl of ABTS$^{\bullet+}$ solution and the absorbance was measured after 40 min at $\lambda_{734\ nm}$ against PBS blank. The final RSC values were calculated and expressed as mg TE/g RW (regression equation $y = 66.131x + 0.8608$; $R^2 = 0.981$).

Oxygen radical absorbance capacity (ORAC) assay. The ORAC method was performed as described by Prior *et al.* (2003), by using fluorescein as a fluorescent probe. The stock solution of fluorescein (95.68 nmol/l) in the PBS, pH 7.4, was prepared. Sample (25 µl) and fluorescein (150 µl) solutions were placed in 96 transparent flat-bottom microplate wells, the mixture was pre-incubated at 37°C for 15 min, followed by a rapid addition of AAPH solution as a peroxyl radical generator (25 µl; 240 mmol/l) using a multichannel pipette. The microplate was immediately placed in the Fluorstar Omega reader, automatically shaken prior to each reading and the fluorescence was recorded every cycle (1 min × 1.1), 90 min in total. The 485 nm excitation and 520 nm emission filters were used.

Raw data were exported from the Mars software to Excel 2003 (Microsoft, Roselle, USA) for further calculations. Antioxidant curves (fluorescence vs time) were first normalised, and from the normalised curves the area under the fluorescence decay curve (AUC) was calculated as follows:

$$AUC = (1 + f_1/f_0 + f_2/f_0 + f_3/f_0 + \cdots + f_i/f_0)$$

where: f_0 = initial fluorescence reading at cycle 0; f_i = fluorescence reading at cycle i

The final $ORAC_{FL}$ values were calculated using a regression equation ($y = 0.1508x + 1.0994$; $R^2 = 0.99$) obtained by means of Trolox solutions (50 to 2500 µmol/l) for calibration. The antioxidant capacity is calculated as mg Trolox per gram of sample (mg TE/g RW).

Identification of flavonoids. The Acquity UPLC system was used consisting of a binary solvent delivery system, autosampler with a 10 µl sample loop, photodiode array (PDA) detector, column manager, and a data station running the Compass acquisition and data software (Waters, Milford, USA) combined with a Bruker maXis UHR-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). An Acquity BEH C18 column (1.7 µm, 100 × 2.1 mm, i.d.) was used for the separation of compounds at 40°C. The mobile phase was initially composed of 92% eluent A (1%, v/v, formic acid solution in ultra-pure water) and 8% eluent B (methanol), followed by a linear gradient from 8% to 60% of eluent B in 12 min, and later on, to 97% B in the following 3.0 min and it was kept at these conditions for the following 3.0 minutes. After the analysis, the initial conditions were re-introduced over 1 minute. Before each new run the column was equilibrated for 2 minutes. The flow rate was 0.3 ml/min and the effluents were monitored at 254 nm. The effluents from the PDA detector were introduced directly into the UHR-QTOF mass spectrometer equipped with an ESI source. Instrument control and data acquisition were achieved using the Compass 1.3 (HyStar 3.2 SR2) software. MS experiments were performed in a negative ionisation mode, the capillary voltage was maintained at +4500 V with the end plate offset at –500 V. Nitrogen was used as the drying and nebulising gas at a flow rate of 10 l/min

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and a pressure of 2 bars. Mass spectra were recorded in a range from 115 to 1000 *m/z*, at a rate of 2.5 Hz. The peak identification was carried out by the obtained accurate masses and calculated molecular formulas comparing them to literature data.

Statistical analysis. The analyses were carried out in triplicate. The results were analysed using the ANOVA programme. Estimated values are expressed as means ± standard deviations (SD). Correlation coefficients were calculated by the MS Excel software using the multiple regression statistic type of analyses.

RESULTS AND DISCUSSION

Total phenolic content and antioxidant capacity. In the present investigation there was a wide range of TPC in the raw material (RW) analysed, as shown in Table 1. The highest TPC was determined in the methanol extract of pure beekeeping products BP (23.3 ± 0.1 mg GAE/g RW) and BB (21.2 ± 0.8 mg GAE/g RW). The mean content of TPC for methanol fractions of all BB samples (pure BB and BBH) as well as BP and BPH mixes ranged from 7.0 ± 2.2 to 6.9 ± 2.5 mg GAE/g RW; the differences between mean TPC were not significant at ($P \leq 0.05$). In the samples of RJ the mean values of TPC were 10.7 ± 0.3 mg GAE/g RW.

The mean content of TPC for SA was 15.4 ± 0.3 mg GAE/g RW and in honey, BBH and BPH mixtures with 0.5% of SA it was lower compared to bee products mixed with vegetable oils, reaching 0.3–4.2 mg GAE/g RW (Table 2).

Several authors determined TPC in methanol, ethanol or water extracts of beebread and data show that this content depends on the methods used for extraction. Japanese researchers found that TPC yielded 0.24 mg/ml in Lithuanian beebread ethanol extract while TPC content extracted from beebread with distilled boiling water or with water at 20°C was 0.20 and 0.45 mg/ml, respectively (Nagai *et al*. 2004). Polyphenol content extracted with ethanol from fresh pollen collected in Poland amounted to 21.30 mg GAE/g RW. The data show that the polyphenol concentration diminished within 12 months of storage (Rzepecka-Stojko *et al*. 2012). TPC in flax oil was reported lower than in hemp oil, 1.14 and 2.45 caffeic acid equivalents/g, respectively (Siger *et al*. 2008). TPC values of vegetable oils were very low. The $DPPH^{\cdot}$ and $ABTS^{\bullet+}$ scavenging capacity for methanolic extracts in beebread samples varied in the range from 1.14 ± 0.09 to 1.46 ± 0.3 mg TE/g RW and from 5.10 ± 1.62 to 5.70 ± 2.30 mg TE/g RW, respectively. Pollen demonstrated slightly higher DPPH and $ABTS^{\bullet+}$ scavenging capacity, from 1.19 ± 2.19 to 1.52 ± 0.052 RW mg TE/g and from 5.37 ± 2.09 to 5.82 ± 1.55 RW mg TE/g, respectively. $ABTS^{\bullet+}$ scavenging in beebread and pollen extracts was higher compared to $DPPH^{\cdot}$ scavenging activity ($P \leq 0.05$). In BB samples ORAC values ranged from 166.0 ± 0.78 to 626.30 ± 0.35 mg TE/g RW and in BP from 133.95 ± 0.77 to 894.04 ± 0.69 mg TE/g RW. The mean differences in DPPH, ABTS, ORAC between methanolic extracts of BB and BP were not significant ($P \leq 0.05$). In general, SA possessed the strongest antioxidant capacity in $DPPH^{\cdot}$ assay, whereas BP in ABTS and ORAC assays. ORAC of SA was rather low (38.73 mg TE/g RW) (Table 2), therefore all additives to the SA increased it up to 153.61–203.46 mg TE/g RW.

Table 2. Total phenolic content, antioxidant activity of pure plant oils, and mixtures of bee products with spirulina (SA) measured in methanol extracts

Sample	Additives	TPC (mg GAE/g)[a]	Antioxidant activity (mg TE/g)[b]		
			DPPH	ABTS	ORAC
SA	–	15.4 ± 0.3	1.84 ± 0.04	4.34 ± 0.98	38.73 ± 0.20
Honey	SA 0.5%	0.3 ± 0.2	0.15 ± 0.35	0.72 ± 0.24	153.61 ± 1.13
BPH	SA 0.5%	3.5 ± 0.3	1.21 ± 2.22	5.01 ± 3.74	106.48 ± 1.04
BBH	SA 0.5%	4.2 ± 0.4	1.50 ± 0.18	5.95 ± 0.96	203.46 ± 1.00
FSO	–	0.3 ± 0.2	0.20 ± 0.02	0.16 ± 0.02	6.08 ± 0.55
SBO	–	0.3 ± 0.2	0.21 ± 0.03	0.10 ± 0.03	1.44 ± 0.72

[a]expressed as mg of gallic acid equivalents (GAE) per 1 g of raw material; [b]expressed as mg Trolox equivalents (TE) per 1 g of raw material; values are mean ± SD of mean of triplicate analyses; FSO – flax seed oil; SBO – sea buckthorn oil; bee pollen (BP) or beebread (BB) mixtures with honey (BPH and BBH) were made by ratio 1 : 2

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Table 3. Correlation matrix between the total phenolic content (TPC) and radical scavenging activity (correlation coefficients *r*) for beebread, bee pollen, and alga spirulina in the assay of methanolic extracts

	TPC	DPPH	ABTS	ORAC
Beebread				
TPC	1			
DPPH	–0.528	1		
ABTS	–0.358	0.874**[l]	1	
ORAC	0.849**[l]	–0.116[n]	–0.01[n]	1
Bee pollen				
TPC	1			
DPPH	–0.621	1		
ABTS	0.001[n]	0.64	1	
ORAC	0.884**[l]	–0.411	0.312[n]	
Alga spirulina				
TPC	1			
DPPH	0.793	1		
ABTS	0.354	0.85	1	
ORAC	–0.777	–0.402	0.023	1

**correlations were statistically significant at the $P \leq 0.05$ and $P \leq 0.01$ level of probability; [l] linear correlation; [n]nonlinear correlation

The correlation of antioxidant capacity with TPC was rather complicated for all analysed samples (Table 3).

A high positive correlation (r = 0.874) was found between DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities for BB extracts. A significant correlation was determined between TPC and ORAC for beebread (r = 0.849) and pollen samples (r = 0.884). A high positive correlation was achieved between ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ scavenging activities for extracts of all tested samples; the coefficients in the case of BB, BP, and SA were 0.874, 0.64, and 0.85, respectively. The statistical analysis did not reveal any correlation between ORAC, DPPH$^{\bullet}$, and ABTS$^{\bullet+}$ in BB extracts samples. TPC and HPLC post-column DPPH$^{\cdot}$ scavenging of Lithuanian pollen was reported previously (Kaškonienė *et al*. 2015) and it was shown that the scavenging of radicals by individual flavonoids differs and there is no correlation between TPC values and radical scavenging activity. Lipophilic components such as fatty acids in beebread and numerous forms of hydrophilic enzymes identified in pollen may affect their antioxidant properties (Čeksterytė *et al*. 2014; Treigytė *et al*. 2014).

Flavonoids identified in pollen and beebread. Flavonoids were studied in pure beekeeping products: beebread and pollen, as well as beebread mixed with honey at a ratio of 1 : 2 (sample A) and pollen mixed with honey 1 : 2 (sample B). Typical chromatograms of the compounds are presented in Figure 1.

Compounds 11, 12, 22, and 23 were common for BBH and BPH. Rhamnetin 3-glucoside and isorhamnetin (or rhamnetin) were found in BPH while isorhamnetin 3-glucoside in pure pollen and BPH. The flavonoids vitexin, catechin-(4a→8)-epicatechin, kaempferol 3-*O*-α-L-(2"-*Z*-*p*-coumaroyl)rhamnoside were detected only in BPH. BBH differed from other samples by the presence of α-phenyl lactic acid (compound 5 in chromatogram A). This compound was not found either in BP or in BB. The concentrations of flavonoids and azelaic acid are summarised in Table 4. Two saccharides (RT = 6.0 and 6.5) were found in pure pollen.

Eight compounds were detected in pure beebread, 3 of them were identified as azelaic acid, vitexin, and kaempferol (or luteolin). It may be observed that pure pollen and its mixture with honey (1 : 2) contain a wider spectrum of different compounds than beebread or beebread mixed with honey 1 : 2.

Most likely, the absence of correlations or weak correlations between the values obtained using different

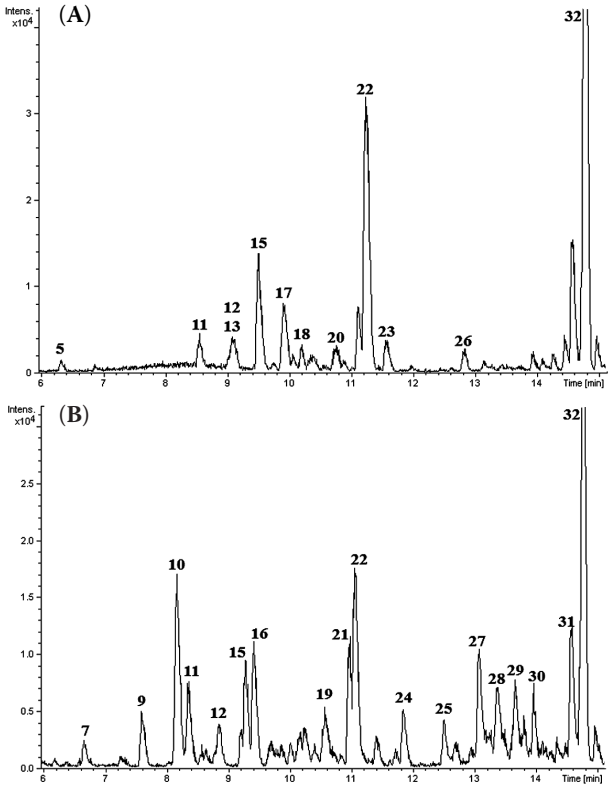

Figure 1. UPLC-MS based peak chromatograms of beebread mixed with honey 1 : 2 (BBH) and in pollen mixed with honey 1 : 2 (BPH); (BBH sample A, BPH sample B)

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Table 4. Compounds identified in beekeeping products by LC-MS

No	RT (min)	Compound	BP	BPH	BBH	BB
			samples assay (n = 3) (mg/g)			
1	6	quercetin 3-*O*-sophoroside	0.16	–	–	–
2	6	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxo-4H-chromen-3-yl 6-*O*-β-D-glucopyranosyl-β-D-glucopyranoside	0.13	–	–	–
3	6	NI[a]	0.35	–	–	–
4	6.2	quercetin dihexoside[a]	0.38	–	–	–
5	6.3	β-phenyl lactic acid[b]			0.11	
6	6.5	5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)-4-oxo-4H-chromen-3-yl 6-*O*-β-D-glucopyranosyl-β-D-glucopyranoside[a]	0.25	–	–	–
7	6.7	quercetin dihexoside[a]	0.42		–	–
8	6.8	NI[c]	0.63	–	–	1.92
9	7.6	rhamnetin 3-glucoside[c]	–	3.70	–	–
10	8.2	isorhamnetin 3-glucoside[c]	0.40	12.62	–	–
11	8.4	quercitrin[c]	–	5.39	2.33	–
12	8.9	azelaic acid[b]	0.11	0.51	0.27	0.18
13	8.9	NI[a]	0.42	2.37	4.11	0.51
14	9.1	NI[b]	0.06	–	–	–
15	9.3	NI[a]	0.16	7.16	10.38	2.77
16	9.4	vitexin isomer[c]	–	8.23	–	–
17	9.8	quercetin[d]	–	–	0.94	0.05
18	10.1	NI[b]	–	–	0.23	–
19	10.6	rhamnetin[e]	–	1.13	–	–
20	10.6	NI[a]	–	4.17	0.84	–
21	10.8	NI[a]	–	7.32	2.45	–
22	11	kaempferol[b]	–	0.92	4.69	0.67
23	11.5	isorhamnetin[e]	–	0.57	0.86	–
24	11.8	tribuloside[a]	–	4.10	–	–
25	12.5	catechin-(4α*8)-epicatechin[a]	–	2.95	–	–
26	12.8	NI[b]	–	–	0.29	–
27	13.0	NI[e]	–	2.14	–	–
28	13.3	NI[e]	–	1.82	–	–
29	13.6	NI[e]	–	1.48		
30	13.9	kaempferol 3-*O*-α-L-(2"-*E*-*p*-coumaroyl-3"-*Z*-*p*-coumaroyl)rhamnoside[a]	–	2.67	–	–
31	14.4	NI[c]	–	–	–	1.30
32	14.5	NI[e]	–	14.63	25.29	2.20
Total identified/not identified			7/5	11/8	6/7	3/5

RT – retention time (min); NI – not identified; compounds quantified as: a – rutin; b – kaempferol; c – vitexin; d – quercetin; e – isorhamnetin

radical scavenging methods in our study may be explained by the qualitative and quantitative differences in individual compounds which may have a higher or a lower contribution to the overall antioxidant capacity. Different flavonoid structures were identified in honey, pollen, and beebread (Baltrušaitytė *et al*. 2007; Isidorov *et al*. 2015). Kaempferol, two forms of quercetin, apigenin, isorhamnetin were identified in beebread. In addition, different forms of sugars such as glucopyranosides and glycosides were also reported in beebread (Isidorov *et al*. 2009). Pollen and beebread contain similar flavonoids and

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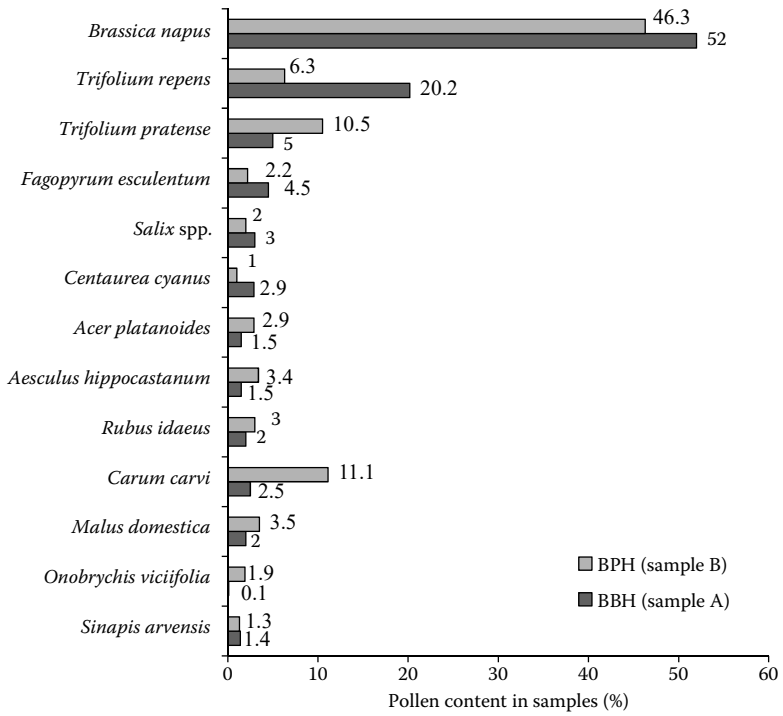

Figure 2. Pollen content (%) in beebread mixed with honey 1 : 2 (BBH) and in pollen mixed with honey 1 : 2 (BPH)

flavonoid glycosides, while their diversity depends on the botanical origin. It was reported that higher antiradical activity of pollen of different botanical origin depends more on flavonoid composition than on TPC (Almaraz-Abarca *et al*. 2004). Flavonoids may serve as biochemical markers of the particular plant source (Tomas-Barberan *et al*. 1989). Quercetin and kaempferol found in pollen are derivatives of flavonol glycosides, which are characterised by a high antiradical activity (Formica & Regelson 1995; von Gadow *et al*. 1997). Screening of radical scavengers by the on-line HPLC-radical scavenging detection identified various flavonoid derivatives possessing different contributions to the antioxidant activity, which also depended on the preparation of material for analysis (Raudonis *et al*. 2010). Therefore, new methods should be developed for the determination of the antioxidant activity for individual flavonoids.

Botanical composition of beebread and pollen. Samples of pure beebread, pollen, and their mixtures with provided additives contained pollen mainly from *Brassica napus* whose content varied in the range from 41.2% to 60.7%.

The botanical composition of samples selected for flavonoid analysis, namely beebread mixed with honey 1 : 2 (sample A) and pollen with honey 1 : 2 (sample B), is shown in Figure 2.

The pollen of *Brassica napus* dominated in both samples, constituting 52.0 and 46.3%, respectively. *Trifolium repens* pollen was secondary – 20.2% in sample A and in sample B it accounted for a minority – 6.3%. The important minor pollen of *Carum carvi* L. in sample B accounted for 11.1% and in sample A, *Trifolium pratense* – 5.0%, *Fagopyrum esculentum* – 4.5%, *Salix* spp. – 3.0%. The content of other pollen did not exceed 3.0%.

CONCLUSIONS

The identification of compounds from phenolic extracts of beekeeping products with the ultra performance liquid chromatography system revealed diversity of their composition and structure. Most of the identified compounds belong to flavonoids.

Pure pollen and pollen mixed with honey 1 : 2 possess a broader spectrum of compounds among which glucopyranosides and flavonoids having different retention time were identified.

The flavonoids quercitrin, kaempferol (or luteolin), isorhamnetin (or rhamnetin) were common for samples of BBH and BPH. Flavonoids in the form of glycosides were not found in BB.

Beekeeping products and mixtures of beekeeping products with additives showed the strongest activity with respect to ORAC radicals compared to DPPH and ABTS radicals. BB and BP extracts possess stronger ABTS radical scavenging activity compared to DPPH$^{\bullet}$.

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The ORAC value was the highest in fresh BP and low in SA – 894.04 and 38.73 TE/g RW, respectively; likewise in vegetable oils FSO and SBO – 6.08 and 1.44 mg TE/g RW, respectively.

A linear significant correlation exists between TPC and ORAC activity for BB and BP extracts as well as between $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ for BB.

$ABTS^{\bullet+}$ and ORAC values were higher for flax seed oil compared to sea buckthorn; however, its content added to the samples had no effect on their antioxidant activity.

References

Almaraz-Abarca N., Campos M.G, Avila-Reyes J.A, Naranjo-Jimenez N., Herrera-Corral J., Gonzalez-Valdez L.S. (2004): Variability of antioxidant activity among honeybee-collected pollen of different botanical origin. Interciencia, 29: 574–578.

Baltrušaitytė V., Venskutonis P.R., Čeksterytė V. (2007): Radical scavenging activity of different floral origin honey and beebread phenolic extracts. Food Chemistry, 101: 502–514.

Brand-Williams W., Cuvelier M.E., Berset C. (1995): Use of a free radical method to evaluate antioxidant activity. LWT-Food Science and Technology, 28: 25–30.

Campos M.G, Cunha P.A. (1994): Free radical scavenger activity of bee pollen. Polyphenols, 94: 415–426.

Campos M.G., Markham K.R., Cunha P.A. (1997): Quality assessment of bee-pollens using flavonoid/phenolic profiles. Polyphenol Communications, 96: 53–54.

Čeksterytė V., Jansen E. (2012): Composition and content of fatty acids in beebread of various floral origin, collected in Lithuania and prepared for storage in different ways. Chemine Technologija, 2: 57–61.

Čeksterytė V., Kazlauskas S., Račys J. (2006): Composition of flavonoids in Lithuanian honey and beebread. Biologija, 2: 28–33.

Čeksterytė V., Kurtinaitienė B., Jansen E., Belova O. (2014): Melissopalynological analysis and biochemical assessment of the nectariferous plants in Lithuania. Baltic Forestry, 20: 218–229.

Formica J.V., Regelson W. (1995): Review of the biology of quercetin and related bioflavonoids. Food Chemical Toxicology, 33: 1061–1080.

Gilroy D., Kauffman K., Hall D., Huang X., Chu F. (2000): Assessing potential health risks from microcystin toxins in blue-green algae dietary supplements. Environmental Health Perspectives, 108: 435–439.

González-Güerca M.C., Almaraz-Abarca N., Ávila-Reyes J.A., Herrera-Corral J., Naranjo-Jiménez N. (2001): Pollen apícola: una alternativa alimenticia y terapéutica. Apitec, 28: 19–23.

Isidorov V.A., Isidorova A.G., Szczepaniak L., Czyżewska U. (2009): Gas chromatographic–mass spectrometric investigation of the chemical composition of beebread. Food Chemistry, 115: 1056–1063.

Isidorov V.A., Bagan R., Bakier S., Święcicka I. (2015): Chemical composition and antimicrobial activity of Polish herbhoney. Food Chemistry, 171: 84–88.

Kasparavičienė G., Ramanauskienė K., Savickas A., Velžienė S., Kalvėnienė Z., Kazlauskienė D., Ragažinskienė O., Ivanauskas K. (2013): Evaluation of total phenolic content and antioxidant activity of different *Rosmarinus officinalis* L. ethanolic extracts. Biologija, 59: 39–44.

Kaškonienė V., Maruška A., Kornyšova O., Charczun N., Ligor M., Buszewski B. (2009): Quantitative and qualitative determination of phenolic compounds in honey. Chemine Technologija, 3: 75–80.

Kaškonienė V., Kaškonas P., Maruška A. (2015): Volatile compounds composition and antioxidant activity of bee pollen collected in Lithuania. Chemical Papers, 69: 291–299.

Kretavičius J., Kurtinaitienė B., Račys J., Čeksterytė V. (2010): Inactivation of glucose oxidase during heat-treatment decrystallization of honey. Zemdirbyste-Agriculture, 97: 116–122.

Kuriakose G.C., Kurup M.G. (2011): Antioxidant and antihepatotoxic effect of *Spirullina laxissima* against carbon tetrachloride induced hepatotoxicity in rats. Food & Function, 2: 190–196.

Louveaux J., Maurizio A., Vorwohl G. (1978): Methods of melissopalinology. Bee World, 59: 139–157.

Molnár S., Kiss A., Virág D., Forgó P. (2013): Comparative studies on accumulation of selected microelements by *Spirulina platensis* and *Chlorella vulgaris* with the prospects of functional food development. Journal of Chemical Engineering & Process Technology, 4: 2–6.

Nagai T., Nagashima T., Myoda T., Inoue R. (2004): Preparation and functional properties of extracts from bee bread. Nahrung, 48: 226–229.

Prior R.L, Hoang H., Gu L., Wu X., Bacchiocca M., Howard L., Hampsch-Woodill M., Huang D., Ou B., Jacob R. (2003): Assays for hydrophilic and lipophilic antioxidant capacity (oxygen radical absorbance capacity (ORAC(FL))) of plasma and other biological and food samples. Journal of Agricultural and Food Chemistry, 51: 3273–3279.

Raudonis R., Bumblauskiene L., Jakstas V., Pukalskas A., Janulis V. (2010): Optimization and validation of post-column assay for screening of radical scavengers in herbal raw materials and herbal preparations. Journal of Chromatography A, 1217: 7690–7698.

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Re R., Pellegrini N., Proteggente A., Pannala A., Yang M., Rice-Evans C. (1999): Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine, 26: 1231–1237.

Riemersma M.A., Rice-Evans C.A., Tyrrell R.M., Clifford M.N., Lean M.E. (2001): Tea flavonoids and cardiovascular health. QJM: An International Journal of Medicine, 94: 277–282.

Rubinson K.A. (1987): Chemical Analysis. Boston, Little, Brown and Company: 178–179, 243–245.

Rzepecka-Stojko A., Stec M., Kurzeja E., Gawrońska E., Pawłowska-Góral K. (2012): The effects of storage of bee pollen extracts on the polyphenol content. Polish Journal of Environmental Studies, 21: 1007–1011.

Schwartz H., Ollilainen V., Piironen V., Lampi A.M. (2008): Tocopherol, tocotrienol and plant sterol contents of vegetable oils and industrial fats. Journal of Food Composition and Analysis, 21: 152–161.

Seo J.C., Choi W.S., Park J.H., Park J.O., Jung K.H., Lee H.J. (2013): Stable isolation of phycocyanin from *Spirulina platensis* associated with high-pressure extraction process. International Journal of Molecular Sciences, 14: 1778–1787.

Sies H. (1997): Oxidative stress: oxidants and antioxidants. Experimental Physiology, 82: 291–295.

Siger A., Nogala-Kalucka M., Lampart-Szczapa E. (2008): The content and antioxidant activity of phenolic compounds in cold-pressed plant oils. Journal of Food Lipids, 15: 137–149.

Singleton V.L., Orthofer R., Lamuela-Raventos R.M. (1999): Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods in Enzymology, 299: 152–178.

Šarić G., Marković K., Vulkević D., Lež E., Hruškar M., Vahčić N. (2013): Changes of antioxidant activity in honey after treatment. Czech Journal of Food Sciences, 31: 601–606.

Tomas-Barberan F.A., Tomas-Lorente F., Ferreres F., Garcia-Viguera F. (1989): Flavonoids as a biochemical markers of the plant origin of bee pollen. Journal of the Science of Food and Agriculture, 47: 337–340.

Treigytė G., Zaikova I., Matuzevičius D., Čeksterytė V., Dabkevičienė G., Kurtinaitienė B., Navakauskienė R. (2014): Comparative proteomic analysis of pollen of *Trifolium pratense*, *T. alexandrinum* and *T. repens*. Zemdirbyste-Agriculture, 101: 453–460.

Varga L., Szigeti J., Kovács R., Földes T., Buti S. (1999): Influence of a *Spirulina platensis* biomass on the microflora of fermented ABT milks during storage (R1). Journal of Dairy Science, 85: 1031–1038.

Vingering N., Oseredczuk M., du Chaffaut L., Ireland J., Ledoux M. (2010): Fatty acid composition of commercial vegetable oils from the French market analysed using a long highly polar column. Oleagineux, Corps gras, Lipides, 17: 185–192.

Von Gadow A., Joubert E., Hansmann C.F. (1997): Comparison of the antioxidant activity of aspalathin with that of other plant phenols of Rooibos tea (*Aspalathus linearis*), α-tocopherol, BHT, and BHA. Journal of Agricultural and Food Chemistry, 45: 632–638.

Wettasinghe M., Shahidi F. (2000): Scavenging of reactive-oxygen species and DPPH free radicals by extracts of borage and evening primrose meals. Food Chemistry, 70: 17–26.

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