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Radical scavenging activity of different floral origin honey and beebread phenolic extracts

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Abstract

Phenolic extracts were isolated from 35 honey and nine beebread samples obtained from different sources in Lithuania by using Amberlite XAD-2 resin. The antioxidant properties of extracts were assessed by the $ABTS^{\cdot+}$ radical cation decolourisation and $DPPH^{\cdot}$ radical scavenging activity. It was found that all honey and beebread extracts were able to scavenge free radicals, however their scavenging activity varied in a wide range, on average between 43.0% and 95.7%. The preliminary screening of phenolic compounds in honey samples was performed by high-performance liquid chromatography with UV and mass spectrometer detectors. The results obtained showed that all samples contain *p*-coumaric acid, kaempferol, chrysin and apigenin. This study demonstrates remarkable variations in antioxidant properties and content of phenolic compounds in honey from different sources; these variations should be considered in using honey as a source of natural dietary antioxidants.

Keywords: Honey; Beebread; Antioxidant activity; DPPH; ABTS; HPLC

1. Introduction

There is a growing demand of natural products in human diet, both due to the possible negative effects of synthetic food additives on human health and to the increased consumer perception of this problem in recent years. Numerous studies demonstrate that a great number of medicinal and aromatic herbs, as well as fruits and leaves of some berry plants biosynthesize phytochemicals possessing antioxidant activity and may be used as a natural source of free radical scavenging compounds (Javanmardi, Khalighi, Kashi, Bais, & Vivanco, 2002; Miliauskas, Venskutonis, & Van Beek, 2004; Sacchetti et al., 2005; Wang & Lin, 2000; Yu, Zhou, & Parry, 2005). The majority of these plants are used by the bees to collect honey nectar; consequently plant origin bioactive components can be transferred to honey. Numerous studies reported that a great number of natural compounds possess health-promoting properties (The National Honey Board, 2002). Honey is known to be rich in both enzymatic and non-enzymatic antioxidants, including glucose oxidase, catalase, ascorbic acid, flavonoids, phenolic acids, carotenoid derivatives, organic acids, Maillard reaction products, amino acids and proteins (Aljadi & Kamaruddin, 2004; Al-Mamary, Al-Meeri, & Al-Habori, 2002; Gheldof & Engeseth, 2002; Gheldof, Wang, & Engeseth, 2002; Schramm et al., 2003). Flavonoids pinobanksin, pinocembrin, quercetin, chrysin, galangin, luteolin and kaempferol were reported in honey (Gheldof et al., 2002; The National Honey Board, 2002), while pinocembrin, pinobanksin and chrysin are characteristic flavonoids of propolis; these flavonoids were determined in the most previously analyzed European honey samples (Yao, Jiang, Singanusong, Datta, & Raymont, 2003b).

It was reported that the composition and antioxidant capacity of honey depend on the floral source used to collect nectar; seasonal and environmental factors, as well as

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processing may also have an effect on honey composition and antioxidant activity (Al-Mamary et al., 2002; Chen, Mechta, Berebaum, Zangerl, & Egeseth, 2000; Frankel, Robinson, & Berenbaum, 1998; Gheldof & Engeseth, 2002; Gheldof et al., 2002; Yao et al., 2003). Some reports showed possible correlations between floral origin and flavonoid profiles (Anklam, 1998; Yao et al., 2004). Predominance of some individual components or a group of compounds in honey is a promising marker for the determination of honey botanical origin. For example, the flavanone hesperitin can be used as a marker for citrus honey; 8-methoxy-kaempferol was the main compound in rosemary, luteolin in lavender and quercetin in sunflower honey (Anklam, 1998; Yao, & Datta et al., 2003).

In general, higher antioxidant capacity was found for darker honey samples (Chen et al., 2000; Frankel et al., 1998; Gheldof & Engeseth, 2002; Nagai, Sakai, Inoue, Inoue, & Suzuki, 2001) as well as in honey with higher content of water (Aljadi & Kamaruddin, 2004; Frankel et al., 1998). Honey color depends on the potential alkalinity and ash content, as well as on the antioxidatively active pigments, such as carotenoids and flavonoids (Frankel et al., 1998).

The antioxidant properties of honey were tested in ground turkey (McKibben & Engeseth, 2002) and turkey breast meat (Antony, Rieck, & Dawson, 2000) to protect against lipid oxidation, in fruit and vegetable homogenates to inhibit enzymatic browning (Chen et al., 2000) and in living organism to retard biologically destructive reactions (Gheldof, Wang, & Engeseth, 2003; Schramm et al., 2003).

The composition of active components in plants depends on various factors, particularly plant bio and chemotype and climatic conditions. Consequently, it can be reasonably expected that honey properties from different locations should be different. Honey production in Lithuania has very long traditions tracking to ancient times; however, its composition and bioactive properties until now have not been studied more comprehensively. The major purpose of this work was to evaluate the radical scavenging activity of different botanical origin Lithuanian honey samples and some other bee products. Although regarded as a first step in characterization of Lithuanian honey and other bee products this study is expected to expand existing knowledge on biological properties of honey and beebread and to assist in more focused design of further research, e.g. aiming at more specified applications of honey and other bee products as natural remedies and/or functional food ingredients.

2. Materials and methods

2.1. Honey samples and their classification

Honey and beebread was obtained from apiarist throughout Lithuania. All samples were collected during the flowering season in 2003, except for the three samples, which were collected in 1999, 2000 and 2001. The floral source of honey samples was determined by the melissophalynological method (Louveaux, Maurizzio, & Vorwohl, 1978; Persano Oddo, Piazza, Sabatini, & Accorti, 1995). Pollen was identified by using previously published data (Burmistrov & Nikitina, 1990; Straka, 1975) and pollen collection of well-known plants, which was prepared for microscoping at the Apicultural Department of the Lithuanian Institute of Agriculture. After the identification of 200–300 pollen and honeydew elements in honey samples, the pollen of plants which do not accumulate nectar and the elements of honeydew were deducted from the total sum. Accordingly, the contribution of nectar-bearing plants to the botanical composition of honey was calculated. The nectar pollen not exceeding 1.0% in the total composition was summed up and considered as a single pollen group. Honey meeting botanical and chemical composition requirements established by the rules of the International Commission for Bee Botany, presently called International Commission for Plant–Bee Relationships was considered as unifloral (Accorti, Persano Oddo, Piazza, & Sabatini, 1986; Louveaux et al., 1978).

The predominant sources of the majority of honey samples were rape and willow. Some honey samples were collected from different flowers (multifloral) and when bees were fed with pine (*Pinus silvestris*), birch (*Betula pendula*) and stinging nettle (*Urtica dioica*) extract additives. Honey samples obtained without the use of plant extracts further are referred as ''natural honey''. The samples of beebread collected during 1999–2003 were also examined. The sources and detailed characterization of honey and beebread samples are listed in Table 1.

2.2. Extraction of phenolic compounds

Extraction was carried out as described previously (Gheldof et al., 2002; Yao et al., 2003; Yao et al., 2004). Sixty grams of Amberlite XAD-2 resin, pore size 9 nm, particle size 0.3–1.2 mm (Supelco, Bellefonte, PA, USA) were soaked in methanol for 10 min, after that most of methanol was decanted and replaced by distilled water. The mixture was stirred, allowed to stand for 5–10 min and was packed into the glass column, 25×2 cm.

Honey and beebread (25–50) g were dissolved in 250 ml of distilled water, and the pH of the solution was adjusted to pH 2.0 by adding concentrated HCl. The solution was filtered slowly through the column with Amberlite XAD-2 resin. The column was washed with 250 ml of acidified water (pH 2 with HCl) and subsequently rinsed with 300 ml of neutral distilled water to remove all sugars and other polar compounds of honey or beebread. The phenol compounds were eluted from the sorbent with 250 ml of methanol (Polskie Odczynniki Chemiczne, Poland). The methanol extracts were concentrated under vacuum at 40 °C in a rotary evaporator Büchi R-114 (Donau, Flawil, Switzerland). The residue was dissolved in 5 ml of distilled water and extracted three times with 5 ml of diethyl ether

Table 1
Characterisation of tested honeys and beebread

Sample code	Data of collection	Botanical composition, %	Municipality	Location
K01-MF	2003 06 03	Fruit tree – 35.6; spring rape (*Brassica napus* L.*ssp. oleifera annua*Metzg.) – 18.8	Kedainiai distr.	Akademija
K02-W	2003 06 09	Willow (*Salix alba* L., *Salix caprea* L.) – 76.1; fruit tree – 10.3	Kedainiai distr.	Krakes, Janiunai
K03-W	2003 06 09	Willow – 68.9 (*Salix alba* L., *Salix caprea* L.); fruit tree – 24.6	Kedainiai distr.	Uzupe, Lazai
K04-W	2003 06 08	Willow – 67.4; fruit tree – 21.7; dandelion (*Taraxacum officinale* L.) – 10.9	Kedainiai distr.	Voluciai
K05-W	2003 06 14	Willow (*Salix alba* L., *Salix caprea* L.); – 55.3 fruit tree–28.3; wild mustard (*Sinapis alba*) – 8.0; dandelion (*Taraxacum officinale*) – 8.4	Kedainiai disr.	Medininkai ir Spitole
K06-MF	2003 06 13	Fruit tree – 33.3; willow (*Salix alba* L., *Salix caprea* L.); – 32.5; dandelion (*Taraxacum officinale* L.) – 24.4	Kedainiai distr.	Degesiai
K07-SR	2003 06 30	Spring rape – 96.8; honeydew – 3.1	Kedainiai distr.	Voluciai
K08-SR	2003 07 07	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 96.6; honeydew – 3.3	Kedainiai distr.	Gudziunai
K09-SR	2003 07 09	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 92.0; fruit tree – 3.5; willow (*Salix alba* L., *Salix caprea* L.) – 2.5	Kedainiai distr.	Degesiai
K10-SR	2003 07 22	Spring rape (*Brassica napus* L. *ssp. oleifera annua*Metzg.) – 48.5; willow (*Salix alba* L., *Salix caprea* L.) – 30.0; cornflower (*Centaurea cyanus*L.) – 5.9; wild mustard (*Sinapis alba* L.) – 5.3	Kedainiai distr.	Paberze
K11-SR	2003 07 24	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 45.9; red clover (*Trifolium pratense*L.) – 24.7; honeydew – 27.0	Kedainiai distr.	Daumantai
K12-SR	2003 07 28	Spring rape (*Brassica napus L. ssp. oleifera annua* Metzg.) – 62.5; wild mustard (*Sinapis alba* L.) – 17.5; cornflower (*Centaurea cyanus* L.) – 7.6	Kedainiai distr.	Medininkai
K13-SR	2003 07 29	Spring rape (*Brassica napus L. ssp. oleifera annua* Metzg.) – 77.5; willow (*Salix alba* L., *Salix caprea* L.) – 5.4; dandelion (*Taraxacum officinale* L.) and fruit tree – 2.7	Kedainiai distr.	Spitole
K14-SR	2003 07 30	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 63.7; willow (*Salix alba* L., *Salix caprea* L.) – 8.8; wild mustard (*Sinapis alba* L.) – 7.2; cornflower (*Centaurea cyanus* L.) – 7.6; fruit tree – 4.8; linden (*Tilia* L.) – 2.8	Kedainiai distr.	Uzupe
K15-SR	2003 07	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 84.7; wild mustard (*Sinapis alba* L.) – 10.5; cornflower (*Centaurea cyanus* L.) – 3.5; honeydew – 2.7	Marijampole distr.	Unknown
K16-SR	2003 07 25	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 89.2; linden (*Tilia cordata* L.)– 7.7; beans (*Vicia faba* L.) – 1.7	Kedainiai distr.	Slapaberze
K17-SR	2003 08 05	Spring rape – 88.0; wild mustard (*Sinapis alba* L.) – 6.9; cornflower – 2.2	Kedainiai distr.	Degesiai
K18-SR	2003 08 06	Rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 54.8; wild mustard (*Sinapis alba* L.) – 24.2; cornflower (*Centaurea cyanus* L.)– 6.7; willow (*Salix alba* L., *Salix caprea* L.) – 4.0; raspberry (*Rubus idaeus* L.) – 4.0; fruit tree – 3.2	Kedainiai distr.	Krakes, Janiunai
K19-SR	2003 08 07	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 71.5; wild mustard (*Sinapis alba* L.) – 14.7; willow (*Salix alba* L., *Salix caprea* L.), and fruit tree – 4.1; cornflower – 2.6 (*Centaurea cyanus* L.)	Kedainiai distr.	Lazai
K20-SR	2003 07 22	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 78.5; willow (*Salix alba* L., *Salix caprea* L.) – 6.2; fruit tree – 6.2; cornflower (*Centaurea cyanus* L.) – 2.4; wild mustard (*Sinapis alba* L.) – 2.9	Kedainiai distr.	Terespolis
K21-SR	2003 08 08	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 74.8; wild mustard (*Sinapis alba* L.) – 5.7; fruit tree – 6.9; caraway (*Carum carvi* L.) – 3.3	Kedainiai distr.	Siaudyne

K22-SR	2003 08 12	Spring rape (*Brassica napus* L. *ssp. oleifera annua*Metzg.) – 73.4; cornflower (*Centaurea cyanus* L.) – 7.3; willow (*Salix alba* L., *Salix caprea* L.) – 5.6; wild mustard (*Sinapis alba*L.) – 6.2	Kedainiai distr.	Gudziunai
K23-SR	2003 08 20	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 71.4; wild mustard (*Sinapis alba* L.) – 13.9; willow (*Salix alba* L., *Salix caprea* L.) – 9.5; dandelion (*Taraxacum officinale*L.)– 5.5	Kedainiai distr.	Medininkai
K24-MF	2003 08 26	Willow (*Salix alba* L., *Salix caprea* L.) – 38.5; fruit tree – 13.6; raspberry (*Rubus idaeus* L.) – 12.4; spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 7.7; honeydew – 6.1; thistle (*Cirsium arvense* Scop.) – 5.9	Silute distr.	Pervalka
K25-MF	2003 07 24	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 42.5; birdsfoot trefoil (*Lotus corniculatus*L.) – 16.6; honeydew – 9.4; linden (*Tilia cordata*L.) – 9.8	Taurage dist.	Pasesuvys
E26-P	2003 07	Honey with pine (*Pinus silvestris*) extract	Unknown	Unknown
E27-B	2003 07	Honey with birch (*Betula pendula*) extract	Unknown	Unknown
E28-SN	2003 07	Honey with stinging nettle (*Urtica dioica*) extract	Unknown	Unknown
C29	2003 08	Unknown (honey from local apiarist)	Vilkaviskis dist.	Svitrunai
C30	2003 07	Unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania)	Unknown	Commercial sample
C31	2003 08	Unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania)	Unknown	Commercial sample
C32	2003 07	Unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania)	Unknown	Commercial sample
C33	2003 05	Unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania)	Unknown	Commercial sample
C34	2003 07	Unknown (grassland and forest honey, EKO agros, R. Maciene, Lithuania)	Unknown	Commercial sample
C35	2003	Unknown (beebread after thermal processing, EKO agros, R. Maciene, Lithuania)	Pakruojis dist.	Commercial sample
C36	2003 09 17	Unknown (beebread with honey and comb, EKO agros, R. Maciene, Rockaiciai, Lithuania)	Pakruojis dist.	Commercial sample
C37	2003 09	Unknown (beebread with honey and comb, EKO agros, R. Maciene, Rockaiciai, Lithuania)	Pakruojis dist.	Commercial sample
C38	2003 09 17	Unknown (beebread with honey and comb, EKO agros, R. Maciene, Isdagieciai, Lithuania)	Pakruojis dist.	Commercial sample
K39-L	2003	Linden (*Tilia cordata* L.) – 65.6; spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 18.2; caraway (*Carum carvi* L.) – 13.0; wild mustard (*Sinapis alba* L.) – 1.6; red clover (*Trifolium pratense* L.) – 1.6; honeydew – 1.7	Kedainiai dist.	Unknown
K40	1999	Red clover (*Trifolium pratense* L.) – 35.4; white clover (*Trifolium repens* L.) – 36.0; wild mustard (*Sinapis alba* L.) – 12.0; spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.)– 11.2; honeydew – 4.2; dandelion (*Taraxacum officinale* L.) – 3.1; willow (*Salix alba* L., *Salix caprea* L.) – 2.3 (beebread and honey mixture (1:1))	Kedainiai dist.	Unknown
K41	2000	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.)– 37.9; caraway (*Carum carvi* L.) – 23.9; cornflower (*Centaurea cyanus* L.)– 16.3; wild mustard (*Sinapis alba* L.) – 7.6; willow (*Salix alba* L., *Salix caprea* L.)– 7.3; red clover (*Trifolium pratense* L.) – 7.0 (beebread and honey mixture (1:1))	Kedainiai dist.	Unknown
K42	2001	Spring rape (*Brassica napus* L. *ssp. oleifera annua*Metzg.) – 46.2; cornflower (*Centaurea cyanus* L.) – 19.9; willow (*Salix alba* L., *Salix caprea* L.) – 12.2; coltsfoot (*Tussilago farfara* L.) – 5.6; honeydew – 5.3; wild mustard – 4.8 (beebread and honey mixture (1:1))	Kedainiai dist.	Unknown
K43	2003	Spring rape (*Brassica napus* L. *ssp. oleifera annua* Metzg.) – 66.8; wild mustard (*Sinapis alba* L.) – 13.6; cornflower (*Centaurea cyanus* L.) – 5.0; red clover (*Trifolium pratense* L.) – 4.5; caraway (*Carum carvi* L.) – 3.2; thistle (*Cirsium arvense* Scop.) – 5.9-3.2; dandelion (*Taraxacum officinale* L.) – 1.8; honeydew – 6.8 (beebread and honey mixture (1:1))	Kedainiai dist.	Unknown
K44	2003	Spring rape (*Brassica napus L. ssp. oleifera annua* Metzg.) – 79.1; red clover (*Trifolium repens* L.) – 9.8; cornflower (*Centaurea cyanus* L.) – 5.7; dandelion (*Taraxacum officinale* L.) – 41.9; willow (*Salix alba* L., *Salix caprea* L.) – 1.8; caraway (*Carum carvi* L.) – 1.7 (beebread and honey mixture (1:1))	Kedainiai dist.	Unknown

W – unifloral willow honey; SR – unifloral spring rape honey; P – pine extract; B – birch tree extract; SN – stinging nettle extract; L – unifloral linden honey; MF – multifloral honey.

(Lachema, Brno, Czech Republic). The extracts were combined and the solvent was removed by flushing with nitrogen. Two replicate extractions were performed for each sample; standard deviation did not exceed 5%. The yield of extracts, expressed as a mean of two extractions was from 10.3 to 348.8 mg/100 g of product. Dried extracts were stored in a refrigerator until further analysis.

2.3. DPPH$^{\cdot}$ radical scavenging assay

The scavenging activity (H/e− transferring ability) against 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH$^{\cdot}$) radical (95%, Sigma–Aldrich Chemie, Steinheim, Germany) was evaluated according to the method of Brand-Williams, Culivier, and Berset (1995) with minor modifications. In the presence of an antioxidant the purple color of DPPH$^{\cdot}$ is fading; the change of absorbency can be followed spectrophotometrically. The solution of 6.5×10^{-5} mol/l DPPH$^{\cdot}$ in methanol was prepared daily before measurement on a UV/vis spectrophotometer Spectronic Genesys 8 (Rochester, USA). Two milliliters of DPPH$^{\cdot}$ solution were mixed with 50 μl of honey or beebread phenolic extract solution in methanol (10 mg/ml) in the 1 cm path length disposable microcuvette (Greiner Labortech, Alpher a/d Rijn, The Netherlands). The final concentration of extract was 0.244 mg/ml. The absorbency of the remaining DPPH$^{\cdot}$ was determined after 16 min at 515 nm. Blank sample contained the same amount of methanol and DPPH$^{\cdot}$. The measurements were performed in triplicate. The radical scavenging activity was calculated by the formula $I = [(A_B - A_A)/A_B] \times 100$; where I = DPPH$^{\cdot}$ inhibition, %; A_B = absorption of a blank sample ($t = 0$ min); A_A = absorption of a tested honey or beebread extract solution at the end of the reaction ($t = 16$ min).

2.4. ABTS$^{\cdot+}$ radical cation decolourisation assay

The antioxidant activity of honey and beebread samples in the reaction with stable ABTS$^{\cdot+}$ radical cation was determined according to Re et al. (1999) method with slight modification. ABTS$^{\cdot+}$ was produced by reacting 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diamonium salt (ABTS) (Fluka Chemie, Buchs, Switzerland) with potassium persulfate ($K_2S_2O_8$). Stock solution of ABTS (2 mM) was prepared by dissolving in 50 ml of phosphate buffered saline (PBS), constituting 8.18 g NaCl, 0.27 g KH_2PO_4, 3.58 g $NaHPO_4 \cdot 11\ H_2O$ and 0.15 g KCl in 1 l of distilled water. The pH of the solution should be 7.4; otherwise it was adjusted with 0.1 M NaOH. ABTS$^{\cdot+}$ was produced by reacting 50 ml of stock solution with 200 μl of 70 mM $K_2S_2O_8$ water solution. The mixture was left to stand in the dark at room temperature for 15–16 hours before use. For the evaluation of antioxidant activity, the ABTS$^{\cdot+}$ solution was diluted with PBS to obtain the absorbency of 0.800 ± 0.030 at 734 nm. Ten microliters of honey or beebread phenolic extracts solution (10 mg/ml) were mixed with 3 ml of ABTS$^{\cdot+}$ solution in the disposable 1 cm path length microcuvette. The final concentration of phenolic extracts was 0.033 mg/ml. The absorbency was read at ambient temperature after 1, 4, 6 and 10 min. PBS solution was used as a blank sample. The measurement was performed in triplicate. The percentage decrease of the absorbance at 734 nm was calculated by the formula $I = [(A_B - A_A)/A_B] \times 100$; where: I = ABTS$^{\cdot+}$ inhibition, %; A_B = absorbency of a blank sample ($t = 0$ min); A_A = absorbency of a tested honey or beebread extract solution at the end of the reaction ($t = 10$ min).

2.5. High performance liquid chromatography (HPLC/UV/MS)

The following HPLC setup was used for the analysis of the extracts: Waters 1525 binary HPLC eluent pump (Millipore, Waters Chromatography, Milford, USA), Hitachi L-7400 UV detector (Merck, Germany) and Waters Micromass ZQ-2000 mass detector. Honey and beebread phenolic compounds were separated on a Synergi MAX-RP analytical column, 250×4.60 mm i.d. (Phenomenex, Torrance, USA) packed with Luna C_{18} stationary phase, particle size 4 μm. The linear binary gradient was used at a flow rate of 0.8 ml/min. The time of HPLC run was over 40 min. Binary mobile phase consisted of a solvent A (ultra pure water with 10% methanol and 1% of acetic acid) and solvent B (100% methanol). Elution from the column was achieved with the following linear gradient: 0–30 min B increased from 30% to 100% and kept constant till 33 min; 33–36 min B decreased back to 30% and kept constant till 40 min. UV detector was operating at 254 nm wavelength.

During the scanning of mass spectra the flow rate was 0.5 ml/min; the injection volume was 10 μl. Mass spectra of phenolic compounds were scanned with ion trap MS after electrospray ionization (ESI) in negative and positive mode. ESI conditions for ionization were as follows: capillary temperature 250 °C, capillary voltage 3 kV, extractor voltage 3 V, tag voltage: (−30, −45, 30, 45) V, sheath gas flow 200 l/h and auxiliary gas flow at 50 l/h.

For HPLC analysis dried extracts of honey and beebread were dissolved in methanol to obtain 0.5% (w/v) solutions, which were filtered through a 0.2 μm Nalgene filter (USA) before analysis. Phenolic compounds were identified by comparison of chromatographic retention times and spectral characteristics of unknown analytes and reference compounds using Mass Lynx 4.0 software (Micromas UR Ltd., UK) and the available literature data (Gheldof et al., 2002; Yao et al., 2003; Yao et al., 2004). The concentrations of identified compounds in the extracts for comparison purpose were expressed by using peak area units (arbitrary units).

2.6. Statistical analysis

All values are expressed as the mean ± standard deviation. Standard deviations were calculated using spreadsheet

software (Excel®). Correlation coefficients (R) to determine the relationship between antioxidant activity and amount of the main source of honey, as well as between antioxidant activities obtained in two different radical scavenging reaction systems were calculated using MS Excel® software (CORREL statistical function).

3. Results and discussion

3.1. General characterization of radical scavenging activity (RSA)

Thirty-five honey samples of different floral origin and nine beebread samples were tested in this study in order to assess their antioxidant properties and, possibly, to find some relationship between RSA and floral origin. The results obtained shows that all tested samples were antioxidatively active, however, their RSA varied in a wide range (Table 2). The RSA of natural honey extracts was from 31.1 ± 4.5 to $86.9 \pm 0.9\%$ in DPPH$^{\cdot}$ reaction system and from 50.4 ± 1.0 to $96.8 \pm 0.7\%$ in ABTS$^{\cdot+}$ reaction system, while that of honey with plant extracts from 80.0 ± 1.6 to $93.0 \pm 1.0\%$ and from 89.5 ± 2.7 to $98.3 \pm 0.7\%$, respectively. The RSA of beebread samples was from 72.5 ± 4.4 to $94.0 \pm 0.3\%$ in ABTS$^{\cdot+}$ reaction system and from 71.1 ± 3.7 to $92.2 \pm 3.0\%$ in DPPH$^{\cdot}$ reaction system.

Due to remarkable differences in antioxidant properties honey and beebread samples were discriminated into several groups according to the ability of their extracts to scavenge free radicals used in the model reaction systems (Fig. 1). The largest group containing about 70% of all tested samples possessed the highest RSA, which was in the range of 70–98%. Only few honey extracts were able to scavenge less than 50% of DPPH$^{\cdot}$ radical.

Most likely, the differences in antioxidant activity between the tested samples depend mainly on a floral source of honey. However, it is well known that the composition of phytochemicals, including antioxidatively active compounds in plants depend on plant species and many other factors; therefore the RSA of monofloral honey samples of rape origin varied in a wide range (Frankel et al., 1998; The National Honey Board, 2002). Only one linden honey sample was available for analysis; the RSA of its extract was the lowest among all tested samples. Some studies showed that honey produced by the bees directly fed with herbal extract additives had higher antioxidant activity comparing to the natural honey (The National Honey Board, 2002). Our results obtained for honey produced with the use of plant extract additives is in agreement with previously reported data. Thus, the RSA of honey with birch, pine and stinging nettle extracts was higher than 90%, except for the sample E28-SN in DPPH$^{\cdot}$ reaction system; the highest inhibition of natural honey extract in the same reaction was $86.9 \pm 0.9\%$ (Table 2).

Beebread samples C35, C36, C37 and C38 and honey with pine and birch extracts had the highest antioxidant activity in DPPH$^{\cdot}$ reaction system (90–94%). Eight beebread samples of the nine showed RSA higher than 80%, while only six extracts of 32 tested natural honey samples had inhibition higher than 80% in DPPH$^{\cdot}$ reaction.

ABTS$^{\cdot+}$ radical cation decolourisation assay is another widely used antioxidant activity screening method, which

Table 2
Radical scavenging activity of honey (K, E) and beebread (C) phenolic extracts in DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ reaction systems. The values represent average ± standard deviation, $n = 3$

Sample code	Inhibition, %		$\frac{DPPH + ABTS}{2}$	Sample code	Inhibition, %		$\frac{DPPH + ABTS}{2}$
	DPPH$^{\cdot}$	ABTS$^{\cdot+}$			DPPH$^{\cdot}$	ABTS$^{\cdot+}$	
K01-MF	80.9 ± 3.8	79.6 ± 1.7	80.3	K23-SR	67.0 ± 2.7	95.1 ± 1.9	82.0
K02-W	82.6 ± 0.2	94.0 ± 0.8	88.3	K24-MF	45.4 ± 1.5	56.9 ± 3.5	51.0
K03-W	77.2 ± 0.5	95.5 ± 0.6	86.4	K25-MF	45.6 ± 0.7	68.0 ± 0.7	56.8
K04-W	79.0 ± 0.9	83.7 ± 3.6	81.4	E26-P	93.0 ± 1.0	98.4 ± 0.7	95.7
K05-W	76.7 ± 0.9	78.4 ± 2.2	77.6	E27-B	90.2 ± 0.5	93.9 ± 1.8	92.0
K06-MF	80.2 ± 2.3	81.9 ± 2.7	81.0	E28-SN	80.0 ± 1.6	89.5 ± 2.7	84.7
K07-SR	75.7 ± 0.6	72.4 ± 1.6	74.0	C29	80.6 ± 0.4	78.6 ± 0.4	79.6
K08-SR	61.6 ± 1.8	65.3 ± 2.8	63.4	C30	70.2 ± 3.3	94.8 ± 0.5	82.5
K09-SR	75.6 ± 1.6	82.3 ± 1.0	79.0	C31	72.4 ± 3.7	76.5 ± 0.2	74.5
K10-SR	63.8 ± 1.7	64.9 ± 3.6	64.4	C32	64.2 ± 1.8	85.3 ± 1.8	74.7
K11-SR	60.5 ± 0.7	61.4 ± 2.2	60.8	C33	83.1 ± 0.1	78.5 ± 2.6	80.8
K12-SR	73.2 ± 0.6	86.8 ± 2.9	80.0	C34	76.5 ± 2.0	80.3 ± 3.1	78.4
K13-SR	86.9 ± 0.9	80.7 ± 1.7	83.8	C35	94.0 ± 0.3	78.3 ± 4.3	86.2
K14-SR	60.4 ± 2.6	59.3 ± 2.5	59.9	C36	93.9 ± 0.6	92.2 ± 3.0	93.0
K15-SR	66.1 ± 3.0	58.3 ± 3.2	62.2	C37	93.0 ± 0.5	91.6 ± 3.0	92.3
K16-SR	39.2 ± 0.6	52.0 ± 1.9	45.6	C38	89.9 ± 0.8	73.2 ± 0.8	81.5
K17-SR	56.4 ± 1.5	59.8 ± 1.7	58.2	K39-L	31.1 ± 4.5	54.8 ± 2.4	43.0
K18-SR	70.5 ± 1.6	89.0 ± 1.9	79.7	K40	80.0 ± 2.7	73.2 ± 3.0	76.6
K19-SR	75.0 ± 1.3	96.8 ± 0.7	85.9	K41	83.4 ± 3.6	83.7 ± 0.9	83.5
K20-SR	72.6 ± 3.3	94.2 ± 3.3	83.4	K42	85.2 ± 1.5	87.7 ± 3.0	86.4
K21-SR	58.7 ± 1.7	78.1 ± 4.2	68.4	K43	72.5 ± 4.4	77.5 ± 0.7	75.0
K22-SR	36.5 ± 2.7	50.4 ± 1.1	43.4	K44	83.5 ± 1.0	71.1 ± 3.7	77.3

MF – multifloral; W – willows; SR – spring rape; P – pine extract; B – birch tree extract; SN – stinging nettle extract; L – linden.

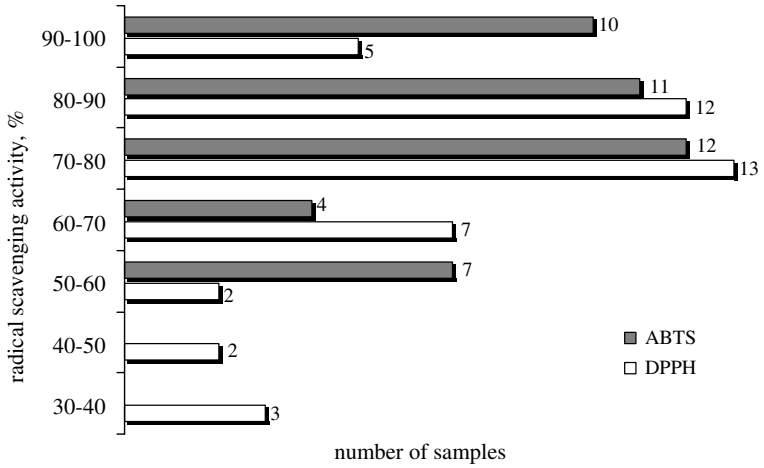

Fig. 1. Distribution of honey samples according their radical scavenging activity in $ABTS^{\cdot+}$ and $DPPH^{\cdot}$ reaction system.

is applicable both for lipophilic and hydrophilic antioxidants. In general, the RSA of honey samples in $ABTS^{\cdot+}$ reaction system was slightly higher comparing to $DPPH^{\cdot}$ reaction; however, it should be noted that extract concentration in $ABTS^{\cdot+}$ reaction was 0.033 mg/l, while in $DPPH^{\cdot}$ it was 0.25 mg/ml. The RSA of 48% of all tested samples exceeded 80% in $ABTS^{\cdot+}$ reaction. The extracts from 10 samples (K02-W, K03-W, K19-SR, K20-SR, K23-SR, E26-P, E27-B, C30, C36, C37) were able to scavenge almost all $ABTS^{\cdot+}$ radicals (90–98%) in the applied reaction assay.

The samples K16-SR, K22-SR and K39-L were the less active, while E26-P, E27-B, C36 and C37 had the highest RSA in both reaction systems. Some tested samples showed lower inhibition in $ABTS^{\cdot+}$ and stronger in $DPPH^{\cdot}$ reaction and on the contrary.

3.2. Antioxidant properties of honey from different sources

The honey was considered as unifloral when more than 45% of pollen was collected from one plant species (Louveaux et al., 1978). The majority of unifloral honey samples were of rape origin (Table 1), 4 samples were of willows and 1 of linden origin. The RSA of rape origin honey samples varied from 36.5 ± 2.7 to $86.9 \pm 0.9\%$ in $DPPH^{\cdot}$ reaction, and from 50.4 ± 1.1 to $95.1 \pm 1.9\%$ in $ABTS^{\cdot+}$ reaction. Honey with the highest amount of rape (K07-SR) did not show the highest antioxidant activity. Ten samples of 17 rape honey samples were more effective in $ABTS^{\cdot+}$ reaction than in $DPPH^{\cdot}$, while three of them (K13-SR, K07-SR, K15-SR) showed lower inhibition of $ABTS^{\cdot+}$; four samples (K08-SR, K10-SR, K11-SR, K14-SR) had almost equal RSA in both reaction systems (Table 2).

The differences in RSA of willow honey samples were less remarkable, most likely, due to fewer samples available for the analysis. All willow's honey samples exhibited quite strong antioxidant activity; the RSA of the extracts isolated from this type honey was from 76.7% to 82.6% in $DPPH^{\cdot}$, and from 78.4% to 95.5% in $ABTS^{\cdot+}$ reaction. All samples were more effective in $ABTS^{\cdot+}$ reaction than in $DPPH^{\cdot}$ reaction.

The antioxidant activity of multifloral honey samples varied from 64.2% to 80.9% in $DPPH^{\cdot}$ radical scavenging assay, and from 76.5% to 81.9% in $ABTS^{\cdot+}$ radical cation decolourisation assay. The radical scavenging activity of six samples of ten of multifloral honeys was similar in both testing methods; inhibition of the samples K24-MF, K25-MF, C30 and C32 was 11–28% lower in $DPPH^{\cdot}$ method, than in $ABTS^{\cdot+}$.

It is interesting to note, that botanical composition of some honey samples (e.g., K07-SR and K08-SR) was quite similar (Table 1), however their antioxidant activity differed by approximately 10%. It is worth noting that the color of these honey samples was different; K07-SR honey was darker than K08-SR. It suggests that botanical species as the main source of honey is not the only factor contributing to its antioxidant activity. It is well known that phytochemical composition of the same botanical species depends on a plant chemotype and various environmental factors; therefore it would be reasonable to perform chemical analysis of plant extracts which were the source of honey samples. Bee-origin metabolism products which could possess the effect on antioxidant properties of honey should also be taken into account. These assumptions provide interesting ideas for further investigations, for instance aiming at finding possible relationships of honey antioxidant properties and its floral origin.

In general, good correlation ($R = 0.716$) was observed between the two applied tests of RSA. However, the results obtained also indicate that the kinetics of radical scavenging reaction in two systems differs (Figs. 2 and 3): faster decline of the curve shows stronger radical scavenging effect in inhibition of free radicals. Thus, in $ABTS^{\cdot+}$ reaction beebread extract C30 during first 2 min of reaction scavenged almost all radicals, while in the $DPPH^{\cdot}$ reaction the absorbency decreased more evenly during the whole period of the reaction and after 16 min reached 70.2%. Beebread extract C36 was almost equally effective in both reaction systems, although the speed of scavenging $ABTS^{\cdot+}$ by

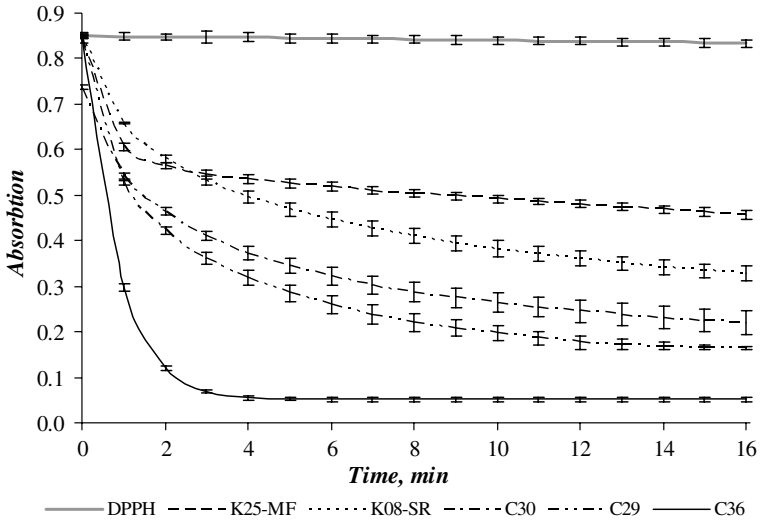

Fig. 2. Kinetics of DPPH· scavenging by the selected honey and beebread extracts as measured by the decrease of absorption at 515 nm.

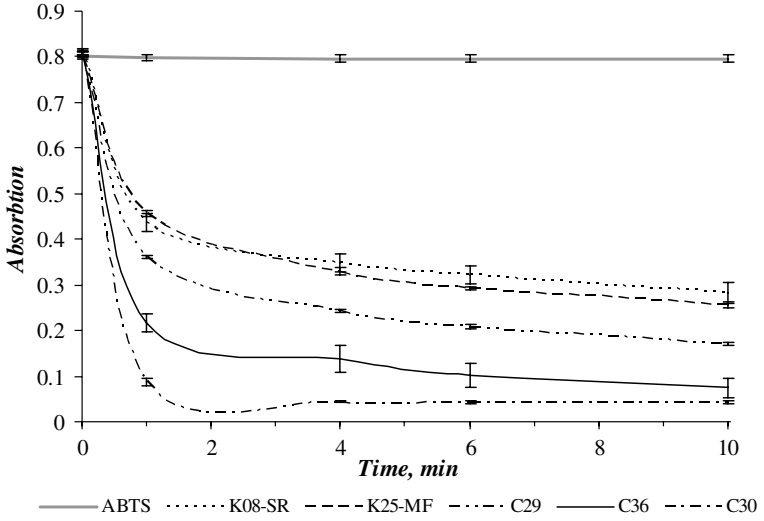

Fig. 3. Kinetics of $ABTS^{\cdot+}$ cation scavenging by the selected honey and beebread extracts as measured by the decrease of absorption at 734 nm.

this extract was slightly slower comparing to DPPH·. To assess the RSA in both reaction systems derivative value which is a mean of the sum of DPPH· and $ABTS^{\cdot+}$ was calculated. This value varied from 43.0% to 95.7%.

There was no correlation between the total amount of rape pollen in the unifloral honey samples and radical scavenging activity of their extracts. Good correlation was determined between the amount of willows pollen in honey samples and RSA of their extracts in both reaction systems; the correlation coefficients were 0.82 and 0.84 in DPPH· and $ABTS^{\cdot+}$ reaction systems, respectively. However, to prove this correlation further measurements are needed, presumably with larger amount of willow honey samples.

It was reported that phenolic compounds are the main components responsible for the antioxidant effects of honey, however, non-phenolic antioxidants are also involved (Aljadi & Kamaruddin, 2004; Gheldof et al., 2002). It should be noted that in this study radical scavenging activity of extracts containing only phenolic compounds extractable from honey on Amberlite XAD-2 resin was determined. Gheldof et al. (2002) showed that the antioxidant activity of the sum of acidified and neutral water phases, methanol phase and water phase after ether extraction was lower than the antioxidant capacity of the whole honey. Probably, the antioxidant components in honey had some synergistic interactions (Gheldof et al., 2002). Therefore, it can be expected that the total antioxidant activity of the tested honey and beebread samples should be higher than the activity defined by the phenolic fraction.

3.3. Preliminary screening of phenolic compounds in honey and beebread extracts

HPLC chromatograms of the phenolic fractions of honey extracts indicate that most of tested honey samples had similar phenolic profiles (Fig. 4). Preliminary screening of honey phenolics showed that *p*-coumaric acid ($R_t = 10.98 \pm 0.28$), kaempferol ($R_t = 19.37 \pm 0.13$), apigenin

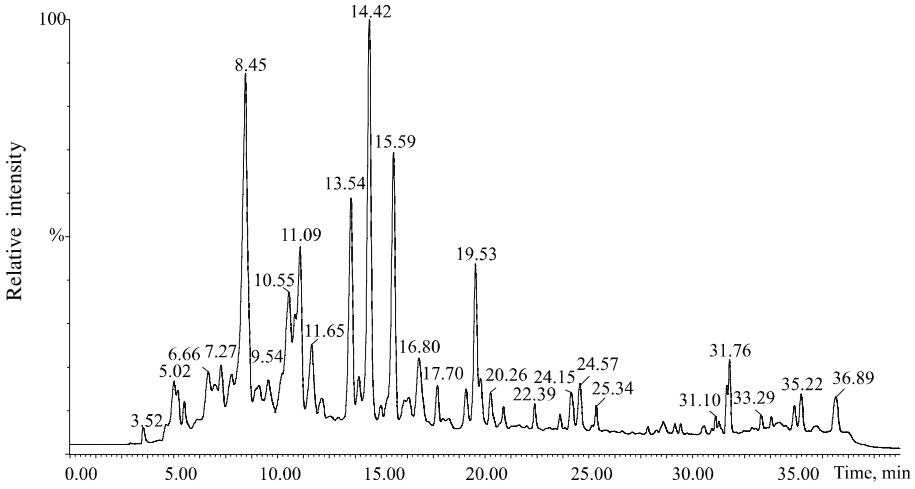

Fig. 4. HPLC/UV profile of K23 (spring rape) honey at $\lambda = 254$ nm.

($R_t = 20.04 \pm 0.34$) and chrysin ($R_t = 24.22 \pm 0.30$) were the main phenolic compounds present in all tested honey and beebread samples. These compounds exhibit antioxidant properties (Amić, Davidović-Amić, Bešlo, & Trinajstić, 2003; Furusawa et al., 2005; Raj Narayana, Sripal Reddy, Chaluvadi, & Krishna, 2001; Torres y Torres & Rosazza, 2001); therefore, they should influence the RSA of the tested honey samples as well. However, the spectrum of honey flavonoids reported in numerous articles is comparatively wide. For instance, such compounds as myricetin, tricetin, quercetin, hesperetin, luteolin, kaempferol, pinocembrin, chrysin, pinobanksin, genkwanin, and galangin were reported as major flavonoids present in honey (Anklam, 1998; Bogdavov, Ruoff, & Persano Oddo, 2004; Gheldof et al., 2002; Tomás-Barberán, Martos, Ferreres, Radovic, & Anklam, 2001; Yao et al., 2003; Yao et al., 2004). Yao et al. (2004) reported that the composition and the levels of flavonoids in honey depend on the floral origin. For instance, the total content of flavonoids in Australian sunflower (*Helianthus annuus*) honey was only 1.79 mg/100 g, quercetin, quercetin 3,3'-dimethyl ether, myricetin, and luteolin being the main components; while the total content of flavonoids in European acacia (*Robinia pseudoacacia*) honey was 4.50 mg/100 g with the highest level of pinobanksin (Tomás-Barberán et al., 2001). Honey may also contain phenolic acids; their composition depends on the plant source as well. Gallic acid was a major compound in New Zealand manuka (Yao et al., 2003) and Australian *Eucalyptus* honey (Yao et al., 2004). Gallic and coumaric acids were dominant in Australian jelly bush (*L. polygalifolium*) honey, which also contained ellagic, chlorogenic, caffeic, ferulic and syringic acids as minor constituents (Yao et al., 2003). Vanillic, syringic, *p*-coumaric, cinnamic, *p*-hydroxybenzoic acids were found in honey samples by Gheldof et al. (2002).

The variations in the concentration of UV detected phenolic compounds in honey samples were assessed by comparing their HPLC peak area. The results obtained clearly show that HPLC peak areas of the UV detected phenolics compounds in honey and beebread extracts and consequently the concentrations of phenolic compounds varied in a very wide range. The content of identified antioxidants in honey and beebread samples expressed in arbitrary units (a.u.) is presented in Figs. 5–8.

The content of *p*-coumaric acid in tested samples varied from 2.4×10^3 to 63.5×10^3 a.u. (Fig. 5). However, the concentration of this phenolic in the 50% of the total analyzed samples was in the range of $(10.0–30.0) \times 10^3$ a.u. The highest content of *p*-coumaric acid was determined in honey produced with the use of birch extract as an additive in bee food. Seven samples of beebread of the tested nine contained more than 20.0×10^3 a.u. of *p*-coumaric acid.

The amount of kaempferol varied from 2.0×10^3 (samples K09-SR, K10-SR, K25-MF) to 119.3×10^3 a.u. (beebread C35). The concentration of kaempferol in 20 analyzed samples was lower than 20×10^3 a.u. The amount of this compound exceeded 40×10^3 a.u. in nine samples, seven of them were the samples of beebread (Fig. 6).

The amount of chrysin in tested samples varied from 0.5×10^3 a.u. to 20.4×10^3 a.u. In 29 samples, the amount of chrysin was lower than 5.0×10^3 a.u. (see Fig. 7). Similar results were obtained for apigenin (Fig. 8); its concentration was lower than 5.0×10^3 a.u. in 28 samples of 44 analyzed. The highest content of apigenin was determined in honey samples with plant extracts (18.9×10^3–31.2×10^3) a.u. As it was already mentioned beebread samples contained only traces of chrysin and apigenin. The content of these compounds in almost all samples of beebread was lower than 5.0×10^3 a.u. (Figs. 7 and 8).

It was reported that the antioxidant capacity of honey depends on a wide range of components including phenolic compounds, peptides, organic acids, enzymes, Maillard reaction products and other minor compounds. However, the contribution of phenolic compounds was reported to be quite significant to the total antioxidant capacity of honey (Gheldof et al., 2002).

HPLC profiles of natural honey extracts analyzed in our study were almost similar; however they differed comparing

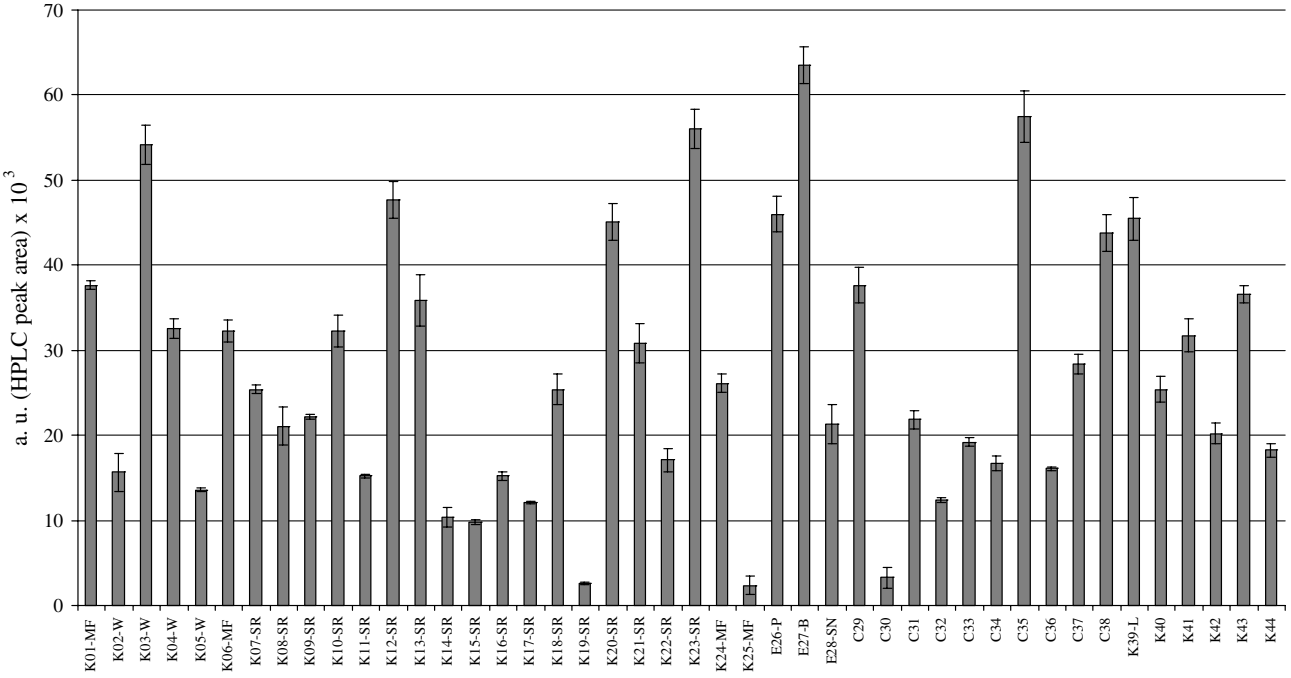

Fig. 5. The content of *p*-coumaric acid in honey and beebread samples ($n = 3$, sample codes as in Table 1).

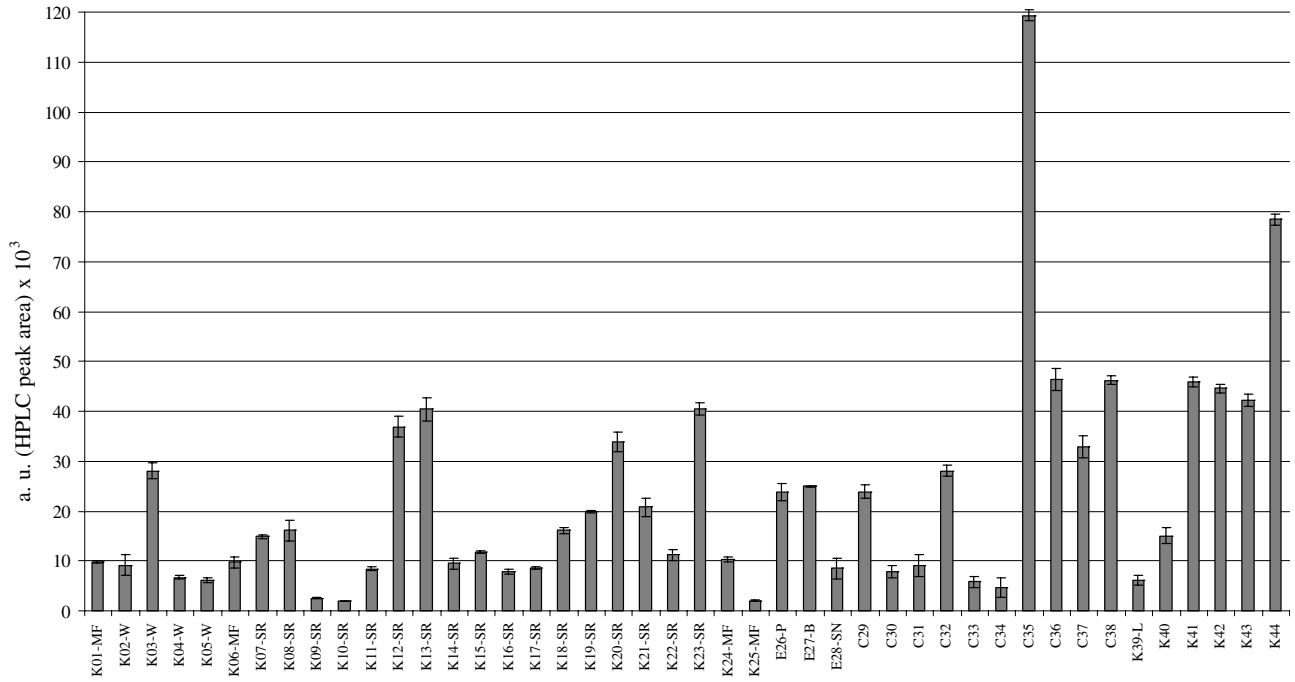

Fig. 6. The content of kaempferol in honey and beebread samples ($n = 3$, sample codes as in Table 1).

to the chromatograms of beebread and honey samples produced with plant extracts (Fig. 9). The preliminary assessment of the content of identified compounds in different samples was performed by comparing the integrated UV peak area at 254 nm. It can be observed that beebread extracts contained more kaempferol than honey; however chrysin and apigenin were present in traces. Honey samples with pine, birch and stinging nettle extracts had higher amounts of apigenin than natural honey samples.

The correlations between the content of identified phenolics compounds (in a.u.) and radical scavenging activity in DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ reaction systems are presented in Table 3. The strongest correlation between these two factors was found for honey samples with plant extracts. However, this correlation was rather weak for other honey and bee bread samples. For instance, the content of apigenin and kaempferol was in a strong correlation between RSA of honey produced with plant extracts in both applied

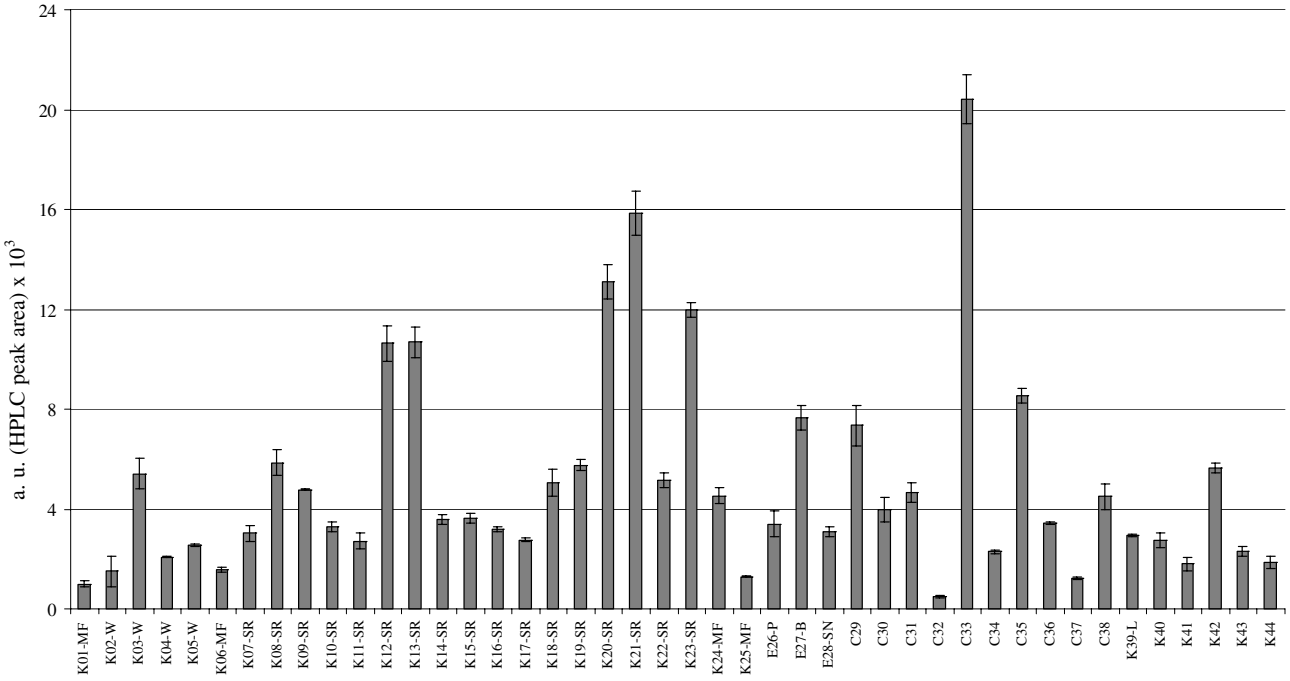

Fig. 7. The content of chrysin in honey and beebread samples ($n = 3$, sample codes as in Table 1).

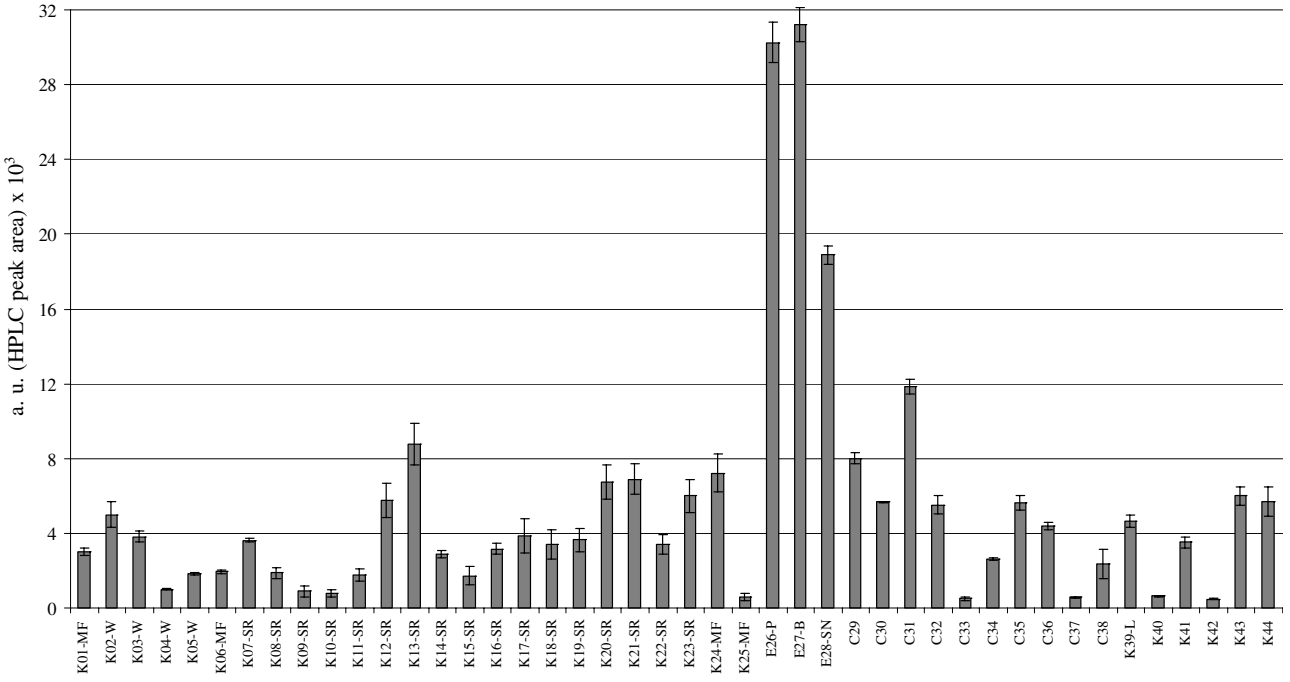

Fig. 8. The content of apigenin in honey and beebread samples ($n = 3$, sample codes as in Table 1).

tests; the correlation coefficients were 0.96 and 0.82 in DPPH$^{\cdot}$ and ABTS$^{\cdot+}$ reaction systems, respectively. On the contrary, the amount of chrysin weakly correlated with antiradical activity of the same honey samples. The amount of *p*-coumaric acid correlated with RSA in DPPH$^{\cdot}$ reaction; lower correlation was obtained for this component with the RSA in ABTS$^{\cdot+}$ reaction (Table 3).

The relationship between the amount of identified antioxidants in natural honey samples and their RSA in both reaction systems yielded a correlation coefficient varying from 0.07 to 0.28, except for kaempferol; its amount better correlated with the RSA in ABTS$^{\cdot+}$ reaction. There was no correlation between the amount of identified phenolics compounds and the RSA in both reactions for beebread samples,

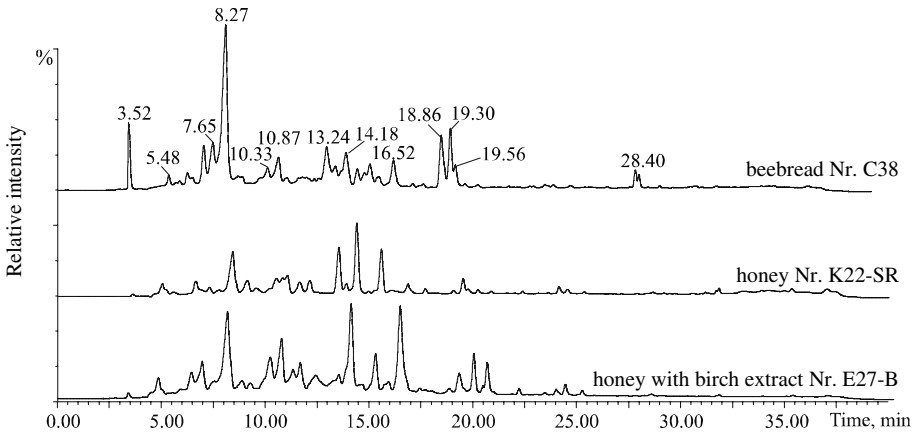

Fig. 9. Comparison of HPLC/UV chromatograms of beebread, natural honey and honey with birch extract.

Table 3
Correlation coefficients between the content of phenolics (in a.u.) and radical scavenging activity in DPPH· and $ABTS^{\cdot+}$ reaction systems

	Between methods	*p*-Coumaric acid	Kaempferol	Chrysin	Apigenin
In honey with plant extracts					
DPPH·	0.94	0.81	0.96	0.37	0.96
$ABTS^{\cdot+}$		0.56	0.82	0.04	0.82
In honey samples					
DPPH·	0.74	0.18	0.28	0.20	0.07
$ABTS^{\cdot+}$		0.27	0.47	0.25	0.22
In bee bread samples					
DPPH·	0.46	0.16	0.37	0.41	-0.15
$ABTS^{\cdot+}$		−0.33	−0.21	−0.06	−0.31

except for kaempferol and chrysin in DPPH· reaction (Table 3). These findings show that other components which were not analyzed in our study should also play an important role in defining RSA of honey and beebread extracts.

The correlation coefficient between the percentage amount of willow pollen in the honey and some identified phenolic compounds was not strong, for *p*-coumaric acid $R = 0.38$, kaempferol $R = 0.30$ and chrysin $R = 0.07$. However, the content of apigenin was in a much better correlation ($R = 0.70$). The correlation coefficients between the amount of rape pollen and the above mentioned phenolics were 0.03, 0.11, 0.11, and 0.18, respectively. It indicates that there was no correlation between these parameters.

4. Conclusions

Assessment of radical scavenging activity of Lithuanian honey and beebread samples revealed that antioxidant properties of these useful products varies in a very wide range; the ratio between the strongest and the weakest radical scavenging extracts was approximately 3:1. Beebread samples had higher antioxidant activity than honey. Consequently, it can be reasonably expected that the effects of bee products on human health should remarkably depend on honey origin. The screening of honey phenolic extracts by HPLC resulted in the identification of *p*-coumaric acid, chrysin, kaempferol and apigenin in all tested samples. Beebread contained higher amount of kaempferol than honey, however, chrysin and apigenin were present in beebread in trace levels. Honey with pine, birch and stinging nettle extracts was richer in apigenin than other, natural honey samples. Preliminary measurements of phenolic compounds in honey and beebread extracts clearly demonstrate that the relationships between floral origin, the concentration of individual phenolic compounds and antioxidant properties are rather complex and further, preferably more focused investigations are needed to determine possible correlations between these three variables.

Acknowledgement

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