

# Content of metals and metabolites in honey originated from the vicinity of industrial town Košice (eastern Slovakia)

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Received: 12 July 2015 / Accepted: 15 October 2015 / Published online: 30 October 2015  
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**Abstract** Composition of three types of honey (mixed forest honey and monofloral—black locust and rapeseed honeys) originated from the vicinity of an industrial town (Košice, Slovak Republic) was compared. Higher content of minerals including toxic metals in forest honey (1358.6 ng Ni/g, 85.6 ng Pb/g, and 52.4 ng Cd/g) than in rapeseed and black locust honeys confirmed that botanical origin rather than the distance for eventual source of pollution (steel factory) affects metal deposition. Benzoic acid derivatives were typically more accumulated in forest but cinnamic acid derivatives and some flavonoids in rapeseed honey (in free and/or glycoside-bound fraction). In terms of quantity, *p*-hydroxybenzoic and *p*-coumaric acids were mainly abundant. Total phenols, thiols, and proteins were abundant in forest honey. Some metals and phenols contributed to separation of honeys based on principal component analysis (PCA). Native amount of 5-(hydroxymethyl)furfural was not related to honey type (~11 µg/g) and was elevated after strong acid hydrolysis (200–350 µg/g) but it did not interfere with the assay of phenols by Folin-Ciocalteu reagent. This is the first report of metals and metabolites in the same study, and data are discussed with available literature. We conclude that black

locust (acacia) honey is the most suitable for daily use and that central European monofloral honeys contain lower amounts of toxic metals in comparison with other geographical regions.

**Keywords** Antioxidants · Food safety · Heavy metals · Mass spectrometry

## Introduction

Monitoring of food composition receives greater attention owing to increasing industrial production and subsequent environmental pollution. Among them, heavy metals are important contaminants naturally occurring in the environment but they are also easily released by various technological processes. Mainly elevated levels of Cd, Pb, Ni, Cr, and Hg are undesirable because of their cytotoxic and carcinogenic effects.

Honey is a unique natural product of honey bees (*Apis mellifera*) typically from the nectar of flowering plants but may also be produced from honeydews of plant-sucking insects (Ajibola et al., 2012). It has a high nutritional value including sugars, antibacterial compounds, and antioxidants (Biesaga and Pyrzyńska 2013). In addition to organic metabolites, honey contains minerals including toxic metals (Pohl 2009): their accumulation is mainly influenced by the botanical origin though differences are not always clear (Silici et al. 2008). Some reports indicated that level of toxic metals may be high in honey, depending on the locality and biological origin (Lachman et al. 2007; Madejczyk and Baralkiewicz 2008).

Phenolic metabolites are important antioxidants both in plants and human diet (Biesaga and Pyrzyńska 2013; Kováčik and Klejdus 2014). Comparison of these phytochemicals in various honeys is scarce, though extensive investigation was reported in individual types (Biesaga and Pyrzyńska 2013;

Responsible editor: Philippe Garrigues

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Rababah et al. 2014; Petretto et al. 2015). Some studies, on the contrary to usefulness of metals, did not show clear differences between various monofloral and multifloral honeys in terms of phenols (Marshall et al. 2014). Interestingly, just a study from Slovak Republic showed that forest honey (plant species not specified) typically contains more total phenols than other types but subsequent profile of individual phenols was not performed (Zalibera et al. 2008).

Storage and processing of honey affects also its nutritional value. In addition, owing to the high amount of sugars, processing of honey gives rise to various sugars-derived metabolites including potentially carcinogenic metabolites (Wu et al. 2009). Among them, 5-(hydroxymethyl)furfural is an important degradation product of hexoses serving as a sensitive marker of honey freshness: its content rises with the age and processing of honey (Biluca et al. 2014; Chakraborti and Bhattacharya 2014).

Owing to the fact that honey samples are often anonymous in terms of botanical origin (Tuzen and Soylak 2005; de Andrade et al. 2014), our study is a specific report involving authentic honey of various types (forest, black locust, and rapeseed) from known localities. Besides, we purposely used honeys from around the industrial town Košice (steel factory), aimed at monitoring whether it will show any impact on metal contents in honey. Additionally, we provide a comparison of analyses of metals and metabolites in all honeys, an observation which has not yet been reported. Methodological aspects of emerging contaminant 5-(hydroxymethyl)furfural are also presented and discussed.

## Materials and methods

### Origin of honey samples, statistical analyses, and equipment

Honey was produced during the year 2011 (provided by Ing. Michal Lovás, Košice, Slovak Republic) using mobile apiary containing several hives. Apiary was moved within localities following vegetation of individual species in the order *Brassica napus* (rapeseed field, locality 1), *Robinia pseudoacacia* (black locust/acacia grove, locality 2), and forest locality (honeydew mixed with blossom including mainly genus *Abies/Pinus* and *Rubus*, locality 3) as shown on Fig. 1. This scheme of honey production was used several years before and the amount of honey exceeded several hundreds of kilograms per locality being further processed by local apicultural association. The identity of honey was verified by look, taste, and smell typical for the given honey and estimated by electrical conductivity measurement.

Individual samples were taken from three individual hives on each locality and stored in closed glass flasks in dark and dry conditions for further analyses (then  $n=3$ ). FW means fresh weight (honey was directly used for sample preparation). One-way ANOVA followed by a Tukey's test (MINITAB



**Fig. 1** Map of the Slovak republic with points indicating the localities where honey was produced. 1 village Geča (rapeseed honey), 2 village Perín (black locust honey), 3 vicinity of the townlet Medzev (forest honey). Data in tables/figures are listed based on typically the highest amount of individual parameters in forest honey

Release 11, Minitab Inc., State College, Pennsylvania, USA) was used to evaluate the significance of differences ( $P<0.05$ ) between various honey types. Principal component analysis (PCA) was performed on level scaled data by PAST software package (ver. 3.02a).

Spectrophotometry was carried out with Agilent/HP DAD UV/Vis 8453 spectrophotometer. Individual metabolites were quantified by using an ACQUITY Ultra Performance LC™ system (Waters, Milford, MA, USA) coupled to both a PDA 2996 photodiode array detector (Waters, Milford, MA, USA) and a Micromass Quattro micro™ API benchtop triple quadrupole mass spectrometer (Waters MS Technologies, Manchester, UK) equipped with an electrospray ionization (ESI) source.

### Assay of electrical conductivity and metals

Conductivity was measured in an aqueous 20 % honey solution (deionized water,  $w/w$ ) at 20 °C (Petretto et al. 2015) using conductometer VARIO Cond (Lašáková et al. 2009).

Metals in honey samples were quantified after wet digestion: 2 g of fresh honey were mineralized in the mixture of ultrapure  $\text{HNO}_3$  and water (10+10 ml) using microwave decomposition (Ethos Sel Microwave Extraction Labstation, Milestone Inc.) at 200 °C over 1 h. The resulting clear solution was quantitatively placed into the glass flasks and diluted to a final volume of 40 ml. All measurements were carried out using an atomic absorption spectrometer AA30 (Varian Ltd.; Mulgrave, Australia) and the air-acetylene flame. Blank (mixture of  $\text{HNO}_3$  and water) was also checked to ensure correctness of metal quantifications. An AMA254 advanced mercury analyzer controlled by WinAMA software (Altec, Prague, Czech Republic) was used for the determination of total mercury concentration by direct analysis according to the manufacturer's instructions. Measurements, accuracy of metal determinations by the addition of known metal concentrations, and accuracy of mineralization using reference material were done as described previously (Kováčik et al. 2012a; Kováčik et al. 2012b).

### Assay of sum of phenols and acid hydrolysis

For the assay of soluble phenols, honey samples were dissolved in the ratio 0.1 g FW per 1 ml of 80 % aqueous methanol followed by centrifugation (4500 rpm) in order to remove eventual insoluble parts from the supernatant. This extract was used for direct measurement of soluble phenols and for acid hydrolysis using three approaches:

(i) Acid hydrolysis A—1 ml of methanolic supernatant was mixed with 1 ml of concentrated HCl (32 %, Sigma-Aldrich) and heated at 60 °C in an oven over 1 h. Then, 1 ml of mixture was evaporated to dryness using SpeedVac and dissolved in 1 ml of 80 % aqueous methanol.

(ii) Acid hydrolysis B—0.9 ml of methanolic supernatant was mixed with 0.1 ml of concentrated HCl and heated at 60 °C in an oven over 1 h. Thereafter, reaction mixture was extracted twice with anhydrous diethyl ether (2×2 ml), evaporated to dryness using SpeedVac and dissolved in 1 ml of 80 % aqueous methanol (Janas et al. 2000).

(iii) Acid hydrolysis C—0.9 ml of methanolic supernatant was mixed with 0.1 ml of concentrated HCl and heated at 60 °C in an oven over 1 h. Phenols were directly measured in this reaction mixture.

Quantification of phenols in all four abovementioned extracts was done using Folin-Ciocalteu phenol reagent (Sigma-Aldrich): the reaction mixture (1.5 ml) contained 30 µl of supernatant, 470 µl of redistilled water, 975 µl of 2 % Na<sub>2</sub>CO<sub>3</sub>, and 25 µl (2 N) of Folin-Ciocalteu reagent. Samples were incubated at 45 °C and absorbance at 750 nm was measured 1 h later. The blank contained 500 µl of redistilled water plus Na<sub>2</sub>CO<sub>3</sub> and the Folin-Ciocalteu reagent. Calculation was based on the calibration curve prepared using gallic acid (Kováčik and Bačkor 2007).

### HPLC quantification of phenolics and 5-(hydroxymethyl) furfural

Phenolics were determined as free compounds and after acid hydrolysis A mentioned above. The method described previously (Grúz et al. 2008) was modified to allow quantification of several flavonoids in addition to phenolic acids. The gradient consisting of solvent A (MeOH) and solvent B (7.5 mM HCOOH) was slightly prolonged as follows: 5 % B for 0.8 min, 5–10 % B over 0.4 min, isocratic 10 % B for 0.7 min, 10–15 % B over 0.5 min, isocratic 15 % B for 1.3 min, 15–21 % over 0.3 min, isocratic 21 % B for 1.2 min, 21–36 % B over 1 min, isocratic 36 % B over 3.8 min, 36–62 % B over 2.4 min, 62–70 % B over 3.1 min, 70–100 % B over 0.5 min, and finally 100–5 % B over 0.5 min. MRM mode was used for quantification of naringenin (271>151), rhamnetin (315>165), and pinobaksin

(271>253). All compounds were identified and quantified by using authentic standards. In addition to phenolics, a major peak found in UV chromatograms was identified as 5-(hydroxymethyl)furfural (5HMF) by comparing its retention time and spectral data with those of authentic standard. 5HMF was quantified by using UV detection at 285 nm both as native (methanolic extract) and after acid hydrolysis as mentioned above. LOD and LOQ of 5HMF were 0.49 and 0.62 ng injected, respectively.

### Measurement of thiols and proteins

For the assay of soluble proteins and non-protein thiols, honey samples were dissolved in the ratio 0.1 g FW per 1 ml of 50 mM sodium phosphate buffer (pH 7.0) followed by centrifugation (4500 rpm) in order to remove eventual insoluble parts from the supernatant. For the quantification of proteins, 100 µl of supernatant was mixed with 900 µl of Bradford's solution (Biorad, USA) and absorbance was measured 20 min later (595 nm); bovine serum albumin was used for calibration curve (Kováčik and Klejdus 2014). Non-protein thiols were quantified using Ellman's reagent (100 µl of supernatant, 800 µl of distilled water, 100 µl of Ellman's reagent—5 mM 5,5'-dithiobis-2-nitrobenzoic acid dissolved in sodium phosphate buffer), and absorbance was measured 20 min later (412 nm); cysteine was used for calibration curve (Kováčik et al. 2014).

## Results and discussion

### Forest honey showed high conductivity and amount of numerous metals

Based on the extensive research, electrical conductivity (EC) is a good criterion to distinguish blossom or mixed honey from honeydew or chestnut honey having EC typically less than 800 and more than 800 µS cm<sup>-1</sup>, respectively (Bogdanov et al. Honey Quality and International Regulatory Standards). Our values from monofloral (blossom) honeys (~200 µS cm<sup>-1</sup>, Table 1) are within the range found in other monofloral honeys (Petretto et al. 2015) including rapeseed and acacia samples from Slovakia (Lašáková et al. 2009). On the other hand, EC over 1200 µS cm<sup>-1</sup> in forest honey is much higher than that from chestnut samples (Petretto et al. 2015) but is within the range of the majority of samples marked as “forest” or “honeydew” originated from the vicinity of Košice mainly (Lašáková et al. 2009). It is concluded that our forest honey certainly contains considerable part of honeydew but is rather mixed with blossom owing to typical smell and taste.

Among dietary (beneficial) elements, only the amount of Ca and Na was not the highest in forest honey (Table 1). Other metals were ca. 3–5 times (Mg, Fe) or >10 times (K, Zn, Mn,

**Table 1** Amount of selected metallic nutrients and electrical conductivity (EC) in honey samples of various botanical and geographical origins

	Forest	Black locust	Rapeseed
Dietary elements			
K (mg g <sup>-1</sup> FW)	3.71±0.20 a	0.34±0.027 b	0.33±0.058 b
Mg (µg g <sup>-1</sup> FW)	65.0±3.32 a	12.5±2.44 b	20.8±3.64 b
Ca (µg g <sup>-1</sup> FW)	24.1±2.66 b	20.3±3.09 b	36.6±4.82 a
Mn (µg g <sup>-1</sup> FW)	15.1±2.30 a	0.44±0.053 b	0.47±0.043 b
Na (µg g <sup>-1</sup> FW)	9.67±0.84 a	8.49±1.10 a	10.3±1.52 a
Fe (µg g <sup>-1</sup> FW)	5.14±0.60 a	1.02±0.21 b	1.09±0.15 b
Cu (ng g <sup>-1</sup> FW)	2015.3±150.2 a	83.8±13.5 b	45.0±4.37 c
Zn (ng g <sup>-1</sup> FW)	1303.7±65.5 a	159.1±12.7 b	167.8±28.5 b
Toxic elements			
Al (µg g <sup>-1</sup> FW)	16.5±2.47 a	4.31±0.38 b	4.83±0.34 b
Ni (ng g <sup>-1</sup> FW)	1358.6±241.9 a	23.4±3.81 c	37.8±5.26 b
Pb (ng g <sup>-1</sup> FW)	85.6±7.19 a	9.40±1.13 b	7.36±0.54 b
Cd (ng g <sup>-1</sup> FW)	52.4±3.52 a	1.17±0.18 b	1.24±0.26 b
Cr (ng g <sup>-1</sup> FW)	32.5±3.51 b	26.8±3.59 b	43.3±3.67 a
Hg (ng g <sup>-1</sup> FW)	2.65±0.38 a	0.90±0.099 b	0.82±0.058 b
EC (µS cm <sup>-1</sup> )	1243.6±54.1 a	217.3±15.0 b	230.8±22.9 b

Forest (locality 3), black locust (locality 2), and rapeseed (locality 1) as indicated in Fig. 1. Data are means±SDs ( $n=3$ ). Values within rows, followed by the same letter(s), are not significantly different according to Tukey's test ( $P<0.05$ )

Cu) more accumulated in the forest honey than in two other types (Table 1). Differences between black locust and rapeseed honeys were rather negligible (ca. 2 times significant difference in terms of Ca and Cu content). These data indicate that distance from the eventual source of pollution (steel factory in Košice) is not the main factor affecting the amount of metals because locality where forest honey was collected is the farthest from the town (locality 3, see Fig. 1). It may therefore be expected that the botanical origin is the main determinant of observed differences considering that various plant species accumulate metals in various quantities which are further accumulated in the pollen and nectar. In this view, higher content of metals in forest honey as mixed type of honey (involving honeydews from trees on the locality as proven by high conductivity mentioned above) may partially explain observed variability. In accordance with our data, typically higher amounts of K, Zn, or Cu (but lower Ca content) in honeydew than in rapeseed honeys were previously observed in Poland (with similar concentration and ratio ca. 10:1 for K amount, Madejczyk and Baralkiewicz 2008). Additionally, Hungarian honeys showed more K, Mn, Cu, and Fe in “forest” (exact type not mentioned) than in acacia/rapeseed honey (Czipa et al. 2015). Also, comparison of the Czech nectar and honeydew honeys revealed higher amount of Zn but less Ca in honeydew type (Lachman et al. 2007) and monofloral (*Rhododendron*) honey contained higher

concentration of Ca but lower concentrations of K, Fe, and Mn than multifloral samples (Silici et al. 2008) that is in accordance with our data if rapeseed or partially black locust (monofloral) and forest honeys are compared (Table 1). Quantities of individual minerals in our Slovak monofloral honeys (rapeseed and black locust) presented here are within the range reported from various countries reaching rather lower limit of the range (Pohl 2009) but samples of forest honey are near the upper limit. In the case of manganese, our forest samples contained ca. 2 times more Mn ( $15.1±2.30$  µg g<sup>-1</sup>) than any report from the Czech Republic or Poland (Lachman et al. 2007; Madejczyk and Baralkiewicz 2008) but other countries reported yet higher values (Pohl 2009). Though source (locality) of individual honeys in various papers could only hardly be detected, it is clear that botanical origin substantially affects composition of mineral nutrients: in the case of the “Mn excess” in our forest honey, we assume it is (mainly) related to occurrence of the genus *Rubus* on the locality (typical smell of honey and observation of honey bees movement) because just species of this genus accumulated high amount of Mn on natural localities (Kula et al. 2012).

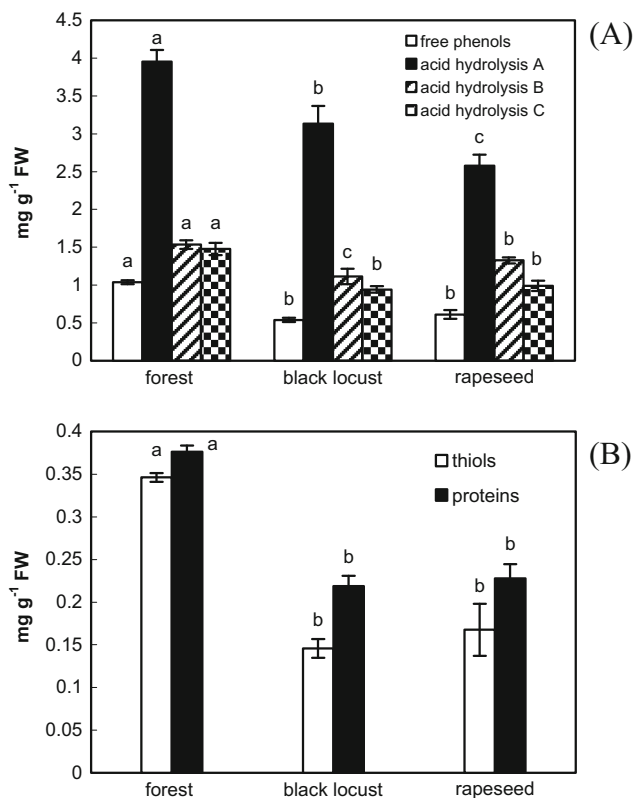
At the level of toxic (or non-beneficial) metals, several interesting results were obtained. Accumulation of Cr and Ni observed here if forest vs. monofloral honeys are compared (Table 1) is within the quantitative range and with similar ratio to values recorded in Poland: Cr did not differ but Ni was more accumulated in honeydew than in rapeseed honey (Madejczyk and Baralkiewicz 2008). High amounts of Ni were also detected in 24 Czech honey samples but we note that differences were less pronounced compared to our data though safety level  $6$  µg g<sup>-1</sup> was not reached (up to  $1.53$  µg g<sup>-1</sup> in honeydew honey, see Lachman et al. 2007 for details). Also, comparison of 20 honey types from Turkey did not reveal such extensive differences (Silici et al. 2008) as observed in the present study and we assume occurrence of Ni-accumulating plant species on the locality 3 (Fig. 1). To support our assumption, monofloral (sunflower) honey revealed only up to  $115$  ng Ni g<sup>-1</sup> even from the industrial area (Citak et al. 2012). Unfortunately, content of Pb or Cd in honeys in the two mentioned studies from Poland and the Czech Republic was not quantified but the range reported for Poland in the extensive review reaches  $8–27$  ng g<sup>-1</sup> for Cd and  $20–70$  ng g<sup>-1</sup> for Pb (Pohl 2009): this fits well with our means for rapeseed and black locust but forest honey exceeds this range and contains several times higher amount than two other honey types (Table 1). In agreement, Hungarian forest honey contained more Pb and Cd than acacia/rapeseed honeys but differences were quantitatively less expressive than observed here (Czipa et al. 2015). Few studies from other geographical regions also reported higher amount of Cd and Pb in honeys from Brazil and Turkey in comparison with our black locust (acacia) and rapeseed honeys but botanical source of those honeys was not mentioned (Tuzen and Soylok 2005; de

Andrade et al. 2014). The amount of Al in forest vs. rapeseed honey observed here (Table 1) is excellently in quantitative agreement with data from Czech honeys where nectar samples contained ca. 4 times less of this metal than honeydew samples (Lachman et al. 2007). Data related to Hg content in honey are scarce. Its amount was not extensively different among various honey types (up to twofold), indicating it is rather environmental contaminant than naturally occurring metal. Our data may be easily compared with analyses of flowers of medicinal plant (chamomile) growing naturally in the field in Košice where higher amount of Hg was detected (even considering expression per grams dry weight 46–197 ng g<sup>-1</sup> DW, Kováčik et al. 2012a). On the other hand, an earlier study from Slovakia reported a Hg content of 1–3 ng g<sup>-1</sup> in honey from uncontaminated areas (Toporcák et al. 1992) that is within the range observed here (Table 1): it seems that honey Hg pollution did not increase with time in Slovakia. We found no threshold data for toxic metals in honey but according to the EU Commission Regulation (EC) No 1881/2006, the lowest contents are set for fresh vegetables: Pb 100–300 ng g<sup>-1</sup>, Cd 50–200 ng g<sup>-1</sup>, and 100 ng Hg g<sup>-1</sup> FW (in dietary supplements). Results of our honey samples are within (forest honey) or below this range (rapeseed and black locust honeys) indicating their food safety (Table 1).

Overall, forest honey contained higher concentrations of majority of detected metals than rather local soil conditions and composition of plant species than the distance from the eventual source of pollution (steel factory near the Košice) affects metallic composition. This conclusion is supported by the fact that differences between mixed honey and two monofloral honeys observed here are more extensive than those found in the Czech Republic or Turkey (Lachman et al. 2007; Silici et al. 2008) while quantitative amounts as well as the ratio of, e.g., K, Cr, or Ni resemble values recorded in Poland (Madejczyk and Baralkiewicz 2008).

**Total phenols and individual phenolics are mainly abundant in forest honey**

Beneficial effects of phenolic compounds on human health mainly owing to their antioxidative properties are well known (Biesaga and Pyrzyńska 2013). Assay of “soluble phenols” or “total phenolic content” (TPC) using Folin-Ciocalteu reagent is frequently used in various matrices including honey. Our data indicate that mixed (forest) honey contained ca. 2 times more TPC than monofloral honeys in methanolic solution (1 vs. 0.5 mg g<sup>-1</sup>, Fig. 2a). This is a good correlation with a unique report from Slovak honeys where forest and multifloral samples typically contained more TPC than black locust (acacia) honey (Zalibera et al. 2008): higher absolute amount of TPC in our samples (ca. twofold) in comparison with the mentioned study could arise from various solvents used for the preparation of honey solution (we used 80 % methanol but



**Fig. 2** Amount of selected metabolites (quantified by spectrophotometry) in honey samples of various botanical and geographical origins. Forest (locality 3), black locust (locality 2), and rapeseed (locality 1) as indicated in Fig. 1. **a** Assay of soluble phenols performed in methanolic solution (free phenols) and various acid hydrolysis methods (acid hydrolysis A, B, and C where A involved the strongest HCl concentration ca. 5 M, see “Materials and methods” section for details). **b** Assay of non-protein thiols (with Ellman’s reagent) and soluble proteins (with Bradford’s reagent). Data are means±SDs (n=3). Values for individual parameters, followed by the same letter(s), are not significantly different according to Tukey’s test (P < 0.05)

water was used by Zalibera et al. 2008). However, such differences between forest/multifloral and black locust samples are probably related to plant species and locality, because various honeys from Florida did not reveal clear difference in relation to botanical origin but TPC values (up to 1 mg g<sup>-1</sup>) are similar to our data (Marshall et al. 2014). Another study from Mediterranean areas also showed TPC content around 0.5 mg g<sup>-1</sup> in various multifloral honeys and rather the impact of harvest time than species (Rababah et al. 2014). We subsequently performed acid hydrolysis in order to identify the amount of phenols bound in glycosides. In addition to standard procedure involving mild acidic conditions (ca. 1 M HCl, Janas et al. 2000) for the release of phenols, we also performed “harsh” hydrolysis (ca. 5 M HCl) owing to the high amount of various sugars in honey. Our data confirmed increase in TPC after mild hydrolysis (marked as acid hydrolysis B and C in Fig. 2a), and the highest amount was detected in forest honey. Comparison of these two hydrolysis types also indicated that extraction of phenols to diethyl ether or direct measurement

from hydrolytic mixture has a relatively negligible impact in various honey types (Fig. 2a). Surprisingly, “harsh” hydrolysis (marked as acid hydrolysis A) showed TPC values 4–6 times higher in all honeys compared to free phenols (Fig. 2a).

Owing to the mentioned observation, subsequent identification and quantification of individual phenols was done in methanolic (free) extracts and in “acid hydrolysis A” extracts. To our knowledge, central European or European authentic honeys were not deeply characterized in terms of individual phenols. Recently, samples from heather honey in Poland were tested but the origin of honey was not specified (Biesaga and Pyrzyńska 2013): acidified methanol showed higher extraction efficiency than acidified water in the mentioned work and quantitative data of detected phenolic acids and flavonoids are usually within the order of our values (Table 2). Comparison of our individual honeys revealed typically the highest amount of benzoic acid derivatives in forest honey but cinnamic acid derivatives were mainly abundant in rapeseed

honey: identical trend was observed at the level of free compounds and after acid hydrolysis (Table 2) though absolute quantity of cinnamic acids was depleted, probably owing to strong acidic conditions. Some flavonoid aglycones did the same, and their sensitivity to sample processing has been reported (Biesaga and Pyrzyńska 2013). Among individual flavonoids, flavanols and flavonols seem to be more accumulated in rapeseed (Table 2). Appearance of flavonol aglycones (kaempferol, rhamnetin) after acid hydrolysis indicates that various glycosides (not included in our spectrum) are present in honeys. Observed difference between forest and rapeseed honey is a relatively good sign because previous comparison of honeys of various botanical origins from Florida did not reveal a clear trend (Marshall et al. 2014). Acid hydrolysis A evoked an extensive increase (up to tenfold) in comparison with free phenols among benzoic acids and flavonols mainly in forest honey, that is in accordance with increase in TPC (cf. Fig. 2a and Table 2). In terms of basic quantity, high amount of

**Table 2** Amount of selected phenolic metabolites (ng g<sup>-1</sup> FW) in honey samples of various botanical and geographical origins

	Free compounds			Acid hydrolysis A		
	Forest	Black locust	Rapeseed	Forest	Black locust	Rapeseed
<b>Benzoic acids</b>						
Gallic	419.3±18.0 a	nd	1.53±0.38 b	nd	nd	nd
Protocatechuic	12443.9±460.2 a	577.8±20.6 c	857.2±46.1 b	25840.2±1589.0 a	924.7±113.8 c	1349.3±84.5 b
<i>p</i> -hydroxybenzoic	3054.5±136.0 a	1569.7±165.1 b	3120.9±108.3 a	9016.5±797.4 a	2194.0±96.3 c	3421.7±102.4 b
<i>m</i> -hydroxybenzoic	290.1±20.8 a	nd	46.8±3.43 b	470.6±49.8 a	nd	94.1±9.96 b
Vanillic	450.9±16.5 b	476.4±23.0 b	934.8±29.2 a	6176.6±252.9 a	3273.5±161.5 b	2934.7±348.6 b
Syringic	734.0±18.9 b	701.6±63.8 b	2096.6±189.0 a	2464.4±207.3 c	8671.2±312.4 b	19378.1±2338.7 a
Salicylic	8175.5±367.8 a	235.1±17.3 c	461.4±17.2 b	42973.0±3348.6 a	1074.3±83.7 c	2148.6±167.4 b
<b>Cinnamic acids</b>						
Chlorogenic	13.7±1.09 a	nd	nd	nd	nd	nd
Caffeic	394.4±11.5 c	536.7±22.4 b	687.5±30.8 a	118.7±10.3 a	53.8±9.37 b	106.2±8.51 a
Ferulic	798.7±42.6 b	1127.6±91.5 a	1291.9±59.6 a	364.2±19.9 b	637.4±35.2 a	747.1±106.0 a
Isoferulic	321.5±31.2 b	398.7±29.3 b	507.1±20.0 a	nd	nd	nd
<i>p</i> -coumaric	1263.6±90.1 b	1350.8±127.6 b	1804.4±75.3 a	519.0±31.4 b	359.3±16.9 c	702.2±54.6 a
<i>m</i> -coumaric	11.5±0.67 a	nd	nd	nd	nd	nd
Sinapic	2.23±0.29 a	2.96±0.21 a	1.23±0.17 b	nd	nd	nd
<b>Flavanones</b>						
Eriodictyol	nd	nd	5.96±0.25 a	28.3±5.80 a	14.1±2.92 b	13.0±1.65 b
Naringenin	7.73±0.38 a	5.11±0.36 b	3.32±0.44 c	nd	nd	nd
<b>Flavanonols</b>						
Pinobanksin	582.6±25.9 b	573.2±9.85 b	787.5±37.2 a	nd	nd	nd
<b>Flavonols</b>						
Kaempferol	nd	nd	nd	290.9±14.2 a	73.0±5.98 c	120.8±15.6 b
Rhamnetin	72.7±6.19 b	163.9±17.5 a	143.8±10.3 a	262.4±33.5 b	491.9±50.4 a	503.5±23.2 a

Forest (locality 3), black locust (locality 2), and rapeseed (locality 1) as indicated in Fig. 1. Data are means±SDs ( $n=3$ ). Values within rows for free compounds or acid hydrolysis A, followed by the same letter(s), are not significantly different according to Tukey's test ( $P<0.05$ ). Acid hydrolysis A denotes hydrolysis with ca. 5 M HCl as shown in Fig. 2

nd not detected

protocatechuic and salicylic acids mainly in forest honey had not yet been reported. In accordance with our data, chlorogenic acid and kaempferol were rather absent while pinobanksin was found in similar range ( $>500 \text{ ng g}^{-1}$ ) in various monofloral honeys from Sardinia (Petretto et al. 2015). On the other hand, common flavonoids usually found in various honeys such as chrysin, quercetin, luteolin, or rutin (Biesaga and Pyrzyńska 2013; Marshall et al. 2014) were not detected in our samples.

**Amount of thiols and proteins in various honeys**

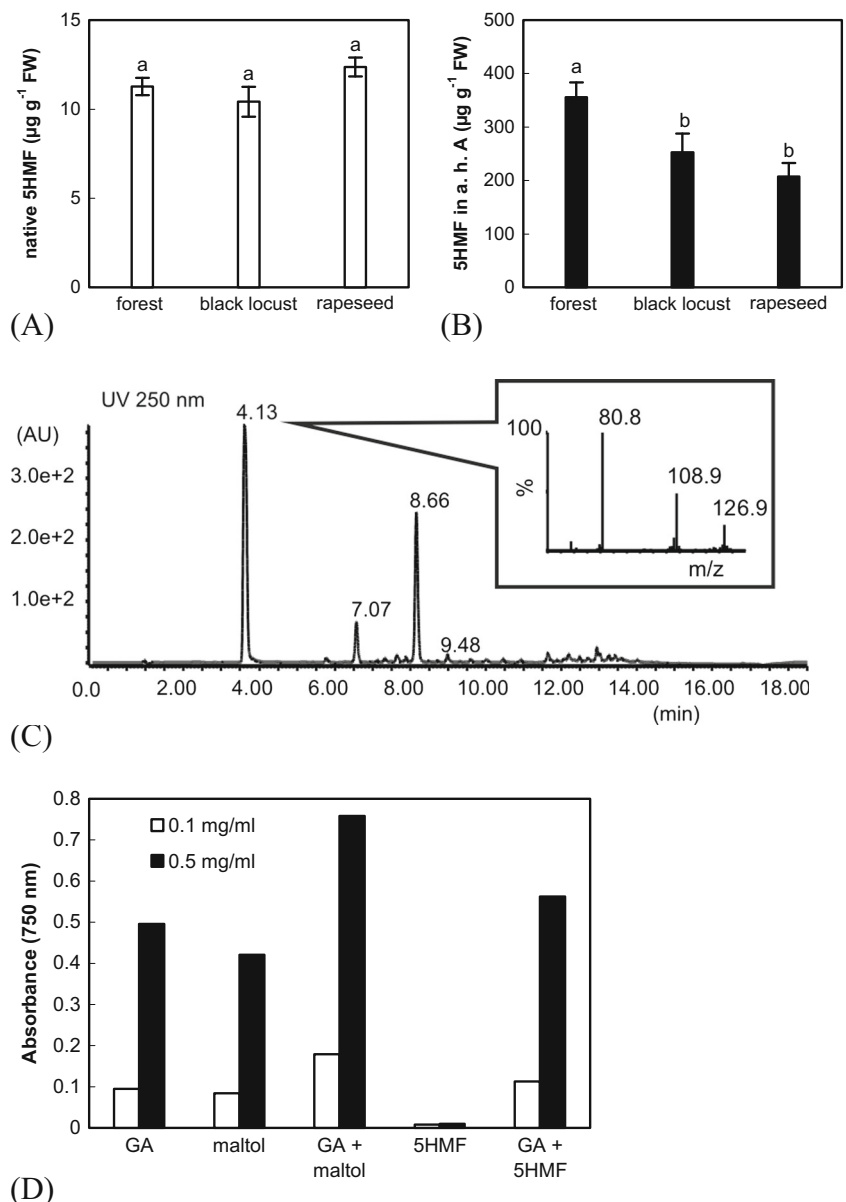
Nitrogenous metabolites (non-protein thiols and soluble proteins) were detected in higher amounts in forest than in monofloral honeys (Fig. 2b). In all honey types, proteins were

slightly more abundant than thiols, which is in accordance with data from various plants where proteins are present in higher quantity (Kováčik et al. 2014). However, absolute quantity of proteins is ca. 30 times lower than in the mentioned study using medicinal plant and even ca. 10 times higher amount of proteins in honeys was reported ( $3\text{--}6 \text{ mg g}^{-1}$ , Ajibola et al. 2012). Further studies using proteomic analyses are needed.

**Quantification of 5-(hydroxymethyl)furfural did not reveal honey-specific amount**

The 5HMF is a common product arising from sugars via Maillard reaction, possessing potential health risk for humans due to its presence in many foods: several reports indicated

**Fig. 3** Quantification of 5-(hydroxymethyl)furfural (5HMF) in honey samples of various botanical and geographical origins. **a** Methanolic extract (native 5HMF). **b** Hydrolysis with HCl (5HMF in acid hydrolysis A, see “Materials and methods” section for details of hydrolysis) and statistics as mentioned in Fig. 2. **c** UPLC-UV chromatogram of honey sample after acid hydrolysis: the major UV-absorbing compound with retention time of 4.13 min was identified as 5HMF by comparison of retention time and spectral data with those of an authentic standard (mass spectra of 5HMF after collision-induced dissociation (CE 23 eV) are given in the inset). **d** Contribution of two sugar derivatives (maltol and 5HMF) to alteration of soluble phenol assay using gallic acid (GA) as reference phenol: stock solutions were prepared in 80 % methanol in two doses (0.1 or 0.5 mg/ml, combined solutions contained both compounds in the given dose) and 30  $\mu\text{l}$  of these solutions was used in the final volume of reaction mixture 1.5 ml (see “Materials and methods” section) giving the shown absorbance. Data are means of three independent replicates



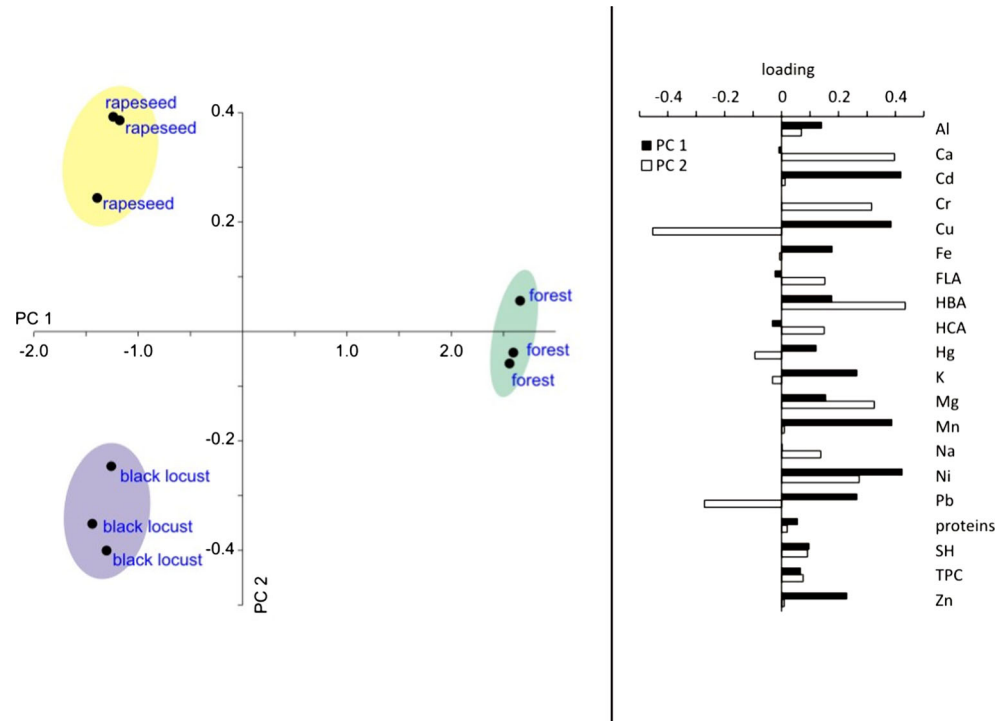
that 5HMF and its subsequent metabolites may exhibit a cytotoxic and carcinogenic effect (see Wu et al., 2009 and the references therein). More generally, 5HMF level indicates freshness of honey and increases with time and storage temperature mainly. For this reason, monitoring of this compound in foods received greater attention in the last few years. In contradiction to our data where native 5HMF was detected in all honey types (Fig. 3a), it was not reported in fresh Brazilian honeys (Biluca et al. 2014). This may be evoked by longer storage of our samples prior to measurement (ca. 3 years) but our values ( $\sim 11 \mu\text{g g}^{-1}$ ) are still low considering estimated daily intake of 5HMF  $\sim 150 \text{ mg}$  (see Wu et al. 2009 and the references therein) and recommended safety level in honey ( $40 \mu\text{g g}^{-1}$ ) according to the EU directive (110/2001). Besides, our values of native 5HMF are several times lower compared to honeys from India where amounts over  $100 \mu\text{g g}^{-1}$  were detected (Chakraborti and Bhattacharya 2014). Subsequent quantification of 5HMF after acid hydrolysis A (strongly acidic conditions) confirmed its high increase in all samples and mainly in forest honey (Fig. 3b), that is in agreement with its generation also through degradation of hexoses in acid media (Biluca et al. 2014). However, our data from acid hydrolysis are still lower than in the mentioned Brazilian honey where elevated temperature over 24 h led to  $695 \mu\text{g g}^{-1}$  5HMF (Biluca et al. 2014). It seems that longer storage of honey evoked relatively negligible increase of 5HMF in Slovak honeys, and further time-dynamic studies should be carried out.

Owing to the appearance of 5HMF after acid hydrolysis, we tested its impact on the reaction with Folin-Ciocalteu reagent (FCR) used for soluble phenols assay because the amount of phenols increased just after acid hydrolysis (cf. Figs. 2a and 3b). Detailed assay showed that 5HMF reacts only weakly with FCR if compared with gallic acid (ca. 5–10 %) and elevates response of gallic acid by only ca. 10 % (GA+5HMF vs. GA, Fig. 3d). On the other hand, maltol, that is a naturally occurring compound but is also formed in some roasted foods, revealed response to FCR comparable with gallic acid and almost doubled GA-evoked absorbance (Fig. 3d). However, maltol was not detected in our honey samples.

### Principal component analysis distinguished honeys based on metals and phenols

Principal component analysis distinguished honeys based on metals and phenols. The PCA displays the separation of different honeys based on the determined parameters (Fig. 4). The loading shows contribution of biochemical parameters to the separation of honey samples along with both PC 1 and PC 2. Briefly, Ni, Mn, and Cd mainly contribute to the separation of forest honey from the remaining two honeys while HBA, Ca, and Cu are mainly correlated with PC 2 and thus contribute to the separation of rapeseed from black locust. The results suggest that the abovementioned parameters can be used to distinguish between different honey types from the analyzed locations. In a study by Cziza et al. (2015), Ca, Cu, and Mn were also found to be among the most discriminating variables which are in accordance with

**Fig. 4** The PCA biplot showing the separation of different honeys based on determined parameters (except for 5HMF). *FLA* sum of flavonoids, *HBA* sum of benzoic acids, *HCA* sum of cinnamic acids, *SH* non-protein thiols, *TPC* total phenolic content





our data. In contrast, we found only weak loading for Na and K while Fermo et al. (2013) found K, Ca, and Na to strongly depend on geographical origin. This discrepancy can be caused by the presence of samples from coastal areas in which higher levels of Na are common if compared to honeys from inland.

### Conclusions

Comparison of authentic honey samples of various botanical origins from the vicinity of an industrial town in Slovakia confirmed that the amount of minerals including toxic metals is mainly affected by the locality, indicating the impact of soil conditions and/or plant species. Exceptionally high content of nickel in forest honey is in agreement with some reports from surrounding countries including Poland and the Czech Republic but is still within the safety limit. Other toxic metals such as Pb and Cd were more accumulated in forest honey than in monofloral samples (black locust and rapeseed) while some dietary elements such as Ca were less accumulated. Subsequent analyses revealed a higher amount of total phenols and nitrogenous metabolites in forest honey, but detailed investigation of individual phenolics showed a variable trend. Native content of potentially toxic 5-(hydroxymethyl)furfural was not related to honey type and was still lower than accepted threshold value for well-stored honey. Overall, sufficient amount of phenols but lower accumulation of some toxic metals in our Slovak black locust (acacia) honey suggest its safe use. Detailed studies of the same type of honey from various localities are our further aim.

**Acknowledgements** JK, corresponding author of this paper born in Košice, dedicates this work to the memory of Ing. Míchal Lovás (1937–2012) who provided honey samples used in the study. The work was financially supported by OP Education for Competitiveness CZ.1.07/2.3.00/30.0017 Postdocs in Biological Sciences at MENDELU (European Social Fund and the state budget of the Czech Republic) and the program “Návrat” for Research, Development, and Innovations (LK21306) funded by the Ministry of Education, Youth and Sports of the Czech Republic.

**Disclosure statement** The authors declare that they have no competing interests.

**Role of the funding source** Sponsors had no involvement in the present study.

### References

Ajibola A, Chamunorwa JP, Erlwanger KH (2012) Nutraceutical values of natural honey and its contribution to human health and wealth. *Nutr Metab* 9:61

Biesaga M, Pyrzyńska K (2013) Stability of bioactive polyphenols from honey during different extraction methods. *Food Chem* 136:46–54

Biluca FC, Betta FD, de Oliveira GP, Pereira LM, Gonzaga LV, Oliveira Costa AC, Fett R (2014) 5-HMF and carbohydrates content in

stingless bee honey by CE before and after thermal treatment. *Food Chem* 159:244–249

Bogdanov S et al. Honey Quality and International Regulatory Standards (Review by the International Honey Commission). [http://www.beekeeping.com/articles/us/honey\\_quality.htm](http://www.beekeeping.com/articles/us/honey_quality.htm)

Chakraborti T, Bhattacharya K (2014) Quality assessment of some Indian honeys in storage through HMF content and invertase activity. *Int J Pharm Pharm Sci* 6:827–830

Citak D, Silici S, Tuzen M, Soylak M (2012) Determination of toxic and essential elements in sunflower honey from Thrace region, Turkey. *Int J Food Sci Technol* 47:107–113

Cziza N, András D, Kovács B (2015) Determination of essential and toxic elements in Hungarian honeys. *Food Chem* 175:536–542

de Andrade CK, dos Anjos VE, Felsner ML, Torres YR, Quinária SP (2014) Relationship between geographical origin and contents of Pb, Cd, and Cr in honey samples from the state of Paraná (Brazil) with chemometric approach. *Environ Sci Pollut Res* 21:12372–12381

Fermo P, Beretta G, Facino RM, Gelmini F, Piazzalunga A (2013) Ionic profile of honey as a potential indicator of botanical origin and global environmental pollution. *Environ Pollut* 178:173–181

Grúz J, Novák O, Strnad M (2008) Rapid analysis of phenolic acids in beverages by UPLC-MS/MS. *Food Chem* 111:789–794

Janas KM, Cvikrova M, PałTgiewicz A, Eder J (2000) Alterations in phenylpropanoid content in soybean roots during low temperature acclimation. *Plant Physiol Biochem* 38:587–593

Kováčik J, Bačkor M (2007) Phenylalanine ammonia-lyase and phenolic compounds in chamomile tolerance to cadmium and copper excess. *Water Air Soil Pollut* 185:185–193

Kováčik J, Klejdus B (2014) Induction of phenolic metabolites and physiological changes in chamomile plants in relation to nitrogen nutrition. *Food Chem* 142:334–341

Kováčik J, Grúz J, Klejdus B, Štork F, Hedbavny J (2012a) Accumulation of metals and selected nutritional parameters in the field-grown chamomile anthodia. *Food Chem* 131:55–62

Kováčik J, Klejdus B, Štork F, Hedbavny J (2012b) Physiological responses of *Tillandsia albida* (Bromeliaceae) to long-term foliar metal application. *J Hazard Mater* 239–240:175–182

Kováčik J, Babula P, Hedbavny J, Klejdus B (2014) Hexavalent chromium damages chamomile plants by alteration of antioxidants and its uptake is prevented by calcium. *J Hazard Mater* 273:110–117

Kula E, Hrdlička P, Hedbavny J, Švec P (2012) Various content of manganese in selected forest tree species and plants in the undergrowth. *Beskydy* 5:19–26

Lachman J, Kolihová D, Miholová D, Košata J, Titěra D, Kult K (2007) Analysis of minority honey components: possible use for the evaluation of honey quality. *Food Chem* 101:973–979

Lašáková D, Nagy J, Kasperová J (2009) Comparison of water content and electric conductivity in honey of various origin. *Folia Veterinaria* 53:31–34

Majejczyk M, Baralkiewicz D (2008) Characterization of Polish rape and honeydew honey according to their mineral contents using ICP-MS and F-AAS/AES. *Anal Chim Acta* 617:11–17

Marshall SM, Schneider KR, Cisneros KV, Gu L (2014) Determination of antioxidant capacities,  $\alpha$ -dicarbonyls, and phenolic phytochemicals in Florida varietal honeys using HPLC-DAD-ESI-MS<sup>n</sup>. *J Agric Food Chem* 62:8623–8631

Petretto GL, Cossu M, Alamanni MC (2015) Phenolic content, antioxidant and physico-chemical properties of Sardinian monofloral honeys. *Int J Food Sci Technol* 50:482–491

Pohl P (2009) Determination of metal content in honey by atomic absorption and emission spectrometries. *Trends Anal Chem* 28:117–128

Rababah TM, Al-Omouh M, Brewer S, Alhamad M, Yang W, Alrababah M, Al-Ghazawi AAM, Al-U Datt M, Alsheyab F, Esoh R, Almajwal A (2014) Total phenol, antioxidant activity, flavonoids, anthocyanins and color of honey as affected by floral origin found in the arid and semiarid Mediterranean areas. *J Food Process Preserv* 38:1119–1128

- Silici S, Uluozlu OD, Tuzen M, Soylak M (2008) Assessment of element levels in Rhododendron honeys of Black Sea region, Turkey. *J Hazard Mater* 156:612–618
- Toporcák J, Legáth J, Kul'ková J (1992) Levels of mercury in samples of bees and honey from areas with and without industrial contamination. *Veterinarni Medicina* 37:405–412
- Tuzen M, Soylak M (2005) Trace heavy metal levels in microwave digested honey samples from Middle Anatolia, Turkey. *J Food Drug Anal* 13:343–347
- Wu JY, Shi ZG, Feng YQ (2009) Determination of 5-hydroxymethylfurfural using derivatization combined with polymer monolith microextraction by high-performance liquid chromatography. *J Agric Food Chem* 57: 3981–3988
- Zalibera M, Staško A, Šleboďová A, Jančovičová V, Čermáková T, Brezová V (2008) Antioxidant and radical-scavenging activities of Slovak honeys—an electron paramagnetic resonance study. *Food Chem* 110:512–521