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ORIGINAL PAPER

Combined approach to studying authenticity markers following spatial, temporal and production practice trends in honey from Croatia

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Abstract

The confirmation of honey authenticity is an ongoing challenge. We investigated new authenticity markers (13 macro and trace elements, total phenolic (TP) content, antioxidant capacity) in 62 unifloral and multifloral honeys from Croatia as loadings for principal component analysis (PCA), taking into account the spatial, temporal and production practice variation and combining them with traditional tools for authentication of the botanical origin (melissopalynological, sensory and physicochemical analyses). PCA as a chemometric tool was compliant with basic statistical testing results (Mann–Whitney *U* test) figuring Ba and Mn, and also pointed to TP, antioxidant capacity parameters, Ca, K and Mg (PC1) as useful markers for discriminating chestnut honey from other unifloral and multifloral honeys. The first PC discerned deciduous honeydew honey sample fairly from nectar honey samples. Although some elements showed regional, seasonal and production practice differences, PCA was not able to discriminate between all groups clearly. Our nutritional assessment based on a calculation of the contribution to the Dietary Reference Value pinpointed deciduous honeydew honey, savory and chestnut honey with the highest daily mineral intake relevance among seven honey types.

Keywords Macro and trace elements · Total phenolic content · Antioxidant capacity · Principal component analysis · Botanical origin · Geographical origin

Introduction

Honey is a highly valued natural product of honeybees whose beneficial components (carbohydrates, organic acids, proteins, amino acids, minerals, polyphenols, vitamins and aroma compounds) are prone to natural and anthropogenic variation [1, 2]. The composition of honey changes primarily due to botanical (nectar from various plant species, secretions from plants or plant-sucking insects) and regional effects (geology, geochemistry, vicinity of sea, climate, environmental pollution status), but to a lower extent also due to temporal (production year) and various production conditions (contamination during processing, beekeeper management practice) [2, 3]. Consequently, numbered

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factors change the quality and impact the price of honey. Forty percent of EU's consumed honey is imported low-cost honey (half of which originates from China) with a large proportion of adulteration cases, 20% of samples non-compliant with Council Directive 2001/110/EC [4] and 14% of samples with added sugar (sampled from EU's external border [5]. Thus Member States, including Croatia, have very high economic and consumer protection interest to promote local products with guaranteed quality and origin. Regarding the number of beehives, Croatia is the twelfth EU's country producing 7–8000 t of honey annually [6, 7], of which 6% is exported. Apparent consumption (production + import–export) in 2017–2019 period ranges in Croatia from 2.1 to 2.2 kg per capita [7–10]. Preferred honey types among consumers (31% multifloral, 27% black locust, 21% meadow, 9% chestnut, 3% lime [11]) follow the main production yields, although varying climatic and pasture conditions in the country enable the production of also sunflower, sage, Christ's thorn and honeydew honey [6] with unique geomorphological and pollen spectrum fingerprint. Mislabeling the botanical and geographical origin of honey because of economic profit has been a problem for decades. To protect consumers and producers from this kind of adulteration and consequently from economic damage and health benefit fraud, stakeholders prescribed compositional criteria, analytical testing and labelling rules for honey products [4, 12]. In addition, there is a constant need for improvements and innovations in origin confirmation by specific and easily applicable methods. Different markers have been explored to this end, e.g., flavonoids and other phenolic compounds, essential and nonessential elements, volatiles, stabile isotope ratios, leptosperin, organic acids, pollen, source plant DNA, etc. [1, 13, 14]. However, the challenge of choosing only one marker compound is its fraudulent addition to honey and as a result, inability to differentiate between native and added marker level [1]. Hence, the multicomponent analysis was suggested as the most promising approach for authenticity confirmation [13]. It would comprise various analytical method producing data and statistical data evaluation techniques (principal component analysis, cluster analysis, linear and square discriminant analysis, artificial neural networks, etc.; reviewed in Pohl [3]) taking into consideration financial and time expenses [1].

Minerals that range from 0.1 to 1% (honeydew honey) in honey mostly due to soil characteristics and the different nectars of honey-producing plants or secretions were explored as markers with chemometric tools for the discrimination of botanical and regional differences (reviewed by Bogdanov et al. [2], Pohl [3], Lazarević et al. [15]). Essential minerals like potassium (K), sodium (Na), calcium (Ca), magnesium (Mg), copper (Cu), iron (Fe), and manganese (Mn) were found to be indicators of such differences [16–29] more often than toxic metals (aluminium, Al; arsenic, As; cadmium, Cd [17, 24, 26, 29]) which are prone to additional variation due to anthropogenic pollution. Minerals, and more pronouncedly, phenolic compounds as natural antioxidant molecules, alongside vitamin C and E, and enzymes (peroxidase, catalase), are responsible for the antioxidant properties of honey. Some honey-containing elements participate in cell/organ antioxidant defense as a necessary part of enzymes that clear free radicals, e.g., superoxide-dismutase containing Zn, Cu, Mn; glutathione-peroxidase with selenium, Se; catalase containing Fe. Hence, the antioxidant capacity of honey together with some element or polyphenolic compound content was previously studied and showed strong association [11, 30, 31], which makes this parameter a promising marker in multicomponent studies of authenticity. Combined element and phytochemical data evaluations involving chemometric methods are scarce in the literature and were previously used in an attempt to authenticate Croatian honeys only in the case of sage honey [32].

We conducted a systematic assessment of honey authenticity markers: (i) macro and trace elements, (ii) non-nutrient phytochemicals (phenols), and (iii) antioxidant capacity, taking into account spatial, temporal and production practice trends across markers. The PCA chemometric method added to our multifaceted approach to classifying honeys from Croatia. In addition, a consumer-relevant overview of mineral intake relevance (based on Dietary Reference Values, [33, 34]) was presented for seven different honey types. This study is supplementary to a recent investigation of harmful, (potentially) toxic anthropogenic pollutants in honeys from Croatia [35].

Materials and methods

Honey sampling, melissopalynological and physicochemical analyses

A total of 62 honey samples (38 chestnut—C (*Castanea sativa* Mill.), three black locust—BL (*Robinia pseudoacacia* L.), one deciduous honeydew honey—H, three sage—SG (*Salvia officinalis* L.), two buckthorn—BU (*Rhamnus* spp.), two savory—SV (*Satureja* spp.) and 13 multifloral—MF) used in this study were donated by beekeepers from different Croatian regions (Fig. 1). Samples harvested in 2018 and 2019 season were put in clean glass jars, labelled, transferred to the laboratory in different periods from harvesting and kept in dark at 4 °C until analysis. Using traditional tools for authentication of botanical origin, qualitative melissopalynological analysis according to the Harmonized methods of melissopalynology [36], sensory [37] and physicochemical analyses (reducing sugar, sucrose and moisture content, electrical conductivity, free acid and hydroxymethylfurfural content, diastase activity [38, 39]) were conducted. Compliance

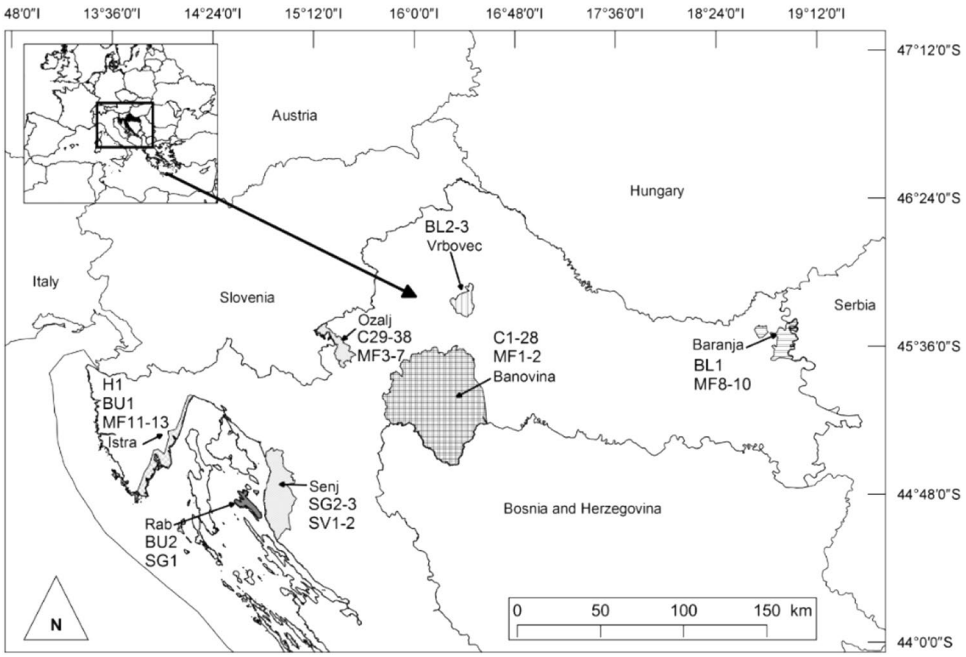

Fig. 1 Honey sampling locations in Croatia with noted sample codes: honey type (C-chestnut, BL-black locust, H-deciduous honeydew honey, SG-sage, BU-buckthorn, SV-savory, MF-multifloral) and ordinal number within the respective honey type

with the international honey standards defined by Council Directive 2001/110/EC [4] and Directive 2014/63/EU [12] was checked. Entire pollen spectrum, morphometry of pollen grains and relative frequency of the pollen types of nectariferous species or honeydew elements was conducted in honey sediment on a Axio Scope A1 (Carl Zeiss, Germany) light microscope at magnification 400–1000 × attached to a digital camera model Axiocam 208 Color (Carl Zeiss, Germany) and coupled to an analysis system (ZEN 3.1 blue edition). The identification was supported by literature data and internal pollen grain reference library (University of Zagreb Faculty of Agriculture).

Total phenolic (TP) content

The total phenolic content was quantified by the Folin–Ciocalteau method as previously described in Beretta et al. [40]. A water solution containing 40% fructose (Kemika, Croatia), 30% glucose (Sigma Aldrich, Germany), 8% maltose (Torlak, Serbia) and 2% sucrose (Fluka, Germany) was made to mimic honey with its main sugar components and was used as the sugar analogue to control for interferences. Before analysis, the sugar analogue and each sample of honey (1 g) were diluted to 5 ml with distilled water. An aliquot (0.1 mL) of 20% (w/v) honey solution was vortexed for 2 min with 1 mL of 10% Folin–Ciocalteau reagent (Sigma-Aldrich, Germany). The absorbance of the reaction mixture was measured at 750 nm against a sugar analogue after incubation at room temperature for 20 min. Solutions of gallic acid (Sigma-Aldrich, Germany) (10–150 mg/L) were used to construct the calibration curve and quantify samples. Results were expressed as mg gallic acid (GA) per kg of honey.

1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was determined by a modified method proposed by Tuberoso et al. [41]. An aliquot (0.2 mL) of 20% (w/v) aqueous honey solution and sugar analogue was mixed with 1.8 mL of methanol (Merck, Germany). Then, 1.5 mL of DPPH (Sigma Aldrich, Germany) methanolic solution (0.18 mmol/L) was added to the honey samples and vortexed vigorously. The mixture was incubated in the dark for 30 min at room temperature. The absorbance was measured at 517 nm against a methanol blank. Radical scavenging capacity was expressed as mmol of the Trolox equivalent per kg of honey (mmol TE/kg) using the appropriate calibration curve of Trolox (Fluka, Germany) (2–20 µmol/L).

Ferric reducing antioxidant power (FRAP)

The reducing capacity of honey samples was assayed according to the adjusted method of Benzie and Strain [42]. An aliquot (0.2 mL) of 5% (w/v) aqueous honey solution and sugar analogue was mixed with 1.8 mL of freshly prepared FRAP reagent, vortexed and incubated at 37 °C for 10 min. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (Sigma-Aldrich, Germany) solution and 20 mM $FeCl_3 \cdot 6H_2O$ (Kemika, Croatia) solution in a 10:1:1 ratio

and thermostated at 37 °C in a warm bath. After incubation, the absorbance was measured at 593 nm against the sugar analogue. Aqueous standard solutions of $FeSO_4 \cdot 7H_2O$ (Sigma-Aldrich, Germany) (0.01–0.4 mM) were used for the calibration curve and the results were expressed as µM Fe(II) of the 10% honey solution.

Macro and trace element analyses

Honey (0.7 g) was acid-digested in an UltraCLAVE IV microwave digestion system (Milestone, Italy) equipped with Teflon vessels and caps. Analytical grade nitric acid (65%, Merck, Germany) was used in sample digestion after purification by a sub-boiling distillation apparatus (duoPUR, Milestone, Italy). Macro (Ca, K, Mg, Na) and trace elements (barium, Ba; chromium, Cr; Cu; Fe; Mn; molybdenum, Mo; selenium, Se; vanadium, V; zinc, Zn) were then quantified by inductively coupled plasma mass spectrometry (Agilent 7500cx, Agilent Technologies, Tokyo, Japan) according to a previously described method [31]. Ultrapure water obtained with a GenPure system (TKA, Germany) was used for the dilution of standard solutions and samples. Standard reference materials (SRM) 1570a Spinach and 1573a Tomato leaves (National Institute of Standards and Technology, USA) were included in each of the two digestion series to control for the quality of the digestion and measurement as the SRM containing a honey matrix is not available commercially. The obtained and certified values of SRMs expressed on a dry mass basis are summarized in Table S1 of the Supplementary material (ESM1) together with the method detection limits (MDL) for each element. Overall recoveries were from 97 to 103% of the certified values. Levels of Cr, Cu, Fe, Mn and Zn in the majority (92%) of honey samples were published earlier [35] categorized according to production characteristics in the context of an investigation of differences in potentially toxic anthropogenic pollutants between organic and conventional honeys. This study, in addition to nine other metal(loid)s, explored their nutritive aspect as essential trace minerals and their eligibility as authenticity biomarkers. All honey element data were expressed on a wet mass basis.

Dietary exposure assessment

We performed a nutritional assessment for honey consumers regarding the intake of macro (Ca, K, Mg, Na) and trace elements (Cu, Fe, Mn, Mo, Se, Zn). Adequate daily amounts of essential elements taken up by food are defined as Dietary Reference Values (DRV [33, 34]) so we presented our daily intake estimation of 10 elements as a percentage of the DRV defined for adults (> 18 years). For Cr, Ba and V, DRVs were not defined because an essential function in human organism for these elements has not been proven or was inconclusive, as for Cr. DRV is an umbrella term for several reference values (population reference intake (PRI), average requirement (AR), adequate intake (AI)), sometimes defined differently for males and females from the general population. In our calculations, we used the highest value for adults. Calculation was based on the following Eqs. (1) and (2).

$$\%\mathrm{DRV} = \frac{\mathrm{EDI}}{\mathrm{DRV}} \times 100 \tag{1}$$

$$\mathrm{EDI} = \frac{\mathrm{c} \times \mathrm{m}}{1000} \tag{2}$$

where DRV is the dietary reference value of the respective element (Ca, PRI 1000 mg/day; Cu, AI 1.6 mg/day; Fe, PRI 16 mg/day; K, AI 3500 mg/day; Mg, AI 350 mg/day; Mn, AI 3 mg/day; Mo, AI 0.065 mg/day; Na, safe and AI 2 mg/day; Se, AI 0.07 mg/day; Zn, PRI 16.3 mg/day; [33, 34]), EDI is the Estimated Daily Intake, c is the mean concentration of the respective element in the respective honey (mg/kg), and m is the amount of honey eaten daily by Croatian consumer (15.1 g [43]). The resulting range of DRV percentages in seven different honeys was then divided to tertiles with the first tertile representing the first third of the calculated % DRV range (marked with a "+" sign), the second tertile marked with a "++" sign and the third tertile being the last third of the % DRV range ("+++" sign). Thus we presented nutritional assessment results in case of consumption of seven honey types as higher (+++) or lower (++ or +) intake of elements, i.e. the consumer would meet the daily requirement for the respective nutrient more (+++) or less (++ or +) adequately.

Statistical analysis and chemometrics

For each evaluated parameter, the mean, median and range of values were calculated as normality (Shapiro–Wilk´s test) and homogeneity of variance (Levene´s test) was not confirmed. Mann–Whitney *U* test and Kruskal Wallis test were used for testing differences in parameters between different honey groups (according to the botanical origin, location, production year or agricultural practice: organic/conventional). Univariate associations of measured markers were assessed using Spearman's rank correlation analysis (r_S, p). Natural clustering of the data (parameters and honey samples) was explored with a chemometric approach. Principal Component Analysis (PCA) was used after the data matrix was autoscaled. PCA is a method of data reduction and pattern recognition which places input data into a reference system characterized by new variables—principal components (PCs). TIBCO Statistica® software, version 13.5.0.17 (TIBCO Software Inc., USA) was used in all statistical analyses.

Results and Discussion

Melissopalynological (pollen spectrum is presented per each honey type in Fig. S1a-g of the Supplementary material), physicochemical (data presented in Table S2 of the Supplementary material) and sensory analyses classified 62 samples according to botanical origin in seven honey types: chestnut (C), black locust (BL), deciduous honeydew honey (H), sage (SG), buckthorn (BU), savory (SV) and multifloral (MF). For 16/62 samples, botanical origin confirmed by melissopalynological, sensory and physicochemical analyses differed from the one proposed by beekeepers at the moment of honey harvest. Alongside revealing the botanical origin, the characteristic pollen spectrum in honeys from a specific location, considering the occurrence frequency of some plant species and their combination, was also proven as a valuable indicator of geographical origin [32]. Five samples were not compliant with the international honey standards defined by Council Directive 2001/110/EC [4] and Directive 2014/63/EU [12] because of excessive sucrose content (sample C22; > 5 g/100 g), moisture (C5 and C11; > 20% H_2O) and hydroxymethylfurfural content (HMF; MF1 and SV2; > 40 mg/kg) (Table S2).

Thirteen macro and trace elements were quantified in all of the 62 honeys (except Cr in four samples). Descriptive statistics and differences between honey types in TP content, parameters of antioxidant capacity (FRAP and DPPH) and element content are presented in Table 1. Potassium was the most abundant in contrast to Se and V as the least abundant elements in honeys, which is in line with previous reports [2, 15, 44]. Taking into account the small number of samples per some honey types, in general, chestnut, deciduous honeydew honey and savory honey had a higher level of the measured parameters compared to the other honey types, while Cr, Fe, Mo and V were similar in all honey types. The origin of the latter metals could be due to wearing stainless steel equipment because of the acidic honey environment [3, 45]. The supremacy of dark honey types in the content of elements, TP content and antioxidant capacity was demonstrated earlier [3, 15, 19, 30, 31, 46, 47] and confirmed here for chestnut, savory and honeydew honey. In addition, previous studies found a significant correlation between element content, TP and antioxidant capacity [19, 30, 31, 48] due to respective antioxidant activity of some elements and phenolic compounds, but also due to their chemical interactions resulting in synergistic antioxidant action [48]. Spearman's correlation analysis in all Croatian honeys ($r_S = 0.67–0.96$, $p < 0.001$; Table S3) corroborated before mentioned associations. Interestingly, total element content, TP, DPPH and FRAP markers were strongly related in multifloral (MF) compared to chestnut (C) honey samples (Table S3) pointing at the importance of nectar and/or pollen species in honey as a main source of elements and polyphenolic compounds [32]. In chestnut samples, Ba, Ca, K, Mg and Mn stood out as main drivers of elemental associations with TP, DPPH and FRAP, while in multifloral honeys those were Ca, Cu, K and Se (Table S4). Chestnut honey stood out with eight times higher Ba and Mn levels compared to the next highest honey type (multifloral). The reason for such a pronounced difference is not apparent but might originate from chestnut plant specificity in accumulating soil Ba and Mn (naturally co-occurring in Ba-Mn oxides) and efficient transport to pollen grains. There are no literature data for *C. sativa*, but Li et al. [49] confirmed high Mn accumulation and transfer rate for *Castanea henryi* (Chinese chestnut) plant. Soil of Banovina region, where the majority of chestnut honeys were harvested, is high in Ba (above Croatian and European median; [50]) because of the natural barite occurrence, so the mechanism of high Ba root absorption and translocation to fruit seen for other nut trees (e.g., black walnut, Brazil nut [51, 52]), might also be present in chestnut. Chestnut honey contains the highest share of pollen grains of all investigated honey types and the grain type within is fairly uniform (> 85% of chestnut pollen, Fig. S1a). Higher Mn in chestnut *vs.* other unifloral honey types was previously reported for Croatian and Italian honeys [31, 53–55], while Ba is an element measured rarely.

Spatial differences in parameters of the respective four honey types are presented in Fig. 2. Differences between the two locations were statistically confirmed for Mg, Mn (higher in Ozalj), Se and V (higher in Banovina) in chestnut (Fig. 2a) and among four locations for all parameters, except Zn and Se, in multifloral honey (Fig. 2b). Higher soil levels of Mg and Mn in the region of Ozalj than in Banovina could explain found differences in honey, while for V soil is less likely to be the main source of honey V because the relation of soil levels in the two regions was opposite to that in honey [50]. The natural release of V to soil and water greatly surpasses the anthropogenic release [56], but in this case, the oil refinery in Sisak (northern edge of Banovina), combustion of fossil fuels, fertilizers or atmospheric deposition might be the cause of the higher V (and Se) in Banovina than Ozalj region. Spatial differences in multifloral honey (Fig. 2b) are somewhat difficult to interpret due to pollen diversity in honeys from different locations: in Banovina and Ozalj samples, chestnut pollen is dominant (52–69% *C. sativa*), in Baranja honey it is rapeseed pollen (56–58% *Brassica napus*) and in Istrian samples a mix of rockrose, ash, wild fruits, Christ's thorn, mustard family and buckthorn pollen prevailed (10–29% *Cistus* spp., *Fraxinus* spp., *Prunus* spp., *Paliurus spina christi*, Brassicaceae, *Rhamnus* spp.; Fig. S1). Thus, a variation in botanical origin could mask possible spatial

Table 1 Differences in parameter levels between honeys of different botanical origin from Croatia (mean ± standard error, median, range in parenthesis)

Parameter	Chestnut (C) N = 38	Black locust (BL) N = 3	Honeydew (H) N = 1	Sage (SG) N = 3	Buckthorn (BU) N = 2	Savory (SV) N = 2	Multifloral (MF) N = 13	All N = 62
TP (mg GA/kg)	203 ± 6[b] 197 (153–287)	65 ± 12.9[ab] 52.6 (52.1–91.1)	234	155 ± 4[a] 153 (149–162)	218 ± 15 (202–233)	193 ± 18 (175–210)	148 ± 18[a] 124 (70.9–299)	183 ± 7 185 (52.1–299)
DPPH (mmol TE/kg)	0.623 ± 0.025[b] 0.619 (0.389–0.970)	0.096 ± 0.009[ab] 0.101 (0.079–0.107)	0.686	0.367 ± 0.046[a] 0.407 (0.275–0.418)	0.602 ± 0.062 (0.541–0.664)	0.664 ± 0.138 (0.526–0.803)	0.318 ± 0.051[a] 0.247 (0.140–0.702)	0.523 ± 0.028 0.544 (0.079–0.970)
FRAP (μmol Fe^{2+}/kg)	367 ± 11[b] 360 (272–510)	221 ± 141 88.6 (72.2–504)	363	200 ± 38[a] 233 (124–242)	302 ± 3 (299–305)	381 ± 6[b] (375–387)	202 ± 25[a] 164 (89.5–363)	316 ± 14 330 (72.2–510)
Elements								
Ba (μg/kg)	702 ± 41[b] 671 (356–1404)	14.4 ± 2.45[ab] 16.4 (9.53–17.3)	63.4	32.7 ± 13.9[a] 19.6 (18.0–60.6)	30.5 ± 4.3[a] (26.3–34.8)	35.6 ± 4.1[a] (31.6–39.7)	90.8 ± 39.8[a] 35.7 (23.1–536)	455 ± 48 483 (9.53–1404)
Ca (mg/kg)	150 ± 6[b] 150 (78.6–213)	12.5 ± 0.6[ab] 12.0 (11.8–13.7)	64.0	33.7 ± 8.4[a] 38.6 (17.3–45.3)	51.3 ± 6.1[a] (45.3–57.4)	45.8 ± 7.0[a] (38.8–52.8)	43.6 ± 6.0[a] 35.5 (21.9–103)	107 ± 8 114 (11.8–213)
Cr (μg/kg)	3.55 ± 0.47 2.41 (<LOD-10.2)	3.15 ± 1.38 2.33 (1.29–5.83)	6.09	6.34 ± 1.71 7.79 (2.92–8.30)	3.52 ± 0.52 (3.00–4.04)	6.50 ± 0.35 (6.15–6.85)	3.30 ± 0.63 2.85 (<LOD-7.29)	3.75 ± 0.35 2.92 (<LOD-10.2)
Cu (μg/kg)	226 ± 6[b] 222 (166–340)	58.0 ± 7.8[ab] 65.7 (42.5–65.8)	661	266 ± 46 284 (179–335)	176 ± 14 (161–190)	557 ± 309 (248–866)	155 ± 20[a] 138 (83.0–308)	221 ± 15 214 (42.5–866)
Fe (mg/kg)	0.663 ± 0.061 0.574 (0.295–2.28)	0.391 ± 0.136 0.331 (0.192–0.651)	1.98	0.267 ± 0.062[a] 0.320 (0.143–0.338)	0.442 ± 0.051 (0.391–0.493)	1.08 ± 0.14 (0.947–1.22)	0.539 ± 0.083 0.450 (0.163–1.148)	0.633 ± 0.050 0.550 (0.143–2.28)
K (mg/kg)	2716 ± 111[b] 2639 (1650–4100)	218 ± 3[ab] 188 (186–281)	2023	600 ± 36[a] 573 (555–672)	1409 ± 155[a] (1254–1564)	958 ± 349[a] (609–1306)	710 ± 101[a] 643 (266–1329)	1962 ± 144 2022 (186–4100)
M (mg/kg)	37.5 ± 1.4[b] 38.1 (23.2–64.6)	10.1 ± 0.4[a] 9.87 (9.60–10.8)	37.9	9.75 ± 2.24[a] 10.0 (5.73–13.5)	9.31 ± 1.10[a] (8.21–10.4)	17.3 ± 3.9[a] (13.4–21.1)	13.5 ± 1.2[a] 11.9 (8.85–22.3)	28.2 ± 1.8 29.7 (5.73–64.6)
Mn (mg/kg)	13.6 ± 1.2[b] 11.9 (4.75–35.1)	0.118 ± 0.030[a] 0.108 (0.071–0.175)	0.878	0.084 ± 0.018[ab] 0.075 (0.058–0.119)	0.276 ± 0.029[a] (0.247–0.304)	0.642 ± 0.186[a] (0.456–0.828)	1.60 ± 0.78[a] 0.609 (0.069–10.4)	8.75 ± 1.08 7.88 (0.058–35.1)
Mo (μg/kg)	2.65 ± 0.30 2.14 (0.739–10.2)	1.12 ± 0.10[a] 1.18 (0.936–1.25)	5.54	1.41 ± 0.05 1.44 (1.32–1.49)	1.38 ± 0.15 (1.23–1.53)	7.57 ± 4.40 (3.17–12.0)	1.78 ± 0.25 1.44 (0.746–3.29)	2.50 ± 0.26 1.97 (0.739–12.0)
Na (mg/kg)	6.81 ± 0.22 6.48 (4.80–10.5)	2.81 ± 1.28[a] 1.88 (1.20–5.35)	15.0	9.50 ± 0.73[a] 9.82 (8.11–10.6)	24.8 ± 14.2[a] (10.6–39.0)	6.86 ± 0.75 (6.11–7.61)	6.31 ± 1.13 5.72 (1.96–14.6)	7.36 ± 0.62 6.45 (1.20–39.0)
Se (μg/kg)	0.959 ± 0.063 0.944 (0.350–1.83)	0.491 ± 0.074[a] 0.557 (0.343–0.574)	1.34	0.370 ± 0.152[a] 0.388 (0.097–0.623)	0.548 ± 0.024 (0.524–0.572)	1.73 ± 0.40 (1.33–2.13)	0.830 ± 0.130 0.894 (0.138–1.73)	0.898 ± 0.056 0.873 (0.097–2.13)
V (μg/kg)	0.803 ± 0.051 0.704 (0.362–1.69)	0.440 ± 0.131 0.322 (0.296–0.702)	4.11	0.448 ± 0.155 0.358 (0.235–0.750)	1.15 ± 0.04 (1.11–1.20)	1.33 ± 0.44 (0.889–1.77)	0.863 ± 0.186 0.598 (0.244–2.25)	0.862 ± 0.076 0.687 (0.235–4.11)
Zn (mg/kg)	0.907 ± 0.079 0.764 (0.492–2.66)	0.293 ± 0.069[a] 0.311 (0.166–0.403)	0.854	0.377 ± 0.057[a] 0.395 (0.270–0.465)	1.19 ± 0.32(0.872–1.51)	0.556 ± 0.065 (0.491–0.621)	0.750 ± 0.125 0.627 (0.273–1.61)	0.815 ± 0.060 0.736 (0.166–2.66)
Total element content (%)	0.293 ± 0.012[b] 0.288 (0.177–0.438)	0.024 ± 0.003[ab] 0.021 (0.021–0.031)	0.214	0.065 ± 0.004[a] 0.062 (0.061–0.074)	0.150 ± 0.016[a] (0.133–0.166)	0.103 ± 0.034[a] (0.068–0.138)	0.078 ± 0.011[a] 0.069 (0.033–0.146)	0.212 ± 0.015 0.218 (0.021–0.438)

Part of data was published in Lazarus et al. (2021); TP—Total phenols; DPPH—1,1-diphenyl-2-picrylhydrazyl radical scavenging activity; FRAP—Ferric reducing antioxidant power

[a]Significantly different from chestnut honey (Mann–Whitney *U* test, $p < 0.05$)

[b]Significantly different from multifloral honey (Mann–Whitney U test, $p < 0.05$)

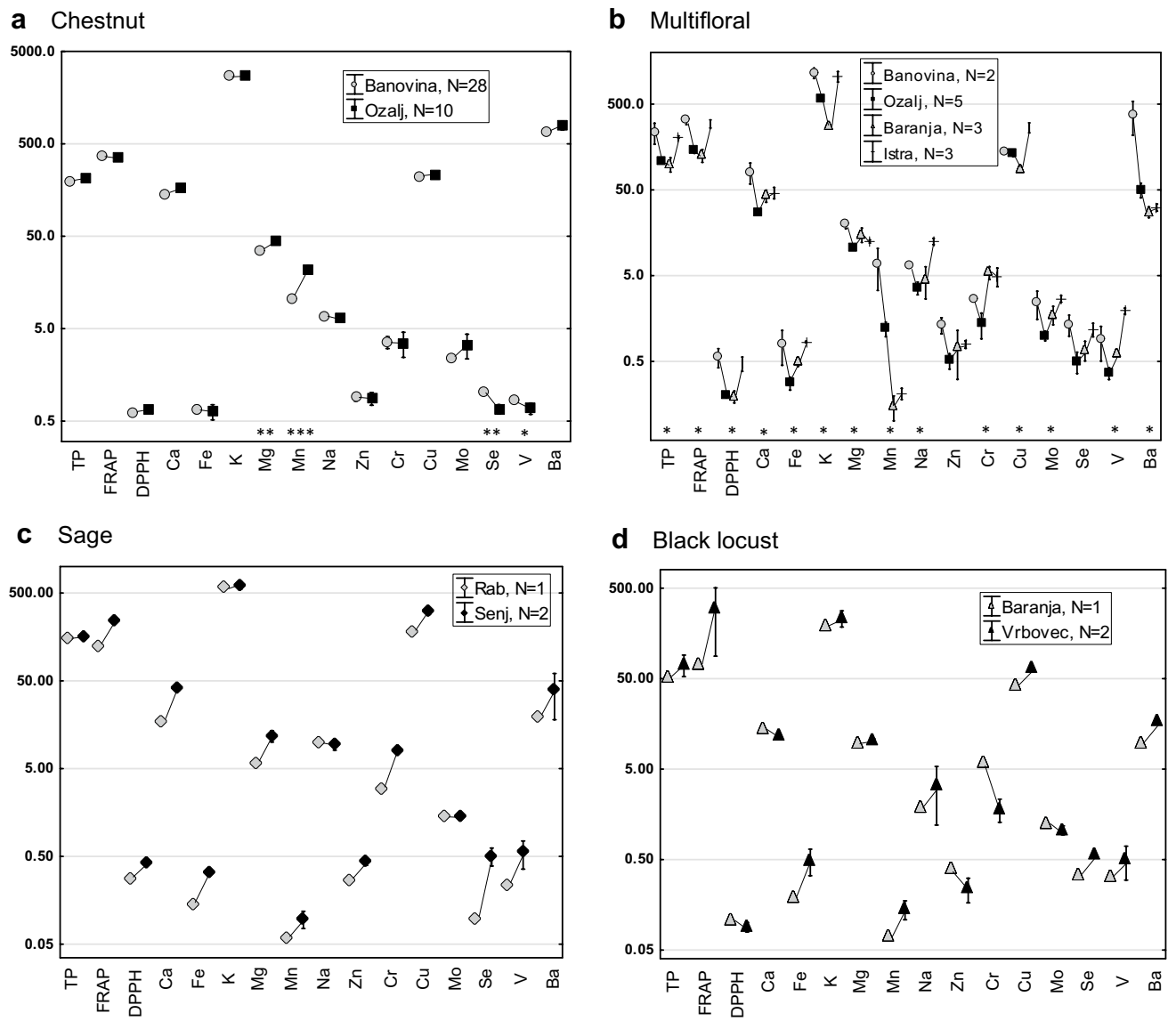

Fig. 2 Spatial categorization of mean (±standard error of mean; whiskers) parameter levels in **a** chestnut, **b** multifloral, **c** sage and **d** black locust honey on a logarithmic scale. Asterixes denote significant differences between locations: $*p<0.05$, $**p<0.01$, $***p<0.001$ (Mann–Whitney *U* test for chestnut, Kruskal–Wallis test for multifloral honey). Units are the same as displayed in Table 1. TP—total phenols; DPPH—1,1-diphenyl-2-picrylhydrazyl radical scavenging activity; FRAP—ferric reducing antioxidant power

differences. Variations between the two locations were also obvious in sage (generally higher in Senj, Fig. 2c) and black locust honeys (Fig. 2d), but the inadequate sample number prevented us from testing the differences statistically. Both in sage and black locust honeys, the dominant pollen was not always from *S. officinalis* (10–26%; natural hypopollenic feature of sage) and *R. pseudoacacia* (28–49%; Fig. S1b and d), respectively, but the samples had a varying content of multiple pollen species which impacted the level of the measured parameters. In conclusion, chestnut honey due to the highest pollen uniformity among honey types would be the best option for studying regional differences in the element and TP content, and the antioxidant capacity of honeys. Other authors explored regional variations of elements in honey samples but failed to discuss the diversity of the pollen spectrum [18, 20, 25, 28, 57]. Previously, the botanical origin was emphasized to influence the element content in honey over other causes of variation (e.g., geographical origin) [26, 58]. However, opposite conclusions with geographical variation completely suppressing the botanical origin as a source of element variation were also reported [57].

A clear temporal trend for respective parameters presented in Fig. 3a-d was not observed. As honey was harvested from the same apiaries in consecutive years, 2018 and

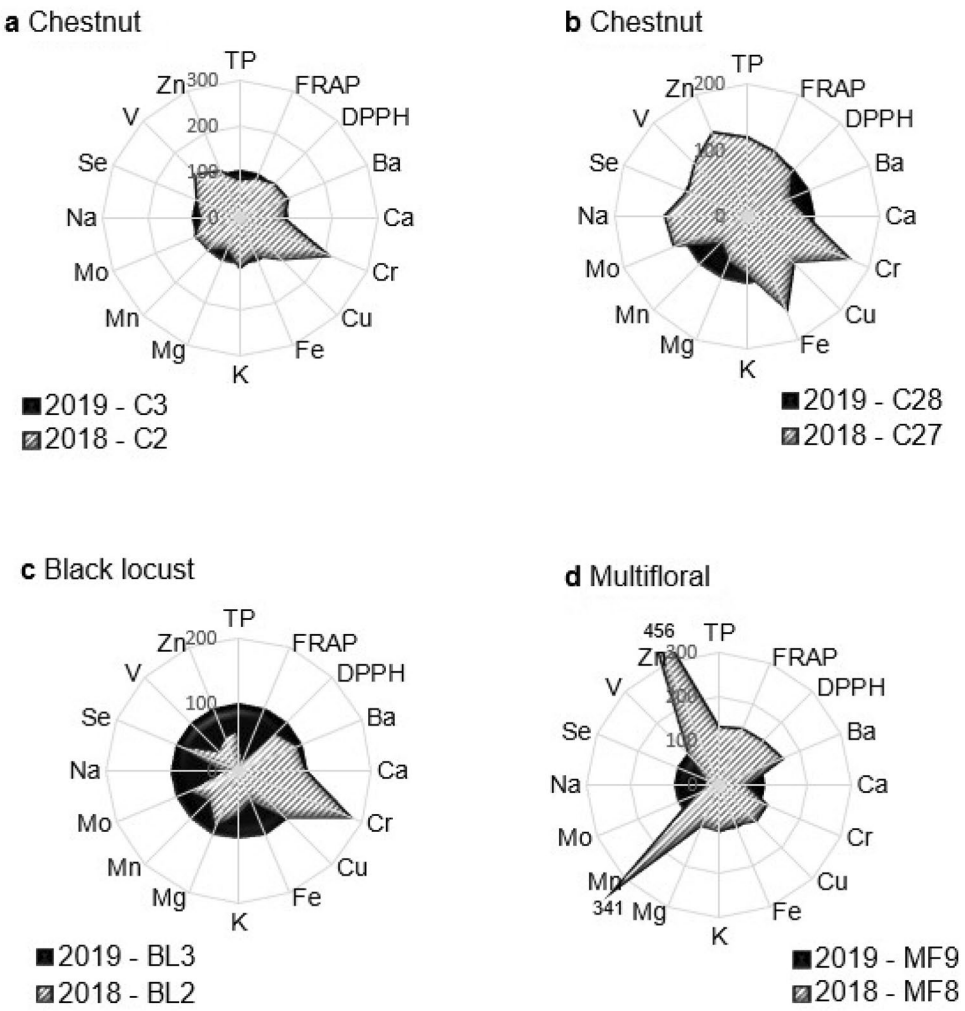

Fig. 3 Relative ratio of parameters in honey produced at the same apiary in 2019 (presented as 100%, black area) and in 2018 (grey area): **a** chestnut samples C2 and C3 from Banovina, **b** chestnut samples C27 and C28 from Banovina, **c** black locust samples BL2 and BL3 from Vrbovec, and **d** multifloral samples MF8 and MF9 from Baranja

2019, and pollen species and ratios were similar between the two samples of the respective honey types, the determined differences can probably be attributed to the microclimate conditions in the two years. The influence of different seasons on pollen composition and nectar production resulting in element variation was reported before [25]. Temporal variation even within the same region (Banovina; samples C2/C3 and C27/C28) showed the opposite direction for e.g., total phenols, Fe and Na when two pairs of samples were compared (Fig. 3a, b). Other reasons, like the age of the honeycomb, previously reported to increase levels of Fe and Na, among other elements [59] could add to the noted variation.

Production conditions (organic *vs*. conventional beekeeping) caused differences only in Cr content ($U = 154$, $p = 0.0014$) when honey types were investigated altogether (Table S5). In our previous report of potentially toxic metal(loid)s, only Cr was higher in organic production conditions in chestnut honey compared to conventional honeys [35]. Chromium in honey could originate from industrial and agricultural activities, but also from wearing honey harvest/production/storage equipment made of metal [3, 60].

In an attempt to categorize our samples based on the measured parameters/markers, we performed a PCA. PCA produced four important principal components (PC) whose eigenvalues were higher than the one covering 77% of the total data variance (Table 2) so we considered this model reliable [17]. In a decreasing order of importance, K, DPPH, Ca, Mg, Ba, FRAP, TP and Mn were the main variables in the most important, PC1, while V and Fe stressed the PC2, Na the PC3, and Zn the PC4 (Table 2). The most important correlations (higher than 0.7) of PCs with parameters are given in bold in Table 2. Figure 4 shows variable/loadings plot (Fig. 4a) and score/sample plot (Fig. 4b) constructed using the first two PCs (62% of total variance) allowing for a visualization of the grouping of parameters (elements, TP content, antioxidant capacity; Fig. 4a) and honey samples (Fig. 4b). Potassium, DPPH, Ca, Mg, Ba, FRAP, TP and Mn being highly correlated with PC1, mainly contribute to the separation of chestnut honey samples from the other

Table 2 Eigenvalues and the proportion of variation explained by the first four principal components (PC) with association coefficients (significant were bolded) obtained by factor analysis[a]

	PC1	PC2	PC3	PC4
Eigenvalue	6.72	3.18	1.28	1.22
Variance (%)	42.02	19.87	7.99	7.61
Variance cumulative (%)	42.02	61.89	69.88	77.49
Association coefficients				
TP	**0.84**	−0.16	0.38	−0.06
DPPH	**0.91**	−0.05	0.24	−0.16
FRAP	**0.86**	−0.08	0.14	−0.08
Ba	**0.88**	0.41	−0.10	−0.02
Ca	**0.90**	0.28	−0.11	0.07
Cr	− 0.12	−0.32	−0.20	−0.34
Cu	0.37	−0.68	−0.09	−0.32
Fe	0.27	**−0.73**	−0.24	0.42
K	**0.93**	0.18	0.00	−0.03
Mg	**0.88**	0.17	−0.23	0.03
Mn	**0.81**	0.43	−0.13	−0.02
Mo	0.22	−0.62	−0.46	−0.18
Na	0.11	−0.43	**0.76**	0.11
Se	0.40	−0.51	−0.22	0.04
V	0.21	**−0.82**	0.16	−0.13
Zn	0.29	−0.23	−0.05	**0.85**

[a]TP—Total phenols; DPPH—1,1–diphenyl–2–picrylhydrazyl radical scavenging activity; FRAP—Ferric reducing antioxidant power; the correlations of PCs with parameters higher than 0.7 are given in bold

honey types along the x-axis, with the samples positioned in the upper right quadrant having the higher content of listed parameters as far as they are from the axis origin (zero), in contrast to samples positioned in the two left quadrants (Fig. 4b). Essential macro and trace elements (K, Ca, Mg and Mn) in honey were proposed before for discrimination of botanical origin [16, 17, 19, 21, 23, 27, 61], but also as indicators of regional differences [18, 20, 22, 25]. These elements originating from geochemical and soil composition characteristics are taken up by plants and carried to honey via nectar [3]. As seen from Fig. 4b, PCA separated some honey types, like black locust or sage, from e.g., savory and honeydew honey, but failed to clearly separate multifloral honeys from other honey types, excluding chestnut. Within the chestnut samples, we were unable to discriminate the Ozalj (C29-38) from the Banovina samples (C1-28). However, we noticed a clustering of different honey types (sage, buckthorn, multifloral, savory and honeydew honey) from the Adriatic coast area in the negative part of PC2 plane. The reason could be in the soil and air element composition characteristic of marine environment, but also in coastal plant species having higher TP content and antioxidant properties as coping mechanisms against climate and environmental challenges, which are then transferred to nectar and honey [32, 62]. The high correlation of Fe and V with the PC2 contributed to the separation of samples along y-axis with the only honeydew sample (H1) extracted in the lower right quadrant due to the highest V and Fe levels. Those two metals are common followers in soil composition [50] so their levels in honey are probably of geogenic origin, although

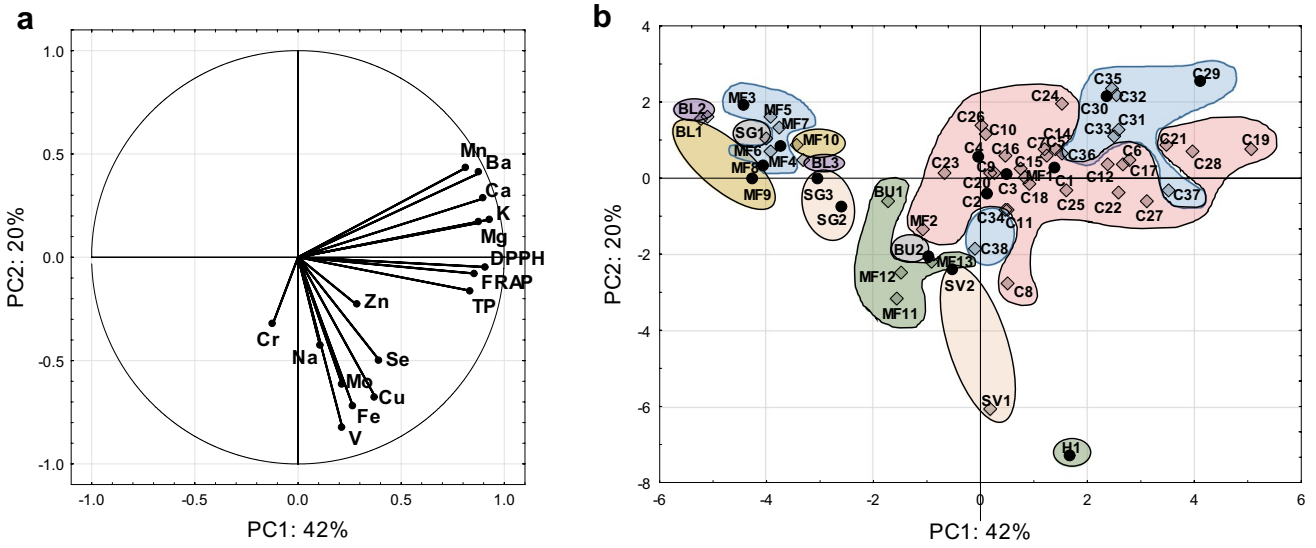

Fig. 4 **a** Loadings/variables plot and **b** score/sample plot for first two principal components. Different letters denote honey type (C-chestnut, BL-black locust, H- deciduous honeydew honey, SG-sage, BU-buckthorn, SV-savory, MF-multifloral) and number identifies sample within the respective honey type. Black circles refer to organic and grey rhombuses to conventional honeys. Colored shapes denote geographic region: violet-Vrbovec, yellow-Baranja, blue-Ozalj, grey-Island Rab, orange-Senj, green-Istra, red-Banovina

they might also originate from industrial activities by the Rijeka oil refinery situated 20 km northeast from the Istra sampling location. While investigating the temporal differences between honey samples, we observed that two chestnut honeys from 2018 (C2 and C27) were located in the negative part of the PC2 plane compared to their 2019 counterparts (C3 and C28) located in the positive part. Visible separation also occurred for black locust samples (BL2/BL3), but was absent for multifloral MF8/ MF9 pair, although both pairs were placed in the positive part of PC2. Finally, there was no clear separation of organic and conventional honeys in the first two PC planes.

Our nutritional assessment of essential element intake based on DRVs showed great variation among the seven different honey types (Table 3). In general, the contribution of honey consumption to an adequate daily intake of essential elements was low (0.01–7% of DRV). Sodium intake can be considered important while ranging 2–26% of the DRV for the seven investigated honey types, and was followed by Mn intake from chestnut honey (7% of DRV). Our estimation also showed that consumers of honeydew honey would benefit the most regarding daily requirements for most essential elements. After honeydew honey, the nutritional value of honeys regarding essential elements and contribution to DRV could be ranked in a descending order: savory, chestnut, sage and buckthorn, multifloral and lastly, black locust honey (Table 3). Bogdanov et al. [2] estimated the intake of vitamins and minerals from honey as marginal but highlighted Cr, Mn and Se as important for children as consumers of honey. In this exposure assessment, we focused on adults as data on daily consumption of honey from a national food consumption study covered only adults. Children will be included in future assessments upon the completion of an ongoing national infant/children study.

Conclusions

This study investigated macro and trace elements, total phenolic content and antioxidant capacity in 62 unifloral and multifloral honeys as markers for classification of honey types taking into account spatial, temporal and production practice trends across markers. The measured markers differed significantly among honey types separating chestnut honey from others regarding especially Ba and Mn levels. PCA results were compliant with basic statistical testing results (Mann–Whitney *U* test) and pointed also to TP, DPPH, FRAP, Ca, K and Mg as useful markers for discriminating chestnut honey from other unifloral and multifloral honeys. In addition, the first principal component discerned the honeydew honey sample fairly from nectar honey samples. Although some elements showed regional, seasonal and production practice differences, PCA was not able to

Table 3 Comparison of essential element contribution to dietary reference values (DRVs) due to daily consumption of seven different honey types from Croatia[a]

	% DRV								All honey types (range of %DRV) 1st tertile 2nd tertile 3rd tertile		
	Chestnut (C)	Black locust (BL)	Honeydew (H)	Sage (SG)	Buckthorn (BU)	Savory (SV)	Multifloral (MF)		+	+ +	+ + +
Ca	+ + +	+	+ +	+	+	+	+		0.019–0.087	0.088–0.16	0.17–0.23
Cu	+	+	+ + +	+ +	+	+ + +	+		0.055–0.24	0.25–0.44	0.45–0.63
Fe	+	+	+ + +	+	+	+ +	+		0.025–0.078	0.079–0.13	0.14–0.19
K	+ + +	+	+ + +	+	+ +	+	+		0.095–0.44	0.45–0.81	0.82–1.17
Mg	+ + +	+	+ + +	+	+	+	+		0.040–0.080	0.081–0.12	0.13–0.16
Mn	+ + +	+	+	+	+	+	+		0.042–2.31	2.32–4.60	4.61–6.89
Mo	+	+	+ + +	+	+	+ + +	+		0.026–0.075	0.076–0.13	0.14–0.18
Na	+	+	+ +	+	+ + +	+	+		2.13–10.0	10.1–18.0	18.1–26.0
Se	+ +	+	+ + +	+	+	+ + +	+ +		0.0080–0.017	0.018–0.028	0.029–0.037
Zn	+ +	+	+ +	+	+ + +	+ + +	+ +		0.027–0.055	0.056–0.083	0.084–0.110

[a]Calculations based on: Ca, PRI 1000 mg/day; Cu, AI 1.6 mg/day; Fe, PRI 16 mg/day; K, AI 3500 mg/day; Mg, AI 350 mg/day; Mn, AI 3 mg/day; Mo, AI 0.065 mg/day; Na, safe and AI 2 mg/day; Se, AI 0.07 mg/day; Zn, PRI 16.3 mg/day (population reference intake (PRI), adequate intake (AI); EFSA 2017; EFSA NDA Panel et al. 2019)

discriminate groups clearly. To sum up, elements, TP and antioxidant capacity markers could be used for authenticity confirmation in chestnut and honeydew honey samples, but for other honey type categorization, help of traditional tools for authentication of the botanical origin (melissopalynological, sensory and physicochemical analyses) is suggested. A consumer-relevant health beneficial ranking of honeys regarding daily intake of essential elements (calculated as %DRV, descending order) would be as follows: honeydew honey, savory, chestnut, sage and buckthorn, multifloral, black locust.

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Authors' contributions ML: Conceptualization, Formal analysis, Investigation, Resources, Writing—Original Draft, Writing—Review & Editing, Visualization, Supervision; BTL: Resources, Validation, Formal analysis, Writing—Review & Editing; AS: Methodology, Validation, Formal analysis; TO: Methodology, Validation, Formal analysis; AJ: Validation, Formal analysis, Investigation; SP: Methodology, Formal analysis, Visualization; MDL: Methodology, Validation, Formal analysis; DB: Methodology, Formal analysis, Writing—Review & Editing.

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Availability of data and materials The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Code availability Not applicable.

Declarations

Conflict of interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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