https://doi.org/10.2478/acph-2024-0031

Original research paper

Phenolic content and antioxidant activity of Croatian and German honey

VALERIJA VUJČIĆ BOK[1,3] https://orcid.org/0000-0003-4507-8082

IVANA ŠOLA[1,*] https://orcid.org/0000-0003-4668-6426

GORDANA RUSAK[1,*] https://orcid.org/0000-0002-8842-7871

ALAN BUDISAVLJEVIĆ[1] https://orcid.org/0000-0002-4709-9760

ROSA NGUYEN[2]

JUTTA LUDWIG-MÜLLER[2] https://orcid.org/0000-0002-9403-5153

ŽELJAN MALEŠ[3] https://orcid.org/0000-0003-1034-2525

[1] *University of Zagreb, Faculty of Science Department of Biology (Botany), 10000 Zagreb, Croatia*

[2] *Technische Universität Dresden, Faculty of Biology, 01069 Dresden, Germany*

[3] *University of Zagreb Faculty of Pharmacy and Biochemistry, Department of Pharmaceutical Botany, 10000 Zagreb, Croatia*

* Correspondence; e-mail: ivana.sola@biol.pmf.hr; gordana.rusak@biol.pmf.hr

ABSTRACT

Since honey has a therapeutic role in the treatment of many diseases, we investigated the content of phenolic compounds and the antioxidant activity in acacia (*Robinia pseudoacacia* L.), chestnut (*Castanea sativa* Mill.) and lime-tree (*Tilia* spp.) honey originated from Croatia and Germany. In total phenols, flavonols and flavonols was observed higher values for Croatian *Castanea* honey compared to German *Castanea* honey. Significant higher values of total flavanols and hydroxycinnamic acids was measured in Croatian *Tilia* honey compared to German *Tilia* honey. For *Robinia* honey, significant higher values of total phenols and flavonols were observed in almost all Croatian honey samples compared to German honey. Croatian honey samples had higher antioxidant activity compared to German honey samples with most tested methods. The highest total phenols, total flavanols, ABTS, DPPH and FRAP values were measured in *Castanea* honey, then in *Robinia* honey and the lowest values in *Tilia* honey samples. With new developed HPLC method, pinobanksin, pinocembrin and chrysin were identified in the most honey samples. Our results imply that both botanical and geographical origin influence the final quality of phenolic compounds and antioxidant activity in honey. High positive correlation between the results of antioxidant activity and polyphenols were detected.

Keywords: antioxidant activity, flavonoids, RP-HPLC, *Castanea sativa* honey, *Robinia pseudoacacia* honey, *Tilia* spp. honey

Accepted July 19, 2024
Published online August 14, 2024

INTRODUCTION

Honey is a natural, sweet and viscous mixture of substances created by honeybees, processing nectar or honeydew (breaking down complex carbohydrates into simpler ones) in their glands with saliva hydrolytic enzymes (1). Main components of honey are water, fructose and glucose and then other sugars (maltose, sucrose and higher sugars), amino acids, proteins, minerals, vitamins, organic acids and polyphenols (2). Flavonoids and phenolic acids are the most common compounds from the polyphenol group and they are the most responsible for honeys natural antioxidant activity and its protective effects on human health (3). In addition to antioxidant properties, honey ingredients have anti-inflammatory, antimicrobial, antimutagenic, antiparasitic and antitumor properties, and an increasing number of studies indicate the importance of the therapeutic role of honey in the treatment of many diseases (4).

Nectar honey can be divided into unifloral or polyfloral honey, depending on whether the grazing of bees is directed mostly towards one or more plant species. More than 100 different types of unifloral honey are known in Europe (5). Persano Oddo *et al*. (5) analyzing the data from the International Honey Commission, reported 10 types of unifloral honey most commonly present in production and commercial availability in the European market.

The composition, quality and biological effects of honey depend on many production parameters. Some of them are plant origin of honey, geographical origin, species of bees that produce honey, climatic conditions as well as the technical process of honey processing, time age of the product and exposure to high temperatures. More precise control of these parameters affects the generation of high prices for honey production, which in turn leads to the problem of increased market share of counterfeits (6).

In order to eliminate counterfeits and ensure standards for honey products, important properties of honey, the method of technological processing and methods for determining the validity of the declaration and product quality are legally defined by Croatian Regulations (7) and in the European Union by Harmonised methods of the European Commission (8, 9). Melisopalinological analysis determines the composition of pollen in honey, and various chemical analyzes define the composition of sugars, water, free acids, water-insoluble substances, hydroxymethylfurfural content and electrical conductivity of honey and enzymatic activity of diastase. In these analyzes, the analysis of the composition of phenolic compounds, which are among the main carriers of antioxidant properties of honey, is not legally required for testing the quality of honey. In the last ten years, there has been an increase in the number of papers examining the composition of non-volatile components, such as polyphenols, which significantly contribute to antioxidant activity. Some of these papers (1, 10–20) have researched Croatian and other honeys from Europe investigating both total and individual polyphenol content as well as antioxidant activity. According to Brščić *et al.* (21), consumers in Croatia prefer unifloral acacia honey the most (56 %), followed by multifloral floral honey (44 %) and meadow honey (35 %). They also prefer unifloral sage (25 %), chestnut (21 %) and linden (16 %) honey. A mild flavor (52 %) and brighter color (44 %) of honey are also preferred by Croatians consumers. Based on these preferences, we decided to use two unifloral honeys in our work: acacia and linden honey, which have mild flavors and brighter colors, and chestnut honey, which has a stronger aroma and darker color. Additionally, these three types of honey were available in both Croatia and Germany.

The aim of our study is to a) determine the polyphenol content and composition by HPLC analysis and by spectrophotometric determination of total soluble phenols (TP) by Foline-Ciocalteau reagent, total flavonoids (TF) by $AlCl_3$ method, total hydroxycinnamic acids (THA) and total flavonols (TFL) by HCl method and the total flavanol (TFLA) content by *p*-

dimethylaminocinnamaldehyde (DMACA) method and antioxidant activity (ABTS: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid), DPPH: 2,2-diphenyl-1-picrylhydrazyl and FRAP: ferric ion reducing antioxidant power) in acacia (*Robinia pseudoacacia* L.) honey, chestnut (*Castanea sativa* Mill.) honey and lime-tree (*Tilia* spp.) honey originated from Croatia and Germany, b) compare the obtained results on the basis of plant botanical and geographical origin. To determine the polyphenol content and composition, we have developed a new HPLC method for detecting flavonoids in honey samples. The novelty of the research lies in the fact that for the first time the same honey type produced in different geographical and climatic regions was compared from Croatia and Germany. So far, the honey samples were compared from the point of botanical origin (11, 18, 19, 22–24), or from the point of production seasons (10). In our study, we took into account both, geographical and botanical origin.

EXPERIMENTAL

Honey samples

Seven samples of honey from the area of Central Europe were collected, four from Croatia and three from Germany. All samples were purchased from beekeepers, only certain samples were available as a commercial product in stores (Table I). All samples have the same method of technological processing, which was checked when buying honey and is legally defined in Europe with the Codex Alimentarius and the European Honey Directive. The origin of each honey is shown on a geographical map created with the help of the program QGIS 2.18 (Fig. 1). Samples were stored two weeks at room temperature in dark place to purification.

Tabale I. Botanical and geographical origin of Croatian and German honey samples and their commercial availability

Botanical origin	Geographical origin	Sample name	Commercial availability
Castanea sativa Mill.	Topusko, Croatia	CsTC	–
Robinia pseudoacacia L.	Topusko, Croatia	RpTC	–
Robinia pseudoacacia L.	Konjščina, Croatia	RpZC	+
Tilia spp.	Bilogora, Croatia	TsBC	–
Castanea sativa Mill.	Brandenburg, Germany	CsBG	+
Robinia pseudoacacia L.	Saska, Germany	RpSG	+
Tilia spp.	Saska, Germany	TsSG	+

119

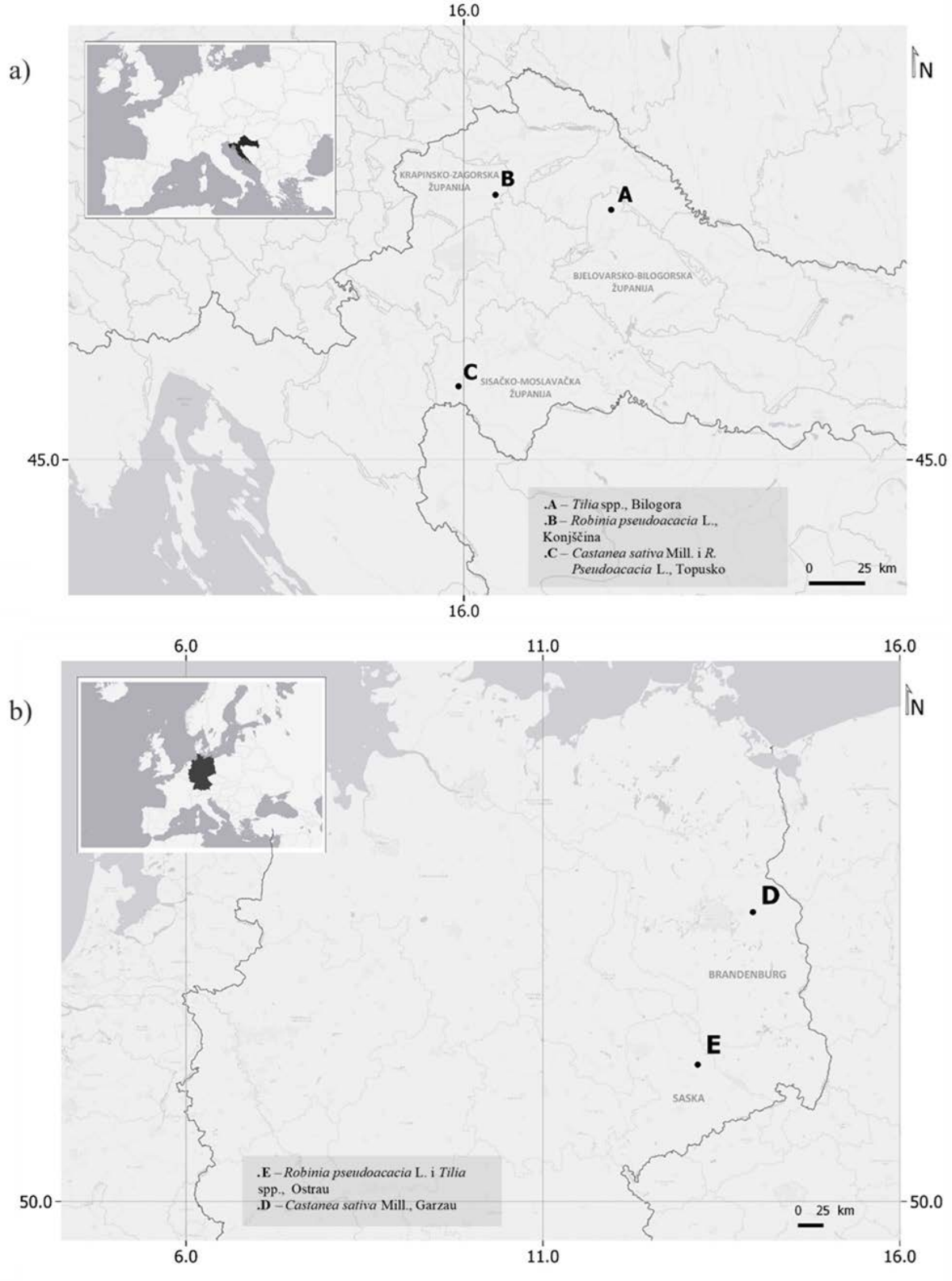


Fig. 1. Geographical origin of sampled honey products in: a) Croatia and b) Germany.

Honey purification

Purification was performed as described in Kenjerić *et al.* (10) with slight modification. Before sampling, each honey is well mixed. Then 25 g of each sample was dissolved in 125 mL 0.01 mol L^{-1} solution of hydrochloric acid. The solution is then vacuum filtrated *via* a polyetersulphonic (PES) filter with pores of 0.2 μm. Chromatography with the aim of extracting phenolic compounds from samples was performed in glass column (25 × 2 cm) with the help of the stationary phase AMBERLITE® XAD® 2, SUPELCO®. About 50 g of stationary phase per sample was washed off 15 minutes by 96 % ethanol (*V/V*), twice dH_2O and once with 150 mL 0.01 mol L^{-1} of hydrochloric acid solution. After passing the samples, the stationary phase with adsorbed compounds was washed with 250 mL 0.01 mol L^{-1} with a solution of hydrochloric acid and 250 mL dH_2O. The elution of compounds from the stationary phase was carried out with 175 mL of 96 % ethanol (*V/V*). The collected fractions are paired on a rotavapor at a temperature range of 32–35 °C with a rotation of 60 rpm. The final masses obtained by extraction were mixed with 96 % ethanol (*V/V*) so that the final mass concentration of each sample was 60 mg mL^{-1}. For further analysis, extracts were prepared at a mass concentration of 10 mg mL^{-1} and were purified three times by centrifugation with 5 min cycles on 15.000 g and 4 °C. The prepared extracts are stored at a temperature of –20 °C until use.

Chemicals and apparatus

Commercial polyphenol standards were purchased from Sigma-Aldrich GmbH (Germany) and Extrasynthese (France). All chemicals and reagents were of analytical grade and supplied by Sigma Aldrich GmbH (Germany) or Kemika (Croatia). RP-HPLC analyses were performed using the Agilent 1100 Series system equipped with a quaternary pump, multiwave UV/Vis detector, autosampler, fraction collector, analytical Zorbax Rx-C18 guard column (4.6 × 12.5 mm, 5 μm particle size) and Poroshell 120 SB-C18 column (4.6 × 75 mm, 2.7 μm particle size) (Agilent Technologies, Waldbronn, Germany). All absorbance measurements of polyphenols

were performed with a NanoDrop 2000c (Thermo Scientific®) and of antioxidant activity with a Fluostar Optima microplate reader (BMG Labtech GmbH, Germany).

Spectrophotometric determination of polyphenols

Total soluble phenols (TP) of honey samples were determined with Foline-Ciocalteau reagent adapted for small volume as described in Vujčić Bok *et al*. (25). A volume 2 µL of tested honey extracts was diluted with 158 µL of distilled water and then 10 µL of Foline-Ciocalteau reagent was added. Afterwards, 30 µL Na_2CO_3 (1.88 mol L^{-1}) was added and the mixture was incubated for 30 min at 45 °C. The absorbance of the mixture was measured at 740 nm. The TP content was calculated from the calibration curve and expressed as gallic acid equivalents (GAE).

The content of total flavonoids (TF) of of honey extracts was determined with $AlCl_3$ adapted for small volume as described in Vujčić Bok *et al*. (25). To dilute the tested solution (2 µL in 80 µL of dH_2O), a volume of 6 µL $NaNO_2$ (5 %) was added. After 5 min incubation, volume of 6 µL $AlCl_3$ (10 %) was added and mixture was incubated at room temperature for additional 6 min. Afterwards, 40 µL NaOH (1 mol L^{-1}) and distilled water were added to final volume of 200 µL. The absorbance of the reaction mixture was read at 520 nm. The TF content was calculated from the calibration curve and expressed as quercetin equivalents (QE).

Total hydroxycinnamic acids (THA) and total flavonols (TFL) of honey extracts were measured as described in Vujčić Bok *et al.* (26) adapted for small volume using caffeic acid and quercetin as standards. Volume of 0.25 mL of the extract was mixed with 0.25 mL HCl (1 g L^{-1}; prepared in ethanol) and 4.55 mL HCl (2 g L^{-1}). The absorbance of the solution was read at 320 and 360 nm, respectively. THA and TFL contents were calculated from the

corresponding calibration curves and expressed as caffeic acid (CAE) and quercetin equivalents (QEE), respectively.

The total flavanol (TFLA) content was determined using *p*-dimethylaminocinnamaldehyde (DMACA) adapted for small volume as described in Rusak *et al.* (27). A volume of 100 µL of tested honey extracts was mixed with 150 µL of DMACA solution (0.1 % in 1 mol L^{-1} HCl in MeOH). After 10 min of incubation at room temperature, absorbance at 640 nm was measured. TFL content was calculated from the calibration curve and expressed as catechin equivalents (CE).

RP-HPLC analysis of flavonoids

Before HPLC analysis, honey samples were hydrolyzed as follows: 150 µL of each extract was mixed with 16,97 µL of HCl (36.5 %, *V/V*) and incubated for 2 h at 80 °C and 300 rpm, stored at –20 °C and centrifuged 15 min on 15.000 g until HPLC analysis.

Qualitative and quantitative RP-HPLC analyses of honey extracts were performed using the Agilent 1100 Series system. The solvents used were: (A) 0.2 % (*V/V*) aqueous glacial acetic acid, and (B) 80 % (*V/V*) methanol + 0.2 % (*V/V*) glacial acetic acid. Gradient profile was (A/B): 85/15 at 0 min, 51.7/48.3 at 20 min, 46.5/53.5 at 24 min, 36.5/63.5 at 30 min, 0/100 at 37.3 min, 0/100 at 40 min. 100/0 at 43 min. Injection volume was 15 µL, the constant flow rate 1.0 mL min^{-1}, and the column temperature was set at 30 °C. The multiwave UV/Vis detector was set at 254, 280, 310, 335 and 360 nm. Phenolic compounds were characterized according to their retention times and UV spectra compared with commercial standards. For the quantitative analyses, calibration curves were obtained by injection of 8 known concentrations (in the range 1-250 µg mL^{-1}) of the mixed 96 % EtOH standard solution in triplicate. The injection volume was 15 µL. The honey extracts were compared with available phenolic standards (pinobanksin,

pinocembrin, chrysin, *p*-coumaric acid, syringic acid, chlorogenic acid and quercetin). The results were expressed as μg mL^{-1} of honey weight.

Antioxidant activity

The ABTS (2,20-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) assay was carried out as described in Radić Brkanac *et al*. (28). A volume of 2 μL of the tested honey extracts was added to 200 μL of ABTS solution and incubated for 6 min at room temperature. The absorbance of the reaction mixture was read at 740 nm. The radical scavenging activity was calculated as percentage of ABTS inhibition as follows: % inhibition = $[(A_0 – A_t)/A_0] \times 100$, where A_0 was the absorbance of the control (blank, without tested solution) and A_t was the absorbance in the presence of the tested solution.

DPPH (1,1-diphenyl-2-picrylhydrazyl) assay was performed as described in Radić Brkanac *et al*. (28); 10 μL of tested honey extracts was added to 190 μL of freshly prepared ethanolic DPPH solution (0.1 mmol L^{-1}) and incubated in the dark for 30 min at room temperature. The decrease in absorbance was measured at 520 nm and the radical scavenging capacity was calculated using the above-mentioned equation.

The ferric reducing antioxidant power (FRAP) assay was performed as described in Radić Brkanac *et al*. (28). The tested honey extracts (10 μL) was mixed with the 190 μL of freshly prepared FRAP reagent (and the absorbance was measured at 595 nm after 4 min of reaction time. The percent of Fe^{3+}-TPTZ reduction was calculated using the formula: % reduction = $[(A_t – A_0)/A_t] \times 100$, where A_0 was the absorbance of the control (blank, without tested solution) and A_t was the absorbance in the presence of the tested solution. Trolox was used as a positive control for all antioxidant activity methods.

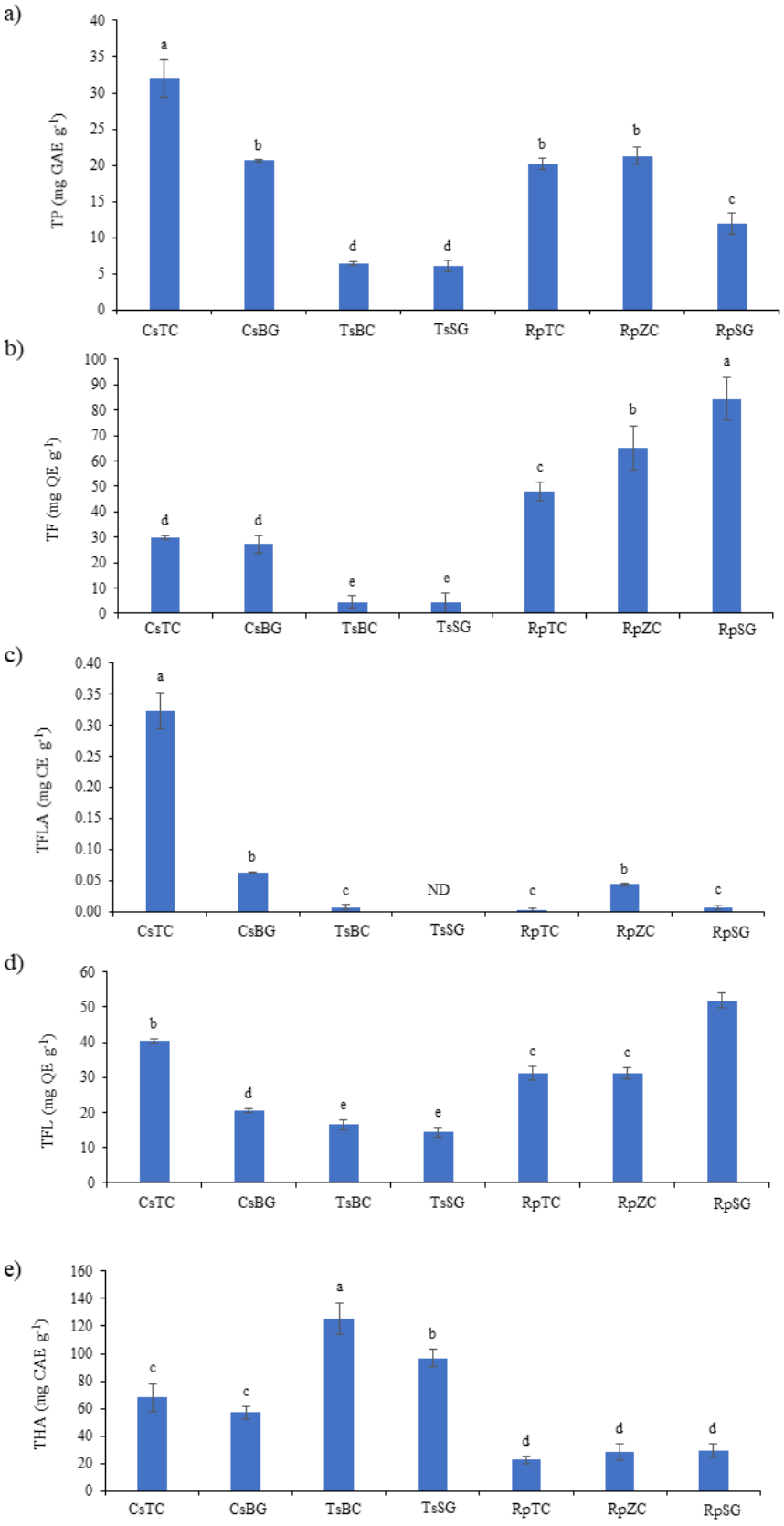
Statistical analysis

All results were evaluated using Statistica 13.3 software package (Stat Soft Inc., USA). RP-HPLC and results from spectrophotometric determination were subjected to one-way ANOVA for comparison of means and significant differences were calculated according to Duncan's multiple range test. The data are presented as the mean ± standard deviations (SD). Pearson's correlation coefficient and Principal component analysis (PCA) between individual and total polyphenols and antioxidant activity were performed. Data were considered statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

Spectrophotometric determination of polyphenols

Total phenols (TP), total flavonoids (TF), total flavanols (TFLA), total flavonols (TFL) and total hydroxycinnamic acids (THA) in Croatian and German honey were presented in Fig. 2.


a)
TP (mg GAE g-1)
40
35
30
25
20
15
10
5
0
a
b
d
d
b
b
c
CsTC
CsBG
TsBC
TsSG
RpTC
RpZC
RpSG
b)
TF (mg QE g-1)
100
90
80
70
60
50
40
30
20
10
0
d
d
e
e
c
b
a
CsTC
CsBG
TsBC
TsSG
RpTC
RpZC
RpSG
c)
TFLA (mg CE g-1)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
b
c
ND
c
b
c
CsTC
CsBG
TsBC
TsSG
RpTC
RpZC
RpSG
d)
TFL (mg QE g-1)
60
50
40
30
20
10
0
b
d
e
e
c
c
CsTC
CsBG
TsBC
TsSG
RpTC
RpZC
RpSG
e)
THA (mg CAE g-1)
160
140
120
100
80
60
40
20
0
c
c
a
b
d
d
d
CsTC
CsBG
TsBC
TsSG
RpTC
RpZC
RpSG

232

Fig. 2. Phenolic content: a) total phenolics (TP); b) total flavonoids (TF); c) total flavanols (TFLA); d) total flavonols (TFL); e) total hydroxycinnamic acids (THA) in Croatian and German honey. Values represent mean ± SD of 3 replicates. Different letters indicate significant difference at $p < 0.05$. *Castanea sativa* honey, Topusko, Croatia – CsTC; *Castanea sativa* honey, Brandenburg, Germany – CsBG; *Tilia* spp. honey, Bilogora, Croatia – TsBC; *Tilia* spp. honey, Saska, Germany – TsSG; *Robinia pseudoacacia* honey, Topusko, Croatia – RpTC; *Robinia pseudoacacia* honey, Konjščina, Zagorje, Croatia – RpZC; *Robinia pseudoacacia* honey, Saska, Germany – RpSG.

The highest TP (Fig. 2a) content was measured in *Castanea sativa* honey originated from Topusko, Croatia and the lowest in *Tilia* spp. honey originated from Bilogora, Croatia and Saska, Germany. Croatian *Castanea* honey had statistically higher values of TP than German *Castanea* honey. Same trend was observed between Craotian *Robinia* honey (Topusko and Konjščina, Zagorje) and German *Robinia* honey from Saska. No significant difference in TP between *Tilia* spp. honey from Bilogora, Croatia and Saska, Germany was detected.

In German *Robinia* honey from Saska were detected highest values of TF (Fig. 2b) and the lowest in *Tilia* spp. honey originated from Bilogora (Croatia) and Saska (Germany). Statistically significant decreased between all *Robinia* honey samples were observed as follows RpSG, RpZC and then RpTC. All *Robinia* honey samples had higher values compared to all *Castanea* and *Tilia* honey samples. *Castanea* honey samples had higher TF values compared to *Tilia* honey samples. No significant difference between all *Tilia* spp. honeys was observed with TF method. Also, no significant difference in TF between all *Castanea sativa* honeys was observed.

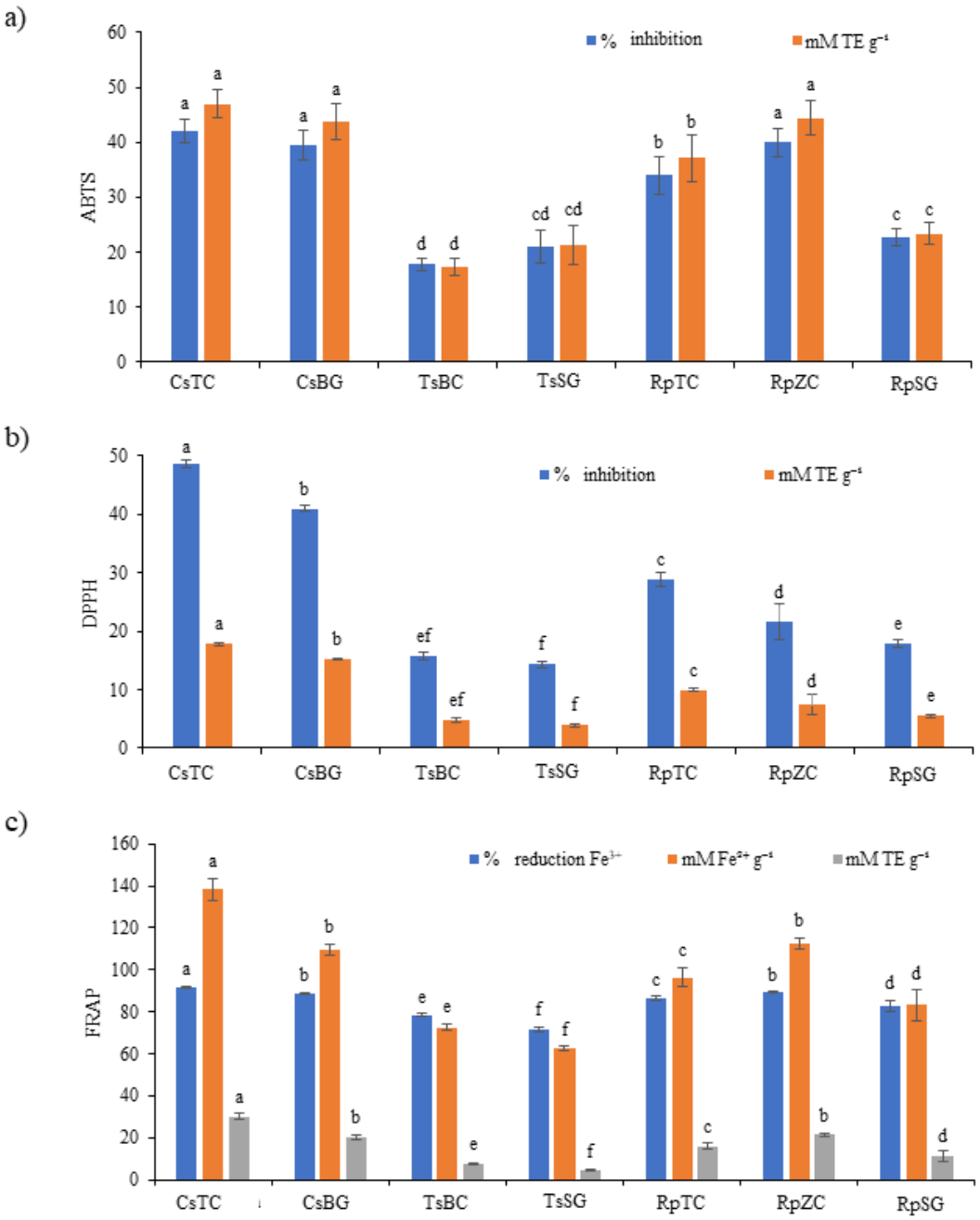
Castanea sativa honey from Topusko (Croatia) had the highest TFLA (Fig. 2c) values. In *Tilia* spp. honey from Saska (Germany), TFLA was not detected. Significant higher values of TFLA were observed between CsTC and CsBG and between RpZC and other *Robinia* honey samples (RpTC and RpSG).

In *Robinia* honey from Saska (Germany) was detected highest values of TFL (Fig. 2d) and the lowest in all *Tilia* spp. honey samples (TsBC and TsSG). German *Robinia* honey (RpSG) had significant higher values than Croatian *Robinia* honey (RpTC and RpZC). Croatian *Castanea* honey (CsTC) had significant higher values than German *Castanea* honey (CsBG). No significant difference in TFL between all *Tilia* spp. honey (TsBC and TsSG) was detected.

Croatian *Tilia* honey (TsBC) had the highest THA (Fig. 2e). The lowest THA values were observed in all *Robinia* honey samples (RpTC, RpZC and RpSG). Significant decline in THA was measured as follows TsBC, and then in TsSG, then in CsTC and CsBG, and then in all *Robinia* honey samples (RpSG, RpZC and RpTC). In Croatian *Tilia* honey significant higher values of THA was observed compared to German *Tilia* honey. No significant difference for THA between all *Castanea* honey samples was observed. Also, no significant difference for THA between all *Robinia* honey samples was observed.

Antioxidant activity

In Fig. 3, antioxidant activity (ABTS; % inhibition and mmol L^{-1} TE g^{-1}, DPPH; % inhibition and mmol L^{-1} TE g^{-1} and FRAP; % reduction Fe^{3+}, mmol L^{-1} Fe^{2+} g^{-1} and mmol L^{-1} TE g^{-1}) of Croatian and German honey were presented.



277

278 Fig. 3. Antioxidant activity: a) ABTS; b) DPPH; c) FRAP in Croatian and German honey.
279 Values represent mean ± SD of 3 replicates. Different letters indicate significant difference at
280 $p < 0.05$. Statistic is performed separate for results presented in % and separate for results
281 presented in mM. *Castanea sativa* honey, Topusko, Croatia – CsTC; *Castanea sativa* honey,
282 Brandenburg, Germany – CsBG; *Tilia* spp. honey, Bilogora, Croatia – TsBC; *Tilia* spp. honey,
283 Saska, Germany – TsSG; *Robinia pseudoacacia* honey, Topusko, Croatia – RpTC; *Robinia*

pseudoacacia honey, Konjščina, Zagorje, Croatia – RpZC; *Robinia pseudoacacia* honey, Saska, Germany – RpSG.

The highest antioxidant activity was measured in *Castanea* honey (CsTC, CsBG) and *Robinia* honey (RpZC) with ABTS method (expressed as a percentage of inhibition and in mmol L^{-1} TE g^{-1}) and the lowest in *Tilia* honey (TsBC). Sample RpZC had statistically higher ABTS values then RpTC sample. Significant decrease in ABTS was observed in *Robinia* honey originated from Croatia (RpZC, RpTC) compared to German honey (RpSG). No significant difference in antioxidant activity measured with ABTS (% inhibition and mmol L^{-1} TE g^{-1}) between all *Castanea* honey samples was observed. Also, no significant difference in antioxidant activity measured with ABTS (% inhibition and mmol L^{-1} TE g^{-1}) between all *Tilia* honey samples was observed.

In Croatian *Castanea* honey (CsTC) was measured the highest antioxidant activity with DPPH method (% inhibition and mmol L^{-1} TE g^{-1}) and lowest in German *Tilia* honey (TsSG). Significant decrease with DPPH (% inhibition and mmol L^{-1} TE g^{-1}) was observed in *Robinia* honey originated from Croatia (RpZC, RpTC) compared to German honey (RpSG). This trend was also observed between Croatian and German *Castanea* honey with DPPH method (% inhibition and mmol L^{-1} TE g^{-1}). Honey sample RpTC had statistically higher DPPH values then RpZC sample.

Castanea honey (CsTC) originated from Croatia had the highest antioxidant activity with FRAP method (% reduction, mmol L^{-1} Fe^{2+} g^{-1} and mmol L^{-1} TE g^{-1}) and lowest had German *Tilia* honey (TsSG). All Croatian honey samples had statistically higher FRAP values (% reduction, mmol L^{-1} Fe^{2+} g^{-1} and mmol L^{-1}TE g^{-1}) then German honey samples.

RP-HPLC flavonoids

With new RP-HPLC method, we identified 3 flavonoids (Fig. 4, Table II) from 7 available phenolic standards (flavonoids: pinobanksin, pinocembrin, chrysin and quercetin; phenolic acids: *p*-coumaric acid, syringic acid and chlorogenic acid). The novelty of the HPLC method refers to a new solvent gradient adapted to the honey. The solvent gradient is described in Experimental. New method is shorter (43 min compared to 60 min in the study of Kenjerić *et al.*(10) and Šarić *et al.* (19); 52 min in the study of Tomás-Barberán *et al.* (22) and still separates both flavonoids and phenolic acids.

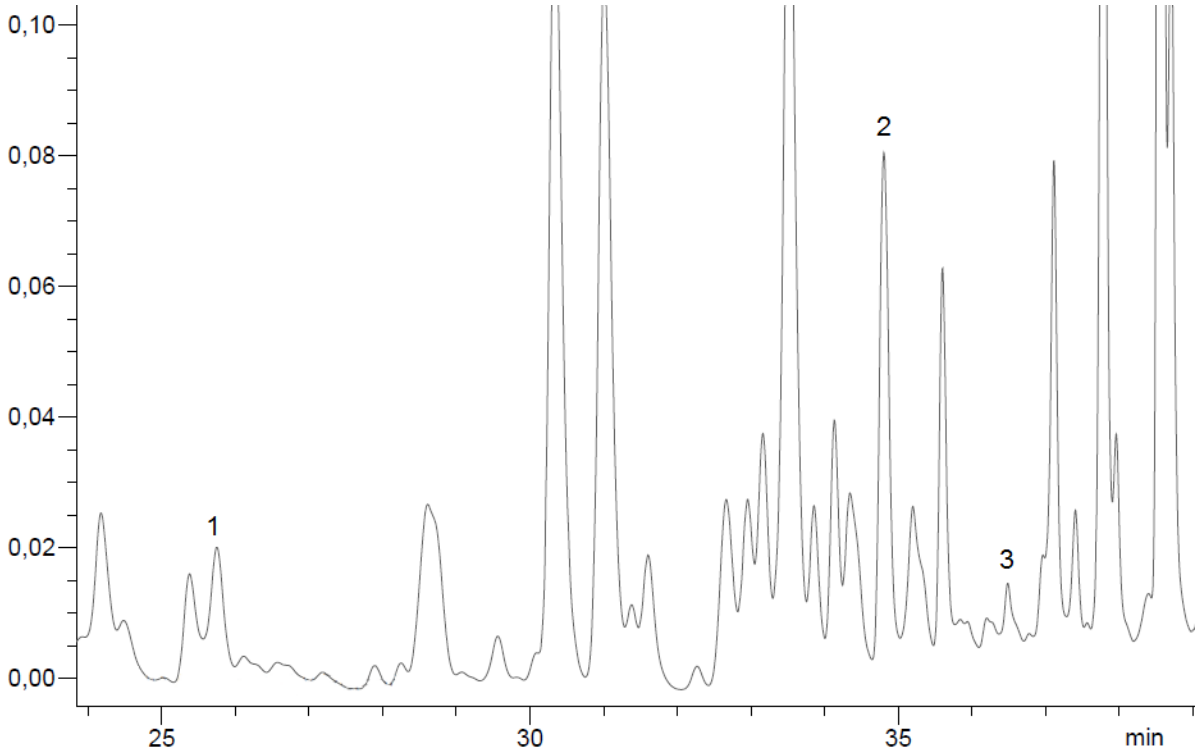


Fig. 4. HPLC profiles of a flavonoids recorded at 280 nm in *Tilia* spp. honey. 1 – pinobanksin, 2 – pinocembrin, 3 – chrysin.

Tabale II. Content of individual and total identified flavonoids by HPLC

Honey sample	Flavonoid (μg mL^{-1})

	Chrysin	Pinocembrin	Pinobanksin	TiF
Castanea sativa honey, Topusko, Croatia – CsTC	9.05[b]	4.24[e]	13.14[c]	26.43[d]
Castanea sativa honey, Brandenburg, Germany - CsBG	8.25[c]	8.42[c]	21.04[a]	37.71[a]
Tilia spp. honey, Bilogora, Croatia – TsBC	6.92[e]	8.27[c]	21.43[a]	36.61[b]
Tilia spp. honey, Saska, Germany – TsSG	4.74[f]	7.89[d]	16.51[b]	29.13[c]
Robinia pseudoacacia honey, Topusko, Croatia – RpTC	7.22[d]	11.70[b]	n.d.	18.91[e]
Robinia pseudoacacia honey, Zagorje, Croatia – RpZC	17.04[a]	19.53[a]	n.d.	36.57[b]
Robinia pseudoacacia honey, Saska, Germany – RpSG	7.20[d]	3.81[f]	n.d.	11.01[f]

Values represent mean of 3 technical replicates (SD < 5 %). Different letters indicate significant difference at $p < 0.05$. TiF – total identified flavonoids; n.d. – not determined

Flavonoids chrysin and pinocembrin were identified in all tested Croatian and German honey samples. Flavonoid pinobanksin is detected only in Croatian and German *Castanea* and *Tilia* honey samples. The highest chrysin and pinocembrin content was measured in Croatian *Robinia* honey sample (RpZC) and lowest in *Tilia* honey sample originated from Saska (Germany) for chrysin and in *Robinia* honey originated from Saska (Germany) for pinocembrin. *Tilia* spp. honey originated from Bilogora (Croatia) and *Castanea sativa* honey originated from Brandenburg (Germany) had the highest pinobanksin content. Total identified flavonids (TiF) were calculated as sum of identified chrysin, pinocembrin and pinobanxin. Sample CsBG had the highest values of TiF and in RpSG were detected lowest values of TiF.

Polyphenols and antioxidant activity

For TP and almost all antioxidant methods (ABTS, FRAP and FRAP) expressed as a percentage of inhibition and in mmol L^{-1}, Croatian *Castanea* honey (CsTC) had the highest

values, followed by German *Castanea* honey (CsBG) and Croatian *Robinia* honey (RpZC), followed by Croatian *Robinia* honey (RpTC), followed by German *Robinia* honey (RpSG), followed by Croatian *Tilia* honey (TsBC) and German *Tilia* honey (TsSG). Same trend was observed for *Castanea* and *Robinia* honey and for *Castanea* and *Tilia* honey, and opposite for *Robinia* and *Tilia* honey in literature (11, 24, 29–31). Based on sensory properties - given the dark color, intense smell and taste, it was expected that the *Castanea* honey will have high values of phenolic compounds and antioxidant activity. Gorjanović *et al*. (23) reported very strong positive correlation between honey color, TP and antioxidant activity (FRAP, ORAC, TEAC and DPPH). The quantitatively high values of the almost all results of the *Castanea* honey can most probably be explained by the fact that chestnut honey is categorized into types of honey with extremely high pollen content (85 %) because *C. sativa* Mill. honey plant is characterized by hyperproduction of pollen and nectar (32) and pollen contributes to the content of proteins, phenolic compounds, vitamins and minerals in honey and its antioxidant capacity (33–35). Also, composition of collected nectar may influence on polyphenols and antioxidant capacity of honey (20). The samples with the lowest antioxidant capacity, the content of total phenols and the examined phenolic subgroups are German and Croatian *Tilia* honey samples. The most probable reason for such results is the fact that *Tilia* honey according to Louveaux *et al*. (32) is grouped into a type of unifloral honey with low pollen content (20–30 %). The obtained results are in accordance with the assumption that *Tilia* honey due to its mild organoleptic properties and yellowish transparent color will have a lower concentration of phenolic compounds and antioxidant capacity compared to *Castanea* honey. The permitted levels of pollen grains in *Tilia* honey can be 10 % if it possesses all the important organoleptic properties. *Robinia* honey is also grouped into a type of unifloral honey with low pollen content (20–30 %) (32). This is in accordance with the lower measured values for most of the tested methods for *Robinia* honey compared to *Castanea* honey. According to the available literature,

there are no results of the spectrophotometric determination of total flavonoids (TF), total flavanols (TFLA), total flavonols (TFL) and total hydroxycinnamic acids (THA) in Croatian and German honey. These methods are rapid and low cost and, in the future, it would be desirable to apply them to determine the composition of polyphenolic groups of compounds because they allow testing purified and unpurified honey samples.

In Croatian *Robinia* honeys, Kenjerić *et al*. (10) detected six flavonoids (quercetin, luteolin, kaempferol, apigenin, chrysin and galangin), and presence of phenolic acids (caffeic acid and *p*-coumaric acid) was also confirmed. Flavonoids myricetin, quercetin, luteolin, kaempferol, apigenin, isorhamnetin, chrysin and galangin were identified in Croatian *Castanea* honey samples by Kenjerić *et al*. (36). According to the available literature, there are no results of the Croatan *Tilia* honey flavonoid profile. Tomás-Barberán *et al.* (22) detected caffeic acid, *p*-coumaric acid, ferulic acid, quercetin, luteolin, kaempferol, pinobanksin, pinocembrin, and chrysin in some German *Robinia* honey samples, caffeic acid, *p*-coumaric acid, pinobanksin, pinocembrin and chrysin were detected in some German *Castanea* honey samples and *p*-coumaric acid, 8-methoxykaempferol and chrysin in German *Tilia* honey sample. In our study we identified chrysin and pinocembrin in all tested Croatian and German honey samples and pinobanksin only in Croatian and German *Castanea* and *Tilia* honey samples. Variability of flavonoid profile and concentrations is to be expected due to the seasons, climatically conditions and other factors.

The values of individual flavonoids obtained by HPLC analysis do not necessarily follow the relationships obtained by measuring total phenols and antioxidant activity. The identified chrysin, pinobanksin and pinocembrin are just some of the compounds that contribute to the total phenol composition and antioxidant activity, so reported values of the mentioned flavonoids give a more specific view of the mutual differences between honey samples. Thus, for example, the sample with the highest concentration of pinobanksin is the Croatian *Tilia*

honey and THA, although antioxidant activity and TP, TF, TFLA and TFL were the lowest compared to other samples. Also, some of flavonoids such as Pcb do not have pronounced antioxidant properties (37).

Based on the detected and identified flavonoids, we can assume the positive biological effects of certain honey samples on human health. Croatian *Robinia* honey originated from Zagorje had a high chrysin content compared to other honey samples which is why it could have a positive effect in anti-inflammatory processes because chrysin inhibits cyclooxygenase-2, the enzyme responsible for inflammation and accompanying pain (38). Same sample had highest pinocembrin content compared to other honey samples, which suggests its potentially beneficial effect on cell protection due to poor blood circulation. According to Khalil *et al*. (37) pinocembrin inhibits the onset of apoptosis in such cells. Croatian *Tilia* honey and German *Castanea* honey had the highest pinobaksin content compared to other samples, which is why it could have a positive effect against tumor formation. According to Silva-Carvalho *et al*. (39), pinobanksin acts by slowing the growth of tumor cells.

In our study *Castanea* honey (CsTC, CsBG) and *Robinia* honey (RpZC and RpTC) had moderate (42.07 %, 39.38 %, 33.99 % and 39.95 %), and all other sample weak (17.73–22.72 %) antioxidant activity in relation to Trolox (82.43 %) by ABTS method. Moderate (40.92-48.56 %) antioxidant activity with DPPH method was observed in *Castanea* honey (CsTC, CsBG) and *Robinia* honey (RpTC; 28.75 %), and all other sample had weak (14.37–21.69 %) antioxidant activity in relation to Trolox (82.06 %). With FRAP method, all tested Croatian and German honey samples showed strong (73.33–94.29 %) antioxidant activity in relation to Trolox (97.45 %). Our classification of antioxidant activity of honey samples is based on the Vujčić *et al*. (40) classification. In this paper antioxidant activity is classified on weak (< 35 %), moderate (35–70 %) and strong (70–100 %) in relation to the positive control (100 %) for herbal originated extracts.

Analyzed Croatian honey samples had higher level of polyphenols and stronger antioxidant activity in comparison to German honey samples. Possible explanation lays in the fact that there is greater diversity of Croatian flora and climatic characteristics are more favorable for beekeeping in Croatia than in Germany. This supports the influence of geographical origin on the quality of honey. If we observe climatic characteristics as the only factor influencing the quality of honey in a geographical area, we can spot that with increase of northern altitude, probably due to the decrease of average annual temperatures, the quality of honey also decreases. This is explained by the lower activity of bees in collecting pollen and nectar at lower temperatures because bees, instead of collecting and producing honey, spend most of their time heating the hive to the optimum temperature of 33–35 °C (41). According to Köppen-Geiger climate classification, Germany is characterized by moderately warm humid climate with warm summers (Cfb), and Croatia by a moderately warm climate with hot summers without drought (Cfa) and with dry summers (Csa) (41). For the period from 1901 to 2000, the Croatian average temperature was 10.90 °C, and the German 8.50 °C (43). It is obvious from the above that Croatia has higher average temperatures compared to Germany, which is logical if we take into account its latitude and the influence of the Mediterranean Sea; the climate certainly remains one of the factors contributing to the difference between Croatian and German honey. An additional argument that is closely related to climatic characteristics is the trend of decreasing diversity of flora from the equator to the north, which affects the quality of honey (44). By reducing the biodiversity of flora in the range of bees, the availability of diverse pollen is reduced, which causes a weak colony due to loss of nutrition and immunodeficiency caused by non-diverse diet of bees (44). According to a direct comparison of flora according to data from 2001, Croatia has 5347 different types of vascular flora, while Germany has 2742 (46). Therefore, the flora of Croatia has 0.07561 species per km^2 of its area, while Germany has

438 0.00771 species per km^2, which means that on one km^2 within the Croatian territory bees will
439 theoretically have 89.80 % more varied pollen.

440

441 *Statistics*

442 Pearson's correlation coefficient between polyphenolic content and antioxidant activity of
443 Croatian and German honey is presented in Table III.

444 *Tabale III. Pearson's correlation coefficient between total and individual polyphenolic content and antioxidant activity of Croatian and*
445 *German honey*

TP	TF	TFLA	TFL	THA	Chr	Pcb	Pbs	TiF	ABTS %	ABTS mmol L^{-1} TE	DPPH %	DPPH mmol L^{-1} TE	FRAP %
1.00													
0.28	1.00												
0.78	–0.09	1.00											
0.43	**0.84**	0.33	1.00										
–0.49	**–0.87**	0.04	–0.66	1.00									
0.47	0.47	0.15	0.22	–0.46	1.00								
0.09	0.19	–0.34	–0.26	–0.32	**0.76**	1.00							
–0.23	**–0.87**	0.17	–0.70	**0.85**	–0.44	–0.37	1.00						
0.01	–0.58	0.04	–0.74	0.49	0.36	0.46	0.63	1.00					
0.94	0.26	0.59	0.24	–0.55	0.60	0.33	–0.20	0.21	1.00				
0.94	0.26	0.59	0.24	–0.55	0.60	0.33	–0.20	0.21	**1.00**	1.00			
0.89	–0.03	**0.80**	0.22	–0.22	0.12	–0.22	0.17	0.10	**0.82**	**0.82**	1.00		
0.89	–0.03	**0.79**	0.21	–0.23	0.14	–0.19	0.18	0.13	**0.83**	**0.83**	**1.00**	1.00	
0.92	0.47	0.58	0.50	–0.60	0.60	0.20	–0.31	0.04	**0.89**	**0.89**	**0.78**	**0.79**	1.00
0.98	0.27	**0.80**	0.42	–0.43	0.55	0.11	–0.16	0.12	**0.93**	**0.93**	**0.87**	**0.88**	**0.94**
0.98	0.27	**0.80**	0.42	–0.43	0.55	0.11	–0.16	0.12	**0.93**	**0.93**	**0.87**	**0.88**	**0.94**

ons are significant at $p < 0.05$

446

TP correlated very strongly ($r > 0.80$) with all antioxidant activity methods (ABTS %: 0.94, ABTS mmol L^{-1} TE 0.94, DPPH % 0.89, DPPH mmol L^{-1} TE: 0.89, FRAP % 0.92, FRAP mmol L^{-1} Fe^{2+}: 0.98 and FRAP mmol L^{-1} TE: 0.98) and strongly ($r > 0.60 < 0.79$) with TFLA (0.78). Positive very strong correlation was observed between TF and TFL (0.84) and negative very strong correlation was observed between TF and THA (–0.87) and TF and Pbs (–0.87). TFLA had very strong (0.80) correlation with DPPH %, FRAP mmol L^{-1} Fe^{2+} and FRAP mmol L^{-1}TE and strong (0.79) with DPPH expressed as mmol L^{-1} TE. THA correlated very strongly with Pbs (0.85) and Chr correlated strongly with Pcb (0.76). All antioxidant methods had very strong (0.82–1) or strong correlation (DPPH % and FRAP %: 0.78, DPPH mmol L^{-1} TE and FRAP %: 0.79) among themselves.

The first (Factor 1) and the second (Factor 2) principal components (PC) described 54.68 % and 23.94 % of the variance (Fig. 5).

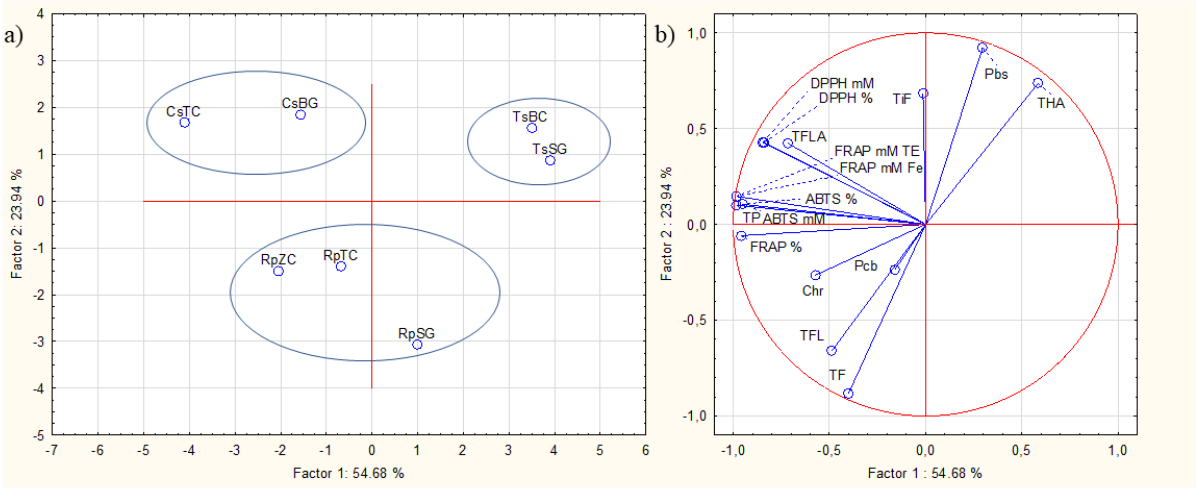


Fig. 5. Principal component analysis of the measured polyphenols and antioxidant activity in the Croatian and German honey. a) Score plot separating the Croatian and German Castanea, Tilia and Robinia honey samples; b) the loading plot of polyphenols and antioxidant activity as

variables. *Castanea sativa* honey, Topusko, Croatia = CsTC; *Castanea sativa* honey, Brandenburg, Germany – CsBG; *Tilia* spp. honey, Bilogora, Croatia – TsBC; *Tilia* spp. honey, Saska, Germany – TsSG; *Robinia pseudoacacia* honey, Topusko, Croatia – RpTC; *Robinia pseudoacacia* honey, Konjščina, Zagorje, Croatia – RpZC; *Robinia pseudoacacia* honey, Saska, Germany – RpSG and TiF – total identified flavonids, Pbs – Pinobanksin, Pcb – pinocembrin, Chr – chrysin, total flavanols – TFLA, total flavonoids – TF, total flavonols – TFL, total hydroxycinnamic acids – THA, total phenols – TP.

Together, the first two PCs represent 78.62 % of the total variability. With PCA plot (Fig. 5a) honey samples were divided on three groups of honey based on their botanical origin. So, the highest distance was detected between *Castanea, Tilia* and *Robinia* honey and the smallest distance was detected between Croatian and German *Tilia* honey (TsBC and TsSG), then in Croatian and German *Castanea* honey (CsTC and CsBG) and then between Croatian (RpZc and RpTC) and German (RpSG) *Robinia* honey. Both, Croatian and German *Castanea* honey (CsTC and CsBG) had strong loadings with most tested total (TP, TFLA and TIF) compounds and antioxidant activity (ABTS: % and mmol L^{-1} TE, FRAP: %, mmol L^{-1} TE and mmol L^{-1} Fe^{2+}, DPPH: % and mmol L^{-1} TE) (Fig. 5b). Croatian (RpZc and RpTC) *Robinia* honey had strong loadings with total (TF and TFL) and individual (Chr and Pcb) polyphenolic compounds (Fig. 5b). Strong loadings with Pbs and THA were detected in Croatian and German *Tilia* honey (TsBC and TsSG) (Fig. 5b).

Correlation analysis confirmed the expected positive correlation between the results of antioxidant methods with the total phenol content (TP) and phenolic subgroup (TFLA). According to Moniruzzaman *et al*. (47) and Flanjak *et al*. (24), phenolic compounds are responsible for antioxidant properties of honey. TF method showed low values of positive and negative correlation coefficients, which are without statistical significance. This can be

489 explained by the non-specificity of this method (48). The THA method shows a negative
490 correlation, *i.e.* an inversely proportional relationship with the results of antioxidant methods.
491 Since hydroxycinnamic acids are powerful antioxidants that can mediate scavenging of harmful
492 reactive oxygen species (49), our correlation results could be the result of certain non-specific
493 reactions.

494 Each honey declared as a unifloral type may contain a different percentage of pollen grains
495 due to the impossibility of direct control of bee grazing, so it is important to define the validity
496 of the declared botanical origin by melisopalinological analysis and by analyzing and defining
497 different markers, *i.e.* specific reference values of certain compounds in honey (50). Overview
498 of the similarities and differences between different Croatian and German *Castanea, Tilia* and
499 *Robinia* honey samples as well as the interrelationships between the measured properties
500 (polyphenol composition and antioxidant activity) were provided by the PCA plots for the
501 purpose of indirectly determining the botanical origin of honey. Three groups of honey
502 (*Castanea, Tilia* and *Robinia*) based on their botanical origin were divided with PCA.
503 According to results of PCA, we can conclude that *Castanea,* is the best quality unifloral honey
504 compared to *Robinia* and *Tilia* honey. Because, *Castanea* honey (CsTC and CsBG) had strong
505 loadings with most tested total (TP, TFLA and TIF) compounds and antioxidant activity
506 (ABTS: % and mmol L^{-1} TE, FRAP: %, mmol L^{-1} TE and mmol L^{-1} Fe^{2+}, DPPH: % and mmol
507 L^{-1} TE). From PCA plots, it can be seen that the different position of *Tilia* honey based on
508 ordinate is most affected by the content of THA and Pbs content, which for these honey samples
509 are the largest compared to other samples, while the position of *Robinia* honey is most affected
510 by TF and TFL and the content of Pcb and Chr. In almost all conducted analyzes, Croatian
511 unifloral types of honey are of better quality than German ones. In PCA plots (Fig. 5a,b),
512 Croatian honey samples are always grouped closely to the most of measured methods. Croatian
513 CsTC samples had smaller distance in PCA plots with TP, TFLA and antioxidant activity

514 methods (ABTS: % and mmol L^{-1} TE, FRAP: %, mmol L^{-1} TE and mmol L^{-1} Fe^{2+}, DPPH: %
515 and mmol L^{-1} TE) compared to German CsBG samples. German CsBG samples had smaller
516 distance in TiF compared to Croatian CsTC samples. *Robinia* honey samples originated from
517 Croatia had smaller distance in PCA plots with TF, TFL, Chr, Pcb while German *Robinia* honey
518 was from the opposite axis of the aforementioned methods. *Tilia* honey originated from Croatia
519 had smaller distance in PCA plots with Pbs and THA compared to *Tilia* honey originated from
520 Germany.

521

522 CONCLUSIONS

523 Through most methods, the sample with the highest antioxidant capacity and the
524 quantitative content of total phenolic compounds is Croatian chestnut honey. According to plant
525 origin, chestnut honey is of higher quality in terms of the quantitative composition of phenolic
526 compounds and antioxidant capacity than acacia and linden honey, and acacia honey is of better
527 quality than linden honey. The above order of unifloral types of honey is most likely caused by
528 the amount of pollen present in the honey because pollen contributes to the final content of
529 phenolic compounds, and thus to the antioxidant capacity of the honey. Additionally, the
530 composition of the collected nectar may influence polyphenols and antioxidant capacity of
531 honey. The analyzed Croatian honey samples are of better quality in terms of the composition
532 of phenolic compounds and antioxidant capacity compared to the German honey samples, most
533 likely due to more favorable climatic characteristics for bee breeding and greater diversity of
534 Croatian flora.

535

536 *Acronyms, abbreviations, symbols.* – ABTS – 2,2 -azinobis(3- ethylbenzothiazoline-6-
537 sulfonic acid), CsBG – *Castanea sativa* honey, Brandenburg, Germany, CsTC – *Castanea*
538 *sativa* honey, Topusko, Croatia, FRAP – Ferric Reducing/Antioxidant Power Assay, RpZC –

Robinia pseudoacacia honey, Konjščina, Zagorje, Croatia, RpSG – *Robinia pseudoacacia* honey, Saska, Germany, RpTC – *Robinia pseudoacacia* honey, Topusko, Croatia, TiF – total identified flavonids, TsBC – *Tilia* spp. honey, Bilogora, Croatia, TsSG – *Tilia* spp. honey, Saska, Germany, TFLA – total flavanols, TF – total flavonoids, TFL – total flavonols, THA – total hydroxycinnamic acids, TP – total phenols.

Acknowledgements. – This work was supported by the University of Zagreb, Croatia (Grant No. 20283112) and by Foundation of the Croatian academy of sciences and arts, Croatia (Grant: Phenolic and antioxidant profile of honeys from certain Croatian regions).

Conflict of interest. – The authors declare that they have no known conflict of interest.

Authors contributions. – Conceptualization, G.R., I.Š. and V.V.B.; investigation, I.Š., V.V.B, A.B. and R.N.; original draft preparation, V.V.B. and A.B.; review and editing, G.R., I.Š. V.V.B, J.L.M. and Ž.M. All the authors have read and agreed to the published version of the manuscript.

REFERENCES

1. M. T. A. Krstonošić, J. M. C. Hogervorst, V. S. Krstonošić and M. P. Mikulić, Phenolic content and *in vitro* antioxidant capacity of mono-and polyfloral honeys originating from Serbia, *Food Feed. Res.* **46**(1) (2019) 83–90; https://doi.org/10.5937/FFR1901083A
2. J. M. Alvarez-Suarez, S. Tulipani, S. Romandini, E. Bertoli and M. Battino, Contribution of honey in nutrition and human health: a review, *Med. J. Nutrition Metab.* **3** (2010) 15–23; https://doi.org/10.1007/s12349-009-0051-6
3. D. Cianciosi, T. Y. Forbes-Hernández, S. Afrin, M. Gasparrini, P. Reboredo-Rodriguez, P. P. Manna, J. Zhang, L. Bravo Lamas, S. Martínez Flórez and P. Agudo Toyos, Phenolic compounds in honey and their associated health benefits: A review, *Molecules* **23**(9) (2018) Article ID 2322 (20 pages); https://doi.org/10.3390/molecules23092322

4. S. Samarghandian, T. Farkhondeh and F. Samini, Honey and health: A review of recent clinical research, *Pharm. Res.* **9**(2) (2017) 121–127; https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5424551/

5. L. Persano Oddo, R. Piro, É. Bruneau, C. Guyot-Declerck, T. Ivanov, J. Piskulová, C. Flamini, J. Lheritier, M. Morlot and H. Russmann, Main European unifloral honeys: descriptive sheets, *Apidologie* **35** (2004) S38–S81; https://doi.org/10.1051/apido:2004049

6. A. Puscas, A. Hosu and C. Cimpoiu, Application of a newly developed and validated high-performance thin-layer chromatographic method to control honey adulteration, *J. Chromatogr.* **1272** (2013) 132–135; https://doi.org/10.1016/j.chroma.2012.11.064

7. Ministry of Agriculture and Forestry, Quality standard of honey and other bees products, *Narodne Novine – Official Journal of The Republic of Croatia*, **20** (2000) 642–652.

8. S. Bogdanov and P. Martin, Honey authenticity: A review, *Mitt. Lebensm. Hyg.* **93** (2002) 232–254.

9. S. Bogdanov, P. Martin and C. Lüllmann, Harmonised methods of the European Honey Com-mission, *Apidologie Extra* **1997** (1997) 53–55.

10. D. Kenjerić, M. L. Mandić, L. Primorac, D. Bubalo and A. Perl, Flavonoid profile of Robinia honeys produced in Croatia, *Food Chem.* **102**(3) (2007) 683–690; https://doi.org/10.1016/j.foodchem.2006.05.055

11. J. Piljac-Žegarac, T. Stipčević and A. Belščak, Antioxidant properties and phenolic content of different floral origin honeys, *J. ApiProduct ApiMed. Sci.* **1** (2009) 43–50; https://doi.org/10.3896/IBRA.4.01.2.04

12. I. Jerković, Z. Marijanović, J. Kezić and M. Gugić, Headspace, volatile and semi-volatile organic compounds diversity and radical scavenging activity of ultrasonic solvent extracts from *Amorpha fruticosa* honey samples, *Molecules* **14**(8) (2009) 2717–2728; https://doi.org/10.3390/molecules14082717

13. I. Jerković and Z. Marijanović, Oak (*Quercus frainetto* Ten.) honeydew honey—approach to screening of volatile organic composition and antioxidant capacity (DPPH and FRAP assay), *Molecules* **15**(5) (2010) 3744–3756; https://doi.org/10.3390/molecules15053744

14. I. Jerković, C. I. Tuberoso, Z. Marijanović, M. Kranjac and M. Malenica-Staver, Antioxidant capacity and chemical profiles of *Satureja montana* L. Honey: hotrienol and syringyl derivatives as biomarkers, *Chem. Biodivers.* **12**(7) (2015) 1047–1056; https://doi.org/10.1002/cbdv.201400183

598 15. P. M. Kuś, I. Jerković, C. I. G. Tuberoso, Z. Marijanović and F. Congiu, Cornflower
599 (*Centaurea cyanus* L.) honey quality parameters: Chromatographic fingerprints,
600 chemical biomarkers, antioxidant capacity and others, *Food Chem.* **142** (2014) 12–18;
601 https://doi.org/10.1016/j.foodchem.2013.07.050
602 16. P. M. Kuś, F. Congiu, D. Teper, Z. Sroka, I. Jerković and C. I. G. Tuberoso, Antioxidant
603 activity, color characteristics, total phenol content and general HPLC fingerprints of six
604 Polish unifloral honey types, *LWT-Food Sci. Technol.* **55**(1) (2014) 124–130;
605 https://doi.org/10.1016/j.lwt.2013.09.016
606 17. D. Bešlo, K. Bešlo, D. Agić, D. Vikić-Topić and B. Lučić, Variations of total phenolic
607 content in honey samples caused by different calibration lines, *Croat. Chem. Acta* **93**(4)
608 (2020) 367–375; https://doi.org/10.5562/cca3805
609 18. G. Šarić, K. Marković, N. Major, M. Krpan, N. Uršulin-Trstenjak, M. Hruškar and N.
610 Vahčić, Changes of antioxidant activity and phenolic content in acacia and multifloral
611 honey during storage, *Food Technol. Biotechnol.* **50**(4) (2012) 434–441;
612 https://hrcak.srce.hr/94500
613 19. G. Šarić, N. Vahčić, D. Bursać Kovačević and P. Putnik, The changes of flavonoids in
614 honey during storage, *Processes* **8**(8) (2020) Article ID 943 (11 pages);
615 https://doi.org/10.3390/pr8080943
616 20. M. Nešović, U. Gašić, T. Tosti, N. Horvacki, B. Šikoparija, N. Nedić, S. Blagojević, L.
617 Ignjatović and Ž. Tešić, Polyphenol profile of buckwheat honey, nectar and pollen, *R.*
618 *Soc. Open Sci.* **7**(12) (2020) Article ID 201576; https://doi.org/10.1098/rsos.201576
619 21. K. Brščić, T. Šugar and D. Poljuha, An empirical examination of consumer preferences
620 for honey in Croatia, *Appl. Econ.* **49**(58) (2017) 5877–5889;
621 https://doi.org/10.1080/00036846.2017.1352079
622 22. F. A. Tomás-Barberán, I. Martos, F. Ferreres, B. S. Radovic and E. Anklam, HPLC
623 flavonoid profiles as markers for the botanical origin of European unifloral honeys, *J.*
624 *Sci. Food Agric.* **81**(5) (2001) 485–496; https://doi.org/10.1002/jsfa.836
625 23. S. Ž. Gorjanović, J. M. Alvarez-Suarez, M. M. Novaković, F. T. Pastor, L. Pezo, M.
626 Battino and D. Ž. Sužnjević, Comparative analysis of antioxidant activity of honey of
627 different floral sources using recently developed polarographic and various
628 spectrophotometric assays, *J. Food Compost. Anal.* **30**(1) (2013) 13–18;
629 https://doi.org/10.1016/j.jfca.2012.12.004
630 24. I. Flanjak, D. Kenjerić, D. Bubalo and L. Primorac, Characterisation of selected Croatian
631 honey types based on the combination of antioxidant capacity, quality parameters, and

chemometrics, *Eur. Food Res. Technol.* **242** (2016) 467–475; https://doi.org/10.1007/s00217-015-2557-0

25. V. Vujčić Bok, I. Šola and G. Rusak, Lemon juice formulations modulate *in vitro* digestive recovery of spinach phytochemicals, *Food Technol. Biotechnol.* (Online) **60**(3) (2022) 293–307; https://doi.org/10.17113/ftb.60.03.22.7104
26. V. Vujčić Bok, M. Gerić, G. Gajski, S. Gagić and A.-M. Domijan, Phytotoxicity of bisphenol A to *Allium cepa* root cells is mediated through growth hormone gibberellic acid and reactive oxygen species, *Molecules* **28**(5) (2023) Article ID 2046 (15 pages); https://doi.org/10.3390/molecules28052046
27. G. Rusak, I. Šola and V. Vujčić Bok, Matcha and Sencha green tea extracts with regard to their phenolics pattern and antioxidant and antidiabetic activity during *in vitro* digestion, *J Food Sci Technol.* **58** (2021) 3568–3578; https://doi.org/10.1007/s13197-021-05086-5
28. S. Radić Brkanac, M. Gerić, G. Gajski, V. Vujčić, V. Garaj-Vrhovac, D. Kremer and A.-M. Domijan, Toxicity and antioxidant capacity of *Frangula alnus* Mill. bark and its active component emodin, *Regul. Toxicol. Pharmacol.* **73**(3) (2015) 923–929; https://doi.org/10.1016/j.yrtph.2015.09.025
29. J. Bertoncelj, U. Doberšek, M. Jamnik and T. Golob, Evaluation of the phenolic content, antioxidant activity and colour of Slovenian honey, *Food Chem.* **105**(2) (2007) 822–828; https://doi.org/10.1016/j.foodchem.2007.01.060
30. S. Maurya, A. K. Kushwaha, S. Singh and G. Singh, An overview on antioxidative potential of honey from different flora and geographical origins, *Indian J. Nat. Prod. Resour.* **5**(1) (2015) 9–19.
31. T. Istasse, N. Jacquet, T. Berchem, E. Haubruge, B. K. Nguyen and A. Richel, Extraction of honey polyphenols: method development and evidence of cis isomerization ubertas academica, *Anal. Chem. Insights* **11** (2016) Article ID ACI-S39739; https://doi.org/10.4137/ACI.S39739
32. J. Louveaux, A. Maurizio and G. Vorwohl, Methods of melissopalynology, *Bee World* **59**(4) (1978) 139–157; https://doi.org/10.1080/0005772X.1978.11097714
33. M. Leja, A. Mareczek, G. Wyżgolik, J. Klepacz-Baniak and K. Czekońska, Antioxidative properties of bee pollen in selected plant species, *Food Chem.* **100**(1) (2007) 237–240; https://doi.org/10.1016/j.foodchem.2005.09.047
34. F. Bonté and A. Desmoulière, Le miel: origine et composition, *Actual. Pharmaceut.* **52**(531) (2013) 18–21; https://doi.org/10.1016/j.actpha.2013.10.004

35. O. Bobis, V. Bonta, A. Varadi, M. Strant and D. Dezmirean, Bee products and oxidative stress: bioavailability of their functional constituents, *Mod. Appl. Bioequiv. Availab*. **1** (2017) P1-5; https://doi.org/10.19080/MABB.2017.01.555565
36. D. Kenjerić, M. L. Mandić, L. Primorac and F. Čačić, Flavonoids in Croatian chestnut (*Castanea sativa*) honey, Međunarodni znanstveno-stručni skup XIII. Ružičkini dani" Danas znanost-sutra industrija", Vukovar, Hrvatska, 16. and 17. September 2010 (2011) 319–325.
37. M. Khalil, S. A. Sulaiman and L. Boukraa, Antioxidant properties of honey and its role in preventing health disorder, *Open Nutraceut. J.* **3** (2010) 6–16; https://doi.org/10.2174/1876396001003010006
38. K. J. Woo, Y.-J. Jeong, H. Inoue, J.-W. Park and T. K. Kwon, Chrysin suppresses lipopolysaccharide-induced cyclooxygenase-2 expression through the inhibition of nuclear factor for IL-6 (NF-IL6) DNA-binding activity, *FEBS letters* **579**(3) (2005) 705–711; https://doi.org/10.1016/j.febslet.2004.12.048
39. R. Silva-Carvalho, V. Miranda-Gonçalves, A. M. Ferreira, S. M. Cardoso, A. J. Sobral, C. Almeida-Aguiar and F. Baltazar, Antitumoural and antiangiogenic activity of Portuguese propolis in *in vitro* and *in vivo* models, *J. Funct. Foods* **11** (2014) 160–171; https://doi.org/10.1016/j.jff.2014.09.009
40. V. Vujčić, S. Radić Brkanac, I. Radojčić Redovniković, S. Ivanković, R. Stojković, I. Žilić and M. Radić Stojković, Phytochemical and bioactive potential of *in vivo* and *in vitro* grown plants of *Centaurea ragusina* L. – Detection of DNA/RNA active compounds in plant extracts via thermal denaturation and circular dichroism: Phytochemical and bioactive characterization of *Centaurea ragusina* L., *Phytochem. Anal.* **28**(6) (2017) 584–592; https://doi.org/10.1002/pca.2708
41. S. A. Corbet, M. Fussell, R. Ake, A. Fraser, C. Gunson, A. Savage and K. Smith, Temperature and the pollinating activity of social bees, *Ecol. Entomol.* **18**(1) (1993) 17–30; https://doi.org/10.1111/j.1365-2311.1993.tb01075.x
42. M. Kottek, J. Grieser, C. Beck, B. Rudolf and F. Rubel, World map of the Köppen-Geiger climate classification updated, *Meteorol. Z.* **15**(3) (2006) 259–263; https://doi.org/10.1127/0941-2948/2006/0130
43. T. D. Mitchell, T. R. Carter, P. D. Jones, M. Hulme and M. New, A comprehensive set of high-resolution grids of monthly climate for Europe and the globe: the observed record (1901–2000) and 16 scenarios (2001–2100), Tyndall centre for climate change research working paper 55 (2004) 25.

699 44. N. M. Waser and J. Ollerton, *Plant-pollinator Interactions: From Specialization to*
700 *Generalization*, University of Chicago Press, Chicago 2006.
701 45. J.-F. Odoux, D. Feuillet, P. Aupinel, Y. Loublier, J.-N. Tasei and C. Mateescu, Territorial
702 biodiversity and consequences on physico-chemical characteristics of pollen collected
703 by honey bee colonies, *Apidologie* **43** (2012) 561–575; https://doi.org/10.1007/s13592-
704 012-0125-1
705 46. T. Nikolić, The diversity of Croatian vascular flora based on the Checklist and CROFlora
706 database, *Acta Bot. Croat.* **60**(1) (2001) 49–67; https://hrcak.srce.hr/160814
707 47. M. Moniruzzaman, C. Yung An, P. V. Rao, M. N. I. Hawlader, S. A. B. M. Azlan, S. A.
708 Sulaiman and S. H. Gan, Identification of phenolic acids and flavonoids in monofloral
709 honey from Bangladesh by high performance liquid chromatography: determination of
710 antioxidant capacity, *BioMed. Res. Int.* **2014** (2014) Article ID 737490 (11 pages);
711 https://doi.org/10.1155/2014/737490
712 48. A. Pękal and K. Pyrzynska, Evaluation of aluminium complexation reaction for flavonoid
713 content assay, *Food Anal. Methods* **7** (2014) 1776–1782;
714 https://doi.org/10.1007/s12161-014-9814-x
715 49. D. Šamec, E. Karalija, I. Šola, V. Vujčić Bok and B. Salopek-Sondi, The role of
716 polyphenols in abiotic stress response: The influence of molecular structure, *Plants*
717 **10**(1) (2021) Article ID 118 (24 pages); https://doi.org/10.3390/plants10010118
718 50. U. M. Gašić, D. M. Milojković-Opsenica and Ž. L. Tešić, Polyphenols as possible
719 markers of botanical origin of honey, *J. AOAC Int.* **100**(4) (2017) 852–861;
720 https://doi.org/10.5740/jaoacint.17-0144
721
722