Evaluation of total phenolic content of Serbian honeys by cyclic voltammetry

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Abstract: In this study, cyclic voltammetry (CV) was applied for determination of total phenolic content in honey samples. Honey samples of diverse botanical source were collected in different geographical regions in Serbia. Cyclic voltammograms taken from -200 to 800 mV at a scan rate 100 mV s^{-1} were used to quantify electrochemical properties of antioxidants present in honeys as well as to deduce antioxidant capacity from the Q_{600} parameter (charge passed to 600 mV). Trolox was used as a standard solution and Q_{600} parameter was expressed as micromoles of Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) equivalents (TE) per kg of honey sample. Good correlations were obtained when total phenolics measured from CVs were compared with the total phenolic content (TPC) determined by well established spectrophotometric technique using Folin-Ciocalteu method and radical scavenging activity (RSA) determined using DPPH·(1,1-diphenyl-2-picrylhydrazyl radical). These results indicated that cyclic voltammetry is highly efficient method and could be an alternative method for rapid determination of total phenolic content.

Keywords: Antioxidant activity, Folin-Ciocalteu method, Q_{600} parameter, radical scavenging activity

INTRODUCTION

Generally, there is a growing interest on the effects of natural antioxidants in food. Polyphenols, i.e. flavonoids and phenolic acids, are considered as one of the important group of components identified in honey having antioxidant activity. Antioxidant activity of honey is closely related to the floral source of honey. Generally, honeys are classified as monofloral (produced by one plant species) and polyfloral (several plant sources). Different honey types were subjected to antioxidant activity tests and have demonstrated significant potential, comparable to the other foodstuff[1].

On the other hand, research on the antioxidant capacity of honey samples originating from Serbia remains scarce. Available literature indicates that until now there have been just few researches to determine both total phenolic content and antioxidant activity of Serbian honeys by spectroscopic metods[2,3,4] and one electrochemical (polarographic) method[5].

Cyclic voltammetry (CV) is well known as a helpful tool to estimate total phenolic content and to monitor antioxidant properties of food rich in polyphenols. CV was shown to be sensitive, convenient, and low costing approach in the quality evaluation of the food products beneficial for human health[6,7]. An evaluation of antioxidant activity of different food products using electrochemical methods were reported[8,9,10,11].

Due to our interest in the quality of the Serbian honey samples and continuing research of these nutritionally important products, in this study we have examined antioxidant potential of honey samples of diverse botanical origin. Samples were collected directly from the beekeepers from different places in Serbia. Two different approaches, spectroscopic and electrochemical techniques to determine antioxidative potential and total phenolic content of selected honey samples were used. Total phenolic content (TPC) was determined by well established spectrophotometric technique using Folin-Ciocalteu method. Antioxidant capacity (RSA) of honey samples was determined using the 2,2-diphenyl-1-picrylhydrazyl radical (DPPH·). Cyclic voltammetry was used to determine the electrochemical response of each sample. To inspect the applicability of cyclic voltammetry in such investigations, electrochemically determined results were correlated with the results obtained by using the spectrophotometric methods already established in the literature.

EXPERIMENTAL

Chemicals and materials

Methanol (HPLC grade), sodium carbonate, potassium chloride, hydrochloric acid, Folin-Ciocalteu reagent, and filter paper (Whatman No.1) were purchased from Merck (Darmstadt, Germany). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma Aldrich (Steinheim, Germany). 2,2-Diphenyl-1-picrylhydrazyl·(DPPH·) was purchased from Fluka AG (Buch, Switzerland). Ultrapure water (ThermoFisher TKA MicroPure water purification system, 0.055 μS cm^{-1}) was used to prepare standard solutions, blanks, and artificial honey (30% glucose, 40% fructose, 10% sucrose, and 20% water, v/v). A sugar analogue of honey was made to check whether the main sugars in honey can interfere in the proposed electrochemical assay. Sugar standards (glucose, fructose, and sucrose) were purchased from Tokyo Chemical Industry (TCI, Europe, Belgium). Syringe filters (13 mm, PTFE membrane 0.45 μm) were purchased from Supelco (Bellefonte, PA, USA). Ethanol (96% by vol) was from J. T. Baker (Deventer, The Netherlands).

Honey samples

A total of 27 honey samples collected from different regions of Serbia (Fig. 1) during the 2009 harvesting season were provided by ‘‘The Association of the Beekeeping Organizations of Serbia’’ (SPOS) (www.spos.info). The botanical origins of the samples were specified by the SPOS based on the information provided by beekeepers and sensory characteristics, and confirmed by physicochemical analyses and chemometrics[12,13]. The honey samples were: acacia (*Robinia pseudoacacia*), sunflower (*Helianthus annuus*), lime (*Tilia cordata*), giant goldenrod (Solidago virgaurea), basil (*Ocimum basilicum*), oilseed rape (*Brassica napus*), buckwheat (*Fagopyrum esculentum*), and polyfloral meadow honey. The number of analyzed samples of each botanical origin is given in Table 1. The honeys were stored at room temperature in dark before analysis.

Fig. 1

Table 1

Instrumentation

Cyclic voltammograms were recorded on a CHI760B instrument (CH Instruments, Austin, Texas, USA). The cell was equipped with GC electrode (Model CHI104), an accessory platinum electrode of larger area (Model CHI221, cell top including Pt wire counter electrode) and an Ag/AgCl reference electrode (Model CHI111). All measurements were taken at ambient temperature. Prior to each run, the surface of the glassy carbon electrode was freshly abraded with 1.0, 0.3 and 0.05 μm alumina powder, rinsed with redistilled water and degreased in ethanol in ultrasonic bath.

An UV/VIS spectrophotometer (GBC UV-Visible Cintra 6) was used for absorbance measurements and spectra recording, using optical cuvettes of 1 cm optical path.

Cyclic voltammograms

Honey samples, 1 g of each, was mixed with 20 mL 0.1 M KCI, homogenized in ultrasonic bath for 10 min at room temperature, then filtered through 0.45 μm PTFE membrane and analyzed for determination of TPC by cyclic voltammetry.

Cyclic voltammograms were recorded in 0.1 M KCl as the supporting electrolyte. Trolox was used as a standard. In order to achieve better similarity with honey matrix, Trolox standard was prepared and recorded in a solution of artificial honey. The solution of artificial honey was

prepared in the same manner as the honey samples (1g of artificial honey in 20 mL of supporting electrolyte). The scan was taken in the potential range between -200 mV and 800 mV with a scan rate 100 mV s^{-1}. Cyclic voltammograms were recorded for Trolox standard in the concentration range 10 to 100 μmol L^{-1}. The obtained calibration curve, Q_{600} = f (concentration of Trolox) was used to calculate Trolox Equivalent Antioxidant Capacity (TEAC) of studied honeys and the results are expressed as micromoles of Trolox Equvivalents per kg of sample (μmol TE kg^{-1}).

Determination of total phenolic content and radical scavenging activity

Samples were prepared according to the slightly modified method proposed by Meda et al.[14]. Each honey sample (5 g) was mixed with 15 mL ultrapure water, homogenized in ultrasonic bath for 15 min at room temperature, transferred to 50 mL volumetric flask, and filled with ultrapure water. The solution was then filtered through 0.45 μm PTFE membrane and analyzed for determination of TPC and RSA.

The TPC was spectrophotometrically determined with a Folin-Ciocalteu method reported by Singleton and Rossi[15], with some modification. Briefly, 0.3 mL of the sample extracts and 6 mL deionized water were mixed with 0.5 mL of Folin-Ciocalteu reagent and solution was incubated 6 min at room temperature. Next, 3 mL of 20% sodium carbonate was added. After 30 min at 40 ^{o}C, absorbance was measured at 765 nm. Gallic acid was used as standard, and calibration curve of gallic acid was prepared in concentration range between 50 and 250 mg L^{-1}. A mixture of water and reagent was used as a blank. The results were expressed as the mg gallic acid equivalent (GAE) per kilogram of honey.

The RSA of the extracts of honey samples was evaluated by modified method of Li et al.[16]. An aliquot of 1.0 mL of extracts (some extracts were diluted ten times) was mixed with 3 mL of methanol solution of DPPH (71 mM). The mixture was left for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH· radical was measured by monitoring continuously the decrease of absorption at 515 nm. RSA was calculated as a percentage of DPPH· discoloration using the equation:

$$RSA\ (\%) = \frac{(A_{DPPH} - A_{sample})}{A_{DPPH}} \times 100$$

where A_{DPPH} is the absorbance of methanol solution of DPPH· radical, A_{sample} is the absorbance in the presence of honey extract. The assays were carried out in triplicate and the results were expressed as mean values.

Statistical analysis

Data of all measurements done in triplicate are expressed as the mean values. Statistical analyses were performed by NCSS software package[17].

RESULTS AND DISCUSSION

In order to quantify electrochemical properties of antioxidants in honey samples cyclic voltammograms were recorded in potential range from -200 to 800 mV, covering all the groups responsible for the antioxidative action. All voltammograms are characterized with one cathodic peak and up to three anodic peaks. All the peak potentials (E_p), peak currents (I_p) and Q_{600} parameter determined from cyclic voltammograms are presented in Table 2. Representative cyclic voltammograms obtained for three honey samples: sunflower honey (H7), lime honey (H13), and polyfloral honey sample (H23) are presented in Fig. 2.

Fig. 2

The results obtained by cyclic voltammetry provide the information on the total antioxidant activity as the total current, obtained as the area under the peak contribution from all of the components that are present, is responsible for the antioxidant activity of the sample.

On the basis of cyclic voltammograms and the position of the specific peaks in different samples and on the basis of the published literature data[18,19,20] one can ascertain dominating compounds. As it can be seen from the results given in Table 2, low intensity current peak appearing at a potential range 120 to 180 mV is found to be characteristic for samples from two regions, Vojvodina and Zlatibor (H7, H8, H20, H23, H25, and H26). This peak could be attributed to the oxidation of ascorbic acid. All peaks in the range of 380 to 480 mV (characteristic for the most of the samples) could be ascribed to the oxidation of compounds having *ortho*-dihydroxy-phenol and gallate group in the structure, which becomes reduced in the reversed scan. Cathodic peak at 350 mV (see Fig. 2 and Table 2) is result of the quinine formation from the oxidation of the *ortho*-dihydroxy-phenol group. As with many phenolic antioxidants, the reaction has the following form:

$$R \leftrightarrows O + 2H^+ + 2e^-$$

where R is the reductant (antioxidant) and O the product of oxidation (oxidant). Formation of ortho-dihidroxy quinone is a feature typical for the antioxidants that react in this potential region (380 to 480 mV). The reversibility of the reaction at the glassy carbon electrode will vary with different antioxidants, and product of the oxidation may be susceptible to further chemical reaction. In all the investigated honey samples the antioxidants

that provide reverse peak were detected, with different peak intensity depending on the concentration.

The third anodic peak detected at potentials between 640 and 670 mV (characteristic for the most of the samples) could be ascribed to the oxidation state of the monophenol group or meta-diphenols on the A-ring of flavonoids or isolated hydroxyl groups, often in one electron process[18,19,20]. Antioxidants which displayed a first anodic peak only at higher potentials were more difficult to oxidize and may be less reactive as antioxidants. As it can be seen from the Table 1, there were no examples with such antioxidants; thus the investigated honey samples contained easily oxidative antioxidants. The absence of corresponding reduction peak also points to the irreversibility of oxidation of reaction products produced in this reaction. It is known that oxidation of monophenol group occurs at high positive potentials forming a phenoxy radical or phenoxonium ion that can successively undergo different secondary reactions in solution. The cathodic peak seen on the reverse scan was matched by new anodic peak that appeared on second cycle in the forward direction. These sets of peaks are likely to be due to the oxidation products of the antioxidants deposited on the electrode surface as a thin film. The same peaks were commonly seen with organic polymers such as polyaniline or polypirrole, formed by oxidation of the respective monomers. The anodic peak that comes from the antioxidant itself was also less intense on the second scan, consistent with a less active electrode surface. Compounds containing monophenol group giving rise to pronounced potential peak and extensively discussed in the literature are phenolic acids such as vanillic acid and *p*-coumaric acid.

Table 2

The results on the total phenolic content and the results of RSA in the honey samples are presented in Table 1, together with the Q_{600} parameter derived from CV. All honey samples were characterized with TPC values ranging between 127.76 mg (acacia – H1) to 887.18 mg (polyfloral – H22) of gallic acid per kg of honey. The average content of total phenolics was in a good agreement with the values given in the literature for the honeys of surrounding regions[21,22,23]. Generally, polyfloral honey samples had the highest values of TPC, while acacia samples showed the lowest values. Such findings are consistent with literature data. Just as an example, here we cite the paper published by Bertoncelj et al.[23] who reported higher TPC values of polyfloral honeys in comparison to monofloral honeys (lime

and sunflower). As it is visible from the Table 1, results of RSA ranged from 1.86% (acacia honey – H4) to 23.20% (polyfloral honey – H22). Among all monofloral honey samples, buckwheat was found to have the highest total phenolic content and radical scavenging activity. This was also found in the study of different monofloral honeys when buckwheat honey was reported to have the highest antioxidant activity[24].

A correlation matrix for these variables shows large positive correlations between all the values. As it is visible from the correlation matrix given in Table 3, Q_{600} parameter derived from CV was strongly correlated with TPC, with correlation coefficient 0.946. Level of significance for each correlation was $p<0.000001$. Such statistically significant correlation clearly indicates the potency of cyclic voltammetry as fast, informative method for the total phenolic content determination.

Table 3

Significant correlations obtained between spectrophotometrically and electrochemically determined total phenolics, TPC and Q_{600}, respectively and RSA indicate that among all active phytochemicals, flavonoids and phenolic acids could be identified as chemicals that account for antioxidant potential of the honey.

CONCLUSION

Serbian honey samples from different botanical origin and geographical regions were studied to determine their total phenolic content and antioxidant capacity. Two methods, spectroscopic and electrochemical, were used for that purpose. Cyclic voltammetry was shown to be a highly attractive alternative method for rapid determination of total phenolic content. Linear dependence between this method and commonly used Folin-Ciocalteu method was high with $r = 0.946$. TPC was compared with the antioxidant activity of honey extracts, and good correlation was obtained. Such simple electrochemical technique could be considered as a valuable method for quality control, not only of honey but also for plant derived food products in general.

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ИЗВОД

Процена садржаја укупних фенола у узорцима српских медова применом цикличне волтаметрије

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Циљ овог рада био је примена цикличне волтаметрије (CV) за одређивање садржаја укупних фенола у узорцима меда. Узорци различитог ботаничког порекла прикупљени су у различитим географским регионима Србије. Циклични волтамограми, снимани од -200 до 800 mV при брзини очитавања од 100 mV s^{-1}, коришћени су за испитивање електрохемијских особина антиоксиданаса присутних у меду, као и да би се одредио антиоксидативни капацитет представљен као Q_{600} параметар (количина наелектрисања измерена на 600 mV). Као стандард је коришћен Тролокс (6-хидрокси-2,5,7,8-тетраметилхроман-2-карбоксилна киселина) и Q_{600} параметар је изражен у Тролокс еквивалентима (TE, μmol kg^{-1} узорка меда). Показано је да су резултати цикличне волтаметрије у доброј корелацији са резултатима који се добијају применом *Folin-Ciocalteu* реагенса, као и са антиоксидативним потенцијалом (*RSA*) који је одређен употребом DPPH· (1,1-дифенил-2-пикрилхидразил) радикала. Резултати указују да је циклична волтаметрија ефикасна метода и може да буде алтернативна метода за брзо одређивање садржаја укупних фенола.

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334 TABLE CAPTIONS

335

336 **Table 1**. Total phenolic content, radical scavenging activity and Q_{600} parameter
337 derived from CV of monofloral and polyfloral honey samples.

338

339 **Table 2**. Peak potentials (E_p), currents (I_p) and Q_{600} parameter determined from cyclic
340 voltammograms of honey samples.

341

342 **Table 3**. Correlation coefficients between TPC, RSA, and Q_{600}.

343

344 **Table 1.** Total phenolic content, radical scavenging activity and Q600 parameter derived from
345 CV of monofloral and polyfloral honey samples.

Sample number	**Botanical origin**	**TPC, mg GAE kg^{-1}**	**RSA, %**	**Q_{600}, μmol TE kg^{-1}**
H1	acacia	127.76	2.18	9.01
H2		279.20	2.93	16.26
H3		328.22	3.34	17.58
H4		281.35	1.86	18.46
H5		368.69	2.51	24.18
H6	sunflower	362.42	5.04	24.62
H7		465.16	9.65	36.92
H8		246.99	5.95	17.58
H9		451.19	10.64	27.25
H10	lime	320.84	4.04	21.98
H11		373.49	4.95	27.03
H12		474.19	6.43	19.78
H13		483.03	10.84	37.36
H14	giant goldenrod	467.11	6.69	24.18
H15		414.99	5.72	26.37
H16	basil	379.63	8.76	15.39
H17		395.46	4.33	28.57
H18	oilseed rape	513.64	13.51	39.56
H19		372.47	9.45	24.18
H20	buckwheat	668.58	14.44	46.15
H21	polyfloral	496.40	5.82	27.47
H22		887.18	23.20	65.93
H23		782.16	13.81	61.54
H24		540.63	7.74	37.36
H25		432.29	5.89	30.77
H26		631.85	11.86	50.55
H27		688.81	18.67	48.35

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Table 2. Peak potentials (Ep), currents (Ip) and Q600 parameter determined from cyclic voltammograms of honey samples.

Sample number	$E_{p,a}$, mV			$I_{p,a}$, μA			$E_{p,c}$, mV	$I_{p,c}$, μA	Q_{600}, μC
	peak 1	peak 2	peak 3	peak 1	peak 2	peak 3			
H1	-	0.422	-	-	1.54	-	0.344	-0.87	0.041
H2	-	0.451	0.658	-	2.24	4.04	0.361	-1.22	0.074
H3	0.384	0.453	0.648	1.17	1.36	2.45	0.366	-0.73	0.080
H4	0.392	0.456	0.640	2.19	2.57	4.19	0.366	-1.49	0.084
H5	0.400	0.470	0.659	0.49	0.63	1.21	0.352	-0.27	0.110
H6	0.395	0.465	0.655	2.24	2.61	4.41	0.359	-1.46	0.112
H7	0.168	0.413	0.645	0.79	1.35	2.75	0.382	-0.83	0.168
H8	0.179	0.424	0.643	0.78	1.42	2.72	0.375	-0.77	0.080
H9	-	0.423	0.642	-	1.78	3.54	0.378	-1.02	0.124
H10	-	0.455	0.654	-	2.01	4.11	0.339	-1.04	0.100
H11	0.394	-	0.621	1.25	-	2.77	0.307	-0.42	0.123
H12	0.359	0.442	0.665	1.53	1.93	3.77	0.328	-0.95	0.090
H13	0.373	0.448	0.651	0.93	1.18	2.66	0.344	-0.56	0.170
H14	0.420	0.480	0.654	2.31	2.77	4.34	0.369	-0.72	0.110
H15	-	0.423	0.672	-	2.56	4.79	0.385	-1.55	0.120
H16	0.419	0.486	-	2.73	3.10	-	0.383	-1.63	0.070
H17	0.395	0.477	-	1.41	1.79	-	0.357	-0.97	0.130
H18	0.403	0.462	0.652	2.51	2.86	4.93	0.363	-1.48	0.180
H19	0.397	0.465	0.667	2.66	3.11	5.38	0.373	-1.76	0.110
H20	0.179	0.393	0.457	1.17	2.21	2.63	0.348	-1.22	0.210
H21	0.385	0.445	-	1.52	1.78	-	0.343	-1.02	0.125
H22	-	0.373	-	-	2.63	-	0.324	-0.13	0.300
H23	0.149	0.382	0.658	4.32	2.32	4.22	0.330	-1.15	0.280
H24	0.386	0.472	-	1.53	1.91	-	0.352	-0.93	0.170
H25	0.122	0.365	0.644	0.98	1.96	3.75	0.333	-1.15	0.140
H26	0.169	0.396	0.475	0.84	1.55	1.91	0.361	-0.86	0.230
H27	0.382	0.465	0.660	2.63	2.96	4.93	0.339	-1.35	0.220

Table 3. Correlation coefficients between TPC, RSA, and Q600.

	TPC	**RSA**	**Q_{600}**
TPC	1		
RSA	0.879	1	
Q_{600}	0.946	0.859	1

352 FIGURE CAPTIONS

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354 **Figure 1**. Geographical regions of Serbia where the 27 honey samples under
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357 **Figure 2**. Cyclic voltammograms of three representative honey samples, H7,
358 H13 and H23, taken from -200 to 800 mV with a scan rate of 100 mV s^{-1}.

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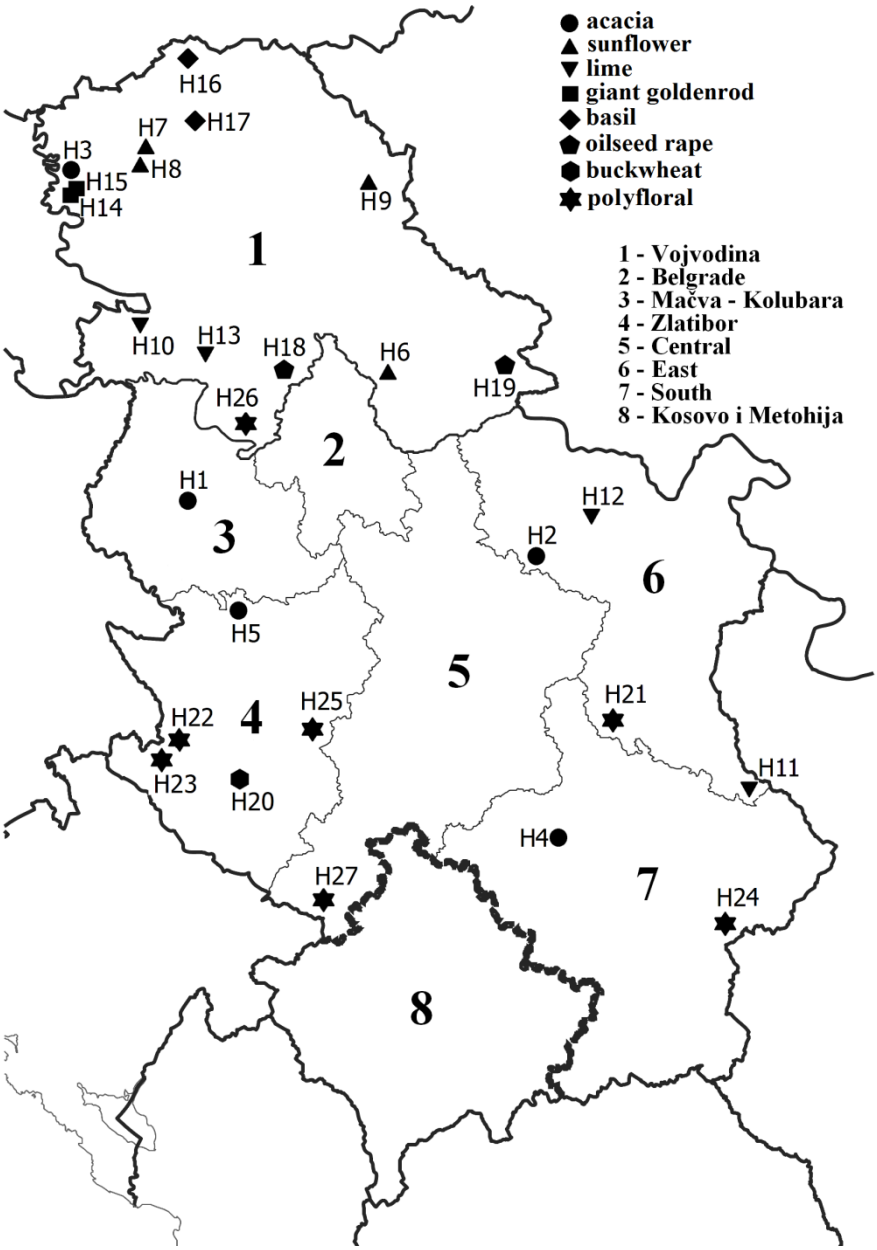
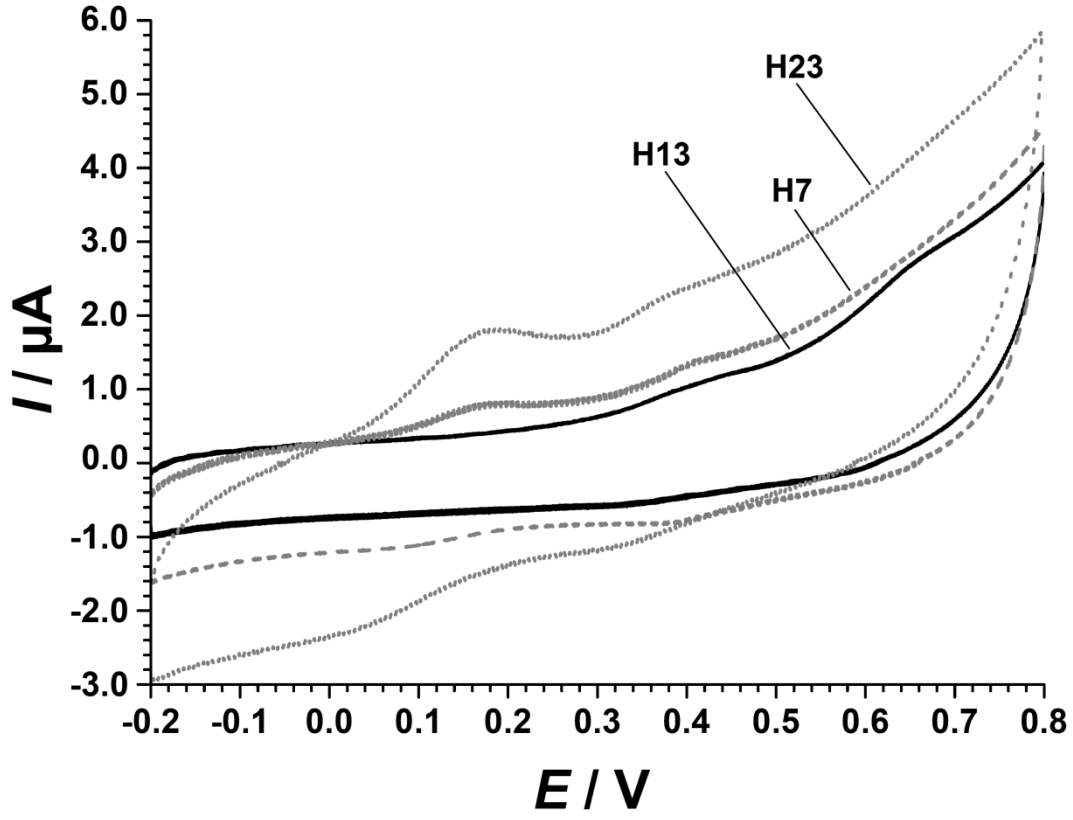


Figure 1. Geographical regions of Serbia where the 27 honey samples under study were collected.



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