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Quality parameters and pattern recognition methods as a tool in tracing the regional origin of multifloral honey

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Abstract: Multifloral honey was characterized in regards mineral to composition, sugar content and basic physicochemical properties. A total of 164 honey samples were collected from different regions of Serbia during the harvesting season 2009. Univariate data analysis (descriptive statistics and analysis of variance), geographic information system and pattern recognition methods (principal component analysis and cluster analysis) were utilized in order to identify the geographical origin of honey. The content of Mg, K, and Cu, electrical conductivity and optical rotation were established as useful indicators in tracing regional differences between honey samples. Samples originating from Zlatibor region were clearly distinguished from those from the rest of Serbia, showing higher K and Mg contents, as well as higher values of optical rotation, electrical conductivity, and free acidity. The influence of the soil composition, and climate conditions, as well as the presence of particular flora on the honey composition is emphasized. The modeling of the geographic origin of honey was attempted by means of linear discriminant analysis.

Keywords: multifloral honey; geographical origin; pattern recognition; Geographic Information System; Serbia.

INTRODUCTION

Honey is a complex mixture because its composition and properties depend not only on the nectar-providing plant species, but also on other factors such as bee species, geographic area, season, mode of storage, and even harvest technology and conditions. The European Council directive 2001/110/EC concerning

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honey allows specific denominations of honey, where the name "honey" can be supplemented by information on the floral, vegetable, regional, territorial or topographical origin. The term "unifloral honey" is used to describe honey in which the major part of the nectar or honeydew is derived from a single plant species. The overwhelming majority of the honeys on the market contain nectar or honeydew from several botanical sources in the foraging area of the beehive and are, therefore, called multifloral or polyfloral honey.

Honey composition is tightly associated to its botanical origin, which is closely related to the geographical area from which the honey originated.[1] The volatile composition is very dependent on the geographical location even for the same plant species, as accumulation of phytochemicals depends on climatic conditions (sunlight and moisture), soil characteristics, and the presence of different minerals arising from soil. This suggests that the chemical composition of the honeys even of the same floral origin may be quite different.[2] Due to the botanical origin given by the particular flora and the ecosystem diversity conditioned by the given territory, honey may have unique characteristics. Indeed, the estimation of honey quality by consumers depends on its organoleptic characteristics, which are strongly dependent on botanical origin of the honey and to some extent on its geographical origin.[3,4]

Considering that honey is a complex natural product, produced by honeybees under relatively uncontrolled conditions, an appropriate characterization of honey samples requires the use of many variables. Thus, multivariate statistical analysis can be applied to find trends or associations among the analyzed data, or to establish a subset of measured parameters allowing honey characterization. Over the last few decades, there have been several reports on the use of multivariate chemometric analysis of the general physicochemical parameters, minerals, sugars, and other constituents, in order to differentiate types of unifloral honeys, honeydew and blossom honeys.[5–7]

Among the parameters tested to assess the provenance of honey, the polyphenolic profile, electric conductivity, acidity, amino acids composition, carbohydrate profile, and pollen proteins were found to be the most useful.[8,9] The mineral composition has also been employed to discriminate honey samples arising from different geographical areas.[10,11] Indeed, due to their stability, minerals seem to be a good criterion for honey classification. The mineral composition of honey is intrinsically connected to the territory of its production and it is closely tied to the flora visited by the bees. In addition, the mineral content of honey is tightly associated to geographical and climatic conditions.[12]

Serbia has very good prerequisites for the development of beekeeping (apiculture), distinguished by heterogeneous relief and climatic conditions and by the existence of various honeybee pastures. Considering the area of wild flora, it would be possible to breed up to 800,000-bee colonies.[13] However, disregarding this possibility, the current utilization of capabilities is only 33.4 %, resulting in annual production of 4000–5000 tons of honey.[14] According to the annual honey production, Serbia would be in the middle of the list of EU member states.[15]

The main goal of this work was to characterize multifloral honeys from a hitherto relatively unexplored geographical area, by using methods easily available to a large number of analytical laboratories dealing in authenticity control of honey in order to establish possible relationships between corresponding chemical variables and the production zone of honey. Thus, the basic physicochemical parameters, minerals, and characteristic mono-, di- and tri-saccharides were evaluated. By the means of several pattern recognition methods, the variables that discriminate honey arising from different regions of Serbia were identified and successful models for further prediction were developed.

EXPERIMENTAL

Sample collection

The total numbers of 164 multifloral honey samples collected from different parts of Serbia (Fig. 1) during the harvesting season 2009, were provided from "The Association of the Beekeeping Organizations of Serbia" (SPOS).[16] The botanical origin of the samples was established by the SPOS based on information provided by the beekeepers and some sensory characteristics of the collected honeys. All samples were in their original packages and were transferred to the laboratory and kept in a refrigerator until analysis.

Physicochemical analysis

Physicochemical parameters (pH, electrical conductivity (*EC*), free acidity (*FA*), optical rotation (*OR*) and moisture) were analyzed using The Harmonized Methods of the International Honey Commission,[17] as described in a previous paper.[13]

Mineral composition

The mineral composition of honey was analyzed by inductively coupled plasm-optical emission spectrometry (ICP-OES). About 0.6–0.7 g of fresh honey was treated with 7 mL of 65 % HNO_3 (Merck, Darmstadt, Germany) and 1 mL of 35 % H_2O_2 (Merck) in polytetrafluoroethylene (PTFE) vessels. A microwave closed digestion system (ETHOS 1, Milestone, Italy) was used for the mineralization process. The final clear solution was made up to 50 mL with bidistilled water. All mineral elements in the digested solutions were determined using an ICP-OES (iCAP 6500 Duo ICP, Thermo Scientific, UK). A multi-element plasma standard solution 4, Specpure, containing 1 g dm^{-3} of each element was analyzed for reference purposes. The results are expressed as mg metal per kg honey (ppm) for the macro elements (K, Mg, Na and Ca) and the micro-elements (Fe, Zn, Cu and Co), or as µg metal per kg honey (ppb) for the trace elements (Cd, Cr and Ni).

Sugar content

The sugar content, *i.e.*, glucose, fructose, sucrose, trehalose, maltose, isomaltose, isomaltotriose, melezitose and turanose with gentiobiose (Gen + Tur), was determined by the means of ion chromatography with amperometric detection. Samples of homogenized honey were weighed (between 0.2 and 0.3 g) and diluted 10,000-fold with ultrapure water (Millipore Simplicity 185 S.A., 67120, Molsheim, France). The solution was filtered (Cronus syringe nylon filter 13 mm, 0.45 µm) and transferred to vials. The honeys were analyzed using an ISC

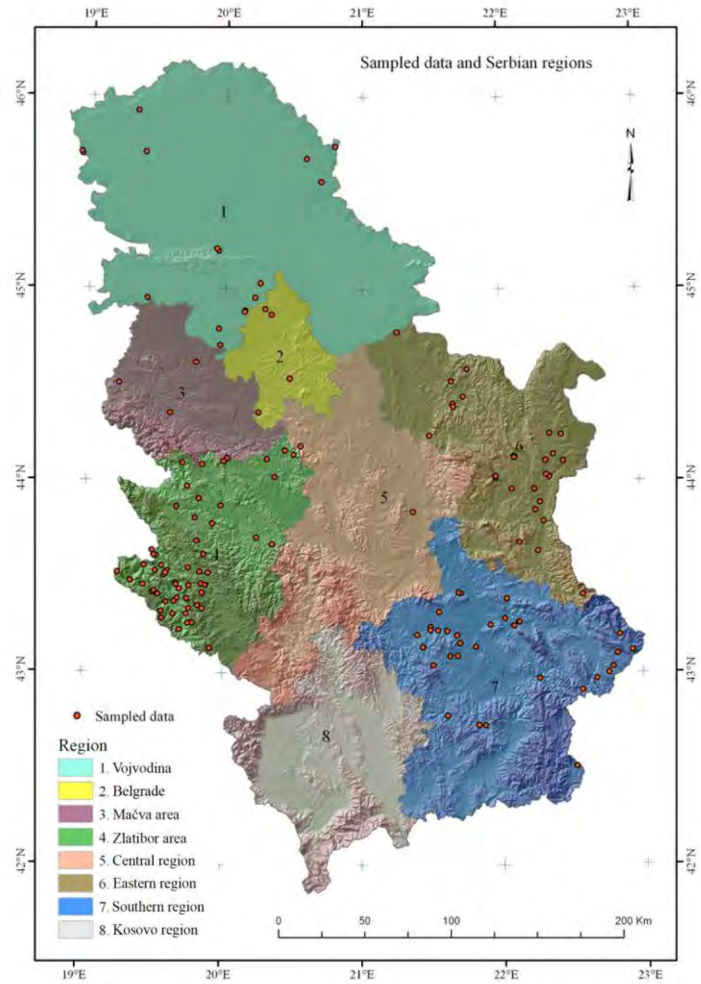

Fig. 1. The regional map of Serbia with marked locations of honey sampling.

3000 DP liquid chromatograph system (Dionex, Sunnyvale, CA, USA) equipped with a quaternary gradient pump (Dionex, Sunnyvale, CA, USA). The carbohydrates were separated on a Carbo Pac® PA10 pellicular anion-exchange column (4×250 mm) (Dionex, Sunnyvale, CA, USA) at 30 °C. Each sample (25 µL) was injected with an ICS AS-DV 50 autosampler (Dionex, Sunnyvale, CA, USA). Sodium hydroxide solution was used as the eluent. Analyses were performed under isocratic mode (52 mM NaOH) with the flow rate set to 1.0 mL min^{-1}. The calibration was performed with standard solutions (D-(+)-Treh, D-(+)-Mel, D-(+)-Tur, D-(–)-Fruc, D-(+)-Sach, D-(+)-Malt, and D-(+)-Glc (Tokyo Chemical Industry, TCI, Europe, Belgium); Gen, iMalt, and iMaltotri (Tokyo Chemical Industry, TCI, Tokyo, Japan)) obtained by the dilution of ten standard sugars in powder form. The evaluation of the sugar content of honey samples was obtained from calibration curves of each sugar contained in the standard solutions. Under these chromatographic conditions, the last sugar was detected after approximately 35 min, and the analysis was ended at 40 min. Results are expressed as percentage of sugar content.

Data analysis

Principal component analysis (PCA) and cluster analysis (CA) were realized by the means of PLS ToolBox, v.6.2.1, for MATLAB 7.12.0.0635 (R2011a). All data were mean centered and scaled to the unit standard deviation prior to any multivariate analysis. For PCA, a singular value decomposition algorithm was employed and in the case of CA, the Ward method for calculating distances was used, since it usually gives clusters of optimal size and form.

Descriptive statistics, Kruskal–Wallis test and linear discriminant analysis (LDA) were performed by means of a demo version of NCSS statistical software (J. Hintze (2001), NCSS and PASS Number Cruncher Statistical Systems, Kaysville, Utah).

Microsoft Office Excel and Access were used to organize the Geographic Information System (GIS) database. Locations of data sampling are vector (point) data. Vector data and the database were integrated, analyzed and printed with GIS software (ArcGIS 9). Geostatistical analyst was used to create a predicted distribution of the attributive data. The Kriging method was used for interpolation.

RESULTS AND DISCUSSION

The results of analysis of physicochemical parameters, mineral and sugar content indicated that some of the honey samples show outlying effects. Regarding the basic physicochemical parameters, these samples met the criteria to be considered as honeydew honeys. According to descriptive sheets for main European unifloral honey,[18] the values of *EC*, pH, *OR* and *FA* for honeydew honeys are in the following ranges, respectively: 0.85–1.63 mS cm^{-1}, 4.4–5.7, $[\alpha]_D^{20}$ 25.2–5, 16.8–37.1 meq kg^{-1}. Several investigators found that honeydew honeys were generally characterized by higher electric conductivity, as well as a higher pH and acidity.[19] According to the Serbian regulations ("Sl. list SCG", No. 45/2003 and "Sl. glasnik RS", No. 43/2013) samples with EC > 1.0 mS cm^{-1}, should be declared as honeydew honey. Consequently, in order to determine the geographical origin of multifloral honey, the 30 samples of honeydew honeys were excluded from the further analysis.

The summarized parameters of descriptive statistics of 134 multifloral honey samples are presented in Table I.

The moisture level had similar values across the regions in the range from 15 to 17 %. In all samples, except of those originating from the Zlatibor region, the pH values agreed with those reported by Arvanitoyannis *et al.*[5] (pH 3.9). The higher pH value measured in the samples from the Zlatibor region (pH 4.31) was in agreement with the values reported for Slovenian multifloral honeys collected from three regions: Alpine, Pannonian and Mediterranean.[8] Furthermore, the electrical conductivity was higher in the honey collected from the Zlatibor area (0.74 mS cm^{-1}) compared to the samples from the rest of Serbia, as well as from the values reported for multifloral honeys in other studies.[5,8,20,21] The free acidity did not show fluctuations among the studied regions and was in agreement with data reported for neighboring countries.[8,20,21] The optical rotation of the samples coming from Serbia was similar to the data reported for Slovenian multifloral honeys, except for the samples collected from the Zlatibor region, which had lower values.

TABLE I. Parameters of descriptive statistics for the investigated honey samples of different geographical origin

Parameter		Vojvodina (17)[a]	Belgrade region (3)	Central Serbia (15)	Eastern Serbia (27)	Southern Serbia (28)	Zlatibor area (38)	Western region (6)[b]
K, mg kg^{-1}	Mean	564.67	609.73	413.26	707.97	547.64	1352.83	597.59
LOQ = 25	*SD*	307.35	201.38	225.85	322.83	253.01	417.77	439.24
µg kg^{-1}	Median	472.40	652.83	336.51	706.14	531.29	1373.05	387.97
	Max.	1211.10	786.07	1058.19	1363.39	1255.74	2559.44	1360.23
	Min.	189.90	390.28	194.48	220.21	164.32	539.69	257.92
Ca, mg kg^{-1}	Mean	84.13	71.04	38.72	57.33	42.99	51.35	72.31
LOQ = 25	*SD*	27.03	32.42	18.17	20.52	14.61	16.20	43.33
µg kg^{-1}	Median	84.60	70.14	35.63	51.58	40.91	50.44	72.67
	Max.	126.84	103.89	85.89	106.40	76.14	100.10	133.13
	Min.	26.99	39.07	16.19	30.53	21.04	17.65	22.08
Mg, mg kg^{-1}	Mean	22.01	15.74	15.62	20.85	17.61	49.69	21.73
LOQ = 10	*SD*	7.69	5.04	8.05	12.50	12.04	19.31	15.68
µg kg^{-1}	Median	21.92	14.70	10.96	17.88	12.69	45.44	16.48
	Max.	44.99	21.22	31.19	66.81	63.01	88.22	46.42
	Min.	7.50	11.30	6.13	8.90	5.94	17.59	5.39
Na, mg kg^{-1}	Mean	19.64	30.00	17.30	18.41	18.36	23.98	16.96
LOQ = 25	*SD*	12.87	23.22	8.30	16.10	12.44	16.15	12.33
µg kg^{-1}	Median	16.76	23.69	16.66	14.80	15.01	20.70	15.55
	Max.	59.12	55.72	30.09	60.85	69.90	91.25	39.21
	Min.	2.59	10.58	1.39	1.74	0.97	7.34	2.90
Zn, mg kg^{-1}	Mean	4.12	0.82	1.65	6.12	2.69	3.71	2.77
LOQ = 2	*SD*	6.38	0.32	1.67	11.84	2.13	6.95	2.37
µg kg^{-1}	Median	1.78	0.76	1.26	2.49	1.83	2.09	1.98
	Max.	27.46	1.16	7.46	61.52	9.54	43.44	7.56
	Min.	0.82	0.53	0.46	0.82	0.71	0.91	1.22
Fe, mg kg^{-1}	Mean	3.49	0.94	1.59	1.97	1.87	2.33	2.40
LOQ = 10	*SD*	5.25	0.17	1.58	1.72	1.41	1.34	1.12
µg kg^{-1}	Median	2.11	0.98	1.18	1.54	1.54	1.96	2.21
	Max.	23.68	1.09	6.78	7.87	8.56	7.26	3.90
	Min.	1.18	0.76	0.38	0.75	0.78	0.79	1.09
Cu, mg kg^{-1}	Mean	0.32	0.27	0.23	0.36	0.27	0.54	0.27
LOQ = 2	*SD*	0.11	0.04	0.09	0.12	0.11	0.18	0.10
µg kg^{-1}	Median	0.28	0.28	0.20	0.34	0.25	0.54	0.24
	Max.	0.56	0.30	0.41	0.69	0.58	1.02	0.44
	Min.	0.18	0.22	0.09	0.18	0.14	0.23	0.17

TABLE I. Continued

Parameter		Vojvodina (17)[a]	Belgrade region (3)	Central Serbia (15)	Eastern Serbia (27)	Southern Serbia (28)	Zlatibor area (38)	Western region (6)[b]
Mn, mg kg^{-1}	Mean	1.51	1.58	2.31	2.05	1.73	4.25	3.97
LOQ = 5	*SD*	2.09	1.49	2.47	1.87	2.16	2.80	5.27
μg kg^{-1}	Median	0.51	1.01	1.21	1.41	0.76	3.64	1.68
	Max.	6.97	3.27	8.49	7.84	8.99	12.15	13.73
	Min.	0.22	0.46	0.20	0.11	0.12	0.78	0.33
Co, mg kg^{-1}	Mean	0.11	0.06	0.02	0.05	0.10	0.07	0.05
LOQ = 2	*SD*	0.16	0.07	0.01	0.05	0.17	0.10	0.07
μg kg^{-1}	Median	0.05	0.02	0.02	0.04	0.04	0.04	0.02
	Max.	0.71	0.15	0.05	0.25	0.83	0.49	0.18
	Min.	0.02	0.02	0.01	0.01	0.01	0.01	0.01
Cr, μg kg^{-1}	Mean	108.16	47.69	32.39	79.95	43.4	39.63	36.09
LOQ = 2	*SD*	105.92	30.34	12.11	91.99	48.9	22.70	49.82
μg kg^{-1}	Median	63.77	45.62	36.33	40.96	34.7	38.38	20.38
	Max.	455.33	79.02	44.24	375.10	222.5	123.22	132.30
	Min.	ND [c]	18.45	ND	ND	ND	ND	ND
Ni, μg kg^{-1}	Mean	71.45	55.95	100.08	77.92	94.3	235.51	60.05
LOQ = 2	*SD*	53.92	28.89	63.71	49.77	100.7	168.28	61.82
μg kg^{-1}	Median	56.14	68.53	81.60	62.18	64.0	173.94	54.30
	Max.	182.94	76.42	230.49	173.82	521.0	956.40	165.57
	Min.	ND	22.90	ND	ND	ND	23.38	ND
Cd, μg kg^{-1}	Mean	4.03	0.59	1.42	2.57	1.9	8.74	2.39
LOQ = 2	*SD*	6.19	0.32	2.45	4.56	2.5	8.90	3.70
μg kg^{-1}	Median	1.35	0.70	0.13	0.35	0.7	6.52	1.15
	Max.	18.55	0.83	8.66	18.07	7.4	42.15	9.70
	Min.	ND	0.23	ND	ND	ND	ND	ND
Moisture, %	Mean	16.79	15.55	16.30	16.46	16.67	17.24	17.80
	SD	1.28	0.25	0.96	1.54	1.24	1.45	1.49
	Median	16.75	15.61	15.99	16.39	16.46	17.12	17.78
	Max.	19.19	15.76	17.67	21.28	20.27	19.93	19.77
	Min.	14.75	15.28	14.72	14.01	14.92	14.01	15.47
EC	Mean	0.37	0.35	0.27	0.40	0.31	0.74	0.35
mS cm^{-1}	*SD*	0.12	0.11	0.12	0.15	0.10	0.14	0.18
	Median	0.34	0.34	0.24	0.35	0.30	0.73	0.28
	Max.	0.61	0.45	0.62	0.68	0.59	0.99	0.63
	Min.	0.19	0.24	0.17	0.18	0.17	0.44	0.18
pH	Mean	3.72	3.87	3.91	3.90	3.88	4.31	3.86
	SD	0.33	0.12	0.31	0.31	0.30	0.25	0.18
	Median	3.76	3.90	4.01	3.91	3.89	4.34	3.96
	Max.	4.22	3.98	4.48	4.58	4.32	4.66	4.00
	Min.	3.07	3.74	3.16	3.45	3.26	3.38	3.58

TABLE I. Continued

Parameter		Vojvodina (17)[a]	Belgrade region (3)	Central Serbia (15)	Eastern Serbia (27)	Southern Serbia (28)	Zlatibor area (38)	Western region (6)[b]
FA	Mean	27.84	25.57	19.33	25.23	23.7	30.88	20.4
meq kg^{-1}	SD	6.66	6.17	7.45	6.89	6.36	6.24	7.41
	Median	31.00	24.50	16.20	23.00	24.0	31.60	18.4
	Max.	37.20	32.20	34.20	38.70	34.2	45.20	30.2
	Min.	11.40	20.00	10.60	15.60	12.8	16.60	13.4
OR / $[\alpha]_D^{20}$	Mean	–14.38	–13.52	–13.48	–12.51	–13.2	–6.50	–14.2
	SD	2.89	1.59	2.52	2.12	2.09	4.84	1.64
	Median	–14.46	–13.04	–13.77	–12.19	–13.0	–6.28	–14.8
	Max.	–10.32	–12.22	–7.67	–8.81	–9.3	6.96	–11.3
	Min.	–20.10	–15.30	–17.22	–17.02	–17.8	–16.83	–15.5
Trehalose	Mean	0.24	0.19	0.14	0.36	0.28	0.30	0.08
%	*SD*	0.31	0.15	0.17	0.32	0.39	0.33	0.07
	Median	0.18	0.24	0.07	0.21	0.13	0.14	0.06
	Max.	1.32	0.32	0.67	1.12	1.76	1.34	0.17
	Min.	0.01	0.02	0.02	0.04	0.03	0.01	0.01
Glucose	Mean	26.41	24.03	24.68	23.29	24.79	24.54	21.52
%	*SD*	3.58	1.99	2.63	2.71	2.80	3.56	2.06
	Median	26.99	23.88	24.92	23.74	24.32	25.06	21.28
	Max.	32.03	26.09	28.37	28.95	29.63	33.56	24.70
	Min.	19.04	22.12	19.25	15.54	19.77	16.97	18.49
Fructose	Mean	36.10	35.89	37.98	34.35	36.81	35.58	31.87
%	*SD*	2.68	3.06	3.57	3.31	2.98	4.54	4.27
	Median	36.82	37.16	37.41	34.17	36.14	35.63	32.22
	Max.	40.71	38.11	43.48	41.11	42.87	44.67	38.09
	Min.	30.81	32.40	32.66	24.64	31.69	24.45	25.08
Sucrose	Mean	3.34	2.19	5.19	4.24	4.48	4.90	3.79
%	*SD*	2.48	1.78	1.36	3.09	3.25	1.36	1.43
	Median	2.46	1.92	5.11	3.10	3.82	5.08	3.90
	Max.	7.53	4.09	8.67	14.36	19.50	7.03	5.71
	Min.	0.12	0.56	2.92	0.31	1.65	1.52	2.18
Isomaltose	Mean	0.64	0.75	0.31	1.01	0.73	0.41	0.37
%	*SD*	0.75	0.65	0.40	0.72	0.58	0.74	0.44
	Median	0.30	1.02	0.07	0.92	0.78	0.16	0.11
	Max.	2.33	1.22	1.06	3.42	1.79	3.64	0.96
	Min.	0.01	ND	ND	0.01	0.03	0.01	0.05
Melezitose	Mean	0.22	0.33	0.31	0.22	0.21	0.36	0.37
%	*SD*	0.13	0.26	0.40	0.06	0.11	0.24	0.44
	Median	0.19	0.19	0.07	0.21	0.19	0.33	0.11
	Max.	0.59	0.63	1.06	0.37	0.58	0.97	0.96
	Min.	0.07	0.16	ND	0.10	0.08	0.01	0.05

TABLE I. Continued

Parameter		Vojvodina (17)[a]	Belgrade region (3)	Central Serbia (15)	Eastern Serbia (27)	Southern Serbia (28)	Zlatibor area (38)	Western region (6)[b]
Gentiobiose plus turanose, %	Mean	0.42	0.47	0.45	0.45	0.34	0.41	0.37
	SD	0.27	0.09	0.36	0.15	0.19	0.17	0.33
	Median	0.44	0.47	0.36	0.42	0.30	0.41	0.25
	Max.	0.91	0.56	1.39	0.77	0.75	0.87	0.97
	Min.	0.02	0.39	0.05	0.13	0.11	0.16	0.06
Isomaltotriose, %	Mean	0.13	0.18	0.13	0.30	0.25	0.18	0.32
	SD	0.13	0.17	0.13	0.16	0.19	0.13	0.09
	Median	0.07	0.19	0.07	0.34	0.24	0.16	0.35
	Max.	0.39	0.35	0.39	0.57	0.87	0.46	0.44
	Min.	ND	ND	ND	ND	0.03	0.01	0.17
Maltose, %	Mean	1.50	1.13	2.11	1.52	1.59	1.35	1.40
	SD	0.90	0.88	0.64	0.72	0.61	0.74	0.90
	Median	1.68	1.60	2.00	1.32	1.61	1.17	1.65
	Max.	3.24	1.68	3.68	3.07	3.45	4.07	2.31
	Min.	0.09	0.12	0.97	0.56	0.59	0.38	0.24

[a]Number of samples; [b]Western region without Zlatibor area; [c]not detected

Twelve minerals were quantified for each honey sample (K, Na, Ca, Mg, Fe, Zn, Mn, Cu, Ni, Cr, Co and Cd). Potassium was the most abundant mineral component, considering all the investigated samples, which agrees with other studies indicating that K is the most common element in honeys.[19,22] The average levels ranged from 400 to 700 mg kg^{-1} for all regions of Serbia, except foe the Zlatibor area, where this parameter reached a value of 1300 mg kg^{-1}. The relatively high contents of potassium reported in the case of Italian (731±397 mg kg^{-1}),[22] Slovenian (1090–1220 mg kg^{-1})[8] and Indian (932.56±0.15 mg kg^{-1})[23] honey did not exceed the values measured in the samples from the Zlatibor region. Calcium and magnesium were the next most common elements, followed by sodium, iron, and zinc. Sodium and magnesium were also present in significant amounts in all the studied samples, but several times lower than the potassium content and 2-time lower than the calcium content. Magnesium was present in higher amount in the samples coming from the Zlatibor mountain area (50 mg kg^{-1}) than in the samples coming from the rest of Serbia (15–22 mg kg^{-1}). The rest of the studied minerals (Zn, Fe, Cu, Mn, Co, Cr, Ni and Cd) were present in minor quantities and some of them could be detected in trace amounts (μg kg^{-1}).

In this study, ten sugars were identified and quantified, which included two monosaccharides, six disaccharides and two trisaccharides (Table I). Similar chromatograms were obtained for all the considered honey samples. The glucose and fructose were clearly the predominant sugars in all samples. A good resolution and separation of all quantified di- and trisaccharides were also observed.

Moreover, the chromatographic method used here was relatively fast (about 30 min) and an approximate sugar profile was obtained.

Fructose was found to be the most abundant sugar component in all samples, followed by glucose. The content of both compounds was in most cases within the limits established by EU legislation (Council Directive 2001/110/EC). Some observed lower values could correspond to mixtures of honeys and honeydew honeys, since many authors found significantly lower glucose and fructose content in honeydew honeys compared with blossom honeys.[22] The ratio of fructose to glucose was between 1.36 and 1.61, indicating that the honey would remain fluid for longer periods. Among the disaccharides, sucrose and maltose were the major components.

Data analysis

According to the D'Agostino *K*-squared, Kolmogorov–Smirnov and the Shapiro–Wilk test for normality of distribution, there was significant deviation from normal distribution for each of the studied variables. The modified Levene equality variance test showed significant heteroscedasticity among the different regions for each variable. Therefore, for statistical evaluation of differences in the results, the Kruskal–Wallis test was employed for each of the variables, taking the appropriate region as a single factor. The results are presented in Table S1 (Supplementary material this paper). In the cases of the observed statistically significant difference between the medians, the Kruskal–Wallis multiple-comparison *Z*-value test was performed. The separated regions are denoted in parentheses (Table S1). Based on the Kruskal–Wallis test, several factors, such as K, Mg, Cu, Ni, and Cd, as well as *EC*, pH and *OR*, were identified as parameters that separate the honey samples from the Zlatibor area from those originating from the rest of Serbia. The other factors are responsible for the differentiation of only limited numbers of regions while the Na, Co, trehalose and gentiobiose plus turanose level do not provide any significant difference among any of the considered regions of Serbia.

A Geographical Information System (GIS) was employed to build the area distribution of each of the studied parameters. A GIS is a system designed to capture, store, manipulate, analyze, manage and present all types of geographical data by merging of cartography, statistical analysis and database technology.[24] The GIS, unlike the other employed statistical methods, provides an estimated geographical distribution of a certain property over the map, therefore being more straightforward and a rather convenient approach. GIS has been introduced in addition to other statistical methods in order to improve the classification performance. The spatial distributions of all parameters are given in Figs. S1–S7 of the Supplementary material.

PCA and CA

Both PCA and CA were performed In order to obtain a basic insight into the data structure, detect possible outliers and identify similarities and specific grouping patterns among the studied samples. PCA resulted in a three-component model that explains 41.35 % of total variance. The first principal component, PC1, accounted for 24.12 % of the overall data variance, the second principal component, PC2, for 9.26 % and the third principal component for 7.97 %. Mutual projections of factor scores and their loadings for the first two PCs are presented in Fig. 2a and b, respectively. Score plot reveals two distinct groups of

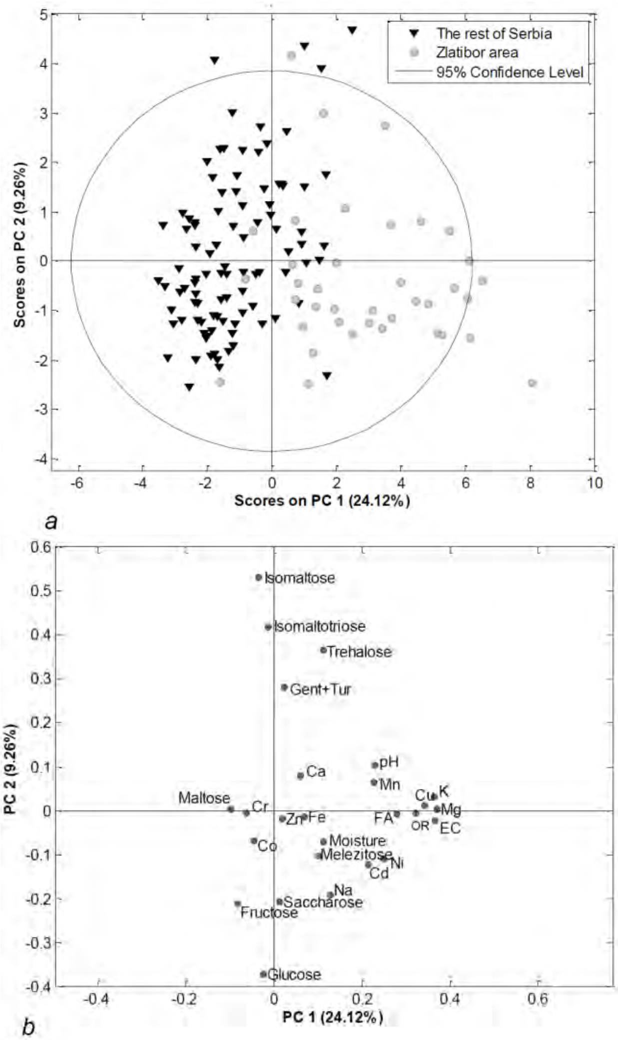

Fig. 2. Principal component analysis: a) score plot, b) loading plot.

samples, separated along the PC1 axis. The majority of the samples originating from Zlatibor region are grouped in the right side of the graph, while those collected from the rest of Serbia form a compact cluster mostly on the opposite side of the PC1 axis. There was no meaningful separation along the PC2 direction. The loading plot implies that the Mg, K, and Cu contents, as well as FA, *OR* and *EC*, are parameters that have the most positive impact on the PC1 direction. The isomaltose, isomaltotriose and glucose contents significantly affect the PC2 component.

Several samples exceeded the limits imposed by the Hotteling T^2 95 % probability ellipse, hence they could be considered as outliers. Among the samples coming from the rest of the Serbian regions, those that could be considered as outliers have higher levels of trehalose and isomaltose. The contents of K, Mg, Cu, and Cd, and the values for *EC* and pH were similar to those obtained for the samples from the Zlatibor region. The three samples from the Zlatibor region that exhibited an outlying effect had very high contents of K, Ca, Mg and Na.

Cluster analysis revealed five clusters at 17 variance weighted distance units (Fig. 3). One cluster consisted entirely of honey samples from the Zlatibor region, while the others accounted for all the samples from the rest of Serbia, as well as a small number of samples from the Zlatibor region. However, any further subclustering according to the proposed regional division of Serbia could not be evidenced.

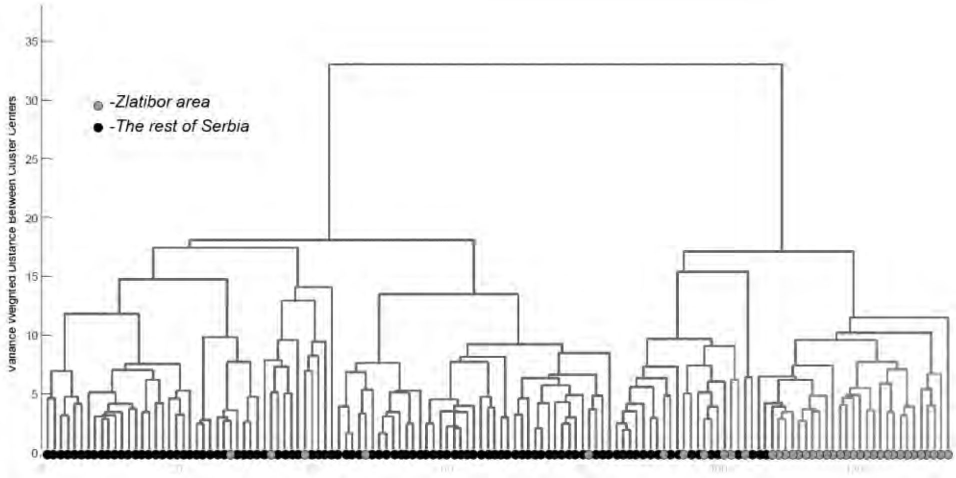

Fig. 3. Dendrogram obtained by the means of hierarchical cluster analysis.

All the employed statistical procedures (Kruskal–Wallis, PCA and CA) as well as GIS (Figs. 4 and S1–S7) confirmed a unique set of parameters (Mg, K, Cu, *EC* and *OR*) that differentiated the samples originating from the Zlatibor area from the rest of the samples collected from Serbia. Different factors could influence a separation among the mentioned areas, such as climate, soil composition, or presence of regional endemic flora, which strongly influences the mineral content in honeys.[10]

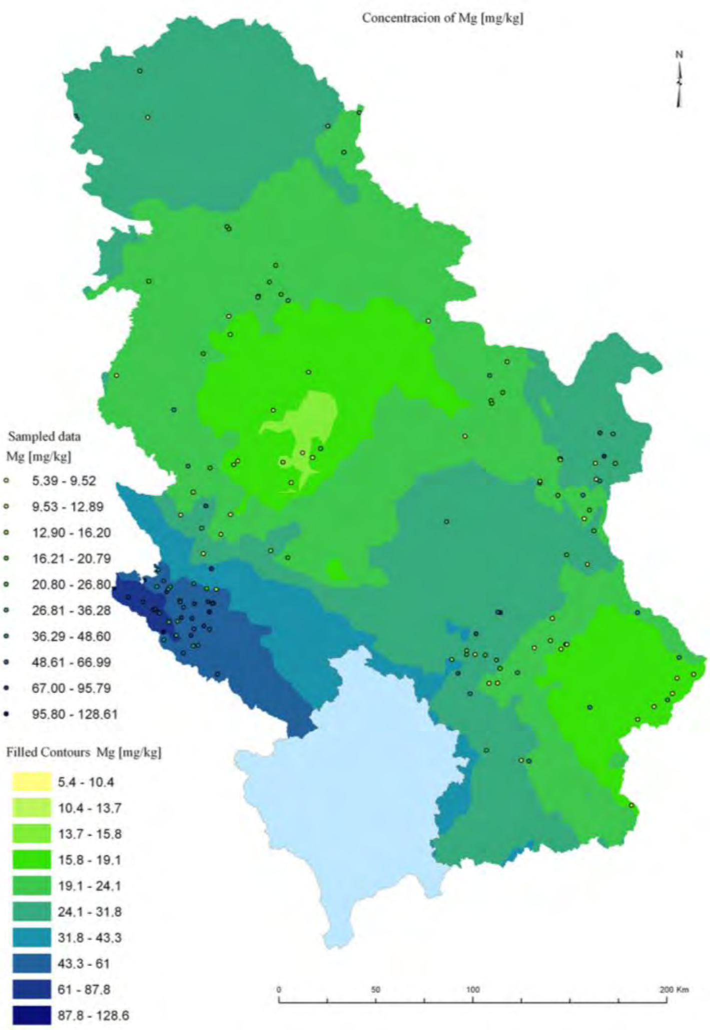

Fig. 4. GIS spatial distribution of Mg content in multifloral honey.

The main soil type of the northern part of Serbia (Vojvodina, the Belgrade region and the Western region, excluding the Zlatibor area) is chernozem, which is characterized by the presence of $CaCO_3$ and high quality calcium mull humus.[25] The two-times higher calcium levels measured in the honey samples obtained from Vojvodina (84.13±27.03 mg kg^{-1}), Belgrade (71.04±32.42 mg kg^{-1}) and the Western region (72.31±43.33 mg kg^{-1}) are in accordance with the soil composition.

The Zlatibor region belongs to the largest serpentine block in the Balkans, which belongs to a group of siliceous rocks that are characterized by calcium deficiency and high concentrations of aluminum, iron, magnesium, nickel, cobalt and chromium and a few plant nutrients.[26] Probably the high amounts of Mg and Ni found in serpentine soils could be responsible for higher amounts of these elements found in honeys from the Zlatibor region.[25,27]

The regional endemics characteristic for the Zlatibor region are *Knautia pancicii*, *Verbascum bosnense*, *Thymus adamovicii* and *Potentilla mollis*. All mentioned plant species are blossom plants and could significantly contribute to distinguishing multifloral honeys originating from the Zlatibor region compared to the rest of Serbia.[26] Unlike other mountain regions of Serbia, Zlatibor area is rich in various coniferous species and other evergreen plants, of which the Serbian spruce (*Picea omorika*) stands out as an endemic one. They represent a reach source of honeydew, together with silver fir (*Abies alba*), European spruce (*P. abies*), Aleppo pine (*Pinus halepensis*) and European larch (*Larix decidua*).

Linear discriminant modeling

LDA was performed in order to establish the best possible mathematical models that would be able to sort unknown samples according to their geographic origin. The linear discriminant analysis was performed on a training set comprised of two types of samples: those from Zlatibor region and the others from the rest of Serbia. The model resulted in one canonical function, with *EC*, and K and Mg levels as the most significant variables that differentiate the two types of samples. Values of standardized canonical coefficients were, respectively: 1.681, –0.750, and 0.680. Two out of 38 (5.26 %) samples originating from Zlatibor region were misclassified, while all samples from the rest of Serbia were properly classified (Tables SII.I–SII.III of the Supplementary material).

In addition, discrimination among the entire set of the studied regions was attempted. The LDA model resulted in six statistically significant canonical functions. The score plot of the first two canonical functions and the first *vs*. the third (Figs. 5a and b) revealed that the samples from the Zlatibor area were completely separated from the other regions. Samples collected from Vojvodina were sufficiently separated from the other regions, with the exception of the samples from the East, which exhibited moderate overlapping. The other regions form well-shaped groups that are mutually overlapped to a certain degree. The factors that discriminate the Zlatibor region from the rest of Serbia are amounts of magnesium (in a positive manner), potassium, and calcium (in a negative manner), and electrical conductivity, with standardized canonical scores of respectively: –0.527,

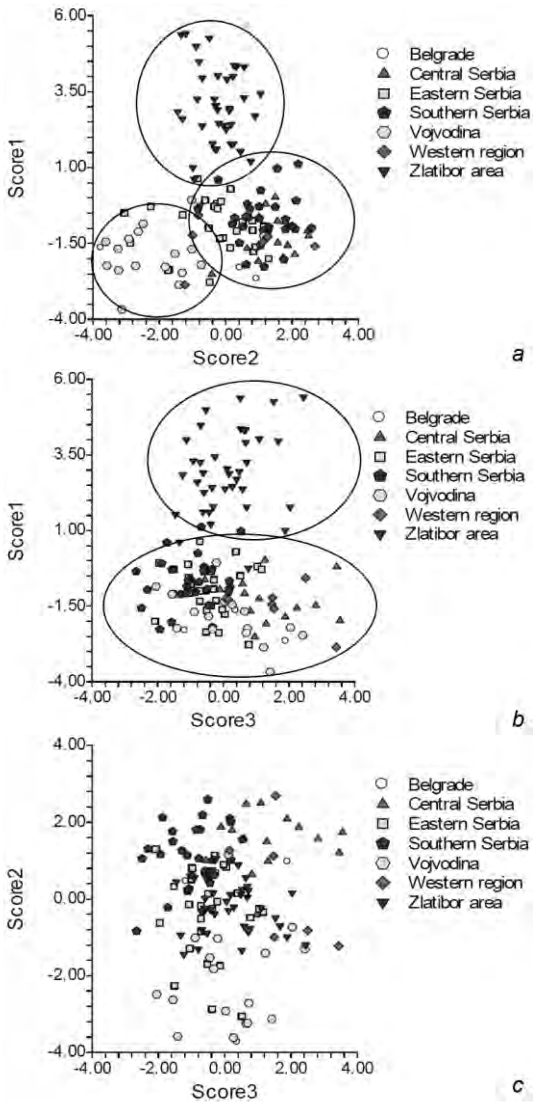

Fig. 5. Linear discriminant analysis, canonical score plots: a) score1/score2, b) score1/score3 and c) score2/score3.

–0.693, 0.643 and 1.602. In addition to the mentioned parameters, Vojvodina was separated from the other regions by the potassium and calcium contents. For these parameters, values of standardized canonical coefficients were respectively: 0.813 and –0.866. A total 36 (94.73 %) of 38 samples from Zlatibor are correctly assigned, 12 (70.58 %) of 17 samples from Vojvodina were also correctly assigned, with two samples being misclassified as Belgrade region, and three assigned to Eastern, Southern and Western region. The rest of the studied samples were misclassified with low to moderate rates (Tables SIII.I–SIII.III of the Supplementary material). This implies that obtained LDA models have quite good predictive power, especially when the number of studied regions is considered.

CONCLUSIONS

The results of this comprehensive study, based on a vast number of genuine honey samples, represent the first attempt to classify multifloral honey over a relatively small area, *i.e.*, the territory of Serbia. Characterization of the studied samples was performed regarding the basic physicochemical properties, mineral composition and sugar content. Univariate (descriptive statistics and analysis of variance) and multivariate (principal component analysis, cluster analysis and linear discriminant analysis) data analyses were performed in order to identify the geographical origin of the honeys.

The following parameters: Mg, K, Cu, electrical conductivity and optical rotation were selected as useful indicators in tracing regional differences between the honey samples. Samples obtained from the Zlatibor region were clearly distinguished from the rest of Serbia, showing higher values of potassium and magnesium contents, as well as higher values of electrical conductivity, pH and free acidity. The influence of the soil composition, and climate conditions, as well as the presence of particular flora on the honey composition was highlighted. Taking into account that there are a small number of articles dealing with the determination of geographical origin of bee products, the presented study provides useful data that could be compared with similar results obtained in the other regions.

SUPPLEMENTARY MATERIAL

Tables S-I–SIII and Figs. S1–S7 are available electronically at http://www.shd.org.rs/ /JSCS/, or from the corresponding author on request.

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И З В О Д

ОДРЕЂИВАЊЕ ГЕОГРАФСКОГ ПОРЕКЛА ПОЛИФЛОРАЛНИХ МЕДОВА ПОМОЋУ ПАРАМЕТАРА КВАЛИТЕТА И МЕТОДА ПРЕПОЗНАВАЊА ОБРАЗАЦА

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Карактеризација полифлоралних медова у погледу минералног састава, садржаја шећера и основних физичко-хемијских параметара извршена је на основу анализе 164 узорка из различитих региона Србије сакупљених током 2009. године. Униваријантна анализа података (дескриптивна статистика и анализа варијансе), заједно са географским информационим системом и мултиваријантном хемометријском анализом (анализа главних компонената и кластерска анализа) примењене су у циљу одређивања географског порекла меда. Утврђено је да су садржај Mg, K и Cu, електрична проводљивост и оптичка ротација параметри који указују на разлике у регионалном пореклу меда. Узорци који потичу из златиборског региона су се јасно раздвојили од узорака из осталих делова Србије на основу већег садржаја K и Mg, као и већих вредности оптичке ротације, електричне проводљивости и слободне киселости. Указано је на утицај састава земљишта, климатских услова и постојања ендемичних врста на састав меда. Моделовање географског порекла меда је урађено применом линеарне дискриминантне анализе.

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