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Geographical origin authentication of honey produced in the region of Rtanj Mountain (Serbia)

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ABSTRACT

This paper represents the melissopalynological analysis, physicochemical characteristics (moisture, pH, electrical conductivity, free acidity, glucose, fructose, hydroxymethylfurfural (HMF), and colour parameters), polyphenol profile, and sensory properties of Rtanj honey samples (76) harvested during 2019–2021 from different locations in the Rtanj Mountain region (eastern Serbia) with the aim to 1) characterise this meadow honey and 2) obtain markers for its differentiating within the geographical origin protection. The melissopalynological analysis of Rtanj honey revealed many types of pollen, of which the most important belong to the Rosaceae family, especially the *Sorbus* group. A relatively low value of the colour parameter L^* (22.7 ± 0.19–59.8 ± 1.75), the positive a^* value of 1.55 ± 0.07–22.3 ± 1.36, and a high content of K (996 ± 376 mg/kg) can be used as markers for its geographical origin authentication, which was confirmed using principal component analysis (PCA). The phenolic profile was evaluated based on the quantification of epicatechin (0.077 ± 0.005 mg/kg), *p*-coumaric acid (0.316 ± 0.004 mg/kg), ferulic acid (0.524 ± 0.002 mg/kg), naringenin (3.386 ± 0.011 mg/kg), luteolin (0.022 ± 0.006 mg/kg), kaempferol (0.628 ± 0.010 mg/kg), and apigenin (0.120 ± 0.002 mg/kg) using high performance liquid chromatography. The colour of Rtanj honey samples varied from extra white to light amber.

1. Introduction

Honey is a supersaturated sugar solution produced by honeybees. It is a globally consumed food known for its beneficial health effects (da Silva, 2016). Increasing interest in honey consumption results from an orientation towards a healthy lifestyle. Its price is strictly related to its botanical and geographical origin, which has to be stated on the label.

The Republic of Serbia is characterised by a long tradition in beekeeping, with a large annual honey production of about 7.000 t and a high average annual export growth rate. These facts point to the necessity of continuous quality control of honey produced in Serbia, with a focus on special honey types with protected botanical and geographical origins (linden honey from Fruška Gora, honey from Homolje, Đerdap honey, and Kačer honey). Honey from the region of Rtanj Mountain (Rtanj honey) became the product with a protected geographical indication certificate obtained at the national level.

The geographical area where Rtanj honey is produced is located in eastern Serbia in the region of Rtanj Mountain (Fig. 1), which is of exceptional geological, geomorphological, biological, historical, and aesthetic value and has been declared a Special Nature Reserve (Zlatković et al., 2014). From the aspect of beekeeping and honey production, it is important to emphasise the meadow phytocenoses of great floristic diversity, with the presence of endemic and relict species, which represent one of the most characteristic centres of diversity in Eastern Serbia. Also, Rtanj is the habitat of species that are considered protected in the flora of Serbia.

Beekeepers for the production of Rtanj honey traditionally use the Carniolan honey bee (*Apis mellifera carnica*), which has shown the best results in the ecological conditions of the defined geographical area. Rtanj honey is polyfloral honey obtained by stationary or migratory

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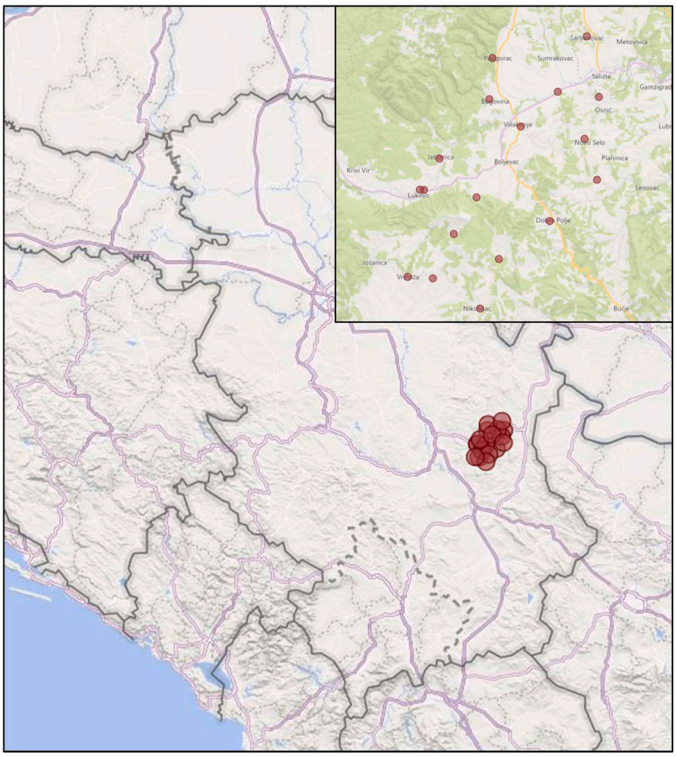

Fig. 1. Map of Serbia indicating the geographic region of Rtanj honey samples.

beekeeping methods in a defined geographical area of production by centrifugation or spinning covered frames with mature honey.

Many studies have proposed different chemical markers to determine the region of honey's origin. Pollen types, chemical composition, mineral content, polyphenol profile, and sensory characteristics of honey are strongly influenced by its geographical origin. Melissopalynological analysis is used to determine the botanical origin of honey, but it also serves as a tool to assess the geographical origin because some pollen types are characteristic of a particular region (Von der Ohe et al., 2004). In the differentiation of honey types, physicochemical parameters are useful indicators (Lazarević et al., 2012), although they are primarily important in honey quality assessment (Bogdanov, 2009; Sakač et al., 2019).

The mineral profile of honey depends on the botanical origin of nectar and pollen, but the soil is also responsible for variations in the content of minerals (Alvarez-Suarez et al., 2010). Therefore, the mineral profile can be used to obtain the fingerprint of the honey's geographical origin.

Polyphenols play a crucial role in determining the effects of the environment on honey characterization since these compounds vary according to the climate and other environmental conditions of the production region (Lo Dico et al., 2019). Silva et al. (2022) stated that an association between physicochemical parameters, phenolic and mineral composition, and the geographical production region is viable.

Sensory properties of honey strongly depend on the plants from which honeybees collected nectar and their geographical origin, because climate and soil determine the arrangement of the chemical components in plants and consequently their content in honey (da Silva et al., 2016; Rodríguez-Flores et al., 2021). Honey odour and flavour support authenticity since they depend on the type of plants visited by the honeybees, processing, and storage conditions (Castro-Vázquez et al., 2009). Honey colour correlates with mineral and phenolic content (Bertoncelj et al., 2007), while sweetness results from simple sugars (glucose, fructose, and sucrose) and depends on the ratio of fructose, glucose, and sucrose in honey, since fructose is 1.2–1.8 times sweeter than sucrose (Cabrera and Santander, 2022).

For all the reasons mentioned above, the objective of this study was to 1) characterise for the first time the uniqueness of Rtanj honey with its protected geographical origin; and 2) obtain a prerequisite for differentiating this product from other meadow honey samples from the regions of Vojvodina and Serbia. This study is the first to present one of the types of honey with the protected geographical indication certificate from Serbia on the scientific stage. The characterisation of Rtanj honey samples harvested in different years (2019–2021) was done in terms of obtaining pollen characteristics, physicochemical parameters (moisture, pH, electrical conductivity, free acidity, glucose, fructose, hydroxymethylfurfural, colour parameters (L^*, a^*, b^*), and minerals), phenolic, and sensory profiles.

2. Material and methods

2.1. Honey samples

The geographical area where Rtanj honey is produced is located in eastern Serbia (1.353 km^2) and includes the areas of two municipalities, Sokobanja and Boljevac (Fig. 1), from which honey samples were collected from the locations marked in Fig. 1.

The harvesting period was 2019–2021. Seventy-six samples were collected from beekeepers who declared their geographical origin. Twenty-five samples were collected in both 2019 and 2020, and twenty-six in 2021. The samples were analysed in the year in which they were produced. They were packed in glass vessels, closed hermetically with the metal lid, and stored at room temperature (22 ± 1 °C) in a dark place until analysis.

2.2. Melissopalynological analysis

Pollen suspended in honey samples was extracted for analysis following the Harmonized Methods of Melissopalynology (Von der Ohe et al., 2004). Qualitative pollen analysis was performed using an Olympus BX51 light microscope at 400 × magnification until a minimum of 500 pollen grains were counted and identified. The identification was done using referent slides and atlases (Beug, 2021; Bucher et al., 2004; Moore and Webb, 1978). Pollen identification was carried out to the lowest possible level: plant species, genus, or family, i.e., type, group, or class of pollen (Beug, 2021; Moore and Webb, 1978). The resulting frequency of recorded pollen grains is expressed as percentages.

2.3. Physicochemical parameters

The physicochemical parameters of honey samples (moisture, pH, electrical conductivity, and free acidity) were determined according to the methods of the AOAC (2000) and Bogdanov (2009).

2.4. Glucose, fructose and sucrose analysis

For determination of sugar composition (glucose, fructose and sucrose) honey sample was dissolved in a 3-fold higher volume of demineralized water, and an aliquot was diluted with a 2-fold higher volume of acetonitrile and kept at −18 °C until analysis. Prior to HPLC analysis, prepared samples were filtered through 0.45 μm pore size PTFE filters (Rotilabo-Spritzenfilter 13 mm, Roth, Karlsruhe, Germany) before injection into the HPLC system. A high-performance liquid chromatography method (obtained with Agilent, Zorbax Carbohydrate 4.6 × 250 mm, 5 μm column, Agilent Technologies Inc., USA) was applied to quantify sugars (glucose, fructose, and sucrose) in honey samples.

HPLC analysis was performed using a liquid chromatograph (Agilent 1200 series), equipped with an evaporative light scattering detector (ELSD), on an Agilent, Zorbax Carbohydrate 4.6 × 250 mm, 5 μm column (Agilent Technologies, USA) with acetonitrile and water (75:25, v/v) as a mobile phase. The flow-rate was 1.100 mL/min. The total run

time was 12 min. The column temperature was 30 °C, and 10 μl of the sample was injected using autosampler.

Identification of glucose, fructose, and sucrose was achieved by comparing their retention time values with those of standards – D-(+)-glucose monohydrate (≥ 99.5%, Sigma-Aldrich), D-(-)-fructose (≥ 99.5%, Sigma-Aldrich), and D(+)-saccharose (≥ 99.7%, CarlRoth). The external standard method was a technique used for quantification.

2.5. Hydroxymethylfurfural (HMF) analysis

The extraction procedure was performed according to the method proposed by Rufián-Henares and De La Cueva (2008), with modifications made by Petisca et al. (2014). Ten grams of sample were suspended in 5 mL of water:methanol (70:30). The mixture was stirred for 1 minute, and then 2.0 mL of Carrez I and Carrez II solutions (Carl Roth GmbH, Germany) were added and centrifuged at 5000 rpm (4 °C) for 15 minutes, recovering the supernatant to a 15-mL flask. Two more consecutive extractions were made with 2 mL of water:methanol (70:30) until collecting 10 mL of supernatant. Two millilitres of this solution were centrifuged at 8000 rpm for 15 minutes before being analysed.

The HPLC method described by Ariffin et al. (2014) and Tomasini et al. (2012) was used to quantify HMF in honey. HPLC analysis was performed using a liquid chromatograph (Agilent 1200 series, Agilent Technologies Santa Clara, CA, USA) with a DAD detector and an Eclipse XDB-C18, 1.8 μm, 4.6 × 50 mm column (Agilent). The column temperature was 30 °C. The injection volume was 2 μL. The mobile phase consisted of two eluents, H_2O (0.1% HCOOH) (A) and methanol (B). The flow rate was 0.75 mL/min. The isocratic elution was applied with the ratio A:B (90:10, v/v). The total run time was 5 min.

2.6. Mineral analysis

The mineral content in honey was determined by AAS after two-phase dry ashing. Around 5 g of honey was weighted in a ceramic crucible and put on a hot plate. The temperature of the hot plate was gradually increased, and when no fumes were observed, the crucible was transferred into a muffle furnace, which was preheated at 550 °C. After 4 h of ashing, the crucible was removed from the furnace and left to cool. In the next phase, 1:1 HNO_3 (5 mL) was added to the crucible. Thereafter, it was put on the hot plate (120–150 °C) in order to evaporate HNO_3 and transferred into the muffle furnace (550 °C) for 4 h. If no clean grey-whitish ash was obtained, the addition of HNO_3 and heating were repeated. After final cooling, the ash was dissolved following the procedure: after careful addition of 1:1 HCl (10 mL), the crucible was heated at 100–110 °C until 5 mL was evaporated. The remaining 5 mL was transferred into a 50-mL flask, which contained the appropriate amount of Cs and La, and water was added to the mark.

The content of K, Na, Ca, Mg, Fe, Cu, and Zn was measured using an atomic absorption spectrophotometer (Varian SPECTRAA-10), with all parameters (wavelength, slit, flame stoichiometry) set by the manufacturer's recommendation. Thousand ppm standards were purchased from AccuStandard (USA), and all calibration curves used were in the linear range (R = 0.999).

2.7. Colour

The colour of honey was measured according to the CIELAB method using a Minolta Chromameter (Model CR-400, Minolta Co., Osaka, Japan). The samples were placed in 20-mm-thick holders and measured against a white background. The weight of the honey sample was 20 g, and the measurement was done at room temperature (23 ± 1 °C).

The CIE $L^*a^*b^*$ coordinates were measured, where L^* is the luminance component and a^* and b^* are colour coordinates related, respectively, to the red/green and yellow/blue spectral ranges.

Colour measurements were taken for each sample in five replications.

2.8. Polyphenol profile analysis

The extraction of polyphenols from honey samples was done according to the method described in the paper of Nyarko et al. (2023). A honey sample (7.5 g) was added to 15 mL of acidified water (pH = 2) and vortexed until the honey was completely dissolved. Honey solution was centrifuged (5804 R, Eppendorf, Hamburg, Germany) at 5000 rpm for 10 min to remove comb, propolis, and particulate matter. The solid-phase extraction (SPE) clean-up step was carried out to remove excess sugars and other interfering matrix components. SPE was performed using a SampliQ C18 ODS (500 mg/6 mL) column. After conditioning with 5 mL of methanol and water, 15 mL of honey solution was passed through the C18 column. Excessive sugars were thoroughly washed with 7.5 mL of water, followed by the subsequent elution of phenolic compounds with 7.5 mL of methanol solution (80% v/v). The resulting extract was dried under a stream of nitrogen at room temperature, and the extract was redissolved in 80% methanol. Finally, the extract was filtrated using a RC syringe filter (0.45 μm) prior to injection into the HPLC system.

The HPLC method described by Maravić et al. (2022) was used to quantify individual polyphenols. HPLC analysis was performed using a liquid chromatograph (Agilent 1200 series, Agilent, Santa Clara, CA, USA) and DAD detector and a Zorbax Eclipse XDB-C18, 1.8 μm, 4.6 × 50 mm column (Agilent, Santa Clara, CA, USA). The column thermostat was set at 30 °C. The injection volume was 5 μL. Separation of polyphenols was achieved with methanol (A) and 1% (v/v) formic acid in water (B) as mobile phases. The flow rate was 1 mL/min. A solvent gradient was 10% A at start; 0–10 min, 10–25% A; 10–20 min, 25–60% A; 20–30 min, 60–70% A. Prior to automatic injection into the HPLC system, the samples were properly diluted in a mixture of mobile phases (A:B, 10:90%, v/v) and filtered through a syringe filter (RC; 0.45 μm). The scanning wavelength was set at 280 nm. Quantification of individual polyphenols was based on external standard calibration with epicatechin, *p*-coumaric acid, ferulic acid, naringenin, luteolin, kaempferol, and apigenin, scanned at 280, 280, 330, 280, 350, 350, and 350 nm, respectively. The results were expressed as mg of compound per kg of honey.

2.9. Sensory profile analysis

Sensory profile analysis was performed with ten sensory panellists (6 women and 4 men, 25–56 years of age) selected, trained, and monitored according to the ISO guidelines (ISO, 8586:2012) and with previous experience in descriptive sensory analysis. During three training sessions (1.5 hours), panellists worked on sensory lexicon development. They were provided with different descriptors found in the literature that were used for sensory profiling of different polyfloral honeys and asked to select those that could be used for Rtanj honey sample differentiation. In the first session, panellists were asked to qualitatively evaluate 15 randomly selected Rtanj honey samples and generate a list of descriptive terms. The panel discussed and refined descriptors and validated the lexicon in order to ensure that the terms and intensity scale effectively differentiated the sensory properties and differences among samples. Each descriptor included in this lexicon was identified in at least one of the honey samples initially assessed. The colour of honey samples was measured visually using the Pfund Honey Color Guide (developed by the US Davis Honey and Pollination Center at the Robert Mondavi Institute) and is expressed as the relative lightness/darkness of amber on a scale in millimetres. Fifteen descriptors related to the honey odour (overall odour, dried herbs, fruity, fresh flower), taste (sweet, sour, bitter), flavour (overall flavour, caramel, fruity, stability), texture (viscosity, pungency, and for those samples that were crystallised, crystal sharpness), and mouthfeel (burning sensations) perceptions were chosen and thoroughly defined for sensory profiling. The intensity of

every sensory descriptor was evaluated on a linear 100-mm scale with end anchors (the left end side indicates minimal perceptible intensity, and the right side indicates maximally perceptible intensity of the descriptor). Every panellist received 30 g of the sample delivered in a 150-mL glass covered with a lid (to prevent loss of valuable volatiles) and labelled with a three-digit random number. A few slices of apple and tap water were used for palate cleansing. Samples were evaluated in duplicate, six samples per session. The evaluation was performed in a controlled sensory laboratory (ISO, 8589:2007). The study was approved by the Ethics Committee of the Institute of Food Technology in Novi Sad, University of Novi Sad, Serbia (Ref. No.).175/I/26–3).

2.10. Statistical analysis

The data were processed statistically using the software packages STATISTICA 10.0 (StatSoft Inc., Tulsa, OK, USA) and XLSTAT 2022.1 (Addinsoft, New York, NY, USA). Results were presented as the mean ± standard deviation of the triplicate analyses for all measurements, except for the colour determination of the samples, which was performed in 5 repetitions (Supplementary table 2a-c). Equations for calibration curves for quantified compounds were presented in Supplementary table 1. A principal component analysis (PCA) was performed on experimental data to determine and visualise the differences between Rtanj honey samples and previously investigated meadow honey samples from the regions of Vojvodina and Serbia (Marić et al., 2021; Sakač et al., 2019). Since the parameters' scales differ greatly, the correlation matrix-based PCA approach was used in the analysis. PCA includes three variables (K, L^*, and a^*), which were shown to be important in preliminary computations (Supplementary table 3). PCA employed a matrix of three variables and thirty samples. Also, confidence ellipses with a 95% confidence interval are included for each set of honey samples in the PCA score plot.

3. Results and discussion

3.1. Melissopalynological analysis of Rtanj honey

The melisopalynological analysis of honey from the Rtanj Mountain area revealed many types of pollen in a total of 76 samples of Rtanj honey, of which the most important belong to the Rosaceae family, especially the *Sorbus* group (Table 1). The diversity of pollen types indicates the floristic richness of the area from which honey samples were collected.

Melissopalynological indicators enabled the characterization of Rtanj honey according to its geographical and botanical origin. Based on the presence and frequency of pollen types recognisable for the Balkan Peninsula flora – *Tilia* genus (linden), *Cornus sanguinea* (red dogwood), *Fraxinus ornus* (manna ash), and *Rhus* pollen group (*Cotinus coggygria* and *Ailanthus altissima*), Rtanj honey was classified as a typical honey of the Balkan region. However, based on the established composition and share of identified types of pollen in Rtanj honey, it cannot be claimed that this type of honey was strictly produced in the Rtanj Mountain region.

Regarding its botanical origin, this honey belongs to the group of polyfloral honeys since it contains different types of pollen from many plant species, shrubs, and trees in the meadow marginal areas and/or

Table 1
Pollen of plant families in Rtanj honey.

Plant family	Pollen frequency (%)						
	Min	Max	> 45	16–45	4–15	< 3	% of honey samples in which pollen was registered
Rosaceae	4.00	33.8	0	69	31	0	100
Rosaceae – *Sorbus* group (*Prunus, Malus, Pyrus, Crataegus, Sorbus*)	1.69	28.3	0	38	31	19	88
Fabaceae	4.84	23.6	0	31	69	0	100
Fabaceae (exclude *Robinia pseudoacacia*, *Gleditsia triacanthos* and *Amorpha fruticosa)*	2.25	22.7	0	19	75	6	100
Tiliaceae	0.35	35.2	0	13	25	50	88
Asteraceae	2.47	18.0	0	6	69	25	100
Ranunculaceae	0.17	16.0	0	6	69	25	100
Lamiaceae	1.38	29.6	0	6	69	25	100
Apiaceae	0.19	40.8	0	6	31	63	100
Boraginaceae	0.49	8.15	0	0	38	56	94
Plantaginaceae	0.34	7.54	0	0	31	69	100
Salicaceae	0.17	15.0	0	0	25	75	100
Poaceae	0.31	4.60	0	0	19	81	100
Crassulaceae	1.23	10.9	0	0	19	19	38
Hypericaceae	0.34	12.3	0	0	6	81	88
Cornaceae	0.17	4.00	0	0	6	75	81
Anacardiaceae	0.17	11.5	0	0	6	69	75
Brassicaceae	0.14	6.76	0	0	6	69	75

Min – minimum value; Max – mximum value.

from extensive types of orchards. The dominant pollen type in all honey samples (22.3%) belonged to the rose family (Rosaceae all.), specifically the *Sorbus* pollen group (*Prunus*, *Pyrus*, *Malus*, *Crataegus*, *Sorbus*) (Table 1). Plants from the *Sorbus* group are recognised as the most frequently present medicinal plants in the region of Rtanj Mountain (Zlatković et al., 2014). Their pollen was detected in every analysed honey sample with large variations in the proportion of pollen (Table 1), which indicates the compatibility of beekeeping practices with the environment.

A notable characteristic of Rtanj honey is the presence of pollen from herbaceous vegetation in both arid and moist meadows, where species from the family Fabaceae dominate (Table 1). By excluding woody species (*Robinia pseudoacacia L.*, *Gleditsia triacanthos L.*, and *Amorpha fruticose L.*), the remaining herbaceous plants consistently featured in each sample, contributing significantly to the pollen content in Rtanj honey (Table 1).

All samples of Rtanj honey, besides the family Fabaceae, are characterised by the presence of pollen from nectar and non-nectar herbaceous plant species from the following families: Asteraceae, Ranunculaceae, Lamiaceae, Apiaceae, Boraginaceae, Plantaginaceae, Poaceae, Crassulaceae, Hypericaceae, and Brassicaceae (Table 1).

Pollen from the mint family (Lamiaceae) was identified in all Rtanj honey samples, ranging from a minimum of 1.38% to a maximum of 29.6%. It is known that monofloral honey types from plants belonging to this family are characterised by a darker colour, such as thyme honey (Oddo and Piro, 2004). Therefore, the high percentage of pollen from Lamiaceae plants in Rtanj honey may imply lower values of the colour parameter L^* of Rtanj honey (Table 2).

The representation of the family Lamiaceae in Rtanj honey is in line with the paper of Zlatković et al. (2014), who listed this family as the most commonly used in traditional medicine in the Rtanj region, represented by 10 mainly traditionally used aromatic plants (e.g., *Mentha pulegium* L., *Thymus praecox* Opiz, *Nepeta cataria* L., and *Origanum vulgare* L.), including the endemic *Satureja kitaibelii*, well-known by its vernacular name Rtanj's tea (Aćimović et al., 2021). The family Lamiaceae was also noted as the predominant plant families in the Zlatibor district, south-western Serbia, which is relatively close to the Rtanj region (Šavikin et al., 2013).

Pollen from plants of the Apiaceae family is present in all samples of Rtanj honey, with the most common proportion being up to 3%. Apiaceae are especially well represented in the regions of Tara Mountain, Kopaonik Mountain, and Ibarska klisura, Serbia (Milosavljević et al., 1999), all of which are in the neighbourhood of the Rtanj Mountain.

3.2. Physicochemical characterisation of Rtanj honey

Phisycochemical parameters of honey represent useful indicators of its quality, which is necessary to be in accordance with EU regulation (Codex Alimentarius, 2019). Among the physicochemical parameters, moisture content, electrical conductivity, pH, free acidity, HMF, and colour parameters were determined and presented in Table 2.

Although the moisture content of Rtanj honey samples was in the wide range of 13.6 ± 0.23–19.2 ± 0.07%, all of them were below the limit (max 20%) recommended by the Codex Alimentarius Commission (2019). The observed variations were the consequence of beekeeping/processing techniques and storage conditions.

Free acidity results from the presence of organic acids in equilibrium with their corresponding lactones and some inorganic ions. Acidity is one of the quality parameters of honey attributed to its freshness (Silva et al., 2022). The maximum level allowed for this parameter is 50.00 meq/kg (Codex Alimentarius, 2019). The free acidity of Rtanj honey ranged from 23.2 ± 0.14–65.6 ± 0.38 meq/kg, with only one sample being above the limit (Table 2). The average free acidity of Rtanj honey (35.7 ± 8.10 meq/kg) was significantly higher than the result published earlier by Sakač et al. (2019) – 19,3 ± 1,88 meq/kg for meadow honey samples from Vojvodina.

Table 2
Physicochemical characteristics, colour parameters, mineral profile and phenolic profile of Rtanj honey.

Physicochemical parameters	Mean value ± SD	Min	Max	Samples exceeding limits
Moisture (%)	16.7 ± 1.18	13.6 ± 0.23	19.2 ± 0.07	0
Acidity (meq/kg)	35.7 ± 8.10	23.2 ± 0.14	65.6 ± 0.38	1
pH	4.20 ± 0.41	3.42 ± 0.10	5.54 ± 0.22	-
Conductivity (mS/cm)	0.51 ± 0.22	0.22 ± 0.07	1.15 ± 0.11	7
Glucose (%)	38.3 ± 1.68	36.0 ± 1.22	39.9 ± 0.37	0
Fructose (%)	43.4 ± 1.38	41.8 ± 0.19	47.8 ± 0.10	0
Sucrose (%)	0.91 ± 0.06	0.81 ± 0.04	1.00 ± 0.03	0
HMF (mg/kg)	4.72 ± 1.00	2.16 ± 0.13	8.12 ± 0.51	0
Colour parameters				
L^*	41.7 ± 8.82	22.7 ± 0.19	59.8 ± 1.75	-
a^*	10.7 ± 6.06	1.55 ± 0.07	22.3 ± 1.36	-
b^*	28.1 ± 11.7	1.72 ± 0.15	45.1 ± 2.03	-
Minerals				
K (mg/kg)	996 ± 376	302 ± 12.6	1341 ± 60.8	-
Na (mg/kg)	207 ± 72.4	122 ± 2.78	339 ± 9.03	-
Ca (mg/kg)	90.3 ± 25.5	54.9 ± 1.78	121 ± 0.88	-
Mg (mg/kg)	53.4 ± 10.7	23.7 ± 0.79	87.0 ± 4.09	-
Fe (mg/kg)	3.20 ± 1.13	1.44 ± 0.07	4.59 ± 0.12	-
Zn (mg/kg)	3.62 ± 1.97	1.47 ± 0.10	6.05 ± 0.16	-
Mn (mg/kg)	2.75 ± 1.04	0.50 ± 0.03	5.67 ± 0.01	-
Cu (mg/kg)	0.94 ± 0.25	0.80 ± 0.02	1.23 ± 0.02	-
Pb (mg/kg)	< 0.25	-	-	-
Cd (mg/kg)	< 0.3	-	-	-
Ni (mg/kg)	< 1	-	-	-
Phenolic profile				
Epicatechin (mg/kg)	0.077 ± 0.005	n.d.	0.113 ± 0.006	-
p-Coumaric (mg/kg)	0.316 ± 0.004	0.291 ± 0.005	0.390 ± 0.010	-
Ferulic acid (mg/kg)	0.524 ± 0.002	0.476 ± 0.001	0.573 ± 0.012	-
Naringenin (mg/kg)	3.386 ± 0.011	3.085 ± 0.003	4.117 ± 0.014	-
Luteolin (mg/kg)	0.022 ± 0.006	n.d.	0.057 ± 0.008	-
Kaempferol (mg/kg)	0.628 ± 0.010	0.527 ± 0.017	0.698 ± 0.003	-
Apigenin (mg/kg)	0.120 ± 0.002	0.106 ± 0.007	0.155 ± 0.006	-

Mean value (n = 76); SD – standard deviation; Min – minimum value; Max – maximum value.

Even though the international legislation on honey (Codex Alimentarius, 2019) does not define pH limits, it is recommended to be low to prevent microbiological growth. The pH values in the examined Rtanj honey samples varied from 3.42 ± 0.10–5.54 ± 0.22 (Table 2), with an average of 4.20 ± 0.41. These values are in line with previously reported results for pH values of meadow honey samples from Serbia

(3.79–4.19 obtained by Sakač et al. (2019) and 3.56–5.54 obtained by Marić et al. (2021)).

The electrical conductivity is related to ash and mineral content (Bergamo et al., 2019). This parameter of Rtanj honey samples ranged from 0.22 ± 0.07–1.15 ± 0.11 mS/cm (Table 2). Out of a total of 76 honey samples, seven had electrical conductivity values above 0.80 mS/cm, which is defined as the limit for floral honey types (Codex Alimentarius, 2019). Although there were samples whose conductivity exceeded the limit, the average value of the electrical conductivity of Rtanj honey was 0.51 ± 0.22 mS/cm, and this value was consistent with previously reported values of meadow honey types from Serbia (Sakač et al., 2019; Đogo Mračević et al., 2020).

HMF is a marker of honey freshness. The content of this Maillard reaction product is defined to be below 40 mg/kg for honey originating from nontropical regions (Codex Alimentarius, 2019). In addition, the amount of up to 10 mg/kg of HMF is recognised as naturally present in honey (Alqarni et al., 2016). As the HMF content of Rtanj honey samples varied between 2.16 ± 0.13 mg/kg and 8.12 ± 0.51 mg/kg, it can be concluded that all Rtanj honey samples were considered fresh (Table 2).

The content of glucose ranged from 36.0 ± 1.22% to 39.9 ± 0.37% for all investigated Rtanj honey samples with a mean value of 38.3 ± 1.68%, while fructose varied between 41.8 ± 0.19% and 47.8 ± 0.10% with a mean value of 43.4 ± 1.38% (Table 2). According to Codex Alimentarius (2019), the total value of glucose and fructose should be above 60%, and all the samples satisfied this recommendation. These results were consistent with previously published content in meadow honey sampled in Serbia and Croatia (63.5%, 64.3%, and 62.1–76.1%, respectively) (Marić et al., 2021; Sakač et al., 2019; Šarić et al., 2008). Sucrose was in the range of 0.81 ± 0.04% to 1.00 ± 0.03% (Table 2), and that content was in accordance with Codex Alimentarius (2019).

Colour is a characteristic of honey that is of great importance for its commercialization, i.e., acceptability and consumer preferences. This parameter of honey varies greatly and is mainly determined by its botanical origin, ash content/mineral composition, honey temperature in the hives, and storage time (Gámbaro et al., 2007; González-Miret et al., 2005). Light types of honey contain lower contents of minerals, and they are generally considered more acceptable, although dark honey is also valued in certain regions (Tuberoso et al., 2014).

The results in Table 2 present the colour parameters of the investigated Rtanj honey samples. The lightness mean value of Rtanj honey samples was 41.7 ± 8.82, with a minimum L^* value of 22.7 ± 0.19 and a maximum L^* value of 59.8 ± 1.75. The measured L^* values of Rtanj honey indicated a darker honey type, which is not characteristic of meadow honey samples collected in Vojvodina (Sakač et al., 2019), Serbia, and the surrounding countries (Marić et al., 2021). The relatively low L^* value of Rtanj honey compared to other meadow honey samples from the region may be a marker for its geographical origin authentication (Fig. 3).

The red tone was present in all Rtanj honey (positive a^* value in all the samples), indicating the presence of polyphenols and carotenoids (da Silva et al., 2016). Being quite opposite from the results for the a^* value of meadow honey samples from the regions of Vojvodina and Serbia (Sakač et al., 2019; Marić et al., 2021), this colour parameter can also be adopted as an indicator of Rtanj honey geographical origin authentication (Fig. 3).

The mean value of yellow hue (b^*) was 28.1 ± 11.7, and this colour parameter was in line with the mean value of meadow honey samples from Vojvodina (31.0 ± 4.65) (Sakač et al., 2019).

The dominant mineral in Rtanj honey samples was K, which is known to be the most abundant mineral in honey (da Silva et al., 2016) and whose content represents more than 70% of the total mineral content in meadow honey samples (Sakač et al., 2019; Vanhanen et al., 2011). Rtanj honey contained twice as much K (mean value of 996 mg/kg) as meadow honey samples from the region of Vojvodina (468 mg/kg) (Sakač et al., 2019). Đogo Mračević et al. (2020) noted that multifloral honeys from Serbia had up to 467 mg/kg K. Therefore, the content of K in Rtanj honey may serve as a striking marker for geographical origin authentication in comparison to meadow honeys from other Serbian regions (Fig. 3). Bilandžić et al. (2017) stated that mineral and trace element concentrations in different honey types depend largely on the elemental composition of flowers with regard to their geographical origin.

Besides K, Na and Ca were the major elements in Rtanj honey (Table 2). In meadow honey samples from Serbia, levels of sodium below 50 mg/kg were previously detected (Đogo Mračević et al., 2020; Marić et al., 2021; Sakač et al., 2019), but the results of Na content in Rtanj honey were above 200 mg/kg on average. The mean value of Ca in Rtanj honey samples (90.3 ± 25.5 mg/kg) was similar to the previously reported range of 63.8–99.9 mg/kg for meadow honey (Alqarni et al., 2014). Sakač et al. (2019) found that the mean value of Ca in meadow honey samples from Vojvodina was 137 mg/kg. Mg content in Rtanj honey samples (mean value of 53.4 ± 24.1 mg/kg) was in line with those published by Đogo Mračević et al. (2020) for meadow honey samples from Serbia (17–61 mg/kg), but higher than Mg amounts in meadow honey from Vojvodina (mean value of 20.1 ± 8.53 mg/kg) reported by Sakač et al. (2019).

Trace elements such as Fe, Zn, Cu, and Mn were also detected in Rtanj honey samples (Table 2). Fe measured in honey samples varied from 1.44 ± 0.07 mg/kg to 4.59 ± 0.12 mg/kg, and these amounts are higher than those for meadow honey characteristic of Vojvodina (0.79–1.59 mg/kg) determined by Sakač et al. (2019) and monofloral Croatian honey types (0.36–7.55 mg/kg) published by Bilandžić et al. (2017). Zinc was detected in Rtanj honey samples within the range of 1.47 ± 0.10 mg/kg to 6.05 ± 0.16 mg/kg and fulfilled the Serbian regulatory standards. The measured contents were similar to the results obtained by Đogo Mračević et al. (2020) for the meadow honey collected in the Serbian region and also comparable with the Zn levels of the honey from the northeast region of Romania published by Oroian et al. (2016).

Mn was registered in Rtanj honey samples, ranging from 0.50 ± 0.03–5.67 ± 0.01 mg/kg. Đogo Mračević et al. (2020) found less Mn in multifloral honey from Serbia (up to 2.05 ± 0.19 mg/kg), while honeydew honey contained 7.96 mg/kg. Since there was an indication that some Rtanj honey samples were from the category of honedew honey (based on the electrical conductivity levels), higher Mn contents were acceptable.

Previously, Cu was not identified in honey samples from Serbia (Đogo Mračević et al., 2020), but Rtanj honey was characterised by a Cu mean value of 0.94 ± 0.25 mg/kg. The concentration of Cu found in Croatian honeys was in the range 0.14–1.39 mg/kg, supporting our results (Bilandžić et al., 2017).

Although mineral contents in honey vary depending on the soil type and can indicate geographical origin (Kaygusuz et al., 2016), only K among all measured minerals can be a predictor in the determination of Rtanj honey's geographical origin.

3.3. Phenolic profile of Rtanj honey

Polyphenols are considered one of the important groups of compounds in honey possessing health properties arising from their antioxidant nature (Hossen et al., 2017; Lo Dico et al., 2019). The main polyphenols in honey are flavonoids, but phenolic acids are also present (Trautvetter et al., 2009). Honey phenolic profile depends on the type of nectar and therefore can serve as floral markers, but it can also be considered an effective tool in establishing honey geographical origin. The content of these antioxidants varies according to honey types, geographical and floral origins, and climate characteristics of the harvesting site (Becerril-Sánchez et al., 2021).

Epicatechin, *p*-coumaric and ferulic acid, as well as naringenin, luteolin, kaempferol, and apigenin were quantified in Rtanj honey (Table 2). Although Gašić et al. (2014) found a wide range of phenolics in polyfloral honeys produced in different regions of Serbia, our results

indicate a relatively poor representation of different polyphenols in Rtanj honey. Namely, only low levels of *p*-coumaric acid (0.29 ± 0.01–0.32 ± 0.01 mg/kg) and ferulic acid (0.48 ± 0.01–0.57 ± 0.01 mg/kg) were found in Rtanj honey. The amount of *p*-coumaric acid in Rtanj honey was much lower than previously reported in Eest Serbia polyfloral honeys by Gašić et al. (2014).

Strikingly higher quantities among polyphenolics were registered in the flavonoids group, especially for naringenin (3.09 ± 0.03–3.39 ± 0.014 mg/kg), which was not found by Gašić et al. (2014) in Serbian honeys. Among the quantified polyphenols in Rtanj honey samples, naringenin was the most abundant phenolic compound, followed by kaempferol, apigenin, epicatechin, and luteolin. Therefore, this compound may be considered the phenolic marker of Rtanj honey. Gašić et al. (2014) registered kaempferol in slightly lower concentrations (0.16–0.59 mg/kg) than Rtanj honey possessed and apigenin (0.13–0.71 mg/kg) in significantly higher concentrations. Luteilin was found in low amounts in Rtanj honey (0.02 ± 0.01–0.06 ± 0.01 mg/kg), while East Serbia's honeys investigated by Gašić et al. (2014) were characterised by somewhat higher levels (0.04–0.13 mg/kg).

3.4. Sensory profile of Rtanj honey

Descriptive sensory analysis is efficiently used for distinguishing various types of food based on their origin or source (Dairou, Sieffermann, 2002). Authentic honey is unique due to its unique sensory properties. The colour of honey is the first indicator of its botanical origin. Each honey varietal can be classified in one of the colour ranges (using a Pfund's colour scale): water white (0–8 mm), extra white (8–17 mm), white (17–34 mm), extra light amber (34–50 mm), light amber (50–85 mm), amber (85–114 mm), and dark amber (114–140 mm) (Silvano et al., 2014)). The colour of Rtanj honey varied from extra white (8–17 mm) to light amber (12–65 mm). This high variability in Rtanj honey colour may be attributed to the variability in pollen types found in the samples, but also to the wide variation in mineral content. Moreover, geographical and climatic conditions, together with temperature and storage conditions before and after processing, may cause the observed honey colour differences as well (Bath and Singh, 1999; Terrab et al., 2004). The sensory profile of Rtanj honey samples is shown in Fig. 2. Broad variability was evident for all measured sensory properties. The taste of Rtanj honey (Fig. 2b) developed from mildly to moderately sour and moderately sweet, often with the appearance of a slightly bitter aftertaste. During Rtanj honey consumption, pungency and a burning sensation in the throat were perceived (Fig. 2c). Depending on the dominant honey plants, the odour and flavour of Rtanj honey represent a harmonious combination of herbal, fruity, and floral notes that can be very subtle, barely noticeable, or moderately pronounced (Figs. 2a and 2c). The odour of Rtanj honey was reminiscent of dried herbs, fermented and processed fruit, with light floral notes (Fig. 2a). The flavour of Rtanj honey was of weak persistence, and during consumption it was noticeable in milk caramel, fried sugar, and fresh and fermented fruits (Fig. 2c). The texture of honey depends not only on temperature and humidity but also on the water-to-sugar ratio. Moreover, the same honey can have a completely distinct texture depending on whether it is given in a liquid or crystallised state. Liquid Rtanj honey had a smooth, buttery texture with moderate to high viscosity, while in its crystallised state it has a sandy consistency due to the presence of moderately large crystals (Fig. 2d).

When grouping the Rtanj honey sensory profile by colour ranges, lighter samples were represented by greater intensity of sweet taste with barely noticeable sourness and without bitterness, while odour and flavour resembled fresh flowers and fruits. These samples showed the highest intensity of burning sensations perceived during swallowing. Darker Rtanj honey samples were accompanied by a decrease in sweetness and an increase in sourness, with a noticeable perception of bitterness and pungency and an increase in the odour and flavour of dried herbs and fruits. A peculiarity of the darkest Rtanj honey samples

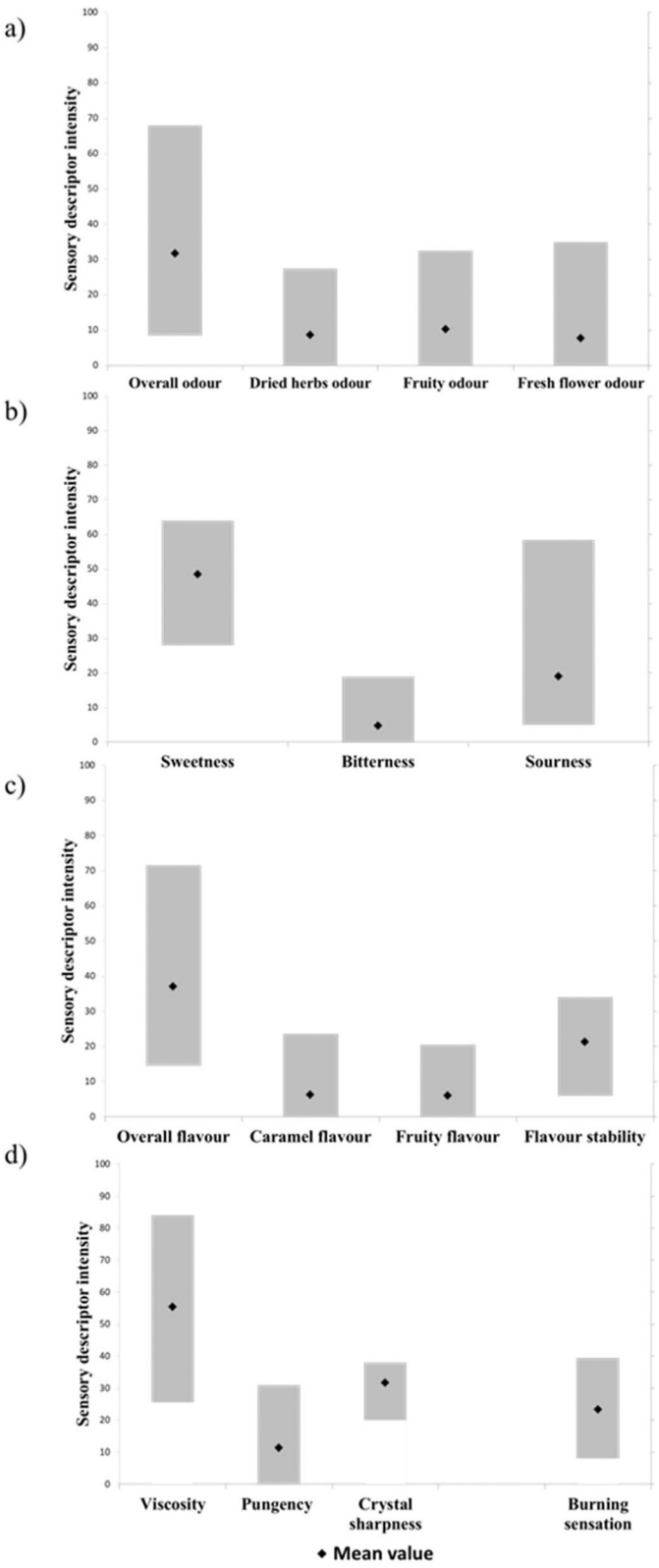

Fig. 2. Range graph – intensity score range for a) odour b) taste c) flavour and d) texture and mouthfeel of Rtanj honey samples.

was the most intense sourness and bitterness, a slight sweetness, and greater intensity of the aromas of the fermented and candied fruits and milky caramel. Similar findings were reported by Cabrera and Santander (2022), who investigated honeys from eastern Formosa province (Argentina). Furthermore, it is important to note that consumers from

various parts of the world have stated that honeys that stand out in the floral-fresh fruit flavour family are the ones they enjoy the most (Anupama et al., 2003).

3.5. Principal component analysis (PCA)

The principal component analysis (PCA) was used to determine and visualise the differences between meadow honey samples from different geographical origins based on colour parameters L^* and a^*, and K content. The choice of these parameters for Rtanj honey samples resulted from a comprehensive analysis of the correlation matrix that encompasses all variables (Supplementary table 3). The chosen parameters of Rtanj honey samples were compared with the findings of our previously published studies in which meadow honey from Vojvodina (Sakač et al., 2019) and Serbia (Marić et al., 2021) was investigated.

The principal components F1 and F2 account for 79.68% and 11.67% of the total variance, respectively, collectively explaining 91.35% of the overall variance. Fig. 3 represents the loading and scoring plot, featuring confidence ellipses with a 95% confidence interval. Notably, the variable L^* exhibits a strong positive correlation with F1 and a minimal correlation with F2. Contrary to this, the other two parameters exhibit negative correlations with F1, with parameter a^* positively correlated with F2 and parameter K negatively correlated with F2.

The samples of Rtanj honey demonstrate a distinct clustering on the negative side of the principal component analysis (PCA), effectively separating them from the other meadow honey samples. In contrast, samples from Vojvodina and Serbia cluster together, exhibiting overlap. This observation leads to the conclusion that parameters K, L^*, and a^* can serve as discriminatory factors for distinguishing Rtanj honey samples from other meadow honey samples from the regions of Vojvodina and Serbia.

4. Conclusion

Seventy-six meadow honey samples harvested during 2019–2021 from different locations in the Rtanj Mountain region (eastern Serbia) were investigated in terms of pollen types, physicochemical parameters, polyphenol profile, and sensory properties. A relatively low value of the colour parameter L^* of Rtanj honey (22.7 ± 0.19–59.8 ± 1.75) and a positive a^* value (red tone) of 1.55 ± 0.07–22.3 ± 1.36 may be the markers for its geographical origin authentication. K content (mean value of 996 ± 376 mg/kg) can also be a predictor in the determination of Rtanj honey's geographical origin.

The evaluation of the phenolic profile revealed epicatechin, *p*-coumaric acid, ferulic acid, naringenin, luteolin, kaempferol, and apigenin as the most abundant phenolics in Rtanj honey.

Rtanj honey was distinguished from other meadow honeys from the region of Vojvodina and Serbia on the basis of colour parameters L^* and a^*, and K content, which was demonstrated by PCA analysis. The limitation of this study is the impossibility of determining markers for its geographical origin based on the complete polyphenolic profile. Therefore, this task will be the next step in the future investigation.

CRediT authorship contribution statement

Marić Aleksandar: Writing – review & editing, Visualization, Validation, Investigation, Formal analysis, Conceptualization. **Maravić Nikola:** Visualization, Validation, Software, Formal analysis, Data curation. **Jovanov Pavle:** Writing – review & editing, Validation, Methodology, Formal analysis, Conceptualization. **Ikonić Predrag:** Supervision, Project administration, Investigation, Conceptualization. **Novaković Aleksandra:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Sakač Marijana:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Conceptualization. **Šikoparija Branko:** Visualization, Validation, Methodology, Investigation, Formal analysis. **Radišić Predrag:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Škrobot Dubravka:** Software, Investigation, Formal analysis, Data curation. **Peulić Tatjana:** Supervision, Resources, Project administration, Investigation, Conceptualization.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

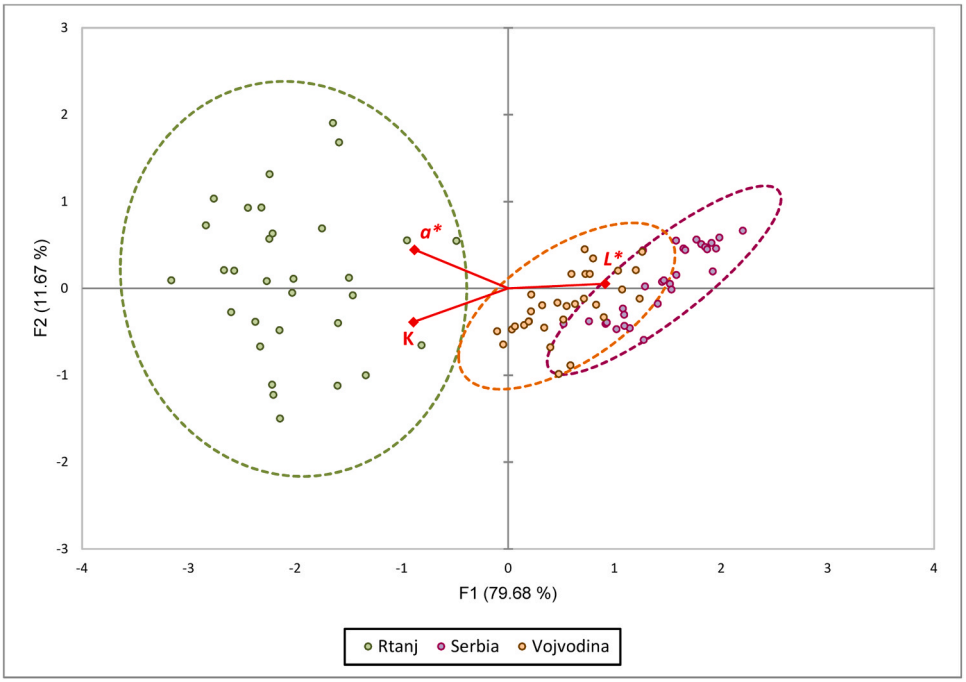

Fig. 3. Principal component analysis (PCA) based on component correlations (L^*, a^*, and K) for the honey samples from Rtanj Mountain, Vojvodina, and Serbia.

Data availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jfca.2024.106088.

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