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Research Article

Pollen, Physicochemical, and Mineral Analysis of Croatian Acacia Honey Samples: Applicability for Identification of Botanical and Geographical Origin

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The aim of the study was to investigate health safety and quality of the Croatian acacia honey, the selected elements in the soil, and whether multivariate methods can provide identification of the origin of honey. The study included 200 acacia honey samples and 100 soil samples from East, Northwest Croatia, and Istria. The proportion of acacia in honey was determined by conducting pollen analysis. Water, free acids, electric conductivity, reducing sugars, saccharose, diastase, and HMF were determined. No significant differences were found using Kruskal-Wallis test regarding the physicochemical parameters ($p = 0.9190$), the mineral content of honey ($p = 0.8955$), or the mineral composition of the soil ($p = 0.8789$). No significant correlation was found between the analyzed elements in honey and soil. Multivariate methods indicated that East Croatia honey samples have higher concentrations of water, HMF, and higher concentrations of measured elements, except for Al. Honey samples from Northwest Croatia are characterized by low concentrations of elements and a higher concentration of saccharose. The Istria samples are richer in reducing sugars, free acids, diastase, higher conductivity, higher content of the acacia pollen grains, and lower concentrations of most metals. Honey from Northwest Croatia and Istria shares the high concentration of Al in honey.

1. Introduction

Honey has been used for centuries as a natural food [1]. The composition of honey depends on the plant species, climate conditions, environmental conditions, and beekeeping practice. Mostly it consists of carbohydrates, water, protein, free amino acids, enzymes, vitamins, minerals, and another 200 or so compounds [2].

Biodiversity of melliferous plant species from different climatic and geographic regions of Croatia provides great potential for honey production, with acacia honey being one of the most common. Therefore, to determine the geographical origin and composition of honey many analytical techniques and parameters have been used in combination with statistical methods. Physicochemical parameters in honey can point to fermentation, sugar adding, heating, honey adulteration attempts, inappropriate storage, and so forth [3, 4]. The water content in honey ranges from 15% to 23% [5–7]. Honey which is placed on the market must not have water content higher than 20% [3, 4]. Permitted value of free acids in honey is <50 mEq of acid/1000 g of honey [3]. Their presence tells us whether the honey is older [8]. Electrical conductivity (mS/cm) is a physical property which greatly depends on the content of mineral substances and acids in honey [9]. Electrical conductivity of floral and blended honey needs to be lower than 0.8 mS/cm [3, 4]. The sum of glucose and fructose or reducing sugars (g/100 g) in honey must be at least 60 g/100 g [3]. Carbohydrates are the main ingredient of honey and their proportion is 73%–83% [7, 10]. Determining the amount of saccharose (≤10 g/100 g) is important for confirming possible adulteration of honey, feeding the bees sugar (saccharose), or directly adding sugar in honey [11]. Diastase activity (DN) represents one of the main parameters in determining the intensity of heating the honey during its processing and storage [12, 13]. Nevertheless, the proportion of HMF in the fresh sieved honey usually does not exceed 10 mg/kg [14, 15].

Macro and micro elements, expressed as a percentage of ash, can be found in floral honey in the amount up to 0.2%. Honey usually contains the following minerals: K, Ca, Na, Mg, Cl, P, Fe, Mn, and others [7]. Mineral content primarily depends on the botanical origin and climatic conditions, but usually also on the type of soil where the honey plant grows [16].

Minerals from the soil are transported through the root system to the honey plant, moving on to nectar, and from there to honey; therefore, the proportion of macro elements, particularly Ca, Mg, Mn, and Na, is influenced by the composition of soil, which is geochemically and geologically determined [17].

The aim and purpose of the study was to provide a review of health safety and quality, of the differences in the pollen analysis, physicochemical parameters, and composition and concentration of twelve elements in the acacia honey from three regions in Croatia (East, Northwest, and Istria). Also, an overview of differences with regard to the composition and concentration of identical elements from the soil from the same locations was given. Moreover, the authors tried to identify to what extent multivariate statistical methods—especially the cluster and PCA analyses—can have a predictive significance for the composition and geographical origin of honey.

2. Materials and Methods

2.1. Materials. The study included 200 samples of acacia honey collected from beekeepers in 2009 and 2010. The samples originated from three Croatian regions: Northwest Croatia, represented by the samples from the Varaždin, Krapina-Zagorje and Bjelovar-Bilogora Counties, with a total of 120 samples; East Croatia, represented by the samples from the Požega-Slavonia, Virovitica-Podravina, Osijek-Baranja, and Vukovar-Srijem Counties, with a total of 40 samples; and Istria region in the county of the same name, also with a total of 40 samples. Moreover, 100 soil samples (Figure 1) were sampled on the same locations (according to the GPS points of the apiary locations), of which there are 60 samples in Northwest and 20 in East Croatia and in Istria, respectively.

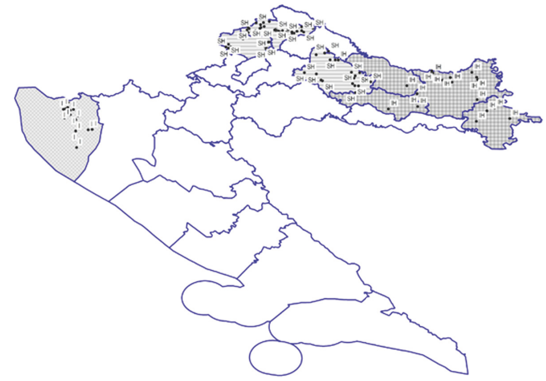

Figure 1: Diagram of the Republic of Croatia with three regions (East Croatia, Northwest Croatia, and Istria) and locations of acacia honey and soil sampling.

2.2. Methods

2.2.1. Pollen Grains. Pollen grains were identified and quantified by applying microscopy (400x magnification) to preparations taken from the honey samples. The proportion of each type of pollen was calculated as a percentage of total pollen. The collection of reference pollen preparations was used for the determination of pollen grains from the samples according to the principle of comparison. Complying with the provisions of the EU directive on the quality of honey [4], of the criteria of expert publications [18], and of the current legislation in the Republic of Croatia [17], the requirement of marking the botanical origin of honey is the presence of the prevailing pollen. The term "unifloral honey" is used to describe those types of honey which originate mainly from one dominant plant species. Unifloral honey can be labeled based on a particular plant type if it contains at least 45% of the pollen grains of the same plant species, with some exceptions; therefore, for acacia honey the amount is 20% *(Robinia pseudoacacia L.)* [18, 19].

2.2.2. Physicochemical Parameters. As it regards physicochemical parameters, water content (%) in honey was determined by a method based on refractometry. Free acids in honey were determined so that the prepared sample was titrated using the solution of 0.1 M of sodium hydroxide to pH 8.30. Electric conductivity in honey is defined as the conductivity of a 20% aqueous solution of honey at 20°C, where 20% refers to the dry weight of honey. The results are expressed in mS/cm. The method for the determination of reducing sugars and saccharose in honey is based on reducing Fehling's solution by titration with a sugar solution from honey and using methylene blue as the indicator. The proportion of saccharose is obtained from the difference in the results obtained before and after the inversion [20]. When determining the diastase activity in honey, the method used is based on the hydrolysis of 1% starch solution from 1 g of honey in one hour at a temperature of 40°C. When determining hydroxymethylfurfural (HMF) in honey using the Winkler method, the procedure is based on the reaction of hydroxymethylfurfural with barbituric acid and p-toluidine, which results in pink, the intensity of which is measured at a wavelength of 550 nm [21, 22].

2.2.3. Determination of Mineral Substances in Honey. Upon the homogenization of honey, the organic matter undergoes a wet chemical decomposition using concentrated nitric acid and hydrogen peroxide in a microwave apparatus (Anton Paar, Multiwave 3000). The method of mass spectrometry for detecting metals in honey is based on inductively coupled plasma (ICP-MS) which leads to ionization. Mass spectrometer is used to detect and identify ions based on their mass/charge ratios [23, 24].

2.2.4. Determination of Mineral Substances in Soil. Soil sampling is defined by a specific procedure. Each sample was taken from a specific point previously defined (according to the Gauss-Krüger coordinates). The samples were extracted from the soil profile at a depth of 0.5 meters and then stored on the ground in PVC containers. In the laboratory, prior to sieving, the samples were dried at ambient temperature and homogenized in an agate mill and then sieved to 2 mm fractions.

Determination of minerals in the soil was conducted by using the samples prepared by extracting the mixture of concentrated acids. Sample preparation was performed in Teflon containers with 10 mL of a solution consisting of a mixture of concentrated acids (HF-hydrofluoric acid, HCl-hydrochloric acid, HNO_3-nitric acid, and $HClO_4$-perchloric acid). The solution is evaporated to dryness at 200°C. Then, the residue is dissolved with 4 mL of 50% HCl, and the sample is heated in a microwave oven. After cooling, the solutions are poured into the polypropylene vials and filled up to reach the volume of 10 mL with 5% HCl. Preparation of the sample in aqua regia extraction: 3 mL of distilled water, 7.5 mL of 6 M HCl, and 2.5 mL of 14 M HNO_3 are added in 1 g of the soil sample at 20°C and it is left to react overnight. After that, the sample is heated to boiling for two hours, and the solution is cooled and filtered. Determination of metal concentrations is also carried out using inductively coupled plasma and mass spectrometry (ICP-MS) [23, 25].

2.2.5. Statistical Analysis. For the purposes of processing and describing the data descriptive statistics, Kruskal-Wallis test and multivariate techniques (Cluster Analysis and Principal Component Analysis) were used. Data analysis was carried out using the statistical software Statistica, version 12.

Cluster Analysis. Clustering or cluster analysis is used to classify data objects, characterized by the values of a set of variables, into groups. The Euclidean distance of two representing objects can be taken as an indicator of similarity/dissimilarity of the investigated variables. The equation for the Euclidean distance between objects i and i' is

$$D_{ii'} = \sqrt{\sum_{j=1}^{m} \left(xij - x_{i'j}\right)^2}, \quad (1)$$

where m is the number of variables.

An agglomerative single-linkage method was used and the results are presented in a form of dendrogram.

Principal Component Analysis (PCA). Principal component analysis reduces a set containing a large number of measured variables to a data set containing fewer new variables. These new variables (principal components, PCs) are linear combinations of the original ones. These linear combinations are chosen to represent a maximum possible proportion of the variability contained in the original data. The results are usually presented in a form of biplots; biplots allow us to investigate a trend in a data table and show relationships or contrasts between objects and between variables, and finally relationships between variables and objects. Through variable reduction and visual display, PCA allows us to observe the sources of variation in an analyzed data matrix.

In general,

$$PC_i = l_{1i}X_1 + l_{2i}X_2 + \cdots + l_{ni}X_n, \quad (2)$$

where PC_i is i-th principal component (PCs) and l_{ji} is the loading of the observed variable X_j [26–28].

Table 1: Pollen analysis of acacia honey from Northwest Croatia, East Croatia, and Istria ($\overline{x}$ = % acacia proportion-*Robinia pseudoacacia L.*).

Northwest Croatia	$\overline{x}$	42.88
	Min–max	22–68
East Croatia	$\overline{x}$	39.11
	Min–max	11–70
Istria	$\overline{x}$	55.20
	Min–max	33–71
Total	$\overline{x}$	43.55
	Min–max	22–71

3. Results and Discussion

Pollen analysis showed that out of 200 analyzed samples of acacia honey, 199 of them (99.5%) had at least a 20% proportion of acacia pollen grains; therefore, according to the legislation, it can be called unifloral honey [19]. The proportion of acacia pollen grains ranged from 39.11% in East Croatia, over 42.88% in Northwest Croatia, and to 55.20% in Istria, which is an average of 43.55%, with a range from 11% (one sample) to 70% (Table 1).

The results of determining the physicochemical parameters are shown in Table 2. The results have shown that all the samples of honey according to the physicochemical

Table 2: Physicochemical indicators in acacia honey from three Croatian regions ($\overline{x}$, σ, range).

	Analysis	Water (%)	Free acids (mEq/1000 g)	Electrical conductivity (mS/cm)	Reducing sugars (g/100 g)	Saccharose (g/100 g)	Diastase (DN)	HMF (mg/kg)
Northwest Croatia	$\overline{x}$	16.78	10.45	0.15	68.93	0.62	10.38	3.56
	σ	1.03	2.20	0.03	1.91	0.52	1.94	2.11
East Croatia	$\overline{x}$	16.94	10.50	0.15	68.46	0.49	11.33	5.92
	σ	1.24	1.95	0.04	2.38	0.41	2.96	2.91
Istria	$\overline{x}$	17.01	11.02	0.18	70.62	0.55	13.75	3.54
	σ	1.06	1.15	0.03	0.91	0.51	2.34	2.97
Total	$\overline{x}$	16.91	10.65	0.16	69.33	0.55	11.82	4.34
	σ	1.11	1.77	0.03	1.73	0.48	2.41	2.66
	Min–max	16.78–17.01	10.45–11.02	0.15–0.18	68.46–70.62	0.49–0.62	10.38–13.75	3.56–5.92
MPC		≤20	<50	0.8	≥60	≤10	≥8	≤40

$\overline{x}$: arithmetic mean; σ: standard deviation.

parameters are safe and of high quality [3]. Kruskal-Wallis test has shown no significant differences among the regions with regard to the physicochemical parameters of honey (p = 0.92).

The water content in our study ranged between 16.78% and 17.01%, which is in accordance with legislation that allows up to 20%. The permitted value of free acid in honey is <50 mEq of acid/1000 g of honey. This means that that the average values obtained in this study, ranging from 10.45–11.02 mEq of acid/1000 g, are within the limits. Average values of electrical conductivity in the samples of this research range from 0.15 to 0.18 mS/cm, which meets the general requirements of a maximum of 0.8 mS/cm. In honey, the sum of the proportions of glucose and fructose or reducing sugars has to be at least 60 g/100 g. We estimated the average of 69.33 g/100 g in the range of 68.46–70.62 g/100 g. All the tested samples have met the requirement for saccharose (up to 10 g/100 g of the sample) and ranged from 0.49 to 0.62 g/100 g. General requirements for the value of diastase activity are 8 DN, whereas our values ranged from 10.38 to 13.75 DN. The proportion of HMF was low (an average of 3.56 to 5.92 mg/kg), which is less than 10 mg/kg, the upper limit for the first class honey [3].

A total of twelve micro and macro elements in acacia honey (K, Ca, Na, Mg, Zn, Al, Fe, Cu, Mn, Ni, Pb, and Cd) in three Croatian regions was examined through a percentage (%) of each macro and micro element, as well as through the range and the average value of each macro and micro element expressed in mg/kg (Table 3). The total proportion of all the elements in honey was an average of 565.55 mg/kg (ranging from 424.04 mg/kg in Northwest Croatia to 765.55 mg/kg in Istria), which makes an average of less than 0.06% (expressed as a percentage of ash) and is within the normal proportion of elements in floral honey to 0.2% [3]. Macro elements (K, Ca, Na, and Mg), thereby, accounted for an average of 554.64 mg/kg or 98.07% of all the proportion of elements. Potential contaminants (Pb and Cd) accounted for a total average of insignificant ≤0.06 mg/kg or ≤0.02% of the content.

The percentage of K ranged from 51.23% to 70.29% ($\overline{x}$% = 57.56%). The determined concentrations were in the range of 258.69 mg/kg to 360.80 mg/kg ($\overline{x}$ = 325.54 mg/kg). The proportion of Ca in the tested samples of honey ranged from 14.81% to 21.04% compared to other minerals. The lowest concentration of Ca expressed in mg/kg was 74.37 mg/kg and the highest 184.39 mg/kg ($\overline{x}$ = 111.24 mg/kg). The proportion of Na in the samples tested in this study was expressed as a percentage from 10.21% to 21.66%. The highest average value of Na was 168.86 mg/kg in East Croatia, and the lowest value of 51.62 mg/kg was recorded in Northwest Croatia ($\overline{x}$ = 95.85 mg/kg). The average value of Mg proportion in the samples of acacia honey in this study ranged from 3.67% to 4.13% ($\overline{x}$% = 3.89%), while the average concentration ranged from 16.82 mg/kg to 30.68 mg/kg ($\overline{x}$ = 22.01 mg/kg). Among micro elements, only Zn had an average proportion exceeding 1% ($\overline{x}$% = 1.26; $\overline{x}$ = 7.12 mg/kg) and Al ($\overline{x}$% = 0.27; $\overline{x}$ = 1.55 mg/kg) and Fe ($\overline{x}$% = 0.22; $\overline{x}$ = 1.23 mg/kg) greater than 0.2%. The proportions of all the other elements that had been tested ranged between 0.06% (Cu) and ≤0.01% (Cd). Kruskal-Wallis test did not show significant differences between the regions with regard to concentrations of twelve elements measured in the honey samples (p = 0.89).

A total of twelve elements in the soil (K, Ca, Na, Mg, Zn, Al, Fe, Cu, Mn, Ni, Pb, and Cd) in three Croatian regions were examined as a percentage of an individual macro or micro element, as well as the range and the average value of an element expressed in mg/kg (Table 4). The total proportion of elements in the soil was an average of 51,306.88 mg/kg (range of 41,205.11 mg/kg in Northwest Croatia to 64,371.18 mg/kg in Istria). Kruskal-Wallis test did not show significant differences between regions with regard to concentrations of twelve metals measured in the soil samples (p = 0.845).

Fe had the highest average concentration in soil ($\overline{x}$ = 24, 985.50 mg/kg, range of 21,355.23 to 30,842.30 mg/kg) and the highest average proportion ($\overline{x}$% = 48.70%) in comparison to the other elements. It is followed by Al, with the concentration in the range of 10,275 mg/kg to 23,350.70 mg/kg ($\overline{x}$ = 15, 864.78 mg/kg) and with the proportion of 28.29%–36.28% ($\overline{x}$% = 30.92%). Ca is in the third place with an average concentration of 4,406.21 mg/kg and the average proportion of 8.58%. It is followed by Mg, which had an average proportion of 6.59% and the average concentration of 3,378.96 mg/kg. Then there is K, with an average concentration of 1,331.39 mg/kg with an average proportion of 2.60%, followed by an average proportion of Mn of about 1.18% and an average concentration of 606.01 mg/kg and an average concentration of Na of 585.92 mg/kg and a proportion of 1.14%. The proportion of all the other elements, including potential contaminants (Cd and Pb), was in the range of 0.15% (Zn) to ≤0.01% (Cd).

Table 5 shows the values of the correlation coefficients between the concentrations of minerals (K, Ca, Na, Mg, Zn, Al, Fe, Cu, Mn, Ni, Pb, and Cd) in acacia honey and soil for three regions in the Republic of Croatia. Although strong positive correlation coefficients were obtained among the concentrations of twelve elements in certain types of honey (e.g., $r = 0.99$ between the Istrian honey and the honey of Northwest Croatia or $r = 0.92$ between the Istrian honey and the honey of East Croatia, $p < 0.05$) as well as strong positive correlations between the concentrations of the twelve elements in soils, no significant correlations were found between the concentrations of the twelve elements in honey and soil.

Cluster analysis of the chemical composition of honey from the three regions of the Republic of Croatia revealed the existence of differences between East Croatia and the other two regions (Figure 2).

The next step was the principal component analysis (PCA) (Figure 3), which "confirmed" the diversity of the composition of honey from East Croatia when compared to the other two regions, primarily in higher concentrations of all the tested elements except for Al, and in higher values of water and HMF, and lower values of pollen proportion, electrical conductivity, free acids, reducing sugars, saccharose, diastase activity, and Al. In contrast, acacia honey from Northwest Croatia was characterized by higher concentrations of saccharose and lower concentrations of almost all of the other remaining parameters, while honey from Istria

TABLE 3: Micro and macro elements in acacia honey from three Croatian regions, according to the proportion percentage (%), average concentrations ($\overline{x}$; σ), and range (mg/kg).

	Elements	K (mg/kg)	Ca (mg/kg)	Na (mg/kg)	Mg (mg/kg)	Zn (mg/kg)	Al (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Cd (mg/kg)	Total (mg/kg)
Northwest Croatia	$\overline{x}$	258.69	74.95	67.07	16.82	3.31	1.66	0.92	0.20	0.16	0.22	0.04	≤0.01	424.04
	σ	64.99	42.30	37.60	8.08	1.23	1.03	0.72	0.09	0.09	0.10	0.02	0.01	
	$\overline{x}$ (%)	62.36	17.13	14.79	4.13	0.77	0.43	0.25	0.05	0.04	0.05	0.01	≤0.01	
East Croatia	$\overline{x}$	360.80	184.39	168.86	30.68	16.46	1.22	1.38	0.51	0.19	0.98	0.08	≤0.01	765.55
	σ	164.54	97.570	97.57	11.76	12.33	0.85	0.97	0.35	0.10	0.65	0.05	0.01	
	$\overline{x}$ (%)	51.23	21.04	21.66	3.67	1.84	0.17	0.20	0.06	0.03	0.11	0.01	≤0.01	
Istria	$\overline{x}$	357.15	74.37	51.62	18.52	1.61	1.76	1.38	0.29	0.15	0.16	0.05	≤0.01	507.06
	σ	96.13	56.02	23.84	12.00	1.27	0.71	1.18	0.22	0.11	0.13	0.03	0.01	
	$\overline{x}$ (%)	70.29	14.81	10.21	3.67	0.32	0.35	0.28	0.06	0.03	0.04	0.01	≤0.01	
Total	$\overline{x}$	325.54	111.24	95.85	22.01	7.12	1.55	1.23	0.33	0.17	0.45	0.05	≤0.01	565.55
	σ	108.55	67.68	53.00	10.62	4.95	0.86	0.95	0.22	0.10	0.30	0.04	0.01	
	$\overline{x}$ (%)	57.56	19.67	16.95	3.89	1.26	0.27	0.22	0.06	0.03	0.08	0.01	≤0.01	
	Min–max	258.69–360.80	74.37–184.39	51.62–168.86	16.82–30.68	1.61–16.46	1.22–1.76	0.92–1.38	0.20–0.51	0.15–0.19	0.16–0.98	0.04–0.08	≤0.01–0.01	
	Min–max%	51.23–70.29	14.81–21.04	10.21–21.66	3.67–4.13	0.32–1.84	0.17–0.43	0.20–0.26	0.05–0.06	0.03–0.04	0.04–0.11	0.01–0.01	≤0.01–0.01	

$\overline{x}$: arithmetic mean; σ: standard deviation.

TABLE 4: Micro and macro elements in the soil from three Croatian regions, according to the proportion percentage (%), average concentrations ($\overline{x}$; σ), and the range of concentrations (mg/kg).

	Elements	K (mg/kg)	Ca (mg/kg)	Na (mg/kg)	Mg (mg/kg)	Zn (mg/kg)	Al (mg/kg)	Fe (mg/kg)	Cu (mg/kg)	Mn (mg/kg)	Ni (mg/kg)	Pb (mg/kg)	Cd (mg/kg)	Total (mg/kg)
Northwest Croatia	$\overline{x}$	631.07	3612.90	470.59	2756.72	74.50	10275.43	22758.97	17.56	566.78	27.79	12.57	0.23	41205.11
	σ	204.01	1712.93	139.24	595.39	20.62	3490.90	7446.24	5.55	270.32	11.92	2.62	0.18	
	$\overline{x}$ (%)	1.51	8.75	1.14	6.61	0.18	24.72	55.56	0.04	1.38	0.07	0.03	0.001	
East Croatia	$\overline{x}$	1877.05	4203.75	794.87	5543.13	69.70	13968.22	21355.23	14.62	485.37	18.10	13.86	0.20	48344.11
	σ	746.89	2227.66	466.75	2339.62	37.14	4766.93	5497.27	4.79	186.05	5.06	6.92	0.09	
	$\overline{x}$ (%)	3.88	8.70	1.64	11.47	0.001	28.89	44.17	0.03	1.00	0.04	0.03	0.00	
Istria	$\overline{x}$	1486.06	5395.99	492.29	1837.02	88.60	23350.70	30842.30	37.59	765.87	50.83	23.64	0.31	64371.18
	σ	909.56	6573.05	132.49	651.04	36.62	18021.97	8740.09	15.97	232.71	18.40	9.15	0.36	
	$\overline{x}$ (%)	2.31	8.38	0.77	2.85	0.14	36.28	47.91	0.06	1.19	0.08	0.04	0.00	
Summary	$\overline{x}$	1331.39	4404.21	585.92	3378.96	77.60	15864.78	24985.50	23.26	606.01	32.24	16.69	0.25	51306.80
	σ	620.15	3504.55	246.16	1195.35	31.46	8759.93	7227.87	8.77	229.69	11.79	6.23	0.21	
	$\overline{x}$ (%)	2.60	8.58	1.14	6.59	0.15	30.92	48.70	0.05	1.18	0.06	0.03	0.00	
	Min–max	631.07–1877.05	3612.90–5395.99	470.59–794.87	1837.02–5543.13	69.70–88.60	10275.43–23350.70	21355.23–30842.30	14.62–37.59	485.37–765.87	18.10–50.83	12.57–23.64	0.20–0.31	
	Min–max %	1.51–3.88	8.38–8.75	0.77–1.64	2.85–11.47	0.001–0.18	28.29–36.28	44.17–55.56	0.03–0.06	1.00–1.38	0.04–0.08	0.03–0.04	0.001–0.001	

$\overline{x}$: arithmetic mean; σ: standard deviation.

Table 5: An overview of the correlation coefficient between the concentrations of minerals (elements) in acacia honey and soil from three Croatian regions.

	NWC-soil	EC-soil	IS-soil	NWC-honey	EC-honey	IS-honey
NWC-soil		0.979145	0.966227	−0.161628	−0.178466	−0.147623
EC-soil	0.979145		0.980134	−0.127430	−0.148898	−0.112774
IS-soil	0.966227	0.980134		−0.157446	−0.180116	−0.140965
NWC-honey	−0.161628	−0.127430	−0.157446		0.967384	0.991399
EC-honey	−0.178466	−0.148898	−0.180116	0.967384		0.926812
IS-honey	−0.147623	−0.112774	−0.140965	0.991399	0.926812	

NWC: Northwest Croatia; EC: East Croatia; IS: Istria.

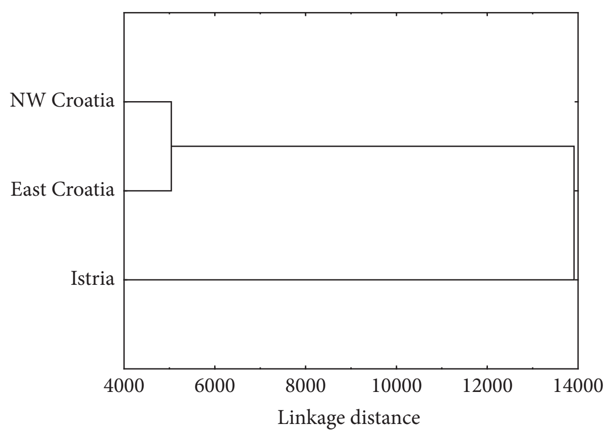

Figure 2: Dissimilarities between the three types of honey in Croatia, according to the analyzed parameters and regions.

was characterized by higher values of pollen proportion, free acids, electrical conductivity, reducing sugars, and diastase activity, with lower concentrations of water, saccharose, HMF, and the majority of examined metals. The "link" between honey from Northwest Croatia and Istria is the higher concentration of Al.

The study included 200 samples of acacia honey from three Croatian regions and 100 soil samples from the same locations. We studied the proportion of acacia pollen, physicochemical parameters, and macro and micro elements in honey and the same selected elements in the soil together with the relationship between them to give an overview of food safety and quality of acacia honey in Croatia. In addition, by applying multivariate statistical methods, we tried to make a contribution to the research of the combination of optimum indicators of the status and quality of honey and tests that could have a predictive effect with regard to determining the composition and the botanical and geographical origin of honey.

By using pollen analysis, it was found that the vast majority of the samples of acacia honey from Croatia (99.5%) had at least a 20% proportion of pollen grains of acacia *(Robinia pseudoacacia L.)*; therefore, according to the legislation, it can be called unifloral honey [3, 10]. Characterization of honey according to the proportion of pollen is already known in the European countries, such as Spain, Italy, and Poland [29–31], and even in other parts of the world, such as India [32], Saudi Arabia [33], and Morocco [34]. There have been a relatively small number of studies of acacia honey on the Croatian territory [35–37], and our research has contributed to the existing knowledge and data that characterize the Croatian acacia honey.

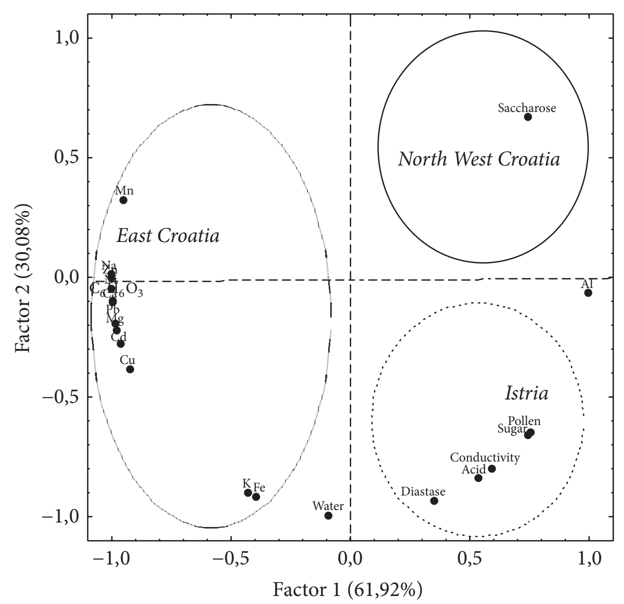

Figure 3: An overview of the PCA (Principal Component Analysis) of the chemical composition of acacia honey from three regions of the Republic of Croatia. Water: water %, Aci: free acids (mEq/100 g), Conductivity: electrical conductivity (mS/cm), Sugar: reducing sugars (g/100 g), Saccharose: saccharose (g/100 g), Diastase: diastase (DN), and HMF: HMF (mg/kg).

Physicochemical parameters were determined according to the criteria laid down in the Croatian legislation and consistent with the directives and recommendations of the EU [3, 4]. All determined physicochemical parameters were within the permitted values and thus speak in favor of high quality and good selection of the acacia honey on the Croatian market taken for this research and, of course, of good beekeeping practices.

Thus, the water content in all the samples was within 20%, and our results are consistent with the results of other researchers on the samples of acacia honey from Croatia (15.40%–16.30%), Romania (17.90%), and Italy (17.10%) [36–39].

The literature shows various values of free acids in the samples of acacia honey. The average values obtained in the study (10.45 to 11.02 mEq/1000 g) are lower than in Slovenia (24 mEq/1000 g), are similar to a Croatian study (10.1–10.7 mEq/1000 g) and a study conducted by Italian authors (11.2 mEq/1000 g), and are moderately higher than in the results of other Croatian researches and one in Romania (6.5–8.4 mEq/1000 g) [36, 38–40].

The average values of electrical conductivity in the samples of this study that range from 0.15 to 0.18 mS/cm comply with the results obtained on 513 samples of the European acacia honey (0.16 mS/cm) and the results of the Romanian and Croatian researchers (0.11 mS/cm-0.22 mS/cm) [36, 37, 39, 41] but are lower than the average values of the Slovenian acacia honey (0.26 mS/cm) [40].

We found the average sum of glucose and fructose (reducing sugars) is 69.33 g/100 g, in the range of 68.46–70.62 g/100 g. On the European research level conducted on 454 samples of acacia honey, the obtained average value is 69.2 g/100 g [35]. Other literature sources from Croatia and Romania also show the results that match this research (67.4–71.5 g/100 g) [37, 39, 42].

All the tested samples have met the requirement regarding saccharose level ranging from 0.49 to 0.62 g/100 g, which is moderately lower than the samples of acacia honey from Romania (1.55 g/100 g), Croatia (from 2.4 to 4.9 g/100 g), and Italy (2.1 g/100 g) [37–39, 41].

The obtained average values of diastase activity in our study ranged from 10.38 to 13.75 DN and are in accordance with the European and previous Croatian results for acacia honey [37, 38].

The proportion of HMF was low (an average of 3.56–5.92 mg/kg), which is less than 10 mg/kg. However, it can be said that, according to this category, honey used in the study is of the first class. In contrast, Šarić et al., who also studied samples of the Croatian acacia honey, obtained values in a wider range (4.7–36.5 mg/kg) [37]. The proportion of HMF in the Moroccan honey ranged from 3.2 to 52.6 mg/kg [43].

A total of twelve micro and macro elements in acacia honey (K, Ca, Na, Mg, Zn, Al, Fe, Cu, Mn, Ni, Pb, and Cd) from three Croatian regions were examined through a percentage (%) of each macro and micro element, as well as through the range and the average value of each macro and micro element expressed in mg/kg. The total proportion of all the elements in honey was 565.55 mg/kg on average, which makes the average less than 0.06% (expressed as ash proportion) and is within the normal proportion of elements in floral honey to 0.2% [3]. Macro elements (K, Ca, Na, and Mg), thereby, accounted for an average of 554.64 mg/kg or 98.07% of all the elements. Potential contaminants (Pb and Cd) accounted for a total average of negligible ≤0.06 mg/kg or ≤0.02%. As expected, K had the largest proportion and its percentage ranged from 51.23% to 70.29% ($\overline{x}\% = 57.56\%$). The determined concentrations were in the range of 258.69 mg/kg to 360.80 mg/kg ($\overline{x} = 325.54$ mg/kg). It was followed by Ca with an average proportion of 19.67%, then by Na with 16.95%, and Mg with 3.89%. Our results are consistent with the results of the mineral composition research of the Slovenian and Romanian honey [39, 40]. Kruskal-Wallis test did not show significant differences between the regions with regard to concentrations of the twelve elements measured in the samples of honey ($p = 0.89$).

Even though the mineral substances in honey are poorly represented with regard to quantity (an average of 0.1–0.2% or less in floral honey), honey contains a number of mineral substances of which some are very important for the proper functioning of the human body. The most common is K, which usually makes 25%–50% of the total proportion of mineral substances. More often, it is common for Na, Ca, and P to appear (10.58). Accordingly, in the Croatian and European regulations the values are prescribed—they allow the maximum proportion of ash to be 0.6% for floral honey [43]. These values are prescribed because the increased proportion of ash can be a sign of honey adulteration with sugar molasses [44]. It is only necessary to point out that the regulations regarding maximum levels of certain contaminants in foodstuff in Croatia prescribe maximum levels for certain contaminants that may be present in certain food, including heavy metals and nonmetals; however, maximum permitted levels of Pb and Cd or other contaminants in honey are not prescribed [3, 4].

In addition to honey, the same twelve elements were determined in the soil (K, Ca, Na, Mg, Zn, Al, Fe, Cu, Mn, Ni, Pb, and Cd), sampled from the apiary sites where the samples of honey were taken for this research. The total proportion of all the elements in the soil was an average of 51,306.88 mg/kg (range of 41,205.11 mg/kg in Northwest Croatia to 64,371.18 mg/kg in Istria). Kruskal-Wallis test did not show significant differences between the regions with regard to the concentrations of the twelve metals measured in the soil samples ($p = 0.845$).

The order is as follows: Fe had the highest average proportion in the soil ($\overline{x}\% = 48.70\%$); it was followed by Al with an average proportion of 30.92%; Ca is in the third place with 8.58%; then the following elements follow: Mg, whose average proportion was 6.59%; then K, with an average proportion of 2.60%; then Mn with around 1.18%; and Na with an approximate proportion of 1.14%. The proportion of all the other elements, including potential contaminants (Cd and Pb), ranged between 0.15% (Zn) and ≤0.01% (Cd). These results are, with some exceptions, more or less consistent with the results of the research conducted by the Geological Survey, when creating the Geological Atlas of Croatia, and are evidently geochemically and geologically specified [45]. Generally, it can be said that the preparations and analyses of the soil samples for mineral composition are a contribution to the obviously correctly conducted procedures of sampling. In addition, the results reveal a good environment condition (in this case its component, the soil) in locations where bees are bred in the Republic of Croatia.

It is considered, as it has already been pointed out, that the proportion of mineral substances in honey largely depends on its botanical origin, but also on the climatic conditions, and the composition of soil on which the honey plant was growing [16]. Moreover, it is also believed that the characteristic soil composition of a particular region can be seen in the mineral composition of honey plants or the mineral composition of its nectar and pollen [46]. Nevertheless, in our study we have not confirmed the aforementioned since no significant correlation between the concentrations of the twelve elements in honey and soil was found. This lack of correlation should certainly point to the fact that the concentrations of elements in honey were not dependent on their concentration in the soil and this may shed new light on the theory regarding the connection between the concentration of minerals in the soil and honey, which should be studied in the future.

Multivariate analyses (cluster and PCA) of the chemical composition of acacia honey from three Croatian regions surprisingly showed good separation of the characteristic features of honey in individual Croatian regions. First, the cluster analysis showed the separation of the characteristics of honey in East Croatia in relation to the other two regions. After that, the PCA analysis confirmed the diversity of the composition of honey from East Croatia with higher concentrations of most elements (all except Al), higher values of water and HMF, and lower values of the proportion of pollen, electrical conductivity, free acids, reducing sugars, saccharose, diastase activity, and Al. Conversely, acacia honey from Northwest Croatia has been characterized by higher concentrations of saccharose and lower concentrations of almost all of the other remaining parameters, while honey from Istria has been characterized by higher values of the proportion of pollen, free acids, electrical conductivity, reducing sugars, and diastase activity, and with the lower concentrations of water, saccharose, HMF, and a majority of examined metals. The "link" between honey from Northwest Croatia and that from Istria is the higher concentration of Al.

4. Conclusion

In conclusion, we could say that, by analyzing pollen, physicochemical parameters, and mineral composition of acacia honey from three Croatian regions and the mineral composition of the soil from the same locations, we presented the high quality of honey and the lack of correlation between the mineral composition of honey and soil. By using multivariate statistical methods, we may have made a contribution to the attempts of optimizing analytical and statistical methods which could have predictive significance for the botanical and geographical composition of honey.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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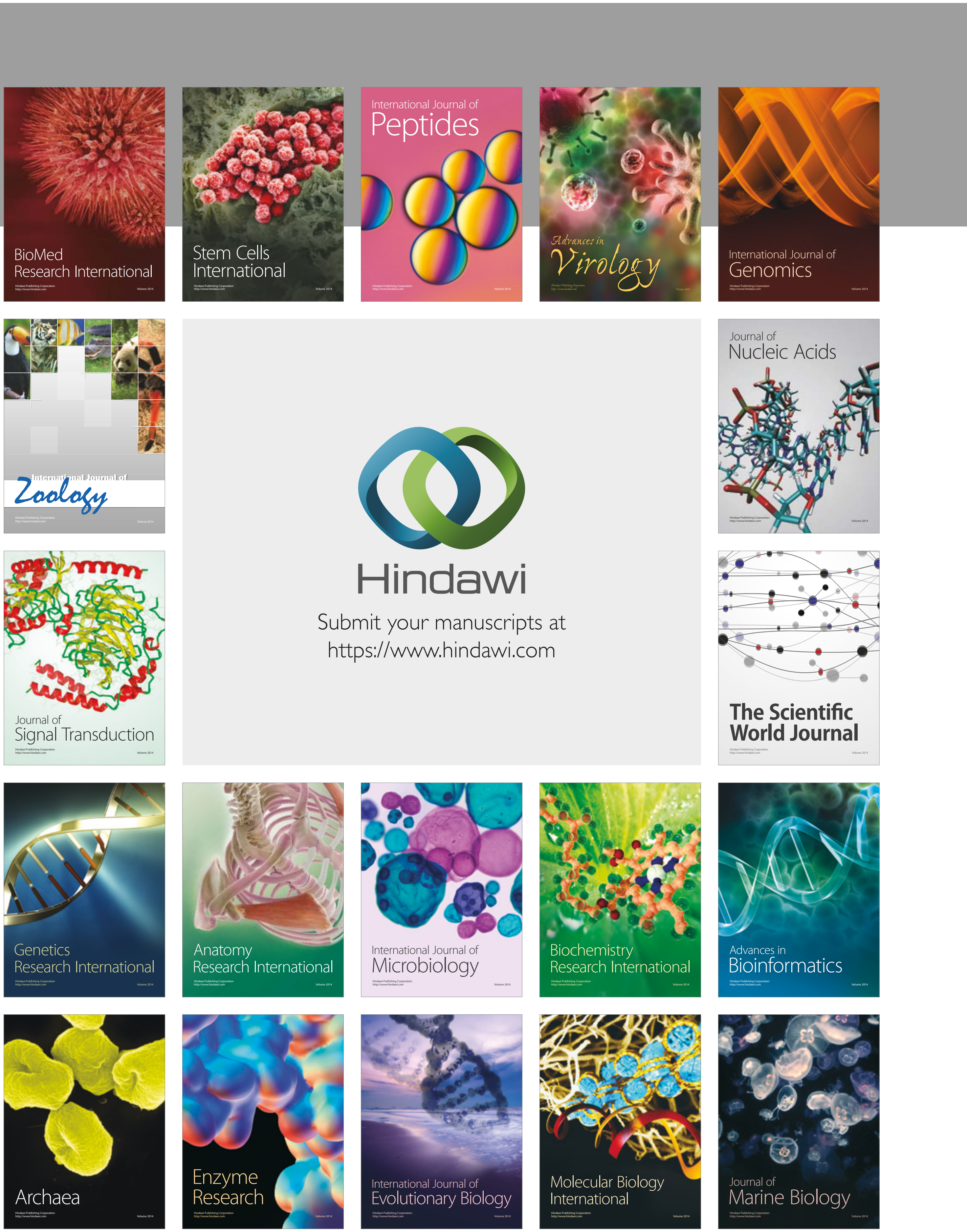
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