Original scientific paper
UDC 638.162(497.5-11)

PROFILE AND DIVERSITY OF PHYSICOCHEMICAL PARAMETERS OF THE BLACK LOCUST HONEY FROM EASTERN CROATIA REGION GIVEN THE TESTING SEASONS

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Abstract

According to its chemical composition, honey is a complex mixture of over 70 ingredients, which enter honey in a variety of ways. Not only that the different types of honey differ, but the honey within each species differs in its composition depending on its herbal and geographical origin, climatic conditions, the type of bees, and the work of the beekeeper. A number of European countries have the national legislation and the reference methods used for local quality control, but they are often specific for a particular country and cannot always be applied in the commercialization of honey for the international sales. Therefore, the profession in accordance with expert associations - International Honey Commission IHC - seeks to harmonize the reference methods and standards for proving the authenticity of botanical origin of monofloral type of honey.

The purpose of this paper is to present the profile of physicochemical parameters of 40 samples of the black locust honey from Eastern Croatia region monitored by the season. Moreover, it will point out their differences within each testing season. Melissopalynological and physicochemical analysis are used to prove the botanical origin of honey, and whether the samples meet the general quality requirements set by the Regulations on the quality of monofloral honey.

Every tested sample meets the requirements set by the Regulations on the quality of monofloral honey regarding physicochemical parameters: water (14.96 - 20.28 %); free acid (8.0 - 16.9 mEq/1000 g); electrical conductivity (0.09 - 0.23 mS/cm); reducing sugars (63.27 - 73.48 g/100 g); sucrose (0.09 - 1.38 g/100 g); diastase (7.26 - 19.92 DN) and hydroxymethylfurfuraldehyde - HMF (1.26 - 14.70 mg/kg). Gained dana were processed by ANOVA) method. The analysis of variance gained an insight into the significance of differences (p - values) within the physicochemical parameters for water and electrical conductivity given the season black locust honey is from $p < 0.05$.

Botanical origin of all the samples, as declared by the manufacturer, was confirmed using the melissopalynological analysis-black locust.

Key words: *Black locust honey, Physicochemical parameters, Melissopalynological analysis, Analysis of variance.*

1. Introduction

According to its chemical composition, honey is a complex mixture of over 70 ingredients, which reach honey in a variety of ways (they are added by bees, originate from plants, or occur by ripening of honey in the comb) (Krell [8]).

Industrial production and falsification of honey is impossible because all the ingredients are still not completely defined. Therefore, honey is a natural food, produced exclusively by bees. Variability and non-existence of two samples of honey that are the same are the characteristics that best describe the chemical composition of honey (Uršulin-Trstenjak *et al.,* [22]). Not only that various types of honey differ, but the honey within each species differs in its composition depending on the plant and geographical origin, climatic conditions, bee races, and the work of the beekeeper (the way honey is processed and stored) (Škenderov and Ivanov [20]; Vahčić and Matković [24]).

The most common ingredients that make up 99% of honey are carbohydrates (mostly fructose and glucose) and water. Other substances (which make only < 1%) are proteins (including enzymes), mineral substances, vitamins, organic acids, phenolic compounds, aroma compounds (volatile compounds), and various chlorophyll derivatives, which are also responsible for sensory and nutritional properties of honey (Singhal *et al.,* [17]; Vahčić and Matković [24]).

Leading experts involved in this issue combine Melissa palynological determination of the type and number of pollen with sensory analysis and physicochemical analysis such as determining the water content, HMF (hydroxymethylfurfural), electrical conductivity, diastasis activity, pH values, and the sugar content (fructose, glucose, sucrose, erlose, raffinose, melesitose) (Persano Oddo and Bogdano [13]; Bogdanov *et al.,* [2]; Uršulin-Trstenjak *et al.,* [23]).

1.2 Aim of the paper

The aim of this paper is to describe the profile of physicochemical parameters of the black locust honey of Eastern Croatia region given the seasons, and to point out their differences within each testing season, after botanical origin was confirmed by melissopalynological analysis of each sample.

2. Materials and Methods

One type of unifloral honey was chosen: black locust honey. Forty samples extracted during the season 1 and season 2 were collected from the beekeepers in the Eastern Croatia region (marked in blue).

Until the analysis was conducted, the samples were stored in controlled conditions, in a dark place at room temperature.

The samples were divided based on the area of their origin and marked with appropriate labels (Table 1 and Figure 2).

After conducting the melissopalynological analysis, physicochemical parameters were determined (water, free acids, electrical conductivity, reducing sugars, sucrose, diastasis, and HMF). Statistical data analysis was performed by analysis of variance (ANOVA).

Pollen grains are identified and quantified using a microscope (at 400x magnification) from the preparation of honey sample. At least 500 pollen grains are counted cumulatively in steps of a hundred in a row, a total of five horizontal rows. The distance between each horizontal row is one visual field. By reviewing the five horizontal rows the whole preparation is encompassed. The share of individual type of pollen is calculated as the percentage of the total pollen number. A collection of reference pollen preparations is used for the determination of pollen grains from the samples based on comparison (DIN 10760:2002-05, [4]).

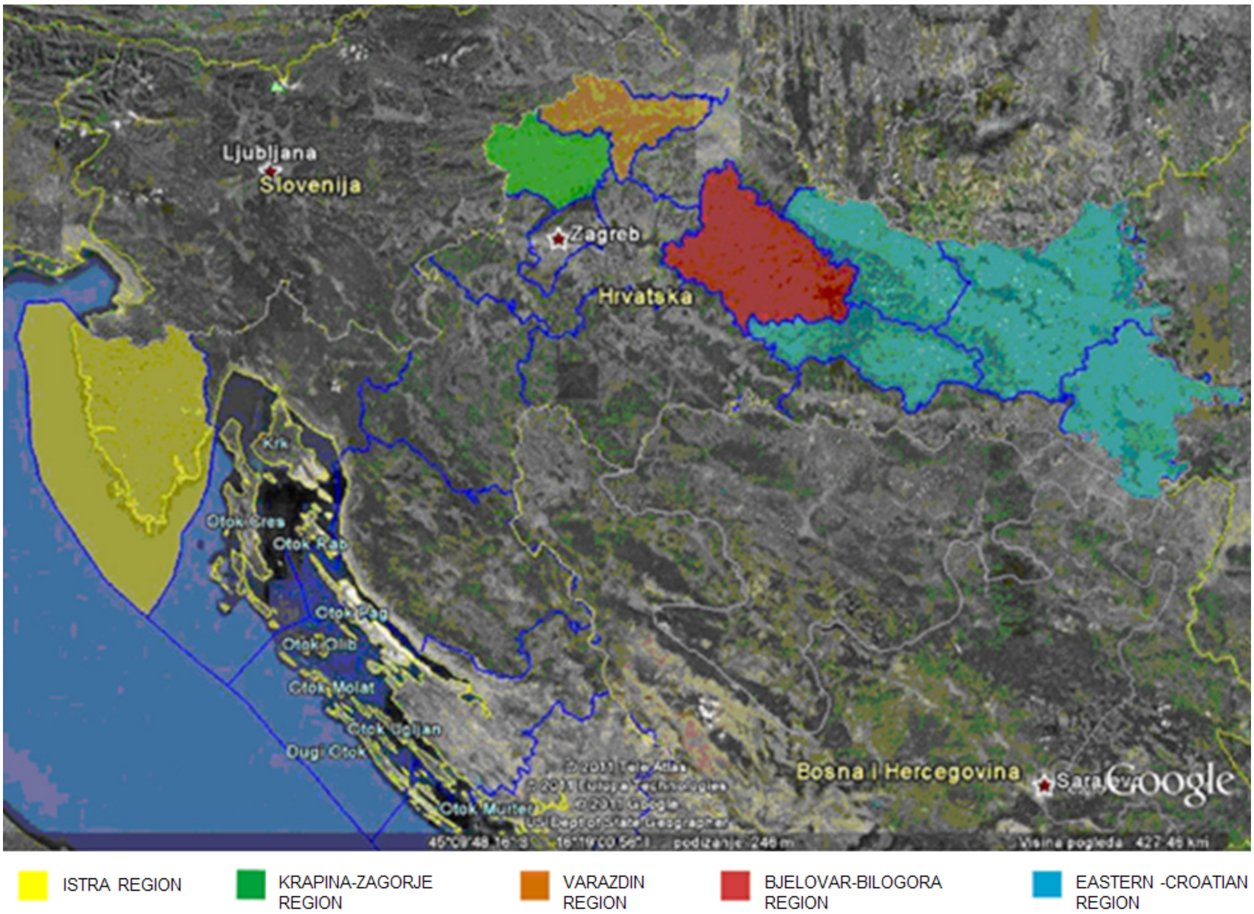

Figure 1. The map of geographical origin of honey (Uršulin-Trstenjak [21])

Table 1. The samples of black locust honey from Eastern Croatia region and their marking further in the text

Eastern Croatian region
IH-1
IH-2
IH-3
IH-4
IH-5
IH-6
IH-8
IH-9
IH-10
IH-11
IH-21
IH-29
IH-35
IH-38
IH-42
IH-46
IH-8
IH-113
IH-128

Figure 2. The map of honey pastures locations in the Eastern Croatia region

Water content in honey is determined by the method prescribed by the International Honey Commission based on refractometry (IHC, [6]).

A prepared sample is titrated using the solution of 0.1M of sodium hydroxide to a pH of 8.30, according to the method prescribed by the International Honey Commission (IHC, [6]).

Electrical conductivity in honey is defined as the conductivity of a 20% aqueous solution of honey at 20 ^{0}C, where 20% refers to the dry weight of honey. The results are expressed in mS/cm (IHC, [6]).

The method is based on the reduction of Fehling's solution by titration with a solution of sugar from the honey, with the use of the methylene blue dye as an indicator. The share of sucrose is obtained from the difference between the results obtained before and after the inversion (IHC, [6]).

The method is based on the hydrolysis of 1% solution of starch by the enzyme from 1g of honey during one hour at a temperature of 40 ^{0}C (IHC, [6]; SLMB, [16]; Bogdanov *et al.*, [1]; DIN 10750:1990, [3]).

The method is based on the reaction of the hydroxymethylfurfural with barbituric acid and p-toluidine, which gives it a pink color, the intensity of which is measured at a wavelength of 550 nm (IHC, [6]; SLMB, [16]).

The mean value, standard deviation and coefficient of variation of the chosen physicochemical parameters in the samples of black locust honey have been calculated. Statistical data analysis included analysis of variance (ANOVA). For this purpose software package STATISTICA 9.1 has been used (StatSoft, [18]).

3. Results and Discussion

3.1 Results

Tables 2 and 3 give an insight into the results of pollen analysis of the black locust honey from the Eastern Croatia region for the seasons 1 and 2.

Table 2. Pollen analysis of the black locust honey from the Eastern Croatia region - season 1

	SAMPLE																			
POLLEN TYPE (%)	IH-1	IH-2	IH-3	IH-4	IH-5	IH-6	IH-7	IH-8	IH-9	IH-10	IH-11	IH-21	IH-29	IH-35	IH-38	IH-42	IH-46	IH-80	IH-113	IH-128
Robinia sp	23	33	27	31	40	59	44	22	30	25	11	48	43	23	38	47	55	49	34	29
Rosaceae	14	9	14	27	23	20	18	11	42	27	20	29	20	22	6	24	12	20	18	24
Brassicaceae	56	42	33	16	17	3	22	53	7	30	47		20	37	38	10	19	12	33	15
Fabaceae	*p*	5	5	4	4	7	5	6	2	12	13	15	5	12	10	9	8	10	5	22
Asteraceae		*p*	*p*	2		*p*	2	1	*p*	*p*	2			*p*	*p*		*p*	*p*	*p*	*p*
Poaceae	*p*		*p*	*p*	*p*	*p*		*p*	*p*	*p*	*p*	*p*	*p*	*p*	*p*			*p*		*p*
Amorpha frutcosae									5											
Castanea sativia			11	7	12				3											
Rhamnaceae				*p*			*p*					*p*			*p*			*p*		
Apiaceae		*p*				*p*	*p*			*p*	*p*				*p*					*p*
Plantago	*p*	*p*	p							*p*					*p*					
Quercus sp	*p*	1	3	4	*p*	1	*p*	*p*			*p*	*p*	4	3	4	*p*	*p*	2	*p*	
Salix sp		*p*			*p*				2	*p*	*p*	*p*		*p*		*p*	*p*	*p*	*p*	
Fraxinus sp	*p*	1	3	2	1		3	1	3			*p*		*p*		*p*			*p*	*p*
Polygonaceae																				
Sambucus sp				3																
Lamiaceae							*p*													
Comus sp	2	5		*p*		2	2	*p*	3	2	3		3	*p*	*p*	5	*p*	3	5	5
Tilia sp		*p*	*p*				*p*													
Loranthus sp			*p*	*p*		*p*		*p*	1											
Cistaceae																				
Taxus							*p*	*p*	*p*											
Pinus sp						*p*		*p*												
Oleaceae																				
Rumix sp			*p*		*p*	*p*		*p*			*p*									
Impatiens			*p*																	
Boraginaceae																				
Vitaceae																				
Moraceae																*p*				
Ericaceae				*p*			*p*													
Fagus sp.																				
Aar sp						*p*						*p*								
Rubiaceae																				
Clusiaceae																				
Caryophyllaceae						*p*														
Convolvulaceae																				
INDET	3	2	1	2	1	3	2	3	1	2	1	4	3	1	1	3	3	2	3	3
Other	2	2	3	2	2	5	2	3	1	2	3	4	2	2	3	2	3	2	2	2

p – smaller than 1%

Table 3. Pollen analysis of the black locust honey from the Eastern Croatia region - season 2

POLLEN TYPE (%)	SAMPLE																			
	IH-1	IH-2	IH-3	IH-4	IH-5	IH-6	IH-7	IH-8	IH-9	IH-10	IH-11	IH-21	IH-29	IH-35	IH-38	IH-42	IH-46	IH-80	IH-113	IH-128
Robinia sp	70	42	66	29	26	61	22	31	57	30	60	43	59	26	25	29	39	48	31	22
Rosaceae	3	*p*	4	25		21	8	11	8	16		26	9	1	4	5	16	21	19	*p*
Brassicaceae	5	22	3	17	59	2	5	42	10	20	18	2	4	2	61	56	4	12	35	72
Fabaceae	13	9	5	5	1	5	3	6	10	5		19	9	64	2	2	17	7	5	1
Asteraceae	*p*	*p*	*p*	3		1	*p*	1	*p*	2	2		*p*	*p*	1		2	*p*	*p*	
Poaceae	*p*		*p*	*p*		*p*		*p*	*p*		*p*	2	*p*	*p*	*p*	*p*	p	1		
Amorpha frutcosae																				
Castanea sativia	*p*	16	9	5			*p*	*p*	2	*p*	3		1							
Rhamnaceae				*p*								*p*						*p*		
Apiaceae			*p*			*p*					*p*		*p*							
Plantago									*p*		*p*		*p*							
Quercus sp	*p*	*p*	2	4		1		*p*		*p*		1	*p*				2	2	1	
Salix sp	3	3	5		10		4		4	11	5	*p*	10	2	2	3	13	1	*p*	*p*
Fraxinus sp				2	*p*			1			5	*p*					p		*p*	
Polygonaceae																				
Sambucus sp				3	*p*		*p*						*p*							
Lamiaceae										*p*										
Comus sp	*p*	1		1		2	*p*	2	3	*p*			1	*p*	*p*		1	3	4	1
Tilia sp		*p*								2			2							
Loranthus sp	*p*		*p*	1		1	*p*	*p*	1											
Cistaceae																				
Taxus	*p*							*p*									p			
Pinus sp						*p*	*p*	*p*							*p*					
Oleaceae											*p*									
Rumix sp						*p*		*p*					*p*				p			
Impatiens															*p*					
Boraginaceae							53						*p*	*p*						
Vitaceae																				
Moraceae			*p*							*p*						*p*				
Ericaceae				*p*																*p*
Fagus sp.	1																			
Aar sp						*p*						*p*								
Rubiaceae																				
Cucurbitaceae																	2			
Caryophyllaceae		*p*	*p*		*p*	*p*	*p*													
Ligustrum	*p*	*p*			*p*								*p*							
INDET	1	3	3	3	2	3	3	3	3	2	3	4	2	3	3	3	2	3	2	2
Other	4	4	3	2	2	3	2	3	2	2	4	3	5	2	2	2	2	2	3	2

p – smaller than 1%

Tables 4 and 5 give an insight into the results of analysis of seven studied physicochemical parameters (water content, free acids, electrical conductivity, reducing sugars, sucrose, HMF and diastase) in the black locust honey from the Eastern Croatia region through seasons 1 and 2.

Table 4. Physicochemical parameters of the black locust honey from the Eastern Croatia region - season 1

	ANALYSES	water (%)	free acid (mEq/1000g)	elctrical conductivity (mS/cm)	reducing sugars (g/100g)	sucrose (g/100g)	diastase (DN)	HMF (mg/kg)
SAMPLES	IH-1	17.72	10.3	0.15	69.86	0.19	12.51	3.00
	IH-2	15.76	10.2	0.14	69.37	0.23	10.82	14.70
	IH-3	16.52	10.3	0.15	67.67	0.00	12.46	5.30
	IH-4	15.04	9.1	0.11	63.27	0.22	10.53	7.50
	IH-5	17.40	10.2	0.14	67.39	0.09	8.63	8.00
	IH-6	15.24	9.9	0.13	68.89	1.02	10.14	4.10
	IH-7	16.48	10.2	0.14	65.44	0.66	8.88	7.60
	IH-8	15.64	10.1	0.13	69.47	0.66	12.68	8.40
	IH-9	16.32	10.9	0.18	67.30	0.52	12.20	13.40
	IH-10	16.48	14.2	0.09	70.57	0.00	19.06	10.70
	IH-21	17.00	8.0	0.11	65.61	0.49	7.26	3.30
	IH-29	16.52	9.1	0.11	67.39	0.27	7.36	6.30
	IH-35	16.60	9.2	0.13	69.08	0.23	9.33	11.00
	IH-38	17.36	11.0	0.14	67.03	0.96	10.68	12.80
	IH-42	15.04	10.5	0.13	67.67	0.44	10.76	4.20
	IH-46	14.96	11.0	0.15	67.39	1.33	10.30	7.20
	IH-80	19.08	8.9	0.12	67.30	0.36	10.80	5.10
	IH-113	15.84	8.9	0.12	68.79	0.26	7.89	7.50
	IH-128	16.40	11.0	0.22	68.23	0.15	13.25	13.80
	$\bar{x}$	16.39	10.17	0.14	67.77	0.43	10.82	8.10
	σ	1.05	1.30	0.03	1.73	0.36	2.68	3.68
	c.v. (%)	6.38%	12.76%	20.32%	2.55%	85.16%	24.80%	45.42%

$\bar{x}$ – Mean value; σ – standard deviation; CV (%) – Coefficient of variation

Table 5. Physicochemical parameters of the black locust honey from the Eastern Croatia region - season 2

	ANALYSES	water (%)	free acid (mEq/1000g)	elctrical conductivity (mS/cm)	reducing sugars (g/100g)	sucrose (g/100g)	diastase (DN)	HMF (mg/kg)
SAMPLES	IH-1	17.04	9.9	0.15	70.57	0.66	10.41	2.62
	IH-2	17.00	10.0	0.18	71.26	0.59	9.64	9.26
	IH-3	16.60	10.0	0.15	71.47	0.89	9.94	3.61
	IH-4	15.36	9.5	0.12	70.57	0.00	10.51	5.70
	IH-6	17.64	11.1	0.15	70.66	0.00	11.83	4.16
	IH-8	20.28	8.2	0.11	68.51	1.38	7.94	1.80
	IH-9	17.80	13.0	0.21	65.78	0.33	15.61	2.06
	IH-10	20.20	32.5	0.51	70.57	0.00	26.02	1.31
	IH-11	15.40	8.0	0.12	63.75	0.48	8.53	3.26
	IH-21	19.36	9.1	0.12	63.43	0.54	9.61	2.05
	IH-29	18.08	16.9	0.23	66.85	0.95	14.58	5.65
	IH-42	18.12	11.0	0.15	70.45	0.11	13.25	3.04
	IH-46	16.04	15.2	0.24	73.48	0.52	19.92	1.26
	IH-80	18.78	8.5	0.13	70.57	1.24	10.70	5.20
	IH-113	17.24	11.1	0.15	70.57	0.00	13.14	2.57
	$\bar{x}$	17.48	10.82	0.16	69.14	0.55	11.83	3.73
	σ	1.42	2.60	0.04	3.03	0.45	3.23	2.14
	c.v. (%)	8.11%	24.07%	26.23%	4.39%	81.57%	27.27%	57.22%

$\bar{x}$ – Mean value; σ – standard deviation; CV (%) – Coefficient of variation

Table 6. The significance of differences (p-values) within certain physicochemical parameters given the season of origin of black locust honey (two-factor analysis of variance of data from Tables 4 and 5)

source ofvariations / parameters	p-value seasons
water	0.0264
free acids	0.2867
electrical conductivity	0.0777
reducing sugars	0.0884
sucrose	0.5861
diastasis	0.0781
HMF	0.0571

3.2 Discussion

The confirmation of the botanical origin of honey as declared by the manufacturer, was performed conducting the melissopalynological or pollen analysis, and by determining the conformity of physicochemical parameters (electrical conductivity, free acids, diastase) with literature data and the requirements of the Regulations. In addition, the share of water, reducing sugars, sucrose and HMF were analyzed to determine whether the samples meet the general quality requirements determined by the Regulations on the quality of unifloral honey (MPRRR, 2009b [12]). According to some studies, botanical origin of honey can be demonstrated by combined analyses regarding electric conductivity, pH values, free acids, and the proportions of sugars fructose, glucose and raffinose (Mateo, Bosch-Reig [10]).

By conducting pollen analysis in the samples of black locust honey during both seasons pollen grains of 36 plant species were found. The most common are those of the family of *Robinia* (black locust family), *Rosaceae* (rose family), *Brassicaceae* (cabbage family), and *Fabaceae* (bean family) (Tables 3 and 4).

Melissopalynological analysis conducted in the first testing season for 19 samples (out of 20) and in the second testing season for 15 samples (out of 20) has helped define botanical origin as defined by the manufacturer - black locust honey. The samples that did not meet the requirements for being classified as unifloral black locust honey are indicated in Tables 3 and 4 in a different color and have not been taken into consideration in the process of further research. In season 1, that is IH-11 (insufficient number of black locust pollen grains) and in season 2, these are the following: IH-5, IH-7, IH-35, IH-38, H-128 (sufficient number of pollen grains from 22% to 26%, but with a large and dominant percentage of other types of pollen grains (MPRRR, 2009b, [12]).

Melissopalynological or pollen analysis, as one of the parameters in determining the botanical origin of honey, was followed by the determination of the physicochemical quality parameters according to the criteria prescribed in the Honey Regulations (MPRRR, 2009a, [11]). All the tested samples have satisfied the requirements of the quality of black locust honey, except for one sample in season 2 – IH-10 (high values of electrical conductivity 0.51 mS/cm, diastase 26.02 DN, and water 20.20%). The values obtained were compared with the literature data (Tables 4 - 5).

Water content in the analyzed samples is below 20% (MPRRR, 2009a [11]) and ranges according to the mean values from 14.96 to 19.36%, which confirms the results of other researchers on samples of black locust honey from Croatia (15.40 to 16.30%), the results of Kenjerić *et al.*, (also from Croatia) monitored through two years (16.50% and 16.10%) (Šarić *et al.*, [19]; Kenjerić *et al.*, [7]). Variance analysis of the data shows that the difference between the seasons is statistically significant ($p < 0.05$).

Permitted value of free acids in honey is < 50 mEq of acid/ 1000g of honey (MPRRR, 2009a [11]), which is shown accordingly by the mean values of the study from 8.0 to 16,9 mEq/1000g. Literature data show various values of free acids in the samples of black locust honey in relation to this research. The obtained average values of this research are lower when compared to those obtained by Golob and Plestenjak (24 mEq/1000g), but same as in Kenjerić *et al.*, (2002 - 10.7 mEq/1000g; 2003 - 10.1 mEq/1000g), and the Italian researchers (11.2 mEq/1000g), or are slightly higher than the results obtained by **Šarić** (7.3 to 8.4 mEq/1000g; 6.45 mEq/1000g) (Golob and Plestenjak [5]; Kenjerić *et al.*, [7]; Persano Oddo and Piro [14]; **Šarić** *et al.*, [19]). The conducted analysis of variance of the data shows that there is no statistically significant difference in the proportion of free acids between the regions ($p > 0.05$).

The mean values of electrical conductivity in the samples of this study ranged from 0.03 to 0.23 mS/cm, which meets the general requirements of a maximum of 0.8 mS/cm (MPRRR, 2009a [11]). These results are also equal to the results obtained in 513 samples of the European black locust honey (0.16 mS/cm), and the average values obtained by Marghitas *et al.*, [9], Kenjerić *et al.*, [7] (0.15 mS/cm; in 2003 0.12 mS/cm), but are lower than the mean values of Slovene black locust of 0.26 mS/cm (Marghitas *et al.*, [9]; Kenjerić *et al.*, [7]; Golob and Plestenjak [5]). Variance analysis of data shows that there is a statistically significant difference in electrical conductivity between the seasons ($p > 0.05$).

The sum of the glucose and fructose share - reducing sugars, or reducing sugars, in honey must be at least 60 g/100 g (MPRRR, 2009a). At the level of the European research on 454 samples of black locust honey, the average value obtained was 69.2 g/100g (Persano Oddo and Piro, [14]), which corresponds to the values obtained in this study - from 63.27 to 73.48 g/100g. There is no statistically significant difference in the proportion of reducing sugars between the regions ($p > 0.05$), as demonstrated by analysis of variance.

All the samples tested met the requirement regarding the sucrose share (up to 10 g/100 g of the sample) for both the seasons 2009 and 2010, in all five regions (MPRRR, 2009a [11]). The share of sucrose ranges from 0.00 to 1.38 g/100g and is lower than the results obtained in the samples of black locust honey by other researchers from Romania (1.55 g/100g), Croatia (from 2.4 to 4.9 g/100g, 2.8 g/100 g) and Italy (2.1 g/100 g) (Marghitas *et al.,* [9]; Šarić *et al.,* [19]; Primorac *et al.,* [15]; Persano Oddo and Piro [14]). Analysis of variance showed no statistically significant difference in the proportion of sucrose given the seasons between the regions ($p > 0.05$).

General requirement for the value of diastase activity is 8 DN, except for species with low enzyme activity (citrus, black locust) where the DN can be < 8, but the proportion of HMF then must be ≤ 15 mg/kg (MPRRR, 2009a). The resulting mean values are from 9.26 to 19.92 DN and are in accordance with the European (10.5 DN) and other Croatian results for black locust honey (Persano Oddo and Piro [14]; Šarić *et al.,* [19]). Analysis of variance for diastase activity shows no statistically significant difference between the seasons ($p < 0.05$).

The share of HMF is low - a very large percentage of honey samples had less than 10 mg/kg (which presents the upper limit for first-class honey), and they ranged from 1.26 to 14.70 mg/kg and are below the mean values obtained by Šarić *et al.,*, also based on the samples of the Croatian black locust honey (from 4.7 to 36.5 mg/kg) (Šarić *et al.,* [19]). Analysis of variance for HMF shows that there is no statistically significant difference in the proportion of HMF between the seasons ($p > 0.05$).

4. Conclusions

Based on the results obtained, the following can be concluded:

- The confirmation of botanical origin of all the honey samples, as declared by the manufacturer, was conducted by:

- melissopalynological or pollen analysis - in testing season, 1 for 19 samples (out of 20) and in testing season 2, 15 samples (out of 20) and by
- confirming the conformity of physicochemical parameters (water, free acid, electrical conductivity, reducing sugars, sucrose, diastase and HMF) - except for one sample in season 2.

- 33 samples out of 40 tested samples meet the requirements of the Regulations on the quality of unifloral honey.

- Using data analysis of variance an insight has been gained into the significance of differences (p-values) among physicochemical parameters - water and electrical conductivity - given the season that black locust honey comes from ($p < 0.05$).

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