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Professional paper

NORTH MACEDONIAN FOREST HONEY: A STUDY OF THE HYDROXYMETHYLFURFURAL, SUGAR PROFILE AND PHYSICAL QUALITY PARAMETERS

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Abstract
Forest honey obtained from North Macedonia during the harvest season 2019 and 2020 was investigated to determine the quality and evaluate its physical-chemical properties. For analysis 45 samples were obtained from various locations and compared against the national and international standards. Physical-chemical properties evaluated were with significant variations ($P<0.05$) between the different regions in terms of moisture content, free acidity, hydroxymethylfurfural (HMF), reducing sugars, sucrose content and electric conductivity. The average values of honey samples for moisture, free acidity, HMF, apparent reducing sugar, sucrose and electric conductivity were: 16.65 %, 24.94 meq/kg, 12.40 ppm, 69.25 g/100g, 1.77 g/100g, and 1.49mS/cm, respectively. This study indicates that honey shows excellent quality properties and that all samples collected were in compliance with the national and international standard limits.

Keywords: HPLC, reducing sugar, quality properties, hydroxymethylfurfural (HMF)

1. Introduction

The physicochemical characteristics of honey are an important indicator of the quality and origin of honey. The physicochemical characteristics of honey depend on the flowers used by the honeybees, as well as regional, beekeeping practices and environmental climatic variations. Many researchers have been carried out physical and chemical properties of honey from different sources. The major quality norm of interest is sucrose content and reducing sugars content, moisture content, electrical conductivity, free acidity, hydroxymethyl furfural (HMF) content and diastase activity (Ahmed *et al*, 2014; Jovanović, 2015; Ajibola, 2015). Beekeeping occupies a special place in Balkan culture, and the richness of forests and untouched land have made honey production a valuable asset for many mountain communities and in North Macedonia. Especially in the Republic of N. Macedonia, this tradition has a unique aspect with the presence of the original bee subspecies *Apis mellifera macedonica*, which is located in the entire historical region of North Macedonia, from eastern Albania through Bulgaria. The relief characteristics in North Macedonia are the main condition for the flow of honey to occur in May and June (in the Lower Areas) and in June and August in mountainous areas, where, if there are conditions in August, the flow of honey occurs on woody species producing black (dark) forest honey (https://www.fondazioneslowfood.com/en/slow-food-presidia/macedonian-honeybee). The Ministry of Agriculture of the Republic of Northern Macedonia announced that more than 7,000 Macedonian families work in beekeeping and that, according to estimates, at the end of 2018, there

were more than 240.000 hives in the country. One hive can give between 18-35 kg of honey per year, depending on the weather. According to the Food and Veterinary Agency, 6.000 hives with over 240.000 bee families have been registered in the country. In 1992, the country recorded a production of 1.600 tons of honey. Today, due to climate change and the increase in pesticide use, honey production has declined.

According to FAO STAT data, in 2018, about 475 tons were produced (http://www.fao.org/faostat/en/#data/QL,; https://www.efe.com/efe/english/destacada/high-altitude-apiculture-in-the-mountains-of-north-macedonia/50000261-4033543).

Primorac et al (2009) conducted that the samples of Macedonian honeydew honey showed statistically significant higher electrical conductivity, proline content, free and total acidity and lower specific rotation, mostly negative values in comparison with Croatian samples. Stankovska *et al* (2006) analyzed 123 honey samples from the Republic of N. Macedonia in terms of cadmium content and founded that only 6 out of 123 samples in total had Cd content higher than 30 $\mu g kg^{-1}$ and only one much higher content of 267.9 $\mu g\ kg^{-1}$. To the best of our knowledge, there is a lack of research and published data on the correlation analysis of the quality parameters of N. Macedonian forest honey, though their production is relatively high. Therefore the aim of this study was to characterize forest honey quality according to physical chemical profile and assessment of correlations of physical and chemical properties of forest honey from N. Macedonia during period of 2019 - 2020.

2. Material and methods

Forty-five (45) honey samples from 16 different locations were provided by beekeepers during the harvest season 2019 and 2020, respectively compared with national and international standards. All samples were stored at suitable conditions until the time of their analysis. Honey samples were classified into sixteen groups based on their origin.

pH and total free acidity were determined according to AOAC Methods (2002). Moisture, electrical conductivity, HMF, sugar and reduced sugars content (HPLC) were analysed according to the European Honey Commission methods (2002). Determination of hydroxymethylfurfural content of honey samples using spectrophotometer. Electrical conductivity of a honey solution at 20% (dry matter basis) was measured at 20 °C in CD2-free deionised distilled water using conductivity meter, and the results were expressed as $\mu S.cm^{-1}$. Free acidity was determined by a titrimetric method: 0.1 M NaOH was added to 10 g honey in 75 mL free CO_2 distilled water until a pH value 8.30. Results were expressed as meq/kg^{-1}. All measurements were performed in triplicate. Moisture was determined by refractometer. All measurements were performed at 20 °C.

Determination of total monosaccharide's content of honey samples using HPLC-RID. The chromatographic analyses for determination of total monosaccharides content of honey samples were carried out a refractive index detector (RID) in the Agilent high performance liquid chromatography (HPLC) equipped with, and evaluated with the ChemStation Software. The chromatographic separation of sugars was achieved in an amine bonded phase column (µ-Bonapak), using acetonitrile/water (84:16) as mobile phase, at a flow rate of 1.0 mL.min-1 and refractive index detection (Ferreira *et al,* 1998). The sample preparation in both determinations was easy, involving only dissolution in deionized water and filtration through 0.45 µm PTFE filter.

2.1. Statistical analysis

Duncan analysis of variance (ANOVA) was used for statistical differences between samples. Correlation analysis and linear regression model were used to identify connectivity and possible causality of variability in significance level $P < 0.05$ using SPSS version 16.0 (SPSS Inc., Chicago, IL, USA.

3. Results and discussion

The physicochemical and chemical properties (moisture content HMF, total monosaccharide's, free acidity, electrical conductivity and sucrose) of honey samples areshown in Table 1. A significant difference was found amonghoney samples on all parameters.The average moisture content ranged from 15.9 to 18.60%. The same findings were conducted in the honey types from Turkey (Kivrak*et al,* 2017).Pires*et al* (2009) considered the moisture to be a useful parameter for describing viscosity of honey linked to climatic factors, the harvesting season, the degreeof maturity and environmental factors. In this study, the highest moisture content was determined on H7, H8, H9 and H12. The moisture content of honey samplesarecorrelated to its botanical origin (Liberato *et al*, 2013).

Table 1. Physical-chemical and chemical properties of forest honey samples.

Samples	Sucrose content (%)	Total mono-saccharides (%)	Moisture content (%)	Electrical conductivity (mS/cm)	Freea acidity (meq/kg)	HMF (mg/kg)
H1	*0.16^{bc}	76.71	16.65	0.41	15.2	12.4
H2	0.12^{a}	64.96	17.6	0.87	32.5	*1.2
H3	2.05^{h}	64.25	17.3	0.24	*11.4	0.78
H4	**0.71^{d}	69.02	17.25	*0.80	30.5	2.25
H5	4.51^{l}	67.25	**17.50	0.75	31	*1.2
H6	**0.72^{d}	66.05	17.65	0.7	44.5	1.78
H7	0.95^{e}	68.11	18.22	0.28	18.9	11.22
H8	4.05^{j}	76.14	18.60	0.62	24.4	2.2
H9	0.20	70.53	18.60	15.22	*11.5	36.35
H10	0.18^{c}	64.51	**17.5	0.31	14.7	17.22
H11	4.46^{k}	74.92	17.90	0.48	23.1	3.05
H12	*0.15^{b}	65.23	18.00	*0.85	42.2	2.01
H13	2.28^{i}	72.52	15.9	0.28	17.4	0.64
H14	1.13^{f}	78.35	*17.2	0.35	22.4	9.26
H15	1.68^{g}	64.35	16.5	1.2	35	3.37
H16	5.02^{m}	65.45	*17.2	0.53	24.3	2.75
Average	1.77	69.25	17.47	1.49	24.9	6.73
P-Value	***0.05***	***0.05***	***0.05***	***0.05***	***0.05***	***0.05***

a–p Different letters in each column correspond to significantly different values ($p < 0.05$).
*Anova – Duncan, *, ** = non significant in the column at P <0.05*

All the honey samples contained less than 20 % moisture content which is regulated for safety against fermentation. This is the maximum value allowed by European and Macedonian legislations for honey (Food and Agriculture Organization, 2001; Rulebook, Macedonia, 2011). Moisture is the key criterion that determines the ability of honey to remain fresh and free of fermentation (Bogdanov *et al*, 1999; Silva *et al,* 2009). According to the legislations, the amount of moisture that flower honeys can contain should be at most 20%. Accordingly, it is seen that all of the analyzed honey samples comply with the Codex Honey (2012/58) in terms of the amount of moisture they contain. The moisture content of honey is the most important factor related to the storage, granulation and fermentation (Ötleş, 1995). Sugars constitute 95% of the dry weight of honey (Kaškonienė *et al,* 2010). As a result of the interaction of sugars in honey with water molecules, the available water required for microorganisms decreases and thus the storage period of honey increases (Derebaşı *et al,* 2014). Similarly, a previous investigation of the honey samples from various locations of Turkey was reported 16 to 20% of moisture content (Can *et al,*2015).

Table 2. Correlations between physic-chemical properties

	Sucrose	Total monosach.	Moisture	Elect. Cond.	Acidity	HMF
Sucrose Pearson correlation Sig. (1-tailed)	1	.152 .575	.011 .969	-.240 .371	-.027 .920	-.418 .107
Total monosach. Pearson correlation Sig. (1-tailed)	.152 .575	1	.004 .988	042 .878	-.338 .201	.158 .559
Moisture Pearson correlation Sig. (1-tailed)	.011 .969	.004 .988	1	.414 .111	.039 .885	.361 .170
Elect. Cond. Pearson correlation Sig. (1-tailed)	-.240 .371	.042 .878	.414 .111	1	-.293 .271	.826 .000
Acidity Pearson correlation Sig. (1-tailed)	-.027 920	.338 .201	.039 .885	-.293 .271	1	- .536* .033
HMF Pearson correlation Sig. (1-tailed)	-.418 .107	.158 .559	.361 .170	.826** .000	-.536* .033	1

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

According to the water content, honey can be classified into: first category (moisture below 17.8%), second (moisture content maximum 18.6%), and third category (max. 20%) (Genç and Dodoloğlu, 2003). Accordingly, among the examined honey samples only 5 samples (H7, H8, H9, H11, H12) belongs to second category and all the remaining honeys are from the first category.

The moisture content in honey varies depends on the climatic conditions, harvested period and the maturity (Ribeiro *et al,* 2014). Moisture content in honey produced in hot and dry areas varies between 16-19%, and the moisture content in honey produced in cold and rainy areas varies

between 17-20%. No microbiological growth occurs if there is less than 171 g/kg of moisture in honey and osmophilic yeasts begin to develop if it is greater moisture than 200 g/kg (Tosi *et al*, 2004)

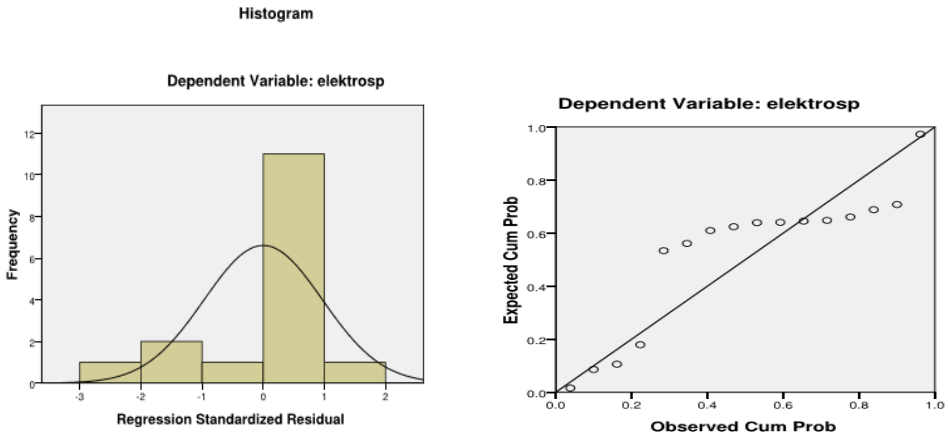

Figure 1. Histogram and frequency distributions between HMF and electrical conductivity

The amount of total monosaccharide (reducing sugar) and sucrose of honey samples are given in Table 1. The amount of total monosaccharide in honey samples was determined between 64.25-78.35% and 69.27% on average. According to the Rulebook of North Macedonia (2011) total monosaccharide amount of honey should be at least 60 g/100 g. According to the results we obtained in the study, the reducing sugar amount of all samples was in compliance with the National Regulations (Rulebook of N. Macedonia, 2011).

Table 3. Regression coefficient between HMF and electrical conductivity and total acidity

Model	Unstandardized Coefficients		Standardized Coefficients	t	Sig.	95 % Confidence Interval for B	
	B	Std. Error	Beta			Lower Bound	Upper Bound
Hmf – Electr.	.325	.059	.826	5.483	.000	.198	.452
Hmf – Acidit.	-.588	.248	-.536	-2.373	.033	-1.119	-.056

The amount of sucrose in honey has been determined as 0.12% (H2) - 5.02 (H16) and 1.77% on average probably due to early harvesting of honey or feeding bees with syrup containing sucrose or directly adding sucrose to honey (Duman Aydın *et al,* 2008; Saxena*et al,* 2010). According to the National Regulations (RNM, 2011), the sucrose content of honey should be maximum 5 g / 100g. According to the results we obtained in the research, the samples numbered 16 were not in accordance with the same regulation (RNM, 2011).

Table 1 shows the results for the titratable acidity of honey samples. Titratable acidity of honey was determined as 11.4-44.5 meq/kg (average 24.94) in terms of gluconic acid. According to the national regulations (2011), the free acidity of honey is allowed to be highest at 50 meq/kg. In the study, it was determined that all honey samples were suitable according Food quality regulation (RNM, 2011) in terms of titratable acidity.

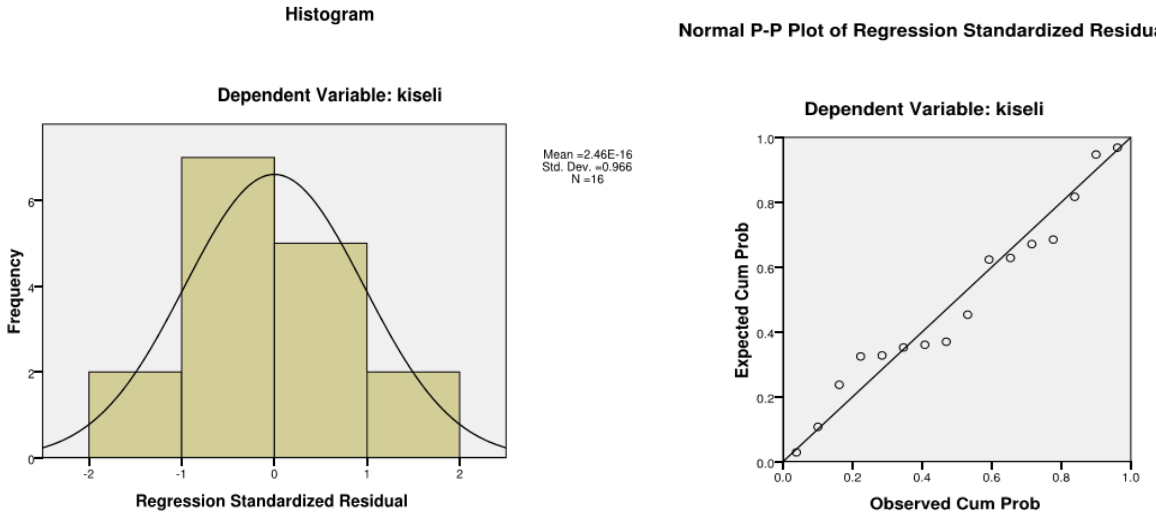

Figure 2. Histogram and frequency distributions between total acidity and HMF

All samples were determined to be suitable. The high sucrose content in the specified sample may indicate that Özcan and Ölmez (2014) found lower amount of reducing sugar at 51.31- 68.30 %. Also, Güler (2005) reported lower average amount of reducing sugar at 68.42% and sucrose at 1.54%. Ribeiro et al. (2014) determined higher amount of reducing sugar at 66.2-80.1 g/100 g, and the amount of sucrose as 2.2- 3.9 g/100 g.

Table 4. Pearson correlation coefficient between total acidity and HMF and HMF and electrical conductivity

	Acidity	HMF		HMF	El. conduct.
Pearson correlation Acidity HMF	1.000 -.536	-.536 1.000	Pearson correlation HMF El. conduct.	1 .826 “	.826** 1
Sig. (1-tailed) Acidity HMF	.016	.016	Sig. (2- tailed) HMF El. conduct.	.000	.000

**. Correlation is significant at the 0.01 level (2-tailed).

In the previous studies on the titration acidity of honey, Silva et al. (2013) reported values from 24.5 to 93.5 meq / kg andManzanares et al. (2014) reported from 16 to 48 meq / kg. Free acidity affects the chemical reactions in honey, contributes to the taste, increases antimicrobial and antioxidant properties (Cavia*et al.* 2007). Titratable acidity was significantly (P<0.05) negatively correlated (r= -0,536) with the HMF content in Table 2, 3 and their distribution relationship in Figure 2 meaning that in higher acidity conditions lower HMF reaction and products are formed.

HMF content in honey samples was determined to be with great variability between 0.64-36.35% and 6.73 % on average. According to the Rulebook of North Macedonia (2011) total HMF content of honey should be highest at 40 mg/kg. HMF is one of the quality parameters of honey, especially at pH 5 and below. According to the Honey Code, the maximum amount of HMF that can be found in honey is 40 mg/kg. (Rulebook of N.M, 2011). 5-hydroxy methyl furfural (HMF) is formed during heat treatment as an intermediate in the Maillard reaction that occurs between reducing sugars such as glucose and fructose in honey and amine groups of free amino acids and protein applied to honey. HMF content is high in honey that has been heat-treated, stored in irregular conditions, and mixed with inverted syrup. In the case of honey produced in tropical climates, the maximum amount of HMF allowed is 80 mg/kg if the production area is indicated on the label (Güney, 2010; Yıldız *et al*, 2010; Windsor *et al*, 2013; Şahin and Akdeniz, 2012).
HMF was significantly ($P<0.01$) positively correlated (r= 0,826) with the electrical conductivity in Table 4 and their distribution relationship in Figure1 indicating that higher HMF compounds promotes higher electrical conductivity activity.
Electrical conductivity in honey samples was also determined to be with great variability between 0.24-15.22 mS/cm and 1.49 mS/cm on average. According to the Rulebook, of North Macedonia (2011) total HMF content of honey should be highest at 0.8 mS/cm and among the examined honey samples only 3 samples (H2, H9, H12, H15) were not suitable according the standard norms.

Conclusions

The results obtained in this study show that honey produced in Northern Macedonia shows excellent quality properties in accordance with national and international standards. The average values of honey samples for moisture, free acidity, HMF, reducing sugar, sucrose, and electrical conductivity were: 16.65 %, 0.32 %, 24.94 meq/kg, 12.40 ppm, 69.25 g/100g, 1.77 g/100 g and 1.49 ms/cm, respectively. According to the regulations on the quality of honey in the North Macedonia, regarding electrical conductivity, only five samples did not meet the criterion. Moisture, titratable acidity, reducing sugar, sucrose, and total sugar of honey samples were found to be statistically significantly different at the level of $P < 0.05$ compared to honey samples. In the future, studies are needed to work on the profile of phenolic substances, antioxidant activity and aroma profile together with pollen profile of Macedonian honey.

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