PHYSICOCHEMICAL PROPERTIES AND HMF LEVEL OF HONEY SAMPLES FROM VARIOUS FLORAL ORIGINS IN ALBANIA

XHULIETA HAMITI, GJYLIZA SHALLARI,

ELDORIDA MOLLA, ELENA MUÇA, PRANVERA LAZO

Department of Chemistry, Faculty of Natural Sciences, University of Tirana

e-mail: xhuljeta.hamiti@fshn.edu.al

Abstract

Many countries have studied characteristics of different mono-floral and poli-floral honeys products about the physicochemical composition, compounds contents and curative properties of each type. We have chosen to study the curative properties and specifically their effects in wounds. The aim of our study is to investigate the physicochemical properties of some honey samples collected from different beekeeping regions in Albania.17 samples of were collected directly from the beekeepers, all registered in associations, during the harvest period in August - September 2022 from different regions of Albania. The first step was to find out some physicochemical properties of honey related to the hydroxymethyl furfural (HMF) level and diastase activity. The physicochemical properties were determined with the methods approved by the International Honey Commission for: pH, refraction index, ash, free acidity, water content, electrical conductivity, diastase activity, and HMF. Each of the physicochemical properties was evaluated using standard, established protocols. Results obtained indicated that the HMF contents differ from 0.3 – 52.1272 mg/kg. The values for diastase activity also seem to be correlated with HMF values. Both these parameters are most important for the freshness of honeys that we will use for our experiments to the wounds. The physicochemical properties (pH, refraction index, ash, free acidity, water content, and electrical conductivity) of honeys showed a significant correlation with HMF and diastase activity concentration.

Key words*: Floral types of honey, physicochemical properties, HMF, Diastase activity, Albania.*

Përmbledhje

Shumë vende kanë mbledhur shumë të dhëna mbi karakteristikat e llojeve të mjaltit mono-floral dhe poli-floral në lidhje me përbërjen fiziko-kimike, përmbajtjen e përbërësve dhe vetitë kuruese të secilit lloj. Qëllimi i studimit tonë është të hetojë vetitë fiziko-kimike të disa mostrave të mjaltit të mbledhura nga rajone të ndryshme bletarie në Shqipëri, në lidhje me efektet curative të mjaltit dhe në vecanti pëefektet curative tëtij në plagë. 17 mostra mjalti u mblodhën direkt në periudhën gusht - shtator 2022 nga rajone të ndryshme të vendit nga bletarë, të cilët janë të regjistruar në shoqatën e bletarëve të Shqipërisë. Faza e pare e studimit tone do të lidhet me përcaktimin e vetive fiziko-kimike të mjaltit dhe shqyrtimi i vlerave të gjetura në lidhje me nivelin e vlerave të hidroksimetil furfuralit (HMF) dhe aktivitetit të diastazës (DN). Përcaktimi i këtyre vetive u krye me metodat e miratuara nga Komisioni Ndërkombëtar i Mjaltit për: pH, indeksin e thyerjes, hirin, aciditetin e lirë, përmbajtjen e ujit, përçueshmërinë elektrike, aktivitetin e diastazës dhe HMF. Secila nga vetitë fiziko-kimike u vlerësua duke përdorur protokolle standarde. Nga rezultatet e marra u vu re që përmbajtja e HMF ndryshon në intervalin e vlerave nga 0.3 – 52.1272 mg/kg. Vlerat për aktivitetin e diastazës gjithashtu duket se janë në korrelacion me vlerat e HMF. Të dy këta parametra nga studime të mëparshme janë konfirmuar si parametra të rëndësishëm për freskinë e mjaltit, gjë e cila është me rëndësi edhe për eksperimentet që do të kryejmë në plagët mbi lëkurë. Vetitë fiziko-kimike (pH, indeksi i thyerjes, hiri, aciditeti i lirë, përmbajtja e ujit dhe përçueshmëria elektrike) e mjaltit treguan një korrelacion të rëndësishëm me vlerat e HMF dhe përqendrimin e aktivitetit të diastazës.

Fjalë kyçe*: mjalte monofloral, veti fiziko-kimike, HMF, aktiviteti i diastazës.*

Introduction

Beekeeping development and honey production in various rural areas of Albania have increased awareness of the consummator for a good and healthy product. It could be solved by quality control analyses of honey for its nutritional values and healing properties. The obtained data will serve as a good start for creating a database regarding the physicochemical properties, chemical composition, organic compound contents, and curative properties of each type of honey, as it is done in various countries.

The physicochemical characteristics of honey, such as the concentration of 5-hydroxymethylfurfural (HMF), a new generated contaminant with genotoxic potential and diastase level (DN), generally reflect the quality and purity of honey. The study of the components of honey, together with information on its botanical and geographical origin and quality relevant parameters, can explain the interactions that occur and shape its physicochemical characteristics. The physical properties of honey consist of a series of characteristic properties that honey acquires during its maturation process but could change during its storage (Frantzana et al., 2019). In addition to the important role of natural honey in traditional medicine, over the past few decades it has been subjected to laboratory and clinical investigations by several research groups and has found a place in modern medicine. The chemical analysis of honey includes the determination of the moisture content, pollen type, electrical conductivity, HMF content, diastase activity, pH, and free acidity. These methods are described in Codex Alimentarious, AOAC, and other approved food quality control standards, but during routine analysis, it is difficulty to be validated (AOAC, 1990). The studies carried out on the chemical composition of different pollen types of honey from different regions (Silva, P.M., et al., 2016) pointed out the HMF, DN number, and sugar content as the most important parameters for the quality control of honey food products. The maximum quality standards for honey's 5-HMF concentration (40 mg/kg) and level 8 Schade units for diastase activity, two of the main enzymes found in honey, have been defined by Codex Alimentary of the World Health Organization and the European Union. To judge the quality of a product, it is required to use the DN number and HMF content (Thrasyvoulou, 1986). In addition, in order to be used for human consumption, honey must meet the requirements set by the European Union on the composition of sugar, where the amount of fructose and glucose cannot be less than 60%, and the amount of sucrose cannot be more than 5%. (AOAC, 1990), (Codex Standard, 1981).

The aim of this study will be the investigation of the comprehensive phenolic compound and amino acid profile, mineral and sugar content, anti-cancer properties, and wound healing properties. This goal will be achieved in collaboration with Trakya University in Turkey, which is known for many publications in this field. But at first, we have started this current study to select the samples that will be analyzed in the second phase based on their

physicochemical parameters, HMF level, and DN number in mono- and multi-floral honey. Our study has begun to analyze the composition, physicochemical properties, HMF, DN number, etc., of 17 honey samples, mono and multi-floral, taken directly from the beekeepers that will be used in wound healing assays.

In our study, we used the Harmonized Methods of the International Honey Commission, taking into account accuracy, precision, and uncertainty. In the accuracy results, we have taken into account the range of the defined parameter and the two accuracy parameters, the repeatability r and the reproducibility R, as these are the decisive parameters for the evaluation of the methods regarding accuracy. The results for the HMF level and the DN number were processed through statistical analysis in relation to the physicochemical parameters to find the factors that affect the freshness of the honey samples.

Materials and methods

1. Honey samples

17 different honey samples were collected from local, experienced beekeepers in different regions of Albania (July – August 2022 period). One sample, the last one, is taken from the market randomly. Samples were stored at room temperature. All honey samples were characterized with the melissopalynological identification method according to their specific botanical variety (Louveaux, et al.,1978).

Melissopalynology characterization of honey or botanical and pollen identification in a sample of honey is done taking into consideration that a honey sample may also contain airborne pollens, spores, and dust due to attraction by the electrostatic charge of bees. Microscopic examination confirmed the origin of the honey claimed by the manufacturer as mono- or poly-floral products.

The determination of moisture is carried out with an Abbe Carl Zeiss digital Biobase at 20^{o}C after waiting 6 minutes for equilibration.

The determination of electrical conductivity was done in a 20% (w/v) honey solution diluted with distilled water. The values are expressed in $mScm^{-1}$ units. The method is based on the original work of Vorwohl. K = 0.9489.

Determination of ash content at a temperature no higher than 600^{o}C, and the residue weighed. The samples dissolved in water were measured for pH values, and the solutions were titrated with a 0.1 N sodium hydroxide solution to pH 8.30.

The determination of HMF content is based on the determination of UV absorbance at 284 nm. In order to avoid the interference of other components at this wavelength, the difference between the absorbance of the clear aqueous honey solution and the same solution after the addition of bisulfite is measured. The HMF level is calculated after subtracting the background absorbance at 336 nm. A developed and fully validated method was applied for the determination of HMF content and diastase activity in honey samples. For the determination of HMF content, quantification was performed using the certified reference material FAPAS T2848QC with a certified value of 31.9 6.01 in order to avoid matrix interferences. The methods LOD and LOQ were determined by the standard deviation of the intercept of the calibration curve and were equal to 2.2 and 6.8 mg/kg, respectively. The calculated LOD was lower than 1/10 of the maximum permissible level of 40 mg/kg, and the LOQ was lower than 1/5 of the maximum permissible as presented in Regulation (EC) No 110/2001 and in Codex Alimentarius (Codex Standard, 12 - 1981; Council Directive, 2001/110/EC).

The diastase activity determination with one standard solution of starch, capable of developing an iodine blue color in a defined range of intensity, is acted upon by the enzyme in the sample under standard conditions. The diminution in blue color is measured at intervals. A plot of absorbance against time, or regression equation, is used to determine the time t_x required to reach the specified absorbance of 0.235. This method is based on the original work of Schade as presented by Codex Alimentarius. The difficulty with methods such as diastase activity is performing an accurate and precise analysis. For this reason, the certified reference material FAPAS T2848QC was used in order to calculate the precision, accuracy, and uncertainty of the proposed methods. The instrumental limits of detection (LOD (mgL-1)) and quantification (LOQ) were calculated. In order to correctly determine the composition of a honey sample, the proposed LODs and LOQs should be less than one tenth and less than one fifth, respectively, of the maximum level in Regulation (EC) 2001/110/EC (Council Directive, 2001/110/EC).

2. Statistical analysis

Statistical analysis was performed with MINITAB 21 software. Descriptive statistics and cluster analysis, were performed in order to examine the level and the variation of the measured parameters and to examine the associations between them based on their similarity evaluated by correlation among chemical parameters of the honey.

Results and discussion

Table 1. The data of physicochemical parameters of honey samples (H%, EC mS/cm, Ash %, Free Acidity meq/kg, HMF mg/kg, DN Schade unit)

Kodi	*Sample*	*Botanic*	*H*	*RIndex*	*EC*	*Ash*	*pH*	*Acidity*	*HMF*	*DN*
1	HK1*	Multifloral	13,2	1,5036	703	1,995	4.29	44.03	0.864	3
2	HDU	Chestnut	13	1,5045	1349	14	5.2	23.29	0.302	3
3	HSH*	Multifloral	13,3	1,5012	787	2,875	4.38	30.77	5.778	3
4	HERZ	Chestnut	13,06	1,5042	140	0.047	4.08	14,45	2.524	3
5	HRR*	Multifloral	13,33	1,4928	593	0.3	4.24	28,29	51.519	7.9
6	HSHLL	R.Acaccia	18,2	1,4909	901	2,44	4.73	21,53	52.127	3
7	HMM	Multifloral	14,6	1,5005	610	4,435	4.58	18,85	1.942	3
8	HMM*	Chestnut	17,66	1,4925	325	0,98	4.11	21,2	7.913	4.8
9	HGJ1*	Multifloral	14,8	1,4998	748	0,725	4.31	45,23	0.057	60
10	HGJ2*	Multifloral	18,46	1,4905	547	2,31	4.01	35,43	8.330	3
11	HDI	Chestnut	16,2	1,4962	854	1,655	4.63	26,53	8.316	4.9
12	HDI*	Multifloral	14,4	1,5006	986	3,555	4.62	28,77	0.329	3
13	HER	Medicago	14,7	1.4997	560	1,275	4.8	37,15	43.457	3
14	HER*	Multifloral	16,6	1,4955	540	1,35	4.33	49,5	44.665	3
15	HP	Arbutus	18,5	1,4845	238	0,0475	3.94	17.14	2.281	3
16	HAMB	Multifloral	16,9	1,4934	355	0,0345	3.84	24.99	18.345	7.9
17	HMO	Multifloral	19,17	1,4887	455	0,0065	3.55	14.5	113.13	3

The results for HMF level and DN number in honey samples are shown in Table 1 together with other physicochemical parameters, such as moisture, refraction index, electrical conductivity (EC), ash content, pH, and free acidity, for comparison reasons.

In general, HMF and DN numbers are used as freshness indicators and are considered among the most important parameters for predicting the quality of honey samples (Thrasyvoulou, 1986).

Our results showed that the content of HMF is present at low concentration levels in most samples, both in mono-floral and multi-floral honey, and is far from the lower limit value of 40 mg/kg set by the Codex. This means that most

of the samples seem to be fresh, unheated, and naturally pure. In general, the contents of HMF in Albanian-type honeys seem to be much lower than those found in the literature, such as Manuka honey, Eucaliptus type honey, Rubus type honey, Echium type honey, Leotondon type honey, and others (Gomes et al., 2010), (Khalil et al., 2010). From Table 1, it can be seen that the last sample HMO in the table seems to have a very high value of HMF, almost two times more than the maximum level allowed by Codex Alimentarius. This sample's results with a very high HMF value indicate that honey must have been stored in bad storage conditions and processed and treated with heat, where the HMF value is much higher than the maximum allowed value of 80 mg/kg.

On the other hand, the other important parameter, DN number, seems to have low values in most of the samples, too. Only the multi-floral type honey HGJ1* from the south of Albania seems to have a high diastase content of 40 Schade units, whereas the others seem to have the lower level of 3 Schade units outlined for raw honey with low natural enzyme content (Table 1). The differences in diastase activity in honey may vary depending on the age of the bees, the nectar collection period, the physiological period of the colony, the large quantity of nectar flow, and its sugar content because a high flow of concentrated nectar leads to a lower enzyme content (Khan et al., 2015). Also, low diastase activity occurs when honeybees are fed artificially. The honeybees were fed with commercial glucose, and the authors found that when the bees were not fed with glucose in excessive amounts, an enzyme deficiency (especially diastase), which is used to convert glucose and fructose, may be promoted. They also proposed that in honeys with low diastase activity, it is essential that they contain no more than 15 mg/kg of HMF in order to prove that the honey has not undergone heat treatment or prolonged storage (Guler et al., 2014).

In our paper, we observed that the sample with the lowest HMF value of 0.0566 mg/kg has the highest level of diastase activity (60.4 Schade units), and the sample with the highest level of HMF of 52.12 mg/kg in the mono-floral honey sample of *Robinia pseudoacaccia* has the lowest diastase activity of around 3 Schade units. This confirms the literature data for the freshness criteria of honey samples, which are to have a low HMF level and a high DN number (Capuano and Fogliano, 2011), (Yücel and Sultanoglu, 2013). In general, using HMF and diastase as criteria to assess the quality of the product,

there are times when some honey samples could be regarded as industrial honey even though they are fresh, unheated, and naturally pure. The level and variability results obtained from the descriptive statistics analysis are presented in Table 2 below.

From this table, physicochemical parameters such as humidity (H), refraction index (RI), electrical conductivity (EC), pH, and free acidity seem to have low (CV% 25%) to moderate (CV% = 38.5%) variability. These values indicate that these parameters are very stable and do not depend on the fact that they are taken from different geographical and botanical areas or that they are mono- or multi-floral.

We can't say the same thing for the other parameters, ash content, HMF level, and DN number, with very high variability, higher than 75% of CV%, followed by high values of skewness and kurtosis, which indicate high asymmetry of the measured data. To explain these data, in Table 2, considering variables of the statistical data such as min, max, and median values for each of them, we see that there is a single data point that goes far outside the average value of each of the group data.

For example, the ash values fluctuate in a narrow range from 0.006 to 4.435%, except for one outliner that corresponds to the sample HDU from the higher area in Albania with an ash content up to 14%. (Figure 1). This honey sample was taken from beekeepers that breed bees in the highest-altitude pasture area in our country. This high amount of ash can be explained by the fact that the vegetation at high altitude is a pasture with special pine and beech trees, which makes the production of honey very aromatic and much sought after by the Albanian consumer.

We can say the same thing for the high variability of the data of two parameters, HMF level and DN number, as important parameters in quality criteria and honey origin, where the freshness of honey is correlated with low HMF level and high DN number values in honey.

So that, regarding the high variability of HMF (CV192%), most of the samples have low values and four of them around the lower permitted value (40 mg/kg) from Codex Alimentarius, except for the HMO sample with the highest value of 113 mg/kg. This fact leads us to draw the conclusion that most of the honey samples fulfill the HMF criteria of freshness.

Respectively for DN number, we can say that the sample HGJ1* with 60 Schade units (the lower limit allowed is 3 Schade units) and a 0.057 HMF value is the freshest honey sample.

Table 2. *The results obtained from descriptive statistics analysis*

Variable	Mean	StDev	CV%	Min	Q1	Median	Q3	Max	Skewness	Kurtosis
H	15.652	2.189	14.0	13	13.3	14.8	17.9	19.2	0.24	-1.52
Ash	2.237	3.303	147.6	0.006	0.174	1.35	2.66	14	3.11	11.11
pH	4.3318	0.4032	9.3	3.55	4.05	4.31	4.63	5.2	0.18	0.25
Acidity	28.33	10.75	38.0	14.45	20	27	36.3	50	0.62	-0.56
RI	1.50	0.0060	0.4	1.48	1.49	1.50	1.50	1.51	-0.31	-0.82
EC	628.9	298.5	47.5	139.9	405	593	821	1349	0.6	0.81
HMF	21.29	30.56	143.5	0.06	1.4	7.91	44	113	1.96	4.15
DN	7.15	13.72	192.0	3	3	3	4.85	60	4.03	16.41

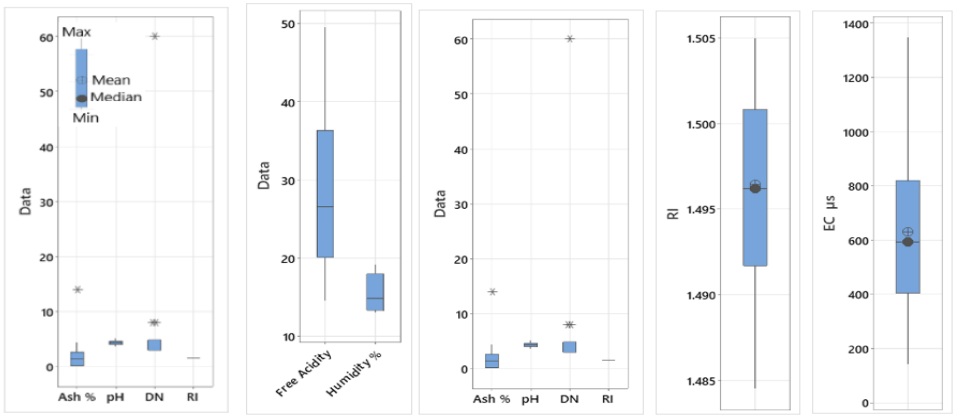


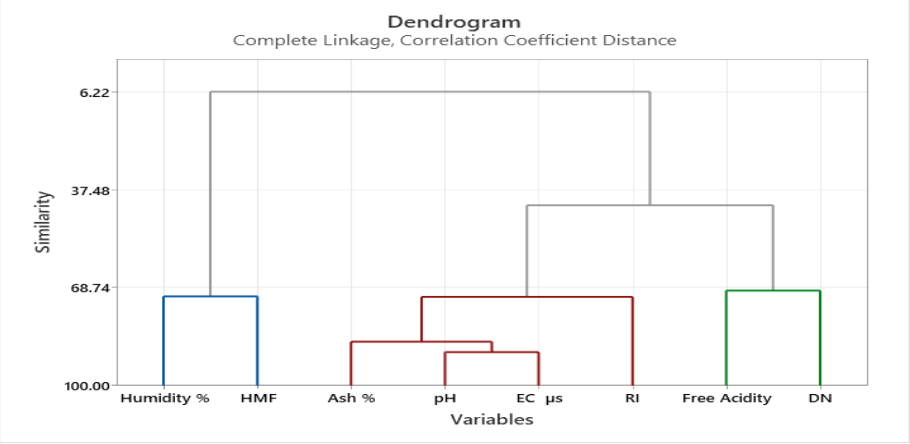
Figure 1. Mean, STDev, Median values for physicochemical parameters (H %, EC mS/cm, Ash %, Free Acidity meq/kg, HMF mg/kg, DN Schade unit)

In this work, the correlation among diastase activity, HMF content, and the other physicochemical parameters was investigated. The multivariate technique of cluster analysis was used to identify possible sources and groupings of different honey types. The HMF and DN number variables were the most significant factors in the classification, according to their values for the stated components.

Also, we tried to make a classification related to freshness, respecting the HMF values and the diastase activity of the honey samples. Classification of

physicochemical properties was achieved with the help of cluster analysis (Figure 2). From this analysis, three main groups were identified. The first group is formed by the values of the HMF content with the values of the moisture percentage with a similarity of almost 70%. This high similarity confirms the fact that high values of HMF, which are increased by reactions leading to the decomposition of fructose and glucose during storage time or heat treatments, are significantly helped by the high amount of water in honey, confirmed also from the literature.

The other one cluster with great interest is the cluster DN and acidity with 68.7 % similarity. Thus, these parameters may be used for indication of the. However, these raw samples with low DN number around 3 Schade unit conform Codex Standard may be identified to have been in bad storage conditions or may be have artificial feeding of the honeybee with carbohydrate supplements.



	Variables
Cluster 1	Humidity % HMF
Cluster 2	Ash % pH RI EC
Cluster 3	Free Acidity DN

Figure 2. *Dendrogram correlations*

Another group of interest is that of ash, pH and EC with 80% similarity. This group confirms the close correlation between EC values with ash and pH

values. From this we conclude that the higher the ash content, the higher the conductivity values will be. Perhaps it would be very valuable in the future to replace the determination of ash content with the faster and easier method of measuring electrical conductivity.

In conclusion, in view of both parameters (HMF and DN), to choose samples with high level of freshness to be used for therapeutic purposes, we can say that 16 honey samples, except the last one, are suitable to be used for our further study.

Conclusions

This study was carried out to determine the physicochemical characteristics of 16 honey samples of different botanical origin which will be used in a further study regarding the therapeutic effects of honey as a product, and specifically for its effects on wound healing. From these honey samples that will be used in the further study, those samples that meet the freshness criterion will be selected, we focused on the precise determination of two indicators such as the level of HMF and diastase activity. These results were obtained from the physicochemical analyzes performed with the methods defined in the Codex Standard for honey Codex Stan 12-1981 and their statistical processing.

From the statistical processing of the data, a satisfactory correlation of similarity up to 70% was seen between the moisture content and the HMF level, which is consistent with previous studies and verifies the freshness of the honey to be selected in the subsequent study. Also, a very good correlation was seen between the diastase activity and the pH value. This corresponds very well with the fact that the number of enzymes is associated with moderate pH values. It should be mentioned that only 4 of the samples had diastase activity above the minimum level set by the Codex Standard for Honey of 3 Schade unit with low natural enzyme content. The level of diastase activity in the other 12 samples was at the level of the minimum limit of quantification (LOD).

In conclusion, in view of both parameters (HMF and DN), to choose samples with high level of freshness to be used for therapeutic purposes, we can say that 16 honey samples, except the last one, are suitable to be used for our further study.

Since this study is being carried out as part of a PhD thesis to select honey

samples that meet the freshness criteria according to the Codex Standard, it was seen that, honey botanical origins were of no particular importance, which is understandable since that freshness of honey is affected by many factors that should be taken into account.

References

Alinorm 01/25, 2001; Council Directive, 2001/110/EC.

AOAC 958.09-1977, 2010; AOAC 980.23, 1983, 1990; Codex Standard, 12-1981.

Capuano and Fogliano (2011)

Frantzana A., Alexeu Dimitra., Panatiogopolou K., Ouzounakis P., Tsilligiri M. (2019). *Therapic properties of honey*. Amerikan Journal of Biomedical Science & Research.

Louveaux, J., Mourizio, A. & Maurizio, & Vorwohl, (1978). *Method of melissopalynology*. Bee world, 59, 139 – 157.

Codex Standard, 12 – 1981.

Council Directive, 2001/110/EC

Gomes, S., Dias, L. G., Moreira. L., Rodrigues, P., & Estevinho, L., (2010); *Physicochemical, microbiological and antimicrobial properties of commercial* honeys from Portugal. Food and Chemical Toxicology, 48, 544-548.

Guler, A., Kocaokutgen, H., Garipoglu, A.V., Onder, H., Ekinci, D., & Biyik, S. (2014). *Detection of aldultery honey produced by honeybee (Apis Mellifera L.) colonies fed with different levels of commercial industrial sugar (C3 and C4 plants) syrups by the carbon isotope ratio analisis*. Food Chemistry. 155 -160.

Khalil, M.I, Sulaiman, S.A, & Gan, S.H. (2010). *High 5-hydroxymethylfurfural concentrations are found in Malasian honey samples stored for more than one year. Food and Chemical Toxicology, 48, 2388-2392.*

Khan, Z.S., Nanda, V., et al., Bhat. M.S. & Khan, A. (2015). *Kinetic studies of HMF formation and diastase activity in two different honeys of Kashmir*.

Silva, P.M., Gouche, C., Gonzaga, L.V., Costa, A.C.O.C., &Fett, R. (2016). *Honey: Honey composition, stability and authenticity*. Food Chemistry, 133, 1640-1645.

Thrasyvoulou, A. (1986). *The use of HMF and diastase as criteria of quality of Greek honey*. Journal of Apicultural.

Yücel and Sultanoglu (2013).

Abbreviations

HMF – 5-hydroxymethylfurfural

DN number – diastase activity

EC – electrical conductivity

RI – index of refraction

H – humidity