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THE PHYSICOCHEMICAL CHARACTERISTICS OF HONEY AND QUANTIFICATION OF SOME ANTI-MICROBIAL AGENTS IN HONEY FROM DIFFERENT SERBIAN REGIONS AS A QUALITY ASSESSMENT TOOL

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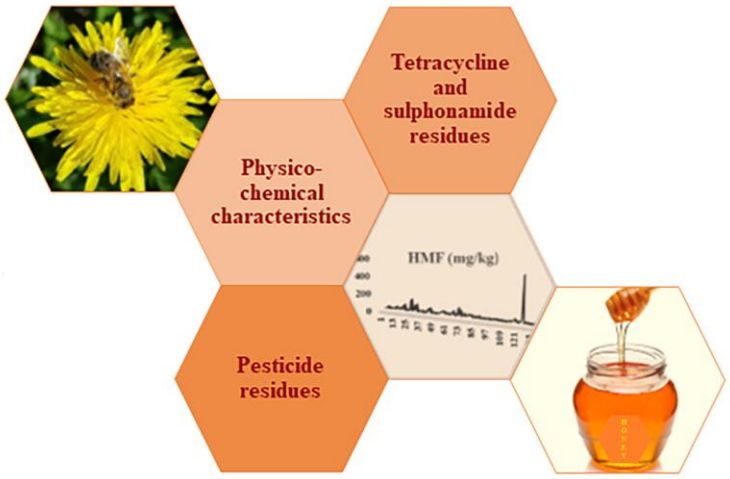
ABSTRACT. Healthy, organic, high-calorie and very tasty – these attributes are often associated with natural products such as honey. The aim of this study is to characterize the 135 honey samples collected in seven regions of central Serbia in terms of their physicochemical parameters (electrical conductivity, moisture, free acids, insoluble matter and reducing sugars, hydroxymethylfurfural, and diastase activity), the possible presence of tetracycline, sulfonamide and pesticide residues, and finally to perform a quality assessment for consumer protection. Considering the physicochemical parameters, it was found that almost all honey samples complied with the European Legislation (EC Directive 2001/110: THE COUNCIL OF THE EUROPEAN UNION, 2001) for the parameters studied. From the point of view of antimicrobial agents, the commercial quality was considered good, and all samples were found to be free of harmful agents such as bacteriostatic, antibiotic and pesticide residues through analysis.



Keywords: geographical origin, pesticide residues, physicochemical parameters, polyfloral honey, unifloral honey.

INTRODUCTION

According to the "Codex Alimentarius Commission", honey is the natural, sweet, viscous, highly complex substance produced by honeybees (CODEX STAN, 2001). Bees produce honey from the nectar of flowering plants, as well as from sweet fruit juices and honeydew (obtained from coniferous and deciduous trees) (MUREȘAN *et al*., 2022; RYBAK-CHMIELEWSKA *et al*., 2013). The quality of the final product depends primarily on the composition and properties of the nectar or honeydew itself. On the other hand, the properties of the nectar are directly related to the geographical area and the periods of nectar collecting. During the processing of nectar/honeydew, the digestive enzymes secreted by the bees are also added, so that the honey is a food with a double origin – both plant and animal.

The characteristic odor, taste and color of honey are due to the components contained in the nectar/honeydew: water-soluble sugars, minerals, enzymes, vitamins, natural colorants, and fragrances. Under the influence of the the external temperature and other factors, a certain loss of water in the nectar leads to an increase in the sugar concentration. The conversion of starch into sucrose – the main component of nectar – is suppressed at low outdoor temperatures. The sugar content in nectar ranges from 10 and 75% (ARONNE *et al*., 2019). The transformation of nectar into honey occurs through the dehydration of nectar, and the conversion of sucrose into glucose and fructose under the influence of enzymes. The primary components of honey are sugars, along with enzymes, amino acids, minerals, and polyphenols, as stated by NAILA *et al*. (2018). Unlike flower honey, honeydew has a higher content of sucrose, dextrin, minerals (iron, copper, manganese, *etc*.) and a lower content of invert sugars (glucose and fructose) due to the absence of fermenting nectar juice. The final product, honey, is hygienically safe and is stored in wax combs.

Besides being used in its original form as a food, honey is also added to many products and is an important component of traditional medicine, both individually and in combination with some other medicinal substances. Researchers argue that honey plays an important role in the treatment of gastrointestinal diseases, diabetes, cardiovascular and neurodegenerative diseases, respiratory diseases, and cancer in addition to having beneficial effects on the human immune system (HOSSEN *et al*., 2017; JOVANOVIĆ *et al.*, 2018).

Nowadays, environmental pollution, either by industrial or agricultural activities (so many chemicals are used to treat agricultural land and crops) is taken up by plants, that is, it can be transferred to neighboring wild vegetation used by bees. On the other hand, preparations used to control honeybee parasites and pathogens have a direct impact on honey quality, so, it is of great interest to increase honey production in uncontaminated areas (GAŠIĆ *et al*., 2014; HLADIK *et al*., 2016; KARISE *et al*., 2017; KUMAR *et al*., 2016; TETTE *et al*., 2016; BILANDŽIĆ *et al*., 2017, SREĆKOVIĆ *et al*., 2019). Geographical and climatic conditions in Serbia are favorable for the production of large quantities of honey (KOSTIĆ *et al*., 2015).

Parameters such as electrical conductivity, free acidity, sucrose, glucose, fructose hydroxymethylfurfural (HMF) content, diastase activity, sulfonamide, tetracycline, and pesticide residues (aldrin, dieldrin, endrin, lindane, sum of dichlorodiphenyltrichloroethane and its derivatives, sum of α and β isomers and endosulphan sulphate, sum of *cis*- and *trans*-chlordane, and sum of heptachlor and heptachlor epoxide) were studied as the main parameters for honey quality evaluation. In this context, monitoring of these parameters in acacia, sunflower, linden, multifloral honey, and honeydew from all over the Central Serbia is crucial to ensure its quality and safety.

In view of all mentioned facts, this study aims to determine and compare the physicochemical and antimicrobial properties of 135 honey samples coming from seven different regions of Central Serbia, with different vegetation.

MATERIALS AND METHODS

Honey samples

A total of 135 honey samples of five different botanical origins (acacia, sunflower, linden, multifloral and honeydew) were collected from seven different regions of Serbia (Tab. 1) during 2016 harvesting season owing to The Association of the Beekeeping Organisations of Serbia (SPOS – abbreviated from serbian name Savez pčelarskih organizacija Srbije). The samples were packaged in 1 L glass containers and brought to the Veterinary Specialist Institute in Kraljevo, Serbia. The geographical origin of the samples (Tab. 1 and Supplementary Material – Tab. S1) was specified by the SPOS, based on the information provided by beekeepers. The samples were stored in the dark at room temperature until subjected to further analysis.

Figure 1. Map of Serbia with honey sampling regions highlighted.

Table 1. Geographical regions and botanical origin of honey.

Serbia regions		Honey type	Number of samples	
1	Braničevo	Acacia	2	4
		Polyfloral	2	
2	Moravica	Acacia	8	23
		Polyfloral	12	
		Sunflower	3	
3	Rasina	Acacia	4	10
		Polyfloral	3	
		Honeydew	1	
		Sunflower	2	
4	Raška	Acacia	5	14
		Polyfloral	8	
		Honeydew	1	
5	Šumadija	Polyfloral	1	1
6	Zaječar	Acacia	14	44
		Polyfloral	26	
		Honeydew	4	
7	Zlatibor	Acacia	12	39
		Polyfloral	17	
		Honeydew	2	
		Sunflower	7	
		Linden	1	

Determination of free acidity

The free acidity of honey samples is the content of all free acids determined by using 0.1 M sodium hydroxide solution (INTERNATIONAL HONEY COMMISSION, 2009, abbreviated IHC, 2009) and expressed in milliequivalents/kg of honey samples (CODEX STAN, 2001). To check the precision and accuracy of the method, the repeatability (r) and root mean square errors (RMS_{bias}) have been calculated from the acidity results obtained from three types of honey and the results of the interlaboratory comparison.

Determination of moisture

The content of water is determined according to the International Honey Commission (IHC, 2009) using the refractometric method, based on the principle that refractive index increases with solids content. Precision and accuracy have been calculated from the results of the analysed honey samples in a similar manner to the parameter considered previously.

Conductivity measurements

Electrical conductivity (μS/cm) was measured in a 20% (w/v) honey solution diluted with freshly distilled water (CODEX STAN, 2001; IHC, 2009) by using an electrical conductivity cell. The precision of the method was determined, and valid data ranged between 20 and 2000 μS/cm.

Determination of insoluble matter

Material insoluble in water was collected in a sintered-glass crucible (pore size 30µ) and the dried residue was weighed (g/100 g) according to the prescribed procedure (CODEX STAN, 2001).

Hydroxymethylfurfural (HMF) determination

The quantitative method for determination of HMF HMF (5-hydroxymethyl-2-furancarboxaldehyde) is proposed by the Association of Official Analytical Chemists (AOAC INTERNATIONAL and LATIMER, 2012, abbreviated AOAC, 2012). Five grams of honey were dissolved in 30 mL of distilled water, quantitatively transferred into a 50 mL volumetric flask, added by 0.5 mL of Carrez solution I (15% solution of potassium ferrocyanide) and 0.5 mL of Carrez solution II (30% solution of zinc acetate) and make up to 50 mL with distilled water. Two drops of ethanol were added to prevent foaming. Then, the solution was filtered using filter paper discarding the first 10 mL of the filtrate. 5 mL aliquots of the filtrate were put into two test tubes; 5 mL of distilled water were added to the first tube to make the "sample solution", and 5 mL of freshly prepared 0.2% sodium bisulphite solution to the second tube for the "reference solution". The absorbance of these solutions was measured by using HACH LANGE DR 5000 UV-visible spectrometer, at 284 (A_{284}) and 336 nm (A_{336}). The HMF content (mg/kg) was calculated by the Equation (1):

$$HMF = (A_{284} - A_{336}) \times 149.7 \quad (1)$$

where 149.7 is a factor calculated by the molecular weight of HMF and the mass of the sample.

For quality assurance, the Veterinary Institute "Kraljevo" set a z-score equal to -1.5 in an interlaboratory proficiency test for HMF content, which is a well-performed value (z-score between -2 and +2).

Diastase activity

The methodology employed to determine the diastatic index follows the guidelines outlined by the AOAC (AOAC, 2012) which involves quantifying the alpha-amylase activity in honey in the presence of starch. Ten grams of honey are weighted into a 50 mL volumetric flask together with 5mL of acetate buffer and 20 mL of distilled water. After complete dissolution of the sample, 3mL of a 0.5 M solution of sodium chloride were added and filled the volumetric flask with distilled water to the meniscus. The standard starch solution was also prepared by using iodide solution. The sample and the starch solutions were placed into a water bath and heated at 40°C for about 15 minutes. Then, 5 mL of starch solution was added into 10 mL of the honey solution; an aliquot was taken every 5 min, quickly added to 10 mL of the iodide solution, and the absorbance was recorded at 660 nm. The readings were plotted as a function of time. According to the official AOAC method, the Diastase Number is calculated as 300 divided by the time (in minutes) needed for the solution to reach an absorbance value of 0.235 (DN expressed in Schade unit) (AOAC, 2012).

For quality assurance, the Veterinary Institute "Kraljevo" set a z-score equal to -1.2 in an interlaboratory proficiency test for diastase activity, which is a well-performed value (z-score between -2 and +2).

Sugar content

Honey samples were analysed for their sugar profiles ('apparent reducing sugars' and 'apparent sucrose') by using Fehling's reagent under specified conditions (CODEX STAN,

2001; IHC, 2009). This method is based on the ability of 'apparent reducing sugars' (glucose and fructose) to reduce copper ions in Fehling's solution causing oxidation of sugars into organic acids. 'Apparent sucrose' is defined as 0.95 of the difference in 'apparent reducing sugars' before and after the hydrolysis procedure proposed by the CODEX STAN (2001). The results are expressed as g invert sugar per 100 g honey and g apparent sucrose per 100 g honey.

Sulfonamide and tetracycline residues

The presence of antibiotic residues (sulfonamide and tetracycline) in honey samples was determined by the microbiological method "Modified method 4 plates" (EUR 15127-EN) (HEITZMAN, 1994). Then, an agar-diffusion test was carried out and the samples were applied to four plates of agar media inoculated by microorganisms. The diffusion of an antibacterial substance is shown by the formation of inhibition zones of microorganism growth. If the sample holds active antimicrobial compounds, the test microbe's growth in the diffusion area will be hindered, leading to the formation of an inhibition zone. The distance between the perimeter of the agar's hole and the boundary of the test microbe's growth defines the width of the inhibition zone. The outcomes were statistically evaluated and shown using descriptive statistical methods.

The presence of pesticide residues

Pesticide standards were obtained from Sigma-Aldrich. Organochlorine pesticides (OCPs) concentration in stock standard solution was 500 μg/mL. The calibration and working solutions were prepared by dilution with acetonitrile on the day of analysis. Stock standard solutions and working solutions of pesticides may be stored in the refrigerator at 4°C: stock standard solution may not be kept for longer than three months and working solution for not longer than a week.

High purity solvents without pesticide residues – *n*-hexane, methanol and acetonitrile – were also obtained from Sigma-Aldrich. Anhydrous magnesium sulphate and sodium acetate were used for the extraction. Dispersive SPE filings Bondesil C18 (pore radius 40 μm) and Bondesil PSA (primary and secondary amine) (pore radius 40 μm), purchased from Merck Darmstadt, Germany, were used for sample purification.

Sample preparation (ANASTASSIADES *et al*., 2003) was based on extraction with acetonitrile in the presence of anhydrous magnesium sulfate and anhydrous sodium acetate. KARTALOVIĆ *et al*. (2016) modified and validated (see the "Supplementary Material", Tables S2-S6) method for sample preparation. Honey samples were thoroughly homogenized and approximately 3 g of the homogenate was weighed into a polyethylene centrifuge tube (50 mL) containing 3 mL of acetonitrile (for extraction) and 3 mL of water. The content was intensely vortex-stirred with 3 g of anhydrous magnesium sulphate and 1 g of anhydrous sodium acetate. Having been stirred for 1 min, the mixture was subsequently centrifuged at 3000 rcf for 5 min. Therefore, 1 mL of the acetonitrile fraction was transferred into a 5 mL tube with 150 mg of anhydrous magnesium sulphate, 100 mg of PSA and 50 mg of C18 cartridge. The tube content was centrifuged at 3000 rcf for 5 min and then purified and a clear extract was obtained. 0.5 mL of the extract was evaporated under nitrogen and reconstituted in hexane. Finally, the sample prepared in this way can be analysed using gas chromatography with mass spectrometry detection (GCMSD).

Gas chromatography with mass spectrometry detection was used for the quantification of pesticide residues in the prepared extracts (Agilent Technology GCMS 7890B/5977A with electron ionization and quadrupole detector). Chromatographic separation was performed on DB-5MS column (30 m × 0.25 mm × 0.25 μm). The column was set at a constant pressure.

The sample volume of 4 μL (spitless mode) was injected at the pressure of 11.36 psi and the carrier gas flow rate of 1.2 mL/min. The carrier gas was helium (99.999% purity). The injector temperature was 250°C, detector temperature was 280°C, and the column temperature was programmed as follows: the initial temperature was 75°C (for 0.5 min) and increased to 300 °C with the velocity of 10 °C/min and it remained the same for 2 min. The total runtime was 25 min.

The target and qualifier abundances were determined by injection of the mixture of pesticide standards under the same chromatographic conditions using the full scan with the mass/charge ratio ranging from *m*/*z* 60 to 500. Standards were prepared in blank matrix extracts to counteract the matrix effect (SANCO, 2017).

The method performance was in calibration range 0.005 – 0.1 mg/kg.

Statistical analysis

All analyses were carried out in triplicate and the mean was expressed in proper units. Statistical analyses were done by using Origin 7.0 SRO software package (OriginLab Corporation, Northampton, MA, USA, 1991-2002) and Microsoft Office Excel 2007 software package. Significant differences were calculated by ANOVA test followed by the least significant difference (LSD) test ($p \leq 0.05$).

RESULTS AND DISCUSSION

Physicochemical characteristics

To investigate potential synthetic nourishment and the quality of honey samples, a physicochemical evaluation was carried out. The freshness of the samples was assessed using HMF and diastase activity, while HMF, sucrose, and diastase activity were utilized to indicate the presence of artificial feeding by honeybees.

Moisture

Humidity is one of the criteria indicating the quality of honey (KAHRAMAN *et al*., 2010). The probability that honey, as a very hygroscopic substance, will ferment during storage is increased with the rise in moisture. Moisture content in honey below 19% as proposed by IHC (2009) allows the stability of honey for a long period of time and retains high-level sensory features and nutritional properties. Fermentation itself is caused by osmophilic microorganisms which possess the capacity to deal with a higher concentration of fructose and dextrose, and this process is easier to start in the presence of larger amounts of water (SILVA *et al*., 2023). As a result of fermentation, alcohol and carbon dioxide are produced. In the presence of oxygen, alcohol can be oxidized to acetic acid and water. As an outcome, all the above-mentioned processes leave a bitter taste of honey.

The values of moisture were found in the following ranges: 13.88–18.60 for acacia honey, 14.28–18.72 for polyfloral honey, 15.48–18.64 for sunflower honey and 14.92–16.90 for honeydew (see the "Supplementary Material", Tab. S1). Based on the results, it was concluded that there were no significant differences in their mean values and these ranges were like those in already published studies (MANZANARES *et al*., 2011), except in one case with the sample designated as 4-6 (Tab. S1). Due to the hygroscopicity of honey, the amount of water is not a constant quantity - it changes during honey storage, depending on the humidity of the air (SEMKIW *et al*., 2008), which may be the reason for the obtained measurement results.

Electrical conductivity and insoluble matter

Electrical conductivity is very useful in the determination of the botanical origin of honey, and it is directly connected with pH, acidity, and the content of ash, minerals, proteins, and other substances in honey. Being a very good indicator of the botanic origin, conductivity is very often used in a routine examination of honey while distinguishing nectar honey from honeydew. Based on the conductivity standards proposed by the CODEX STAN (2001), the floral honey and blend of floral and honeydew honeys should exhibit less than 800 μS/cm and honeydew should exhibit more than 800 μS/cm, and the results presented in this study are in correlation with these parameters, except in two honey samples (honeydew, designated as 3-5 and 6-11), Table S1. Honeydew is obtained by processing the honeydew, secreted by aphids and scale insects that live on the tips of branches and leaves. Honeydew is a sweet liquid secreted in the form of the excrement of aphids and scale insects that suck plant sap. These insects secrete honeydew in the form of droplets on leaves and other plant parts, where it is collected by bees and processed into honey. Besides the high content of amino acids, carbohydrates, polyhydroxy alcohols and enzymes, honey contains some organic acids and significant amounts of minerals found in honeydew, which contribute to the higher electrical conductivity of honeydew, compared to other types of honey. Minerals are present in nectar honey at 0.1–0.2% and in honeydew at over 1%. When considering the mineral composition of honey, it is necessary to consider its botanical and geographical origin, but also the environmental conditions in which the bees have grazed, as well as honey processing methods (BOGDANOV *et al*., 2007). The specific geological and geochemical characteristics of the soil of a certain region are also reflected in the mineral composition of honey plants, i.e., in the mineral composition of their nectar and pollen. The mineral content also depends on the botanical origin of the nectar and climatic conditions. On the other hand, soil composition and climatic conditions determine the structure and occurrence of the botanical species of honey plants in a certain geographical area (BILANDŽIĆ *et al*., 2014).

One of the purity criteria for honey is the content of water-insoluble solids. Using this parameter, it is possible to identify total pollen remains, bee remnants, pieces of honeycomb and impurities, preferably below the permitted maximum of 0.1 g/100 g of honey (MAC, maximum allowable concentration). Also, centrifuging honey from honeycombs containing brood or pollen, low-quality filtration or sedimentation, and storage in open vessels lead to an increase in insoluble substances and a deterioration in honey quality. When presenting the results, it is necessary to consider the considerable interlaboratory variation – between 26 and 85% (CODEX STAN, 2001). The results in Table S1 show the values of the water-insoluble solids between 0.01 and 0.095 g/100 g in all honey samples, which is in accordance with those proposed by the "Codex Alimentarius Commission".

Acidity

One of the important indicators of honey quality is the presence of acids. Acids in honey directly affect sugar fermentation (alcoholic fermentation produces organic acids) and inhibit the growth of microorganisms, which, consequently, generate lower or higher bactericidal activity (GOMES *et al*., 2010). Additionally, many organic acids in honey exist in the form of esters and directly affect both the taste and smell of honey. It is known that the acids in honey originate from nectar/honeydew, but, due to enzymatic activity, they also occur during storage. The International Honey Commission has allowed a maximum acidity of 50 meq/kg (CODEX STAN, 2001). None of the samples exceeded the limit allowed (Table S1), which indicated the freshness of all honey samples.

Under conditions of repeatability assessment, the results of honey acidity in the samples of acacia, polyfloral honey and honeydew were compared. The mean value, standard

deviation, coefficient of variation (also known as the relative standard deviation), and repeatability coefficient for each sample, based on six measurements, were calculated. The obtained values are given in Table 2.

Table 2. The repeatability of the method.

Sample	Result (mmol/k)	Mean value (mmol/kg)	Standard deviation (mmol/kg)	Relative standard deviation (%)	Repeatability coefficient (mmol/kg)
Acacia	19.43 19.69 19.31 19.76 19.98 19.89	19.677	0.26	1.33	0.73
Polyfloral	21.19 21.00 22.10 21.49 21.80 22.48	21.68	0.559	2.58	1.57
Honeydew	38.49 38.00 37.79 38.09 37.50 37.07	37.82	0.494	1.31	1.38

The precision of the analytical method was evaluated by the content of the analyte in the sample and the relative standard deviation. Maximum values of RSD in relation to the concentration of the analyte are shown in Table 3 (AOAC, 1998). Based on the results (Table 2) and the data in Table 3, the precision criteria are satisfied.

Table 3. Analytical method precision validation.

The content of the analyte in the sample	Max RSD [%]
100%	1.3
≥10%	1.8
≥1%	2.7
≥0,1%	3.7
100 ppm	5.3
10 ppm	7.3
1 ppm	11
100 ppb	15
10 ppb	21
1 ppb	30

The accuracy of the method was determined based on the results of the interlaboratory comparison. The acidity in honey was determined in the interlaboratory comparison organized

by the Scientific Veterinary Institute of Serbia (**1**) and the Public Health Institute in Zajecar (**2**) (Table 4). It can be concluded that the accuracy is appropriate because z-values are acceptable in both cases (Table 4). Based on the results shown in Table 4, RMS_{bias} is calculated by using the next Equation (MAGNUSSON, 2017):

$$RMS_{bias} = \sqrt{\frac{\sum (bias)^2}{n}} = 0.022 \quad (2)$$

Root mean square errors is 0.022 (2.2%).

Table 4. Interlaboratory comparison of acidity results.

	1	**2**
Value obtained in Specialized Veterinary Institute "Kraljevo" (mmol/kg)	17.61	8.68
Assigned value (mmol/kg)	18.09	8.83
Difference (mmol/kg)	-0.48	-0.15
Bias relative value	-0.0265	-0.0169
Bias %	-2.65	-1.69
Z		-0.3

Hydroxymethylfurfural (HMF)

HMF can be formed either by Maillard reaction, or during the dehydration of fructose and glucose under acid conditions. Its value is virtually absent or very low in fresh honey (NOZAL *et al*., 2001). The process of HMF formation is faster at a higher temperature and lower pH value. For this reason, the increased mass concentration of HMF is the indicator of high temperature applied in the phase transition from crystallized honey to its liquid state. Additionally, its mass concentration increases during long storage, as well as due to inappropriate storage conditions (GOMES *et al*., 2010). "Codex Alimentarius" and European Legislation (CODEX ALIMENTARIUS, 2017; COUNCIL DIRECTIVE, 2001) defined the maximum value of HMF at 40 mg/kg, with an exception in the case of lower diastase activity, which will be discussed further. HMF values of the honey samples in our study were mainly below the suggested value (Figure 2a): in several samples, it was slightly higher, but in one case (sample designated as 7-30) the value of HMF was extremely large pointed out to honey falsification.

Enzymatic activity

Diastase activity is one of the most important composition criteria for honey, responsible for converting nectar and honeydew to honey, giving it the unique character and functionnality (together with other enzymes, such as invertase, glucose oxidase, catalase, and acid phosphatase) and it is a very sensitive indicator of how honey has been handled. Diastase (amylase) activity is used as a measure of honey freshness, since diastase activity decreases in heated/old honey and, consequently, the potential health benefits of honey could be reduced (PITA-CALVO and VAZQUEZ, 2017). In raw honey, there are bee enzymes and nectar enzymes. The content of diastase enzyme in honey can vary depending on the origin of the honey and the region from which it has been collected. Thus, the heating of honey as well as the long-term storage of honey inactivate diastase, and since it is the most stable enzyme at high temperatures, its absence means that there are no other enzymes in honey (invertase, phosphatase, catalase). Serbian legislation as well as European regulations prescribe diastase activity as one of the parameters of honey quality assessment. CODEX STAN (2001) established a minimum diastase activity value of 8 on the Schade scale.

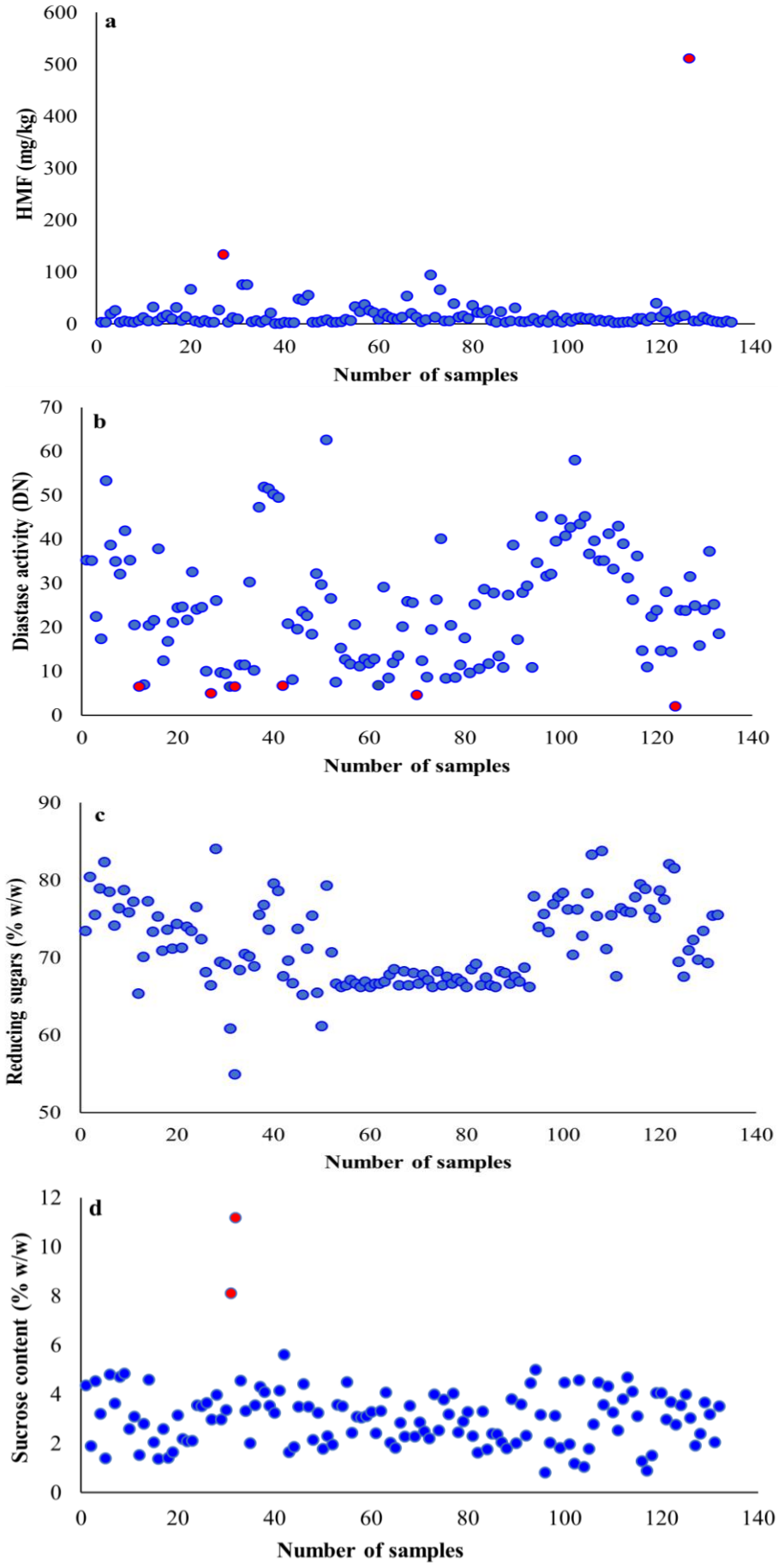


Figure 2. a) HMF, b) diastase activity, c) reducing sugars and d) sucrose content of the honey samples.

For honey with low natural enzyme content (*e.g.*, honeydew), a value between 3 and 8 is allowed if the content of HMF is not above 15 mg/kg (CODEX ALIMENTARIUS, 2017). Our results suggest that most honey samples have fulfilled the criteria except in seven cases from different regions (Figure 2b, Table S1): three samples of acacia honey (designated as 4-6, 6-19, 7-30), three samples of polyfloral honey (2-8, 2-23, 3-4) and a honeydew honey (sample 3-5).

Reducing sugars and sucrose

The main constituents of honey are carbohydrates (primarily, fructose and glucose) making around 80% of all honey components (KAHRAMAN *et al*., 2010). Water and sugar make up around 99% of the honey content. Except for monosaccharides, in honey is generally identified 11 disaccharides (of which sucrose is the main one) and 12 oligosaccharides. Most of these saccharides are not present in nectar due to different biochemical processes. Carbohydrates mostly affects the physical properties of honey such as viscosity, density, stickiness, the tendency to crystallization, and hygroscopicity. Also, sucrose is a very important parameter used to detect honey adulteration. The determination of sugars is related to the quality of honey, as well as its botanical and geographical origin. In accordance with Codex standards, the minimum reducing sugars content in nectar honey is 60% and around 45% in honeydew, while the allowed sucrose maximum is 5%, except in acacia honey where the maximum value is 8% and in honeydew is 10% (CODEX STAN, 2001).

In this study, we determined reducing sugars and sucrose, and the following mean values were obtained: 71.69 and 3.15 for acacia honey, 71.48 and 3.01 for polyfloral honey, 76.90 and 2.77 for sunflower honey, and 65.97 and 3.92 for honeydew, respectively (Figures 2c and 2d, Tab. S1). The results are in accordance with the Codex Standards, except in two cases with slightly higher values for sucrose (samples designated as 3-4 and 3-5) (CODEX STAN, 2001).

According to the Statistical Office of the Republic of Serbia (2019), the annual production of honey in Serbia ranges from 5000 to 12000 t in the past ~~five~~ few years (MRAČEVIĆ *et al*., 2020). Among the various kinds of honey produced in Serbia, some of the prominent ones include acacia, polyfloral, sunflower, linden, and honeydew honey (MRAČEVIĆ *et al*., 2020). The EU market could be attracted to Serbian honey, according to LAZAREVIĆ *et al*. (2012), therefore it is crucial to ensure that it meets the quality standards required by the European Union. The Council Directive 2001/110/EC outlines both broad and detailed features of honey that are crucial for assessing its genuineness, including levels of moisture, free acidity, electrical conductivity, diastase activity, sugar content (fructose, glucose, and sucrose), and hydroxymethylfurfural content. As a result of the determination of the above-mentioned specific ingredients of honey, it can be stated that the results obtained in this study are very similar to those previously reported for Serbian honey (LAZAREVIĆ *et al*., 2012; SAKAČ *et al*., 2019; MRAČEVIĆ *et al*., 2020) which is, further, in accordance with the quality standards required by the European Union.

Potential honey contamination with sulphonamide and tetracycline residues

Antibiotics and sulfonamides are used to prevent or treat diseases of all animal species (JUAN-BORRAS *et al*., 2016). Unprofessional conduct in the administration of antibiotics and sulfonamides, and non-adherence to prescribed doses and drug-withdrawal periods (the time required to excrete the residue of administered antibiotics and sulfonamides until an acceptable level – MRL, maximum residue limit) lead to the occurrence of residues of antibiotics and sulfonamides in foodstuffs of animal origin. Minimum amounts of residues of antibiotics/sulfonamides in animal products such as honey may inflict considerable harm to people consuming such kind of food.

Antibiotics are specific products of microbial metabolism with high physiological activity against certain types of microorganisms. Besides natural antibiotics obtained by microbial biosynthesis, there are synthetic and semi-synthetic antibiotics obtained by chemical modification of antibiotics or strictly synthetic chemicals (DEMAIN, 2009). Tetracyclines, the inhibitors of protein biosynthesis, are often applicable due to their broad-spectrum action. It has become imperative to detect tetracycline residues in animal products to safeguard consumers and hinder the proliferation of antibiotic resistance (TARAPOULOUZI *et al.,* 2013).

Sulfonamides are a special type of synthetically produced chemical substances, antimicrobial agents containing a sulfonamide group. They also inhibit the synthesis of folic acid and nucleotides. Sulfonamide is a structural analog of *p*-aminobenzoic acid which is necessary for the folic acid synthesis in bacteria. Sulfonamides inhibit the bacterial growth (bacteriostatic effect) but do not kill bacteria (no bactericidal effect). They are also frequently used in combination with certain antibiotics.

To control the residues of tetracycline and sulfonamides in honey samples, we used a screening method, such as a microbiological test, in our study. It was confirmed that no residues of tetracycline and sulfonamide were detected in all honey samples – no zone of inhibition was observed (Table 5) (according to the Reg EC N 396/2005). For this reason, a quantitative method and the use of liquid chromatography with mass spectrometry were not necessary.

Organochlorine pesticide residues and honey contamination

The use of pesticides in the treatment of various bee diseases without approved and controlled protocols could contaminate the environment, harm animal species, and endanger human health, such as carcinogenic risks. The use of toxic substances in beehives poses the risk of contamination of honey and other bee products (JUAN-BORRAS *et al*., 2016).

The basic properties of organochlorine pesticides include their persistence, chemical stability, fat solubility, and a relatively low level of biotransformation and biodegradation.

Therefore, residues of 12 organochlorine pesticides were investigated in 135 honey samples from seven different regions of Serbia. This study showed that the honey samples were free from contamination (Table 5) – no insecticide residues were detected in the samples. The maximum residue level of 5 μg/kg was not exceeded in all samples. The analysis of the honey samples confirmed their harmlessness and gave an overview of the quality of honey from this area. However, the presence of pesticides may vary from year to year, as the reproduction of parasites and their treatment depends on numerous factors. Therefore, the monitoring of possible contamination of honey should be constantly in order to make out a proper evaluation of this highly valued natural product.

CONCLUSION

Honey is a favorite natural product, highly valued for both – nutritional and medicinal properties. It is often considered to be a healthy and organic food, enjoyed by many people around the world. However, despite its widespread popularity, the quality of honey is not always guaranteed, and it is important to have an official confirmation of its properties. Our study was conducted to determine the quality of honey in Serbia. The main objective of the study was to assess the enzyme activity (diastase), the content of hydroxymethylfurfural, sulfonamide, tetracycline, and pesticide residues (aldrin, dieldrin, endrin, lindane, total dichlorodiphenyltrichloroethane and derivatives, total of *cis* and *trans* isomers and endosulfan sulfate, sum of *cis* and *trans* chlordane, and sum of heptachlor and heptachlor epoxide) in 135 honey samples from seven different regions of Serbia, and to determine the physicochemical properties of five types of honey.

Table 5. Tetracycline, sulfonamide and pesticide residues in honey samples.

Parameter	Region 1 (Braničevo)	Region 2 (Moravica)	Region 3 (Rasina)	Region 4 (Raška)	Region 5 (Šumadija)	Region 6 (Zaječar)	Region 7 (Zlatibor)	MRL, mg/kg (REG EC N 2005)
Sulfonamide[1]	-	-	-	-	-	-	-	Without residue
Tetracycline[1]	-	-	-	-	-	-	-	0.10
Aldrin[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Dieldrin[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Endrin[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Lindane[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Σ DDT[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Σ Endosulfan[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Σ Chlordane[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01
Σ Heptachlor[2]	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	<0.005	0.01

[1]HPLC SOP – High Pressure Liquid Chromatography, Standard Operating Procedure (HEITZMAN, 1994);
[2]GC/MS SOP – Gas Chromatography/ Mass Spectrometry, Standard Operating Procedure;
Σ DDT = sum of dichlorodiphenyltrichloroethane and derivates;
Σ Endosulfan = sum of α and β isomers and endosulfan sulfhate;
Σ Chlordane = sum of *cis* and *trans* chlordane;
Σ Heptachlor = sum of heptachlor and heptachlor epoxide;
MRL – Maximum Residue Limit.

The study found that the quality characteristics of most of the tested honey samples met the international honey regulations recommended by the INTERNATIONAL HONEY COMMISSION (2009). However, there were a few honeydew honeys which did not meet the standards. The electrical conductivity of the honeydew honey was found to be lower than the minimum value of 0.8 mS cm^{-1}, which may indicate fraud or a mixture with blossom honeys. Additionally, some of the honey samples had diastase activity lower than the permissible limit of 8 Schade units. This suggests that long storage periods or heating during processing or storage may have occurred. It is important to note that honey with low diastase activity may not have the same nutritional or medicinal properties as honey with higher activity levels. Despite these concerns, honey remains a valuable and versatile product, used not only as a raw food but also in cosmetics and pharmaceuticals. It is important to continue monitoring the quality of honey to ensure that consumers receive the best possible product.

It is crucial to monitor and detect the presence of xenobiotics in food products intended for human consumption since they pose a significant threat to human health. Therefore, our investigation focused on monitoring and surveillance to ensure the safety of food products. The results of our study revealed that all honey samples met the allowed concentrations for tetracycline and sulfonamide residues, indicating the absence of hazardous substances.

The transfer of pesticide residues to honey and other bee products is a pressing issue that requires urgent attention as it can cause genetic mutations and cellular degradation. Our study utilized GC/MS to investigate pesticide residues in honey samples from various regions in Serbia, and the results indicated that all investigated pesticide residues were below the acceptable minimal concentrations of less than 0.005 mg/kg. These findings provide valuable insights into the safety and quality of honey products and underscore the importance of monitoring and surveillance to ensure their safety for human consumption.

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THE PHYSICOCHEMICAL CHARACTERISTICS OF HONEY AND QUANTIFICATION OF SOME ANTI-MICROBIAL AGENTS IN HONEY FROM DIFFERENT SERBIAN REGIONS AS A QUALITY ASSESSMENT TOOL

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Supplementary Material

Table S2. Examined organochlorine pesticides and their retention times.

Pesticide	Retention time (min)
α-HCH	12.305
β-HCH	12.918
γ-HCH (Lindane)	13.007
δ-HCH	13.563
Heptachlor	14.460
Aldrin	15.150
Heptachlor epoxide	15.934
trans Chlordane	16.393
α-Endosulfan	16.624
cis Chlordane	16.667
pp' DDE	16.068
Dieldrin	17.117
Endrin	17.524
Endosulfan	17.701
pp' DDD	17.851
Endrin aldehyde	18.061
Endosulfansulphate	18.496
pp' DDT	18.550
Methoxychlor	18.803
Endrin ketone	19.344

Table S3. Retention time, molecule weight and ions important for the analysis.*

Pesticide	RT (min)	MW	T	Q1	Q2
α-HCH	11.28	290.8	183	181	219
β-HCH	12.47	290.8	219	181	183
γ-HCH (Lindane)	12.57	290.8	181	183	109
δ-HCH	13.74	290.8	109	219	183
Heptachlor	15.74	370	272	235	237
Aldrin	17.40	362	263	220	291
Heptachlor epoxide	19.55	386	353	81	355
trans Chlordane	20.88	406	373	375	-
α-Endosulfan	21.46	404	195	159	133
cis Chlordane	21.71	406	373	375	-
pp' DDE	22.84	378	79	277	239
Dieldrin	23.09	316	246	176	211
Endrin	23.80	378	263	191	226
Endosulfan	24.26	404	195	157	159
pp' DDD	24.90	318	235	165	237
Endrin aldehyde	25.065	378	67	345	-
Endosulfansulphate	25.97	420	272	274	387
pp' DDT	26.26	352	235	165	200
Methoxychlor	26.88	344	227	165	184
Endrin ketone	27.46	240	317	67	-

*Retention time – RT; molecular mass – MW; primary, target, ion – T; secondary and tertiary ion – Qualifier Ions, Q1 and Q2.

Table S4. SIM program applied for analysis.

Time (min)	Pesticide/ Group of pesticide	m/z	Total dwell time
10.78	α-HCH, β-HCH, γ-HCH, δ-HCH	181, 219, 109	150
14.98	Heptachlor	100, 237, 272	150
16.66	Aldrin	66, 263, 293	150
18.84	Heptachlor epoxide	81, 353, 237, 263	200
20.37	*cis/trans* Chlordane, Endosulfan I	373, 237, 272, 195, 237, 170	300
22.38	Dieldrin, *pp'* DDE	79, 263, 246, 176, 318	250
23.45	Endrin, Endosulfan II	81, 67, 263, 245, 195, 237, 243	350
24.62	*pp'* DDD, Endrin aldehyde	235, 165, 67, 173, 250	250
25.29	*pp'* DDT, Endosulfansulphate	165, 235, 237, 275, 387, 422	300
26.56	Methoxychlor	227, 152	100
27.14	Endrin ketone	67, 317, 345	150

Table S5. Validation data for the listed pesticides.

Pesticide	Precision (%)	Reproducibility (%)	Accuracy (%)	Linearity (r^2)	LOQ (mg/kg)	LOD (mg/kg)
α-HCH	1.20874	12.31710	112.75304	0.99853	0.00451	0.00135
β-HCH	5.85603	7.58655	112.36659	0.99672	0.00457	0.00137
γ-HCH (Lindane)	7.35402	9.05507	100.80005	0.99768	0.00496	0.00149
δ-HCH	4.74408	14.03158	112.41577	0,99792	0,00100	0.00030
Heptachlor	8.94628	17.16411	118.17588	0.99603	0.00358	0.00108
Aldrin	3.51919	11.58078	103.24248	0.99847	0.00451	0.00135
Heptachlor epoxide	7.87525	6.83867	96.08820	0.99787	0.00210	0.00063
trans Chlordane	10.21934	5.10399	98.62626	0.99825	0.00357	0.00107
α-Endosulfan	6.13672	14.86338	95.19121	0.99792	0.00237	0.00071
cis Chlordane	12.81811	4.76600	94.15898	0.99810	0.00320	0.00096
pp' DDE	11.21875	12.58085	96.76469	0.99871	0.00483	0.00145
Dieldrin	7.26587	12.52852	89.40030	0.99796	0.00459	0.00138
Endrin	9.24630	12.86689	101.47781	0.99408	0.00341	0.00102
Endosulfan	9.64287	10.62710	93.91650	0.99781	0.00459	0.00138
pp' DDD	5.21497	3.62948	113.30032	0.99524	0.00430	0.00129
Endosulfansulphate	5.65242	12.30613	113.54725	0.99534	0.00489	0.00147
pp' DDT	6.10376	9.66530	103.42179	0.99479	0.00466	0.00140
Endrin aldehyde	3.71780	13.36805	116.29021	0.99129	0.00490	0.00147
Methoxychlor	2.94677	13.30149	94.04154	0.99664	0.00465	0.00140
min	**1.20874**	**3.62948**	**89.40030**	**0.99129**	**0.00100**	**0.00030**
max	**12.81811**	**17.16411**	**118.17588**	**0.99871**	**0.00496**	**0.00149**

*r – correlation coefficient; LOQ – limit of quantification; LOD – limit of detection.

Table S6. Data from internal control for spike sample of honey (true value of spike sample is 0.05 mg/kg).

Matrix	N	Xsr	Stdev	RSD (%)	Bias (%)	Recovery (%)
Honey	15	0.052	0.005	9.62	3.960	103.960