doi: 10.7251/ZSAN2302333M **Original scientific paper**

ASSESSMENT OF TOTAL FLAVONOID CONTENT, ANTIOXIDANT ACTIVITY AND COLOR OF DIFFERENT HONEY SAMPLES

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Abstract: *The aim of this paper was to examine and analyze color intensity, total flavonoid content and antioxidant activity, and determine the correlation between them in different honey samples from different geographical areas. A total of 12 honey samples were analyzed, 5 were collected from the area of the city of Prijedor (3 samples of linden honey, 1 acacia honey and 1 flower honey), 1 sample each from the municipality of Jezero (meadow honey) and Mrkonjić Grad (meadow honey) and from the place Čelarevo in the Republic of Serbia (sunflower honey), while the remaining 4 honey samples were purchased in the store (2 meadow, 1 flower and 1 acacia honey). The color intensity was determined using the Pfund method, and the values ranged from 19.24 mm (store-bought acacia honey) to 201.1 mm (meadow honey from municipality of Jezero). The content of total flavonoids was obtained using the method of forming a complex with aluminum chloride, and catechin (CE) was used as a standard. The obtained results for flavonoids ranged from 0.044 mg CE/100 g of honey (sunflower honey) to 54 mg CE/100 g (acacia honey from the territory of Prijedor). The DPPH test was used to determine antioxidants, and the obtained results, expressed as IC_{50} (mg/ml), showed that the flower honey produced in the territory of the city of Prijedor has the highest antioxidant capacity (10.1 mg/ml). The data were processed in Excel 2016, and statistical analysis showed a positive weak correlation between color intensity and total flavonoid content (r^2=0.18), a negative moderate correlation between color intensity and antioxidant activity (r^2=-0.42) and weak negative correlation between flavonoids and antioxidant activity (r^2=-0.14).*

Key words: *honey, color, flavonoids, antioxidant activity*

Introduction

Besides the fact that natural honey plays an important role in traditional medicine, during the last few decades, it has been subjected to laboratory and clinical tests by several research groups and has found a place in modern medicine as well [1]. Several studies prove the effectiveness of honey for various medicinal purposes, due to its antibacterial [2, 3], anti-inflammatory, antioxidant [4, 5], antiviral, [6, 7], anti-fungal [8] and anti-cancer properties [9]. These activities are mainly attributed to phenolic compounds in honey, such as flavonoids, which have antioxidant properties and radical scavenging activities [10]. Antioxidants are substances that protect the body's cells from damage caused by unstable molecules known as free radicals. If free radicals overpower the body's ability to regulate them, a condition known as oxidative stress occurs [11]. Oxidative stress is a redox-sensitive phenomenon that occurs when reactive oxygen species (ROS) accumulate in a living

organism faster than the rate of detoxification of the organism. It can lead to symptoms of premature aging and cause acute mortality at higher doses [12]. Honey is a good source of natural antioxidants [13], and the antioxidants present in it, such as vitamins, enzymes and other bioactive compounds (phenols and flavonoids), with antioxidant activity, can eliminate the production of ROS. The levels of enzymes (such as catalase) and vitamins (such as vitamins C and E) play a key role in the antioxidant capacity of honey. The percentage of these components of honey determines the quality [14]. Depending on geographical and climatic conditions, different types of honey contain a wide range of phytochemicals [15], even when honey is produced from the same plant and geographical origin, honey can vary in texture, color and composition depending on the type of bee, soil, weather conditions, and even the age of the bees, which greatly affects the enzyme activity that produces honey [16]. Methods of quantitative analysis of flavonoid compounds are expressed by spectrophotometric (for example, they can be determined using the method of aluminum chloride (AlCl3) and 2-4 dinitrophenylhydazine) and chromatographic techniques [17]. A number of analytical methods are available for determining the antioxidant properties of honey, and in recent years, the tests used to assess the antioxidant activity of honey are: DPPH test (diphenyl-1-picrylhydrazyl)-free radical scavenging activity test, FRAP (Ferric Reducing Antioxidant Power), ORAC (The Oxygen Radical Absorbance Capacity), ABTS [2, 2-azino-bis (3 ethylbenzothiazoline-6-sulfonic acid) diammonium salt], TEAC [6-hydroxy-2,5, 7,8-tetramethylchroman-2-carboxylic acid, known as Trolox - antioxidant capacity equivalent] [18]. An assay based on the use of the DPPH radical is among the most popular spectrophotometric methods [19] and the use of the DPPH assay together with various other useful methods such as FRAP and ORAC is preferred because they can more accurately represent the antioxidant properties of honey [18]. Color is one of the most important sensory properties of honey for consumers. Honey originating from different plant species has different colors, but there can be variability within them as well, if it originates from different geographical locations [20]. The color of honey is influenced by several factors: (phenols, carotenoids, sugars, minerals, pollen), water content, plant and geographical origin, temperature and weather conditions, etc. The color of honey can be assessed in different ways, the most famous is the Pfund scale [21]. Al-Farsi et al., [22] and Mendoza-Bacilio et al., [23] observed a strong correlation between color, flavonoids and antioxidants of honey.

The aim of this paper was to examine and analyze color intensity, total flavonoid content and antioxidant activity, and determine the correlation between them in different honey samples from different geographical areas.

Material and Methods

Material

A total of 12 honey samples were analyzed, 5 were collected from the area of the city of Prijedor, Bosnia and Herzegovina (BiH), (3 samples of linden honey, 1 acacia honey and 1 flower honey), 1 sample each from the municipality of Jezero (meadow honey), BiH and Mrkonjić Grad (meadow honey), BiH and from the place

Čelarevo in the Republic of Serbia (sunflower honey), while the remaining 4 samples of honey were purchased in the store (2 meadow, 1 flower and 1 acacia honey) in BiH. The samples were stored at room temperature until analysis. The experiment was performed during November and December 2022, in the Laboratory for Sanitary Chemistry in College of Health Sciences in Prijedor.

Methods

Color intensity was determined according to the method of Al-Farsi et al., [22]. The samples were diluted up to 50% with distilled water and then centrifuged at 3200 rpm/5 minutes. Absorbance was measured at 635 nm using a spectrophotometer (Jenway 6305 UV/VIS, 190-1000nm, UK) and color intensity was determined using the Pfund scale, according to the following equation:

$$\mathbf{Pfund = -38.70 + 371.39 * Abs}$$

where is:
Pfund = honey color value on the Pfund scale (mm);
Abs = absorbance reading value at 635 nm wavelength.

The total flavonoids content in honey samples was determined by the method according to Al-Farsi et al. [22]. 1 ml of diluted honey sample is mixed with 4 ml of distilled water and 0.3 ml of 5% sodium nitrite (NaNo2). After five minutes, 0.3 ml of 10% AlCl3 is added, and after six minutes, 2 ml of 1M sodium hydroxide (NaOH). The volume of the mixture was increased with distilled water to 10 ml, and the absorbance was measured with the help of a spectrophotometer (Jenway 6305 UV/VIS, 190-1000nm, UK), at 510 nm. Concentrations of total flavonoids were obtained from a curve (y=0.0034x+0.0187, R2=0.9942), which was made using catechin (CE) as a standard. The results are expressed in mg CE/100g.

Antioxidant activity was determined using the DPPH test, according to the method of Reshma et al., [24]. Honey concentrations from 600 mg to 1 g/ml were made, and from each dilution 0.1 ml was mixed with 1.9 ml of DPPH (130 µM) made in ethanol, and 1 ml of acetate buffer (100 mM, pH=5.5). For the blank, honey was used in the same concentrations, which contains all reagents except DPPH. The samples were mixed and incubated for 90 minutes in a water bath at a temperature of 25°C. Absorbance was measured at 517 nm, and the obtained results were expressed as IC_{50} (mg/ml).

Results and discussion

Table 1 shows data on the type of tested honey, geographical origin and color, and in order to classify the colors, they are presented according to the Pfund scale (water white, extra white, white, extra light amber, light amber, amber and dark amber).

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Table 1. Type, geographical origin, color and classified colors of honey based on the Pfund scale

Type of honey	Geographical origin	The color of honey (mmPfund) ± Sd	Label of Honey color*
Home-produced honey			
Meadow honey	Jezero-BiH	201.21 ± 0.002	Dark Amber
Linden honey	Prijedor-BiH	35.95± 0.005	Extra Light Amber
Linden honey	Prijedor-BiH	78.28±0.007	Light Amber
Linden honey	Prijedor-BiH	55.63±0.003	Light Amber
Acacia honey	Prijedor-BiH	30.80±0.001	White
Flower honey	Prijedor-BiH	42.26±0.003	Extra Light Amber
Sunflower honey	Čelarevo-Serbia	37.06±0.005	Extra Light Amber
Meadow honey	Podrašnica, Mrkonjić Grad-BIH	45.98±0.008	Extra Light Amber
Commercial types of honey			
Meadow honey	-	89.8±0.005	Amber
Meadow honey	-	112.08±0.003	Amber
Flower honey	-	69±0.003	Light Amber
Acacia honey	-	19.24±0.010	White

*appropriate color label and applied ranges of each color on the Pfund scale were used according to the standards (United States Standards for Grades of Extracted Honey) issued by the USDA-(The U.S. Department of Agriculture) [25].

The color of the tested samples varied from white (in the samples of acacia honey purchased in the store, 19.24 mm) to dark amber (in the sample of domestic meadow honey, from the municipality of Jezero - 201.21 mm). Živković et al., [26] determined that acacia honey has a color intensity of 20 mm, which is similar to the results of this research (Table 1), where domestic acacia honey has a value of 30.80 mm, and purchased 19.24 mm. Domestic meadow honey, from the area of the village of Podrašnice, which belongs to Mrkonjić Grad, has a color intensity of 45.98 mm, while meadow honey from an area 11 km away, in the municipality of Jezero, has a value of 201.21 mm, which shows that the color of honey can vary even without regardless of the small distance of geographical areas. The color variability of the color of honey is influenced by a number of factors, such as: processing, handling, storage, age of the honey, but also conditions of color measurement [21]. Purchased meadow honey (both samples) have the Amber color label and color values of 89.8 and 112.08 mm (Table 1), which is similar to the results of the research by Živković et al., [26], which determine the same color label for meadow honey, and an intensity of 98 mm. The color of linden honey samples ranged from Extra Light Amber, which is consistent with the research of Pauliuc et al., [27] and Light Amber, which is consistent with the research of Živković et al., [26]. The sample of sunflower honey has a color value of 37.06 mm (Table 1), which is within the range of research according to Alba et al., [28], whose investigated values for sunflower honey range

from 36.9-82.9 mm. The color intensity in the domestic flower honey sample is 42.26 mm, which is lower compared to the values reported in the study by Al-Farsi et al., [22], while the purchased flower honey with a color intensity of 69 mm is in the range of reported values [22].

Table 2 shows the total content of flavonoids and antioxidants in the tested honey samples.

Table 2. Flavonoids and antioxidant activity of honey samples

Samples	Geographical origin	Total flavonoids (mg/100g) ± Sd	DPPH IC_{50} mg/ml
Home-produced honey			
Meadow honey	Jezero-BiH	51.9 ± 0.001	131.2
Linden honey	Prijedor-BiH	18.1 ± 0.001	543.6
Linden honey	Prijedor-BiH	19.6 ± 0.003	262.8
Linden honey	Prijedor-BiH	12.2 ± 0.003	149.7
Acacia honey	Prijedor-BiH	54 ± 0.001	578.5
Flower honey	Prijedor-BiH	48.1 ± 0.002	10.1
Sunflower honey	Čelarevo-Serbia	0.044 ± 0.002	692
Meadow honey	Podrašnica, Mrkonjić Grad-BiH	47.5 ± 0.003	623.7
Commercial types of honey			
Meadow honey	-	10.7 ±0.001	169.7
Meadow honey	-	23.1 ±0.001	388.6
Flower honey	-	18 ± 0.009	427.1
Acacia honey	-	31.9 ± 0.001	310.7

The highest flavonoid content of 54 mg/100g was recorded in a sample of domestic acacia honey, while in sunflower honey the value of 0.044 mg/g represents the lowest value. Albu et al [28] determined the total content of flavonoids in sunflower honey (2.52 mg quercetin (QE)/100 g, which is significantly higher compared to the results obtained in this research. Šarić et al., [29] in their research observed changes in the antioxidant activity and phenolic components of honey during storage, and recorded total flavonoid values in acacia honey from 8.29 to 29.65 mg (QE) per 100 g of honey during storage of two months. Those values are lower compared to the results obtained in this research, with the amount of total flavonoids in domestic acacia honey of 54 mg CE/100g and purchased honey of 31.9 mg/ CE/100 g (Table 2). Domestic flower honey has significantly more flavonoids (48.1 mg CE/100 g) compared to the values measured in purchased flower honey (18 mg CE/100 g), which is also the case for meadow and acacia honey (Table 2). According to Polak-Sliwinska and Tanska [30], the content of total flavonoids in water samples is 2.75 mg QE/100 g, while in ethanol 1.29 mg QE/100 g, which is generally lower compared to linden honey samples in this research. The higher content of flavonoids may be a consequence of botanical and geographical differences, as well as climatic and environmental factors such as humidity, temperature and soil composition [22]. Antioxidant analysis was determined using the DPPH test, via the IC_{50} parameter, which represents the concentration of material necessary to inhibit 50% of free

radicals. Therefore, a lower IC_{50} value in honey indicates a greater amount of antioxidants and the ability to neutralize free radicals and vice versa. The highest antioxidant activity was shown by flower honey, made in household conditions (10.1 mg/l), which significantly differs in antioxidant content from other honey samples, but also from commercial flower honey with an IC_{50} of 427.1 mg/l. Domestic meadow honey shows a higher antioxidant activity (131.2 mg/l) compared to commercial meadow honey samples (169.7-388.6 mg/l), which was shown to be the opposite for acacia honey (Table 2). There are numerous studies related to the analysis of antioxidants in honey, for example Stagos et al., [31] were examined the antioxidant activity in honey from acacia, mint and plants, whose mean values were 5.5 mg/ml (ABTS test), while Al-Fars et al., [22] determined antioxidant values in Sidr honey samples, which ranged from 33.8 to 72.3 mg/l, and for flower samples from 91.2 to 190.1 mg/l (DPPH test), whose values are significantly higher than the results obtained in this research. The sunflower honey sample showed the lowest antioxidant activity of 692 mg/ml (Table 2).

The correlation between total flavonoid content, color and radical scavenging activity in honey extracts was analyzed (Table 3).

Table 3. Correlation between color, flavonoids, and antioxidants

	Color	***Flavonoids***	***IC_{50}***
Color	1		
Flavonoids	0.18	1	
IC 50	-0.42	-0.14	1

According to the results in Table 3, there is a positive weak correlation between color intensity and total flavonoid content (r2=0.18), which according to Al-Farsi et al., [22] indicates that darker honey has a higher amount of flavonoids. For example, a sample of meadow honey from the municipality of Jezero in Bosnia and Herzegovina had the darkest color, a high flavonoid content of 51.9 mg/100g and a lower IC_{50} value of 131.2 mg/l, i.e. a significant amount of antioxidants (Table 2). It is followed by commercial samples of meadow honey, which have a slightly lighter honey color, a smaller amount of flavonoids, but also a lot of antioxidants, with the exception of meadow honey from the area of Mrkonjić Grad, the village of Podrašnica, which has a color labeled Extra Light Amber, a lot of flavonoids, but an IC_{50} of 623.7 mg/l. Also, an exception is acacia honey, which has the color label White, and a significant amount of flavonoids (Table 2). A negative moderate correlation was also observed between color intensity and DPPH (IC_{50}) (r2=-0.42), indicating that a darker honey sample reduces the IC_{50} value [24], and a weak negative correlation was recorded between flavonoids and DPPH (IC_{50}) (r2=-0.14), that is, as the amount of flavonoids increases, the IC_{50} decreases.

Conclusion

The tested parameters: color, flavonoids and antioxidants, vary depending on the type of honey. Flower honey produced under household conditions, originating

from Prijedor, Bosnia and Herzegovina, showed a dark color, a significant amount of flavonoids and a small IC50 value, i.e. the highest amount of antioxidants among the tested samples, which significantly sets it apart from other honey samples, as well as from commercial flower honey. The lowest antioxidant activity was recorded in sunflower honey from Čelarevo in Serbia, with the lowest amount of flavonoids, and light color, which indicates that darker colored honey is superior to lighter colored honey. All three samples of linden honey come from the same geographical area, two had the color label Light Amber, while one had a lighter shade of Extra Light Amber and had similar values for flavonoids and antioxidants, except for 1 sample that had a high IC_{50} value, which indicates variability within the same type of honey, but also within the same geographical area. Domestic and commercial acacia honey had the color label White, a considerable amount of flavonoids, but high IC_{50} values. The tested samples of meadow honey (homemade and purchased) showed a significant level of antioxidants, with the exception of one sample, which can be attributed to the measurement conditions. A positive correlation was observed between color and flavonoids, while a negative correlation was recorded between color and antioxidants, and flavonoids and antioxidants.

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PROCJENA UKUPNOG SADRŽAJA FLAVONOIDA, ANTIOKSIDATIVNE AKTIVNOSTI I BOJE RAZLIČITIH UZORAKA MEDA

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Sažetak: *Cilj ovog rada bio je ispitati i analizirati intenzitet boje, ukupan sadržaj flavonoida i antioksidativnu aktivnost, te utvrditi povezanost između njih u različitim uzorcima meda, sa različitih geografskih područja. Ukupno je analizirano 12 uzoraka meda, 5 je prikupljeno sa područja grada Prijedora (3 uzorka meda od lipe, 1 bagremov med i 1 cvjetni), po 1 uzorak iz opštine Jezero (livadski med) i Mrkonjić Grada (livadski med) te iz mjesta Čelarevo u Republici Srbiji (med od suncokreta), dok su ostala 4 uzorka meda nabavljena u trgovini (2 livadska, 1 cvjetni i 1 bagremov med). Intenzitet boje određen je pomoću Pfund metode, a vrijednosti su se kretale od 19,24 mm (bagremov med-kupovni) do 201,1 mm (livadski med sa teritorije opštine Jezero). Sadržaj ukupnih flavonoida dobijen je primjenom metode stvaranja kompleksa sa aluminijum hloridom, a katehin (CE) je upotrijebljen kao standard. Dobijeni rezultati za flavonoide su se kretali od 0,044 mg CE/100 g meda (med od suncokreta), do 54 mg CE/100 g (bagremov med sa teritorije Prijedora). Za određivanje antioksidanasa korišten je DPPH test, a dobijeni rezultati, koji su izraženi kao IC_{50} (mg/ml), su pokazali da cvjetni med proizveden na teritoriji grada Prijedora ima najveći antioksidativni kapacitet (10,1 mg/ml). Podaci su obrađeni u Excel-u 2016,*

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a statistička analiza pokazala je pozitivnu slabu korelaciju između intenziteta boje i ukupnog sadržaja flavonoida (r^2=0,18), negativno umjerenu korelaciju između intenziteta boje i antioksidativne aktivnosti (r^2=-0,42) i slabo negativnu korelaciju između flavonoida i antioksidativne aktivnosti (r^2=-0,14).

Ključne riječi: *med, boja, flavonoidi, antioksidativna aktivnost*