FLUORIMETRIC PROFILES, FLAVONOID AND POLYPHENOLS CONTENT OF ACACIA, MEADOW AND HONEYDEW HONEY SAMPLES AND THEIR CORRELATION WITH COLOUR INTENSITY OF HONEY

ORIGINAL SCIENTIFIC PAPER

Nahida Srabović[1]✉, Aida Smajlović[1], Adaleta Softić[1], Nađa Špralja[2], Nermina Ramić[3]

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[1]University of Tuzla, Faculty of Pharmacy, Department of Biochemistry, Urfeta Vejzagića 8, 75000 Tuzla, Bosnia and Herzegovina
[2]Tefarm, Pharmacy and Medical Supply Center, 74 260 Tešanj, Bosnia and Herzegovina
[3]O.S. Pharm Pharmacy, 75260 Kalesija, Bosnia and Herzegovina

✉ nahida.srabovic@untz.ba

ABSTRACT:
Introduction: Previous studies have showed that fluorimetric analysis may be used as a simple, rapid, low cost and reliable method for authentication botanical origin of honey. Primary aims of this study were to record fluorescence spactra of acacia, meadow and honeydew honey samples and to determine content of flavonoid and polyphenoles in relation to colour intensity of honey.
Material and methods: Fluorescence spectra of honey samples were recorded. Spectrophotometric analysis was used to determine flavonoid and polyphenols content. The honey color scoring was developed by the authors as the arbitrary system.
Results: Acacia honey showed high fluorescence emission intensity after an excitation at 340 nm, 390 nm and 440 nm. Meadow honey showed fluorescence after excitation at 390 nm and 440 nm, while fluorescence, caused by excitation at 340 nm, was absent. Honeydew honey showed low intensity of fluorescence at 440 nm excitation while fluorescence was absent at 340 nm and 390 nm excitation, respectively. Statistically significant difference was found for flavonoids and polyphenols levels, between honeydew and acacia honey. Statistically significant difference in polyphenols levels between meadow and acacia honey was found. There was no statistically significant difference of flavonoids and polyphenols between samples of meadow and honeydew honey.
Conclusion: Fluorimetric profiles, flavonoid and polyphenols content, together with colour intensity of honey may be useful in authenitication of botanical origin of honey.

KEYWORDS: florescence spectra, honey, botanical origin, flavonoids, polyphenols

INTRODUCTION

Honey is a natural product derived from honeybees from the nectar of flowers with a wide range of minor constituents with antioxidant properties such as flavonoids, certain enzymes (glucose oxidase, catalase), amino acids and proteins. Generally, antioxidants play an important role in food preservation and human health by combating damage caused by oxidizing agents. Over the past decades numerous methods and analyses were developed for determining botanical origin of honey and its correlation with phenolic and flavonoid contents as antioxidants of honey [1]-[5].

Numerous different methods which are considered to be the traditional methods for authenticating the botanical origin of honey require considerable sample preparation, highly skilled personnel, and are also time-consuming and costly [6]. Previous studies have showed that fluorescence spectroscopy in honey analyses has many advantages compared to other methods such as better sensitivity, little sample preparation and rapid, simple and low-cost usage [7], [8]. Primary aim of this study was to record fluorescence spectra which were, according to our previous study, critical for estimation of botanical origin analyzed acacia, honeydew and meadow honey and also to determine content of flavonoids and phenolic compounds, strong fluorophores and well established markers of botanical origin of honey [6], in relation to colour intensity of honey.

MATERIAL AND METHODS

The honey samples which fluorimetric profiles coincided with the manufacturer's statements on the botanical origin were included in the study: 73 different honey samples (11 acacia, 25 meadow, 9 honey-

dew, 4 lime, 2 heather, 1 sunflower and 21 mixed honeys). The number of 63 honey samples was collected from individual producers from Bosnia and Herzegovina and 10 samples were commercially available.

FLUORIMETRIC ANALYSIS

Fluorescence spectra of honey samples were recorded at spectrofluorimeter RF-5301 PC (Shimadzu, Japan). Fluorescence emission spectra from 290 nm to 650 nm were recorded after excitation of the honey samples at different wavelengths: 220 nm, 270 nm, 310 nm, 340 nm, 350 nm, 390 nm, 440 nm, 450 nm and 460 nm. The entrance and exit slits for the excitation light-beam were both 1.5 nm. Honey samples, used for recording fluorescence spectra, were prepared in the following manner: 20 g of honey were incubated 8 h in a water bath at 40°C, cooled at room temperature and then used for recording fluorescence spectra in 1 cm quartz cell, without dilution and filtering.

ESTIMATION OF TOTAL PHENOLIC COMPOUNDS

For the determination of total phenolic compounds, a modified method of Singleton and Rossi [9] was used. The amount of 5.0 g of honey was treated with 50 mL of distilled water, mixed and filtered through a qualitative filter. 500 μL of this solution was mixed with 2.5 mL Folin – Ciocalteau reagent (0.2N) for 5 min. and then 2 mL of Na_2CO_3 solution (75 g/L) was added. The samples were incubated at room temperature in the dark for 2 hrs and the absorbance of the samples was measured at 760 nm. For the blank solution, methanol was used in place of honey; a stock solution of gallic acid (1 mg/mL) was prepared for further dilution. The linearity obtained was R^2 = 0.9987 (Y= 6.9156x). Results were expressed as mg gallic acid equivalent (GAE) per 100 g of sample.

DETERMINATION OF FLAVONOIDS

For flavonoids determination, 1mL of honey solution (1 mg/mL) was mixed with 0.3 mL of 5% $NaNO_2$ and after 5 minutes 0.3 mL of 10% $AlCl_3$ was added. The honey samples were mixed, incubated for 6 minutes and neutralized with 2.0 mL of 1M NaOH solution. The absorbance was read at 510 nm. Quercetin was used to calculate the standard curve. A linearity of 0.998 (R^2) was obtained (Y= 0.378x – 0.002), and result expressed as mg quercetin equivalent (QE) per 100 g of sample [8].

COLOR SCORING

The honey color scoring was developed by the authors as the arbitrary system. The color intensity was estimated by four independent observers. All honey samples were classified into four groups: very bright, bright, medium dark and dark.

STATISTICAL ANALYSIS

For statistical analysis of the results SPSS software, release 20.0. (SPSS Inc., Chicago, IL, USA) was used. Mann-Whitney U test was used to compare differences in flavonoids and polyphenols levels between honey samples of different botanical origin. Correlations between flavonoids, polyphenols and color intensity were defined by Spearman rank correlation analysis. In all tests, two-sided *P* below 0.05 was considered significant.

RESULTS AND DISCUSSION

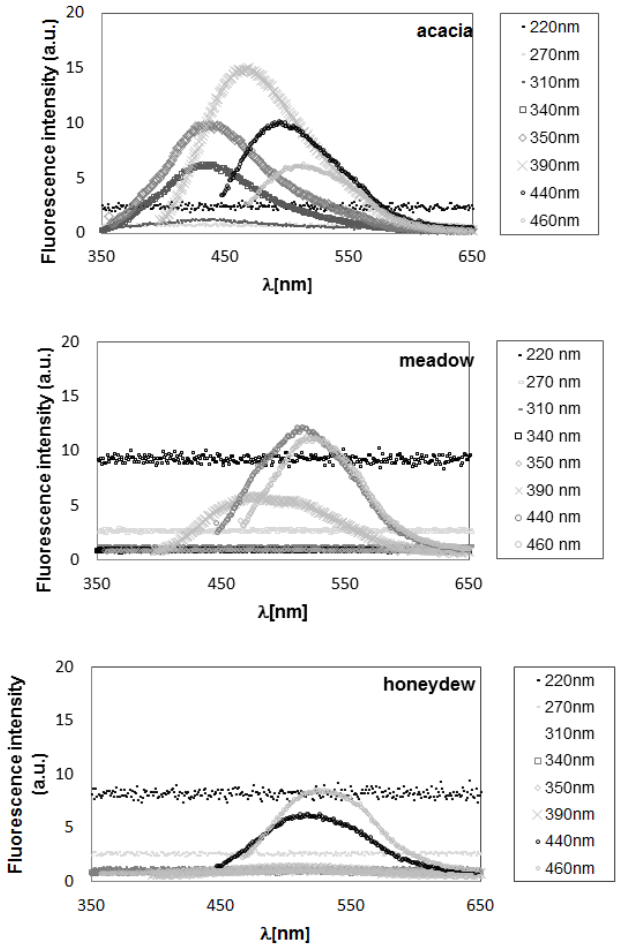

Figure 1. Fluorescence emission spectra of natural acacia, meadow and honeydew honey from Bosnia and Herzegovina recorded from 290 nm to 650 nm, at different excitation wavelengths: 220 nm, 270 nm, 310 nm, 340 nm, 350 nm, 390 nm, 440 nm, 450 nm and 460 nm.

According to our previous research, honey samples of same botanical origin had very similar set of fluorimetric emission spectra [7], [8]. In most of the tested samples, the obtained fluorimetric profiles coincided with the manufacturer's statements on the botanical origin of honey. Also, by determining the concentration of proline in honey samples, those samples in which the proline was not detected were excluded from the study as they were genuine [10]. Therefore, a further investigation included 9 samples of acacia honey, 12 meadow samples and 11 samples of honeydew honey.

Fluorimetric profiles of acacia, meadow and honeydew honey are presented in Figure 1. Acacia honey showed high fluorescence emission intensity after an excitation at 340 nm, 390 nm and 440 nm. Meadow honey showed fluorescence at excitation at 390 nm and 440 nm, while fluorescence emission, caused by excitation at 340 nm, was absent. Honeydew honey showed low intensity of fluorescence at excitation at 440 nm while fluorescence intensity was absent at excitation at 340 nm and 390 nm. These excitation wavelengths and resulting fluorimetric profiles represent a possible algorithm for fast characterization of botanical origin of honey samples. In further experiment, fluorescence emission profiles were used to differentiate honey samples into three groups: acacia, meadow and honeydew. Samples which fluorimetric profiles were of mixed character were not included in the further analysis.

Intrinsic fluorophores and their specific microenvironments in honey produce a complex excitation-emission pattern which varies among samples. Dramićanin and colleges have found five spectral regions of high emission intensities for natural honeys [11]. Our results of fluorescence emission spectra after excitations at 310 nm, 340 nm and 350 nm, for acacia honey were in accordance with the fourth spectral region, obtained after the excitation from 310 nm to 360 nm [11]. However, according to our results, fluorescence spectra of honeydew and meadow samples showed very low fluorescence emission when excited in this region. Moreover, the fluorescence emission after an excitation at 310 nm, 340 nm and 350 nm, together with fluorescence emission at 420 nm after the excitation between 220 nm and 400 nm [7], represents one of the key results for identification of acacia honey.

In Table 1 the descriptive statistics for proline, flavonoid and polyphenol values in three analyzed honey groups are presented.

Table 1. Descriptive statistics for proline, flavonoids and polyphenols in different types of honey

Honey		Sample No	Median ± SD	Range
Acacia	Proline (mg/kg)	9	452.60 ± 214.10	190.00 – 778.80
	Flavonoids (mg QE/100g)	7	15.49 ± 0.39	8.45 – 19.89
	Polyphenols (mg GAE/100g)	9	222.00 ± 72.92	98.00 – 298.00
Meadow	Proline (mg/kg)	12	694.60 ± 340.30	279.60 – 1229.20
	Flavonoids (mg QE/100g)	12	19.45 ± 16.23	5.81 – 67.42
	Polyphenols (mg GAE/100g)	12	443.00 ± 168.52	164.00 – 654.00
Honeydew	Proline (mg/kg)	11	665.60 ± 386.14	331.60 – 1441.80
	Flavonoids (mg QE/100g)	9	22.53 ± 9.81	11.97 – 44.54
	Polyphenols (mg GAE/100g)	11	388.00 ± 147.10	240.00 – 642.00

Acacia had the lowest levels and the honeydew honey had the highest levels of flavonoids and polyphenols (Table 1). Obtained levels of polyphenols in honeydew honey are consistent with the previously published data [12] which showed higher levels of phenolic compounds in honeydew honey in relation to other honey samples. When honeydew and acacia honey were compared, highly statistically significant difference was detected for flavonoids and polyphenols. We found statistically significant difference in polyphenol levels between meadow and acacia honey samples which is also in accordance with results ob-

tained by other authors who found that meadow honeys had significantly higher total phenolic content than acacia honey [13]. In this research we have not found statistically significant difference in the flavonoids content or in the content of polyphenols between samples of meadow and honeydew honey (Table 2).

Table 2. Comparison of levels of flavonoids and polyphenols measured in three different types of honey

	Flavonoids		Polyphenols	
Honey	Man Whitney U	P value	Man Whitney U	P value
Acacia *vs* honeydew	8.5	0.012*	6.5	0.001**
Acacia *vs* meadow	28.5	0.261	23.5	0.028*
Meadow *vs* honeydew	42	0.422	60	0.74
(*) statistically significant difference (**) highly statistically significant difference				

Table 3. Correlations of flavonoids, polyphenols and color intensity in different honey types

Sample		Spearman correlation coefficient	P value
Flavonoids *vs* polyphenols	Acacia	0.342	0.45
	Meadow	0.441	0.151
	Honeydew	0.874	0.002*
All honey samples	Color intensity *vs* flavonoids	0.608	0.001**
	Color intensity *vs* polyphenols	0.546	0.001**
(*) statistically significant difference (**) highly statistically significant difference			

The results of correlations between flavonoids, polyphenols and color intensity are presented in Table 3. Correlation between flavonoids and polyphenols was significant in the honeydew samples. On the other hand, some authors [14] found low correlation (R=-0.38) between total phenolic and total flavonoid content in the honeydew samples analyzed.

Based on the color intensity, all honey samples were classified into four groups: very bright (N=5), bright (N=8), medium dark (N=8) and dark (N=11). In all analyzed samples, statistically significant correlation was found between color intensity and the flavonoid levels, and between color intensity and polyphenols, respectively (Table 3). Our results are consistent with the results of Pontis et al. [15] who also found significant correlation between color and flavonoid and phenolic content of the honey samples which they investigated.

CONCLUSION

Obtained results suggest that fluorimetric profile, flavonoid and polyphenols content, together with colour intensity of honey may be useful in authentication of botanical origin of honey. Further investigation is necessary for developing unique algorithm for fast characterization of botanical origin of honey.

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