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VARIABILITY ESTIMATION OF THE PROTEIN AND PHENOL TOTAL CONTENT IN HONEY USING FRONT FACE FLUORESCENCE SPECTROSCOPY COUPLED WITH MCR-ALS ANALYSIS

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It has been explored to what extent the ratio of the two main fluorophores in honey, originating from proteins and phenolic compounds, change between the honey extraction stage and packaging in the jars. Fluorescence spectroscopy combined with multivariate curve resolution alternating least squares (MCR-ALS) was used to determine the ratio of the spectral components originating from phenolics and proteins $(Ph/Pr)_{fl}$*, as a ratiometric indicator of variability in selected Lime tree (Tilia L.) honey samples. Spectrophotometric quantification of phenols and proteins in the honey samples was also performed and their ratio* $(Ph/Pr)_{sp}$ *calculated. The values of the* $(Ph/Pr)_{fl}$ *ratio and honey protein content after packaging depended on the quality of homogenization before packing in jars. Colorimetric and fluorometric results for phenols and proteins were in compliance. The examples are the values 3.34, 3.30 and 9, 3.14 for* $(Ph/Pr)_{fl}$ *and corresponding values 2.64, 2.18 and 12.75, 2.31 for* $(Ph/Pr)_{sp}$*, in all pairs, the first value presenting the sample after extraction and the second value the sample after packing in jars. Both methods show that in the former case there is no change in the phenol/protein ratio, and in the latter case the ratio decreased.*

Keywords: *honey, fluorescence spectroscopy, multivariate curve resolution alternating least squares, phenol and protein quantification, ratio metric indicator, melissopalynological analysis.*

ОЦЕНКА ИЗМЕНЧИВОСТИ ОБЩЕГО СОДЕРЖАНИЯ БЕЛКА И ФЕНОЛА В МЕДЕ С ПОМОЩЬЮ ФЛУОРЕСЦЕНТНОЙ СПЕКТРОСКОПИИ В СОЧЕТАНИИ С АНАЛИЗОМ РАЗРЕШЕНИЯ МНОГОМЕРНЫХ КРИВЫХ МЕТОДОМ ЧЕРЕДУЮЩИХСЯ НАИМЕНЬШИХ КВАДРАТОВ

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Исследовано изменение соотношения двух основных флуорофоров, выделенных из белков и фенольных соединений липового меда, между стадией экстракции меда и его упаковкой в банки. Соотношение спектральных компонент, соответствующих фенольным и белковым соединениям $(Ph/Pr)_{fl}$*, определено на основе данных флуоресцентной спектроскопии в сочетании с алгоритмом разрешения многомерных кривых методом чередующихся наименьших квадратов (MCR-ALS). Проведено спектрофотометрическое определение количества фенолов и белков в образцах меда и рассчитано их соотношение* $(Ph/Pr)_{sp}$*. Отношение* $(Ph/Pr)_{fl}$ *и содержание белка в меде после упаковки зависят от качества гомогенизации перед упаковкой в банки. Колориметрические и флуорометрические резуль-*

таты измерений соотношения фенол/белок согласуются: $(Ph/Pr)_{fl}$ = 3.34, 3.30 и 9, 3.14 и $(Ph/Pr)_{sp}$ = = 2.64, 2.18 и 12.75, 2.31; во всех парах первое значение для образца сразу после извлечения, второе — после упаковки в банки. Оба метода показывают, что в первом случае соотношение фенол/белок не изменяется, во втором уменьшается.

Ключевые слова: *мед, флуоресцентная спектроскопия, алгоритм разрешения многомерных кривых методом чередующихся наименьших квадратов, количественное определение фенола и белка, показатель отношения, мелиссопалинологический анализ.*

Introduction. Various methods for studying honey quality have been in research focus over the past years [1]. Having in mind a notable increase in honey bee (*Apismellifera L.*) colony loses [2], it is of great interest to develop fast and reliable methods for examining effects that diseases, parasites, pesticides, and poor nutrition have on the health status of managed honey bees. It is of interest to develop fast and reliable methods for screening of honey variability.

Fluorescence is a nondestructive, sensitive, and fast method for analysis of fluorescent compounds contained in very low amounts (nanomolar concentrations) in the samples. It can be used for structural or concentration studies and for analytical or diagnostic purposes [3]. The fluorescence spectra, in combination with appropriate statistical methods, may provide useful fingerprints in food analysis [4]. The suitability of front face geometry in fluorescence spectroscopy has already been demonstrated for characterization of different solid-state samples [4–6] leading to its application in differentiation and classification of different honey types with respect to their botanical [7] and geographic origin [8].

The aim of this study was to test to what extent data obtained from steady-state fluorescence spectroscopy could be used for analysis of variability in honey foraged by different colonies but in the same geographical area. Also, the possibility to identify differences resulting from homogenization before packaging has been explored. We applied steady-state fluorescence spectroscopy in combination with multivariate curve resolution alternating least squares (MCR-ALS) for spectral analysis [9] in order to differentiate Lime (*Tilia L.*) honey samples from the Fruška Gora Mountain, collected from different beekeepers. We studied changes of the emission spectra of these samples immediately after honey extraction and after packing in honey jars. We determined the ratio of the spectral components of two main fluorophores in honey, originating from proteins and phenolic compounds, as a ratiometric indicator of variability in honey samples foraged from individual beekeepers. Phenolic compounds in honey originate from nectar, while proteins mainly originate from bees (2/3 of the total honey proteins), but pollen suspended in nectar could also contribute to protein content (1/3 of the total honey proteins) [10]. In order to confirm the contribution of proteins and phenols to the analyzed spectral components, we have also quantified proteins and phenols in each sample and performed melissopalynological analysis to determine the concentration of pollen grains and contribution of *Tilia* pollen.

Experimental. Folin–Ciocalteu reagent, gallic acid, and 85% H_3PO_4 were obtained from Sigma-Aldrich (St Louis, USA). Sodium carbonate solution, Coomassie Brilliant Blue G-250, and 95% ethanol were purchased from Fluka Analytical (Buchi, Switzerland). Bovine serum albumin was obtained from Biowest (Nuaillé, France).

Samples of Lime tree (*Tilia* L.) honey, foraged on Fruška Gora Mountain in 2015, were obtained from 17 beekeepers. Each producer provided a sample immediately after extraction from combs and, one month later, a sample from the same batch after packing into jars. All samples were stored at room temperature in the dark before analysis. Beekeepers that provided honey samples for the analysis were involved in the certification process for allowance to use Protected Designation of Origin (PDO) for "Fruškagora Lime honey" and thus their production was approved for good health status of their colonies, no adulteration and mixing with honey collected in previous forage. In addition, the analyzed honey has a high contribution of Lime tree nectar, which is indicated by the contribution of *Tilia* pollen [11].

Qualitative and quantitative melissopalynological analysis have been performed following the harmonized methods of melissopalynology [12]: 10 g of the sample was diluted in dH_2O, centrifuged, and the resulting sediment transferred to a microscopic slide. After mounting with glycerine-jelly, slides were analyzed using light microscope at ×400 magnification. The percentage of *Tilia* pollen as pollen concentration expressed in number of pollen grains per 10 g of honey (PG/10 g) are reported after counting pollen at approximately 2.5% of the slide surface.

The fluorescence spectra of the honey samples were recorded using an Fl3-221 P spectrofluorimeter (Jobin Yvon, Horiba, France), equipped with a 450W Xe lamp and a photomultiplier tube. The sample was

placed in the solid sample holder, in front-face configuration. The illumination's incident angle was set to 35° to minimize light reflections, scattered radiation, and depolarization phenomena. Rayleigh masking was applied in order to reduce Rayleigh scattering from the solid sample, which limits the sensitivity and accuracy of the measurement. Fluorescence steady-state emission spectrum of the mixtures such as honey may be a sum of two or more individual components corresponding to various fluorophores – emitting structural entities. In order to determine the number and emission profiles of components in an integral spectrum, measurement of series of emission spectra at different excitation wavelengths in a wavelength range is performed, thus obtaining excitation-emission matrices (EEMs) that are subsequently analyzed by using advanced statistical methods [13–15]. The fluorescence emission spectra in the range from 280 to 550 nm were recorded with excitation wavelengths of 270 to 370 nm. The integration time was 0.1 s, the wavelength increment in excitation measurements was 5 nm, and emission increment was 1 nm. A spectral band width of 2 nm was employed for both the excitation and emission slits.

An excitation-emission matrix (EEM) was generated for further statistical analysis by using the chemometric algorithm Multivariate curve resolution-alternating least squares (MCR-ALS). MCR-ALS has been used to resolve the overlapping signals and to extract the number of components and their spectral profiles for each excitation-emission matrix (EEM). The aim of MCR-ALS is to mathematically decompose the spectra of mixtures into the spectra of related components. It can identify a model from the empirical data, without any a priori assumption about the nature or composition of the system under investigation. Spectroscopic data are arranged in the matrix $\mathbf{D}(r \times c)$, where r is the number of emission wavelengths, while c is the number of measured spectra. MCR decomposition of the experimental matrix is performed according to the equation

$$\mathbf{D} = \mathbf{C}\mathbf{S}^{\mathrm{T}} + \mathbf{E},$$

where $\mathbf{C}(r \times n)$ is the matrix of the emission spectra of the components (fluorophores, the number of which is n); $\mathbf{S}(c \times n)$ is the matrix of concentration profiles, which represent the excitation spectra of the components; $\mathbf{E}(r \times c)$ is the matrix of residuals. Since there is an infinite number of matrices **C** and **S** that can generate the same matrix of data **D**, the spectra are extracted in such a way that they do not have any physical meaning, and it is necessary to perform rotation of the obtained solution. Considering the nature of the fluorescence spectra, non-negativity and uni-modality, it is necessary to incorporate these constraints in the algorithm for the MCR ALS in order to obtain solutions with a physical meaning. The software Unscrambler X 10.4 was used to handle the EEM data.

Samples were prepared according to a slightly modified method proposed by Gašić et al. [16]. Each honey sample (5 g) was mixed with 10 mL distilled water, homogenized in an ultrasonic bath for 15 min at room temperature, transferred to a 50 mL volumetric flask, and filled to the mark with ultrapure water. The total phenolic content was spectrophotometrically determined by the Folin–Ciocalteu method with some modifications [17]. Briefly, 0.3 mL of the sample solution and 6 mL deionized water were mixed with 0.5 mL of Folin–Ciocalteu reagent and incubated for 6 min at room temperature. After addition of 3 mL of 20% sodium carbonate solution, the sample was kept at 40°C for 30 min before the absorbance was measured at 765 nm. Gallic acid was used as the standard, and the calibration curve of gallic acid was prepared in the concentration range between 0 and 250 m/L. A mixture of water and Folin–Ciocalteu reagent was used as the blank. The results are expressed as gallic acid equivalent (GAE) per kg of honey.

Total protein content was determined using the Bradford procedure [18]. Honey samples (5 g) were diluted with distilled water (10 mL). Coomassie brilliant blue (200 μL) was added to the 5 μL honey solution. Coomassie brilliant blue forms a protein-dye complex. After 5 min of incubation, the absorbance was measured at 595 nm against an albumin standard solution of bovine serum (10-100 μg/0.1 mL), and the total protein content was quantified and expressed as g/kg of honey.

Results and discussion. After extraction, honey from many bee colonies is stored in barrels. Before packing in the jars, honey from one or many barrels has to be homogenized properly. Therefore the differences between the honey samples after extraction and after packing in the honey jars are expected to come from improper homogenization of honey and/or from different properties of honey colonies.

The contribution of *Tilia* pollen (Table 1) confirmed that all honey samples could be labeled as unifloral Lime tree honey [19]. In samples 1, 2, 3, 7, 11, 14, and 16 the difference in *Tilia* pollen contribution exceeded 10% reproducibility of the method [15], indicating that for those samples there is a discrepancy in the contribution of Lime tree nectar, most likely resulting from inappropriate homogenization prior to packaging. The decrease seen in sample 2 lowered the contribution of *Tilia* pollen below the threshold required for labeling the product as PDO "Fruškogorski lipov med" [14].

TABLE 1. Results of Melissopalynological Analysis Showing Pollen Concentration and Contribution of Lime Tree (*Tilia*) Pollen in Honey Samples from Fruška Gora Mountain, Collected from Different Beekeepers in 2015, after Extraction and after Packing

Sample	Pollen concentration (PG/10 g)		Frequency of *Tilia* pollen (%)	
	after extraction	after packing	after extraction	after packing
1	24778	18407	92.4	61.4
2	18630	21741	69.4	24.9
3	11778	18481	66.8	80.8
4	41815	51741	81.2	79.0
5	**22370**	**1768**	**83.6**	**81.5**
6	26630	23000	90.5	93.7
7	12630	9852	86.4	73.5
8	33556	11648	77.9	73.3
9	20778	16852	88.2	92.8
10	22370	6815	83.9	83.5
11	19667	18815	79.5	88.6
12	**4800**	**18759**	**76.5**	**79.9**
13	24852	25407	76.5	82.4
14	22037	10148	69.9	58.1
15	7395	5537	75.3	74.4
16	42481	24074	72.5	81.5
17	4796	7679	87.5	80.1
Mean	21257	17101	79.9	75.8
Standard deviation	11151	11394	7.7	16.1
Coefficient of variation	0.52	0.67	0.10	0.21

N o t e. The samples for which contribution of lime tree pollen dropped below the threshold for PDO "Fruškagora Lime honey" [11] are written in bold.

Determined pollen concentrations are considered as an estimate for classifying honey samples into one of the five groups related to the type of major nectar source and applied extraction method [15]. All samples belong to either Class I or Class II, which corresponds to unifloral honey that was extracted by centrifuging combs. Having in mind 30% reproducibility of the method for these honey classes, only in samples 5 and 12 did packing result in a notable decrease of pollen concentrations that would lead to change in pollen class.

Figure 1a shows the excitation-emission landscapes for the honey sample from one of the beekeepers. Such spectral series allowed studying the main emitting compounds in honey, which are the base for estimation of differences between the honey samples. In all honey emission spectra, there were two broad characteristic maxima, the one at about 340–360 nm and the other at about 415–450 nm. The former can be assigned to the proteins in honey, which mainly (2/3 of total honey proteins) originate from bees and partly (1/3 of total honey proteins) originate from pollen [13]. The latter maximum is assigned to various phenolic compounds that are contained in honey.

The results of MCR-ALS analysis of the emission profiles from Fig. 1a are shown in Fig. 1b. The emission spectrum of honey contains four components with the maxima 340, 370, 415, and 450 nm. They can be linked to the presence of fluorophores in honey: the 340 nm component to the proteins, the 370 nm component to syringic acid, and the 415–450 nm component to various phenolic compounds – hydroxycinnamic acids, chlorogenic acid, caffeic acid, coumarins, and stilbenes [20–22]. The positions of emission maxima of the phenolic components varied for various honey samples, but they were in the same emission range. Thus those components were used as the phenolic fingerprint for further calculations. In certain honey samples, there was only one component in the phenolic range of the spectrum (the samples 1 and 17), and it was used for further calculations. The maximum position of the protein component varied for various honey samples, but it was in the same emission range and was used as protein fingerprint. The component at 370–380 nm rarely occurred in honey samples and was not used in further analysis. The corresponding loadings (excitation profiles) of the components from Fig. 1b are shown in Fig, 1c. The excitation profiles are in correlation with the quantum yields of the corresponding fluorophores. We calculated the area of the characteristic

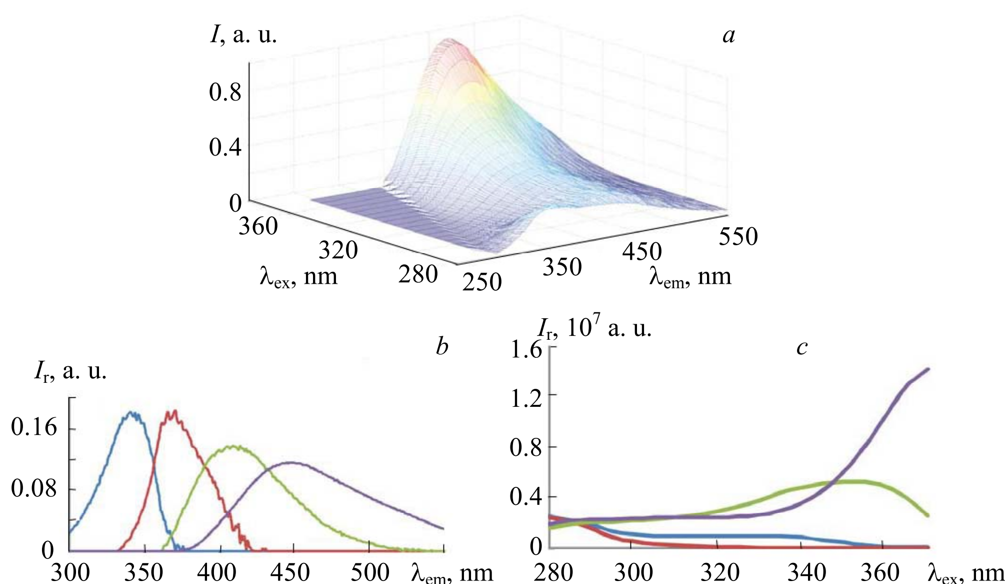

Fig. 1. Fluorescence spectra of a honey sample with pronounced protein part (the sample 13 after packing in the jar), as an example: a) excitation-emission matrix for the raw spectra; b) emission spectra of the pure components obtained by applying the MCR-ALS method in the analysis of the raw spectra; c) excitation spectra profiles (loadings) corresponding to the spectral components from (a), obtained by the MCR-ALS method. Colors of the loading curves match colors of the corresponding emission components.

protein and phenolic components. In order to determine the exact area of the components, we multiplied the spectra of components with the corresponding loadings. We further determined the ratio $(Ph/Pr)_{fl}$ = [total phenolic components area/protein component area] as a fluorometric ratiometric indicator of honey samples from various beekeepers (Table 2). Also, total phenol and total protein quantification results obtained by using spectrophotometric tests, totPhC and totPrC, along with corresponding $(Ph/Pr)_{sp}$ = [total phenol/total protein] ratio are presented in the same table for comparison.

The fluorometric estimation of the phenol/protein relative content enables one to get an insight into these minor but important honey components. As saccharides, the main component of honey, are not fluorescently active, and thus do not interfere in the emission spectra with proteins and phenols, the proposed fluorometric approach may be advantageous over existing methods used for protein and phenol quantification in honey, in terms of sensitivity and speed (parallel determination without pre-sample preparation). Protein studies in honey samples are relatively limited, mainly due to the low amount of proteins in honey (0.1–0.5%), the difficulties in extracting honey proteins from the sugar-rich environment, and because of the obstructions in protein characterization by conventional methods. Bradford's method for measuring total proteins has disadvantages regarding the different affinity of the CBB reagent for binding to proteins of different properties [23] and its affinity to phenolic compounds [24], while the new fluorometric method relies on the specific amino acid fluorophore (tryptophan, Trp) emission. The Folin Ciocalteu method for the determination of total phenols is not completely selective [25]. It has been shown that in addition to phenols, numerous organic and inorganic compounds, including proteins, may react. On the other hand, the peak from phenolic compounds in the emission spectra is clearly separated from the protein part of the spectrum, which enables their parallel determination.

In certain cases, it was not possible to determine $(Ph/Pr)_{fl}$ ratio (Table 2, samples 4 after packing, 7 after extraction and packing, and 12 and 13 after extraction) since the protein component was not observed after MCR analysis. Although in these samples proteins were observed by the spectrophotometric method (Table 2), their emission was below detectability level. This may be due to the protein environments in the particular honey samples. One can see that in seven out of thirteen samples where the protein component could be resolved, the result of phenol/protein ratio obtained from the emission spectra is in compliance with the corresponding ratio obtained from the spectrophotometric quantification of proteins and phenols (samples 1, 2, 6, 9, 11, 16, and 17). In some cases (samples 3, 5, 8, 10, 14, and 15) the result of the fluorometric ratio-

TABLE 2. Results of Fluorometric and Spectrophotometric Analysis of the Lime (*Tilia L.*) Honey Samples from the Same Locality on the Fruška Gora Mountain, Collected from Different Beekeepers in 2015

Sample	$(Ph/Pr)_{fl}$ after extraction	$(Ph/Pr)_{fl}$ after packing	TotPrC (g/kg) after extraction	TotPrC (g/kg) after packing	TotPhC (g/kg) after extraction	TotPhC (g/kg) after packing	$(Ph/Pr)_{sp}$ after extraction	$(Ph/Pr)_{sp}$ after packing
1	**9**	**3.14**	0.05	0.25	0.64	0.57	**12.75**	**2.31**
2	**2.26**	**6.59**	0.38	0.55	0.68	0.99	**1.78**	**1.81**
3	6.37	5.11	0.41	0.33	0.82	0.76	1.99	2.30
4	5.00	**-**	0.41	0.32	0.67	0.66	1.64	2.07
5	1.76	0.38	0.40	0.30	0.67	0.66	1.67	2.21
6	**3.34**	**3.30**	0.33	0.38	0.88	0.83	**2.64**	**2.18**
7	–	–	0.35	0.28	0.67	0.62	1.94	2.25
8	3.52	2.39	0.41	0.13	0.63	0.35	1.53	2.65
9	**3.4**	**1.55**	0.30	0.40	0.63	0.66	**2.13**	**1.66**
10	5.01	3.33	0.47	0.41	0.68	0.65	1.46	1.60
11	**0.62**	**3.56**	0.28	0.19	0.72	0.73	**2.56**	**3.93**
12	–	2.21	0.30	0.42	0.69	0.64	2.28	1.53
13	–	4.29	0.40	0.27	0.71	0.78	1.77	2.83
14	5.52	1.00	0.57	0.22	0.82	0.76	1.43	3.44
15	6.47	5.83	0.52	0.41	0.75	0.92	1.44	2.42
16	**2.59**	**5.56**	0.46	0.51	0.63	0.73	**1.37**	**1.42**
17	**2.59**	**0.78**	0.28	0.29	0.63	0.65	**2.26**	**2.20**

N o t e. The data after extraction and after packing in the jars are presented: the ratio of phenolics to protein emission spectral components $(Ph/Pr)_{fl}$, total phenol and total protein content quantified spectrophotometrically, and their ratio $(Ph/Pr)_{sp}$. "–" indicates that protein component was not detected after analysis of emission spectra. The samples for which change in the $(Ph/Pr)_{fl}$ ratio from the "after extraction" to the "after packing" step is in accordance with the $(Ph/Pr)_{sp}$ ratio, are written in bold.

metric analysis was not in compliance with the corresponding $(Ph/Pr)_{sp}$ ratio. Both types of disagreements are related to the characteristics of the samples, i.e., to their emission spectra where the protein part of the spectrum was weakly expressed, as shown by the example of emission spectra and corresponding MCR analysis for sample 7 at Fig. 2. The absence of the protein component after MCR analysis or poor expression of the protein maximum in the raw spectra in the "after extraction" samples may be related to the honey characteristics from particular hives [13], and in the "after packaging samples" it may be related to inappropriate homogenization of honey coming from different hives, before packing in the jars. As a possible source of disagreements, we also have in mind flaws of the colorimetric methods for total protein and total phenol determination mentioned above.The above analysis shows that the possible limit of applicability of the fluorometric method for determining the ratio of phenols and proteins may be an extremely weak expression of either protein or phenol signal in the emission spectra (mainly related to the characteristics of honey samples), which is a limitation for the success of MCR analysis.

The spectrophotometric protein and phenol quantifications, as well as fluorometric ratiometric analysis results, show that there were notable differences in protein content in the samples from the same producer after extraction and samples after packaging. How does one explain these differences? For samples 1 and 2, the notable increase in the content of proteins corresponds to the notable change in nectar contributions. The decrease in frequency of *Tilia* pollen (Table 1) indicates the higher contribution of nectar from other plant species, which is expected to lead to a qualitative and quantitative change of protein fraction coming from plants [26]. For sample 2 after packaging, the contribution of *Robinia pseudoacacia* pollen notably increased (from 4% to 18%, data not shown in Table 1), which indicates a notable increase of false acacia nectar in the honey. Having in mind that false acacia forage takes place before linden forage, the increase in its nectar contribution could be related to inappropriate homogenization with earlier collected honey. On the other hand, in samples 8, 13, 14, although there is a decrease in protein content, *Tilia* nectar contribution did not change notably. Only in sample 14 was there an obvious decrease in total pollen concentration, which inevitably leads to a decrease in quantity of pollen coming from plants. A similar situation where a notable

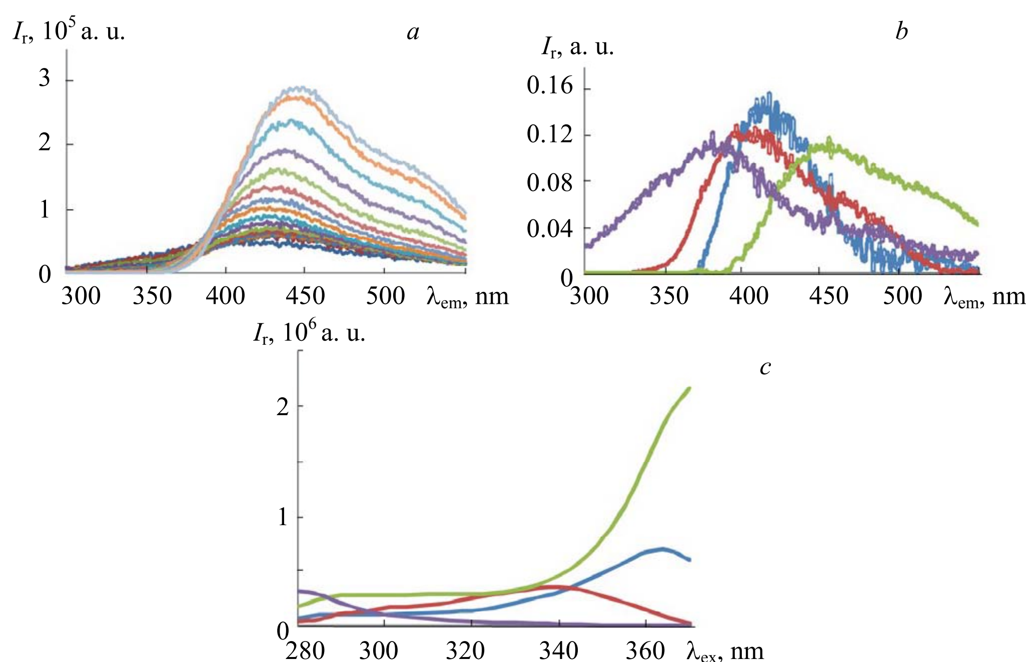

Fig. 2. Fluorescence spectra of a honey sample with poorly pronounced protein part (sample 7 after packing in the jar): a) excitation-emission matrix for the raw spectra; b) emission spectra of the pure components obtained by applying the MCR-ALS method in the analysis of the raw spectra; c) excitation spectra profiles (loadings) corresponding to the spectral components from (b) obtained by the MCR-ALS method. Colors of the loading curves match colors of the corresponding emission components.

decrease (>50%) in pollen concentration corresponds to a decrease in protein content is seen in samples 5, 8, 10 and 15, while the opposite situation is seen in sample 12 where a notable increase (more than 50%) in pollen concentration corresponds to an increase in protein content. Such situation also indicates the importance of the protein component coming from plants. For samples 8 and 13, there is no notable difference in the result of melissopalynological analysis; thus we can only assume that inappropriate homogenization of honey extracted from different hives could have affected the protein component coming from bees.

The results of this study show that fluorometric ratiometric analysis may be used for fast and reliable screening of honey sample variability and for the selection of samples for further, more detailed analysis. Future experiments on marker proteins from bees and from pollen are planned to confirm the presented fluorometric approach.

Conclusion. Here we proposed a fast and reliable method to study honey sample variability based on the fluorescence characteristics of two main fluorophores in honey, proteins and phenolic compounds. The area of the characteristic protein and phenolic components in the emission spectra $(Ph/Pr)_{fl}$ of lime honey were calculated as a ratiometric indicator of the honey samples foraged from various beekeepers. Different values of the $(Ph/Pr)_{fl}$ ratio after extraction and after packaging, as well as the absence of protein component after MCR analysis or poor expression of the protein maximum in the raw spectra, may be related to variability in characteristics of honey originating from different bee colonies, or to inappropriate homogenization of honey before packing in the jars. The results show that fluorometric ratiometric analysis may be used for fast and reliable screening of honey sample variability and for selection of the samples for further, more detailed analysis.

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