

## Research Article

# Detection of Adulterated Honey by Fluorescence Excitation-Emission Matrices

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Honey is a frequent target of adulteration through inappropriate production practices and origin mislabelling. Current methods for the detection of adulterated honey are time and labor consuming, require highly skilled personnel, and lengthy sample preparation. Fluorescence spectroscopy overcomes such drawbacks, as it is fast and noncontact and requires minimal sample preparation. In this paper, the application of fluorescence spectroscopy coupled with statistical tools for the detection of adulterated honey is demonstrated. For this purpose, fluorescence excitation-emission matrices were measured for 99 samples of different types of natural honey and 15 adulterated honey samples (in 3 technical replicas for each sample). Statistical *t*-test showed that significant differences between fluorescence of natural and adulterated honey samples exist in 5 spectral regions: (1) excitation: 240–265 nm, emission: 370–495 nm; (2) excitation: 280–320 nm, emission: 390–470 nm; (3) excitation: 260–285 nm, emission: 320–370 nm; (4) excitation: 310–360 nm, emission: 370–470 nm; and (5) excitation: 375–435 nm, emission: 440–520 nm, in which majority of fluorescence comes from the aromatic amino acids, phenolic compounds, and fluorescent Maillard reaction products. Principal component analysis confirmed these findings and showed that 90% of variance in fluorescence is accumulated in the first two principal components, which can be used for the discrimination of fake honey samples. The classification of honey from fluorescence data is demonstrated with a linear discriminant analysis (LDA). When subjected to LDA, total fluorescence intensities of selected spectral regions provided classification of honey (natural or adulterated) with 100% accuracy. In addition, it is demonstrated that intensities of honey emissions in each of these spectral regions may serve as criteria for the discrimination between natural and fake honey.

## 1. Introduction

Honey is a pure natural food produced by bees from the nectar of flowers. Its main components are different types of carbohydrates, water, and some minor constituents, such as pollen grains, proteins, amino acids, lipids, alkaloids, enzymes, and flavoring components. The composition and concentrations of minor constituents are unique characteristics of honey, and some of them can be used to differentiate honey samples by their geographical and botanical origins, as well as to define their quality and authenticity [1].

According to European Union standards [2], honey is a pure product, in which no components can be added

or removed from. According to the botanical origin, honey can be categorized into two groups: honeydew and nectar honey types. Nectar honey in turn is divided into polyfloral and monofloral honey. Polyfloral honey contains nectar from different plant species, while monofloral is from single plant species with more than 45% of the total pollen in honey [3]. However, a minimum percentage of pollen in monofloral honey may be different for various types of honey. For example, the Sunflower pollen should not be less than 45% of the total pollen, but for acacia and linden, it should not be less than 30% in honey [4]. Eucalyptus honey and chestnut honey, having overrepresented pollen grains, can show a pollen frequency of 70% to 90% [5].

According to the Serbian regulations, acacia honey, linden honey, and sunflower honey are defined as monofloral when pollen concentration is over 20%, 25%, and 40%, respectively.

As a relatively expensive food product, honey is a frequent target of adulteration through inappropriate production practices and its origin mislabelling [6]. This presents a serious issue on markets and violates consumer rights. Much effort, thus, is nowadays directed for the development of characterization methods for honey to fight this problem. For example, to prove the geographical and botanical origin of honey, the microscopic analysis of pollen grains [7–9] is a common method. However, it is time-consuming method and requires work of an experienced operator. Other methods, according to European Union legislation [2], include physicochemical analysis [10–12], proton nuclear magnetic resonance ( $^1\text{H}$  NMR) imaging [13], sensory analysis [14], chromatography [15–17], and electric-tongue analysis [18, 19]. Unfortunately, these methods are time and labor consuming, require highly skilled personnel, and lengthy sample preparation.

Optical spectroscopy methods are less demanding in this respect, and many of them are successfully applied in food analyses [20, 21]. Near infrared (NIR) spectroscopy in combination with chemometric methods was used for the quantitative and qualitative detection of beet syrup adulteration of honey [22], adulteration of honey with mixtures of fructose and glucose [23], and adulteration of honey with HFCS (high-fructose corn syrup) [24]. The application of mid-FTIR spectroscopy coupled with the SVM (support vector machine) algorithm provided the determination of the botanical origin of Serbian unifloral honey [25]. Fluorescence spectroscopy offers advantages over classic methods of food analysis, such as minimal sample preparation, high sensitivity and specificity of classification [26], and rapid and relatively simple use of instrumentation. This technique is suitable for the analysis and authentication of food products such as milk [27], cheese [28], cereal flours [29], and wine [30]. Geographical and botanical origin of honey samples have been authenticated by fluorescence spectroscopy [31]. Recently, parallel factor analysis (PARAFAC) has been used to fully describe fluorescence of honey [32] and cereal flour [33]. Since there is no quantitative fluorescence marker for the detection of fake honey, fluorescence of honey intrinsic fluorophores must be utilized for the analysis.

Here, we aimed at exploring differences in fluorescence between natural honey and adulterated honey which arise due to dissimilar composition of fluorescence species that are present in them. For this purpose, we measured fluorescence excitation-emission matrices of 114 samples of natural and fake honey samples (in three technical replicas) aiming at developing the fluorescence-based technique for the fast and nondestructive detection of honey adulteration. Principal component and linear discriminant analysis of characteristic honey emission features were utilized to describe observed differences and to build and test the classification model.

## 2. Materials and Methods

**2.1. Samples.** In this research, a total of 114 samples, 99 natural honey samples (45 acacias, 11 lindens, 14 sunflowers,

and 29 meadow mixes) and 15 fake honey samples, were obtained from the Association of the Beekeeping Organizations of Serbia (SPOS, [www.spos.info](http://www.spos.info)). Fake honey samples were manufactured during winter feeding of bee colonies with a sucrose solution, which was later converted into artificial honey by bees. Honey samples were stowed in the original packages at 20–22°C until analysis.

**2.2. Measurements of Fluorescence Excitation-Emission Matrices.** Room temperature fluorescence excitation-emission matrices (EEMs) were obtained by a Perkin Elmer Fluorescence Spectrophotometer LS45 in a front face measurement configuration. The instrument was equipped with a Xe lamp for excitations and a R928 PMT for the detection of emission radiation. EEMs were recorded over the 270–640 nm emission range (at 0.5 nm intervals) and 240–500 nm excitation range (with a 5 nm step). Emission intensities were automatically normalized to the excitation intensity by the instrument. Honey samples were liquefied at 40°C and pipetted into 3 mL quartz cuvettes before measurements. Contributions to measured signal intensity from the first and second order Rayleigh scattering were removed and replaced with interpolated values. The use of interpolated values to remove scattering contribution has been shown to provide better and more meaningful results when dealing with EEMs compared to the data deletion [34].

**2.3. Data Analysis.** Data analyses comprised the calculation of spectral domain volumes below EEM intensity surfaces, testing of the statistical significance of differences observed between spectral characteristics of natural and fake honey samples by a *t*-test, investigating differences by principal component analysis (PCA) and the classification of samples using a linear discriminant analysis (LDA) of EEM data. Analyses were performed using the R software package (R Foundation for Statistical Computing, Vienna, Austria).

Spectral domain volumes (spectral domain being represented by maximal and minimal values of excitation and emission wavelengths,  $\lambda$ ) are numerically calculated from the measured emission intensity  $I(\lambda_{\text{exc}}, \lambda_{\text{em}})$  using the following formula [35]:

$$\int_{\lambda_{\text{exc}}^{\min}}^{\lambda_{\text{exc}}^{\max}} \int_{\lambda_{\text{em}}^{\min}}^{\lambda_{\text{em}}^{\max}} I(\lambda_{\text{exc}}, \lambda_{\text{em}}) d\lambda_{\text{em}} d\lambda_{\text{exc}} = \Delta\lambda_{\text{em}} \Delta\lambda_{\text{exc}} \sum_{j=1}^M \sum_{i=1}^N I(\lambda_{\text{exc}}^j, \lambda_{\text{em}}^i), \quad (1)$$

where  $\lambda_{\text{exc}}^j = \lambda_{\text{exc}}^{\min} + j\Delta\lambda_{\text{exc}}$ ,  $\lambda_{\text{em}}^i = \lambda_{\text{em}}^{\min} + i\Delta\lambda_{\text{em}}$ ,  $\Delta\lambda_{\text{exc}} = (\lambda_{\text{exc}}^{\max} - \lambda_{\text{exc}}^{\min})/M$ ,  $\Delta\lambda_{\text{em}} = (\lambda_{\text{em}}^{\max} - \lambda_{\text{em}}^{\min})/N$ , and  $M$  and  $N$  are the numbers of measurement intervals for excitation and emission, respectively.

Principal component analysis (PCA) is a method used for reducing data dimensionality and identifying differences between analysed samples as well as investigating and visualizing variations found in a data set [36]. To apply PCA, three-way data array must be unfolded into two-dimensional

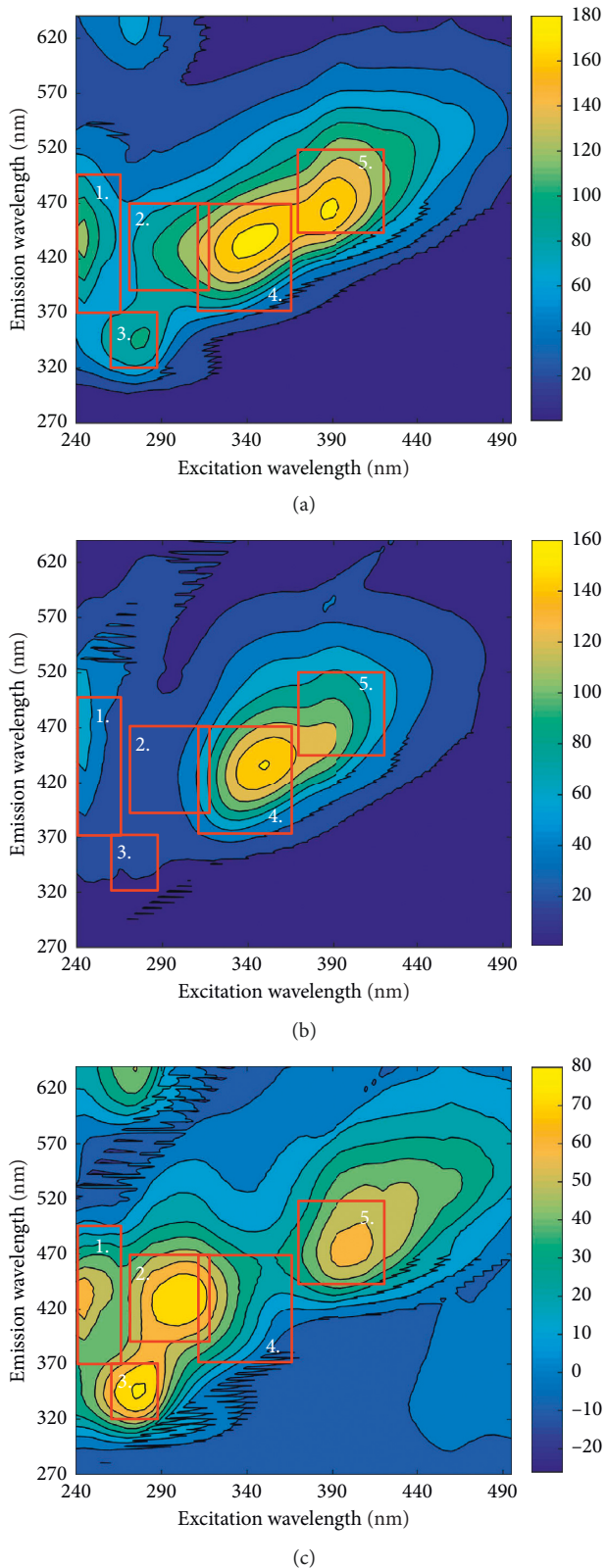


FIGURE 1: Fluorescence excitation-emission matrices of (a) natural and (b) fake honey and (c) their difference spectrum. The discussed spectral regions are marked with a full line

TABLE 1: Spectral regions showing characteristic fluorescence of honey fluorophores.

Spectral domain	$\lambda_{ex}$ (nm)	$\lambda_{em}$ (nm)	Fluorophore
1st	240–265	370–495	Phenolic compounds
2nd	280–320	390–470	Phenolic compounds
3rd	260–285	320–370	Aromatic amino acids
4th	310–360	370–470	Phenolic compounds
5th	375–435	440–520	Maillard reaction products

TABLE 2: Statistical analysis mean values, standard deviation: SD, and statistical significance of difference between means:  $p$  of five spectral domain volumes (marked on Figure 1) in EEMs of 99 natural and 15 fake honey.

Spectral domain	Honey	Mean*	SD*	$p^{**}$
1st	Natural	$1.38 \times 10^5$	3399	$4.90 \times 10^{-13}$
	Fake	$6.13 \times 10^4$	8688	
2nd	Natural	$9.42 \times 10^4$	2580	$2.03 \times 10^{-11}$
	Fake	$4.14 \times 10^4$	6597	
3rd	Natural	$5.70 \times 10^4$	1895	$7.97 \times 10^{-12}$
	Fake	$1.73 \times 10^4$	4844	
4th	Natural	$3.37 \times 10^5$	6844	$5.44 \times 10^{-13}$
	Fake	$1.83 \times 10^5$	17494	
5th	Natural	$3.08 \times 10^5$	5191	$3.12 \times 10^{-21}$
	Fake	$1.40 \times 10^5$	13268	

\*Values are in counts  $\times$  nm<sup>2</sup>. \*\*Decision on statistical significance is made in a traditional way based on a probability value for the null hypothesis.  $p$ : >0.05, not significant; 0.05 to 0.01, significant; 0.01 to 0.001, very significant; <0.001, extremely significant.

(2D) matrix. This is achieved by unfolding initial data array (114 samples  $\times$  741 emissions  $\times$  53 excitations) into a 2D matrix (114 samples  $\times$  39273 parameters (emissions  $\times$  excitations)). After the analysis, the calculated loadings can be refolded back into a three-way data array. PCA was applied on unfolded EEM spectra to explore differences between natural and fake honey and to determine which parts of EEM spectrum contribute the most to those differences. This was carried out by investigating calculated PCA scores and loadings.

Linear discriminant analysis (LDA) is a linear classification method with a goal to find one or more linear functions of the input variables which, then, can be used for the sample classification. Fisher’s algorithm [37] is used for the building LDA classification model utilizing the spectral domain volumes as input data. K-fold cross-validation was used to calculate classification error. Samples were divided three times in such way that two thirds of samples were used for the training of the classification model, while the one third was used for model testing. From three tastings, the mean classification error was derived.

### 3. Results and Discussion

Figure 1 shows EEM spectra of natural and fake honey samples as well as the difference spectrum in forms of contour maps.

Intrinsic fluorophores and their specific microenvironments in honey produce a complex excitation-emission pattern which varies among samples. As presented in

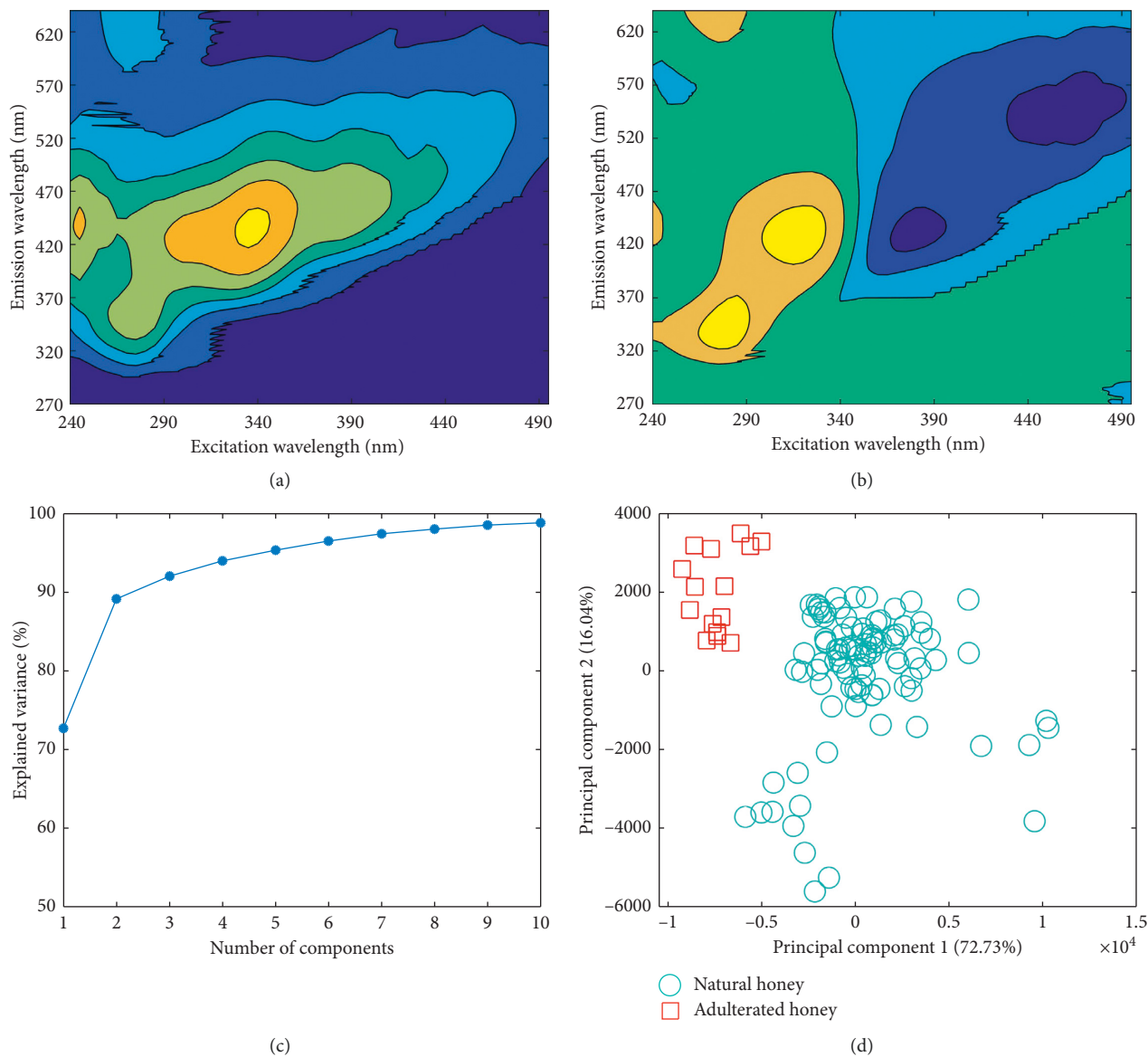


FIGURE 2: Results of PCA of unfolded honey EEM spectra: (a) loadings of the first principal component, (b) loadings of the second principal component, (c) cumulative variance plot, and (d) the PCA score plot.

Table 1, fluorescence EEMs of honey are characterized by 5 spectral regions of high emission intensities: (1) excitation: 240–265 nm, emission: 370–495 nm; (2) excitation: 280–320 nm, emission: 390–470 nm; (3) excitation: 260–285 nm, emission: 320–370 nm; (4) excitation: 310–360 nm, emission: 370–470 nm, and (5) excitation: 375–435 nm, emission: 440–520 nm (marked on Figure 1 with a full line). These emissions come from the aromatic amino acids, phenolic compounds, and fluorescent Maillard reaction products, such as furosine and hydroxymethylfurfural [32]. One should note that hydroxymethylfurfural is an indicator for the evaluation of honey storage and heat damage and that its concentration varies between natural and adulterated honey. The total emission intensities over these spectral regions are quantified according to (1) for each sample, and differences between natural and fake honey samples are tested on the statistical significance using *t*-test (Table 2).

Differences in fluorescence of natural and fake honey samples are extremely significant in all spectral regions ( $p < 0.001$ ). Therefore, the total fluorescence intensity in each spectral region may serve as a criterion for the detection of fake honey.

PCA was performed for the further study of differences between fluorescence responses of honey and fake honey samples; results are presented in Figure 2. Loadings of the first and second principal components are given in Figures 2(a) and 2(b) as contour plots (PCA analysis was applied to unfolded spectra; the obtained loadings were transformed into two-dimensional matrix for the presentation). These plots confirmed that variations of fluorescence between samples are largest in the selected spectral regions. Variations in the first, third, and fourth regions are described by the first principal component (Figure 2(a)), while variations in the second, third, and fifth are described by the second

TABLE 3: LDA classification and cross-validation errors.

	Classification error (%)	Cross-validation error (%)
Natural honey	0	0
Fake honey	0	0

principal component. From the cumulative variance plot (Figure 2(c)), one can observe that the first and second principal components account for almost 90% of variations in honey EEM data. The score plot of the first and second principal components of natural and adulterated honey samples' EEMs (Figure 2(d)) clearly demonstrates the distinctive separation between two groups of samples.

The quality of fluorescence-based discrimination between natural and fake honey samples was evaluated by a linear discriminant analysis (LDA) of 5 spectral domain volumes. LDA is a method used to find one or several linear functions (linear latent variables) of the data features that can be used for separation between two or more groups [37, 38]. For estimation of classification performance, LDA classification errors were obtained and are given in Table 3. For this study, the 3-fold split of data were used as the cross-validation method (data set was divided differently three times, while each time two thirds of the samples were used for the classification training and one third for the model testing). Perfect classification and cross-validation (0% error) is achieved for the set of 114 honey samples. Therefore, as expected from the previous results, the obtained confusion and prediction matrix showed correct classification of all natural honey samples (99/99) and fake (15/15) honey samples. There were zero samples misclassified. This result was expected since all spectral domain volumes showed extremely significant differences between natural and fake honey samples. As mentioned before, standard methods used for detection of honey adulteration such as isotopic, chromatographic, or thermal analysis are often destructive, time-consuming, and usually expensive. Therefore, based on our results, one can conclude that fluorescence EEMs can be used to detect fake honey in fast and nondestructive manner and that this method has a potential for the detection of adulteration of lower percentages. This would be the subject of the future study.

#### 4. Conclusions

To conclude, fluorescence excitation-emission spectroscopy can be effectively used for the nondestructive and fast detection of adulterated honey specimens. Differences in fluorescence of natural and adulterated honey samples are extremely significant in five spectral regions due to differences in concentrations and local environments of aromatic amino acids, phenolic compounds, furosine, and hydroxymethylfurfural, as is demonstrated by statistical testing and PCA. By quantifying fluorescence responses and subjecting them to the statistical classification technique, for example, LDA, it is possible to detect adulterated honey with 100% accuracy. Such accuracy suggests that fluorescence excitation-emission spectroscopy

may be a promising method for the low-level adulteration of honey, which is the subject of our future work.

#### Data Availability

Data are available upon request to Lea Lenhardt Acković (lea.lenhardt@gmail.com).

#### Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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