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# Authentication of the Botanical Origin of Honey by Front-Face Fluorescence Spectroscopy. A Preliminary Study

KASPAR RUOFF,*,†,§ ROMDHANE KAROUI,‡ ERIC DUFOUR,‡ WERNER LUGINBÜHL,† JACQUES-OLIVIER BOSSET,† STEFAN BOGDANOV,† AND RENATO AMADÒ§

Swiss Bee Research Centre/Agroscope Liebefeld-Posieux, Schwarzenburgstrasse 161, CH-3003 Berne, Switzerland, UR Typicité des Produits Alimentaires, ENITA Clermont Ferrand, site de Marmilhat, F-63370 Lempdes, France, and Swiss Federal Institute of Technology (ETH), Institute of Food Science and Nutrition, ETH-Zentrum, Schmelzbergstrasse 9, CH-8092 Zurich, Switzerland

The potential of front-face fluorescence spectroscopy for the authentication of unifloral and polyfloral honey types ($n = 57$ samples) previously classified using traditional methods such as chemical, pollen, and sensory analysis was evaluated. Emission spectra were recorded between 280 and 480 nm (excit: 250 nm), 305 and 500 nm (excit: 290 nm), and 380 and 600 nm (excit: 373 nm) directly on honey samples. In addition, excitation spectra (290−440 nm) were recorded with the emission measured at 450 nm. A total of four different spectral data sets were considered for data analysis. After normalization of the spectra, chemometric evaluation of the spectral data was carried out using principal component analysis (PCA) and linear discriminant analysis (LDA). The rate of correct classification ranged from 36% to 100% by using single spectral data sets (250, 290, 373, 450 nm) and from 73% to 100% by combining these four data sets. For alpine polyfloral honey and the unifloral varieties investigated (acacia, alpine rose, honeydew, chestnut, and rape), correct classification ranged from 96% to 100%. This preliminary study indicates that front-face fluorescence spectroscopy is a promising technique for the authentication of the botanical origin of honey. It is nondestructive, rapid, easy to use, and inexpensive. The use of additional excitation wavelengths between 320 and 440 nm could increase the correct classification of the less characteristic fluorescent varieties.

**KEYWORDS: Honey; unifloral; botanical origin; authentication; front-face fluorescence spectroscopy; *Robinia pseudoacacia*; *Rhododendron ferrugineum*; *Castanea sativa*; *Brassica napus* var. *oleifera***

## INTRODUCTION

According to the Codex Alimentarius Standard for Honey (*1*) and the EU Council Directive (*2*) relating to honey, the use of a botanical designation of honey is allowed if it originates predominantly from the indicated floral source. At the current stage of knowledge, a reliable determination can be achieved by a global interpretation of sensory, pollen, and physicochemical analyses carried out by an expert (*3*, *4*). As several analytical methods are simultaneously necessary for a reliable authentication of unifloral honeys, such work is time-consuming and costly. Thus, there is a need for new methods that allow a rapid and reproducible authentication of the botanical origin of honey at low cost (*5*). The use of front-face fluorescence spectroscopy seems to be a promising approach.

Fluorescence spectroscopy provides information on the presence of fluorescent molecules and their environment in biological samples. Food products contain numerous intrinsic fluorophores and are therefore suitable for fluorescence spectroscopy investigations. Honey contains small amounts of proteins, peptides, and free amino acids, which include tryptophan, tyrosine, and phenylalanine residues. Following excitation of a protein solution at 290 nm, characteristic fluorescence emission spectra of tryptophan residues can be recorded. When fluorescence of tryptophan, tyrosine, and phenylalanine has to be considererd, the excitation wavelength is set at 250 nm and the fluorescence emission is recorded between 280 and 480 nm. However, numerous fluorescent compounds such as nucleic acids and polyphenols found in food products may fluoresce following excitation in the 250−280 nm range. Food products also contain vitamins, some of which are fluorescent compounds. Riboflavin is, due to its conjugated double bonds, a good fluorescent probe, exhibiting fluorescence emission spectra (400−640 nm) following excitation at 380 nm.

As compared to the spectroscopic techniques based on absorption, fluorescence spectroscopy offers a 100−1000-fold higher sensitivity. It provides information on the presence of

* Author to whom correspondence should be addressed [telephone +41 (0)31 323 82 38; fax +41 (0)31 323 82 27; e-mail kaspar.ruoff@alp.admin.ch].
† Swiss Bee Research Centre/Agroscope Liebefeld-Posieux.
‡ UR Typicité des Produits Alimentaires.
§ Swiss Federal Institute of Technology (ETH).

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fluorescent molecules and their environment in organic materials.

With classical right-angle fluorescence spectroscopy, the measurements are carried out in dilute solutions where the absorbance is below 0.1. At a higher absorbance rate, a decrease of fluorescence intensity and a distortion of emission spectra are observed due to the inner filter effect. To overcome such problems, front-face fluorescence spectroscopy was developed (*6*) where only the surface of the material is illuminated and examined. The emitted photons are collected at an angle of 56° to the surface of the sample, to minimize artifacts generated by the photons of excitation reflected from the sample (*7*). This technique allows a quantitative investigation of fluorophores in powders as well as in concentrated or even opaque samples.

Foods have complex matrices containing many different fluorophores. Their signals could overlap and make it impossible to measure the concentration of a single compound. Nevertheless, the shape of normalized fluorescence spectra in combination with multivariate statistics can be used for characterizing and identifying different foods. This has already been shown for processed milk (*8*), authenticating the geographical origin of cheese (*9*), as well as studying cheese ripening and structure (*10*−*12*).

Unifloral honeys are well known to contain numerous polyphenols (*13*−*16*) as well as other fluorophores such as amino acids (*17*, *18*). Some of them have already been proposed as tracers for unifloral honeys, for instance, ellagic acid for heather honey from *Erica* and *Calluna* species (*19*) or hesperetin for Citrus honeys (*20*, *21*). As polyphenols are strong fluorophores, fluorescence spectroscopy should be helpful for authenticating the botanical origin of honey.

Also, fluorescent amino acids have been proposed as markers for unifloral honeys. Phenylalanine and tyrosine were found to be characteristic for lavender honeys and allowed a differentiation from eucalyptus honeys (*22*). Tryptophan and glutamic acid were used for the differentiation between honeydew and blossom honeys (*23*). Therefore, the aim of the current work is to study the fluorescence characteristics of seven different varieties and to develop a rapid, nondestructive, low-cost, and reliable method for authentifying unifloral honeys.

## MATERIALS AND METHODS

**Sampling and Botanical Classification by Reference Methods.** A total of 57 honey samples produced in Switzerland between 1998 and 2001 were collected and stored at 4 °C until analysis. To classify these honey samples, the following measurands were determined according to the harmonized methods of the European Honey Commission (*24*): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (*25*, *26*). On the basis of these analytical results and sensorial evaluation by four experts, the honey samples were assigned to one of the seven following honey types: acacia (*Robinia pseudoacacia*) ($n = 7$), alpine rose (*Rhododendron ferrugineum*) ($n = 5$), sweet chestnut (*Castanea sativa*) ($n = 9$), rape (*Brassica napus* var *oleifera*) ($n = 10$), honeydew ($n = 8$), alpine polyfloral ($n = 7$), and lowland polyfloral honeys ($n = 11$).

**Fluorescence Spectroscopy.** An aliquot part of 20 g of the honey samples was liquefied at 40 °C for 8 h, then allowed to cool to room temperature and pipetted into a 1 cm quartz cuvette. The latter was placed in the sample holder for the recording of the fluorescence spectra, which was done by using a FluoroMax-2 (Spex-Jobin Yvon, F-91165 Longjumeau) spectrofluorometer equipped with a variable angle front-surface accessory, with the incident angle of the excitation radiation set to 56°. All spectra were corrected for instrumental distortions in excitation using a rhodamine cell in the reference channel.

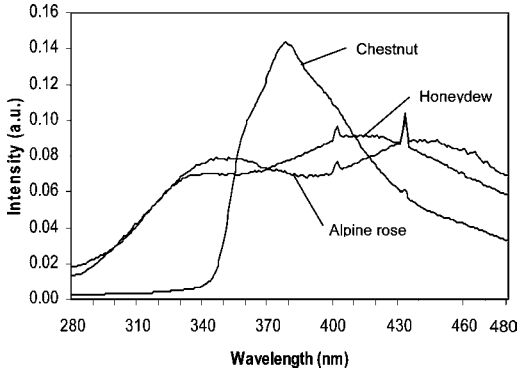


**Figure 1.** Normalized fluorescence spectra of different honey types (excitation at 250 nm).

Using the excitation wavelengths of 250, 290, and 373 nm, the fluorescence emission spectra were recorded from 280 to 480 nm (increment 1 nm; slits: at excitation, 3.5 and at emission, 2.0), 305−500 nm (increment 1 nm; slits: at excitation, 2.5 and at emission, 2.0), and 380−600 nm (increment 2 nm; slits: at excitation and emission, 1.5), respectively. Fluorescence excitation spectra were recorded with excitation wavelength from 290 to 440 nm and measurement of light emission at 450 nm (increment 1 nm; slits: at excitation, 2.0 and at emission, 1.5). Three spectra were recorded using different aliquots of each sample.

**Processing of Spectra and Multivariate Analysis.** First, a normalization of each spectrum was done to reduce the residual scattering effects according to Bertrand and Scotter (*27*) using the formulas:

$$c_i = F_i/\text{norm}$$

and

$$\text{norm} = \sqrt{\sum_{j=1}^{n} F_j^2}$$

where $c_i$ is the normalized value at the emission wavelength $i$, $F_i$ is the raw fluorescence intensity at the emission wavelength $i$, $F_j$ is the fluorescence at wavelength $j$, and $n$ is the number of data points for each spectrum.

Principal component analysis (PCA) was used to eliminate the spectral collinearity, random noise, and to reduce the number of variables for subsequent analysis. It was performed on two different data sets. The subsequent linear discriminant analysis (LDA) was performed on the PC covering at least 99% of the total spectral variability (SYSTAT Version 10.2).

## RESULTS AND DISCUSSION

**Fluorescence Spectra of Different Honey Types.** The recording of fluorescence spectra at various excitation and emission wavelengths was performed to study the differences between the seven honey types. **Figures 1**−**3** show their normalized fluorescence (emission) spectra. Every spectrum is more or less typical for a given honey type. As most of the spectra represent very similar shapes and can therefore visually hardly be distinguished, only a few of the most different spectra are shown in the figures. The various spectra were recorded using different aliquots of the same sample.

For the spectra recorded following excitation at 250 nm (**Figure 1**), all honey types except chestnut honey exhibit broad and overlapping emission bands with at least two maxima located between 320−390 and 390−460 nm, respectively. The very characteristic fluorescence spectrum of chestnut honey shows a much narrower band with at least three shoulders and a maximum at approximately 380 nm. The two small peaks at

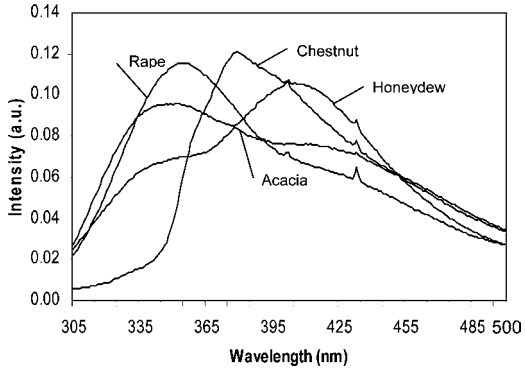


**Figure 2.** Normalized fluorescence spectra of different honey types (excitation at 290 nm).

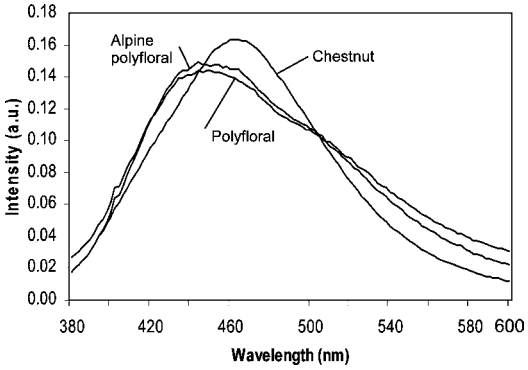


**Figure 3.** Normalized fluorescence spectra of different honey types (excitation at 373 nm).

402 and 433 nm observed in all honey types (**Figures 1** and **2**) are artifacts probably due to instrumental interferences.

The most significant differences between the spectra of the honey types under investigation were found at the excitation wavelength of 290 nm (**Figure 2**). The spectra of chestnut and honeydew honeys show maxima at about 375 and 410 nm, respectively, and express completely different shapes. Yet all spectra show at least two broad overlapping emission bands.

At the excitation wavelength of 373 nm (**Figure 3**), the spectrum of chestnut honey again clearly differs from those of the other honey types investigated, but the shapes of the latter are much more similar, including a maximum at about 450 nm. The emission spectra shown in **Figures 1**−**3** are due to numerous fluorescent compounds occurring in the various honey types in different concentrations and in different environments leading to the various forms of these spectra.

When the excitation spectra were scanned from 290 to 440 nm with emission measured at 450 nm, several shoulders were observed between 330 and 370 nm (**Figure 4**). For most honey types, the maxima were located at about 370 nm, chestnut honey showing an additional maximum at about 390 nm. Chestnut, alpine rose, and acacia honeys were shown to be the most distinctly different.

It has been shown that chestnut honey, as compared to the other honey types analyzed in this study, contains relatively high amounts of hydroxycinnamates such as caffeic, *p*-coumaric, and ferulic acids as well as unidentified flavonoids (*13, 28*). Chestnut honey may also contain more phenylalanine than the other honey types analyzed in this study (*18*). This may explain the differences in the fluorescence spectra.

**Linear Discriminant Analysis (LDA) on the Fluorescence Spectra.** LDA was performed on the principal component scores of each type of spectra as well as on the combination of the four different types of spectra. To build a classification model that potentially holds for single measurements of given samples, all of the three spectra recorded per sample (instead of mean spectra) were statistically treated as independent objects to include instrumental measurement uncertainty and variation from replicate measurements (pure spectral random noise is eliminated by PC data reduction). Chemometric evaluation using a single type of spectra resulted in rather poor classification rates except for chestnut honey (**Table 1**). The average rate of correct classifications was about 70% ranging from 36% for lowland polyfloral up to 100% for chestnut and rape honeys. The most useful type of spectra for the discrimination between different honey types was the emission spectra recorded following excitation at 290 nm and the excitation spectra (290−440 nm). The spectra recorded at an excitation wavelength of 290 nm allow a correct classification of chestnut and rape honey. With a rate of 80%, these conditions were also the best ones for the authentication of alpine rose honey. Considering emission spectra recorded in the 380−600 nm range, honeydew honey could be recognized with a probability of 92% from the other honey types. The excitation spectra (290−440 nm) were most useful for the authentication of acacia and alpine honeys (**Table 1**).

Combining the individually reduced data of the four types of spectra significantly improved the rate of correct classification of all honey types. The LDA was based on 19 principal components. The average classification rate rose to 94% (**Table 2**). Acacia, alpine rose, alpine polyfloral, and chestnut honey varieties were correctly assigned.

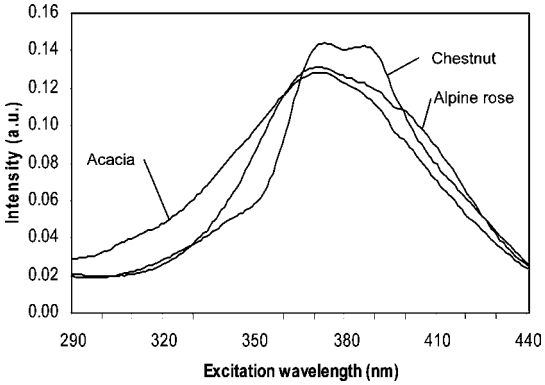


**Figure 4.** Normalized fluorescence spectra of different honey types (emission at 450 nm).

**Table 1.** Percentage of Correct Classification by Using Single Data Sets at Different Excitation and Emission Wavelengths and by Combining the Data of the Four Different Wavelengths (Jack-knifed Classification by the "Leave One Out" Method)[a]

| honey type | rate of correct classification (%) | | | | |
|---|---|---|---|---|---|
| | by using a single data set | | | | combining the four |
| botanical origin | 250 nm | 290 nm | 373 nm | 450 nm | data sets |
| acacia ($n = 7$) | 57 | 71 | 71 | 81 | 100 |
| alpine rose ($n = 5$) | 60 | 80 | 53 | 73 | 100 |
| alpine polyfloral ($n = 7$) | 76 | 57 | 52 | 86 | 100 |
| honeydew ($n = 8$) | 75 | 75 | 92 | 83 | 96 |
| chestnut ($n = 9$) | 100 | 100 | 100 | 100 | 100 |
| lowland polyfloral ($n = 10$) | 36 | 45 | 55 | 48 | 73 |
| rape ($n = 10$) | 80 | 100 | 60 | 70 | 97 |

[a] The spectra were recorded after excitation at wavelengths of 250, 290, and 373 nm or by measuring the emission at 450 nm when scanning the excitation from 290 to 440 nm ($n$ = number of samples).

**Table 2.** Jack-knifed Classification of the Combination of Four Data Sets ($n$ = Number of Samples)

| honey type | acacia | alpine rose | alpine polyfloral | honeydew | chestnut | polyfloral | rape | correct (%) |
|---|---|---|---|---|---|---|---|---|
| acacia ($n = 7$) | 21 | | | | | | | 100 |
| alpine rose ($n = 5$) | | 15 | | | | | | 100 |
| alpine polyfloral ($n = 7$) | | | 21 | | | | | 100 |
| honeydew ($n = 8$) | | | | 23 | | 1 | | 96 |
| chestnut ($n = 9$) | | | | | 27 | | | 100 |
| lowland polyfloral ($n = 10$) | | 3 | | | 3 | 24 | 3 | 73 |
| rape ($n = 10$) | | | | | | 1 | 29 | 97 |
| total ($n = 57$) | 21 | 18 | 21 | 23 | 30 | 26 | 32 | 94 |

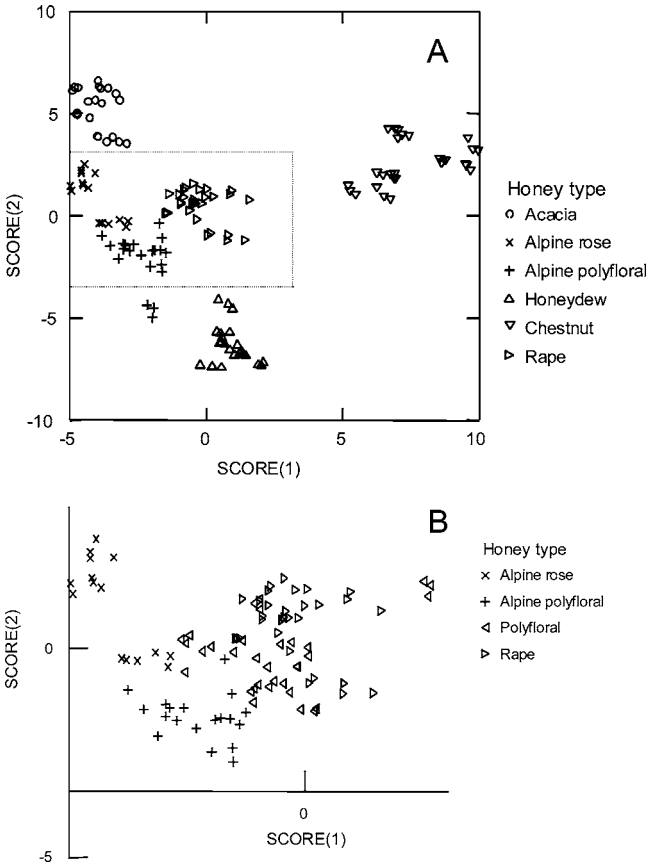


**Figure 5.** (**A**) Scatter plot of canonical discriminant scores without lowland polyfloral honey; dashed line designates enlargement. (**B**) Enlargement from scatter plot of canonical discriminant scores with lowland polyfloral honey samples included.

Honeydew and rape honeys reached with 96% and 97%, respectively, high rates of correct assignment. The lowest correct classification rate (73%) was found for the lowland polyfloral honeys. Three samples (nine spectra) were wrongly assigned (**Table 2**). One was assigned to alpine rose honey, a second to the chestnut honey, and a third to rape honey.

The higher rate of misclassifications for the lowland polyfloral honeys can be well explained by the fact that the latter generally consist of small amounts of nectar or honeydew produced by various plants species. Therefore, they do not have distinct physical or chemical properties that would correspond to their fluorescence characteristics. Rape is one of the most important nectar producing plants in Switzerland, and most lowland polyfloral honeys contain considerable amounts of this honey type. This explains why some lowland polyfloral honeys are misclassified as unifloral rape honeys and vice versa (see **Table 2** and **Figure 5**). One spectrum of a honeydew honey sample was classified as a lowland polyfloral honey.

**Table 3.** Validation of the Discriminant Function (Combining the Four Data Sets, $n$ = Number of Samples)

| honey type | honeydew | chestnut | polyfloral | rape | correct (%) |
|---|---|---|---|---|---|
| honeydew ($n = 2$) | 6 | 0 | 0 | 0 | 100 |
| chestnut ($n = 3$) | 0 | 9 | 0 | 0 | 100 |
| lowland polyfloral ($n = 3$) | 0 | 0 | 9 | 0 | 100 |
| rape ($n = 3$) | 0 | 0 | 3 | 6 | 67 |
| total ($n = 11$) | 6 | 9 | 12 | 6 | 91 |

Furthermore, the scatter plot of the scores of the first two discriminant functions from the LDA revealed some interesting characteristics of the honey samples analyzed. As numerous plant species contribute to the characteristics of lowland polyfloral honeys, their corresponding cluster is the least homogeneous and is located between the clusters of alpine polyfloral and the rape honeys (**Figure 5B**). One sample of lowland polyfloral honey was located among the rape honey samples. However, the pollen analysis revealed a relative frequency of 57% of *Brassica* sp. pollen, in fact, just below the 70% threshold set in Switzerland for a unifloral rape honey based on traditional methods. Another so-called lowland polyfloral honey was found between the clusters of rape and chestnut honey (**Figure 5B**). It was classified as lowland polyfloral honey as it predominantly consisted of apple and rape nectar but had also a minor contribution of chestnut. Alpine rose honey seemed to consist of two clusters that may be explained as follows: the samples which contain more alpine rose pollen, and are thus assumed to be purer honeys, are located in the neighborhood of acacia honeys. As the other alpine rose honey samples contain less alpine rose pollen, they are expected to have nectar contributions from other alpine plant species. Consequently, they are located close to the group of alpine polyfloral honeys but were still, based on the classical criteria, considered as unifloral alpine rose honeys. Moreover, one sample of alpine polyfloral honeys lies in the figure closer to the group of honeydew honeys. Its pollen analysis revealed that it contains a considerable amount of raspberry (*Rubus* sp.) honey.

Despite the limited number of samples, another principal component matrix was generated for the honey types represented by 8−11 samples, that is, honeydew, chestnut, lowland polyfloral, and rape, to evaluate the potential of the discriminant function generated, by randomly assigning samples to a calibration and a validation set. About two-thirds of the spectra were used as calibration spectra to build the model, and about one-third of the spectra were used as validation spectra for the evaluation of performance of the model. The classifications of the samples in the validation group are shown in **Table 3**. All samples but one (a sample of rape honey; three spectra) were correctly classified.

This preliminary study shows that front-face fluorescence spectroscopy combined with chemometrics offers a promising approach for the authentication of the botanical origin of honey.

The technique is nondestructive, rapid, easy to use, and not expensive. It neither needs any particular sample preparation nor special qualification of the personnel. The current results show that there is a strong correlation between the classic methods for the authentication of different honey types and the fluorescence characteristics of the honey samples studied.

Unifloral honeys with very characteristic fluorescence spectra, such as chestnut honey, can be easily recognized using only one of the single spectra recorded. Honey types having less characteristic spectra, such as alpine polyfloral or lowland polyfloral honeys, need a combination of several spectra for a reliable authentication. To a certain extent, the use of complementary excitation spectra (probably between 320 and 440 nm) could help to increase the correct classification rate of less typical honey varieties. However, these preliminary findings should be confirmed with a larger set of samples and additional honey types.

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