CHAPTER 7

# Authentication of the Botanical and Geographical Origin of Honey by Front-Face Fluorescence Spectroscopy*


## ABSTRACT

Front-face fluorescence spectroscopy, directly applied on honey samples, was used for the authentication of eleven unifloral and polyfloral honey types (n = 371 samples) previously classified using traditional methods such as chemical, pollen, and sensory analysis. Excitation spectra (220 – 400 nm) were recorded with the emission measured at 420 nm. In addition emission spectra were recorded between 290 and 500 nm (excitation: 270 nm) as well as between 330 and 550 nm (excitation: 310 nm). A total of four different spectral data sets were considered for data analysis. Chemometric evaluation of the spectra included principal component analysis (PCA) and linear discriminant analysis (LDA), the error rates of the discriminant models were calculated by the Bayes' theorem. They ranged from < 0.1% (polyfloral and chestnut honeys) to 9.9 % (fir honeydew honey) by using single spectral data sets and from < 0.1% (metcalfa honeydew, polyfloral and chestnut honeys) to 7.5 % (lime honey) by combining two data sets. This study indicates that front-face fluorescence spectro-scopy is a promising technique for the authentication of the botanical origin of honey and may also be useful for the determination of the geographical origin within the same unifloral honey type.


## 7.1 INTRODUCTION

According to the Codex Alimentarius Standard (*1*) and the European Union Council Directive (*2*) relating to honey, the use of a botanical designation of honey is allowed if it originates predominately from the indicated floral source. Honey may also be designated by the name of a geographical region if it was produced within the area referred to (*1, 2*).

The vast majority of the honeys on the market contain significant nectar or honeydew contributions from several plant species and are therefore called polyfloral or multifloral honeys. Normally they are just designated with the word "honey". Probably no honey produced by free bees flying is purely unifloral. The term unifloral honey is used to describe honey in which the major part of nectar or honeydew is derived from a single plant species. Honey composition, flavour and colour varies considerably depending on the botanical source it originates from (*3*).

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The physical, chemical and pollen analytical characteristics of the most important European unifloral honeys have been described in various papers (*3-7*). On contrary to the unifloral honeys the polyfloral honeys do not exhibit distinct physical or chemical characteristics apart from a huge variability, which makes their authentication particularly difficult.

The interest in the production of unifloral honeys is caused by higher consumer preference for some honey types generating a commercial concern of the beekeepers. The recent interest in the therapeutic or technological use of certain honey types may also contribute to the demand of a reliable determination of the botanical origin.

### 7.1.1 BOTANICAL ORIGIN

A number of new analytical techniques combined with multivariate data analysis have been proposed for the determination of the botanical origin of honey. They are for example based on physical and chemical measurands determined during quality control of honey (*8, 9*) or the former combined with the determination of mineral content (*10*), as well as carbohydrate composition (*11*), amino acid composition (*12*), mass-spectrometry or metal oxide semiconductor based gas sensors (*13, 14*), differential scanning calorimetry (*15*), pyrolysis mass spectrometry (*16*), raman (*17*) and near-infrared spectroscopy (*18*).

Many of the methods mentioned above allow to clearly discriminate between several types of unifloral honeys, but none of these methods accounts for the polyfloral honeys that represent the majority of the honeys produced. This means that these methods may not be useful in analytical practice, as the great challenge in honey analytics is not to distinguish between several unifloral honey types but to discriminate the minority of unifloral honeys from the overwhelming majority of polyfloral honeys on the market. This also explains why until now none of these proposed methods are commonly used for the determination of the botanical origin of honey.

Only a single ion chromatographic method has been tested in the presence of polyfloral honeys and showed a potential to discriminate between several unifloral as well as polyfloral honey samples by first classifying the honey samples into two groups by colour measurements (*19*). However, only very few samples were analysed in this study and it remains to be verified if this methodology is useful in analytical practice.

Currently, a reliable determination of the botanical and geographical origin can only be achieved by a global interpretation of sensory, pollen and physico-chemical analyses carried out by experts (*4, 20, 21*). However, the uncertainty related to the interpretation of pollen analytical results, originating from a number of different factors demands the development of new analytical methods (*22*).

### 7.1.2 GEOGRAPHICAL ORIGIN

Pollen analysis is currently used to determine the geographical origin of honey as pollen in honey reflect the vegetation type where the nectar has been collected by the bees. In the past many analytical methods such as amino acid composition (*23, 24*), raman spectroscopy (*17*), mineral content (*25, 26*), sugar or mineral composition combined with common chemical quality control data (*27-29*) together with multivariate data evaluation have been proposed for the determination of the geographical origin.

Unfortunately in most of the above quoted studies the botanical origin of the honey samples was not determined, or the discrimination between the geographical origins was not verified on samples of the same botanical origin. Generally the sample sets analysed were small or limited to a small geographical area. The distinctions found are therefore rather due to differences of the vegetation type between the geographical regions and thus to the botanical origin of honey (30). A geographical discrimination will therefore be found when the differences are related to the vegetation type present in these areas.

As several analytical methods have to be used together for a reliable authentication of the botanical origin, such a work is time consuming and costly. Very specialised expertise is needed for the interpretation of the pollen spectrum used for the determination of the geographical origin of honey. Thus, there is a real need for new methods that allow a rapid and reproducible authentication of the botanical and geographical origin of honey at low cost (*21, 31*).

### 7.1.3 FLUORESCENCE SPECTROSCOPY

Compared to spectroscopic techniques based on absorption, fluorescence spectroscopy offers a 100 to 1000-fold higher sensitivity. It provides information on the presence of fluorescent molecules and their environment in inorganic and organic materials. In addition, front-face fluorescence spectroscopy allows an investigation of fluorophores in powders as well as in concentrated or opaque samples (*32, 33*).

Honey is known to contain fluorophores such as polyphenols (*34-37*) and amino acids (*38, 39*). Fluorescence spectroscopy should therefore be helpful for authenticating the botanical origin of honey. More detailed information on fluorescence spectroscopic applications to honey and other food can be found in our previous study that already showed that front-face fluorescence spectroscopy is a promising approach for the determination of the botanical origin of honey (*40*).

The aim of the current work was to study the fluorescence spectroscopic characteristics of eleven honey types and to develop a rapid, low-cost and reliable method for the authentication of unifloral and polyfloral honeys. As the physical and chemical characteristics of honey may be changed by adulteration the potential of fluorescence spectroscopy was also studied on this subject. As minor nectar contributions from plant species other than the unifloral source may contribute to regional characteristics of unifloral honeys the potential of fluorescence spectroscopy for the determination of the geographical origin of honey was studied as well.

## 7.2 MATERIALS AND METHODS

### 7.2.1 SAMPLING AND BOTANICAL CLASSIFICATION BY REFERENCE METHODS

A total of 371 honey samples produced between 1998 and 2004 were collected and stored at 4 °C until analysis. They originated predominantly from Switzerland (CH) but samples from Germany (D), Italy (I), Spain (E), France (F), Slovenia (SLO) and Denmark (DK) were also included.

To classify these honey samples corresponding to their botanical origin the following measurands were determined according to the harmonised methods of the European Honey Commission (*41*): electrical conductivity, sugar composition, fructose/glucose ratio, pH-value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (*42, 43*).

Based on these analytical results, the honey samples were assigned to one of the following eleven honey types, according to the criteria of Persano and Piro (3): acacia (*Robinia pseudoacacia*) (CH, n = 14; D, n = 4; F, n= 3); alpine rose (*Rhododendron* spp.) (CH, n = 14; I, n = 5); chestnut (*Castanea sativa*) (CH, n = 21; I, n = 5; F, n = 3); rape (*Brassica* spp.) (CH, n = 22); fir honeydew (*Abies* and *Picea* spp.) (CH, n = 56; D, n = 63; SLO, n = 2); oak honeydew (*Quercus* spp.) (E, n = 8); honeydew from *Metcalfa pruinosa* (I, n = 14); heather (*Calluna vulgaris*) (D, n = 21; DK, n = 2); lime (*Tilia* spp.) (CH, n =14; D, n = 9; I, n = 4); dandelion (*Taraxacum* s.l.) (CH, n = 10; D, n = 7; I, n = 2) and polyfloral honeys (CH, n = 68). In the heterogenous group of the polyfloral honeys nectar or honeydew contributions from all of the above-mentioned sources were represented.

### 7.2.2 ADULTERATED HONEYS

In order to evaluate the potential of fluorescence spectroscopy to detect beet sugar adulteration, an artificial honey was produced by feeding two colonies after the nectar flow, in autumn, with a sucrose solution of 62.5 g/100 g generally used as winter feed of bee colonies in Switzerland. The sucrose solution was converted into artificial honey by the bees and left to ripen in the combs until extraction. To evaluate the possibility to detect honey adulteration by fluorescence spectroscopy six chestnut and six acacia honey samples were adulterated with 50 % of the artificial honey produced.

### 7.2.3 FLUORESCENCE SPECTROSCOPY

An aliquot of 20 g of the honey samples was liquefied at 55 °C for 8 h, allowed to cool to room temperature and poured into a 1 cm quartz cuvette. The latter was placed into the sample holder of a Perkin Elmer LS 50 B Luminescence Spectrometer (Perkin Elmer, Beaconsfield, UK) equipped with a variable angle front-surface accessory, with the incident angle of the excitation radiation set to 56°. Spectra were recorded at a scan rate of 150 nm/min and saved as ASCII textfiles. Instrumental artifacts were corrected in excitation using a rhodamine cell in the reference channel.

### 7.2.4 METHOD DEVELOPMENT

In order to find additional wavelength ranges with specific emission or excitation for the honey types of interest in addition to those already used in the preliminary study (*40*), the following ranges were studied. An excitation scan between 220 and 440 nm and recording of the fluorescence intensity at 420 and 490 nm was carried out. Six further emission scans were recorded with wavelengths between 220 – 600 nm, with excitation wavelengths being 210, 270 nm, 310 nm, 350 nm, 390 nm and 440 nm, respectively. The following three instrumental settings yielded the most discriminating fluorescent spectra for the ten types of unifloral honeys studied: excitation scan between 220 and 440 nm with the fluorescence emission measured at 420 nm, (method A); using the excitation wavelenghts of 270 nm and 310 nm, fluoresecence emission spectra were recorded from 290 to 500 nm, (method B) and from 330 to 550 nm, (method C), respectively. The excitation slit-width was set to 10 nm and the scan speed to 150 nm/min for all of the three methods. Two spectra were recorded using different aliquots of each sample. The spectra of the honey types studied are shown in the **Figure 1**.

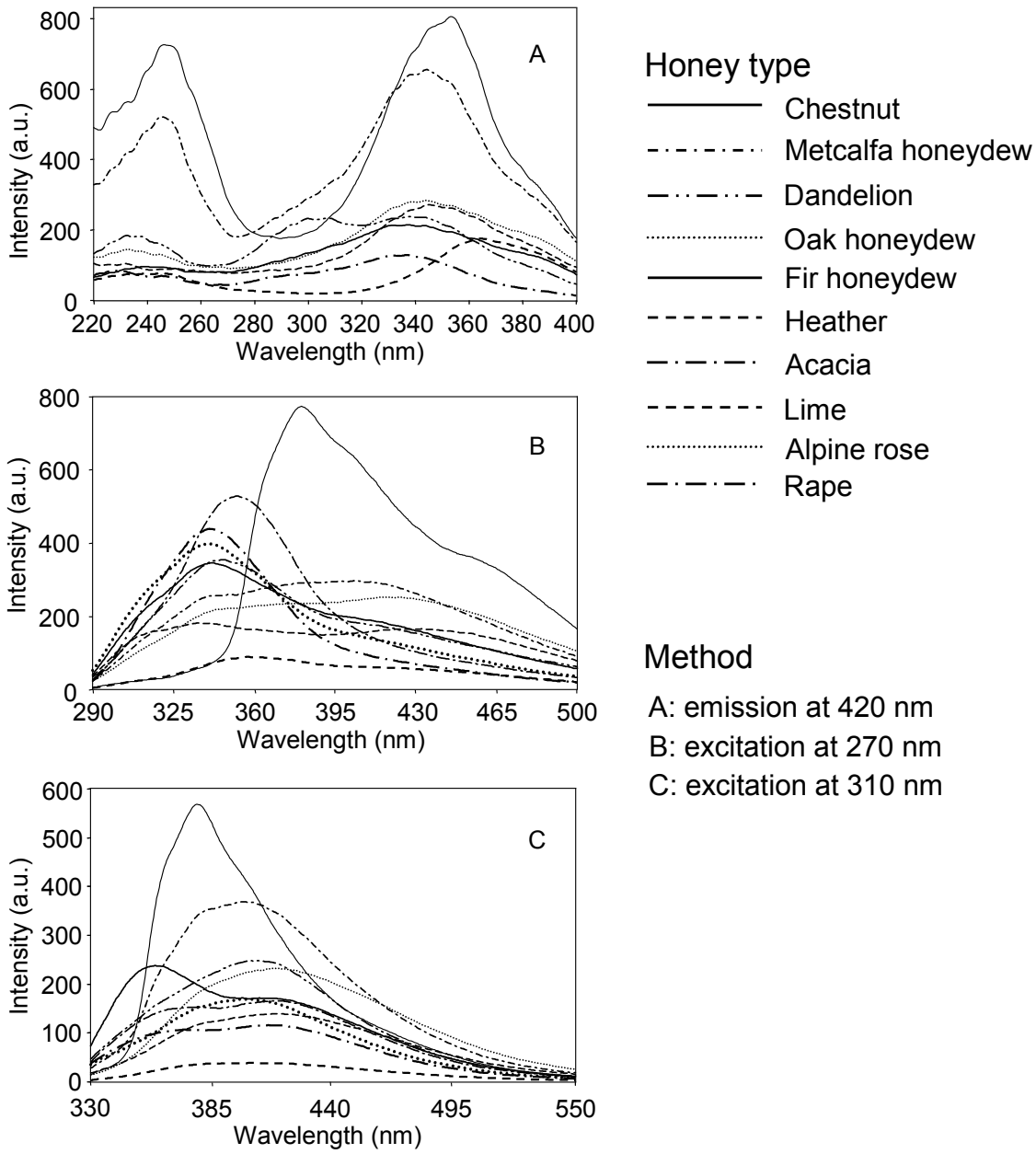


**Figure 1**. Fluorescence spectra of different honey types

A control honey sample for the evaluation of instrumental stability and determining the intermediate precision of the method was prepared by heating an acacia honey for 20 min up to 100 °C, then filtered to remove the pollen grains and partitioned into 2 ml glass vials, then stored at -20 °C until analysis. The intermediate precision was determined by recording spectra of the control honey sample on 18 days of analysis within 1.5 month. The small coefficients of variation indicate that instrumental conditions were reasonably stable over the duration of the measurements (**Table 1**).

### 7.2.5 PROCESSING OF SPECTRA AND MULTIVARIATE ANALYSIS

The spectra were converted into the GRAMS spc-format (GRAMS/32 AI Vs. 6.0, Thermo Galactic, Salem NH, U.S.A.) for more convenience in the visual examination and data reduction. It was found that a normalisation of the spectra was not necessary and that the consideration of the fluorescence intensities can even improve the possibilities in discriminating the different unifloral honeys (*40*).

To avoid random noise resulting from instrumental effects, only the following spectral ranges were used for multivariate analysis: method A: 224- 398 nm; method B: 290-500 nm and method C: 333- 547 nm. These ranges were also used for the combination of the spectra.

After elimination of spectral outliers, principal component analysis (PCA) was applied to eliminate the spectral collinearity and to reduce the number of variables to 20 PC's (using the PLSplus/IQ Add-on of GRAMS/32 AI Vs. 5.09). This was performed separately for each type of spectra and each combination of different types of spectra.

In linear discriminant analysis (LDA), the 20 initial PC's were further reduced by backward elimination of principal components on the basis of their partial F-values in the discriminant models (SYSTAT® Version 11, Systat Software Inc., Richmond, USA). The models were then optimised for maximum correct classification in jackknife classification. To account for the limited precision of single measurements, both spectra of each sample were used in the model of single types (A, B, and C) of spectra rather than the average. In the models using combined spectra, averaged spectra were used. The validation was carried out using spectra of one third of the samples selected randomly and not present in the group of samples used to build the model.

### 7.2.6 GEOGRAPHICAL ORIGIN

The applicability of fluorescence spectroscopy for the determination of the geographical origin of honey was evaluated for the honey types where samples originating from different countries were available. The differences resulting from the geographic origin were studied within the groups of unifloral honeys by using MANOVA (SYSTAT® Version 11, Systat Software Inc.) as well as LDA and are visualised by plots of the canonical discriminant scores (**Figure 3** and **4**).

## 7.3 RESULTS AND DISCUSSION

### 7.3.1 REPEATABILITY

The repeatability of the three different methods was determined by a six-fold measurement at the maximum intensity ($I_{max}$) of an acacia honey. With coefficients of variation ($cv_r$) between 1.1 and 2.6 % the methods showed a good repeatability which stayed in the same range over 43 days while the intermediate precision was determined (**Table 1**).

**Table 1.** Repeatability and intermediate precision of the three fluorescence spectroscopic methods

| | Method | | |
|---|---|---|---|
| *Repeatability (n = 6)* | A | B | C |
| Average $I_{max}$ (au)* | 179.8 | 185.8 | 100.9 |
| reproducibility $s_r$ (au) | 1.9 | 2.1 | 2.6 |
| coefficient of variation $cv_r$ (%) | 1.1 | 1.1 | 2.6 |
| Repeatability limit (r) (au) | 5.4 | 5.9 | 7.5 |
| Relat. repeatability limit (%) | 3 | 3.2 | 7.4 |
| *Intermediate precision (n = 18)* | | | |
| Average $I_{max}$ (au) | 203 | 195 | 106 |
| Laboratory reproducibility $s_L$ (au) | 7.2 | 2.7 | 2.4 |
| Relat. laboratory reproducibility $v_L$ (%) | 3.6 | 1.4 | 2.2 |

*arbitrary units (au)

### 7.3.2 FLUORESCENCE SPECTRA OF DIFFERENT HONEY TYPES

The recorded fluorescence spectra at three different excitation and emission wavelengths for the ten unifloral honey types considered are displayed in **Figure 1** (for better legibility the spectra of alpine rose and rape honey are not shown in **Figure 1 A** as they are visually very similar to fir honeydew honey). Every spectrum is typical for a given honey type. The spectra obtained by the different methods were recorded using different aliquots of the same sample.

Excitation spectra were scanned from 220 to 400 nm with the emission measured at 420 nm (method A, **Figure 1 A**). For most honey types two maxima at about 240 nm and between 340 and 360 nm, respectively were observed, while lime honey exhibits its second maximum at about 365 nm. Most of the honey types investigated had their intensity within the same order of magnitude except for chestnut honey that shows a nearly two-fold intensity at the maximum. Metcalfa honeydew honey is also characterised by a more intense fluorescence. Dandelion honey shows an additional shoulder at about 300 nm.

For the spectra recorded using excitation at 270 nm (method B, **Figure 1 B**), all honey types except chestnut, rape and lime honeys exhibited broad and overlapping emission bands including at least two maxima located between 330-350 nm and 400-440 nm, respectively. The very characteristic fluorescence spectrum of chestnut honey showed a much narrower band with two shoulders and a maximum at approximately 380 nm. Rape and lime honeys showed both maxima at about 350 nm while the latter had a broader emission between 400 and 500 nm. Alpine rose honey showed a shoulder at about 310 nm and a maximum at about 340 nm. For heather, fir honeydew, dandelion, acacia, rape and alpine rose honeys the intensities at the maxima ranged between 150 and 520 arbitrary units while chestnut exhibited a considerably higher intensity of about 800 units. The lowest intensity was detected for lime honey. However, the intensities were found to vary considerably within the honey types.

Using an excitation wavelength at 310 nm (method C, **Figure 1 C**) the spectra of chestnut honey again clearly differed from the other honey types investigated, especially by the two-fold intensity compared to the others having a maximum at about 380 nm. Most of the honey types exhibited a maximum at about 400 nm and an intensity in the range from 100 to 200 arbitrary units. Lime honey showed again the lowest intensity. Rape and acacia honeys were characterized by a shoulder at 365 nm. The maximum of the spectra of fir honeydew honeys was located at about 355 and showed a shoulder at 420 nm. The band of the chestnut honey spectrum was narrower than by using method B and less intense, but nevertheless the most intense among the spectra recorded by method C. The spectra of metcalfa honeydew honeys expressed a broad band with an intensity of about 350 arbitrary units, being thus the second most intense spectra.

It has been reported that chestnut honey, compared to the other honey types analysed in this study, contains high amounts of hydroxycinnamates such as caffeic, p-coumaric and ferulic acids as well as unidentified flavonoids (*34, 44*). Chestnut honey may also contain more phenylalanine than the other honey types analysed in this study (*39*). The fluorescence of 2-aminoacetophenone, the main volatile component of chestnut honey, may also explain the characteristic spectra (*45, 46*).

Interestingly heather honey commonly known to contain high amounts of phenolic compounds (*47*) does not show spectra of high fluorescence intensity compared to the other honey types. This may be due to scattering, reflection and interference effects resulting from the numerous air bubbles present in heather honey.

### 7.3.3 LINEAR DISCRIMINANT ANALYSIS (LDA) APPLIED TO THE FLUORESCENCE SPECTRA: BOTANICAL ORIGIN

LDA was performed on the principal components of each type of spectra as well as on the combination of the two most significant types of spectra. In the evaluation of single spectra the highest average classification rate (weighted according the number of samples) of 85 % in validation was obtained for the method A (**Table 2**). The rates of correct classification were similar in both jackknife classification and validation, demonstrating that the models used were robust. Throughout the three methods studied the classification rate for the polyfloral honeys was with only 42 to 63 % very low. This can be explained by the lack of specific physical and chemical characteristics of this honey type. Thus the polyfloral honeys are classified into the groups of unifloral honeys with the smallest Mahalanobis distance (**Table 3**).

For the method A the lowest classification rate of 80 % was observed for metcalfa honeydew honey. Twenty percent of the samples were misclassified as chestnut honey. This can be explained by the important nectar contribution of chestnut often present in metcalfa honeydew honeys. In spite of the low number of samples (n = 5) used for validation, the 20 % of misclassification arises from a single misclassified sample. In the validation step all samples of acacia, alpine rose, chestnut, lime, dandelion and rape honeys were correctly classified. No validation was done for the oak honeydew honey due to the low number of samples available. In the jackknife classification some difficulties occurred to assign alpine rose and acacia honeys (**Table 3**). Some samples of heather honey were also misclassified to rape and polyfloral honeys.

**Table 2.** Percentage of correct classification by using single data sets at different excitation and emission wavelengths and by combining of the data of the methods (jackknife classification by the "leave one out" method and validation with independent samples)

[1]Method A: excitation scanned between 220 and 400 nm, emission measured at 420.
[2]Method B: excitation at 270 nm, emission measured between 290 and 500 nm.
[3]Method C: excitation at 310 nm, emission measured between 330 and 500 nm.

| | Rates of correct classification by the different methods (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | A[1] | | B[2] | | C[3] | | Combination of spectra from methods A and B | |
| Honey type | Jackknife | Validation | Jackknife | Validation | Jackknife | Validation | Jackknife | Validation |
| Acacia | 95 | 100 | 90 | 79 | 85 | 75 | 90 | 100 |
| Alpine rose | 87 | 100 | 50 | 80 | 63 | 50 | 93 | 100 |
| Heather | 98 | 88 | 100 | 100 | 91 | 100 | 100 | 100 |
| Chestnut | 97 | 100 | 96 | 100 | 96 | 100 | 96 | 100 |
| Lime | 96 | 100 | 98 | 100 | 98 | 100 | 95 | 100 |
| Dandelion | 100 | 100 | 97 | 100 | 100 | 100 | 100 | 100 |
| Rape | 88 | 100 | 100 | 100 | 93 | 43 | 95 | 100 |
| Fir honeydew | 92 | 86 | 91 | 84 | 84 | 76 | 96 | 97 |
| Metcalfa honeydew | 93 | 80 | 100 | 100 | 100 | 75 | 92 | 100 |
| Oak honeydew | 100 | | 100 | | 78 | | 100 | |
| Polyfloral | 57 | 50 | 47 | 50 | 42 | 43 | 63 | 55 |
| Average (weighted) | 87 | 85 | 84 | 83 | 80 | 73 | 90 | 91 |

Interestingly a few samples of fir honeydew honey were classified as polyfloral or lime honeys. This could indicate that the value of 0.8 $mScm^{-1}$ in electrical conductivity is not always adequate to discriminate between polyfloral and honeydew honeys. Lime honeys very often contain some honeydew honey, which complicates their characterisation.

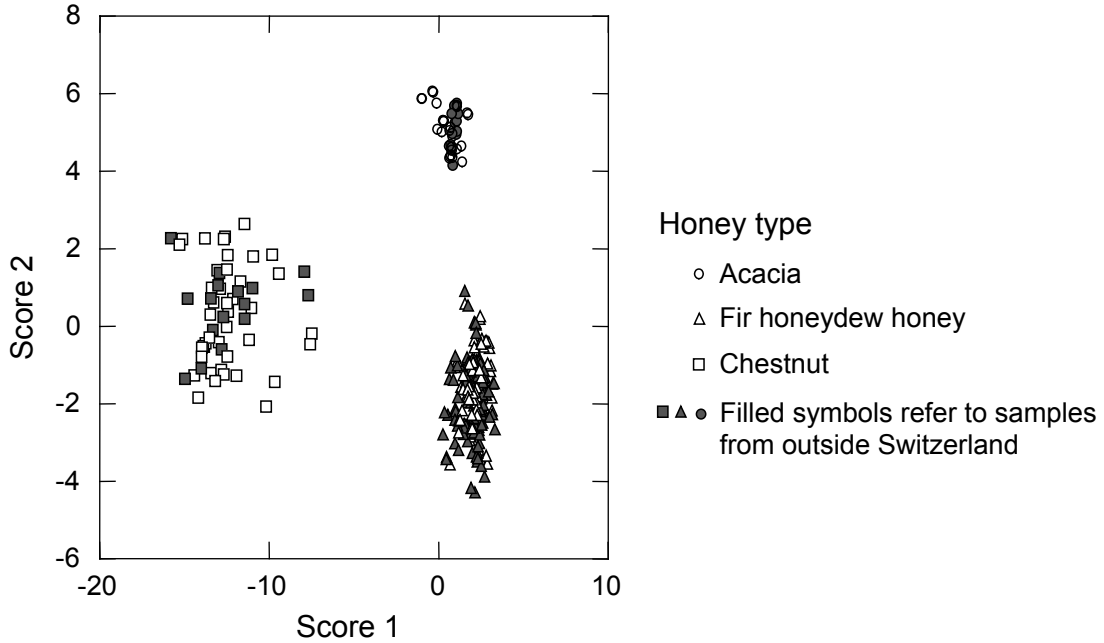


**Figure 2.** Scatterplot of canonical discriminant scores from method A (for better legibility only the spectra of three honey types are displayed)

**Table 3**. Jackknife classification and validation tables for the honey samples classified by LDA on the spectra of method A

| | Jackknife classification rate for method A (%) | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Acacia | Alpine rose | Heather | Chestnut | Lime | Dandelion | Rape | Fir honeydew | Metcalfa honeydew | Oak honeydew | Polyfloral |
| Acacia (n=21) | 95 | **5** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Alpine rose (n = 19) | **11** | 87 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **3** |
| Heather (n = 23) | 0 | 0 | 98 | 0 | 0 | 0 | **0** | 0 | 0 | 0 | **2** |
| Chestnut (n = 29) | 0 | 0 | 0 | 97 | 0 | 0 | 0 | 0 | 2 | 2 | 0 |
| Lime (n = 26) | 0 | 0 | 0 | 0 | 96 | 0 | 0 | 0 | 0 | 0 | **4** |
| Dandelion (n = 18) | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 |
| Rape ( n = 24) | 0 | 0 | 0 | 0 | 0 | 0 | 88 | 0 | 0 | 0 | **12** |
| Fir honeydew (n = 120) | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 92 | 0 | 0 | **8** |
| Metcalfa honeydew (n = 14) | 0 | 0 | 0 | **7** | 0 | 0 | 0 | 0 | 93 | 0 | 0 |
| Oak honeydew (n = 8) | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 |
| Polyfloral (n = 65) | 0 | **9** | 2 | **3** | **9** | **5** | **7** | **9** | 0 | 0 | 57 |

| | Classification rate in validation for method A (%) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Acacia | Alpine rose | Heather | Chestnut | Lime | Dandelion | Rape | Fir honeydew | Metcalfa honeydew | Polyfloral |
| Acacia (n=7) | 100 | **0** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Alpine rose (n = 6) | **0** | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | **0** |
| Heather (n = 8) | 0 | 0 | 88 | 0 | 0 | 0 | **6** | 0 | 0 | **6** |
| Chestnut (n = 10) | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lime (n = 9) | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 | **0** |
| Dandelion (n = 6) | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | 0 | 0 |
| Rape (n = 7) | 0 | 0 | 0 | 0 | 0 | 0 | 100 | 0 | 0 | **0** |
| Fir honeydew (n = 40) | 0 | 0 | 0 | 0 | 3 | 0 | 0 | 86 | 0 | **11** |
| Metcalfa honeydew (n = 5) | 0 | 0 | 0 | **20** | 0 | 0 | 0 | 0 | 80 | 0 |
| Polyfloral (n = 22) | 0 | **7** | 0 | **9** | **0** | **0** | **21** | **14** | 0 | 50 |

Even though samples originated from different geographical origins were correctly classified according to their botanical origin. Irrespective of their geographical origin the fluorescent characteristics of honey from various botanical origins seem to be uniform, as samples from outside Switzerland group among the samples from Switzerland (**Figure 2**, for better legibility the scores of only three different honey types are displayed).

The overall discriminating potential of method B is comparable to method A (**Table 2**). However for the discrimination between alpine rose and acacia honeys more difficulties were encountered using method B than method A. In spite of the fact that the two groups were mingled, some samples of alpine rose honey were even misclassified as polyfloral honeys (data not shown).

The potential of the method C for the classification of both unifloral and polyfloral honeys by using a single discriminant model was clearly inferior to that of the methods A and B. Beside the difficulties already mentioned for alpine rose and acacia honeys a considerable number of samples belonging to the groups of rape and honeydew honeys were not correctly classified in validation (**Table 2**).

To evaluate whether the rate of correct classification could be further increased by combining two of the most promising types of spectra, the ones of method A and B were averaged and concatenated for each sample. The rate of correct classification increased for alpine rose, fir honeydew and even for polyfloral honeys compared to the results obtained by using the individual methods A and B (**Table 2**).

The classification tables revealed that polyfloral honeys were very often classified into the groups of the unifloral honeys while the latter were rarely misclassified into the one of the polyfloral honeys. This observation lead to the development of a two step procedure. In the first step the sample was attributed to one of the eleven honey types considered using an overall discriminant model including all honey types. In the second step this classification was verified by using one or several two-group models consisting of a group formed by samples of a given unifloral honey versus a group called "non-unifloral" consisting of all the other samples. Each two-group model was separately built using LDA backward elimination and forward selection. For the verification of the classification by the first model at least the two-group model of the corresponding honey type was used. In addition one to six two-group models (indicated by bold numbers in **Table 3**) were used when a misclassification rate of higher than 3% was calculated in jackknife classification or validation tables of the overall model.

The classification rates for the unifloral honeys in the two-group models were generally > 90 % while the classification rate for the polyfloral honeys ranged between 48 and 75 % (**Table 4**). However, as far as the polyfloral honeys are concerned this is not very important, as we are principally interested in the authentication of unifloral honeys. The high rates of correct classification for both, the unifloral and non-unifloral groups considered by the two-group models indicate that the botanical origin can be reliably determined by this procedure. The respective error rates of this two-step procedure using the methods A and B as well as the combination of the two former types of spectra were calculated by applying Bayes' theorem on the conditional probabilities of disjoint events.

**Table 4a.** Jackknife and validation tabels for the honey samples classified by the two-group discriminant models of methods A and B.

**Method A**

| | Jackknife classification | | | | Validation | |
|---|---|---|---|---|---|---|
| | Unifloral | | Non-Unifloral | | Unifloral | |
| | n | Correct class. (%) | n | Correct class. (%) | n | Correct class. (%) |
| Acacia | 21 | 100 | 343 | 96 | 7 | 100 |
| Alpine rose | 19 | 100 | 345 | 90 | 6 | 83 |
| Heather | 23 | 98 | 341 | 99 | 8 | 88 |
| Chestnut | 29 | 97 | 335 | 99 | 10 | 90 |
| Lime | 26 | 100 | 338 | 97 | 9 | 100 |
| Dandelion | 18 | 100 | 346 | 98 | 6 | 100 |
| Rape | 24 | 91 | 343 | 93 | 7 | 100 |
| Fir honeydew | 120 | 95 | 244 | 93 | 40 | 88 |
| Metcalfa honeydew | 14 | 100 | 350 | 100 | 5 | 100 |
| Oak honeydew | 8 | 94 | 356 | 99 | | |
| Polyfloral | 65 | 74 | 300 | 65 | 22 | 48 |

**Method B**

| | Jackknife classification | | | | Validation | |
|---|---|---|---|---|---|---|
| | Unifloral | | Non-Unifloral | | Unifloral | |
| | n | Correct class. (%) | n | Correct class. (%) | n | Correct class. (%) |
| Acacia | 21 | 100 | 341 | 96 | 7 | 100 |
| Alpine rose | 16 | 97 | 346 | 89 | 5 | 80 |
| Heather | 23 | 100 | 340 | 95 | 8 | 100 |
| Chestnut | 28 | 96 | 335 | 99 | 9 | 100 |
| Lime | 26 | 100 | 336 | 95 | 8 | 100 |
| Dandelion | 19 | 100 | 343 | 94 | 6 | 100 |
| Rape | 22 | 100 | 340 | 98 | 7 | 100 |
| Fir honeydew | 120 | 92 | 242 | 92 | 40 | 84 |
| Metcalfa honeydew | 12 | 100 | 350 | 98 | 4 | 88 |
| Oak honeydew | 8 | 100 | 354 | 95 | | |
| Polyfloral | 68 | 65 | 294 | 72 | 22 | 75 |

**Table 4b.** Jackknife and validation tabels for the honey samples classified by the two-group discriminant models on the combination of the spectra A and B

**Combination of spectra from methods A and B**

| | Jackknife classification | | | | Validation | |
|---|---|---|---|---|---|---|
| | Unifloral | | Non-Unifloral | | Unifloral | |
| | n | Correct class. (%) | n | Correct class. (%) | n | Correct class. (%) |
| Acacia | 20 | 100 | 327 | 97 | 7 | 100 |
| Alpine rose | 15 | 100 | 332 | 91 | 7 | 100 |
| Heather | 23 | 100 | 324 | 100 | 8 | 100 |
| Chestnut | 27 | 96 | 320 | 100 | 9 | 100 |
| Lime | 26 | 100 | 321 | 96 | 9 | 100 |
| Dandelion | 19 | 95 | 328 | 98 | 6 | 100 |
| Rape | 21 | 100 | 326 | 98 | 7 | 100 |
| Fir honeydew | 117 | 97 | 230 | 93 | 39 | 100 |
| Metcalfa honeydew | 12 | 100 | 335 | 98 | 4 | 100 |
| Oak honeydew | 8 | 100 | 339 | 99 | | |
| Polyfloral | 59 | 69 | 288 | 71 | 15 | 75 |

Method A gave again the most promising results with an error probability (wrong classification of a sample of unknown botanical origin) < 5 % for all honey types except for fir honeydew where it was 10 % (Table 5). The error probabilities by using method B were higher for all honey types compared to those using method A except for the determination of metcalfa honeydew. By using the combination of the spectra of methods A and B, the error probability could be reduced to < 5 % (in validation) for the eleven honey types studied. It is interesting to notice that the error probabilities of the honey types that express the highest variability in physical and chemical characteristics such as lime and fir honeydew honeys are the highest in fluorescence spectroscopy as well. This can be interpreted that fluorescence spectroscopy reproduces well the characteristics of classical criteria.

**Table 5**. Error probabilities for the classification of unifloral and polyfloral honeys by the different methods

| | Error probability | | | | | |
|---|---|---|---|---|---|---|
| | Method A | | Method B | | Combination of spectra from methods A and B | |
| Honey type | Jackknife | Validation | Jackknife | Validation | Jackknife | Validation |
| Acacia | 0.029 | 0.006 | 0.109 | 0.030 | 0.022 | 0.005 |
| Alpine rose | 0.016 | 0.003 | 0.058 | 0.018 | 0.009 | 0.001 |
| Heather | 0.044 | 0.013 | 0.050 | 0.051 | 0.003 | 0.003 |
| Chestnut | 0.003 | $< 10^{-3}$ | 0.034 | 0.053 | $< 10^{-3}$ | $< 10^{-3}$ |
| Lime | 0.067 | 0.037 | 0.096 | 0.054 | 0.075 | 0.039 |
| Dandelion | 0.037 | 0.008 | 0.075 | 0.072 | 0.021 | 0.019 |
| Rape | 0.003 | 0.002 | 0.046 | 0.070 | 0.033 | 0.042 |
| Fir honeydew | 0.088 | 0.099 | 0.107 | 0.090 | 0.047 | 0.045 |
| Metcalfa honeydew | 0.040 | 0.004 | 0.004 | 0.002 | $< 10^{-3}$ | $< 10^{-3}$ |
| Oak honeydew | 0.044 | | 0.050 | | 0.006 | |
| Polyfloral | $< 10^{-3}$ | $< 10^{-3}$ | 0.034 | 0.031 | $< 10^{-3}$ | $< 10^{-3}$ |

### 7.3.4 GEOGRAPHICAL ORIGIN

Differences in geographical origin were studied within the groups of samples of the same botanical origin when samples were available from at least two countries. Interestingly a statistically significant difference was found by MANOVA between the geographical origins of all honey types studied (**Table 6**). The lime honey samples originating from Switzerland, Germany and Italy formed groups in the plot of discriminant scores according to their geographical origin (**Figure 3**). The samples could also be correctly classified by LDA according to their geographical origin except for one Swiss sample that was classified to German provenience (data not shown). But the classification according to the geographical origin could only be observed within the groups of honeys of the same botanical origin. An LDA model of acacia, lime, dandelion and fir honeydew honeys of German and Swiss origin failed to classify the samples according to their geographical provenience (**Table 7**). This clearly indicates that the characteristics resulting from the botanical source are considerably stronger than the geographical aspects. The sample set of the lime honeys was small, a larger sample set would possibly lead to a less pronounced difference.

Table 6. Results from MANOVA for the geographical origin of the different unifloral honeys (method A).

| Honey type, Country | Wilks' Lambda | p |
|---|---|---|
| Acacia (CH, D, F) | 0.009 | $< 10^{-3}$ |
| Alpine rose (CH, I) | 0.027 | $< 10^{-3}$ |
| Fir honeydew (CH, D) | 0.696 | $< 10^{-3}$ |
| Chestnut (CH, F, I) | 0.001 | $< 10^{-3}$ |
| Lime (CH, D, I) | 0.004 | $< 10^{-3}$ |
| Dandelion (CH, D, I) | 0.023 | $< 10^{-3}$ |

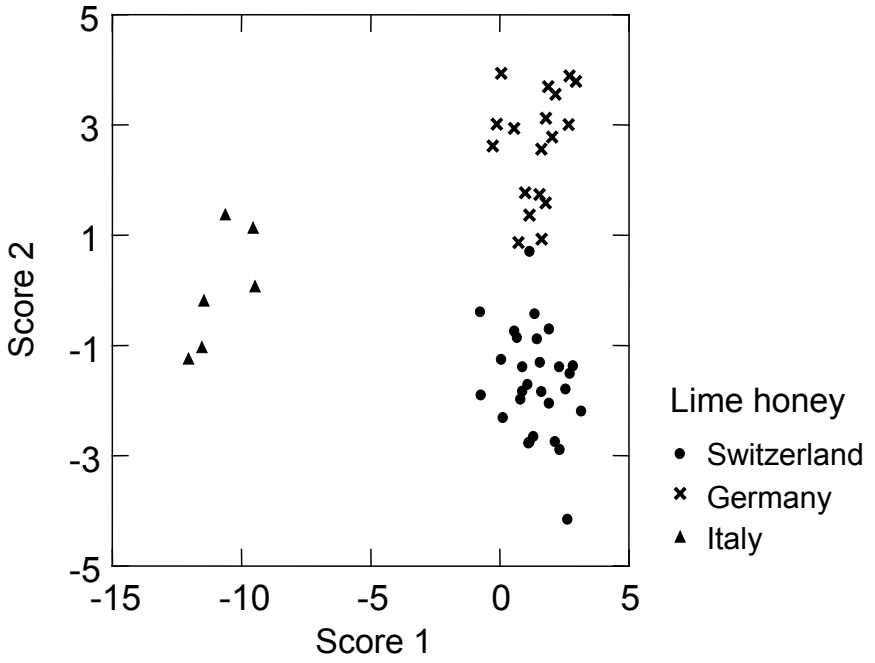


**Figure 3.** Scatterplot of canonical discriminant scores of lime honeys of different geographic origin (method A)

This may be illustrated on the example of the fir honeydew honeys from Germany and Switzerland where a classification according to geographical origin was not possible (**Figure 4**). However, the samples of fir honeydew honeys originated from an area of approximately 300 km in diameter belonging to Switzerland and Germany and therefore having a very similar vegetation.

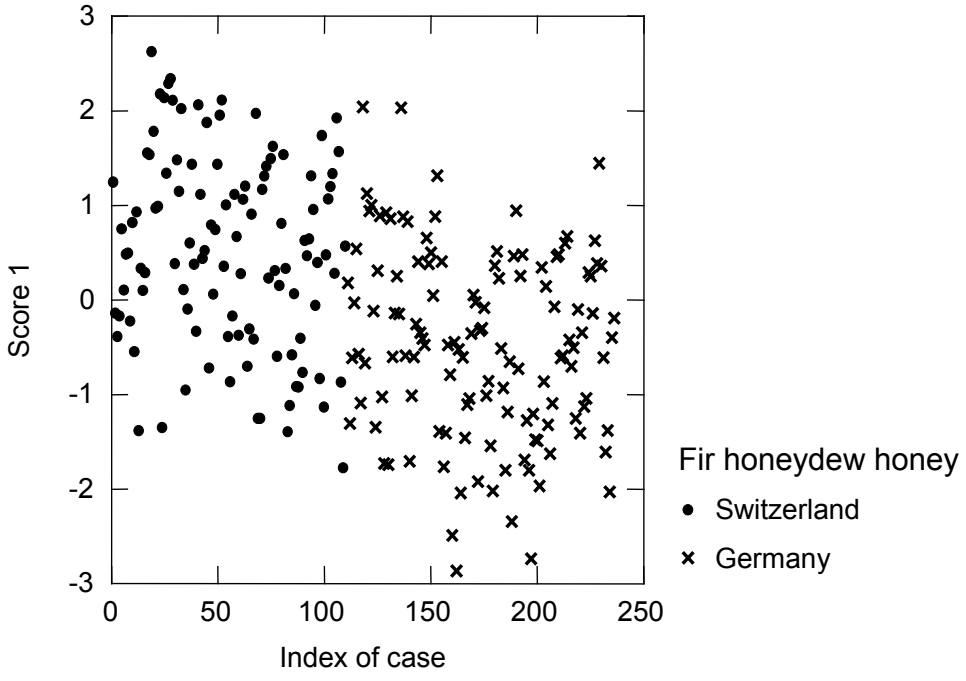


**Figure 4.** Scatterplot of the canonical discriminant score of honeydew honeys from spruce and fir of German and Swiss provenience (method A)

In future studies it should be verified if the geographical origin of honey could be determined by fluorescence spectroscopic techniques based on the minor contributions of accompanying flora that may be different in areas distant enough. The chemometric models should also be validated with samples of polyfloral provenience.

**Table 7.** Percentage of correct classification according to the geographical provenience by using the data set of method A

| | Jackknifed classification matrix* | | |
|---|---|---|---|
| | Switzerland | Germany | Correct (%) |
| Switzerland | 117 | 69 | 63 |
| Germany | 59 | 109 | 65 |
| Total | 176 | 178 | 64 |

*Jackknife classification by the "leave one out" method on samples from acacia, lime, dandelion and honeydew honeys from spruce and fir.

### 7.3.5 ADULTERATION BY FEEDING OF BEES

The acacia and chestnut honey samples adulterated with as much as 50 % of artificial honey did not show any comprehensible changes in the spectra compared to the pure samples in any of the three methods studied. Generally the spectra of the adulterated samples remained in the range of the natural variation of the corresponding unifloral honeys. A detection of honey adulteration is therefore not possible except if the adulterant contains a characteristic fluorophore.

### 7.3.6 CONCLUSION

While absolutely pure unifloral honeys do not exist, the definition of unifloral honey is in fact based on the points of view and the descriptions of different analysts. However a consensus has been reached using the physical, chemical and pollen analytical characteristics of the unifloral honeys considered as internationally recognised criteria already published (*3-6*).

Of capital importance is certainly to ensure a uniform honey quality that can be recognised by consumers preferring a given type of honey. Currently the determination of the botanical origin of honey relies on the judgement of experienced experts who base their decision on the criteria of several analytical measurands. The challenge of new analytical methods that do not need such an expertise is to mathematically model and reproduce this decision making process. As the definition of a unifloral honey is ultimately a matter of opinion, absolutely correct classification by chemometric models can therefore not be expected as these models are trained by uncertain sample sets as reference.

As the characteristic physical and chemical differences between unifloral and polyfloral honeys are small and only very few compounds are specific to a given type of honey, the chemometric approach based on a fingerprint seems to be more promising than the search for individual marker compounds.

This study shows that front-face fluorescence spectroscopy combined with chemometrics offers a promising approach to the authentication of the botanical origin of honey and that the problems related to the determination of the polyfloral honeys can be overcome by the successive use of at least two mathematical models. The current results show that classifications based on classical criteria commonly used for the determination of the botanical origin of honey can be very well reproduced by front-face fluorescence spectroscopy and chemometrics. It depends on the cer-

tainty needed whether to base the classification on the single spectra of type A or to combine the spectra of methods A and B.

Of course the proposed fluorescence spectroscopic method needs a considerable amount of preliminary work to establish the chemometric models based on samples of known botanical origin. Once the classification models have been set the technique enables a rapid determination of the botanical origin without particular sample preparation and special qualification of laboratory personnel. It remains to be tested by future studies if these models can be transferred from one instrument to another, like in infrared spectroscopy when normalised fluorescence spectra are used (*40*) or the instruments are calibrated with reference materials.

In addition, the present work clearly shows that fluorescence characteristics of honey are much more depending on their botanical origin than on the geographical origin. Therefore the former should be determined before proposing a method for the determination of the geographical origin of honey. Such a method must be tested as well with samples of the same botanical origin.

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