

## Authentication of the Botanical Origin of Honey by Near-Infrared Spectroscopy

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Fourier transform near-infrared spectroscopy (FT-NIR) was evaluated for the authentication of eight unifloral and polyfloral honey types ( $n = 364$  samples) previously classified using traditional methods such as chemical, pollen, and sensory analysis. Chemometric evaluation of the spectra was carried out by applying principal component analysis and linear discriminant analysis. The corresponding error rates were calculated according to Bayes' theorem. NIR spectroscopy enabled a reliable discrimination of acacia, chestnut, and fir honeydew honey from the other unifloral and polyfloral honey types studied. The error rates ranged from  $<0.1$  to 6.3% depending on the honey type. NIR proved also to be useful for the classification of blossom and honeydew honeys. The results demonstrate that near-infrared spectrometry is a valuable, rapid, and nondestructive tool for the authentication of the above-mentioned honeys, but not for all varieties studied.

**KEYWORDS:** Honey; botanical origin; FT-NIR; chemometrics

### INTRODUCTION

The vast majority of the honeys sold 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-flying bees 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, flavor, and color vary considerably depending on the botanical source it originates from (1). According to the Codex Alimentarius Standard for Honey (2) and the European Union Council Directive (3) related to honey, the use of a botanical designation of honey is allowed if it originates predominately from the indicated floral source and possesses the corresponding sensorial, physical, chemical, and microscopic properties.

The physical, chemical, and pollen analytical characteristics of the most important unifloral honeys have been described in various papers (1, 4–6). On the contrary to unifloral honeys, polyfloral honeys do not express distinct physical or chemical characteristics apart from a huge variability, which makes their authentication particularly difficult.

Interest in the production of unifloral honeys is founded on a higher consumer preference for some honey varieties, leading to a commercial interest of the beekeepers. Recent applications in therapeutic or technological use of certain honey varieties may also account for the requirement of a reliable determination of the botanical origins (7–10).

Up to now a reliable authentication of the botanical origin can be achieved only by experts by a global interpretation of sensory, pollen, and physicochemical analyses that include at least measurement of electrical conductivity and sugar composition (4, 11, 12). A specific analytical method has to be applied for each measurand of interest, thus resulting in laborious and expensive analyses. Especially the uncertainty related to the interpretation of pollen analytical results, originating from plant morphological differences, variable ratios of pollen and nectar from different plant species, the activity of the bees, or even honey processing and filtration as well as new plant cultivars and sources such as honeydew without any relationship with pollen production, lead to the search for new analytical methods (13).

In the past decades near-infrared spectrometry (NIR) has become a rapid and well-established technique for the quantitative and qualitative analysis of food. It has been successfully applied in both transmission and transmittance modes to the quantitative analysis of honey. Accurate predictions were obtained for fructose, glucose, sucrose, maltose, water, and ash content as well as for the fructose/glucose and glucose/water

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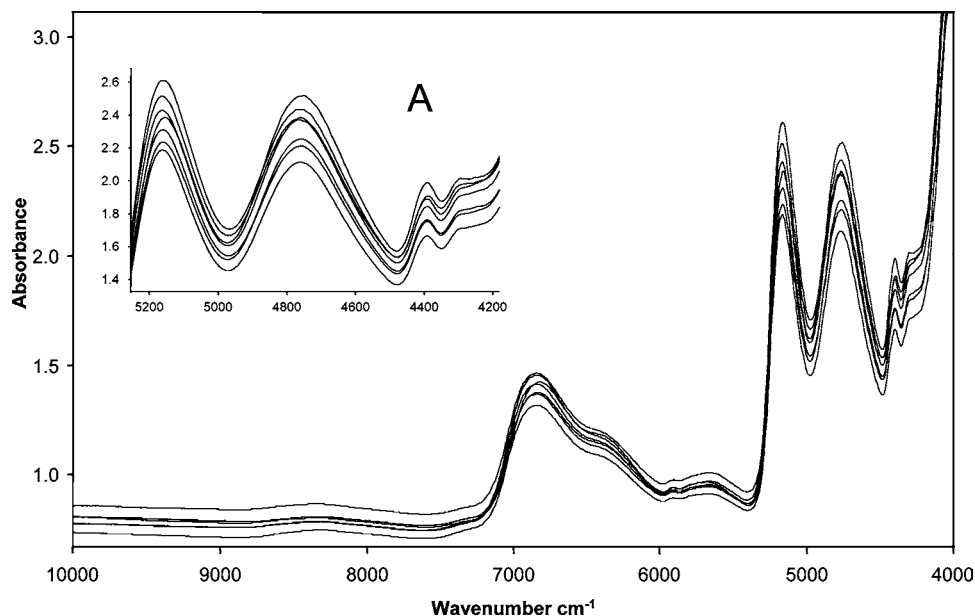


Figure 1. FT-NIR spectra of seven unifloral honey types [(A) enlargement of the region between 4160 and 5260  $\text{cm}^{-1}$ ].

ratios in honey samples from different crops (14–20). Furthermore, the physical characteristics of honey such as electrical conductivity, color, and polarimetric properties have also been successfully calibrated (20, 21).

The potential of NIR spectroscopy for the determination of the botanical origin of honey was recently evaluated using a reflectance probe (22). Principal component analysis (PCA) was used for data reduction. Linear discriminant analysis (LDA) was applied for the classification of the honey types studied. Over 80% of acacia, chestnut, and rape honeys were correctly assigned to the corresponding honey type on the basis of the spectra and Mahalanobis distance in cross-validation, whereas only a third of the heather honeys considered were correctly classified. Half of the samples of various other unifloral origins were incorrectly assigned to the groups mentioned above and the other half of the samples were not assigned to a group. However, the number of samples per honey type was very restricted as 13 different unifloral honeys from 9 European countries were studied on a total of only 51 samples. No discrimination into groups according to geographical origin was found (22). These encouraging preliminary results should be validated with a larger set of samples.

Although NIR spectroscopy would allow one to clearly discriminate between several types of unifloral honeys, this does not mean that the methodology will be useful in analytical practice because the great challenge in honey analytics is not to distinguish between several unifloral honey types but to discriminate the minority of  $\approx 20\%$  of unifloral honeys from the overwhelming majority of  $\approx 80\%$  of polyfloral honeys on the market. Unfortunately, polyfloral honeys have so far not been considered in most of the recently developed analytical methods proposed for the authentication of the botanical origin of honey (22–32).

The aim of the present work was to investigate eight unifloral and polyfloral honey types by using Fourier transform near-infrared (FT-NIR) spectroscopy in transfection mode in order to develop a rapid and reliable method for the authentication of unifloral and polyfloral honeys.

## MATERIALS AND METHODS

### Sampling and Botanical Classification by Reference Methods.

A total of 364 honey samples produced between 1998 and 2004 were collected and stored at 4 °C until analysis. They originated predominantly from Switzerland (CH); a few samples from Germany (D) were also included.

To classify these honey samples, the following measurands were determined according to the harmonized methods of the European Honey Commission (33): electrical conductivity, sugar composition, fructose/glucose ratio, pH value, free acidity, and proline content. Pollen analysis was carried out according to DIN 10760 (34, 35).

On the basis of these analytical results, the honey samples were assigned to one of the following eight honey types according to the criteria of Persano and Piro (1): acacia (*Robinia pseudoacacia*) (CH,  $n = 19$ ; D,  $n = 4$ ); alpine rose (*Rhododendron* spp.) (CH,  $n = 14$ ); sweet chestnut (*Castanea sativa*) (CH,  $n = 27$ ); rape (*Brassica napus* var. *oleifera*) (CH,  $n = 25$ ); fir honeydew (*Picea* spp. and *Abies* spp.) (CH,  $n = 52$ ); lime (*Tilia* spp.) (CH,  $n = 13$ ; D,  $n = 7$ ); dandelion (*Taraxacum* s.l.) (CH,  $n = 20$ ; D,  $n = 4$ ); and polyfloral honeys (CH,  $n = 179$ ). In the heterogeneous group of the polyfloral honeys nectar or honeydew contributions form all of the above-mentioned sources were represented.

**NIR Spectroscopy.** The honey samples were liquefied in a heating cabinet at 50 °C for 9 h and then allowed to cool to room temperature before analysis. NIR spectra were recorded using a Büchi NIRLab N-200 spectrometer equipped with an MSC 100 measuring cell with a rotating sample holder (Büchi Labortechnik AG, Flawil, Switzerland) to level out effects of sample inhomogeneity. About 10 g of liquefied honey was poured into a clean glass Petri dish and covered with the transfection plate, so defining a 0.3 mm layer of honey between the bottom of the Petri dish and its surface and acting as reflector. Sixty-four scans with a resolution of 4  $\text{cm}^{-1}$  were recorded in transfection mode for each spectrum in the wavenumber range between 4000 and 10000  $\text{cm}^{-1}$ . Figure 1 shows a typical FT-NIR spectrum of honey. Three replicates of each sample were averaged to one average spectrum. The repeatability was determined by a 10-fold measurement of the absorbance of a polyfloral honey sample.

**Processing of Spectra and Multivariate Analysis.** To exclude random variability resulting from instrumental effects, the following spectral range was used for multivariate analysis: 4112–9947  $\text{cm}^{-1}$ . After elimination of spectral outliers, PCA was applied to eliminate the spectral collinearity and to reduce the number of variables to 20 PCs (using GRAMS/32 AI with the PLSplus/IQ Add-on, vs. 5.09, Thermo Galactic, Salem, NH).

**Table 1.** Jackknife Classification and Validation Tables for the Honey Samples As Classified by LDA (All Honey Types Considered Separately)

	acacia	alpine rose	fir honeydew	chestnut	dandelion	lime	rape	polyfloral	correct
Jackknife Classification Rate (%)									
acacia ( <i>n</i> = 20)	85	10	0	0	0	0	0	5	85
alpine rose ( <i>n</i> = 11)	9	45	0	0	0	27	9	9	45
fir honeydew ( <i>n</i> = 49)	0	0	90	0	2	2	0	6	90
chestnut ( <i>n</i> = 26)	0	0	0	96	0	4	0	0	96
dandelion ( <i>n</i> = 23)	0	9	0	0	39	9	17	26	39
lime ( <i>n</i> = 18)	0	11	0	0	0	44	0	44	44
rape ( <i>n</i> = 24)	0	4	0	0	29	0	63	4	63
polyfloral ( <i>n</i> = 172)	3	4	9	9	10	3	14	48	48
								weighted av	60
Classification Rate in Validation (%)									
acacia ( <i>n</i> = 7)	71	14	0	0	0	0	0	14	71
alpine rose ( <i>n</i> = 3)	0	100	0	0	0	0	0	0	100
fir honeydew ( <i>n</i> = 16)	0	0	88	0	0	0	0	13	88
chestnut ( <i>n</i> = 8)	0	13	0	75	0	13	0	0	75
dandelion ( <i>n</i> = 7)	0	29	0	0	29	14	0	0	29
lime ( <i>n</i> = 6)	0	17	0	0	0	83	0	0	83
rape ( <i>n</i> = 8)	0	0	0	0	50	0	50	0	50
polyfloral ( <i>n</i> = 57)	9	19	7	5	9	32	0	19	19
								weighted av	45

In LDA, the 20 initial PCs were further reduced by backward elimination on the basis of their partial *F* values in the discriminant models (SYSTAT version 11, Systat Software Inc., Point Richmond, CA). The validation was accomplished with spectra of a third of the samples selected randomly and not present in the group of samples used to build the model.

## RESULTS AND DISCUSSION

### NIR Spectra of Different Honey Types and Repeatability

**Limits.** The repeatability limit ( $r_{IR}$ ) of the FT-NIR measurements was calculated on the basis of 10 subsequent analyses of different aliquots of the same polyfloral honey sample determined at the maximum absorbance at  $4761\text{ cm}^{-1}$ . The average of the maximum intensity of 2.236 au, a standard deviation of 0.069, a coefficient of variation of 3.1%, and a  $r_{IR}$  of 0.195 were found, indicating a satisfying repeatability of the method.

The NIR spectra of the seven unifloral honeys studied are shown in **Figure 1**. Each spectrum displayed is a typical individual spectrum of the given honey type. Visible to the naked eye are mostly differences in absorbance intensity. Characteristic differences in shape were observed between 4200 and  $7100\text{ cm}^{-1}$ . The largest variation among the spectra of the honey types considered were observed in C–O and C–C stretching regions of the saccharides between 4200 and  $5200\text{ cm}^{-1}$  (**Figure 1A**).

**LDA.** When LDA was performed on the eight different honey types, only chestnut and fir honeydew honeys were correctly classified with a rate of  $\geq 90\%$  in jackknife classification (**Table 1**). Some of the acacia honey samples were misclassified as alpine rose or polyfloral honeys, but were nevertheless correctly classified to 85%. Generally, a considerable number of samples were misclassified to groups of unifloral and polyfloral honeys, showing rates of correct classification of only 39–63% in jackknife classification. Dandelion honey showed with 39% the lowest jackknife classification rate. The samples were predominantly misclassified to polyfloral and rape honeys. Rape honey samples were vice versa often misclassified as dandelion honeys, which resulted in a jackknife classification rate of only 63%. Nectar contributions from dandelion and rape are prevalent in Swiss blossom honeys and may explain the misclassifications between polyfloral, rape, and dandelion honeys. Lime honeys showed with 44% a low rate of correct classification as well.

Nearly half of the lime honey samples were assigned to the polyfloral honeys. This may be explained by the variable chemical composition of this honey type as it often contains different amounts of honeydew and thus nonuniform physical and chemical characteristics, similar to polyfloral honeys containing nectar and honeydew.

In validation the classification rates for all honey types diminished even more for all honey types except alpine rose and lime honeys. Probably this was due to the small number of samples in validation that happened to be very characteristic. Only 19% of the polyfloral honeys were correctly classified; samples were misclassified to all groups except rape honey. Especially the high rate of misclassification of the polyfloral honeys into the groups of unifloral honeys makes it impossible to use the developed model for the determination of the eight unifloral and polyfloral honey types studied. The results show that NIR spectra contain too little information for a discrimination of most of the honey types considered.

If only unifloral honeys were considered for classification, all of the honey types studied showed correct classification rates in jackknife classification and validation of  $> 80\%$  except for dandelion (43%) and rape honey (63%) (detailed results are not shown). These findings indicate that analytical methods considering only the unifloral honeys (see Introduction) are too optimistic.

The observation that acacia, chestnut, and fir honeydew honeys could be nevertheless distinguished from the other unifloral and polyfloral honeys led to the idea to reduce the model to just four groups including acacia, chestnut, and honeydew honeys and a so-called pooled group combining samples of polyfloral, alpine rose, lime, rape, and dandelion honeys. The LDA carried out showed that the above-mentioned unifloral honeys could be well distinguished from the samples of the pooled group (**Table 2**). The classification rates for the three unifloral honeys were considerably higher compared to the ones found for the model considering all honey types as separate groups (**Table 1**). The rates were similar in jackknife classification and validation, indicating that these models were robust. Again, the unifloral honeys could be well distinguished from each other by this overall model. Misclassifications happened only between the pooled group and the unifloral honeys.

**Table 2.** Jackknife Classification and Validation Tables for the Honey Samples As Classified by LDA (Samples of Dandelion, Alpine Rose, Lime, Rape, and Polyfloral honeys Were Combined in the Pooled Group)

	acacia	fir honeydew	chestnut	pooled group	correct
Jackknife Classification Rate (%)					
acacia ( <i>n</i> = 20)	95	0	0	5	95
fir honeydew ( <i>n</i> = 49)	0	92	0	8	92
chestnut ( <i>n</i> = 26)	0	0	96	4	96
pooled group ( <i>n</i> = 248)	3	7	6	84	84
				weighted av	87
Classification Rate in Validation (%)					
acacia ( <i>n</i> = 7)	86	0	0	14	86
fir honeydew ( <i>n</i> = 16)	0	88	0	13	88
chestnut ( <i>n</i> = 8)	0	0	88	13	88
pooled group ( <i>n</i> = 81)	7	5	9	79	79
				weighted av	81

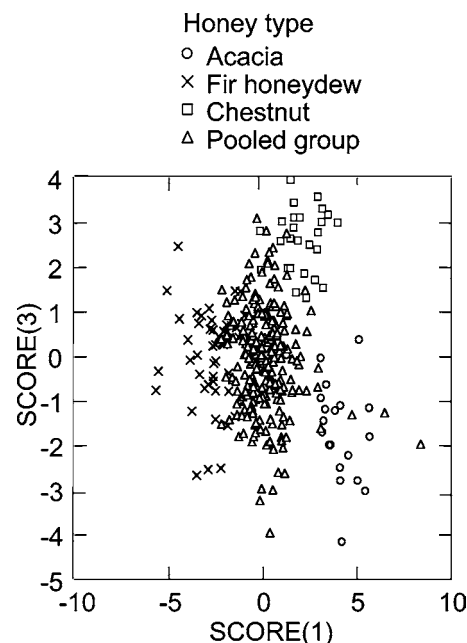
**Table 3.** Jackknife and Validation Tables for the Honey Samples Classified by the Two-Group Discriminant Models

	jackknife classification				validation			
	unifloral		non-unifloral		unifloral		non-unifloral	
	<i>n</i>	correct classification (%)	<i>n</i>	correct classification (%)	<i>n</i>	correct classification (%)	<i>n</i>	correct classification (%)
acacia	20	95	323	96	7	86	81	93
fir honeydew	49	92	294	94	16	94	81	91
chestnut	26	100	317	93	8	100	81	85
pooled group	248	79	95	87	81	65	31	84

**Table 4.** Error Probabilities for the Classification of Acacia, Chestnut, and Fir Honeydew honeys and Samples Belonging to the Pooled Group, Calculated by Bayes' Theorem

honeytype	error probability	
	jackknife	validation
acacia	0.026	0.048
fir honeydew	0.036	0.048
chestnut	0.035	0.063
pooled group	<10 <sup>-3</sup>	0.001

The results in jackknife classification and validation (**Table 2**) revealed that honeys from the pooled group were often classified into the groups of acacia, chestnut, and honeydew honeys. This observation led to the development a two-step procedure. In the first step the samples were classified to one of the four groups by an overall discriminant model. In the second step this classification was verified by using several 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. 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 four two-group models (fields with italic numbers in **Table 2**) were used when a misclassification rate of >3% was calculated in jackknife classification or validation tables of the overall model. The probabilities for misclassification were calculated by applying Bayes' theorem on the conditional probabilities of disjoint events. The error probabilities cannot be directly taken from **Table 2**; they quantify only the conditional probabilities of correct classification given the corresponding honey type. By Bayes' theorem was calculated the posterior probability of finding the correct honey type given a distinct classification by

**Figure 2.** Scatterplot of the canonical discriminant scores.

the discriminant model, and the error rate is simply the complement to 1. The classification rates for the unifloral honeys in the two-group models were >90% (**Table 3**). 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 of these three unifloral honey types can be reliably determined according to this procedure. The classification rate for the samples of the pooled group was with 79 and 65%, respectively, considerably lower. However, this is not very important, as we are principally interested in the authentication of unifloral honeys and the correct classification rate of 87 and 84%, respectively, shows that unifloral honeys are rarely assigned to the pooled group.

If a sample is assigned to the same honey type by the overall and the two-group models, it is very likely that it belongs to this type of honey. If the classifications of the two models do not agree, the sample has to be considered to belong to the pooled group. When the sample is assigned to the same honey type by both the overall model and the corresponding two-group model and is, moreover, considered to belong to the non-unifloral groups in all of the other two-group models tested, the honey sample belongs almost certainly to the honey type indicated by the overall model. The respective error rates of this two-step procedure were calculated by using Bayes' theorem.

Indeed, the approach in two steps allowed further improvement in the reliability in discrimination of acacia, fir honeydew, and chestnut honeys from the other honey types considered in the pooled group. The error probabilities calculated by using Bayes' theorem (misclassification of a sample of unknown botanical origin) were found to be generally <6% (**Table 4**). NIR spectroscopy can therefore be used for the determination of acacia, chestnut, and honeydew honeys. The display of the first and third linear discriminant scores shows that these three unifloral honeys form distinct groups that do not overlap at all. However, some overlap occurs between the unifloral honeys and samples of the pooled group (**Figure 2**). The interference of the samples of the pooled group, especially of the polyfloral honeys, with the unifloral honeys is characteristic and may be explained by their similar physical and chemical compositions.

**Table 5.** Jackknife Classification and Validation Tables for Blossom and Fir Honeydew Honeys As Classified by LDA

	blossom	fir honeydew
Jackknife Classification Rate (%)		
blossom ( $n = 294$ )	94	6
fir honeydew ( $n = 49$ )	8	92
Classification Rate in Validation (%)		
blossom ( $n = 96$ )	93	7
fir honeydew ( $n = 16$ )	6	94

According to the current standards (2, 3) honeys can be classified into blossom and honeydew honeys according to the electrical conductivity (honeydew honeys having values  $>0.8$  mS  $\text{cm}^{-1}$ ). However, some blossom honey types, for example, lime, chestnut, and heather honeys, are excluded from these classifications although expressing conductivity values  $>0.8$  mS  $\text{cm}^{-1}$ . Therefore, there is a need for alternative methods for the discrimination between blossom and honeydew honeys.

When the same samples were assigned to only two groups, that is, into blossom and fir honeydew honeys, the samples were correctly classified at rates of  $>90\%$  both in jackknife classification and validation (Table 5). NIR spectroscopy seems therefore to present a promising approach for the determination of the two main honey types.

This study shows that NIR spectroscopy combined with chemometrics offers a promising approach for the authentication of certain unifloral honeys and that the problems related to the determination of the polyfloral honeys can be handled by the successive use of at least two mathematical models. The methodology permits the discrimination of acacia, chestnut, and fir honeydew honeys, expressing the most characteristic chemical compositions among the honey types studied. This means that NIR spectroscopy and the mathematical models developed agree with the characterization based on the classical criteria for the above-mentioned honey types.

However, the recorded NIR spectra generally show too small specific characteristics to allow a determination of the botanical origin of the eight unifloral and polyfloral honey types studied. The potential of the method could possibly be improved by measurement in transmission mode with a shorter path length where sharper bands and less saturated spectra in the region between 4000 and 7500  $\text{cm}^{-1}$  nm were obtained (16).

Another way to gain more specific information would be to use an instrument scanning the spectrum from the visible to the near-infrared regions as color measurements have been shown to be useful for the authentication of some types of honey (24, 36). However, this approach may not help to solve problems related to the main obstacle in the determination of the botanical origin of honey, the discrimination between polyfloral and unifloral honeys, because the color of polyfloral honeys is highly variable.

In addition to the possibility to determine the botanical origin of honey, the same spectra can be used to obtain quantitative information on several measurands important for routine quality control. Using partial least-squares regression models, calibrations proved satisfying accuracies for the determination of water, glucose, fructose, sucrose, the total monosaccharide contents as well as the fructose/glucose and glucose/water ratios (37).

A drawback of the current method is that before the botanical origin can be determined routinely, a considerable amount of work has to be carried out to build the chemometric models involved. The possibility of transferring the corresponding

models or the spectra between different instruments and laboratories should be verified by future studies.

In conclusion, the results demonstrate that NIR spectrometry is a valuable, rapid, and nondestructive tool for the determination of the botanical origin of some honey types and for the quantitative analysis of measurands related to the main components in honey.

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