CHAPTER 5

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

ABSTRACT

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 applying principal component analysis (PCA) and linear discriminant analysis (LDA). The corresponding error rates were calculated by 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 lower than 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 non-destructive tool for the authentication of the above mentioned honeys, but not for all varieties studied.

5.1 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, flavour and colour varies 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 predominantly 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 contrary to unifloral honeys the polyfloral honeys do not express distinct physical or chemical characteristics apart from a huge variability, which makes their authentication particularly difficult.

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Ruoff, K.; Luginbühl, W.; Bogdanov, S.; Bosset, J. O.; Estermann, B.; Ziolko, T.; Amadò, R. Authentication of the Botanical Origin of Honey by Near-Infrared spectroscopy. *J. Agric. Food Chem.* **2006**, 54, 6867-6872.

The interest in the production of unifloral honeys is 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 also account for the requirement of reliable determination of the botanical origins (*7-10*).

Up to now a reliable authentiction of the botanical origin can only be achieved by experts by a global interpretation of sensory, pollen and physico-chemical 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, filtration as well as new plant cultivars and sources such as honeydew without any relationship with pollen production, lead to search for new analytical methods (*13*).

In the last decades near-infrared spectrometry (NIR) has become a rapid and well established technique for quantitative and qualitative analysis of food. It has been successfully applied both in transmission and transflectance modes to the quantitative analysis of honey. Accurate predictions were obtained for fructose, glucose, sucrose, maltose, water and ash contents as well as for the fructose/glucose and glucose/water ratios in honey samples from different crops (*14-20*). Furthermore physical characteristics of honey such as electrical conductivity, colour and polarimetric properties have also been successfully calibrated (*20-21*).

The potential of near-infrared 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 (*Robinia pseudoacacia*), chestnut (*Castanea sativa*) and rape (*Brassica* spp.) honeys were correctly assigned to the corresponding honey type on the basis of the spectra and mahalanobis distance in cross-validation, while only a third of the hea-ther (*Calluna vulgaris*) 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 only 13 different unifloral honeys from nine 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 near-infrared spectroscopy would allow 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 approximately 20 % of unifloral honeys from the overwhelming majority of about 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 FT-NIR spectroscopy in transflection mode in order to develop a rapid and reliable method for the authentication of unifloral and polyfloral honeys.

5.2 MATERIALS AND METHODS

5.2.1 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 harmonised 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*).

Based on 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), chestnut (*Castanea sativa*) (CH, n = 27), rape (*Brassica* spp.) (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 heterogenous group of the polyfloral honeys nectar or honeydew contributions from all of the above-mentioned sources were represented.

5.2.2 NEAR-INFRARED 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 a 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 transflection plate so defining a 0.3 mm layer of honey between the bottom of the Petri dish and its surface and acting as reflector. 64 scans with a resolution of 4 cm^{-1} were recorded in transflection mode for each spectrum in the wavenumber range between 4000-10000 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 ten-fold measurement of the absorbance of a polyfloral honey sample.

5.2.3 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 cm^{-1}. 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 GRAMS/32 AI with the PLSplus/IQ Add-on, Vs. 5.09, Thermo Galactic, Salem NH, U.S.A.).

In LDA, the 20 initial PC's were further reduced by backwards elimination on the basis of their partial F-values in the discriminant models (SYSTAT® Version 11, Systat Software Inc., Richmond, USA). 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.

5.3 RESULTS AND DISCUSSION

5.3.1 NIR-SPECTRA OF DIFFERENT HONEY TYPES AND REPEATABILITY LIMITS

The repeatability limit (r_{IR}) of the FT-NIR measurements was calculated based on 10 subsequent analyses of different aliquots of the same polyfloral honey sample determined at the maximum absorbance at 4761 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 satifying repeatability of the method.

The near-infrared 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 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 cm^{-1} (**Figure 1**, enlargement A).

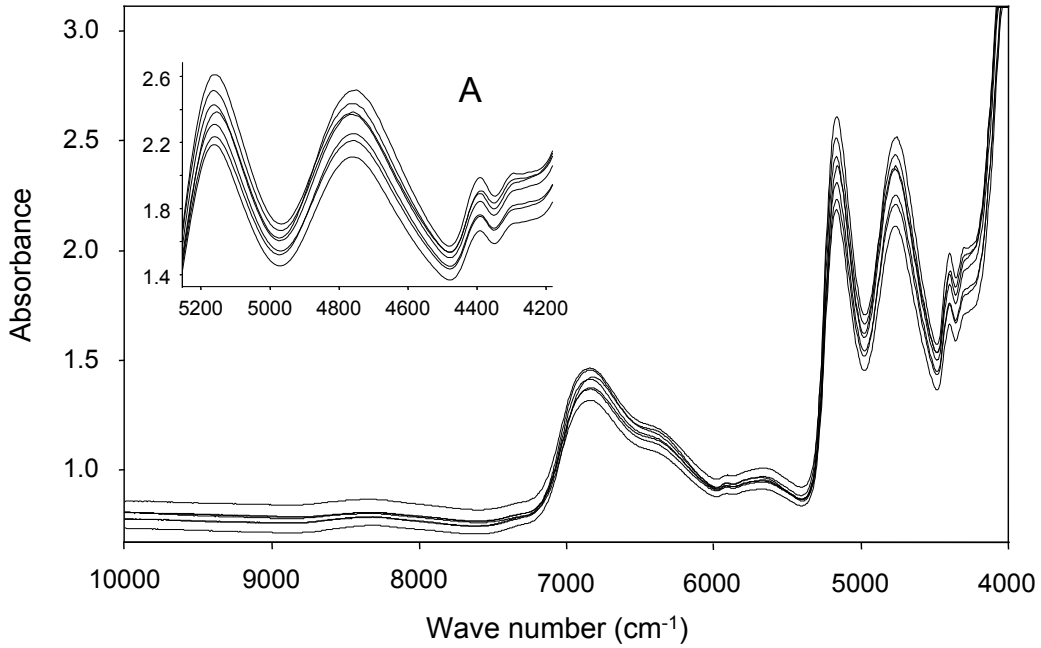

Figure 1. FT-NIR spectra of 7 different honey types (A: enlargement of the region between 4160 and 5260 cm^{-1})

5.3.2 LINEAR DISCRIMINANT ANALYSIS

When LDA was performed on the eight different honey types only chestnut and fir honeydew honeys were correctly classified with a rate of 90 % or higher 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.

Table 1. Jackknife classification and validation tables for the honey samples as classified by LDA (all honey types considered separately).

	Jackknife classification rate (%)								
	Acacia	Alpine rose	Fir honeydew	Chestnut	Dandelion	Lime	Rape	Polyfloral	Correct
Acacia (n = 20)	85	10	0	0	0	0	0	5	85
Alpine rose (n = 11)	9	46	0	0	0	27	9	9	45
Fir honeydew (n = 49)	0	0	90	0	2	2	0	6	90
Chestnut (n = 26)	0	0	0	96	0	4	0	0	96
Dandelion (n = 23)	0	9	0	0	39	9	17	26	39
Lime (n = 18)	0	11	0	0	0	44	0	44	44
Rape (n = 24)	0	4	0	0	29	0	63	4	63
Polyfloral (n = 172)	3	4	9	9	10	4	14	48	48
								Weighted average	60

	Classification rate in validation (%)								
	Acacia	Alpine rose	Fir honeydew	Chestnut	Dandelion	Lime	Rape	Polyfloral	Correct
Acacia (n = 7)	71	14	0	0	0	0	0	14	71
Alpine rose (n = 3)	0	100	0	0	0	0	0	0	100
Fir honeydew (n = 16)	0	0	88	0	0	0	0	13	88
Chestnut (n = 8)	0	13	0	75	0	13	0	0	75
Dandelion (n = 7)	0	29	0	0	29	29	14	0	29
Lime (n = 6)	0	17	0	0	0	83	0	0	83
Rape (n = 8)	0	0	0	0	50	0	50	0	50
Polyfloral (n = 57)	9	19	7	5	9	32	0	19	19
								Weighted average	45

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 except for 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 higher than 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 jackknifed 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 only happened between the pooled group and the unifloral honeys.

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 fir honeydew honeys. This observation lead 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 (indicted by bold numbers in **Table 2**) were used when a misclassification rate of higher than 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.

Table 2. Jackknife classification and validation tables for the honey samples as classified by LDA (the samples of dandelion, alpine rose, lime, rape and polyfloral honeys were combined in the pooled group).

	Jacknife classification rate (%)				
	Acacia	Fir honedew	Chestnut	Pooled group	Correct
Acacia (n = 20)	95	0	0	**5**	95
Fir honeydew (n = 49)	0	92	0	**8**	92
Chestnut (n = 26)	0	0	96	**4**	96
Pooled group (n = 248)	**3**	**7**	**7**	84	84
				Weighted average	87

	Classification rate in validation (%)				
	Acacia	Fir honeydew	Chestnut	Pooled group	Correct
Acacia (n = 7)	86	0	0	**14**	86
Fir honeydew (n = 16)	0	88	0	**13**	88
Chestnut (n = 8)	0	0	88	**13**	88
Pooled group (n = 81)	**7**	**5**	**9**	79	79
				Weighted average	81

The error probabilities cannot be directly taken from **Table 2**, they only quantify the conditional probabilities of correct classification given the corresponding honey type. By Bayes' theorem the posterior probabilities of finding the correct honey type given a distinct classification by the discriminant model was calculated, and the error rate is simply the complement to 1. The classification rates for the unifloral honeys in the two-group models were higher than 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 by this procedure. The classification rate for the samples of the pooled group was with 79 % respectively 65 % 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 % respectively 84 % 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 model 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 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 Bayes' theorem.

Table 3. Jackknife and validation table for the honey samples classified by the two-group discriminant models

	Jackknife classification				Validation			
	Unifloral		Non-Unifloral		Unifloral		Non-Unifloral	
	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)	n	Correct class. (%)
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

Indeed, the approach in two steps allowed to further improve 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 using Bayes' theorem (misclassification of a sample of unknown botanical origin) were found to be generally lower than 6 % (**Table 4**). Near-infrared spectroscopy can therefore be used for the determination of acacia, chestnut and honeydew honeys. The display of the first and the 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 composition.

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.

	Error probability	
Honeytype	Jackknife	Validation
Acacia	0.022	0.045
Fir honeydew	0.031	0.044
Chestnut	0.030	0.058
Pooled group	$< 10^{-3}$	0.001

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 >0.8mScm^{-1}). However some blossom honey types e.g. lime, chestnut and heather honeys are excluded from these classifications although expressing conductivity values >0.8mScm^{-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, i.e. into blossom and fir honeydew honeys, the samples were correctly classified at rates of over 90 % both in jacknifed classification and validation (**Table 5**). Near-infrared spectroscopy seems therefore to present a promising approach for the determination of the two main honey types.

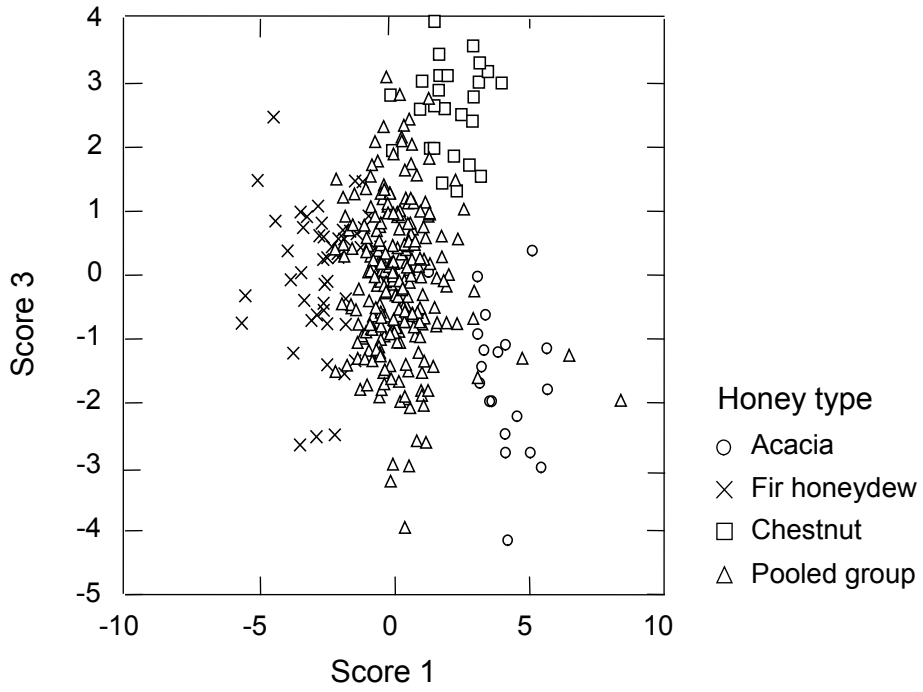

Figure 2. Scatterplot of the canonical discriminant scores

Table 5. Jackknife classification and validation tables for blossom and fir honeydew honeys as classified by LDA

	Jackknife classification rate (%)	
	Blossom	Fir honeydew
Blossom (n = 294)	94	6
Fir honeydew (n = 49)	8	92

	Classification rate in validation (%)	
	Blossom	Fir honeydew
Blossom (n = 96)	93	7
Fir honeydew (n = 16)	6	94

This study shows that near-infrared 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 to discriminate acacia, chestnut and fir honeydew honeys, expressing the most characteristic chemical compositions among the honey types studied. This means that near-infrared spectroscopy and the mathematical models developed agree with the characterisation 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 measuring in transmission mode with a shorter path length where sharper bands and less saturated spectra in the region between 4000 and 7500 cm^{-1} nm were obtained (*16*).

Another way to gain more specific information would be to use an instrument scanning the spectrum from visible to the near infrared region as colour 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 colour 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 the 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 routinely determined , a considerable amount of work has to be carried out to build the chemometric models involved. The possibility to transfer the corresponding models or the spectra between different instruments and laboratories should be verified by future studies.

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

ACKNOWLEDGEMENT

The authors thank the Swiss Federal Office of Public Health, Narimpex AG, and the Swiss Beekeepers' Association for their financial support. Mrs. Birgit Lichtenberg-Kraag is acknowledged for the supply of honey samples from Germany.

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