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^{1}H Nuclear Magnetic Resonance Spectra of Chloroform Extracts of Honey for Chemometric Determination of Its Botanical Origin

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In this work, we present a new NMR study, coupled with chemometric analysis, on nonvolatile organic honey components. The extraction method is simple and reproducible. The ^{1}H NMR spectra of chloroform extracts acquired with a fast and new pulse sequence were used to characterize and differentiate by chemometric analysis 118 honey samples of four different botanical origins (chestnut, acacia, linden, and polyfloral). The spectra collection, processing, and analysis require only 30 min. The ^{1}H spectrum provides a fingerprint for each honey type, showing many characteristic peaks in all spectral regions. Principal component analysis (PCA) and projection to latent structures by partial least squares-discriminant analysis (PLS-DA) were performed on selected signals of the spectra to discriminate the different botanical types and to identify characteristic metabolites for each honey type. A distinct discrimination among samples was achieved. According to the distance to model criterion, there was no overlap between the four models, which proved to be specific for each honey type. The PLS-DA model obtained has a correlation coefficient R^2 of 0.67 and a validation correlation coefficient Q^2 of 0.77. The discriminant analysis allowed us to classify correctly 100% of the samples. A classification index can be calculated and used to determine the floral origin of honey as an alternative to the melissopalinology test and possibly to determine the percentage of various botanical species in polyfloral samples. Preliminary data on the identification of marker compounds for each botanical origin are presented.

KEYWORDS: Honey; floral origin; NMR; multivariate statistical analysis

INTRODUCTION

Honey is defined as "the natural sweet substance produced by *Apis mellifera* bees from the nectar of plants or from secretions of living parts of plants or excretions of plant-sucking insects on the living parts of plants, which the bees collect, transform by combining with specific substances of their own, deposit, dehydrate, store, and leave in honeycombs to ripen and mature" (*1*). There have been many reported beneficial effects from the use or consumption of honey, including antimicrobial properties (*2*, *3*), antioxidant effects (*4*, *5*), wound healing effects (*6*), and prevention of life-threatening pathologies such as diabetes, cardiovascular deseases (*7*), and cancer (*8*). Honeybees and their products can also be employed as potential bioindicators of environmental contamination (*9*).

The composition and properties of a particular honey sample depend strongly on the type of flowers visited by the bees, as well as on the climatic conditions in which the plants grow and on contributions of the beekeeper (*10*, *11*).

The Codex Alimentarius Standard (*12*) and the European Union Council Directive (*1*) specify that the term "honey" may be completed by a reference to the origin, whether blossom or plant, provided the product comes predominantly from the indicated source and has the appropriate organoleptic, physicochemical, and microscopic properties corresponding to that origin.

The interpretation of "predominantly" remains ambiguous, and the definition of unifloral or polyfloral nowadays is not based on physical−chemical parameters but on melissopalynological analysis. Usually, honey is considered unifloral when the pollen frequency of one plant is over 45% (*13*). For honey samples with under-represented pollen grains (e.g., lavender, citrus, and rosemary) botanical classification may be achieved with a percentage pollen frequency of only 10−20%. Melissopalynology, i.e., the identification and quantification of pollen grains contained in honey, has been traditionally used to ascertain the botanical origin of honeys, although with some limitations (*14*). Specifically, melissopalynology requires trained analysts and the previous knowledge of pollen morphology. In spite of these problems, palynological analysis remains the reference method nowadays.

In the last few decades, specific chemical and physical properties of honey have been used to determine its botanical origin (*14*−*16*) and new analytical techniques have been proposed toward this aim. They are based, for example, on the determination of total flavonoids, profile of volatiles, and amino acid and carbohydrate composition.

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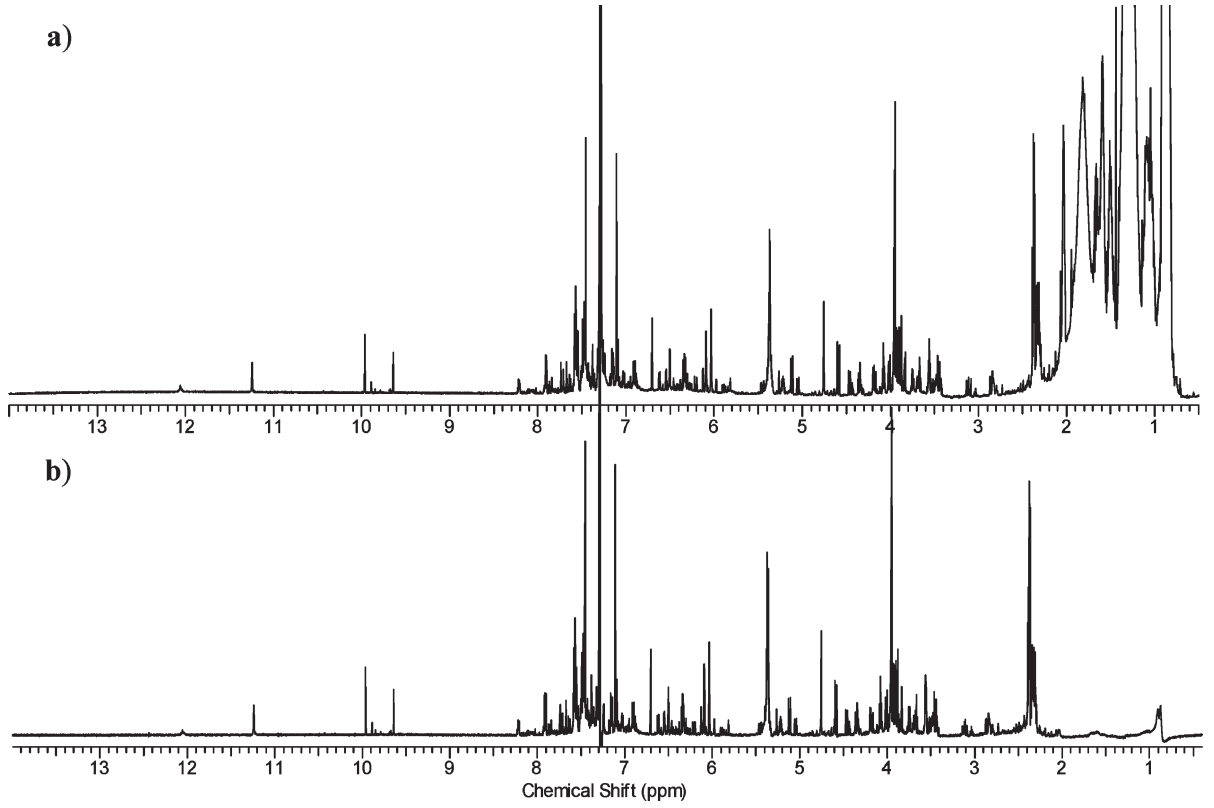

Figure 1. Comparison between complete 1D ^{1}H spectra of a honey sample obtained with (**a**) a single pulse sequence in 50 min and (**b**) the modified DPFGSE sequence in 25 min.

An improvement in the determination of the botanical origin can certainly be achieved by the application of the multivariate analytical approach. Several applications to honey classification have been reported (*17*−*21*). For example, attempts have been made to use some physical and chemical properties (*22*) or the mineral content (*23*) of honey. Although interesting results were obtained, most of these studies were based on a fairly limited number of honey samples or used a combination of parameters based on several independent measurements.

Recently, also nuclear magnetic resonance (NMR) techniques have been proposed to identify and classify honey of different floral sources (*24*, *25*) or geographic origins (*26*, *27*). One of the main advantages of this technique is that structural and quantitative information can be obtained on a wide range of chemical species in a single NMR experiment. NMR is frequently applied to food samples that can be directly examined as liquids (*28*), but very simple extraction or sample preparation procedures may also be used (*29*). In the case of honey, the botanical or geographical differentiations were based on different carbohydrate composition. Consonni and Cagliani (*27*) showed that this parameter can be used to distinguish honeys of different geographical origins and hinted at the possibility of separating acacia and polyfloral honeys. The ^{13}C spectrum was suggested to be the best probe of carbohydrate composition. Lolli et al. (*25*) classified samples of different botanical origins by using 2D HMBC experiments coupled with multivariate statistical analysis, by dissolving the samples in water or DMSO. In only one NMR study, conducted at 300 MHz, the solid-phase methanol extracts were analyzed (*24*). In this paper, we show that ^{1}H NMR spectra of organic extracts can be used as a "fingerprint" to differentiate the botanical origin of honey. The use of high fields also allows the identification and characterization of some of these compounds as botanical markers.

In this work, we present a new NMR study, coupled with chemometric analysis, on nonvolatile organic compounds. ^{1}H spectra of chloroform extracts of honey were analyzed with a fast and new pulse sequence (*30*). The extraction method is simple, and reproducible, and it yields an extract that has never been analyzed before. The advantage of this approach is to eliminate the compounds most present in the honey mixture, i.e., the carbohydrates, and to retain the aroma compounds and those hydrophobic substances that differ the most in honeys of various sources. Also, the extraction procedure yields a concentrated solution amenable to fast NMR analysis. A total of 118 samples of acacia, linden, chestnut, and polyfloral honeys were analyzed. By using principal component analysis (PCA) and supervised techniques (projection to latent structures by partial least squares-discriminant analysis, PLS-DA), a classification model according to floral origin was obtained, with high predictability power. A classification index can be calculated and used to determine floral origin of honey as an alternative to the melissopalinology test and possibly to determine the percentage of various botanical species in polyfloral samples.

During this analysis, we were able to isolate and identify some molecular markers of these botanical origins.

MATERIALS AND METHODS

Samples. A total of 118 honey samples of different botanical origins were analyzed. Among them, 93 were obtained directly from the producers with certified origin, while 25 were commercial products.

Samples of four different botanical origins were obtained from Veneto apiaries: 28 acacia (*Pseudoacacia robinia L.*), 23 chestnut (*Castanea sativa*), 22 linden (*Tilia spp.*), and 20 polyfloral honeys. Commercial samples included 7 acacia, 8 chestnut, 4 linden, 5 polyfloral, and 1 declared chestnut−linden honey.

Sample Preparation. Portions of samples (6 g) were weighted in a centrifuge tube and dissolved with 15 mL of deionized water. Fifteen milliliters of $CHCl_3$ were added, and the mixture was mechanically stirred for 10 min. The biphasic mixture was then centrifuged at 10 000 rpm for 15 min at 4 °C. The lower chloroform phase was collected, and the solvent

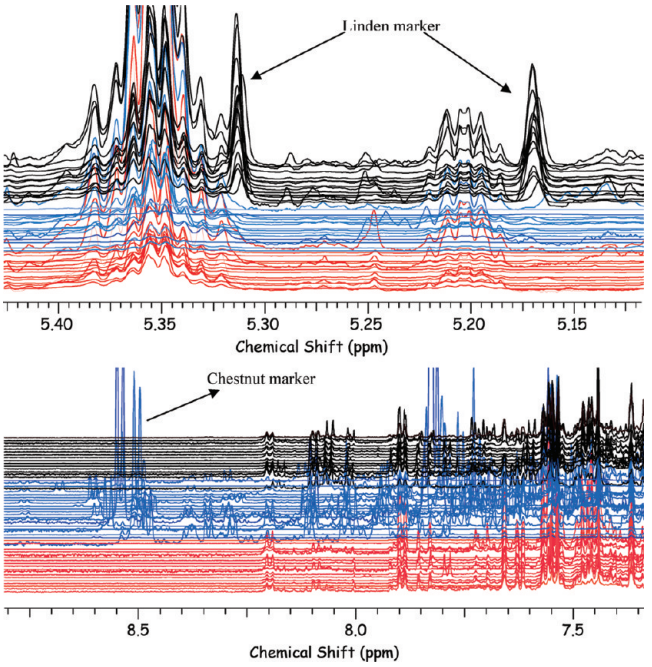

Figure 2. Tile plot of all spectra of chestnut, linden, and acacia honeys: region between 5.10 and 5.45 ppm (top) and between 7.2 and 9.0 ppm (bottom), with the arrows indicating proton signals of markers of linden and of chestnut. Red, blue, and black colors indicate acacia, chestnut, and linden honeys, respectively.

was evaporated under a gentle stream of nitrogen. The solid residue was dissolved in 600 μL of $CDCl_3$ and put in an NMR tube.

To identify markers from chestnut and acacia honey, separations were conducted using a silica gel column eluted with $CHCl_3$ and a gradient of 0−5% MeOH.

NMR Analysis. Spectra were recorded on a Bruker Avance 600 DMX instrument, operating at 600.09 MHz for ^{1}H and equipped with a 5 mm TXI *xyz* gradient inverse probe.

The 1D spectra were acquired using a modified double pulsed field gradient spin echoes (DPFGSE) sequence (*30*). Specifically, the cluster [*G*−*S*−*G*], where *G* represents a pulsed field gradient and *S* is a generic element (usually a 180° soft pulse), was modified by the addition of an inversion hard pulse after the first gradient (that is, $G-\pi-S-G$) and incorporating an inversion Reburp pulse of 2 kHz sweep width and 10 ms duration centered at 1 ppm. The introduction of a π pulse in the DPFGSE sequence allowed us to remove the highest signals present in the 0−2 ppm region. All gradient pulses were followed by a 100 μs recovery delay. The typical acquisition parameters of this experiment were as follows: temperature, 298 K; recycle time, 2 s; spectral window, 6000 Hz; number of scans, 256; data points, 32K; receiver gain, 8K. The parameters for the 1D spectra obtained with the standard single-pulse sequence were as follows: temperature, 298 K; recycle time, 2 s; spectral window, 6000 Hz; number of scans, 1024; data points, 32K; receiver gain, 256.

Data were processed using the ACD software (ACD/Specmanager 7.00 sofware, Advanced Chemistry Development Inc., 90 Adelaide Street West, Toronto, Ontario, Canada M5H 3V9). Fourier transformation was performed after zero-filling the FID data to 128K points and after apodization using a decreasing exponential with line broadening of 0.5 Hz. The spectra were phased and baseline-corrected using the ACD manual routine, and the ^{1}H NMR chemical shifts were referenced to the residual $CHCl_3$ signal at 7.27 ppm. Each ^{1}H spectrum was segmented into identical intervals ("buckets") of 0.04 ppm, and the signal intensity in each interval was integrated. The spectra were normalized to the total sum of integral covering the δ interval 13−1.8 and excluding the δ region 7.26−7.28, which contains the residual solvent peak. The resulting normalized integrals composed the data matrix that was submitted to multivariate analysis.

Identification of markers for each botanical origin was obtained through 1D and 2D spectra. The following parameters were used.

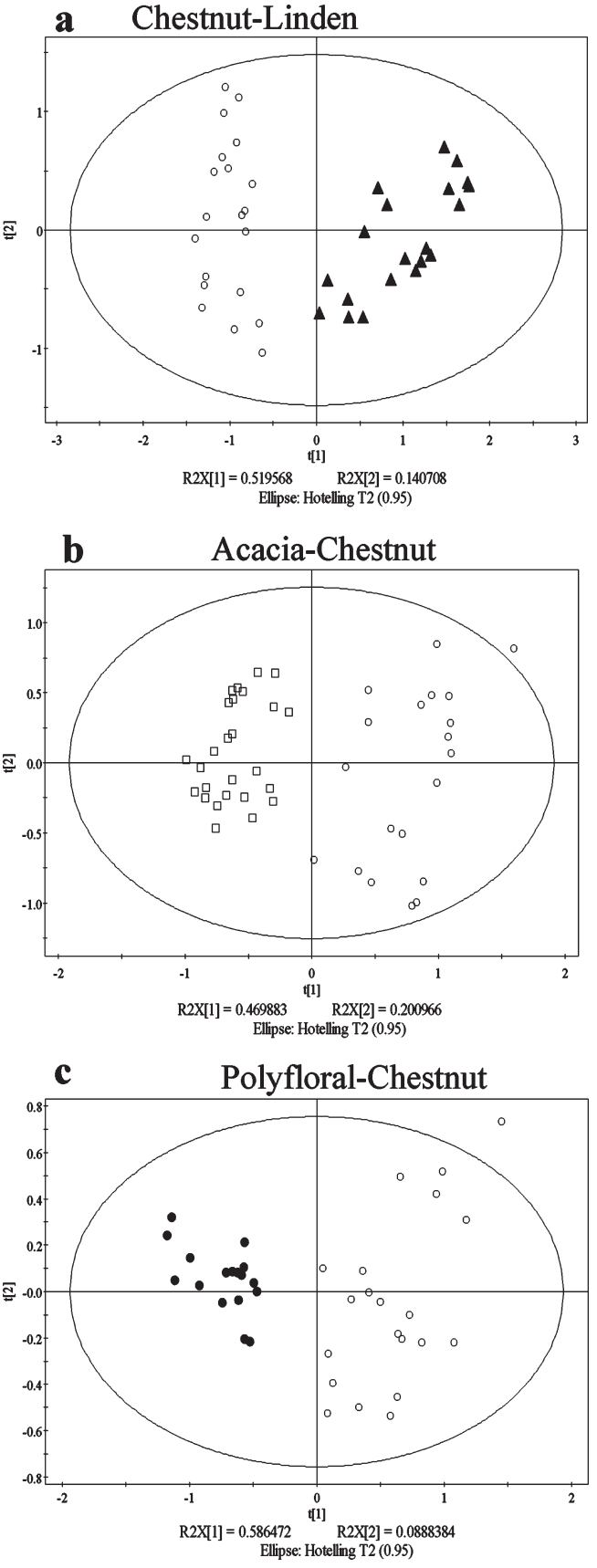

Figure 3. PCA score plots of the analysis performed on the 85 samples of the training set. Two botanical origins were compared at a time. As an example, the results obtained comparing chestnut honey with the other three types of honeys are reported: (**a**) chestnut−linden; (**b**) acacia−chestnut; (**c**) polyfloral−chestnut.

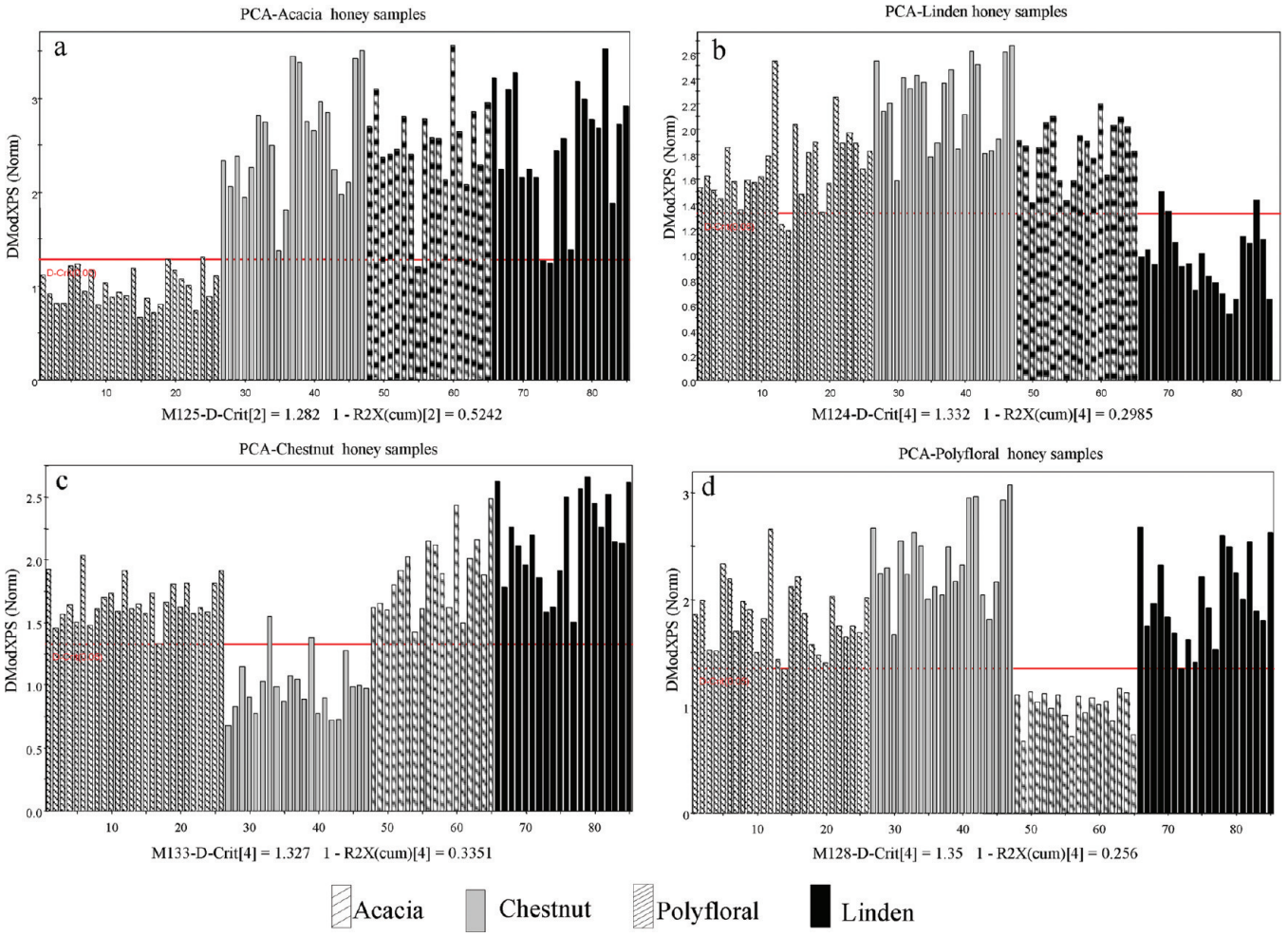

Figure 4. Distance to the model (DmodXPS) plots for the training set: (**a**) DModX plot taking acacia honey as a reference; (**b**) DModX plot taking linden honey as a reference; (**c**) DModX plot taking chestnut honey as a reference; (**d**) DModX plot taking polyfloral honey as a reference. The red lines represent the maximum tolerable distance (Dcrit) for the considered data set. Moderate outliers have DModX values larger than Dcrit.

(i) 1D selective TOCSY spectra were recorded with a 180° Gaussian shaped pulse of 79.8 ms, a TOCSY mixing time of 70 ms, 32 scans, and 16K data points (*31*).

(ii) $^{1}H-^{1}H$ TOCSY spectra were recorded in the time-proportional phase incrementation (TPPI) mode, with a spectral window of 10 ppm in both dimensions, 2048 × 512 data points, 2 s relaxation delay, 70 ms mixing time, and 32−128 scans.

(iii) $^{1}H-^{1}H$ COSY spectra were recorded in magnitude mode, with a spectral window of 10 ppm in both dimensions, 2048 × 512 data points, 2 s relaxation delay, and 16 scans.

(iv) $^{1}H-^{1}H$ NOESY spectra were recorded in the TPPI mode, with a spectral window of 10 ppm in both dimensions, 2048 × 512 data points, 2 s relaxation delay, 1.2 s mixing time, and 128−256 scans.

(v) HMQC spectra were recorded in the TPPI mode, with a spectral window of 10 ppm (^{1}H) and 220 ppm (^{13}C), 1 s relaxation delay, 1024 × 256 data points, and 128−256 scans.

(vi) HMBC spectra were recorded in the TPPI mode, with a spectral window of 10 ppm (^{1}H) and 220 ppm (^{13}C), 1 s relaxation delay, 1024 × 256 data points, and 600−800 scans.

Statistical Analysis. Principal component analysis (PCA) and PLS-DA using "mean centering" as data pretreatment (*32*) was conducted using the software SIMCA-P11 (Umetrics, Umea, Sweden).

Data were visualized by plotting either the PC scores, where each point in the score plot represents an individual sample, or the loading plot, which permits us to identify the spectral regions with the greatest influence on the separation and clustering of the samples and, therefore, to deduce which compounds are responsible for such clustering (markers).

To validate the robustness of the discrimination, the samples were divided into a training set and a test set. The latter was composed of two randomly selected samples from each botanical class and of the samples purchased in commercial stores for a total of 33 samples (i.e., 9 acacia, 10 chestnut, 6 linden, 7 polyfloral, and 1 declared chestnut−linden). The statistical analysis was performed on the training set composed of 85 samples: 26 acacia, 21 chestnut, 20 linden, and 18 polyfloral honeys.

SIMCA-P calculates normalized prediction distances of the samples to the models (DmodX) whose critical values (Dcrit) were computed with 0.95 confidence intervals. The distance of each sample to each of the PC models was computed and plotted in a DmodX plot (*33*). This approach was used to assess the classification performance of samples by predicting class membership and to evaluate the specificity of the models.

A PLS-DA model was also applied, separating the samples into classes according to their origin. The validity of the PLS-DA model was assessed using the correlation coefficient R^2 and the cross-validation correlation coefficient Q^2. The latter was derived using the default option of SIMCA-P. The same training set and test set were used as in the PCA.

RESULTS AND DISCUSSION

Spectral Analysis. Representative spectra of a honey chloroform extract are reported in **Figure 1**. The spectrum of **Figure 1a** was obtained with the standard single-pulse sequence in 50 min. The spectrum is dominated by signals in the 0−2 ppm region, which originate from protons of hydrocarbon chains belonging to high-molecular-weight *n*-alkanes or linear fatty acids already identified as honey constituents (*34*). These signals are not relevant for our study; they hide resonances of other molecules that may be markers

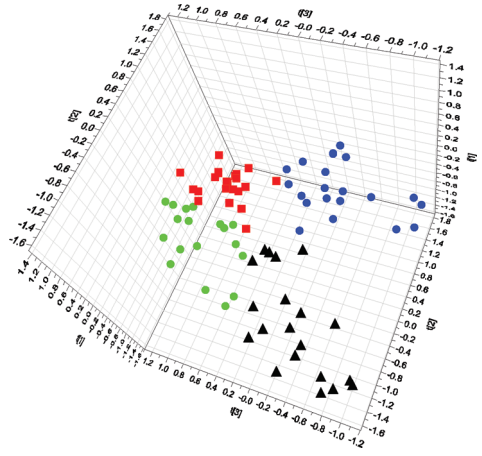

Figure 5. PLS-DA 3D score plot of the honey classification model. The axes of the plot are PLS-DA components 1−3. Red, blue, black, and green colors indicate acacia, chestnut, linden, and polyfloral honeys, respectively.

of botanical origin and, furthermore, limit the usable receiver gain value so that the very weak signals are inaccurately sampled and poor integrated intensities result. Removal of the strongest signals, by means of the sequence described in Materials and Methods (*30*), allowed an increase of the receiver gain, which resulted in shorter acquisition times, improved digitization of the small amplitude peaks, and lower integration errors.

A typical spectrum obtained with the modified DPFGSE sequence is reported in **Figure 1b**; it was obtained in only 25 min and has the same S/N ratio of the spectrum of **Figure 1a**.

The spectrum appears very crowded in the entire spectral range and provides global information about the complex extraction mixture, although deciphering the chemical content of such samples from the NMR data is less straightforward. In general, the advantage of NMR is that all types of compounds give rise to signals simultaneously, so that the NMR spectrum represents a fingerprint of the matrix under study. The choice of chloroform as a solvent offers great advantages compared to other solvents previously used in NMR studies of honey. The residual chloroform signal is very sharp and hides a very small region at 7.26 ppm which does not influence the analysis. On the other hand, solvents such as DMSO and MeOH are less suitable, since they present large signals in very important areas (around 3.4 ppm for MeOH and around 2.5 ppm for DMSO).

Superimposing the spectra of all the analyzed samples reveals the regions in which signals present only in one honey type are clearly visible. As an example, two expansions of the spectra are reported in **Figure 2**, in which signals exclusively from linden samples (top) and chestnut samples (bottom) appear.

To evaluate the differences between the various botanical origins, a chemometric analysis was carried out using SIMCA-P, as described in Materials and Methods.

Evaluation of the Discriminant Ability of the NMR Spectra. As a first step, an unsupervised approach by means of PCA was applied to the training set. PC analysis was performed, comparing two botanical origins at a time. The plots of the first two PCs of all the PCAs demonstrate that very good discrimination of honeys according to their botanical origins is reached. All R^2 and Q^2 values were over 70% and 60%, respectively. As an example, the comparisons of chestnut honey with the other honey types are reported in **Figure 3**.

This procedure provided the variables responsible for sample separation and produced a final data matrix with optimal sample clusterization.

Table 1. Classification List Reprojected onto the PLS-DA Models Performed by Considering All Training Set Samples.[a]

sample		probability of class membership			
botanical origin	geographic origin	acacia	chestnut	linden	polyfloral
		Training Set			
acacia	Veneto	**0.77**	−0.15	0.23	0.14
acacia	Veneto	**0.80**	0.09	0.08	0.03
acacia	Veneto	**0.97**	−0.03	−0.02	0.07
acacia	Veneto	**0.96**	−0.03	0.02	0.06
acacia	Veneto	**1.02**	0.30	0.12	−0.44
chestnut	Veneto	0.03	**1.20**	−0.15	−0.08
chestnut	Veneto	0.23	**0.91**	−0.10	−0.04
chestnut	Veneto	−0.08	**0.95**	−0.05	0.18
chestnut	Veneto	0.05	**0.61**	0.26	0.08
chestnut	Veneto	0.01	**1.16**	−0.12	−0.05
linden	Veneto	0.26	0.02	**0.90**	−0.18
linden	Veneto	0.02	0.08	**0.90**	−0.01
linden	Veneto	0.04	−0.06	**0.93**	0.08
linden	Veneto	−0.32	0.38	**0.97**	−0.03
linden	Veneto	−0.04	0.08	**0.74**	0.22
polyfloral	Veneto	0.13	0.11	−0.13	**0.90**
polyfloral	Veneto	−0.24	0.17	0.03	**1.05**
polyfloral	Veneto	−0.15	0.22	0.24	**0.69**
polyfloral	Veneto	0.07	0.05	0.00	**0.87**
polyfloral	Veneto	0.06	0.14	0.06	**0.74**
		Test Set			
acacia*	Veneto	**0.62**	−0.05	0.20	0.23
acacia*	Veneto	**1.04**	0.07	−0.16	0.05
acacia	Abruzzo	**0.86**	0.00	0.32	-0.18
acacia	France	**0.85**	−0.05	−0.24	0.44
acacia	Italy	**1.06**	−0.12	−0.12	0.18
acacia	France	**0.93**	−0.13	0.12	0.08
acacia	Veneto	**0.62**	−0.13	0.22	0.20
acacia	Italia	**0.53**	−0.11	0.10	0.49
acacia	Italy	0.43	−0.09	0.09	***0.57***
chestnut*	Veneto	−0.21	**0.87**	0.29	0.05
chestnut*	Veneto	−0.19	**0.71**	0.32	0.16
chestnut	Italy	−0.05	**1.47**	−0.43	0.02
chestnut	France	−0.08	**0.69**	0.12	0.27
chestnut	Italy	−0.17	**0.93**	−0.08	0.31
chestnut	Piedmont	−0.49	**1.10**	0.24	0.14
chestnut	Italy	−0.26	**1.01**	−0.03	0.28
chestnut	Veneto	0.04	**0.76**	0.22	-0.02
chestnut	Veneto	0.06	**1.14**	−0.27	0.08
chestnut	France	0.03	0.28	0.20	***0.48***
linden*	Italy	0.04	0.08	**0.70**	0.19
linden*	Veneto	0.54	−0.08	**0.99**	-0.45
linden	Italy	−0.04	0.05	**1.20**	-0.21
linden	Italy	−0.03	0.01	**0.80**	0.22
linden	France	0.30	0.35	**0.52**	0.42
linden	Italy	0.15	−0.1	0.40	***0.55***
polyfloral*	Veneto	0.00	0.02	0.20	**0.79**
polyfloral*	Veneto	0.09	0.12	0.05	**0.74**
polyfloral	Austria	0.33	0.08	0.16	**0.42**
polyfloral	Italy	0.48	0.00	−0.03	**0.55**
polyfloral	Italy	0.55	−0.12	−0.09	**0.66**
polyfloral	Lithuania	0.22	0.18	0.02	**0.62**
polyfloral	Veneto	0.24	0.25	0.12	**0.63**
chestnut−linden	Piedmont	−0.29	**0.89**	0.41	−0.01

[a] The results of only five training set samples per botanical origin are reported for clarity. The results of all test samples are reported. The eight test samples randomly selected from those obtained directly from the producers with certified origin are marked with an asterisk. The complete classification list is given in the Supporting Information.

By modeling each class separately through PCA, it became evident that each botanical origin is characterized by specific

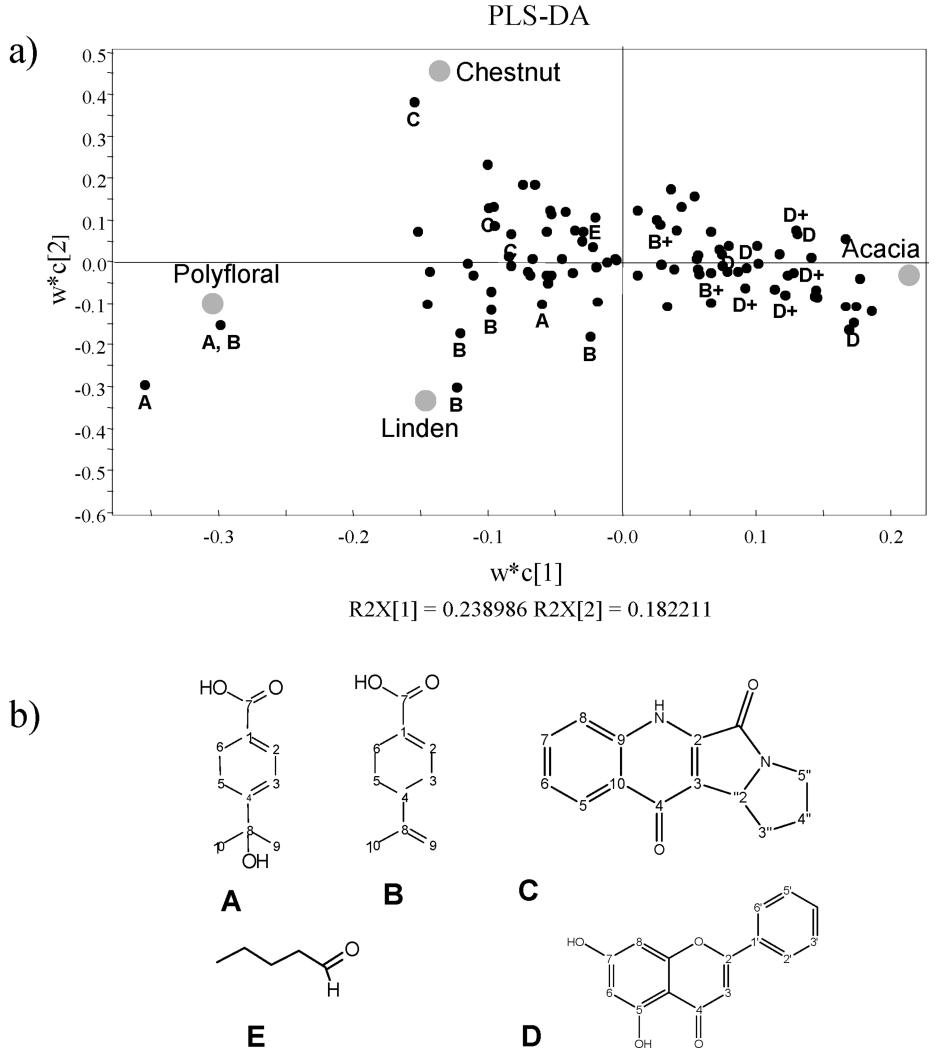

Figure 6. (**a**) PLS-DA weight plot for the 85 samples of the training set. The identified markers are indicated with letters. (**b**) Structures of identified markers.

resonances. The specificity of the models was demonstrated with the DmodX criterion and the critical value (Dcrit) with 95% confidence intervals. These parameters show the distance of each sample to the model in the X space (*33*).

In **Figure 4**, all the samples were compared using the Dcrit generated for each of them. We found that almost all acacia observations of the prediction set are predicted within the critical distance. Moreover, the other three species are far outside the tolerance interval of the acacia model. This means that the PCA model trained on acacia recognizes honeys of this botanical origin in the classification phase, and it is specific for this species. Few ambiguities exist (five false positive and four false negative results). In each of these cases, though, the correct classification is obtained by comparing all the models.

The good discrimination obtained with the PC analysis prompted us to perform a PLS-DA classification on the training test to derive a model with high prediction ability. The PLS-DA model obtained shows high discrimination, with $R^2 = 0.67$, $Q^2 = 0.77$, and $K = 0.87$ (**Figure 5**). The robustness of the PLS-DA model was evaluated using the classification list shown in **Table 1**. Each sample was classified by means of a "probability of class membership" indicative of its representativeness. The results of correct and ambiguous classifications obtained for both training and test set are displayed in **Table 1**: when the "probability of class membership" is larger than 0.5 (highlighted in bold), the object is considered correctly predicted; the samples incorrectly predicted are indicated in bold italics. All the samples belonging to the training set and the eight test samples (labeled with an asterisk in **Table 1**) randomly selected from those directly obtained from the producers with certified origin were correctly classified (see Table SI-1 in the Supporting Information). Also, all the commercial test samples fit the model space defined by the training set by using Hotelling's T^2 test and the distance to the model test. Two samples, declared to be acacia and linden, respectively, were classified as polyfloral, although the "probability of class membership" for the origin specified in the label was higher than that of the other monofloral types. The honey sample declared to contain both linden and chestnut honeys is correctly classified; in fact, it is evident by the class index that both botanical origins are present.

The variables responsible for this discrimination are shown in the weight plot (**Figure 6a**) that graphs together the *x*-variable weights "*w*" and the *y*-variable weights "*c*" (*35*), showing the relationships between the selected variables and the four botanical origins. Some of these resonances are labeled with letters and correspond to the identified compounds reported in **Figure 6b**.

Characterization of Botanical Markers. The identification of the compounds that give rise to resonances characteristic of the different botanical origins was undertaken using both 1D and 2D NMR techniques.

To identify markers of linden honey, TOCSY, COSY, NOESY, HMQC, and HMBC spectra were acquired directly

Table 2. ^{1}H and ^{13}C NMR (ppm) Assignment of Compound **C**

position	integration	^{1}H	^{13}C	COSY	HMBC	NOESY
1			175.1			
2			126.3			
3						
4			139.8			
5	1	7.57	118.4		124.2, 132.8, 127.5	
6	1	7.42	124.2		118.4, 127.5, 132.8	7.57, 8.44
8	1	8.44	126.3		132.8, 139.8, 175.1	7.42
7	1	7.69	132.8		126.3,139.8	7.42, 7.57
9			127.5			
2′			167.0			
2″	1	4.80	63.0	2.63, 1.26	29.26 (weak), 126.3, 167.0	2.42, 2.45, 2.63, 3.52
3″a	1	1.26	29.26	2.42, 2.45, 2.63, 4.80	63, 126.3	
3″b	1	2.63	29.26	1.26, 2.42, 2.45, 4.80	41.52	4.80, 1.26
4″a	1	2.42	29.26	1.26, 2.63, 3.52, 3.66	63.0	2.62, 3.52, 4.80
4″b	1	2.45	29.26	1.26, 2.63, 3.52, 3.66	63.0	1.26, 3.68
5″a	1	3.52	41.52	2.42, 2.45, 3.66	29.26	2.42, 3.66
5″b	1	3.66	41.52	2.42, 2.45, 3.52	29.26, 167.0	2.45, 3.52
HN		9.74				7.57

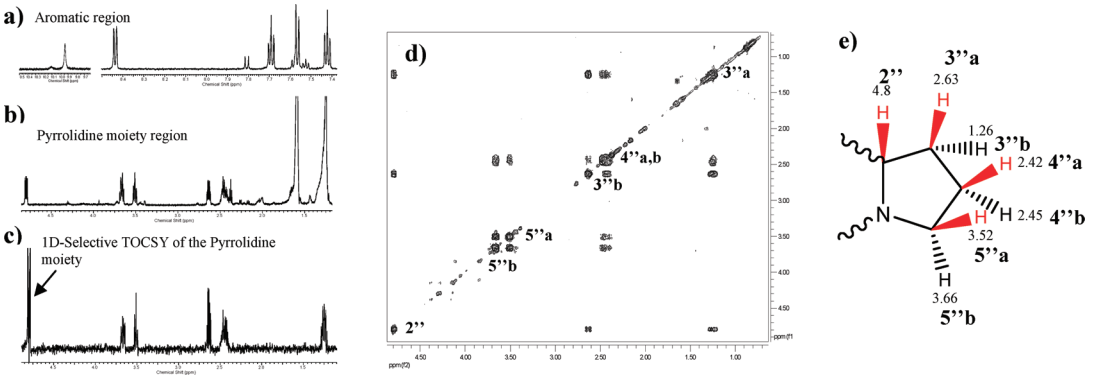

Figure 7. Expansions of the aromatic region (**a**) and of the pyrrolidine moiety region (**b**) of the purified chestnut marker. (**c**) Selective TOCSY spectrum, obtained with a 70 ms mixing time and a selective 180° Gaussian shaped pulse of 79.8 ms centered at 4.08 ppm. All the resonances belonging to the pyrrolidine moiety are present. (**d**) Portion of a COSY spectrum with the assignment of the pyrrolidine moiety. (**e**) Structure and chemical shift assignment of the pyrrolidine moiety.

on an organic extract. We identified two principal terpene acids from chemical shift information, mass spectroscopy data, and literature data (*36*): i.e., 4-(1-hydroxy-1-methylethyl)cyclohexa-1,3-dienecarboxylic acid (**A**) and 4-(1-methylethenyl)cyclohexa-1,3-dienecarboxylic acid (**B**). In all the analyzed linden honey samples, signals from compound **B** were more intense than those from compound **A**. The mass spectroscopy data and the ^{1}H and ^{13}C assignments are reported in the Supporting Information (Figure SI-1 and Table SI-2).

The identification of markers for chestnut and acacia honeys was conducted after purification of the organic extracts through silica gel column chromatography. Many fractions were analyzed, and some of them were identified. Here, we present preliminary results.

A fraction from acacia honey returned a HRESi(+)MS pseudomolecular ion of m/z 254.05 corresponding to the molecular formula $C_{15}H_{10}O_4$. The ^{1}H NMR resonances of this fraction (the assignment is reported in Table SI-3 of the Supporting Information) allowed us to identify this compound as crysin (**D**). This substance is present in higher quantity in this honey but is also present in the other honeys. The compound labeled with the letter **E** in **Figure 6a** corresponds to hexanal.

The chestnut marker (**C**) was isolated and identified as the compound recently detected by Beretta et al., who characterized it in DMSO (*37*). Our NMR spectra were obtained in $CDCl_3$, and the complete assignment is reported in **Table 2**. Our assignment agrees with that of Beretta et al., except for protons 3″ and 4″. This point is illustrated in **Figure 7**, where a selection of the COSY spectrum is shown. The NOESY correlations allowed us to obtain also the stereospecific assignment, shown in **Figure 7e**. In Figures SI-2 and SI-3 (Supporting Information), the UV spectrum and the mass spectrum are reported, respectively. Interestingly, the UV spectrum is identical with that of an unidentified marker of chestnut honey recently reported (*38*).

In conclusion, we presented a simple method to determine the botanical origin of honey, characterized by rapid sample preparation and short acquisition and processing time of the spectra. The PLS-DA approach permitted a discrimination of samples by calculating a specific model for each honey type with pratically no overlap. The results are promising in the perspective of developing similar models suitable to identify honeys of other botanical origins. The method we developed is able to distinguish also polyfloral honeys, allowing a complete classification of all the honey types analyzed. Each honey sample can be classified by means of the "probability of class membership", reported in **Table 1** and in Table SI-1 (Supporting Information). Specifically, we chose a value of 0.50 as the cutoff to assign a sample to that class. The possible presence of other botanical types is indicated by a high value of the corresponding class.

Many of the methods mentioned in the Introduction allow one to clearly discriminate between several types of unifloral honeys, but polyfloral honeys, which represent the majority of the honeys produced, are rarely considered. Only two methods applied discriminant analysis on a high number of samples (*20*, *22*). Devillers et al. (*22*) obtained 100% of correct classification on eight different monofloral honey types, but they used data from eight independent measurements (conductivity, pH, free acidity, and percentages of fructose, glucose, and raffinose). On the basis of front-face fluorescence spectroscopy, Ruoff et al. (*20*) concluded that the classification rates for the unifloral honeys were generally >90%, whereas the classification rate for the polyfloral honeys ranged between 48 and 75%. This method seems a promising approach to discriminate between several unifloral as well as polyfloral honey samples using a single spectroscopic measurement. Our method is also based on a single spectroscopic measurement, and it shows two principal advantages with respect to that proposed by Ruoff et al.: i.e., reduced sample preparation time and better molecular characterization of the components.

To be used in practice, it would be necessary to extend the domain of application of the method we propose to other categories of unifloral honeys and to expand the database. Work is in progress in this direction, also with the aim to identify new chemical entities as botanical markers.

Supporting Information Available: Tables and figures giving additional characterization and test data. This material is available free of charge via the Internet at http://pubs.acs.org.

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