JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Classification of Italian Honeys by 2D HR-NMR

Massimo Lolli,[†] Davide Bertelli,*[,†] Maria Plessi,[†] Anna Gloria Sabatini,[§] and Cinzia Restani[#]

Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia, Via Campi 183, 41100 Modena, Italy; Consiglio per la Ricerca e Sperimentazione in Agricoltura-Istituto Nazionale di Apicoltura, Via di Saliceto 80, 40128 Bologna, Italy; and Centro Interdipartimentale Grandi Strumenti, Università degli Studi di Modena e Reggio Emilia, Via Campi 213/A, 41100 Modena, Italy

The importance of honey has been recently increased because of its nutrient and therapeutic effects, but the adulteration of honey in terms of botanical origin has increased, too. The floral origin of honeys is usually determined using melisso-palynological analysis and organoleptic characteristics, but the application of these techniques requires some expertise. A number of papers have confirmed the possibility of characterizing honey samples by selected chemical parameters. In this study high-resolution nuclear magnetic resonance (HR-NMR) and multivariate statistical analysis methods were used to identify and classify honeys of five different floral sources. The 71 honey samples (robinia, chestnut, citrus, eucalyptus, polyfloral) were analyzed by HR-NMR using both ^{1}H NMR and heteronuclear multiple bond correlation spectroscopy (HMBC). Spectral data were analyzed by application of unsupervised and supervised pattern recognition and multivariate statistical techniques such as principal component analysis (PCA) and general discriminant analysis (GDA). The use of $^{1}H-^{13}C$ HMBC coupled with appropriate statistical analysis seems to be an efficient technique for the classification of honeys.

KEYWORDS: HR-NMR; HMBC; honey; botanical origin; multivariate statistical analysis; classification

INTRODUCTION

Honey is the natural product obtained by honey bees (*Apis mellifera* L.) from the nectar of flowers or from secretions of other living parts of the plants or excretions of sucker insects. The composition and properties of honey depend on the botanical origin of the nectars or secretions.

The Italian annual nectar and honeydew honey production was estimated at about 14200 tons for the year 2006 (*1*). Italy ranks fifth among the world import traders of honey, displaying an individual consumption of about 400 g/year. In fact, only 85000 apiculturists for a total of 1157000 hives are present in Italy, but this value is accompanied by the highest number of varieties of honeys in Europe. The large variety of melliferous sources also enables Italy to produce many characteristic unifloral and a high number of polyfloral nectar honeys. Among unifloral honeys, robinia (*Robinia pseudoacacia* L.), citrus (*Citrus* spp.), chestnut (*Castanea sativa* Miller.), and eucalyptus (*Eucalyptus globulus* spp.) honeys are relevant for their production levels, distribution, and consumer preference; besides, many other unifloral honeys are produced in more limited amount. Each product is unique on the basis of the nature, amount, and combination of the various components that give to each honey a unique and individual organoleptic character. The control and characterization of quality and botanical origin of unifloral honeys are of great importance and interest in apiculture. Today the most important techniques to determine or certify the unifloral origin of honeys are the melisso-palynological analysis and the evaluation of organoleptic characteristics. To avoid errors these controls must be conducted by a group of very expert testers and, if the classification of honeys is attempted only on the basis of the above-mentioned parameters, it can sometimes be imprecise or ambiguous.

Various analytical techniques were used in the past to determine the floral source of honey, that is, determination of saccharides, phenolic compounds, amino acids, proteins, physicochemical parameters, or aroma compounds (*2*–*13*); however, the experimental method to determine this could be improved. In our previous paper the application of diffuse reflectance infrared Fourier transform spectroscopy (DRIFTS) to Italian honeys was considered. The results demonstrated that DRIFTS seems to be an effective method for this purpose (*14*).

Nuclear magnetic resonance (NMR) spectroscopy has been widely applied in chemistry and biochemistry to identify organic compounds and to structurally analyze biopolymers. In recent

* Corresponding author [telephone +39(0)592055147; fax +39(0) 2055131; e-mail bertelli.davide@unimore.it].

[†] Dipartimento di Scienze Farmaceutiche, Università degli Studi di Modena e Reggio Emilia.

[§] Consiglio per la Ricerca e Sperimentazione in Agricoltura-Istituto Nazionale di Apicoltura.

[#] Centro Interdipartimentale Grandi Strumenti, Università degli Studi di Modena e Reggio Emilia.

10.1021/jf072763c CCC: $40.75
Published on Web 01/19/2008

years, high-resolution NMR was applied to research or confirm the presence of particular components or contaminants in foods (*15–17*) and to verify the variations in the composition as a consequence of natural (enzymatic hydrolysis, crystallization, aging) (*18, 19*) or artificial processes (cooking, refinement) (*20*).

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. Moreover, foods are highly labile systems, subjected to biochemical and chemical changes; NMR offers a powerful method to monitor these changes. High-resolution NMR is obviously more readily applied to food samples that can be directly examined as liquids, such as fruit juices and purees, beverages, wines, and oils (*17, 21–24*), but very simple extraction or sample preparation procedures may also be used.

In recent years the use of much higher magnetic fields and the greater sensitivity and spectral dispersion that they bring have stimulated interest in 1D and 2D NMR spectroscopy as a routine method for the analysis of complex mixtures (*21, 25, 26*).

There is a great scientific and commercial interest around the world in the characterization of unifloral honeys. The objective of this study was to determine the feasibility of the classification of Italian honeys based on NMR spectra obtained by heteronuclear multiple bond correlation (HMBC) and multivariate statistical analysis of spectroscopic results. Applications of NMR spectroscopy to honey analysis go back a short time and are in general few (*27–31*), and the 2D NMR, in particular, to our knowledge, has never been previously applied to honey in the attempt to classify samples of different botanical origins; only TOCSY was applied to honey to quantify amino acids (*30*).

MATERIALS AND METHODS

Materials and Sample Preparation. α-D-(+)-Glucose (anhydrous, 96%), β-D-(+)-glucose (minimum 97%), and D-(−)-fructose (minimum 99%) were purchased from Sigma-Aldrich (Milan, Italy). Seventy-one honey samples consisting of 14 robinia (*R. pseudoacacia* L.) honeys, 7 citrus species honeys, 10 chestnut (*C. sativa* Miller.) honeys, 6 eucalyptus (*E. globulus* spp.) honeys, and 34 polyfloral honeys were collected by CRA-INA [Consiglio per la Ricerca e sperimentazione in Agricultura-Istituto Nazionale di Apicoltura (Council for the Research in Agriculture-Italian National Institute for Apiculture, Bologna, Italy)] during the years 2005 and 2006. To ensure and certify the botanical origin of honeys, CRA-INA performed preventively the melissopalynological and organoleptic analyses of the samples.

To prepare samples, 1 g, exactly weighed, of each honey was dissolved in 100 mL of ultrapure demineralized water, obtained by a Milli-Q Plus 185 system from Millipore (Milford, MA); after the complete dissolution, two aliquots of 20 mL each were transferred into two glass plates and freeze-dried, using an Edwards Modulyo freeze-dryer (Edwards High Vacuum International) (Crawley, U.K.), until the complete removal of water. At the end of this process, the samples appeared as very hygrophilous thin films adhering to the bottom of the glass plates and were maintained under vacuum in a desiccator over phosphoric anhydride until analysis. One of the films obtained from freeze-drying was dissolved in 0.6 mL of deuterium oxide [99.9 atom % D Sigma-Aldrich (Milan, Italy)], the other in the same volume of (methyl sulfoxide)-d_6 [99.9 atom % D Sigma-Aldrich (Milan, Italy)] and transferred to Wilmad NMR tubes, 5 mm, Ultra-Imperial grade, 7 in. L, 526-PP purchased from Sigma-Aldrich. The honey solutions were immediately analyzed and were used for both 1D and 2D NMR experiments.

NMR Spectroscopy. 1D and 2D NMR spectra of standards and honey samples, on D_2O and DMSO-d_6 solutions, were acquired with a Bruker FT-NMR Avance 400 (Ettlingen, Germany), and all of the NMR experiments were performed at 300 K. D_2O and DMSO-d_6 were selected as solvents due to their high solvent capacity with respect to the principal components of honey. The ^{1}H NMR spectra were measured at 400.13 MHz with presaturation for water suppression. The number of data points was 16K, the acquisition time 1.71 s, the delay time 10 s, and the numbers of scans 8. Spectral width was 2003.205 Hz. Total acquisition time was 99 s.

The ^{13}C NMR spectra were measured at 100.61 MHz. The number of data points was 64K; an acquisition time of 5.66 s and a delay time of 20 s gave a total repetition time of 25.66 s; 160 scans were accumulated with a proton inverse gated decoupling, and 4 dummy scans were also added. Spectral width was 8049.666 Hz. Total acquisition time was 70 min and 19 s.

The ^{1}H−^{13}C HMBC spectra are a 2D H-1/C-13 correlation via heteronuclear zero and double-quantum coherence optimized on long-range couplings with low-pass *j*-filter to suppress one-bond correlations, no decoupling during acquisition, and gradient pulses for selection. The acquisition parameters were as follows: number of scans, 8; dummy scans, 16; number of data points, 4K in *F*2 (^{1}H) and 256 in *F*1 (^{13}C); spectral width, 2003.205 Hz in *F*2 (^{1}H) and 8049.666 Hz in *F*1 (^{13}C); digital resolution, 0.4891 Hz in *F*2 (^{1}H) and 31.4441 Hz in *F*1 (^{13}C); acquisition time, 1.022 s; delay time, 1.0 s; HMBC delay time, 65 ms. Total acquisition time was 72 min and 42 s. The choice of ^{1}H−^{13}C HMBC instead of other 2D NMR techniques depends on the fact that it furnishes a relatively high number of well-defined signals related to the correlations not only of the principal monosaccharides but also of the principal disaccharides; this technique permits also long-range correlation to be obtained of hydroxyls that are not detected, for example, by a heteronuclear single quantum correlation (HSQC) sequence; moreover, HMBC does not need a phasing process, avoiding in this way the introduction of a source of variability during the spectral calculation. Also, sensitivity appears to be sufficient to the aim of this study because signals are acquired on the proton channel, preventing partially the low sensitivities typical of ^{13}C spectroscopy. Undoubtedly the disadvantage of HMBC is that it is a time-consuming approach with respect to the simpler ^{1}H NMR.

Assignment of NMR Signals. At first, the NMR spectra of honey were analyzed by referring to NMR spectra of standard substances. The ^{1}H and ^{13}C signals of principal sugars (glucose, fructose, maltose, and sucrose) were assigned in such a way.

Spectral Calculations. *^{1}H NMR.* The ^{1}H NMR spectra were used as intensity and were not integrated because of their high complexity and the high number of overlapping signals. Each spectra generated a 16K data file; these files were collected in a data set consisting of 16K spectral variables and 71 samples. Then, to reduce the number of data points, all zones of the spectra without signal are deleted and the resolution of spectra was reduced, obtaining a spectrum with 585 data points for spectra acquired in D_2O and 626 data points for spectra acquired in DMSO-d_6.

^{1}H−^{13}C HMBC. The application of this technique to honey samples generates spectra that are too complicated to be visually analyzed, and in this case the solution to this problem is to analyze the data by chemometric methods. To apply chemometric analysis to HMBC spectra, the integration of the signals was performed. Volume integration of spectral correlations was calculated by the spectrometer software. This program adds together the intensity of the points located in previously manually defined areas surrounding the correlations; all spectra were processed using the same map of regions of interest. The selection of signals to be integrated was performed by considering their presence in the spectra of all the samples and the absence of overlapping zones between two or more adjacent signals; moreover, only the signals with an integration volume of >10 volume units were used as variables in the statistical analysis. In the spectra obtained from D_2O, 87 regions of interest were defined to calculate integrals and used for chemometric analysis, instead of the 98 regions defined for the spectra obtained from DMSO-d_6. The selected signals originate not only from glucose and fructose but also from principal disaccharides present in honey. To apply statistical analysis two different data sets were created, one for the data of D_2O ^{1}H−^{13}C HMBC and one for the DMSO-d_6 ^{1}H−^{13}C HMBC spectra of honeys. The first data set consists of 87 variables and 71 samples, and the second one consists of 98 variables and 71 samples.

Statistical Analysis. To achieve a reliable differentiation between different honey types, unsupervised and supervised pattern recognition

procedures were applied to the data sets. The most important use of these chemometric methods is to represent the *n*-dimensional data set in a smaller number of dimensions, usually two or three. This allows the observations of groupings of cases, which can define the structure of the data set; therefore, principal component analysis (PCA) (*32, 33*) and general discriminant analysis (GDA) (*34*) were used in this work in the attempt to classify honeys according to their NMR profile. PCA allows a large portion of the total variance of data to be expressed with a smaller number of variables, which can be used to graphically represent the population of samples and to identify the most significant of original factors. PCA is a multivariate procedure which rotates the data such that maximum variabilities are projected onto the axes. Essentially, a set of correlated variables is transformed into a new set of uncorrelated variables, which are ordered by reducing variability. Uncorrelated variables are linear combinations of the original variables, and the last of these variables can be removed with minimum loss of real data. GDA is a supervised technique used to determine whether a given classification of cases into a number of groups is an appropriate one; therefore, each sample is preventively assigned to a group, and a model was searched and computed to maximize the classification. It can be used, for instance, to test whether a particular clustering of cases obtained from an unsupervised technique is a likely one. Also, this analysis, after a model was computed, can be used in the attempt to classify unknown samples. In this work we apply a GDA based on the general linear model. To avoid the risk of overfitting, the number of variables for GDA was reduced, considering only the signal which presented a factorial weight during PCA > |0.7|. After the construction of the model, to evaluate the classification performance, the leave-one-out method (*35*) was used as a validation procedure. Before PCA and GDA were performed, all data were normalized by subtracting the mean and dividing it by the standard deviation.

All statistical calculations were performed using Statistica 6.1 for Windows (StatSoft Italia, Vigonza, Italy).

RESULTS AND DISCUSSION

D_2O ^1H NMR Spectrum of Honey. In **Figure 1a** are present not only the principal signals of glucose and fructose but also some signals of the main disaccharides (maltose and sucrose). Two main regions of the D_2O ^1H NMR spectra of honey are evident, the first at mid-low-frequency between δ_H 3.00 and 4.20, which is very rich in signals, and the second one between δ_H 4.40 and 5.40, in which the following signals are present: residual water; anomeric protons of glucose (δ_H 4.55, β-glucopyranose; δ_H 5.12, α-glucopyranose), maltose (δ_H 5.30, of nonreducing residue); and, partially overlapped with these, the anomeric proton of the α form of a reducing residue of maltose (δ_H 5.15) (the signal related to the β anomeric form of the reducing residue of maltose expected at about δ_H 4.60 is not visible because it is near the water suppression zone); and the anomeric proton of glucose residue of sucrose (δ_H 5.34, β-fructofuranosyl-α-glucopyranoside). The assignments of the major signals originating from the principal monosaccharides present in honey in the D_2O ^1H NMR spectrum are summarized in **Table 1**.

DMSO-d_6 ^1H NMR Spectrum of Honey. The DMSO-d_6 ^1H NMR spectrum of honey (**Figure 1b**) is characterized by three main regions. The first is a mid-low-frequency region between δ_H 3.00 and 4.20, which is very similar to that described for the D_2O spectrum. A second region between δ_H 4.40 and 5.80 presents the following signals: hydroxyls and anomeric hydroxyl of fructose (δ_H 5.68, α-fructofuranose; δ_H 5.40, β-fructofuranose; δ_H 5.20, β-fructopyranose); anomeric protons of glucose; hydroxyls of α-maltose and sucrose. The last region between δ_H 6.00 and 6.80 contains the anomeric hydroxyls of glucose and α and β maltose (δ_H 6.66 and 6.30). The assignments of the major signals originating from the principal monosaccharides present in honey in the DMSO-d_6 ^1H NMR spectrum are summarized in **Table 1**.

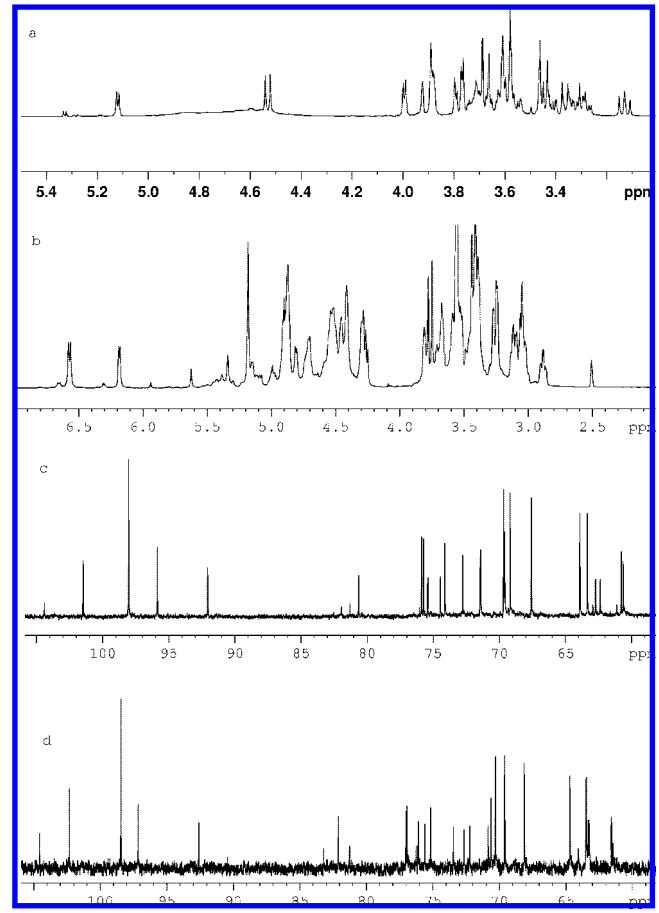

Figure 1. 1D spectra of a robinia honey: (**a**) D_2O ^1H NMR spectrum; (**b**) DMSO-d_6 ^1H NMR spectrum; (**c**) D_2O ^{13}C NMR spectrum; (**d**) DMSO-d_6 ^{13}C NMR spectrum.

D_2O ^{13}C NMR Spectrum of Honey. Two main regions of the D_2O ^{13}C NMR spectrum of honey (**Figure 1c**) were characterized: a low-frequency region between δ_C 60 and 85 ppm and a region between δ_C 90 and 105 ppm, in which the signals of anomeric carbons of all sugars are present. Weak signals related to the anomeric carbons of reducing and nonreducing residues of maltose (δ_C 100.8 and δ_C 96.6 respectively) and sucrose (δ_C 103.65 fructose residue; δ_C 92.14 glucose residue) are also present in the majority of samples. The assignments for the principal monosaccharides of the D_2O ^{13}C NMR spectrum are summarized in **Table 1**.

DMSO-d_6 ^{13}C NMR Spectrum of Honey. The DMSO-d_6 ^{13}C NMR spectrum of honey shown in **Figure 1d** is similar to that obtained with D_2O. However, the chemical shifts of the carbons achieved using DMSO-d_6 are higher than those obtained with D_2O. The assignments for the principal monosaccharides of the D_2O ^{13}C NMR spectrum are summarized in **Table 1**.

$^1H-^{13}C$ HMBC Spectrum of Honey. HMBC spectroscopy is particularly useful because it connects protons with carbons via two or three bonds, couplings that can supply more information about connectivities. Signals related to protons of principal mono- and disaccharides of honey are present. The spectra obtained with DMSO-d_6 are richer in signals because of the presence of correlation points caused by hydroxyls. A D_2O $^1H-^{13}C$ HMBC spectrum of a robinia honey and an example of a DMSO-d_6 $^1H-^{13}C$ HMBC spectrum of a robinia honey are respectively shown in **Figures 2** and **3**.

Results of Statistical Analysis. *^1H NMR.* The statistical analysis of spectra does not produce acceptable results. PCA is

Table 1. Assignment of ^{1}H and ^{13}C NMR Signals of α-Glucopyranose, β-Glucopyranose, α-Fructofuranose, β-Fructofuranose, and β-Fructopyranose Using D_2O and DMSO-d_6 as Solvents

	chemical shift (δ)				
	D_2O		DMSO-d_6		
compound	^{1}H	^{13}C	^{1}H	^{13}C	assignment
α-glucopyranose	5.12	92.2	4.96; 6.18	92.8	C1H; 1OH
	3.55	72.5	3.52; 4.52	72.6	C2H; 2OH
	3.73	73.4	3.43; 4.83	73.4	C3H; 3OH
	3.39	69.7	3.05; 4.70	70.3	C4H; 4OH
	3.83	72.6	3.71	72.6	C5H
	3.65, 3.40	61.5	3.60, 3.33; 4.49	61.5	C6H; 5OH
β-glucopyranose	4.55	95.9	4.52; 6.58	97.1	C1H; 1OH
	3.21	74.1	3.18; 4.88	75.2	C2H; 2OH
	3.54	75.7	3.49; 4.93	77.0	C3H; 3OH
	3.29	69.6	3.21; 5.09	70.8	C4H; 4OH
	3.37	75.8	3.29	76.9	C5H
	3.80, 3.72	60.8	3.67, 3.62; 4.70	61.6	C6H; 5OH
α-fructofuranose	3.57	63.8	3.30; 5.13	63.9	C1H; 1OH
	—	104.4	—; 5.68	104.6	C2; 2OH
	from 3.90 to 4.15	81.9	3.90–4.15; ND*	82.2	C3H; 3OH
	from 3.90 to 4.15	75.4	3.90–4.15; ND*	76.3	C4H; 4OH
	from 3.90 to 4.15	81.3	3.90–4.15	82.5	C5H
	3.89, 3.62	61.0	3.72, 3.44; ND*	62.8	C6H; 5OH
β-fructofuranose	3.56	62.7	3.44; 4.80	63.1	C1H; 1OH
		101.5	—; 5.40	102.3	C2; 2OH
	from 3.90 to 4.15	75.9	3.90–4.15; ND*	76.5	C3H; 3OH
	from 3.90 to 4.15	75.3	3.90–4.15; ND*	76.2	C4H; 4OH
	from 3.90 to 4.15	81.8	3.90–4.15	82.4	C5H
	3.79, 3.61	62.4	3.65, 3.40; ND*	63.2	C6H; 5OH
β-fructopyranose	3.40, 3.50	63.9	3.25, 3.41; 4.54	64.7	C1H; 1OH
	—	98.0	—; 5.20	98.4	C2; 2OH
	3.72	67.6	3.56; 4.27	68.0	C3H; 3OH
	3.74	69.5	3.56; 4.37	70.5	C4H; 4OH
	3.81	69.2	3.63; 4.29	69.9	C5H; 5OH
	3.92, 3.61	63.3	3.78, 3.42	63.5	C6H

*Signals not detectable.

not able to group samples according their botanical origin. In the case of D_2O spectra the first two components explain only 51.2% of total variance; this percentage increases to 64% for DMSO-d_6 spectra. The use of GDA does not notably improve the results; in fact, for both D_2O and DMSO-d_6 spectra only the samples of *Eucalyptus* honey are well clustered. The obtained models permit correct classification of only 25 and 27% of the samples, respectively, during the cross-validation. The difficulty in using the monodimensional spectra to classify honeys is probably due to the high number of overlapping signals, which generate a high level of collinearity.

^{1}H−^{13}C HMBC. (a) PCA. In **Figure 4** are reported the PCA results related to the data set obtained from D_2O ^{1}H−^{13}C HMBC spectra of honeys; as evident, this statistical method is only marginally able to group samples as a function of botanical origin. The first two components explain 67.53% (PC1, 48.17%; PC2, 19.36%) of the total variance. The application of PCA to the data set obtained from DMSO-d_6 ^{1}H−^{13}C HMBC spectra

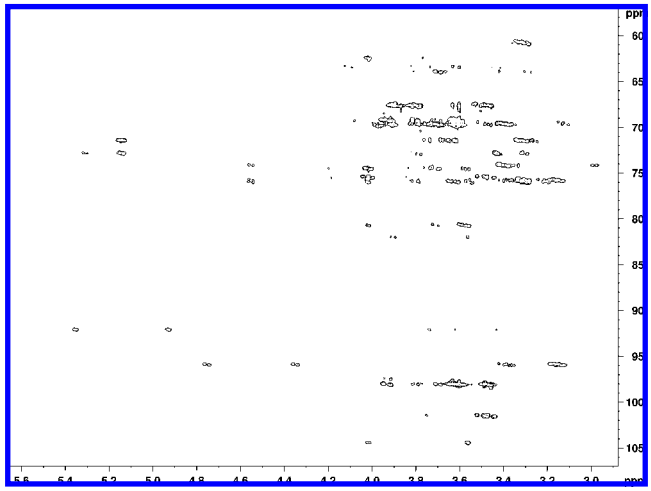

Figure 2. 2D D_2O ^{1}H−^{13}C HMBC spectrum of a robinia honey.

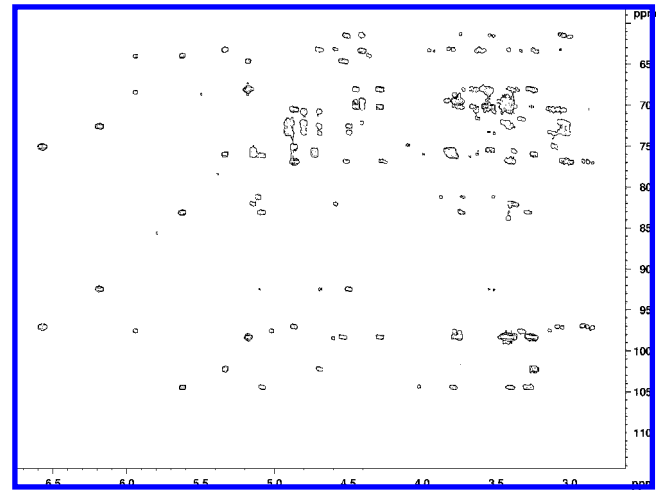

Figure 3. 2D DMSO-d_6 ^{1}H−^{13}C HMBC spectrum of a robinia honey.

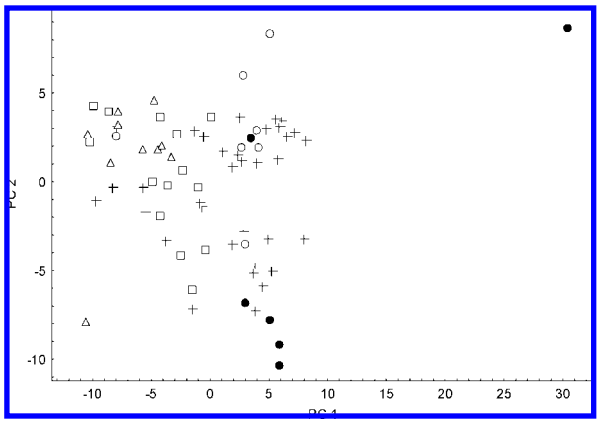

Figure 4. Principal component plot of PC1 and PC2 for the data set obtained by D_2O $^1H-^{13}C$ HMBC spectrum of honey showing the distribution of polyfloral honeys (+), eucalyptus honeys (●), chestnut honeys (△), citrus species honeys (○), and robinia honeys (□). The amount of variation described by PC1 represents 48.17% of the data set variation; PC2 represents 19.36%.

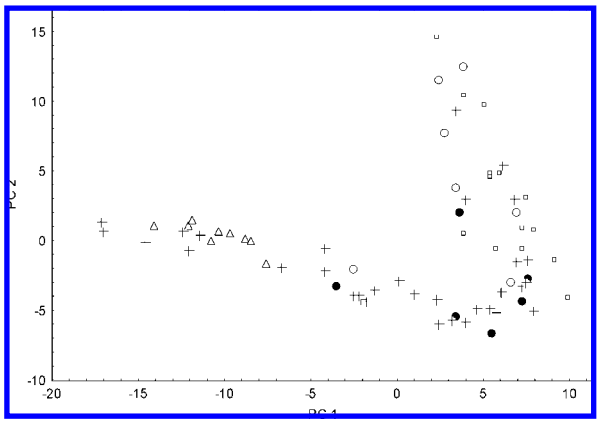

Figure 5. Principal component plot for the data set obtained by DMSO-d_6 $^1H-^{13}C$ HMBC spectrum of honey showing the distribution of polyfloral honeys (+), eucalyptus honeys (●), chestnut honeys (△), citrus species honeys (○), and robinia honeys (□). The amount of variation described by PC1 represents 58.84% of the data set variation; PC2 represents 22.28%.

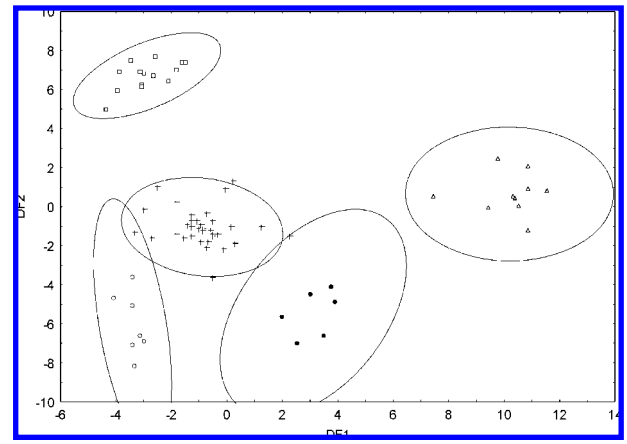

Figure 6. Score plot of the first two canonical functions for the data set obtained by D_2O $^1H-^{13}C$ HMBC spectrum of honey showing separation of honeys in five different clusters: polyfloral honeys (+), eucalyptus honeys (●), chestnut honeys (△), citrus species honeys (○), and robinia honeys (□).

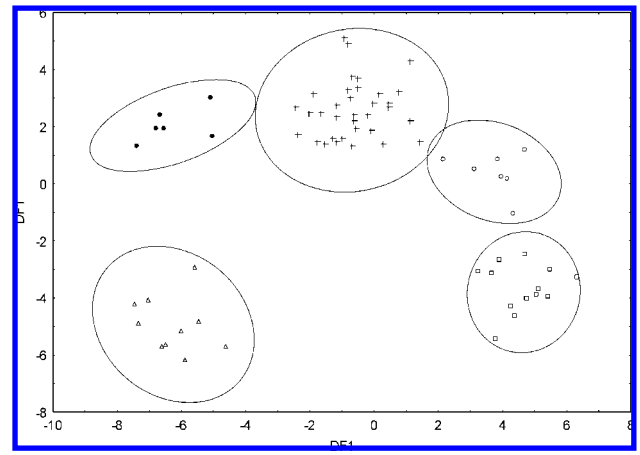

Figure 7. Score plot of the first two canonical functions for the data set obtained by DMSO-d_6 $^1H-^{13}C$ HMBC spectrum showing separation of honeys in five different clusters: polyfloral honeys (+), eucalyptus honeys (●), chestnut honeys (△), citrus species honeys (○), and robinia honeys (□).

of honeys permits the percentage of explained variance to be increased. In fact, in this analysis the first two components explain 81.12% (PC1, 58.84%; PC2, 22.28%) of the total variance; nevertheless, the quality of classification does not seem to improve notably (**Figure 5**). For both data sets the most correlated signals are verified, in order to assign those to the appropriate compound. With regard to the NMR spectra obtained with D_2O, PC1 is especially correlated to the signals of fructose and, in particular, β-fructofuranose. The contribution of β-fructopyranose is not relevant. For glucose, the most important signals belong to α-glucopyranose. Considering PC2, glucose and fructose seem to have comparable importance. The significant signals are certainly related to β-fructopyranose instead of α-fructofuranose and β-fructofuranose, whereas α-glucopyranose and β-glucopyranose seem to have the same influence. With regard to the NMR spectra obtained with DMSO-d_6, PC1 is especially correlated to hydroxylic signals and, in particular, with those belonging to β-fructopyranose. For glucose, the most correlated signals for PC1 are those of α-glucopyranose. On the contrary, PC2 is significantly related to β-fructopyranose and β-glucopyranose protonic signals. In this case α-fructofuranose is the sugar with minor statistical weight. Considering the above-reported results, the better performance obtained from DMSO-d_6 spectra is probably due to the presence in these spectra of the signals of hydroxyls, which appear very correlated with extracted components.

(b) GDA. The factorial reduction of variables had produced for D_2O $^1H-^{13}C$ HMBC spectra of honey a data set containing only 49 variables. In this case (**Figure 6**) the model is able to group the 71 samples in five evident clusters (robinia, chestnut, citrus, eucalyptus, polyfloral). Looking at the posteriori probabilities, all samples were correctly classified. During the cross-validation procedure the model presents a predictive capacity of 92%. The most important variables for DF1 are strongly related to the signals belonging to β-glucopyranose and less to β-fructopyranose and α-glucopyranose; DF2 is particularly related to the signals of α-glucose besides β-fructopyranose.

Considering the data sets obtained from DMSO-d_6 $^1H-^{13}C$ HMBC spectra of honeys (**Figure 7**), the factorial reduction of variables had produced a data set with 52 variables and the quality of results is better than that obtained from D_2O. A prediction ability of 97% was obtained for the model by cross-validation. The most important variables for DF1 are the signal of hydroxyls and in particular those of β-glucose. Considering

DF2, glucose and fructose seem to have comparable importance. The significant signals are certainly correlated to hydroxyls of α-fructofuranose instead of β-fructopyranose and β-fructofuranose, whereas α-glucopyranose and β-fructofuranose seem to have little influence. In this case the weight of the signals related to correlation of hydroxyls confirms the utility of $^{1}H-^{13}C$ HMBC, which permits the detection of the long-range coupling of these protons.

Conclusion. This investigation demonstrates that $^{1}H-^{13}C$ HMBC coupled with appropriate multivariate statistical analysis seems to be an efficient and versatile technique for the classification of the different botanical origins of honey samples. Between the two chemometric techniques, the supervised GDA demonstrated the highest efficiency in the attempt to construct a model for the prediction of honey origin if associated with a preventive factorial selection of most significant variables. The use of D_2O gives good results, but the use of DMSO-d_6 enables better results to be achieved; this is due to the possibility of using signals related to hydroxyls.

ABBREVIATIONS USED

HMBC, heteronuclear multiple bond correlation; HR NMR, high-resolution nuclear magnetic resonance; D_2O, deuterium oxide; DMSO-d_6, (methyl sulfoxide)-d_6; PC1, principal component 1; PC2, principal component 2; PCA, principal component analysis; GDA, general discriminant analysis; 1D, one-dimensional; 2D, bidimensional; CF1, canonical function 1; CF2, canonical function 2.

ACKNOWLEDGMENT

We thank the Centro Interdipartimentale Grandi Strumenti of the University of Modena and Reggio Emilia for the use of the Bruker Avance 400 spectrometer.

LITERATURE CITED

(1) Osservatorio Nazionale della Produzione e Mercato del Miele [National Observatory on Honey Production and Commercialization]. Andamento produttivo e di mercato del miele in Italia, rapporto annuale 2006; http://www.osservatoriomiele.org/relazione2006.pdf (accessed May 2007).

(2) Abu-Tarboush, H. M.; Al-Kahtani, H. A.; El-Sarrage, M. S. Floral type identification and quality evaluation of some honey types. *Food Chem.* **1993**, *46*, 13–17.

(3) Andrade, P.; Ferreres, F.; Gil, M. I.; Tomas-Barberan, F. A. Determination of phenolic compounds in honeys with different floral origin by capillary zone electrophoresis. *Food Chem.* **1997**, *60*, 79–84.

(4) Azeredo, L. D. C.; Azeredo, M. A. A.; Souza de, S. R.; Dutra, V. M. L. Protein contents and physicochemical properties in honey samples of *Apis mellifera* of different floral origin. *Food Chem.* **2003**, *80*, 249–254.

(5) Conte, L.; Gracco, L.; Piasenzotto, L. Solid phase microextraction (SPME) applied to honey quality control. *J. Sci. Food Agric.* **2003**, *83*, 1037–1044.

(6) Anklam, E. A review of the analytical methods to determine the geographical and botanical origin of honey. *Food Chem.* **1998**, *63*, 549–562.

(7) Hermosin, I.; Chicon, R. M.; Cabezudo, M. D. Free amino acid composition and botanical origin of honey. *Food Chem.* **2003**, *83*, 263–268.

(8) Latorre, M. J.; Pena, R.; Pita, C.; Botana, A.; Garcia, S.; Herrero, C. Chemometric classification of honeys according to their type. II. Metal content data. *Food Chem.* **1999**, *66*, 263–268.

(9) Nozal, M. J.; Bernal, J. L.; Toribio, L.; Alamo, M.; Diego, J. C.; Tapia, J. The use of carbohydrate profiles and chemometrics in the characterization of natural honeys of identical geographical origin. *J. Agric. Food Chem.* **2005**, *53*, 3095–3100.

(10) Radovic, B. S.; Careri, M.; Mangia, A.; Musci, M.; Gerboles, M.; Anklam, E. Contribution of dynamic headspace GC-MS analysis of aroma compounds to authenticity testing of honey. *Food Chem.* **2001**, *72*, 511–520.

(11) Serrano, S.; Villajero, M.; Espejo, R.; Jodral, M. Chemical and physical parameters of andalusian honey: classification of citrus and eucalyptus honeys by discriminant analysis. *Food Chem.* **2004**, *87*, 619–625.

(12) Benedetti, S.; Mannino, S.; Sabatini, A. G.; Marcazzan, G. L. Electronic nose and neural network use for the classification of honey. *Apidologie* **2004**, *35*, 397–402.

(13) Ruoff, K.; Luginbuhl, W.; Kunzli, R.; Bogbanov, S.; Bosset, J. O.; von der Ohe, K.; Amado, R. Authentication of the botanical and geographical origin of honey by front-face fluorescence spectroscopy. *J. Agric. Food Chem.* **2006**, *54*, 6858–6866.

(14) Bertelli, D.; Plessi, M.; Sabatini, A. G.; Lolli, M.; Grillenzoni, F. Classification of italian honeys by mid infrared diffuser reflectance spectoscopy (DRIFTS). *Food Chem.* **2007**, *101*, 1565–1570.

(15) Arvanitoyannis, I. S.; Chalhoub, C.; Gotsiou, P.; Lydakis-Simantiris, N.; Kefalas, P. Novel quality control methods in conjunction with chemometrics (multivariate analysis) for detecting honey authenticity. *Crit. Rev. Food Sci.* **2005**, *45*, 193–203.

(16) Pereira, G. E.; Gaudillere, J. P.; Van-Leeuwen, C.; Hilbert, G.; Lavallie, O.; Maucourt, M.; Deborde, C.; Moing, A.; Rolin, D. ^{1}H NMR and chemometrics to characterize mature grape berries in four wine-growing areas in Bordeaux, France. *J. Agric. Food Chem.* **2005**, *53*, 6382–6389.

(17) Kosir, I. J.; Kidric, J. Identification of amino acids in wines by one- and two-dimensional nuclear magnetic resonance spectroscopy. *J. Agric. Food Chem.* **2001**, *49*, 50–56.

(18) Clark, S.; Barnett, N. W.; Adams, M.; Cook, I. B.; Dyson, G. A.; Johnston, G. Monitoring a commercial fermentation with proton nuclear magnetic resonance spectroscopy with the aid of chemometrics. *Anal. Chim. Acta* **2006**, *563*, 338–345.

(19) Belloque, J.; Carrascosa, A. V.; Fandino, R. L. Changes in phosphoglyceride composition durino storage of ultrahigh-temperature milk, as assessed by ^{31}P-nuclear magnetic resonance: possibile involvement of thermoresistant microbial enzymes. *J. Food Prot.* **2001**, *64*, 850–855.

(20) Duarte, I. F.; Delgadillo, I.; Gil, A. M. Study of natural mango juice spoilage and microbial contamination with *Penicillium expansum* by high resolution H-1 NMR spectroscopy. *Food Chem.* **2006**, *96*, 313–324.

(21) Belton, P. S.; Delgadillo, I.; Gil, A. M.; Casuscelli, F.; Colquhoun, I. J.; Tennis, M. J.; Spraul, M. High field proton NMR studies of apple juices. *Magn. Reson. Chem.* **1997**, *35*, S52–S60.

(22) Bosco, M.; Toffanin, R.; Palo, D.; Zatti, L.; Segre, A. High resolution ^{1}H NMR investigation of coffee. *J. Sci. Food Agric.* **1999**, *79*, 869–878.

(23) Vlahov, G.; Chepkwony, P. K.; Ndalut, P. K. ^{13}C NMR characterization of triacylglycerols of *Moringa oleifera* seed oil: an "oleic-vaccenic acid" oil. *J. Agric. Food Chem.* **2002**, *50*, 970–975.

(24) Thomas, M. E.; Robert, G. B. High-resolution proton NMR spectroscopy of milk, orange juice, and apple juice with efficient suppression of the water peak. *J. Agric. Food Chem.* **1986**, *34*, 834–837.

(25) Charlton, A. J.; Farrington, W. H. H.; Brereton, P. Application of ^{1}H NMR and multivariate statistics for screening complex mixtures: quality control and authenticity of instant coffee. *J. Agric. Food Chem.* **2002**, *50*, 3098–3103.

(26) Fan, T. W. M. Metabolite profiling by one- and two-dimensional NMR analysis of complex mixtures. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *28*, 161–219.

(27) Lindner, P.; Bermann, E.; Gamarnik, B. Characterization of citrus honey by deuterium NMR. *J. Agric. Food Chem.* **1996**, *44*, 139–140.

(28) Mazzoni, V.; Bradesi, P.; Tomi, F.; Casanova, J. Direct qualitative and quantitative analysis of carbohydrate mixtures using ^{13}C NMR

spectroscopy: application to honey. *Magn. Reson. Chem.* **1997**, *35*, S81–S90.
(29) Cabras, P.; Angioni, A.; Tuberoso, C.; Floris, I.; Reniero, F.; Guillou, C.; Ghelli, S. Homogentisic acid: a phenolic acid as a marker of strawberry-tree (*Arbutus unedo*) honey. *J. Agric. Food Chem.* **1999**, *47*, 4064–4067.
(30) Sandusky, P.; Raftery, D. Use of selective TOCSY NMR experiments for quantifying minor components in complex mixtures: application to the metabonomics of amino acids in honey. *Anal. Chem.* **2005**, *77*, 2455–2463.
(31) Cotte, J. F.; Casabianca, H.; Lheritier, J.; Perrucchietti, C.; Sanglar, C.; Waton, H.; Grenier-Loustalot, M. F. Study and validity of C-13 stable carbon isotopic ratio analysis by mass spectrometry and H-2 site-specific natural isotopic fractionation by nuclear magnetic resonance isotopic measurements to characterize and control the authenticity of honey. *Anal. Chim. Acta* **2007**, *582*, 125–136.
(32) Burt, C. The factorial analysis of qualitative data. *Br. J. Psychol.* **1950**, *3*, 166–185.
(33) Velicer, W. F.; Jackson, D. N. Component analysis vs. factor analysis: some issues in selecting an appropriate procedure. *Multivariate Behav. Res.* **1990**, *25*, 1–28.
(34) McLachlan, G. J. *Discriminant Analysis and Statistical Pattern Recognition*; Wiley: New York, 1992.
(35) Henrion, R.; Henrion, G. Uberwachte klassifikation. In *Multivariate Datenanaly*; Springer-Verlag: Berlin, Germany, 1994; pp 71–73.

Received for review September 17, 2007. Accepted December 20, 2007. The Fondazione Cassa di Risparmio di Modena is gratefully acknowledged for the financial support given for the acquisition of the Bruker Avance 400 spectrometer.

JF072763C