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Detection of Honey Adulteration by Sugar Syrups Using One-Dimensional and Two-Dimensional High-Resolution Nuclear Magnetic Resonance

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The importance of honey adulteration detection has recently increased owing to the limited production levels in recent years and the relative high price of honey; therefore, this illegal practice has become more and more attractive to producers. Hence, the need has arisen for more effective analytical methods aiming at detecting honey adulteration. The present research presents an effective method to detect adulteration in honey falsified by intentional addition of different concentrations of commercial sugar syrups, using one-dimensional (1D) and two-dimensional (2D) nuclear magnetic resonance (NMR) coupled with multivariate statistical analysis. Sixty-three authentic and 63 adulterated honey samples were analyzed. To prepare adulterated honeys, seven different sugar syrups normally used for nutrition of bees were used. The best discriminant model was obtained by 1D spectra, and leave-one-out cross-validation showed a predictive capacity of 95.2%. 2D NMR also furnished acceptable results (cross-validation correct classification 90.5%), although the ^{1}H NMR sequence is preferable because it is the simplest and fastest NMR technique.

KEYWORDS: HR-NMR; HMBC; honey; adulteration; multivariate statistical analysis

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 productions were estimated at about 20000 tons for the year 2009 (latest available data) and 13000−14000 for the year 2008. In 2008, in particular, yield level was well below the production of previous years, with a production loss of about 30%. This significant decline occurred mainly due to high winter mortality of colonies, widespread poisoning phenomena, and the consequent depopulation of hives, which considerably reduced the Italian beekeeping heritage. The bad weather conditions observed in Italy during the years 2008 and 2009 also contributed (*1*−*3*).

In recent years, the general drop in production and the consequent increase in market prices have encouraged falsification practices, through the intentional addition of inexpensive sugar syrup to honey, and have made honey adulteration particularly attractive. This falsification/adulteration procedure is often difficult to detect, owing to the fact that the sugar compositions of these low-cost syrups were sometimes close to those of authentic honey. In the literature, several methods were proposed to detect honey adulteration through sugar profile analysis. Cordella et al. used two types of microscopy techniques and the mass spectrometry of ^{13}C (^{13}C-IRMS) to develop their control method (*4*). Elflein et al. proposed to measure the differences between ^{13}C/^{12}C stable carbon isotope ratios of protein and sugar compounds with a combination of elemental analyzer−isotope ratio mass spectrometry and liquid chromatography−isotope ratio mass spectrometry (δ^{13}C-EA/LC-IRMS) (*5*). Morales et al. reported a method previously validated to study the oligosaccharide profile of honey and detect adulterations of corn syrups and high-fructose corn syrups in genuine honey samples by high-performance anion-exchange chromatography pulsed amperometric detection (HPAEC-PAD) (*6*). Also, a new methodology based on GC-MS was described by Ruiz-Matute et al. (*7*). However, the most common technique used to determine the adulteration of honey is attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR), often coupled with multivariate analysis (*8*−*10*). Recently, also differential scanning calorimetry (*11*) and site-specific natural isotopic fractionation nuclear magnetic resonance (NMR) (*12*) were proposed as analytical tools for adulteration detection.

The objective of this study was to determine the feasibility of honey adulteration detection using one-dimensional (1D) and two-dimensional (2D) NMR spectra and multivariate statistical analysis.

NMR spectroscopy has been widely applied in chemistry and biochemistry to identify organic compounds and to structurally

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analyze biopolymers. In recent years, high-resolution NMR was applied to research or confirm the presence of particular components in foods (*13*) and to verify the variations in the composition as a consequence of natural (enzymatic hydrolysis, crystallization, aging) or artificial processes (cooking, refinement) (*14*−*18*).

NMR spectroscopy shows a number of advantages compared to other analytical techniques: it is fast (<5 min is required to acquire a ^{1}H NMR spectrum); it needs no calibration with internal standards or components extraction prior to the analysis; and it shows remarkable selectivity and identifies unknown compounds at a molecular level. One of the main advantages of this technique is its ability to furnish structural and quantitative information on a wide range of chemical species in a single NMR experiment, with excellent repeatability and reproducibility. Moreover, foods are highly labile systems, subjected to biochemical and chemical changes, and 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: fruits juices and purees, beverages, wines, oils, etc. (*13*, *19*−*21*), 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 spectroscopic resolution that they bring have stimulated interest in 1D and 2D NMR spectroscopy as a routine method for the analysis of complex mixtures (*22*, *23*) such as of honey (*24*−*29*); however, to the best of our knowledge, no previous studies were focused on the detection of honey adulteration, which involves the addition of sugar syrups, using simple 1D and 2D high-resolution NMR directly on honey samples without any sample pretreatment.

MATERIALS AND METHODS

Materials and Sample Preparation. Seven different sugar solutions, marketed as specific products for the apicultural practice, were purchased in all. Besides, 63 authentic honeys of different botanical sources (robinia, eucalyptus, citrus, chestnut, and multiflower) were collected by Consiglio per la Ricerca e la Sperimentazione in Agricoltura-Unità di Ricerca di Apicoltura e Bachicoltura, Bologna, Italy (CRA-API), during the year 2008. All of the honey samples were analyzed as the authentic one and randomly adulterated by adding 10, 20, or 40 g/100 g of one of the seven commercial sugar syrups. Such honey adulteration was carried out to obtain three different adulterated samples for each sugar solution for each level of syrup addition; consequently, 63 adulterated samples were obtained in total (21 at 10%, 21 at 20%, and 21 at 40%). To prepare samples for NMR analysis, 200 mg exactly weighed of each authentic or adulterated honey was directly dissolved in 600 μL of methyl sulfoxide-d_6 (Sigma-Aldrich, Milan, Italy) (DMSO-d_6) and transferred to a Wilmad NMR tube, 5 mm, Ultra-Imperial grade (Sigma-Aldrich). DMSO-d_6 was selected as solvent not only because of its high dissolving capacity with respect to the principal components of honey but also because it permitted the acquisition of the signal of hydroxylic groups, obviously very abundant in honey samples. A droplet of tetramethylsilane (TMS) was added to each NMR sample as reference. The honey solutions were immediately analyzed and were used for both 1D and 2D NMR experiments.

Gas Chromatographic Determination of Sugar in Syrups. All of the syrups used as adulterant were analyzed by CRA-API using a well-consolidated GC method after silanization to determine the principal saccharides profile. The silanizing agent was obtained by mixing 5 parts of pyridine (Sigma-Aldrich) with 2 parts of hexamethyldisilazane (Sigma-Aldrich) and 1 part of chlorotrimethylsilane (Sigma-Aldrich). To prepare samples, 3 g of each syrup was dissolved in distilled water and added to 5 mL of a 10% (w/v) mannitol (Sigma-Aldrich) solution as internal standard. The samples were filled to 500 mL in a volumetric flask and mixed. One hundred microliters of sample was transferred to a conical-bottom test tube and allowed to dry under nitrogen flow at 50 °C. Then, 500 μL of silanizing solution was added, and the tube was sealed with a screw-on plug. The solution was well stirred in an ultrasonic bath and kept overnight in a shaker. The next day, the solution was dried under nitrogen flow at 75 °C, and the residue was redissolved in 500 μL of hexane, stirred, and centrifuged at 5000 rpm for several seconds, and then 0.6 μL was injected in the gas chromatograph. The chromatographic separation was performed using a Varian 3900 gas chromatograph (Varian, Palo Alto, CA) equipped with a flame ionization detector (FID) system, and the column used was a 25 m × 0.32 mm i.d., 0.1 μm film thickness, DB-5 capillary column (Agilent Technologies, Milan, Italy), with the following instrumental conditions: injector and detector temperatures, 250 °C; oven temperature, increased from 70 to 140 °C at 49 °C/min and then to 300 °C at 60 °C/min; carrier gas, hydrogen at 4 mL/min. Concentrations were calculated using the internal standard method, considering the response factors obtained by analyzing a standard sugar solution prepared by dissolving in water in a 100 mL volumetric flask 2 g, exactly weighed, of each sugar in the presence of mannitol as internal standard. The results were expressed as grams of sugar per 100 g of sample and were reported as mean of triplicate injections of all the samples.

NMR Spectroscopy. 1D and 2D NMR spectra of authentic and adulterated honey samples were acquired with a Bruker FT-NMR Avance 400 spectrometer (Ettlingen, Germany), and all of the NMR experiments were performed at 300 K. The ^{1}H NMR spectra were measured at 400.13 MHz using the presaturation technique for water signal suppression. The acquisition parameters of this experiment were as follows: time domain (number of data points), 16K; acquisition time, 1.71 s; delay time, 10 s (this value was established from preliminary T1 measurament and different real optimization experiments on real samples to ensure the complete relaxation of all nuclei in all kinds of honey considered); number of scans, 8. Spectral width was 2003.205 Hz. Total acquisition time was 99 s.

The ^{1}H−^{13}C heteronuclear multiple bond correlation (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, with no decoupling during acquisition and using gradient pulses for selection. The acquisition parameters were as follows: number of scans, 8; dummy scans, 16; time domain (number of data points), 4K in the acquisition or direct HMBC dimension F2 (^{1}H) and 256 in indirect HMBC dimension F1 (^{13}C); spectral width, 2003.205 Hz in F2 (^{1}H) and 8049.666 Hz in F1 (^{13}C); digital resolution, 0.4891 Hz in F2 (^{1}H) and 31.4441 Hz in F1 (^{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 chemical shifts were reported as δ_H (ppm) relative to TMS.

Spectroscopic Calculations. *^{1}H NMR.* The ^{1}H NMR spectra were used as intensity and were not integrated, owing to their high complexity and the high number of overlapping signals. Each spectrum generated a file containing 16K data points corresponding to time domain, that is, the number of points acquired and digitalized by the instrument along the spectral width of 2003.205 Hz to obtain the free induction decay (FID) and then converted in spectrum by Fourier transform; these files were collected in a data set consisting of 16K spectroscopic variables and 126 samples.

Before statistical analyses are performed, some spectroscopic preprocessing should be performed. First, spectra were phased and calibrated using the TMS signal. Then, to reduce the inhomogeneous proton NMR chemical shift of signals along the spectra, due to small pH changes and intermolecular interactions, all spectra were aligned using the toolbox Icoshift 1.0 for Matlab (Mathworks Inc., Natick, MA) (*30*). At least, to reduce the number of data points, all of the spectroscopic regions devoid of signals were deleted and the spectroscopic resolution was reduced, obtaining a data set with 3150 data points for each spectra.

^{1}H−^{13}C HMBC. The application of this technique to honey samples generated spectra that were too complicated to be visually analyzed, and in this case the problem solution was to analyze the data by chemometric methods. To apply chemometric analysis to HMBC spectra, the integration of the signals was performed. Volume integration of spectroscopic 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 previously defined. The integrated signal selection was performed by considering the signals' presence in the spectra of all the samples and the absence of overlapping adjacent signals; moreover, only the signals with an integration volume of >10 volume units were used as variables in the statistical analysis. In this way 138 regions of interest were defined to calculate integrals and used for chemometric analysis.

Statistical Analysis. To apply statistical analysis, two different data sets were created, the first one related to the data of ^{1}H NMR and the second one concerning the ^{1}H−^{13}C HMBC spectra of honeys. The first data set consists of 3150 variables and 126 samples, and the second one consists of 138 variables and 126 samples.

To achieve a reliable differentiation between authentic and adulterated samples, unsupervised and supervised statistical 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, factor analysis (FA) (*31*) and general discriminant analysis (GDA) (*32*) were used in this work in the attempt to identify adulterated honeys according to their NMR fingerprint. The main applications of FA technique are (1) to *reduce* the number of variables and (2) to *detect structure* in the relationships between variables, that is, to *classify variables*. Therefore, FA applied as a data reduction or structure detection method allows the expression of a large portion of total variance of data 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. In our study this technique was used to identify the most significant variables to be introduced in the models. 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 is searched and computed to maximize the classification. Besides, after a model is computed, this analysis can be used in the attempt to classify unknown samples. In this work, we applied a GDA based on general linear model. To avoid the risk of overfitting, the number of variables for GDA was reduced by performing a preliminary factorial selection of variables (FA) followed by a forward stepwise method to select the most significant variables to be introduced in the models. After the construction of the model, to evaluate the classification performance, the leave-one-out method (*33*) was used as a cross-validation procedure. Before FA and GDA were performed, all data were normalized.

All statistical calculations were performed using Statistica 6.1 for Windows (StatSoft Italia, Vigonza, Italy) and SPSS 13.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Considering that, in real cases, it is very difficult to know which kind of sugar syrup was employed as adulterant and also to have the authentic sample, to verify the feasibility of using NMR analysis combined with chemometrics as a practical tool to detect honey adulteration, the sampling procedure above-reported was employed. Different sugar solutions, marketed as specific products for the apicultural practice, were used to adulterate several authentic honeys of different botanical sources (robinia, eucalyptus, citrus, chestnut, and multiflower) to reduce the influence of botanical origin.

In this way, we introduced in the models the spectroscopic variability related not only to the sugar solutions used but also to the botanical origin of honey that NMR is able to detect as previously demonstrated (*25*); therefore, an effective model should be able to distinguish between these two kinds of variability to obtain good classification results in the detection of honey adulteration.

Syrup Composition. In **Table 1** the compositions of syrups used as adulteration agent are reported. As evident, the considered solutions present variable compositions. °Brix values (20 °C) of these sugar syrups ranged between 68.5 and 79.0 (**Table 1**). Considering these results, the presence of solids other than sugars dissolved in syrups was evident. MANOVA test performed between groups of spectra of samples prepared with different syrups considering the °Brix levels as discriminant parameter demonstrated that no significant effect on the classification of adulterated honeys was present for $P = 0.05$. Syrup (Syr) 1 shows high content of glucose, maltose, and maltotriose; however, it has very low content of fructose. This fact suggests that it derives from starch hydrolysis. Syr 2, Syr 6, and Syr 7 with high contents of glucose, maltose, and maltotriose, present also a high content of fructose, suggesting that they derive from hydrolyzed starch, treated with isomerase. Syr 5 is very rich in fructose; also in this case it is possible to indicate starch as starting material. Syr 3 and Syr 4 seem to be inverted sugar solutions, with the difference that in Syr 4 the presence of sucrose is evident. The high compositional variability of this kind of product shows that it is very difficult to identify a typical product used as adulterant. Therefore, these results justify our experimental plan, which was to simultaneously and randomly enter all of the syrups in the model.

Table 1. Composition of Syrups Used as Adulteration Agents (Grams per 100 g)

		syrup						
	t_R (min)	1	2	3	4	5	6	7
fructose	12.3	1.3	24.4	33.5	31.4	45.6	21.8	18.4
glucose	13.0	26.3	22.9	33.4	26.5	17.0	26.4	23.5
raffinose	18.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
sucrose	20.8	<0.1	<0.1	<0.1	13.2	0.2	<0.1	<0.1
trehalose	21.8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
maltose	23.1	25.8	19.3	<0.1	<0.1	7.1	17.1	16.0
gentiobiose	23.8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
isomaltose	24.1	0.3	0.6	<0.1	<0.1	0.2	0.2	0.4
melezitose	28.8	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
maltotriose	30.3	10.3	3.5	<0.1	<0.1	2.9	7.6	7.4
panose	31.3	0.2	0.5	<0.1	<0.1	<0.1	0.3	0.2
maltotetraose	38.3	0.6	0.4	<0.1	<0.1	0.1	0.4	0.4
total		64.8	71.6	66.9	71.1	73.1	73.8	66.3
°Brix (20 °C)		72.5	78.5	69.5	72.5	76.5	79.0	73.0

DMSO-d_6 ^{1}H NMR Spectrum of Authentic and Adulterated Honeys. In **Figure 1** is shown a typical spectrum of an authentic honey. The DMSO-d_6 ^{1}H NMR spectrum of a honey, regardless of the botanical origin, is characterized by three main regions. A midlow field region between δ_H 3.00 and 4.20 contains the signal of aliphatic protons except for anomeric protons. A second region between δ_H 4.40 and 5.80 presents the following signals: hydroxyls and anomeric hydroxyls of fructose (δ_H 5.68, α-fructofuranose; δ_H 5.35, β-fructofuranose; δ_H 5.20, β-fructopyranose); anomeric protons of glucose (δ_H 4.96, β-glucopyranose; δ_H 4.52, β-glucopyranose). The last region between δ_H 6.00 and 6.80 contains the anomeric hydroxyls of glucose (δ_H 6.58, β-glucopyranose; δ_H 6.18, α-glucopyranose) and α- and β-maltose (δ_H 6.65 and 6.30) if sufficiently concentrated. The assignments of the major signals originating from the principal monosaccharides present in honey in the DMSO-d_6 ^{1}H NMR spectrum were already performed and reported in our previous work (*25*). From a comparison of the spectrum of an authentic honey with that of an adulterated one (**Figure 2**) it should be immediately noted that some signals were present only in the adulterated honey spectrum or at least were of greater intensity. In particular, in the highlighted zones the signals of anomeric hydroxyls of α- and β-maltose are well evident (δ_H 6.65 and 6.30), such as signals related to other hydroxyls of maltose, sucrose, or other oligosaccharides (δ_H 5.40, 5.10, 4.80, and 4.70).

^{1}H−^{13}C HMBC Spectrum of Adulterated Honey. The choice of ^{1}H−^{13}C HMBC instead of other 2D NMR techniques depended 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 of hydroxyls that are not detected, for example, by a heteronuclear single quantum correlation (HSQC) sequence to be obtained. Moreover, HMBC does not need a phasing process, avoiding in this way the

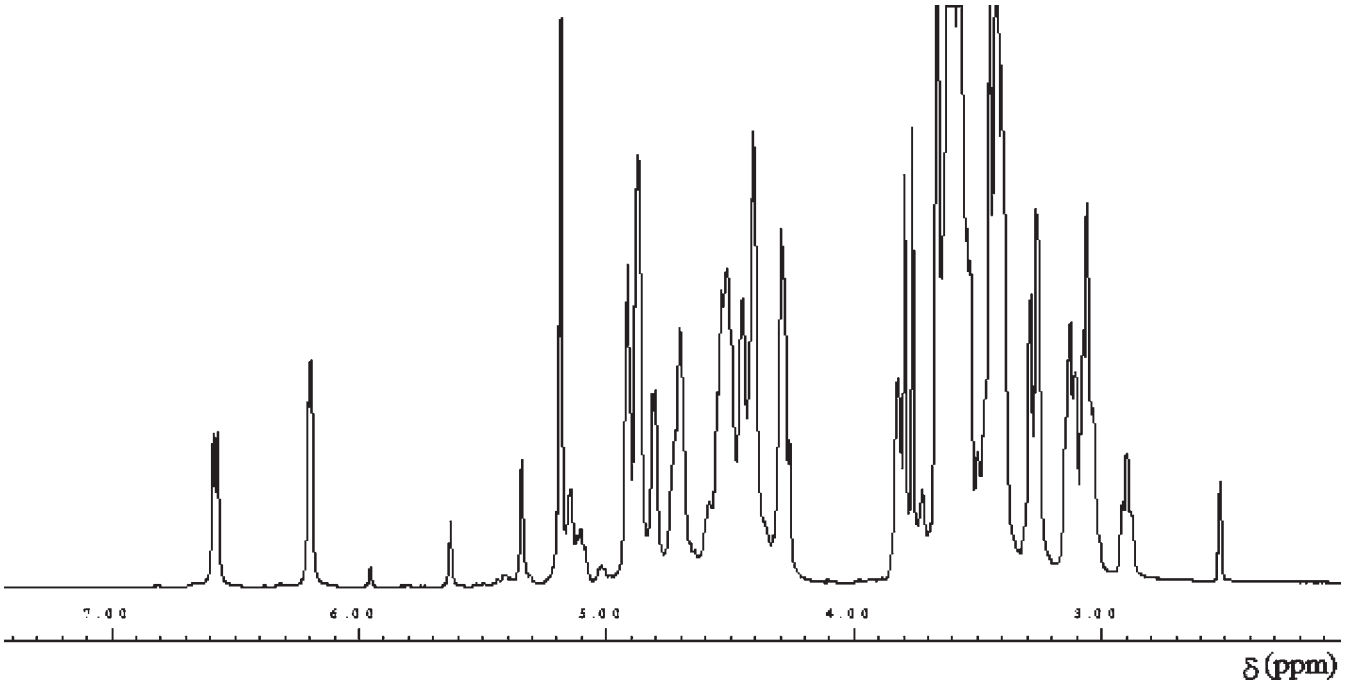

Figure 1. 1D DMSO-d_6 ^{1}H NMR spectrum of an authentic honey.

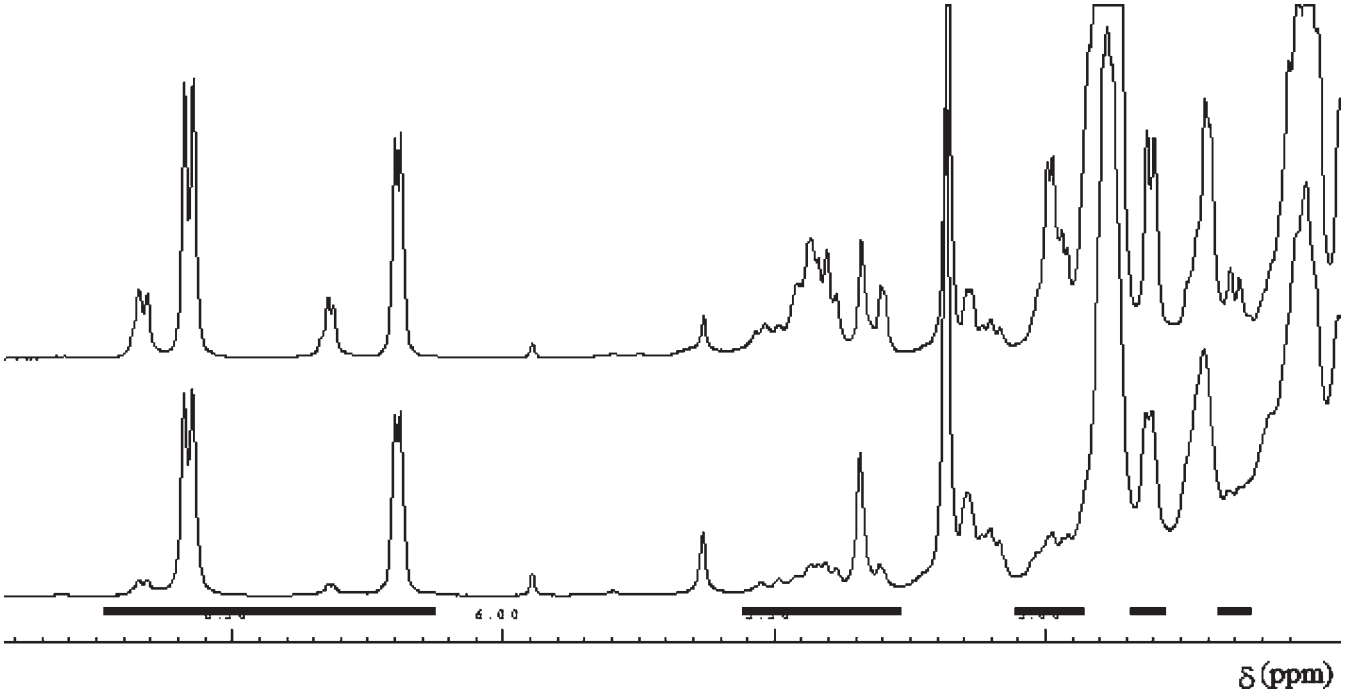

Figure 2. Comparison between significant spectroscopic regions of an authentic (lower spectrum) and a 40% adulterated honey (upper spectrum). The highlighted spectroscopic regions are those containing the most correlated variables with CF1 and CF2 during GDA.

introduction of a source of variability during the spectroscopic calculation. Also, sensitivity appears to be sufficient for the aim of this study because signals are acquired on the proton channel, preventing partially the low sensitivity typical of ^{13}C spectroscopy. Undoubtedly, the disadvantage of HMBC is that it is a time-consuming approach with respect to the simplest ^{1}H NMR.

HMBC spectroscopy is a particularly useful technique because it connects protons with carbons via two or three bonds, couplings that can supply more information about connectivities. The spectra obtained with DMSO-d_6 show a higher number of signals owing to the presence of correlation points also caused by hydroxyls. A ^{1}H−^{13}C HMBC spectrum of an adulterated honey is shown in **Figure 3**. Also, in this spectrum some signals related to disaccharides are well evident in the encircled areas between δ_H 6 and 6.6 corresponding to anomeric hydroxyls of maltose and in areas surrounding δ_H 5.4 and between δ_H 4.5 and 5 corresponding to hydroxyls of maltose, sucrose, and other oligosaccharides.

Statistical Analysis. *^{1}H NMR.* With regard to the data set obtained by 1D spectra, the statistical analysis provided acceptable results. The GDA model was performed on a data set

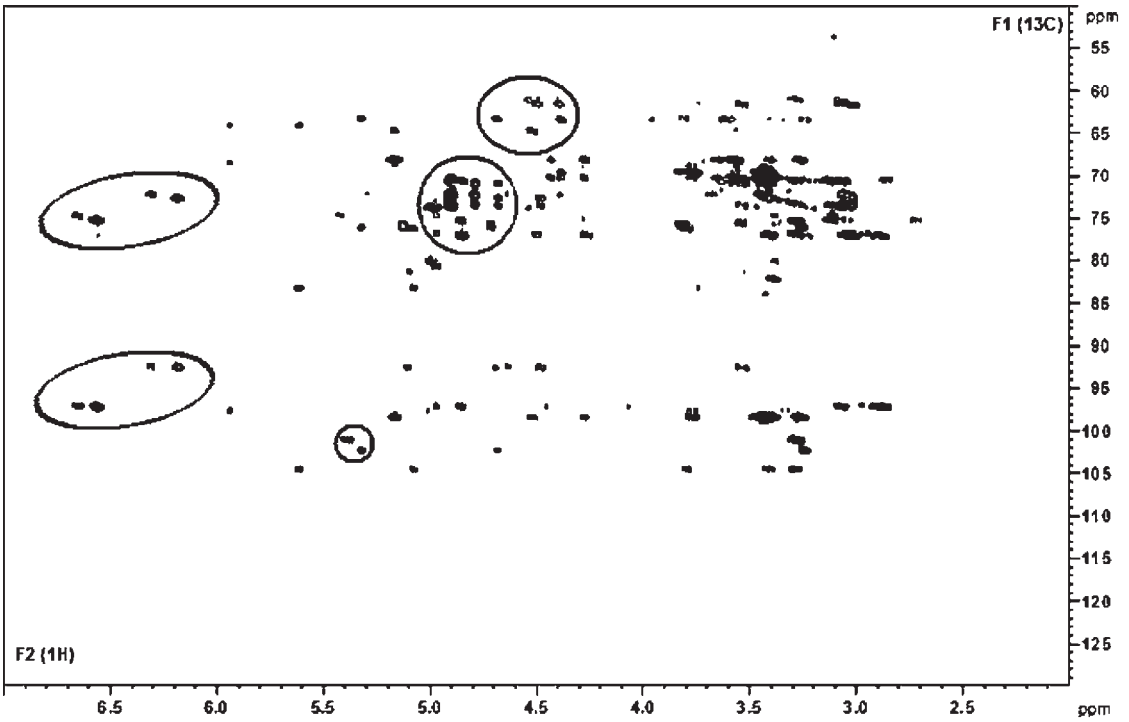

Figure 3. 2D DMSO-d_6 $^1H-^{13}C$ HMBC spectrum of an adulterated honey.

obtained by applying a preventive factorial selection of the spectroscopic variables. Only the variables with a factorial weight during FA > |0.7| were retained, obtaining in this way a reduced data set of 315 variables. On this reduced data set a forward stepwise GDA was performed, choosing $P < 0.01$ as selection criterion for model entry; in this way 55 variables were introduced in the model. The first and second canonical functions explained the 73.1 and 24.9% of the total variance, respectively. The most correlated variables were those in the anomeric spectroscopic region (δ_H 6.10−6.60 for signals related to glucose and maltose and around δ_H 5.00 for signals related to fructose and sucrose) (**Figure 2**). As evident from **Figure 4A**, the GDA model was able to identify sufficiently separated clusters corresponding to the authentic and adulterated honeys. In particular, CF1 was able to discriminate between different adulteration levels. From the validation results reported in **Table 2**, is evident that the 96.8% of original cases were correctly classified by the model and, during cross-validation, 95.2% of samples were exactly assigned to their groups. In particular, it should be noted that the model was able to correctly classify all of the authentic honeys and that all of the misclassifications ($n = 6$) were referred to adulterated honeys. In particular, the method was perfectly able to identify the adulteration, and it was sufficiently accurate to correctly classify adulterated honeys in accordance with the syrup addition levels, with a prediction capacity of 90.5% (only 6 of 63 cases were misclassified).

$^1H-^{13}C$ HMBC. In **Figure 4B** and in **Table 3** the results obtained by HMBC analysis are reported. A forward stepwise GDA was performed on the complete data set of 138 regions of interest using $P < 0.01$ as selection criterium; in this way 22 regions were selected and introduced in the models. The first and second CFs explained 98.6% (89.3 and 9.8%, respectively) of the total variance. This difference in explanatory capacity with respect to results obtained from 1D NMR was why CF2 failed to adequately separate clusters. The most significant regions were those related to anomeric signals of glucose and maltose δ_H 6.60 and 6.10 in F2 (^1H) and those belonging to hydroxyls of different

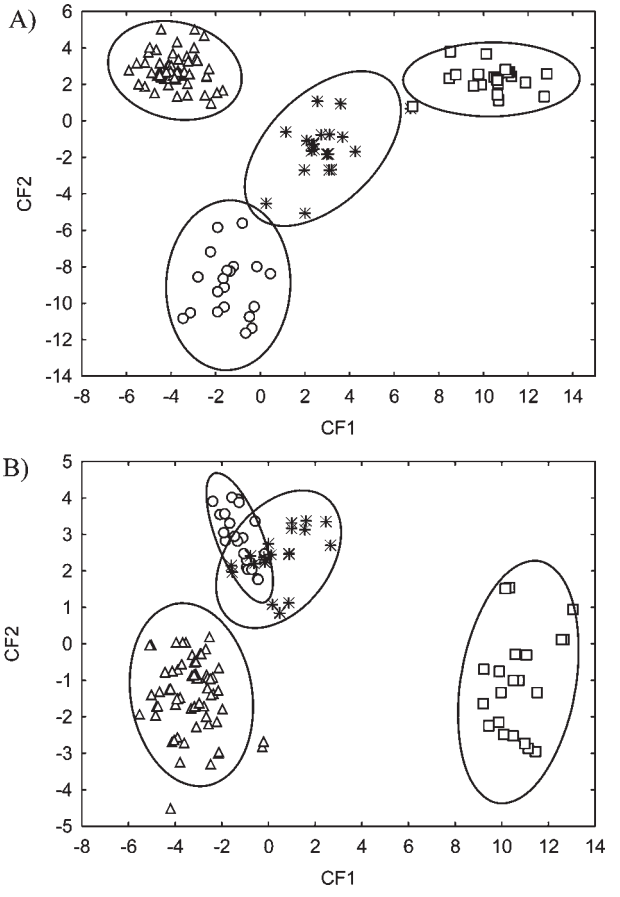

Figure 4. (**A**) Score plot of the first two canonical functions for the data set obtained by DMSO-d_6 ^1H NMR spectra. (**B**) Score plot of the first two canonical functions for the data set obtained by DMSO-d_6 $^1H-^{13}C$ HMBC [10% adulterated honeys (○), 20% adulterated honeys (∗), 40% adulterated honeys chestnut honeys (□), authentic honeys (△)]. The confidence ellipses coefficient is set to 95%.

Table 2. Classification Results Using ^{1}H NMR[a]

			predicted group membership adulteration level				
			10%	20%	40%	authentic	total
original	count	adulterated 10%	19	2	0	0	21
		adulterated 20%	1	19	1	0	21
		adulterated 40%	0	0	21	0	21
		authentic	0	0	0	63	63
	%	adulterated 10%	90.5	9.50	0	0	100
		adulterated 20%	4.75	90.5	4.75	0	100
		adulterated 40%	0	0	100	0	100
		authentic	0	0	0	100	100
cross-validate	count	adulterated 10%	19	2	0	0	21
		adulterated 20%	2	18	1	0	21
		adulterated 40%	0	1	20	0	21
		authentic	0	0	0	63	63
	%	adulterated 10%	90.5	9.50	0	0	100
		adulterated 20%	9.50	85.75	4.75	0	100
		adulterated 40%	0	9.50	90.5	0	100
		authentic	0	0	0	100	100

[a] Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case. 96.8% of original grouped cases were correctly classified. 95.2% of cross-validated grouped cases were correctly classified.

Table 3. Classification Results Using HMBC[a]

			predicted group membership adulteration level				
			10%	20%	40%	authentic	total
original	count	adulterated 10%	16	5	0	0	21
		adulterated 20%	4	17	0	0	21
		adulterated 40%	0	0	21	0	21
		authentic	0	0	0	63	63
	%	adulterated 10%	76.2	23.8	0	0	100
		adulterated 20%	19.1	80.9	0	0	100
		adulterated 40%	0	0	100	0	100
		authentic	0	0	0	100	100
cross-validate	count	adulterated 10%	16	5	0	0	21
		adulterated 20%	5	16	0	0	21
		adulterated 40%	0	0	21	0	21
		authentic	1	1	0	61	63
	%	adulterated 10%	76.2	23.8	0	0	100
		adulterated 20%	23.8	76.2	0	0	100
		adulterated 40%	0	0	100	0	100
		authentic	1.60	1.60	0	96.8	100

[a] Cross-validation is done only for those cases in the analysis. In cross-validation, each case is classified by the functions derived from all cases other than that case. 92.8% of original grouped cases were correctly classified. 90.5% of cross-validated grouped cases were correctly classified.

sugar moieties between δ_H 5.40 and 4.50 in F2 (^{1}H) and are highlighted in **Figure 3**.

As evident from **Figure 4B**, this approach appeared to be less satisfactory than the ^{1}H NMR, which was confirmed also by cross-validation results. Considering the graphical results, the method was able to perfectly cluster samples adulterated at 40% and authentic honeys, whereas results for the samples adulterated with 10 and 20% of syrup were less satisfactory. With regard to the cross-validation results, 92.8% of original grouped cases and only 90.5% of cross-validated grouped cases were correctly classified. Although these results apparently appear worse than those obtained from 1D spectra, also in this case all of the authentic honeys samples, except for two which were confused and were classified as adulterated with 10 or 20% of syrup, were correctly classified. Moreover, no adulterated sample was identified as authentic (**Table 3**).

In conclusion, considering the obtained results, it can be concluded that the use of high-resolution NMR coupled with an appropriate data processing procedure and multivariate statistical methods enabled development of sufficiently effective and appropriate models for honey adulteration detection. The sample preparation step is very simple and quick, and it is certainly comparable to those necessary to prepare samples for other techniques (i.e., FTIR methods). Other NMR applications, in particular, the very effective site-specific natural isotopic fractionation, require a sample preparation step considerably more complex and time-consuming with respect to 1D and 2D direct measurements. Therefore, it is interesting to note that the best results were obtained using the ^{1}H NMR sequence, which represents the simplest and fastest NMR technique with experimental times comparable to those of other well-established spectrometric procedures.

The developed models seem to be particularly related to signal belonging to hydroxyls of sugars and in particular to those of anomeric hydroxyls, as suggested by the fact that the most significant signals were identified in the spectroscopic region between δ_H 5.00 and 7.00. The experimental plan employed demonstrated that the models were sufficiently robust, also considering the variability due to botanical source and the different compositions of sugar solutions; therefore, it seems to be very promising to be applied on completely unknown samples in real cases of honey adulteration. This result was also demonstrated by the fact that the misclassified honey samples were of different botanical origins (three robinia, two citrus, and one chestnut honey) and were adulterated with different syrups (syrups 1, 7, 6, 6, 5, and 4, respectively). Moreover, it should be considered, for further applications, that any increase of database size can only improve the obtained results.

The classification results are absolutely comparable to those obtained by other authors using different techniques; in particular, the proposed model has a predictive capacity certainly comparable to that obtained using FTIR (*9*). Moreover, the NMR technique has the undoubted advantage of allowing the individuation and the assignment of anomalous signals to specific molecular structures. This fact will allow, after the construction of a sufficient database, the identification with high accuracy of the origin and kind of sugar syrup using its molecular fingerprint. Such an approach appears to be very difficult using a less informative technique such as FTIR. In fact, NMR allows discrimination between different signals of very similar chemical moieties of very similar substances. Besides, NMR will allow the performance, if necessary, of a simultaneous quantification of different sugars in an effort to properly estimate the level of adulteration with precision and accuracy. This work should be the basis for all of these kinds of future applications. Finally, due to the high cost of instrumentation and complexity of the technique itself, NMR instruments are certainly less common and available in control laboratories than other more popular techniques. However, in the coming years, also due to the falling prices for basic and advance instrumentation, the already evident dissemination of high-field instruments will continue. In our opinion it is therefore probable that the NMR techniques will continue to spread in the future also as routine techniques for the analysis of honey.

ABBREVIATIONS USED

HMBC, heteronuclear multiple bond correlation; F1, indirect HMBC dimension; F2, acquisition or direct HMBC dimension;

FA, factor analysis; GDA, general discriminant analysis; CF1, canonical function 1; CF2, canonical function 2.

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