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Geographical Characterization of Polyfloral and Acacia Honeys by Nuclear Magnetic Resonance and Chemometrics

ROBERTO CONSONNI* AND LAURA R. CAGLIANI

Istituto per lo Studio delle Macromolecole, Laboratory, NMR, CNR, v. Bassini 15, 20133 Milan, Italy

The importance of geographical origin determination is an increasing and pressing requirement for all foods. Honey is one of the largest studied foods due to its nutritional and medicinal properties in a correct diet. In this paper, a total of 41 honey samples (polyfloral and acacia) from different countries have been analyzed in terms of ^{1}H NMR spectroscopy coupled with multivariate statistical methods. Unsupervised principal component analysis resulted as an efficient tool in distinguishing ^{1}H NMR spectra of polyfloral and acacia honey samples and for geographical characterization of the latter ones. Hierarchical projection to latent structures discriminant analysis was successfully applied for the discrimination among polyfloral honey samples of different geographical origins. ^{13}C NMR spectroscopy was applied to honey samples with the aim to investigate possible sugar isoforms differentiation. Our preliminary data indicated a different isoforms ratio between βFP and βFF only for polyfloral Argentinean samples, while Hungarian samples showed resonance shifts for some carbons of αFF, βFP, βFF, and αGP isoforms for both varieties. These data confirmed the potentiality of ^{13}C spectroscopy in food characterization, especially in sugar-based foods.

KEYWORDS: Honey; PCA; hierarchical PLS-DA; ^{1}H and ^{13}C NMR; geographical and botanical characterization

INTRODUCTION

According to the Codex Alimentarius Commission (*1*), honey is defined as a natural sweet substance produced by honey bees and is made up of water and sugars, mainly fructose and glucose. Other minor components like proteins, free amino acids, flavors and aromas, pigments, vitamins, and many volatile compounds are present, constituting the organoleptic and nutritional properties of honeys. Honey is a very popular and appreciated product all around the world because of its readily available source of energy and also because of its antibacterial and antioxidant activity (*2, 3*). The large variety of honey available nowadays in the European markets, whether multi- or polyfloral type, presents large differences in physical, chemical, and organoleptic characteristics because of the variable percentage of different plants from which they are obtained. On the opposite, uni- or monofloral honeys are constituted of nectar belonging to a single plant in an extent of at least 10−20 (i.e., citrus, arbutus, lavender, thymus, and rosemary) or 45% for the others (i.e., eucalyptus or castanea) (*4–6*).

Italian production of nectar and honeydew in 2007 was as high as 20000 tons. Unfortunately, adulteration was largely performed (mainly due to the lower price of honey from China or Latin America). Thus, it is necessary to control the quality and authenticity to preserve the production area, to develop particular standards of quality, and to protect consumers from commercial speculation (*7*). Differences in price and quality also persist between countries within Europe and even among regions of the same country. In particular, the European Union Commission is encouraging the development of new analytical methods to control and verify the quality specification for different honeys and to characterize the geographical origin.

Following the Codex Alimentarius Standard for honey (*1*) and the Council directive (*8*), the use of honey botanical designation is allowed according to floral or plant source, whereas the use of the geographical origin is allowed when produced exclusively within the area declared in the label. The council directive (*8*) established that the product label should report the place where the honey was gathered. Honey originating from different countries, either from the European Community (EC) or not, should be indicated with the mentions "blend of EC honeys", "blend of non-EC honeys", or "blend of EC and non-EC honeys".

Actually, botanical and geographical origin is evaluated by pollen analysis (melissopalynology) because pollen reflects the vegetation from which the nectar was collected. However, for a correct evaluation of the botanical origin, sensory and several physicochemical analyses like color, flavor, pH and total acidity, electrical conductivity, optical activity, moisture, sugar profile, proline amount, and invertase and diastase activity are needed (*9, 10*). Melissopalynology presents some limitations (*11*): a great knowledge of pollen morphology and specialized

* To whom correspondence should be addressed. Tel: 02-23699578. Fax: 02-23699620. E-mail: roberto.consonni@ismac.cnr.it.

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professional personnel are required to achieve reliable results. Besides, the pollen analysis is quite time-consuming due to pollen scarcity, and fraudulent additions could invalidate the results (*12*). Nevertheless, a geographical characterization of Greek thyme honey has been recently obtained with a discriminant analysis protocol (*13*). Many other studies were performed to establish new methodologies and different analytical methods, mostly combined with chemometric analysis, to assess the geographical origin of honey, with either different or equal botanical origin (*14–21*).

Among all of the analytical methods applied in food characterization, NMR has achieved during last years general acceptance as a powerful method (*22, 23*) due to its noninvasive characteristics, high reproducibility, and sensitivity demonstrated in a large range of applications. NMR studies, focused on structure determination of specific compounds in honey, were already present in the literature (*24–27*), and recently, a classification method based on heteronuclear NMR experiments was proposed (*28*) with the attempt to classify different botanical origins of honey samples. In this work, we present a study focused on the geographical differentiation of polyfloral and acacia honeys from different countries (EC and non-EC) by using ^{1}H NMR combined with multivariate statistical protocols. Furthermore, a ^{13}C NMR-based approach showed the possibility to analyze the isoforms of the most abundant sugars, giving promising results in honey characterization. The main advantage of our methodology resumes a rapid and sensible analysis with very simple sample preparation.

MATERIALS AND METHODS

NMR Analysis. Twenty-three samples of polyfloral and 18 samples of acacia honey of different geographical origin were analyzed. Among the polyfloral honey samples, 15 were of certain origin: three from Argentina, 12 from EC countries, and precisely seven from Hungary and five from Italy. Another eight samples were purchased directly on the market: one belonged to Argentina, three to EC countries (one from Italy, one from Hungary, and one a blend of different EC countries), while four were blends of honeys from EC and non-EC countries. All acacia honey samples were of certain origin: 15 were from Italy, while three were from Hungary. ^{1}H NMR spectra were recorded on a Bruker DMX 500 spectrometer (Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany) operating at 11.7 T and equipped with a 5 mm reverse probe with *z*-gradient. Two replicates were taken for each sample, prepared in double after mixing to minimize possible variability: about 100 mg of honey was dissolved in 600 μL of deuterated water (Sigma-Aldrich, 99.96 atom % D, Milan, Italy) for each sample. The measured pH ranged between 3.5 and 4.5 for all analyzed samples. All spectra were recorded at 300 K, with 7500 Hz spectral width and 32 k of data points. Solvent suppression was achieved by applying a presaturation scheme with low-power radiofrequency irradiation. An exponential function with LB = 0.3 was applied before Fourier transformation, and phase and baseline were manually corrected with ACD/Spec Manager (ACD Laboratories, version 8.12, Toronto, Canada) software. Spectra were referenced and scaled to TSP (trimethylsilyl 2,2,3,3-$^{2}H_4$ propionate) external standard and reduced to integrated regions (buckets) of equal width (0.04 ppm) over the spectral region 10.49 to 0.13 ppm. Spectra alignment resulted in no significant resonance shifts for all signals when the anomeric α-glucose proton was used as a reference: the bucket size provided for possible small resonance shifts. ^{13}C spectra were acquired with samples prepared by dissolving 200 mg in 500 μL of DMSO-$^{2}H_6$ solvent (Cambridge Isotope Laboratories, Inc. 99.9 atom % D, Andover, United States), with 1024 transients and 32786 points covering 30000 Hz and a pulse width of 13 μs (90° pulse) and 5 s for relaxation between each scan. A standard inverse-gated decoupling pulse sequence from a Bruker library was adopted to avoid the heteronuclear nuclear Overhauser effect derived from proton decoupling. Spectra were processed by applying an exponential line broadening of 1 Hz for sensitivity enhancement before Fourier transformation; phases, baselines, and references were made by using the solvent signal as a chemical shift standard (TOPSPIN1.2 Bruker Biospin GmbH Rheinstetten, Karlsruhe, Germany).

Statistical Methods. NMR data converted into Excel worksheet (Microsoft) were imported into SIMCA-P 11 (Umetrics, Umea, Sweden) for statistical analysis. Principal component analysis (PCA) and hierarchical projection to latent structures discriminant analysis (PLS-DA) were performed with "mean centering" and "unit variance" (UV), respectively, as data pretreatment.

PCA analysis is a well-known data compression technique that searches for the main variability of the original data set, without any preconceived ideas, on the possible relationship between samples (observations) and responses (variables) (*29*). Measuring *N* variables (NMR signals in our case) for each sample, the data can be represented into a *N* dimensional space; however, the correlations among variables could reduce the dimensionality into a small number of factors or components that are descriptive of the maximum variation within the data. These components (principal components) can be displayed graphically as a "score" plot, useful to observe any grouping in the original data set, representing the new coordinates for the observations. Coefficients by which the original variables are multiplied to obtain the PCs are called "loadings", and these coefficients define the orientation of the PC's plane with respect to the original data set.

^{1}H NMR honey spectra were characterized by strong sugar signals and very small signals, thus supporting the possible use of the "hierarchical approach" (*30*). When hierarchical models are concerned, the whole X-matrix was divided into block A, corresponding to a region from 2.97 to 5.49 ppm (excluding the water region from 4.65 to 4.85 ppm) dominated by strong sugar signals and into block B from 0.13 to 2.97 ppm and from 5.49 to 10.49 ppm, collecting small signal intensities for amino and organic acids. Each block was modeled applying PCA; the two sets of score vectors consisting of four orthogonal variables, "super variables", were used to build up the data matrices of the upper level, and PLS-DA (*31*) with UV scaling as data pretreatment was performed. PLS-DA is based on the application of the PLS method to the $X-Y$ matrices where the response is formed by dummy variables corresponding to the different classes. The *Y*-matrix is generated by the following simple rule: when the sample *i* belongs to the class *j*, the value of the Y_{ij} element will be one, while the other elements of the row *i* will be equal to zero. This representation allows the application of PLS regression as a discriminating tool making use of previous information about class separation in the training set. Among 15 polyfloral honey samples of certain origin, 13 constituted the training set, while the remaining 10 samples constituted the test set. Acacia honey samples did not require a classification in both training and test sets because they are easily discriminated by PCA.

RESULTS AND DISCUSSION

^{1}H NMR provides a simple method to obtain global information about complex samples in a single experiment maintaining the natural ratio of the substances present. **Figure 1A** represents a typical ^{1}H NMR spectrum of polyfloral honey water solution, showing dominant resonances of main components content. Different spectral regions are characterized by specific compound resonances, like the methyl amino acid region (0−2 ppm), sugars region (3−5.5 ppm), and aromatic region (6−10 ppm). Among all resonances, several compounds can be readily identified and resumed in **Figure 1B−D**. In the sugar region, specific signals of glucose, 5.17 and 4.58 ppm (α and β anomeric protons, respectively), and of α-fructose, 4.04 and 3.97 ppm (H_5 and H_4, respectively), were recognized. Other sugars, with very low anomeric proton signal intensity, were identified by the addition of standard solution: sucrose at 5.35 ppm, turanose at 5.24 ppm, and raffinose at 5.37 and at 4.94 ppm (**Figure 1C**). ^{1}H NMR spectra of acacia samples were similar to those of polyfloral honey, showing the same water-soluble compounds content but in different concentrations. In particular, the comparison among proton spectra of the two varieties

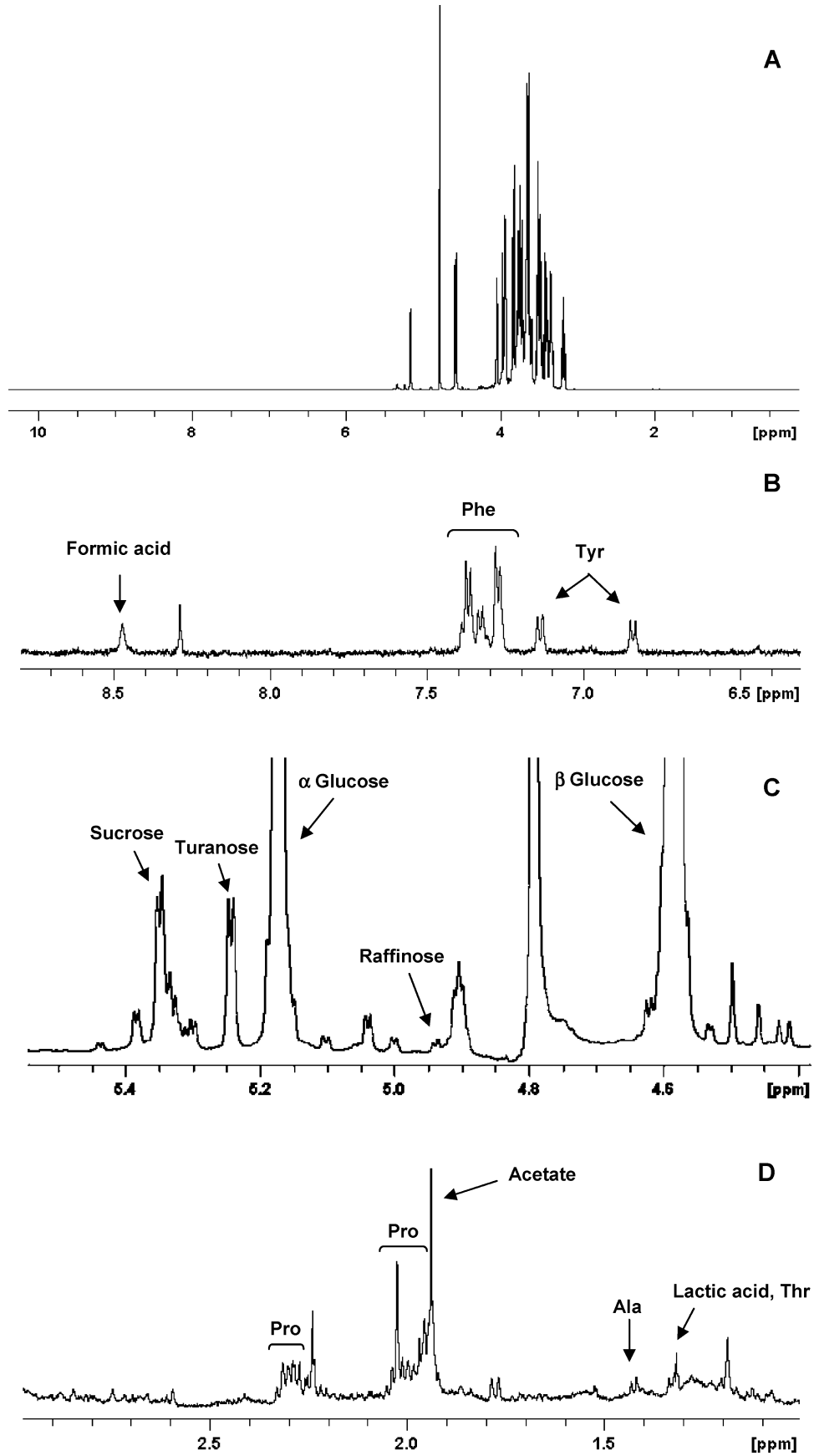

Figure 1. (**A**) Complete ^{1}H NMR spectrum of polyfloral honey sample dissolved in water. Expansions of the aromatic (**B**), anomeric (**C**), and aliphatic (**D**) regions showing principal spin system assignments.

revealed an evidently larger fructose and sucrose content in acacia honey than in polyfloral samples. Analysis of ^{13}C spectra recorded with very high S/N ratio (8960 scans) did not reveal the presence of low abundant sugars detected with other techniques (*32*), due to low sensibility of the ^{13}C nucleus. In this latter case, the use of more sensible techniques, like HPLC-

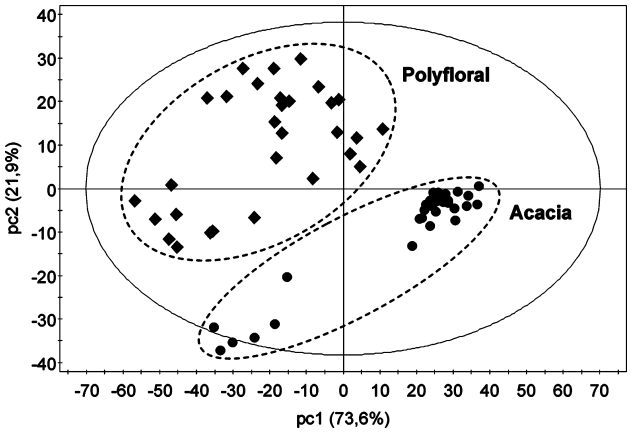

Figure 2. PCA score plot performed by considering 15 polyfloral (filled diamond) and 18 acacia (filled circle) honey samples of certain origin. Pc1 = 73.6% and pc2 = 21.9%. $R^2X = 99.9\%$ and $Q^2 = 99.4\%$.

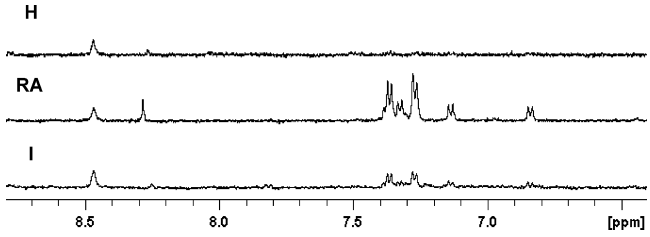

Figure 3. Expansions of ^{1}H NMR aromatic regions of polyfloral honeys spectra of certain origins. From the top are represented typical Hungarian (H), Argentinean (RA), and Italian (I) honeys.

MS, coupled with the high specificity of NMR method, would improve the samples characterization.

The low field region highlighted the content of tyrosine (Tyr), phenil alanine (Phe), and formic acid (**Figure 1B**), while the amino acids region resulted characterized by the dominant presence of proline (Pro). Other amino and organic acids like alanine (Ala), threonine (Thr), acetate, and lactic acid were also detected (**Figure 1D**).

PCA analysis was performed considering all honey samples of certain origin: 15 polyfloral and 18 acacia. The first two PCs explaining 95.5% of the total variance lead to a clear differentiation among samples according to the different variety, as depicted in the score plot of **Figure 2**. Acacia samples resulted from the corresponding loading plot (data not shown) to be enriched in sugar content with respect to polyfloral honey samples.

Concerning polyfloral honey samples, PCA performed on a full spectrum (data not shown) did not lead to a clear sample separation according to the geographical origin. Accurate inspection of ^{1}H NMR regions of low abundant resonances, such as aromatic one, revealed clear different compounds content due to aromatic amino acids and formiate. In particular, Argentinean honeys were much more rich in Phe and Tyr with respect to Italian and Hungarian ones (**Figure 3**). This suggests the use of a hierarchical approach (*33*) to preserve the information for very low NMR signals otherwise strongly reduced by the more intense sugar signals when the complete spectrum is considered. We performed hierarchical PLS-DA by using 13 polyfloral honeys of certain origin samples constituting the training set: the first two PCs were scored in the plot of **Figure 4**, modeling both selected regions of the ^{1}H NMR spectrum independently. A clear differentiation of samples according to the geographical origin was achieved. The model diagnostics were summarized by the fit goodness, R^2 (91.9%), and the

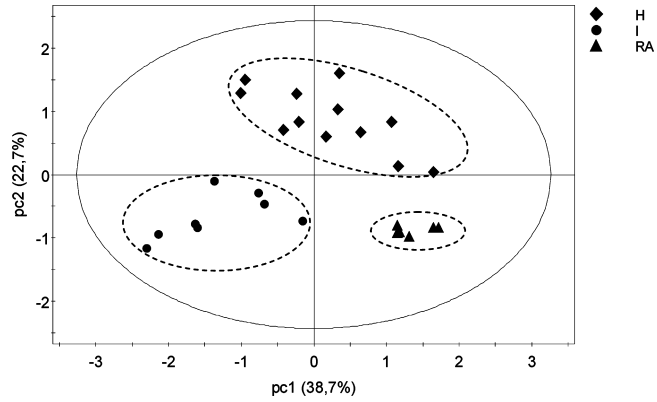

Figure 4. Hierarchical PLS-DA score plot performed by considering 13 polyfloral honey samples of certain origins, constituting the training set. Filled symbols represent honey samples from Hungary (diamond), Italy (circle), and Argentina (triangle). Pc1 = 38.7% and pc2 = 22.7%. $R^2X = 91.9\%$, $R^2Y = 79.7\%$, and $Q^2 = 72.7\%$.

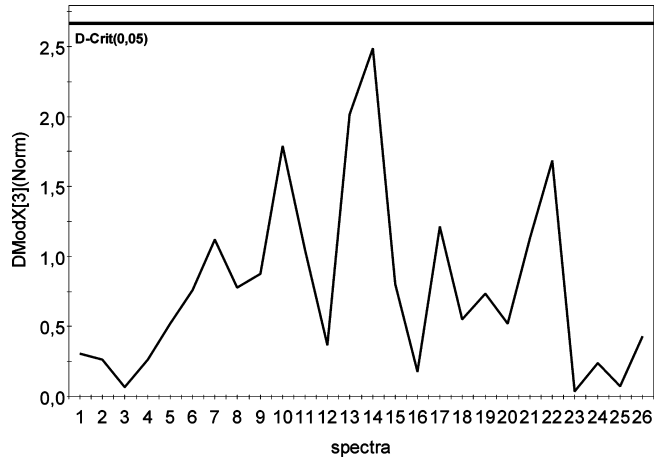

Figure 5. Residual standard deviation plot (DModX) for 13 training set polyfloral samples for a hierarchical PLS-DA model. All observations were largely below the *D* critical value of 0.05.

prediction goodness parameter, Q^2 (72.7%); the cross-validation (CV) was used to estimate the predictive ability of the model (*34*). In this score plot, a very low sample variability between replicates can be evaluated by observing the close proximity of the observations, thus supporting both the strong reproducibility of the NMR method and the sample homogeneity.

The model validation can be estimated by the use of a residual matrix. It expresses the deviations between the original values and projections, by the use of residual standard deviations that can be computed for the observations. These residuals were also known as DModX, and the corresponding plot is presented in **Figure 5**. This plot enabled the evaluation of outliers in the hierarchical PLS-DA model: all of the observations were largely below the *D* critical value, thus indicating no outliers detection and confirming the model validity.

Test set samples were reprojected into the PLS-DA model to check whether they could be classified with respect to the training set samples. In the bidimensional score plot (**Figure 6**) of the predicted *t* scores (tPS), filled and open symbols represented training and test set samples, respectively. Test set samples from Italy (I, open circle) were correctly predicted, resulting within the other Italian training set samples. The same held true for Argentina (RA, open triangle) and Hungary (H, open diamond) test set samples. Noticeably, samples of mixed origin (EC and non-EC countries, inverted open triangle) were clustered in the middle of the score plot, thus confirming their

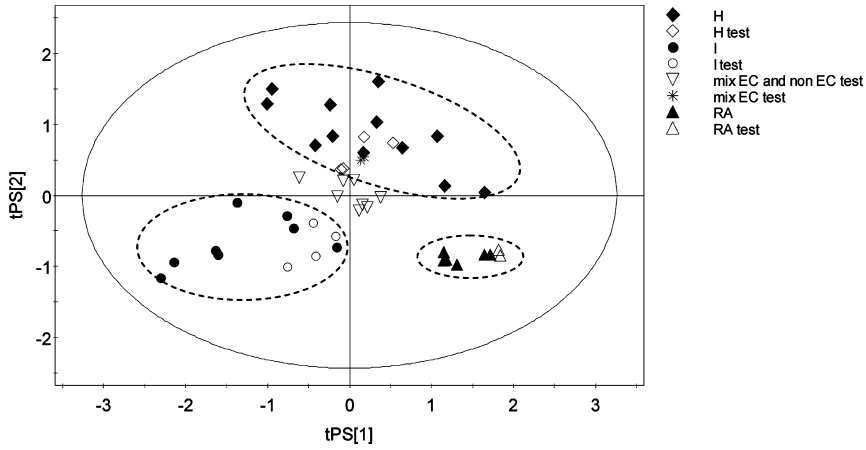

Figure 6. Hierarchical PLS-DA performed considering 13 polyfloral honeys of certain origins (training set) with reprojection of polyfloral test set sample scores (10 samples). Filled symbols represent training set honey samples from Hungary (H, diamond), Italy (I, circle), and Argentina (RA, triangle), while open symbols represent test set honey samples from Hungary (H, diamond), Italy (I, circle), and Argentina (RA, triangle) from different EC countries (star) and from different EC and non-EC countries (inverted triangle).

Table 1. Classification List for All Polyfloral Honey Test Set Samples Reprojected onto the Hierarchical PLS-DA Model Performed by Considering 13 Polyfloral Honey Samples as a Training Set[a]

	prediction classes		
test set spectra countries	Argentina	Hungary	Italy
Argentina	**0.88**	0.12	0.00
Argentina	**0.90**	0.08	0.02
Hungary	0.10	**0.65**	0.24
Hungary	0.11	**0.67**	0.23
Hungary	0.01	**1.07**	−0.08
Hungary	−0.10	**1.10**	0.00
Italy	0.46	−0.28	**0.82**
Italy	0.46	−0.14	**0.68**
Italy	0.47	−0.02	**0.55**
Italy	0.37	0.05	**0.58**
mix EC	0.08	**0.80**	0.12
mix EC	0.07	**0.83**	0.10
mix EC and non-EC	0.35	0.31	0.34
mix EC and non-EC	0.34	0.35	0.31
mix EC and non-EC	0.20	0.45	0.35
mix EC and non-EC	0.15	**0.57**	0.27
mix EC and non-EC	0.04	**0.54**	0.42
mix EC and non-EC	0.29	0.44	0.28
mix EC and non-EC	0.34	0.44	0.21
mix EC and non-EC	0.16	**0.61**	0.23

[a] Values higher than 0.6 indicate a probability higher than 10% to belong to the class.

mixed content. Blended samples from EC countries (star) resulted in the Hungary grouping, most likely due to its nonbalanced mixed composition, enriched in Hungarian honey. The robustness of hierarchical PLS-DA model could be evaluated by the classification list, showed in **Table 1**. Each sample of the training set was classified by means of a "classification score" indicative of its representativeness. When the probability of belonging to a class is higher than 10%, the object is correctly predicted and the score is larger than 0.6. In our case, all samples belonging to a single country were correctly classified, thus indicating a good model. The classification score obtained from mixed samples reflected their blend composition.

An unsupervised PCA approach enabled a clear geographical differentiation of the 18 acacia honey samples between Italian and Hungarian origins (**Figure 7**). Six PCs explained 99.7% of the total variance with $Q^2 = 98.1\%$. The corresponding loading plot (data not shown) indicated a generally larger content of all water-soluble compounds for Italian samples with respect to foreign samples. Our determination resulted in agreement with

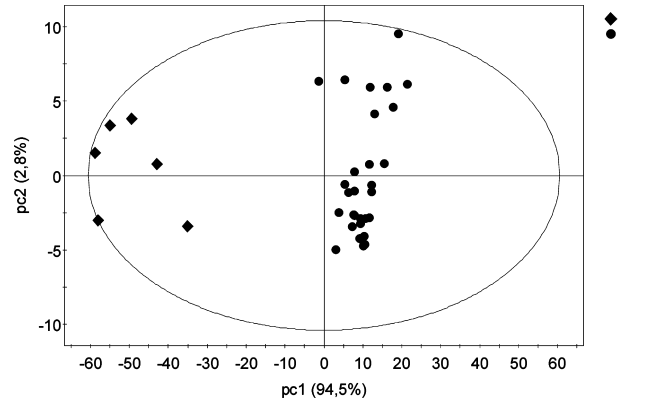

Figure 7. PCA score plot performed considering all 18 acacia honey samples. Filled symbols represent honey samples from Hungary (diamond) and from Italy (circle). Pc1 = 94.5% and pc2 = 2.8%. $R^2 = 99.7\%$ and $Q^2 = 98.1\%$.

what was found previously by using volatile and semivolatile organic compounds for acacia honey samples by means of electronic nose and neural network determination (*17*), and an even better discrimination was achieved with ^{1}H NMR of water-soluble compounds. Honey samples were also investigated by means of ^{13}C NMR spectroscopy, to evaluate the presence of different sugar isoforms. Quantitative analysis obtained by integration of ^{13}C signals for both fructose and glucose (**Figure 8A**) led to F/G ratio for polyfloral (medium value 1.1) and acacia (medium value 1.5) honey samples, confirming the larger presence of fructose in the latter honey variety, as observed with other techniques (*35*). ^{13}C NMR spectra of honey samples revealed the dominant signals of fructose and glucose in their different tautomeric isoforms: In particular, fructose presents α,βFF and α,βFP forms and glucose only α,βGP forms. A water solution of glucose and fructose showed the sugar forms in their "naturally" occurring distribution (*36*), in particular, 2.4, 4.8, 23, and 69.8% of abundance for αFP, αFF, βFF, and βFP, respectively, while glucose presents the pyranose tautomeric forms with 37 and 63% of abundance for αGP and βGP forms, respectively. The analysis of all honey samples performed in organic solvent (DMSO-$^2H_6$) revealed the presence of a small deviation with respect to "natural" isoforms ratio for fructose. Only in RA polyfloral honey (**Figure 8A**), the ratio between βFP and βFF isoforms is significantly almost equal to one, thus suggesting a possible identification marker. It is known that

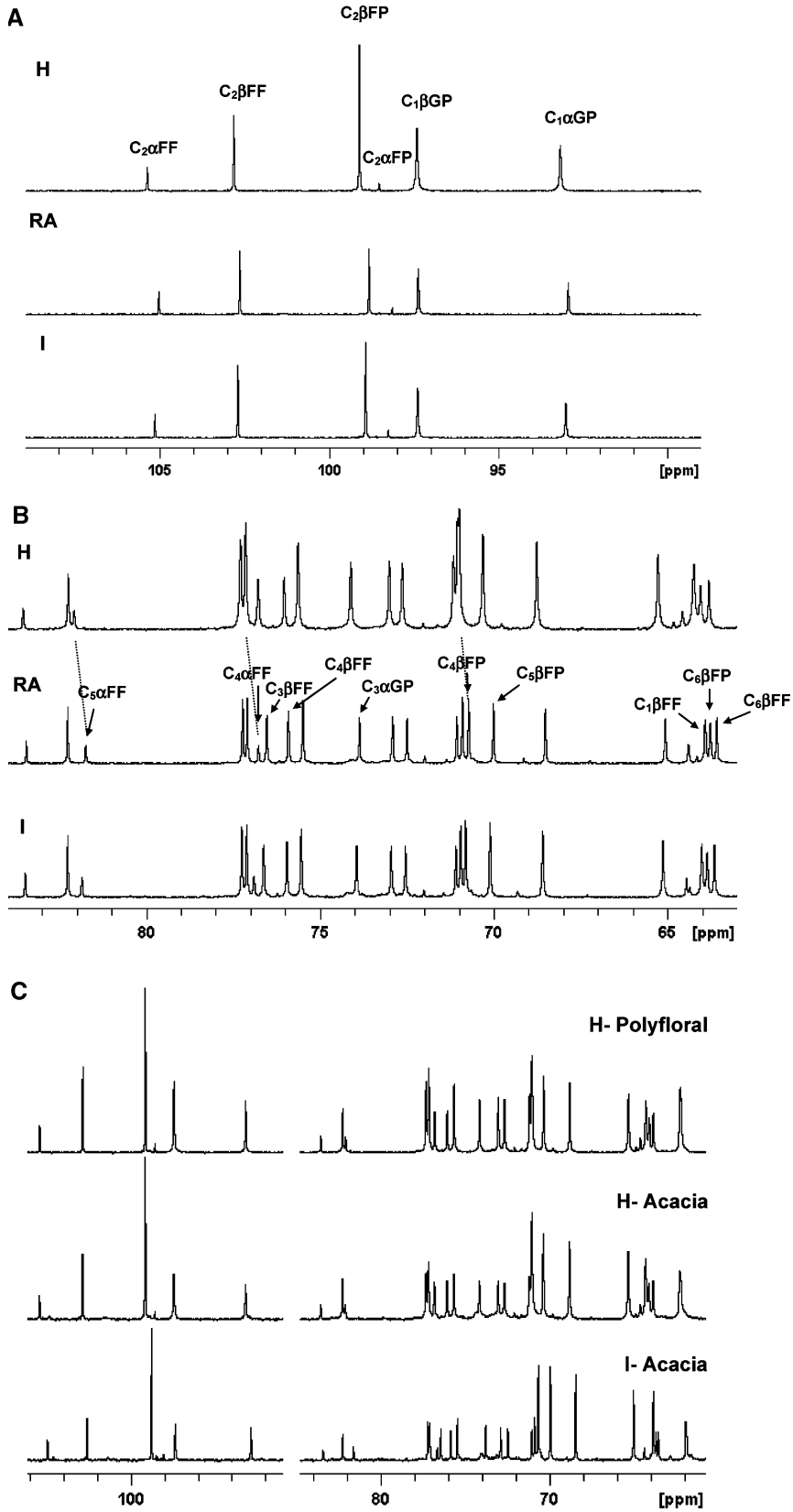

Figure 8. Low (**A**) and high field (**B**) regions of ^{13}C NMR spectra of polyfloral honey samples: from the top are represented a typical Hungarian (H), Argentinean (RA), and Italian (I) honeys. All anomeric signals (**A**) and carbon signals, which presented a significant shift in Hungarian samples with respect to the others (**B**), are indicated. (**C**) ^{13}C NMR spectra of Hungarian polyfloral and acacia and Italian acacia honey samples are compared.

thermal degradation of sugars and in particular of the most abundant βFP isoform leads to furanic derivatives. Among them, the most referenced is HMF (5-hydroxymethyl-2-furfurale), whose concentration is fixed by law below 40 mg/kg (*7*). The reduction in concentration of the βFP isoform observed for RA samples would justify a very large amount of HMF, detectable by ^1H NMR: our unpublished results indicated that the proton NMR detection limit for HMF was as low as 10 mg/kg, and this made us confident to exclude thermal degradation. Interestingly, specific carbons of only H polyfloral and acacia honey

samples experienced large shifts with respect to the same carbons of the other samples when central line of DMSO-$^2H_6$ solvent is aligned: in particular C_2, C_4, and C_5 of αFF; C_2, C_4, C_5, and C_6 of βFP; C_1 C_3, C_4, and C_6 of βFF; and C_1 and C_3 of αGP showed the largest deviations with respect to the medium values observed for RA and I honey samples (**Figure 8B**,**C**. This suggested that the aforementioned fructose and glucose isoforms most likely carried different substituents only in the Hungarian honey samples.

^{1}H NMR spectroscopy is here suggested as a valid tool for food characterization and the combination with chemometric techniques largely improves the capability of sample classification. The simple sample preparation and the high quality results obtained represent a valid alternative to other complex and time-consuming analysis. Among possible multivariate statistical tools, the hierarchical PLS-DA demonstrated the high efficiency in NMR data analysis with the aim of classification capability, as already demonstrated (*33*). The high selectivity achievable with ^{13}C NMR spectroscopy indicated its feasible use for food authentication, as previously reported (*37, 38*). The advantage of ^{13}C NMR with respect to other more sensible techniques (i.e., HPLC-MS) lies on the possibility to identify sugar isoforms otherwise not detectable. In particular, our data concerning sugar isoforms, both βFP and βFF ratio and αFF, βFP, βFF, and αGP shifts, suggest possible geographical markers for RA and H honey samples, respectively. Noticeably, the isoforms shifts are independent from the honey variety. To confirm our preliminary ^{13}C NMR results, a larger sample data set is also in preparation with the aim to control a possible correlation between our data and different productions.

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LITERATURE CITED

(1) Codex Alimentarius Commission. Codex standard 12, Revised Codex Standard for Honey. *Stan. Stan. Methods* **2002**, *11*, 1−8.
(2) Bogdanov, S. Nature and origin of the antibacterial substances in honey. *LWT Food Sci. Technol.* **1997**, *30*, 748–753.
(3) Perez, E.; Rodriguez-Malaver, A. J.; Vit, P. Antioxidant capacity of Venezuelan honey in wistar rat homogenates. *J. Med. Food* **2006**, *9*, 510–516.
(4) Conti, M. E.; Stripeikis, J.; Campanella, L.; Cucina, D.; Tudino, M. D. Characterization of Italian honeys (Marche Region) on the basis of their mineral content and some typical quality parameters. *Chem. Cent. J.* **2007**, *1*, 14–23.
(5) Persano Oddo, L.; Piazza, M. G.; Sabatini, A. G.; Accorti, M. Characterization of unifloral honeys. *Apidologie* **1995**, *26*, 453–465.
(6) Perez-Arquillué, C.; Conchello, P.; Arino, A.; Juan, T.; Herrera, A. Physicochemical attributes and pollen spectrum of some unifloral Spanish honeys. *Food Chem.* **1995**, *54*, 167–172.
(7) Bogdanov, S.; Martin, P. Honey authenticity. *Mitt. Geb. Lebensmittelunters. Hyg.* **2002**, *93*, 232–252.
(8) Council Directive 2001/110/EC of 20 December 2001 relating to honey. *Off. J. Eur. Commun.* **2002**, *L10*, 47−52.
(9) Von Der Ohe, W.; Persano Oddo, L.; Piana, M. L.; Merlot, M.; Martin, P. Harmonized methods of melissopalynology. *Apidologie* **2004**, *35*, S18–S25.
(10) Bogdanov, S.; Ruoff, K.; Persano Oddo, L. Physicochemical methods for characterization of unifloral honeys: A review. *Apidologie* **2004**, *35*, S4–S17.
(11) Molan, P. C. The limitation of the methods of identifying the floral source of honeys. *Bee World* **1998**, *79*, 59–68.
(12) Baroni, M. V.; Chiabrando, G. A.; Costa, C.; Wunderlin, D. A. Assessment of the floral origin of honey by SDS-page immunoblot techniques. *J. Agric. Food Chem.* **2002**, *50*, 1362–1367.
(13) Karabournioti, S.; Thrasyvoulou, A.; Eleftheriou, E. P. A model for predicting geographical origin of honey from the same floral source. *J. Apic. Res.* **2006**, *45*, 117–124.
(14) Cometto, P. M.; Faye, P. F.; Di Paola Naranjo, R. D.; Rubio, M. A.; Aldao, M. A. J. Comparison of free amino acid profile in honey from three Argentinian regions. *J. Agric. Food Chem.* **2003**, *51*, 5079–5087.
(15) Gonzalez Paramas, A. M.; Gomez Barez, J. A.; Garcia-Villanova, R. J.; Rivas Palà, T.; Ardanuy Albajar, R.; Sanchez Sanchez, J. Geographical discrimination of honeys by using mineral composition and common chemical quality parameters. *J. Sci. Food Agric.* **2000**, *80*, 157–165.
(16) Alissandrakis, E.; Tarantilis, P. A.; Harizanis, P. C.; Polissiou, M. Comparison of the volatile composition in thyme honeys from several origins in Greece. *J. Agric. Food Chem.* **2007**, *55*, 8152–8157.
(17) Benedetti, S.; Mannino, S.; Sabatini, A. G.; Marcazzan, G. L. Electronic nose and neural network use the classification of honey. *Apidologie* **2004**, *35*, 397–402.
(18) Ruoff, K.; Luginbuhl, W.; Kunzli, R.; Bogdanov, S.; Bosset, J. O.; Von Der Ohe, K.; Von Der Ohe, W.; Amadò, R. Authentication of the botanical and geographical origin of honey by front-face fluorescence spectroscopy. *J. Agric. Food Chem.* **2006**, *54*, 6858–6866.
(19) Ruoff, K.; Luginbuhl, W.; Kunzli, R.; Iglesias, M. T.; Bogdanov, S.; Bosset, J. O.; Von der Ohe, K.; Von der Ohe, W.; Amadò, R. Authentication of botanical and geographical origin of honey by Mid-Infrared spectroscopy. *J. Agric. Food Chem.* **2006**, *54*, 6873–6880.
(20) Woodcock, T.; Downey, G.; Kelly, J. D.; O'Donnell, C. Geographical classification of honey samples by near-infrared spectroscopy: A feasibility study. *J. Agric. Food Chem.* **2007**, *55*, 9128–9134.
(21) 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. Nutr.* **2005**, *45*, 193–223.
(22) Karoui, R.; De Baerdemaeker, J. A review of the analytical methods coupled with chemometrics tools for the determination of the quality and identity of dairy products. *Food Chem.* **2007**, *102*, 621–640.
(23) Alam, T. D.; Alam, M. K. Chemometric analysis of NMR spectroscopy data: A review. *Annu. Rep. NMR Spectrosc.* **2004**, *54*, 41–80.
(24) Ferreres, F.; Andrade, P.; Tomas-Barberan, F. A. Natural occurrence of abscisic acid in heather honey and floral nectar. *J. Agric. Food Chem.* **1996**, *44*, 2053–2056.
(25) Frerot, E.; Velluz, A.; Decorzant, E.; Naef, R. From linden flower to linden honey. Part 2. Glycosidic precursors of cyclohexa-1,3-diene-1-carboxylic acids. *Chem. Biodiversity* **2006**, *3*, 94–100.
(26) Blunt, J. W.; Munro, M. H. G.; Swallow, W. H. Carbon-13 NMR analysis of tutin and related substances: application to the identification of minor components of toxic honey. *Aust. J. Chem.* **1979**, *32*, 1339–1343.
(27) 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.
(28) Lolli, M.; Bertelli, D.; Plessi, M.; Sabatini, A. G.; Restani, C. Classification of Italian honeys by 2D HR-NMR. *J. Agric. Food Chem.* **2008**, *56*, 1298–1304.
(29) Jackson, J. E. *User's Guide to Principal Components*; Wiley and Sons Eds.: New York, 1991.
(30) Wold, S.; Kettaneh, N.; Tjessem, K. Hierarchical multiblock PLS and PC models for easier model interpretation and as an alternative to variable selection. *J. Chemom.* **1996**, *10*, 463–482.

(31) Wold, S.; Johansson, E.; Cocchi, M. PLS-partial least squares projections to latent structures. In *3D QSAR in Drug Design*; Kubinyi, H., Ed.; ESCOM Science: Leiden, 1993; pp 523−550.
(32) Nozal, M. J.; Bernal, J. L.; Toribio, L.; Alamo, M.; Diego, J. C. The use of carbohydrate profile and chemometrics in the characterization of natural honeys of identical geographical origin. *J. Agric. Food Chem.* **2005**, *53*, 3095–3100.
(33) Consonni, R.; Cagliani, L. R.; Benevelli, F.; Spraul, M.; Humpfer, E.; Stocchero, M. NMR and chemometric methods: A powerful combination for characterization of balsamic and traditional balsamic vinegar of Modena. *Anal. Chim. Acta* **2008**, *611*, 31–40.
(34) Stone, M. Cross-validation: A review. *Math. Oper. Stat.* **1978**, *9*, 127–139.
(35) Mendes, E.; Brojo Proenca, E.; Ferreira, I. M. P. L. V. O.; Ferreira, M. A. Quality evaluation of Portuguese honey. *Carbohydr. Polym.* **1998**, *37*, 219–223.
(36) 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.
(37) Consonni, R.; Cagliani, L. R.; Rinaldini, S.; Incerti, A. Analytical method for authentication of traditional balsamic vinegar of Modena. *Talanta* **2008**, *75*, 765–769.
(38) Vlahov, G.; Del Re, P.; Simone, N. Determination of geographical origin of olive oils using 13C nuclear magnetic resonance spectroscopy. I—Classification of olive oils of the Puglia region with denomination of protected origin. *J. Agric. Food Chem.* **2003**, *51*, 5612–5615.

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