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NMR Characterization of Saccharides in Italian Honeys of Different Floral Sources

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ABSTRACT: The saccharide profiles of 5 different botanical species in 86 Italian honey samples were investigated by ^{1}H and ^{1}H−^{13}C NMR spectroscopy. Nineteen saccharides were identified in the aqueous extracts, namely, fructose, glucose, gentiobiose, isomaltose, kojibiose, maltose, maltulose, melibiose, nigerose, palatinose, sucrose, turanose, erlose, isomaltotriose, kestose, maltotriose, melezitose, raffinose, and maltotetraose. PCA performed on NMR spectral regions, in particular between 4.400 and 5.700 ppm and the fructose signal at 4.050 ppm, revealed a partial sample grouping. The score contribution plots derived from PCA performed using the mean values for the buckets of the anomeric region for each floral source allowed the identification of saccharides characterizing different honeys. OPLS-DA models were further evaluated to confirm the previous findings. OPLS-DA models were also built to highlight differences between polyfloral and high mountain polyfloral honeys and between high mountain polyfloral and rhododendron honeys, both collected at high altitude; S-plots highlighted the characteristic saccharides.

KEYWORDS: *honey, saccharides, botanical origin, NMR, PCA, OPLS-DA, S-plot*

1. INTRODUCTION

Honey is a natural and healthy food produced by honeybees from the nectar of several plants, and it is considered one of the most complex foods produced by nature. Saccharides constitute the majority of compounds in honey, and water, proteins, amino acids, organic acids, vitamins, flavonoids, and alkaloids are present in lower amounts. In honey, as already observed for other foods,[1] minor components are the determinants of specific organoleptic characteristics, whereas the carbohydrate content is responsible for the energy value and the physicochemical properties, mainly viscosity and crystallinity.

In honey, saccharides primarily consist of monosaccharides such as glucose and fructose (30% and 38%, respectively), which in combination with other monosaccharides constitute 70% of the total carbohydrate content in honey, whereas only 10−15% of carbohydrates were disaccharides linked by glycosidic bonds in different positions and configurations.[2] In addition, other saccharides, such as tri- and tetrasaccharides, could be present in small amounts. Several studies have been devoted to determine the carbohydrate profile of honey.[3−5] These investigations typically involve the use of chromatographic methods, sometimes in combination with mass spectrometry;[6] in this latter work, a total of 16 disaccharides and 12 trisaccharides were identified in honey. The analysis of the complex mixture of the carbohydrates present in honey usually requires specific chromatographic procedures, especially in the case of disaccharide separations. Only recently, a two-dimensional GC-TOF-MS method was successfully applied[7] to overcome the coelution problem encountered with anomeric structures. Nevertheless, the time-consuming sample preparation, which involves chemical derivatization, and the demanding analytical requirements make it difficult to perform metabolite characterization of honeys for either geographical or botanical assessments. In contrast, nuclear magnetic resonance (NMR) requires a minimal sample amount and minimal processing procedures. Thus, NMR is a feasible alternative for the investigation of complex mixtures for both structural characterization and chemical compound identification. Several studies concerning the use of NMR for honey characterization have been published,[8,9] but only a few of these studies combined NMR with chromatographic techniques.[10] Some of these previous investigations focused on carbohydrate adulteration[11−13] or carbohydrate determination,[14−17] whereas others were devoted to the botanical or geographical characterization of honey. All of these past studies analyzed specific classes of chemical compounds, including alkaloids,[18] amino acids,[19] organic acids,[20] aromatic compounds,[21] organic extractives,[22] and possible marker compounds,[23−25] but none took into account the total carbohydrate content.

The first NMR study for the analysis of saccharides in honey was published in late 1997[26] in which ^{13}C NMR spectroscopy was applied to the saccharide analysis of artificial and natural honey samples. Although there were intrinsic limitations due to the very low NMR sensitivity of the ^{13}C nucleus, the researchers were able to quantify 10 different saccharides. The present study aims to investigate, for the first time, the potential of applying ^{1}H NMR and chemometrics for the analysis of the saccharide contents of Italian honeys of different botanical origins. Furthermore, we focused our efforts on the differentiation of two highly valued honeys that are both collected at over one thousand meters of altitude, namely, high mountain polyfloral and rhododendron honey, and on the differentiation of two types of polyfloral honeys, namely, polyfloral and high mountain polyfloral honeys. The presented approach resulted in a particularly convenient method because of the minimal sample preparation required. This method could be a valid alternative to chromatographic or melissopalyno-

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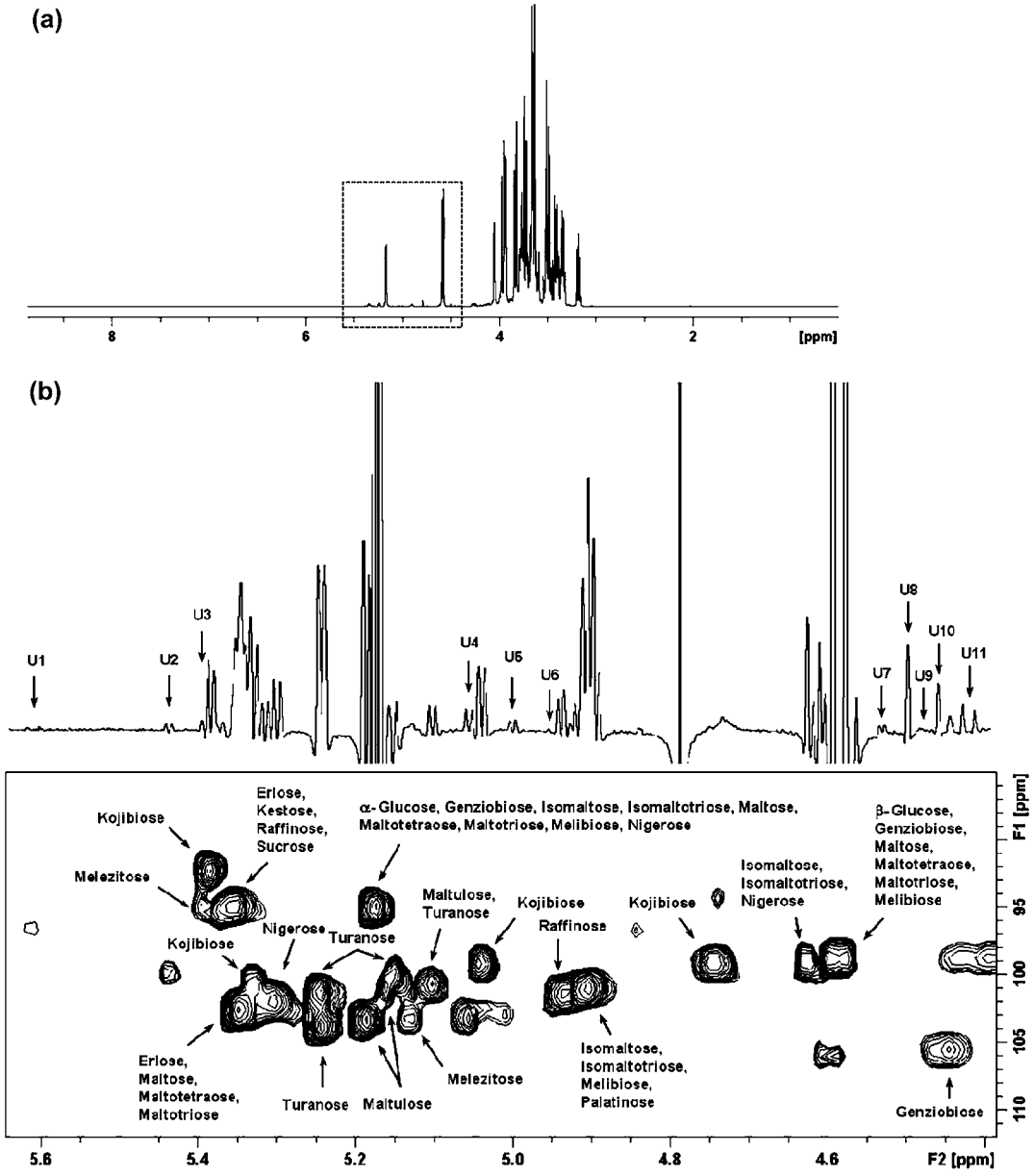

Figure 1. (A) ^{1}H NMR spectrum of the aqueous extract of a chestnut honey sample, with the selection of the anomeric region. (B) Anomeric region of 1D ^{1}H NMR spectrum processed with a Gaussian function (LB = −5 Hz and GB = 0.2) and the corresponding HSQC expansion with assignment of saccharides. Unassigned resonances are indicated by the letter U.

logical methods for establishing the quality of honey in terms of botanical origin and for detecting carbohydrate frauds.

2. MATERIALS AND METHODS

2.1. NMR Sample Preparation. A total of 86 Italian uncrystallized honey samples of different botanical origins provided by trusted producers were analyzed. These samples included 23 acacia samples (*Robinia pseudoacacia*), 20 rhododendron samples (*Rhododendron ferrugineum*, typically collected at 1400/1700 m of altitude), 18 polyfloral samples produced at over 1000 m of altitude (defined as high mountain polyfloral honey), 16 chestnut samples (*Castanea sativa*), and 9 polyfloral samples. Two replicates were performed for each sample to minimize possible inhomogeneity. About 100 mg of honey was dissolved in 600 μL of deuterated water (Sigma-Aldrich, 99.96 atom % D, Milan, Italy). ^{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 inverse probe with a z-gradient. All spectra were acquired at 300 K, with a 7500 Hz spectral width over 32 K data points for 1D spectra. Solvent suppression was achieved by applying a water presaturation scheme with low-power radiofrequency irradiation (typically 60 db of attenuation for 1.2 s). An exponential function with line broadening = 0.3 Hz was applied before Fourier transformation performed on 32 K data points; the phase and baseline of spectra were manually corrected with TOPSPIN software (Bruker Biospin GmbH, version 1.3, Rheinstetten, Karlsruhe, Germany). All spectra were calibrated with the anomeric signal of α-glucose at 5.173 ppm, resulting in a very good overlap with no significant resonance shifts for all other signals. Only the spectral region between 4.400 and 5.700 ppm and the fructose signal at 4.050 ppm were considered for statistical analysis; the region between 4.640 and 4.860 ppm containing the residual water signal was excluded. Intelligent bucketing was performed according to saccharide assignments in the selected spectral areas, yielding a total of 30 buckets (variables) that were further normalized to the total integral areas with ACD/NMR software (ACD Laboratories, version 11, Toronto, Ontario, Canada).

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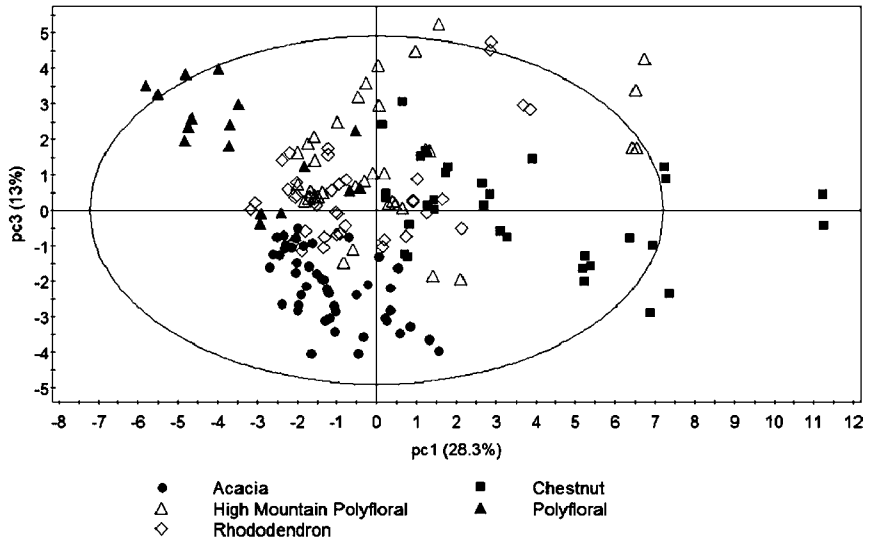

Figure 2. Score plot of PCA performed by considering Italian honey samples of five different botanical origins analyzed in duplicate. Five total explained components, R^2X = 81% and Q^2_{cum} = 58.7%.

The acquisition parameters for 2D $^1H-^{13}C$ HSQC (heteronuclear single quantum coherence) spectra were as follows: number of scans, 256; number of data points, 2K in *F*2 (^1H) and 512 in *F*1 (^{13}C); spectral width, 7500 Hz in *F*2 (^1H) and 31442 Hz in *F*1 (^{13}C). Spectra were also processed with a Gaussian function (line broadening = −5 Hz and Gaussian broadening = 0.2) applied before Fourier transformation to improve the spectral resolution and to resolve signal overlap. Pure standards for the 19 saccharides were purchased from Sigma-Aldrich (Milan Italy).

2.2. Multivariate Data Analysis. Principal component analysis (PCA) and orthogonal projection to latent structures-discriminant analysis (OPLS-DA) were performed with unit variance as the data pretreatment. OPLS-DA optimizes the model complexity by removing the systematic variations in the X data that are not related to Y. This classification method is well suited to maximize separations among different predefined classes of samples and has an intrinsic prediction power. The model validation was performed using the permutation test, in which a total of 200 models were calculated by randomizing the order of Y variables in the corresponding PLS-DA models. The obtained Q^2 and R^2 values, describing the predictive ability and the reliability of the fitting, respectively, were plotted and compared with the Q^2 and R^2 values obtained from the real model. Statistical data analysis was performed with SIMCA-P+ 12 software (Umetrics, Umea, Sweden).

3. RESULTS AND DISCUSSION

3.1. Determination of the Saccharide Content by ^1H NMR. The typical ^1H NMR spectrum of honey in aqueous solution is dominated by intense resonances from saccharides, whereas less-concentrated compounds are responsible for the signals in the aliphatic and aromatic regions of the spectrum (Figure 1A).

The anomeric sugar region, typically between 4.4000 ppm and 5.7000 ppm, is the most representative and interesting part of the ^1H NMR spectrum for evaluating the saccharide contents of the analyzed botanical honey species (Figure 1B). The two primary monosaccharides, glucose in its α and β pyranosidic forms (signals at 5.1732 ppm and 4.5860 ppm, respectively) and fructose in its β pyranosidic form (doublet at 4.0524 ppm), clearly dominate. In addition, 10 disaccharides, 6 trisaccharides, and one tetrasaccharide were also identified, in agreement with the results of Anklam and De La Fuente,[27,28] who identified approximately 25 saccharides by GC analysis. The assignment of anomeric protons for all 19 of these saccharides was achieved by recording 1D ^1H and 2D HSQC spectra for each single saccharide and by the use of spiking experiments. Furthermore, the application of the Gaussian function before Fourier transformation of the ^1H NMR spectra allowed better identification of signals that overlapped in the 1D spectra. Complete overlap was observed for the anomeric signals of sucrose, erlose, and maltose. Nevertheless, the use of HSQC spectra and the unambiguous positioning of resonances outside the anomeric region allowed us to confirm the presence of these saccharides. The anomeric signal of kestose overlapped with the anomeric proton of raffinose at 5.3729 ppm. Nevertheless, an isolated resonance of kestose at 4.2284 ppm allowed its identification; this saccharide was detected at very low levels in all samples. The anomeric signals of five other trisaccharides were detected; these five trisaccharides were erlose (5.3505 ppm), isomaltotriose (5.1874 ppm, 4.8988 ppm, and 4.6194 ppm), maltotriose (5.3383 ppm, 5.1732 ppm, and 4.5860 ppm), melezitose (5.3924 ppm and 5.1316 ppm), and raffinose (5.3729 ppm and 4.9397 ppm). Among the signals of these trisaccharides, the maltotriose resonances were completely overlapped with other anomeric signals. Only the spiking experiment allowed the identification of maltotriose by analyzing the resonance pattern centered at 5.3383 ppm in the 1D ^1H NMR spectrum processed with the Gaussian function. Ten disaccharides, identified as gentiobiose (5.1732 ppm, 4.5860 ppm, and 4.4455 ppm), isomaltose (5.1874 ppm, 4.9055 ppm, and 4.6194 ppm), kojibiose (5.3841 ppm, 5.3304 ppm, and 5.0404 ppm), maltose (5.3505 ppm, 5.1732 ppm, and 4.5860 ppm), maltulose (5.1874 ppm, 5.1536 ppm, and 5.1034 ppm), melibiose (5.1732 ppm, 4.9251 ppm, and 4.6150 ppm), nigerose (5.3159 ppm, 5.3003 ppm, 5.1732 ppm, and 4.6115 ppm), palatinose (4.9183 ppm), sucrose (5.3505 ppm), and turanose (5.2444 ppm, 5.1536 ppm, and 5.1034 ppm) were recognized. Finally, we observed the anomeric signals for the single tetrasaccharide present: maltotetraose (5.3567 ppm, 5.1732 ppm, and 4.5860 ppm). The chemical shift values measured for the identified saccharides were compared with those presented in the literature; this comparison showed that there was very good agreement between these values.[29] On the basis of the aforementioned published data for both proton and

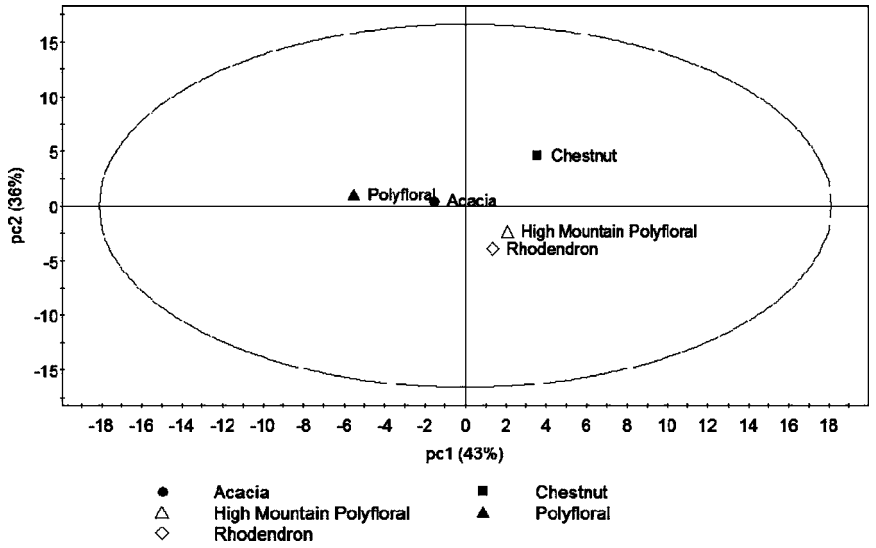

Figure 3. Score plot of PCA performed by considering the mean values for all buckets of each botanical origin of honey. Two total explained components, R^2X = 79% and Q^2_{cum} = 31.9%.

carbon resonances, we could safely exclude the presence of laminaribiose, the expected anomeric signals of which were completely buried under the glucose signals with the exception of anomeric proton resonances at 4.662 and 4.680 ppm (105.61 and 105.53 ppm, respectively, for anomeric carbon resonances); these resonances were never observed in our samples. The same result was obtained for the corresponding cross-peak in the HSQC spectra of the honey samples; this was in contrast with the results of other authors who detected laminaribiose and neo-kestose in French chestnut honey[4] by HPLC and GC analysis. We did not detect these saccharides in Italian honey samples. The trehalose signals were also completely buried under the glucose signal, thus preventing its identification. Spiking experiments performed for cellobiose revealed the presence of an isolated doublet at 4.5968 ppm. The accurate analysis of our samples did not reveal the presence of this signal, thus enabling us to exclude the presence of this disaccharide. An anomeric signal for panose was identified at 5.3458 ppm; this signal partially overlapped with the anomeric signal of kojibiose, and the other resonances were completely buried under the signals for other saccharides, thus preventing the discrimination of these resonances. No definitive results were obtained by HSQC analysis either.

The same holds true for other saccharides such as trehalulose, which was detected in Spanish honeys by GC-MS analysis,[6] and for leucrose and inulobiose, which were detected in Czech honeys by GC-TOF-MS.[7] We could not determine the contents of these saccharides in our samples. Finally, in the anomeric sugar region, 11 unidentified compounds were also detected, namely, U1 (5.6108 ppm) U2 (5.4381 ppm), U3 (5.3924 ppm), U4 (5.0570 ppm), U5 (5.0013 ppm), U6 (4.9533 ppm), U7 (4.5316 ppm), U8 (4.4984 ppm), U9 (4.4814 ppm), U10 (4.4601 ppm), and U11 (4.4218 ppm).

3.2. Multivariate Analysis of ^{1}H NMR Data. With the single aim of exploring possible correlations between the saccharide content and the botanical origins of honey, PCA analysis was initially performed on all 86 samples (172 ^{1}H NMR spectra). This model yielded five components that explained 81% of the total variance, with Q^2_{cum} = 58.7%. A clustering tendency for all species was visible after scoring the first and the third PCs of the model (Figure 2). Chestnut honeys were grouped on the right side of the plot, acacia honeys were centered in the mid lower left region of the score plot, and polyfloral honeys partially overlapped with high mountain polyfloral and rhododendron samples, which were all grouped in the middle; this latter overlap is most likely due to the similar proveniences of these honeys with respect to altitude. The corresponding loading plot (data not shown) suggested that fructose and turanose were the characteristic saccharides of acacia samples, and chestnut was largely enriched in all other saccharides. It was not possible to define specific saccharides that were characteristic of the other botanical species. For clearer data interpretation, the mean values were calculated for the buckets within the same variety. Hence, a single representative honey sample was plotted for each botanical origin. This new PCA model yielded two components explaining 79% of the total variance, with Q^2_{cum} = 31.9%. The corresponding score scatter plot (Figure 3) revealed the similarity of the high mountain polyfloral and rhododendron honeys, which were grouped in the lower right region of the score plot; chestnut honey was located in the top right region, acacia honey was located in the middle, and polyfloral honey was located in the left region of the score plot. This last PCA was useful in defining the saccharide profiles of honeys from different botanical sources through the analysis of the score contribution plots (Figure 4). These plots, represented the bucket (saccharide) contribution to sample differentiation with respect to the average values and highlighted the saccharide content that was characteristic of each botanical origin. An arbitrary level of sensitivity was adopted for the contribution plot analysis, and when a bucket with multiple assignments was present, unambiguous single buckets were use to solve the assignment. A generally greater saccharide content for chestnut honey with respect to all other varieties was detected; in particular, chestnut honey was enriched in unknown compound U1 (bucket at 5.5816 ppm), kojibiose (buckets at 5.3225 and 5.0203 ppm), nigerose (bucket at 5.2777 ppm), maltulose (buckets at 5.1429 and 5.0831 ppm), U4 (bucket at 5.0486 ppm), U5 (bucket at 4.9782 ppm), U6 (bucket at 4.9481 ppm), raffinose (bucket at 4.9306 ppm), isomaltose and isomaltotriose (buckets at 4.8517, 4.6220, and 4.6158 ppm), U9 (bucket at 4.4746 ppm), U10 (bucket at 4.4523 ppm), gentiobiose

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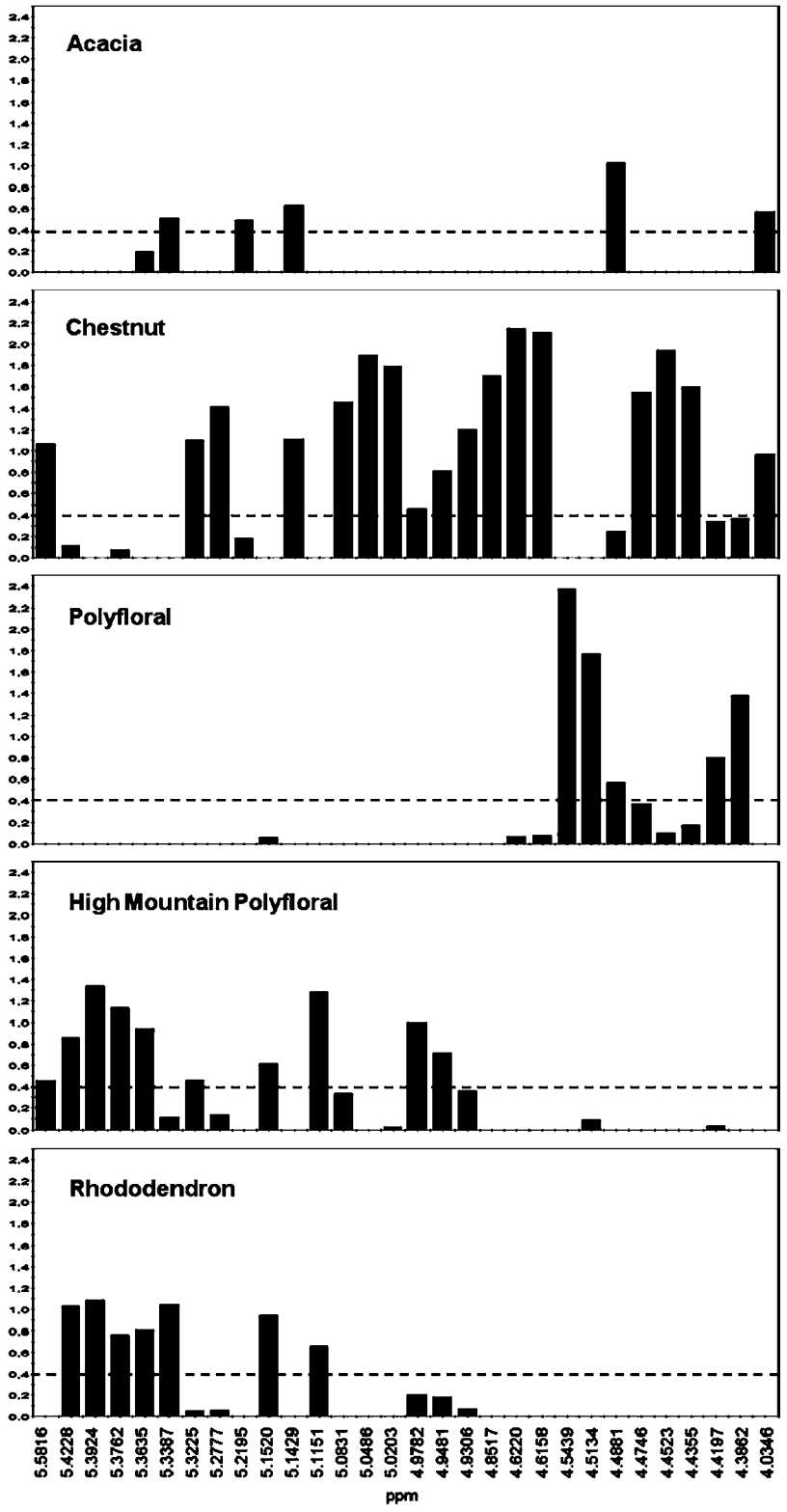

Figure 4. Contribution plots extracted from the PCA performed by considering the mean values for all buckets of the five botanical origins. Dotted lines indicate the chosen sensitivity level. The ppm values of each single considered bucket are reported.

(buckets at 4.4523 and 4.4355 ppm), and fructose (bucket at 4.0346 ppm). High mountain polyfloral honey was characterized by high levels of U1 (bucket at 5.5816 ppm), U2 (bucket at 5.4228 ppm), U3 (bucket at 5.3924 ppm), melezitose (buckets at 5.3924, 5.3762, and 5.1151 ppm), kestose (bucket at 5.3635 ppm), maltotriose and maltotetraose (bucket at 5.3225 ppm), α-glucose (bucket at 5.1520 ppm), U5 (bucket at 4.9782 ppm), and U6 (bucket at 4.9481 ppm). Rhododendron honey was enriched in U2 (bucket at 5.4228 ppm), U3 (bucket at 5.3924 ppm), melezitose (buckets at 5.3924, 5.3762, and 5.1151 ppm), kestose (bucket at 5.3635 ppm), maltose and erlose (bucket at 5.3387 ppm), and α-

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Table 1. List of Assigned Buckets Considered for the Statistical Analysis[a]

		acacia		chestnut		polyflower		hm polyflower		rhododendron	
ppm	bucket assignment	SCP	OPLS-DA	SCP	OPLS-DA	SCP	OPLS-DA	SCP	OPLS-DA	SCP	OPLS-DA
5.5816−5.6497	**U1*[a]**			×				×			
5.4228 −5.4567	**U2***							×	×	×	×
5.3924−5.4228	MLZ + U3							×	×	×	×
5.3762−5.3924	KJB + MLZ							×	×	×	×
5.3635−5.3762	RFF + KST							×		×	×
5.3387−5.3635	MLT + SCR + ML3 + ML4 + ERL	×	×							×	×
5.3225−5.3387	KJB + ML4 + ML3			×				×			
5.2777−5.3225	**NGR***			×	×						
5.2195−5.2777	**TRN***	×	×								
5.1520−5.2195	αGL + IMT + MTL + NGR + GNZ + IM3 + ML3 + ML4 + MLT + TRN + MLB							×	×	×	×
5.1429−5.1520	TRN+ MTL	×	×	×							
5.1151−5.1429	**MLZ***							×	×	×	×
5.0831−5.1151	TRN+ MTL			×	×						
5.0486−5.0831	**U4***			×	×						
5.0203−5.0486	**KJB***			×	×						
4.9782−5.0203	**U5***			×				×	×		
4.9481−4.9782	**U6***			×				×	×		×
4.9306−4.9481	**RFF***			×	×						
4.8517−4.9306	IMT + IM3 + PLT + MLB			×	×						
4.6220−4.6505	IMT + IM3			×	×						
4.6158−4.6220	NGR + IMT + IM3			×	×						
4.5439−4.6158	βGL + IMT + NGR + GNZ + IM3 + ML4 + ML3 + MLT + MLB					×	×				
4.5134−4.5439	**U7***					×	×				
4.4881−4.5134	**U8***	×	×			×					
4.4746−4.4881	**U9***			×	×						
4.4523−4.4746	GNZ + U10			×	×						
4.4355−4.4523	**GNZ***			×	×						
4.4197−4.4355	GNZ + U11					×					
4.3862−4.4197	**U11***					×	×				
4.0346−4.0742	FRC + TRN + PLT	×	×	×							

[a]For each floral source of honey, the characterizing buckets were indicated (SCP, score contribution plot derived from PCA analysis in Figure 2; OPLS-DA from the corresponding classification one-versus-all models). Abbreviations: ERL, erlose; FRC, fructose; αGL and βGL, α and β glucose; GNZ, genziobiose; IM3, isomaltotriose; IMT, isomaltose; KJB, kojibiose; KST, kestose; ML3, maltotriose; ML4, maltotetraose; MLB, melibiose; MLT, maltose; MLZ, melezitose; MTL, maltulose; NGR, nigerose; PLT, palatinose; RFF, raffinose; SCR, sucrose; TRN, turanose; U1−11, unknown compounds. [a]Asterisks and bold font indicate a bucket including a single saccharide resonance.

glucose (bucket at 5.1520 ppm). The bucket at 5.3387 ppm included several saccharides, such as maltose, maltotriose, maltotetraose, sucrose, and erlose; for the correct assignment of the saccharides, a combined analysis of the HSQC spectra for the honeys of all botanical origins and of single-saccharide spectra was performed to identify possible unique carbohydrate resonances. In the case of rhododendron honey, we verified that the bucket at 5.3387 ppm was mainly affected by erlose and maltose.

Acacia honey was enriched in sucrose (bucket at 5.3387 ppm), turanose (buckets at 5.2195, 5.1429, and 4.0346 ppm), unknown compound U8 (buckets at 4.4881 ppm), and fructose (buckets at 4.0346 ppm). Finally, polyfloral honey was enriched in β-glucose (bucket at 4.5439 ppm), U7 (bucket at 4.5134 ppm), U8 (bucket at 4.4881 ppm), and U11 (bucket at 4.4197 and 4.3862 ppm).

With the aim of further validating our findings obtained from the analysis of the score contribution plots, one-versus-all OPLS-DA models were performed. In these models, each single floral source was considered a single class and was scored against all other floral sources, which constituted the second class. These models, which were checked for their noncausality as described in Materials and Methods, were used to extract the representative saccharides able to characterize each single floral source by the use of S-plot. The OPLS-DA models for acacia, chestnut, rhododendron, polyfloral, and high mountain polyfloral honeys yielded overall quality of fits (R^2Y) and overall cross-validation coefficients (Q^2Y) of 75.7% and 71.9%, 77% and 72.5%, 50.5% and 41.3%, 63.6% and 58.9%, and 30.2% and 24.3%, respectively. On the basis of this comparison (summarized data in Table 1), we could conclude that the characteristic saccharides for the different honeys were as described: acacia honey, sucrose, turanose, fructose and U8; chestnut honey, nigerose, kojibiose, raffinose, isomaltose, isomaltotriose, gentiobiose, U4, U9, and U10; polyfloral honey, β-glucose, U7 and U11; high mountain polyfloral honey, melezitose, α-glucose, U2, U5, U6, and U3; and rhododendron honey, melezitose, α-glucose, maltose, erlose, kestose U2, and U3.

A more in-depth investigation of saccharides affecting the differentiation of polyfloral and high mountain polyfloral honeys and of the two high mountain samples, rhododendron

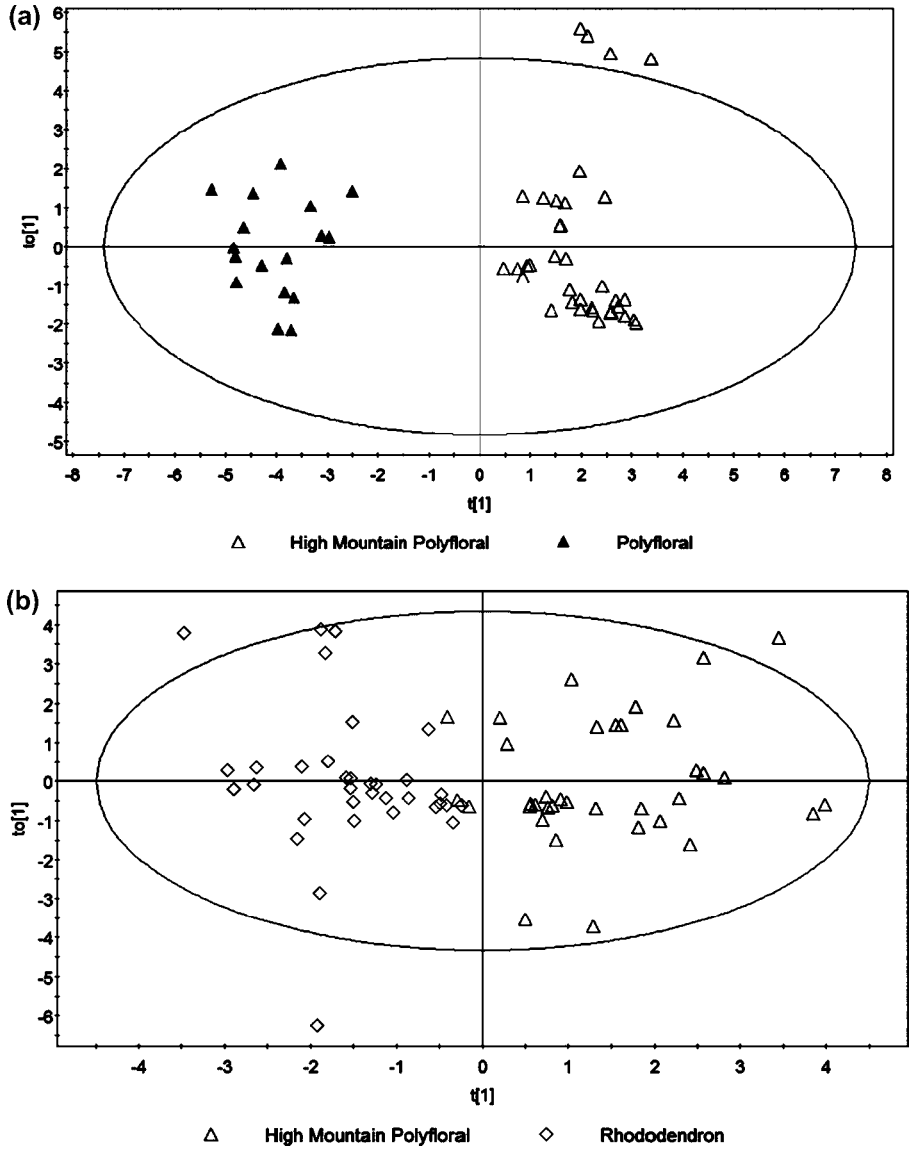

Figure 5. (A) Score plot of the OPLS-DA model performed by considering polyfloral and high mountain polyfloral honey samples. One predictive and five orthogonal explained components, R^2X = 84.5%, R^2Y = 93.5%, and Q^2Y = 89.5%. (B) Score plot of the OPLS-DA model performed by considering high mountain polyfloral and rhododendron honeys. One predictive and one orthogonal explained components, R^2X = 25.2%, R^2Y = 51.2%, and Q^2Y = 39.6%.

and high mountain polyfloral, was performed with OPLS-DA models for pairs of floral sources. The model including polyfloral and high mountain polyfloral honeys (Figure 5A) resulted in one predictive component and five orthogonal latent components, with an overall quality of fit (R^2Y) of 93.5% and an overall cross-validation coefficient (Q^2Y) of 89.5%. The second model, which included rhododendron and high mountain polyfloral honeys (Figure 5B), resulted in one predictive component and one orthogonal latent component, with an overall quality of fit (R^2Y) of 51.2% and an overall cross-validation coefficient (Q^2Y) of 39.6%. The noncausality for both models was assessed with the use of the permutation test, which yielded R^2 and Q^2 values that were substantially lower than the values for the corresponding original models. The relative contributions of buckets in well-clustered polyfloral and high mountain polyfloral honey samples could be easily extracted from the corresponding S-plot (data not shown): kojibiose (buckets at 5.0203, 5.3762, and 5.3225 ppm), nigerose (bucket at 5.2777 ppm), isomaltose and isomaltotriose (bucket at 4.8517 ppm), unknown compounds U2, U4, U5, and U6 (buckets at 5.4228, 5.0486, 4.9782, and 4.9481 ppm, respectively), and raffinose (buckets at 5.3635 and 4.9306 ppm) were characteristic of high mountain polyfloral samples, whereas β-glucose (bucket at 4.5439 ppm), gentiobiose (bucket a t4.4523, 4.4355, and 4.4197 ppm), and unknown compounds U7, U8, U9, and U11 (buckets at 4.5134, 4.4881, 4.4746, and 4.3862 ppm, respectively) were characteristic of polyfloral samples. The S-plot derived from the OPLS-DA model performed on rhododendron and high mountain polyfloral honeys indicated that rhododendron honey was characterized by erlose and sucrose (bucket at 5.3387 ppm), U2 (bucket at 5.42228 ppm), turanose (bucket at 5.2195 ppm), and isomaltose and isomaltotriose (bucket at 4.8517 ppm). High mountain polyfloral was characterized by gentiobiose (4.4523, 4.4355, and 4.4197 ppm), kojibiose (bucket at 5.0203 ppm), fructose (bucket at 4.0346 ppm), and U4, U5, U7, U8, U9, and

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U11 (buckets at 5.0486, 4.9782, 4.5134, 4.4881, 4.4746, and 4.3862 ppm, respectively).

In the present study, for the first time, the identification of 19 saccharides in aqueous extracts of Italian honeys of 5 different botanical origins was performed by 1D proton, 2D HSQC, and spiking NMR experiments. The multivariate statistical analysis performed on the NMR data led to characterize honeys from different botanical origins on the basis of relative differences in the saccharide content. Chestnut honey was the most enriched in the 19 identified saccharides relative to all other investigated honey species. Moreover, the differentiation between two particular types of honeys collected at high altitude, namely, rhododendron and high mountain polyfloral, and between two types of polyfloral honeys, namely, polyfloral and high mountain polyfloral, was investigated, highlighting the characteristic saccharides of each honey.

The NMR approach presented herein could be used for establishing the botanical origins of honeys. Furthermore, adulteration by carbohydrate addition could also be identified by analyzing the different ratios of the saccharides. In addition, as already noted by other authors, the primary advantages of this methodology with respect to other analytical approaches, such as GC or HPLC methods, are improved reproducibility of NMR and the lack of sample derivatization or chemical treatment, thus limiting the experimental time and improving the results. Finally, the palynological composition of honeys appears to be not particularly efficient for the botanical discrimination of their origins.[30] From this point of view, NMR determination could adequately support these investigations of honey sources. Our data, even if preliminary, suggest that the saccharide content could be employed to characterize honey samples and to construct an identity card of saccharides for each floral source.

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