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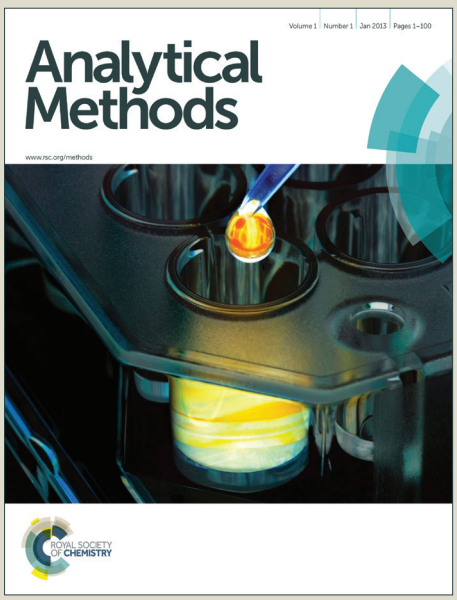

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Identification and quantitative determination of carbohydrate molecules in Greek honey by employing ^{13}C NMR spectroscopy

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Abstract

A methodology based on ^{13}C NMR spectroscopy was employed to detect and quantify fourteen mono-, di- and trisaccharide molecules' in authentic Greek honey samples with no prior separation. Unambiguous assignment of ^{13}C NMR chemical shifts has been achieved by means of two dimensional NMR techniques using sugar model compounds. The quantitative ^{13}C NMR method was rigorously validated (accuracy, linearity, range, limit of detection, etc.) using either single sugar molecules, or artificial mixtures of isoglucose (glucopyranose and fructose) and global mixtures of fourteen model compounds. Subsequent integration of appropriate signals in the ^{13}C NMR spectra allowed the quantification of these compounds. The present methodology has been applied to authentic Greek honey samples and provided quantitative results for 28 sugar tautomers. Observed differentials in the content of these biomarkers amongst the various honey samples of different botanical origin is expected to form the basis for the development of a sensitive method that can be used to obtain valuable information about honey authenticity.

Keywords: Honey, sugars, ^{13}C NMR, quantitative analysis

1. Introduction

Honey is produced by honeybees from the nectar of different plants as well as from honeydew, or from secretions of other living parts of the plants. It is one of the most complex foods in nature, and is the only sweetening agent that can be used by the humans without processing. Besides its very high nutritive value, honey is endowed by therapeutic properties.[1,2] It demonstrates anti-microbial and anti-inflammatory activity, stimulating the body's immune system to fight infections and other diseases. Furthermore, its anti-oxidant capacity prevents significantly oxidative reactions to occur within the human body. It has been found[1,3,4] that honey has a significant antioxidant content measured by means of its capacity to scavenge free radicals.

Honey is reported as a concentrated aqueous solution containing about 200 substances including a complex mixture of sugars that constitute about 95 to 97% of the dry weight of honey.[2] Major components are the two monosaccharides fructose, glucose and water, fructose being the dominant sugar. Besides the two main sugar components, honey contains about 25 oligosaccharides (di-, tri-, and tetra-saccharides). The rest 5% to 3% of its composition comprises small amounts of other constituents, such as minerals, proteins, vitamins, organic acids, amino acids, flavonoids, phenolic acids, enzymes and other phytochemicals. The composition of honey depends heavily on the floral source, and can vary for the same floral source due to seasonal climatic variations or to different geographical origin. This fact has been exploited to investigate the botanical and geographical origin of honey, as well as the detection of honey adulteration.[5-7] Various constituents of honey, e.g. amino acids, trace elements, aroma compounds in combination with multivariate statistical methods has been used in the past for the classification of honey samples.[5,7] Major

analytical techniques employed for these studies were simple gas chromatography (GC) and gas chromatography coupled with mass spectrometry (GC-MS). [5,6,8]

^{1}H NMR spectroscopy has found application to the analysis of honey only in the last few years[9-15] despite the fact that this technique has a number of advantages for food analysis[16] compared to GC and GC-MS. Among these are the lack of any sample pre-treatment, the non-invasive approach, the relatively easy and quick data acquisition, and its ability to provide a large number of metabolites in a single experiment. However, the richness of information makes the ^{1}H NMR spectra highly complex even at high magnetic fields (≥ 14.1 T) to be analyzed or compared by simple procedures. This means that metabolic profiling by which food metabolites are identified and used for subsequent statistical analysis is not applicable. Therefore NMR fingerprinting in combination with multivariate statistical analysis have been used as an alternative tool for the authentication of honey.[9-13,17] Nevertheless, NMR fingerprinting suffers from the fact that does not reveal the identity of those constituents that play an important role in the detection of fraud or facilitates the discrimination of samples from different flora and/or originated from different geographical areas.[16]

The deficiency of ^{1}H NMR for metabolic profiling can be overcome by using ^{13}C NMR spectroscopy. The respective ^{13}C spectra obtained under proton decoupling show singlets for non-equivalent carbon-13 nuclei spread over a larger chemical shifts range (~250 ppm) relative to of ^{1}H NMR spectrum (~15 ppm). This property makes easier the recognition and integration of sugar resonances in honey appropriate for metabolic profiling. This appears to be feasible even for sugars that are present in different tautomers due to their mutarotation in water (e.g. fructose and glucose). ^{13}C NMR spectroscopy has been used largely for structural studies[18,19] and the

investigation of the motional behavior[20] of mono- and oligosaccharide molecules in solution, as well as the study of their mutarotation in aqueous solutions and other solvents.[21-23] To the best of our knowledge, only two reports have been published[24,25] using ^{13}C NMR in the analysis of carbohydrates in honey. The first investigation[24] in 1988 was carried out after HPLC separation of mono- and oligosaccharides from honey followed by borohydride reduction of the latter, and quantification of the oligosaccharide fraction using ^{13}C NMR spectroscopy. The second investigation[25] in 1997 aimed at the direct application of ^{13}C NMR to the analysis of carbohydrate molecules in honey. Nevertheless, the low sensitivity and resolution of the experiments performed on a 200 MHz spectrometer hindered the identification of most of the sugar tautomers, and did not allow the quantification of a number of di-, and trisaccharides in several honey samples. Moreover, no method validation was reported in both studies.

The present work aims at the determination of a larger number of carbohydrates in authentic Greek honey, comprising mono-, di-, and trisaccahrides and their tautomers using ^{13}C NMR experiments conducted on a 500 MHz NMR spectrometer. The accuracy and precision of this analytical method, as well as the properties of linearity, detection and quantification limits will be thoroughly examined.

2. Material and methods

2.1 Samples

Greek honey samples were purchased from local producers and originated from flowers of different plants (pine, chestnut, spruce, thyme, heather, citrus, and polyfloral). All honey samples were stored in the dark in screw-cap bottles at room temperature (18-25 ^{o}C) from the time of acquisition to the NMR analysis.

2.2 Chemicals

The monosaccharide model compounds D-glucose (purity 99.5%) (**1**) and D-fructose (99%) (**2**); the disaccharides maltose (*α*-D-Glc*p*(1-→4)-D-Glc*p*) (99%) (**3**), isomaltose (*α*-D-Glc*p*(1-→6)-D-Glc*p*) (98%) (**4**), nigerose (*α*-D-Glc*p*(1→3)-D-Glc*p*) (≥95%) (**5**), sucrose (*α*-D-Glc*p*(1→2)-*β*-D-Fru*f*) (98%) (**6**), turanose **(***α*-D-Glc*p* (1→3)-D-Fru*f*) (98%) (**7**), and maltulose (*α*-D-Glc*p*(1→4)-D-Fru*f*) (≥98%) (**8**); the trisaccharides erlose (*α*-D-Glc*p*(1→4)-*α*-D-Glc*p*(1→2)-*β*-D-Fru*f*) (97%) (**9**), maltotriose (*α*-D-Glc*p*(1→4)-*α*-D-Glc*p*(1→4)-D-Glc*p*) (≥95%) (**10**), isomaltotriose (*α*-D-Glc*p*(1→6)-*α*-D-Glc*p*(1→6)-D-Glc*p*) (98%) (**11**), panose (*α*-D-Glc*p*(1→6)-*α*-D-Glc*p*(1→4)-D-Glc*p*) (98%) (**12)**, melezitose (*α*-D-Glc*p*(1→3)-*β*-D-Fru*f*(2→1)-*α*-D-Glc*p*) (99%) (**13**), and 1-kestose (*α*-D-Glc*p*(1→2)-*β*-D-Fru*f*(1→2)-*β*-D-Fru*f*) (≥98%) (**14**) were purchased from Sigma-Aldrich (Athens, Greece). **Fig. 1** illustrates the chemical structures and the numbering system of the fourteen sugar model compounds. 1, 4-dioxane and deuterated solvents used in the present study were purchased from Sigma-Aldrich (Athens-Greece) as well.

2.3 Sample preparation

For ^{1}H and ^{13}C NMR experiments, 10-20 mg of each model compound, depending on its molecular mass, was dissolved in 0.4 mL of D_2O (100% deuterated). The artificial mixture of sugars (see below) was prepared by adding the 14 sugar model compounds of the present study in D_2O at concentrations similar to the average values found in honey. The samples of honey were prepared in triplicate dissolving 15 mg of honey in 0.4 mL of D_2O. Sodium-3-trimethylsilyl-d_4 propionate (TSP) was used as an internal reference for chemical shifts (δ 0.00), whereas 1,4-dioxane (δ 67.15) was the internal reference for quantification purposes. All samples were allowed to stand at rest to fully equilibrate prior to ^{13}C NMR experiments.

2.4 NMR experiments

All NMR experiments were conducted on an AMX500 spectrometer operating at 500.1, and 125.7 MHz for proton, and carbon-13 nuclei, respectively. The spectrometer was equipped with 5 mm inverse probes. The probe temperature was 26 ± 1 ^{o}C. The TopSpin 3.0 software provided by Brūker-Biospin was used for all processing procedures described below.

2.4.1 One dimensional NMR spectra

^{1}H NMR spectra for the model compounds were acquired with the following acquisition parameters: time domain 32 K; 90^{o} pulse width 9.6 μs; spectral width 12 ppm; acquisition time 2.7 s, and relaxation delay 1 s. 32 scans and 4 dummy scans were accumulated for model compounds. Line broadening of 0.3 Hz for sensitivity enhancement was used. ^{13}C NMR spectra for model compounds were acquired with the following acquisition parameters: time domain 32 K; 90^{o} pulse width 10.3 μs; relaxation delay 2 s. 64 scans and 4 dummy scans were accumulated for model compounds. For the artificial mixture and the samples of honey the data points and the number of scans were increased to 64 K and 512, respectively, to obtain a better resolution and sensitivity. An exponential line broadening of 1.0 Hz was applied before Fourier transform. Each ^{1}H and ^{13}C NMR spectrum was phase corrected manually and a fourth-order polynomial function was used for base-line correction. To ensure quantitative ^{13}C NMR spectra, the magnitude of the recycling time (acquisition time + relaxation delay) was set five times the longest spin-lattice relaxation time (T_1) measured for sugar components in isoglucose and artificial mixtures (see below) and in honey including the T_1 value of the internal standard 1,4-dioxane by using the null method. The longest ^{13}C T_1 value in D_2O was measured for the internal standard (5.5 s) as expected. Therefore, the recycling time for a

quantitative ^{13}C NMR experiments was set to 28 s. The total time needed to obtain ^{13}C NMR data from a single honey sample is 50 min.

2.4.2 Two dimensional NMR experiments

In order to assign ^{13}C chemical shifts especially of model sugars (turanose, maltulose etc.) heteronuclear 2D NMR experiments (HSQC and HMBC) were employed. For the carbon assignment high resolution in the indirect dimension was desired - especially in the case of crowded spectral regions e.g. carbons C6, C6′ and C1′. Phase-sensitive ge-2D multiplicity-edited HSQC experiments using PEP and inversion, refocusing and matched sweep adiabatic pulses with gradients in back-inept (hsqcedetgpsisp2.3) were acquired with 2500 Hz and 10 kHz of spectral widths and number of data points 2K and 1K for ^{1}H and ^{13}C dimension, respectively. 4 transients were collected for each point with 16 dummy scans and relaxation delay of 2 s. The spectra were zero-filled to a final size of 4K × 2K prior to Fourier transformation. Magnitude-mode ge-2D HMBC experiments using low-pass *J*-filter (hmbcgplpndqf) were conducted with spectral widths of 2500 Hz and 10 kHz, and 2K and 1K data points for ^{1}H and ^{13}C dimensions, respectively. 4 transients were collected for each point with 16 dummy scans and relaxation delay of 1.5 s. The spectra were zero-filled to a final size of 4K × 2K prior to Fourier transformation.

3. Results and Discussion

3.1 Mutarotation of sugars

The composition of reducing sugars in various solvents, but especially in aqueous solutions has been extensively studied by different techniques preferably by ^{13}C carbon NMR.[21-23] These substances undergo the so-called mutarotation and form two or more species known as ‘tautomers’ or simply ‘forms’. Mutarotation occurs when

the hemiketal ring opens and closes, sometimes with a different α-/β- configuration than the original molecule. For example, D-glucose is present in aqueous solution into two forms, namely α-D-glucopyranose and β-D-glucopyranose, differing in the configuration of the anomeric carbon. On the other hand, fructose was detected in four forms: α-D-fructopyranose, β-D-fructopyranose, α-D-fructofuranose, and β-D-fructofuranose at different proportions in water and in dimethylsulfoxide.[26,27] ^{1}H NMR spectroscopy has revealed the presence of the hydrated and non-hydrated acyclic forms in solutions of sugars.[28,29] The acyclic forms exist in traces, and therefore their presence cannot be detected by ^{13}C NMR spectroscopy without prior isotopic enrichment.[28,30] The composition of tautomers varies widely amongst the various reducing sugars and for the same sugar in different solvents.[21,22] For instance, the composition of the two stable tautomers α-D- and β-D-glucopyranose of D-glucose changes from 38% and 62% in aqueous solution, respectively, to 47% and 50%, in pyridine,[21] respectively. The knowledge of the sugars composition in aqueous solutions is crucial for the accurate determination of their content in honey. **Table 1** contains the composition of the tautomeric forms of the sugars of the present study in aqueous solutions as determined by ^{13}C NMR spectroscopy. These results, which agree very well with those found in literature,[21,22,31] were obtained after equilibration lasted from one to three weeks. As can be seen in this table, not all sugars showed the phenomenon of mutarotation. The trisaccharides erlose, 1-kestose and melezitose were present in single forms in water even after one month of equilibration. The concentration of the various forms of each sugar was determined, either from the integral of each carbon signal with respect to the known concentration of the internal reference (1, 4 –dioxane) or by averaging the integrals of all carbon signals resolved in the ^{13}C spectra. No significant differences were observed between the two methods.

3.2 ^{13}C NMR experiments

First, the assignment of ^{13}C NMR chemical shifts of all sugar model compounds used in the present study was performed on the basis of the chemical shifts reported in the literature.[18,19,32-39] This assignment was verified by us upon conducting a number of 2D NMR experiments. As an example, **Fig. 2** depicts the HSQC and HMBC spectra of turanose. In the HMBC spectra cross-peaks proving 1-3 glycosidic linkage between sugars units are indicated e. g C1-H3' and H1-C3'. ^{13}C NMR chemical shifts of the fourteen model compounds and their tautomers relative to 1, 4-dioxane are reported in **Table S1** (Figures and Tables numbering preceded with an S are provided as supporting material). Generally, the values of chemical shifts are consistent with the literature data, except for carbons C5, C1′, C4′, and C6' for turanose forms 7a-FF and 7b-FF; C3′ and C4' maltulose 8a-FF. **Fig. 3** shows the ^{13}C NMR spectrum of an isoglucose mixture, i.e. a mixture of 55% fructose and 45% glucose, which has been used as a substitute for sugar in beverages, processed foods, cereals and baked goods, as well as for adulteration of honey. The spectrum was recorded after the completion of tautomerization. The higher resolution offered by the 11.7 T magnetic field allowed the observation of almost all carbon resonances for the two tautomers of D-glucose and four tautomers of D-fructose. **Fig. 4** illustrates the ^{13}C NMR spectrum of an artificial mixture of the fourteen sugar molecules after the completion of mutarotation. The concentration of each sugar in the mixture was nearly equal to the average percentage concentration found in honey.[40,41] No significant chemical shifts changes were observed for each tautomer in isoglucose and artificial mixtures relative to those of the individual model compounds in its own solutions, indicating that the ^{13}C chemical shifts of a single sugar tautomer are not influenced by the presence of other sugars in the mixture. This finding is important for the detection of sugars in honey,

which, as already mentioned, is a complex mixture of sugars. The spectrum shows a complex pattern of resonances due to the presence of twenty eight tautomers. Taking into consideration that the chemical shifts of the non-reducing sugars of the α- and β-anomers of D-maltose, D-isomaltose, D-nigerose, D-maltotriose and D-isomaltotriose are identical, as well as those of the carbon signals of two non-reducing sugars within each of the anomers of the trisaccharides D-maltotriose, and D-isomaltotriose, the expected number of signals in the spectrum of **Fig. 4** is three hundreds in total. However, several of the tautomer resonances are overlapped, limiting thus the number of the observed resonances to one hundred and four in total. The spectrum in **Fig. 4** can be divided into five regions for a better visualization of the various resonances. The five ^{13}C NMR subspectra, **Figs S1a** to **Fig. S1e**, are provided as supporting materials. The resonances of the five subspectra are numbered continually from high to low frequencies, and their assignment is depicted in **Table S2**. The most interesting region is that of the anomeric carbons (δ 91-106) of all sugars, and the region δ 78-86, where carbons C3 and C5 of the fructofuranosyl rings resonate. Integration of ^{13}C signals within these regions allows the direct quantitative determination of a number of tautomers of D-fructose, D-turanose, D-maltulose, D-melezitose, D-nigerose, 1-kestose. Indirect quantitative determination of tautomers of different sugar molecules that give overlapping signals in the spectra was achieved from other signals, which were unique for each model compound. The percentage composition of each tautomer for mono-, di-, and tri-saccharide molecules of **Fig. 4** is summarized in **Table 1**, as mentioned earlier.

3.3 Validation of the present ^{13}C NMR methodology

3.3.1 Quantification

The applicability of the ^{13}C NMR method to quantitative analysis was rigorously examined by (a) comparing the amount of each sugar as determined by ^{13}C NMR in water solution with the previously weighed amount, (b) by correlating the amounts of (i) glucose and fructose components in isoglucose, and (ii) the amounts of sugar molecules in the complex artificial mixture determined by ^{13}C NMR with the actual weighed amounts. **Table 2** shows the amount of the trisaccharide erlose calculated from each signal in the ^{13}C spectrum, which should be compared with the actual amount of 31.12 μmol dissolved in D_2O. The last column of **Table 2** gives the percentage of the calculated amount relative to the actual quantity. The average value from all carbons was 99.40%, and 100% from the anomeric carbons alone. Integration of the carbon-13 resonances in the spectra in **Figs. 3** and **4** allowed the quantitative determination of each sugar component in isoglucose and the artificial mixture, respectively. With the aid of the internal standard 1, 4-dioxane, absolute concentration for each sugar tautomer in the mixture was measured. The concentration of each tautomer was the average value obtained from the integration of several of its carbon signals resolved in the corresponding ^{13}C spectra. Overlapped signals were not used for quantitative analysis, although the use of deconvolution gave satisfactory results. In such cases, the amount of each sugar was calculated from signals that were unique for this particular sugar. The concentration of each sugars tautomers in isoglucose and the artificial mixture are summarized in **Table S3** and **S4**, respectively. Regression analysis of these data resulted in excellent correlations for both the isoglucose and the artificial mixture as shown in **Figs. S2** and **S3**, respectively. The following regression equations describe the data in **Figs. S2** and **S3**:

Isoglucose:

$$C_{NMR} = -0.0245(\pm 0.3663) + 0.9958(\pm 0.0025) * C_{Actual} \quad R^2 = 0.9999 \quad p < 10^{-6} \qquad (1)$$

Artificial mixture:

$$C_{\mathrm{NMR}} = -0.5003(\pm 0.2991) + 0.9927(\pm 0.0014) * C_{\mathrm{Actual}} \quad R^2 = 0.9999 \quad p < 10^{-6} \qquad (2)$$

When the regressions were forced through the origin (Z-regression), similar slopes and standards errors were obtained, implying that the intercepts in these relationships are not significant.

Isoglucose: $C_{NMR} = 0.9957(\pm 0.0014) * C_{Actual} \quad R^2 = 0.9999$ (3)

Artificial mixture: $C_{\mathrm{NMR}} = 0.9922(\pm 0.0013) * C_{\mathrm{Actual}} \quad R^2\, 0.9999$ (4)

Moreover, composite Z-regression equations (3) and (4) indicates that the overall correspondence between the NMR estimates for the sugar components in isoglucose and the artificial mixture and the actual values is close to a 1:1 ratio, while the low errors involved in the slopes (values in parentheses) of the regression equations obtained from the residuals support the fact that the deggree of the uncertainty involved in the regression analysis is low.

3.3.2 Repeatability and reproducibility

The repeatability for the ^{13}C NMR measurements was calculated by recording six consecutive spectra on the same day (intraday experiments), and using the same solution containing 0.0225 g of standard D-glucose dissolved in 1.00 mL of water, whereas the reproducibility was estimated by performing measurements on different days (inter-day experiments) on six different samples of the same concentration (78.06 μmol/mL) of D-glucose and using the same experimental protocol for each measurement. The results of the statistical analysis of the data in **Table 3** show that the performance of the present methodology for sugars determination in honey is successful. The repeatability will be further examined later, while quantifying sugars in samples of honey.

3.3.4 Linearity and range

An additional requirement for the present analytical method is its ability (within a given range) to obtain test results of variable data (e.g., integrals) that must be directly proportional to the concentration (amount of analyte) in the sample. In other words, the working sample concentration and samples tested for accuracy should be in the linear range. To determine linearity, ^{13}C NMR spectra for a series of solutions were prepared according to the experimental protocol described previously upon adding small amounts of standard D-glucose at a concentration range covering the lower and the upper concentration (from 2.5 to 1650 μmol/mL) of sugars found in honey and keeping fixed the concentration of the internal standard (1, 4-dioxane). The form of the highest concentration (β-D-glycopyranose) was used, whereas the average integral over the six carbon signals was used. When the ratios of the average signal integrals of β-D-glycopyranose over the signal integral of the internal standard at various concentrations were plotted against the corresponding concentrations, linear relationships were observed (**Fig. S4**) with very good correlation coefficients (R = 0.9999). Also, very good linearity is observed when the absolute concentration of β-D-glycopyranose is plotted against that calculated one from the ^{13}C NMR spectrum as shown in **Fig. S4**. Further support to this finding is the correlation observed between the concentrations of β-D-glycopyranose as determined by ^{13}C NMR (from 2.41 to 1627 μmol/mL) against the signal-to-noise ratio (S/N). A very good linear relationship (R = 0.9999) was observed between the β-D-glycopyranose concentration and the S/N ratio over the whole concentration range (**Fig. S5**) described by the following regression equations:

$$C_{NMR} = 0.8043(\pm 0.0018) * (S/N) \quad R^2 = 0.9999 \qquad (5)$$

These results prove that the present NMR methodology has a very good linearity over the whole average concentration range of sugars components found in honey.

3.3.5 Limit of detection (LOD) and limit of quantification (LOQ)

LOD is estimated from the minimum concentration at which the analyte can reliably be detected. This concentration corresponds to a signal-to-noise ratio of about 3. The lowest sugar concentration measured by ^{13}C NMR that corresponds to *S*/*N* ratio of 3.1 is 2.41 μmol/mL (**Fig. S5**). From these results, and taking into account that 2.5 g of β-D-glycopyranose was dissolved in 1.00 mL of water, the LOD of ^{13}C NMR spectroscopy was calculated to be 0.017 g/100 g of honey for monosaccharides (MW = 180.16), 0.033 g/100 g for disaccharides (MW = 342,30), and 0.049 g/100 g for trisaccharides (MW = 504.44). The LOQ is considered to be 10 times the LOD. Therefore, the LOQs for mono-, di-, and trisaccharides are 0.17 g/100 g, 0.33 g/100 g, and 0.49 g/100 g, respectively.

3.4 Detection and quantification of sugars in authentic honeys of Greek origin

Fig. 5 shows stacked ^{13}C NMR spectra of seven types of authentic honey samples from thyme, chestnut, spruce, citrus, pine, heather, and polyfloral. The spectra can be separated as before into diagnostic regions containing signals of the pyranosyl anomeric carbons (δ 91-106), signals from the furanosyl anomeric carbons and the primary C6 carbons (δ 60 – 69), and signals of the remaining ring carbons (δ 69.5-86). The assignment of all carbon signals and their attribution is based on the assigned ^{13}C NMR spectra of the isolated model compounds, the isoglucose mixture and in particular on the artificial mixture of all sugar model compounds. A few signals were unidentified and they were attributed to sugar components of honey not included in this study. The quantification of the fourteen sugars and their tautomers (in total 28 molecules) was achieved preferably upon integration of signals that were not overlapped in combination with the known concentration of the internal standard. When this was not feasible, the amount of each sugar tautomer contributing to a

particular overlapping signal has been calculated from other signals in the spectrum, which were unique for each sugar tautomer. For example, the concentration of *β*-D-nigerose can be calculated from the signal of C5′ at *δ* 76.25, which is overlapped by the carbon signal C5 of *β*-D-glucose, the latter tautomer being calculated from its non-overlapped signal at *δ* 74.70, as shown below:

δ 76.25 *β*-D-glucopyranose + *β*-D-nigerose = 26.63 *μ*mol

δ 74.70 *β*-D-glucopyranose = 26.50 *μ*mol

β-D-nigerose = 0.13 *μ*mol

The concentration of the tautomers in g/100 g of honey for the seven samples of honey is summarized in **Table 4**. Also, this Table lists the standard deviations (in parentheses) of the analyses, since each honey variety has been analyzed in triplicate. The standard deviations, ranging from 0.01 to 0.05 g/100 g, support our previous conclusion about the robustness of the present analytical method for the sugars determination in honey.

The number of honey samples is not sufficient to allow any definite conclusions about their sugar content. Nevertheless, two general remarks can be made at this stage. Two sugar tautomers were not detected in all honey samples of the present study. These are *α*-D-fructopyranose molecule and the tautomer *8*α-FF of D-maltulose (**Table 4**). The absence of the D-fructose tautomer may be attributed to its low concentration (≤ 2%) in honey, but this argument does not seems to hold for the D-maltulose tautomer, which is contained in a higher percentage (~8%). However, it is too early to ascribe the label of biomarkers to these molecule without further research. Second, concrete differences were observed amongst the honey varieties regarding individual concentrations and range of concentrations for certain carbohydrate molecules (**Table 4**). For instance, the concentration of D-turanose ranged from 1.07 g/100g to 2.35

g/100g, the higher concentration observed for the spruce variety and the lowest for the pine honey. Another important disaccharide in honey is sucrose. A higher content of this sugar (1.11 - 1.71 g / 100g) was found in samples of pine, flowers and chestnut, while the sucrose content in other honey samples was well below the range of 0.1 - 0.18 g / 100g. In this respect, the sugar content as reflected on the ^{13}C signal intensities may be considered as a useful index for subsequent metabonomic studies leading to botanical discrimination of honey samples.

4. Conclusions

In summary, this study demonstrated the potential of ^{13}C NMR spectroscopy to detect and quantify a large number of simple and complex carbohydrate molecules in honey samples in a single experiment. Differences in sugar concentrations and in some cases sugar composition observed in honey samples of different botanical origin could pave the way of using these biomarkers as useful indices for classification/prediction studies.

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Captions of Figures

Fig. 1 Chemical structures and numbering system of sugar molecules. The first number is the serial number of each sugar followed by the letters α and β denoting the tautomeric form of the reducing sugar. GP = glucopyranose, FP = fructopyranose, FF = fructofuranose.

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Fig. 5. 125 MHz ^{13}C NMR spectra of Greek honey samples originated from five different botanical sources: (A) thyme; (B) chestnut; (C) spruce; (D) citrus trees; (E) pine; (F) heather; (G) polyfloral.

Table 1. Equlibrium tautomeric composition of aqueous solutions of sugars of this study at ambient temperature[a].

Tautomer	**wt%**	**Tautomer**	**wt%**
α-D-glycopyranose (1*α*-GP)	37,5	turanose (7α-FF)	20.0
β-D-glycopyranose (1*β*-GP)	62.5	turanose (7*β*-FF)	40.2
α-D-fructopyranose (2*α*-FP)	2.0	maltulose (*8*β-FP)	62.2
β-D-fructopyranose (2*β*-FP)	70.0	maltulose (*8*α-FF)	8.2
α-D-fructofuranose (2*α*-FF)	5.0	maltulose (*8*β-FF)	29.6
β-D-fructofuranose (2*β*-FF)	23.0	erlose (9*β*-FF)	100
α-D-maltose (3*α*-GP)	38.9	*α*-D-maltotriose (10*α*-GP)	37.1
β-D-maltose (3*β*-GP)	61.1	*β*-D-maltotriose (10*β*-GP)	62.9
α-D-isomaltose (4*α*-GP)	41.2	*α*-D-isomaltotriose (11*α*-GP)	35.0
β-D-isomaltose (4*β*-GP)	58.8	*β*-D-isomaltotriose (11*β*-GP)	65.0
α-D-nigerose (5*α*-GP)	38.4	*α*-D-panose (12*α*-GP)	25.1
β-D-nigerose (5*β*-GP)	61.6	*β*-D-panose (12*β*-GP)	74.9
sucrose (6*β*-FF)	100	melizitose (13*α*-GP)	100
turanose (7*β*-FP)	39.8	1-kestose (14*β*-FF)	100

[a]the tautomeric form of the reducing sugar is denoted in parentheses (see Fig. 1).

Table 2. ^{13}C NMR quantitative results of the trisaccharide erlose

Carbons	δ (ppm)	^{13}C NMR quantities	% Difference
	***α*-D-Glc*p*(1→4)**		
C1	100.32	31.39	100.87
C2	72.30	31.52	101.29
C3	73.53	31.03	99.71
C4	69.86	31.04	99.74
C5	73.23	31,17	100.16
C6	61.02	30.80	98.97
	***α*-D-Glc*p*(1↔2)**		
C1’	92.49	31.43	101.00
C2’	71.35	31.06	99.81
C3’	73.44	31.05	99.78
C4’	77.32	30.20	97.04
C5’	71.51	31.06	99.81
C6’	60.69	31.13	100.03
	β*-D-Fru*f		
C1’’	61.87	31.13	100.03
C2’’	104.16	30.16	97.92
C3’’	77.01	30.69	98.63
C4’’	74.57	30.53	98.10
C5’’	81.91	30.87	99.19
C6’’	62.88	30.26	97.24

Table 3. Repeatability[a] and reproducibility[a] of the ^{13}C NMR methodology for the determination of sugars in honey.

A/A	Repeatability	Reproducibility
1	34,677	34.642
2	34,702	34.699
3	34,680	34.703
4	34,672	34.688
5	34,710	34.676
6	34,685	34.696
Ave	34.700	34.684
SD	0.015	0.023
%CV	0.043	0.066

[a]Calculated using solutions of 34.691 μmol/mL of the β-D-glucopyranose tautomer prepared from 0.010 g of D-glucose dissolved in D_2O after equilibration, and taking into account that the equilibrium composition of β-D-glucopyranose is 62.5% (Table 1).

Table 4. Quantification (g / 100 g of honey) of sugar tautomers contained in various Greek honey samples by employing ^{13}C NMR spectroscopy.

Tautomer	Thyme	Pine	Polyfloral	Heather	Spruce	Chestnut	Citrus
α-D-glycopyranose (1*α*-GP)	15.27 (±0.03)[a]	10.16 (±0.05)	15.07 (±0.07)	15.39 (±0.01)	9.92 (±0.02)	11.79 (±0.01)	16.14 (±0.01)
β-D-glycopyranose (1*β*-GP)	26.50 (±0.02)	14.76 (±0.02)	26.37 (±0.04)	21.65 (±0.01)	16.52 (±0.01)	19.46 (±0.03)	23.95 (±0.04)
Total D-glucose	41.77 (±0.01)	24.92 (±0.03)	41.44 (±0.03)	37.04 (±0.01)	26.44 (±0.01)	31.25 (±0.02)	40.09 (±0.02)
α-D-fructopyranose (2*α*-FP)	0.55 (±0.01)	n. d.	n.d.	0.76 (±0.04)	n. d.	0.67 (±0.02)	1.08 (±0.03)
α-D-fructofuranose (2*α*-FF)	3.81 (±0.06)	2.11 (±0.02)	4.75 (±0.02)	3.62 (±0.01)	1.51 (±0.01)	1.92 (±0.01)	3.98 (±0.01)
β-D-fructofuranose (2*β*-FF)	13.15 (±0.02)	10.91 (±0.05)	13.31 (±0.03)	12.15 (±0.01)	7.02 (±0.01)	8.98 (±0.01)	12.96 (±0.01)

β-D-fructopyranose (2*β*-FP)	25.58 (±0.01)	19.37 (±0.03)	25.78 (±0.01)	24.67 (±0.02)	21.34 (±0.01)	21.77 (±0.01)	25.41 (±0.02)
Total D-fructose	43.09 (±0.01)	32.39 (±0.03)	43.84 (±0.02)	41.20 (±0.01)	29.87 (±0.01)	33.34 (±0.01)	43.43 (±0.01)
α-D-maltose (3*α*-GP)	0.60 (±0.02)	0.55 (±0.00)	0.55 (±0.01)	0.76 (±0.01)	0.55 (±0.02)	0.55 (±0.00)	1.17 (±0.02)
β-D-maltose (3*β*-GP)	0.94 (±0.03)	0.84 (±0.03)	0.90 (±0.01)	1.16 (±0.03)	0.86 (±0.00)	0.87 (±0.01)	0.76 (±0.02)
Total D-maltose	1.54 (±0.03)	1.49 (±0.03)	1.45 (±0.01)	1.96 (±0.03)	1.41 (±0.02)	1.42 (±0.01)	1.93 (±0.02)
turanose (7*β*-FP)	0.39 (±0.01)	0.46 (±0.02)	0.79 (±0.01)	0.75 (±0.01)	0.97 (±0.01)	0.63 (±0.01)	0.86 (±0.02)
turanose (7*β*-FF)	0.20 (±0.01)	0.21 (±0.01)	0.36 (±0.01)	0.35 (±0.01)	0.46 (±0.01)	0.30 (±0.01)	0.45 (±0.01)
turanose (7α-FF)	1.05	0.40	0.86	0.70	0.92	0.65	0.73

	(±0.01)	(±0.01)	(±0.01)	(±0.01)	(±0.01)	(±0.02)	(±0.01)
Total D-turanose	1.64 (±0.01)	1.07 (±0.01)	2.01 (±0.01)	1.80 (±0.01)	2.35 (±0.01)	1.58 (±0.01)	2.04 (±0.01)
α-D-isomaltose (4α-GP)	0.47 (±0.05)	0.79 (±0.01)	0.16 (±0.01)	0.41 (±0.01)	0.38 (±0.03)	0.41 (±0.01)	0.44 (±0.01)
β-D-isomaltose (4β-GP)	0.80 (±0.05)	1.26 (±0.03)	0.24 (±0.01)	0.77 (±0.00)	0.59 (±0.03)	0.77 (±0.01)	0.67 (±0.01)
Total D-isomaltose	0.14 (±0.02)	2.06 (±0.02)	0.40 (±0.01)	1.18 (±0.01)	0.96 (±0.03)	1.18 (±0.01)	1.1 (±0.01)
α-D-nigerose (5α-GP)	0.12 (±0.01)	0.11 (±0.01)	0.31 (±0.02)	0.32 (±0.01)	0.33 (±0.01)	0.12 (±0.01)	0.25 (±0.01)
β-D-nigerose (5β-GP)	0.19 (±0.02)	0.17 (±0.03)	0.54 (±0.01)	0.54 (±0.01)	0.55 (±0.01)	0.24 (±0.01)	0.41 (±0.01)
Total D-nigerose	0.31 (±0.01)	0.28 (±0.03)	0.85 (±0.01)	0.86 (±0.01)	0.88 (±0.01)	0.35 (±0.01)	0.66 (±0.01)

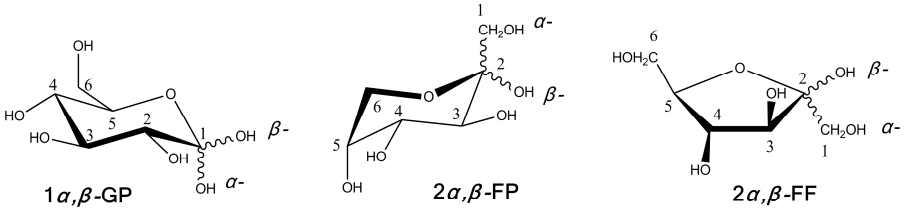
maltulose (*8*β-FF)	1.24 (±0.02)	0.18 (±0.00)	1.05 (±0.01)	0.74 (±0.03)	1.04 (±0.01)	0.24 (±0.01)	0.81 (±0.01)
maltulose (*8*α-FF)	0.03	--	--	--	--	--	--
maltulose (*8*β-FP)	0,66 (±0.01)	0.3 (±0.01)	0.61 (±0.01)	0.46 (±0.01)	0.52 (±0.01)	0.46 (±0.01)	0.43 (±0.01)
Total D-maltulose	1.93 (±0.01)	0.48 (±0.01)	1.66 (±0.01)	1.20 (±0.02)	1.56 (±0.01)	0.70 (±0.01)	1.23 (±0.01)
sucrose (6*β*-FF)	0.1 (±0.00)	1.71 (±0.02)	1.11 (±0.01)	0.17 (±0.02)	0.18 (±0.01)	1.29 (±0.01)	0.15 (±0.01)
erlose (9*β*-FF)	0.41 (±0.01)	0.64 (±0.03)	0.73 (±0.02)	0.22 (±0.01)	0.62 (±0.01)	1.21 (±0.01)	0.82 (±0.01)
melisitose (13*α*-GP)	0.14 (±0.01)	0.10 (±0.01)	0.17 (±0.00)	0.09 (±0.00)	8.44 (±0.01)	0.1 (±0.00)	0.22 (±0.01)
1-kestose (14*β*-FF)	0.20 (±0.01)	0.19 (±0.02)	0,11 (±0.00)	1.96 (±0.01)	0.18 (±0.01)	0.22 (±0.00)	0.20 (±0.01)

α-D-maltotriose (10α-GP)	0.01 (±0.01)	>0.01 (±0.00)	0.01 (±0.00)	>0.01 (±0.00)	0.01 (±0.00)	>0.01 (±0.00)	0.01 (±0.00)
β-D-maltotriose (10β-GP)	0.01 (±0.01)	0.01 (±0.00)	0.01 (±0.01)	0.01 (±0.00)	0.02 (±0.00)	0.01 (±0.00)	0.01 (±0.00)
Total D-maltotriose	0.02 (±0.01)	0.01 (±0.00)	0.02 (±0.01)	0.01 (±0.00)	0.03 (±0.00)	0.01 (±0.00)	0.02 (±0.00)
α-D-isomaltotriose (11α-GP)	0.05 (±0.01)	0.06 (±0.01)	0.02 (±0.01)	0.07 (±0.00)	0.01 (±0.00)	0.01 (±0.00)	0.01 (±0.00)
β-D-isomaltotriose (11β-GP)	0.09 (±0.01)	0.03 (±0.00)	0.05 (±0.01)	0.13 (±0.00)	0.02 (±0.00)	0.01 (±0.00)	0.03 (±0.00)
Total D-isomaltotriose	0.13 (±0.02)	0.08 (±0.01)	0.07 (±0.01)	0.02 (±0.00)	0.03 (±0.00)	0.02 (±0.00)	0.04 (±0.00)
α-D-panose (12α-GP)	0.07 (±0.01)	0.08 (±0.01)	0.02 (±0.00)	0.03 (±0.01)	0.16 (±0.01)	0.04 (±0.01)	0.07 (±0.01)
β-D-panose (12β-GP)	0.02	0.17	0.05	0.09	0.30	0.08	0.13

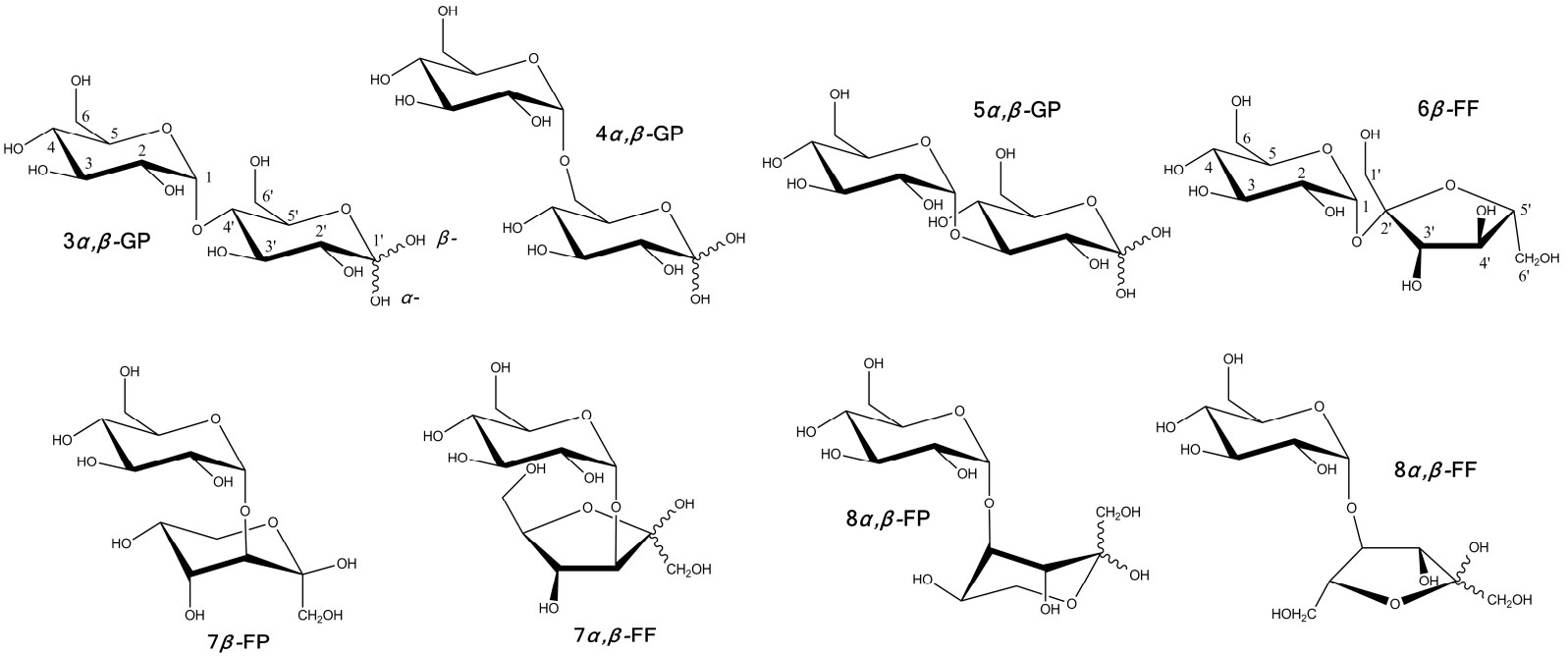
	(±0.01)	(±0.01)	(±0.00)	(±0.01)	(±0.01)	(±0.01)	(±0.01)
Total D-panose	0.09	0.25	0.08	0.12	0.45	0.12	0.20
	(±0.01)	(±0.03)	(±0.00)	(±0.01)	(±0.01)	(±0.01)	(±0.01)

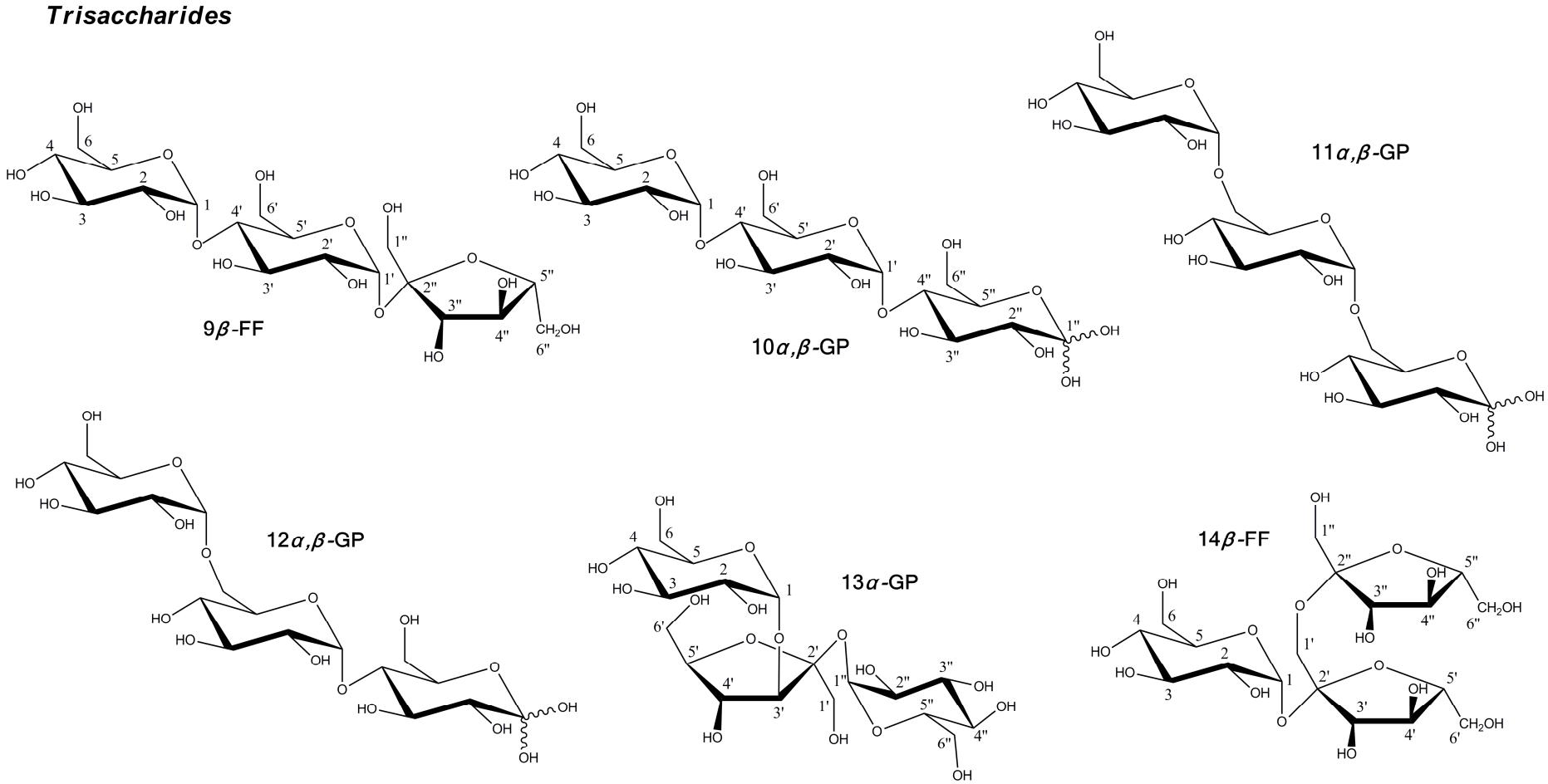
[a]Numbers in parentheses denote standard deviation of three measurements.

Monosaccharides



Disaccharides





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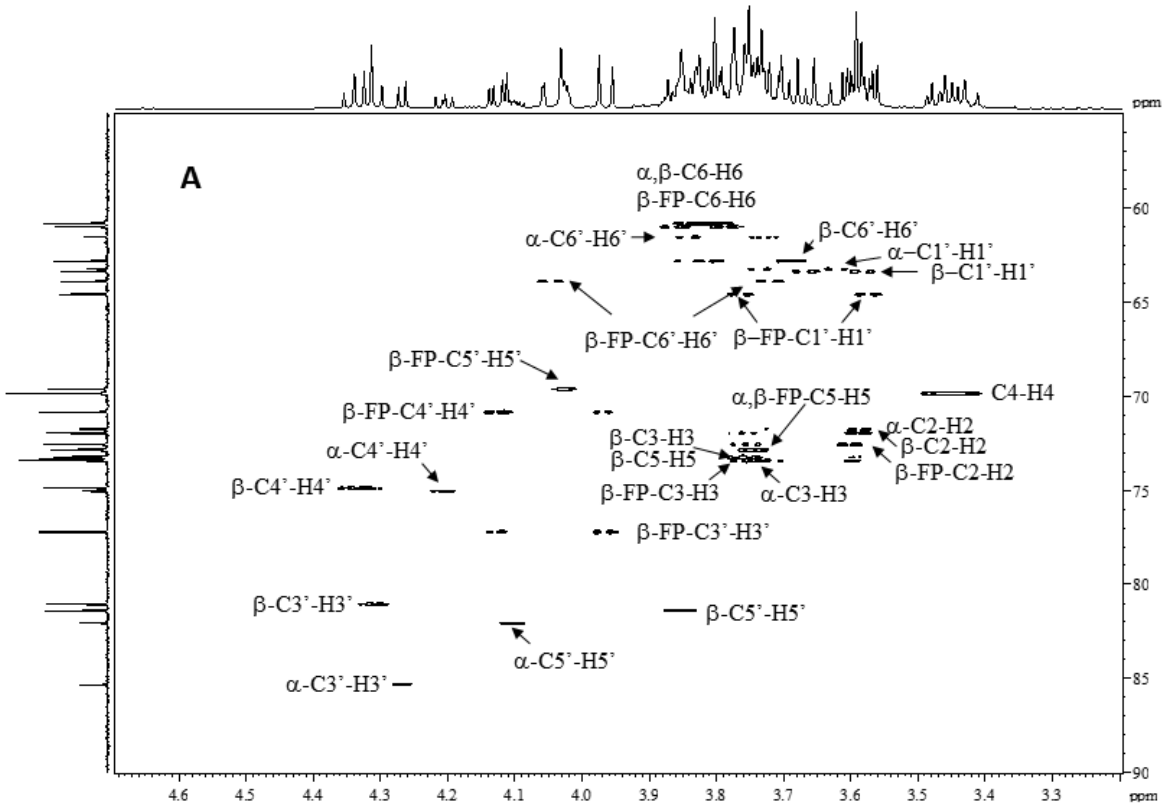

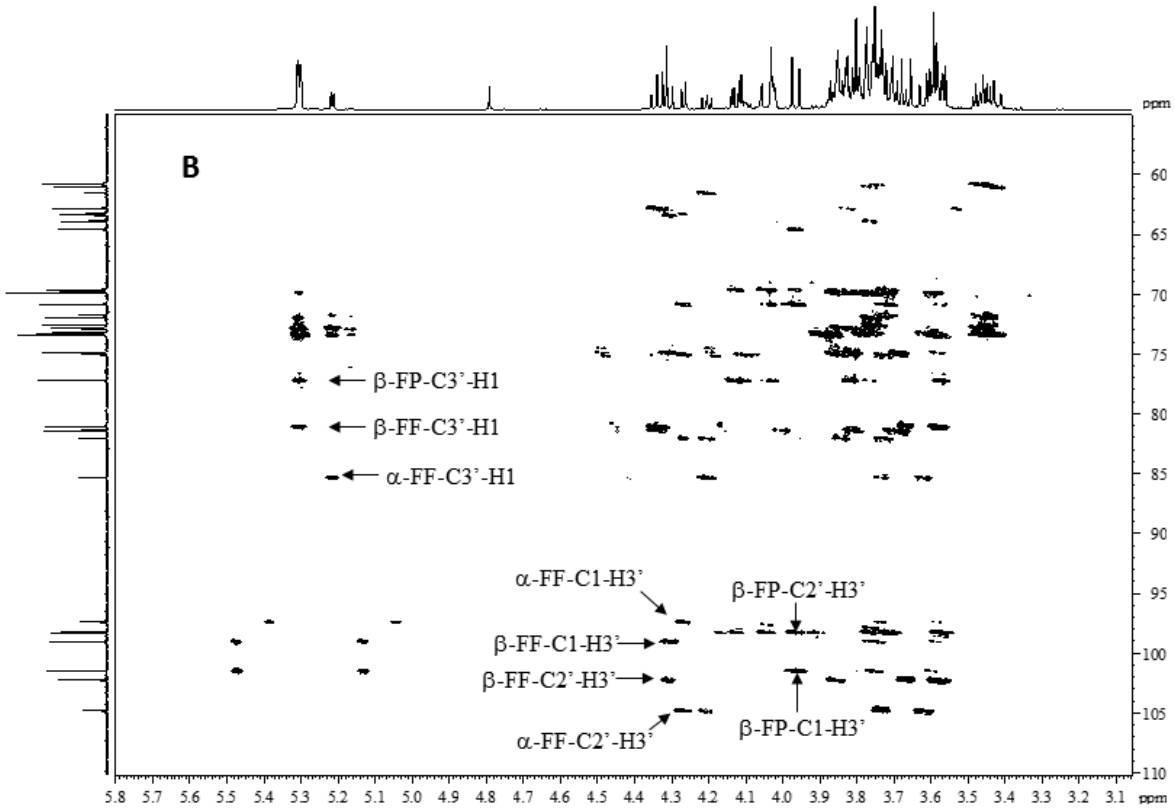

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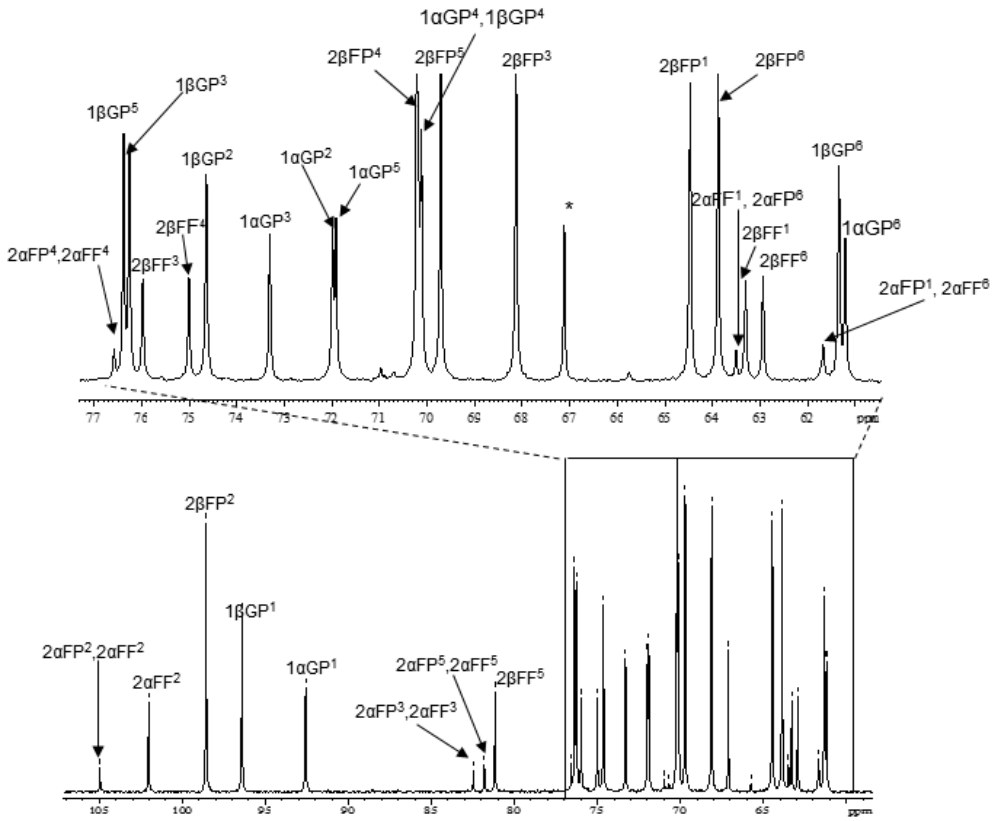

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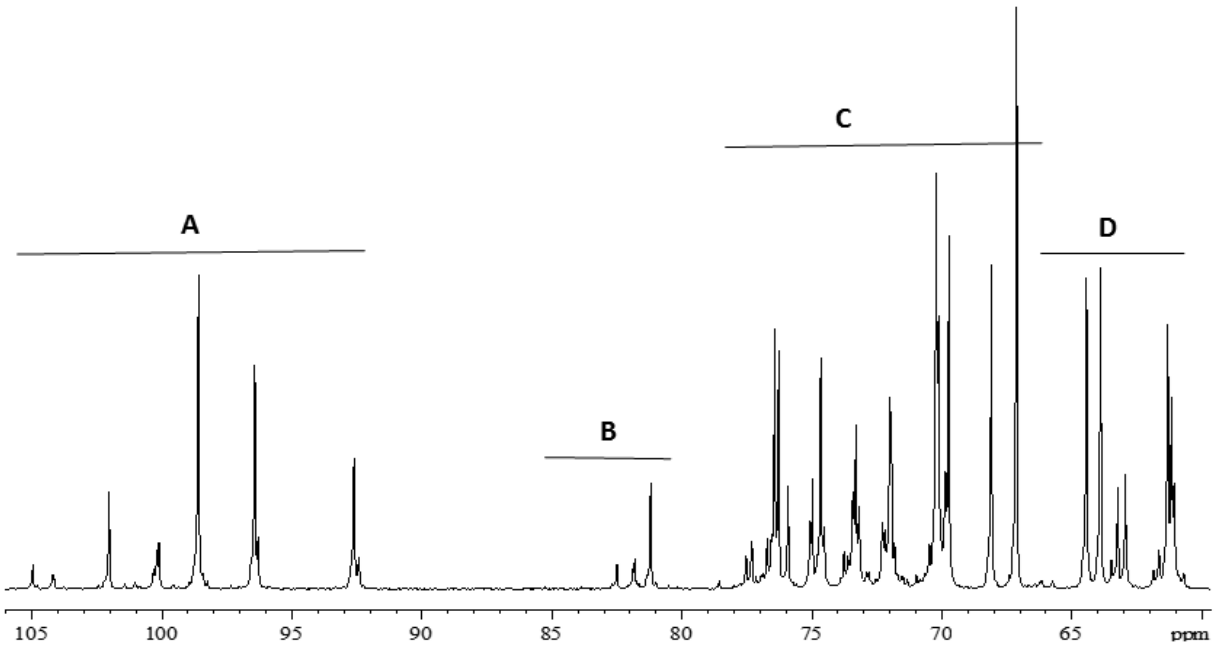

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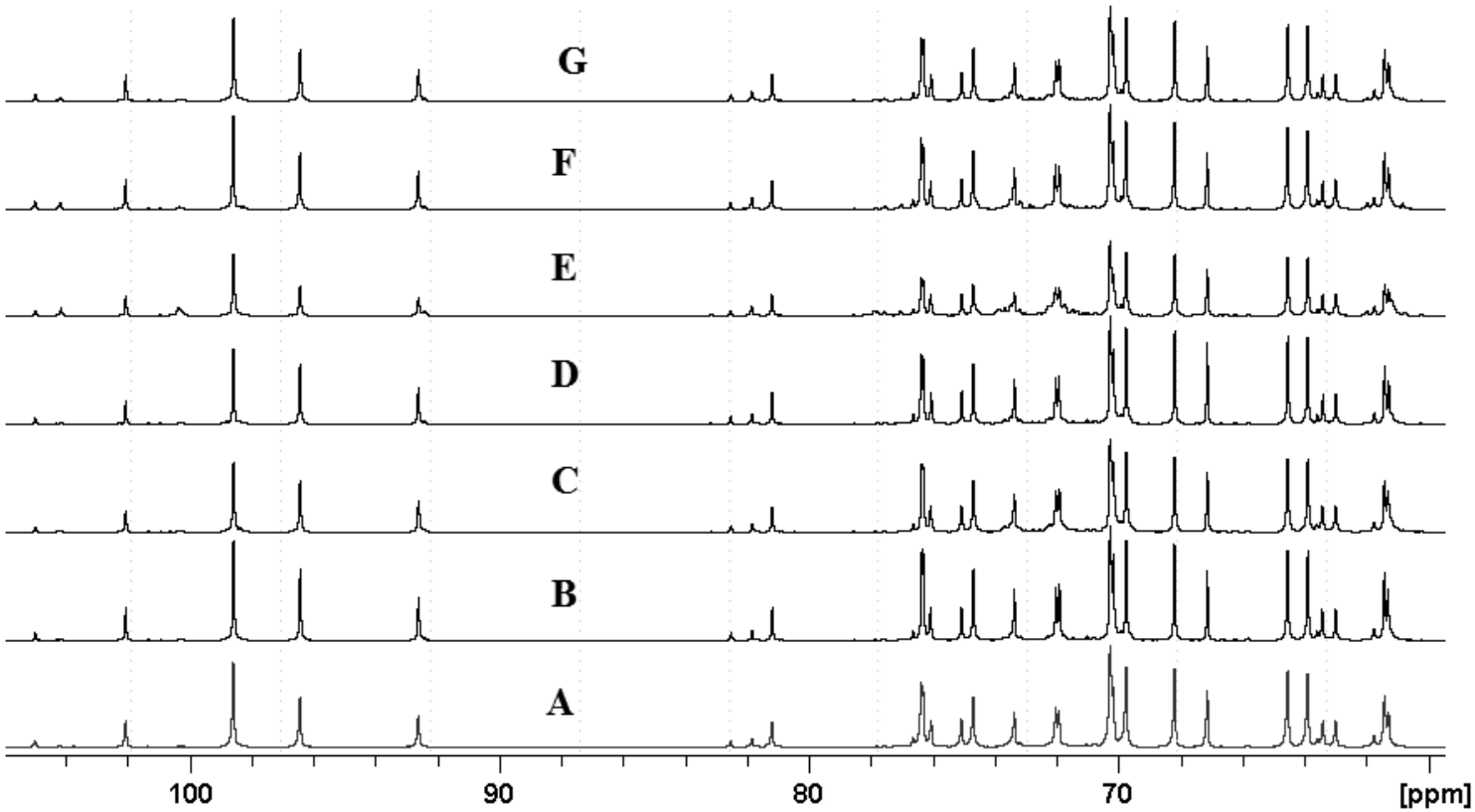

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