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Direct Qualitative and Quantitative Analysis of Carbohydrate Mixtures Using ^{13}C NMR Spectroscopy: Application to Honey

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A method is described which allows the identification of individual carbohydrates of multi-component artificial mixtures using the computer-aided analysis of their ^{13}C NMR spectra, without previous separation. Quantitation of mono-, di- and trisaccharides was carried out after improvement of the experimental procedure and using an internal standard. The procedure was then applied to authentic honeys of different floral types, harvested in Corsica (France). Several oligosaccharides (identification of some of these is not easy by chromatographic techniques) were observed at levels ranging from 0.4 to 3.3%. In addition, the fructose/glucose ratio, which has an influence on crystallization, was easily obtainable. © **1997 John Wiley & Sons, Ltd.**

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INTRODUCTION

Carbohydrate analysis is of considerable importance in the food and pharmaceutical industries, where a knowledge of the qualitative and quantitative distribution of sugars in raw materials is of great interest for quality control.[1] For this purpose, several techniques have commonly been applied, such as gas chromatography (GC)[1–3] and high-performance liquid chromatography (HPLC),[4–6] sometimes coupled on-line with mass spectrometry (GC–MS,[7] HPLC–MS[8]) or Fourier transform infrared spectrometry (GC–FT-IR).[9] Nevertheless, all these analytical procedures suffer from several drawbacks. Concerning GC, the poor volatility of carbohydrates necessitates a time-consuming chemical pretreatment of the samples before injection[1,7,9] (e.g. methylation, acylation, trimethylsilylation, oximation). HPLC, which allows the analysis of underivatized or thermolabile carbohydrates, also requires sample preparation of natural mixtures (e.g. centrifugation and/or filtration, precipitation, extraction).[5] In recent years, the detection of saccharides has been improved by the use of ion chromatography coupled with amperometric or photometric detection.[5,10,11] More recently capillary electrophoresis (CE) with direct or inverse UV detection has been suggested for the analysis of carbohydrates.[5,12]

The aforementioned techniques have been tried in order to determine the composition of honey, an important product in food nutrition, which mainly consists of carbohydrates plus water and various other compounds.[13,14] Honey is characterized by a high content of the two monosaccharides glucose and fructose (42–85%).[15,16] However, both the presence and the ratio of several di- and trisaccharides (total amount $<10\%$) vary considerably from one type of honey to another. Some workers have suggested that oligosaccharide profiles could allow a possible correlation with the botanical and geographical origin of honeys.[17–19]

In the field of carbohydrate research, ^{13}C NMR spectroscopy has been widely employed for molecular dynamics[20,21] and structural studies.[22,23] However, from the analytical point of view, only a few papers have described the use of NMR for the analysis of natural mixtures of carbohydrates, even though this technique has been widely used for the identification and quantitation of individual components of complex mixtures.[24–26] About 20 years ago, Blunt and Munro[27] developed a procedure for the qualitative and quantitative analysis of sugars such as glucose, fructose, *myo*-inositol, pinitol and sequoyitol, extracted from various tissues of *Pinus radiata*. Tamate and Bradbury[28] identified and quantified glucose, fructose, maltose, sucrose and raffinose in tropical root crops. De Bruyn and Van Loo[29] identified sucrose and two trisaccharides, 1-kestose and neokestose, present in plant extracts. Swallow and Low[30] identified several oligosaccharides including 6-kestose and kelose in a commercial beet medium invert syrup. In addition, Rapp and Markowetz[31,32] carried out the determination of glucose, fructose, saccharose, rhamnose, xylose, ribose, mannitol and inositol in wines and fruit juices. More recently, Vogels *et al.*[33] identified glucose, fructose and mannose in German white wines.

Only a few reports have been published on the application of NMR to the analysis of carbohydrates in honey. Low *et al.*[34] investigated the qualitative and quantitative analysis of minor disaccharides found in

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two Canadian honeys. Nevertheless, ^{13}C NMR analysis was carried out only after HPLC separation of fructose and glucose on the one hand, and of all oligosaccharides on the other, followed by borohydride reduction of the latter. Maltose, isomaltose, sucrose, kojibiose, turanose, palatinose, gentiobiose, neotrehalose and nigerose were then identified on the basis of the 'fingerprint' peaks of their reduced forms. For quantitation, they used a relaxation reagent and *O*-methyl-D-ribose as an internal standard. Nevertheless, there were limitations to the identification of some reduced trisaccharides, which exhibited several overlapped signals with the corresponding reduced disaccharides and so were not identified or quantified. More recently, Rapp and Markowetz[35] described in a short report the direct identification of glucose, fructose, threalose, sucrose, turanose, maltose, nigerose and maltotriose in one sample of sunflower honey.

As part of our ongoing work on the identification of individual components of complex natural mixtures, using the computer-aided analysis of their ^{13}C NMR spectra,[36,37] we studied the potential of this methodology for the direct identification and quantitation of mono-, di- and trisaccharides present in honeys.

RESULTS AND DISCUSSION

Our goal was to carry out both qualitative and quantitative determinations from the analysis of the same spectrum. In this context, we were interested in developing an experimental procedure which would allow the best compromise between good resolution of the signals and accurate quantitation. The selected method was validated by checks performed on artificial mixtures.

Qualitative procedure

We adapted to carbohydrates a methodology based on the computer-aided analysis of the ^{13}C NMR spectrum of a complex mixture (essential oil, bio-oil) developed in our laboratory.[36,37] In this procedure, an individual component is identified by comparison of the signals of the mixture spectrum with those of pure reference spectra compiled in a laboratory-made library. Each compound is identified by taking into account the three following parameters, directly available from the computer program: (i) the number of observed carbons with respect to the number of expected signals, (ii) the number of overlapped signals of carbons which possess the same chemical shift and (iii) the difference in the chemical shift of each signal in the mixture and in the reference.

In this context, it was of great importance to obtain reproducible chemical shift values between the mixture spectrum and the reference spectra and to minimize the number of overlapped signals. We therefore began simultaneously (i) to define the optimum experimental and acquisition conditions to record NMR spectra (solvent, concentration, low-power decoupling of the proton band, pulse width, FID treatment) and (ii) to create a ^{13}C NMR spectral data library. We then checked the experimental procedure on artificial mixtures of carbohydrates before its application to six honeys of different botanical origin.

The experimental procedure was improved by recording the spectra using deuterium oxide as internal lock signal in H_2O–D_2O solution (75:25, v/v). We chose as an internal standard the usual 1,4-dioxane, which exhibits a single resonance at 67.12 ppm and so is easily identified in the ^{13}C NMR spectrum of the mixture. Moreover, it does not overlap with any resonance of saccharides. We pulsed under the whole spectral width of 12 500 Hz (0–250 ppm) since we observed that spectra recorded under the carbohydrate characteristic resonance region (60–110 ppm) gave no significant differences. Simultaneously, we optimized the signal treatment parameters: all spectra (pure compounds, artificial mixtures, honeys) were treated with Gaussian and Lorenzian multiplication of the FID applied before Fourier transformation (see Experimental). Applied to our mixtures of carbohydrates, this treatment led to the best resolution of the signals, which is of great importance for the identification of minor components, without decreasing the accuracy of quantitation (see below, Quantitative procedure).

References. We measured the spectra of 35 commercially available saccharides. We propose to detail here the spectra of ten of these (two monosaccharides, five disaccharides and three trisaccharides) usually present in honeys.

D-Glucose and D-glucose reducing-end di- and trisaccharides (*Fig. 1*). D-Glucose in solution exists naturally, because of the mutarotational equilibrium phenomenon, as a mixture of six isomers, i.e. the two α- and β-anomers in pyranose form (α-GP and β-GP) associated with very small quantities of α- and β-anomers in furanose form, the corresponding aldehyde and hydrated aldehyde (*gem*-diol). The furanose, aldehyde and *gem*-diol forms, which are present in a very small proportion (0.14–0.0024%), have been determined by Maple and Allerhand[38] using ultra-high-resolution NMR and a concentrated solution of ^{13}C-labeled D-glucose. In the standard spectrum of a dilute solution of D-glucose, 12 signals were observed and assigned to the anomeric forms α-GP and β-GP.[39,40]

Similarly, the spectra of the disaccharides nigerose, maltose and isomaltose (Fig. 1) exhibited 19, 20 and 15 signals, respectively, instead of the 24 expected, corresponding to 5, 4 and 9 overlaps, respectively, between the two α-GP and β-GP anomeric forms.[41,42]

Isomaltotriose is a D-glucose reducing-end trisaccharide. In its spectrum, only 19 resonances were observed instead of the 36 expected, taking into account the two α-GP and β-GP forms due to the similarity of the two glucose moieties. It should be pointed out that the anomeric carbon of each form was clearly identified.[42] Finally, the spectrum of the non-reducing trisaccharide melezitose exhibited only 18 signals as expected.[41]

D-Fructose and D-fructose reducing-end di- and trisaccharides (*Fig. 2*). D-Fructose can potentially exist in solution in five tautomeric forms, i.e. the two α- and β-pyrans (α-FP, β-FP), the two α- and β-furans (α-FF, β-FF) and the open-chain form (acyclic keto

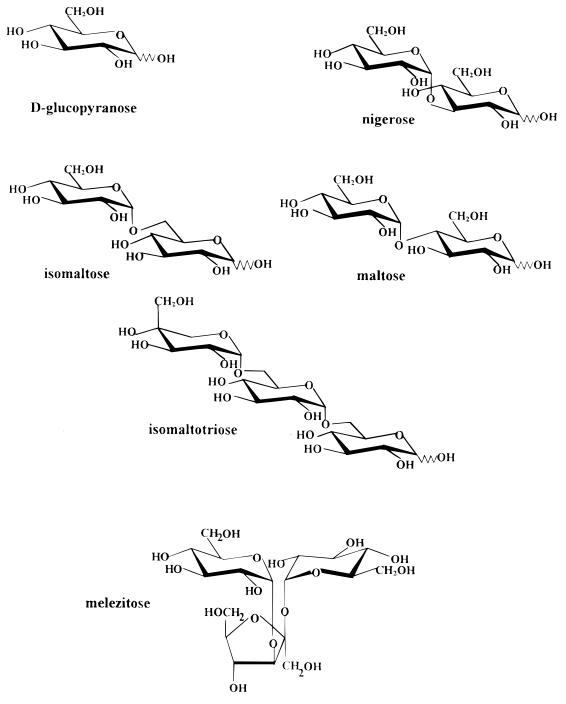

Figure 1. D-Glucose and D-glucose reducing and non-reducing-end di- and trisaccharide anomeric forms: α, axial OH; β, equatorial OH.

form).[40,43,44] Under our experimental conditions, the fructose spectrum exhibited 22 signals instead of the 24 expected, corresponding to the four cyclic forms. The minor α-FP form was clearly identified. Conversely, the keto form was not detected, in agreement with literature data.[40,44]

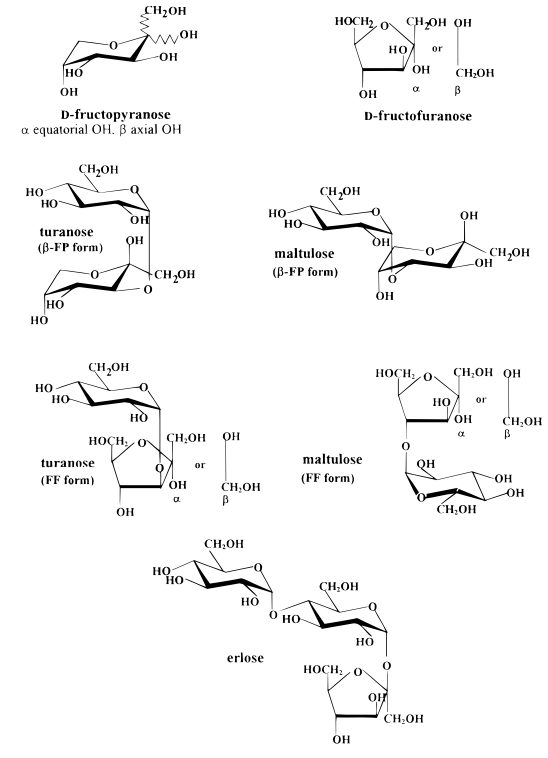

Figure 2. D-Fructose and D-fructose reducing and non-reducing-end di- and trisaccharides.

Maltulose and turanose are D-fructose reducing-end disaccharides. In our spectra of dilute solutions of these compounds, 29 and 32 signals were observed, respectively, corresponding to the α- and β-furan forms and to the β-pyran form.[41,43,44] No signals were detected for the α-pyranose and the ketonic form.

Finally, the spectrum of erlose, a D-fructose non-reducing-end trisaccharide, was characterized by the presence of 18 signals corresponding to the β-FF form.

It should be pointed out that the presence of overlapped signals for α- and β-anomeric forms of the same saccharide did not constitute a drawback. Associated with their intensities, it could improve the identification of these sugars, especially when they are present in a low ratio.

Artificial mixtures. To check our experimental procedure, we recorded the spectra of several artificial mixtures. We detail here the results for one of these, a mixture M1 of five mono-, di- and trisaccharides, in proportions close to their natural concentrations in honey (Table 1). D-Fructose, D-glucose, isomaltose, turanose and melezitose were mixed in the relative proportions 45:41:4:5:5 (w/w). In the spectrum of this mixture, all the carbons were observed for all isomeric forms of D-fructose, D-glucose, isomaltose and melezitose and for the two major forms of turanose. The number of overlapped signals ranged from 0 to 1 for the monosaccharides (the α-GP form of glucose excepted) and from 0 to 6 for the disaccharides and reached 8 for the trisaccharide. The chemical shift differences between the mixture and the reference are $\leqslant$0.05 ppm for 99 carbons out of the 102 observed (0.06 ppm for the other three). Comparable results were obtained for other artificial mixtures. From these results, we drew the following conclusions:

(i) The number of observed isomeric forms and detected peaks were sufficient to allow the unambiguous identification of the five saccharides in the mixture.

Table 1. ^{13}C NMR identification of D-fructose, D-glucose, isomaltose and turanose isomeric forms and melezitose in the artificial mixture M1

Components[a]	^{13}C NMR[b]	OS[c]
D-Fructose:		
α-FF	6/6	1
β-FF	6/6	1
α-FP	6/6	0
β-FP	6/6	1
D-Glucose:		
α-GP	6/6	5
β-GP	6/6	1
Isomaltose:		
α-GP	12/12	0
β-GP	12/12	2
Turanose:		
β-FF	12/12	6
β-FP	12/12	1
Melezitose	18/18	8

[a] FF = fructofuranose; FP = fructopyranose; GP = glucopyranose.
[b] Number of observed carbons with respect to the number of expected signals.
[c] Number of overlapped signals of carbons which possess the same chemical shift.

Table 2. Equilibrium composition of selected saccharides as calculated either from the mean values of resonance intensities of all carbons or from peak intensities of anomeric carbon in ^{13}C NMR spectra[a]

Standard	Tautomeric form[b]	All carbons (%)	Anomeric carbons (%)	Literature data (%)	(%)	(%)	(%)
D-Fructose	α-FF	5.2	4.8	5[c]	5[c]		
	β-FF	22.7	23.0	23[c]	23[c]		
	α-FP	1.9	2.4	2[c]	0[c]		
	β-FP	70.2	69.8	70[c]	72[c]		
D-Glucose	α-GP	37.5	37.0	34[c]	36[c]	37.3[c]	38.8[d]
	β-GP	62.5	63.0	66[c]	64[c]	62.6[c]	60.9[d]
Maltulose	α-FF	8.2	8.9	7.6[e]	12.1[e]		
	β-FF	29.1	26.7	30.7[e]	22.4[e]		
	β-FP	62.7	64.4	61.5[e]	64.0[e]		
Turanose	α-FF	17.7	17.0	20.0[f]			
	β-FF	43.4	42.1	41.0[f]			
	β-FP	38.9	40.9	39.0[f]			
Maltose	α-GP	39.4	39.7				
	β-GP	60.6	60.3				
Isomaltose	α-GP	41.8	42.1				
	β-GP	58.2	57.9				
Nigerose	α-GP	39.1	38.6				
	β-GP	60.9	61.4				
Isomaltotriose	α-GP	—[g]	36.4				
	β-GP	—[g]	63.6				

[a] Each standard in H_2O–D_2O (75:25, v/v) solution at 27 °C (spectra were recorded three times to ensure reproducibility).
[b] FF = fructofuranose; FP = fructopyranose; GP = glucopyranose.
[c] Ref. 40 and references cited therein.
[d] Ref. 38 and references cited therein.
[e] Ref. 43 and references cited therein.
[f] Ref. 49 and references cited therein.
[g] Quantitation was carried out taking into account only the intensities of anomeric carbons, owing to the large number of overlapped signals of the anomeric forms (see text).

(ii) The anomeric carbons of all isomeric forms of the five molecules were detected in the mixture spectrum. No overlap of these peaks with any other was observed.
(iii) The number of overlapped signals is always less than or equal to half of the number of resonances for each form, except for the α-GP form of D-glucose. Nevertheless, there is no problem in the identification of this sugar, taking into account either the peak intensities of this form (D-glucose is a major component) or the resonances of the β-GP form.
(iv) The difference in concentration (D-fructose and D-glucose on the one hand, di- and trisaccharides on the other) had an insignificant influence on chemical shift variations between the mixture and the reference spectra.

Quantitative procedure

Several techniques have been developed for the quantitation of individual components of a mixture using ^{13}C NMR spectroscopy: waiting a period of $5T_1$ (or 5–6 s for saccharides) before applying another pulse allows complete relaxation of carbon nuclei;[45] the gated decoupling technique induces the suppression of the nuclear Overhauser enhancement (NOE) and is useful when nuclei have different spin–lattice relaxation times (T_1);[46] the addition of paramagnetic species allows a shortening of the relaxation time by means of an electronic relaxation mechanism;[46] and the use of an internal standard permits quantitation even in presence of other (unknown) substances.[46]

Although all these procedures were successfully carried out alone or combined, some inconveniences occurred. On the one hand, both the use of gated decoupling technique and waiting for a period of $5–6T_1$ result in a considerable lengthening of the time required to obtain an acceptable signal-to-noise ratio,[27] and on the other hand, the use of paramagnetic species leads to prejudicial broadening of peak resonances.

Taking into account these considerations, we chose to record the spectra without using inverse gated decoupling or a relaxation reagent and we preferred the internal standard method.

We mentioned that 1,4-dioxane is the usual standard in the qualitative analysis of carbohydrates, but its spin–lattice relaxation time T_1 (5.5 s measured by the inversion–recovery method) is longer than those of saccharides (typically 0.5–1 s[28]). It has been suggested that the best approach for the analysis of complex mixtures containing nuclei with a wide range of T_1s is a compromise between the aforementioned typical quantitative

procedures and a rapid train of short pulses.[47] It is known that a small flip angle provides a smaller difference in the steady-state magnetization than a larger angle in presence of nuclei having different T_1 values.[27,48]

Therefore, in order to be able to use dioxane for quantitative measurements, we chose a pulse width corresponding to a 30° flip angle. In our hands, this experimental procedure associated with a spectral width of 0–250 ppm and a short recycling time led to reasonable time of analysis despite the utilization of a routine medium-field spectrometer (4.7 T).

Otherwise, accurate peak area measurements which are commonly used to access the ratio of the different nuclei of a pure compound or the ratio of individual components of simple mixtures are often not reliable with complex mixture spectra, since the signals are not always well differentiated, owing to the large number of resonances. Consequently, the ratios of different resonances (saccharides *vs.* 1,4-dioxane) were obtained from the peak heights, this procedure being checked on artificial mixtures.

References. In order to check our quantitative procedure, evaluation of the ratios of isomeric forms for D-fructose, D-glucose, maltulose and turanose, already described in the literature,[38,40,43,49] was carried out using the above-mentioned parameters. The procedure was then applied to maltose, isomaltose, nigerose and isomaltotriose.

Each reference spectrum was recorded after mutarotation was completed (a few hours after dissolution in a D_2O–H_2O mixture). Quantitative evaluation was based on the relative peak heights taking into account for each isomer, either the mean value of all carbons or only the anomeric peak heights (Table 2).

The results in Table 2 revealed that the tautomeric form percentages obtained for D-fructose, D-glucose, maltulose and turanose were in agreement with literature data. We found that the ratios obtained for the disaccharides maltose, isomaltose and nigerose and the trisaccharide isomaltotriose were close to those determined for the two anomeric forms of D-glucose itself.

Artificial mixtures. We investigated first the quantitative analysis of a mixture of D-fructose and D-glucose and then two more complex artificial mixtures of these monosaccharides with three different di- and trisaccharides.

Artificial mixture of D-fructose and D-glucose (M2). We measured the spectrum of a non-equimolar mixture M2 of D-fructose and D-glucose (59.5:40.5). In Table 3 are reported the results obtained taking into account either the mean value of the peak heights of all carbons or only the anomeric peak heights. Four spectra were recorded to ensure reproducibility. We observed that the isomeric form ratios do not differ significantly from those obtained for the same references studied as pure compounds (Table 2), whatever the method employed. Finally, the fructose to glucose (F/G) ratio was also correctly estimated, the relative error ranging from −2.0 to +4.1% considering either all the carbons or only the anomeric carbons.

It should be pointed out that the ratio of different forms for both sugars and the F/G ratio were easily and quickly obtained taking into account only the signal heights of anomeric carbons. This latter evaluation is of great interest for the quality control of honey since it has an effect on crystallization during ageing.[13]

Artificial mixtures of mono-, di- and trisaccharides. Since our final goal was to apply ^{13}C NMR spectroscopy to

Table 3. ^{13}C NMR spectrum of a mixture of D-fructose and D-glucose: tautomeric form relative ratios (%); F/G ratio[a]

			M2			
	All carbons[b]			Anomeric carbons[b]		
Components/parameter[c]	Range (%)	Mean (%)	Ref.	Calc.	Range (%)	Mean (%)	Ref.
α-FF	5.7–6.2	5.8	5.2		4.8–5.5	5.2	4.8
β-FF	22.8–24.0	23.1	22.7		21.1–22.4	21.7	23.0
α-FP	1.9–2.5	2.2	1.9		1.9–2.2	2.0	2.4
β-FP	67.7–69.6	68.8	70.2		70.6–72.0	71.0	69.8
F (%)	58.9–59.4	59.1		59.5	60.2–60.4	60.3	
RE (%)		−1.0 to −0.2				+1.2 to +1.5	
α-GP	37.3–38.5	38.0	37.5		35.2–37.6	36.6	37.0
β-GP	61.5–62.7	62.8	62.5		62.4–64.8	63.4	63.0
G (%)	40.6–41.0	40.8		40.5	39.5–39.8	39.6	
RE (%)		+0.2 to +1.2				−2.5 to −1.7	
F/G	1.44–1.46	1.45		1.47	1.51–1.53	1.52	
RE (%)		−2.0 to −0.7				+2.7 to +4.1	

[a] D-fructose and D-glucose contents (%) were measured either from the mean values of the resonance intensities of all carbons or from anomeric carbons peak intensities.
[b] Four spectra were recorded to ensure reproducibility. The ratios were measured from the intensities; Range and Mean refer to the four analyses; Ref., tautomeric form relative ratio (%) estimated in Table 2; calc., mass percentage of each sugar calculated taking into account the weight of each sugar with a precision of 0.1 mg.
[c] FF = Fructofuranose; FP = fructopyranose; GP = glucopyranose. RE = relative error. G = glucose content measured. F = fructose content measured. Values of the repeatability of the measurement for the four analyses given at 95%: F, 59.1 ± 0.3% (all carbons); F, 60.3 ± 0.1% (anomeric carbons); G, 40.8 ± 0.3% (all carbons); G, 39.6 ± 0.2% (anomeric carbons).

investigate the quantitative composition of saccharides in honey, we measured the spectra of two artificial mixtures of D-fructose, D-glucose and three di- and trisaccharides, isomaltose, turanose and melezitose, on the one hand (mixture M1), and maltulose, maltose and isomaltotriose on the other (mixture M3), in relative proportions close to those found in honey. Since honey not only contains carbohydrates but also water and various compounds (proteins, enzymes, etc.),[13] it was of interest to access directly the mass of each sugar in the mixture.

Quantitative analyses of both mixtures were therefore carried out in the presence of a precise amount of 1,4-dioxane (internal standard), taking into account the signal heights of anomeric carbons (Fig. 3, Tables 4 and 5). For each sugar we calculated the following: (i) the molar relative ratio of each component; (ii) the corresponding amount of each sugar, expressed as mass (mg) mS_m, determined by comparison of the peak height of the anomeric carbon (I_S) with that of 1,4-dioxane (I_d) using the equation $mS_m = (I_S/I_d)n_d M_S \times 4$ (where n_d = number of moles of 1,4-dioxane and M_S = the molecular weight of sugar); and (iii) the relative error (RE) between the quantitative experimental determinations and the initial weight expressed as a percentage.

Tables 4 and 5 summarize the results obtained from the analysis of M1 and M3. Duplicate analyses were carried out in order to establish the reproducibility and led to comparable results. Comparison of the results revealed that both methods allowed reliable quantitative determinations of the components. The relative errors ranged from −12.0 to +7.2% for M1 and from −14.3 to +11.1% for M3, except for the trisaccharide isomaltotriose (+22.0 to +27.8%). Nevertheless, we observed that such a high relative error was obtained for a trisaccharide that was present in the mixture at a very low ratio and that possesses two anomeric forms (i.e. two signals with very low intensity). Conversely, melezitose, which is a non-reducing trisaccharide, gave a reasonable relative error for M1 (*ca.* 5%).

The analysis of the same mixtures was also carried out using both the gated decoupling technique and peak area integrals and did not lead to more accurate results.

It appears from the analysis of artificial mixtures that a correct evaluation of the quantity of sugar was obtained using the procedure developed and with 1,4-dioxane as an internal standard, so we applied it to honey.

Direct ^{13}C NMR identification and quantitation of sugars in authentic honeys

We applied the aforementioned procedure to the direct identification and quantitation of carbohydrates present in six authentic honeys of different floral types (mixed floral, *Castanea*, *Robinia*, *Asphodelus*, *Anthyllis* and *Clementina*), harvested in various regions of Corsica (France) (Table 6).

We identified 5–8 carbohydrates (12–15 distinct isomeric forms) in each sample. The resonances of all carbons, except one, were observed for all isomeric forms of all identified sugars, except the minor forms of minor components, i.e. the α-FF form of maltulose and the α-FF form of turanose. In the spectrum of some samples, a few unassigned resonances, with very low intensities, belonged to carbohydrates which are missing from our spectral data library. The number of overlapped signals were in the range 0–3 (α-GP form of glucose excepted), 1–9 and 6–11 for the mono-, di- and trisaccharides, respectively. Nevertheless, as seen with artificial mixtures, the large number of overlapped signals for some of the isomeric forms of a few carbohydrates, which resulted from the similarity of their saccharide backbones, does not constitute a drawback for identification. In fact, the monosaccharide glucose was one of the two major compounds and the peak intensities are much higher than any other. For oligosac-

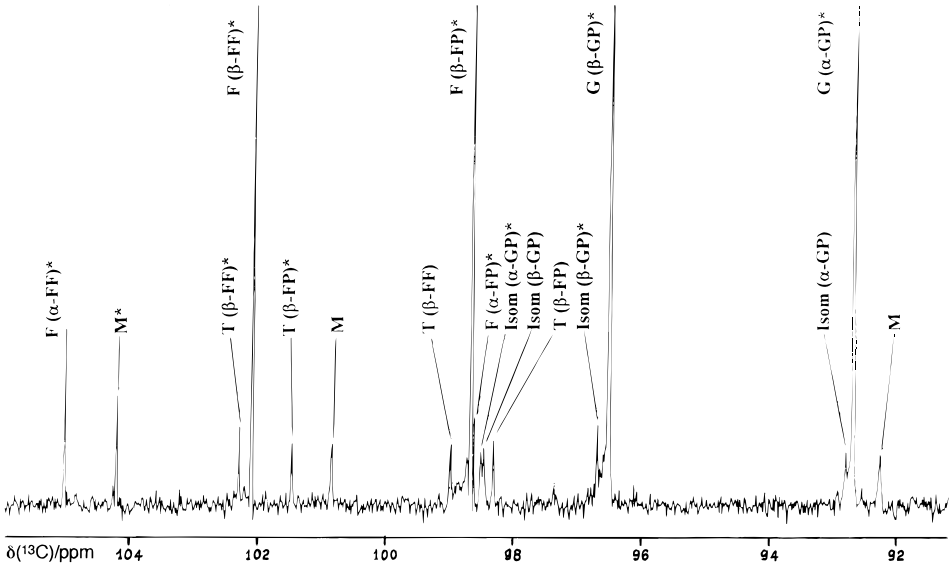

Figure 3. ^{13}C NMR spectrum of the anomeric region of the artificial mixture M1. F = fructose; G = glucose; T = turanose, Isom = isomaltose; M = melezitose, FF = fructofuranose; FP = fructopyranose; GP = glucopyranose. The asterisks indicate resonances used for quantitation of each form of each sugar.

Table 4. ^{13}C NMR quantitation of individual components of mixture M1 measured either using the molar relative ratios (%) or 1,4-dioxane as internal standard (mass estimations)

			Molar relative ratio (%)[c]			Mass (mg)/1,4-dioxane[d]		
Standard	Tautomeric form[a]	I[b]	% S_m	% S_c	RE (%)	mS_m	mS_c	RE (%)
D-Fructose	α-FF	0.8						
	β-FF	3.9						
	α-FP	0.5						
	β-FP	9.7	47.8	48.5	−1.4	103.8	102.0	+1.8
D-Glucose	α-GP	5.3						
	β-GP	8.9	45.5	44.2	+2.9	99.1	93.1	+6.4
Isomaltose	α-GP	0.3						
	β-GP	0.4	2.2	2.5	−12.0	8.9	9.8	−9.2
Turanose	β-FF	0.4						
	β-FP	0.3	2.6	2.8	−7.1	11.9[e]	11.1	+7.2
Melezitose	α-GP	0.6	1.9	2.0	−5.0	11.0	11.6	−5.4

[a] FF = fructofuranose; FP = fructopyranose; GP = glucopyranose.
[b] I = anomeric carbon peak intensity of each isomeric form of each sugar.
[c] % S_m = molar relative ratio (%) measured for each sugar; a correction factor was applied since the minor anomeric form (α-FF) of turanose is not detected and estimated at 17.0% in the standard turanose solution (Table 2). % S_c = molar relative ratio (%) calculated for each sugar weighted with a precision of 0.1 mg. RE = relative error.
[d] mS_m = mass (mg) measured by ^{13}C NMR for each sugar with 1,4-dioxane as internal standard. mS_c = mass (mg) of each sugar weighed with a precision of 0.1 mg.
[e] A correction factor was applied for the mass (mg) measured for turanose (α-FF form undetected).

charides, several remaining carbons, including all the anomeric ones, were assigned to only one molecule. The chemical shift variations between the mixture and the references were in all cases $\leqslant 0.05$ ppm.

The quantitation of the carbohydrates was carried out with 1,4-dioxane as an internal standard, taking into account the peak height of the anomeric carbon for each form of each component (Fig. 4). For maltulose and turanose, the minor α-FF form was undetected. We calculated the effective content of the carbohydrate using a correcting factor, based on the ratio of each form in the spectrum of the pure compound (Table 2).

The total content of carbohydrates ranged from 60.4 to 71.0%. Among these, as expected, fructose (31.5–

Table 5. ^{13}C NMR quantitation of individual components of mixture M3 measured either using the molar relative ratios (%) or 1,4-dioxane as internal standard (mass estimations)

			Molar relative ratio (%)[c]			Mass (mg)/1,4-dioxane[d]		
Standard	Tautomeric form[a]	I[b]	% S_m	% S_c	RE (%)	mS_m	mS_c	RE (%)
D-Fructose	α-FF	0.8						
	β-FF	3.4						
	α-FP	0.5						
	β-FP	9.3	48.2	48.5	−0.6	135.0	128.9	+4.7
D-Glucose	α-GP	3.8						
	β-GP	7.3	36.9	36.1	+2.2	106.6	95.9	+11.1
Maltulose	β-FF	0.6						
	β-FP	1.2	6.5	6.6	−1.5	35.5[e]	33.3	+6.6
Maltose	α-GP	0.7						
	β-GP	1.1	6.0	7.0	−14.3	33.6	35.3	−4.8
Isomaltotriose	α-GP	0.3						
	β-GP	0.4	2.3	1.8	+27.8	18.8	15.4	+22.0

[a] FF = fructofuranose; FP = fructopyranose; GP = glucopyranose.
[b] I = anomeric carbon peak intensity of each isomeric form of each sugar.
[c] % S_m = molar relative ratio (%) measured for each sugar; a correction factor was applied since the minor anomeric form (α-FF) of maltulose is not detected and estimated at 8.9% in the standard maltulose solution (Table 2). % S_c = molar relative ratio (%) calculated for each sugar weighted with a precision of 0.1 mg. RE = relative error.
[d] mS_m = mass (mg) measured by ^{13}C NMR for each sugar with 1,4-dioxane as internal standard. mS_c = mass (mg) of each sugar weighed with a precision of 0.1 mg.
[e] A correction factor was applied for the mass (mg) measured for maltulose (α-FF form undetected).

Table 6. ^{13}C NMR quantitative analysis of carbohydrates (%) in six honeys[a]

Components	Honey floral type					
	Mixed floral	*Castanea*	*Robinia*	*Asphodelus*[b]	*Anthyllis*	*Clementina*
Fructose	35.6	37.1	32.4	32.7	31.5	33.4
Glucose	26.8	23.0	25.9	22.7	28.7	28.4
Turanose	2.2[c]	1.7[c]	0.8[c]	1.8[c]	1.7[c]	1.8[c]
Maltulose	0.8[c]	1.0[c]	0.6[c]	0.7[c]	0.6[c]	1.8[c]
Maltose			0.7	0.9		
Isomaltose	1.4				1.7	
Nigerose					2.6	2.3
Isomaltotriose	2.5	1.7		1.7		3.3
Melezitose				1.1		
Erlose		0.4		1.7		
Carbohydrates	69.3	64.9	60.4	63.3	66.8	71.0
Oligosaccharides	6.9	4.8	2.1	7.9	6.6	9.2
F + G	62.4	60.1	58.3	55.4	60.2	61.8
F/G	1.3[3]	1.6	1.2[5]	1.4[4]	1.1	1.1[7]

[a] All percentages are expressed with respect to fresh matter (raw honey).
[b] Repeatability of the measurement given at 95% for four analyses of *Asphodelus* honey: fructose 32.2 ± 1.7%; glucose, 24.6 ± 1.8%; turanose, 1.9 ± 0.2%; maltulose, 0.9 ± 0.2%; maltose, 0.7 ± 0.2%; isomaltotriose, 1.8 ± 0.2%; melezitose, 1.0 ± 0.2%; erlose, 1.4 + 0.3%.
[c] Correction factors applied since the minor anomeric forms (α-FF) of maltulose and of turanose are not detected and estimated at 8.9% and 17.0%, respectively, in the solution of maltulose and turanose (Table 2).

37.1%) and glucose (22.7–28.7%) were the main components. These two monosaccharides accounted for 55.4–62.4% of the total content of honey and 87.0–96.5% of the identified carbohydrates. These results fitted perfectly with literature data, particularly those of White[14] and those of Sabatini *et al.*[15] and Stefanini[16] relative to North American and Italian honeys, respectively. The F/G ratio, which is easily obtainable by NMR, ranged from 1.1 to 1.6 and is strongly dependent on the floral type of honey.[15] Our calculated ratios for *Castanea* and *Clementina* are in agreement with literature data.[15]

The remaining carbohydrates consisted of a mixture of di- and trisaccharides. Their contents ranged from 2.1 to 9.2% of the total carbohydrates, in agreement with literature reports.[50] Two disaccharides, maltulose and turanose, are present in all samples and the trisaccharide isomaltotriose in four samples. Three other disaccharides (maltose, isomaltose and nigerose) and two trisaccharides (melezitose and erlose) were detected in one and two samples, respectively.

Some of these di- and trisaccharides whose identification and quantitation are not easy by chromatographic[50,51] techniques (co-elution), even after

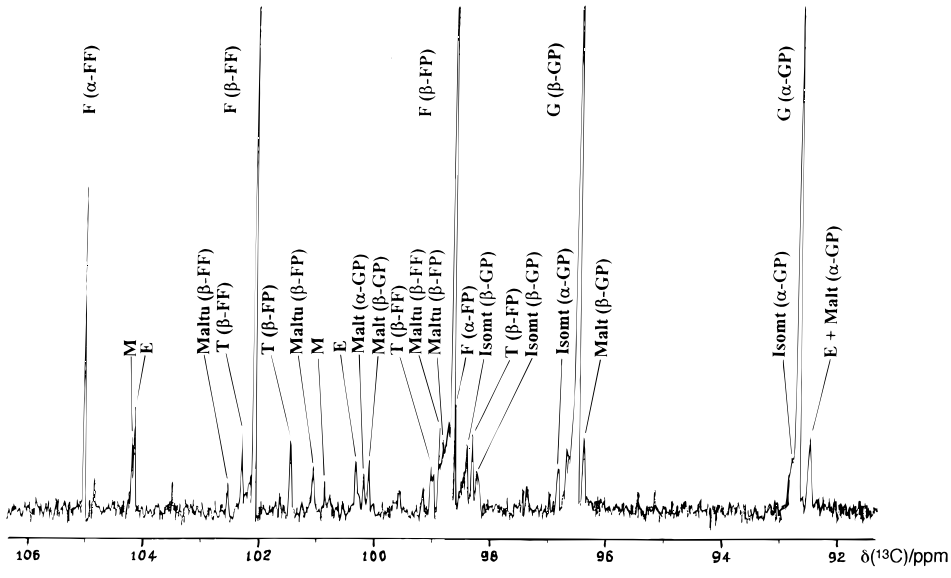

Figure 4. ^{13}C NMR spectrum of the anomeric region of *Asphodelus* honey. F = fructose; G = glucose; M = melezitose; E = erlose; Maltu = maltulose; T = turanose; Malt = maltose; Isom = isomaltotriose; FF = fructofuranose; FP = fructopyranose; GP = glucopyranose.

the reduction of the oligosaccharide fraction, were observed in several samples at a proportion ranging from 0.4 to 3.3%. Particularly maltulose and turanose, present in all samples analysed, needed specific conditions[51] (maltulose) or a special chromatographic column[17] (turanose) in order to be identified from the other oligosaccharides.

CONCLUSION

The computer-assisted analysis of the ^{13}C NMR spectrum of a carbohydrate mixture is useful for the identification and quantitation of its individual components. The experimental procedure was checked on artificial mixtures and then applied to authentic honeys of different botanical origins. The F/G ratio was readily obtainable and minor oligosaccharides were identified down to levels as low as 0.4% without any physical or chemical treatment of the samples.

EXPERIMENTAL

Standard saccharides and reagents

The following mono-, di- and trisaccharides whose ^{13}C NMR spectra are presented here (Figs 1 and 2) were purchased from Sigma: D-glucose, D-fructose, maltose [*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranoside], isomaltose [*O*-α-D-glucopyranosyl-(1 → 6)-D-glucose], turanose [*O*-α-D-glucopyranosyl-(1 → 3)-D-fructose], maltulose [*O*-α-D-glucopyranosyl-(1 → 4)-D-fructose], nigerose [*O*-α-D-glucopyranosyl-(1 → 3)-D-glucopyranose], isomaltotriose [*O*-α-D-glucopyranosyl-(1 → 6)-*O*-α-D-glucopyranosyl-(1 → 6)-D-glucose], melezitose [*O*-α-D-glucopyranosyl-(1 → 3)-*O*-β-D-fructofuranosyl-(2 → 1)-α-D-glucopyranoside] and erlose [*O*-α-D-glucopyranosyl-(1 → 4)-D-glucopyranosyl-β-D-fructofuranose]. The other saccharides compiled in the spectral data bank were also obtained from Sigma.

Deuterium oxide (99.8% D_2O) was obtained from Euriso-Top and 1,4-dioxane from Janssen.

^{13}C NMR spectra

All the ^{13}C NMR spectra were recorded on a Bruker AC 200 Fourier transform spectrometer operating at 50.323 MHz for ^{13}C, equipped with a 10 mm probe, in H_2O–D_2O (75:25, v/v), with all shifts referred to internal 1,4-dioxane ($\delta = 67.12$ ppm downfield from internal TMS), with the following parameters: pulse width (PW) 3 μs (flip angle 30°), acquisition time 1.3 s (relaxation delay time $D_1 = 2$ s) for a 32K data table with a spectral width (SW) of 12 500 Hz (250 ppm), composite phase decoupling (CPD) of the proton band, digital resolution of 0.763 Hz per point; Lorenzian and Gaussian multiplication of the FID were applied before Fourier transformation (LB = −0.35 Hz, GB = 0.1 Hz).

To create the spectral data bank, *ca.* 35 spectra of mono-, di- and trisaccharides and related reduced derivatives were recorded. Each compound was diluted to 0.25 mol l^{-1} and 1000 or 2000 scans were acquired (2000 scans were required for compounds such as D-fructose, maltulose and turanose which possess minor forms).

The spectra of honey were recorded with 300 mg of raw material diluted in 2 ml of H_2O–D_2O (75:25, v/v). To obtain a good signal-to-noise ratio, 5000 acquisitions were accumulated.

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