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Identification and quantitative determination of carbohydrates in ethanolic extracts of two conifers using ^{13}C NMR spectroscopy

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Abstract—We developed a method for the direct identification and quantification of carbohydrates in raw vegetable extracts using ^{13}C NMR spectroscopy without any preliminary step of precipitation or reduction of the components. This method has been validated (accuracy, precision and response linearity) using pure compounds and artificial mixtures before being applied to authentic ethanolic extracts of pine needles, pine wood and pine cones and fir twigs. We determined that carbohydrates represented from 15% to 35% of the crude extracts in which pinitol was the principal constituent accompanied by arabinitol, mannitol, glucose and fructose.

Keywords: ^{13}C NMR spectroscopy; Quantitative determination; Carbohydrates; Pinitol; *Pinus nigra* Arnold ssp. *laricio* Poiret; *Abies alba* Miller

1. Introduction

Since the pioneering work of Berthelot,[1] the alcoholic extracts of conifers have been known to contain carbohydrates including saccharides, cyclitols and inositols. Most studies reported on the identification of individual components and/or the biological activities of the alcoholic extracts are from various pines (*Pinus radiata*, *Pinus pinaster*, *Pinus densiflora*)[1–6] and from fir (*Abies pindrow*).[7]

Knowledge of the qualitative and quantitative distribution of sugars in natural matrices (e.g., fruits and vegetables) is of particular importance because it provides information on freshness, maturity, storability and on the optimization of technological processes. Consequently, analysis of carbohydrates is widespread in various industries (food, pulp and paper, etc.). During the last four decades, several chromatographic methods involving GC and/or HPLC have been developed for the identification and the quantification of carbohydrates in natural mixtures.[8–11] All the chromatographic methods require a clean-up procedure (mainly separation of carbohydrates from other compounds). Moreover, a preliminary step of derivatization is unavoidable for GC[3,8,9] and is often required for HPLC with respect to the detection technique (UV detection for instance).[9–11] Conversely, only a few studies have reported on the use of NMR spectroscopy, which is known as a powerful technique for structure elucidation of pure compounds, for the identification and quantitative determination of carbohydrates in natural mixtures.

The first study dates to 1976. Blunt and Munro[12] in their excellent pioneering work identified and quantified five saccharides in the ethanolic extract of dried *P. radiata* using a low field Fourier transform spectrometer (1.9 T) and 2-hydroxymethyl-2-methylpropane-1,3-diol as internal standard. However, reliable results were obtained only after preliminary precipitation of the oligomers, and the method needed a correcting factor for each compound. Glucose, fructose, pinitol, *myo*-inositol and sequoyitol accounted for 0.3–3.6% of the extract.

In 1985, Tamate and Brabury[13] determined the content of various mono- and disaccharides in a solvent extract of sweet potato using the method of external

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standardization applied to ^{13}C NMR spectroscopy. No optimized pulse sequence was developed because it was not useful using external standardization. The ratio between the height of the peak of the anomeric carbon of each sugar and the height of the peak of 1,4-dioxane was measured. Quantification was obtained using a linear calibration relationship between this ratio and the concentration of the compound. Each identified carbohydrate (fructose, sucrose, maltose and glucose) accounted for 0.2–0.5% of the extract.

A few years later, Low et al.[14] identified and quantified the minor disaccharides found in honey using a spectrometer operating at 9.4 T after two preliminary sample preparation steps: first elimination of glucose and fructose by HPLC and then reduction of the disaccharides. Identification and quantification were based on the signal of the anomeric carbon of the reduced sugars after the addition of a relaxation reagent and using methyl β-D-ribofuranoside as the internal standard. The percentages of maltose, sucrose, kojibiose, turanose, palatinose, gentiobiose, neothrealose, nigerose and isomaltose were then given relatively to the maltose concentration. According to the authors, the main limitation of this procedure concerned the simultaneous identification of reduced di- and trisaccharides whose anomeric carbons exhibited very close chemical shifts.[14]

More recently, we developed a method that allowed direct identification and quantification of sugars in honey and anhydrosugars in biomass pyrolysis liquids using a routine spectrometer (4.7 T) and 1,4-dioxane as the internal standard.[15,16] The quantification, carried out using a small pulse angle and a short recycling time, was obviously done on the basis of not fully relaxed spectra. The experimental procedure (precision, accuracy, linearity) was validated with pure compounds and mixtures of these compounds. Beside the two major saccharides, glucose and fructose (23.0% and 37.1%, respectively), we identified and quantified turanose, maltulose, maltose, isomaltose, nigerose, isomaltotriose, melezitose and erlose (0.6–2.6% each), in various honeys from Corsica.[16] Following the same procedure, we also identified and quantified three anhydrosugars, levoglucosan (6.5%), levomannosan and levogalactosan (0.6% each), in the carbohydrate fraction of a biomass pyrolysis liquid.[15]

In the course of our ongoing work on the determination of the essential oils, resins and extracts of Corsican resinous, we investigated the ethanol extracts of *Pinus nigra* Arnold ssp. *laricio* Poiret and *Abies alba* Miller. To avoid multi-step sample preparation before analysis (precipitation of the oligomers, reduction of the carbohydrates, etc.), we developed a procedure using ^{13}C NMR spectroscopy, which allowed the direct identification and quantification of the carbohydrates in ethanolic extracts of pine and fir.

2. Results and discussion

2.1. Identification of components

In our laboratories, a computerized procedure based on ^{13}C NMR spectroscopy, which allows the identification of components of natural mixtures, has been developed and applied to several families of compounds: phenols in biological oils,[17] sugars in honeys,[16] mono-, di- and triterpenes in essential oils, solvent extracts and resins.[18–20] For each family of compounds, a specific spectral library, including spectra recorded in-house and literature data, was built up and is constantly increased with new data. Identification of the components using ^{13}C NMR is carried out by comparison of chemical shift values of the mixture spectra with those of reference spectra. These spectra are compiled in several libraries devoted to different families of compounds, using a laboratory-made software (a microprogram developed using Access®). Each compound is identified by taking into account three parameters, which are directly available from the computer program: (i) the number of observed signals with respect to those expected, (ii) the difference between the chemical shift of each signal in the mixture and in the reference ($\Delta\delta$), (iii) the number of overlapped carbons signals belonging to two components, which fortuitously possess the same chemical shift.[16,19]

Using a comparative method, it is essential to obtain reproducible chemical shifts between reference spectra and mixture spectra; this means that all the spectra must be recorded using the same solvent. The ethanolic extracts were prepared from various parts of *P. nigra* Arnold ssp. *laricio* Poiret: A (needles), B (wood) and C (cones) and of *A. alba* Mill. D (twigs). The extracts being not totally soluble in H_2O, we recorded the ^{13}C NMR spectrum in a mixture of DMSO-d_6 and H_2O (50:50; v:v). An adequate spectral data library was built up by recording the spectra of approximately 40 pure carbohydrates (common sugars, polyols and inositols) in the same solvent.

The computerized comparison of the resonances of the ^{13}C NMR spectra of the raw extracts A–D with those of the references allowed the identification of eight compounds: glucose and fructose (whose isomeric forms were observed in the ^{13}C NMR spectra), four linear polyols (mannitol, arabinitol, xylitol and erythritol) and two cyclohexane polyols (*myo*-inositol and pinitol, the latter being a monomethylated derivative) (Fig. 1).

Glucose and pinitol were identified in all extracts A–D. The three principal isomeric forms of fructose (α- and β-fructofuranose and β-fructopyranose) were identified in the pine needles and pine wood extracts (A and B) and fir twig extract (D). Five other polyols were also identified in the extracts (A–D): *myo*-inositol in pine needles (A), arabinitol in the pine wood and pine cones

(B and C), mannitol in pine cones (C) and xylitol and erythritol in fir twigs (D).

In all these extracts, each compound was identified by the observation of all of its signals. The chemical shift variations ($\Delta\delta$) between the reference spectra and the extract spectrum were less than 0.06 ppm for all resonances. The number of overlapped signals was less than or equal to two for each isomeric form of the carbohydrates. Therefore, each component was identified by confirmation of at least two-thirds of the signals belonging solely to that molecule.

2.2. Quantitative procedure

Whatever the technique used, quantitative determination of a component in a mixture needs the comparison of the signals of that compound with those of an internal standard, or the utilization of calibration curves (external standardization). Several methods have been developed for the quantitation of individual components of a mixture using ^{13}C NMR spectroscopy with an internal standard. One is to wait a period of $5T_1$ of the longest T_1 value (component and internal standard) before applying another pulse, combined with a 90° pulse angle to ensure complete relaxation of each carbon.[21,22] Applying the aforementioned parameters with the gated decoupling technique, which provides the suppression of the nuclear Overhauser enhancement (NOE), is well known as a standard sequence for quantitative NMR measurements and it has been widely used.[23–25] However, this procedure is time consuming and this could prove prohibitive, particularly when a routine spectrometer is used.

Quantitative determination can also be done using a rapid train of short pulses, because for smaller flip angles (smaller pulse widths) there is less change in the steady-state magnetization as T_1 is varied, than for larger flip angles. However, the choice of a low flip angle would necessitate longer accumulation times to recover an adequate S/N ratio.[12,26,27] Finally, it has been suggested that a good approach for the quantitative analysis of complex mixtures containing nuclei with a wide range of T_1's is a compromise between the aforementioned procedures (90° pulse angle, gated decoupling, $5T_1$ of relaxation delay on the one hand and a rapid train of short pulses on the other hand).[28,29] Such a procedure should be validated with representative reference compounds (in pure form or as artificial mixtures).[15,18,20]

To carry out a quantitative determination of carbohydrates using ^{13}C NMR, we first measured the T_1 values of the carbons of pure compounds (the T_1's values ranged from 0.2 to 1.4 s in 50:50 DMSO-d_6–H_2O). Then, we looked for an internal standard with the following properties: a high degree of molecular symmetry to minimize the number of peaks belonging to the internal standard; signals that do not overlap with the sample; carbon T_1 ranging from 0.2 to 1.4 s. After various tests, we choose 1,6-hexandiol as internal standard (T_1 of all the methylenic carbons = 1.0 s).

We determined and plotted the percentage of recovered signal S/N (%) as a function of the pulse angle α, for the carbons with T_1's = 0.2 s and 1.4 s (lowest and highest values for protonated carbons of carbohydrates and internal standard) according to the work of Becker et al. (Fig. 2).[30]

Using a medium field spectrometer (9.4 T) for the analysis of a complex natural mixture, we took advantage of the high resolution reached with the standard time domain of 128 K data table, which decreases the number of overlapping signals. The subsequent acquisition time of 2.7 s (total recycling time, 2.8 s) allowed the utilization of the largest flip angle of 90°. Indeed, this pulse sequence gave an acceptable difference of steady-state magnetization (8% and 6%) between carbons of carbohydrates and those of 1,6-hexandiol. It also allowed, for the same S/N ratio, the recovery of a maximum of restored magnetization and the reduction of the number of accumulated scans (and consequently a reduction in spectrometer use time, Fig. 2). The accuracy, precision and response linearity of such a procedure ($\alpha = 90°$ and total recycling time of 2.8 s for 128 K data table) were validated by several experiments carried out on pure compounds and artificial mixtures and are reported below.

2.2.1. Reference compounds: accuracy and response linearity of the experimental procedure. We first compared the amounts of pure pinitol, glucose, fructose, mannitol, *myo*-inositol and erythritol diluted in DMSO-d_6–H_2O (50:50, v:v) at different concentrations and measured by NMR, with the weighed ones. From the ^{13}C NMR spectra, we calculated the mean value of the areas of the carbons of each carbohydrate (A_C) and that of the methylenes of 1,6-hexandiol (A_R), used as internal standard. The calculated amount m_C (mg) of each carbohydrate was determined using Eq. 1:

$$m_C = \frac{2A_C \times M_C \times m_R}{A_R \times M_R} \quad (1)$$

where m_R is the amount (mg) of 1,6-hexandiol, M_R is the molecular weight of 1,6-hexandiol and M_C is the molecular weight of the carbohydrate of interest. Factor 2, due to the symmetry of 1,6-hexandiol, was used for all the compounds except mannitol, *myo*-inositol and erythritol, which are also symmetrical molecules. We compared the amounts of carbohydrates measured by NMR with the weighed ones, in the range of ~1–18 mg of carbohydrate diluted in 0.5 mL of solvent. The relative errors were less than 6% for 80% of the experiments and lower than 10% for the remaining experiments (Tables 1–4).

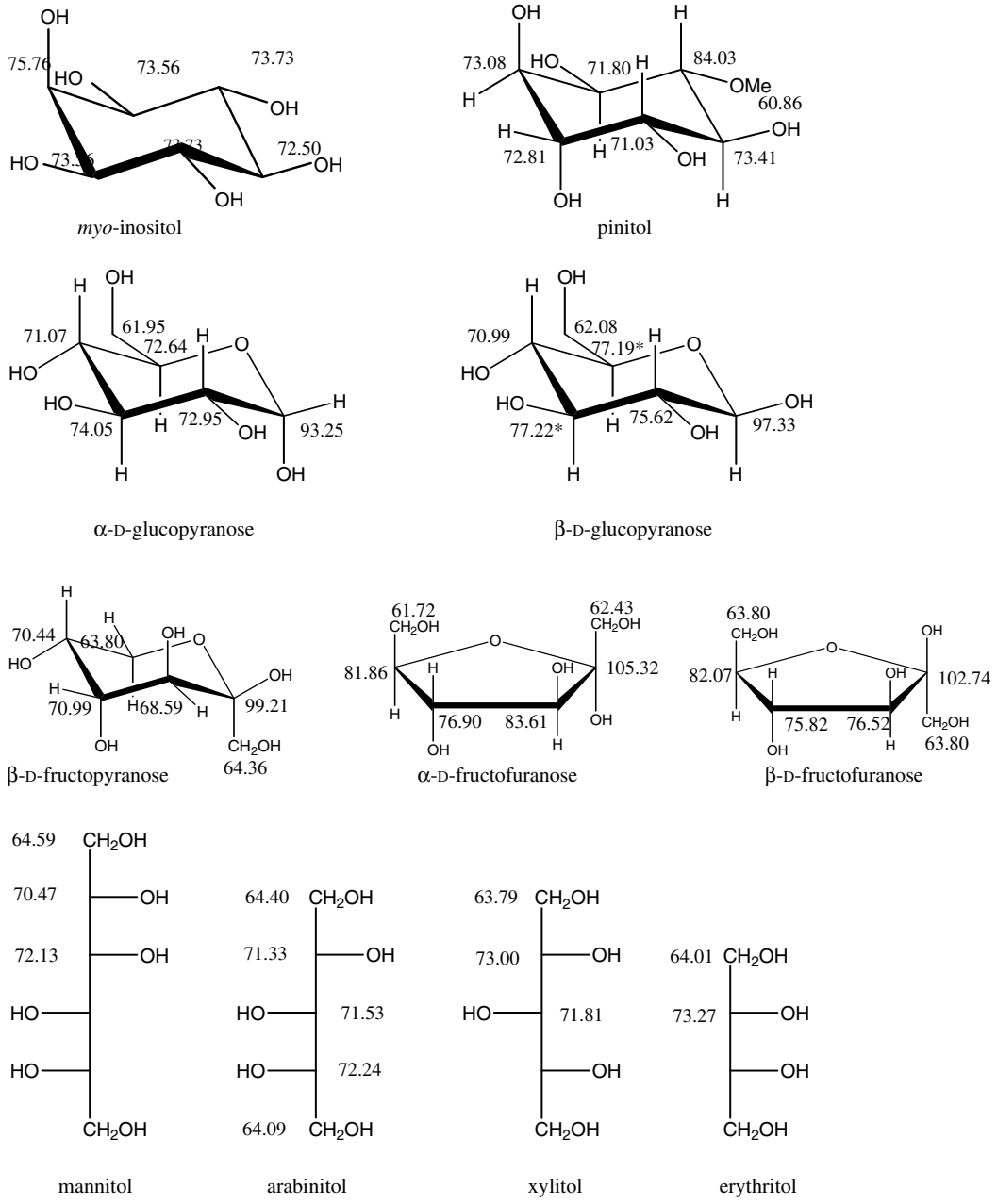

Figure 1. Structure and assignment of the chemical shifts of carbohydrates identified in the ethanolic extracts of *Pinus nigra* ssp. *laricio* and *Abies alba* Miller. Beside each carbon is reported the chemical shift (ppm) in DMSO-d_6–H_2O (v:v; 50:50), $*$ data can be interchanged.

To express the amounts of each carbohydrate measured by ^{13}C NMR (m_C) as a function of the weighed ones (m_W), we drew calibration lines for the six compounds (Fig. 3). The response linearity of the experimental procedure was clear from the linear determination factor, R^2, which ranged from 0.994 to 0.999.

2.2.2. Artificial mixture: accuracy and precision of the experimental procedure. We recorded the spectrum of a mixture (M1) consisting of pinitol, D-glucose, D-fructose, *myo*-inositol and mannitol and the mass of each component was calculated according to Eq. 1. The results, which are reported in Table 5, showed that the reliable determination of the mass of the five components in the mixture was available from the ^{13}C NMR spectrum. The relative errors, less than 6%, attested of the accuracy of the procedure. In addition, the values of the repeatability (using the Student test bilateral coefficient 't' = 4.60), measured from five spectra recorded under identical conditions and given using a confidence interval of 99%, ranged from ±2.8% to ±7.2%, which demonstrated the precision of the measurements (Fig. 4).

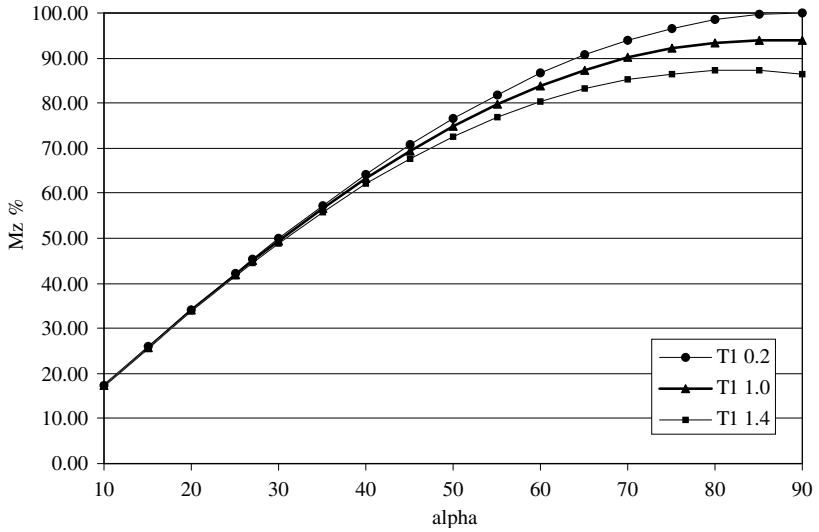

Figure 2. Plot of S/N (%) versus flip angle for selected values of T_1 and total recycling time of 2.8 s as plotted[30] from $\frac{S}{N} = \frac{M_0(1-e^{-\tau/T1})\sin\alpha}{\sqrt{\tau}(1-e^{-\tau/T1}\cos\alpha)}$ where τ is the minimum total recycling time of 2.7 s using a 128 K data table (acquisition time = 2.6 s and relaxation delay = 0.1 s).

Table 1. Quantitative determinations of pure pinitol and *myo*-inositol by ^{13}C NMR spectroscopy using 1,6-hexandiol as internal standard[a]

	(1) Pinitol						(2) *myo*-Inositol				
A_R[b]	97.6	98.5	96.7	96.4	95.9	95.4	99.1	98.5	99.8	98.8	98.6
A_C[c]	3.5	5.5	10.8	16.7	23.1	28.8	6.7	11.5	18.0	31.9	39.2
m_C[d]	1.1	2.3	4.5	7.0	9.7	12.2	0.9	1.6	2.5	4.5	5.5
m_W[e]	1.2	2.4	4.7	7.1	9.5	11.8	0.9	1.7	2.6	4.3	5.1
RE[f]	8.3	4.2	4.3	1.4	2.1	3.4	0.0	5.9	3.8	4.7	7.8

[a] Amount (m_R) of 1,6-hexandiol used for (1) was 9.3 mg and for (2) was 7.7 mg. Molecular weight of 1,6-hexandiol (M_R) = 118 g/mol; molecular weight (M_C) of pinitol = 194 g/mol and *myo*-inositol = 180 g/mol.
[b] A_R: mean area of the methylenes of 1,6-hexandiol.
[c] A_C: mean area of the protonated carbons of carbohydrates.
[d] m_C: mass (mg) of each carbohydrate measured by ^{13}C NMR using Eq. 1.
[e] m_W: weighed quantity (mg) of each carbohydrate.
[f] RE: relative error between m_C and m_W expressed in percent.

Table 2. Quantitative determinations of pure D-glucose by ^{13}C NMR spectroscopy using 1,6-hexandiol as internal standard[a]

	(3) D-Glucose									
	αGP	βGP	αGP	βGP	αGP	βGP	αGP	βGP	αGP	βGP
A_R[b]	100.9		98.4		98.7		100.0		98.9	
A_C[c]	6.1	1.9	10.5	6.1	14.3	11.4	17.3	18.3	19.1	21.9
m_C[d]	1.4	0.4	2.5	1.5	3.4	2.7	4.1	4.3	4.5	5.2
m_{CT}[e]	1.9		4.0		6.1		8.4		9.7	
m_W[f]	2.0		3.9		5.8		7.8		9.7	
RE[g]	5.0		2.6		5.2		7.7		0.0	

[a] Amount (m_R) of 1,6-hexandiol used was 5.0 mg. Molecular weight of 1,6-hexandiol (M_R) = 118 g/mol; molecular weight (M_C) of D-glucose = 180 g/mol.
[b] A_R: mean area of the methylenes of 1,6-hexandiol.
[c] A_C: mean area of the protonated carbons of D-glucose.
[d] m_C: mass (mg) of D-glucose measured by ^{13}C NMR using Eq. 1.
[e] m_{CT}: sum of the mass of α-glucopyranose (αGP) and β-glucopyranose (βGP).
[f] m_W: weighed quantity (mg) of D-glucose.
[g] RE: relative error between m_C (or m_{CT}) and m_W expressed in percent.

2.3. Application to the quantitative determination of carbohydrates in ethanolic extracts of pine and fir

The results obtained with the pure compounds and the mixture indicated that quantitative determination of carbohydrates can be carried out using spectra acquired with the selected parameters. Therefore, we applied the same procedure to the ethanol extracts of *P. nigra* ssp. *laricio* and *A. alba* Miller. The amount of each carbohydrate (m_C, mg) was calculated according to Eq. 1

Table 3. Quantitative determinations of pure D-fructose by ^{13}C NMR spectroscopy using 1,6-hexandiol as internal standard[a]

	(4) D-Fructose														
	αFF	βFF	βFG	αFF	βFF	βFG	αFF	βFF	βFG	αFF	βFF	βFG	αFF	βFF	βFG
A_R[b]		98.2			98.7			98.4			97.9			97.8	
A_C[c]	2.1	8.3	11.5	3.6	15.8	28.4	6.8	20.0	40.3	7.3	29.1	51.9	8.1	31.7	68.1
m_C[d]	0.3	1.3	1.8	0.6	2.4	4.4	1.1	3.1	6.3	1.1	4.5	8.1	1.3	5.0	10.6
m_{CT}[e]		3.4			7.4			10.4			13.8			16.8	
m_W[f]		3.5			7.0			10.5			13.9			17.4	
RE[g]		2.9			5.7			1.0			0.7			3.4	

[a] Amount (m_R) of 1,6-hexandiol used was 9.1 mg. Molecular weight of 1,6-hexandiol (M_R) = 118 g/mol; molecular weight (M_C) of D-fructose = 180 g/mol.
[b] A_R: mean area of the methylenes of 1,6-hexandiol.
[c] A_C: mean area of the protonated carbons of D-fructose.
[d] m_C: mass (mg) of D-fructose measured by ^{13}C NMR using Eq. 1.
[e] m_{CT}: sum of the mass of α-fructofuranose (αFF), β-fructofuranose (βFF) and β-fructopyranose (βFP).
[f] m_W: weighed quantity (mg) of D-fructose.
[g] RE: relative error between m_C (or m_{CT}) and m_W expressed in percent.

Table 4. Quantitative determinations of pure mannitol and erythritol by ^{13}C NMR spectroscopy using 1,6-hexandiol as the internal standard[a]

	(5) Mannitol					(6) Erythritol				
A_R[b]	99.7	97.3	97.9	97.3	97.9	98.8	97.3	98.7	97.5	97.1
A_C[c]	11.0	21.5	31.5	43.1	55.7	15.6	31.2	49.0	62.9	73.5
m_C[d]	1.0	2.1	3.0	4.1	5.2	1.4	2.9	4.4	5.7	6.7
m_W[e]	1.1	2.1	3.2	4.3	5.4	1.4	2.8	4.1	5.5	6.9
RE[f]	9.1	0.0	6.3	4.7	3.7	1.4	3.3	6.5	4.0	2.5

[a] Amount (m_R) of 1,6-hexandiol used for (5) was 5.9 mg and for (6) was 8.6 mg. Molecular weight of 1,6-hexandiol (M_R) = 118 g/mol; molecular weight (M_C) of mannitol = 180 g/mol and erythritol = 122 g/mol.
[b] A_R: mean area of the methylenes of 1,6-hexandiol.
[c] A_C: mean area of the protonated carbons of carbohydrates.
[d] m_C: mass (mg) of each carbohydrate measured by ^{13}C NMR using Eq. 1.
[e] m_W: weighed quantity (mg) of each carbohydrate.
[f] RE: relative error between m_C and m_W expressed in percent.

and the subsequent mass percentage of each component (% m_C) was calculated according to Eq. 2, where m_E is the amount of crude extract (Table 6):

$$\% \, m_C = \frac{m_C \times 100}{m_E} \quad (2)$$

We found that carbohydrates represented 36%, 18%, 16% and 16.5% of the crude extracts A, B, C and D, respectively. Pinitol was the major component (accompanied by glucose and fructose) of pine needles and pine wood extracts (A and B) and in the fir twig extract (D) in which it accounted for 13%, 9% and 5% of the extract, respectively. The pine cone extract (C) also contained pinitol (5%) but it is noticeable that the two polyols, arabinitol and mannitol, represented 6% and 3% of the mixture, respectively. The other polyols, *myo*-inositol (present only in the pine needle extract), xylitol and erythritol (found only in the fir twig extract), are minor components, with proportions ranging from 0.5% to 1%.

From these results, we conclude that ^{13}C NMR spectroscopy, following the computerized method developed in our laboratories, is an efficient tool for the direct identification of carbohydrates in crude solvent extracts. Taking into account the advantages (high sensitivity and resolution) and the constraint (standard acquisition time) imposed by the utilization of a modern medium field spectrometer, an optimized pulse sequence ($\alpha = 90°$, decoupling of the proton band, total recycling time, 2.7 s) led to reliable quantitative determinations of the identified compounds with an analysis time of 45 min.

3. Experimental

3.1. ^{13}C NMR spectra

All the ^{13}C NMR spectra were recorded on a Bruker (Wisembourg, France) AVANCE 400 Fourier Transform spectrometer operating at 100.13 MHz for ^{13}C, equipped with 5 mm probe (Bruker probe, 5 mm, dual $^{1}H/^{13}C$). The spectra were recorded in DMSO-d_6–H_2O (50:50, v:v), with all shifts (d) referenced to TMS, with the following parameters: pulse width = 8 ms (flip angle 90°); acquisition time = 2.6 s for 128 K data table with a

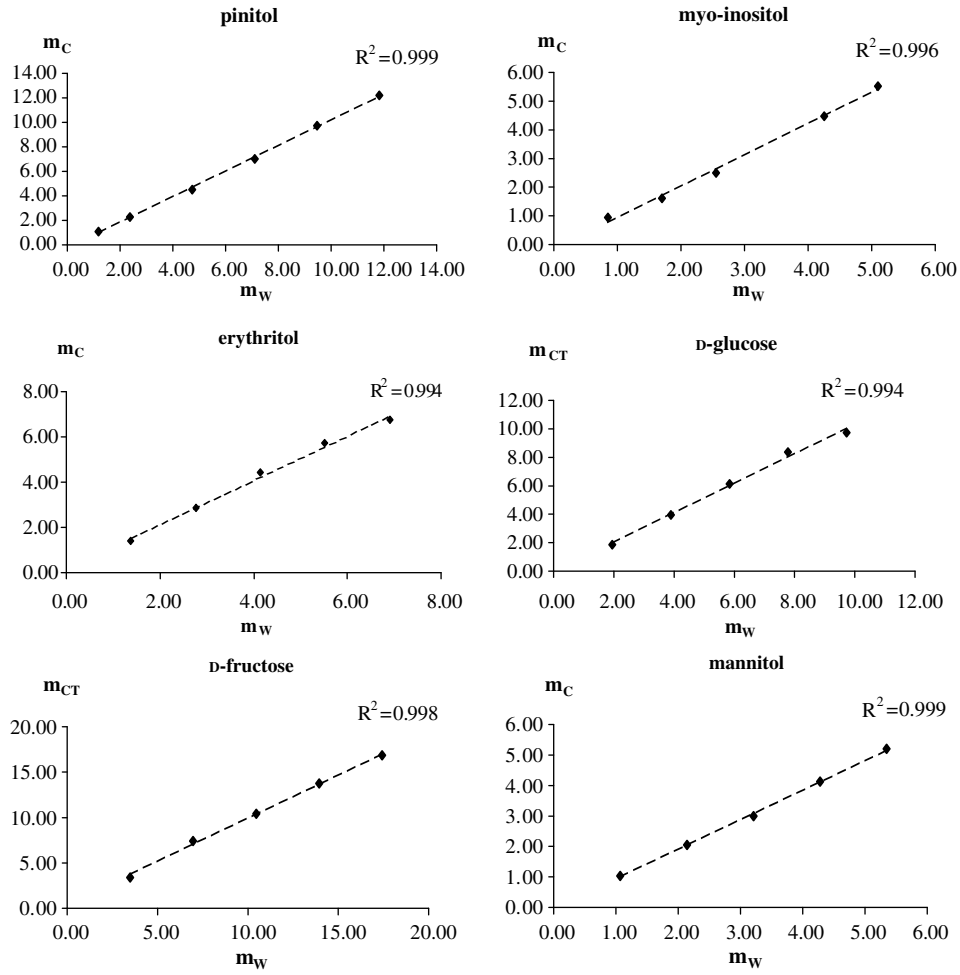

Figure 3. Calibration lines of pinitol, D-glucose, D-fructose, *myo*-inositol, mannitol and erythritol. m_W: weighed amount (mg), m_C: calculated amount (mg) using ^{13}C NMR spectroscopy according to Eq. 1, m_{CT}: total calculated amount of the tautomeric forms of glucose and fructose.

Table 5. ^{13}C NMR quantitation of individual components of mixture M1 measured using 1,6-hexandiol as the internal standard[a]

Components	A_C[b]	m_C[c]	m_W[d]	RE[e]	R[f] (%)
Pinitol	38.2	6.2	6.5	4.6	6.0 ± 3.6
D-Glucose[h]		5.0	4.8	4.2	5.0 ± 7.2
αGP[g]	1.3	2.1			
βGP[g]	2.3	2.9			
D-Fructose[i]		4.7	5.0	6.0	4.7 ± 3.1
αFF[g]	2.5	0.4			
βFF[g]	10.7	2.7			
βFP[g]	17.9	1.6			
myo-Inositol	20.6	1.5	1.5	0.0	1.5 ± 2.8
Mannitol	20.7	1.6	1.6	0.0	1.6 ± 4.9

[a] Amount of 1,6-hexandiol used in the experiment was 4.7 mg; peak intensity of its methylenes (A_R) = 95.9 and molecular weight (M_R) = 118 g/mol. For pinitol, D-glucose, D-fructose, *myo*-inositol and mannitol, M_C = 194 g/mol, 180 g/mol, 180 g/mol, 180 g/mol and 180 g/mol, respectively; A_D: mean intensity of the methylenes of 1,6-hexandiol.

[b] A_C: mean intensity of the protonated carbon of carbohydrates.

[c] m_C: mass (mg) of carbohydrates measured by ^{13}C NMR according to Eq. 1.

[d] m_W: weighed quantity (mg) of carbohydrates.

[e] RE: relative error between m_C and m_W expressed in percent.

[f] R: repeatability of m_C given at 99% (measured from five analyses).

[g] Abbreviations: αGP (α-glucopyranose), βGP (β-glucopyranose), αFF (α-fructofuranose), βFF (β-fructofuranose), βFP (β-fructopyranose).

[h] m_C = sum of the mass of tautomeric forms αGP, βGP.

[i] m_C = sum of the mass of tautomeric forms αFF, βFF, βFP.

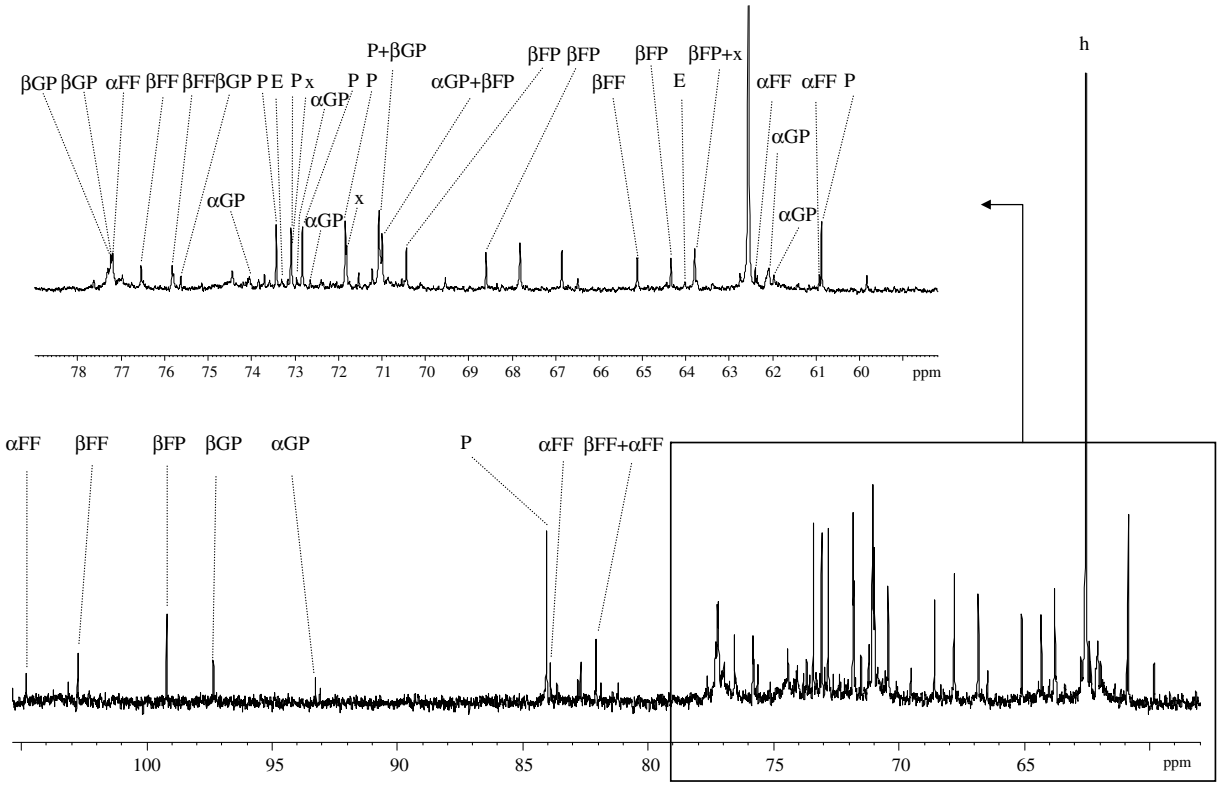

Figure 4. Part of the ^{13}C NMR spectrum of sample D. h: 1,6-hexandiol; αFF: α-fructofuranose, βFF: β-fructofuranose, βFP: β-fructopyranose, αGP: α-glucopyranose, βGP: β-glucopyranose, E: erythritol, P: pinitol, xylitol.

Table 6. ^{13}C NMR quantitation of carbohydrates in authentic extracts of *Pinus nigra* ssp. *laricio* (A: needle, B: wood, C: cone) and *Abies alba* (D: twigs)[a]

Components	A			B			C			D		
	A_C[b]	m_C[c]	%[d]	A_C[b]	m_C[c]	%[d]	A_C[b]	m_C[c]	%[d]	A_C[b]	m_C[c]	%[d]
Pinitol	27.9	6.6	13	15.2	4.7	9	7.0	1.6	5	7.8	1.9	5
D-Glucose[e]		5.9	12		2.2	4		0.6	2		1.8	5
αGP[f]	11.4	2.5		3.9	1.1		—	—		2.0	0.5	
βGP[f]	15.5	3.4		4.0	1.1		2.6	0.6		5.7	1.3	
D-Fructose[g]		4.7	10		2.1	4	—	—	—		1.7	5
αFF[f]	1.6	0.4		0.3	0.1					—	—	
βFF[f]	7.1	1.6		2.4	1.3					4.5	1.0	
βFP[f]	12.2	2.7		4.6	0.7					3.1	0.7	
myo-Inositol	2.6	0.6	1	—	—	—	—	—	—	—	—	—
Mannitol	—	—	—	—	—	—	5.7	1.2	3	—	—	—
Arabinitol	—	—	—	2.3	0.6	1	11.4	2.1	6	—	—	—
Xylitol	—	—	—	—	—	—	—	—	—	2.5	0.3	1
Erythritol	—	—	—	—	—	—	—	—	—	1.6	0.1	0.5
Total			36			18			16			16.5

[a] Amount of 1,6-hexandiol (m_R) used: A, 7.1 mg; B, 9.2 mg; C, 7.1 mg; D, 7.5 mg; molecular weight of 1,6-hexandiol (M_R) = 118 g/mol; molecular weight (M_C) of pinitol = 194 g/mol, D-glucose, D-fructose, *myo*-inositol and mannitol = 180 g/mol, arabinitol = 152 g/mol, xylitol = 96 g/mol, erythritol = 64 g/mol; analyzed mass of the fraction m_F = A, 49.6 mg; B, 53.2 mg; C, 34.9 mg; D, 37.7 mg. A_R: mean area of the methylenes of 1,6-hexandiol A, 99.5; B, 98.7; C, 100.0 and D, 99.2.

[b] A_C: mean area of the protonated carbon of carbohydrates.

[c] m_C: mass (mg) of carbohydrates measured by ^{13}C NMR according to Eq. 1.

[d] %: mass percentage of carbohydrates measured by ^{13}C NMR according to Eq. 2.

[e] m_C = sum of the mass of tautomeric forms of αGP, βGP.

[f] Abbreviations: αGP (α-glucopyranose), βGP (β-glucopyranose), αFF (α-fructofuranose), βFF (β-fructofuranose), βFP (β-fructopyranose).

[g] m_C = sum of the mass of tautomeric forms αFF, βFF, βFP.

spectral width of 25,000 Hz (250 ppm); composite pulse decoupling (CPD, Waltz-16) of the proton channel; digital resolution = 0.183 Hz/pt. The number of acquisitions accumulated was 256 for reference spectra and 1024 for mixture spectra.

3.2. T_1 measurements

The longitudinal relaxation delays of the ^{13}C nuclei (T_1 values) were determined for each protonated carbon of each compound (pinitol, D-glucose, D-fructose, mannitol, *myo*-inositol and erythritol) and for the methylenes of 1,6-hexandiol by the inversion-recovery method, using the standard sequence: 180°-τ-90°-$D1$, with a relaxation delay $D1$ of 20 s. Each delay of inversion (τ) was thus taken into account for the computation of the corresponding T_1 using the function $I_p = I_0 + p \cdot e^{-\tau/T1}$.

3.3. Calibrations lines

A weighed amount of 1.2–11.8 mg of pinitol, 2.0–9.7 mg of D-glucose, 3.5–17.4 mg of D-fructose, 1.1–5.4 mg of mannitol, 0.9–5.1 mg of *myo*-inositol and 1.4–6.9 mg of erythritol was dissolved in 2.0 mL of DMSO-d_6–H_2O (50:50, v:v), containing 9.3 mg, 5.0 mg, 9.1 mg, 5.9 mg, 7.7 mg and 8.6 mg of 1,6-hexandiol, respectively.

3.4. Artificial mixture M1

Pinitol (6.5 mg), D-glucose (4.8 mg), D-fructose (5.0 mg), *myo*-inositol (1.5 mg) and mannitol (1.5 mg), purchased from Aldrich, were diluted in 2.0 mL of DMSO-d_6–H_2O (50:50, v:v), containing 1,6-hexandiol (4.7 mg).

3.5. Ethanol extracts

The dried plant material (A: 191 g of pine needles, B: 133 g of pine wood and D: 131 g of fir twigs) was ground to a fine powder. The powders of A, B and D were placed in a Soxhlet extractor. The continuous Soxhlet extraction was carried out with pentane; the residue was then extracted with ethyl acetate and then with ethanol. The ethanol extract was submitted to filtration through decolourizing charcoal, and concentrated in vacuo to give a syrup: A, 7.52 g; B, 2.54 g; D, 5.68 g. The dried cones C of *Pinus* (92.0 g) were crushed into pieces of 0.5–1 cm and extracted by maceration with methanol during 24 h at room temperature. The methanol extract was concentrated in vacuo to give a brown powder. This powder was successively extracted (solid–liquid extraction) with pentane, ethyl acetate and ethanol. The ethanol extract was submitted to a filtration on decolourizing charcoal, and concentrated in vacuo to give a syrup: C 1.00 g.

The spectra were recorded with 49.6 mg of A, 53.2 mg of B, 34.9 mg of C and 37.7 mg of D dissolved in 0.5 mL of DMSO-d_6–H_2O (50:50, v:v), containing 7.1 mg, 9.2 mg, 7.1 mg and 7.5 mg of 1,6-hexandiol, for A–D, respectively.

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