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Monosaccharide composition of glycans based on Q-HSQC NMR

Guilherme Lanzi Sassaki[a,*], Marco Guerrini[b], Rodrigo Vassoler Serrato[c], Arquimedes Paixão Santana Filho[a], Juliane Carlotto[a], Fernanda Simas-Tosin[a], Thales Ricardo Cipriani[a], Marcello Iacomini[a], Giangiacomo Torri[b], Philip Albert James Gorin[a]

[a] Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, Curitiba 81531-980, Brazil
[b] Instituto di Richerche Chimiche e Biochimiche G. Ronzoni, Milan 20133, Italy
[c] Setor Litoral, Universidade Federal do Paraná, Matinhos 83260-000, Brazil

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ABSTRACT

Glycans have essential functions related to structural architecture and specific cell surface phenomena, such as differentiation, biosignalling, recognition and cell–cell interaction, with the carbohydrate structure determining main function in the cell. Due to the importance of the primary structure, the monosaccharide composition is crucial to show the glycan structure. We now present a method for complex carbohydrates based on NMR spectroscopy, which has shown to give similar results to those obtained by the classic GC–MS-carboxy-reduction/deuterium labeling approach. Quantitative HSQC, through J_{C-H} dependence showed 155 Hz as the best value for $^{1}H/^{13}C$ anomeric aldoses, allowing milli-microM detection using conventional inverse probe heads. Combining the quantification of native monosaccharide units of the glycan and those from the hydrolyzed product, a strong correlation occurs between the molecular mobility of the monosaccharide units, giving rise to some insights on the dynamic properties of the parent glycan.

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1. Introduction

The biological properties of glycans have been studied extensively, with their carbohydrate moiety having essential roles, contributing to structural architecture, cell integrity, growth, and specific cell surface phenomena, such as differentiation, biosignalling, recognition and interaction with endogenous or exogenous substances (Pinto, Barreto-Bergter, & Taborda, 2008). Due to their related function, the chemical structure determination of native glycoconjugates in terms of their monosaccharide and oligosaccharide chains is essential (Dembitsky, 2004; Pinto et al., 2008; Varki, Freeze, & Manzi, 1995). The latter has been determined by several chromatographic methods, namely thin layer chromatography (TLC), high performance liquid chromatography (HPLC), high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), capillary zone electrophoresis (CZE) and mainly by gas chromatography–mass spectrometry (GC–MS) (Carpita & Shea, 1989; Kakehi & Honda, 1989; Mariño, Bones, Kattla, & Rudd, 2010; Paulsen, Olasfsdóttir, & Ingólfsdóttir, 2002; Pinto et al., 2008; Sassaki et al., 2008; Zanetta, Timmerman, & Leroy, 1999). However, determining all the structural and functional properties of carbohydrates is difficult and laborious, and generally requires different techniques to achieve desirable results. The strategy depends on the problems to be answered and also the sample amount. Thus, mass spectrometry (MS) techniques for analysis of glycan have become very popular. Combining it with chromatography, chemical and enzyme reactions, allied to tandem MS, it is possible to cover the major information for structural purposes. However, determining conformation, anomeric configuration and sugar binding is quite complicated using MS alone. For these reasons, in order to characterize an unknown glycan, NMR spectroscopy provides all the experimental strategies for structural and conformational determination. Recently, (Lundborg, Fontana, & Widmalm 2011; Souza, Rietkerk, Selin, & Lankhorst, 2013) used 1D and 2D NMR experiments to determine the sugar components in many polysaccharides. Due to the recent developments of cryogenic technology, high field magnets and probe design, the sensitivity of NMR spectroscopy has widened to carry out sub-nanomolecular analyses, giving rise to new perspectives for NMR users in the carbohydrate field. Due to the particular characteristics and NMR versatility, it is now possible to identify glycoconjugates in complex biological

* Corresponding author. Present address: Departamento de Bioquímica e Biologia Molecular, Universidade Federal do Paraná, P.O. Box 19046, Curitiba-PR 81531-980, Brazil. Tel.: +55 41 3361 1577; fax: +55 41 3266 2042.
E-mail address: sassaki@ufpr.br (G.L. Sassaki).

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Table 1
Mutarotation values and $^{3}J_{H1-H2}$ obtained by independent ^{1}H NMR experiments, $^{1}H/^{13}C$ chemical shifts and J_{C-H} by HSQC.

[a]Monosaccharide	Mutarotated mixture (%)		$^{3}J_{H1-H2}$ (Hz)		δ (ppm)	δ (ppm)
	α	β	α	β	α (J_{C-H} Hz)	β (J_{C-H} Hz)
Glc*A*p	48.4	51.6	3.7	8.0	5.255/95.12(172)	4.681/98.86(163)
Man*p*	66.2	33.8	1.7	1.1	5.163/96.88(170)	4.881/96.53(161)
ManNAc*p*	54.1	45.9	1.5	1.6	5.111/95.94(172)	5.011/95.82(162)
Glc*p*	37.5	62.5	3.8	7.9	5.214/94.90(171)	4.629/98.72(162)
ManHep	32.2	67.8	3.6	7.8	5.214/95.17(170)	4.548/99.06(160)
Ara*p*[b]	33.6	60.3	3.5	7.7	5.221/95.44(169)	4.498/99.60(161)
Ara*f*[b]	3.1	5.6	4.4	–	5.204/103.82(173)	5.285/100.44(173)
GlcNAc*p*	55.1	44.9	3.5	8.4	5.187/93.67(172)	4.700/97.76(162)
2-deoxy-Glc	48.3	51.7	3.6	9.8	5.362/94.14(171)	4.919/96.25(162)
GlcHep	11.2	88.8	4.0	8.4	5.127/95.90(171)	4.843/96.84(163)
Xyl*p*	35.0	65.0	3.7	7.8	5.180/95.07(170)	4.556/99.40(162)
$ManNH_2p$[b]	37.6	58.5	1.3	1.6	5.382/93.22(170)	5.191/93.87(164)
$ManNH_2f$[b]	1.4	2.4	5.5	5.6	5.541	5.528
$GlcNH_2$	62.5	37.5	3.6	8.4	5.431/91.99(171)	4.927/95.56(161)
$GalNH_2p$[b]	57.3	40.2	3.7	8.5	5.457/92.13(171)	4.859/95.99(162)
$GalNH_2f$[b]	1.6	0.8	4.9	2.5	5.543	5.489
Fuc*p*[b]	28.6	67.2	3.9	7.9	5.180/95.07(170)	4.533/99.00(160)
Fuc*f*[b]	1.7	2.3	4.2	2.9	5.251	5.204
Rib*p*[b]	20.5	58.9	2.1	6.5	4.914/96.60(167)	4.849/96.33(162)
Rib*f*[b]	13.3	7.4	1.8	3.9	5.231/103.76(173)	5.362/101.7(173)
Gal*p*[b]	32.2	62.4	3.7	7.8	5.246/95.12(172)	4.573/96.71(161)
Gal*f*[b]	2.0	3.2	4. 8	3.3	5.255/97.76(173)	5.194/101.23(173)
Rha*p*[b]	62.4	37.6	1.7	1.1	5.095/96.78(170)	4.849/96.33(162)
GalA*p*[b]	42.1	48.1	3.8	7.9	5.314/95.16(171)	4.603/99.00(161)
GalA*f*[b]	3.9	5.8	4.8	3.6	5.242	5.195

[a] Monosaccharide standards belong to the D-series relative to D-glyceraldehyde. NMR measurements were performed after 24 h of mutarotation to reach the equilibrium at 27 °C.
[b] For the same monosaccharide the pyranoside and furanoside ring forms. The latter is present at least 2% in the equilibrium mixture.

samples, detect contaminants in pharmaceutical products, perform carbohydrate interaction with proteins and develop quantitative analysis of complex carbohydrates (Guerrini et al., 2008; Mayer & Meyer, 1999; Sassaki et al., 2011; Torri & Guerrini, 2008). We now present a method in which monosaccharide composition of complex carbohydrates was determined by quantitative-HSQC through J_{C-H} dependence for the anomeric carbons solely by NMR spectroscopy, which showed similar results to those obtained from the GC–MS-carboxy reduction approaches.

2. Materials and methods

2.1. Monosaccharide analysis

Polysaccharide samples (5 mg) were hydrolyzed with 2 M TFA at 100 °C for 8 h. The solution was then evaporated, and the residue dissolved in D_2O for NMR analysis (400 μL) and adjusted to pH 3.5 using a diluted D_2O-H_2SO_4 solution. After ^{1}H and Q-HSQC experiments, the monosaccharides were reduced with

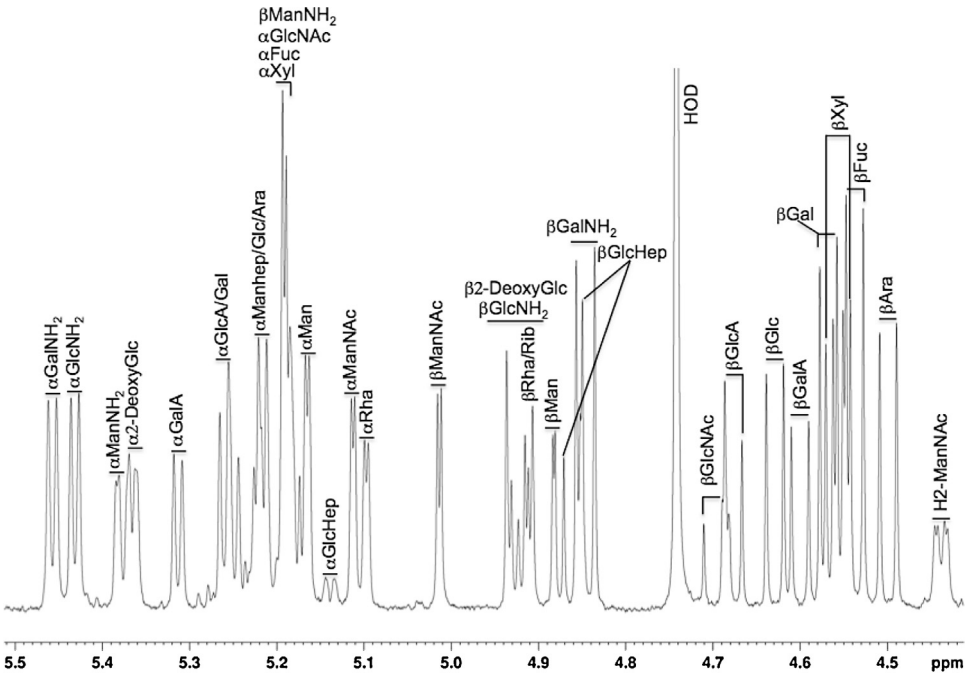

Fig. 1. Partial ^{1}H NMR spectrum of the anomeric region of 18 monosaccharides recorded at 400 MHz in D_2O at 27 °C, using the pulse program noesygppr1d.2: chemical shifts referenced to TMSP-d_4 ($\delta = 0$).

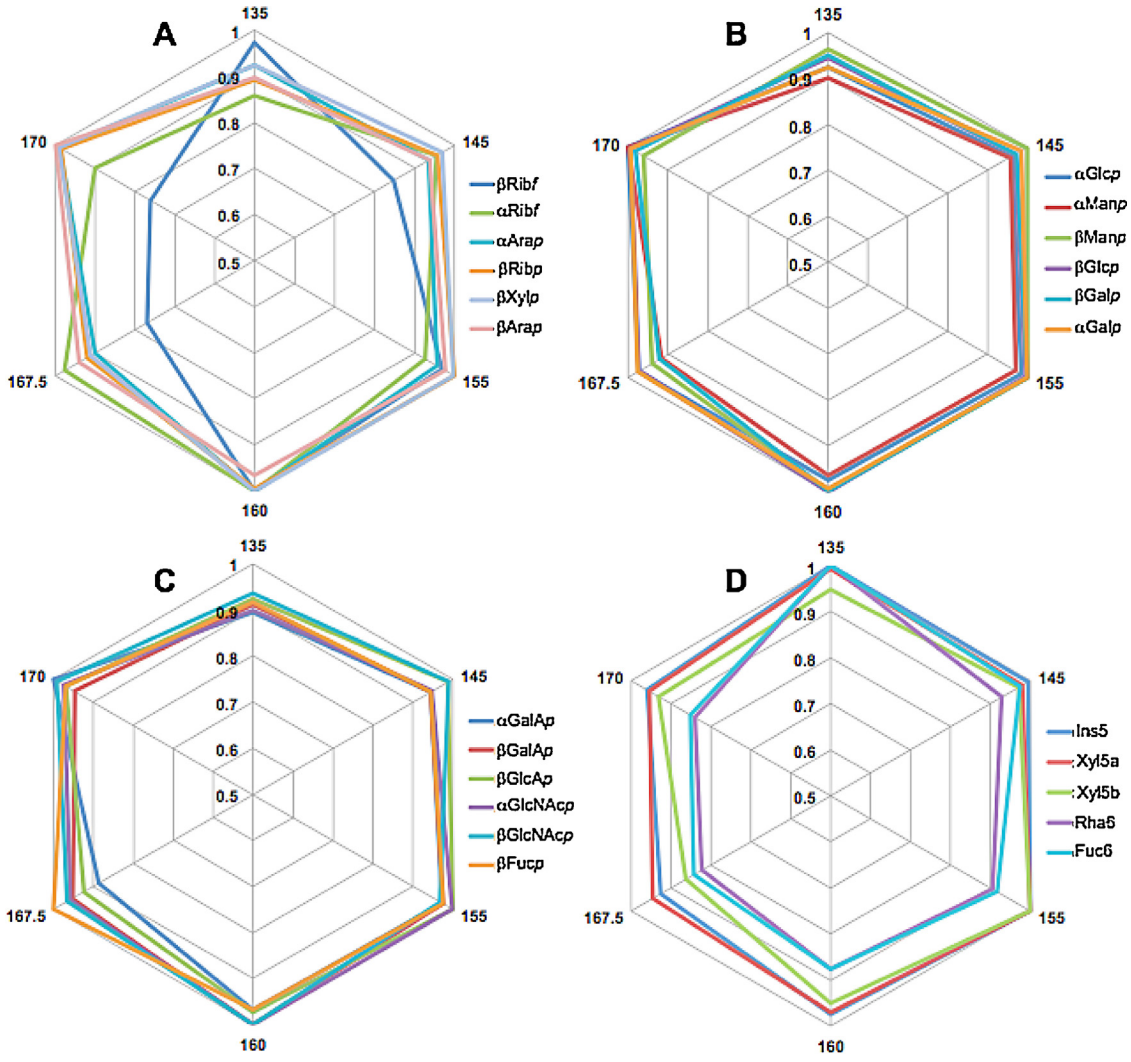

Fig. 2. Visualization of J_{C-H} dependence on Q-HSQC. Peak volumes were normalized to 1 and the data were plotted for some anomeric carbons and those of C6-Fuc, C5-Ins and C5-Xyl. J_{C-H} dependence of pentoses (A); hexoses (B); uronic and amino sugars (C); CH, CH_2 and CH_3 (D).

2 mg $NaBH_4$ yielding alditols, which were acetylated in Ac_2O-pyridine (1:1 v/v, 0.5 mL) at room temperature for 12 h (Wolfrom & Thompson, 1963a,b). The resulting alditol acetates were extracted with $CHCl_3$, and analyzed by GC–MS (Varian, Saturn 2000R-3800 gas chromatograph coupled to a Varian Ion-Trap 2000R mass spectrometer), using a DB-225-MS column (30 m × 0.25 mm × 0.25 μm) programmed from 50° to 220 °C at 40 °C/min, with He as carrier gas. Components were identified by their typical retention times and electron ionization (EI-70 eV) spectra. The uronic acid content of polysaccharides was determined using the colorimetric *m*-hydroxybiphenyl method (Filisetti-Cozzi & Carpita, 1991). Carboxy-reduction of polysaccharides (10 mg) was carried out by

Table 2
Determination of the monosaccharide composition by Q-HSQC and GC*-Filisetti-Cozzi.

Monosaccharide	RG-I-AC NMR	RG-I-AC GC*	Gum-AC NMR	Gum-AC GC*	PA-AC NMR	PA-AC GC*	EPS PPe8 NMR	EPS PPe8 GC*
Man	5.6	6.8	0.0	0.9	0.0	0.0	0.0	0.0
Glc	13.7	10.5	0.0	1.8	0.0	0.0	44.0	44.2
Ara	14.2	13.5	56.0	53.3	38.0	40.0	0.0	0.0
Xyl	1.0	0.5	0.0	0.0	26.0	23.0	0.0	0.0
Fuc	3.1	1.1	0.0	0.0	0.0	0.0	0.0	0.0
Gal	24.1	27.4	32.0	30.0	11.0	7.0	0.0	0.0
Rha	11.6	8.6	6.0	4.0	0.0	0.0	45.0	44.6
GalA	26.7	21.0	0.0	0.0	0.0	0.0	0.0	0.0
GlcA	0.0	0.0	6.0	10.0	26.0	30.0	11.0	10.2

NMR measurements were performed at 27 °C after pH adjustment to 3.5. (RG-I-AL) rhamnogalacturanan *from Arctium lappa*. (Gum-AC) glucuronoarabinogalactan from *Anadenanthera colubrine*. (PA-AC) glucuronoarabinoxylan from *Ananas comosus*. (EPS-PPe8) exopolysaccharide from *Burkholderia tropica*.

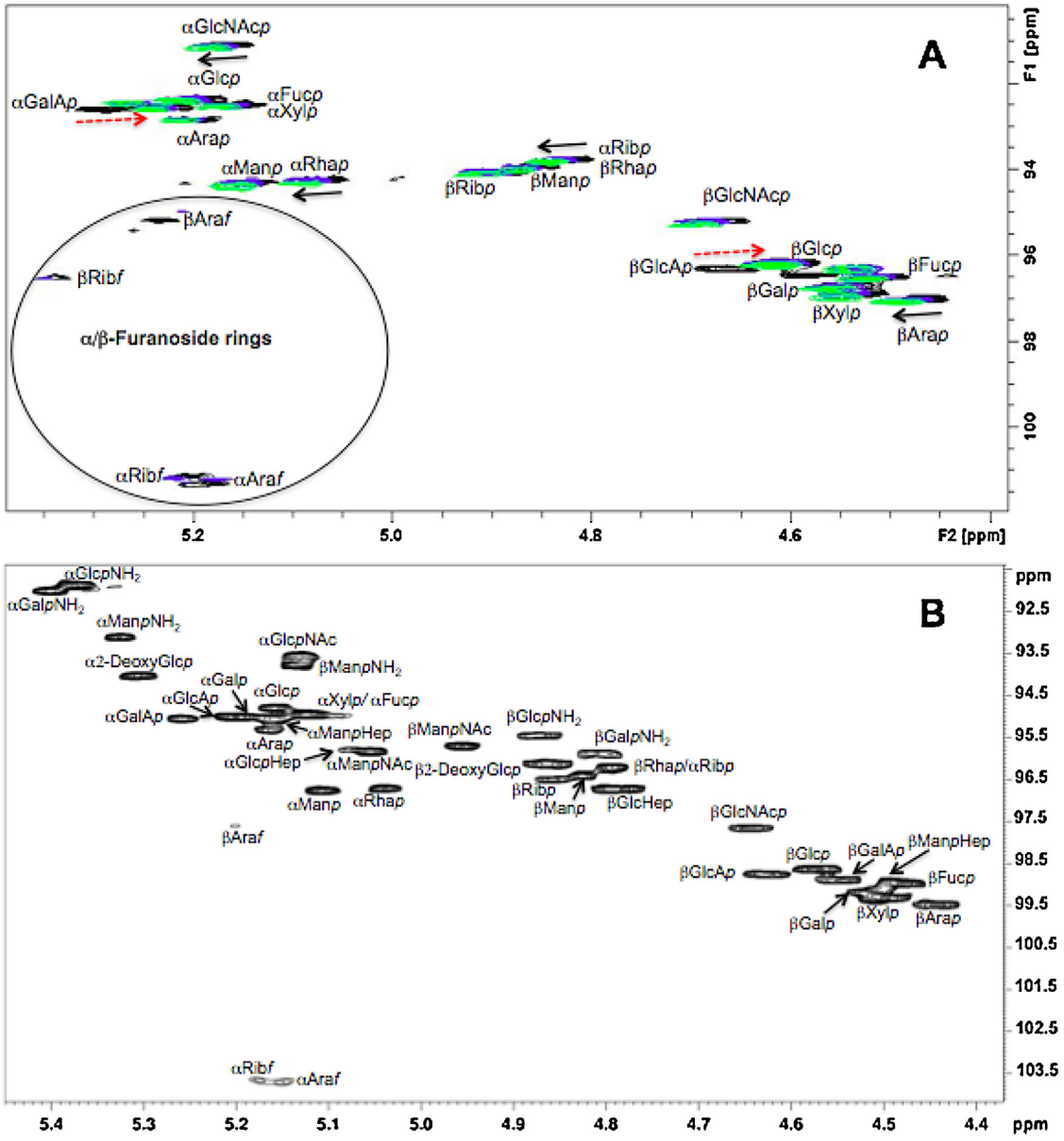

Fig. 3. (A) Partial overlaid 2D Q-HSQC spectra at different pH values, (black, pH 3.5), (blue, pH 6.0), (green, pH 9.0). The black circle shows the furanose anomeric protons. The arrows show chemical shift perturbations due to pH variation. (B) Partial 2D Q-HSQC (1024 × 400, ns = 32) spectrum of the anomeric region of 18 monosaccharides (5 mM) recorded in a 400 MHz magnetic field in D_2O at 27 °C and pH 3.5, chemical shifts referenced to TMSP-d_4 ($\delta = 0$), using a J_{C-H}-coupling of 155 Hz. (For interpretation of the references to color in figure legend, the reader is referred to the web version of the article.)

the carbodiimide method (Taylor & Conrad, 1972), using $NaBH_4$ as the reducing agent, giving polysaccharides with carboxyl groups reduced to primary alcohols.

2.2. NMR spectroscopy

The 18 monosaccharides samples from the D-series (1 mg/mL) were dissolved in D_2O and stocked for 24 h to allow mutarotation to reach equilibrium. Spectra were obtained at 27 °C, at pH 3.5, 7.0 and 9.0, containing 0.001% of TMSP-d_4 (2,2,3,3-tetradeuterium-3-trimethylilsilylpropionate) as internal standard ($\delta = 0$). All spectra were obtained with a Bruker 400 MHz AVANCE III NMR spectrometer using a 5 mm inverse gradient probe (broad band Inverse probe, BBI). 1D ^1H NMR were performed after 90° (p1) pulse calibration by evolution until 360° using a start p1 of 4 us plus increment of 2 us (p1 6.4–7.0 μs), calculation of offset (1885.0–1885.6 Hz) to obtain a spectrum width of 4795 Hz, using 16 scans to give a signal/noise (S/N) ratio of at least 1000:1 for the anomeric region (90° pulse, relaxation delay = 10.0 s, number of time domain points = 65,536 and acquisition time = 6.832 s). Integration of H-1 areas was performed without tube spinning and respecting a HDO signal with a medium half line width varying from 1.0 to 1.2 Hz and TMSP 0.8 to 1.0 Hz (Heikkinen, Toikka, Karhunen, & Kilpela, 2003), presaturation of residual HDO was carried out with the pulse program noesygppr1d.2, which included presaturation during relaxation delay (10 ms) and spoil gradient, using a relaxation delay = 2.0 s, number of time domain points = 65,536 and acquisition time = 6.832 s. 2D-MNR Q-HSQC, quantitative heteronuclear correlation via double inept transfer with decoupling during acquisition, using sensitivity improvement trim pulses in inept transfer and shaped pulses for all 180° pulses on the ^{13}C channel (hsqcetgpsisp2.2 on Bruker spectrometers). The experiment set up was based on the peak volume, which is dependent on the correlation peak to the relation between the polarization transfer delay (D) and the

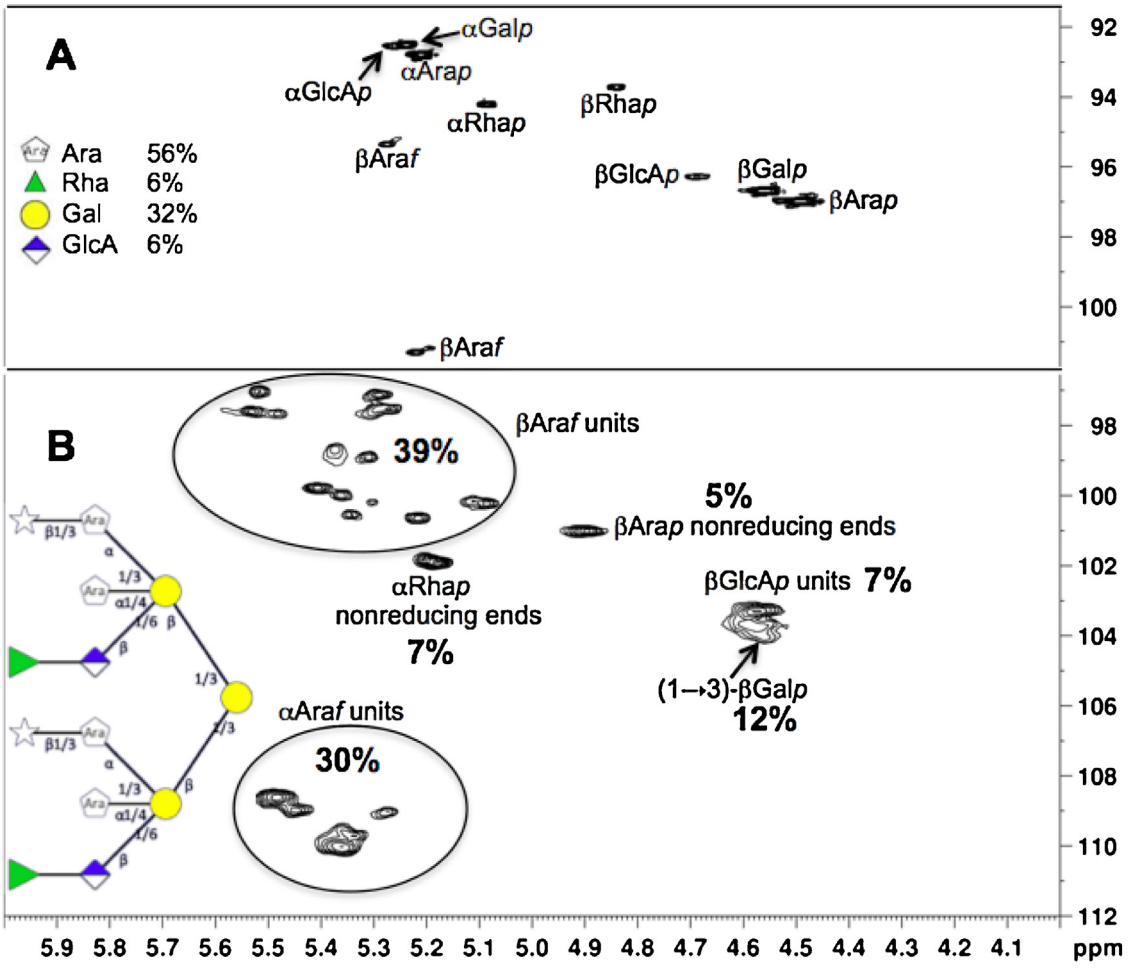

Fig. 4. Partial 2D Q-HSQC spectra from *Anadenanthera colubrina*. Hydrolyzed gum (A), native gum (B).

J_{C-H}-tune for each atom and also by the size of molecule, which is affected by T_2 (Heikkinen et al., 2003; Torri & Guerrini, 2008). Optimization of J_{C-H}-coupling was performed at 135, 145, 155, 165, and 170 Hz. The spectral widths for Q-HSQC were 3595 Hz (^{1}H) and 5031 Hz (^{13}C), experiments being recorded for quadrature detection in the indirect dimension, using 8 scans per series of 1 K × 400 W data points with zero filling in F1 (2 K) prior to Fourier transformation. A recycle delay of 1.14 s (1 s relaxation delay and 0.14 s acquisition time) was found sufficient to provide quantitative results in the monosaccharide (20, including NeuNAc and Ins) mixtures and the same value was used for the hydrolyzed polysaccharide samples, resulting in an overall experimental time of 1 h. However, we also performed experiments varying from 7 min (1 scan) to 3.30 h (32 scans) at the same resolution.

2.3. Monosaccharide composition by Q-HSQC

The hydrolyzed glycan solutions were evaporated under a N_2 stream in a fume hood and dissolved in 0.5 M NH_4OH (200 μL), held at 50 °C for 5 min in reinforced Pyrex tubes with 4 mL Teflon lined screw cap vessels. The product was dried as described above, giving rise to monosaccharides and also to de-lactonized uronic acids. The residual material was then dissolved in 500 μL D_2O and adjusts to pH 3.5 using a diluted D_2O-H_2SO_4 solution prior to NMR analyses. Relative monosaccharide quantification was performed by integration of the anomeric cross peaks and normalized to percentage values. The identification was based on the chemical shift map obtained from a standard mixture containing 5 mM of all 18 monosaccharides of interest.

3. Results and discussion

Interpretation of mutarotation was the starting point for quantification, since the sum of the area of the anomers has to be identical or similar of those from Q-HSQC. Interestingly, very little information data about the mutarotation of sugars has been found, and classical articles of Pigman (Pigman & Isbell, 1968) and Isbell (Isbell & Pigman, 1969) furnishing the possible equilibrium states for each monosaccharide in aqueous solution as well as the fundamental equation which describes their mutarotational behavior. Nonetheless, the data from the pyranose and furanose anomers in solution were mainly for Glc, Man and Gal. Since complex carbohydrates show a myriad of monosaccharide units, a widespread study concerning sugar equilibrium in solution and some other NMR parameters were determined herein (Table 1). It is important to note that mutarotation for some *eq-eq* OH1–OH2 showed great variation for α- and β-anomers. The amino sugars showed an inversion of the equilibrium in comparison to their Glc, Gal and Man precursors. This behavior was less intense for the *N*-acetylated forms. In the case of uronic acids, the equilibria of the anomers are similar, tending to the β-configuration, and the same profile was observed for 2-deoxy-Glc. The main difference between the anomers was observed for GlcHep*p*, which showed to favor the β-anomer. The NMR α,β ^{1}H/^{13}C chemical shifts, $^3J_{H1-H2}$ and J_{C-H} were measured and they are in agreement with previous classic

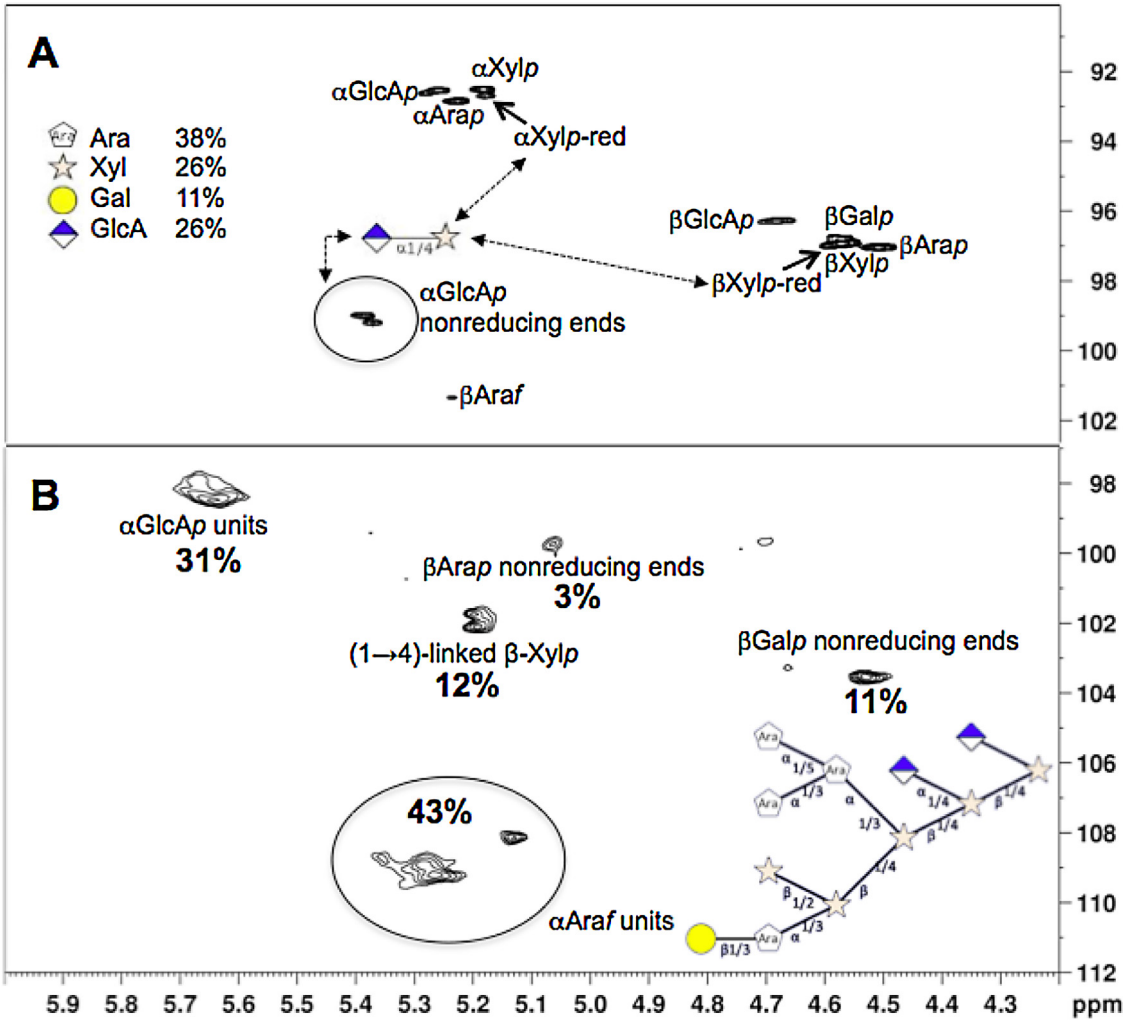

Fig. 5. Partial 2D Q-HSQC spectra from *Ananas comosus*. Hydrolyzed pineapple gum (A), Native gum (B).

studies of Chambat, Joseleau, Lapeyre, and Lefebvre (1978), Gorin and Mazurek (1975) and Perlin and Casu (1969). The combination of all NMR parameters led us to perform a reliable quantification based that the sum of all the anomeric ^{1}H from 1D ^{1}H NMR spectrum should be similar to those from HSQC, which is very potent to resolve much signal overlapping. Thus a mixture containing an equimolar (5 mM) amount of Glc, Rha, Ara and GlcA was prepared for quantification experiments (^{1}H NMR and Q-HSQC). The correlation between the experiments was very good, relative standard deviation (RSD) was calculated from three replicates and values of 1.3–1.5 were obtained when a $J_{C–H}$ of 155 Hz was used. Coefficient of variance (CV) was also very low, being less than 2% for all anomeric signals. In order to evaluate and quantify the monosaccharides via ^{1}H NMR spectra, a mixture of 18 monosaccharides was analyzed (Fig. 1). The spectrum, however, showed many overlapping peaks that prevented any quantification, since, in some cases, 4 component peaks overlapped. For quantification proposes, the pulse sequence (noesygppr1d.2) at 27 °C gave the best separation and maintenance of the peak heights near to residual water, which avoids loss of intensity for the *ax–ax* H1–H2.

Since the direct ^{1}H NMR method revealed the impossibility of a precise quantification for a simple mixture of monosaccharides due to peak overlapping, the use of 2D HSQC has been proved effective for peak separation. In order to obtain the best performance and reproducibility during quantification, a variation of $J_{C–H}$ dependence for the anomeric region was evaluated (Fig. 2). It was possible to observe that the α and β anomers from all monosaccharides were near $J_{C–H}$ 155–160 Hz. Peak volumes were normalized with a maximum value of 1.00 at a specific $J_{C–H}$ for each monosaccharide. The results showed values near of 1.00, becoming between 0.94 and 1.00, the exception being for ^{1}H/^{13}C correlations belonging to CH, CH_2, and CH_3 groups of the non-anomeric carbons, in this case the best $J_{C–H}$ was from 135 to 145 Hz, giving an average value between 0.92 and 1.00, using a $J_{C–H}$ of 155 Hz these carbons varying from absolute values of 0.90 to 1.00. Torri and Guerrini (2008) showed that glycosaminoglycans can be quantified by HSQC,[12] and also proposed that a $J_{C–H}$ of 155 Hz is the best for analyzing heparin and its low molecular derivatives, such as enoxaparin, tinzaparin and dalteparin (Torri & Guerrini, 2008). However, they warned that sub- or over-estimation can occur in the peak volumes, due to T_2 differences in fast and slow tumbling molecules, this effect being described by $V_c \propto \exp(-2D/T_2)$. In the case of monosaccharide analysis, this effect can be neglected due to long and similar T_2 values, with more robust and precise quantifications.

In order to determine the monosaccharide composition by Q-HSQC, polarization transfer delay was adjusted to $J_{C–H}$ 155 Hz and relaxation delay was 1. Determination of optimum separation also depends on other sample parameters such as pH, concentration and temperature. Since the mutarotation equilibrium is strongly affected by the final pH solution (Pigman & Isbell, 1968), different

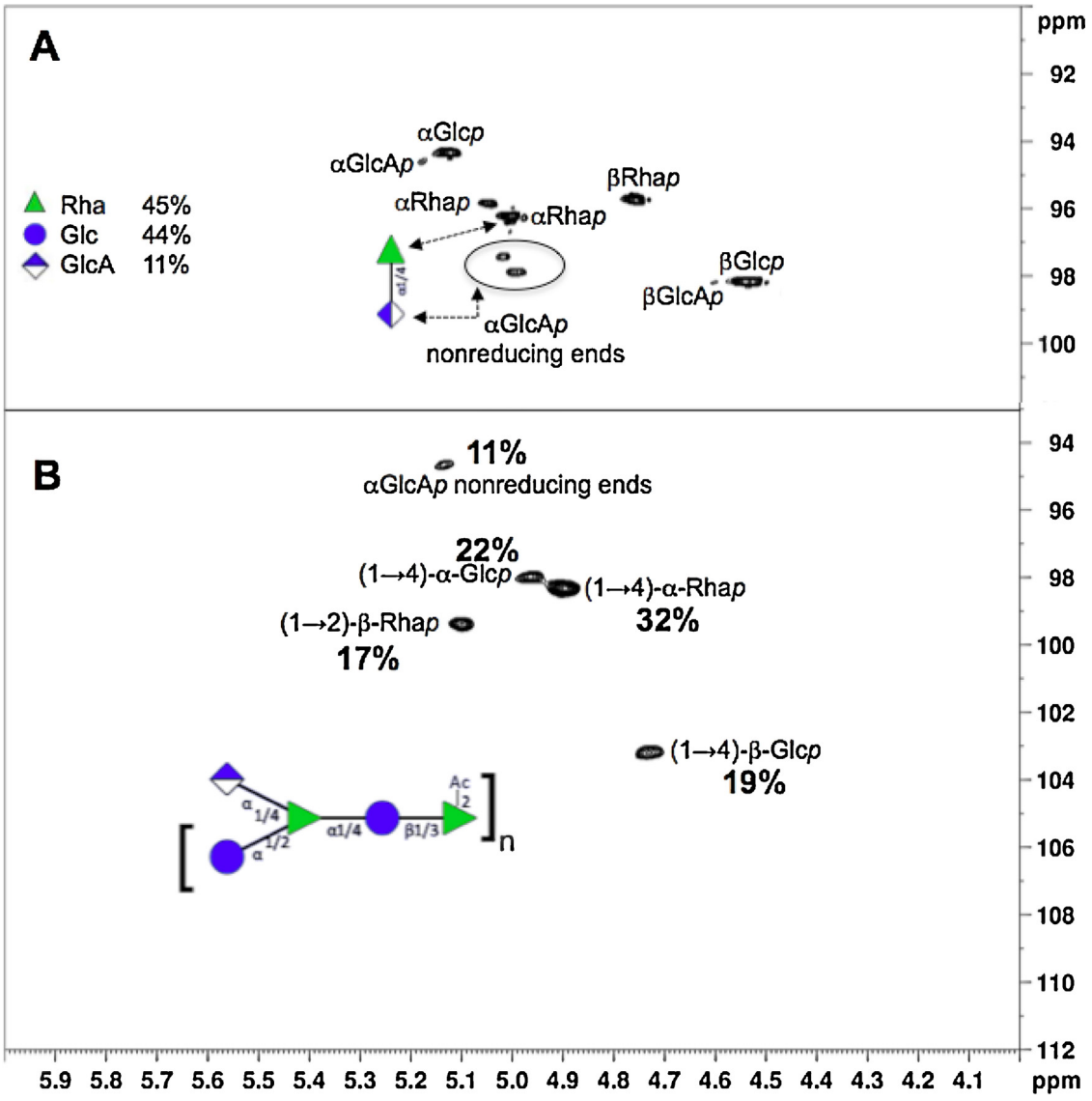

Fig. 6. Partial 2D Q-HSQC spectra of EPS PPe8 from *Burkholderia tropica*. Hydrolyzed EPS (A), native EPS (B).

pHs were tested and evaluated. The Q-HSQC experiments showed different anomeric populations and ring configurations at pH 3.5, 6.0, and 9.0 (Fig. 3A).

Differences in the chemical shifts deviations (CSD) and ring conformation were observed at different pHs. The experiment gave different distributions of the anomers as expected, although, this phenomenon did not interfere with quantification. Special attention should be paid to the formation of furanose rings at pH 3.5, which was shown to be absent at pH 9.0. At first inspection, it was naturally supposed that pH 9.0 would be the right choice, although it was found that many peaks overlapped for complex mixture analyses, mainly when containing uronic acids. After a better evaluation the solution with pH 3.5 showed fewer overlapped peak correlations and a good CSD for identification and quantification of a standard solution containing 18 monosaccharides (Fig. 3B).

After testing all important samples and NMR parameters, the determination of monosaccharide composition by Q-HSQC was performed for glycan samples. For this, very well characterized complex polysaccharides were tested and the total acid hydrolysis was carried out as described previously (Delgobo, Gorin, Tischer, & Iacomini, 1999; Serrato et al., 2008; Simas-Tosin et al., 2013). The quantitative NMR experiments clearly showed the influence of the flexibility and rigidness in the native glycan structures before hydrolysis (Fig. 4A and B). The peak volume is significantly altered by T_2 differences. The same behavior was found for highly substituted polysaccharides of pineapple gum (Fig. 5A and B). Curiously, for the exopolysaccharide from *Burkholderia tropica* (EPS PPe8) the comparison between the native and hydrolyzed samples (Fig. 6A and B) showed similar monosaccharide composition, indicating that glycosyl units had similar and homogenous T_2s along the polymer. These results are very interesting from a qualitative view of the dynamic properties of the native glycans in solution. These experimental observations were confirmed using lactose and melibiose standard solutions, since they are fast tumbling molecules, having long similar T_2s. Q-HSQC of both showed similar results for the native disaccharides and their hydrolyzed products, corroborating with the expected dynamic properties of small molecules.

The complete bona fide of the Q-HSQC also included a comparison with the traditional determination of uronic acid content, carboxy-reduction of glycans and GC–MS analysis of the reduced samples, (Table 2). For all tested glycans, the correlation between the NMR and the GC–MS-Filisetti-Cozzi protocol (Filisetti-Cozzi &

Carpita, 1991) showed very similar results, giving robustness for quantification by Q-HSQC. The main differences were observed for GalA and GlcA, this behavior being explained by the resistance of the biouronic acids during hydrolysis, which can underestimate its quantity by GC–MS. These results are very clear, since in the 2D NMR spectra of the hydrolyzed polysaccharides, it is possible to observe for EPS PPe8 and pineapple gum from *Ananas comosus* the presence of aldobiouronic acids (Figs. 5a and 6a). It is also important notice that for more precision during integration, we have respect a signal noise ratio of 10 fold for the smallest peak after extract the 1D projections from HSQC.

4. Conclusion

In conclusion, we developed a concise method for estimation and quantification of complex mixtures of monosaccharides through 2D NMR Q-HSQC. It showed robustness and flexibility to choose the best polarization transfer delay for quantification, for anomeric $^{1}H/^{13}C$ a J_{C-H} 155 Hz appears the best value for different aldoses. The detection for routine analysis was applied easily at 5 mM concentration in BBI-400 MHz NMR equipment. Preliminary test on 600 MHz-QXI probe, showed ~5 fold gain by Q-HSQC at the same conditions, allowing mM detection. Moreover, we also reproduced the "old experiments" of mutarotation looking with "new eyes", showing how complex sugar analyses in solution can be and use these variations as aids for CSD analytical studies. Also, we are able to observe some dynamic properties due a strong correlation between the quantification of native monosaccharide units of the glycan and those from the hydrolyzed form. Finally, our approach also permits the quantification of non-total hydrolyzed glycan-containing resistant oligosaccharides, became unique for such structures. To analyze the latter, normal carboxy-reduction-deuterium labeling, followed by methylation and comparison with the native structure, is needed.

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