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Structural characterization of the acetylated heteroxylan from the natural hybrid *Paulownia elongata*/*Paulownia fortunei*

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Abstract—The heteroxylan from the hybrid *Paulownia elongata*/*Paulownia fortunei* is an *O*-acetyl-(4-*O*-methylglucurono)xylan with an acetylation degree (DS) of 0.59 and a molecular weight (M_w) of 29 kDa. The heteroxylan backbone is composed by (1→4)-linked β-D-xylopyranosyl units (Xyl*p*) partially ramified with terminal (1→2)-linked 4-*O*-methyl-α-D-glucuronosyl (MeGlc*p*A) and a small proportion of α-D-glucuronosyl (Glc*p*A) residues in a molar ratio of Xyl*p*:(MeGlc*p*A+Glc*p*A) of 20:1. Roughly half of the β-D-xylopyranosyl units in the backbone are acetylated: 3-O-acetylated (22 mol %), 2-O-acetylated (23 mol %) or 2,3-di-O-acetylated (7 mol %). ESI-MS and MALDI-MS studies of partially hydrolyzed heteroxylan revealed a random distribution of *O*-Ac and MeGlc*p*A within the backbone. However, the frequency of substitution with *O*-Ac along the backbone is not uniform and the molecular regions that did not contain MeGlc*p*A substituents possessed an acetylation degree significantly lower than the average DS of the xylan.

Keywords: *Paulownia*; Xylan; Structural analysis; Electrospray; MALDI; NMR spectroscopy

1. Introduction

Paulownia is a genus of about 20 species native to China and South-East Asia and cultivated since as early as 1000 BC. Its characteristics of rot resistance, dimensional stability and a very high ignition point ensure the popularity of this timber in the world market.[1] *Paulownia* is also known as a fast growing, short-rotation timber crop valuable also for the production of chemical pulp.[2,3] These attractive characteristics explain the recent activity on *Paulownia* cultivation in South Europe, more specifically on the Iberian Peninsula. The first experimental plantation of the natural hybrid *Paulownia elongata*/*Paulownia fortunei* in northern Portugal occurred in 2003. Plantations of several *Paulownia* species (mainly *Paulownia fortunei* L.) have been present in Spain since 2000. Under the appropriate conditions, a 5–7 years old tree can reach about 15–20 m high and annual production is as high as 150 tons/hectare.[4]

The growing interest in *Paulownia* as a promising chemical feedstock has stimulated studies on the structural features of its main constituents. Besides cellulose, xylan is a massive carbohydrate constituent that determines the basic properties of cellulosic pulp (yield and physical properties) as well as the parameters and mode of the chemical processing of this wood for different needs.[4,5] In particular, xylo-oligosaccharides (XOS) may represent an interesting chemical feedstock being isolated from wood by hydrothermal treatment.[5] The structural features of *Paulownia* xylan are not yet known and are the topic of the present study.

The heteroxylan was isolated from the hybrid *Paulownia elongata*/*Paulownia fortunei* by extraction of peracetic holocellulose with dimethyl sulfoxide (Me_2SO) and thoroughly characterized by wet chemistry, NMR spectroscopy, and mass spectrometry methods. Some features of the primary structure of these polysaccharides, regarding the regularity of substitution of the main xylan backbone with glucuronic residues and acetyl groups, have been proposed.

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2. Experimental

2.1. Wood preparation and analysis

A wood sample of the hybrid *Paulownia elongata*/*Paulownia fortunei* (3.5 years-old tree) was harvested from a plantation in the north of Portugal (plantation of Barros e Filhos Lda., Monção). Several trees of about 6.0–7.5 m high were cut and the stem body between 0.5 and 2.0 m high was air dried, chipped and ground to 40–60 mesh. Wood sawdust (10 g, 40–60 mesh) was extracted for 1 h with 250 mL of water under reflux. After air drying, water-extracted sawdust was extracted with acetone for 6 h in a Soxhlet extractor. The determination of acid-insoluble lignin (Klason lignin) with 72% H_2SO_4 and ash content were determined by standard TAPPI methods.[6] The cellulose content was assessed using the Kürschner and Hoffer method via extraction of lignin and hemicelluloses with ethanol–HNO_3 (4:1, v/v) consecutively four times under reflux for 1 h (solution-to-wood ratio of 50).

2.2. Isolation of heteroxylan

The wood holocellulose was obtained from extractives-free sawdust by delignification with peracetic acid. The sawdust (5.0 g) was treated for 25 min with 11% (w/v) of peracetic acid solution (200 mL) at 85 °C. After delignification, the holocellulose was filtered off on a porous glass filter, washed with acetone and further with warm water and air-dried. The holocellulose yield was 60.5%. The isolation of acidic heteroxylans was carried out by two consecutive extractions with Me_2SO (1.5 g of holocellulose with 90 mL of Me_2SO in each assay) at 60 °C for 24 h and further precipitation of resulted extracts in an excess of 7:2:1 EtOH–MeOH–water acidified by HCOOH. The complete precipitation of heteroxylan was accomplished in 3 days at 4 °C. The heteroxylan was isolated by centrifugation, washed four times with anhydrous MeOH and quickly dried under vacuum at room temperature.

2.3. Carbohydrate analysis

The heteroxylan was subjected to Saeman hydrolysis (treatment with 72% H_2SO_4 at 20 °C for 3 h, followed by 1.5 h hydrolysis with diluted 1 M H_2SO_4 at 100 °C) and neutral monosaccharides were determined as alditol acetate derivatives by gas chromatography.[7] Uronic acids were determined colorimetrically with *m*-phenylphenol by modification of the method described by Blumenkrantz and Asboe-Hansen.[8]

2.4. Size-exclusion chromatography (SEC)

The xylan sample was dissolved in a small amount of 10% LiCl solution in *N*,*N*-dimethylacetamide (DMAC) at 60 °C and further diluted with DMAC to a xylan concentration of about 0.5% (5 mg/mL). SEC analysis was carried out on two PLgel 10 μm MIXED B 300 × 7.5 mm columns protected by a PLgel 10 μm pre-column (Polymer Laboratories, UK) using a PL-GPC 110 system (Polymer Laboratories). The columns, injector system and the detector (RI) were maintained at 70 °C during the analysis. The eluent (0.1 M LiCl solution in DMAC) was pumped at a flow rate of 0.9 mL/min. The analytical columns were calibrated with pullulan standards (Polymer Laboratories, UK) in the range 5.8–100 kDa. The injected volume was 100 μL.

The water-soluble polysaccharides were analyzed on the same equipment, but using two columns in series (Plaguagel-OH MIXED 8 μm, 300 × 7.5 mm) and a guard column (Plaguagel-OH 8 μm), supplied by Polymer Laboratories Ltd, UK. The injector and columns were maintained at 36 °C to decrease solvent viscosity and peak broadening. The mobile phase was HPLC grade water (J.T. Baker Chem. Co.) containing 0.1 M $NaNO_3$ and 0.02% NaN_3, with a flow rate of 0.9 mL/min. The injected volume was 100 μL and the sample concentrations of 0.4–0.5%. The analytical columns were calibrated with pullulan standards (Polymer Laboratories, UK) in the range 0.8–48 kDa.

2.5. Mild acid hydrolysis and fractionation of XOS

The heteroxylan sample (30 mg) was treated with CF_3COOH (50 mmol/L) at 100 °C for 90 min. The xylo-oligosaccharides were then separated by semi-preparative HPLC (pump Knauer K-1001, RI detector Knauer K-2401) using a Shodex sugar KS 2002 (Showa Denko K. K.) column (300 mm × 20 mm) at 30 °C and ultra-pure water (pH 6.5) as eluent, at a flow rate of 2.80 mL/min. The injected sample volume was 500 μL.

2.6. ESI-MS, ESI-MS/MS and MALDI-MS analyses

Electrospray ionization mass spectra (ESI-MS) and tandem mass spectra (ESI-MS/MS), acquired in positive mode, were carried out on a Micromass (Manchester, UK) Q-TOF2 hybrid tandem mass spectrometer. Samples were introduced at a flow rate of 10 μL/min into the ESI source. In the MS and MS/MS experiments the time-of-flight (TOF) mass resolution was set to approximately 9000. For ESI analysis, oligosaccharides were diluted in MeOH–water–formic acid (50:50:0.1, v/v/v). The cone voltage was 35 V, and the capillary voltage was 3 kV. The source temperature was 150 °C. The data was processed using MassLynx software (version 4.0). MS/MS spectra were obtained using argon as the collision gas with the collision energy set between 35 and 65 V.

Sample preparation for Matrix Assisted Laser Desorption Ionization (MALDI) analysis was performed by mix-

ing 5 μL of the oligosaccharide mixture dissolved in water to 20 μL of 2,5-dihydroxybenzoic acid (DHB) dissolved in a solvent mixture composed by acetonitrile–aqueous TFA (0.1%, v/v) (70:30, v/v). From this mixture, 0.3 μL was deposited on top of a layer of crystals of 2-chloromercaptobenzothiazole (CMBT) formed by deposition of 0.5 μL of CMBT solution in tetrahydrofuran–MeOH–water (1:1:1, v/v/v). Positive ion MALDI mass spectra were acquired with a MALDI-TOF/TOF Applied Biosystems 4800 ProteomicsAnalyser (Applied Biosystems, Framingham, MA, USA) instrument equipped with a nitrogen laser emitting at 337 nm and operating in reflectron mode. In each spectrum, 1500 shots were averaged.

2.7. NMR spectroscopy

One-dimensional ^{1}H NMR spectra were recorded in D_2O (30 °C) on a Bruker AMX 300 spectrometer operating at 300.13 MHz. Sodium 3-(trimethylsilyl)propionate-d_4 was used as internal standard (δ 0.00). The relaxation delay was 12 s, r.f. 90°-pulse width of 10.2 μs and about 600 pulses were collected.

Two-dimensional ^{1}H–^{1}H TOCSY (Total Correlation Spectroscopy) spectra ($\tau_{mix} = 0.050$ s) were acquired at a spectral width of 2185 Hz in both dimensions on a Bruker AMX 300 spectrometer at 60 °C. The relaxation delay was 2.0 s. For each FID, 128 transients were acquired, the data size was 1024 in $t_1 \times 512$ in t_2. Homonuclear two-dimensional nuclear Overhauser effect (NOESY) spectra were acquired on a Bruker DRX 500 MHz NMR spectrometer operating at 500.13 MHz and using $\tau_m = 0.5$ s. The phase sensitive ^{1}H-detected HSQC (Heteronuclear Single Quantum Coherence) spectrum was acquired over a F1 spectral weight of 12,000 Hz and a F2 width of 2000 Hz with a 2048×1024 matrix and 128 transients per increment. The delay between scans was 2 s and the delay for polarization transfer was optimized for $^{1}J_{CH} = 150$ Hz.

Paulownia wood was analyzed by solid-state ^{13}C CP-MAS NMR as a fine powder (<60 mesh). ^{13}C NMR spectra were recorded at 100.6 MHz (9.4 T) on a Bruker Avance 400 spectrometer. A 7-mm double bearing Bruker rotor was spun in air at 5.0 kHz. In all experiments, the ^{1}H and ^{13}C 90° pulses were ~4 μs. The CP-MAS spectra were recorded with a 5 s recycle delay and a 2 ms contact time. The number of scans was 8000.

3. Results and discussion

3.1. Chemical composition of wood

The general chemical composition of the hybrid *Paulownia elongata*/*Paulownia fortunei* (further as *Paulownia* for simplicity) is presented in Table 1. *Paulownia* wood is characterized by a relatively low holocellulose content, which can be explained by a high proportion of water-soluble oligosaccharides. According to the brief sugar and SEC analyses, water-soluble oligosaccharides of $M_w < 1500$ Da are composed essentially of glucose (Glc), galactose (Gal), mannose (Man) and arabinose (Ara). No significant amount of xylose (Xyl) was found. Aiming to diminish the contamination of xylan with water-soluble polysaccharides, wood was extracted with hot water before the holocellulose isolation. Additionally, acetone extraction was applied to improve wood accessibility in delignification with peracetic acid.

Table 1. Chemical composition of *Paulownia*

Wood component	Amounts in wood, % w/w
Holocellulose	60.5
Lignin	23.6
Cellulose (Kürschner and Hoffer)[a]	38.9
Extractives (acetone)	2.7
Ash	0.4
Water-soluble polysaccharides	9.7
Sugars analysis	
Rha	1.6
Ara	1.0
Xyl	18.0
Man	2.1
Gal	4.4
Glc	36.6
UA (as galacturonic acid)	5.0

[a] Without correction on the residual pentosans.

Chemical analysis of the wood, and sugar analysis, clearly showed that xylan is the principal hemicellulose of *Paulownia* and the second most abundant polysaccharide after cellulose (Table 1). The significant amount of xylan was additionally confirmed by a solid-state ^{13}C NMR spectrum of wood extracted with water and acetone, which showed the characteristic signal around 63 ppm assigned to C-5 in [→4)-β-D-Xyl*p*-(1→] (Fig. 1). Signals of similar intensity at 21 and 172 ppm, assigned to methyl and carbonyl carbons of acetyl moieties, respectively, indicate the significant acetylation degree of *Paulownia* hemicelluloses. Comparing the intensity of carbon signals at 63 and 21 ppm, it may be proposed that roughly half of xylan internal units are acetylated.

3.2. Isolation and chemical analysis of heteroxylan

The xylan of approximately 62% (w/w) yield, based on total amount of xylan in wood, was isolated from peracetic acid-treated holocellulose by two consecutive extractions with Me_2SO. The high purity of the isolated xylan was confirmed using neutral monosaccharide analysis, which revealed the presence of mainly xylose (Xyl) and uronic acid residues, although a small proportion of glucose (Glc) and mannose (Man) was also present. Only trace amounts of galactose (Gal), arabinose (Ara) and rhamnose (Rha) were detected (Table 2). The presence of Glc and Man in the sugar analysis

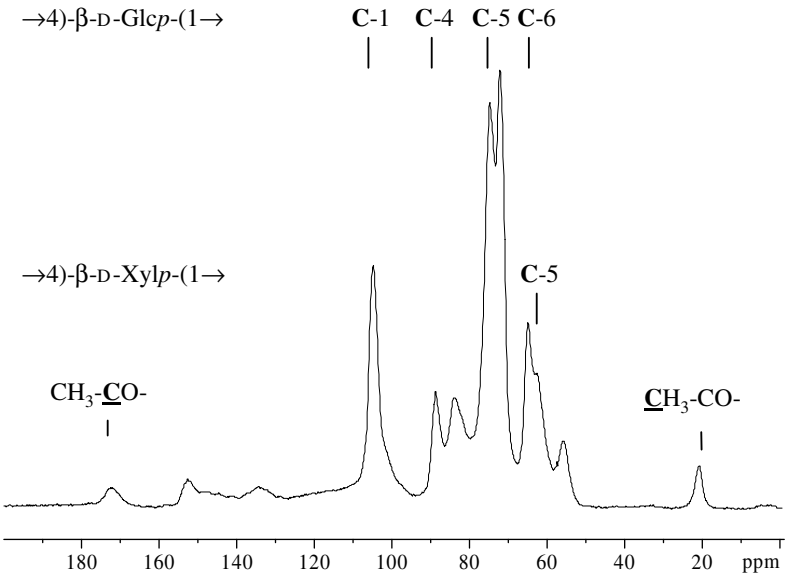


Figure 1. ^{13}C CP-MAS NMR spectrum of extracted *Paulownia* wood.

Table 2. Carbohydrate composition of *Paulownia* heteroxylan

Monosaccharide	% (w/w) in heteroxylan
Rha	0.4
Fuc	—
Ara	0.1
Xyl	87.0
Man	3.0
Gal	1.0
Glc	2.5
UA[a]	6.0

[a] All kinds of uronosyl units (UA) were calculated as MeGlc*p*A.

may indicate the contamination of xylan with glucomannan, which was difficult to remove by gradual re-precipitation from the Me_2SO solution. The molecular weight (M_w) of xylan was about 29 kDa as revealed by SEC analysis.

3.3. Structural analysis

The particular structural features of *Paulownia* xylan were assessed employing a set of NMR techniques and based on known signal assignments in xylans from angiosperms.[9–11] Thus, the proton and carbon chemical shifts were assigned using ^{1}H–^{1}H (TOCSY) and ^{1}H–^{13}C (HSQC) correlation NMR spectroscopy as shown in Table 3. The fact that all of α-D-glucuronosyl moieties are (1→2)-linked to [→4)-β-D-Xyl*p*-(1→] units was additionally confirmed using long-range proton–proton correlations in the NOESY spectra. No reliable correlations were found between protons in [→4)-β-D-Xyl*p*-(1→] and in Man*p* or Glc*p* units demonstrating that the latter residues are not the part of xylan. Hence the *Paulownia* xylan may be considered as a heteroxylan

Table 3. Proton/carbon chemical shifts (δ, ppm) in heteroxylan from *Paulownia*

Structural unit	Assignments					
	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	
					ax	eq
Xyl*p* (isol.)	4.47/102.9	3.28/73.7	3.55/74.5	3.80/77.4	3.40/63.9	4.10/63.9
Xyl*p* (Xyl*p*-Xyl*p*-Ac)	4.44/103.6	3.21/73.8	3.50/74.6	3.78/77.5	3.38/64.0	4.06/64.0
Xyl*p*-3Ac	4.57/102.4	3.49/71.9	4.98/76.3	3.93/76.5	3.47/63.8	n.d./63.8
Xyl*p*-2Ac	4.68/101.2	4.68/74.6	3.80/72.5	3.87/77.2	3.45/63.8	n.d./63.8
Xyl*p*-2,3Ac	4.80/100.5	4.82/74.6	5.16/74.0	4.06/76.6	3.54/63.9	n.d./63.9
Xyl*p*-3Ac-2Glc*p*A	4.73/102.1	3.70/75.8	5.06/75.8	3.98/77.2	3.50/n.d.	n.d./n.d.
MeGlc*p*A	5.27/98.9	3.57/72.3	3.88/73.7	3.27/83.2	n.d./n.d.	—[a]
Glc*p*A	5.25/n.d.	3.61/n.d.	3.82/n.d.	3.52/n.d.	n.d./n.d.	—[a]
Man*p*-2Ac	4.90/n.d.	5.48/n.d.	4.01/n.d.	3.60/n.d.	n.d./n.d.	—[a]

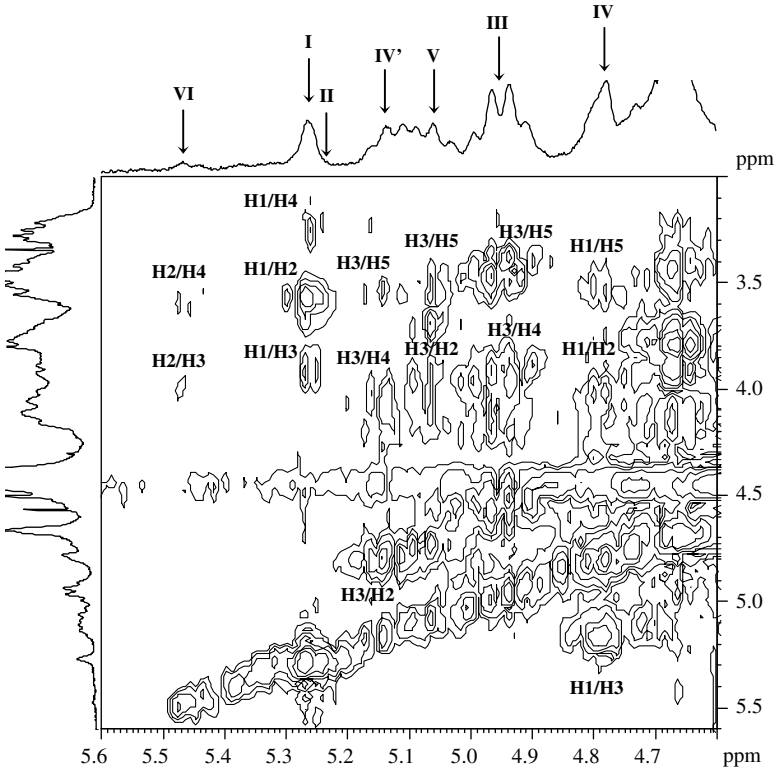
Abbreviations used: Xyl (isol.), non-acetylated Xyl*p* in the backbone isolated from other acetylated Xyl*p* units; Xyl*p* (Xyl*p*-Ac), Xyl*p* linked with neighbouring acetylated Xyl*p*; Xyl*p*-3Ac, 3-O-acetylated Xyl*p*; Xyl*p*-2Ac, 2-O-acetylated Xyl*p*; Xyl*p*-2,3Ac, 2,3-di-O-acetylated Xyl*p*; Xyl*p*-3Ac-2Glc*p*A, MeGlc*p*A 2-O-linked and 3-O-acetylated Xyl*p*; MeGlc*p*A-2Gal*p*, Gal*p* 2-O-linked MeGlc*p*A; Man*p*-2Ac, 2-O-acetylated Man*p*.
n.d.—non-determined.
[a] Not relevant.

with the main backbone composed of partially acetylated [→4)-β-D-Xyl*p*-(1→] units and ramified at O-2 with terminal 4-*O*-methyl-α-D-glucuronic acid (MeGlc*p*A) residues.

Apparently, a small proportion (less than 10 mol % from the total amount) of terminal glucuronic residues is not methylated (Glc*p*A). This suggestion was made based on a series of cross-signals found in the TOCSY spectrum indicating the presence of uronic moieties slightly different from MeGlc*p*A (Fig. 2, Table 3). The displacement of the H-4 resonance to downfield from 3.27 to 3.52 ppm, H-2 from 3.57 to 3.61 ppm and C-3 from 3.88 to 3.82 ppm is indicative of the presence of terminal glucuronic units, which are non-methylated at O-4.[12] This also justifies the observed chemical shift of the anomeric proton at 5.25 ppm (H-1 in Glc*p*A) in addition to a major resonance at 5.27 ppm (H-1 in MeGlc*p*A). The chemical shift of 5.24 ppm was previously reported for the anomeric proton of terminal [α-D-Glc*p*A-(1→] linked to the heteroxylan backbone.[13]

The correlations to proton at 5.48 ppm (Fig. 2) were assigned, according to the literature data,[14] to partially acetylated internal mannopyranose units [→4)[2-*O*-Ac]-β-D-Man*p*-(1→] in the composition of glucomannan. Partial acetylation of glucomannan in angiosperms is rather uncommon and deserves more detailed study in the future.

The proportions of structural units with different substitution patterns in the xylan backbone were assessed using quantitative ^{1}H NMR spectroscopy.[9–11] Figure 3 shows the ^{1}H NMR spectrum of heteroxylan and the designation of regions used for the integration of protons from structural fragments according to assignments presented in Table 3. Non-acetylated, 3-O- and 2-O-acetylated internal xylose residues and glucopyranosyluronic residues were assessed based on their anomeric proton resonances, whereas the amounts of 2,3-di-O-acetylated and 3-O-acetylated internal xylose residues O-2 substituted with MeGlc*p*A were estimated based on H-3 resonances in the corresponding structures (Fig. 3). The balance of acetyl groups was determined by comparison of the integral of acetyl group protons at 2.05–2.25 ppm and integrals of characteristic protons in acetylated xylopyranosyl rings. The results of the distribution of different structural units in the heteroxylan, expressed per 100 xylose residues, are presented in



I – MeGlc*p*A-(1→ **IV, IV' –** 4)[3-*O*-Ac][2-*O*-Ac]-β-D-Xyl*p*-(1→

II – Glc*p*A-(1→ **V –** 4)[4-*O*-Me-α-D-Glc*p*A-(1→2][3-*O*-Ac]-β-D-Xyl*p*-(1→

III – 4)[3-*O*-Ac]-β-D-Xyl*p*-(1→ **VI –** 4)[2-*O*-Ac]-β-D-Man*p*-(1→

Figure 2. TOCSY spectrum (D_2O, 60 °C) of the xylan from *Paulownia* wood.

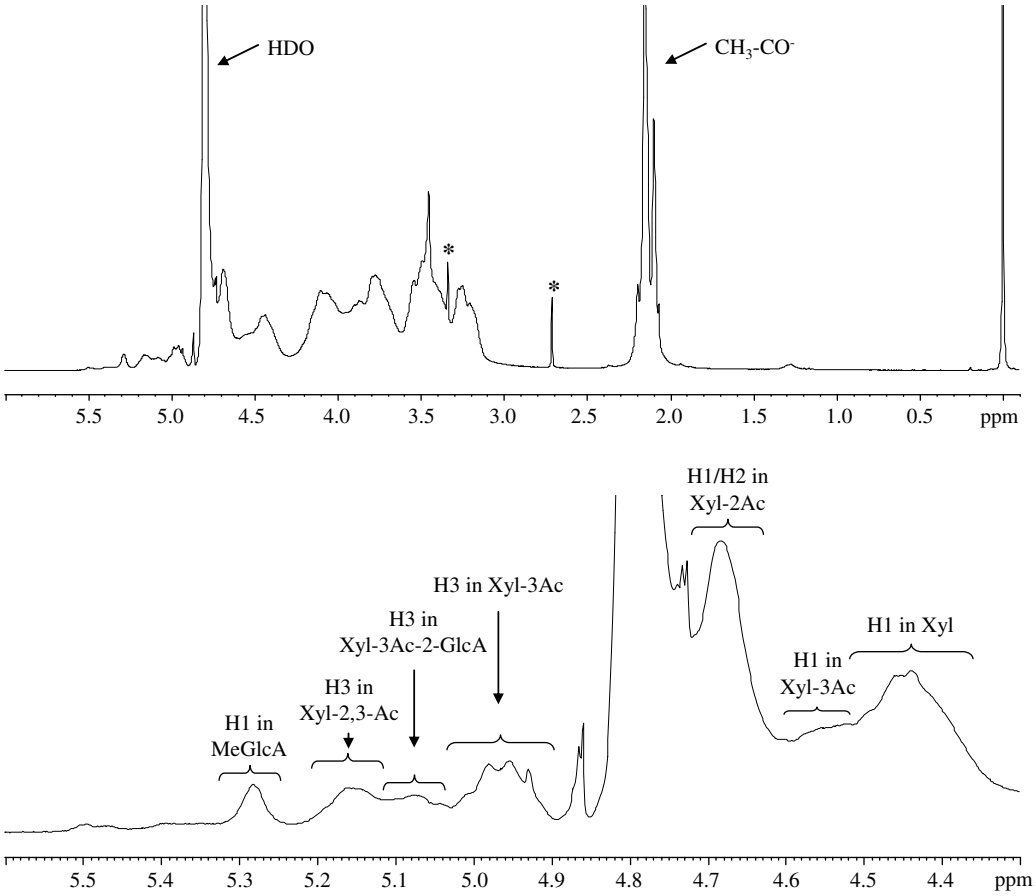


Figure 3. ^{1}H NMR spectrum (D_2O, 30 °C) of heteroxylans from *Paulownia* wood (top image) and the expanded region of anomeric protons (bottom image). Solvent impurities are marked by asterisk.

Table 4. Accordingly, 52 mol % of Xyl*p* residues are acetylated and possess a degree of substitution (DS) of 0.59. This result is coherent with degree of acetylation estimated based on wood analysis by ^{13}C CP-MAS NMR spectroscopy as discussed above.

The most frequent acetylated Xyl*p* units were suggested to be those solely 2-O-acetylated (23 mol %) followed by 3-O-acetylated residues (17 mol %). The proportion of 2,3-di-O-acetylated residues was relatively low (7 mol %). Similar proportions of [→4)[4-*O*-Me-α-D-Glc*p*A-(1→2)][3-*O*-Ac]-β-D-Xyl*p*-(1→] and [4-*O*-Me-α-D-Glc*p*A-(1→] residues in the heteroxylan indicate that the majority of the β-D-Xyl*p* units ramified at O-2 with MeGlc*p*A residues are 3-O-acetylated (Table 4). Similar conclusions were previously reached for heteroxylans isolated from other angiosperms.[9–11]

Generally, the acetylation degree of *Paulownia* heteroxylan was close to the acetylation degree reported previously for *Eucalyptus*,[9] *Populus*[10] and *Acacia*[15] heteroxylans (DS = 0.58–0.61), but was certainly higher than in heteroxylans from *Betula* and *Fangus*[11] (DS ≈ 0.40). In contrast, the frequency of substitution of the main backbone with MeGlc*p*A residues in heteroxylans from *Paulownia* (Xyl*p*:(MeGlc*p*A+Glc*p*A) ≈ 20:1) was similar to that found in *Betula* and *Fangus*[11] (Xyl*p*:MeGlc*p*A ≈ 15–18:1), but significantly lower than in *Eucalyptus*,[9] *Populus*[10] and *Acacia*[15] (Xyl*p*:MeGlc*p*A ≈ 10–11:1). A particular feature of *Paulownia*

Table 4. Relative content in acetyl groups in structural units of *Paulownia* heteroxylans

Structural fragment and short designation	Relative abundance (per 100 Xyl*p* units)
→4)-β-D-Xyl*p*-(1→(**Xyl*p***)	48
→4)[3-*O*-Ac]-β-D-Xyl*p*-(1→(**Xyl*p*-3Ac**)	17
→4)[2-*O*-Ac]-β-D-Xyl*p*-(1→(**Xyl*p*-2Ac**)	23
→4)[3-*O*-Ac][2-*O*-Ac]-β-D-Xyl*p*-(1→(**Xyl*p*-2,3Ac**)	7
→4)[3-*O*-Me-α-D-Glc*p*A-(1→2)][3-*O*-Ac]-β-D-Xyl*p*-(1→(**Xyl*p*-3Ac-2MeGlc*p*A**)	5
4-*O*-Me-α-D-Glc*p*A-(1→(**MeGlc*p*A**)	5

heteroxylan is the unusually high frequency acetylation of Xyl*p* units at O-2 (27 mol %), when compared to that in heteroxylans of other angiosperms (13–18 mol %).[9–11,15]

3.4. Partial acid hydrolysis of heteroxylan and fractionation of XOS

Aiming to assess the distribution of MeGlc*p*A and acetyl moieties along the backbone, heteroxylan was partially hydrolyzed to produce a series of XOS, which, after fractionation was analyzed by mass spectrometry. The conditions of acid hydrolysis (50 mM CF_3COOH, 1 h, 100 °C) were selected to obtain a series of XOS with relatively low molecular weight (<3000 Da), but without significant deacetylation. Under these conditions, only a small proportion of acetyl groups (<20%) is hydrolyzed.[16] The XOS obtained were fractionated using preparative ligand exchanged/size exclusion chromatography (LEX/SEC)[16] and subjected to analysis by ESI-MS, ESI-MS/MS and MALDI-MS. Five fractions (F1–F5) were isolated as shown in the elution chromatogram (Fig. 4). Fractions F1 and F2 contained acidic XOS with one or more uronosyl units, whereas fractions F3–F5 were constituted of neutral XOS. The proportion of acidic and neutral oligosaccharides was about 75/25 (w/w). The XOS of fraction F1 possessed a higher molecular weight than those of F2 and were harder to assess with MS techniques. F4 and F5 were composed of dimers and monomers, respectively, and were not relevant for assessing the distribution of MeGlc*p*A and acetyl moieties along the backbone. Hence, the most suitable fractions for the structural studies by MS were F2 and F3. These fractions were subsequently analyzed by ESI-MS, ESI-MS/MS and MALDI-MS in more detail.

3.5. Mass spectrometry analysis

ESI-MS and MALDI-MS are recognized techniques for structural studies of complex oligosaccharides.[17] In particular, these are frequently employed for the study of xylans.[18–21] When ESI-MS and MALDI-MS are acquired in a positive mode, oligosaccharides ionize preferentially as sodium adducts, $[M+Na]^+$, even without the addition of any sodium salt.[17,22] ESI-MS and MALDI-MS spectra provided information on the molecular mass of oligomers and their relative abundance through the detection of the corresponding $[M+Na]^+$ ions. Electrospray tandem mass spectrometry (ESI-MS/MS) also allows the assessment of distribution patterns of acetyl groups and MeGlc*p*A residues in XOS[16] and structural elucidation of complex XOS.[23] ESI-MS and MALDI-MS are complementary techniques, where low molecular weight XOS are predominantly identified by ESI-MS while the high molecular weight XOS are easier to assess by MALDI-MS.

ESI-MS and MALDI-MS spectra of acidic and neutral XOS from *Paulownia* heteroxylan are presented in Figures 5 and 6. In general, coherent data were obtained by both techniques. The identified neutral XOS from F3 are resumed in Table 5. These were linear oligomers composed of Xyl*p* units with polymerization degree (DP) ranging from 2 to 20. Among these XOS, a series of non-acetylated oligomers ($Xylp_{2,4-14}$) were identified in addition to the acetylated ones possessing from 1 to 9 acetyl groups, depending on DP (Table 5). The analysis of the relative intensities of $[M+Na]^+$ ions of acetylated XOS with the same DP (Figs. 5 and 6 and Table 5) showed that the ions of major intensity correspond to oligomers with acetylation degree of ~0.25–0.30, much lower than the average DS (~0.59) detected in heteroxy-

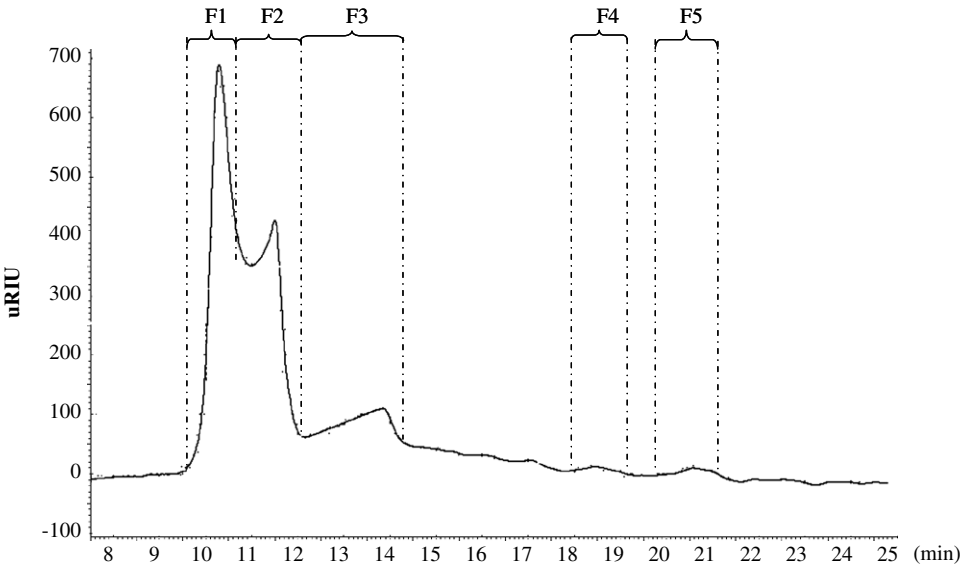


Figure 4. LEX/SEC chromatogram of xylo-oligosaccharides released after partial acid hydrolysis of *Paulownia* heteroxylan. Collected fractions are depicted as F1–F5.

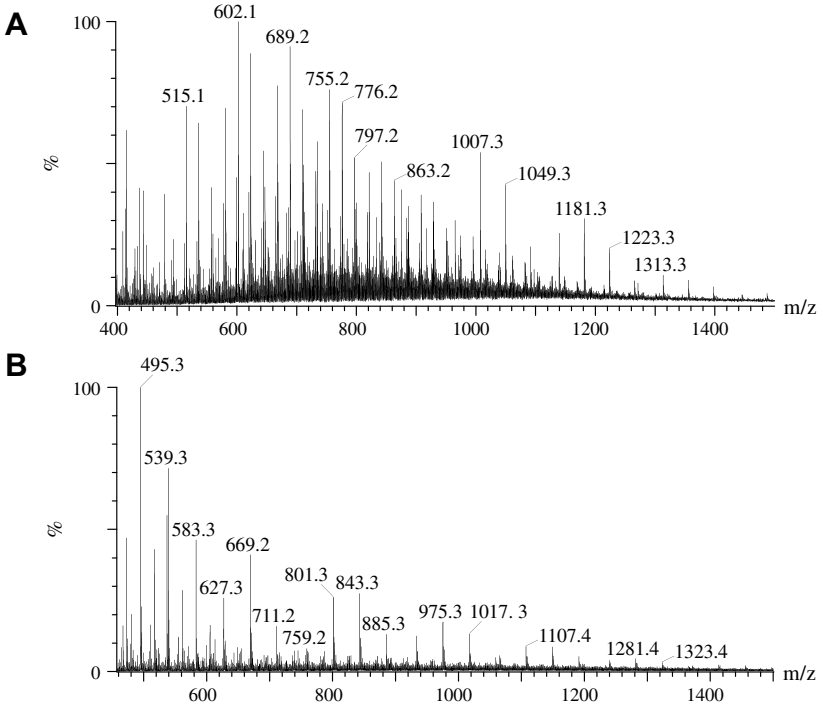


Figure 5. ESI-MS spectra (positive ion-mode) of (A) neutral xylo-oligosaccharides (fraction F3); (B) acidic xylo-oligosaccharides (fraction F2).

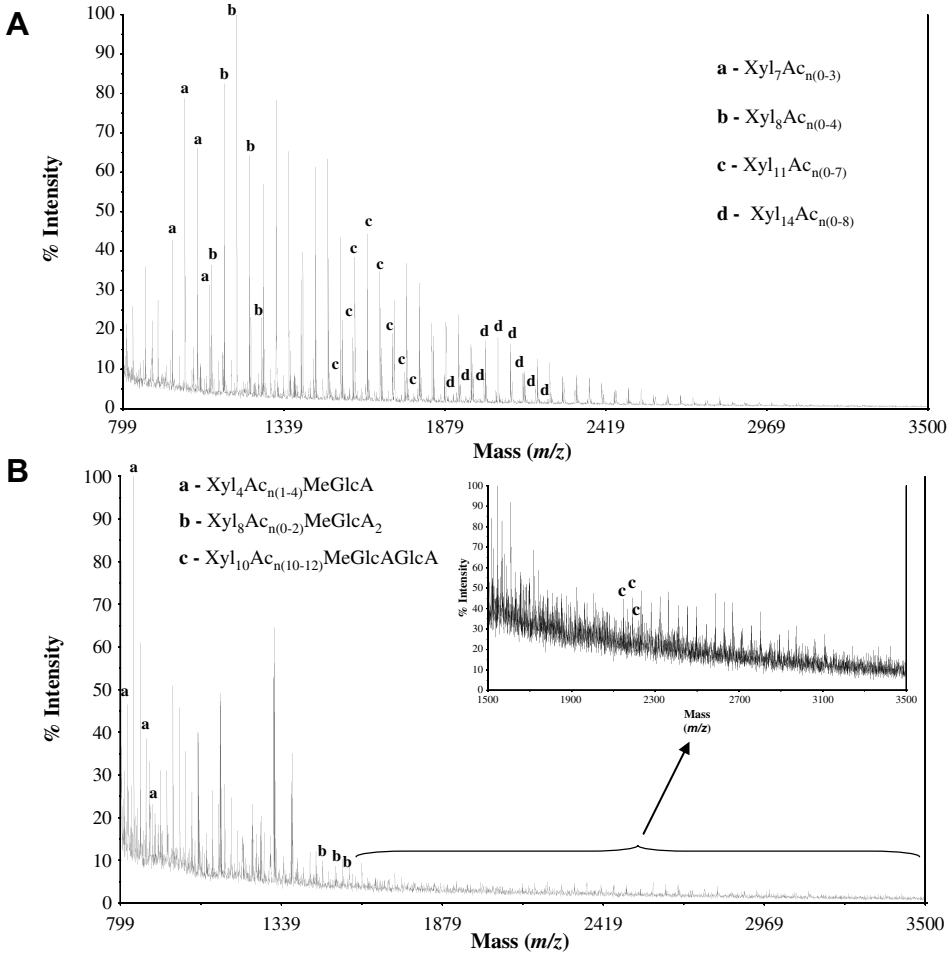


Figure 6. MALDI-MS spectra (positive ion-mode) of (A) neutral xylo-oligosaccharides (fraction F3); (B) acidic xylo-oligosaccharides (fraction F2).

Table 5. Neutral xylo-oligosaccharides identified in the ESI-MS and MALDI-MS spectra of fraction F3

$[M+Na]^+$	Number of *O*-Ac groups (*n*)									
	0	1	2	3	4	5	6	7	8	9
$Xylp_mAc_n$										
$Xylp_2Ac_n$	305[a]	347[a]	389[a]							
$Xylp_3Ac_n$		**479**[a]	521[a]	563[a]						
$Xylp_4Ac_n$	569[a]	**611**[a]	653[a]	695[a]						
$Xylp_6Ac_n$	833[a]	875[a]	917	959[a]						
$Xylp_7Ac_n$	965[a]	**1007**[a]	1049[a]	1091[a]						
$Xylp_8Ac_n$	1097[a]	1139[a]	**1181**[a]	1223[a]	1265[a]					
$Xylp_9Ac_n$	1229	1271	**1313**[a]	1355	1397	1439				
$Xylp_{10}Ac_n$	1361	1403	1445	**1487**	1529	1571	1613			
$Xylp_{11}Ac_n$	1493	1535	1577	**1619**	1661	1703	1745	1787		
$Xylp_{12}Ac_n$	1625	1667	1709	**1751**	1793	1835	1877	1919		
$Xylp_{13}Ac_n$	1757	1799	1841	**1883**	1925	1967	2009	2051	2093	
$Xylp_{14}Ac_n$	1889	1931	1973	2015	**2057**	2099	2141	2183	2225	
$Xylp_{15}Ac_n$		2063	2105	2147	**2189**	2231	2273	2315	2357	
$Xylp_{16}Ac_n$			2237	2279	**2321**	2363	2405	2447	2489	2531
$Xylp_{17}Ac_n$		2327	2369	2411	**2453**	2495	2537	2579	2621	
$Xylp_{18}Ac_n$			2501	2543	2585	**2627**	2669	2711	2753	2795
$Xylp_{19}Ac_n$						**2759**	2801	2843	2885	
$Xylp_{20}Ac_n$			2765			**2891**			3017	

[a] $[M+Na]^+$ ions observed in both ESI-MS and MALDI-MS spectra; major abundance ions are depicted in bold.

lan. This feature cannot be explained simply by deacetylation during the partial hydrolysis and certainly indicate that neutral oligosaccharides possessed significantly lower substitution with *O*-Ac than a whole heteroxylan.

The analysis of $Xylp_mAc_n$ ($m = 3$–7, $n = 0$–3) by ESI-MS/MS, according to previously published methodology,[16] showed the random location of acetyl groups in the XOS. For example, in the tetramer $Xylp_4Ac_2$ the simultaneous presence of several isomeric structures was suggested: Xyl*p*-Xyl*p*Ac-Xyl*p*-Xyl*p*Ac, Xyl*p*-Xyl*p*Ac-Xyl*p*Ac-Xyl*p* and Xyl*p*-Xyl*p*-Xyl*p*-Xyl*p*Ac_2. ESI-MS/MS data on the structures of partially acetylated XOS, presented in Table 5, indicate the random/irregular distribution of *O*-Ac along the heteroxylan backbone.

The identified acidic XOS from F2 are summarized in Tables 6 and 7. These were grouped into three main series corresponding to XOS with one MeGlc*p*A ($Xylp_mAc_n$MeGlc*p*A), two MeGlc*p*A ($Xylp_mAc_n$MeGlc*p*A_2) and XOS with one MeGlc*p*A and one Glc*p*A ($Xylp_mAc_n$MeGlc*p*AGlc*p*A). The acetylated XOS with

Table 6. Acidic xylo-oligosaccharides identified in the ESI-MS and MALDI-MS spectra of fraction F2

	Number of *O*-Ac groups (*n*)									
	0	1	2	3	4	5	6	7	8	9
$Xylp_mAc_nMeGlcpA$										
$Xylp_1Ac_n$MeGlc*p*A	363[a]	**405**[a]								
$Xylp_2Ac_n$MeGlc*p*A	495[a]	**537**[a]								
$Xylp_3Ac_n$MeGlc*p*A	627[a]	**669**[a]	711[a]							
$Xylp_4Ac_n$MeGlc*p*A	759[a]	801[a]	**843**[a]	885[a]	927					
$Xylp_5Ac_n$MeGlc*p*A		933[a]	975[a]	**1017**[a]	1059					
$Xylp_6Ac_n$MeGlc*p*A		1065[a]	1107[a]	1149[a]	**1191**[a]	1233		1317		
$Xylp_7Ac_n$MeGlc*p*A		1197	1239[a]	1281[a]	**1323**[a]	1365				
$Xylp_8Ac_n$MeGlc*p*A		1329	1371	1413	**1455**	1497				
$Xylp_9Ac_n$MeGlc*p*A			1503	1545	1587	**1629**		1713		
$Xylp_{10}Ac_n$MeGlc*p*A				1677	1719	**1761**	1803			
$Xylp_{11}Ac_n$MeGlc*p*A			1767	1809	1851	1893	**1935**	1977		
$Xylp_mAc_nMeGlcpA_2$										
$Xylp_5Ac_n$MeGlc*p*A_2				**1207**						
$Xylp_6Ac_n$MeGlc*p*A_2	1213	1255								
$Xylp_7Ac_n$MeGlc*p*A_2	1345			1471						
$Xylp_8Ac_n$MeGlc*p*A_2	1477	1519	1561							
$Xylp_9Ac_n$MeGlc*p*A_2	1609	1651	1693							
$Xylp_{10}Ac_n$MeGlc*p*A_2	1741	1783	1825	1867						
$Xylp_{11}Ac_n$MeGlc*p*A_2		1915	1957	1999						
$Xylp_{12}Ac_n$MeGlc*p*A_2	2005	2047	2089							

[a] $[M+Na]^+$ ions observed in both ESI-MS and MALDI-MS spectra; major abundance ions are depicted in bold.

Table 7. Acidic xylo-oligosaccharides with mixed uronic moieties identified in MALDI-MS spectra of fraction F2

$[M+Na]^+$	Number of *O*-Ac groups (*n*)								
	9	10	11	12	13	14	15	16	17
$Xylp_mAc_nMeGlcpAGlcpA$									
$Xylp_6Ac_n$MeGlc*p*AGlc*p*A	1577			1703					
$Xylp_7Ac_n$MeGlc*p*AGlc*p*A									
$Xylp_8Ac_n$MeGlc*p*AGlc*p*A			1925						
$Xylp_9Ac_n$MeGlc*p*AGlc*p*A	1973	2015							
$Xylp_{10}Ac_n$MeGlc*p*AGlc*p*A		2147	2189	2231					
$Xylp_{11}Ac_n$MeGlc*p*AGlc*p*A	2237	2279	2321	2363	2405				
$Xylp_{12}Ac_n$MeGlc*p*AGlc*p*A		2411	2453	2495	2537				
$Xylp_{13}Ac_n$MeGlc*p*AGlc*p*A	2501	2543	2585	2627	2669	2711			
$Xylp_{14}Ac_n$MeGlc*p*AGlc*p*A		2675	2717	2759	2801				
$Xylp_{15}Ac_n$MeGlc*p*AGlc*p*A				2891		2975		3059	
$Xylp_{16}Ac_n$MeGlc*p*AGlc*p*A				3023	3065	3107			
$Xylp_{17}Ac_n$MeGlc*p*AGlc*p*A			3113		3197				3065
$Xylp_{18}Ac_n$MeGlc*p*AGlc*p*A					3329				
$Xylp_{19}Ac_n$MeGlc*p*AGlc*p*A									
$Xylp_{20}Ac_n$MeGlc*p*AGlc*p*A						3635			

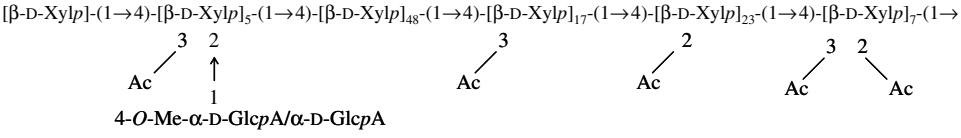
mixed uronic substituents, which was (MeGlc*p*A and Glc*p*A) of relatively low abundance, possessed a rather significant DS with *O*-Ac (>0.70) and were identified only by MALDI-MS (Table 7). These MS data confirm the results of NMR analysis recognizing the presence of both MeGlc*p*A and Glc*p*A in the heteroxylan from *Paulownia*.

A series of acidic XOS with one MeGlc*p*A residue showed the most abundant signals both in ESI-MS and in MALDI-MS spectra (Figs. 5 and 6). DP of these XOS ranged from 1 to 12 (Table 6). The analysis of the most intensive molecular ions derived from acetylated acidic XOS of the same DP (Figs. 5 and 6 and Table 6) revealed the DS with *O*-Ac of at least 0.50–0.60, that is, almost twice than that of neutral XOS. Moreover, XOS with the same DP possessed significant variation in a number of attached *O*-Ac (Table 6). For example, $Xylp_6Ac_n$MeGlc*p*A contained from 1 to 7 acetyl groups. This fact supports the random acetylation within acidic XOS. It may be also assumed that the acetylation frequency of structural XOS containing MeGlc*p*A substituents was higher that those free of MeGlc*p*A groups. As far as the distribution of MeGlc*p*A residues along the XOS backbone is concerned, this should be irregular because XOS of very disperse backbone lengths monosubstituted with MeGlc*p*A were detected (Table 6). Additionally, a short chain XOS with two acidic residues, such as $Xylp_5Ac_3MeGlcpA_2$, were identified, together with relatively long fragments with only one acidic residue, $Xylp_{11}Ac_7$MeGlc*p*A (Table 6).

The MALDI-MS spectrum of F2 contains an overlapped series of peaks belonging to XOS with one and two MeGlc*p*A ($Xylp_mAc_n$MeGlc*p*A and $Xylp_mAc_n$MeGlc*p*A_2, respectively) (Fig. 6). The signals of ions of the $Xylp_mAc_nMeGlcpA_2$ series were of much lower intensity than those of the $Xylp_mAc_n$MeGlc*p*A series and were analyzed essentially by MALDI-MS. It was difficult, sometimes, to distinguish clearly all the XOS in the $Xylp_mAc_nMeGlcpA_2$ series (especially those with high *O*-Ac content) and to select major ions. However, in general, these oligosaccharides contained a significant proportion of acetylated Xyl*p* units and showed a random distribution of MeGlc*p*A residues along the backbone, similar to XOS of the $Xylp_mAc_n$MeGlc*p*A series.

4. Conclusion

The results of the present study allow us to define the xylan from the hybrid *Paulownia elongata*/*Paulownia fortunei* as an *O*-acetyl-(4-*O*-methylglucurono)xylan with a formal structure, based on 100 Xyl*p* units, as presented below:



A small proportion of glucuronosyl residues (less than 10% from the total amounts) in the heteroxylan is not methylated.

The results of ESI-MS and MALDI-MS studies of partially hydrolyzed heteroxylan revealed the random distribution patterns of *O*-Ac and MeGlc*p*A in the back-

bone, and the occurrence of *O*-Ac residues along the backbone was not uniform. In particular, a significant proportion of XOS from xylan did not contain Me-Glc*p*A substituents and these XOS possessed relatively low acetylation degrees (DS ≈ 0.2–0.3). Some proportion of XOS did not contain any substituents at all. Simultaneously, the XOS containing MeGlc*p*A residues showed much higher DS with *O*-Ac (DS ≈ 0.6–1.0) than the neutral XOS. These facts may indicate the eventual heterogeneous structure of xylan determined either by its different morphological origin or by the biosynthesis specificity in the cell wall.

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