

# Journal of Visualized Experiments

## Sequencing of plant wall heteroxylans using enzymic, chemical (methylation) and physical (mass spectrometry, nuclear magnetic resonance) techniques.

--Manuscript Draft--

<b>Manuscript Number:</b>	JoVE53748R4
<b>Full Title:</b>	Sequencing of plant wall heteroxylans using enzymic, chemical (methylation) and physical (mass spectrometry, nuclear magnetic resonance) techniques.
<b>Article Type:</b>	Methods Article - JoVE Produced Video
<b>Keywords:</b>	cell wall; Arabinoxylan; 2 aminobenzamide; Endoxylanase; Reducing end and Internal region oligosaccharides; Methylation; Glycosyl sequence; MALDI-TOF-MS; ESI-QTOF-MS; ESI-MSn NMR
<b>Manuscript Classifications:</b>	1.11.562: Plant Cells; 4.9.698: Polysaccharides; 95.51.30: plants
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<b>Abstract:</b>	<p>This protocol describes the specific techniques used for the characterization of reducing end (RE) and internal region glycosyl sequence(s) of heteroxylans. De-starched wheat endosperm cell walls were isolated as an alcohol-insoluble residue (AIR)1 and sequentially extracted with water (W-sol Fr) and 1M KOH containing 1% NaBH4 (KOH-sol Fr) as described by Ratnayake et al., (2014)2. Two different approaches (see summary in Figure 1) are adopted. In the first, intact W-sol AXs are treated with 2AB to tag the original RE backbone chain sugar residue and then treated with an endoxylanase to generate a mixture of 2AB-labelled RE and internal region reducing oligosaccharides, respectively. In a second approach the KOH-sol Fr is hydrolyzed with endoxylanase to first generate a mixture of oligosaccharides which are subsequently labelled with 2AB. The enzymically released ((un)tagged) oligosaccharides from both W- and KOH-sol Frs are then methylated and the detailed structural analysis of both the native and methylated oligosaccharides is performed using a combination of MALDI-TOF-MS, RP-HPLC-ESI-QTOF-MS and ESI-MSn. Endoxylanase digested KOH-sol AXs are also characterized by nuclear magnetic resonance (NMR) that also provides information on the anomeric configuration. These techniques can be applied to other classes of polysaccharides using the appropriate endo-hydrolases.</p>
<b>Author Comments:</b>	
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in	

your cover letter.	
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	

**Sequencing of plant wall heteroxylans using enzymic, chemical (methylation) and physical (mass spectrometry, nuclear magnetic resonance) techniques.**

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**KEYWORDS:**

Cell wall, Heteroxylan, 2 aminobenzamide, Endoxylanase, Reducing end and Internal region oligosaccharides, Methylation, Glycosyl sequence, MALDI-TOF-MS, ESI-QTOF-MS, ESI-MS<sup>n</sup> NMR.

**SHORT ABSTRACT:**

This protocol describes the specific techniques used for the structural characterization of reducing end (RE) and internal region glycosyl sequence(s) of heteroxylans by tagging the RE with 2 aminobenzamide prior to enzymatic (endoxylanase) hydrolysis and then analysis of the resultant oligosaccharides using mass spectrometry (MS) and nuclear magnetic resonance (NMR).

**LONG ABSTRACT:**

This protocol describes the specific techniques used for the characterization of reducing end (RE) and internal region glycosyl sequence(s) of heteroxylans. De-starched wheat endosperm cell walls were isolated as an alcohol-insoluble residue (AIR)<sup>1</sup> and sequentially extracted with water (**W-sol Fr**) and 1M KOH containing 1% NaBH<sub>4</sub> (**KOH-sol Fr**) as described by Ratnayake *et al.* (2014)<sup>2</sup>. Two different approaches (see summary in Figure 1) are adopted. In the first, intact **W-sol AXs** are treated with 2AB to tag the original RE backbone chain sugar residue and then treated with an endoxylanase to generate a mixture of 2AB-labelled RE and internal region reducing oligosaccharides, respectively. In a second approach, the **KOH-sol Fr** is hydrolyzed with endoxylanase to first generate a mixture of oligosaccharides which are subsequently labelled with 2AB. The enzymically released ((un)tagged) oligosaccharides from both **W-** and **KOH-sol Frs** are then methylated and the detailed structural analysis of both the native and methylated oligosaccharides is performed using a combination of MALDI-TOF-MS, RP-HPLC-

ESI-QTOF-MS and ESI-MS<sup>n</sup>. Endoxylanase digested **KOH-sol** AXs are also characterized by nuclear magnetic resonance (NMR) that also provides information on the anomeric configuration. These techniques can be applied to other classes of polysaccharides using the appropriate endo-hydrolases.

## INTRODUCTION:

Heteroxylans are a family of polysaccharides that are the predominant non-cellulosic polysaccharides of the primary walls of grasses and the secondary walls of all angiosperms<sup>3-6</sup>. The xylan backbones differ in their types and patterns of substitution with glycosyl (glucuronic acid (GlcA), arabinose (Araf)) and non-glycosyl (O-acetyl, ferulic acid) residues depending upon tissue type, developmental stage and species<sup>7</sup>.

Walls from wheat (*Triticum aestivum* L.) endosperm are composed primarily of arabinoxylans (AXs) (70%) and (1→3)(1→4)-β-D-glucans (20%) with minor amounts of cellulose and heteromannans (2% each)<sup>8</sup>. The xylan backbone may be variously un-substituted and predominantly mono-substituted (primarily O-2 position and to a lesser extent O-3 position) and di-substituted (O-2 and O-3 positions) with α-L-Araf residues<sup>9</sup>. The reducing end (RE) of heteroxylans from dicots (for example, *Arabidopsis thaliana*)<sup>10</sup> and gymnosperms (for example, spruce (*Picea abies*))<sup>11</sup> contains a characteristic tetrasaccharide glycosyl sequence; -β-D-Xylp-(1→3)-α-L-Rhap-(1→2)-α-D-GalpA-(1→4)-D-Xylp. To understand heteroxylan biosynthesis and function (biological and industrial), it is important to fully sequence the xylan backbone to understand the types and the patterns of substitutions as well as the sequence of the reducing end (RE).

Specific techniques used for the structural characterization of reducing end (RE) and internal region glycosyl sequence(s) of heteroxylans are described in this manuscript. The techniques rely on fluorophore tagging (with 2 aminobenzamide (2AB)) the reducing end (RE) of the heteroxylan chain prior to enzymatic (endoxylanase) hydrolysis. This approach, particularly for the RE sequencing, was first reported by the York laboratory<sup>10, 12-13</sup> but is now extended to include the internal region sequencing and is a combination of established techniques that is equally adaptable to all heteroxylans independent of their source of isolation. This approach can also be applied to other classes of polysaccharides using (where available) the appropriate endo-hydrolases.

In the present study, de-starched wheat endosperm cell walls were isolated as an alcohol-insoluble residue (AIR) and sequentially extracted with water (**W-sol Fr**) and 1M KOH containing 1% NaBH<sub>4</sub> (**KOH-sol Fr**) as described in Ratnayake et al. (2014)<sup>2</sup>. The released oligosaccharides from both **W-** and **KOH-sol Frs** are then methylated and the detailed structural analysis of both the native and methylated oligosaccharides is performed using a combination of MALDI-TOF-MS, ESI-QTOF-MS-coupled with HPLC with the online chromatographic separation using a RP C-18 column and ESI-MS<sup>n</sup>. Endoxylanase digested **KOH-sol** AXs was also characterized by nuclear magnetic resonance (NMR).

## PROTOCOL:

### 1. Labelling of the reducing end (RE) sugar residue of W-sol AXs with 2-aminobenzamide (2AB)

1.1 Incubate **W-sol** AXs with 2AB (0.2M) in the presence of 1M NaBH<sub>3</sub>CN (sodium cyanoborohydride) (pH 5.5) for 2 h at 65°C to convert the reducing ends of the polysaccharide backbone chains to their fluorescent derivatives.

Note: CAUTION: The following step should be performed in the fume hood as NaBH<sub>3</sub>CN releases poisonous cyanide gas when it is in contact with water.

1.1.1 Weigh out NaBH<sub>3</sub>CN (62.8 mg) and dissolve in water (1 ml) in a microcentrifuge tube (1.5 ml) to prepare a 1M NaBH<sub>3</sub>CN solution. Dissolve 2AB reagent (27.2 mg) in 1M NaBH<sub>3</sub>CN solution (1 ml) by heating at 65°C and adjust the pH of the reaction mixture (0.2 M 2AB, 1 M NaBH<sub>3</sub>CN) to pH 5.5 with 10% acetic acid.

1.1.2 Add 200 µL of reaction mixture (0.2M 2AB, 1M NaBH<sub>3</sub>CN) to **W-sol** AXs (1 mg) in a glass tube with a cap and mix using a vortex mixer. Incubate for 2 hrs at 65°C in a fume hood. Cool the suspension to room temperature (RT) and add 4 vols. of absolute ethanol.

1.1.3 Place the suspension in a cold storage (4°C) overnight to precipitate polysaccharides.

1.1.4 Centrifuge (1500 x g, 10 min, RT) to remove supernatant. Wash the pellet extensively with absolute ethanol (x4), acetone (x1) and methanol (x1), centrifuging between each wash. Vacuum dry at 40°C overnight. Note: Extensive washing also removes residual 2AB.

### 2. Generation of xylo-oligosaccharides from 2 AB labelled W-sol AXs

2.1 Dissolve 2AB labelled **W-sol** AXs (1 mg) in 500 µl of sodium acetate buffer (100 mM, pH 5) in a microcentrifuge tube (1.5 ml). Add 4 units of endoxylanase (GH 11, [M1]) and incubate at 37°C for 16 hrs.

2.2 Destroy enzyme activity by heating the reaction mixture for 10 min in a boiling water bath. Cool the suspension to RT and transfer to glass tube with a cap. Add 4 vols. of absolute ethanol and place the suspension in a cold storage (4°C) overnight to precipitate any undigested polysaccharides.

2.3 Centrifuge (1500 x g, 10 min, RT) to separate undigested polysaccharides (pellet) and endoxylanase generated xylo-oligosaccharides (supernatant). Decant supernatant into a clean glass tube and place it into a warm water bath (40°C).

2.4 Evaporate the ethanol under a stream of nitrogen gas to an end point volume (~500 µL). Freeze the supernatant at -80°C for 4 hrs and dry the frozen supernatant in a freeze dryer to recover xylo-oligosaccharides.

### 3. Generation of xylo-oligosaccharides from KOH-sol AXs and 2AB labelling

3.1 Treat **KOH-sol** AXs with endoxylanase (GH 11, [M1]) to generate xylo-oligosaccharides as described above (sections 2.1 - 2.4).

3.2 Treat endoxylanase generated xylo-oligosaccharides from **KOH-sol** AXs with 2AB reaction mixture (0.2M 2AB, 1M NaBH<sub>3</sub>CN) as described above (sections 1.1.1 - 1.1.2).

3.3 Decant supernatant into a clean glass tube and place it into a warm water bath (40°C). Evaporate the ethanol under a stream of nitrogen gas to an end point volume (~500 µL). Freeze the supernatant at -80°C for 4 hrs and dry the frozen supernatant in a freeze dryer to recover xylo-oligosaccharides.

### 4. MALDI-TOF-MS

#### 4.1 Preparation of MALDI-matrix solution

4.1.1 Add a small scoop of 2, 5-dihydroxybenzoic acid (DHB) to 50% acetonitrile (500 ml) containing 0.1% formic acid in a tube (MALDI-matrix solution). Mix using a vortex, if it dissolves quickly, add another small scoop of DHB. Note: Ideal concentration of MALDI-matrix solution is 10 µg µl<sup>-1</sup>.

#### 4.2 Preparation of MALDI-target plate

4.2.1 Deposit the aqueous oligosaccharide (native) samples (5-10 µg) (**W-sol** and/or **KOH-sol**) onto a MALDI-target plate. Add 0.3 µl MALDI-matrix solution using a separate tip and mix by pipetting up and down. Allow the mixture to dry at RT.

Note: Properly dried samples should consist of long needle-shaped crystals pointing toward the center of the spot. If the deposit is sticky and/or smeary the sample may either be too concentrated or consist of salts- such deposits are unlikely to produce good spectra and the sample should be purified further.

4.2.2 Introduce the target plate into the MS source and operate in positive (+ive) ion mode. Adjust the accelerator voltage to 19.0 kV at ion source 1 and 16.3 kV at ion source 2. Adjust the laser power to greater than 70 %. Select the sample spot on the MALDI-target plate and click start to begin laser shots.

Note: All areas of the target will not yield signals. A particular spot will only give a signal for a few laser shots due either to depletion of the sample/matrix mixture or characteristics of the crystal.

4.2.2.1 Move the laser to different areas of the target during acquisition and average approximately 200 random spectra to obtain satisfactory signal-to-noise.

### 5. ESI-QTOF-MS

5.1. Analyze endoxylanase-generated oligosaccharides (native) using nano-HPLC coupled with an electrospray ionization (ESI) quadrupole time of flight (QTOF) MS instrument with online chromatographic separation using a RP C-18 column (75  $\mu\text{m}$   $\times$  150 mm; 3.5  $\mu\text{m}$  bead size).

5.1.1 Transfer the endoxylanase-generated aqueous oligosaccharides mixture into a vial and place into the HPLC auto sampler. Program the elution gradient of 5-80% with the mobile phases 0.1 % (v/v) formic acid in water and 0.1 % (v/v) formic acid in acetonitrile, respectively, over 60 min.

5.1.2 Adjust the flow rate to 0.2  $\mu\text{L}/\text{min}$ . Adjust the positive-ion mode in the scan range of 300 - 1600  $m/z$  and a scan-rate of 0.5 scans/second using the ESI source conditions as follows: curtain gas 10, GS1 4, source temperature 100  $^{\circ}\text{C}$ , ion spray voltage 2300 V, and de-clustering potential 50 V. Run the LC chromatographic program and elute oligosaccharides. The resultant total ion chromatogram (TIC) is saved automatically by the software.

5.1.3 Open the saved TIC and select extract chromatogram. Type the expected masses (e.g. 271, 403, 535, 667, 799 and 931  $m/z$ ) at the command line. Scan chromatogram by clicking Enter. Process the selected ion scans of the ESI-QTOF MS chromatogram data using software according to the manufacturer's instructions<sup>14</sup>.

## 6. ESI-MS<sup>n</sup>

6.1 Insert 1 to 2  $\mu\text{L}$  of per-O-methylated oligosaccharide (methylated as described by Pettolino *et al*<sup>1</sup>) sample in 50% acetonitrile into a nanospray tip using a syringe. Trim the nano-spray tip using a glass cutter to fit into the discrete nano-spray holder attached to the MS.

6.2 Set the mass according to the expected mass range (200-1500  $m/z$ ) and curtain gas to 10, ionspray voltage at 1900 V and polarity to positive.

6.3 Press the acquire button to open relevant window, enter data file name and adjust the collision energy (up and/or down in the compound tab) to obtain a total ion scan (ESI-MS<sup>1</sup>). Then press the STOP button.

6.4 Change scan type from product ion to fragment a peak of interest. Enter the mass of interest (e.g. 885  $m/z$ ) to fragment and adjust the mass range (200-900  $m/z$ ). Press the acquire button and adjust the collision energy (up and/or down in the compound tab) to achieve entire fragmentation of parent ion (885  $m/z$ ) and acquire a fragment ion scan (ESI-MS<sup>2</sup>).

6.5 Enter the mass of fragment ion of interest (e.g. 711  $m/z$ ) and adjust the mass range (200-720  $m/z$ ). Press the acquire button and adjust the collision energy to achieve entire fragmentation of precursor ion (711  $m/z$ ) and acquire a fragment ion scan (ESI-MS<sup>3</sup>).

## 7. <sup>1</sup>H NMR spectroscopy

7.1 Dissolve endoxylanase generated mixture of oligosaccharides (**KOH-sol**, native form) (~500  $\mu\text{g}$ ) in D<sub>2</sub>O (1.0 ml, 99.9%) in a plastic test tube (15 ml). Freeze the suspension at -80 $^{\circ}\text{C}$  for 4 hrs and dry the frozen suspension in a freeze dryer to recover xylo-oligosaccharides.

7.2 Repeat 7.1 twice in order to fully exchange the H<sub>2</sub>O with D<sub>2</sub>O.

7.3 Dissolve dried oligosaccharides in D<sub>2</sub>O (0.6 ml, 99.9%) and add 0.5 µl of acetone (5% in D<sub>2</sub>O) as an internal standard. Transfer the deuterated oligosaccharides into the NMR sample tube.

7.4 Hold the NMR tube containing sample by the top and insert the sample tube in a plastic spinner. Place the spinner in the sample depth gauge. Push or pull the sample tube to adjust the depth of the sample to ensure that the center line of the sample top and bottom depth gauges are equal.

7.5 Remove the depth gauge and insert the sample into the auto sampler attached to a 600 MHz NMR spectrometer equipped with a cryo-probe.

7.6 Login and open spectrometer control software. Enter the sample file name. Open an existing dataset and then use the “**edc**” command to save it to under a new name. Type the position number of the sample in the auto sampler and press ENTER.

Note: Pressing the ENTER button will drop the sample tube gently to the magnet bore where it will be positioned at the top of the probe.

7.7 Set the desired sample temperature by typing “**edte**” at the command line. Wait for the sample temperature to reach the desired value before proceeding to the next step. Enter “**lock**” at the command line and select appropriate solvent (D<sub>2</sub>O). Wait for the “**lock finished**” message to appear at the bottom of the window.

7.8 Type “**atmm**” at the command line and click “**optimize**” at the top of the atmm menu bar. Choose start for tuning and matching of the probe for the selected channel (<sup>1</sup>H in this case).

7.9 Type “**topshim**” at the command line for the shimming process in which minor adjustments are made to the magnetic field until uniform magnetic field is achieved around the sample. Acquire the signal by typing “**zg**” at the command line and enter.

7.10 Analyze spectra using software<sup>15</sup> according to the manufacturer’s instructions with <sup>1</sup>H chemical shift referenced to an internal standard of acetone at 2.225 ppm.

## REPRESENTATIVE RESULTS:

Endoxylanase digestion of 2AB-labelled **W-sol** AXs generates a mixture of 2AB-labelled RE oligosaccharides and a series of un-labeled (without 2AB label) oligosaccharides derived from the internal regions of the xylan chain (Figure 1; from Ratnayake *et al.*<sup>2</sup>). A series of chromatographic approaches is then employed to fractionate the complex mixture of isomers. Finally, MS techniques are utilized to identify the isomeric structures that are then sequenced by MS<sup>n</sup> techniques. Here we present a representative, rather than comprehensive, example of the approach.

The signals in the MALDI-TOF-MS spectrum of oligosaccharides derived from 2AB-labelled



native **W-sol** AXs (Figure 2A) include a highly abundant pseudo-molecular ion series at  $m/z$  701, 833 and 965 representing a series of unlabeled neutral internal region oligosaccharides with 5-7 pentosyl residues ( $P_{5-7}$ ), respectively. A series of signals at  $m/z$  745, 877 and 1009, designate that an unlabeled acidic oligosaccharide series, with  $P_{4-6} + \text{HexA}_1$  (Hexuronic acid), is also present in this fraction (Figure 2A). Pseudo-molecular ions at  $m/z$  821 and 953 indicate the presence of the 2AB-labelled original RE oligosaccharides  $P_{5-6} + 2\text{AB}$ , respectively.

The ESI-QTOF-MS analysis for the native oligosaccharides with an on-line chromatographic fractionation of oligosaccharides by RP C-18 HPLC is then performed. Figure 2 (B&C), shows the selected ion scans, extracted from ESI-QTOF-MS total ion chromatogram (TIC), of oligosaccharides released by endoxylanase from **W-sol Fr** AXs. The signals include a pseudo-molecular ion series assigned as  $[M + \text{NH}_4]^+$  at  $m/z$  696, 828, 960, 1092, 1224 and 1356 representing a series of internal region neutral oligosaccharides with 5-10 pentosyl residues ( $P_{5-10}$ ), respectively (Figure 2B). Several isomeric structures are possible for an oligosaccharide of a defined mass (see below ESI-MS<sup>n</sup> analysis). Hence the multiple peaks for each of the molecular ion scans are possible as observed in Figure 2B. Pseudo-molecular ions assigned as  $[M + \text{H}]^+$  at  $m/z$  271, 403, 535, 667, 799 and 931 indicate the presence of a 2AB labelled RE oligosaccharide series  $P_{1-6} + 2\text{AB}$ , respectively (Figure 2C). The signals detected as  $[M + \text{H}]^+$  ions at  $m/z$  613, 745, 877, 1009, 1141 and 1273 indicate the presence of an acidic oligosaccharides with  $P_{3-8} + \text{HexA}_1$ , respectively (Figure 2C). In the commelenid monocots, the xylan backbone can also be substituted with phenolic acids, primarily ferulic acid (and also *p*-coumaric acid), which has the same molecular mass as glucuronic acid and can be detected in **W-sol** AXs of wheat endosperm cell walls. However, further analysis of **W-** and **KOH-sol** AXs using ESI-MS<sup>n</sup> (and compositional analyses by GC-MS of TMS derivatives following methanolysis; not shown here) confirm the acidic oligosaccharides with  $P_{3-8} + \text{HexA}_1$  in wheat endosperm AXs.

The signals assigned as  $[M + \text{H}]^+$  ions of ESI-Q-TOF full scan spectrum of the region between 3.10-3.48 min (Figure 2D) includes the series of 2AB labelled RE oligosaccharides:  $m/z$  271, 403, 535, 667, 799, 931, 1063 and 1195 ( $P_{1-8} + 2\text{AB}$ , respectively). A series of pseudo-molecular ions in the ESI-Q-TOF full scan spectrum of the region between 3.59-4.05 min (Figure 2E) represent the internal region acidic oligosaccharides observed as both  $[M + \text{Na}]^+$  ions:  $m/z$  613, 745, 877, 1009 and 1141 ( $P_{3-7} + \text{HexA}_1$ , respectively) and  $[M + \text{NH}_4]^+$  ions:  $m/z$  740, 872, 1004, 1136 and 1268 ( $P_{4-8} + \text{HexA}_1$ , respectively).

In order to sequence the individual oligosaccharides, we performed ESI-MS<sup>n</sup> on the per-O-methylated oligosaccharides rather than on the native oligosaccharides obtained from **W-sol** and **KOH-sol** AXs since it is challenging to unequivocally assign structures by sequencing native oligosaccharides. In addition, it also requires greater quantities of material. Methylation of the oligosaccharides was carried out as described by Pettolino *et al.*<sup>1</sup>. The ESI-MS<sup>n</sup> investigations performed on the RE neutral oligosaccharide alditols derived from **KOH-sol** AXs, and 2AB labelled neutral RE oligosaccharide derived from **W-sol** AXs are described below as an example to assist in the interpretation of the spectra and deduced structures. The same approach can be applied to all the oligosaccharides generated from enzymic hydrolysis. The fragment ions in the ESI-MS<sup>n</sup> spectra were identified as Y and B ions according to Domon & Costello.<sup>16</sup> Un-methylated hydroxyl group(s) generated during gas-phase fragmentation of per-O-methylated oligosaccharides in MS<sup>n</sup> provides a 14Da mass difference “scar” that can be used to identify the

branching pattern and the glycosyl sequences.<sup>12-13</sup> Each scar generated by the fragmentation event is marked as a solid line (Figures 3 & 4). As several isomeric structures are possible for a defined mass, then in these isomeric structures, Y and B ions are labelled in red and black, respectively.

The ESI-MS<sup>2</sup>, ESI-MS<sup>3</sup> and ESI-MS<sup>4</sup> spectra of per-O-methylated RE neutral oligo-glycosyl alditol generated from the fragmentation of the pseudo-molecular ion  $m/z$  885 (P<sub>4</sub>+Xyl<sub>ol</sub>) is shown in Figure 3. The ESI-MS<sup>2</sup> spectrum includes the abundant Y ions at  $m/z$  711, 551 and 391 generated by the loss of one, two and three pentosyl residues, respectively, from the parent ion. The abundant  $m/z$  711 ion can be generated by either the loss of a non-reducing terminal end Xyl residue or by the loss of a terminal side chain Ara residue. The diagnostic Y  $m/z$  391 ion in the resulting spectrum can be generated from the loss of three non-reducing end Xyl residues from the RE oligosaccharide which has a side chain Ara residue on the RE Xyl<sub>ol</sub> residue or the RE oligosaccharide which has a side chain Ara residue on the 2<sup>nd</sup> Xyl residue from the RE Xyl<sub>ol</sub> residue. Although there is a formal possibility that other structures, such as Xyl<sub>4</sub>-Xyl<sub>ol</sub>, and (Ara)Xyl-Xyl-Xyl-Xyl<sub>ol</sub> would give rise to this fragment ion these structures are excluded from consideration as the specificity of the endo-xylanase used to cleave the polysaccharide would either degrade or not cleave at the glycosidic linkage adjacent to a branch point, respectively. Correspondingly, the diagnostic Y  $m/z$  551 ion can be generated from the loss of two non-reducing end Xyl residues from the RE oligosaccharide which has the side chain Ara residue on either the RE Xyl<sub>ol</sub> residue or the RE oligosaccharide which has the side chain Ara residue on the penultimate Xyl residue. Thus, four possible isomeric structures can be proposed (Figure 3: I, II, III and IV). The abundant Y ion  $m/z$  377 (see Figure 3: Ia and IIa) and B ion  $m/z$  503 (see Figure 3: Ia, Ib, IVa and IVb) generated from further fragmentation of isomeric precursor  $m/z$  711 ion are also observed in this spectrum. The ESI-MS<sup>3</sup> spectrum (Figure 3) recorded by the fragmentation of isomeric precursor  $m/z$  711 ions included a major peak at  $m/z$  537 (Y ion of P<sub>2</sub>+Xyl<sub>ol</sub> with two scars; generated from the precursor ion Ia, Ib, IIa, IIb, IIIa, IIIb, IVa and IVb) and  $m/z$  391 (Y ion of P<sub>1</sub>+ Xyl<sub>ol</sub> with one scar; generated from the precursor ion IIb, IIIa, IIIb, IVa and IVb). These two major peaks ( $m/z$  537 and  $m/z$  391) can be generated by the loss of one and two non-reducing terminal Xyl residues, respectively, from the isomeric precursor  $m/z$  711 ion generated from the fragmentation of the pseudo-molecular parent ion  $m/z$  885 (P<sub>4</sub>+ Xyl<sub>ol</sub>) during ESI-MS<sup>2</sup>. Relatively lower abundance peaks at  $m/z$  377 (Y ion of P<sub>1</sub>+ Xyl<sub>ol</sub> with two scars; generated from the precursor ion Ia and IIa) and 551 (Y ion of P<sub>2</sub>+ Xyl<sub>ol</sub> with one scar; generated from the precursor ion Ib and IIb) were also observed in this spectrum.

The ESI-MS<sup>4</sup> of the fragmentation of the isomeric precursor  $m/z$  551 ions included a major peak at  $m/z$  377 (Y ion of P<sub>1</sub>+Xyl<sub>ol</sub> with two scars) and  $m/z$  391 (Y ion of P<sub>1</sub>+ Xyl<sub>ol</sub> with one scar) generated from the precursor ion of structures I and II. Therefore the collective evidence suggested that the RE glycosyl sequence consists of the Ara side chain attached to the RE Xyl<sub>ol</sub> residue (diagnostic fragmentation pathway  $m/z$  885→711→551→391; Figure 3 II), Ara side chain attached to both RE Xyl<sub>ol</sub> and the penultimate (1<sup>st</sup> Xyl residue from RE Xyl<sub>ol</sub>) Xyl residue (diagnostic fragmentation pathway  $m/z$  885→711→537→391; Figure 3 III), Ara side chain attached to the penultimate (1<sup>st</sup> Xyl from RE Xyl<sub>ol</sub>) Xyl residue (diagnostic fragmentation pathway  $m/z$  885→711→537→377; Figure 3 I) and the Ara side chain attached on the 2<sup>nd</sup> Xyl residue from the RE Xyl<sub>ol</sub> (Figure 3 IV).

The presence of these ions confirms the proposed isomeric structures I, II, III and IV and the

neutral RE oligosaccharide structure of:  $-\text{[Araf-(1}\rightarrow\text{3)]}_{(+/-)}\text{-Xylp-(1}\rightarrow\text{4)-[Araf-(1}\rightarrow\text{3)]}_{(+/-)}\text{-Xylp-(1}\rightarrow\text{4)-[Araf-(1}\rightarrow\text{3)]}_{(+/-)}\text{-Xylp}$ .

The ESI-MS<sup>2</sup> spectrum of per-O-methylated 2AB labelled RE oligosaccharide generated from the fragmentation of the quasi-molecular  $[\text{M}+\text{Na}]^+$  ion at  $m/z$  871 ( $\text{P}_4+2\text{AB}$ ) is shown in Figure 4. This spectrum includes the most abundant Y ion at  $m/z$  697 ( $\text{P}_3+2\text{AB}$  with one scar),  $m/z$  537 ( $\text{P}_2+2\text{AB}$  with one scar) and  $m/z$  377 ( $\text{P}_1+2\text{AB}$  with one scar), generated by the loss of either one, two or three non-reducing pentosyl residues, respectively. The  $m/z$  697 ion can be generated by either the loss of a non-reducing terminal end Xyl residue or by the loss of a terminal Ara residue whereas the  $m/z$  537 ion can only be generated by the loss of two terminal Xyl residues. The diagnostic fragmentation pathway ( $m/z$  871 $\rightarrow$ 697 $\rightarrow$ 537 $\rightarrow$ 377) suggested that the existence of linear un-branched xylan backbone oligosaccharide(s) at the RE corresponding to the quasi-molecular ion  $m/z$  871 ( $\text{P}_4+2\text{AB}$ ). However the linear un-branched xylosyl backbone ( $\text{P}_4+2\text{AB}$ ) is susceptible to site specific endoxylanase for further digestion. Therefore two isomeric  $m/z$  697 ions can exist. Accordingly two possible isomeric structures are proposed (Figure 4: I and II). In these isomeric structures Y and B ions are labelled in red and black, respectively. The ESI-MS<sup>3</sup> spectrum recorded from the fragmentation of isomeric precursor  $m/z$  697 ion generates  $m/z$  523 ion (Y ion of  $\text{P}_2+2\text{AB}$  with two scars; Figure 4, structures Ia, Ib, IIa and IIb),  $m/z$  363 ion (Y ion of  $\text{P}_1+2\text{AB}$  with two scars; Figure 4, structure IIa) and Y ion at  $m/z$  377 ( $\text{P}_1+2\text{AB}$  with one scar; Figure 4, structures Ia and Ib). The fragment ion at  $m/z$  377 can only arise from the proposed structure I and the fragment ion at  $m/z$  363 can only arise from the proposed structure II. The simultaneous presence of these two ions confirms the proposed structures I and II. Thus the RE glycosyl sequence of the xylan chain of wheat endosperm AXs consist of an Ara branch attached to the RE Xyl residue (fragmentation pathway  $m/z$  871 $\rightarrow$ 697 $\rightarrow$ 523 $\rightarrow$ 363) and/or penultimate Xyl residue (fragmentation pathway  $m/z$  871 $\rightarrow$ 697 $\rightarrow$ 523 $\rightarrow$ 377).

### NMR analysis of the AXs

MS-based analyses do not provide information on either the anomeric configuration ( $\alpha/\beta$ ) or the D/L configuration of the sugars that must be obtained by other approaches, including enzymic and physical (e.g. NMR). For heteroxylans with the characteristic RE reduced tetrasaccharide ( $\text{Xyl-Rha-GalA-Xyl}_0$ ), the NMR spectrum contains anomeric signals leading to the identification and sequencing of this RE oligosaccharide. We describe the use of 600-MHz 1D <sup>1</sup>H-NMR spectroscopy as a single step method to determine the complete glycosyl sequence of the wheat endosperm AX RE oligosaccharide on the **KOH-sol Fr**, including the anomeric configuration ( $\alpha/\beta$ ) and the D/L configuration of the sugars. Resonances were assigned on the basis of published assignments of wheat AX oligosaccharides<sup>17-18</sup> (Figure 5, Table 1). The <sup>1</sup>H-NMR spectrum of the AX extracted from wheat endosperm is dominated by the anomeric chemical shifts at 5.39, 5.27 and 5.22 ppm that are assigned to the proton of a terminal  $\alpha$ -L-Araf residue, attached to O-3 position ( $\text{T-}\alpha\text{-L-Araf}\rightarrow\text{3}^{\text{S}}$ ) of the singly branched (1,4)- $\beta$ -Xylp backbone residues and both O-3 and O-2 positions ( $\text{T-}\alpha\text{-L-Araf}\rightarrow\text{3}^{\text{D}}$  and  $\text{T-}\alpha\text{-L-Araf}\rightarrow\text{2}^{\text{D}}$ ) of doubly branched (1,4)- $\beta$ -Xylp backbone residues, respectively (Figure 5 and Table 1).

The signals at 5.41 are assigned to the ( $\text{T-}\alpha\text{-L-Araf}\rightarrow\text{3}^{\text{S+D}}$ ) H1 signal of  $\alpha$ -L-Araf side chain attached to the O-3 position of the singly branched  $\beta$ -D-Xylp residue with adjoining doubly branched  $\beta$ -D-Xylp. The signal at 5.29 is assigned to the ( $\text{T-}\alpha\text{-L-Araf}\rightarrow\text{3}^{\text{D+D}}$ ) H1 signal of  $\alpha$ -L-

Araf side chain attached to the O-3 position of the doubly branched  $\beta$ -D-Xylp residue with adjoining doubly branched  $\beta$ -D-Xylp. The signal at 5.24 is assigned to the (T- $\alpha$ -L-Araf $\rightarrow$ 2<sup>D+D</sup>) H1 signal of  $\alpha$ -L-Araf side chain attached to the O-2 position of the doubly branched  $\beta$ -D-Xylp residue with adjoining doubly branched  $\beta$ -D-Xylp.

### Figure Legends:

**Figure 1.** Summary of Experimental Approach. A summary of the strategy employed in generating, purifying and sequencing the reducing end (RE) and internal region oligosaccharides of wheat endosperm arabinoxylans (AXs) is shown. This figure has been reproduced with permission from Ratnayake *et al* (2014)<sup>2</sup>.

**Figure 2.** MALDI-TOF MS (A) and ESI-QTOF MS (B-E) analysis of native oligosaccharides released by endoxylanase from 2AB labelled **W-sol** AXs as outlined in Figure 1.

MALDI-TOF MS spectrum: (A) (The signals are identified as  $[M+Na]^+$  adduct ions); Selected ion scans of the ESI-QTOF MS chromatograms: B = P<sub>5-10</sub> derived from internal region oligosaccharides (The signals are identified as  $[M + NH_4]^+$  adduct ions); C = P<sub>1-6</sub>+2AB derived from RE oligosaccharides (The signals are identified as  $[M+H]^+$  adduct ions) & P<sub>3-8</sub>G derived from acidic oligosaccharides (The signals are identified as  $[M+Na]^+$  adduct ions); D = ESI-QTOF full scan spectrum: region between 3.10-3.48 min, E = ESI-QTOF full scan spectrum: region between 3.59-4.05 min (The signals are identified as both  $[M+Na]^+$  and  $[M + NH_4]^+$  adduct ions); P = pentosyl unit (either Ara or Xyl); G = uronosyl residue (GlcA); RE = reducing end oligosaccharides; 2AB = 2 aminobenzamide; EIC: extracted ion chromatogram.

**Figure 3.** The ESI-MS<sup>2</sup>, ESI-MS<sup>3</sup> and ESI-MS<sup>4</sup> spectra of per-O-methylated RE neutral glycosyl alditol (P<sub>4</sub>+Xyl<sub>01</sub>) -  $m/z$  885. The signals are assigned as the  $[M + Na]^+$  pseudo-molecular ion adducts. As several isomeric structures for a defined mass are possible then in isomeric structures Y and B ions are labelled in red and black, respectively. Each “scar” generated by the fragmentation event is marked as a solid line. X = Xylosyl residue; A = Arabinosyl residue. The ESI-MS<sup>3</sup> spectra has been reproduced with permission from Ratnayake *et al* (2014)<sup>2</sup>.

**Figure 4.** The ESI-MS<sup>2</sup> and ESI-MS<sup>3</sup> spectra of per-O-methylated 2AB labelled neutral RE oligosaccharide (P<sub>4</sub>+2AB) -  $m/z$  871. The signals are assigned as the  $[M + Na]^+$  pseudo-molecular ion adducts. As several isomeric structures for a defined mass are possible in isomeric structures, Y and B ions are labelled in red and black respectively. Each “scar” generated by the fragmentation event is marked as a solid line. X = Xylosyl residue; A = Arabinosyl residue. This figure has been reproduced with permission from Ratnayake *et al* (2014)<sup>2</sup>.

**Figure 5.** Anomeric region of the 600-MHz 1D <sup>1</sup>H-NMR spectrum of the AX oligosaccharides generated by endoxylanase treatment of the **KOH- Sol Fr.** <sup>1</sup>H chemical shift referenced to an internal standard of acetone at 2.225 ppm. T- $\alpha$ -L-Araf $\rightarrow$ 3<sup>S</sup>: H1 signal of  $\alpha$ -L-Araf side chain attached to the O-3 position of the singly branched  $\beta$ -D-Xylp residue; T- $\alpha$ -L-Araf $\rightarrow$ 2<sup>D</sup>: H1 signal of  $\alpha$ -L-Araf side chain attached to the O-2 position of the doubly branched  $\beta$ -D-Xylp residue; T- $\alpha$ -L-Araf $\rightarrow$ 3<sup>D</sup>: H1 signal of  $\alpha$ -L-Araf side chain attached to the O-3 position of the doubly branched  $\beta$ -D-Xylp residue; T- $\alpha$ -L-Araf $\rightarrow$ 3<sup>S+D</sup>: H1 signal of  $\alpha$ -L-Araf side chain attached to the O-3 position of the singly branched  $\beta$ -D-Xylp residue with adjoining doubly branched  $\beta$ -D-Xylp;

T- $\alpha$ -L-Araf $\rightarrow$ 2<sup>D+D</sup>: H1 signal of  $\alpha$ -L-Araf side chain attached to the O-2 position of the doubly branched  $\beta$ -D-Xylp residue with adjoining doubly branched  $\beta$ -D-Xylp; T- $\alpha$ -L-Araf $\rightarrow$ 3<sup>D+D</sup>: H1 signal of  $\alpha$ -L-Araf side chain attached to the O-3 position of the doubly branched  $\beta$ -D-Xylp residue with adjoining doubly branched  $\beta$ -D-Xylp; 2- $\alpha$ -L-Araf $\rightarrow$ 3<sup>S</sup>: H1 signal of 2- $\alpha$ -L-Araf side chain residue attached to the O-3 position of the singly branched  $\beta$ -D-Xylp residue;

**Table 1:** <sup>1</sup>H-NMR signals of the xylo-oligosaccharides generated by endoxylanase treatment of the wheat endosperm KOH-sol Fr.

## DISCUSSION:

Most matrix phase cell wall polysaccharides have seemingly randomly substituted backbones (with both glycosyl and non-glycosyl residues) that are highly variable depending upon the plant species, developmental stage and tissue type<sup>3</sup>. Since polysaccharides are secondary gene products their sequence is not template derived and there is therefore no single analytical approach, such as exists for nucleic acids and proteins, for their sequencing. The availability of purified linkage-specific hydrolytic enzymes has provided a powerful tool to degrade polysaccharides to oligosaccharides that can then be chromatographically fractionated, and when used in combination with chemical and physical techniques completely sequenced. The challenge is to then re-assemble these complex mixtures into the original polysaccharide sequence- one that is still to be successfully addressed.

Here we describe an approach (whose order of application can be varied) that relies on the integration of established enzymic, chemical and physical techniques for the structural characterization of both the reducing end (RE) and internal region glycosyl sequence(s) of heteroxylans. An additional complementary technique not described here that has proved very useful for characterizing oligosaccharides is PACE (polysaccharide analysis by carbohydrate gel electrophoresis) developed by the Dupree<sup>19</sup> group and it could easily be integrated into this protocol if the equipment is available. Furthermore, variations on the LC chromatography can also be useful, such as tandem inline hydrophilic interaction chromatography (HILIC) followed by RP chromatography offering the possibility of separating both untagged/tagged oligosaccharides in a single step. The techniques rely on tagging (with 2 aminobenzamide (2AB)) the reducing end (RE) of the heteroxylan chain prior to enzymatic (endoxylanase) hydrolysis. Two different approaches (see summary in Figure 1) are adopted. In the first, intact **W-sol** AXs are treated with 2AB to tag the original RE backbone chain sugar residue and then treated with an endoxylanase to generate a mixture of 2AB-labelled RE and internal region reducing oligosaccharides, respectively. In a second approach the **KOH-sol Fr** is hydrolyzed with endoxylanase to first generate a mixture of oligosaccharides which are subsequently labelled with 2AB. In this latter scenario the original RE of the **KOH-sol** AX would not be labelled with 2AB since it had been reduced to the glycosyl-alditol during the alkali extraction that contained the reductant, sodium borohydride (NaBH<sub>4</sub>). Therefore, the 2AB-labelled oligosaccharides generated post-xylanase digestion, will originate from “internal” oligosaccharides and the original RE oligosaccharide will contain a RE alditol without a 2AB tag (see Figure 1). This approach can also be applied to other classes of polysaccharides using (where available) the appropriate endo-hydrolases.

The MS-based approach is significantly enhanced by methylation of the oligosaccharides generated after endoxylanase treatment since the un-methylated hydroxyl group(s) generated during gas-phase fragmentation of per-O-methylated oligosaccharides in MS<sup>n</sup> provides a 14Da mass difference “scar” that can be used to assist in the identification of the branching pattern and the glycosyl sequence.<sup>5-6</sup> The identity of the pentosyl residues (and any sugar residue) cannot be made from the MS data alone but comes from having a knowledge of the composition of the molecule; where this is not available then the relevant monosaccharide and linkage analyses must be performed prior to making these assignments in MS<sup>n</sup>. Furthermore the signals corresponding to the RE acidic oligosaccharide alditol, generated from **KOH-sol Fr** (Xyl<sub>3</sub>-MeGlcA-Xylitol: *m/z* 761) and the characteristic dicot xylan RE glycosyl sequence (Xyl<sub>2</sub>-Rha-GalA-Xylitol: *m/z* 761), if present, are not able to be distinguished as both have the same molecular mass in native form but can be distinguished from their MS fragmentation (MS<sup>n</sup>) spectra which is best performed on the methylated oligosaccharides. Finally, MS-based techniques are unable to provide information on either the anomeric configuration ( $\alpha/\beta$ ) of the glycosidic linkage or the D/L configuration of the sugars- this must be determined by alternate methods, including enzymic and physical (e.g. NMR).

## Acknowledgments

This project was supported by funds from Commonwealth Scientific and Research Organisation Flagship Collaborative Research Program, provided to the High Fibre Grains Cluster via the Food Futures Flagship. AB also acknowledges the support of an Australia Research Council (ARC) grant to the ARC Centre of Excellence in Plant Cell Walls (CE110001007).

**Disclosures:** We have nothing to disclose.

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Figure 1

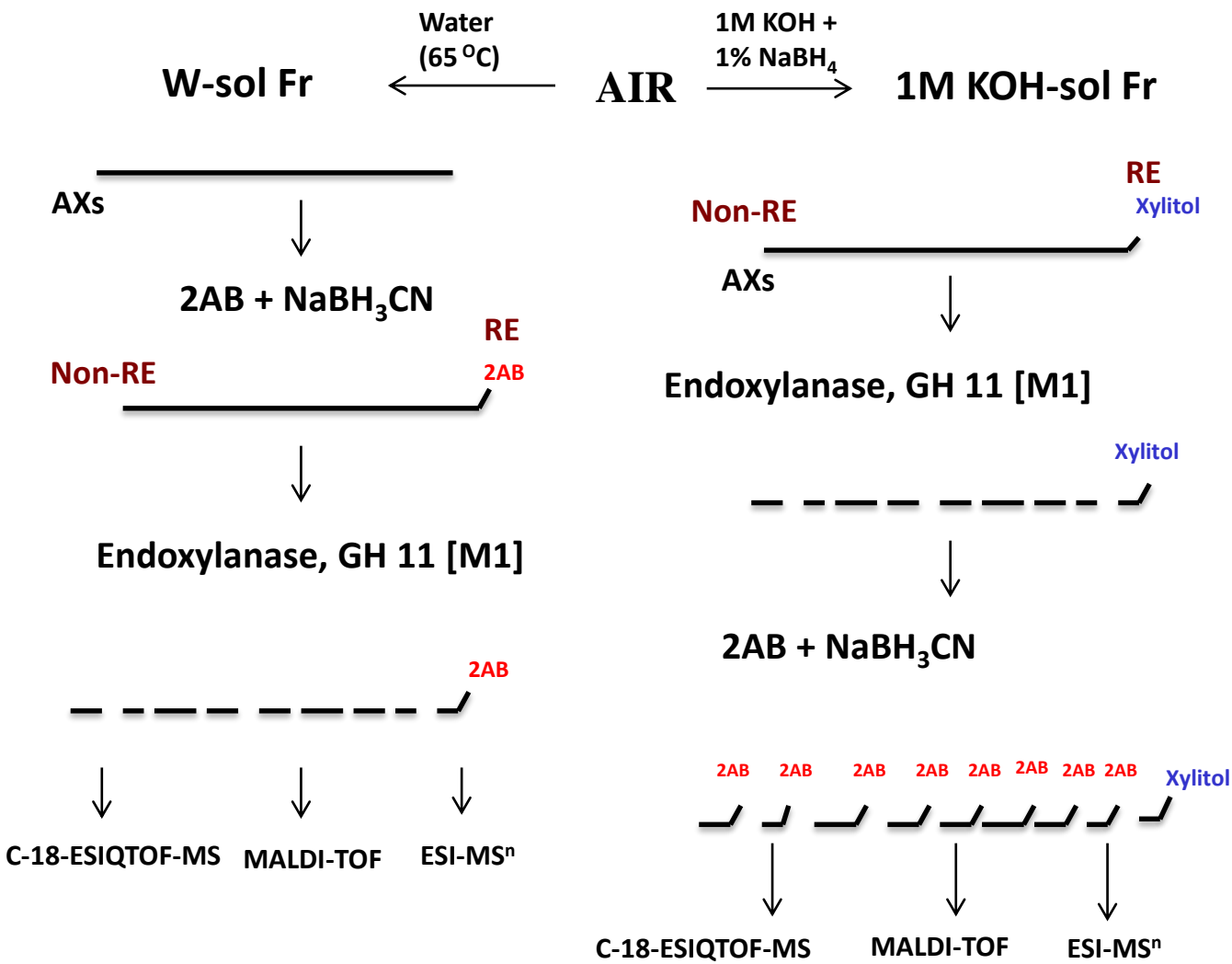




Figure 2  
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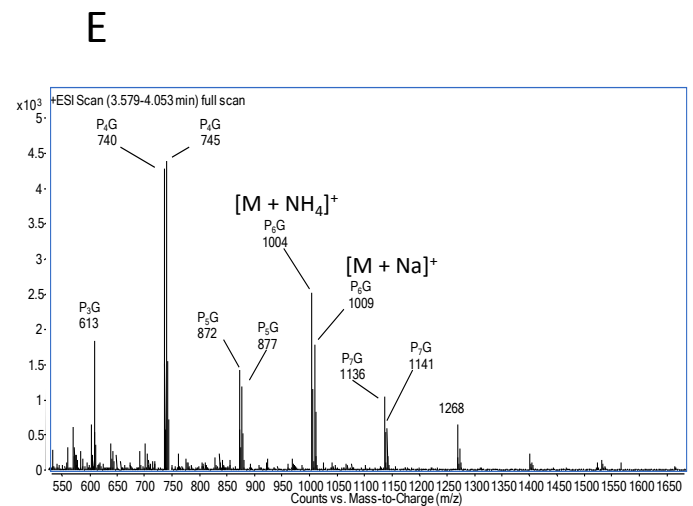
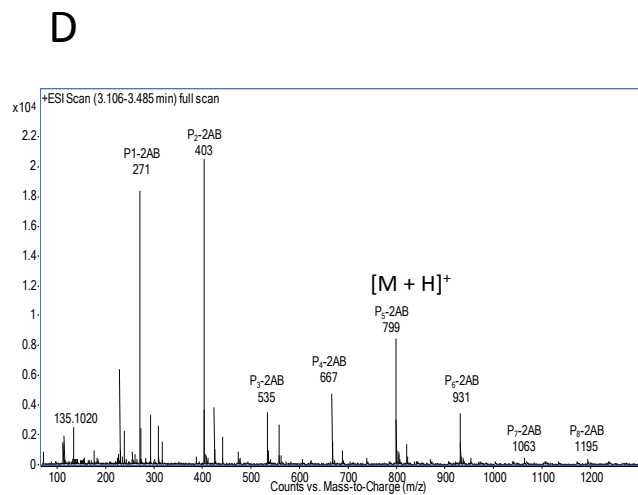
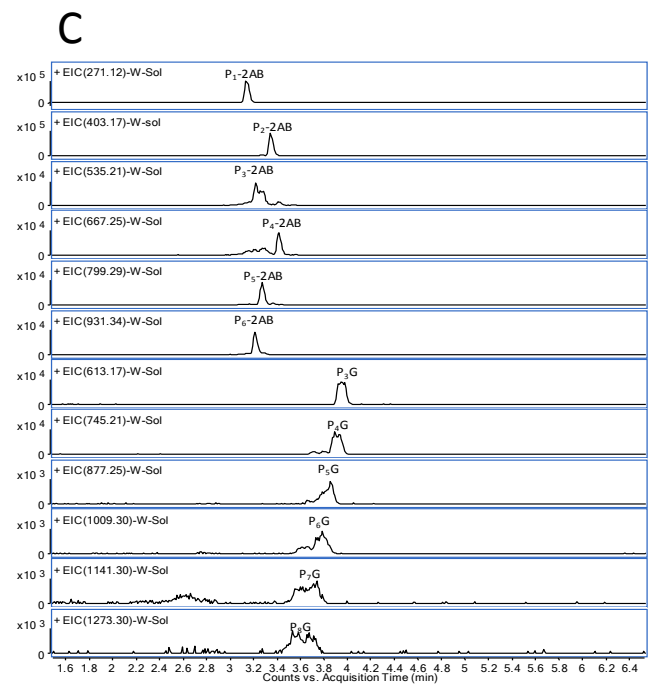
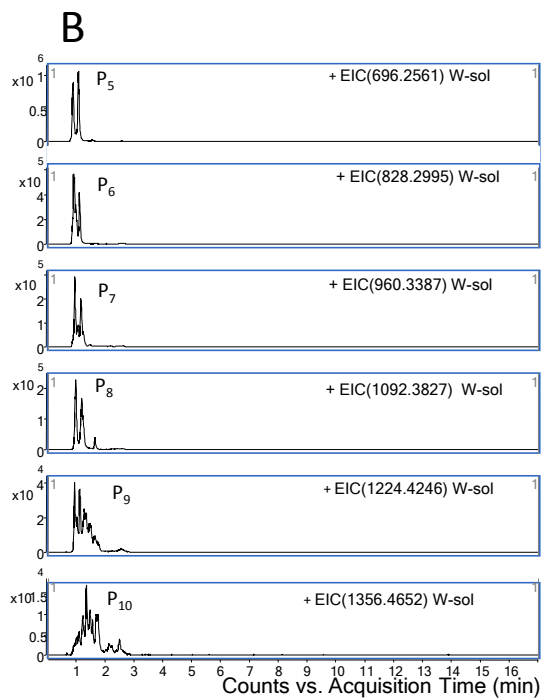
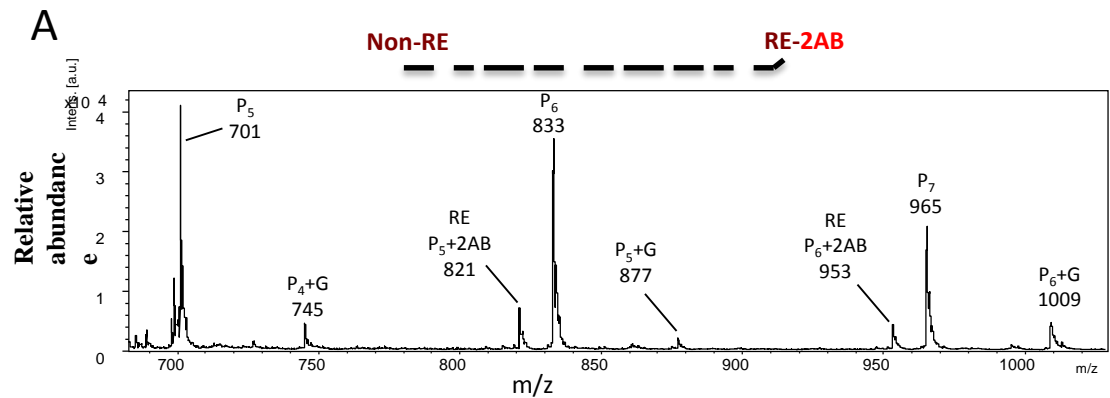




Figure 4

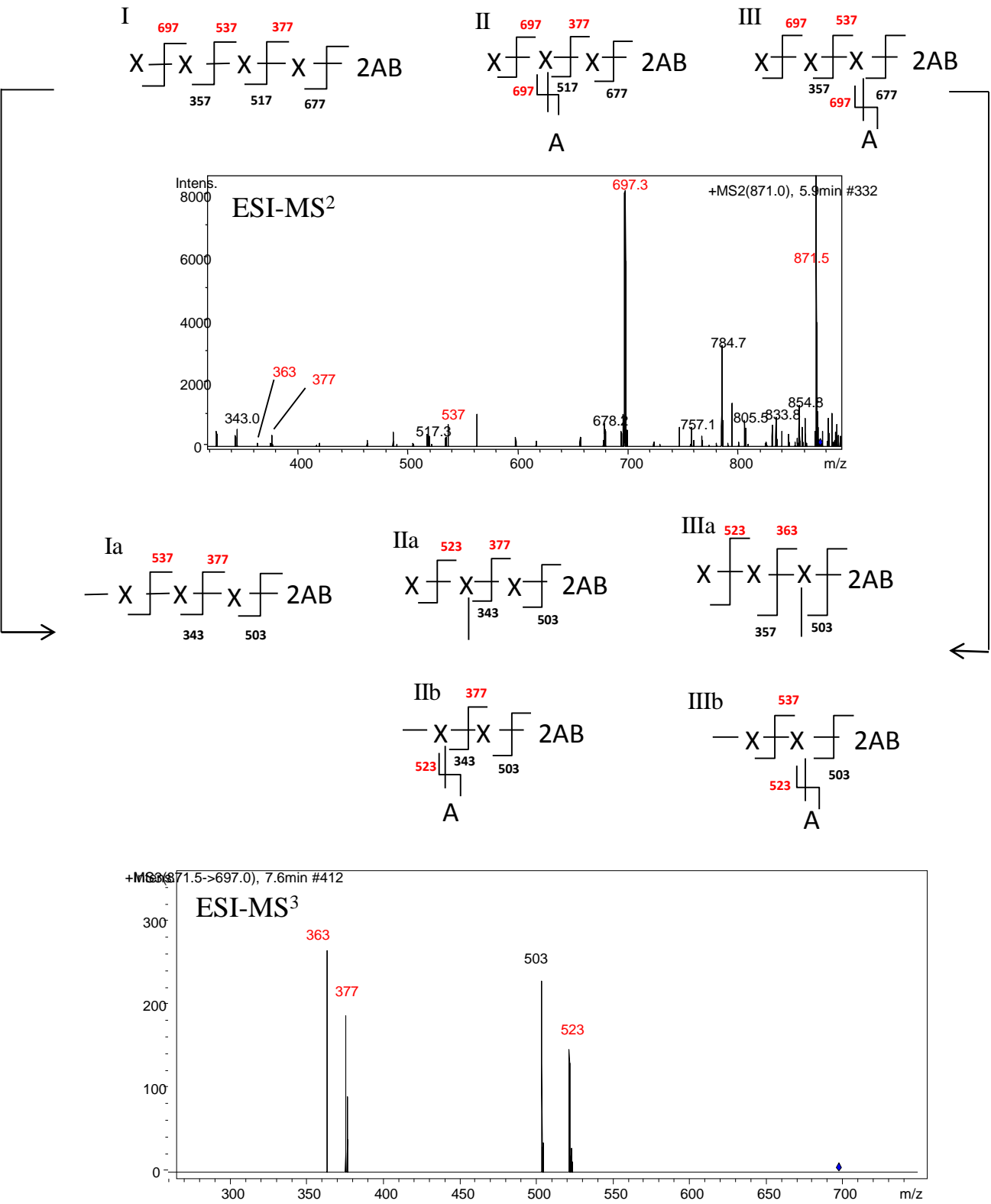


Figure 5

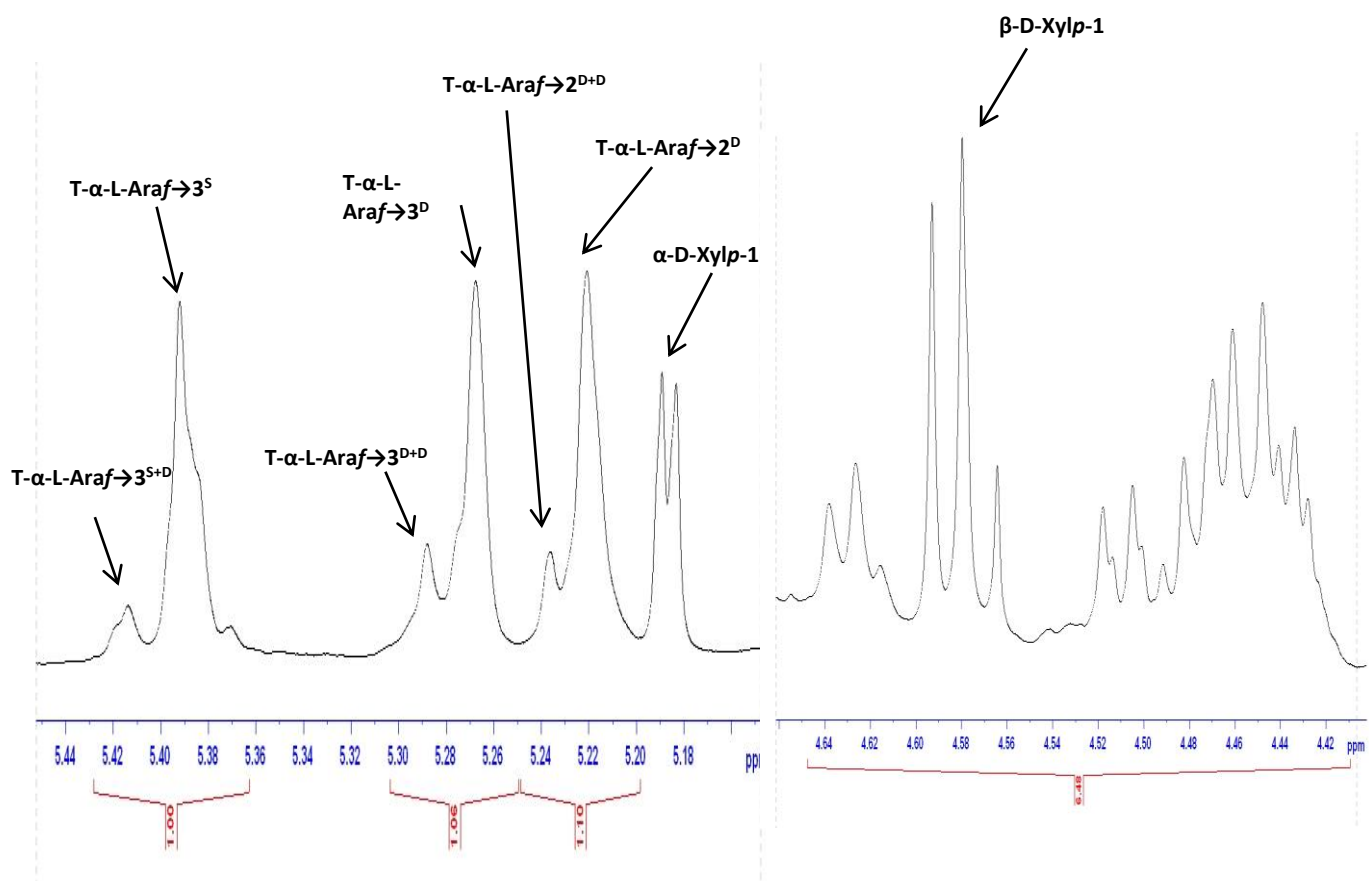


Table 1. <sup>1</sup>H-NMR signals of the xylo-oligosaccharides generated by endoxylanase treatment of the wheat

Sugar residues	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5 <sub>eq</sub> /C-5	H-5 <sub>ax</sub> /C-5
T-α-L-Araf → 3 <sup>S</sup>	5.396/107.6	4.16	3.95	4.3	3.82	3.72
T-α-L-Araf → 3 <sup>S+D</sup>	5.415/	4.18	3.95			
T-α-L-Araf → 2 <sup>D</sup>	5.223/	4.16	3.97		3.82	3.74
T-α-L-Araf → 2 <sup>D+D</sup>	5.243/	4.16	3.98			
T-α-L-Araf → 3 <sup>D</sup>	5.272/108.9	4.18	3.96	4.26	3.79	3.74
T-α-L-Araf → 3 <sup>D+D</sup>	5.298/	4.18	3.96			
2-α-L-Araf → 3 <sup>S</sup>	5.548/106.4	4.27	4.06	4.3	3.82	
α-Xylp (Reducing)	5.185/91.9	3.55	3.72			
β-Xylp (Reducing)	4.580/96.6	3.29	3.48	3.64	4.06	3.38
β-4-Xylp	4.470/	3.32	3.58			
β-4-Xylp <sup>+S</sup>	4.461	3.31	3.56	3.75	4.08	3.36
β-4-Xylp <sup>+D</sup>	4.448	3.31	3.56	3.75	4.08	3.36
β-3,4-Xylp	4.518/	3.44		3.85		
<sup>S+</sup> β-3,4-Xylp	4.514/	3.47	3.75	3.86	4.14	3.43
<sup>D+</sup> β-3,4-Xylp	4.505					
β-3,4-Xylp <sup>+S</sup>	4.492	3.45	3.74			
β-3,4-Xylp <sup>+D</sup>	4.482	3.45	3.74			
β-2,3,4-Xylp	4.638					
<sup>S+</sup> β-2,3,4-Xylp	4.627	3.59	3.87	3.88		
<sup>D+</sup> β-2,3,4-Xylp	4.616					
β-2,3,4-Xylp <sup>+S</sup>	4.593					
β-2,3,4-Xylp <sup>+D</sup>	4.593					

Chemical shifts are reported relative to internal acetone, δ 2.225.

<sup>S</sup> = Singly branched β-Xylp

<sup>S+D</sup> = Singly branched β-Xylp + Doubly branched β-Xylp

<sup>D</sup> = Doubly branched β-Xylp

<sup>D+D</sup> = Doubly branched β-Xylp + Doubly branched β-Xylp

endosperm KOH-sol Fr.

Table of specific Materials / Equipments:

Name of the Materila/equipments	Company	Catalog Number	Comments
2 aminobenzamide (2AB)	Sigma-Aldrich (www.sigmaaldrich.com)	A89804	
sodium borohydride (NaBH <sub>4</sub> )	Sigma-Aldrich (www.sigmaaldrich.com)	247677	Hazardous, handle with care
sodium cyanoborohydride (NaBH <sub>3</sub> CN)	Sigma-Aldrich (www.sigmaaldrich.com)	156159	Hazardous, handle with care
endo-1,4-β-Xylanase M1 (from Trichoderma viride) (120101a)	Megazyme (www.megazyme.com)	E-XYTR1	
Deuterium Oxide (D <sub>2</sub> O)	Sigma-Aldrich (www.sigmaaldrich.com)	151882	
Freeze dryer (CHRIST-ALPHA 1-4 LD plus)			
RP C18 Zorbax eclipse plus column	Agilent		(2.1×100 mm; 1.8 μm bead size)
MicroFlex MALDI-TOF MS (Model - MicroFlex LR)	(Bruker Daltonics, Germany)		
(ESI) -(QTOF) MS (Model # 6520)	(Agilent, Palo Alto, CA )		
ESI-MS <sup>n</sup> - ion-trap (Model # 1100 HCT)	(Agilent, Palo Alto, CA).		
Bruker Avance III 600 MHz -NMR	Bruker Daltonics, Germany		
Topspin (version 3.0)-Biospin- software	Bruker		
GC-MS (Model # 7890B)	Agilent		



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Article Title:

SEQUENCING OF PLANT WALL HETEROXYLANS USING ENZYMIC, CHEMICAL (METHYLATION) AND PHYSICAL (MASS SPECTROMETRY, NUCLEAR MAGNETIC RESONANCE) TECHNIQUES.

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22<sup>nd</sup> Sep 2015

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Dear Editor,

**Re: Sequencing of plant cell wall heteroxylans using enzymic, chemical (methylation) and physical (mass spectrometry, nuclear magnetic resonance) techniques by Ratnayake S & Bacic A.**

The manuscript has been revised accordingly to the editorial and reviewers' comments, with the changes that address each of the comments typed in red.

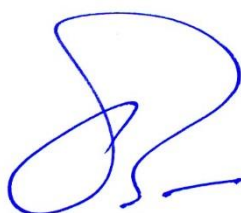
The major concerns of all three reviewers can be summarized as follows:

- insufficient explanation of how each analysis contributes to the delineation of the structure of the heteroxylan; and
- lack of mass fragmentation analysis on the oligosaccharides (including structural isomers) separated by chromatography system.

We believe both these major concerns have now been addressed, as well as most of the others raised by the reviewers - see responses below in red. Furthermore, results of an additional fragmentation analysis of the per-O-methylated fluorophore tagged RE oligosaccharides have been included in the manuscript.

We trust you will now find the revised manuscript acceptable for publication.

Regards,



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## Editorial comments:

1) All of your previous revisions have been incorporated into the most recent version of the manuscript. In addition, Editor may have made formatting changes and minor copy edits to your manuscript. On the JoVE submission site, you can find the updated manuscript under "file inventory" and download the microsoft word document. **Please use this updated version for any future revisions and track all changes using the track changes function in Microsoft Word.**

2) The following commercial language was removed from the protocol: "Eppendorf tube" was replaced with "microcentrifuge tube".

Au Response: OK

3) Grammar/Formatting:

-1.1.4/2.4/2.6/4.2.2/6.4/7.9/7.13/7.14/ – Please correct the run on sentence.

Au Response: Done.

4) Additional detail is required for 2.6 – What temperature is the water bath? “Warm” is not specific.

Au Response: A specific temperature value (40C) has been added.

5) Please adjust the highlighting to identify 2.75 pages or less of text (which includes headings and spaces) that should be visualized to tell the most cohesive story of your protocol steps. We suggest filming only one of the MS analyses. Also note that some of your protocol steps may be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

Au Response: Done, Some protocol steps have been combined as requested. The color of the text has been changed to red to highlight protocol steps.

6) All figure legends should have a title and a brief description in the legend (Figure 1 does not.)

Au Response: Done.

7) Discussion: What is the significance of this technique with respect to other methods?

Au Response: this has been addressed in the revised “Discussion”.

8) JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Au Response: Done.

9) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammatical errors. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Au Response: Done

10 ) Please disregard the comment below if all of your figures are original.

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Au Response: Permission to re-use the following figures has been obtained:

1. Figure 1 has been taken from Ratnayake *et al* (2014)<sup>2</sup>, Figure 1.
2. The ESI-MS<sup>3</sup> spectra has been taken from Ratnayake *et al* (2014)<sup>2</sup>, part of Figure 5.
3. Figure 4 has been taken from Ratnayake *et al* (2014)<sup>2</sup>, Supplemental Figure S3.

Permission correspondence is attached as supplementary files.

## Reviewers' comments:

### Reviewer #1:

#### *Manuscript Summary:*

Structural study of plant polysaccharide is important for the understanding about cell wall biosynthesis, structure and function, and their degradation. However, it has been a great challenge due to structural heterogeneities in plant polysaccharides that are delineated according to species, tissue types and developmental phases. The present study describes the structural studies of xylooligosaccharides that were enzymatically released from the wheat arabinoxylan primarily using mass spectrometry, and further studied with nuclear magnetic resonance spectroscopy. The major impact of this study lies on the modification of oligosaccharide reducing end by either tagging a fluorophore or reduction to alditol for characterizing the internal or reducing end glycosyl sequence. The detailed description of the fluorophore tagging and its product cleanup will be valuable since this method is increasingly used in characterizing plant derived oligosaccharides. Moreover, the derivatized oligosaccharides can be separated on a chromatography system to distinguish isomeric structures.

#### *Major Concerns:*

There is a major drawback on the work flow of this methodological paper that the mass fragmentation analysis was not performed on the oligosaccharides separated by chromatography system, and instead the per-O-methylated oligosaccharides that introduced to an ESI-ITMS by direct diffusion were analyzed. Mass fragmentation analysis on the per-O-methylated oligosaccharides has been widely described and the method is known. In addition, the deduced structures can be a mixture of isomers. Therefore, this work can be more valuable if examples on the fragmentation analysis of those column separated oligosaccharides are shown. Additionally, the discussion can also be more informative if the results on fragmentation analysis of the fluorophore tagged and per-O-methylated oligosaccharides are compared.

Au response: Endoxylanase digestion of wheat heteroxylan resulted in xylo-oligosaccharides comprising up to 12 pentosyl units (DP 12, unpublished data). Structural heterogeneity of these xylo-

oligosaccharides includes a large number of isomeric structures. There is no single chromatographic system that can separate these complex xylooligosaccharides and this is the underlying motivation for developing and describing the approach, a combination of enzymic, chemical and physical, we have outlined in this manuscript. The online RP C-18 column allowed classes of oligosaccharides to be fractionated which could be identified from the QTOF-MS extracted ion chromatogram analyses which recognises the individual oligosaccharides (Figure 2 B&C). The complete characterization of these individual oligosaccharides can then be achieved by the isolation and fragmentation of interested oligosaccharides using ESI-MS<sup>n</sup> experiments as described. The results of an additional fragmentation analysis of the per-O-methylated fluorophore tagged RE oligosaccharides have now been included in the 'Representative Results' to demonstrate the utility of this use.

*Minor Concerns:*

P2 L55: primary wall and secondary wall of grasses?

Au response: Correct and this is already implied in the sentence. No change required.

P2 L98: How much oligosaccharides were tagged with 2AB since 'quantitatively convert' was mentioned?

Au response: 1 mg was used and is now included. All those chains with a reducing end will be reduced. We have removed "quantitatively" as we are not able to make this measurement.

P3 L100: Sodium cyanoborohydride will release poisonous cyanide gas when it is in contact with water. Thus, a safety precaution should be highlighted.

Au response: a safety message is included.

P3 L106: Is mixing needed?

Au Response: yes and now added to protocol.

P4 L134: How is the cleanup procedure for the 2AB-tagged oligosaccharides? Sections 1.1.5 described the cleanup of 2AB-tagged polymeric xylans using 4 volume ethanol. Are the cleanup procedures different for oligosaccharide compared to the polymer?

Au Response: Thank you for picking up this error. The cleanup procedure for oligosaccharides has been revised (Section 3.2) as it is different to that required for the polymer.

P4 L135: (Section 1.1.1-1.1.5)

Au Response: The cleanup procedure for oligosaccharide has been revised.

P4 L136: Method for per-O-methylation of xylooligosaccharides should be included.

Au Response: this is exhaustively described in Pettolino et al (2012)- reference "1" and this has now been referenced.

P8 L296: Product ion m/z 391 can be derived from linear Xyl<sub>4</sub>-Xyl-ol or (Ara)Xyl-Xyl-Xyl-Xyl-ol or Xyl-(Ara)Xyl-Xyl-Xyl-ol. Why they are not determined as the possible structures?

Au Response: The structure (Ara)Xyl-Xyl-Xyl-Xyl-ol cannot be excluded based upon the MS<sup>n</sup> analyses, however, it is not a possibility as the specificity of the endoxylanase enzyme does not generate oligosaccharides without an unsubstituted pentosyl residue at the non-reducing end.



The linear structure Xyl<sub>4</sub>-Xyl-ol is also a formal possibility, however, this oligosaccharide would be further digested by the endo-xylanase to Xyl<sub>(1-3)</sub>-Xyl-ol oligosaccharides. Therefore it would not be detected and thus was not considered as a possible structure.

The structure Xyl-(Ara)Xyl-Xyl-Xyl-ol is possible and is included in Figure 3.

P10 L395: 'The complete structural characterization.....' At this point, the column separated oligosaccharides were not analyzed further with mass fragmentation analysis to identify the possible structure. Thus, the 2-AB tagging cannot be considered as the method for complete structural characterization. Please rephrase the sentence.

**Au Response:** "complete" has been removed as suggested.

Table 1: H-1 for terminal alpha-GlcpA in proton NMR should be around 5.3ppm

**Au Response:** Correct but as terminal alpha-GlcpA was not detected in our structure it has been removed from Table 1.

*Additional Comments to Authors:*

N/A

## **Reviewer #2:**

### *Manuscript Summary:*

The article describes a series of techniques used to determine the structure of heteroxylans present in plant cell walls. In the article the authors give details of their version of the procedure of Mazumder and York that uses MSN analysis of per-O-methylated oligosaccharides and NMR to determine branching patterns of the heteroxylan. The article also uses 2 aminobenzamide to label the reducing end of the heteroxylan and oligosaccharides produced by enzymatic digestion to aid in the identification of the reducing end structures and improve reversed phase chromatography of the oligosaccharides. NMR analysis is used to identify specific sugars and linkages.

### *Major Concerns:*

The article would benefit from a more narrative approach to the results section. It would be very helpful to the reader for the authors to explain how each analysis contributes to the delineation of the structures. One example is that it is not clear what the LC-MS analysis contributes. If the results section presented each technique with a preamble about its purpose and followed by showing what was concluded about the heteroxylan structure the reader would likely be better able to use these techniques. The current version indicates the identity of fragments but doesn't address what the presence of these species indicates about the structure of the heteroxylan. The MSn and NMR data are discussed with respect to the final structure but it would be good to include the MALDI-TOF and LC-MS data in the discussion.

**Au Response:** As suggested, and within the constraints of the JoVE guidelines, a more descriptive interpretation of the spectra to explain the presence of structural species and the deduced structures with an additional fragmentation analysis (2AB labelled neutral RE oligosaccharide derived from W-sol AXs) have been included in the manuscript.

The method allows assignment of the structures at the RE on the heteroxylan (an important and distinguishing structural feature) and sequences the oligosaccharides released by endo-xylanase digestion. The order of these internal region oligosaccharides in the polymer cannot be determined from this (or any other) approach except by doing shorter enzyme hydrolysis times that result in larger stretches of oligosaccharides that can be further sequenced using the combination of approaches described in this manuscript.

**[Editorial recommendation:** Please keep JoVE's manuscript requirements in mind as you address the above comment and similar comments. Results should be discussed in the "Representative Results" section, not in the Discussion.]

*Minor Concerns:*

HexA1 used without definition.

**Au Response:** defined on page 6- "hexuronic acid".

Figure 2A should indicate in the legend that peaks are sodium adducts.

**Au Response:** the adducts are a mixture of  $M+H^+/Na^+/NH_4^+$  adducts & these have been indicated as appropriate in the figure legend.

Define EIC (extracted ion current)

**Au Response:** defined in Figure 2 legend.

Line 287 Open solid line is confusing and perhaps solid line is sufficient.

**Au Response:** The word 'open' has been deleted

*Additional Comments to Authors:*

N/A

**Reviewer #3:**

*Manuscript Summary:*

The manuscript describes methods for sequencing of plant wall heteroxylans polysaccharides by a combination of enzymatic digestions, permethylation and MS and NMR techniques. The authors use two methods 1. Tag with 2AB the polysaccharide and then digest it with endoxylanase and perform MS and NMR analysis, 2. Reduce, then digest polysaccharide with endoxylanase and then tag and perform same analysis. In the two methods 1 is suppose to give information on reducing end oligosaccharide as 2AB-oligos, the other will give the reducing end oligosaccharide as xylitol residue at the reducing end since it was initially reduced. Also newly released internal residues in example 2 will be all be labeled with 2-AB, whereas in example 1 only reducing end oligo is labeled with 2-AB. The differences between labeled and unlabeled MS spectra and fragmentation of these oligos will reveal information on both reducing end and internal structure of plant wall heteroxylans. MS analysis after permethylation has given diagnostic fragments that allows for sequence information. H-NMR analysis gives alpha beta configuration and D and L information.

*Major Concerns:*

The authors explained the methodology well and the idea behind labeling with 2-AB before and after xylanase digestions. They perform MS analysis after permethylation and also LC-MS using C18



column and H-NMR. They had also previously performed glycosyl composition. However, they failed to explain the significance of the data. The manuscript seems to be description of method, and then list of MS spectra and subsequent assignment of m/z ions to oligosaccharides and fragment ions formed during MSn analysis. The results were stated but not explained or discussed. It seems to be list of protocols used, and data obtained with no discussion of obtained data to xylan structures and biosynthesis.

**Au Response:** We agree but the manuscript was prepared according to the JoVE's manuscript requirements as this is a methodology paper. However, a more descriptive interpretation of the spectra to explain the presence of structural species and the deduced structures with an additional fragmentation analysis (2AB labelled neutral RE oligosaccharide derived from W-sol AXs) has been included in the manuscript.

**[Editorial recommendation:** Please keep JoVE's manuscript requirements in mind as you address the above comment and similar comments. Results should be discussed in the "Representative Results" section, not in the Discussion.]

*Minor Concerns:*

There are no minor revisions, the manuscript will need to explain the results better, and assignment of M/z is not enough.

**Au Response:** changes have been made throughout the text to improve the flow/descriptions and a more descriptive interpretation of the spectra has been included.

Apparently the method used has already been published by other authors as stated in the introduction. What is novel about this paper? What did the authors do that was different or significant?

If this is a method paper then the novelty should be discussed?

**Au Response:** the novelty is the order in which the combination of enzymic/chemical/physical techniques have been applied to enable comprehensive sequencing of internal and reducing end oligosaccharides derived from a polysaccharide by enzymic digestion

*Additional Comments to Authors:*

N/A

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