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Differentiation of isomeric oligosaccharide structures by ESI tandem MS and GC–MS

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Abstract—A mixture of arabinoxylan oligosaccharides from wheat seedling was permethylated and analyzed by electrospray ion trap MS and GC–MS. The presence of isomeric structures differing in degree of branching and position of the branched residue along the xylose backbone was demonstrated for oligosaccharides with four and five monosaccharide residues. No isomeric structures were found for oligosaccharides with three monosaccharide residues. Linkage analysis by GC–MS reveals that xylose residues were substituted with single arabinoxyl residues at C-3.

Keywords: Plant oligosaccharides; Isomeric structures; Permethylation; Gas chromatography; MS

1. Introduction

In higher plants the three main polysaccharides of the cell wall are cellulose, pectin, and hemicellulose. In cereals the hemicellulose fraction is composed mainly of arabinoxylans. Arabinoxylans are complex highly heterogeneous polysaccharides consisting of a linear β-(1 → 4) linked xylopyranose (Xyl*p*) backbone to which α-L-arabinofuranose (Ara*f*) units are attached via α-(1 → 3) and/or α-(1 → 2) linkages.[1] Although arabinoxylans from various cereals and/or different plant tissues share the same basic chemical structure their high degree of microheterogeneity prevent the assignment of a single structure to arabinoxylans. They differ in the degree of branching (DB), the distribution of the Ara*f* residues along the xylan backbone as well as in the presence and proportion of the different types of linkage of the Ara*f* residues in the Xyl*p* ring.[1]

Information on the nature, configuration, relative content of monosaccharide residues, and the type and amount of specific linkages have been obtained for arabinoxylans using ^{13}C NMR.[2–6] However, a drawback of the NMR-based strategy is that information about the residue sequence in the chain cannot be obtained.[1] Information on this structural characteristic for arabinoxylans has been obtained using a multistep procedure, which include digestion with specific endoxylanases, a chromatography separation step for fractionation of the produced oligosaccharides followed by analysis of the isolated oligosaccharides by a combination of ^{1}H NMR, monosaccharide composition, methylation analysis, and FABMS.[7–12] Although successful this approach requires sample amounts in the micromole range. By MS it is possible to obtain information on the monosaccharide sequence, branching pattern, and the presence of modifying chemical groups with oligosaccharide amounts on the picomole level. In addition, MS allows the analysis of

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oligosaccharides mixtures. Due to the potential high heterogeneity of carbohydrates[13] the presence of isomeric structures in highly complex mixtures is very likely to occur. Thus, the capacity to analyze mixtures of isomeric compounds is a desirable feature in any analytical technique for carbohydrate characterization. Despite the fact that separation of isomers is not possible by MS identification of oligosaccharide isomeric structures based on their fragmentation pattern has been reported using MALDI-TOF-PSD,[14,15] ESI-MS,[16] and ESI-ITMS.[17,18] However, in those reports the possibility for differentiation between individual structures within mixtures of isomeric compounds was not demonstrated. ESI-ITMS with multiple MS steps have been used to characterize a mixture of two isobaric oligosaccharide structures from chicken ovalbumin[19] and more recently to characterize mixtures of pectin oligosaccharides differing in the position of the methylated monosaccharide.[20–22] The possibility to analyze mixtures of isomeric oligosaccharides by ESI-ITMS and obtain structural information from their fragmentation mass spectra is of particular importance since at present no other analytical method including NMR has this capacity.

Recently, we reported the use of permethylation followed by analysis by ESI-ITMS with multiple steps of MS to differentiate isomeric structures from a mixture of arabinoxylan oligosaccharides with degree of polymerization (DP) of 6.[23] However, the positions of the Ara*f* substitution in the Xyl*p* ring could not be assigned. In the present study, application of this strategy to the analysis of arabinoxylan oligosaccharides with DP 3, 4, and 5 is reported. The analyses revealed the presence of isomeric oligosaccharides differing in the distribution of the Ara*f* substituting residues along the Xyl*p* backbone and/or degree of branching for structures with DP 4 and 5. In addition, linkage analysis by GC–MS of the partially methylated acetyl alditols (PMAA) showed that Xyl*p* residues were singly substituted at position C-3.

2. Results and discussion

2.1. ESI-MS^n analysis of the non-permethylated sample

Due to the large molecular weight of plant arabinoxylans (AX), digestion with endoxylanase A, which specifically cleaves the β-(1→4) linkages of the xylose backbone, was needed in order to obtain AX oligosaccharides with masses amenable to MS analysis. Analysis with the ESI-QTOF instrument with CID of the signals at *m/z* 437.3 (DP 3), 569.3 (DP 4), and 701.3 (DP 5) from the enzyme digest of AX 70–80% produced a series of abundant fragment ions spaced by 132 Th. (The fragmentation spectrum for the signal at *m/z* 701.3 is shown in Fig. 1.) These signals can be identified as Y, B, ^{2,4}A (M−90), and ^{0,2}A (M−60) fragment ions according to the nomenclature introduced by Domon and Costello.[24] The fragment ions ^{2,4}A and ^{0,2}A arising from cross-ring cleavages indicate that the monosaccharide units were (1→4) linked.[25–27] From these spectra it was not possible to obtain information regarding the presence of branching arabinoxyl residues and their possible distribution along the xylopyranose backbone. These spectra were identical to the fragmentation spectra of linear non-permethylated commercial xylooligosaccharides of DP 3, 4, 5 (data not shown). The reason is that both component monosaccharides in arabinoxylans (xylose and arabinose) are isobaric and fragmentation cannot differentiate between mass losses due to loss of xylose from the linear backbone from loss of branching arabinose residues. However, if all free hydroxyl groups are methylated a different number of methyl groups will be introduced on the xylose residues depending on whether they are unsubstituted or substituted with arabinoxyl residues. Upon fragmentation the branched xylose residues will be distinguished by a decrement of 14 Th per branching point. Sample permethylation of the enzyme digest of AX 70–80% followed

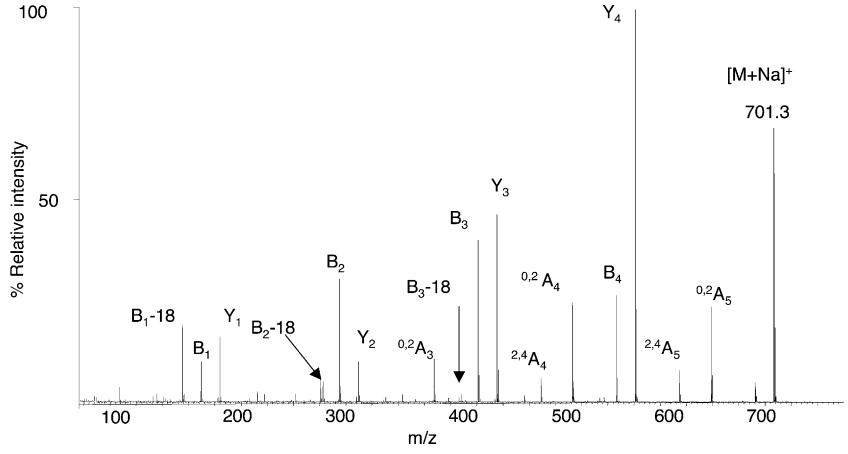


Figure 1. ESI-QTOF MS^2 spectrum of the non-permethylated oligosaccharide with DP 5 from the enzyme digest of AX 70–80%.

by ESI-ITMS with CID analysis was explored as a method to distinguish between different isomeric structures.

2.2. ESI-MS^n analysis of the permethylated sample

The analysis of the signals at m/z 549.3 (Fig. 2a), 709.6 (Fig. 2b), and 869.6 (Fig. 2c) from the permethylated enzyme digest of AX 70–80% by ESI-ITMS with CID produce spectra where most of the intense signals can be identified as Y and B fragment ions. Additional fragment ions in these spectra are identified as ^{1,5}X, ^{2,4}A, ^{3,5}A, and internal fragment ions. The observed cross-ring cleavages ^{1,5}X and ^{3,5}A confirm the presence of $(1\rightarrow4)$ glycosidic linkages for these oligosaccharides.

For the peak at m/z 549.3 no evidence of the presence of isomeric structures in the mixture was obtained from the MS^2 spectra. The oligosaccharide structure inferred from the interpretation of the spectrum is represented by structure I in Scheme 1. This conclusion is supported by the fact that this spectrum was similar to the spectrum produced by a permethylated commercial linear xylooligosaccharide with DP 3 (data not shown). The presence of fragment ions from the precursor ions at m/z 709.6 (Fig. 2b) and 869.6 (Fig. 2c) with m/z values 14 Th lower than the expected value for fully methylated residues indicates the presence of branched Xyl*p* residues. These MS^2 spectra differ from those produced by the permethylated linear xylooligosaccharides with DP 4 and 5 (data not shown). From the interpretation of MS^2 spectra, the presence of three different isomeric oligosaccharides for signal at m/z 709.6 (Fig. 2b) and five for signal at m/z 869.6 (Fig. 2c) can be inferred (these numbers include the oligosaccharides with Xyl*p* residues substituted at position C-2 with Ara*f*). For simplicity only the isomeric oligosaccharides with Xyl*p* substituted with Ara*f* at position C-3 are shown for signals at m/z 709.6 and 869.6. In Scheme 1 the two isomers for the signal at m/z 709.6 (structures II and III) are shown. For the signal at m/z 869.6 the two isomeric oligosaccharides with Xyl*p* substituted with Ara*f* at position C-3 are shown in Scheme 1 (structures IV and V). The structure VI (Scheme 1) represents the double Ara*f* substituted oligosaccharide for the signal at m/z 869.6.

For the ion at m/z 709.6 no further isolation and fragmentation steps were necessary to confirm the presence of the structures II and III. Nevertheless, MS^3 analysis of the fragment ion at m/z 535.2 (data not shown) was performed to extend the low mass limit and get more clear information on low mass fragment ions. From the interpretation of the MS^2 spectra of the ion at m/z 869.6 is not possible to establish unambiguously the

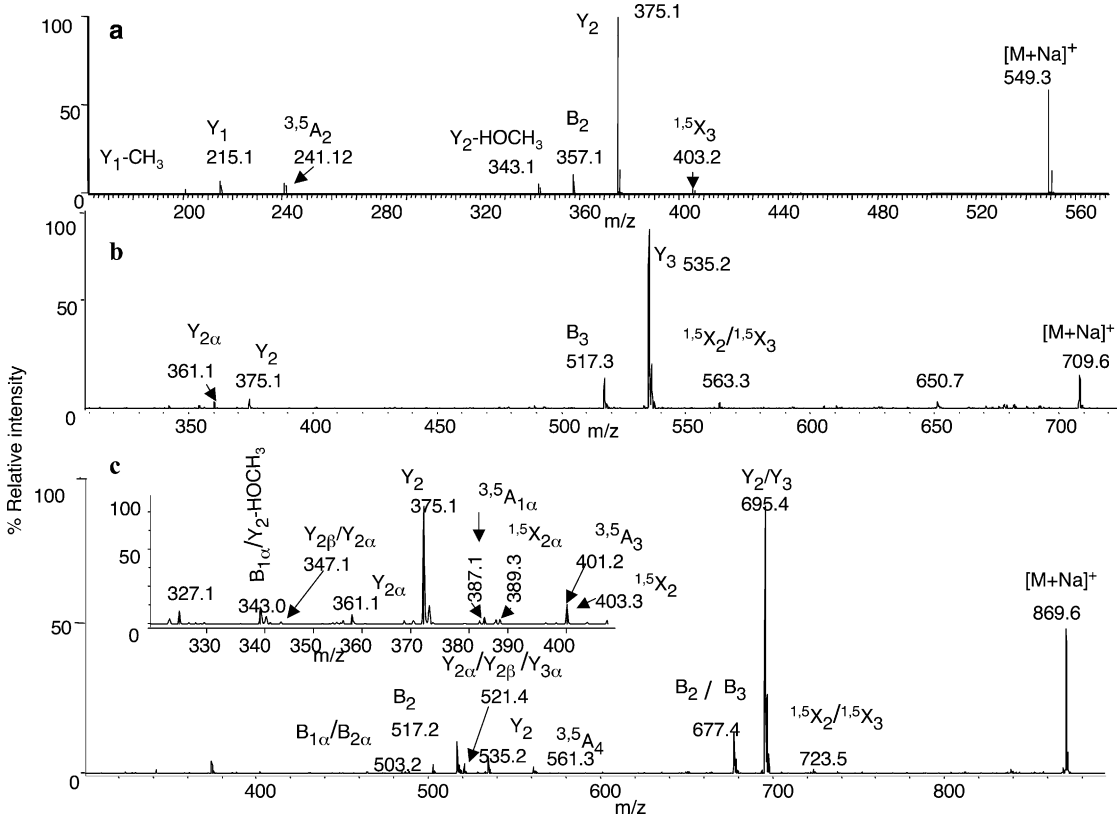
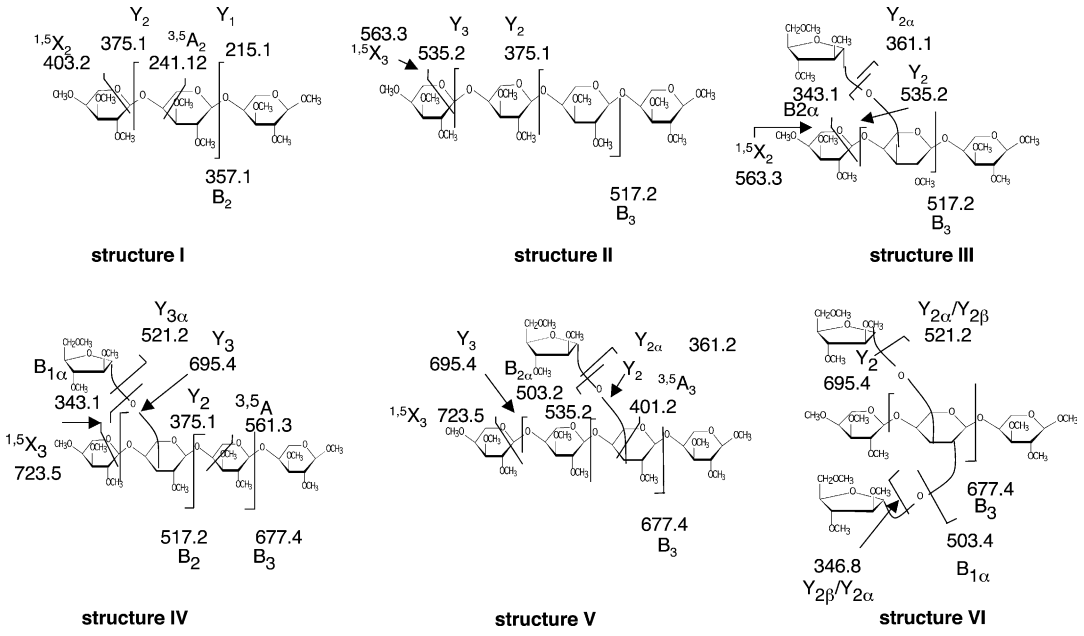


Figure 2. (a) ESI-IT MS^2 spectrum of the ion at m/z 549.3 (polymer with DP 3) from the permethylated enzyme digest of AX 70–80%. (b) ESI-IT MS^2 spectrum of the ion at m/z 709.6 (polymer with DP 4) from the permethylated enzyme digest of the AX 70–80%. (c) ESI-IT MS^2 spectrum of the ion at m/z 869.6 (polymer with DP 5) from the permethylated enzyme digest of AX 70–80%; inset: (top left) zoom of the low mass region.



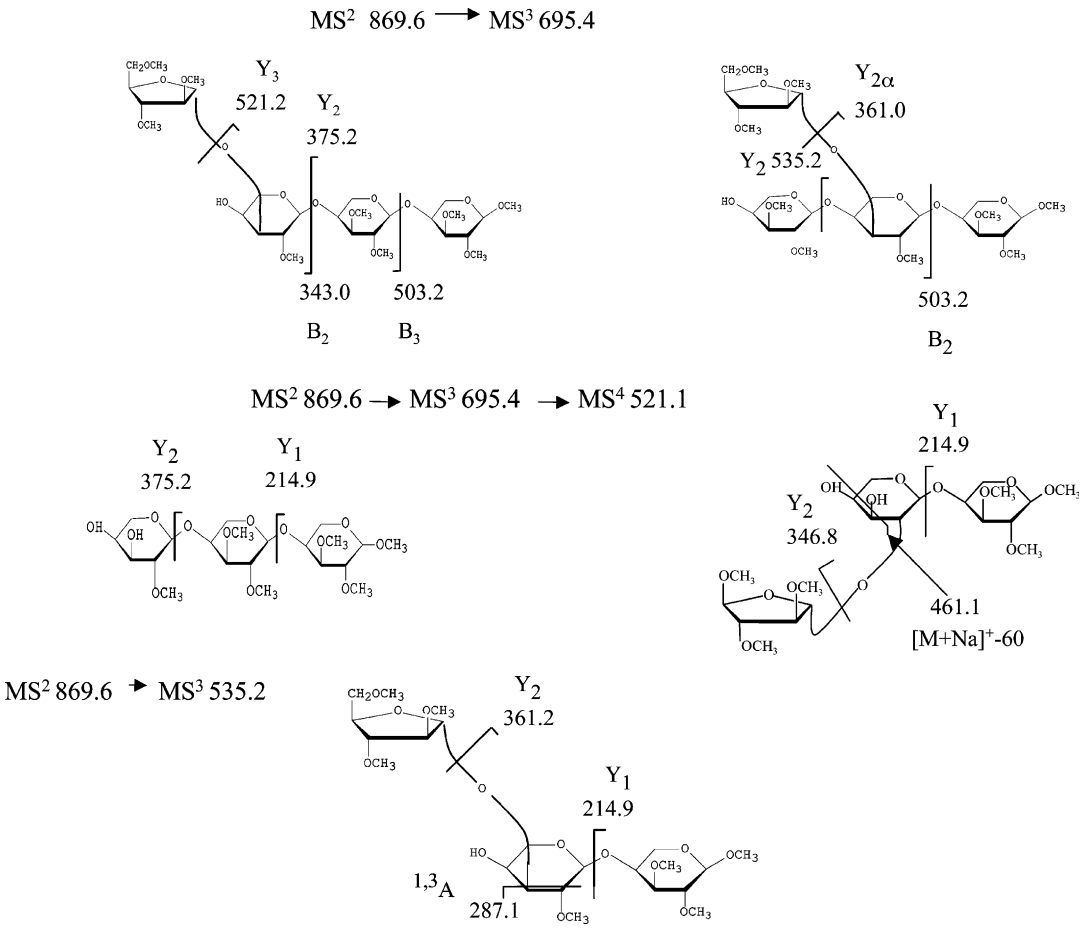
Scheme 1. Possible structures derived by MS^2 of the ions at m/z 549.3, 709.6, and 869.6 (Fig. 2). Structure I represents the possible structure for the ion at m/z 549.3, structures II and III represent the possible structures for the ion at m/z 709.6, and structures IV, V, and VI represent the possible structures derived by MS^2 analysis of the ion at m/z 869.6.

complete set of structures present due to the inability of establishing the origin of the fragment ion at m/z 375.1. For example, the signals at m/z 695.4, 535.2, and 375.1 in the fragmentation spectra (Fig. 2c) suggest the presence of a linear structure and the signals at m/z 695.4, 521.4, and 375.1 suggest a structure with one branching arabinoxyl residue. Another source of uncertainty is the low intensity of some fragment ions in the low mass region of the spectra, for example, the presence of the structure VI is only suggested by the fragment ion at m/z 347.1 and the presence of the structure V is suggested by the presence of the ion at m/z 361.1. As can be seen clearly from the spectrum (inset Fig. 2c) the intensity of both ions is almost at base line level preventing unambiguous confirmation of the presence of these structures in the MS^2 step.

The reduced intensity of some ions in the low mass region of the spectra is a consequence of the reduced trapping efficiency for fragment ions below 30% of the m/z of the precursor ion (low mass cutoff), which are not trapped under the applied CID conditions. Using fragment ions with lower m/z than the precursor ion to perform further sequential isolation/fragmentation steps (MS^n) allows extension of the accessible mass range toward ions of lower mass by decreasing the 30% cutoff value. Furthermore, using additional tandem MS steps to analyze the fragmentation pathway of selected fragment ions permit the identification of precursor ions of specific fragments ion confirming the presence of specific oligosaccharides structures in the mixture. Thus additional MS^n steps were performed to establish the origin and/or confirming the presence of ions 347.1, 361.1, and 375.1 and in this way establish the presence of specific oligosaccharide structures.

The fragment ion at m/z 695.4 was selected for MS^3 analysis. The resulting spectrum is shown in Figure 3a and the structures derived from its interpretation in Scheme 2. The observed signals can be identified as Y and B fragment ions and their presence supports the previously proposed structures IV and V in the MS^2 spectra with the same limitations for unequivocal confirmation of the presence of these structures for the reasons above mentioned. The presence of the structure VI cannot be confirmed from this spectrum since the ion at m/z 347.1 is not present. In a third step, the fragment ion at m/z 521.2 derived from the fragment ion at m/z 695.4 was selected as precursor ions for MS^4 analyses. The MS^4 spectrum for the ion at m/z 521.2 is shown in Figure 3b and its interpretation is shown in Scheme 2. In this spectrum the observed signals are Y fragment ions. The presence of the signal at m/z 346.8 corroborates the presence of the structure VI whereas the signal at m/z 375.1 confirms the presence of the structure IV. To confirm the presence of the structure V, the fragment ion at m/z 535.2 was selected as precursor fragment ion for further analysis. The low intensity of this fragment ion in the MS^3 spectra of the ion at m/z 695.4 (Fig. 3a) prevents its isolation in the MS^3 step; hence it was isolated in the MS^2 step. The resulting MS^3 spectrum is shown in Figure 3c and its interpretation in Scheme 2. The interpretation confirmed the presence of the structure V in the mixture of oligosaccharides.

The analysis with ESI-MS^n of the signals at m/z 709.6 and 869.6 produced evidence for the presence of oligo-



Scheme 2. Possible structures derived by MS^3 of the ion at m/z 695.4 (Fig. 3a), by MS^4 of the ion at m/z 521.2 (Fig. 3b), and by MS^3 of the ion at m/z 535.2 (Fig. 3c).

saccharide structures differing in the degree of branching and in the distribution of the Ara*f* substituents along the xylose chain. No signals corresponding to cross-ring cleavages were identified in the MS^2 spectra, which permit assignment of the branching positions for single substituted Xyl*p* residues. Moreover, MS^3 and MS^4 spectra were devoid of any cross-ring cleavages. Therefore, despite the use of several MS^n steps it was not possible to localize the position of substitution (C-2 or C-3) on the Xyl*p* ring for structures with a single Ara*f* substitution.

2.3. GC–MS analysis

Information on the position of branching in the Xyl*p* residues was obtained from the analysis of PMAA derivatives by GC–MS. The resulting chromatogram is shown in Figure 4 and the detected PMAA derivatives are presented in Table 1. Since the derivatives 1,3,4,5-tetra-*O*-acetyl-(1-deuterio)-2-*O*-methyl xylitol (originating from C-3 singly substituted Xyl*p*) and 1,2,4,5-tetra-*O*-acetyl-(1-deuterio)-3-*O*-methyl xylitol (originating from C-2 singly substituted Xyl*p*) have very close retention times they cannot be resolved under most chromatographic conditions.[2,28] However, their presence can be inferred from the fragment ions characteristic for these two derivatives: m/z 118 (for 2-*O*-methyl xylitol) and m/z 129 (for 3-*O*-methyl xylitol).[12,29–32] In our analysis only the fragment ion at m/z 118 (for 2-*O*-methyl xylitol) indicating C-3 singly substituted Xyl*p* was observed. No indication was observed of the presence of C-2 substituted Xyl*p* residues.

The presence of single substituted Xyl*p* residues at position C-3 have been previously reported for wheat arabinoxylans from endosperm,[4,12,31,33,34] bran,[35] and beeswing bran.[28] Substitution of the Xyl*p* residue at position C-2 has also been reported for wheat arabinoxylans from endosperm although this type of linkage is less abundant.[31,36] Our results provide the first evidence of single substituted Xyl*p* residues at position C-3 for arabinoxylans from wheat seedlings. However, the presence of low levels of C-2 single substituted Xyl*p* residues in the AX 70–80% fraction cannot be completely ruled out since its detection may has been affected by incomplete hydrolysis of the oligosaccharides, or selective losses during preparation of the volatile PMAA derivatives. In

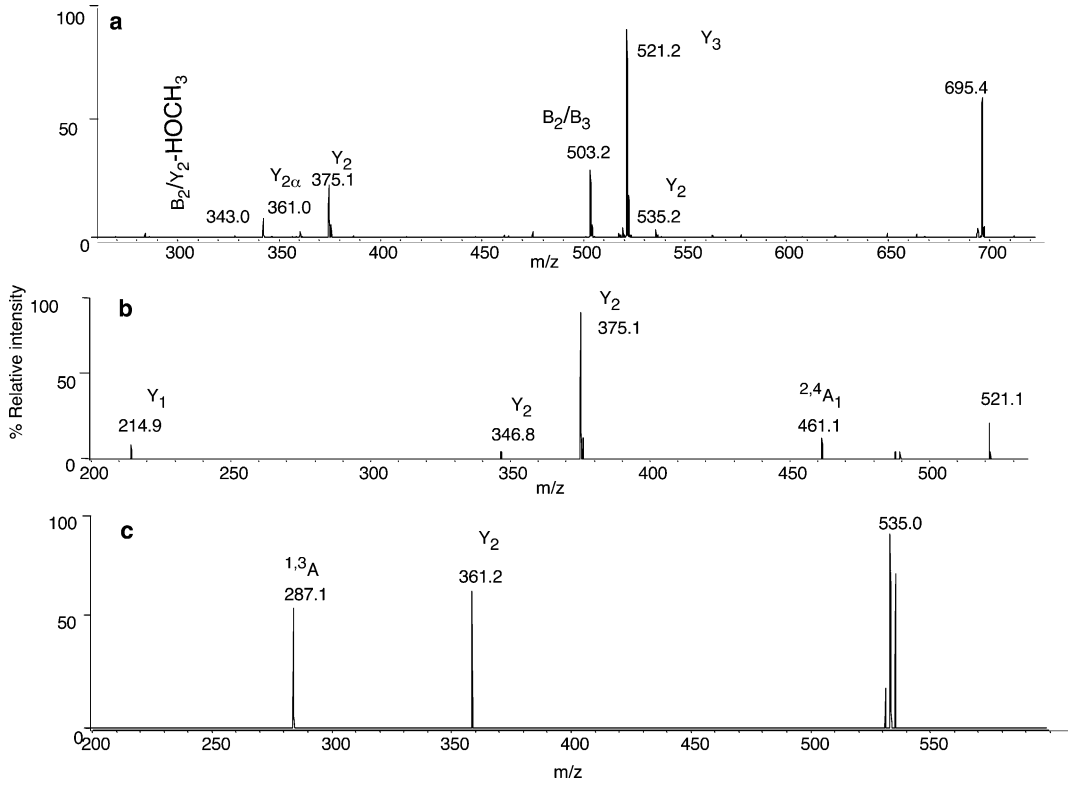


Figure 3. (a) ESI-IT MS^3 spectrum of the ion at m/z 695.4 from the MS^2 spectrum Figure 2c. (b) ESI-IT MS^4 spectrum of the ion at m/z 521.2 from the MS^3 spectrum Figure 3a. (c) ESI-IT MS^3 spectrum of the ion at m/z 535.2 in the spectrum Figure 2c.

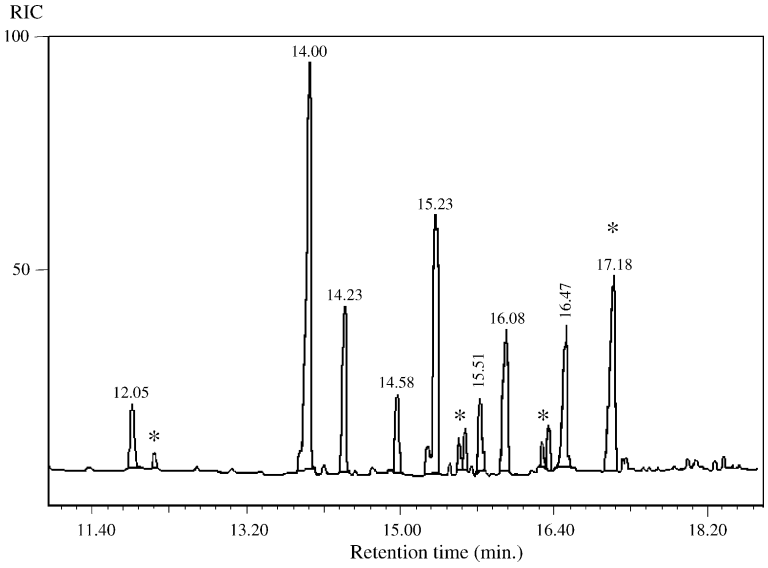


Figure 4. Gas chromatogram of the PMAA derivatives for the enzyme digest of AX 70–80%. The numbers above each peak represent the retention time of the derivatives identified in Table 1. The peaks identified with asterisk are nonidentified contaminants.

addition since variation in the content of C-2 substituted Xyl*p* residues have been reported between arabinoxylans fractions precipitated with different ethanol or ammonium sulfate percent the presence of C-2 substituted Xyl*p* residues in the arabinoxylans fractions precipitated with lower percent of ethanol cannot be ruled out.[12,31]

Undermethylation at some positions of the monosaccharide residues has also been described as a source of artifacts giving rise to a wrong assignment of

Table 1. PMAA derivatives identified by GC–MS analysis of the enzyme digest of the sample AX 70–80%

Retention time[a] (min)	PMAA derivatives	Linkage
14.00 ± 0.01	1,4-Di-*O*-acetyl-(1-deuterio)-2,3,5-tri-*O*-methyl pentitol	Terminal arabinose
14.23 ± 0.01	1,5-Di-*O*-acetyl-(1-deuterio)-2,3,4-tri-*O*-methyl pentitol	Terminal xylose
15.23 ± 0.02	1,4,5-Tri-*O*-methyl-(1-deuterio)-2,3-di-*O*-methyl pentitol	Nonsubstituted 1,4-linked xylose
16.08 ± 0.01	1,3,4,5-Tetra-*O*-acetyl-(1-deuterio)-2-*O*-methyl pentitol	1,4-Linked xylose substituted at position 3 with arabinosyl residues
15.51 ± 0.01	1,2,3,4,5-Penta-*O*-acetyl-(1-deuterio)-methyl pentitol	1,4-Linked xylose substituted at positions 2 and 3 with arabinosyl residues
14.58 ± 0.01	1,4,5-Tri-*O*-acetyl-2,3,6-tri-*O*-methyl hexitol	Nonsubstituted 1,4-linked hexose
16.47 ± 0.03	1,5-Di-*O*-2,3,4,6-tetra-*O*-methyl hexitol	Terminal hexose

[a] Average ± standard deviation.

monosaccharide linkage pattern.[32] This can be excluded in our work since analysis of the permethylated samples by ESI-ITMS prior to preparation of the PMAA derivatives for GC–MS shows that permethylation reaction has been complete. Moreover, in our case the permethylation was performed on the oligosaccharide mixture and not on the intact polysaccharide. This most likely has decreased any steric hindrance for the methylation efficiency due to conformational effects of the polysaccharide chain.

PMAA derivatives from hexose were also identified by GC–MS analysis. This is consistent with the detection of linear oligosaccharides of hexose in ESI-ITMS analysis as described previously.[23] These oligosaccharides are most likely produced by nonspecific endoxylanase A degradation of β-D-glucans present in the sample. The sum of the PMAA derivatives of pentoses identified confirmed the structural features already identified by ESI-ITMS for wheat oligosaccharides.

2.4. Concluding remarks

This study has demonstrated that a combination of enzymatic digestion with specific endoxylanase, sample permethylation followed by MS^n analysis with ESI-ITMS and linkage analysis by GC–MS can be used to analyze unseparated mixtures of arabinoxylan oligosaccharides, and to identify isomeric structures. Information on the type of linkage of Xyl*p* residues, the distribution of the Ara*f* residues along the Xyl*p* backbone, and the degree of branching were obtained with ESI-ITMS. The GC–MS analysis provided evidences for the presence of Ara*f* residues linked at position C-3 of the Xyl*p* residues in arabinoxylans from wheat seedlings and confirmed the conclusion derived from the analysis of the arabinoxylan oligosaccharide mixture by ESI-ITMS.

NMR is considered to be the most powerful tool for structural characterization of carbohydrates.[37] However, complete structural characterization by NMR requires a carbohydrate sample with a high degree of purity. The traditional approach has been to separate out the different components of carbohydrate mixtures for individual structural analysis. This approach however present some limitations, for example, separation of isomeric structures is a laborious procedure or simply impossible in some cases and minor components can be lost or cannot be recovered in amounts sufficient for NMR analysis. In addition when limited amounts of sample are available analyses by NMR can be very time consuming and may become impossible.[37] To overcome these limitations different approaches have been reported including NMR analysis of carbohydrate mixtures and nanoprobe ^{1}H NMR. However, analysis of carbohydrates mixtures has been so far limited to mixtures of 2–3 structures because analysis of more complex mixtures decreased the amount of structural data that can be obtained for each individual component.[38–40] In the arabinoxylan mixture used to generate the data presented in this work, a total of 22 signals were observed by MALDI-TOF,[23] assuming a similar degree of heterogeneity for oligo-/polysaccharides with DP higher than 6 to that observed for oligosaccharides with DP 3–6 the number of different structures in the mixture can be around 10^2 a fact which preclude the direct analysis of this mixture by NMR. As an alternative to the use of NMR, MS with CID has been used for the characterization of pectins,[20,41–49] xylans,[50–52] amylopectins.[53–57] The advantages of MS are speed of analysis, sensitivity, and capacity to analyze oligosaccharides mixtures. In addition the fragmentation pathways as well as fragmentation mechanisms of carbohydrates by CID are well characterized.[25,58–60] From the present results and those previously[23] reported it seems that analyses by ESI-ITMS of mixtures of isomeric arabinoxylan oligosaccharides is limited to structures with DP 6 due to the number of MS steps that can be performed on the ion trap mass spectrometer. Owing to the complexity of the oligosaccharide mixture analyzed in this work structural characterization of oligosaccharides with DP > 6 will require a chromatographic fractionation step before ESI-ITMS analysis.

3. Experimental

3.1. Cell wall fractionation

Preparation of the cell wall and fractionation by sequential ethanol precipitation to obtain arabinoxylan samples was performed as described previously.[23] The process produces a series of arabinoxylan fractions labeled according to the ethanol percentage needed to achieve precipitation, for example, AX 10%, AX 10–20%, etc. The arabinoxylan fraction precipitated with 70–80% ethanol concentration (AX 70–80%) was used to generate the data presented in this work.

3.2. Enzymatic digestion

Ten microliters of a solution of endoxylanase A in 50 mM sodium acetate pH 5.0 (1 mU/μL 1 U releases 1 μmol reducing sugar per min) was added to 250 μL (~6 μg/μL) of arabinoxylan sample AX 70–80% in the same buffer solution and the sample incubated at 37 °C for 24 h. The enzymatic reaction was stopped by freezing the samples at −20 °C.

3.3. Permethylation

Permethylation was performed according to the method of Ciucanu and Keres.[61] Enzymatically digested arabinoxylans were dried in a 4 mL glass tube, to which 250 μL Me_2SO–NaOH slurry and methyl iodide (250 μL) were added. The mixture was mixed vigorously and then incubated at room temperature for 60 min. With the glass tube placed in a small ice bath for cooling, the reaction was quenched by slow dropwise addition of water (500 μL). Samples were then extracted with 1 mL aliquots of chloroform and the extracted organic phase rinsed with 2 mL of water. The extract was dried under nitrogen, re-dissolved in methanol, and stored at −20 °C until use.

3.4. Preparation of the partially methylated acetyl alditols (PMAA) derivatives

For preparation of the PMAA derivatives 50 μg of the enzyme digest of AX 70–80% were permethylated and then hydrolyzed for 2 h at 110 °C with 100 μL of 2 M trifluoroacetic acid. The sample was cooled to room temperature and then dried down under a stream of argon. The residual trifluoroacetic acid was eliminated by adding 250 μL of isopropanol and then evaporating the sample under a stream of argon. This step was repeated once more and then the sample was reduced with 250 μL of 1 M $NaBD_4$ in 1 M NH_4OH at room temperature for 18 h. The reaction was stopped with glacial acetic acid and the sample was evaporated with a stream of Ar, followed by multiple evaporations with 1% acetic acid/methanol (v/v) and a final evaporation with methanol. Sample acetylation was performed by addition of 100 μL of pyridine and 100 μL of acetic acid anhydride followed by incubation for 2 h at 100 °C. After this step 100 μL of hexane was added and the solvent removed with a stream of Ar. The sample was stored at −20 °C until used. For GC–MS analyses the sample was dissolved in 50 μL of acetone.

3.5. Mass spectrometric analysis

3.5.1. ESI-MS. The mass spectrometric analysis was performed as previously described.[23] Briefly, ESI mass spectra were acquired in positive ion mode using either a quadrupole time of flight instrument QTOF-1 (Micromass, Manchester, UK) or a quadrupole ion trap. Both instruments are equipped with nanoESI ion sources. For analysis in the QTOF instrument samples were sprayed from palladium/gold coated borosilicated glass needles (Protana Engineering A/S, Odense, Denmark) and the voltage applied on the needle and the nitrogen (N_2) backpressure adjusted to produce a stable electrospray. The source temperature was 40 °C and nitrogen was used as a drying gas. For collision induced dissociation (CID) experiments argon was used as the collision gas and the collision energy adjusted to reduce the intensity of the precursor ion to 50% of its original intensity.

ESI-ion trap analyses were performed using an Esquire LC ion trap (IT) mass spectrometer (Bruker Daltonic, Bremen, Germany). Palladium/gold coated borosilicated glass needles from Protana Engineering A/S (Odense, Denmark) were used for sample analysis. Data acquisition was performed in standard resolution mode with a scan speed of 13,000 Da/s. A flow of nitrogen of 2 mL/h with a temperature of 40 °C was used to assist in the drying process and a backpressure of nitrogen was applied to the needle to start the spray process. For CID experiments an isolation window of 6 Th was used and the collision adjusted to reduce the intensity of the precursor ion to 50% of its initial intensity. Commercial available linear xylooligosaccharides (Megazyme International, Ireland) with DP 3, 4, and 5 were used to produce reference MS^n spectra of linear structures.

3.5.1.1. Sample preparation for ESI-MS. Non-permethylated samples for nanoESI analysis were desalted by the previously reported microcolumn purification procedure.[20] Briefly, 0.5 μL of enzymatically digested arabinoxylan sample was applied to microcolumns filled with AG-50W-X8 resins (H^+ form) and the pass

through mixed in the glass needle with 1 μL of 50% ACN/10 mM sodium acetate (v/v). For analysis of the permethylated arabinoxylan solution 5 μL of the permethylated solution was diluted with 15 μL of absolute methanol and 5 μL of 10 mM sodium acetate solution. One to two microliters of this solution was used for nanoESI analysis.

3.6. GC–MS

Linkage analysis was performed using a VARIAN gas chromatograph (Varian Inc., Walnut Creek, CA) coupled to a Finnigan MAT (Palo Alto, CA) SSQ710-single stage quadrupole mass spectrometer. The sample (1 μL) was introduced in the splitless injection mode in a HP-5MS column (30 m × 0.25 mm, 0.25 μm film thickness, Agilent Technologies) using helium as carrier gas. The PMAA derivatives were separated using the following temperature gradient: 80 °C for 5 min, 80–170 °C at 11 °C/min, 170–240 °C at 14 °C/min, 240 °C held for 10 min. Samples were ionized by electrons impact at 70 eV. Mass spectra were acquired by scanning the quadrupole from m/z 35 to m/z 500 with a scan rate of 0.5 s. Detector and injector temperatures were both at 250 °C analyses were performed in triplicate and the PMAA derivatives were identified as previously reported.[32,62,63]

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References

1. Izydorczyk, M. S.; Biliaderis, C. G. *Carbohydr. Polym.* **1995**, *28*, 33–48.
2. Ebringerova, A.; Hromadkova, Z.; Petrakova, E.; Hricovini, M. *Carbohydr. Res.* **1990**, *198*, 57–66.
3. Bengtsson, S.; Aman, P. *Carbohydr. Polym.* **1990**, *12*, 267–277.
4. Hoffman, R. A.; Roza, M.; Maat, J.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Polym.* **1991**, *15*, 415–430.
5. Izydorczyk, M. S.; Biliaderis, M. S. *Carbohydr. Polym.* **1992**, *17*, 237–247.
6. Vinkx, C. J. A.; Reynaert, H. R.; Grobet, P. J.; Delcour, J. A. *Cereal Chem.* **1993**, *70*, 311–317.
7. Hoffmann, R. A.; Leeflang, B. R.; de Barse, M. M.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **1991**, *221*, 63–81.
8. Hoffman, R. A.; Geijtenbeek, T.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **1992**, *223*, 19–44.
9. Vietor, R. J. Ph.D. thesis, Wageningen Agricultural University, The Netherlands, 1992.
10. Bengtsson, S.; Aman, P.; Andersson, R. E. *Carbohydr. Polym.* **1992**, *17*, 277–284.
11. Gruppen, H.; Kormelink, F. J.; Voragen, A. G. *J. Cereal Sci.* **1993**, *18*, 111–128.
12. Izydorczyk, M. S.; Biliaderis, M. S. *Cereal Chem.* **1993**, *70*, 641–646.
13. Laine, R. A. *Glycobiology* **1994**, *4*, 759–767.
14. Garozzo, D.; Nasello, V.; Spina, E.; Sturiale, L. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1561–1566.
15. Yamagaki, T.; Mitsuishi, Y.; Nakanishi, H. *Tetrahedron Lett.* **1998**, *39*, 4051–4054.
16. Reinhold, V. N.; Reinhold, B. B.; Chan, S. In *Biological Mass Spectrometry: Present and Future*; Seyama, Y., Ed.; Wiley: Chichester, UK, 1994.
17. Konig, S.; Leary, J. A. *J. Am. Soc. Mass Spectrom.* **1998**, *9*, 1125–1134.
18. Viseux, N.; de Hoffmann, E.; Domon, B. *Anal. Chem.* **1998**, *70*, 4951–4959.
19. Weiskopf, A. S.; Vouros, P.; Harvey, D. J. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 1493–1504.
20. Korner, R.; Limberg, G.; Christensen, T. M.; Mikkelsen, J. D.; Roepstorff, P. *Anal. Chem.* **1999**, *71*, 1421–1427.
21. Kester, H. C.; Benen, J. A.; Visser, J.; Warren, M. E.; Orlando, R.; Bergmann, C.; Magaud, D.; Anker, D.; Doutheau, A. *Biochem. J.* **2000**, *346*(pt 2), 469–474.
22. Mutenda, K. E.; Korner, R.; Christensen, T. M.; Mikkelsen, J.; Roepstorff, P. *Carbohydr. Res.* **2002**, *337*, 1213–1223.
23. Matamoros Fernández, L.; Obel, N.; Scheller, H. V.; Roepstorff, P. *J. Mass Spectrom.* **2003**, *38*, 427–437.
24. Domon, B.; Costello, C. E. *Glycoconjugate J.* **1988**, *5*, 397–409.
25. Zhou, Z.; Ogden, S.; Leary, J. A. *J. Org. Chem.* **1990**, *55*, 5444–5446.
26. Hofmeister, G. E.; Zhou, Z.; Leary, J. A. *J. Am. Chem. Soc.* **1991**, *113*, 5964–5970.
27. Lemoine, J.; Fournet, B.; Despeyroux, D.; Jennings, K. R.; Rosenberg, R.; de Hoffman, E. *J. Am. Soc. Mass Spectrom.* **1992**, *4*, 197–203.
28. Brillouet, J.-M.; Joseleau, J.-P. *Carbohydr. Res.* **1987**, *159*, 109–126.
29. Shibuya, N.; Iwasaki, T. *Phytochemistry* **1985**, *24*, 285–289.
30. Vietor, R. J.; Angelino, S. A.; Voragen, A. G. *J. Cereal Sci.* **1992**, *15*, 213–222.
31. Gruppen, H.; Hamer, R. J.; Voragen, A. G. *J. Cereal Sci.* **1992**, *16*, 53–67.
32. Carpita, N. C. In *Analysis of Carbohydrates by GLC and MS*; McGinnis, G. D., Ed.; CRC Press: Boca Raton, FL, 1989.
33. Perlin, A. S. *Cereal Chem.* **1951**, *28*, 382–393.
34. Gruppen, H.; Hoffmann, R. A.; Kormelink, F. J.; Voragen, A. G.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **1992**, *233*, 45–64.
35. Shiiba, K.; Yamada, H.; Hara, H.; Okada, K.; Nagao, S. *Cereal Chem.* **1993**, *70*, 209–214.
36. Izydorczyk, M. S.; Biliaderis, M. S. *Carbohydr. Polym.* **1994**, *24*, 61–71.

37. Duus, J.; Gotfredsen, C. H.; Bock, K. *Chem. Rev.* **2000**, *100*, 4589–4614.
38. Spellman, M. W.; Basa, L. J.; Leonard, C. K.; Chakel, J. A.; O'Connor, J. V.; Wilson, S.; van Halbeek, H. *J. Biol. Chem.* **1989**, *264*, 14100–14111.
39. Spellman, M. W.; Leonard, C. K.; Basa, L. J.; Gelineo, I.; van Halbeek, H. *Biochemistry* **1991**, *30*, 2395–2406.
40. Manzi, A. E.; Norgard-Sumnicht, K.; Argade, S.; Marth, J. D.; van Halbeek, H.; Varki, A. *Glycobiology* **2000**, *10*, 669–689.
41. Schols, H. A.; Mutter, M.; Voragen, A. G.; Niessen, W. M.; van der Hoeven, R. A.; van der Greef, J.; Bruggink, C. *Carbohydr. Res.* **1994**, *261*, 335–342.
42. Brull, L.; Huisman, M.; Schols, H.; Voragen, F.; Critchley, G.; Thomas-Oates, J.; Haverkamp, J. *J. Mass Spectrom.* **1998**, *33*, 713–720.
43. Korner, R.; Limberg, G.; Mikkelsen, J. D.; Roepstorff, P. *J. Mass Spectrom.* **1998**, *33*, 836–842.
44. Limberg, G.; Korner, R.; Buchholt, H. C.; Christensen, T. M.; Roepstorff, P.; Mikkelsen, J. D. *Carbohydr. Res.* **2000**, *327*, 293–307.
45. van Alebeek, G. J.; Zabotina, O.; Beldman, G.; Schols, H. A.; Voragen, A. G. *J. Mass Spectrom.* **2000**, *35*, 831–840.
46. van Alebeek, G.; Schols, H.; Voragen, A. G. *Carbohydr. Polym.* **2001**, *46*, 311–321.
47. Bonnin, E.; Le Goff, A.; Korner, R.; Vigouroux, J.; Roepstorff, P.; Thibault, J. F. *Biochim. Biophys. Acta* **2002**, *1596*, 83–94.
48. van Alebeek, G. J.; Christensen, T. M.; Schols, H. A.; Mikkelsen, J. D.; Voragen, A. G. *J. Biol. Chem.* **2002**, *277*, 25929–25936.
49. Mutenda, K. E.; Korner, R.; Christensen, T. M.; Mikkelsen, J.; Roepstorff, P. *Carbohydr. Res.* **2002**, *337*, 1213–1223.
50. Jacobs, A.; Larsson, P. T.; Dahlman, P. *Biomacromolecules* **2001**, *2*, 979–990.
51. Deery, M. J.; Stimson, E.; Chappell, C. G. *Rapid Commun. Mass Spectrom.* **2001**, *15*, 2273–2283.
52. Jacobs, A.; Dahlman, O. *Biomacromolecules* **2001**, *2*, 894–905.
53. Richardson, S.; Nilsson, G. S.; Bergquist, K. E.; Gorton, L.; Mischnick, P. *Carbohydr. Res.* **2000**, *328*, 365–373.
54. Richardson, S.; Cohen, A.; Gorton, L. *J. Chromatogr. A* **2001**, *917*, 111–121.
55. van der Burgt, Y. E.; Bergsma, J.; Bleeker, I. P.; Mijland, P. J.; van der Kerk van Hoof, A.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **2000**, *329*, 341–349.
56. van der Burgt, Y. E.; Bergsma, J.; Bleeker, I. P.; Mijland, P. J.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **2000**, *327*, 423–429.
57. van der Burgt, Y. E.; Bergsma, J.; Bleeker, I. P.; Mijland, P. J.; van der Kerk-van Hoof, A.; Kamerling, J. P.; Vliegenthart, J. F. *Carbohydr. Res.* **2000**, *325*, 183–191.
58. Hofmeister, G. E.; Zhou, Z.; Leary, J. A. *J. Am. Chem. Soc.* **1991**, *113*, 5964–5970.
59. Lemoine, J.; Strecker, G.; Leroy, Y.; Fournet, B.; Ricart, G. *Carbohydr. Res.* **1991**, *221*, 209–217.
60. Lemoine, J.; Fournet, B.; Despeyroux, D.; Jennings, K. R.; Rosenberg, R.; de Hoffman, E. *J. Am. Soc. Mass Spectrom.* **1992**, *4*, 197–203.
61. Ciucanu, I.; Keres, F. *Carbohydr. Res.* **1984**, *131*, 209–216.
62. Izydorczyk, M. S.; Macri, L. J.; MacGregor, A. W. *Carbohydr. Polym.* **1998**, *35*, 259–269.
63. Marry, M.; Cavalier, D. M.; Schnurr, J. K.; Netland, J.; Yang, Z.; Pezeshk, V.; York, W. S.; Pauly, M.; White, A. R. *Carbohydr. Polym.* **2003**, *51*, 347–356.