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ARTICLE

Characterization of Oligomeric Xylan Structures from Corn Fiber Resistant to Pretreatment and Simultaneous Saccharification and Fermentation

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Corn fiber, a byproduct from the corn industry, would be a good source for bioethanol production if the hemicellulose, consisting of polymeric glucoronoarabinoxylans, can be degraded into fermentable sugars. Structural knowledge of the hemicellulose is needed to improve the enzymatic hydrolyses of corn fiber. Oligosaccharides that resisted a mild acid pretreatment and subsequent enzymatic hydrolysis, representing 50% of the starting material, were fractionated on reversed phase and size exclusion material and characterized. The oligosaccharides within each fraction were highly substituted by various compounds. Oligosaccharides containing uronic acid were accumulated in two polar fractions unless also a feruloyl group was present. Feruloylated oligosaccharides, containing mono- and/or diferulic acid, were accumulated within four more apolar fractions. All fractions contained high amounts of acetyl substituents. The data show that complex xylan oligomers are present in which ferulic acid, diferulates, acetic acid, galactose, arabinose, and uronic acids were combined within an oligomer. Hypothetical structures are discussed, demonstrating which enzyme activities are lacking to fully degrade corn glucuronoarabinoxylans.

KEYWORDS: Xylan; bioethanol; hemicellulose; ferulate; MALDI-TOF-MS; oligosaccharides

INTRODUCTION

There is great interest in the use of plant biomass to generate biofuels. To prevent competition with food crops, there is a shift from the use of first (starch and sugar rich) to second (lignocellulose rich) generation feedstocks, which are often industrial byproducts or residual plant materials. The hemicellulose present in the second-generation feedstocks should be utilized, next to cellulose, in view of economics. Important second-generation feedstocks worldwide are byproducts of the corn-processing industry, such as corn fiber (*1*).

The hemicellulose in corn mainly consists of complex and highly substituted glucuronoarabinoxylans (GAX) (*2*, *3*). The backbone of these GAX consists of β-D-(1→4)-linked xylopyranosyl units, which are highly substituted with monomeric α-L-arabinofuranosyl residues. These are linked at the O-2 and/or O-3 position of the xylose. Also, (4-*O*-methyl)-α-D-glucuronic acid and oligomeric side chains consisting of arabinose, xylose, and galactose (mainly at the terminal position) (*4*−*8*) can be part of GAX. Additionally, GAX containing ferulic acid substituents have been isolated from corn bran (*8*, *9*) in which the ferulic acid moieties are ester linked to the O-5 position of the arabinofuranosyl residues (*8*, *10*). Next to ferulic acid monomers, di-, tri-, and tetraferulates have been reported to be present in corn bran xylans (*11*−*13*). These studies indicate ferulate cross-links between xylan structures. Besides ferulic acid, also *O*-acetyl groups can be present (*14*, *15*). Acetyl groups linked at the O-2 and/or O-3 position of the xylose backbone residues have been described for hemicellulose from wood and grasses (*16*−*18*). Acetyl groups could also be attached to the arabinose residues as reported for an isolated xylan from bamboo shoots (*19*).

To obtain bioethanol in an efficient way from corn byproducts, the hemicellulose should be hydrolyzed into fermentable monosaccharides. Enzymatic hydrolysis of the GAX is preferred over chemical degradation because it is more environmentally friendly. Also, it requires less energy and results in fewer byproducts such as furfural (*20*), hindering fermentation reactions. However, due to the high complexity of the GAX and lack of knowledge on the exact structures present, no complete enzyme toolbox is available yet for complete degradation of GAX to monosaccharides.

As described above, several studies have been performed to determine structural features of corn hemicellulose, but it is particularly interesting to determine the structural features of the GAX that resist enzymatic hydrolysis, using industrial and experimental enzymes. Within this paper corn fiber was treated with hemicellulases after which the enzyme-resistant oligomeric GAX structures were isolated and studied. Corn fiber was pretreated by a mild dilute-acid treatment and enzymatically hydrolyzed by hemicellulases followed by fermentation to remove the fermentable monosaccharides (glucose, xylose, and arabinose) present. The remaining oligosaccharides, being resistant to enzymatic hydrolysis, were fractionated, and the sugar composition and amounts of acetic and ferulic acid were determined within each fraction. Subsequently, MALDI-TOF-MS analysis was

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used to tentatively characterize the oligosaccharides that were present.

MATERIALS AND METHODS

Enzyme-Resistant Corn Oligosaccharides. Corn fibers from a dry fractionation plant were milled, using a Retsch ZM200 mill (Retsch Gmbh, Haan, Germany) equipped with a 1 mm sieve. The fibers were dispersed in a sulfuric acid solution to final fiber and sulfuric acid contents of 11 and 0.22% (w/w), respectively, and pretreated for 30 min at 140 °C in an 1 L autoclave (benchtop reactor 4520; Parr Instrument Co., Moline, IL). Heating and cooling of the autoclave were forced within 5 min, by leading steam or cold water through tubing inside the autoclave. After the pretreatment, the pH was adjusted to 5 by adding KOH (5 M), and a pasteurization step for 10 min at 95 °C was performed. To determine the amounts of soluble and insoluble hemicellulose, the pretreated material was centrifuged (2000*g*, 10 min, room temperature). Total arabinose plus xylose contents (as representatives of the hemicellulose content) in the supernatant and pellet were determined as their alditol acetates by gas chromatography according to the method of Englyst (*21*), using inositol as an internal standard. The supernatant, pellet, and starting material were hydrolyzed according to the Seaman hydrolysis, using 72% (w/w) sulfuric acid at 30 °C for 1 h followed by a hydrolysis with 1 M sulfuric acid at 100 °C for 3 h. The amounts of xylose and arabinose detected were expressed as percentages of the amounts of xylose and arabinose present in the starting material (corn fiber) to determine how much of the hemicellulose became soluble after the pretreatment.

The supernatant (pH 5), containing the soluble hemicellulose, obtained after pretreatment was subjected to enzymatic hydrolysis and a fermentation by yeast, also referred to as simultaneous saccharification and fermentation (SSF). A commercial enzyme preparation, Spirizyme, and some experimental (hemi)cellulases described previously (*22*), including two α-arabinofuranosidases (*M. giganteus* GH51 and *H. insolens* GH43), one endoxylanase (*H. insolens* GH10) and one β-xylosidase (*T. reesei* GH3) obtained from Novozymes (Bagsvaerd, Denmark), were added to hydrolyze the accessible starch, cellulose, and hemicellulose. Additionally, two experimental Nedalco yeast strains (Bergen op Zoom, The Netherlands) were added to consume the fermentable monosaccharides released by the enzymes. The resulting product was heated to inactivate the yeast and filtered ($595^1/_2$ filter paper, ∅ 240 mm, Schleicher & Schuell, Dassel Germany), resulting in a final filtrate (2 L in total). To determine the degree of polymerization (DP) of the oligosaccharides present in the filtrate, it was analyzed by high-performance size exclusion chromatography (HPSEC). A Dionex Ultimate 3000 system (Dionex, Sunnyvale, CA) equipped with a set of four TSK-Gel super AW columns (Tosoh Bioscience, Tokyo, Japan) in series, guard column (6 mm i.d. × 40 mm) and separation columns 4000, 3000, and 2500 (6 mm i.d. × 150 mm), was used. Elution was performed with filtered aqueous 0.2 M sodium nitrate at 40 °C at a flow rate of 0.6 mL/min and followed by refractive index detection (Shodex RI, Showa Denko K.K., Kawasaki, Japan).

The final filtrate was applied to an Extract Clean C18 column (10 g of C18 material, 75 mL column volume, Grace, Deerfield, IL) in portions of 30 mL. The column was successively rinsed with water (40 mL) and methanol (80 mL) (J. T. Baker, Deventer, The Netherlands) resulting in two fractions: a water fraction, containing polar and/or charged oligosaccharides, and a methanol fraction, containing the more apolar oligosaccharides. The methanol fraction was evaporated under vacuum. As precipitation during the evaporation was observed, water was added to the methanol fraction to keep the oligosaccharides in solution. After evaporation of the methanol, both fractions were lyophilized before further fractionation.

Fractionation of Enzyme-Resistant Corn Oligosaccharides. *Polar and/or Charged Oligosaccharides*. The water fraction obtained after solid phase extraction (SPE) was separated on an AKTA explorer instrument (GE Healthcare, Uppsala, Sweden) equipped with a home-packed size exclusion Bio-Gel P2-column (900 × 26 mm; 200−400 mesh, Bio-Rad Laboratories, Hercules, CA). The column was thermostated at 60 °C, and elution was performed with distilled water (60 °C) at a flow rate of 1.0 mL/min. For each run 1 g of the water fraction dissolved in 5 mL of water was applied onto the column. Fractions of 8 mL were collected. The total sugar content of each fraction was analyzed by an automated orcinol/sulfuric acid assay (*23*), using an autoanalyzer (Skalar Analytical BV, Breda, The Netherlands), which resulted in an elution profile for the Bio-Gel P2 elution. Arabinose was used as a standard. Fractions were also analyzed for their oligosaccharide profile by high-performance anion exchange chromatography (HPAEC) as described below. Fractions were combined on the basis of the Bio-Gel P2 elution profile and the oligosaccharide profiles analyzed on HPAEC, resulting in six final fractions: W-1 (7% (w/w) of dry matter (DM)), W-2 (11% (w/w) of DM), W-3 (12% (w/w) of DM), W-4 (22% (w/w) of DM), W-5 (12% (w/w) of DM), and W-6 (14% (w/w) of DM). The average recovery of the DM applied on the Bio-Gel P2 column was 78% (w/w) (n = 2).

Apolar Oligosaccharides. Portions of the methanol fraction obtained after SPE (±250 mg dissolved in 2.5 mL of water, in total 2 g) were reapplied to a smaller C18 SPE column (tC18, 5 g of column material, 20 mL column volume, Waters, Milford, MA). Fractions were collected by subsequent elution with 10, 20, 30, 40, 50, and 100% (v/v) methanol. Each fraction was vacuum evaporated and lyophilized, resulting in six final fractions: M-1 (10% (w/w) of DM), M-2 (8% (w/w) of DM), M-3 (12% (w/w) of DM), M-4 (27% (w/w) of DM), M-5 (18% (w/w) of DM), and M-6 (11% (w/w) of DM). The total recovery was 86% (w/w) based on DM.

Protein Quantification. The nitrogen content of samples was determined using the combustion (DUMAS) method on a FlashEA 1112 series nitrogen and protein analyzer (Thermo Scientific, Waltham, MA). The instructions of the supplier were followed, and methionine was used as a standard. To convert nitrogen values to protein contents, a conversion factor of 6.25 was used.

Saccharide Analysis by HPAEC. *Monosaccharide Composition of the Oligosaccharides*. To determine the saccharide composition, samples (90−150 μg of DM) were dried at 40 °C under vacuum and methanolyzed with 1 mL of 2 M HCl in dry methanol for 16 h at 80 °C. Subsequently, samples were dried under a stream of air followed by hydrolysis with 2 N TFA at 121 °C for 1 h. Samples were again dried under a stream of air and dissolved in 1 mL of H_2O. The monosaccharides were analyzed by a HPAEC system (ICS-3000 system, Dionex) equipped with a CarboPac PA1 column (2 mm i.d. × 250 mm, Dionex), a CarboPac PA1 guard column (2 mm i.d. × 50 mm), and a pulsed amperometric detector (Dionex). The system was controlled by Chromeleon software (Dionex). The flow rate was 0.3 mL/min, and the mobile phase consisted of (A) H_2O, (B) 0.1 M NaOH, and (C) 1 M NaOAc in 0.1 M NaOH. The elution profile was as follows: first 30 min, isocratic at 100% A; 30−30.1 min, linear from 100% A to 100% B; 30.1−45.0 min, C linearly from 0 to 40%; 45.0−45.1 min, C linearly from 40 to 100%; 45.1−50.0 min, isocratic at 100% C; 50−50.1 min, C linearly from 100 to 0% and B from 0 to 100%; 50.1−58.0 min, isocratic at 100% B; 58.0−58.1 min, A linearly from 0 to 100%, followed by reconditioning of the column for 15 min. From 0 to 32 min and from 50.2 to 73 min postcolumn addition of 0.5 M NaOH at 0.1 mL/min was performed to detect the eluted saccharides. The samples were analyzed before and after hydrolysis to correct for the monosaccharides that were already present. The total amount of oligosaccharides was calculated by subtracting the total amount of monosaccharides present before hydrolysis from the total amount of monosaccharides present after hydrolysis.

Oligosaccharide Profile. The oligomeric profiles of all fractions (0.2−1.0 mg of DM/mL) were analyzed by using the same HPAEC equipment and column as described above, using another elution gradient. The flow rate was 0.3 mL/min, and 20 μL was injected. The mobile phase consisted of (A) 0.1 M NaOH and (B) 1 M NaOAc in 0.1 M NaOH. The elution profile was as follows: 0−45 min, B linearly from 0 to 40%; 45.0−45.1 min, B linearly from 40 to 100%; 45.1−48.0 min, isocratic at 100% B; 48.0−48.1 min, B linearly from 100 to 0%, followed by reconditioning of the column for 12 min.

Determination of Degree of Acetylation. About 10 mg of each fraction was saponified with 0.5 mL of 0.4 N NaOH in isopropanol/H_2O (1:1) for 3 h at room temperature. The acetic acid content was determined with an Ultimate 3000 system (Dionex) equipped with a Shodex RI detector and an Aminex HPX 87H column (300 mm × 7.8 mm) (Bio-Rad). Elution was performed with H_2SO_4 (5 mM) at a flow rate of 0.6 mL/min and a column oven temperature of 40 °C.

Determination of Degree of Feruloylation and Coumaroylation. For each fraction about 5 mg was dissolved in 200 μL of methanol. Subsequently, 5 mL of 0.5 M KOH (flushed with N_2) was added, and samples were put under N_2 at room temperature in the dark for 16 h. After 16 h, the pH was adjusted to ±2 by the addition of 0.75 mL of HCl (6 M).

The ferulic acid and coumaric acid released were extracted by 4 mL of ethyl acetate twice. The total ethyl acetate fraction was dried under N_2, and the residue was dissolved in 1 mL of methanol. The recoveries ($n = 5$) were 79% (±2 SE) and 102% (±2 SE) for ferulic acid and coumaric acid, respectively.

Analysis was performed on an Acella UHPLC system (Thermo Scientific) equipped with a photodiode array detector and coupled to an LTQ XL ion trap mass detector equipped with electrospray ionization source (Thermo Scientific). The system was controlled by Xcalibur software. Analysis was performed on a Hypersyl GOLD, 1.9 i.d. mm × 150 mm column with 1.9 μm particle size (Thermo Scientific). The mobile phase was composed of (A) H_2O + 1% (v/v) acetonitrile + 0.2% (v/v) acetic acid and (B) acetonitrile + 0.2% (v/v) acetic acid. The flow rate was 0.4 mL/min, and the column temperature was 30 °C. The elution profile was as follows: first 5 min, isocratic 0% B; 5−23 min, linear from 0 to 50% B; 23−24 min, linear from 50 to 100% B; 24−27 min, isocratic at 100% B; 27−28 min, linear from 100 to 0% B, followed by reconditioning of the column for 7 min. Spectral data were collected from 200 to 600 nm, and quantification was performed at 320 nm. Ferulic and coumaric acid contents were identified and quantified on the basis of standards.

MS data were collected in the negative mode with an ion spray voltage of 3.5 kV, a capillary voltage of −20 V, and a capillary temperature of 350 °C. Full MS scans were made within the range m/z 150−1500, and MS_2 data of the most intense ions were obtained.

Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). Each fraction (±5 mg/mL) was analyzed before and after alkali treatment to determine the presence of ester-linked moieties. Alkali treatment was performed by mixing 50 μL of a fraction with 50 μL of 0.1 N NaOH before incubation for 16 h at 4 °C. Subsequently, 50 μL of 0.1 M acetic acid was added. Samples were desalted by adding a spatula Dowex resin (AG 50W-X8 resin, H^+,100−200 mesh, Bio-Rad) to 30 μL of sample. After this, 5 μL of sample was mixed with 5 μL of sodium acetate buffer (30 mM), and from this 1 μL was mixed with 2 μL of matrix solution (2,5-dihydroxybenzoic acid, 10 mg/mL H_2O/ACN (3:7)) on a stainless steel metal plate. The samples were crystallized under a stream of air and analyzed on an Ultraflex workstation (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm laser and controlled by the software Flexanalysis. Analysis was performed in the positive mode with a laser intensity set at 32%. To calibrate the mass spectrometer, a mixture of maltodextrins (300−3000 Da) was used. After a delayed extraction time of 120 ns, the ions were accelerated with a 25 kV voltage and detected using reflector mode.

RESULTS AND DISCUSSION

Separation of Charged/Polar and Apolar Enzyme-Resistant Oligosaccharides. After an acidified corn fiber suspension had been heated in water at 140 °C for 30 min and then centrifuged, about 95% (w/w) of all hemicellulosic xylose and arabinose from the starting material was present as soluble mono- and oligosaccharides in the supernatant. The subsequent enzyme treatment resulted in partial hydrolysis of these oligosaccharides into xylose and arabinose monomers. Most of these monomers were removed during the fermentation. The remaining oligosaccharides after enzyme treatment, fermentation, and filtration (OS_{ER}) represented 50% (w/w) of the amount of arabinose and xylose that was present in the starting material. Size exclusion chromatography of this filtrate showed that the degree of polymerization was ≤12 (no further results shown). The structural characteristics of these oligosaccharides are of interest to determine the reason for their resistance toward enzymatic hydrolysis.

On the basis of MALDI-TOF-MS and HPAEC analyses (data not shown), the number of different enzyme-resistant oligosaccharide (OS_{ER}) structures in the filtrate was too large to be able to directly characterize them in detail. Therefore, a first separation of the OS_{ER} was performed with reversed phase material (C18), which is known to retain apolar groups such as ferulic acid (*24*) and, therefore, most likely also oligosaccharides containing ferulic acid. After a successive elution with water and methanol, full recovery of material, based on DM, was obtained.

Table 1. Sugar Composition of the Enzyme-Resistant Oligosaccahrides (OS_{ER}) and the Total Amount of OS_{ER} Present within Each Subfraction (% (w/w) of DM) Obtained after Fractionation of the Final SSF Filtrate from Corn Fiber on Bio-Gel P2 (W-1−6) and Reversed Phase Material (M-1−6)

	Xyl	Ara	Gal	Glc	Rha	Man	GalA	GlcA	total oligosaccharides
W-1	14.2	3.1	3.6	1.5	2.1	5.4	8.2	2.7	40.8
W-2	18.7	0.9	3.1	4.7	3.1	1.2	1.9	11.1	41.6
W-3	8.9	2.3	2.7	8.3	0.1	1.4	0.0	2.0	25.7
W-4	2.3	0.6	2.5	3.5	0.0	0.6	0.3	0.2	10.0
W-5	15.6	10.3	5.6	2.6	0.0	0.6	0.3	0.2	35.1
W-6	2.4	0.9	0.4	1.8	0.0	0.1	0.2	0.0	5.9
M-1	24.0	5.1	2.2	16.0	0.1	1.3	1.0	1.0	50.5
M-2	16.5	3.3	3.6	23.6	0.0	0.7	0.4	0.3	48.4
M-3	30.5	10.0	5.4	3.2	0.0	0.4	0.4	1.9	51.9
M-4	22.7	7.5	3.1	1.8	0.0	0.3	0.4	1.1	36.9
M-5	25.1	10.2	3.3	1.7	0.0	0.2	0.4	0.9	41.7
M-6	17.8	6.5	2.1	2.7	0.0	0.3	0.2	0.8	30.4

From the total amount of OS_{ER} applied, 74% (w/w) ended up in the water fraction and the other 26% (w/w) in the methanol fraction. Analysis of both fractions showed that they contained 27 and 42% (w/w) carbohydrates and 11 and 13% (w/w) protein for the water and methanol fractions, respectively. The remaining part of the water fraction is represented by furfural and salts, which are formed/added during the pretreatment. Neither furfural (*25*) nor salts are retained by reversed phase material. Also, ester-linked acetyl groups and acetic acid represents part of the DM (quantified and discussed later). Part of the acetyl groups might have been hydrolyzed into acetic acid due to pretreatment conditions.

Next to proteins and sugars, the methanol fraction also contains ester-linked feruloyl and acetyl groups (quantified and discussed later). Other phenolic compounds might also be present. Both the water and methanol fractions were further fractionated on the basis of size by elution on Bio-Gel P2 or polarity by a batchwise elution on reversed phase column material, respectively. These fractionations resulted in 12 subfractions in total.

Sugar Composition and Distribution of the 12 Enzyme-Resistant Oligosaccharide Subfractions. The total amount of oligosaccharides and the sugar composition (% (w/w) of DM) of each subfraction are presented in **Table 1**. All subfractions contained 30−50% (w/w) of oligosaccharides on the basis of DM, with the exception of subfractions W-4 (10% (w/w) oligosaccharides) and W-6 (5.9% (w/w) oligosaccharides). In subfraction W-6 all free monosaccharides were accumulated, resulting in a total sugar content of 61% (w/w) of DM.

In general, xylose was the predominant sugar as expected with GAX as main corn hemicellulose (**Table 1**). Some subfractions also contained relatively high amounts of galactose and/or glucose, which were sometimes even more abundant than xylose (W-4 and M-2). Galactose can be part of oligomeric side chains as described for corn hemicellulose (*4*). However, it is questionable if the amounts detected in certain subfractions all originate from hemicellulosic fragments. Cellulodextrins that have been accumulated within certain subfractions could very well account for part of the glucose. In addition, both glucose and mannose could also be products from the fermentation because yeast is known to produce glucomannans as exopolysaccharide, which can easily be extracted (*26*).

Besides neutral sugars, also uronic acids were present, which were mainly accumulated within subfractions W-1 and W-2. Bio-Gel P2 material is known to contain some negative charge,

resulting in some exclusion of uronic acids (*27*) and thereby earlier elution as expected on the basis of their size. Subfraction W-1 contained mainly galacturonic acid (GalA), whereas W-2 was rich in glucuronic acid (GlcA).

Within cereals both GlcA and its methylated form (MeGlcA) can be present (*2*), and usually one of these forms predominates. For corn both MeGlcA and GlcA have been reported to be present (*28*), but information on their ratio is lacking. Quantification or identification has often not been possible due to the use of colorimetric methods that cannot distinguish between both forms (*3*, *29*) and/or the lack of use of MeGlcA as a standard. We obtained MeGlcA by incubation of glururonoxylooligosaccharides (aldouronic acids, Sigma, St. Louis, MO) with an α-glucuronidase. Within the water fraction, only GlcA was present on the basis of HPAEC analysis and comparison to the GlcA and MeGlcA standards (results not shown). The GalA in the W-1 fraction might originate from pectin fragments as also rhamnose was present in this fraction. However, GalA as a part of GAX should also not be excluded. Recently, the presence of a sequence of glycosyl residues, containing GalA and rhamnose at the reducing ends of glucuronoxylans from wood, spruce, and *Arabidopsis*, has been reported (*30*).

Table 2. Distribution of the Monosaccharides (mono) and Oligosaccharides (oligo) among All Water (W-1−6) and Methanol (M-1−6) Subfractions Expressed as a Percentage of the Monosaccharides and Oligosaccharides That Were Present in the Final Filtrate from Corn Fiber That Was Obtained after Saccharification and Fermentation (SSF)

	water		methanol	
subfraction	mono	oligo	mono	oligo
1	0.7	9.2	1.1	5.7
2	0.9	13.8	0.2	4.0
3	4.0	9.2	0.3	6.6
4	1.0	6.9	0.7	11.2
5	5.1	13.4	0.4	8.1
6	91.2	2.6	0.1	3.8
total	102.9	55.1	2.8	39.4

On the basis of the amount of monosaccharides and OS_{ER} within each subfraction and the distribution of the DM among the subfractions, the absolute amounts of mono- or oligosaccharides within each subfraction were calculated. These values were expressed as a percentage of the monomers (predominantly arabinose) and OS_{ER} that were present in the initial supernatant (pretreated and subjected to SSF) (**Table 2**). The recovery of monosaccharides was 105.7% and that for oligosaccharides, 94.5%. Most of the monosaccharides (91% (w/w) of total monosaccharides) ended up in the W-6 subfraction. The OS_{ER} were more equally distributed among all 12 subfractions, and only 5.5% (w/w) of the OS_{ER} was lost during fractionation.

Sugar and Ester-Linked Substituents of OS_{ER}. *Sugars*. Quantification of the substituents is important to determine the degree of substitution (DS) of the xylan, which reflects the complexity with respect to enzyme degradation. To calculate the DS within the subfractions, all xylose was expressed as backbone components. The amounts of arabinose, galactose, GlcA, acetic acid, ferulic acid, and coumaric acid were expressed as moles of substituent per 100 mol of xylose residues (**Figure 1**), also referred to as $DS_{Substituent}$.

On the basis of these data all subfractions are heavily substituted with multiple substituents. In general, the DS_{Ara} ranged from 20 to 40. Subfraction W-5 stood out against the other subfractions with a DS_{Ara} of 66. The methanol subfractions generally contained a bit more galactose (DS_{Gal} 13−29) compared to the water subfractions (DS_{Gal} 7−18). Also, for this sugar substituent one subfraction (W-4) stood out with a DS_{Gal} of 89. As mentioned earlier, GlcA mainly accumulated within the

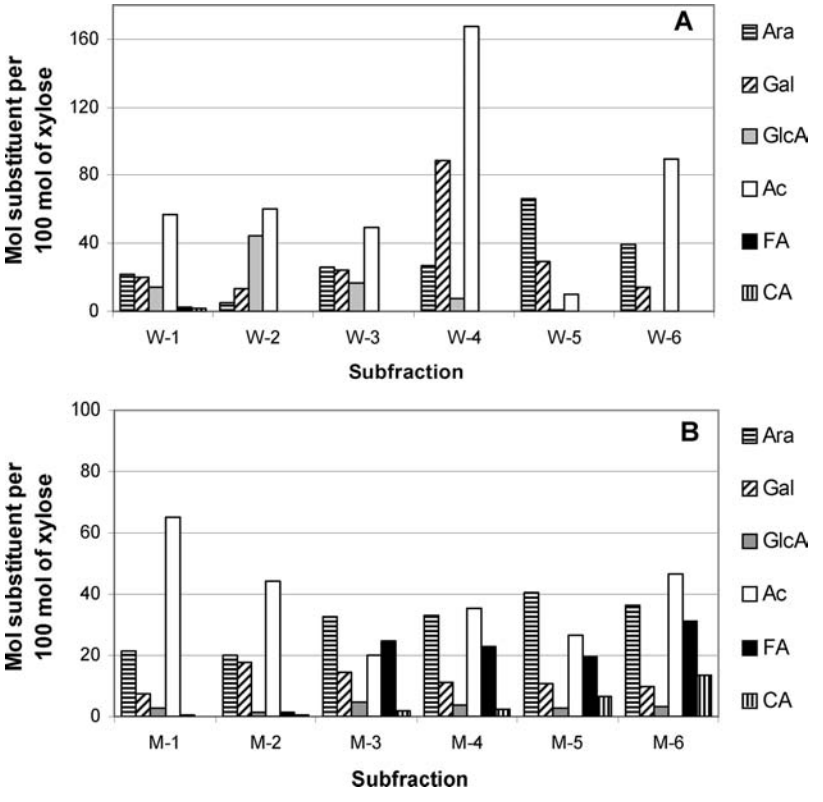

Figure 1. Substituents that are present within each water (**A**) and methanol (**B**) subfraction expressed as moles of substituent per 100 mol of xylose residues. Abbreviations: Ara, arabinose; Gal, galactose; GlcA, glucuronic acid; Ac, acetyl; FA, ferulic acid; CA, coumaric acid.

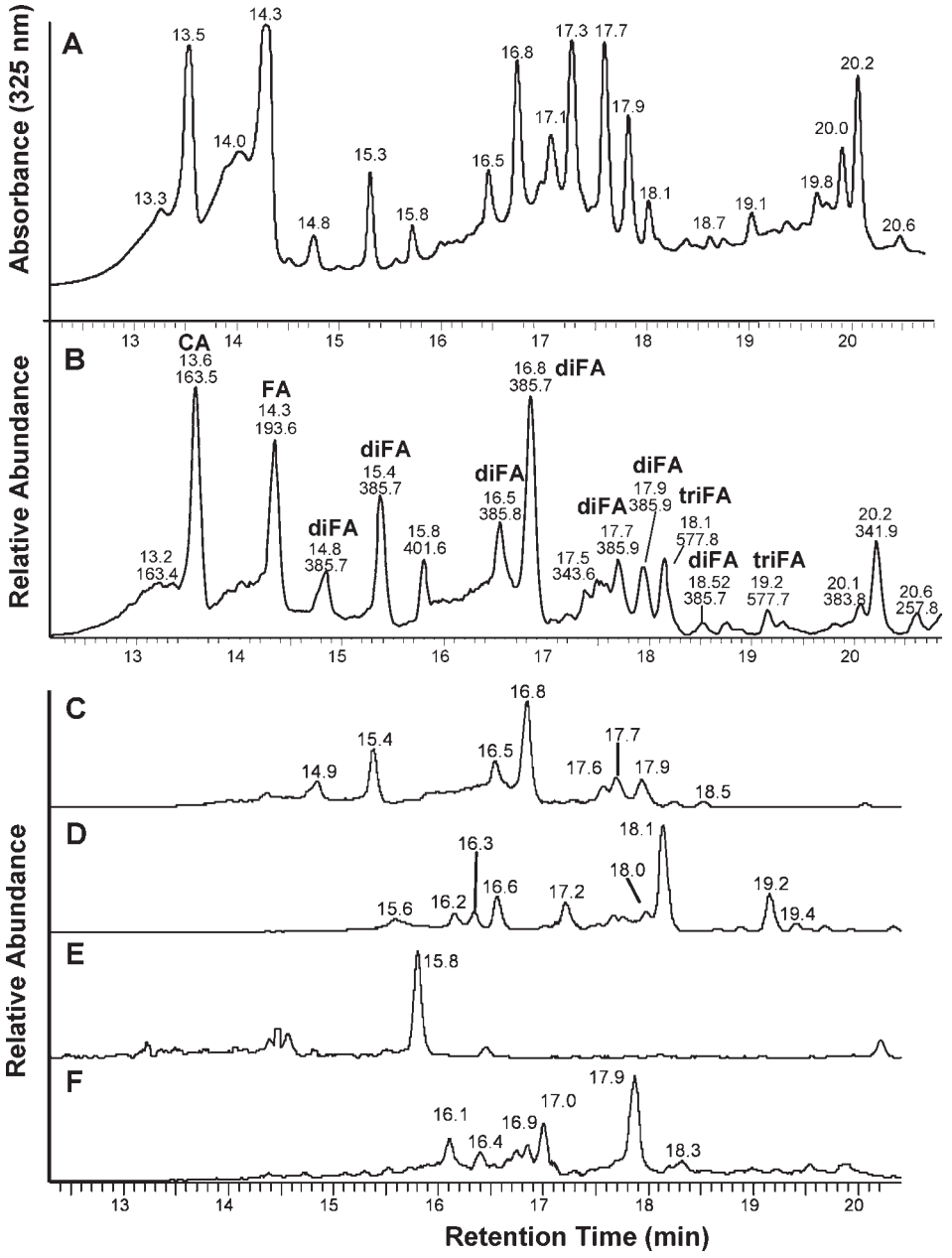

Figure 2. Analysis of an ethyl acetate extract from alkali-treated resistant oligosaccharides from corn fiber (subfraction M-6) by RP-UHPLC-UV-MS: (**A**) UV chromatogram; (**B**) base peak MS spectrum; (**C**−**F**) elution profiles of the *m*/*z* values 385, 577, 403, and 595, respectively, which were subtracted from the MS data. Retention times (and *m*/*z* values beneath them) are given. Abbreviations: CA, coumaric acid; FA, ferulic acid; diFA, diferulate; triFA, triferulate.

first couple of water subfractions with the highest DS_{GlcA} of 44 within W-2.

O-Acetyl. Besides sugar substituents, also *O*-acetyl groups were highly abundant with the highest value for subfraction W-4: 167. Attachment of the acetyl to sugar residues other than xylose (*19*) should be considered to explain such a high DS_{Ac}.

Phenolic Acids. The ester-linked phenolics ferulic acid and coumaric acid were highly abundant within subfractions M-3−6. This showed that oligosaccharides, containing these apolar phenolics, are retained by reversed phase material and eluted with ≥30% (v/v) methanol/water. The degrees of ferulic acid substitution within fractions M-3−6 were similar, but the coumaric acid substitution increased.

Next to monomeric ferulic acid also several di- and triferulates were detected on the basis of UV (**Figure 2A**) and MS detection (**Figure 2B**) after alkali treatment of the subfractions. Ethyl acetate extracts obtained before alkali treatment did not contain di- and triferulates, which confirms they are ester linked to the OS_{ER}. Seven different diferulate structures have been described to be present in grass cell walls, which have a molecular mass of 386 Da (*31*). On the basis of the *m*/*z* values from the base peak profile (**Figure 2B**), seven peaks corresponded to diferulates (*m*/*z* 385 $[M - H]^-$) and two peaks corresponded to triferulates (*m*/*z* 577 $[M - H]^-$). Next to the diferulates described (*31*), formation of a tetradehydrofuran due to water addition has been reported for both di- and triferulates (*11*, *32*), resulting in molecular masses of 404 and 596, respectively. Panels **C**−**F** of **Figure 2** represent spectra of the occurrence of *m*/*z* 385, 577, 403, and 595 in time, respectively, that have been extracted from the total MS data that were recorded (scans from 150 to 1500 Da). Indeed, more triferulates could be distinguished compared to the base peak profile, and new peaks that correspond to di- and triferulates, containing one tetrahydrofuran ring (*m*/*z* of 403 and 595, respectively), were detected (**Figure 2E**, **F**). All of these

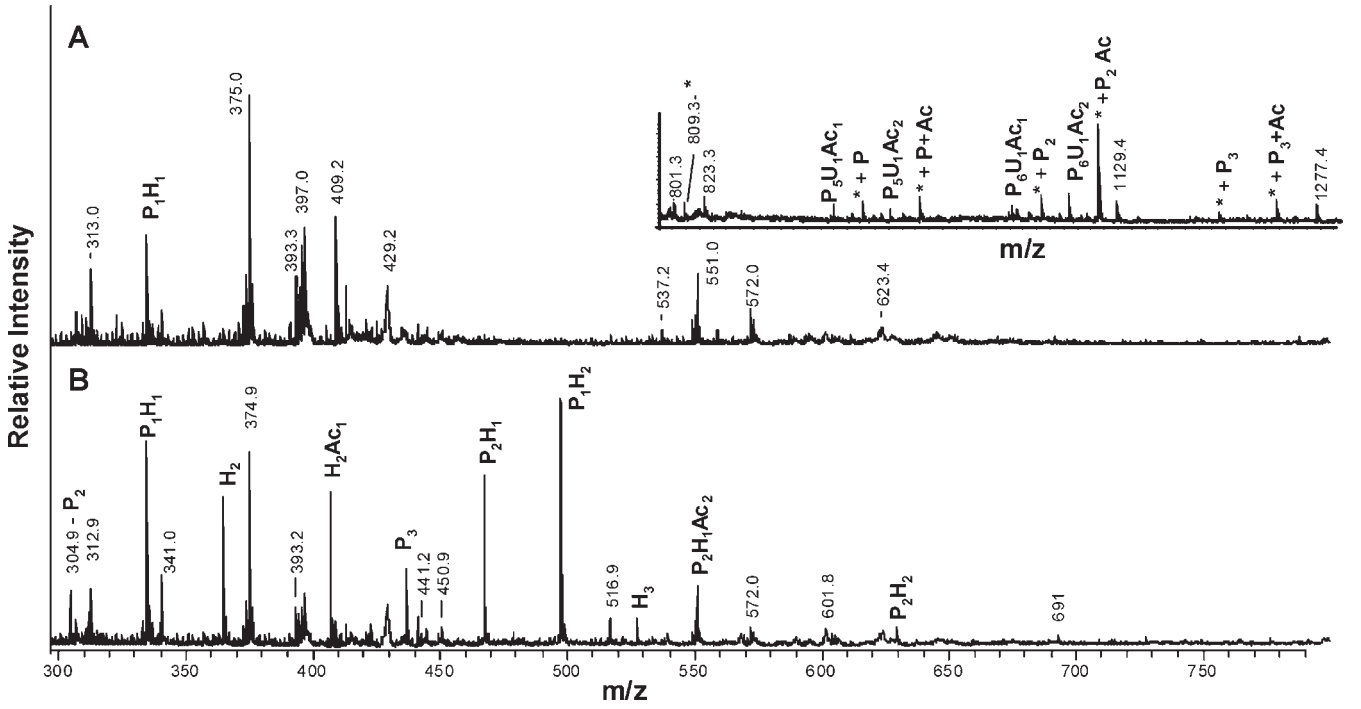

Figure 3. MALDI-TOF mass spectrum of (**A**) W-1 and (**B**) W-4. The *m*/*z* values detected in a higher mass range are given in the inset. If possible, peaks were tentatively annotated (P, pentose; H, hexose; U, uronic acid; Ume, *O*-methylated uronic acid; Ac, acetyl group).

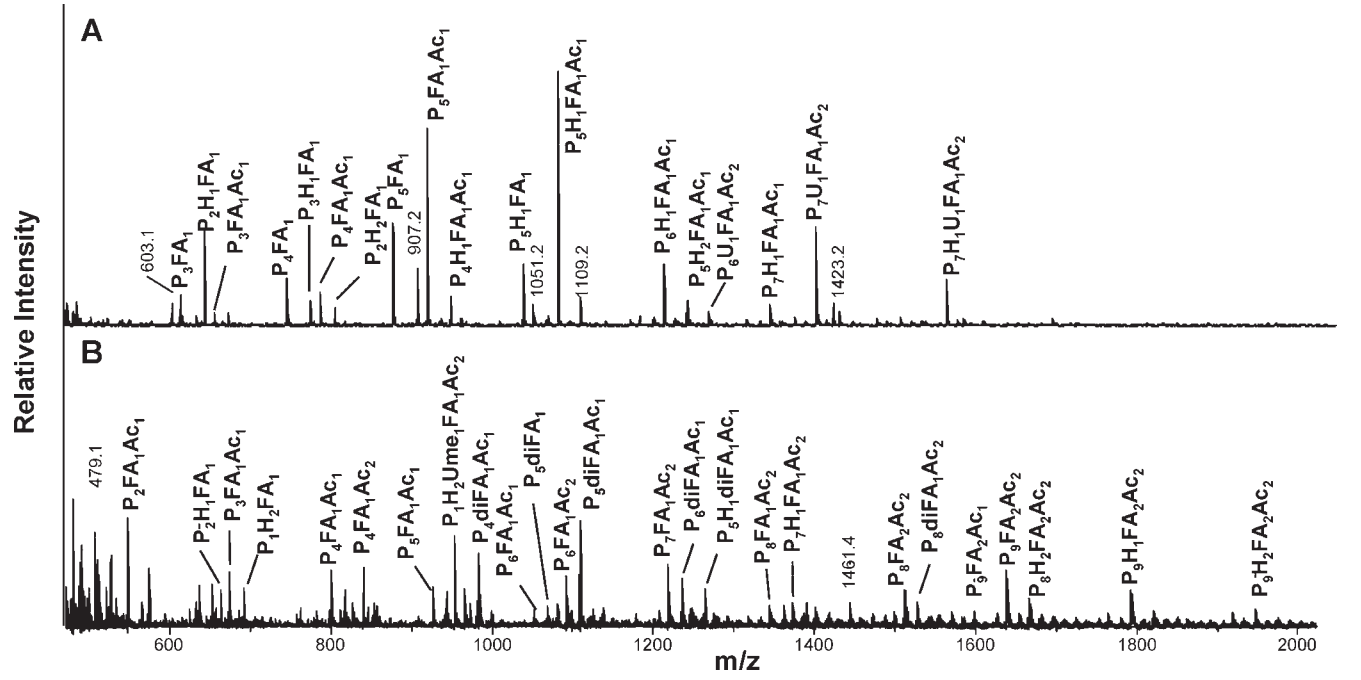

Figure 4. MALDI-TOF mass spectrum of (**A**) M-3 and (**B**) M-5. If possible, peaks were tentatively annotated (P, pentose; H, hexose; U, uronic acid; Ume, *O*-methylated uronic acid; FA, ferulic acid; diFA, diferulate; Ac, acetyl group).

di- and triferulates have not been accounted for in the quantification due to the lack of standards and poor resolution. This means that the degree of substitution is even larger than indicated in **Figure 1**. From **Figure 2C**−**F** 9 different diferulates, including tetrahydrofuran, and 15 different triferulates, of which 6 were tetrahydrofurans, were detected. The 8 diferulates from **Figure 2** might include isomeric structures because only 4 particular diferulates have been reported as main diferulates present in many plant sources, including corn (*31*, *33*).

Annotation of the Enzyme-Resistant Oligosaccharides Present. MALDI-TOF-MS was used to characterize the oligosaccharides in more detail. Only oligomers of 300 Da and higher are detectable by this MS technique. To verify the presence of acetic acid, ferulic acid, and coumaric acid by MALDI-TOF-MS, all subfractions were analyzed before and after alkali treatment. In this way it is possible to discriminate between uronic acids and ferulic acids (FA), which have similar *m*/*z* values. Furthermore, a distinction between pentoses and hexoses can be made. On the basis of these data most OS_{ER} were tentatively annotated. **Figures 3** and **4** show examples of MALDI-TOF-MS spectra, and **Tables 3** and **4** summarize the compositions of all oligosaccharides that were detected and could be tentatively identified.

W-1−6 Subfractions. In general, the DP decreases from W-1 to W-6 (**Table 3**), as expected after fractionation from Bio-Gel P2. MALDI-TOF-MS confirmed that oligosaccharides that contain uronic acids were accumulated within subfractions W-1 and W-2 (**Table 1**). For example, within the OS_{ER} from W-1, oligosaccharides corresponding to $P_5U_1Ac_{1-2}$ (m/z 919 + 961) and $P_6U_1Ac_{1-2}$ (m/z 1051 + 1093) were present (**Figure 3**). This annotation was supported by the presence of P_5U_1 (m/z 877) and P_6U_1 (m/z 1009) after alkali treatment. Fractions W-3, W-4, and W-5 contained oligosaccharides that were similar in their compositions. As an example, the mass spectra of W-4 are given in **Figure 3**. The pentose series P_{2-3} is assumed to be a combination of xyloses and arabinoses based on the sugar analysis (**Table 1** and **Figure 1**). Additionally, these structures were not hydrolyzed during enzyme treatment, which would have been expected if they consisted of only xylose. The hexose series could represent cellulodextrins as all fractions contained glucose (**Table 1**). The detection of pentose oligomers with hexose substitution (P_5H_1 or $P_2H_1(Ac_2)$ in subfractions W-2, W-4, and W-5) suggests the presence of oligomeric side chains, containing xylose, arabinose (*7*), and galactose (*5*, *8*). However, several oligosaccharides contained more hexoses than pentoses (P_1H_6 or $P_{1-2}H_5$ in subfraction W-2). These structures might represent a part where GAX is linked to other polysaccharides. Not all peaks that were detected could be annotated. For subfraction W-1, hardly any peak detected in the lower mass range (< 800) could be identified. Furthermore, a series of unidentified peaks were detected that had mass differences of pentose units (+132 Da) or acetyl groups (+42 Da), which starts at m/z 809 indicated by the asterisk (**Figure 3**). After alkali treatment, these peaks disappeared and masses lacking one or more acetyl groups (-42_n) appeared (e.g., 809 − 42 = 767) with again differences of 132 Da (pentose) (m/z 767, 899, 1031, 1163).

From **Table 3** it can be estimated that at least 38 different oligosaccharides are present, reflecting the complexity of the OS_{ER} that are left in the water subfractions

M-1−6 Subfractions. The separation on reversed phase material was not based on DP, but highly depended on the presence of acetyl groups, ferulic acid, and diferulates. Many OS_{ER} could be identified tentatively (**Table 4**), which showed that with each methanol subfraction the oligomeric complexity increases. The OS_{ER} present in M-1 and M-2 had similar compositions and differed only in their DP, which was higher in M-2. The masses detected in M-2 matched with pentose series P_{2-6} (m/z 569, 701, and 833), but after alkali treatment, all peaks disappeared and peaks of 42 or 84 Da lower appeared. This shows that both M-1 and M-2 were composed of pentose/hexose combinations with one or two acetyl groups, in which the hexoses dominated. On the basis of the sugar composition of these two subfractions (**Table 1**) it is likely that the oligosaccharides are composed of xylose and glucose units. Within subfractions M-3−6 no oligosaccharides without a FA or a diFA substituent were detected. The MALDI-TOF mass spectra of subfractions M-3 and M-5 are given in **Figure 4**. Besides a ferulic acid group, subfraction M-3 contains pentose oligomers with or without one hexose and in most cases one or two additional acetyl groups. After alkali treatment, indeed a pentose series (sometimes with one additional hexose) appeared, which additionally could contain a substituent with a m/z of 176. This could indicate that either saponification was not complete and still one ferulic acid group is left or a uronic acid group is present. After alkali treatment, the oligosaccharides were not retained anymore on reversed phase material. This means that the m/z of 176 more likely represents a uronic acid rather than a ferulic acid. Therefore, oligosaccharides (m/z 1269, 1401, and 1563) with two m/z 176 substituents, of which one is alkali labile, contain both a ferulic acid and a uronic acid group. OS_{ER} detected within M-4 were similar in their compositions compared to M-3 (**Table 4**). Within the M-5 subfraction the DP is higher compared to M-3 and M-4. The degree of acetylation is also higher. Even more, hardly any oligosaccharide without an acetyl group is present, which explains the increased affinity toward reversed phase material. Furthermore, several oligosaccharides (m/z 1533, 1623, 1665, 1695, 1827, and 1989) containing a diferulate (+ 368 Da) were present. The composition of OS_{ER} detected within the M-6 subfraction

Table 3. Oligosaccharide Compositions Present in Each Water Subfraction (W-1−6) Tentatively Identified on the Basis of Masses (m/z) Detected with MALDI-TOF-MS before and after Removal of Ester-Linked Moieties[a]

W-1	W-2	W-3	W-4	W-5	W-6
$P_{1-3}H_5$	H_{5-7}	H_{4-7}	P_{2-3}	P_2	P_1
$P_{5-6}U_1Ac_{1-2}$	$H_{5-6}Ac_1$	$P_4{}_{-6}Ac_1$	H_{2-3}	H_2	H_1
$P_4U_1Ac_1$	$P_{1-2}H_5$	P_1H_3	H_2Ac_1	H_2Ac_1	
	P_1H_6	P_2H_{2-3}	$P_{1-2}H_{1-2}$	P_1H_{1-2}	
	P_5H_1	$P_1H_{3-4}Ac_1$	$P_2H_1Ac_2$	P_2H_1	
	$P_{5-6}H_1Ac_1$			$P_2H_1Ac_2$	
	$P_2H_1Ac_2$				
	$P_{1-2}Ume_1Ac_1$				
	$P_{2/4}U_1Ac_1$				

[a] For example, $P_{1-2}H_5$ means that both P_1H_5 and P_2H_5 have been identified. Abbreviations: P, pentose; H, hexose; Ac, acetyl; U, uronic acid; Ume, methylated uronic acid.

Table 4. Oligosaccharide Compositions Present in Each Methanol Subfraction (M-1−6) Tentatively Identified on the Basis of Masses (m/z) Detected with MALDI-TOF-MS before and after Removal of Ester-Linked Moieties[a]

M-1	M-2	M-3	M-4	M-5	M-6
P_4	P_4	$P_{3-5}FA_1$	$P_4{}_{-7}FA_1Ac_1$	$P_2H_1FA_1$	$P_4{}_{-9}FA_1Ac_2$
P_2H_3	P_3H_5	$P_2{}_{-6}H_1FA_1$	$P_8FA_1Ac_2$	$P_1H_2FA_1$	$P_6{}_{-8}U_1FA_1Ac_2$
P_4Ac_1	P_4Ac_1	$P_2H_2FA_1$	$P_4{}_{-7}H_1FA_1Ac_1$	$P_2{}_{-6}FA_1Ac_1$	$P_7{}_{-8}H_1U_1FA_1Ac_2$
H_3Ac_2	H_3Ac_2	$P_3{}_{-7}FA_1Ac_1$	$P_5{}_{-6}U_1FA_1$	$P_4{}_{-8}FA_1Ac_2$	$P_6{}_{-8}H_2U_1FA_1Ac_2$
$P_{1-2}H_{3-4}Ac_{1-2}$	$P_1H_{3-4}Ac_{1-2}$	$P_4{}_{-7}H_1FA_1Ac_1$	$P_6H_1U_1FA_1$	$P_7H_1FA_1Ac_2$	$P_7{}_{-8}H_3U_1FA_1Ac_2$
	$P_2H_3Ac_1$	$P_5H_2FA_1Ac_1$	$P_2{}_{-5}diFA_1$	$P_9U_1FA_1Ac_1$	$P_3{}_{-6}diFA_1Ac_1$
	$P_3H_4{}_{-5}Ac_1$	$P_6{}_{-7}H_1U_1FA_1$	$P_3{}_{-8}diFA_1Ac_1$	$P_9U_1FA_1Ac_1$	$P_3{}_{-5}H_1{}_{-2}diFA_1Ac_1$
	$P_2H_4{}_{-5}Ac_{1-2}$	$P_6{}_{-7}U_1FA_1Ac_2$	$P_6{}_{-7}H_1diFA_1Ac_1$	$P_8{}_{-9}H_2U_1FA_1Ac_2$	
				$P_9H_1U_1FA_1Ac_2$	
				$P_1H_2Ume_1FA_1Ac_1$	
				P_5diFA_1	
				$P_4{}_{-8}diFA_1Ac_1$	
				$P_4{}_{-5}H_1diFA_1Ac_1$	

[a] For example, $P_2H_4{}_{-5}Ac_{1-2}$ means that $P_2H_4Ac_1$, $P_2H_4Ac_2$, $P_2H_5Ac_1$, and $P_2H_5Ac_2$ have been identified. Abbreviations: P, pentose; H, hexose; Ac, acetyl; U, uronic acid; Ume, methylated uronic acid; FA, ferulic acid; diFA, diferulate.

showed high similarity with the M-5 fraction. Only the number of OS_{ER} containing diferulates or two acetyl groups was higher.

In comparison to the water subfractions, the methanol subfractions are even more complex, with around 90 different oligosaccharide structures that were present (**Table 4**).

In conclusion, it can be stated that many complex xylan-based oligosaccharides, representing 50% (w/w) of the starting material, survived pretreatment, enzymatic hydrolysis, and fermentation. These structures have in common that several substituents such as ferulic acid, diferulates, acetic acid, galactose, arabinose, and uronic acids are combined within one oligomer. To fully hydrolyze such oligomers, novel accesory enzymes, which are highly tolerant to the presence of other different substituents, are required. Fractionation resulted in 12 fractions containing structurally different enzyme-resistant oligosaccharides. These fractions will be used for further analysis to fully characterize the oligosaccharides.

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