Carbohydrate Polymers 86 (2011) 722–731

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Characterization of substituents in xylans from corn cobs and stover

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ARTICLE INFO

Article history:
Received 23 March 2011
Received in revised form 28 April 2011
Accepted 11 May 2011
Available online 18 May 2011

Keywords:
Corn stover
Cobs
Xylan
Ferulic
Coumaric acid

ABSTRACT

Structural knowledge on hemicellulose from corn cobs and stover is helpful to better understand their position within the plant cell wall architecture as well as their enzymatic saccharification. In this research different extracts were prepared with water, 1 M and 4 M alkali. Most of the xylans were yielded in the 1 M alkali fractions. The xylans were substituted with arabinose and glucuronic acid; 9 and 5 (cobs) and 14 and 9 (stover) per 100 xylosyl residues, respectively. Also esterlinked groups were present, like acetic, ferulic and coumaric acids; 49, 4 and 6 (cobs) and 39, 2 and 5 per 100 xylosyl residues (stover), respectively. Incubation with a well-characterized endoxylanase showed a more blockwise distribution of side-groups for stover xylans compared to cobs. Analysis of soluble fractions of dilute acid treated cobs and stover showed that especially feruloylated oligomers were resistant.

1. Introduction

Sustainable production of fuels or chemicals should preferably involve agricultural and forest biomasses, which are not competitive with the production of food and feed (Schubert, 2006). Examples of such feedstocks are corn fibers, cobs and stover, all three being byproducts of the currently existing ethanol production based on starch from kernels. The interest in such byproducts as a source for production of fuel ethanol has been described frequently. A good example is our recent paper (Van Eylen, Van Dongen, Kabel, & de Bont, 2011), which showed that a recombinant xylose-fermenting *Saccharomyces cerevisiae* was able to produce ethanol from all xylose and glucose released from corn fibers, cobs and stover. Still, a bottleneck is the maximal release of fermentable sugars from the rigid biomass structure, usually accomplished by combining a pretreatment with an enzymatic saccharification. In this perspective, the architecture of the plant cell wall including carbohydrate composition of the biomasses studied is an important research aspect.

Corn fibers, cobs and stover are composed of mainly lignin, cellulose and the hemicellulose xylan. Xylans per definition consist of a backbone of β-1,4-xylosyl residues, and can be substituted with for example α-L-arabinofuranose, α-D-(4-*O*-methyl) glucuronic acid, and *O*-acetyl esters (Scheller & Ulvskov, 2010). Furthermore, xylans are shown to be interlinked with other xylan structures and even with lignin via ferulic acid bridges connected to the arabinofuranose substituents on the xylan backbone. The exact type and amount of substitutions depend on the kind of feedstock and type of plant tissue studied (Ishii, 1997).

Information about the general compositions of corn fiber or corn pericarp has been presented frequently, but only a few papers describe detailed xylan structures (Muralikrishna & Rao, 2007; Saulnier, Vigouroux, & Thibault, 1995).

Recent work is by Appeldoorn, Kabel, Van Eylen, Gruppen, and Schols (2010) describing detailed xylan structures originating from corn fibers. Not only attention was paid to carbohydrate substituents on the xylan backbone, but also the type and amounts of esterified acetic and ferulic acids present were highlighted. The main conclusions from this paper were that corn fiber xylan structures are very complex, and difficult to saccharify.

For corn cobs and stover detailed structural characterizations of the xylan structures present are rather rare. An exception for corn cobs is the work of Hromádková, Kováciková, and Ebringerová (1999), whereas for corn stover, in recent work of Naran, Black, Decker, and Azadi (2009) attention was paid to the structural elucidation of the xylan structure.

In this paper we focus on the characterization of xylan structures from corn cobs and corn stover with the use of various chromatographic and mass spectrometric techniques. The distribution of carbohydrate substituents present was determined by the aid of an endoxylanase with known substrate specificity. Also, attention was paid to the presence of *O*-acetyl, ferulic and coumaric acid esters in corn cobs and stover.

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doi:10.1016/j.carbpol.2011.05.007

2. Experimental

2.1. Feedstock materials

Corn cobs, 95% (w/w) dry matter (DM), and stover, 90% (w/w) DM, were kindly provided by ADM (Decatur, IL, USA). The cobs and stover were dry milled by using a Retsch ZM200 Mill (Retsch GMBH, Haan, Germany) equipped with a 1.0 mm sieve before use.

2.2. Preparation of water and alkali extracts

Milled cobs (30 g) and stover (30 g) were suspended in distilled water, 400 mL and 800 mL, respectively, and extracted for 24 h at 65 °C under continuous stirring. After centrifugation (10,000 × *g*; 30 min; 20 °C), the residues were re-extracted with distilled water at 65 °C. All supernatants were combined and collected as water soluble solids (WSS). The corresponding residues were recovered as water unextractable solids (WUS). Subsequently, WUS (10 g) were suspended in 200 mL 1 M KOH containing 0.26 M $NaBH_4$, for 24 h at room temperature under continuous stirring. After centrifugation (10,000 × *g*; 30 min; 20 °C), the residues were re-extracted once with 1 M KOH with 0.26 M $NaBH_4$ for 24 h at room temperature under continuous stirring. The supernatants were combined and coded '1 M KOHss'. The residues were re-extracted three times with 4 M KOH containing 0.26 M $NaBH_4$. The supernatants were combined and coded '4 M KOHss'. The final residues were collected and coded 'Res'. All fractions were neutralized with acetic acid, dialyzed against distilled water and freeze-dried.

2.3. Preparation of dilute acid extracts

Dilute acid extractions were performed with milled corn feedstocks at temperatures of 160–180 °C by using a 1 L Parr (Moline, IL, USA) bench scale high pressure reactor. To increase the heating rate, a steam coil was installed in the reactor vessel. During the heating phase steam at 20 bar was sent through the coil. During the treatment temperature was controlled electrically (PID-controller). After the desired treatment time, the feedstock was rapidly cooled by sending water (15 °C) through the coil. Typical maximum heating and cooling rates were 87 and 60 °C min^{-1}, respectively. During treatment the feedstock was continuously stirred at 300 rpm. The conditions for pretreatment of cobs were: 1% (w/w) sulphuric acid, based on DM, 10 g DM feedstock per 100 g total mass; 10 min at 160 °C. The conditions of pretreatment of stover were: 2% (w/w) sulphuric acid, based on DM, 7.5 g DM feedstock per 100 g total mass; 10 min at 180 °C. The pretreatment conditions were chosen as such that a high percentage of oligomers ended up in soluble state (Van Eylen et al., 2011). Samples were centrifuged (1500 × *g*; 15 min; 25 °C) and the supernatants were collected. Residues were washed three times with distilled water, and the washing water was added to the supernatants. Washed residues are referred to as 'Solid'. The combined supernatants and washing water are referred to as 'Liquid'. Solids and Liquids were freeze-dried.

2.4. Neutral sugar composition

The neutral sugar composition was determined by gas chromatography (GC) according to Englyst and Cummings (1984), using inositol as internal standard. The samples were treated with 72% (w/w) H_2SO_4 (1 h, 30 °C) followed by hydrolysis with 1 M H_2SO_4 for 3 h at 100 °C. The constituent sugars released were analyzed as their alditol acetates using GC.

2.5. Uronic acid content

The uronic acid content was determined as anhydro-uronic acid by an automated m-hydroxydiphenyl assay (Blumenkranz & Asboehan, 1973) with an auto-analyzer (Skalar Analytical BV, Breda, The Netherlands). Glucuronic acid (Fluka AG, Buchs, Switzerland) was used as a reference in a concentration range from 12.5 to 200 μg mL^{-1}.

2.6. Acetic acid content

Feedstock material, and corresponding WUS and WSS-fractions (10 mg) were saponified, in duplicate, by addition of 1 mL 0.4 M NaOH in isopropanol/water (1:1 (v/v)). Also, material was analyzed to which no alkali was added in order to correct for the presence of free acetic acid. Samples (100 μL) were subjected to high performance liquid chromatography (HPLC), using a Dionex (Sunnyvale, USA) Ultimate 3000 UPLC-system equipped with a Shodex RI-101 (Kawasaki, Japan) refractive index (RI) detector and an Animex HPX 87H column (300 mm × 7.8 mm) (Bio-Rad, Hercules, USA) plus pre-column (Voragen, Schols, & Pilnik, 1986). Elution was performed by using 5 mM H_2SO_4 as eluent at a flow rate of 0.6 mL min^{-1} at 40 °C.

2.7. Ferulic and coumaric acid content

Feedstock material, WUS and Liquid (10 mg DM per sample) cobs and stover were put in a tube (16 mm × 100 mm) (Corning Incorporated, New York, USA), to which subsequently 200 μL methanol and 5 mL KOH (0.5 M, flushed with N_2) was added. The tubes were flushed with N_2, closed and put in dark surroundings at room temperature (20 °C).

After 16 h, 0.75 mL HCl (6 M) was added to adjust the pH-value <2. Ferulic and coumaric acid were extracted by using 4 mL ethylacetate twice.

The ethylacetate fraction was dried using N_2 at 20 °C, and the residue was re-dissolved in 1 mL methanol. 180 μL was transferred to vials, from which 10 μL was subjected to ultra-high performance liquid chromatography (U-HPLC). Also, material was analyzed to which no alkali was added in order to determine the free ferulic and coumaric acid content. U-HPLC analysis was performed as described by Appeldoorn et al. (2010).

2.8. Lignin content

To 300 mg DM feedstock, WUS and WSS from cobs and stover, 3 mL 72% (w/w) H_2SO_4 was added. Samples were hydrolyzed for 1 h at 30 °C. After this prehydrolysis, 37 mL distilled water was added to each sample and samples were put in a boiling water bath for 3 h. Each half hour samples were shaken. Samples were filtered over G4 glass filters, which were dried overnight and from which the mass was determined before analysis. The residues were washed until they were free of acid and dried in the filter overnight at 105 °C. The weight of the dried residues was taken as the content of acid insoluble lignin (AIL).

Acid soluble lignin (ASL) was determined by measuring the UV-absorbance of the filtrate at 205 nm. Samples were diluted with 1 M H_2SO_4 until absorption values, relative to a solution of 1 M H_2SO_4, were between 0.1 and 0.8. The ASL was calculated as: ASL (% w/w)) = $((A \times B \times C)/(D \times E)) \times 100\%$, where A was the absorption relative to 1 M H_2SO_4, B was the dilution factor, C was the volume of the filtrate (0.040 L), D was the extinction coefficient of lignin (110 g L^{-1} cm^{-1}, which is consistent with the value used in the TAPPI procedure and which represents an average of the values found for different woods and pulps) and E was the weight of dry matter of sample before acid hydrolysis (0.30 g).

2.9. Protein content

Protein content was calculated from the nitrogen content of the material, using a nitrogen conversion factor of 6.25, D-methionine (Acros Organics, New Jersey, USA) was used as a reference. All samples were prepared and analyzed in triplicate using a Flash EA 1112 Nitrogen Analyzer (Thermo Scientific, Rockford, IL, USA).

2.10. Starch content

Feedstock material (10 mg mL^{-1}, 50 mM NaOAc, pH6) was heated for 1.5 h at 85 °C, after which the α-amylase Termamyl (Novozymes A/S, Bagsvaerd, Denmark), 0.10% (v/w) enzyme liquid on DM, was added. After cooling down to 30 °C, the amyloglucosidase Spirizyme Plus FG (Novozymes A/S, Bagsvaerd, Denmark), 0.10% (v/w) enzyme liquid on DM, was added. Samples were incubated head-over-tail during 20 h at 30 °C. Incubations were stopped by heating at 100 °C for 5 min, after which the amount of glucose was determined by HPAEC.

2.11. HPSEC; analysis of molecular weight distribution

High-performance size-exclusion chromatography (HPSEC) was performed on three TSK-gel columns (6.0 mm × 15.0 cm per column) in series (SuperAW4000, SuperAW3000, SuperAW2500; Tosoh Bioscience, Stuttgart, Germany), in combination with a PWX-guard column (Tosoh Bioscience, Stuttgart, Germany). Elution took place at 40 °C with 0.2 M sodium nitrate at 0.6 mL min^{-1}.

The eluate was monitored using a Shodex RI-101 (Kawasaki, Japan) refractive index (RI) detector as well as by measuring UV-absorption at 280 nm, 320 nm and 480 nm using an Ultimate 3000 RS Variable Wavelength Detector (Dionex, Sunnyvale, USA). Calibration was performed by using pullulans (Associated Polymer Labs Inc., New York, USA) with a molecular weight in the range of 0.18–788 kDa.

2.12. HPAEC; analysis of mono- and oligosaccharides

High-performance anion-exchange (HPAEC) was performed on a Dionex (Sunnyvale, USA) Ultimate 3000 UPLC-system equipped with a CarboPac PA-1 column (2 mm × 250 mm ID) in combination with a CarboPac PA guard column (2 mm × 50 mm ID) and PAD detection. The system was controlled by Chromeleon software. For analysis of oligosaccharides in solution, elution (0.3 mL min^{-1}) was performed with a combination of linear gradients with two types of eluents, A: 0.1 M NaOH and B: 1 M NaOAc in 0.1 M NaOH. The elution profile was as following: 0–40 min: 0–40% B, followed by a cleaning step (5 min 100% B) and equilibration step (15 min 100% A).

For analysis of monosaccharides, elution (0.3 mL min^{-1}) was performed with a combination of linear gradients with three types of eluents, A: 0.1 M NaOH, B: 1 M NaOAc in 0.1 M NaOH, C: water. The elution profile was as following: 0–30 min at 100% C, 30–35 min at 100% B; 35–42 min at 100% A; 42–60 min 100% C. Post-column alkali (0.5 M NaOH, 0.1 mL min^{-1}) was added for detection purposes.

2.13. MALDI–TOF mass spectrometry

For matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI–TOF MS) an Ultraflextreme instrument (Bruker Daltonics, Bremen, Germany) was used. The mass spectrometer was calibrated with a mixture of maltodextrins (Avebe, Veendam, The Netherlands). The samples were mixed with a matrix solution (1 μL sample in 1 μL matrix), after desalting the samples with anion-exchange material (AG 50W-X8 Resin; Biorad, Hercules, USA). The matrix solution was prepared by dissolving 10 mg of 2,5-dihydroxybenzoic acid (Bruker Daltonics, Bremen, Germany) in 1 mL mixture of acetonitrile–water (300 μL:700 μL). Samples (1 μL) were put on a target plate and allowed to dry at room temperature under a stream of dry air.

2.14. Furfural and hydroxymethylfurfural

The contents of furfural and hydroxymethylfurfural (HMF) were quantified by using a Shimadzu ('s-Hertogenbosch, The Netherlands) HPLC system with column oven CTO-10-A-vp and Auto injector SIL-10AD-vp equipped with a guard column (Bio-Rad H cartridge) and an Aminex HPX-87H column (300 mm × 7.8 mm) (Bio-Rad, Hercules, USA).

Elution took place at 80 °C with 10 mM Na_2HPO_4 at 0.6 mL min^{-1}. The eluate was monitored using a refractive index detector RID-10A (Shimadzu, 's-Hertogenbosch, The Netherlands).

2.15. Enzyme digestions

Detailed knowledge on xylan structure and carbohydrate substitution was obtained by the aid of glycosyl hydrolase (GH) family 10 endoxylanase from Aspergillus awamori (EC-number 3.2.1.8), with known substrate specificity (Kormelink, Gruppen, Viëtor, & Voragen, 1993). Alkali extracts from cobs and stover (4 mg mL^{-1}) were digested with this GH10 endoxylanase with a dose of 0.11 mg enzyme protein/g DM, at 30 °C for 24 h by using an Eppendorf rotator for mixing ('head-over-tail'). Besides, partial degradation with this endoxylanase was studied by addition of only 1.2 μg enzyme protein/g DM and subsequent incubation at 30 °C for 5 h head-over-tail. After incubation, samples were centrifuged (10,000 × *g*, 10 min, 20 °C) and analyzed by HPSEC as described under Section 2.11.

Next to this endoxylanase, the commercially available enzyme mixture Cellic CTec2 (Novozymes A/S, Bagsvaerd, Denmark) was used for digestion from alkali extracts and Liquids of cobs and stover. Cellic CTec2 is commercial enzyme cocktail available on the market to saccharify lignocellulosic materials. More information can be found on the website of its producer (www.bioenergy.novozymes.com). The alkali extracts (4 mg mL^{-1}) were incubated with a dose of 6.3 mg enzyme protein/g DM, for 24 h, head-over-tail at 30 °C. Besides, Liquids (5 mg mL^{-1}) were digested with Cellic CTec2 with a dose of 6.3 mg enzyme protein/g DM, for 24 h, head-over-tail at 50 °C. Samples were centrifuged (10,000 × *g*, 10 min, 20 °C) and analyzed by reversed phase chromatography coupled to mass spectrometry (Section 2.16).

2.16. Reversed phase analysis on Liquids of cobs and stover

Reversed phase chromatography was performed by using an Acella U-HPLC system (Thermo Scientific, Rockford, IL, USA), equipped with a photodiode array detector and coupled to a LTQ XL Iontrap mass detector equipped with electrospray ionization source (Thermo Scientific Rockford, IL, USA). The system was controlled by Xcalibur software. Analysis was performed on an Aquasil C18 column (150 mm × 2.1 mm) with 3 μm particle size (Thermo Scientific, Rockford, IL, USA). The mobile phase was composed of A: water and B: methanol. The flow rate was 200 μL min^{-1} and the column temperature was 30 °C. The elution profile was as following: 0–70 min: 0–28% B, 70–80 min: 28–100% B; followed by a cleaning step (10 min: 100% B) and equilibration step (20 min: 0% B). Spectral data were collected from 200 to 600 nm. MS data were collected in the positive mode with an ion spray voltage of 3.5 kV, a capillary voltage of +48 V and a capillary temperature of 250 °C. Full MS scans were made within the range *m*/*z* 150–2000.

Table 1
Composition of cobs and stover feedstock (FS) and the corresponding water-soluble solids (WSS) and water-unsoluble solids (WUS) fractions, including extraction yields.

	Corn cobs % (w/w) on dry matter			Corn stover % (w/w) on dry matter		
	FS	WSS	WUS	FS	WSS	WUS
Yield[a]	100	5.8	89	100	4.9	84
Sugars (total)	68	43	67	57	15	69
Glucose[b] (from which starch)	34 (1.8)	29 (<1.8)	34 (<1.8)	30 (0.6)	2.9 (<0.6)	40 (<0.6)
Xylose[b]	28	5.0	28	19	2.7	21
Arabinose[b]	2.4	1.5	2.2	2.7	2.1	3.0
Galactose[b]	0.8	1.3	0.7	1.0	2.6	1.1
Rhamnose[b]	0.7	0.2	0.2	1.1	0.4	0.3
Mannose[b]	0.1	3.9	0.2	0.5	1.3	0.4
Uronic acids[b]	1.8	2.0	2.0	2.2	2.6	2.7
Esters						
Acetic acid	4.3	0.6	1.9	2.4	4.9	1.5
Ferulic acid	1.1	Na[c]	1.2	0.2	Na[c]	0.4
Coumaric acid	1.7	Na[c]	2.0	1.1	Na[c]	1.7
Lignin						
Acid insoluble	17	5.3	21	29	24	28
Acid soluble	1.3	Na[c]	Na[c]	1.9	Na[c]	Na[c]
Protein ($N \times 6.25$)	1.0	3.7	1.7	4.2	14.6	3.5

[a] Expressed as g dry material (DM) based on 100 g of feedstock (FS).
[b] Expressed as anhydro-units in polymers.
[c] Not analyzed.

3. Results and discussion

3.1. Characterization of corn feedstocks and corresponding fractions

3.1.1. Composition of corn feedstocks

Cobs and stover were analyzed for their carbohydrate composition including content of esterified acetic, ferulic and coumaric acid, and content of protein, and lignin. The results are presented in Table 1.

Our data are in agreement with previously reported compositions of cobs and stover (Kabel, Carvalheiro, et al., 2002; Pellerin, Gosselin, Lepoutre, Samain, & Debeire, 1991; Templeton, Sluiter, Hayward, Hames, & Thomas, 2009), although it would have been interesting to have extended this work to a wider range of samples from e.g. different corn varieties or from e.g. the same variety taken in different stages from plant growth.

3.1.2. Composition of water soluble and unsoluble solids (WSS and WUS)

The type of carbohydrates from the corn cobs and stover that are water soluble, were determined by extractions using water of 65 °C. The resulting WSS and remaining WUS were characterized (Table 1). In water only 5.8% (w/w) and 4.9% (w/w) of DM was extracted from cobs and stover feedstock, respectively. The yield of carbohydrates, which were mainly represented by glucuronoarabinoxylans (GAX), was less than 4% (w/w) in the cobs and stover WSS. In the remaining WUS fractions over 85% (w/w) of GAX was collected, and these WUS fractions were used for further extraction of cobs and stover xylans.

As shown in Table 1, both WUS fractions were rich in ferulic and coumaric acid esters, with 1.2% (w/w) ferulic and 2.0% (w/w) coumaric acid for cobs, and with 0.4% (w/w) ferulic and 1.7% (w/w) coumaric acid for stover. An indication of the degree of substitution (DS) of xylans by ferulic and coumaric acid esters is given by the DS-X_{FA} and DS-X_{CA} (shown in Table 2), which will be discussed in detail in Section 3.2.1.

3.1.3. Composition of alkali extracts

GAX are known to be extractable by using alkali solutions (Gruppen, Kormelink, & Voragen, 1993). To be able to focus on the structural characteristics of GAX from cobs and stover, the WUS fractions of both cobs and stover were extracted by using 1 M and 4 M KOH, sequentially. $NaBH_4$ was added to prevent alka-

Table 2
Sugar yields and molar sugar composition of water-unsoluble solids (WUS), the corresponding alkali fractions 1 M KOHss, 4 M KOHss, and residue (Res) from cobs and stover.

	Yield DM[a]	Yield GAX[b]	Total sugar content[c]	Molar composition[d]							DS-X[e]				
				A	X	M	Gal	R	Glc	UA	A	UA	Ac	FA	CA
Cobs			72	3.3	37	0.1	0.9	0.9	38	1.8	9	5	49	4	6
WUS	100	100	67	3.6	46	0.3	0.9	0.3	45	2.5	8	5	21	3	7
1 M KOHss	33	71	72	9.6	83	0.2	1.5	0.1	1.8	3.6	12	4	0	0	0
4 M KOHss	3.1	6.5	76	8.4	79	0.2	1.8	0.2	7.4	3.2	11	4	0	0	0
Res[f]	46	25	89	2.0	20	0.2	0.3	0.4	76	1.1	10	6	0	0	0
Stover			59	5.0	36	0.7	1.6	1.9	46	3.1	14	9	39	2	5
WUS	100	100	68	5.0	35	0.5	1.5	0.4	54	3.4	15	10	23	2	7
1 M KOHss	34	59	51	12	74	0.7	2.6	0.2	3.9	6.2	16	8	0	0	0
4 M KOHss	2.8	5.5	62	14	64	0.7	3.6	0.4	9.4	8.4	21	13	0	0	0
Res[f]	48	24	86	2.9	13	0.5	0.6	0.5	81	1.8	23	14	0	0	0

[a] Expressed as g/100 g WUS (dry matter based).
[b] Glucuronoarabinoxylans (GAX) calculated as the sum of uronic acids, arabinose and xylose, based on the GAX originally present in the WUS.
[c] Percentage (w/w) dry matter based.
[d] Expressed as anhydro-units in polymers, A: arabinose, X: xylose; M: mannose; Gal: galactose; R: rhamnose; Glc: glucose; UA: uronic acid.
[e] Degree of substitution: mol substituent per 100 mol of xylose, A: arabinose; UA: uronic acid; Ac = acetyl; FA: ferulic acid; CA = coumaric acid.
[f] Residue.

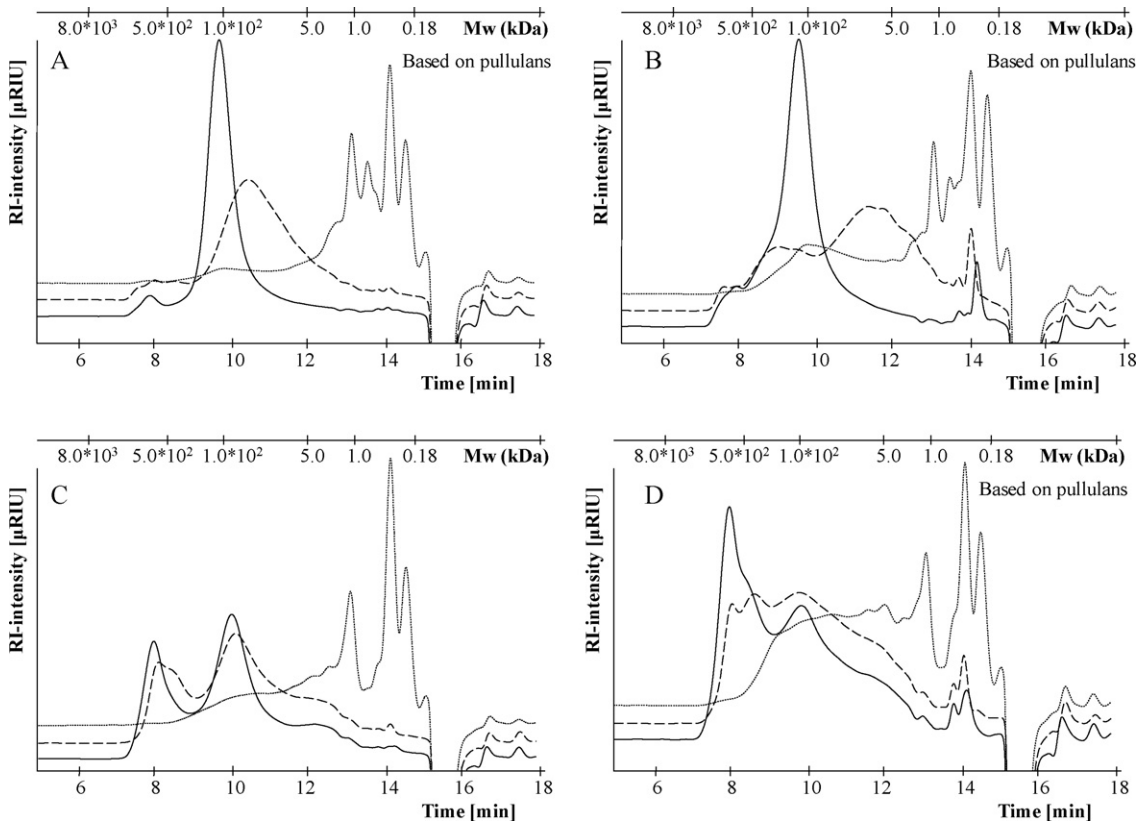

Fig. 1. Molecular weight distribution of 1 M KOHss cobs (A), 4 M KOHss cobs (B), 1 M KOHss stover (C) and 4 M KOHss stover (D), before hydrolysis (solid line), partial hydrolysis (dashed line), and endpoint hydrolysis (round dotted line) with endoxylanase; refractive index (RI) detection.

line peeling (Fry, Willis, & Paterson, 2000). A disadvantage of using alkali is that esterified substituents are removed. This issue was dealt with by using Liquids from a dilute acid treatment that were studied with a focus on esterified xylan structures, as described under Section 3.3.

In Table 2 the GAX extraction yields are shown. Most of the GAX were extracted in the 1 M KOHss fractions, 71% (w/w) and 59% (w/w) for cobs and stover, respectively. Subsequently, the 4 M KOHss fractions yielded another 6.5% (w/w) of GAX for cobs and 5.5% (w/w) for stover. Both in the 1 M and 4 M KOHss, a noticeable amount of glucosyl residues was present. It could be either originating from (xylo-)glucans or being present as xylan side-chains. In the residues (Res) the remaining of the GAX was collected, 25% (w/w) and 24% (w/w) for cobs and stover, respectively, next to most of the cellulose.

3.2. Structural features of xylans in the alkali extracts

The xylan structures as present in the alkali extracts from cobs and stover were studied in more detail.

3.2.1. Degree of substitution on the xylan backbone

To understand the type and amounts of substituents of the xylans from cobs and stover, first, the DS is calculated from the molar sugar composition, and presented in Table 2. The DS gives an indication of substitution as the number of moles of substituents per 100 mol of xylosyl residues. For example, the degree of arabinose substituents, indicated as DS-X_A, is expressed as the number of moles of arabinose per 100 mol xylosyl residues.

From the calculated DS-values, it was concluded that the stover alkali extracted xylans contained more arabinose and uronic acid substituents compared with the alkali extracted cobs xylans. Maybe more remarkable was that based on our findings (Table 2) for the alkali extracts of both cobs and stover more than 75 out of 100 xylosyl residues were not substituted. Here we have to point out that by using alkali the ester groups are lost.

DS-X_{FA} and DS-X_{CA} showed that xylans present in the original feedstock were surprisingly high feruloylated and also coumaroylated (Table 1). Besides, DS-X_{Ac} values showed that the original xylans also contained many acetyl substituents. These DS-X_{Ac} values for cobs and stover were quite high (49 and 39 acetyl groups per 100 xylosyl residues, respectively) and were in agreement with earlier reported values (Kabel, Carvalheiro, et al., 2002; Selig, Adney, Himmel, & Decker, 2009).

3.2.2. Endoxylanase degradation

Digestions with an endoxylanase with known substrate specificity can be used to obtain an indication about the distribution of the substituents over the xylan backbone.

Hereto, a purified endoxylanase I (GH-family 10) was used for incubation with the 1 M and 4 M KOHss fractions from cobs and stover. This endoxylanase cleaves the β-1,4-glycosidic linkages between the xylosyl residues in the backbone of xylans, but its activity is known to be hindered by the presence of substituents. So, the more substituents are present, and the more these substituents are neighboring each other, the less 'free' xylan sites are available for the endoxylanase to cut, and the less low molecular weight material will remain after digestion. Both partial and complete degradations were analyzed. The molecular weight (M_w) distributions are presented in Fig. 1.

Both the 1 M KOHss and the 4 M KOHss fractions of cobs consist of one large xylan population with a M_w of approximately 100 kDa, based on pullulan standards, while a minor population was present with a M_w higher than 500 kDa. The partial degradation showed

Table 3
Distribution of total sugars, glucuronoarabinoxylans (GAX), and glucose over soluble ('Liquid') and insoluble ('Solids') fractions from pretreated cobs and stover.

	Yield total sugars (%, w/w)	Yield GAX[a] (%, w/w)	Yield glucose/glucan (%, w/w)
Cobs	100	100	100
Liquid total	53	76	15
Monomers	7.2	13	7.4
Oligomers	46	64	7.7
Solids total	47	24	85
Stover	100	100	100
Liquid total	36	70	6.5
Monomers	7.4	17	0.4
Oligomers	28	53	6.1
Solids total	64	30	94

[a] GAX: glucuronoarabinoxylans, calculated as sum of glucuronic acid, arabinose and xylose.

that the 500 kDa population was more difficult to degrade than the 100 kDa population. Nevertheless, for the 1 M KOHss fraction of cobs, at the endpoint, also the 500 kDa population was degraded in rather low M_w material. The 4 M KOHss fraction of cobs was much less degraded, resulting in a relatively large population of larger xylan structures with a M_w between 5 and 500 kDa. The latter indicated that for the xylans in the 4 M KOHss of cobs, although still a considerable part of the xylans were degraded rather well, a condensed part of the material was highly substituted.

Stover xylans, Fig. 1C and D, differed from cobs xylans in the fact that both the 1 M and 4 M KOHss extract contained a relative larger portion of a population with a M_w larger than 500 kDa. These differences in degradability give rise to the hypothesis that the substituents are distributed in such a way over the stover xylan backbone, that corresponding attack and degradation by the endoxylanase is more difficult than in case of degradation of cobs xylans. This can be ascribed either to the way of distribution (more blockwise) and/or a higher degree of substitution (which is in agreement with calculated DS-values shown in Table 2) for stover xylans.

The M_w distribution of 1 M KOHss stover with and without endoxylanase (Fig. 1C) was also detected by using UV at a wavelength of 320 nm (Fig. 2).

Fig. 2 shows that the larger xylan population (M_w > 500 kDa) also had a good UV response at 320 nm, and that this population was rather well degraded by the endoxylanase. The same was observed for the 4 M KOHss fraction of stover (data not shown). Cobs hardly showed these complexes at 320 nm. UV-absorption at 320 nm is known to be specific for ferulic acid and lignin (Hartley, Akin, Himmelsbach, & Beach, 1990). So, it was speculated that stover xylans in the 1 M and 4 M KOHss extracts partly linked

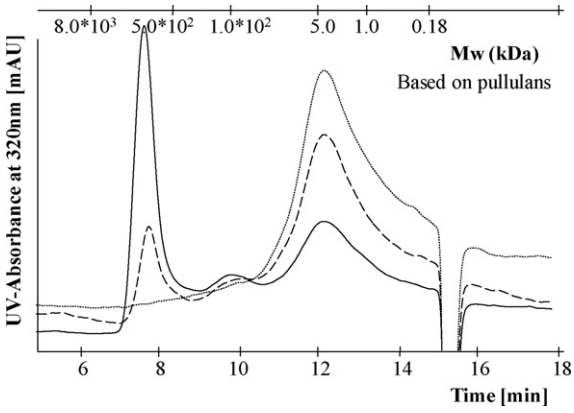

Fig. 2. Molecular weight distribution of 1 M KOHss stover, with no hydrolysis (solid line), partial endoxylanase digested (dashed line), and endpoint digested (round dotted line); UV (320 nm) detection.

covalently (non-esterified) with lignin- or ferulic acid like structures. Such lignin–carbohydrate complexes (LCCs) are found in many plant species, but are, generally, poorly defined structures. In such LCCs lignin is directly or indirectly covalently bound to carbohydrates, and the resulting complexes present a barrier to biological degradation (Jeffries, 1997). The oligomeric fragments formed by endoxylanase hydrolysis of the alkali extracts were monitored by using HPAEC and MALDI–TOF MS. Endoxylanase digest of wheat flour arabinoxylan was used as a reference (Kormelink et al., 1993). The HPAEC elution patterns and the main masses from the MALDI TOF mass spectra (data not shown) are presented in Fig. 3. The endoxylanase used released xylose (X1), xylobiose (X2) and xylotriose (X3) in all alkali extracts for both feedstocks. In addition, by combining the MALDI–TOF mass spectra of endo-digested 1 M KOHss extract of cobs and stover with the HPAEC patterns, also the presence of a series of 4-*O*-methyl-glucuronic acid containing pentose-oligomers was shown. In the 4 M KOHss extracts of both cobs and stover mainly non-substituted pentose-oligomers were identified, which seemed unexpected based on the conclusions in Section 3.2. Most likely, the substituted xylan structures left over after the endoxylanase digestion (Fig. 1B and D) were still too large to be detectable in the mixture by either HPAEC or MALDI–TOF MS.

3.3. Characterization of Liquids after dilute acid pretreatment

Liquids from dilute acid pretreated corn cobs and stover were studied with a focus on the presence of esterified *O*-acetyl, ferulic, and coumaric acids on the xylan backbone. Appeldoorn et al. (2010) already showed that for corn fibers such a dilute acid pretreatment preserves a large part of the esterified groups originally present. In Table 3 it is presented that in the Liquid of both feedstocks more than half of the GAX was collected as oligomers, and about 15% as monomers, while only a quarter of the GAX remained in the Solids.

Glucose was by far the most dominant sugar in the remaining Solids, presumably present as cellulose. In Table 4 molar ratios expressed as moles of substituents per 100 mol of xylosyl or arabinosyl residues are shown for the feedstocks, the Liquids and the Solids for cobs and stover. These ratios have been corrected for the presence of free monomers. Table 4 shows that xylans present in cobs feedstock were highly acetylated, with a DS-X_{Ac} of 49, indicating that on average half of the xylose moieties was substituted with an acetyl moiety. This is in agreement with the content of esterified acetic acids for corn cobs as published by Kabel, Carvalheiro, et al. (2002). Xylans present in the Liquid of cobs, had a DS-X_{Ac} of 39. Compared to cobs the number of acetyl substituents of stover was slightly lower with a DS-X_{Ac} of 39 for the stover feedstock, and a DS-X_{Ac} of 29 for the stover Liquid.

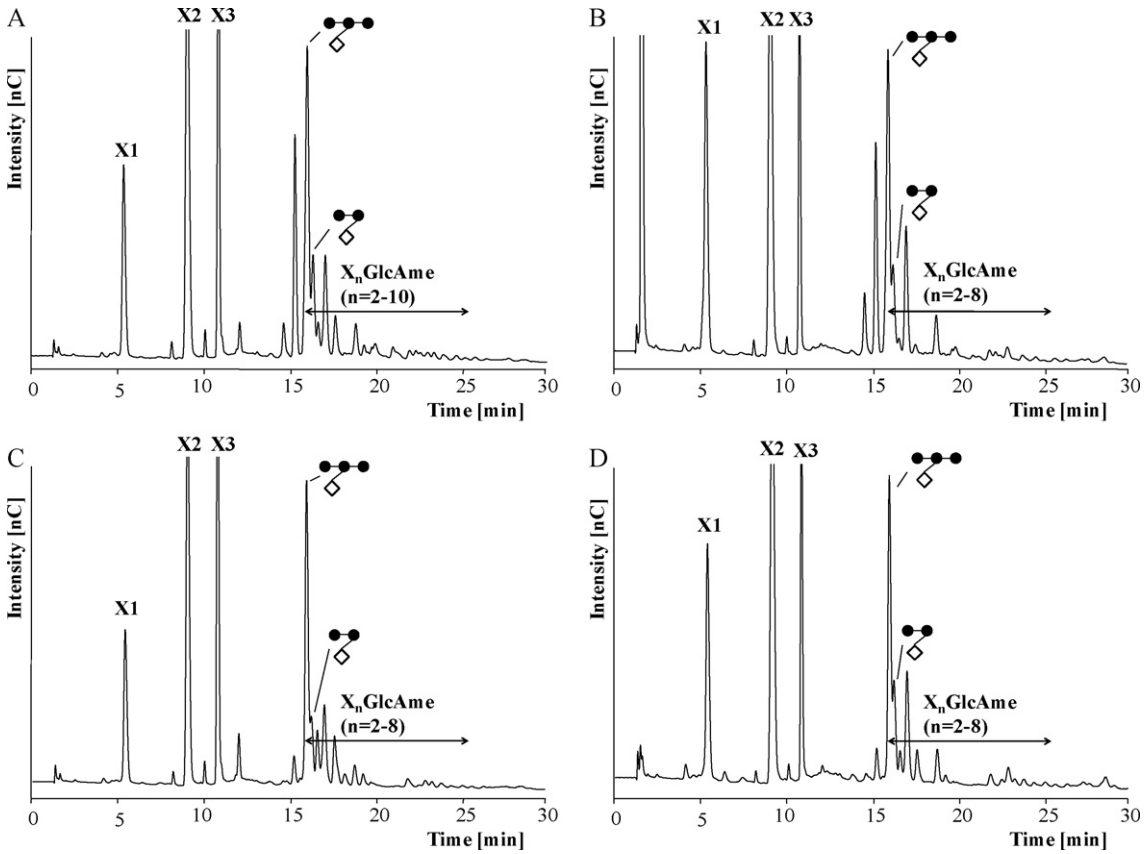

Fig. 3. HPAEC elution patterns of endoxylanase digested (endpoint) alkali fractions; 1 M KOHss cobs (A), 4 M KOHss cobs (B), 1 M KOHss stover (C), 4 M KOHss stover (D). X1: xylose; X2: xylobiose; X3: xylotriose; $GlcA_{me}$: 4-*O*-methyl-glucuronic acid (●: xylose; ◊: arabinose).

Next to acetic acid, ferulic and coumaric acids were also present as ester substituents of the corn xylans, with cobs xylans slightly higher substituted with both ferulic and coumaric acid (Table 3). The amount of free ferulic and coumaric acid esters was less than 5% of the original amount of ferulic and coumaric acid esters present, whereas much more xyloses (10–14%) and arabinoses (50–57%) were released as monomers. The degradability of the esterified xylan structures in the Liquids of cobs and stover was studied by using the commercial enzyme preparation Cellic CTec2, which was helpful for further structural elucidation.

The HPSEC-elution patterns of the Liquid of cobs and stover before and after incubation with Cellic CTec2 are shown in Fig. 4. For both incubations an endpoint was reached. The amount of oligomeric material before and after Cellic CTec2 was recognized by determination of the area under the RI-graph (Kabel, Bos, Zeevalking, Voragen, & Schols, 2007). From the xylans present in the Liquid of cobs (Fig. 4A), 37% remained in structures with a M_w between 240 and 5000 Da, whereas for stover Liquid this percentage was even 68% (Fig. 4B). Reversed phase U-HPLC–MS was carried out to obtain more detailed information about the oligomeric structures in the Liquids, with and without digestion by using Cellic CTec2. In Figs. 5 and 6, the results are shown for Liquid of cobs and stover, respectively. The C18-column used is known to elute non-esterified pentoses in the first 5 min, while acetylated pentose-oligomers elute between 5 and 60 min (Kabel, Schols, & Voragen, 2002). After 60 min even more hydrophobic pentose-oligomers will elute, for example feruloylated oligomers. Fig. 5A shows that in the Liquid of cobs a series of acetylated pentose-oligomers is present. After incubation with the commercial enzyme preparation most of the acetylated oligomers were degraded (Fig. 5B), suggesting the presence of acetyl xylan esterases in Cellic CTec2. However, some peaks corresponding to small oligomers (DP 2 and 3) with one acetyl group remained. More remarkable was that Fig. 5C showed that in the Liquid of cobs also various feruloylated pentose-oligomers were present. Saponification of corresponding samples and subsequent identification by use of MALDI–TOF MS (data not shown) made clear that the masses given correspond to feruloylated oligomers and not to coumaroylated oligomers, which presence was expected based on the relative high coumaric acid contents of both feedstocks (Table 1). This suggests that esterlinked coumaric acids were attached to larger or other structures, which could not be detected in the experimental set-up used in our research. Fig. 5D showed that esterlinked ferulic acid groups were not released by using Cellic CTec2, and therefore mainly ferulic acid esterified xylo-oligosaccharides accumulated.

Table 4
Molar sugar ratios of oligo- and polysaccharides from cobs and stover before and after pretreatment.

	DS-X					DS-A
	A[a]	UA[a]	Ac[a]	FA[a]	CA[a]	FA[a]
Cobs	9	5	49	4	6	45
L[c]	4	4	39	2	1	56
S[c]	9	3	8	Na[b]	Na[b]	Na[b]
Stover	14	9	39	2	5	12
L[c]	3	7	29	1	1	32
S[c]	13	5	15	Na[b]	Na[b]	Na[b]

[a] Mol substituents per 100 mol xylosyl/arabinosyl residues; X: xylose; A: arabinose; UA: uronic acid; Ac: acetyl; FA: ferulic. acid; CA: coumaric acid.
[b] Not analyzed.
[c] L: Liquid; S: Solids.

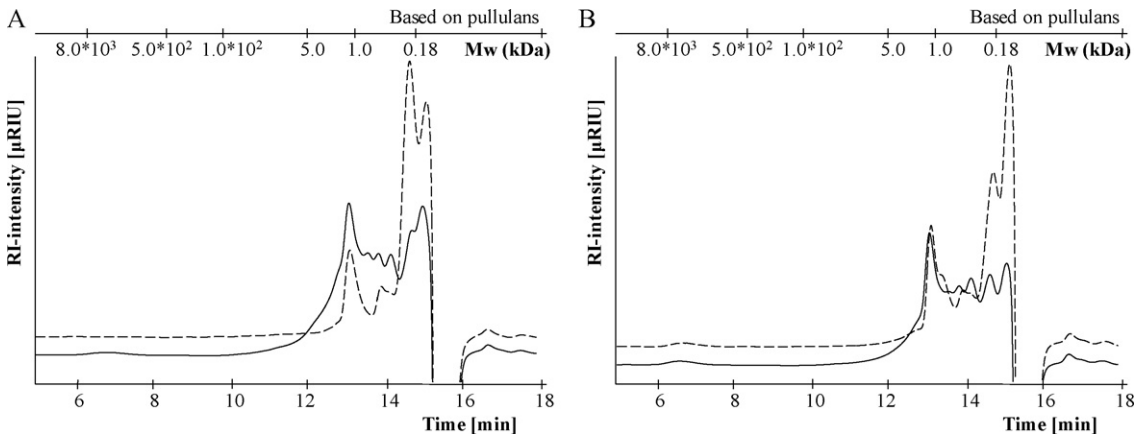

Fig. 4. Molecular weight distribution of pretreated cobs ('Liquid') (A), and stover ('Liquid') (B) before (solid line) and after endpoint (round dotted line) digestion with Cellic CTec2; refractive index (RI) detection.

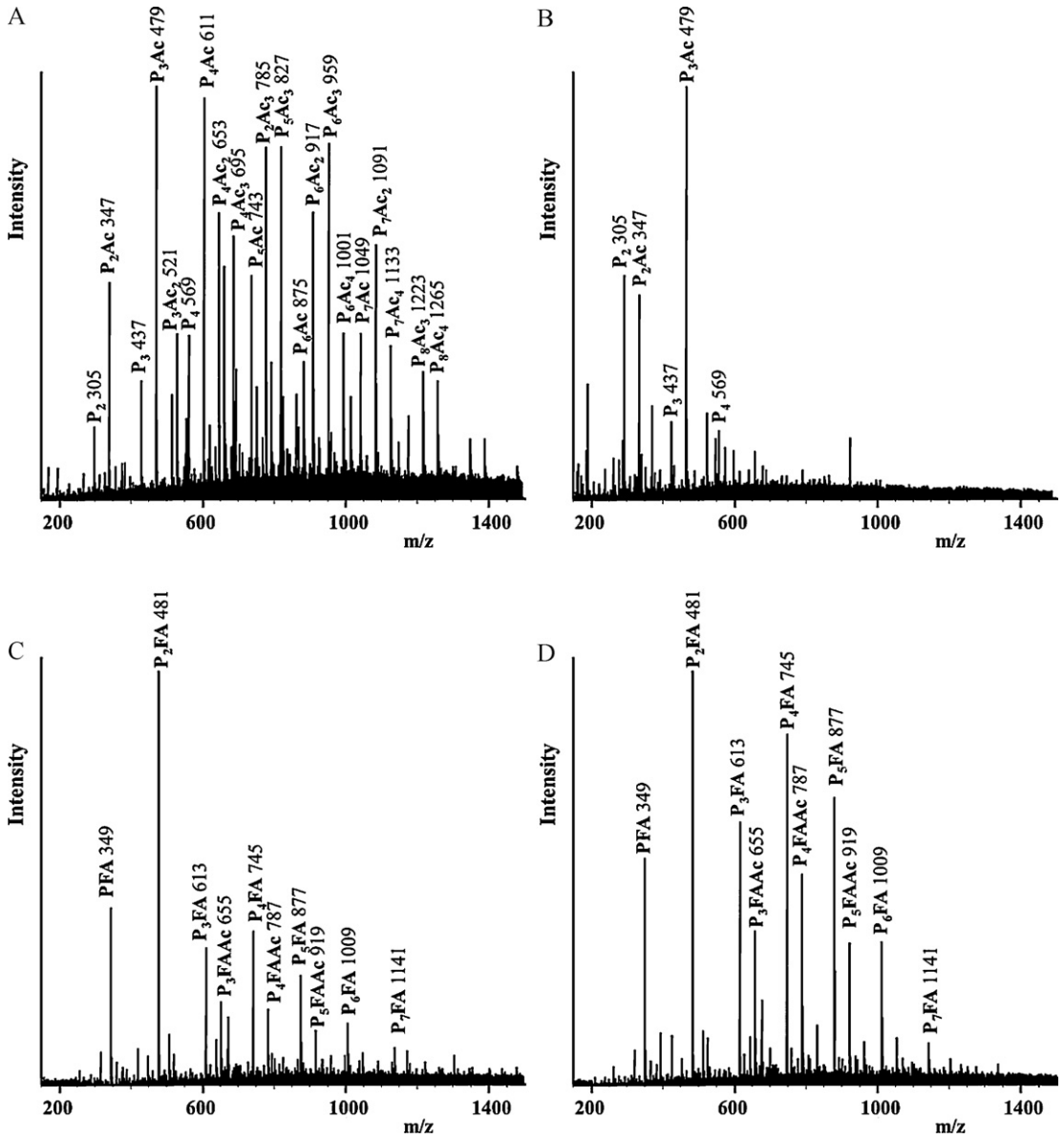

Fig. 5. Mass spectra of Liquid cobs obtained after elution from Aquasil C18 column. Elution-time 0–60 min (A), and 60–80 min (C). Cellic CTec2 digested Liquid cobs, elution time 0–60 min (B), and 60–80 min (D). P: pentose; Ac: acetyl; FA: ferulic acid (masses represent sodium adducts).

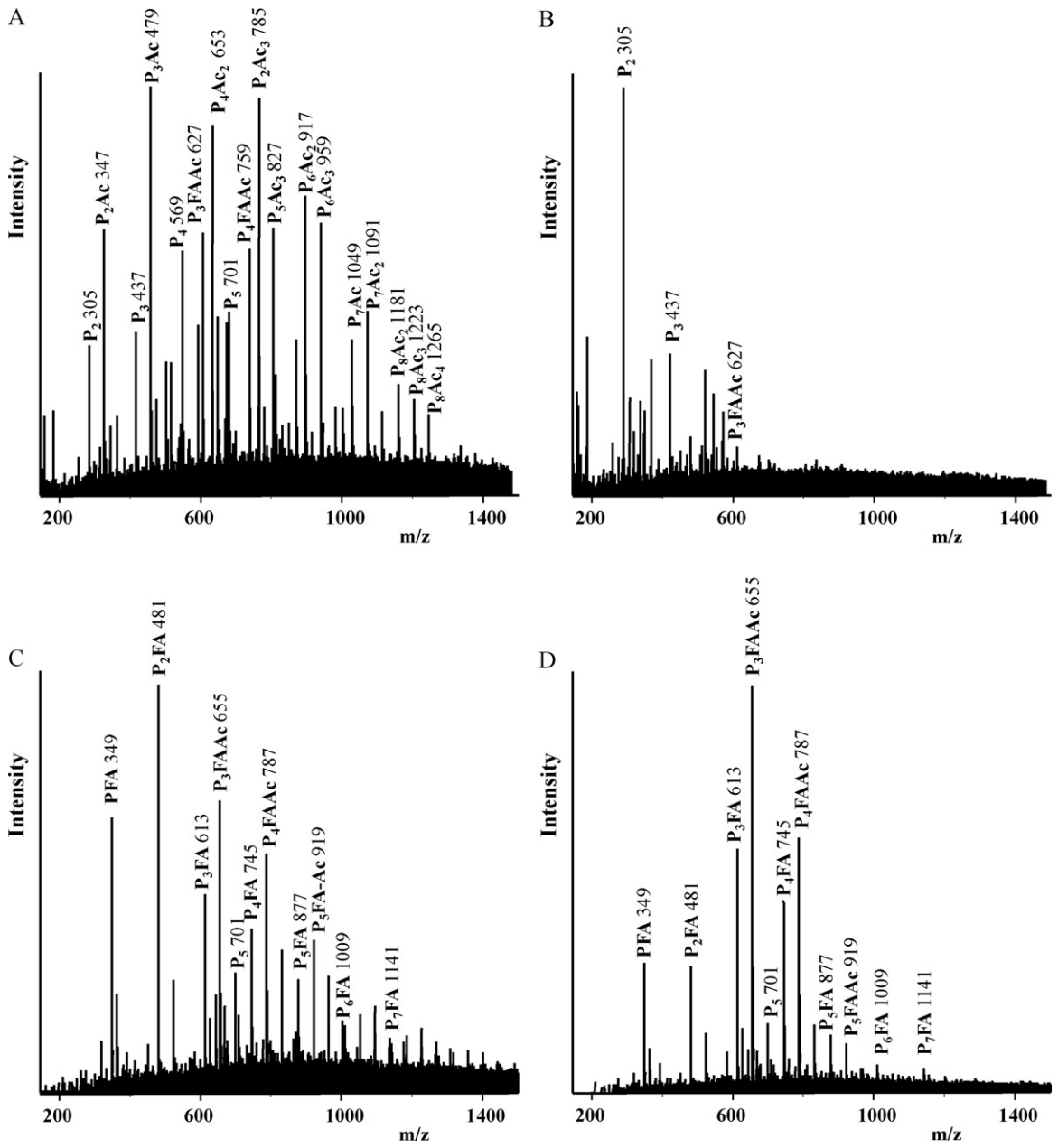

Fig. 6. Mass spectra of Liquid stover obtained after elution from Aquasil C18 column. Elution-time 0–60 min (A), and 60–80 min (C). Cellic CTec2 digested Liquid stover, elution time 0–60 min (B), and 60–80 min (D). P: pentose; Ac: acetyl; FA: ferulic acid (masses represent sodium adducts).

These observations were similar for stover Liquid before and after incubation with Cellic CTec2 (Fig. 6).

In summary, oligomers present in both Liquid of cobs and stover were highly acetylated and feruloylated. Presence of esterified coumaric acid was analyzed for both corn feedstocks, but coumaroylated xylan structures could not be detected by the methods applied here. The evidence of acetylated and feruloylated xylan structures in cobs and stover is shown in this paper for the first time.

4. Conclusions

In this research xylan structures from cobs and stover were studied in detail. Alkali extracted xylans present in stover were more heavily substituted with arabinose and (4-*O*-methyl) glucuronic acids than those present in cobs. Also, the results indicated that stover xylans were covalently bound to either lignin or ferulic acid like structures, while this was not as evident for the cobs. The higher degree of substitution for stover xylans compared to cobs was in agreement with a lower degradability by using a well-defined endoxylanase. Remaining xylan structures after dilute acid pretreatment were also studied. Due to pretreatment 50–65% GAX were released as soluble oligomers from both cobs and stover. It was shown for the first time by using reversed phase chromatography coupled to mass spectrometry that these remaining oligomers were highly esterified with *O*-acetyl esters, and with ferulic acid esters, or with a combination of both acetic- and ferulic acid. Coumaric acid was shown to be present in both feedstocks as well, but oligomeric xylan structures with esterified coumaric acid could not be observed by use of the methods applied in our paper. In future, it would be interesting to extend this work to a larger number of samples.

Acknowledgements

This work was financially supported by the Dutch Ministry of Economic Affairs via an EOS-KTO grant (www.senternovem.nl/eos).

The authors would like to thank Jan van Ee (ADM, Amsterdam, The Netherlands) for providing corn cobs and corn stover, Henk Schols (Wageningen University, Wageningen, The Netherlands) and Jan de Bont (Nedalco, Bergen op Zoom, The Netherlands) for their kind help in reading and optimizing the manuscript.

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