Carbohydrate Polymers 94 (2013) 791–799

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

Profiling of soluble neutral oligosaccharides from treated biomass using solid phase extraction and LC–TOF MS

Ramin Vismeh[a,c], James F. Humpula[b,c], Shishir P.S. Chundawat[b,d], Venkatesh Balan[b,c], Bruce E. Dale[b,c], A. Daniel Jones[a,c,e,*]

[a] Department of Chemistry, Michigan State University, East Lansing, MI, USA
[b] Biomass Conversion Research Laboratory, Department of Chemical Engineering and Materials Science, Michigan State University, Lansing, MI, USA
[c] DOE Great Lakes Bioenergy Research Center, Michigan State University, East Lansing, MI, USA
[d] DOE Great Lakes Bioenergy Research Center, University of Wisconsin, Madison, WI, USA
[e] Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI, USA

ARTICLE INFO

Article history:
Received 16 October 2012
Received in revised form 1 February 2013
Accepted 5 February 2013
Available online 13 February 2013

Keywords:
Ammonia fiber expansion (AFEX) pretreatment
Arabinoxylans
Corn stover
Glucans
Porous graphitized carbon (PGC)
Solid phase extraction (SPE)

ABSTRACT

Thermochemical pretreatments of cellulosic biomass are known to improve cell wall enzymatic digestibility, while simultaneously releasing substantial amounts of soluble oligosaccharides. Profiling of oligosaccharides released during pretreatment yields information essential for choosing glycosyl hydrolases necessary for cost-effective conversion of cellulosic biomass to desired biofuel/biochemical end-products. In this report we present a methodology for profiling of soluble neutral oligosaccharides released from ammonia fiber expansion (AFEX™)-pretreated corn stover. Our methodology employs solid phase extraction (SPE) enrichment of oligosaccharides using porous graphitized carbon (PGC), followed by high performance liquid chromatography (HPLC) separation using a polymeric amine based column and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). For structural elucidation on the chromatographic time scale, nonselective multiplexed collision-induced dissociation was performed for quasi-simultaneous acquisition of oligosaccharide molecular and fragment masses in a single analysis. These analyses revealed glucans up to degree of polymerization (DP) 22 without modifications. Additionally, arabinoxylans up to DP = 6 were detected in pretreated biomass extracts (post-enzymatic digestion). Cross-ring fragment ion abundances were consistent with assignment of linkages between sugar units in glucans and also xylose backbone in arabinoxylans as 1–4 linkages. Comprehensive profiling of soluble oligosaccharides also demonstrated decreases in levels of acetate esters of arabinoxylan oligosaccharides with concomitant increases in nonacetylated oligosaccharides that were consistent with earlier observations of 85% release of acetate esters by AFEX™ pretreatment.

1. Introduction

The biosynthesis of carbohydrate polymers represents one of the most prolific biochemical transformations on Earth. It has been estimated that natural plant biosynthesis generates more than 10^{11} tons of biomass per year (Duchesne & Larson, 1989; Pauly & Keegstra, 2008) with around half of this consisting of carbohydrate polymers cellulose and hemicelluloses. Carbohydrate polymers are responsible for plant cell wall structure and strength, storage of biochemical energy in the form of starch, and production of materials with far-reaching application including gelling and emulsifying agents and as drug delivery agents (Sinha & Kumria, 2001). Humankind exploits only a small fraction of this biomass, and as a result, cell wall polysaccharides are attractive renewable resources. Abundance alone makes cell wall polysaccharides attractive renewable feedstocks for bioenergy, and specialty products.

Plant cell wall oligosaccharides are materials of daunting complexity, being composed of various (5 or 6 carbon) sugar monomers with different degrees and positions of branching, assorted chemical modifications including acetylation and feruloylation (Ishii, 1997), and heterogeneity in molecular mass. These

Abbreviations: AFEX, ammonia fiber expansion; AFEXTCS, AFEX-treated corn stover; AP1, aperture 1; AX, arabinoxylan; CID, collision-induced dissociation; DP, degree of polymerization; ESI, electrospray ionization; HILIC, hydrophilic interaction chromatography; HPAEC, high performance anion-exchange chromatography; IM, ion mobility; MALDI, matrix-assisted laser desorption ionization; PGC, porous graphitized carbon; SPE, solid phase extraction; TOF, time-of-flight; UTCS, untreated corn stover.

* Corresponding author at: Department of Biochemistry and Molecular Biology, 603 Wilson Road, Biochemistry Room 212, Michigan State University, East Lansing, MI 48824, USA. Tel.: +1 517 432 7126; fax: +1 517 353 9334.
E-mail addresses: vismehra@msu.edu (R. Vismeh), humpulaj@egr.msu.edu (J.F. Humpula), chundawa@msu.edu (S.P.S. Chundawat), balan@egr.msu.edu (V. Balan), bdale@egr.msu.edu (B.E. Dale), jonesar4@msu.edu (A.D. Jones).

0144-8617/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.02.005

factors have strong influence on their solubility and digestibility (Scheller & Ulvskov, 2010). Conversion of complex polysaccharides, particularly those from cellulosic biomass, to fermentable monosaccharides is often inefficient owing to chemical modifications including formation of diferulate crosslinks that take place in vivo during cell wall assembly. Yields of fermentable products are improved following application of hydrolytic and ammonolytic pretreatments including acid, alkali, and AFEX™, which remove acetyl and phenolic acid esters from modified polysaccharides. AFEX involves treating biomass with liquid ammonia in a pressurized reactor at temperatures of 100 °C and above. The process causes physical changes in cellulose crystallinity and partial depolymerization of hemicelluloses. Recycling of ammonia leaves behind a glycopolymer-rich material with reduced recalcitrance to hydrolysis (Sousa, Chundawat, Balan, & Dale, 2009). In order to better predict yields of conversion of cell wall biomass to fermentable sugars and to optimize design of efficient biorefineries, comprehensive profiling in combination with yield determination of mono- and poly-saccharides generated by pretreatment and during enzymatic processing is needed that goes beyond destructive conversion of glycopolymers to monomeric sugars.

Profiling of oligosaccharides derived from processing of cell walls starts best by defining the molecular masses of the individual components in a mixture of oligosaccharides. Modern mass spectrometry readily provides molecular mass information through application of soft ionization methods including matrix-assisted laser desorption ionization (MALDI) (Harvey, 1999) or electrospray ionization (ESI) (Reinhold, Reinhold, & Costello, 1995). When soft ionization is combined with CID to generate fragment ions, the resulting information allows for characterization of sequences of sugar monomers, linkages between sugars based on cross-ring fragment masses, and presence of branching. This approach has been exploited to investigate structures of oligosaccharides derived from plant tissues (Fernandez, Obel, Scheller, & Roepstorff, 2003, Fernandez, Obel, Scheller, & Roepstorff, 2004; Harrison et al., 2011; Maslen, Goubet, Adam, Dupree, & Stephens, 2007; Van Dongen, Van Eylen, & Kabel, 2011). Most of these reports describe characterization of products of enzymatic digestions using soft ionization and MS/MS, frequently employing permethylation to improve information content in the mass spectra. One report demonstrated detection of fructans with DP > 100 using HPLC based on PGC and electrospray ionization (Harrison et al., 2011). More recently, the combination of mass spectrometry with ion mobility (IM) separations of ions allowed for discrimination of oligosaccharides by shape, and not just mass (Munisamy, Chambliss, & Becker, 2012). Despite great advances in IM technology, this approach has yet to achieve resolution of the vast array of isomeric oligosaccharides without prior physical separation.

Another fast and powerful tool for structural identification/confirmation of analytes employs quasi-simultaneous acquisition of exact masses at high and low collision energies in a single analysis without mass filtering (MS^E) (Plumb et al., 2006). Fast data acquisition provided by time-of-flight mass spectrometry allowed for extension of this technique to use more than two collision conditions, termed multiplexed collision-induced dissociation. This approach yields CID mass spectra using multiple collision energies on the chromatographic time scale, and has driven discoveries of new plant metabolites and genes responsible for metabolite accumulation (Gu, Jones, & Last, 2010; Schilmiller et al., 2010). To our knowledge, multiplexed CID has not been reported for oligosaccharides.

Separation of oligosaccharides before mass spectrometry is essential for comprehensive oligosaccharide profiling owing to the complexity of plant oligosaccharide mixtures. Some notable successes in separation of oligosaccharides were achieved through use of hydrophilic interaction chromatography (HILIC) (Churms, 1996; Karlsson, Swerup, & Sandberg, 2008.; Leijdekkers, Sanders, Schols, & Gruppen, 2011). High-pH anion exchange chromatography (HPAEC) has emerged as another common carbohydrate separation method that takes advantage of partial ionization of oligosaccharides at elevated pH (Cataldi, Campa, & De Benedetto, 2000; Guignard et al., 2005; Lee, 1996; van der Hoeven et al., 1998), but the common nonvolatile mobile phase additives that achieve high pH are incompatible with electrospray ionization unless they are removed post-column. PGC has proved to be a suitable chromatographic stationary phase for retention of very polar compounds owing to both hydrophobic and electronic-type interactions between the analyte and the PGC surface (Hennion, 2000; West, Elfakir, & Lafosse, 2010). PGC columns have been used to enrich or separate various sugars and sugar polymers including sugar phosphates from *Arabidopsis thaliana* (Antonio et al., 2007), cell wall oligosaccharides (Westphal, Schols, Voragen, & Gruppen, 2010) and human milk oligosaccharides (Strum, Aldredge, Barile, & Lebrilla, 2012; Zhang, Xie, Hedrick, & Lebrilla, 2004). PGC separations offer the advantage that mobile phases are often compatible with mass spectrometry analyses.

About a decade ago, the introduction of the Prevail Carbohydrate ES column (Alltech), a polymeric column with amine groups, yielded separations that resolved a variety of neutral mono- and oligosaccharides. This column yields separations similar to normal phase chromatography using water and acetonitrile as solvents, and these are compatible with ESI mass spectrometry. Most of the applications of this column reported to date focused largely on analyses of mono- and disaccharides (Agblevor, Murden, & Hames, 2004; Kalay et al., 2012; Slimestad & Vagen, 2006; Vinjamoori, Byrum, Hayes, & Das, 2004; Wan & Yu, 2007).

As mentioned above, pretreatment of biomass improves yields of conversion of carbohydrates to fermentable sugars, but the fundamental relationships between the severity of pretreatment and the digestibility of products remains uncertain. Pretreatment processes release complex mixtures of substances including phenolics, Maillard reaction products, and mono- and oligosaccharides (Chundawat et al., 2010). Initial efforts that profiled soluble carbohydrates using a Bio-Rad Aminex 42-A column resolved carbohydrates with DP < 5, but larger oligomers were not resolved. Furthermore, chromatographic peak areas of oligosaccharides detected using refractive index and mass spectrometric detection did not account for the total sugar monomers yielded by acid hydrolysis. This finding led us to suspect that losses of oligosaccharides were occurring during sample processing, and a strategy for oligosaccharide enrichment was pursued using SPE based on PGC as a prelude to LC/MS profiling.

In the current study, soluble neutral oligosaccharides including xylans and glucans were profiled in extracts of AFEX™-pretreated corn stover (AFEXTCS). Enrichment of larger oligomers using PGC–solid phase extraction (PGC–SPE) followed by analytical separation using a Prevail Carbohydrate ES column coupled to multiplexed collision-induced dissociation mass spectrometry provided a fast technique that yielded rich information for comprehensive profiling of neutral soluble oligosaccharides in extracts of untreated and pretreated plant material.

2. Results and discussion

2.1. Optimization of conditions for PGC–SPE and enrichment of oligosaccharides

Initial LC/MS profiling of carbohydrates released from corn stover during AFEX™ processing detected low mass (DP < 6) oligosaccharides, but mass balance calculations suggested that the analytical result failed to account for more than 75% of glucans.

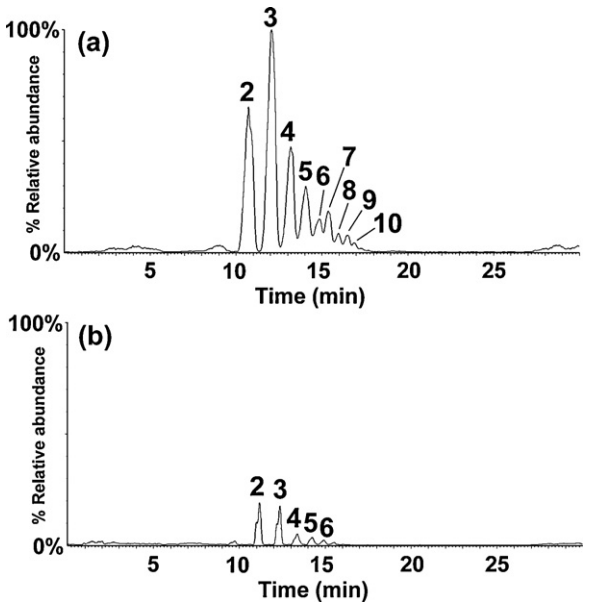

Fig. 1. LC/ESI-TOF-MS extracted ion chromatograms of $[M+Cl]^-$ ions from oligomers of hexose (DP = 2–13) in corn syrup (a) and non-retained portion of corn syrup after passing through a PGC–SPE cartridge (b). Peaks are labeled with the DP value for each oligosaccharide. Scaling of the *y*-axis was performed to the same absolute signal for both chromatograms.

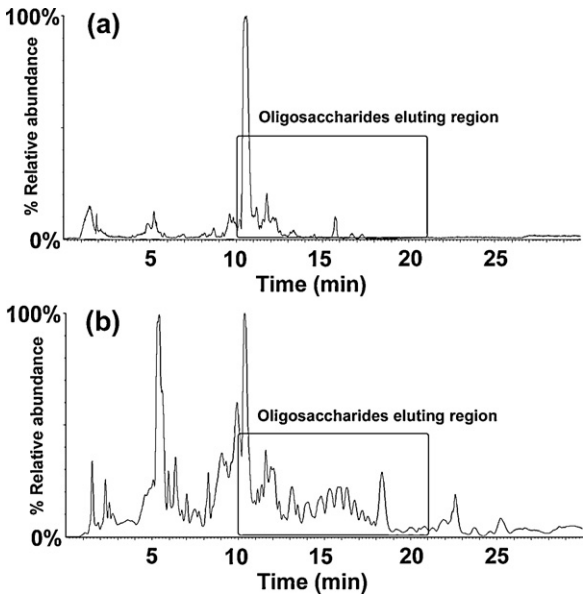

Fig. 2. LC/ESI-TOF-MS total ion chromatograms of (a) unprocessed AFEXTCS extract and (b) PGC–SPE enriched oligosaccharides from the same extract. Scaling of the *y*-axis was performed to the same absolute signal for both chromatograms.

Working from the assumption that matrix constituents might suppress ionization, a fractionation scheme was developed, based on PGC–SPE (1000 mg cartridge), to remove matrix components and enhance the range of detected oligosaccharides. Based on the PGC–SPE manufacturer's guidelines, it was anticipated that the retention capacity of a PGC–SPE cartridge would be 10–50 mg of sugars. The performance of this fractionation was tested by loading 7 mL of a 6 g/L aqueous corn syrup (food grade, derived from corn starch) solution (42 mg total sugars) onto the PGC cartridge. The original solution and the non-retained material were analyzed with LC/TOF-MS in negative ion mode. Fig. 1a shows the extracted ion LC/MS chromatogram of $[M+Cl]^-$ adduct ions with masses corresponding to corn syrup hexose oligomers (Megherbi, Herbreteau, Faure, & Salvador, 2009), and Fig. 1b shows the corresponding chromatogram of the non-retained material. Comparing the total peak areas for $[M+Cl]^-$ ions from the identified hexose oligomers (DP = 2–13) showed size-dependent retention of oligosaccharides, ranging from 67% for DP = 2 to 100% for DP = 10 and beyond, and are consistent with a recent independent report (Westphal et al., 2010). To determine the effect of pH on retention of sugars, corn syrup was diluted with aqueous formic acid or ammonium hydroxide to pH of 2.3, 6.7 and 11.3. Increasing pH from 2.3 to 6.7 yielded 20–30% more retention as based on retention times for individual oligohexoses. However, further increase to pH 11.3 only yielded an additional 5% increase in retention, mainly for sugars of DP < 5.

In order to select solvent for elution of oligosaccharides from PGC cartridges, corn syrup hexose oligomers were again used for method development and optimization. A range of solvents have been suggested for elution of retained solutes from PGC, and selection of solvents was guided by a desire to elute in solvents compatible with LC separation and mass spectrometric detection. Though acetonitrile has been used to elute oligosaccharides from PGC chromatography columns (Westphal et al., 2010), our efforts compared oligomer recoveries using acetonitrile with methanol. After loading 7 mL of 6 g/L corn syrup to a PGC–SPE cartridge, elution with 20 mL methanol yielded > 99% recovery of hexose oligomers from DP = 6 to 13 as determined using LC/MS, whereas elution with the same amount of acetonitrile yielded only ~80% of the hexose oligomers loaded on column.

AFEXTCS includes a complex range of substances including phenolics and various polar constituents (Chundawat et al., 2010) that can compete for retention on SPE cartridges. Based on preliminary surveys, it was observed that loading more than 6 mL of AFEXTCS led to undesirable breakthrough of short-chain oligosaccharides (DP = 2–4). Therefore, 6 mL of extract was loaded onto each PGC–SPE cartridge. After loading, 5.0 mL of water was used to elute salts and less retained substances, followed by elution of oligosaccharides using 30 mL methanol. A total of 48 mL AFEXTCS extract was loaded on 8 PGC–SPE cartridges in parallel, and methanol-eluted material (240 mL) was combined and reduced to 6 mL (an 8-fold concentration relative to the original extract) under vacuum for LC/ESI-TOF-MS analysis.

When unprocessed AFEXTCS extracts were concentrated by a factor of 8 by evaporation, LC/ESI-TOF-MS analyses of the solutions failed to detect oligohexoses larger than DP = 5. During solvent evaporation of the crude extracts, substantial quantities of precipitate were formed, and this process was suspected to remove larger oligosaccharides by sedimentation as the solvent evaporated. Filtered crude extracts (0.2 μm syringe filters) generated insoluble material upon standing at 25 °C for 24 h. It is proposed that colloidal particles in the crude extracts, that are currently of unknown composition, adsorb oligosaccharides and coalesce to form insoluble precipitates. However, when freshly prepared extract was loaded onto PGC cartridges, oligosaccharides were retained and eluted using methanol, with colloidal particles being retained in the column. Fig. 2a and b compares the total ion LC/MS chromatograms of AFEXTCS extract and the enriched oligosaccharides from the same extract. The results demonstrate that enriching larger oligosaccharides through removal of adsorptive constituents improves stability of oligosaccharide-containing solutions, yields dramatic increases in recoveries of DP > 4, and allows for improved profiling of oligosaccharides released from biomass.

2.2. Hexose oligomers from the AFEXTCS extract

Neutral oligosaccharides eluted between 10 and 21 min using the Prevail Carbohydrate ES column, and ESI mass spectra were

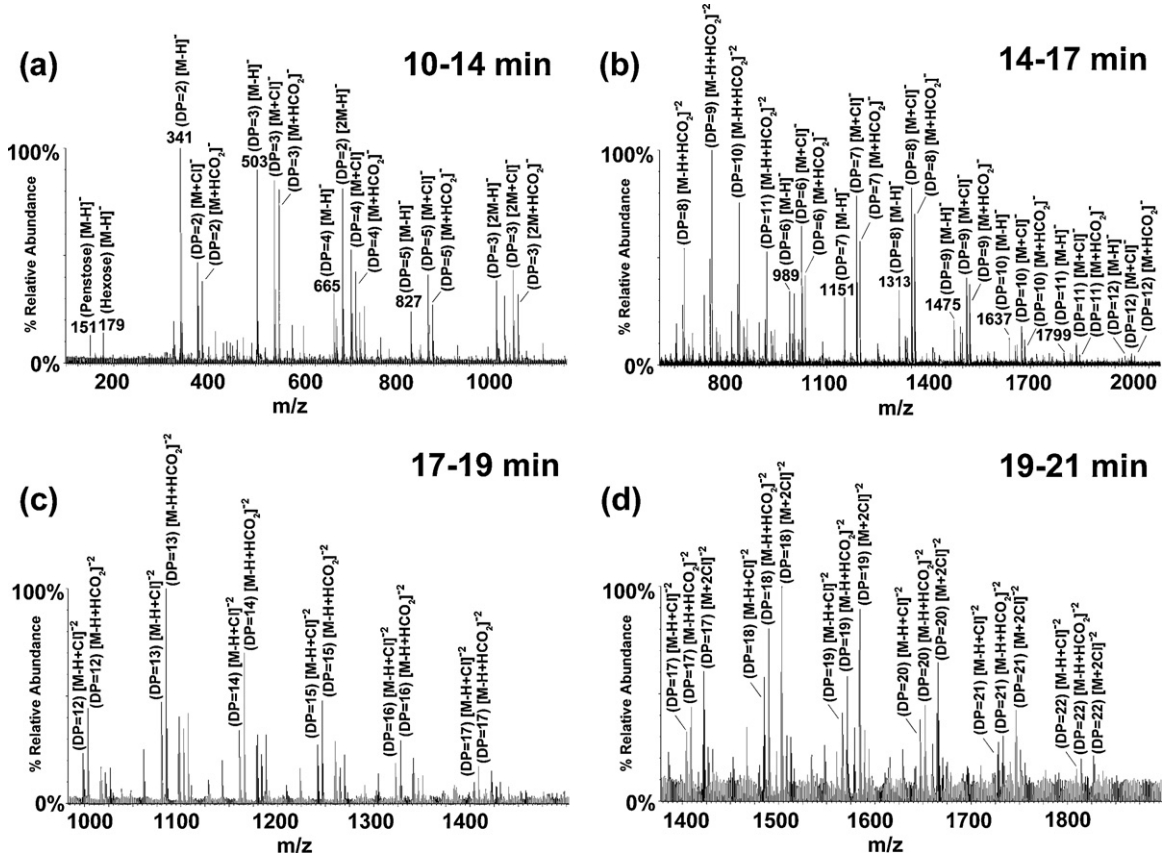

Fig. 3. LC/ESI-TOF-MS spectra of PGC–SPE enriched oligosaccharides from AFEXTCS extract (Fig. 2b) averaged over retention time windows of (a) 10–14 min, (b) 14–17 min, (c) 17–19 min and (d) 19–21 min. These spectra were acquired using the lowest collision potential (15 V). Only $[M-H]^-$ ions are labeled with corresponding *m/z* values.

generated in both positive and negative ion modes. For representation of a more comprehensive assessment of oligosaccharide content, spectra were summed over portions of this retention time window (Fig. 3). Combinations of accurate mass measurements with observations of a series of ions differing in mass by 162 Da and generation of fragment ions using nonselective multiplexed CID (Schilmiller et al., 2010) suggested presence of hexose oligomers of DP = 2–22 in the enriched oligosaccharide fraction from AFEXTCS extract.

Oligosaccharides that dominated the mass spectra generated multiple ions indicative of individual molecular masses, including $[M-H]^-$, $[M+HCO_2]^-$ and $[M+Cl]^-$ ions for smaller hexose oligomers (DP < 10) and doubly charged species including $[M-H+Cl]^{2-}$, $[M-H+HCO_2]^{2-}$ and $[M+2Cl]^{2-}$ for larger oligomers (DP > 10). Fig. 4 demonstrates separation of oligomers in the form of extracted ion chromatograms for singly- and doubly-charged chloride adducts of oligohexoses up to DP = 22 detected in AFEXTCS enriched fractions. Based on peak areas, abundances of hexose oligomers showed monotonic decrease with increasing DP. It is recognized that oligosaccharides will differ in their response factors in the mass spectrometer, and until authentic standards are available for more oligosaccharides in this range, these values should be treated as estimates of absolute oligosaccharide yields.

While molecular masses of the dominant constituents corresponded to oligohexoses, the identities of monomeric subunits were not distinguished from molecular mass alone. To identify the subunit composition of the unprocessed AFEXTCS extract, acid hydrolysis was performed followed by analysis of monomeric sugars using HPLC and refractive index detection. The hydrolyzed material showed increases, relative to the nonhydrolyzed AFEXTCS extract, in concentrations of glucose, arabinose and xylose (Table 1). Since glucose was the only hexose detected in the hydrolysis products, it was concluded that the overwhelming majority of hexose oligomers in the AFEXTCS extract were glucans.

2.3. Non-selective CID spectra of glucans

MS/MS spectra of oligosaccharides reveal valuable information about sequence, linkage and branching in the form of characteristic fragment ions arising from cleavage of the glycosidic bonds (B, C, Y, and Z ions, Fig. 5a) and yielding sequence information, and cross-ring fragmentation (A and X ions, Fig. 5a) indicative of linkage type (Domon & Costello, 1988). Consecutive cleavages of the glycosidic bonds leads to sequence information but not branching or linkage type between the sugar units. However, type of linkage between sugar units can often be identified from characteristic cross-ring fragment ion masses. Fig. 5 shows three CID spectra acquired at different collision energies for the oligosaccharide eluting at retention time 16.1 min (Fig. 4, DP = 8). At the lowest collision energy (15 V), the mass spectrum displays singly- and doubly-charged ions indicative of the oligosaccharide molecular mass. Mass spectra obtained under elevated CID voltages (aperture 1 (AP1); Fig. 5c and

Table 1
Carbohydrate content in nonhydrolyzed (free sugar concentration) and acid hydrolyzed (total) AFEXTCS extract determined using HPLC and refractive index detection ($N = 3$; uncertainty = standard deviation).

	Glucose (g/L)	Xylose (g/L)	Arabinose (g/L)
Free sugars	0.12 ± 0.02	0.09 ± 0.01	0.07 ± 0.00
Total sugars	0.54 ± 0.04	1.99 ± 0.09	0.48 ± 0.01
Oligomeric sugars	0.42 ± 0.02	1.90 ± 0.08	0.41 ± 0.01

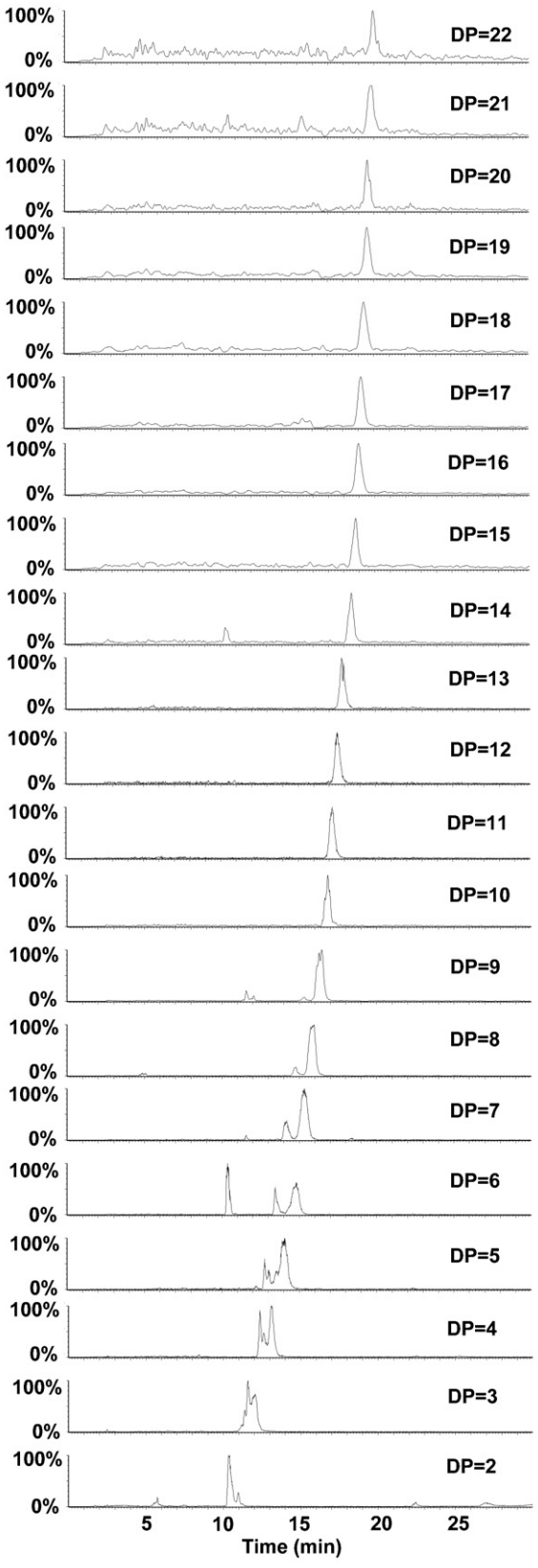

Fig. 4. LC/ESI-TOF-MS extracted ion chromatograms ($[M+Cl]^-$ for DP = 2–10 and $[M-H+Cl]^{-2}$ for DP = 11–22) of hexose oligomers from the PGC–SPE enriched AFEXTCS extract.

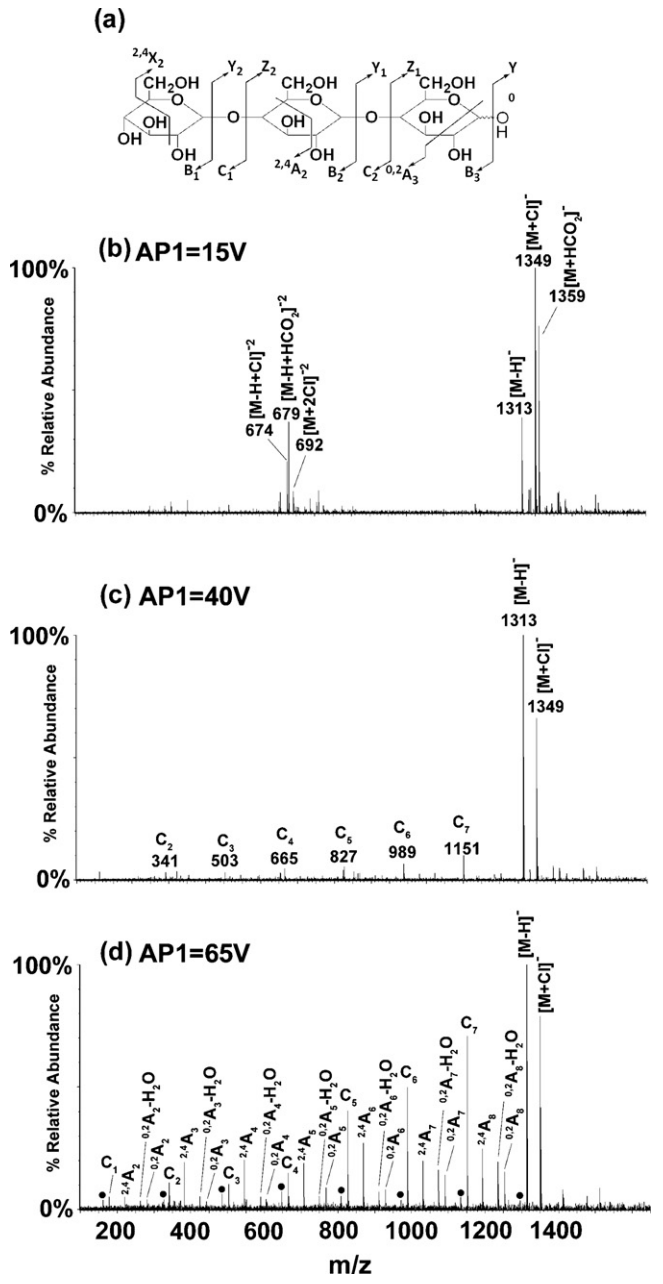

Fig. 5. Structure of cellotriose, showing the Domon and Costello nomenclature for fragment ions (a) LC/ESI-TOF-MS spectra of the glucan eluting at 16.1 min (DP = 8) at three different AP1 (CID) voltages in negative ion mode: (b) 15 V, (c) 40 V, and (d) 65 V. Peaks (d) labeled with black circles exhibit masses consistent with their annotation as B ions formed upon loss of water from corresponding C ions. ^{0,2}A and ^{2,4}A fragment ions have *m/z* values of corresponding C ion minus 60 or 120 units respectively.

d) of 40 and 65 V respectively were quasi-simultaneously acquired in a single analysis. Acquisition of mass spectra under multiple conditions from a single injection can be achieved because of the fast acquisition speed of the TOF analyzer (approx. 2×10^4 spectrum transients/s. As expected, more fragment ions were observed for glucan with DP = 8 at higher AP1 voltages. At an intermediate collision energy (40 V), the most prominent fragment ions correspond to C-type fragments, and their masses provide a useful indication of oligosaccharide sequences, and their monotonic increase in abundance with chain length suggest a linear oligosaccharide (Fernandez et al., 2004). At the highest collision energy (65 V), cross-ring (A-series) fragments were of sufficient abundance to

suggest a linear oligosaccharide, consistent with 1,4-linkages based on the consistent presence of ^{0,2}A and ^{2,4}A fragment ions (Garozzo, Giuffrida, Impallomeni, Ballistreri, & Montaudo, 1990; Hofmeister, Zhou, & Leary, 1991; Pasanen, Janis, & Vainiotalo, 2007; Spengler, Dolce, & Cotter, 1990; Zhou, Ogden, & Leary, 1990) which are 60 and 120 Da lighter than the C-series ions, and the absence of ^{0,3}A fragment ions that would have indicated 1,6-linkages (Garozzo et al., 1990; Spengler et al., 1990; Zhou et al., 1990). Neutral losses of 78 Da corresponding to ^{0,2}A-H_2O were also observed from $[M-H]^-$ precursors in β-1,4-linked oligosaccharides in previous studies (Garozzo et al., 1990; Pasanen et al., 2007; Quemener, Ordaz-Ortiz, & Saulnier, 2006). Fragments from reducing and non-reducing end in the CID spectra in Fig. 5-d could not be distinguished, as no derivatization was performed to identify the reducing end.

Although the multiplexed CID approach is non-selective (e.g. all ions are subjected to CID, and no specific *m/z* value was selected for fragmentation), the CID spectrum in Fig. 5d exhibits all of the abundant fragments generated by selective CID of *m/z* 1313 ($[M-H]^-$) using a linear ion trap mass analyzer (Q-Trap 3200). One of the major benefits of performing non-selective CID derives from collisional activation of all adduct ions and different charge states at the same time, which increases the yield of fragment ions. This is specifically helpful for larger oligosaccharides that ionize in a more diverse collection of singly- and doubly-charged pseudomolecular forms owing to the presence of various anions in the biomass extracts or mobile phase (Fig. 3a–d).

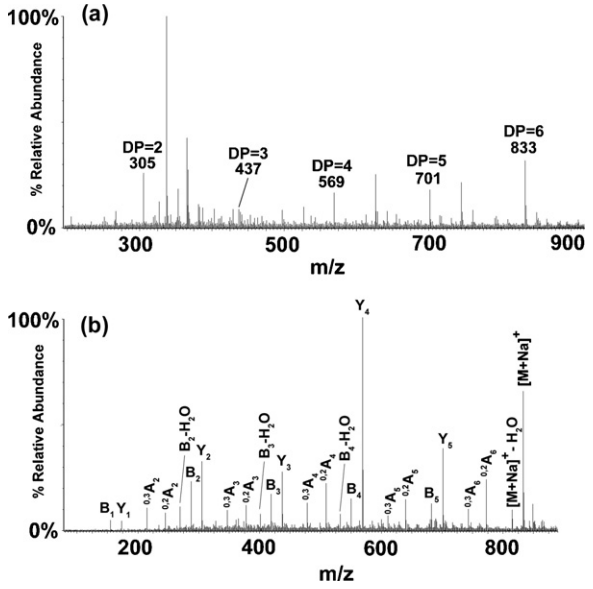

Fig. 6. (a) ESI-TOF-MS spectrum of endoxylanase-digested AFEXTCS extract analyzed using flow injection in positive ion mode showing $[M+Na]^+$ ions from arabinoxylans ranging from DP = 2 to DP = 6; (b) LC/ESI-TOF-MS spectrum of arabinoxylan with DP = 6 at elevated AP1 (CID) voltage (65 V) in positive ion mode.

2.4. Pentose oligomers from the AFEXTCS extract

Despite results from acid hydrolysis of AFEXTCS extract that determined 1.9 g/L of xylose and 0.4 g/L of arabinose in oligomeric forms (Table 1), LC/MS results yielded no clear evidence for pentose oligomers in either the original extract of the PGC-enriched fraction. Based on these findings, it was concluded that in contrast to glucans, soluble arabinoxylans released by AFEX™ treatment have high DPs (perhaps beyond the mass range acquired) or significant heterogeneity among numerous chemical forms, and need to be digested enzymatically to reduce molecular mass and complexity before detection using mass spectrometry. Characterization of more complex plant oligosaccharides has relied on enzymatic digestion (Hoffman et al., 2005; Izydorczyk & Biliaderis, 1995; Kabel, Carvalheiro, et al., 2002; Teleman, Nordstrom, Tenkanen, Jacobs, & Dahlman, 2003) to reduce the complexity of the oligosaccharide forms to allow individual digestion products to be characterized. In keeping with this tradition, AFEXTCS extract was treated with endo-(1,4)-β-xylanase in order to cleave (1,4)-β linkages between the xylose units in high DP arabinoxylans. After enzymatic digestion, a flow injection analysis of AFEXTCS extract showed ions consistent with a range of arabinoxylans from DP = 2 to DP = 6. Fig. 6a displays a mass spectrum of endoxylanase digest of AFEXTCS extract using positive mode ESI. Identification was based on accurate mass measurements and comparison with commercial arabinoxylans from oat spelt and birch wood after the same enzymatic digestion process. Both ESI positive (Fernandez et al., 2004; Wang et al., 2009) and negative (Quemener et al., 2006; Wang et al., 2009) modes were used for ionization of arabinoxylans in previous reports; however, positive mode ESI generated more abundant ions for digested arabinoxylans in AFEXTCS extract.

Support for structure annotation of the endoxylanase product was generated from multiplexed CID mass spectra. Fig. 6b shows a CID mass spectrum of an arabinoxylan (DP = 6) identified from the AFEXTCS extract after enzymatic digestion at high CID voltage (65 V). Similar CID spectra for $[M+Na]^+$ of arabinoxylans with DP = 6 were reported by Fernandez et al. (2003). Although formation of ^{0,2}A (loss of 60) and ^{0,3}A (loss of 90) fragment ions was consistent with (1–4) linked pentose units (Fernandez et al., 2003; Hofmeister, Zhou and Leary, 1991; Lemoine, Strecker, Leroy, Fournet, & Ricart, 1991; Lemoine et al., 1993; Zhou et al., 1990) branching information could not be obtained as no derivatization (e.g. *O*-methylation) nor further selective MS^n were performed. However, based on the ratio of xylose to arabinose in corn stover cell walls (Table 1), it is of high probability that DP = 6 contains at least one arabinose unit, and the prominence of the Y_4 fragment ion relative to other Y-ions is consistent with this conclusion. In summary, based on acid hydrolysis, accurate mass measurements, non-selective CID spectra and literature precedent, oligomers of pentose from the AFEXTCS extract were assigned as arabinoxylans with (1–4) linked backbone.

It is known that *O*-acetyl units are attached to hemicellulose arabinoxylans in different hardwoods (Ishii, 1991; Kabel, Carvalheiro, et al., 2002; Kabel, Schols, & Voragen, 2002; Selig, Adney, Himmel, & Decker, 2009). Cell wall acetylation has appreciable impact on enzymatic digestion as was shown in corn stover digestibility after different biomass treatment processes (Selig et al., 2009). The acetyl groups in acetylated cell wall glycopolymers are estimated to account for 35 mg/g dry weight in corn stover (Chundawat et al., 2010). AFEX™ treatment yielded > 85% removal of acetyl groups via formation of acetic acid and acetamide through hydrolysis and ammonolysis reactions respectively (Chundawat et al., 2010; Humpula et al., 2011). Solvolytic formation of acetic acid or acetamide depends on the severity of AFEX™ pretreatment conditions, which are otherwise mild compared to acid pretreatment methods (Chundawat et al., 2010). In order to assess release of soluble arabinoxylans and removal of acetyl groups, untreated corn stover (UTCS) and AFEXTCS were both digested with endo-(1,4)-β-xylanase for 12 h, and released arabinoxylans were profiled using LC/ESI-TOF-MS. A tentative comparison of peak areas of acetylated and non-acetylated digested arabinoxylans showed removal of >85% of the acetyl groups on digested arabinoxylans (DP = 2–6) after AFEX™ treatment (this estimate is for arabinoxylans that were cleaved by the endo-(1,4)-β-xylanase to DP = 2–6). Evidence of acetyl group removal is presented in Fig. 7, which shows four extracted ion chromatograms for acetylated and unmodified arabinoxylans in AFEXTCS and UTCS after enzymatic digestion. Fig. 7a documents that arabinoxylans with DP = 3 are more abundant in AFEXTCS compare to UTCS, which indicates that AFEX™ treatment

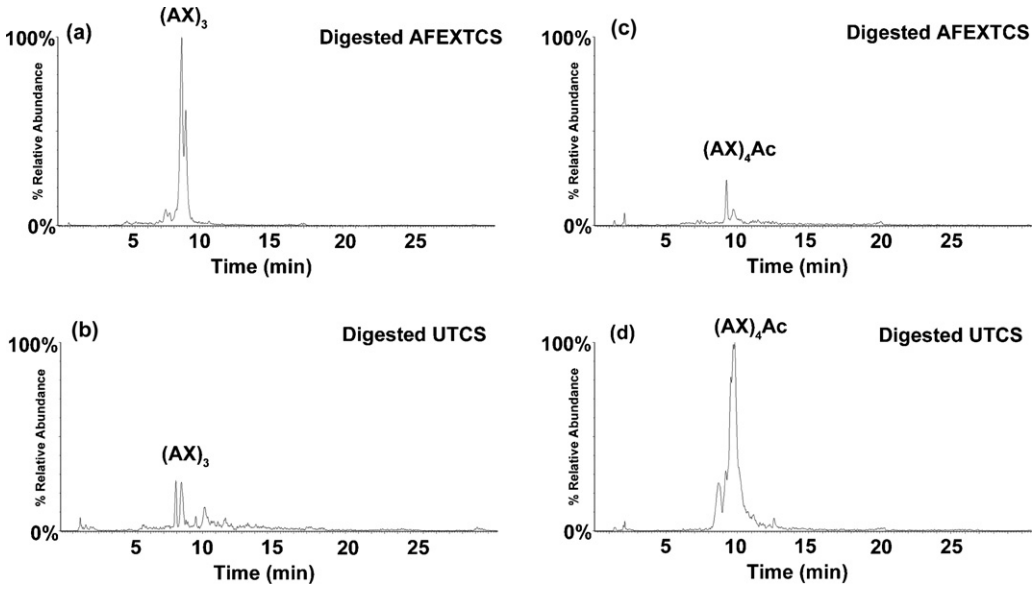

Fig. 7. Positive ion mode LC/ESI-TOF-MS extracted ion chromatograms for arabinoxylans (AX) with DP = 3 (*m/z* 437) from endoxylanase digested AFEXTCS (a) and digested UTCS (b), LC/ESI-TOF-MS extracted ion chromatograms (*m/z* 611) for arabinoxylans with DP = 4 and one acetyl group (Ac) in digested AFEXTCS (c) and digested UTCS (d). Scaling of the *y*-axis was performed to the same absolute signal for chromatograms (a) and (b) and also for (c) and (d).

released fermentable sugars from hemicellulose, as was demonstrated earlier using hydrolysis to monosaccharides (Chundawat et al., 2010; Selig et al., 2009). On the other hand, comparison of levels of an acetylated arabinoxylan (DP = 4) in UTCS and AFEXTCS digests documents that AFEX™ efficiently removed most of the acetyl groups under this specific condition, since the peak area in AFEXTCS (Fig. 7c) is less than 10% of the peak in UTCS (Fig. 7d). Similar reductions in amounts of poly-acetylated arabinoxylans were also observed.

3. Conclusions

Identification of oligosaccharides released from pretreated biomass serves as an important step toward optimization of enzymatic digestion processes and reduction of the cost of biofuel production. The presented methodology here presents a simple, quick and powerful approach for enrichment, separation and identification of soluble neutral oligosaccharides. Here this approach was applied to profile glucans and arabinoxylans from the AFEXTCS hydrolysates, but the strategy can be applied for characterization of large neutral oligosaccharides from other sources as well. The Prevail Carbohydrate ES phase offers great utility for separation of large oligosaccharides including isomers, and the use of multiplexed CID provides acquisition of CID spectra for all separated oligosaccharides in a single LC/MS analysis, producing information-rich spectra that lead to rapid structure annotations. Although not reported here, this technique has potential use in combination with oligosaccharide derivatization for fast and reliable characterization of complex oligosaccharides, and efforts are already underway to profile arabinoxylans released from cellulosic biomass by AFEX™ and related processes.

4. Methods

4.1. AFEX™ treatment

Corn stover (harvested in 2002 at the Kramer farm in Wray, CO, USA) was incubated in a reactor under pressure with liquid ammonia at 130 °C with 60% moisture. The loading ratio was 1:1 (w/w) NH_3-to-biomass, and total residence time was 15 min. Detailed protocols can be found in our previous publications (Chundawat et al., 2010).

4.2. Enzyme hydrolysis

50 mg of oat spelt xylan mixture (Sigma–Aldrich, USA), 50 mg of birch wood xylan mixture (Sigma–Aldrich, USA), 50 mg of AFEXTCS and UTCS and 0.5 mL of AFEXTCS extract were all separately incubated with 25 μL of food grade xylanase, RE4 (40 mg/mL, from Genencor, USA) for 12 h at 50 °C. Final volume in all cases were 1 mL using sodium acetate buffer at pH = 5. In each case, samples were injected directly for LC/ESI-TOF-MS analysis without further treatment.

4.3. Enrichment of oligosaccharides by PGC–SPE

PGC cartridges with bed weights of 1000 mg used for enrichment of oligosaccharides were HyperSep Hypercarb SPE columns from Thermo Fisher Scientific Inc. (USA). For evaluating retention capacity of cartridges, 7 mL of diluted (6 mg/mL total sugar) corn syrup (Kroger, USA) was loaded to one cartridge and non-retained materials after loading were analyzed using LC/ESI-TOF-MS for comparison with the diluted corn syrup sample. Methanol and acetonitrile were both tested for eluotropic strength and methanol showed to be a stronger eluent for elution of retained oligosaccharides on the PGC cartridges. For enriching oligosaccharides in corn stover hydrolysate, 6 mL of hot water extract of AFEXTCS (20 mL water/g treated biomass) was loaded onto each cartridge (48 mL total of extract was loaded to 8 cartridges in parallel). 5 mL water was used to wash non- and poorly-retained compounds from the PGC cartridges that were not collected. After washing, 30 mL of methanol was used to elute retained oligosaccharides. Eluants (total of 240 mL from 8 cartridges) were evaporated under vacuum and pooled to yield a final volume of 6 mL. This concentrated fraction was injected directly for LC/ESI-TOF-MS analysis.

4.4. LC/ESI-TOF-MS separation and identification of oligosaccharides

Oligosaccharides from all extracts (enriched, enzyme digest or non-processed) were analyzed using a Prevail Carbohydrate ES column (150 mm × 2 mm, 5 μm; Alltech Associates Inc., Deerfield, IL, USA) coupled to the mass spectrometer. The LC/MS system used in this work consisted of three Shimadzu HPLC pumps (LC-20AD) coupled to a Waters LCT Premier Time-of-Flight Mass Spectrometer (TOF-MS). Gradient elution was performed based on solvent A (0.15% aqueous formic acid) and B (acetonitrile) over 30 min. Total solvent flow was maintained at 250 μL/min, and gradient elution was performed using the following solvent compositions: initial, 95% B, held for 1 min; linear gradient to 70% B at 8 min and then to 50% B at 18 min; hold at 50% B until 25 min, sudden increase to initial condition at 25.01 min and final hold at this composition until 30 min. Positive and negative mode electrospray ionization were performed along with multiplexed collision induced dissociation (CID) by switching among three different AP1 voltages (15, 40, and 65 V). Processing of LC/MS data was accomplished using MassLynx v. 4.1 software.

4.5. Acid hydrolysis and analysis of monosaccharides

Monomeric carbohydrate concentrations were measured before acid hydrolysis, while oligosaccharide concentrations were calculated by taking sugar concentrations measured after acid hydrolysis and subtracting the monomeric sugar concentrations. For acid hydrolysis, 500 μL of extract and 17.6 μL of 72% sulfuric acid were placed in tightly capped 10 mL culture tubes which were vortexed and placed in a preheated block heater (EL-02 Elite, Major Science, Saratoga, CA) at 121 °C for 1 h, after which they were cooled on ice to room temperature. For sugar analysis, the fractions were filtered using 0.22 μm PES syringe filters (Whatman Inc., Piscataway, NJ), and were analyzed using a Prominence HPLC system (Shimadzu, Columbia, MD) using an Aminex HPX-87H HPLC carbohydrate analysis column, a Cation H guard column (Bio-Rad, Hercules, CA) and a RID-10A refractive index detector (Shimadzu, Kyoto, Japan). 5 mM aqueous H_2SO_4 was the mobile phase, delivered at a flow rate of 0.6 mL/min and 50 °C. The injection volume was 10 μL. Yields of xylose after hydrolysis were corrected using a carbohydrate recovery standard in order to account for xylose degradation resulting from the hydrolysis conditions. Typical xylose recoveries were about 80–90% using these conditions.

Acknowledgements

This work was funded by Great Lakes Bioenergy Research Center (http://www.greatlakesbioenergy.org/) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, through Cooperative Agreement DEFC02-07ER64494 between The Board of Regents of the University of Wisconsin System and the U. S. Department of Energy. Michigan AgBioResearch provided additional support. The authors thank Lijun Chen, Beverly Chamberlin, and Dr. Scott Smith from RTSF Mass Spectrometry and Metabolomics Core at Michigan State University for technical support.

References

Agblevor, F. A., Murden, A., & Hames, B. R. (2004). Improved method of analysis of biomass sugars using high-performance liquid chromatography. *Biotechnology Letters*, *26*, 1207–1210.

Antonio, C., Larson, T., Gilday, A., Graham, I., Bergstroem, E., & Thomas-Oates, J. (2007). Quantification of sugars and sugar phosphates in Arabidopsis thaliana tissues using porous graphitic carbon liquid chromatography-electrospray ionization mass spectrometry. *Journal of Chromatography A*, *1172*, 170–178.

Cataldi, T. R. I., Campa, C., & De Benedetto, G. E. (2000). Carbohydrate analysis by high-performance anion-exchange chromatography with pulsed amperometric detection: The potential is still growing. *Fresenius Journal of Analytical Chemistry*, *368*, 739–758.

Chundawat, S. P. S., Vismeh, R., Sharma, L. N., Humpula, J. F., Sousa, L. D., Chambliss, C. K., et al. (2010). Multifaceted characterization of cell wall decomposition products formed during ammonia fiber expansion (AFEX) and dilute acid based pretreatments. *Bioresource Technology*, *101*, 8429–8438.

Churms, S. C. (1996). Recent progress in carbohydrate separation by high-performance liquid chromatography based on hydrophilic interaction. *Journal of Chromatography A*, *720*, 75–91.

Domon, B., & Costello, C. E. (1988). A systematic nomenclature for carbohydrate fragmentations in FAB-MS MS spectra of glycoconjugates. *Glycoconjugate Journal*, *5*, 397–409.

Duchesne, L. C., & Larson, D. W. (1989). Cellulose and the evolution of plant life. *Bioscience*, *39*, 238–241.

Fernandez, L. E. M., Obel, N., Scheller, H. V., & Roepstorff, P. (2003). Characterization of plant oligosaccharides by matrix-assisted laser desorption/ionization and electrospray mass spectrometry. *Journal of Mass Spectrometry*, *38*, 427–437.

Fernandez, L. E. M., Obel, N., Scheller, H. V., & Roepstorff, P. (2004). Differentiation of isomeric oligosaccharide structures by ESI tandem MS and GC–MS. *Carbohydrate Research*, *339*, 655–664.

Garozzo, D., Giuffrida, M., Impallomeni, G., Ballistreri, A., & Montaudo, G. (1990). Determination of linkage position and identification of the reducing end in linear oligosaccharides by negative-ion fast atom bombardment mass-spectrometry. *Analytical Chemistry*, *62*, 279–286.

Gu, L. P., Jones, A. D., & Last, R. L. (2010). Broad connections in the Arabidopsis seed metabolic network revealed by metabolite profiling of an amino acid catabolism mutant. *Plant Journal*, *61*, 579–590.

Guignard, C., Jouve, L., Bogeat-Triboulot, M. B., Dreyer, E., Hausman, J. F., & Hoffmann, L. (2005). Analysis of carbohydrates in plants by high-performance anion-exchange chromatography coupled with electrospray mass spectrometry. *Journal of Chromatography A*, *1085*, 137–142.

Harrison, S., Fraser, K., Lane, G., Hughes, D., Villas-Boas, S., & Rasmussen, S. (2011). Analysis of high-molecular-weight fructan polymers in crude plant extracts by high-resolution LC–MS. *Analytical and Bioanalytical Chemistry*, *401*, 2955–2963.

Harvey, D. J. (1999). Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. *Mass Spectrometry Reviews*, *18*, 349–450.

Hennion, M. C. (2000). Graphitized carbons for solid-phase extraction. *Journal of Chromatography A*, *885*, 73–95.

Hoffman, M., Jia, Z. H., Pena, M. J., Cash, M., Harper, A., Blackburn, A. R., et al. (2005). Structural analysis of xyloglucans in the primary cell walls of plants in the subclass asteridae. *Carbohydrate Research*, *340*, 1826–1840.

Hofmeister, G. E., Zhou, Z., & Leary, J. A. (1991). Linkage position determination in lithium-cationized disaccharides—tandem mass-spectrometry and semiempirical calculations. *Journal of the American Chemical Society*, *113*, 5964–5970.

Humpula, J. F., Chundawat, S. P. S., Vismeh, R., Jones, A. D., Balan, V., & Dale, B. E. (2011). Rapid quantification of major reaction products formed during thermochemical pretreatment of lignocellulosic biomass using GC–MS. *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, *879*, 1018–1022.

Ishii, T. (1991). Acetylation at O-2 of arabinofuranose residues in feruloylated arabinoxylan from bamboo shoot cell-walls. *Phytochemistry*, *30*, 2317–2320.

Ishii, T. (1997). Structure and functions of feruloylated polysaccharides. *Plant Science*, *127*, 111–127.

Izydorczyk, M. S., & Biliaderis, C. G. (1995). Cereal arabinoxylans: Advances in structure and physicochemical properties. *Carbohydrate Polymers*, *28*, 33–48.

Kabel, M. A., Carvalheiro, F., Garrote, G., Avgerinos, E., Koukios, E., Parajo, J. C., et al. (2002). Hydrothermally treated xylan rich by-products yield different classes of xylo-oligosaccharides. *Carbohydrate Polymers*, *50*, 47–56.

Kabel, M. A., Schols, H. A., & Voragen, A. G. J. (2002). Complex xylo-oligosaccharides identified from hydrothermally treated eucalyptus wood and Brewery's spent grain. *Carbohydrate Polymers*, *50*, 191–200.

Kalay, H., Ambrosini, M., van Berkel, P. H. C., Parren, P., van Kooyk, Y., & Vallejo, J. J. G. (2012). Online nanoliquid chromatography–mass spectrometry and nanofluorescence detection for high-resolution quantitative N-glycan analysis. *Analytical Biochemistry*, *423*, 153–162.

Karlsson, G., Swerup, E., & Sandberg, H. (2008). Combination of two hydrophilic interaction chromatography methods that facilitates identification of 2-aminobenzamide-labeled oligosaccharides. *Journal of Chromatographic Science*, *46*, 68–73.

Lee, Y. C. (1996). Carbohydrate analyses with high-performance anion-exchange chromatography. *Journal of Chromatography A*, *720*, 137–149.

Leijdekkers, A. G. M., Sanders, M. G., Schols, H. A., & Gruppen, H. (2011). Characterizing plant cell wall derived oligosaccharides using hydrophilic interaction chromatography with mass spectrometry detection. *Journal of Chromatography A*, *1218*, 9227–9235.

Lemoine, J., Fournet, B., Despeyroux, D., Jennings, K. R., Rosenberg, R., & Dehoffmann, E. (1993). Collision-induced dissociation of alkali-metal cationized and permethylated oligosaccharides—influence of the collision energy and of the collision gas for the assignment of linkage position. *Journal of the American Society for Mass Spectrometry*, *4*, 197–203.

Lemoine, J., Strecker, G., Leroy, Y., Fournet, B., & Ricart, G. (1991). Collisional-activation tandem mass-spectrometry of sodium adduct ions of

methylated oligosaccharides—sequence-analysis and discrimination between Alpha-NeuAc-(2–3) and Alpha-NeuAc-(2–6) linkages. *Carbohydrate Research*, *221*, 209–217.

Maslen, S. L., Goubet, F., Adam, A., Dupree, P., & Stephens, E. (2007). Structure elucidation of arabinoxylan isomers by normal phase HPLC-MALDI-TOF/TOF-MS/MS. *Carbohydrate Research*, *342*, 724–735.

Megherbi, M., Herbreteau, B., Faure, R., & Salvador, A. (2009). Polysaccharides as a marker for detection of corn sugar syrup addition in honey. *Journal of Agricultural and Food Chemistry*, *57*, 2105–2111.

Munisamy, S. M., Chambliss, C. K., & Becker, C. (2012). Direct infusion electrospray ionization—ion mobility—high resolution mass spectrometry (DIESI-IM-HRMS) for rapid characterization of potential bioprocess streams. *Journal of the American Society for Mass Spectrometry*, *23*, 1250–1259.

Pasanen, S., Janis, J., & Vainiotalo, P. (2007). Cello-, malto- and xylooligosaccharide fragmentation by collision-induced dissociation using QIT and FT-ICR mass spectrometry: A systematic study. *International Journal of Mass Spectrometry*, *263*, 22–29.

Pauly, M., & Keegstra, K. (2008). Cell–wall carbohydrates and their modification as a resource for biofuels. *Plant Journal*, *54*, 559–568.

Plumb, R. S., Johnson, K. A., Rainville, P., Smith, B. W., Wilson, I. D., Castro-Perez, J. M., et al. (2006). UPLC/MS^E; a new approach for generating molecular fragment information for biomarker structure elucidation. *Rapid Communications in Mass Spectrometry*, *20*, 1989–1994.

Quemener, B., Ordaz-Ortiz, J. J., & Saulnier, L. (2006). Structural characterization of underivatized arabino-xylo-oligosaccharides by negative-ion electrospray mass spectrometry. *Carbohydrate Research*, *341*, 1834–1847.

Reinhold, V. N., Reinhold, B. B., & Costello, C. E. (1995). Carbohydrate molecular-weight profiling, sequence, linkage, and branching data—ES-MS and CID. *Analytical Chemistry*, *67*, 1772–1784.

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. In S. Merchant, W. R. Briggs, & D. Ort (Eds.), *Annual review of plant biology* (pp. 263–289). Palo Alto: Annual Reviews.

Schilmiller, A., Shi, F., Kim, J., Charbonneau, A. L., Holmes, D., Jones, A. D., et al. (2010). Mass spectrometry screening reveals widespread diversity in trichome specialized metabolites of tomato chromosomal substitution lines. *Plant Journal*, *62*, 391–403.

Selig, M. J., Adney, W. S., Himmel, M. E., & Decker, S. R. (2009). The impact of cell wall acetylation on corn stover hydrolysis by cellulolytic and xylanolytic enzymes. *Cellulose*, *16*, 711–722.

Sinha, V. R., & Kumria, R. (2001). Polysaccharides in colon-specific drug delivery. *International Journal of Pharmaceutics*, *224*, 19–38.

Slimestad, R., & Vagen, I. M. (2006). Thermal stability of glucose and other sugar aldoses in normal phase high performance liquid chromatography. *Journal of Chromatography A*, *1118*, 281–284.

Sousa, L. D., Chundawat, S. P. S., Balan, V., & Dale, B. E. (2009). 'Cradle-to-Grave' assessment of existing lignocellulose pretreatment technologies. *Current Opinion in Biotechnology*, *20*, 339–347.

Spengler, B., Dolce, J. W., & Cotter, R. J. (1990). Infrared-laser desorption mass-spectrometry of oligosaccharides—fragmentation mechanisms and isomer analysis. *Analytical Chemistry*, *62*, 1731–1737.

Strum, J. S., Aldredge, D., Barile, D., & Lebrilla, C. B. (2012). Coupling flash liquid chromatography with mass spectrometry for enrichment and isolation of milk oligosaccharides for functional studies. *Analytical Biochemistry*, *424*, 87–96.

Teleman, A., Nordstrom, M., Tenkanen, M., Jacobs, A., & Dahlman, O. (2003). Isolation and characterization of O-acetylated glucomannans from Aspen and Birch wood. *Carbohydrate Research*, *338*, 525–534.

van der Hoeven, R. A. M., Hofte, A. J. P., Tjaden, U. R., van der Greef, J., Torto, N., Gorton, L., et al. (1998). Sensitivity improvement in the analysis of oligosaccharides by on-line high-performance anion-exchange chromatography ion spray mass spectrometry. *Rapid Communications in Mass Spectrometry*, *12*, 69–74.

Van Dongen, F. E. M., Van Eylen, D., & Kabel, M. A. (2011). Characterization of substituents in xylans from corn cobs and stover. *Carbohydrate Polymers*, *86*, 722–731.

Vinjamoori, D. V., Byrum, J. R., Hayes, T., & Das, P. K. (2004). Challenges and opportunities in the analysis of raffinose oligosaccharides, pentosans, phytate, and glucosinolates. *Journal of Animal Science*, *82*, 319–328.

Wan, E. C. H, & Yu, J. Z. (2007). Analysis of sugars and sugar polyols in atmospheric aerosols by chloride attachment in liquid chromatography/negative ion electrospray mass spectrometry. *Environmental Science & Technology*, *41*, 2459–2466.

Wang, J., Yuan, X. P., Sun, B. G., Cao, Y. P., Tian, Y., & Wang, C. T. (2009). On-line separation and structural characterisation of feruloylated oligosaccharides from wheat bran using HPLC-ESI-MS^n. *Food Chemistry*, *115*, 1529–1541.

West, C., Elfakir, C., & Lafosse, M. (2010). Porous graphitic carbon: A versatile stationary phase for liquid chromatography. *Journal of Chromatography A*, *1217*, 3201–3216.

Westphal, Y., Schols, H. A., Voragen, A. G. J., & Gruppen, H. (2010). Introducing porous graphitized carbon liquid chromatography with evaporative light scattering and mass spectrometry detection into cell wall oligosaccharide analysis. *Journal of Chromatography A*, *1217*, 689–695.

Zhang, J. H., Xie, Y. M., Hedrick, J. L., & Lebrilla, C. B. (2004). Profiling the morphological distribution of O-linked oligosaccharides. *Analytical Biochemistry*, *334*, 20–35.

Zhou, Z. R., Ogden, S., & Leary, J. A. (1990). Linkage position determination in oligosaccharides—MS/MS study of lithium-cationized carbohydrates. *Journal of Organic Chemistry*, *55*, 5444–5446.