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Chromatographic determination of 1, 4-β-xylooligosaccharides of different chain lengths to follow xylan deconstruction in biomass conversion

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Abstract Xylooligosaccharides released in hydrothermal pretreatment of lignocellulosic biomass can be purified for high-value products or further hydrolyzed into sugars for fermentation or chemical conversion. In addition, characterization of xylooligosaccharides is vital to understand hemicellulose structure and removal mechanisms in pretreatment of cellulosic biomass. In this study, gel permeation chromatography was applied to fractionate xylooligosaccharides produced from birchwood xylan according to their specific degree of polymerization (DP). Then, each fraction was identified by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) and matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF–MS); and their concentrations were determined by a down-scaled post-hydrolysis method. Based on PAD responses and sugar concentrations for each fraction, a series of response factors were developed that can be used to quantify xylooligosaccharides of DP from 2 to 14 without standards. The resulting approach can profile xylooligosaccharides and help gain new insights into biomass deconstruction.

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Introduction

For the majority of lignocellulosic feedstocks for production of bioethanol and other biofuels, heteroxylans are the predominant component in hemicellulose, the second most abundant polysaccharide in nature [6, 9]. When heated up with water, the structure of biopolymers within plant cell walls is disturbed, and heteroxylans are depolymerized into numerous xylooligosaccharides with different chain lengths [8, 15, 26]. With the growing commercial importance of hemicellulose, these soluble oligosaccharides can be either purified for high-value-added products such as ingredients in functional foods, cosmetics, and pharmaceuticals due to their prebiotic activity [22] or further hydrolyzed into fermentable sugars as platform molecules for biofuels [23–25]. However, the reactions of heteroxylans to soluble xylooligosaccharides and monomeric sugars also involve degradation to byproducts, which reduce sugar recovery and inhibit or prevent subsequent bioconversion processes [1, 16]. Detailed study of xylooligosaccharide hydrolysis kinetics and degradation pathways is essential to understand hemicellulose decomposition mechanisms and can aid in designing low-cost processes.

Unfortunately, quantification of xylooligosaccharides is very challenging, and the conventional post-hydrolysis method conducted with 4 wt% acid at 121 °C for 1 h remains dominant for quantifying oligosaccharides in liquid hydrolyzates [19]. However, this approach only determines the total equivalent monomeric xylose concentration and does not characterize the distribution of

xylooligosaccharide concentrations according to chain length. It also suffers from sugar degradation during the procedure, and although sugar recovery standards can be applied in an attempt to account for such losses, it introduces additional uncertainties into the measurement [27].

HPAEC-PAD promises to be a sensitive and selective tool for analysis of complex carbohydrates without derivatization [2, 11, 18] and has proved effective in separating xylooligosaccharides released from hydrothermal pretreatment of corn stover [26]. The HPAEC-PAD takes advantage of the weakly acidic properties of carbohydrates in alkaline eluents (pH > 13) to separate carbohydrates and detects the corresponding aldehyde and hydroxyl groups by electro-oxidation reactions under a multistep potential waveform [10, 12]. PAD detection has been suggested to directly detect oligosaccharides at oxide-free surfaces [12] by absorbing analyte molecules on a gold electrode surface where they are anodically oxidized under a positive potential. However, previous studies have shown that the PAD response of carbohydrates is a complicated function of certain chemical and physical variables such as pK_a and structural features such as molecular dimensions and spatial structure [2, 17]. For a series of oligosaccharides with a range of DPs, such factors can result in different reaction rates for electro-catalytic oxidation on the electrode surface and further impact PAD responses. Thus, corresponding sugar standards are needed to quantify oligosaccharide fractions with different DPs. Unfortunately, because xylooligosaccharide standards are only available for DPs less than 6, accurate quantification of higher DP xylooligosaccharides released by biomass pretreatment has not been possible with HPAEC-PAD.

This study sought to calibrate the HPAEC-PAD technique by developing a series of coefficients we term response factors to more precisely characterize xylooligosaccharides according to their DP when standards are not available. First, xylooligosaccharides from hydrothermally hydrolyzed birchwood xylan were separated into individual DP fractions by size exclusion GPC, and the purity of these fractions was checked by HPAEC-PAD and MALDI-TOF–MS. A sketch of the major steps in this integrated system is shown in Fig. 1. The PAD responses of these isolated fractions were then compared to their sugar concentrations quantified by a downscaled post-hydrolysis approach, and a series of response factors were established for xylooligosaccharides with DP from 2 to 14. These response factors allow fast and more precise quantification of xylooligosaccharides produced by lignocellulosic biomass pretreatment on HPAEC-PAD without expensive, and in many cases, unavailable, xylooligosaccharide standards. They also provide valuable new insight into the mechanism of sugar release and new opportunities to advance pretreatment and other biomass deconstruction technologies.

Materials and methods

Materials

Birchwood xylan (Lot No. 038K0751) was purchased from Sigma–Aldrich (St. Louis, MO, USA) and was measured to have a xylan content of ~85 % by a two-step acid hydrolysis method [19]; d-xylose with a xylose purity >99 % was also obtained from Sigma–Aldrich (Batch No. 1403673). Xylobiose, xylotriose, and xylotetraose standards (Cat No. O-XBI, O-XTR, O-XTE) of over 95 % purity were purchased from Megazyme International Ireland Ltd. (Bray Business Park, Bray, Co. Wicklow, Ireland). Sodium acetate (Cat No. S7545) and 50 wt% sodium hydroxide solution (Cat No. 72064) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

Xylooligosaccharide production

Xylooligosaccharide hydrolyzate was produced by hydrothermal pretreatment of 15 % (w/v) birchwood xylan at 200 °C for 15 min in 6-in.-long cylindrical tube reactors made of Hastelloy C276 (Industrial Alloys Plus Inc., Utica, KY, USA). This condition was previously found to maximize xylooligosaccharide concentrations and minimize degradation [7]. After the 15-min reaction time, the tubes were quenched quickly in room temperature water. The hydrolyzate was then filtered through a glass fiber filter (Fisherbrand, Cat No. 09-804-110A). To avoid further degradation or precipitation of higher DP xylooligosaccharides, fresh stock was always generated just before analysis.

Xylooligosaccharide separation by GPC

To fractionate the xylooligosaccharide mixture into its individual components according to their DP, a 5-cm inner diameter by 1-m-long low-pressure glass column with an acrylic water jacket (Cat No. XK50/100, GE Healthcare, Piscataway, NJ, USA) was filled with ultra fine Biogel P-4 (Bio-Rad, Hercules, CA). Gel preparation was based on the instruction manual by Bio-Rad. After that, 400 ml of degassed, deionized (DI) water was slowly poured along the inner wall of the glass column to fill 20 % of the column. The gel slurry was then slowly poured evenly into the column in the same way as water to avoid splashing and trapping air bubbles. After a 5-cm-high bed was formed, the column outlet was opened to allow water to flow until all the gel was packed. A 7-cm I.D. adjustable-length flow adapter (GE Healthcare, Piscataway, NJ, USA) was then connected to a peristaltic pump (Masterflex, Model 7518-00). Degassed DI water was pumped through the column at a flow rate of 1.6 ml/min overnight to complete

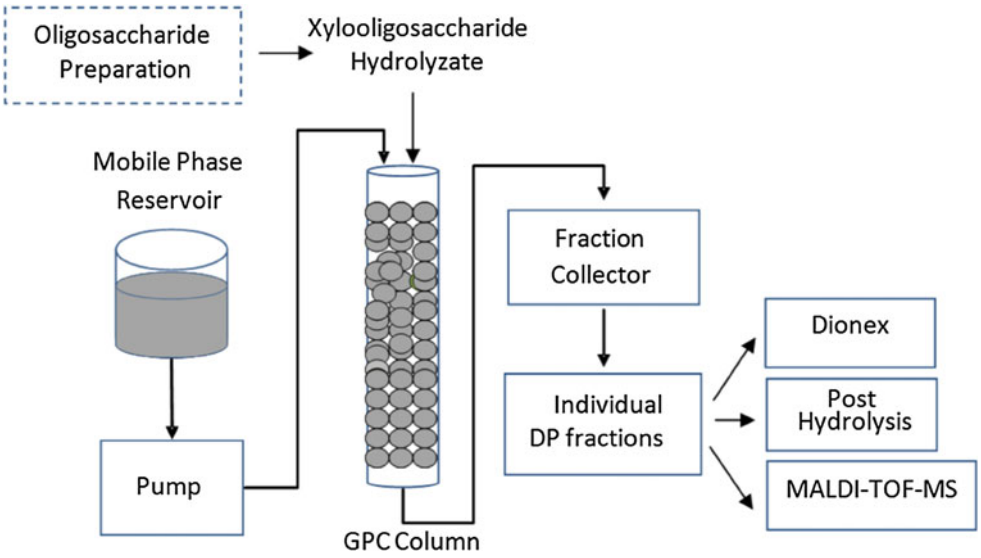

Fig. 1 Diagram of the integrated chromatographic system used to isolate and characterize xylooligosaccharides resulting from hydrothermal pretreatment of birchwood xylan

packing. A Fisher Scientific immersion circulator (Pittsburgh, PA) was used to heat water to 50 °C and pump it through the 7-cm I.D. × 100-cm acrylic water jacket (GE Healthcare, Piscataway, NJ, USA). After the column reached equilibrium at the flow rate and temperature, 10 ml of xylooligosaccharide hydrolyzate prepared as above was injected into the column. The outflow from the column passed into a Waters Fraction Collector II (Waters Corporation, Milford, MA, USA) to automatically collect sample fractions at 6-min intervals.

Xylooligosaccharide identification

Unfractionated xylooligosaccharide hydrolyzate and xylooligosaccharide fractions separated by the GPC system and collected by the Waters fraction collector were analyzed by the HPAEC-PAD technique on a Dionex DX-600 Ion Chromatograph system equipped with a ED50 electrochemical detector, a CarboPac PA100 (4 × 250 mm) anion exchange column, and a guard cartridge (Dionex Corp., Sunnyvale, CA, USA). The mobile phases were operated in the gradient mode from 50 to 450 mM of sodium acetate through 150 mM sodium hydroxide (Dionex Application Note 67). An example Dionex IC chromatogram of the distribution of chain lengths for unfractionated xylooligosaccharide hydrolyzate resulting from the preparation method described earlier is shown in Fig. 2. Peaks of xylose, xylobiose, xylotriose, and xylotetraose were determined by retention time of corresponding commercial sugar standards. Assignment of chromatographic peaks for xylooligosaccharides with DP > 4 was based on the generally accepted assumption that retention time of a homologous series of xylooligosaccharides increases with DP [3] and that each successive peak represents an oligosaccharide with one more xylose residue than for the previous peak. Xylooligosaccharide fractions that displayed the highest purity in the Dionex analysis were selected for each DP from 2 to 14. A Bruker Microflex LT mass spectrometer in the positive-ion mode was employed to further confirm the purity of the selected xylooligosaccharide fractions on MALDI-TOF–MS. One µl of a mixture prepared by mixing 5 µl of each sample with 5 µl 10 mM NaCl was then added to 1 µl DHB (dihydroxybenzoic acid matrix 10 mg/ml in 50 % acetonitrile) on a stainless-steel MALDI target plate and dried with warm air.

Determination of isolated xylooligosaccharide concentrations

Concentrations of the individual isolated xylooligosaccharide DP fractions collected with the GPC were measured according to the NREL post-hydrolysis method [19]. However, due to the small volumes of each fraction that could be collected, these measurements were scaled down by a factor of 100 in our custom-made 96-well plates made of Hastelloy 276 steel [20]. An amount of 400 µl of liquid sample was transferred into each vial, followed by 14 µl of 72 wt% sulfuric acid to bring the acid concentration to 4 wt%. The resulting mixture was then hydrolyzed at 121 °C for 1 h to breakdown the oligosaccharides to xylose [19]. Sugar recovery standards containing xylose at a concentration close to that in the samples were also run in parallel to estimate sugar degradation in calculating the final xylose concentration. The validity of the downscaled method for quantification of sugars was previously established [5]. Monomeric sugars were analyzed with a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA, USA) equipped with a refractive index detector (Waters 2414). The sugars were separated on an Aminex

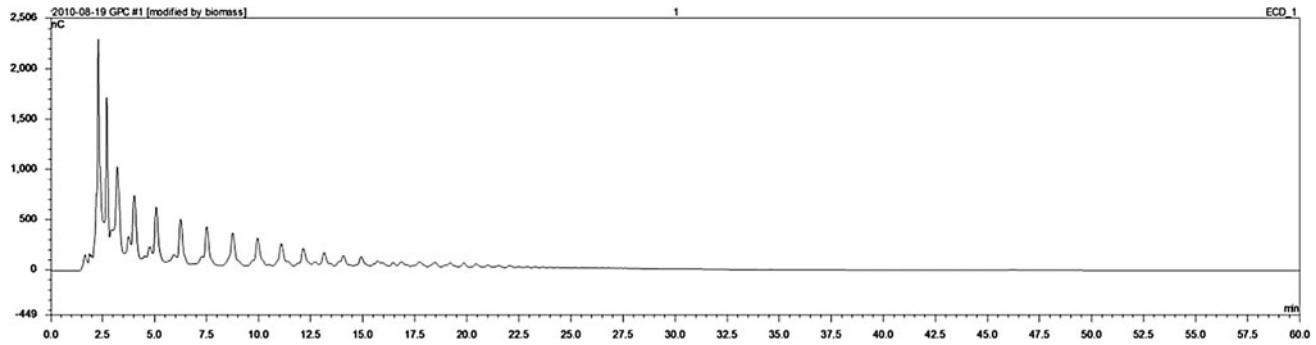

Fig. 2 Example chromatogram from HPAEC-PAD analysis of xylooligosaccharide hydrolyzate produced by hydrothermal pretreatment of birchwood xylan as detected by the Dionex IC system coupled with a CarboPac PA-100 column

HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) heated to 65 °C with 0.005 M sulfuric acid as the eluent at 0.6 ml/min in an isocratic mode.

Definition and calculation of response factors

PAD response factor α_n to quantify higher DP xylooligosaccharides for which no standards are available was determined through calibration with existing xylose standard based on basic HPLC quantitative analysis theory:

$$\frac{H_n}{C_n} = \alpha_n \times \frac{H_1}{C_1} \quad (1)$$

in which C_1 is the concentration of the xylose standard in g/l, H_1 is the corresponding xylose peak height from the HPAEC-PAD chromatogram, C_n is the concentration of the xylooligosaccharide of DP_n as measured by downscaled post hydrolysis, and H_n is the peak height measured for that xylooligosaccharide. To calculate α_n, a linear curve passing through the origin was fit to C_n versus H_n measurements for three different injection concentrations of each xylooligosaccharide DP fraction. As shown in Eq. (2), the slope of the resulting calibration curve, M_n, relates the concentration C_n to the PAD response H_n for each DP xylooligosaccharide.

$$C_n = M_n \times H_n \quad (2)$$

If n equals 1, Eq. (2) corresponds to a xylose calibration curve:

$$C_1 = M_1 \times H_1 \quad (3)$$

By dividing Eq. (3) by Eq. (2), the response factor α_n defined above can be calculated as the ratio of M_1 to M_n for each DP xylooligosaccharide:

$$\frac{M_1}{M_n} = \frac{C_1}{H_1} \times \frac{H_n}{C_n} = \alpha_n \quad (4)$$

In this study, all sugar concentrations were measured in g/l.

Results and discussion

Isolation and characterization of individual DP xylooligosaccharides

After injection of the xylooligosaccharide hydrolyzate into the GPC column, the fraction collector was set to collect liquid samples in 15-ml plastic test tubes at 6-min intervals. The first isolated xylooligosaccharide fraction was detected at around 770 min and identified as DP 14 based on the retention time from the Dionex IC chromatogram of mixed DP xylooligosaccharide hydrolyzate (Fig. 2). Injection of 10 ml of xylooligosaccharide hydrolyzate into the GPC resulted in the best separation of xylooligosaccharide fractions over a DP range from 14 to 2 during the retention period from 770 to 1,560 min. Based on analysis of the results from the Dionex IC system, 13 individual fractions corresponding to xylooligosaccharide from DP 2 to 14 were selected for subsequent experiments. As the chromatograms in Fig. 3 show, these fractions were selected to maximize the proportion of the dominant DP xylooligosaccharide in each fraction. The separation purity of each DP fraction was calculated based on Eq. (5) to gauge the separation efficacy of the GPC system:

$$\textit{Separation purity} = \frac{\textit{Peak area of desired DP xylooligosaccharide}}{\textit{Sum of peak areas in chromatogram}} \times 100\,\% \quad (5)$$

The separation purity values calculated in this manner are shown in Fig. 4. Although the purity for DP 14 was somewhat low at 87 %, the separation purities calculated for xylooligosaccharide fractions from DP 13 to 2 were all above 90 %, indicating good purity of the samples in this DP range.

We assumed that the xylooligosaccharides were composed of xylose as backbone (dehydrated molecular weight of 132) with few glucuronic acid residues (GlcA,

Fig. 3 Analytical HPAEC-PAD chromatograms of 13 individual DP xylooligosaccharide fractions isolated from GPC system, from right to left corresponding to **a** DP 14–11, **b** DP 10–6, and **c** DP 5–2

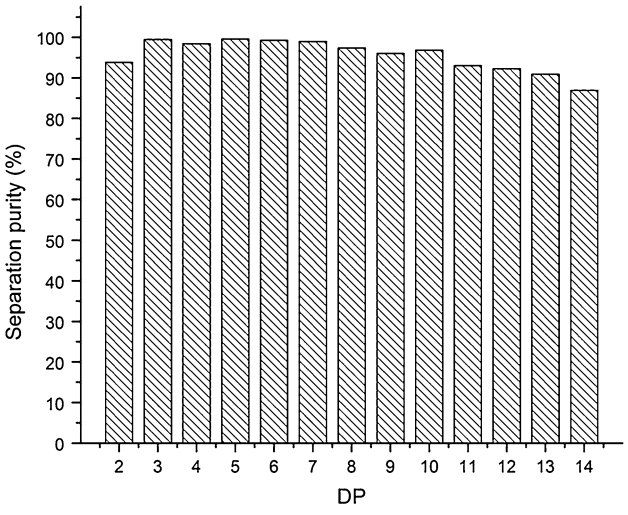

Fig. 4 Separation purities of individual DP xylooligosaccharide fractions isolated by the GPC system

dehydrated molecular weight of 176). However, to test these assumptions, we developed mass spectrograms for each fraction, as illustrated in Fig. 5 for xylooligosaccharide fractions with DP 4, 7, 10, and 13. The neutral oligosaccharides gave pseudo-molecular ions $[M + Na]^+$ and oligosaccharides containing GlcA gave $[M + Na]^+$ and $[M + 2Na]^+$ ions. Results from MALDI-TOF–MS supported the successful isolation of the selected xylooligosaccharide fractions by GPC with reasonably high purity. Thus, they should serve as good standards to calculate response factors for xylooligosaccharides.

Concentrations of isolated xylooligosaccharide fractions

As shown in Fig. 6, the concentrations of isolated DP fractions covered a range of 0.5–1.1 g/l. Although concentrations of lower DP xylooligosaccharide fractions,

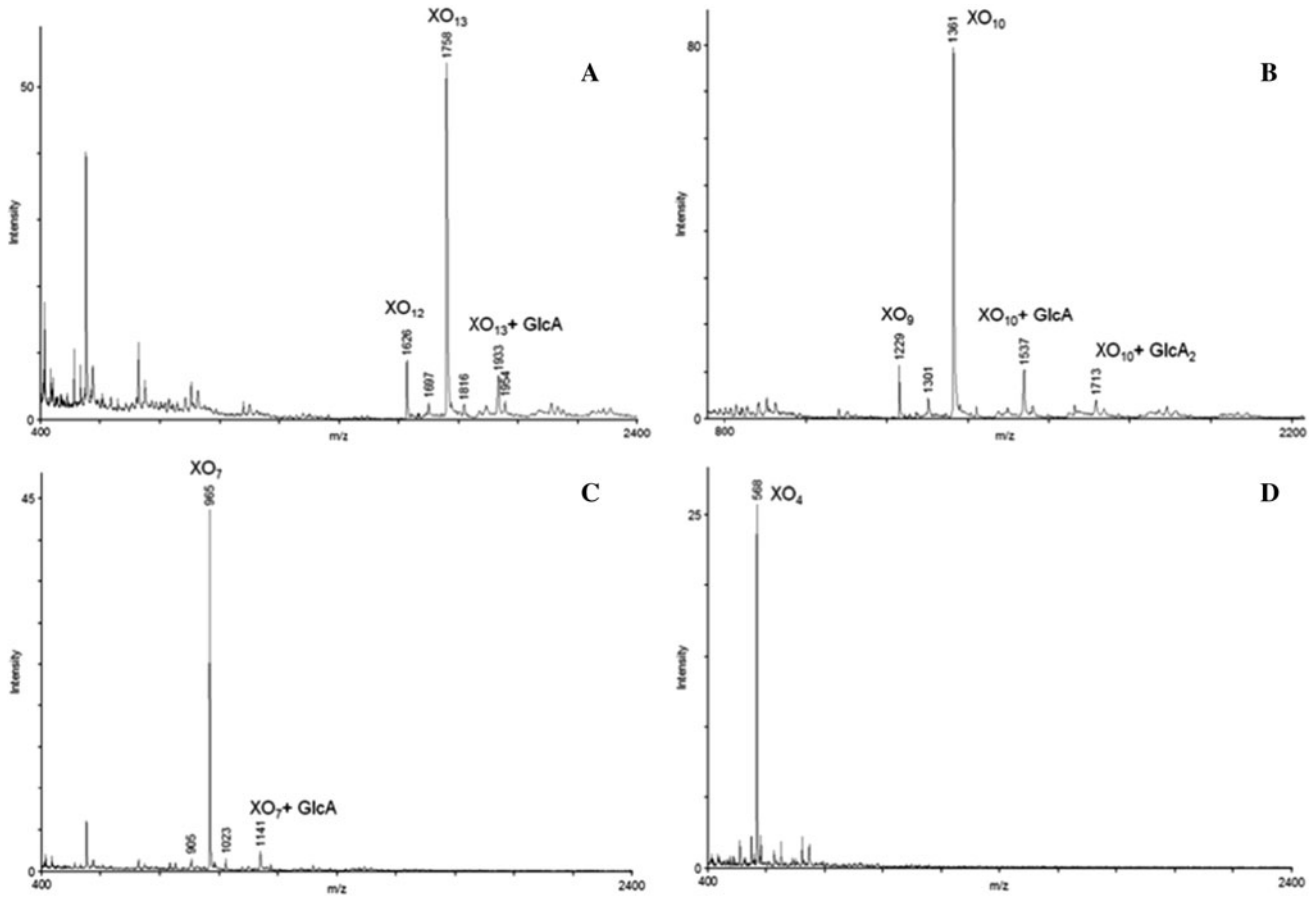

Fig. 5 Selected MALDI-TOF–MS spectra of individual DP xylooligosaccharides isolated by the GPC system: **a** DP 13, **b** DP 10, **c** DP 7, and **d** DP 4

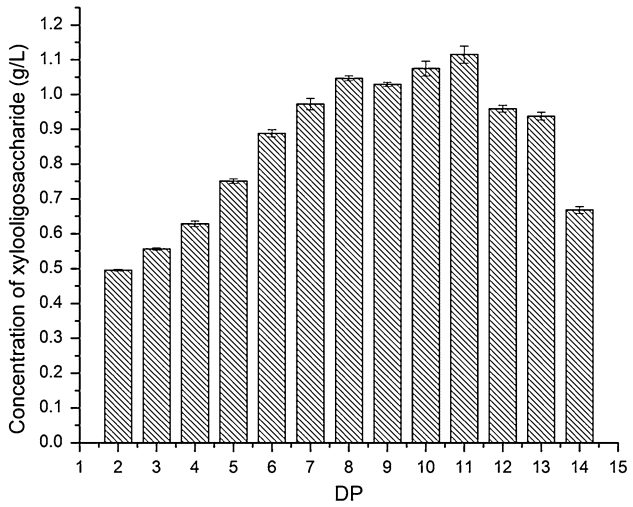

Fig. 6 Concentration of isolated xylooligosaccharide fractions over a DP range from 2 to 14 as determined by downscaled post-hydrolysis. The *error bars* represent the standard deviation for three replicates

such as xylobiose and xylotriose, were lower than those of higher DP fractions, these results did not represent the actual concentration distribution in mixed DP xylooligosaccharide hydrolyzate produced by hydrothermal pretreatment.

Xylooligosaccharide response factors

Each DP fraction was analyzed by Dionex IC for application of three different injection concentrations (including two- and fourfold dilution with DI water) to obtain the linear fit calibration curves described earlier. Table 1 shows the linear fit slope (M_n, according to Eq. 2) and the squares of correlation coefficients (R^2) based on peak height and peak area. Slope values based on peak height showed a significant decreasing trend with DP; thus, the PAD response per unit concentration of xylooligosaccharides decreased as chain length increased. The R^2 values for the slopes calculated from peak heights also showed very good linear relationships for xylose standard and xylooligosaccharides up to DP 10, demonstrating that values calculated from the response factor agreed well with experimental data. For xylooligosaccharides from DP 11–14, the R^2 values resulting from a linear fit of peak height were not as good as those for lower DPs but still

Table 1 Slopes and squares of the correlation coefficients for lines drawn from the origin to each concentration and its PAD response for the range of xylooligosaccharide DPs considered

	Peak height based[a]		Peak area based[b]	
DP	Slope	R^2	Slope	R^2
1	7,394.8	0.9899	1,113.9	0.7816
2	5,345.8	0.9755	920.7	0.7233
3	3,307.5	0.9249	649.9	0.6620
4	2,023.8	0.9947	562.0	0.7321
5	1,441.1	0.9463	405.0	0.5616
6	919.3	0.8544	316.6	0.6854
7	804.3	0.9715	303.9	0.6846
8	629.7	0.9588	288.3	0.6986
9	601.4	0.9340	238.8	0.7146
10	513.1	0.9574	191.8	0.6667
11	401.2	0.7675	131.4	0.6329
12	448.0	0.7583	153.6	0.6138
13	373.7	0.7214	119.6	0.6039
14	309.1	0.6359	86.2	0.6023

The slopes on the left are based on response values calculated according to the height of the peaks while those on the right side are calculated based on the area under the curve

[a] Linear relationship between concentration and peak height

[b] Linear relationship between concentration and peak area

followed a predictable trend of higher DP xylooligosaccharides having lower PAD responses.

As also shown in Table 1, slope values based on peak area, decreasing with DP, are consistent with results based on peak height. The corresponding results for R^2, however, indicate that the linear fit through zero was not good for the isolated xylooligosaccharide fractions. Thus, PAD response based on peak height provided a better fit for the calculation model defined in this study than those based on peak area.

Response factors were quantitatively calculated from the measured slopes for each DP fraction according to Eq. (4). As shown in Fig. 7a, response factors based on peak height decreased according to an exponential pattern from xylose to the xylooligosaccharide with DP 14. Because xylose was selected as the calibration basis, its response factor value α_1 was set as 1. The PAD detection response of xylooligosaccharides dropped dramatically from 1 to 0.1 as the DP increased from 1 to 7. This result indicates that even if the PAD peak height of xylooligosaccharide of DP 7 was only one-tenth of that for xylose, the mass concentrations of these two samples might be similar. The decreasing trend of response factors became milder after DP of 8, dropping from α_8 of 0.085 to α_{14} of 0.042.

Response factors based on peak area were also calculated and are shown in Fig. 7b. Although both sets of α_n

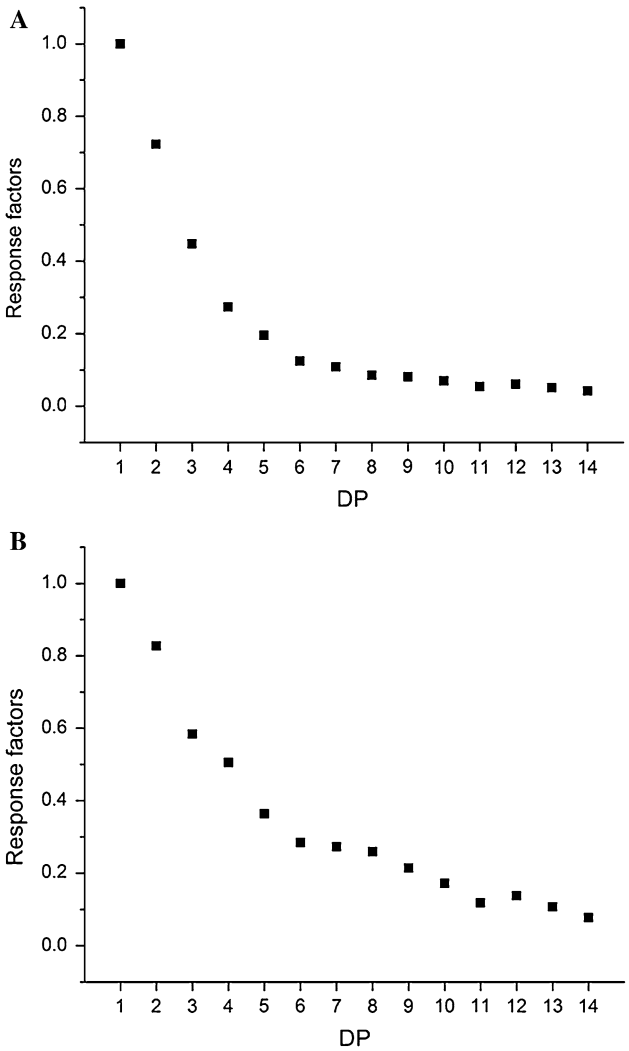

Fig. 7 Response factors based on PAD response peak height (**a**) and peak area (**b**) for xylooligosaccharides from DP 2–14, with xylose as the sugar standard

also dropped with increasing oligosaccharide chain length, the values did not follow a smooth trend with increasing DP in addition to not showing good linearity for the PAD response according to the definition earlier. Thus, use of response factors based on peak area is not recommended for application of this analytical method.

The numerical value of each response factor depended on the sugar standard from which other response factors were calibrated. For example, if we chose xylobiose as the standard, Eq. (4) would be modified to Eq. (6) for calculation of α_n:

$$\frac{M_2}{M_n} = \frac{C_2}{H_2} \times \frac{H_n}{C_n} = \alpha_n \tag{6}$$

According to Eq. (6), the value of α_2 would be set equal to 1, and the response factor for DP 7 would shift to 0.15

instead of the value of 0.1 calculated when xylose was used as the basis, as shown in solid triangles in Fig. 8. Thus, the ratio between any two of response factors remained the same. This result allows flexibility in choosing a sugar standard other than xylose if interference by other compounds corrupts the xylose peak, with commercial xylooligosaccharide sugar standards currently available over a DP range from 2 to 6. However, because lower DP oligosaccharides cost less, it is generally advisable to use the lowest DP possible while still assuring good accuracy.

Consistent with our response factor results for xylooligosaccharides, Koch et al. [13] found an exponential decline in PAD response per μg of glucan chains with increasing DP, and Timmermans et al. [21] found a similar pattern for fructooligosaccharides derived from inulin. However, the factor responsible for such a sharp decline in PAD response has not been well defined. Johnson and LaCourse suggested aldehydes, including reducing sugars, were anodically detected by PAD during the potential region of −0.6–0.2 V [12]. Because there is only one reducing aldehyde group per oligosaccharide molecule, PAD responses of xylooligosaccharides could be in inversely proportion to molecular weight and thus decline with DP. Based on this assumption, a series of response factors for xylooligosaccharides covering DP 2 to 14 were calculated and compared to our measured response factors, as shown in solid squares in Fig. 8. Alternatively, Koch et al. [13] proposed that the ratio of the most acidic hydroxyl group in the C_1 position on the reducing end to other hydroxyl groups in oligosaccharide molecule is larger for short chains than for long ones, resulting in more effective oxidation at the gold electrode for the former.

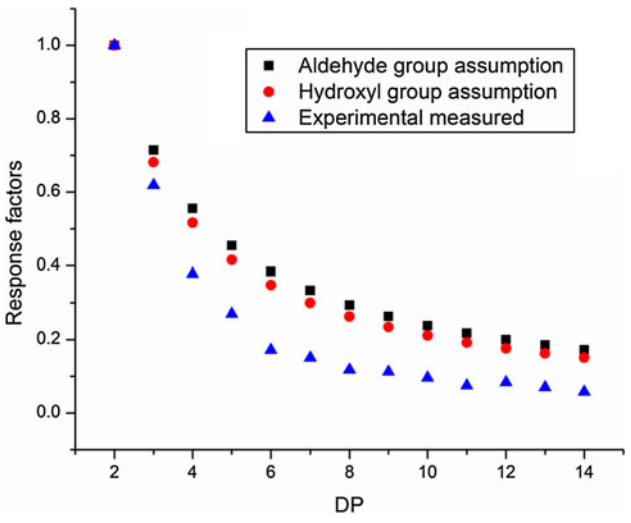

Fig. 8 Comparison of two assumption models with the experimental measured response factors in this study based on PAD peak height for xylooligosaccharides from DP 2–14, with xylobiose as sugar standard basis

Corresponding response factors calculated from this assumption are also presented in Fig. 8 (solid cycles). Although all three response factor series follow a similar pattern, our experimentally measured response factors had a lower PAD response than predicted by either mechanism. Several explanations could be offered to account for the lower value of experimentally measured response factors compared to those calculated by either "ideal assumption." First, random coiling of xylooligosaccharides of higher DP could reduce detection of reducing aldehyde groups or hydroxyl groups, lowering the detection response. Second, the presence of methyl groups [14] and side-chain substitution by GlcA could hinder interactions between xylooligosaccharide molecules and the gold PAD electrode. Diffusion of analyte molecules might also impact surface reaction on the PAD electrode [4], and diffusion coefficients measured for our selected individual DP xylooligosaccharide fractions decreased with DP (data not shown).

The response factors determined in this study can provide a relatively accurate basis for application of HPAEC-PAD technology for fast quantification of the degree of polymerization of xylooligosaccharides released from biomass pretreatment and enzymatic hydrolysis. Although the response factors are currently limited to xylooligosaccharides with DP less than 14, further study could extend the range of accurate PAD response factors as well as improve the detection response for higher DP xylooligosaccharides.

Conclusions

High-purity xylooligosaccharide fractions with DP values from 2 to 14 were chromatographically isolated from hydrothermal pretreatment hydrolyzate of birchwood xylan. PAD responses measured for each fraction dropped with increasing DP. Response factors calculated from PAD response values and corresponding xylooligosaccharide concentrations also dropped with DP. Response factors calculated from PAD peak heights followed a more consistent trend than those based on area and provided a good basis for accurately calculating xylooligosaccharide concentrations for DP values up to 14. Experimentally determined response factors followed the trend predicted from hydroxyl and aldehyde group models but were lower, particularly for higher DP.

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