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ORIGINAL PAPER

Separation and purification of xylose oligomers using centrifugal partition chromatography

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Abstract Xylose oligomers, which have a prebiotic effect, have been used as additives to human and animal food. These oligomers are also the primary intermediate in hemicellulose degradation during the pretreatment of biomass. Centrifugal partition chromatography (CPC) was used in this study to separate and purify xylan-derived oligomers from birchwood xylan. The xylan was partially hydrolyzed to achieve varying degrees of polymerization at 130°C using 0.98% aqueous sulfuric acid for 20 min with a 2.5% solid loading. The CPC solvent system consisting of dimethyl sulfoxide (DMSO), tetrahydrofuran (THF), and water in a 1:6:3 volumetric ratio was used because of its ability to dissolve xylose oligomers of different degrees of polymerization. The CPC was operated in the ascending mode with the water- and DMSO-rich bottom phase acting as the stationary phase, while the THF-rich top phase was the eluent. This paper delineates a method for the production and purification of xylose monomer and xylose oligomers (up to xylopentaose) using CPC. The amount and purity of compounds collected from the CPC fractionation based on 1 g of birchwood xylan were 25.26 mg of xylose at 91.86% purity, 10.71 mg of xylobiose at 85.07% purity, 4.15 mg of xylotriose at 54.71% purity, 5.03 mg of xylotetraose at 38.33% purity and 3.31 mg of xylopentaose at 30.43% purity.

Keywords Xylose · Centrifugal partition chromatography · Oligomers · Pretreatment · Separation

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Introduction

Xylose oligomers are chains of xylose molecules linked with β-1,4-bonds with the degree of polymerization greater than 2 [13]. Xylose oligomers can be produced from xylan either by chemical or enzymatic hydrolysis or by a combination of both [1, 2, 17, 22, 26]. Xylose oligomers have been reported to have a prebiotic effect on humans [17, 23] and inhibit the growth of pathogens in human intestines [23]. In addition to pharmaceutical applications, xylose oligomers have been used in fortified foods, anti-obesity diets, animal feeds, as agricultural ripening agents, and as yield enhancers because of their lower sweetness and high stability over a wide pH range (2.5–8.0) [22]. Xylose oligomers are also used as standards for describing hemicellulose degradation, which is a key operation in the production of cellulosic biofuels [9, 11, 12]. Characterization of the kinetics of oligomer conversion to xylose is crucial because monomeric xylose can yield furfural [18, 20], a by-product which must be eliminated, or at least minimized, if ethanol yields are to be maximized. Furfural inhibits the fermentation of carbohydrate monomers into ethanol, where it reduces the specific growth rate, specific ethanol productivities, and the cell-mass on glucose and ATP [19].

Several techniques have been reported for the purification of xylan-derived xylose oligomers. For example, Zhu et al. [27] employed charcoal absorption followed by elution with ethanol. Corn stover and cobs were first pretreated with aqueous ammonia; and the delignified xylan-rich substract was then hydrolyzed with enzymes yielding oligomers of various degrees of polymerization (DP). To purify the oligomers, activated carbon was added to the oligomer supernatant at 1–10% w/w. The combined mixture was subsequently eluted with various ethanol:water ratio ranging from 0 to 50% ethanol concentration. The highest yield of xylose oligomers was obtained with the 15–30% ethanol elution. However, the oligomers were not characterized because they were quantified via the 4% sulfuric acid method, which accounts for the oligomers by determining the increase of sugar monomers that are obtained after hydrolyzing all the oligomers into their monomeric form [27]. In another study by Akpinar et al. [2], ultrafiltration techniques were employed to separate and purify xylose oligomers. Hydrolysate from the enzymatic hydrolysis of cotton stalk xylan was filtered through a 10-kDa molecular weight cut-off membrane to separate high-molecular-weight polysaccharides and enzymes, followed by filtration through a 1–3 kDa molecular weight cut-off membrane to further fractionate the xylose oligomers. The permeate from the 1 and 3-kDa membranes contained a mixture of xylose oligomers, of which 43.3 and 81.6%, respectively, were reported to have a DP higher than 5. Chromatographic separation of the xylose oligomers was also reported by Jacobs et al. [10]. Water hydrolysates from microwave-pretreated spruce and aspen wood chips were fractionated using a size exclusion column and characterized by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) mass spectrometry (MS).The study showed that acetylated oligomers were common in softwood hemicelluloses and would co-elute with other oligomers, thereby reducing the purity of each collected fractions. The removal of acetyl substituent was performed by alkaline hydrolysis in the presence of ammonium hydroxide. Unfortunately, the resulting purity of each fractionated compound was not reported in the study.

Centrifugal partition chromatography (CPC) is a form of chromatographic separation which uses a liquid–liquid biphasic system without a solid support to maintain the stationary phase. Two immiscible liquids, prepared by mixing two or more solvents, are used as mobile and stationary phases [3, 14]. The advantages of CPC are its ability to separate broad ranges of molecular weight compounds; the samples can be recovered by flushing the system, since the stationary phase is also made up of liquid; and, the technique is well suited for preparative separation or purification because of the large volume of stationary phase [4]. CPC has been widely used as a purification tool for many compounds including flavonoids [5], flavonolignans [6], and macrolide antibiotics [24]. The use of CPC has also been extended to the purification of apple-derived catechin oligomers [21]. Operating in ascending mode with the solvent system of hexane:methyl acetate:acetonitrile:water in a 1:1:1:1 volumetric ratio, Shibusawa et al. [21] was able to fractionate apple catechin oligomers up to a chain length of 10. However, the total mass and the corresponding purity of each DP were not reported in the study.

The aim of this work was to determine a solvent system that could be used in the CPC for fractionating xylan-derived oligomers. The selection of the best solvent for xylose oligomers, as proposed by Foucalt and Chevolot [7], was used as the starting point. The fractions collected from the CPC were subsequently analyzed for purity as well as overall yield.

Materials and methods

Materials

Birchwood xylan, xylose (DP1), arabinose, mannose, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO). Xylobiose (DP2), xylotriose (DP3), xylopentaose (DP5), and xylohexaose (DP6) were purchased from Megazyme (Wicklow, Ireland). Glucose, butanol, and heptane were obtained from Alfa-Aesar (Ward Hill, MA). Tetrahydrofuran (THF) was purchased from J.T. Baker (Philllipsburg, NJ). Acetonitrile, ethyl acetate, hexane, methanol, and sulfuric acid were purchased from EMD Chemicals (Gibbstown, NJ). Chloroform and isopropanol were obtained from EM Science (Gibbstown, NJ). Ethanol was purchased from Pharmco-Aaper (Shelbyville, KY or Brookfield, CT). $CaCO_3$ was obtained from Fisher Scientific (Fair Lawn, NJ). Water was prepared with a Direct-Q system (Millipore, Billerica, MA). All solvents were of HPLC grade.

Sample preparation

Xylan hydrolysis was carried out using 500 mg of birchwood xylan in 20 ml of 0.98% aqueous sulfuric acid in a thick-walled stainless-steel reactor (interior diameter of 14.22 mm, wall thickness of 5.59 mm, length of 200 mm, for a total chamber volume of 32 ml). The loaded reactor was submerged in an industrial fluidized sand bath manufactured by Techne Ltd (Burlington, NJ) at 130°C for 20 min. These hydrolysis conditions were selected as optimum from experiments using 0.98% sulfuric acid at 130°C for reaction times of 20–60 min, and experiments

using water at 130, 180, and 240°C for 5–120 min. The fluidized sand bath was preheated 2 h prior to the experiment, and the hydrolysis began once the reactor was submerged in the fluidized sand bath. After hydrolysis, the reactor was quenched with cool water for 1 min. The reactors were then stored in a 4°C cold room for 30 min. After cooling, the hydrolysate (which was mainly slurry) was recovered, and volume and pH of the hydrolysate were recorded. The hydrolysate was then neutralized to pH of 5.5–6 using $CaCO_3$, while being continuously stirred for 12–24 h. A small quantity of the sample was filtered using a 0.45-μm PTFE syringe filter (VWR International, West Chester, PA), followed by filtration with a 0.2-μm nylon syringe filter (National Scientific, Rockwood, TN), and then analysis using high-performance liquid chromatography (HPLC). The remaining aliquot was filtered through a 1-μm PTFE syringe filter (Whatman, Florham Park, NJ) and then lyophilized into a dried powder form in a Labconco (Kansas City, MO) freeze-drier system.

HPLC identification and quantification

Analysis and quantification of monomers were performed using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) fitted with a SP-G precolumn and a SP0810 column (Shodex, Kawasaki, Japan). The samples were analyzed essentially as described by Martin et al. [16]. Xylose, arabinose, glucose, and mannose were quantified using the peak area generated from the individual calibration curves.

Analysis and quantification of xylose oligomers were performed using a Waters Alliance HPLC system (Model 2695, Waters Corporation, Milford, MA) employing a Micro-Guard De-Ashing precolumn and Aminex HP-87A column (Bio-Rad, Hercules, CA). The sample was processed at an eluent flow rate of 0.2 ml/min. The amount of xylose oligomers in each sample was quantified using peak height calibrated from the injection of DP2, DP3, and DP5 standards. Because of the prohibitive cost of oligomer standards, additional oligomers were not purchased as reference compounds. The quantification of xylotetraose (DP4) and DP6 was based on peak height compared to DP2 as reported by Li et al. [12] and Yang and Wyman [25]. Monomer and oligomers compounds were detected using a refractive index (RI) detector (model 2414, Waters Corporation, Milford, MA), with the column temperature set at 85°C.

CPC separation

CPC solvent selection

Approximately 15 mg of freeze-dried birchwood xylan extract was reconstituted in 2 ml of each of the solvents listed in Table 1. The sample was vortexed and allowed to settle for 12–24 h before being filtered through a 0.2-μm nylon syringe filter. The filtrate was dried under nitrogen and, when dried, reconstituted in 1 ml of water. The samples were again filtered through a 0.2-μm nylon syringe filter and analyzed by HPLC for the presence of xylose monomer and oligomers.

Table 1 Solvent affinity study for xylose oligomers

Solvent	Suitability for CPC solvent	Detectable compounds
Water	Excellent	Detectable from xylose (DP1) to xylose 12-mer (DP12)
Acetonitrile	Poor	Detectable of DP1 only
Buthanol	OK	Detectable from DP1 to xylotriose (DP3) only
Chloroform	Poor	Detectable of DP1 only
DMSO	Excellent	Detectable from DP1 to DP12
Ethanol	OK	Detectable from DP1 to DP3 only
Ethyl Acetate	No	None
Heptane	No	None
Methanol	Good	Detectable from DP1 to xylohexaose (DP6) only
Hexane	No	None
Isopropanol	No	None

Measurement of partition coefficients

The measurement of partition coefficients was adopted from the procedure of Wang-Fan et al. [24]. The CPC separation was performed with a two phase solvent system consisting of DMSO, THF, and water. Based on the exact volumetric ratio of each solvent, 30 ml of the solvent system was prepared, continuously stirred for 6 h, and allowed to separate into two phases for 10 h at room temperature. The time required for the solvent system to form two distinct phases, as well as the volume of each phase, was recorded. After the solvent system settled, 3 ml of each of the upper and lower phases were added to 20 mg of freeze-dried birchwood xylan extract. The sample was vortexed and allowed to settle at room temperature for 2 h. Then, 1 ml of top and bottom phases were sampled, dried under nitrogen and reconstituted in water to be quantitatively analyzed by HPLC. The partition coefficient (log K) of the solute was calculated using the following equation [24]:

$$\log K = \log[C]_{top} - \log[C]_{bottom} = \log A_{top} - \log A_{bottom} \quad (1)$$

where, $[C]_{top}$ and $[C]_{bottom}$ are the concentrations of the oligomers in the top and bottom phases, respectively, and A_{top} and A_{bottom} are the HPLC peak areas of the oligomers in the top and bottom phases, respectively. Ideally the

value of K should be between 0.5 and 2.0 to achieve an efficient CPC separation [15].

Solvent system and sample preparation

For the CPC runs, the solvent system was prepared in a 2-L separatory funnel, allowing for full mixing and equilibration. The upper phase, mostly THF, and the lower phase, mostly water and DMSO, were allowed to settle for at least 10 min before being collected in separate bottles. The DMSO- and water-rich lower phase was used as the stationary phase, while the THF-rich upper phase was used as the mobile phase. Using the solvent system and the CPC in this manner led to the operation of the CPC in the ascending mode. With respect to sample preparation, 0.5–1.0 g of equivalent crude birchwood xylan that had been hydrolyzed, neutralized and dried was reconstituted in 10 ml of the DMSO:THF:water solvent system. The sample was then filtered through a 1-μm PTFE syringe filter before being injected into the CPC system.

CPC setup

The CPC separation was performed using a bench scale CPC from Kromaton Technologies (Angers, France) [6], but fitted with an evaporative light scattering detector (ELSD) manufactured by SofTA Corporation (Westminster, CO). The solvents were pumped into the CPC with a Waters 510 pump (Waters, Milford, MA). When the system was operated in the ascending mode, the 200-mL column was first filled with lower phase at 8.5 ml/min with the rotor spinning at 200 rpm. Once the rotor was completely filled with the stationary phase, which took approximately 30 min, the CPC rotor speed was increased to 1,000 rpm. At that point, the upper phase was introduced into the CPC rotor at a flow rate of 8.5 ml/min. The mobile phase volume was determined once the top phase exited the CPC rotor. A 10-ml sample was injected into the 10-ml sample loop and then introduced into the CPC rotor; this was the start of the CPC run. The flow rate was set at 8.5 ml/min and fractions were collected every 1 min, for 120 min, using a Waters Fraction Collector III (Milford, MA). The eluent was monitored by an ELSD with the following setup: 50 psig air pressure using ultra pure nitrogen, 25°C spray chamber temperature, and 55°C drift tube temperature. From the CPC run, it was determined that the mobile and stationary phases had volumes of 131 and 69 ml, respectively, with the operating pressure at approximately 250 psig.

Analysis of CPC fractions

The oligomers fractions collected from CPC run were analyzed for monomer and oligomers concentrations by the HPLC method described previously. The yield was calculated as the mass of oligomers detected by HPLC collected from CPC fractions divided by the equivalent mass of crude birchwood xylan that was injected into CPC. The CPC fractions were grouped based on the most common concentrated compounds to determine the purity of each compound. The purity was determined using the peak area of the specific compound divided by the total peak area (DP1 to xylose 12-mer (DP12)).

Results and discussion

Xylose oligomers identification

The xylose monomer, also termed DP1, and three other xylose-oligomer standards, DP3, DP5, and DP6, were analyzed using HPLC to determine their retention times, as shown in Fig. 1a.

Xylose monomer and oligomers production

Birchwood xylan was hydrolyzed at 130°C for 50 min using 0.98% aqueous sulfuric acid, and a 2.5% solid loading. As expected, the HPLC analysis of the hydrolyzed samples showed that the monomer composition of birchwood xylan was predominantly composed of xylose, as shown in Fig. 1c. Birchwood xylan was then partially hydrolyzed for only 20 min under the same conditions to prevent complete hydrolysis of xylan into xylose monomers. This was important because the composition of the sample that was to be separated in the CPC needed to contain a high concentration of oligomers. By using this technique, a series of xylose oligomers could be produced and detected by HPLC as shown in Fig. 1b. The identities of DP1, DP2, DP3, DP5, and DP6 were confirmed by co-chromatography with authentic standards. The identities of other peaks such as DP4 and xyloheptaose (DP7) were then deduced based on their relative retention times. Thus, for CPC purification and separation purposes, the sample was hydrolyzed for 20 min.

CPC solvent selection

The results of various solvent affinities of dissolving xylose oligomers are shown in Table 1; with DMSO identified as the best candidate. Because water was not among the solvents proposed by Foucalt and Chevolot [7], DMSO was taken as the starting point for establishing the required solvent system. However, two other solvents had to be identified so that a two phase system could be obtained. Water was chosen as one of the solvents because of its polar nature, and THF was selected because of its

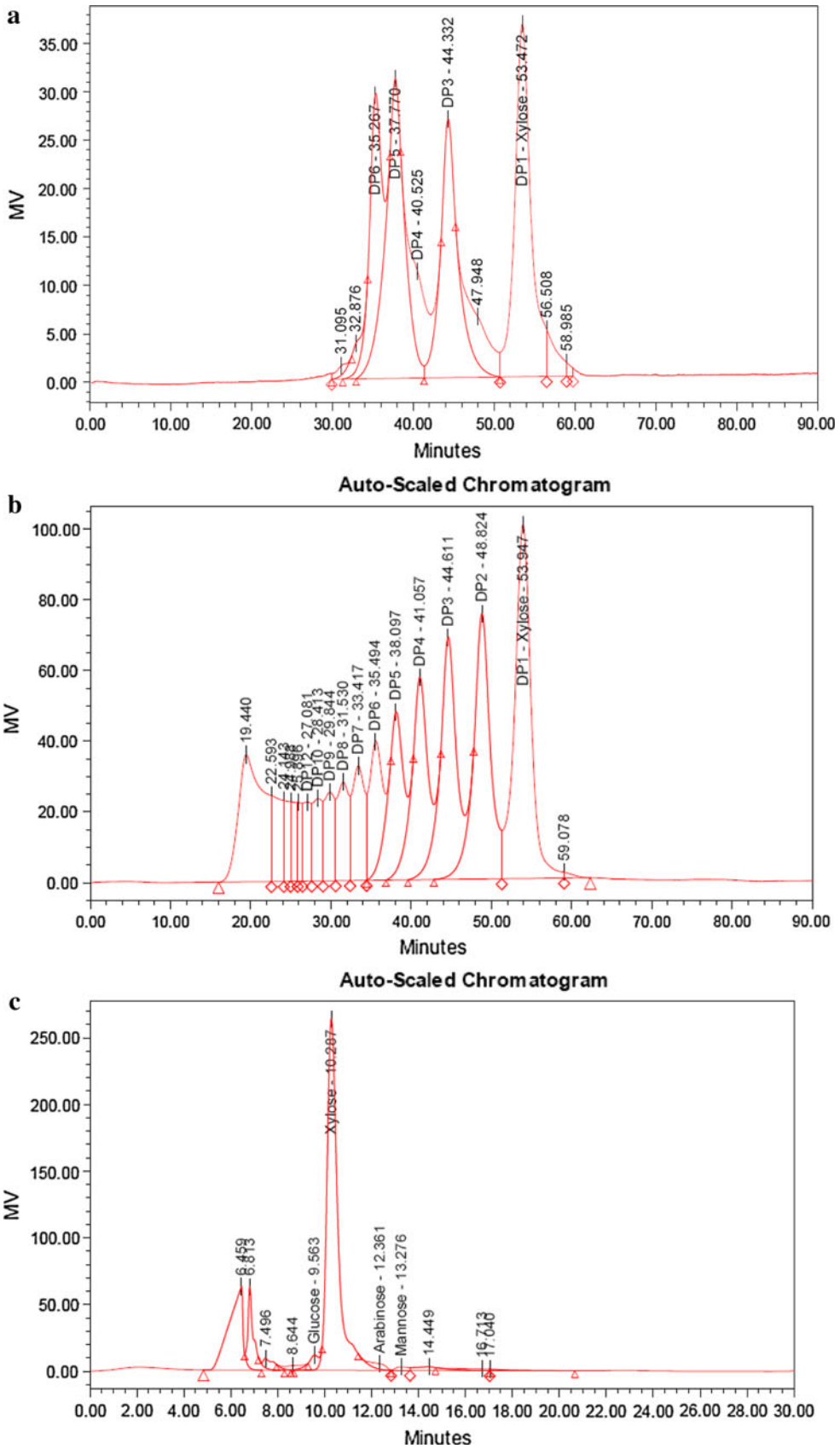

Fig. 1 HPLC chromatograms for **a** xylose (DP1), xylotriose (DP3), xylopentaose (DP5), and xylohexaose (DP6) standards, **b** birchwood xylan sample hydrolyzed at 130°C for 20 min, and **c** 50 min

Table 2 Properties of various dimethyl sulfoxide, tetrahydrofuran, and water solvent combinations

D:T:W[a] ratio	Settling time (s)	Top phase (% volume)	Number of replicates	Partition coefficient				
				Xylose	Xylobiose	Xylotriose	Xylotetraose[b]	Xylopentaose
1:6:3	60	42.90	3	0.25 ± 0.01	0.15 ± 0.02	0.09 ± 0.01	0.01 ± 0.02	0
1:7:2	70	67.10	3	0.11 ± 0.00	0.04 ± 0.00	0	0	0
2:6:2	>180	NA	NA	NA	NA	NA	NA	NA
3:3:4	∞	NA	NA	NA	NA	NA	NA	NA
3:5:2	40	42.90	3	0.13 ± 0.01	0.04 ± 0.01	0.01 ± 0.01	0	0
3:6:1	∞	NA	NA	NA	NA	NA	NA	NA
4:2:4	∞	NA	NA	NA	NA	NA	NA	NA
6:2:2	∞	NA	NA	NA	NA	NA	NA	NA

[a] *D* dimethyl sulfoxide, *T* tetrahydrofuran, *W* water

[b] Xylotetraose oligomers are deduced from relative position on the high pressure liquid chromatography chromatogram

lipophilic qualities. Interestingly, the combination of water, DMSO and THF was also proposed by Foucalt et al. [8] for the purification of macrolide antibiotics. The ratio of water, DMSO and THF had to be determined. Experimental results showing the K values determined in solvent systems of DMSO:THF:water prepared in various proportions are shown in Table 2. The solvent system of 1:6:3 DMSO:THF:water was selected because it partitioned the xylan-derived oligomers into two distinct phases with a settling time of 60 s. The K values of the oligomer reference compounds were calculated for DMSO:THF:water 1:6:3 as shown in Table 2. Although not within the ideal range of $0.50 < K < 2.00$, DMSO:THF:water prepared in a ratio of 1:6:3 presented the starting point for the purification of xylose oligomers. With this solvent composition, the partition coefficients of DP1, DP2, DP3, and DP4 were determined as 0.25, 0.15, 0.09, and 0.01, respectively. Thus, DMSO:THF:water in a ratio of 1:6:3 was selected as the solvent system for CPC separation.

Xylose oligomers fractionation and quantification

Xylose oligomers were prepared, as shown in Fig. 1b, and injected onto the CPC for fractionation. Each injection contained 0.5–1.0 g of equivalent crude birchwood xylan, which had been hydrolyzed, neutralized, and dried, that contained an assortment of xylose oligomers. A typical CPC run is presented in Fig. 2; oligomers eluted between 35 and 78 min. The collected fractions were dried under a nitrogen stream and reconstituted in water for HPLC analysis. The identity and quantity of the distinct oligomers present in the corresponding fractions were determined by HPLC analysis. The identity of each oligomer was confirmed by co-chromatography with the available oligomer standards. Figure 3 presents the HPLC chromatograms of the corresponding CPC fractions, which were collected at 38, 45, 53, 69, and 74 min, respectively. As seen in Table 3, the

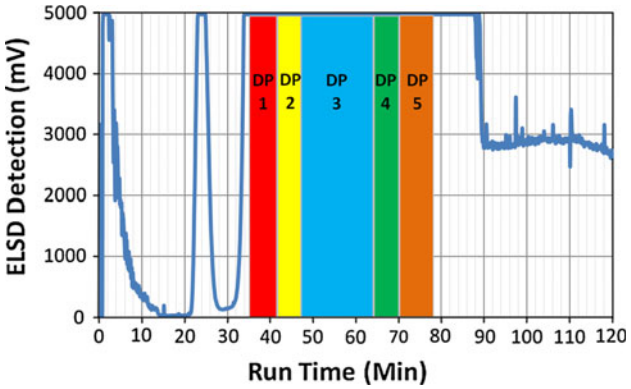

Fig. 2 Centrifugal partition chromatography (CPC) chromatogram for hydrolyzed birchwood xylan at 8.5 ml/min flow rate, 1,000 rpm, ascending mode, and evaporative light scattering detector (ELSD) at 25°C spray chamber temperature, 55°C drift tube temperature, and 50 psig air pressure using ultra pure nitrogen

amount and purity of compounds collected from the CPC fractionation based on 1 g of birchwood xylan were 25.26 mg of DP1 at 91.86% purity, 10.71 mg of DP2 at 85.07% purity, 4.15 mg of DP3 at 54.71% purity, 5.03 mg of DP4 at 38.33% purity, and 3.31 mg of DP5 at 30.43% purity.

As in the study by Li et al. [12], the results presented in this paper used peak height rather than peak area for the quantification of the xylose oligomers. Peak height was preferred because of the wide oligomer peak base, as seen in Fig. 1b, where DP3 had a 10-min peak base. DP1 and oligomers, ranging from DP2 to DP5, were the predominant compounds in the sample. DP1, DP2, and DP3 were successfully separated, as seen in Fig. 3a–c, respectively. To further the work by Li et al. [12], which was centered on the detection of oligomers, our study was aimed at purifying DP of various lengths. Although higher degree of oligomers were not separated as successfully as presented in Fig. 3d, e, these fractions contained valuable oligomers

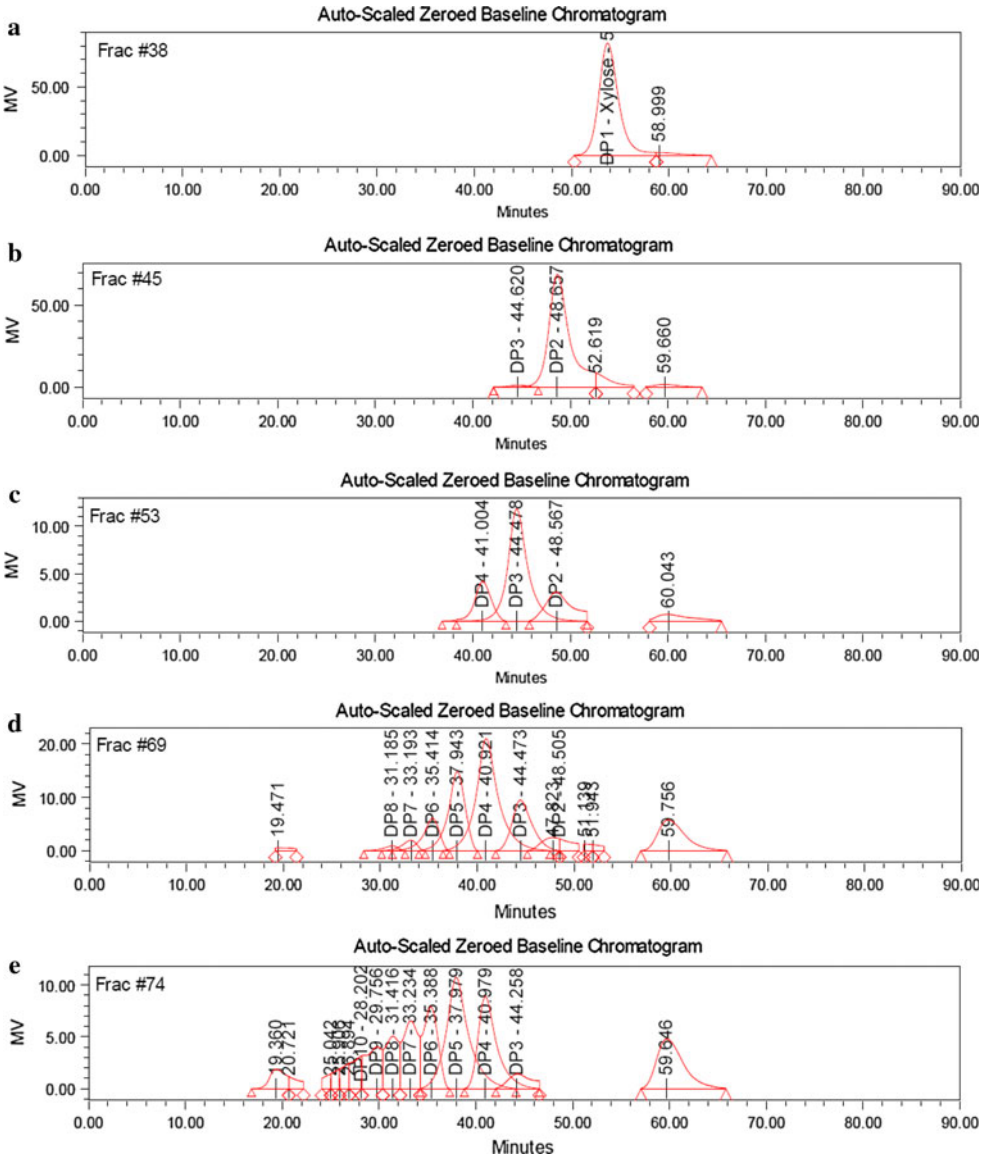

Fig. 3 HPLC chromatograms of the fractions collected from the CPC exercise presented in Fig. 2. **a** Chromatogram of xylose (DP1)-rich fraction collected between 37 and 38 min. **b** Chromatogram of xylobiose (DP2)-rich fraction collected between 44 and 45 min (DP2). **c** Chromatogram of xylotriose (DP3)-rich fraction collected between 52 and 53 min. **d** Chromatogram of xylotetraose (DP4)-rich fraction collected between 68 and 69 min. **e** Chromatogram of xylopentaose (DP5)-rich fraction collected between 73 and 74 min

Table 3 Yield of the CPC fractionated xylose monomer and oligomers

Fractionated compounds	Yield of each compound (mg/g xylan)	Purity[b] (%)
DP1—Xylose	25.26 ± 3.35	90.08 ± 2.52
DP2—Xylobiose	10.71 ± 1.91	83.01 ± 2.92
DP3—Xylotriose	4.15 ± 0.59	50.76 ± 5.59
DP4—Xylotetraose[a]	5.03 ± 0.71	35.77 ± 3.63
DP5—Xylopentaose	3.31 ± 2.08	31.19 ± 1.07
Total yield	48.46 ± 1.95	

[a] Xylotetraose oligomers are deduced from relative position on the high-pressure liquid chromatography chromatogram

[b] Purity is calculated as the peak area of the compound divided by the sum of peak area of DP1–DP12 (xylose 12-mer)

in sufficient quantity (in mg scale) for in-house use. The production of fractionated oligomers, which are significantly more economical than purchasing directly from the supplier, will be utilized for our subsequent elucidation of the depolymerization kinetics of oligomers with the objective of maximizing ethanol yield from the hemicelluloses by minimizing by-product formation. Future work will focus on improving the partition coefficient and the separation resolution of each individual oligomer by exploring other solvent systems in CPC.

Conclusions

The solvent system of DMSO, THF, and water in a 1:6:3 volumetric ratio was selected in this study because of the

ability of the solvent to dissolve xylose oligomers of different degrees of polymerization. The CPC was operated in the ascending mode with the water- and DMSO-rich bottom phase acting as the stationary phase, eluted by the THF-rich top phase. The amount and purity of compounds collected from the CPC fractionation based on 1 g of birchwood xylan were 25.26 mg of DP1 at 91.86% purity, 10.71 mg of DP2 at 85.07% purity, 4.15 mg of DP3 at 54.71% purity, 5.03 mg of DP4 at 38.33% purity, and 3.31 mg of DP5 at 30.43% purity. Future work will focus on improving the yield and purity of each compound by exploring other solvent systems in CPC.

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