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BIOENERGY/BIOFUELS/BIOCHEMICALS

Separation of xylose oligomers using centrifugal partition chromatography with a butanol–methanol–water system

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Abstract Xylose oligomers are the intermediate products of xylan depolymerization into xylose monomers. An understanding of xylan depolymerization kinetics is important to improve the conversion of xylan into monomeric xylose and to minimize the formation of inhibitory products, thereby reducing ethanol production costs. The study of xylan depolymerization requires copious amount of xylose oligomers, which are expensive if acquired commercially. Our approach consisted of producing in-house oligomer material. To this end, birchwood xylan was used as the starting material and hydrolyzed in hot water at 200 °C for 60 min with a 4 % solids loading. The mixture of xylose oligomers was subsequently fractionated by a centrifugal partition chromatography (CPC) with a solvent system of butanol:methanol:water in a 5:1:4 volumetric ratio. Operating in an ascending mode, the butanol-rich upper phase (the mobile phase) eluted xylose oligomers from the water-rich stationary phase at a 4.89 mL/min flow rate for a total fractionation time of 300 min. The elution of xylose oligomers occurred between 110 and 280 min. The yields and purities of xylobiose (DP 2), xylotriose (DP 3), xylotetraose (DP 4), and xylopentaose (DP 5) were 21, 10, 14, and 15 mg/g xylan and 95, 90, 89, and 68 %, respectively. The purities of xylose oligomers from this solvent system were higher than those reported previously using tetrahydrofuran:dimethyl sulfoxide:water in a 6:1:3 volumetric ratio. Moreover, the butanol-based solvent system improved overall procedures by facilitating the evaporation of the solvents from the CPC fractions, rendering the purification process more efficient.

Keywords Xylose oligomers · Centrifugal partition chromatography · Separation · Purification · Hydrolysis

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Introduction

Xylose oligomers are made up of xylose molecules with the degree of polymerization (DP) ranging from 2 to 10, linked together by β-1-4 bonds [11]. Xylose oligomers are produced from lignocellulosic feedstocks through enzymatic hydrolysis, chemical fractionation, or hydrolytic degradation [21]. Because of their stability over a wide pH range (2.5–8.0), and their non-cariogenic and prebiotic properties, xylose oligomers have been used in food applications, such as fortified foods, anti-obesity diets, and novel foods [21]. Xylose oligomers are also found in pharmaceutical applications, such as the treatment of gastrointestinal infections, osteoporosis, otitis, and pruritus cutaneous, and agricultural applications, such as ripening agents and feeds for domestic animals [21].

In lignocellulosic biomass processing to produce bioethanol, xylose oligomers are the intermediate products of xylan depolymerization into the xylose monomer. The presence of xylose oligomers has been shown to inhibit the enzymatic hydrolysis of polysaccharides, such as glucan

and xylan, into their respective monomeric forms [8]. Although the mechanism of the inhibition is not fully understood, an increasing concentration of xylose oligomers is inhibitory to the yield and conversion rates of polysaccharides [17]. Several studies have examined the depolymerization kinetics of xylose oligomers, with the goal of maximizing the conversion of xylose oligomers into monomers, while minimizing the production of by-products, such as furfural and formic acid [5, 7, 15]. These studies were either carried out with high-value purified reference compounds or with crude biomass hydrolyzates. Lau et al. [9] reported on the production of in-house prepared oligomers xylobiose (DP 2) to xylopentaose (DP 5) by centrifugal partition chromatography (CPC).

Specifically, CPC is a type of liquid–liquid separation utilizing a constant-gravity field produced by the rotation of a rotor [4]. When introduced into the CPC system, the solute distributes between the two partially miscible liquid phases that are separated by their densities [12]. The setup of CPC operations, where one of the two phases acts as the stationary phase and the other acts as the mobile phase, allows the solute to elute in either the ascending or descending mode. In the ascending mode, the lighter mobile phase enters the rotor at its periphery, flows through the stationary phase, and exits through its center. In the descending mode, the denser mobile phase enters the rotor at the center, travels through the stationary phase, and exits at its periphery [12]. CPC applications have been documented for the separation of flavonoids [1, 3, 4, 14, 18], sugars [9, 19], lipids [22], and antibiotics [23].

To purify xylose oligomers, Lau et al. [9] successfully used CPC with a tetrahydrofuran (THF):dimethyl sulfoxide (DMSO):water solvent system in a 6:1:3 volumetric ratio, in an ascending mode. Unfortunately, the use of this specific solvent system, as reported by Lau et al. [9], had a number of disadvantages. First, the purity of the xylose oligomers, ranging from 83 % for xylobiose (DP 2) to 31 % for xylopentaose (DP 5), was not sufficient to be used in depolymerizatoin studies. Secondly, DMSO was very difficult to evaporate, requiring copious amounts of nitrogen gas, thereby adding to the production costs. Furthermore, it was very difficult to completely remove DMSO from the samples; this was deemed problematic because these residues promoted the dehydration of xylose, thereby falsely increasing the yield of furfural [2, 16], thwarting the subsequent kinetic study.

The goal of our study was to improve the purity and yield of xylose oligomer separation using CPC by exploring other solvent systems. The solvent systems proposed by Foucault and Chevolot [4] served as a starting point. The solvent systems used in our study were prepared according to the method used by Wang-Fan et al. [23], while the selection criteria were based on the list described by Marston and Hostettmann [13]. The purity of the fractionated xylose oligomers was verified by high-performance liquid chromatography with a refractive index detector (HPLC-RI) and electrospray ionization–mass spectrometry (ESI–MS).

Materials and methods

Materials

Birchwood xylan and xylose (DP 1) were purchased from Sigma-Aldrich (St. Louis, MO). Xylobiose (DP 2), xylotriose (DP 3), xylotetraose (DP 4), xylopentaose (DP 5), and xylohexaose (DP 6) were purchased from Megazyme (Wicklow, Ireland). All solvents were of HPLC grade.

Sample preparation

The hot water hydrolysis procedure was carried out using 800 mg of birchwood xylan mixed with 20 mL of water. The mixture was loaded into a thick-walled stainless-steel reactor (interior diameter 14.22 mm, wall thickness 5.59 mm, length 200 mm, for a total chamber volume of 32 mL) and heated to 200 °C in an industrial fluidized sand bath manufactured by Techne Ltd (Burlington, NJ) for 60 min. After hydrolysis, the reactor tube was quenched in tap water for 1 min and stored in a cold room at 4 °C for 30 min. After cooling, the hydrolysate was transferred to a 50-mL beaker and neutralized to pH 6–8 using calcium carbonate while being continuously stirred for 2 h. The neutralized hydrolysate was subsequently filtered through a 1-μm PTFE syringe filter (Whatman, Florham Park, NJ). A small quantity of the filtered sample was filtered through a 0.2-μm nylon syringe filter (National Scientific, Rockwood, TN) before being analyzed by HPLC. The remaining aliquot was placed in clean glass tubes, and dried using an Automatic Environmental SpeedVac System (SpeedVac model AES 1010; Savant, Ramsey, MN) on the lowest heat setting. The dried birchwood xylan hydrolysate was subsequently used for the solvent study as well as the CPC fractionation for the purification of xylose oligomers.

HPLC identification and quantification

Analysis and quantification of xylose oligomers were performed using a Waters Alliance HPLC system (model 2695, Waters Corp, Milford, MA) employing a Micro-Guard De-Ashing pre-column and Aminex HPX-42A column (Bio-Rad, Hercules, CA), with the column temperature set at 85 °C. Samples were processed at an eluent flow

rate of 0.2 mL/min. Xylose oligomers in each sample were quantified using calibration curves generated using purchased DP 2, DP 3, DP 4, DP 5, and DP 6 standards. The xylose monomer and oligomers were detected using a RI detector (model 2414; Waters Corp). Quantification of the xylose monomer and oligomers were based on peak height, as reported by Li et al. [10] and Yang and Wyman [24]. Analysis and quantification of furfural and formic acid were performed using a Waters 2695 HPLC system equipped with an Bio-rad Micro-Guard cation H refill cartridges guard column and Bio-Rad Aminex HPX-87H column heated to 55 °C. The eluent was 0.005 M aqueous sulfuric acid at a flow rate of 0.6 mL/min. Detection was obtained with a Waters 2996 Photodiode Array Detector set at 280 nm for furfural and 210 nm for formic acid. Samples were quantified based on peak area using calibration curves generated using purchased standards.

CPC separation

Solvent selection

Eight solvent systems, listed in Table 1, were prepared to explore the potential solvent combinations that can be used to separate xylose oligomers. After a solvent system was identified based on the partition coefficients of xylose oligomers in the solvent systems listed in Table 1, a second set of 12 solvent systems, listed in Table 2, were prepared to determine the optimum volumetric ratio of solvents within the solvent systems for use in the CPC fractionations.

Each solvent system was prepared in an 18- to 20-mL volume, according to the volumetric ratio of its individual solvent components. The solvent mixtures were continuously stirred for 18 min at 1,400 rpm using a vortex mixer (VWR, Radnor, PA). After mixing, the solvents were

Table 1 Partition coefficients for xylose monomer and oligomers in various solvent systems that contained alcohol components as compared to the DMSO:THF:water system reported by Lau et al. [7]

Solvent system	Volumetric ratio in solvent system	DP 4	DP 3	DP 2	DP 1
DMSO:THF:water	1:6:3	0.00 ± 0.01	0.02 ± 0.00	0.05 ± 0.00	0.12 ± 0.00
BuOH:EtOH:water	4:1:4	0.01 ± 0.01	0.03 ± 0.00	0.07 ± 0.00	0.15 ± 0.00
Chloroform:MeOH:water	7:13:8	37.90	29.52 ± 13.85	11.37 ± 3.93	4.77 ± 1.37
BuOH:acetic acid:water	4:1:5	0.02	0.04	0.07	0.15
BuOH:formic acid:water	3:6:14			0.02	0.07
Heptane:ethyl acetate:EtOH:water	4:5:3:3	0	0	0	0.01 ± 0.00
Heptane:ethyl acetate:MeOH:water	6:1:6:1	0	0	0	0.01 ± 0.01
Heptane:ethyl acetate:MeOH:water	2:1:2:1	0	0	0	0.01 ± 0.01

DP Degree of polymerization, *DMSO* dimethyl sulfoxide, *THF* tetrahydrofuran, *BuOH* butanol, *EtOH* ethanol, *MeOH* methanol

Table 2 Partition coefficients for the xylose monomer (DP 1) and oligomers (DP 2–DP 5) in various butanol–methanol/ethanol–water solvent combinations

Solvent system	Volumetric ratio in solvent system	Settling time (s)	DP 5	DP 4	DP 3	DP 2	DP 1
BuOH:EtOH:water	4:1:4	45	–	0.009 ± 0.001	0.022 ± 0.002	0.058 ± 0.003	0.127 ± 0.006
BuOH:EtOH:water	5:1:4	36	–	0.006 ± 0.000	0.015 ± 0.001	0.044 ± 0.003	0.104 ± 0.009
BuOH:EtOH:water	4:1:5	41	–	0.008 ± 0.000	0.021 ± 0.001	0.056 ± 0.000	0.124 ± 0.003
BuOH:EtOH:water	6:1:3	70	–	0.007	0.019	0.049	0.113
BuOH:EtOH:water	3:1:6	51	–	0.008 ± 0.002	0.023 ± 0.003	0.059 ± 0.002	0.129 ± 0.001
BuOH:EtOH:water	4:2:4	NA[a]					
BuOH:MeOH:water	4:1:4	59	0.010 ± 0.000	0.018 ± 0.000	0.041 ± 0.001	0.087 ± 0.003	0.166 ± 0.001
BuOH:MeOH:water	5:1:4	48	0.008 ± 0.001	0.016 ± 0.001	0.038 ± 0.002	0.081 ± 0.004	0.158 ± 0.004
BuOH:MeOH:water	4:1:5	56	0.006 ± 0.001	0.012 ± 0.002	0.028 ± 0.001	0.068 ± 0.002	0.138 ± 0.004
BuOH:MeOH:water	6:1:3	NA[a]					
BuOH:MeOH:water	3:1:6	61	0.003 ± 0.004	0.011 ± 0.000	0.027 ± 0.002	0.063 ± 0.003	0.135 ± 0.002
BuOH:MeOH:water	4:2:4	NA[a]					

[a] NA the solvent mixture did not form two distinct phases, but remained as one homogenous phase

allowed to settle for 19 h so that they could form two distinct phases. One milliliter of the solvent system from the top and the bottom phases, respectively, of each of the settled solvents was added to 40 mg of dried hydrolyzed birchwood xylan, which was prepared using the method listed in the sample preparation section. The mixture was vortexed, filtered through a 0.45-µm PTFE syringe filter (VWR International, West Chester, PA), and allowed to settle for 4 h. After settling, 0.4 mL of top phase was collected, reconstituted in 0.5 mL water, and labeled as the upper phase. Similarly, 0.4 mL of the bottom phase was collected, reconstituted in 0.5 mL water, and labeled as the lower phase. Both the upper and lower phases were filtered through 0.2-µm nylon syringe filters and analyzed by HPLC for xylose oligomers.

Measurement of partition coefficients

The measurement of partition coefficients of xylose oligomers was adopted from the work reported by Wang-Fan et al. [23]. The partition coefficient (log K) of the xylose oligomers was calculated using the equation:

$$K = [C]_{\mathrm{upper}} / \log[C]_{\mathrm{lower}} \quad (1)$$

where $[C]_{\mathrm{upper}}$ and $[C]_{\mathrm{lower}}$ are the concentration of xylose oligomers in the upper and lower phases, respectively.

Solvent preparation for CPC fractionation

For the CPC fractionation, the solvent system consisting of butanol:methanol:water was prepared in a 2-L separatory funnel, allowing for full mixing. After mixing, the solvent was allowed to settle for 2 h to form two distinct phases. The upper phase, which contained mainly butanol, and the lower phase, which contained mainly water, were collected in two separate bottles. For sample preparation, 640 mg of hydrolyzed birchwood xylan, which was dried in clean glass tubes, was reconstituted with 6 mL of the lower phase and 4 mL of the upper phase from the butanol:methanol:water solvent system. The sample was then vortexed, filtered through a 1-µm PTFE syringe filter, and injected into the CPC system.

CPC setup

The CPC separation was performed using a bench scale CPC from Kromaton Technologies (Angers, France) fitted with an evaporative light scattering detector (ELSD) (SofTA Corp, Westminster, CO) and a UV detector (model VUV24; Reflect Scientific, Orem, UT) equipped with a preparatory flow cell, at 254 nm wavelength. In the ascending mode, the water-rich lower phase became the stationary phase and was pumped into the 200-mL column using a Waters 510 pump (Waters Corp) at 8.5 mL/min while the rotor was spinning at 200 rpm. Once the column was filled with the lower phase, which took about 30 min, the pump was stopped. The rotor speed was increased to 1,000 rpm before the butanol-rich upper phase (the mobile phase) was introduced into the CPC rotor at 8.5 mL/min. The stationary phase volume was determined once the upper phase exited from 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 fractionation process. The flow rate was maintained at 4.89 mL/min, and fractions were collected immediately after the start of the CPC fractionation using a Waters Fraction Collector III (Waters Corp). Each fraction was collected over a 2-min period, equivalent to 9.78 mL, for a total duration of 240 min. The ELSD was set up with a 50 psig with ultra-pure nitrogen, a spray chamber temperature of 25 °C, and a drift tube temperature of 55 °C.

Analysis of CPC fractions

The collected CPC fractions were dried using the Automatic Environmental SpeedVac System described in section Sample preparation, reconstituted in 0.5 mL of water, filtered through a 0.2-µm nylon syringe filter, and analyzed by HPLC for composition. The amount of xylose oligomers was determined by multiplying the volume of the consolidated xylose oligomer fractions by the concentration of xylose oligomers, as determined by the HPLC analysis. The purity of xylose oligomers was determined by taking the ratio of peak area of the xylose oligomers and dividing by the sum of peak areas of DP 1 to xylose 12-mers (DP 12). No peaks corresponding to higher DPs were detected by the HPLC setup.

ESI–MS analysis

Electrospray ionization mass spectrometry was performed on a quadrupole time-of-flight mass spectrometer. Samples were mixed with methanol (with 0.1 % formic acid) and directly infused into the ESI source via a syringe pump. Mass spectra were obtained in the positive ion mode.

Results and discussion

Xylose monomer and oligomers production

The hydrolysis reported in Lau et al. [9] was performed using dilute acid at 130 °C with 0.98 % v/v sulfuric acid for 20 min. Although these conditions generated a suite of oligomers, processing problems were encountered due to the addition of calcium carbonate necessary to neutralize the acid, which resulted in the formation of calcium sulfate. The presence of calcium sulfate resulted in irreversible

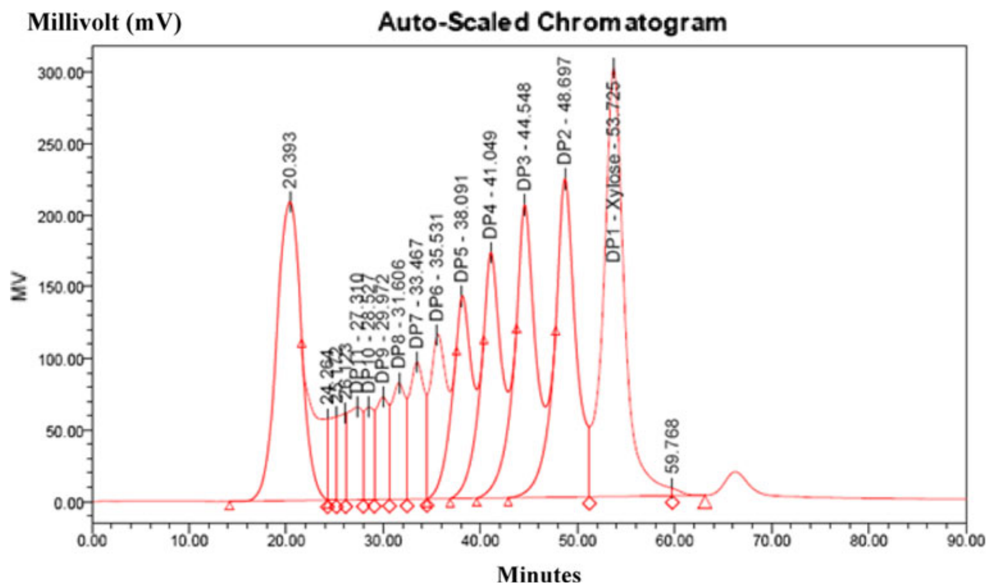

Fig. 1 High-performance liquid chromatography (HPLC) chromatogram of hydrolyzed birchwood xylan containing the xylose monomer (*DP 1*) and oligomers (*DP 2*, *DP 3*, *DP 4* and higher) analyzed using the Bio-Rad Aminex HPX-42A column heated to 85 °C. The sample was processed with water as the mobile phase at a flow rate of 0.2 ml/min. The compound was detected using a refractive index (RI) detector. DP 1, DP 2, DP 3, and DP4 were detected at retention times of 54, 49, 45, and 41 min, respectively

degradation of the silver resin and a high operating pressure of the HPLC column. Hydrolysis in water avoids the generation of calcium sulfate, thereby prolonging the operating life of the HPLC column. Thus, we hydrolyzed birchwood xylan in water at 200 °C for 60 min, with a 4 % solid loading (0.8 g in 20 mL of water). The resulting hydrolysate contained various xylose oligomers, ranging in length from DP 2 to DP 11, as determined by HPLC analysis (Fig. 1). The hydrolysis condition was selected based on a series of preliminary experiments to optimize the yield of xylose oligomers. At this optimum hydrolysis condition, the yields of the xylose monomer (DP 1), and various oligomers (DP 2, DP 3, DP 4, and DP 5), based on 1,000 mg of birchwood xylan, were 94, 60, 25, 36, and 43 mg/g birchwood xylan, respectively.

CPC solvent selection

Based on the results of their solvent affinity study of xylose oligomers, Lau et al. [9] reported that alcohols, such as methanol, ethanol, and butanol, are good solvents for xylose oligomers, although not as good as water and DMSO. The table of solvent possibilities reported by Foucault and Chevolot [4] indicated that the CPC solvent system used to separate oligomers could be combinations of methanol, ethanol, butanol, heptane, toluene, chloroform, ethyl acetate, and water. Therefore, we prepared seven solvent systems, containing combinations of these solvents and used these systems to determine the partition coefficients of oligomers standards, as shown in Table 1. As a control, we used the solvent combination DMSO:THF:water at a volumetric ratio of 1:6:3, as previously reported by Lau et al. [9] (see Table 1). Although none of the solvent systems resulted in partition coefficient values within the range of 0.5 and 2.0, as recommended by Marston and Hostettmann [13], we did observe that solvent systems based on butanol and chloroform could potentially separate xylose oligomers.

Based on these results, we prepared various combinations of butanol, water, ethanol, and methanol for testing as solvent systems. Of the seven solvent systems tested, butanol:ethanol:water at a 4:1:4 volumetric ratio, chloroform:methanol:water at 7:13:8 volumetric ratio, and butanol:acetic acid:water at 4:1:5 volumetric ratio yielded similar partition coefficients for xylose oligomers to that obtained with the DMSO:THF:water solvent system. When compared to the lower DP oligomers, DP 4 and DP 5 showed less affinity to the aqueous phase, as reflected by their lower partition coefficients. The upper phase of the chloroform:methanol:water solvent system consisted primarily of the aqueous phase, and thus resulted in partition coefficients for xylose oligomers which were >1, an indication of the higher solubility of xylose oligomers in the aqueous upper phase. The solvent system of chloroform:methanol:water was not considered further because of chloroform toxicity issues, leading to handling and storage constraints. Similarly, the solvent system composed of butanol:acetic acid:water was not further explored because of the need to neutralize the CPC fractions with calcium carbonate, creating an unnecessary preparation step. Thus, the solvent system composed of butanol:ethanol:water was further considered for oligomer separation by CPC.

Having established that the solvent system butanol:ethanol:water could separate xylose oligomers, we conducted subsequent experiments to refine the volumetric ratio of the solvents. Twelve combinations of butanol, water, and

Table 3 Separation factors for xylose monomer (DP 1) and oligomers (DP 2–DP 5) in various butanol–methanol/ethanol–water solvent combinations

Butanol:methanol/ethanol:water solvent system	Volumetric ratio in solvent system	DP 4/DP 5	DP 3/DP 4	DP 2/DP 3	DP 1/DP 2
BuOH:EtOH:water	4:1:4		2.39	2.67	2.18
BuOH:EtOH:water	5:1:4		2.69	2.94	2.35
BuOH:EtOH:water	4:1:5		2.53	2.59	2.24
BuOH:EtOH:water	6:1:3		2.76	2.53	2.30
BuOH:EtOH:water	3:1:6		2.77	2.51	2.19
BuOH:EtOH:water	4:2:4				
BuOH:MeOH:water	4:1:4	1.87	2.32	2.11	1.89
BuOH:MeOH:water	5:1:4	2.04	2.40	2.13	1.95
BuOH:MeOH:water	4:1:5		2.27	2.41	2.03
BuOH:MeOH:water	6:1:3				
BuOH:MeOH:water	3:1:6		2.51	2.32	2.15

either ethanol or methanol were prepared at various volumetric ratios and evaluated in terms of their xylose oligomer partition coefficients. The results of these experiments in terms of partition coefficient and corresponding settling time of the solvent systems are presented in Table 2. Of the 12 solvent systems tested, butanol:ethanol:water at a 4:2:4 volumetric ratio and butanol:methanol:water at either a 6:1:3 or 4:2:4 volumetric ratio did not form two distinct phases, rendering these solvents unsuitable for CPC applications. However, butanol:methanol:water either prepared at either a 4:1:4 or 5:1:4 volumetric ratio yielded the highest partition coefficients for xylose oligomers, indicating that these two solvent systems could be used for the purification of xylose oligomers. The partition coefficients of DP 1, DP 2, DP 3, DP 4, and DP 5 in the butanol:methanol:water solvent system at a 4:1:4 volumetric ratio were 0.166, 0.087, 0.041, 0.018, and 0.010, respectively, and 0.158, 0.081, 0.038, 0.016, and 0.008, respectively, in the butanol:methanol:water solvent system at a 5:1:4 volumetric ratio. The partition coefficients of oligomers determined using the methanol-based systems yielded values closer to those reported by Marston and Hostettmann [13].

The optimum solvent system has not only to have partition coefficients in the range of 0.5 and 2.0, but also a settling time, defined as the time necessary for the solvent mixture to form two distinct phases, of <30 s, ensuring satisfactory retention of the stationary phase [13]. In our experiments, only the butanol:methanol:water solvent systems prepared either at a 4:1:4 or 5:1:4 volumetric ratio had settling times of 59 and 48 s, respectively, which was quicker than the 60 s reported by Lau et al. [9] using the DMSO:THF:water solvent system at a volumetric ratio of

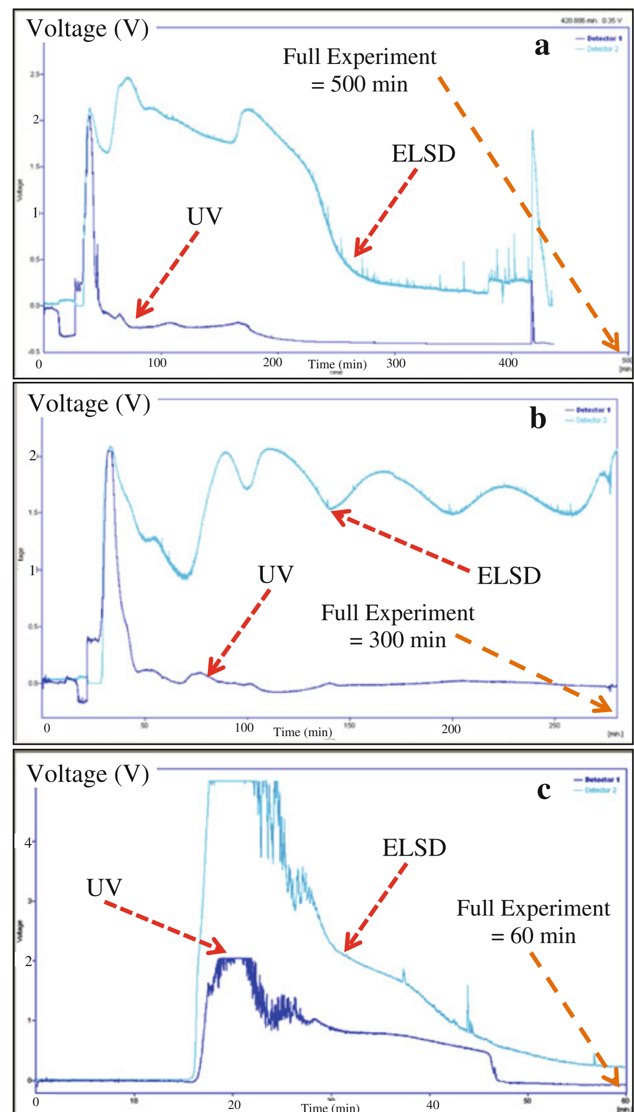

Fig. 2 Centrifugal partition chromatography (CPC) chromatogram for hydrolyzed birchwood xylan using the butanol:methanol:water solvent system: **a** at a 4:1:4 volumetric ratio in ascending mode, with the water-rich phase in the rotor, **b** at a 5:1:4 volumetric ratio in ascending mode, **c** at a 5:1:4 ratio in descending mode, with the butanol-rich phase in the rotor. The CPC was set at 4.89 ml/min flow rate, 1,000 rpm, with an evaporative light scattering detector (*ELSD*) and a UV detector at 254 nm wavelength

1:6:3. Lastly, for a solvent system to be appropriate, the separation factor between two adjacent compounds must be >1.5 [13]. The separation factor is determined by dividing the partition coefficients of two adjacent compounds. As shown in Table 3, with the exception of the butanol:ethanol:water 4:2:4 and butanol:methanol:water 6:1:4 solvent systems, all solvent preparations fulfilled the criteria established by Marston and Hostettmann [13], with separation factors of between 1.87 and 2.94. Based on these three criteria, we identified the butanol:methanol:water solvent system at volumetric ratios of 4:1:4 and 5:1:4 ratio

Table 4 Yield of the centrifugal partition chromatography-fractionated xylose monomer and oligomers

Solvent system	Fractionated compounds	Elution time (min)	Yield of each compound (mg/g xylan)	Purity (%)[a]
Butanol:methanol:water in a 4:1:4 volumetric ratio	Xylobiose (DP 2)	76–84	12.53	81.17
	Xylotriose (DP 3)	88–104	9.60	70.98
	Xylotetrose (DP 4)	108–124	14.20	62.41
	Xylopentaose (DP 5)	128–156	21.21	51.85
	Total yield		57.54	
Butanol:methanol:water in a 5:1:4 volumetric ratio	Xylobiose (DP 2)	110–130	20.99 ± 2.91	94.62 ± 7.14
	Xylotriose (DP 3)	160–180	9.61 ± 0.38	89.65 ± 13.04
	Xylotetrose (DP 4)	220–240	13.98 ± 3.44	88.62 ± 7.88
	Xylopentaose (DP 5)	262–280	14.54	68.06
	Total yield		59.13	

[a] Purity is calculated as the peak area of the compound divided by the sum of peak area of DP 1–DP 12 (xylose 12-mer)

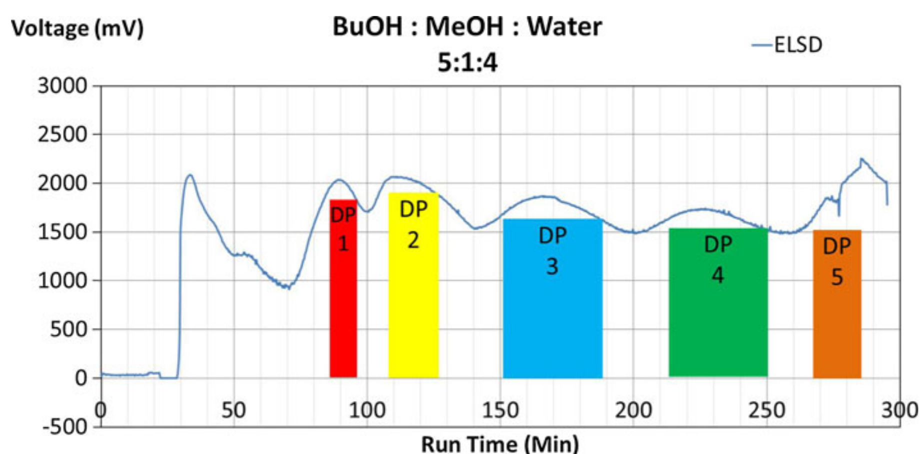

Fig. 3 Centrifugal partition chromatography chromatogram for hydrolyzed birchwood xylan using the butanol:methanol:water solvent system at a 5:1:4 volumetric ratio. The Kromaton CPC operated at 4.89 ml/min flow rate, rotating at 1,000 rpm, in the ascending mode. The ELSD was set at a spray chamber temperature of 25 °C, a drift tube temperature of 55 °C and a 50 psig air pressure using ultra-pure nitrogen. The high-performance liquid chromatography (HPLC) chromatograms of the individual fractions of the xylose monomer and oligomers are shown in Fig. 4

as the optimum solvent systems for the purification of xylose oligomers using CPC.

CPC solvent selection

The ELSD and UV chromatograms of the CPC fractionation xylose oligomers in butanol:methanol:water at a 4:1:4 volumetric ratio are presented in Fig. 2a. The volumes of the mobile and stationary phases were 123 and 77 mL, respectively. The maximum operating pressure during the 400-min CPC fractionation was 420 psig. The CPC fractions were subsequently analyzed by HPLC and consolidated according to the composition of the oligomers. The elution times of DP 1, DP 2, DP 3, DP 4, DP 5, and DP 6, were 60–70, 76–84, 88–104, 108–124, 128–156, and 160–190 min, respectively. The purity and yield of xylose oligomers were calculated and are summarized in Table 4. The purities of DP 2, DP 3, DP 4, and DP 5, were 81, 71, 62, and 52 %, respectively, while their respective yields were 13, 10, 14, and 21 mg/g of birchwood xylan. In contrast to the CPC fractions produced in the DMSO:THF:water solvent system [9], our current CPC fractions were easily dried without nitrogen, thereby decreasing experimental costs.

Although the butanol:methanol:water solvent system prepared at a volumetric ratio of 4:1:4 improved the purity

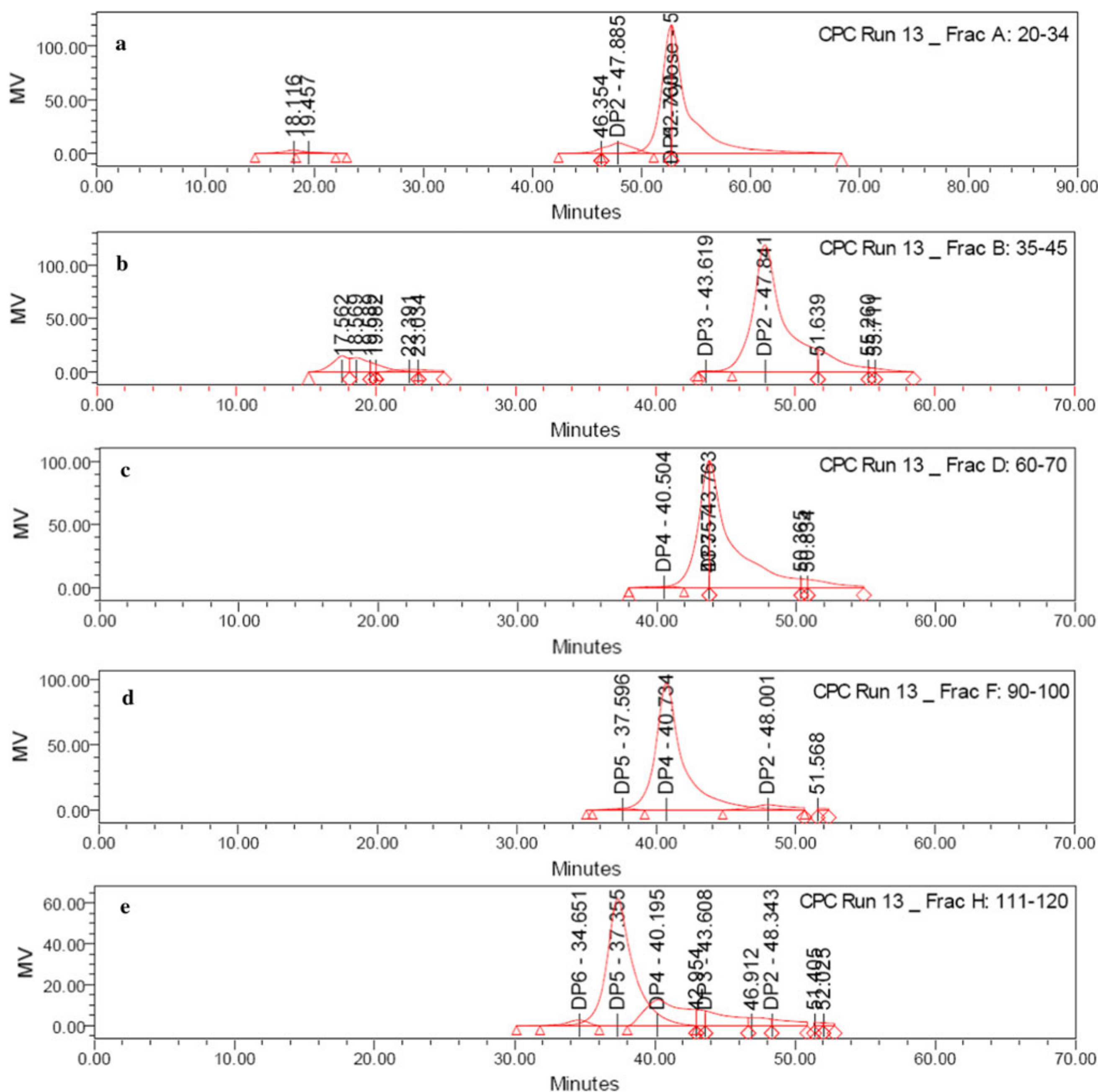

Fig. 4 High-performance liquid chromatography chromatograms of the fractions collected from the CPC fractionation presented in Fig. 3. Chromatogram of: **a** xylose-rich fraction collected between 80 and 108 min, **b** xylobiose (*DP2*)-rich fraction collected between 110 and 130 min, **c** xylotriose (*DP3*)-rich fraction collected between 160 and 180 min, **d** xylotetraose (*DP4*)-rich fraction collected between 220 and 240 min, **e** xylopentaose (*DP5*)-rich fraction collected between 262 and 280 min

of the recovered xylose oligomers over a similar separation in DMSO:THF:water, the purities of the separated compounds could be improved. We therefore tested the 5:1:4 butanol:methanol:water solvent system for xylose oligomer purification under similar operating conditions as those used for the 4:1:4 butanol:methanol:water solvent system. The ELSD and UV chromatograms of the CPC experiment are presented in Fig. 2b. The volumes of the mobile and stationary phases were 109 and 91 mL, respectively, with the maximum operating pressure reaching 440 psig during the 300-min CPC fractionation. Similarly, the CPC fractions were analyzed by HPLC and consolidated according to oligomer composition. The elution times of DP 1, DP 2, DP 3, DP 4, and DP 5, were 80–108, 110–130, 160–180,

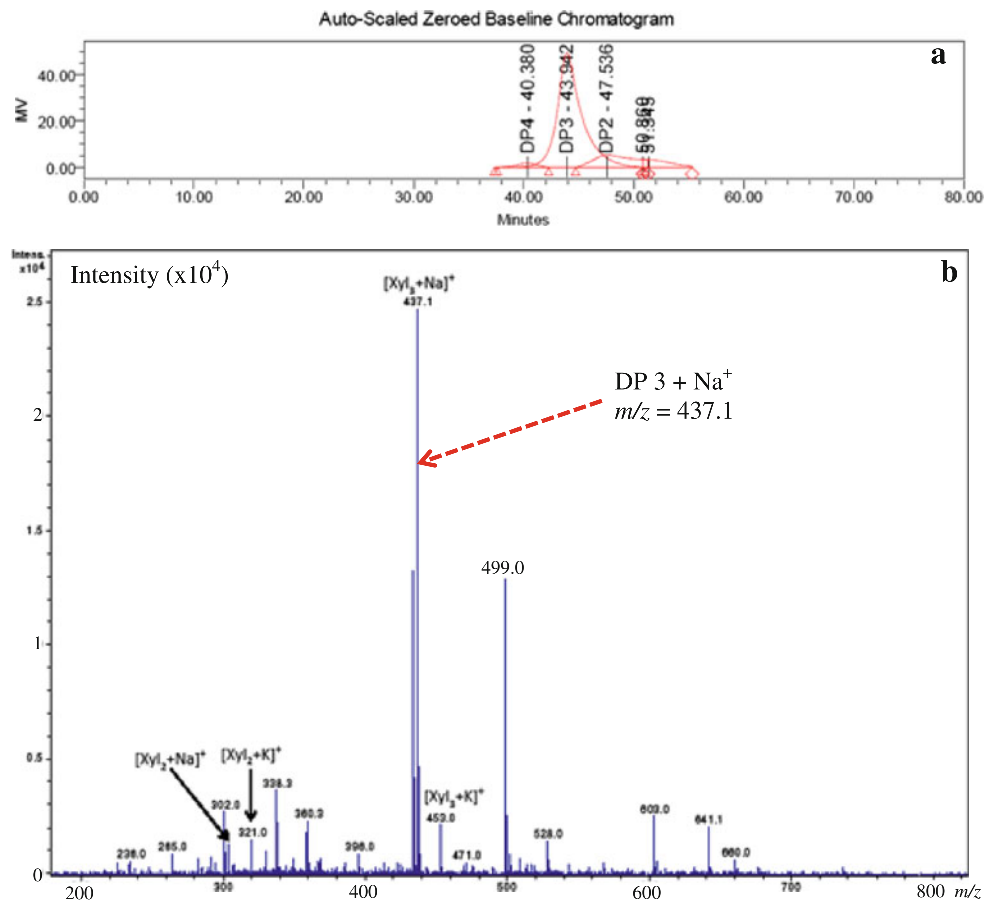

Fig. 5 High-performance liquid chromatography chromatogram (**a**) and electrospray ionization mass spectrometry (**b**; ESI–MS) spectra of CPC-fractionated DP 3. The ESI–MS spectra verify the presence of DP 3 as indicated in the HPLC analysis

220–240, and 262–280 min, respectively. The purity and yield of xylose oligomers were calculated and are summarized in Table 4. The purities of DP 2, DP 3, DP 4, and DP 5, were 95, 90, 89, and 68 %, respectively, while the respective yields were 21, 10, 14, and 15 mg/g of birchwood xylan.

The ELSD chromatogram for the CPC fractionation experiments with the corresponding HPLC chromatograms are presented in Figs. 3 and 4. The purity of the xylose oligomers collected using the butanol:methanol:water solvent system at a 5:1:4 volumetric ratio was higher than that of the butanol:methanol:water solvent system at a 4:1:4 volumetric ratio; also, the fractionation using the former solvent system resulted in a more distinct peak separation compared to that of the latter solvent system. However, the elution times for fractionation using the 5:1:4 butanol:methanol:water solvent system were longer than those of the 4:1:4 butanol:methanol:water solvent system by an average of 80 min; this can be attributed to the lower partition coefficients for xylose oligomers. On the other hand, considering the improvement in the purity of the obtained xylose oligomers, the longer fractionation time of the 5:1:4 butanol:methanol:water solvent system was justified.

In order to further reduce the overall fractionation time, the butanol:methanol:water solvent system at a volumetric 5:1:4 ratio was also tested in the descending mode, meaning that the butanol-rich upper phase was now the stationary phase, while the water-rich lower phase served as the mobile phase. The strategy was aimed at reducing the overall fractionation time by eluting the xylose oligomers in reverse order, which would result in the higher DP xylose oligomers being eluted first, followed by the lower DP xylose oligomers, and lastly by xylose. Interestingly, the total fractionation time was reduced to 60 min. Unfortunately, the xylose oligomers were not well separated, even when the collection time was reduced from 2 min to 10 s per sample fraction, and this condition was not further explored.

The CPC fractions were analyzed by ESI–MS to confirm the identity of the oligomers based on molecular weight. Through MS analysis, the molecular weight of the CPC fractions containing DP 3, DP 4, and DP 5 had significant

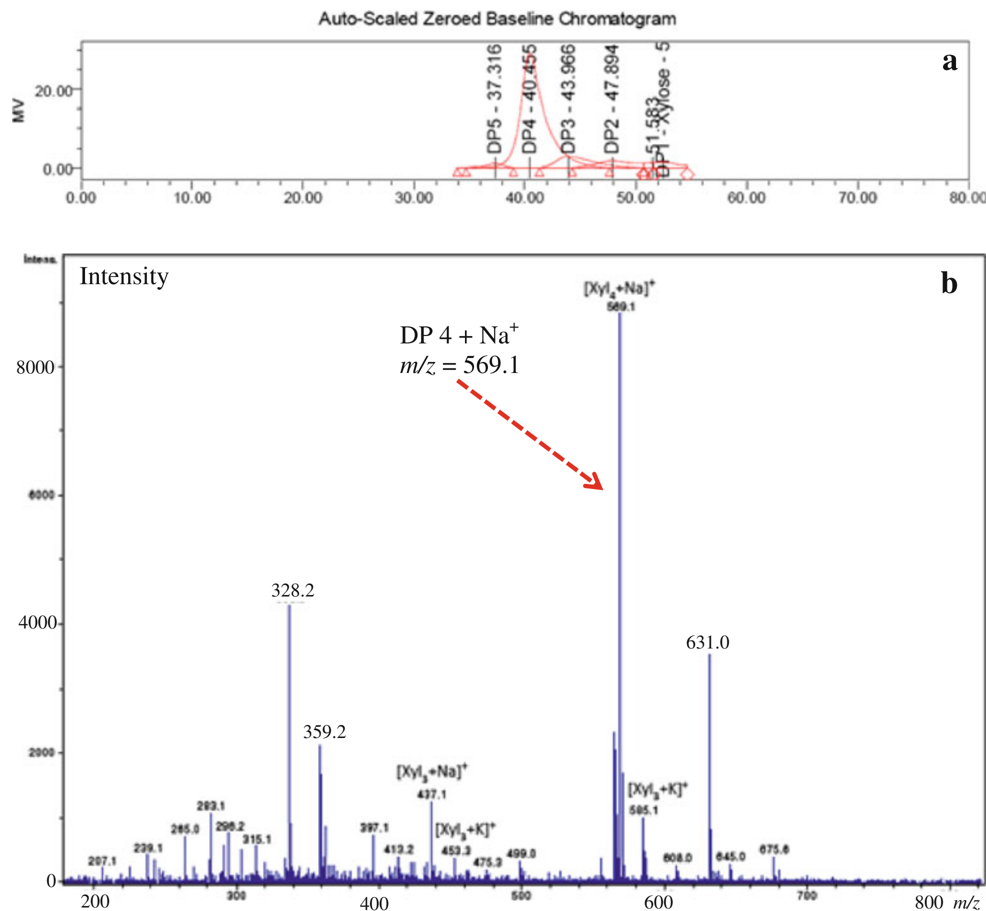

Fig. 6 High-performance liquid chromatography chromatogram (**a**) chromatogram and ESI–MS (**b**) spectra of CPC-fractionated DP 4. The ESI–MS spectra verify the presence of DP 4 as indicated in the HPLC analysis

ions at m/z of 437, 569 and 701, respectively, corresponding to the molecular weight of the individual xylose oligomers with the sodium ion. Similarly, the ionized standards were observed as the M + 23 rather than the M + 1 ions, which is rather common for natural products which contain excess sodium. Therefore, the presence of xylose oligomers was confirmed by ESI–MS to match the results from the HPLC analysis. The results presented in Figs. 5, 6 and 7 confirmed that the CPC fractionated compounds were indeed DP 3, DP 4, and DP 5 oligomers.

The fractionated CPC compounds were also analyzed for the presence of other impurities. Through HPLC–UV analysis, formic acid concentrations in DP 2, DP 3, and DP 4 were determined to be 0.42, 0.00, and 0.71 g/g xylose oligomer, respectively. However, furfural, which was present in the initial hydrolyzed birchwood xylan mixture, was not detected in the fractionated xylose oligomers. Both formic acid and furfural were most likely formed from the degradation of the xylose monomer during hot water hydrolysis [5, 7, 15]. However, 4-*O*-methyl-D-glucuronic acid, which is a common constituent in xylan [6, 20], was not detected in the fractionated CPC samples, as analyzed by MS analysis (results not presented).

In sum, the solvent system of butanol:methanol:water at a 5:1:4 volumetric ratio in an ascending mode was deemed suitable for the purification of xylose oligomers by CPC.

Conclusions

Xylose oligomers with a DP between 2 and 5 were successfully fractionated using CPC with a solvent system of butanol:methanol:water in 5:1:4 volumetric ratio. Operated in ascending mode, xylose oligomers were eluted from the CPC between 110 and 280 min after the introduction of the sample. The purities of DP 2, DP 3, DP 4, and DP 5 were 95, 90, 89, and 68 % respectively, which is an improvement over the previously reported CPC work using the THF:DMSO:water solvent system in a 6:1:3 solvent ratio. The total yields of xylose oligomers were 59 mg/g

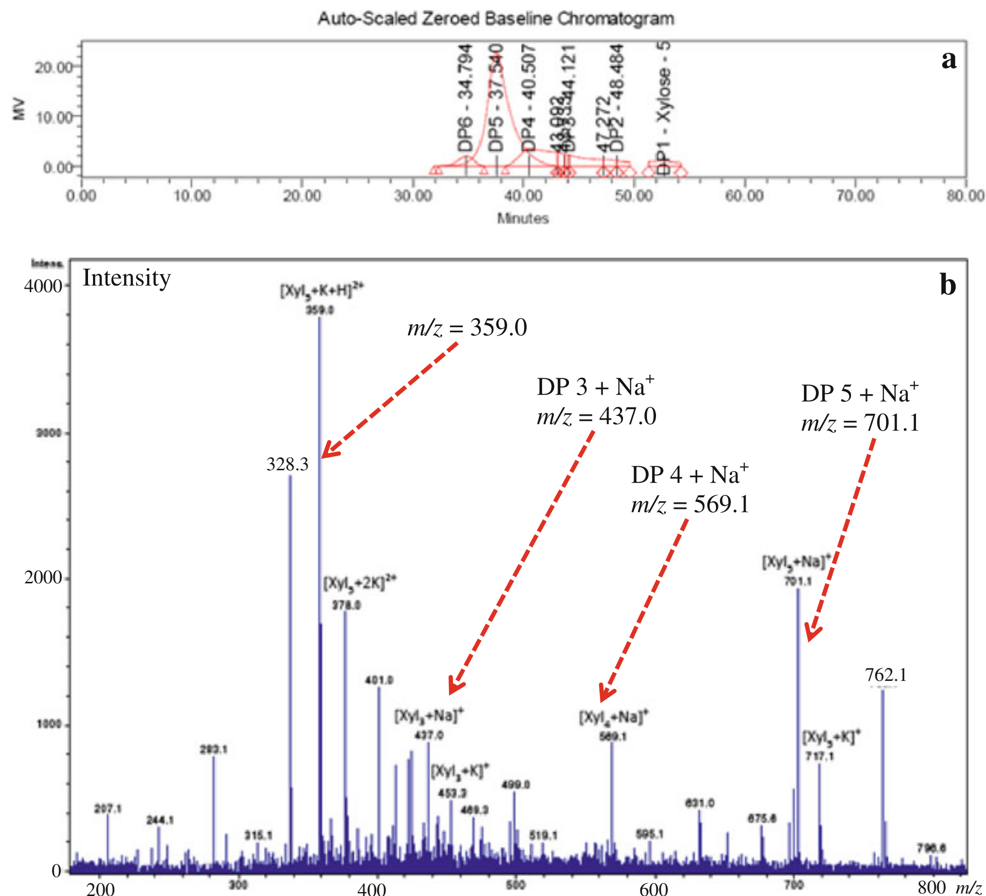

Fig. 7 High-performance liquid chromatography chromatogram (**a**) and ESI–MS spectra (**b**) of CPC-fractionated DP 5. The ESI–MS spectra verify the presence of DP 5 as indicated in the HPLC analysis

birchwood xylan. The presence of xylose oligomers in the CPC fractions was verified using ESI–MS.

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