Efficient Analysis of Monosaccharides and Oligosaccharides from Hydrolyzed Hemicellulose of *Spartina anglica*

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Spartina anglica, a plant that controls coastal erosion, is widely distributed throughout the world and is rich in cellulose, hemicellulose, and lignin. The hemicellulose from *Spartina anglica* can be extracted and hydrolyzed into monosaccharides and xylooligosaccharides under acid or enzymatic digestion conditions. In this study, an effective PMP(1-phenyl-3-methyl-5-pyrazolone)-derivatized HPLC (High performance liquid chromatography) method was developed for monitoring monosaccharides and xylooligosaccharides of *Spartina anglica*. With phosphate buffer (0.04 M, pH 8.06) as mobile phase A, and acetonitrile as mobile phase B, in which the elution gradient was set as A:B/79:21, the monosaccharides (glucose, xylose and arabinose) and xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose) could be separated completely using the C18 column. This provides an economical, rapid, and efficient method for process monitoring in the bioconversion of *Spartina anglica*.

Keywords: Spartina anglica; Hemicellulose; Xylooligosaccharides; PMP derivatization; HPLC

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INTRODUCTION

Spartina anglica was introduced to China in 1980s for coastal erosion control. This plant is resistant to salt, water, and high-pH soil. To prevent the expansion of tidal flats, Fujian province introduced *S. anglica* from abroad in the 1980s. It has played a vital role in creating land with silt, thus protecting river banks. However, rapid propagation and tenacious vitality make it grow out of control in the coastal beaches, which results in severe destruction of original ecosystems. Moreover, aquatic plants and others have difficulty surviving in this area. Although *S. anglica* can be converted into fuel and other high-value products, an analysis method of monosaccharide and oligosaccharides has not yet been established.

Spartina anglica is rich in cellulose, hemicellulose, and lignin. Traditionally, natural cellulose raw material treatment is mainly for the utilization of cellulose, but the hemicellulose and lignin have not been fully utilized, resulting in great waste of resources and environmental pollution. Cellulose, hemicellulose, and lignin are polymers with complex spatial structures. In natural cellulose materials, they aggregate into complex supramolecular compounds. Most lignin exists in intercellular layers and forms a solid binding net framework with hemicellulose, which provides a protective layer on cellulose. Therefore, it is quite difficult to separate the three components completely for their utilization in industry.

As a homogeneous glycan, cellulose is connected by beta-1,4 glycosidic bond with

D-glucose, in which the main hydrolysates are cellobiose and glucose (Lin and Tanaka 2006). Unlike cellulose, hemicelluloses exists as heterogeneous glycan with more complex components. Hemicellulose from different biomass sources has different components, but the major carbohydrates include D-glucose, D-xylose, L-Arabia sugar, D-mannose, D-galactose, L-rhamnose, 4-O-methyl glucuronic acid, and galactose uronic acid (Peng *et al.* 2012). Hemicellulose plays an important role in the process of lignocellulose hydrolysis (Zhang *et al.* 2012a, b). Xylan and arabino-glycan of lignocellulose can be transformed into functional sugar such as xylooligosaccharides and arabinose by acid or enzymatic hydrolysis (Lavarack *et al.* 2002). Hence, the production of xylose or xylooligosaccharides from *S. anglica* has a huge market prospect. HPLC, gas chromatography (GC), and ion chromatography are common methods for the analysis and determination of carbohydrates (Arfelli and Sartini 2014). However, there is still no well-established method to analyze the complex lignocellulosic biomass hydrolyzed products, especially the xylooligosaccharides.

Saccharides lack ultraviolet absorption and are usually detected by HPLC with a refractive index differential detector (RID). HPLC-RID has low sensitivity to saccharides and poor separation effect on xylooligosaccharides (Brienzo *et al.* 2010; Thamyres *et al*. 2020). Because carbohydrates are difficult to volatilize and detect by GC only using indirect methods, carbohydrates generally are derivatized into volatile compounds and then detected. However, derivatization is complicated and only suitable for monosaccharide detection, and there have not been many reports on the detection of oligosaccharide. The compound 1-phenyl-3-methyl-5-pyrazolone (PMP) increases the ionization degree of sugar and detection sensitivity (Honda *et al.* 1989). Therefore, PMP pre-column derivatization HPLC has been widely applied to monosaccharide analysis (You *et al.* 2009). To date, this method has not been applied to simultaneous analysis of monosaccharides and oligosaccharides in *Spartina* biomass.

In the previous reports, residual PMP reagent after derivatization reaction was generally degraded by adopting multiple extraction and drying. The PMP derivatives were easily lost, and the operation was complicated, which increased the chance of system error. In this paper, under the optimized conditions, since PMP is eluted first and the drying step can be omitted, the above disadvantages were overcome by directly analyzing the extracted sample. The newly established method yielded monosaccharides and oligosaccharides from *S. anglica* hydrolysis from a simplified process that improved separation efficiency. In this paper, a PMP derivatization HPLC method was established to analyze monosaccharides and oligosaccharides from hydrolysate of *Spartina anglica* hemicellulose.

EXPERIMENTAL

Extraction of Hemicellulose

Spartina anglica raw material was harvested from the seashore of Ningde, Fujian Province, China. It was dried and crushed into powder. The powder was soaked with 2% NaOH and 1% H_2O_2 for 2 h at 60 °C, then filtered and extracted with 2% NaOH for 2 h at 100 °C. The liquid from both steps was combined, concentrated, and precipitated with 2 volumes of 95% ethanol. The sediment was washed twice with 70% ethanol, air-dried, and used as the crude hemicellulose (Thamyres *et al*. 2020). The yield of crude hemicellulose was obtained as 22.5%.

Hemicellulose Hydrolysis

Two grams of crude hemicellulose powder were resuspended in 100 mL citrate buffer (50 mM, pH 5.0) containing 0.02% azide. The dosage of xylanase (Genuo, China) preparation was 200 IU/g dry matter. One unit (1 U) enzyme equals the amount of enzyme degradation and release 1 μmol reducing sugar per min from xylan solution (5 mg/ mL) at 37 °C and pH 5.5. Hydrolysis was performed in flasks in a water bath rolling at 50 °C for 0.5 h. Hydrolysis samples were boiled for 10 min, centrifuged at 8000 rpm for 5 min, filtered, and stored at 4 °C for further analysis.

Purity and Structural Characterization of Hemicellulose

Hemicellulose (0.3 g) was added into 4% H_2SO_4 and incubated at 120 °C for 1 h. The solution was neutralized with calcium carbonate and centrifuged at 12000 rpm for 5 min. The supernatant was analyzed by HPLC (2695-2996, Waters, Milford, MA, USA) with Aminex (HPX-87H, BioRad, Hercules, CA, USA). The assay conditions were as follows: mobile phase of 5 mM H_2SO_4, at a speed of 0.6 mL/min, sample injection volume of 20 μL, column temperature at 35 °C, RID as monitor, and runtime for 15 min.

KBr powder was dried to constant weight in electro-thermostatic oven (DHG9123A, Shanghai Jing Hong laboratory equipment Co., Ltd., Shanghai, China). Samples and KBr were added into agate mortar with mass ratio of 1:100, which were ground at the infrared lamp. Milled powder was pressed to slice in the tablet compression machine. The slice was measured in the infrared spectrometer (Nicolet 330, ThermoFisher Scientific, Waltham, MA, USA) at scanning wavelength of 4000 to 400 cm^{-1}.

Preparation of PMP Derivatives of Saccharides

Monosaccharides derivatized by PMP have strong absorption peak at 245 nm, and the structure of derivatives are so stable that they have little interference for analysis (Honda *et al.* 1989; Zhang *et al.* 2003). The mechanism of PMP derivatization is shown in Fig. 1, with the reaction of PMP and glucose as an example.

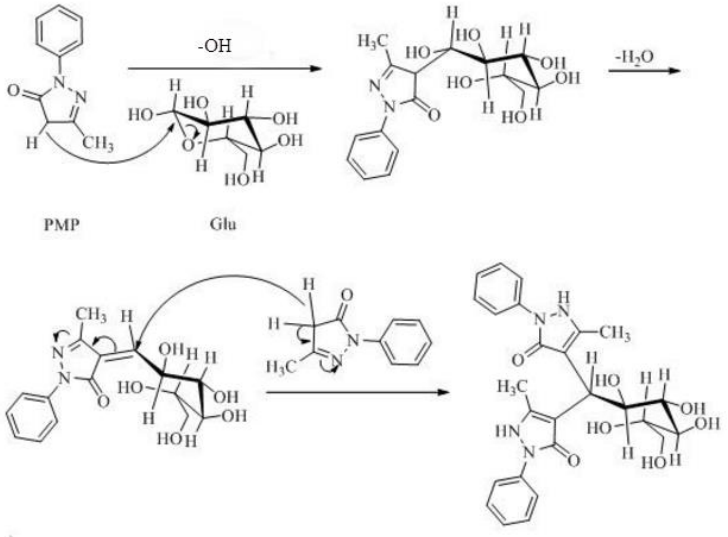


Fig. 1. Process and mechanism of PMP derivatization

Saccharides standard solution (Purity>90%, Megazyme, Bray, Ireland) or hydrolysate of *Spartina anglica* hemicelluloses (100 μL) were added into NaOH solution, and mixed with 100 μL 0.5 M PMP (AR, Acros Organics, Geel, Belgium)-methanol solution. The reaction was sustained for 30 min at 70 °C. The solution was cooled to room temperature, and 100 μL of 0.3 M HCl was added to neutralize it. The extraction was performed with 1 mL of $CHCl_3$, and organic solvent was removed after vortex oscillation. This process was repeated three times, and the extract was centrifuged at 12,000 rpm for 5 min. The supernatant was diluted to a certain ratio and tested with 0.22 μm membrane filter.

Optimization of Separation Condition for HPLC

HPLC separation conditions were previously described (Li *et al.* 2013). The testing conditions were as follows: C_{18} chromatographic column (Shiseido, Tokyo, Japan), UV detection wavelength at 245 nm, mobile phase A of phosphate buffer (0.04 M, pH 8.00), mobile phase B of acetonitrile, and gradient elution(A:B = 79:21). The velocity was adjusted from 0.5 mL/min to 0.4 mL/min for better separation.

RESULTS AND DISCUSSION

Purity and Structural Characterization of Hemicellulose

Hemicellulose is a complex structure with the main chain and side chain. The main chain of hemicellulose consists of D-xylose. The side chain of hemicellulose usually consists of L-arabinose, D-glucose, D-mannose, L-rhamnose, 4-O-methylglucuronic acid, and galactose uronic acid. After the crude hemicellulose (0.3 g) was completely hydrolyzed by H_2SO_4, 0.01473 g glucose, 0.1928 g xylose, and 0.01974 g arabinose were obtained according to calculation based on the standard work curve (Fig.2). The results showed that arabinose and glucose were the main sugar groups of side chains. The mass content of hemicellulose reached 62.35%, which indicates a high purity of hemicellulose and possibility of application in the industry.

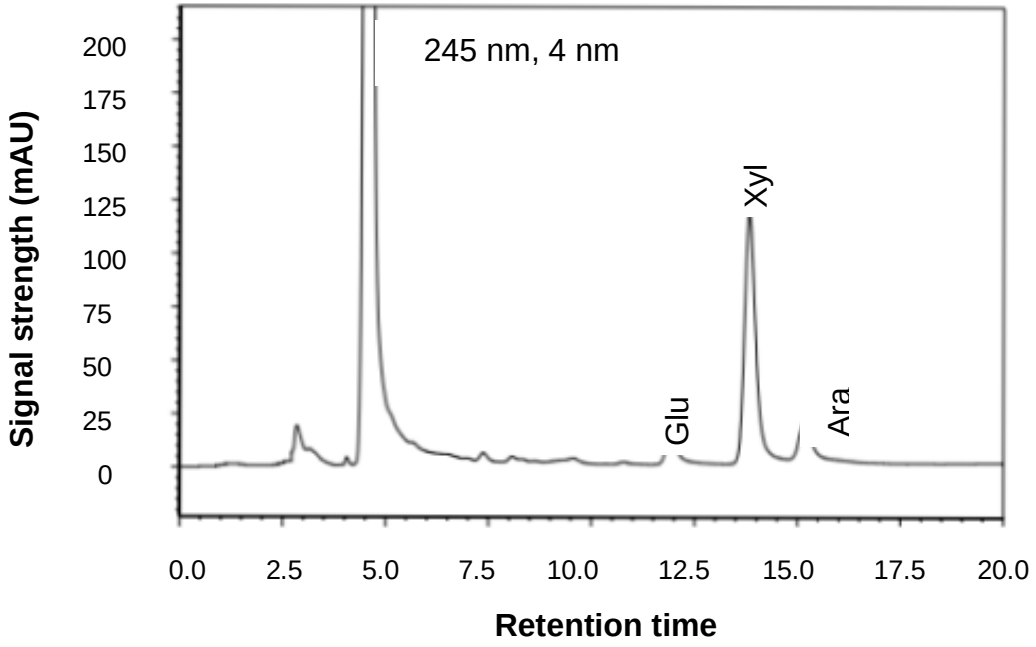


Fig. 2. HPLC analysis of hemicellulose hydrolysate from *Spartina anglica*

FT-IR Analysis

FT-IR is a common technique used to determine the structure of polysaccharides. The absorption region changes as chemical groups interact at different molecular levels. Hemicellulose is a polysaccharide with a complex infrared absorption peak, and its typical peaks are observed at 1200 to 900 cm^{-1}. Infrared spectrum results showed the hemicellulose extracted from *Spartina anglica* had a typical xylan structure (Fig. 3). Cao *et al.* (2015) assumed that the bands at 1504 cm^{-1}, 1240 cm^{-1}, and 1040 cm^{-1} showed the relative concentration in lignin, cellulose, and hemicellulose, respectively. The strong absorption peak at 1040 cm^{-1} indicated the presence of hemicellulose, as attributed to the C-O bond stretching vibration of C-O-C. The lowest frequency peak at wavenumber 3450 cm^{-1} showed the strongest activity, which can be attributed to vibration of O(6)H-O(3) bond (Agarwal *et al.* 2011). Wavenumber 1639 cm^{-1} was related to absorbed water (Zhang *et al.* 2015). The acromion at 991 cm^{-1} showed the existence of arabia sugar side chain. The absorption at 897 cm^{-1} was the frequency vibration and ring frequency vibration of C-1 group, which indicated that the main connections between hemicellulose units were β-glycosidic bonds (Bian *et al.* 2012).

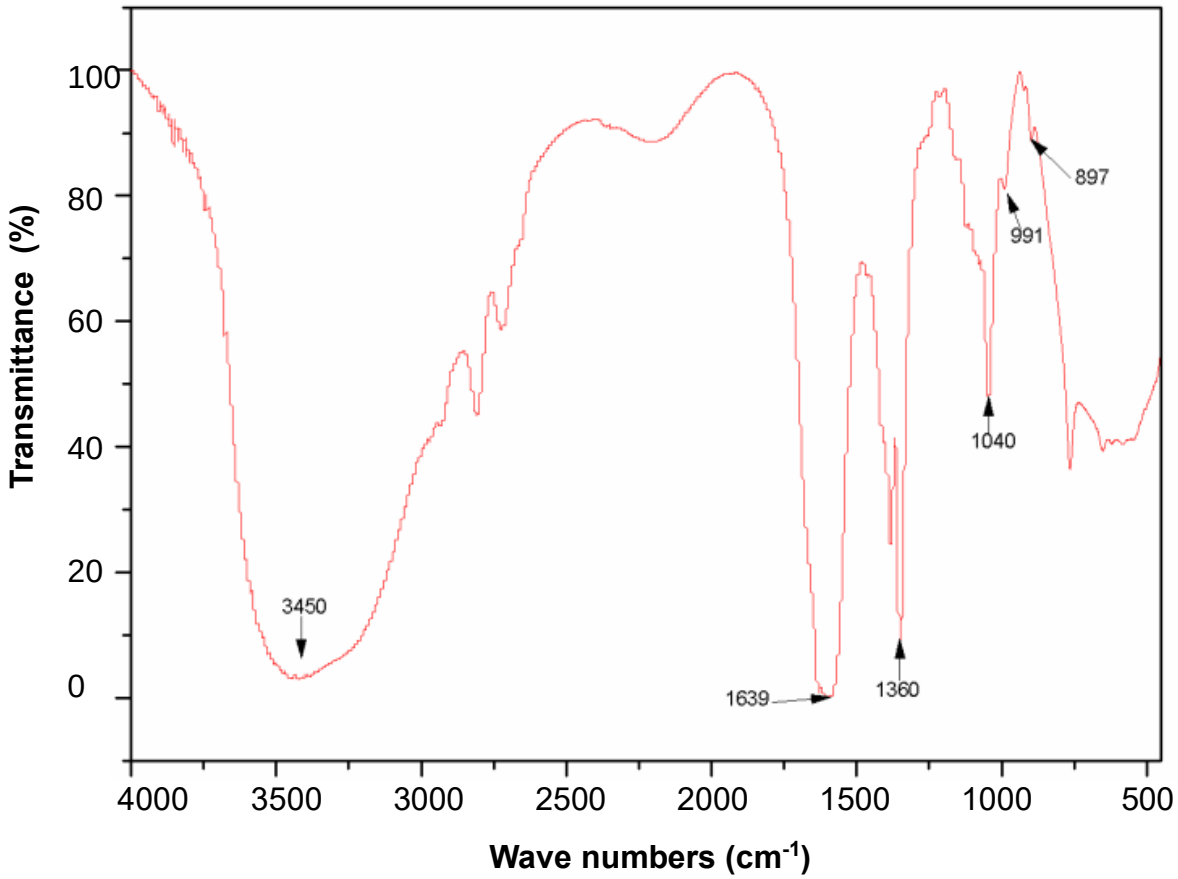
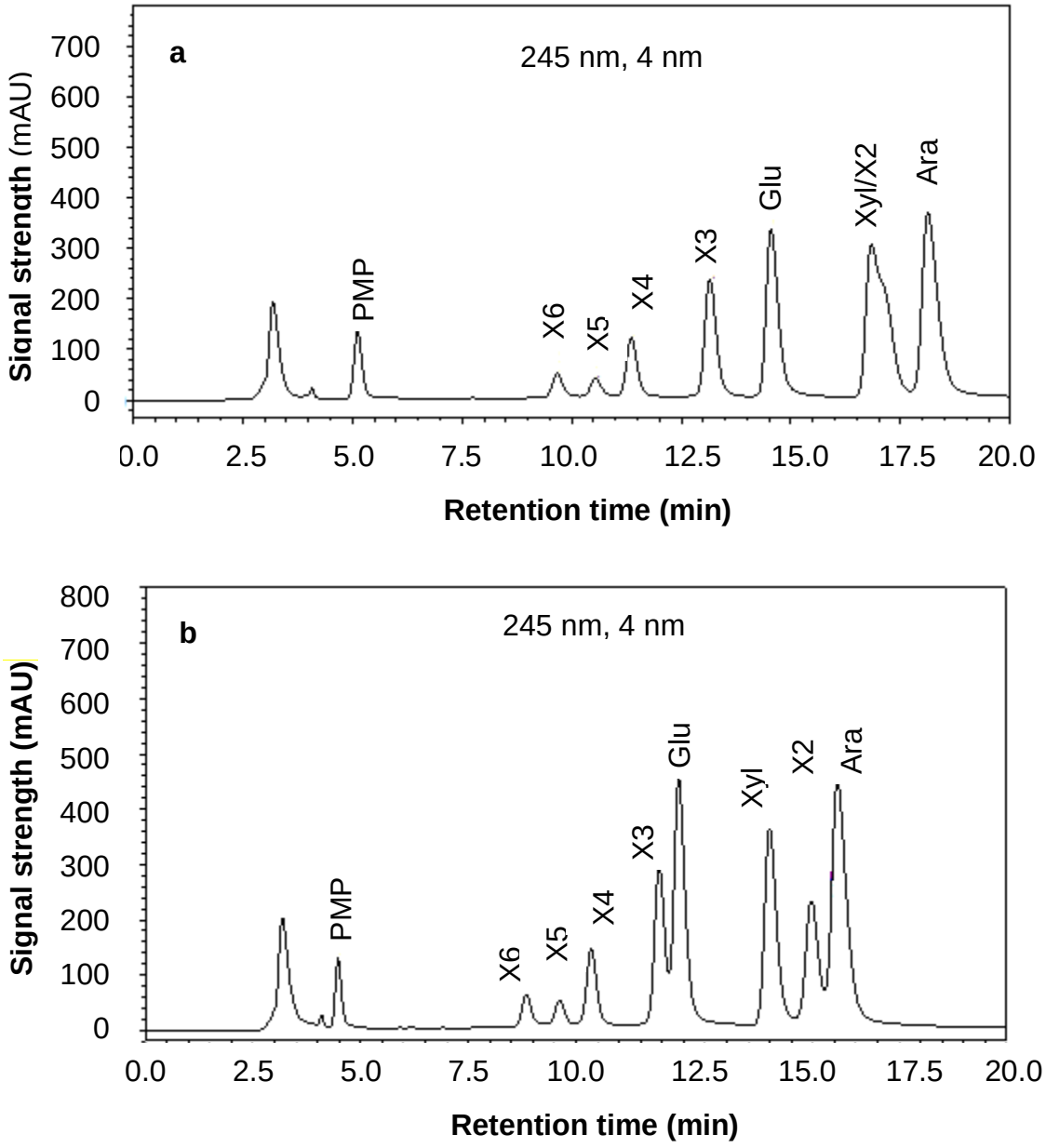


Fig. 3. FT-IR spectra of isolated hemicellulose from *Spartina anglica*

PMP Derivatization and Separation Conditions

Hemicellulose is mainly composed of xylan, which can be used to produce high value-added xylo-oligosaccharides (XOS) at industrial scale by enzymatic hydrolysis. However, rapid analysis of complex monosaccharides and oligosaccharides in hydrolysates

of lignocellulosic biomass has remained a challenge. The commonly used methods for analysis of saccharides from lignocellulosic biomass are HPLC with a refractive index detector and GC analysis of alditol acetate derivatives (Krull and Inglett 1980; Slavin and Marlett 1983). The HPLC method is less sensitive for low concentrations of saccharides and gives a low resolution of oligo-saccharides.



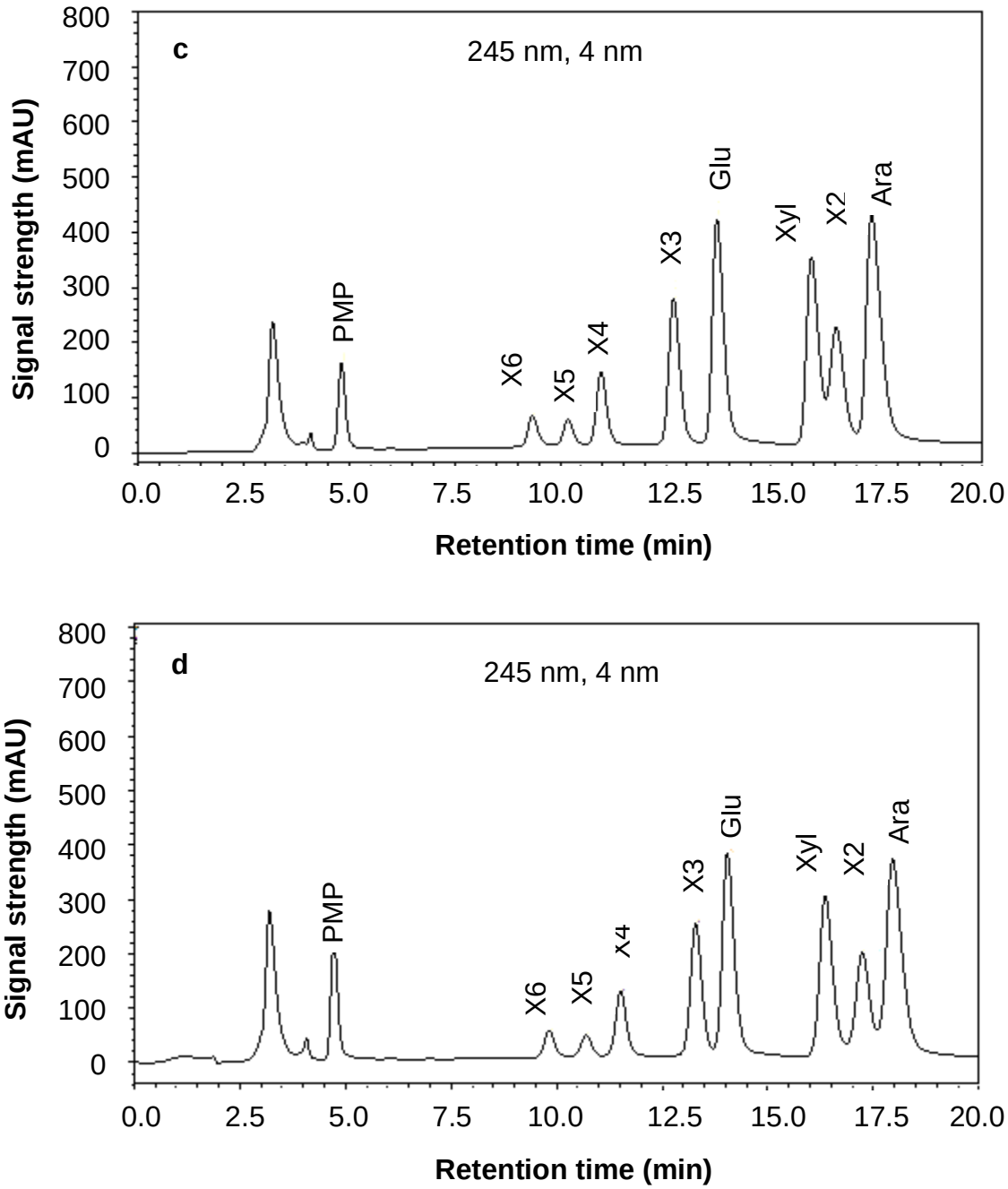


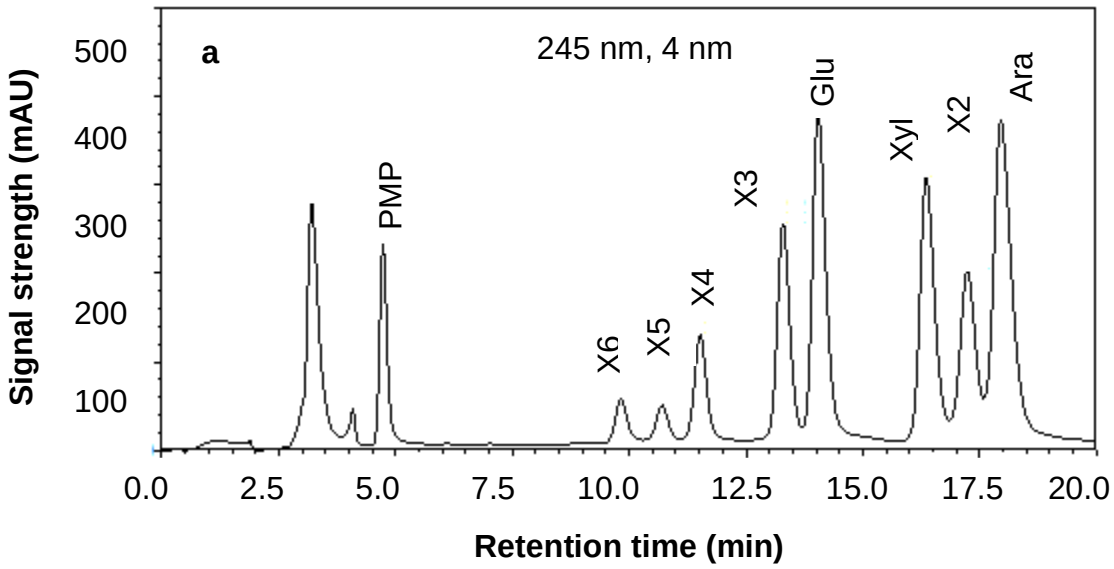
Fig. 4. Effect of mobile phase pH value on the separation of eight standard saccharides derivatives. a: pH 8.00; b: pH 8.10; c: pH 8.05; d: pH 8.06

With consideration of the possible composition of hemicellulose, the standard monosaccharides solution (glucose, xylose, and arabinose) and xylooligosaccharides solution (xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose) were prepared and derivatized with PMP. Linear regression was performed based on the measurement value calculated on the concentration of standard samples. Diluted standard solution was injected gradually to determine the minimum detection limit by calculating the concentration of standard solution when the noise-signal ratio was three.

As shown in Fig. 4, PMP derivatized xylose and xylobiose could not be separated when the mobile phase pH was 8.00. Although baseline separation of both was achieved until pH 8.10, xylotriose and glucose were not separated. However, according to the information shown in the chromatograms, it was possible to achieve separation within the pH range 8.00 to 8.10. The eight kinds of PMP derivatized carbohydrate were separated completely at pH values up to 8.06. The retention time of PMP reagent was shorter than all the PMP derivatives, which will not interfere with the separation of PMP derivatives. This assay condition established the separation of monosaccharides (glucose, xylose, and arabinose) and xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose), simplified the process steps, and improved efficiency.

HPLC Analysis for Hemicellulose Hydrolysate

The enzymolysis products of hemicellulose from *Spartina anglica* consist of monosaccharides and xylooligosaccharides. As shown in Fig. 5b, xylose, xylobiose, xylotriose, xylotetraose, xylopentaose, and xylohexaose were detected compared with the controlled trial (Fig. 5a). No glucose was detected, which suggested a high purity of hemicellulose. The full enzymolysis products were separated at pH 8.06. Xylobiose was the main xylooligosaccharide in the enzymolysis product.



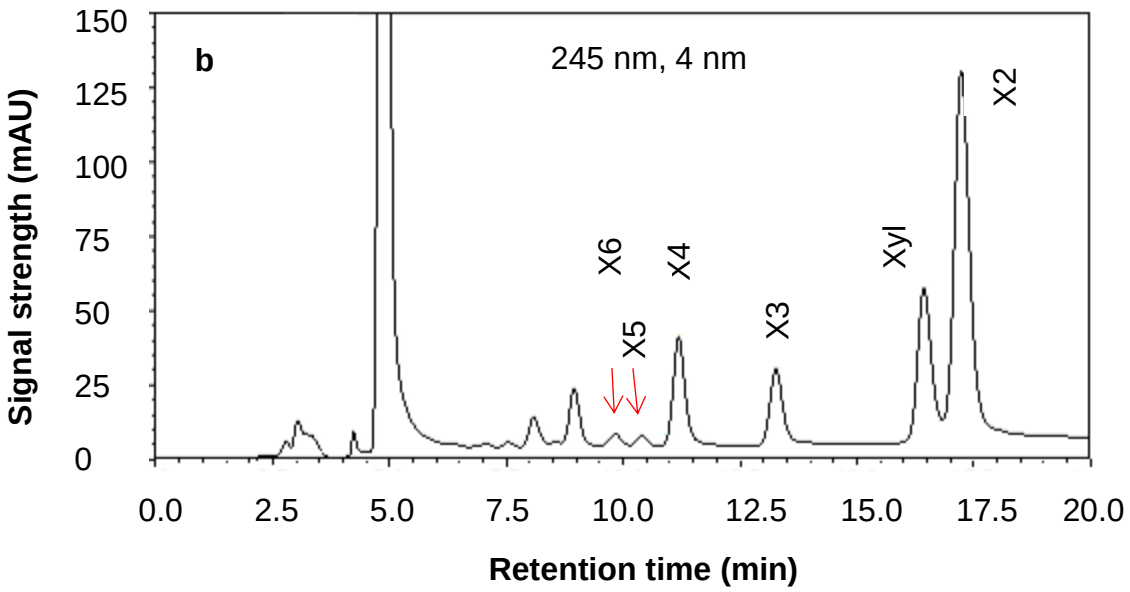


Fig. 5. Analysis of enzymatic hydrolysates of *Spartina anglica* hemicelluloses by HPLC. a: chromatogram of the standard XOS (X_2~X_6) derivatives; b: chromatogram of enzymatic hydrolysates of *Spartina anglica* hemicelluloses

Regression Equation, Correlation Coefficient, the Minimum Detection Limit, and Precision Analysis using HPLC

To test the accuracy of established method at short period, seven consecutive repeated experiments were performed, and the average relative standard deviations were calculated. Eight standard sugar regression equation, coefficient of determination (R^2), minimum detection limit (LOD), and average relative standard deviation (RSD) are shown in Table 1. Six kinds of standard sugar showed good linearity (R^2=0.9916 to 0.9995) at 0.12 to 9.5 mM, sensitivity (LOD 1.60 to 2.27 μM), and precision (RSD of six kinds of standard sugar < 1.36%). To evaluate the effect of PMP pre-column derivatization-HPLC method in natural production of xylooligosaccharides, this method was used to analyze the hemicellulose hydrolysis product of *Spartina anglica* using commercial xylanase. Xylotetraose, xylotriose, xylose, and xylobiose were separated and detected according to this method. Moreover, a small amount of xylopentaose and xylohexaose were detected at the same time (Fig. 5b).

Table 1. Regression Equations, Coefficient (R^2), Limit of Detection (*LOD*) and Average Relative Standard Deviations (*RSD*) of the Proposed HPLC Method

Saccharides	Regression equations	R^2	LOD (μM)	Average RSD (%)
X2	$y=4.8\times10^6x-140000$	0.9938	1.60	1.36
X3	$y=3.5\times10^6x-19387$	0.9996	1.69	1.13
X4	$y=2.7\times10^6x-63461$	0.9998	1.94	1.65
X5	$y=1.8\times10^6x-66009$	0.9998	1.86	1.20
X6	$y=9.8\times10^5x+22584$	0.9944	1.74	1.30
Glu	$y=4.5\times10^6x+760000$	0.9951	2.27	1.15
Xyl	$y=7.1\times10^6x+657379$	0.9990	1.66	1.18
Ara	$y=9.3\times10^6x+79040$	0.9925	1.68	1.14

CONCLUSIONS

1. This study established a method to extract hemicellulose from *Spartina anglica*. The hemicellulose belongs to a kind of xylan and arabino-glycan. The purity of hemicellulose obtained by this method is at least 62.4%, which could be used in the production of bio-based product.
2. A PMP-derivatization method was developed for simultaneous separation and analysis of monosaccharides and xylooligosaccharides. The method is suitable for use in the process monitoring of bioconversion. Three monosaccharides (glucose, xylose, and arabinose) and five xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose) were simultaneously separated and analyzed, by using a C18 chromatographic column with UV detection wavelength at 245 nm.
3. The PMP-derivatized method was successfully applied to analysis the enzymolysis products of hemicellulose from *Spartina anglica*. The enzymolysis products are consist of monosaccharide (xylose) and xylooligosaccharides (xylobiose, xylotriose, xylotetraose, xylopentaose, xylohexaose).

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