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Solid Phase Extraction and Separation of Polysaccharides from Marine Plant by Ionic Liquid-Modified Polymers

HENG ZHANG, BAOKUN TANG and KYUNG HO ROW*

Department of Chemistry and Chemical Engineering, Inha University, 253, Yonghyun-Dong, Nam Gu, Incheon 402 751, Republic of Korea

*Corresponding author: Fax: +82 32 8724046; Tel: +82 32 8607470; E-mail: rowkho@inha.ac.kr

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Ionic liquid-modified polymers were applied to the separation of laminarin and fucoidan by solid phase extraction from natural plant sources. The most suitable sorbent for polysaccharide separation was identified from the adsorption behaviour of the targets on different ionic liquid modified polymers. The proper elution solvents for both laminarin and fucoidan were determined from practical tests. The sorbents performed stably and selectively, showing potential applications in the separation of other polysaccharides or other biomacromolecules.

Keywords: Solid phase extraction, Ionic liquid modified sorbents, Polysaccharides.

INTRODUCTION

Laminarin (Fig. 1a), which is composed of glucose monomers joined mainly by â (1,3) glycosidic bonds with some â (1,6) branching, is the major polysaccharide of algae[1,2]. Depending on the degree of polymerization, the molecular weight of laminarin was found to be approximately 5000 Da[3]. Several studies reported the bioactivities and specific applications of laminarin, such as a therapeutic agent for immune disorders and antibacterial, antioxidation and intestinal protection[4-6].

Fucoidan (Fig. 1b), another major storage polysaccharide distributed in seaweed, is highly sulphated. In general, á-1,3-linked sulphated L-fucose forms the main skeleton of fucoidans and a repeating sequence of alternating á(1 → 4) and á(1→ 3) glycosidic bonds also exist[7]. Some studies reported that fucoidan has a molecular weight of approximately 100 kDa[8]. Fucoidan has a wide range of physiological and biological properties, which suggest a potential role in the treatment of many diseases, such as cerebral ischemia, Alzheimer's disease, myocardial infarction, cardiovascular disorders, renal disease and cancer[9-14]. In view of these specific biological activities, both laminarin and fucoidan isolated from marine plants have attracted a great deal of attention. However, most current researches on polysaccharides put an emphasis on their structures or bioactivities, but neglect the importance of separation and purification of polysaccharides before further studies.

Several methods, such as ethanol precipitation and gel permeation chromatography have been used for separation and purification of polysaccharides. The traditional ethanol precipitation method is time-consuming and requires large amount of solvents. The gel filtration chromatography is a complicated as well as expensive technique. Solid-phase extraction (SPE) is a high performance and convenient technology for separating bioactive compounds from plants with low cost, low consumption of solvent and being environmental friendly, but little attention has been paid to its use in polysaccharides separation. To improve the separation efficiency of solid-phase extraction, the selection of an appropriate sorbent is extreme important[15]. Thus, the sorbents with specific functional groups has been investigated and employed.

In recent years, ionic liquids (ILs) and related materials have been used as media for not only green synthesis, but also for analysis applications. They interact with target compounds *via* anion exchange, hydrogen bonding, π-π, or hydrophobic interactions[16]. These ionic liquid-based materials, poly(ionic liquid)s (PILs) have attracted attention because they have the properties of both ionic liquids and polymers and have a number of innovative applications. Although poly(ionic liquid)s applied to separating monosaccharides through hydrogen bonding and hydrophobic interactions[17], the lack of attention was paid to its potential as sorbent for isolation of polysaccharides. Comparing with monosaccharides, separation of polysaccharides is more difficult due to their complicated structures. By thorough considerations of the structures of polysaccharides, the ion exchange nature of poly(ionic liquid) may be empolyed for the separation of these compounds due to the appearance of sulfonic acid group in fucoidan. In this case, a blank polymer

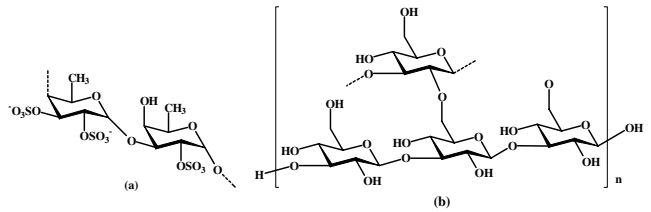

Fig. 1. Structures of (a) Fucoidan and (b) Laminarin

was prepared and different imidazoles including imidazole, methylimidazole, carboxyl-imidazole and amino-imidazole have potentials to separate these polysaccharides by anion exchange and hydrogen bonding interactions. The application of ionic liquid-based polymers for solid phase extraction of polysaccharides with detection by high performance liquid chromatography (HPLC) was proposed and evaluated. All necessary conditions were investigated systematically to optimize the process. Furthermore, this fast, convenient and efficient isolation method was used to separate laminarin and fuoidan from *Laminaria japonica*.

EXPERIMENTAL

The seaweed species, *Laminaria japonica*, was acquired from a local market in Incheon, Korea. The fucoidan and laminarin standards were purchased from Sigma Aldrich (St. Louis, MO, USA). Acetonitrile, methanol, ethanol, toluene and sodium nitrate were supplied by Duksan Pure Chemical Co. Ltd. (Ansan, Korea). Hydrochloride acid was obtained from Daejung (Gyonggi-do, South Korea). 2,2'-Azo*bis*isobutyro-nitrile (AIBN) was purchased from Junsei Chemical Co. (Tokyo, Japan). Imidazole (98 %), 1-methylimidazole (99 %), 1-(3-aminopropyl) imidazole, 1-imidazoleacetic acid, 4-(chloromethyl)styrene (90 %) and divinylbenzene (50 %) were acquired from Tokyo Chemical Industry Co. Ltd. (Tokyo, Japan). Distilled water was filtered using a vacuum pump (Division of Millipore, Waters, USA) and filter (HA-0.45, Division of Millipore, Waters, USA). The samples were filtered (Minisart RC 15, 0.45 µm, Goettingen, Germany) before being injected into the HPLC system.

HPLC analysis: The HPLC system consisted of a YL9112 Isocratic pump (Young Lin Co., Anyang, Korea), RI detector (RI750F, Young Lin Instrument Co., Anyang, Korea) and integrated data system (Clarity Chromatography Software, version 2.3, DateApex, EU). The injection valves with 20 µL sample loops were used. HPLC was performed using a Waters Ultrahydrogel™ WATO 11530 size exclusion column (300 × 7.8 mm i.d.) and a waters ultrahydrogel™ WATO 11565 guard column (40 × 6 mm i.d.) from waters (Milford, MA, USA). The mobile phase was water. The flow-rate and injection volume were set to 0.6 mL min^{-1} and 10 µL, respectively.

Preparation of ionic liquid modified polymer: 4-(Chloromethyl)styrene (monomer, 5.08 g), divinylbenzene (crosslinker, 2.60 g), 8.0 mL of ethanol (porogen) and AIBN (initiator, 0.076 g) were mixed under a nitrogen atmosphere with rapid stirring. The emulsion solution underwent polymerization by heating to 70 °C for 24 h. After complete polymerization, the obtained blank polymer was filtered and washed three times with ethanol to remove the coagulated and soluble impurities.

The blank polymer (1 g) and different ionic liquid modifiers (1.5 g) were added to 35 mL of ethanol. After heating the sample for 12 h under reflux, the obtained modified polymers (Fig. 2; imidazole polymer (PIm), methylimidazole polymer (PImM), carboxyl-imidazole polymer (PImCOOH) and amino-imidazole polymer ($PImNH_2$)) were cooled to room temperature. After washing sequentially with toluene, ethanol and methanol, the chemically bonded polymers were oven-dried for the subsequent experiments. Table-1 listed the synthesized polymers.

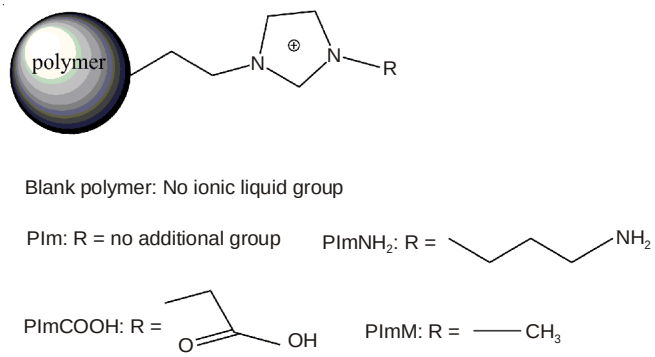

Fig. 2. Chemical structure of all the ionic liquid-modified silica sorbents

Adsorption: The static method was used to evaluate the adsorbent capacity of the different synthesized particles. 1 mL of standard mixtures of laminarin and fucoidan and a mass of 20 mg synthesized materials were added to the vials with constant stirring at room temperature for 2 h to obtain the equilibrium adsorption. As a result, the amounts of polysaccharides adsorbed on the synthesized materials were calculated by subtracting the concentrations of unabsorbed laminarin and fucoidan from the initial amounts of these polysaccharides.

Procedure of solid-phase extraction: 200 mg of the ionic liquid modified polymers was packed into empty cartridges and preconditioned with 5 mL ethanol and methanol. 0.2 mL of the polysaccharide standard mixtures were loaded onto the packed cartridges. After loading and eluting the targets in the solid-phase extraction cartridges with different solvents to separate the polysaccharides, the filtrates were evaporated to dryness for 80 °C and reconstituted in 0.2 mL of the mobile phase for further HPLC detection. To extract the polysaccharides from marine plants, 0.10 mol L^{-1} of hydrochloric acid was used as the extraction solvent and heated at a sample-to-solvent ratio of 1/30 g mL^{-1} for 80 °C and 4 h.

Characteristic analysis: The hydrogen, carbon and nitrogen contents were measured by elemental analysis performed on an EA1112 (Italy). The fourier transform infrared (FT-IR, Vertex 80 V Bruker, USA) spectra were measured in the range of 4000-400 cm^{-1} at a scan rate of 20 scans min^{-1}. A KBr pellet was used for FT-IR analysis.

RESULTS AND DISCUSSION

Performance evaluation: Table-2 listed the elemental analysis results of the blank polymer and $PImNH_2$. Under this condition, H, C and N % represent the percentage of hydrogen, carbon, nitrogen, as determined by elemental analysis. As a result, the percentage of nitrogen of $PImNH_2$ was significantly

TABLE-1
POLYMERS USED IN THIS EXPERIMENT

No.	Ionic liquid monomer types	Monomer/crosslinker ratio (mmol mmol^{-1})	Propogen amount (mL mmol^{-1})	Ionic liquid/Blank polymer ratio (g g^{-1})
P1	Imidazole	2/1	0.2	1/1
P2	1-Methylimidazole			
P3	1-(3-Aminopropyl) imidazole			
P4	1-Imidazole acetic acid			
P5	1-(3-Aminopropyl) imidazole	1/3		
P6		1/2		
P7		1/1		
P8		3/1		
P9		4/1		
P10		3/1	0.2	1/1
P11			0.3	1/1.5
P12			0.4	1/2
P13			0.5	1/2.5
P14			0.6	1/3
P15				
P16				
P17			0.2	
P18				
P19				

TABLE-2
ELEMENTAL ANALYSIS OF POLYMERS

Materials	C (%)	H (%)	N (%)
Blank polymer	76.38	6.78	0.41
$PImNH_2$	74.15	7.55	7.93

greater than the blank polymers without the ionic liquid modifiers.

Fig. 3 shows the FT-IR spectra of the blank polymer and $PImNH_2$. The peaks at 1600, 1585, 1500 and 1450 cm^{-1} were assigned to four vibrations of C=C bonds in benzene. This shows that both the blank polymer and $PImNH_2$ contain benzene. The band at 1562 cm^{-1} was assigned to imidazole groups[18]. The peak observed in the FT-IR spectra of $PImNH_2$ at 3500-3300 cm^{-1} was assigned to the stretching vibrations of NH and NH_2. These spectra confirmed that the synthesis of the amino-imidazolium polymer was successful.

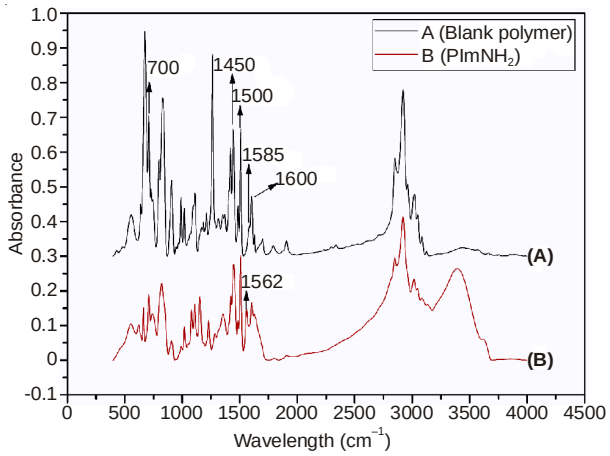

Fig. 3. FT-IR spectra of (A) blank polymer and (B) $PImNH_2$

Optimization of ionic liquid modified sorbents: First of all, the calibration curves were constructed according to the areas of the chromatographic peak areas at six increasing concentrations, ranging from 0.50 to 2 mg mL^{-1}. The measurement at each concentration was repeated 3 times with good linearity. The linear correlation equations were y = 82.632x – 1.8142 (r^2 = 0.998) for fucoidan and y = 91.018x – 4.2508 (r^2 = 0.999) for laminarin.

The adsorption capacities of the sorbents were introduced. In addition to examining the interactions between the target compounds and sorbent, the absorption capacity can be used to help select the optimal sorbent for separating fucoidan and laminarin. The amounts of fucoidan and laminarin adsorbed onto the different sorbents were obtained using the following equation:

$$Q = \frac{(C_o - C)V}{m}$$

where Q (mg g^{-1}) is the amount adsorbed, m (g) is the mass of the sorbent, C_o (mg mL^{-1}) is the initiator concentration, V (mL) is the volume of the sample solvent and C (mg mL^{-1}) is the unabsorbed concentration.

The functional groups of the polymers affect the selectivity towards and adsorption capacities of the target compounds. Efficient solid-phase extraction is essential for evaluating the adsorption abilities of the sorbents modified with different ionic liquids as functional monomers. As shown in Fig. 4a, the order of the amounts adsorbed on the different ionic liquids-modified materials was $PImNH_2$ > PImM > PImCOOH > PIm for fucoidan and PIm > PImM > PIm > $PImNH_2$ for laminarin. The amount of fucoidan adsorbed onto $PImNH_2$ was greater than the others because of the ionic interactions from the amino groups and the extra hydrogen bonding[19]. On the other hand, the amount of laminarin adsorbed onto $PImNH_2$ was the lowest due to the high hydrophilicity, which made it easier to separate one from the other. In this case, P3 was found to be the best choice.

A series of materials with different monomer/cross linker ratios (1/3, 1/2, 1/1, 2/1, 3/1, 4/1 mmol mmol^{-1}) were prepared for further study. According to the results, the amounts adsorbed were highest when the monomer/cross linker ratio was 3/1 mmol mmol^{-1} (Fig. 4b). The pore size affects the arrangement

of functional groups located within the pores[20]. The pore size of the polymers was lower with large amounts of cross linker, which has a relationship with the distribution of active sites. In view of steric hindrance, the target compounds cannot interact with the functional groups effectively if the functional groups are too close. Therefore, P8 was selected for further study. The volume of porogen per amount of monomer and cross linker was tested in the range, 0.2-0.6 mL $mmol^{-1}$ (Fig 4c). No obvious change with increasing ratio was observed for both laminarin and fucoidan. Therefore, P10 was considered to be the optimum for achieving the highest level of fucoidan and laminarin adsorption.

An excess of functional monomer will result in an excessive number of binding sites and steric mismatch. Furthermore, insufficient monomer will reduce the self-assembly and selectivity[18]. As shown in Fig. 4d, the ratios of ionic liquid and blank polymer were tested from 1/1 to 1/3 g g^{-1}. The amount of polysaccharides adsorbed increased with increasing ratio until 1/1.5 g g^{-1}; the amount decreased with further increases in the ionic liquid ratio. Multiple interactions between the targets and functional groups of the ionic liquids would result in one target compound molecule being bound to several functional groups, thereby reducing the adsorption capacity. As a result, P16 was found to be the optimal material for polysaccharide separation.

Optimization of the solid-phase extraction procedure: A mixture of fucoidan and laminarin was prepared in water and 0.2 mL of an aqueous solution was loaded onto the $PImNH_2$ cartridges. Subsequently, 1 mL of the different solvents was applied to separate the two polysaccharides. The amounts of polysaccharides in the eluent were detected by HPLC. The amount of laminarin adsorbed onto $PImNH_2$ was the lowest and approached zero, which made it much easier to identify the elution solvent for laminarin. First, as shown in Table-3, solvents with different polarities were chosen to elute laminarin. These organic solvents had the ability to elute most of the laminarin and a small amount of the fucoidan from the $PImNH_2$ cartridge. When water was used, a small amount of fucoidan was detected in the eluent from the $PImNH_2$ cartridge but most of the laminarin had been eluted. Therefore, water was considered to be optimal.

After using water to elute laminarin, different salts and acids were arranged to elute fucoidan. Considering that fucoidan contains sulfonic groups in its structure, the anion exchange interaction between fucoidan and $PImNH_2$ is a major interaction. Sodium nitrate, nitric acid and sodium chloride solutions were dissolved in water at a concentration of 1 mol L^{-1}. A comparison of the amounts fucoidan eluted by the different salts and acids revealed the salts to be better than the acids.

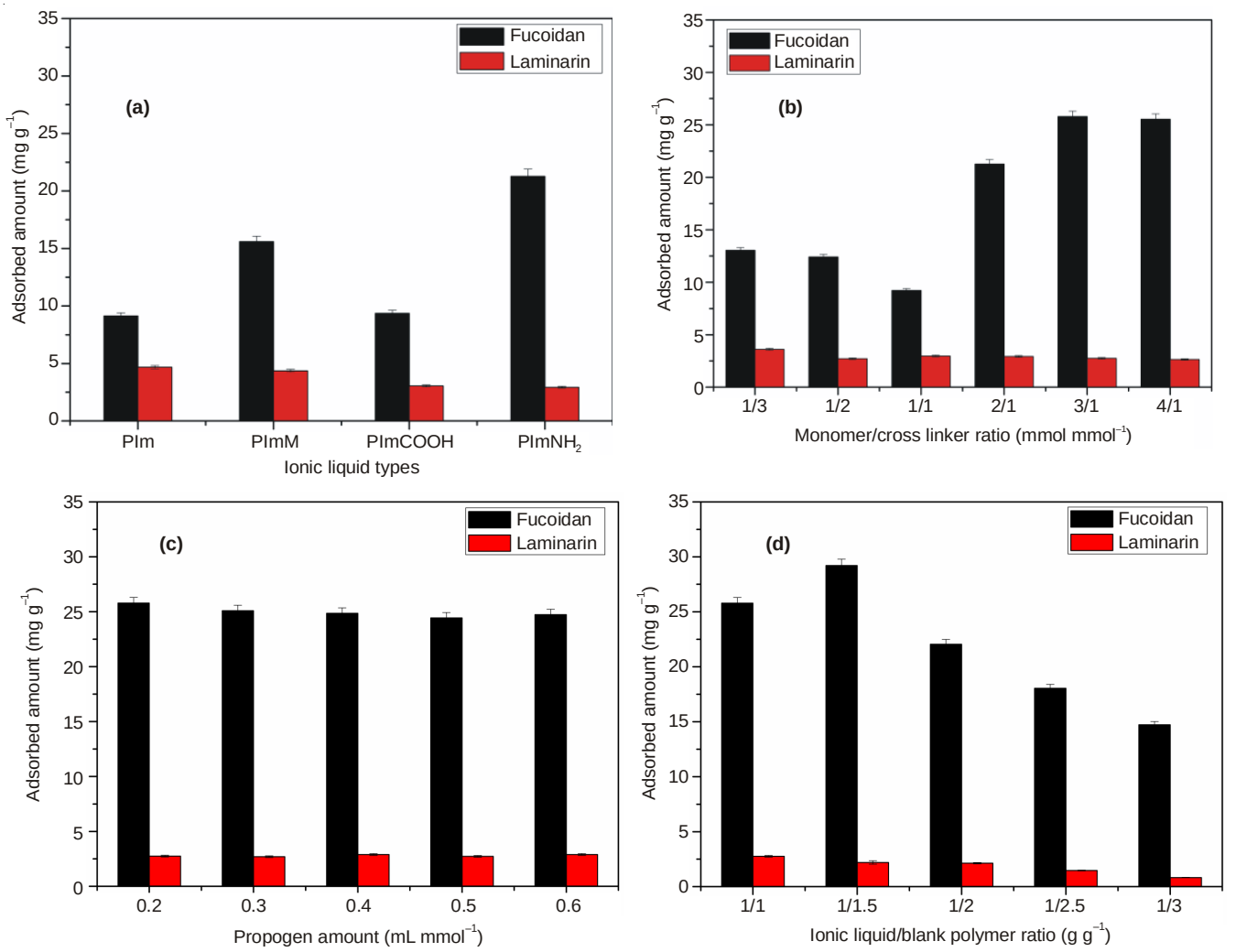

Fig. 4. Effect of the types (a) and, monomer/cross linker ratios (b), amounts (c) of the porogen and blank polymer/ILs ratios (d) of the modified polymers on the amounts of flavonoids adsorbed on them

TABLE-3
ELUTION EFFICIENCY OF DIFFERENT SOLVENTS FOR TWO POLYSACCHARIDES

Solvent	Loaded (mg mL^{-1})	Elution 1 (mg mL^{-1})		Elution 2 (mg mL^{-1})	
		Fucoidan	Laminarin	Fucoidan	Laminarin
Methanol	1.00	0.02	0.28	-	-
Water		0.05	0.75	-	-
Acetonitrile		0.02	0.39	-	-
0.4 mol/L HCl		0.13	0.49	-	-
1 Mol/L $NaNO_3$	1.00	Water		0.86	0.05
1 Mol/L NaCl				0.42	0.07
1 Mol/L HNO_3				0.23	0.18

The sodium nitrate solution was found to be the best of the salt solutions for eluting fucoidan.

Analytical performance: To evaluate the proposed method, a group of experiments was designed to examine the linearity, precision, limit of detection (LOD) and other properties. The LODs of laminarin and fucoidan based on a signal-to-noise ratio of 3 were 0.18 and 0.15 mg mL^{-1}, respectively. Both targets showed good linearity. Repeatability assays were conducted by five injections of standard mixtures of the two polysaccharides after solid-phase extraction over a five-day period. The relative standard deviations (RSDs) of fucoidan and laminarin were 1.8 and 3.2 %, respectively. These results confirmed the stability of the proposed method and its potential applications.

Application of the proposed method in separating the plant extract: After loading 0.2 mL of the extracted solution of *Laminaria japonica*, the $PImNH_2$ cartridge with the sample was eluted sequentially with water (2 mL) and a 1 mol L^{-1} sodium nitrate solution (2 mL). The two polysaccharides were separated successfully, as shown in Fig. 5. Approximately 52.68 and 248.21 mg g^{-1} of fucoidan and laminarin, respectively, were obtained, confirming that the proposed method can separate the two polysaccharides successfully.

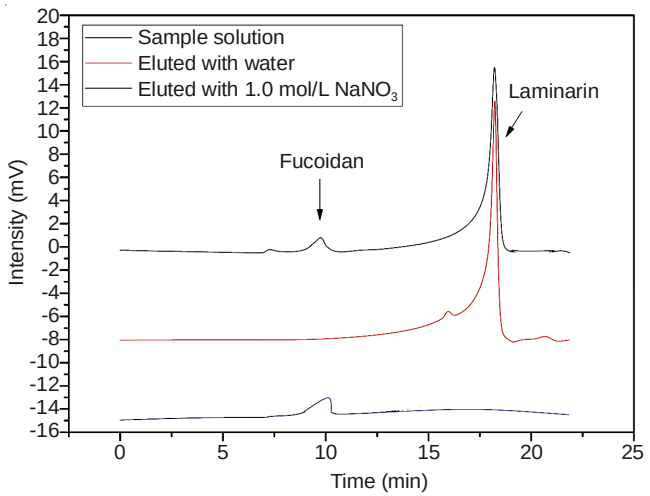

Fig. 5. Chromatogram of the polysaccharides from *Laminaria japonica* separated by optimized $PImNH_2$

Conclusion

A simple solid-phase extraction associated with a size exclusion chromatography method for separating laminarin and fucoidan in seaweeds was proposed and validated. To develop the adsorption capacity, a range of sorbents with different synthesized ratios were evaluated. $PImNH_2$ was selected as the selective sorbent for the separation of fucoidan and laminarin, which are found widely in seaweed. Two polysaccharides were separated under the optimized solid-phase extraction conditions with good linearity, precision and low deviation error. The material used was stable and applicable. These results suggest that the developed method would be an efficient and potential method for separating other polysaccharides from various samples.

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