High-performance Anion Exchange Chromatography with Pulsed Amperometric Detection for Simultaneous Determination of Monosaccharides and Uronic Acids

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Abstract: A method for analysis of eight monosaccharides and two uronic acids in polysaccharide and wood hemicellulose by high-performance anion-exchange chromatography with pulsed amperometric detection was established. The chromatographic separation was carried out on a CarboPac PA20 anion-exchange column using NaOH and NaOAc (50–200 mM) as eluent at the flow rate of 0.5 ml min⁻¹. The separation was completed in 30 min. Detection limits of 2.5–14.4 µg l⁻¹ (25 µl injection, 3 times of baseline noise) were achieved under the optimized conditions. Relative standard deviations (RSDs) for seven consecutive determinations of 5 mg l⁻¹ eight monosaccharides and two uronic acids were 0.3%–1.5%. The method had been applied for the determination of eight monosaccharides and two uronic acids in hydrolysates of polysaccharide and wood hemicellulose.

Key Words: Monosaccharide; Uronic Acid; High performance anion exchange chromatography; Pulsed amperometric detection

1 Introduction

Carbohydrate is one of the most abundant compounds in nature and also the main energy source to maintain the life of all creatures and vegetables. Besides, sugars are raw materials for organisms to produce other compounds. So, it is of great significance to analyze sugars for the investigation of bionomy.

Sugar compounds cannot be detected by absorption due to lack of chromophore in the molecular structure. The traditional means for quantification of sugars was phenol-sulfate colorimetry^[1] or improved phenol-sulfate colorimetry^[2] for total amount analysis and then paper chromatography, silica gel thin chromatography^[2], or gas chromatography (GC)^[3] was used for separate monosaccharides quantification in polysaccharides. Besides, high performance liquid chromatography (HPLC)^[4] and capillary electrophoresis (CE)^[5] were also commonly used techniques to separate monosaccharides and oligosaccharides. Since

most sugars are neutral, polar, and hydrophilic compounds, they must be derivatized to hydrophobic or volatile compounds before separation by HPLC or GC. However, derivatization was often affected by the complex matrix in real samples and the derivatization was not complete or would result in the production of multiple derivatization products. While refraction index and low wavelength UV detector was sensitive to eluent composition and sample matrix, the application of the gradient elution method was limited.

The methods for determination of uronic acids, the acidic derivants of sugars, were traditional sulfuric acid-carbazole colorimetry^[1,3,6] and meta-hydroxydiphenyl method^[2,7], which could only quantify the total amount of uronic acids. The amount of uronic acids was calculated by the content of galacturonic acid or glucuronic acid. So the determination results were not the real values of the specific kind of uronic acids. Furthermore, the neutral monosaccharide, neutral pentose, and neutral hexose in real samples had the same

Received 28 December 2005; accepted 9 May 2006

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This work was supported by the National Natural Science Foundation of China (Nos. 20577058, 20475060) and the National Basic Research Program of China (No. 2003CB415001).

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absorbance wavelength as uronic acids. So, deviation was possible for the determination of uronic acids with the sulfuric acid-carbazole colorimetry method. Although the improved sulfuric acid-carbazole colorimetry method was suitable for the identification of uronic acids with relatively precise results, the content and composition of neutral carbohydrates should be measured before determination, which were so complicated and time-consuming thus restricting the application of this method. The separation of monosaccharides and uronic acids could be achieved by CE, but the sensitivity was limited by injection volume and UV-detector with the detection limits higher than 12 mg l^{-1} ^[5]. Pre- or on-column derivatization was used in the method of reversed phase chromatography in conjunction with UV detection which had the detection limits in the range of $30\text{--}100 \text{ mg l}^{-1}$ ^[4,9] for the two uronic acids.

Recently, high performance anion exchange chromatography coupled with pulsed amperometric detection had been used to directly analyze the contents of carbohydrates. This new technology had readily solved the above-mentioned problems. It was based on the difference of ion-exchange interaction between sugars and stationary phase caused by pK_a of sugars at acidity higher than pH 12. The method had the advantage of a no need of derivatization, simplicity, high sensitivity, organic solvent freedom, and extraordinarily low detection limit of pmol.

In this study, an effective and sensitive method was proposed to analyze eight monosaccharides and two uronic acids in polysaccharide and wood hemicellulose hydrolysate by high performance anion exchange chromatography coupled with quadruple-potential waveform-pulsed amperometric detection. The chromatographic separation was completed in 30 minutes on a Dionex CarboPac PA20 anion-exchange column using NaOH and NaOAc eluents (50–200 mM) at the flow rate of 0.5 ml min^{-1} .

2 Experimental

2.1 Instrument

Dionex ICS-2500 ion chromatograph system was used throughout the experiment. It consists of a GS50 gradient pump with online degassing, an ED50A electrochemical detector (Au electrode), an AS50 autosampler, a LC30 chromatography oven (30°C), and an injection valve with $25 \mu\text{l}$ injection loop. CarboPac PA20 ($150 \text{ mm} \times 3 \text{ mm i.d.}$, Dionex) anion-exchange column was used for sugar separation. Chromeleon 6.5 software was used to record and process the data. Ultrapure water purification system (Barnstead, IA, USA) was used to produce $18.2 \text{ M}\Omega \text{ cm}$ water.

2.2 Standards and reagents

All chemicals were of reagent grade except where specified.

Standard reference materials of fucose, rhamnose, arabinose, galactose, glucose, xylose, mannose, ribose, glucuronic acid, and galacturonic acid were provided by Sigma. Sodium hydroxide solution of 50% (w/w) was from Alfa Aesar. Anhydrous sodium acetate was obtained from Dionex (AAA-Direct Certified). Sodium azide was obtained from Beijing Chemical Corporation, China. All solutions including standard solutions, sample solutions, and the mobile phase were prepared by highly purified water produced by water purification system.

The standard stock solutions of eight monosaccharides and two uronic acids at 1000 mg l^{-1} were made by dissolving appropriate amounts of sugar in water. In order to prevent microorganisms from changing the concentration, the aqueous solution containing $20 \text{ mg l}^{-1} \text{ NaN}_3$ was used to prepare standard solutions at low concentration by diluting the corresponding stock solutions. All solutions were stored in -20°C refrigerators.

The mobile phase consisted of water, 250 mM NaOH, and 1 M NaOAc. Mobile phase of 250 mM NaOH was prepared by diluting 13.1 ml of 50% (w/w) NaOH into 987 ml of purified water that was filtered through a $0.22 \mu\text{m}$ nylon filter. Mobile phase of 1 M NaOAc was obtained by dissolving 82.0 g of anhydrous sodium acetate in water and filtering the solution through a $0.22 \mu\text{m}$ nylon filter. Both mobile phases should be stored under nitrogen circumstance with the pressure of 6–8 psi to prevent absorption of carbon dioxide from atmosphere.

2.3 Sample preparations

The polysaccharides and wood hemicellulose hydrolysate were diluted, doubled, and decupled, respectively, followed by passing through a $0.22\text{-}\mu\text{m}$ nylon filter before injection.

3 Results and discussion

3.1 Simultaneous separation of eight monosaccharides and two uronic acids

Carbohydrates are weak acids with pK_a above 11. The use of NaOH as an eluent makes sugars in their anionic form. The pellicular resin of anion-exchange column CarboPac PA20 is stable in the pH range of 0–14 and is capable of separating sugars fast with low concentration of NaOH solution. As weak acid organic compounds, sugars are separated on an anion-exchange column following the order of monosaccharide, oligosaccharide, and polysaccharide. Generally, the elution sequence of monosaccharide is related to the pK_a value, and in the same condition, the rate of elution increases with the value

of pK_a . Uronic acids are the acidic derivatization product of the corresponding monosaccharide. The carboxyl groups in the molecular structure makes them more acidic with even lower pK_a compared to their corresponding sugars, so their retention on the column is longer than the corresponding sugars. The pK_a values of eight monosaccharides and two uronic acids are shown in Table 1. The results of the experiment indicate that 20 min is enough for the separation of eight monosaccharides with the eluent of 2 mM NaOH in isocratic elution. While for the uronic acids, it should be around 60 min with at least 200 mM NaOH. The eluent of NaOAc can be used to separate acidic and hydrophobic amino acids for its stronger elution ability than that of NaOH^[13]. In this experiment, it was found that uronic acids can be easily eluted using 50–200 mM NaOAc in gradient elution. Under the optimal condition, eight monosaccharides and two uronic acids are well separated in 30 min using gradient elution, and the condition of eluent is shown in Table 2. Each time after the separation, 200 mM NaOH should be used to equilibrate the separation column for 20 min in order to flush the possible carbonate from the column thus insuring reproducibility of the retention time. Therefore, the total run time for analyzing these compounds is 50 min.

Table 1 The pK_a value of eight monosaccharides and two uronic acids (in water, 25 °C)^[14,15]

Compound	pK_a	Compound	pK_a
Glucuronic acid	3.20	Glucose	12.28
Galacturonic acid	3.48	Arabinose	12.34
Ribose	-	Galactose	12.35
Mannose	12.08	Rhamnose	-
Xylose	12.15	Fucose	-

Table 2 Gradient conditions for anion-exchange separation of monosaccharides and uronic acids

Time (min)	H ₂ O (%)	250 mM NaOH (%)	1 M NaOAc (%)	Curve ^a
0.0	99.2	0.8	0	
20.0	99.2	0.8	0	
20.1	94.2	0.8	5	5
30.0	79.2	0.8	20	5
30.1	20	80	0	5
50.0	20	80	0	

^a Curve 5 is a linear gradient.

3.2 Amperometric detection of eight monosaccharides and two uronic acids

The electrochemistry detection of sugars is based on the

electrochemical response of hydroxyl groups of saccharides in the alkali condition (pH >12). As the derivatives of sugars, uronic acids with several hydroxyl groups can also be detected in the same situation. The detection waveform of sugars pass through three developmental stages, direct current (constant potential) amperometric detection, tripotential pulsed amperometric detection, and quadruple-potential amperometric detection. In direct current amperometric detection mode, the electrode does not participate in the electrochemical reaction. The oxidation products are easily adhered on the surface of the electrode thus lead to electrode surface passivation and produce poor reproducibility. The tripotential pulsed amperometric detection mode uses three different potentials in each cycle to oxidize the sugars (detection of sugars), to cleanse the electrode using positive potential (removing all oxidized products away from the surface of electrode), and then to use negative potential (reducing the Au oxidation product to Au), respectively. Because of the use of positive potential to cleanse the electrode, the sunken phenomena and electrochemical erosion of the surface of the gold electrode that occurs will decrease the sensitivity^[16].

Table 3 Quadruple waveform for detection of carbohydrate

Time (s)	0.00	0.20	0.40	0.41	0.42	0.43	0.44	0.50
Potential (V, Ag/AgCl)	0.1	0.1	0.1	-2.0	-2.0	0.6	-0.1	-0.1
Integration		Start	End					

While the quadruple-potential amperometric detection uses four potentials in each cycle to oxidize the analytes to its oxidation products (detection of sugars), to cleanse the electrode using negative potential (removing all oxidized products away from the surface of electrode), to oxidize the electrode using positive potential and to activate the electrode (reducing the Au oxidation product to Au), respectively. It is applied to the experiment owing to its high sensitivity, good repeatability, and also the elimination of electrochemical erosion and sunken phenomena on the surface of the gold electrode. The waveform of quadruple-potential ampere detection for sugars is shown in Table 3.

3.3 Analytical performance

Under the above-mentioned optimized conditions of separation and detection, eight monosaccharides and two uronic acids were identified and the chromatogram is shown in Fig.1. The contents of eight monosaccharides acids were all around 5 mg l⁻¹ and the values of galacturonic acid and glucuronic acid were 19.68 and 25.18 mg l⁻¹, respectively (shown in Table 4). The relative standard deviations (RSDs) of peak areas for the 10

analytes by seven successive injections were in the range of 0.3%–1.5%. Based on a three times of signal-to-noise ratio, the detection limits of the analytes ranged from 2.5 to 14.4 $\mu\text{g l}^{-1}$.

Fig.1 Chromatogram for separation of eight monosaccharides and two uronic acids

1. Fucose; 2. rhamnose; 3. arabinose; 4. galactose; 5. glucose; 6. xylose; 7. mannose; 8. ribose; 9. galacturonic acid; 10. glucuronic acid.

Eight monosaccharides: 5 mg l^{-1} , galacturonic acid: 19.68 mg l^{-1} , glucuronic acid: 25.18 mg l^{-1} .

Table 4 Contents of monosaccharides and uronic acids in polysaccharides and lumber hemicellulose hydrolysates

Peak No.	Compound	Contents in polysaccharide hydrolysate	Contents in lumber hemicellulose hydrolysate
		(mg l^{-1})	(mg l^{-1})
1	Fucose	0.50	ND
2	Rhamnose	0.74	ND
3	Arabinose	0.83	48.3
4	Galactose	3.75	30.5
5	Glucose	24.03	55.9
6	Xylose	0.91	124.9
7	Mannose	3.46	24.6
8	Ribose	14.98	ND
9	Galacturonic acid	0.70	ND
10	Glucuronic acid	ND	ND

ND: Not detected

3.4 Sample analysis

Duplicate samples of polysaccharide hydrolysate and wood hemicellulose hydrolysate were injected for investigation. Eight monosaccharides and galacturonic acid were detected in polysaccharide with the concentration in the range of 0.50–24.03 mg l^{-1} . The concentrations of arabinose, galactose, glucose, xylose, and mannose in the wood hemicellulose hydrolysate ranged from 24.6 to 124.9 mg l^{-1} .

4 Conclusions

A simple, effective, and sensitive method was set up to simultaneously analyze eight monosaccharides and two uronic acids using high-performance anion-exchange chromatography with pulsed amperometric detection. The interferences in traditional methods for determination of monosaccharides and uronic acids were resolved. The method was applied to identify monosaccharides and uronic acids in polysaccharides and wood hemicellulose hydrolysate and it could also be used in a number of matrices, such as grain, legume, vegetables, fruits, national drugs, coastal water, and sediment.

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