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Determination of carbohydrates by hydrophilic interaction chromatography with pulsed amperometric detection using postcolumn pH adjustment

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ABSTRACT

An HPLC method for the determination of carbohydrates using a postcolumn pH adjustment technique with lithium hydroxide solution followed by pulsed amperometric detection was developed. In conjunction with hydrophilic interaction chromatography, the technique was used to determine reducing sugars, non-reducing sugars and sugar alcohols. Detection limits from 0.7 to 2.7 ng with a signal-to-noise ratio of 3 were achieved. The relative standard deviation for peak area was better than 2.7%. The postcolumn pH adjustment technique enabled gradient elution to be used.

INTRODUCTION

A number of chromatographic modes such as borate-complex anion exchange [1], anion exchange [2,3], ligand exchange [4], size exclusion and hydrophilic interaction [5] have been applied to carbohydrates. Hydrophilic interaction chromatography succesfully separates monosaccharides, disaccharides and higher oligosaccharides (DP3–DP10) with isocratic elution. The advantage of this mode is simplicity. Recently, a hydrophilic interaction method using a ligand-exchange column [6] was developed to achieve the separation of different monosaccharides and sugar alcohols simultaneously. Today, hydrophilic interaction chromatography is widely used to separate carbohydrates.

Carbohydrates generally cannot be detected by absorption in the ultraviolet and visible regions and refractive index (RI), absorbance [7,8], fluorescence [9–11] and electrochemical [12,13] detection methods have been used. The RI detector is not sensitive enough to detect amounts < 100 ng. Absorbance and fluorescence detection are highly sensitive, but need precolumn or postcolumn derivatization procedures. Electrochemical detection using pulsed amperometry enables permits the direct detection of sub-nanogram amounts of carbohydrates.

Pulsed amperometric detection (PAD) of carbohydrates was first reported by Hughes and Johnson [14]. Alkaline solutions were used to ionize carbohydrates, which were subsequently detected by pulsed amperometry using a flow-injection system. Pulsed amperometry removed oxidation products from the working electrode.

Rocklin and Pohl [15] applied pulsed amperometry to the HPLC of carbohydrates. In their method, reducing and non-reducing sugars were separated as anions using an ion-exchange column with an alkaline eluent and the eluates were detected with a pulsed amperometric detector. However, PAD has not been used with hydrophilic interaction chromatography of carbohydrates owing to the high concentration of acetonitrile in the mobile phase.

In this study, we developed a method for carbo-

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hydrate analysis by hydrophilic interaction chromatography with PAD using postcolumn pH adjustment. In addition to using isocratic elution, we performed hydrophilic interaction chromatography with gradient elution.

EXPERIMENTAL

Reagents and chemicals

D-Glucose, D-fructose, sucrose, maltose, D-xylose and xylitol were obtained from Wako (Osaka, Japan) and maltotriose, maltotetraose, maltopentaose, maltohexaose and maltoheptaose from Nacalai Tesque (Kyoto, Japan). HPLC-grade acetonitrile and amino acid analysis-grade lithium hydroxide were obtained from Wako. Deionized, distilled water was used.

HPLC system and conditions

The HPLC system consisted of a Model HP79855A autosampler (Hewlett-Packard, Waldbronn, Germany), a Model HP79852A gradient pump (Hewlett-Packard), a Model LC100T column oven (Yokogawa Analytical Systems, Tokyo, Japan), a Model LC100P reagent pump for pH adjustment (Yokogawa Analytical Systems) and a Model HP1049A electrochemical detector (Hewlett-Packerd).

The column used for isocratic elution was an Asahipak NH2P-50 (particle size 5 μm, 250 mm × 4.6 mm I.D.), (Asahi Chemical Industry, Tokyo, Japan) packed with polyamine-bonded polyvinyl alcohol gel [16]. The mobile phase was acetonitrile–water (75:25,v/v) at a flow-rate of 1.0 ml/min. The reagent solution for postcolumn pH adjustment was 0.6 mol/l lithium hydroxide at a flow-rate 0.8 ml/min. The temperature of the column oven was 40°C.

The column used with gradient elution was an Excelpak CHA-P44 (particle size 6 μm, 150 mm × 6.0 mm I.D.), (Yokogawa Analytical Systems) packed with highly cross-linked sulphonated polystyrene gel (Zn^{2+} form). Mobile phase A was acetonitrile–water (80:20, v/v) and mobile phase B was water, with a linear gradient from 5 to 40% B in 30 min at a flow-rate of 1.0 ml/min. The postcolumn conditions were as same as in isocratic elution. The oven temperature was 70°C.

The eluates from the column were mixed with the reagent solution for pH adjustment. Carbohydrates were ionized in alkaline solution (pH > 13) and introduced into the electrochemical detector. A gold working electrode was used to oxidize the carbohydrates and the potentials used in the electrochemical detector were detection potential E_1 = 0.15 V (T_1 = 30 ms), oxidative cleaning potential E_2 = 0.65 V (T_2 = 15 ms) and reductive cleaning potential E_3 = −0.95 V (T_3 = 20 ms).

RESULTS AND DISCUSSION

Lithium hydroxide, tetra-*n*-butylammonium hydroxide, sodium hydroxide and potassium hydroxide were evaluated as potential pH-adjustment agents in the presence of acetonitrile-containing mobile phases. Tetra-*n*-butylammonium hydroxide is expensive and sodium and potassium hydroxide caused phase separation when mixed with acetonitrile. When lithium hydroxide was used, the peak areas were not affected when either isocratic or gradient elution was performed. To determine the effect of the concentration of acetonitrile in the mobile phase, 10 μg of glucose were applied to an Asahipak NH2P-50 column. The flow-rate of the mobile phase was 1.0 ml/min and 0.4 mol/l lithium hydroxide at a flow-rate of 1.0 ml/min was used as the postcolumn pH agent. Changes in acetonitrile concentration did not significantly affect detection (Fig.1). Therefore, lithium hydroxide was chosen as the alkaline solution for pH adjustment.

The optimum postcolumn conditions required using the lithium hydroxide solution were investigated. The concentration of lithium hydroxide affected the peak responses of carbohydrates. Usu-

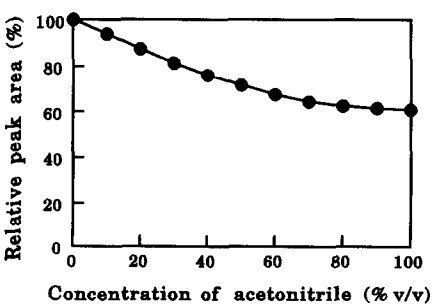

Fig. 1. Dependence of relative peak area of glucose on acetonitrile concentration in water.

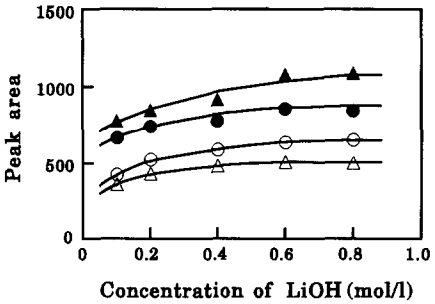

Fig. 2. Effect of concentration of lithium hydroxide on peak areas. ▲ = Fructose; ● = glucose; ○ = sucrose; △ = maltose.

ally, alkali concentrations of 0.1–0.4 mol/l are recommended [15], but we studied higher concentrations of lithium hydroxide. Chromatography was carried out with the Asahipak NH2P-50 column using an acetonitrile–water (75:25, v/v) isocratic mobile phase at a flow-rate of 1.0 ml/min. The flow-rate of lithium hydroxide solution was 0.8 ml/min. Portions of 5 μg each of fructose, glucose, sucrose and maltose were injected. Maximum peak areas were achieved at lithium hydroxide concentrations between 0.6 and 0.8 mol/l (Fig.2). The postcolumn flow-rate also altered the detector response. Lower flow-rates resulted in higher peak areas (Fig.3). However, the baseline noise increased at flow-rates of less than 0.4 ml/min. This was due to the low back-pressure on the postcolumn pump. The optimum flow-rate was 0.6–0.8 ml/min. In this study, a lithium hydroxide concentration of 0.6 mol/ml and a flow-rate of 0.8 ml/min were adopted as the optimum conditions for postcolumn pH adjustment.

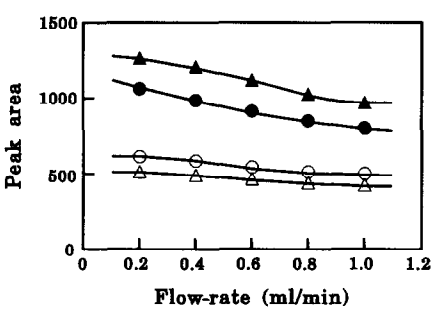

Fig. 3. Effect of postcolumn flow-rate of lithium hydroxide on peak areas. ▲ = Fructose; ● = glucose; ○ = sucrose; △ = maltose.

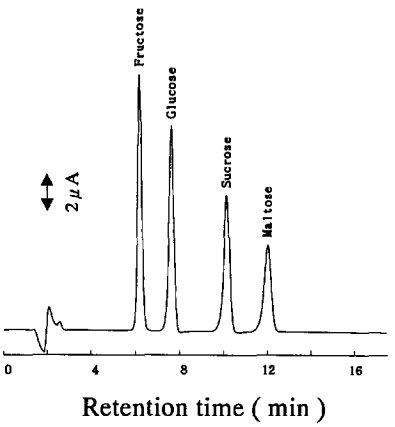

Fig. 4. Chromatography of a standard mixture of carbohydrates on the Asahipak column.

Fig. 4 shows a chromatogram of 1 μg each of fructose, glucose, sucrose and maltose standards. As reported in Table I, satisfactory reproduciblities were obtained, as reflected by the relative standard deviations (R.S.D.). The calibration graphs for all the carbohydrates were linear (40 ng–5 μg). The detection limits for fructose, glucose, sucrose and maltose were 0.9, 0.7, 2.1 and 2.7 ng (Table I), respectively, at a signal-to-noise ratio of 3. The limit of detection of sucrose was comparable to that observed with the reducing sugars.

In hydrophilic interaction chromatography, carbohydrates have generally been detected using RI detectors. However, the RI detector is not as sensitive as the PAD. Another disadvantage of RI detection is that it can only be used with isocratic elution. Chromatography with gradient elution was performed on the Excelpak CHA-P44 column. Mobile

TABLE I

CHARACTERIZATION OF THE DETERMINATION OF CARBOHYDRATES ON THE ASAHIPAK NH2P-50 COLUMN

Compound	R.S.D. ($n=10$) (%)	Correlation γ	Detection limit (ng)
Fructose	1.8	0.999	0.9
Glucose	2.1	1.000	0.7
Sucrose	0.8	0.998	2.1
Maltose	2.7	1.000	2.7

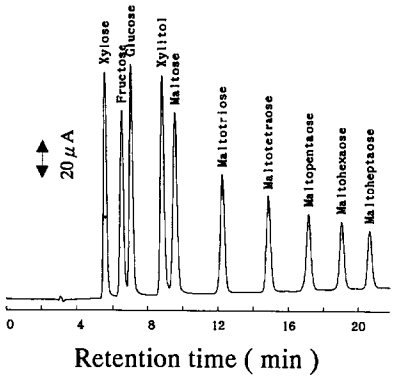

Fig. 5. Chromatography of a standard mixture of carbohydrates using gradient elution.

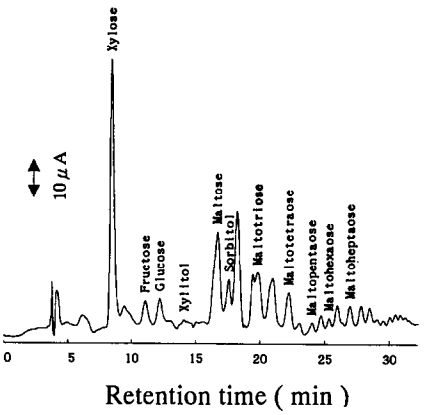

Fig. 6. Chromatography of carbohydrates in beer using gradient elution.

phase A was acetonitrile–water (80:20, v/v) and mobile phase B was water. Monosaccharide and oligosaccharides standards were separated by applying a linear gradient from 5 to 40% mobile phase B in 30 min. Fig. 5 illustrates the separation of the standards analysed with this gradient method. The standard sample contains 5 μg each of xylose, fructose, glucose and xylitol and 10 μg of other carbohydrates. The R.S.D. (n = 5) was better than 0.5% for retention times and 1.1–2.8% for peak areas (Table II). These results indicate that the proposed method can be used with gradient elution in hydrophilic interaction chromatography.

TABLE II

RELATIVE STANDARD DEVIATIONS FOR RETENTION TIMES AND PEAK AREAS OF CARBOHYDRATES AFTER GRADIENT ELUTION

Compound	R.S.D. (%) (n = 5)	
	Retention time	Peak area
Xylose	0.27	2.7
Fructose	0.32	2.8
Glucose	0.29	2.5
Xylitol	0.33	2.6
Maltose	0.46	2.8
Maltotriose	0.45	2.6
Maltotetraose	0.37	2.4
Maltopentaose	0.33	2.2
Maltohexaose	0.29	1.1
Maltoheptaose	0.37	2.2

The method was applied to the analysis of beer carbohydrates. Fig. 6 shows a chromatogram of carbohydrates in beer. Glucose to maltoheptaose were well separated in 30 min.

CONCLUSIONS

A sensitive and simple method for the determination of reducing and non-reducing sugars by hydrophilic interaction chromatography has been developed. This HPLC system is based on postcolumn pH adjustment using lithium hydroxide followed by pulsed amperometric detection. It is possible that the method might also be applicable to the determination of alcohols and polyols.

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