

## Immobilization of Styrene-acrylamide Co-polymer on Either Silica Particles or Inner Surface of Silica Capillary for the Separation of D-Glucose Anomers

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Styrene-acrylamide co-polymer was immobilized on porous partially sub-2  $\mu\text{m}$  silica monolith particles and inner surface of fused silica capillary (50  $\mu\text{m}$  ID and 28 cm length) to result in  $\mu\text{LC}$  and CEC stationary phases, respectively, for separation of anomeric D-glucose derivatives. Reversed addition-fragmentation transfer (RAFT) polymerization was incorporated to induce surface polymerization. Acrylamide was employed to incorporate amide-functionality in the stationary phase. The resultant  $\mu\text{LC}$  and CEC stationary phases were able to separate isomers of D-glucose derivatives with high selectivity and efficiency. The mobile phase of 75/25 (v/v) acetonitrile (ACN)/water with 0.1% TFA, was used for HPLC with a packed column (1 mm ID, 300 mm length). The effects of pH and ACN composition on anomeric separation of D-glucose in CEC have been examined. A mobile phase of 85/15 (v/v) ACN/30 mM sodium acetate pH 6.7 was found the optimized mobile phase for CEC. The CEC stationary phase also gave good separation of other saccharides such as maltotriose and Dextran 1500 (MW~1500) with good separation efficiency (number of theoretical plates ~300,000/m).

**Key Words :** Styrene-acrylamide copolymer, Surface polymerization, Chromatographic stationary phases, D-Glucose, Anomeric separation

### Introduction

Oligosaccharides play important roles in many biological processes, but the structural elucidation of oligosaccharides remains a major challenge due to the complexities of their structures.<sup>1</sup> Glycomics is the comprehensive study of all glycans expressed in biological systems and, glycosylation is highly sensitive to the biochemical environment and has been implicated in many diseases including cancer.<sup>2</sup> Glyco-protein analysis requires determining both the sites of glycosylation as well as the glycan structures associated with each site.<sup>3</sup> Recent advances have led to the development of new analytical methods including especially mass spectrometry to provide powerful methods in complicated tasks such as determining oligosaccharide composition, figuring out the glycosylation site, and discovering several potentially promising markers of several types of cancer and diseases.<sup>1-3</sup>

The power of glycosylation analysis is dramatically enhanced when chromatography and mass spectrometry are coupled in the analysis. Various glycosylation analysis strategies including pretreatments of samples, chromatography, and mass spectrometry have been well introduced in some review articles.<sup>4-11</sup>

HPLC and HPCE methods have been developed for the high-resolution and reproducible quantitation of carbohydrates.<sup>12,13</sup>

The high performance anion exchange chromatography-pulsed amperometry (HPAEC-PAD) system was developed for common use in separation and detection of saccharides.<sup>10</sup> This method was proved to be useful for determination of composition of monosaccharides in a sugar sample and

separation of saccharides of low molecular weights in general, but it showed poor capability for separation of saccharide isomers and was hardly compatible with mass spectrometry owing to use of a strong base as the mobile phase.<sup>10</sup>

The most popular C18 columns in general HPLC are hardly usable in separation of bare saccharides (carbohydrates) since saccharides have too high polarity and show little retention and resolution in a C18 column. C18 columns are somewhat useful for separation of derivatized saccharides, but still show poor performance in separation of isomers. Two highly prevailing types of stationary phase for separation of saccharides are porous graphite carbon (PGC) columns<sup>8,11</sup> and hydrophilic interaction chromatography (HILIC) columns.<sup>5</sup>

PGC columns show good performance in separation of saccharide isomers, but their separation efficiency is known to be inferior to that of C18 columns.

In HILIC, the polarity of stationary phase is quite high so that increased retention of saccharides is enabled to yield improved resolution owing to the strong solute-stationary phase interactions.<sup>5</sup> A variety of commercially available stationary phases are made by chemically bonding polar moieties to silica porous particles such as polysuccinimide (Poly GLYCOPLEX), polyaspartamide (Polyhydroxyethyl A), polyamine (Hypersil APS-2), propylamine (Nucleosil NH2), some amides (glycoSep N and GlycoSep C), carbamoyl ligand (TSK amide 80), and zwitterionic sulfovetaine (ZIC-HILIC).

Glucose is an important building block of all forms of sugar (saccharides) from various sources. Separation of glucose by various chromatographic techniques has been well documented in the literature,<sup>14-20</sup> NH<sub>2</sub>-containing silica based columns prevailing in small scale separation.<sup>14-16,20</sup>

Sulfonated cross-linked styrene divinylbenzene cation exchange resin is widely used in industrial chromatographic separation.<sup>17-19,21</sup>

Mutarotation of carbohydrate anomers is a fundamental phenomenon in chemistry.<sup>22-24</sup> Anomers are in equilibrium each other in aqueous phases. Glucose has crystalline  $\alpha$  and  $\beta$  anomers. When dissolved in water, these equilibrate both with an open aldehyde form and with each other. When solid pure glucose in the form of D- $\alpha$ -glucopyranose is dissolved in water some of it converts partially into  $\beta$ -form by mutarotation, resulting in an equilibrium mixture of 36% of  $\alpha$  and 64% of  $\beta$ -anomers at 25 °C.<sup>24</sup>

The method development for anomeric separation has been crucial since one of the anomers is mostly responsible for the biological activities of compound and also each anomer has its specific uses.<sup>25</sup>  $\beta$ -D-glucose is selectively responsible for many of the glucose functions such as being the main blood circulating sugar, being used for diabetes test.  $\beta$ -D-Glucose-6-phosphate,  $\beta$ -D-glucosamine, *N*-acetyl- $\beta$ -D-glucosamine, *N*-acetylmuramic acid, etc., are some of its derivatives responsible for many important functions like treatment of osteoarthritis, affecting reactions of immune system with HIV and tumor, incorporation to antibiotic formulations, affecting bilirubin solubility, etc. Therefore  $\beta$ -D-glucose enrichment is important from clinical, immunological and pharmaceutical point of view. Of course, D-glucose is the main precursor of  $\beta$ -D-glucose.

There have been rare cases where anomeric separation of D-glucose was observed in CEC<sup>26</sup> and HPLC,<sup>27</sup> and the resolution of the CEC study was far better than that of HPLC study. In the CEC study, the aminoalkyl-phase monolithic CEC column was prepared, and the column showed baseline separation of D-glucose anomers in the mobile phase of high acetonitrile content.<sup>26</sup> In the HPLC study, a silica monolith column was used to show some anomeric separation of D-glucose but with chromatographic resolution inferior to 1.<sup>27</sup> Monolithic silica support stationary phases have been reported to be efficient for separation of carbohydrates.<sup>27,28</sup>

All the stereochemical anomers of D-glucose-based saccharides arise from the two anomers of D-glucose, thus the general performances of a stationary phase for structural investigation of such saccharides may be evaluated by the separation efficiency and resolution of the D-glucose anomers.

This study reports some exhaustive work on the anomeric separation between  $\alpha$ -D-glucose and  $\beta$ -D-glucose in HPLC and CEC where good resolution and enhanced separation efficiency were obtained using the stationary phases immobilized with styrene-acrylamide co-polymer. Here we demonstrate very high separation efficiency and improved resolution of D-glucose anomers in comparison to the previous studies.<sup>26,27</sup> The strategy for our phases was to incorporate the characteristics of both PGC and HILIC phases. The PGC phases have planar  $sp^2$  character and the HILIC phases, the capability of hydrogen bonding. The polystyrene part of our mobilized copolymer provides the planar  $sp^2$  character, and the polyacrylamide part, the hydrogen bonding capability for the stationary phase. The optimization of elution para-

meters such as eluent composition and pH was carried out to get the best separation especially in CEC. The CEC column was also found to be efficient for separation of other saccharides such as maltotriose and Dextran 1500 (average M.W. = 1500).

In this study, styrene-acrylamide copolymer was immobilized on porous partially sub-2  $\mu\text{m}$  silica monolith particles and on the inner surface of fused silica capillary through RAFT polymerization. Details of RAFT polymerization can be found somewhere else.<sup>29,30</sup> There have been some reports on catalytic effects of organometallic compounds for isocyanate-hydroxyl reaction in homogeneous systems.<sup>31</sup> The idea of using similar catalysts in a heterogeneous isocyanate-hydroxyl reaction as in that study led us to carry out the reaction between 4-chloromethylphenylisocyanate (4-CPI) and silica silanol groups with some catalyst. Dibutyl tin dichloride (DBTDC) was chosen for its good solubility in the reaction solvent. The resultant HPLC and CEC stationary phases gave efficient and better separation resolution of D-glucose enantiomers as compared to previously reported phases.<sup>26,27</sup>

Synthesis of partially sub-2  $\mu\text{m}$  silica monolith particles by adoption of subtle formulation of reaction mixture, multiple heating steps followed by calcination and their chromatographic applications have been developed in our laboratory.<sup>32,33</sup>

Due to enhanced sensitivity in detection of sugar units by binding a chromophore to result in products with strong UV absorption, derivatization techniques of sugars in HPLC and CEC analysis have attracted much attention.<sup>34,35</sup> Amination is one of the methods reported for pre-column derivatization, where an aromatic amine is introduced to the aldehydic group at the reducing end of sugar unit. ABEE (*p*-amino benzoic ethyl ester) derivatization has been getting special attention,<sup>36,37</sup> and is used in this study. Only terminal aldehyde group can be derivatized (aminated or reductively aminated). When D-glucose is reductively aminated, the product is composed of one isomer while there will be two anomers when it is only aminated. Thus D-glucose is aminated while oligosaccharides are reductively aminated in this study.

## Experimental

**Chemicals and Materials.** Glacial acetic acid, urea, polyethylene glycol (PEG) 10000, tetramethylorthosilicate (TMOS), acrylamide, 4-chloromethylphenylisocyanate (4-CPI), styrene, sodium cyanoborohydride, sodium diethyl dithiocarbamate, anhydrous THF, D-glucose, maltotriose anhydrous toluene, ABEE (*p*-amino benzoic ethyl ester), and dibutyl tin dichloride (DBTDC) were purchased from Sigma-Aldrich (St. Louis, MO, USA) HPLC grade methanol, acetonitrile, acetone, and water were obtained from Mallinckrodt Baker (Phillipsburg, NJ, USA). Dextran 1500 was obtained from Fluka. All the reagents were used as received. Screen frits (1.6 mm diameter, 0.08 mm thickness, and 0.5  $\mu\text{m}$  pore size) were obtained from Valco (Houston, TX, USA). Glass lined stainless steel tubing (30 cm, 1.0 mm ID, 1/8 inch OD) and

silica capillary (50  $\mu\text{m}$  ID, 365  $\mu\text{m}$  OD) were purchased from Grace (Deerfield, IL, USA).

**Synthesis of Ground Silica Monolith Particles.** Synthesis of partially sub-2  $\mu\text{m}$  porous silica monolith particles by a renovated sol-gel procedure with multi-step heating followed by grinding and calcination has been reproduced according to the method reported previously.<sup>33</sup> A mixture of 1620 mg PEG and 1650 mg Urea in 15 mL 0.01 N acetic acid in a Teflon vial, was magnetically stirred for 10 minutes in ice/water bath followed by the addition of 5 mL TMOS and the stirring was kept on for 40 min. The solution was incubated at 40  $^{\circ}\text{C}$  in an oven for 48 h then at 120  $^{\circ}\text{C}$  in an autoclave for 48 h. The residual water due to sol-gel process was decanted off and the solid cake of monolith was dried at 70  $^{\circ}\text{C}$  for 20 h, ground with mortar and pestle for 10 min, and calcined at 550  $^{\circ}\text{C}$  for 50 h.

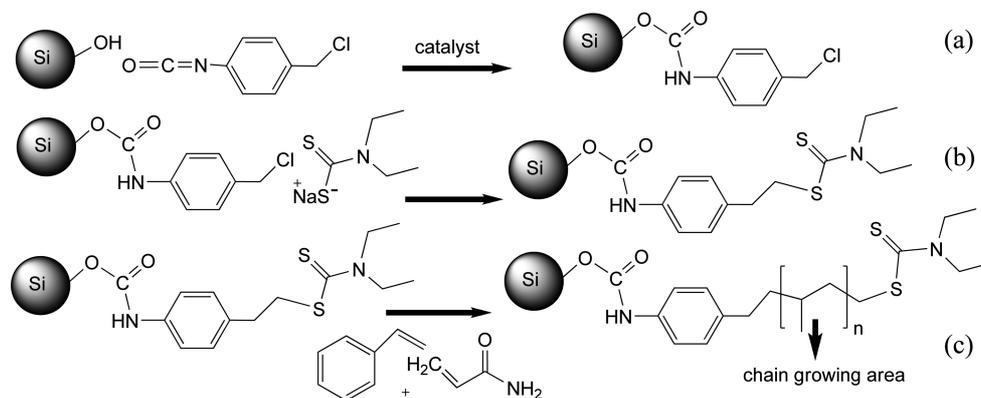
**Synthesis of Initiator Bound Silica Monolith.** 4-Chloromethylphenylisocyanate 300 mg and DBTDC 100 mg, were dissolved in 30 mL anhydrous toluene and sonicated for 10 min. Then 1 g calcined silica monolith particles dried at 120  $^{\circ}\text{C}$  overnight was suspended in it; the reaction mixture was treated at 80  $^{\circ}\text{C}$  for 50 h. The halogen terminated silica particles were washed with toluene and acetone, filtered and air dried. A mixture of 800 mg sodium diethyldithiocarbamate in 20 mL anhydrous THF was sonicated for 10 min,  $\text{N}_2$ -purged for 20 min being followed by the addition of halogen-attached silica particles and the whole reaction mixture was treated at 55  $^{\circ}\text{C}$  overnight under stirring. The initiator attached silica monolith was washed with THF, MeOH/water (60/40 v/v %) and acetone in sequence and was stored in vacuum desiccator. Illustration of reaction scheme is shown in Figure 1.

**Synthesis of Styrene-acrylamide Copolymer Immobilized Silica Monolith Particles (RAFT polymerization).** 20 mL Toluene (anhydrous) taken in 50 mL RB-flask was  $\text{N}_2$ -purged for 10 min. The initiator attached silica particles were suspended in it and refluxed at 110  $^{\circ}\text{C}$ . Meanwhile dropwise addition of monomer mixture was taken place *via* dropping funnel. The monomer mixture was composed of 4 mL styrene and 100 mg acrylamide taken in 4 mL (1:1) mixture of anhydrous toluene and chloroform. Radical polymerization was carried out under reflux and stirring at

110  $^{\circ}\text{C}$  for 30 h under  $\text{N}_2$ -environment. The resultant styrene-acrylamide co-polymer immobilized silica stationary phase was subjected to washing with chloroform, toluene and acetone, filtered and dried at 60  $^{\circ}\text{C}$ . The overall procedure of synthesis is shown in Figure 1.

**Styrene-acrylamide Immobilized Open Tubular CEC Capillary (50  $\mu\text{m}$  ID) Column.** Pretreatment of fused silica capillary was carried out as follows. The capillary was filled with 1 M NaOH and kept for 24 h at room temperature, then at 55  $^{\circ}\text{C}$  overnight, washed with water and 0.1 M HCl followed by water to pH 7, acetone for 1 hr, and dried under nitrogen at 120  $^{\circ}\text{C}$  for 2-3 h to expose silanol functionalities. The capillary was then treated with a solution of 25 mg of 4-CPI in 2.5 mL toluene (anhydrous) and 20 mg of DBTDC (catalyst) at 80  $^{\circ}\text{C}$  for 40 h. The capillary was flushed with toluene for 5 h, and acetone overnight, and dried with a flow of  $\text{N}_2$  for 30 min. The capillary was then treated with 100 mg sodium diethyl dithiocarbamate dissolved in 3 mL anhydrous THF at 55  $^{\circ}\text{C}$  for 15 h, followed by washing with THF for 6 h, acetone for 2 h, and  $\text{N}_2$ -dried for 30 min. In-situ RAFT co-polymerization was carried out using a monomer mixture of 0.6 mL styrene, 50 mg acrylamide, 2 mL anhydrous toluene and 1 mL chloroform at 100  $^{\circ}\text{C}$  for 30 h. The reaction mixture was degassed by sonication followed by  $\text{N}_2$ -purging for 10 min, and was kept flowing through the capillary with a syringe through a 0.2- $\mu\text{m}$  Whatman (Maidstone, England) syringe filter. The column was flushed with chloroform at room temperature, toluene at 50  $^{\circ}\text{C}$  followed by acetone to remove the residual reaction mixture and free oligomers. Prior to CEC-operation the capillary was flushed with mobile phase to get the stationary phase in equilibrium with mobile phase. A detection window was created at a distance of 8.4 cm from the outlet end by burning the polyimide coating. The total length and effective length of the open tubular column were 36.4 and 28.0 cm, respectively. The capillary column was finally installed in the instrument for analysis.

**Derivatization of Saccharides.** ABEE tagged maltotriose and Dextran1500 were obtained *via* reductive amination by reacting an aliquot of 100  $\mu\text{L}$  (10 mg saccharide/1 mL water) with 80  $\mu\text{L}$  acetic acid, 80  $\mu\text{L}$  of 1.4 M  $\text{NaBH}_3\text{CN}$ , and 400  $\mu\text{L}$  of 0.6 M ABEE in methanol while D-glucose



**Figure 1.** Reaction Schemes for synthesis of halogen-ligand bound silica (a), Initiator attached silica (b), and poly (styrene-acrylamide) bound silica (c).

was aminated in the absence of  $\text{NaBH}_3\text{CN}$ . The reaction mixture was treated at  $80\text{ }^\circ\text{C}$  for 2 h, cooled to room temperature and extracted with chloroform many times to remove the non-reacted ABEE.<sup>36,37</sup> The Derivatized saccharides were filtered through a  $0.2\text{ }\mu\text{m}$  syringe filter and the stock solutions were stored below  $4\text{ }^\circ\text{C}$ .

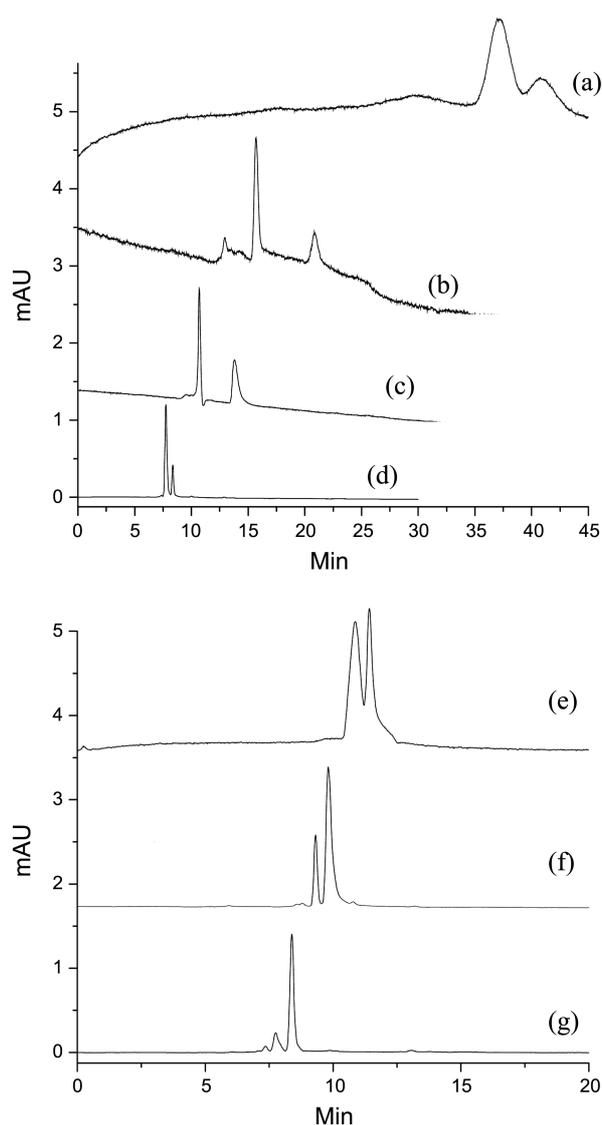
#### Instrumentation.

**HPLC:** A 10AD pump (Schimadzu, Japan), a Valco (Houston, TX, USA) CI4W.05 injector with 50 nL injection loop, a membrane degasser (Schimadzu DGU-14A), a UV-VIS capillary window detector (Jasco UV-2075), a homemade 1.0 mm I.D glass lined micro-column were assembled to construct the  $\mu\text{LC}$  system. The software Multichro 2000 from Youlingisul (Korea) was used for acquisition and processing of chromatographic data. Packing of micro columns was carried out according to the procedure published<sup>33,38-40</sup> with some modification. The column was assembled as reported<sup>33</sup> by placing  $0.5\text{ }\mu\text{m}$  commercial screen frit in the 1/8" outlet of a reducing union (1/8"-1/16"), and a glass lined stainless steel column (1 mm, ID and 30 cm, long) was fitted to the outlet, and the tubing was connected to the packer. The slurry was prepared by suspending the 300 mg stationary phase in methanol by sonication for 20 min and the supernatant was removed. This procedure was repeated thrice. The final 3.3 mL slurry (methanol + stationary phase) was fed into reservoir. A pressure of 16,000 psi for 5 min, 11,000 psi for 10 min and 8,000 psi for 30 min was applied in sequence with mechanical vibration. After packing, the inlet union was installed with a frit, and the 1/16" outlet of the outlet union was installed with a capillary ( $50\text{ }\mu\text{m}$  I.D.,  $365\text{ }\mu\text{m}$  O.D.) with an aid of PEEK sleeve, and was connected to the capillary window detector. A piece of short Teflon tubing was used to connect the two capillary ends. D-glucose was derivatized according to the procedure discussed in the above section and stored at  $4\text{ }^\circ\text{C}$ . The sample was further diluted with corresponding mobile phase for injection.

**CEC:** CEC experiments were performed on an Agilent (Waldbronn, Germany) HP<sup>3D</sup>CE system with a diode array detector and the Chemstation data processing software. The stock buffer solutions (30 mM sodium acetate) were prepared in distilled water and kept in a refrigerator. Later, the pH of acetate buffer was controlled by mixing pure acid (acetic acid) to the corresponding stock solution to get the desired pH followed by acetonitrile addition to get the final mobile phase. Freshly derivatized D-glucose, maltotriose and dextran samples were made each time when a new mobile phase was used. All the samples and eluents were filtered through a  $0.2\text{-}\mu\text{m}$  Whatman (Maidstone, England) syringe filter. The column was equilibrated with the running mobile phase for at least 30 min. Samples were injected by positive potential mode (7-10 kV) with injection times 3-8 s. The detection wavelength, capillary temperature and applied potential were set to 214 nm,  $25\text{ }^\circ\text{C}$  and 30 kV, respectively.

### Results and Discussion

**HPLC.** Examination of chromatographic performance in



**Figure 2.** Chromatograms of D-glucose in various mobile phases. 0.1% TFA was included in a, b, c, and d while 10 mM ammonium acetate buffer at pH 6.5 was incorporated in e, f, and f.  $\beta$ -D-glucose eluted first and  $\alpha$ -D-glucose later. ACN/aqueous buffer volume ratio: (a) 95/05, (b) 85/15, (c) 75/25, (d) 60/40 (e) 80/20, (f) 75/25 (g) 60/40. Detection wavelength was 214 nm and flow rate was  $15\text{ }\mu\text{L}/\text{min}$ . For c,  $N_\beta = 22,400$ ,  $N_\alpha = 7,200$ ,  $R = 6.0$ .

separation of D-glucose anomers was carried out using two sets of mobile phases, one containing TFA, and the other containing ammonium acetate. Both sets of mobile phases showed that water content in the mobile phase has a prominent effect on the retention times of D-glucose anomers. Retention time is decreased with increase of water content due to hydrogen-bonding of D-glucose with water as shown in Figure 2. The mobile phase of 80/20 (v/v) acetonitrile/water with 0.1% TFA, was found to be the best choice ( $R=6.0$ ) (Fig. 2). Migration pattern of D-glucose anomers studied in ammonium acetate buffer also showed the same trend with water content variation but the separation-resolutions were lower than those of TFA containing mobile phases and the separation efficiencies of both mobile phases

were comparable.

The pH-dependent mutarotation of 1-thioaldopyranoses in aqueous media has been reported.<sup>41</sup> 1-Thio-D-mannopyranose and 1-thio-D-altropyranose showed very strong pH dependence where the anomeric equilibrium ratios changed dramatically from a preference for the  $\beta$ -anomer at lower pH to the  $\alpha$ -anomer at higher pH.<sup>41</sup>

Thus it is believed that  $\beta$ -D-glucose was eluted first followed by  $\alpha$ -D-glucose as shown in Figure 2 since  $\beta$ -D-glucose should be the major anomer in lower pH (the eluent had 0.1% TFA). However,  $\alpha$ -D-glucose was the anomer of higher abundance at pH 6.5 as shown in F and G of Figure 2 and at pH 6.7 in Figure 3. It seems that the relative abundances of anomers are governed by not only pH but also eluent composition according to Figure 2 and Figure 3.

Furthermore, it is suspected that the elution order of anomers may be varied depending upon pH in CEC as demonstrated in Figure 4 and Figure 5. On slight increase of pH from the 6 level (Fig. 4) to the 7 level (Fig. 5), the elution pattern was inverted with serious degradation of separation resolution. Very sophisticated charge distribution of the hydroxyl and amine groups of aminated D-glucose anomers and their different interactions with the stationary phase and with the electrostatic fields owing to the different stereochemistry might cause inversion of elution order of anomers, which is not clear at present. This issue requires more exhaustive investigation in the future.

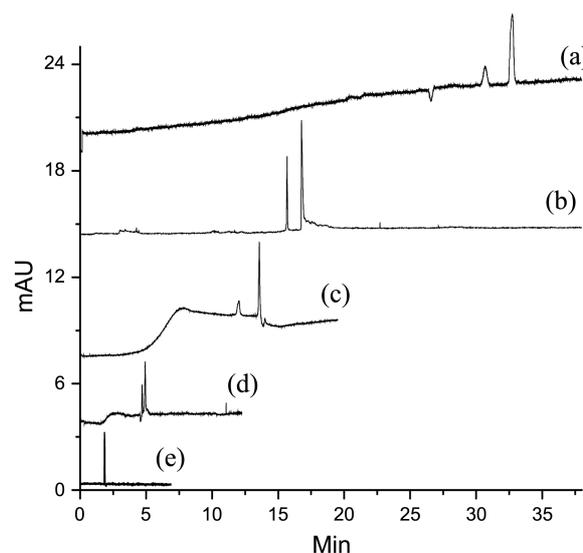
#### Capillary Electrochromatography.

##### Effect of Eluent Water Content on Anomeric Separation.

The water content in the mobile phase critically affects migration times of D-glucose anomers. Higher water content results in shorter retention times probably owing to strong increased hydrogen bond interaction between the mobile phase and the solute as well as influence of water disturbing the hydrogen bonding between the acrylamide containing stationary phase and D-glucose. The role of hydrogen-bonding is vital in organic rich mobile phases.<sup>42</sup> 85/15 acetonitrile/30 mM, sodium acetate pH 6.7 gave the best separation resolution (Fig. 3(b), Fig. 4(c), Table 1). High separation efficiency (Tables 1, 2) may be partially due to low viscosity of the eluent of high acetonitrile content.<sup>34</sup> The marked decrease in retention times (Fig. 4) and poor anomer selectivities at higher pH are due to high EOF, and this effect is more pronounced in pH range above 8 (data not shown). Higher water contents over 15% resulted in degraded anomeric separation and water contents lower than 15% also gave lower resolution and lower separation efficiency.

In an eluent of high water content, retention times of both anomers are reduced to cause peak overlap while in an eluent of low water content, retention times are increased to cause band broadening as shown in Figure 3. Thus there should be an optimum water composition (15%) to give the best chromatographic resolution.

**Effect of pH on Anomeric Separation.** The increase in EOF will reduce the retention times of anomers, and the increase in EOF is, in turn, caused by increase of pH<sup>43,44</sup> owing to increased ionization of the unreacted silanol groups



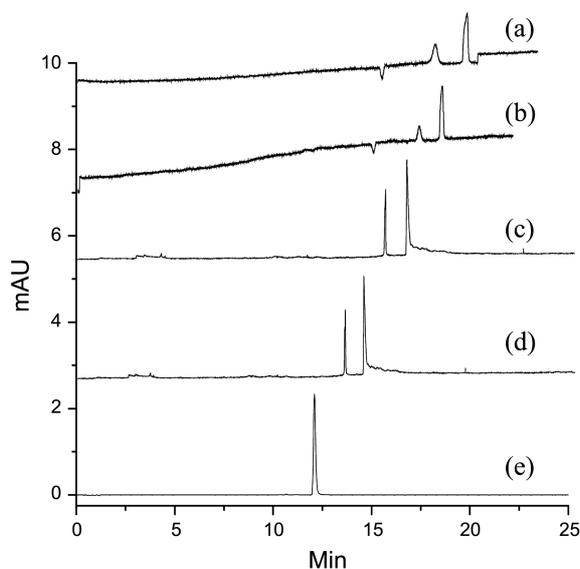
**Figure 3.** Effect of eluent water content on anomeric separation of D-glucose in acidic acetate buffer (30 mM sodium acetate buffer at pH 6.7).  $\beta$ -D-glucose eluted first and  $\alpha$ -D-glucose later. (a) 5% (b) 15% (c) 20% (d) 30%. Sample injection: 8 kV 5 s. Applied potential was 30 kV. The resolution “R” values are 5.0, 6.5, 6.0 and 1.7 for a, b, c, and d respectively. The retention time of EOF marker (acetone) in d eluent is 2.2 min (e).

**Table 1.** Number of theoretical plates and resolutions of aminated D-glucose anomers at different compositions of CEC eluent. pH was set at the optimized value 6.7

ACN/30 mM sodium acetate composition (v/v %)	$N_1/m$	$N_2/m$	Resolution (R)
95/5	185,500	206,800	5.0
85/15	248,000	301,200	6.5
80/20	172,000	188,400	6.0

of the stationary phase. At lower pHs, the fraction of ionized silanol groups will be reduced to decrease the EOF and will give long retention times while the OH groups of the saccharides may be somewhat positively charged. Positively charged analytes may undergo silanophilic interaction with unreacted silanol groups, a phenomenon that may lead to band broadening and loss of resolution between the separated analytes.<sup>45</sup> As pH was increased, the density of negative charge of the silanol groups was increased giving rise to high EOF and hence reduced retention times (Fig. 4). Even a small change in pH caused considerable variations in migration times (Fig. 4). As the pH moved to further higher pH (alkaline pH) the separation performance was swiftly degraded owing to too fast EOF (Fig. 5). The two anomers were congested at pH higher than 8 and the capability of anomeric separation was lost (data not shown). Enhancement of EOF with increase of pH is also evident based on retention times of acetone: 12 min at pH 6.9 (Fig. 4), 6.5 min at pH 7.8 (Fig. 5), and 3.9 min at pH 8.8 (data not shown).

**Separation of Other Saccharides.** The CEC stationary phase of current study proved effective for the separation of



**Figure 4.** Migration of D-glucose anomers at different pHs. (a) pH 5.8, (b) pH 6.3 (c) pH 6.7 and (d) pH 6.9. Mobile phase composition: 85/15 (v/v) acetonitrile/30 mM sodium acetate. Sample injection: 8 kV 5 s. Applied potential: 30 kV.  $\beta$ -D-glucose eluted first and  $\alpha$ -D-glucose later. The resolution “R” values are 5.1, 5.2, 6.5 and 5.9 for a, b, c and d respectively. Migration time of acetone (EOF-marker) in d eluent was 12.3 min (e).

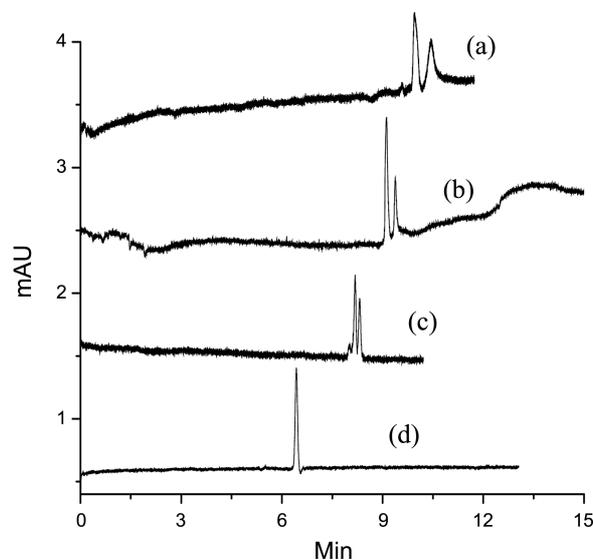
**Table 2.** Number of theoretical plates and resolutions of aminated D-glucose anomers at different pHs of CEC eluent

pH of mobile phase 85/15 ACN/30 mM sodium acetate	$N_1/m$	$N_2/m$	Resolution (R)
pH = 5.8	191,800	209,200	5.1
pH = 6.3	182,600	188,600	5.2
pH = 6.7	248,000	301,200	6.5
pH = 6.9	211,700	211,000	5.9

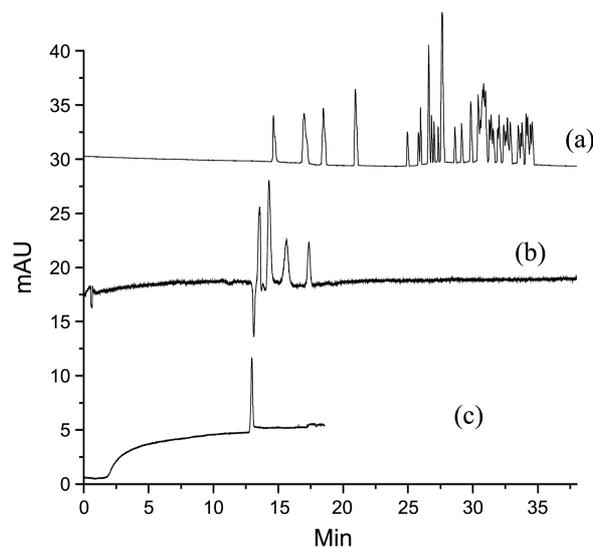
other saccharides such as maltotriose and Dextran 1500 consisting of many sugar molecules of different molecular weights. The numbers of theoretical plates for a few peaks of maltotriose and dextran were found close to one million.

### Conclusion

An extensive study of anomeric separation of aminated D-glucose has been carried out with styrene-acrylamide copolymer immobilized HPLC and CEC stationary phases at various pHs and mobile phase compositions. The HPLC and CEC study showed that chromatographic characteristics like resolution, selectivity and separation efficiency of sugars are highly dependent on pH of the mobile phase. The separation resolution and efficiency of aminated D-glucose anomers in the optimized elution conditions were much better than those of previous studies in the literature. In addition, The CEC stationary phase gave efficient separation of isomeric saccharides for maltotriose and Dextran 1500. Realizing good separation performances for a saccharide sample com-



**Figure 5.** Comparison of migration of D-glucose anomers at different alkaline pHs: (a) pH 7.2, (b) pH 7.5 (c) pH 7.8 Mobile phase: 85/15 (v/v) acetonitrile/30 mM sodium acetate. Sample injection: 5 kV 3 s. Applied potential: 30 kV. First eluting peak is suspected to be  $\alpha$  isomer. The resolution “R” values are 2.6, 2.9 and 1.8 for a, b, and c, respectively. Migration time (d) of acetone (EOF-marker) was 6.5 min in c eluent.



**Figure 6.** Electropherograms of Dextran 1500 (average M.W = 1500) (a), maltotriose (b) and acetone (c). Mobile phase: 85/15 (v/v) acetonitrile/30 mM sodium acetate pH 6.7. Sample injection: 5 kV 3 s. Applied potential: 30 kV.

posed of many stereochemical isomers requires not only high separation efficiency but also improved capability of anomeric separation. This study demonstrates the possibility of such realization by adopting the strategy of incorporating both planar  $sp^2$  and hydrogen bond characters in the stationary phase in the format of copolymer. Further study for stationary phases of even better performances is under away.

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