# Structural Analysis of Chromophore-Labeled Disaccharides by Capillary Electrophoresis Tandem Mass Spectrometry Using Ion Trap Mass Spectrometry

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Disaccharides tagged with *p*-aminobenzoic acid (ABA) were separated by capillary electrophoresis (CE) and analyzed on-line with negative ion electrospray ionization tandem mass spectrometry (ESI/MS/MS). The formation of glycosylamine instead of reductive amination was selected as the derivatization reaction. In negative ion ESI, the glycosylamine approach provides more information on linkage and anomeric configuration than reductive amination. In CE analysis of ABA-labeled disaccharides, $\alpha$-cyclodextrin (CD) was found to play a crucial role in the separation of linkage isomers. Although ammonium acetate/$\alpha$-CD provided the best resolution of linkage isomers, the borate buffer was superior to $\alpha$-CD in the separation of disaccharides with the same linkage but different anomeric configuration and/or monosaccharide composition. Both $\alpha$-CD and borate suppressed the ion signal in ESI, and operational conditions were successfully obtained using 10 mM $\alpha$-CD or 10 mM borate. (J Am Soc Mass Spectrom 2000, 11, 292–300)   © 2000 American Society for Mass Spectrometry

The growing interest in biological functions of carbohydrates has prompted the need of high resolution separation techniques for the structural analysis of complex carbohydrates. In recent years, capillary electrophoresis (CE) has emerged as an alternative technique to conventional analytical techniques (gas chromatography, thin-layer chromatography, and high-performance liquid chromatography) for carbohydrate analysis. The separation of carbohydrates by CE, especially capillary zone electrophoresis (CZE) has often required the in situ conversion of these compounds into charged species. Charged species like borate [1–11] or metal ions [12] have been used as in situ complexation reagents in the separation of carbohydrates by CZE. Furthermore, since carbohydrates are weakly acidic compounds, they can also be separated as anions under strong alkaline conditions [13–15].

Another difficulty encountered in the analysis of sugars is the lack of their inherent chromophore in the ultraviolet-visible region. To visualize carbohydrates for UV or fluorescence detection, introducing a chromophore into the molecules has received considerable attention [2–11, 16, 17]. In recent years, derivatization of carbohydrate has been used not only to enable sensitive detection by UV or fluorescence but also to improve the resolution of multicomponents samples in CE analysis. Efficiencies in excess of one million plates per meter have been achieved by labeling carbohydrates with 8-aminonaphthalene-1,3,6-sulfonate [8].

Reductive amination is the most popular labeling procedure and has been used in the analysis of carbohydrates by CE with UV detection [18, 19]. This procedure reduces the reducing sugar and produces an open ring product. In the labeling of oligosaccharides, in addition to the very popular reductive amination procedure, glycosylamine approach has been reported [20–22]. Unlike reductive amination, the reducing sugar maintains its closed ring structure as in glycosylamine. Based on our previous study [20, 21], closed ring derivatives (glycosylamines) provided improved structural information on linkage and anomeric configuration information compared to open ring products under negative ion FAB/CID or ESI/in source CID.

On-line coupling of CE and mass spectrometry (CE/MS) is a very promising technique for carbohydrate analysis. To obtain more structural information, disaccharides were derivatized with the glycosylamine approach and studied by CE/MS/MS. The utilities and limitations of this approach are discussed.

## Experimental

### Chemicals

Glc$\alpha$1–2Glc, Glc$\alpha$1–3Glc, Glc$\beta$1–3Glc, Man$\alpha$1–3Man, Glc$\alpha$–4Glc, Glc$\beta$1–4Glc, Gal$\beta$1–4Glc, Gal$\beta$–4Man,

Glc$\alpha$1–6Glc, Glc$\beta$1–6Glc, Gal$\alpha$1–6Glc, Gal$\beta$1–6Gal were obtained from Sigma Chemical (St Louis, MO). Sodium tetraborate anhydrous, ammonium acetate, and ammonium hydroxide were purchased from Janssen (Belgium). *p*-Aminobenzoic acid ethyl ester (ABEE), *p*-aminobenzoic acid (ABA), and *m*-aminobenzoic sulfonic acid (ABS) were purchased from Aldrich Chemical (St Louis, MO). Deionized (18 Mohms) water (Milli-Q water system, Millipore, Bedford, MA) was used in the preparation of the samples and buffer solution. HPLC grade 2-propanol was obtained from J. T. Baker (Phillipsburg, NJ).

### Preparation of Chromophore-Labeled Oligosaccharides

The procedure of Reinhold et al. [22] was adopted for the preparation of glycosylamine derivatives. For ABA glycosylamines, the sugars (up to 1 mg) were dissolved in 20 $\mu$L water and 80 $\mu$L reagent solution (prepared by dissolving 1 g of ABA in 0.5 mL methanol at 50 °C) and reacted at 50 °C for 6 h. The solution was washed with 2 mL of ethyl acetate to remove the excess ABA reagent before the CE separation.

### Apparatus

The CZE system was made in-house using a CZE 1000R (Spellman, Plainview, NY) high voltage power supply. CE columns were fused-silica capillary (Polymicro Technologies, Phoenix, AZ) 50 $\mu$m i.d., 375 $\mu$m o.d., and 92 cm length (84 cm to detector). A small area of the polyimide coating was burned off to form a window for UV detection. The detection (UV-C Rainin, Emeryville, CA) wavelength was set at 254 nm. Before use, the capillary column was washed with 1 M sodium hydroxide followed by water and running buffer. The capillary was then equilibrated with the running buffer for 20 min. To avoid analyte adsorption, the capillary column needs to be rinsed with 0.1 N sodium hydroxide between analysis.

Mass spectra were acquired using a LCQ quadrupole ion trap mass spectrometer equipped with an electrospray ionization source (Finnigan MAT; San Jose, CA). The spray needle voltage was set to −4.5 kV and the spray was stabilized with a nitrogen sheath gas operating at 30 lb/in.$^2$. The stainless-steel inlet capillary was heated to 200 °C. Helium was admitted to the vacuum system to a pressure of 1 mtorr, and the instrument was operated at a background pressure of $2 \times 10^{-5}$ torr. In MS/MS experiments, a mass range of *m/z* 160–480 was scanned. The automatic gain control was used to set the maximum injection time at 800 ms. In no case did a CE peak produce sufficient ions to trigger the automatic gain control of the ion trap.
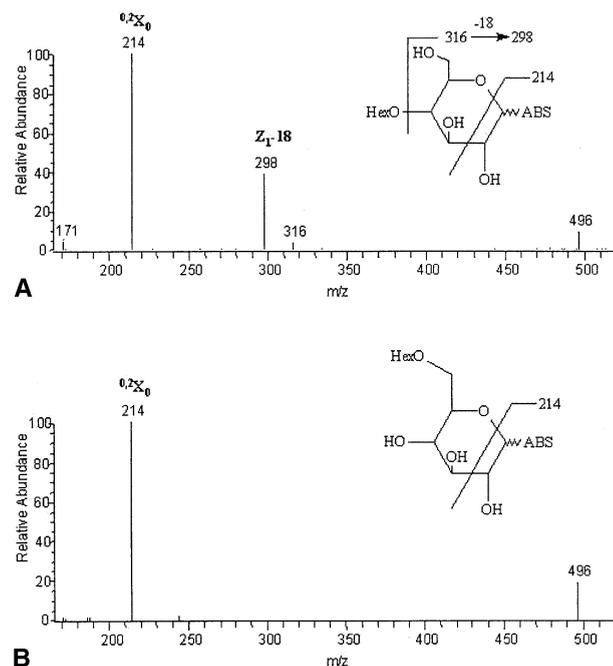


**Figure 1.** Negative ion ESI product ion mass spectra of ABS-labeled (**A**) Glc$\beta$1–4Glc and (**B**) Glc$\beta$1–6Glc. The major product ions were assigned based on the nomenclature proposed by Domon and Costello [26].

## Results and Discussion

### Choice of Chromophore

UV or fluorescence sensitivity, structural information from product ion spectra, and CE separation efficiency were the important factors in the selection of suitable chromophore in our CE/MS/MS study. Mainly for the CE separation, a charged compound in aqueous solution, *m*-aminobenzoic sulfonic acid (ABS), was selected as the chromophore. Disaccharides were labeled with the glycosylamine approach because such a derivative (glycosylamine) provided much more linkage information than the corresponding reductive amination derivative [20, 21].

According to our previous study [21], the ESI mass spectra of the ABEE-tagged disaccharides of different linkages are similar in the positive ion mode but quite different in the negative ion mode. Therefore, the mass analysis was performed in the negative ion mode. The negative ion ESI/MS/MS spectra of ABS-labeled Glc$\beta$1–4Glc and Glc$\beta$1–6Glc are shown in Figure 1a,b, respectively. The differences between these two spectra are the absence of the peaks at *m/z* 316 and 298 in the 1–6 linked disaccharide. The results indicate that a 1–4 linkage can be differentiated from a 1–6 linkage on the basis of the presence or absence of the *m/z* 316 and 298 ions, but the lack of a characteristic ion in a 1–6 linked disaccharide might make the assignment ambiguous, especially if these two sugars are not separated by CE. An alternative derivatization reagent, *p*-aminobenzoic acid (ABA) was then evaluated. In comparison with
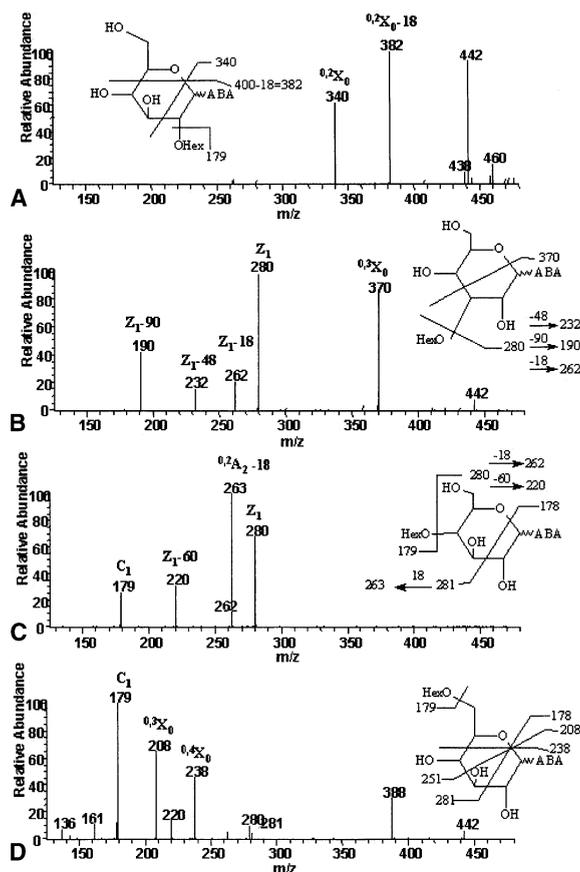
**Figure 2.** Negative ion ESI product ion mass spectra of ABA-labeled (**A**) Glc$\beta$1–2Glc, (**B**) Glc$\beta$1–3Glc, (**C**) Glc$\beta$1–4Glc, and (**D**) Glc$\beta$1–6Glc. The major product ions were assigned based on the nomenclature proposed by Domon and Costello [26].

ABS derivatives, ABA-labeled disaccharides yielded many characteristic fragment ions under negative ion ESI/MS/MS (Figure 2). These fragments are presumed to arise from cross-ring cleavage ions on the reducing end monosaccharide and were critical for the differentiation of different glycosidic linkages. The characteristic fragment ions for all ABA-labeled disaccharides investigated are listed in Table 1. In general, the product

ion spectra are similar to ABEE derivatives in ESI/in source CID [21] and ABBE derivatives in FAB/CID [20]. A notable difference was the observation of a peak at $m/z$ 388, one of the characteristic ions in ABA-tagged 1–6 linked disaccharide. The corresponding fragment ion was not detected in ABEE derivatives [21] or ABBE derivatives [20].

Determination of the anomeric configuration by mass spectrometry directly is limited mainly because stereoisomers often give very similar mass spectra. Traeger et al. [23] and our group [21] differentiated the anomeric configuration by the relative abundance of selected ions in negative ion ESI mass spectra. In this study, similar to that of the ABEE derivative [21], the relative abundance of some fragment ions was found to be sensitive to the anomeric configurations. For example, among the three major fragment ions, the intensities of the $m/z$ 370 and 190 ion varied synchronously relative to the intensity of the $m/z$ 280 ion when the anomeric configuration of the 1–3 linked disaccharide was changed. In replicate injections obtained over six consecutive days, the ion ratios of $m/z$ 280/(370 + 190) $\pm$ 2$\sigma$ for Glc$\beta$1–3Glc–ABA and Glc$\alpha$1–3Glc–ABA were 1.01 $\pm$ 0.21 and 0.47 $\pm$ 0.19, respectively. The significant difference between these two values provides a reliable method for the detection of the anomeric configuration of 1–3 linked disaccharides. Although anomeric configurations of 1–4 and 1–6 linked disaccharides could be determined by the ion ratio as for 1–3 linked disaccharides, examination of the product ions of 1–4 and 1–6 linked disaccharides with different anomeric configuration (Table 1) showed that the peak at $m/z$ 221 was detected in both 1–4 and 1–6 linked disaccharides of $\alpha$ configuration but not in $\beta$ configuration. In ABEE derivatives, the anomeric configuration of 1–4 and 1–6 linked disaccharides was determined by the relative abundance of certain ions [21]. The determination of the anomeric configuration by the presence or absence of the peak at $m/z$ 221, as proposed in this study, is easier than by the relative abundance of selected fragment ions as suggested for the ABEE derivatives [21].

**Table 1.** Specific fragment ions for ABA-labeled disaccharides ($m/z$)

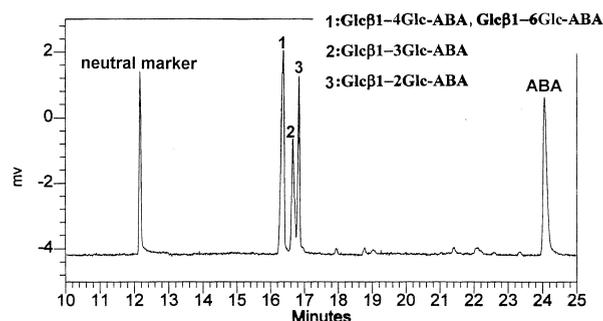| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Glc$\beta$1–2Glc | | | | | | 340 | 382 |
| Glc$\alpha$1–3Glc | 190 | | | 232 | | 370 | |
| Glc$\beta$1–3Glc | 190 | | | 232 | | 370 | |
| Man$\alpha$1–3Man | 190 | | | 232 | | 370 | |
| Glc$\alpha$1–4Glc | | | 221 | | 263 | | |
| Glc$\beta$1–4Glc | | | | | 263 | | |
| Gal$\beta$1–4Glc | | | | | 263 | | |
| Gal$\beta$1–4Man | | | | | 263 | | |
| Glc$\alpha$1–6Glc | | 208 | 221 | 238 | | | 388 |
| Glc$\beta$1–6Glc | | 208 | | 238 | | | 388 |
| Gal$\alpha$1–6Glc | | 208 | 221 | 238 | | | 388 |
| Gal$\beta$1–6Gal | | 208 | | 238 | | | 388 |

**Figure 3.** CE/UV electropherogram of ABA-tagged disaccharides of different linkage. Condition: 20 kV, UV detection at 254 nm. Running buffer: 10 mM borate, pH 10.0. Neutral marker: mesityl oxide.

## CE Analysis of ABA-Labeled Disaccharides of Different Linkages

The most commonly used background electrolyte in the electrophoretic separation of carbohydrates is borate buffer [10, 11]. Under alkaline conditions, borate ions form complexes with vicinal hydroxyl groups, thus imparting a negative charge to carbohydrates and making them amenable to migration under an electric field. The electropherogram of ABA-labeled disaccharides obtained by using 10 mM pH 10.0 borate buffer is shown in Figure 3. The mobility of ABA-labeled disaccharides depends on the stability of the complexes formed with borate. The ABA-labeled Glc$\beta$1–2Glc formed the most stable complex with borate and thus had the highest mobility (the longest retention time) among the four disaccharides. The ABA-labeled Glc$\beta$1–4Glc and Glc$\beta$1–6Glc coeluted and formed the least stable complexes with borate. Borate concentration up to 50 mM has been studied to improve the resolution of Glc$\beta$1–4Glc–ABA and Glc$\beta$1–6Glc–ABA, however, no improvement was observed.

Ammonium acetate, one of the widely used buffers for CE-ESI/MS analysis, was then studied to enhance separation of linkage isomers. The migration times of the four ABA-labeled glucose disaccharides were identical when using 50 mM ammonium acetate. This observation can be accounted for by the fact that these ABA-labeled isomers not only have identical molecular masses and very similar pKa values, but also lack of selective interaction between the ammonium acetate and the isomers of ABA-labeled glucose disaccharides. Thus, it is found that the separation of ABA-labeled disaccharides with different linkage can not be achieved by varying the pH of buffer over a limited range (pH 5.5 to 6.5).

Cyclodextrins (CDs) are frequently employed as a running buffer modifier in CE. Separation of ABA-labeled linkage isomers with addition of $\alpha$-CD, $\beta$-CD, or $\gamma$-CD into ammonium acetate was studied. It was found that the resolving power was greatly enhanced with the addition of $\alpha$-CD in ammonium acetate. The hydrophobic interior and cavity size of $\alpha$-CD might facilitate the formation of inclusion complexes with the ABA derivatives and improve the separation. The electropherograms obtained by using 50 mM ammonium acetate containing 10 mM $\alpha$-CD at different pH are shown in Figure 4. The best electropherogram was obtained using a pH 5.5 buffer (Figure 4b). The ABA-labeled Glc$\beta$1–2Glc, Glc$\beta$1–4Glc, Glc$\beta$1–3Glc, and Glc$\beta$1–6Glc eluted past the detector in the order of increasing electrophoretic mobility and, hence, in the order of decreasing interaction with $\alpha$-CD. The concentration of $\alpha$-CD on separation efficiency was also studied. No enhancement of the resolving power was observed with concentrations of $\alpha$-CD above 10 mM. In comparison with borate buffer, although Glc$\beta$1–3Glc–ABA and Glc$\beta$1–4Glc–ABA were still not baseline resolved, the overall separation power of the ammonium acetate/$\alpha$-CD was superior to borate electrolyte for the analysis of linkage isomers.

Despite the fact that the in-source CID spectra provide similar information as MS/MS product ion spectra, MS/MS has the advantage of reducing background ions and resulting in a total ion electropherogram (TIE) of higher signal/noise ratio. The on-line CE-ESI/MS/MS analysis of a mixture of ABA-tagged disaccharides of different linkages is given in Figure 5. Ions at $m/z$ 340, 263, 190, and 208, which represented 1–2, 1–4, 1–3, and 1–6-linked disaccharides, were selected for the reconstructed ion electropherograms. The CE peak width at half height measured from the electropherogram is approximately 13 s. Although 1–3 and 1–4 linked disaccharides had very similar migration times, the appearance of characteristic product ions provided significant differentiation between these two isomers.

It is well known that the ionization efficiency of the electrospray is deteriorated by the presence of other species in the sprayed solution, such as electrolyte or other matrix molecules [24]. A $\alpha$-CD concentration of 10 mM lowered the ESIMS signal of ABA-labeled disaccharides by a factor of approximately 2. However, the use of $\alpha$-CD enabled the separation of ABA-labeled disaccharides of different linkage, otherwise impossible with only ammonium acetate buffer.

## CE Analysis of ABA-Disaccharides of Different Anomeric Configuration and Monosaccharide Composition

Although ammonium acetate/$\alpha$-CD buffer was superior to borate buffer in separating disaccharides of different linkages, carbohydrates of the same linkage but different anomeric configuration or monosaccharide composition could not be resolved with the ammonium acetate/$\alpha$-CD buffer. Borate has been reported to provided enhanced separation of these isomers [10, 11]. The CE/UV electropherograms of mixtures of 1–6, 1–4, and 1–3 linked disaccharides using 10 mM pH 10.0 borate buffer are shown in Figure 6a,b,c, respectively. As can be seen, most disaccharides could be separated
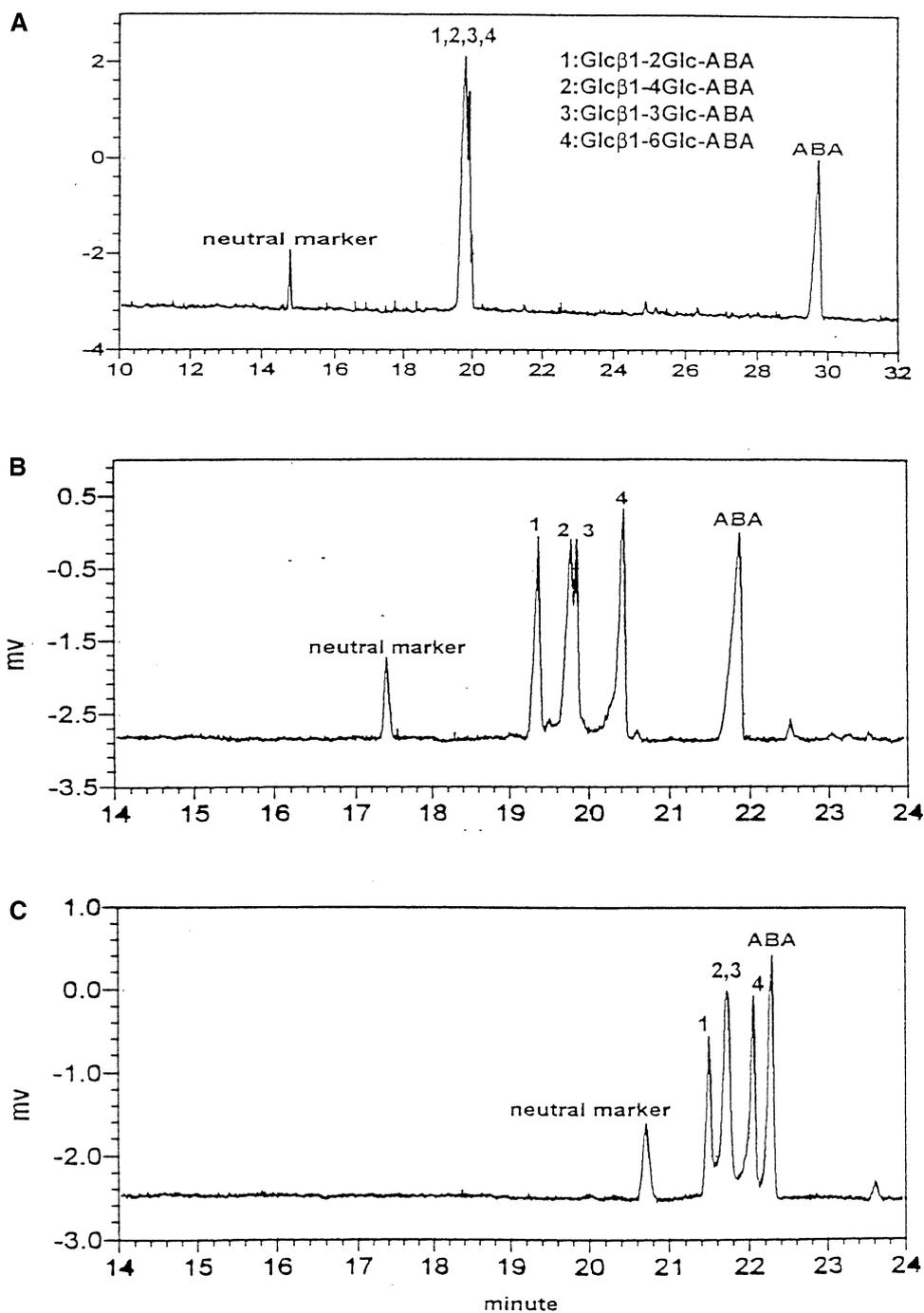
**Figure 4.** The CE/UV electropherograms of ABA-tagged Glc$\beta$1–2Glc, Glc$\beta$1–3Glc, Glc$\beta$1–4Glc, and Glc$\beta$1–6Glc. Condition: 20 kV, UV detection at 254 nm. Running buffer: 50 mM ammonium acetate containing 10 mM $\alpha$-CD at pH: (**A**) 6.5, (**B**) 5.5, and (**C**) 4.5.

except for Glc$\alpha$1–6Glc–ABA and Glc$\beta$1–6Glc–ABA. These two anomeric isomers could not be separated even though the concentration of borate was increased to 50 mM. High concentration of borate has been reported to be unsuitable for ESI analysis [25], consequently, a buffer with 10 mM borate at pH 10.0 was used for the analysis of ABA-labeled disaccharides of

different anomeric configuration and/or monosaccharide composition by CE-ESI/MS/MS.

The stability of sugar–borate complex depends strongly on the number of vicinal hydroxyl groups. The 1–4 linked disaccharides, Glc$\alpha$1–4Glc–ABA and Glc$\beta$1–4Glc–ABA formed the least stable complexes with borate. In comparison, Gal$\beta$1–4Glc–ABA has a higher
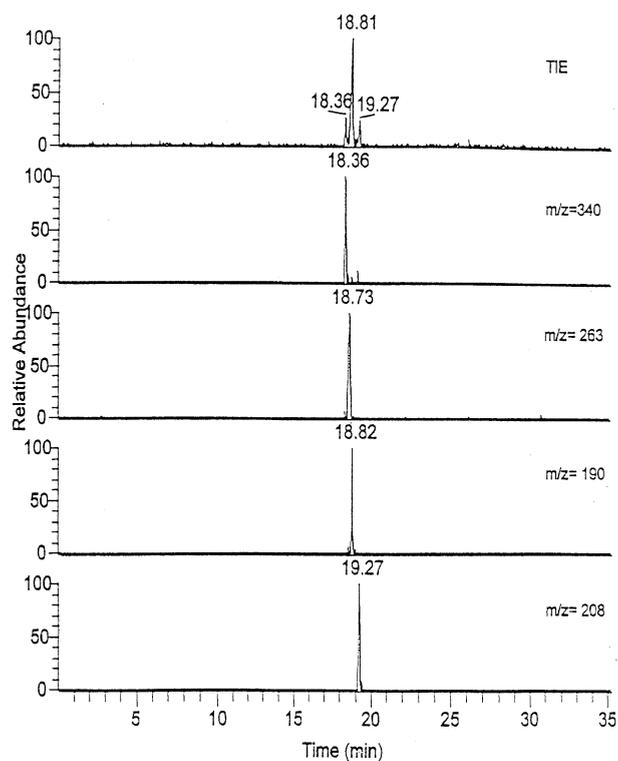
**Figure 5.** The CE/MS/MS mass electropherogram of ABA-tagged Glcβ1–2Glc, Glcβ1–3Glc, Glcβ1–4Glc, and Glcβ1–6Glc mixture. Condition: 20 kV, running buffer: 50 mM ammonium acetate containing 10 mM α-CD at pH 5.5. Make up solution (sheath liquid) was water-2-propanol (20:80) containing 0.5% ammonia.

electrophoretic mobility due to the galactose moiety, which enables complexation of the *cis*-oriented hydroxyl groups at C-3 and C-4. The Galβ1–4Man–ABA has the highest mobility because both the terminal galactose and the reducing end mannose have *cis*-oriented hydroxyl groups. This rational can be also used to explain the elution order of 1–3 and 1–6 linked disaccharides.

The CE-ESI/MS/MS electropherograms of 1–3, 1–4, and 1–6-linked ABA labeled disaccharides using 10 mM borate at pH 10.0 are shown in Figures 7, 8, and 9, respectively. The TIE and mass electropherograms of 1–3 linked ABA-labeled disaccharides are shown in Figure 7, the three ions at *m/z* 370, 232, 190 are characteristic of a 1–3 linkage. The mass electropherograms and TIE of four 1–4 linked disaccharides are shown in Figure 8. The characteristic ion of a 1–4 linked disaccharide is the ion at *m/z* 263. Because the anomeric configuration of a 1–4 linked disaccharide could be determined according to the presence or absence of the ion at *m/z* 221, the second peak (15.41 min) was assigned as the α form of 1–4 linked disaccharide but the others were all attributed to the β form. Figure 9 shows the mass electropherograms using the ions at *m/z* 388, 238, 208, and 221. The first three are specific product ions of a 1–6 linkage. The last one (*m/z* 221) is a specific product ion of the α form of 1–6 and 1–4-linked ABA-labeled
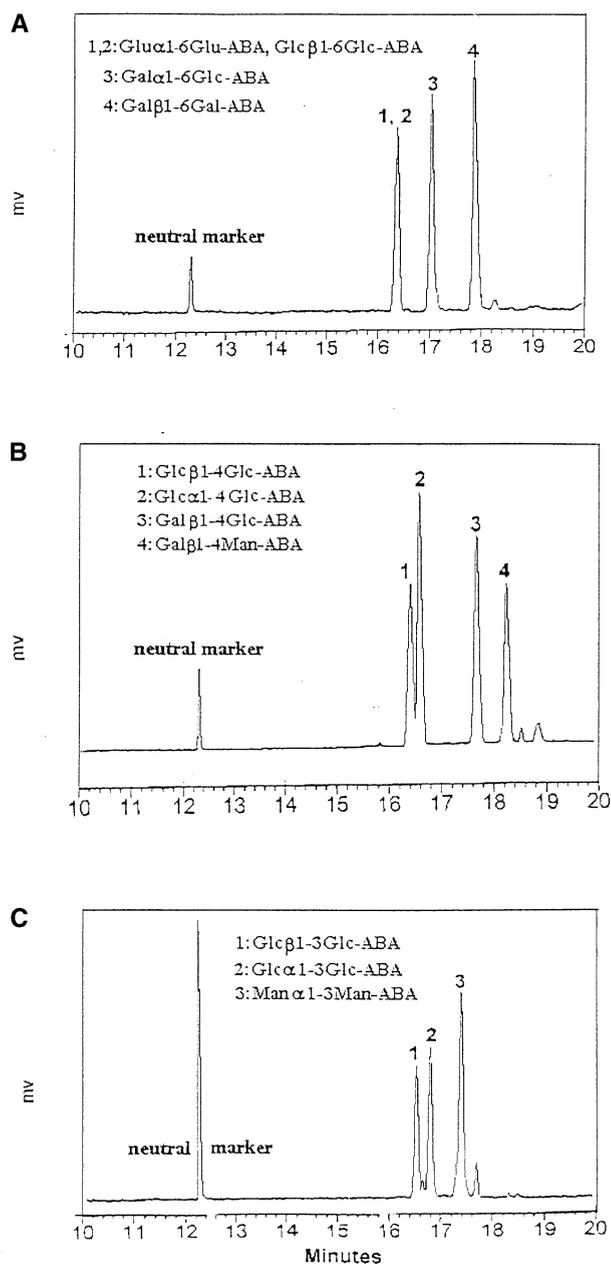


**Figure 6.** The CE/UV electropherogram of ABA-tagged (**A**) Glcα1–6Glc, Glcβ1–6Glc, Galα1–6Glc, and Galβ1–6Gal; (**B**) Glcα1–4Glc, Glcβ1–4Glc, Galβ1–4Glc, and Galβ1–4Man; and (**C**) Glcα1–3Glc, Glcβ1–3Glc, and Manα1–3Man. Conditions were the same as in Figure 3.

disaccharides. According to the mass electropherogram of *m/z* 221, peak 1, 2 were assigned as the α form of 1–6 linked disaccharides (there are two disaccharides in peak 1 and one of them is due to the α-form disaccharide), whereas peak 3 was attributed to the β form of a 1–6 linked disaccharide.

The selection of the glycosylamine approach rather than the popular reductive amination for labeling the reducing end of disaccharides was mainly due to the fact the negative ion MS/MS mass spectrum of a closed ring (glycosylamine) derivative was much more char-
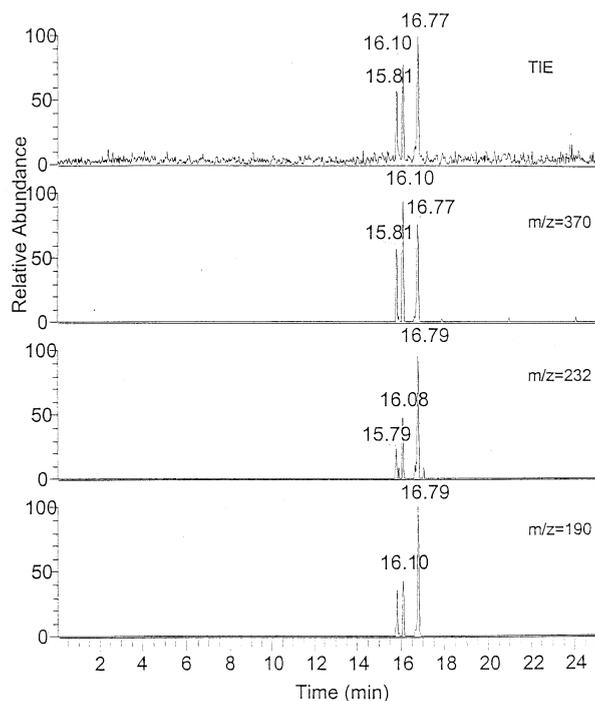
**Figure 7.** The CE/MS/MS mass electropherogram of ABA-tagged Glcα1–3Glc, Glcβ1–3Glc, and Manα1–3Man mixture. Running buffer: 10 mM borate, pH 10.0. Make up solution (sheath liquid) was water-2-propanol (20:80) containing 0.5% ammonia.
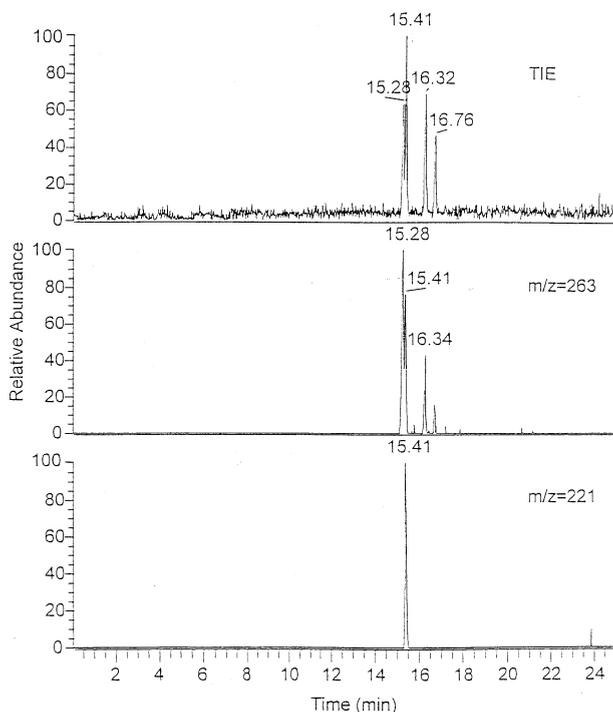


**Figure 8.** The CE/MS/MS mass electropherogram of ABA-tagged Glcα1–4Glc, Glcβ1–4Glc, Galβ1–4Glc, and Galβ1–4Man mixture. Conditions were the same as in Figure 7.

acteristic than that of an open ring (reductive amination) derivative. However, one limitation of this approach is that disaccharides derivatized with glycosylamine can produce two anomers. Anomers of 1–2, 1–4, and 1–6-linked ABA disaccharides can be separated by using ammonium acetate/α-CD buffer system, which might make the electropherogram more complicated. Fortunately, the yield of the minor anomer of most derivatives could be significantly reduced by setting the reaction temperature to 50 °C.

ABEE and ABA have been used as the derivatization reagent of reductive amination in CE/UV analysis [10, 11] using borate as the running buffer. Although ABEE derivatives exhibit lower electrophoretic mobilities than ABA derivatives, the migration order was generally not affected by the different derivatization agent. ABEE derivative was not chosen in this study mainly because of two reasons: (1) The derivatives cannot be separated with ammonium acetate/α-CD electrolyte, most likely due to the lack of carboxyl group. (2) Because of the acidic carboxyl groups, the sensitivity of ABA derivatives in negative ion ESI is approximately 30-fold higher than the neutral ABEE derivatives.

## Conclusions

ABA provides the charge and the chromophore for the sensitive detection of disaccharides in ESI/MS and CE/UV, respectively. The product ion spectra of the closed ring (glycosylamine) derivative provides more information on anomeric configuration and linkage than those of the popular reductive amination approach. Running buffer containing ammonium acetate/α-CD provided better resolution than borate in the separation of linkage isomers. The interaction between α-CD and the derivatized sugar was believed to play an important role in the separation of linkage isomers. Although ammonium acetate/α-CD provided better results in the analysis of linkage isomers, running buffer containing borate was found to be superior to ammonium acetate/α-CD in the separation of disaccharides of the same linkage but different anomeric configuration and/or monosaccharide composition. Both α-CD and borate were found to reduce the analyte signal under ESI. To reduce the effect of ion signal suppression, less concentrated α-CD or borate, providing acceptable CE resolution should be used. Because of the lack of standards, the potential of this approach to the analysis of oligosaccharides remains to be explored. Preliminary results indicated that the linkages of linear oligosaccharides such as maltotriose, isomaltotriose, and maltopentaose could be analyzed with this approach.
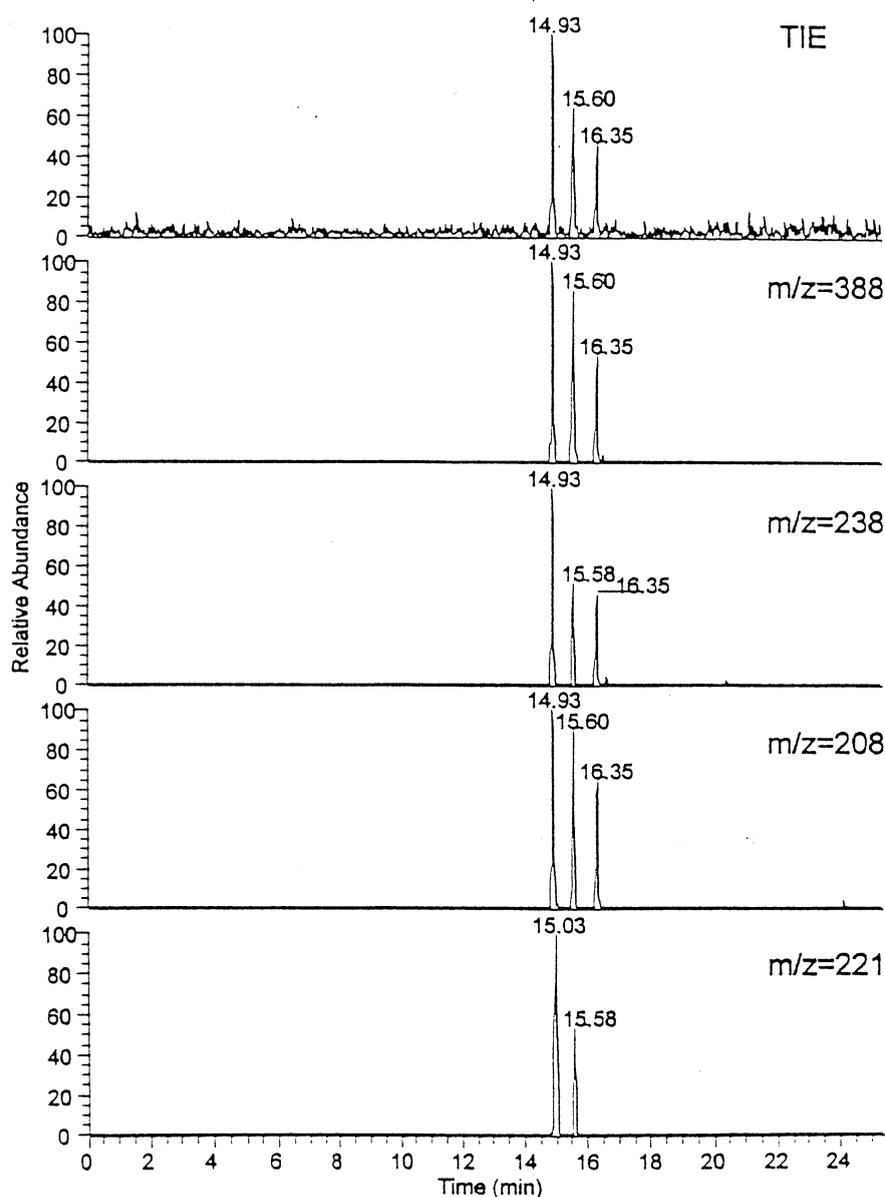
## Acknowledgment

**Figure 9.** The CE/MS/MS mass electropherogram of ABA-tagged Glcα1–6Glc, Glcβ1–6Glc, Galα1–6Glc, and Galβ1–6Gal mixture. Conditions were the same as in Figure 7.

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