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Differentiation of underivatized monosaccharides by atmospheric pressure chemical ionization quadrupole time-of-flight mass spectrometry

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RATIONALE: Differentiation of underivatized monosaccharides is essential in the structural elucidation of oligosaccharides which are closely involved in many life processes. So far, such differentiation has been usually achieved by electrospray ionization mass spectrometry (ESI-MS). As an alternative to ESI-MS, atmospheric pressure chemical ionization mass spectrometry (APCI-MS) should provide complementary results.
METHODS: A quadrupole time-of-flight (QTOF) mass spectrometer with accurate mass measurement ability was used with an APCI heated nebulizer ion source because we believe that a recently published article using a single quadrupole mass spectrometer assigned incorrect identities for APCI ions from hexoses. Using APCI-QTOF, the MS^2 and *pseudo*-MS^3 mass spectra of 11 underivatized monosaccharides were obtained under various collision voltages. The mass spectra were carefully interpreted after accurate mass measurement.
RESULTS: Differentiation of three hexoses was achieved by different MS^2 spectra of their $[M+NH_4]^+$ and $[M-H]^-$ ions. The MS^2 spectra of the $[M+NH_4]^+$ ions were also used to distinguish methyl α-D-glucose and methyl β-D-glucose, while the *pseudo*-MS^3 spectra of the $[M+H]^+$ ions were utilized to differentiate the three hexosamine and *N*-acetylhexosamine stereoisomers. Unique $[M+O_2]^-$ ions were observed and their distinctive fragmentation patterns were utilized to differentiate the three hexosamine stereoisomers.
CONCLUSIONS: Although ESI coupled with single or triple quadrupole and ion trap mass spectrometers has been widely utilized in the differentiation of monosaccharides, this report demonstrated that APCI-QTOF-MS had its own advantages in achieving the same goal. Copyright © 2012 John Wiley & Sons, Ltd.

Extensive studies have shown that oligosaccharides are closely involved in many life processes by modulating proteins or mediating a variety of interactions among cells and molecules.[1,2] An understanding of these biological processes is closely associated to the knowledge of their structures. The structural elucidation of oligosaccharides usually includes the differentiation of the monosaccharide stereoisomers, the determination of the oligosaccharide sequence, and the identification of the linkage location and branching sites.[3]

Mass spectrometry has been widely used in structural elucidation of oligosaccharides due to its high accuracy, analytical versatility and high sensitivity.[4] In earlier studies, fast atom bombardment mass spectrometry (FAB-MS) was utilized to determine the sequence and linkage position[5,6] of both derivatized and underivatized oligosaccharides.[6] Later, electrospray ionization mass spectrometry (ESI-MS) became the predominant technique for the analysis of oligosaccharides.[7–20] In particular, the differentiation of underivatized monosaccharides including hexoses,[13–20] hexosamines,[10,12,20] *N*-acetylhexosamines[12,20] and methyl D-glucopyranosides[16,20] was achieved by investigating the fragmentation behavior of their protonated/deprotonated ions and/or their metal,[11–16] ammonium[18,20] and $NH_2NH_2H^+$ adducts.[19] The tandem mass spectra were usually acquired using ion trap[11,12,14,15,19,20], triple-quadrupole[13,16,18] or quadrupole time-of-flight (QTOF)[17] mass spectrometers.

Although atmospheric pressure chemical ionization mass spectrometry (APCI-MS) has not so far been widely used in the analysis of monosaccharides, both Liang *et al.*[21] and Ullah *et al.*[22] showed that APCI-MS had advantages over ESI-MS in quantification due to there being fewer matrix effects. Recently, Choi and Kim[23] reported the differentiation of glucosamine, mannosamine and galactosamine by APCI-MS using the fragmentation patterns of either their $[M+H]^+$ or $[M_{d6}+D]^+$ ions when H_2O and D_2O were, respectively, used as the solvent. They also then reported the differentiation of galactose from glucose and mannose by APCI-MS using the fragmentation patterns of their $[M+H_2O]^+$ ions, and the differentiation of glucose from galactose and mannose by APCI-MS using the fragmentation behavior of their $[M_{d5}+D_2O+D]^+$ ions.[24] According to Choi and Kim,[23,24] the difference between H_2O and D_2O resulted in different types of ionization products from hexoses, but not from hexosamines. This contradiction could be caused by the incorrect assignment of the ionization products from hexoses, i.e. $[M+H_2O]^+$ and $[M_{d5}+D_2O+D]^+$ ions. As ubiquitous trace amounts of ammonia are present in the atmosphere[25] and ammonia can interact with hexoses through hydrogen bonds,[26] the identity of the ionization products from

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hexoses could be $[M+NH_4]^+$ and $[M_{d5}+ND_4]^+$. A possible incorrect assignment could be a result of inaccurate mass measurement with the single quadrupole mass spectrometer used in the study because the nominal *m/z* values of the $[M+NH_4]^+$ and $[M+H_2O]^+$ ions, and the $[M_{d5}+ND_4]^+$ and $[M_{d5}+D_2O+D]^+$ ions were identical.

In this study the use of APCI-MS has been explored for the analysis of eleven underivatized monosaccharides (Supplementary Fig. S1, see Supporting Information). A quadrupole time-of-flight (QTOF) mass spectrometer was selected for the analysis due to its accurate mass measurement ability. The identities of the ionization products of the underivatized monosaccharides and their corresponding product ions were assigned after accurate mass measurement. Differentiation of underivatized monosaccharides by APCI-QTOF-MS was achieved for three hexoses, two methyl D-glucopyranosides, three hexosamines and three *N*-acetylhexosamines.

EXPERIMENTAL

Chemicals

Water (H_2O) and methanol were HPLC grade and purchased from Fisher Scientific (Suwanee, GA, USA). Deuterium oxide (D_2O) and ammonium acetate (NH_4Ac) were purchased from Sigma-Aldrich (St. Louis, MO, USA). D-(+)-Glucose (Glc), D-(+)-mannose (Man), D-glucosamine hydrochloride (GlcN·HCl), D-mannosamine hydrochloride (ManN·HCl), *N*-acetyl-D-mannosamine (ManNAc), *N*-acetyl-D-galactosamine (GalNAc) and methyl α-D-glucopyranoside (MeαGlc) were also purchased from Sigma-Aldrich. D-Galactosamine hydrochloride (GalN·HCl) and methyl β-D-glucopyranoside hemihydride (MeβGlc·1/2H_2O) were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). D-(+)-Galactose (Gal) was purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA, USA). *N*-Acetyl-D-glucosamine (GlcNAc) was purchased from Ferro Pfanstiehl Laboratories Inc. (Waukegan, IL, USA). Individual monosaccharides were dissolved either in H_2O, D_2O, methanol, or in methanol containing 10 mM NH_4Ac. The concentration of the monosaccharide was either 0.1 mM for positive-ion or 0.5 mM for negative-ion mass spectrometric experiments.

Mass spectrometry

Mass spectrometric analysis was performed using a QSTAR Elite quadrupole time-of-flight (QTOF) mass spectrometer equipped with an APCI heated nebulizer ion source and an integrated syringe pump (AB Sciex, Foster City, CA, USA). Nitrogen (Airgas, Bowling Green, KY, USA) was used as the nebulizer, auxiliary, curtain and collision gas. The mass spectrometric parameters in the instrumental control panel were briefly optimized to minimize in-source fragmentation. In positive-ion MS mode, they were: nebulizer current, 1.0 μA; heater temperature, 250 °C; auxiliary gas pressure, 40 psi; nebulizer gas pressure, 40 psi; curtain gas pressure, 40 psi; collision gas pressure: 3 psi; declustering potential, 30 V; focusing potential, 100 V; declustering potential 2: 10 V. In positive-ion MS^2 mode, all the mass spectrometric parameters remained the same except that collision gas pressure was adjusted to 4 psi. The collision voltage was varied over an appropriate range and is described in detail in the Results and Discussion section. In negative-ion MS and MS^2 modes, the absolute values of the spectrometric parameters remained the same as in positive-ion mode, but the voltages were inverted. The accumulation time of each mass spectrometric scan was 1 s. Data acquisition was performed in multiple-channel acquisition (MCA) mode and all mass spectra obtained were an accumulation of 10 scans. The monosaccharide solutions were infused into the mass spectrometric system using a gastight syringe (Hamilton, Reno, NV, USA). The infusion flow rate was either 30 μL min^{-1} in positive-ion mode or 50 μL min^{-1} in negative-ion mode. Each monosaccharide solution was analyzed in five replicates.

RESULTS AND DISCUSSION

Ionization products of hexoses by positive-ion APCI

Figure 1 shows an APCI-QTOF mass spectrum of 1.0 mM Glc in H_2O. In addition to an adduct ion at nominal *m/z* 198, other ions at nominal *m/z* 180, 163, 145 and 127 were observed due to inevitable in-source fragmentation in the APCI process.[27] A similar mass spectrum with ions at the same nominal *m/z* values was reported by Choi and Kim[24] using APCI single quadrupole mass spectrometry, though the adduct ion at nominal *m/z* 198 was observed to be the base peak, which might be a result of different ambient environment and/or instrumental conditions. A MS^2 mass spectrum of the ion at nominal *m/z* 198, similar to the APCI-QTOF mass spectrum in Fig. 1, was also reported by Zhu and Sato[20] using ESI ion trap mass spectrometry. Among the three studies, there is an obvious disagreement in the identities of the ions at nominal *m/z* 198 and 180, i.e. $[M+H_2O]^+$ and $M^{+\cdot}$ by Choi and Kim vs. $[M+NH_4]^+$ and $[M+NH_4-H_2O]^+$ by us in this study, and Zhu and Sato.[20] However, there is an agreement in the elemental compositions of the ions at *m/z* 163, 145 and 127 being $[C_6H_{10}O_5+H]^+$, $[C_6H_8O_4+H]^+$ and $[C_6H_6O_3+H]^+$ (accurate *m/z* values: 163.0607, 145.0501 and 127.0395,

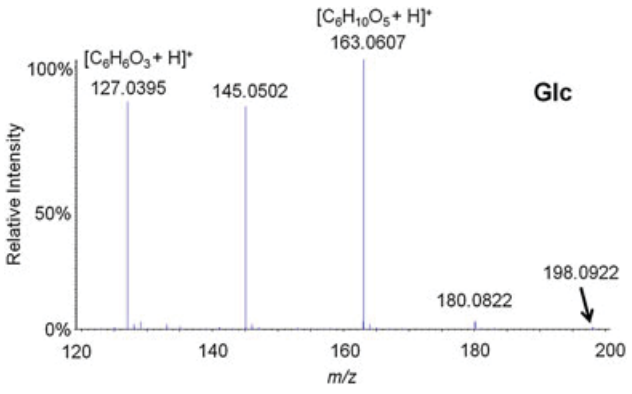

Figure 1. Positive-ion APCI-QTOF mass spectrum of 1.0 mM Glc in H_2O. The mass spectrum was calibrated using the $[C_6H_{10}O_5+H]^+$ and $[C_6H_6O_3+H]^+$ ions with theoretical *m/z* values of 163.0607 and 127.0395, respectively. The *m/z* values of other peaks measured by APCI-QTOFMS are indicated in the figure.

respectively). As a result of this agreement, the mass spectrum shown in Fig. 1 could be calibrated using the peaks representing the $[C_6H_{10}O_5 + H]^+$ and $[C_6H_6O_3 + H]^+$ ions. The mass measurement accuracy obtained by APCI-QTOF-MS was then verified by the experimental and accurate *m/z* value of the peak representing the $[C_6H_8O_4 + H]^+$ ion, i.e. 145.0502 vs. 145.0501. Because the experimental *m/z* value of the adduct ion was 198.0922, it was obviously more reasonably assigned as $[M + NH_4]^+$ with an accurate *m/z* value of 198.0978, rather than $[M + H_2O]^+$ with an accurate *m/z* value of 198.0740. With regard to the ion with an experimental *m/z* value of 180.0822, its identity was obviously more reasonably assigned as $[M + NH_4 - H_2O]^+$ with an accurate *m/z* value of 180.0872, rather than $M^{+\cdot}$ with an accurate *m/z* value of 180.0634. The mass differences were 5.6 and 5.0 milli *m/z* units for the $[M + NH_4]^+$ and $[M + NH_4 - H_2O]^+$ ions, respectively. These differences were slightly larger than that for the $[C_6H_8O_4 + H]^+$ ion, i.e. 0.1 milli *m/z* units. This was because the *m/z* values of the $[M + NH_4]^+$ and $[M + NH_4 - H_2O]^+$ ions were beyond the calibration range, i.e. *m/z* 163.0607 to 127.0395. Because the adduct ion was confirmed to be $[M + NH_4]^+$, the identities of the ions at *m/z* 163.0607, 145.0502 and 127.0395 were $[M + NH_4 - H_2O - NH_3]^+$, $[M + NH_4 - 2H_2O - NH_3]^+$ and $[M + NH_4 - 3H_2O - NH_3]^+$, respectively.

APCI-QTOF mass spectra of 1.0 mM Man and Gal in H_2O were also acquired and calibrated similarly. Their adduct ions were also verified to be $[M + NH_4]^+$. The APCI-QTOF mass spectra of 1.0 mM Glc, Man and Gal in D_2O were further acquired and calibrated as described. Their adduct ions were confirmed to be $[M_{d5} + ND_4]^+$. In order to enhance the abundance of the $[M + NH_4]^+$ ion relative to the other in-source fragmentation ions, 0.1 mM Glc, Man and Gal were prepared in methanol containing 1, 10 and 100 mM NH_4Ac. The best result was obtained with methanol containing 10 mM NH_4Ac.

Differentiation of Glc, Man and Gal

The positive-ion APCI-QTOF mass spectra of Glc, Man and Gal did not show significant differences. Therefore, their $[M + NH_4]^+$ ions were selected as precursor ions for further MS^2 experiments. Figure 2(A) shows positive-ion APCI-QTOF-MS^2 spectra of the $[M + NH_4]^+$ ions at *m/z* 198.098 generated from 0.1 mM Glc, Man and Gal in methanol containing 10 mM NH_4Ac. Accurate mass measurement determined that the product ions at nominal *m/z* 181, 180, 163 and 145 were $[M + NH_4 - NH_3]^+$, $[M + NH_4 - H_2O]^+$, $[M + NH_4 - H_2O - NH_3]^+$ and $[M + NH_4 - 2H_2O - NH_3]^+$, respectively. Glc can be differentiated from Man and Gal due to the obviously larger relative intensity of peaks at *m/z* 180 to 163. Figure 2(B) shows the relative intensity of the peaks at *m/z* 180 to 163 of Glc, Man and Gal at different collision voltages from 7 to 14 V. As the collision voltage increased from 7 to 14 V, the relative intensity of peaks at *m/z* 180 to 163 from Glc decreased from 1.7 to 0.2, while they were below 0.3 and almost identical for both Man and Gal. Thus, Glc can be better differentiated from Gal and Man at lower collision voltages, e.g. 8 V in Fig. 2(A).

In order to further differentiate Man from Gal, negative-ion APCI-QTOF mass spectra of 0.5 mM Man and Gal in methanol were acquired. The ionization products of hexoses by

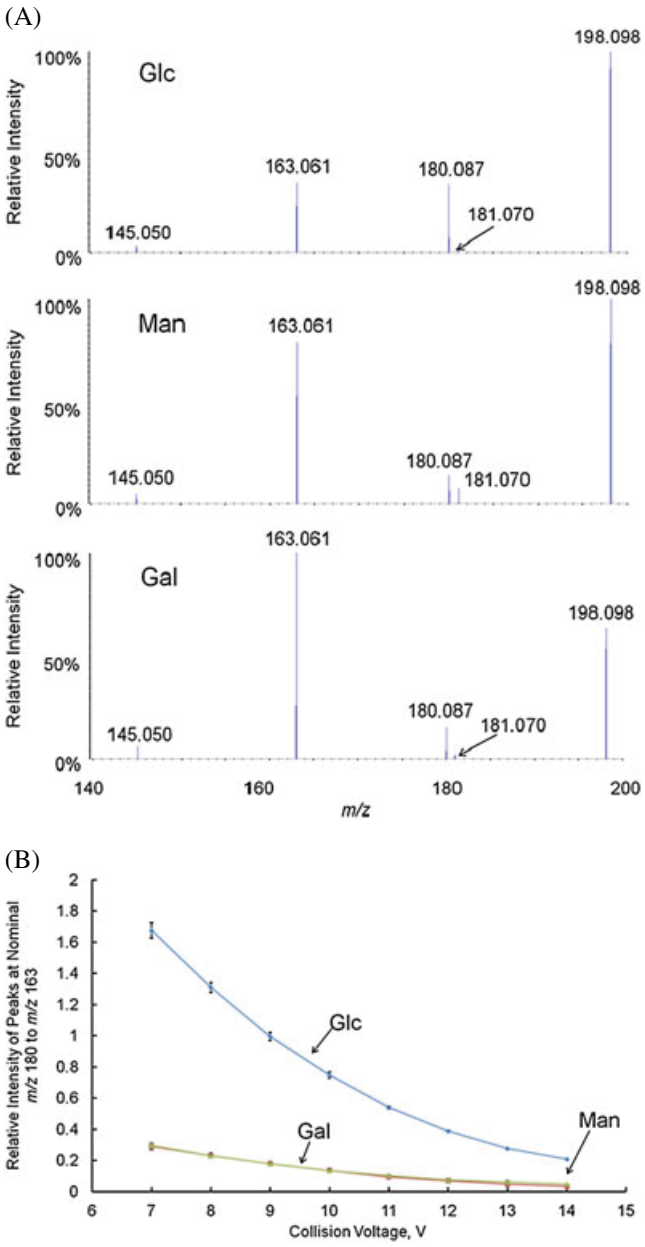

Figure 2. (A) Positive-ion APCI-QTOF-MS^2 spectra of the $[M + NH_4]^+$ ions at *m/z* 198.098 generated from 0.1 mM Glc, Man, and Gal in methanol containing 10 mM NH_4Ac. Collision voltage was 9 V. Product ions are identified as $[M + NH_4 - NH_3]^+$(*m/z* 181), $[M + NH_4 - H_2O]^+$ (*m/z* 180), $[M + NH_4 - H_2O - NH_3]^+$ (*m/z* 163) and $[M + NH_4 - 2H_2O - NH_3]^+$ (*m/z* 145). (B) Relative intensities of peaks at nominal *m/z* 180 to 163 in the APCI-QTOF-MS^2 spectra at different collision voltages for Glc, Man and Gal.

negative-ion APCI were $[M + O_2]^-$ ions (data not shown) due to the generation of $O_2^{-\cdot}$ during the APCI process and its hydrogen-bond interaction with the hexoses.[28] Abundant deprotonated molecules, i.e. $[M - H]^-$, and other in-source fragment ions, e.g. $[M - H - H_2O]^-$, were also detected at *m/z* 179.056 and 161.044, respectively. The $O_2^{-\cdot}$ ion has a $\Delta_{acid}G$ value of 1450.5 kJ/mol,[29] while the hexoses have free energies of acidity for the hexopyranose forms in the 1407–1419 kJ/mol range (based on G3(MP2)B3

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calculations; J. E. Bartmess, manuscript in preparation). Therefore, dissociation of the $[M+O_2]^-$ ions may be largely responsible for the observation of abundant deprotonated hexose molecules.[30] The deprotonation process induced by O_2^- is expected to occur at the most acidic site of the hexoses, presumably the anomeric -OH on C_1 for Glc and the -OH groups on C_4 for Gal, while the acidities of the -OH groups on C_1, C_2 and C_4 are close for Man.[30,31] The gas-phase acidities of hexoses could also be affected due to possible ring-opening process of their anions.[32] Nevertheless, the negative-ion APCI-QTOF-MS spectra of Man and Gal did not show significant differences.

The $[M-H]^-$ ions from Man and Gal were selected as precursor ions for further MS^2 experiments. Figure 3(A) shows negative-ion APCI-QTOF-MS^2 spectra of the $[M-H]^-$ ions at *m/z* 179.056 generated from 0.5 mM Man and Gal in methanol. Accurate mass measurement determined that the product ions at nominal *m/z* 161, 119 and 89 were $[M-H_2O-H]^-$, $[M-C_2H_4O_2-H]^-$ and $[M-C_3H_6O_3-H]^-$, respectively.

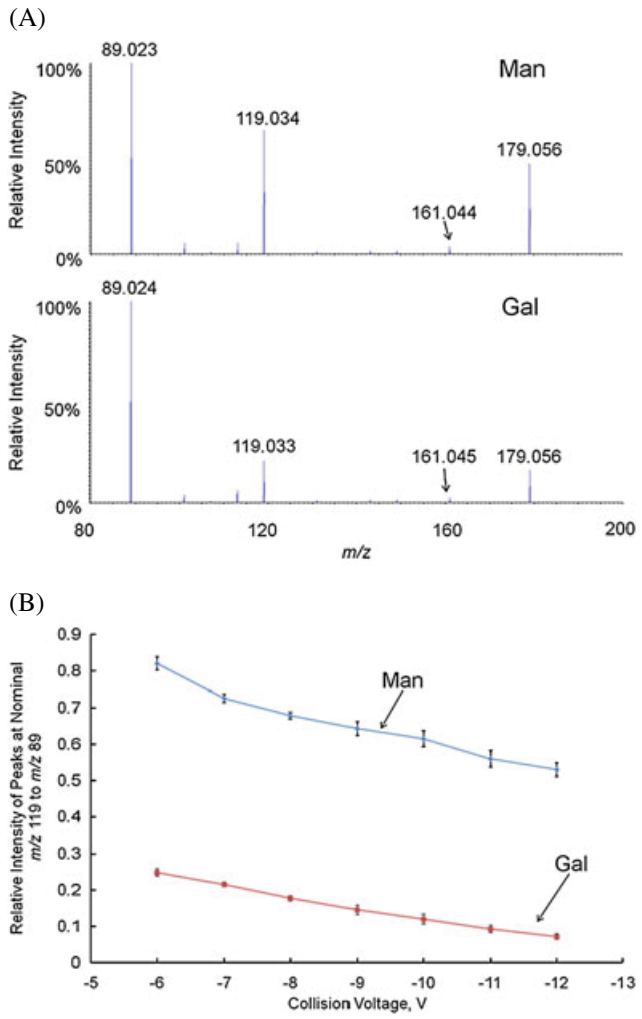

Figure 3. (A) Negative- ion APCI-QTOF-MS^2 spectra of the $[M-H]^-$ ions at *m/z* 179.056 generated from 0.5 mM Man and Gal in methanol. Collision voltage was −10 V. Product ions are identified as $[M-H_2O-H]^-$ (*m/z* 161), $[M-C_2H_4O_2-H]^-$ (*m/z* 119) and $[M-C_3H_6O_3-H]^-$ (*m/z* 89). (B) Relative intensities of peaks at nominal *m/z* 119 to 89 in the APCI-QTOF-MS^2 spectra at different collision voltages for Man and Gal.

Man can be differentiated from Gal due to the obviously larger relative intensity of the peaks at *m/z* 119 to 89. Figure 3(B) shows the relative intensity of the peaks at *m/z* 119 to 89 of Man and Gal at different collision voltages from −6 to −12 V. The relative intensities of the peaks at *m/z* 119 to 89 were above 0.5 from Man and below 0.25 from Gal. Thus, Man can be differentiated from Gal.

Differentiation between Me*α*Glc and Me*β*Glc

In order to study the fragmentation behavior of monosaccharides influenced by different anomeric configurations, anomers of methyl D-glucopyranoside, i.e. Me*α*Glc and Me*β*Glc, were selected. Me*α*Glc and Me*β*Glc differ from Glc in that the anomeric hydroxyl group is replaced with a methoxy group. Consequently, they are able to maintain their own configuration in solvent due to the substantially increased energy barrier for the mutarotation process.

The positive-ion APCI-QTOF mass spectra of Me*α*Glc and Me*β*Glc (data not shown) exhibited a relatively larger intensity of the peaks representing $[M+NH_4]^+$ ions at *m/z* 212.113 from Me*α*Glc than from Me*β*Glc under the same mass spectrometric conditions. It is possible that the anomeric oxygen in the axial position is a more favorable configuration than that in the equatorial position when hydrogen bonds are formed between methyl D-glucopyranosides and NH_4^+. Such a difference was not observed among Glc, Man and Gal as the result of the fast mutarotation at room temperature in their solutions.[1]

However, the difference observed between the mass spectra of Me*α*Glc and Me*β*Glc was not sufficient to distinguish them. Therefore, their $[M+NH_4]^+$ ions were selected as precursor ions for further MS^2 experiments. Figure 4(A) shows positive-ion APCI-QTOF-MS^2 spectra of the $[M+NH_4]^+$ ions at *m/z* 212.113 generated from 0.1 mM Me*α*Glc and Me*β*Glc in methanol containing 10 mM NH_4Ac. Accurate mass measurement determined that the peaks at *m/z* 212, 195, 180, 163 and 145 were $[M+NH_4]^+$, $[M+NH_4-NH_3]^+$, $[M+NH_4-CH_4O]^+$, $[M+NH_4-CH_4O-NH_3]^+$ and $[M-CH_6O_2+H]^+$, respectively. The observation of $[M+NH_4-CH_4O]^+$ rather than $[M+NH_4-H_2O]^+$ supports a previous deduction that the anomeric center is the most active site of the monosaccharides.[18] Me*α*Glc could be differentiated from Me*β*Glc due to the greater abundance of $[M+NH_4-CH_4O]^+$ ions than $[M+NH_4-NH_3]^+$ ions. Figure 4(B) shows the relative intensity of peaks at *m/z* 180 to 195 of Me*α*Glc and Me*β*Glc at different collision voltages from 8 to 14 V. As the collision voltage increased from 8 to 14 V, the relative intensity of the peaks at *m/z* 195 to 180 from Me*α*Glc increased from 1.7 to 4.9, while it was below 0.4 for Me*β*Glc. Therefore, Me*α*Glc can be better differentiated from Me*β*Glc at higher collision voltages, e.g. 14 V in Fig. 4(B).

In the negative-ion APCI-QTOF mass spectra of 0.5 mM Me*α*Glc and Me*β*Glc in methanol (data not shown), abundant peaks were observed for both $[M+O_2]^-$ ions at *m/z* 226.0646 and $[M-H]^-$ ions at *m/z* 193.0732. As described in the last section, this is quite different from the negative-ion APCI-QTOF mass spectra of hexoses, where the dissociation of $[M+O_2]^-$ ions to form much more abundant $[M-H]^-$ ions was favorable. This is probably because the most acidic hydrogen[30,31] on the anomeric oxygen in D-glucose has been replaced by a methyl group in methyl D-glucopyranosides. A higher energy barrier

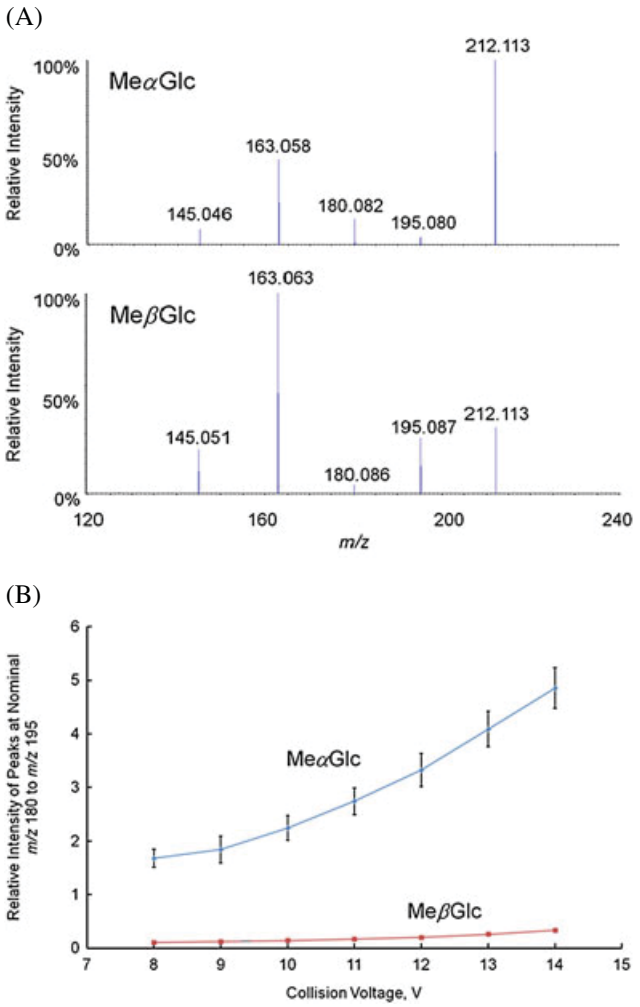

Figure 4. (A) Positive-ion APCI-QTOF-MS^2 spectra of the $[M+NH_4]^+$ ions at *m/z* 212.113 generated from 0.1 mM Me*α*Glc and Me*β*Glc in methanol containing 10 mM NH_4Ac. Collision voltage was 11 V. Product ions are identified as $[M-2H_2O+H]^+$ (*m/z* 144), $[M-3H_2O+H]^+$ (*m/z* 126), $[M-CH_8O_4+H]^+$ (*m/z* 96), $[M-C_2H_8O_4+H]^+$ (*m/z* 84) and $[M-C_3H_8O_4+H]^+$ (*m/z* 72). (B) Relative intensities of peaks at nominal *m/z* 180 to 195 in the APCI-QTOF-MS^2 spectra at different collision voltages for Me*α*Glc and Me*β*Glc.

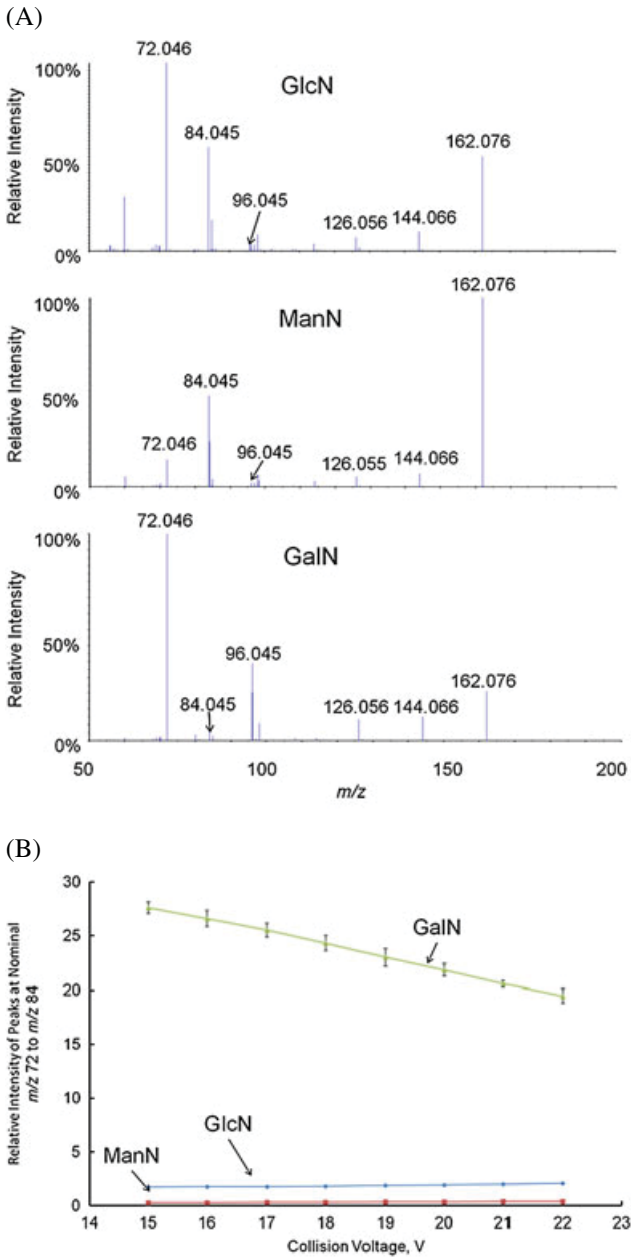

Figure 5. (A) Positive-ion APCI-QTOF-MS^2 spectra of the $[M-H_2O+H]^+$ ions at *m/z* 162.076 generated from 0.1 mM GlcN, ManN and GalN in methanol containing 10 mM NH_4Ac. Collision voltage was 18 V. Product ions are identified as $[M-2H_2O+H]^+$ (*m/z* 186) and $[M-C_2H_8O_4+H]^+$ (*m/z* 126). (B) Relative intensities of peaks at nominal *m/z* 72 to 84 in the APCI-QTOF-MS^2 spectra at different collision voltages for GlcN, ManN and GalN.

is therefore created in the dissociation process of the $[M+O_2]^-$ ions of methyl D-glucopyranosides to $[M-H]^-$ ions. Consequently, much more abundant $[M+O_2]^-$ ions were observed in the negative-ion APCI-QTOF mass spectra of Me*α*Glc and Me*β*Glc.

The APCI-QTOF-MS^2 spectra of the $[M+O_2]^-$ and $[M-H]^-$ ions generated from 0.5 mM Me*α*Glc and Me*β*Glc in methanol showed no significant differences.

Differentiation of GlcN, ManN, and GalN

Positive-ion APCI-QTOF-MS spectra of 0.1 mM GlcN·HCl, ManN·HCl and GalN·HCl in methanol containing 10 mM NH_4Ac showed abundant $[M+H]^+$ ions at *m/z* 180 (data not shown). However, unlike the corresponding hexoses, peaks representing $[M+NH_4]^+$ ions were not detected. This is because the proton affinity (PA) of NH_3, i.e. 853.6 kJ/mol,[33] is much less than that of GlcN, ManN or GalN, i.e. 937.7, 942.0, and 940.8 kJ/mol,[11] respectively. Therefore, the $[M+H]^+$ ions of GlcN, ManN and GalN could be formed by abstracting protons from NH_4^+. Even if the $[M+NH_4]^+$ ions were temporarily formed by collision, they would be ready to undergo dissociation to form $[M+H]^+$ and NH_3, a favorable process when an analyte molecule has a much stronger PA value than that of NH_3.[26] Other in-source fragment ions were also observed in positive-ion APCI-QTOF mass spectra of 0.1 mM GlcN·HCl, ManN·HCl and

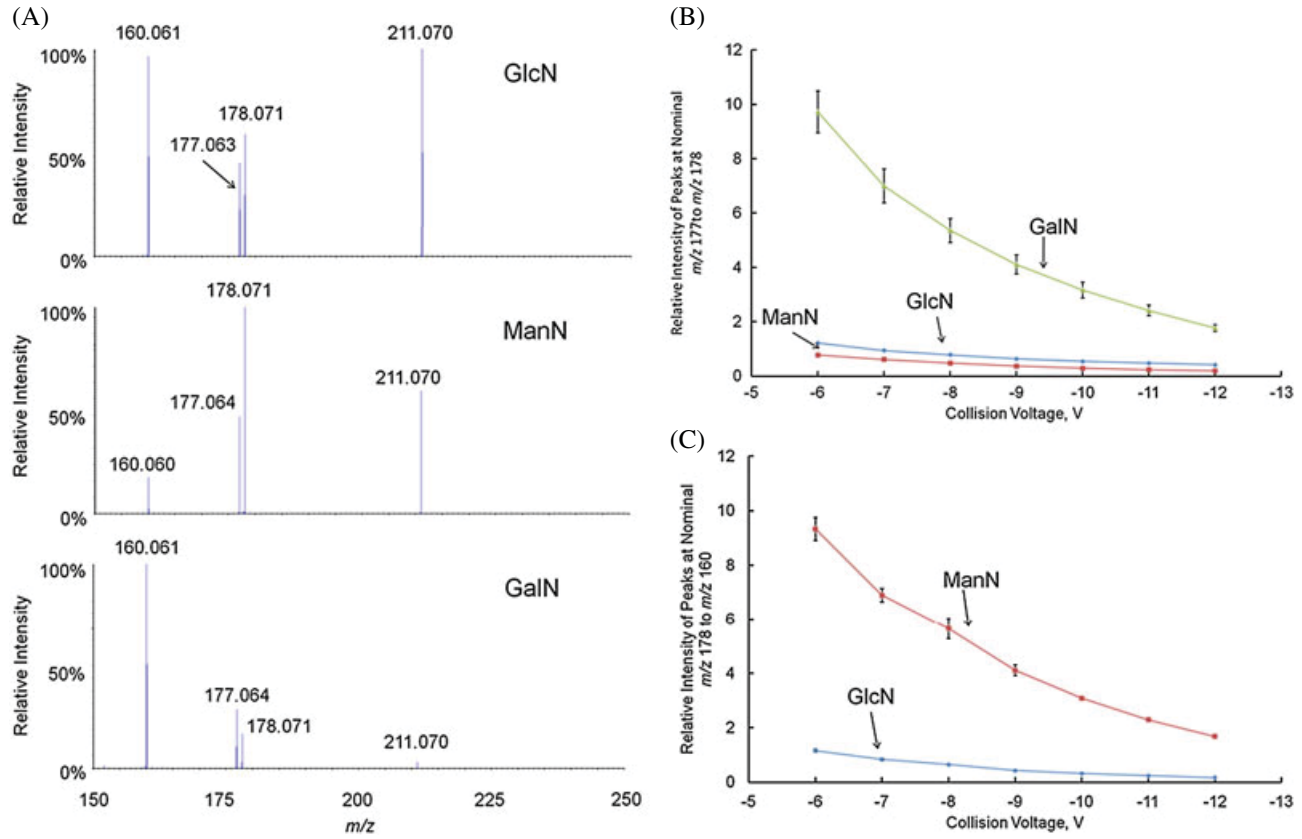

Figure 6. (A) Negative-ion APCI-QTOF-MS^2 spectra of the $[M+O_2]^-$ ions at *m/z* 211.070 generated from 0.5 mM GlcN, ManN and GalN. Collision voltage was −8 V. Product ions are identified as $[M-H]^-$ (*m/z* 178), $[M-2H]^-$ (*m/z* 177), and $[M-H-H_2O]^-$ (*m/z* 160). (B) Relative intensities of peaks at nominal *m/z* 177 to 178 in the APCI-QTOF-MS^2 spectra at different collision voltages for GlcN, ManN and GalN. (C) Relative intensities of peaks at nominal *m/z* 178 to 160 in the same spectra under same collision voltages as in (B).

GalN·HCl in methanol containing 10 mM NH_4Ac. Accurate mass measurement determined that the major ions were $[M-H_2O+H]^+$, $[M-2H_2O+H]^+$ and $[M-3H_2O+H]^+$ with theoretical *m/z* values of 162.076, 144.066 and 126.055.

Neither the positive-ion APCI-QTOF mass spectra of GlcN, ManN and GalN nor the MS^2 spectra of their $[M+H]^+$ ions (data not shown) showed any significant differences. Conventional MS^3 spectra, commonly acquired by using ion trap mass spectrometers,[12,15,20,34] could not be acquired by using QTOF mass spectrometers. However, *pseudo*-MS^3 experiments could be performed by utilizing the in-source fragment ions as the precursor ions as in previous studies.[35,36] In our case, the *pseudo*-MS^3 spectra of their $[M+H]^+$ ions could be acquired when the abundant in-source fragment ions, i.e. $[M-H_2O+H]^+$ ions at *m/z* 162.076, were utilized as the precursor ions. Figure 5(A) shows positive-ion APCI-QTOF-MS^2 spectra of the $[M-H_2O+H]^+$ ions at *m/z* 162.076 generated from 0.1 mM GlcN, ManN and GalN in methanol containing 10 mM NH_4Ac. Accurate mass measurement determined that the identities of product ions at nominal *m/z* 144, 126, 96, 84 and 72 were $[M-2H_2O+H]^+$, $[M-3H_2O+H]^+$, $[M-CH_8O_4+H]^+$, $[M-C_2H_8O_4+H]^+$ and $[M-C_3H_8O_4+H]^+$, respectively. Abundant $[M-CH_8O_4+H]^+$ ions at *m/z* 96 were only detected for GalN, while peaks representing $[M-C_2H_8O_4+H]^+$ ions at *m/z* 84 were observed with large relative intensity for GlcN and ManN, but not for GalN. In addition, peaks representing $[M-C_3H_8O_4+H]^+$ ions at *m/z* 72 were the most abundant for both GlcN and GalN, but not for ManN. Figure 5(B) shows the relative intensity of peaks at *m/z* 72 to 84 of GlcN, ManN and GalN at different collision voltages from 15 to 22 V. As the collision voltage was increased from 15 to 22 V, the relative intensity of the peaks at *m/z* 72 to 84 from GalN decreased from 27 to 19, while they were between 1.5 and 2.5 for GlcN and below 0.5 for ManN. Therefore, GlcN, ManN and GalN were clearly distinguished.

The negative-ion APCI-QTOF mass spectra of 0.5 mM GlcN·HCl, ManN·HCl and GalN·HCl in methanol showed chloride adducts, i.e. $[M+{}^{35}Cl]^-$ ions at *m/z* 214.056 and $[M+{}^{37}Cl]^-$ at *m/z* 216.051, with the characteristic 3 to 1 isotopic ratio (data not shown). $[M+O_2]^-$ ions of GlcN, ManN and GalN at *m/z* 211.072 were also observed with a much lower abundance. This could be because O_2^- formed less stable hydrogen bonds with hexosamines than Cl^-, and this was also reflected in the negative-ion APCI-QTOF-MS^2 spectra of $[M+O_2]^-$ and $[M+{}^{35}Cl]^-$ ions where no product ions were observed beyond *m/z* 100 for the $[M+{}^{35}Cl]^-$ ions.

Figure 6(A) shows negative-ion APCI-QTOF-MS^2 spectra of the $[M+O_2]^-$ precursor ions at *m/z* 211.070 generated from 0.5 mM GlcN·HCl, ManN·HCl and GalN·HCl in methanol. Accurate mass measurement determined that the product ions at nominal *m/z* 178, 177 and 160 were $[M-H]^-$,

$[M-2H]^-$ and $[M-H-H_2O]^-$, respectively. The $[M-2H]^-$ ions can best be rationalized as loss of hydrogen peroxide from the $[M+O_2]^-$ ions. The relative intensities of the $[M-2H]^-$ ions at *m/z* 177 were larger than those of the $[M-H]^-$ ions at *m/z* 178 for GalN, while the opposite was observed for ManN and GlcN. Meanwhile, ManN showed smaller relative intensity of the $[M-H-H_2O]^-$ ions at *m/z* 160, different from GlcN and GalN. Figure 6(B) shows the relative intensities of peaks at *m/z* 177 to 178 of GlcN, ManN and GalN at different collision voltages from −6 to −12 V. As the collision voltage increased from −6 to −12 V, the relative intensity of the peaks at *m/z* 177 to 178 from GalN decreased from 10 to 1.6, while they were below 1.2 for both GlcN and ManN. Figure 6(C) shows the relative intensities of the peaks at *m/z* 178 to 160 of GlcN and ManN under different collision voltages from −6 to −12 V. As the collision voltage increased from −6 to −12 V, the relative intensities of the peaks at *m/z* 178 to 160 were between 1.5 and 9.5 for ManN and less than 1 for GlcN. Therefore, differentiation of three hexosamine isomers was also successful using negative-ion APCI-QTOF-MS.

Differentiation of GlcNAc, ManNAc, and GalNAc

Positive-ion APCI-QTOF mass spectra of 0.1 mM GlcNAc, ManNAc and GalNAc in methanol containing 10 mM NH_4Ac were obtained (data not shown). Similar to hexosamines, abundant $[M+H]^+$ ions at *m/z* 222.096 were observed, but $[M+NH_4]^+$ ions were absent (The PAs of GlcNAc, ManNAc and GalNAc are 925.7, 926.1 and 927.8 kJ/mol, respectively,[37] which are much higher than that of NH_3, i.e. 853.6 kJ/mol.) Other in-source fragment ions were also observed. Accurate measurement determined that the major fragment ions were $[M-H_2O+H]^+$, $[M-2H_2O+H]^+$ and $[M-3H_2O+H]^+$ with theoretical *m/z* values of 204.087, 186.076 and 168.066.

Neither the positive-ion APCI-QTOF mass spectra of GlcN, ManN and GalN nor the MS^2 spectra of their $[M+H]^+$ ions (data not shown) showed any significant differences. Figure 7 (A) shows positive-ion APCI-QTOF-MS^2 spectra of the $[M-H_2O+H]^+$ ions at *m/z* 204.087 generated from 0.1 mM GlcNAc, ManNAc and GalNAc in methanol containing 10 mM NH_4Ac. Accurate mass measurement determined that the product ions at nominal *m/z* 186 and 126 were $[M-2H_2O+H]^+$ and $[M-C_2H_8O_4+H]^+$, respectively. Figure 7(B) shows the relative intensities of the peaks at *m/z* 186 to 126 of GlcN, ManN and GalN at different collision voltages from 9 to 15 V. As the collision voltage increased from 9 to 15 V, the relative intensities of the peaks at *m/z* 186 to 126 from GlcNAc decreased from 3 to 0.3, while they decreased from 1.3 to 0.1 for both ManNAc and GalNAc. Thus, GlcNAc can be differentiated from ManNAc and GalNAc at lower collision voltages, e.g. 9 V in Fig. 7(B). To further distinguish ManNAc and GalNAc, positive-ion APCI-QTOF-MS^2 spectra of the $[M+H-3H_2O]^+$ ions at *m/z* 168.066 were studied and these are shown in Fig. 8(A). Accurate mass measurement determined that the product ions at nominal *m/z* 138 and 126 were $[M-CH_8O_4+H]^+$ and $[M-C_2H_8O_4+H\ ]^+$, respectively. Figure 8(B) shows the relative intensities of the peaks at *m/z* 138 to 126 of ManNAc and GalNAc under different collision voltages from 7 to 13 V. As the collision voltage increased from 7 to 13 V, the relative intensities of the peaks at *m/z* 138 to 126 from GalNAc increased

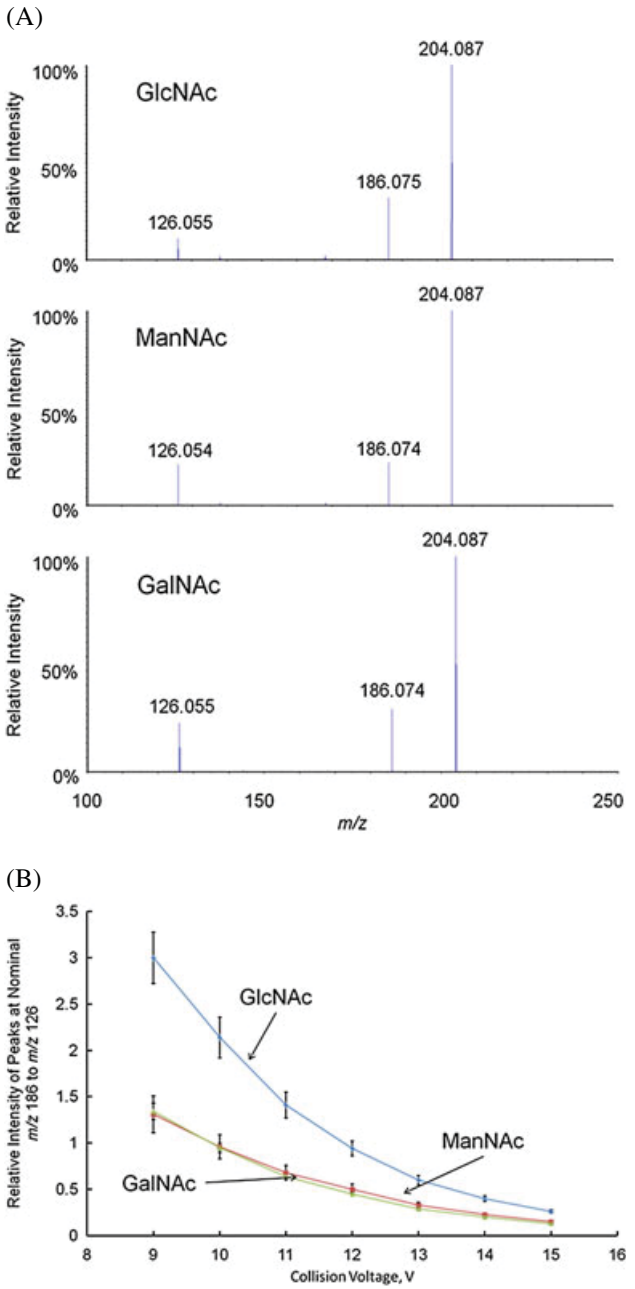

Figure 7. (A) Positive-ion APCI-QTOF-MS^2 spectra of the $[M-H_2O+H]^+$ ions at *m/z* 204.087 generated from 0.1 mM GlcNAc, ManNAc and GalNAc in methanol containing 10 mM NH_4Ac. Collision voltage was 9 V. Product ions are identified as $[M-2H_2O+H]^+$ (*m/z* 186) and $[M-C_2H_8O_4+H]^+$ (*m/z* 126). (B) Relative intensities of peaks at nominal *m/z* 186 to 126 in the APCI-QTOF-MS^2 spectra at different collision voltages for GlcNAc, ManNAc and GalNAc.

from 1 to 2.9, while they were below 0.5 from ManNAc. As a result, GlcNAc, ManNAc and GalNAc could be successfully distinguished by positive-ion APCI-QTOF-MS.

Negative-ion APCI-QTOF-MS spectra of 0.5 mM GlcNAc, ManNAc and GalNAc in methanol were also obtained, but no significant differences were observed. Abundant $[M-H]^-$ ions at *m/z* 220.083 and trace amount of $[M+O_2]^-$ ions at nominal *m/z* 253 were obtained. MS^2 spectra of the $[M+O_2]^-$, $[M-H]^-$

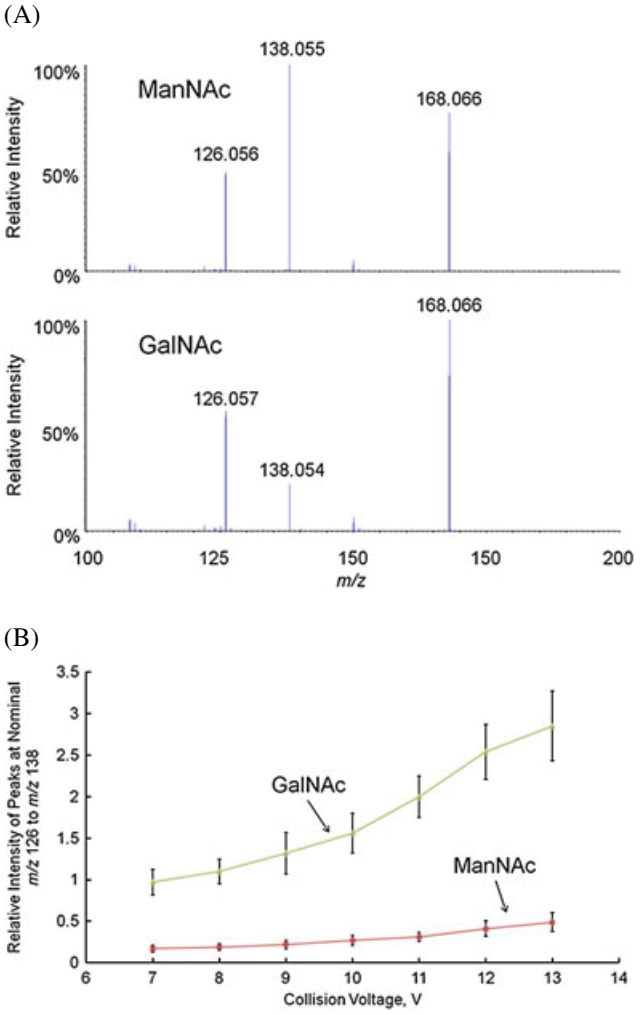

Figure 8. (A) Positive-ion APCI-QTOF-MS^2 spectra of the $[M - 3H_2O + H]^+$ ions at *m/z* 168.066 generated from 0.1 mM GlcNAc, ManNAc and GalNAc in methanol containing 10 mM NH_4Ac. Collision voltage was 9 V. Product ions are identified as $[M - 2H_2O + H]^+$ (*m/z* 186) and $[M - C_2H_8O_4 + H]^+$ (*m/z* 126). (B) Relative intensities of peaks at nominal *m/z* 126 to 138 in APCI-QTOF-MS^2 spectra at different collision voltages for GlcNAc, ManNAc and GalNAc.

and in-source fragment ions were also acquired, but the differentiation of GlcNAc, ManNAc and GalNAc was not achieved after careful inspection of the fragmentation patterns.

CONCLUSIONS

APCI-QTOF has been proven to be a convenient and efficient technique for the differentiation of underivatized monosaccharides. For the hexoses, Glc was differentiated from Man and Gal through the different MS^2 spectra of their $[M + NH_4]^+$ ions; Man was further differentiated from Gal through the different MS^2 spectra of their $[M - H]^-$ ions. For methyl D-glucopyranosides, MeαGlc was differentiated from MeβGlc through the different MS^2 spectra of their $[M + NH_4]^+$ ions. For the hexosamines, GlcN, ManN and GalN were differentiated by either the different *pseudo*-MS^3 spectra of their $[M + H]^+ \rightarrow [M + H - H_2O]^+$ ions or the different MS^2 spectra of their $[M + O_2]^-$ ions. For the *N*-acetylhexosamines, GlcNAc, ManNAc and GalNAc were differentiated by the different *pseudo*-MS^3 spectra of their $[M + H]^+ \rightarrow [M + H - H_2O]^+$ ions.

Although ESI has been widely utilized in the differentiation of monosaccharides, this report demonstrated that APCI could also be used to achieve the same goal. With ESI, $[M + NH_4]^+$, $[M + H]^+$, metal adduct and $[M + Cl]^-$ ions of monosaccharides were usually produced. In comparison with ESI, APCI is unable to produce metal adduct ions, but it could generate more abundant $[M - H]^-$ and unique $[M + O_2]^-$ ions due to the generation of O_2^- in the APCI process and its strong basicity. Both $[M - H]^-$ and $[M + O_2]^-$ ions were proven to be useful in the differentiation of certain underivatized monosaccharides.

Although single or triple quadrupole and ion trap mass spectrometry have been predominantly used in the analysis of (oligo- and mono-)saccharides, this report demonstrated that QTOF-MS could be advantageous due to its accurate mass measurement ability. While QTOF mass spectrometers are unable to perform MS^n analysis, it appeared to us that their *pseudo*-MS^3 capability could also meet the requirements as $MS^{n(>3)}$ analyses have been rarely used in the analysis of (oligo- and mono-)saccharides.

SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article.

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