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OLIGOSACCHARIDE ANALYSIS VIA ANION ATTACHMENT USING
NEGATIVE MODE ELECTROSPRAY (ES) AND MATRIX ASSISTED LASER
DESORPTION / IONIZATION (MALDI) MASS SPECTROMETRY

A Thesis

Submitted to the Graduate Faculty of the
University of New Orleans
in the partial fulfillment of the
requirements for the degree of

Master of Sciences
in
The Department of Chemistry

by
Yanjie Jiang

B.S., Nankai University of China, 1996

May 2004

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ABSTRACT

Eleven tested anions were able to form adducts with neutral oligosaccharides at low cone voltage in negative ion mode electrospray mass spectrometry. Among them, fluoride and acetate have the abilities to significantly enhance the absolute abundance of $[M-H]^-$ for neutral oligosaccharides. The chloride adduct has the best stability among all the adduct species investigated. For the above three anions, CID of adduct species may be used for structural determination of neutral oligosaccharides. In the presence of F^- and Ac^- , simultaneous detection of acidic oligosaccharides and neutral oligosaccharides was achieved. The ratio of Cl^- : non-Cl-containing product ions obtained in CID spectra of chloride adducts of disaccharides was used to differentiate anomeric configurations of disaccharides. Density functional theory (DFT) was employed to evaluate the optimized structures of chloride adducts of disaccharides.

The formation and decomposition of chloride adducts with oligosaccharides of different acidities were investigated in MALDI mass spectrometry.

CHAPTER I INTRODUCTION

The carbohydrates, also called saccharides, are the most abundant biological molecules and they are much more heterogeneous in structure than other biological molecules. It is well known that saccharides act as a major energy source (e.g. glucose and starch) and as a material that offers structural solidity (e.g. cellulose). Another interesting feature of saccharides is that they appear to play important roles in cell-surface recognition phenomena (1, 2). An understanding of the saccharide structure is essential for understanding the varied functions of carbohydrates in biological systems.

Mass spectrometry has emerged as an important technique for oligosaccharides analysis (3-17). Important innovations came through the introduction of Electrospray (ES) and Matrix Assisted Laser Desorption/ionization (MALDI). So far, oligosaccharide studies using mass spectrometry were mostly focused on the positive mode via adduct formation with H^+ or with metal ions, among which the group I (18-22), group II (23) and first row transition metal (24-26) were primarily used. It was found that lithium ion may more efficiently ionize oligosaccharides as compared to other alkali metal ions, and the fragments derived from cross-ring cleavage may provide linkage information (18, 19). The stereochemistry of glycosidic bond

configurations was studied by tandem mass spectrometry of diastereomeric cobalt-glucosyl-glucose disaccharide complexes (26).

Compared to the extensive number of studies done in positive mode mass spectrometry, fewer studies have been done to analyze oligosaccharides in the negative mode, with the exception of the sialylated oligosaccharides which contain at least one neuraminic acid moiety that readily deprotonates. Unlike the sialylated oligosaccharides, due to the lack of an acidic group, neutral oligosaccharides exhibit a low tendency to form $[M-H]^-$ by deprotonation. It has been reported (27, 28) that the use of nanoscale electrospray (tens of nL/min) can improve sensitivity for neutral oligosaccharides relative to conventional microliter/min flow ES. Pfenninger et al. (29, 30) employed negative mode nanospray together with quadrupole ion trap mass spectrometry to analyze underivatized neutral oligosaccharides from human milk and found that deprotonated molecular ions of neutral oligosaccharides follow distinct fragmentation rules. Chai et al. (31) investigated the fragmentation of neutral underivatized oligosaccharides using negative ES-MS with collision induced dissociation (CID) on a Q-TOF instrument. Trager et al. (32) showed that negative mode electrospray mass spectrometry can be used to characterize glycosidic bond linkages in underivatized disaccharides. Wheeler et al. (16) investigated the ES fragmentation spectra of 12 sialylated carbohydrates on a Q-TOF mass spectrometer. Often, sialylated oligosaccharides are detected with the best sensitivity in the negative mode, while the asialylated species perform best in the positive ion mode (33).

Biological samples are most often comprised of neutral oligosaccharides and acidic oligosaccharides. Most often, they are separated and analyzed independently from one another. Huang et al. (34) developed an HPLC/MS method that allows direct analysis of underivatized sialylated and neutral oligosaccharide via online ES mass spectrometry in the positive mode. Anion attachment is a promising method for analyzing neutral oligosaccharides and acidic oligosaccharides at the same time.

There have been few studies of the oligosaccharide analysis in the negative mode via the anion attachment approach. Wong et al. (35) reported the use of bisulfate as a dopant for oligosaccharides analysis in MALDI. Both the bisulfate adduct $[M+HSO_4]^-$ and the bisulfate-containing derivative $[M+HSO_4-H_2O]^-$ were observed for neutral oligosaccharides with four or more sugar units. Zhu et al. (36) investigated the use of chloride anion for the ranking of gas-phase basicities and chloride affinities of monosaccharides, and it was also found that the decomposition of chloride adducts can give glycosidic bond linkage information for disaccharides. Cai et al. (37) found that chloride anion can form stable anionic adducts with neutral oligosaccharides in MALDI-TOF. The current study is aimed at comparing the use of different anions for anion attachment and oligosaccharide analysis. The ability to distinguish α - and β -linked disaccharides via an anion attachment approach is also explored. Density functional theory calculations were employed to assist us in understanding the optimized anion disaccharide adduct structures.

CHAPTER II. EXPERIMENTAL

2.1. Chemicals

Maltose, cellobiose, gentiobiose, isomaltose, laminaribiose, trehalose and raffinose were obtained from Sigma Chemical Co. (St. Louis, MO). 3-sialyllactose was obtained from Glyko (Novato, CA). Ammonium chloride, ammonium bromide, ammonium fluoride, ammonium bisulfate, ammonium acetate, ammonium trifluoroacetate, ammonium oxalate, ammonium tartrate, ammonium dihydrogen phosphate, sodium nitrate and citric acid were obtained from Aldrich (Milwaukee, WI). All materials were used as received without further purification.

For electrospray experiment, both of the saccharides and the salts were prepared separately at 1mM in methanol/water = 9 : 1. The above solutions were mixed in a 1 :1 ratio to make a final solution concentration of 0.5 mM for both the disaccharides and salts.

For MALDI experiment, the oligosaccharide were prepared as 1 mM aqueous solution. Ammonium chloride and ammonium bromide were prepared at 1mM in methanol/water = 9 : 1 solution. Harmine matrix was purchased from Aldrich (Milwaukee, WI) and was prepared by saturating in ethanol.

2.2. Instrumentation

All electrospray mass spectrometry experiments were performed on a Quattro II triple quadrupole mass spectrometer (Micromass, Inc., Manchester, UK) equipped with an electrospray source. The ES capillary voltage was maintained at -2.5 kV. Sample solutions were introduced into the electrospray source at a flow rate of 3.3 $\mu\text{L}/\text{min}$ via a syringe pump. Nitrogen gas was used as both nebulizing gas and drying gas to improve ionization efficiency. Argon gas at a pressure of 0.23 - 0.25 mtorr was used as the collision gas to acquire collision induced dissociation (CID) spectra. Collision energies are given in the laboratory frame of reference (Elab). In comparison studies of various disaccharides, because all employed disaccharides have the same molecular weights, constant Elab values also mean uniform values in the center-of-mass frame of reference (Ecom). Mass spectra were acquired and processed using MassLynx software (version 3.0, Micromass).

All MALDI experiments were performed on a Voyager-Elite MALDI rTOF mass spectrometer (Applied Biosystems, Framingham, MA) equipped with a pulsed N₂ laser ($\lambda = 337 \text{ nm}$). The accelerating voltage was set at 20 kV. The sample was loaded into the wells of a gold-coated 100-well sample plate by thin-layer method. 1 μL of matrix solution was first deposited into the well on sample plate and dried to form a matrix layer. 1 μL of the oligosaccharide and chloride mixture was then deposited on

top of the matrix layer, and allowed to dry. Each acquired mass spectrum represents an average of 100-150 laser shots.

2.3. Density Functional Theory Calculations

Individual disaccharide structures were generated using PC SPARTAN Plus version 2.0 (Wavefunction, Inc., Irvine, CA) and energy minimized using the MMFF94 force field to obtain low energy starting structures. To build the chloride adduct structures, chloride ion was placed arbitrarily away from the disaccharide, and the preliminary structure was refined using a MMFF94 force field. The input structures were submitted to geometry optimization at the B3LYP/6-31G* level in Gaussian 98, Revision A.3 (38) (Gaussian, Inc., Pittsburgh, PA). For all adduct calculations, the multiplicity was set to 1 and the charge state was set to -1.

CHAPTER III. RESULTS AND DISCUSSIONS

3.1 oligosaccharide analysis in Electrospray (ES) mass spectrometry

Most oligosaccharides are not charged in aqueous solutions near neutral pH, unlike peptides and oligonucleotides, so the ionization efficiency is intrinsically low. In the positive mode, many reports have demonstrated that certain metal cations, especially group I metal cations (18-22), can aid the ionization of oligosaccharides via the formation of metal adducts. Also, the decomposition of such adducts may give richer information about the oligosaccharide structure than that of the protonated molecule. However, the number of reports detailing anion attachment approaches in the negative mode is far fewer than cation attachment with metal cations in the positive ion mode. In this work, the first investigation was focused on comparing different anions for neutral oligosaccharide analysis.

3.1.1. Anion selection

Using cellobiose as the model oligosaccharide, eleven anions, i.e., chloride, bromide, fluoride, bisulfate, acetate, trifluoroacetate, oxalate, tartrate, dihydrogen phosphate, nitrate and citrate, were tested for their abilities to form adducts with this disaccharide. Setting the cone voltage at 15 volts, all the tested anions can form observable adducts with cellobiose, and the obtained signal intensities are shown in Table 1. The ES-MS/MS studies investigating decompositions of [cellobiose + anion]⁻ adducts (Figure 1) show that F⁻ and Ac⁻ adducts gave purely disaccharide-related product ions. While Cl⁻ adducts produced both Cl⁻ and disaccharide-related product ions upon CID. All the other tested anions only gave the respective anions as product ions, hence, they are not useful for detailed disaccharide structure determinations. For this reason, the studies that follow were focused on the performance of F⁻, Ac⁻, and Cl⁻ as attaching anions to cellobiose.

Table1 adduct formation and decomposition of cellobiose with inorganic and organic acid anions(a)

	Structure	Molecular weight	Gas phase basicity (kJ/mol)(f)	[M-H] ⁻ (d)	[M+anion] ⁻ (d)	upon	CID(c)
						Anion(e)	Analyte related(e)
cellobiose		342	~1372	+	nd	nd	*
Fluoride	F ⁻	19	1529±0.84	++	++	nd	*
Chloride	Cl ⁻	35/37	1372.8±0.84	+	+++	*	*
Bromide	Br ⁻	79/81	1331.8±0.84	+	+++	*	nd
Nitrate(b)	NO ₃ ⁻	62	1329.7±0.84	+	+++	*	nd
Bisulfate	HSO ₄ ⁻	97	1265±10	++	+++	*	nd
Dihydrogen phosphate	H ₂ PO ₄ ⁻	97	1351±21	+	+++	*	nd
Acetate	CH ₃ COO ⁻	59	1427±8.4	++	++	nd	*
Trifluoro-acetate	CF ₃ COO ⁻	113	1328±8.4	+	+++	*	nd
Oxalate	HOOC-COO ⁻	89	N/A	++	+++	*	nd
Tartrate	HOOC-CH(OH)-CH(OH)-COO ⁻	149	N/A	++	++	*	nd
Citric acid	HOOC-CH ₂ -C(OH)(COOH)-CH ₂ -COO ⁻	191	N/A	++	++	*	nd

(a) ammonium salts are used as the anion source

(b) sodium salt is used as the anion source

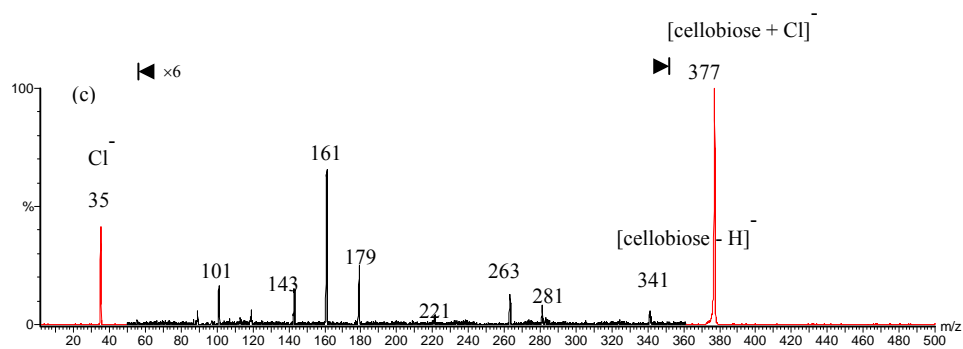
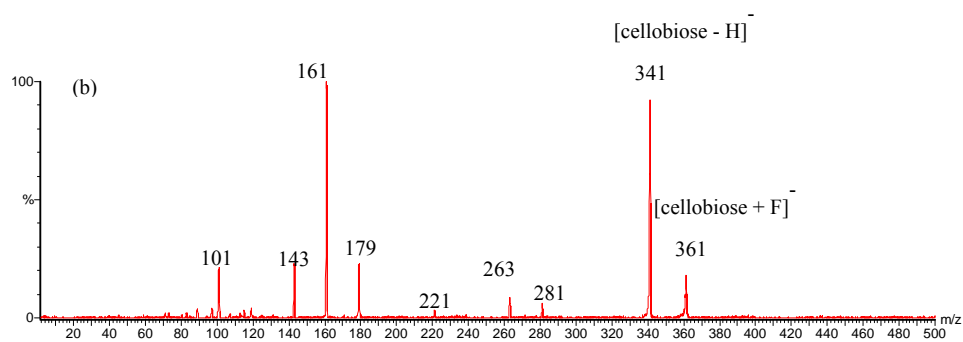
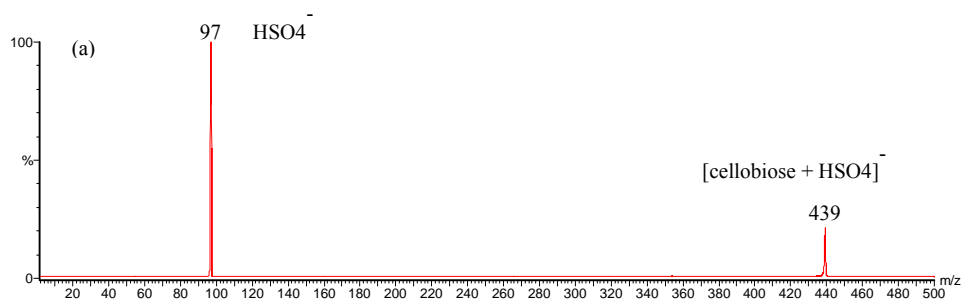
(c)all MS/MS CID experiments were performed under the same conditions. Cone voltage was set at 15V, collision voltage at 30V and the collision cell pressure at 2.4×10^{-4} mbar.

(d) + stands for the absolute intensity $< 10^5$, ++ stands for the absolute intensity $< 10^6$, +++ stands for the absolute intensity $< 10^7$.

(e) * stands for appearance, nd for not detected.

(f) For the gas phase basicities of anions, see ref 41.

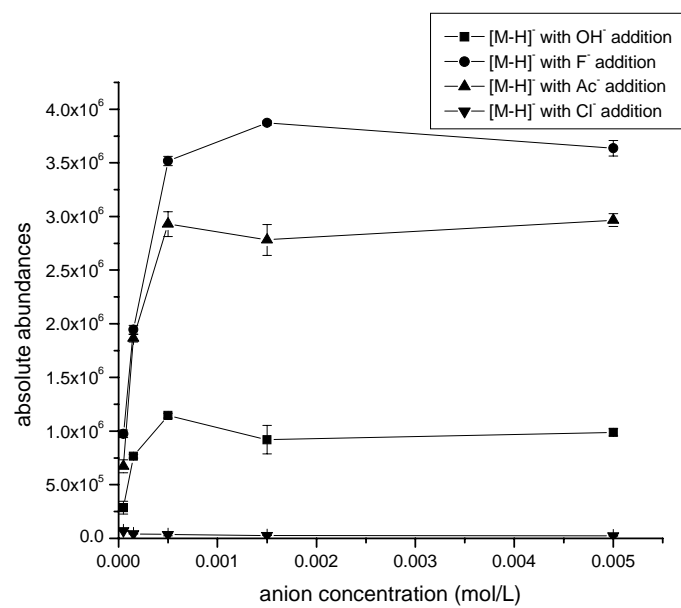
Figure 1. ES-MS/MS spectra of anionic adducts of cellobiose with different attaching anions (a) [cellobiose + HSO₄]⁻ as precursor ion, (b) [cellobiose + F]⁻ as precursor, (c) [cellobiose + Cl]⁻ as precursor.



3.1.2. Anion concentration study

Prior to the anion comparison, the anion concentration effect was evaluated. Figure 2 shows the trend of absolute abundances of $[M-H]^-$ in response to a changing anion concentration from 5×10^{-5} to 5×10^{-3} mol/L while holding cellobiose concentration at 5×10^{-4} mol/L with the cone voltage fixed at 20 V. The ratios of anion concentration versus cellobiose concentration were 1:10, 3:10, 1:1, 10:3 and 10:1 for every anion investigated. For OH^- , F^- and Ac^- as attaching anions, the absolute abundance of $[M-H]^-$ increased linearly with rising anion concentration up to 5×10^{-4} mol/L. Further augmentation of the anion concentration did not result in a clear increase in abundance of $[M-H]^-$ for any of the tested anions. For Cl^- , the absolute abundance of $[M-H]^-$ barely changed in response to an increasing anion concentration. Overall, this experiment indicated that an anion concentration of 5×10^{-4} mol/L gave the optimal signal intensity for solutions containing cellobiose at this same concentration, so the anion versus disaccharide ratio was set at 1:1 for later experiments.

Figure 2. Absolute abundances of deprotonated cellobiose, $[M-H]^-$, versus anion concentration. Cellobiose was held at a constant final concentration of 5×10^{-4} mol/L, anion concentration was set at 5×10^{-5} , 1.5×10^{-4} , 5×10^{-4} , 1.5×10^{-3} or 5×10^{-3} mol/L. Each point was the average of three measurements and error bars represent the standard deviation.



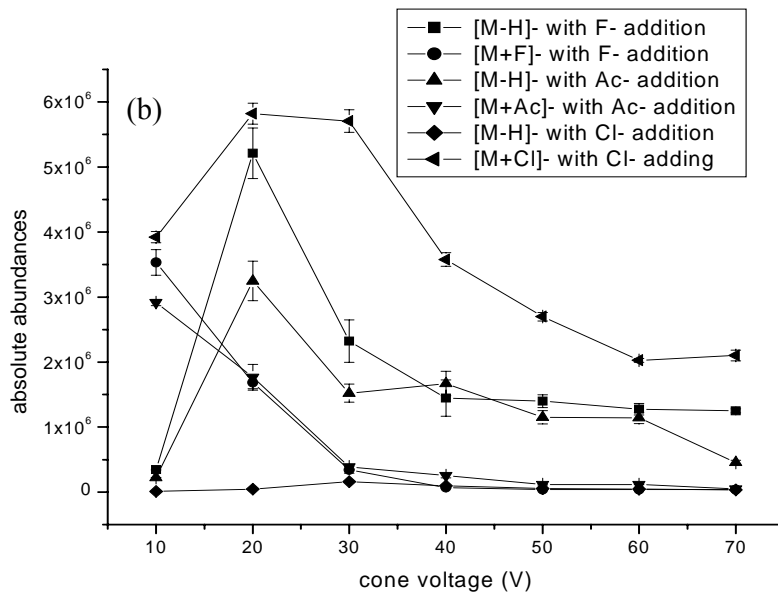
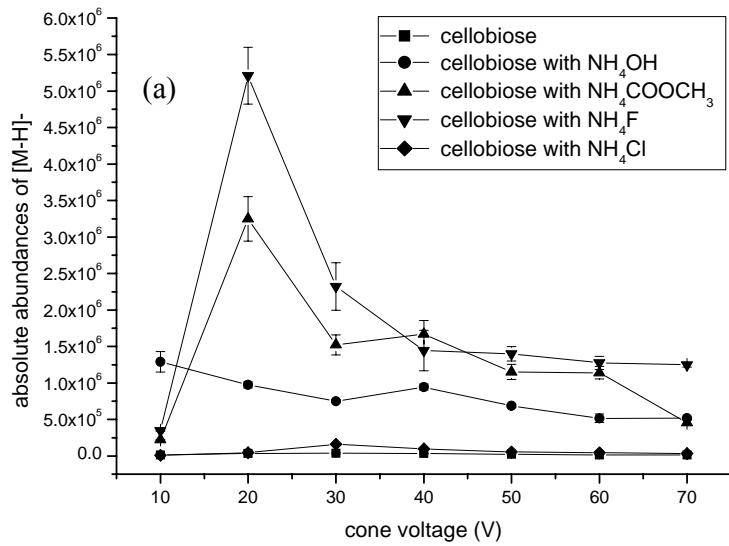
3.1.3. Anion comparison

When attaching anions are present, the employed cone voltage exhibited a large impact on the appearance of mass spectra. Figure 3a shows the absolute abundances of $[M-H]^-$ as a function of cone voltage. Five different solutions were tested, i.e., pure cellobiose solution (5×10^{-4} mol/L), and cellobiose mixed with ammonium salts of OH^- , CH_3COO^- , F^- , and Cl^- (cellobiose and the salt were each prepared at 5×10^{-4} mol/L in all cases). For the pure cellobiose solution, the best sensitivity for $[M-H]^-$ was obtained using cone voltages from 20 volts to 40 volts. At cone voltages below 20 volts, the transmission of all ions from the source to the quadrupole was low, while at cone voltages higher than 40 volts, decomposition of $[M-H]^-$ became substantial. Addition of base to sample solutions is often practiced to aid the formation of $[M-H]^-$ for analytes which do not readily undergo deprotonation. In this study, the effect of base addition to the solution was tested. It was found that the abundance of $[M-H]^-$ in the presence of OH^- was increased about 10 to 20 times relative to that of the pure cellobiose solution run under the same conditions. The influence of F^- addition was different from that of base addition. In the presence of F^- , using a cone voltage of 10 volts, the signal of $[M-H]^-$ was about 10 times higher than that obtained from pure cellobiose solution. Most notably, with a cone voltage setting of 20 volts, the abundance of $[M-H]^-$ was the highest in all the experiments performed in this study, i.e., 100 times higher than found with the pure cellobiose solution under the same instrumental conditions. With further increases in the cone voltage, the abundance of

$[M-H]^-$ decreased due to the decomposition of $[M-H]^-$. It should be noted that the presence of F^- gave a higher intensity peak for $[M-H]^-$ than the addition of OH^- . The general trend and tendencies observed during Ac^- addition were similar to those observed for F^- addition except that the absolute abundance of $[M-H]^-$ did not achieve the same high values at cone voltages of 20 volts and 30 volts. In contrast, the presence of Cl^- cannot increase the $[M-H]^-$ signal intensity, but instead, a high abundance $[M+Cl]^-$ peak is observed (Figure 3b). It can thus be concluded that the presence of OH^- , F^- or Ac^- can each increase the peak intensity of $[M-H]^-$, with F^- addition offering the most dramatic increase, followed by Ac^- , then OH^- . Of course, increases in $[M-H]^-$ abundance will benefit MS/MS studies aimed at structural elucidation of oligosaccharides.

The ability of F^- and Ac^- to increase the abundance of $[M-H]^-$ is proposed to result from the decomposition of the corresponding adducts involving loss of the protonated anion (neutral molecule). For the F^- example, Figure 3b shows that at a cone voltage of 10 volts, $[M+F]^-$ was the predominant peak relative to $[M-H]^-$. After increasing the cone voltage to 20 volts, the peak intensity of $[M+F]^-$ dropped off quickly while the peak intensity of $[M-H]^-$ climbed dramatically. Combining the information derived from Figure 3a that, in the absence of F^- , the intensity of $[M-H]^-$ did not increase significantly upon changing the cone voltage from 10 volts to 20 volts, it can be concluded that in the presence of F^- , the high abundance of $[M-H]^-$ is attributable to the decomposition of $[M+F]^-$ via HF loss. This conclusion is supported by CID

Figure 3. Ion abundance vs. cone voltage of deprotonated molecules and anionic adducts of cellobiose arising from five different solutions, i. e., pure cellobiose solution (5×10^{-4} mol/L), and cellobiose mixed with ammonium salt of OH^- , CH_3COO^- , F^- , and Cl^- (cellobiose and the salt were each prepared at 5×10^{-4} mol/L in all cases). (a) Absolute abundances of deprotonated cellobiose $[\text{M-H}]^-$ versus cone voltage, (b) Absolute abundances of deprotonated cellobiose $[\text{M-H}]^-$ and cellobiose adduct with anions (A) $[\text{M+A}]^-$ versus cone voltage. Each point was the average of three measurements and error bars show the standard deviation.



studies that show loss of neutral HF from $[M+F]^-$ (Figure 1b). At a cone voltage of 20V, the internal energy of $[M+F]^-$ was high enough to cause the adduct to undergo substantial decomposition to form $[M-H]^-$, but low enough to prevent consecutive decomposition of $[M-H]^-$ resulting in the highest abundance of $[M-H]^-$ in the whole study. Under such optimized conditions, employing addition of NH_4F at 5×10^{-5} mol/L, cellobiose displayed a limit of detection ($S/N = 3$) of 1.3×10^{-8} mol/L (flow rate of $1.7 \mu\text{L}/\text{min}$ for an average of 1 minute acquisition time (22 fmol consumed)).

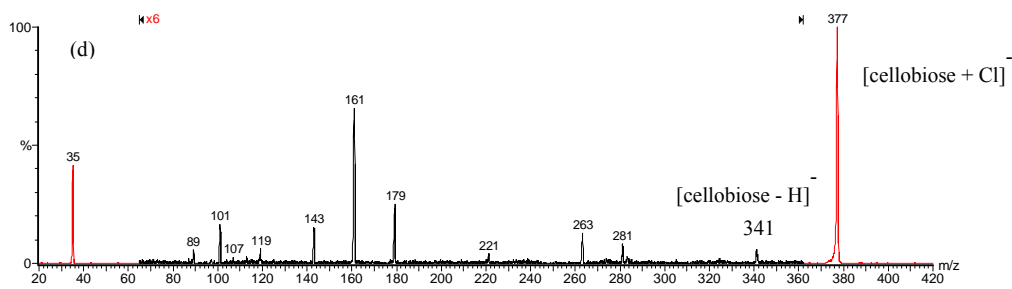
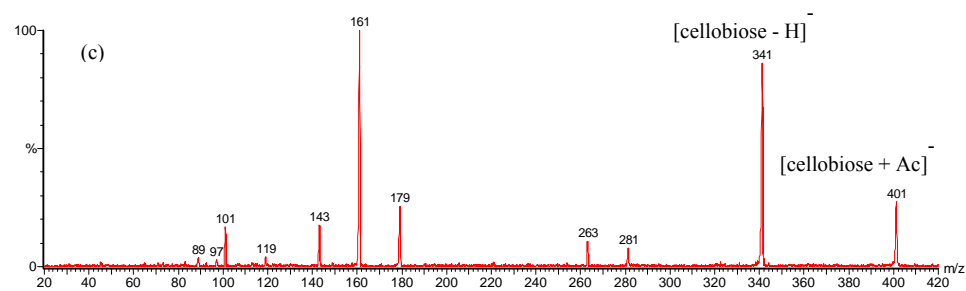
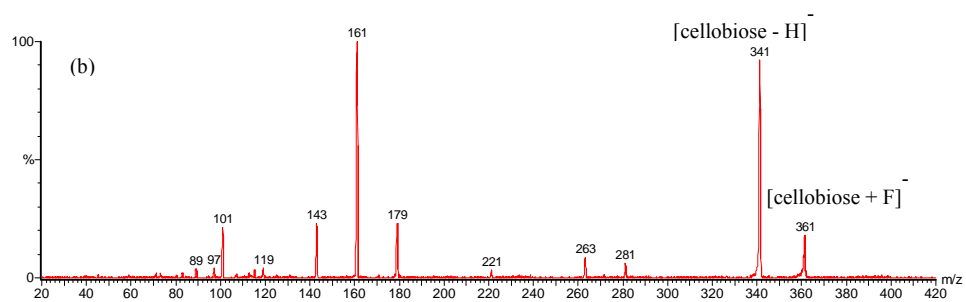
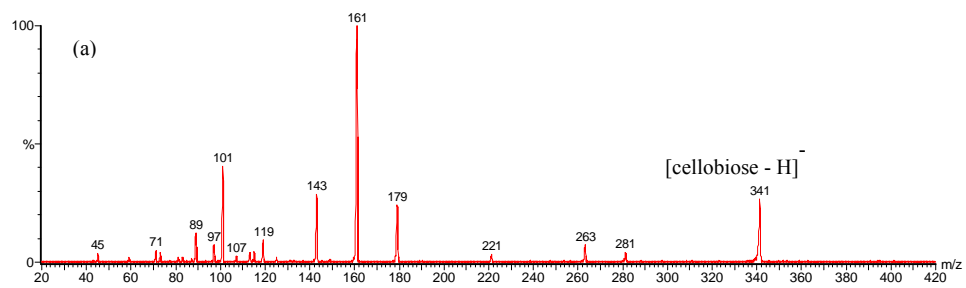
The stability of anionic adducts varied for the different attaching anions (Figure 3a). Chloride adduct appeared to be the most stable adduct species. Notably, under all the conditions tested, chloride adduct was always the predominant species compared to $[M-H]^-$ in agreement with previous studies (39, 40). The underlying reason for this phenomenon has been attributed to the degree of matching between the proton affinity of the deprotonated molecules ($[M-H]^-$), vs. that of the anion.

3.1.4. CID of anionic adducts of disaccharides for linkage differentiation

ES-MS/MS studies revealing product ion spectra of $[M+F]^-$ and $[M+Ac]^-$ of cellobiose gave purely disaccharide related product ions, while $[M+Cl]^-$ produced both Cl^- and disaccharide related product ions upon CID. The decomposition of fluoride adducts was a straight-forward process, with F^- showing strong attraction for one proton leading to loss of HF, thus leaving $[M-H]^-$ and the possibility for consecutive decompositions. The decomposition of chloride adducts revealed a competition between Cl^- formation and disaccharide-related product ion formation. This contrast can be explained by considering that F^- had a higher gas phase basicity (Table 1) (41) than the disaccharide, so F^- had a higher tendency to attract the proton than the disaccharide. On the other hand, Cl^- has a gas phase basicity similar to that of the disaccharide, so the competition for the available proton was more even.

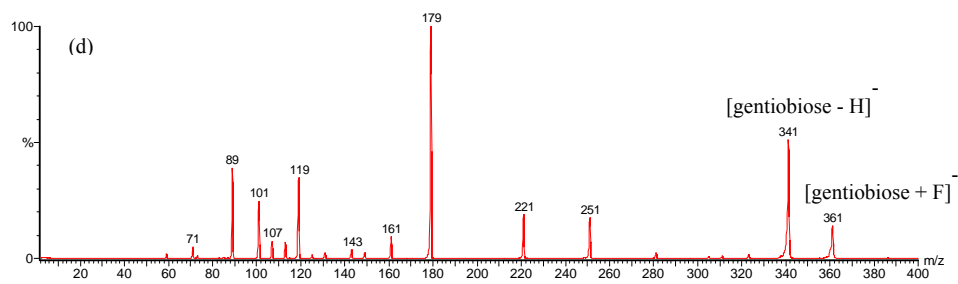
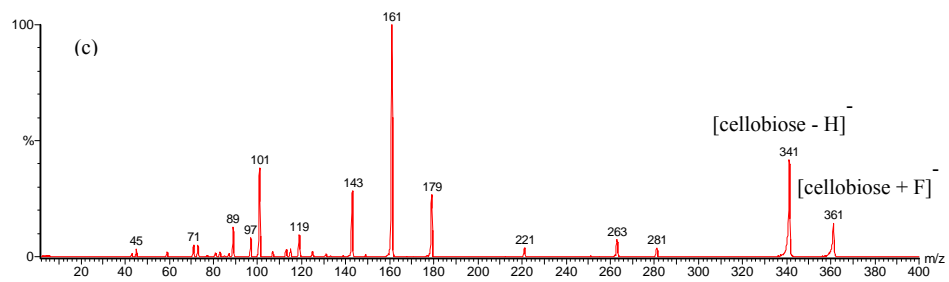
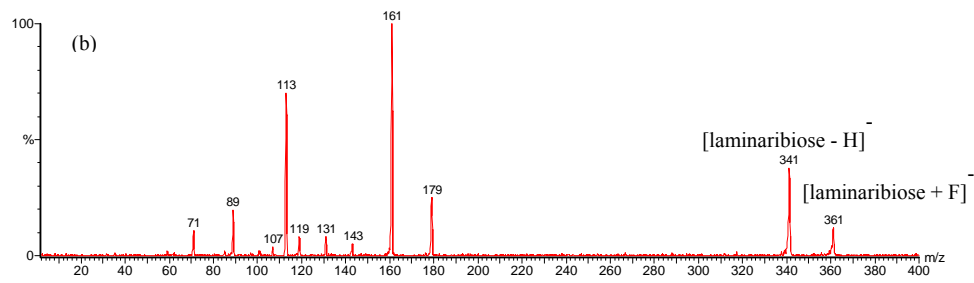
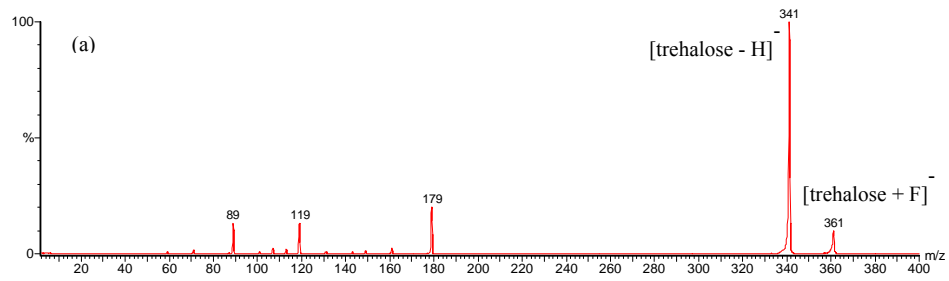
As mentioned previously, F^- , Cl^- and Ac^- adducts may undergo decomposition to produce structurally-informative fragment ions. Figure 4 shows that the fragmentation patterns pertaining to the analyte structure were similar to each other for all adducts investigated. Furthermore, the fragmentation patterns were quite similar with that of $[M-H]^-$, which indicated that the first step of the decomposition for each adduct was the loss of the protonated anion (neutral) prior to consecutive decomposition of $[M-H]^-$.

Figure 4. ES-MS/MS spectra of deprotonated cellobiose $[M-H]^-$ and anionic adducts of cellobiose employing the following precursor ions: (a) $[M-H]^-$ (b) $[M+F]^-$ (c) $[M+Ac]^-$ and (d) $[M+Cl]^-$.



F⁻ has been used to form adducts with glucopyranosyl-glucose disaccharides with different linkage positions and the decomposition of each adduct was studied (Figure 5). The fragmentation pattern of [M+F]⁻ was quite similar to the fragmentation pattern of [M-H]⁻ upon in-source decomposition reported by Trager et al. (32), which reinforces the notion that the first step of decomposition of [M+F]⁻ is loss of HF. In addition, the CID product ion spectrum of [M+F]⁻ shows great similarity with that of [M+Cl]⁻ except for the appearance of Cl⁻ in the latter (36). Thus, the decomposition of [M+F]⁻ can also be used for linkage position differentiation by inspection of the peaks characteristic of cross-ring cleavages appearing at m/z values higher than m/z 179, and the intensity ratio of m/z 161 versus m/z 179 (glycosidic bond cleavages at either side of the oxygen). The high signal-to-noise ratio in CID spectra obtained using [M+F]⁻ as parent ions was largely due to the high abundances of the precursor adducts as compared with [M-H]⁻, and to the lack of competition during the first decomposition step, unlike the case of [M+Cl]⁻.

Figure 5. ES-MS/MS spectra of fluoride adducts of various glucopyranosyl-glucose disaccharides having different glycosidic bond linkages: (a) trehalose (Glc α 1-1Glc) (b) laminaribiose (Glc β 1-3Glc) (c) cellobiose (Glc β 1-4Glc) and (d) gentiobiose (Glc β 1-6Glc)



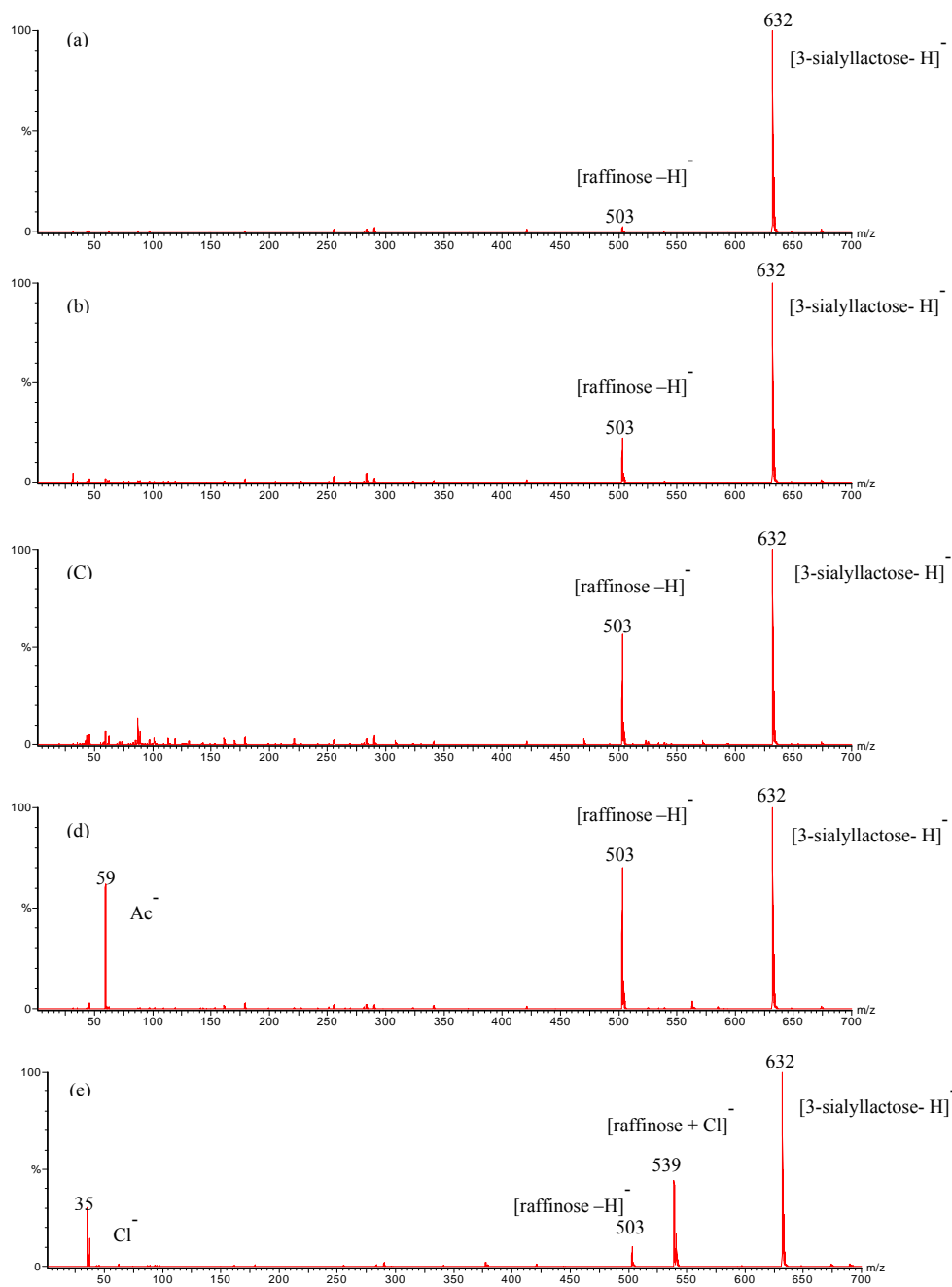
3.1.5. Anion attachment for simultaneous analysis of neutral and acidic oligosaccharides

In the oligosaccharide pool from biological sources, it is often the case that neutral oligosaccharides and acidic oligosaccharides exist simultaneously. Most of the reports treated the analysis of neutral and acidic oligosaccharides separately. Because F^- and Ac^- addition can increase the intensity of $[M-H]^-$ at cone voltages higher than 20 volts, these two anions can be used for the simultaneous analysis of neutral and acidic oligosaccharides in negative mode mass spectrometry.

Raffinose was chosen as a model neutral oligosaccharide and 3-sialyllactose as a model acidic oligosaccharide; the simultaneous detection of these compounds is demonstrated in Figure 6. Previous studies have indicated that a cone voltage between 20 volts and 40 volts allows the detection of $[M-H]^-$ for oligosaccharides, so for this study, the cone voltage was set at 40 volts. With the two oligosaccharides present in equal concentrations (5×10^{-4} mol/L), Figure 6a shows that raffinose (mol. wt. = 503) was barely detectable compared with 3-sialyllactose (mol. wt. = 632) in pure aqueous solution. Figure 6b shows that the signal intensity of raffinose was enhanced to 1/5 of the intensity of 3-sialyllactose with the addition of base (5×10^{-4} mol/L NaOH). Figure 6c and 6d show that the response of deprotonated raffinose was about 2/3 that of 3-sialyllactose upon addition of F^- and Ac^- (5×10^{-4} mol/L). F^- or Ac^- addition was

thus superior to OH^- in that the response was better, and the caustic nature of OH^- addition may affect analyte stability in the solution.

Figure 6. ES-MS spectra of raffinose and 3-sialyllactose at the concentration of 5×10^{-4} mol/L (a) in pure aqueous solution (b) adding 5×10^{-4} mol/L OH^- (c) adding 5×10^{-4} mol/L F^- (d) adding 5×10^{-4} mol/L Ac^-



3.1.6. Chloride adducts for differentiating anomeric configuration of disaccharides

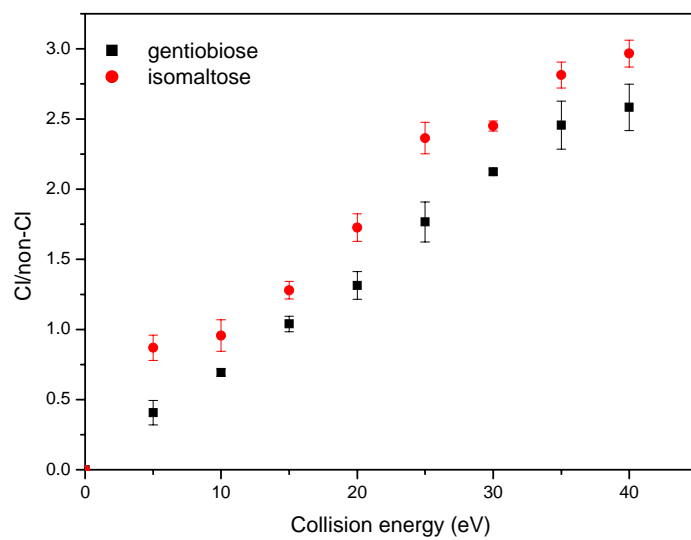
A study was undertaken to investigate whether ES-MS/MS of chloride adducts could be used as a means to differentiate stereoisomeric disaccharides. Two pairs of disaccharides were obtained: gentiobiose and isomaltose, plus maltose and cellobiose. These isomeric pairs differ only in the anomeric configuration at the glycosidic bond. Upon low-energy CID, there are two lowest energy possibilities for chloride adducts of these disaccharides to undergo decompositions. One pathway is to simply form chloride (pathway 1), while the other pathway is loss of HCl to leave $[M-H]^-$ (pathway 2) which may undergo consecutive decomposition. For each disaccharide pair, Figures 7a and 7b show that the ratio of Cl^- / non- Cl^- product ions is heavily dependent on the CID conditions. In both figures, as the collision voltage was increased, the ratio of Cl^- / non- Cl^- rose. At low collision energy (E_{lab}), the ratio of Cl^- / non- Cl^- product ions was below 1, which indicated that pathway 1 was less favorable than pathway 2. At high collision energy, the ratio of Cl^- / non- Cl^- product ions was above 1, which indicated that pathway 1 had become more favorable than pathway 2. Considering the numerous available electropositive hydrogens (e.g. each hydroxyl hydrogen on the disaccharides), it is reasonable to assume that a distribution of structures of chloride adducts exists, with Cl^- attached to various hydroxyl hydrogens, including conformations exhibiting multiple hydrogen bonding (40). However, in order to decompose via loss of neutral HCl in preference to departure of Cl^- , chloride may be required to be situated near to the most acidic proton (the anomeric reducing

end hydroxyl hydrogen). At low collision energy, it is conceivable that energy was consumed in reorienting the Cl^- closer to the most acidic hydrogen via movement along the potential energy surface of the ion-dipole complex. This mechanism can account for the tendency to lose neutral HCl at low collision energy. At high collision energy, however, it is rationalized that the excess energy present tended to favor Cl^- departure before it could reach the most acidic proton, leading to a higher rate of Cl^- loss vs. HCl loss at high collision energy.

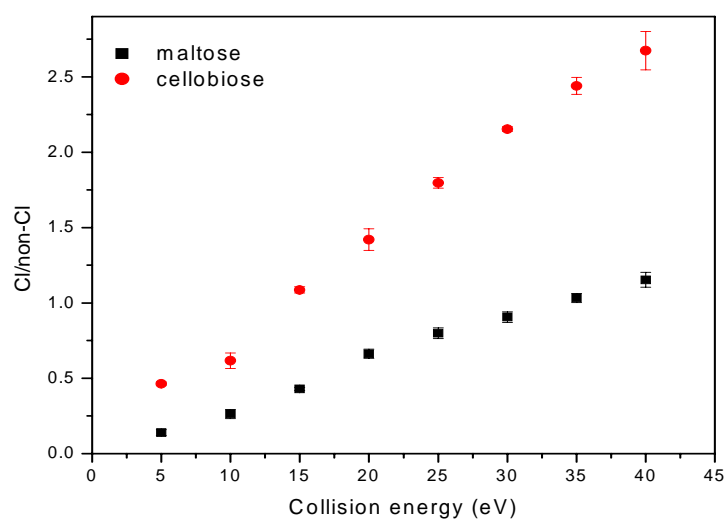
For the 1-6 linkage disaccharide tested, the Cl^- / non-Cl was always higher for the α isomer (isomaltose) than for the corresponding β isomer (gentiobiose) at every collision energy (Figure 7a). Conversely, for the 1-4 linkage disaccharide tested, the Cl^- / non-Cl was always higher for the β isomer (cellobiose) than the corresponding α isomer (maltose, Figure 7b). The differentiation of anomeric configuration is thus possible based upon the different tendency to form Cl^- vs. $[\text{M-H}]^-$ and consecutive decompositions.

Figure 7. The relative ratio of Cl/non-Cl product ions versus collision energy (laboratory frame of reference) for anomeric pairs: (a) gentiobiose (Glc β 1-6Glc) and isomaltose (Glc α 1-6Glc) (b) cellobiose (Glc β 1-4Glc) and maltose (Glc α 1-4Glc).

(a)



(b)



3.1.7. Density functional theory calculation

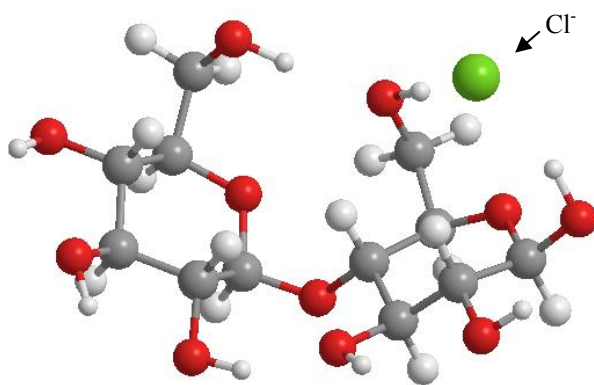
Computer modeling is a good approach to visualize the interaction between a neutral analyte and the attaching anion. Efforts have been made to study halide adducts of olefins (42), chloride adducts of acetylene (43) and halide adducts of α -D-glucose (40). The aim in this study was to gain insight into the probable attaching position of chloride anion onto cellobiose and maltose disaccharides. Carbohydrates have both extensive hydrogen bonding possibilities and anomeric effects, so modeling results are sensitive to the level of calculation. B3LYP/6-31G* was chosen because it has been used previously to study the minimum energy conformation of monosaccharides (44-49) and it is a higher level calculation compared to the HF/6-31G* level. The HF/6-31G* level of theory was employed to calculate relaxed potential energy surfaces for 12 analogs of disaccharides (50). In the latter study, the analogs were made by replacing glucose with tetrahydropyran, thus hydrogen bonding effects were ignored. It was found that the torsional energy and the simple bulk of ring structures were major factors in determining favored disaccharide conformations.

In this study, the structures of chloride adducts with cellobiose and maltose were optimized at B3LYP/6-31G* level using Gaussian 98, Revision A.3 (38). The initial adduct structures were built via PC SPARTAN plus version 2.0 on a template of cellobiose or maltose optimized by a MMFF94 conformer search. Chloride anion was placed away from the disaccharide and another MMFF94 conformer search was

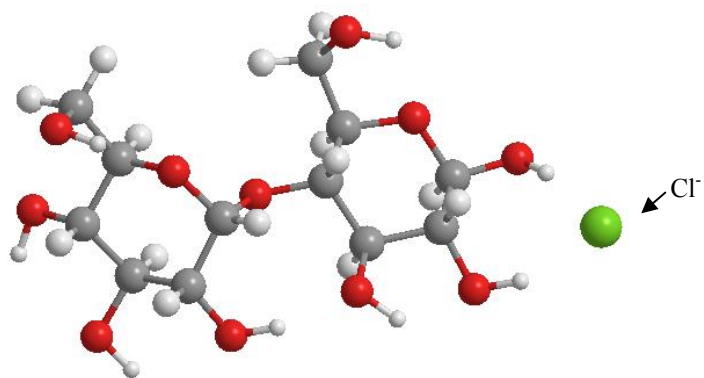
performed. The conformer search gave several geometries depending upon the initial placement of the anions. The adduct structures of different geometries were further optimized by B3LYP/6-31G* calculations with the parameters set at the same criteria. Four pathways were checked for force and displacement convergence. Only certain input structures can give four-pathway convergence (maximum energy, RMS energy, maximum displacement, and RMS displacement), which marks a successful optimization procedure. Figure 8 shows the optimized structures for chloride adducts of cellobiose and maltose. Computer modeling suggests that Cl⁻ exhibits different favored binding positions for the two anomeric configurations. But, each time, the binding involved the hydrogen of the anomeric hydroxyl group in the reducing ring. This may be rationalized by considering that the electronegative chloride interacts with the most acidic hydrogen in the disaccharide molecule. Indirect evidence for this assertion was also given by the observation that the chloride adduct had a similar product ion spectrum as compared to [M-H]⁻, hence the first step in chloride adduct decomposition was [M-H]⁻ formation via loss of HCl; the least energy would be consumed in the latter loss when Cl⁻ was bound to the most acidic proton of the disaccharide. In addition, the optimized structure indicated that multiple hydrogen bonding was contributing to the high stability of the chloride adducts.

Figure 8. Computer modeling structure of the chloride adduct optimized at B3LYP/6-31G* level using Gaussian 98, Revision A.3. (a) chloride adduct with cellobiose (b) chloride adduct with maltose. In each case, the reducing end is on the right.

(a)



(b)

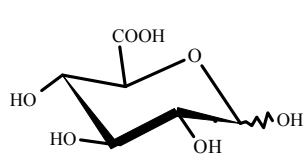
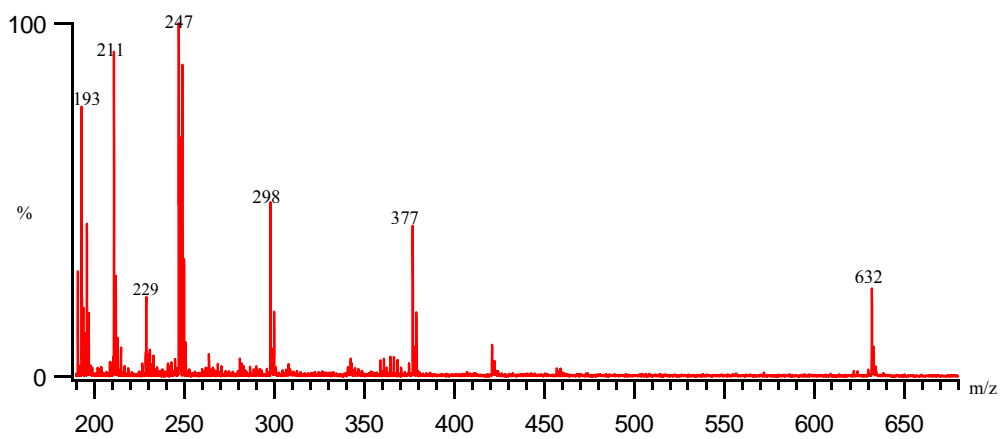


3.2. Oligosaccharide analysis in MALDI mass spectrometry

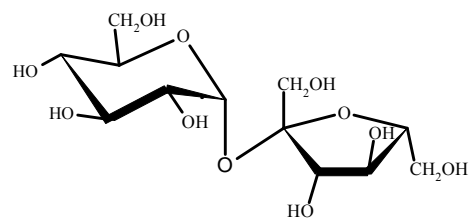
The previous part on ES mass spectrometry study showed that chloride adducts of disaccharides appeared to be the most stable species among all the anionic adducts (figure 3b). In addition, investigations by our group have found that chloride can form stable anionic adducts using MALDI-TOF (37). The effective matrixes for such purpose have gas phase acidities lower than or close to that of HCl (1373 kJ/mol). In this study, the influence of the oligosaccharide acidity, and the competition for available chloride anions in MALDI have been investigated.

Three oligosaccharides, D-glucuronic acid, sucrose, and 3-sialyllactose were chosen as model compounds (Figure 9). Sucrose is a neutral disaccharide, D-glucuronic acid contains one carboxylic acid group in the C6 of the sugar ring and 3-sialyllactose has one carboxylic acid group in the C2 of the sugar ring, which is a typical kind of acidic oligosaccharide in biological systems. An aqueous mixture of D-glucuronic acid : sucrose : 3-sialyllactose : NH_4Cl : HCl = 1 : 1 : 1 : 2.5 : 2.5 was prepared, then a 1 μL aliquot of the mixture was deposited on a dried layer of harmine that had been predeposited on a MALDI sample plate. The negative ion reflectron MALDI spectrum of the mixture is shown in Figure 9, which clearly indicates that under the same condition, 3-sialyllactose (containing one sialic acid moiety) exclusively forms $[\text{M}-\text{H}]^-$ (m/z 632), neutral sucrose forms only $[\text{M}+\text{Cl}]^-$ (m/z 377, 379), but D-glucuronic acid (containing one carboxylic acid moiety) forms both

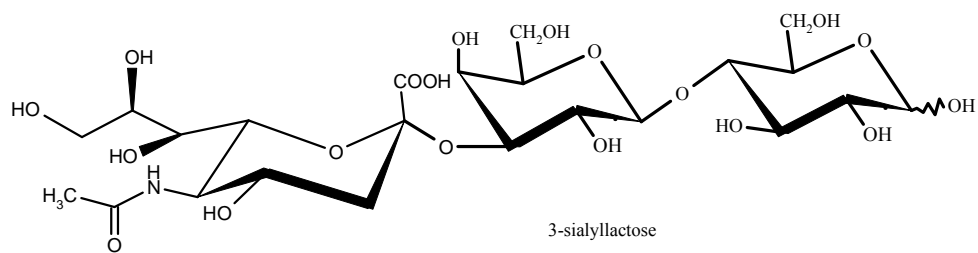
Figure 9 MALDI (reflectron mode) mass spectrum of D-glucuronic acid, sucrose, and 3-sialyllactose mixed with HCl and ammonium chloride, using the “thin layer” method in sample preparation and harmine as the matrix. Ions at m/z 193 and m/z 229 correspond to $[M-H]^-$ and $[M+Cl]^-$ of D-glucuronic acid; m/z 377 corresponds to $[sucrose + Cl]^-$; m/z 632 corresponds to $[3-sialyllactose - H]^-$. $[Harimine - H]^-$ appears at m/z 211, while $[harimine + Cl]^-$ appears at m/z 247 (saturated).



D-glucuronic acid

GB[M-H]⁻ ~ 1331 kJ/mol

Sucrose

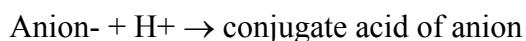
GB[M-H]⁻ ~ 1373 kJ/mol

3-sialyllactose

GB[M-H]⁻ ~ 1265 kJ/mol

$[M-H]^-$ (m/z 193) and $[M+Cl]^-$ (m/z 229, 231). This observation showed that acidity of the oligosaccharide plays an important role in the formation of $[M+Cl]^-$ in MALDI. Chloride adduct ions appearing at m/z 298 and 300 contain one nitrogen, they are the result of chloride attachment to neutral mass 263, which is the remainder of the N-acetylneuraminic acid (sialic acid) moiety subsequent to loss of HCOOH.

One way of evaluating the acidity of the compound is by the value of the gas phase basicity of its corresponding anion. The gas phase basicity of the anion is defined as the negative of the free energy change ($-\Delta G_{\text{anion-}}$) of the reaction:



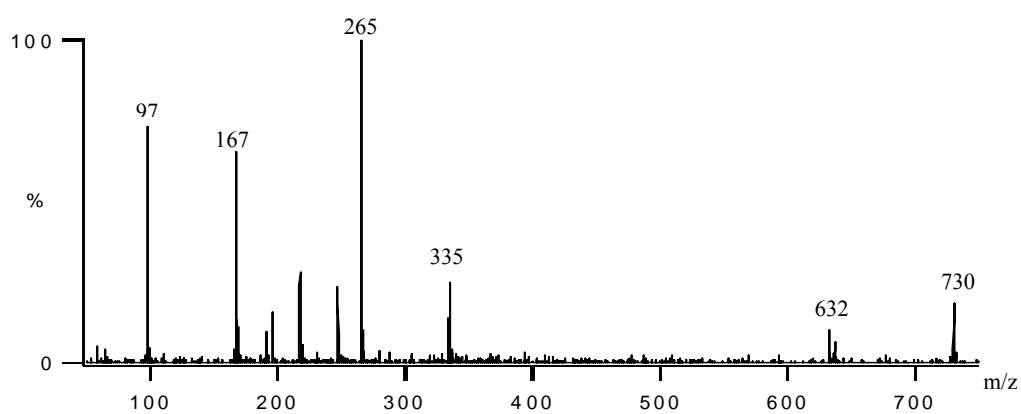
The higher the gas phase basicity of the anion, the less acidic is the acid. For the anions used in this project, they appeared in the references as: $GB_{Cl^-} \sim 1373$ kJ/mol, $GB_{Br^-} \sim 1331$ kJ/mol and $GB_{HSO_4^-} \sim 1265$ kJ/mol (Table 1).

A previous study (36) has shown that upon CID, the chloride adduct of sucrose yields chloride and $[M-H]^-$ in approximately equal abundances under low-energy collision conditions. This implies that $[\text{sucrose} - H]^-$ has a gas-phase basicity very close to that of chloride. The gas-phase basicities of $[3\text{-sialyllactose} - H]^-$ and $[D\text{-glucuronic} - H]^-$ are not available in the literature. We have evaluated the gas-phase basicities of $[3\text{-sialyllactose} - H]^-$ and $[D\text{-glucuronic} - H]^-$ via MALDI-PSD and ES-MS/MS approaches. 3-sialyllactose does not form $[M+Cl]^-$ in MALDI with harmine as matrix, but it does form $[M + HSO_4]^-$ with THAP as matrix

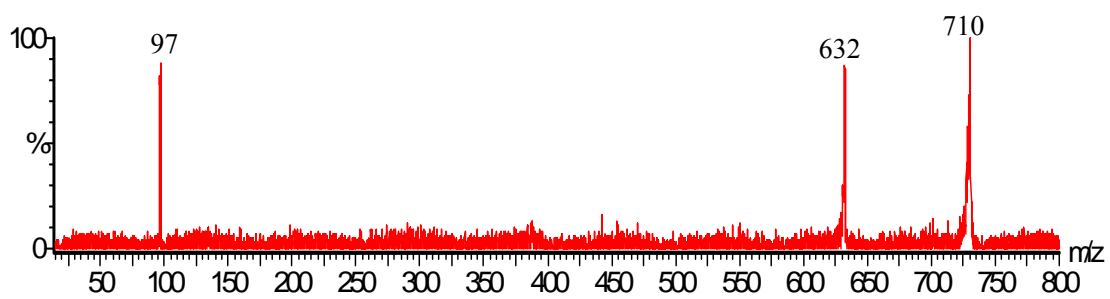
Figure 10 (a) MALDI mass spectrum of 3-sialyllactose mixed with H_2SO_4 , Ions at m/z 632 and m/z 730 correspond to $[\text{M}-\text{H}]^-$ and $[\text{M}+\text{HSO}_4]^-$ of 3-sialyllactose; m/z 97 corresponds to HSO_4^- ; $[\text{THAP} - \text{H}]^-$ appears at m/z 167, while $[\text{THAP} +\text{HSO}_4]^-$ appears at m/z 265; m/z 335 is believed to be the dimer of THAP.

(b) ES-MS/MS mass spectrum of $[\text{3-sialyllactose} + \text{HSO}_4]^-$, m/z 97 corresponds to HSO_4^- and m/z 632 corresponds to $[\text{3-sialyllactose} -\text{H}]^-$

(a)



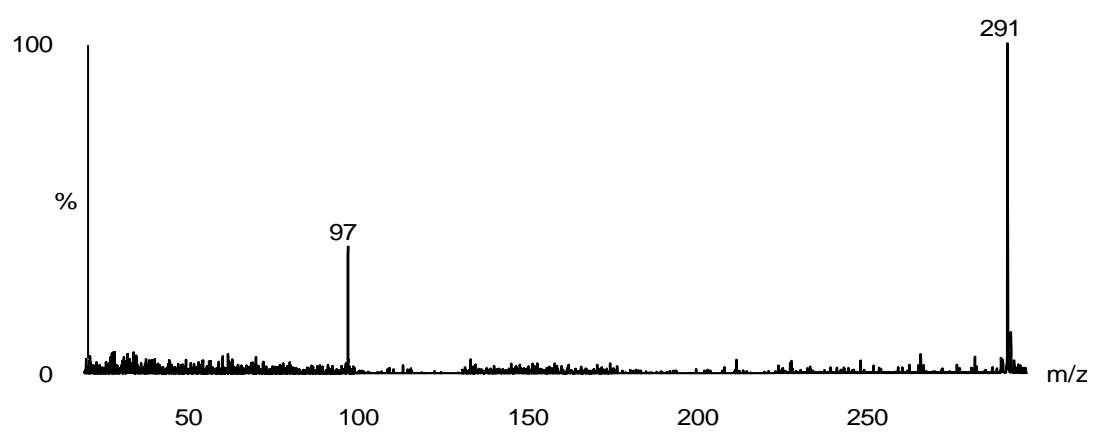
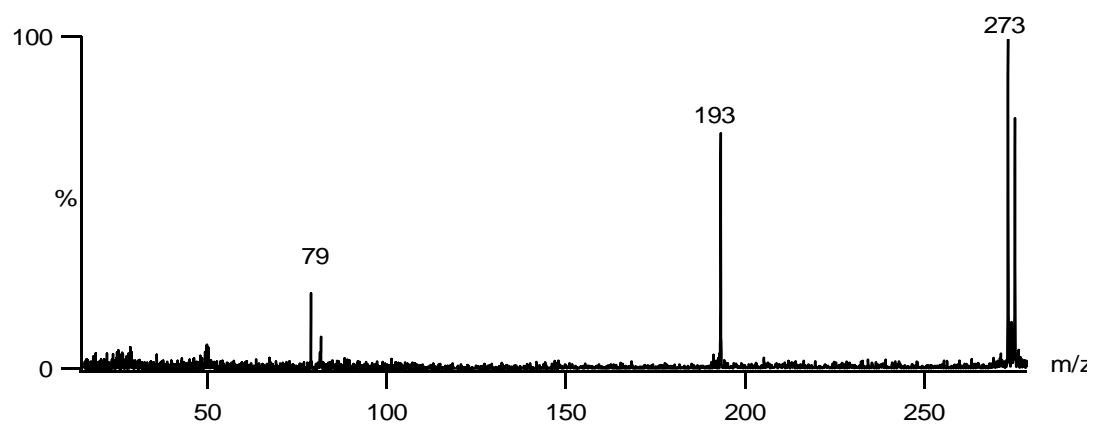
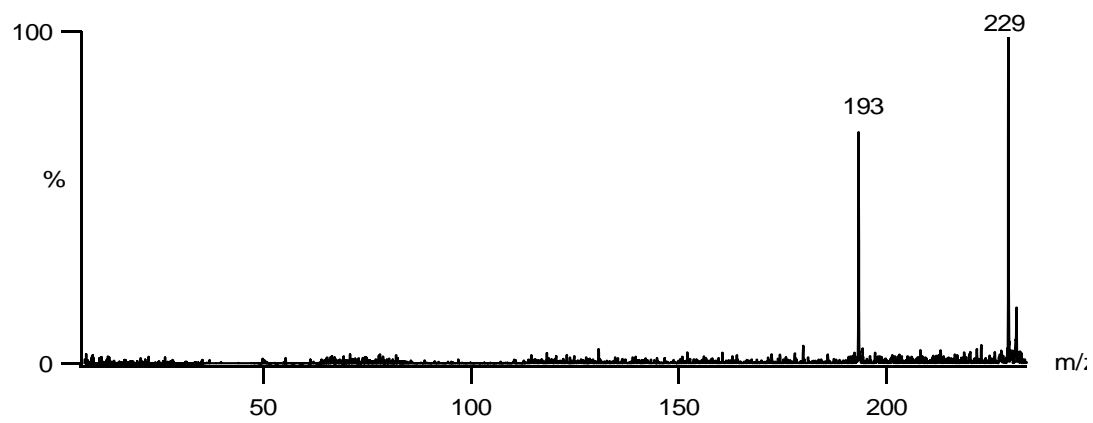
(b)



(Figure 10a). However, the abundance of $[3\text{-sialyllactose} + \text{HSO}_4]^-$ formed in MALDI is not high enough for a PSD experiment. Instead, we turned to ES-MS and ES-MS/MS for the evaluation of the $[\text{M-H}]^-$ gas-phase basicity of 3-sialyllactose. In electrospray under the same conditions that favor adduct formation, 3-sialyllactose does not form observable adducts with chloride, but it does form adducts with bisulphate. ES-MS/MS experiments reveal that upon CID, the precursor ion $[3\text{-sialyllactose} + \text{HSO}_4]^-$ gives both $[\text{M-H}]^-$ and bisulphate as product ions, and the two fragment ions have nearly the same abundances (Figure 10b). These compiled results provide evidence that $[3\text{-sialyllactose} - \text{H}]^-$ has a gas-phase basicity very close to that of bisulphate (1265 kJ/mol) .

As for D-glucuronic acid, it forms adducts with chloride, bromide, and bisulphate in MALDI. PSD spectra in Figure 11 show that $[\text{D-glucuronic acid} + \text{Cl}]^-$ yields $[\text{D-glucuronic acid} - \text{H}]^-$ as the major product ion (Figure 11a), $[\text{D-glucuronic acid} + \text{HSO}_4]^-$ yields bisulphate as the major product ion (Figure 11c), while $[\text{D-glucuronic acid} + \text{Br}]^-$ yields both bromide and $[\text{D-glucuronic acid} - \text{H}]^-$ as product ions (Figure 11b). ES-MS/MS experiments were also performed on the three adducts of D-glucuronic acid and corroborative results were obtained. It can thereby be concluded that D-glucuronic acid has a gas-phase basicity not far from that of bromide (1332 kJ/mol). In addition to allowing approximate evaluation of this gas-phase basicity, Figure 11 reinforces the point that chloride adducts yield

Figure 11 PSD spectra of D-glucuronic acid adducts with: (a) chloride, (b) bromide and (c) bisulphate anions. Upon decomposition, $[\text{D-glucuronic acid} + \text{Cl}]^-$ at m/z 229 and 231 yields $[\text{D-glucuronic acid} - \text{H}]^-$ at m/z 193; $[\text{D-glucuronic acid} + \text{Br}]^-$ at m/z 273 and 275 yields both $[\text{D-glucuronic acid} - \text{H}]^-$ at m/z 193, and bromide anion at m/z 79 and 81; while $[\text{D-glucuronic acid} + \text{HSO}_4]^-$ at m/z 291 yields only bisulphate anion at m/z 97.



structurally-informative product ions upon decomposition. This is clearly not the case for decomposition of bisulphate adducts.

For the three compounds 3-sialyllactose, D-glucuronic acid and sucrose, the $[M-H]^-$ gas-phase basicities follow the trend: $GB [sucrose - H]^- > GB [D\text{-glucuronic acid} - H]^- > GB [3\text{-sialyllactose} - H]^-$. From the pattern of chloride adduct formation for these three compounds shown in the conventional MALDI mass spectrum (Figure 9), it can be rationalized that under the same condition, relatively acidic compounds (low $[M-H]^-$ gas-phase basicity), such as 3-sialyllactose, will not form stable adducts with chloride. Instead, they will form $[M-H]^-$. On the other hand, neutral compounds such as sucrose will form adducts with chloride in overwhelming preference to $[M-H]^-$. Lastly, mildly acidic compounds, such as D-glucuronic acid, can yield both chloride adducts and $[M-H]^-$ in the conventional MALDI mass spectrum. In the chloride attachment approach, oligosaccharides, from neutral to acidic compounds, can be detected as $[M-H]^-$ or $[M+Cl]^-$, or both forms in the same run in MALDI-TOF.

CHAPTER IV. CONCLUSIONS

Fluoride anion and acetate anion were found to have the ability to significantly increase $[M-H]^-$ formation for neutral oligosaccharides, even in the presence of acidic oligosaccharides which may severely suppress the formation of $[M-H]^-$ from neutral oligosaccharides in ES mass spectrometry. Addition of these anions may find potential application in the analysis of oligosaccharide mixtures present in biological samples that contain both neutral oligosaccharides and acidic oligosaccharides. The CID product ion spectra of fluoride, acetate and chloride adducts with oligosaccharides showed great similarity with the CID spectra of $[M-H]^-$ of the same disaccharide, so the decomposition of these anionic adducts of disaccharides was believed to be initialized by loss of the protonated neutral anion to form $[M-H]^-$. The chloride adduct also tended to decompose by forming Cl^- as a product ion. The B3LYP/6-31G* level optimized chloride adduct structure showed that the source of the proton implicated in HA loss most probably came from the hydrogen of the anomeric hydroxyl group in the reducing ring. In addition, the abundance ratio of Cl^- /non- Cl^- product ions from disaccharide adduct decomposition was shown to be useful for differentiating the anomeric configuration of glycosidic bond linkages.

In negative ion MALDI, highly acidic oligosaccharides do not form adducts with chloride anions, but mildly acidic saccharides (e.g., containing a carboxylic acid

group) form both deprotonated molecules and chloride adducts, and each may provide structural information concerning the oligosaccharide upon decomposition.

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