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Improved LC-MS/MS of Heparan Sulfate Oligosaccharides via Chip-based Pulsed Make-up Flow

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Abstract

Microfluidic chip-based hydrophilic interaction chromatography (HILIC) is a useful separation system for liquid chromatography-mass spectrometry (LC-MS) in compositional profiling of heparan sulfate (HS) oligosaccharides; however, ions observed using HILIC LC-MS are low in charge. Tandem MS of HS oligosaccharide ions with low charge results in undesirable losses of SO₃ from precursor ions during collision induced dissociation. One solution is to add metal cations to stabilize sulfate groups. Another is to add a non-volatile, polar compound such as sulfolane, a molecule known to supercharge proteins, to produce a similar effect for oligosaccharides. We demonstrate use of a novel pulsed make-up flow (MUF) HPLC-chip. The chip enables controlled application of additives during specified chromatographic time windows and thus minimizes the extent to which non-volatile additives build up in the ion source. The pulsed MUF system was applied to LC-MS/MS of HS oligosaccharides. Metal cations and sulfolane were tested as additives. The most promising results were obtained for sulfolane, for which supercharging of the oligosaccharide ions increased their signal strengths relative to controls. Tandem MS of these supercharged precursor ions showed decreased abundances of product ions from sulfate losses yet more abundant product ions from backbone cleavages.

Introduction

Heparan sulfates (HS) are linear, acidic polysaccharides belonging to the glycosaminoglycan (GAG) family. Each mature HS chain is biosynthesized as a polysaccharide of repeating disaccharide units (4GlcAβ1-4GlcNAcα1-) that undergoes a series of subsequent modification reactions.¹ Because these reactions are not template driven, mature HS chains are extremely polydisperse, with varying sulfation patterns, acetylation compositions, epimerization sites and chain lengths.² This heterogeneity and the essential roles HS plays in physiology drive the need for effective methods for correlation of HS structure and function.³ Detailed structural information on HS is needed to for basic biomedical research, development of therapeutics, and biomarker discovery efforts. Such efforts include chemical or chemoenzymatic synthesis of HS oligosaccharides^{4–7}, generation of structural libraries,⁸ metabolic engineering of HS biosynthesis⁹ and biomarker discovery.¹⁰ For each of these applications, it is necessary to correlate structure with biological function in the context of the heterogeneity that characterizes the HS compound class. As a result, it is desirable to

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develop methods to produce as much structural information as possible in a high throughput manner. Such analytical data will inform purification efforts appropriate with the goals of the project in question.

In previous work, we used HILIC LC-MS to profile compositions of HS oligosaccharides and other GAGs as part of comparative glycomics experiments.^{11–17} Recently, chip-based HILIC LC-MS with a post-column make-up flow (MUF) has shown great utility for the profiling of HS oligosaccharides.^{11, 12} The integration of a make-up flow on the HPLC-chip improved the spray stability generated from HILIC mobile phase over the course of a gradient elution for the separation of HS oligosaccharides. In addition, using organic solvent as the make-up solution enables use of a single spray voltage that is lower than that required to achieve stable spray for a mobile phase of high aqueous content during the entire HILIC gradient. This results in the enhanced capability of the HILIC LC-MS system to analyze longer HS oligosaccharides that elute at high aqueous gradient where electrospray would otherwise be unstable. This increased stability enables glycomics experiments requiring extended periods of stable instrument performance.

While the LC-MS compositional profiling information of HS oligosaccharides is useful, there is a clear need to extend this capability to the tandem MS dimension. Electron detachment dissociation (EDD) shows great potential for determination of fine structure of purified HS and other GAG oligosaccharides using static infusion.^{18–20} The technique requires several minutes of spectral acquisition and is most appropriate for detailed analysis of purified oligosaccharides, rather than high throughput structural analysis. Negative ion electron transfer dissociation (NETD) has been demonstrated to produce similar product ion patterns on GAG oligosaccharides as EDD with similar extended spectral acquisition times.²¹ Another option for tandem MS is to permethylate the oligosaccharides,^{22, 23} followed by chemical desulfation and acetylation of the formerly sulfated sites.²⁴ The advantage of this approach, as demonstrated for chondroitin sulfate oligosaccharides, is that the derivatized products are stable and compatible with reversed phase LC/MS. The derivatization steps result in formation of some degree of side products, and it may be that this approach is also most appropriate for purified oligosaccharides.

HILIC chromatography has the advantage that it is directly compatible with tandem MS of native GAG oligosaccharides. HILIC LC-MS/MS is sensitive relative to EDD/NETD methods and does not require derivatization and workup. It is possible to use this approach to acquire tandem mass spectra in high throughput. Unfortunately, tandem MS of HS oligosaccharide precursor ions produces a mixture of product ions resulting from competing backbone dissociation versus those from losses of SO₃, and this is particularly true when using collision induced dissociation.^{25–29} This competition favors backbone cleavages as negative ion charge state increases (in absolute value) and as metal cation adducts are included in the precursor ions. The use of cations to facilitate tandem MS of saccharides has been appreciated since the early 1990s.^{30–34} Their application to LC-MS/MS experiments is limited by their potential to coat the source optics, cause loss of robust instrument performance and to increase mass spectral complexity.

Sulfolane and other reagents with low solution phase basicities and volatilities have been shown to increase the charge state of protein and protein complexes when added to the electrospray solution and have been utilized to facilitate top-down MS analysis in the positive ionization mode.^{35, 36} On-line LC/MS requires continuous infusion of spray solution, and there is concern that such non-volatile and highly viscous molecules will coat the MS ion source and optics and negatively impact the instrument response over time. Effective use of either type of additive would be facilitated by the ability to target the post-column addition precisely during the LC-MS runs.

The present work describes use of a chip-based system for automated pulsing of additives to the LC effluent to maximize backbone fragmentation of HS oligosaccharides in tandem MS. This system features software-controlled pulsing of additives at specified times during the LC elution. Using this system, the efficacies of a series of metal cations for pairing with HS oligosaccharides and improving the quality of the on-line tandem mass spectra were evaluated. The utility of sulfolane to supercharge negative ions during LC-MS analyses was also shown using the pulsed MUF chip. The pulsed MUF chip represents a means of introducing additives to LC effluents in a manner that improves the value of HS tandem mass spectra and minimizes quantity of non-volatile additives that enter the ion source.

Experimental Section

Materials

Porcine intestinal mucosa HS was purchased from Celsus Laboratories, Inc. (Cincinnati, OH). Heparin lyase I and II from *Flavbacterium heparinum* were purchased from IBEX (Montreal, QC). Arixtra (GlcNS(6S)-GlcA-GlcNS(3S,6S)-IdoA(2S)-GlcNS(6S), C₃₁H₄₃N₃Na₁₀O₄₉S₈) was purchased from Organon Sanofi-Synthelabo LLC (West Orange, NJ). Arixtra was prepared by dialysis using a 100 Da molecular weight cutoff membrane filter before LC-MS analysis. Sulfolane, metal salts and other reagents were purchased from Sigma-Aldrich.

Heparan Sulfate Oligosaccharides Preparation

Digestion of porcine intestinal mucosa HS (350 µg) was performed in a 1 mL solution consisting of 500 µL digestion buffer (100 mM NaCl, 20 mM Tris-HCl, 1 mM Ca(OAc)₂, pH 7.4) at 37 °C. Aliquots of 20 mIU heparin lyase I and 50 mIU heparin lyase II were added and incubated overnight and the UV absorbance at 232 nm was recorded for benchmarking complete digestion. Partial digestion was then performed in the same solution system and the digestion progress was monitored until the UV absorbance at 232 nm reached 30% of completion. The digestion was then stopped by heating at 100 °C for 10 min. The digestion products were dried by centrifugal evaporation and reconstituted in water and purified/profiled using a SuperdexTM Peptide PC 3.2/30 column (GE Healthcare, Piscataway, NJ). The column was equilibrated and operated using 50 mM ammonium acetate in 10% acetonitrile. The fraction corresponding to degree of polymerization (dp)4 and dp6 HS oligosaccharides were collected for MS analysis.

Pulsed MUF Chip-based Amide-HILIC LC-MS

Chip-based LC-MS was conducted using an Agilent (Santa Clara, CA) chip cube system³⁷ coupled with a 6520 quadrupole time-of-flight mass spectrometer (Q-TOF) operating in negative mode. Amide-80 stationary phase (5 µm particle size, 80 Å pore size, Tosoh Bioscience, Montgomeryville, PA) was packed into the analytical column (75 µm × 43 mm) and the enrichment column (160 nL) of an HPLC-chip by Agilent Technologies (Santa Clara, CA). Compared to the previously described MUF chip¹², the pulsed MUF chip has an additional additive flow path and a modified flow design for the control of pulsing status (Figures 1 and S-1). The additive flow path has a 100 nL additive loop, which can be superimposed on the MUF flow for pulsing additives. A syringe pump was used to fill the additive loop. The HPLC-chip source interfaced by an HPLC-chip cube was coupled to an Agilent 1200 series HPLC system with three pumps as previously described.¹²

The HILIC mobile phases were as follows: solvent A was 10% acetonitrile, 50 mM formic acid (pH adjusted to 4.4 by ammonium hydroxide); solvent B was 95% acetonitrile and 5% solvent A. HS oligosaccharides from SEC fractions were loaded onto the trapping column with an initial solvent composition of 85% B at 4 µL/min for 9.5 min. The trapping column

was then placed in-line with the analytical column and a gradient from 85% B to 0% B was run over a period of 39 min with a flow rate of 200 nL/min. The trapping column and analytical columns were then washed with 0% B for 10 min. The gradient was returned to the initial condition after 10 min and was maintained for a 7 min equilibration period. The MUF composition was 95% acetonitrile pumped at 200 nL/min over the course of the entire run.

Pulsing Additives During Online LC-MS

The inlet of the pulsed flow utilized the direct infusion port and the waste was brought out using a previously unused port on the stator.³⁷ Pulsed runs were conducted using either metal cations (500 μ M in 50% acetonitrile) or sulfolane (105 mM in acetonitrile). The additive loop was pre-primed with additive solution using a syringe pump at a flow rate of 120 μ L/min and the flow was maintained during the pulsing period to enable re-filling of the loop for multiple pulses in the LC-MS run. For the established pump flow rate and additive loop volume, the delay time between the outer rotor valve action and pulse effect was determined to be 35 s. Valve action time points for pulsing were specified in the Agilent MassHunter Workstation Data Acquisition software according to the retention time of target HS oligosaccharide compositions and the delay time. Additives were programmed to be pulsed during the central peak period of a specific composition of HS oligosaccharide. In practice, the duration a pulse event on the chip system was in the range of 48–54 sec plus 6–10 sec of tailing (using trace ion). The analytes (HS oligosaccharides) average 60 sec peak width on the chip-HILIC chromatograph and are well matched to the observed pulsed MUF time range.

Online Collision Induced Dissociation (CID) Tandem MS of HS Oligosaccharides

Instrument tuning was optimized manually to minimize losses of SO_3 in the MS mode using HS-IS ($\Delta\text{HexA}_2\text{S-GlcNS}_6\text{S}$), a trisulfated disaccharide standard. Tandem mass spectra were acquired using the “Targeted MS/MS” mode in the Agilent MassHunter Workstation Data Acquisition software. For sulfolane pulsed runs, major HS oligosaccharides with the same composition but different charge states were selected as targeted precursor ions. In cation pulsed runs, cation adducts and unadducted ions were selected as targets. The m/z , charge state and retention time values of targeted precursor ions were specified in the targeted list table for automated online tandem MS acquisition. The retention times at which additives were pulsed in was determined from a preceding LC-MS run. For each precursor ion, collision energy was set as a ramp from 0 to 30 V with an increment of 5 V throughout the retention time period. The isolation width was set to medium (~ 4 u) or narrow (~ 1.3 u) depending on the isotopic cluster width.

Nomenclature

HS oligosaccharide composition is presented as [ΔHexA ,HexA,GlcN,Ac, SO_3] (ΔHexA : 4,5-unsaturated hexuronic acid; HexA: hexuronic acid; GlcN: glucosamine; Ac: acetate; SO_3 : sulfate), denoting the number of the corresponding residues. Product ions from tandem MS are labeled using the conventional carbohydrate fragmentation nomenclature with revision for HS oligosaccharides. Specifically, HS oligosaccharides without modification of sulfation (S) and/or acetylation (Ac) are considered as the backbone for fragmentation nomenclature and the modification is followed in parenthesis, such as $^{0,2}\text{A}_4$ (1Ac, 3S) and Y_2 (2S).

Results

HPLC-Chip with Pulsed Make-up Flow (MUF)

In order to make best use of the beneficial effects of additives while minimizing potential problems associated with their low volatilities, a modified MUF HPLC-chip was devised. The newly designed pulsed MUF HPLC-chip comprised two extra chip layers compared to the standard MUF chip¹²: one layer with an additive loop and the other distribution chip layer to coordinate flow paths and ports (Figure S-1). The flow paths of this chip enabled pulsing of additives stored in the additive loop via the MUF. The outer rotor was employed to control the status of pulsing and loading.³⁷ At the loading position, exogenous additives were introduced into and temporarily stored in the additive loop. By programmed switching of the valve of the outer rotor, the additive loop was superimposed on the MUF flow and additive was then delivered to the MUF flow. The pulsed MUF flow was then merged with the LC flow at the HPLC-chip tip. (Figure 1)

Effect of Pulsing Metal Cations During HILIC LC-MS of HS Oligosaccharide

Divalent alkaline earth metal cations, several divalent transition metal cations and monovalent alkaline metal cations were tested for their propensity to form adducts with highly sulfated degree of polymerization (dp) 4 and dp6 HS oligosaccharides. The cation solution was pulsed according to the time that the most highly sulfated dp4 and dp6 eluted. Table 1 shows the cation adduct formation efficiency of highly sulfated dp4 and dp6 species of different charge states. For a given oligosaccharide composition, cation adducts with lower charge states were more abundant than those with higher charge states. In addition, the abundances of cation adducted ions increased in proportion to the number of sulfate groups in the HS oligosaccharides. Adducted ions containing more than one metal cation were generally not observed. The overall signal intensities did not change significantly in cation pulsed relative to control data. As shown in Figures S-2 and S-3, the mass spectra showed the presence of both adducted and unadducted ions. This phenomenon divided the ion signal for a given oligosaccharide composition and was thus a disadvantage.

Cobalt cation adducts, which have the highest overall cation formation efficiency and simplest isotopic distribution observed, were targeted for tandem MS. Although metal cationization with one cobalt ion equivalent stabilized one sulfate group, product ions resulting from SO₃ loss were much more abundant than those from backbone dissociation (Figure S-4). The conclusion from these studies was that the low charge state of the precursor ions observed using HILIC LC-MS resulted in abundant losses of SO₃ even in the presence of cation adducts. As a result, effort was made to assess the effect of charge state manipulation on tandem MS of HS oligosaccharides.

Effects of Pulsing Sulfolane During HILIC LC-MS of HS Oligosaccharides

In initial experiments, infusion of a sulfolane containing solution into the ion source using nano-electrospray resulted in a 90% decrease in signal intensity over an 8 hour period. The pulsed makeup flow chip enabled addition of sulfolane in acetonitrile solution during the time region in which dp4 and dp6 HS oligosaccharides elute. Figure 2 shows that average charge states, represented as absolute values (the charge state in the text refers to the absolute value in the negative mode), increased in the sulfolane pulsed LC-MS data when compared to those observed for control data for a series of HS dp4 and dp6. For these data, [1,1,2,1,2] and [1,1,2,1,3] were subjected to pulsed addition of sulfolane, demonstrating that the ESI conditions produced the same intensities as in the control. The extent to which charge states increased correlated with increasing number of sulfate groups and length of the oligosaccharide backbone (Figure S-5). In addition, ion intensities increased by 3~9-fold in the pulsed sulfolane data, as shown in Figure 3. When the fold-changes in ion abundances

were normalized to those in control data, it was clear that the increase in abundance was negatively correlated to the number of sulfate groups for a given oligosaccharide length (Figure S-6). The abundance increases were also higher for dp6 than for dp4. Examples of extracted mass spectra from a designated time period of control and pulsed sulfolane addition data are compared on the same intensity scales in Figures S-7 and S-8, demonstrating the improved sensitivity. The extent of ammonium adduction as a percent of all ions produced was diminished in the presence of sulfolane, relative to the control mass spectra.

Pulsed Addition of Sulfolane for Online LC-MS/MS of HS Oligosaccharides

Figure 4 shows the tandem MS spectra of [1,1,2,1,3] with different charge states from sulfolane pulsing. With respect to the acquisition of tandem mass spectra, the collision energy ramp used in the present work relies on the fast data acquisition rate of the TOF instrument and the relatively broad peaks of the HILIC chromatogram. This acquisition method not only eliminated the need for optimizing collision energies but also maximized the structural information resulting from a single LC-MS/MS run. When doubly charged, the trisulfated tetrasaccharides underwent abundant losses of one SO₃ group and ions produced from backbone dissociation were relatively low in abundance (22% of the sum of total intensities). By contrast, triply charged [1,1,2,1,3] showed very low abundance product ions from SO₃ loss from precursor ion (Figure 4B). The abundances of product ions from glycosidic and cross-ring dissociation were high, with 64% of the total (Figure 4C). Online tandem MS of [1,1,2,1,4] (Figure 5) showed improvement with increasing precursor ion charge state similar to that of [1,1,2,1,3]. The [1,1,2,1,4]²⁻ precursor ion produced two abundant ions from loss of SO₃ groups and generated very few other product ions. As charge state increased, the abundances of ions produced from SO₃ losses decreased. When the precursor ion charge equaled the number of sulfate groups in the [1,1,2,1,4]⁴⁻ precursor ion, ions produced from SO₃ losses were very low in abundance. At the same time, the percent abundance of product ions from backbone cleavages increased with the charge state, with [1,1,2,1,4]²⁻ at 20% and [1,1,2,1,4]³⁻ at 68% (Figure 5D). Tandem MS of the more highly sulfated heparin-like dp4, [1,1,2,0,5], showed the same trend of improvement (Figure 6). The figure compares the tandem MS profiles of the 2-, 3- and 4- precursor ions. Although for the 4- precursor there was still one abundant ion resulting from SO₃ loss from the precursor ion, the percentage of backbone cleavages had increased to 55% compared with 20% from doubly charged precursor ion. A 5- precursor ion was observed but at an intensity below the threshold for effective tandem MS. The tandem mass spectra were acquired using collision energy steps across the elution of the chromatographic peak and the resulting mass spectra are presented as an average of all tandem mass spectra collected. This method of spectral acquisition maximizes the types of product ions observed and avoids over-fragmentation of oligosaccharides.

Despite increased abundances of product ions from backbone cleavages as the precursor ion charge state increases, it is likely that some of the backbone cleavages were produced from secondary collision from SO₃ loss species, especially when the sulfate groups of the precursor ions were not highly charged. In cases where the precursor ions contain sufficient charge to deprotonate all sulfate groups, product ions resulting from dissociation to sulfate groups may still occur for some ion populations. This was apparent from the observation of HSO₄⁻ at *m/z* 97 in most tandem mass spectra. Nonetheless, the ability to increase oligosaccharide charge states through pulsed addition of sulfolane significantly improved the abundances of product ions from backbone dissociation relative to those observed for lower charge state precursor ions.

Discussion and Conclusions

We demonstrate that the HPLC-chip with pulsed post-column MUF enables facile manipulation of the LC effluent prior to the electrospray ionization (ESI) emitter. This is especially desirable for the scenario in which additives such as high concentration metal salts or non-volatile organic chemicals (sulfolane). Such additives have low volatilities and may build up on the ion source optics after continuous use. By transient pulsing only at the designated retention time, the extent to which non-volatile additives build up in the source is minimized. It is expected that pulsed MUF chip workflows will be applicable to LC-MS/MS analysis methods where ESI charge state manipulations are desirable.

Metal cation adducts can be easily obtained using direct infusion ESI and have shown great potential for improving the tandem MS of HS oligosaccharides. By using post-column transient pulsing, metal cation adducts were observed during LC-MS. Despite this, the abundances of adducted ions decreased as the observed charge states increased. Thus, the extent of observed dissociation to SO₃ groups was comparatively lower using metal cations than that for pulsed sulfolane. Multiple cation adduction was not observed, limiting the extent of sulfate stabilization. The metal salt concentrations used here were relatively high for the LC-MS system; however, the efficiency of adduct formation was still not adequate to eliminate SO₃ losses and the salt solution used also resulted in a degree of clogging of the spray tip, presumably due to salt precipitation. In addition, cation adduction complicated the mass spectra and made clean isolation of precursor ions from low m/z region very difficult. In summary, although we were able to obtain cation adducted ions in LC-MS by post-column pulsing, the usefulness of these adducts during negative HILIC LC-MS/MS remains to be defined in the future.

Supercharging has been shown previously for the ionization of proteins and protein complexes using compounds including nitrobenzyl alcohol, nitrobenzonitrile and sulfolane in the positive mode.³⁸ We have shown that for the ionization of carbohydrates in the negative mode, supercharging is also achieved by the addition of sulfolane. In addition to supercharging, ammonium adduction is minimized and overall signal strengths are increased in the presence of sulfolane. The extent to which sulfolane shifts the charge state distribution towards higher absolute values depends on the number sulfate groups on the HS oligosaccharides. While the charge state increase is greatest for the most highly sulfated oligosaccharides, deprotonation of all sulfate groups was not observed, mainly due to the charge-charge repulsion effect. Despite this, in contrast to metal cation adduction, the effects of enhanced ionization by pulsing sulfolane appear promising for the online LC-MS/MS of HS oligosaccharides. The ability to supercharge precursor ions minimizes the extent of SO₃ losses and maximizes the abundances of ions from backbone dissociation. Some degree of SO₃ losses during tandem MS seems unavoidable, as the same phenomena have also been observed in electron-based dissociation of lowly sulfated HS saccharides.^{39, 40}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Two working conditions of the pulsed make-up flow (MUF) of the HPLC-chip. *Left:* Loading position. The additive loop was filled with additive solutions via a syringe pump. *Right:* Pulse Position. After the rotor valve changed, the additive loop was superimposed on the MUF by the changes of flow paths (Figure S-1) within the chip, the additive solution was introduced into the MUF and merged with LC effluent. Control of the two positions is programmable by specifying the valve action time in the acquisition software.

Figure 2.

Average charge states of major HS dp4 and dp6 oligosaccharides in control and sulfolane

pulsed runs. Average charge states were calculated by $\frac{\sum q_i I_i}{\sum I_i}$, from i to n, where q_i is the charge of i-th charge state, I_i is the intensity of the i-th charge state and n is the number of observed charge states in one mass spectrum of pulse affected scans.³⁵ HS oligosaccharide composition code: [Δ HexA,HexA,GlcN,Ac,SO₃], denoting the number of the corresponding residues (See methods.). [1,1,2,1,2] and [1,1,2,1,3] were not subjected to sulfolane pulsing.

Figure 3.

Ionization response of a series of HS dp4 and dp6 oligosaccharides in control and sulfolane pulsed runs. Equal quantities of samples were injected for the control and pulsed runs. Each bar shows the sum of the intensities of all charge states and adducted forms of the designated oligosaccharide composition (total intensity). The diamond symbols show the fold change of ionization signals calculated as the ratio of the total intensity of a designated HS oligosaccharide composition in the control run *versus* that in the sulfolane pulsed run. [1,1,2,1,2] and [1,1,2,1,3] in the pulsed run were not subjected to sulfolane pulsing.

Figure 4. Online LC-CID MS/MS of double charged (A) and triply charged (B) [1,1,2,1,3] from sulfolane pulsed run. Major peaks are labeled and the diamond shows the precursor ions. The percent ion abundance of product ions resulting from oligosaccharide backbone cleavages is shown in C.

Figure 5.

Online LC-CID MS/MS of $[1,1,2,1,4]$ with sulfolane pulsing with charge state 2-(A), 3-(B) and 4-(C). Backslash(\) is used to separate alternative fragment assignments. The percent ion abundance of product ions resulting from oligosaccharide backbone cleavages is shown in D.

Figure 6. Online LC-CID MS/MS of [1,1,2,0,5] from sulfolane pulsed run with charge state 2-(A), 3-(B) and 4-(C). The product ions percentage abundance from backbone cleavages is shown in D.

Cation adduct formation efficiency of major highly sulfated HS oligosaccharides by pulsing cation acetate solutions via make-up flow. For the divalent and monovalent cations tested, the efficiency was calculated as the ratio of the abundance of the adduct ion versus the total abundance of the same saccharide composition. The gray scale provides visualization of the data: the darker of the cell, the higher of the value, thus the higher the cation formation efficiency.

Table 1

<u>Composition</u>		[1,1,2,0,5]		[1,1,2,0,6]		[1,1,2,1,4]		[1,2,3,1,5]	
Charge		3-	2-	3-	2-	3-	2-	3-	2-
Be ²⁺		10%	14%	16%	22%	2%	13%	12%	
Mn ²⁺		10%	32%	13%	47%	5%	25%	17%	
Cu ²⁺		1%	2%	3%	11%	1%	2%	12%	
Ni ²⁺		34%	31%	57%	60%	7%	25%	39%	
Zn ²⁺		5%	12%	2%	17%	1%	8%	11%	
Si ²⁺		13%	34%	18%	71%	9%	41%	22%	
Ba ²⁺		8%	28%	15%	67%	1%	12%	8%	
Co ²⁺		27%	42%	34%	64%	8%	41%	33%	
Na ⁺		3%	18%	3%	25%	7%	17%	7%	
Cs ⁺		5%	19%	3%	15%	0%	19%	2%	