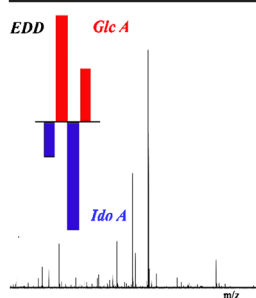


# Single Stage Tandem Mass Spectrometry Assignment of the C-5 Uronic Acid Stereochemistry in Heparan Sulfate Tetrasaccharides using Electron Detachment Dissociation

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**Abstract.** The analysis of heparan sulfate (HS) glycosaminoglycans presents many challenges, due to the high degree of structural heterogeneity arising from their non-template biosynthesis. Complete structural elucidation of glycosaminoglycans necessitates the unambiguous assignments of sulfo modifications and the C-5 uronic acid stereochemistry. Efforts to develop tandem mass spectrometric-based methods for the structural analysis of glycosaminoglycans have focused on the assignment of sulfo positions. The present work focuses on the assignment of the C-5 stereochemistry of the uronic acid that lies closest to the reducing end. Prior work with electron-based tandem mass spectrometry methods, specifically electron detachment dissociation (EDD), have shown great promise in providing stereo-specific product ions,

such as the  $B_3 - CO_2$ , which has been found to distinguish glucuronic acid (GlcA) from iduronic acid (IdoA) in some HS tetrasaccharides. The previously observed diagnostic ions are generally not observed with 2-*O*-sulfo uronic acids or for more highly sulfated heparan sulfate tetrasaccharides. A recent study using electron detachment dissociation and principal component analysis revealed a series of ions that correlate with GlcA versus IdoA for a set of 2-*O*-sulfo HS tetrasaccharide standards. The present work comprehensively investigates the efficacy of these ions for assigning the C-5 stereochemistry of the reducing end uronic acid in 33 HS tetrasaccharides. A diagnostic ratio can be computed from the sum of the ions that correlate to GlcA to those that correlate to IdoA. **Keywords:** Glycosaminoglycans, Heparan sulfate, Uronic acids, Stereochemistry, FTICR, Electron detachment dissociation

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## Introduction

A large portion of the extracellular matrix and basement membranes are comprised of proteoglycans, composed of proteins covalently attached to the class of carbohydrates called glycosaminoglycans (GAGs) [1–3]. GAGs are linear negatively charged biopolymers, the basic building blocks of which consist of a repeating disaccharide sequence of an amino sugar and a uronic acid or galactose [4, 5]. They are categorized as either keratan sulfate (KS), chondroitin sulfate (CS),

dermatan sulfate (DS), hyaluronan (HA), or heparan sulfate (HS) depending on their disaccharide repeating unit [1–6]. Among these classes of GAGs, HS is the most structurally complex [6, 7]. They are initially synthesized in the Golgi apparatus as alternating disaccharide units of D-glucuronic acid and *N*-acetylated glucosamine [6, 8]. C5-epimerization of glucuronic acid (GlcA) to iduronic acid (IdoA) occurs during the biosynthesis of HS followed by a series of sulfo modifications [7, 8]. Sulfo modifications may occur at the 2-*O* position of the uronic acid, and the *N*-, 3-*O*, and 6-*O* positions of the glucosamine unit [8]. These structural modifications often do not go to completion, producing HS chains with varying sequences of sulfation, acetylation, and IdoA/GlcA content [6, 9]. Despite these varying structural modifications, specific structural motifs on HS chains have been reported to bind target proteins with high specificity. These categories of proteins, called

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heparan sulfate binding proteins (HSBPs), include chemokines, cytokines, blood coagulation factors, such as serine proteases, cell adhesion proteins, growth factors, and morphogenetic factors [7, 10, 11]. Documented physiologic processes influenced by HS interactions with proteins include growth and development, cancer, inflammation, viral infectivity, and blood coagulation [12–15]. The numerous biological functions of this bio-molecule continue to inspire research into structure–function relationships of HS oligomers. However, this research has been hampered by their enormous microheterogeneity and limited availability requiring very sensitive and robust analytical methods for their analysis.

Advanced analytical methods like nuclear magnetic resonance (NMR) spectroscopy have been used to determine sulfo modifications and the C-5 stereochemistry of the uronic acid in GAGs [16, 17]. However, the quantity and purity of GAGs extracted from natural sources are often not suitable for NMR analysis [18]. These drawbacks make mass spectrometry an excellent alternative for GAG analysis. Negative electrospray ionization mass spectrometry offers an excellent platform for GAG analysis, offering high sensitivity, throughput, and accuracy [19, 20]. However, tandem mass spectrometry of GAGs, especially heparan sulfate, is often challenging due to variations in oligomer length, hexuronic acid stereochemistry, and sulfation heterogeneity [6, 21]. ESI-MS is able to determine the length, degree of sulfation of GAGs, and other features affecting the elemental composition. To determine sites of sulfation, *N*-acetylation, and hexuronic acid stereochemistry, more advanced methods are required. Recent advances in tandem mass spectrometric applications to GAGs using collision-induced dissociation (CID) [22, 23], infrared multiphoton dissociation (IRMPD) [24], electron induced dissociation (EID) [25], electron detachment dissociation (EDD) [26–28], and negative electron transfer dissociation (NETD) [29] have addressed some of the challenges encountered during their structural analysis. Inherent sulfo decomposition of the labile sulfate half ester group present in GAGs hinders structural characterization. This phenomenon occurs mostly during the ionization and ion activation stages of the experiment. Chemical derivatization [30], deprotonation of the acidic groups [23, 31, 32], and metal cation exchange [22, 23] have been reported to effectively reduce sulfo decomposition depending on the degree of sulfation. Recent CID MS/MS reports on Arixtra and highly sulfated heparan sulfate GAGs showed that one can obtain very rich and structurally informative product ion coverage by adding dilute NaOH to the spray solution [33, 34]. Electron-based activation methods, especially EDD, have shown great potential in providing highly informative cross-ring products as well as the corresponding glycosidic cleavages, which are essential for localization of sulfo positions [25–27]. Although mass spectrometry methodologies continue to gain ground in assigning sites of sulfo modifications, a remaining challenge has been the inability to discriminate diastereomers that differ by the

chirality of the uronic acid C-5 center. Zaia and coworkers were the first to address this challenge. Using collision induced dissociation, they were able to differentiate chondroitin sulfate (CS) from dermatan sulfate (DS) in mammalian extracellular matrix, and quantitatively assign the amount of the diastereomers present in mixtures [35]. The ability to assign the C-5 stereochemistry in uronic acid residues of 4-*O*-sulfo chondroitin sulfate epimers with varying oligomer lengths (dp4-dp10) based on diagnostic cross-ring ions  $^{2,4}A_n$  and  $^{0,2}X_n$  in CID mass spectra has also been reported by Kailemia et al. [36]. Compared with heparan sulfates, chondroitin sulfates have a well-defined sulfation pattern; hence, the former requires a more sensitive activation method for stereochemistry assignments. EDD results for HS tetrasaccharides reported by Wolff et al. showed the possibility of obtaining a stereospecific ion  $B_3'-CO_2$  for assigning the C-5 stereochemistry for moderately sulfated tetramers (0–0.25 sulfates per disaccharide) [18]. Gas-phase separation of epimeric mixtures of HS tetrasaccharides using field asymmetric ion mobility spectrometry (FAIMS) followed by EDD fragmentation confirmed the presence of the  $B_3'-CO_2$  ion for the GlcA-containing epimer [37]. Recent EDD reports, however, showed that the presence of a sulfo group at the 2-*O* position of the uronic acid hinders production of the  $B_3'-CO_2$  ion in GlcA-containing epimers [38]. More recent work on the assignment of the C-5 stereochemistry for 2-*O*-sulfated HS tetramers (0.5–2.5 sulfates per disaccharide) revealed the possibility of assigning the C-5 stereochemistry of HS tetrasaccharides using a ratio combination of selected ions obtain from EDD-PCA experiments [38]. Such analyses are useful when epimeric compounds are available. The scarcity of naturally occurring epimeric HS samples has motivated the development of a technique that assigns the stereochemistry of these tetramers without reference to their isomers. Here we present for the first time a more general approach in assigning the C-5 hexuronic stereochemistry for 33 HS tetrasaccharide standards from a single stage EDD tandem mass spectrum.

## Experimental

### *Sample Preparation*

Thirty-three heparan sulfate tetrasaccharides standards were synthesized using a modular approach [39]. All the compounds examined had their compositions confirmed using FTICR MS accurate mass measurement and had their structures confirmed by  $^1H$  NMR, HSQC, and COSY. Supplementary Figures 1–4 show the chemical structures of all 33 compounds.

### *Mass Spectrometry Analysis*

EDD experiments were performed on a 9.4T Bruker Apex Ultra QeFTMS (Billerica, MA, USA) with a hollow cathode (HeatWave, Watsonville, CA, USA), which serves as the

source of electrons for EDD; 0.1 mg/mL of each standard were injected at a rate of 120  $\mu\text{L/h}$  in 50:50 methanol:H<sub>2</sub>O and ionized by a metal electrospray capillary (#G2427A; Agilent Technologies, Santa Clara, CA, USA). Where necessary, 0.1 mM NaOH was added to the spray solvent to enhance the intensity of preferred sodium adducted precursor ions for analysis. All the HS tetrasaccharides were analyzed in the negative ion mode. Each EDD experiment was repeated three times with almost similar results for each HS standard examined.

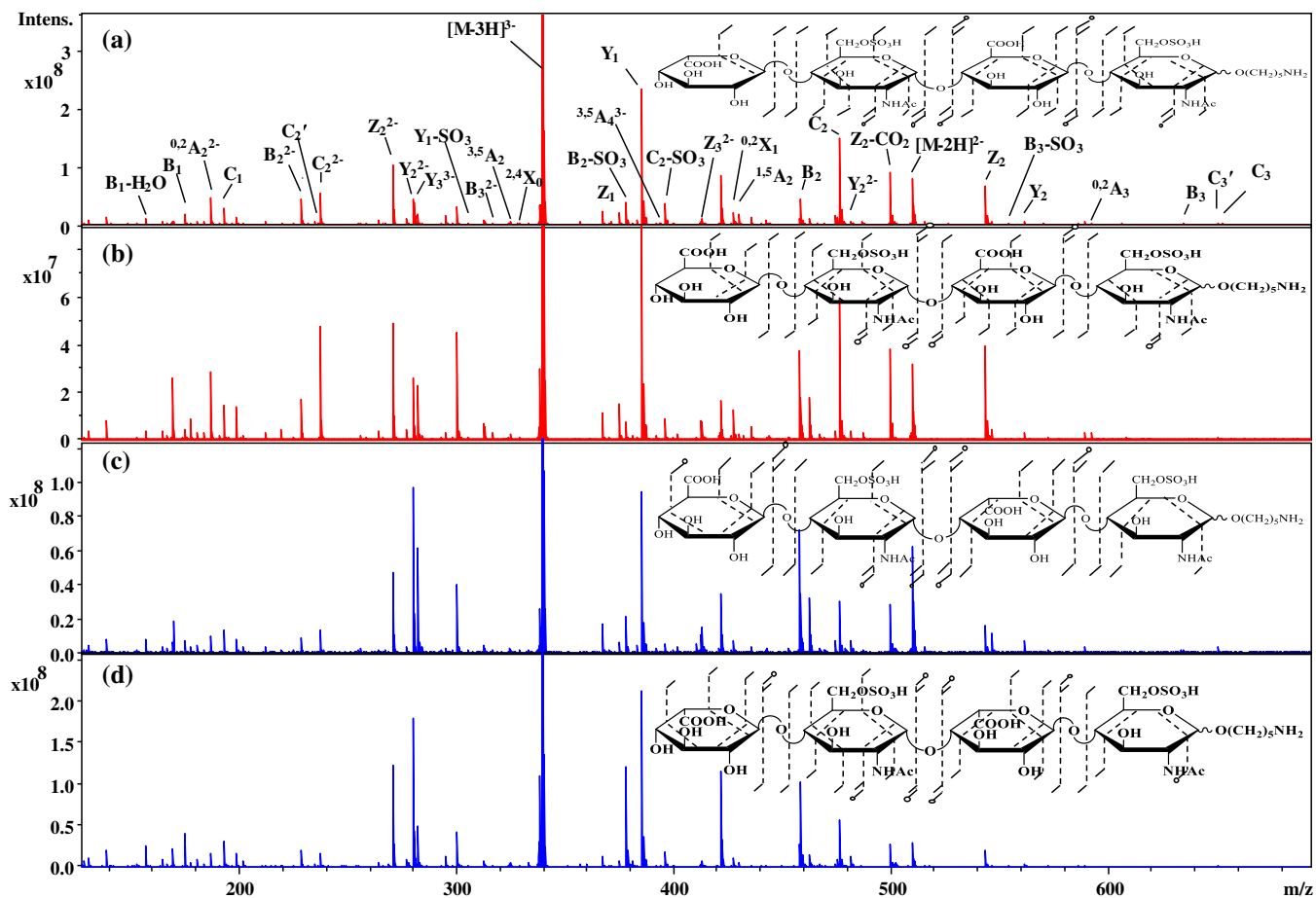
For the EDD experiment, multiply charged precursor ions were isolated in the external quadrupole and accumulated for 1–4 s before injection into the FT-ICR cell. Precursor ion selections were refined using in-cell isolation with a coherent excitation frequency (CHEF) event. These ions were then irradiated with 19 eV electrons for 1 s. The extraction lens was set to  $-18.5 \pm 0.5$  V with the cathode heater at 1.5 A. Twenty-four acquisitions were signal averaged per spectrum; 512 K points were acquired for each spectrum, padded with one zero fill, and apodized using a sine bell window. Internal calibration was achieved using confidently assigned glycosidic product ions as internal calibrants, providing mass accuracy of <1 ppm. We report peaks with S/N >10 due to the large number of low-intensity product ions formed by EDD. All cross-ring and

glycosidic product ions generated from the EDD experiment were assigned using accurate mass measurement and GlycoWorkbench [40]. These ions are reported using the Domon and Costello nomenclature [41].

## Results and Discussion

### Determination of Sulfo Positions

Electron detachment dissociation has been shown to provide excellent product ion coverage for the structural analysis of glycosaminoglycans. Figure 1 shows abundant glycosidic and cross-ring product ions for the epimeric di-sulfated HS standards epimers, labeled (2b, 2f, 2d, and 2g). EDD fragmentation of the  $[M - H]^{3-}$  precursor ion produced almost identical fragmentation for these compounds as one would expect for these epimers (Figure 1). These compounds differ only in the C-5 hexuronic acid stereochemistry and have both their glucosamine unit sulfated at the 6-O position. We are able to unambiguously assign the sites of sulfation on these epimers with a combination of cross-ring and glycosidic product ions. The mass difference between product ions  $C_1$  and  $^{3,5}A_2$ , and  $C_3$



**Figure 1.** EDD mass spectra with structural annotations for four epimeric synthetic di-sulfated HS tetrasaccharides: **(a)** 2b-(I doA-GlcNAc6S-GlcA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), **(b)** 2f-(GlcA-GlcNAc6S-GlcA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), **(c)** 2d-(GlcA-GlcNAc6S-I doA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), and **(d)** 2g-(I doA-GlcNAc6S-I doA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>)

and  $^{3,5}A_4$ , confidently identifies the two 6-*O* sulfo groups on the second and fourth glucosamine units towards the reducing end for HS standards 2b, 2d, and 2g (Figure 1). A similar assignment of the 6-*O* sulfo group on the second glucosamine unit from the non-reducing end can be made using the  $C_1$  and  $^{3,5}A_2$  for compound 2f; however, the sulfate group on the reducing end sugar is assigned using the mass difference between cross-ring ions product ions  $^{2,4}A_4$  and  $^{0,2}A_4$ . We also show in Figure 2, efficient EDD fragmentation for tri-sulfated epimers 3b-(IdoA-GlcNS6S-GlcA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 3h-(GlcA-GlcNS6S-IdoA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) for the  $[M - 4H + Na]^{3-}$  precursor ion. The *N*- and 6-*O* sulfo groups on the second residue near the non-reducing ends can be assigned using the mass difference between  $^{0,2}A_2$  and  $B_2$ , and  $^{2,4}A_2$  and  $^{0,2}A_2$  product ions, respectively. The *N*-sulfo group on the reducing end glucosamine is assigned using cross-ring product ion  $^{0,2}X_0$ . We have included a comprehensive mass list (*m/z* and intensity) showing similar fragmentation efficiency for triplicate EDD experiment for all the 33 standards in the accompanying [supplemental material](#).

### Assignment of the Reducing End Hexuronic Acid Stereochemistry

Electrospray ionization mass spectra for all HS standards considered for this work produced abundant multiply charged ions for the EDD experiment. Figure 3 shows the general structure

for the synthetic heparan sulfate tetrasaccharides epimers with an aminopentyl linker on the anomeric carbon. This modification provides a mass shift for the resulting reducing end fragments ions that enable confident assignment of product ions that would otherwise exhibit identical masses for reducing end and non-reducing end products. In this figure, arrows highlight the C-5 carbon of the uronic acid residue that is the focus of this investigation, namely the acidic sugar closest to the reducing end.

ESI-MS of the mono-sulfated synthetic heparan sulfate standards yield abundant doubly charged ions molecular ions,  $[M - 2H]^{2-}$ . With this charge state, two of the three acidic protons are removed during ionization. Close examination of the EDD spectra for the GlcA-containing standards 1a and 1b, Figure 4a, reveals results consistent with previous EDD reports on modestly sulfated HS tetrasaccharides [35, 40]. Diagnostic ion  $B_3'$ -CO<sub>2</sub> indicative of glucuronic acid in less sulfated HS GAGs (0–0.25 sulfates per disaccharide) reported for naturally extracted and synthetically produced HS tetrasaccharides [18, 21] are observed for only samples 1a (IdoA2S-GlcNAc-GlcA-GlcNAc-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 1b (IdoA-GlcNAc6S-GlcA-GlcNAc-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), Figure 4a, occurring at *m/z* 589.0972. An expanded mass spectra region for the stereospecific ion  $B_3'$ -CO<sub>2</sub> is shown in Figure 4a. An intense  $B_3'$  ion relative to its original  $B_3$  ion is a feature also reported for being diagnostic for GlcA-containing HS [18]. These ions for standards 1a and 1b stand in stark contrast to standards 1d (GlcA-GlcNAc-IdoA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 1f (IdoA-GlcNAc-IdoA-

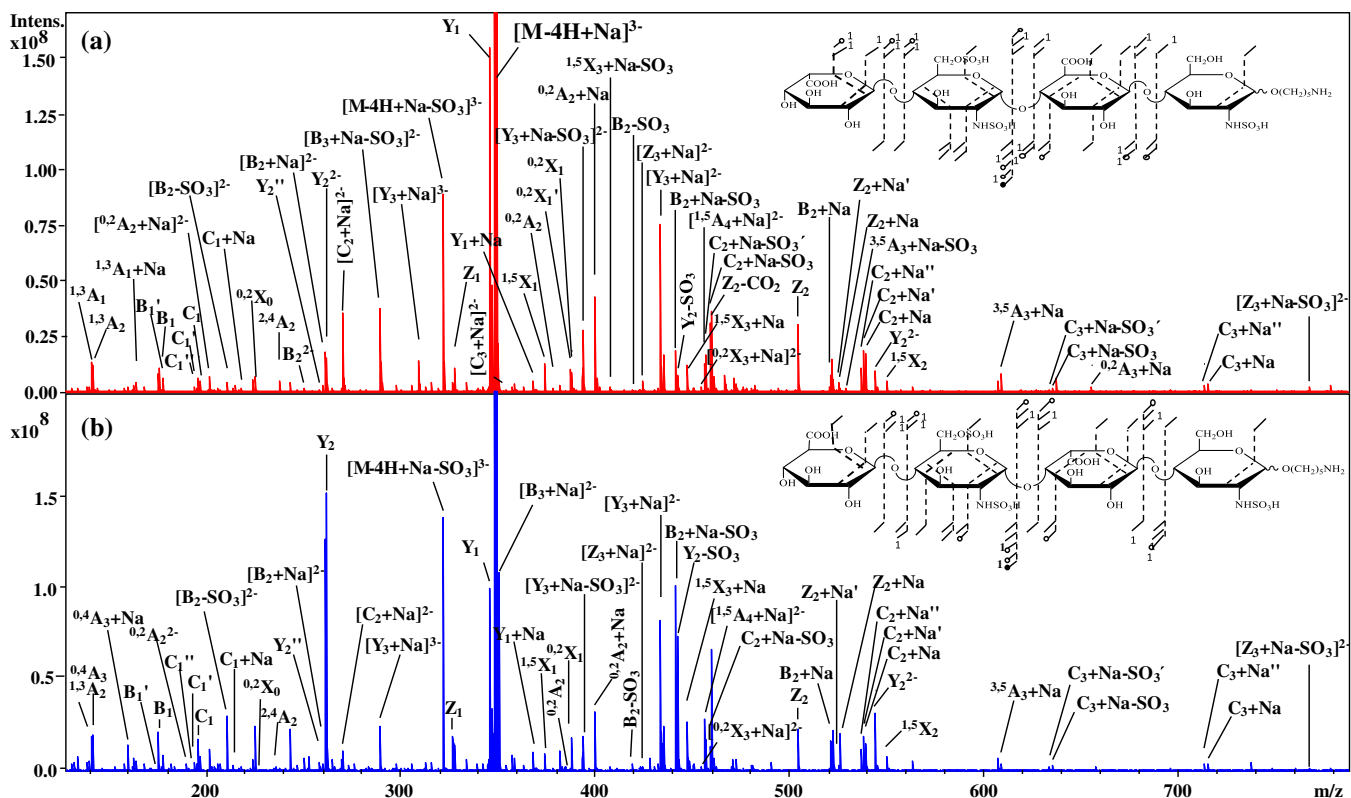
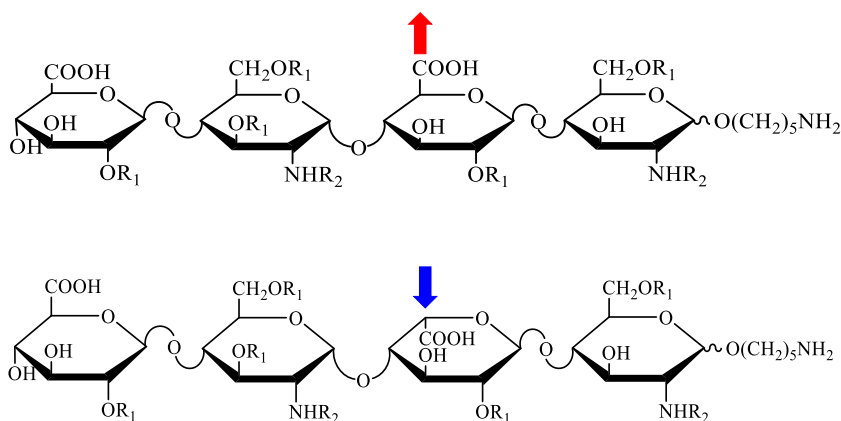


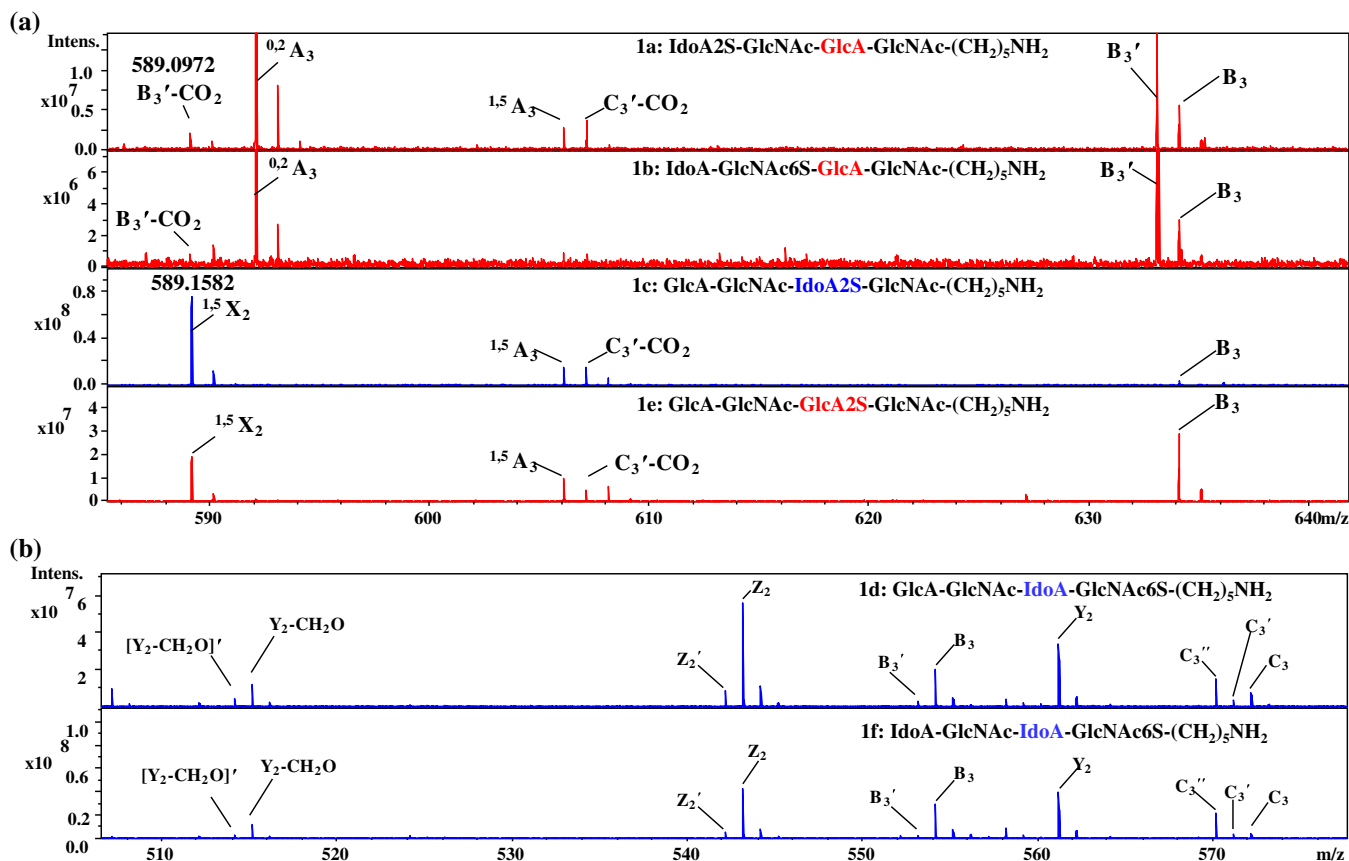
Figure 2. EDD mass spectra and annotated structures for the tri-sulfated HS tetrasaccharides: (a) 3b-(IdoA-GlcNS6S-GlcA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), and (b) 3h-(GlcA-GlcNS6S-IdoA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) for the  $[M - 4H + Na]^{3-}$  precursor ion



**Figure 3.** General structure for the synthetic heparan sulfate tetrasaccharide epimers with an anomeric aminopentyl linker, where  $R_1 = \text{H}, \text{SO}_3\text{H}$  and  $R_2 = \text{H}, \text{SO}_3\text{H}, \text{Ac}$

GlcNAc6S-( $\text{CH}_2$ )<sub>5</sub>NH<sub>2</sub>), with the  $B_3'$  ion with a lower intensity relative to its  $B_3$  ion (Figure 4b). The absence of the  $B_3'$  ion for standard 1c (GlcA-GlcNAc-IdoA2S-GlcNAc-( $\text{CH}_2$ )<sub>5</sub>NH<sub>2</sub>) and 1e (GlcA-GlcNAc-GlcA2S-GlcNAc-( $\text{CH}_2$ )<sub>5</sub>NH<sub>2</sub>) further confirmed the influence of the 2-*O* sulfation on the uronic acid in the formation of the  $B_3'$ -CO<sub>2</sub> for 1e as reported earlier [38]. The non-specificity of this previously reported ion over a wide range of sulfo modifications has been the main motivation for

this work. Principal component analysis of EDD results from our most recent work revealed several ions that could be used to differentiate GlcA2S from IdoA2S. These ions included,  $B_3$ ,  $Y_1$ ,  $C_2$ , and  $Z_2$  fragments, found to be diagnostic for GlcA2S, whereas  $Y_2$  and  $^{1,5}X_2$  were diagnostic for IdoA2S. We combine these ions into a diagnostic ratio formula below (Equation 1) capable of assigning the C-5 stereochemistry of HS tetrasaccharide standards.



**Figure 4.** Expanded EDD spectra for the  $[M - 2H]^{2-}$  precursor ion for the mono-sulfated HS standards showing the region for the stereospecific ion  $B_3'$ -CO<sub>2</sub> (a) for compounds 1a, 1b, 1c, and 1e  $m/z$  570–640, and (b) compounds 1d and 1f  $m/z$  509–580



$$\begin{aligned} \text{DR} &= \log\left(\frac{1}{3}\left(\frac{\sum(\text{GlcA})}{\sum(\text{IdoA})}\right)\right) \\ &= \log\left(\frac{1}{3}\left(\frac{\sum(B_3, Y_1, C_2, Z_2)}{\sum(Y_2, {}^{1,5}X_2)}\right)\right) \end{aligned} \quad (1)$$

Equation 1 computes the ratio of the sum of intensities of ions that are statistically validated as diagnostic for the presence of GlcA to the sum of intensities of ions diagnostic of IdoA. The factor of one-third applied to each sum was determined empirically to produce a positive value for the diagnostic ratio (DR) when GlcA is present at the second residue from the reducing end, and negative values when IdoA is present in the same position. The DR values computed for all the standards include fragments with sulfo losses from the selected ions. The discussion below examines the results for a comprehensive set of HS standards with 1 to 4 sulfo modifications.

### Diagnostic Ratio Results for Mono-Sulfated HS Standards

Diagnostic ratio (DR) results for all six HS standards for the mono-sulfated tetrasaccharides labeled 1a to 1f are shown in Figure 5. For a common precursor ion selection  $[M - 2H]^{2-}$ , at  $m/z$  469.6327 and subsequent electron irradiation, we are able to confidently resolve the C-5 hexuronic acid stereochemistry for the residue closest to the reducing end, for singly sulfated standards. All the GlcA-containing standards showed positive diagnostic ratio results, whereas those for IdoA were negative. The lowest DR results obtained for these set of HS tetramers GlcA was  $0.61 \pm 0.07$  (GlcA-GlcNAc-GlcA2S-GlcNAc-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and the highest for mono-sulfated standards containing IdoA was  $-0.38 \pm 0.09$  (IdoA-GlcNAc-IdoA-

GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>). Epimeric pair 1c (GlcA-GlcNAc-IdoA2S-GlcNAc-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 1e (GlcA-GlcNAc-GlcA2S-GlcNAc-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) with 2-*O* sulfo modification on the central uronic unit are clearly differentiated, as shown in Figure 5. We also note the non-reducing end stereochemistry has a negligible impact on the DR results for the mono-sulfated set of compounds. This is evident comparing the diagnostic ratio results, which were  $-0.44 \pm 0.03$  for standards 1d (GlcA-GlcNAc-IdoA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and  $-0.38 \pm 0.09$  for 1f (IdoA-GlcNAc-IdoA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>).

### Diagnostic Ratio Results for Di-Sulfated HS Standards

ESI-MS of the di-sulfated HS standards produced abundant  $[M - 3H]^{3-}$  ions at  $m/z$  339.4050 and 325.4015 depending on the disaccharide repeating sequence. These tetrasaccharides have four ionizable protons, two on the sulfo groups and two on the carboxyl groups. Figure 6 shows the diagnostic ratio results obtained for all the eight HS standards examined upon EDD fragmentation of the  $[M - 3H]^{3-}$  precursor ion. Again, we show the standards containing GlcA residues towards the reducing end have positive diagnostic ratio values relative to those containing IdoA residues. The broad selection of standards allowed for the applicability of diagnostic ratio formula to discriminate epimers. Standards 2b (IdoA-GlcNAc6S-GlcA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 2d (GlcA-GlcNAc6S-IdoA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) differing in the C-5 stereochemistry for the non-reducing end and central uronic acid residues were unambiguously resolved with diagnostic ratio values  $0.64 \pm 0.01$  and  $-0.22 \pm 0.01$ , respectively. The diagnostic ratio results for the remaining epimer pairs, [2c (GlcA-GlcNAc-IdoA2S-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>):  $-0.33 \pm 0.05$ , and 2e (GlcA-GlcNAc-

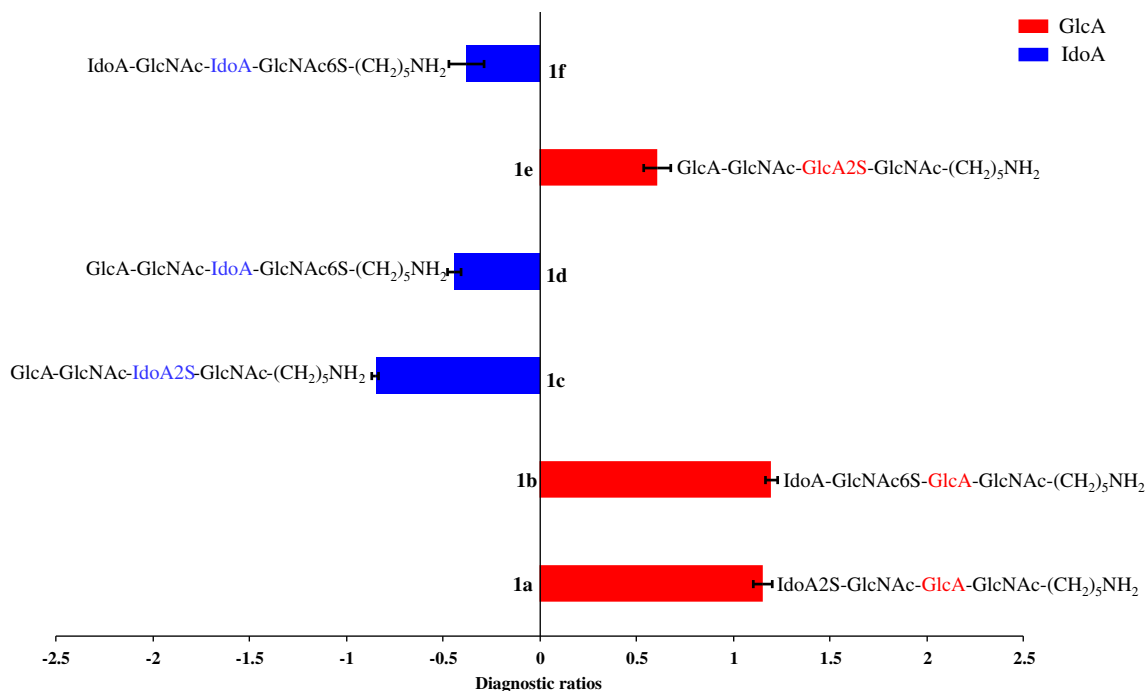


Figure 5. EDD diagnostic ratio results for the mono-sulfated HS tetrasaccharides standards (1a–1f), for the  $[M - 2H]^{2-}$  precursor ion

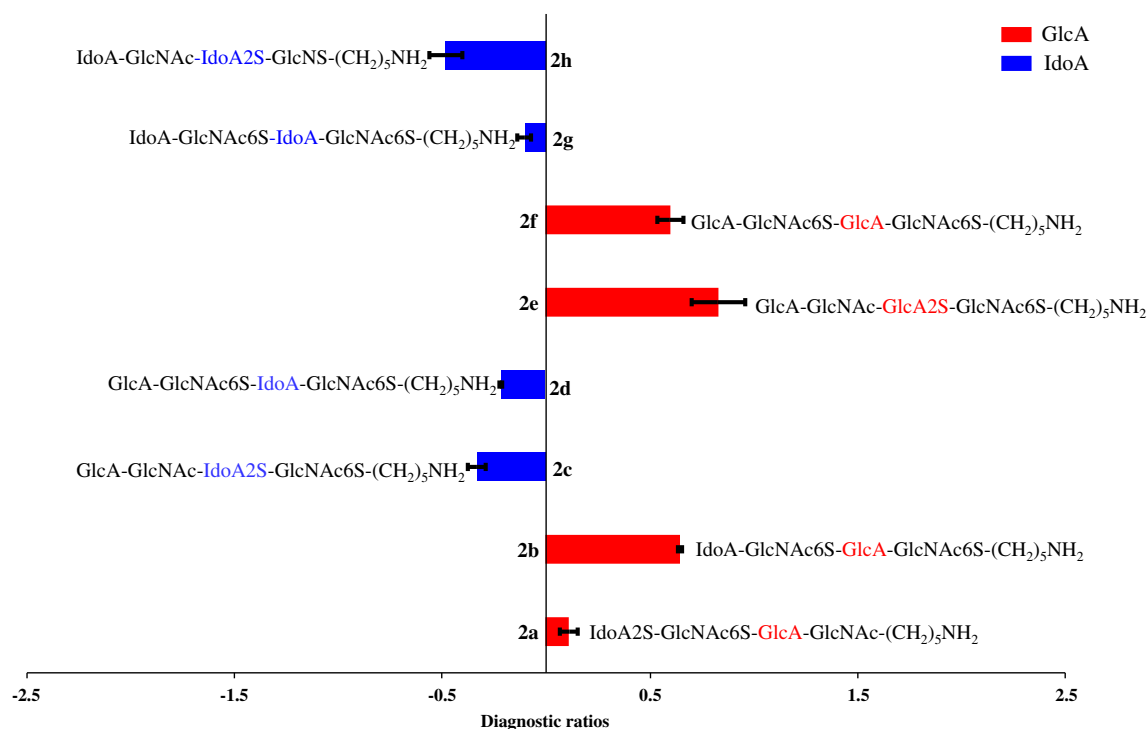


Figure 6. EDD diagnostic ratio results for the di-sulfated HS tetrasaccharides standards (2a–2h) for the  $[M - 3H]^{3-}$  precursor ion

GlcA2S-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>:  $0.83 \pm 0.11$ ] and [2f (GlcA-GlcNAc6S-GlcA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>:  $0.60 \pm 0.06$  and, 2g (IdoA-GlcNAc6S-IdoA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>:  $-0.11 \pm 0.03$ ] further established the usefulness of the formula to discriminate epimers. The overall contribution of the non-reducing end

uronic acid stereochemistry is again observed to have very minimal impact on the diagnostic ratio values as we compare standards 2b and 2f (Figure 6). Standard 2h (IdoA-GlcNAc-IdoA2S-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), which has a different disaccharide repeating sequence compared with the seven di-sulfated

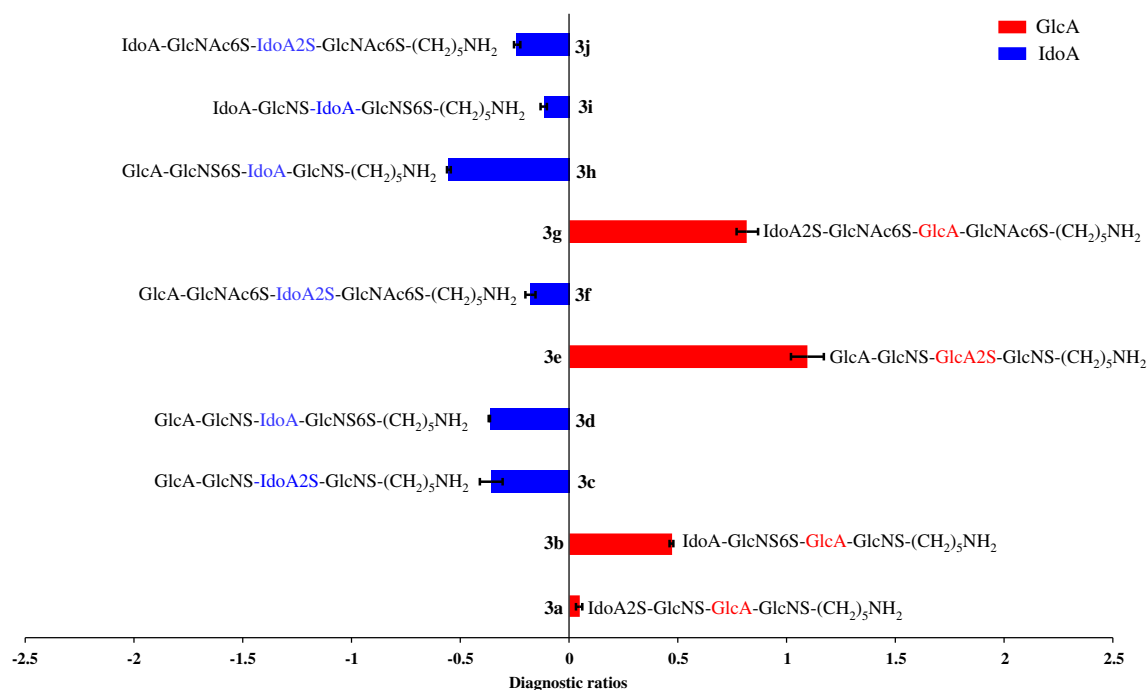


Figure 7. EDD diagnostic ratio results for the tri-sulfated HS tetrasaccharides standards (3a–3j), for the  $[M - 4H + Na]^{3-}$  precursor ion

standards produced results consistent with the IdoA-containing tetramers. The lowest diagnostic ratio value recorded for the GlcA-containing standards was  $0.11 \pm 0.04$  for 2a (GlcA2S-GlcNAc6S-GlcA-GlcNAc-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and the highest for with IdoA was  $-0.11 \pm 0.03$  recorded for 2g (IdoA-GlcNAc6S-IdoA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>).

### Diagnostic Ratio Results for Tri-Sulfated HS Standards

Previous EDD and CID reports have shown that the presence of sodium counter ions can provide a means to control stereochemical dependent fragmentation [21, 36]. We found that to be very useful for the tri- and tetra-sulfated HS standards. For the tri-sulfated HS standards, the  $[M - 4H + Na]^{3-}$  precursor ion at  $m/z$  345.3775 was selected for the EDD experiment. This ionized state allows for all the sulfo groups and a carboxylic group to be ionized. Figure 7 shows diagnostic ratio results for 10 HS tetrasaccharides standards. The C-5 stereochemistry of the central uronic acid is clearly resolved using the respective diagnostic ratios for the isomers. The lowest diagnostic ratio recorded for the GlcA-containing isomers was  $0.05 \pm 0.01$  for standard 3a (IdoA2S-GlcNS-GlcA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), and the closest to zero for those with IdoA was  $-0.12 \pm 0.01$ , recorded for sample 3i (IdoA-GlcNS-IdoA-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>). The ability to resolve epimers for the tri-sulfated HS standards using the diagnostic ratio formula is tested with standards 3b (IdoA-GlcNS6S-GlcA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 3h (GlcA-GlcNS6S-IdoA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), and 3c (GlcA-GlcNS-IdoA2S-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 3e (GlcA-GlcNS-GlcA2S-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), as shown on Figure 7. Even

though standards 3f (GlcA-GlcNAc6S-IdoA2S-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), 3g (IdoA2S-GlcNAc6S-GlcA-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), and 3j (IdoA-GlcNAc6S-IdoA2S-GlcNAc6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) have different repeating disaccharide units compared with the other seven isomers, EDD fragmentation of the  $[M - 4H + Na]^{3-}$  precursor ion ( $m/z$  373.3846) for 3f, 3g, and 3j produced diagnostic ratio results consistent for assigning their hexuronic acid stereochemistry. We observe a minor diagnostic ratio difference between standards 3f ( $-0.12 \pm 0.01$ ) and 3j ( $-0.24 \pm 0.01$ ), which differ only at the non-reducing end uronic acid. However, this minor difference had no impact on the assignment of the stereochemistry of the central uronic acid.

### Diagnostic Ratio Results for the Tetra-Sulfated HS Standards

Highly sulfated HS GAGs are difficult to characterize due to loss of the labile sulfo groups. However, we show, in Figure 8, reproducible diagnostic ratio results for these highly sulfated tetramers for the  $[M - 5H + 2Na]^{3-}$  precursor for all seven isomers (standards 4a–4g) and  $[M - 5H + Na]^{4-}$  for 4h and 4i. The selected precursor ion for the diagnostic ratio analysis ensured at least all sulfo groups are ionized. The diagnostic ratio values obtained for this degree of sulfation allowed for confident assignment of their respective C-5 uronic acid stereochemistry. The lowest diagnostic ratio recorded for the GlcA-containing tetra-sulfated standards was  $0.066 \pm 0.006$  and the closest to zero for the IdoA standards was  $-0.06 \pm 0.01$ , recorded for 4a (IdoA2S-GlcNS6S-GlcA-GlcNS-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 4f (IdoA-GlcNS6S-IdoA-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), respectively. Epimeric pairs 4c (GlcA-GlcNS-

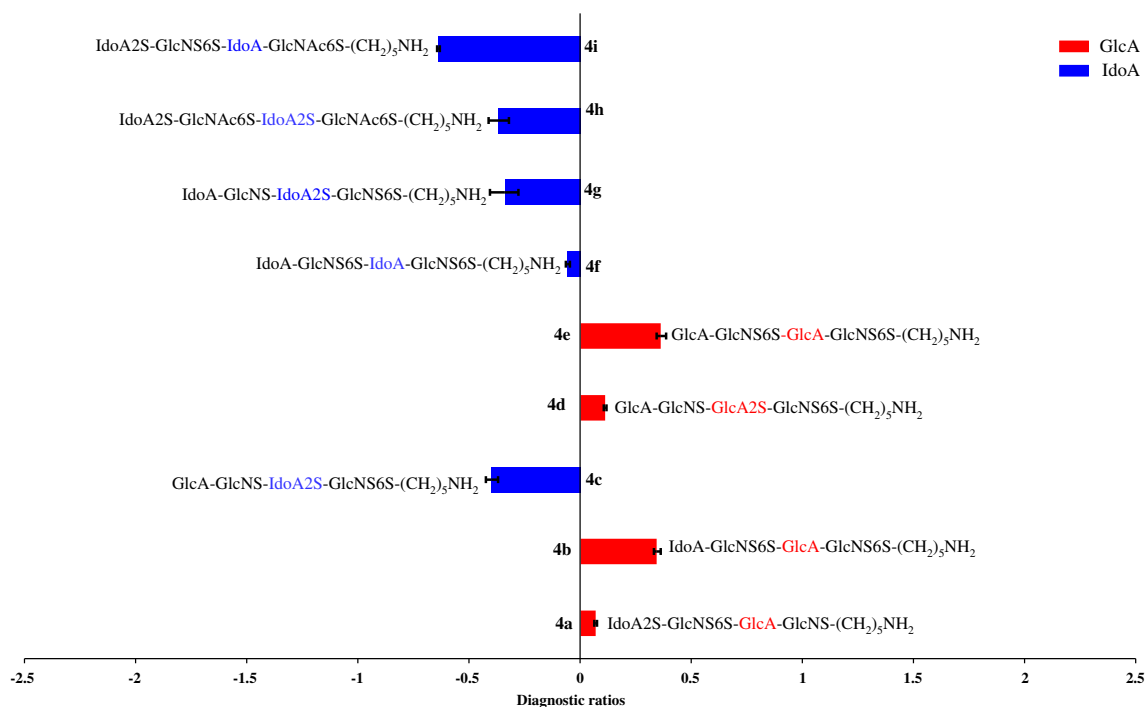


Figure 8. EDD diagnostic ratio results for the tetra-sulfated HS tetrasaccharides standards 4a–4g for the  $[M - 5H + 2Na]^{3-}$  precursor ion and  $[M - 5H + Na]^{4-}$  for standards 4h and 4i



IdoA2S-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 4d (GlcA-GlcNS-GlcA2S-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) are clearly resolved as shown in Figure 8, with diagnostic ratio values  $-0.40 \pm 0.03$  and  $0.109 \pm 0.007$ , respectively. Epimeric standards 4b (IdoA-GlcNS6S-GlcA-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 4f (IdoA-GlcNS6S-IdoA-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) are also differentiated unambiguously based on the diagnostic ratio values  $0.35 \pm 0.01$  and  $-0.06 \pm 0.01$ , respectively. We again compare the diagnostic ratio results for standards 4b (IdoA-GlcNS6S-GlcA-GlcNS6S) and 4e (GlcA-GlcNS6S-GlcA-GlcNS6S) differing only at the non-reducing end uronic acid. Their respective diagnostic values,  $0.35 \pm 0.01$  and  $0.36 \pm 0.02$ , again indicated the non-reducing end uronic acid stereochemistry had little or negligible impact on the diagnostic ratio results. Standards 4c (GlcA-GlcNS-IdoA2S-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>) and 4g (IdoA-GlcNS-IdoA2S-GlcNS6S-(CH<sub>2</sub>)<sub>5</sub>NH<sub>2</sub>), also differing at the non-reducing end uronic acid residue, produced almost similar diagnostic ratio values as shown in Figure 8. A mass list (mass (*m/z*) – intensity table) for the ions used for the DR calculations for all 33 HS standards are included in the [Supplemental Material](#).

## Conclusions

In this study, we have demonstrated the capability to assign the C-5 hexuronic acid stereochemistry from a single stage tandem mass spectrum using EDD. The diagnostic ratio provides the means to assign the stereochemistry of the uronic acid near the reducing end for HS tetrasaccharides. For all 33 tetramers that were examined, the diagnostic ratio was positive for GlcA near the reducing end, whereas those having IdoA residues near the reducing end had negative DR values. The smallest absolute value of DR for an IdoA-containing tetrasaccharide standard was  $-0.06 \pm 0.01$ , and for a GlcA was  $0.05 \pm 0.01$ . These data show that the diagnostic ratio clearly distinguishes the uronic acid stereochemistry, not by comparison to a standard, but with a number derived directly from the data.

The applicability of this approach to typical analytical problems faced by glycosaminoglycan researchers remains to be demonstrated. Generally speaking, researchers are confronted with mixtures of GAG oligomers, and these would need to be resolved before the type of analysis presented in this paper could be performed, as the diagnostic ratio only has significance for single component samples. Secondly, the diagnostic ratio presented here has been demonstrated on tetramers that are alkylated at the reducing end. This modification breaks the symmetry of the structure and allows one to easily distinguish reducing end from non-reducing end fragments. This derivatization can be performed on real-world samples, but will require an extra step in the work-up procedure. Finally, this approach has been demonstrated only for assigning the stereochemistry of the uronic acid residue closest to the reducing end. Future work will focus on extending this approach to assign the

C-5 hexuronic acid stereochemistry of additional residues in longer chain HS GAGs using EDD.

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