=SUPPORTING INFORMATION

Mass Spectrometric Method for Determining the Uronic Acid Epimerization in Heparan Sulfate Disaccharides Generated using Nitrous Acid and PGC

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This supporting information includes additional results and information as described in the text of the main article: (1) Detailed experimental protocol; (2) Figure S-1, (3) Figure S-2 shows the hydrazinolysis and HONO depolymerization of HS and the resulting HexA-2,5-anhydromannose disaccharides; (4) Figure S-3 shows the HONO- and heparin lyase-derived disaccharide structures; (5) Figure S-4 shows the extracted ion chromatograms of HS HONO-derived dp2 from bovine tissue samples, (6) Figure S-5 shows the mass spectra of the HONO-dervied disaccharides from HSPIM, (7) Figure S-6 shows the extracted ion chromatograms of HS heparitinase derived-dp2 from HSBK, HSPIM and heparin analyzed by SEC-MS, (8) Table S-1 shows the relative quantities of the nine HexA-aMan$_R$ disaccharides from HSBK, HSPIM, and heparin extracted from the PGC, (9) Table S-2 shows the comparison of the disaccharide abundances from HSBK, HSPIM, and heparin obtained by extensive deaminative cleavage analyzed by PGC-MS and exhaustive enzymatic digestion analyzed using SEC-MS; (10) Figure S-7 shows the EDD spectra of the purified mono-sulfated HONO-derived dp2s; (11) Figure S-8 shows the EID spectra of the purified mono-sulfated HONO-derived dp2s, and (12) Figure S-9 illustrates the chemical *O*-desulfation of the purified mono-sulfated dp2s.

Experimental Section

Materials – Porcine intestinal mucosa heparin, bovine kidney HS, bovine liver β-glucuronidase, and recombinant human α-L-iduronidase were purchased from Sigma-Aldrich (St. Louis, MO). Porcine intestinal mucosa HS was purchased from Celsus Laboratories (Cincinnati, OH). Heparin lyases I, II and III from *Flavobacterium heparinum* were purchased from Ibex (Montreal, QC). Heparan sulfate disaccharides were from Sigma and V-Labs (Covington, LA). K5 polysaccharide and epimerized *N*-sulfated K5 polysaccharide were from Iduron (Manchester, UK). Reagents used for LC-MS were of HPLC grade and obtained from Fisher Scientific (Fairlawn, NJ).

Hydrazinolysis and Nitrous Acid (HONO) Depolymerization –For the hydrazinolysis, 10-15 μg oligosaccharide was dissolved in 20 μL of a 70% hydrazine solution containing 1% (w/w) hydrazine sulfate. The mixture was heated at 97^{o}C for 4 hours. After cooling the reaction in an ice-cold bath, the hydrazine was evaporated in a stream of air at room temperature and lyophilized to remove any residual hydrazine. After repeated evaporation to dryness, the sample was desalted using a PD-10 cartridge (GE Healthcare, Piscataway, NJ), and lyophilized.

To the dried deacetylated sample, 20 μL of cold HONO pH 1.5 reagent (0.5M H_2SO_4 and 0.5M $Ba(NO_2)_2$ with volume ratio of 1:1) was added. After 10 min, the pH of the solution was adjusted to 4 with 1M Na_2CO_3. 20 μL of HONO pH 4 reagent (5.5 M $NaNO_2$ and 0.5M H_2SO_4 with 5:2 volume ratio) was added. After 15 min, the reaction was terminated by adjusting the pH to 8.5 using 1M Na_2CO_3. The HONO-treated cleavage products were reduced with 0.5 M $NaBH_4$ for 8 h at 55 ^{o}C. After incubation, samples were acidified to ~pH 4 with acetic acid and then neutralized to pH 7 by addition of ammonia.

Enzymatic Digestion – HS from bovine kidney (HSBK) and porcine intestinal mucosa (HSPIM) as well as heparin were digested with 3.2 milliunits of heparin lyase I, 3.2 milliunits of heparin lyase II, and 1.6 milliunits of heparin lyase III in a final volume of 50 μL of 50 mm Tris/HCl buffer, pH 7.45, in the presence of 5 mm $CaCl_2$. Half of the total amount of the enzymes were added and the solutions were incubated at 37^{o}C for 24 hrs. The rest of the enzymes were then added and incubation was continued for another 24 hrs. The digests were analyzed using size exclusion chromatography (SEC) LC-MS without further treatment.

Chemical O-Desulfation – For 6-*O*-desulfation the pyridinium salt of the HONO-derived dp2s was prepared by passing the sample (~10 μg) through a column with AG cation exchange resins (dimensions, supplier) followed by addition of 1 μL pyridine to the eluant. After lyophilization, 20 μL of pyridine and 2 μL of *N*-methyl-*N*-(trimethylsilyl)-trifluoroacetamide (MTSTFA) were added and the mixture was heated at 100°C for 15min.

2-*O*-desulfated disaccharides were prepared by dissolving 10 μg quantity of the HONO-derived dp2s in 0.5 mL of 1.0 M NaOH, and the solution was frozen and lyophilized. The resulting *O*-desulfated disaccharides were desalted using SEC.

Liquid Chromatography/Mass Spectrometry – The PGC column (Hypercarb Column, 5 um, 150 mm × 4.6 mm) was purchased from Thermo Electron Corporation (Waltham, MA). The samples were separated using a Waters Acquity Ultra Performance LC system (Milford, MA). Solvent A was 0.1% formic acid adjusted to pH 5.5 by ammonia and solvent B was solvent A in 90% acetonitrile. The gradient used was 0-15% B in 4 min and 15% B over 50 min. The column was then washed with 100% B for 10 min and equilibrated with 100% A for 15 min. The flow rate was 0.5 mL/min, split down before the mass spectrometer to approximately 100 μL/min. Disaccharides eluting from the PGC column were analyzed using an Applied Biosystems QSTAR Pulsar-I (Q-TOF) mass spectrometer (Carlsbad, CA) operating in negative ion mode. The ionization of the LC flow was accomplished by a TurboIonSpray source with capillary voltage set at -3500, nebulizer gas at 60, curtain gas at 35, turbo gas at 30, temperature at 100, and the sprayer position optimized to give least sulfate loss for ΔHexA2S-GlcNS6S standard (<15%).

The SEC column (Superdex™ peptide PC 3.2/30) was purchased from GE Healthcare (Piscataway, NJ). For disaccharide analysis, the mobile phase (12.5 mm formic acid, pH adjusted to 4.4 using ammonia, in 10% acetonitrile) was delivered isocratically by a Waters Acquity UltraPerformance LC system at 0.015 ml/min. Disaccharides eluting from the SEC column were analyzed using an Applied Biosystems QSTAR Pulsar-I (Q-TOF) mass spectrometer operating in negative ion mode. The ionization of the LC flow was accomplished by a TurboIonSpray source with capillary voltage set at -3500, nebulizer gas at 40, curtain gas at 25, and turbo gas at 0.

Tandem Mass Spectrometry – All tandem mass spectra were obtained using a 12 Tesla Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometer (SolariX, Bruker Daltonics, Billerica, MA) The samples were diluted to 0.1 mg/mL in 50% methanol and directly infused to the mass spectrometer via a nano ESI source operated in negative ion mode. The capillary voltage was 1100 V, the drying gas flow was 2.5 L/min, and the ion transfer capillary temperature was set at 180 °C. The mass spectra were acquired over the *m/z* range of 36 to 1000. Electron detachment dissociation (EDD) and electron-induced dissociation (EID) experiments were performed by fragmenting after quadrupole isolation using the following parameters: heater current 1.63 A, electron pulse length 2 ms, electron bias -19.25 to -20 V, and ECD lens -19 to -18.5 V. The collision cell ion accumulation time was set to 2 seconds. For each spectrum, 200 scans were averaged. Background spectra were acquired by setting the electron bias to 0 V. DataAnalysis 4.0 (Bruker Daltonics) was used for data analysis.

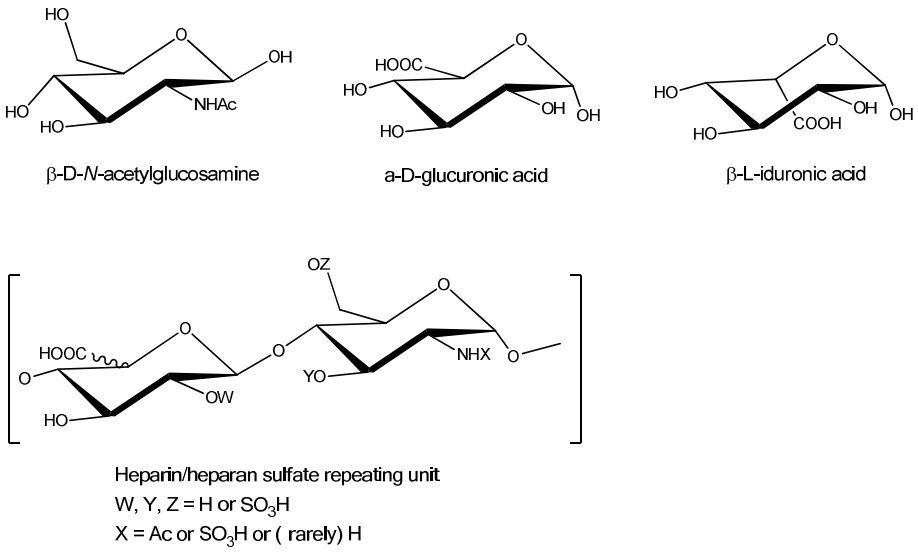

Figure S-1. Structures of monosaccharides and the disaccharide repeating unit found in heparan sulfate and heparin. Note that glucuronic acid and iduronic acid are epimers. A uronosyl-C5-epimerase acts on chains during biosynthesis to change the stereochemistry of the C5 position.

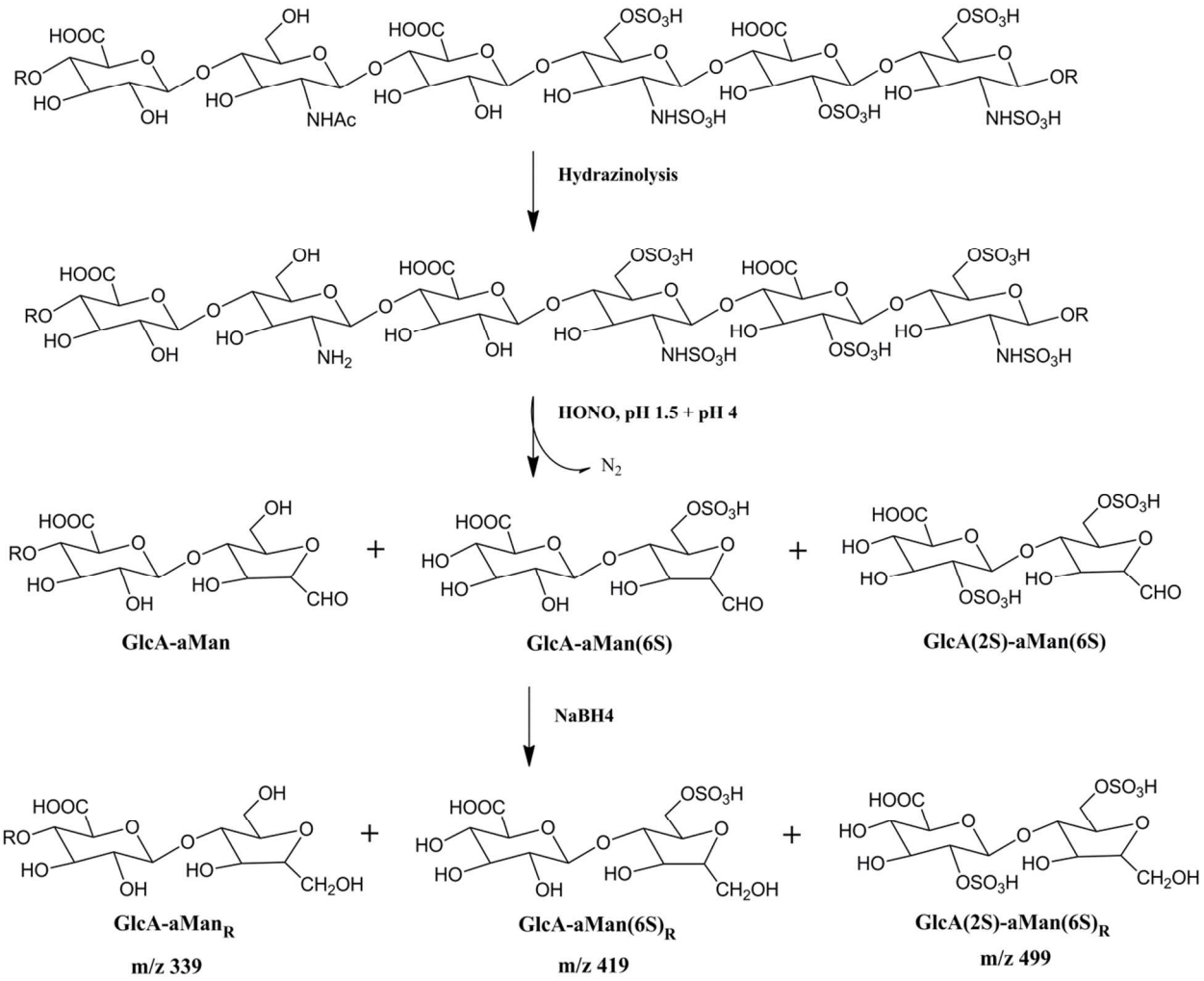

Figure S-2. Scheme showing hydrazinolysis and HONO depolymerization of HS.

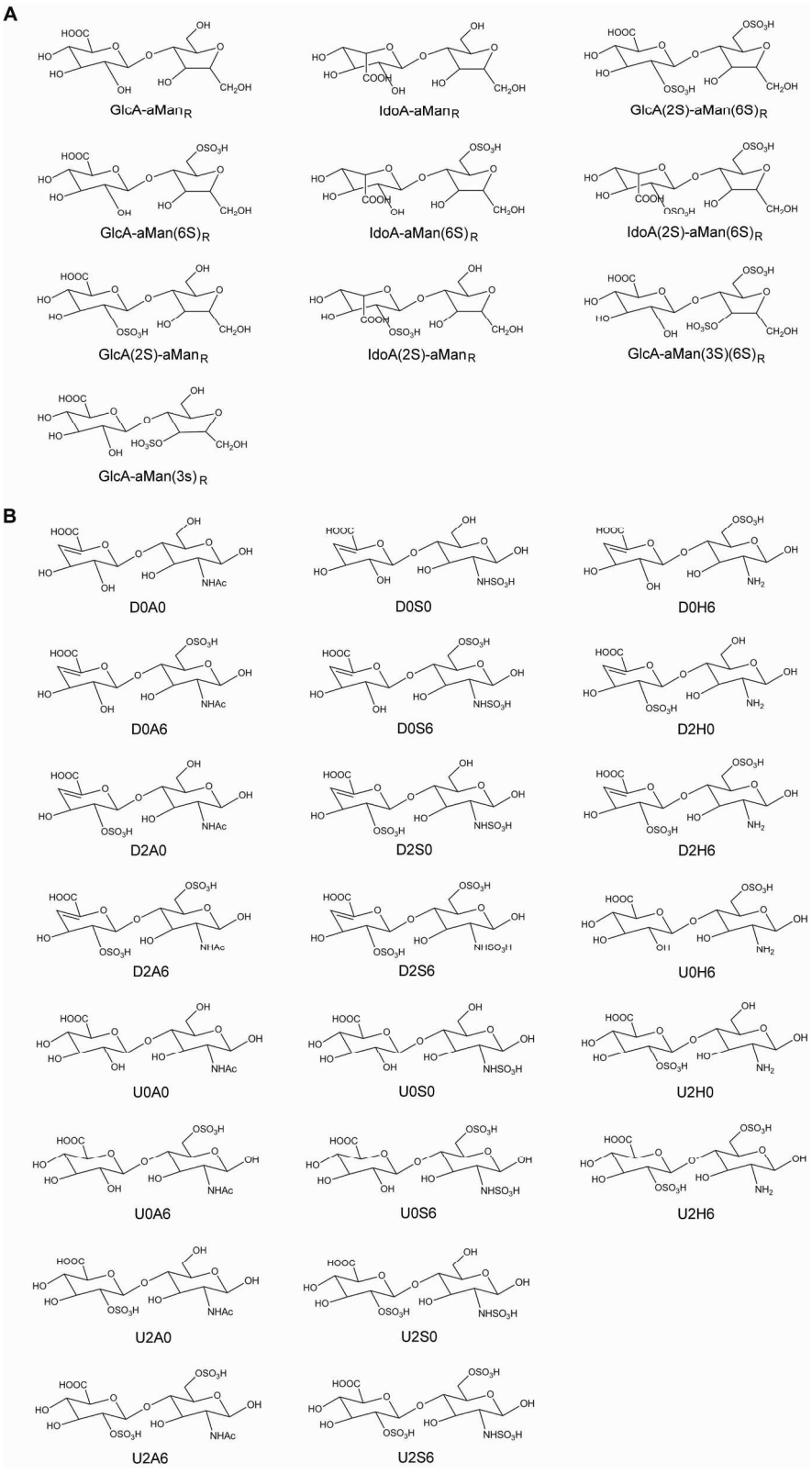

Figure S-3. Structures of disaccharides derived from (A) HONO depolymerizaytion and (B) heparin lyase digestion.

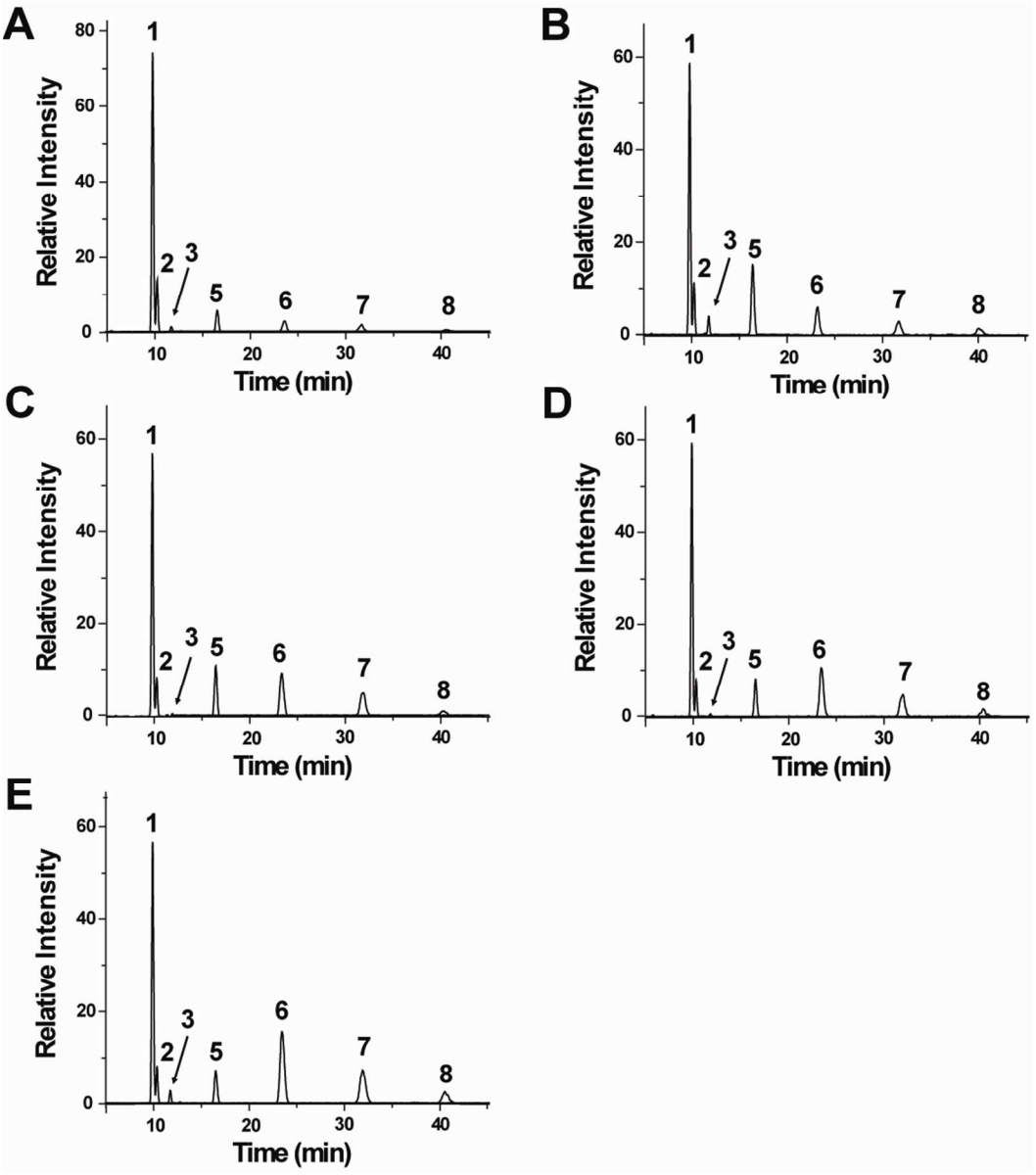

Figure S-4. Total ion chromatogram of HONO-derived dp2s analyzed by PGC-MS from bovine organ tissue samples: (A) Aorta, (B) Intestine, (C) Lung, (D) Kidney A, and (E) Kidney B. HS from Kidney A and B were recovered by ion-exchange chromatography and eluted with 1.0 M and 1.25 M NaCl, respectively. All samples only have seven peaks: two non-sulfated (peaks 1 and 2), four mono-sulfated (peaks 3 and 5-7), and one di-sulfated dp2 (peak 8).

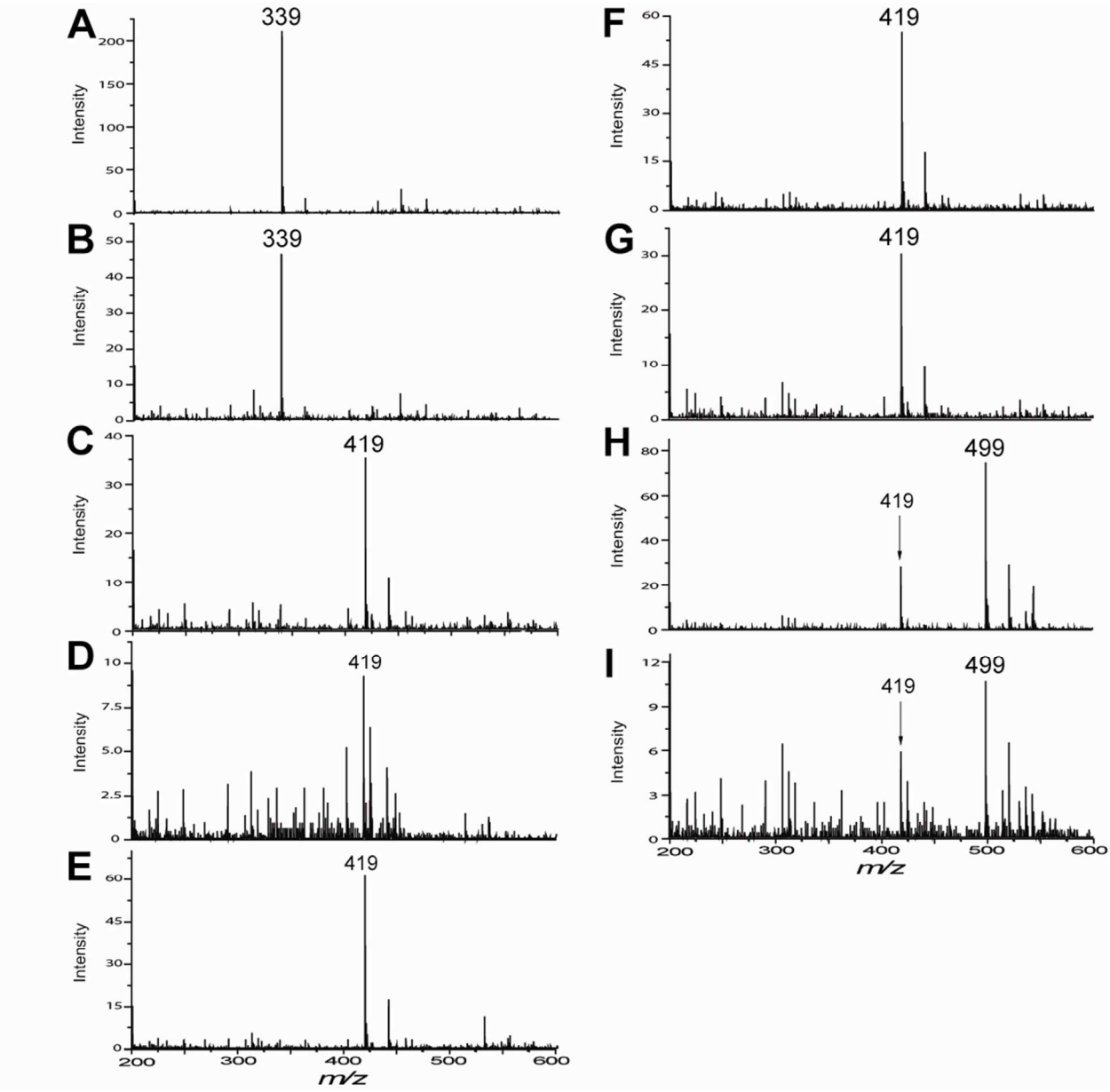

Figure S-5. Mass spectra of the HONO-dervied disaccharides from HSPIM. (A) Peak 1, (B) Peak 2, (C) Peak 3, (D) Peak 4, (E) Peak 5, (F) Peak 6, (G) Peak 7, (H) Peak 8, and (I) Peak 9.

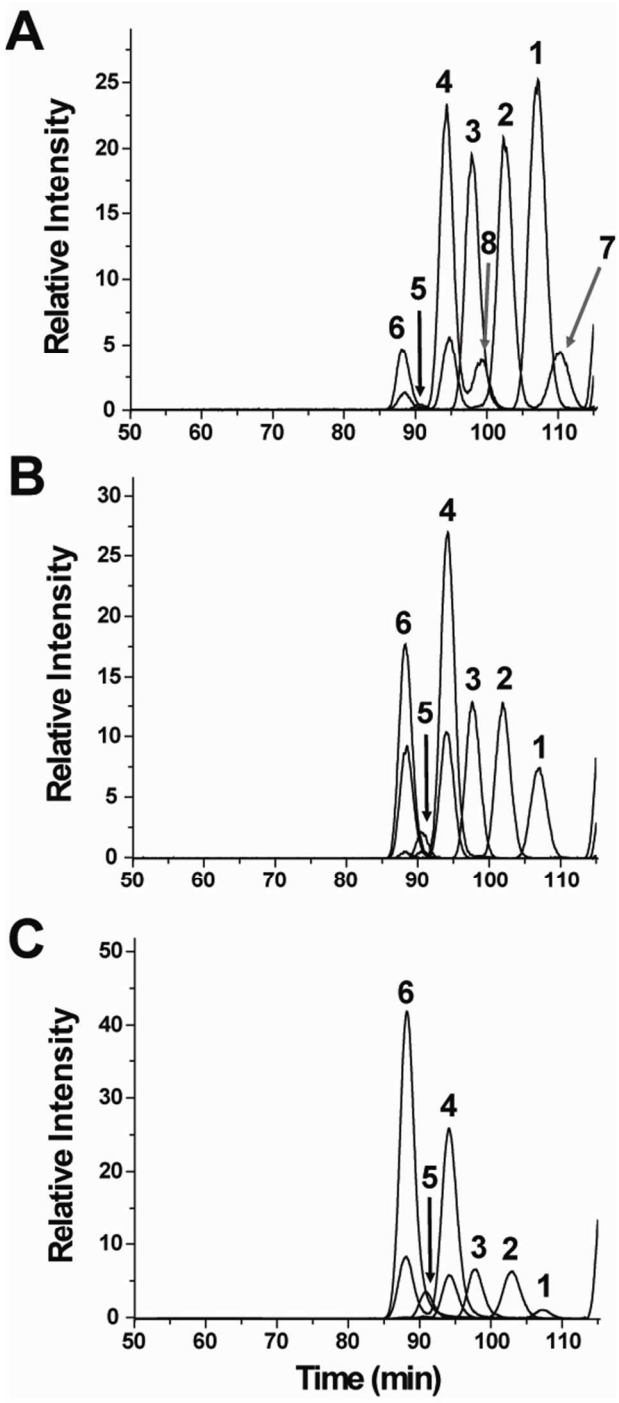

Figure S-6. Extracted ion chromatogram of heparin lyase-derived dp2s analyzed by SEC-MS from (A) HSBK, (B) HSPIM, and (C) heparin. The disaccharide peaks are: *1*, D0A0; *2*, D0S0; *3*, D2A0 or D0A6; *4*, D2S0 OR D0S6; *5*, D2A0; *6*, D2S6; *7*, D2H0 OR D0H6; *8*, D2H6.

Table S-1. Relative quantities of the nine HexA-aMan$_R$ disaccharides extracted from the PGC-MS data

Disaccharide	HSBK	HSPIM	Heparin
1	39 ± 3	17 ± 1	3.6 ± 0.5
2	4.1 ± 0.4	2.7 ± 0.3	0.8 ± 0.2
3	0.8 ± 0.2	7.1 ± 0.9	8.2 ± 0.9
4	0.2 ± 0.1	2.0 ± 0.7	3.7 ± 0.4
5	11 ± 1	8.6 ±0.6	9 ± 0.4
6	12 ± 1	10 ± 1	5.1 ± 0.3
7	15 ± 1	13 ± 1	3.9 ± 0.3
8	18 ± 2	35 ± 3	62 ± 3
9	0	4.4 ± 0.4	7.9 ± 0.7

Table S-2. Comparison of the disaccharide abundances from HSBK, HSPIM, and heparin obtained by extensive deaminative cleavage analyzed by PGC-MS and exhaustive enzymatic digestion analyzed in triplicate using SEC-MS

Disaccharide	HSBK		HSPIM		Heparin	
	HONO/ PGC-MS	Lyases/ SEC-MS	HONO/ PGC-MS	Lyases/ SEC-MS	HONO/ PGC-MS	Lyases/ SEC-MS
Non-sulfated[a] or [D0A0 + U0A0 + D0S0 + U0S0][b]	43 ± 3	39 ± 3	20 ± 2	17 ± 2	3 ± 1	4 ± 1
Mono-sulfated[a] or [D2A0/D0A6 + U2A0/U0A6 + D2S0/D0S6 + U2S0/U0S6 + D2H0/D0H6 + U2H0 + U0H6][b]	39 ± 4	47 ± 4	41 ± 4	46 ± 2	27 ± 3	30 ± 4
Di-sulfated[a] or [D2A6 + U2A6 + D2S6 + U2S6 + D2H6 + U2H6][b]	18 ± 2	14 ± 2	39 ± 3	36 ± 3	70 ± 4	66 ± 5

[a] These dps2 are derived from deaminative cleavage with HONO.

[b] These dp2s are derived from enzymatic digestion with heparin lyases I, II and III.

Non-reducing end descriptor: U = saturated, D = Δ4,5-unsaturated uronic acid

Uronic acid *O*-sulfation: 0 = no sulfation, 2 = 2-*O*-sulfation

Hexosamine descriptor: A = *N*-acetylated, S = *N*-sulfated, H = free amine

Hexosamine *O*-sulfation: 0 = no sulfation, 6 = 6-*O*-sulfation.

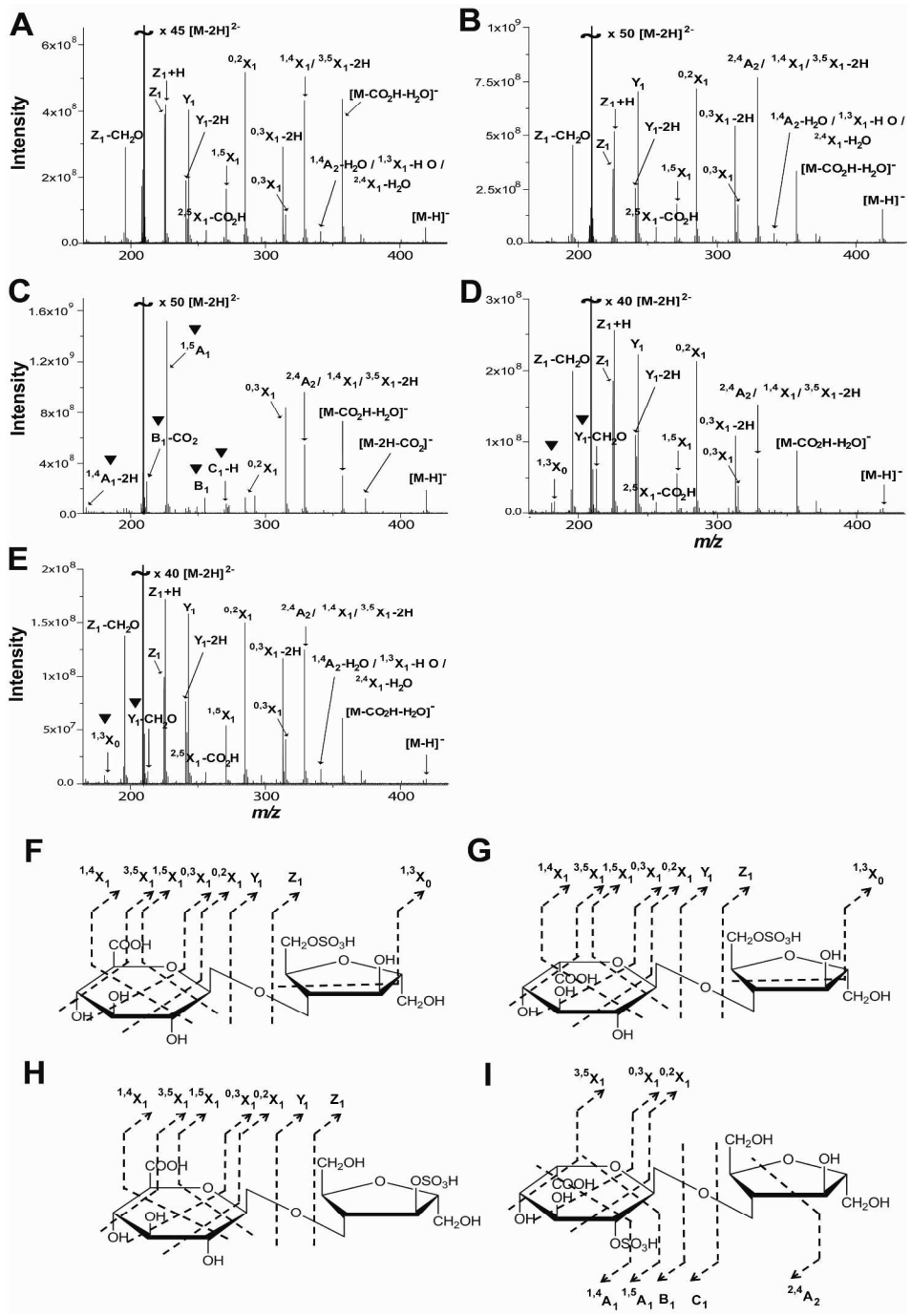

Figure S-7. EDD spectra of the $[M-2H]^{2-}$ of the purified mono-sulfated HONO-derived dp2s. (A) MS^2 of peak 3, (B) MS^2 of peak 4, (C) MS^2 of peak 5, (D) MS^2 of peak 6, and (E) MS^2 of peak 7. The peak assignments for the disaccharides (F) GlcA-aMan(6S)$_R$, (G) IdoA-aMan(6S)$_R$, (H) GlcA-aMan(3S)$_R$, and (I) IdoA(2S)-aMan$_R$ are shown. The symbol ▼ above the peak labels indicates product ions unique to some isomeric disaccharides. Ambiguous assignments are indicated separated by a forward slash.Fragmentation of peaks 3 (A) and 4 (B) matches those of either F and I. Mass spectra of peak 5 indicates it has structure I. Fragmentation pattern of peaks 6 and 7 matches those of structures F and G.

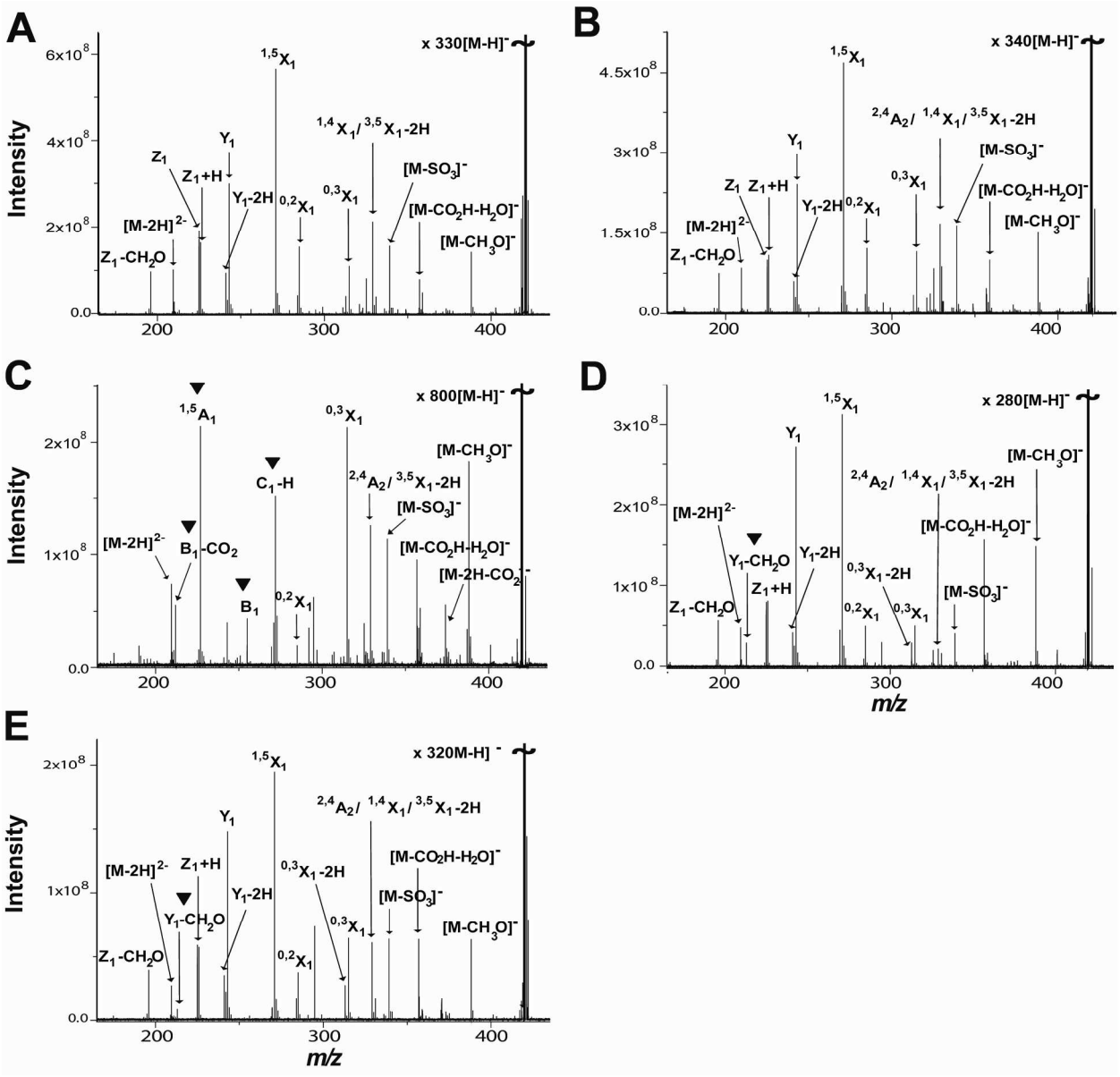

Figure S-8. EID spectra of the [M-H]$^-$ of the purified mono-sulfated HONO-derived dp2s. (A) MS^2 of peak 3, (B) MS^2 of peak 4, (C) MS^2 of peak 5, (D) MS^2 of peak 6, and (E) MS^2 of peak 7. The symbol ▼ above the peak labels indicates product ions unique to some isomeric disaccharides.

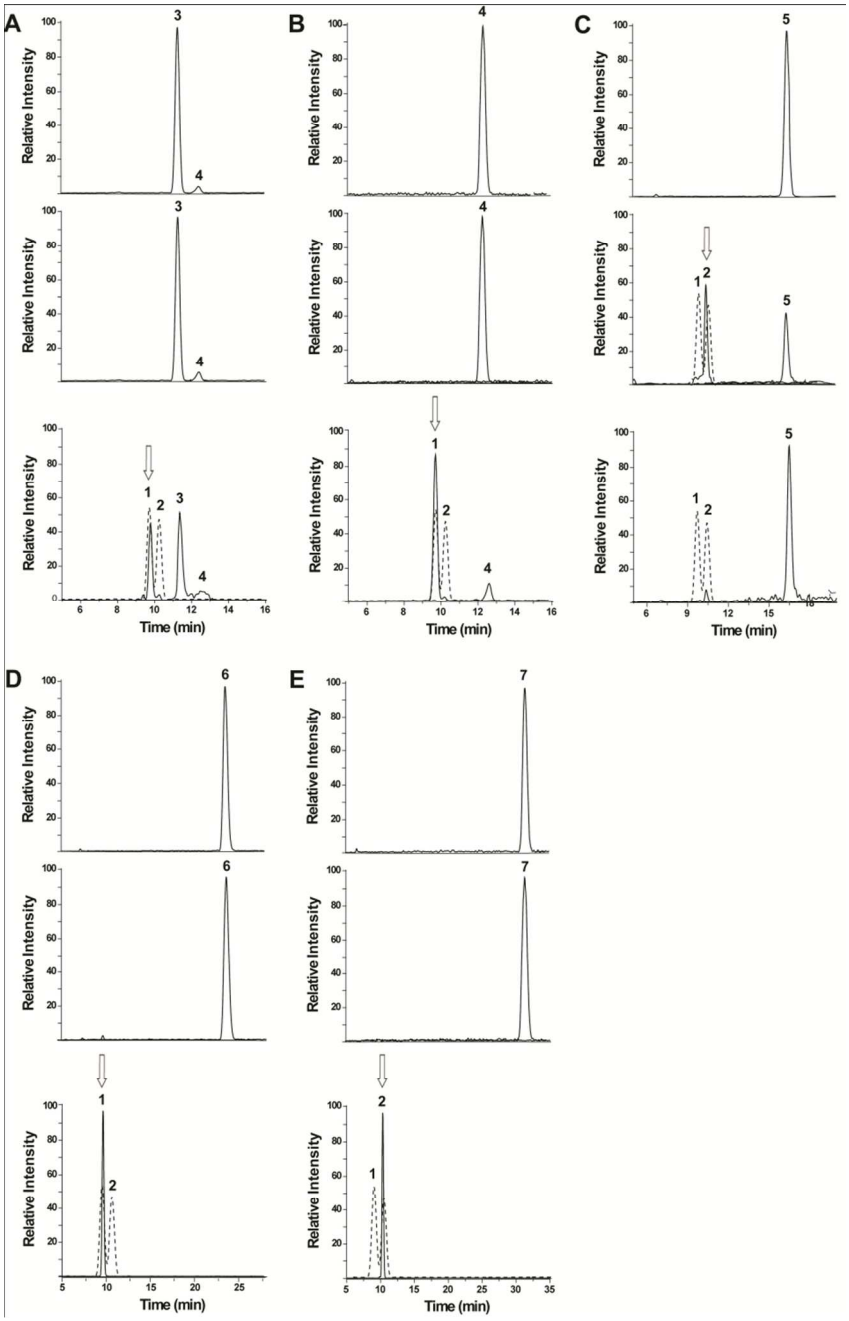

Figure S-9. Chemical *O*-desulfation of the purified mono-sulfated dp2s (A) peak 3, (B) peak 4, (C) peak 5, (D) peak 6, and (E) peak 7. The EIC of *m/z* 339 from the epimerized K5 polysaccharide are shown in dotted lines. The top panel represents the EIC of *m/z* 419 for the purified mono-sulfated dp2. The middle panel shows the effect of 2-*O*-desulfation. Only peak 5 showed a decrease in intensity after 2-*O*-desulfation. Also, there is an increase in peak 2, which has been identified as IdoA-aMan$_R$. The bottom panel shows the effect of 6-*O*-desulfation on the purified HONO derived-dp2s. Among the five mono-sulfated dp2s, peak 5 showed minimal 6-O-desulfation. For peaks 3, 4 and 6, there is a significant decrease in the mono-sulfated dp2 and an increase in the peak 1, GlcA-aMan$_R$. In contrast, the decrease in peak 7 resulted in an increase in peak 2, IdoA-aMan$_R$.