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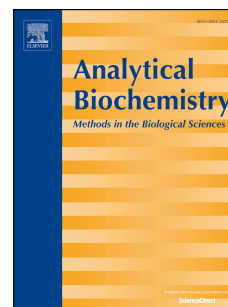
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Heparan sulfate disaccharide measurement from biological samples using pre-column derivatization, UPLC-MS and single ion monitoring

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Title

Heparan sulfate disaccharide measurement from biological samples using pre-column derivatisation, UPLC-MS and single ion monitoring

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1.0 Introduction

Glycosaminoglycans (GAGs) are long unbranched polysaccharide chains composed of repeating disaccharide subunits of alternating uronic acid and glycosamine (GlcN) residues. These molecules have a large net negative charge as a result of a complex arrangement of N- and/or O-linked sulfate binding on the sugar moieties. Heparin is the best known GAG and is expressed exclusively by mast cells [1]. Other GAGs include heparan sulfate (HS), chondroitin sulfate (CS), dermatan sulfate (DS) and keratan sulfate (KS). These are ubiquitously expressed on the extracellular matrix and bound, via serine residues, to protein cores on many cell surfaces in the form of proteoglycans (PGs) [2].

Whilst heparin is well known for its anticoagulant properties, there is increasing evidence that heparin and other GAGs can influence cell functions through a diverse range of activities that include the regulation of cell survival, growth, differentiation and angiogenesis and invasion [3, 4, 5]. Although the molecular mechanism of the action of GAGs is not well understood, accumulating evidence suggests that the effects of heparan sulfate glycosaminoglycans may be attributable at least in part to their binding of the growth factors and regulating of their signaling [6, 7].

GAGs show considerable variation in chain length and degree of sulfation on either sugar in the disaccharide subunit. In the case of HS, the disaccharide units are differentially sulfated on the C-6 carbon or N position of the GlcN, on C-2, or occasionally on C-3, of the GlcA/IdoA subunit [8]. The polysaccharide chain length coupled with the variation in the extent of sulfation, give rise to hundreds of potential polysaccharide structural combinations.

GAGs have been implicated in a wide variety of pathology including respiratory failure [9], placental malaria [10] and cancer [11]. HS and CS have been shown to be important for the initial attachment of Merkel cell polyomavirus (MCV) to cells while 6-O-sulfated and N-sulfated HS is important for the reporter vector entry [12]. Determining the composition of these GAGs is important in understanding their role. Depolymerization of GAGs and analysis of their disaccharides is an important step in the characterization of GAGs. Depolymerization can be achieved using specific lyases which cleave the glycosidic linkage between a hexosamine and a hexuronic acid thus introducing an unsaturated bond (absorbing at 232 nm) between the C4 and C5 of the hexuronic acid. GAGs can also be depolymerized chemically by mild hydrazinolysis and subsequent deaminative cleavage with nitrous acid [13]. This method retains the original epimeric nature of the hexuronic acid but loses information about the N-sulfation or N-acetylation because the resulting disaccharide comprise a hexuronic acid linked to a 2,5-anhydrohexose bearing an aldehyde group [13].

Enzymatic depolymerization of heparin/heparan sulfate (hep/HS) with heparinases give rise to twelve disaccharides out of which only eight are commonly seen in normal organisms [14,

15]. The other four (N-unsubstituted glucosamine) are rare and are believed to result from the loss of labile N-sulfate groups during isolation or incomplete modification (N-deacetylase/N-sulfotransferase) during hep/HS biosynthesis [14]. These twelve hep/HS disaccharides are commercially available.

Previous techniques used for the compositional analysis of GAG disaccharides include liquid chromatography (LC) with UV [16], fluorescence [17], and MS detection. These GAG disaccharides are hydrophilic and hence will not be well retained on a reverse phase (RP) column. Underivatized GAG disaccharides can be analyzed directly using a variety of separation methods including hydrophilic interaction liquid chromatography (HILIC)-MS. However, co-elution of disaccharides has been reported [13]. To overcome these issues, GAG disaccharides may be derivatized with a hydrophobic fluorophore which increases their retention on RPLC and may improve MS sensitivity. Derivatizing reagents employed for this include 4,4-difluoro-5,7-dimethyl-4-bora-3a-diaza-s-indacene-3-propionic acid (BODIPY) and 2-aminobenzamide [14]. Retention of GAG disaccharides and oligosaccharides may also be improved using ion pair RPLC with reagents such as tetrabutylammonium [18], n-hexylamine, and n-pentylamine. [19] However, each of these has specific additional drawbacks that make analysis of GAG disaccharides problematic, particularly when analyzing biological samples. These require long run times and some of the non-volatile ion pairing reagents contaminate the MS interface [20]. Capillary electrophoresis with laser-induced fluorescent detection [21] and fluorophore-assisted carbohydrate electrophoresis (FACE) [22] have also been employed in GAG disaccharide analysis. However, both of these methods could suffer from poor reproducibility of migration times.

There is therefore a need to develop a more rapid, selective and sensitive method to quantify levels of GAG disaccharides in biological samples. In this article, we report a method of rapid separation and detection of twelve heparin/heparan sulfate disaccharides and an internal standard in 11 min. This method demonstrates excellent reproducibility, which does not require the specialized separation techniques needed for the separation of native disaccharides and has been successfully applied to the comparison of disaccharide expression in a variety of human cancer cell lines. Derivatization-based methods frequently report the use of highly toxic reducing agents such cyanoborohydride. The method described uses the less toxic 2-methylpyridine borane. This analysis employs an optimized selected ion recording (SIR) precolumn RP-UPLC-MS method based on 2-aminoacridone derivatization without the need to remove excess unreacted reagent. We have applied this method to the analysis of disaccharide levels in a variety of human cell lines.

2.0 Materials and methods

2.1 Materials

Actinase (from *Streptomyces griseus*), 2-aminoacridone (AMAC), 2-methylpyridine borane complex, heparan sulfate (sodium salt from bovine kidney), anion exchange spin columns, 3 kDa, and 10 kDa molecular weight cut off filters were purchased from Sigma-Aldrich, (Poole, UK). Twelve unsaturated heparin/HS disaccharides (Δ UA,2S - GlcNS,6S; Δ UA,2S-GlcNS; Δ UA,2S - GlcNAc,6S; Δ UA-GlcNS,6S; Δ UA-GlcNS; Δ UA-GlcNAc; Δ UA,2S- GlcNAc; Δ UA-GlcNAc,6S; Δ UA,2S - GlcN; Δ UA,2S-GlcN, 6S; Δ UA-GlcN, 6S; Δ UA-GlcN) and an internal standard (Δ UA,2S - GlcNCOEt,6S) were purchased from Iduron (Manchester, UK). Heparinases (from *Flavobacterium heparinum*) I, II, and III were also purchased from Iduron (Manchester, UK). Acetic acid, LC-MS grade dimethyl sulfoxide (DMSO), LC grade ammonium acetate, methanol, and UPLC water were purchased from Fisher Scientific, (Loughborough, UK).

2.2 Cell culture

GAGs were extracted from a variety of human cell lines grown in our laboratory. These included cell lines from ovarian adenocarcinoma (OVCAR-3), metastatic breast adenocarcinoma (MDA-468), acute myeloid leukaemia (MOLM-13), choriocarcinoma (BeWo) and a colon cancer cell line (HCT116). Cells were incubated at 37 °C in media containing 10 % fetal bovine serum , 1 % penicillin and streptomycin (Thermofisher, Hemel Hempsted, UK) in an atmosphere containing 5 % CO₂. All cells were cultured using RPMI 1640 medium except HCT116 cells which were cultured using DMEM (Sigma) and BeWo cells were cultured using DMEM:F12. All cell lines were purchased from LGC standards (Teddington, UK) with the exception of MOLM-13 cells (DSMZ, Braunschweig, Germany) and were cultured for at least two passages prior to GAG analysis.

2.3 Extraction and enzymatic depolymerization of heparin/HS disaccharides from cells

We used an adaptation of the extraction method reported by Han, et al. [23] and Volpi, et al. [24] for the extraction of heparin/HS from cells. Briefly, cells grown to confluence were scraped gently from the flask and washed in 100 mM phosphate buffered saline. They were then incubated with actinase (20 mg/mL) for 18 h to proteolyse intracellular and membrane

bound proteins. The resulting solution was filtered to remove particulates, and the peptides removed using a 10 kDa molecular weight cut-off filter. The retentate was lyophilized and re-suspended in 8 M urea with 2 % CHAPS. Anion exchange spin columns were used to extract GAGs from this mixture, washed with 200 mM NaCl and subsequently eluted with 16 % NaCl. This solution was desalted using a 3 kDa molecular weight cut-off filter, lyophilized, and re-suspended in 50 mM phosphate buffer (pH 7.0). Time-controlled enzymatic depolymerization of the GAGs was achieved by the addition of 3 mIU of heparinase I, II, and III at 37 °C for 48 h as described above. The solution was then heated in boiling water for 10 min and centrifuged at 12,000 x g for 10 min to remove denatured proteins. The supernatant was collected and 0.5 µL of internal standard (Δ UA,2S - GlcNCOEt,6S, 1 mg/mL) was added. This was then lyophilized, derivatized with AMAC and analyzed using UPLC-MS.

In order to determine the recovery of disaccharides from this extraction procedure, heparan sulfate standards (5 and 10 µg) were extracted and disaccharides quantified as described above (extracted HS). In addition, the same amount of HS was directly digested with heparinases without prior extraction and then further processed as above (unextracted HS). For each HS subunit the recovery was determined as the amount detected from extracted HS as a percentage from that obtained from unextracted HS.

2.4 Derivatization of heparin/HS disaccharide standards

The heparin/HS disaccharides were derivatized pre-column using AMAC employing a modification of the method reported by Volpi, et al. [24]. Briefly, solutions containing heparin/HS disaccharides were lyophilized and re-dissolved in a solution of 0.1 M AMAC in a mixture of DMSO and acetic acid (17 : 3, v/v). The mixture was then incubated at room temperature for 15 min and then an equal volume of 2-methylpyridine borane complex (1 M) in acetonitrile was added. This mixture was centrifuged at 10,000 x g for 1 min at room temperature and incubated at 45 °C for 4 h. Prior to injection the sample volume was adjusted to 200 µL with a mixture of DMSO : water (1 : 1, v/v).

2.5 Optimization of AMAC derivatization of heparin/HS disaccharides

To determine the optimal concentration of AMAC, 12 heparin/HS disaccharides and the internal standard (0.5 µg each) were incubated with different concentrations of AMAC over a range of 0.53 – 4.20 mg/mL using the procedure described in Section 2.4. In order to determine the optimum temperature for derivatization, the optimal AMAC concentration (2.1

mg/mL) was used to derivatize a mixture of 12 heparin/HS disaccharides containing 0.5 µg of each disaccharide as described above. The samples were then incubated at 37, 45, or 65 °C for 4 h and subsequently analyzed. To optimize incubation time a mixture of 12 heparin/HS disaccharides was derivatized under optimal conditions of AMAC concentration and incubation temperature for 2, 4, 6, and 8 h and subsequently analyzed as described above.

2.6 Optimization of UPLC-MS conditions

Individual heparin/HS disaccharides were derivatized using optimized conditions described in Section 2.5 and analyzed using UPLC-MS as described above. A mixture of 12 AMAC-derivatized heparin/HS disaccharides was injected at a concentration of 1 µg/mL for the optimization of desolvation temperature, cone and extractor voltages. MS detection was based on the most intense ions observed from trisulfated and disulfated disaccharides while single ions were seen for the mono and non-sulfated disaccharides. Desolvation temperature, cone voltage and extractor voltage were varied between 300 – 400 °C, -15 – 75 V and -1 – 4 V respectively. For all further experiments, the optimized conditions for AMAC concentration, incubation conditions, desolvation temperature, and extractor voltage were 2.1 mg/mL, 4h, 45 °C for 4 h, 350 °C and 3 V respectively. Individual cone voltages were used for each of the derivatized disaccharides.

2.7 UPLC-MS conditions

A Waters Acquity H-Class UPLC system with an SQ Detector 2 was used for the LC-MS analysis. Separation was performed using an ACQUITY BEH Shield RP 18 column (1.7 µm, 2.1 x 150 mm; Waters, Ireland, UK) maintained at 45 °C. Separation was achieved using gradient elution. Solvent A and B were 80 mM ammonium acetate and methanol respectively and were delivered at a flow rate of 0.3 mL/min. The gradient was as follows; Solvent A and 12 % B for 1.7 min followed by a linear gradient of 12 – 15 % B from 1.7 - 5 min, 15 -30 % B from 5 - 10 min, 30 – 100 % B from 10 – 10.7, 100 % B from 10.7 – 13.3 mins, 100 – 12 % B from 13.3 – 13.7 mins and 12 % B from 13.7 – 18 mins. An aliquot (5 µL) of each sample was injected onto the column.

Eluates were detected using a single quadrupole mass spectrometer equipped with an ESI source operated in negative ion mode. The capillary voltage was set to -3 kV; source temperature, 150 °C; source gas flow, 800 L/hr and cone gas flow 50 L/hr. The MS probe position was optimized to 4 mm vertical and 5.1 mm horizontal for the detection of AMAC-

derivatized heparin/HS disaccharides. Data was acquired using selected ion recording (SIR) and was analyzed using the MassLynx® software version 4.1.

2.8 Linearity, Limits of detection, quantification and reproducibility

A mixture of 12 Heparin/HS disaccharides and an internal standard (Δ UA,2S - GlcNCOEt,6S) were derivatized and analyzed in duplicate using optimum conditions at a range of concentrations (0.019-10 μ g/mL) of each disaccharide. Simple least squares regression analysis was used to determine linearity. The limits of detection and quantification were the lowest concentration that produced a signal:noise ratio of at least 3:1 and 10:1 respectively. Reproducibility was determined at concentrations of 0.625, 1.25, and 5 μ g/mL and was defined as the coefficient of variation from five consecutive injections.

3.0 Results and discussion

Prior to derivatization studies, we conducted extensive studies to determine the optimum conditions for efficient separation of disaccharide subunits using HPLC. We found that effective separation of all thirteen disaccharides could be achieved in 11 min. With additional column reconditioning prior to analysis of the next sample, this gives a total run time of 18 min.

3.1 Optimization of Derivatization conditions

Figure 1a shows the effect of AMAC concentration on ion intensity (LC-peak area). We found that the optimum concentration was 2.1 mg/mL. This concentration achieved the highest LC-peak area for all except two of the disaccharides (Δ UA–GlcN and Δ UA,2S – GlcNS). This concentration was used for all subsequent experiments. Interestingly, lower LC-peak areas were observed at higher AMAC concentrations. This may be attributed to signal suppression due to excess unreacted AMAC. It was also observed that the same concentration of different disaccharides produced significantly different LC-peak areas. It seems likely that this is due to differences in their efficiencies of ionization with Δ UA,2S – GlcNAc and Δ UA,2S – GlcNS,6S producing the highest and lowest LC-peak areas respectively with a ten-fold difference between them.

The optimum temperature for derivatization was found to be 45 °C. Above this temperature a reduction in signal intensity was observed with all disaccharides except for Δ UA - GlcN, Δ UA –GlcN,6S, Δ UA,2S–GlcN and Δ UA,2S–GlcN,6S (Fig 1b). Subsequent analysis revealed that this reduction in signal intensity of the N-substituted disaccharides at higher temperatures was due to the loss of sulfonic groups during derivatization resulting in the formation of N-unsubstituted HS disaccharide forms. Thus, Δ UA,2S-GlcNS, 6S \rightarrow Δ UA, 2S-GlcN, 6S, Δ UA,2S-GlcNS \rightarrow Δ UA,2S-GlcN, Δ UA-GlcNS, 6S \rightarrow Δ UA-GlcN, 6S, and Δ UA-GlcNS \rightarrow Δ UA-GlcN. Some loss of these sulfonic groups was observed at 45 °C. Previous studies have noted that GAG N-sulfate groups are labile and may be lost during derivatization [14]. This breakdown is demonstrated using Δ UA, 2S-GlcNS,6S as an example (Figure 1c). At 45°C, Δ UA, 2S-GlcN,6S was formed during derivatization. When the temperature was increased to 65°C, three N-unsubstituted (Δ UA, 2S-GlcN, 6S, Δ UA-GlcN, 6S, and Δ UA, 2S-GlcN) and one N-substituted HS disaccharide (Δ UA-GlcNS) were formed. We did not see any loss of sulfo groups from N-unsubstituted disaccharides during derivatization.

The reduction in the MS signal for the non-sulfated N-substituted disaccharide (Δ UA–GlcNAc) may be due to the breakage of the glycosidic bond linking Glc and GlcNAc with increasing derivatization temperature. The resulting non-sulfated GlcNAc–AMAC residue would only be minimally ionized in the ESI negative ion mode employed in this work.

For all subsequent experiments, an incubation temperature of 45 °C was used for derivatization in order to optimize the signal intensity and minimize the loss of sulfonic groups. Figure 1d shows single ion recording chromatograms of Δ UA – GlcNS at three different incubation temperatures. It was noted that the LC-peak for this disaccharide split with no baseline separation. A likely explanation for this phenomenon is anomeric interconversion. Similar findings have been reported previously [13]. These authors found that interconversion between the α and β forms occurred with one form predominating at increased temperature. In the present study significant interconversion was detected at 65 °C but only minimal interconversion occurred at 45 °C.

The effect of AMAC incubation time is shown in Figure 1e. As expected, the lowest LC-peak areas for all the disaccharides were observed at 2 h. The LC-peak areas of the N-substituted disaccharides peaked at 4 h with most of them decreasing thereafter. The exceptions to this trend were the N-unsubstituted disaccharides with their LC-peak areas increasing with increased incubation time. The reason for this observation can be attributed to the loss of the labile N-sulfo group of the N-substituted GAG disaccharides with prolonged exposure to heat during derivatization. This resulted in the formation of the N-unsubstituted disaccharides during derivatization with a resultant increase in their amounts. The N-unsubstituted disaccharides produced in this way cannot be distinguished from the ones originally present in the sample during separation and MS detection since they have the same mass and retention times.

In summary, the optimum AMAC derivatization conditions for this method is 0.210 μ g of AMAC with 4 h incubation at 45 °C.

3.2 Optimization of MS conditions

Initial MS conditions were as follows; desolvation temperature, cone voltage and extractor voltage were between 350°C, -50 V and -2.5 V respectively. Using these conditions, we were able to detect all thirteen derivatized HS disaccharides tested. The molecular weight and disaccharide ions detected using selected ion recording (SIR) of AMAC-derivatized heparin/HS disaccharides are shown in Table 1. For several of these, multiple ions were

detected which represent loss of sulfonic groups which are likely to result from in-source fragmentation. It is notable that with a single exception the same disaccharide subunits that showed loss of sulfonic groups during high temperature derivatization above also show multiple ions during ionization. Figure 2b also shows MS spectra for two of these (Δ UA,2S–GlcNS,6S and Δ UA,2S–GlcNAc,6S) confirming this. For others, only single ions were detected by MS. It was observed during optimization of derivatization conditions that certain disaccharides are more labile than others. Notably, doubly charged species were seen for disaccharides with more than one sulfate group. This can be explained by the loss of protons from disaccharides with two or more sulfonic acid groups and can be linked to the pH of the mobile phase.

Figure 2a shows the effect of different cone voltages (-15 to -75 V) on the LC-peak area of derivatized HS disaccharide subunits. The optimum voltage was found to be in the range -20 to -55 V depending on the subunit. Table 2 shows the optimal cone voltage for each subunit. These voltages were used in subsequent experiments. Lower cone voltages were optimum for lower m/z values and vice versa. Optimum cone voltage may be impacted by many factors. In-source fragmentation is certainly an important factor here. In-source fragmentation at higher cone voltages in ESI-MS operated in the negative mode has previously been reported [25].

As a group, the disaccharides that were detected using their $[M-2H]^{2-}$ species had lower m/z values and required lower cone voltages for optimal signal intensities (LC-peak areas) with signal intensity decreasing with further increase in cone voltage. This can be explained by a reduction in in-source fragmentation thus preserving the parent ion, and promoting the formation of $[M-2H]^{2-}$ species at a lower cone voltage. Notably, in-source fragmentation is promoted at high cone voltages. This is clearly seen in Figure 2b using Δ UA,2S – GlcNS,6S and Δ UA,2S – GlcNAc,6S at a higher concentration (100 μ g/mL) as examples. The signal intensity of the other disaccharides detected using their $[M-H]^-$ and $[M-SO_3-H]^-$ species increased with increasing cone voltage, peaked, and decreased afterwards. The decrease most likely resulting from an increased in-source fragmentation of the monitored ion at higher cone voltages. Our results can be explained by both an increase in in-source fragmentation at higher cone voltages and differential ionization.

Figure 2c and d show the effect of extractor voltage and desolvation temperature on signal intensity. It is clear that the optima are 3V and 350°C respectively for all derivatized subunits. It can be seen that there is more efficient solvent evaporation with increasing temperature up to 350°C. However, further temperature increase leads to in-source fragmentation and consequent loss of signal. This can clearly be seen in the spectra of Δ UA,2S –

GlcNS,6S (Fig 2e). This was studied at a higher concentration and a cone voltage of -60 V where fragmentation is promoted in order to show this. With increasing temperature, the ion assigned to $\Delta\text{UA},2\text{S} - \text{GlcNS},6\text{S}$ with the loss of two sulfonic group ($[\text{M}-2\text{SO}_3-\text{H}]^-$, m/z 610) relative to that with the loss of one sulfonic group ($[\text{M}-\text{SO}_3-\text{H}]^-$, m/z 690) is increased.

3.3 Chromatography of GAG subunits

Based on these results further analysis was carried out using the optimum cone voltage for each derivatized HS disaccharide with an extractor voltage -3 V and desolvation temperature of 350 °C. From the MS analysis alone there were a number of disaccharides with m/z values close to 610 ($\Delta\text{UA},2\text{S} - \text{GlcNS}$, $\Delta\text{UA}-\text{GlcNS},6\text{S}$, $\Delta\text{UA}-\text{GlcNS}$, $\Delta\text{UA},2\text{S}-\text{GlcN}$ and $\Delta\text{UA}-\text{GlcN}, 6\text{S}$). In addition, $\Delta\text{UA},2\text{S}-\text{GlcNAc}$ and $\Delta\text{UA}-\text{GlcNAc},6\text{S}$ both had similar m/z values (~652). However, using the UPLC conditions described each of these eluted at a different retention time and each can be easily quantified. Figure 3 shows the single ion recording (SIR) chromatograms of a mixture of AMAC-derivatized HS disaccharides run using the optimized conditions described above. It was notable that the separation and quantification of the thirteen HS disaccharides was achieved within approximately 11 min with good LC-peak shape. We are not aware of any previous reports of LC-MS-based methods of GAG disaccharide analysis with such rapid analysis time. The run time of the current method is significantly shorter than previous studies of AMAC-derivatized GAG disaccharides LC-MS analysis. These have reported run times of 50-60 min [14, 24].

The derivatized disaccharides eluted in order of increasing hydrophobicity with the tri-sulfated disaccharide eluting first followed by di- and then mono-sulfated subunits and the non-sulfated subunits eluted last. The only exception to this was the mono-sulfated $\Delta\text{UA} - \text{GlcNS}$ which eluted within the retention window of the di-sulfated subunits. This order of elution has been reported previously [14]. It is interesting to note that within the elution window for di-sulfated disaccharides, those which are N-sulfated eluted before those which are n-acetylated. This same pattern was also observed for the mono-sulfated subunits. This order of elution can also be explained by differences in hydrophobicity between n-sulfated and n-acetylated subunits. Differences in hydrophobicity may also explain the observation that, in general, 6S sulfated subunits eluted prior to 2S subunits.

The analysis of underivatized GAG disaccharides either by direct MS infusion or with specialized separation techniques including strong anion exchange [17], graphitized carbon LC [26] and HILIC columns [13] has been reported previously. The key advantage of these methods is that loss of disaccharides during derivatization is avoided but there are several disadvantages associated with direct analysis. Separation using graphitized carbon has been shown to affect the recovery of the trisulfated disaccharide because these species are

disproportionally retained and co-elution of disaccharides has been reported with HILIC [13]. In addition, a sample solvent of high organic concentration is required for HILIC chromatography, which may result in solubility issues as GAG disaccharides are polar. With ion exchange chromatography, the zwitterionic nature of N-unsubstituted disaccharides may result in poor retention [27]. Analysis of underivatized GAG disaccharides from commercially available GAG oligosaccharides and purified samples by tandem MS without LC separation has also been reported [28, 29, 30, 31]. These methods are complex and require a relatively expensive tandem MS to achieve the collision induced dissociation (CID) necessary to yield diagnostic ions for the differentiation of sulfational positional isomers. In addition, many of these direct analysis methods may be significantly affected by the numerous substances present in biological samples if GAGs are not purified before analysis. Size exclusion chromatographic methods have also been reported for the analysis of underivatized GAG disaccharides [27, 32]. However, these methods also require tandem MS in order to resolve sulfational positional isomers which co-elute [13] in addition to long run times of over 100 minutes. A previous study has analysed GAG disaccharides from heparin sulphate using direct SEC-LC/MS and MS/MS also revealed Δ UA-GlcNAc to be the most abundant disaccharide [32]. They found that 10 μ g starting material was required in order to quantify disaccharides. However, the indirect LC/MS analysis with AMAC used in our study required only 5 μ g starting material and required a significantly shorter run time. Thus, our method overcomes these challenges and consequently a significantly shorter run time, good resolution and peak shape is achieved. In addition, there is no co-elution of disaccharides, the recovery of highly sulfated disaccharides is not problematic, and the relatively expensive tandem MS is not required. Derivatization with AMAC increases the hydrophobicity of these polar HS disaccharides. Increasing hydrophobicity increases ESI response as the hydrophobic ions have an increased affinity for the droplet surface thus entering the gas phase more readily [33, 34]. Hydrophobic analytes can also be well separated from potentially ion suppressing compounds and salts [34] thus reducing interference from any contaminating material that maybe be present in biological samples. Our method also permits UV/fluorescent detection due to AMAC derivatization thus can be adapted for use in laboratories without mass spectrometers.

3.4 Method validation

There was a clear linear relationship between the subunit concentration and the LC-peak area for each subunit across the range of concentrations tested (0.02-10 μ g/mL). The limits

of detection and quantification varied between different subunits (Table 3) and ranged from 0.02 - 0.15 and 0.02 - 0.31 $\mu\text{g/mL}$ respectively. The mono-sulfated $\Delta\text{UA},2\text{S} - \text{GlcNAc}$ showed the greatest ionization capacity with an LOD of 0.02 $\mu\text{g/mL}$. The linearity (r^2) of this method (0.9747 – 1) is similar to previously reported [14], however, our LODs were higher. The precision (CV) of this method, based on five repeat measures at three concentrations ranged from 0.64 – 9.02 % at 0.625 $\mu\text{g/mL}$, 0.54 – 3.20 % at 1.25 $\mu\text{g/mL}$, and 0.39 – 1.57% at 5 $\mu\text{g/mL}$. There was little variation between disaccharide subunits but the precision was lowest at lower concentrations. This precision is within the range (<15%) accepted by the US Food and Drug Administration (FDA) and European Medicines Agency (EMA) for bioanalytical methods [35, 36]

Table 4 shows the recovery of heparan sulfate disaccharides following extraction of HS as described in the methods. Six subunits were quantifiable from HS after extraction. As expected, the recovery is better with higher amounts of starting material (10 μg) compared to 5 μg irrespective of disaccharide. The highest disaccharide recovery is seen for $\Delta\text{UA} - \text{GlcNAc}$ as expected because it is the most abundant disaccharide seen in the HS standard. This bias in recovery may influence the compositional analysis of the disaccharides recovered from different sources. The reason for this poor recovery may be linked to the numerous steps involved in the extraction, depolymerization, and derivatization of these GAG disaccharides.

3.5 Heparin/HS disaccharide composition of HS standard and cell lines

We found that the non-sulfated $\Delta\text{UA}-\text{GlcNAc}$ was the most abundant disaccharide present in HS standard comprising, about half of all disaccharide subunits (Fig 5, Table 4). $\Delta\text{UA}-\text{GlcNAc},6\text{S}$ comprised about 20 % of HS and the disaccharides $\Delta\text{UA},2\text{S}-\text{GlcNS}$ and $\Delta\text{UA},2\text{S}-\text{GlcNAc}$ were the least abundant representing 0.5 and 0.7% respectively. Compositional analysis of porcine intestinal mucosal HS by fluorescent and UV detection of heparan sulfate was previously carried by Deakin & Lyon [17]. They also found that $\Delta\text{UA}-\text{GlcNAc}$ was the most abundant disaccharide. Our other results were in broad agreement; the only exceptions being that we have found much higher levels of $\Delta\text{UA}-\text{GlcNAc},6\text{S}$ and much lower concentrations of $\Delta\text{UA}-\text{GlcNS},6\text{S}$ (21.8% and 12% respectively, Table 5). We were also able to detect an additional disaccharide not seen in the other study ($\Delta\text{UA},2\text{S}-\text{GlcNAc}$). An advantage of our method is the use of a combination of retention time and m/z that provides an unequivocal identification of each HS disaccharide. In addition, we believe that the additional use of an internal standard improved reproducibility between runs. Finally, we have used single ion monitoring for each disaccharide. This selective approach may also enhance sensitivity. Thus, this difference may be due to the increased sensitivity of our

method or the difference in the source of HS as we used HS from bovine kidney. The relative abundance of disaccharides present in heparan sulfate is also broadly in agreement with that reported by Gill et al. [13] with the non-sulfated $\Delta\text{UA-GlcNAc}$ and monosulfated $\Delta\text{UA-GlcNAc,6S}$ as the most abundant disaccharides present. However, we found that the disulfated $\Delta\text{UA,2S-GlcNS}$ was significantly less common (0.5% of total) compared to previous reports (8.3% of total, [13]).

In comparison to the HS the cell lines generally comprised significantly less of the non-sulfated HS disaccharide $\Delta\text{UA-GlcNAc}$ and the trisulfated $\Delta\text{UA,2S-GlcNS,6S}$. We are unaware of any previous reports quantifying expression of specific HS disaccharides in human cell lines. However, the composition of HS disaccharides in this study was broadly in agreement with a previous report that has examined expression from a canine kidney cell line (MDCK) [18] in that the most abundant cell line was $\Delta\text{UA-GlcNAc}$. However, their study did not examine the absolute concentrations of HS disaccharides. There were significant differences in the heparan sulfate disaccharide composition between the various cell lines tested. For the majority of cell lines the non-sulfated $\Delta\text{UA-GlcNAc}$ was the most abundant disaccharide (Fig 4, Table 5) as is also the case for heparan sulfate itself. The only exception to this was in the breast cancer cell line MDA-468 where the monosulfated $\Delta\text{UA-GlcNAc,6S}$ had a slightly higher concentration.

Many studies have demonstrated regulatory effects of glycosaminoglycans on cell function including proliferation, differentiation and invasion [3, 37, 38]. In addition, several studies have examined the effects of specific HS and CS disaccharides on cell function. Poh et al. [39] found that CS disaccharides inhibit aggressive triple negative breast cancer cell viability. Previous studies have reported that addition of specific HS disaccharides, notably monosulfated $\Delta\text{UA-GlcNS}$ significantly increased the production of the hepatocyte growth factor (HGF) *in vitro* and *in vivo* [40, 41]. HGF is known to contribute to the tumorigenic phenotype in many cancers and it is interesting to note that relative expression of this disaccharide was elevated in many of the cancer cell lines tested here compared to HS. In order to understand how specific disaccharide expression may be associated with tumour development it will be useful to apply the methodology described here to compare their expression in tissues from various cancer stages and with tissues from unaffected individuals.

4.0 Conclusion

We have used this methodology to show the rapid separation and detection of AMAC derivatized heparan sulfate disaccharides with a total run time of only 18 min. The method employed SIR in contrast to the commonly used TIC methods. Extracted ion chromatograms have been shown to be about 10 times more sensitive than TIC. We have shown that there are differences in both the total amount and composition of disaccharides between different tissue types. Further studies are needed to examine the significance of these differences.

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Table 1: Heparin/HS disaccccharide structural codes, MS ions, and assignment. Extractor voltage: 3 V, and desolvation temperature: 350°C. MS ions in bold were the most intense seen and used for DS MS detection.

HS DS structural code	MS ion (DS + AMAC)	Assignment
ΔUA,2S – GlcNS,6S	769.9	[M-H] ⁻
	690.2	[M-SO ₃ -H] ⁻
	610.0	[M-2SO ₃ -H] ⁻
	384.5	[M-2H]²⁻
	344.5	[M-SO ₃ -2H] ²⁻
	304.7	[M-2SO ₃ -2H] ²⁻
ΔUA,2S – GlcNS	690.2	[M-H] ⁻
	610.0	[M-SO₃-H]⁻
	344.0	[M-2H] ²⁻
	304.5	[M-SO ₃ -2H] ²⁻
ΔUA,2S – GlcNAc,6S	732.3	[M-H] ⁻
	652.4	[M-SO ₃ -H] ⁻
	365.7	[M-2H]²⁻
ΔUA – GlcNS,6S	690.1	[M-H] ⁻
	610.0	[M-SO₃-H]⁻
	344.0	[M-2H] ²⁻
	304.5	[M-SO ₃ -2H] ²⁻
ΔUA – GlcNS	610.3	[M-H]⁻
ΔUA – GlcNAc	572.3	[M-H]⁻
ΔUA,2S – GlcNAc	652.3	[M-H]⁻
ΔUA – GlcNAc,6S	652.2	[M-H]⁻
ΔUA,2S – GlcNCOEt, 6S	372.6	[M-2H]²⁻
ΔUA,2S – GlcN	610.3	[M-H]⁻
ΔUA,2S – GlcN, 6S	344.6	[M-2H]²⁻
ΔUA – GlcN, 6S	610.2	[M-H]⁻
ΔUA – GlcN	530.2	[M-H]⁻

Table 2: Optimal cone voltage for the different heparin/HS DS

HS DS subunit	Optimal cone voltage (-V)
Δ UA,2S – GlcNS,6S	20
Δ UA,2S – GlcNS	55
Δ UA,2S – GlcNAc,6S	25
Δ UA – GlcNS,6S	55
Δ UA – GlcNS	55
Δ UA – GlcNAc	50
Δ UA,2S – GlcNAc	55
Δ UA – GlcNAc,6S	55
Δ UA,2S – GlcN	50
Δ UA,2S – GlcN,6S	20
Δ UA – GlcN,6S	55
Δ UA – GlcN	50

Table 3: Monitored MS ions, retention time, limits of detection, limits of quantitation, r^2 , slope, and intercept of the method. LOD and LOQ were determined using signal:noise 3:1 and 10:1 respectively.

HS DS structural code	Monitored MS ion (m/z)	Retention time (min)	LOD ($\mu\text{g/ml}$)	LOQ ($\mu\text{g/ml}$)	r^2	Slope	Intercept
$\Delta\text{UA}, 2\text{S} - \text{GlcNS}, 6\text{S}$	384.5	2.5	0.07	0.11	0.9999	1269.6	-83.46
$\Delta\text{UA}, 2\text{S} - \text{GlcNS}$	610.0	4.0	0.08	0.14	0.9998	1955.1	-124.63
$\Delta\text{UA}, 2\text{S} - \text{GlcNAc}, 6\text{S}$	365.7	6.1	0.09	0.18	0.9999	1720.2	-115.7
$\Delta\text{UA} - \text{GlcNS}, 6\text{S}$	610.0	3.2	0.08	0.17	0.9999	1947.9	-79.96
$\Delta\text{UA} - \text{GlcNS}$	610.3	5.4	0.12	0.17	0.9993	2457.7	-243.67
$\Delta\text{UA} - \text{GlcNAc}$	572.3	10.1	0.06	0.09	0.9998	3051.4	-150.75
$\Delta\text{UA}, 2\text{S} - \text{GlcNAc}$	652.3	8.7	<0.02	0.02	0.9999	12007	144.94
$\Delta\text{UA} - \text{GlcNAc}, 6\text{S}$	652.2	7.6	0.13	0.19	0.9997	2756.2	-357.42
$\Delta\text{UA}, 2\text{S} - \text{GlcNCOEt}, 6\text{S}$	372.6	8.3	0.07	0.13	1	2678.5	-130.26
$\Delta\text{UA}, 2\text{S} - \text{GlcN}$	610.3	8.0	0.15	0.31	0.9747	1031.3	386.21
$\Delta\text{UA}, 2\text{S} - \text{GlcN}, 6\text{S}$	344.6	5.8	0.15	0.31	0.9785	686.75	262.55
$\Delta\text{UA} - \text{GlcN}, 6\text{S}$	610.2	7.2	0.15	0.31	0.9876	1435.3	303.37
$\Delta\text{UA} - \text{GlcN}$	530.2	9.4	0.15	0.31	0.9843	1381.2	341.58

Table 4: Recovery of Hep/HS DS following digestion, derivatization, and separation using the final protocol described above.

HS standard (μ g)	Recovery (%)					
	Δ UA,2S – GlcNS,6S	Δ UA2S – GlcNS	Δ UA – GlcNS,6S	Δ UA – GlcNS	Δ UA – GlcNAc	Δ UA – GlcNAc,6S
5	26.32	33.33	18.18	27.78	42.86	24.14
10	26.67	35.71	30.77	39.02	43.02	33.33

Table 5. Composition of disaccharide subunits present in the cell lines tested. Top panel indicates concentrations (ng/mg dry weight). Bottom panel represents the relative abundance expressed as a percentage of total for each cell line.

ng/mg dry weight					
DS subunit	OVCAR	MOLM-13	MDA-468	HCT-116	BeWo
Δ UA _{2S} -GlcNS _{6S}	241.2 (3.11)	1197.5 (5.19)	110.2 (5.22)	256.0 (3.49)	203.9 (0.27)
Δ UA _{2S} -GlcNS	97.0 (5.76)	235.6 (6.32)	ND	78.2 (1.69)	ND
Δ UA-GlcNS _{6S}	74.9 (1.42)	84.0 (14.48)	61.7 (1.46)	98.4 (2.67)	65.2 (2.24)
Δ UA-GlcNS	210.8 (8.86)	252.6 (14.11)	109.9 (2.08)	153.3 (1.56)	197.9 (2.66)
Δ UA-GlcNAc	309.0 (3.95)	1095.1 (99.68)	121.3 (0.47)	259.8 (2.63)	326.9 (3.39)
Δ UA _{2S} -GlcNAc	11.6 (0.63)	14.1 (0.40)	ND	ND	4.2 (0.94)
Δ UA-GlcNAc _{6S}	113.2 (1.31)	177.0 (0.67)	150.9 (7.04)	180.8 (4.80)	90.6 (0.95)
Total	1058 (3.58)	3056 (7.26)	554 (3.25)	1027 (2.81)	889 (1.74)

% total for cell line						
DS subunit	OVCAR	MOLM-13	MDA-468	HCT-116	BeWo	HS
Δ UA _{2S} -GlcNS _{6S}	22.8 (0.29)	39.2 (0.17)	19.9 (0.94)	24.9 (0.34)	22.9 (0.03)	8.2 (0.10)
Δ UA _{2S} -GlcNS	9.2 (0.55)	7.7 (0.21)	ND	7.6 (0.16)	ND	0.5 (0.01)
Δ UA-GlcNS _{6S}	7.1 (0.13)	2.7 (0.47)	11.1 (0.26)	9.6 (0.26)	7.3 (0.25)	8.7 (0.23)
Δ UA-GlcNS	19.9 (0.84)	8.3 (0.46)	19.8 (0.37)	14.9 (0.15)	22.3 (0.30)	12.6 (0.38)
Δ UA-GlcNAc	29.2 (0.37)	35.8 (0.32)	21.9 (0.08)	25.3 (0.26)	36.8 (0.38)	48.0 (0.44)
Δ UA _{2S} -GlcNAc	1.1 (0.06)	0.5 (0.01)	ND	ND	0.5 (0.11)	0.7 (0.01)
Δ UA-GlcNAc _{6S}	10.7 (0.12)	5.8 (0.02)	27.2 (1.27)	17.6 (0.47)	10.2 (0.11)	21.3 (0.19)

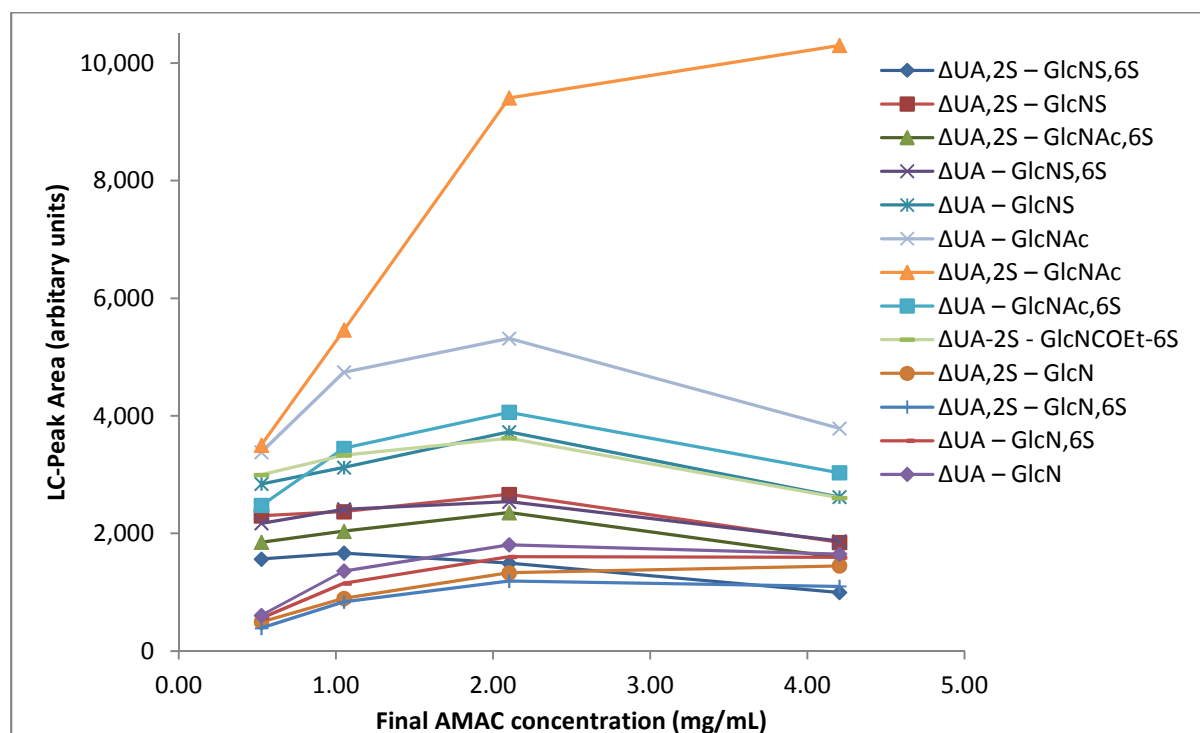


Figure 1a: LC-peak area as a function of the concentration of AMAC used for derivatization. Heparin/HS DS concentration: 2.5 $\mu\text{g/mL}$.

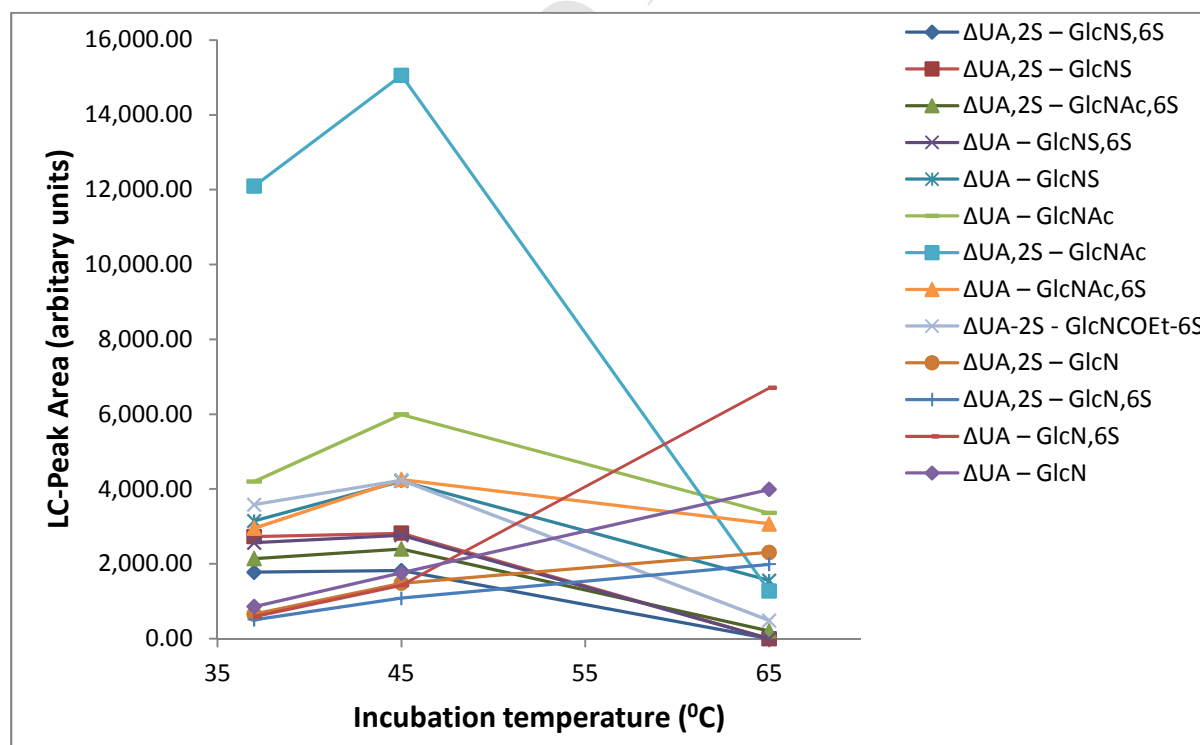


Figure 1b: LC-peak area as a function of derivatization temperature of AMAC-derivatized heparin/HS DS standard. Heparin/HS DS concentration: 2.5 $\mu\text{g/mL}$.

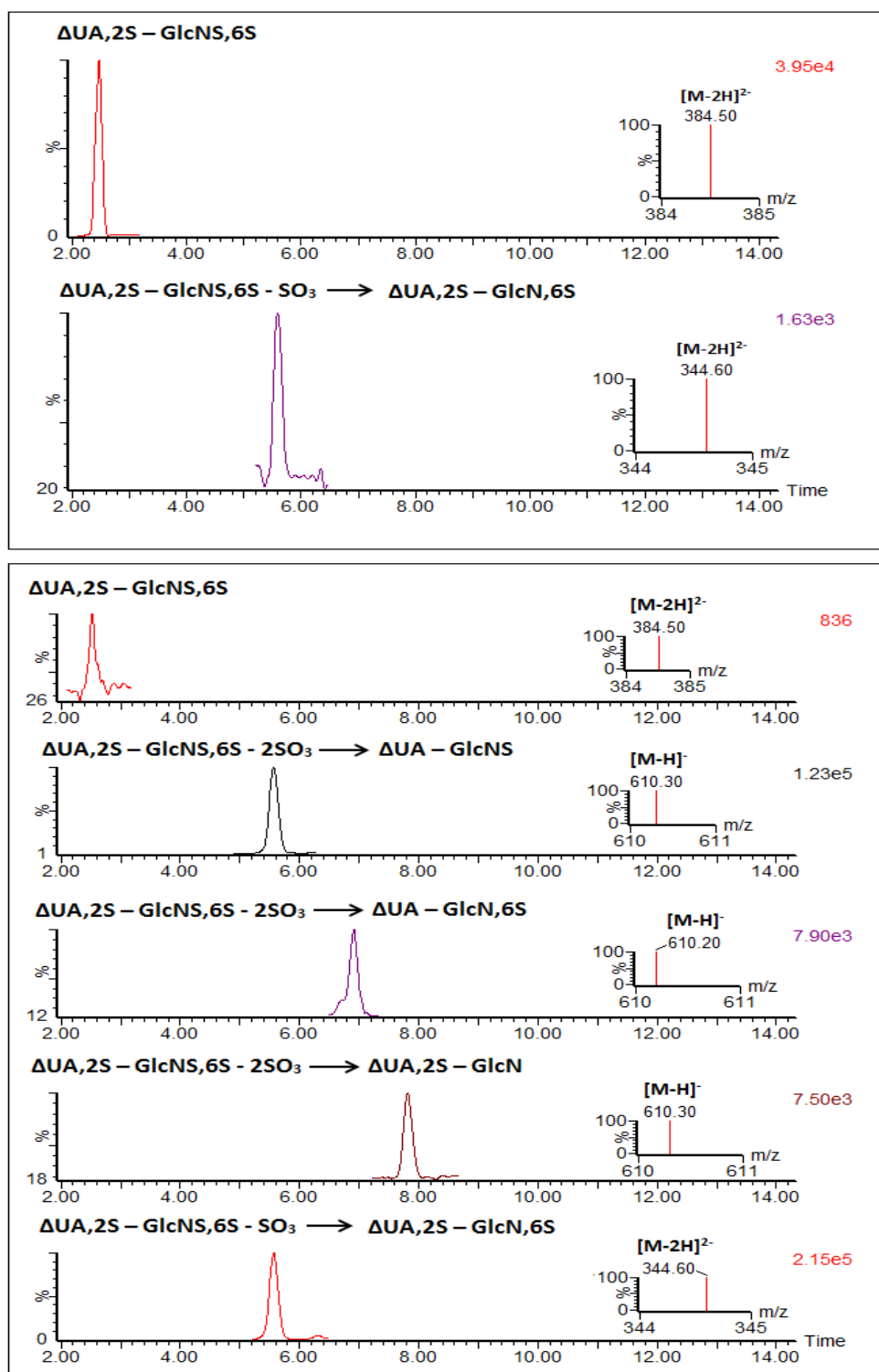


Figure 1c: Selected ion recording (SIR) chromatogram of AMAC-derivatized $\Delta\text{UA},2\text{S} - \text{GlcNS},6\text{S}$ derivatized at 45 °C (top box) and 65 °C (bottom box) for 4 h showing loss of SO_3^- groups resulting in the formation of other DS during derivatization. Injection vol. 3 μL

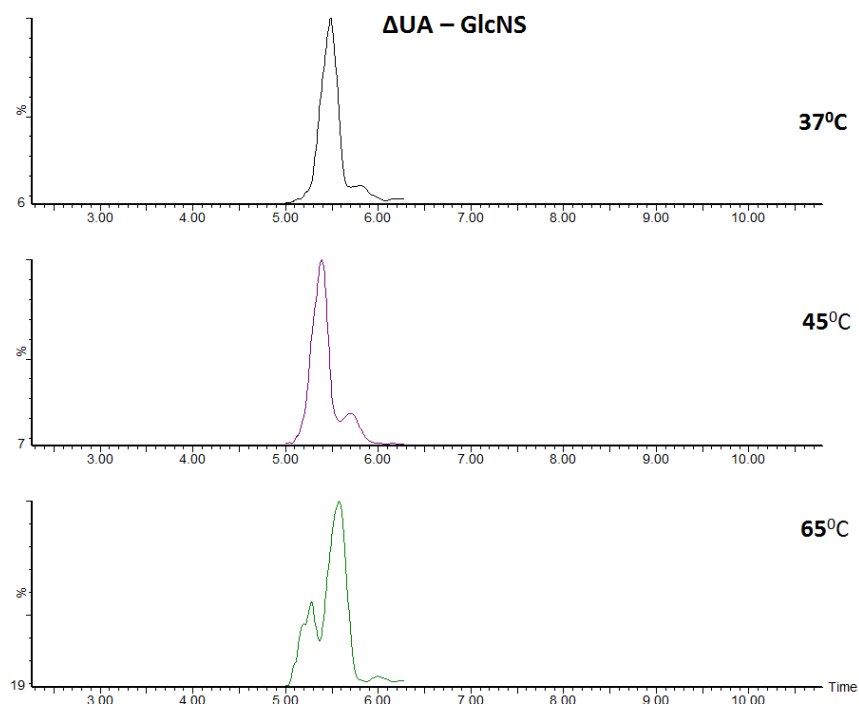


Figure 1d: SIR chromatogram of $\Delta\text{UA} - \text{GlcNS}$ when derivatized at different temperatures. Top: 37 °C, middle: 45 °C, and bottom: 65 °C.

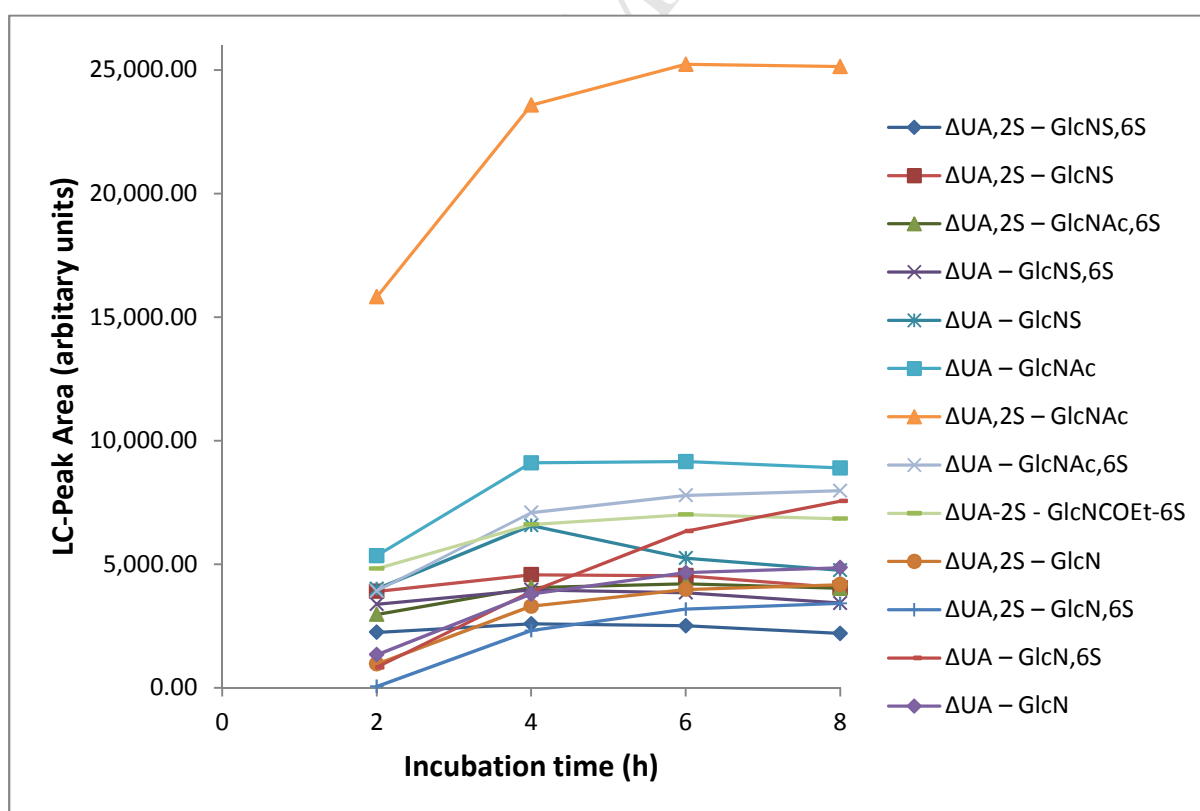


Figure 1e: LC-peak area against derivatization incubation time of AMAC-derivatized heparin/HS DS standard. Heparin/HS DS concentration: 2.5 $\mu\text{g/mL}$.

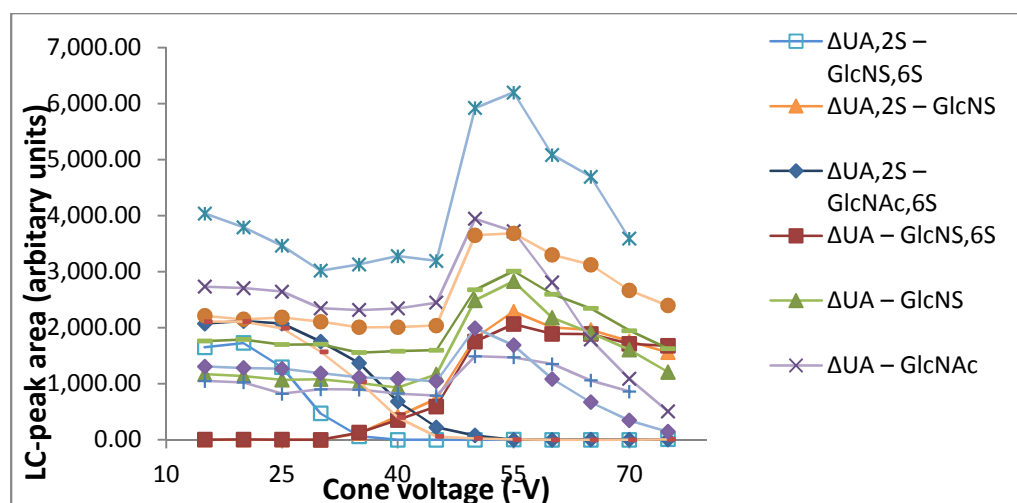


Figure 2a: LC-peak area of AMAC derivatized heparin/HS DS standard (1 $\mu\text{g/mL}$) as a function of cone voltage.

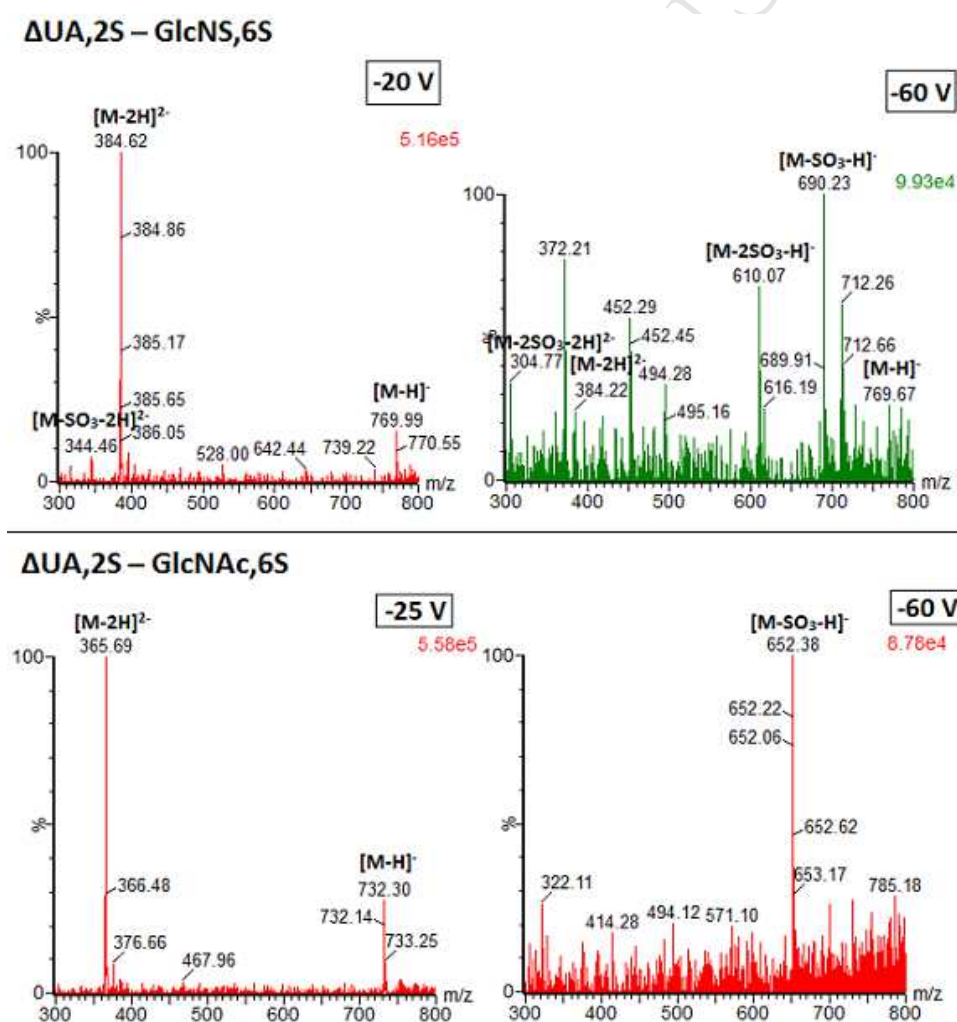


Figure 2b: Mass spectrum of 100 $\mu\text{g/mL}$ AMAC-derivatized $\Delta\text{UA},2\text{S} - \text{GlcNS},6\text{S}$ (Top) obtained at a cone voltage of -20 and -60 V and $\Delta\text{UA},2\text{S} - \text{GlcNAc},6\text{S}$ (bottom) obtained at -25 and -60 V

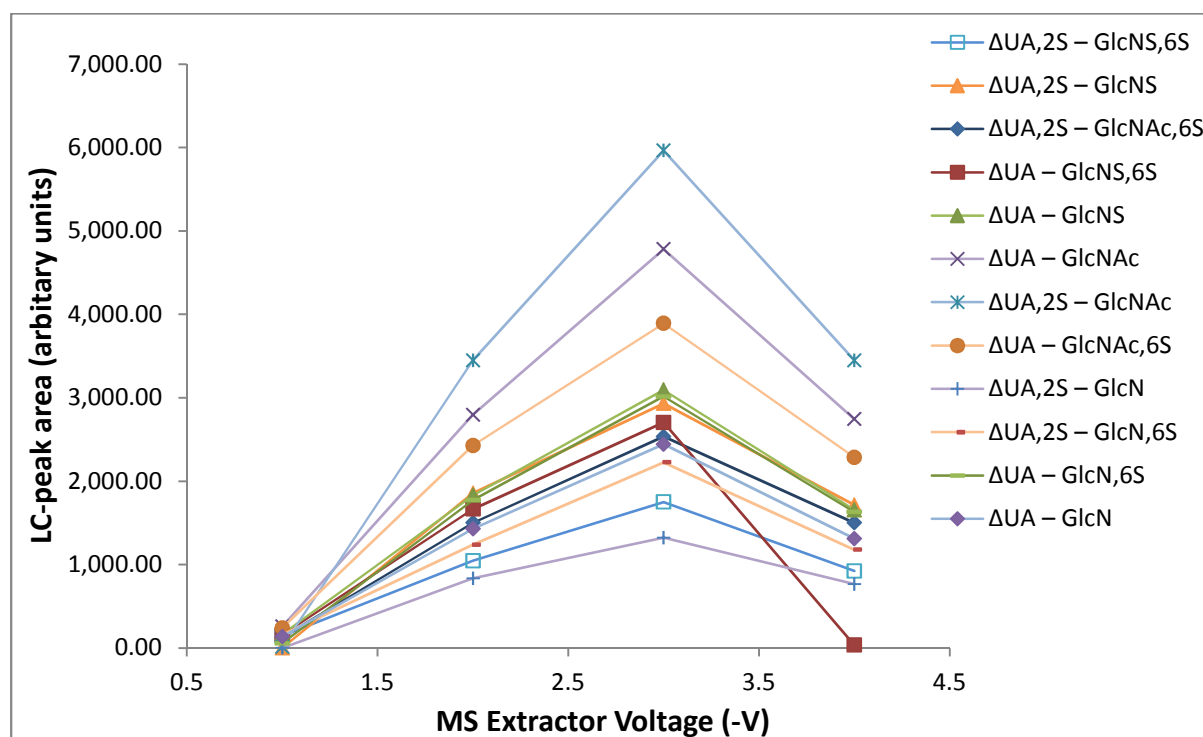


Figure 2c: LC-peak area against MS extractor voltage of AMAC-derivatized heparin/HS DS. Concentration: 1 $\mu\text{g/mL}$.

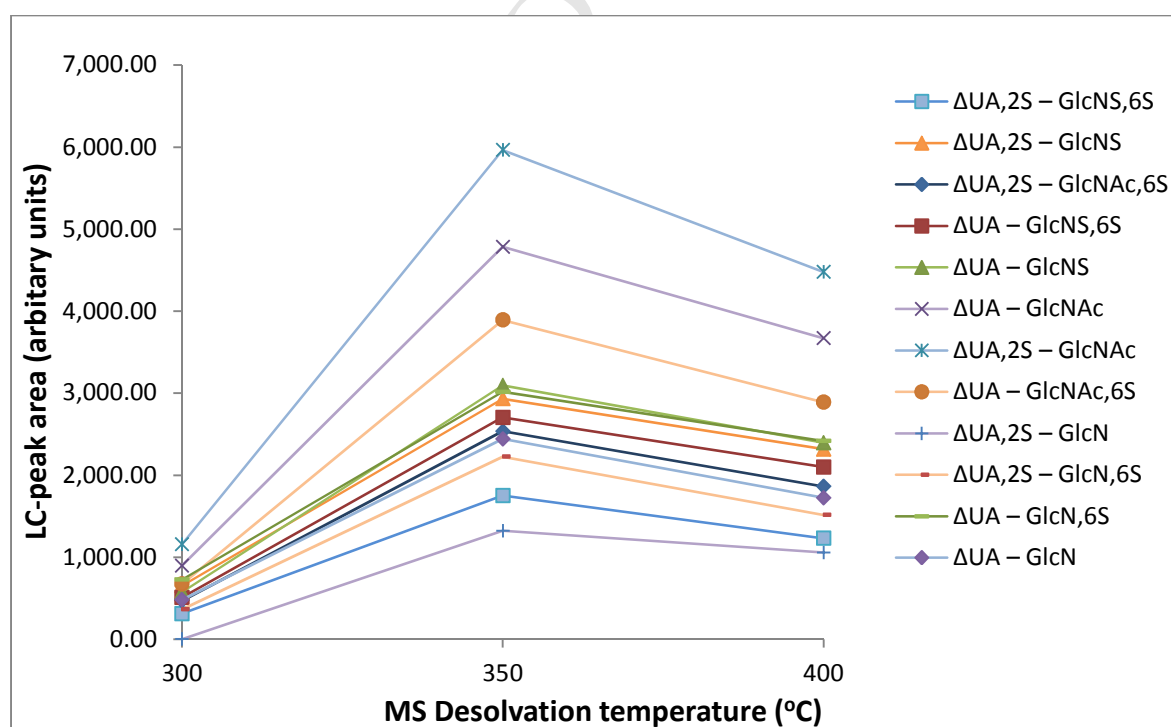


Figure 2d: LC-peak area as a function of desolvation temperature of AMAC-derivatized heparin/HS DS. Concentration: 1 $\mu\text{g/mL}$.

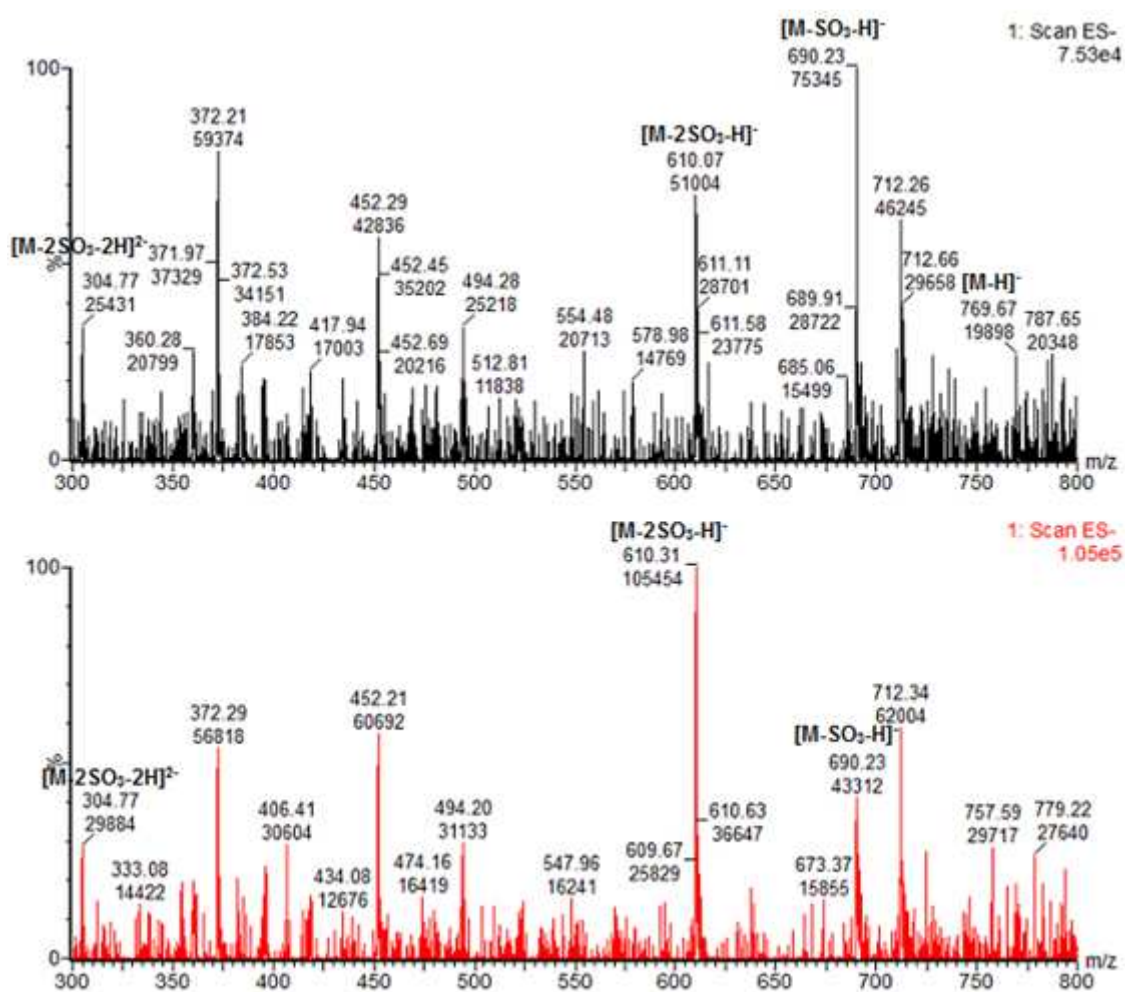


Figure 2e: Mass spectrum of AMAC-derivatized Δ UA,2S – GlcNS,6S obtained at a cone voltage of -60 V and desolvation temperature of 350 °C (top) and 400 °C (bottom). Top number: m/z , bottom number: intensity

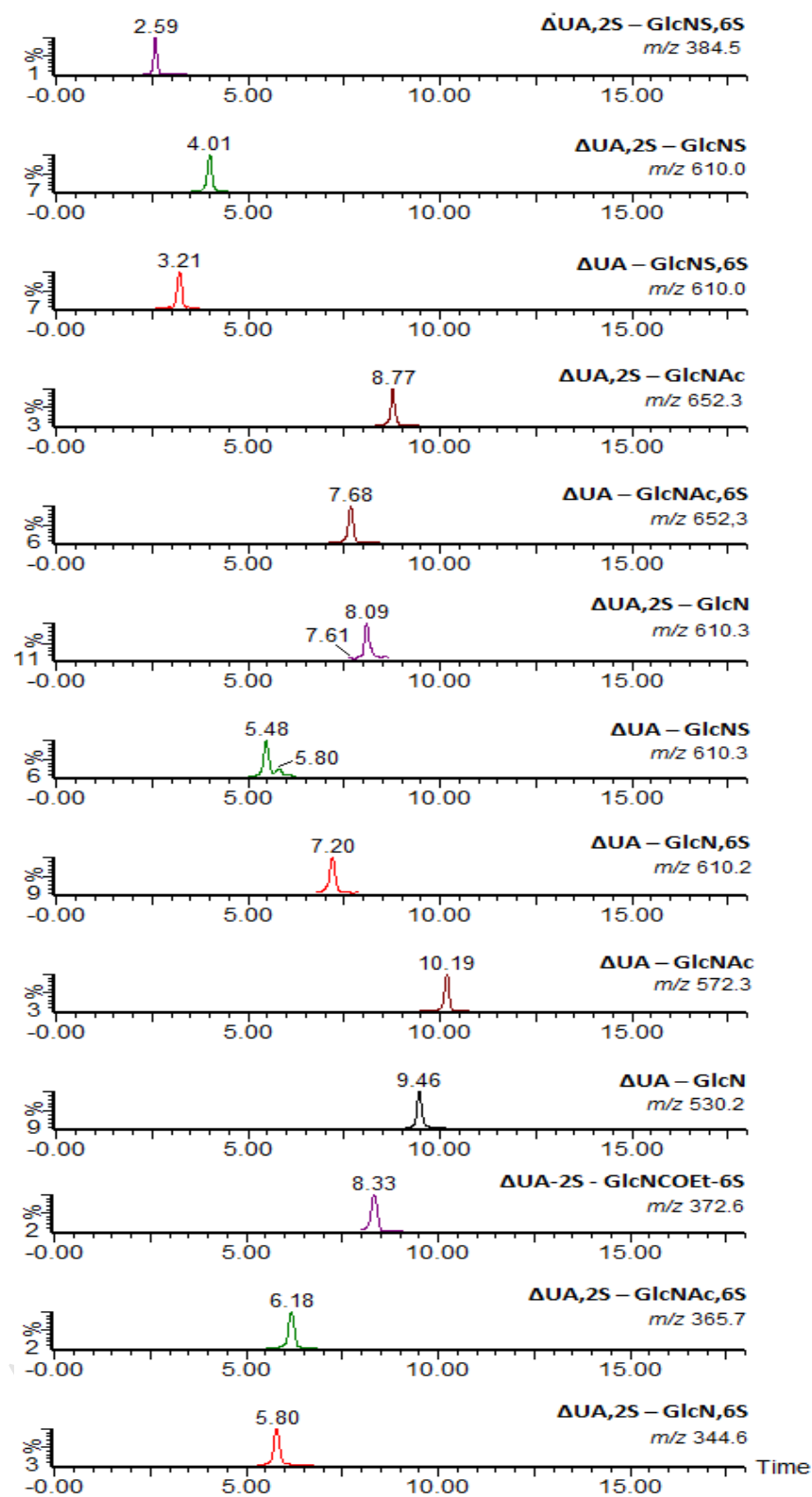


Figure 3: The SIR chromatograms of AMAC-derivatized heparin/HS DS using optimal derivatization and MS conditions. 10 μ g/mL, injection volume: 5 μ L

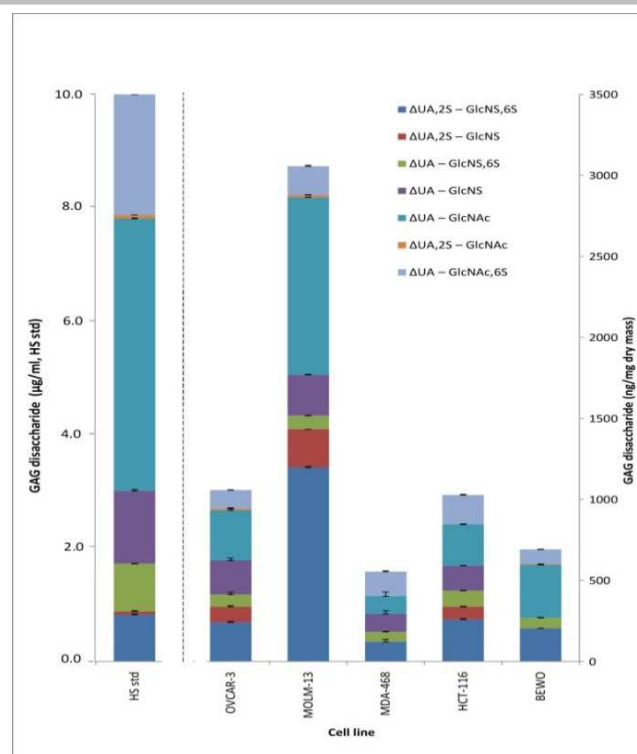


Figure 4: Bar chart and table showing the concentrations of recovered heparin/HS DS composition of heparan sulphate standard ($\mu\text{g/ml}$) and different cell lines (ng/mg dry weight).